UNIVERSITY OF ADELAIDE LIBRARY
4.9.89

The Intestinal Immune Response to *Giardia* in the Rat

Agnes Phyllis Waight Sharma

Department of Microbiology and Immunology
University of Adelaide,
Adelaide, 5000
Australia

A Thesis Submitted for the Degree of Doctor of Philosophy.

September, 1988.

The Intestinal Immune Response to *Giardia* in the Rat

Agnes P. Waight Sharma

I dedicate this thesis to my husband and to my children for their love and patience. Thank you.

Abstract

An experimental model of giardiasis was developed in the rat. Two morphologically identical isolates of *Giardia duodenalis* were used in these studies. One was isolated from a colony of mice from a municipal zoo and the other from a laboratory rat. The rat isolate produced a chronic infection in all seven inbred rat strains tested while the mouse isolate in each case produced an acute infection. Balb/c mice were found to be relatively resistant to primary infections with either isolate but C3H/HeJ mice became infected chronically with the mouse isolate and experienced a prolonged infection with the rat isolate. Infections with both isolates were chronic in congenitally hypothymic nude rats. Natural or metronidazole- induced termination of primary infections with either organism was followed by a high level of immunity to re-infection. Thus, infection with *G. duodenalis* in rats offered a model for giardiasis based on an organism related closely to human *G. lamblia.*

Using a sensitive ELISA, specific serum and bile anti- *Giardia* IgM and IgA responses were studied in DA ($RT1^{av1}$) rats at various times during primary and secondary infections. Antibody responses to both organisms were similar. Only IgA anti-*Giardia* antibodies were detected in bile whereas both IgM and IgA antibodies were detected in serum. Biliary IgA antibody titers increased throughout the course of the primary infection and remained at high levels for at least 10 weeks. Biliary IgA titers increased 16-fold during the secondary infection with both isolates. Serum IgA anti-*Giardia* titers also increased but more slowly than the titers in bile. Serum IgM antibody responses were observed against both organisms during the primary and secodnary infections. Immune bile, but not normal bile, led to a substantial fall in fecal cyst excretion when infused into the duodena of conscious rats infected with the mouse isolate, that is, in the acute infection. However, despite the presence of surface-bound IgA *in vivo* on trophozoites of the chronic infection (rat isolate) and the presence of specific biliary IgA antibodies, immune bile had no effect on the course of infection in these animals. The findings suggest that secretory antibodies are protective only in some infections with *Giardia.*

Comparative studies using the two rodent isolates were undertaken in order

to gain a better understanding of antigens involved in the immune response as well as to determine the degree of sharing of common antigens. Both were identical with each other by isoenzyme analysis. Cross-protection by primary infection with the heterologous isolates suggested the existence of common antigens. Trophozoites harvested from the intestinal lumen during primary infections with both isolates of *Giardia* were examined for surface-bound IgA by immunofluorescence microscopy. The fraction of trophozoites with bound IgA increased with time after infection, reaching approximately 80% by 10-15 days post-infection. IgA antibodies were thus detected bound to trophozoites isolated from the gut at approximately the same time they appeared in bile. At any stage in the infection, all trophozoites could be labelled *in vitro* with hyperimmune bile. Furthermore, the biliary antibodies were extensively cross-reactive against heterologous trophozoite antigen. The data demonstrated that some of the secretory IgA immune response in infected rats was directed against surface antigens of the trophozoites. Similar results were obtained with both isolates of *G. duodenalis*. This suggests either that (a) some trophozoites are inaccessible to antibody during early infection, (b) the trophozoites are heterogenous with respect to their dominant surface antigens and/or (c) the antibody response proceeds by sequential activation of clones of B cells making antibody against the repertoire of parasite antigens.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University and to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis. The author consents to the thesis being made available for photocopying and loan, if applicable and if accepted for the award of the degree.

Agnes Phyllis Waight Sharma

Acknowledgements

I wish to express my deepest appreciation and gratitude to my supervisor,Dr. Graham Mayrhofer, for his guidance and encouragement throughout the course of this project. My heartfelt thanks also go to Professor Derrick Rowley for giving me the opportunity to undertake this project in the Department of Microbiology and Immunology. I am also grateful to past and present workers in Labs 4 and 8 for making it such a pleasant environment in which to work.

I am indebted to Dr. Connor Thomas for his computer expertise and patience displayed whilst I compiled my thesis using *Microsoft Word* and LaTeX.

I also thank Ann Hallett for providing assistance in the care of the experimental animals and Gary Penny for his photographic expertise.

I acknowledge the receipt of a University of Adelaide Postgraduate Scholarship during the period of my candidature.

Publications

Material contained in this thesis has been published in the following papers.

Waight Sharma A. & Mayrhofer G. (1988) A comparative study of infections with rodent isolates of *Giardia duodenalis* in inbred strains of rats and mice and in hypothymic nude rats. *Parasite Immunology*. **10**: 169–179.

Waight Sharma A. & Mayrhofer G. (1988) Biliary immune responses in giardiasis. *Parasite Immunology*. **10**: 181-191.

Mayrhofer G. & Waight Sharma A. (1988) The secretory immune response in rats infected with rodent *Giardia duodenalis* isolates and evidence for passive protection with immune bile. *Adv. Giardia Res.* Ed. P.M. Wallis & B.R. Hammond. Calgary: University of Calgary Press. In Press.

Abbreviations

Ab	antibody
ADCC	antibody-dependent cell-mediated cytotoxicity
ATCC	American Type Culture Collection
BSA	bovine serum albumin
C	centigrade
CIE	counterimmunoelectrophoresis
CRP170	cysteine-rich protein 170 kDa
DEA	diethanolamine
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
ELISA	enzyme-linked immunosorbent assay
E-S	excretory-secretory
FcR	receptor for Fc portion of immunoglobulins
FCS	fetal calf serum
g	gram(s)
GluNAc	N-acetyl-D-glucosamine
HCl	hydrogen chlorine
IF	immunofluorescence
IgA	immuoglobulin A
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
in	inch(es)
kDa	kilodalton
kg	kilogram(s)

mg	milligram
min	minute(s)
ml	millimeter(s)
mm	millimeter(s)
MoAb	monoclonal antibody
MVB	microvillus border
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	normal phosphate buffered saline
PBS_m	phosphate buffered saline, 400 mOsm/kg
PI	protease inhibitors
PNPP	p-nitro-phenylphosphate disodium
PO-1	Portland strain of *Giardia lamblia*
PP	Peyer's patches
QEH	Queen Elizabeth Hospital
RT	room temperature
SC	secretory component
SDS	sodium dodecyl sulphate
S.E.M.	standard error of the mean
SEM	scanning electron microscopy
sIgA	secretory immunoglobulin A
SPF	specific pathogen free
SRBC	sheep red blood cells
TYI-S-33	trypticase, yeast extract, iron-serum medium
μg	microgram(s)
WB	isolate of *Giardia*
WGA	wheat germ agglutinin

Contents

1 Introduction 1

1.1 Historical Background 1

1.1.1 Discovery of the parasite 1

1.1.2 Nomenclature 2

1.1.3 Classification 3

1.2 Characteristics of the Parasite 4

1.2.1 Life cycle 4

1.2.2 Structure and morphology 4

1.2.3 Cultivation of *Giardia in vitro* 7

1.3 Clinical features of giardiasis 9

1.4 Pathology and pathogenesis of giardiasis 9

1.4.1 Colonization and attachment 9

1.4.2 Penetration of the epithelium 11

1.4.3 Mechanisms of diarrhea and malabsorption 12

1.5 Diagnosis of giardiasis 13

1.5.1 Stool specimens 13

1.5.2 Intestinal fluid examination 13

1.5.3 Serodiagnosis of giardiasis 14

1.6 Epidemiology 14

1.6.1 General 14

1.6.2 Transmission of *Giardia* 16

1.6.3 Is Giardiasis a Zoonosis? 17

1.7 Treatment . . . 19
1.8 Antigens of *Giardia* . . . 20
1.8.1 Cyst antigens . . . 22
1.8.2 Differentiation antigens . . . 22
1.8.3 Trophozoite antigens . . . 23
1.8.4 Antigenic variation . . . 28
1.9 Host immune response in giardiasis . . . 31
1.9.1 General properties of anti-giardial immunity . . . 31
1.9.2 The role of humoral immune responses . . . 32
1.9.3 The role of cell-mediated immune responses . . . 37
1.9.4 The role of non-lymphoid cells in giardiasis . . . 39
1.10 Anti-*Giardia* vaccine . . . 40
1.11 Secretory IgA . . . 41
1.11.1 Synthesis and transport into the intestinal lumen . . . 41
1.11.2 T cell regulation of the IgA response . . . 42
1.11.3 Biliary sIgA . . . 43
1.11.4 The function of secretory IgA . . . 44
1.12 Aims of Thesis . . . 45

2 Materials & Methods 46
2.1 Experimental Animals . . . 46
2.1.1 Adult rats and mice . . . 46
2.1.2 Infant mice . . . 46
2.2 Source of *Giardia* isolates . . . 47
2.2.1 *G. lamblia* Portland-1 (PO-1) strain . . . 47
2.2.2 *G. duodenalis* rat strain . . . 47
2.2.3 *G. duodenalis* mouse strain . . . 47
2.3 Maintenance of parasites . . . 47
2.3.1 PO-1 *Giardia* . . . 47
2.3.2 Rodent *Giardia* . . . 47

2.4 Experimental infection 48
2.4.1 Cyst isolation 48
2.4.2 Cyst purification 48
2.4.3 Inoculation with cysts 49
2.4.4 Quantitation of cyst excretion 49
2.4.5 Drug termination of primary infections 49
2.5 Morphological studies of trophozoites 50
2.5.1 Preparation of trophozoites 50
2.5.2 Staining with Trichrome stain 50
2.6 Surgical techniques 50
2.6.1 Bile duct cannulation 50
2.6.2 Passive bile transfers 51
2.7 Preparation of antisera 51
2.7.1 Preparation of rat antiserum to PO-1 trophozoites 51
2.7.2 Preparation of rabbit antiserum to rat immunoglobulin . . . 54
2.8 Isoenzyme studies 55
2.9 Excystation of *Giardia* cysts 55
2.10 Rodent *Giardia* growth experiments 56
2.10.1 Experimental media 56
2.10.2 Culture procedures 57
2.11 *In vitro* culture of *Giardia* 58
2.12 Preparations of crude membranes of PO-1 trophozoites 59
2.13 Freezing of trophozoites 59
2.14 Trophozoites harvested from intestines 59
2.15 Localization and distribution of *Giardia* in the intestines of infected rats 61
2.16 Encystation of PO-1 trophozoites *in vitro* 61
2.17 Determination of neuraminidase activity in *Giardia* 62
2.18 Enzyme-linked immunosorbent assay (ELISA) 63
2.18.1 Preparation of conjugates 63

2.18.2 *Giardia* antigen 64

2.18.3 Procedure 64

2.19 Indirect Immunofluorescence 65

2.20 Protein profiles 66

2.21 Surface proteins of *Giardia* trophozoites 66

2.21.1 Biotinylation of trophozoite surface proteins 66

2.21.2 SDS-PAGE 67

2.21.3 Western Transfer 67

2.21.4 Visualization of biotinylated proteins 67

2.22 Preparation of *Giardia* nucleic acid 68

3 Kinetics of *Giardia duodenalis* Infections **70**

3.1 Introduction 70

3.2 Isolation of rodent isolates of *G. duodenalis* 71

3.2.1 Mouse isolate 72

3.2.2 Rat isolate 73

3.3 Preliminary experiments 73

3.3.1 Mouse isolate 73

3.3.2 Rat isolate 75

3.4 Infection of normal inbred rat strains with the mouse isolate 77

3.4.1 Primary infection 77

3.4.2 Secondary infection 77

3.5 Infection of normal inbred rat strains with the rat isolate 81

3.5.1 Primary infection 81

3.5.2 Secondary infection 81

3.6 Infection of inbred strains of mice 86

3.6.1 Infection with the mouse isolate of *G. duodenalis* 86

3.6.2 Infection with the rat isolate of *G. duodenalis* 88

3.7 Infection of hypothymic nude rats 88

3.8 Discussion 91

4 Biliary and Serum Antibody Responses in Rats Infected with *Giardia duodenalis* **96**

4.1 Introduction 96

4.2 Attempts to culture the rodent isolates of *Giardia* 97

4.3 Standardization of ELISA 98

4.3.1 Determination of specificities of conjugates 100

4.3.2 Antigen coating conditions 100

4.3.3 Determination of endogenous alkaline phosphatase 104

4.3.4 Crossreactivity with media components 104

4.4 Antibody responses in rats during primary and secondary infection with *Giardia*. 104

4.4.1 Serum and biliary antibody responses in rats 106

4.5 Immunoglobulin levels in bile from CBH rnu/rnu and CBH rnu/+ rats during infection with the mouse isolate of *G. duodenalis* 111

4.6 Discussion 111

5 *In vivo* Microenvironments during Infection with *Giardia duodenalis* **118**

5.1 Introduction 118

5.2 Distribution of trophozoites and cysts in the intestines of infected rats 119

5.2.1 Trophozoite numbers 119

5.2.2 Localization of trophozoites and cysts 120

5.3 *In vitro* and *in vivo* reactions of specific IgA antibodies with trophozoites 124

5.3.1 Preliminary experiments 124

5.3.2 Binding of IgA antibodies to trophozoites recovered from normal DA rats, CBH rnu/rnu and CBH rnu/+ rats 126

5.4 Determination of neuraminidase activity in trophozoites 127

5.5 Induction of encystation of trophozoites *in vitro* 128

5.6 Discussion 129

6 Passive Transfer of Immunity with Immune Bile **143**

6.1 Introduction 143
6.2 Passive protection with immune bile 144
6.2.1 The effect of immune bile on an established infection 144
6.2.2 The effect of immune bile in prevention of infection 145
6.3 Discussion 148

7 Comparison of the Mouse and Rat Isolates of *Giardia duodenalis* 155
7.1 Introduction 155
7.2 Morphology of the isolates 156
7.3 Isoenzyme studies 156
7.4 Cross-protection studies *in vivo* 156
7.5 *In vitro* cross-reactivity 159
7.5.1 ELISA 159
7.5.2 Immunofluorescence assay 159
7.6 Comparison of protein profiles 161
7.7 Comparison of surface proteins of rodent isolates with PO-1 164
7.8 Occurrence of *Giardia* virus 164
7.9 Discussion 168

8 General discussion 171
8.1 Speciation of *G. duodenalis* isolates 171
8.2 Rat strain susceptibility to *Giardia* infection 173
8.3 Anti-*Giardia* immune response in rats 174
8.4 Protective immunity 176
8.5 The role of mucus in *Giardia* infection 178
8.6 Future studies 179
8.6.1 Analysis of the specificity of biliary IgA antibodies 180
8.6.2 Molecular biology studies 181

9 Bibliography 183

List of Tables

1.1 *Giardia* specific surface proteins 27

3.1 Major histocompatibility haplotypes of rat & mouse strains 80

3.2 Proportion of rats excreting cysts during secondary infection of inbred strains with mouse and rat isolates of *G. duodenalis* 95

4.1 Cultural conditions used in attempts to axenize *G. duodenalis in vitro* 99

4.2 Specificity of alkaline-phophatase rat conjugates as determined by ELISA 101

4.3 Comparison of E-S and sonicate antigens of *G.lamblia* PO-1 trophozoites in ELISA 103

4.4 Titres of anti-*Giardia* IgG antibodies in pooled bile from DA rats . . 115

4.5 Antibody response to mouse *G.duodenalis* in nude CBH rats and heterozygous littermates 116

4.6 Levels of IgA and IgG in bile of nude CBH rats and heterozygous littermates 117

5.1 Distribution of trophozoites in intestinal mucus of DA rats infected with *G. duodenalis* 125

5.2 Distribution of cysts between free luminal fluid and mucus in rats infected with *G. duodenalis* 139

5.3 Binding of IgA antibodies to *G. duodenalis* trophozoites *in vivo* and *in vitro* 140

5.4 Proportion of trophozoites with bound IgA in the free and mucus-associated fractions of trophozoites harvested from the intestines of DA rats 141
5.5 Fluorescence assay of *G. duodenalis* trophozoites from gut lumen of CBH nudes and heterozygotes 142

6.1 Effect of intra-intestinal infusion of immune bile in infections of the rat isolate 149

7.1 Enzymes identified in the *G. duodenalis* isolates. 158
7.2 Cross-reactivity of biliary IgA antibodies in bile with both isolates of *G. duodenalis* 160
7.3 Binding of IgA antibodies to homologous and heterologous *G. duodenalis* trophozoites *in vitro* 162

List of Figures

2.1 Diagramatic representation of the intra-intestinal infusion of bile . . 52
2.2 Photograph showing rats after insertion of cannula 53

3.1 Percoll separation of cysts of *S. muris* and *Giardia* 74
3.2 Infection in F344 rats inoculated orally with Mouse isolates from two different sources 76
3.3 Daily cyst release in F344 infected with the rat isolate 78
3.4 Weekly cyst release in F344 infected with the rat isolate 79
3.5 Daily cyst pattern of inbred rats infected with the mouse isolate . . 82
3.6 Primary and secondary infection of inbred rats with the mouse isolate 83
3.7 Daily pattern of cyst ecretion in inbred rats infected with the rat isolate 84
3.8 Weekly cyst excretion by inbred rats infected with the rat isolate . . 85
3.9 Daily cyst excretion by mice infected with *G. duodenalis* isolates . . 87
3.10 Weekly cyst excretion by mice infected with *G. duodenalis* isolates . 89
3.11 Infection of nude rats by the mouse isolate 90

4.1 Different numbers of PO-1 trophozoites binding to ELISA wells . . . 102
4.2 Specific antibody titres in DA rats infected with the mouse isolate . 107
4.3 Specific antibody titres in DA rats infected with the rat isolate . . . 109

5.1 Total number of trophozoites in the small intestine of rats infected with *G. duodenalis* 121
5.2 Localization of trophozoites and cysts in intestinal tract 123
5.3 Peanut lectin agglutination 130

5.4 Phase-contrast microscopy of unfixed and unstained cysts induced *in vitro* 131

6.1 The effect of passive intra-intestinal infusion with immune bile on the course of infection with the mouse isolate in DA rats (**Experiment 1**)146

6.2 The effect of passive intra-intestinal infusion with immune bile on the course of infection with the mouse isolate (**Experiment 2**) . . . 147

6.3 The effect of intra-intestinal infusion with immune bile in preventing infection with the mouse isolate 150

7.1 Trichrome staining of trophozoites showing morphology 157

7.2 Immunofluorescent staining of *Giardia* trophozoites. 163

7.3 Comparison of protein profiles of rodent isolates of *G. duodenalis*. . 165

7.4 SDS-PAGE analysis of surface-biotinylated trophozoites of *Giardia*. . 166

7.5 Gel electrophoresis of nucleic acid preparation of three strains of *Giardia* showing the presence of the ds RNA 167

Chapter 1

Introduction

1.1 Historical Background

1.1.1 Discovery of the parasite

.....wherein I have sometimes also seen animalcules a-moving very prettily; some of 'em a bit bigger, others a bit less, than a blood-globule, but all of one and the same make. Their bodies were somewhat longer than broad, and their belly, which was flatlike, furnisht with sundry little paws, wherewith they made such a stir in the clear medium and among the globules, that you might e'en fancy you saw a pissabed running up against a wall; and albeit they made a quick motion with their paws, yet for all that they made but slow progress.

Anton van Leevenhoek (cited in Dobell 1920)

These words written by van Leevenhoek in 1681 are generally attributed to be the first reported observation of the species of *Giardia* found in man (Dobell 1920), although he made no drawings of the organism he saw in his own stools. Van Leevenhoek postulated it was the cause of his diarrhea and abdominal cramps. For almost 300 years this correct assumption was overlooked and *Giardia lamblia* was generally thought to be a non-pathogenic commensal in the human intestine.

1.1.2 Nomenclature

During the 19th century different groups were mostly preoccupied with taxonomic aspects of this organism. There was no agreement on nomenclature, a state existing even today. One hundred and seventy-eight years after van Leevenhoek's letter, Lambl rediscovered the human organism in 1859 and called it *Cercomonas intestinalis*, but both generic and species names were homonyms (reviewed in Filice 1952). Ignoring this fact, most Europeans still call the human species *Giardia intestinalis* (Levine 1979). In 1875 Davaine found the form in the rabbit, calling it *Hexamita duodenalis*. *Hexamita* was not accepted (Filice 1952) but the species name *duodenalis* was retained for the form in the rabbit (Hegner 1922). Shortly after, in 1879, Grassi discovered the mouse parasite, first calling it *Dimorphus muris* then in 1888 changing it to *Megastoma entericum*, the name he gave to the human form in 1881. However, as *Megastoma* had already been given to a tunicate in 1814, the new species name he introduced for the human parasite was not universally adopted (Corradetti 1935; reviewed in Filice 1952).

The genus *Giardia* itself was named in 1882 by Joseph Kunstler who discovered the tadpole protozoan which he called *Giardia agilis*. Blanchard introduced the generic name *Lamblia* in 1888 in honor of Lambl, accredited by some to be the discoverer of the protozoan. The frog species was called *Giardia agilis* and the human one *Lamblia intestinalis* (reviewed in Levine 1979), the name used by Eastern Europeans today.

In 1915, after much confusion about species names, particularly *intestinalis* and *enterica*, Stiles proposed that the name *Giardia lamblia* be used for the form occurring in man if the various forms in certain mammals are separate species; at the same time he recommended that if they are identical to that in man, then *duodenalis* would be the correct species name (Kofoid & Christiansen 1915). At that time it was believed these forms were separate so Kofoid accepted Stiles' suggestion and offered *G. lamblia* as the name for that in man, a term favored by Americans today.

1.1.3 Classification

In 1924 Nieschulz recognized three groups of species based on the shape of median bodies, a classification later adopted by Filice (1952). Filice felt it was impossible to distinguish on a species level between forms in man, in rabbit and certain other animals and that *duodenalis* rather than *lamblia* be the correct trivial name, as suggested by Stiles (Kofoid & Christiansen 1915), for the third species in the genus. He suggested that instead of sub-generic rank, the type species be recognized as three distinct species (Filice 1952) with all species having the same median body morphology as suggested by Nieschulz: *G. agilis* (with long teardrop-shaped median bodies) from amphibia; the *G. muris* group (with double, rounded, separate small median bodies) and the *G. duodenalis* group (with median bodies resembling the claw of a claw-hammer). New species would be limited to forms that could be distinguished from existing species by morphological differences and all other forms would be races of these species.

According to this classification, the species infecting man would in fact be of the race *lamblia* and the form from the laboratory rat of the race *simoni* (Lavier 1924, cited by Filice 1952) of the third species, *G. duodenalis*. However, the number of species in the genus has not yet been established; two extreme positions are held: those who favor Filice and those who assign new species for *Giardia* from each host.

In this thesis the human species will be referred to as *Giardia lamblia*, following the suggestion of Stiles (Kofoid & Christiansen 1915), not because I believe the available evidence separates clearly the forms as species (as discussed above) but rather for clarity. The rat isolates will be referred to as either rat or mouse *Giardia duodenalis* according to the host of origin and *G. muris* will describe the mouse *Giardia* (Filice 1952).

1.2 Characteristics of the Parasite

1.2.1 Life cycle

The life-cycle is relatively simple involving a trophozoite and a cyst stage. Infection normally follows the ingestion of cysts although, experimentally, cultured trophozoites have been used to orally infect mice (Hill *et al.* 1983), dogs (Hewlett *et al.* 1982), gerbils (Belosevic *et al.* 1983), rabbits (Schleinitz *et al.* 1983) and even humans (Nash *et al.* 1987b), suggesting that trophozoites are less fragile than previously thought. After passage through the acid environment of the stomach, excystment factors there (Bingham & Meyer 1979; Schaefer III *et al.* 1984) promote excystation of ingested cysts in the small intestine. Immediately after excystment, the organism divides into two daughter cells (Buchel *et al.* 1987; Coggins & Schaefer III 1984, 1986; Rice & Schaefer III 1981) and the cells then continue to multiply by binary fission. Although most authors have reported that *Giardia* trophozoites are found in the small intestine, the exact position wherein they live most abundantly varies. The life cycle of the parasite is completed by encystment but the factors inducing this change are less well understood. The cysts are excreted in the faeces to complete the cycle.

1.2.2 Structure and morphology

1.2.2.1 Trophozoite

Early descriptions of the morphology of the trophozoite and cyst were given in the mid-nineteenth century by Lambl, Cunningham and Grassi (reviewed in Erlandsen & Feely 1984; Feely *et al.* 1984). When viewed with the light microscope, the trophozoite resembles a pear cut in half lengthwise with the broad end anterior and the narrow end caudal (Feely *et al.* 1984). There are four pairs of flagella arranged in bilateral symmetry: two emerge anterolaterally, two posterolaterally, two ventrally and two caudally. There is a median body unique to this genus. Its function is unknown although there is speculation it could serve as a depot of cytoskeletal

proteins for mobilization prior to mitosis or encystment (Feely *et al.* 1984). On the ventral surface there is a large sucking disc, termed adhesive disc by Friend (1966), made up by a complex cytoskeleton of concentrically coiled microtubules, microfilaments and associated fibrous structures (Friend 1966; Holberton 1973; Holberton & Ward 1981). The disc cytoskeleton contains tubulin and giardin (Crossley & Holberton 1983, 1985), the latter being a rod-shaped filamentous protein and present only in the ventral disc. A ventrolateral flange lies immediately below the exit points of the flagellae. Contractile proteins localized in the periphery of the ventral disc (Feely *et al.* 1982) would suggest their participation in attachment to the substratum (Crossley & Holberton 1983; Erlandsen *et al.* 1978; Feely *et al.* 1984). The dorsal side of the trophozoite is convex whereas the ventral surface is somewhat concave. The organism is binucleate; and although the nuclei are similar in morphology, it is not yet known whether they are identical functionally. Recent evidence suggests that the organism is diploid (Andrews *et al.* 1988a; Meloni & Thompson 1987).

Electron microscopic profiles of plasma membranes from trophozoites (Clark & Holberton 1986) have shown that they are asymmetric, with a thin fuzzy filamentous coat on the external surface. Although *Giardia* is a typical eucaryote, it lacks mitochondria (Friend 1966). In both trophozoites and cysts, there are numerous membrane-bound ovoid vacuoles (100-400 nm wide) distributed peripherally adjacent to the plasmalemma. In trophozoites, they are found along both the dorsal and ventral surfaces except in the region of the ventral disc (Feely *et al.* 1984; Friend 1966). Their function has not been resolved and has been the subject of several studies (Bockman & Winborn 1968; Feely & Dyer 1987; Friend 1966; Lindmark 1988). The presence of acid phosphatase and other lysosomal enzymes in these organelles suggest they are lysosomal in nature (Feely & Dyer 1987; Lindmark 1980, 1988). Chitin synthetase in these vacuoles would confirm their role in cyst wall formation (Friend 1966) but this enzyme has yet to be identified in these organelles. They could also have a digestive function as they have been seen to take up ferritin (Bockman & Winborn 1968).

1.2.2.2 Cysts

Detailed information on light microscopic structure of the cyst is given by Filice (1952). Ultrastructural studies show that the cysts are elliptically shaped, ranging in size from 6–10 μm and enclosed within a cyst wall made of chitin (Ward *et al.* 1985). Chitin is not present on the surface of the trophozoite (Ward *et al.* 1987). The cyst wall is generally tightly applied to the surface of the organism (Feely *et al.* 1984), although gaps of variable size appearing between the cell membrane of the trophozoite and the cyst wall have been reported (Nemanic *et al.* 1979; Sheffield & Bjorvatn 1977). The cytoplasm of the cyst contains either two or four nuclei (Boeck 1919; Feely 1988; Filice 1952), the latter called *copulation cysts* by Boeck (1919). It also contains basal bodies and axonemes of flagella, axostyles associated with the caudal flagella, median bodies and fragmented portions of the ventral disc. Two ventral discs are clearly observed in cysts containing four nuclei (Feely 1988) apparently due to the presence of two differentiated trophozoites enclosed within the cell wall.

In the periphery of the cyst cytoplasm an extensive network of vacuoles and tubules exists just beneath the cell membrane. The vacuoles resemble those seen in the dorsal cytoplasm of the trophozoite (Friend 1966); and, as mentioned above, their exact function has not yet been defined although there is increasing evidence they are lysosomal in nature (Feely & Dyer 1987; Lindmark 1988). A role in excystation has been suggested (Lindmark 1988) as it has been observed that during excystation *in vitro* these vacuoles coalesce at the point of trophozoite emergence (Coggins & Schaefer III 1986; Feely *et al.* 1984) and may cause the actual rupture of cyst wall.

1.2.2.3 Symbionts

Symbionts of *Giardia* appear to be more common than is generally appreciated (Feely *et al.* 1988). Bacterial endosymbionts have been reported in the cytoplasm of both trophozoites (Boeck 1919; Nemanic *et al.* 1979; Radulescu *et al.* 1982; Wenrich 1940) and cysts (Nemanic *et al.* 1979) of *G. muris*, in the trophozoites of

G. microti (Feely *et al.* 1984; 1988) and in trophozoites (Nemanic *et al.* 1979) and cysts (Radulescu *et al.* 1982) of *G. lamblia*. Ectosymbiotic bacteria attached to both dorsal and ventral surfaces of *G. muris* trophozoites (Feely *et al.* 1984) and mycoplasma-like organisms on surfaces of trophozoites from prairie vole, laboratory rat and beaver (Feely *et al.* 1988) have also been described.

Virus-like particles have been seen in the cytoplasm of trophozoites of *G. muris* from mice (Brug 1942 cited in Feely *et al.* 1984, 1988), rats (Feely *et al.* 1988) and hamsters (Sogayar & Gregorio 1986) and in *G. duodenalis* from rats (Sogayar & Gregorio 1986). A double-stranded (ds) RNA virus has also been detected in the nucleus of human *Giardia* (Wang & Wang 1986) and has recently been confirmed in trophozoites of other human isolates and in *Giardia* from cat, guinea pig, sheep and beaver (De Jonckheere & Gordts 1987; Miller *et al.* 1988a, 1988b).

As yet no correlation has been demonstrated between the virulence potential of a *G. lamblia* strain and the presence or absence of bacteria or virus. The ultrastructure of trophozoites and cysts show no signs of abnormality (Feely *et al.* 1988). There is speculation the endosymbionts may possibly play a role in (a) host specificity or range of infectivity (Feely *et al.* 1988; Nemanic *et al.* 1979), (b) pathogenic effects of *Giardia* (Miller *et al.* 1988a, 1988b; Nemanic *et al.* 1979) or (c) the expression of different antigenic phenotypes as occurs in *Trichomonas vaginalis* where the presence of a double-stranded RNA virus is associated with the expression of certain surface antigens (Wang *et al.* 1987).

1.2.3 Cultivation of *Giardia in vitro*

Although *Giardia* was the first intestinal protozoan to be recognized (Dobell 1920), methods for long-term cultivation did not become available until the 1960s (reviewed in Meyer & Radulescu 1984). The early *in vitro* systems were either xenic or monoxenic cultures. Credit for the first description of a reproducible method of *Giardia* culture goes to Karapetyan (1960; 1962), who was successful in growing both human and rabbit *Giardia* up to 5 months but only in the presence of yeast (monoxenically). Following Karapetyan's report (1962), various other workers were

successful in establishing monoxenic cultures of *Giardia* (cited in Meyer & Radulescu 1984).

Axenization of *Giardia* from the rabbit and chinchilla (Meyer 1970) was rapidly followed by the axenization of *Giardia* from humans (Meyer 1976) and guinea pigs (Fortess & Meyer 1976). Inconsistent growth in the media employed led Visvesvara (1980) to adapt *Giardia* trophozoites to grow in a modified TPS-1 medium originally developed for *E. histolytica* (Diamond 1968). Others were also successful in establishing cultures of human *Giardia* in this medium (Bhatia & Warhurst 1981; Gillin & Diamond 1980; Gordts *et al.* 1985; Kasprzak & Majewska 1983, 1985), including clonal growth (Gillin & Diamond 1980), although constituents of TPS-1 varied from batch to batch in their ability to support growth of *G. lamblia* (Farthing *et al.* 1983; Kasprzak & Majewska 1985). This in turn led to *Giardia* being adapted to grow in TYI-S33 medium (Gillin & Diamond 1981a), a second medium developed for the axenic cultivation of *E. histolytica* (Diamond *et al.* 1978). Human *Giardia* has been cultured from duodenal aspirates (Gordts *et al.* 1985; Meyer 1976). Two groups independently showed that bile-supplemented TYI-S33 medium (Farthing *et al.* 1983; Keister 1983) dramatically promoted growth with marked reduction in generation time (Farthing *et al.* 1983; Keister 1983). Comparative growth studies have shown that there are differences in growth of particular strains and that the growth depends on the medium used (Gillin & Diamond 1981a; Kasprzak & Majewska 1985). Like tissue culture cells, *Giardia* trophozoites are amenable to cryopreservation (Lyman & Marchin 1984; Meyer & Chadd 1967; Phillips *et al.* 1984).

G. muris has never been established in culture. Very few reports have described attempts at culturing this parasite (Meyer & Radulescu 1984; Schaefer III *et al.* 1984; Wallis & Wallis 1986). Culture of rodent representatives of *G. duodenalis* has not been reported either, although *G. duodenalis* from other animals has been cultivated successfully either from trophozoites or from cysts after *in vitro* excystation (Bingham & Meyer 1979; Gasser *et al.* 1987a, 1987b; Kasprzak & Majewska 1983; Wallis & Wallis 1986; Wenman *et al.* 1986). Mongolian gerbils (Wallis

& Wallis 1986) and baby mice (Mayrhofer, unpublished) are also used as animal models to excyst and host *Giardia* spp. isolated from humans and other mammals.

1.3 Clinical features of giardiasis

The clinical features of giardiasis vary from asymptomatic carriers to symptomatic patients (reviewed in Wolfe 1984). In the normal human, *Giardia* infection usually has a limited duration of four to six weeks and few or no clinical symptoms (Brodsky *et al.* 1974; Jokipii & Jokipii 1977; Rendtorff 1954). When symptoms occur, they are commonly an increased frequency of bowel action, abdominal distention and discomfort and occasionally lassitude. A proportion of symptomatic patients have severe diarrhea and pass bulky soft, yellow offensive stools. They experience weight loss, abdominal distension and discomfort and may have chronic diarrhea with intestinal malabsorption manifested by impaired absorption of d-xylose, fat and vitamin B^{12} and steatorrhea (Wright *et al.* 1977b). The factors involved in the pathogenesis of the chronic infections and malabsorption that occur in some patients are not understood, although recent evidence suggest that differences between isolates account for some features of individual infections (Nash *et al.* 1987b). Patients with immune deficiencies are likely to have malabsorption and abdominal symptoms (Ament *et al.* 1973) because such patients experience difficulties in clearing the organisms. Occasionally, extra-intestinal manifestations have been reported in association with *Giardia* infections (Roberts-Thomson *et al.* 1982; Smith 1985).

1.4 Pathology and pathogenesis of giardiasis

1.4.1 Colonization and attachment

Colonization of the small intestine is facilitated by trophozoite attachment to the microvillus surface on mucosal epithelial cells. In the murine model of giardiasis, trophozoites colonize the proximal 25% of the small intestine (Owen *et al.* 1979) with the largest number of organisms present in the region of the mid jejunum

(Gillon *et al.* 1982a). In the latter study relatively few organisms were found in the duodenum, distal jejunum and ileum. There is practically no information on the distribution of the parasite along the small intestine in man.

In order for the trophozoites to cause the intestinal dysfunction observed in giardiasis, it has been postulated that there is a requirement for attachment of the trophozoite to the microvillous border (MVB) of the intestinal epithelial cells (reviewed in Erlandsen & Feely 1984). The first direct evidence of interaction of the trophozoite with the MVB was in fact reported in studies of rat giardiasis (Erlandsen 1974). By scanning electron microscopy (SEM) studies, large numbers of trophozoites were seen adhering to the intestinal villi and physical impression of the MVB of the epithelial cells were shown to be mirror images of the surface of the ventral disc of the trophozoite. Similar lesions were demonstrated in the mouse model (Owen *et al.* 1979) and in human giardiasis (Erlandsen & Feely 1984; Poley & Rosenfield 1982). Large numbers of trophozoites have been seen lodged in mucus within the unstirred layer (Balasz & Szatloczky 1978; Das *et al.* 1988; Owen *et al.* 1979; Poley & Rosenfield 1982; Rodrigues Da Silva *et al.* 1964).

Several different theories have been put forward to explain the mechanism of attachment. The most popular are (a) the production of a reduced pressure beneath the ventral disc by the action of ventral flagella (Holberton 1973, 1974); (b) the involvement of contractile proteins (identified in trophozoites by Feely *et al.* 1982); and (c) the recognition and adherence mediated by specific host and parasite surface membrane determinants (Farthing *et al.* 1986b). This matter remains unresolved.

Attachment of the parasite to the substratum by the ventral suction disc does not account for the selective colonization of the proximal small intestine or the preferential attachment to certain cell types. In the murine model of giardiasis, the trophozoites attach preferentially to microvilli of the less differentiated enterocytes at the bases of villi, rather than to the mature cells at the villus tip (Owen *et al.* 1979). This suggests that the trophozoites might attach, detach and re-attach via specific cell-cell interaction mediated by complementary molecules. However, the

observed distribution might also be accounted for by mechanical factors related to mucus flow and passage of digesta, as well as to other microenvironmental factors influencing growth and differentiation. It is possible that trophozoites adhere to mucus by a receptor-mediated interaction.

A plasma membrane-bound surface lectin, Taglin (to denote trypsin-activated *Giardia* lectin) has been described in human and murine trophozoites (Ward *et al.* 1987). This may be involved in adherence of the parasite to surface of enterocytes or to mucus *in vivo* (Lev *et al.* 1986a). The lectin is most avid for mannose-6-phosphate, is activated by trypsin, and is active within a very narrow pH range, the optimum being 6.5 which is the average pH of the small intestine where *Giardia* thrives (Farthing *et al.* 1986b; Lev *et al.* 1986a, 1986b; Ward *et al.* 1987). There is paucity of evidence supporting the role of lectin-mediated attachment to either the epithelial cells or to mucus.

1.4.2 Penetration of the epithelium

There is no general agreement as to whether trophozoites penetrate the epithelium. The organism is generally believed to be noninvasive. Most biopsy specimens do not demonstrate invasive organisms and the actual frequency of invasion is unknown since most patients do not undergo intestinal biopsy. Histological studies, however, have demonstrated the presence of trophozoites within the mucosa in humans (Brandborg *et al.* 1967; Morecki & Parker 1967; Saha & Ghosh 1977). In the murine model Lupasco *et al.* (1970) found the organism in the mucosa only when overwhelming infection was present whereas Owen *et al.* (1979) only found invasion in areas of necrosis or where mechanical trauma had occurred. *In vitro* studies have yielded conflicting results (Chavez *et al.* 1986; Meyer & Radulescu 1979; Radulescu *et al.* 1980) possibly due to the use of different *Giardia* strains and different target cells. Nevertheless, the parasite or antigenic fragments must gain access to tissues under normal conditions since it is well known that systemic antibody response to the parasite occurs in patients (Ridley & Ridley 1976; Wright *et al.* 1977a) and in experimental animals (Anders *et al.* 1982; Erlich *et al.* 1983). Whether this

is limited to the Peyer's patches (PP) (Owen *et al.* 1979) and how frequently the organism enters by this route is also unknown.

1.4.3 Mechanisms of diarrhea and malabsorption

None of the hypotheses put forward to explain the pathogenicity of *Giardia* has proved satisfactory (Smith 1985). It has been shown that trophozoites do not produce an enterotoxin similar to the cholera-toxin or the *Escherichia coli* classes of toxin (Smith *et al.* 1982c). Likewise, a mechanical barrier to absorption created by a dense blanket of *Giardia* covering the mucosa is an unlikely explanation for either the diarrhea or the malabsorption associated with giardiasis. As discussed above trophozoites preferentially attach at the bases of villi and less frequently at the distal villus (Owen *et al.* 1979), leaving the remainder of the intestine available for normal absorption. Bacterial overgrowth linked to infection with *G. lamblia* has been reported but the use of antibiotics does not lead to improvement in symptoms (Wright *et al.* 1977b). Bile salt deconjugation by the parasite leading to steatorrhea has also been disproved. *G. lamblia* does not have the capacity to deconjugate bile acids *in vitro* (Smith *et al.* 1981b).

Other hypotheses put forward to explain some aspects of diarrhea and malabsorption warrant further investigation. Elaboration of prostaglandins and leukotrienes (eicosanoid products of arachidonic metabolism) in the small intestine by trophozoites could contribute to the pathogenesis of the disease as these products affect intestinal motility and secretion (Blair & Weller 1987). *Giardia* trophozoites can take up arachidonic acid, a polyunsaturated fatty acid derived from ingested food in the small intestine. The presence of hydrolytic enzymes in lysosomal-like vacuoles on the periphery of trophozoites could lead to brush border injury when trophozoites lie adjacent to the MVB (Feely & Dyer 1987).

1.5 Diagnosis of giardiasis

Although *G. lamblia* is the most common intestinal parasite in the developed world, also afflicting millions of people in developing countries, it is sometimes difficult to diagnose (Danciger & Lopez 1975; Jokipii & Jokipii 1977; Pickering 1985). Clinically, the majority of patients are asymptomatic (Gill & Jones 1985; Walia *et al.* 1986) and symptomatic patients do not always present with a typical clinical picture of giardiasis easily recognizable by the general physician.

1.5.1 Stool specimens

Diagnosis is primarily made by direct microscopic examination of stool or stool concentrates for the presence of cysts or trophozoites. For definitive diagnosis, at least 3 samples should be submitted to the laboratory, as a single negative stool specimen cannot rule out infection because cyst excretion is frequently intermittent in human infections (Danciger & Lopez 1975).

Wet mounts of faecal specimens may be stained using Lugol's iodine or fixed smears can be stained with Trichrome stain (Thornton *et al.* 1983). Stool concentrates are made either by formol-ether concentration or by zinc-sulfate flotation. Recently, coproimmunodiagnosis for *Giardia* faecal antigen has been described, using counterimmunoelectrophoresis (CIE) (Craft & Nelson 1982; Rosoff & Stibbs 1986; Vinayak *et al.* 1985) and enzyme-linked immunosorbent assay (ELISA) (Green *et al.* 1985; Nash *et al.* 1987a; Ungar *et al.* 1984), but these methods are not in general use.

1.5.2 Intestinal fluid examination

When parasites cannot be found in suspected cases following multiple stool examinations, examination of duodenal or upper jejunal fluid may prove valuable. There are mixed reports on the usefulness of intestinal fluid examination, with some investigators reporting it to be more reliable than stool examinations in making the diagnosis (Kamath & Murugasu 1974; Rosenthal & Liebman 1980) while others

have shown that stools can be positive when duodenal fluid and biopsies are negative (Naik *et al.* 1978; Nash *et al.* 1987b).

The *string test* can permit definitive diagnosis of giardiasis in the physician's office: microscopic examination of sample of duodenal fluid obtained on string may reveal trophozoites. Use of this simple test may eliminate some of the difficulties in diagnosing giardiasis (Jones 1986; Rosenthal 1985).

1.5.3 Serodiagnosis of giardiasis

At present, no acceptable serological test exists for the diagnosis of giardiasis. Indirect immunofluorescence (IF) (Ridley & Ridley 1976; Visvesvara *et al.* 1980; Winiecka *et al.* 1984; Wittner *et al.* 1983) and ELISA (Haralabidis 1984; Smith *et al.* 1981a; Wittner *et al.* 1983) using axenically grown trophozoites as antigen have been used for the detection of antibodies in serum. These studies have had discrepant results ranging from perfect distinction to complete overlap of titres in patients and controls. Therefore, no method has reproducibly been capable of diagnosing giardiasis as compared to the clinically more relevant distinction. Recently, Goka *et al.* (1986) detected serum IgM and IgG antibodies by ELISA in patients with diarrhea in the U.K. and South India. This study suggests that current infections can be distinguished from previous infections by the presence of IgM antibodies in the former. Jokipii *et al.* (1988) were also unable to serologically diagnose the disease by looking for cyst-specific antibodies in patients with primary giardiasis. The titres in individual patients were not distinguishable from those in healthy controls who presumably had never been infected with *Giardia*.

1.6 Epidemiology

1.6.1 General

The epidemiology of *Giardia* is diverse. The disease is endemic throughout the world, being common not only in the tropics (Antia *et al.* 1966; Boreham *et al.*

1981; Chavalittamrong *et al.* 1978; Islam *et al.* 1983) but also in temperate zones (Brodsky *et al.* 1974; Farthing *et al.* 1986a; Harter *et al.* 1982; Meyer & Jarroll 1980). Prevalence rates throughout any country are usually determined for a select group and may not be representative of the country. Nevertheless, in any group looked at, most studies have shown higher rates of infection in the young than in adults.

In the developing countries, rates in the range of 8-20% are common in South America, the Caribbean, Africa, the Middle East and South-East Asia (Farthing *et al.* 1986a; Mata 1978; W.H.O. 1986). In areas where giardiasis seems to be endemic it is quite common to detect infection in the native population with no evidence of clinical involvement (Abdel-Hafez *et al.* 1986; Mason & Patterson 1987; Walia *et al.* 1986), whereas newcomers are most frequently infected with a high percentage becoming symptomatic (Speelman & Ljungstrom 1986). Giardiasis can be acquired at a very young age (Farthing *et al.* 1986a; Islam *et al.* 1983; Walia *et al.* 1986) with the overall prevalence being highest in children under 10 years of age (Gilman *et al.* 1985).

In the more developed countries of North America and Europe, prevalence rates are slightly lower but follow the same pattern, in that the highest rates occur in children under the age of ten years (Meyer & Jarroll 1980; Neringer *et al.* 1987; W.H.O. 1980). In Australia, trophozoites have been identified in duodenal aspirates in 2% of adults, although the actual group(s) studied was not specified (Roberts-Thomson 1985). In Australian Aboriginal children under the age of 6 years in South-west Australia, *G. lamblia* was recorded in 26% of asymptomatic children above 4 months of age (Gill & Jones 1985). 18% of a group of hospitalized Aboriginal children with diarrhea in Perth were found to excrete *Giardia* cysts, while 24% of a control group of children without diarrhea also excreted cysts (Gracey *et al.* 1983).

In South Australia 822 cases were notified to South Australian Health Commission in 1986 (South Australian Health Commission 1986). The annual numbers are increasing and this could be due either to greater awareness of the disease among general practitioners, increased requests for laboratory diagnosis or an in-

creased testing of contacts. Alternatively, the incidence in the community may be increasing due to changes in the community affecting the exposure especially of toddlers to infection. It is notified at a relatively uniform rate throughout the year in South Australia.

Giardia in children in day care centers can be transmitted directly (by person-to-person spread) or indirectly (by fomites or through ingestion of contaminated food or drink). Since 1974, the incidence of *Giardia* has increased dramatically in day-care centers (Black *et al.* 1977; Boreham & Shepherd 1984; Keystone *et al.* 1978; Pickering *et al.* 1984; Woo & Paterson 1986). Children of all ages are affected, but the attack rate has been highest for children less than three years of age. Lack of infection control methods in understaffed situations contributes to the spread of giardiasis (Pickering *et al.* 1986). These should be enforced continuously in order to reduce transmission of the parasite.

1.6.2 Transmission of *Giardia*

1.6.2.1 Waterborne transmission

Reports of outbreaks of giardiasis among American and other travellers to Leningrad (Craun 1984) and to Portugal (cited in Craun 1984) have confirmed the relationship between epidemic giardiasis in tourists and the consumption of tap water (Jokipii & Jokipii 1974). One of the first recognized waterborne outbreaks of giardiasis in the United States occurred in Aspen, Colorado, in December 1965 - January 1966 (Craun 1984). The source was traced to sewage contamination of wells. Two outbreaks of water-borne giardiasis have been reported from Western Europe recently: Bristol, U.K. (Jephcott *et al.* 1986) and Mjovik, Sweden (Neringer *et al.* 1987). The municipal water supply was implicated in both instances. Boreham & Phillips (1986) have found no evidence that giardiasis is a waterborne infection in Australia.

1.6.2.2 Foodborne transmission

Reports of foodborne giardiasis outbreaks are rare (Barnard & Jackson 1984; Osterholm *et al.* 1981; Petersen *et al.* 1988). Circumstantial evidence that foodborne giardiasis occurred was provided when a Christmas pudding, contaminated by rodent faeces, was found to contain *Giardia* cysts. A family of four ate the pudding and three of them developed mild diarrhea (cited in Barnard & Jackson 1984) implying at the same time that rodent *Giardia* can be transmitted to humans. In the other two reported outbreaks, food was presumably contaminated during preparation. The vehicle of transmission in one case was home-canned salmon (Osterholm *et al.* 1981) while in the other it was a noodle salad (Petersen *et al.* 1988); both prepared by symptomatic individuals, the latter actively excreting cysts.

1.6.2.3 Sexual transmission

Cases of intestinal protozoan infections in homosexual men have been reported since 1968 (Kean *et al.* 1979; Meyers *et al.* 1977; Most 1968). Venereal transmission is on the increase and is not limited to the homosexual population (reviewed in Owen 1984). The occurrence of subclinical or asymptomatic giardiasis is an important factor in the sexual spreading of this disease. A high percentage of asymptomatic homosexual carriers has been reported in North America (Keystone *et al.* 1980), Sweden (Hakansson *et al.* 1984) and Finland (Ranki *et al.* 1985).

1.6.3 Is Giardiasis a Zoonosis?

Giardia has been detected in a number of domestic animals: calves (Deshpande & Shastri 1981; Gasser *et al.* 1987a); horses (Mayhew & Greiner 1986); llama and lambs (Kiorpes *et al.* 1987); cats and dogs (Collins *et al.* 1987; Kirkpatrick & Farrell 1984; Swan & Thompson 1986) as well as other animals such as beavers and voles (Wallis *et al.* 1984, 1986).

Beavers have been implicated in the United States as a source of contamination of water supplies (Davies & Hibler 1979; Dykes *et al.* 1980; Navin *et al.*

1985) based on the fact that cysts isolated from the beavers appeared morphologically similar to cysts excreted by humans. It is quite conceivable that the mountain streams or creeks are in fact contaminated by human *Giardia* cysts from hitchhikers and campers (Barbour *et al.* 1976) and these are later ingested by other humans who come along. Cysts are hardy and are known to survive for long periods of time particularly in cooler waters (Fontaine *et al.* 1984; Wallis *et al.* 1986).

However, no experimental studies documenting animal-to-human *Giardia* transmission have been published. The question of whether mice may serve as reservoirs of infection for human beings is not new. In 1892, an attempt was actually made to infect man with mouse *Giardia* by Moritz & Holzl (mentioned in Simon 1922). Cysts from mice were fed to a tuberculous patient but the attempt was unsuccessful. One of the few documented cases of cross-transmission is that between primates and handlers in an American zoo (cited in Woo 1984).

Evidence supporting cross infection with at least some strains of *G. duodenalis* between humans and animals is accumulating. Cross-transmission studies have confirmed that the human strain of *Giardia* will produce infection in animals (Davies & Hibler 1979; Hewlett *et al.* 1982; Kirkpatrick & Green 1985; Woo & Paterson 1986). Thus, a wide range of mammalian species are susceptible to the human *Giardia*.

Wenman *et al.* (1986) isolated and cultured *G. duodenalis* trophozoites from several animals (domestic and wild) in Alberta, Canada, and compared them with a human isolate by immunoblotting analysis in an attempt to determine the role of mammals in human infections. All strains shared similar protein profiles and important protein antigens. This conservation across isolates from several host mammal species suggested that *Giardia* strains are not unique to each host species and that there could be a wide range of potential reservoirs *of G. duodenalis* infection (Wenman *et al.* 1986). More recently, isoenzyme and DNA analysis of isolates of *Giardia* from humans and lower animals have shown a remarkable degree of genetic heterogeneity as well as important similarities among some isolates (Thompson *et al.* 1988). With respect to the zoonotic potential, it was observed

that all feline isolates were genetically identical or very similar to each other and to many human isolates, indicating that cats are possible reservoirs of infection for humans. In contrast, isolates from a dog and a rat were distinct genetically from all human isolates, suggesting that dogs may be a less likely source of infection for humans. However, Boreham & Phillips (1986) were unable to find any correlation between infection and the possession of household pets in an epidemiological study conducted in Mt. Isa, Queensland, Australia.

1.7 Treatment

No specific anti-giardial chemotherapeutic agent has been manufactured. The drugs currently in use are synthetic anti-microbial agents originally manufactured for other organisms. The oldest available anti-*Giardia* drug is Quinacrine (Atabrine), an anti-malarial agent, known to be highly effective and the drug of choice in the U.S.A. (Craft *et al.* 1981; reviewed in Lerman & Walker 1982) but not generally available in Australia. Several 5-nitroimidazole derivatives, originally intended for the treatment of trichomoniasis, have come into clinical use in the treatment of giardiasis: metronidazole (Flagyl) (Jokipii & Jokipii 1978; Mendelson 1980; Wright *et al.* 1977b); tinidazole (Fasigyn) (Jokipii & Jokipii 1978); and ornidazole (Tiberal) (Wolfe 1984).

Metronidazole is the drug of choice in several countries except in the U.S.A where its use has been discouraged because of early reports that it caused lung tumors in animals and is mutagenic to some bacteria (Wolfe 1984). Tinidazole is effective in single dosage, in contrast to metronidazole, with infrequent side effects (Bassily *et al.* 1987; Mendelson 1980) and *in vitro* was found to be more active than metronidazole against *G. lamblia* (Jokipii & Jokipii 1980). A nitrofuran derivative, Furazolidone, is a bactericidal antimicrobial agent with broad-spectrum activity used for the treatment of gastrointestinal infections including enteric fever and other salmonelloses and shigelloses. It is commonly used in the treatment of giardiasis, especially in children, in several countries including Australia (Boreham *et al.* 1988;

Craft *et al.* 1981; Mendelson 1980; Wolfe 1984).

Recently, a possible therapeutic role for intravenous administration of human gammaglobulin with anti-*Giardia* activity for patients with giardiasis refractory to conventional drug therapy has been suggested (Arvind *et al.* 1988). Clinical observations have indicated that the prevalence of giardiasis in patients with agammaglobulinemia has decreased since the implementation of replacement therapy with intravenous pooled human gammaglobulin preparations. Preliminary observations have suggested that the the presence of anti-*Giardia* IgG antibodies in tested preparations of pooled gammaglobulins may explain the decreased prevalence of giardiasis in treated agammaglobulinemic patients.

The treatment of asymptomatic cyst excretors is controversial. Because of the potential for development of intermittent chronic symptoms in carriers and since infected children, foodhandlers or male homosexuals may be sources of infection in the community some physicians make a strong case for treating all infected persons without contraindications such as pregnancy (Wolfe 1984). However, treatment of all symptomless *G. lamblia* infections in a hyperendemic area is of questionable value because of rapid reinfection (Gilman *et al.* 1988). In such hyperendemic areas treatment is not an effective public health measure particularly when resources are limited.

Recent studies indicate that drug resistant isolates of *Giardia* may be responsible for the failure rate in clearing the infection (McIntyre *et al.* 1986). Organisms isolated from patients who responded poorly to therapy were found to be least sensitive to furazolidone *in vitro*. Considerable differences occur *in vitro* in sensitivity to metronidazole (Boreham *et al.* 1988; McIntyre *et al.* 1986).

1.8 Antigens of *Giardia*

Despite the relatively simple life cycle of *Giardia* there are a number of considerations that create problems in an immunological approach to the study of antigens of *Giardia*. As already mentioned, the vast majority of people who harbor *Gia-*

rdia display no clinical symptoms, with the parasite apparently living harmlessly inside the host. The minority who exhibit symptoms usually fall into one of two categories: those who experience an acute, self-limiting infection (Brodsky *et al.* 1974; Rendtorff 1954) or those who become chronically infected (Heap 1974). The question therefore arises as to whether all strains of *G. lamblia* have the potential to cause disease or whether distinct substrains exist. Some might not cause disease under any conditions (Aggarwal *et al.* 1983; Nash *et al.* 1987b). In the former case, the problem becomes that of identifying either the stimuli that result in activation of pathogenicity, or alternatively, the host factors that predispose individuals to either form of disease. In the latter situation it becomes important to identify and to distinguish between the virulent and avirulent varieties of the organism.

Antigenic analysis of *Giardia* is in an embryonic stage and, therefore, information about the antigens of *Giardia* is not very extensive. Problems in the study of antigens important to the host-parasite relationship stem, at least in part, from (a) a lack of an axenic *in vitro* culture system in which the complete life cycle of the parasite can be maintained and (b) the probability that during the lengthy process of adaptation to axenic culture, selection pressures may operate to cause quantitative and qualitative changes in the surface antigens. High levels of encystation have not been achieved *in vitro* so far in any of the media employed to culture *Giardia*, although induction of encystation has been reported (Gillin *et al.* 1987), whereas it has been possible to excyst trophozoites *in vitro* (Feely 1986; Rice & Schaefer III 1981). Antigens present in different amounts in virulent and avirulent strains or in those causing acute versus chronic infections are of particular interest. In some studies (Torian *et al.* 1984; Ungar & Nash 1987) part of the immune response was found to be directed against intracellular antigens and these antibodies would be irrelevant for protection as they cannot recognize the living parasite. Considering the rapid rate of turnover of the membrane of the *Giardia* (Farthing *et al.* 1985a; Nash *et al.* 1983; Roberts-Thomson & Anders 1984) and the differentiation process from the trophozoite to cysts, it seems likely that different antigens may be present both in and on the *Giardia* at different stages. This may confuse studies

that commence with the premise that all trophozoites in an *in vivo* population will have similar antigens.

1.8.1 Cyst antigens

Cyst antigens have been used serologically by different groups in diagnosing giardiasis, without actually defining any cyst-specific antigen (Ridley & Ridley 1976; Vinayak *et al.* 1978; Wright *et al.* 1977a). They were based on the detection of undefined cyst antigens, some of which might cross-react with antigens of other intestinal protozoa (Riggs *et al.* 1983) or unknown immunogens (Jokipii *et al.* 1988).

As mentioned in Section 1.5.1, antigen detection methods in coprodiagnosis of giardiasis have been developed including CIE (Craft & Nelson 1982; Vinayak *et al.* 1985) and ELISA (Green *et al.* 1985; Nash *et al.* 1987a). More recently, a *G. lamblia*-specific stool antigen (GSA 65) was isolated from stools of patients (Rosoff & Stibbs 1986). Immunoreactivity with monospecific antiserum revealed the presence of this antigen in cyst wall and on trophozoites. Its role in the encystation process is unknown. It is possible this antigen could well have been among those detected in other studies on coprodiagnosis (Craft & Nelson 1982; Vinayak *et al.* 1985).

1.8.2 Differentiation antigens

Research on differentiation antigens of *Giardia spp.* is practically non-existent. This is hampered by the lack of an *in vitro* model of encystation due to incomplete knowledge of the regulatory mechanisms which control it or the biochemical reactions that are responsible for the synthesis of the cyst cell wall (Gillin *et al.* 1987; Gillin *et al.* 1988; Ward *et al.* 1985). Recent studies of differentiation antigens during encystation of another intestinal protozoan flagellate have identified a stage-specific sialoglycoprotein in encysting cells of *Entameba spp.* (Avron *et al.* 1987; Chayen *et al.* 1985; Chayen *et al.* 1988).

In this context, analysis of the surface carbohydrate moieties of trophozoites

have demonstrated N-acetyl-glucosamine (GluNAc) on surface glycoproteins (Erlich *et al.* 1983; Hill *et al.* 1981; Ward *et al.* 1987) and two distinct subsets of trophozoites have been identified on the basis of wheat germ agglutinin (WGA) binding (Ward *et al.* 1988). It is suggested they may represent either varying stages of differentiation from trophozoite to cyst or, alternatively, cell cycle variations in the expression of surface carbohydrate moieties. Gillin *et al.* (1987) were able to induce a subset of trophozoites to encyst and these exhibited increased levels of chitin synthetase activity as compared with non-encysting cultures. This suggests that chitin synthesis is developmentally regulated. If the encystation process could be induced *in vitro*, evidence could perhaps be provided that the newly excysted trophozoite may be more susceptible to immune attack than the more mature trophozoite.

1.8.3 Trophozoite antigens

Advances in the culture of the parasites *in vitro* (Section 1.2.3) and the application of modern technology (monoclonal antibodies, molecular biological techniques and isozyme electrophoresis) have accelerated research on this organism during the last decade. The antigenic complexity was revealed when Visvesvara demonstrated that antibodies to several trophozoite antigens were produced by rabbits immunized with axenic trophozoites and by hyperimmune human sera (reviewed in Visvesvara & Healy 1984). Antigens from cat and guinea pig (Visvesvara & Healy 1984) isolates and from *G. muris* (Roberts-Thomson 1987) reacted extensively with human sera indicating antigenic cross-reactivity. Indirect IF studies have indicated that the external parasite membrane is an important site for antigens (Visvesvara & Healy 1980; Visvesvara *et al.* 1980).

Current interest is, therefore, being paid to identifying antigens on the parasite membrane that could be accessible to the host immune response (Einfield & Stibbs 1984; Smith *et al.* 1982b; Torian *et al.* 1984; Visvesvara *et al.* 1980). In most cases, the identification of antigens is mainly by biochemical or immunochemical analysis of proteins or glycoproteins on polyacrylamide gels after surface labelling. Assignment of functional roles to these molecules, with respect to the biology of

the parasite and its interaction with the host, has not yet been achieved. Table 1.1 summarizes the *Giardia*-specific proteins that have been identified as quantitatively important surface antigens.

Four major surface proteins have been detected in trophozoites and their apparent molecular weights determined. A 170 kilodalton (kDa) protein has been shown to be present on the surface of WB and WB-like *Giardia* isolates and released into the medium as an exretory-secretory (E-S) antigen (Nash *et al.* 1983; Nash & Aggarwal 1986). A monoclonal antibody (MoAb) to the 170kDa antigen differentiated *Giardia* possessing this antigen from other isolates and was also found to be cytotoxic to the trophozoites expressing the antigen (Nash & Aggarwal 1986). Immunoblot analysis of patients' sera demonstrated that none of the sera reacted with the antigens corresponding to the polydisperse E-S products on the surface of WB nor with 170kDa protein suggesting they may not be major antigens in natural human infection (Taylor & Wenman 1987). These latter two components could be precipitated by antibodies that agglutinate or are adsorbed by fixed trophozoites. More recently this antigen has been shown to be cysteine-rich and undergoes antigenic variation (see Section 1.8.4).

A membrane protein varying in molecular weight (75-88kDa) has been described by several groups (Clark & Holberton 1986; Edson *et al.* 1986; Einfield & Stibbs 1984; Erlich *et al.* 1983; Mayrhofer *et al.*, unpublished data; Nash *et al.* 1983). Sera from *G. muris*-infected BALB/c mice recognise a surface-labelled antigen of 82kDa, but it is immunoprecipitated to a lesser extent by sera from infected mice of the strain C3H/He, which have a defective ability to spontaneously eliminate *Giardia* infections (Erlich *et al.* 1983). Einfeld & Stibbs (1984) used immunoblotting to detect an 82kDa antigen common to four *G. lamblia* strains from different countries. Edson *et al.* (1986) described an 88kDa surface protein which was immunogenic in humans. Mayrhofer *et al.* (unpublished) have detected antibodies against the 82 kDa protein of a *G. lamblia* isolate in the serum of the patient from whom the organism was isolated. This is in contrast to Taylor & Wenman (1987) who demonstrated that in natural human infection none of the sera

they tested reacted with the 82kDa protein . There is conflicting evidence about whether the 82kDa is a glycoprotein (Clark & Holberton 1986; Einfield & Stibbs 1984). Recently, Mayrhofer *et al.* (unpublished data) obtained evidence that the 82kDa antigen undergoes variation (Section 1.8.4).

Proteins ranging in molecular weight 52-58kDa have been described (Clark & Holberton 1986; Einfield & Stibbs 1984; Taylor & Wenman 1987; Torian *et al.* 1984; Wenman *et al.* 1986) which could be tubulin (Torian *et al.* 1984), a principal component of microtubules present in cytoskeletons of all eucaryotic cells and been shown to be a constituent of *Giardia* membranes (Clark & Holberton 1986). MoAbs, specific for the trophozoite flagella and disc, recognized proteins in the 53-55kDa range on the surface of the parasite (Torian *et al.* 1984) and reacted in immunoblots with purified bovine brain tubulin. The identity of this protein was further confirmed when MoAbs to yeast spindle tubulin reacted with this protein (Crossley *et al.* 1986).

Membrane proteins in the 30kDa range have been detected in both *G. muris* and *G. lamblia* (Butscher & Faubert 1988; Crossley *et al.* 1986; Erlich *et al.* 1983; Taylor & Wenman 1987). Immunoprecipitation analyses demonstrated that sera from immunized and challenged BALB/c mice recognize a complex of 4 acidic proteins of approximately 32kDa (Gm32) better than sera from immunized and challenged C3H/He mice. However, solublized trophozoite antigens containing this Gm32 protein complex did not confer protection in BALB/c mice when used as vaccines (Erlich *et al.* 1983). A 31kDa surface protein was detected in the WB strain of *G. lamblia* (Taylor & Wenman 1987). These authors observed that this was the major surface antigen recognized during human infection and concluded it might be a disc component, giardin, described by Crossley & Holberton (1983). It is possible that this is the same protein that has been described by other groups (Einfield & Stibbs 1984; Nash *et al.* 1983; Wenman *et al.* 1986). However, Crossley *et al.* (1986) also report a 30kDa ventral flagellar membrane protein against which anti-giardin sera does not react. Clark & Holberton (1986) described a group of 5 polypeptides ranging in molecular weight 32kDa to 38kDa and suggested that

they were intrinsic membrane proteins and not exposed at the surface in plasma membranes of *G. lamblia*. Cytotoxic MoAbs raised against *G. muris* recognized major glycoproteins of 30kDa and 36kDa (Butscher & Faubert 1988) on the surface of trophozoites of both *G. muris* and WB *G. lamblia*, although the reaction with the latter was weaker indicating either that the epitope shared on *G. lamblia* is different or that it is expressed in smaller amounts.

Thus, cell-surface labeling of trophozoites and precipitation with polyvalent antisera (Einfield & Stibbs 1984; Moore *et al.* 1982; Smith *et al.* 1982b; Wenman *et al.* 1986) or MoAbs against *Giardia* membranes (Butscher & Faubert 1988; Nash & Aggarwal 1986), have identified both different and apparently common or cross-reactive giardial antigens (Nash & Keister 1985; Smith *et al.* 1982b; Ungar & Nash 1987). In a comparison of *Giardia* isolates from domestic and wild animals with a human isolate from the same geographical location the most consistent antigen was a 52kDa (Wenman *et al.* 1986). Other conserved antigens of molecular weights 62kDa, 38kDa & 31kDa were also detected. These could prove to be major inter-species antigens that are conserved among the *Giardia* species. Most studies are in agreement that most of the antigens that are different between isolates appear to be on the surface of the organism while the common antigens are usually internal or somatic (Torian *et al.* 1984; Ungar & Nash 1987). MoAbs produced against internal antigens did not cause IF when living homologous trophozoites were studied (Torian *et al.* 1984). These antisera also failed to react with surfaces of heterologous living *Giardia* trophozoites (Ungar & Nash 1987) but reacted in an ELISA using sonicated trophozoites and in an IF assay using formalin-fixed trophozoites.

Other indications that *Giardia* isolates from man are heterogenous comes from studies on isoenzymes (Andrews *et al.* 1988a; Baveja *et al.* 1986; Bertram *et al.* 1983; Meloni *et al.* 1987) and differences in DNA sequences (Nash *et al.* 1985). These differences could account for differences of the surface antigens of *Giardia* (Nash & Keister 1985). A more recent method used to define shared or unique antigens of *Giardia* was to study the translation products of the RNA of various isolates (Aggarwal & Nash 1987b). Differences in translated products were noted

Table 1.1 *Giardia* specific surface proteins[a]

Major	Minor	Reference
82; 32	25-32	Erlich *et al.* 1983
94-225[b]	16-94	Nash *et al.* 1983
82	24; 30	Einfield & Stibbs 1984
	53; 105	
	180	
94-225[b]	16-94	Nash & Keister 1985
75	15-22	Clark & Holberton 1986
	54/58	
88		Edson *et al.* 1986
170[c]		Nash & Aggarwal 1986
31; 52	38; 62	Wenman *et al.* 1986
31	27; 28	Taylor & Wenman 1987
	55/56	
	80	
170[c]		Adam *et al.* 1988
30; 36		Butscher & Faubert 1988
82; 85		Mayrhofer *et al.*
65		(unpublished data)

[a]Molecular weight expressed in kilodaltons

[b]Polydisperse E-S products

[c]Released as an E-S antigen

between isolates that are indeed different as shown previously by DNA banding patterns, surface antigens, E-S products and biologic behaviour in gerbils (Aggarwal & Nash 1987a; Nash *et al.* 1983, 1985; Nash & Keister 1985). Nothwithstanding these differences in the translation products of the two isolates used, sera from gerbils infected with either isolate recognized cross-reacting antigens on the other. Therefore, this study confirmed that there are common antigens shared by two different *Giardia* isolates and may be of biological importance and of use in serological diagnosis of *Giardia* infections in humans.

Some of the common antigenic determinants may be carbohydrate. As previously mentioned, sugars are present on the surface of some trophozoites (Erlich *et al.* 1983; Hill *et al.* 1981; Ward *et al.* 1988). GluNAc has been identified as the major saccharide moiety on glycoproteins on the trophozoite surface (Ward *et al.* 1988). Studies on the surface carbohydrate residues of *Giardia* cysts identified the polysaccharide chitin as the major structural component of the cyst wall (Ward *et al.* 1985). At least 2 of the major surface proteins described may be glycoproteins (Butscher & Faubert 1988; Einfield & Stibbs 1984).

Caution needs to be exercised when comparing parasites that have been maintained in the laboratory for many years. Not only are accidental contaminations possible and likely, but it is probable that field isolates consist of mixed populations of parasites, with some overgrowing the others with time in culture. Furthermore, recent studies indicate that the surface antigens are markedly different between trophozoites of the same cultured line when grown *in vitro* and after a period of adaptation in gerbils (Aggarwal & Nash 1988) or in baby mice (Mayrhofer & Ey, unpublished).

1.8.4 Antigenic variation

Antigenic variation is a powerful survival strategy adapted by certain pathogens including parasitic protozoa that are extra-cellular or free-living for all or part of their existence. It is exemplified by the African trypanosomes, which are by far the best characterized and most studied system of this kind (Cross 1978, 1984; Donelson

& Rice-Ficht 1985). Variant antigens have also been identified on the surfaces of several species of bacteria including *Neisseria gonorrhoeae* (Stern *et al.* 1986) and the *Borrelia* (Barbour *et al.* 1982), on the flagella of *Salmonella* (Silverman *et al.* 1979) and on fimbriae of *E. coli* (Eistenstein 1981). The *Borrelia*, which are procaryotes, exhibit a spectacular form of antigenic variation, strikingly similar to that displayed by a eucaryotic protozoan, the African trypanosome (Barbour *et al.* 1982). The malaria variant antigens are distinguished from the above by being expressed on the surface of an infected host cell by organisms that are predominantly intra-cellular (Howard 1984).

Antigenic variation in *Giardia* has only recently been shown *in vitro* (Adam *et al.* 1988; Mayrhofer *et al.*, unpublished data) and *in vivo* (Aggarwal & Nash 1988). The WB isolate of *G. lamblia* expresses a cysteine-rich 170-kDa (CRP 170) surface antigen that undergoes antigenic variation, perhaps explaining some of the differences in surface labelling and antigenic profile reported in the literature (Adam *et al.* 1988). *In vitro* studies have shown that clones of *G. lamblia* are able to generate organisms with varying antigens (Nash *et al.*, unpublished, cited in Aggarwal & Nash 1988). Exposure of clones possessing a 170 kDa surface antigen to a homologous cytotoxic MoAb kills all but a few of the trophozoites which no longer possess this antigen. Subsequent exposure of the new population to a cytotoxic MoAb specific for the new antigens results in the survival of trophozoites possessing another set of antigens. By indirect IF with MoAbs against surface antigens, Mayrhofer *et al.* (unpublished data) were able to show that a human isolate of *Giardia*, Ad-1, exhibited reciprocal expression of a major 82 kDa surface protein and a complex consisting of an 82kDa protein and a 65 kDa protein. The variant surface antigens of *Giardia* show stable expression for a limited number of generations. The entire population does not shift from expression of one antigen to another but the new antigen is selected either by cultural selection pressures (Mayrhofer *et al.*, unpublished data) or by antiserum (Nash *et al.*, unpublished and cited in Adam *et al.* 1988), the latter being similar to the situation in trypanosomes (Doyle *et al.* 1980).

In vivo evidence of antigenic variation has been recently shown. Gerbils

were inoculated with defined *G. lamblia* clones (Aggarwal & Nash 1988) and the surface antigens of the intestinal trophozoites were studied at different times during infection. It was demonstrated that *G. lamblia* changes its surface antigen(s) *in vivo* within 7 days following inoculation and appears to maintain the same set of surface antigens during the course of the infection. This isolate (WB) undergoes varying changes *in vitro* and causes a self-limiting infection in gerbils (Aggarwal & Nash 1987a) whereas gerbils infected with a strain that undergoes rapid change *in vitro* (GS/M) develop a chronic infection. It remains to be seen whether this latter isolate also undergoes variation *in vivo*.

Antigenic variation in *Trypanosoma brucei*, (Borst & Cross 1982; Donelson & Rice-Ficht 1985), *N. gonorrhoeae* (Stern *et al.* 1986) and *B. hermsii* (Plasterk *et al.* 1985) is associated with DNA rearrangement. The most common mechanism is duplicative transposition as exemplified by *T. brucei* and *N. gonorrhoeae*. Each organism has a repertoire of silent genes for the variant antigen, Variant Surface Glycoprotein in *T. brucei* and the pilus and opacity proteins in *N. gonorrhoeae*. A silent gene is duplicated and the extra copy is placed into an expression site, (near the telomere of the chromosome) displacing the previously expressed gene, and a new variant antigen is then expressed. *B. hermsii* (Plasterk *et al.* 1985) also has a repertoiere of silent genes and one expression site for the Variable Major Protein, but in this case, antigenic change may involve a mechanism other than duplicative transposition.

Adam *et al.* (1988) have isolated a portion of the gene encoding the *Giardia* CRP 170 antigen from a λ gt11 expression library and have used this cloned gene fragment as a probe to examine the mechanism of antigenic variation in *Giardia*. They have obtained evidence that suggests that the gene encoding the CRP170 antigen is subject to extensive rearrangement. In the case of the proteins observed by Mayrhofer *et al.* (unpublished data), it has not been determined whether the variable expression relates to antigenic variation as a mechanism adopted for immune evasion or whether it reflects differentiation of the trophozoite populations.

T. vaginalis, a protozoan flagellate pathogenic for man, has the ability to

alternate between two different phenotypes associated with the presence of a ds RNA virus (Wang *et al.* 1987). The WB isolate used by Nash does not possess the ds RNA virus (cited in Adam *et al.* 1988) ruling out this sort of mechanism for antigenic variation, at least in this strain. The isolate used by Mayrhofer *et al.* (unpublished data) does possess the virus. However, as mentioned earlier (Section 1.2.2.3), a correlation between phenotypes of *Giardia* and the presence or absence of ds RNA has yet to be defined.

Although it is difficult at this stage to document precisely the relevance of antigenic variation to the course of a natural *Giardia* infection, the phenomenon must be considered as likely to contribute to the chronicity of natural infections. Variation in antigenic structure of surface molecules would provide a mechanism that could allow *Giardia* to evade the immune response directed against its outer membrane.

1.9 Host immune response in giardiasis

1.9.1 General properties of anti-giardial immunity

The natural course of giardial infection in hyperendemic areas illustrates both the complexity of immune response to *Giardia* and the nature of the host-parasite interaction. Children have the greatest prevalence and suffer the most severe clinical disease (Farthing *et al.* 1986a; Gilman *et al.* 1985; Walia *et al.* 1986). With increasing age, children and young adults suffer fewer episodes. By adulthood the clinical effects of the disease are rarely apparent. This was first suggested by studies of giardiasis outbreaks (Istre *et al.* 1984) in which individuals repeatedly exposed to *G. lamblia* had a lower incidence of infection and symptoms than newly exposed individuals. This indicated that prior exposure imparts partial resistance to reinfection. It also indicates that *Giardia* has a remarkable capacity to reinitiate multiple infections in a previously immunized host. The immunological basis for the slow acquisition of natural immunity is unknown and it remains to be determined whether

reinfection represents further infection with the same strain of *Giardia* or whether successive infections involve new strains.

The chronicity of many infections raises the question of the effectiveness of immune responses against *Giardia* parasitizing the gastrointestinal tract. In fact, the only direct evidence of protective immunity in man comes from the volunteer study of Rendtorff (1954), using a single *Giardia* isolate. Whether this isolate was typical of all *Giardia* is unknown and whether it would protect against other isolates remains to be tested. Consideration must be given to whether *Giardia* actively suppress the immune response (Belosevic *et al.* 1985b; Brett 1983) or are able to evade it in some circumstances. The capacity of *Giardia* parasites to undergo antigenic variation (Section 1.8.4) may account for the chronicity seen in some individuals, but the possibility that chronicity is a particular property of specific *Giardia* strains or results from specific immunological deficiencies in some individuals must also be borne in mind.

1.9.2 The role of humoral immune responses

1.9.2.1 Immunoglobulin levels in giardiasis

Infection with *Giardia* does not normally produce increased levels of serum immunoglobulins in normal patients although elevated levels of IgA have been reported (Janoff *et al.* 1988; Roberts-Thomson *et al.* 1982) with mildly depressed levels of IgG (Roberts-Thomson *et al.* 1982). Levels of IgE are either within normal values (Geller *et al.* 1978) or increased (El-Badrawy *et al.* 1983; Farthing *et al.* 1984) while reports on levels of IgM are rare (Janoff *et al.* 1988). Roberts-Thomson & Anders (1981) found no statistically significant correlation between total serum IgA and IgG and anti-*Giardia* IgA or IgG levels. Low immunoglobulin levels in intestinal secretions in humans were reported by Zinneman & Kaplan (1972) and Popovic *et al.* (1974), but this was not substantiated by the work of Jones & Brown (1974). An increase in the numbers and percentages of lamina propria plasma cells with cytoplasmic IgM, IgG and IgA has been demonstrated in some patients (Thompson *et*

al. 1977). Giardiasis is more common in immunodeficient, hypogammaglobulinemic individuals (Ament & Rubin 1972; Hughes *et al.* 1971; Zinneman & Kaplan 1972)). Reduced or absent circulating IgA is present in this group. However, whether giardiasis is more common in selective IgA deficiency remains controversial. Serum immunoglobulin levels have not been reported in animal models.

1.9.2.2 Systemic antibody responses in giardiasis

Although there have been many descriptions of systemic antibody responses to infection with *Giardia* in man (Ridley & Ridley 1976; Roberts-Thomson 1987; Smith *et al.* 1981a; Visvesvara *et al.* 1980), it is difficult to assess the significance of these responses either in relation to the course of the infection as seen in man or in relation to the data gained from studies in experimental animals (Anders *et al.* 1982; Snider *et al.* 1985; Snider & Underdown 1986). Circulating anti-*Giardia* IgG and IgA antibodies were shown to be present in individuals with symptomatic giardiasis (Baveja & Warhurst 1983; Roberts-Thomson 1987; Smith *et al.* 1981a; Visvesvara *et al.* 1980). It is clear that the intestinal infection elicits systemic antibodies capable of reacting with antigens prepared from the parasite (Goka *et al.* 1986; Jokipii *et al.* 1988; Roberts-Thomson & Anders 1981); in some cases, titres have increased with infection and declined with treatment in symptomatic patients (Jokipii *et al.* 1988). Such antibodies may be useful diagnostically, but their relationship to immunity is obscure. The presence of specific IgM antibodies appears to be a good indicator of current infection (Baveja & Warhurst 1983; Goka *et al.* 1986), while IgG and IgA antibodies may persist after infection has been eliminated (Jokipii *et al.* 1988; Roberts-Thomson 1987; Roberts-Thomson & Anders 1981). It is expected that many intracellular *Giardia* antigens (released during parasite degradation or as E-S antigens) (Farthing *et al.* 1985a; Nash *et al.* 1983, 1985) elicit specific antibodies that have no effect on parasite growth.

1.9.2.3 Secretory antibody in giardiasis

Recent work has attempted to examine the importance of secretory antibody in parasite clearance and in the development of protective immunity. As mentioned above, the association of secretory IgA deficiency with giardiasis (Popovic *et al.* 1974; Zinneman & Kaplan 1972) is controversial. The importance of the secretory immune response in human giardiasis has received little attention. Secretory IgA antibody was shown to be present on the surface of *G. lamblia* trophozoites in human jejunal biopsies (Briaud *et al.* 1981). As mentioned above, normal levels of secretory IgA have been reported in duodenal aspirates from infected patients (Jones & Brown 1974; Naik *et al.* 1979). Anti-*Giardia* sIgA and IgG were detected in human milk (Miotti *et al.* 1985) which may protect breast-fed infants as suggested by epidemiological studies (Farthing *et al.* 1986a).

The importance of secretory immunity in murine giardiasis has been clearly demonstrated. Anti-*Giardia* sIgA and IgG antibodies have been reported in mouse intestinal secretions (Anders *et al.* 1982; Heyworth 1986; Snider *et al.* 1985; Snider & Underdown 1986) and on the surface of *G. muris* trophozoites (Heyworth 1986; Roberts-Thomson & Anders 1984). Support for the importance of secretory immunity in clearance of parasites comes from the protective effects of immune milk for suckling mice infected with *G. muris* (Andrews & Hewlett 1981) as discussed below. Elegant experiments by Snider *et al.* (1985) have shown the importance of B cells in the secretory immune response. Treatment of immunocompetent mice with anti-IgM antisera reduced both serum and intestinal immunoglobulin concentrations. When these animals were experimentally infected with *G. muris* they were unable to clear the infection, which otherwise they would have been capable of doing. This inability to clear the infection was associated with an important defect in secretory immunity (Snider *et al.* 1985).

The sequential response of PP B-cell subsets to *G. muris* infection in Balb/c mice has recently been demonstrated by Carlson *et al.* (1986). PP sIgM B cells were shown to multiply rapidly in response to *G. muris* infection, increasing above control levels by 4 days after infection and reaching a maximum at 7 days. PP sIgA

B cells, on the other hand, responded later and did not reach a maximum until 11–14 days after infection. This parallels the appearance of IgA on trophozoites 10 days after infection (Heyworth 1986) and support the theory that antigens could be involved in the process of B-cell immunoglobulin isotype switching (discussed below).

1.9.2.4 Protective immunity in giardiasis

The capacity of antibodies to prevent parasite growth or activate immune process that lead to parasite destruction can be studied by passive transfer of antibodies to naive hosts that are then challenged with the parasite, or by a variety of *in vitro* tests for anti-*Giardia* activity. Mice can be passively protected by inoculation of an IgM monoclonal antibody that reacts with a surface antigen of *G. muris* trophozoites (Butscher & Faubert 1988). It was suggested by these authors that the mechanism of protection via the MoAb could be agglutination of flagella and prevention of attachment. This suggestion is supported by the fact that all the monoclonals (specific for surface antigens on trophozoites) raised to the *G. muris* trophozoites were observed to agglutinate the trophozoites and flagella in the absence of complement. However, polyclonal antiserum from infected and challenged BALB/c mice recognizing a similar surface antigen when injected i.p. had no effect on cyst excretion (Erlich *et al.* 1983). It has been suggested that the discrepancy could be due to the fact that the polyclonal serum used by Erlich *et al.* (1983) may have contained lower quantities of the cytotoxic antibodies compared to the ascites fluid used by Butscher & Faubert (1988). It may also have contained antibodies of different (IgG) isotype. Parenteral administration of serum containing high levels of antibody in transfer experiments in mice was unsuccessful in conferring protection (Roberts-Thomson & Mitchell 1978; Underdown *et al.* 1981). However, this route of administration may not elevate antibody levels in intestinal secretions.

Immunity is transferred from immune mothers to their offspring via the milk (Andrews & Hewlett 1981; Stevens & Frank 1978; Underdown *et al.* 1981), where specific anti-*Giardia* IgA and IgG antibodies have been demonstrated. To be pro-

tected, neonates had to be suckling immune milk at the time of challenge and for 3-5 days after challenge. Protection was not sustained after weaning and immune milk was largely ineffective for established infections (Andrews & Hewlett 1981). However, a role for cells present in milk could not be excluded. Experimental passive transfer of immunity has been demonstrated with lymphocytes (Mitchell *et al.* 1982; Roberts-Thomson & Mitchell 1978) which may be essential for antibody production, as discussed below (Section 1.9.2.6). Experimental human volunteer studies (Rendtorff 1954) and epidemiological studies (Farthing *et al.* 1986a; Gilman *et al.* 1985) demonstrate protective immunity is acquired following infection with *Giardia*. However, as mentioned above (Section 1.9.1), age-specific prevalence continues to rise throughout childhood into adolescence, suggesting either that protective immunity is not acquired following a single infection or successive infections may involve new strains.

1.9.2.5 *In vitro* antibody tests in giardiasis

In vitro tests for evaluation of the importance of antibodies with anti-*Giardia* activity have been reported. Kaplan *et al.* (1985) demonstrated that both sera and an IgG fraction from rabbits immunized against *G. muris* whole trophozoites, as well as milk and an IgA fraction, enhanced the adherence of the parasite to murine neutrophils and macrophages and increased phagocytosis. Both lysis and immobilization of trophozoites have been described in the presence of sera from infected susceptible and resistant strains of mice (Belosevic & Faubert 1987). Axenically cultured human trophozoites were killed by human sera containing antibodies and complement (Hill *et al.* 1984) and by strain-specific MoAb (Nash & Aggarwal 1986). More recently, MoAbs against *G. muris* were able to kill trophozoites in the presence of guinea-pig complement (Butscher & Faubert 1988). The effector mechanisms that are activated by binding of specific anti-*Giardia* antibodies to the surface of the cell have not been identified *in vivo*. It is unlikely that complement plays a role in the lumenal environment. The importance *in vivo* of ADCC or antibody-dependent phagocytosis (opsonization) has not been established (Kaplan

et al. 1985).

1.9.2.6 The role of T cells in antibody production in giardiasis

Congenitally hypothymic nude mice develop prolonged and fatal disease (Stevens *et al.* 1978), a process that can be reversed by reconstitution with T cells from normal mice and be even more rapid if the donor mice have acquired protective immunity to *Giardia* (Roberts-Thomson & Mitchell 1978; Stevens *et al.* 1978; Vinayak *et al.* 1980a); it is possible this is due to the fact that they have been supplied with helper/inducer T cells required for the production of intestinal antibody (Kawanishi & Strober 1983). There is evidence to suggest that the impaired ability of nude mice to clear *Giardia* infection might result from a deficiency of T_h cells leading to defective production of trophozoite-specific antibody (Carlson *et al.* 1987). Clearance of *Giardia* seems to require T cells of the helper/inducer phenotype in mice (Carlson *et al.* 1986a, 1986b; Heyworth *et al.* 1986, 1987). As discussed below (Section 1.11.2), T_h lymphocytes are believed to play an important stimulatory role in the production of IgA antibodies by B lymphocytes (reviewed in Mestecky & McGhee 1987).

1.9.3 The role of cell-mediated immune responses

Most of our understanding of the cellular immune response in giardiasis has derived from the work in the mouse model of giardiasis. There is no evidence that there is an increased incidence of giardiasis in patients with disorders known to be associated purely with impaired cellular immunity (Janoff *et al.* 1988). Experimental infection can be modified by drugs which impair general lymphocyte function such as corticosteroids (Nair *et al.* 1981) and cyclosporin A, which affects T lymphocyte function specifically (Belosevic *et al.* 1986b).

1.9.3.1 *In vivo* evidence for involvement of lymphocytes in giardiasis

The role of lymphocytes in the eradication of the disease has been studied in recent years. In the *G. muris*-model, delayed-type hypersensitivity was shown by a radiometric ear assay after prior immunization with trophozoites and treatment with cyclophosphamide (Anders *et al.* 1982). Infiltration of the small intestinal epithelium with lymphocytes occurs approximately 2 weeks after initiation of infection and coincides with a decline in trophozoite or cyst numbers within the intestinal lumen (Gillon *et al.* 1982a; Kanwar *et al.* 1984; MacDonald & Ferguson 1978; Upadhyay *et al.* 1986). As in the murine model, increase levels of intraepithelial lymphocytes have been described in human patients (Ferguson *et al.* 1976; Wright & Tomkins 1977). Increased numbers of lymphocytes have not been demonstrated in the intestinal lumen in murine giardiasis (Heyworth *et al.* 1985a, 1985b). Luminal T cells have been observed in contact with *Giardia* trophozoites (Owen *et al.* 1979) suggesting that direct cytotoxicity may contribute to parasite eradication (Kanwar *et al.* 1986a).

Results of recent studies, however, do not support the view that intraluminal lymphocytes play a part in clearance of *G. muris* infections (Heyworth *et al.* 1985a). Identical numbers of intraluminal Lyt2$^+$ (cytotoxic T lymphocytes) cells in *Giardia*-infected Balb/c mice and nude mice suggests that *G. muris* is not cleared by cytotoxic T lymphocytes. In addition, there was no evidence of lymphocyte attachment to *Giardia* trophozoites in contrast to the observations of Owen *et al.* (1979). These studies argue against lymphocyte-mediated killing of these parasites *in vivo*. T-cell subsets appear to have a regulatory influence on the production of intestinal anti-*Giardia* antibodies (Section 1.11.2).

1.9.3.2 *In vitro* cellular reactivity in giardiasis

The capacity of lymphocytes to respond *in vitro* to an extract of solubilized axenic trophozoites of *G. lamblia* was examined (Roberts-Thomson & Anders 1984; Smith *et al.* 1982c). Lymphocytes from patients with acute giardiasis did not proliferate in response to *Giardia* antigens whereas those from a patient with chronic giardiasis

showed a positive response (Smith 1984). In murine giardiasis, intraepithelial and lamina propria lymphocytes exhibit direct cytotoxicity against *Giardia* (Kanwar *et al.* 1986a). Understanding of the seemingly complex interactions could soon be possible using T-cell clones, monoclonal antibodies and purified giardial antigens.

1.9.4 The role of non-lymphoid cells in giardiasis

Macrophages as effector cells have been studied in both the murine model and in human giardiasis (Belosevic & Faubert 1986a; Kaplan *et al.* 1985; Owen *et al.* 1981; Radulescu & Meyer 1981). Peripheral blood monocytes from uninfected individuals have been shown to kill cultured trophozoites *in vitro* (Smith *et al.* 1982a), although this has recently been challenged on the basis of technical problems with the thymidine release assay used to measure *Giardia* killing (Aggarwal & Nash 1986). Phagocytosis of invading *G. muris* macrophages within the lamina proporia of the small intestinal epithelium was demonstrated by Owen *et al.* (1981) in both normal and nude mice. This process may both eliminate parasites and facilitate presentation of parasite antigens to T_h cells. Except for the studies reported by Owen *et al.* (1981), all have reported enhancement of killing in the presence of antibody. Recent *in vitro* studies have shown that trophozoites of *G. lamblia* are ingested and killed by human macrophages (Hill & Pearson 1987). In this context, *G. lamblia* trophozoites had been demonstrated to trigger the respiratory burst in human macrophages resulting in the production of H_2O_2 and O_2^- which appear to participate in macrophage killing of this organism (Smith 1985).

Prolonged *G. muris* infection in mice deficient in mast cells (W^f/W^f) in contrast to controlled infections in normal littermates has been described (Erlich *et al.* 1983). It is possible mediators released by mast cell degranulation may have direct effects on parasites, effects on vascular endothelium permitting diapedesis of effector cells and effects on gastrointestinal motility (Roberts-Thomson 1987). However, unlike comparable studies with nematode parasites, resistance was not restored to W^f/W^f mice by reconstitution with normal bone marrow cells. Further studies on the role of mast cells in giardiasis is required. To date, no studies support

a role for neutrophils and eosinophils in the elimination of *G. muris* infection in mice.

1.10 Anti-*Giardia* vaccine

At this time, no vaccines are generally available for any human parasite . *Giardia* is antigenically complex and there is no information about which antigens are protective, even in the *G. muris*-model. The identification of protective antigens of *G. lamblia* is made more difficult by lack of a suitable animal model. Moreover, it is not yet known whether or not both humoral and cell-mediated responses of the host required and whether they are directed against the same host-protective antigenic molecules or determinants. The challenge facing those interested in developing a defined-antigen vaccine for immunoprophylaxis is to identify host-protective effector mechanisms and the parasite target antigens of these host responses not to mention appropriate presentation and delivery of vaccine molecules. The use of recombinant DNA technology, coupled to immunological techniques, provides powerful tools for obtaining protective antigens (or peptides) in large quantities, if these antigens can be identified. Complementary DNA libraries have been constructed from *Giardia* and cDNA clones expressed in *Escherichia coli*. The *Giardia* gene products were identified with antisera raised against whole parasites and against partially purified antigen(s) (Upcroft *et al.* 1987).

An obvious question is whether individuals could be protected by vaccination as it is not known how long the host might remain resistant if placed in parasite-free environment. Resistance to infection is developed in the face of continuous challenge (see above). Persons at risks to whom the vaccine would be given would include children and travellers to or campers in endemic areas. However, giardiasis is not a life-threatening disease *per se*. Until the mechanisms of protective immunity have been fully elucidated and the protective antigens identified, as discussed above, an anti-*Giardia* vaccine is not of practical importance.

1.11 Secretory IgA

1.11.1 Synthesis and transport into the intestinal lumen

In recent years, a considerable amount of information has accumulated on the IgA system. Recent results (Delacroix 1985 cited in Mestecky & McGhee 1987) indicate that the combined synthesis of systemic and secretory IgA (sIgA) (approximately 66mg/kg/day) places IgA as the predominant immunoglobulin produced in humans. Mucosal surfaces represent the largest area of contact of the immune system with the environment. The mucosal IgA response is thought to be generated predominantly in the PP, but it is expressed in the intestinal mucosa.

It is now well established that PP B cells are the source of intestinal lamina propria IgA plasma cells (Craig & Cebra 1971; Husband & Gowans 1978). The majority of cells in the germinal centers of secondary follicles in PP bear surface IgA (Butcher *et al.* 1982). However, unlike germinal centers in other secondary lymphoid tissue, B cell development and differentiation into plasma cells rarely occur in this tissue (Craig & Cebra 1971) not because such cells have an intrinsic inability to become plasma cells (they can be induced to produce immunoglobulin *in vitro*), but because of rapid migration out of the patch (Bienenstock & Dolezel 1971; Kagnoff 1977). It is not known what causes this rapid exit from PP. Lymphoblasts or memory cells leave PP and enter the mesenteric lymph node. They then home by unknown mechanisms (reviewed in Woodruff & Clarke 1987) to the lamina propria of the intestinal tract where they differentiate into IgA plasma cells producing antibodies specific for the ingested antigen (reviewed in Mestecky & McGhee 1987; Pierce & Gowans 1975). Once in the lamina propia the mature plasma cells have a half-life of 4 to 5 days (Mattioli & Tomasi 1973).

Recently, evidence has been provided that an endothelial cell surface molecule that is selectively expressed in mucosal organs (lamina propria and the mammary gland) is required for lymphocyte homing to mucosal lymphoid tissues. A MoAb defining a 58-66kDa endothelial cell protein antigen (*addressin*) expressed by small vessels in the lamina propria significantly inhibited the migration of mesenteric node

immunoblasts to the intestinal lamina propria (Streeter *et al.* 1988). Analysis of the nature and regulation of this endothelial-cell *addressin* may lead to a better understanding of lymphocyte trafficking.

Finally, in the lamina propia polymeric IgA is transported into the gut lumen through the columnar epithelial cells via a specific receptor, secretory component (SC), which is located on the basal surface of these cells (Mostov *et al.* 1984). SC is produced by epithelial cells found on mucosal surfaces of a variety of secretory organs including the gastrointestinal tract (Brandtzaeg 1985). It is also present on hepatocytes of certain mammalian species, including the rat, and is involved in transport of IgA from the blood to the bile (Orlans *et al.* 1979; Socken *et al.* 1979) (see Section 1.11.3). The SC on the sIgA molecule confers some resistance to proteolysis, an important characteristic because of the environment in which sIgA exerts its activity (Lindh 1975).

1.11.2 T cell regulation of the IgA response

The IgA response is considered to be T cell dependent (reviewed in Mestecky & McGhee 1987), since athymic nude mice exhibit depressed serum levels of IgA, neonatally thymectomized rabbits do not undergo IgA responses and human T cell dysfunctions contribute to this isotype-specific immunodeficiency in some individuals. On the other hand, athymic (nude) rats have similar levels of biliary sIgA to those of thymic rats and they make similar biliary IgA responses to *Brucella abortus*, although they do not respond to sheep erythrocytes (SRBC) (Andrew & Hall 1982a). However, nude rats are known to have a small population of lymphocytes which bear T cell markers (Vaessen *et al.* 1986; Vos *et al.* 1983) and may therefore be poor models for studying thymus dependency of IgA responses.

T cells with receptors for the Fc portion of IgA (Tα) have been identified in a number of species. Recent studies would suggest that the IgA FcR on T cells is important in the induction and differentiation of IgA$^+$ B cells. Antigen-specific T cell clones derived from PP greatly augmented the IgA response of PP IgA$^+$ B cells and that these cloned T cells bore FcR for IgA (Kawanishi *et al.* 1982; Kiyono *et al.*

1982, 1984). T cells with a T helper phenotype isolated from murine PP were found to induce B cells from PP to switch from expression of surface IgM to surface IgA (Kawanishi *et al.* 1982, 1983a, 1983b). This contrasted with a switch from surface IgM to surface IgG by splenic B cells in the presence of spleen derived T cell clones (Kawanishi *et al.* 1983a). T cells can also influence the proliferation and terminal differentiation of B cells through the production of soluble factors (Kawanishi *et al.* 1983b; Kiyono *et al.* 1985), now termed IL-4 and IL-5, respectively (Kinashi *et al.* 1986; Noma *et al.* 1986). Recent evidence indicates that dendritic cells in PP may play a role in generating IgA responses by focusing the T cells which are involved in polyclonal PP B cell activation leading to IgA production (Spalding *et al.* 1984).

1.11.3 Biliary sIgA

It is well documented that the liver actively secretes sIgA into the bile. In the rat, bile is a convenient source of sIgA and free secretory component (Lemaitre-Coelho *et al.* 1977). IgA is transported from blood to bile in the rat (Jackson *et al.* 1978; Orlans *et al.* 1978) by a rapid active transport mechanism in the liver (Birbeck *et al.* 1979; Orlans *et al.* 1979; Reynolds *et al.* 1980). In rats large amounts of IgA (38.2 mg/kg/day) are removed from the circulation and transported into bile. Thus the liver clears daily 24 times the entire plasma pool of polymeric IgA (Delacroix 1985, cited in Mestecky & McGhee 1987).

Polymeric IgA combines with SC exposed on the sinusoidal surface of hepatocytes and is transported through the cytoplasm to be discharged into the bile canaliculus (Orlans *et al.* 1979; Socken *et al.* 1979) in a manner analagous to the transport of IgA across human enterocytes (Brandtzaeg 1985).This hepatobiliary transport system is less active in man and appears to involve transport via the biliary epithelium rather than the hepatocytes (Dooley *et al.* 1982; reviewed in Underdown & Schiff 1986). Furthermore, in addition to the rat, hepatobiliary transport is efficient in only two other species studied: the rabbit and the chicken (reviewed in Underdown & Schiff 1986). Manning *et al.* (1984) investigated the origins of biliary immunoglobulins in the rat. They concluded that the major part of

biliary IgA is derived from intestinal lymphoid tissues via the thoracic duct lymph and the remainder from local synthesis in the liver. IgA-producing cells have been shown to migrate to the liver (Jackson *et al.* 1985) where their secreted product are transported into the biliary tract. SC-mediated hepatobiliary transport is not restricted to free polymeric IgA as small immune complexes (molecular weight $<10^6$) are also transported from the circulation into the bile (Peppard *et al.* 1981; Socken *et al.* 1981). These studies suggest that the continual process of hepatic uptake of polymeric IgA and IgA-containing immune complexes may represent a natural pathway of disposal of absorbed antigens.

1.11.4 The function of secretory IgA

A number of studies have demonstrated that the biological function of sIgA is to prevent initiation of infection by organisms that use the mucosal surface as their primary location of attachment during development of disease. While sIgA is neither opsonic nor possesses bacteriocidal activities (reviewed in Underdown & Schiff 1986), it can efficiently cross-link antigens, prevent adhesion and inactivate toxins. Also, sIgA can render bacteria mucophilic (Magnusson & Stjernstrom 1982) and this function may enhance the *flypaper* effect of surface mucus (see below). Most clinical evidence for a significant defense function of sIgA antibodies comes from studies of viral infections.

Purified sIgA antibodies have been shown to prevent attachment of bacteria to mucosal surfaces. Adhesion of oral *Streptococci* to buccal epithelial cells was inhibited *in vitro* (Williams & Gibbons 1972). *In vivo* evidence of anti-adhesion acitivity was provided by Fubara & Freter (1973) who demonstrated that increased levels of anti-*Vibrio cholerae* sIgA caused a five-fold reduction of bacterial attachment to the ileal wall in mice that had been previously immunised orally with *V. cholerae*. Secretory IgA also prevented adhesion of *E. coli* to the human urinary tract epithelium (Svanborg-Edan & Svennerholm 1978) and of *Salmonella* spp. to the luminal wall of the intestine (Magnusson & Stjernstorm 1982). Rabbits orally immunised with purified CFA/I fimbriae of ETEC were protected against challenge

with virulent ETEC expressing the homologous fimbriae (Evans *et al.* 1982) and this protection correlated with increase anti-CFA/I sIgA levels. It was highly specific since protection was not provided against challenge (ETEC) expressing heterologous fimbriae. Recent studies have shown that affinity-purified, anti-M protein (of group A streptococci) sIgA, when administered intranasally, protected mice against systemic infection after intranasal challenge with group A streptococci. In contrast, anti-M protein serum immunoglobulin administered intranasally was not protective at this site (Bessen & Fischetti 1988).

Immune reactions at the epithelial surface may stimulate the release of mucus from goblet cells and thereby reinforce the mechanical barrier against penetration of microorganisms (reveiwed in Miller 1987). The *flypaper* effect of this barrier may be further enhanced by interaction between sIgA antibodies and mucus. Antigens present in immune complexes that are trapped in the mucus layer are more rapidly degraded by proteolytic enzymes than free antigens (Walker *et al.* 1982). In humans it has been proposed that IgA1 is miscible with mucus because of structural homologies (Clamp 1980) but the situation in rats is unknown.

1.12 Aims of Thesis

The aim of the thesis was to establish a model of giardiasis in the rat in order to study intestinal immune responses with particular emphasis on secretory IgA. The rat is amenable to experimentation with easier access to bile than in the mouse. Chapter 3 describes the establishment of this model by determining the susceptibility to infection and the course of the disease in various inbred strains of specific pathogen free rats as well as evidence of acquired immunity. In Chapters 4 and 5, anti-*Giardia* immune responses in bile and serum as well as in the intestinal environment are described. Chapter 6 describes the passive transfer of immunity with immune bile while in Chapter 7 the rodent *G. duodenalis* isolates are compared.

Chapter 2

Materials & Methods

2.1 Experimental Animals

2.1.1 Adult rats and mice

Unless stated otherwise, age-matched female inbred specific pathogen free (SPF) rats and mice (6–8 weeks old) were purchased from the Animal Resources Centre, Western Australia. Animals from the above source were at all times free from any metazoan or protozoan infections. Experiments done to determine specific biliary and serum antibodies were carried out in eight to 10 week old specific pathogen free (SPF) female inbred DA (RTI^{av1}) rats obtained from Gilles Plains Animal Resources Centre, South Australia. All animals were acclimatised to a clean conventional animal room for up to 10 days after arrival and were provided with autoclaved water, cages and bedding and irradiated food.

2.1.2 Infant mice

A breeding colony of conventional LAC mice was maintained in the Medical School Animal House of the University of Adelaide. The infant mice were used at 1–3 days of age to infect with rodent *Giardia* for harvesting trophozoites 8–10 days later (Section 2.12).

2.2 Source of *Giardia* isolates

2.2.1 *G. lamblia* Portland-1 (PO-1) strain

The Portland-1 (PO-1) strain of *G. lamblia* was originally isolated in Portland, Oregon, and was kindly provided by Dr. E. A. Meyer in 1981.

2.2.2 *G. duodenalis* rat strain

The rat strain was isolated from a ginger-hooded inbred laboratory rat, GHA, obtained from the conventional animal house at the Queen Elizabeth Hospital (QEH), Woodville, South Australia. The GHA rat was produced originally from mixed Lewis, Wistar and "chocolate browns" at the QEH (Festing & Staats 1973) and is available only at the QEH.

2.2.3 *G. duodenalis* mouse strain

The mouse isolate came from a naturally infected non- laboratory colony of random-bred mice from a municipal zoo.

2.3 Maintenance of parasites

2.3.1 PO-1 *Giardia*

PO-1 strain was cultured *in vitro* (see Section 2.10.1) when required. Otherwise it was stored in liquid nitrogen after freezing.

2.3.2 Rodent *Giardia*

The rat isolates were maintained by serial passage through CBH rnu/rnu nude rats at 3–4 month intervals. During the course of this work the nude rat colony at the Animal Resources Center in Western Australia was closed by infection for about a

year. During this period the rodent isolates were passaged in DA rats at shorter intervals until nude rats once more became available.

2.4 Experimental infection

2.4.1 Cyst isolation

Cysts were isolated from faeces of rodents by a method incorporating techniques described by Roberts-Thomson *et al.* (1976a) and by Coggins & Schaefer III (1986). Faeces were homogenized in distilled water containing 0.01% Tween 20 (Sigma Chemical Company, St. Louis, Mo.) on a magnetic stir plate (Coggins & Schaefer III 1986), then overlaid on 1M sucrose and concentrated by centrifugation (400g for 15 min at 18–20 C) (Roberts-Thomson *et al.* 1976a), followed by resuspension in distilled water containing Tween 20. The cysts were washed twice; and, if necessary, the flotation on sucrose was repeated, followed by three washes in distilled water containing Tween 20.

2.4.2 Cyst purification

If required, cysts were further purified by a modification of the method described by Sauch (1984). This involved sedimentation at unit gravity through a continuous Percoll (Pharmacia Fine Chemicals, Sweden) gradient ranging in density from 1.01 to 1.03 g/ml. The working solutions of Percoll were made aseptically by the one-step procedure for diluting Percoll as recommended by the manufacturer. Low density Percoll solution (1.01 g/ml) was made by adding 3.1 ml of undiluted Percoll (1.13 g/ml) and 10 ml of 1.5 M NaCl to 86.9 mls of distilled water without Tween 20. High-density Percoll (1.03 g/ml) was made by adding 18.5 ml of undiluted Percoll and 10 mls of 1.5 M NaCl to 71.5 mls of distilled water. The pH of the solutions were not adjusted and they were stored at 4 C.

A 12 ml continuous linear gradient was formed in a 25 ml Universal bottle by the use of a gradient maker. Up to 1 ml of sample of the crude isolate was

carefully layered onto the gradient, forming a sample layer less than 2 mm thick. Care was taken that the concentration of cysts never exceeded 5×10^6/ml, in order to avoid streaming (Sauch 1984). After a sedimentation time of 1.5–2.5 hours at room temperature, the cyst band was removed and washed three times with distilled water without Tween 20 (500g for 5 min at 4 C). The preparation appeared free of contaminants when viewed by phase microscopy. Purified cysts were stored in distilled water containing antibiotics (200 μg/ml gentamicin, [*Garamycin*, Schering Corporation, U.S.A.]; 200 μg/ml benzyl penicillin, [*Crystapen*, Glaxo Australia, Boronia, Vic.]; and 2μg/ml amphotericin B, [*Fungizone*, E.R. Squibb, Melbourne, Vic.]) at 4 C and used within 3–5 days after isolation.

2.4.3 Inoculation with cysts

Cysts were counted in a hemocytometer by phase-contrast microscopy and adjusted to allow intragastric intubation of 1000 cysts in 0.2 ml for mice (using an 18-gauge blunt needle) or 5000 cysts in 0.3-0.5 ml for rats (using a pediatric nasogastric tube).

2.4.4 Quantitation of cyst excretion

To quantitate cyst excretion, non-fasting individually marked mice or rats were placed in separate cages and the faeces excreted were collected over a 2 hour period at the same time each day (8 a.m. to 10 a.m.). Cysts were concentrated over 1M sucrose as described (Section 2.4.1) and resuspended in 1 ml of distilled water and counted as described. The counts are expressed as the $\log_{10}$ geometric mean of the cysts per gram of faeces for each group of animals.

2.4.5 Drug termination of primary infections

Infections in rats were terminated after 10 weeks by administration of metronidazole, 50 mg. by intragastric intubation on 3 consecutive days. This treatment was effective in all cases as judged by cessation of cyst excretion.

2.5 Morphological studies of trophozoites

2.5.1 Preparation of trophozoites

Trophozoites were obtained by excystation from purified cysts. After initial concentration from a faecal suspension (see Section 2.4.1), the cysts were purified and stored as described (see Section 2.4.2). Trophozoites were excysted essentially as described in Section 2.9.

2.5.2 Staining with Trichrome stain

Smears were prepared by resuspending trophozoites in fetal calf serum (FCS; Flow Laboratories, North Ryde, N.S.W.) diluted 1:1 with phosphate-buffered saline (PBS, Dulbecco's solution A, pH 7.2) and then partially air drying small aliquots onto subbed microscope slides. Before completely dry, the smears were fixed with Schaudinn's fixative (1:2 [v/v] 95% ethyl alcohol:aqueous saturated mercuric choloride; 5 ml glacial acetic acid/100 ml) and, in order to define the median bodies, they were stained using the Trichrome technique as described by Wheatley (1951)

2.6 Surgical techniques

2.6.1 Bile duct cannulation

Bile was obtained by cannulating the bile duct as described by Lambert (1965). Rats were anesthetized and a mid-line incision was then performed. The bile duct was cannulated as near the porta hepatis as possible with a medical grade polyethylene tubing (Dural Plastics & Engineering, Dural, N.S.W.; 0.4 mm internal diameter, 0.8 mm external diameter). This ensured freedom of collection from pancreatic secretions. After recovery from anesthesia, the rats were kept in Bollman metabolic cages. Bile was collected without protease inhibitors (PI) in sterile 100-ml bottles in an ice bath over a period ranging from 4 hours to 3-4 days. Bile samples were snap frozen in dry ice-ethanol and stored at -100 C until used either in ELISA assays

(Chapter 4), for immunofluorescence (Chapter 5), in passive transfer experiments (Chapter 6) or for immunoprecipitations (Chapter 7).

2.6.2 Passive bile transfers

Rats were anesthethized with ether and abdominal incision was made. Intraduodenal infusion of bile was achieved through a cannula consisting of medical grade polyethylene tubing (0.4 mm internal diameter, 0.8 mm external diameter), tipped with 2.5 cm of soft silicone rubber tubing, (Silastic, Dow-corning; 0.012 in. internal diameter, 0.025 in. external diameter). An anterior midline abdominal incision was made under ether anesthesia. The cannula was passed through the posterior abdominal wall on the left side and into the stomach through a puncture in the antral region (Figure 2.1). The cannula was fed through the pylorus and anchored with a purse string suture as it entered the stomach. The end of the silicone tubing was adjusted to lie in proximity to the entry of the common bile duct. The animals were held unanesthetized and with free access to food and water in Bollman metabolic cages. Infusion was accomplished with the cannula connected to a peristaltic pump delivering 0.5 ml per hour. Rats were first infused with saline for 3–4 hours before infusing bile either prior to infection or after infection with *Giardia*. At the conclusion of the period of bile infusion, cannulae were removed by quick traction and the animals were returned to individual holding cages.

2.7 Preparation of antisera

2.7.1 Preparation of rat antiserum to PO-1 trophozoites

Five Porton rats were immunized intra-peritoneally (ip) with a total of 2.43×10^6 of PO-1 trophozoites in Complete Freund's Adjuvant (CFA). Trophozoites were prepared by harvesting cells as described in Section 2.10. The washed cells were adjusted to 3×10^7/ml normal PBS and emulsified with CFA. Each rat received 300 μl of the suspension. Three weeks later, rats were boosted with 3×10^6 PO-1

Figure 2.1 Diagramatic representation of the intra-intestinal infusion of bile. The arrowhead indicates entry of the cannula through a puncture in the anterior antral region of the stomach. The cannula was fed through the pylorus and the end of the silicone tubing was positioned to be at the level of the entry of the common bile duct. A purse-string suture secured the cannula as it entered the stomach wall.

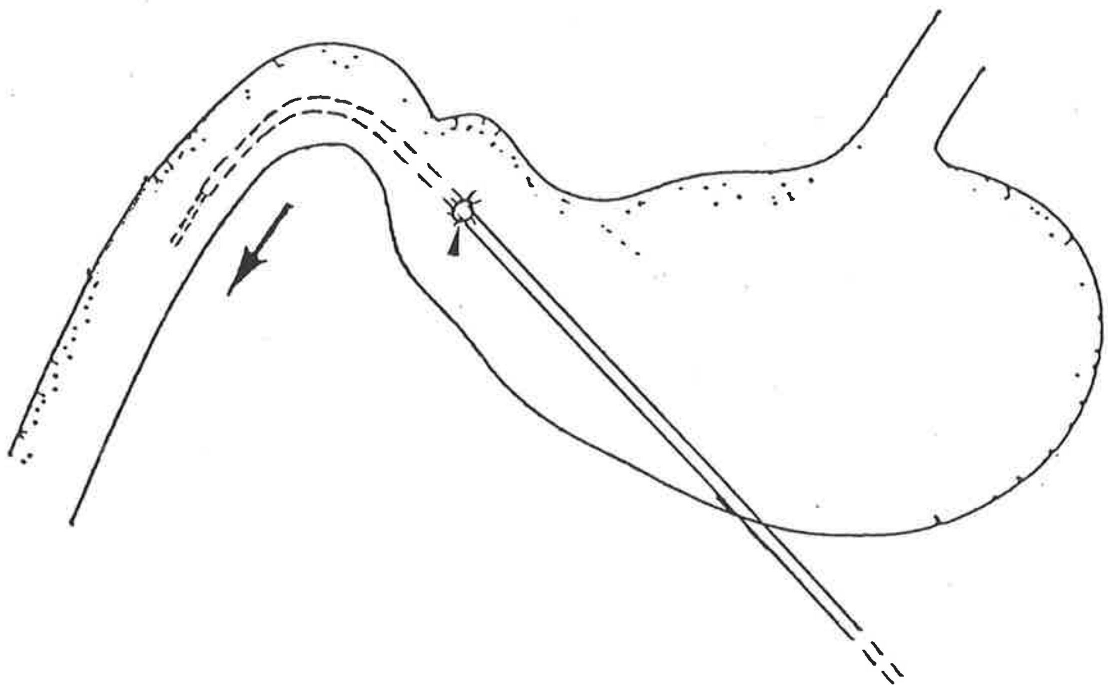
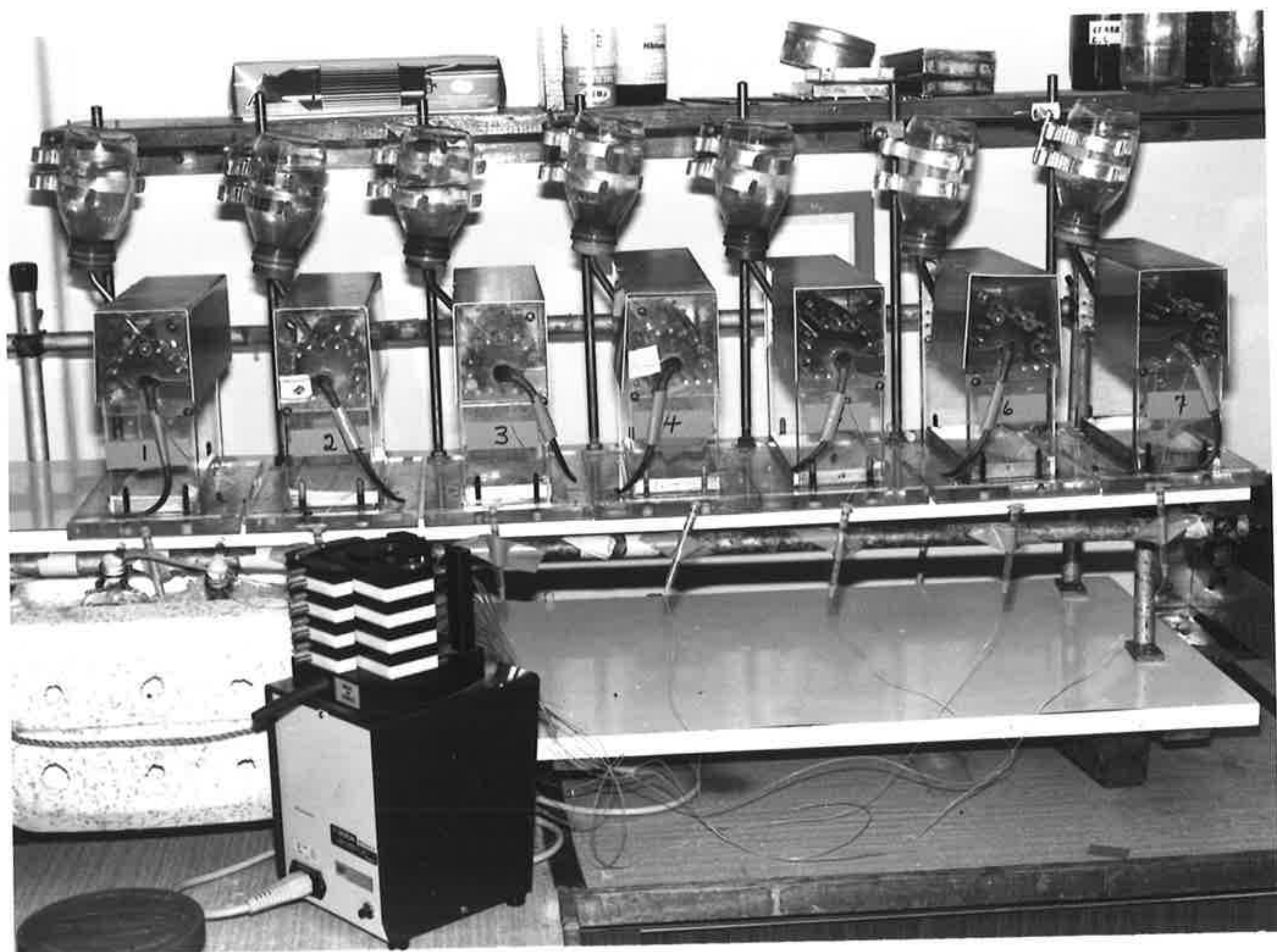


Figure 2.2 Photograph showing rats in Bollman cages after insertion of cannulae. Infusion of bile was accomplished with a peristaltic pump, delivering 0.5 ml of bile per hour and commenced 3–4 hours after conclusion of the operation. At the conclusion of the infusion, cannulae were removed by quick traction and the animals were returned to individual holding cages.


1
2
3
4
6
7

trophozoites ~~emulsified in CFA~~ by intravenous (iv) injection. Three weeks after the second immunization, the rats were injected iv with 3×10^6 organisms plus 2×10^6 organisms ip without CFA. This was followed a week later by a fourth immunization using the same protocol as for the third immunization.

All rats were bled by cardiac puncture 7 days following the last immunization. The blood was allowed to clot for one hour at 37 C followed by 3–4 hours at 4 C to allow the clot to retract. The sera were collected after spinning in an Eppendorf centrifuge at room temperature for 5 min to remove residual erythrocytes. The sera were pooled, heat-inactivated (56 C, 30 min) and stored at -20 C until required.

2.7.2 Preparation of rabbit antiserum to rat immunoglobulin

Rabbit anti-rat IgA was raised against IgA purified from rat thoracic duct lymph (courtesy of Dr. Graham Mayrhofer). The IgA was purified by S300 gel filtration, hydroxyapatite chromatography and ion exchange chromatography on FPLC. Rabbits were immunized ip by receiving 250 μg of IgA emulsified in CFA in a total of five sites (50 μg in 0.2 ml/site). Rabbits were boosted four weeks later by ip injection of a further 250 μg of IgA in CFA in five different sites as before followed by 100 μg IgA in 0.7 ml saline (without CFA) given iv.

Rabbits were bled from the marginal ear vein 10 days after the last boost and serum was collected as described above. The antiserum was absorbed by passage through 2 Sepharose 4B columns, one coated with normal rat serum and the other with purified rat IgG (all classes). Anti-IgA antibody was immunopurified by adsorption to an IgA-Sepharose 4B column and eluted with 3M sodium thiocyanate. $F(ab)_2$ fragments were prepared from these purified antibodies and analysed for binding rat immunoglobulins by radial immunodiffusion.

2.8 Isoenzyme studies

Isoenzyme analysis was performed on trophozoites freshly excysted as described below (Section 2.9). Aliquots containing 5 $\times$ 10^7 trophozoites were snap-frozen in dry ice-acetone and transported on dry ice for enzyme analysis at the Evolutionary Biology Unit of the South Australian Museum. Analysis was performed on sonicates of the orgamisms, as described elsewhere (Richardson *et al.* 1986), utilising electrophoresis on cellulose acetate gels.

2.9 Excystation of *Giardia* cysts

Cysts were isolated by initial concentration over 1 M sucrose and purified by repeated sedimentation at unit gravity through Percoll gradients as described above (Section 2.4). The cysts were held in distilled water containing penicillin (200 μg/ml), gentamicin (200 μg/ml) and amphotericin B (2 μg/ml) for 3 days and were shown to be bacteriologically sterile. Excystation was performed essentially as described (Coggins & Schaefer III 1984; Schaefer III *et al.* (1984).

Excystation is divided into three steps, including (1) induction, (2) wash and (3) incubation. Induction was done in 12 ml screw cap tubes containing 0.5 ml cyst suspension (2–5 x 10^6 per ml) and 10 ml of induction medium (5 ml 1x Hanks', pH 7.2, supplemented with 17 mM glutathione, 29 mM L-cysteine-HCl [reducing solution] and 5 ml 50 mM $NaHCO_3$) with incubation in a 37 C water bath for 30 min. The pH of the reducing solution was 2 and never adjusted. The mixing of the reducing solution and bicarbonate always resulted in CO_2 evolution so the cap to the tube was screwed on quickly to prevent loss of the CO_2.

Following induction, the cysts were pelleted by centrifugation at RT at 300 g. After aspiration of the supernatant, the cysts were resuspended in 10 ml trypsin/Tyrode wash solution (0.5% w/v trypsin [Sigma] dissolved in 1x Tyrode's solution [Parker 1950], pH 8.0) by vortexing and centrifuged at 300 g for 5 min. The washed cysts were resuspended in 10 ml trypsin/Tyrode's solution (also the

incubation solution) and incubated for 45 min in a 37 C water bath. Following incubation, the prepartion was centrifuged at 300 g for 5 min and the pellet was resuspended in appropriate solutions depending on their use.

Excysted trophozoites were used for (a) morphological studies (Chapter 7), (b) isoenzyme analysis (Chapter 7), (c) as antigens for coating ELISA plates for determination of biliary IgA antibodies in infected rats (Chapter 4) and (d) in attempts to culture the rodent isolates (Chapter 4).

2.10 Rodent *Giardia* growth experiments

2.10.1 Experimental media

The following basic culture media were employed in these studies.

Media 1: Meyer's Medium (M3) (Meyer 1970) devised to establish culture of cat *Giardia*, consists of two components: A and B, to which reducing solution is added. Component A: Hank's balanced salt solution (HBSS), 140 mls; yeast (Difco) 2 g; L-cysteine hydrochloride (Sigma), 0.2 g and agar (Oxoid), 0.1 g. This solution was autoclaved. Component B was made by combining the following sterile components: Eagle's Minimum Essential Medium (MEM, Flow Labs.) 15 mls; FCS, 20 mls; penicillin, 200 μg/ml and gentamicin, 200 μg/ml. A and B were mixed in sterile container and 0.75% $NaHCO_3$ added to bring the solution to pH 6.6. Reducing solution was prepared as described in Section 2.9. At the time the medium was used, 0.3 ml of reducing solution added to 7.0 ml of M3 and pH adjusted to 6.8.

Media 2: This was a modification of the HSP-3 (HBSS, serum, trypticase) medium described by Bingham & Meyer (1979). Trypticase (BBL, Baltimore), 0.5 g; glucose, 25 mg; L-cysteine HCl, 50 mg; HBSS, 42.5 mls; FCS, 10 mls; MEM, 3.75 mls; 1M $NaHCO_3$, 0.75 mls; penicillin and gentamicin, as above. The volume was brought up to 100 mls with deionized water (Millipore). The pH was adjusted to 6.85 with 1M $NaHCO_3$ and the solution was filter-sterilized.

Media 3: Serum-free medium of Wieder *et al.* (1983). This consists of the following: lipoprotein cholesterol solution (Pentex, Miles Lab.), 0.2 ml; Bovine serum albumin (BSA, Fraction V, Flow), 0.05 g; ox-bile (Oxoid), 25 mg; trypticase, 1 g; yeast, 0.5 g; glucose 0.5 g; NaCl, 0.1 g; L-cysteine HCl, 0.1 g; ascorbic acid, 0.03 g; ammonium ferric citrate (1 mg/ml), 1.15 mls; MEM, 3.35 mls; KH_2PO_4, 3o mg; K_2HPO_4, 50 mg; penicillin and gentamicin, as for Media 1. Water was added to a volume of 50 mls and the pH adjusted to 6.86 with 2N NaOH.

Media 4: TYI-S33 (trypticase, yeast, iron-serum) of Diamond *et al.* (1978) developed for *E. histolytica* and currently applied to the growth of human isolates of *Giardia*. This medium is composed of the following components: trypticase, 2 g; yeast, 1 g; glucose 1 g; L-cysteine HCl, 0.2 g; NaCl, 0.2 g; ascorbic acid, 20 mg; ox-bile, 10 mg; ammonium ferric citrate, 2.28 mg; MEM, 6.7 mls; K_2HPO_4, 100 mg; KH_2PO_4, 60 mg; FCS, 10 mls; penicillin and gentamicin, as above. The volume was made up to 100 mls with deionized water, adjusted to required pH (usually pH 7.04) and the solution was filter-sterilized.

Media 5: BI-S-33 as described by Kasprzak & Majewska (1985). This medium is made up of basically the same ingredients as TYI-S33 (above), except that the trypticase and yeast components are replaced by Biosate (BBL), a peptone mixture consisting of 2 parts trypticase:1 part yeast (by weight). 3 g per 100 ml of medium is used.

2.10.2 Culture procedures

Rodent *Giardia* trophozoites used to attempt growth *in vitro* were obtained from the following sources: excysted trophozoites (as described in Section 2.9) or trophozoites harvested from the intestines of nude rats and baby mice (Section 2.14).

(a) Washed excysted trophozoites were resuspended in 15 ml of growth media (M3, HSP-3, TYI-S33 and serum-free medium). Each medium was supplemented with the following percentages of FCS: 5, 10, 15, 20 or 25. Bile was added to M3 and HSP-3: either ox-bile (0.075 %) or fresh rat bile (0.75%, 0.4%, 1%, 3% or 6%). The ox-bile present in normal TYI-S33 medium was replaced by fresh rat bile as

above. Only the pH of TYI-S33 was adjusted: pH 6.8 and pH 6.6 in addition to the normal pH of 7.04. The redox potential of TYI-S33 medium was varied (by modifying the concentrations of L-cysteine HCl) from an Eh of +100 to -235. All cultures were set up at 37 C.

(b) Intestinal trophozoites from CBH nude rats were prepared as described (Section 2.14, Method 1). The experiments using these trophozoites as inocula employed two media: TYI-S33 or BI-S-33 with the following modifications. (1) Replacement of ox-bile by either fresh rat bile (0.75%) or glycocholic acid (sodium salt, Sigma, 20 mg or 40 mg/100 ml). (2) Decrease in redox potential by increasing the concentration of L-cysteine HCl (0.240 mg). All cultures were incubated at 37 C.

(c) Trophozoites of both rodent *G. duodenalis* harvested from the intestines of baby mice (Section 2.14, Method 3) were used to inoculate normal TYI-S33 medium. Trophozoites were washed and the pellet resuspended in 15 ml medium and incubated at 37 C. No further modifications were made.

2.11 *In vitro* culture of *Giardia*

The medium used for culturing *Giardia* PO-1 was TYI-S-33 (Trypticase, yeast extract, iron-serum) nutrient broth originally developed for *Entameba histolytica* by Diamond *et al* (1978) and subsequently modified by the addition of bile (Farthing *et al* 1983; Keister 1983). The components of this medium are described in Section 2.10.1.

Cells are grown at 37 C in 15 ml TYI-S33 medium in screw-capped tubes. Log-phase (48–72 hour) cultures of trophozoites are harvested. The medium and debris is removed and replaced with ice-cold PBS_m and incubated for 10–15 minutes in an ice-bath. The tubes are inverted gently 10–15 times to dislodge attached trophozoites. The cells are pelleted at 1000 rpm for 10 min at 4 C. The cells are then pooled in 50 ml centrifuge tubes (Falcon) and washed 2–3 times with ice-cold PBS_m. Before the last wash the cells are counted in a hemocytometer under phase

microscopy and cells adjusted to required concentration for subsequent processing

2.12 Preparations of crude membranes of PO-1 trophozoites

Log phase trophozoites were harvested as described above (Section 2.11). After the last wash in PBS_m, the cells were resuspended to 1–2 × 10^7 trophs/ml in distilled H_2O containing 10 mM Tris (pH 7.5) and PI. The suspension was incubated for 1 hour at 4 C. The membranes were pelleted at 8000g for 15 min at 4 C in an SM 24 rotor. The supernatant was decanted and the membrane pellet was washed twice with PBS as above. The pellet was resuspended in 100–200 μl of PBS_m for use in the neuraminidase assay described below (Section 2.17)

2.13 Freezing of trophozoites

Washed cells were collected in 1 ml of TYI-S-33 medium containing 30% FCS and 10% Dimethyl sulphoxide (DMSO, BDH England). They were frozen in a controlled rate freezer (Phillips) and stored in liquid Nitrogen until required.

2.14 Trophozoites harvested from intestines

Method 1. Mucosal scrapings were obtained from the jejunum and the ileum of infected CBH rnu/rnu rats that were fasted overnight. The small intestine was excised, placed in a Petri dish and slit lengthwise. The faecal debris was removed gently and the mucosa was scraped with a fine forceps. The scrapings were resuspended in cold medium in a small 50-mm Petri dish (Falcon Plastics) and aspirated several times with a Pasteur pipette to break up tissue fragments and dislodge the trophozoites. After transferring to a centrifuge tube, the contents were centrifuged at 200g for 1 min to sediment large tissue fragments. The supernatant was used to inoculate 15 ml screw-capped Falcon tubes containing media (Section 2.10.2).

Method 2. Rats were fasted for 5 hours, with access to sterile water, in cages with perforated bases. Trophozoites were harvested from the intestinal lumen by the technique described by Heyworth *et al.* (1985). The samll intestine was ligated at the pylorus and at the distal end of the ileum. The intestine was further ligated to separate it into three segments: duodenum, jejunum and ileum. Each section was cut and transferred to individual sterile Petri dishes and the serosa was washed in ice cold PBS (pH 7.2, 400 mosmol/kg, containing 0.1% sodium azide, NaN_3) to remove free blood. One end of each section was cannulated with a blunt-ended needle attached to a 10 ml syringe filled with cold PBS. Ten millilitres of cold PBS were injected into the lumen and left for 5 min to allow trophozoites to detach. After excision of the ligated end of each section, the contents of the lumen were flushed out and washings collected in a Petri dish. The flushed material was centrifuged at 450g for 5 min at 4 C in 50 ml centrifuge tubes and washed once in cold PBS. The pellet were resuspended in 1 ml of cold PBS and counted in a hemocytometer. When used for IF assay, the concentration was adjusted to 10^6 cells/ml.

Method 3. Infected baby mice were sacrificed 10–12 days after infection. In some cases, infected baby mice were given two 25-mg doses of clindamycin by gastric intubation 48 and 24 h before they were sacrificed in order to reduce the number of intestinal bacteria. The animals were killed by cervical dislocation and the peritoneal cavity opened. The entire small intestine was removed and opened longitudinally by using scissors, and placed into sterile 5-ml bottles containing approvimately 4 mls of TYI- S33 medium. The bottles were left on ice for at least 45 minutes after which they were shaken to dislodge any attached trophozoites. All the contents, except the large pieces of intestine, were removed and pooled into 50-ml Falcon centrifuge tubes. The trophozoites were separated from intestinal bacteria or other debris by pouring the contents of one Falcon tube into a 60-ml tissue culture flask and allowing the trophozoites to adhere to the flasks using the method of Feely and Erlandsen (1981).

2.15 Localization and distribution of *Giardia* in the intestines of infected rats

The trophozoites and cysts present in the intestine of infected rats were obtained using Method 2 as described in Section 2.14. In order to determine the distribution in the different segments of the small intestine, the total number of either trophozoites or cysts was calculated by adding the sub-totals of each segment. To obtain separate populations in each segment, the lumen was flushed as described above (Section 2.13) and the wash collected after 5 min. This yielded the population found in the luminal fluid. The lumen of each segment was flushed again with 10 mls of ice-cold PBS and left standing on ice for 20 minutes. The flushings were collected. The segments were then cut longitudinally and the mucus scraped gently. This was mixed with the fluid from the second flushings. The mucus fragments were dispersed gently with a Pasteur pipette. This material was transferred to a centrifuge tube and washed in cold PBS.

2.16 Encystation of PO-1 trophozoites *in vitro*

Method 1. The encystation of PO-1 trophozoites was first attempted using the encystation medium (AEM) developed for *E. histolytica* by Rengpien & Bailey (1975). This medium consisted of trypticase (2.5g), yeast (2.5g), KH_2PO_4 (125mg), K_2HPO_4 (275mg), dialyzed FCS (25 mls) and water (up to 500mls). Log phase cultures of PO-1 cells were harvested as described above (Section 2.11). Washed trophozoites were resuspended in 4 mls of AEM medium and incubated at 37 C for 4 days.

Method 2. The second medium used for induction of cysts was that described by Avron *et al.* (1986). This consisted in lowering the osmotic pressure of TYI-S33 and depletion of nutrients from the medium. Trophozoites were harvested as described above and washed thoroughly to remove traces of nutrients. They were then resuspended in the encystation medium at a concentration of 5×10^5 cells/ml.

TYI-S33 growth medium without serum (100%) was diluted with sterile distilled water (final volume 100 ml) to concentrations of: 0%, 47%, 59%, 70%, 81% and 100%. To each solution was added 10% FCS. The osmotic pressure of each solution measured by an osmometer was 8, 38, 121, 155, 202 and 343, respectively. These cultures were incubated for 7 days at 37 C.

Method 3. The third series of experiments involved induction by means of mucin (hog gastric mucin, Sigma; bovine submaxillary gland mucin, type 1, Sigma), L-fucose (Calbiochem), N-acetylneuraminic acid (Sigma) and N-acetylglucosamine (Sigma). Harvested and washed trophozoites (at the concentrations used in Method 1) were incubated in TYI-S33 medium containing these sugars at varying concentrations. Mucin was used at concentrations ranging from 2 mg/ml to 10 mg/ml and the other sugars at three concentrations: 1 mM, 0.5 mM and 0.2 mM. All the cultures were set up (in in 2 ml Wheaton vials and incubated for 7 days at 37 C. At daily intervals, one vial per inductive agent was harvested by chilling on ice for 10 min and pelleting the cells by centrifugation at 1000 rpm for 10 minutes at 4 C. The cultures were examined for the presence of cyts under phase microscopy.

2.17 Determination of neuraminidase activity in *Giardia*

This was performed essentially as described by Pereira (1983). PO-1 *Giardia* trophozoites were used. Whole trophozoites were harvested as described in Section 2.11. The washed cells were resuspended in 500 μl PBS containing gentamicin (200 μg/ml) and PI (10 μl of 2 mg/ml). Cell concentrations used in the assay ranged from 8×10^6 to 10^6 trophozoites per 100 μl. The TYI-S33 medium of 6 culture tubes were obtained by chilling the tubes for 10 mins at 4 C and pelleting the cells. The medium was decanted and concentrated 100-fold using positive pressure and an Amicon ultrafiltration unit (Amicon, Danvers). Membranes were prepared as described in Section 2.12.

Freshly donated human blood was used as the source of human erythrocytes.

The cells were washed 4 times by centrifugation in normal PBS (0.15 M NaCl; 0.01 M sodium phosphate, pH 7.4) at 4 C. After the last wash, the supernatant was removed and 10% suspension in sterile PBS (containing gentamicin) was made. 50 μl of 10% human RBC was mixed with 100 μl of sample (whole cells, membrane preparations of trophozoites or concentrated culture filtrate) containing PI. Controls used included human RBC alone and *Giardia* samples without RBC. The reactions were carried out in 1.5 ml plastic test tubes (Microtube, Eppendorf) at 37 C for 2 hours in a shaker to maintain cells in suspension. The reaction was stopped by centrifuging samples for 30 seconds in an Eppendorf microfuge at room temperature. The RBC-mixture was washed twice in PBS containing 2 mg/ml BSA. Pellets were resuspended in 250 μl PBS-BSA for titration against peanut lectin (PNA, Sigma) agglutination.

For hemagglutination, doubling dilutions of PNA (using PBS-BSA as diluent) were made up in eppendorf tubes (1/2 - 1/4096) and 20 μl of each transferred to wells of a 96-well microtitre plate (Titertek). 20 μl of RBC-sample mixture was then added to appropriate wells, mixed and left at room temperature for 30 mins before determining titre.

2.18 Enzyme-linked immunosorbent assay (ELISA)

2.18.1 Preparation of conjugates

Antibody was conjugated with alkaline phosphatase (from Calf Intestinal mucosa VII-S, Sigma, Missouri, U.S.A.) by the one step glutaraldehyde procedure (Avrameas 1969). 1 mg of antibody was mixed with 1 mg of alkaline phosphatase and dialysed overnight at 4 C against 2 liters of PBS/Mg/Zn with 2 changes of dialysate. 0.11 volume of 0.25% glutaraldehyde diluted in isophosphate buffer (pH 7.4) was added, mixed and the mixture allowed to react for 3 hours at room temperature. 0.2 ml of 1M lysine (pH 7) was added and dialysed overnight at 4 C

against 2 L PBS/Mg/Zn containing 0.05% NaN_3. 0.1 volume of 20mg/ml BSA was added and the solution was diluted in enzyme diluent to exactly 12.5 ml. 12.5 ml of glycerol was then added to yield a 25 ml stock conjugate (i.e. 1 mg Ab/25 ml) and 5 ml aliquots were dispensed and stored at 4 C. For use, the stock was diluted 1:20 in glycerol/enzyme diluent (1:1) to give a working solution which was further diluted in enzyme diluent in the desired concentration.

The conjugate of rabbit anti-rat IgM with alkaline phosphatase was prepared as above, using immuno-purified antibody kindly provided by Dr. D.W. Mason (Oxford, England).

2.18.2 *Giardia* antigen

Antigens for coating ELISA plates were prepared from trophozoites either excysted from purified cysts of the respective isolates of *G. duodenalis* or from axenic PO-1 trophozoites. To prepare antigen for use in the ELISA, the cysts were excysted as described in Section 2.9. Excystation was routinely greater than 75%. Excysted trophozoites were suspended in phosphate-buffered saline (PBS, 400 mOsm/kg), adjusted to 2 x 10^6 per ml. and sonicated in a Branson Sonifier Cell Disrupter B15. Axenic PO-1 trophozoites were harvested as described in Section 2.11. After the final wash, the cells were adjusted to the desired concentration for coating wells (see Section 4.3.2).

2.18.3 Procedure

ELISA was performed in round-bottomed vinyl microtitre plates (Costar, Data Packaging Corporation, Cambridge, Mass.) to which all assay reagents were added in volumes of 100 μl per well. *Giardia* antigen solution was incubated in wells for 1 hour at 37 C and a further incubation overnight at 4 C. After washing with normal PBS containing 0.05 % Tween 20 and 0.05 % sodium azide (PBS-Tween 20 buffer), all wells were post-coated with 1 % in PBS-Tween 20 for 6-8 hours at 4 C. All subsequent incubations, except for substrate, were at 4 C; and all wells were

washed 4 times prior to addition of test samples and subsequent reagents.

To assay antibodies in serum or bile (see Section 4.4.1, duplicate 2-fold serial dilutions in PBS-Tween 20 containing 1 % BSA were incubated overnight in wells coated with the homologous trophozoite antigen preparation. After washing, bound antibody was detected by a further incubation overnight with 1:400 dilutions of alkaline-phosphatase-antibody conjugates previously found to be optimal with the relevant immunoglobulin isotype (Table 4.2).

Substrate (p-nitro-phenylphosphate disodium, Sigma, PNPP) in 10 % diethanolamine buffer was added to all the washed wells and incubated for 4 hours at 37 C. The optical density (OD)in the wells was read at 405 nm using a Titertek Multiscan automated spectrophotometer after initial absorbance readings were adjusted to zero using unreacted substrate preparation. The antibody titre of any given sample was defined as the reciprocal of the dilution which produced a mean OD of 0.150, representing twice the mean OD produced by conjugates alone reacting in antigen-coated wells without added rat antibodies.

2.19 Indirect Immunofluorescence

To detect IgA antibodies acquired *in vivo*, 400 μl aliquots of cell suspensions were added to 0.5 ml polyethylene tubes (Eppendorf, FRG) and centrifuged for 5 min at 1000 rpm. Two hundred microlitres of rabbit anti-rat IgA (prepared as described in Section 2.7.2) diluted 1:20 in TYI-S33 culture medium containing 0.5% NaN_3 was added to the cells. This and subsequent incubations were carried out for 1 hour on ice. Cells were then centrifuged, washed once with cold PBS and incubated for 1 hour with 50 μl of 1:20 dilution (in TYI-S33) of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (kindly provided by Dr. Peter Ey). After centrifugation and washing with PBS, cells were fixed by addition of 25 μl of 10% formaldehyde in PBS, placed on microscope slides and examined with an Olympus fluorescence microscope. Fluorescent and non-fluorescent trophozoites were counted and the percentage of fluorescent organisms calculate.

To show that bile contained homologous and heterologous secretory IgA antibodies against surface antigens of *Giardia* trophozoites, cells were incubated with immune bile (1:10). Biliary IgA antibody bound to the trophozoites were detected by the IF assay described above. Negative controls included (a) substitution of medium for either rabbit anti-rat IgA or immune bile, (b) omission of conjugate and (c) either normal rabbit serum or pooled normal bile from SPF rats.

2.20 Protein profiles

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12% gel. 20 μl of whole cells (2.5×10^5) (prepared as described in Section 2.11 & 2.13, Method 3)were solubilized in sample buffer by boiling for 3 min at 100 C and immediately loaded for electrophoresis. Gels were electrophoresed at 25 mA constant current for 3–4 hours. Protein staining was achieved by incubation overnight at room temperature, with gentle agitation, in 0.06% (w/v) Coomassie Brilliant Blue G250 (dissolved in 5% [v/v] perchloric acid). Destaining was accomplished with several changes of 5% (v/v) acetic acid for 24 hours, with gentle agitation.

Size markers (Bio-Rad) were transferrin (76 kDa), BSA (67 kDa), horseradish peroxidase (40 kDa) and bovine gamma globulin (25 kDa).

2.21 Surface proteins of *Giardia* trophozoites

2.21.1 Biotinylation of trophozoite surface proteins

The method used was a modification of that described by Cole *et al.* (1987). Rodent *Giardia* trophozoites (prepared as described in Section 2.13, Method 3) or PO-1 trophozoites (prepared as described in Section 2.11) were resuspended at 1.5×10^6/ml in PBS_m, and 1 ml aliquots transferred to eppendorf tubes, spun at 1000 rpm for 10 minutes at 4 C to pellet cells. To each pellet (1.5×10^6 cells) was added 100 μl of freshly-prepared 2 mg/ml (200 g; 0.1 M) NHSS-biotin (BRESA, Adelaide, South Australia) and labelling was achieved by incubating on ice for 15 minutes

with occasional gentle mixing. Then 100 μl of cold 300 mM glycine in water was added to stop the reaction by incubating a further 2 minutes on ice. The volume was then increased by adding 1 ml of ice-cold PBSm. The suspensions from each eppendorf tube was aliquoted into 6 eppendorf tubes pre-marked with 20 μl mark; the cells are spun at 1000 rpm for 5 minutes at 4 C. The supernatant removed to the 20 μl mark and snap frozen in dry ice-methanol and stored at-100 C until used.

2.21.2 SDS-PAGE

The SDS-PAGE system of Lugtenberg *et al.* (1975) was used with 12% gels. The biotinylated pellets (2.5×10^5 cells) were solubilized by adding 20 μl of SDS sample buffer and boiling at 100 C for 3 minutes. The samples (40 μl) were then loaded immediately for electrophoresis.

2.21.3 Western Transfer

After electrophoresis, proteins were transferred onto nitrocellulose (Hibond C., Amersham) at 90 V for 16 hr at 4 C following the method of Towbin *et al* (1979). After transfer,the nitrocellulose sheet was blocked for 15 min at RT with 100 ml of 1% Blotto, 0.1% Tween 20 in PBS (PBS-T) with gentle mixing.

2.21.4 Visualization of biotinylated proteins

The biotinylated proteins were visualized by a modification of the peroxidase system as described by Graham & Karnovsky (1966) and modified by Cole *et al.* (1987). The blocked nitrocellulose was washed 4 times with PBS-T followed by one wash with PBS (5 min each wash). The nitrocellulose was then incubated with streptavidin-biotinylated horseradish peroxidase complex (strep-bioHRP complex, Amersham, U.K., cat. no. RNP 1051) diluted in PBS containing 0.1% BSA (PBS-BSA) (12.5 μl in 5–10 ml, 1/400) for 30 minutes at RT. Excess streptavidin complex was removed by washing 5 times as above. The staining was carried out in two steps. The nitrocellulose was incubated with 7.5 mg diaminobenzidine (DAB,

Sigma, U.S.A), 5 mg $CoSO_4$ in 15 ml of PBS for 5 minutes at RT. This was followed by staining for further 5–10 minutes with 10 μl (30%) H_2O_2 added to 7.5 mg DAB, and 5 mg of $CoSO_4$ in PBS. It was then washed well with water and blotted immediately to minimise background darkening and stored at RT for photography next day.

2.22 Preparation of *Giardia* nucleic acid

DNA and ds RNA were isolated from *Giardia* either grown to log phase (PO-1, see Section 2.11) or from *Giardia* harvested from the intestines of neonatally infected baby mice (rodent, see Section 2.13, Method 3). Washed trophozoites were either used immediately or stored as frozen pellet at -100 C. The extraction procedure was essentially that described by Davis *et al.* (1986). Washed pellets ($\sim 10^7$/ml) were resuspended in 100 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) and 100 μl of lysis buffer (1%SDS-TE) was added immediately followed by 1 mg of proteinase K (Boehringer-Mannheim). The solution was mixed gently. One ml of TE saturated phenol (pH 7.5) was added and mixed; this was followed by the addition of 1 ml chloroform:isoamyl alcohol (24:1) and mixing gently. The mixture was incubated at 55 C for 5 min then in an ice water bath for 5 min. The sample was spun for 2 min at 3000g at room temperature to separate phases. The upper aqueous phase was transferred to a new tube and the extraction repeated. The aqueous phase was transferred in equal volumes to 2 autoclaved microfuge tubes. 2.5 volumes of absolute alcohol was added to the aqueous phase and mixed. The mixtures were frozen for 10 min on dry ice and microfuged for 10 min at 4 C. The supernatant was discarded and each pellet was resuspended in 25 μl autoclaved H_2O after which samples were pooled in a new microfuge tube.

For elimination of RNA, RNAse A (Boehringer-Mannheim), boiled previously to eliminate DNAse, was added to samples and incubated for 15 minutes at 100 C prior to electrophoresis. The nucleic acid species were separated by electrophoresis on horizontal 0.8% (w/v) agarose gels (Seakem HGT). Gels were run

at 100 V for 1.5–2 hours in TBE buffer (67 mM Tris base, 22 mM boric acid and 2 mM EDTA, final pH 8.8). Hind III digested λ phage DNA (BRESA, Adelaide) was used as size markers. After electrophoresis, the gels were stained in distilled water containing 2 μg/ml ethidium bromide. Nucleic acid bands were visualized by trans-illumination with UV light and photographed on Polaroid 667 positive film.

Chapter 3

Kinetics of *Giardia duodenalis* Infections

3.1 Introduction

Most of the experimental work on the immunology of giardiasis has been carried out in mice, using isolates presumed to be *G. muris*. *G. muris* is a species distinct from *G. duodenalis* on morphological grounds (Filice 1952) and it does not infect humans. This may affect its value as an experimental model for human giardiasis, although the *G. muris*-model has provided important information about acquired resistance to *Giardia* infection.

There may therefore be value in using animal isolates of *G. duodenalis* as models of human disease. This would allow studies of infection in the natural host with organisms that are potentially more closely related to *G. lamblia* than is *G. muris*. The most readily available parasite-host combination is *G. duodenalis* (*simoni*) in the rat (Boeck 1919; Hegner 1927a; Potter 1928). As an experimental animal, the rat offers the advantages over mice of easy access both to the thoracic duct lymph and to the bile for studies on secretory immunity, which is well understood in this species (Manning *et al.* 1984).

The aim of experiments described in this Chapter was to assess inbred rats as hosts for *Giardia*. They were designed to see (a) if there are differences in the

infections produced in different rat strains; (b) whether acquired immunity occurred and (c) whether athymic rats have increased susceptibility to infection with *Giardia*. Several strains of inbred rats were examined to determine whether strain differences in the course of infection could be detected, as has been found in mice infected with *G. muris* (Roberts-Thomson & Mitchell 1978). It was then planned to exploit these differences in the manner described by Erlich *et al.* (1983) in an attempt to define protective antigens. Two morphologically identical rodent isolates of *G. duodenalis* (see Chapter 7) have been studied; although at first sight it appears strange to have used two apparently similar strains of *G. duodenalis*, one from mice and the other from rats, the results have shown fortuitiously a rather dramatic difference in the duration of infection.

The pattern of primary and secondary infection with the two organisms in seven inbred strains of rats were therefore studied and compared with infections in homozygous and heterozygous hypothymic nude rats. For comparison with studies in *G. muris* (Belosevic *et al.* 1984b; Brett & Cox 1982; Roberts-Thomson & Mitchell 1978), the isolates were also studied in key strains of mice. The two isolates produce different patterns of infection in rats and they appear to offer a good model with which to study secretory immunity during acute and chronic giardiasis.

3.2 Isolation of rodent isolates of *G. duodenalis*

At commencement of the project, *G. muris* was unavailable from any laboratory in Australia. Wild strains of *G. muris* were therefore looked for in the various rodent colonies available in Adelaide. It was then planned to develop a rat model using this organism and to compare it with a *G. lamblia*-rat model if the latter could be established (Craft 1982). Initial attempts to identify a source of *G. muris* in the University Animal House failed, although infections with other parasites were detected. As it had been shown previously that treatment of mice with corticosteroids could produce recrudescence of occult infections (Nair *et al.* 1981), mice were selected at random and treated with prednisolone. Cyst excretion was still not

observed. Eventually, two sources of *Giardia* cysts were identified in other colonies, and these were assumed initially to be *G. muris*.

3.2.1 Mouse isolate

The mouse isolate came from a naturally infected non-laboratory colony of random-bred mice maintained at the Whyalla municipal zoo. These mice were infected with *Giardia* and *Spironucleus muris* (synonym, *Hexamita muris*), making it difficult to obtain purified *Giardia* cysts from primary faecal isolates. Although both organisms produce cysts, the cysts differ in size. *Giardia* cysts measure 9 × 6 μm and *S. muris* ca. 6 × 3 μm (Sauch 1984). Initial attempts to purify *Giardia* cysts by repeated sucrose flotation followed by attempts to either pick them up using a dissecting microscope and a drawn out pipette or to retain them selectively on Millipore filters (5μm–9μm) failed.

The *Giardia* cysts were purified eventually by a modification of a simple unit gravity velocity sedimentation procedure as described by Sauch (1984). This used a Percoll density gradient that allows separation of *Giardia* and *S. muris* cysts on the basis of their size differences. The viability of *Giardia* cysts is not affected by exposure to sedimentation in Percoll, as assessed by infectivity for mice (Sauch 1984). Cysts were harvested initially by centrifugation over 1M sucrose as described by Roberts-Thomson *et al.* (1976a) and the process was repeated twice in order to obtain crude isolates not heavily contaminated with faecal debris. The cysts were re-suspended and overlaid on Percoll gradients as described in Section 2.4.2. Bacteria and fine-sized debris remained at the top of the gradient while *Giardia* cysts were well separated after 1.5–2.5 h from the faster sedimenting faecal debris and the slower sedimenting *S. muris* cysts and bacteria (Fig 3.1). The fractions containing *Giardia* cysts were pooled and the process was repeated 3 times to ensure isolation of pure *Giardia* cysts. Subsequent morphological studies on the trophozoites of this organism identified it as *G. duodenalis* (Figure 7.1).

3.2.2 Rat isolate

The rat isolate of *Giardia* was obtained from GHA rats from the QEH as described (Section 2.3.2). These rats were also infected with a mixture of parasites including *Giardia* and *S. muris*. The *Giardia* cysts were purified from faecal suspensions by velocity sedimentation in Percoll gradients as described above for the mouse isolate. As with the mouse isolate, the organism from rats was identified as *G. duodenalis* by morphological examination of the trophozoite (Figure 7.1).

3.3 Preliminary experiments

Initial experiments using the mouse isolate were hampered by the difficulty in obtaining animals from local breeding facilities that were free from intestinal parasites. Presumed SPF mice and rats turned out to be contaminated with two strains of *Entameba*; in some instances, several parasites were present including *S. muris*, although the animals were free of*Giardia*. The source of *S. muris* in these instances was not the inocula, as SPF mice could be obtained free of parasites from a facility in Western Australia and when these were infected with the same cysts they remained free of *S. muris*. Animals from the Animal Resource Centre in Western Australia were used for most of the subsequent experiments. To avoid contamination with the various parasites endemic in the Medical School Animal House, a separate room was set up outside of the Animal House and was maintained independently for the duration of the project.

3.3.1 Mouse isolate

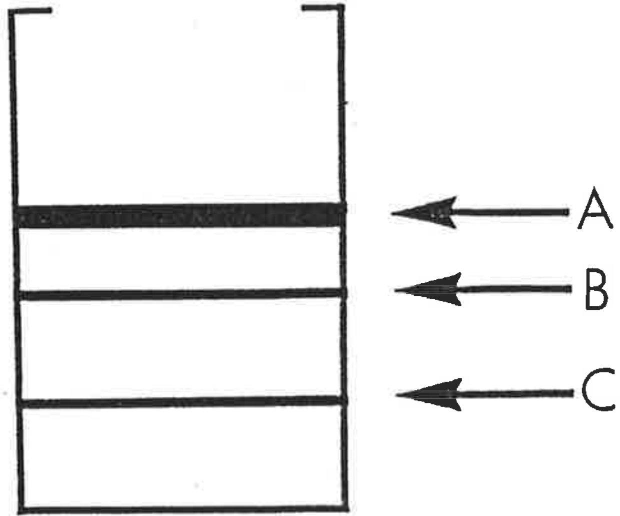
Once the *Giardia* cysts were separated from *S. muris*, they were tested for both viability and mono-infection in 6 female 4 week old SPF C3H/HeJ mice. The mice were monitored daily and found to be infected only with *Giardia* cysts. These animals remained free of *S. muris* during the 10 weeks of observation. Two mice were selected at random during the second week of infection and sacrificed. The

Figure 3.1 Diagrammatic representation of the separation by Percoll density gradient sedimentation of cysts of *S. muris* and *Giardia*. For details of separation see Section 2.4.2.

A Bacteria and faecal debris.

B *S. muris* cysts.

C *Giardia* cysts.


A
B
C

intestinal contents were examined and contained only *Giardia* trophozoites. *Giardia* was subsequently passaged in C3H/HeJ mice until rats free of intestinal parasites were obtained from Western Australia.

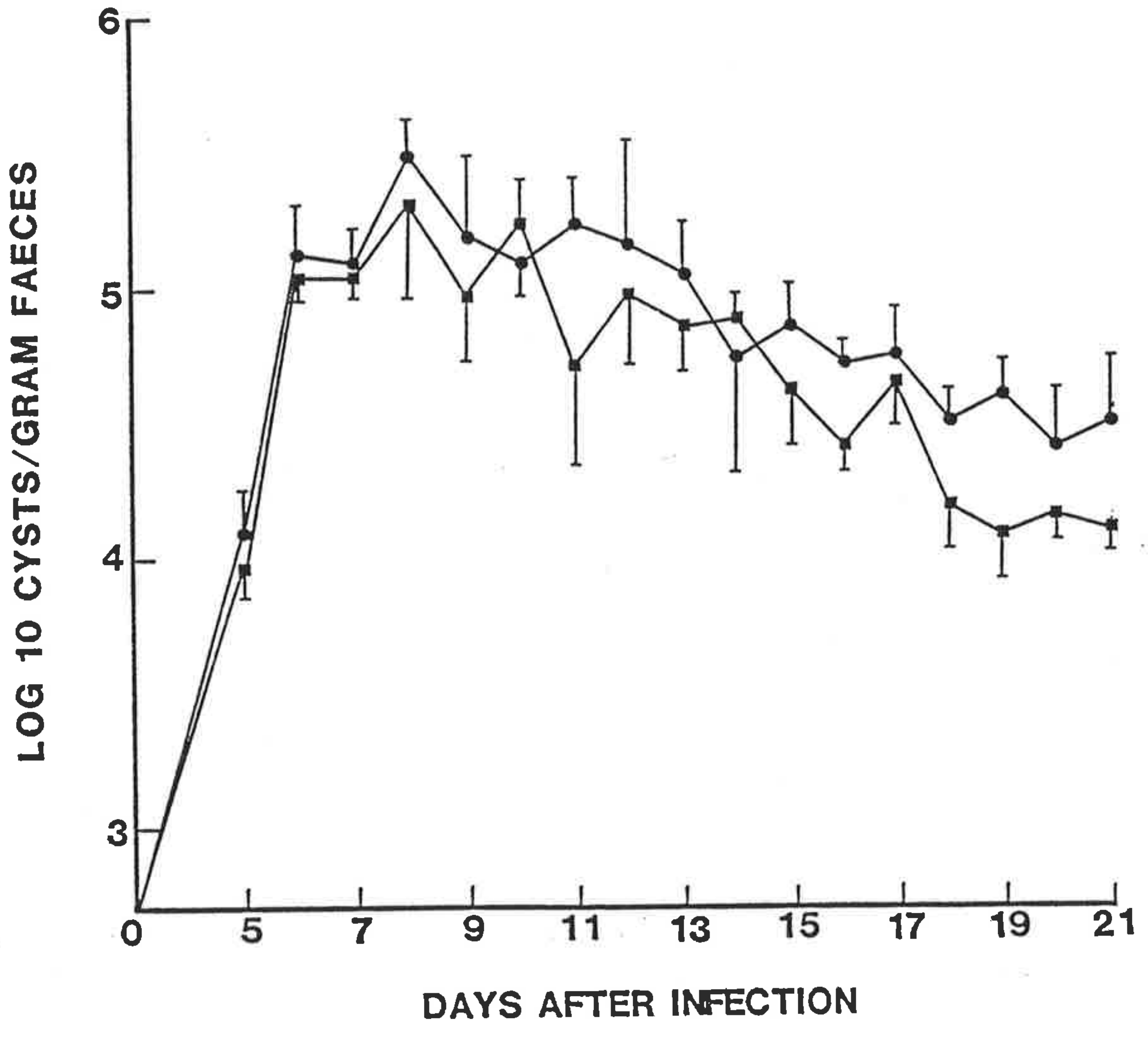
After four passages in C3H/HeJ mice an experiment was set up to determine (a) whether rats could be infected with this isolate; and (b) whether there was any difference between the kinetics of infection with the mouse-passaged cysts and infection with cysts from organisms that had been passaged through rats. Two groups of 9 week old F344 rats, each containing 6 rats, were infected as follows: Group 1 with cysts immediately isolated from C3H/HeJ mice and Group 2 with cysts obtained from F344 rats of Group 1.

Oral inoculation of F344 rats with 5×10^3 *Giardia* cysts resulted in a pattern of infection assessed by the number of cysts excreted per gram of faeces over a 2 hour period. The kinetics of infection with both types of cysts are shown in Figure 3.2. Cysts were first detected at 5 days after infection and peak cyst excretion was observed during the second week of the infection. The results indicated (1) that it was possible to infect rats with cysts that had originated in mice and (2) that following adaptation in rats, the kinetics of infection was similar to that caused by cysts produced in mice.

3.3.2 Rat isolate

Purified cysts of the rat isolate of *G. duodenalis*, from the GHA rat (Section 3.2.2), were used to infect two female SPF F344 rats in order to assess whether the separation process had been successful. These two F344 rats were housed separately and kept isolated to avoid contamination with any other intestinal parasites. By the tenth day of infection it was apparent that both rats were infected only with *Giardia*. The infection was monitored by cyst excretion daily for 21 days (Figure 3.3) and at weekly intervals thereafter up to 20 weeks (Figure 3.4). Although they were still excreting cysts at this time, they were sacrificed at 24 weeks to examine the contents of the small intestine. At this time, they had a pure infection with *Giardia*. These two F344 rats were the source of cysts of the rat isolate used in a

Figure 3.2 Daily pattern of infection in F344 rats inoculated orally with mouse *G. duodenalis* cysts from two different sources. (●) mouse cysts that were rat-adapted by one passage in F344 rats before infecting rats; (■) mouse cysts that were passaged four times in C3H/HeJ mice. Each point is the $\log_{10}$ geometric mean ± S.E.M. of cysts released by 6 rats after infection with the two sources of cysts.



6
5
4
3
LOG 10 CYSTS/GRAM FAECES
0
5
7
9
11
13
15
17
19
21
DAYS AFTER INFECTION

number of subsequent experiments, until nude rats became available to maintain the parasite stock.

3.4 Infection of normal inbred rat strains with the mouse isolate

3.4.1 Primary infection

Groups of 6 rats from each of 7 inbred strains (Table 3.1) were infected intragastrically with 5×10^3 cysts per rat. The pattern of daily cyst excretion did not vary markedly among strains of rats (Figure 3.5 where, for clarity, only the pattern of five rats are shown). All strains became infected and cyst excretion was detected in each after a prepatent period of 5–6 days. Typically, peak levels were observed during week 2 (Figure 3.5) of the infection, followed by a gradual decline in cyst output. Figure 3.6 shows that clearance of cysts occurred from 3–5 weeks after infection in most strains and that excretion of cysts ceased after 5–6 weeks. There were also no major differences in the ability of the rats to eliminate the *Giardia*, although low cyst counts persisted in WF rats for up to 7 weeks after infection.

3.4.2 Secondary infection

Experiments were then conducted in order to determine whether immunity to reinfection follows a primary infection and if the degree of resistance varied among rat strains. To overcome the difficulty of measuring resistance in a parasitized host, the rats were drug-cured of their primary infection (Underdown *et al.* 1981). Ten weeks after the primary infection (see above) all rats were treated with metronidazole for three consecutive days (Section 2.4.5). These rats were then rested for a period of 2 weeks, during which time no rats excreted cysts, thus confirming the efficacy of eradication of the primary infection with metronidazole. The rats were then challenged with the same dose of cyst (5×10^3) as in the primary infection. Evidence of a high degree of resistance was shown by low daily cyst excretion and

Figure 3.3 The daily pattern of cyst release in two F344 rats each inoculated with 5×10^3 cysts of rat *G. duodenalis* isolated and purified from GHA rat (Section 2.2.2). Horizontal dotted line indicate the limits of cyst detection in the faeces.

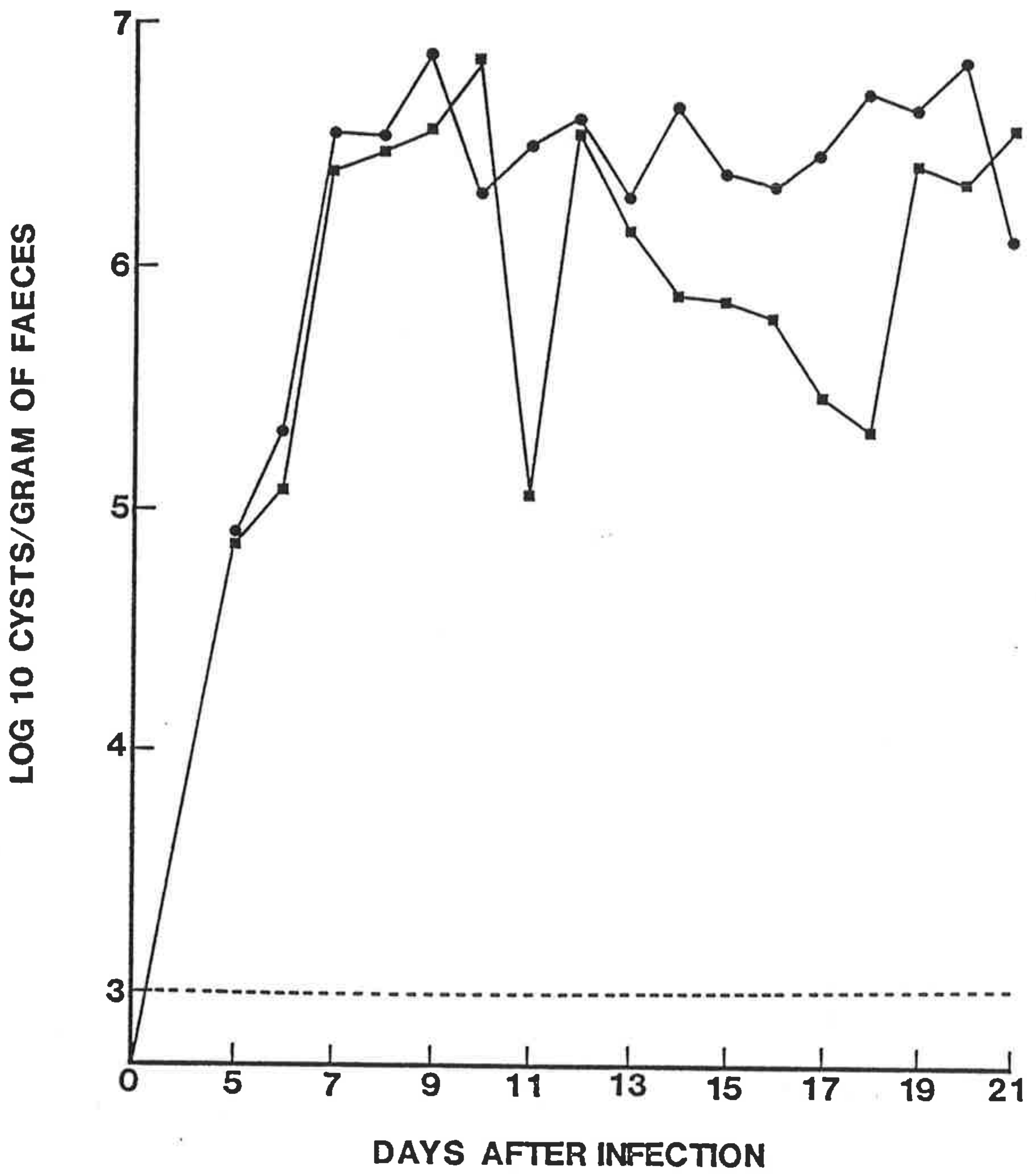

LOG 10 CYSTS/GRAM OF FAECES
7
6
5
4
3
0
5
7
9
11
13
15
17
19
21
DAYS AFTER INFECTION

Figure 3.4 The weekly pattern of cyst release in two F344 rats (same rats as in Figure 3.3) infected with rat *G. duodenalis*. These two rats continued excreting cysts for a further 4 weeks at which time they were sacrificed. The horizontal dotted line indicates the limit of cyst detection.

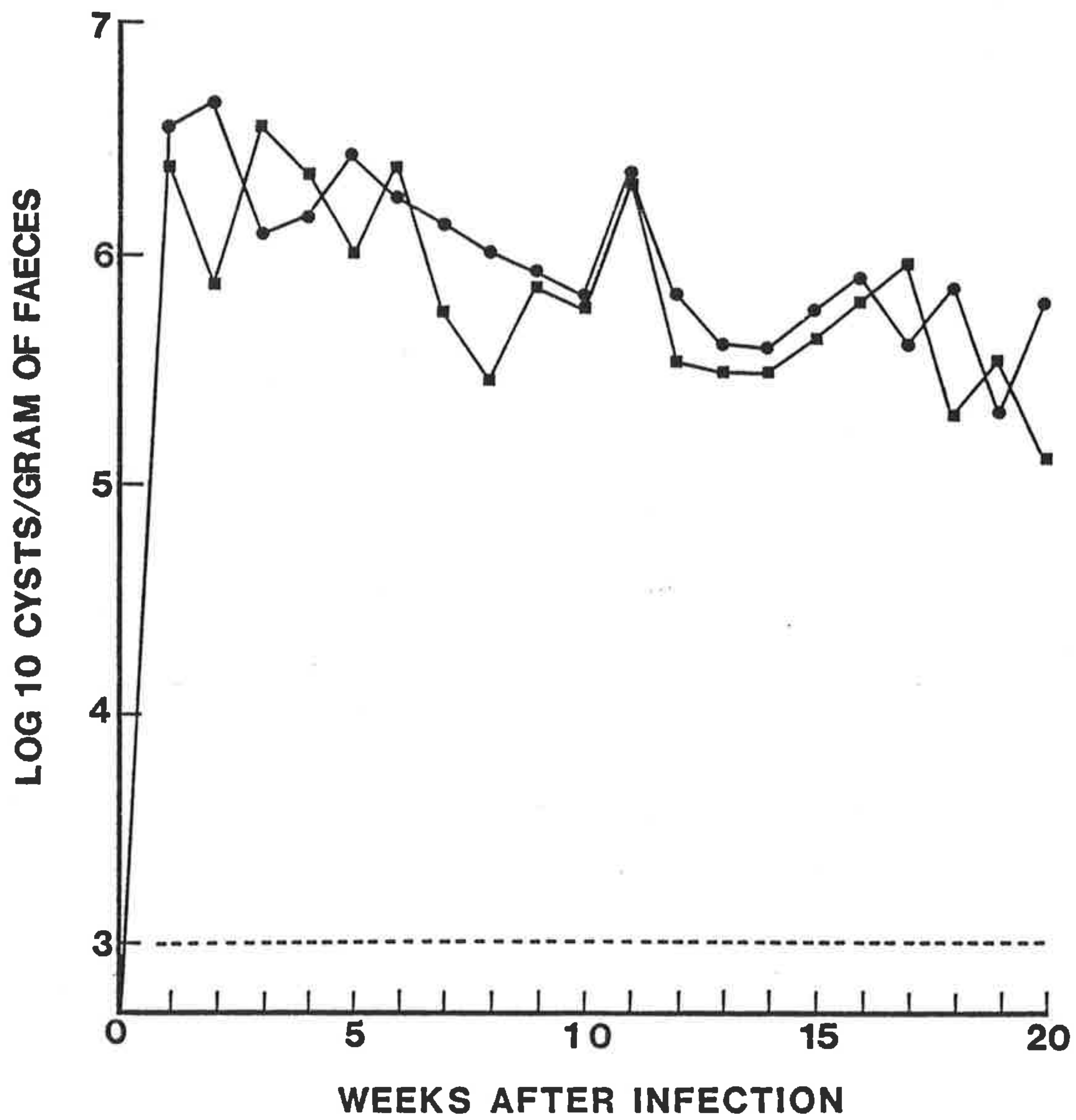

LOG 10 CYSTS/GRAM OF FAECES
7
6
5
4
3
0
5
10
15
20
WEEKS AFTER INFECTION

Table 3.1 Major histocompatibility haplotypes of rat[a] & mouse[b] strains.

		Haplotype	Strain
Rat	Standard	$RT1^{n}$	BN
		$RT1^{u}$	WF
		$RT1^{c}$	PVG/c
		$RT1^{u}$	WAG
		$RT1^{u}$	LOU/M
	Variant	$RT1^{av1}$	DA
		$RT1^{lv1}$	F344
Mice		$H\text{-}2^{k}$	C3H/HeJ
		$H\text{-}2^{d}$	Balb/c

[a]Gunther & Stark (1978)

[b]Staats (1972)

by rapid resolution of the secondary infection (Figure 3.6). Some rats in each strain failed to excrete any cysts after the secondary infection (Table 3.2), suggesting total resistance to reinfection in these animals.

3.5 Infection of normal inbred rat strains with the rat isolate

3.5.1 Primary infection

The prepatent period of 5–6 days was the same as that found after infection with the mouse isolate and the peak level of cyst excretion was also similar (Figure 3.7). The rat isolate produced a chronic infection lasting for longer than 10 weeks, with high levels of cyst excretion in all of the rat strains tested (Figure 3.8). However, in contrast to the findings with the mouse isolate, there was no resolution of the infection in any of the strains. As mentioned earlier (Section 3.3.2), cyst excretion was observed to continue for at least 6 months after infection in F344 rats (Figure 3.4) without any clearance.

3.5.2 Secondary infection

Similar experiments as with the mouse isolate were undertaken in order to determine the degree of resistance, if any, in the rat strains undergoing a primary infection with the rat isolate. The primary infection was terminated with metronidazole and the drug-cured rats were challenged 2 weeks later with 5×10^3 cysts. Cyst excretion was monitored daily as before. Resistance to reinfection was shown by lower cyst counts and cessation of cyst excretion within 14 days after challenge (Figure 3.8). Some animals in all strains except WAG and LOU/M did not excrete cysts during the second infection (Table 3.2).

Figure 3.5 Daily cyst pattern of excretion by rats of five normal inbred strains infected with 5×10^3 of the mouse isolate of *G. duodenalis*. (△) WF; (□) PVG/c; (●) F344; (▲) BN; (■) LOU/M. Each point represents the $\log_{10}$ geometric mean ± S.E.M. of cysts released by 6 rats for each strain. The dotted line indicates the limit of detection of cysts in the faeces.

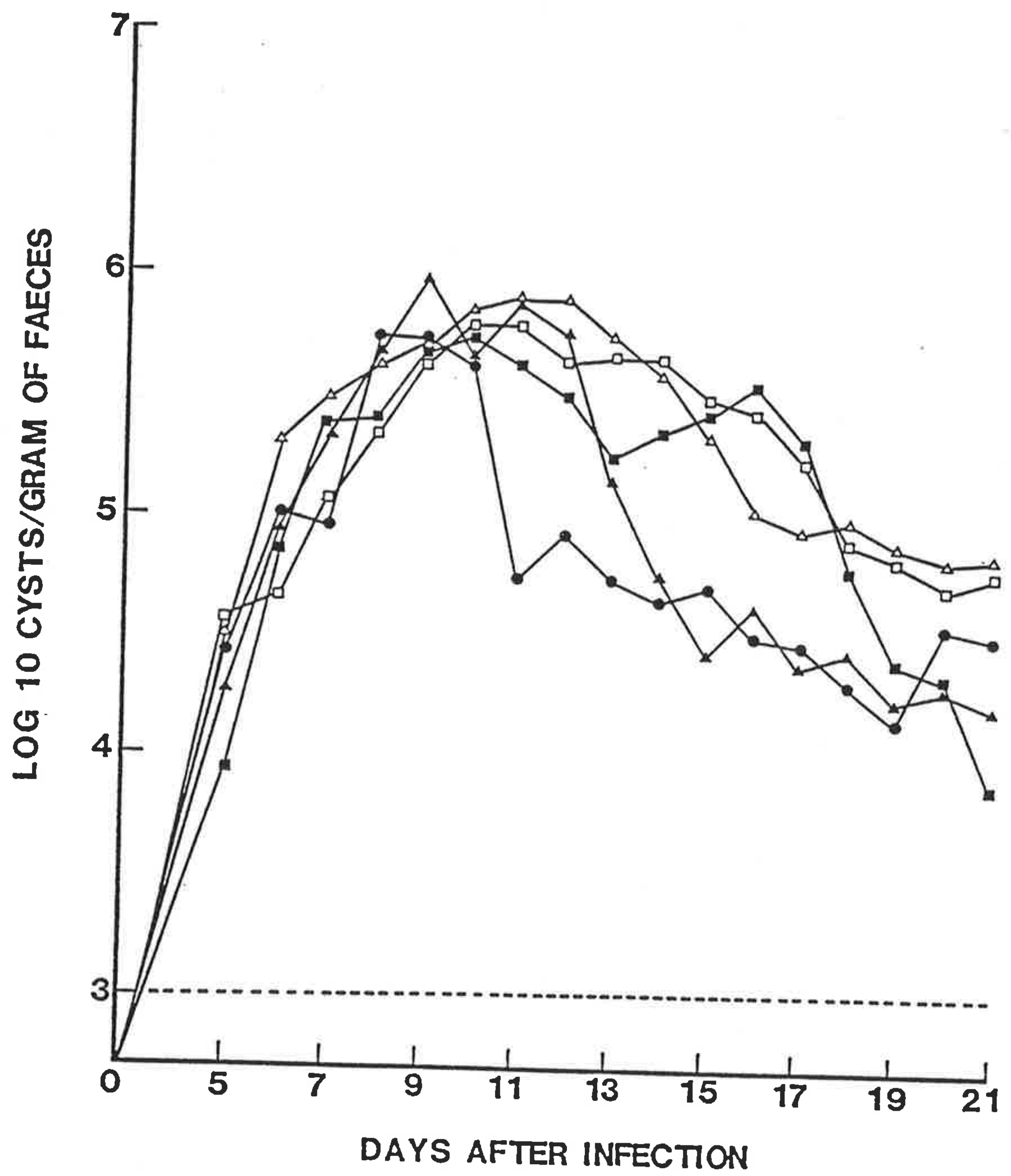

LOG 10 CYSTS/GRAM OF FAECES
7
6
5
4
3
0
5
7
9
11
13
15
17
19
21
DAYS AFTER INFECTION

Figure 3.6 Weekly cyst excretion by rats of seven normal inbred strains and by hypothymic CBH rnu/rnu rats after primary and secondary infection with 5×10^3 cysts of the mouse isolate of *G. duodenalis*. (●) Course of primary infections; (■) course of secondary infection. The primary infection was treated with metronidazole at 10 weeks and the secondary infection was commenced after a further 2 weeks. Limits of cyst detection in faeces are indicated by horizontal dotted lines. Points indicate $\log_{10}$ geometric means $\pm$ S.E.M. of cyst excretion by five animals in the primary infection and the $\log_{10}$ geometric mean $\pm$ S.E.M. for those animals that excreted cysts during the secondary infection.

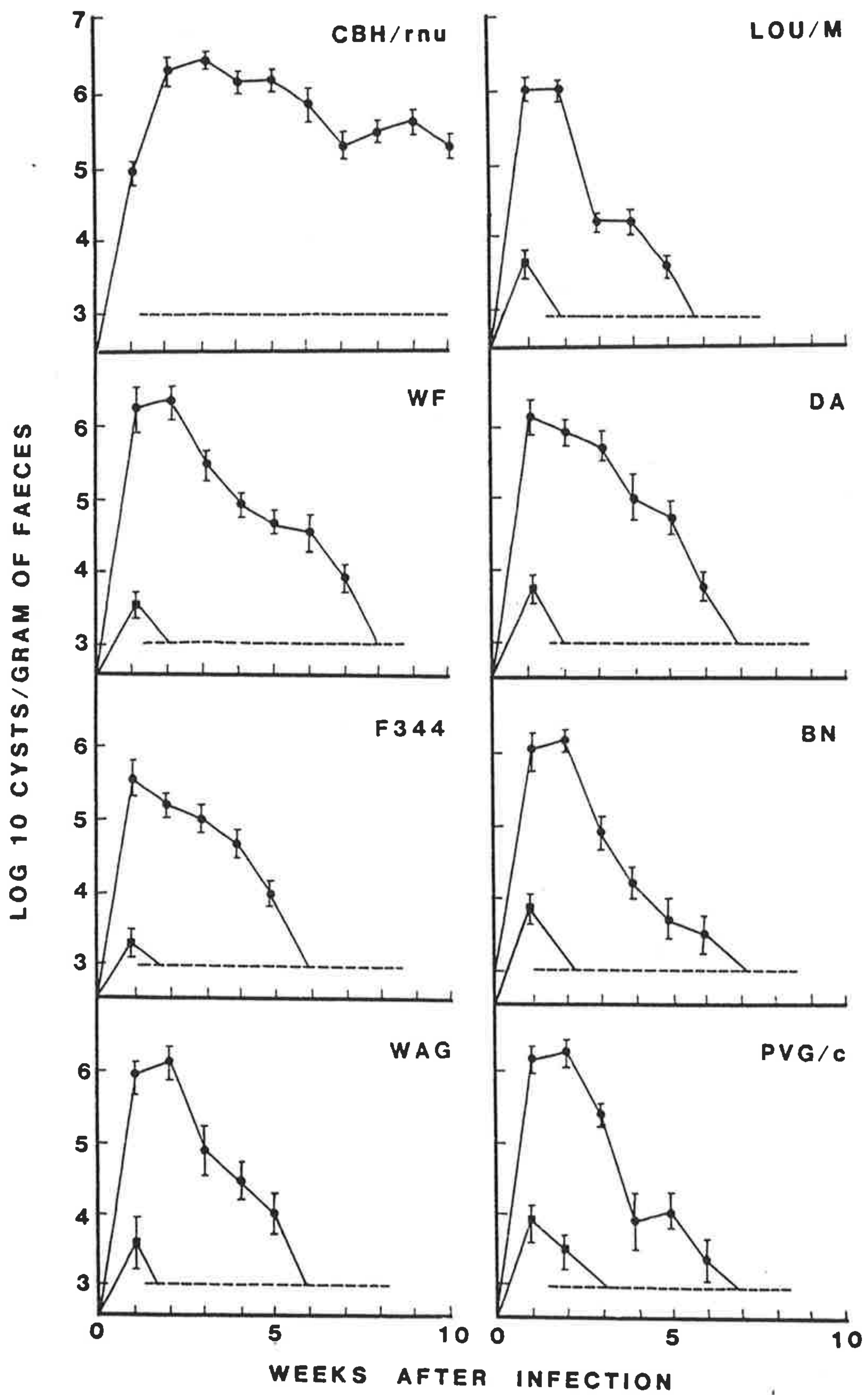
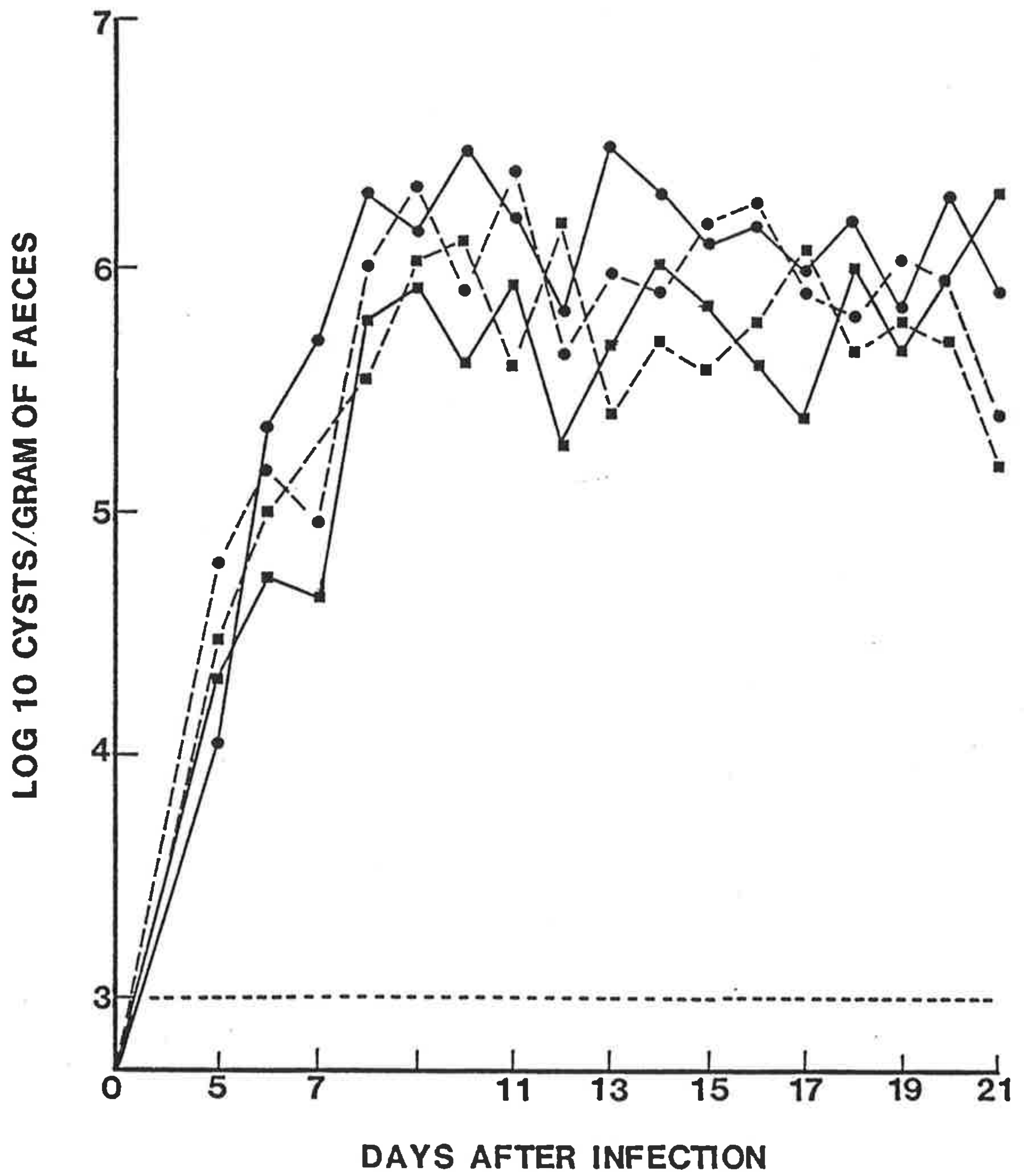
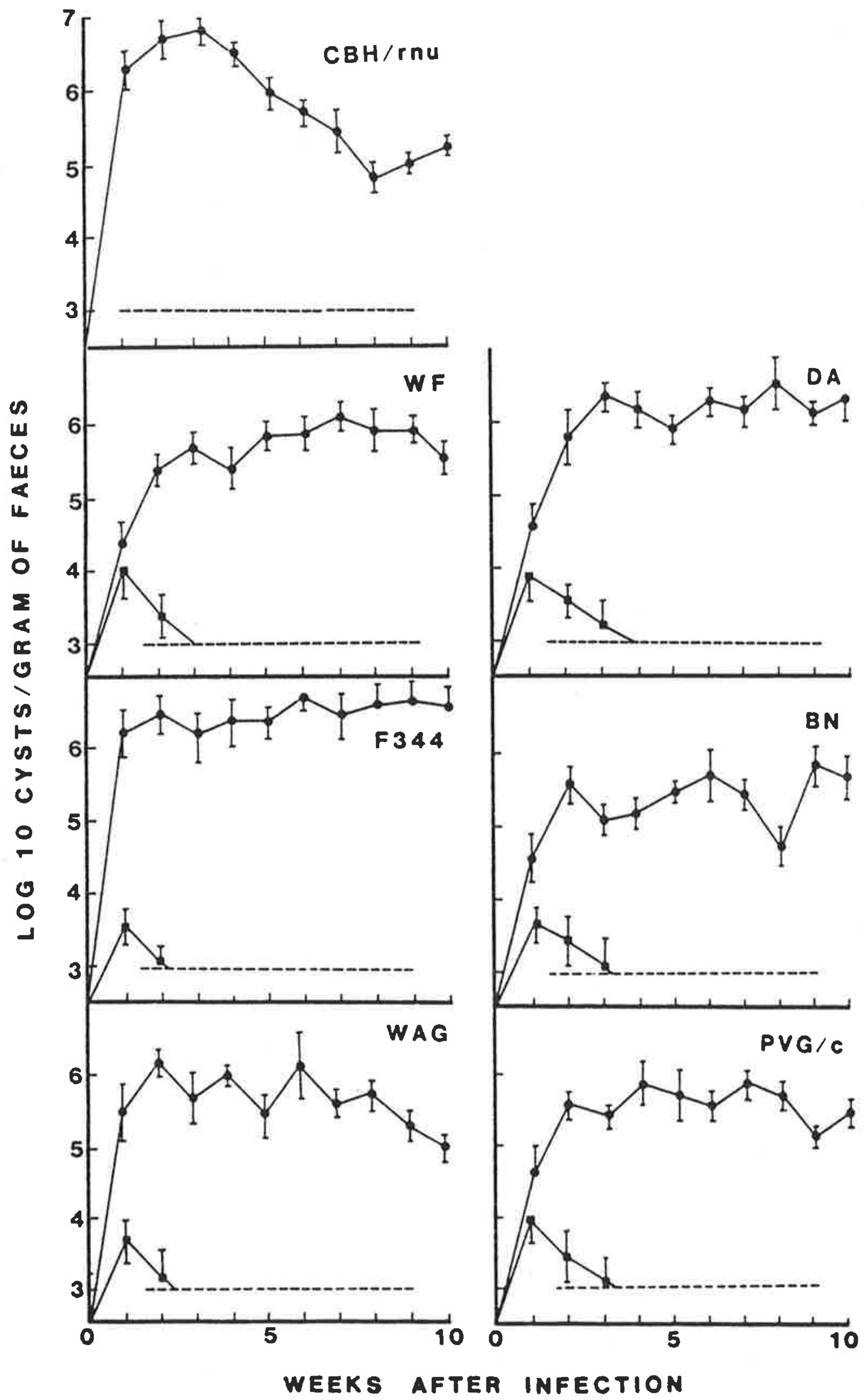

CBH/rnu
LOU/M
WF
DA
F344
BN
WAG
PVG/c
LOG 10 CYSTS/GRAM OF FAECES
WEEKS AFTER INFECTION
7
6
5
4
3
0
5
10

Figure 3.7 Daily pattern of cyst excretion with the rat isolate of *G. duodenalis* in 4 normal inbred rat strains. (■—■) DA; (●—●) WAG; (●- - -●) PVG/c; (■- - -■) BN. All other details are as described in Figure 3.5.



7
6
5
4
3
LOG 10 CYSTS/GRAM OF FAECES
0
5
7
11
13
15
17
19
21
DAYS AFTER INFECTION

Figure 3.8 Weekly cyst excretion by rats of seven normal inbred strains and by hypothymic CBH rnu/rnu rats after primary and secondary infection with 5×10^3 cysts of the mouse isolate of *G. duodenalis*. (●) course of primary infection; (■) course of secondary infection. The primary infection was treated with metronidazole at 10 weeks and the secondary infection was commenced after a further 2 weeks. Limits of cyst detection in faeces are indicated by indicated by horizontal dotted lines. Points indicate $\log_{10}$ geometric means ± S.E.M. of cyst excretion by five animals in the primary infection and the $\log_{10}$ geometric mean ± S.E.M. for those animals that excreted cysts during the secondary infection.


CBH/rnu
WF
DA
F344
BN
WAG
PVG/c
LOG 10 CYSTS/GRAM OF FAECES
WEEKS AFTER INFECTION
7
6
5
4
3
0
5
10

3.6 Infection of inbred strains of mice

3.6.1 Infection with the mouse isolate of *G. duodenalis*

When the mouse isolate was first isolated it was assumed that it was *G. muris* since it had been obtained from wild mice. Therefore, an experiment was set up to determine whether the kinetics of infection were comparable to that originally described by Roberts-Thomson & Mitchell (1978) for an isolate of *G. muris* obtained originally from a golden hamster (Roberts-Thomson *et al.* 1976a). Six Balb/c and 6 C3H/HeJ mice were infected and the pattern of cyst excretion was monitored daily for up to 21 days and thereafter at weekly intervals for up to 15 weeks.

The daily cyst excretion during a primary infection of the two strains of mice are shown in Figure 3.9A. All six mice in both groups became infected. The latent period was four days with cysts being detectable on the fourth day of infection in both groups of mice. Cyst excretion increased reaching a maximum on day 10 for Balb/c and during days 9–12 for C3H/HeJ. Maximal cyst excretion did not differ significantly in the two groups. By day 12 C3H/HeJ mice had significantly higher cyst counts than Balb/c. This difference continued until the end of the experiment.

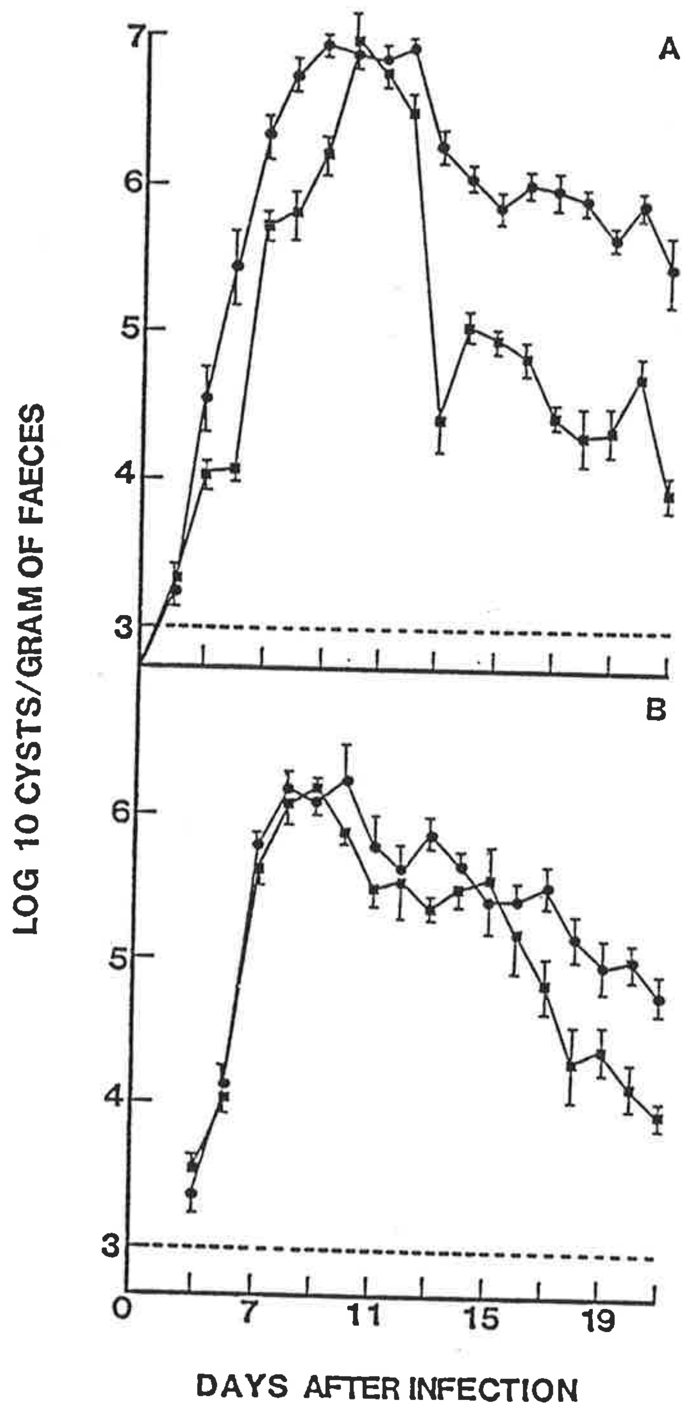
The patent period in Balb/c mice was of relatively short duration, with a decline in cyst excretion commencing 1–2 weeks after infection and reaching completion by approximately 7 weeks. In contrast, C3H/HeJ mice continued to excrete cysts for the duration of the experiment (9 weeks), although in gradually declining numbers (Figure 3.10A).

The kinetics of cyst excretion after infection with the mouse isolate in these two strains of mice therefore resembled that produced by *G. muris* infection in the same strains (Roberts- Thomson & Mitchell 1978; Underdown *et al.* 1981). A difference was that C3H/HeJ mice infected with the mouse isolate eventually ceased excreting cysts after a period of 15 weeks (not shown). They were therefore able eventually to eliminate a primary infection with this organism. This observation was originally attributed to either the use of different strains of *G. muris* or different sub-strains of C3H/He mice used in the two investigations. It is now known that

Figure 3.9 Daily cyst excretion by mice infected with the *G. duodenalis* isolates. (●) Balb/c mice infected with 1000 cysts; (■) C3H/HeJ mice infected with 1000 cysts. Horizontal dotted lines indicate the limits of cyst detection in faeces. Points are $\log_{10}$ geometric means ± S.E.M. of cyst excretion by five animals.

A Infections with the mouse isolate.

B Infections with the rat isolate.



A
B
LOG 10 CYSTS/GRAM OF FAECES
7
6
5
4
3
0
7
11
15
19
DAYS AFTER INFECTION

the mouse isolate of *Giardia* used in this experiment is not of the *G. muris*-type but rather of the *G. duodenalis*-type.

3.6.2 Infection with the rat isolate of *G. duodenalis*

Figure 3.9b shows the daily pattern of cyst excretion in both strains of mice. In contrast to the mouse isolate, the patent periods with the rat isolate were short in both strains of mice (Fig 3.10b). Balb/c mice ceased to excrete cysts by 5 weeks after infection, while elimination in C3H/HeJ mice was slower but virtually complete by 10 weeks after infection.

3.7 Infection of hypothymic nude rats

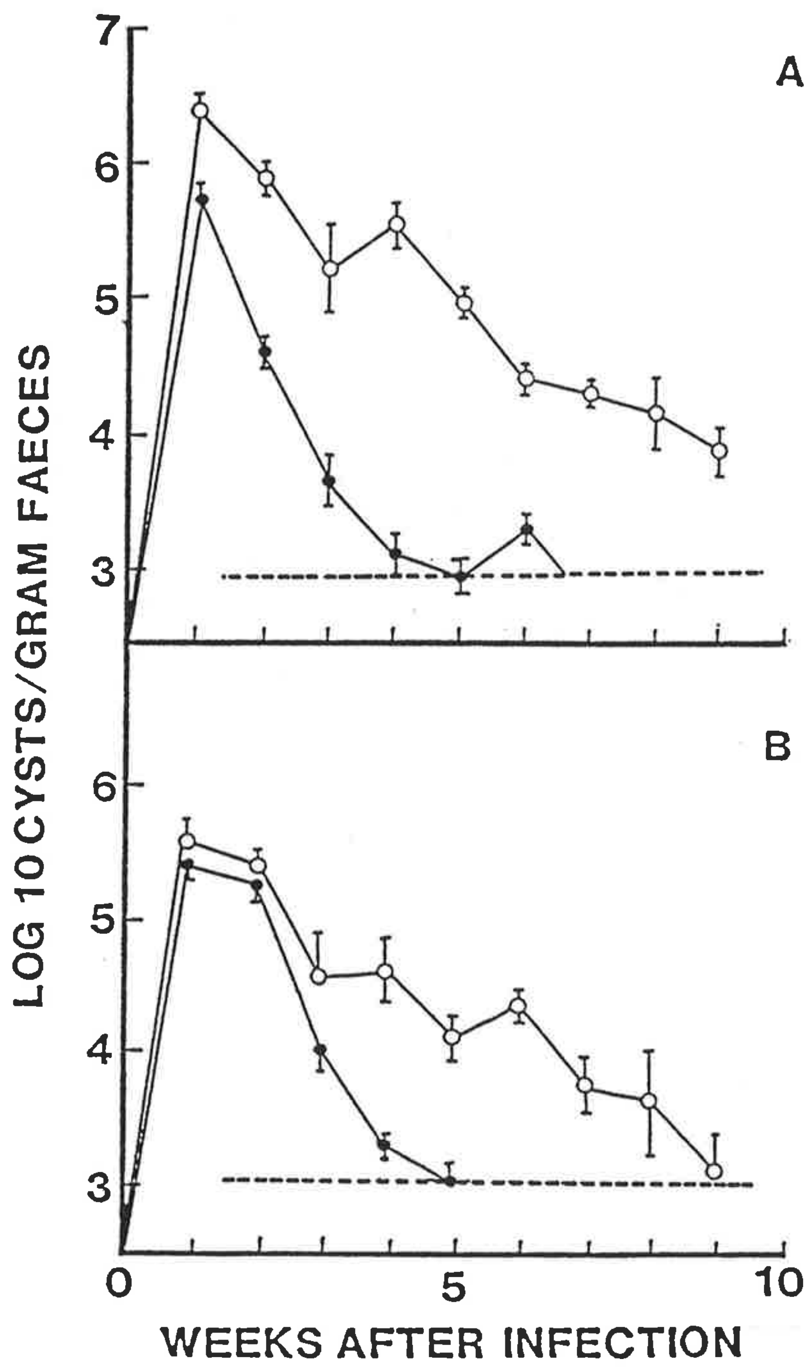
Infections with both isolates in CBH rnu/rnu rats led to excretion of cysts throughout the 10 week period illustrated in Figures 3.6 and 3.8. The persistent infection produced by the mouse isolate in hypothymic animals was in marked contrast to the self-limiting infections that were observed in euthymic strains. Cyst excretion in animals infected with both isolates was observed at similar levels even after 8 months of infection in one instance, although usually the animals were not kept for this long.

CBH rnu/rnu (nude) rats and heterozygous (+/nu) thymus-intact littermates were infected with the mouse isolate of *G. duodenalis* and the course of infection was compared. Figure 3.11 shows the pattern of cyst excretion for 3 nude rats and 3 heterozygous littermates. Cyst excretion in homozygous rats rose to peak levels during the second week of infection and persisted at similar levels for the remainder of the observation period of 9 weeks, confirming previous results as shown in Figure 3.6. Maximum cyst excretion occurred in heterozygous animals also during the second week of infection and then started declining by the fourth week. There was neither observable clinical illness nor mortality in any of the experimental animals.

Figure 3.10 Weekly cyst excretion by mice (same as in Figure 3.9) infected with the *G. duodenalis* isolates. (●) Balb/c; (○) C3H/HeJ. Other details are as described in the legend for Figure 3.9.

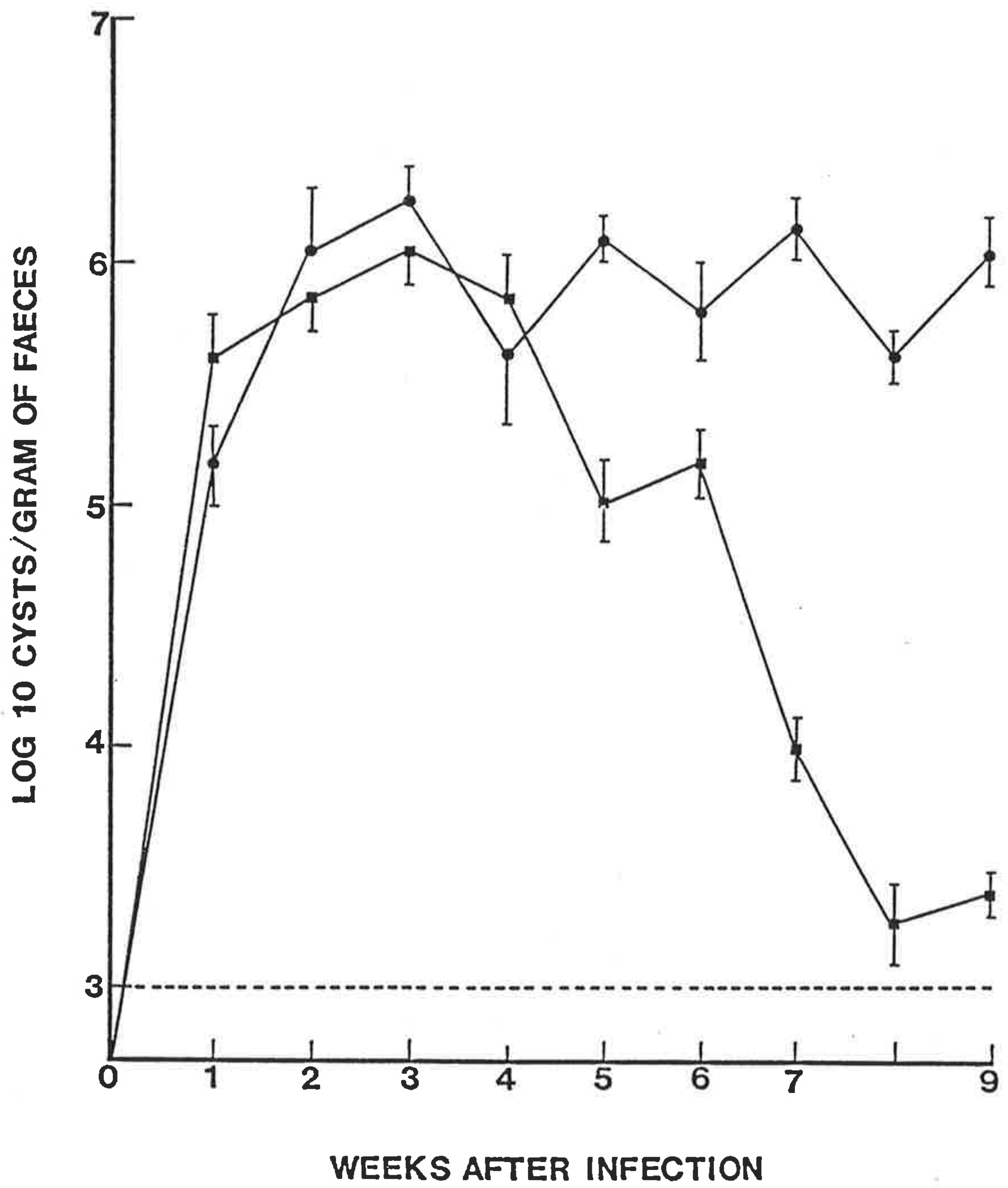
A Infections with the mouse isolate.

B Infections with the rat isolate.



A
B
LOG 10 CYSTS/GRAM FAECES
7
6
5
4
3
0
5
10
WEEKS AFTER INFECTION

Figure 3.11 The course of infection with the mouse isolate of *G. duodenalis* compared in CBH rnu/rnu (nude) rats (●) and intact heterozygous littermate CBH +/rnu rats (■). Rats were infected with 5×10^3 cysts; each point represents the $\log_{10}$ geometric mean ± S.E.M. of cyst excretion by four animals in each group. The horizontal dotted line represents the limits of cyst detection.



LOG 10 CYSTS/GRAM OF FAECES
7
6
5
4
3
0
1
2
3
4
5
6
7
9
WEEKS AFTER INFECTION

3.8 Discussion

Rats have been used as models of infection with *G. lamblia* (Anand *et al.* 1980; Craft 1982; Sehgal *et al.* 1976) from human sources and with *G. muris* (Majewska & Kasprzak 1983). Successful infection with *G. lamblia* has also been reported in mice (Kanwar *et al.* 1985a). Most investigators who have attempted to infect animals with *G. lamblia* have observed an intermittent pattern of cyst release (Belosevic *et al.* 1983; Craft 1982; Hewlett *et al.* 1982) that is said to be characteristic of *Giardia* infection in humans (Danciger & Lopez 1975; Rendtorff 1954) and that infection of most animals is not age-dependent (Belosevic *et al.* 1983; Sehgal *et al.* 1976). These observations have suggested that animal models using *G. lamblia* could be alternatives to the *G. muris*-mouse model in which to study several aspects of giardiasis. However, most workers have been unable to obtain regular infection of either mice or rats with human isolates of *Giardia* and even in baby mice, infections are short-lived (Hill *et al.* 1983). The gerbil is the only available animal which reliably accepts human *Giardia* cysts or trophozoites (Aggarwal & Nash 1987a) and susceptibility to infection with *G. lamblia* is higher in this rodent (Belosevic *et al.* 1983) than the reported susceptibility of either rats or dogs (Hewlett *et al.* 1982; Sehgal *et al.* 1976). Nevertheless, as far as is known, the gerbil is not the natural host or reservoir of *G. lamblia* and is unavailable for use in Australia.

Although it was originally the intention in the present study to use organisms with the morphology of *G. muris*, these could not be identified in animals at my disposal. However, isolates with the morphological characteristics of *G. duodenalis* were obtained from both mice and rats. The two isolates were indistinguishable by light microscopy (Figure 7.1). The mouse and rat isolates are therefore related organisms; and for comparative purposes and as a model for human giardiasis, they may offer advantages over *G. muris* because they belong to the same morphological group as *G. lamblia*.

The role of genetic influences and particularly MHC (RT1) genes in influencing susceptibility to infection with *Giardia* has not been studied before in rats.

In the current work, based on a number of strains of inbred rats, no differences in susceptibility to either isolate of *G. duodenalis* was detected. This is in contrast to the findings in mice (Belosevic *et al.* 1984b; Olveda *et al.* 1982; Roberts-Thomson & Mitchell 1978) where important strain differences were observed in the handling of infections with *G. muris*. These findings suggest that rat genetic factors are less important in defining susceptibility to *Giardia* infections in rats, although large numbers of strains would need to be examined to support this view. Conversely, the nature of the parasite determines the kinetics of infection in all strains of rats. Studies in the *G. muris*-model indicated that resistance to infection was influenced by several genes, although genes mapping to the H-2 locus did not appear to be involved. In humans, there was a higher frequency of giardiasis in persons with HLA haplotypes including A1 and B12 antigens (Roberts-Thomson *et al.* 1980), as well as individuals of blood group A (Barnes & Kay 1977; Zisman 1977).

Despite their morphological similarities, the two isolates differ markedly in the infections that they produce in mice and in rats. The most striking difference is in the kinetics of the infections that they produce in rats. In all of the rat strains, the rat isolate produced a chronic infection that showed no evidence of resolving. This may indicate that the organism has been selected for survival in the rat. In contrast, the mouse isolate produced a shorter-lived infection in all rat strains.

Comparison of infections with the mouse isolate in the congenic pair CBH rnu/+ and CBH rnu/rnu shows that gross T-cell deficiency has a profound effect on the natural history of *Giardia* infections. Infections were long-lived in CBH rnu/rnu rats, similar to the chronic infections produced by *G. muris* in nude mice (Boorman *et al.* 1973; Roberts-Thomson & Mitchell 1978; Stevens *et al.* 1978). However, in contrast to the findings with nude mice and *G. muris* (Boorman *et al.* 1973; Roberts-Thomson & Mitchell 1978), there was no mortality associated with the infection in the nude rats used in this study.

Infections in mice with the mouse isolate were similar to those described for *G. muris* (Roberts-Thomson *et al.* 1976b). Mice of the Balb/c strain became quickly resistant to the primary infection, while C3H/HeJ mice exhibited a more

chronic infection, although this was eventually eliminated. In the case of the rat isolate, this infection in C3H/HeJ mice was less chronic than was the case for the mouse isolate. However, in this case, the difference between infections in Balb/c and C3H/HeJ was less marked.

The mouse isolate therefore produces a more chronic infection in the homologous host, although the effect of host origin was less conspicuous than with the rat isolate. It is of some interest that the C3H/HeJ mouse exhibits only a quantitative defect in its ability to eliminate these *G. duodenalis* isolates, compared with its almost qualitative inability to eliminate *G. muris* (Roberts-Thomson & Mitchell 1978). Furthermore, these mice showed only marginal impairment in their ability to recover from infection with the rat isolate. This suggests that C3H/HeJ mice have difficulty in mounting an immune response to the protective antigens of some *Giardia*, rather than a serious deficiency at the effector level (Erlich *et al.* 1983).

Acquired resistance to reinfection has been demonstrated in mice or gerbils infected with *Giardia* (Belosevic *et al.* 1984b; Roberts-Thomson & Mitchell 1978; Roberts-Thomson *et al.* 1976b) and has also been observed here in rats infected with isolates of *G. duodenalis*. Partial (Brett & Cox 1982; Kanwar *et al.* 1985a; Roberts-Thomson *et al.* 1976b) and complete (Belosevic & Faubert 1983b; Faubert *et al.*. 1983; Underdown *et al.* 1981) protection against challenge depended on the time after the primary infection when challenge occurred, although it was suggested that the difference could be due to superimposing a secondary infection on an existing primary infection (Underdown *et al.* 1981). An intriguing but unexplained observation is that after drug-cure chronically infected C3H/HeJ mice become resistant to re-infection (Underdown *et al.* 1981). It is not clear whether elimination of the parasite unmasks previously frustrated or suppressed immunity or whether different immune responses are involved on the one hand in preventing establishment of infection and on the other in elimination of existing infection. Resistance of rats to re-infection with the rat isolate was similar, at least superficially. Following drug treatment to eliminate the primary infection, all strains of rats showed a high degree of resistance to re-infection when challenged 2 weeks later. Resistance has

been found to be complete if the interval between drug treatment and re-infection is extended to 4 weeks (data not shown). How elimination of the primary infection reveals underlying protective immunity is unclear, but determining the mechanism may hold the key to understanding how the host-parasite relationship is tipped in favor of the parasite in chronic giardiasis.

In conclusion, *G. duodenalis* isolates from rodents appear to offer reproducible models for studying the immune response to *Giardia*, including the role of immunity in controlling the infection and, in particular, the importance of secretory antibodies in the process. The different infections produced by the isolates in rats suggests that parasite factors as well as host factors influence the outcome of infection and comparative studies between these two organisms may help to identify virulence determinants. The animals showed no clinical signs related to the infection and there were no deaths. Diarrhea in the rat only occurs with severe disturbance of gastrointestinal function, due to the very efficient absorption of fluid in the large intestine in this species. Detailed metabolic studies would be required to assess the possible pathological effects of infection with *Giardia* and at this stage it is not clear whether the organism should be considered a pathogen in rats.

Table 3.2 Proportion of rats excreting cysts during secondary infection of inbred strains with mouse and rat isolates of *G. duodenalis*

Inbred Rat	Proportion of rats excreting cysts	
Strains	Mouse isolate	Rat isolate
F344	3/6	3/6
WAG	4/6	6/6
LOU/M	5/6	6/6
DA	3/6	2/6
BN	4/6	3/6
PVG/c	4/6	2/6

Chapter 4

Biliary and Serum Antibody Responses in Rats Infected with *Giardia duodenalis*

4.1 Introduction

A high degree of immunity to reinfection after clearance of a primary infection exists in experimental animals (Faubert *et al.* 1983; Roberts Thomson *et al.* 1976b) including rats as demonstrated in Chapter 3, yet the mechanisms involved in clearance of *Giardia* from the intestine are poorly understood. Recent studies have indicated that clearance is likely to involve antibody (Andrews & Hewlett 1981; Snider *et al.* 1985). Specific secretory antibodies have been demonstrated in intestinal fluid (Anders *et al.* 1982; Snider *et al.* 1985; Snider & Underdown 1986), in milk (Andrews & Hewlett 1981; Kaplan *et al.* 1985) and on the surfaces of trophozoites (Briaud *et al.* 1981; Heyworth 1986; Roberts-Thomson & Anders 1984), the latter suggesting that parasite-specific antibodies bind to trophozoites in the intestinal lumen.

The findings in hypothymic rats (Chapter 3) and mice (Roberts-Thomson & Mitchell 1978; Stevens *et al.* 1978) suggest an overall thymus-dependency of the protective immune response in giardiasis. Reconstitution of nude mice with syngeneic lymphoid cells resulted in progressive reduction of the number of cyts being excreted

and accelerated resolution of infection occurred when the transferred lymphoid cells were obtained from mice which had previously been exposed to *Giardia* (Roberts-Thomson *et al.* 1978). It remains to be established whether immune T lymphocytes are involved directly as effector cells or whether other thymus-dependent processes are involved. These could include T lymphocyte help for protective secretory antibody production (Kawanishi & Strober 1983) or for proliferation of mucosal mast cells (Mayrhofer & Fisher 1979; Erlich *et al.* 1983).

The objectives of experiments described in this Chapter were (a) to culture the rodent isolates of *Giardia in vitro* for use as antigen in a serological assay; (b) to develop an ELISA for the detection and comparison of antibody responses against the two *G. duodenalis* isolates in bile and in serum of normal DA rats and (c) to compare the responses in normal inbred DA rats with hypothymic (nude) rats and their heterozygous littermates.

4.2 Attempts to culture the rodent isolates of *Giardia*

As mentioned before (Section 1.2.3) rodent (rats, mice and voles) isolates of *Giardia* have never been established in culture (Meyer & Radulescu 1984; Schaefer III *et al.* 1984; Wallis & Wallis 1986). Nevertheless, several attempts were made to culture the rodent isolates of *G. duodenalis* used in this study (see Section 2.10) in the hope that this could provide a source of antigens that would be readily available for analysis (summarized in Table 4.1). Three sources of trophozoites were used: (a) Excysted *in vitro*, (b) trophozoites harvested from intestinal scrapings of nude rats and (c) trophozoites obtained from baby mice treated with gentamicin before being sacrificed.

Attempts to grow trophozoites obtained from cysts or from nude rat gut were unsuccessful. The trophozoites did not stick well to the culture tubes and did not survive for more than 48 hrs. However, when trophozoites were taken from baby mice, they were observed to adhere to the culture tube forming a monolayer

during the first 72 hrs similar to that observed with human cultures. However, during the next 24 hours the trophozoites lost the ability to attach and by the 5th day in culture very few trophozoites adhered to the wall, although numerous cells were seen swimming around in the characteristic fashion of *Giardia* trophozoites. During the first 36 hours, dividing cells were observed. In spite of these encouraging observations, the trophozoites eventually died off by the 6th day. Obviously the requirements of rodent *Giardia* appear to be different from those obtained from humans.

Because the rodent isolates could not be cultured, alternative sources of antigens were used in this study. Firstly, the axenic strain of *G. lamblia*, Portland-1 (PO-1), was used. PO-1 was originally isolated in 1976 (Meyer 1976) from a woman with an 8-yr history of diarrheal disease. It was cultured axenically and has been deposited with the American Type Culture Collection (ATCC) in 1980, with the identification ATCC 30888 (Bertram *et al.* 1983). This isolate has an intriguing history. Based on isoenzyme patterns, it was suggested that it might have been overgrown by a cat isolate prior to its deposition with the ATCC (Bertram *et al.* 1983). It was selected for use in this study because it was felt its antigens might be related more closely to the rodent isolates of *G. duodenalis* than would alternative sources of trophozoites of human. In the event that it proved impossible to obtain large quantities of *G. duodenalis* antigens then the antibody responses in infected rats could be carried out using PO-1 as antigen. PO-1 was in fact only used for comparative studies of cross-reactivity with the rat isolates. The second source of antigen for used in ELISA assays was obtained from excysted *G. duodenalis*, obtained *in vitro* from cysts purified from rat faeces (Section 2.9).

4.3 Standardization of ELISA

The ELISA is technically a very simple, sensitive and reproducible assay in which to quantitate antibodies to *Giardia*. It is an objective assay and can detect smaller amounts of antibody than is possible by immunofluorescence, an advantage that

Table 4.1 Cultural conditions used in attempts to axenize *G. duodenalis in vitro*[1].

Source of Trophozoites	Media[2]	Attachment[3]	Survival in hours
Excysted[4]	M3	–	<24
	HSP-3	–	<24
	TYI-S33[5]	–	<24
	Serum-free	–	<24
Mucosal scrapings (Rats)	BI-S33[5]	+	48
	BI-S33[6]	+	48
	TYI-S33[5]	+	36
Intestinal (Baby mice)	TYI-S33	+++	120–144

[1] The media listed were modified as described in Section 2.10.

[2] M3 (Meyer 1970); HSP-3 (Bingham & Meyer 1979); TYI-S33 (Keister 1983);. Serum free medium (Wieder *et al.* 1983); BIS-33 (Kasprzak & Majewska 1985) (Section 2.10.1)

[3] Attachment to the wall of tube: -, none; +, about 25% confluent; +++, about. 75% forming a monolayer for the first 72 hours, becoming detached during the next 24 hours, so that by the 5th day few cells were attached

[4] Cysts isolated from faeces and excysted according the to the method of Schaefer III *et al.* (1984), (see Section 2.9).

[5] TYI-S33 and BIS-33 media with ox-bile (0.075%) or with ox-bile replaced by fresh rat bile (Section 2.10.2).

[6] BI-S33 medium containing glycocholic acid instead of bile (Section 2.10).

makes it more applicable for studies of antibodies in fluids where the antibodies might be present in low titre.

4.3.1 Determination of specificities of conjugates

The working strengths and specificities of the alkaline phophatase-labeled anti-rat conjugates were determined using plates (Costar) coated with purified rat IgG, IgM, IgA, or IgE (10 μg/ml). Dilutions of each conjugate (1/100–1/1200) were reacted with the rat immunoglobulins at 37 C for varying periods. The anti-IgA and anti-IgM conjugates were isotype-specific (Table 4.2), as practically no cross-reaction was found even against high concentrations of heterologous Igs (100 μg/ml) coated onto the plate (data not shown).

4.3.2 Antigen coating conditions

Rat anti-PO-1 serum and PO-1 trophozoites were used to standardize the ELISA. In Figure 4.1, the results of using different numbers of cultured trophozoites to coat the wells are shown. Employing concentrations of 10^3–10^6 trophozoites per well as antigen, it can be seen that there was little difference in antibody binding in wells coated with 2×10^5 to 10^6 cells. With less trophozoites than this, the binding was less and sensitivity was reduced. Binding to intact and to sonicate antigens were also compared. Under the conditions of coating the wells, no difference was seen between the two preparations and there appeared to be no advantage in including protease inhibitors while preparing the sonicate or not (Table 4.3).

The effectiveness of three available microtitre plates were also compared during standardization of the ELISA. It was found that the optical density (OD) readings of negative controls in Costar plates were lower (data not shown) than in the Linbro polyvinyl or the Titertek polystyrene microtitre plates. Therefore, the assays using PO-1 as antigens were carried out in polyvinyl Costar plates (Lot No. 3175) using 2×10^5 intact trophozoites per well without protease inhibitors. In assays utilizing *G. duodenalis* as antigen, the same concentration of excysted

Table 4.2 Specificity of alkaline-phophatase rat conjugates as determined by ELISA[a]

Rat Immuno-globulin	Dilution of conjugates								
	Anti-Rat IgA			Anti-Rat IgM			Anti-Rat $F(ab)_2$		
	200	400	800	400	600	800	200	400	800
IgA	1.420[b]	**0.920**	0.570	0.100	0.089	0.063	1.500	**1.017**	0.620
IgG	0.019	0.014	0.011	0.079	0.066	0.014	**1.187**	0.602	.300
IgM	0.150	0.090	0.065	1.500	**1.070**	0.920	>2.000	1.937	**1.020**
IgE	0.025	0.019	0.014	0.092	0.077	0.025	>2.000	1.670	0.720

[1] Dilutions of each conjugate (1/100–1/1200) were reacted against all classes of rat Igs using plates coated with purified rat IgG, IgM, IgA or IgE and incubated for varying periods at 37 C.

[b] Optical density (OD) at 405 nm. Results shown are for the dilutions nearest the highest dilution of the conjugates giving an absorbance value of about 1.000 with the rat Ig solutions at 10 *u*g/ml after 4 hours of substrate incubation.

Figure 4.1 Antibody binding to different numbers of *G. lamblia* PO-1 trophozoites per well as detected by ELISA and read at OD 405nm. Intact washed cells in PBS_m were used to coat the ELISA plates at 10^6 (■- - -■) 5×10^5 (□—□), 2×10^5(●- - -●), 10^5 (▲—▲), 10^4 (▲- - -▲) and 10^3 (●—●). They were tested with rat anti-PO-1 serum. Normal SPF rat serum was tested against 10^6 (○- - -○).

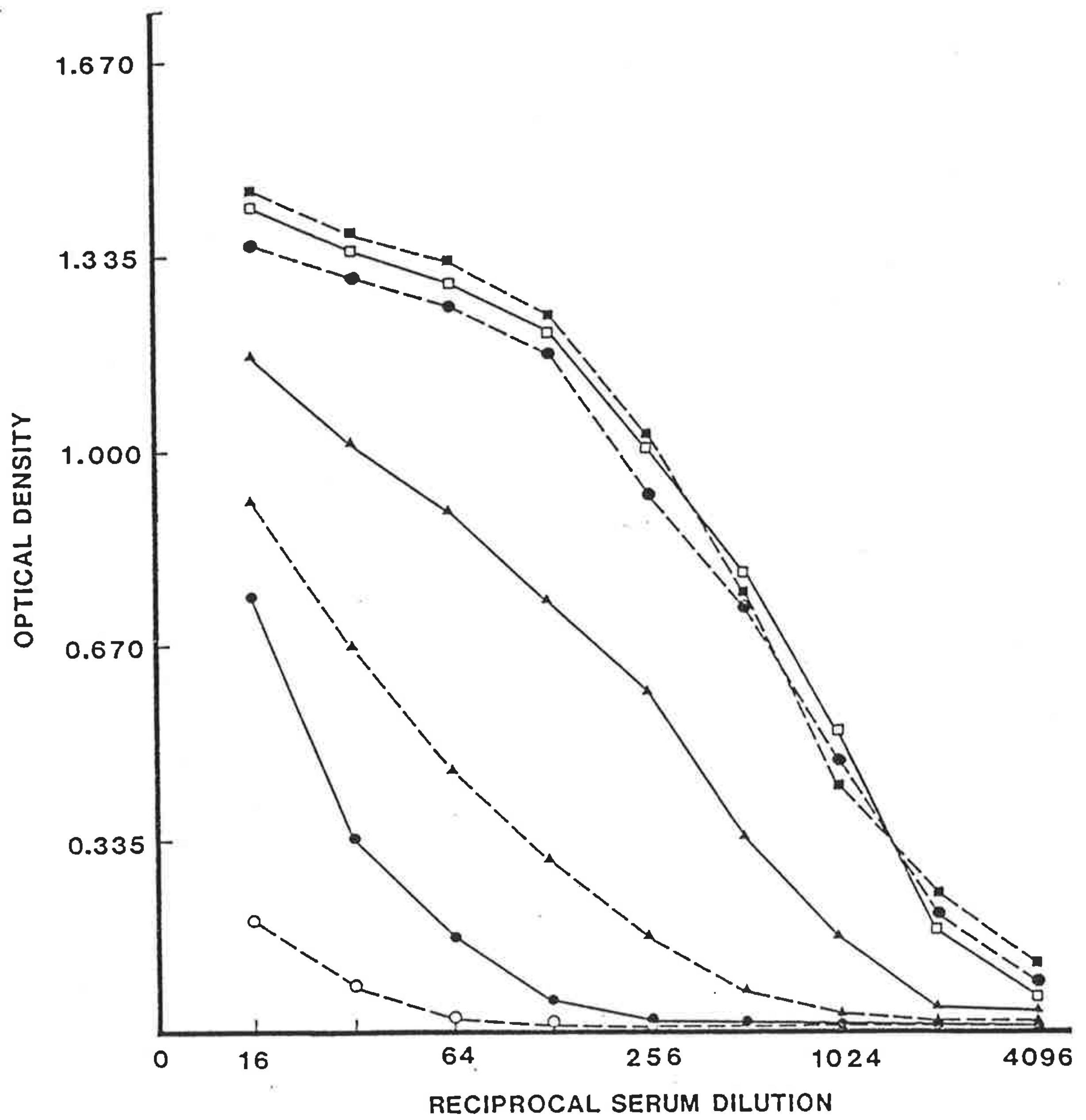

1.670
1.335
1.000
0.670
0.335
OPTICAL DENSITY
0
16
64
256
1024
4096
RECIPROCAL SERUM DILUTION

Table 4.3 Comparison of excretory-secretory antigens from trophozoites and antigens from sonicates of *G. lamblia* PO-1 trophozoites as sensitizing antigens for ELISA[a].

Dilution of Sera[b]	Intact Cells	Sonicate with PI[c]	Sonicate without PI[c]
64	0.968[d]	0.952	0.960
128	0.895	0.875	0.884
256	0.785	0.750	0.793
512	0.601	0.585	0.595
1024	0.498	0.510	0.480
2048	0.396	0.418	0.426
NRS[e]	0.092	0.110	0.096

[a] Intact cells or sonicates of trophozoite suspensions (2 x 10^6/ml) prepared as described in Section 2.17.3.

[b] Dilution of rat anti-PO-1 in ELISA well.

[c] Protease inhibitors (PI): 10 μl of 2 mg/ml cocktail.

[d] Optical density at 405 nm after 4 hours of substrate incubation and using rat anti-F(ab)$_2$-alkaline phosphatase conjugate (1:400). Each point is the mean of triplicate wells.

[e] Normal rat serum at 1:64 dilution.

trophozoites per well were used in Costar plates belonging to the same lot number. In this case the cells were sonicated in order to disrupt any cysts that did not excyst and no protease inhibitors were added.

4.3.3 Determination of endogenous alkaline phosphatase

Endogenous alkaline phophatase contributes considerably to background noise thus limiting both the sensitivity and specificity of ELISA. Therefore experiments were carried out to determine whether trophozoites or bile contained endogenous enzyme. Cells, sonicates or bile (diluted two-fold) were incubated with DEA buffer containing PNPP and absorbances (405 nm) were measured 90 minutes after the addition of the substrate. OD readings of <0.100 were obtained, indicating the absence of endogenous enzyme.

4.3.4 Crossreactivity with media components

The rat anti-PO-1 antiserum was tested for antibody specific for polypeptides from TYI-S33. This was investigated because PO-1 was grown in this medium and it was possible that the trophozoites used to raise the antiserum could have been contaminated with medium components. However, the OD readings recorded when the medium or its individual components were reacted against the antiserum were all <0.020, indicating that the antiserum possesses no detectable activity for polypeptides that might be present in the medium.

4.4 Antibody responses in rats during primary and secondary infection with *Giardia*.

Except for the experiments carried out in hypothymic (nude) rats and their heterozygous littermates, all subsequent experiments were carried out in DA rats. DA rats were chosen primarily because of easy availability and also, as discussed in Chapter 3, there were no clear differences in susceptibiltiy of different rat strains or

RT1 haplotypes to infection with either of the isolates. Two separate experiments were conducted:

(1) to analyse the antibody responses in DA rats using both isolates of *G. duodenalis*.

(2) to investigate the antibody responses in hypothymic (nude) rats and their heterozygous littermates using the mouse isolate of *G. duodenalis*.

Experiment 1. Two groups consisting of 35 rats each were infected intragastrically with 5000 cyts: the first group with the mouse isolate and the second with the rat isolate. In addition, 10 uninfected rats served as controls (5 at the beginning and 5 at the end of the experiment). The bile ducts of 5 randomly selected animals from each group were cannulated on days 10, 15, 21 and 42 after infection and blood was also collected on each occasion. The remaining animals in each group at day 42 were then treated with metronidazole as described in Section 2.4.5. Four weeks after treatment, tbe bile ducts of 5 randomly selected rats were cannulated and blood collected. The remainder were challenged with the homologous *Giardia* isolate. Bile and blood were collected from rats on days 10 and 15 after the secondary infection. The faeces of these rats were clear of cysts at the time of re-infection. None of the animals excreted cysts during the 14 day period following secondary challenge with either organism. Five of the uninfected control rats were sampled at the commencement of the experiment and the remainder at the conclusion. These animals were found during the course of the experiment to be excreting cysts of *Entameba* spp. They had been obtained from an SPF facility in South Australia (Section 2.1.1). At no time have any other parasites, including *Giardia* spp., been observed in animals from this source.

Experiment 2. The experiment with nude rats was carried out using only the mouse isolate of *G. duodenalis* because of the limited availability of these animals. Four nude (CBH/rnu) rats and 4 heterozygous littermates were

infected intra-gastrically with 5 x 10^3 cysts. Bile and sera were collected at two time-points: 3 weeks and 6 weeks after infection. Uninfected nude (2) and heterozygous (2) rats served as controls. The time course of infection in nudes and their littermates were described in Figure 3.11.

4.4.1 Serum and biliary antibody responses in rats

Individual serum and bile samples from rats infected with either the mouse or the rat *Giardia* isolates were assayed for specific anti-*Giardia* antibodies against homologous trophozoite antigens by isotype specific ELISA (see Section 2.18). The mean IgA and IgM antibody titres at various times after primary and secondary infections with the two isolates in DA rats are shown in Figures 4.2 and 4.3. Pools of equal volumes of individual bile samples at each time point were used to determine IgG titres, due to limited amounts of available antigen (Table 4.4). Anti-*Giardia* antibody titres in both sera and bile of nude rats and their heterozygous littermates are shown in Table 4.5. Prior to infection, the DA rats used in these experiments had low level significant titers of IgM antibody in serum and of IgG and IgA antibody in bile. These low levels of specific or cross-reactive antibodies appear related to the source of animals. SPF rats of the same strain from a different source (Animal Resources Centre, Western Australia) were found to have insignificant levels of serum and biliary antibodies (data not shown). The antigens responsible for generating the antibodies are not known. As indicated above, there is no evidence that the breeding colony from which the rats were obtained is infected with Giardia species. It is possible that *Giardia* and *Entamoeba* share cross-reactive antigens (Haralabidis 1984), but this possibility has not been explored.

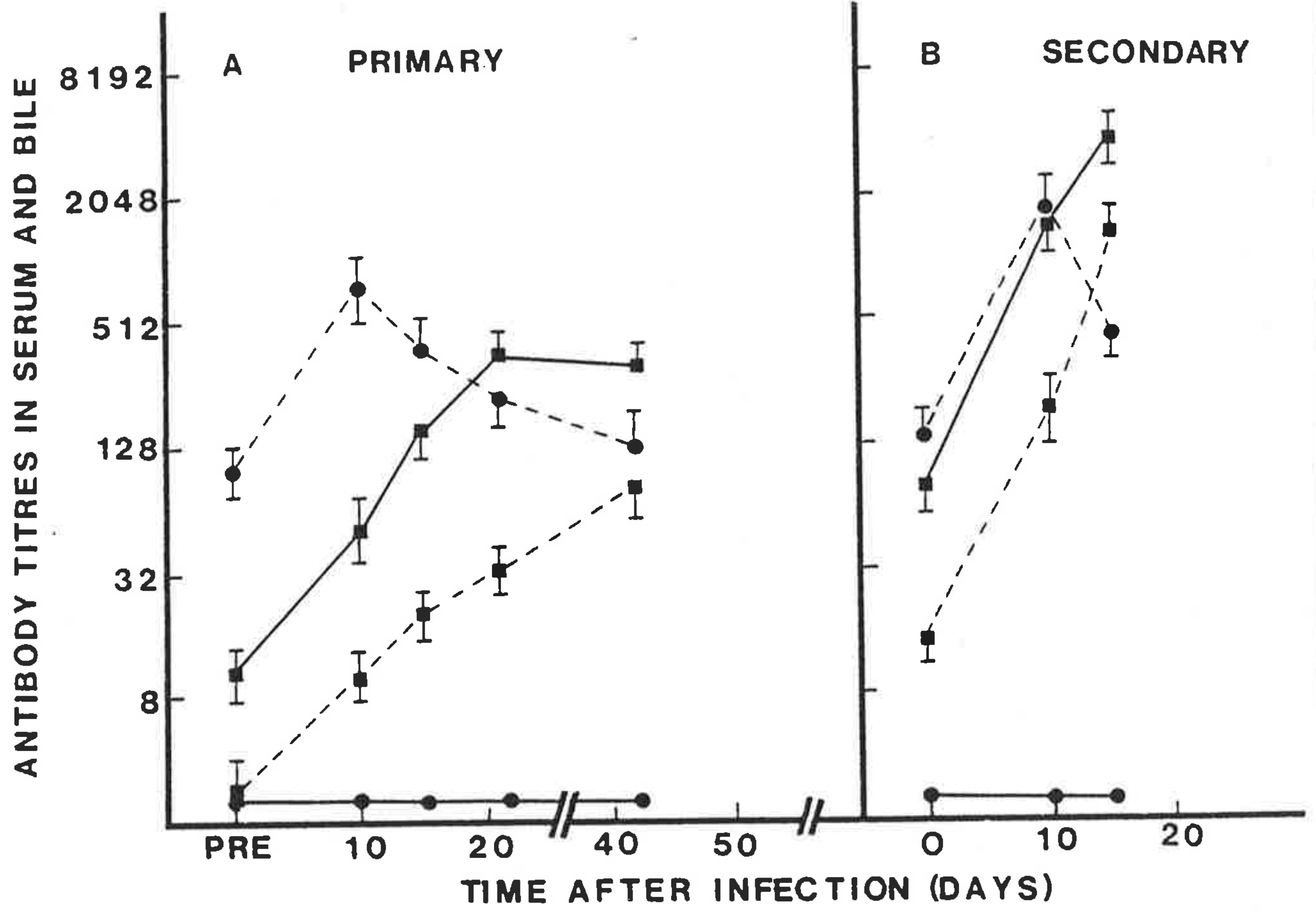
4.4.1.1 Serum antibodies

A rise in the titre of IgM anti-Giardia antibodies in serum was detected at 10 days after primary infections with either organism (Figures 4.2 and 4.3). The responses to the two isolates in DA rats were similar and the IgM antibody titres then de-

Figure 4.2 Specific antibody titres, against homologous trophozoite antigens, in the sera and bile of female DA rats infected with the mouse isolate of *G. duodenalis*. The primary infection with 5 x 10^3 cysts was terminated after 6 weeks with metronidazole and the animals were re-infected with the same dose of cysts 4 weeks later. Antibody titres were measured using isotype-specific ELISA assays. (Dotted lines) measurements on sera. (Solid lines) measurements on bile, (■) IgA antibodies, (●) IgM antibodies. Points are mean ± SEM for 5 animals.

(a) Primary infection.

(b) Secondary infection.


A
PRIMARY
B
SECONDARY
8192
2048
512
128
32
8
ANTIBODY TITRES IN SERUM AND BILE
PRE
10
20
40
50
0
10
20
TIME AFTER INFECTION (DAYS)

clined and had reached starting levels by 6 weeks after infection. An early rise in IgM antibody levels in DA rats was also seen during the secondary response to reinfection.

Levels of IgM antibody in the serum of CBH nude rats were not determined due to limited quantity of antigen available (Table 4.5). Prior to infection specific IgM levels in the sera of CBH rnu/+ were negligible. By the third week of infection, although they had increased, levels were lower than the corresponding time in DA rats and had decreased to lower levels by the 6th week.

Specific IgG antibodies to *Giardia* were not determined in the sera of DA rats but were assayed in CBH nude rats and their phenotypically normal littermates (Table 4.5). Prior to infection no specific IgG antibody activity was detected in the sera of both these groups of animals. By the third week, when they were next assayed, specific IgG antibodies had appeared in both with levels slightly higher in rnu/+ than in rnu/rnu rats.

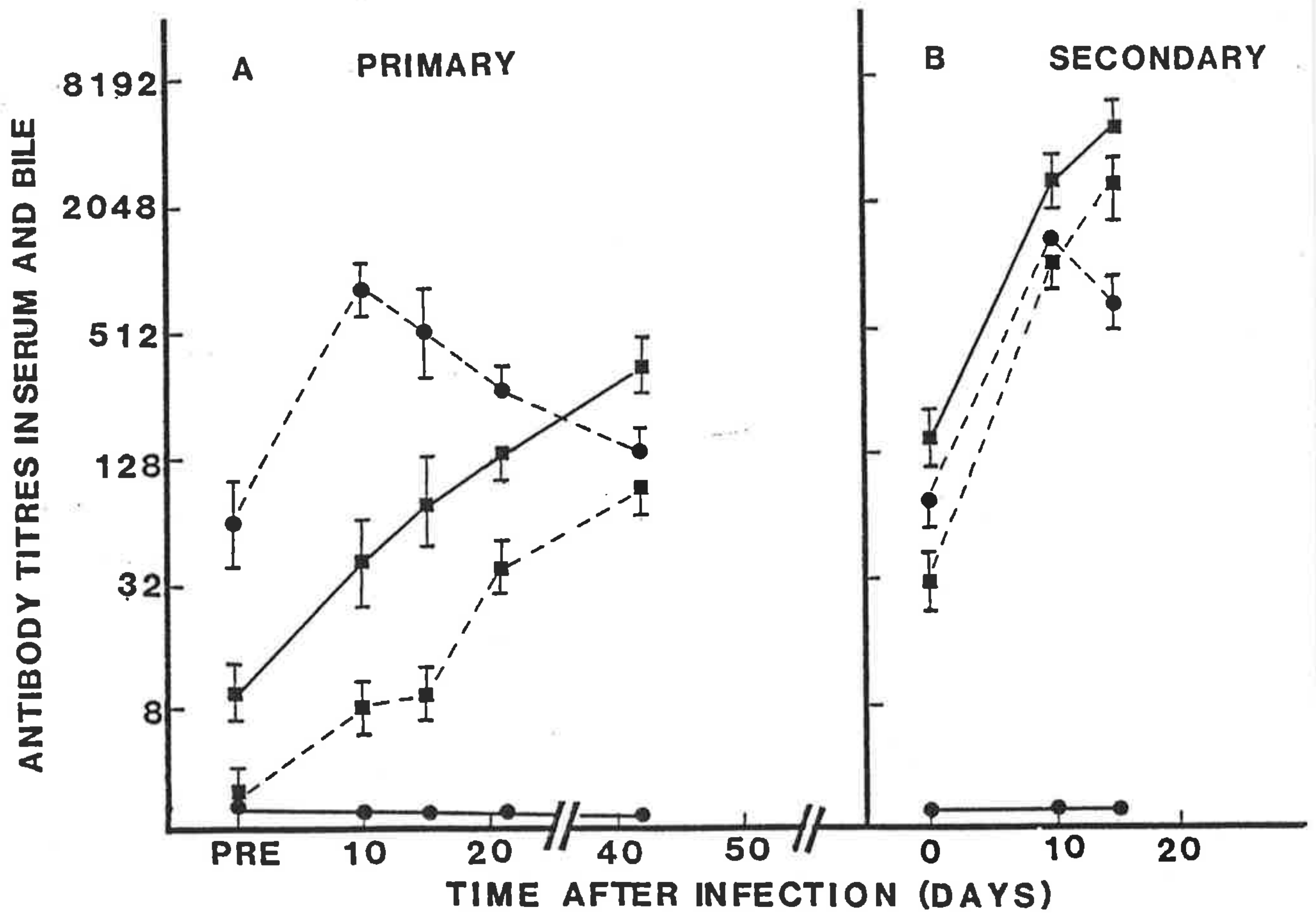
No further change was seen in nude rats in the following 3 weeks. In contrast, the level in the sera of heterozygotes had increased 13-fold. It was not possible to determine the day on which the peak value was actually reached due to the small numbers of rats used in these experiments, but it should be noted that the animals were continuously exposed to antigen from the intestinal infection throughout this period.

Specific IgA antibodies were not detectable in sera prior to infection in either DA rats, nudes and heterozygotes. Serum levels of IgA anti-*Giardia* antibodies in DA rats increased progressively and with similar kinetics throughout the primary responses to infection with the two isolates. They remained above baseline levels after clearance of the organisms with metronidazole, suggesting that the levels were not underestimated due to the presence of serum antigen during infection. Secondary infections provoked larger and more rapid IgA antibody responses in the sera of DA rats. Low levels of IgA antibody were detected in the sera of nude rats at three weeks after infection with no appreciable rise seen in the following three weeks. In contrast, specific IgA first detected at 3 weeks in the heterozygotes,

Figure 4.3 Specific antibody titres, against homologous trophozoite antigens, in the sera and bile of female DA rats infected with the rat isolate of *G. duodenalis*. All other details as described in legend to Figure 4.2. (Dotted lines) measurements on sera, (Solid lines) measurement on bile. (●) IgA antibodies, (■) IgM antibodies. Points are mean ± standard error for 5 animals.

(a) Primary infection.

(b) Secondary infection.


A
PRIMARY
B
SECONDARY
ANTIBODY TITRES IN SERUM AND BILE
8192
2048
512
128
32
8
PRE
10
20
40
50
0
10
20
TIME AFTER INFECTION (DAYS)

increased almost 4-fold by the sixth week of infection.

4.4.1.2 Biliary antibody responses

IgM antibodies were not detected in bile at any time during the primary or secondary infections with either isolate. In contrast, both IgG and IgA antibodies were present. The titres of anti-*Giardia* IgG antibody in pooled bile from DA rats undergoing primary and secondary infection with both isolates (Table 4.5) were lower than for IgA antibodies and continued to rise after IgM antibodies had peaked and declined. Like the IgA response, secondary infection provoked rapid increases in IgG antibody levels, greater than that elicited by the primary infection. The titers of specific IgG antibody in bile of heterozygotes during theprimary infection with the mouse isolate (Table 4.5) was similar to that seen in the DA rats whereas the titers in nude bile were much lower during the third and sixth weeks of infection. There was only a two-fold difference between the third and sixth week of infection in nudes.

IgA antibody levels in bile rose during primary infections with both isolates in DA rats, in parallel with the increases of IgA antibodies already described in serum. The titres of antibody reached during infections with the two isolates were similar and in both cases exceeded serum titres approximately 4-fold. Biliary IgA antibody titres declined during the four weeks after treatment with metronidazole but did not reach pre- infection levels. Challenge with the homologous organism caused a large secondary biliary IgA antibody response in DA rats.

Before infection with the mouse isolate, no IgA antibody activity was detectable in the bile of either the nude or the heterozygous rats. By the third week after infection, specific IgA antibodies were detected in both groups although there was an 11-fold difference between them (Table 4.5). The levels increased by the sixth week in the rnu/+ in contrast to that seen in rnu/rnu rats in which levels decreased three-fold.

4.5 Immunoglobulin levels in bile from CBH rnu/rnu and CBH rnu/+ rats during infection with the mouse isolate of *G. duodenalis*

The observation of IgA antibody activity noted in bile paralleled the level of IgA present in bile in both nudes and heterozygous littermates (Table 4.6) in that IgA concentrations in bile of the heterozygotes increased by the sixth week above levels seen in the third week. In contrast, the level of IgA concentration in the bile of nude rats increased 6-fold by the third week and declined in concentration by the sixth week of infection.

In nude rats, IgG levels did not change at three weeks of infection but by the sixth week had risen slightly (Table 4.6), again paralleling the observation noted in the 2-fold rise in antibody activity of this isotype in bile of these rats (Table 4.5). In contrast, the IgG levels in the heterozygous rats had increased greater than 2-fold by three weeks and the levels were increased by the sixth week of infection (Table 4.6), similar to the IgG antibody activity (Table 4.5)

4.6 Discussion

Studies of growth stimulation by whole bile or bile components in medium with serum (Farthing *et al.* 1983, 1985b; Gillin *et al.* 1986; Keister 1983) support the idea that biliary lipids are important for growth of *Giardia in vivo* and may help to determine the specific colonization of the small intestine by this pathogen. More than 50 years ago, Hegner & Eskridge (1937) showed that administration of sodium glycocholate and sodium taurocholate resulted in increased numbers of trophozoites in the small intestine of rats but sodium glycocholate was the more effective stimulant. Biliary diversion studies in the rat *in vivo* had also strongly suggested the dependency of *Giardia* growth on luminal bile (Bemrick 1963). These observations justified the use of either fresh rat bile or sodium glycocholate in the

studies of growth stimulation of the rodent isolates. However, even when combined with the other cultural conditions as described in Section 2.10, it proved impossible to initiate an *in vitro* culture of these organisms.

Previous work has shown the appearance of anti-*Giardia* IgA antibodies in the bile of rats inoculated intra-duodenally with axenic trophozoites of the Portland strain of *G. lamblia* (Loftness *et al.* 1984). The results reported here comprise the first description of a biliary response to a natural *Giardia* infection. In rats, up to 90 % of the IgA in the intestinal lumen is delivered via the bile (Lemaitre-Coelho *et al.* 1977, 1978). Therefore, the measurement of biliary antibody responses can be expected to reflect the overall secretory immune reponse against the parasite. Secretory antibodies have been detected in the intestines of mice infected with *G. muris* (Heyworth 1986; Roberts-Thomson & Anders 1984). In this species, IgA is transported directly across the gut epithelium and biliary transport is relatively insignificant (Underdown 1986).

Specific antibodies have been measured by an isotype-specific ELISA in sera and bile from DA rats infected with each of the rodent isolates of *G. duodenalis*. The findings show that primary and secondary infections with the two isolates of *G. duodenalis* lead to transient IgM antibody responses in serum, followed closely by sustained IgA antibody responses. There was a parallel rise in biliary IgA antibody titers, consistent with the known origin of most of the IgA in bile from blood-borne IgA (Jackson *et al.* 1978; Orlans *et al.* 1978). In addition, the increase in titers after secondary challenge confirm the existence of immunological memory in secretory immunity (Andrew & Hall 1982b). IgG antibodies were also detected in bile, consistent with other studies reporting the presence of this antibody class in response to intestinal infections (Jackson & Cooper 1981). No IgM antibodies were detected in bile at any time, although it is possible that an early transient response (Jackson & Walker 1983; Dahlgren *et al.* 1986) could have been missed.

In view of the extended course of *Giardia* infections, it is more likely that IgA antibodies rather than IgM antibodies could be involved in elimination of the parasite. Furthermore, both IgG and IgM immunoglobulins are subjected to rapid

degradation in the intestinal lumen (Haneberg 1974; Haneberg & Endresen 1976; Richman & Brown 1977); therefore, a protective function of either in the gastrointestinal environment is questionable. In contrast, sIgA retains antibody activity in the lumen due to its resistance to proteolysis. This resistance is due to the covalent binding of SC (Lindh 1975) whereas in sIgM there is lack of covalent binding, with SC being present in a more *open* quarternary structure than in sIgA (Brandtzaeg 1975).

The finding of specific antibodies in the bile and sera of nude rats, albeit in low titers, was surprising since infection of nudes with intestinal parasites is associated with an absence of specific antibody response either in serum or bile suggesting that parasite-specific antibody responses in intact rats are thymus-dependent (Rose *et al.* 1984; Vos *et al.* 1983). This is consistent with the lack of specific response observed in nude rats with other T-dependent antigens such as SRBC or tetanus toxoid (Andrew & Hall 1982; Vos *et al.* 1980b). However, this contrasts with the normality of their responses to T-independent antigens in which essentially normal titres of specific antibodies appear in bile (Andrew & Hall 1982a) and in serum (Vos *et al.* 1980). It is noteworthy that IgA levels in the bile of nude rats are higher than those in thymus-bearing littermates (Bazin *et al.* 1980; this study). This could be due to T-independent responses to different antigenic stimuli by the intestinal flora. Functional assessment of nude rats indicates a normal B cell response is present (Vos *et al.* 1980).

Low but significant numbers of cells bearing T-cell markers can be found in young animals (Brooks *et al.* 1980; Festing *et al.* 1978). Recent studies have shown that nude rats at older ages reveal large numbers of cells with T-cell markers (Schuurman *et al.* 1985; Vaessen *et al.* 1986). It is possible that some of the cells are functional T cells, possibly helper T cells (T_h), which in cooperation with B cells, can produce low level IgA antibodies to environmental antigens including *Giardia*; or *Giardia* could trigger primary B cells directly to produce IgA in the absence of T cells suggesting that, whereas T cells may not be essential for IgA production, their presence is necessary to achieve normal levels in serum and bile.

It is also possible that the gradual trend towards reduced cyst excretion in the nude rats (Figure 3.11) reflects the gradual effects of the inefficient immune response in these animals.

The mechanisms of protective immunity in giardiasis is still poorly understood. The chronic infections in hypothymic rats suggest a role for T lymphocytes, as has been suggested from studies on murine giardiasis (Roberts-Thomson & Mitchell 1978; Stevens *et al.* 1978). Nude mice are unable to clear infection with *G. muris*, yet when reconstituted with lymphocytes either from naive or immune mice they clear the infection in a normal fashion (Roberts-Thomson & Mitchell 1978). Presumably, as discussed earlier (Section 1.8.2.2), these cells contained T_h which resulted in the production of specific IgA antibodies. Both nude mice (Heyworth 1986) and mice treated with anti-mμ (Snider *et al.* 1985) have impariment of intestinal antibody production. There is evidence to suggest that $T_h$cells play a part in clearance of the organism, since nude mice have been found to have a more profound deficiency of T_h (L3T4) cells leading to a defective production of trophozoite-specific antibodies (Carlson *et al.* 1987). In addition, administration of anti-L3T4 MoAb (which selectively depletes T_h cells) to previously immunocompetent Balb/c markedly impairs their capacity to eliminate *Giardia* (Heyworth *et al.* 1987). It remains to be seen whether depletion of T_h cells in Balb/c mice leads to impairment of the IgA antibody response to *G. muris* antigens. Furthermore, the link is at present only circumstantial between deficiency of T_h cells, defective IgA antibody responses and impaired expulsion of *Giardia*.

As discussed above, the two isolates of *G. duodenalis* are, in general terms, comparably immunogenic. There is no clear evidence that the different infections produced by the isolates can be accounted for simply in terms of the magnitude of the secretory antibody response, although peak levels of IgA antibodies in bile were reached earlier during the less chronic infection produced by the mouse isolate.

Table 4.4 Titres of anti-*Giardia* IgG antibodies in pooled bile collected from DA rats[1].

	Days after Infection				
		Primary		Secondary	
Days	Pre	20	40	10	20
Mouse *Giardia*	12[2]	16	66	294	1042
Rat *Giardia*	12	18	72	388	1229

[1] Equal volumes of bile from 3 rats for each time-point were pooled and asayed for IgG antibody activity against the homologous *G. duodenalis* isolate in an indirect ELISA as described in Material and Methods.

[2] Each point is the mean titre of duplicate determinations and expressed as reciprocal serum dilution.

Table 4.5 Antibody response to mouse *G. duodenalis* in nude CBH rats and heterozygous littermates.

		ELISA titres					
	Weeks after	rnu/rnu			rnu/+		
Sample	Infection	IgM	IgG	IgA	IgM	IgG	IgA
Bile	Pre[a]	–[b]	–	–	–	–	–
	3	–	4[c]	14	–	18	153
	6	–	9	12	–	82	316
Serum	Pre	ND[d]	–	–	–	–	–
	3	ND	12	6	46	21	11
	6	ND	11	4	15	282	42

[a] Uninfected control rats.

[b] Negative reaction in a 1:2 dilution.

[c] Each point is the mean titre of two rats expressed as reciprocal serum dilution.

[d] Not determined.

Table 4.6 Levels of IgA and IgG in bile of nude CBH rats and heterozygous littermates as a function of time after infection with the mouse isolate of *G. duodenalis*[a]

Weeks after Infection	Immunoglobulin levels (μg/ml)[b]			
	IgA		IgG	
	rnu/rnu	rnu/+	rnu/rnu	rnu/+
Pre[c]	13.7[d]	42.5	15.7	18.5
3	87.2	61.1	15.8	39.4
6	38.2	$>$ 160	23.7	77.4

[a] Levels of IgA and IgG were determined by an isotype-specific ELISA using plates coated with immunopurified rat reagents

[b] The IgA and IgG concentrations were calculated by referral to a standard curve using immunopurified rat IgA and IgG, respectively

[c] Uninfected normal controls

[d] Each point is the mean of duplicate determinations for 2 rats

Chapter 5

In vivo Microenvironments during Infection with *Giardia duodenalis*

5.1 Introduction

In attempting to explain the different patterns of infection in DA rats with what appear to be very similar morphological strains of *G. duodenalis* (see Chapter 8), it was felt that examination of the small intestinal microenvironment could provide clues to the different mechanisms involved in colonization by the two organisms. The previous chapter has clearly illustrated that infection with these gut lumen-dwelling parasites leads to specific antibody responses in both serum and bile which are similar in this model to those observed in the murine model (Anders *et al.* 1982; Andrews & Hewlett 1981; Loftness *et al.* 1984; Underdown *et al.* 1981). There have been reports of the detection of anti-*Giardia* antibody (IgA or other isotype) in intestinal secretions other than bile (Anders *et al.* 1982; Snider *et al.* 1985; Snider & Underdown 1986) and on trophozoites harvested from the intestine of mice (Heyworth 1986; Roberts-Thomson & Anders 1984)) and from patients (Briaud *et al.* 1981).

Studies in the murine model have provided compelling evidence that clear-

ance of intestinal *Giardia* infections is likely to be antibody dependent. Elegant studies by Snider *et al.* (1985) have shown that treatment of immunocompetent mice with anti-IgM antisera from birth leads to prolonged infection with *G. muris* and is associated with defective secretory immunity as well as systemic humoral immunity. Recent studies in nude mice have strongly suggested that impaired clearance of *G. muris* may be due to defective production of anti-trophozoite antibodies secondary to helper/inducer T cell deficiency (Heyworth *et al.* 1987).

In the present chapter the aims were (a) to determine whether there were differences in the delivery of secretory antibody against surface antigens during the course of infection with the two isolates of *G. duodenalis* in DA rats; (b) to compare the delivery of intestinal antibody against surface antigens during the response of the DA rats and CBH rnu/+ rats to infection with the mouse isolate of *G. duodenalis* to that observed in CBH rnu/rnu nude rats and (c) to correlate the distribution of trophozoites and cysts in the small intestine during the course of the infection and with the presence of specific antibodies on their surfaces.

5.2 Distribution of trophozoites and cysts in the intestines of infected rats

5.2.1 Trophozoite numbers

Trophozoite numbers in the small intestines of DA rats were studied in two groups of animals: group 1 comprised of 15 rats infected with the mouse isolate, and group 2 also comprised of 15 rats infected with the rat isolate. The trophozoites were harvested from the small intestine as described in Section 2.14 and 2.15. By day 7, large numbers of trophozoites were present in both groups of rats and they had increased in similar proportions by day 10 (Figure 5.1). Peak trophozoite numbers were observed at day 15 in rats infected with the mouse isolate, while in rats infected with the rat isolate a plateau was reached between days 10-21. Trophozoite numbers had started to decline by day 21 in the case of infection with the mouse isolate and

by day 42 trophozoite numbers were greater than 1000 fold lower than at the peak level on day 15 post-infection. In contrast, rats infected with the rat isolate had undiminished numbers of trophozoites at 6 weeks of infection.

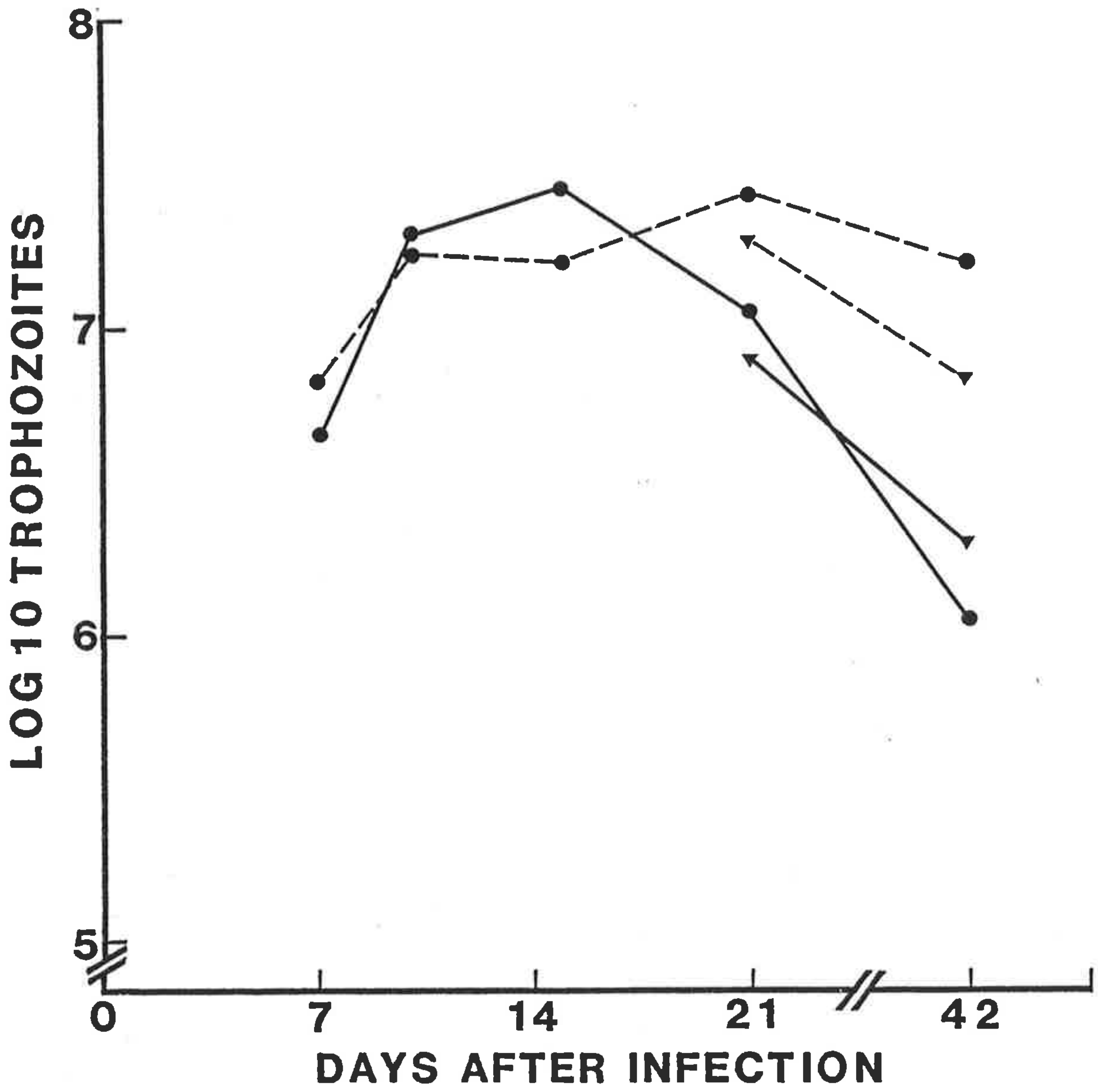
Figure 5.1 also shows the total numbers of trophozoites in the small intestine of CBH rats (4 rnu/rnu and 4 rnu/+) infected with the mouse isolates. At both time points the numbers of trophozoites were higher in the rnu/rnu as compared to the rnu/+. However, observations were made only at two time points and peak trophozoite numbers were not determined. However, this might be expected to have occurred between days 10–21 for the heterozygotes, while indefinite infection of rnu/rnu rats has already been described, based on observations of cyst excretion (Figure 3.11).

5.2.2 Localization of trophozoites and cysts

The distribution of trophozoites and cysts in the small intestine (using the methods as described in Section 2.15) in DA rats (the same animals described in Section 5.2.1) at different time points during infection with both isolates of *G. duodenalis* is shown in Figure 5.2. At no time were there any trophozoites or cysts detected in the duodenum of these rats. In infections with the rat isolate approximately 1000 fold more trophozoites were recovered from the jejunum compared with the ileum by the seventh day after infection but by the tenth day, when infection was maximal, the trophozoites were evenly distributed between the jejunum and the ileum. The situation remained unchanged for the rest of the period of observation. In contrast, in the infections caused by the mouse isolate the jejunal segment was the site of of maximum trophozoite numbers throughout the 6-week period, although large numbers of trophozoites were also recovered from the ileum.

Encystation is an important physiological process by which trophozoites are lost from the intestine and it is conceivable that changes in the intestinal environment during the immune response to the parasite could favor encystation and tip the balance in favor of the host. For this reason, the location of cysts and correlation with trophozoite distribution was studied in the same animals. Cysts were

Figure 5.1 Total number of trophozoites in the small intestine of rats infected with either the mouse isolate or the rat isolate of *G. duodenalis* (5 x 10^3) cysts. (●- - -●) DA rats infected with the rat isolate; (●—●) DA rats infected with the mouse isolate; (▲- - -▲) CBH rnu/rnu rats infected with the mouse isolate and (▲—▲) CBH rnu/+ rats infected with the mouse isolate. CBH rats were assayed only at days 21 and days 42. Each point represents the mean number of trophozoites for three animals except for those of the rnu/rnu and rnu/+ when only two rats were tested at each time point.


8
7
6
5
LOG 10 TROPHOZOITES
0
7
14
21
42
DAYS AFTER INFECTION

observed in the intestine from day 7 in both infections. Throughout the infection, large numbers of cysts were present in both segments of the small intestine, although they tended to be more numerous in the ileum. An equilibrium in the proportion of trophozoites and cysts was reached between 15 days and 6 weeks after infection with the rat isolate. At most time points, trophozoites outnumbered cysts by 1000 fold in both segments of intestine.

In the case of infection with the mouse isolate, the proportion of cysts and trophozoites at 15 days after infection was similar in both segments to that observed with the rat isolate. However, by 3 weeks after infection, the proportion of cysts in the ileum had risen and by 6 weeks cysts outnumbered trophozoites in this segment. At this stage, the total numbers of cysts and trophozoites had fallen significantly.

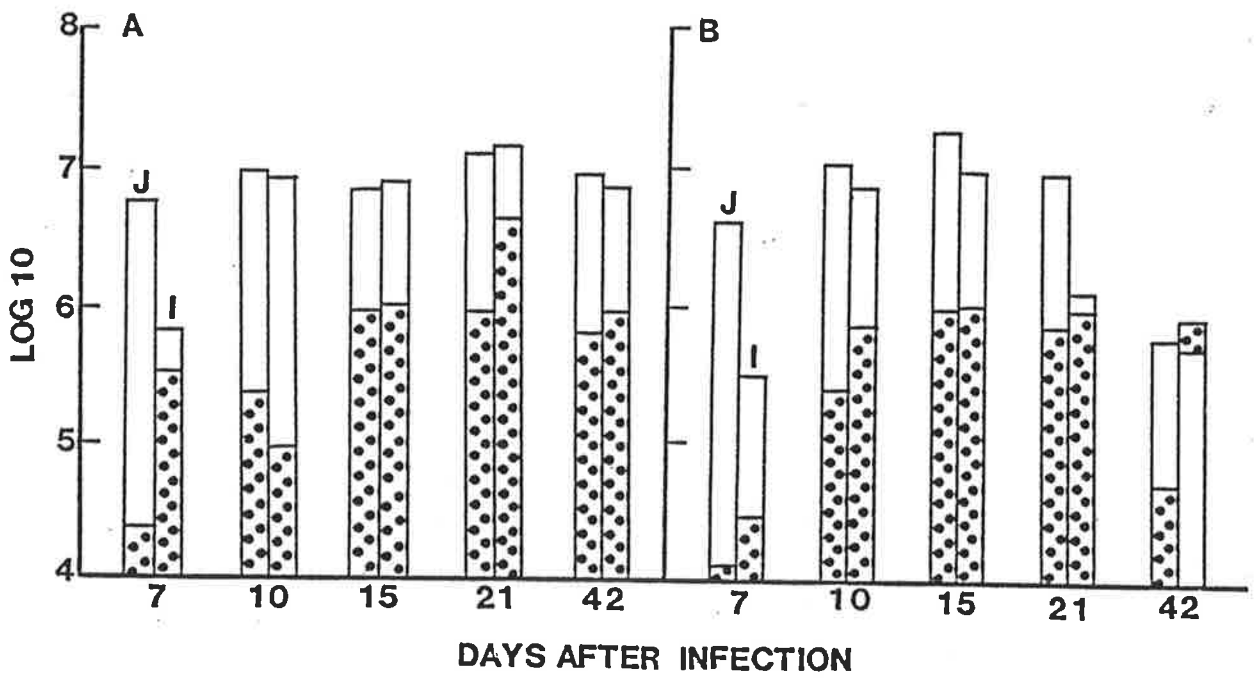
Trophozoites and cysts were divided into two separate populations: those found in the luminal wash (that is, intestines were flushed gently with 10 ml of ice-cold PBS_m as described in Section 2.14 and collected within five minutes) and those associated with mucus (collected after allowing the intestinal segments to stand on ice for 20 minutes). Tables 5.1 and 5.2 summarize the distribution of these populations for trophozoites and cysts, respectively. The greater proportion of trophozoites were associated with clouds of debris and mucus, except on days 7 and 10 after infection. In fact, trophozoites in various stages of encystation were observed in the mucus layer at all stages of the infection. As the infections progressed, the percentages associated with mucus increased and there were no apparent differences between the two infections.

In the case of cysts, the majority were associated with mucus at all time points and free cysts were rare at any time in the jejunum. The proportion of mucus-associated cysts also increased as the infection progressed and the picture was similar for both infections. The distribution of cysts and trophozoites was also examined in CBH rnu/rnu and CBH rnu/+ rats. As in DA rats, trophozoites and cysts were never seen in the duodenum. The distribution of trophozoites and cysts between free luminal fluid and mucus was not determined in these animals, but it was observed that in animals killed at 3 weeks and 6 weeks post-infection the

Figure 5.2 Localization of *G. duodenalis* trophozoites and cysts in the intestinal tract of infected DA rats. (□) trophozoites; (▣) cysts. The small intestine was cut into three parts: the duodenum; J, the jejunum; and I, the ileum. Cysts and trophozoites were enumerated in hemocytometer chambers by phase-microscopy. Total bar heights represent $\log_{10}$ of the geometric mean for three animals examined for the particular intestinal segment. At no time were either trophozoites or cysts observed in the duodenal segment.

(a) Rat isolate.

(b) Mouse isolate.



A
B
8
7
6
5
4
LOG 10
J
I
J
I
7
10
15
21
42
7
10
15
21
42
DAYS AFTER INFECTION

majority were present in association with mucus.

5.3 *In vitro* and *in vivo* reactions of specific IgA antibodies with trophozoites

5.3.1 Preliminary experiments

Before experiments were performed to detect whether specific biliary IgA antibodies were directed against surface antigens of trophozoites, a preliminary experiment was carried out with PO-1 trophozoites to determine whether incubation with bile would have a deleterious effect on cells. Trophozoites were incubated at either 37 C or 4 C for one hour with several dilutions of bile. At both temperatures and at all dilutions there was no lysis of cells; however, the motility of trophozoites (assessed visually by phase-contrast microscopy) was affected at 37 C. Movement was sluggish after incubation at 37 C and some clumping of cells occurred, particularly at the lower dilutions of bile. However, this phenomenon was not observed when the assays were carried out at 4 C.

Immune bile raised against the rat isolate of *G. duodenalis* was used in this preliminary work. Since this pooled immune bile cross-reacted in ELISA with PO-1 antigens, the agglutination and decreased motility observed at 37 C could have been due to the presence of cross-reacting antibodies. Belosevic & Faubert (1987) have observed that immune serum both lyses and immobilizes *G. muris* trophozoites *in vitro* and this has also been observed using heat-inactivated rabbit anti-PO-1 antiserum in this laboratory. Lysis was correlated with the presence of complement (Butscher & Faubert 1988; Hill *et al.* 1984) whereas immobilization appeared to be complement-independent. It is unlikely that complement proteins are present in bile. These observations have not been followed up but would be an interesting area for future work.

Secretory IgA antibodies that had bound to trophozoites *in vivo* were examined in a preliminary experiment by indirect IF on organisms harvested from the

Table 5.1 Distribution of trophozoites in intestinal mucus at various times after infection of DA rats with either the mouse or the rat isolate of *G. duodenalis*[a]

Days after	Mouse Isolate		Rat Isolate	
Infection	Jejunum	Ileum	Jejunum	Ileum
7	19	11	25	4
10	31	23	30	21
15	29	47	21	41
21	39	37	35	33
42	46	23	42	32

[a] Rats were infected and killed as described in Materials and Methods. Trophozoites were enumerated in hemocytometer chambers by phase-contrast micrsocopy. The data show the mean % of the trophozoites observed in association with mucus of 3 rats at each time point.

intestines of rats infected with the two isolates of *G. duodenalis*. For each isolate, three rats were infected and the trophozoites were recovered after sacrifice at 7, 10 and 28 days after administration of cysts. The examination at 7 days was expected to precede the IgA antibody response in bile (Figures 4.2 and 4.3) and therefore to provide a negative control for non-specific binding of IgA to the trophozoites.

The results (data not shown) show that very few trophozoites obtained 7 days after infection had detectable surface-bound IgA. By 10 days after infection, more than 70% of the trophozoites reacted with anti-IgA. The proportion of trophozoites bearing IgA on their surfaces had increased slightly when examined 28 days after infection. Nearly all cells were stained after trophozoites were incubated with immune bile, confirming the presence in the bile of secretory IgA antibodies directed at trophozoite surface antigens. Controls for autofluorescence and for non-specific binding of rabbit and rat immunoglobulins were negative in each case.

5.3.2 Binding of IgA antibodies to trophozoites recovered from normal DA rats, CBH rnu/rnu and CBH rnu/+ rats

Indirect IF on trophozoites harvested from DA rats infected above (Section 5.2.1) confirmed results obtained in the preliminary experiment (Section 5.3.1), as shown in Table 5.3. The IF was performed as described in Section 2.19. By the tenth day of infection more than 65% had detectable surface-bound IgA antibodies. The proportion of cells bearing IgA on their surfaces increased as the infection progressed. At any given time during the primary infection, there was always a subpopulation of trophozoites that had no detectable bound antibody. This was also true when cells were exposed to bile from a contemporary infection. This contrasted with reactions with homologous hyperimmune bile when essentially all trophozoites were labelled. When the proportion of trophozoites with bound IgA during infection with either isolate was analysed according to whether or not they were mucus-associated, there were no significant differences observed between the free and mucus-bound popula-

tions at any time during infection (Table 5.4). With both isolates no fluorescence of cysts were seen at any stage during the infection.

Trophozoites from CBH rnu/+ rats showed surface fluorescence after incubation with antibodies against rat IgA. The percentages of fluorescent cells detected at the two time points tested were slightly lower than at the corresponding time points during infection with the mouse isolate in DA rats (Table 5.5). Prior incubation with contemporary bile from CBH rnu/+ donors did not increase the proportion of trophozoites with bound IgA. In contrast, only small percentages of trophozoites from nude rats showed evidence of *in vivo* binding of IgA antibodies at both times (Table 5.5). Furthermore, incubation with contemporary bile from infected nude rats did not increase the proportion of trophozoites with bound IgA.

5.4 Determination of neuraminidase activity in trophozoites

Because of the observations made in the series of experiments described in this chapter regarding the association of trophozoites with mucus, it was felt worthwhile to investigate whether *Giardia* trophozoites possessed neuraminidase. This enzyme could be involved in the destruction of mucin and thus allow the parasite to colonize the mucus layer. The procedure followed was a simple one described for rapid screening of neuraminidase activity and applied to another protozoan, *T. cruzi* (Pereira 1983). This assay is based on the ability of peanut lectin to agglutinate mature human red blood cells only after desialylation of the erythrocytes. A correlation has been demonstrated between the amount of sialic acid released from the red cells with the lectin hemagglutination titer. The assay was performed using the PO-1 strain of *G. lamblia* as described in Section 2.17. Figure 5.3 demonstrates that no enzyme activity was detected by this method when either whole trophozoïtes, membranes or concentrated culture filtrates were assayed.

5.5 Induction of encystation of trophozoites *in vitro*

Several attempts were made over the four-year period of this study to induce encystation of the PO-1 strain of *G. lamblia* in order to characterize the different cell surface antigens of the parasite during the developmental stages of encystation as well as during excystation. Excystation (see Section 2.9) (of either human *Giardia* or *G. duodenalis* rodent isolates) was performed successfully always with a yield greater than 75%.

Initial attempts at encystation followed the methodology described for *Entameba* spp (see Section 2.16). These included with or without modifications: (a) the use of the axenic encystation medium (AEM) developed for *E. invadens* (Rengpien & Bailey 1975); (b) lowering the osmotic pressure and depletion of nutrients from the medium as used for *E. invadens* (Avron *et al.* 1986) and *E. histolytica* (Chayen *et al.* 1988). Although these procedures did induce a small percentage of PO-1 trophozoites to encyst, the numbers undergoing encystation were not greater than in control cultures using either TYI-S33 or PBS_m. *Entameba* inhabits the large bowel and perhaps the encystation factors there differ from those of the small intestine, which *Giardia* inhabits.

Since a large proportion of both trophozoites and cysts were associated with mucus, it was felt that factors in the mucus could play a role in encystation. Indeed, trophozoites in various stages of encystation were observed in the mucus layer (see Section 5.2.2). Therefore, experiments were carried out to observe whether mucin and sugars present in the mucin (N-acetyl-glucosamine; L-fucose; sialic acid) could act as stimuli for encystation. When trophozoites were incubated with either hog stomach or submandibular gland mucin at several concentrations (as described in Section 2.16), commencement of encystation of trophozoites was apparent within thirty minutes at 37 C (Figure 5.4). Maximal induction of encystation (approximately 30% completion) occurred at 72 hours with mucin at a concentration of 8 mg/ml, although induction was apparent at all concentrations used. Figure 5.4

shows cysts of PO-1 induced with mucin (8 mg/ml). In addition, even a low concentration of L-fucose (2 mM) caused the appearance of cysts during the five days of incubation. At no time was there 100% encystation although, when transferred to the encystation medium, most trophozoites began to do so, as judged by the appearance of increased refractility of the dorsal surface. Many of these refractile organisms at some point either disintegrate during the encystation process or remain in bizarre shapes covered by presumptive cyst wall, with flagella protruding and beating vigorously. In fact, it was observed that the flagella beats vigorously during encystation, reminiscent of *Chilomastix* encystation (personal observation) and the cyst forms consequently rotate at a fast rate. The flagella do not appear to be enclosed in the cyst wall but rather get sheared off just before closure of the cyst wall in the region of the adhesive disc.

During the course of this work, Gillin *et al* (1987) reported the induction of encystation *in vitro* by exposure of trophozoites to primary bile salts. The yield was increased in slightly alkaline medium, with an optimum at pH 7.8 (Gillin *etal.* 1988). However, the exact stimuli that causes the induction of chitin synthesis by chitin synthetase, which has been reported in *Giardia* (Gillin *et al.* 1987), is unknown. More recently, two populations of trophozoites, based on WGA binding *in vitro*, has been demonstrated and N-acetyl-glucosamine has been described on the surface of one of these populations (Ward *et al.* 1988). Whether or not these cells represent trophozoites in varying stages of differentiation from trophozoite to cyst is unknown.

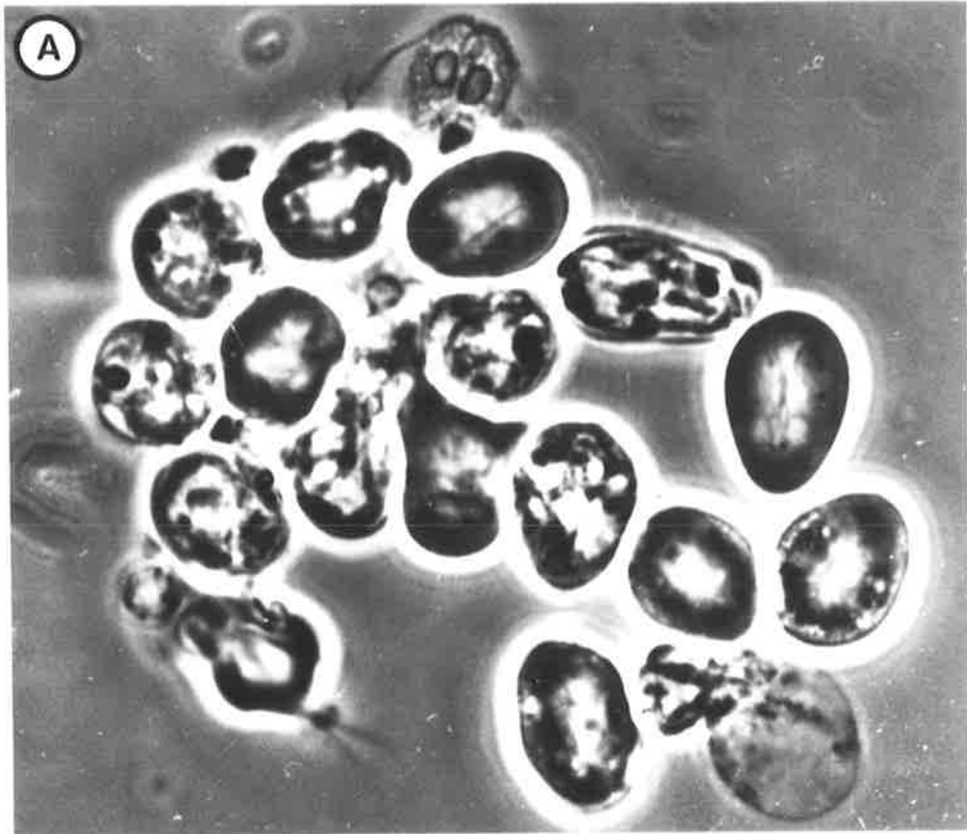
5.6 Discussion

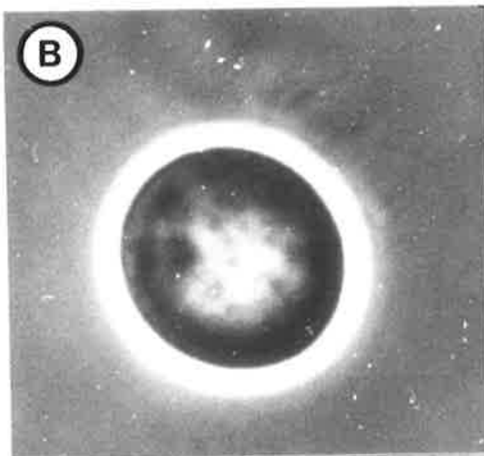
The precise distribution of trophozoites along the small intestine in humans has not been determined for practical reasons. It is generally believed that giardiasis is an infection of the duodenum and jejunum in humans (Ament & Rubin 1972; Saha & Ghosh 1977) because the most accurate method of diagnosis is duodenal fluid aspiration or biopsy. Localization studies carried out in rats have found trophozoites in

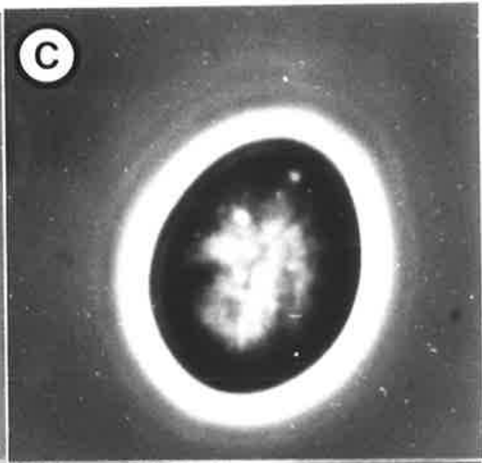
Figure 5.3 Peanut lectin agglutination titers against human red blood cells after incubation with 100 μU of *V. cholerae* neuraminidase (C, D); whole trophozoites of PO-1 (E); membrane preparations of trophozoites of PO-1 (F); culture medium supernatant concentrated 100× (G). Negative control wells contain no enzymes (A, B).

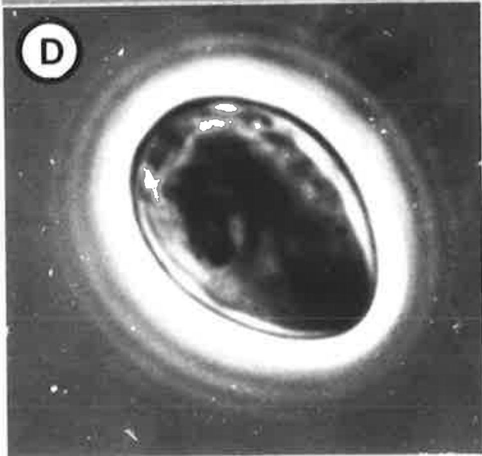
A
B
C
D
E
F
G
H

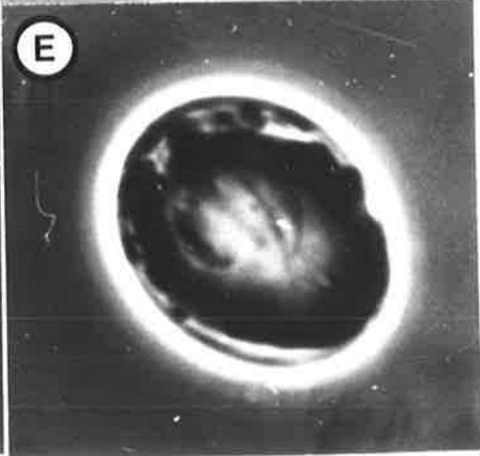
Figure 5.4 Phase-contrast microscopy of unfixed and unstained cysts induced *in vitro*, showing typical cyst refractility (halo around each cyst) and morphology. PO-1 trophozoites were incubated for 5 days in 2 mls of TYI-S33 medium supplemented with 8 mg/ml of mucin. Fully formed cysts (b-e) were usually seen clumped together (a) as depicted in this photograph.


A


B


C


D


E

duodenum, jejunum and ileum, and each area has been claimed to be the preferential habitation for the organism (Armaghan 1937; Hegner & Eskridge 1937, 1938; Kofoid *et al.* 1937). But these studies have been criticised for their limitations (Olveda *et al.* 1982).

In the *G. muris*-model, the greatest proportion of trophozoites occurs in the upper segments of the intestine, including the duodenum (Belosevic & Faubert 1983; Gillin *et al.* 1987; Olveda *et al.* 1982; Owen *et al.* 1979). However, in the latter study the actual numbers of trophozoites in the different regions of the gut were not determined (Owen *et al.* 1979).

Belosevic & Faubert (1983) have shown a significant posterior movement of the trophozoite population during infection with *G. muris*, leading the authors to suggest that it is related to density of population at different stages of infection. During the early phase, when the numbers were low, the majority of the parasites were located in the proximal 25% of the small intestine as reported by Owen *et al.* (1979). It is unknown whether this migration posteriorly is due to space availability in the gut or due to the immunological response of the host or changes in other factors influencing local microenvironments.

In this study, colonization was confined to the jejunum and ileum, although early colonization of the duodenum in the first week after infection cannot be ruled out. At 7 days post-infection, the majority of the trophozoites were recovered from the jejunum. Subsequently, approximately equal numbers were recovered from the jejunum and the ileum at most time points. These findings support to some extent the caudal drift of the population with time as described by Belosevic & Faubert (1983a) and show that in the rat, *G. duodenalis* colonizes most of the small intestine. No major differences were observed between the pattern of colonization by the two *G. duodenalis* isolates, except during the clearance phase of the infection with the mouse isolate.

The observations in this study that the majority of trophzoites are associated with mucus complements the results described by others (Gault *et al.* 1987; Owen *et al.* 1979; Poley & Rosenfield 1982; Rodrigues Da Silva *et al.* 1964). Owen *et*

al. (1979) demonstrated that relatively few trophozoites were found in intestinal washes. Ultrastructural studies revealed that trophozoites were mainly within the unstirred layer, either attached to the surface or within the mucus coat. Flushing the gut did not dislodge the latter population and did not alter the mucosal distribution (Owen *et al.* 1979). Owen's observations are therefore comparable with the observations described in this chapter. Incubating the intestine in ice-cold PBS_m for 20 minutes (which, by analogy, with *in vitro* observations, should result in detachment of trophozoites) and vigorous shaking released the trophozoites, great numbers of which were seen still attached to mucus strands. This made them difficult to count accurately in counting chambers. Nevertheless, the counts demonstrate that the majority of trophozoites or cysts, especially in the latter part of the infection, are found associated with mucus.

Cysts have been detected in the gut of suckling mice infected with *G. muris* and found in every intestinal segment including the duodenum (Gillin *et al.* 1987; Hill *et al.* 1983), although no mention was made of any association with mucus. This is confirmed by the findings of cysts in the jejunal and ileal segments of DA rats infected with *G. duodenalis*. Although not quantitated, cysts were also observed in the intestines of both CBH rnu/rnu and rnu/+ rats infected with the mouse isolate. To an even greater extent than with trophozoites, cysts were found to be almost entirely associated with the mucus layer. Very little is known about the regulatory mechanisms which control encystment of *Giardia* or the biochemical reactions that are responsible for the synthesis of the cyst wall. Recent reports suggest bile salts and an optimum pH of 7.8 are involved (Gillin *et al.* 1987; Gillin *et al.* 1988). The observations of the association between trophozoites and cysts with mucus in rats suggested that the microenvironment might itself be a stimulus for encystation. Microscopic examination of the mucus layer suggests that encystation occurs in this environment and the *in vitro* experiments with mucin and mucin-associated sugars provide preliminary evidence that mucin may be inductive. Only a proportion of the trophozoite population may be responsive to undergo encystation at a given time. Clearly, the proportion of receptive trophozoites and factors which alter them

could have dramatic effects on the size of the resident populations.

The demonstration of IgA bound to trophozoites confirms the observations of specific IgA antibodies in the infected rats and suggests that the luminal trophozoites are accessible to these antibodies. Intestinal IgA responses to infection have been described in *G. muris*-model (Anders *et al.* 1982; Heyworth 1986; Roberts-Thomson & Anders 1984; Snider *et al.* 1985). It is clear that there is a sharp rise in the proportion of trophozoites with surface-bound IgA from about the time when IgA antibodies can be detected in bile. Appropriate controls indicate that this is due to specific binding by IgA antibodies to trophozoite antigens. Several observations can be made. Firstly, at least part of the IgA antibody response in bile is directed against surface antigens of the trophozoites, in confirmation of a previous observation (Loftness *et al.* 1984). Secondly, approximately equal proportions of trophozoites from the two infections were labelled at any given time. Mechanisms by which trophozoites of the rat isolate evade the immune response are therefore more subtle than bulk modulation of complexed surface antigens or release of soluble antigens to compete for specific antibody (Nash *et al.* 1983). Thirdly, there is a significant population of trophozoites without bound antibody even during the late stages of infection with either isolate. A similar finding has been reported in the *G. muris*-model (Heyworth 1986), although in this study the proportion of IgA positive trophozoites was always less than 30%. Whether this reflects heterogeneity among the trophozoites (in terms of antigenicity or ability to modulate surface antigens) or the presence of micro-environments protected from antibody is not known, but the fact that these cells can bind antibodies from hyperimmune bile *in vitro* indicates that they bear antigens against which the host can eventually mount an immune response.

The small proportion of IgA^+ trophozoites obtained from nude rats complements the findings reported for nude mice (Heyworth 1986). Nude rats also have an impaired ability to clear *Giardia* (Figure 3.11) and have only low levels of specific IgA antibodies in bile. It is possible that inadequate production of trophozoite-specific antibodies leads to impaired clearance of the parasite. It is not known

whether in nude rats there is a deficiency of helper/inducer T lymphocytes as has been demonstrated in the nude mice (Carlson *et al.* 1987).

It has been suggested that IgA has an affinity for mucus (Clamp & Creeth 1984) and that microorganims bound by IgA antibodies also have increased affinity for the mucus layer (Magnusson & Stjernstrom 1982). Since a large proportion of the trophozoites were associated with mucus, an analysis was made of *in vivo* binding of IgA to the free and mucus-associated fractions of trophozoites harvested from the gut. Because it was difficult to perform assays on the trophozoites enmeshed in mucus, attempts were made to solubilize the mucus with several concentrations of N-acetyl-cysteine (Mucomyst). However, gentle mechanical shearing proved to be better, although it was not possible to entirely dissolve the mucus. The apparent lack of success could have been due to the fact that, although it has been shown that rat goblet cell mucin contains 34 S-S bonds per molecule, it is not reduced to subunits by sulfhydryl reagents (Forstner *et al.* 1973). Mucus strands or *flecks* covered with trophozoites were common. Nevertheless, no differences could be observed in the proportion of IgA and trophozoites between those attached to the mucus and those not associated with mucus.

The role that neuraminidase plays during the process of infection is unclear in both trypanosomiasis and amebiasis (Pereira 1983; Udezulu & Leitch 1987). However, with *V. cholerae* infections there is an apparent correlation between neuraminidase activity and the severity of the clinical signs and symptoms (Kabir *et al.* 1984). The enzyme cleaves terminal sialic acid residues from carbohydrate modified proteins. It could help parasites to penetrate the mucus layer covering the gut mucosa and the enzyme may also assist penetration of target cells or cause modification of cells that play a role in controlling a parasite infection. Both *T. cruzi* and *E. histolytica* shed the enzyme into the medium when cultured (Pereira 1983; Udezulu & Leitch 1987). *E. histolytica* is an invasive pathogen inhabiting the colon. It is possible that colonic mucins differ in composition from those mucins found in the small intestine particularly in rats (Forstner *et al.* 1973). Small intestinal mucins contain fewer sialic acid residues. *Giardia* is non-invasive and inhabits the small in-

testine and it appears not to require neuraminidase in order to penetrate the mucus barrier. In fact, the rodent *G. duodenalis* appear to associate preferentially with the mucus layer. It might be speculated that the role of the lectin that has been described on *Giardia* (Section 1.4.1) might be to facilitate this interaction.

In conclusion, it is likely that clearance of *Giardia* is dependent on anti-trophozoite antibody although the mechanism is not known. Recent observations in the *G. muris*-model supports the concept that clearance by cells is unlikely. In infected Balb/c and nude mice there are identical numbers of intraluminal phagocytes and cytotoxic/suppressor T cells (Heyworth *et al.* 1985a). In addition, mice with natural killer cell deficiency (beige mice) clear the infection normally (Heyworth *et al.* 1986).

Giardia have an intimate association with mucus and it is possible that the parasite prefers this microenvironment and exploits it to complete its life cycle. However, mucus has been shown to be important as an effector mechanism during the rapid expulsion of *Nippostrongylus brasiliensis* in immune rats (reviewed in Miller 1987) and *Trichinella spiralis* (Lee & Ogilvie 1982; Miller 1987). It is possible that changes in composition of mucus in immune animals, increase in mucus flow rate or interaction between mucus and IgA-coated trophozoites could facilitate the immune clearance of the trophozoites. On the other hand, these changes could achieve the same effect by trigerring more trophozoites into encystation, either by enhancing the physiological stimulus for differentiation or by creating an environment that is in some way hostile to the organisms. The increased ratio of cysts to trophozoites during the late phase of the infection with the mouse isolate would be consistent with this mechanism. Finally, the immobilizing effects of secretory antibody might reduce the ability of the trophozoite to hold their position against the flow of mucus and lead to gradual attrition of the population.

An explanation is required for why all trophozoites were not immunoglobulin positive during infections with either isolate of *G. duodenalis*. Similar observations have been described by Heyworth (1986) for *G. muris* infections. Recent reports suggest that antigenic variation occurs in *Giardia* (Adam *et al.* 1987) and that

it is not only an *in vitro* phenomenon (Aggarwal & Nash 1988). It has also been shown that gerbils develop either acute or chronic infections depending on the strain of *G. lamblia* isolate with which they are infected (Aggarwal & Nash 1987). The strain WB causes an acute infection and shows no change in surface antigens *in vivo*. In contrast, the strain causing the chronic infection (GS/M) changes its surface antigens *in vitro* more rapidly than does the WB isolate. It has yet to be demonstrated that the strain GS/M undergoes antigenic variation *in vivo*; if this is so, this could explain the development of chronic infections in gerbils with this organism (Aggarwal & Nash 1987). Whether or not antigenicv variation occurs with the two *G. duodenalis* isolates is unknown, although the data on binding of IgA antibodies *in vivo* are consistent with this sort of mechanim. If this is the case, it would appear that in hyperimmune animals the immune response eventually overhauls the antigen repertoire of the parasite.

An alternative hypothesis is also possible - the tempo of the immune response to various surface antigen may be different, leading to an antibody repertoire that only gradually recognizes the whole population. Nevertheless, this explanation requires that the trophozoite population is diverse antigenically with respect to its major surface antigens.

There is no satisfactory explanation from this study for the difference in the kinetics of infection between the two isolates of *G. duodenalis*. In terms of antibody titers in bile (Chapter 4), kinetics of the antibody response and proportion of trophozoites labelled by antibody, the infections are suprisingly very similar. Furthermore, the association of the two infections with mucus is also similar, as were the proportions of antibody-labelled trophozoites in the free and mucus-associated populations. Other explanations must be sought for the eventual elimination of the infeciton with the mouse isolate and the persistence of infection with the rat isolate.

Finally, it is worth considering the immunity to reinfection with the two organisms. A similar situation has been described in C3H/He mice, which are resistant to reinfection with *G. muris* (Underdown *et al.* 1981). A possible explanation for resistance to reinfection is that the immune host has antibodies against the ma-

jority of antigenic variants and that naive trophozoites (in contrast to the selected population during a chronic infection) are unable to generate new variants before they are cleared by immune effector mechanisms.

Table 5.2 Distribution of cysts between free luminal fluid and mucus at various times after infection of DA rats as a function of time after infection with either isolate of *G. duodenalis*[a]

Days after	Mouse Isolate		Rat Isolate	
Infection	Jejunum	Ileum	Jejunum	Ileum
7	30	42	28	40
10	20	61	15	70
15	37	48	32	50
21	27	61	26	68
42	20	75	13	80

[a] The data show the mean percentage of the total number of cyts observed as a function of time. All other details are as described for Table 5.1.

Table 5.3 Binding of IgA antibodies to *G. duodenalis* trophozoites *in vivo* and *in vitro*

Time of infection (days)	Proportion of trophozoites with bound IgA (%)[a]					
	IgA Bound by Rat Isolate			IgA Bound by Mouse Isolate		
	in vivo[b]	*in vitro*		*in vivo*[b]	*in vitro*	
		Pooled Bile[c]	Contemporary Bile[d]		Pooled Bile[c]	Contemporary Bile[d]
7	3	76	nd[e]	2	82	nd
10	65.7	93	30.3	66.8	95	34.2
15	79.3	96	74	75.7	nd	76
21	80.2	100	76.5	80.7	98	82.1
42	77.5	nd	69.3	78.6	100	79.4

[a] Each data point refers to the mean percentages of 3 rats.

[b] IgA bound to trophozoites when isolated from the intestines of infected rats; detected by Rabbit anti-Rat IgA (1:20) and FITC-conjugated Goat anti-Rabbit IgG (1:20).

[c] IgA bound to trophozoites after additional incubation with pooled homologous immune bile (1:10), followed by detection of bound IgA by the indirect immunofluorescence assay.

[d] IgA bound to trophozoites after additional incubation with pooled contemporary immune bile (1:10), followed by detection of bound IgA by the indirect immunofluorescence assay.

[e] Not determined

Table 5.4 Proportion of trophozoites with bound IgA (%) in the free and mucus-associated fractions of trophozoites harvested from the intestines of DA rats infected with either isolate of *G. duodenalis*[a]

Days after	Rat Isolate		Mouse Isolate	
infection	Free	Mucus Associated	Free	Mucus Associated
7	3	2	2	2
10	69	62	65	69
15	78	80	72	79
21	81	79	78	83
42	73	82	79	78

[a] Fractions of trophozoites were obtained as described in Section 2.15. The proportion of trophozoites with bound IgA in each fraction are presented as percentages. The fluorescence assay (Section 2.19) was performed on the trophozoites described in Table 5.1.

Table 5.5 Fluorescence assay of *G. duodenalis* trophozoites from intestinal lumen of CBH rnu/rnu and rnu/+ rats[a]

Weeks after infection	Percentage-positive trophozoites[b]			
	rnu/rnu		rnu/+	
	IgA bound *in vivo*[c]	IgA bound *in vitro*[d]	IgA bound *in vivo*[c]	IgA bound *in vitro*[d]
3	4	7	77	65
6	3	5	78	62

[a] Rats infected with the mouse isolate of *G. duodenalis*. Each point represents the means of 2 rats.

[b] Mean number of trophozoites counted per slide was 225.

[c] IgA bound to trophozoites when isolated from the intestines of infected rats; detected by rabbit anti-rat IgA (1:20) and FITC-conjugated goat anti-rabbit IgG (1:20).

[d] IgA bound to trophozoites after additional incubation with homologous contemporary bile (1:10), followed by detection of bound IgA by the indirect immunofluorescence assay (as described in Section 2.19).

Chapter 6

Passive Transfer of Immunity with Immune Bile

6.1 Introduction

Experimental infection with *Giardia* resulted in resistance to subsequent infection in rats, as demonstrated in Chapter 3. This is consistent with observations in other animal models of giardiasis (Belosevic *et al.* 1984b; Roberts-Thomson *et al.* 1976b; Underdown *et al.* 1981) as discussed in that chapter. The protective immunity induced following a primary infection can be duplicated in Balb/c, but not in C3H/He mice, by immunization with either a sonicate of whole trophozoites (Roberts-Thomson & Mitchell 1979) or with exo-antigens (Mitchell *et al.* 1982) emulsified in Freund's complete adjuvant.

Attempts to transfer resistance to infection with either single (Roberts-Thomson & Mitchell 1978) or multiple (Underdown *et al.* 1981) injections of serum containing high levels of antibody were unsuccessful whereas transfer of lymphoid cells from immune donors to athymic mice led to rapid clearance (Roberts-Thomson & Mitchell 1978). However, a role for antibody is not excluded by these experiments, since parenteral administration of immune serum may not produce high levels of antibody in the gut. In addition, it is highly possible that reconstitution with lymphoid cells supplies T_h cells required for production of intestinal antibody (as

discussed in Section 1.11.2)

Studies on secretory immunity in giardiasis have focused on the contribution of milk antibodies to protective immunity. Neonatal mice are protected by milk from immune mothers, whereas mice suckling on non-immune mothers were not protected (Andrews & Hewlett 1981; Stevens & Frank 1978; Underdown *et al.* 1981). This complements the observation in humans that significant protection of breast-fed babies (Islam *et al.* 1983; Miotti *et al.* 1985; Nayak *et al.* 1987) is possibly associated with high levels of specific IgA present in breast milk (Miotti *et al.* 1985; Nayak *et al.* 1987).

The experiments described in this chapter were carried out to ascertain whether it was possible to demonstrate directly that passive transfer of secretory antibody in the form of immune bile could protect against infection with the *G. duodenalis* isolates.

6.2 Passive protection with immune bile

6.2.1 The effect of immune bile on an established infection

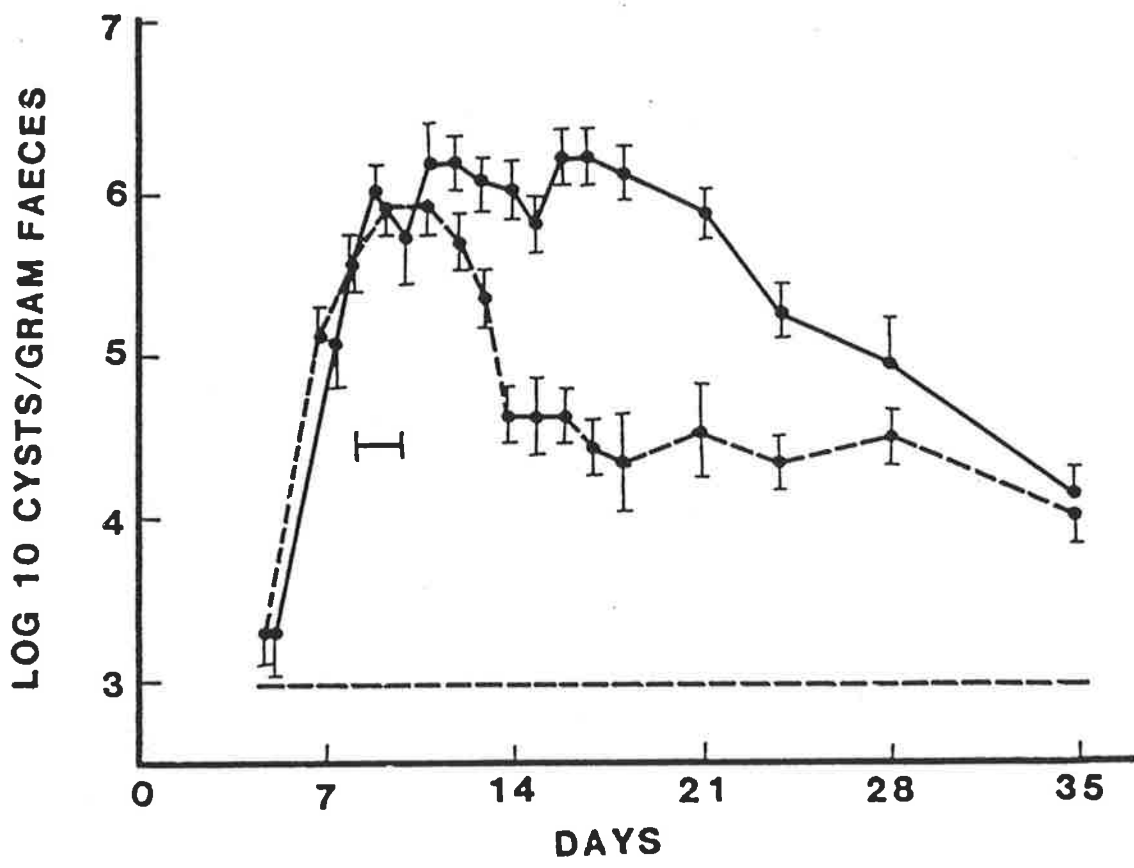
In this experiment, 2 groups of 8 rats were infected: Group 1 with the mouse and Group 2 with the rat isolate of *G. duodenalis*. Cannulae were inserted into the duodena of the animals (as described in Section 2.6.2) on day 8 after infection (Figure 2.1). Four rats in each group received approximately 12 ml of bile per day from a pool of homologous immune bile collected from the animals undergoing both primary and secondary infections as described in Section 4.5. The ELISA titre of IgA antibody in the pools of homologous immune bile for the mouse and rat isolates were 2048 and 2254, respectively. The remaining 4 rats received the same volume of bile, but from a pool of uninfected donors. The rate of bile infusion (12 ml/day) approximated the normal daily bile output. Infusion was continued for a period of 84 hours, after which the cannulae were removed and the animals were returned to holding cages.

Faecal cyst excretion during the course of infection with the mouse isolate is shown in Figure 6.1. Infusion of control bile from uninfected donors had no effect on the rate of cyst excretion which was similar to that described previously (Figure 3.6). In contrast, in animals receiving immune bile there was a very significant decline in cyst excretion, commencing towards the end of the infusion period. Animals that had received immune bile continued to excrete fewer cysts until the end of the infection, when cyst excretion declined in both groups. The experiment with the mouse isolate was repeated and immune bile was infused for a longer period (from day 8 to day 14). Figure 6.2 shows that in 2 animals receiving immune bile, there was a significant decline in cyst output commencing during the period of bile infusion. Non-immune bile had no effect on the cyst excretion of 3 control rats. In contrast, neither immune bile nor control bile had any effect on the infection with rat isolate, as assessed by cyst excretion (Table 6.1).

6.2.2 The effect of immune bile in prevention of infection

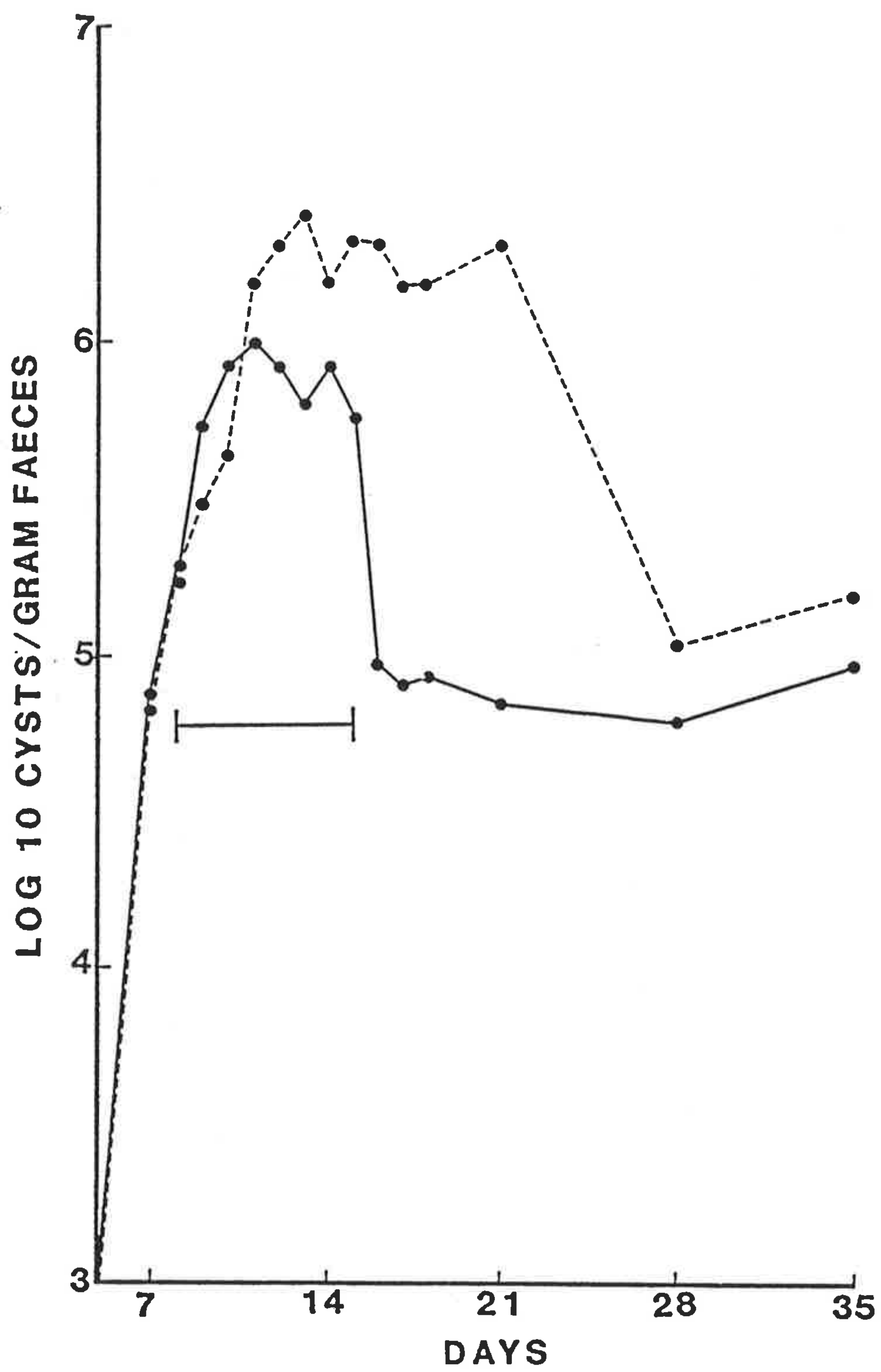
Experiments were carried out to determine whether intra-intestinal infusion of immune bile could prevent establishment of an infection. Two groups of rats were cannulated 24 hours before infection with *Giardia*: Group 1 comprised of 8 rats (4 experimental and 4 controls); Group 2 comprised of 7 rats (4 experimental and 3 controls). As before, the rats were first perfused for 3 hours with saline. Infusion of homologous immune bile was then started 24 hours prior to infection with either the rat (Group 1) or the mouse (Group 2) isolate. After infection, bile infusion was carried out for a further 50 hours. Cannulae were then removed and the rats were returned to their cages. Monitoring of cyst excretion was started at day 5 and continued daily for 2 weeks in the group of rats infected with the rat isolate. Cyst counts were performed daily for 2 weeks and then weekly up to the 4th week after infection with the mouse isolate. Table 6.1 shows that immune bile had no effect on cyst excretion in animals infected with the rat isolate. In contrast, in the animals infected with the mouse isolate, immune bile induced a delayed onset of

Figure 6.1 The effect of passive intra-intestinal infusion with immune bile on the course of infection with the mouse isolate in female DA rats (**Experiment 1**). The rats in each group were infected with 5000 cysts and 7 days later a cannula was introduced into the duodenum. Immune bile (Dotted line) or control normal bile (Solid line), was infused at the rate of 12 ml per day for the period indicated by the horizontal bar. The cannulae were then withdrawn and cyst excretion was monitored for the period indicated. Horizontal dotted line indicates limits of cyst detection. Points are mean ± S.E.M. for 4 animals.



LOG 10 CYSTS/GRAM FAECES
7
6
5
4
3
0
7
14
21
28
35
DAYS

Figure 6.2 The effect of passive intra-intestinal infusion with immune bile on the course of infection with the mouse isolate in female DA rats (**Experiment 2**). Immune bile (solid line) or control normal bile (dotted line), was infused for the period iindicated by the horizontal bar. All other details are as described for Figure 6.2.



LOG 10 CYSTS/GRAM FAECES
7
6
5
4
3
7
14
21
28
35
DAYS

cyst excretion as well as significantly lower cyst counts which gradually increased to control levels by the fourth week of infection (Figure 6.3).

6.3 Discussion

Direct evidence was sought for a role of secretory antibody in immunity against *Giardia*. A technique was devised to allow passive transfer of bile, which can be obtained easily and is a major source of intestinal IgA in rats (Lemaitre-Coelho *et al.* 1977, 1978). Immune bile, containing IgA against the homologous isolate, was infused into the duodena of animals infected with either of the two isolates. Infusion of immune bile, but not of bile from uninfected rats, led to a marked decrease in cyst excretion in the rats with established primary infections with the mouse isolate. When infusion of immune bile was started prior to infection, the effect was not only to delay the onset of cyst excretion but also to result in a significant reduction in cyst output. This sort of study is limited by the amounts of bile available and the time for which animals can be restrained. Nevertheless, the evidence is compelling that immune bile delivered in physiological amounts can reduce the parasite load. This effect is attributed to the content of IgA antibody in the bile. In contrast, infusion of control bile had no apparent effect on cyst excretion. In the case of infections with the rat isolate of *G. duodenalis,* homologous immune bile had no effect in either preventing establishment of infection or in reducing cyst excretion from an established infection.

These experiments are the first, with the exception of feeding immune milk, to use a passive transfer technique to examine directly the protective effects of secretory antibody in *Giardia* infection. The technique has some limitations with respect to the length of time for which bile infusions can be continued ethically and the fact that cyst counts rather than parasite load was monitored. Nevertheless, it has been successful in demonstrating an effect of secretory antibodies on *Giardia* infections. It is of considerable interest that the protective effect of antibody mirrors the ability of the active immune response to control the infection - that is,

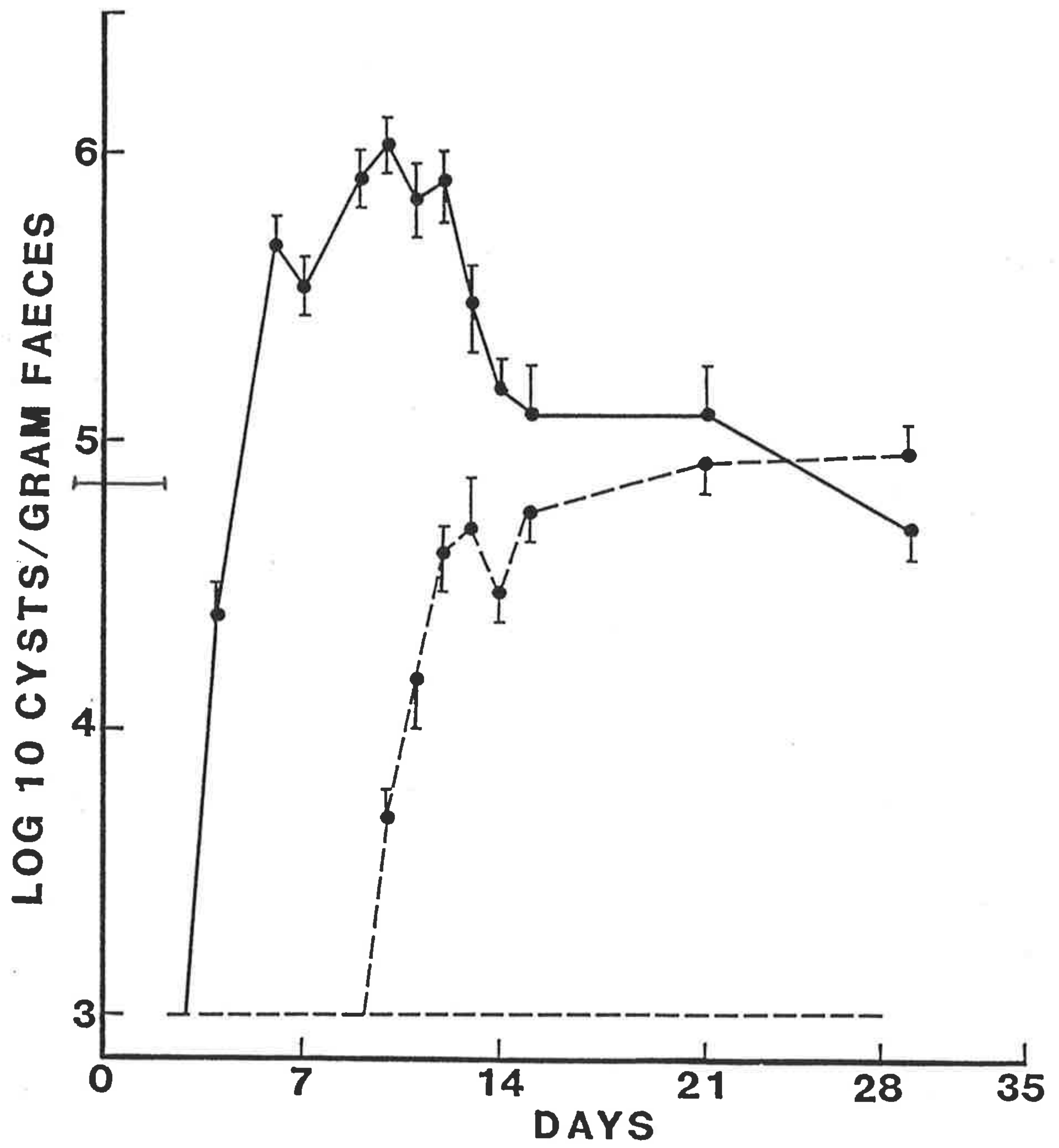
Table 6.1 Effect of intra-intestinal infusion of immune bile in infections with the rat isolate of *G. duodenalis*

Days after infection	Effect on prevention[a]		Effect on established infection[b]	
	Immune	Control	Immune	Control
7	4.30 ± .022	4.50 ± .076	4.62 ± .084	4.32 ±.113
8	5.10 ± .081	5.24 ± .042	5.14 ± .113	5.29 ± .054
9	5.29 ± .051	5.31 ± .062	5.33 ± .083	5.40 ± .086
10	5.37 ± .106	5.42 ± .113	5.62 ± .059	5.59 ± .101
11	5.57 ± .091	5.61 ± .098	5.73 ± .101	5.64 ± .112
12	5.68 ± .090	5.76 ± .102	5.89 ± .046	5.78 ± .065
13	6.27 ± .077	6.01 ± .083	6.21 ± .069	6.34 ± .078
14	6.33 ± .101	6.19 ± .068	6.29 ± .081	6.40 ± .058

[a] Rats received either immune or non-immune bile 24 hours prior to infection with the rat isolate of *G. duodenalis*. Each data point refers to the mean $\log_{10}$ ± S.E.M. cysts per gram of faeces of 4 rats.

[b] Rats received either immune or non-immune bile on day 8 for 3 days after infection with the rat isolate of *G.duodenalis*. Each data point refers to the mean $\log_{10}$ ± S.E.M. cysts per gram of faeces of 4 rats for the immune bile and 3 rats for the non-immune bile.

Figure 6.3 The effect of intra-intestinal infusion with immune bile in preventing the establishment of infection with the mouse isolate of *G. duodenalis* in female DA rats. Immune bile (Dotted line) or control normal bile (Solid line), was infused for the period indicated by the horizontal bar. All other details are as described for Figure 6.2.


LOG 10 CYSTS/GRAM FAECES
6
5
4
3
0
7
14
21
28
35
DAYS

passive antibody affected the course of infection with the mouse isolate but not the infections with the rat isolate.

The effect of passive immune bile on the infection with the mouse isolate was to suppress cyst excretion. However, it fell short of complete elimination of the parasite. This is not unexpected when it is considered that the period of bile infusion was short in comparison with the length of the primary infection with this organism. In the face of an active immune response, elimination of the parasite is gradual and occurs over the space of several weeks. The effects noted are therefore very significant and contrast with the ineffectiveness of immune milk on established infections with *G. muris* in baby mice (Andrews & Hewlett 1981). Nevertheless, it is interesting that following cessation of bile infusion, cyst excretion did not rise back to control levels. The reasons for this are unknown, but perhaps the most likely is that the period of passive infusion has overlapped with the onset of the primary active immune response, with the result that attrition of trophozoite numbers has followed the normal kinetics for a primary infection, though commencing from the levels which remained after administration of passive antibody.

The failure of passive immune bile to affect either establishment of infection with the rat isolate or the kinetics of an existing infection requires further consideration. Despite the chronicity of primary infection with this organism, it is nevertheless the case that following drug cure, animals are solidly immune against reinfection. If this immunity depends on secretory antibody, then it might be expected that pooled hyperimmune bile would be passively protective. While it can always be suggested that the amount of antibody transferred was inadequate, the observations raise questions about the importance of other factors in resistance to this isolate. These factors could be other modalities of the adaptive immune response, changes in the composition of mucus or the presence of a particular sort of inflammatory exudate in the mucosa of the gut. The antibody response to the rat isolate may also be qualitatively different and the parasite may respond differently to antibodies directed against it. Nevertheless, the results in Chapter 5 indicate that hyperimmune bile raised against this isolate binds with essentially all of the

trophozoite population (and is no less reactive than homologous hyperimmune bile on trophozoites of the mouse isolate, see Chapter 7). Furthermore, the two isolates are cross-protective (Chapter 7), arguing that the isolates share common protective antigens. It appears that the difference in the host-parasite relationship between the two isolates will turn out to be quite subtle and that it will provide important insights into the causes (host and/or parasite) of chronic infections with *Giardia.*

The experiments on passive transfer of immune bile described in this chapter do not provide evidence of antigenic variation *in vivo*, but it is possible that IgA antibodies in bile specific for surface antigens in the trophozoites led to the emergence of new antigens *in vivo*. Pooled immune bile possesses a repertoire of antibodies recognizing antigens on the surface of trophozoites harvested at different stages of the infection. In the *G. lamblia*-gerbil model, it has been shown that the WB isolate, changes its antigens *in vivo* only within the first 7 days and this isolate causes a self-limiting infection (Aggarwal & Nash 1987a) whereas gerbils infected with a strain (GS/M) that undergoes rapid change *in vitro* develop a chronic infection. It remains to be seen whether this latter isolate also undergoes variation *in vivo*.

The results reported here complement those of Butscher & Faubert (1988) who demonstrated a marked reduction in intestinal parasite burden in Balb/c mice treated i.p. with a cytotoxic MoAb specific for a surface antigen on *G. muris*. Mice were injected i.p. 24 hours prior to and after infection as well as on days 3 and 5. The parasite burden was determined on day 8 when trophozoites present in the gut were counted. Those treated had nearly three times fewer trophozoites early in the acute phase of the infection than did mice treated with negative ascites. The *in vitro* effect of the MoAb was to agglutinate trophozoites and a similar effect *in vivo* could have prevented attachment to the substratum. In the environment of the gut lumen, complement-mediated cytotoxicity is an unlikely explanation. Kaplan *et al.* (1985) reported that specific IgA antibody against *G. muris*, when mixed with phagocytic cells, was able to render trophozoites noninfective. This raises the question about the possible role of cells and opsonic or ADCC mechanisms in the protective immune responses against *Giardia*, although this mechanism is unlikely

in the intestinal lumen.

Although the importance of bile as a route of secretion of IgA antibodies into the intestine differs between rats and man, the convenience of bile in rats for measurement and collection of IgA antibodies is obvious. The mode of action of secretory antibodies in reducing the trophozoite population in the duodenum is unknown, but they are likely to interfere with attachment to the mucosa and thereby increase the susceptibility of the trophozoite to elimination by peristalsis or to damage by intestinal contents as has been postulated for *Eimeria tenella* sporozoites (Davis & Porter 1979). *In vitro* effects of antibody on trophozoites include opsonization (Hill *et al.* 1984; Owen *et al.* 1981; Radulescu & Meyer 1981), agglutination and immobilization (Butscher & Faubert 1988; Mayrhofer, unpublished data; Radulescu & Meyer 1981) and lysis (Hill *et al.* 1984). It is likely that secretory antibodies, whether in the bile or in the intestinal mucus, would have similar adverse effects upon the parasite.

Several lines of evidence suggest that protection against giardiasis may be acquired passively by neonates suckling milk from immune mothers (Andrews & Hewlett 1981; Kaplan *et al.* 1985; Miotti *et al.* 1985; Stevens & Frank 1978; Underdown *et al.* 1981). The protection in the suckling mice was lost after weaning and immune milk was ineffective for established infections (Andrews & Hewlett 1981). The secretory immune system was implicated in this protection since IgA antibodies were detected in milk (Andrews *et al.* 1979; Andrews & Hewlett 1981) although the role of cells was not excluded. However, antibody-independent killing of trophozoites by non-immune human milk (Gillin *et al.* 1983a, 1983b; Gillin *et al.* 1985; Hernell *et al.* 1986) due to release of free fatty acids from milk triglycerides by the action of bile salt-stimulated lipase (Gillin *et al.* 1985; Hernell *et al.* 1986) has been demonstrated *in vitro*. Normal human duodenal and upper jejunal fluid has also been shown to be toxic for *Giardia* trophozoites *in vitro* (Das *et al.* 1988). However, infusion of non-immune bile appeared to have no effect on the course of infection in the experiments described in this chapter. All control rats had normal levels of cyst excretion.

In conclusion, the involvement of locally produced antibodies in immunity to *Giardia* is likely for a number of reasons. Firstly, these parasites are normally confined to the lumen of the intestine. Secondly, the results obtained from the measurements of IgA antibodies throughout the course of infections show that there is a local antibody response and that IgA antibodies do bind to the trophozoites. Thirdly, the lack of secretory antibody response in nude rats, in which infection with the mouse isolate is chronic, is consistent with a role for IgA antibodies in limiting infection in the immunologically intact host. As discussed elsewhere, similar finding in nude mice (Stevens *et al.* 1978) and in hypogammaglobulinemic anti-IgM suppressed mice (Snider *et al.* 1985) also argue in favor of the importance of antibody. The response with the mouse isolate fulfills this requirement thereby consolidating the case for implicating the secretory immune system in the protective response to *Giardia*.

Chapter 7

Comparison of the Mouse and Rat Isolates of *Giardia duodenalis*

7.1 Introduction

Comparative studies between the *G. duodenalis* isolates may provide important clues to explain their biological behavior. The different infections produced by the isolates in rats suggest that parasite factors as well as host factors influence the outcome of infection. As discussed in Section 1.8.3, recent studies have shown diversity among many human and other mammalian *Giardia* isolates from diverse geographical areas (Nash *et al.* 1983, 1985; Smith *et al.* 1982b; Wenman *et al.* 1986). In addition, *G. lamblia* isolates possessing different surface antigens (Nash *et al.* 1985; Nash & Keister 1985) result in different patterns of infection (Aggarwal & Nash 1987a; Nash *et al.* 1987b) indicating that factors peculiar to different *Giardia* isolates influence the course of infection, either directly or by inducing different types of host responses.

The experiments described in this chapter were carried out in order to compare the two isolates, in an effort to explain the different patterns of infections; both were also compared, whereever possible, with the PO-1 isolate of *G. lamblia*.

7.2 Morphology of the isolates

It was believed initially that the mouse and rat isolates represented *G. muris* and *G. duodenalis*, respectively. However, examination of Trichrome-stained fixed specimens (prepared as described in Section 2.5) showed that trophozoites from the two isolates were indistinguishable. Morphometry was not performed, but they appear similar in shape and size (Figure 7.1, top). Both had median bodies with the 'claw hammer' morphology characteristic of *G. duodenalis* (Figure 7.1, bottom).

7.3 Isoenzyme studies

Enzyme analysis was carried out at the Evolutionary Biology Unit of the South Australian Museum (see Section 2.8). Evidence supporting a close relationship between the isolates came from comparison of the electrophoretic mobilities of the 27 enzymes shown in Table 7.1. Although comparison with *G. muris* was not undertaken, the identity of the isoenzymes at this number of loci in the two isolates suggests a very close genetic relationship and is consistent with the morphological identification of both as *G. duodenalis*. Differences in the electrophoretic mobilities of enzymes between individuals or between species (i.e. isoenzymes) reflect corresponding structural differences between the genes that encode those enzymes. No differences were noted between the rat and mouse isolates for any of the 27 enzymes examined and identity at this number of loci suggests strongly both organisms belong to the same species. Furthermore, there were no differences at any of these loci when the two rodent isolates were compared with two *G. lamblia* isolates Adelaide-1 and PO-1. The rodent isolates of *G. duodenalis* therefore appear to be closely related to *G. lamblia*.

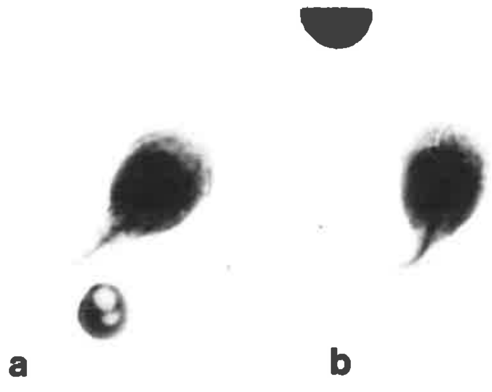
7.4 Cross-protection studies *in vivo*

The two rodent isolates of *G. duodenalis* produce infections with quite different kinetics, despite the apparent close relationship (Section 7.3). The kinetics of anti-

Figure 7.1 Examples of trophozoites excysted *in vitro* from the rodent isolates and stained with Trichrome. Top figures: over developed to show that both have similar shape and size. Bottom figures: show that both have median bodies characteristic of *G. duodenalis*. (x 1345)

a Mouse isolate.

b Rat isolate.


a
b

a
b

Table 7.1 Enzymes identified in the *G. duodenalis* isolates.

Enzyme	E.C. no.	Enzyme	E.C. no.
Aconitase	4.2.1.3	Hexokinase	2.7.1.1
Acid Phosphatase	3.1.3.2	Malate Dehydrogenase	1.1.1.27
Adenosine Deaminase	3.5.4.4	Malic Enzyme	1.1.1.40
Alcohol Dehydrogenase	1.1.1.1	Mannose-Phosphate Isomerase	5.3.1.8
Aldolase	4.1.2.13	Nucleoside Phosphorylase	2.4.2.1
Enolase	4.2.1.11	Peptidase (Valine-leucine)	3.4.11 or 13
Fructose-Diphosphatase	3.1.3.11	Phosphoglycerate Mutase	2.7.5.3
Glyceraldehyde-3-Phosphate Dehydrogenase	1.2.1.12	6-Phosphogluconate Dehydrogenase	1.1.1.44
Glutamate Dehydrogenase	1.4.1.3	Phosphoglycerate Kinase	2.7.2.3
Glutamate-Oxalacetate Transaminase	2.6.1.1	Phosphoglucomutase	2.7.5.1
Glucose-6-Phosphate Dehydrogenase	1.1.1.49	Sorbitol Dehydrogenase	1.1.1.14
Glycerophosphate Dehydrogenase	1.1.1.8	Triose-Phosphate Isomerase	5.3.1.1
Glucose-Phosphate Isomerase	5.3.1.9	Uridine Monophosphate Kinase	2.7.4.?
Glutathione Reductase	1.6.4.2		

body production against each organism is nevertheless similar (Chapters 4 & 5). It was therefore interesting to determine whether the rat isolate lacks certain protective antigens that are present on the mouse isolate. Experiments were undertaken to examine whether significant protection could be induced against infection with the mouse isolate by antigens of the rat isolate and vice versa. Two groups of DA rats were used, each containing six animals. Group 1 was infected with the mouse isolate and Group 2 with the rat isolate. Primary infections were verified by cyst excretion (data not shown). Both groups of animals were drug-cured of their infection after six weeks and were challenged by the heterologous isolate four weeks after treatment. Prior to challenge, the faeces of these animals were free from cysts and cysts were not detected on daily monitoring for three weeks following challenge. Therefore, infection with each isolate conferred protection against challenge with the heterologous organism.

7.5 *In vitro* cross-reactivity

7.5.1 ELISA

When pooled immune bile from animals infected with *G. duodenalis* was compared in ELISAs using plates coated with either homologous or heterologous antigens (sonicates of excysted organisms, see Section 2.18.2), similar titres were recorded irrespective of antigen source (Table 7.2). In addition, infection with both isolates produced antibodies that cross-reacted with PO-1 antigens, although the titres were less than those recorded on plates coated with *G. duodenalis* antigens (Table 7.2).

7.5.2 Immunofluorescence assay

These assays were performed (as described in Section 2.19) using living trophozoites harvested from the intestines of infected DA rats used in experiments described in Chapter 5. The organisms were labelled with either homologous or heterologous immune bile, the patterns of immunofluorescence observed were uniform surface

Table 7.2 Cross-reactivity of biliary IgA antibodies in pooled bile as detected in ELISA employing both isolates of *G. duodenalis*[a]

Antigens	Mouse biliary IgA	Rat biliary IgA
Mouse[b]	2121 ± 50	1998 ± 35
Rat[b]	2052 ± 31	2254 ± 22
PO-1[c]	748 ± 38	897 ± 40

[a] Equal volumes of bile from rats infected with either isolate of *G. duodenalis* were pooled separately and assayed for IgA antibody activity against the homologous and heterologous isolate and against PO-1 *G. lamblia*. Data are means of triplicate determinations expressed as reciprocal serum dilutions ± S.E.M.

[b] Sonicate of excysted trophozoites of the rodent isolates of *G. duodenalis* prepared as described in Section 2.18.2.

[c] E-S antigens of trophozoites of PO-1 as described in Section 2.18.2.

fluorescence or diffuse fluorescence (apparently due to labeling of cytoplasmic antigens in dead cells).

The data shown in Table 7.3 demonstrates that similar percentages of trophozoites harvested from the intestines of DA rats reacted with the homologous and heterologous pooled immune bile. Figure 7.2 shows photomicrographs of bound biliary IgA antibodies on trophozoites of the rat isolate (Figure 7.2 a & b) and of PO-1 trophozoites (Figure 7.2c). When pooled heterologous immune bile was reacted with the rat isolate the intensity of fluorescence was equal to that observed with the combination of homologous bile and trophozoites (Figure 7.2a). Pooled immune bile from rats infected with rat isolate reacted with PO-1 trophozoites but with a lesser intensity (Figure 7.2c). Similarly, when trophozoites of the mouse isolate was reacted with heterologous immune bile, the intensity of fluorescence was equal to that of the mouse trophozoites incubated with homologous immune bile (data not shown). Pooled immune bile from the mouse isolate also reacted with PO-1 trophozoites with lesser intensity as described above with immune bile from the rat isolate. The results of the IF confirm that both rodent isolates share common antigens with the PO-1 strain of *Giardia*. None of the isolates reacted with non-immune bile or serum.

7.6 Comparison of protein profiles

The proteins in whole cell lysates of trophozoites of all three isolates were examined by SDS-PAGE and the Coomassie blue-stained gel (as described in Section 2.20) is shown in Figure 7.3. Protein profiles revealed close similarity between the three isolates. In both rodent isolates, Coomassie-staining bands ranging in moleuclar weight from approximately 90 kDa to 14 kDa were visualized compared to 130 kDa to 14 kDa range of proteins in PO-1. Minor polypeptide differences were detected between the rodent isolates and PO-1: major proteins, 76 kDa and 61 kDa, observed in PO-1 (lane D) are absent or negligible in the rodent isolates. No differences were detected between the mouse (lane B) and the rat isolate (lane C). Major proteins

Table 7.3 Binding of IgA antibodies to homologous and heterologous *G. duodenalis* trophozoites *in vitro*

Time of Infection (days)	Proportion of trophozoites with bound IgA (%) *in vitro*[a]			
	IgA bound by rat isolate		IgA bound by mouse isolate	
	Homologous bile[b]	Heterologous bile[c]	Homologous bile[b]	Heterologous bile[c]
7	76	72	82	79
10	93	87	95	92
15	96	89	ND[d]	ND[d]
21	100	96	98	92
42	ND[d]	ND[d]	100	94

[a] Each data point refers to the means of 3 rats per time point. The trophozoites were harvested from the intestines of rats infected with the *G. duodenalis* as described in Chapter 5.

[b] IgA bound to trophozoites by incubation with pooled homologous immune bile (1:10), followed by detection of bound IgA by rabbit anti-rat IgA (1:20) and FITC-conjugated goat anti-rabbit IgG (1:20).

[c] IgA bound to trophozoites by incubation with pooled heterologous immune bile (1:10) followed by the detection system as described in b.

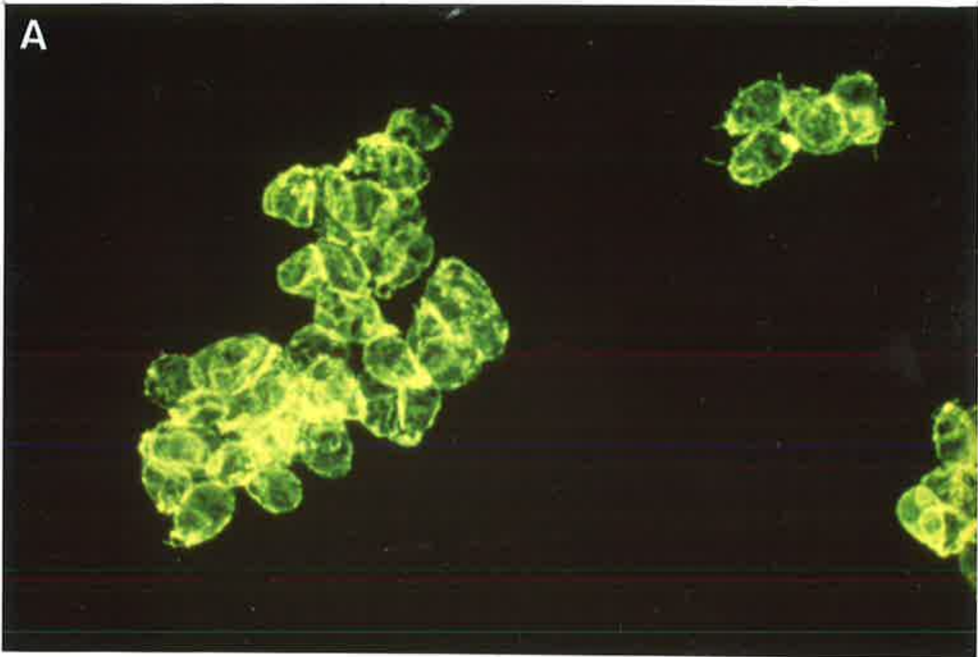
[d] Not determined.

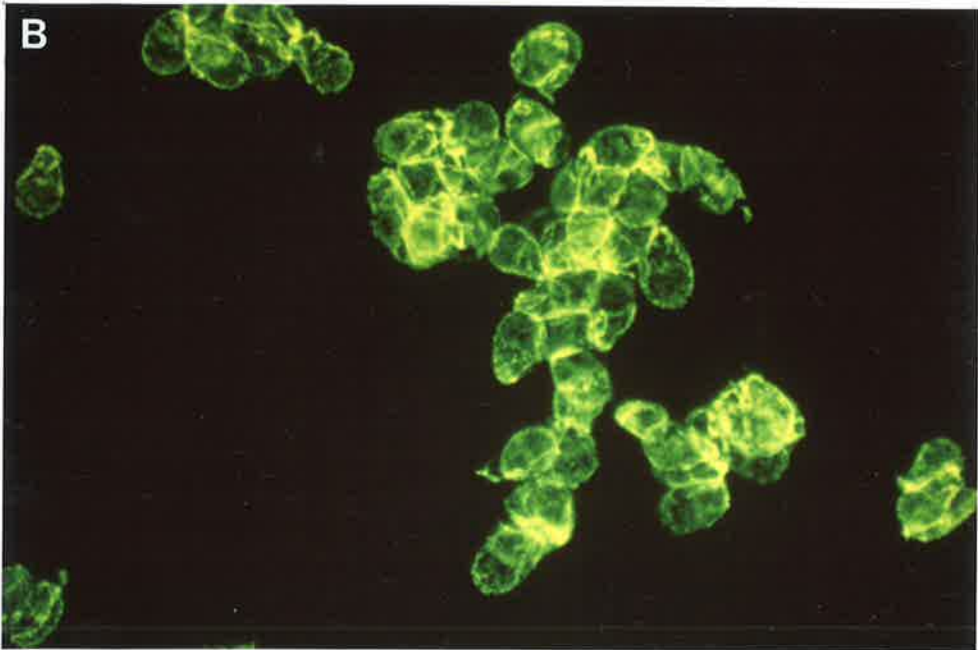
Figure 7.2 Immunofluorescent staining of *Giardia* trophozoites with pooled immune bile. *G. duodenalis* trophozoites were isolated from DA rats infected with the rat isolate and passively coated with antibodies by incubation in homologous or heterologous bile from DA rats infected with the rat and the mouse isolate of *G. duodenalis*, respectively. PO-1 trophozoites were harvested from axenic culture and coated with antibodies by incubation with pooled immune bile as described below (C). Trophozoites were kept at 4 C throughout the staining procedure. Bile was used at a dilution of 1:10 and IgA bound to trophozoites was detected by rabbit anti-rat IgA (1:20) and FITC-conjugated goat anti-rabbit IgG (1:20). Fluorescent cells were detected with a UV light source with a (IF-490) filter.

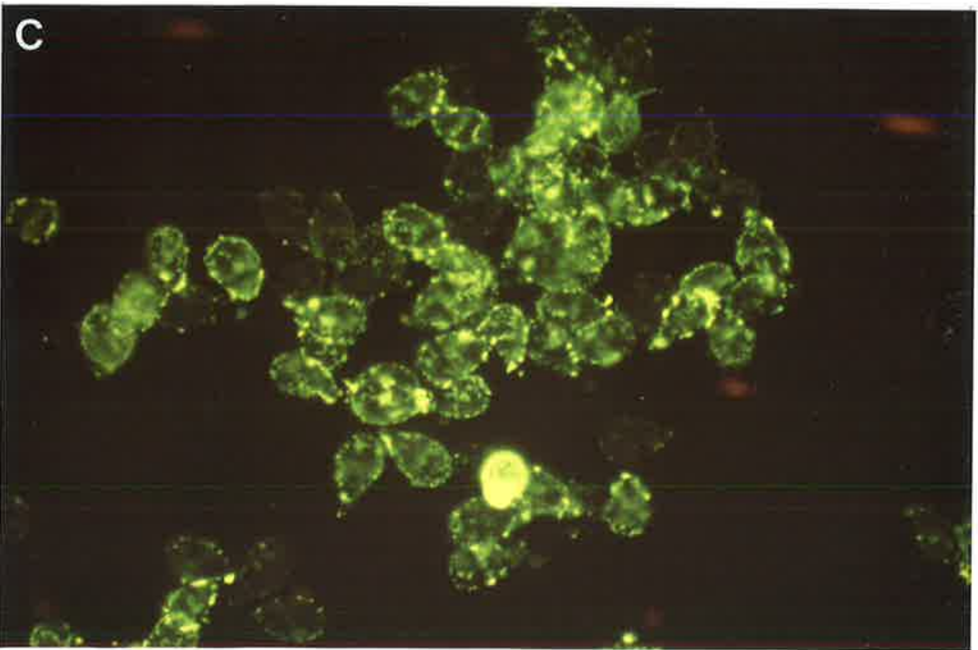
A Rat isolate trophozoites reacted with pooled homologous immune bile.

B Rat isolate trophozoites reacted with pooled heterologous immune bile.

C PO-1 trophozoites reacted with pooled immune bile from rats infected with the rat isolate.


A


B


C

common to the rodent isolates were a 51 kDa and a doublet of 29.5-32 kDa. Minor proteins common to all three organisms include proteins of molecular weights 92 kDa, 84 kDa, 61 kDa, 45 kDa and 43 kDa.

7.7 Comparison of surface proteins of rodent isolates with PO-1

SDS-PAGE analysis of biotinylated trophozoites (as described in Section 2.21) of PO-1 revealed a prominently labelled component having a molecular weight of 92 kDa (Figure 7.4). Other less heavily labeled components (arrows) were detected at 76 kDa, 61 kDa, 45 kDa. The 32 kDa corresponding to the higher molecular weight component of the doublet was also labelled in PO-1 trophozoites. Comparison of the SDS-PAGE profile of the biotinylated trophozoites of the rodent isolates revealed that the major proteins 51 kDa and the doublet 29.5-32 kDa are surface exposed proteins in both organisms. In both rodent isolates the 92 kDa and 84 kDa were labeled less heavily.

7.8 Occurrence of *Giardia* virus

Both of the rodent *Giardia* and also the PO-1 isolate were examined to detect evidence of viral RNA. A 7kb (kilobase) double stranded (ds) RNA has been described in several isolates of *Giardia* and has been shown to be the genome of an infectious virus (Miller *et al.* 1988a, 1988b; Wang & Wang 1986). Nucleic acid preparations (prepared as described in Section 2.22) from all three isolates contained a 7kb species after electrophoresis on agarose gels and staining with ethidium bromide (Figure 7.5a). It was separated clearly from cellular RNA, but was nevertheless sensitive to treatment with RNAse (Figure 7.5b). These observations indicate that the rodent isolates both carry virus, as does in the *in vitro* cultured PO-1 isolate.

Figure 7.3 Comparison of protein profiles of rodent isolates of *G. duodenalis*. Whole cell lysates were prepared in sample buffer and analysed by 12% SDS-PAGE. Molecular weights are shown in kilodaltons. Protein standards with the indicated molecular weights: transferrin, 76kDa; bovine serum albumin, 67kDa; bovine gamma globulin, 53kDa; horseradish peroxidase, 40KDa and bovine gamma globulin, 25kDa. The positions of proteins are indicated by arrows, from top to bottom: *left*, 78kDa, 74kDa, 43kDa, 20.5kDa, 19.5kDa, 16.5kDa; *right*, 90kDa, 78kDa, 62kDa, 51kDa, 43kDa, 36kDa, 30kDa and 20kDa.

Lane A Protein standards

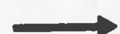
Lane B Mouse isolate

Lane C Rat isolate

Lane D PO-1 axenic isolate

76
67
53

25



A B C D

Figure 7.4 SDS-PAGE analysis of surface-biotinylated trophozoites of *G. duodenalis* isolates and PO-1. Lanes A & E, biotinylated standards of the indicated molecular weights (in kDa; top to bottom: Lane A,; Lane E,.). Lanes B, C, & D, profiles of biotinylated trophozoites solubilized in SDS sample buffer. The arrows indicate the biotinylated components. Lane D, mouse isolate; Lane C, rat isolate; and Lane B, PO-1.

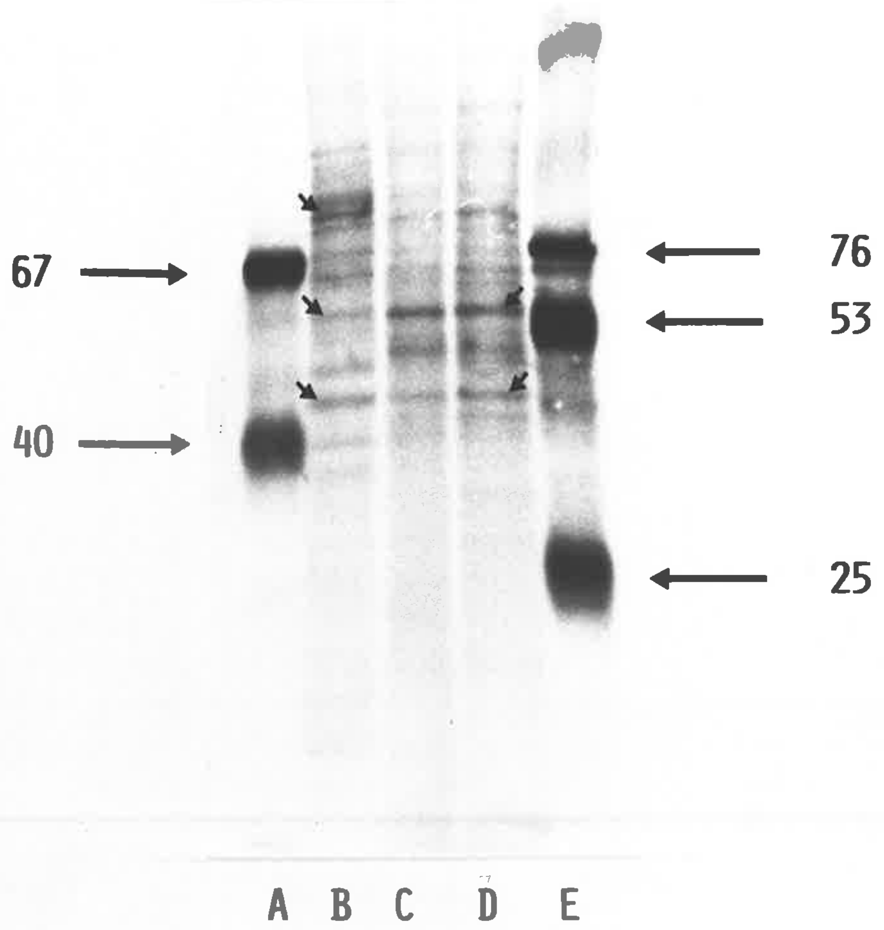
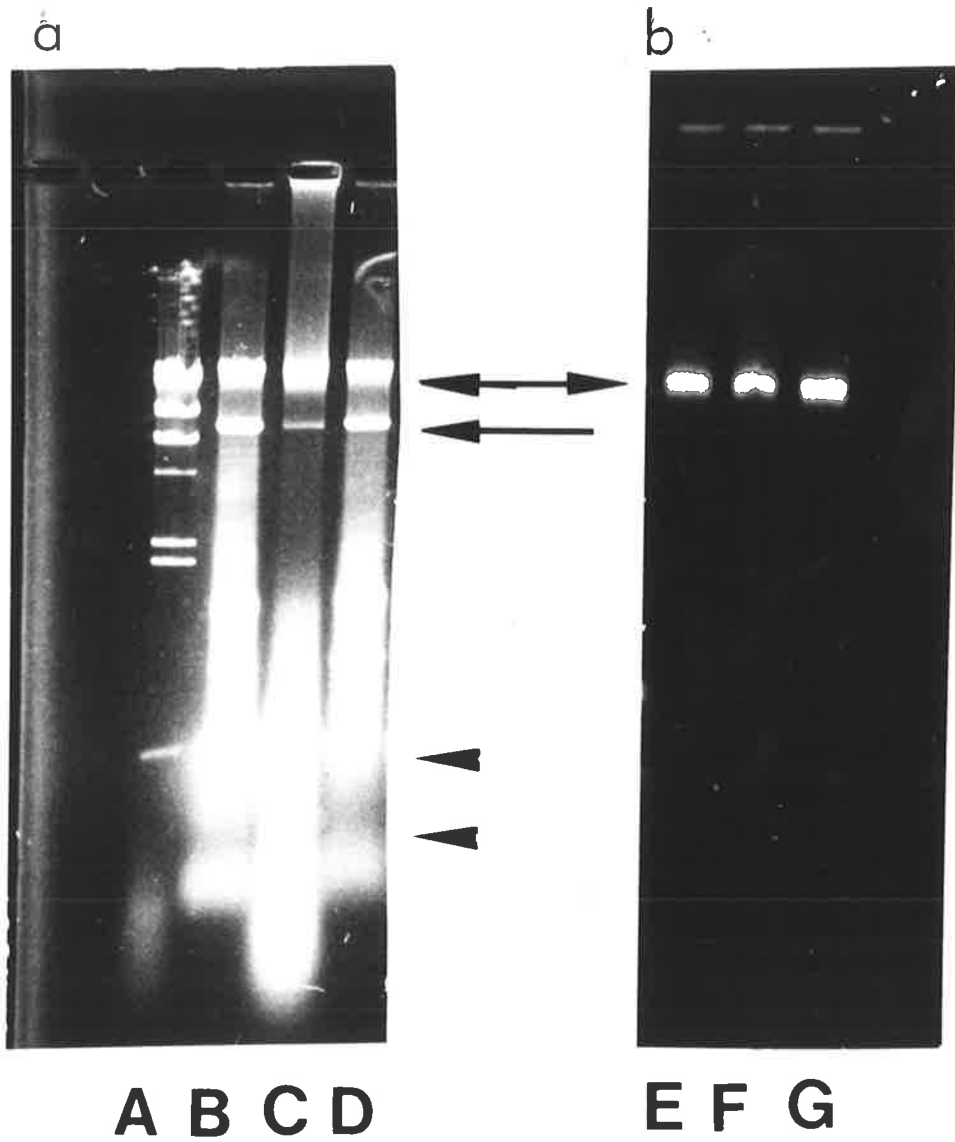

67
40
76
53
25
A B C D E

Figure 7.5 Gel electrophoresis of nucleic acid preparation of three strains of *Giardia*. Lane A, λ DNA Hind III digest as size marker; lanes B & E, mouse isolate; lanes C & F, rat isolate; lanes D & G., PO-1 isolate. Large arrow indicates position of the 7-kb RNA band and the double-headed arrow, the nuclear DNA. Cellular RNA forms a diffuse band, indicated by an arrowhead, which is RNAse sensitive.

a Without RNAse treatment.

b With RNAse treatment.


a
b
A B C D
E F G

7.9 Discussion

The identification of isolates used for experimental purposes in the literature has not always been rigorous. It cannot be assumed that all infections in mice are due to *G. muris* or that *G. muris* only infects mice. In the present work, organisms with the morphology of *G. muris* could not be obtained from animal stocks available in Adelaide. However, as discussed in Chapter 3, isolates with the morphological characteristics of *G. duodenalis* were obtained from both mice and rats. The two isolates were indistinguishable by microscopy and they were also identical when examined for the elctrophoretic mobilities of 27 enzymes. The latter findings suggest the two isolates are very closely related and preliminary studies have shown that there is also isoenzyme identity with two human isolates, PO-1 (Bertram *et al.* 1983) and Adelaide-1. The mouse and the rat isolates are therefore closely related organisms. It is also likely that some human isolates are closely related to *Giardia* that infect other mammalian species, as suggested by the recent studies of Meloni *et al.* (1988). Recent experiments (Andrews & Mayrhofer, unpublished) have shown that *G. muris* differs from Adelaide-1 and the rodent isolates at approximately 80% of the enzyme loci examined and is therefore related only distantly to *G. duodenalis*.

The cross-protection experiments showed that a high level of immunity against the heterologous isolate is generated by primary infection with either *G. duodenalis*. This provided evidence that these organisms are not only similar in isoenzyme profiles but that they also possess common protective antigens. The data obtained from the serological analyses in this study confirm that common antigens exist between the two isolates. The bile from the mouse isolate cross-reacted avidly with some antigens of the rat isolate in both ELISA and IF assays and the reverse was also true. The cross-protection studies suggest that the protective antigens must lie among those common antigens. However, it is conceivable that different members of this group could be responsible for immunity to each isolate. Alternatively, the protective antigens responsible for the more rapid elimination of the mouse isolate may not be immunodominat or they may be expressed

in low amounts. The shared immunodominant antigen might not be responsible for generating protecitve immunity and could in fact assist the parasite to resist immune attack.

The present study demonstrates the presence of an RNA band, probably corresponding to the ds RNA virus (De Jonckheere & Gordts 1987; Miller *et al.* 1988a, 1988b; Wang & Wang 1986). This is in agreement with the demonstration of viral-like particles in *G. duodenalis* from rats (Sogayar & Gregorio 1986) and in *G. muris* from rats (Feely *et al.* 1988) as well as more recent information that ds RNA has been detected in *G. muris* (Lindmark, unpublished data - cited in Feely *et al.* 1988).

The picture which emerges from these studies is that the two isolates of *G. duodenalis* are similar antigenically as well as genetically, In this respect, it seems valid to use the isolates as a model in which to examine some the factors that predispose to chronicity in giardiasis. The studies have not identified differences in either major surface proteins or major antigens. It is necessary to consider whether the antigens responsible for clearance of the mouse isolate are quite minor either in a quantitative sense or with respect to immunogenicity. It is noteworthy that the studies were performed with hyperimmune biliary antibodies, whereas bile from a primary infection may, in retrospect, have been more discriminating in defining antigens that lead to natural termination of infections with the mouse isolate. The result from the cross-protective study support this possibility and argue that the differences in the immune response to the two isolates are quantitative or temporal, rather than qualitative.

However, it is also possible that non-immunological factors interact and influence the effectiveness of the immune response to the organism. These parasite-determined factors could be related to mechanisms of immune evasion, such as antigenic variation (Section 1.8.1.4), or alternative, they could be behavioral and influence such things as the accessibility of the trophozoite to antibodies or the effects of the antibodies on the association between the trophozoites and their preferred substratum. It seems likely that the notion that the presence of protective

antigen determines the outcome of infection may be too simple by itself to account for the differences in the pattern of infection between these two related organisms.

Chapter 8

General discussion

8.1 Speciation of *G. duodenalis* isolates

Important findings with regard to speciation of *G. duodenalis* have emerged from this study. The speciation of those *Giardia* that infect mammals presents a considerable problem because there is general similarity in the morphology of isolates from various sources. Until recently, the main criteria that have been used to assign species to isolates have been the host of origin and the morphology of the organism. Host specificity is now recognized not to be absolute and although it appears to be restricted (Woo 1984), its value in taxonomy is still unclear. Morphological considerations allowed Filice (1952) to subdivide isolates from mammals into a minimum of two tentative species, *G. muris* and *G. duodenalis* (see Section 1.1.3). *G. muris* infects mice principally but it also has been found in rats (Grant & Woo 1978a) and in hamsters (Roberts-Thomson *et al.* 1976a). The morphology of its median body is distinctive and it may well constitute a single species. Organisms with the morphological characteristics of *G. duodenalis* have been isolated from many different mammalian species. In most cases, the host specificity of such isolates is either uncertain or unknown.

The classification of mammalian isolates into 2 species must be regarded as a temporary expedient. With respect to *G.muris*, more recent investigations of host specificity suggest that this morphological species is heterogenous (Bemrick 1961;

Grant & Woo 1978). Morphometric studies on *G. duodenalis* isolates from a number of mammalian species suggest heterogeneity in this morphological species also and there is support for this conclusion from the results of isoenzyme analysis (Andrews *et al.* 1988a; Bertram *et al.* 1983; Meloni *et al.* 1988) and studies on the genome using restriction endonucleases and hybridization with cloned *Giardia* DNA (Nash *et al.* 1985). However, there is still insufficient information to be able to decide whether each of the two morphological species is comprised of an indeterminate number of races or whether each encompasses organisms sufficiently different to be designated separate species.

Biochemical taxonomy has been used to address problems of classification of a number of protozoan parasites. Comparison of electrophoretic mobility of enzymes has been used to resolve these speciation problems (Andrews *et al.* 1988b; Kreutzer *et al.* 1983; Tait 1985) as this technique can provide genetic evidence necessary to provide a species-level taxonomy. The technique has been applied to *Giardia* and differences among isolates have been detected (Andrews *et al.* 1988a; Baveja *et al.* 1986; Bertram *et al.* 1983; Meloni *et al.* 1988). Andrews *et al.* (1988a) have examined 50 different enzymes for suitability as genetic markers and 4 distinct groups of *G. lamblia* were detected among the 29 stocks examined. This provides preliminary evidence that *G. lamblia* is not a single species.

The two isolates of *Giardia* used in this work appeared similar in morphology belonging to the *duodenalis* group (Filice 1952) as evidenced by 'claw hammer' morphology of the median body characteristic of this group (Section 7.2). When examined for electrophoretic mobility of 27 enzymes (Table 7.1), they were identical suggesting the two isolates are very closely related genetically. Furthermore, both were identical at all loci to two isolates of *G. lamblia* from humans. Although this cannot be taken to imply that the human and rat isolates are genetically identical or that cross-infections can occur, it does emphasize the close relationship between all of the organisms and the need to keep an open mind to the question of zoonotic transmission of giardiasis. Other isozyme studies from this laboratory (Andrews & Mayrhofer, unpublished) indicate that *G. muris* is in fact a separate species and

related distantly to *G. duodenalis*.

8.2 Rat strain susceptibility to *Giardia* infection

Genetic differences in the response of mice to various intracellular protozoan infections are well documented (Bradley 1980). However, only a few studies have examined genetic differences in the susceptibility of mice to the extracellular gut-dwelling protozoan, *Giardia* (Belosevic *et al.* 1984b; Roberts-Thomson & Mitchell 1978; Roberts-Thomson *et al.* 1980). These workers demonstrated that there were marked differences in the duration of the infection among different strains of mice. Roberts-Thomson & Mitchell (1978) demonstrated that in most mouse strains tested, the infection is self-limiting. Mice are resistant to reinfection, with the degree of resistance being greater in those strains in which the duration of the primary infection was short (Roberts-Thomson & Mitchell 1978). In contrast, persistent infection was observed in hypothymic nude mice (Roberts-Thomson & Mitchell 1978; Stevens *et al.* 1978), normal C3H/He mice (Roberts-Thomson & Mitchell 1978) and mast cell deficient (W^f/W^f) mice (Roberts-Thomson *et al.* 1981). However, Belosevic *et al.* (1984b) did not observe chronic infection in C3H/He mice possibly due to different substrains of C3H/He mice. Both groups of workers concluded that susceptibility to prolonged infection with *G. muris* is under the control of several genes (Belosevic *et al.* 1984b; Roberts-Thomson *et al.* 1980). The genes controlling this trait did not appear to be linked to the H-2 locus.

In contrast to the above reports, there were no differences in inbred rat strains tested in this study for susceptibility to infection with *G. duodenalis* isolates. This suggests that genetic factors are less important in the rat in defining susceptibility to infection. However, larger numbers of rat strains would have to be examined to support this view. All rats tested were resistant to reinfection with the homologous isolate of *G. duodenalis*. None of the normal inbred rat strains used have a specific immunological defect and, therefore, their failure to eliminate a primary infection with the rat isolate may reflect either specific immunological unresponsiveness to

Giardia antigens of this isolate. Alternatively, the rat isolate may lack key antigens or possess the ability to evade the immune response in all of the strains of rats. The latter possibility is the more likely explanation, because infection with the rat isolate does induce resistance to reinfection with either isolate and, as discussed below subsequent experiments demonstrated the presence of specific immunological responses to antigens of both isolates.

Although related by morphology and at all enzyme loci tested, the two rodent isolates of *G. duodenalis* cause very different infections in rats (Chapter 3). The infection produced by the mouse isolate was acute. This isolate also produced an acute infection in BALB/c mice similar to that described for *G. muris* (Roberts-Thomson & Mitchell 1978). In contrast, the rat isolate produced a chronic infection in rats, with no evidence of resolution after many weeks. In Balb/c mice, this isolate produced an acute infection similar to that caused by the mouse isolate, although in C3H/HeJ mice the infection with the rat isolate was more chronic, though resolving in approximately 12 weeks.

8.3 Anti-*Giardia* immune response in rats

Evidence was obtained in rats of a competent anti-*Giardia* immune response to the rat isolate. When persistently infected rats were cured by treatment with metronidazole, they were resistant to challenge not only with the homologous isolate (Chapter 4) but also with the heterologous isolate (Chapter 7). Anti-*Giardia* immune responses to the rat isolate were compared with the responses of rats to the mouse isolate. Studies reported in this thesis have shown that IgA and IgG antibodies, but not IgM, appear in the bile of rats during primary infections with the two isolates of *G. duodenalis* (Chapter 4). Titres of IgA antibodies were similar in infections with the two isolates, and in both cases secondary infections produced a boost in antibody levels. It is clear that this isotype also contributes to the secretory antibody response. This confirms the observations of others that IgG appear in bile after oral as well as parenteral administration of antigen (Andrew & Hall

1982a; Jackson & Cooper 1981).

IF studies on trophozoites immediately after isolation provided evidence of binding of IgA antibodies with specificities for trophozoite surfaces. The intensity of staining on trophozoites of the rat isolate was equivalent to that observed on trophozoites of the mouse isolate at an equivalent stage of the infection. Therefore, measurements of total anti-*Giardia* antibody levels failed to discriminate clearly between the two isolates of *G. duodenalis*.

The picture which is emerging is that of *Giardia* causing a localized infection and presenting antigenic stimuli to the intestinal antibody defence system. During the course of the infection, antigens must become available to local and perhaps also peripheral lymphoid tissue. Antibodies enter the circulation and some are secreted into bile. However, some circulating antibodies of the IgM isotype are produced which appear to be largely irrelevant to protection, although they may have some transient or *back-up* importance. However, the response of the local secretory IgA system is rapid, producing antibody at the site of parasitic attack (Chapters 4 & 5). In rats, this antibody reaches the intestine mainly via the bile, but in other species such as man, local secretion would be more important. It is evident that this system has the potential to play an essential part in the protective immune response to *Giardia*.

It is against this background of local immunity that the functional significance of the serum antibody response must be defined. Normal infections with *Giardia* in rats stimulate a serological response giving rise to IgM and IgA antibodies (it is expected that IgG antibodies can also be detected in sera [Anders *et al.* 1982] of infected rats although these were not assayed in the sera of the rats used in this study, as discussed in Chapter 4). These antibodies could mediate a variety of potentially protective effects such as lysis, agglutination, opsonization or immobilization (Belosevic & Faubert 1987; Hill *et al.* 1984; Smith *et al.* 1983).

However, for serum antibodies to be effective by any mechanism they must be made available in adequate concentrations at the site of infection together with accessory factors such as complement components. Transudation of serum antibody

does not significantly contribute to the overall level of antibody in secretions (Orlans *et al.* 1978). There remains the possiblity, however, that a humoral response may be essential to prevent trophozoites being carried in the blood stream to other organs since giardial infections have been reported to involve occasionally extra-intestinal sites (Roberts-Thomson *et al.* 1982; Smith 1985), although penetration of the epithelial layer is still a matter of controversy (see Section 1.4.2). In this way, serum antibodies could be seen as having a *back-up* function, perhaps having a transient importance or becoming involved only when invasion threatens. Serum antibodies can have anti-trophozoite effects, either directly (Nash & Aggarwal 1986), through the action of complement (Belosevic & Faubert 1987; Butscher & Faubert 1988; Hill *et al.* 1984) or by opsonization and subsequent phagocytosis by macrophages (Kaplan *et al.* 1985; Radulescu & Meyer 1981). Complement-mediated lysis and phagocytosis could have importance within the mucosa, but it seems unlikely that either are significant factors within the gut lumen. However, direct cytotoxic effects of antibody, together with other effects such as prevention of adherence, immobilization or agglutination are all possible mechanisms by which antibodies of any isotype could provide protection. Biliary IgG antibodies, presumably derived from serum, could therefore contribute to protection provided that they are not degraded within the gut lumen.

8.4 Protective immunity

Mechanisms of protective immunity in giardiasis remains largely unresolved. Experimental evidence suggests that antibodies play some part in protection because B-cell depleted normal animals are unable to clear infection they otherwise normally would have (Snider & Underdown 1986). Some protection against infection is also afforded by treatment with monoclonal antibodies against a surface antigen of *G. muris* (Butscher & Faubert 1988).

In this thesis, experiments are described that were undertaken to test directly the hypothesis that secretory antibody is protective in immunity to *Giardia*

(Chapter 6). A technique of passive infusion of bile was developed that allows for observations to be made on the rate of cyst excretion by recipients of biliary antibodies. This technique allows for the delivery of secretory antibodies in a physiological form and at a physiological rate (0.5 ml bile/hr) into the small intestine by controlled infusion via a cannula inserted into the recipient's duodenum (Section 2.4.2). Approximately 0.5-1.0 mg IgA is secreted in rat bile per hour (Lemaitre-Coelho *et al.* 1977; Manning *et al.* 1984; Peppard 1979). Bile contains from 0.5 to 0.8 mg/ml of sIgA. Therefore, this technique infuses approximately 0.3 to 0.4 mg of IgA per hour. Non-specific effects of bile were controlled by comparison of the responses to immune bile versus normal bile (Chapter 6).

A protective effect of infusion of immune bile was observed in infections with the mouse isolate. Immune bile led to decreased rates of cyst excretion whether it was infused during the establishment phase in naive hosts or after infection had become patent. The effect fell short of complete eradication of cysts from the faeces. This was not entirely unexpected in view of the practical limitations on the period for which bile could be infused, and the relatively long time course of the primary infection with this organism. Elimination of infection by the active immune response appears to involve gradual attrition of the trophozoite population and for this reason the substantial effects of the immune bile infusions are more remarkable. The fact that cyst excretion remained low following the period of passive infusion may be due to the onset of the developing active immune response. The kinetics of clearance of this residual infection followed the same pattern of clearance as that observed in the control infection.

No protection was obtained against the rat isolate by infusion of homologous immune bile. Results obtained in Chapter 3 have shown that prior infection with this isolates leads to resistance to reinfection. Therefore, it was expected that immune bile would lead to prevention of establishment of infection. The explanation for this could be that the rat isolate has a greater ability to evade the immune response, perhaps due to a more rapid rate of antigenic change (Aggarwal & Nash 1988); the period of bile infusion would then have been inadequate to overcome

this and produce a detectable effect. It is also possible that mechanisms other than humoral immunity may be important in resistance to infection with this organism. The findings are in accordance with the greater potential of the rat isolate to establish more persistent infections in rats.

Studies of secretory antibody responses in nude rats also provide indirect evidence for the importance of secretory antibody. IgA and IgG antibody responses to infection were markedly reduced in these animals compared with phenotypically normal rats. The results support the suggestions by Heyworth *et al.* (1987) that the defective clearance of *Giardia* in thymus-deficient animals is secondary to a deficiency of T cell help for IgA antibody responses.

8.5 The role of mucus in *Giardia* infection

Trophozoites have been observed to be associated with mucus *in vivo*. They penetrate the mucus gel (Das *et al.* 1988; Erlandsen & Feely 1984; Owen *et al.* 1979) and have a marked affinity for mucus strands (Das *et al.* 1988). This is borne out by the experiments described in Chapter 5 in which the majority of both trophozoites and cysts is associated with mucus. Recent reports support the idea that association with mucus is beneficial to trophozoites in several ways. Intestinal mucus promotes growth of *G. lamblia in vitro* in medium simulating the upper small intestine (Gault *el al.* 1987) although it was not determined whether and how intestinal mucus is metabolized by *Giardia*. Small, but not large, intestinal mucus promoted attachment of trophozoites *in vitro* (Zenian & Gillin 1985) and also protected them from toxic factors in, or generated by, intestinal fluid (Das *et al.* 1988; Reiner *et al.* 1986; Zenian & Gillin 1987). These studies suggest that small intestinal mucus favors the growth and specific colonization of the small intestine by *Giardia*.

In this context, it is tempting to speculate that trophozoites interact with mucus via a receptor-mediated mechanism. As discussed in Section 1.4.1, a plasma membrane-bound surface lectin has been described in human and murine trophozoites (Farthing *et al.* 1986b; Lev *et al.* 1986a, 1986b; Ward *et al.* 1987). Although

the function of the lectin has been discussed mainly in terms of adherence to the mucosa, it may be involved equally in adherence of the parasite to mucus *in vivo*. This hypothesis awaits further clarification. On the other hand, the effect of sIgA might be amplified by association with mucus. SIgA in apical mucin might have a functional role in immunity to giardiasis by attaching to the surface of trophozoites and inhibiting or delaying their attachment to the mucosa. SIgA has been shown to make bacteria mucophilic (Magnusson & Stjernstrom 1982). Attachment to the mucus blanket could therefore aid in the clearance of the parasite from the intestine. Further studies are required to test the efficiency with which sIgA antibodies increase the association of trophozoites with rat intestinal mucus. This can be done by incubating trophozoites in dilutions of sIgA antibodies in the presence of graded concentrations of mucus or determining whether sIgA increases affinity of the organism for mucin columns.

By the same token, it is possible mucus provides stimuli that induce increased encystation of trophozoites, as described in Chapter 5. This possibility is also suggested by the observations that cysts were always seen enmeshed in mucus and never free in the intestinal lumen of infected rats. If sIgA antibodies increased the association of trophozoites with mucus, accentuation of the encystation process could lead to attrition of the vegetative population.

However, it was not possible technically to differentiate whether trophozoites with bound sIgA were more frequent in the free and mucus-associated fractions of trophozoites harvested from the intestines of DA rats infected with the two isolates of *G. duodenalis* (Table 5.4).

8.6 Future studies

The studies reported in this thesis have not identified the unique characteristics of the two *G. duodenalis* isolates which account for the differences in the infections that they produce. It is clear that they share major surface proteins as well as being similar genetically (Chapter 7). They nevertheless behave differently biologically;

one causing an acute infection and the other a chronic infection.

However, both isolates are capable of inducing a protective immune response against both homologous and heterologous challenge infections. This, together with the evidence from immunofluorescence studies (Chapter 7), suggests that the organisms share common surface antigens and that at least some of the shared antiges are potentially protective. Furthermore, both isolates induce secretory and serum antibody responses that have similar magnitudes and kinetics (Chapter 4) and similar proportions of trophozoites exhibit bound IgA antibody at various stages throughout infection (Chapter 5). The trophozoite population in each case is clearly heterogenous with respect to surface antigens but in each case, the immune response appears to 'mature' at a similar rate to gradually encompass most antigen specificities.

The two isolates of *G. duodenalis* offer an interesting model in which to explore the factors which determine the effectiveness of the host's immune response in eliminating *Giardia*. The challenge is now on the one hand to dissect the host's immune response to determine exactly against which antigens antibody is being produced at various stages during infection and on the other to identify which antigens are cross-reactive, which are unique between the isolates and ultimately, which are protective. Furthermore, parasite-determined factors such as ability/facility to vary surface antigens as well as micro-ecological preferences of the two isolates must be considered.

Further studies required to detect subtle differences between the isolates could include the following:

8.6.1 Analysis of the specificity of biliary IgA antibodies

This could be accomplished by immunoblotting and immunoprecipitation analyses of surface-labeled trophozoite antigens, analogous to the studies described by Erlich *et al.* (1983). These experiments could establish whether there is a qualitative difference in the antibody responses of the chronically infected rats versus the acutely infected animals. Such studies, using homologous and heterologous biliary antibod-

ies and appropriate cross-absorptions, could establish whether antigens would be detected that were present on the mouse isolate and absent from the rat isolate. The problem of defining specific surface antigens that are protective is a difficult one. Genetic approaches using site-directed mutagenesis to modify or delete surface antigens are at present a distant possibility. Classical serological approaches involving absorptions are impractical unless the *G. duodenalis* isolate can be adapted to culture. Perhaps the most promising approach might be to raise monoclonal antibodies to candidate antigens (preferably of the IgA isotype) and to use these passively to assess protection.

8.6.2 Molecular biology studies

Molecular biological techniques could be employed to further assess the closeness of the relationship between the two isolates of *G. duodenalis*. Such studies could include analysis of restriction endonuclease patterns of the DNA from the isolates as well as Southern blot hybridization using cloned DNA fragments from the isolates (Adam *et al.* 1988; Nash *et al.* 1985). However interesting the findings of the studies might be, they are unlikely to point to the genetic differences responsible for the biological characteristics of the organisms.

If the comparative studies of surface antigens described above yielded candidate protective antigens peculiar to the mouse isolate, a longer term strategy would be to attempt to clone the genes for those antigens. Cloned DNA could then be used to probe the genome of the rat isolate to determine whether related genes are present in this organism. Ultimately one would wish to introduce the cloned material into the rat isolate and to gain expression so that the effects on the nature of the infection could be examined. However, there have been no reports of transfection in *Giardia* and such studies would require a large committment to working out basic methods for transfection and for selection of transfectants. It seems likely that immunological techniques will provide the shorter route to identification of protective antigens. Once defined, recombinant DNA methods could be applied to cloning the relevant genes, assist in obtaining sequence and allow production of large amounts

of product for further characterization, for immunization and for absorption studies.

Chapter 9

Bibliography

Abdel-Hafez M.M.A, El-Kady N., Bolbol A.S. & Baknina M.H. (1986) Prevalence of intestinal parasitic infections in Riyadh district, Saudi Arabia. *Ann. Trop. Med. Parasitol.* **80**: 631–634.

Adam R.D., Aggarwal A., Lai A.A., Dela Cruz V., McCutchan T. & Nash T.E. (1988) Antigenic variation of cysteine-rich protein in *Giardia lamblia*. *J. Exp. Med.* **167**: 109–118.

Aggarwal A., Bhatia A., Naik S.R. & Vinayak V.K. (1983) Variable virulence of isolates of *Giardia lamblia* in mice.*Ann. Trop. Med. Parasitol.* **77**: 163–167.

Aggarwal A. & Nash T.E. (1986) Lack of cellular cytotoxicity by human mononuclear cells to *Giardia*. *J. Immunol.* **136**: 3486–3488.

Aggarwal A. & Nash T.E. (1987a) Comparison of two antigenically distinct *Giardia lamblia* isolates in gerbils. *Am. J. Trop. Med.* **36**: 325–332.

Aggarwal A. & Nash T.E. (1987b) *Giardia lamblia*: RNA translation products. *Exp. Parasitol.* **64**: 336–341.

Aggarwal A. & Nash T.E. (1988) Antigenic variation of *Giardia lamblia in vivo*. *Infect. Immun.* **56**: 1420–1423.

Allen A., Bell A., Mantle M. & Pearson J.P. (1982) The structures and physiology of gastrointestinal mucus. *Adv. Exp. Med. Biol.***144**: 115–133.

Ament M.E. & Rubin C.E. (1972) Relation of giardiasis to abnormal intestinal structure and function in gastrointestinal immunodeficiency syndromes. *Gastroenterology.* **62**: 216–226.

Ament M.E., Ochs H.D. & Davis S.D. (1973) Structure and function of the gastrointestinal tract in primary immunodeficiency syndromes. A study of 39 patients. *Medicine.* **52**: 227–248.

Anand B.S., Kumar M., Chakravarti R.N., Sehgal A.K. & Chhutlani P.N. (1980) Pathogenesis of malabsorption in *Giardia* infection: an experimental study in rats. *Trans. R. Soc. Trop. Med. Hyg.* **74**: 565–569.

Anders R.F., Roberts-Thomson I.C. & Mitchell G.F. (1982) Giardiasis in mice: analysis of humoral and cellular immune response to *Giardia muris*. *Parasite Immunology.* **4**: 47–57.

Andrew E. & Hall J.G. (1982a) IgA antibodies in bile of rats. I. Some characteristics of the primary response. *Immunology.* **45**: 169–175.

Andrew E. & Hall J.G. (1982b) IgA antibodies in the bile of rats. II. Evidence for immunological memory in secretory immunity. *Immunology.* **45**: 177–182.

Andrews R.H., Adams M., Boreham P.F.L., Mayrhofer G. & Meloni B.P. (1988a) *Giardia intestinalis*: electrophoretic evidence for a species complex. *Int. J. Parasitol.* In press.

Andrews J.S. Jr., Ellner J.J. & Stevens D.P. (1979) Identification of anti-*Giardia* antibodies in murine breast milk. *Clin. Res.* **27**: 340A.

Andrews R.H., Handman E., Adams M., Baverstock P.R. & Mitchell G.F. (1988b) Genetic characterization of *Leishmania* isolates at 37 enzyme loci. *Int. J. Parasitol.* **18**: 445–452.

Andrews J.S. Jr., & Hewlett E.L. (1981) Protection against infection with *Giardia muris* by milk containing antibody to giardia. *J. Infect. Dis.* **143**: 242–246.

Antia F.P., Desai H.G., Jeejeebhoy K.N., Kane M.P. & Borkar A.V. (1966) Giardiasis in adults: incidence, symptomatology and absorption studies. *Indian J. Med. Sci.* **20**: 471–477.

Armaghan V. (1937) Biological studies on the *Giardia* of rats. *Am. J. Hyg.* **26:** 237–258.

Arvind A.S., Butcher P.D., Webster A.D.P. & Farthing M.J.G. (1988) Anti-*Giardia* IgG activity in intravenous gammaglobulin preparations: possible therapeutic implications. *Gastroenterology.* **94:** A14.

Avrameas S. (1969) Coupling of enzymes to proteins with glutaraldehyde. Use of the conjugate for the detection of antigens and antibodies. *Immunochemistry.* **6**: 53–66.

Avron B., Chayen A., Stolarsky T., Schauer R., Reuter G. & Mirelman D. (1987) A stage-specific sialoglycoprotein in encysting cells of *Entameba histolytica. Mol. Biochem. Parasitol.* **25:** 257–266.

Avron B., Stolarsky T., Chayen A. & Mirelman D. (1986) Encystation of *Entameba invadens* IP-1 is induced by lowering the osmotic pressure and depletion of nutrients from the medium. *J. Protozool.* **33**: 522–525.

Balasz & Szatloczky E. (1978) Electron microscopic examination of the mucosa of the small intestine in infection due to *Giardia lamblia. Pathol. Res. Pract.* **163:** 251–260.

Barbour A.G., Nichols C.R. & Fukushima T. (1976) An outbreak of giardiasis in a group of campers. *Am. J. Trop. Med. Hyg.* **25**: 384–389.

Barbour A.G., Tessier S.L. & Stoenner H.G. (1982) Variable major proteins of *Borrelia hermsii. J. Exp. Med.* **156**: 1312–1324.

Barnard R.J. & Jackson G.J. (1984) *Giardia lamblia.* The transfer of human infections by foods. In: *Giardia and Giardiasis, Biology, Pathogenesis and Epidemiology.* Eds. S.L. Erlandsen & E.A. Meyer. New York: Plenum Press. pp. 365–378.

Barnes G.L. & Kay R. (1977) Blood groups in giardiasis. *Lancet.* **1**: 808.

Bassily S., Farid Z., El-Masry N.A. & Mikhail E.M. (1987) Treatment of intestinal *Entameba histolytica* and *Giardia lamblia* with metronidazole, tinidazole and ornidazole: a comparative study. *J. Trop. Med.* **90**: 9–12.

Baveja U.K., Jyoti A.S., Kaur M., Agarwal D.S., Anand B.S. & Nanda R. (1986) Isoenzyme studies of *Giardia lamblia* isolated from symptomatic cases. *Aust. J. Exp. Biol. Med. Sci.* **64**: 119–126.

Baveja U.K. & Warhurst D.C. (1983) Humoral response in giardiasis. *J. Commun. Dis.* **15**: 256–260.

Bazin H., Maldaque P.M., Schonne E., Crabbe P.A., Bouldon H. & Heremans J.F. (1971) The metabolism of different immunoglobulin classes in irradiated mice. V. Contribution of the gut to serum IgA levels in normal and irradiated mice. *Immunology.* **20:** 571–595.

Bazin H., Platteau B., Pauwels R. & Capron A. (1980) Immunoglobulin production in nude rats with special attention to the IgE isotype. *Ann. Immunol.* **131:** 31–37.

Befus D. & Bienenstock J. (1982) Factors involved in symbiosis and host resistance at the mucosa-parasite interface. *Prog. Allergy.* **31:** 76–77.

Belosevic M. & Faubert G.M. (1983a) *Giardia muris*: correlation between oral dosage, course of infection and trophozoite distribution in the mouse small intestine. *Exp. Parasitol.* **56**: 93–100.

Belosevic M. & Faubert G.M. (1983b) Temporal study of acquired resistance in infections of mice with *Giardia muris*. *Parasitology.* **87**: 517–524.

Belosevic M. & Faubert G.M. (1986a) Killing of *Giardia muris* trophozoites *in vitro* by spleen, mesenteric lymph node and peritoneal cells from susceptible and resistant mice. *Immunology.* **59**: 269– 275.

Belosevic M. & Faubert G.M. (1986b) Comparative studies of inflammatory responses in susceptible and resistant mice infected with *Giardia muris*. *Clin. Exp. Immunol.* **65**: 622–630.

Belosevic M. & Faubert G.M. (1987) Lysis and immobilization of *Giardia muris* trophozoites *in vitro* by immune serum from susceptible and resistant mice. *Parasite Immunology.* 9: 11–19.

Belosevic M., Faubert G.M., Guy R. & MacLean J.D. (1984a) Observations on natural and experimental infections with *Giardia* isolated from cats. *Can. J. Comp. Med.* **48**: 241–244.

Belosevic M., Faubert G.M. & MacLean J.D. (1985a) Suppression of primary antibody response to sheep erythrocytes in susceptible and resistant mice infected with *Giardia muris*. *Infect. Immun.* **47**: 21–25.

Belosevic M., Faubert G.M. & MacLean J.D. (1985b) *Giardia muris* induced depression of the primary immune response in spleen and mesenteric lymph node cell cultures to sheep red blood cells. *Parasite Immunol.* **7**: 467–478.

Belosevic M., Faubert G.M. & MacLean J.D. (1986b) The effects of cyclosporin A on the course of infection with *Giardia muris* in mice. *Am. J. Trop. Med. Hyg.* **35**: 496–500.

Belosevic M., Faubert G.M., MacLean J.D., Law C. & Croll N.A. (1983) *Giardia lamblia* infections in mongolian gerbils: an animal model. *J. Infect. Dis.* **147**: 222–226.

Belosevic M., Faubert G.M., Skamene E. & MacLean J.D. (1984b) Susceptibility and resistance of inbred mice to *Giardia muris*. *Infect. Immun.* **44**: 282–286.

Bemrick W.J. (1961) The host specificity of *Giardia* from laboratory strains of *Mus musculus* and *Rattus norvegicus*. *J. Parasitol.* **48**: 287–290.

Bemrick W.J. (1963) The effect of bile flow on *Giardia duodenalis* race *simoni* in the intestine of a laboratory strain of *Rattus norvegicus*. *J. Parasitol.* **49**: 956–959.

Bemrick W.J. (1984) Some perspectives on the transmission of *Giardias*. In: *Giardia and Giardiasis, Biology, Pathogenesis and Epidemiology*. Eds. S.L. Erlandsen & E.A. Meyer. New York: Plenum Press. pp. 379–400.

Bertram M.S., Meyer E.A., Lile J.D. & Morse S.A. (1983) A comparison of isozymes of five axenic *Giardia* isolates. *J. Parasitol.* **69**: 793–801.

Bessen D. & Fischetti V.A. (1988) Passive acquired mucosal immunity to group A streptococci by secretory immunoglobulin A. *J. Exp. med.* **167**: 1945–1950.

Bhatia V.N. & Warhurst D.C. (1981) Hatching and subsequent cultivation of cysts of *Giardia intestinalis* in Diamond's medium. *J. Trop. Med.* **84**: 45.

Bienenstock J & Dolezel J. (1971) Peyer's patches. Lack of specific antibody-containing cells after oral and parenteral immunization. *J. Immunol.* **106:** 938–945.

Bingham A.K. & Meyer E.A. (1979) *Giardia* excystation can be induced *in vitro* in acidic solutions. *Nature.* **277**: 301–302.

Birbeck M.S.C., Cartwright P., Hall J.G., Orlans E. & Peppard J. (1979) The transport by hepatocytes of immunoglobulin A from blood to bile visualized by autoradiography and electron microscopy. *Immunology.* **37:** 477–484.

Black R.E., Dykes A.C., Sinclair S.P. & Wells J.G. (1977) Giardiasis in day-care centers: evidence of person-to-person transmission. *Pediatrics.* **60**: 486–491.

Blair R.J. & Weller P.F (1987) Uptake and esterification of arachidonic acid by trophozoites of *Giardia lamblia. Mol. Biochem. Parasitol.* **25:** 11–18.

Bockman A. & Winborn W.B. (1968) Electron microscopic localization of exogenous ferritin within vacuoles of *Giardia muris. J. Parasitol.* **15:** 26–30.

Boeck W.C. (1919) Studies on *Giardia microti. Univ. Calif. Publ. Zool.* **19**: 85–134.

Boland C.R. & Owen R.L. (1982) Are lactating mothers a source of giardiasis? *N. Eng. J. Med.* **307**: 820.

Boorman G.A., Lina P.H.C., Zurcher C. & Nierwerkerk H.T.M. (1973) *Hexamita* and *Giardia* as a cause of mortality in congenitally thymusless (nude) mice. *Clin. Exp. Immunol.* **15:** 623–627.

Boreham P.F., Dondey J. & Walker R. (1981) Giardiasis among children in the city of Logan, Southeast Queensland. *Aust. Pediatr. J.* **17**: 209–212.

Boreham P.F. & Phillips R.E. (1986) Giardiasis in Mount Isa, Northwest Queensland. *Med. J. Aust.* **144**: 524–528.

Boreham P.F.L., Phillips R.E. & Shepherd R.W. (1988) Altered uptake of metronidazole *in vitro* by stocks of *Giardia intestinalis* with different drug sensitivities. *Trans. Roy. Soc. Trop. Med. Hyg.* **82:** 104–106.

Boreham P.F. & Shepherd R.W. (1984) Giardiasis in child care centers. *Med. J. Aust.* **141**: 263.

Borst P. & Cross G.A.M. (1982) Molecular basis for trypanosome antigenic variation. *Cell.* **29**: 291–303.

Borst P. & Greaves D.R. (1987) Programmed gene rearrangements altering gene expression. *Science.* **235**: 658–667.

Bradley D.J. (1980) Genetic control of resistance to protozoal infections. In: *Genetic control of natural resistance to infection and malignancy.* Eds. E. Skamene, P.A.L. Kongshavn & M. Landy. New York: Academic Press, Inc. pp. 9–28.

Brandborg L.L., Tankersley C., Gottlieb S., Barancik M. & Sartor V.E. (1967) Histological demonstration of mucosal invasion by *Giardia lamblia* in man. *Gastroenterology.* **52**: 143–150.

Brandtzaeg P. (1975) Human secretory immunoglobulin M. An immunochemical and immunohistochemical study. *Immunology.* **29**: 559–569.

Brandtzaeg P. (1985) Role of J chain and secretory component in receptor-mediated glandular and hepatic transport of immunoglobulins in man. *Scand. J. Immunol.* **22**: 111–146.

Brett S.J. (1983) Immunodepression in *Giardia muris* and *Spironucleus muris* infections in mice. *Parasitology.* **87**: 507–515.

Brett S.J. & Cox F.E.G. (1982) Immunological aspects of *Giardia muris* and *Spironucleus muris* infections in inbred and outbred strains of laboratory mice: a comparative study. *Parasitology.* **85**: 85–99.

Briaud M., Morichau-Beauchant M., Matuchansky C., Touchard G. & Babin P. (1981) Intestinal immune response in giardiasis. *Lancet.* **2**: 358.

Brodsky R.E., Spencer H.C.Jr. & Schultz M.G. (1974) Giardiasis in American travelers to the Soviet Union. *J. Infect. Dis.* **130**: 319–323.

Brooks C.G., Webb P.J., Robins R.A., Robinson G., Baldwin R.W. & Festing M.F.W. (1980) Studies on the immunobiology of rnu/rnu 'nude' rats with congenital aplasia of the thymus. *Eur. J. Immunol.* **10**: 58–65.

Brown P.J., Charley-Poulain J. & Pery P. (1981) *Nippostrongylus brasiliensis* infection in the rat. I. Production of bile IgA and serum IgG antibodies by adult rats. *Vet. Immunol. Immunopathol.* **2**: 343–352.

Buchel L-A., Gorenflot A., Chochillon C., Savel J. & Gobert J-G. (1987) *In vitro* excystation of *Giardia* from humans: a scanning electron microscopy study. *J. Parasitol.* **73**: 487–493.

Butcher E.C., Rouse R.V., Coffman R.L., Nottenburg C.N., Hardy R.R. & Weisman I.L. (1982) Surface phenotype of Peyer's patch germinal center cells: implications for the role of germinal centers in B-cell differentiation. *J. Immunol.* **129:** 2698–2707.

Butscher W.G. & Faubert G.M. (1988) The therapeutic action of monoclonal antibodies against a surface glycoprotein of *Giardia muris*. *Immunology.* **64:** 175–180.

Carlson J. R., Heyworth M.F. & Owen R.L. (1986a) Response of Peyer's patch lymphocyte subsets to *Giardia muris* infection in Balb/c mice. I. T-cell subsets. *Cell. Immunol.* **97**: 44–50.

Carlson J.R., Heyworth M.F. & Owen R. L. (1986b) Response of Peyer's patch lymphocyte subsets to *Giardia muris* infection in Balb/c mice. II. B-cell subsets: enteric antigen exposure is associated with immunoglobulin isotype switching by Peyer's patch B cells. *Cell. Immunol.* **97**: 51–58.

Carlson J.R., Heyworth M.F. & Owen R.L. (1987) T-lymphocyte subsets in nude mice with *Giardia muris* infection. *Thymus*. **9**: 189–196.

Chavalittamrong B., Charoenviohya S., Tuchinda P. & Suntornpoch. (1978) Prevalence of *Giardia lamblia* in children attending an outpatient department of Siriraj Hospital. *S.E. Asian J. Trop. Med. Publ. Hlth.* **9:** 51–54.

Chavez B., Knaippe F., Gonzalez Mariscal L. & Martinez Palomo A. (1986) *Giardia lamblia*: electrophysiology and ultrastructure of cytopathology in cultured epithelial cells. *Exp. Parasitol.* **61:** 379–389.

Chayen A., Avron B. & Mirelman D. (1985) Changes in cell surface proteins and glycoproteins during the encystation of *Entameba invadens*. *Mol. Biochem. Parasitol.* **15**: 83–93.

Chayen A., Avron B., Nuchamowitz Y. & Mirelman D. (1988) Appearance of sialoglycoproteins in encysting cells of *Entameba histolytica*. *Infect. Immun.* **56**: 673–681.

Clamp J.G. (1981) The role of mucus secretions in the protection of the gastrointestinal mucosa. *Curr. Topics Vet. Med. Animal Aci.* **12**: 389–397.

Clamp J.R. & Creeth J.M. (1984) Some non-mucin components of mucus and their possible biological roles. *Ciba Foundation Symposium.* **109**: 121–136.

Clark J.T. & Holberton D.V. (1986) Plasma membrane isolated from *Giardia lamblia*: identification of membrane proteins. *Eur. J. Cell. Biol.* **42**: 200–206.

Coggins J. R. & Schaefer III F.W. (1984) *Giardia muris*: scanning electron microscopy of *in vitro* excystation. *Exp. Parasitol.* **57**: 62–67.

Coggins J.R. & Schaefer III F.W. (1986) *Giardia muris*: ultrastructural analysis of *in vitro* excystation. *Exp. Parasitol.* **61**: 219–228.

Cole S.R., Ashman L.K. & Ey P.L. (1987) Biotinylation: an alternative to radioiodination for the identification of cell surface antigens in immunoprecipitates. *Mol. Immunol.* **24**: 699–705.

Collins G.H., Pope S.E., Griffin D.L., Walker J. & Connor G. (1987) Diagnosis and prevalence of *Giardia* spp. in dogs and cats. *Aust. Vet. J.* **64**: 89–90.

Corradetti A. (1935) *Giardia enterica* (Grassi 1881) the correct name for the *Giardia* of man. *J. Parasitol.* **21**: 310–311.

Craft J.C. (1982) Experimental infection with *Giardia lamblia* in rats. *J. Infect. Dis.* **145**: 495–498.

Craft J.C., Murphy T. & Nelson J.D. (1981) Furazolidone and quinacrine: comparative study of therapy for giardiasis in children. *Am. J. Dis. Child.* **135**: 164–166.

Craft J.C. & Nelson J.D. (1982) Diagnosis of giardiasis by counterimmunoelectrophoresis of feces. *J. Infect. Dis.* **145**: 499–504.

Craig S.W. & Cebra J.J. (1971) Peyer's patches: an enriched source of precursors for IgA-producing immunocytes in the rabbit. *J. Exp. Med.* **134:** 188–200.

Craun G.F. (1984) Waterborne outbreaks of giardiasis. In: *Giardia and Giardiasis, Biology, Pathogenesis and Epidemiology*. Eds. S.L. Erlandsen & E.A. Meyer. New York: Plenum Press. **pp.** 243–261.

Cross G.A.M. (1978) Antigenic variation in trypanosomes. *Proc. R. Soc. Lond.* **202:** 55–72.

Cross G.A.M. (1984) Structure of the variant glycoproteins and surface coat of *Trypanosoma brucei. Phil. Trans. R. Soc. Lond.* **307:** 3–12.

Crossley R. & Holberton D.V. (1983) Characterization of proteins from the cytoskeleton of *Giardia lamblia. J. Cell Sci.* **59**: 81–103.

Crossley R. & Holberton D.V. (1985) Assembly of 2.5 nm filaments from giardin, a protein associated with cytoskeleton microtubules in *Giardia. J. Cell. Sci.* **78**: 205–231.

Crossley R., Marshall J., Clark J.T. & Holberton D.V. (1986) Immunocytochemical differentiation of microtubules in the cytoskeleton of *Giardia lamblia* using monoclonal antibodies to alpha-tubulin and polyclonal antibodies to associated low molecular weight proteins. *J. Cell. Sci.* **80**: 233–252.

Dahlgren U.I.H., Ahlstedt S. & Hanson L.A. (1986) Hepatic production of biliary IgM in the rat. *Scand. J. Immunol.* **24**: 615–619.

Dahlgren U., Ahlstedt S., Hedman L, Wadsworth C. & Hanson L.A. (1981) Dimeric IgA in the rat is transferred from serum into bile but not into milk. *Scand. J. Immunol.* **14:** 95–98.

Danciger M. & Lopez M. (1975) Numbers of *Giardia* in the feces of infected children. *Am. J. Trop. Med. Hyg.* **24**: 237–242.

Das S., Reiner D.S., Zenian J., Hogan D.L., Koss M.A., Wang CS & Gillin F.D. (1988) Killing of *Giardia lamblia* trophozoites by human intestinal fluid *in vitro*. *J. Infect. Dis.* **157**: 1257–1260.

Davies R.B. & Hibler C.P. (1979) Animal reservoirs and cross-species transmission of *Giardia*. In: *Waterborne transmission of giardiasis*. Eds. W. Jakubowski & J.C. Hoff. Cincinnati: Environmental Protection Agency. pp. 104–126.

Davis L.G., Dibner M.D. & Battey J. (1986) *Basic methods in molecular biology*. New York: Elsevier. pp. 136–138.

Davis P.J. & Porter P. (1979) A mechanism for secretory IgA-mediated inhibition of the cell penetration and intracellular development of *Eimeria tenella*. *Immunology*. **36:** 471–477.

De Carneri I. & Trane F. (1977) Oral infection of mice with trophozoites of *G. muris* *Am. J. Trop. Med. Hyg.* **26:** 566–567.

De Jonckheere J.F. & Gordts B. (1987) Occurrence and transfection of a *Giardia* virus. *Mol. Biochem. Parasitol.* **23**: 85–89.

Deshpande P.D. & Shastri U.V. (1981) Incidence of *Giardia* infection in calves in Maharashtra State, India. *Trop. Anim. Health. Prod.* **13:** 34.

Diamond L.S. (1968) Techniques of axenic cultivation of *Entameba histolytica* Schaudinn, 1903 and *Entameba histolytica*-like ameba. *J. Parasitol.* **54:** 1047–1056.

Diamond L.S., Harlow D. & Cunnick C.C. (1978) A new medium for the axenic cultivation of *Entameba histolytica* and other *Entameba*. *Trans. R. Soc. Trop. Med. Hyg.* **72**: 431–432.

Dobell C. (1920) The discovery of the intestinal protozoa of man. *Proc. Roy. Soc. Med.* **13**: 1–15.

Donelson J.E. & Rice-Ficht A.C. (1985) Molecular biology of trypanosome antigenic variation. *Microbiol. Rev.* **49**: 107–125.

Dooley J.S., Potter B.J., Thomas H.C. & Sherlock S. (1982) A comparative study of the biliary secretion of human dimeric and monomeric IgA in the rat and in man. *Hepatology*. **2:** 323–327.

Doyle J.J., Hirumi H., Hirumi K., Lupton E.N. & Cross G.A.M. (1980) Antigenic variation in clones of animal infective *Trypanosoma brucei* derived and cloned *in vitro*. *Parasitology*. **80:** 359–370.

Dykes A.C., Juranek D.D., Lorenz R.A., Sinclair S., Jakubowski W. & Davies R. (1980) Municipal waterborne giardiasis: an epidemiological investigation. Beavers implicated as a possible reservoir. *Ann. Intern. Med.* **92**: 165–170.

Edson C.M., Farthing M.J.G., Thorley-Lawson D.A. & Keusch G.T. (1986) An 88,000-Mr *Giardia lamblia* surface protein which is immunogenic in humans. *Infect. Immun.* **54**: 621–625.

Einfeld D.A. & Stibbs H.H. (1984) Identification and characterization of a major surface antigen of *Giardia lamblia. Infect. Immun.* **46**: 377–383.

Eistenstein B.I. (1981) Phase variation of type I fimbriae in *Escherichia coli* is under transcriptional control. *Science*. **214:** 337–339.

El-Badrawy F., El-Hady K. & Makled M.K. (1983) Determination of immunoglobulin E (IgE) and complement III (C3) in children with giardiasis. *J. Egyptian Soc. Parasitol.* **13:** 261–269.

Erlandsen S.L. (1974) Scanning electron microscopy of intestinal giardiasis: lesions of the microvillous border of villus epithelial cells produced by trophozoites of *Giardia*. In: *Scanning Electron Microscopy/1973*. Ed. O. Johari. Chicago: IIT Research Institute. pp. 775–782.

Erlandsen S.L. & Feely D.E. (1984) Trophozoite motility and the mechanism of attachment. In: *Giardia and Giardiasis, Biology, Pathogenesis and Epidemiology.* Eds. S.L. Erlandsen & E.A. Meyer. New York: Plenum Press. pp. 33–64.

Erlandsen S.L., Schollmeyer J.V., Feely D.E. & Chase D.G. (1978) Analysis of the attachment and release of *Giardia muris*: evidence for the presence of contractile proteins and their distribution in the adhesive disc and associated with flagella. *J. Cell Biol.* **79:** 264a.

Erlich J.H., Anders R.F., Roberts-Thomson I.C., Schrader J.W. & Mitchell G.F. (1983) An examination of differences in serum antibody specificities and hypersensitivity reactions as contributing factors to chronic infection with the intestinal protozoan parasite, *Giardia muris*, in mice. *Aust. J. Exp. Biol. Med. Sci.* **61**: 599–615.

Evans D.G., de la Cabada F.J. & Evans D.J. (1982) Correlation between intestinal immune response to colonization factor antigen/I and acquired resistance to enterotoxigenic *Escherichia coli* diarrhea in an adult rabbit model. *Eur. J. Clin. Microbiol.* **1:** 178–185.

Farthing M.J.C., Chong S. & Walker-Smith J.A. (1984) Acute allergic phenomena in giardiasis. *Lancet.* **2:** 1428.

Farthing M.J.G., Edson C.M., Prokopec J., Inge P.M.G. & Goka A.J.K. (1985a) Identification of *Giardia* surface antigens and their release during *in vitro* growth. *Gut.* **26**: A552.

Farthing M.J.G., Keusch G.T. & Carey M.C. (1985b) Effects of bile and bile salts on growth and membrane lipid uptake by *Giardia lamblia.* Possible implications for pathogenesis of intestinal disease. *J. Clin. Invest.* **76**: 1727–1732.

Farthing M.J.G., Mata L., Urrutia J.J. & Kronmal R.A. (1986a) Natural history of *Giardia* infection of infants and children in rural Guatemala and its impact on physical growth. *Am. J. Clin. Nutr.* **43**: 395–405.

Farthing M.J.G., Pereira M.E.A & Keusch G.T. (1986b) Description and characterization of a surface lectin from *Giardia lamblia. Infect. Immun.* **51:** 661–667.

Farthing M.J.G., Varon S.R. & Keusch G.T. (1983) Mammalian bile promotes growth of *Giardia lamblia* in axenic culture. *Trans. R. Soc. Trop. Med. Hyg.* **77**: 467–469.

Faubert G.M., Belosevic M., Walker T.S., MacLean J.D. & Meerovitch E. (1983) Comparative studies on the pattern of infection with *Giardia* spp. in mongolian gerbils. *J. Parasitol.* **69**: 802–805.

Feely D.E. (1986) A simplified method for *in vitro* excystation of *Giardia muris. J. Parasitol.* **72**: 474–475.

Feely D.E. (1988) Morphology of the cyst of *Giardia microti* by light and electron microscopy. *J. Protozool.* **35:** 52–54.

Feely D.E., Chase D.G., Hardin E.L. & Erlandsen S.L. (1988) Ultrastructural evidence for the presence of bacteria, viral-like particles and mycoplasma-like organisms associated with *Giardia* spp. *J. Protozool.* **35:** 151–158.

Feely D.E. & Dyer J.K. (1987) Localization of acid phosphatase activity in *Giardia lamblia* and *Giardia muris* trophozoites. *J. Protozool.* **34:** 80–83.

Feely D.E. & Erlandsen S.L. (1981) Isolation and purification of *Giardia* trophozoites from rat small intestine. *J. Parasitol.* **67**: 59–64.

Feely D.E., Erlandsen S.L. & Chase D.G. (1984) Structure of trophozoite and cyst. In: *Giardia and Giardiasis, Biology, Pathogenesis and Epidemiology*. Eds. S.L. Erlandsen & E.A. Meyer. New York: Plenum Press. **pp.** 3–31.

Feely D.E., Schollmeyer J.V. & Erlandsen S.L. (1982) *Giardia* spp.: Distribution of contractile proteins in the attachment organelle. *Exp. Parasitol.* **53:** 145–154.

Ferguson A., McClure J.P. & Townley R.R.W. (1976) Intraepithelial lymphocyte counts in small intestinal biopsies from children with diarrhea. *Acta Pediatr. Scand.* **65**: 541–546.

Festing M.F.W., May D., Connors T.A., Lovell D. & Sparrow S. (1978) An athymic nude mutation of the rat. *Nature.* **274:** 365–366.

Festing M. & Staats J. (1973) Standardized nomenclature for inbred strains of rats (4th listing). *Transplantation.* **16:** 221–229.

Filice F.P. (1952) Studies on the cytology and life history of *Giardia* from the laboratory rat. *Univ. Calif. Publ. Zool.* **57**: 53–146.

Florey H.W. (1955) Mucin and the protection of the body. *Proc. R. Soc. London.* **143**: 147–158.

Fontaine J.-P., Delage A. & Lauraire M.-Cl. (1984) Sur l'epidemiologie de la giardase. *Ann. Parasitol.* **59:** 541–554.

Forstner J.F., Jabbal I. & Forstner G. (1973) Goblet cell mucin of rat small intestine. Chemical and physical characterization. *Can. J. Biochem.* **51**: 1154–1166.

Fortess E. & Meyer E.A. (1976) Isolation and axenic cultivation of *Giardia* trophozoites from the guinea pig. *J. Parasitol.* **62**: 689.

Friend D.S. (1966) The fine structure of *Giardia muris*. *J. Cell Biol.* **29**: 317–332.

Freter R. (1972) Parameters affecting the association of vibrios with the intestinal surface in experimental cholera. *Infect. Immun.* **6**: 134–141.

Fubara E.S. & Freter R. (1973) Protection against enteric bacterial infection by secretory IgA antibodies. *J. Immunol.* **111**: 395–403.

Gasser R.B., Eckert J. & Rohrer L. (1987a) Isolation of *Giardia* from Swiss cattle and cultivation of trophozoites *in vitro*. *Parasitol. Res.* **73**: 182–183.

Gasser R.B., Eckert J. & Rohrer L. (1987b) Infectivity of Swiss *Giardia* isolates to jirds and mice and *in vitro* cultivation of trophozoites originating from sheep. *Parasitol. Res.* **74**: 103–111.

Gault M.J., Gillin F.D. & Zenian A.J. (1987) *Giardia lamblia*: stimulation of growth by human intestinal mucus and epithelial cells in serum-free medium. *Exp. Parasitol.* **64**: 29–37.

Geller M., Geller M., Flaherty D.K., Black P. & Madruga M. (1978) Serum IgE levels in giardiasis. *Clin. Allergy.* **8**: 69–71.

Gill J.S. & Jones H.I. (1985) Intestinal parasites and bacteria in Aboriginal children in South West Australia. *Aust. Pediatr. J.* **21**: 45–49.

Gillin F.D. (1987) *Giardia lamblia*: the role of conjugated and unconjugated bile salts in killing by human milk. *Exp. Parasitol.* **63**: 74–83.

Gillin F.D. & Diamond L.S. (1980) Clonal growth of *Giardia lamblia* trophozoites in a semisolid agarose medium. *J. Parasitol.* **66**: 350–352.

Gillin F.D. & Diamond L.S. (1981a) *Entameba histolytica* and *Giardia lamblia*: growth responses to reducing agents. *Exp. Parasitol.* **51**: 382–391.

Gillin F.D. & Diamond L.S. (1981b) *Entameba histolytica* and *Giardia lamblia*: effects of cysteine and oxygen tension on trophozoite attachment to glass and survival in culture media. *Exp. Parasitol.* **52**: 9–17.

Gillin F.D., Gault M.J., Hofmann A.F., Gurantz D. & Sauch J.F. (1986) Biliary lipids support serum-free growth of *Giardia lamblia*. *Infect. Immun.* **53**: 641–645.

Gillin F.D. & Reiner D.S. (1982) Attachment of the flagellate *Giardia lamblia*: role of reducing agents, serum, temperature and ionic composition. *Mol. Cell. Biol.* **2**: 369–377.

Gillin F.D., Reiner D.S. & Boucher S.E. (1988) Small intestinal factors promote encystation of *Giardia lamblia in vitro*. *Infect. Immun.* **56:** 705–707.

Gillin F.D., Reiner D.S. & Gault M.J. (1985) Cholate-dependent killing of *Giardia lamblia* by human milk. *Infect. Immun.* **47**: 619–622.

Gillin F.D., Reiner D.S., Gault M.J., Douglas H., Das S., Wunderlich A. & Sauch J.F. (1987) Encystation and expression of cyst antigens by *Giardia lamblia in vitro*. *Science.* **235**: 1040–1043.

Gillin F.D., Reiner D.S. & Wang C.-S. (1983a) Human milk kills parasitic intestinal protozoa. *Science.* **221**: 1290–1292.

Gillin F.D., Reiner D.S. & Wang C.-S. (1983b) Killing of *Giardia lamblia* trophozoites by normal human milk. *J. Cell. Biochem.* **23**: 47–56.

Gillon J. (1984) Giardiasis: review of epidemiology, pathogenetic mechanisms and host responses. *Quarterly J. Med.* **209**: 29–39.

Gillon J. (1985) Clinical studies in adults presenting with giardiasis to a gastrointestinal unit. *Scott. Med. J.* **30:** 89–95.

Gillon J., Al Thamery D. & Ferguson A. (1982a) Features of small intestine pathology (epithelial cell kinetics, intraepithelial lymphocytes, disaccharidases) in a primary *Giardia muris* infection. *Gut.* **23**: 498–506.

Gillon J., Andre C., Descos L., Minaire Y. & Fargier M.C. (1982b) Changes in mucosal immunoglobulin-containing cells in patients with giardiasis before and after treatment. *J. Infect.* **5:** 67–72.

Gilman R.H., Brown K.H., Visvesvara G.S., Mondal G., Greenberg B., Sack R.B., Brandt F. & Khan M.U. (1985) Epidemiology and serology of *Giardia lamblia* in a developing country, Bangladesh. *Trans. R. Soc. Trop. Med. Hyg.* **79**: 469–473.

Gilman R.H., Marquis G.S., Miranda E., Vestequi M. & Martinez H. (1988) Rapid infection by *Giardia lamblia* after treatment in a hyperendemic third world community., *Lancet.* **1:** 343–345.

Goka A.J.K., Rolston D.D.K., Mathan V.I. & Farthing M.J.G. (1986) Diagnosis of giardiasis by specific IgM antibody enzyme-linked immunosorbent assay. *Lancet.* **2**: 184–186.

Goldsmid J.M., Wellock R. & Adams M. (1984) Giardiasis in Tasmania. *Aust. Pediatr. J.* **20:** 259.

Gordts B., Hemelhof W., Van Tilborgh K., Retore P., Cadranel S. & Butzler J.-P. (1985) Evaluation of a new method for routine *in vitro* cultivation of *Giardia lamblia* from human duodenal fluid. *J. Clin. Microbiol.* **22**: 702–704.

Gracey M. (1973) Enteric disease in young Australian aborigines. *Aust. New Zeal. J. Med.* **3:** 576–579.

Gracey M., Burke V. & Robinson J. (1983) Patterns of intestinal infection in Australian aboriginal children. *Ann. Trop. Pediatr.* **3:** 35–39.

Graham R.C. Jr. & Karnovsky M.J. (1966) The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* **14:** 291–302.

Grant D.R. & Woo P.K. (1978a) Comparative studies of *Giardia* spp. in small mammals in southern Ontario. I. Prevalence and identity of the parasites with a taxonomic discussion of the genus. *Can. J. Zool.* **56**: 1348–1359.

Grant D.R. & Woo P.K. (1978b) Comparative studies of *Giardia* spp. in small mammals in southern Ontario. II. Host specificity and infectivity of stored cysts. *Can. J. Zool.* **56**: 1360–1366.

Grant D.R. & Woo P.K. (1979) Comparative studies of *Giardia* spp in small mammals in southern Ontario. III. Duration and cyst production in natural and experimental infections. *Can. J. Zool.* **57**: 307–313.

Green E.L., Miles M.A. & Warhurst D.C. (1985) Immunodiagnostic detection of *Giardia* antigen in faeces by a rapid visual enzyme-linked immunosorbent assay. *Lancet.* **2**: 691–693.

Gunther E. & Stark O. (1978) The major histocompatibility system of the rat. *Transplant. Proc.* **11**: 1550–1553.

Hakansson C., Thoren K., Norkrans G. & Johannisson G. (1984) Intestinal parasitic infection and other sexually transmitted diseases in asymptomatic homosexual men. *Scand. J. Infect. Dis.* **16**: 199–202.

Hall J., Orlans E., Reynolds J., Dean C., Peppard J., Gyure L. & Hobbs S. (1979) Occurrence of specific antibodies of the IgA class in the bile of rats. *Int. Arch. Allergy Appl. Immun.* **59**: 75–84.

Haneberg B. (1974) Immunoglobulins in feces from infants fed human or bovine milk. *Scand. J. Immunol.* **3**: 191–197.

Haneberg B. & Endresen C. (1976) Fragments of immunoglobulins in human faeces. *Acta. Pathol. Microbiol. Scand.* **84**: 31–36.

Haralabidis S. Th. (1984) Immunodiagnosis of giardiasis by ELISA and studies on cross-reactivity between the anti-*Giardia lamblia* antibodies and some heterologous parasitic antigens and fractions. *Ann. Trop. Med. Parasitol.* **78**: 295–300.

Hart G., Haidaris C.G. & So M. (1987) Purification and characterization of a neuraminidase, a developmentally regulated antigen of *Trypanosoma cruzi*. In: *Molecular Strategies of Parasitic Invasion*. Alan R. Liss, Inc. pp. 213–222.

Harter L., Frost F. & Jakubowski W. (1982) *Giardia* prevalence among one to three year old children in two Washington State counties. *Am. J. Publ. Hlth.* **72**: 386–388.

Heap T. (1974) Giardiasis: a common cause of prolonged diarrhea in adults. *Med. J. Aust.* **2**: 592–595.

Hegner R.W. (1922) A comparative study of the giardias living in man, rabbit and dog. *Am. J. Hyg.* **2**: 406–434.

Hegner R.W. (1927a) Excystation and infection in the rat with *Giardia lamblia* from man. *Am. J. Hyg.* **7**: 433–447.

Hegner R.W. (1927b) The viability of cysts of *Giardia lamblia* from man in the stomach of the rat. *Am. J. Hyg.* **7**: 782–785.

Hegner R.W. & Eskridge L. (1937) The influence of bile salts on *Giardia* infections in rats. *Am. J. Hyg.* **26**: 186–192.

Hegner R.W. & Eskridge L. (1938) Localization of *Giardia muris* in rats. *J. Parasitol.* **24**: 511–515.

Hernell O., Ward H., Blackberg L. & Pereira M.E.A. (1986) Killing of *Giardia lamblia* by human milk lipases: an effect mediated by lipolysis of milk lipids. *J. Infect. Dis.* **153**: 715–720.

Hewlett E.L., Andrews J.S., Ruffier J. & Schaefer III F.W. (1982) Experimental infection of mongrel dogs with *Giardia lamblia* cysts & cultured trophozoites. *J. Infect. Dis.* **145**: 89–93.

Heyworth M.F (1986) Antibody response to *Giardia muris* trophozoites in mouse intestine. *Infect. Immun.* **52**: 568–571.

Heyworth M.F., Carlson J.R. & Ermak T.H. (1987) Clearance of *Giardia muris* infection requires helper/inducer T lymphocytes. *J. Exp. Med.* **165**: 1743–1748.

Heyworth M.F., Kung J.E. & Eriksson E.C. (1986) Clearance of *Giardia muris* infection in mice deficient in natural killer cells. *Infect. Immun.* **54:** 903–904.

Heyworth M.F., Owen R.L. & Jones A.L. (1985a) Comparison of leukocytes obtained from the intestinal lumen of Giardia-infected immunocompetent mice and nude mice. *Gastroenterology.* **89**: 1360–1365.

Heyworth M.F., Owen R.L., Seaman W.E., Schaefer III F.W. & Jones A.L. (1985b) Harvesting of leukocytes from intestinal lumen in murine giardiasis and preliminary characterization of these cells. *Dig. Dis. Sci.* **30**: 149–153.

Hill D.R., Burge J.J. & Pearson R.D. (1984) Susceptibility of *Giardia lamblia* trophozoites to the lethal effect of human serum. *J. Immunol.* **132**: 2046–2051.

Hill D.R., Guerrant R.L., Pearson R.D. & Hewlett E.L. (1983) *Giardia lamblia* infection of suckling mice. *J. Infect. Dis.* **147**: 217–221.

Hill D.R., Hewlett E.L. & Pearson R.D (1981) Lectin binding by *Giardia lamblia*. *Infect. Immun.* **34**: 733–738.

Hill D.R. & Pearson R.D. (1987) Ingestion of *Giardia lamblia* trophozoites by human mononuclear phaocytes. *Infect. Immun.* **55:** 3155–3161.

Holberton D.V. (1973) Fine structure of the ventral disk apparatus and the mechanism of attachment in the flagellate *Giardia muris*. *J. Cell Sci.* **13**: 11–14.

Holberton D.V. (1974) Attachment of *Giardia* - a hydrodynamic model based on flagellar activity. *J. Exp. Biol.* **60**: 207–221.

Holberton D.V. & Ward A.P. (1981) Isolation of the cytoskeleton from *Giardia*. Tubulin and a low-molecular weight protein associated with microribbon structures. *J. Cell Sci.* **47**: 139–166.

Howard R.J. (1984) Antigenic variation of bloodstage malaria parasites. *Phil. Trans. R. Soc. Lond.* **307:** 141–158.

Hughes W.S., Cerda J.J., Hotizapple P. & Brooks F.P (1971) Primary hypogammaglobulinemia and malabsorption. *Ann. Intern. Med.* **74:** 903–910.

Husband A.J. & Gowans J.L. (1978) The origin and antigen dependent distribution of IgA-containing cells in the intestine. *J. Exp. Med.* **148:** 1146–1160.

Islam A., Stoll B.J., Ljungstrom I., Biswas J., Nazrul H. & Huldt G. (1983) *Giardia lamblia* infections in a cohort of Bangladeshi mothers and infants followed for one year. *J. Pediatr.* **103**: 996–1000.

Istre G.R., Dunlop T.S., Gaspard G.B. & Hopkins R.S. (1984) Waterborne giardiasis at a mountain resort: evidence for acquired immunity. *Am. J. Publ. Hlth.* **74**: 602–604.

Jackson G.D.F. & Cooper G.N. (1981) Immune responses of rats to live *Vibrio cholerae*: secretion of antibodies in bile. *Parasite Immunol.* **3**: 127–135.

Jackson G.D.F., Lemaitre-Coelho I., Vaerman J.-P., Bazin H. & Beckers A. (1978) Rapid disappearance from serum of intravenously injected rat myeloma IgA and its secretion into bile. *Eur. J. Immunol.* **8**: 123–126.

Jackson G.D.F. & Walker P.G. (1983) The transient appearance of IgM antibodies in the bile of rats injected with *Salmonella enteritidis*. *Immunol. Lett.* **7**: 41–45.

Jackson G.D.F., Walker P.G., Schiff J.M., Barrington P.J., Fisher M.M. & Underdown B.J. (1985) A role for the spleen in the appearance of IgM in the bile of rats injected intravenously with horse erythrocyte. *J. Immunol.* **135**: 152–157.

Janoff E.N., Smith P.D. & Blaser M.J. (1988) Acute antibody responses to *Giardia lamblia* are depressed in patients with acquired immunodeficiency syndrome. *J. Infect. Dis.* **157**: 798–804.

Jephcott A.E., Begg N.T. & Baker I.A. (1986) Outbreak of giardiasis associated with mains water in the United Kingdom. *Lancet.* **1**: 730–732.

Jokipii L. & Jokipii A.M.M. (1974) Giardiasis in travelers: a prospective study. *J. Infect. Dis.* **130**: 295–299.

Jokipii A.M.M. & Jokipii L. (1977) Prepatency of giardiasis. *Lancet.* **1**: 1095–1097.

Jokipii A.M.M. & Jokipii L. (1978) Comparative evaluation of two dosages of tinidazole in the treatment of giardiasis. *Am. J. Trop. Med. Hyg.* **27**: 758–761.

Jokipii L. & Jokipii A.M.M. (1980) *In vitro* susceptibility of *Giardia lamblia* trophozoites to metronidazole and tinidazole. *J. Infect. Dis.* **141**: 317–325.

Jokipii L., Miettinen A. & Jokipii A.M.M. (1988) Antibodies to cysts of *Giardia lamblia* in primary giardiasis and in the absence of giardiasis. *J. Clin. Microbiol.* **26**: 121–125.

Jones E.G. & Brown W.R. (1974) Serum and intestinal fluid immunoglobulins in patients with giardiasis. *Am. J. Dig. Dis.* **19**: 791–796.

Jones J.E. (1986) String test for diagnosing giardiasis. *Am. Fam. Physician.* **34**: 123–126.

Kagnoff M.F. (1977) Functional characteristics of Peyer's patch lymphoid cells. IV. Effect of antigen feeding on the frequency of antigen-specific B cells. *J. Immunol.* **118:** 992–997.

Kamath K.R. & Murugasu R. (1974) A comparative study of four methods for detecting *Giardia lamblia* in children with diarrheal disease and malabsorption. *Gastroenterology.* **66**: 16–21.

Kanwar S.S., Ganguly N.K., Mahajan R.C. & Walia B.N.S. (1985a) Acquired resistance to *Giardia lamblia* infection in mice. *Trop. Geogr. Med.* **37**: 32–36.

Kanwar S.S., Ganguly N.K., Walia B.N.S. & Mahajan R.C. (1985b) Changes in mouse splenic lymphocyte sub-population during primary and secondary course of *Giardia lamblia* infection. *Indian J. Med. Res.* **82**: 393–397.

Kanwar S.S., Ganguly N.K., Walia B.N.S. & Mahajan R.C. (1984) Enumeration of small intestinal lymphocyte population in *Giardia lamblia* infected mice. *J. Diarrheal Dis. Res.* **2**: 243–248.

Kanwar S.S., Ganguly N.K., Walia B.N.S. & Mahajan R.C. (1986a) Direct and antibody-dependent cell-mediated cytotoxicity against *Giardia lamblia* by splenic and intestinal lymphoid cells in mice. *Gut.* **27**: 73–77.

Kanwar S.S., Samra H., Ganguly N.K. & Mahajan R.C. (1986b) Comparative evaluation of *Giardia lamblia* infection in mouse and rat. *Indian J. Med. Res.* **84**: 577–581.

Kaplan B.S., Uni S., Aikawa M. & Mahmoud A.A.F. (1985) Effector mechanisms of host resistance in murine giardiasis: specific IgG and IgA cell-mediated toxicity. *J. Immunol.* **134**: 1975–1981.

Karapetyan A. (1960) Methods of *Lamblia* cultivation. *Tsitologiia* **2:** 379–384.

Karapetyan A. (1962) *In vitro* cultivation of *Giardia duodenalis. J. Parasitol.* **48**: 337–340.

Kasprzak W. & Majewska A.C. (1983) Isolation and axenic growth of fresh *Giardia intestinalis* strains in TPS-1 medium. *Trans. R. Soc. Trop. Med. Hyg.* **77:** 223–224.

Kasprzak W. & Majewska A.C. (1985) Improvement in isolation and axenic growth of *Giardia intestinalis* strains. *Trans. R. Soc. Trop. Med. Hyg.* **79**: 551–557.

Kawanishi H. & Strober W. (1983) T cell regulation of IgA immunoglobulin production in gut-associated lymphoid tissues. *Mol. Immunol.* **20**: 917-930.

Kawanishi H., Saltzman L.E. & Strober W. (1982) Characteristics and regulatory functions of murine ConA-induced, cloned T-cells obtained from Peyer's patches and spleen: mechanisms regulating isotype-specific immunoglobulin production by Peyer's patch B Cells. *J. Immunol.* **129:** 475–483.

Kawanishi H., Saltzman L.E. & Strober W. (1983a) Mechanisms regulating IgA class-specific immunoglobulin production in murine gut-associated lymphoid tissues. I. T-cells derived from Peyer's patch that switch sIgM B cells to sIgA B cells *in vitro*. *J. Exp. Med.* **157:** 433–450.

Kawanishi H., Slatzman L.E. & Strober W. (1983b) Mechanisms regulating IgA class-specific immunoglobulin production in murine gut-associated lymphoid tissues. II. Terminal differentiation of post-switch sIgA-bearing Peyer's patch B cells. *J. Exp. Med.* **158:** 649–669.

Kean B.H., William D.C. & Luminais S.K. (1979) Epidemic amebiasis and giardiasis in a biased population. *Br. J. Vener. Dis.* **55:** 375–378.

Keister D.G. (1983) Axenic culture of *Giardia lamblia* in TYI-S-33 medium supplemented with bile. *Trans. R. Soc. Trop. Med. Hyg.* **77**: 487–488.

Keystone J.S., Keystone D.L. & Proctor E.M. (1980) Intestinal parasitic infections in homosexual men: prevalence, symptoms and factors in transmission. *Can. Med. Assoc. J.* **123**: 512–514.

Keystone J.S, Krajden S. & Warren M.R. (1978) Person-to-person transmission of *Giardia lamblia* in day-care nurseries. *Can. Med. Assoc. J.* **119**: 241–248.

Kinashi T., Harada N., Severinson E., Tanabe T., Sideras P., Konishi M., Azuma C., Tominaga A., Bergstedt-Lindqvist S., Takahashi M., Matsuda F., Yaoita Y., Takatsu K. & Honjo T. (1986) Cloning of complementary DNA encoding T-cell replacing factor and identity with B-cell growth factor. *Nature*. **324:** 70–73.

Kiorpes A.L., Kirkpatrick C.E. & Bowman D.D. (1987) Isolation of *Giardia* from a llama and from sheep. *Can. J. Vet. Res.* **51**: 277– 280.

Kirkpatrick C.E. & Farrell J.P. (1984) Feline giardiasis: observations on natural and induced infections. *Am. J. Vet. Res.* **45:** 2182–2188.

Kirkpatrick C.E. & Green IV G.A. (1985) Susceptibility of domestic cats to infections with *Giardia lamblia* cysts and trophozoites from human sources. *J. Clin. Microbiol.* **21:** 678–680.

Kirkpatrick C.E. & Skand D.L. (1985) Giardiasis in a horse. *J. Am. Vet. Med. Assoc.* **187:** 163–164.

Kiyono H., Cooper M.D., Kearney J.F., Mosteller L.M., Michalek S.M., Koopman W.J. & McGhee J.R. (1984) Isotype specificity of helper T cell clones. Peyer's patch Th cells preferentially collaborate with mature IgA B cells for IgA responses. *J. Exp. Med.* **154:** 798–811.

Kiyono H., McGhee J.R., Mosteller L.M., Eldridge J.H., Koopman W.J., Kearney J.F. & Michalek S.M. (1982) Murine Peyer's patch T cell clones. Characterization of antigen-specific helper T cells for immunoglobulin A responses. *J. Exp. Med.* **156:** 1115–1130.

Kiyono H., MostellerBarnum L.M., Pitts A.M., Williamson S.I., Michalek S.M. & McGhee J.R. (1985) Isotype-specific immunoregulation. IgA binding factors produced by Fcα receptor-positive T cell hybridomas regulate IgA responses. *J. Exp. Med.* **161:** 731–747.

Kofoid C.A. & Christiansen E.B. (1915) On the life history of *Giardia*. *Proc. Natl. Acad. Sci.* **1**: 547–552.

Korman S.H., Le Blancq S.M., Spira D.T., El On J., Reifen R.M. & Deckelbaum R.J. (1986) *Giardia lamblia*: identification of different strains from man. *Z. Parasitenkd.* **72**: 173–180.

Kreutzer R.D., Semko M.E., Hendricks L.D. & Wright N. (1983) Identification of *Leishmania* spp. by multiple isozyme analysis. *Am. J. Trop. Med. Hyg.* **32:** 703–715.

Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227**: 680–685.

Lambert R. (1965). In: *Surgery of the disgestive system in the rat.* Springfield: C.C. Thomas. Chapter 13.

Lee G.B. & Ogilvie B.M. (1982) The intestinal mucus barrier to parasites and bacteria. *Adv. Exp. Med. Biol.* **144**: 247–248.

Lemaitre-Coelho I., Jackson G.D.F. & Vaerman J.-P. (1977) Rat bile as a convenient source of secretory IgA and free secretory component. *Eur. J. Immunol.* **7**: 588–590.

Lemaitre-Coelho I., Jackson G.D.F. & Vaerman J.-P. (1978) Relevance of biliary IgA antibodies in rat intestinal immunity. *Scand. J. Immunol.* **8**: 459–463.

Lerman S.J. & Walker R.A. (1982) Treatment of giardiasis: literature review and recommendations. *Clin. Pediatr.* **7:** 409–414.

Lev B., Ward H., Keusch G.T. & Pereira M.E.A. (1986a) Lectin activation in *Giardia lamblia* by host protease: a novel host-parasite interaction. *Science.* **232**: 71–73.

Lev B., Ward H. & Pereira M.E.A. (1986b) Prolectin activation in *Giardia lamblia.* In: *Microbial lectins and agglutinins: properties and biological activity.* Ed. D. Mirelman. New York: John Wiley & Sons. **pp.** 301–317.

Levine N.D. (1979) *Giardia lamblia*: classification, structure, identification. In: *Waterborne transmission of giardiasis.* Ed. W. Jakubowski & J.C. Hoff. Ohio: U.S. Environmental Protection Agency. **pp.** 2–8.

Lewis P.D. Jr., Belosevic M., Faubert G.M., Curthoys L. & Maclean J.D. (1987) Cortisone-induced recrudescence of *Giardia lamblia* infections in gerbils. *Am. J. Trop. Med. Hyg.* **36**: 33–40.

Lichtman S., Sherman P. & Forstner G. (1986) Production of secretory immunoglobulin A in rat self-filling blind loops. Local secretory immunoglobulin A immune response to luminal bacterial flora. *Gastroenterology.* **91**: 1495–1502.

Lindh E. (1975) Increased resistance of immunoglobulin A dimers to proteolytic degradation after binding of secretory component. *J. Immunol.* **114:** 284–286.

Lindmark D.G. (1980) Energy metabolism of the anaerobic protozoan *Giardia lamblia. Mol. Biochem. Parasitol.* **1:** 1–12.

Lindmark D.G. (1988) *Giardia lamblia:* localization of hydrolase acitivities in lysosome-like organelles of trophozoites. *Exp. Parasitol.* **65:** 141–147.

Lindmark D.G. & Jarroll D.L. (1982). Pyrimidine metabolism in *Giardia lamblia* trophozoites. *Mol. Biochem. Parasitol.* **5:** 291–296.

Ljungstrom I., Stoll B. & Islam A. (1987) *Giardia* infection during pregnancy and lactation. *Trans. R. Soc. Trop. Med. Hyg.* **81**: 161.

Lloyd S. & Soulsby E.J.L. (1978) The role of IgA immunoglobulins in the passive transfer of protection to *Taenia taeniaeformis* in the mouse. *Immunology.* **34**: 939–945.

Loftness T.J., Erlandsen S.L., Wilson I.D. & Meyer E.A. (1984) Occurrence of specific secretory immunoglobulin A in bile after inoculation of *Giardia lamblia* trophozoites into rat duodenum. *Gastroenterology*. **87**: 1022–1029.

LoGalbo P.R., Sampson H.A. & Buckley R.H. (1982) Symptomatic giardiasis in three patients with X-linked agammaglobulinemia. *J. Pediatr.* **101**: 78–80.

Lopez Brea M. (1982) *Giardia lamblia*: incidence in man and dogs. *Trans. R. Soc. Trop. Med. Hyg.* **76:** 565.

Lugtenberg B., Meijers J., Peters R., Van der Hoek P. & Van Alphen L. (1975) Electrophoretic resolution of the 'major outer membrane proteins ' of *Escherichia coli* K12 into four bands. *FEBS Lett.* **58**: 254–258.

Lupasco G.H., Radulescu S. & Cernat M.J. (1970) The presence of *Lamblia muris* in the tissues and organs of mice spontaneously infected. *J. Parasitol.* **56**: 444–445.

Lyman J.R. & Marchin G.L. (1984) Cryopreservation of *Giardia lamblia* with dimethyl sulfoxide using a Dewar bottle. *Cryobiology* **21**: 170–176.

MacDonald T.T. & Ferguson A. (1978) Small intestinal epithelial cell kinetics and protozoal infections in mice. *Gastroenterology.* **74**: 496–500.

Magnusson K.E. & Stjernstrom I. (1982) Mucosal barrier mechanisms. Interplay between secretory IgA (sIgA), IgG and mucins on the surface properties and association of *Salmonellae* with intestine and granulocytes. *Immunology.* **45**: 239–248.

Majewska A.C. & Kasprzak W. (1983) The rat as an animal model of giardiasis. *Acta Protozool.* **22**: 71–77.

Manning R.J., Walker P.G., Carter L., Barrington P.J. & Jackson G.D.F. (1984) Studies on the origins of biliary immunoglobulins in rats. *Gastroenterology.* **87**: 173–179.

Mason P.R. & Patterson B.A. (1987) Epidemiology of *Giardia lamblia* infection in children: cross-sectional and longitudinal studies in urban and rural communities in Zimbabwe. *Am. J. Trop. Med. Hyg.* **37**: 277–282.

Mata L.J. (1978) The children of Santa Maria Cauque: a prospective field study of health and growth. MIT, Cambridge, Massachusetts. **pp.** 228–253.

Mattioli C.A. & Tomasi T.B. (1973) The life span of IgA plasma cells from the mouse intestine. *J. Exp. Med.* **138**: 452–460.

Mayhew I.G. & Greiner E.C. (1986) Protozoal diseases. *Vet. Clin. N. Am.* **2**: 439–459.

Mayrhofer G. & Fisher R. (1979) Mast cells in severely T-cell depleted rats and the response to infestation with *Nippostrongylus brasiliensis. Immunology.* **73**: 145–155.

McIntyre P., Boreham P.F.L., Phillips R.E. & Shepherd R.W. (1986) Chemotherapy in giardiasis: clinical responses and *in vitro* drug sensitivity of human isolates in axenic culture. *J. Pediatr.* **108:** 1005–1010.

Meloni B.P., Lymbrey A.J. & Thompson R.C.A. (1988) Isoenzyme electrophoresis of 30 isolates of *Giardia* from humans and lower animals. *Am. J. Trop. Med. Hyg.* **38**: 65–73.

Meloni B.P. & Thompson R.C.A. (1987) Comparative studies on the axenic *in vitro* cultivation of *Giardia* of human and canine origin: evidence for intraspecific variation. *Trans. Roy. Soc. Trop. Med.* **81:** 637–640.

Mendelson R.M. (1980) The treatment of giardiasis. *Trans. R. Soc. Trop. Med. Hyg.* **74:** 438–439.

Mestecky J. & McGhee J.R. (1987) Immunoglobulin A (IgA): molecular and cellular interactions involved in IgA biosynthesis and immune response. *Adv. Immunol.* **40:** 153–245.

Meyer E.A. (1970) Isolation and axenic cultivation of *Giardia* trophozoites from the rabbit, chinchilla and cat. *Exp. Parasitol.* **27**: 179–183.

Meyer E.A. (1976) *Giardia lamblia*: isolation and axenic cultivation. *Exp. Parasitol.* **39**: 101–105.

Meyer E.A. & Chadd J.A. (1967) Preservation of *Giardia* trophozoites by freezing. *J. Parasitol.* **53:** 1108–1109.

Meyer E.A. & Jarroll E.L. (1980) Giardiasis. *Am. J. Epidemiol.* **111:** 1–12.

Meyer E.A. & Radulescu S. (1979) *Giardia* and giardiasis. *Adv. Parasitol.* **17:** 1–47.

Meyer E.A. & Radulescu S. (1984) *In vitro* cultivation of *Giardia* trophozoites. In: *Giardia and Giardiasis, Biology, Pathogenesis and Epidemiology*. Eds. S.L. Erlandsen & E.A. Meyer. New York: Plenum Press. **pp.** 99–109.

Meyers J.D., Kuharic H.A. & Holmes K.K. (1977) *Giardia lamblia* infection in homosexual men. *Br. J. Vener. Dis.* **53:** 54–55.

Miller H.R.P. (1987) Gastrointestinal mucus, a medium for survival and for elimination of parasitic nematodes and protozoa. *Parasitology.* **94**: S77–S100.

Miller R.L., Wang A.L. & Wang C.C. (1988a) Identification of *Giardia lamblia* isolates susceptible and resistant to infection by the double-stranded RNA virus. *Exp. Parasitol.* **66:** 118–123.

Miller R.L., Wang A.L. & Wang C.C. (1988b) Purification and characterization of the *Giardia lamblia* double-stranded RNA virus. *Mol. Biochem. Parasitol.* **28:** 189–196.

Miotti P.G., Gilman R.H., Pickering L.K., Ruiz Palacios G., Park H.S. & Yolken R.H. (1985) Prevalence of serum and milk antibodies to *Giardia lamblia* in different populations of lactating women. *J. Infect. Dis.* **152**: 1025–1031.

Mitchell G.F., Anders R.F., Brown G.V., Handman E., Roberts-Thomson I.C., Chapman C.B., Forsyth K.P., Kahl L.P. & Cruise K.M. (1982) Analysis of infection characteristics and antiparasite immune response in resistant compared with susceptible hosts. *Immunol. Rev.* **61:** 137–188.

Moore G.W., Sogandares Bernal F. Dennis M.V., Root D.M., Beckwith D. & Van Voorhis D. (1982) Characterization of *Giardia lamblia* trophozoite antigens using polyacrylamide gel electrophoresis, high performance liquid chromatography, and enzyme-labeled immunosorbent assay. *Vet. Parasitol.* **10**: 229–237.

Morecki R. & Parker J.G. (1967) Ultrastructural studies of the human *Giardia lamblia* and subjacent jejunal mucosa in a subject with steatorrhea. *Gastroenterology.* **52**: 151–164.

Most H. (1968) Manhattan: a 'tropic isle'? *Am. J. Trp. Med. Hyg.* **17:** 333–354.

Mostov K.E., Friedlander M. & Blohel G. (1984) The receptor for transepithelial transport of IgA and IgM contains multiple immunoglobulin-like domains. *Nature.* **308:** 37–43.

Mueller J.C., Jones A.L. & Brandborg L.L. (1973) Scanning electron microscope observation in human giardiasis. *Scanning Electron Microscopy 1973.* 557–564.

Myler P.J., Allen A.L., Agabian N. & Stuart K. (1985) Antigenic variation in clones of *Trypanosoma brucei* grown in immune-deficient mice. *Infect. Immun.* **47:** 684–690.

Naik S.R., Kumar L., Naik S. (1979) Immunological studies in giardiasis. *Ann. Trop. Med. Parasitol.* **73:** 291–292.

Naik S.R., Rau N.R., Vinayak V.K., Narayanan V.A., Zunzurwade S., Sehgal S.C. & Talwar P. (1978) Presence of *Candida albicans* in normal and in *Giardia lamblia* infected human jejunum. *Ann. Trop. Med. Parasitol.* **72**: 493–494.

Nair K.V., Gillon J. & Ferguson A. (1981) Corticosteroid treatment increases parasite numbers in murine giardiasis. *Gut.* **22**: 475–480.

Nash T. & Aggarwal A. (1986) Cytotoxicity of monoclonal antibodies to a subset of *Giardia* isolates. *J. Immunol.* **136**: 2628–2632.

Nash T.E., Gillin G.D. & Smith P.D. (1983) Excretory-secretory products of *Giardia lamblia*. *J. Immunol.* **131**: 2004–2009.

Nash T.E., Herrington D.A. & Levine M.M. (1987a) Usefulness of an ELISA for detection of *Giardia* antigens in feces. *J. Clin. Micro.* **25**: 1169–1171.

Nash T.E., Herrington D.A., Losonsky G.A. & Levine M.M. (1987b) Experimental human infections with *Giardia lamblia*. *J. Infect. Dis.* **156**: 974–984.

Nash T.E. & Keister D.B. (1985) Differences in excretory-secretory products and surface antigens among 19 isolates of *Giardia*. *J. Infect. Dis.* **152**: 1166–1171.

Nash T.E., McCutchan T., Keister D., Dame J.D., Conrad J.D. & Gillin F.D. (1985) Restriction-endonuclease analysis of DNA from 15 Giardia isolates obtained from humans and animals. *J. Infect. Dis.* **152**: 64–73.

Navin T.R., Juranek D.D., Ford M., Minedew D.J., Lippy E.C. & Pollard R.A. (1985) Case-control study of waterborne giardiasis in Reno, Nevada. *Am. J. Epidemiol.* **122**: 269–275.

Nayak N., Ganguly N.K., Walia B.N.S., Wahi V., Kanwar S.S. & Mahajan R.C. (1987) Specific secretory IgA in the milk of *Giardia lamblia*-infected and uninfected women. *J. Infect. Dis.* **155**: 724–727.

Nemanic P.C., Owen R.L., Stevens D.P. & Mueller J.C. (1979) Ultrastructural observations on giardiasis in a mouse model. II. Endosymbiosis and organelle distribution in *Giardia muris* and *Giardia lamblia*. *J. Infect. Dis.* **140:** 222–228.

Neringer R., Andersson Y. & Eitrem R. (1987) A waterborne outbreak of giardiasis in Sweden. *Scand. J. Infect. Dis.* **19**: 85–90.

Noma Y., Sideras P., Naito T., Bergstedt-Lindqvist S., Azuma C., Severnson E., Tanabe T., Kinashi T., Matsuda F., Yaoita Y. & Honjo T. (1986) Cloning of cDNA encoding the murine IgG1 induction factor by a novel strategy using SP6 promoter. *Nature.* **319:** 640–646.

Olveda R., Andrews J.S. & Hewlett E.L. (1982) Murine giardiasis: localization of trophozoites and small bowel histopathology during the course of infection. *Am. J. Trop. Med. Hyg.* **31**: 60–66.

Orlans E., Peppard J., Fry J.F., Hinton R.N. & Mullock B.M. (1979) Secretory component as the receptor for polymeric IgA on rat hepatocytes. *J. Exp. Med.* **150:** 1577–1581.

Orlans E., Peppard J., Reynolds J. & Hall J. (1978) Rapid active transport of immunoglobulin A from blood to bile. *J. Exp. Med.* **147**: 588–593.

Osterholm M.T., Forfang J.C., Ristinen T.L., Dean A.G., Washburn J.W., Godes J.R., Rude R.A. & McCullough J.C. (1981) An outbreak of foodborne giardiasis. *N. Engl. J. Med.* **304:** 24–28.

Owen R.L. (1980a) Ultrastructural basis of *Giardia* function. *Trans. R. Soc. Trop. Med. Hyg.* **74**: 429–433.

Owen R.L. (1980b) The immune response in clinical and experimental giardiasis. *Trans. R. Soc. Trop. Med. Hyg.* **74**: 443–445.

Owen R.L. (1984) Direct fecal-oral transmission of Giardiasis. In: *Giardia and Giardiasis, Biology, Pathogenesis and Epidemiology*. Eds. S.L. Erlandsen & E.A. Meyer. New York: Plenum Press. **pp.** 329–339.

Owen R.L. Allen L. & Stevens D.P. (1980) Intraepithelial trapping of *Giardia* by macrophages in Peyer's patches of normal and nude mice - an intestinal defense and antigen processing mechanism. *Gastroenterology.* **78**: 1232.

Owen R.L., Allen C.L. & Stevens D.P. (1981) Phagocytosis of *Giardia muris* by macrophages in Peyer's patch epithelium in mice. *Infect. Immun.* **33**: 591–601.

Owen R.L., Nemanic P.C. & Stevens D.P. (1979) Ultrastructural observations on giardiasis in a murine model. I. Intestinal distribution, attachment and relationship to the immune system of *Giardia muris*. *Gastroenterology.* **76**: 757–769.

Parker R.C. (1950) *Methods of Tissue Culture.* 2nd Ed. New York: Paul B. Hoeber, Inc. pp. 77–78.

Peppard J.V. (1979) Quantitative estimation of IgA in rats: a comparison of two methods. *J. Immunol. Methods.* **31:** 129–139.

Peppard J.V., Jackson L.E. & Hall J.G. (1983) The occurrence of secretory IgM in the bile of rats. *Clin. Exp. Immunol.* **53**: 623– 626.

Peppard J.V., Orlans E., Andrew E. & Payne A.W.R. (1982) Elimination into bile of circulating antigen by endogenous IgA antibody in rats. *Immunology.* **45:** 467–472.

Peppard J., Orlans E., Payne A.W.R. & Andrew E. (1981) The elimination of circulating complexes containing polymeric IgA by excretion in the bile. *Immunology.* **42:** 83–89.

Pereira M.E.A. (1983) A rapid and sensitive assay for neuraminidase using peanut lectin hemagglutination: application to *Vibrio cholerae* and *Trypanosoma cruzi*. *J. Immunol. Methods.* **63**: 25–34.

Petersen L.R., Carter M.L. & Hadler J.L. (1988) A foodborne outbreak of *Giardia lamblia*. *J. Infect. Dis.* **157:** 846–848.

Phillips R.E., Boreham P.F.L. & Shepherd R.W. (1984) Cryopreservation of viable *Giardia intestinalis* trophozoites. *Trans. R. Soc. Trop. Med. Hyg.* **78**: 604–606.

Pickering L.K. (1985) Problems in diagnosing and managing giardiasis. *Pediatr. Infect. Dis.* **4:** S6–S10.

Pickering L.K., Bartlet A.V. & Woodward W.E. (1986) Acute infectious diarrhea among children in day care: epidemiology and control. *Rev. Infect. Dis.* **8:** 539–547.

Pickering L.K., Woodward W.E., DuPont H.L. & Sullivan P. (1984) Occurrence of *Giardia lamblia* in children in day-care centers. *J. Pediatr.* **104:** 522–526.

Pierce N.F. & Gowans J.L. (1975) Cellular kinetics of the intestinal immune response to cholera toxoid in rats. *J. Exp. Med.* **142:** 1550–1563.

Plasterk R.H.A., Simon M.I. & Barbour A.G. (1985) Transposition of structural genes to an expression sequence on a linear plasmid causes antigenic variation in the bacterium *Borrelia hermsii*. *Nature.* **318:** 257–263.

Poley J.R. & Rosenfield S. (1982) Malabsorption in giardiasis: presence of a luminal barrier (mucoid pseudomembrane). A scanning and transmission electron microscopic study. *J. Pediatr. Gastroenterol. Nutr.* **1:** 63–80.

Popovic O., Pendic B., Paljm A., Andrejevic M. & Trpkovic D. (1974) Giardiasis, local immune defense and responses. *Eur. J. Clin. Invest.* **4:** 380.

Potter L.A. (1928) Two species of *Giardia* from the rat. *Am J. Hyg.* **8:** 77–84.

Quinn (1971) The epidemiology of intestinal parasites of importance in the United States. *South. Med. Bull.* **59:** 29–30.

Radulescu S., Iancu L., Simionescu O. & Meyer E.A. (1976) Serum antibodies in giardiasis. *J. Clin. Pathol.* **29:** 863.

Radulescu S. & Meyer E.A. (1981) Opsonization *in vitro* of *Giardia lamblia* trophozoites. *Infect. Immun.* **32:** 852–856.

Radulescu S., Rau C., Petrasincy D., Gaicu N. & Meyer E.A. (1980) Behavior and cytopathogenicity of *Giardia lamblia* in cell cultures. *Arch. Roumaines Pathol. Exp. Micro.* **39:** 163–170.

Radulescu S., Smolinschi M., Rau C. & Meyer E.A. (1982) *Giardia* spp. cysts, possible vectors of enterobacteria. *Mol. Biochem. Parasitol.* (Suppl.) **p:** 578.

Ranki A., Valle S.-L., Antonen J., Suni J., Jokipii L., Jokipii A.M.M., Saxinger C. & Krohn K. (1985) Immunosuppression in homosexual men seronegative for HTLV III. *Can. Res.* **45**: 4616S–4618S.

Reiner D.S., Wang C.S. & Gillin F.D. (1987) Human milk kills *Giardia lamblia* by generating toxic lipolytic products. *J. Infect. Dis.* **154**: 825–832.

Rendtorff R.C. (1954) The experimental transmission of human intestinal protozoan parasites. II. *Giardia lamblia* cysts given in capsules. *Am. J. Hyg.* **59**: 209–220.

Rengpien S. & Bailey G.B. (1975) Differentiation of *Entameba*: a new medium and optimal conditions for axenic encystation of *E. invadens*. *J. Parasitol.* **61**: 24–30.

Reynolds J., Gyure L., Andrew E. & Hall J.G. (1980) Studies of the transport of polyclonal IgA antibody from blood to bile in rats. *Immunology.* **39**: 463–467.

Rice E.W. & Schaefer III F.W. (1981) Improved *in vitro* excystation procedure for *Giardia lamblia* cysts. *J. Clin. Micro.* **14**: 709–710.

Richardson B.J., Baverstock P.R. & Adams M. (1986) *Allozyme Electrophoresis: a handbook for systematic and population studies*. Academic Press, Sydney.

Richman L.K. & Brown W.R. (1977) Immunochemical characterization of IgM in intestinal fluids. *J. Immunol.* **119**: 1515–1519.

Ridley M.J. & Ridley D.S. (1976) Serum antibodies and jejunal histology in giardiasis associated with malabsorption. *J. Clin. Path.* **29**: 30–34.

Riggs J.L., Dupuis K.W., Nakamura K. & Spath D.P. (1983) Detection of *Giardia lamblia* by immunofluorescence. *Appl. Environ. Microbiol.* **45**: 698–700.

Roberts-Thomson I.C. (1985) Giardiasis. In: *Infectious diarrhea in the young. Strategies for control in humans and animals.* Ed. S. Tzipori. Amsterdam : Elsevier Science Publishers B.V. pp. 316–319.

Roberts-Thomson I.C. (1987) Giardiasis: the role of immunological mechanisms to host-parasite relationships. In: *Immunopathology of the small intestine*. Ed. M.N. Marsh. New York: John Wiley & Sons Ltd. pp. 209–222.

Roberts-Thomson I.C. & Anders R.F. (1981) Serum antibodies in adults with giardiasis. *Gastroenterology.* **80**: 1262

Roberts-Thomson I.C. & Anders R.F. (1984) Cellular and humoral immunity in Giardiasis. In: *Giardia and Giardiasis, Biology, Pathogenesis and Epidemiology.* Eds. S.L. Erlandsen & E.A.Meyer. New York: Plenum Press. **pp.** 185–200.

Roberts-Thomson I.C., Anders R.F. & Bhathal P.S. (1982) Granulomatous hepatitis and cholangitis associated with giardiasis. *Gastroenterology.* **83:** 480-483.

Roberts-Thomson I.C., Anders R.F., Underdown B.J. & Mitchell G.F. (1981) *Giardia muris* in inbred mouse strains. *Gastroenterology.* **80:** 1263.

Roberts-Thomson I.C. & Mitchell G.F. (1978) Giardiasis in mice. I. Prolonged infections in certain mouse strains and hypothymic (nude) mice. *Gastroenterology.* **75**: 42–46.

Roberts-Thomson I.C. & Mitchell G.F. (1979) Protection of mice against *Giardia muris* infection. *Infect. Immun.* **24**: 971–973.

Roberts-Thomson I.C., Mitchell G.F., Anders R.F., Tait B.D., Kerlin P., Kerr-Grant A. & Cavanagh P. (1980) Genetic studies in human and murine giardiasis. *Gut.* **21**: 397–401.

Roberts-Thomson I.C., Stevens D.P., Mahmoud A.A.F. & Warren K.S. (1976a) Giardiasis in the mouse: an animal model. *Gastroenterology.* **71**: 57–61.

Roberts-Thomson I.C., Stevens D.P., Mahmoud A.A.F. & Warren K.S. (1976b) Acquired resistance to infection in an animal model of giardiasis. *J. Immunol.* **117**: 2036–2037.

Rodrigues Da Silva J., Gomes J., Coutinho S., Braga D.L. & De Figueiredo N. (1964) Histopathologic findings in giardiasis: a biopsy study. *Am. J. Dig. Dis.* **9:** 355–365.

Rohrer L., Winterhalter K.H., Eckert J. & Kohler P. (1986) Killing of *Giardia lamblia* by human milk is mediated by unsaturated fatty acids. *Antimicrob. Agents Chemo.* **30**: 254–257.

Rose M.E., Peppard J.V. & Hobbs S.M. (1984) Coccidiosis: characterization of antibody responses to infection with *Eimeria nieschulzi*. *Parasite Immunology*. **6**: 1–12.

Rosekrans P.C.M., Lindeman J. & Meijer C.J.L.M. (1981) Quantitative histological and immunohistochemical findings in jejunal biopsy speciments in giardiasis. *Virchow's Archiv. A. Pathol. Anat. Histopath.* **393**: 145–151.

Rosenthal P. (1985) Collection of duodenal bile in infants and children by the string test. *J. Pediatr. Gastroenterol. Nutr.* **4:** 284–285.

Rosenthal P. & Liebman W.M. (1980) Comparative study of stool examinations, duodenal aspirations and pediatric Entero-Test for giardiasis in children. *J. Pediatr.* **96:** 278–279.

Rosoff J.D. & Stibbs H.H. (1986) Isolation and identification of a *Giardia lamblia*-specific stool antigen (GSA 65) useful in coprodiagnosis of giardiasis. *J. Clin. Microbiol.* **23**: 905–910.

Saha T.K. & Ghosh T.K. (1977) Invasion of small intestinal mucosa by *Giardia lamblia* in man. *Gastroenterology*. **72**: 402–405.

Sauch J.F. (1984) Purification of *Giardia muris* cysts by velocity sedimentation. *Appl. Environ. Microbiol.* **48**: 454–455.

Schaefer III F.W., Rice E.W. & Hoff J.C. (1984) Factors promoting *in vitro* excystation of *Giardia muris* cysts. *Trans. R. Soc. Trop. Med. Hyg.* **78**: 795–800.

Schleinitz P., Justus P., Stenzel P., Owen R. & Meyer E. (1983) Successful introduction of culture adapted *Giardia* into a rabbit model: ultrastructural features. *Gastroenterology*. **84:** 1301.

Schrank G.D. & Verwey W.F. (1976) Distribution of cholera organisms in experimental *Vibrio cholerae* infections: proposed mechanisms of pathogenesis and antibacterial immunity. *Infect. Immun.* **13**: 195–203.

Schuurman H.J., Vos J.G., Broekhuizen R., Brandt C.J.W.M. & Kater l. (1985) *In vivo* biological effect of allogeneic cultured thymic epithelium on thymus-dependent immunity in athymic nude rats. *Scand. J. Immunol.* **21:** 21–30.

Sehgal A.K., Grewal M.S., Chakravarti R.N., Broor S.L., Deka N.C. & Chhuttani P.N. (1976) Experimental giardiasis in albino rats. *Ind. J. Med. Res.* **64**: 1015–1018.

Sheffield H.G. & Bjorvatn B. (1977) Ultrastructure of the cyst of *Giardia lamblia. Am. J. Trop. Med. Hyg.* **26**: 23–30.

Sherman P., Fleming N., Forstner J., Roomi N. & Forstner G. (1987) Bacteria and the mucus blanket in experimental bowel bacterial overgrowth. *Am. J. Pathol.* **126**: 527–534.

Silberberg A. & Meyer F.A. (1982) Structure and function of mucus. *Adv. Exp. Med. Biol.* **144**: 53–74.

Silverman M., Zieg J., Helmen M. & Simon M. (1979) Phase variation in *Salmonella*: genetic analysis of a recombinational switch. *Proc. Natl. Acad. Sci.* **76**: 391–395.

Simon C.E. (1922) A critique of the supposed rodent origin of human giardiasis. *Am. J. Hyg.* **2**: 406–434.

Sminia T. & Jeurissen S.H.M. (1986) The macrophage population of the gastrointestinal tract of the rat. *Immunobiology.* **172**: 72–80.

Smith P.D. (1984) Human immune responses to *Giardia lamblia.* In: *Giardia and Giardiasis, Biology, Pathogenesis and Epidemiology.* Eds. S.L. Erlandsen & E.A. Meyer. New York: Plenum Press. **pp.** 201–218.

Smith P.D. (1985) Pathophysiology and immunology of giardiasis. *Ann. Rev. Med.* **36**: 295–307.

Smith P.D., Elson C.O., Keister D.B. & Nash T.E. (1982a) Human host response to *Giardia lamblia.* I. Spontaneous killing by mononuclear leukocytes *in vitro. J. Immunol.* **128**: 1372–1376.

Smith P.D., Gillin F.D., Brown W.R. & Nash T.E. (1981a) IgG antibody to *Giardia lamblia* detected by enzyme-linked immunosorbent assay. *Gastroenterology.* **80**: 1476–1480.

Smith P.D., Gillin F.D., Kaushal N.A. & Nash T.E. (1982b) Antigenic analysis of *Giardia lamblia* from Afghanistan, Puerto Rico, Ecuador and Oregon. *Infect. Immun.* **36**: 714–719.

Smith P.D., Gillin F.D., Spira W.M. & Nash T.E. (1982c) Chronic giardiasis: studies on drug sensitivity, toxin production and host immune response. *Gastroenterology.* **83**: 797–803.

Smith P.D., Horsburgh C.R. & Brown W.R. (1981b) *In vitro* studies on bile acid deconjugation and lipolysis inhibition by *Giardia lamblia. Dig. Dis. Sci.* **26**: 700–704.

Smith P.D., Keister D.B. & Elson C.O. (1983) Human host response to *Giardia lamblia*. II. Antibody-dependent killing *in vitro*. *Cell. Immunol.* **82**: 308–315.

Smith P.D., Keister D.B., Wahl S.M. & Meltzer M.S. (1984) Defective spontaneous but normal antibody-dependent cytotoxicity for an extracellular protozoan parasite, *Giardia lamblia*, by C3H/HeJ mouse macrophages. *Cellular Immunology.* **85**: 244–251.

Snider D.P., Gordon J., McDermott M.R. & Underdown B.J. (1985) Chronic *Giardia muris* infection in anti-IgM-treated mice. I. Analysis of immunoglobulin and parasite-specific antibody in normal and immunoglobulin-deficient animals. *J. Immunol.* **134**: 4153–4163.

Snider D.P. & Underdown B.J. (1986) Quantitative and temporal analyses of murine antibody response in serum and gut secretions to infection with *Giardia muris*. *Infect. Immun.* **52**: 271–278.

Socken D.J., Jeejeebhoy K.N., Bazin H. & Underdown B.J. (1979) Identification of secretory component as an IgA receptor on rat hepatocytes. *J. Exp. Med.* **150:** 1538–1548.

Socken D.J., Simms E.S., Nagy B.R., Fisher M.M. & Underdown B.J. (1981) Secretory component-dependent hepatic transport of IgA antibody-antigen complexes. *J. Immunol.* **127:** 316–319.

Sogayar M.I.L. & Gregorio E.A. (1986) Cytoplasmic inclusions in *Giardia*: an electron microscopy study. *Ann. Trop. Med. Parasitol.* **80**: 49–52.

South Australian Health Commission (1986) Infections and notifiable disease in South Australia. Epidemiology Branch Communicable Disease Control Unit.

Spalding D.M., Williamson S.I., Koopman W. & McGhee J.R. (1984) Preferential induction of polyclonal IgA secretion by murine Peyer's patch dendritic cell-T cell mixtures. *J. Exp. Med.* **160:** 941–946.

Speelman O. & Ljungstrom I. (1986) Protozoal enteric infections among expatriates in Bangladesh. *Am. J. Trop. Med. Hyg.* **35**: 1140–1145.

Spencer L., Gyure L.A. & Hall J.G. (1983) IgA antibodies in the bile of rats. III. The role of intrathoracic lymph nodes and the migration pattern of their blast cells. *Immunology.* **48**: 687–693.

Staats J.C. (1972) Standardized nomenclature for inbred strains of mice. Fifth listing. *Cancer Res.* **32:** 1609–1646.

Stern A., Brown M., Nickel P. & Meyer T.F. (1986) Opacity gene in *Neisseria gonorrhoeae*: control of phase and antigenic variation. *Cell.* **47:** 61–71.

Stevens D.P. & Frank D.M. (1978) Local immunity in murine giardiasis: is milk protective at the expense of the maternal gut? *Trans. Assoc. Am. Phys.* **91**: 268–272.

Stevens D.P., Frank D.M. & Mahmoud A.A.F. (1978) Thymus dependency of host resistance to *Giardia muris* infection: studies in nude mice. *J. Immunol.* **120**: 680–682.

Streeter P.R., Berg E.L., Rouse B.T.N., Bargatze R.F. & Butcher E.C. (1988) A tissue-specific endothelial cell molecule involved in lymphocyte homing. *Nature.* **331:** 41–46.

Svanborg-Eden C. & Svennerholm A.M. (1978) Secretory immunoglobulin A and G antibodies prevent adhesion of *Escherichia coli* to human urinary tract epithelial cells. *Infect. Immun.* **22:** 790–797.

Swan J.M. & Thompson R.C. (1986) The prevalence of *Giardia* in dogs and cats in Perth. W. Australia. *Aust. Vet. J.* **63:** 110–112.

Tagliabue A., Nencioni L., Villa L., Keren D.F., Lowell G.H. & Boraschi D. (1983) Antibody-dependent cell-mediated antibacterial activity of intestinal lymphocytes with secretory IgA. *Nature.* **306**: 184–186.

Tait A. (1985) Genetics of protein variation in populations of protozoa. In: *Ecology and genetics of host-parasite interactions.* Ed. D. Rollinson & R.M. Anderson. London: Academic Press, Inc. **pp.** 185–203.

Taylor G.D. & Wenman W.M. (1987) Human immune response to *Giardia lamblia* infection. *J. Infect. Dis.* **155**: 137–140.

Thompson R.C.A., Meloni B.P. & Lymbery A.J. (1988) Humans and cats have genetically-identical forms of *Giardia*: evidence of a zoonotic relationship. *Med. J. Aust.* **148:** 207–209.

Thompson A., Roland R., Hecker R., Gibson G.E. & Reid D.P. (1977) Immunoglobulin-bearing cells in giardiasis. *J. Clin. Pathol.* **30** : 292–294.

Thornton S.A., West A.H., DuPont H.L. & Pickering L.K. (1983) Comparison of methods for identification of *Giardia lamblia*. *Am. J. Clin. Pathol.* **80:** 858–860.

Torian B.E., Barnes R.C., Stephens R.S. & Stibbs H.H. (1984) Tubulin and high-molecular-weight polypeptides as *Giardia lamblia* antigens. *Infect. Immun.* **46**: 152–158.

Towbin H., Staehelin T. & Gordon S. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* **76**: 4350–4354.

Udezulu I.A. & Leitch G.J. (1987) A membrane-associated neuraminidase in *Entameba histolytica* trophozoites. *Infect. Immun.* **55**: 181–186.

Underdown B.J., Roberts-Thomson I.C., Anders R.F. & Mitchell G.F. (1981) Giardiasis in mice: studies on the characteristics of chronic infection in C3H/He mice. *J. Immunol.* **126**: 669–672.

Underdown B.J. & Schiff M.J. (1986) Immunoglobulin A: strategic defense initiative at the mucosal surface. *Ann. Rev. Immunol.* **4**: 389–417.

Ungar B.L.P. & Nash T.E. (1987) Cross-reactivity among different *Giardia lamblia* isolates using immunofluorescent antibody and enzyme immunoassay techniques. *Am. J. Trop. Med. Hyg.* **37**: 283–289.

Ungar B.L.P., Yolken R.H., Nash T.E. & Quinn T.C. (1984) Enzyme-linked immunosorbent assay for the detection of *Giardia lamblia* in fecal specimens. *J. Infect. Dis.* **149**: 90–97.

Upadhyay P., Ganguly N.K., Walia B.N.S. & Mahajan R.C. (1986) Kinetics of lymphocyte subpopulation in intestinal mucosa of protein deficient *Giardia lamblia* infected mice. *Gut.* **27**: 386–391.

Upcroft J.A., Capon A.G., Dharmkrong-At A., Healey A., Boreham P.F.L. & Upcroft P. (1987) *Giardia intestinalis* antigns expressed by *Escherichia coli*. *Mol. Biochem. Parasitol.* **26**: 267–276.

Vaerman J.-P., Derijck-Langendries A., Rits M. & Delacroix D. (1985) Neutralization of cholera toxin by rat bile secretory IgA antibodies. *Immunology* **54**: 601–603.

Vaessen L.M.B., Broekhuizen R., Rozing J., Vos J.G. & Schuurman H.-J. (1986) T-cell development during ageing in congenitally athymic (nude) rats. *Scand. J. Immunol.* **24**: 223–235.

Van der Heijden, S.W. & Bianchi A.T.G. (1987) Contribution of immunoglobulin-secreting cells in the murine small intestine to the total 'background' immunoglobulin production. *Immunology.* **62**: 551–555.

Van der Ploeg, Lex H.T., Schwartz D.C., Cantor C.R. & Borst P. (1984) Antigenic variation in *Trypanosoma brucei* analyzed by electrophoretic separation of chromosome-sized DNA molecules. *Cell.* **37**: 77–84.

Vinayak V.K., Aggarwal A., Bhatia A., Naik S.R. & Chakravarti R.N. (1980a) Adoptive transfer of immunity in *Giardia lamblia* infection in mice. *Ann. Trop. Med. Parasitol.* **75**: 265–267.

Vinayak V.K., Ficai K.K., Chandna R., Venkateswarlu K. & Mehta S. (1985) Detection of *Giardia lamblia* antigen in the feces by counterimmunoelectrophoresis. *Pediatr. Infect. Dis.* **4**: 383–386.

Vinayak V.K., Jain P. & Naik S.R. (1978) Demonstration of antibodies in giardiasis using the immunodiffusion technique with *Giardia* cysts as antigen. *Ann. Trop. Med. Parasitol.* **72**: 581–582.

Vinayak V.K., Sharma G.L., Aggarwal A. & Naik S.R. (1980b) Acquired resistance in experimental giardiasis. *Indian J. Med. Res.* **72**: 210–213.

Vinayak V.K.; Venkateswarlu K., Khanna R. & Mehta S. (1987) Hypogammaglobulinaemia in children with persistent giardiasis. *J. Trop. Pediatr.* **33**: 140–142.

Visvesvara G.S. (1980) Axenic growth of *Giardia lamblia* in Diamond's TPS-I medium. *Trans. R. Soc. Trop. Med. Hyg.* **74**: 213–215.

Visvesvara G.S. & Healy G.R. (1980) The possible use of an indirect immunofluorescent test using axenically grown *Giardia lamblia* antigens in diagnosing giardiasis. In: *Waterborne Transmission of Giardiasis.* Eds. W. Jakubowski & J.C. Hoff. Ohio: U.S. Environmental Protection Agency. **pp.** 53–63.

Visvesvara G.S. & Healy G.R. (1984) Antigenicity of *Giardia lamblia* and the current status of serologic diagnosis of giardiasis. In: *Giardia and Giardiasis, Biology, Pathogenesis and Epidemiology.* Eds. S.L. Erlandsen & E.A. Meyer. New York: Plenum Press. **pp.** 219–232.

Visvesvara G.S., Smith P.D., Healy G.R. & Brown W.R. (1980) An immuinofluorescence test to detect serum antibodies to *Giardia lamblia. Ann. Int. Med.* **93**: 802–805.

Vos F.G., Kreeftenberg J.G., Kruijt B.C., Kruizinga W. & Steerenberg P.A. (1980) The athymic nude rat. II. Immunological characteristics. *Clin. Immunol. Immunopathol.* **15:** 229–237.

Vos J.G., Ruitenberg E.J., van Basten N., Buys J., Elgersma A. & Kruizinga W. (1983) The athymic nude rat. IV. Immunocytochemical study to detect T-cells and immunological and histopathological reactions against *Trichinella spiralis. Parasite Immunology.* **5:** 195–215.

Walia B.N.S., Ganguly N.K., Mahajan R.C., Kumar D., Madan I.J., Gambhir S.K. & Kanwar S.S. (1986) Morbidity in preschool giardia cyst excretors. *Trop. Geogr. Med.* **38**: 367–370.

Walker W.A., Lake A.M. & Block K.J. (1982) Immunologic mechanisms for goblet cell mucus release: possible role in mucosal host defense. In: *Recent advances in mucosal immunity.* Ed. W. Strober, L.A. Hanson & K.W. Sell. New York: Raven Press. **pp.** 331–342.

Wallis P.M., Buchanan-Mappin J.M., Faubert G.M. & Belosevic M. (1984) Reservoirs of *Giardia* spp. in Southwestern Alberta. *J. Wildl. Dis.* **20:** 279–283.

Wallis P.M. & Wallis H.M. (1986) Excystation and culturing of human and animal *Giardia* spp by using gerbils and TYI-S33 medium. *Appl. Environ. Microbiol.* **51**: 647–651.

Wallis P.M., Zammuto R.M. & Buchanan-Mappin J.M. (1986) Cysts of *Giardia* spp. in mammals and surface waters in Southwestern Alberts. *J. Wildl. Dis.* **22:** 115–118.

Wang A.L. & Wang C.C. (1986) Discovery of a specific double-stranded RNA virus in *Giardia lamblia. Mol. Biochem. Parasitol.* **21**: 269–276.

Wang A.L., Wang C.C. & Alderete J.F. (1987) *Trichomonas vaginalis* phenotypic variation occurs only among trichomonads infected with the double-stranded RNA virus. *J. Exp. Med.* **166:** 142–150.

Ward H.D., Alroy J., Lev B.I., Keusch G.T. & Pereira M.E.A. (1985) Identification of chitin as a structural component of *Giardia* cysts. *Infect. Immun.* **49**: 629–634.

Ward H.D., Alroy J., Lev B.L., Keusch G.T. & Pereira M.E.A. (1988) Biology of *Giardia lamblia.* Detection of N-acetyl-glucosamine as the only surface saccharide moiety and identification of two subsets of trophozoites by lectin binding. *J. Exp. Med.* **167**: 73–88.

Ward H.D., Lev B.I., Kane A.V., Keusch G.T. & Pereira M.E.A. (1987) Identification and characterization of Taglin, a mannose-6-phosphate binding, trypsin-activated lectin from *Giardia lamblia*. *Biochemistry*. **26**: 8669–8674.

Webster A.D.B. (1980) Giardiasis and immunodeficiency diseases. *Trans. R. Soc. Trop. Med. Hyg.* **74**: 440–443.

Weisz-Carrington P. Schrater A.F., Lanm M.E. & Thorbecke G.J. (1979) Immunoglobulin isotype in plasma cells of normal and athymic mice. *Cell. Immunol.* **44:** 343–351.

Wenman W.M., Meuser R.U., & Wallis P.M. (1986) Antigenic analysis of *Giardia duodenalis* strains isolated in Alberta. *Can. J. Microbiol.* **32**: 926–929.

Wenrich D.H. (1940) Observations on parasites and inclusion bodies in certain intestinal protozoa. *Science.* **92:** 416–417.

Wheatley W.B. (1951) A rapid staining procedure for intestinal amoebae and flagellates. *Am. J. Clin. Pathol.* **21**: 990–991.

Wieder S.C., Keister D.B. & Reiner D.S. (1983) Mass cultivation of *Giardia lamblia* in a serum-free medium. *J. Parasitol.* **69**: 1181–1182.

William D.C., Shookhoff H.B., Felman Y.M. & DeRamos S.W. (1978) High rate of enteric protozoal infections in selected homosexual men attending a venereal disease clinic. *Sex. Transm. Dis.* **5:** 155–157.

Williams R.C. & Gibbons R.J. (1972) Inhibition of bacterial adherence by secretory immunoglobulin A: a mechanism of antigen disposal. *Science.* **177:** 697–699.

Winiecka J., Kasprzak W., Kociecka W., Plotkowiak J. & Myjak P. (1984) Serum antibodies to *Giardia intestinalis* detected by immunofluorescence using trophozoites as antigen. *Trop. Med. Parasitol.* **35**: 20–22.

Wittner M., Maayan S., Farrer W. & Tanowitz H.B. (1983) Diagnosis of giardiasis by two methods. Immunofluorescence and enzyme-linked immunosorbent assay. *Arch. Pathol. Lab. Med.* **107**: 524–527.

Wolfe M.S. (1984) Symptomatology, Diagnosis and Treatment. In: *Giardia and Giardiasis, Biology, Pathogenesis and Epidemiology.* Eds. S.L. Erlandsen & E.A. Meyer. New York: Plenum Press. **pp.** 147–162.

Woo P.K. (1984) Evidence for animal reservoirs and transmission of Giardia infection between animal species. In: *Giardia and Giardiasis, Biology, Pathogenesis and Epidemiology*. Eds. S.L.Erlandsen & E.A. Meyer E.A. New York: Plenum Press. **pp.** 341–364.

Woo P.T.K. & Paterson W.B. (1986) *Giardia lamblia* in children in day-care centres in southern Ontario, Canada, and susceptibility of animals to *G. lamblia*. *Trans. R. Soc. Trop. Med. Hyg.* **80**: 56–59.

Woodruff J.J. & Clarke L.M. (1987) Specific cell-adhesion mechanisms determining migration pathways of recirculating lymphocytes. *Ann. Rev. Immunol.* **5**: 201–222.

World Health Organisation. (1980) Parasite-related diarrheas. *Bull. W.H.O.* **58**: 819–830.

World Health Organisation. (1986) Major parasitic infections: a global review. *Wld. Hlth. Statist. Quart.* **39**: 145–160.

Wright S.G. (1980) Giardiasis and malabsorption. *Trans. R. Soc. Trop. Med. Hyg.* **74**: 436–437.

Wright S.R. (1983) Parasites and travellers' diarrhea. *Scand. J. Gastroenterol.* **18**: 25–29.

Wright S.G., Moody A.H., Tomkins A.M. & Ridley D.S. (1977a) Fluorescent antibody studies in giardiasis. *Gut.* **18**: A986.

Wright S.G. & Tomkins A.M. (1977) Quantification of the lymphocytic infiltrate in jejunal epithelium in giardiasis. *Clin. Exp. Immunol.* **29**: 408–412.

Wright S.G., Tomkins A.M. & Ridley D.S. (1977b) Giardiasis: clinical and therapeutic aspects. *Gut.* **18**: 343–350.

Zenian A. & Gillin F.D. (1985) Interactions of *Giardia lamblia* with human intestinal mucus: enhancement of trophozoite attachment to glass. *J. Protozool.* **32**: 664–668.

Zenian A.J. & Gillin F.D. (1987) Intestinal mucus protects *Giardia lamblia* from killing by human milk . *J. Protozool.* **34**: 22–26.

Zinneman H.H. & Kaplan A.P. (1972) The association of giardiasis with reduced intestinal secretory immunoglobulin A. *Amer. J. Dig. Dis.* **17**: 793–797.

Zisman M. (1977) Blood group A and giardiasis. *Lancet.* **2**: 1285.