MODULATION OF *STAPHYLOCOCCUS AUREUS* ADHERENCE TO CULTURED HUMAN ENDOTHELIAL CELLS BY CYTOKINES

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SUMMARY

Adhesion of microorganisms to host tissues is the first step of microbial invasion and colonization of body tissues. The binding of *Staphylococcus aureus* (*S. aureus*) to endothelial cells can initiate the pathogenetic mechanism of acute endocarditis, a fulminant life-threatening disease, which leads to the rapid destruction of infected valves and metastatic bacterial dissemination. Since a variety of cytokines are released during this infection, it is possible that they contribute to either the prevention or promotion of the pathogenic process by altering endothelial cell adhesion properties for the bacteria. The objective of the present study was to determine if cytokine treatment of endothelial cells altered their adhesiveness for the bacteria and to compare this with adhesion of neutrophils.

Endothelial cells from human umbilical veins were treated with a number of cytokines to determine whether this treatment affected their adhesiveness for *S. aureus*. Preincubation of human umbilical vein endothelial cells with pathophysiological concentrations of either tumor necrosis factor (TNF), interleukin-1β (IL-1β) or lymphotoxin (LT) significantly reduced their ability to adhere *S. aureus*. Under these conditions the cytokines caused an increase in the adhesion of human neutrophils. These effects of cytokines occurred in a concentration related manner and were preincubation time dependent. Responses were observed within 1h and the degree of bacterial adherence continued to decrease up to 4h incubation.

The mechanisms by which these cytokines depressed bacterial-endothelial cell adherence were partly studied. The TNF-, IL-1β- and LT-induced changes in bacterial-endothelial cell adhesion were dependent on new protein and RNA synthesis as they could be prevented by the inhibitors, cycloheximide and actinomycin D. The enhanced neutrophil adherence induced by TNF, IL-1β and LT was similarly sensitive to these inhibitors. The depression of bacterial adherence induced by these cytokines was reversed by the platelet activating factor (PAF) antagonist WEB 2086, suggesting that PAF plays a role in the cytokine-induced alteration in bacterial-endothelial cell adherence. Phorbol myristate acetate (PMA), an activator of protein kinase C (PKC) also depressed the bacterial-endothelial cell adherence and dramatically enhanced neutrophil adherence. However while pretreatment of endothelial cells with the PKC inhibitor, staurosporine, totally inhibited the PMA-induced enhancement of neutrophil adherence, it had no effect on the PMA-induced decrease of bacterial adherence. These results suggest that the PMA-induced effects on bacterial adherence are independent of PKC as are those induced by TNF and LT since the effects of these cytokines were similarly not sensitive to staurosporine.

A plausible mechanism for the TNF-, IL-1β- and LT-induced decrease in bacterial adhesion to HUVEC is proposed, based on the ability of these cytokines to stimulate production of plasminogen activator (PA) which is responsible for conversion of plasminogen to plasmin. This enzyme then cleaves the integrin molecules such as fibrinogen from the surface of endothelial cells. Since these molecules are responsible for binding *S. aureus* , their destruction results in decreased bacterial adhesion.

The other cytokines studied, IL-2, IL-4, IFN-γ and IL-8 had no effect on the adhesion of *S. aureus* or neutrophils to endothelial cells. However, these cytokines affected the ability of TNF, IL-1β and LT to alter the bacterial-endothelial cell adhesion. IFN-γ, IL-4 or IL-8 inhibited the ability of TNF, IL-1β or LT to decrease bacterial adhesion to endothelial cells. The TNF- and IL-1β-induced enhancement of neutrophil adhesion was either unaffected by IFN-γ or diminished to varying degree by IL-4 or IL-8. The LT-induced increase of neutrophil adhesion was augmented by either IFN-γ, IL-4 or IL-8.

The data suggest that the cytokines TNF, LT and IL-1β may play an important role in defence against bacteria by inhibiting bacterial adhesion to surfaces such as the endothelium. It was also evident that these activities were under the influence of other cytokines.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Z. H. Huang

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ABBREVIATIONS

AA	Arachidonic acid
aFGF	Acidic fibroblast growth factor
bFGF	Basic fibroblast growth factor
E. coli	*Escherichia coli*
G-CSF	Granulocyte coloning stimulating factor
GM-CSF	Granulocyte macrophage coloning stimulating factor
HSA	Human serum albumin
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1
ICAM-2	Intercellular adhesion molecule-2
Ig	Immunoglobulin
IL-1	Interleukin-1
IL-1ra	Interleukin-1 receptor antagonist
IL-2	Interleukin-2
IL-3	Interleukin-3
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-12	Interleukin-12
LAK	Lymphokine activated killer cells
LAL assay	Limulus Amebocyte Lysate assay
LT	Lymphotoxin

MNL	Mononuclear leukocytes
NF-κB	Nuclear factor-κB
NK cells	Natural killer cells
PA	Plasminogen activator
PAF	Platelet activating factor
PAI	Plasminogen activator inhibitor
PCA	Procoagulant activity
PDGF	Platelet derived growth factor
PGI_2	prostaglandin I_2
PHA	Phytohaemagglutinin
PKA	Protein kinase A
PKC	Protein kinase C
PLA_2	Phospholipid A_2
PLP fixative	Perodate, lysine, paraformaldehyde fixative
PMA	Phorbol myristate acetated
PMB	Polymyxin B
PTK	Protein tyrosine kinase
RT	Room temperature
S.aureus	*Staphylococcus aureus*
TGF-β	Transforming growth factor beta
TNF	Tumor necrosis factor
VCAM	Vascular cellular adhesion molecule

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CHAPTER 1

INTRODUCTION

1.1 General introduction

Attachment of microorganisms to host tissues leads to a range of host responses in an attempt to eradicate the microorganisms from the body. Inflammation is the hallmark of microbial tissue invasion which is associated with the delivery of the plasma and cellular components of the blood to the infectious sites. The recruitment of leukocytes aids the control of the pathogen usually involving a variety of intercellular mediators. These mediators include polypeptide cytokines which are released by the inflammatory cells and possibly by tissue cells. Cytokines have a range of activities which either depress or promote particular cellular responses, contributing to a network of activities essential for host survival and maintenance of the normal haemostasis of the host. It is therefore of major importance to further define the physiological and pathophysiological function of these cytokines.

The endothelium is known to play an active, rather than being a "passive", part in host inflammatory responses. Studies on cytokine-endothelial cell interaction will lead to a better appreciation of the role of the endothelium in hemostatic processes. Studies of the effects of cytokines on the interaction of the endothelium with bacteria are of major importance in trying to elucidate the role of cytokines in immunity to bacteria.

1.2 Characteristics of Human Endothelial Cells

Endothelial cells constitute a continuous monolayer lining between the circulating blood and the vascular tissue. In a normal adult, endothelial cells occupy a surface of greater than 1000 m^2 and weigh over 100g. The properties of endothelial cells can be classified as follows: procoagulation, anticoagulation, fibrinolysis, endothelial derived and endothelial targeted growth regulation, synthesis of connective tissue components, modulation of inflammatory and immune reactions and special metabolic functions. Endothelial cells not only serve as a barrier between the bloodstream and subendothelial tissues, but also act as a target and source of some biological molecules which influence the structure and functional integrity of the circulation.

1.2.1 Synthesis of matrix proteins and glycosaminoglycans

Endothelial cells lie on the subendothelial extracellular matrix via their basement membrane. The basement membrane consists of a group of glycoproteins and glycosaminoglycans, eg., fibronectin, collagen, laminin, thrombospondin, heparan sulfate, chondroitin sulfate, dermatan sulfate, elastin and microfibrils (Jaffe, 1988). It has been shown that endothelial cells synthesize and release matrix components to the extracellular matrix, as well as to the bloodstream. These molecules may contribute to endothelial cell proliferation, migration, and adhesion properties (Jaffe, 1987). Moreover, endothelial cells not only regulate the synthesis of these membrane proteins, but also modify their degradation by releasing collagenase (Gross, 1982; Kalebic et al, 1983).

1.2.2 Fibrinolysis

Endothelial cells can synthesize and secrete plasminogen activator (PA) [tissue type PA (t-PA) and urokinase type PA (u-PA)] which converts plasminogen, a plasma glycoprotein, into the active fibrinolytic enzyme, plasmin. Endothelial cells also release a PA inhibitor (PAI) to regulate the activity of PA (Loskutoff, 1977; 1983). In subconfluent cultures of endothelial cells, the amount of PAI exceeds that of PA, whereas with confluent endothelial cell cultures PA concentrations are much higher than PAI (Levin et al, 1979). Some studies have shown that TNF, IL-1, LT, PMA, and bFGF induce the production of PA (Victor et al, 1990; Niedbala et al, 1991). The cytokine-induced production of PA is inhibited by the protein and RNA synthesis inhibitors, cycloheximide and actinomycin D respectively (Niedbala et al, 1991; Gross et al, 1982; Montesano et al, 1986; Saksela et al, 1987). Addition of IFN-γ to the TNF- or IL-1α-treated endothelial cells antagonizes the production of PA and the PA-mediated degradation of extracellular matrix without affecting PAI activity (Wojta et al, 1992; Niedbala et al, 1992). The activities of PA and PAI are very important in a variety of biological processes such as clot fibrinolysis, placental implantation, and migration of normal and malignant cells.

1.2.3 Von Willebrand protein production

Endothelial cells synthesize and release von Willebrand protein which acts as a carrier for coagulation factor VIII in the circulation (Jaffe et al, 1973). Von Willebrand protein is stored and concentrated in the

Weibel-Palade body in the endothelial cytoplasm (Weibel and Palade, 1964; Wagner et al, 1982; Warhol et al, 1984; Reinders et al, 1984). The presence of this protein in the endothelial cells is distinctive and it is absent in other vascular cell types (Jaffe et al, 1973). Stimulation of endothelial cells by either thrombin, IL-1 or endotoxin leads to the rapid release of von Willebrand protein and its expression on the endothelial cell surface mediating the adhesion of platelets to endothelial cells (Levine, 1982; Schorer et al, 1985).

1.2.4 Inflammatory responses

Endothelial cells form a barrier which leukocytes cross by adherence and transmigration to reach the extravascular space. It has been shown that leukocyte extravasation is mediated by the interaction of surface molecules on both endothelial cells and leukocytes. Some leukocyte products, such as cytokines, can regulate the leukocyte adhesion to endothelial cells by modulating the expression of surface molecules on both the endothelial cells and leukocytes (see 1.4).

1.3 The Effects of Cytokines on Human Endothelial Cells

Cytokines are a group of endogenous biologically active polypeptides, secreted by a variety of cells, which form a network in the regulation of host cellular functions acting in an autocrine, paracrine and endocrine manner. These proteins are regarded as the fourth form of soluble intercellular signalling molecules, along with

neurotransmitters, endocrine hormones and autacoids (Nathan et al, 1991).

One of the major functions of cytokines is to promote host immunity against microbial invasion, and removal and repair of damaged tissues (Ferrante et al, 1992). The uncontrolled and persistent production of cytokines such as which occurs in exacerbated inflammation can lead to pathophysiological responses resulting in severe morbidity and mortality. These molecules form a network characterized by a cascade of interaction leading to synergistic or antagonistic host responses. Their effects are dependent on cytokine concentration, the type and activation stage of the target cell, the composition and integrity of the surrounding matrix, and the composition of the cytokine population in the local environment (Elias et al, 1992).

A number of cytokine inhibitors have been described, but recently major effort has gone into characterizing two of these. One is a receptor antagonist of IL-1. The inhibitor can be produced by the same cells as those which produce IL-1 (eg. macrophages). This interleukin-1 receptor antagonist (IL-1ra), like IL-1, interacts with the IL-1 receptor, but fails to induce a response and at the same time blocks IL-1 binding (Dinarello, 1991). The other inhibitor acts on TNF and is actually a cleaved fragment of the extracellular protein of either the 55 KDa or 75 KDa peptide of the TNF receptor (Wallach et al, 1991). This is readily shed probably via enzymatic cleavage and can be found in body fluids where it can bind to and neutralize the activity of TNF (Dayer, 1991; Seckinger et al, 1987a, 1990).

1.3.1 Tumor necrosis factor (TNF) and Lymphotoxin (LT)

TNF is a polypeptide (non-glycosylated) which forms a trimer with a total molecular mass of 52 KDa (Fiers, 1991). LT, in contrast to TNF, is a glycoprotein in nature existing as a trimer with molecular mass of 64 KDa. LT, also known as TNF-β, is closely related in structure and function to TNF (TNF-α). LT is encoded by a gene immediately adjacent to the TNF gene, and displays a 50% amino acid homology to TNF. TNF is relatively stable in detergents, organic solvents and acid conditions, but sensitive to proteases while LT is highly unstable in detergents, acids and organic solvents, but relatively resistant to proteases (Aggarwal, 1989). TNF is produced mainly by activated macrophages and monocytes, but also by activated T lymphocytes, mast cells, neutrophils, keratinocytes, astrocytes, microglial cells and endothelial cells. In contrast, LT is exclusively produced by the activated lymphocytes. It has been found that TNF receptors are widely distributed on nearly all type of cells. At present two types of TNF receptor, TNF-R p55 and TNF-R p75 have been recognized. Endothelial cells have two types of TNF receptors (Mackay et al, 1993). Of considerable importance is the finding that LT and TNF recognize the same receptors. It has been shown that TNF and LT bind to a 55 KDa receptor with the same affinity, but TNF binds to 75 KDa receptor with higher affinity than LT. But the LT binding to endothelial cells was half-maximal at a concentration of 800 pM which was 10 times higher than the concentration required for TNF (Locksley et al, 1987). This may account for the similarities and differences in the biological activities of TNF and LT (Schall et al, 1990; Smith et al, 1990).

Both LT and TNF are cytotoxic or cytostatic for some tumor cells (Gray et al, 1984). These two cytokines activate the functions of macrophages, enhance neutrophil adhesion, phagocytosis, respiratory burst and cytotoxicity, induce adipocyte lipolysis and bone resorption, increase the expression of HLA class II antigens, have antiviral activity and promote B cell growth (Trinchieri et al, 1987; Wong et al, 1986; Shalaby et al, 1985; Pujio-Borrell et al, 1987; Patton et al, 1986; Canalis, 1987; Kehrl et al, 1987; Ferrante, 1992; Kumaratilake et al, 1991; Ferrante et al, 1992,1988a,1988b). TNF and LT also have similar effects on endothelial cells. They induce production of procoagulant activity, IL-1, coloning stimulating factors (CSFs) and platelet activating factor (PAF), induced expression of HLA class I antigen, cause the alteration in endothelial cell morphology and modulate the expression of endothelial adhesion molecules (Cotran et al, 1989).

Because of the differences in their receptor-binding affinities, there are some differences in their biological activities. TNF is usually more active than LT on a variety of cell types, including endothelial cells. TNF is an inducer for production of IL-1, CSFs, IL-6, IL-8 and platelet derived growth factor (PDGF), whereas LT is a very poor inducer of IL-1 and granulocyte macrophage coloning stimulating factor (GM-CSF) production (Poter, 1990). In the induction of endothelial cell adhesion molecules, higher concentration and longer preincubation time are required for LT compared with TNF to reach similar activity (Yonehara et al, 1989).

1.3.2 Interleukin-1 (IL-1)

IL-1 consists of two well characterised species, IL-1α and IL-1β of similar size of 17 KDa. IL-1 is primarily produced by monocytes/macrophages activated by many agents including microbial products, complement component, clotting components and inflammatory agents. There are two types of IL-1 receptor. Type I is expressed on T cells and cells of mesenchymal lineage, type II is mainly expressed on B cell and cells of the myelomonocytic lineage. Both IL-1α and IL-1β trigger the same receptors (Dinarello, 1991). Recently another form of IL-1 has been described, an IL-1 receptor antagonist (IL-1ra). IL-1ra can bind to both types of IL-1 receptors, but it competitively inhibits the IL-1 binding and is unable to induce a response (Seckinger et al, 1987b).

IL-1 is a cytokine with broad proinflammatory activities. It stimulates the proliferation of T cells and thymocytes and promotes antibody production by antigen-stimulated B cells and induces fever and production of acute phase proteins. IL-1, TNF and LT have overlapping activities on various tissues including endothelial cells. All these cytokines induce procoagulant activity and increase the production of PGI_2, PGE_2, tissue factor, von Willebrand Factor and PAF by endothelial cells (Bevilacqua, 1984; 1986; Naworth, 1986; Mantovani et al, 1989). These cytokines have also been reported to increase the synthesis and the release of plasminogen activator and the plasminogen activator inhibitor *in vitro* (Victor et al, 1990; Niedbala et al, 1991).

Like TNF and LT, IL-1 augments the adhesion of neutrophils, monocytes, lymphocytes and tumor cells to cytokine-treated endothelial cells (Contran et al, 1989). The cytokine-induced increase in leukocyte adhesion correlates with the expression of endothelial cell adhesion molecules (see 1.4.3). Treatment of endothelial cells with IL-1 can induce the production of IL-6, IL-8, GM-CSF, PDGF. In addition, TNF induces IL-1 production by endothelial cells (Mantovani et al, 1989). IL-1 also alters the morphological features of endothelial cells, changing them from polygonal epithelial-like cells to spindle fibroblastic-like cells. This may contribute to leukocyte migration across endothelial cells (Stolpen et al 1986).

1.3.3 Interferon-γ (IFN-γ)

IFN-γ, a 45 KDa glycoprotein dimer which contains two protein chains of 20 KDa and 25 KDa, is produced by activated T helper lymphocytes (Th_1) and natural killer cells (NK cells). Its receptor is expressed on leukocytes and many other cell types.

IFN-γ is well known for its ability to activate mononuclear phagocytes and enhance the expression of MHC class II antigens on these cells. Like TNF, IFN-γ also exhibits cytotoxicity activity on some cell types and inhibits lipoprotein lipase production which contributes to the development of cachexia (Billiau et al, 1992). On endothelial cells, IFN-γ induces the expression of MHC class II (HLA-DR, DP and DQ), and increases (and synergizes with TNF in) the expression of MHC class I (HLA-A,B,C) (Collins et al, 1984; Pober et al, 1986). Like TNF, LT and IL-1, IFN-γ can also alter the endothelial cell morphology (Stolpen et

al, 1986). IFN-γ can increase the TNF, LPS and IL-1 induced IL-6 production by endothelial cells (Leeuwenberg et al, 1990).

1.3.4 Interleukin-2 (IL-2)

IL-2 is a 15-17 KDa peptide, produced by activated T cells, which promotes the proliferation and differentiation of lymphocytes. Although it has been shown that endothelial cells from patients receiving either IL-2 or IL-2 in combination with lymphokine-activated killer (LAK) therapy, have increased expression of ICAM-1, E-selectin and HLA-DQ antigen (Cotran et al, 1989), this was postulated to be an indirect effect of IL-2 since administration of IL-2 can boost the endogenous synthesis of other cytokines by monocytes or lymphocytes, such as, IFN-γ, LT and TNF which are capable of promoting the expressions of ICAM-1, E-selectin and HLA-DQ on the surface of endothelial cells. *In vitro* IL-2 itself has no direct effect on endothelial cell antigen expression (Cotran et al, 1989).

1.3.5 Interleukin-4 (IL-4)

IL-4 is a 15-19 KDa glycoprotein. Its receptor is expressed on macrophages, lymphocytes, and a variety of cell lines, such as haematopoietic, fibroblast, lymphoid, and mast cell lines. Two types of IL-4 receptors have been described; those of high affinity and those of low affinity. Endothelial cells have the high affinity receptor for IL-4 (Paul et al, 1991).

This cytokine promotes proliferation of T and B cells, class switching during immunoglobulin production and expression of MHC class II

antigens. On nonlymphoid hematopoietic cells, IL-4 acts in different ways. It has been shown that IL-4 inhibits the growth of macrophages and macrophage-mediated killing of parasites (Kumaratilake et al, 1993). The antitumor effect of IL-4 has also been demonstrated (Paul, 1991). On endothelial cells, IL-4 has been shown to increase the expression of 1.4C3 antigen and is synergistic with either TNF or IL-1 in the expression of this antigen (Thornhill et al, 1990). The production of IL-6 by endothelial cells is also promoted by IL-4 (Howells et al, 1991). IL-4 itself does not alter the endothelial cell morphology. However, it synergizes with the TNF- and IFN-γ-induced morphological changes on endothelial cells (Thornhill et al, 1990).

1.3.6 Interleukin-8 (IL-8)

Chemokine IL-8, also known as neutrophil activation peptide, is an 8.4 KDa protein. It is released by LPS activated monocytes/macrophages, mitogen stimulated-lymphocytes and virus-infected fibroblasts. The IL-8 receptor is distributed on neutrophils, lymphocytes and monocytes. It has been shown that IL-8 activates neutrophil functions, such as chemotaxis, degranulation, production of superoxide anion and enhancement of neutrophil adhesion to unstimulated and cytokine-stimulated endothelial cells (Schroder et al, 1992). It has also been found that TNF and IL-1 promote endothelial cells to produce IL-8 (Gimbrone et al, 1989). This endothelial-derived IL-8 acts as a leukocyte adhesion inhibitor (Herbert et al, 1990). It reduces the neutrophil adhesion and migration across TNF and IL-1 activated endothelial cells, and enhances the detachment of adhered neutrophil from endothelial cells (Luscinskas et al, 1992).

1.3.7 Granulocyte colony stimulating factor (G-CSF) and granulocyte macrophage colony stimulating factor (GM-CSF)

G- and GM-CSF are two glycoprotein haematopoietic growth factors with molecular weights of 24 KDa and 23-29 KDa respectively. These two cytokines are produced predominantly by activated lymphocytes, macrophage as well as endothelial cells, fibroblast, mesothelial cells, etc (Demetri et al, 1991; Gasson, 1991). GM-CSF has been shown to stimulate neutrophil and macrophage degranulation, cytotoxicity, parasite and tumor cell killing and oxidative metabolism (Gasson, 1991). Endothelial cells possess high affinity receptors for G- and GM-CSF (Bussolino et al, 1989). Stimulation of endothelial cells with G/GM-CSF induced endothelial migration and proliferation which was attributed to wound healing (Bussolino et al, 1991). Exposure of endothelial cells to both cytokines altered the cell shape and cytoskeletal reorganization which was consistent with the endothelial cell migration function (Bussolino et al, 1991). These two mediators failed to induce procoagulant activity, expression of E-selectin, HLA antigens and the production of PAF. The other two colony stimulating factors, IL-3 and M-CSF had none of these functions (Bussolino et al, 1991). It has also been reported that GM-CSF and IL-3 promote monocyte adhesion to endothelial cells and plastic surfaces (Gamble et al, 1989; Elliott et al, 1990).

1.3.8 Transforming growth factor β (TGF-β)

TGF-β is a homodimeric polypeptide (25 KDa) released by platelets as well as activated macrophages, lymphocytes, neutrophils and synovial

fibroblasts. Three isoforms of TGF-β (TGF-β_1, TGF-β_2 and TGF-β_3) have been identified in mammalian species (Wahl, 1992). TGF-β can be immediately secreted by platelets at the site of injury or immunological challenge, and active TGF-β is usually cleared from the circulation within minutes (Assoian et al, 1983; Wakefield et al, 1991). TGF-β receptors have been widely distributed on most cell types. Three types of TGF-β receptor with molecular sizes of 280, 85 and 72 KDa have been reported to be expressed on endothelial cells. It appears that type III receptor (280 KDa) is expressed on subconfluent endothelial culture and has high affinity for the binding of TGF-β_1 and TGF-β_2. The type I and II (72 and 85 KDa respectively) receptors are mainly expressed on confluent culture, have high affinity for TGF-β_1 but low affinity for TGF-β_2 (Wahl, 1992; Muller et al, 1987; Suzuki et al, 1991). It was shown that TGF-β acted as a chemoattractant for neutrophils and monocytes (Reibman et al, 1991; Adams et al, 1991) and it has been suggested that the enhancement of monocyte migration into tissues by TGF-β is mediated by an increase in the expression of integrin receptors on monocytes, increase in monocyte adhesiveness for the matrix and augmentation of the production of type IV collagenase for monocyte diapedesis (Wahl, 1992). TGF-β also stimulates monocytes to produce cytokines, such as TNF, IL-1, IL-6, PDGF and bFGF all of which play a part as a positive feedback to TGF-β production (Wahl, 1992). Some studies have suggested that the rapid release and accumulation of TGF-β could induce the differentiation of Th_1 phenotype (Swain et al, 1991). TGF-β also regulates fibroblast proliferation and the synthesis and degradation of the extracellular matrix which may contribute to tissue repair during inflammation (Roberts et al, 1990). In contrast to the proinflammatory properties of TGF-β, especially in the early stages of the inflammatory response,

TGF-β can also have suppressive activity on the ability of mediators to activate or induce differentiation in inflammatory cells. This transition of pro- to antiinflammatory responsiveness may limit the host's inflammatory response and minimize the overt damage (Wahl, 1992).

TGF-β inhibits the growth and/or differentiation of endothelial cells, epithelial cells, NK cells, lymphokine activated killer cells (LAK cells) and CD_4^+ lymphocytes, but promotes the differentiation of CD_8^+ lymphocytes which produce the cytokines IL-4, IL-5 and IL-10 with the additional immunosupressive actions (Wahl, 1992). TGF-β also inhibits IL-1 receptor expression and induces the production of IL-1 receptor antagonist (Dubois et al, 1990; Turner et al, 1990), suppresses hematopoiesis and blocks colony formation of early progenitors, resulting in a reduction in the number of mature leukocytes (Hatzfeld et al, 1991).

Treatment of endothelial cells with TGF-β was shown to antagonize the IL-1- and TNF-induced increase of adhesion of neutrophils, lymphocytes and tumor cells to endothelial cells (Gamble et al, 1988; 1991; Bereta et al, 1992). It is also evident that TGF-β (β_1 and β_2) inhibits the TNF- and IL-1-induced expression of E-selectin and E-selectin mRNA levels in endothelial cells, but not the expression of VCAM-1 and ICAM-1. This effect of TGF-β is additive with IL-4 (Gamble et al, 1993). TGF-β has been reported to inhibit the growth of proliferating endothelial cells and PMA-induced tube formation (Suzuki et al, 1991). TGF-β enhances the production and deposition of PAI and decreases PA activity, whereas bFGF increase PA activity of endothelia cells (Chaudhury et al, 1991).

1.3.9 Interferon-α (IFN-α)

IFN-α is produced by a wide range of cells in response to a range of stimuli (Murray et al, 1988). Like IFN-γ, IFN-α induce the expression of HLA-DP, DQ, DR antigens on endothelial cells (Pober et al, 1986). Treatment of endothelial cells with IFN-α and IFN-γ also enhances the synthesis and expression of leu 13 antigen, a 16 KDa leukocyte antigen present on T and B cell surfaces. The function of this antigen expression is not clear (Jaffe et al, 1989). Another study showed that IFN-α inhibits T cell binding to unstimulated endothelial cells and IFN-γ induced T cell binding to endothelial cells by decreasing the expression of ICAM-1 antigen (Eguchi et al, 1992).

1.3.10 Interleukin-6 (IL-6)

IL-6 (IFN-β_2, B cell stimulatory factor and hybridoma/plasmacytoma growth factor) is a 26 KDa protein (Hirano et al, 1990). The cytokine is released by activated monocytes, lymphocytes, fibroblast and endothelial cells (Bauer et al, 1988; Kohase et al, 1986; Shalaby et al, 1989; Espevik et al, 1990). Stimulation of endothelial cells with LPS, TNF, LT IFN-γ and IL-1 induces the production of IL-6. The effects of TNF and IL-1 on the induction of IL-6 were synergistic with IFN-γ and antagonized by TGF-β and glucocorticoids (Shalaby et al, 1989; Waage et al, 1990). IL-6 has been shown to induce the synthesis of acute phase proteins from hepatocytes, promote the terminal differentiation of B cells for Ig synthesis, augment the T cell response, enhance the growth of multiple myeloma and synergises with IL-3 in promoting the proliferation of haematopoietic progenitors (Hirano et al, 1990). It

has also been shown that IL-6 enhances the expression of PDGF mRNA in endothelial cells. PDGF is a cytokine which stimulates the proliferation and migration of vascular smooth muscle cells and fibroblasts as well as being a chemoattractant for monocytes and neutrophils. This may contribute to the pathogenesis of vascular disease, such as atherosclerosis (Calderon et al, 1992).

The effects of cytokines on endothelial cells are summarised in Table 1.1. In this study we have focused on the cytokines, TNF, LT, IL-1, IL-2, IFN-γ, IL-4 and IL-8.

1.4. Modulation of Expression of Endothelial Cell Adhesion Molecules by Cytokines and Other Mediators

In the early 1980's, Pober and colleagues found that cultured endothelial cells which were pretreated with medium conditioned by lectin-activated peripheral blood lymphocytes expressed new bioactive molecules and new functions but this medium did not injure endothelial cells. On the basis of this observation, they redefined the term "endothelial cell activation" to distinguish it from the sublethal injury with consequent dysfunction. Endothelial cell activation describes the changes which the cells undergo at the level of expression of specific gene products that endow them with new morphological and antigenic characteristics and to perform new functions (Pober, 1988).

Table 1.1 The Effects of Cytokines on Endothelial Cells

The effects of cytokines	TNF	LT	IL-1	IL-2	IFN-α	IFN-γ	IL-4	IL-6	IL-8	TGF-β	GM-CSF
Angiogenesis	+									+	+
Tumor cell adhesion	+		+							-	
Production of ECM proteins	+		+							+	
Procoagulant activity	+	+	+								
Neutrophil adhesion	+	+	+			+	-		-	-	
Lymphocyte adhesion	+	+	+			+	+			-	
NK cell adhesion	+		+								
Monocyte adhesion	+	+	+			+					+
MHC class I antigen	+	+			+	+					
MHC class II antigens						+					
Expression of ICAM-1	+	+	+			+	-				
Expression of E-selectin	+	+	+			+	-				
Expression of VCAM-1	+		+				+				
Alteration of integrin molecules	+	+	+								
Production of IL-1	+	±	+								
Production of IL-6	+	+	+			+					
Production of IL-8	+	+	+								
Production of GM-CSF	+	±	+								
Production of PAF	+	+	+								
Production of PDGF	+	+	+								
Alteration of endothelial morphology	+	+	+			+	+				+

+: Induce
-: Inhibit
±: Marginally effective

Endothelial cell activation has been shown to occur both *in vitro* and *in vivo*. *In vivo*, the vascular endothelial cells from patients with lymphoma, acute granulomatous lymphadenitis, septic shock, or vascular leak syndrome and from animals undergoing intradersmal injection of cytokines, have been shown to display increased expression of ICAM-1, ELAM-1 and HLA-DQ/DP antigens (Contran et al, 1989). *In vitro*, it is also evident that cytokines can regulate endothelial adhesion properties by modulating the expression of certain surface molecules (Pober et al, 1991).

1.4.1 Intercellular adhesion molecules (ICAM)

ICAM-1 (CD54) and ICAM-2 belong to the immunoglobulin superfamily. They were identified as the ligands for lymphocyte function-related antigen-1 (LFA-1 or CD11a/CD18) (Springer, 1990; de Fougerolles et al, 1991). ICAM-2 shows a 35% homology to ICAM-1, but lacks an RGD (Arg-Gly-Asp) sequence (Staunton et al, 1989). ICAM-2 has two immunoglobulin-like domains while ICAM-1 has five. Both forms of ICAM are constitutively expressed on resting endothelial cells, and the ICAM-2 level is higher than ICAM-1. Stimulation of endothelial cells with IL-1, TNF, LT, IFN-γ and PMA increases the expression of ICAM-1, but not ICAM-2 (Pober et al, 1991; Wertheimer et al, 1992; de Fougerolles et al, 1991). The recognition of ICAM-1 by LFA-1 is partially responsible for neutrophil, but not lymphocyte, adhesion to endothelial cells (Fig. 1.1).

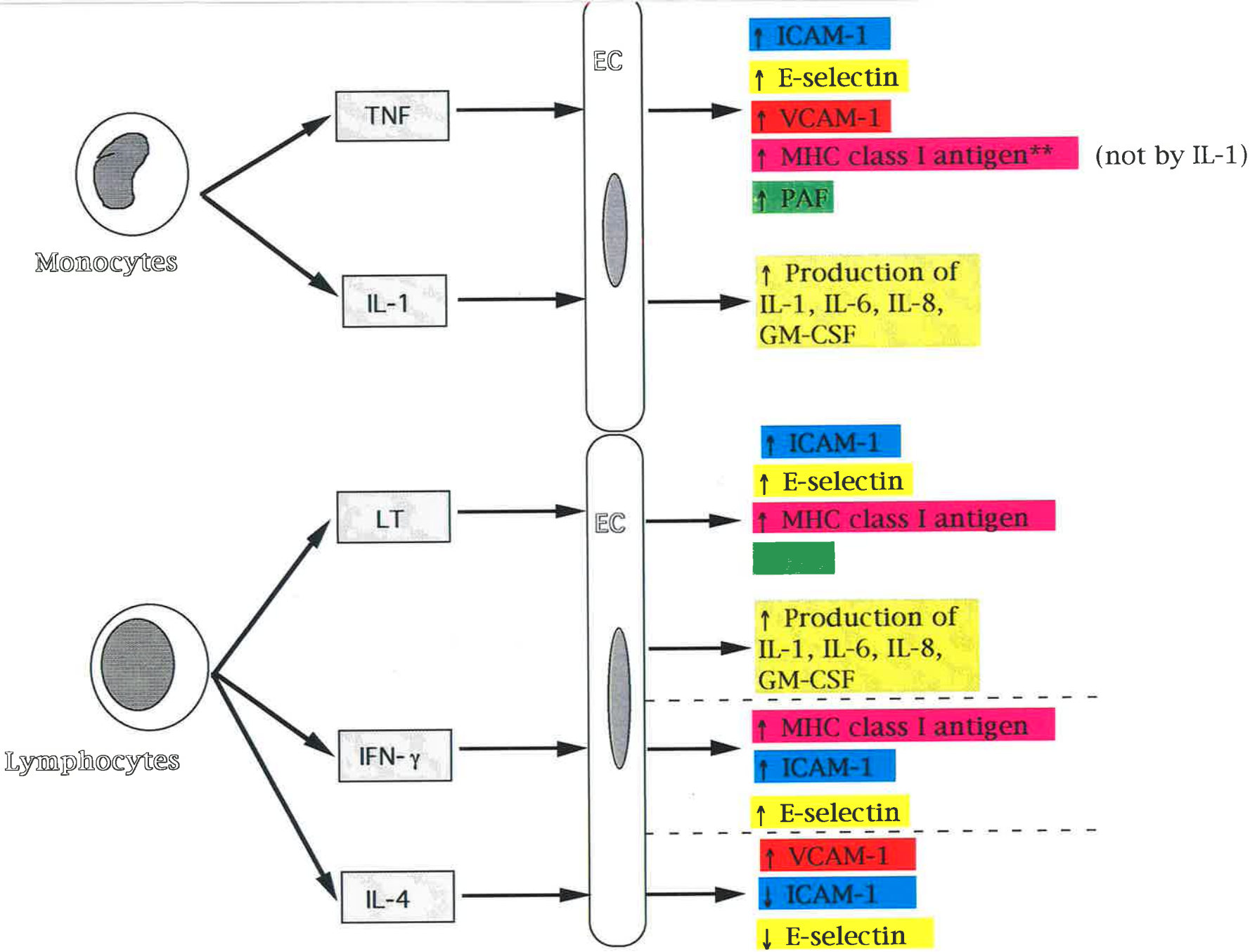


Fig.1.1 Effects of cytokines on the expression of endothelial cell adhesion molecules for the adhesion of leulocytes.

1.4.2 E-selectin

As a member of the selectin family, E-selectin has an N-terminal lectin-like domain followed by an epidermal growth factor-like domain and complement binding protein-like domain. E-selectin is not expressed on the surface of resting endothelial cells. However, its expression can be induced by the stimulation of endothelial cells with TNF, LT, IL-1 and LPS. IFN-γ can stabilize the expression of E-selectin without prolonging the period of synthesis. The ligand of E-selectin has been proposed to be the sialylated form of the Lewis X glycan presented on cell surface glycoproteins and lipids of neutrophils, monocytes and some tumor cells. Thus, the expression of E-selectin is involved in leukocyte adhesion to vascular cells (Pober et al, 1991). IL-4 and TGF-β have been found to inhibit the TNF- and IL-1-induced E-selectin expression which is associated with inhibition of neutrophil and a subset of lymphocyte adhesion to endothelial cells (Thornhill et al, 1990; Gamble et al, 1993). Infection of endothelial cells by cytomegalovirus induced the expression of E-selectin which enhanced neutrophil adhesion to infected endothelial cell monolayers (Span et al, 1991)

1.4.3 P-selectin (CD62)

P-selectin is another member of the selectin family. Like E-selectin, P-selectin is not normally expressed on the endothelial cell surface, but stored in the Weibel-Palade bodies. Shortly after activation of endothelial cells with thrombin, histamine, PAF and phorbol esters, P-selectin is rapidly translocated to the plasma membrane of endothelial cells where it mediates adhesion of monocytes, neutrophils, and

lymphocytes to the endothelial cells (Pober et al, 1991; Albelda, 1991). The ligand for P-selectin remains to be clearly defined.

1.4.4 Vascular cell adhesion molecule-1 (VCAM-1)

VCAM-1 is another member of immunoglobulin superfamily. It has six repeated immunoglobulin-like extracellular domains compared to ICAM-1 and ICAM-2 which have 5 and 2 immunoglobulin-like domains respectively. VCAM-1 is expressed much less than ICAM-1 on resting endothelial cells. This molecule becomes clearly apparent on the surface of endothelial cells after activation by TNF, IL-1, LPS and IL-4. etc. The ligand for VCAM-1 has been proposed to be VLA-4, a b_1 integrin molecule. The recognition and binding of VCAM-1 to VLA-4 contributes to the delayed recruitment of lymphocytes and monocytes to inflammatory foci (Fleming, 1991).

1.4.5 Integrin receptors

Endothelial cells line blood vessels with their abluminal surface to subendothelial matrix, and their luminal surface to blood. The cells synthesize and express integrin receptors on their abluminal surface as well as their apical surface (Conforti et al, 1992). The former receptors mediate attachment of endothelial cells to adjacent cells and substratum, which modulate endothelial cell proliferation, migration and permeability properties. On cultured endothelial cells, the integrin receptors for matrix proteins, such as fibronectin, laminin, collagens, fibrinogen, von Willebrand Factor and vitronectin have been identified (Conforti et al, 1989; Languino et al, 1989; Kramer et al, 1990; Cheresh et al, 1987; Dejana et al, 1988). The latter receptors cause

adhesion of plasma proteins and circulating cells, which may participate in thrombosis and inflammatory responses. The integrin receptor, $\alpha_v\beta$ was detected on the luminal surface of endothelial cells. It is the main receptor for vitronectin, but it can also promote endothelial cells to bind fibrinogen, thrombospondin, thrombin and von Willebrand Factor (Conforti et al, 1992). Many of these integrin receptors recognize the site encompassing a tripeptide RGD which is a common sequence in many matrix proteins and is suggested to play an important role in cell adhesion (Ruoslahti et al, 1986).

Human endothelial cells have been reported to synthesize and release fibronectin, collagen, laminin, heparan sulfate, vitronectin etc., into the bloodstream (Berge et al, 1992; Jaffe et al, 1988; Sage, 1984). These molecules constitute subendothelial matrix and can also be located onto cell surface via their receptors. Furthermore, heparan sulfate can interact with collagen and fibronectin both of which are of importance in cell adhesion (Heremans et al, 1990; Koliakos et al, 1989).

Klein et al (1992) reported that treatment of endothelial cells with proinflammatory mediators, TNF, IL-1 and LPS for 4h significantly reduced the cell-associated heparan sulfate and dermatan sulphate, which mediated the binding of endothelial cells to the surface proteins of *S. aureus* (Liang et al, 1992). Activated endothelial cells were found to completely lose their surface negative charge. Another study showed that TNF and IL-1 downregulated the expression of $\alpha_6\beta_1$ integrin (laminin receptor) by selectively depressing the expression of the α_6 subunit. This affected the adhesion of endothelial cells to laminin (Defilippi et al, 1992). The combined treatment of endothelial

cells with TNF and IFN-γ resulted in a 70% decrease of vitronectin receptor ($\alpha_v\beta_3$) by reducing the β_3 subunit synthesis (Defilippi et al, 1991).

The adhesion molecules are found not only on the surface of endothelial cells but also in serum as soluble factors. The soluble forms of ICAM-1, E/P-selectin and VCAM-1 have all been found in supernatants of endothelial cell cultures stimulated with cytokines. It has also been speculated that shedding of adhesion molecules may decrease the enhanced adhesion in activated endothelial cells. The soluble molecules may also act as competitive inhibitors to reduce ligation of circulating cells to endothelial cells (Pigott et al, 1992). The detailed function of these soluble molecules remains to be explored.

1.5 Microbial Adhesion to Host Tissues and Pathogenesis of Infection

Adhesion of microorganisms to host tissues is the first and critical step in pathogenicity of microbial infection (Beachey, 1981). Shortly after birth, microorganisms start to heavily colonize the skin and mucosal surfaces of the upper respiratory tract and the gastrointestinal tract (Beachey, 1980). While some organisms appear as indigenous parasites in a symbiotic balance with the host, other pathogenic microorganisms escape from the nonspecific host defences and the eradication by the soluble factors in the secretion. To produce infection, these pathogenic organisms must adhere to host tissues then colonize and invade tissues which is followed by damage of host

tissue, and possibly bacterial dissemination. Therefore, attachment of microorganism to host tissue is a virulence factor in microbial infection.

1.5.1 The theory of bacterial adhesion: adhesin-receptor interaction

It is well appreciated that the attachment of bacteria to host tissues is a highly selective process. Beside bacterial tissue tropism, genetic and species specificity, an important consideration in bacterial-host interaction is the adhesin-receptor theory. In the early 1980s, Beachy suggested that once bacteria survive the nonspecific defences, the adhesins on the bacterial surface specifically bind to their complementary receptors on the host tissue surface in a lock and key manner (Beachey, 1981). It was evident that the adhesion of bacteria to host tissue was inhibited by the addition of adhesin or receptor analogues, by enzymes which specifically destroy the receptors or adhesins, and by antibodies against the adhesin or receptor components. Purified receptors or bacterial adhesins bind to their complementary components on the bacterial or host tissue surface, and block the adhesion of bacteria to host tissues. This theory may apply with the interaction of staphylococci to host tissues, such as epithelial cells and endothelial cells.

1.5.2 Interaction of S. aureus *with host tissue cells*

S. aureus is a highly virulent organism which causes a variety of severe infections in the skin (after trauma), soft tissues, joints, bones and in the cardiovascular and respiratory systems.

1.5.2.1 Adhesion of S. aureus *to connective tissue proteins.*

S. aureus has been shown to bind to several connective tissue proteins with high affinity. These connective tissue proteins including fibronectin, collagens, laminin, vitronectin, fibrinogens and heparan sulfate form the constituents of eukaryotic cell surfaces and of basement membranes (Herrmann et al, 1988; Chhatwal et al, 1987; Spezale et al, 1986; Switalski et al, 1983; Liang et al, 1992). Binding proteins for fibronectin, fibrinogen, collagen and heparan sulfate on the staphylococcal surface have also been identified (Boden et al, 1989; Ryden et al, 1982; Switalski et al, 1989; Liang et al, 1992).

1.5.2.2 Adhesion of S. aureus *to epithelial cells*

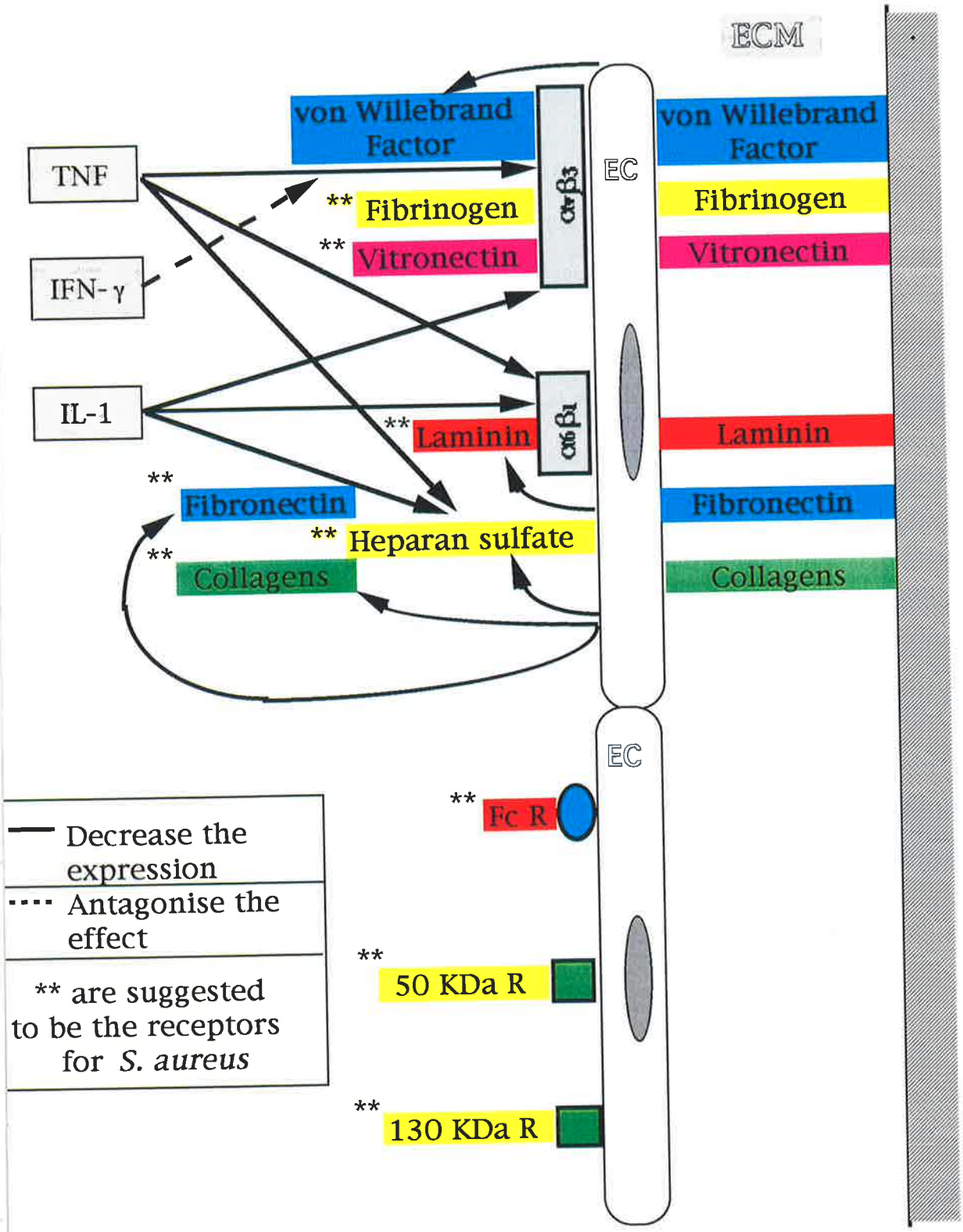
The adhesion of *S. aureus* to epithelial cells may occur on the skin and mucosal surfaces. Previous *in vitro* studies have shown that the adhesion of staphylococci to nasal epithelial cells was mediated by teichoic acid, a major cell well component of staphylococci. Treatment of epithelial cells with teichoic acid was shown to reduce by bacterial adhesion to epithelial cells (Aly et al, 1980). Aly et al (1977) demonstrated that heat treatment (80°C) of staphylococci only slightly decreased their adhesion to epithelial cells. They also showed that attachment of staphylococci to nasal cells was determined by the source of host cells. The affinity of staphylococci was greater for cells from staphylococcal carriers than noncarriers (Aly et al, 1977). Bacterial adhesion was significantly increased with the keratinization of epithelial cells, and in patients with atopic dermatitis, which is characterised by the hyperkeratinization and dryness of the skin (Bibel et al, 1982). Two types of staphylococcal receptors on the nasal

epithelial cells have been proposed to mediate staphylococcal adhesion. One is expressed on epithelial cells which are not fully keratinized and was not blocked by staphylococcal teichoic acid. The other is presented only on keratinized cells and was secondary and supplementary to the above, and is blocked by teichoic acid. The increased adhesion of *S.aureus* to epithelial cells in patients with atopic dermatitis has been suggested to be associated with new expression of teichoic acid inhibitable receptors or induced expression of early type receptor for *S. aureus* , which was responsible for the high bacterial counts (Aly et al, 1982).

1.5.2.3 Adhesion of S. aureus *to human endothelial cells*

The interaction of staphylococcus with endothelial cells has been well investigated in the last decade (Fig 1.2). Staphylococcus is the predominant pathogen causing acute endocarditis. The infection usually occurs at the normal cardiac valves resulting in the rapid destruction of the infected valves, metastatic bacterial dissemination, and high morbidity and mortality.

In 1985, Ogawa et al developed an *in vitro* model of the infection of human endothelial cells with staphylococci. Their studies showed that the infection of endothelial cells with *S. aureus* occurs in a series of steps: bacterial adhesion, endocytosis of bacteria, bacterial intracellular proliferation, and disruption of endothelial cells (Ogawa et al, 1985; Lowy, 1988, a,b). The adhesion of *S. aureus* to human endothelial cells was dependent on the interaction time, bacterial inocula and endothelial cell number (Ogawa et al, 1985; Vercelotti et al, 1984).



g. 1.2 Modulation of endothelial cell integrin receptors during inflammation
: receptor. EC: endothelial cells. ECM: subendothelial extracellular matrix.

Tompkins et al (1992) reported that the adhesion of *S. aureus* to endothelial cells was significantly increased when the staphylococci used were harvested from the exponential phase growth. The increased adhesion was related to increased expression of a group of staphylococcal binding proteins on the bacterial surface. These staphylococcal binding proteins were shown to be trypsin sensitive and of molecular sizes of 30, 55-57, 70, 85 KDa. Treatment of endothelial cells with protein A, another staphylococcal cell wall component, failed to reduce the adhesion of *S. aureus* to endothelial cells.

Infection of staphylococci also modulates the function of endothelial cells. It was found that there was an induction of the expression of Fc receptors on the endothelial cells following the staphylococcal infection (Bengualid et al, 1990). The Fc receptors were not detected on uninfected endothelial cells. The increased Fc receptor expression was dependent on the bacterial inoculum and phagocytosis of bacteria. It has been suggested that the induced Fc receptors may contribute to the development of vasculitis during the staphylococcal-endovascular infection. Tompkins et al (1990) isolated an endothelial membrane protein which could bind *S. aureus*. This molecule was synthesized and expressed on the endothelial cell surface, and was shown to be trypsin sensitive and periodate insensitive with a molecular mass of 50 KDa. Addition of this purified protein significantly blocked the adhesion of *S. aureus* to endothelial cells by binding to staphylococci. The study with porcine cardiac endothelial cells showed that an 130 kDa glycoprotein partly mediated this specific binding of *S. aureus* (Johnson, 1993).

Another approach to investigating the staphylococcal infection of endothelial cells is to focus on the serum factors which promote the interaction. Some soluble macromolecules such as fibrinogen, fibronectin and vitronectin are found in the plasma and can be deposited on the endothelial surface by their specific receptors (Cheresh et al, 1987). Cheung et al (1991) suggested that plasma fibrinogen which accumulates on endothelial cell surfaces acts as a bridging molecule mediating the *S. aureus* adhesion to endothelial cells. However, they failed to show that other macromolecules, such as vitronectin and fibronectin, could play the same role.

It was also shown that heparitinase was able to upregulate the adhesion of *S. aureus* to endothelial cells. In contrast, acidic fibroblast growth factor (aFGF) and concanavalin A decreased the bacterial adhesion (Lowy et al, 1988; Johnson, 1993).

1.6 Circulating Cytokine Levels and Pathophysiology

There are a number of clinical reports which have shown that elevated serum cytokine levels occur in the patients suffering from a variety of diseases. For example, high serum level of TNF, IL-1 and IL-6 have been found in the case of cerebral malaria and also in sepsis, burn injury and trauma (Stevenson et al, 1990; Billiau et al, 1991; Durum et al, 1990). High TNF and IL-8 levels have been found in the meningeal inflammation caused by meningococci (Ramilo et al, 1990; Girardin, 1992; Arditi et al, 1990; Halstensen et al, 1993). The concentration of G-CSF has been found to be higher in the elderly with respiratory and urinary tract infections (Kawakami et al, 1990). The concentration of

serum TNF has been found to be elevated in children with acute lower respiratory tract infections (Nohynek et al, 1991). In addition, the cytokines, TNF, IL-1β, IL-6 and IL-8 all have been detected in high concentration in patients with sepsis and septic shock (Billiau et al, 1991). The level of circulating cytokines was found to correlate with the severity of the disease. The groups of patients with high serum cytokine levels were usually shown to have high mortality. This is why it has been proposed that the administration of cytokine monoclonal antibodies may improve the outcome of these diseases.

1.7 Concluding Remarks

Bacterial adhesion to the surface of tissues such as endothelium is vital to the pathogenicity of infections. The interaction of the bacteria with the tissue cells occurs via adhesin-receptor interactions which still remain to be clearly defined. Since cytokines can modulate the expression of tissue surface antigens and the mediators are produced during bacterial infections, they could play a central role in the bacterial-host tissue cell interactions. In this study we have addressed this question by investigating the effects of a group of cytokines on adhesion of *S. aureus* to human endothelial cells. These effects on bacterial adhesion were compared to effects on neutrophil adherence to similarly treated endothelial cells.

1.8 Specific Aims

1) To develop an *in vitro* assay to measure adhesion of *S. aureus* to

human umbilical vein endothelial cells.

2) To characterize the effects of cytokines on adhesion of *S. aureus* to endothelial cells.
3) To compare the effects of cytokines on adhesion of *S. aureus* with their effect on adhesion of neutrophils.
4) To determine the effect of different combinations of cytokines on adhesion of *S. aureus* or neutrophils to cytokine-treated endothelial cells.
5) To study the mechanism of the effects of cytokines on adhesion of *S. aureus* to endothelial cells.

1.9 Hypothesis

During the course of a bacterial infection, cytokines are produced which act on endothelial cells to alter their adhesion properties for *Staphylococcus aureus*.

CHAPTER 2

MATERIALS AND METHODS

2.1 Preparation and Culture of Human Umbilical Vein Endothelial Cells (HUVEC)

Endothelial cells were prepared from human umbilical cord veins by a modified method of Jaffe et al (1973). Following delivery, umbilical cords were immediately stored at 4°C in Hanks' Balanced Salt Solution (HBSS) supplemented with Streptomycin (50μg/ml) and Penicillin (50IU/ml). The cords were processed within 12h after delivery under sterile conditions. Firstly the cords were inspected and sections of the cords with clamp marks or holes were excised. After washing the lumen with 50 ml of warm HBSS, the ends of the cords were cannulated with 16 gauge blunt needles each connected to a syringe. Then the needles were secured by clamping the ends of the cords with clamps. The lumen of each vein was perfused with 0.1% collagenase in HBSS (PH=7.2, type VIII, Sigma) which was prewarmed to 37°C and millipore filtered (0.2μm filter, Sartorius, Gottingen, Germany) (Fig. 2.1). After 20 min digestion, the cell-enriched perfusate was collected and the lumen was perfused with another 50 ml of HBSS and the second perfusate also collected. HUVEC suspensions from 2-3 cords were pooled, centrifuged at 600g for 5 min and then washed twice with HBSS. Then the HUVEC were resuspended in endothelium complete medium.

HUVEC were cultured in 75cm^2 culture flask (Corning, New York, USA) at 37°C, 5% CO_2 incubator. After overnight incubation, the contaminating cells were removed by rinsing the flask twice with HBSS. Examination of endothelial cells by microscopy under phase contrast showed that the cells had the typical cobblestone morphology. The culture medium was changed every 2-3 days and endothelial cells usually became confluent

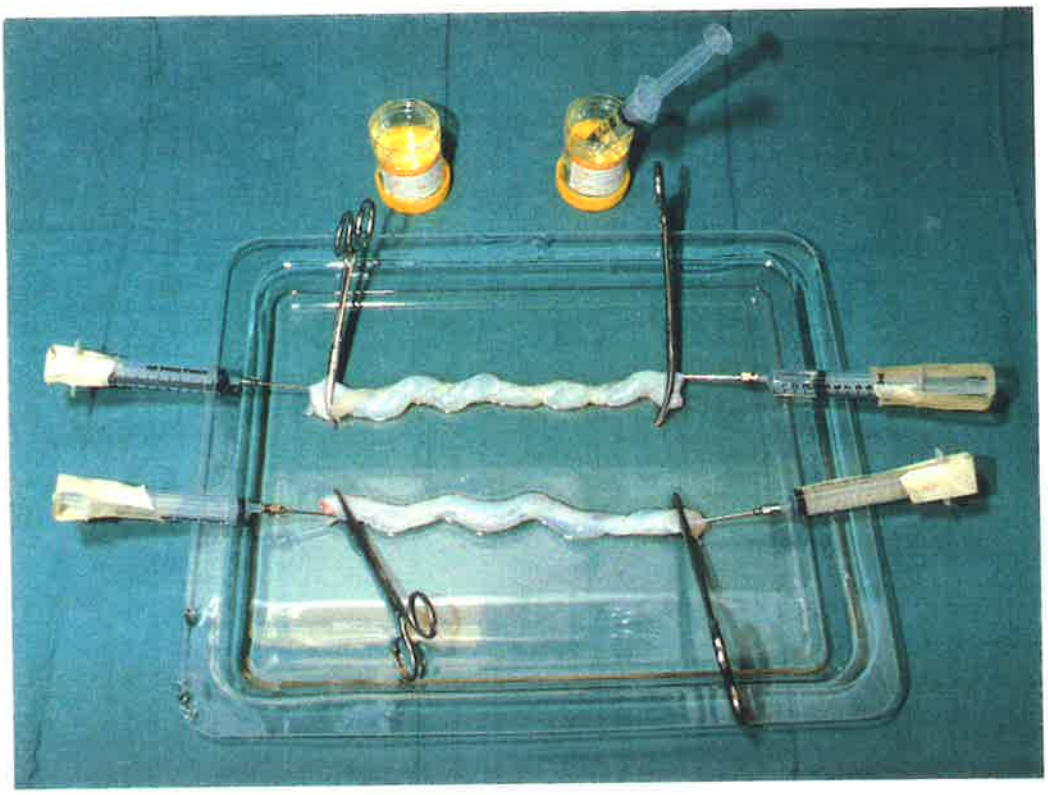


Fig. 2.1 Photograph of the procedure for preparing HUVEC from umbilical cords

after 3-4 days of culture (Fig. 2.2). HUVEC were passaged by treating the culture for 3-4 minutes at room temperature with 0.05% trypsin (ICN-Flow, Sydney) in calcium- and magnesium- free HBSS containing 0.02% EDTA. The detached endothelial cells were harvested, washed twice and then resuspended in the endothelium complete medium. The cells were either subcultured 1 in 2 into 75 cm^2 flasks or seeded into 96 flat bottom well of microtitre plates at the cell density of 5×10^4/well for measuring bacterial or neutrophil adhesion. All culture vessels were precoated with 1% gelatin for 2h at 37°C. In 96-well microtitre plates, a "tight" monolayer of endothelial cells was achieved two days following seeding. This was confirmed by staining the cells with 0.25% Rose Bengal (Sigma) in HBSS and examination under phase contrast microscopy. HUVEC were always used within their third passage. Endothelial cells were further characterized by the presence of Factor VIII related antigen which was detected by a peroxidase-conjugated rabbit anti-human von Willebrand Factor antibody (Dakopatts, Glostrup, Denmark) (see 2.7).

2.2 Culture and Labelling of *Staphylococcus aureus*

Staphylococcus aureus NCTC 6571 (protein A positive) was obtained from the National Collection of Type Culture (London) and maintained on blood agar by weekly subculture. The bacteria were labelled by a method adapted from that of Ogawa (Ogawa et al, 1985). The bacteria were inoculated into Todd Hewitt Broth (Oxoid, England) containing 20 μCi/ml ^{3}[H]-thymidine (Amersham, Sydney) and incubated at 37°C under agitation. After 18-20h incubation, the organisms were pelleted by centrifugation at 2400g for 5 min and washed twice in cold HBSS. Then the bacteria were resuspended in HBSS supplemented with 0.1% Human

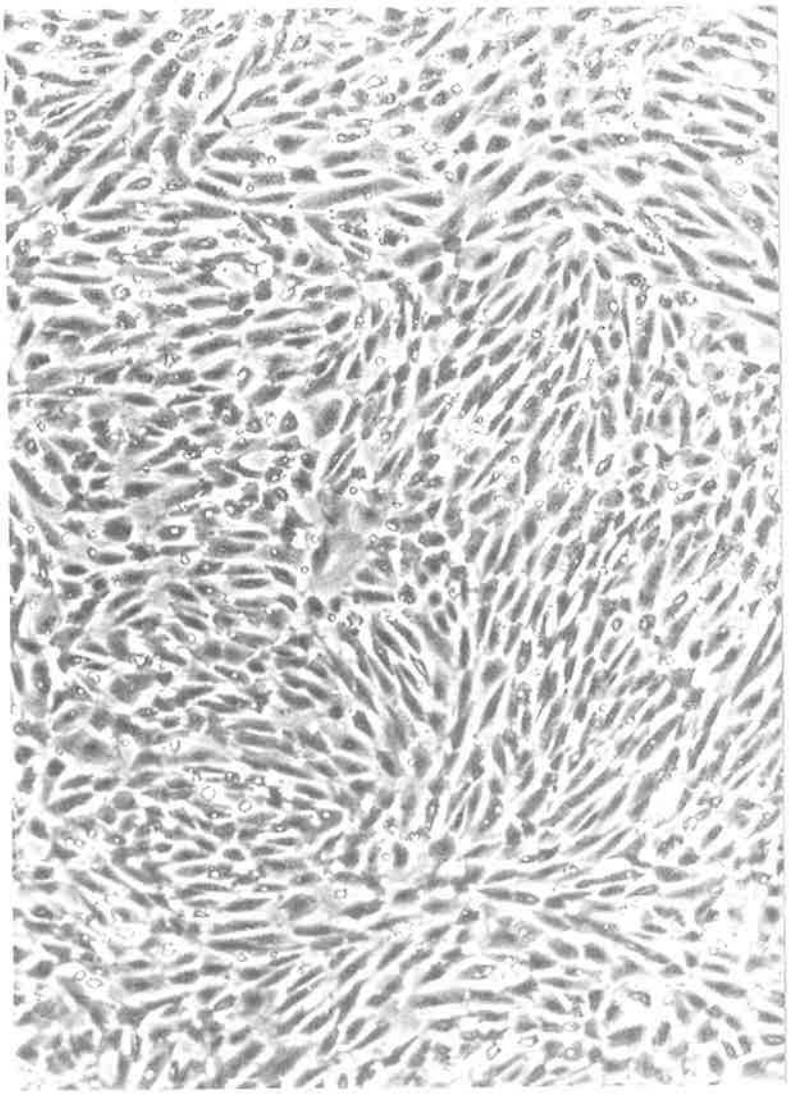


Fig. 2.2 Photomicrograph of a confluent culture of endothelial cells (passage 0) on day 3. Endothelial cells appear as homogeneous, closely apposed and polygonal population with a centrally located nucleus and obscure cell borders. (x 100 magnification)

Serum Albumin (HSA). Single bacterial suspensions were prepared by repeated aspiration through a 26 gauge needle. The bacterial concentration was adjusted to 1-1.8×10^9/ml following an estimate by counting in a haemocytometer chamber as well as spectrophotometrically using an ELISA reader (Dynatech MR 7000, Chantilly, VA, USA) at OD_{620} about 0.4-0.6 (Fig. 2.3). The bacterial inoculum was 1-3×10^9 CFU/ml verified by inoculating the bacteria onto blood agar plate for colony growth.

2.3 Preparation of Neutrophils and Mononuclear Leukocytes (MNL)

Human neutrophils were prepared from the peripheral blood of healthy donors as previously described (Ferrante et al, 1982). Blood was drawn into 25 IU/ml heparin-containing tubes. Six millilitres of blood was layered onto 4ml Hypaque-Ficoll medium (density 1.114) in clear polystyrene tubes (Disposable Product, Adelaide), and centrifuged at 200-400g for 30 min at RT. After centrifugation, the leukocytes were separated into two distinct bands and red blood cells sedimented to the bottom (Fig. 2.4). Neutrophils were collected from the lower leukocyte band and washed twice in M199 (Cell Image Ltd, Adelaide). Then neutrophils were resuspened in HBSS and adjusted to a concentration of 5×10^6 cells/ml. The preparation consisted of 96%-99% neutrophils, and the viability of neutrophils was >99% (Fig. 2.5).

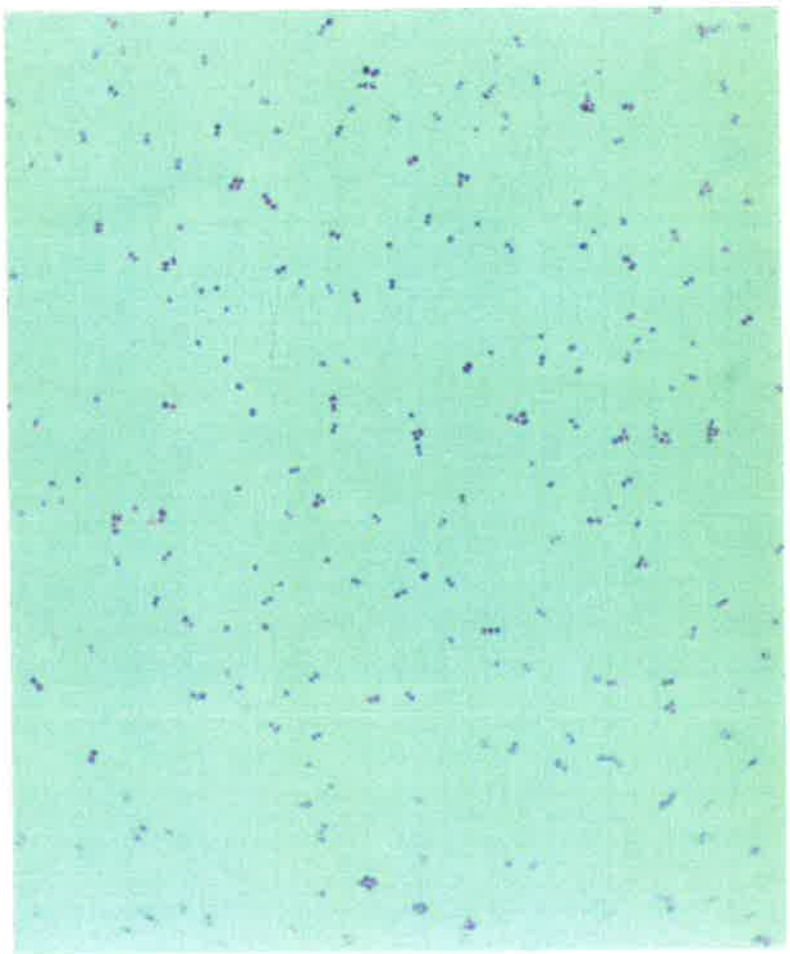


Fig. 2.3 Photomicrograph showing a typical Staphylococcus aureus (NCTC 6571) suspension labelled with ^{3}[H]-thymidine and staining positive for the gram stain (x 100 magnification).

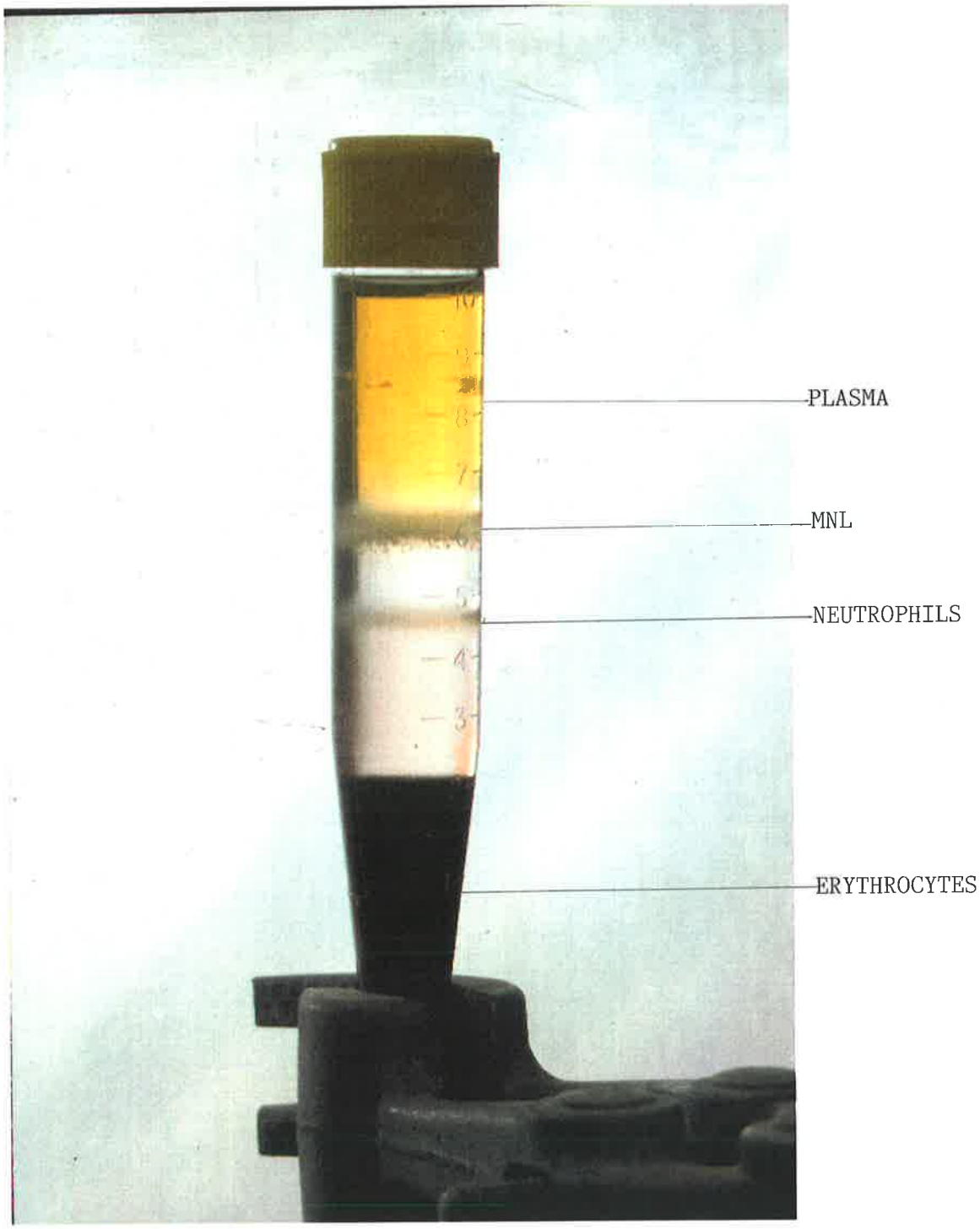


Fig. 2.4 Fractionation of leukocytes by the rapid single step method. Neutrophils were collected from the lower band of the leukocytes population.

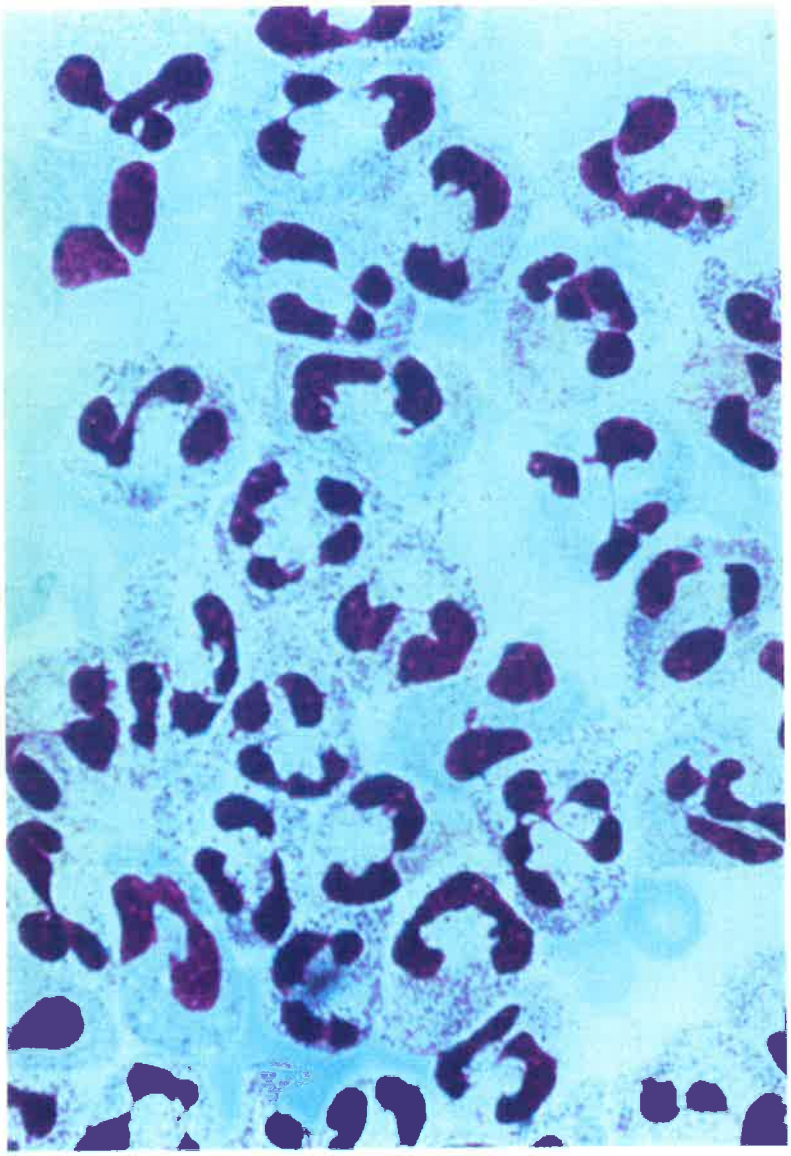


Fig. 2.5 A typical smear of the neutrophil preparation separated by the single step method

2.4 *Staphylococcus aureus* Adhesion Assay

The adhesion of *Staphylococcus aureus* to HUVEC was measured by the method of Ogawa (Ogawa et al, 1985). Confluent HUVEC in 96-well microtitre plates were treated with different cytokines or stimulators for the time indicated in Results. After the preincubation, the monolayer was washed with assay buffer (HBSS supplemented with 0.1% HSA) and brought to 4°C. To each well was added 50µl of assay buffer and 25µl of 1.5×10^9/ml ^{3}[H] thymidine labelled *S. aureus*.. The plate was centrifuged at 1300g for 5 min and placed at 4°C for 20 min on a rotation platform. The interaction of *S. aureus* with HUVEC was terminated by aspirating the nonadherent bacteria followed by two sequential washes of the monolayer with assay buffer. The nonadherent bacteria together with the two washes were pooled into the scintillation vials. The adherent bacteria and HUVEC were lysed by adding 200µl of 1M NaOH for 3h at RT. The lysates and the washes were transferred into scintillation vials and the radioactivity determined in a liquid scintillation spectrophotometer (Beckman LS 3801, California, USA). The adherence was calculated as :

$$\% \text{ Adherence} = \frac{\text{cpm of adherence to treated-HUVEC}}{\text{total cpm added}}$$

Previous studies (Ogawa et al, 1985; Vercellotti et al, 1984) have suggested that adhesion of *S. aureus* to human endothelial cells is dependent on the bacterial inocula and endothelial cell number. Under our experimental conditions, the adhesion of *S. aureus* to endothelial cells was shown to occur within minutes and reached plateau by 5 min (Fig. 2.6). Therefore, 20 min was chosen as the interaction time for adhesion of *S. aureus* to HUVEC.

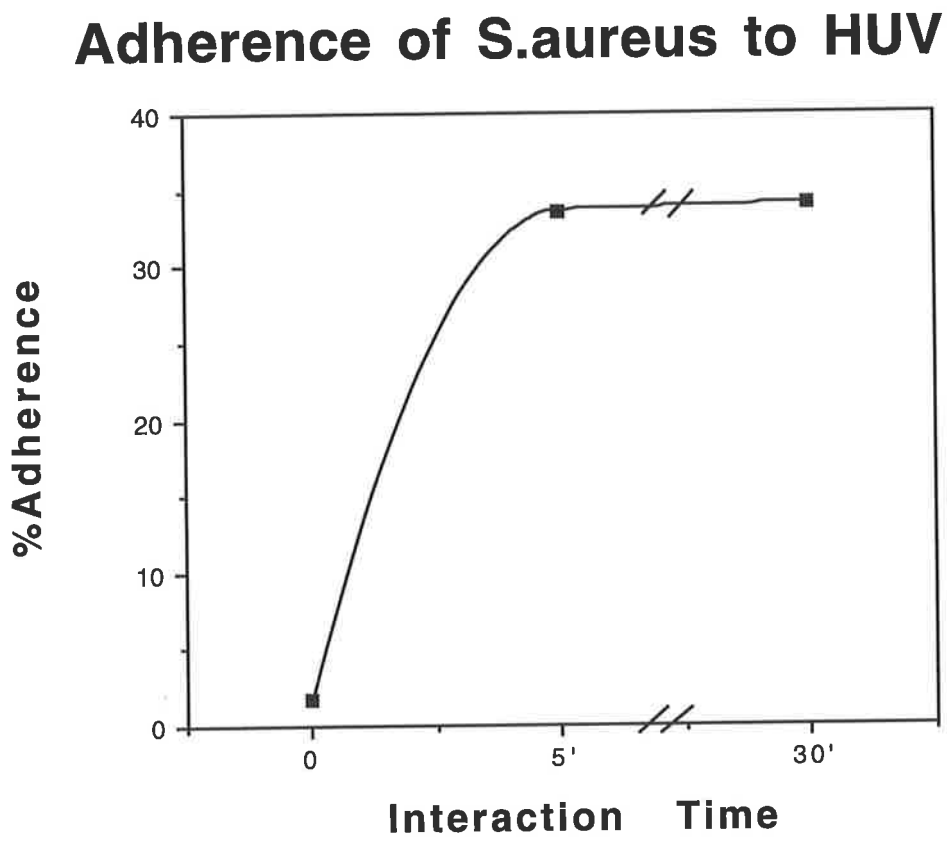


Fig. 2.6 The effect of adhesion time on the interaction of *S.aureus* to HUVEC. The adherence was measured by counting the ^{3}[H] thymidine labelled adherent bacteria. The results presented as the mean of three experiments.

2.5 Neutrophil Adhesion Assay

The assay was conducted according to the method of Gamble et al (1988). HUVEC monolayers were pretreated with the designated cytokines or mediators for the time stipulated in the Results. The wells were washed once with prewarmed HBSS and replenished with 100µl of assay buffer. Then 100µl of 5×10^6/ml freshly prepared neutrophils was added. The mixture was incubated at 37°C, in a 5% CO_2 air mixture and high humidity for 30 min with occasional agitation. At the end of the incubation, the nonadherent neutrophils were removed by two sequential washes in assay buffer. The adherent neutrophils and HUVEC were stained by 0.25% Rose Bengal in HBSS for 5 min at RT. The background stain was washed off with assay buffer. The stain taken up by HUVEC and neutrophils was released by treating the cells with Ethanol/PBS(1:1) for 50 min at RT. The reaction was measured at OD_{570} in an ELISA plate reader (Dynatech MR 7000, Chantilly VA, USA). The adherence of neutrophils was expressed as:

Adherence (OD_{570})

= (OD_{570} of neutrophils+HUVEC)-(OD_{570} of HUVEC alone)

2.6 Preparation of MNL Conditioned Medium

Mononuclear leukocyte (MNL) conditioned medium was prepared as described elsewhere (Ferrante et al, 1986). MNL were prepared from whole blood by centrifugation over Hypaque-Ficoll (see section 2.3). MNL

were harvested from the upper leukocyte band and washed twice with M199. The cells were adjusted to a concentration of 1×10^6/ml in RPMI 1640 supplemented with 2.5% heat-inactivated human AB serum in the presence or absence of Phytohaemagglutinin (PHA) (1μg/ml). After incubation at 37°C with 5% CO_2 for 24h, the cells were washed once with M199 and resuspended in fresh medium with 5% human AB serum and reincubated for a further 48h. After incubation the supernatant was collected, centrifuged at 600g for 10 min and filtered through 0.2μm filter. The conditioned medium was aliquoted and stored at -70°C.

2.7 Measurement of Cytokines

The cytokines in the conditioned medium were measured by an enzyme-linked immunosorbent assay (Ferrante et al, 1990). Microtitre plates (Dynatch Laboratories, Chantilly, VA, USA) were coated with the IgG fraction of goat anti-mouse IgG for 18-20h at 4°C. Then the murine monoclonal antibodies against either TNF, LT, IL-1β, IFN-γ or IL-2 were added to the respective wells. After incubation, at 37°C for 3h, the conditioned medium or control recombinant cytokine standards were added for 18-20h at 4°C. Then the rabbit anticytokine antiserum was added to the wells and plates incubated for 3h at 37°C. This was followed by the addition of sheep anti-rabbit immunoglobulin horseradish peroxidase conjugate (Silenus, Melbourne, Australia) and incubation for 3h at 37°C. The wells were washed and the substrate 2.2'-azino-di-[3-ethylbenzthiazoline sulphonate-(6)] (Boehringer Manheim, Sydney, Australia) was applied to develop the colour reaction for 45-60 min at 37°C. The plate was read at A_{414} in an ELISA reader (Dynatech,

Chantilly, VA, USA). Each assay was cytokine specific and could not detect another cytokine.

2.8 Von Willebrand Factor Staining for Endothelial Cells

Staining of endothelial cells with rabbit antiserum to human von Willebrand Factor was conducted essentially as described in the instruction of DAKOPATTS kit (Dakopatts, Glostrup, Denmark) with some modification. HUVEC were seeded into 6-well tissue culture tray or plastic slide chamber (Corning, New York, USA) which were precoated with 1% gelatin for 1h. After rinsing the wells with HBSS, methanol or PLP (periodate, lysine, paraformaldehyde) fixative was added to the wells for 20 min in the presence of 0.6% H_2O_2 to remove the endogenous peroxidase. Then 10% normal rabbit serum was added to the wells as plastic coating agent. After 20 min the excess serum was aspirated and peroxidase-conjugated rabbit anti-human von Willebrand Factor IgG was added (1/500 dilution). After 2h the wells were gently washed and DAB (Sigma) solution was applied for 5 min. Finally the cells were counterstained with either Mayer's Haemotoxylin, 0.25% Rose Bengal or Giemsa and mounted. It was found that endothelial cells were 99%-100% positive for Factor VIII related antigens (Fig. 2.7).

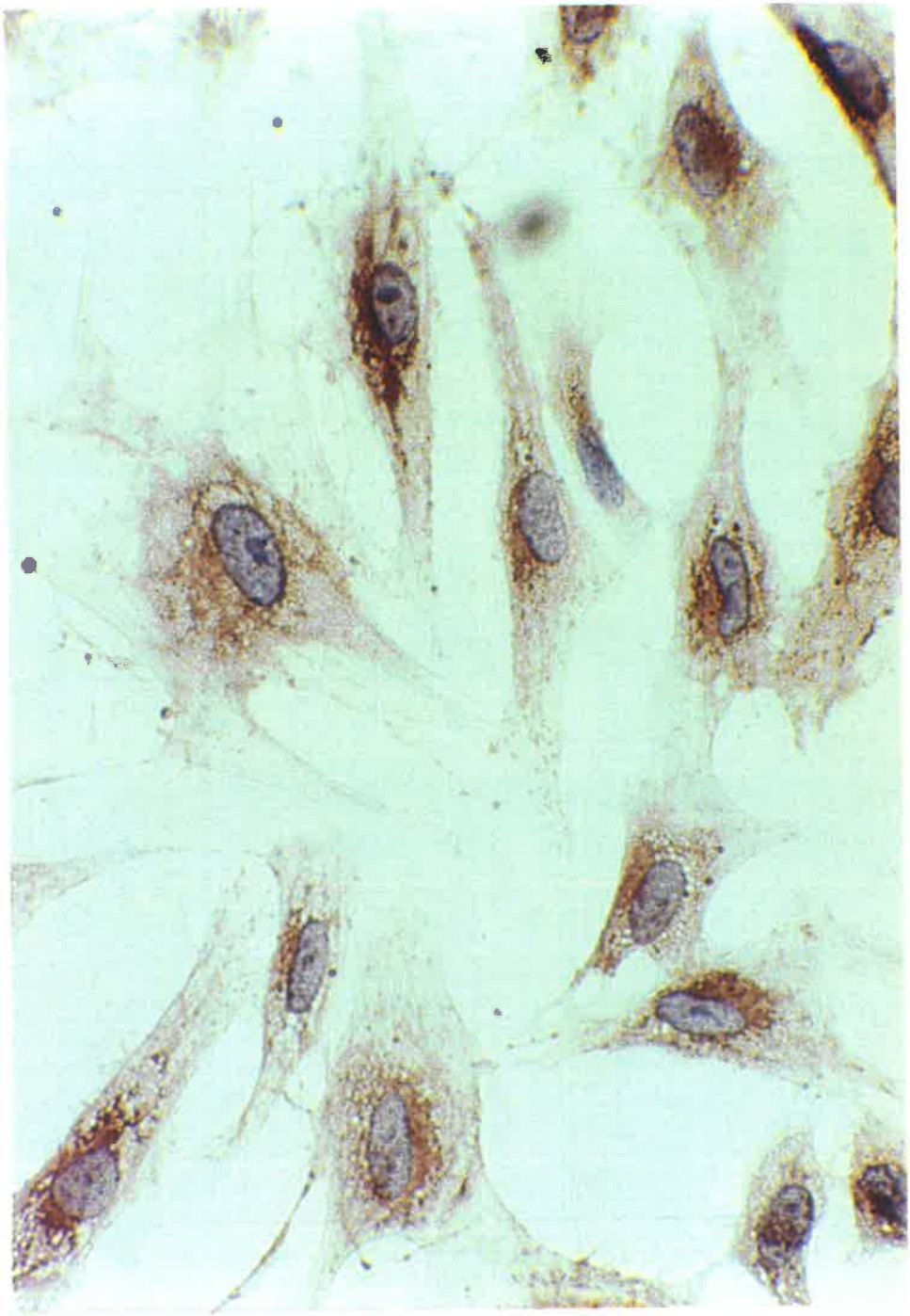


Fig. 2.7 Specific staining for endothelial cells. Subconfluent endothelial cells were stained with peroxidase conjugated rabbit anti-human Factor VIII antiserum. 99%-100% of endothelial cells were positive for the stain.

2.9 Materials

2.9.1 Media and buffers

The tissue culture media, RPMI 1640, HBSS and M199 were prepared by reconstitution of the powders (Muticell, Cytosystems, Sydney) in sterile pyrogen-free water. All batches were quality controlled before experimental use and no endotoxin could be detected by the Limulus Amebocyte Lysate (LAL) assay.

Endothelium complete medium consisted of RPMI 1640 with 20% heat-inactivated human AB serum, 0.9% TES (N-tris[Hydroxymethyl]-methyl-2-aminoethane-sulfonic acid), 0.4% $NaHCO_3$, 0.26% D-glucose, 4mM L-glutamine, 50μg/ml Streptomycin and 50U/ml Penicillin.

The assay buffer used in the experiments was HBSS without phenol red supplemented, with 0.1% human serum albumin.

The assay buffer and medium were all filtered through 0.2μm filter (Sartorius, Gottingen, Germany). The osmolarity and pH of the medium and buffer were adjusted to the physiological range.

2.9.2 Cytokines and their monoclonal antibodies

Human recombinant TNF, LT and IFN-γ were the gifts from Dr. GR Adolf (Genentech, Austria) with the specific activities of 5×10^7 U/mg, 1-2 $\times10^8$ U/mg and 2 $\times10^7$ U/mg respectively. The purities of these cytokine preparations were all >99%. The endotoxin contamination of these cytokine preparations was reported to be <1 EU/mg for TNF, <0.1ng/ml for LT and <1.25 EU/ml for IFN-γ. IL-1β and IL-4 were provided from Dr.

Y. Hirai, Otsuka Pharmaceutical Co Itd and Dr. K. Hama, Mimase Research Institute, Japan. IL-2 and some IL-1β were purchased from Boehringer Mannheim, Australia. IL-8 was obtained from Sandoz Forschungs Institut, Austria. The purities of IL-1β, IL-2, IL-4 and IL-8 preparations were all >99%. The specific activities for IL-1β, IL-2, IL-4, IL-8 were 2 $\times 10^7$ U/mg or 1×10^8 U/mg, 2 $\times 10^6$ U/mg, 1×10^6 U/mg, 0.6-1.7mg/ml respectively, and endotoxin contamination was <10 EU/ml for IL-1β, <10 EU/ml for IL-2 and <0.05 EU/ml for IL-8.

The monoclonal antibodies employed in the experiments were supplied by Dr. GR Adolf (anti-TNF and anti-LT) or Cistron Biotechnology, USA (anti-IL-1β). The murine monoclonal antibodies raised against the recombinant TNF, LT and IL-1β were immunoglobulin G subclass antibodies and purified from ascitic fluid, with the neutralizing activities of $>5 \times 10^5$ NU/ml for TNF, 500 NU/ml for IL-1β and 1mg for 1ng LT.

2.9.3 Other reagents

Cycloheximide, actinomycin D, platelet activating factor (PAF), 4β-phorbol-12-myristate-13-acetate (PMA) and staurosporine were obtained from Sigma Chemical Company (St. Louis, MO., USA). WEB 2086 was supplied by Boehringer Ingelheim (Ingelheim, FRG). Polymyxin B (PMB) was purchased from Grand Island Biological Company, NY, USA.

2.10 Presentation of Data and Statistics

The data shown in graphs are presented as three to six experiments, each experiment was conducted in triplicate. Each experiment was run with

endothelial cells from a different set of cords. In neutrophil studies each experiment was conducted with neutrophils from a different donor. All data have been calculated and expressed as a percentile of noncytokine treated endothelial control. The data are presented as mean±sem of 3-6 experiments. In all cases the data were analysed by the Student's t-test for paired data (Software: statwork, USA). $P<0.05$ was taken as the level of significance. The 95% confidence interval of the basal adherence of bacteria to endothelial cells was from 32.1% to 41.4%; the basal adherence of neutrophils to endothelial cells was from 0.088 to 0.142 optical density units.

2.11 Summary of Experimental Plan (Fig. 2.8)

The experimental procedures have been outlined in Fig. 2.8.

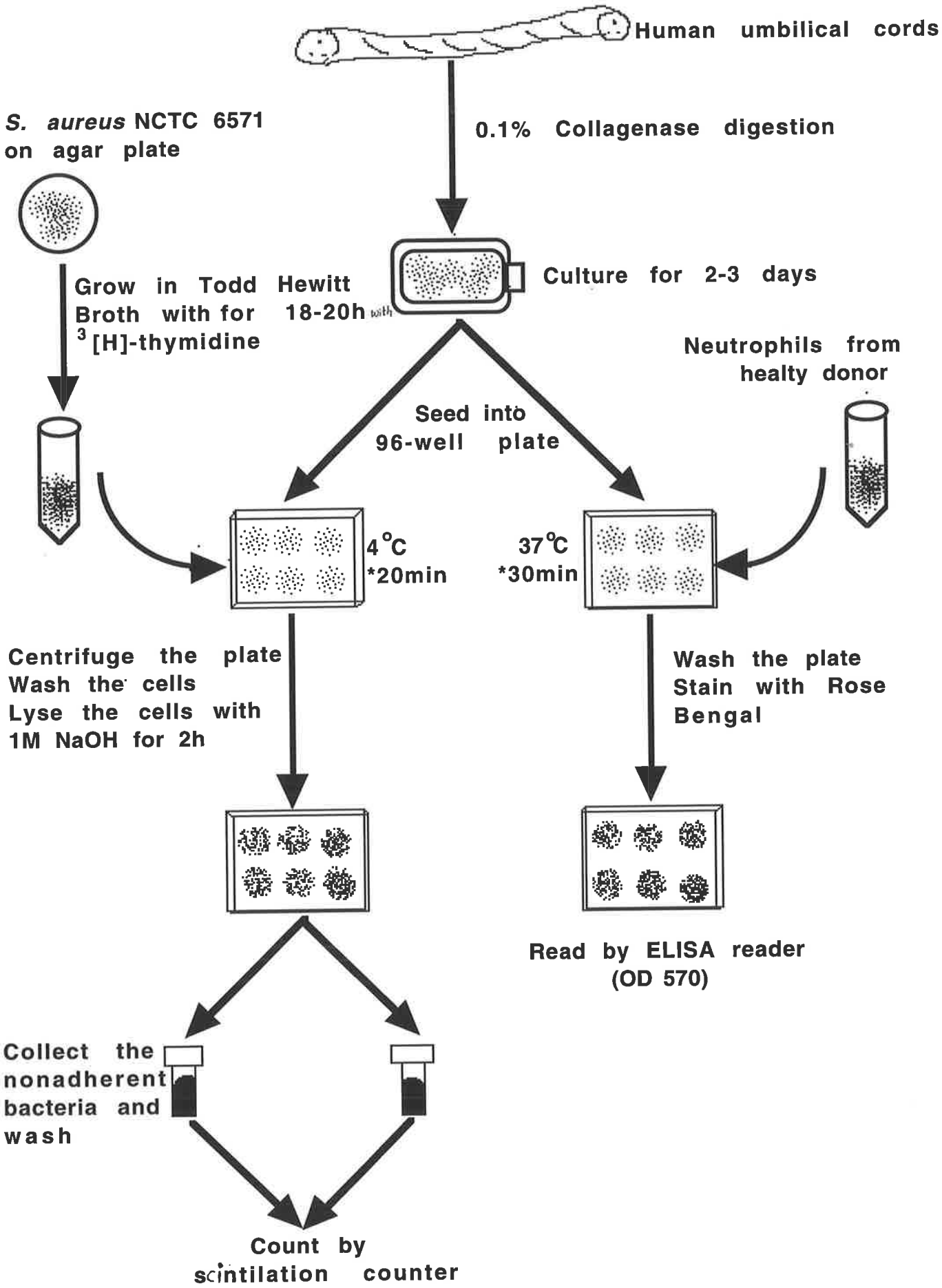


Fig 2.8 Summary of exprerimental plan

CHAPTER 3

ALTERATION OF

S. aureus ADHESION TO HUVEC

BY MONOKINES; TNF and IL-1β

3.1 Introduction

Monocytes and macrophages respond by producing a variety of functional mediators when stimulated with lymphokines or microbial antigens such as LPS. Two important molecules produced by these cells are TNF and IL-1 which are known to play important roles in regulating the inflammatory response (Chaplin et al, 1992).

TNF is a polypeptide cytokine which forms a trimer of a total molecular mass of 52 KDa in its physiologically active state. According to proposed models of TNF-receptor interaction, the TNF trimer interacts mutually with three receptor units on the surface of cells causing a localized aggregation of receptors (Sprang and Eck, 1992). Although the mechanism of TNF-induced signal transduction remains to be defined, it is evident that TNF-receptor interaction triggers a myriad of cellular events. Two distinct cellular receptors for TNF have been identified and cloned. These receptors have a conserved cysteine-rich repeat extracellular domain like that formed in nerve growth factor receptor. Their intracellular domains are quite distinct. The receptors have apparent molecular mass of 55 KDa and 75 KDa (Sprang and Eck, 1992). Although they are usually co-expressed on most tissues, the relative amounts of each receptor type can vary markedly. Endothelial cells have high affinity binding sites for TNF (Shalaby et al, 1985). The TNF binding is half-maximal at a TNF concentration of 100 pM and at saturation 1500 molecules are bound per endothelial cell (Nawroth et al, 1990). Two types of TNF receptors are expressed on the surface of endothelial cells (Espevik et al, 1990; Mackay et al, 1993).

IL-1 consists of two species of cytokine, IL-1α and IL-1β. Each IL-1 is synthesized as a pro-IL-1, 31 KDa precursor peptide and then cleaved by proteases to generate the mature form of 17 KDa peptides in the extracellular fluid. The amino acid homology between the two cytokines is about 25% (Dinarello, 1991). There are two types of IL-1 receptors. The type I is expressed on T cells and cells of mesenchymal lineage; and the type II is mainly expressed on B cell and cells of the myelomonocytic lineage. These two types of IL-1 receptor are glycoproteins with molecular mass of 80 KDa and 63-68 KDa respectively, which belong to Ig superfamily. Both IL-1α and IL-1β trigger the same receptors (Dinarello, 1991). Endothelial cells express the type I receptor for IL-1, but not an appreciable level of type II receptor (Colotta et al, 1993). It has also been found that the type II receptor of IL-1 which is released into biological fluids retains the binding ability for IL-1 and acts as a negative regulator to control the activity of IL-1 (Giri et al, 1990).

TNF and IL-1 show highly overlapping biological activities on endothelial cells (Table 3.1). Both of these monokines have been shown to increase endothelial procoagulant activity, induce the production of PGI_2, IL-1, IL-6, IL-8, CSFs, and enhance the expressions of ICAM-1, E-selectin and VCAM-1 which mediate adhesion of neutrophils, monocytes and lymphocytes (including NK cells) to endothelial cells. The attachment of cancer cells to endothelial cells was also shown to be augmented by stimulation of endothelial cells with TNF and IL-1. This latter effect was mediated by upregulating the synthesis and expression of integrin receptor on endothelial cells. It has also been

Table 3.1 The effects of TNF and IL-1 on endothelial cells

Effects	TNF	IL-1
Receptors for cytokine	+	+
Cytotoxicity	+/-	-
Arachidonate metabolism	+	+
Induction of PAF	+	+
Procoagulant activity	+	+
Plasminogen activator/plasminogen activator inhibitor	+	+
Hyperadhesiveness for leukocytes	+	+
Hyperadhesiveness for tumor cells	+	+
Induction of the expression of ICAM-1	+	+
Induction of the expression of E-selectin	+	+
Induction of the expression of VCAM-1	+	+
Modulation of the expression of Integrin	+	+
Production of IL-1	+	+
Production of IL-6	+	+
Production of IL-8	+	+
Production of GM-CSF	+	+
Production of PGI2/E2	+	+
Production of PDGF	+	+
Induction of the expression of HLA class I antigens	+	-
Morphological changes	+	+

+: active
+/-: marginally active
-: inactive

found that activation of HUVEC by TNF induced the production of IL-1 by these cells (Mantovani et al, 1989, Lafrenie et al, 1992).

Clinical studies have shown that IL-1 and TNF are elevated in the serum of patients suffering from septic shock, bacterial meningitis, burn injury, trauma, acute listeriosis and acute lower respiratory infection (Ramilo et al, 1990; Billiau et al, 1991; Nohynek et al, 1991; Vassalli, 1992; Dinarello, 1991). It was therefore of interest to see if TNF and IL-1 regulate bacterial adhesion to tissues since this process plays a vital role in promoting bacterial persistence and pathogenesis. In all studies adherence of neutrophils was used as a positive and comparative control for the activity of cytokines.

3.2 The Effect of Mononuclear Leukocyte (MNL) Conditioned Medium on Adhesion of *S. aureus* to HUVEC

Human mononuclear leukocytes (MNL) stimulated with the mitogen, phytohaemagglutinin (PHA), have been shown to produce high amounts of TNF and LT (Ferrante et al, 1990). As shown in Fig 3.1 we found this medium to contain 10198pg/ml of TNF, 4738pg/ml of LT, 7746pg/ml of IFN-γ, 85pg/ml of IL-2 and 406pg/ml of IL-1β. This conditioned medium was used as a source of cytokines expected to be produced during infection *in vivo*. The ability of this conditioned medium to alter endothelial cell properties in relation to bacterial adhesion was examined. HUVEC were treated for 4h with 20% (v/v) of the conditioned medium, then the cells were tested for bacterial binding. The results showed that treatment of endothelial cells with the conditioned medium reduced the degree of *S. aureus* adhesion

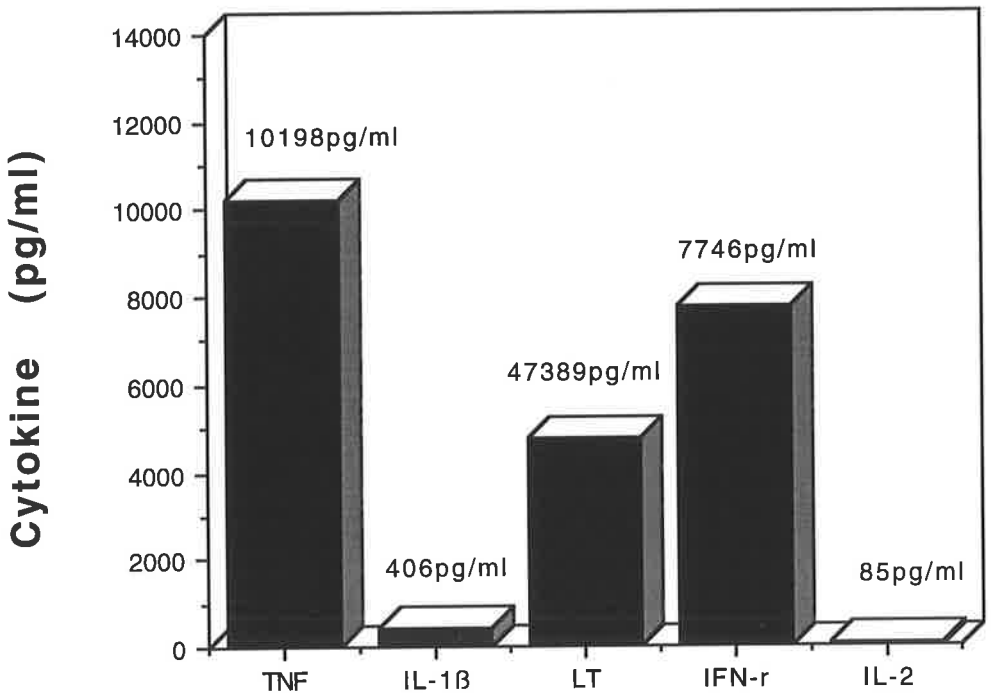


Fig. 3.1 The concentration of cytokines in PHA-stimulated MNL conditioned medium. The cytokine concentrations were measured by enzyme-linked immunosobent assay (ELISA).

(Fig. 3.2). The adhesion was reduced to 60% of control level. In contrast endothelial cells treated with this conditioned medium showed a moderate increase in neutrophil adhesion (Fig. 3.2). There was a four fold increase in neutrophil adherence. Thus it is evident the pretreatment of HUVEC with conditioned medium increases their ability to adhere neutrophils but depresses their binding of *S.aureus*.

3.3 The effect of TNF on adhesion of *S. aureus* to HUVEC

3.3.1 The effect of TNF

TNF was suspected to be one of the cytokines in the conditioned medium responsible for the decrease of *S. aureus* adhesion to HUVEC. Thus the effect of recombinant human TNF was examined in this system. HUVEC were pretreated for 4h at 37°C with 400U/ml of TNF and then tested for ability to adhere bacteria. The results showed that TNF caused a 25% decrease in bacterial adhesion ($P<0.05$) (Fig. 3.3). In association with this decrease there was a marked increase in neutrophil adhesion (Fig. 3.3).

TNF caused a decrease in bacterial adhesion in a concentration-related manner from 4-400U/ml (Fig. 3.4), the decrease was highest at 400U/ml. Enhancement of neutrophil adhesion was maximal at 4U/ml suggesting that there may be a difference in the concentration of TNF required to trigger the decrease in bacterial adherence and neutrophil adherence.

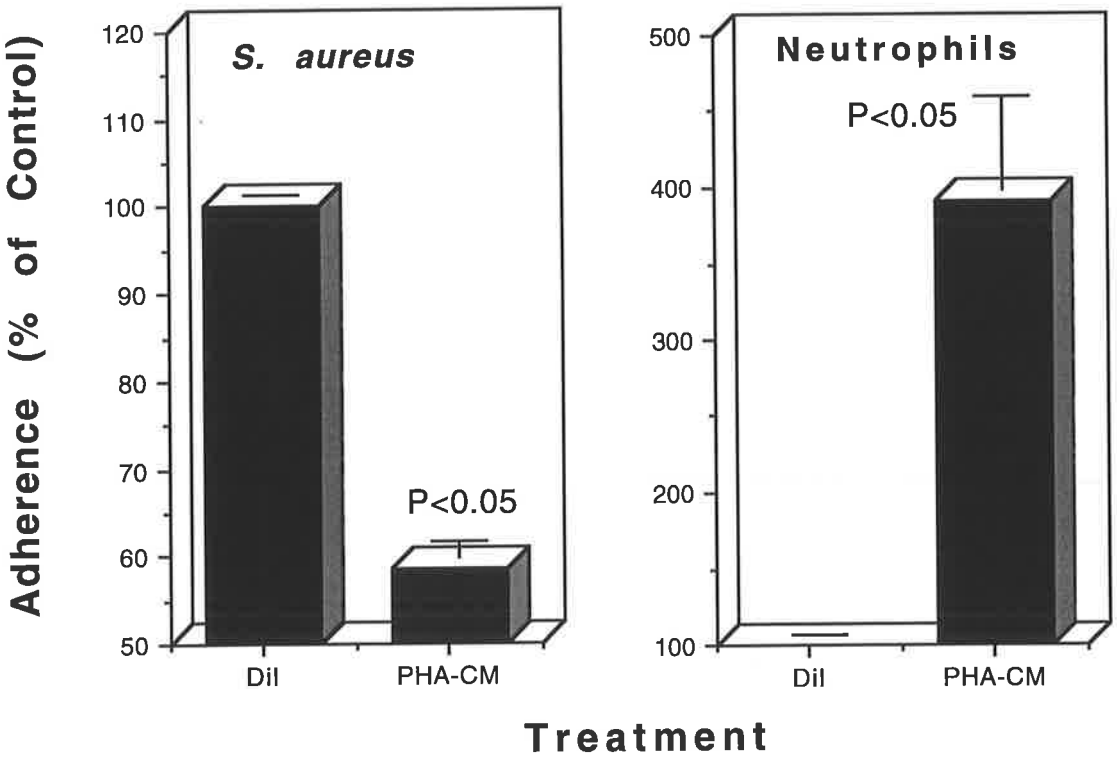


Fig. 3.2 The effect of PHA-stimulated MNL conditioned medium (PHA-CM) on adhesion of *S. aureus* or neutrophils to HUVEC. HUVEC were treated with 20% conditioned medium or diluent (dil) for 4h and washed before the addition of *S. aureus* or neutrophils. The results are presented as the mean±sem of three experiments, each conducted in triplicate. P value is the difference from the non-conditioned medium treatment.

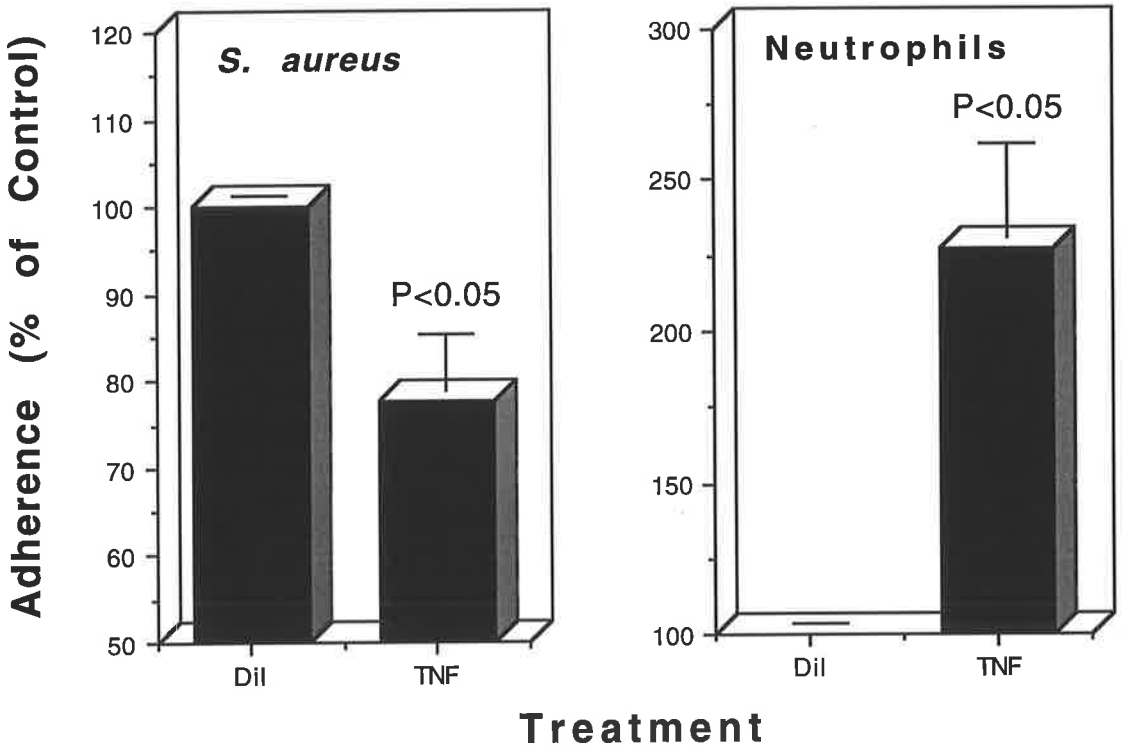


Fig. 3.3 The effect of TNF on either adhesion of *S. aureus* or neutrophils to HUVEC. HUVEC were treated with 400U/ml of TNF or diluent (dil) for 4h and washed before the addition of *S. aureus* or neutrophils. The results are presented as the mean±sem of five experiments, each conducted in triplicate. P value is the difference from the diluent treatment.

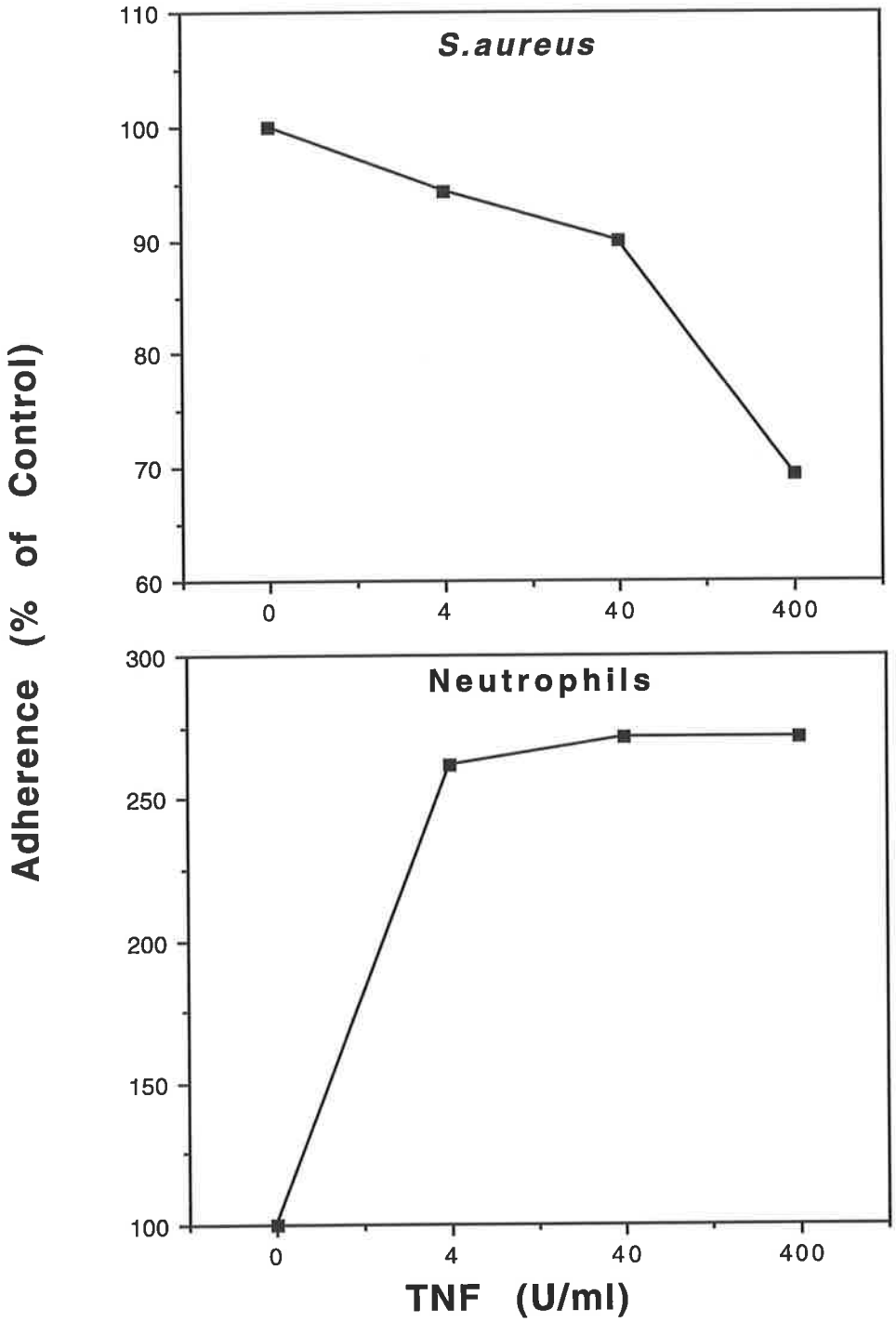


Fig. 3.4 The effect of varying concentrations of TNF on either adhesion of *S. aureus* or neutrophils to HUVEC. The HUVEC were treated with different concentrations of TNF for 4h, washed and then *S. aureus* or neutrophils were added. The results are the mean of three experiments each conducted in triplicate.

3.3.2 *Effect of heat-inactivation, treatment with polymyxin B (PMB) or anti-TNF monoclonal antibodies on the activity of the TNF preparation.*

To ensure that the effect of the TNF preparation was due to the cytokine and not a contaminant, such as LPS, the TNF preparation was treated in various ways to exclude this possibility.

Boiling the TNF preparation for 1h is known to inactivate the cytokine while leaving the LPS activity intact. The results showed that when the TNF preparation was boiled, it lost both the abilities to cause a decrease in bacterial adhesion as well as the increase in neutrophil adhesion to HUVEC (Fig. 3.5).

It has also been established that PMB abolishes the biological effects of LPS, but not the activity of TNF. When the TNF preparation was treated with 10μg/ml of PMB and added to the HUVEC, it was just as active as that not treated with PMB in inducing the decrease in bacterial adhesion and increase in neutrophil adhesion to HUVEC (Fig. 3.6).

Finally, the ability of an anti TNF neutralizing monoclonal antibody to prevent the alteration in the adhesion function of HUVEC was examined. The result showed that pretreating the TNF preparation with the monoclonal antibody reduced the TNF-induced decrease in bacterial adhesion and increase in neutrophil adhesion (Fig. 3.7). When considering the effects of the addition of the monoclonal antibody alone, it is apparent that treating the TNF-preparation with the anti

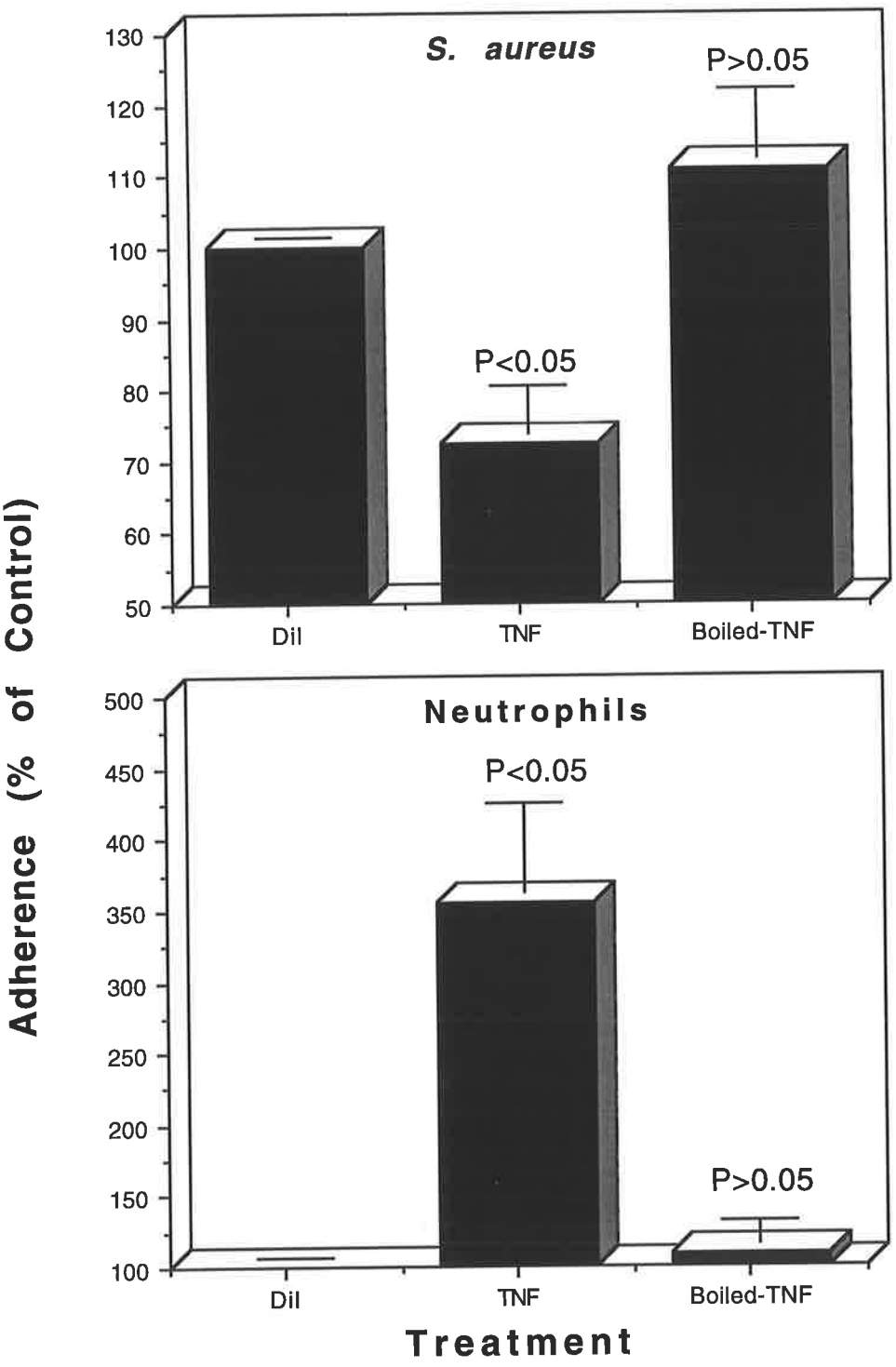


Fig. 3.5 The effect of heat-inactivation of TNF on its ability to alter the adhesion of *S. aureus* or neutrophils to HUVEC. The HUVEC were treated with 400U/ml of TNF or boiled TNF for 4h and then *S. aureus* or neutrophils were added. The results are the mean±sem of five experiments, each conducted in triplicate. P value is the difference from the diluent treatment.

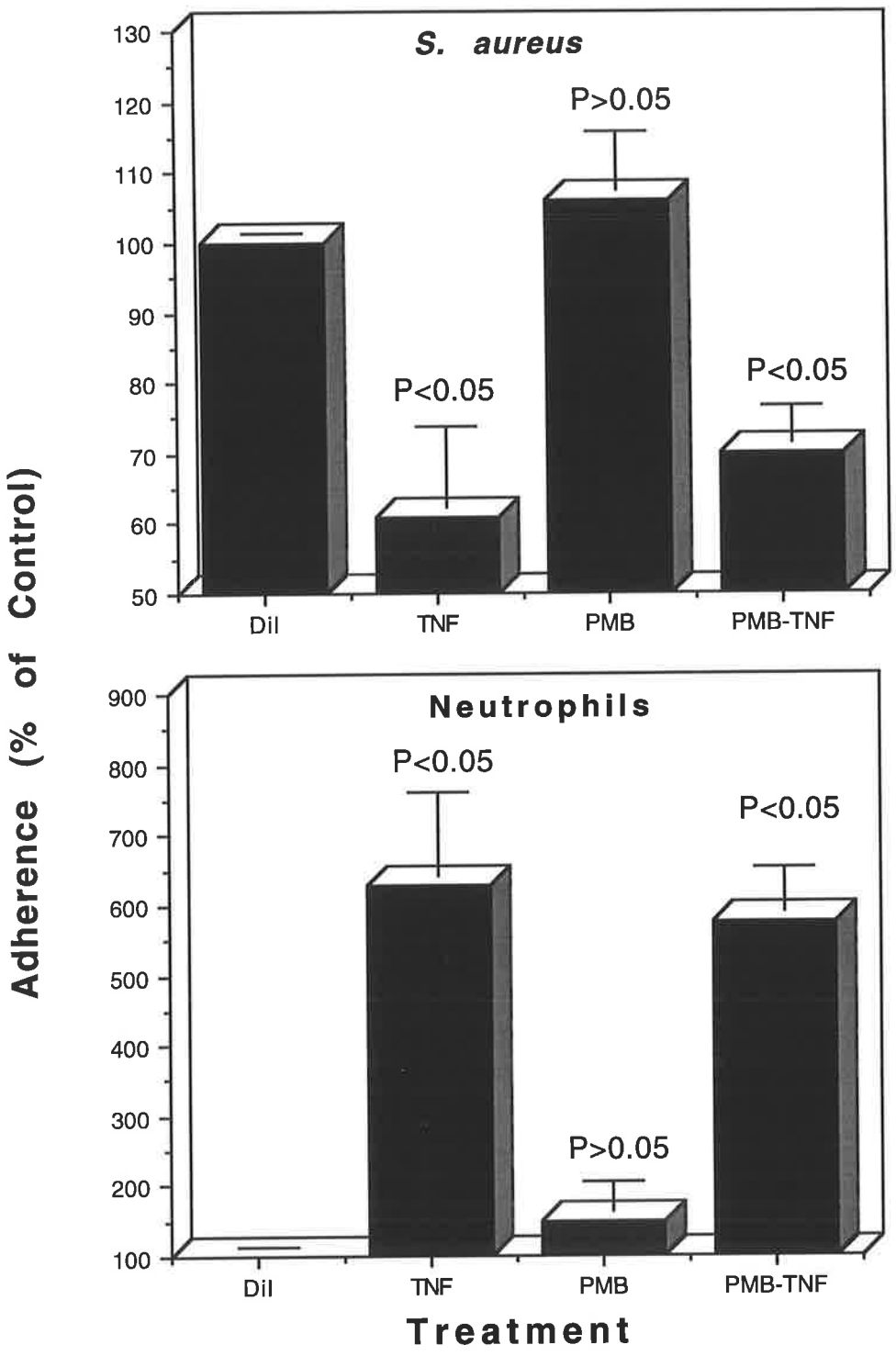


Fig. 3.6 The effect of PMB treatment of TNF preparation on its ability to alter the adhesion of *S. aureus* or neutrophils to HUVEC. The HUVEC were incubated with 400U/ml of TNF or PMB (10μg/ml) treated TNF for 4h, washed and then *S. aureus* or neutrophils were added. The results are presented as the mean±sem of three experiments, each conducted in triplicate. P value is the difference from the diluent treatment.

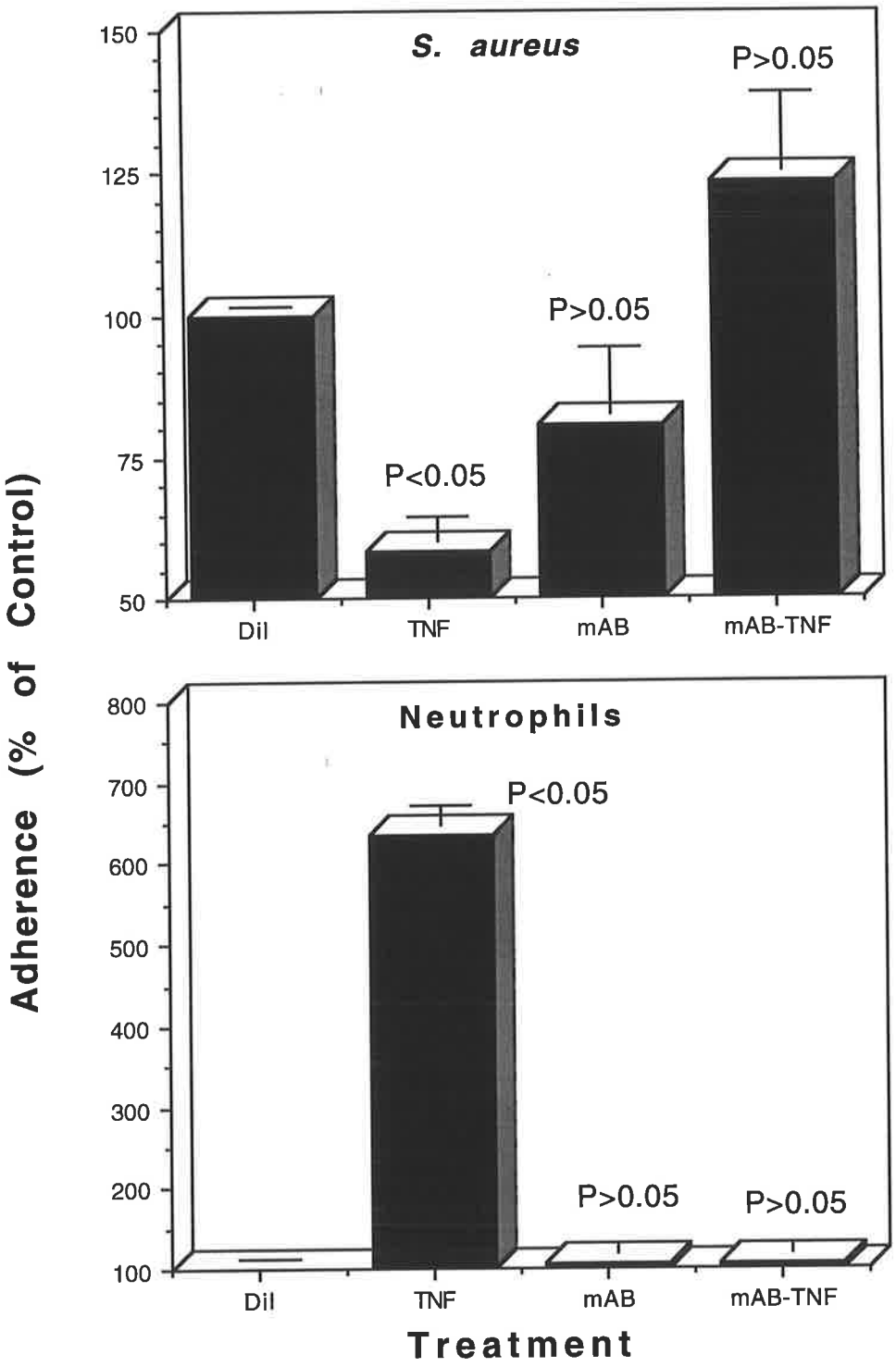


Fig. 3.7 The effect of anti-TNF monoclonal antibody (mAB) treatment of the TNF preparation on its ability to alter the adhesion of *S. aureus* or neutrophils to HUVEC. The TNF (400U/ml) preparation was treated with anti-TNF (1:50 dilution) for 30 min and then added to the HUVEC. After 4h incubation the endothelial monolayers were washed and then *S. aureus* or neutrophils were added. The results are the mean±sem of three experiments, each conducted in triplicate. P value is the difference from diluent treatment.

TNF antibody totally abolishs its effects on the decrease of bacterial adherence and increase of neutrophil adherence. (Fig. 3.7).

3.3.3 The effect of varying the TNF pretreatment time

HUVEC were treated with 400U/ml of TNF for varying times 0-4h and then examined for ability to adhere either bacteria or neutrophils. The results showed that the effects of TNF were dependent on preincubation time. The TNF-induced decrease in bacterial adhesion was clearly evident by 1h pretreatment, but was most marked with 4h pretreatment (Fig. 3.8). The increase in neutrophil adhesion induced by TNF was quite marked by 1h pretreament with further slight increases with increasing pretreatment time, up to 4h (Fig. 3.8).

3.3.4 The importance of protein and RNA synthesis on the TNF-induced effects on HUVEC.

Investigations were conducted to determine whether the effects of TNF on bacterial adhesion required the expression of new RNA and protein. The HUVEC were pretreated with 4μg/ml of actinomycin D, an inhibitor of RNA synthesis, and 10μg/ml of cycloheximide, an inhibitor of protein synthesis. The results showed that both actinomycin D and cycloheximide abolished the TNF-induced decrease in bacterial adhesion (Fig. 3.9 and Fig. 3.10). The TNF-induced enhancement of neutrophil adhesion was moderately decreased by cycloheximide and markedly decreased by actinomycin D (Fig. 3.9 and Fig. 3.10).

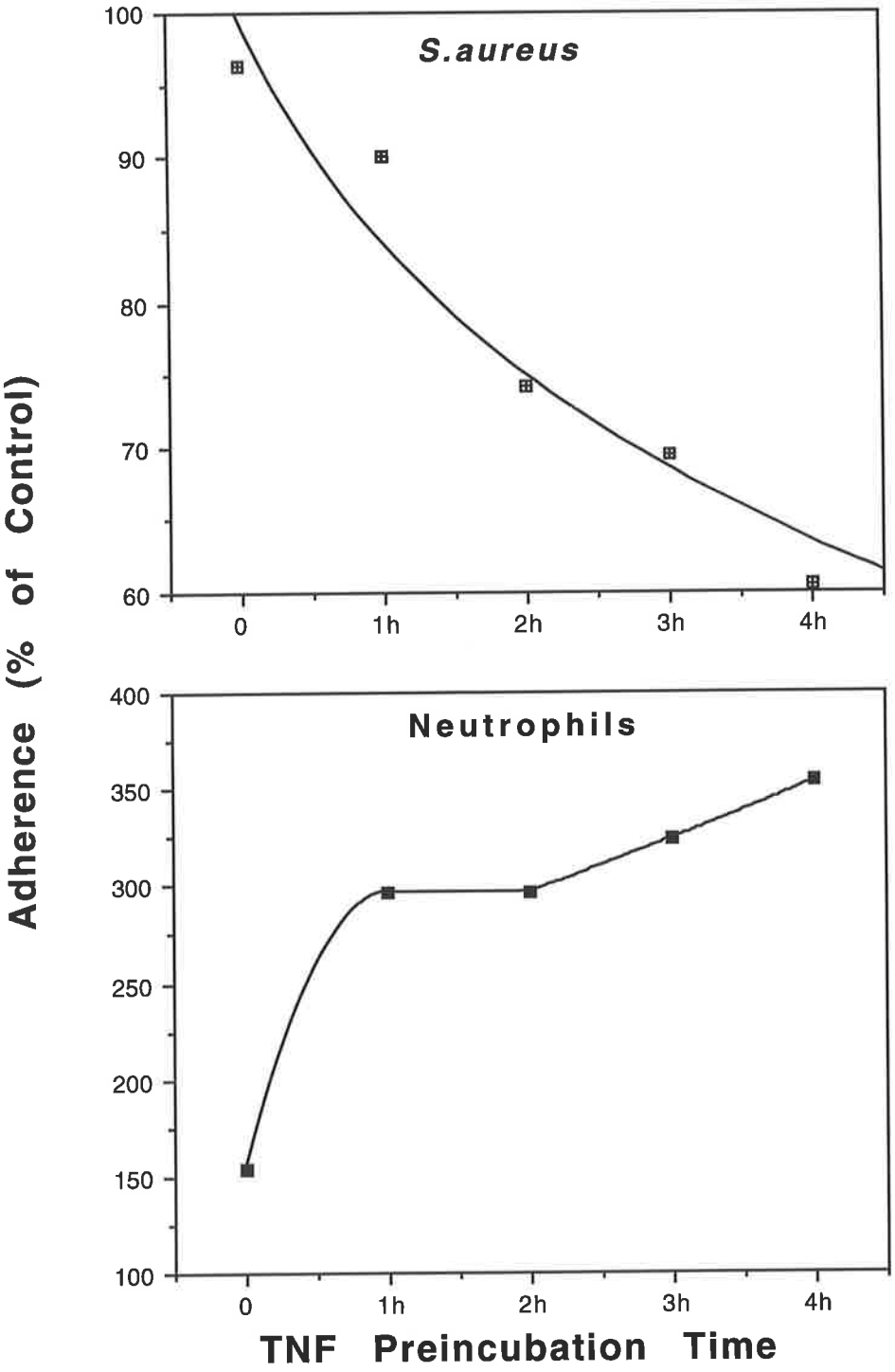


Fig. 3.8 The effect of preincubation time of TNF on adhesion of *S. aureus* or neutrophils to HUVEC. The HUVEC were treated with 400U/ml of TNF for 1h to 4h, washed and then *S. aureus* or neutrophils were added. The results are the mean of three experiments, each conducted in triplicate.

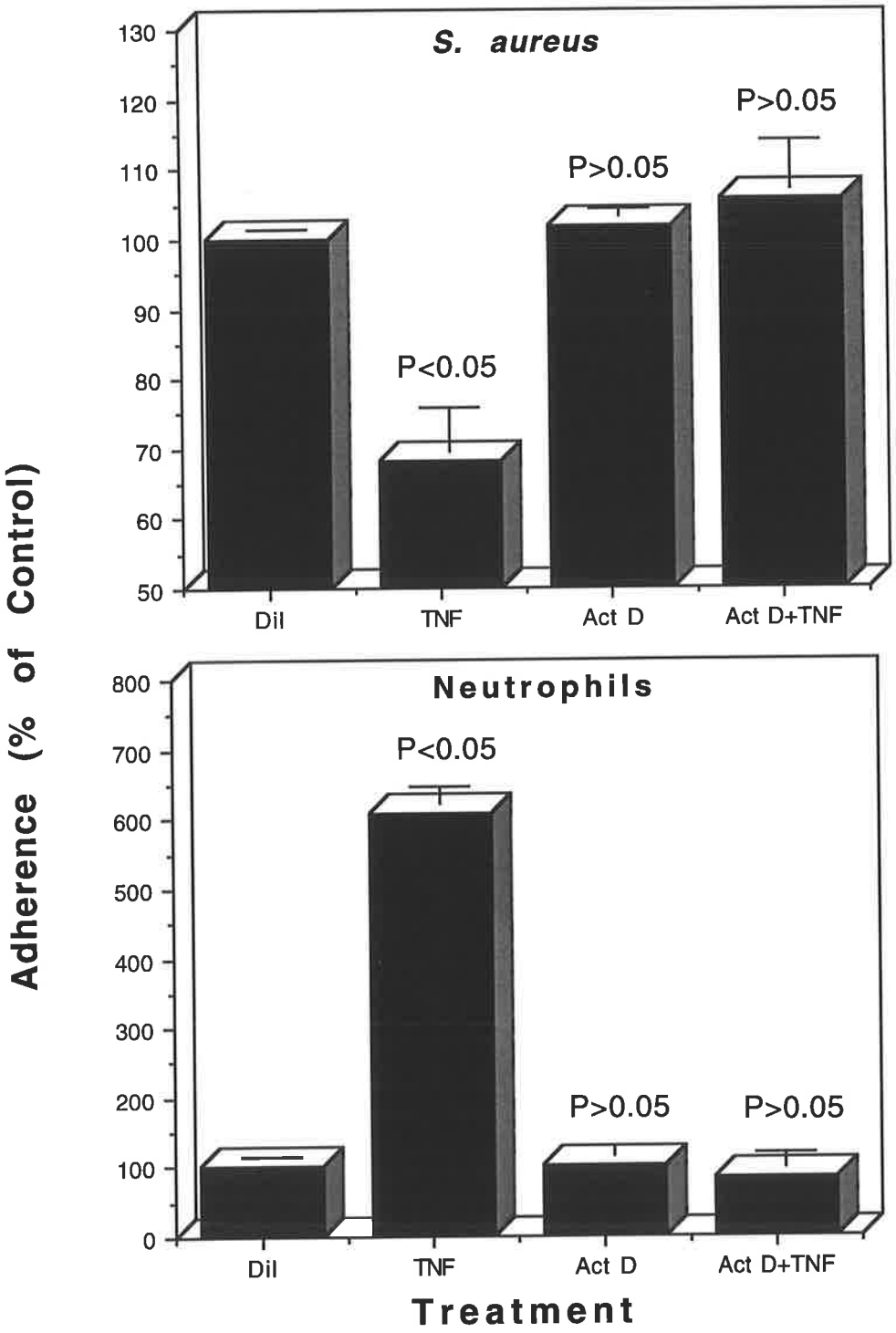


Fig. 3.9 the effect of actinomycin D (Act D) on the TNF-induced alteration of adhesion of *S. aureus* or neutrophils to HUVEC. The HUVEC were incubated with 400U/ml of TNF in the presence or absence of 4μg/ml actinomycin D for 4h and washed before the addition of *S. aureus* or neutrophils. The results are presented as the mean±sem of four experiments, each conducted in triplicate. P value is the difference from the diluent treatment.

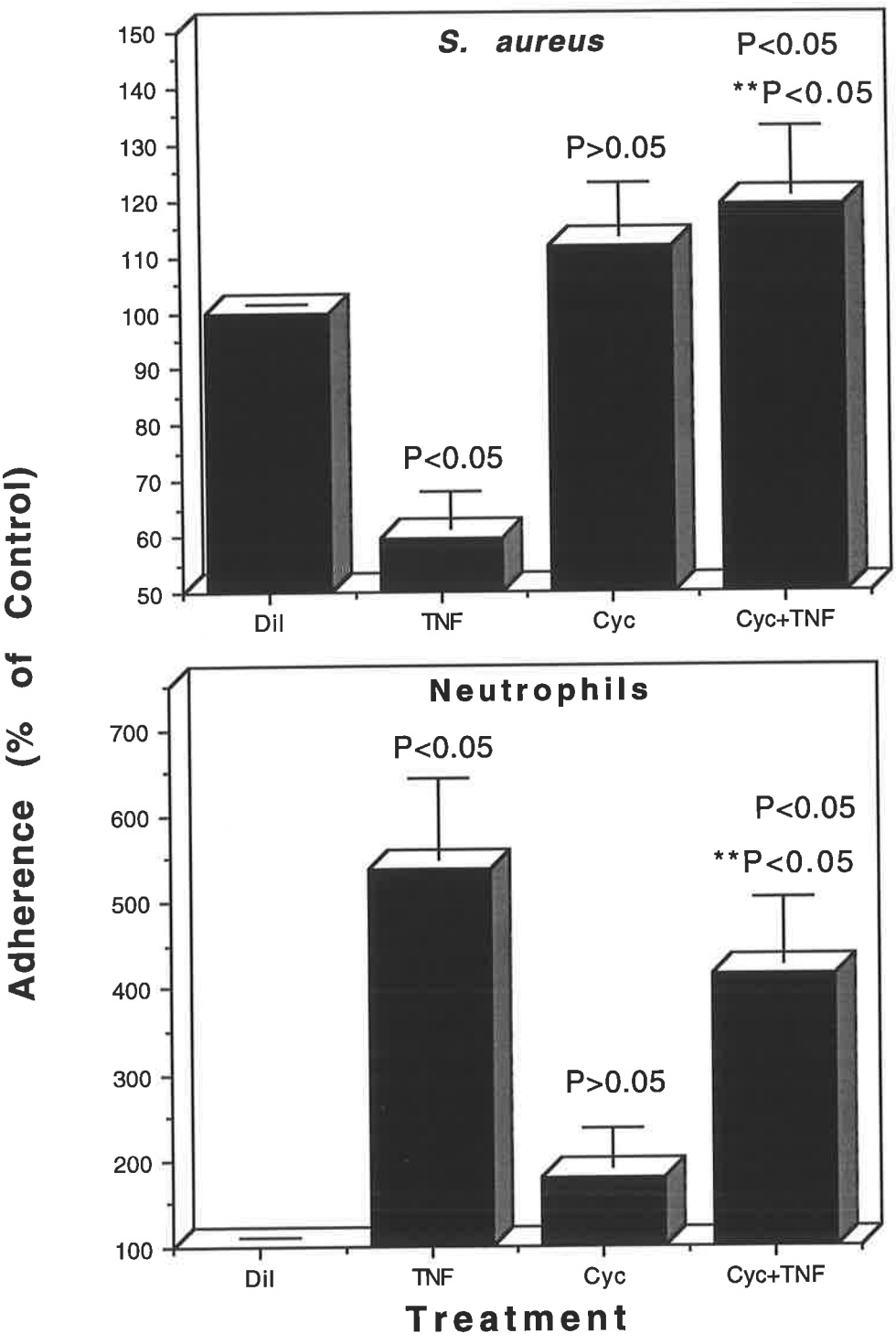


Fig. 3.10 The effect of cycloheximide (Cyc) on the TNF-induced alteration in the adhesion of *S. aureus* or neutrophils to HUVEC. Cycloheximide (10μg/ml) was added to the monolayers 1h prior to addition of 400U/ml of TNF or diluent for another 4h incubation. Then the monolayers were washed, *S. aureus* or neutrophils were added. The results are presented as the mean±sem of three experiments, each conducted in triplicate. P value is the difference from the diluent treatment **P value is the difference from the TNF treatment.

3.4 The Effect of IL-1β on Adhesion of *S. aureus* to HUVEC

3.4.1 The effect of IL-1β

Endothelial cells were pretreated with 1ng/ml of IL-1β for 4h at 37°C, washed and then *S. aureus* or neutrophils were added to the monolayers. The results presented in Fig. 3.11 show that IL-1β depressed adhesion of *S. aureus* to HUVEC. In contrast, the adhesion of neutrophils to HUVEC was enhanced by IL-1β treatment. The effects of IL-1β were concentration-related (Fig. 3.12). The IL-1β-induced neutrophil adhesion to endothelial cells showed marked enhancement at 4pg/ml with only a slight increase with further increase of IL-1β up to 400pg/ml. Although the IL-1β induced decrease of *S. aureus* adhesion was evident at 4pg/ml, the maximal effect was seen at 4000pg/ml (Fig. 3.12). Thus it is evident that the sensitivity for the enhancement of neutrophil adherence is much greater than that for the decrease in bacterial adherence.

3.4.2 The effect of varying the IL-1β pretreatment time

To determine the time required for the IL-1β induced changes, endothelial cells were preincubated with 1ng/ml of IL-1β at 37°C for varying time periods up to 4h. At the selected time points they were examined for their ability to adhere *S. aureus* or neutrophils. The results showed that over this time period, a decrease in bacterial adhesion was evident at 1h and the maximal effect was reached at 4h. This coincided with the time-dependent effects on neutrophil adhesion (Fig. 3.13). These results related to IL-1-induced time-dependent

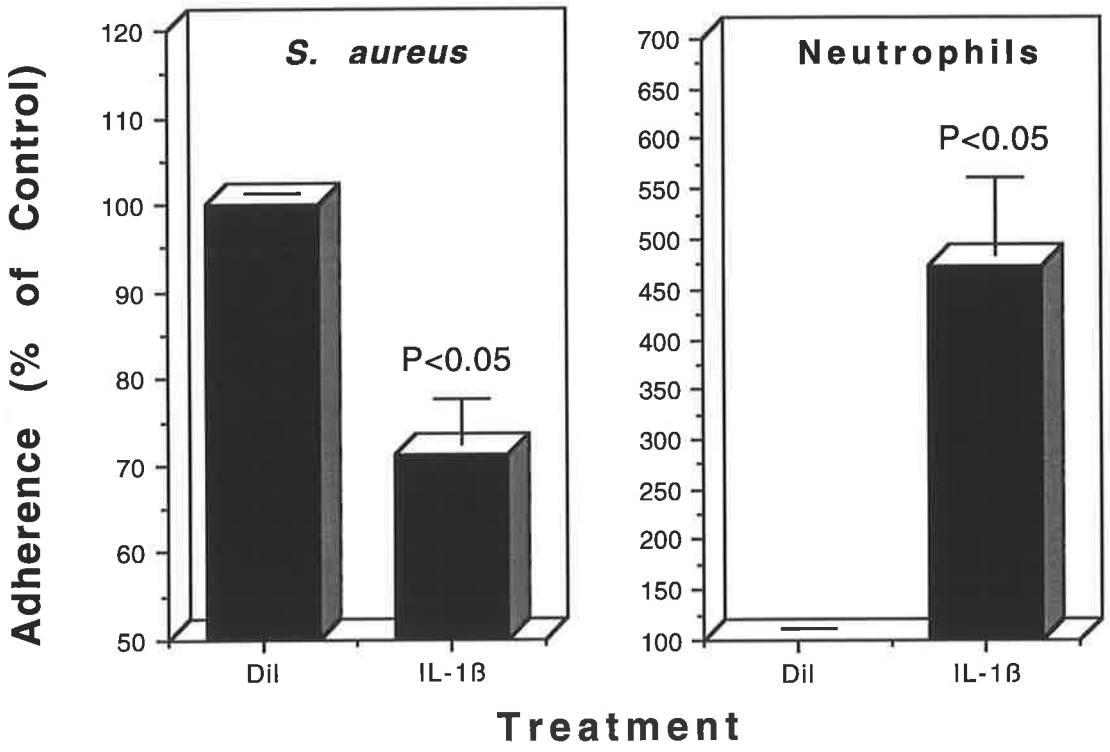


Fig. 3.11 The effect of IL-1β on adhesion of either *S. aureus* or neutrophils to HUVEC. HUVEC were treated with 1ng/ml of IL-1β or diluent (dil) for 4h and washed before the addition of *S. aureus* or neutrophils. The results are presented as the mean±sem of four experiments, each conducted in triplicate. P value is the difference from the diluent treatment.

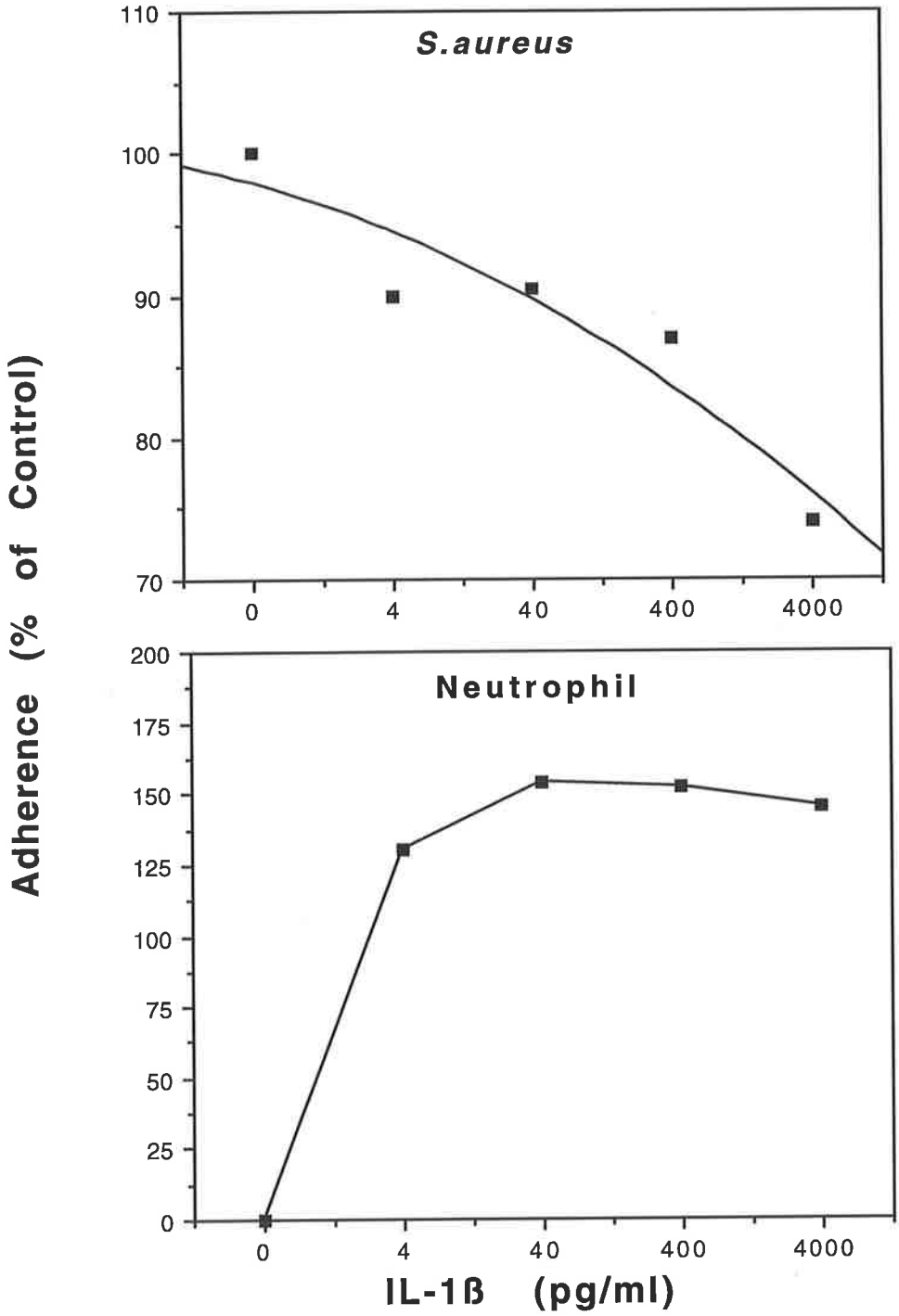


Fig. 3.12 The effect of varying concentrations of IL-1β on adhesion of either *S. aureus* or neutrophils to HUVEC. The HUVEC were treated with different concentrations of IL-1β for 4h. Then the endothelial monolayers were washed followed by addition of *S. aureus* or neutrophils. The results are the mean of three experiments, each conducted in triplicate.

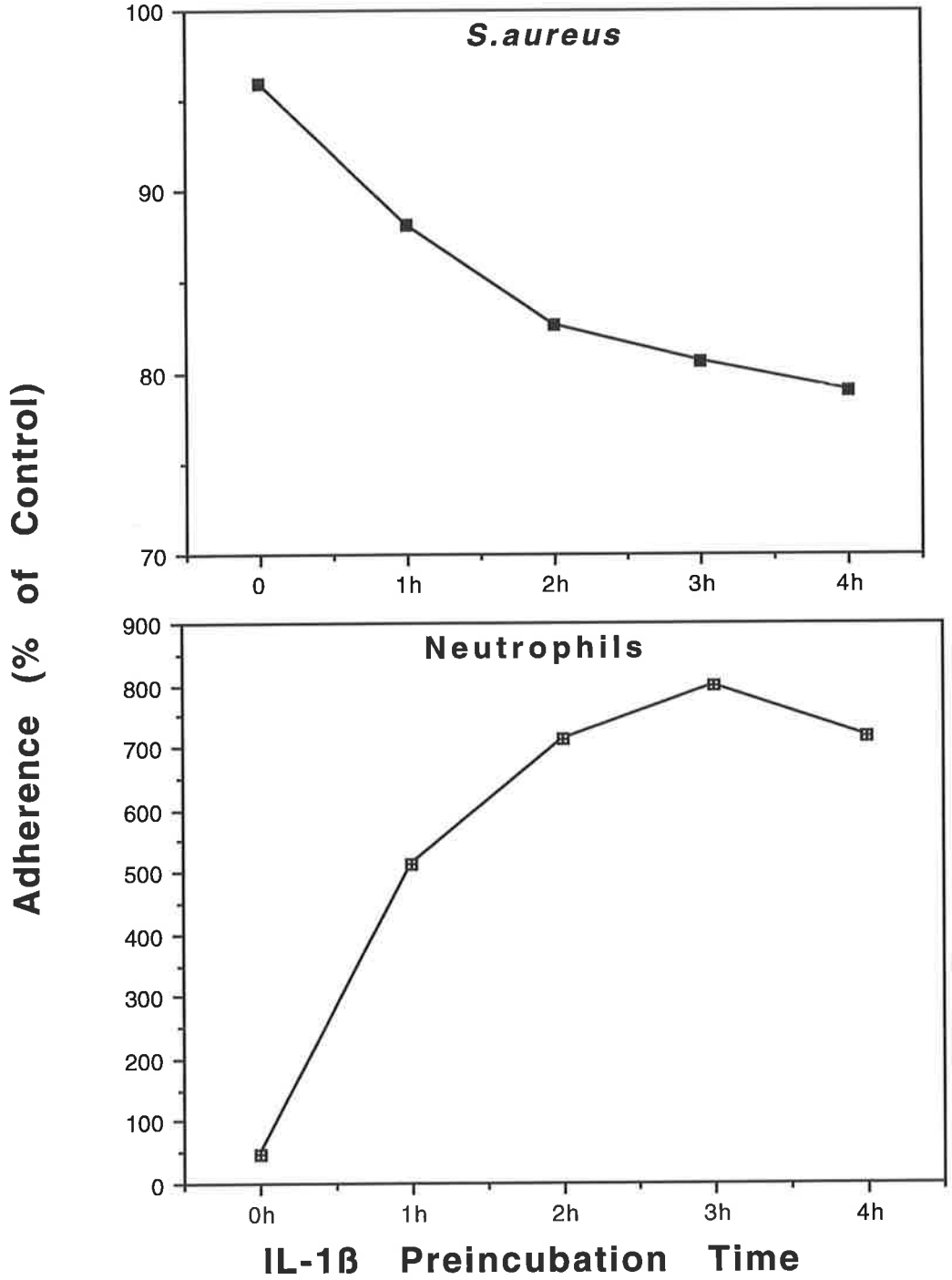


Fig. 3.13 The effect of IL-1β preincubation time on adhesion of *S. aureus* or neutrophils to HUVEC. The HUVEC were treated with 1ng/ml of IL-1β for 1h to 4h. Then the endothelial cell monolayers were washed and *S. aureus* or neutrophils added. The results are the mean of three experiments, each conducted in triplicate.

changes for the decreased bacterial adherence and increased neutrophil adherence parallel the results seen with TNF (section 3.3.3).

3.4.3 The effect of heat-inactivation, PMB treatment and anti-IL-1β monoclonal antibody treatment on the activity of the IL-1β preparation.

To exclude the possibility that other substances in the IL-1β preparation may have been responsible for the effects on HUVEC, we examined the sensitivity of the preparation to boiling and PMB, and neutralization with an anti IL-1β monoclonal antibody.

When the IL-1β preparation was boiled for 60 min, it lost most of its activity of altering the adhesion of *S. aureus* to endothelial cells as well that of modulating neutrophil adhesion (Fig. 3.14). In another group of experiments, the IL-1β preparation was treated with 10μg/ml of PMB to inactivate any LPS activity which could be contributing to the biological effects of the IL-1β preparation. The results showed that the PMB-treated IL-1β preparation was just as active as the nontreated preparation in altering bacterial adhesion and neutrophil adhesion to HUVEC (Fig. 3.15).

In the third aspect of this study, we used a monoclonal antibody, which specifically neutralises the activity of IL-1β to try to show that it is only IL-1β in the preparation which is responsible for the activity on endothelial cells. Pretreating the IL-1β preparation with the anti-IL-1β monoclonal antibody abolished its effect on bacterial adhesion and

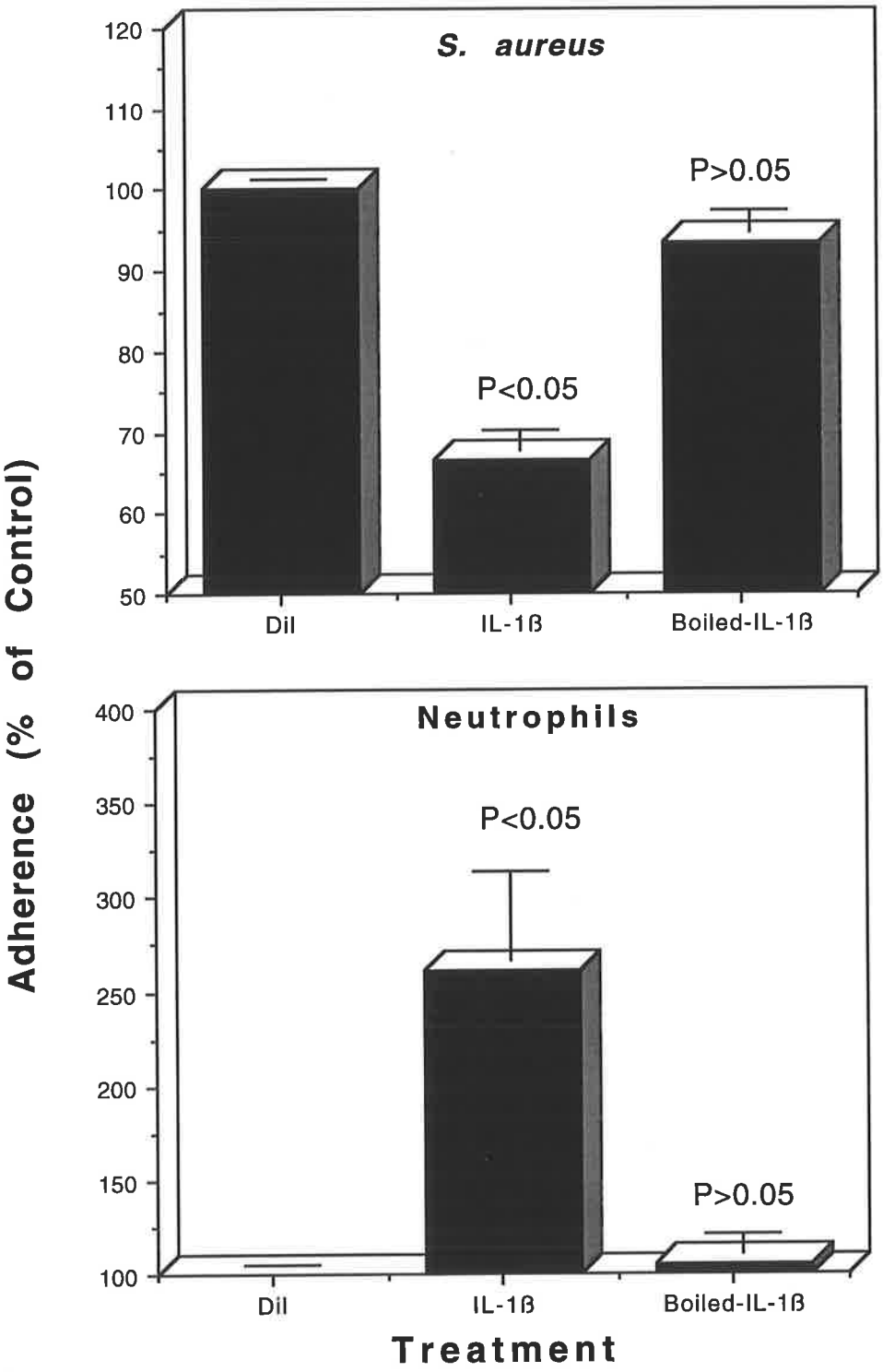


Fig. 3.14 The effect of heat-inactivation of IL-1β preparation on its ability to alter the adhesion of *S. aureus* or neutrophils to HUVEC. The HUVEC were treated with 1ng/ml of IL-1β or boiled IL-1β for 4h and then *S. aureus* or neutrophils were added. The results are the mean±sem of four experiments, each conducted in triplicate. P value is the difference from the diluent treatment.

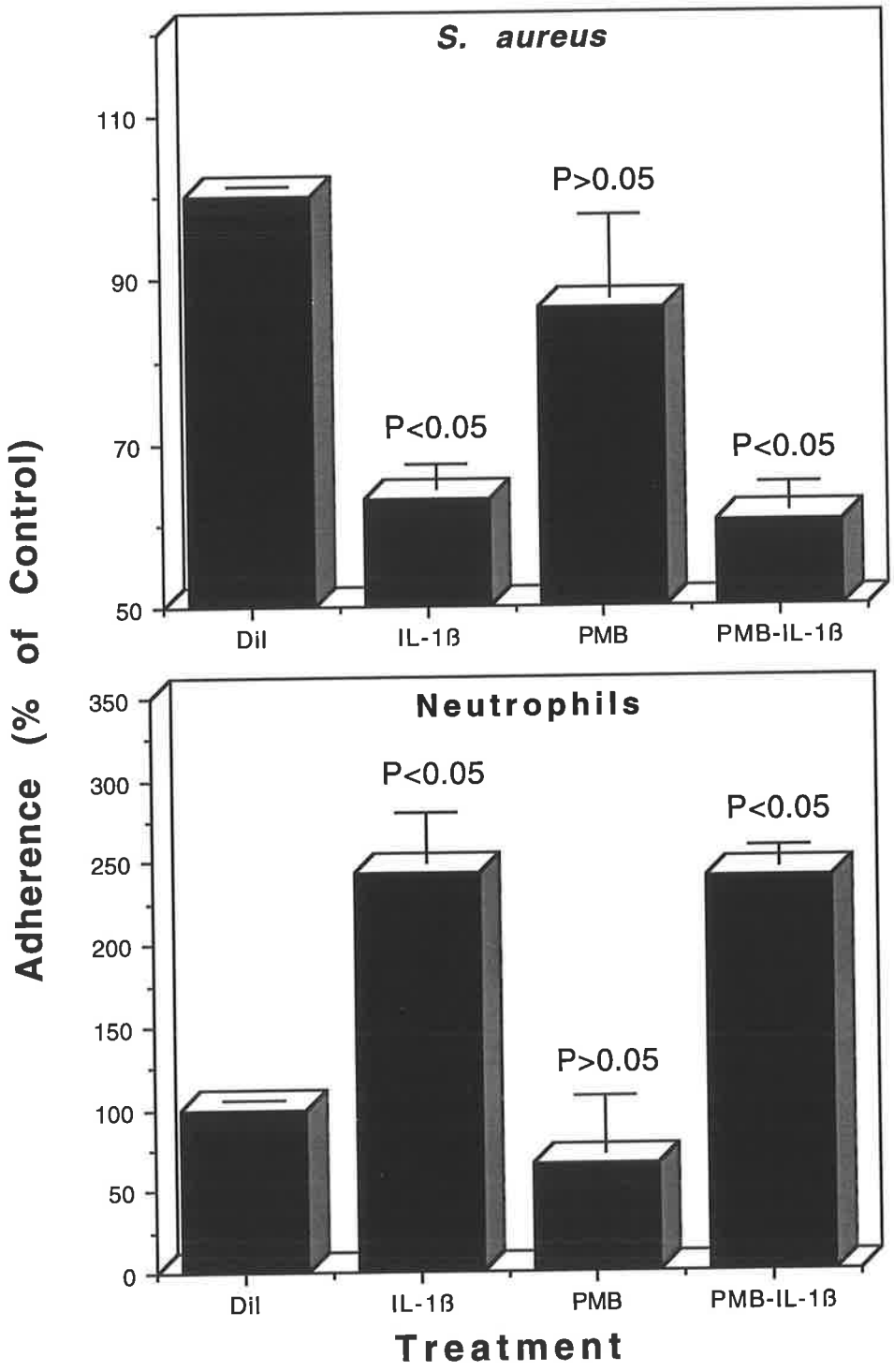


Fig. 3.15 The effect of PMB treatment of IL-1β preparation on its ability to alter the adhesion of *S. aureus* or neutrophils to HUVEC. The HUVEC were incubated with 1ng/ml of IL-1β or PMB (10μg/ml) treated IL-1β for 4h, washed and then *S. aureus* or neutrophils were added. The results are presented as the mean±sem of four experiments, each conducted in triplicate. P value is the difference from the diluent treatment.

significantly reduced its enhancement of neutrophil adhesion (Fig. 3.16).

The data shows that it is the IL-1β in the cytokine preparation which is responsible for altering the bacterial and neutrophil adherence to endothelial cells.

3.4.4 The importance of protein and RNA synthesis on the IL-1β –induced effects on HUVEC.

The importance of RNA synthesis and protein synthesis on the IL-1β induced changes on HUVEC was examined using the RNA and protein synthesis inhibitors actinomycin D and cycloheximide respectively. Endothelial cells were treated with either 10μg/ml of cycloheximide 1h prior to the additional 4h incubation with IL-1β, or 10μg/ml of actinomycin D simultaneously with the addition of IL-1β. The results suggested that cycloheximide and actinomycin D prevented the IL-1β-induced decrease in bacterial adhesion as well as the enhancement of neutrophil adhesion (Fig. 3.17). HUVEC treated with cycloheximide did not show the depressed *S.aureus* adhesion induced by IL-1β (Fig. 3.18). Similarly the IL-1β induced enhancement of neutrophil adhesion was prevented by treating the cells with cycloheximide.

3.5 Summary

The data showed that the monokines, TNF and IL-1β altered the adhesion of *S. aureus* to HUVEC. In contrast to the enhancing effects

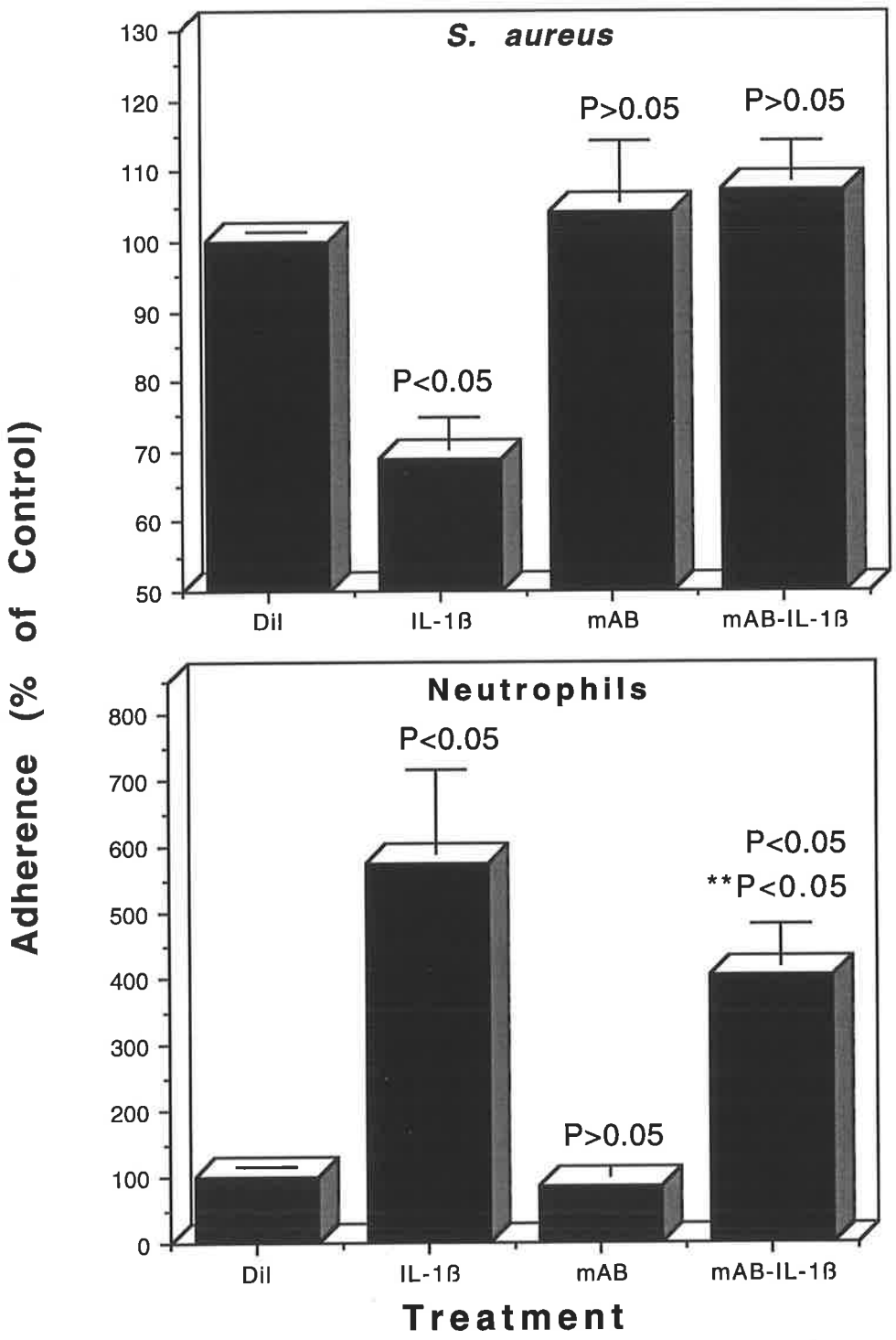


Fig.3.16 The effect of anti-IL-1β monoclonal antibody (mAB) treatment of IL-1β on its ability to alter the adhesion of *S. aureus* or neutrophils to HUVEC. The IL-1β preparation (1ng/ml) was treated with the anti-IL-1β (1:100 dilution) for 30 min and then added to the HUVEC. After 4h incubation the endothelial monolayers were washed and then *S. aureus* or neutrophils were added. The results are the mean±sem of four experiments, each conducted in triplicate. P value is the difference from the diluent treatment. **P<0.05 is the difference from the nonaddition of mAB.

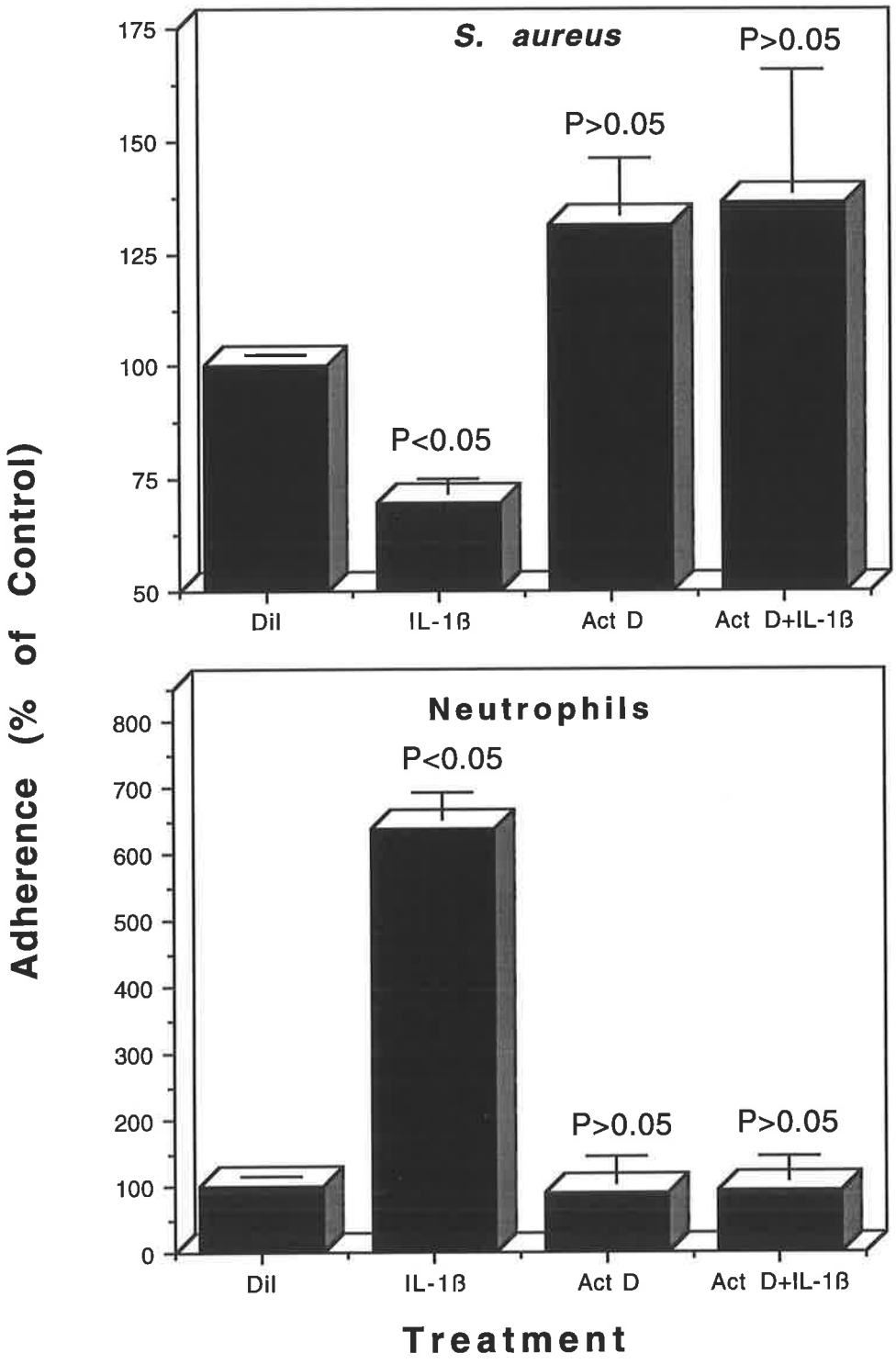


Fig. 3.17 The effect of actinomycin D (Act D) on the IL-1β-induced alteration in adhesion of *S. aureus* or neutrophils to HUVEC. The HUVEC were incubated with 1ng/ml of IL-1β in the presence or absence of 4μg/ml of actinomycin D for another 4h incubation. Then the monolayers were washed and *S. aureus* or neutrophils added. The results are presented as the mean±sem of four experiments, each conducted in triplicate. P value is the difference from the diluent treatment.

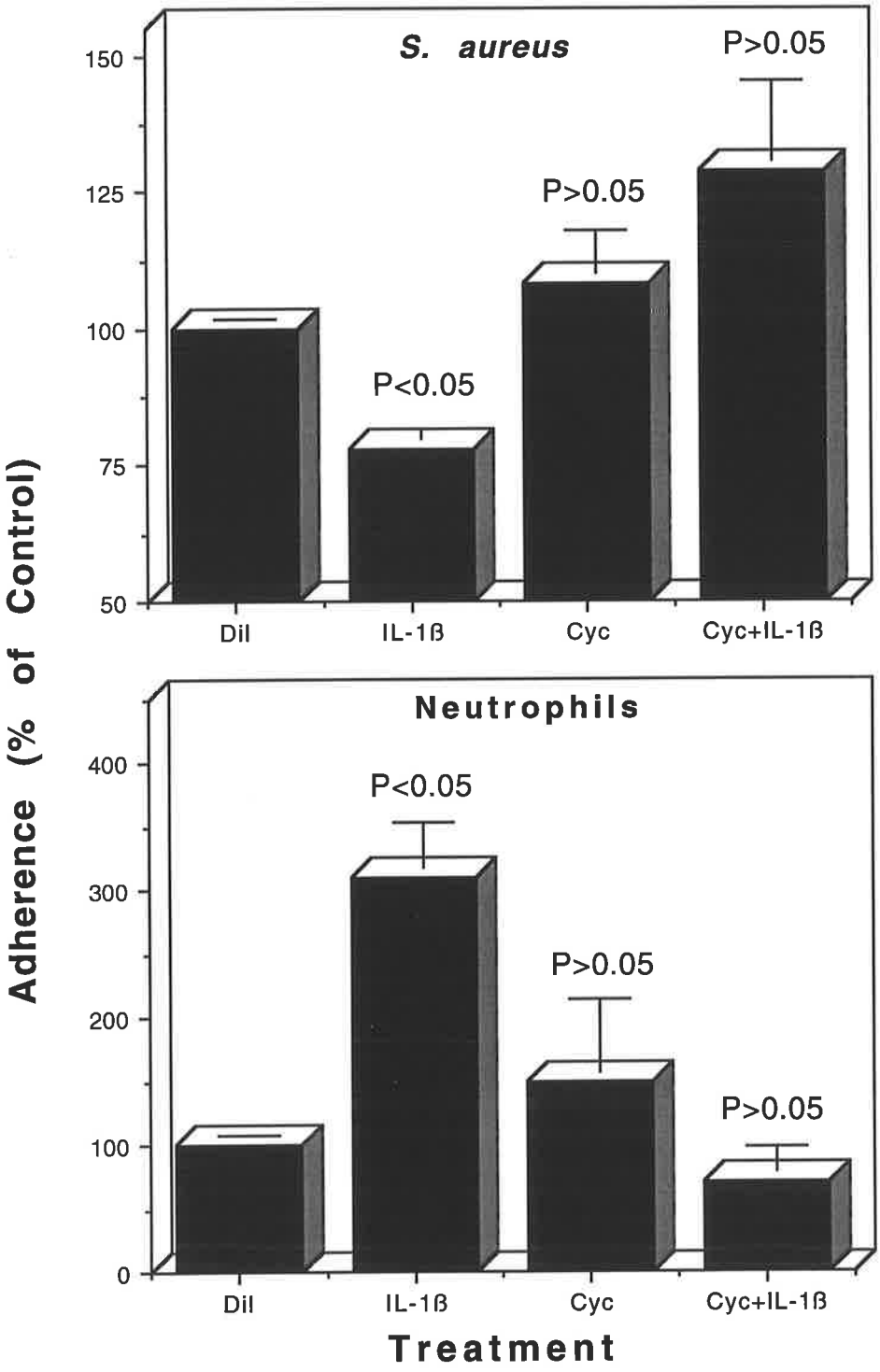


Fig. 3.18 The effect of cycloheximide (Cyc) on IL-1β induced alteration of the adhesion of *S. aureus* or neutrophils to HUVEC. Cycloheximide (10μg/ml) were added to the HUVEC 1h prior to addition of 1ng/ml of IL-1β or diluent for another 4h incubation. Then the monolayers were washed and *S. aureus* or neutrophils added. The results are presented as the mean±sem of three experiments, each conducted in triplicate. P value is the difference from the diluent treatment.

of these cytokines on neutrophil adhesion to endothelial cells, pretreating endothelial cells with TNF or IL-1β decreased the binding of *S. aureus* to the cells. It was evident that the recombinant TNF and IL-1β were responsible for these effects since the activities of the cytokines were abolished by boiling but not affected by PMB treatment and could be neutralised by anti-TNF or anti-IL-1β monoclonal antibodies.

TNF-or IL-1β-induced decreases in bacterial adhesion became evident at 4U/ml and 4pg/ml respectively, and the maximal effects were reached at 400U/ml for TNF and at 4ng/ml for IL-1β. There was a clear difference in the concentration required to alter the adherence of *S. aureus* and that required to alter the adherence of neutrophils. At 4U/ml of TNF or 4pg/ml of IL-1β almost maximal enhancement of neutrophil adhesion occurred. This suggests that the mechanisms for the alteration of *S.aureus* adhesion and neutrophil adhesion induced by both TNF and IL-1β primarily correlate. It is possible that this is a function of the varied signal tranduction mechanisms which TNF/IL-1 can utilize in TNF/IL-1 sensitive cells. Studies on varying pre-exposure time of cytokines to HUVEC showed that with IL-1β, the depression in *S.aureus* adherence and enhancement of neutrophil adherence were essentially co-incidental. The alterations were evident within 1h and reached maximal effects in 3h. Studies with TNF showed differences between the alteration in bacterial adherence and neutrophil adherence. The TNF-induced enhancement of neutrophil adherence was reached by 1h pretreatment time and only increase by a further 16% between 1-4h. However the TNF-induced decrease in bacterial adherence continued to decrease up to 4h pretreatment time.

Both the decrease in bacterial adhesion and the increase in neutrophil adhesion caused by TNF or IL-1β were inhibited by the inhibitors of RNA and protein synthesis, showing the dependency of these effects on the expression of new messages and new proteins.

CHAPTER 4

THE EFFECTS OF LYMPHOKINES

ON BACTERIAL ADHESION TO HUVEC

4.1 Introduction

Products of activated T cells, lymphokines, have been shown to alter endothelial cell antigenic expression which contributes to the recruitment of leukocytes by certain tissues (Haskard et al, 1986; Cotran et al, 1989). Among these lymphokines, LT, IL-4, IFN-γ and IL-2 are important in the regulation of adhesiveness of endothelial cells for circulating leukocytes.

Based upon the present tentative characterization of Th cells into Th_1 and Th_2 cells, LT (TNF-β) is produced by activated Th_1 cells. This cytokine has similar effects to TNF on endothelial cells and shares the same receptors on these cells (Table 4.1). Like TNF, LT induces the production of IL-1 and platelet activating factor in endothelial cells, increases the expression of procoagulant activity and enhances the expression of MHC class I antigens and of the adhesion molecules which promote leukocyte binding to endothelial cells. However, recombinant human LT has less binding affinity for the TNF receptor on endothelial cells compared to TNF. This has been believed to be responsible for the higher concentration of LT necessary to induce similar activities, such as the production of IL-1, haematopoietic growth factors and the augmentation of neutrophil adhesion to endothelial cells (Locksley et al, 1987; Broudy et al, 1987).

IFN-γ is produced by activated Th_1 lymphocytes. The cytokine activates endothelial cells causing an increase in expression of ICAM-1 and MHC class I antigen and a *de novo* induction of MHC class II antigens (Pober, 1986; 1983). The TNF-, IL-1- and LPS-induced expression of E-

Table 4.1 The effects of TNF and LT on endothelial cells

Effects	TNF	LT
Receptor binding	++	+
Cytotoxicity	+/-	-
Induction of PAF	++	+
Procoagulant activity	+	+
Plasminogen activator/plasminogen activator inhibitor	+	+
Hyperadhesiveness for leukocytes	++	+
Hyperadhesiveness for tumor cells	+	NR
Induction of the expression of ICAM-1	+	+
Induction of the expression of E-selectin	+	+
Induction of the expression of VCAM-1	+	NR
Modulation of the expression of Integrin	+	NR
Production of IL-1	++	+
Production of IL-6	++	NR
Production of IL-8	+	NR
Production of GM-CSF	++	+
Production of PGI2/E2	+	+
Production of PDGF	+	+
Induction of the expression of HLA class I antigens	+	+
Morphological changes	+	+

+/-: marginally active
+: active
++: very active
NR: not reported

selectin on endothelial cells can be accelerated and prolonged by the addition of IFN-γ.

IL-2 is also a product of activated Th_1 lymphocytes. Its receptor is subdivided into three classes: α chain alone (low affinity), β chain alone (moderate affinity) and α plus β chain (high affinity). The main role of IL-2 is to promote lymphocyte proliferation. There is no evidence to show that IL-2 directly alters the expression of endothelial surface molecules. While, *in vivo* studies have shown that endothelial cells from patients who were undergoing IL-2, or LAK therapy, displayed increased expression of ICAM-1, E-selectin and HLA-DQ antigens, this was suggested to result from the effects of the products of IL-2 activated lymphocyte (Cotran et al, 1989).

IL-4 is a product of activated Th_2 cells, which was originally recognized to promote lymphocyte proliferation, B cell class switching and the expression of MHC class II antigens (Roehm et al, 1984, Snapper et al, 1987, Howard et al, 1982, Spits et al, 1988). More recently it became evident that IL-4 inhibited the respiratory burst, killing of parasites and the production of TNF, IL-1, IL-6 and IL-8 by monocytes (Abramson et al, 1990, Hart et al, 1989, Gibbons et al, 1990, Kumaratilake et al, 1992, Standiford et al, 1990). IL-4 has been shown to act on endothelial cells to increase the expression of VCAM-1 which contributes to the recruitment of lymphocytes into tissues (Masinovsky et al, 1990). Like IFN-γ, IL-4 had no effect on stimulating endothelial cell adhesion of neutrophils (Thornhill et al, 1990).

Studies with this group of cytokines will increase our understanding of the role of activated T lymphocytes on bacterial-endothelial cell

interaction. The data will also be of further interest since these cytokines are differently produced by subsets of Th lymphocytes.

4.2 The Effect of LT on Adhesion of *S. aureus* to HUVEC

4.2.1 The effect of LT

Pretreatment of endothelial cells with 400U/ml of recombinant human LT for 4h resulted in significant inhibition of adhesion of *S. aureus* to HUVEC (Fig. 4.1). The adhesion of neutrophils to similarly treated endothelial cells was augmented. The effect of this cytokine was concentration-dependent (Fig. 4.2). Depressed bacterial adhesion occurred at 4U/ml reaching maximal effects at 400U/ml. At 4U/ml neutrophil adhesion was increased, but unlike the altered effects on bacterial adhesion, the enhancement of neutrophil adhesion by LT progressively increased to 4000U/ml (Fig. 4.2). Comparing these results with the effects of TNF, it is evident that in the enhancement of neutrophil adherence, LT is less active than TNF (Chapter 3). The TNF effect had reached maximal effects at 4U/ml while only a small effect was seen at the same concentration for LT. However, on the adherence of bacteria, LT was found to be more effective than TNF in reducing bacterial adherence. Thus a major proportion of effects was seen with 4U/ml of LT compared to very little effect with TNF. This illustrates some of the purported difference between the two cytokines even though these bind to the same receptor.

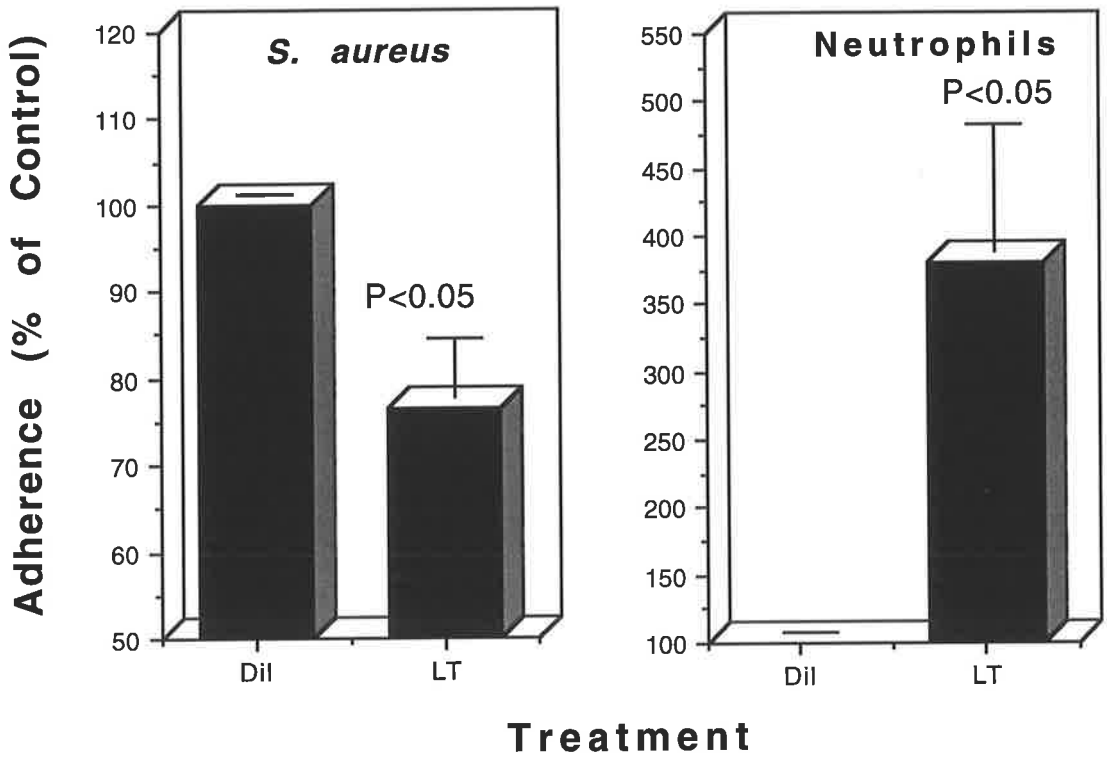


Fig. 4.1 The effect of LT on adhesion of either *S. aureus* or neutrophils to HUVEC. HUVEC were treated with 400U/ml of LT or diluent (dil) for 4h and washed before the addition of *S. aureus* or neutrophils. The results are presented as the mean±sem of four experiments, each conducted in triplicate. P value is the difference from the diluent treatment.

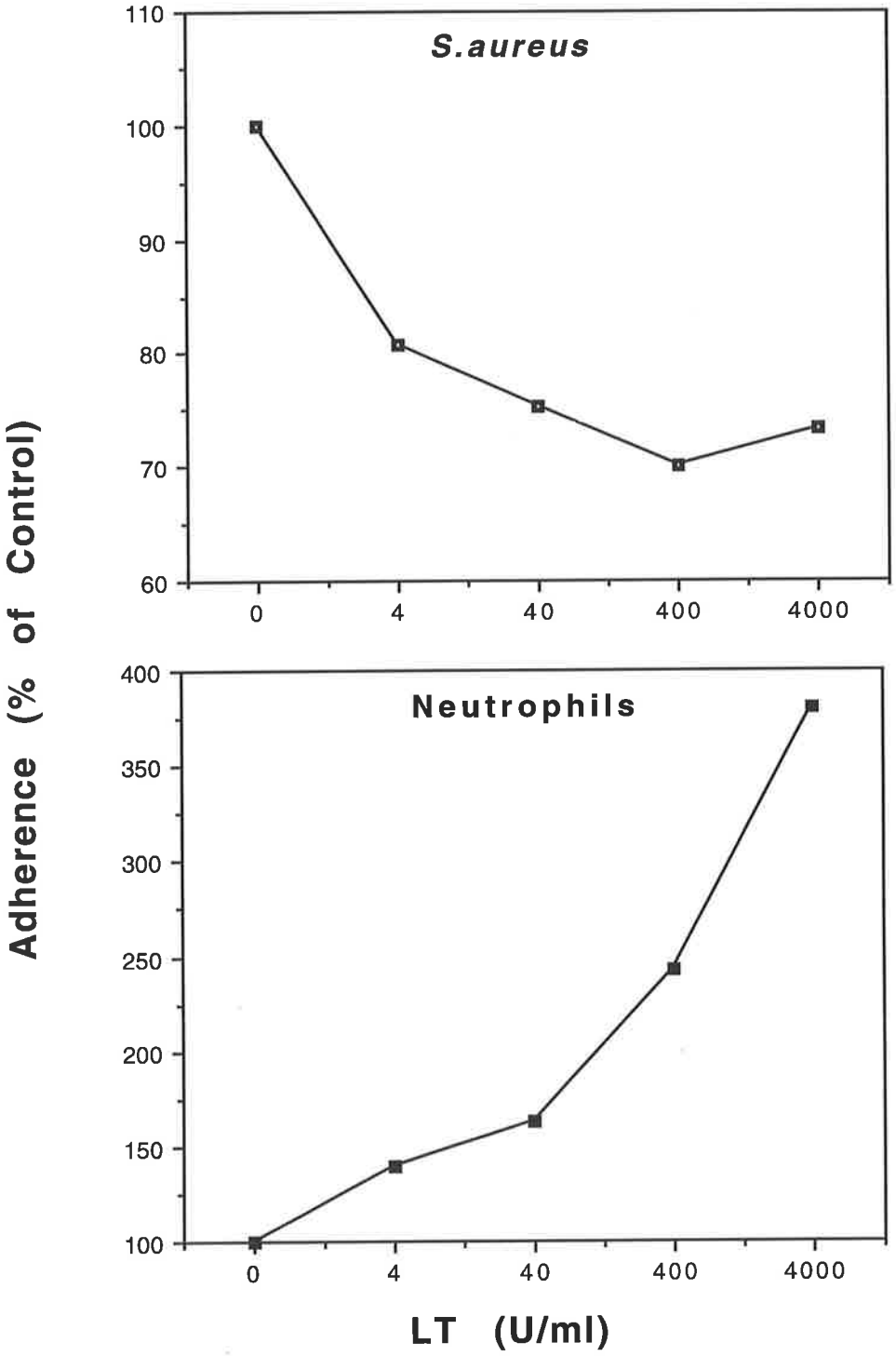


Fig. 4.2 The effect of varying concentrations of LT on adhesion of *S. aureus* or neutrophils to HUVEC. The HUVEC were treated with different concentrations of LT for 4h. Then the monolayers were washed followed by the addition of *S. aureus* or neutrophils. The results are the mean of four experiments, each conducted in triplicate.

4.2.2 *The effect of varying the LT pretreatment time*

To examine the effect of varying cytokine preincubation time on HUVEC, endothelial cells were treated with 400U/ml of LT for different times, then tested for bacterial and neutrophil adherence. Bacterial adhesion was progressively inhibited with an increase in LT preincubation time (Fig 4.3). The inhibition was seen at 1h and continued to increase to 3-4h. Neutrophil adhesion was also increased over the same preincubation time (Fig. 4.3).

4.2.3 *The effects of heating, treatment with PMB and anti-LT monoclonal antibody treatment on the activity of LT*

LPS can alter the expression of surface antigens on endothelial cells and could be a potential contaminant which contributes to the effects seen with the LT preparation. The following experiments were essentially designed to exclude this possibility. The LT preparation which had been boiled for 60 min failed to depress HUVEC-bacterial adhesion or to increase neutrophil adhesion (Fig. 4.4). Treatment of the LT preparation with 10μg/ml of PMB to inhibit any LPS activity, neither affected the cytokine-induced decrease of bacterial adherence nor the cytokine-induced enhancement of neutrophil adhesion (Fig. 4.5). When the preparation was treated with an anti-LT monoclonal antibody, its ability to alter both bacterial adhesion and neutrophil adhesion to endothelial cells was almost totally abolished (Fig 4.6).

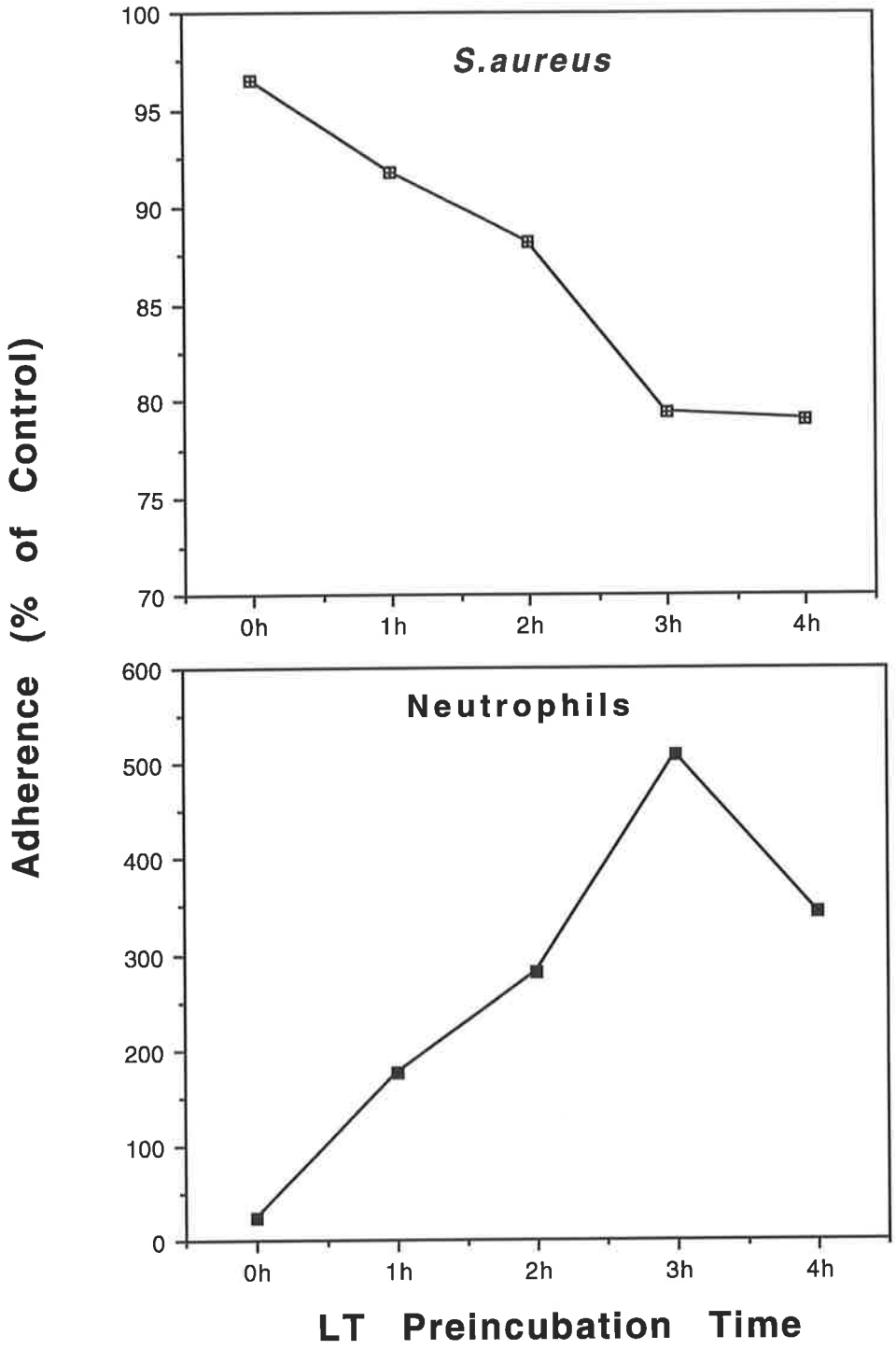


Fig. 4.3 The effect of LT preincubation time with HUVEC on their adhesion of *S. aureus* or neutrophils. The HUVEC were treated with 400U/ml of LT for 1h to 4h. Then the monolayers wells were washed and *S. aureus* or neutrophils were added. The results are the mean of four experiments, each conducted in triplicate.

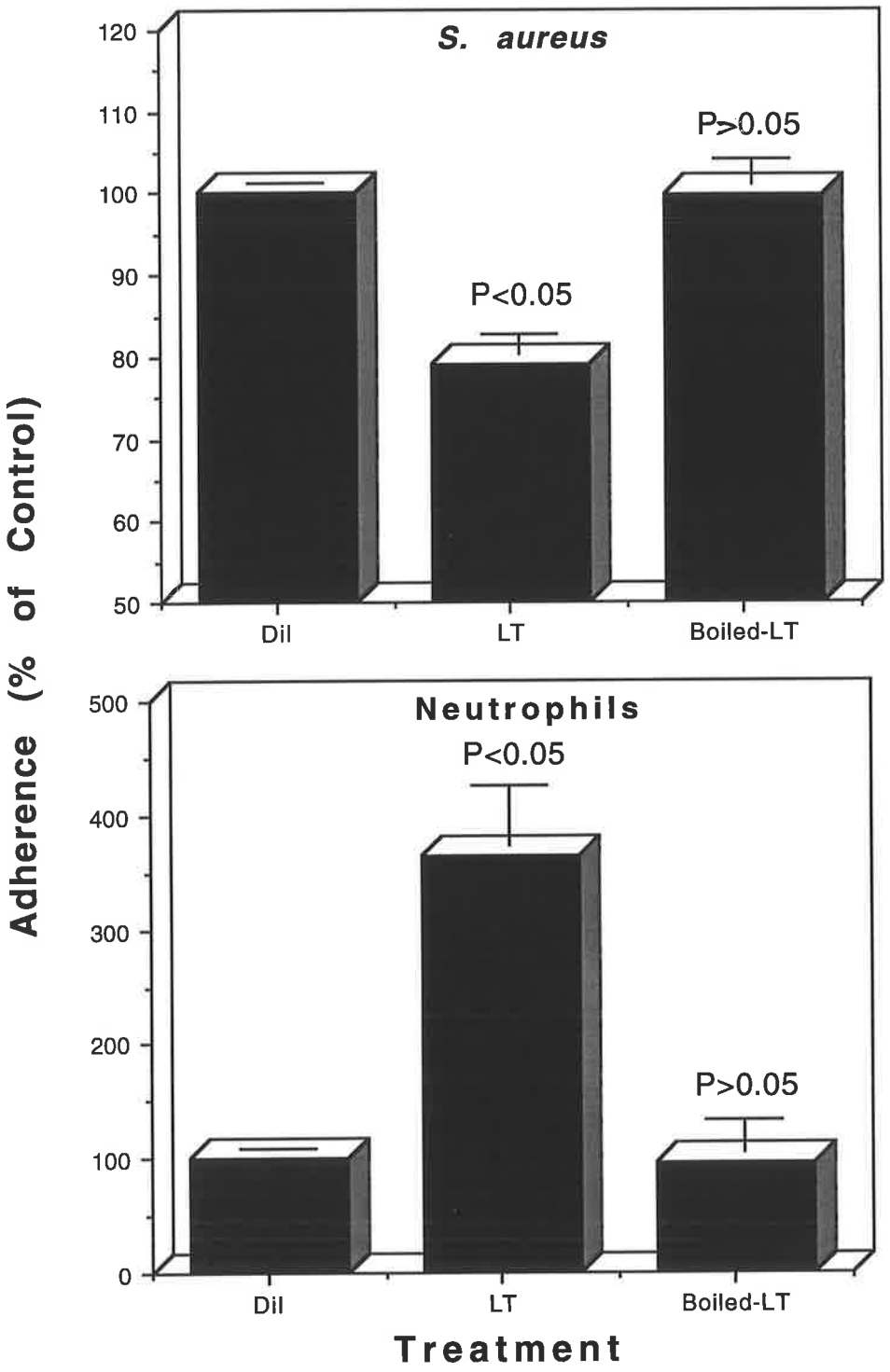


Fig. 4.4 The heat-inactivation of the LT preparation on its ability to alter the adhesion of *S. aureus* or neutrophils to HUVEC. The HUVEC were treated with 400U/ml of LT or boiled LT for 4h and then *S. aureus* or neutrophils added. The results are the mean±sem of four experiments, each conducted in triplicate. P value is the difference from the diluent treatment.

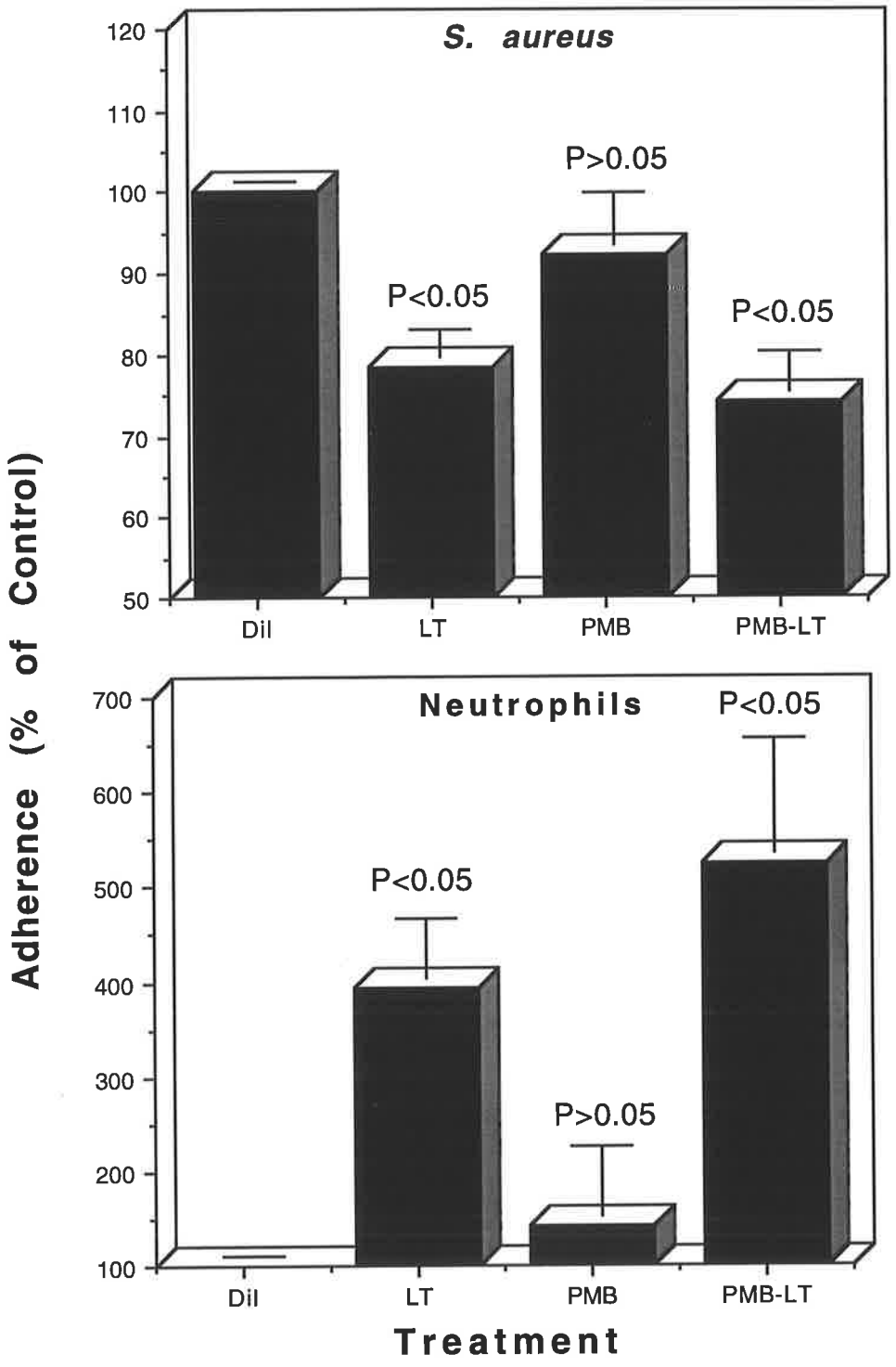


Fig. 4.5 The ability of PMB-treated LT preparation to alter the adherence of *S. aureus* or neutrophils to HUVEC. The HUVEC were treated with 400U/ml of LT or PMB (10μg/ml) treated LT for 4h, washed and then *S. aureus* or neutrophils were added. The results are presented as the mean±sem of three experiments, each conducted in triplicate. P value is the difference from the diluent treatment.

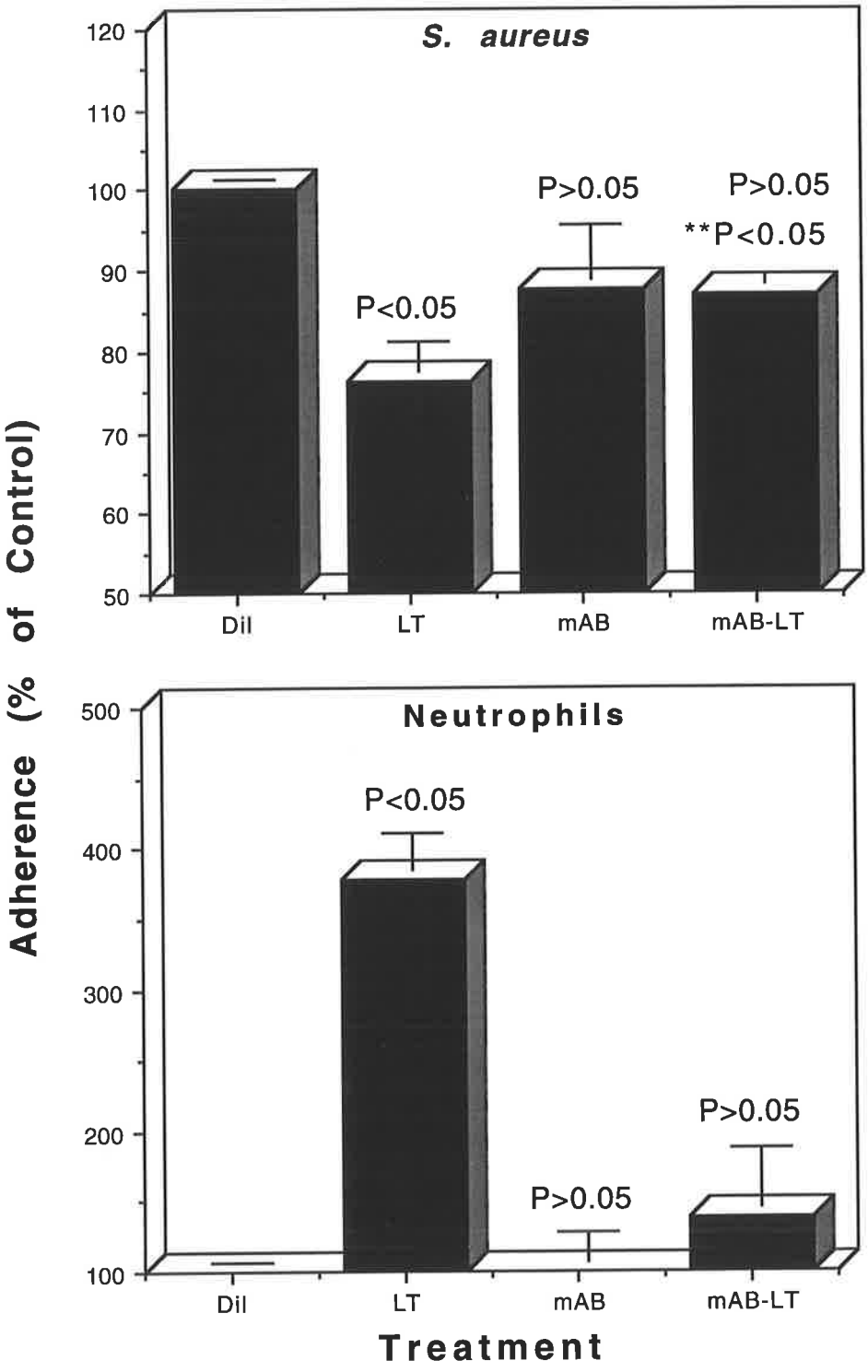


Fig. 4.6 The ability of anti-LT monoclonal antibody (mAB) treated LT preparation on adhesion of *S. aureus* or neutrophils to HUVEC. The LT (400U/ml) preparation was treated with anti-LT (1:50 dilution) for 30 min and then added to the HUVEC. After 4h incubation the monolayers were washed and then *S. aureus* or neutrophils added. The results are the mean±sem of three experiments, each conducted in triplicate. P value is the difference from the diluent treatment. **P value is the difference from the nonaddition of mAB.

4.2.4 *The importance of protein and RNA synthesis on the LT-induced effects on HUVEC*

Endothelial cells were pretreated with 10μg/ml of cycloheximide for 1h before addition of LT to inhibit protein synthesis, or treated simultaneously with LT and 4μg/ml of actinomycin D to inhibit RNA synthesis. Then the cells were tested for adhesion of bacteria or neutrophils. The results showed that both the LT-induced depression of bacterial adhesion and enhancement of neutrophil adhesion were sensitive to these inhibitors (Fig. 4.7 and Fig. 4.8). These results suggested that *de novo* synthesis of RNA and protein are both important for the LT-induced changes on endothelial cells in relation to adhesion of bacteria and neutrophils.

4.3 Effects of Interferon-γ, Interleukin-2 and Interleukin-4 on Adhesion of *S. aureus* to HUVEC

The results showed that treatment of endothelial cells with 4-4000U/ml of IFN-γ for 4h at 37°C did not alter adhesion of *S. aureus* or neutrophils to endothelial cells (Fig. 4.9). Lack of effect was also seen in adhesion of bacteria or neutrophils to endothelial cells pretreated with 1-100U/ml of IL-2 for 4h at 37°C (Fig. 4.10). Furthermore, 100U/ml of IL-4 had no effect on adhesion of *S. aureus* or neutrophils to cytokine-treated endothelial cells (Fig. 4.11).

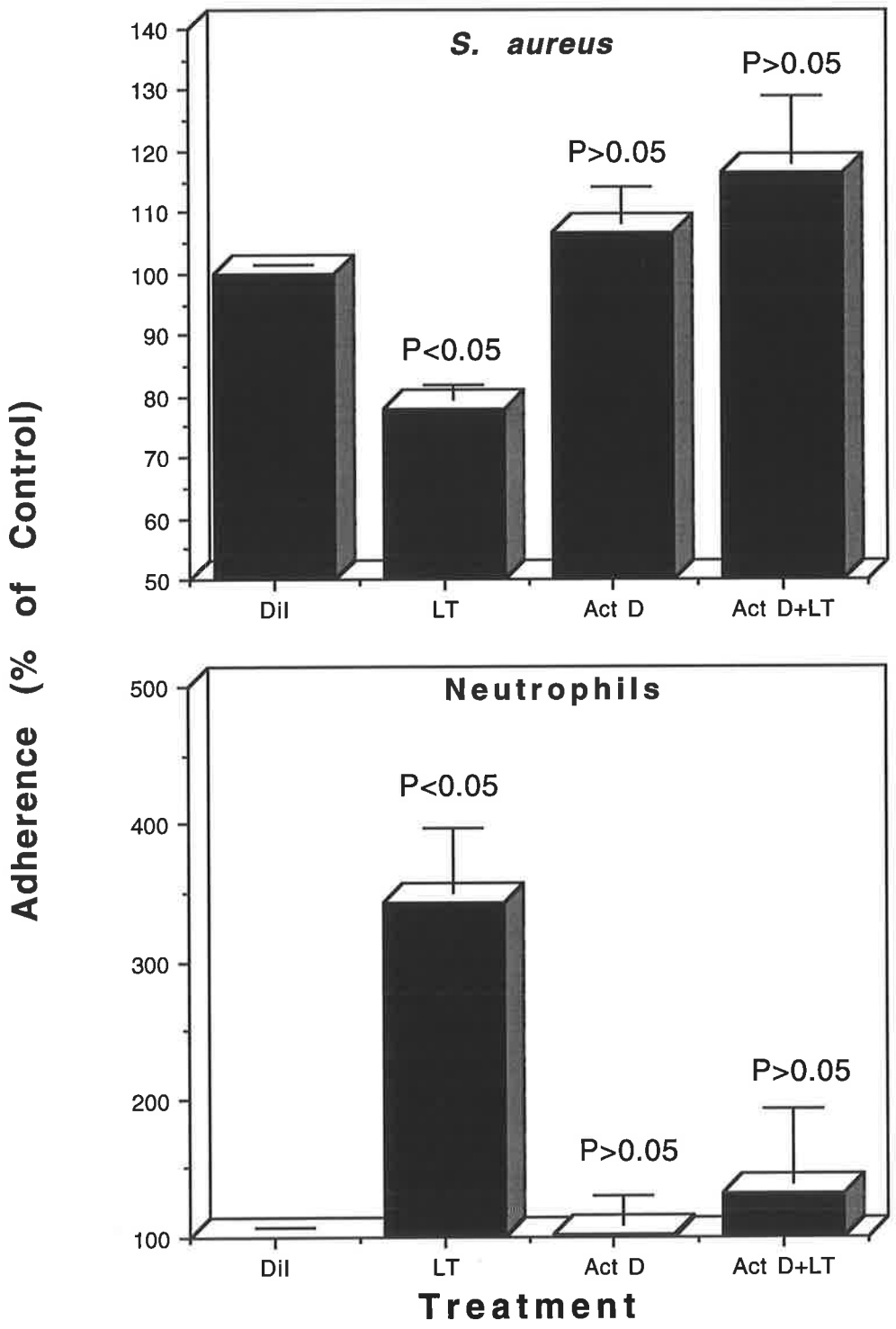


Fig. 4.7 The effect of actinomycin D (Act D) on the LT-induced alteration of the adhesion of *S. aureus* or neutrophils to HUVEC. The HUVEC were incubated with 400U/ml of LT in the presence or absence of 4μg/ml actinomycin D for another 4h incubation. Then the monolayers were washed and *S. aureus* or neutrophils added. The results are presented as the mean±sem of five experiments, each conducted in triplicate. P value is the difference from the diluent treatment.

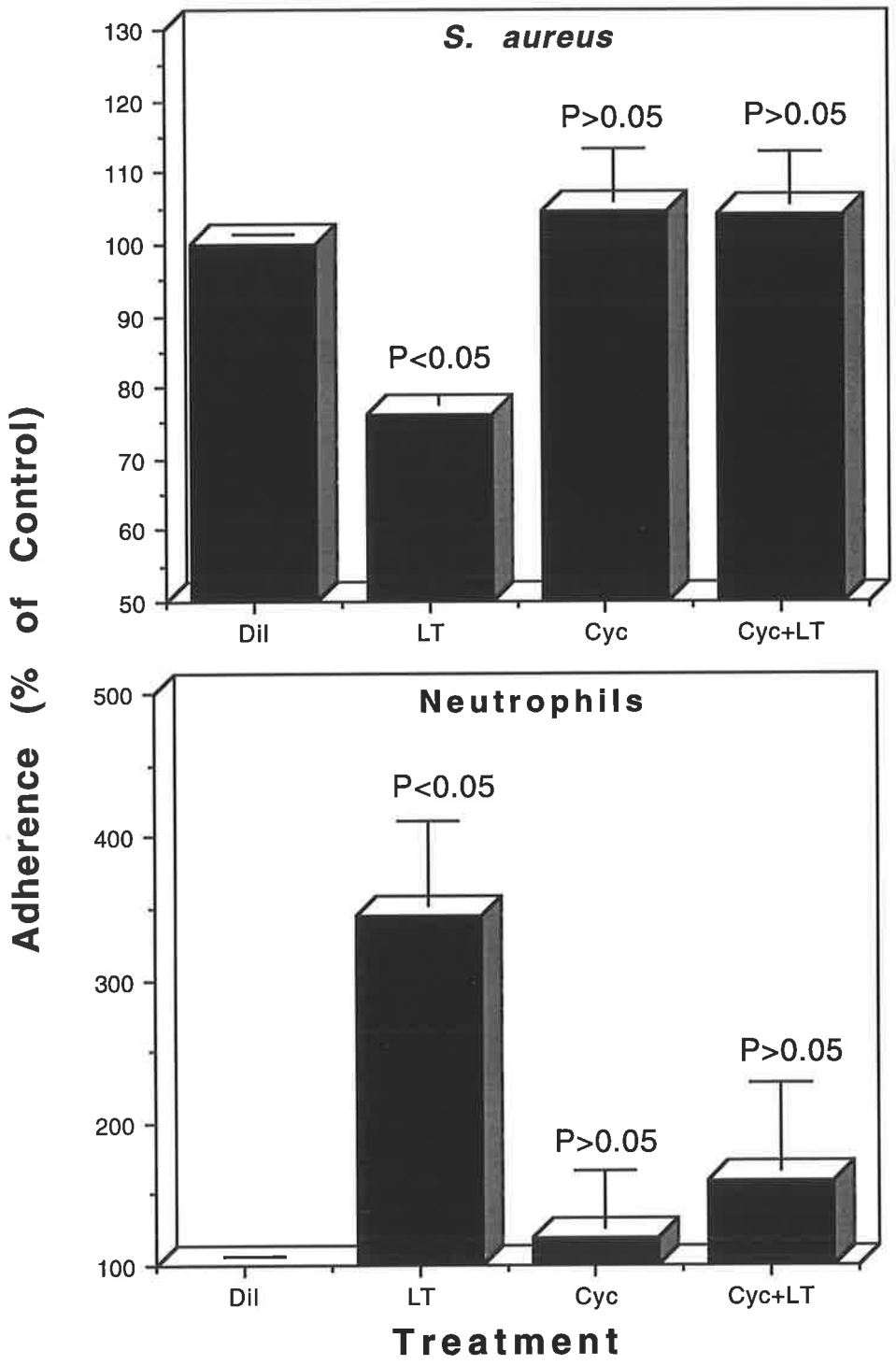


Fig. 4.8 The effect of cycloheximide (Cyc) on the LT-induced alteration of the adhesion of *S. aureus* or neutrophils to HUVEC. Cycloheximide (10μg/ml) was added to the HUVEC 1h prior to additon of 400U/ml of LT or diluent for another 4h incubation. Then the monolayers were washed and *S. aureus* or neutrophils added. The results are presented as the mean±sem of three experiments, each conducted in triplicate. P value is the difference from the diluent treatment.

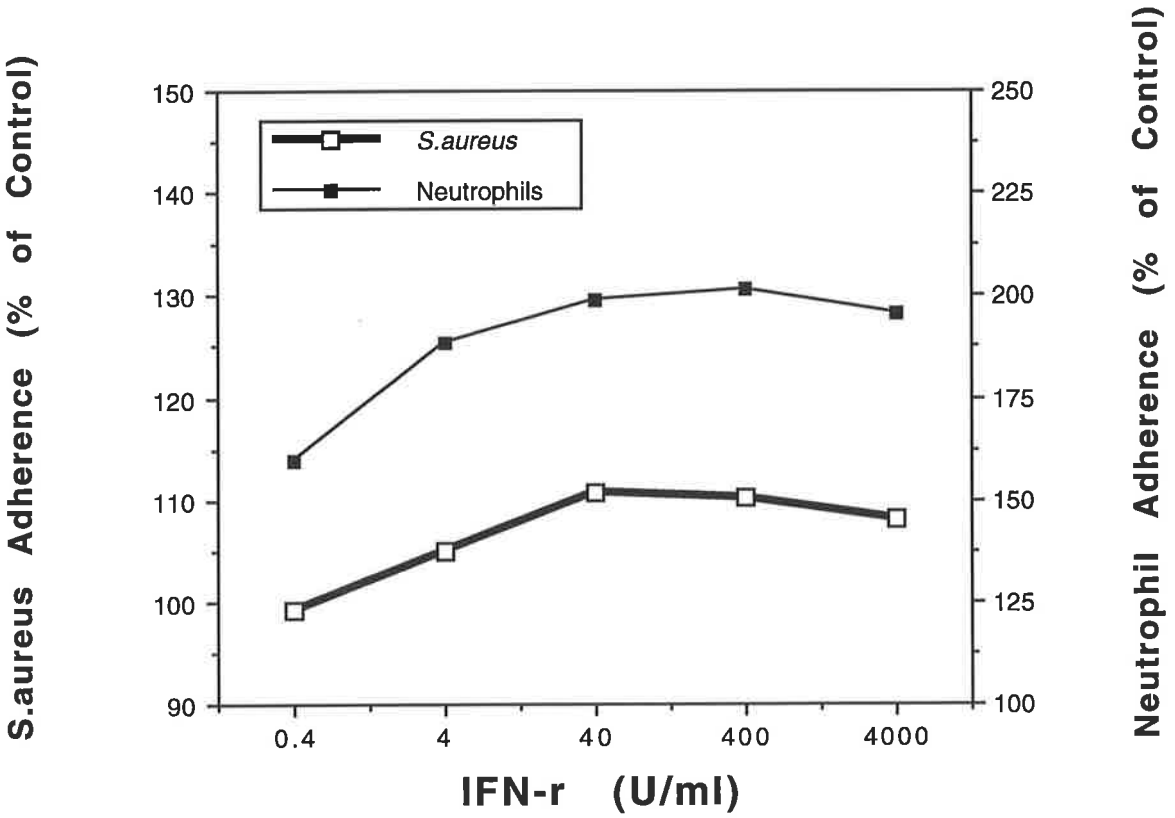


Fig. 4.9 The effect of IFN-γ on adhesion of *S. aureus* or neutrophils to HUVEC. HUVEC were incubated with varying concentrations of IFN-γ for 4h. Then the monolayers were washed and *S. aureus* or neutrophils added. The results are presented as the mean of three experiments, each conducted in triplicate.

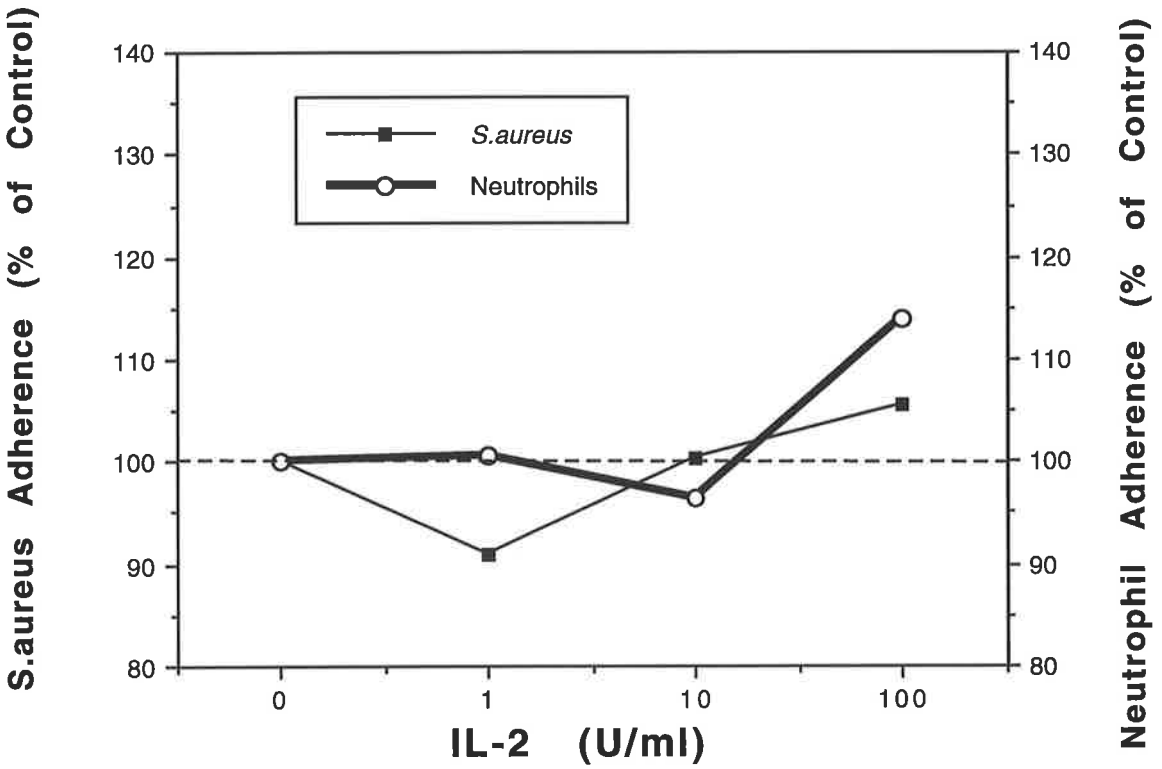


Fig. 4.10 The effect of IL-2 on adhesion of *S. aureus* or neutrophils to HUVEC. HUVEC were incubated with varying concentrations of IL-2 for 4h. Then the monolayers were washed and *S. aureus* or neutrophils added. The results are presented as the mean of three experiments, each conducted in triplicate.

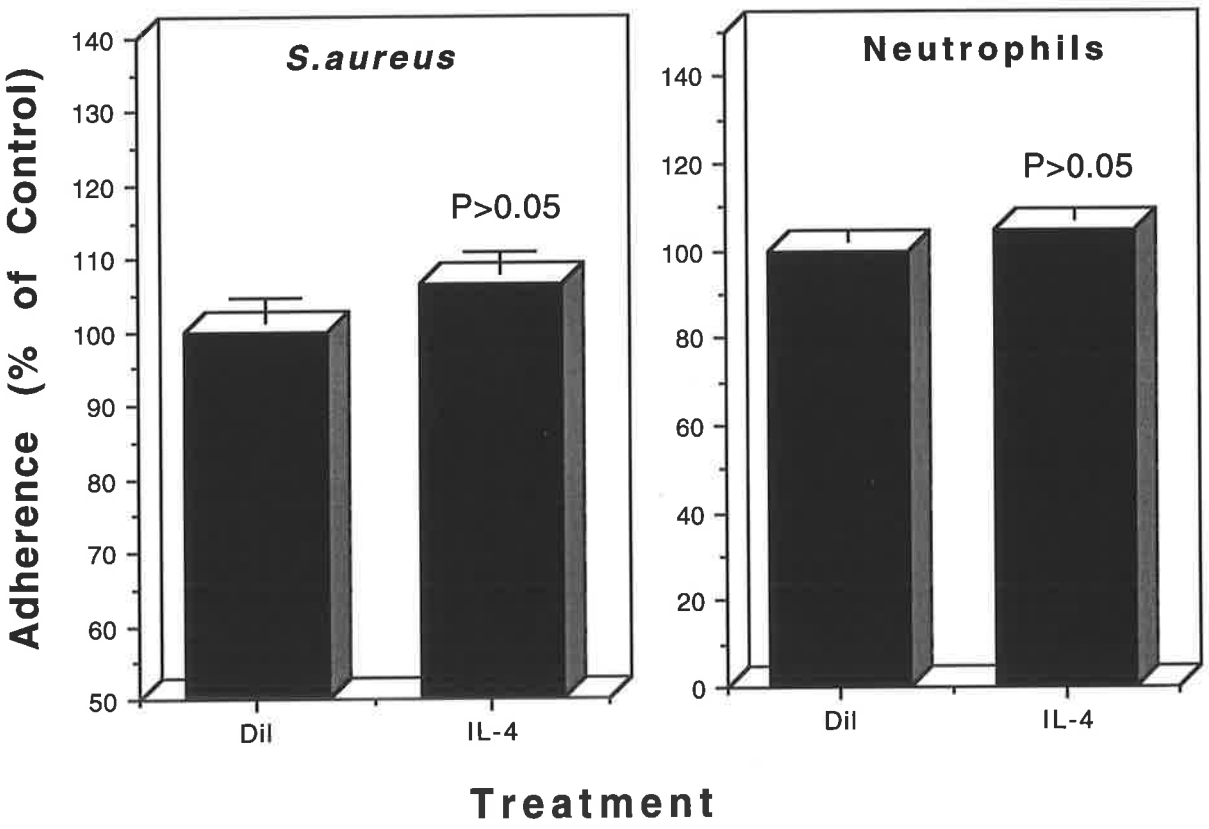


Fig. 4.11 The effect of IL-4 on adhesion of *S. aureus* or neutrophils to HUVEC. HUVEC were incubated with 100U/ml of IL-4 for 4h. Then the monolayers were washed and *S. aureus* or neutrophils added. The results were presented as the mean±sem of three experiments, each conducted in triplicate. P value is the difference from the diluent treatment.

4.4 Summary

The data showed that LT decreased adhesion of *S. aureus* to HUVEC. The ability of LT to induce these changes in endothelial cells was preincubation time-dependent and concentration-dependent. LT was effective at 4U/ml. It is of interest that TNF which binds to the same receptor as LT required much higher concentration to achieve similar effects on bacterial adherence. However, with respect to neutrophil-endothelial adhesion, higher concentrations of LT were required compared with TNF. These activities of LT were attributed to the cytokine itself and not a contaminant in the preparation since the activity could be abolished by boiling and the addition of anti-LT monoclonal antibodies, and it was not sensitive to PMB. Both the LT-induced depression of bacterial adhesion and enhancement of neutrophil adhesion were highly sensitive to the RNA inhibitor, actinomycin D and protein synthesis inhibitor, cycloheximide suggesting an important role for new RNA and protein synthesis in the expression of the LT-induced changes on HUVEC.

The other lymphokines, products of activated T lymphocytes (IL-2, IL-4 and IFN-γ), had no effect on adhesion of *S. aureus* or neutrophils to HUVEC.

CHAPTER 5

THE COMBINATION EFFECTS OF CYTOKINES ON ADHESION OF *S.aureus* TO HUVEC

5.1 Introduction

Cytokines are usually seen in the context of a network involving the interplay between different cytokines having similar or different effects on target tissues. It is therefore of importance to know whether the addition of a combination of cytokines to HUVEC influences their bacterial adhesion properties. It has been previously reported that cytokines can be synergistic or be antagonistic in their biological effects on a variety of tissues. Studies along these lines are important since they will give a better perspective on the regulation of the inflammatory responses by cytokines in bacterial infections.

5.2 The Effects of IFN-γ on the TNF-, LT- and IL-1β-induced Alteration of *S. aureus* Adhesion to HUVEC

IFN-γ activates endothelial cells and increases the expression of ICAM-1 and MHC class I antigen and the *de novo* induction of MHC class II antigens (Pober et al, 1986; 1983). This cytokine also shows synergistic or antagonistic effects with other cytokines in the regulation of endothelial cell functions. Although IFN-γ does not induce the expression of E-selectin on endothelial cells, it upregulates and prolongs the expression of E-selectin on TNF- and IL-1α-treated endothelial cells by increasing the stability of this molecule. IFN-γ also abrogates the refractory period for restimulating the expression of E-selectin (Pober et al, 1990; Leeuwenberg et al, 1990). The expression of ICAM-1 is also synergistically increased by the combination of IFN-γ and TNF (Pober et al, 1990). IFN-γ is synergistic with TNF, IL-1 and LPS in the production of IL-6 by endothelial cells (Leeuwenberg et al, 1990). Alteration of endothelial cell morphology is caused by treatment

with IFN-γ and this effect is synergistic with either TNF or LT (Stolpen et al 1986). IFN-γ increases TNF receptor number on several tumor cell lines (Aggarwal et al, 1985), although not on endothelial cells (Johnson et al 1990). Wojta et al (1992) and Niedbala et al (1992) demonstrated that IFN-γ prevented the TNF- and IL-1-induced expression of urokinase-type plasminogen activator in endothelial cells.

In this study, the effects of IFN-γ on the TNF-, LT- and IL-1β-induced alteration in bacterial and neutrophil adhesion to HUVEC were examined. HUVEC pretreated for 4h with 400U/ml of IFN-γ showed no significant changes in their ability to adhere either bacteria or neutrophils (Fig. 5.1). However, IFN-γ was found to inhibit the ability of TNF to alter bacterial but not neutrophil adhesion to HUVEC (Fig. 5.1). In these experiments the HUVEC were pretreated with 400U/ml of IFN-γ together with 400U/ml of TNF for 4h. Under these conditions the effect of IFN-γ was to prevent the TNF-induced decrease in bacterial adhesion but not the TNF-induced enhancement of neutrophil adhesion to endothelial cells (Fig. 5.1).

Similarly, studies on the effects of IFN-γ on the changes induced by IL-1β also showed that when the HUVEC were coincubated for 4h with 400U/ml of IFN-γ and 1ng/ml of IL-1β, the IL-1β-induced decrease in bacterial adherence did not occur (Fig. 5.2). In contrast, IFN-γ did not affect the IL-1β-induced enhancement of neutrophil adhesion (Fig. 5.2).

Not surprisingly, these effects of IFN-γ were also seen with LT, which binds to the same receptor as TNF. HUVEC treated with LT showed a

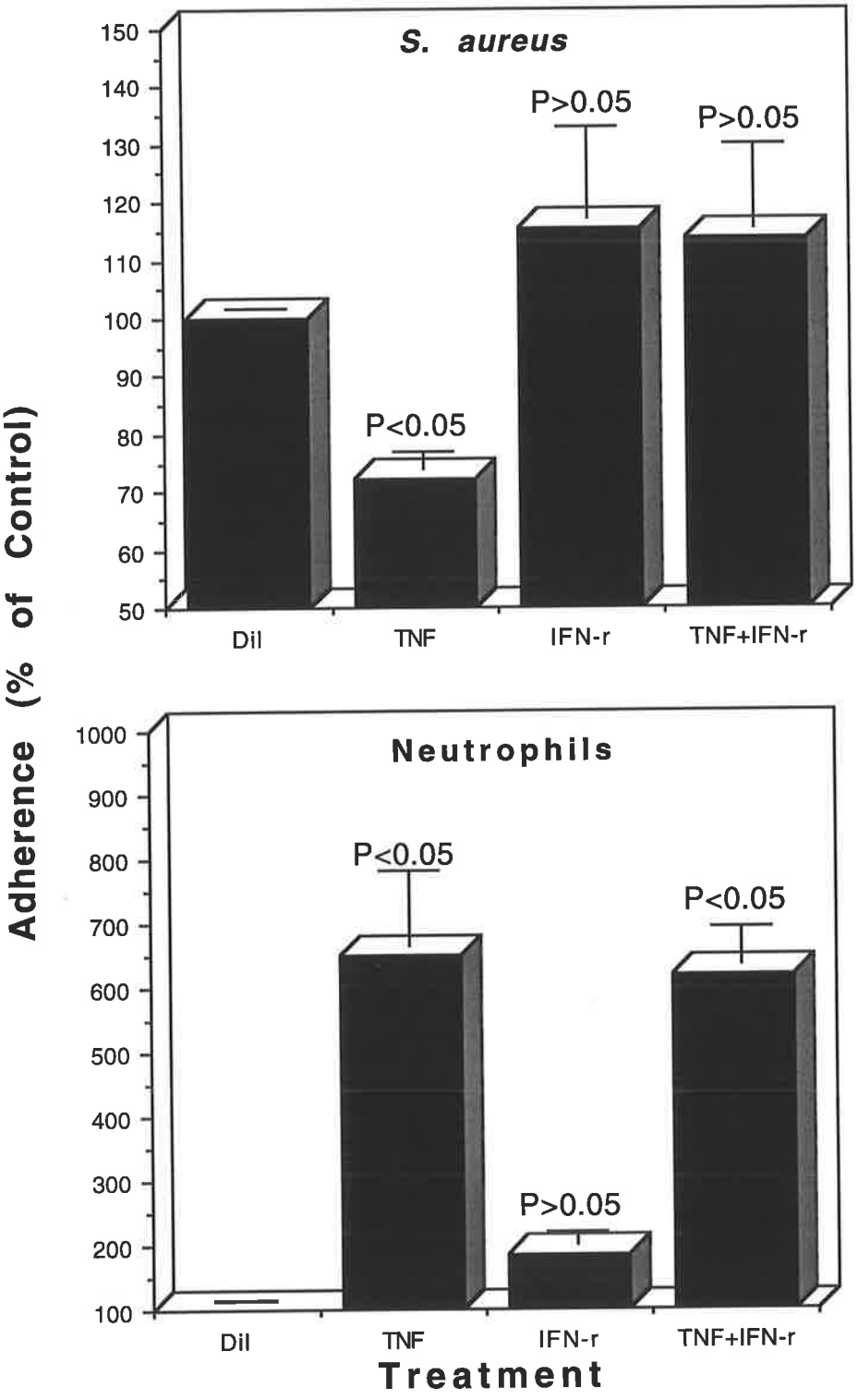


Fig.5.1 The effect of IFN-γ on the TNF-induced decrease in adhesion of *S. aureus* or increase in adhesion of neutrophils to HUVEC. HUVEC were incubated with 400U/ml of TNF in the presence or absence of 400U/ml of IFN-γ for 4h. Then the monolayers were washed and *S. aureus* or neutrophils added. The results are the mean±sem of three experiments, each conducted in triplicate. P value is the difference from the diluent treatment.

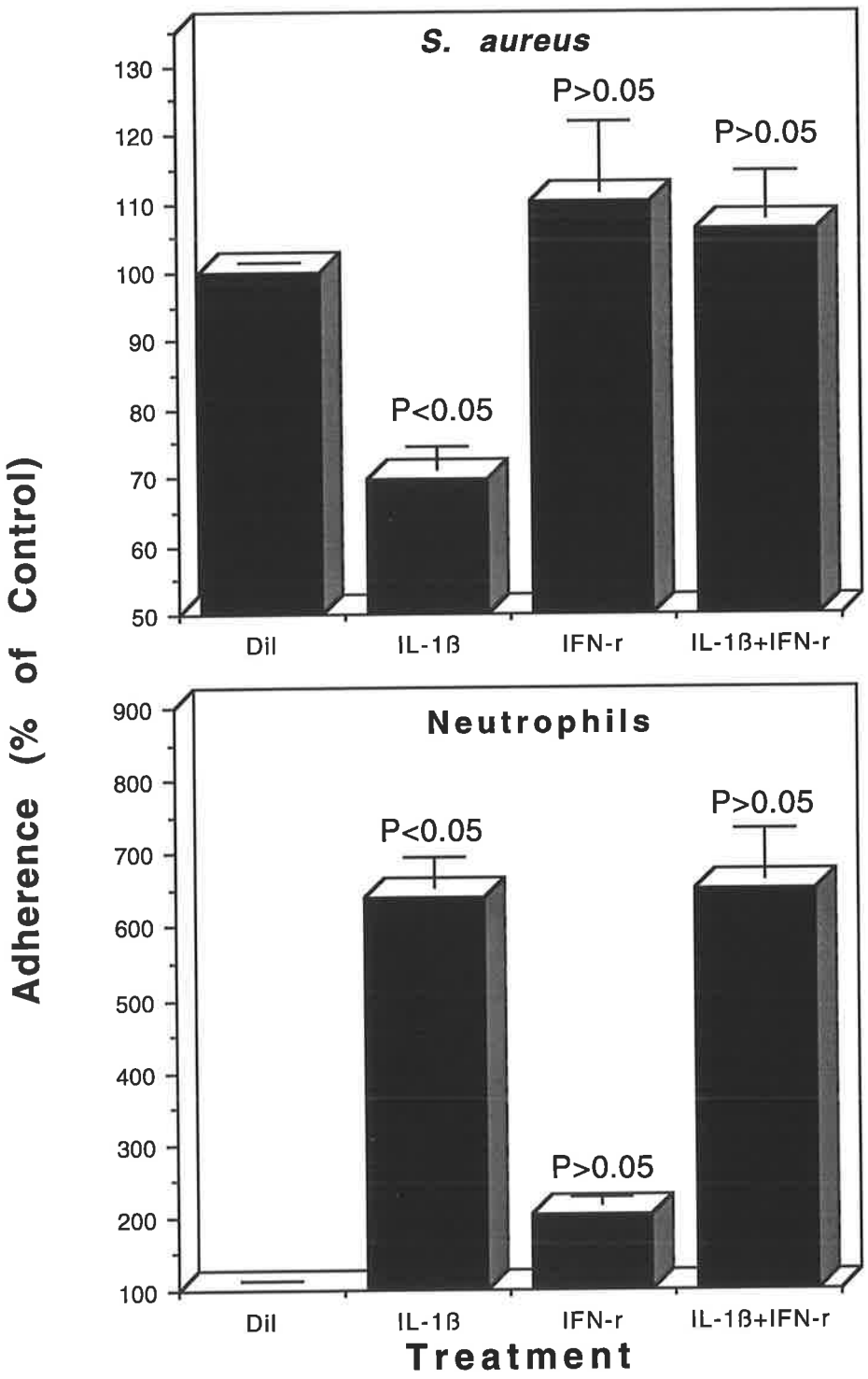


Fig. 5.2 The effect of IFN-γ on the IL-1β-induced decrease in adhesion of *S. aureus* or increase in adhesion of neutrophils to HUVEC. HUVEC were incubated with 1ng/ml of IL-1β in the presence or absence of 400U/ml of IFN-γ for 4h. Then the monolayers were washed and *S. aureus* or neutrophils added. The results are the mean±sem of three experiments, each conducted in triplicate. P value is the difference from the diluent treatment.

a significant decrease in bacterial adhesion and a significant increase in neutrophil adhesion. The effects of LT on bacterial adherence were lost when endothelial cells were coincubated with 400U/ml of LT and 400U/ml of IFN-γ (Fig. 5.3). Such IFN-γ treatment had no significant effect on the LT induced enhancement of neutrophil adhesion. (Fig. 5.3).

IFN-γ inhibited the TNF-, LT- and IL-1β-induced depression of bacterial adhesion in a concentration-related manner (Fig. 5.4, 5.5, 5.6). With 400U/ml of TNF, the inhibitory effects were seen at 0.4U/ml of IFN-γ and total inhibition at 4-4000U/ml (Fig 5.4). This concentration-related effect of IFN-γ was also seen with IL-1β (Fig. 5.5) and LT (Fig. 5.6). At the concentration range of 0.4U/ml-4000U/ml of IFN-γ, the cytokine had essentially no significant effect on the TNF-, IL-1β-, or LT-induced enhancement of neutrophil adhesion (Fig. 5.4, 5.5, 5.6).

5.3 Inhibition of the TNF-, LT- and IL-1β-induced Alteration of *S. aureus* Adhesion to HUVEC by IL-4

IL-4 is a lymphokine with diverse functions. The cytokine has been reported to alter the expression of VCAM-1 on endothelial cells to promote lymphocyte adhesion to and transmigration through endothelial cells (Masinovsky et al, 1990). However, IL-4 depresses the TNF-, IL-1β- and LPS-induced expression of E-selectin and the TNF-, IL-1β- and IFN-γ-induced expressions of ICAM-1 by endothelial cells (Thornhill et al, 1990). Previously we have shown that IL-4 does not affect bacterial adhesion to HUVEC (chapter 4) and here we have

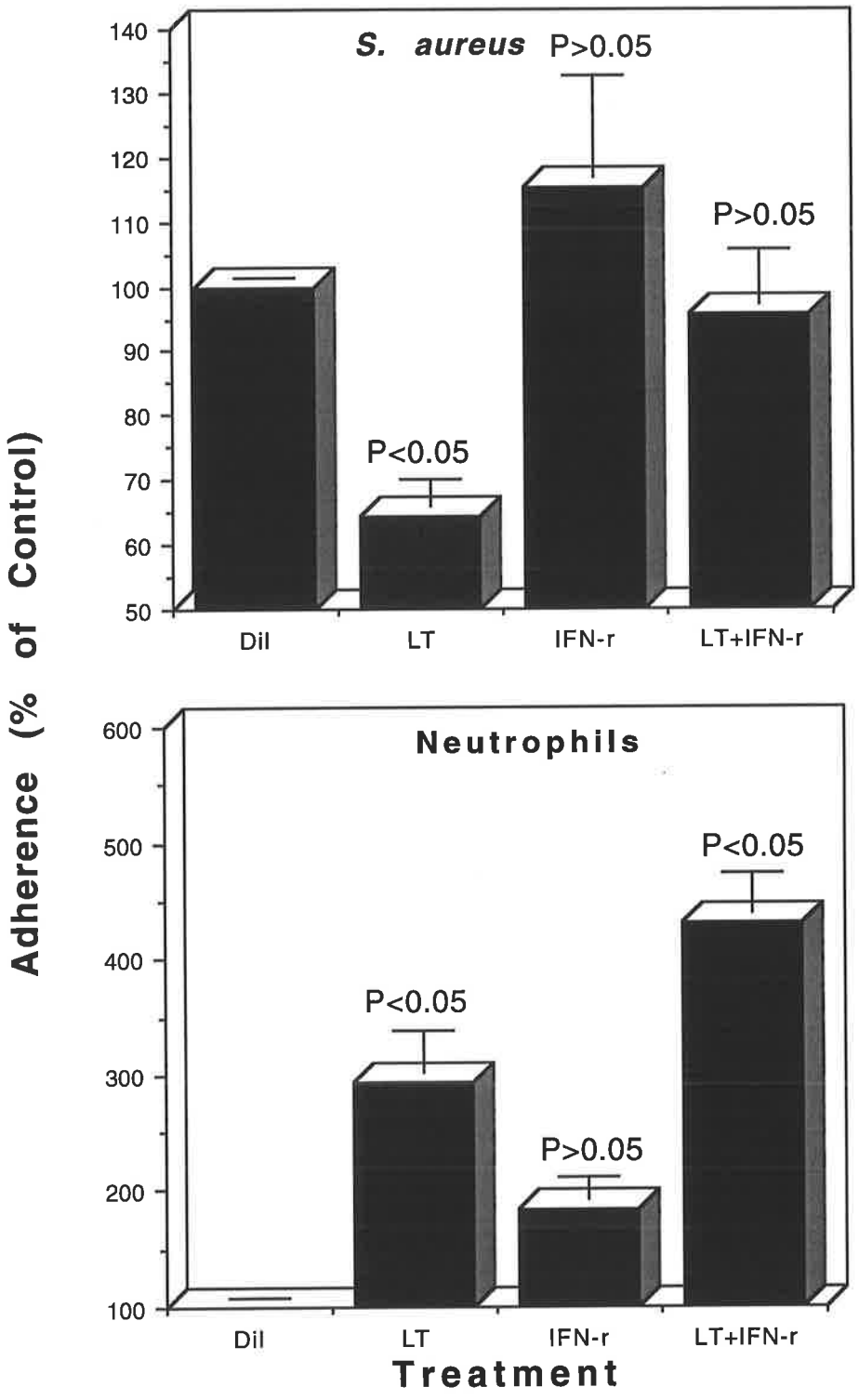


Fig. 5.3 The effect of IFN-γ on the LT-induced decrease in adhesion of *S. aureus* or increase in adhesion of neutrophils to HUVEC. HUVEC were incubated with 400U/ml of LT in the presence or absence of 400U/ml of IFN-γ for 4h. Then the monolayers were washed and *S. aureus* or neutrophils added. The results are the mean±sem of three experiments, each conducted in triplicate. P value is the difference from the diluent treatment.

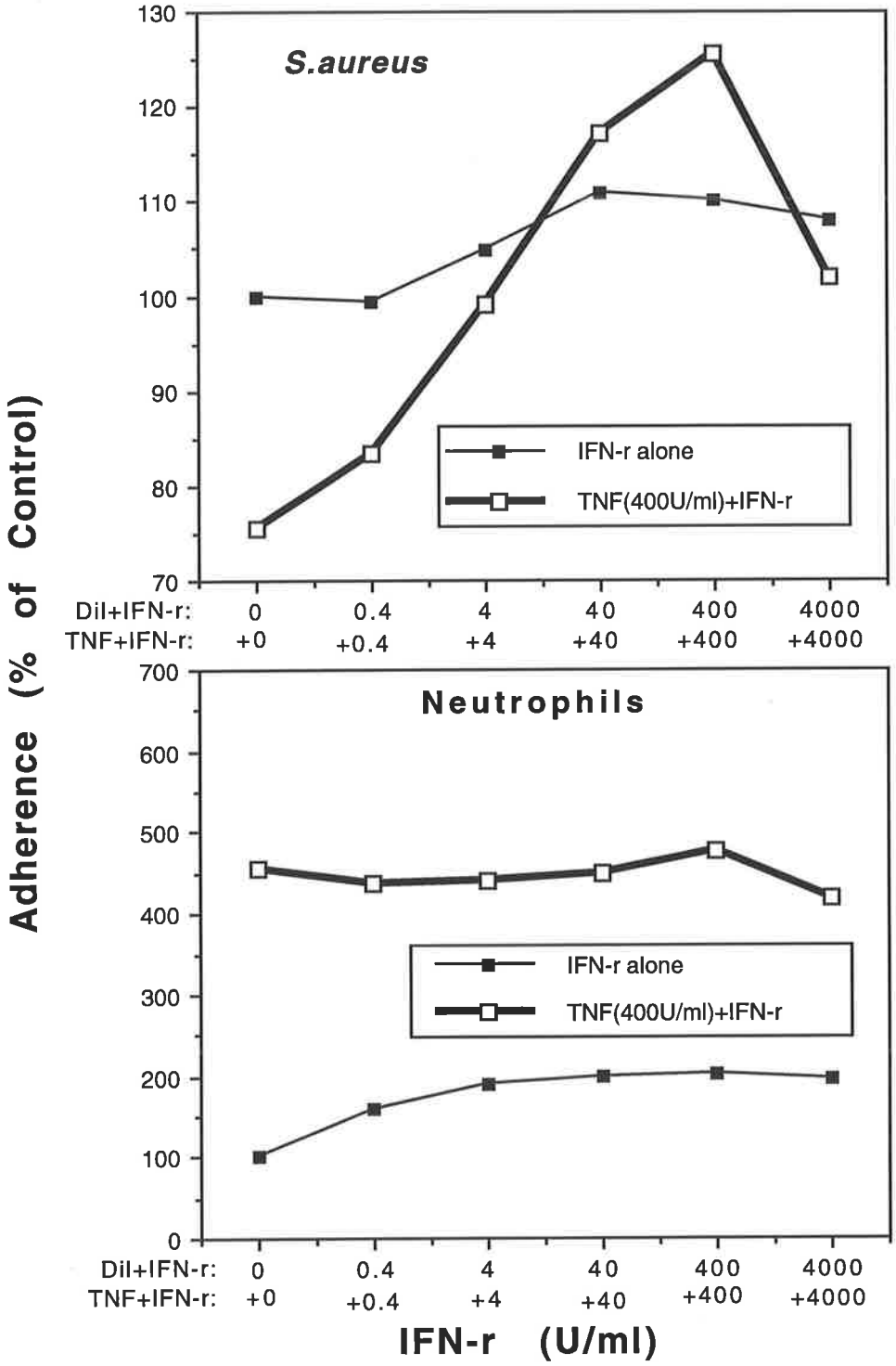


Fig. 5.4 The effect of different IFN-γ concentrations on the TNF-induced decrease in adhesion of *S. aureus* or increase in adhesion of neutrophils to HUVEC. HUVEC were incubated with 400U/ml of TNF in the presence or absence of 0-4000U/ml of IFN-γ for 4h. Then the monolayers were washed and *S. aureus* or neutrophils added. The results are the mean of four experiments, each conducted in triplicate.

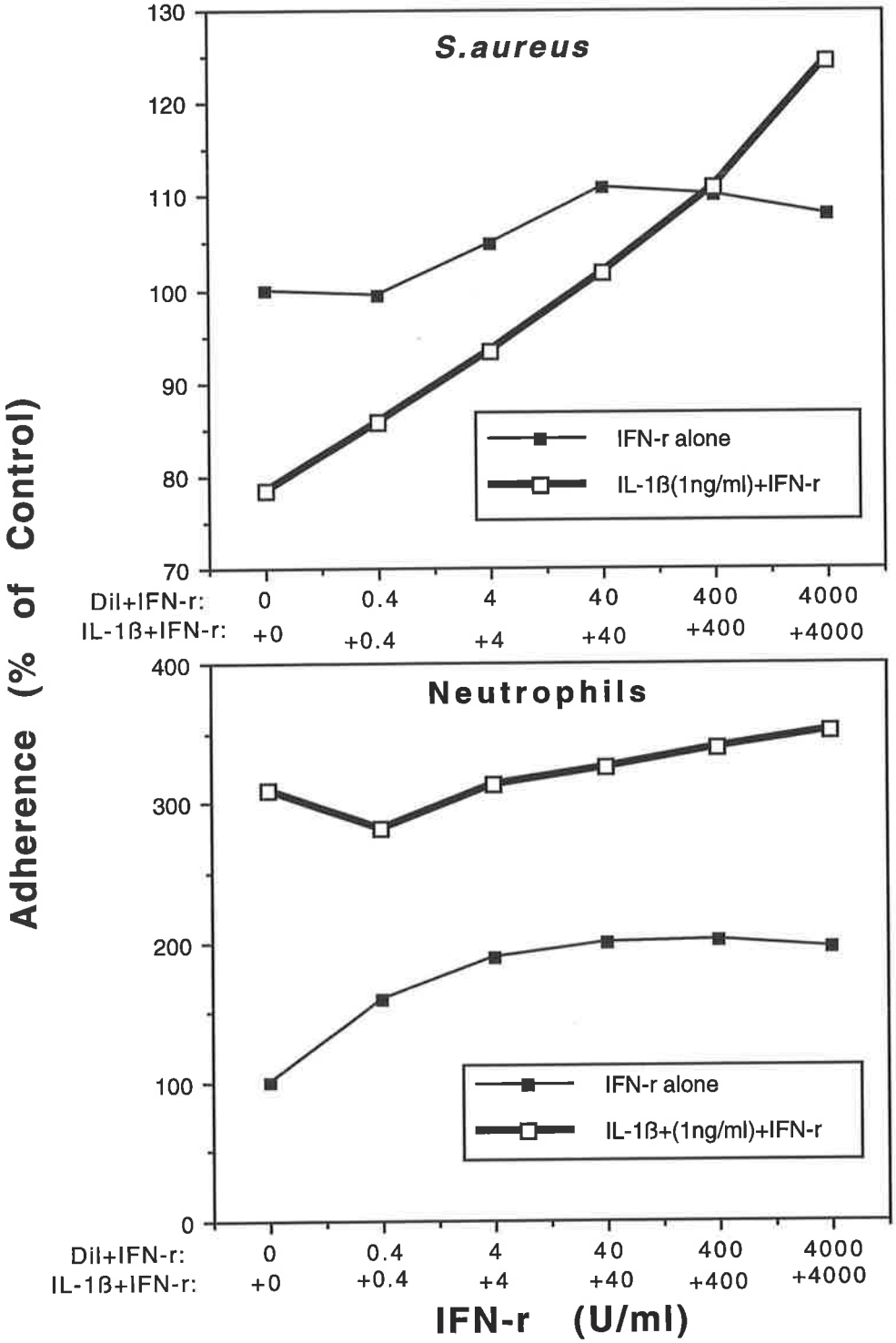


Fig. 5.5 The effect of different IFN-γ concentrations on the IL-1β-induced decrease in adhesion of *S. aureus* or increase in adhesion of neutrophils to HUVEC. HUVEC were incubated with 1ng/ml of IL-1β in the presence or absence of 0-4000U/ml of IFN-γ for 4h. Then the monolayers wells were washed and *S. aureus* or neutrophils added. The results are the mean of four experiments, each conducted in triplicate.

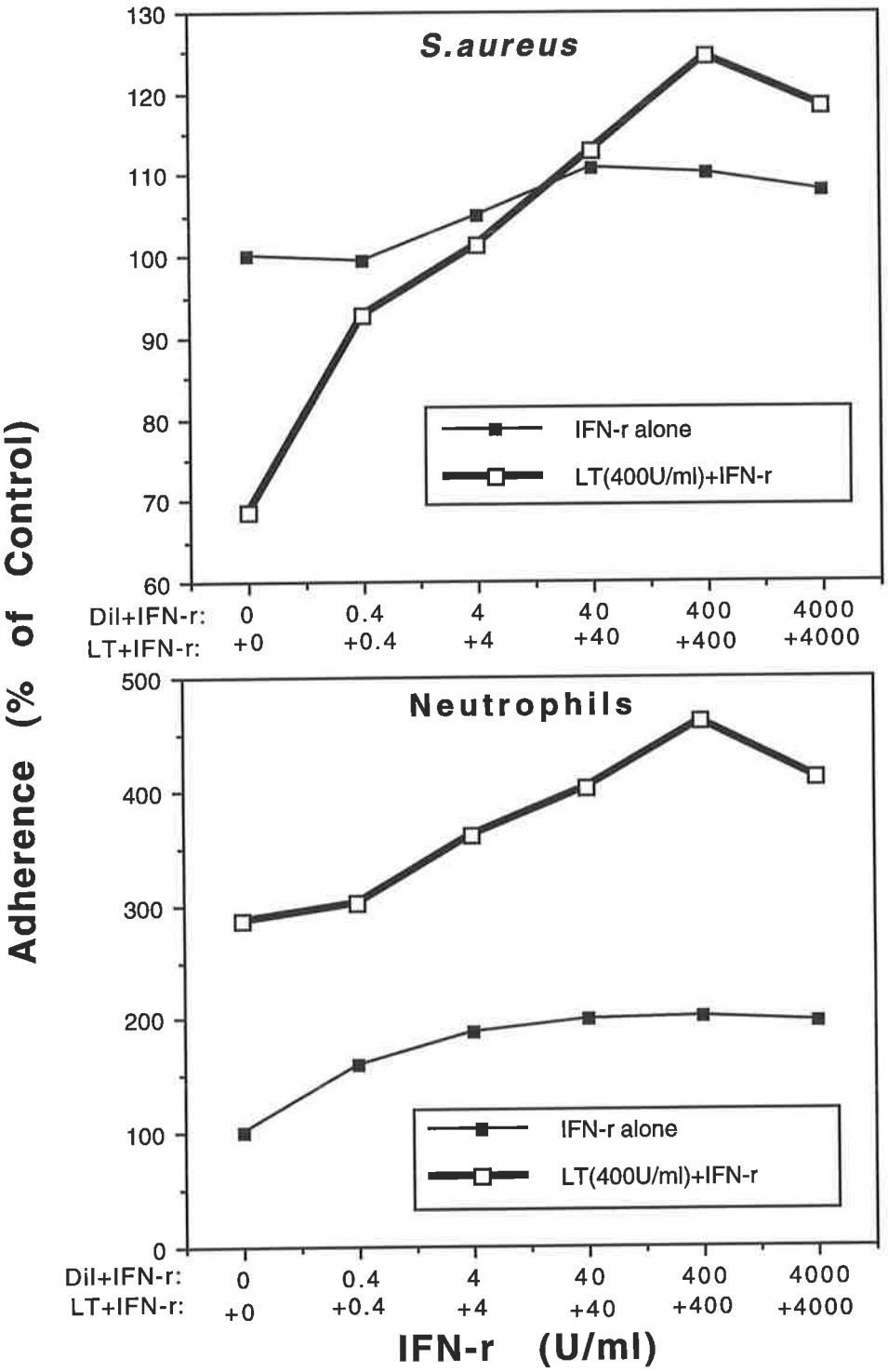


Fig. 5.6 The effect of different IFN-γ concentrations on the LT-induced decrease in adhesion of *S. aureus* or increase in adhesion of neutrophils to HUVEC. HUVEC were incubated with 400U/ml of LT in the presence or absence of 0-4000U/ml of IFN-γ for 4h. Then the monolayers were washed and *S. aureus* or neutrophils added. The results are the mean of four experiments, each conducted in triplicate.

examined whether IL-4 may regulate the expression of immunity to infection by altering the action of other cytokines on bacteria-endothelial cell adherence.

Endothelial cells treated with 100U/ml of IL-4 for 4h showed no change in their ability to adhere bacteria. Preincubation of endothelial cells with IL-4 and either of TNF (400U/ml), IL-1β (1ng/ml), or LT (400U/ml) for 4h reduced the expected TNF-, IL-1β- and LT-induced decrease of *S. aureus* adhesion to HUVEC (Fig. 5.7). In addition, both the TNF- and IL-1β-induced alteration of neutrophil adhesion were also significantly diminished by IL-4. Yet this cytokine enhanced the LT-induced increase of neutrophil adhesion (Fig. 5.8).

5.4 Inhibition of TNF-, LT- and IL-1β-induced Alteration of *S. aureus* Adhesion to HUVEC by IL-8

IL-8 is produced by activated monocytes, lymphocytes, neutrophils and cytokine activated endothelial cells (Yoshimura et al, 1987; Schroder et al, 1988; Bazzoni et al, 1991; Gimbrone et al, 1989). The cytokine was first described as a neutrophil activating peptide which induced shape changes, degranulation and adhesion of neutrophils to endothelial cells (Baggiolini et al, 1989). IL-8 released locally by endothelial cells acts as a neutrophil adhesion inhibitor which prevents neutrophil attachment to TNF- and IL-1β-treated endothelial cells (Gimbrone et al, 1989). An increased level of circulating IL-8 is found in patients with sepsis (Hack et al, 1992). Therefore, it is of interest to know whether IL-8 could modify bacterial adhesion to endothelium.

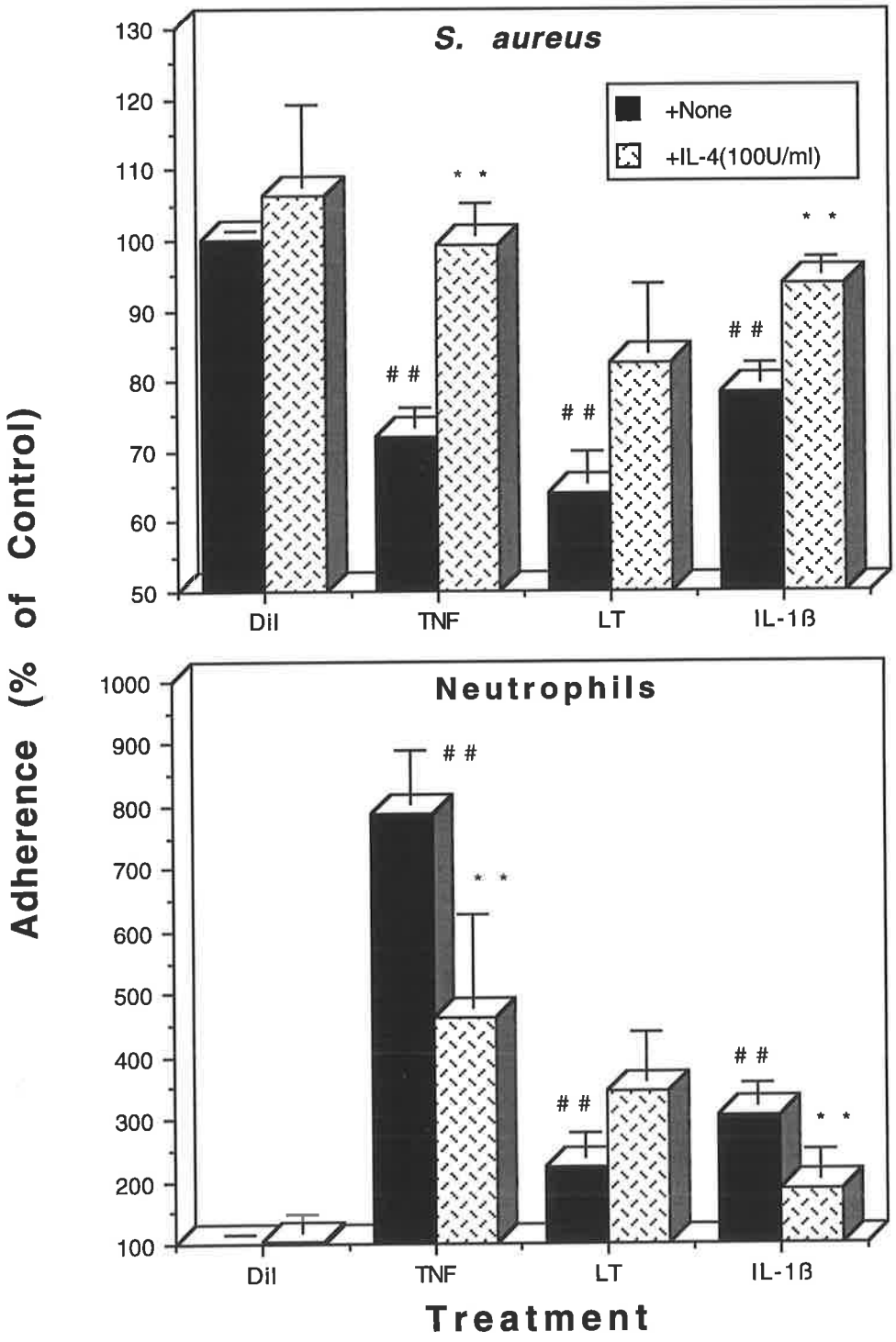


Fig. 5.7 The effect of IL-4 on the TNF-, LT- and IL-1β-induced decrease in adhesion of *S. aureus* or increase in adhesion of neutrophils to HUVEC. HUVEC were incubated with 400U/ml of TNF, 400U/ml of LT or 1ng/ml of IL-1β in the presence or absence of 100U/ml of IL-4 for 4h. Then the monolayers were washed and *S. aureus* or neutrophils added. The results are the mean±sem of three experiments, each conducted in triplicate. ##:P<0.05 is the difference from the diluent treatment. **:P<0.05 is the difference from the nonaddition of IL-4.

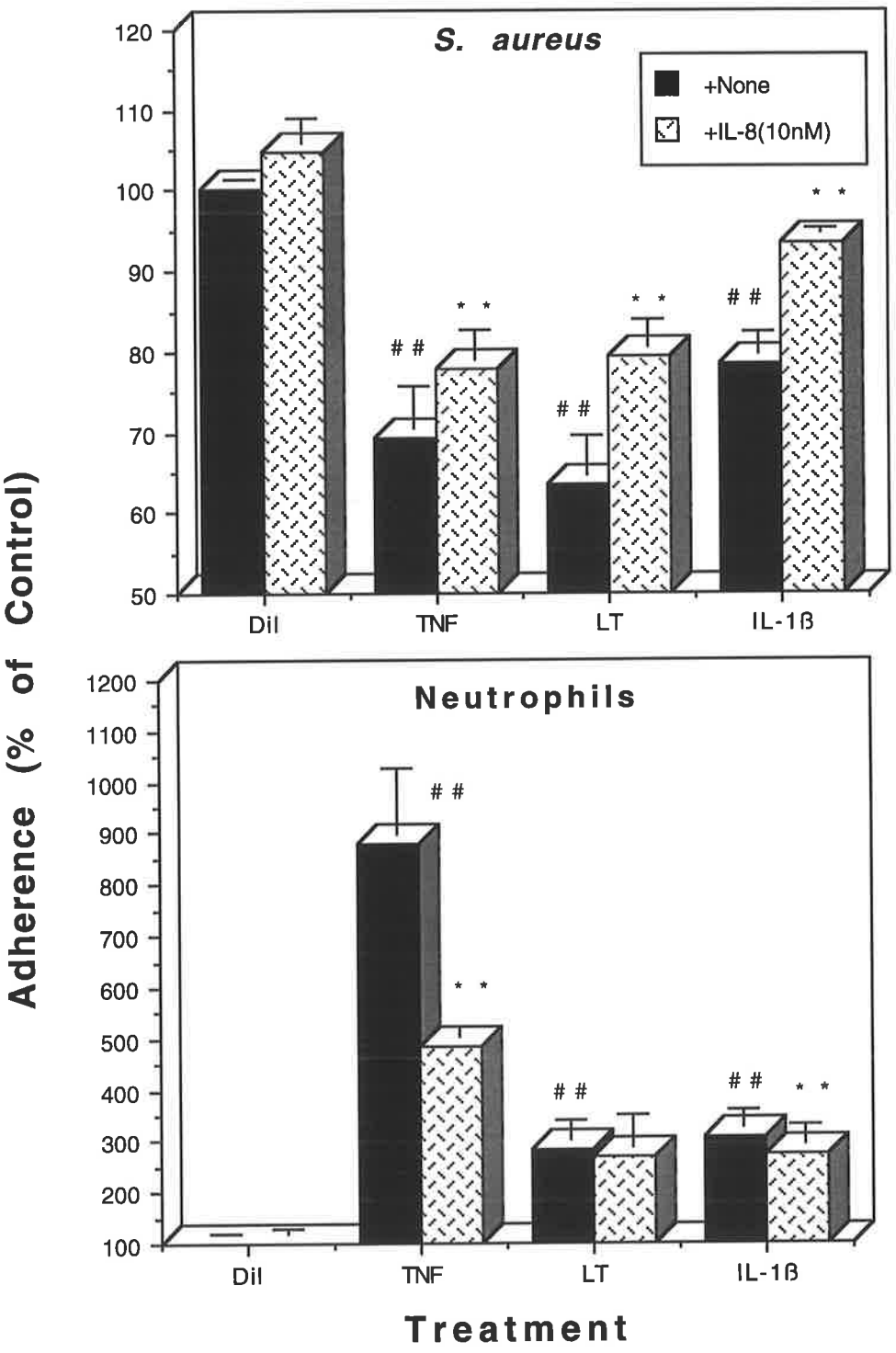


Fig. 5.8 The effect of IL-8 on the TNF-, LT- and IL-1β-induced decrease in adhesion of *S. aureus* or increase in adhesion of neutrophils to HUVEC. HUVEC were incubated with 400U/ml of TNF, 400U/ml of LT or 1ng/ml of IL-1β in the presence or absence of 10nM of IL-8 for 4h. Then the monolayers were washed and *S. aureus* or neutrophils added. The results are the mean±sem of three experiments, each conducted in triplicate. ##P:<0.05 is the difference from the diluent treatment. **:P<0.05 is the difference from the nonaddition of IL-8.

Endothelial cells were treated with optimal concentration of TNF, LT or IL-1β in the presence of 10nM of IL-8 for 4h, and then tested for ability to adhere *S. aureus* or neutrophils. The results presented in Fig. 5.8 show that 10nM of IL-8 moderately inhibited the TNF-, LT- and IL-1β-induced depression of the bacterial adhesion to HUVEC. IL-8 also inhibited the TNF- and IL-1β–, but not LT-induced enhancement of neutrophil adhesion.

5.5 The Effect of Combined Addition of TNF and IL-1β on Adhesion of *S. aureus* to HUVEC.

TNF and IL-1β can be both produced by activated macrophages in the response to bacterial endotoxin and other microbial products. Although these two factors act on endothelial cells via different receptors, their biological properties on tissues are strikingly similar. Both of these monokines increase the production of procoagulant activity by endothelial cells and increase adhesion of neutrophils, monocytes and lymphocytes to endothelial cells by modifying the expressions of E-selectin and ICAM-1 on these cells. TNF and IL-1β also stimulate the production of PGI_2, IL-6, IL-8 and GM-CSF in endothelial cells. Both TNF and IL-1 have been reported to alter endothelial structural integrity which may contribute to the extravasation of leukocytes to underlying tissues (Locksley et al, 1987; Cotran et al, 1989). It was reported that IL-1α suppressed the TNF-induced enhancement of neutrophil adhesion to nylon wool microcolumns (Seow et al, 1987). IL-1β (as little as 0.1ng/ml) inhibited the TNF- and LT-induced increases in RNA and protein synthesis of endothelial cells (Cavender et al, 1988) Here we have examined the

effect of adding TNF and IL-1β to endothelial cells on the ability of these cells to adhere *S. aureus*.

The results (Fig. 5.9) showed that treatment of endothelial cells with a combination of 400U/ml of TNF and 1ng/ml of IL-1β did not result in any differences in the effects seen with either of the cytokines alone. The neutrophil adhesion to endothelial cells treated with both cytokines was also similar to that seen with either of the cytokines alone, although there was some evidence that the higher adhesion seen with TNF was reduced to that of IL-1β when the cytokines were used in combination.

5.6 Summary

The major findings from these studies was that, although some cytokines as single mediators do not alter adhesion of S. *aureus* to endothelial cells, they can still affect the ability of endothelial cells to respond to other cytokines which do alter the bacterial adhesion properties of the tissue. While IFN-γ, IL-4 and IL-8 had no effect on bacterial adhesion when each was added individually to HUVEC, these cytokines affected the abilities of TNF, LT and IL-1β to alter bacterial adhesion to endothelial cells. IL-4 and IL-8 significantly reduced the decreases of bacterial adhesion induced by TNF, LT and IL-1β, and IFN-γ abolished the effects of TNF LT and IL-1β. Measuring neutrophil adhesion under the same conditions showed that IFN-γ had no effect on the TNF-, LT- and IL-1β-induced enhancement of neutrophil adhesion to HUVEC. However IL-4 and IL-8 did show a moderate and significant reduction in the TNF- and IL-1β-induced

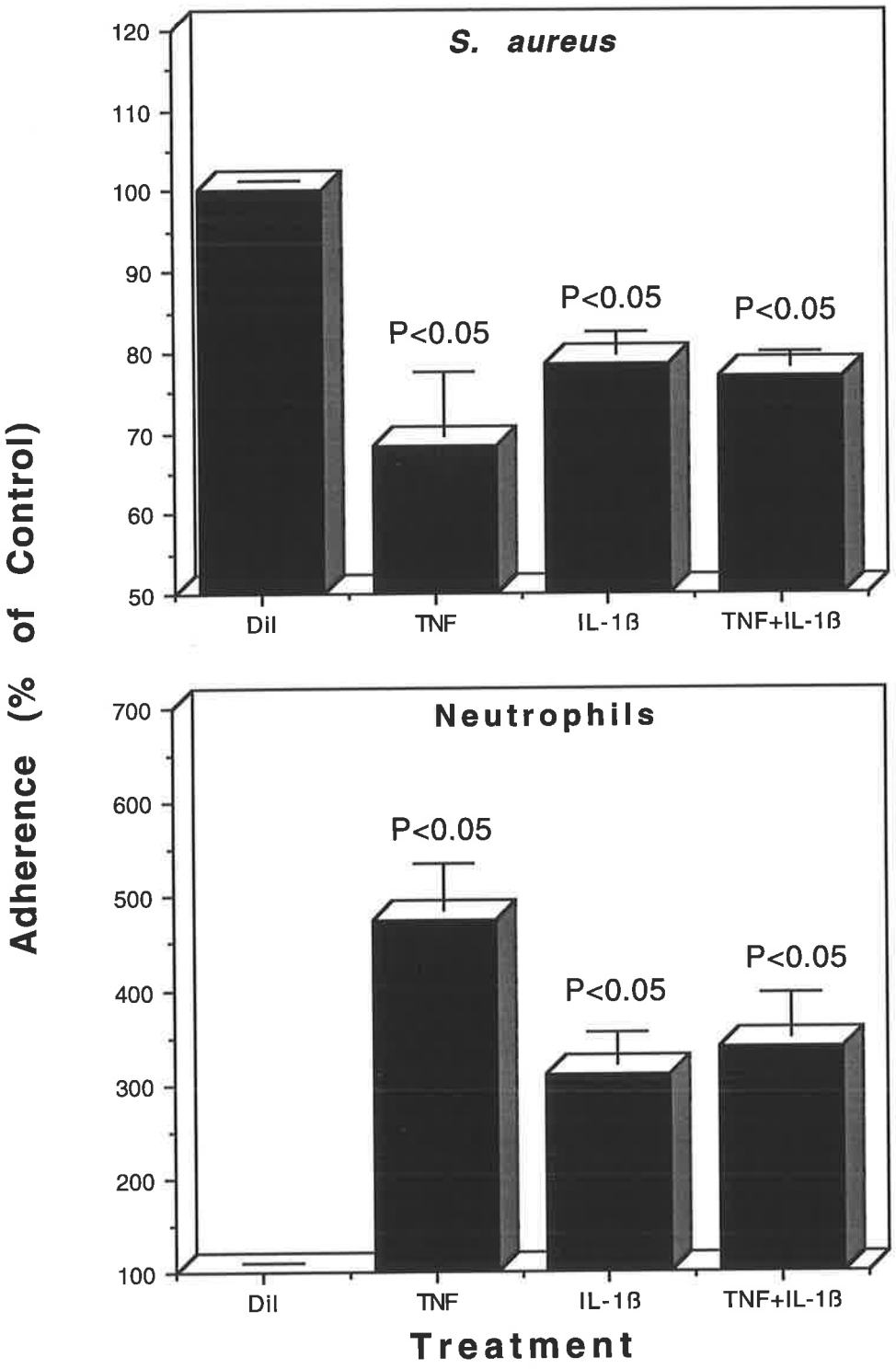


Fig. 5.9 The effect of combined addition of IL-1β and TNF on the decrease in adhesion of *S. aureus* or increase in adhesion of neutrophils to HUVEC. HUVEC were incubated with 400U/ml of TNF, and (or) 1ng/ml of IL-1β for 4h. Then the monolayers were washed and *S. aureus* or neutrophils added. The results are the mean±sem of three experiments, each conducted in triplicate. P value is the difference from the diluent treatment.

increase in neutrophil adhesion. The combined addition of TNF and IL-1β to HUVEC did not alter the effects produced by the individual cytokines.

CHAPTER 6

MECHANISMS OF TNF, LT AND IL-1β INDUCED ALTERATION OF BACTERIA-ENDOTHELIAL CELL ADHESION: THE ROLE OF PAF AND PROTEIN KINASE C

6.1 Introduction

Activation of endothelial cells by cytokines, TNF, LT and IL-1, leads to the alteration of these cells' adhesive property towards neutrophils (Cotran et al, 1989). These cytokines also stimulate endothelial cells to produce PAF which has been considered as a signal for the recruitment of leukocytes to the inflammatory sites (Zimmerman et al, 1992). It is also evident that PMA, an activator of PKC, like TNF also increases the expression of endothelial cell adhesion molecules, ICAM-1 and E-selectin, associated with the augmentation of neutrophil adhesion to endothelial cells (Ritchie et al, 1991; Gudewicz, 1989). In addition, the production of PAF by endothelial cells can be induced via the activation of PKC by PMA (Whatley et al, 1989).

PAF, a potent phospholipid autacoid, was first described as an activator of platelets (Benveniste et al, 1972). On endothelial cells, PAF is not constitutively expressed but can be induced within minutes following cell stimulation (Bussolino et al, 1988). The synthesis of PAF can occur either by the *de novo* pathway or the remodelling pathway (Lee et al, 1989). The *de novo* pathway continuously generates a basal level of PAF. This pathway is usually not available to stimulation The remodelling pathway is responsible for the production of excess amounts of PAF following stimulation with a variety of stimuli, such as, TNF, IL-1, Leukotriene C_4/D_4, thrombin, histamine, bradykinin, etc. (Bussolino et al, 1988; Zimmerman et al, 1992). This pathway involves two important enzymes: phospholipase A_2 (PLA_2) and acetyl-ConA-dependent acetyltransferase. The PLA_2 hydrolyses the PAF precursor to Lyso-PAF, the second step is the acetylation of Lyso-PAF to PAF by acetyltransferase. The PKC activator, PMA, has been shown

to increase the production of PAF by activating PLA_2 which enhances the production of the immediate PAF precursor (Whatley et al, 1989). The activity of acetyltransferase in endothelial cells can be upregulated by TNF, IL-1 and calcium ionophore A23187 (Bussolino et al, 1988). PAF is degraded by acetylhydrolases which can control the intracellular and plasma PAF levels (Blank et al, 1981; Farr et al, 1980).

It has been shown that cytokines including TNF, LT and IL-1 induce the synthesis of PAF in endothelial cells (Bussolino et al, 1990). Eighty percent of the induced PAF in endothelial cells remains cell associated and the remainder released (Camussi et al, 1987). The synthesis of PAF by TNF-, IL-1- and LT-treated endothelial cells requires new protein and RNA synthesis (Bussolino et al, 1988).

PKC is involved in signal transduction for a variety of cell surface acting stimuli. While the role of PKC in the TNF- and IL-1-induced endothelial cell activation is not clearly understood, treatment of endothelial cells with PMA seems to induce similar effect to TNF or IL-1 on endothelial cells which induces an increase in neutrophil adhesion, expression of endothelial cell adhesion molecule ICAM-1 and production of PAF (Gudewicz, 1989; Lane et al, 1989; Heller et al, 1991). However, alternative pathway(s), beside activation of PKC, have also been found to be involved in the alteration of endothelial cell properties (Ritchie et al, 1991).

In this study, the role of PAF as a mediator in the cytokine-induced alteration of HUVEC for adhesion of bacteria was examined. The

studies were extended to determine the possible role of PKC in the mechanisms by which cytokines alter this endothelial cell function.

6.2 Effect of the PAF Antagonist WEB 2086 on the Cytokine-Induced Alteration of Bacterial Adhesion to HUVEC

It has been shown that addition of PAF receptor antagonists during the adhesion of neutrophils to endothelial cells partially inhibits the IL-1-induced neutrophil adhesion to the cells but not the TNF-induced neutrophil adhesion (Breviario et al, 1988; Zimmerman et al, 1990). WEB 2086, a PAF receptor antagonist, inhibits ^{3}[H]-PAF binding to endothelial cells (Korth et al, 1989). Treatment of TNF- and IL-1-activated endothelial cells with WEB 2086 prevented the neutrophil transmigration, but not the neutrophil adhesion to endothelial cells (Kuijpers et al, 1992). In our laboratory it has been shown that WEB 2086 inhibits PAF-induced human neutrophil respiratory burst and degranulation (Bates et al, 1991). This antagonist was tested for its effects on the TNF-, LT- and IL-1β-induced decrease of bacterial adhesion to HUVEC. In conjunction with its effect on bacterial adhesion, its effect on neutrophil adhesion was also examined.

HUVEC were pretreated with either 400U/ml of TNF, 400U/ml of LT or 1ng/ml of IL-1β for 4h, then washed and further treated with 40μM of WEB 2086 for 5 min. The HUVEC were then tested for ability to adhere either *S. aureus* or neutrophils. The results showed that WEB 2086 inhibited the decreased bacterial adhesion to HUVEC induced by TNF, LT and IL-1β (Fig. 6.1). In relation to neutrophil adhesion, WEB 2086

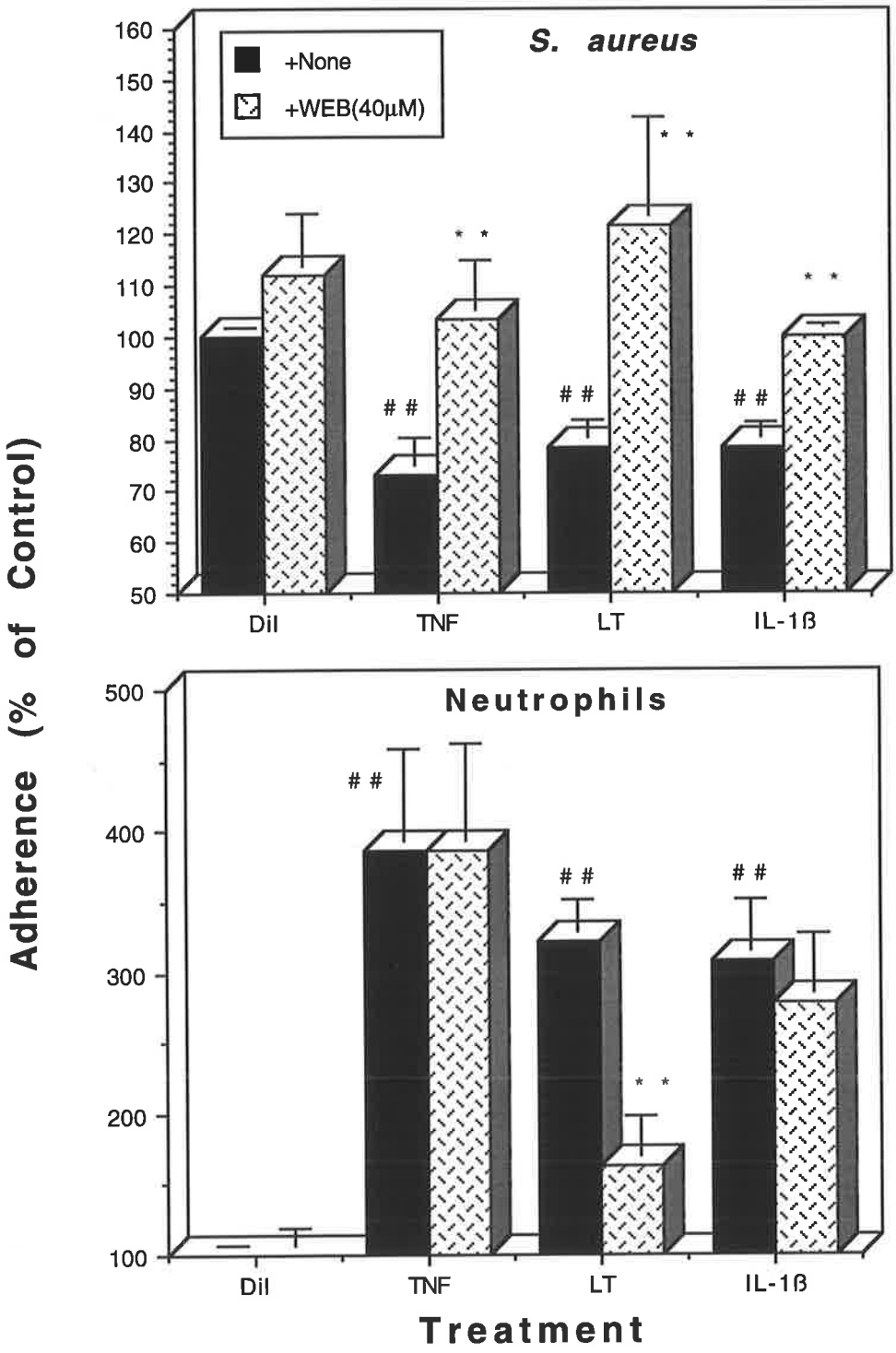


Fig. 6.1 The effect of WEB on the TNF-, LT- and IL-1β-induced decrease in adhesion of *S. aureus* or increase in adhesion of neutrophils to HUVEC. HUVEC were incubated with 400U/ml of TNF, 400U/ml of LT or 1ng/ml of IL-1β for 4h. Then the endothelial cell monolayers were washed and 40μM of WEB was added for 5 min. The results are the mean±sem of three experiments, each conducted in triplicate. ##:P<0.05 is the difference from the diluent treatment. **:P<0.05 is the difference from the nonaddition of WEB.

treatment of HUVEC only affected the enhancement induced by LT ($P<0.05$), but not the changes induced by TNF and IL-1β (Fig. 6.1).

6.3 The Role of Protein Kinase C in the Cytokine-induced Decrease in Bacterial Adherence to Endothelial Cells

In the first aspect of this study we established whether activation of PKC resulted in decreased bacterial adherence to HUVEC. HUVEC were pretreated with PMA, a PKC activator, and then examined for ability to adhere either *S. aureus* or neutrophils. Varying concentrations of PMA (0.001μM-10μM) were tested. The results presented in Fig. 6.2 showed that HUVEC treated for 60 min with PMA had a depressed ability to bind the bacteria. But PMA-treated HUVEC showed enhanced adhesiveness for neutrophils (Fig 6.2). The optimal PMA-induced changes were seen at 0.1μM PMA for both decreased bacterial adherence and increased neutrophil adherence (Fig 6.2).

These results raise the possibility that the cytokines, TNF and LT, may use PKC as part of the signal transduction system to induce the decreased bacterial adherence. To test this we used the PKC inhibitor staurosporine. This inhibitor was standardized on neutrophils by measuring its effects on the PMA-induced respiratory activity. Staurosporine inhibited to more than 95% the PMA-induced superoxide production in human neutrophils (data not presented).

HUVEC were treated for 60 min with 10nM of staurosporine and then tested for ability to respond to TNF and LT. The results showed that

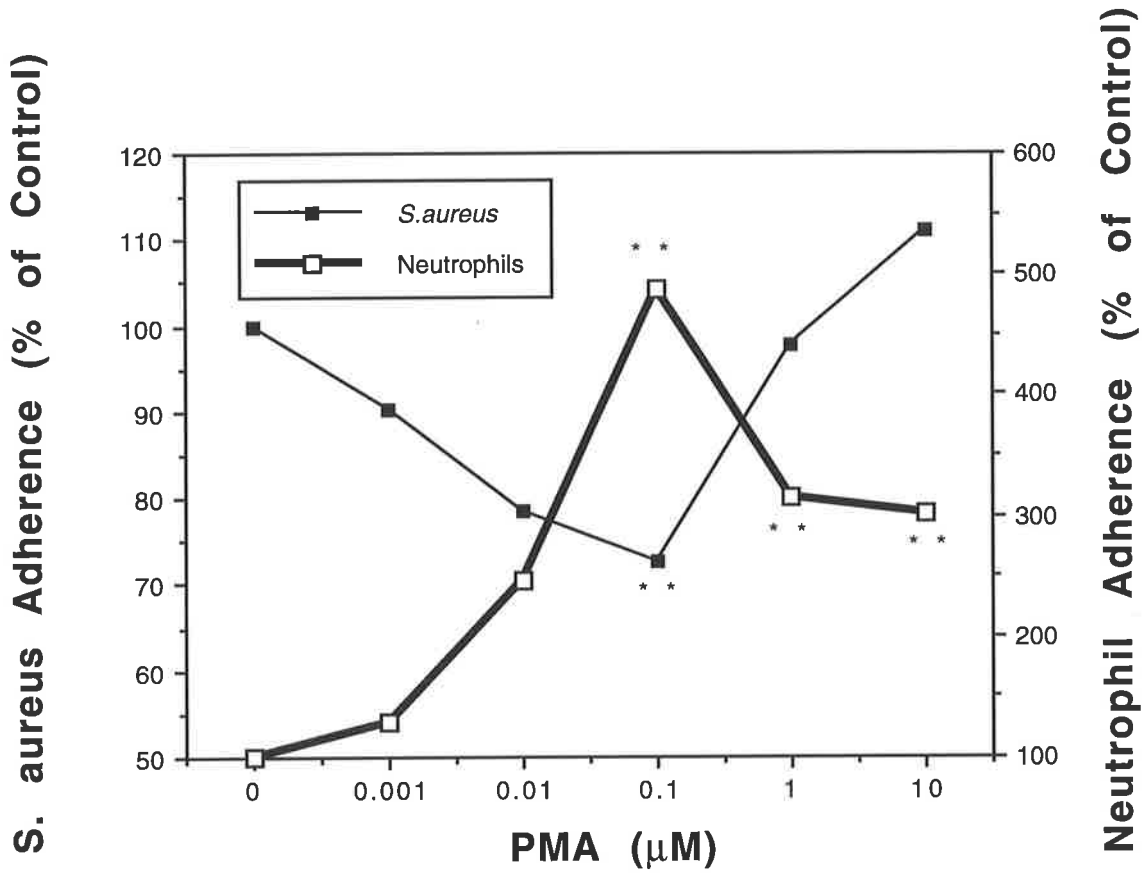


Fig. 6.2 The effect of different PMA concentrations on adhesion of *S. aureus* or neutrophils to HUVEC. HUVEC were incubated with 0.001-10μM of PMA for 60 min. Then the monolayers were washed and *S. aureus* or neutrophils were added. The results are the mean of three experiments, each conducted in triplicate. **:$P<0.05$ is the difference from the diluent treatment.

this inhibitor had no effect on the TNF- and LT-induced depression of bacterial adherence (Fig 6.3). Similarly there was no effect on the TNF- and LT-induced enhancement of neutrophil adherence. Interestingly, while the PMA-induced depression of bacterial adherence was not affected by staurosporine, the enhancement of neutrophil adhesion was abolished (Fig 6.3).

6.4 Summary

The data suggest that TNF-, LT- and IL-1-induced decrease in bacterial adherence to HUVEC involves a common mechanism since in all three cases their activities were inhibited by WEB 2086s, a receptor antagonist of PAF. The results therefore imply a role for this molecule in the cytokine-induced effects on the bacterial adhesion to HUVEC. However, the mechanisms for cytokine-induced alteration of neutrophil adherence to endothelial cell may be different since only the LT-induced effects were inhibited by WEB 2086.

A role for PKC in the cytokine-induced depression of bacterial adherence to HUVEC was ruled out by the findings first that treatment of HUVEC with a PKC inhibitor, staurosporine, did not alter their ability to show the cytokine-induced changes in bacterial adherence. This finding was consistent with the result which showed that the PMA-induced depressed bacterial adherence was not inhibited by staurosporine. Similarly increased neutrophil adherence induced by the cytokines was not sensitive to staurosporine. Staurosporine was active in the system since the PMA-induced enhancement of neutrophil adherence was inhibited by this agent. The optimal

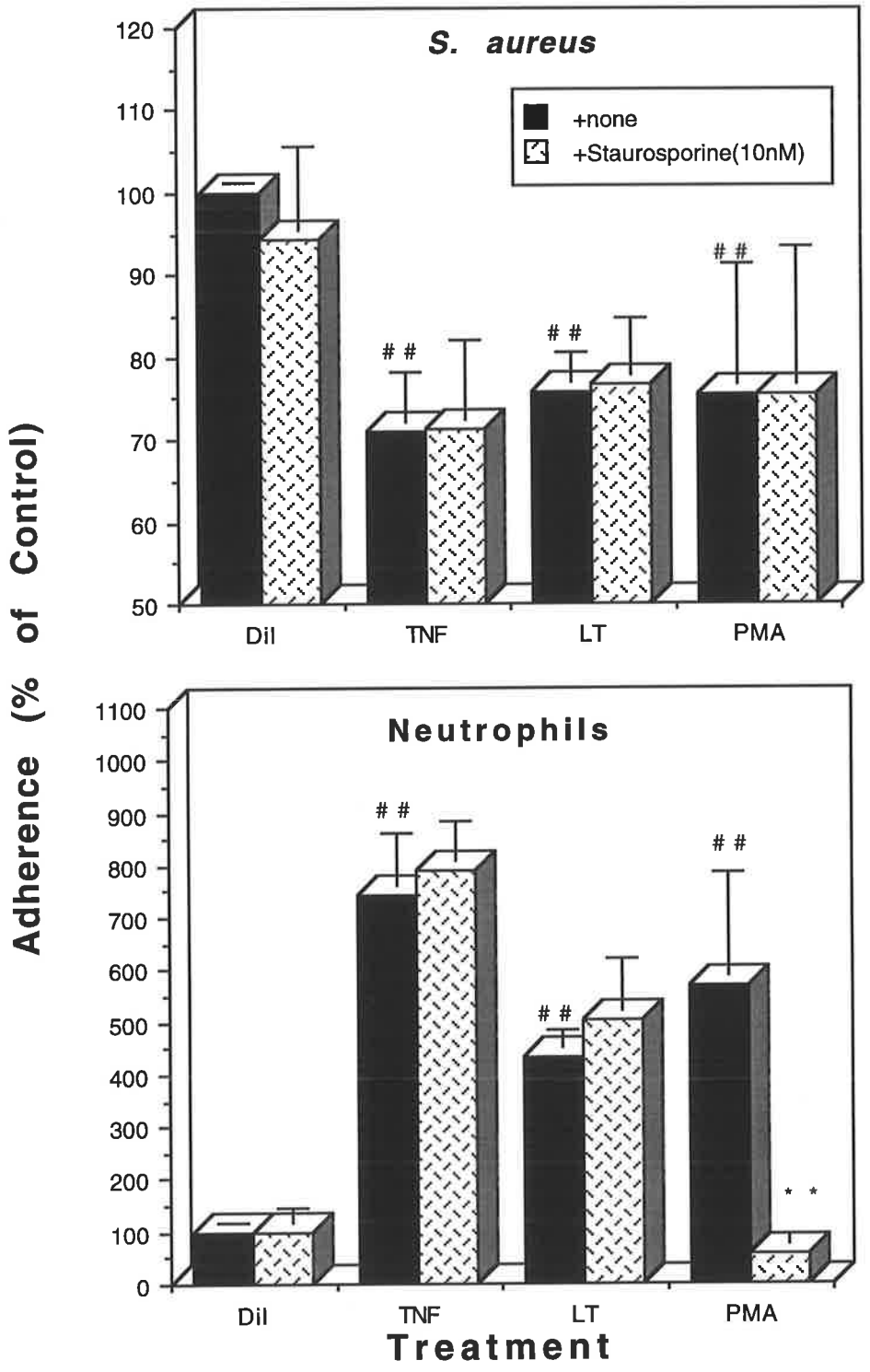


Fig. 6.3 The effect of staurosporine on the TNF-, LT- and PMA-induced alteration of adhesion of *S. aureus* or neutrophils to HUVEC. HUVEC were incubated with 400U/ml of TNF, 400U/ml of LT or 0.1μM of PMA in the presence or absence of 10nM of staurosporine for 4h, 4h and 60 min respectively. Then the monolayers were washed and *S. aureus* or neutrophils added. The results are the mean±sem of three experiments, each conducted in triplicate. ##:$P<0.05$ is the difference from the diluent treatment. **:$P<0.05$ is the difference from the nonaddition of staurosporine.

concentration of PMA which induced the maximal increase in the adherence of neutrophils was concomitant with the maximal decrease in the adherence of bacteria to HUVEC.

CHAPTER 7

DISCUSSION

7.1 Introduction

Adherence of *S. aureus* to endothelial cells is a critical step in the pathogenesis of endocarditis and vascular injury. When pathogenic bacteria gain access to the blood stream, there is a risk of septicemia which could lead to endocarditis, abscess formation and metastatic complications. During bacterial infections, the inflammatory cells and local tissue cells release a range of polypeptide cytokines in response to the invading pathogens. Amongst the range of properties and roles of these cytokines is their ability to increase neutrophil killing of *S. aureus* (Ferrante, 1992) and their alteration of endothelial cell functions (Cotran et al, 1989). The relationship between various cytokines in the cytokine network operating at foci of infection with the respect to the ability of *S. aureus* to adhere to and colonize the endothelial tissue was the focus of this study.

7.2 Induction of Cytokines by *S. aureus*

Staphylococcus has been shown to directly stimulate leukocytes to produce both TNF and IL-1β (Ferrante et al, 1990; Dinarello CA, 1991). Others have demonstrated that products of these bacteria, namely toxic shock syndrome toxin-1 (TSST-1) and enterotoxin B, C are potent inducers of TNF, IL-1β and LT, both *in vitro* and *in vivo* (See et al, 1992; Fisher et al, 1990; Ikejima et al, 1989; Okusawa et al, 1981). Besides its pathogenic implications, cytokine production during a staphylococcal infection may aid the host defence system by activating the neutrophil bactericidal activity (Ferrante, 1992) and, as our study indicates, by limiting colonization of *S. aureus* of endothelium.

7.3 Inhibition of Bacterial Adhesion by Cytokines

The unique finding in this study was that some cytokines can decrease the ability of bacteria to adhere to endothelial cells. Of the cytokines examined (TNF, LT, IL-1β, IFN–γ, IL-2, IL-4 and IL-8), only TNF, LT and IL-1β were found to induce changes in the ability of HUVEC to adhere *S. aureus* (Table 7.1; Fig. 7.1). Treatment of endothelial cells with concentrations of cytokines normally found during infection resulted in significant decreases in bacterial adherence to HUVEC. These results suggest that one role of cytokines in defence against infection may be that of inhibition of bacterial adherence and colonization of endothelial tissues. This is in addition to which previous findings suggest that TNF, LT and IFN–γ may play an important role in host defence by activating the neutrophil bactericidal activity against staphylococcus (Ferrante, 1992). Since monocytes and macrophages are the major cells producing TNF and IL-1β, our results imply an important role for this cell type in modifying endothelial bacterial adherence (Fig 7.1). Activated T lymphocytes may also be important since LT (TNF-β) is specifically produced by this cell type. Receptors for TNF and IL-1β are present on endothelial cells (Shalaby et al, 1990, Dinarello CA, 1991) and LT binds to the same receptors as TNF. IL-1β is also released by many different tissues including endothelial cells (Cotran et al, 1989). Thus local production of cytokines by host tissues may also contribute to defence against bacteria by inhibiting microbial attachment to tissue surfaces.

The TNF-induced decreased bacterial adherence were correlated with an increased neutrophil adherence and were seen over a similar concentration

Table 7.1 The effects of cytokines on *S. aureus* and neutrophil adhesion to HUVEC

Cytokines	*S. aureus* adhesion	Neutrophil adhesion
TNF	↓	↑
LT	↓	↑
IL-1	↓	↑
IL-2	–	–
IL-4	–	–
IL-8	–	–
IFN-γ	–	–

↑: Increase

↓: Decrease

–: No effect

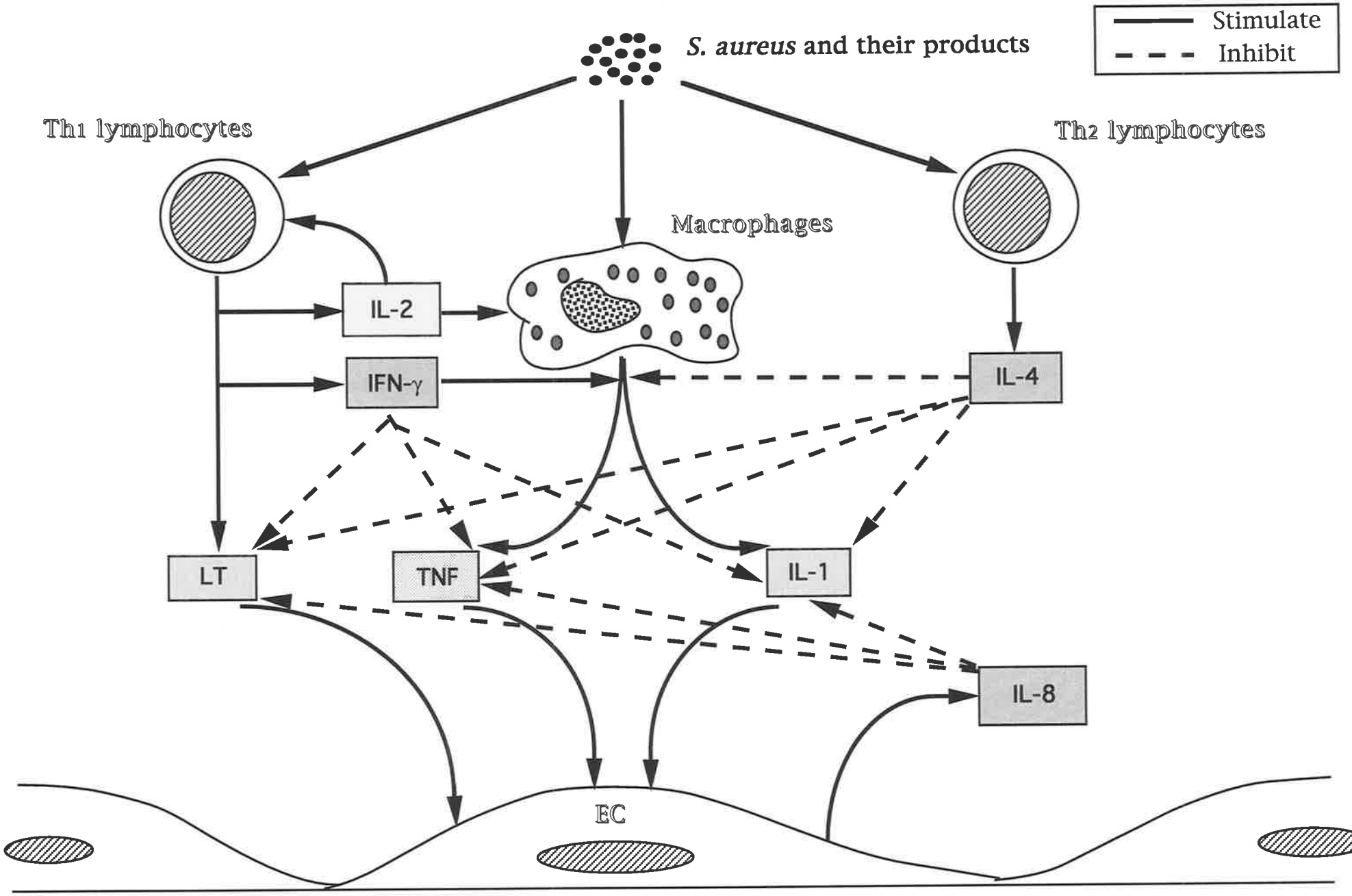


Fig. 7.1 The effects of cytokines on the modulation of *S. aureus* adhesion to endothelial cells (EC).

range of 4-400U/ml. This was also the case for IL-1β (4-4000pg/ml) and LT (4-400U/ml).

The HUVEC changes with respect to bacterial adherence were highly dependent on cell-cytokine pretreatment time, with increasing activity over a 4h period. The effects on both neutrophil adherence and alteration of bacterial adherence were evident by 1h preincubation time. These results suggested that the effects of cytokines on bacterial adherence may require the induction of new proteins.

Downregulation of endothelial-bacterial adherence was not caused by a toxic effect of TNF, LT and IL-1β on HUVEC. The release of the cytoplasmic enzyme lactate dehydrogenase (LDH) was used as a marker of the integrity of HUVEC and it was found that none of the cytokines caused the release of LDH. This is consistent with previous reports (Schemer et al, 1989). The studies on bacterial adherence were run in parallel with studies on neutrophil adherence and in this case all three cytokines enhanced neutrophil adhesion to endothelial cells, providing further support that the alteration in bacterial adhesion induced by the cytokines was not associated with toxicity.

7.4 The Importance of Protein and RNA Synthesis

Treatment of endothelial cells with either the protein synthesis inhibitor cycloheximide, or RNA synthesis inhibitor actinomycin D prevented the cytokine-induced downregulation of bacterial adhesion to HUVEC. The effects of the protein and RNA synthesis inhibitors were also reflected in their inhibition of the TNF-, IL-1β- and LT-induced enhancement of neutrophil adherence. Thus it appears that the stimulation of endothelial cells with TNF,

IL-1 and LT induces synthesis of new proteins and RNA which in turn contribute to the downregulation of the bacterial adhesion and the enhancement of neutrophil adhesion to endothelial cells. The results therefore suggest that the cytokines induce new gene transcription which in turn alters the expression of molecules responsible for the adhesion of leukocytes and bacteria to HUVEC (Fig 7.3).

7.5 Comparison of Effects of Cytokines on Bacterial versus Neutrophil Adherence

Our findings (Table 7.1) that treatment of endothelial cells with TNF, IL-1β and LT decreased the adhesion of bacteria to endothelial cells, and increased the neutrophil adhesion to endothelial cells suggested that the adhesion of bacteria or neutrophils to TNF-, IL-1β– and LT-treated endothelial cells occurs by different mechanisms. It has been reported that treatment of endothelial cells with TNF, IL-1β and LT for 4h enhances the expression of the adhesion molecules ICAM-1 and E-selectin. The induction of these molecules is inhibited by cycloheximide and actinomycin D, suggesting a requirement for new RNA and protein synthesis in endothelial-dependent neutrophil adhesion (Pober et al, 1987). ICAM-1 is the ligand for β_2 integrin receptors and E-selectin is the ligand of the sialylated form of the Lewis X glycan on the surface of neutrophils. The ligation of these molecules promotes neutrophils from a rolling stage along the vessel wall to a stable binding state to the cytokine-activated endothelial cells (Pober et al, 1991; Butcher, 1991). Our results suggest that the cytokine-induced expression of the endothelial cell adhesion molecules while correlating with the increased neutrophil adhesion to endothelial cells is unrelated to the alteration in the cytokine-induced decrease in bacterial adhesion. Therefore the

binding of staphylococcal adhesins to their counter receptors on the host cell surfaces is unlikely to be facilitated by ICAM-1 and E-selectin.

7.6 Mechanisms of Adhesion of Staphylococci to Endothelial Cells

It has been shown that the adhesion of Staphylococci to endothelial cells is mediated by binding of bacterial adhesin(s) to its counter receptors on the host tissue cells (eg. endothelial cells) in a key and lock manner (Beachey, 1981). Previous studies have shown that staphylococci bind to surfaces coated with fibronectin, fibrinogen, vitronectin, collagen, heparan sulfate and laminin, etc (Herrmann et al, 1988; Chhatwal et al, 1987; Spezale et al, 1986; Switalski et al, 1989; Liang et al, 1992). These macromolecules are constitutive components of plasma and subendothelial extracellular matrix. Endothelial cells synthesize and release some of the integrin molecules, such as fibronectin and vitronectin (Berge et al, 1992; Jaffe et al, 1978). It has also been shown that endothelial cells synthesize and express receptors for these integrins on their basal and luminal membranes (Cheresh, 1987; Conforti et al, 1992). The former may mediate the endothelial-matrix adhesion which is important in endothelial cell growth and migration. The latter is responsible for binding integrin molecules from the blood stream which act as bridging molecules to facilitate the adhesion of *S. aureus* to endothelial cells (Cheung et al, 1991). A recent study suggests that fibrinogen fragments, which are broken down by the proteolytic enzyme plasmin, lose their adhesion-promoting activity for adhesion of *S. aureus* to central venous catheters (Vaudaux et al, 1993). This finding may have a bearing on the understanding of mechanisms by which cytokine decreases bacterial adhesion to HUVEC.

7.7 Mechanisms by which TNF, LT and IL-1 Alter the Staphylococcal Binding Properties of Endothelial Cells

It has been documented that alteration of endothelial cells by TNF is associated with loss of fibronectin from the endothelial cell submatrix (Stolpen et al, 1986). Klein et al (1992) showed that treatment of endothelial cells with TNF, IL-1 and LPS for 4h reduced the cell associated heparan sulphate and dermatan sulphate. These molecules have been shown to bind to the surface proteins of *S. aureus* (Liang et al, 1992). The cytokine-activated endothelial cells were found to completely lose their negative charge on the cell surface as a result of the loss of surface sulphated glycosaminoglycans. Defippi et al (1992) reported that activation of endothelial cells by TNF and IL-1β downregulated the expression of a laminin receptor by decreasing the expression of α_6 subunit, but not the β_1 subunit. It is possible that the TNF-, IL-1- and LT-induced decrease in bacterial adhesion to endothelial cells is due to a downregulation or shedding of certain integrin receptor(s) by the endothelial cells.

Vascular endothelial cells play a major role in the regulation of extrinsic fibrinolysis so as to maintain an unclotted surface for blood components. Previous studies showed that endothelial cells modulate fibrinolysis by the synthesis and release of tissue plasminogen activator (t-PA), urokinase plasminogen activator (u-PA) and plasminogen activator inhibitor (PAI) (Loskutoff, 1977; 1983). Plasminogen is a plasma glycoprotein which can accumulate on endothelial cells by attaching to its binding protein on the surface of endothelial cells (Dudani et al, 1991). PA converts the plasminogen to a broad spectrum serine protease, plasmin. PAI, an inhibitor of PA, controls

the activation of plasminogen activator (Saksela, 1985; Hekman et al, 1987). Some studies have shown that TNF, IL-1α/β, LT, PMA and bFGF induce the production of PA in a time and cytokine concentration dependent manner (Victor et al, 1990; Niedbala et al, 1991) (Fig 7.2). The production of PA by endothelial cells was inhibited by the protein and RNA synthesis inhibitors, cycloheximide and actinomycin D respectively (Niedbala et al, 1991). In view of these findings and our results on cytokine-induced depression of bacterial adhesion, it is highly feasible that treatment of endothelial cells with TNF, LT, IL-1 or PMA enhances the conversion of plasminogen to plasmin as a result of an increase in synthesis and release of PA by the cytokine-stimulated endothelial cells. This plasmin could cleave the integrin protein responsible for the binding of *S. aureus* and thus lead to downregulation of bacterial adhesion to endothelial cells. This suggestion is supported by evidence that PMA-stimulated endothelial cells have fibrinolytic activity as fibronectin fragments with RGD (Arg-Gly-Asp) sequence have been detected in the supernatant of PMA-treated endothelial cell cultures (Odekon et al, 1991). Vaudaux et al (1993) have showed that plasmin-digested fibrinogen coating of polyurethane cannulas prevents *S. aureus* adherence. Furthermore, TNF, IL-1β, LT and PMA alter the endothelial cell morphological rearrangement from a cobblestone to a spindle pattern which is associated with the reduction of the stainable fibronectin following stimulation of the cells with TNF (Stolpen et al, 1986; Groenewegen et al, 1985; Montesano et al, 1985). It is also feasible that an increase in PA activation may give rise to these changes.

Some aspects of the mechanisms by which TNF, IL-1β and LT induced changes in bacterial-endothelial adhesion were also studied. It has been shown that activation of endothelial cells by cytokines such as TNF, IL-1 and LT induces membrane associated PAF expression (Bussolino, et al, 1990). Maximal

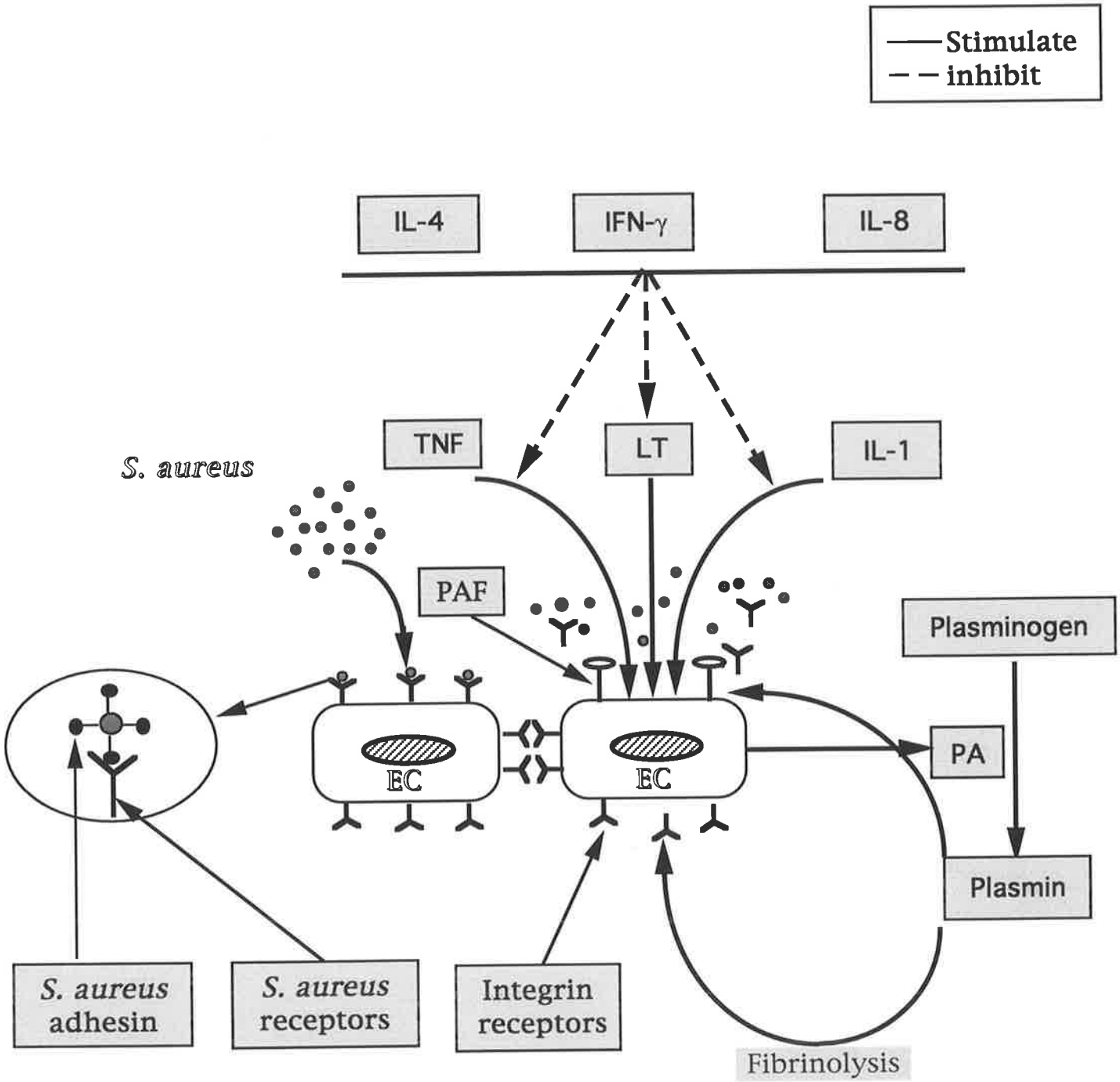


Fig 7.2 Proposed machanisms of cytokine effects on adhesion of *S. aureus* to endothelial cells (EC). Stimulation of EC with TNF, LT and IL-1 increase plasminogen activator (PA) production which converts plasminogen to plasmin, an active fibrinolytic enzyme. This results in altering the receptors for *S. aureus* on EC leading to decrease the adhesion of *S. aureus* to EC.

expression of PAF occurred after 4h treatment with cytokines. This is similar to our findings of the time required for the cytokine-induced decrease in bacterial adhesion. Moreover, pretreating the TNF-, IL-1β– and LT-altered endothelial cells with the PAF antagonist, WEB 2086, reversed the cytokine-induced decrease in bacterial adhesion. Treatment of endothelial cells with PMA is also known to induce the production of membrane PAF (Whatley et al, 1989), and we found that adhesion of bacteria to endothelial cells was also decreased by pretreating the cells with PMA. These findings suggest that PAF may be involved in the modulation of the adhesion of bacteria to endothelial cells induced by TNF, LT and IL-1. However, it has been reported that IL-1β does not induce the production of PAF (Dejana et al, 1987). Thus, PAF might not be the only molecule involved in the cytokine-induced modulation of the adhesion of bacteria to endothelial cells.

Our findings show that PMA, which is known to activate protein kinase C (PKC), also decreases endothelial cell adhesion of staphylococci. However, staurosporine, a PKC inhibitor, did not inhibit the TNF-, IL-1β- and LT-induced changes in endothelial-bacterial adherence suggesting that PKC is not involved. Thus the effects of TNF, LT and IL-1 on alteration of endothelial cell-bacterial adhesion are likely to involve pathways other than activation of PKC. For the adhesion of neutrophils, both a PKC dependent and PKC independent pathway have been reported for cytokine-activated endothelial cells (Ritchie et al 1991). This is consistent with our finding that staurosporine blocked only the PMA-induced enhancement of neutrophil adhesion to PMA-activated endothelial cells but not the TNF-, IL-1- and LT-induced effects.

TNF-, LT- and IL-1-induced signal transduction mechanisms in endothelial cells still remain ill-defined. It has been shown that TNF receptors consist of at

least two types, a 75 KDa and 55 KDa peptide which differ in function (Winzen et al, 1993). Many cell types coexpress both types. Human endothelial cells have been shown to express both types of TNF receptors (Clauss et al, 1992; Thorp et al, 1992, Mackay et al, 1993). LT shares the same receptor(s) with TNF and elicits similar biological activities on endothelial cells. It has been postulated that the binding of TNF or LT to the 55 KDa receptor is mainly responsible for induction of signal transduction (Pfizenmaier et al, 1992; Mackay et al, 1993) (Fig. 7.3). It is also evident that the triggering of the 55 KDa TNF receptor (and 75 KDa receptor?) on endothelial cells by TNF induces the expression of the adhesion molecules, E-selectin, ICAM-1 and VCAM-1, which play an important part in the recruitment of leukocytes to sites of infection (Dudani et al, 1992; Mackay et al, 1993). The receptor for IL-1 on endothelial cells has also been identified, being an 80 KDa glycoprotein (type I receptor) belonging to the immunoglobulin superfamily (Dinarello, 1990). The proposed pathway of action for TNF/LT and IL-1 are similar to each other. The ligation of TNF receptor(s) (mainly 55 KDa) or IL-1 receptor (type I) causes the activation of a pertussis toxin-sensitive G proteins. Then, the G protein coupled phospholipase A_2 (PLA_2) is activated, resulting in the production of lyso-PAF and arachidonic acid from the 1-alky-2-acyl-*sn*-glycero-3-phosphocholine. The lyso-PAF is the immediate precursor of PAF. TNF, LT and IL-1 also activate the enzyme, acetyltransferase, which promotes the induction of PAF from the lyso-PAF followed by the translocation of this molecule to the membrane of endothelial cells. Eighty percent of the PAF produced remains membrane-associated and the remainder released into the medium. The other product of this pathway is arachidonic acid which mediates the synthesis of prostaglandin (PGI_2) and leukotrienes, two groups of important autacoids in the inflammatory responses. The production of PGI_2 has a negative feedback effect on the

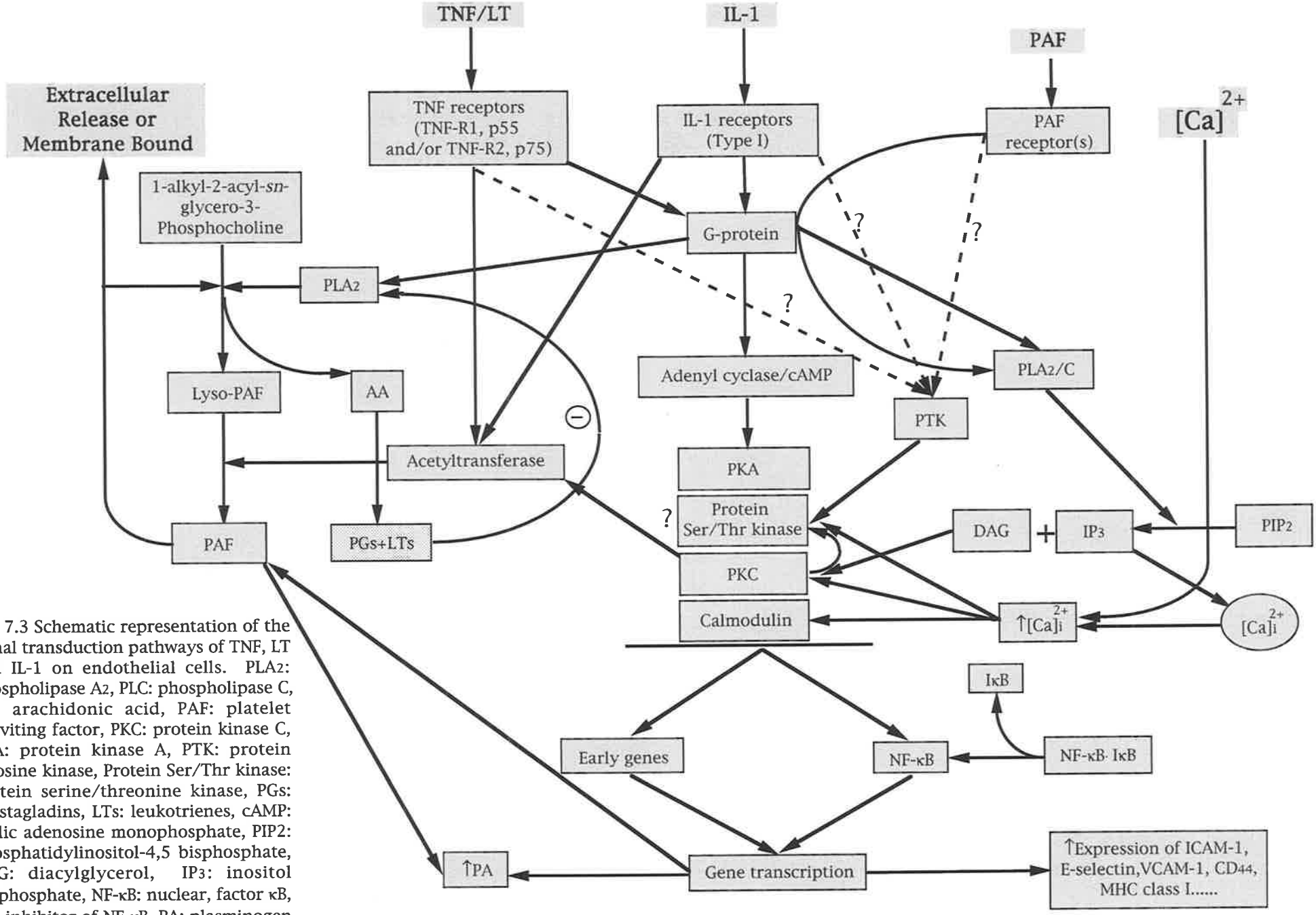


Fig. 7.3 Schematic representation of the signal transduction pathways of TNF, LT and IL-1 on endothelial cells. PLA2: phospholipase A2, PLC: phospholipase C, AA: arachidonic acid, PAF: platelet activiting factor, PKC: protein kinase C, PKA: protein kinase A, PTK: protein tyrosine kinase, Protein Ser/Thr kinase: protein serine/threonine kinase, PGs: prostagladins, LTs: leukotrienes, cAMP: cyclic adenosine monophosphate, PIP2: phosphatidylinositol-4,5 bisphosphate, DAG: diacylglycerol, IP3: inositol trisphosphate, NF-κB: nuclear, factor κB, IκB: inhibitor of NF-κB, PA: plasminogen activator.

activation of PLA_2 to prevent the overproduction of PAF and PGI_2 (Zimmerman et al, 1985).

TNF/LT-, and IL-1-induced gene expression is mediated by activation of transcription factors, such as the nuclear factor (NF)-κB (Fig 7.3). NF-κB has been shown to be exclusively activated through the 55 KDa TNF receptor, which correlates with the altered expression of ICAM-1, E-selectin, VCAM-1, MHC class I and CD 44 on endothelial cells (Mackay et al, 1993). NF-κB becomes dissociated from its inhibitor, I-κB, and translocates to the nucleus where it binds to its cognate DNA sequences (Pfizenmaier et al, 1992; Dinarello, 1991; Mackay et al, 1993). Studies have shown that activators of PKC and PKA are similar to TNF, LT and IL-1 in the activation of NF-κB. This may explain the similarity of PMA with these three cytokines in their alteration of endothelial cell properties and functions, such as the promotion of neutrophil adhesion, the expression of ICAM-1 and E-selectin, and the induction of plasminogen activator, etc, (Ritchie et al, 1991; Gudewicz, 1989; Lane et al, 1989). From our finding, TNF, LT, IL-1 and PMA all decreased the adhesion of *S. aureus* to endothelial cells. However, while the PKC inhibitor, staurosporine, has been reported to abolishe the PKC-dependent activation of NF-κB, it did not interfere with the TNF-induced effect (Pfizenmaier et al, 1992). This is also consistent with reports that the PKC inhibitor blocks only the PKC-induced expression of E-selectin and ICAM-1 on endothelial cells rather than the TNF-induced expression of adhesion molecules (Ritchie et al, 1991). Thus it can be postulated that the effects of TNF, LT and IL-1 on endothelial cell-bacterial and endothelial-neutrophil adherence involves the activation of the transcriptional factor NF-κB, which is independent of PKC activation

7.8 The Effects of Other Cytokines on Bacterial Adherence

Although the cytokines, IL-2, IL-4 IL-8 and IFN-γ alone had no effect on the bacterial adherence to HUVEC, some abrogated the effects of TNF, LT and IL-1β on this process. Our results showed that IL-4, IL-8 and IFN–γ inhibited the TNF-, IL-1β- and LT-induced decrease in bacterial adhesion to endothelial cells. However, the TNF, IL-1β and LT-induced neutrophil adhesion to endothelial cells was only affected by IL-4 and IL-8. The TNF- and IL-1β-induced enhancement of neutrophil adhesion was inhibited by IL-4 and only slightly inhibited by IL-8. In addition the LT-induced neutrophil adhesion was slightly increased by IL-4, IFN–γ and IL-8. The reasons for this difference between the TNF and LT are not clear since both cytokines interact with the same receptor(s) (Table 7.2). Nevertheless, it is worthnoting that there are many reported differences in the biological effects between TNF and LT (Porter, 1990).

IL-4 has been shown to decrease the TNF- and IL-1-induced expression of E-selectin and ICAM-1 but it also induces the expression of VCAM-1 on endothelial cells, This leads to a diminishing neutrophil adhesion and promotion of lymphocyte adhesion (Thornhill et al, 1990).

IFN–γ has been found to act synergistically with TNF in inducing expression of MHC class I antigens on the endothelial cells (Doukas et al, 1990). IFN–γ increased the rate and duration of expression of E-selectin on TNF- and IL-1-stimulated endothelial cells (Doukas et al 1990; Leeuwenberg et al, 1990). This seems to agree with our results which showed that IFN–γ did not affect the TNF-, IL-1- and LT-induced enhancement of neutrophil adhesion to endothelial

Table 7.2 Effects of IFN-γ, IL-4 and IL-8 on the TNF-, LT and IL-1β induced adherence of *S. aureus* or neutrophils to endothelial cells

Cytokines	*S. aureus* adhesion	Neutrophil adhesion
Effects of IFN-γ on		
TNF	I	N
LT	I	N
IL-1	I	N
Effects of IL-4 on		
TNF	I	I
LT	I	N
IL-1	I	I
Effects of IL-8 on		
TNF	I	I
LT	I	N
IL-1	I	I

I: Inhibit

N: No effect

cells. Although IFN–γ has been reported to increase the number of TNF receptors on several cell lines, it does not affect TNF receptor number and affinity on endothelial cells (Aggarwal et al, 1985; Johnson et al, 1990). Addition of IFN–γ to the TNF- or IL-1-treated endothelial cells has been shown to antagonize the production of PA and the PA-mediated degradation of extracellular matrix without increasing the PAI activity (Wojta et al, 1992; Niedbala et al, 1992). This may explain the inhibitory effects of IFN-γ on the ability of TNF and IL-1 to alter endothelial cell-bacterial adherence.

IL-8 has been found to inhibit neutrophil adhesion to TNF- and IL-1-activated endothelial cells and possibly functions to protect the endothelial cells from neutrophil-mediated injury (Gimbrone et al, 1989; Herbert et al, 1990). Luscinskas et al (1992) suggested that the IL-8-induced downregulation of neutrophil adhesion to cytokine-activated endothelial cells was not a result of an effect on the expression of endothelial cell adhesion molecules but involved the downregulation of neutrophil adhesion molecules. In our system, we cotreated the endothelial cells with IL-8 and either TNF, LT or IL-1β for 4h, washed the cells and then tested for the adhesion of bacteria or neutrophils. Under these conditions, we found a decrease in TNF- and IL-1β–induced neutrophil adhesion to endothelial cells. Concurrently, the adhesion of bacteria to similarly treated endothelial cells was reversed by the addition of IL-8. It is therefore evident that the IL-8 inhibition of TNF-, IL-1- and LT-induced decrease in bacterial adhesion is unlikely to involve alteration in the expression of endothelial cell adhesion molecules.

7.9 The Cytokine Network and Bacterial Adhesion to the Endothelium

Bacterial invasion results in the rapid production of TNF and IL-1, usually from infiltrating macrophages. This provides a first line defence whereby these cytokines can participate in activating phagocytic cells (Ferrante, 1992). From our present studies it is evident that protection against infection by these cytokines may be through their ability to inhibit adhesion to tissues, such as the endothelium. The development of an immune response can then lead to the release of a spectrum of cytokines which may also function to add or take over the roles of TNF and IL-1. While keeping in mind that to understand clearly the operation of a cytokine network in infection and immunity is often met with great limitation, we have nevertheless attempted to place some perspective on aspects of the cytokine network which could be operating during an *S. aureus* infection.

There is evidence to show that subpopulations of T helper cells (CD_4^+ cells) may produce different cytokines (Fig. 7.4). Th cells can be tentatively classified as Th_1 and Th_2 cell types. Th_1 cells have been found to produce LT, IL-2 and IFN–γ while Th_2 cells produce IL-4, IL-5, IL-6 and IL-10. Both types of Th cells produce the common cytokines, TNF, GM-CSF and IL-3 (Fig. 7.4) (Mosmann et al, 1989). The stimulation of Th_1 cells particularly in the acute stage of the infection, would result in production of LT and IFN–γ which have antagonistic effects in relation to endothelial-bacterial adherence; IFN–γ abrogates the effects of LT. However under these circumstances IFN–γ would also stimulate production of TNF and IL-1β from macrophages (Billiau, 1992) which most likely leads to a downregulation of the bacterial adherence to endothelial tissue. During the chronic stage of infection, production of IL-4 by Th_2 cells not only

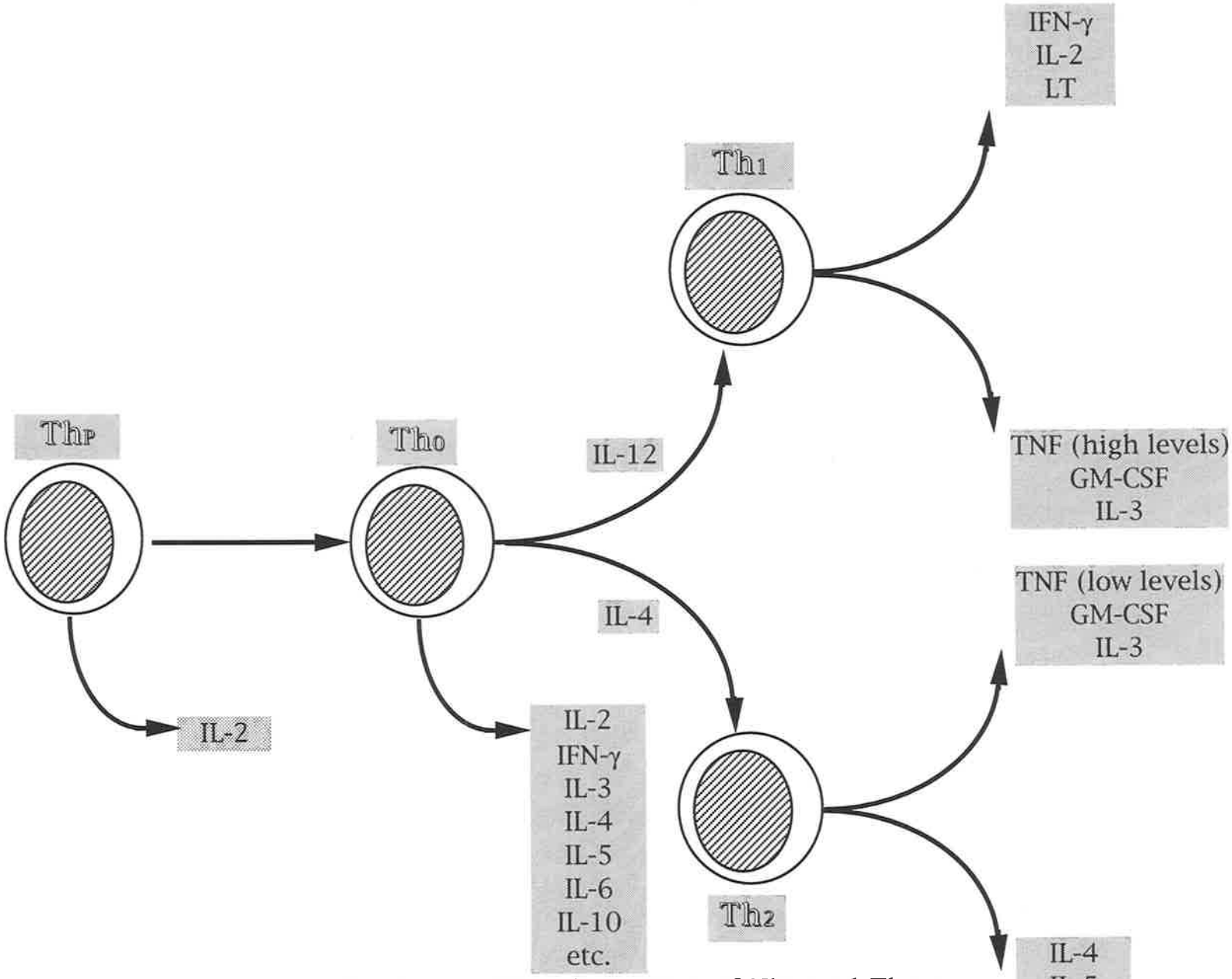


Fig 7.4 Cytokine production by T cell subsets. This distinction of Th1 and Th2 was primarily from the mouse immune system and still remains to be appropriately characterized in humans.

inhibits production of TNF and IL-1β (Hart et al, 1989) but also abrogates the effects of these cytokines. It would thus seem that a switch from the acute stage to the chronic stage of an infection and the switch from Th_1 to Th_2 lymphocyte types leads to a non-protective response, although the host may be spared from the potential pathophysiologic events by IL-4-mediated inhibition of TNF and IL-1 production by macrophages. IL-4 depresses the tissue damaging properties of macrophages (Hart et al, 1989; Essner et al, 1989; Kumaratilake et al, 1992).

7.10 Conclusions

Bacterial infections lead to the production of a variety of polypeptide cytokines. Some of these cytokines are likely to play a vital role in the inflammatory responses of the body's defence system. While cytokines have generally been considered as mediators which activate phagocytic cells to increase bacterial phagocytosis (Ferrante, 1992), the data in this study suggests that cytokines such as TNF, LT and IL-1β may also function to decrease bacterial colonization of tissues by inhibiting bacterial adhesion to tissue surfaces. All three cytokines reduced the ability of cultured human umbilical vein endothelial cells (HUVEC) to bind *S. aureus*. Similarly treated HUVEC, however, showed enhanced ability to bind neutrophils, suggesting that the mechanism of these two types of effects induced by these cytokines occurs via different mechanisms. With respect to the adhesion of neutrophils and bacteria, both are dependent on new protein and RNA synthesis. PAF is involved in the TNF-, IL-1- and LT-induced depression of bacterial adhesion to endothelial cells and the mechanisms used is independent of activation of PKC. It is also speculated that the depressed bacterial adhesion caused by these cytokines is associated with activation of plasminogen activator in endothelial cells, which leads to fibrinolysis and

reduction of the surface receptor(s) for integrin molecule(s), leading to reduced binding of staphylococcus. The modulation of neutrophil adhesion properties is associated with increased expression of ICAM-1 and E-selectin on endothelial cells

While the cytokines IL-4, IFN–γ and IL-8 had no effect on the endothelial-bacterial adherence, these cytokines were capable of abrogating the effects of TNF, IL-1 and LT, suggesting that they may play a regulatory role on the cytokine-induced decrease in bacterial adherence to endothelial cells.

This study suggested that the monokines TNF and IL-1β, and the lymphokine, LT, participate in host defence by preventing bacterial adhesion to tissue surfaces and that this activity of these cytokines can be regulated by other cytokines.

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