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Bacterial Modulins: a Novel Class of Virulence Factors Which Cause Host Tissue Pathology by Inducing Cytokine Synthesis

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INTRODUCTION

The past two decades have seen much of the pathophysiology of infectious diseases explained in terms of the induction of proinflammatory cytokines and the consequent host response to these potent local hormones (103). The pathological role of cytokines in infections has been most clearly delineated in the lethal condition known as gram-negative septic shock, which is estimated to kill 150,000 people annually in the United States (170). The pathological effect is believed to be due to the release of endotoxin from gram-negative bacteria and the subsequent induction of the synthesis of a variety of so-called proinflammatory cytokines—interleukin-1 (IL-1), IL-6 and tumor necrosis factor (TNF). These cytokines can, in turn, induce the synthesis of other mediators such as chemotactic cytokines (e.g., IL-8), cell lipid-derived prostanoids and leukotrienes, and endothelial cell adhesion molecules (230). The consequence of the synthesis of this range of proinflammatory molecules is a marked decrease in heart function, a lowering of blood pressure, and the blocking of the vasculature of the major organs with leukocytes. These changes result in failure of the major organs and rapid death of the patient (170). Septic shock is an acute condition, but the same proinflammatory cytokines are also involved in the pathology of chronic infectious diseases such as leprosy, tuberculosis (103), and the ubiquitous periodontal diseases (116).

Paradoxically, the same cytokines are also crucial in the induction of the acute-phase response, which is an integral part of our innate defense against infecting microorganisms (103, 195). The importance of cytokines in the defense against extracellular and intracellular bacteria is being brought into sharper focus by genetic manipulation of embryos. It is now possible, by use of the technique of homologous recombination, to disrupt specific genes in mice so that animals cannot synthesize the active protein encoded by that gene (95). These mice have a genetic loss of function and are referred to as knockout mice. A growing number of types of cytokine knockout mice have been generated during the past few years, and many are hypersusceptible to intracellular bacteria but fail to respond to endotoxin (13a, 100, 225). Of particular interest are a growing number of cytokine knockouts (e.g., of IL-2 or IL-10) in which the animals die of an inappropriate inflammatory response to their own commensal bacteria (123, 193, 223), suggesting that these cytokines (which have anti-inflammatory actions) are normally employed in blocking the inflammatory response to our resident bacteria.

Whether we are dealing with the induction of proinflammatory or anti-inflammatory cytokines in response to bacteria, it is clear that certain components or products of bacteria must act to trigger their synthesis. Endotoxin and one of the components of endotoxin, lipopolysaccharide (LPS), have been instrumental in the development of cytokine biology over the past 30 to 40 years (35). However, the emphasis on endotoxin/LPS has obscured the fact that components of both gram-negative and gram-positive bacteria, other than LPS, have the capacity to induce cytokine synthesis. During the past 5 to 10 years, the range of bacterially derived components (proteins, carbohydrates, and lipids) able to stimulate cytokine synthesis has begun to be recognized and explored. Some of these molecules are extremely potent inducers of cytokine synthesis, demonstrating activity at pico- to femtomolar concentrations. The biological roles of this group of bacterial cytokine-stimulating components and products remain to be clarified. One simplifying theme in the increasingly complex field of bacterial pathogenicity is the concept of the virulence factor. Bacteria produce a wide variety of factors which promote their ability to invade the host, spread in the host, and survive host defense responses. These diverse molecules have been grouped into four classes: adhesins, invasins, aggressins, and impedins. Adhesins are bacterial proteins which allow the bacterium to bind to a variety of cells, mainly epithelia, as the first step to entering the host. Invasins are bacterial proteins which allow bacteria to enter eukaryotic cells. Aggressins are molecules such as toxins and proteases which damage the host or actively promote the spread of infections, and impedins are bacterial components which inhibit the operation of the host defense mechanism without actually doing any damage (the biology of virulence factors is reviewed in detail in reference 152a). Given the biological properties of cytokines, it is likely that these bacterial cytokine-inducing molecules are also virulence factors. However, they do not fit into the classes of virulence factors just described. With the understanding that such cytokine-inducing components would modulate cell activity, with pathological consequences, we have suggested the term "modulin" (analogous to impedin, aggressin, etc.) to describe this class of molecule (Fig. 1). Endotoxin and LPS would therefore be members of this class of virulence factor. We will review the current literature describing the cytokine-inducing activity of modulins in the context of cytokine biology and host-bacterium interactions generally.

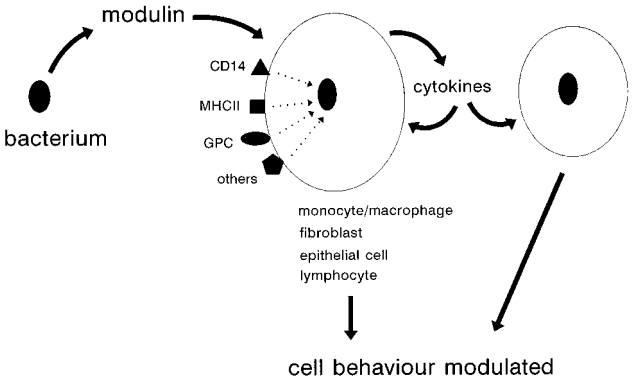

FIG. 1. Diagrammatic representation of the action of a modulin. These molecules would interact with eukaryotic cells (e.g., monocytes/macrophages, fibroblasts, epithelial cells, and lymphocytes) via the CD14 or other cell-selective surface receptors (including major histocompatibility complex class II [MHC II] [57], a G-protein-coupled [GPC] glycoprotein receptor [174], and other non-CD14-coupled receptors) to induce cytokine synthesis. The arrows represent the different intracellular signalling pathways induced via the different receptors. The cytokines produced by this interaction would then act in an autocrine or paracrine manner to modulate host cell behavior. We propose that in situations in which the commensal microflora is in normal contact with host cells, the action of the modulins would be to induce a cytokine network which controls the host inflammatory response to the bacteria. In this case, the modulins act not as virulence factors but as homeostatic regulators.

CYTOKINE BIOLOGY

This section provides a brief introduction to cytokine biology for the reader who has no more than a limited knowledge of this complex area of eukaryotic biology. Readers who are au fait with this topic should go to the section on LPS and cytokine induction, below.

Cytokine research was initiated by those pioneering workers who were trying to understand the mechanisms responsible for fever, which is part of the acute phase response to infection. William Welch, who later became dean of Johns Hopkins Medical School, suggested in 1888 that fever may be the result of the release of "ferments" by host cells in response to bacteria or their products. He proposed that these ferments then

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TABLE 1. History of the nomenclature of IL-1[a]

Experimental finding	Yr	Synonym for IL-1
Peritoneal exudate cells release a substance producing fever	1948	Granulocyte pyrogen
Injection of endotoxin into rabbits induces a plasma protein inducing fever	1955	Endogenous pyrogen (EP)
Material from leukocytes produces an acute-phase response	1969	Leukocytic endogenous mediator (LEM)
Activity stimulates lymphocyte proliferation	1972	Lymphocyte-activating factor (LAF)
Monocyte factor induces prostaglandin and collagenase synthesis	1977	Mononuclear cell factor
Factor stimulates cartilage breakdown	1979	Catabolin
International Lymphokine Workshop	1979	Interleukin-1

[a] See references 3 and 35.

acted on the temperature-controlling centers of the brain to produce fever (reviewed by Atkins [3]). However, this prescient hypothesis was not tested in Popperian fashion for another 60 to 70 years, when the pioneering work of individuals such as Bennett, Beeson, Atkins, and Kampschmidt demonstrated that leukocytes exposed to bacterial pyrogens such as endotoxin would release a heat-labile (in contrast to the heat-stable endotoxin) pyrogen. This heat-labile pyrogen was termed endogenous pyrogen (Table 1) to distinguish it from bacterial exogenous pyrogens, and attempts to purify it continued throughout the 1960s and 1970s (reviewed in references 35 and 36). In the 1960s, immunologists became interested in protein factors which controlled leukocyte function. These factors were named lymphokines, and prominent among them was an activity termed lymphocyte-activating factor (166). Scientists working on the pathogenesis of the crippling joint disease rheumatoid arthritis also described proteins which were implicated in the inflammation and tissue destruction which constitute this disease. These were named mononuclear cell factor (MCF) and catabolin (86). In the late 1970s, it was realized that all these activities were due to the one protein, which, in 1979, was named interleukin-1 and became the prototypic molecule for a family of proteins now given the general title "cytokine" (100) (Table 1).

Cytokines—a Working Definition

All living systems consist of complex interacting control networks which exist in a state of homeostasis: i.e., they maintain a stable internal environment. This idea was propounded by the great 19th century physiologist Claude Bernard, and his work found its embodiment in the discovery of the endocrine glands and their secretions; this developed into the medical speciality of endocrinology. Endocrine hormones, such as the pituitary hormone thyroid-stimulating hormone, which is produced by cells in the anterior pituitary, enters the blood and circulates until it reaches the follicular cells of the thyroid, where it binds to specific cell surface receptors and induces specific cell activation, leading to release of the thyroid hormones such as thyroxine (Fig. 2). Endocrine hormones can be proteins, peptides, or steroids.

In contrast, cytokines are largely inducible proteins or glycoproteins with a molecular mass of >5 kDa, which can be secreted by any cell in the body, with the possible exception of erythrocytes, and which can bind to and activate a range of cells. LPS is able to induce most cell populations to synthesize a range of cytokines. Much of the impetus to the study of cytokines has been based on the perception that they are major mediators of tissue pathology in diseases ranging from asthma to zoonoses (100). Over the past decade or so, it has become clear that cytokines are an integral part of Bernard's concept of homeostasis of the milieu interieur (7). However, unlike endocrine hormones (which act largely at a distance), cytokines generally act at the local level, with the range of interactions between cells and cytokines being described, by analogy with endocrine hormones, as paracrine, autocrine, and even intracrine factors (63, 100) (Fig. 2).

Biological Actions of Cytokines

The enormous interest in cytokines, which has been growing apace over the past two decades, is, in part, the result of the incredible potency and range of actions exhibited by these molecules and the recognition that they are involved in all aspects of disease pathology (21). This range of actions is illustrated graphically in Fig. 3. Cytokines generally have no inherent, e.g., enzymatic, activity, and they produce their biological effects only when they bind to their specific, high-affinity receptors on the surface of target cells, inducing specific intracellular signalling pathways, and eventually resulting in the switching on of particular sets of genes. These genes encode the synthesis of a variety of proteins including (depending on the cell population) cell-to-cell adhesion receptors, proteases, acute-phase proteins, lipid-metabolizing enzymes, nitric oxide (NO) synthase, and cytokines—including the cytokine initially stimulating the cell (Fig. 4). Cell stimulation can result in cell division and cell differentiation. As well as activating cells, cytokines can inhibit cell division or induce apoptosis (programmed cell death) (23). Another important function of one class of cytokines is the stimulation of chemokinesis (i.e., directed cell movement), a mechanism important in combating

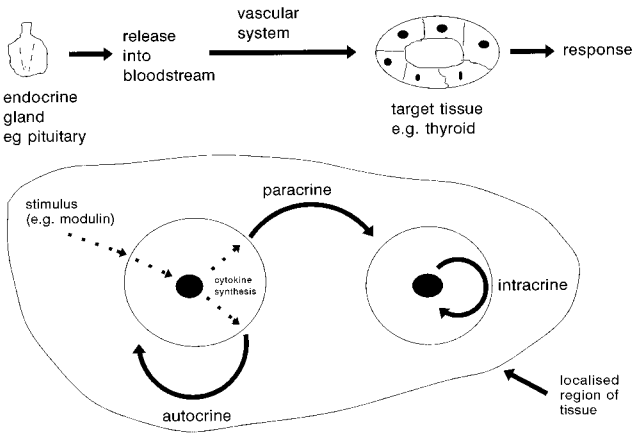

FIG. 2. Diagrammatic comparison of endocrine hormones and cytokines, highlighting the general "action-at-a-distance" properties of the former and the general local action of the latter. It is only in rare cases that cytokines are found in biologically significant amounts in the blood. In terms of these local actions, cytokines can demonstrate intracrine (within the cell), autocrine (self-stimulation), and paracrine (*para*: beside) interactions with cells. It is clear from the huge numbers of cytokines being discovered that this local signalling process is crucial for the maintenance of cell, tissue, organ, and organismal homeostasis.

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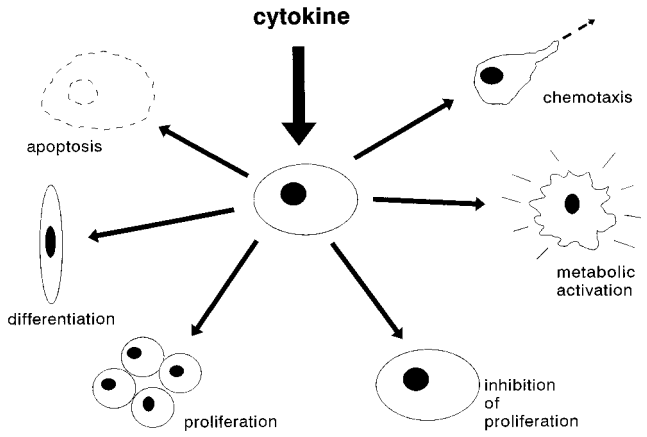

FIG. 3. Diagrammatic representation of the range of biological actions that cytokines have on eukaryotic cells. These actions include the activation of cell metabolism and synthesis. This can lead to induction of the synthesis of a range of proteins including cyclooxygenase II, proteolytic enzymes, NO synthase, and various adhesion receptors. In addition, cytokines can have marked effects on the eukaryotic cell cycle, causing proliferation, inhibition of cell proliferation, or apoptosis. A growing class of cytokines are the small proteins known as chemokines, which induce directed leukocyte migration.

infection (152). The affinity of cytokine receptors for their ligands is generally extremely high (10^{-9} to 10^{-15} M^{-1}), and most cells have only very small numbers of receptors (hundreds to a few thousand) (24, 100). Furthermore, only a percentage of these receptors need to be occupied to produce a maximal response. Thus, very low levels of cytokines can induce biological effects in vivo.

Cytokine Nomenclature

One of the most confusing aspects of cytokine biology is the nomenclature. The first cytokine had at least six different names before it was given the internationally agreed name of interleukin-1 (Table 1). The second cytokine discovered was a T-cell growth factor which was named interleukin-2. The term "interleukin" means "between leukocytes." However, it soon

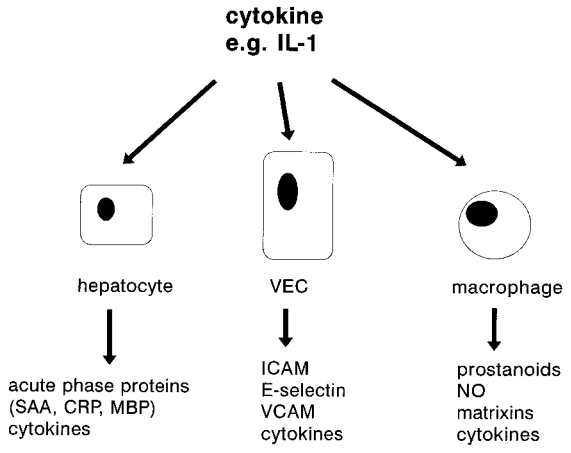

FIG. 4. The range of proteins, enzymes, adhesion receptors, and lipid mediators produced by cells in response to stimulation by proinflammatory cytokines such as IL-1. Abbreviations: SAA, serum amyloid A; CRP, C-reactive protein; MBP, mannose-binding protein; VEC, vascular endothelial cell; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule; NO, nitric oxide. For further details of the effects of IL-1 on specific cell populations, see references 37 and 100.

TABLE 2. The six cytokine families[a]

Family	Example(s)
Interleukins	IL-1 to IL-16
Cytotoxic cytokines	TNF-α, TNF-β
Colony-stimulating factors	IL-3, G-CSF, M-CSF, GM-CSF
Interferons	IFN-α, IFN-β, IFN-γ
Growth factors	Platelet-derived growth factor (PDGF)
Chemokines	IL-8, Rantes

[a] For more details, see reference 100.

became apparent that IL-1, for example, could be made by cells other than leukocytes (e.g., keratinocytes, osteoblasts, and fibroblasts) and could stimulate a wide range of cells in addition to leukocytes. Many cytokines have been named on the basis of the first biological activity ascribed to the cytokine. For example, tumor necrosis factor alpha (TNF-α) has an enormous range of proinflammatory actions but is now generally regarded as having weak cytotoxic (necrotic) activity (54). Likewise, transforming growth factor β (TGF-β) is a very potent inhibitor of epithelial cell division. Thus, it is not possible to define the actions of a cytokine on the basis of its name. Cytokine biology has surely become the richest source of acronyms. The recently published *Dictionary of Cytokines* (100) has entries on several hundred cytokines or cytokine-like activities. With each cytokine having one or more cell surface receptors, there are possibly 400 to 500 proteins making up the assembly of current cytokines.

Over the past 16 years, since the introduction of the term "interleukin," cytokines have been grouped into the following categories: interleukins, tumor necrosis factors, colony-stimulating factors, interferons, growth factors, and chemokines. Examples of these various cytokine "families" are provided in Table 2. However, it must be realized that the interleukins, for example, as a group share little in terms of gene structure, molecular structure, or biological activity (reviewed in reference 100). A more logical grouping of cytokines is based on the type of receptors they bind, because this defines, to some extent, common intracellular signalling pathways (100) (Table 3).

Pathological Actions of Cytokines

While cytokines are increasingly viewed as agents involved in homeostatic regulation, the initial impetus for their study was their perceived pathological activities. As described above, IL-1 was initially discovered as a protein capable of inducing pyrexia (endogenous pyrogen). During the 1980s, many more actions were described, and these are listed in Table 4 and reviewed in detail in references 37 and 83. Parallel work on

TABLE 3. Cytokine receptor superfamilies[a]

Receptor superfamily[b]	Example(s)[c]
Hematopoietic receptor SF	IL-3, IL-4, IL-5, IL-9, IL-12, G-CSF, GM-CSF, EPO
Immunoglobulin receptor SF	IL-1/6, FGF, PDGF, M-CSF
Protein tyrosine kinase SF	EGF, IGF
Nerve growth factor SF	NGF, TNF-α, TNF-β
G-protein-coupled receptor SF	Chemokines
Interferon receptor SF	IFN-α, IFN-β, IFN-γ, IL-10
Complement control protein SF	IL-2

[a] Modified from reference 100.
[b] SF, superfamily.
[c] EPO, erythropoietin; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; IGF; insulinlike growth factor; NGF, nerve growth factor.

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TABLE 4. Biological actions of IL-1, IL-6, and TNF

Biological action	Action displayed by:		
	IL-1	IL-6	TNF
Endogenous pyrogen	+	+	+
Induction of acute-phase proteins	+	+	+
Activation of T- and B-lymphocytes	+	+	+
Stimulation of immunoglobulin synthesis	−	+	−
Stimulation of fibroblast proliferation	+	+	+
Stimulation of cyclooxygenase II induction	+	−	+
Stimulation of cartilage breakdown	+	−	+
Activation of endothelial cells	+	−	+
Stimulation of murine bone breakdown	+	+	+
Induction of endothelial adhesion molecules	+	−	+
Induction of septic shock-like syndrome	+	−	+
Induction of IL-1, TNF, and IL-8	+	−	+
Induction of IL-6	+	−	+
Induction of hyperalgesia	+	−	+

TNF demonstrated a very similar pattern of activity (230), whereas the cytokine IL-6 shares only some of these proinflammatory actions (114) (Table 4). Many of the reported actions of these cytokines are clearly related to combating bacterial infections (103). Thus, they are all pyrogens, and the increase in core temperature that they induce is believed to promote the antibacterial activity of leukocytes (36). All three cytokines (i.e., IL-1, IL-6, and TNF) have the capacity to stimulate the liver to synthesize acute-phase proteins, with IL-6 being the most potent. Examples of acute-phase proteins are serum amyloid A and mannose-binding protein, and their synthesis can be increased up to 1,000-fold by exposure to proinflammatory cytokines. Their function is largely that of opsonizing bacteria to aid in their removal by the body's phagocytic cells (103).

Infections require the rapid mobilization of leukocytes to the sites colonized by bacteria. This extravasation of leukocytes from the lumen of the blood vessel into the tissue is dependent on the existence of separate families of cell surface receptors on circulating leukocytes and on vascular endothelial cells (Fig. 5). These adhesion receptors are, in turn, dependent on proinflammatory cytokines for their synthesis and/or activation. Local proinflammatory cytokine production stimulated, for example, by release of modulins from bacteria induces the synthesis of a range of adhesion receptors by local vascular endothelial cells. These receptors include the selectin family (P-selectin and E-selectin), which recognize carbohydrate antigens (such as sialyl Lewis-X) on leukocytes and are responsible for the slowing of leukocytes and for the phenomenon of leukocyte rolling (along the endothelial surface) at sites of inflammation. The other major vascular endothelial adhesion receptors are the intercellular adhesion molecules (ICAMs) and vascular cell adhesion molecules (VCAMs). These bind with high affinity to counterreceptors on the leukocyte cell surface termed β_2-integrins, which are a family of heterodimeric proteins. It is this interaction between the integrins and the ICAMs which halts the circulating (but by now rolling) leukocytes and allows them to enter the underlying connective tissue by the process known as diapedesis. Circulating leukocytes constitutively express β_2-integrins, but these proteins are normally in a nonfunctional conformation. However, they can be activated by exposure to chemotactic factors such as IL-8, which also act to attract leukocytes into the connective tissue surrounding the blood vessels (115).

The central argument of this review is that many components of bacteria have the capacity to stimulate a range of cell populations to synthesize cytokines and thus tell the body that it has been invaded. To date, most attention has focused on LPS as the warning component. We also propose that com-

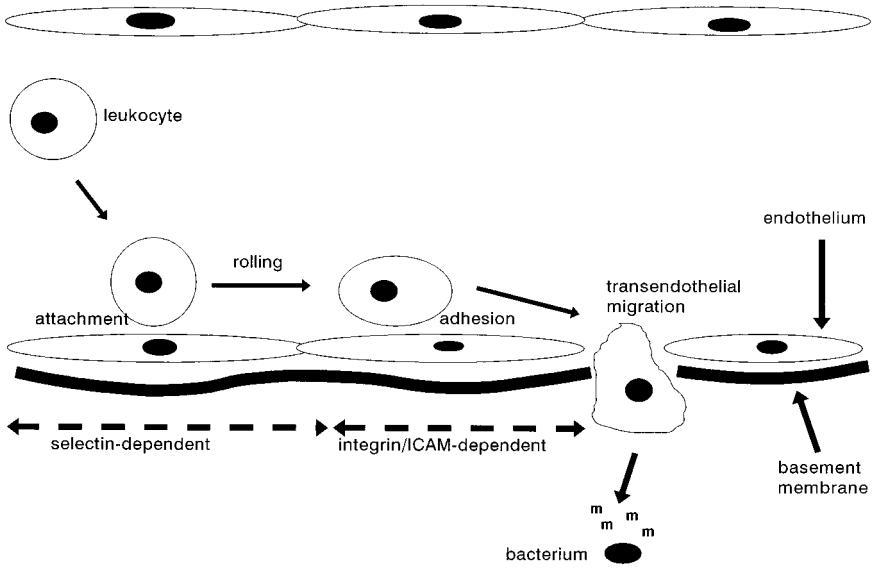

FIG. 5. Highly simplified diagram showing the interaction between a circulating leukocyte and the vascular endothelium in the lumen of a blood vessel. The release of bacterial modulins (denoted by m) is the initial event. This will induce the local production of proinflammatory cytokines by resident cells (e.g., fixed-tissue macrophages or fibroblasts) including IL-1, TNF, and IL-8. These cytokines and certain modulins (e.g., LPS) can induce endothelial cells to release and synthesize adhesion molecules. The selectins (P-selectin and L-selectin) are involved in the initial binding events between the circulating leukocytes and the endothelial surface and involve low-affinity binding to carbohydrate residues on the leukocyte cell surface. This slows the leukocyte as it rolls along the endothelial cell surface. Cytokines such as IL-8 and TNF induce conformational changes in the structure of adhesive counterreceptors (the heterodimeric β_2-integrins) on these rolling leukocytes. This results in the high-affinity interaction between these β_2-integrins and endothelial adhesion receptors such as intercellular adhesion molecule (ICAM-1), which stops the leukocyte and sets in train the next step in leukocyte emigration—diapedesis, in which the leukocyte moves through the endothelial cell barrier into the surrounding connective tissue and moves along the chemotactic gradient to the source of the bacteria.

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TABLE 5. Cytokines with proinflammatory actions

Cytokine[a]	Major inflammatory activity
Chemokines (IL-8, etc.)	Leukocyte chemotaxis
IL-2	T-cell proliferation
IL-3	Stimulation of hematopoietic stem cells
IL-5	Eosinophil differentiation
IL-9	B-cell activation
IL-11	Induction of acute-phase synthesis
G-CSF	Expansion of granulocyte population
M-CSF	Expansion of macrophage population
GM-CSF	Induction of granulocyte/macrophage populations
PDGF	Fibroblast chemotaxis, monocyte/neutrophil activation
TGF-β	Neutrophil chemoattractant
FGF	Fibroblast activation
IFNs	Macrophage activation, cytokine synthesis

[a] PDGF; platelet-derived growth factor; FGF, fibroblast growth factor.

mensal bacteria induce the synthesis, by the host, of a range of cytokines which either inhibit the inflammatory response to such commensal organisms or induce a state of inflammation which is self-limiting and recognized as healthy. This idea will be developed in the next two sections.

In addition to the above effects, the three proinflammatory cytokines under discussion (IL-1, IL-6, and TNF) can activate T and B lymphocytes, and IL-1 is a potent stimulator of hematopoiesis (37). Thus, so far, the actions described for IL-1, IL-6, and TNF have all been what one could term host protective, and this is certainly one of their key roles. However, these proinflammatory cytokines also have activities which are damaging to the integrity of the organism, and this can result from an accentuation of their normal protective function. For example, if the pyrexia is too prolonged or the core temperature rises too high, damage can ensue. As described above, LPS can have lethal effects by activating leukocyte adhesion receptors in the major organs, leading to vascular clogging, anoxia, and failure of the affected organ. Indeed, administration of IL-1 or TNF to animals can induce a septic shock-like state (37). IL-1 and TNF also have profound effects on mesenchymal cells, i.e., the connective tissue cells of the connective tissue element of the organs and tissues, including fibroblasts, found in all tissues, osteoblasts of bone, and chondrocytes of the cartilage of the joints. Such cells are important in both the production and turnover of the connective tissue extracellular matrices present in all tissues. It is now firmly established that the cells of connective tissue respond to very low levels of IL-1 and TNF, undergoing metabolic changes which result in the loss of extracellular tissue matrices. For example, exposure of chondrocytes to IL-1 inhibits the synthesis of connective tissue components such as collagen and induces the synthesis of a family of enzymes, the matrixins (which include enzymes such as collagenase and gelatinase), which are able to hydrolyze all the components of the extracellular matrix. The obvious consequence of this process is the loss of extracellular matrix around activated cells (85). The mesenchymal cells also respond to proinflammatory cytokines by inducing the synthesis of the enzyme cyclooxygenase 2, which produces the prostaglandins—a family of lipidic mediators with proinflammatory properties (86, 87). These various local cellular and systemic responses to proinflammatory cytokines (as delineated in Table 4) illustrate why these molecules have been classified as the key pathological mediators in many diseases. Currently, much effort is being expended to inhibit the synthesis or actions of TNF and IL-1, and phase III clinical trials of a neutralizing antibody to TNF are showing clinical efficacy in treating rheumatoid arthritis, although the success of such potential drugs in treating patients with septic shock is open to question (reviewed in references 84 and 88).

The term "proinflammatory cytokines" has been used so far to describe only a very limited number of proteins. However, many cytokines either are found at sites of inflammation or have proinflammatory actions either in vitro or in vivo. A list of cytokines involved in inflammation is provided in Table 5.

Among the puzzles of cytokine biology are (i) the enormous redundancy of many of the cytokines (Table 4) and (ii) the capacity of cytokines for interactive behavior, which results in complex networks of interactions between cells, cytokines, and other noncytokine mediators. Thus, cytokines produced by one cell can induce neighboring cells to synthesize cytokines, including the initiating cytokine. Cytokines can even "feed back" to induce the cell producing the cytokine to make more of that cytokine and/or to make other cytokines. As discussed below, some cytokines can feed back to inhibit cytokine synthesis. This process creates a network of interactions between cells and cytokines, which can lead to induction of the synthesis and release of a wide range of mediators. In addition to cytokines, this sequence of interactions can, as described above, lead to the production of prostanoids, nitric oxide, proteases, and various cell surface proteins including adhesion molecules and cell surface proteases. This complex of interactions between cytokines and cells has been termed the cytokine network and involves both stimulatory and inhibitory events.

Anti-Inflammatory Cytokines

The discussion so far has dealt with the pathological actions of cytokines, in particular their proinflammatory tissue-destructive behavior. However, in any complex system, there are usually both positive and negative signals. Most of the cytokines discovered during the period between the early 1970s and the late 1980s were proinflammatory. It is only within the last 10 years that cytokines which can inhibit tissue inflammation have been discovered (Table 6). The best known anti-inflammatory cytokine is a member of the IL-1 family. Until 1990, this family consisted of two members, IL-1α and IL-1β, which, although they demonstrated only 20 to 30% sequence homology, both bound with high affinity to a common receptor (37). In 1990, a third member of this family, termed IL-1 receptor antagonist (IL-1ra), was discovered (47). This protein binds to the IL-1 receptor but fails to trigger an agonist response, and thus it acts as a true receptor antagonist (74). An increasing number of cytokines have been discovered to have activities which oppose or downregulate inflammatory processes. Among these are IL-4, TGF-β, IL-10, IL-12, and IL-13 (Table 6). These cytokines are believed to have a modulating function over the immune and inflammatory events which occur following infection or trauma, allowing resolution of the

TABLE 6. Cytokines with anti-inflammatory actions

Cytokine	Anti-inflammatory activity
IL-1ra	Antagonizes cellular actions of IL-1
IL-4	Inhibits macrophage synthesis of IL-1, TNF, and prostaglandins
IL-10	Inhibits proinflammatory cytokine production by T cells and macrophages
IL-13	Inhibits macrophage activation and proinflammatory cytokine production
TGF-β	Has many inhibitory actions on macrophages and lymphocytes

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TABLE 7. Phenotypic changes in cytokine gene knockouts

Cytokine[a]	Phenotype
IL-1β	No clearly definable change
IL-1 type I receptor	No clearly definable change
IL-1β-converting enzyme	Resistant to endotoxic shock
IL-2	Ulcerative colitis in response to commensal gut flora
IL-4	Deficiency in Th2 response
IL-6	Major deficiency in acute-phase response, susceptible to *Listeria* spp.
NF-IL-6	Inhibition of macrophage bactericidal function
IL-8 receptor	Deficiency in neutrophil chemotaxis/accumulation of neutrophils and B cells in lymphoid organs
IL-10	Chronic enterocolitis
TGF-β	Multifocal inflammatory disease
IFN-α/β receptor	Susceptibility to viral infection
IFN-γ	Not susceptible to influenza virus but susceptible to mycobacterial infection
IFN-γ receptor	Susceptible to mycobacterial and listerial infections
G-CSF	Neutropenic and susceptible to *Listeria* spp.
GM-CSF	Accumulation of surfactants in lung, but no effects on hematopoiesis
Lymphotoxin	Lack of lymph nodes and Peyer's patches
MIP-1α	Resistance to coxsackievirus-induced myocarditis
TNF type I receptor	Susceptible to LPS plus galactosamine and to *Listeria* spp.

[a] NF-IL-6, transcriptional factor upregulating IL-6 synthesis.

inflammation and return of the tissue to its normal state. The anti-inflammatory actions of these cytokines have been studied largely with cells in culture or in vivo by injecting large amounts of the cytokine under study. IL-10, for example, has recently been shown to have anti-inflammatory effects in humans (19). Another approach recently taken has been to create cytokine gene knockouts (reviewed in reference 75). A growing number of such knockouts have added to our understanding of the role that cytokines play in the inflammatory response to bacteria.

Cytokine Gene Knockouts and Susceptibility to Infection

One of the problems of producing gene knockouts is that it is impossible to predict the effect of abrogating any particular protein. For example, knockout of the growth-promoting cytokine TGF-α results in mice whose major phenotype is wavy hair (137). In a growing number of examples, the knockout of a cytokine gene results in mice that are susceptible to microorganisms. For example, mice deficient in the gene for gamma interferon (IFN-γ) remained healthy in the absence of pathogens. However, they were susceptible to normally sublethal doses of the intracellular bacterium *Mycobacterium bovis* (27). Knockout of NF-IL-6, the major transcriptional control element of the IL-6 gene, renders mice highly susceptible to infection by *Listeria monocytogenes* (223). A similar finding has been reported for mice lacking the type I TNF receptor (192). Mice lacking one of the TNF receptors or the enzyme IL-1β-converting enzyme (ICE) (134, 192), which is responsible for the cleavage of active IL-1β from an inactive pro-form (such that mice lacking this enzyme essentially lack the ability to produce IL-1β), do not develop septic shock when given an injection of endotoxin. However, it has not been established whether these mice are more susceptible to infections. In experiments that are the corollary of the above experiments, introduction of the human IL-10 gene into mice reduced the mortality associated with lethal endotoxemia (190).

The consequences of most of these cytokine knockouts are therefore explicable in terms of the known functions of the cytokines. However, some of the cytokine-deficient mice show unexpected responses. Mice lacking the cytokine IL-2, which functions largely as a T-cell growth factor, develop normally in the first month after parturition. In the next month, 50% of the animals die and the remainder all develop an inflammatory bowel disease which closely resembles the severe human disease ulcerative colitis. The cause of this colitis became clear when knockout mice were kept under germ-free conditions. In the absence of a normal commensal bowel flora, no disease was evident (193). A similar picture has emerged for animals in which the gene for IL-10 has been abrogated. These animals develop inflammation of the whole gastrointestinal tract (and not just the colon as in the IL-2-deficient mice), and this inflammation is much reduced in animals kept under specific-pathogen-free (not germ-free) conditions (123). Animals deficient in TGF-β1 develop a multifocal inflammatory lesion, although it has not been established that it is a response to commensal bacteria (203). The main phenotypic changes in cytokine knockout mice are detailed in Table 7. These knockout mice show the importance of certain cytokines in our response to infection, and the possible use of cytokines as antimicrobial agents has recently been reviewed (124).

LPS AND CYTOKINE INDUCTION

Having provided an overview of cytokines for the nonspecialist, we will now consider the components and products of bacteria which have the capacity to stimulate cytokine synthesis. We will begin with LPS. This article is not intended to review in detail the biology of LPS, although this molecule is the most widely studied modulin. There are a number of recent reviews on LPS and on LPS-induced cytokine induction (35, 145, 186). However, it is important to understand how LPS induces cytokine synthesis in order to compare and contrast this mechanism (or mechanisms) with those of the various other modulins produced by gram-negative and gram-positive bacteria. To this end, the structure and function of LPS and the mechanism by which it activates eukaryotic cells are described.

LPS and Endotoxin: Definition and Composition

LPS has become the most popular reagent for stimulating cytokine production and is used by scientists throughout the whole range of biomedical specialities. This universal usage of LPS has led to its being regarded as a homogeneous reagent with uniform properties; this, as this review will take care to correct, is not the case. Before discussing the structure and properties of LPS, it is important to define what we are talking

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about. For the general scientific "public," the terms "endotoxin" and "LPS" are interchangeable. This belief and, as we will discuss, the complex nature of the interaction between LPS and its receptor(s) have led to the many apparently contradictory claims about the ability of LPS from any particular bacterium to stimulate the synthesis of cytokines. This has arisen largely because of the confusion regarding nomenclature and the fact that it is extremely difficult to standardize the purity and physical state of LPS. Hitchcock et al. (93) have clarified the nomenclature as follows. The term lipopolysaccharide (LPS) "should be reserved for purified bacterial extracts which are reasonably free of detectable contaminants, particularly protein." Such preparations may be obtained by using the Westphal extraction procedure as a starting point (245). In contrast, the term endotoxin should be used to refer to "products of extraction procedures which result in macromolecular complexes of LPS, protein and phospholipid." Complexes of this type are normally obtained by extraction of bacteria with trichloroacetic acid, butanol, or EDTA. The thesis being forwarded in this review is that many proteinaceous components of the bacterial cell wall are potent inducers of cytokine synthesis. Thus, the activity of endotoxin may be due as much to these as to the LPS. One major problem in the cytokine literature is that the purity of the LPS or endotoxin used by investigators is taken for granted and remains inadequately specified or even ignored. Even if investigators were concerned about this problem, it is difficult to define a "pure" preparation of LPS by criteria applied to other macromolecules. Molecular weight is not particularly useful, because even a single cell contains LPS molecules with different O-antigenic side chain lengths. Despite this, it is important that investigators attempt, as far as is possible, to ensure that their preparations are free of cytokine-inducing contaminants which could either enhance or diminish the cytokine-inducing activity of LPS. Most investigators do not produce their own LPS but obtain it from commercial suppliers, believing that the product is pure. Our own experience is that commercial preparations can contain substantial amounts of protein (in one case up to 60%).

LPS is an amphiphilic molecule that, above the critical micelle concentration, forms aggregates, which decreases its solubility. The physical state of the LPS can markedly affect its biological activities. This has been clearly shown with the LPS extracted from *Salmonella* spp., which exists as a negatively charged molecule bound to amines such as putrescine and spermine. Removal of these bases by electrodialysis followed by neutralization with triethylamine or metal ions alters both the solubility and biological activity of the LPS. Thus, the triethylamine salt of *Salmonella abortus-equi* LPS is more toxic in mice and pyrogenic in rabbits than is the corresponding partially soluble calcium salt. In contrast, the calcium salt is more toxic in rats (59).

Standard for LPS. Since a particular preparation of LPS may give very different results in different assays, it is important that researchers have at least a notion of the relative potencies of the preparation being used. Although there is no pharmacopeial standard for LPS, there is an international standard for endotoxin, calibrated in international units of biological activity, based on the potency of the preparation in *Limulus* amoebocyte lysate tests. This preparation was also calibrated in rabbit pyrogen tests and serves as a useful starting point for standardization of preparations of LPS/endotoxin for experimental use (171).

Structure of LPS

The architecture of LPS (Fig. 6) is built up of three separate "building blocks": (i) lipid A, (ii) an inner core region, and (iii) the O-specific side chain. These separate building blocks have widely different compositions and structures, and this is reflected in their biological activities and functions.

Lipid A. It has been recognized for many years that the "endotoxic" activities of LPS are largely due to the lipid A region, although activity can be modulated by other regions of the molecule (15, 185). Lipid A is the general term for a family of β(1-6)-linked disaccharides to which are attached medium- to long-chain fatty acids (10- to 28-carbon chain length) linked to the sugar residues by ester or amide linkages and containing glycosidic and nonglycosidic phosphoryl groups. The lipid A molecules from bacteria can differ in terms of the sugar residues; the nature, chain length, number, and location of acyl residues; the degree of phosphorylation; and the nature of the substituents on phosphate groups (185).

The structural requirements for proinflammatory cytokine induction have been examined. Active molecules must have the disaccharide, two phosphoryl groups, and six fatty acids in a defined location such as is found in *Escherichia coli* LPS. Indeed, the fatty acids are important determinants of activity, with the major variables being number, location, chain length, and stereochemistry. *E. coli* lipid A with five or four fatty acid residues are, respectively, 10- and 100-fold less potent at inducing proinflammatory cytokine synthesis than is the hexaacyl *E. coli* lipid A (2, 112, 136a, 185, 221a).

Core region. The core region consists of an inner and an outer core. The biological activities of the outer core have not been investigated in any detail. Although it is immunogenic and functions as a phage receptor, it does not appear to be directly involved in cytokine induction. The inner core is characterized by the presence of the unusual sugars heptose and 2-keto-3-deoxyoctonic acid (KDO), and these have been reported to exert an effect on the biological activities of lipid A, including its capacity to induce cytokine synthesis. For example, Cavaillon and coworkers (13, 15) have reported that lipid A must be linked to at least one KDO residue in order to induce monocytes/macrophages to synthesize IL-1. IL-1 induction appears to be dependent on the presence of the carboxyl group of KDO (16, 70). However, this finding has not been supported by the work of other groups (see, e.g., reference 136a). It has also been reported that the heptose-heptose-KDO from a *Salmonella minnesota* mutant and the core oligosaccharides from *Bordetella pertussis* and *S. minnesota* are able to release IL-1 from human monocytes (71, 125, 136a) but only when they are present at high concentrations.

O-antigenic side chain. Although the O-antigenic side chain has a number of important biological functions, its role in the cytokine-inducing activities of LPS is uncertain. It does not appear to be essential for the induction of IL-1 synthesis by monocytes/macrophages. However, Mannel and Falk (143) reported that the polysaccharide portion of the LPS molecule was essential for optimal TNF production by human peripheral blood mononuclear cells (PBMCs). In contrast, Feist et al. (52) demonstrated that R-type LPSs from *S. minnesota* were more potent than were S-type LPSs in stimulating TNF release.

LPS Receptor

Given the enormous amount of attention that has been devoted to the biology of LPS, it is surprising that in late 1995 the receptor for this molecule has not been conclusively identified. Two lines of evidence support the belief that cells have LPS receptors and that this complex bacterial product does not

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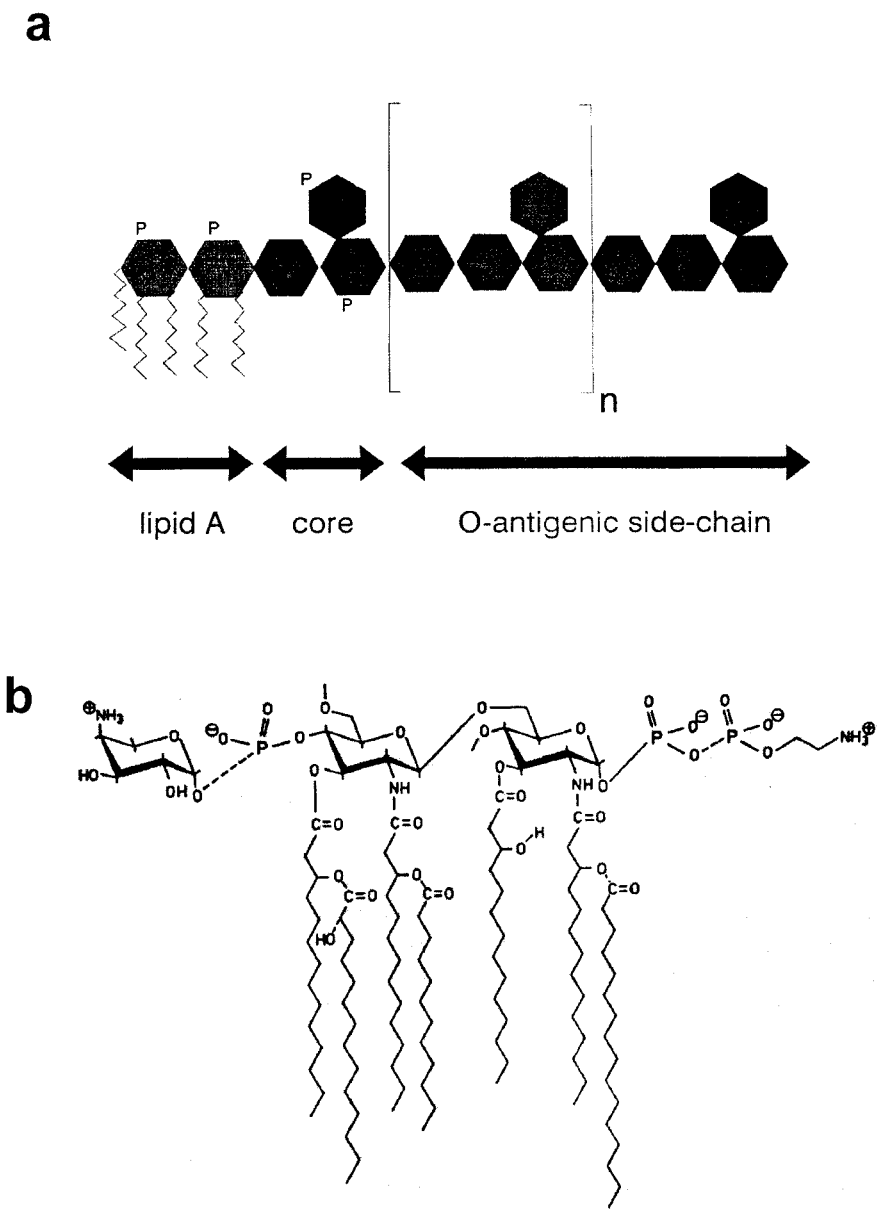

FIG. 6. (a) General structure of LPS. The lipid A portion consists of a disaccharide with fatty acid and phosphate (P) substituents. This is attached to a core region characteristically containing heptose and KDO. The O-antigenic side chain consists of repeating oligosaccharide units. (b) Detailed structure of the lipid A of *Salmonella minnesota*. The general chemical structure of lipid A is highly conserved and consists of a β(1–6)-linked D-glucosamine disaccharide phosphorylated at position 1 on the right-hand residue and position 4 on the left-hand sugar. The disaccharide also has a variable number of fatty acid substituents. The most commonly observed fatty acid is β-hydroxymyristic acid, a C_{14} saturated fatty acid, which is substituted on the amino groups. The hydroxyl groups are also esterified with other long-chain fatty acids, such as lauryl, myristic, and palmitic acids.

activate cells by some bizarre nonspecific mechanism. The first is the discovery of LPS antagonists. In the early to mid-1980s, it was reported that lipid X, a monosaccharide biosynthetic precursor of lipid A isolated from a variant of *E. coli*, acted as an LPS antagonist in vitro (29) and could protect animals against LPS-induced lethal toxicity (175). Nontoxic lipid A molecules from *Rhodobacter capsulatus* and *R. sphaeroides* also behave as LPS antagonists (191). A stable endotoxin antagonist (E5531), which was able to inhibit LPS-induced cytokine and nitric oxide production by human monocytes in vitro with 50% inhibitory concentrations (IC_{50}s) of <1 nM, has recently been described and was also effective in inhibiting LPS-induced TNF-α production and associated lethality in mice (20). The findings that biological actions of LPS can be antagonized and that antagonists show structure-activity relationships strongly suggest that the ultimate reaction of LPS with cells is via some type of recognizable receptor. However, as described below, the nature of the receptor system recognizing LPS must be extremely complex.

Parallel studies have used chemical cross-linking agents to produce a covalently linked complex between radiolabelled LPS (or lipid A) and its putative cell surface receptor(s). After formation of the covalent complex, the cell membranes were solubilized, the cell proteins were fractionated, and the molecular mass of the radiolabelled complex was determined. Morrison and coworkers (130–132, 189), using this cross-linking technique, identified an 80-kDa cell surface protein as the LPS receptor on a variety of cells responsive to LPS, including B

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and T lymphocytes and macrophages. Erythrocytes did not possess this receptor. Cross-linking was blocked by unlabelled LPS. Dziarski (42, 43) also reported a similar surface receptor which bound LPS and peptidoglycan. However, Dziarski has now reported that this "receptor" is in fact cell-bound albumin, originating from the culture medium in which cells were grown (43). The albumin binds very strongly to many, although not all, cell types by a mechanism that has not yet been established. Thus, the biological significance of the 80-kDa receptor is at present uncertain. Other proteins capable of being cross-linked to labelled LPS have had reported molecular masses of 95, 65, 55, 25, and 18 kDa (236). The nature of these proteins has not been defined, and it is possible that the true LPS receptor is a multimeric molecule (such as the IL-2 receptor) or a set of interacting surface receptors or even a range of distinct receptors recognizing different forms of LPS.

Two established cell surface proteins have been demonstrated to bind LPS. Wright and Jong (249) reported that all three β_2-integrins (leukocyte adhesion molecules consisting of two proteins, one common to all β_2-integrins [CD18] and the other unique to each integrin [CD11a/b/c]) were capable of binding to LPS. However, the finding that CD18-deficient cells responded to LPS suggested that these were not signalling receptors (248). A more recent study, in which CD11c/CD18 has been transfected into Chinese hamster ovary (CHO) cells, which normally do not respond to LPS and do not have this integrin receptor, has revealed that transfected cells do respond to LPS (101).

While the search for the LPS receptor was under way, a number of discoveries radically altered our thinking of the way in which LPS interacts with cells. The first was the discovery that acute-phase serum contained a protein (termed LPS-binding protein [LBP]) which bound to LPS (228). LBP is a 60-kDa glycoprotein, synthesized by liver hepatocytes, which binds to the lipid A region of LPS (229), forming a complex which can be 1,000-fold more active than free LPS (147). Levels of LBP in normal rabbit serum range from <0.5 to 10 μg/ml, and those in acute-phase serum may reach 50 μg/ml; in human acute-phase serum, 300 μg/ml has been recorded (236). LBP shows sequence homology with the mammalian proteins cholesteryl ester transfer protein, which is involved in transporting lipids between plasma lipoprotein particles, and a bactericidal protein produced by polymorphonuclear leukocytes (PMNs), bactericidal/permeability-increasing protein, which also facilitates lipid transfer between proteins (66, 198). However, whereas LBP forms a complex with greater "endotoxic" activity, bactericidal/permeability-increasing protein forms a complex in which the biological activity of LPS is neutralized. Indeed, bactericidal/permeability-increasing protein is bactericidal (48). LBP also functions to opsonize LPS-bearing particles such as gram-negative bacteria and LPS-coated erythrocytes (251). By using LPS-coated erythrocytes, it was found that LBP promoted the binding of the erythrocytes to macrophages and that this was dependent on the plasma membrane protein CD14 (237, 250). CD14, a 50- to 55-kDa glycoprotein, is an example of a glycosylphosphatidylinositol-anchored membrane glycoprotein expressed predominantly on myeloid cells. It has also recently been shown to exist as a soluble protein in the plasma (reviewed in references 236, 237, and 260). Transfection of a CD14-negative murine pre-B lymphocyte cell line (70Z/3), which is relatively unresponsive to LPS-LBP complexes, with CD14 increased responsiveness by up to 1,000-fold (128). It is now believed that CD14 forms at least part of an LPS receptor but there are a number of problems in accepting this hypothesis. The affinity of binding to LPS is quite low, with an apparent dissociation constant of between 10^{-8} and 10^{-7} M^{-1} (113). This value would appear to be too low for an agonist-receptor interaction in which the agonist can show activity at picogram-per-milliliter concentrations. The second major problem with the hypothesis is the fact that CD14 is unable to induce intracellular signalling. One possible explanation is that the CD14 is only part of a larger receptor complex which contains the intracellular signalling domain(s). However, there is still considerable confusion about the nature of the other possible cell surface LPS-binding proteins and receptors.

CD14 is present in serum at concentrations up to 4 μg/ml (69). This soluble form of the molecule has now been shown to act both as an agonist (79, 176) with cells that do not express CD14 and as a competitive antagonist of cells in which this glycoprotein is membrane bound (78).

It is now possible to see a pattern emerging in the literature with regard to the interaction of LPS with plasma proteins. For example, it is now established that LBP interacts with LPS and CD14 in a complex manner which can (i) transfer LPS from LPS-containing micelles to CD14 and, in doing so, accelerate the binding of LPS to CD14 (72); (ii) transfer LPS from micelles to high-density lipoprotein (HDL) particles (253); and (iii) transfer LPS from LPS-CD14 complexes to HDL (252). Once bound to HDL, the LPS is then in a biologically inactive state. CD14 acts to increase the rate of transfer of LPS to HDL, and thus it appears (at least from the current literature) that CD14 plays a pivotal role in determining the activity of LPS, being able to act as a receptor, an antagonist, and a catalyst of LPS inactivation (for a recent review, see reference 247a). This conclusion has been strengthened by the report that LPS acts to induce the synthesis of CD14 and raise levels of CD14 in mice (51) and that transgenic mice expressing human CD14 are hypersensitive to LPS (53).

LPS-Induced Transmembrane Signalling

The problem of transferring information from the outside of the cell to the inside has been solved by the evolution of the cell surface receptor and its linkage to intracellular proteins which amplify the message by the generation of what are termed second messengers. Cells contain three types of cell surface receptors (ion channel, G-protein coupled, and enzyme [e.g., tyrosine kinase] linked), but we are concerned only with the last two, because these are involved in the induction of cytokine synthesis. The G-protein receptors consist of seven transmembrane α-helices and interact with a family of trimeric G-proteins whose function is to transmit the signal from the receptor into the cell. This is done by activating additional enzymatic systems, such as adenylyl cyclase to form cyclic AMP or the phospholipase C-inositol trisphosphate system which upregulates cellular calcium levels and produces diacylglycerol. The ultimate result of the generation of these second messengers is the activation of cellular kinases which phosphorylate serine or threonine residues on proteins. The tyrosine kinase type of receptor either has an intracellular kinase domain and can directly phosphorylate proteins or is linked to a tyrosine kinase. The recent editorial by Krebs gives a brief historical overview of protein phosphorylation (121). It is now the accepted paradigm that intracellular signalling is controlled by the pattern of phosphorylation and dephosphorylation of proteins, and many different protein kinases and protein phosphatases are present in eukaryotic cells.

As may be expected from a molecule with the profound cellular effects that LPS possesses, LPS appears to stimulate many pathways of intracellular activation, including ion channel receptors (177). However, this brief discussion will be re-

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stricted to the transduction mechanisms involved in the rapid stimulation of cytokine gene transcription, which occurs probably within minutes to hours of LPS interacting with the cell. These transduction pathways appear largely to be related to binding to the CD14 receptor (146), although evidence is accumulating to suggest that non-CD14 pathways also exist (50). Initial examination of LPS-induced signalling suggested that G-protein-coupled receptors were activated. The LPS-induced activation of pertussis toxin-sensitive G-proteins (102, 257) and of protein kinase C (22, 201, 224) has also been reported. There is obviously a complex relationship between the various intracellular signalling pathways. This is shown by the finding that calcium/calmodulin antagonists differentially regulate LPS-inducible cytokine genes. Thus, IL-1β transcription was strongly suppressed while IL-1α transcription was only weakly inhibited and transcription of the chemokine IP-10 was elevated (164). This shows the presence in the activated cell of a calcium-dependent, a calcium-independent, and an inversely dependent calcium signalling pathway.

That LPS induces tyrosine kinase activation was initially shown by Weinstein et al. (242) by Western blotting (immunoblotting), which revealed that cellular proteins became phosphorylated on tyrosine residues within 5 min of LPS activation and that one of these proteins was identified as a mitogen-activated protein kinase. These are serine/threonine kinases which undergo phosphorylation in cells following exposure to mitogens (243). Inhibition of tyrosine kinase has been shown to block LPS-induced TNF-α synthesis (182, 201), and Reimann et al. have recently suggested a scheme whereby LPS binding to CD14 induces an intracellular kinase cascade involving five different kinases (182). This kinase pathway ensures that the cell surface signal eventually activates specific gene transcription. As can be appreciated, the mechanisms by which LPS induces gene transcription via the CD14 receptor are now being shown to be extremely complex. To add to this complexity, there is evidence that CD14-independent signalling can also take place (182, 201).

An intriguing recent suggestion is that LPS stimulates cells by mimicking the action of ceramide, a lipid second messenger produced when cells are stimulated by proinflammatory cytokines such as IL-1 or TNF. Ceramide is produced by the hydrolysis of sphingomyelin by a sphingomyelin-specific form of phospholipase C and in turn activates a ceramide-activated protein kinase and a ceramide-activated protein phosphatase. Thus, Joseph and coworkers (104a) showed (i) that there was a close structural similarity between ceramide and a portion of the lipid A molecule and (ii) that LPS stimulated ceramide-activated protein kinase in a ceramide-independent manner. It was further shown that macrophages from the C3H/HeJ LPS-insensitive mouse failed to respond to cell-permeable analogs of ceramide or sphingomyelinase, demonstrating the importance of this pathway in LPS-induced cell activation (3a).

One obvious consequence of improved understanding of the nature of the intracellular signaling induced by LPS is that it may be possible to use this information in the development of therapeutic agents. For example, it has recently been shown that a selective tyrphostin inhibitor of a tyrosine kinase acting on the 42-kDa MAP kinase ($p42^{MAPK}$) inhibits LPS-induced TNF-α synthesis in vitro and protects mice against LPS-induced lethality (161).

CYTOKINE-STIMULATING CELL COMPONENTS OTHER THAN LPS

Only within the past few years has it become appreciated how many components and products of bacteria have the capacity to stimulate cytokine synthesis. These components, like LPS, may have relevance to human disease. For example, gram-positive bacteria are also causative agents of septic shock, and 150,000 patients each year are estimated to die from the release of cytokine-inducing gram-positive molecules (170, 195). The current literature on these cytokine-inducing molecules will be reviewed, and their mechanism of action will be related to our current understanding of the mechanisms by which LPS stimulates cellular cytokine synthesis, as reviewed above. The main reason for making such a comparison is to define the range of cellular mechanisms that bacterial components and products utilize to induce cytokine synthesis. If they stimulate cells by the same pathways that LPS utilizes, they will therefore be inhibited by the LPS-binding antibiotic polymyxin B, by neutralizing antibodies to CD14, and by antagonists of LPS. In addition, they should not stimulate a response in the LPS-unresponsive C3H/HeJ strain mouse. However, if any particular bacterial component or product fails to meet these criteria, it must be acting by a receptor and/or postreceptor mechanism distinct from that of LPS. Thus, when possible, each section below will relate the biological activity of the modulin under discussion to that of LPS. Each section will also include data on the potency of each preparation to give the reader an idea of the level of activity of each type of modulin in relation to the diverse range of potencies reported for LPS. The natures of the various types of cytokine-stimulating bacterial modulins and an approximate estimate of their potency are provided in Table 8. It must be emphasized that in almost all of the studies reported, care has been taken to ensure that the cytokine-inducing activity is not due to the presence of contaminating LPS.

TABLE 8. Cytokine-inducing bacterial components other than LPS

Component or product	Lowest concentration inducing cytokine synthesis (ng/ml)	Mechanism[a]
Porins	10	NL
LAP	1	NL
Fimbrial proteins	10	NL
Surface-associated proteins	0.01	NL
Protein A	1,000	?
Heat shock proteins	10	?
Lipoproteins	0.5	?
Glycoproteins	0.05	?
Lipids	0.1	?
Cell surface polysaccharides	2,500	NL/L
Peptidoglycan	100	?/L
Peptidoglycan fragments	50	NL
Teichoic acids	1,000	NL
LAM	100	L
Exotoxins	0.03	NL
Superantigens	10	NL
LPS	Wide range of potencies reported (pg/ml to μg/ml)	

[a] NL, the mechanism of cytokine stimulation is clearly different from that of LPS. L, available evidence suggests that the component stimulates cytokine synthesis by an LPS-like mechanism. For example, the activity of the component can be inhibited by anti-CD14 antibodies. NL/L, some reports demonstrate that certain preparations of this form of modulin act in an LPS-independent manner whereas other reports show that other preparations of the modulin (e.g., from other bacteria with different structures) act in a CD14-dependent manner.

Proteins

Outer membrane proteins. Approximately 50% of the dry mass of the outer membrane of gram-negative bacteria consists

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of proteins and more than 20 immunochemically distinct proteins (termed outer membrane proteins [OMPs]) have been identified in *E. coli*. Apart from their structural role, OMPs have also been shown to have other functions, particularly with regard to transport, and have been classified as permeases and porins. Furthermore, several OMPs have been shown to be potent inducers of cytokine synthesis.

(i) Porins. Porins are OMPs which form trimers that span the outer membrane and contain a central pore with a diameter of about 1 nm. These porins (e.g., OmpC and OmpF of *E. coli*) are permeable to molecules with molecular masses lower than approximately 600 Da. Apart from their transport functions, OMPs are major antigens, and there are now several reports of their ability to stimulate cytokine synthesis.

Isolated porins from *Salmonella typhimurium* (81), *Yersinia enterocolitica* (235), or *Helicobacter pylori* (234) have been shown to stimulate monocytes and lymphocytes to release a range of proinflammatory and immunomodulatory cytokines including IL-1, IL-4, IL-6, IL-8, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IFN-γ. The porins from *S. typhimurium* showed similar dose responses to the LPS from this organism in their capacity to stimulate the release of TNF and IL-6, with a rectilinear dose response over the range 10 ng/ml to 1 μg/ml (maximal response). In contrast, the porins were relatively inactive in stimulating IL-1α release from monocytes or IFN-γ release from human lymphocytes (60). The porins from *Y. enterocolitica* stimulated the release of IL-1α, IL-6, and TNF-α from human monocytes in a dose-dependent fashion over the concentration range 100 ng/ml to 5 μg/ml, with statistically significant release at 100 ng/ml, but they were less effective at stimulating the release of IL-8, IFN-γ, and GM-CSF (235). In spite of inducing the above cytokines, the porins were unable to stimulate IL-3 and IL-4 release even at concentrations as high as 20 μg/ml.

Injection of 0.3 to 30 μg of purified *S. typhimurium* porins into the paws of rats induced a dose-dependent edema which was maximal at 2 to 3 h and still present at 5 h. Similar doses of LPS failed to produce an edematous response. Edema was unaffected in animals which had had their complement levels depleted, demonstrating that inflammation was not associated with complement activation. However, it could be somewhat decreased by indomethacin and was significantly reduced by dexamethasone. Rat peritoneal cells incubated with porins released histamine but little prostacyclin, suggesting that porins have little ability to induce the prostanoid-producing enzyme cyclooxygenase II (62). Porins were also shown to kill D-galactosamine-sensitized LPS-responsive and LPS-unresponsive mice. A 100-ng amount of porin was sufficient to kill 80 to 90% of animals, a similar potency to that of LPS from *S. typhimurium*. The lethal effect of the porin preparation could be completely blocked by preadministration of a neutralizing antiserum to TNF-α but was not abolished by polymyxin B. Porins were also pyrogenic in rabbits and elicited a localized Shwartzman reaction when used as the sensitizing and eliciting agent. Both the fever and the Shwartzman reaction were unaffected by the administration of polymyxin B, indicating that LPS did not contribute to the biological responses (61).

The capacity of the porins to induce the synthesis of many proinflammatory cytokines and their stability to proteolysis suggest that the porins could play a role in the virulence of bacteria.

(ii) Lipid A-associated proteins. Some methods of extracting LPS from bacteria, for example with trichloroacetic acid or butanol, result in an LPS-OMP complex to which the term "endotoxin" should be applied. The proteins associated with the LPS are known as lipid A-associated proteins (LAP) or endotoxin-associated proteins and are known to have biological activities distinct from those of LPS. For example, endotoxins, unlike protein-free LPSs, are mitogenic for lymphocytes from LPS-unresponsive C3H/HeJ mice (206). It was subsequently demonstrated that the B-cell mitogenicity of butanol-extracted endotoxin was attributable to its LAP constituent (65, 217). The properties of LAP have been extensively reviewed by Hitchcock and Morrison (94). LAP appear to be important virulence factors, since immunization of *Salmonella*-hypersusceptible mice with LAP-LPS complexes but not with LPS protects against the lethality of *S. typhimurium* infection (110). Furthermore, LAP from several species have potent adjuvant activity (214–216). Thus, LAP may be key mediators of leukocyte behavior during infections, and, indeed, Hogan and Vogel (95) have suggested that LAP represent a "second signal" for the activation of macrophages. However, the role of this second signal in the responsiveness of the host to gram-negative bacteria needs to be more fully investigated.

Johns et al. (104) have reported that preparations of LAP from *S. typhimurium* have IL-1-like properties. This conclusion was based on the finding that injection of LAP into mice induces the acute-phase reactant serum amyloid A (SAA), a protein requiring a cytokine such as IL-1 for induction. However, unlike protein-free LPS, addition of LAP to macrophages did not induce "SAA-inducing activity" in culture, an activity which is assumed to be due to the presence of IL-1. LAP were also found to be active in the lymphocyte-activating factor assay and to act as a costimulatory factor for the proliferation of resting human T lymphocytes. LAP were also shown to be capable of inducing the formation of granulopoietic colonies when added to human peripheral blood and bone marrow progenitor cells which had been depleted of accessory cells (monocytes and T and B lymphocytes) (12). These workers also confirmed the great stability of the LAP inasmuch as heating to 100°C for 30 min or exposure to trypsin or pronase for 24 h at 37°C did not decrease the biological activity of these proteins. These findings have been largely confirmed by Porat et al. (173), who isolated LAP from *E. coli*. However, these workers reported that in contrast to the studies of Johns and coworkers (12, 104), T cells partially mediated the effect of the LAP and that the formation of granulocyte/macrophage colonies induced by LAP could be blocked by a neutralizing antiserum to IL-1β. Porat et al. also reported that the active protein in the LAP preparation had a molecular mass of 17 kDa (173). It is not known whether these differences in biological activity are because the LAP are from different bacterial species.

Direct comparison of the cytokine-stimulating activity of LAP and LPS has been reported for three bacterial species. Mangan et al. (142) have compared the capacities of LPS and LAP from *S. typhimurium* to stimulate IL-1 synthesis. Significant amounts of mRNA for IL-1β were induced in human monocytes exposed to 1 ng of LAP per ml, and in a comparison with the ability of LAP and LPS to induce these cells to release IL-1, the former was consistently three to four times more active. We have compared the ability of LAP and protein-free LPS from *Actinobacillus actinomycetemcomitans*, an organism associated with various forms of inflammatory periodontal disease, to stimulate the release of IL-1β, IL-6, or TNF-α from human monocytes or human gingival fibroblasts. LAP from this organism induced the release of IL-6 but not IL-1β or TNF-α from human gingival fibroblasts over the concentration range from 10 ng/ml to 10 μg/ml, with significant release at 10 ng/ml (181). In contrast, LPS from this organism was unable to stimulate IL-6 release even at 10 μg/ml. At higher concentrations (100 ng/ml), the LAP also induced the release of IL-6 and

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IL-1β but not TNF-α from human monocytes. We have also demonstrated that LAP from *Porphyromonas gingivalis*, one of the causative organisms of periodontitis, are potent stimulators of IL-6 release from human gingival fibroblasts (179). Significant release of IL-6 was found at a concentration of 10 ng/ml, whereas LPS from this organism was 1 log unit less active in this respect. The LAP and the LPS from this organism were equally active at inducing IL-6 release from myelomonocytic cells.

In summarizing the very limited studies of this fascinating group of proteins, the claim by Johns et al. (104) that preparations of LAP have IL-1-like activity deserves consideration. This was also the conclusion from our studies of a surface-associated fraction from the oral bacterium *A. actinomycetemcomitans*, which are discussed below (76).

(iii) Other outer membrane proteins. A preparation containing OMPs of *Shigella flexneri* (1 μg/ml was the lowest dose tested) induces murine macrophages to release TNF-α and IL-6 (25). The possible interactions between OMPs and other virulence factors, such as LPS, remain to be clarified. However, a 39-kDa OMP from *Proteus mirabilis* which is mitogenic for B cells (119) has been shown to inhibit the LPS-induced production of macrophage oxygen-derived free radicals as well as the LPS-induced synthesis of IL-1 by murine macrophages (240). Thus, in addition to inducing cytokine synthesis, at least one OMP appears to be able to downregulate bacterially induced cytokine synthesis.

Fimbrial proteins. Fimbriae (also known as pili) are rod-shaped structures originating in the cytoplasmic membrane and are composed of a hydrophobic protein termed pilin. Their main function is to enable the bacterium to adhere to host cells or to other bacteria by means of specific receptors. A bacterium may be able to elaborate a number of fimbriae, each with a specific adhesin at its tip to enable it to adhere to particular host cell receptors. Fimbriae are found mainly on gram-negative bacteria, although they have also been detected on streptococci and actinomycetes.

Injection of isolated *E. coli* pili into mice elicited a local stimulation of IL-6 release (136), and in in vitro studies, pili from *E. coli* were shown to stimulate epithelial cell lines to release IL-6 (80, 81, 122, 219). *E. coli* S-fimbriae at 10 μg/ml stimulated the release of IL-6 from renal epithelial cells; lower concentrations were not tested (122). These studies have been reviewed recently (80). A 14-kDa fimbrial protein (at 1 μg/ml) from *Salmonella enteritidis* was able to stimulate the release of IL-2 from T cells of mice immunized with the organism (163).

Several studies have demonstrated the ability of fimbriae from *P. gingivalis* to stimulate cytokine synthesis in various cell types. *P. gingivalis* fimbriae (1 μg/ml) stimulated human gingival fibroblasts to release IL-1β (162). IL-1β was also produced by mouse macrophages which were stimulated in a dose-dependent manner by *P. gingivalis* fimbriae (1 to 8 μg/ml) (73). The fimbrial protein also induced accumulation of IL-1β mRNA in the macrophages of "LPS-unresponsive" C3H/HeJ mice. At 4 μg/ml, fimbriae from this organism were able to induce IL-1β and GM-CSF gene expression in mouse embryonic calvarial bone cells (107). Matsushita et al. (148) reported that a 55-kDa cell surface protein, possibly a fimbrial protein, from the periodontopathogenic organism *Prevotella intermedia* was able to stimulate the release of IL-1α, IL-1β, IL-6, and IL-8 from human PBMCs when it was present at a concentration of 0.1 μg/ml but that TNF release required the fimbrial protein to be present at >1 μg/ml. The potencies of the fimbriae were generally similar to those of LPS from the organism. Human gingival fibroblasts were less responsive to the fimbrial protein—even concentrations as high as 100 μg/ml stimulated the release of only low levels of IL-1β and IL-6. The protein was also able to induce secretion of IL-6 and TNF-α from macrophages obtained from LPS-unresponsive C3H/HeJ mice, suggesting activation by an LPS-independent pathway.

Other cell wall-associated proteins. (i) Unidentified surface-associated proteins. Our own work has shown that gentle saline extraction of a number of oral gram-negative bacteria or *Staphylococcus aureus* releases a largely proteinaceous fraction which, on the basis of electron-microscopic examination of the cells before and after extraction, appears to consist of material loosely associated with the cell walls of these organisms. This material (which we have termed surface-associated material [SAM]) has a number of potent biological actions. An interesting although controversial finding was that SAM from *A. actinomycetemcomitans* exhibited IL-1-like activity in bioassays for IL-1 (76). SAMs from a number of oral bacteria have subsequently been found to be capable of stimulating the release of cytokines (IL-1, IL-6, IL-8, and TNF) from various human cell populations including monocytes, gingival fibroblasts, and neutrophils (180, 181). Of particular interest was the finding that with some oral bacteria, the SAMs were considerably more potent than the corresponding LPSs and were indeed as potent as (the potent) LPS from *E. coli*. For example, SAM from *A. actinomycetemcomitans* at a concentration of 10 pg/ml was able to stimulate IL-6 production by human PBMCs. Highly purified *E. coli* LPS, as expected, stimulated IL-6 secretion from human gingival fibroblasts by an IL-1-dependent mechanism, whereas IL-6 release stimulated by the SAM (at concentrations as low as 10 ng/ml) was independent of IL-1, since it was unaffected by the presence of IL-1ra or neutralizing antibodies to IL-1α and IL-1β. Neutralizing antibodies to TNF, which block TNF-induced IL-1 synthesis, also failed to inhibit IL-6 release. These data suggest that the SAM is inducing IL-6 gene transcription in mesenchymal cells by a novel mechanism. On the basis of its sensitivity to trypsin and heat, the active component of SAM was assumed to be proteinaceous, and isolation of the active moiety has revealed that the active component is a 2-kDa peptide (181a). The SAM at concentrations as low as 10 ng/ml also stimulated IL-1β and TNF-α release from human PBMCs.

Soluble surface-associated proteins from other organisms have the capacity to activate myelomonocytic cells and to induce cytokine synthesis. For example, Mai et al. (140) reported that *Helicobacter pylori*, an organism associated with gastric lesions, could stimulate human monocytes to express the cell surface class II major histocompatibility molecule HLA-DR and the IL-2 receptor and could also induce the synthesis of both IL-1 and TNF. The presence of as few as 1,000 intact bacteria was sufficient to induce significant cytokine synthesis. Extraction of LPS-free surface-associated proteins and LPS revealed that the former fraction was significantly more potent and efficacious in stimulating cytokine synthesis. There has been increasing interest in this organism in recent years, and a number of recent reports have suggested that *H. pylori* releases factors that can stimulate epithelial cells to produce leukotrienes and IL-8 (127) and also a protein which activates neutrophils (49). As described for oral bacteria, the surface-associated protein fraction contains a number of distinct proteins, and thus the potency of the active moiety is probably high. In an attempt to identify the component of *H. pylori* responsible for stimulating IL-8 release from epithelial cells, Huang et al. (99) concluded that a surface protein (possibly the CagA protein) was the most likely candidate.

The SR protein of *Streptococcus mutans* has also been reported to stimulate human monocytes to release IL-1, IL-6, and TNF in the presence of polymyxin B (208).

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(ii) Protein A. Most strains of *Staphylococcus aureus* have a 42-kDa surface protein which is able to interact nonspecifically with the Fc region of mammalian immunoglobulins. This protein, known as protein A, has also been shown to stimulate (at 1 to 10 μg/ml) human mononuclear cells to release IL-1, IL-4, IL-6, TNF-α, and IFN-γ (233) and was more active than other cell wall constituents, including muramyl dipeptide, muramic acid, and teichoic acid.

Heat shock proteins. Exposure of many bacterial species to environmental stress is known to induce the synthesis of a family of proteins known as heat shock proteins or stress proteins. The main function of these molecules is to ensure the correct folding of proteins, but they are also antigenic, and a number of studies have demonstrated their ability to stimulate cytokine release in vitro. Friedland et al. (56) reported that the 65-kDa heat shock protein from *Mycobacterium leprae*, at a concentration of 10 ng/ml, induced the synthesis of mRNA for TNF in a human monocytic cell line. The protein at a concentration of 10 μg/ml was also capable of stimulating the release of IL-6 and IL-8 from monocytes. Another heat shock protein from this organism, with a molecular mass of 71 kDa, also stimulates murine intraepithelial lymphocytes to release IL-3, GM-CSF, IFN-γ, and IL-6 but not IL-2, IL-4, IL-5 or TGF-β (5). Subsequently, heat shock proteins from a number of other bacterial species (*E. coli*, *Legionella pneumophila*, *M. leprae*, *Mycobacterium bovis*) have been shown to stimulate the accumulation of mRNAs for IL-1α, IL-1β, IL-6, GM-CSF, and TNF-α in murine macrophages (183). Given the capacity of these heat shock proteins to bind to other components, all the studies described were carefully controlled to ensure that the cytokine-inducing activity was not due to contaminating LPS.

Lipoproteins

Lipoproteins are found in the bacterial cytoplasmic membrane and are also common constituents of the cell wall of both gram-negative and gram-positive bacteria. Indeed, the Braun lipoprotein of *E. coli*, which is responsible for anchoring the outer membrane to the peptidoglycan layer, is one of the most abundant proteins in this organism. There are several reports concerning the cytokine-inducing ability of lipoproteins. A hydrophobic 48-kDa membrane lipoprotein from *Mycoplasma fermentans* has been reported to stimulate the release of IL-1β and TNF from human peripheral blood monocytes in a dose-dependent manner over the concentration range from 10 ng/ml to 1.0 μg/ml (120). The TNF-stimulating ability of the lipoprotein was abrogated by proteinase treatment, whereas a lipoprotein lipase removed some but not all of its cytokine-inducing ability. Anti-CD14 monoclonal antibodies had no effect on the cytokine-inducing ability of the lipoprotein, demonstrating that induction of cytokine release does not occur via binding to this putative LPS receptor.

A membrane preparation (0.5 to 50 ng/ml) extracted from *Mycoplasma arginini* and consisting of five lipoproteins was able to stimulate in a dose-dependent manner the release of IL-1, TNF-α, and IL-6 from human monocytes (90). Addition of polymyxin B had no effect on cytokine release. Most (approximately 80%) of the IL-1 remained cell associated.

Cytokine-stimulating ability is not confined to lipoproteins of mycoplasmas, because a surface lipoprotein (OspA) from *Borrelia burgdorferi*, the causative agent of Lyme disease, has been shown to stimulate (at a concentration of 500 ng/ml) the induction of mRNA for IL-1, IL-6, IL-12, IFN-β, and TNF-α in macrophages from LPS-responsive (BALB/c) and LPS-unresponsive (C3H/HeJ) mice (139). A subsequent study showed that lipid modification of the protein was essential for its cytokine-stimulating activity (244). OspA (at 100 ng/ml) could also induce IL-6 release from human PBMCs (220).

The importance of the lipid in these lipoproteins has been emphasized by the finding that the synthetic lipopeptide *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-2*RS*-propyl]-(*R*)-cysteinyl-alanyl-glycine (Pam_3Cys-Ala-Gly), an analog of the N terminus of a bacterial lipoprotein, stimulated the synthesis of IL-1, IL-6, and TNF-α by murine macrophages (77). Shimizu et al. (202) have demonstrated that the cytokine-inducing potency of a number of synthetic peptides (which are analogs of the N terminus of the *E. coli* lipoprotein) is affected by structural differences in the glycerol moiety. The synthetic lipopeptide CGP 31362, a structure derived from a gram-negative lipopeptide, stimulates LPS-unresponsive murine macrophages to release TNF-α by a process which involves protein tyrosine phosphorylation and mitogen-activated protein kinases (41). Thus, LPS and lipopeptides both appear to stimulate similar intracellular signal transduction pathways, which may be related to their ability to mimic ceramide (104a).

Glycoproteins

There is an increasing number of reports of the presence of glycoproteins in prokaryotes, and a number of cell wall glycoproteins from *Cytophaga johnsonae* have been shown to potently stimulate the release of TNF from a mouse macrophage cell line; concentrations as low as 50 pg/ml significantly stimulate TNF synthesis (30).

Lipids

Apart from LPS and its associated lipid A, few bacterial lipids have been investigated for cytokine-stimulating ability. Nevertheless, an uncharacterized, protein-free, lipid/polyol isolated from the membranes of *Mycoplasma fermentans* has been shown at concentrations as low as 100 pg/ml to stimulate the release of IL-6 and TNF-α from murine macrophages. Periodate treatment of the extract resulted in loss of most of the cytokine-stimulating activity, demonstrating the importance of the polyol moiety of the molecule in cell activation (157). A purified membrane preparation from *M. fermentans*, consisting of a dipalmitoyl- and a stearoyl-palmitoyl-glycerodiphosphatidylcholine, has also been shown to stimulate human monocytes to secrete TNF-α (194).

Polysaccharides

Cell surface polysaccharides. Capsular polysaccharides play important roles in bacterial virulence by, for example, hindering phagocytosis or inhibiting complement activation and complement-mediated killing. They are relatively poor immunogens and may lower the antibody response of the host to the bacterium. It is certain that cell surface and cell-associated carbohydrates will play other roles, and in this context there have been a number of recent reports that polysaccharides from bacterial cell walls and capsules can stimulate cytokine synthesis. Purified capsular polysaccharides from two serotypes (serotypes 5 and 8) of *Staphylococcus aureus* have been shown to stimulate the release of IL-1β, IL-6, IL-8, and TNF-α from human PBMCs; IL-8 from the human epithelial KB cell line; and IL-6 and IL-8 from human endothelial cells (207). However, cytokine release was evident only at polysaccharide concentrations of 10 μg/ml and above. Binding of the polysaccharides was demonstrated to occur in a dose-dependent, saturable fashion and was enhanced by calcium ions but inhibited by serum. The results of competitive binding assays suggested that both polysaccharides bind to the same receptor. The *Streptococcus*

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mutans serotype f polysaccharide (a rhamnose-glucose polymer) at a concentration of 25 μg/ml stimulates the release of TNF-α from human monocytes. Although the polysaccharide bound to both CD11b and CD14 on the monocyte cell surface, only binding to CD14 elicited cytokine release. However, the presence of heat-inactivated human serum inhibited cytokine production. The blood component responsible for inhibition was shown to be mannan-binding protein, which forms a complex with the polysaccharides which then binds to the C1q receptor prior to uptake by the monocyte (209). It is possible that this polysaccharide, whose binding is inhibited by anti-CD14, binds to one component of the LPS receptor.

Takahashi et al. (222) have shown that the serotype-specific polysaccharides from *A. actinomycetemcomitans* induce IL-1 release from murine macrophages at concentrations ranging from 12.5 to 100 μg/ml. In contrast, the polysaccharide group-specific antigens of *Porphyromonas gingivalis* were far more potent in that IL-1β was released from human PBMCs at a concentration of 100 ng/ml (159). The mucoid exopolysaccharide (an alginate containing mannuronic and guluronic acid residues) of *Pseudomonas aeruginosa*, at a concentration of 2.5 μg/ml, was able to stimulate the release of IL-1 from mouse macrophages (26). Surprisingly, addition of polymyxin B resulted in enhanced production of IL-1. On a weight basis, the polysaccharide was more potent than LPS from the same organism.

The relationship between the chemical structures of defined polysaccharides and cytokine synthesis has been reported by Otterlei et al. (168). Their studies showed that certain polysaccharides were as potent as (albeit fairly inactive) *E. coli* LPS, and they concluded that β(1-4)-linked polyuronic acids probably bind to the same receptor as LPS.

At least one study has shown that polysaccharides are able to induce cytokine production in vivo (141). Injection of type III or group-specific polysaccharides of group B streptococci into neonatal rats resulted in increased levels of circulating TNF-α, with the peak level induced by the group-specific polysaccharide being almost three times greater than that induced by the type III polysaccharide.

Peptidoglycan. As reviewed by Schwab (199, 200), peptidoglycan has long been recognized to have potent immunomodulatory actions. In terms of the ability of this structurally important cell wall component to induce cytokine synthesis, attention has focused largely on the synthetic muramyl dipeptide, *N*-acetyl-muramyl-L-alanyl-D-isoglutamine (MDP), the smallest common structural unit of peptidoglycans. MDP and its analogs can stimulate the synthesis of a variety of cytokines by a range of cell populations, and the literature has been well reviewed (18, 167). However, MDP is a synthetic molecule which is not a product of bacterial or eukaryotic degradation of peptidoglycan, and therefore results obtained with this molecule will not be discussed.

There have been few studies of the cytokine-stimulating ability of peptidoglycan. This is probably largely because of the complications involved in dealing with this material, which is generally insoluble and which, depending on the method of preparation and dissolution, has a wide range of molecular masses and structures (although such problems have not prevented investigations with LPS). Purified cell walls from a number of gram-positive bacteria have been reported to stimulate the release of IL-6 and TNF-α from human PBMCs at a concentration of 100 ng/ml (92). Cytokine release was unaffected by polymyxin B or by the presence of anti-CD14 monoclonal antibodies, except for *Streptococcus pyogenes*. Fractionation of the cell walls revealed, in each case, that most of the cytokine-stimulating activity was attributable to the peptidoglycan components (92).

A soluble peptidoglycan (molecular weight, ~125,000) obtained from the supernatant of cultures of *Staphylococcus aureus*, grown in the presence of penicillin, was able to induce the release of IL-1 and IL-6 from human PBMCs in a dose-dependent manner over the range 1 to 30 μg/ml (241). In this respect, the peptidoglycan was almost 1,000-fold less active than LPS from *Salmonella friedenau*. As was found for peptidoglycan from *Streptococcus pyogenes* (92), anti-CD14 monoclonal antibodies were able to block peptidoglycan-induced cytokine release.

In a comparative study of the capacity of whole *Staphylococcus aureus*, *S. epidermidis*, and their respective peptidoglycans to stimulate TNF-α release from human monocytes, it was demonstrated that whereas whole cells and the peptidoglycan were able to stimulate the release of this cytokine, the former were more effective stimuli than were the isolated cell wall components (227). Thus, 10^7 staphylococcal cells, corresponding to 0.1 μg of peptidoglycan, produced the same amount of TNF as did 1 to 10 μg of purified peptidoglycan. The explanation of these differences in activity could relate to degradation of the peptidoglycan during isolation, to differences in steric interaction between whole-cell peptidoglycan and isolated material, or to synergistic interactions between peptidoglycan and other cell wall components in whole bacteria. Indeed, degradation of the peptidoglycan by enzymes or by sonication decreased its capacity to induce TNF synthesis (108, 227).

Monocytes are not the only cells which respond to peptidoglycans by releasing cytokines. Lichtman et al. (135) have shown that rat Kupffer cells can be induced to release IL-1 and TNF-α by exposure to peptidoglycan-polysaccharide from *Streptococcus pyogenes*. However, the potency of the peptidoglycan-polysaccharide was quite low, with microgram-per-milliliter concentrations required to induce detectable amounts of cytokine and 100 μg/ml required for maximum release. LPS from *E. coli* was a far more potent inducer of cytokine release from the Kupffer cells, and it is interesting to compare the mechanisms of cytokine induction by these two components. Cytokine induction is preceded by endocytosis in both cases, since cytochalasin B blocked cytokine release by peptidoglycan-polysaccharide and LPS. However, microtubule function appears to be more important in LPS-stimulated cytokine release, because this was reduced considerably by addition of colchicine. Since nisoldipine, a calcium channel blocker, inhibited cytokine production by both components, this would imply that calcium influx acts, in some manner, as the second messenger with both agonists. It is of interest that taxol, an anticancer agent and microtubule stabilizer, has a similar activity profile to LPS when added to myelomonocytic cells (144).

Peptidoglycan fragments. Peptidoglycans released in vivo will be degraded by host enzymes, and such breakdown products may, in turn, have cytokine-inducing activity. Two recent papers report that *N*-acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramyl-L-alanyl-D-isoglutamyl-*m*-diaminopimelyl-D-alanine [G(Anh)MTetra], a naturally occurring breakdown product of peptidoglycan, at concentrations as low as 50 ng/ml, induces the synthesis of IL-1β, IL-6, and G-CSF by human monocytes (39, 40). This finding confirms the initial study of Dinarello and Krueger (38). The synthesis of all three cytokines can be blocked by inhibitors of protein kinase C but not by inhibitors of protein kinase A or tyrosine kinases. Interestingly, the transcriptional control of these three cytokines showed distinct differences. In run-on transcription assays, [G(Anh)MTetra] markedly increased IL-1β transcription, whereas it had much less effect on the transcription rate of the gene for IL-6, and

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the increased level of mRNA for G-CSF was shown to be due to stabilization of the mRNA transcripts. By using the protein synthesis inhibitor cycloheximide, it was shown that IL-6 mRNA expression depended on the synthesis of new protein but that this was not the case for IL-1β or G-CSF.

Bordetella pertussis, the causative organism of whooping cough, releases a low-molecular-mass (921-Da) peptidoglycan fragment, called tracheal cytotoxin, which damages airway epithelium and contributes to disease pathology. Addition of tracheal cytotoxin to respiratory epithelial cells resulted in the rapid accumulation of intracellular IL-1α but without release of this cytokine. It has been proposed that this upregulation of intracellular IL-1 may contribute to epithelial cell pathology in pertussis (82). This is but one of a growing number of examples in which toxins, lysins, etc., also show the capacity to induce cytokine synthesis.

Water-soluble peptidoglycan fragments from *Staphylococcus epidermidis* have also been reported to stimulate the proliferation of spleen mononuclear cells from various strains of mice and the synthesis of various cytokines including IL-1 and GM-CSF (165).

The nature of the receptors on mammalian cells responsive to peptidoglycan has not been defined, although there is evidence that peptidoglycan binds to the LPS receptor (42, 43, 45). However, Dziarski (42, 43) has shown that neither muramyl dipeptide nor [G(Anh)MTetra] competes for LPS-binding sites on murine macrophages, a finding confirmed by LeContel et al. (126). However, these data must now be interpreted in the light of Dziarski's recent claim that the 70-kDa LPS and peptidoglycan receptor is, in fact, cell-bound serum albumin (44).

Teichoic Acids

Anionic polymers such as teichoic and lipoteichoic acids (LTAs) are major components of the walls of gram-positive bacteria. Teichoic acids consist of chains of glycerol, ribitol, mannitol, or sugars linked by phosphodiester bonds and are attached to muramic acid residues in the peptidoglycan. D-Alanine and L-lysine are common substituents of the chains, which usually contain approximately 40 residues. LTAs consist of chains of glycerol phosphate, with D-alanine and sugar substituents, attached to a glycolipid (or diglyceride) in the cytoplasmic membrane. Both teichoic acids and LTAs are antigenic and often constitute the major somatic antigens of gram-positive bacteria. LTAs are amphiphilic molecules with a number of biological activities. In some ways, LTAs can be regarded as the gram-positive equivalent of LPSs, although the latter have a much greater potency and range of biological activities.

Riesenfeld-Orn et al. (184) reported that LTA from pneumococci stimulated human monocytes to release IL-1 but not TNF. In contrast, the LTA from *Streptococcus faecalis* stimulated murine mononuclear cells to release both cytokines, with deacylation of the LTA abrogating the cytokine-stimulating activity (232). In comparative studies of the LTAs from a range of gram-positive bacterial species, Bhakdi et al. (9) reported that there were major differences in their capacities to stimulate human monocyte cytokine synthesis (specifically IL-1β, IL-6, and TNF-α). LTAs from several enterococcal species were capable of stimulating the release of similar amounts of all three cytokines to those induced by a crude preparation of *E. coli* LPS, although the potency of the LTA was significantly lower than that of the LPS. In contrast, LTAs from organisms such as *Staphylococcus aureus*, *Streptococcus mutans*, and *Leuconostoc mesenteroides* were inactive. In agreement with the findings of Tsutsui et al. (232), deacylation abolished this activity. Addition of polycations (poly-L-arginine or poly-L-lysine) to monocytes stimulated with LTA abolished the stimulatory activity, although the polycations had no effect on the cytokine-stimulating activity of LPS. Analysis of the kinetics of cytokine stimulation revealed that very short exposure of cells to LTA (5 to 30 min) was sufficient to trigger cytokine production, suggesting that the cellular receptor for LTA is distinct from that of LPS and is perhaps the macrophage scavenger receptor (145). This can also be inferred from the report that LTA stimulation of cytokine production by human monocytes is not inhibited by blockade of CD14 (92). The cytokine-stimulating LTAs were also shown to be able to stimulate murine bone marrow macrophages to release the potent cell-cell-stimulating gas, nitric oxide (98). While Bhakdi et al. (9) failed to find that LTA from *Staphylococcus aureus* stimulated cytokine production, Standiford et al. (211) reported that LTA from this organism and from *Streptococcus pyogenes* stimulated human monocytes to produce IL-8. In a comparative study of LPS, peptidoglycan, and teichoic acid (the last two components from *Staphylococcus epidermidis*), it was found that the teichoic acid could stimulate the production of IL-1β, IL-6, and TNF-α, although only at concentrations of 10 to 100 μg/ml (149). Thus, the conclusion from the literature is that the teichoic acids are weak stimulators of cytokine synthesis, requiring microgram-per-milliliter concentrations for activity. Nevertheless, evidence for the possible involvement of LTA-induced cytokine release in an infectious process comes from the study of Danforth et al. (28), who investigated the expression of macrophage inflammatory protein 1α (MIP-1α), a macrophage-activating and chemotactic cytokine, in endocardial samples from patients with acute *Staphylococcus aureus* endocarditis. Cell-associated MIP-1α expression was detected immunohistochemically in neutrophils, macrophages, and fibroblasts. Furthermore, human PBMCs treated with LTA in vitro stimulated the release of MIP-1α.

Gram-positive bacteria can produce a septic shock-like condition, and as many people die each year from gram-positive as from gram-negative sepsis. LPS is obviously a very potent inducer of cytokine synthesis. Peptidoglycans and teichoic acids are believed to be the equivalent components, inducing cytokine-induced shock in patients with gram-positive sepsis. However, given the relatively weak cytokine-inducing activity of peptidoglycan and teichoic acids in the studies described above, perhaps we have to look at other gram-positive bacterial components as the inducers of the shock-like state.

Mycobacterial Cell Wall Components

In the genus *Mycobacterium*, the peptidoglycan layer is covered by lipid-rich layers, so that up to 60% of the dry weight of the cell wall may consist of lipids, rendering it extremely hydrophobic. A variety of lipids, glycolipids, and lipoproteins have been isolated from mycobacterial cell walls, and several of these contain mycolic acids, which are unique to the mycobacteria, nocardiae, and corynebacteria.

Lipoarabinomannan. Lipoarabinomannan (LAM), a major cell wall component of *Mycobacterium* spp., exhibits immunoregulatory and anti-inflammatory effects which favor the survival of the mycobacteria. These effects include suppression of T-lymphocyte proliferation through interference with antigen processing (154), inhibition of macrophage activation by IFN-γ (204, 205), and scavenging of oxygen-derived free radicals (17). Moreno et al. (154, 155) were the first to report that LAM had the capacity to stimulate human blood monocytes and activate murine peritoneal macrophages to release TNF-α. Maximal release was found at a concentration of 10 μg/ml, with signif-

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icant release at a concentration of 100 ng/ml. A surprising finding was that polymyxin B, a well-known inhibitor of LPS activity, bound to and inactivated LAM, suggesting a similarity in structure between LAM and LPS. Treatment of the LAM with dilute alkali significantly diminished its TNF-stimulating activity, suggesting that the *o*-acyl groups may be responsible for stimulation of cytokine synthesis (155). In a more detailed study, Barnes et al. (4) showed that LAM stimulated human PBMCs to transcribe mRNA for cytokines normally thought of as being macrophage products, i.e., IL-1α, IL-1β, IL-6, IL-8, GM-CSF, TNF, and IL-10. In contrast, LAM did not stimulate the transcription of cytokines normally associated with lymphocytes, i.e., IFN-γ, IL-2, IL-3, and IL-4, although the whole bacterium *Mycobacterium tuberculosis* was capable of stimulating the transcription of these cytokine genes. Lipomannans and phosphatidylinositol mannoside were capable of stimulating the same cytokine profile as was LAM. Deacylation of LAM almost totally inhibited its capacity to induce cytokine synthesis, showing that the activity was associated with the phosphatidylinositol portion of the molecule.

Evidence is mounting to support the hypothesis that LAM and LPS have very similar modes of action on cells. Thus, the stimulation of IL-8 release from the human monocyte cell line THP-1 by LAM is inhibited by anti-CD14 antibodies (175a). Leukocyte β_2-integrin receptors may also bind LAM (196). It has also been demonstrated that LAM and LPS activate human monocyte IL-6 gene expression by an identical pathway involving NF-κB and NF-IL-6 (259). Interestingly, LAM preparations from virulent or attenuated strains of mycobacteria differ markedly in their biological actions, with the former but not the latter being unable to stimulate murine resident peritoneal macrophages to release TNF-α (1). The LAM from an avirulent mycobacterium, strain H37Ra, is able to stimulate the transcription of the immediate-early response genes c-*fos*, JE, and KC and to stimulate the production of TNF-α. In contrast, LAM from a virulent strain, Erdman, failed to trigger these genes or the production of TNF-α (187, 188). This capacity to produce LAM, which is incapable of stimulating a macrophage response, is now seen as a key determinant in mycobacterial virulence, and the structural basis of the differences is under investigation.

Other mycobacterial components. Other components of mycobacteria have recently been shown to stimulate cytokine synthesis. These include a 58-kDa protein that is able to stimulate human monocyte TNF-α synthesis (238) and the 65-kDa mycobacterial heat shock protein, which has been reported to stimulate TNF production (56). In addition, purified protein derivative of tuberculin from *Mycobacterium tuberculosis* has been reported to preferentially stimulate a Th1 pattern of cytokine production from human peripheral blood (31). Lipomannan and phosphoinositolmannoside at 100 ng/ml have also been shown to stimulate the release of IL-8 from human PBMCs in the presence of polymyxin B (258).

EXTRACELLULAR PRODUCTS

Proteases

As a result of their tissue-damaging potential, proteases are well recognized as virulence factors in disease. However, in recent years, bacterial and viral proteases have been found to interact with the cytokine network in both positive and negative ways. For example, the potent proinflammatory cytokine IL-1β is initially synthesized as an inactive 31-kDa precursor, which has to be proteolytically cleaved by a specific proteinase, ICE, which acts at Asp-116 and Ala-117 to produce biologically active IL-1β (247). Exotoxin B, a conserved extracellular cysteine protease of *Streptococcus pyogenes*, cleaves the inactive IL-1β precursor between His-115 and Asp-116 to produce biologically active IL-1 (106). This bacterial protease could therefore stimulate the production of IL-1β locally and induce tissue pathology. In contrast, vaccinia virus contains a gene (*crmA*) for a 38-kDa serpin which inhibits the activity of ICE, thus inhibiting IL-1β production (178). *Pseudomonas aeruginosa* produces an alkaline protease and elastase, both of which can inactivate a range of human cytokines including IL-1, IL-2, IFN-γ, and TNF-α (109, 169), and a protease from *Legionella pneumophila* has been reported to inactivate IL-2 and cleave CD4 on human T cells (153). Many bacteria produce proteases and also protease inhibitors, which may play roles in the control of cytokine networks.

Exotoxins

Many species of bacteria secrete potent exotoxins. A wide variety of cells or tissues constitute the targets for these molecules, which display an equally wide range of actions. Nevertheless, there is increasing evidence that the exotoxins have one property in common—their capacity to induce cytokine release in cells that are not necessarily their prime target.

The Shiga-like toxin type 1 at 100 ng/ml has been shown to induce the release of IL-1α, IL-6, and TNF-α in macrophages from C3H/HeN mice. TNF-α release was unaffected by polymyxin B and also occurred in macrophages from LPS-unresponsive C3H/HeJ mice, suggesting that Shiga-like toxin type 1 uses a different signalling pathway from LPS in inducing cytokine release (226).

Streptococcus pyogenes erythrogenic toxin A at 200 ng/ml was able to stimulate the release of IL-1α, IL-1β, IL-6, IL-8, TNF-α, and TNF-β from human PBMCs, the most abundant cytokine produced being IL-8 (158). In contrast, monocytes exposed to erythrogenic toxin A released only small quantities of TNF-α and did not produce IL-1α, IL-1β, or IL-6. These findings imply that in contrast to LPS, erythrogenic toxin A requires the presence of T lymphocytes to elicit the release of cytokines from monocytes.

The edema toxin of *Bacillus anthracis* (which consists of an "edema factor" together with a "protective antigen") can stimulate the release of IL-6 but not TNF-α from human monocytes. Of particular interest was the finding that the toxin also inhibited the LPS-induced release of TNF-α from human monocytes (96).

Another toxin able to downregulate cytokine release is the α-hemolysin of *E. coli*. This toxin inhibits the release of IL-1β, IL-6, and TNF-α from human leukocytes as a result of calcium-dependent binding of the toxin to the cells (118). This pore-forming toxin also caused the rapid release of large amounts of IL-1β from cultured monocytes but not from freshly isolated cells. The effects appeared to be due not to stimulation of IL-1 synthesis but to stimulation of the processing and export of preformed cytokine (11). Monocytes did not release TNF-α in response to this toxin. A similar finding was reported with the pore-forming staphylococcal α-toxin (10). The *E. coli* hemolysin is a potent inducer of phosphoinositide hydrolysis in human polymorphonuclear leukocytes (67), and both the *E. coli* and staphylococcal toxins induce vascular endothelial cells to release nitric oxide (218). A recent report has highlighted the synergism that exists between LPS and *E. coli* hemolysin (239). Newly discovered toxins such as the mitogenic factor from streptococci also have the capacity to stimulate cytokine synthesis (160).

Listeriolysin O, a 58-kDa thiol-activated hemolysin, is the

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best-characterized virulence factor of *Listeria monocytogenes* and is necessary for the intracellular survival of the organism. Two recent reports have established that this toxin can potently induce murine macrophages to synthesize both IL-1 mRNA transcripts and protein (231, 256). A toxin from *Bordetella pertussis*, termed pertussis toxin, which is a protein, has also been shown to enhance IL-4 production, which may explain the ability of this toxin to upregulate immunoglobulin E responses (156).

Two of the most potent stimulators of proinflammatory cytokine synthesis are pneumolysin from *Streptococcus pneumoniae* and toxin B from *Clostridium difficile*, an organism causing pseudomembranous colitis. The former toxin has recently been reported to stimulate human monocytes to produce both IL-1β and TNF-α, with 3 pg of this toxin per ml being sufficient to induce the synthesis of both IL-1 and TNF and 10 pg/ml producing more than 50% of the maximal response. Since pneumolysin has a molecular mass of 56 kDa, 3 pg/ml is equivalent to a molar concentration of approximately 5×10^{-14} M. Pneumolysin was more potent than LPS and produced a greater maximal response. At a concentration of 1 ng/ml, pneumolysin produced three to four times the amount of cytokine that was induced by a concentration of 50 ng of LPS per ml (97). Purified toxin B stimulated human monocytes to produce IL-1, TNF, and IL-6 (55). The maximal stimulation of TNF and IL-6 synthesis was produced by 300 pg of toxin B per ml. Since toxin B has a molecular mass of 269 kDa, this is equivalent to a molar concentration of 10^{-12} M. Toxin B and LPS showed synergistic interactions in the induction of the synthesis of IL-α, IL-6, and TNF. A second toxin from this organism, toxin A, also induced cytokine release but was approximately 1,000 times less potent than toxin B. The receptor for toxin A has been identified as a galactose- and *N*-acetylglucosamine-containing glycoprotein coupled to a G-protein (174). Toxin B has recently been shown to possess monoglucosyltransferase activity, and one of its actions on eukaryotic cells is to glucosylate a GTPase involved in actin cytoskeleton regulation (105). It is not clear if this activity is related to cytokine induction.

Superantigens

The ability of certain bacterial exotoxins to activate large proportions of the peripheral T-lymphocyte populations of humans and mice, as opposed to the small numbers of lymphocytes activated by processed peptide antigens, led Kappler and Marrack to name them superantigens (246). Most superantigens isolated to date are produced by *Staphylococcus aureus* and by streptococcal species. Among the superantigens released by *S. aureus* are the group of enterotoxins consisting of six different serotypes (staphylococcal enterotoxins A to E [SEA to SEE]), toxic shock syndrome toxin 1, and an exfoliative toxin (8). *Streptococcus pneumoniae* produces pyrogenic exotoxins: SPEA, SPEB, and SPEC (8). Two gram-negative bacteria, *Y. enterocolitica* (213) and *Pseudomonas aeruginosa* (129), are also reported to produce superantigens. To date, most studies have concentrated on the superantigens from *S. aureus*.

Injection of a superantigen such as SEB into mice results in the rapid appearance of a variety of cytokines, i.e., TNF, IL-1, IL-6, and IFN-γ in the serum; in D-galactosamine-sensitized mice, the injection of 2 μg of SEB per mouse resulted in 50% mortality, which could be blocked by administration of a neutralizing antibody to TNF-α (151). The mechanism of cytokine generation in vivo is obviously complex, involving antigen-presenting cells, B lymphocytes, and T lymphocytes. However, it appears that in cell culture, binding of the superantigens to class II molecules is the trigger for cytokine production (57) and that the subsequent synthesis of cytokines such as IL-1 is dependent on protein tyrosine phosphorylation (197). The involvement of the major histocompatibility complex is clearly seen when class II-deficient mice are used, these animals being resistant to the effects of staphylococcal enterotoxins (212). In studies in which the dose-dependent activation of cytokine synthesis by SEA has been determined in cultures of murine monocytes, 200 ng/ml was sufficient to induce maximal production of TNF-α, with an IC_{50} of <50 ng/ml. Both toxic shock syndrome toxin 1 and SEB, at a concentration of 100 ng/ml each, have also been reported to stimulate the release of IL-12 (a proinflammatory cytokine with immunoregulatory functions) from human PBMCs (133). Thus, these superantigens can be potent inducers of cytokine synthesis (68).

A number of investigations have also demonstrated that there are synergistic interactions between superantigens and endotoxins which could be important in the induction of tissue pathology (6, 68, 89, 133, 212).

Other Extracellular Proteins

An uncharacterized protein (>150 kDa) isolated from the supernatant of cultures of *Streptococcus mitis* was able to stimulate the release of TNF-α, IL-6, IFN-γ, and thymocyte-activating factor in human peripheral blood cells when used at a concentration of 0.2 μg/ml (approximately 1 nM). The cytokine-stimulating activity was abrogated by protease and heat treatment. This factor also stimulated IL-6 and thymocyte-activating protein, presumably IL-1, release from human gingival fibroblasts and human umbilical vascular endothelial cells. The protein was as potent as LPS from *Salmonella abortus-equi* in stimulating TAF from human umbilical vein endothelial cells (221).

BACTERIAL ADHESION

The first stage of an infectious process involves adhesion of the organism to host tissue. The mechanism of adhesion and the bacterial components involved in the process have long been the subject of extensive investigation. However, only recently has it been reported that adhesion of an organism to host cells may trigger cytokine synthesis. Whether or not the cytokines released as a result of this interaction actually play a role in adhesion remains to be determined. However, their overproduction may have pathological consequences for the host. Attachment of *Salmonella typhimurium* to monolayers of human intestinal epithelium has been shown to result in the synthesis of the potent chemoattractant IL-8 and in the transepithelial migration of human polymorphonuclear leukocytes (150). Such migration was found not to be due to the classical formyl-peptide-induced directed migration pathway, and neutralization of the IL-8 did not block polymorphonuclear leukocyte transmigration, suggesting that a novel transcellular chemotactic factor is induced by interaction of bacteria with epithelial cells.

Yamamoto et al. (254) have also reported that binding of *Legionella pneumophila* to the surface of murine macrophages (treated with cytochalasin D to prevent phagocytosis) resulted in increased levels of mRNA for IL-1α, IL-1β, IL-6, TNF-α, and GM-CSF but not IFN-β.

BACTERIAL INVASION

A number of bacteria enter and survive within mammalian cells, and it has been adduced in recent years that cytokines and/or their receptors are involved in the process of cell inva-

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sion. For example, the invasion of intestinal epithelial cells by *Salmonella typhimurium* requires the phosphorylation of the epidermal growth factor receptor (58). It has also been shown that the invasion of fibroblasts by *Shigella flexneri* induces the production of IFN-β, while isogenic variants which are not invasive do not stimulate cytokine synthesis. This suggests that bacterial uptake may require IFN synthesis (91). Similarly, Eckmann et al. (46) concluded from their studies of the invasion of epithelial cells by *Salmonella* spp. and *Listeria monocytogenes* that the process of cell entry, and not simply the presence of the bacteria, was the signal inducing invaded cells to synthesize and secrete IL-8. As epithelial cells are the first site of entry of bacteria, the activation of IL-8 synthesis would act as an early-warning system at a time when bacterial products are unavailable to stimulate circulating leukocytes.

It has also been reported that the invasin protein of *Yersinia pestis* interacts with β_1-integrins and acts as a costimulatory signal for the proliferation of CD4 lymphocytes, a process accompanied by the synthesis of TNF-α and IFN-γ (14).

Recently it has been shown that endothelial cells also respond to bacterial invasion by releasing cytokines (255). Internalization of *Staphylococcus aureus* by human umbilical vein endothelial cells resulted in the synthesis of mRNA for IL-6 after 3 h and then IL-1β mRNA after 12 h. Treatment of the endothelial cells with cytochalasin D, an inhibitor of endocytosis but not of bacterial adhesion, inhibited IL-6 and IL-1β gene expression, demonstrating that cytokine induction was a consequence of bacterial invasion rather than adhesion.

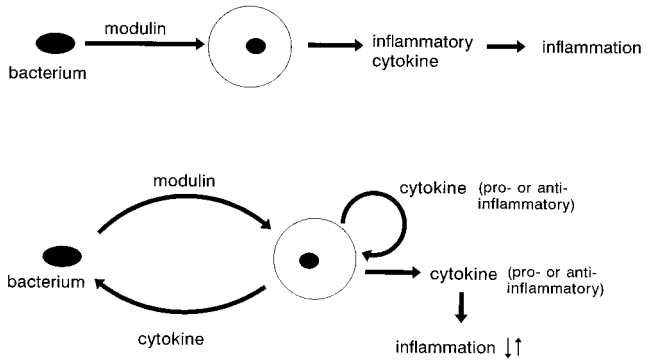

FIG. 7. The conventional paradigm of the interaction of bacterial products with host cells is shown in the top diagram and is a direct linear sequence of interactions. In the bottom schema, this interaction is seen to be much more complex. Modulins interact with host cells to induce a cytokine network which may either upregulate or downregulate inflammation. In the case of our own normal microflora, we suggest that the interaction of the modulins from these bacteria with host cells sets up a cytokine network which is anti-inflammatory. Recent information from the study of cytokine knockout mice has shown that the removal of a key modulatory cytokine (e.g., IL-2 or IL-10) results in severe inflammation in response to the normal microflora. In addition to the cytokines induced by the modulins influencing host cell behavior, there is now evidence to suggest that these cytokines can interact directly with bacteria. Such interactions may either remove cytokines (by binding to cell surface receptors), thus impeding the host inflammatory response, or enhance the growth of bacteria, thus acting to the advantage of the microorganism.

SUMMARY AND CONCLUSIONS

Cytokines are potent signalling molecules which have important homeostatic regulatory functions, particularly with regard to the response of the organism to bacteria, multicellular parasites, and viruses. As reference to the bibliography will support, until the late 1980s LPS was the only bacterial component firmly established as being able to stimulate eukaryotic cells to synthesize and release cytokines. During the same period, it was established that the response of mammals to LPS is a complex one, involving the interaction of LPS with a range of proteins such as LBP, bactericidal/permeability-inducing protein, septin, CD14, HDLs, and possibly many other, as yet undiscovered, eukaryotic proteins. It is the complex between LPS and LBP/CD14 which is the active stimulator of cellular cytokine synthesis, free LPS being much less active in this respect. Thus, mammals and presumably other members of the kingdom *Animalia* have evolved systems for detecting, amplifying the response of, and eventually disposing of one component of gram-negative bacteria, i.e., LPS. The potency of LPS as an inducer of cytokine synthesis has resulted in the widely accepted paradigm that bacteria (particularly pathogenic organisms) induce cytokine synthesis via released LPS/endotoxin and that such cytokines then act to limit the infection as well as producing the symptoms of the infection. This is a simple linear relationship, as shown in Fig. 7. However, in recent years, additional information has become available which suggests that the interactions between bacteria and their host may be more complex. The central theme of this review is that LPS is not the only component/product of the bacterial cell which can induce eukaryotic cells to produce cytokines, and we can see that during the past 10 years, a large number of reports have established that many proteins, some carbohydrates, and a few lipids can induce a range of murine and human cells to produce cytokines.

It is important for the reader to note that in virtually all of the reports reviewed in this article, the authors had used appropriate controls (e.g., the failure of polymyxin B to inhibit cytokine-inducing activity, the inhibition of the cytokine-inducing activity by heat or protease, and/or the ability to stimulate monocytes from C3H/HeJ LPS-unresponsive mice) to demonstrate that the activity of the bacterial component under test was not due to contaminating LPS. Therefore, we are not simply reviewing a mass of literature on LPS-contaminated bacterial components.

It is clear that some of the modulins, in addition to LPS, act on cells by binding to CD14. Examples of this are LAM, peptidoglycan from *Staphylococcus aureus*, and polysaccharides from *Streptococcus mutans* (175a, 247a). This has given rise to the speculation that CD14 is a pattern recognition receptor evolved to recognize both gram-negative and gram-positive bacteria (175a). However, although not examined in detail, many of these non-LPS cytokine-inducing molecules act by mechanisms distinct from those used by LPS. For example, their activity is not blocked by anti-CD14 antibodies but is inhibited by heat or proteolytic digestion or they activate monocytes from the LPS-unresponsive C3H/HeJ mouse. Indeed, a few of the reports suggest that bacteria possess molecules that are able to downregulate the cytokine-inducing actions of LPS. The only clearly identified receptors for the modulins are major histocompatibility complex class II (57), which binds superantigens, and a G-protein-coupled glycoprotein to which *Clostridium difficile* toxin A binds (174). One of the major questions that this review raises is how many receptors are needed to recognize this large, and increasing, number of bacterial cytokine inducers. Does each modulin have its own receptor, or, like CD14, are there other pattern-recognizing receptors on the eukaryotic cell surface that are able to recognize a range of modulins (175a)? Given the number of modulins that have been identified, the interactions between them and eukaryotic cells, plus the cytokines they induce, must be extremely complex, raising the possibility of a supernetwork.

Thus, we now have a developing picture in which bacteria

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possess many molecules, both components and expressed products, able to interact with eukaryotic cells to induce (or possibly inhibit) cytokine synthesis. One of the surprising findings from this review of the literature was the potency of some known toxins as stimulators of cytokine synthesis. They were at least as potent as the most potent preparations of LPS. The possibility that these molecules have been misclassified or that they have dual functions which depend on the amounts produced by bacteria deserves consideration.

The large and growing number of bacterial molecules shown to be able to interact with eukaryotic cells to induce cytokine synthesis suggests that there is a more complex interplay between the prokaryotic and eukaryotic worlds than has previously been perceived. It is surely unlikely that all these cytokine-inducing bacterial components are there simply to warn the host of the presence of bacteria. A plausible hypothesis is that these cytokine-inducing molecules form part of an interactive signalling system. A number of pieces of evidence in support of this hypothesis have been published in recent years. Indeed, there is a dawning of the appreciation that bacterial virulence is not simply a reflection of the attributes of the bacterium but also a synthesis of the interaction between the bacterium and the host (reviewed in reference 195). We have already discussed the consequences of genetically disabling cytokine genes, with the finding that certain transgenic knockout animals develop inflammation in response to their normal commensal microflora (123, 193) (Table 7). From these studies, we suggest that the normal role of IL-2 (possibly IL-4) and IL-10 is to downregulate the inflammatory response to the bowel microflora and that these may only be two of many cytokines involved in regulating our response to the enormous microbiota colonizing the multicellular host. Indeed, we go further and speculate that many of the cytokine-inducing proteins produced by bacteria are evolutionary precursors of mammalian cytokines, so that perhaps they should be named bacteriokines, although we shy away from introducing a second term in this review. This idea is not as speculative as it may seem. There is now very good evidence for the production of cytokine-like molecules (or cytokine network-modulating molecules) by various viruses. Cowpox virus is the most interesting virus in this respect, producing a range of proteins whose functions would appear to be to suppress the inflammatory response to virally infected cells. Among the cytokine-modulating molecules produced by this virus is a protein which inhibits ICE (178), the protease which generates the active form of IL-1β, and a soluble IL-1 receptor which prevents the binding of IL-1β, but not IL-1α or IL-1ra, to the type I IL-1 receptor (210). This clearly tells us something about the way the cowpox virus perceives the two forms of IL-1, with IL-1β being the antiviral cytokine. A number of other viruses produce homologs of cytokines: e.g., BCRF-1 is a homolog of the anti-inflammatory cytokine IL-10 (98), and a range of soluble cytokine receptors are also produced by viruses (64). The function of these cytokine-like molecules, as stated above, is to inhibit antiviral inflammatory/immune system mechanisms. Given that the size of the bacterial genome is log units larger than that of most viruses, how many more cytokine-like proteins are bacteria likely to produce?

In addition to the possibility that commensal or pathogenic bacteria produce cytokines to downregulate host responses, information suggesting that eukaryotic cytokines can interact with bacteria is beginning to emerge. For example, two papers have suggested that cytokines can selectively bind to bacteria. *Shigella flexneri* has receptors for TNF-α (138), and *E. coli* has receptors for IL-1 and TNF-α (172). The role of such receptors is not clear, but Porat et al. have reported that IL-1 will stimulate the growth of pathogenic strains of *E. coli* (172), although this has not been substantiated by another group (111). Also, cytokines such as IL-2 can stimulate the growth of a range of bacteria (32–34). If this two-way traffic of information does occur, it opens up new horizons for the interaction of the prokaryotic and eukaryotic worlds. Indeed, just as this review was completed, a report appeared that two pathogenic strains of *E. coli* produce proteins capable of inhibiting mitogen-stimulated human PBMCs from synthesizing the lymphokines IL-2, IL-4, IL-5, and IFN-γ but not the cytokines IL-1β, IL-6, IL-8, IL-10, or IL-12 or the chemokine Rantes (117). The ability to block local IL-2 production may account for the inflammation induced by enteropathogenic *E. coli* because it removes one section of the local gut cytokine network inhibiting responses to the normal bowel microflora. We suspect that this will be only the first of many reports delineating the capacity of bacterial cells to control the cytokine network either as a virulence mechanism or as part of a homeostatic control system for the maintenance of a host-bacterium symbiosis.

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