PEPTIDOGLYCAN AND THE PEPTIDOGLYCAN-DEGRADING N-ACETYLMURAMYL-L-ALANINE AMIDASE IN HUMAN TISSUES

PEPTIDOGLYCAAN EN HET PEPTIDOGLYCAAN AFBREKEND N-ACETYLMURAMYL-L-ALANINE AMIDASE IN HUMANE WEEFSELS

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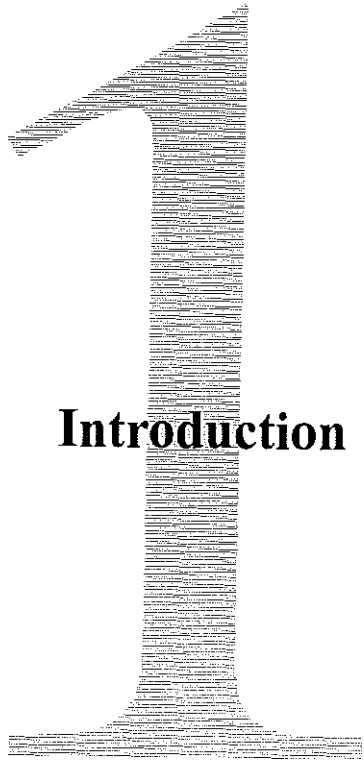


Introduction

Normal flora as well as pathogenic bacteria, can induce acute and chronic inflammations in humans. Probably as a result of the therapeutic efficacy of antibiotics in the past decades there has been relatively little interest in the mechanisms underlying bacterially induced inflammation. However, because of the increase in the incidence of antibiotic resistant bacteria, the subject is gaining interest. It is important to investigate the inflammatory mechanisms in order to provide new tools for clinicians as they will have to treat the inflammatory symptoms as well as the infection.

One of the major components present in Gram-positive bacteria is peptidoglycan (PG). It has been shown that PG possesses inflammatory properties similar to LPS. This suggests that PG is involved in the pathogenesis of inflammation induced by Gram-positive bacteria and possibly also Gram-negative bacteria. A description of the recent work done to test this hypothesis is given in the first part of this introduction. In the second part of this chapter special attention is given to the detection of PG in tissues. The presence of PG in tissues is a prerequisite for the induction of inflammation by PG products. The presence of PG in tissues implicates the presence of PG degrading systems, necessary to prevent the inflammation. In the last part of this chapter, an overview is given on the PG degrading systems available in humans.

1.1 INFLAMMATORY PROPERTIES OF PEPTIDOGLYCAN

PG is present in the cell walls of most bacteria and is the major constituent of Gram-positive cell walls (Fig. 1, upper part). It is composed of alternating N-acetyl glucosamine (GlucNAc) and N-acetyl muramic acid (MurNAc) forming long sugar chains which are interlinked by peptide side chains resulting in a large, complex macromolecule (Fig. 2). This bag-shaped molecule surrounds the cell and gives it the strength to withstand the turgor pressure exerted by the cytoplasm.

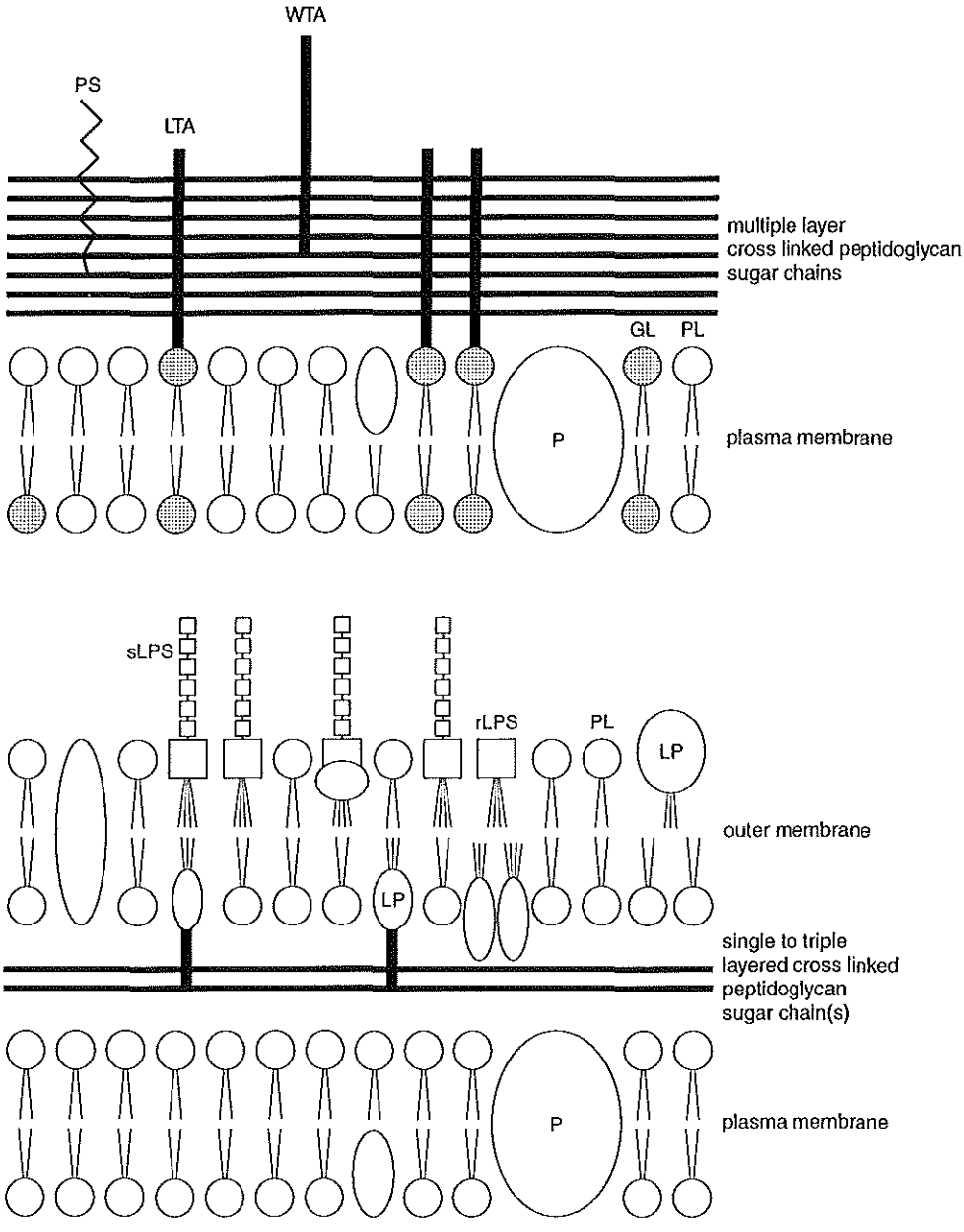
During a bacterial infection PG and several other cell wall components are implicated in the pathogenesis of the inflammation. In Gram-negative infections, endotoxin (a lipopolysaccharide-protein complex from the outer membrane; Fig. 1, lower part) is a well known activator of the innate immune system. During Gram-positive infections, when no endotoxin is produced, PG is able to induce similar effects as endotoxin. The activation of complement [1-3], macrophages and monocytes [4], but also the activation of granulocytes [5,6] and upregulation of adhesion molecules on endothelial cells [7] are shared properties of endotoxin and PG. Pro-inflammatory cytokines such as IL-1, IL-6,

IL-8 and TNF-α can mimic the whole spectrum of toxicity caused by endotoxin and PG. This may explain why septic shock caused by Gram-positive bacteria and Gram-negative bacteria have common cytokine induced characteristics [8]. Gupta et al. [9] recently described that the tyrosine phosphorylation of several proteins and the activation of kinases in macrophages were identical upon stimulation with PG or endotoxin, which supports the hypothesis that PG and endotoxin activate macrophages through similar mechanisms.

IL-1 and TNF-α appear to play predominant roles in the normal physiologic inflammatory response. Many of the physiological functions of IL-1 overlap the functions of TNF-α, both stimulating the non-specific host response. However, when excessive concentrations occur, the beneficial responses exerted by these cytokines are often outweighed by adverse physiologic effects. In sepsis, these two cytokines mediate the septic state by influencing vascular resistance and permeability, cardiac function and bone marrow function. They also stimulate the production of other inflammatory cytokines. Particularly important is the local release of IL-8, which recruits and activates neutrophils, resulting in tissue damage and organ dysfunction [10]. In addition to IL-8, TNF-α and IL-1 induce the secretion of other mediators, including IL-6, platelet-activating factor, prostaglandins and leukotrienes [11,12]. The induction of inflammatory cytokines is therefore an important mechanism in the pathogenesis of PG-induced inflammation.

To study the inflammatory properties of PG, experimental models for pertussis, meningitis, sepsis and arthritis have been applied. A summary of these models as provided in this introduction gives an idea on the wide range of inflammatory properties exerted by PG in vitro and in vivo.

Figure 1. Gram-positive and Gram-negative cell wall composition.
Upper part Diagrammatic representation of a generalised Gram-positive cell wall-plasma membrane complex. The plasma membrane is shown as being composed of protein (P), phospholipid (PL), glycolipid (GL), lipoteichoic acid (LTA) and wall teichoic acid (WTA), which is connected to the multiple layered peptidoglycan sugar chains. The composition may vary between different Gram-positive bacteria.
Lower part Diagrammatic representation of a generalised Gram-negative bacterial cell envelope. The inner leaflet of the bilayer of the outer membrane is shown to be composed of phospholipid (PL), protein (P) and lipoprotein (LP) which is covalently linked to the thin peptidoglycan layer in the periplasmic space between the plasma membrane and the outer membrane. Smooth and rough variants of lipopolysaccharide (sLPS and rLPS) are present in the outer membrane. The three regions of LPS are depicted as black lines (lipid A, fatty acids coupled to disaccharide diphosphate units), black rectangle (core polysaccharide) and open squares (units of O-polysaccharides repeated up to 25 times). LP is present in the outer membrane. The composition may vary between different Gram-negative bacteria.


WTA
PS
LTA
multiple layer
cross linked peptidoglycan
sugar chains
GL
PL
P
plasma membrane
sLPS
rLPS
PL
LP
outer membrane
LP
single to triple
layered cross linked
peptidoglycan
sugar chain(s)
P
plasma membrane

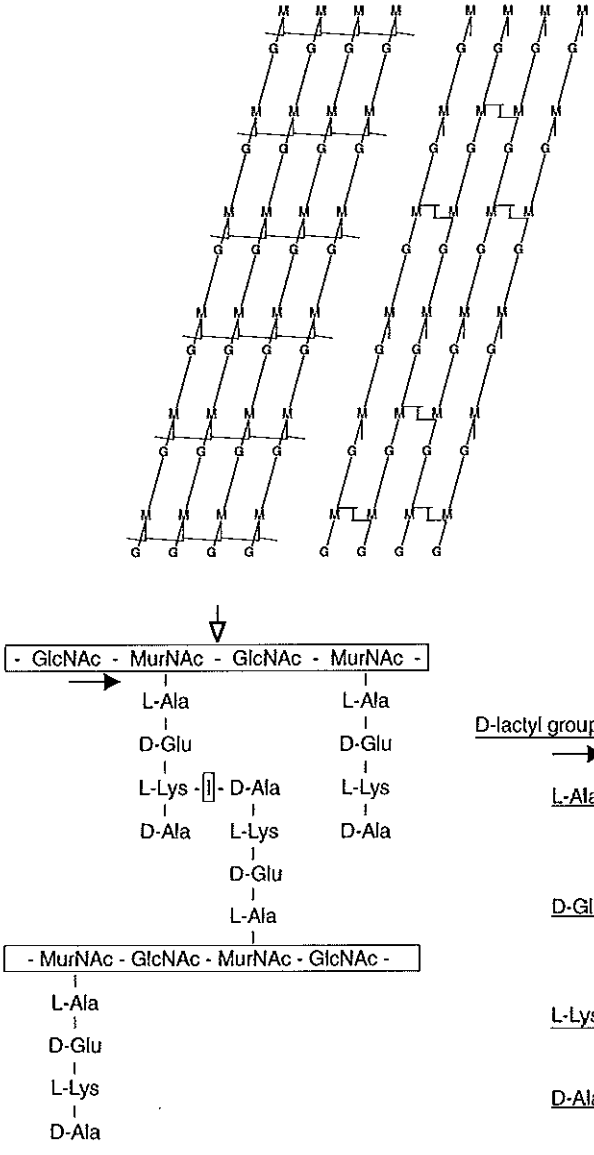
1.1.1 In vitro models for inflammation

Most of the in vitro models to study the pathogenesis of inflammation caused by bacterial products use peripheral blood monocytes, whole blood or macrophage cell lines. Gold et al. [13] and Chedid et al. [14] showed for the first time that PG products were able to induce release of IL-1 from purified monocytes in vitro. Purified high molecular weight PG from *Staphylococcus epidermidis* stimulated IL-1, IL-6 and TNF-α release by human peripheral blood monocytes [15]. High molecular weight PG was required for the induction of chemiluminescence, an activation marker, in human polymorphonuclear leukocytes. Chemiluminescence induced by low molecular weight PG obtained after sonication of the isolated cell walls, was much lower (<20%) than with high molecular weight PG [6]. Heumann et al. [16] stimulated human monocytes with purified cell wall preparations containing mainly PG from 10 different Gram-positive bacteria, which were all able to induce TNF-α release.

Vowels et al. [17] investigated the production of IL-8, IL-1β and TNF-α by human monocyte cell lines and freshly isolated peripheral blood monocytes after stimulation with PG- containing culture supernatants of *Propionibacterium acnes*, implicated in the pathophysiology of acne vulgaris. Fractionation of the supernatants by selective dialysis showed that most of the original cytokine inducing activity was present in the fractions with a molecular weight between 3 and 30 kDa. After treatment of the culture supernatants with lysozyme, the IL-8 and TNF-α production were approximately 50% lower than was found with untreated culture supernatants. This shows that the molecular weight of PG is important for the induction of cytokines in these *in vitro* assays. The studies of Dobrina et al. [7] showed that the CD11/CD18 adhesion complex was upregulated after a 4 hour incubation of human endothelial cells with purified PG from the Gram-negative *Leptospira icterohaemorrhagiae*, resulting in an increased cell adhesiveness for neutrophilic granulocytes.

Small muramyl peptides, the monomers of macromolecular PG, are also able to induce cytokine release by monocytes. It was demonstrated that the anhydro disaccharide tetrapeptide (Fig. 3) strongly induced IL-1 and IL-6 mRNA production in human monocytes [18]. The same product was investiga-

Figure 2. Upper part Alternating N-acetyl glucosamine (G) and N-acetyl muramic acid (M) are connected by peptide bonds resulting in a cross-linked network. Gram-positive peptidoglycan (left) is more extensively cross-linked than Gram-negative (right) peptidoglycan.
Lower part Detailed structures of muramyl peptides. The interpeptide bridges, **I**, are variable between strains. N-acetylmuramyl-L-alanine amidase (NAMLAA) hydrolyses the lactate bond between N-acetyl muramic acid (MurNAc) and the first aminoacid of the peptidoglycan chain L-alanine (---►). Lysozyme hydrolyses the bond between MurNAc and GlucNAc (--->). Some bacteria can contain an extra O-acetyl group on the C-6 atom of MurNAc (box).


- GlcNAc - MurNAc - GlcNAc - MurNAc -
L-Ala
D-Glu
L-Lys - i - D-Ala
D-Ala
L-Lys
D-Ala
D-Glu
L-Ala
L-Ala
D-Glu
L-Lys
D-Ala
- MurNAc - GlcNAc - MurNAc - GlcNAc -
L-Ala
D-Glu
L-Lys
D-Ala
D-lactyl group
MurNAc
GlcNAc
L-Ala
D-Glu
L-Lys
D-Ala

ted in a different test system. *Bordetella pertussis*, the causative organism of whooping cough, was used in an in vitro model with cultured hamster trachea epithelial cells [19]. A PG-containing fraction inhibited DNA synthesis in these cells. The component responsible for this effect was found to be the anhydro-disaccharide tetrapeptide subunit of PG, also called tracheal cytotoxin (TCT) [20]. Wilson et al. [21] found that loss of ciliated cells in human epithelial biopsies could be induced by TCT. In later studies Nixon Heis et al. [22] showed that intracellular IL-1 is produced by tracheal epithelial cells after TCT treatment and hypothesized that TCT toxicity might be mediated through IL-1 and nitric oxide production. In a structure-activity study the peptide side chain, which is normally linked to MurNAc in PG (Fig. 2), was shown to be the minimal structure of TCT which was fully toxic in the assay using tracheal epithelial cells (Fig. 3) [23].

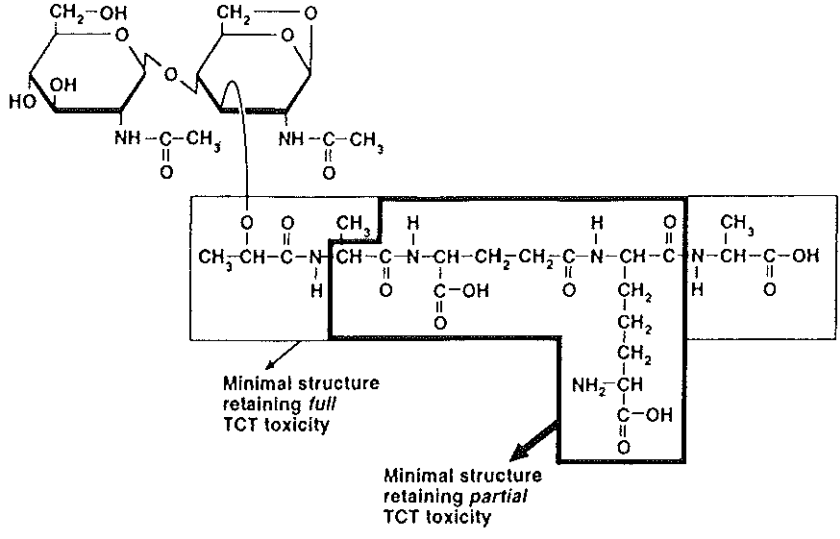


Figure 3. Tracheal cytotoxin (TCT). The larger box contains the lactyltetrapeptide portion of TCT, which is sufficient to reproduce the TCT toxicity of the complete molecule. The smaller box contains the smallest fragment shown to elicit measurable toxicity in respiratory epithelial cells [23].

In conclusion, the combined results of the in vitro studies show that PG, derived from normal flora bacteria or pathogens, is capable of inducing most of the effects seen in local or systemical inflammation.

1.1.2 In vivo models for inflammation

The in vivo studies on chronic inflammation are mostly performed using peptidoglycan polysaccharide complexes (PG-PS). Systemic inflammations caused by these less well-defined complexes show that PG-PS can induce multisystem disease [24]. An animal model for chronic, erosive arthritis in rats can be used to study the involvement of PG in the pathogenesis of the disease. In susceptible rats, e.g. Lewis rats, a single intraperitoneal (i.p.) injection of cell wall fragments (CWF) causes an acute polyarthritis, followed by a chronic persistent arthritis [25]. In vitro degradation of CWF by mutanolysin (a PG degrading enzyme from *Streptomyces globisporus*) resulted in loss of the arthropathic properties of the cell walls, indicating the potent role of PG in the pathogenesis of this disease in Lewis rats [26,27].

Studies of classic adjuvant induced arthritis, where rats are immunized subcutaneously with PG products ground in mineral oil, have demonstrated a role for the chemically defined muramyl dipeptide (MDP) subunits of PG [28]. The arthropathic activity of MDP depends on the mineral oil used and the amino acids coupled to the muramic acid [29]. However, when injected i.p. in aqueous suspension, neither PG nor its subunit MDP induced chronic arthritis [30]. Although in the i.p. route of arthritis induction in rats the use of high molecular weight PG is essential, lysozyme-solubilized CWF are still capable of inducing arthritis in the adjuvant model [31 and chapter 5], indicating that high molecular weight PG is not a prerequisite for arthritis induction in this model. Therefore, the adjuvant arthritis model was used in chapter 5 as a tool to investigate the inflammatory properties of CWF after enzymatic degradation.

A structure-activity study was performed by Tuomanen et al [32] and Burroughs et al. [33]. They used a rabbit model to study the pathogenesis of bacterial meningitis by injecting intracisternally 17 different muramyl peptides derived from PG of the Gram-negative *Haemophilus influenzae*, together representing 96% of the total PG. The ability to induce leukocytosis, blood-brain barrier permeability and brain edema varied between different muramyl peptides, suggesting that each muramyl peptide interacts with host defenses in a specific and individual manner.

Another property of muramyl peptides is the sleep-promoting effect [reviewed in 34]. The PG subunit MDP was shown to enhance slow-wave sleep in rabbits [35] and other mammals. IL-1 can mimic these somnogenic effects and it was hypothesized that the properties of MDP are mediated through IL-1, which cytokine, as described above, is released by monocytes upon activation by PG during a bacterial infection.

1.1.3 Other pro-inflammatory components of bacterial cell walls

In Gram-positive bacteria, PG accounts for more than half of the cell wall mass [36], while in Gram-negative bacteria the cell wall is no more than 3 PG layers thick [37]. In Gram-positive bacteria (lipo)teichoic acids (LTA) and poly-saccharides account for most of the other half of the cell wall mass. In Gram-negative bacteria smooth and rough variants of (lipo)polysaccharides (LPS) and (lipo)proteins form the bulk of the cell wall besides the PG (Fig. 1). A summary of all the known cell wall components in Gram-negative and Gram-positive bacteria which possess inflammatory capacities is given in Table 1. LPS and LTA have been shown to possess similar inflammatory properties as PG. Endotoxin, a complex of LPS and (lipo)proteins, has been shown to possess a higher inflammatory potency than LPS, LTA or PG [39,40]. Since PG is a major inflammatory component of Gram-positive bacteria, this thesis emphasizes the role of PG in the pathogenesis of inflammation.

Table 1. Inflammatory cell wall components from Gram-positive and Gram-negative bacteria

Gram-positive	Gram-negative	Gram-positive and negative
Teichoic acids	Lipopolysaccharide	Cell surface proteins
Lipoteichoic acids	Lipid A	Surface-associated proteins
Lipoarbinomannans	Lipid A-associated proteins	Fimbriae and pili
Purified proteins	Outer membrane proteins	Lipopeptides
Mycobacterial heat shock protein	Porins	Lipoproteins
Protein A		Muramyl peptides
		Peptidoglycan
		Polysaccharides
		Toxins

From reference 35.

1.2 DETECTION OF PEPTIDOGLYCAN IN INFLAMED AND NORMAL TISSUES

It is hypothesized that PG is involved in the pathogenesis of inflammation, as for instance in arthritis. The presence of PG in relevant tissues, however, is a prerequisit for this hypothesis. Most studies investigating the presence of PG in tissues focus on the detection of muramic acid (Mur), the

characteristic aminosugar (after release of the N-acetyl group of MurNAc by acid hydrolysis) in the glycan backbone of PG. However, also radioactively labeled PG-PS has been used in an arthritis model [41]. After i.p. injection into rats, [^{125}I]-PG-PS localized in liver and spleen as high molecular weight PG and in synovia as partially degraded lower molecular weight PG [41].

Gilbert and Fox [42] measured the levels of rhamnose polysaccharide and Mur in tissues using a combination of gas chromatography and mass spectrometry. They used streptococcal PG-PS in the i.p. model of arthritis induction and found a time-dependent decrease of Mur levels in liver and limbs of rats. Mur was still detectable 63 days after injection in both liver and limbs. In the same arthritis model a fluorescein-labeled streptococcus-specific antibody was used to detect the presence of streptococcal antigens in tissues by immunohistochemical techniques. The streptococcal cell wall material accumulated rapidly in the liver, spleen, lymph nodes and synovia where it remained detectable for more than 90 days [43].

In humans the first results were obtained by Martin et al. [44], who showed the presence of PG monomers in human urine after penicillin administration. This indicates that small PG fragments, released upon antibiotic treatment [45], can be cleared from the circulation by the kidneys. Mur was also detected in the synovial fluids of patients with acute inflammatory arthritis of unclear origin but who had a history of bacterial disease [46], providing support for the idea that these PG products present in the synovia are able to play a role in the pathogenesis of the inflammation.

Although Fox and Fox [47] were not able to demonstrate Mur in normal serum, there is accumulating evidence that also without introduction of PG by injection or infection, tissues of rats and humans contain PG. In peripheral blood leukocytes from 21 of 98 healthy human subjects, Mur could be detected using a combination of gas chromatography and mass spectrometry [48]. Leukocytes from umbilical vein blood from 41 newborns were Mur negative. Since newborns lack gut flora, intestinal absorption of bacteria or of their degradation products was believed to be the most likely explanation for the presence of Mur in the peripheral blood leukocytes. Immunohistochemical studies using a monoclonal antibody against PG-PS revealed the presence of PG-PS containing cells in spleen of conventional rats [49]. Using immunohistochemistry the presence of PG-PS was also detectable in the bowel-wall in humans and a connection with Crohns disease has been proposed [50]. Sen and Karnovsky [51] found Mur in brain, liver, and kidneys of conventional rats. The last three studies indicate that PG is present in tissues even without an inflammation, which suggests that the PG found in tissues can originate from

normal flora bacteria and might therefore be capable of maintaining a chronic inflammation.

Together with the work presented in chapters 2 and 3 of this thesis, these results show that PG is detectable in human tissues, which supports the hypothesis that PG can play a role in the pathogenesis of inflammations with unknown etiology.

1.3 PEPTIDOGLYCAN PERSISTENCE VERSUS DEGRADATION; THE HUMAN ENZYMES

1.3.1 Persistence of peptidoglycan

When invading bacteria or their PG-containing CWF enter our system, the CWF are rapidly cleared through the action of hydrolytic enzymes present in serum and phagocytic cells. Nevertheless, PG does occur in human tissues and it has been shown in animal models that injected PG can persist for a long time. The inflammatory properties of PG and its persistence in tissues justifies to study the properties of PG in relation with its resistance to hydrolytic degradation.

To investigate the ability of PG to persist in tissues, PG from different sources was injected i.p. into rats. The injected PG could be detected in macrophages for more than 90 days. Lichtman et al. [52] showed that PG laden macrophages were able to induce inflammation in vivo. They transplanted livers of rats, injected i.p. with PG, into a second rat with preinjured joints, resulting in a reactivation of arthritis. This finding suggests that PG present in macrophages can play a role in the pathogenesis of inflammation.

Rat macrophages and human monocytes were shown to process streptococcal cell walls in vitro [53]. Similar results were obtained using *Bacillus subtilis* PG and a mouse macrophage cell line [54]. Macrophages were also shown to digest radioactively labeled cell walls from staphylococci. These cell wall laden macrophages then excreted low-molecular weight PG fragments, which were shown to possess somnogenic and pyrogenic activities in rabbits [55].

The relation between persistence in tissues and resistance to degradation by lysozyme was clearly shown when PG-PS from group A and group D streptococci were compared [41]. Group A PG-PS were more resistant to in vitro lysozyme degradation and were also shown to persist longer in vivo than group D PG-PS.

The experiments using animal models all indicate that macrophages

play an important role in clearing and degrading circulating high molecular weight PG, but also that CWF can persist in macrophages, retaining their inflammatory properties.

1.3.2 Peptidoglycan degrading enzymes in humans

In humans, three different hydrolytic enzymes are known to degrade peptidoglycan; lysozyme, N-acetylglucosaminidase and N-acetylmuramyl-L-alanine amidase. The relationship between the structure of PG and its resistance to degradation by lysozyme has been the subject of several investigations. The resistance of PG to degradation by lysozyme was shown to depend upon the degree of O-acetylation [56]. This can be explained by the finding that the presence of O-acetyl groups on the glycan backbone (Fig. 2) of PG results in a weaker affinity of lysozyme for PG.

The relation between the in vivo inflammatory capacity and the susceptibility to lysozyme degradation was shown using the i.p. route of arthritis induction in rats. The most resistant PG preparations were the most potent inducers of arthritis [57,58]. Most interestingly, the amount of O-acetyl groups present in PG can be influenced by treatment with antibiotics. *Staphylococcus aureus* treated with Clindamycin resulted in PG with an increased number of O-acetyl groups and this PG was less sensitive to lysozyme degradation [59]. This might be an important side effect of treatment with Clindamycin and similar antibiotics.

N-acetylglucosaminidase is a common enzyme present in human sera and other tissues [60]. Its primary function is the degradation of oligosaccharides containing ß-glucosamide linkages, present on many glycoproteins. Glucosaminidase activity was also shown to be present in a granule extract from human neutrophils, which was shown to be capable of hydrolysing free N-acetylglucosamine (GlucNAc) groups from non-reducing ends of the PG-backbone [61]. The effect of N-acetylglucosaminidase degradation of PG on the inflammatory properties of PG has not been investigated. It is, however, reasonable to assume that this enzyme has very little effect on these properties because it can only hydrolyse one GlucNAc molecule from the long sugar chain of macromolecular PG. The properties of muramyl peptides are also not likely to be affected by N-acetylglucosaminidase since it has been shown that GlucNAc is not essential for the inflammatory properties of muramyl peptides [34].

N-acetylmuramyl-L-alanine amidase (NAMLAA) was first described by Pelzer in 1963, who showed that its enzymatic activity was present in *Escherichia coli* [62]. It was shown that NAMLAA specifically hydrolyses the

lactamide bond which links MurNAc of the polysaccharide chains to L-alanine of the peptide side chain (Fig. 2). Since then NAMLAA enzymes have been purified from *Staphylococcus areus* [63], *Bacillus subtilis* [64] and bacteriophage T7 [65]. The involvement of the enzyme in autolysis of pneumococci was shown [66] and the lysis was shown to be dependent on the presence of teichoic acids [67]. Autolytic microbial enzymes like NAMLAA were not involved in the lysis after the pneumococci were ingested by phagocytes [68]. The first bacterial gene encoding NAMLAA has been identified in *Staphylococcus aureus* [69] and recently this enzyme has been shown to possess cluster dispersing activity [70]. Mutants without the gene were shown to grow in giant clusters, suggesting that NAMLAA in these cells is involved in cell separation after cell division. Cytoplasmic NAMLAA was also shown to be involved in beta-lactamase regulation and cell wall recycling [71].

In human sera NAMLAA activity was reported for the first time by Ladešić et al. [72] and the enzymatic activity of the enzyme was determined by Valinger et al. [73]. They showed that NAMLAA has the same enzymatic hydrolytic activity as the bacterial enzyme. The result of such hydrolysis is degradation of macromolecular PG to small PG products, which can be excreted in the urine [44,45]. A good colorimetric method for the detection of NAMLAA activity in serum has been described by Hazenberg and De Visser [74]. The detection limit of this assay corresponds to the activity in 200 times diluted human serum and this assay proved to be highly valuable for the characterization of NAMLAA from human serum, as is described in chapter 4. Further studies on the purification and characterization of the enzyme have been performed by Tomasic et al. [75], Mollner and Braun [76], de Pauw et al.

Table 2. Localization of PG degrading enzymes in human body fluids

Human body fluids	lysozyme	N-acetylglucosaminidase	NAMLAA
saliva	+	?	-
cerebrospinal fluid (without pathological signs)	±	?	-
milk	++	?	-
synovial fluid	?	?	±
serum	+	+	+
urine	-	?	-
tears	++	?	-

-: undetectable, ±: lower than in serum, +: equal to serum, ++: higher than in serum.

[77] and Vanderwinkel et al. [78,79], but the purification to homogeneity of NAMLAA remained problematic. Therefore the biological role of this human enzyme could not be investigated before.

1.3.3 Localization of peptidoglycan-degrading enzymes

The localization of lysozyme [80], N-acetylglucosaminidase [60] and NAMLAA might be important for the biological role of these enzymes. Table 2 gives a summary of the localization in human body fluids. The precise localization of NAMLAA and the comparison with lysozyme distribution in human mononuclear cells is described in chapter 6 of this thesis.

1.4 SCOPE OF THE THESIS

PG polymers and monomers have potent biological effects. A major factor herein is the ability of PG to persist in tissues. The presence of PG in tissues is a prerequisite for the induction of perpetuating effects in vivo. To investigate whether PG is present in human tissues, we used a monoclonal antibody, recognizing PG, for immunohistochemical studies. The immunohistochemical detection of PG products in synovial tissues of rheumatoid arthritis patients and healthy controls is described in chapter 2. The extraction of a high molecular weight carbohydrate enriched fraction from human spleens and the biochemical determination of Mur, the characteristic aminosugar of PG in this extract, is described in chapter 3.

The persistence of PG in tissues depends on its degradability by hydrolytic enzymes. In this respect, lysozyme has been investigated extensively by other investigators. The relatively new mammalian NAMLAA, which cleaves the peptide side chain of the PG sugar backbone, has so far not been very well characterized. We hypothesize that NAMLAA has a potent role in the inactivation of PG products. In order to investigate this hypothesis, NAMLAA was purified and biochemically characterized (chapter 4). The biological activity of NAMLAA in relation to the inflammatory properties of NAMLAA digested PG was then investigated using the purified NAMLAA (chapter 5).

The localization of NAMLAA might be important for the biological function of this enzyme. In order to study the localization of NAMLAA, a monoclonal antibody has been prepared against NAMLAA and this antibody has been used for immunohistochemical studies and electron microscopy (chapter 6). The results in chapter 7 compare the presence of NAMLAA and lysozyme in cerebrospinal fluid and sera of bacterial meningitis patients and

healthy controls.

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Presence of bacterial flora derived antigen in synovial tissue macrophages and dendritic cells

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ABSTRACT

In previous studies, using an animal model, human bacterial flora derived peptidoglycan polysaccharides were shown to be arthropathic after a single subcutaneous injection. A prerequisite for proof of the hypothesis that bacterial products from the normal resident flora are involved in the immune reaction of human chronic polyarthritis of unknown etiology, is the presence of these antigen's in synovial tissue. 2E9, a monoclonal antibody we developed against intestinal peptidoglycan polysaccharides was used in a histochemical study in rats and stained macrophages in the spleen red pulp. In this study human synovial tissues from 10 RA and 20 non-RA patients were stained with 2E9. We found that 8 out of 10 RA had 2E9 positive macrophages and dendritic cells in their synovia. A significant difference was observed with the control group in which 7 out of 20 were positive. No positive cells or staining of the matrix were found in the cartilage of 6 RA patients. These results show that exogenous bacterial antigens are present in synovial tissue macrophages and dendritic cells. It was concluded that the unknown antigen in the immune reaction in RA is not necessarily endogenous.

INTRODUCTION

There is evidence that supports a central position for the T cell in rheumatoid arthritis (RA) [1]. Specific $CD4^+$ T cells react with antigenic peptides presented by Major Histocompatibility Complex (MHC) class II molecules on antigen presenting cells. The result is that activated T cells stimulate other cells, leading to the production of effector molecules such as cytokines and degradative enzymes. Synovial MHC class II dependent T cell activation is considered the primary driving force in development of RA, the antigen in the trimolecular, MHC-(super)antigen-T cell receptor complex, however, is not identified. Although, tentative candidates are generally believed to be endogenous, exogenous antigens could not be excluded.

We hypothesize that bacteria or bacterial products from the normal resident flora are implicated in the immune reaction of chronic human polyarthritis of unknown etiology like RA. In previous studies, using a rat model, the arthropathic properties of the most abundant groups of bacteria in the human flora were investigated. Cell wall fragments (CWF) from obligate anaerobic *Eubacterium* and *Bifidobacterium* species, with peptidoglycan as a major component, induced a severe chronic polyarthritis after a single intraperitoneal

or subcutaneous (in oil) injection [2,3,4]. Although the faecal flora of RA patients and healthy subjects was different, *Eubacterium* and *Bifidobacterium* were present in both flora in a very high number (10^9-10^{10} per gram stool). CWF of *Eubacterium aerofaciens* strains isolated from the flora of patients and controls were arthropathic [5]. Soluble peptidoglycan polysaccharide complexes originating from the obligate anaerobic flora were purified from human intestinal contents by gel filtration. The complexes isolated from ileostomy fluid, that proved to be less degraded by intestinal enzymes, induced chronic arthritis in rats after injection in the base of the tail [6]. From that study we concluded that the human intestinal flora contains soluble bacterial cell wall derived products with arthropathic properties.

If RA is a T cell dependent immune response to peptidoglycan-polysaccharides of the bacterial flora, these antigens must be present in synovial tissues. An immunohistochemical study with monoclonal antibody (2E9) directed against intestinal peptidoglycan polysaccharides, which was developed in our laboratory, showed that the red pulp of rat spleen is scattered with positively staining macrophages [7]. Further studies with human tissues showed that peptidoglycan-polysaccharides positive cells (2E9, positive) were present in bowel wall and spleen [8,9]. Peptidoglycan was also isolated from the human spleen and biochemical characterized [9]. The latter showed that the presence of peptidoglycan is human tissues is probably normal feature. In the present study we investigated the presence of bacterial antigens in human synovial tissues using this unique monoclonal antibody. The detection of bacterial product in synovial tissues in RA is, to our knowledge, a novel finding.

MATERIALS AND METHODS

Patients

Synovial tissues were obtained at the time of reconstructive surgery from the knees or hips of 10 patients of definite or classical RA according to the ARA criteria [10]. Non-rheumatoid synovial tissue was obtained from knee biopsies of 25 patients with a non-inflammatory knee injury. The average ages of the RA patients (3 males and 7 females) and of the control group (20 males) were 68 and 28, respectively.

Cartilage was obtained from another group of 6 RA patients. The average age of these RA patients (all female) was 61. Synovial tissues and cartilage were stored at –70°C until use.

Preparation of cryostat sections

Frozen serial sections (6 µm) were cut on a Reichert-Jung 2800 Frigocut cryostat. The synovial tissue sections were placed on poly-L-lysine-coated (Sigma, St. Louis, MO) microscopic slides and the cartilage sections were placed on 3-amino-propyltriethoxysilane-coated slides (Sigma, St. Louis, MO). After drying in the air for 1 hour the sections were stored at -20°C.

Immunohistochemistry

To study the general morphology the thawed sections were dipped in phosphate-buffered saline (PBS) with 4% formaldehyde and stained with hematoxylin and eosin. For immunohistochemistry the slides were fixed for 10 minutes in acetone. After rinsing in PBS with 0.2% bovine serum albumin (BSA), the sections were incubated for 1 hour at room temperature with 70 µl diluted monoclonal antibody. The monoclonal antibodies were used as listed in Table 1. 2E9 is a monoclonal antibody with the IgG3 isotype and NS7, with the same isotype, is used as a negative control monoclonal antibody. 2E9 is directed against intestinal flora derived peptidoglycan polysaccharides from human and rat faeces [7]. Inhibition assays with ELISA showed that peptidoglycan from *Escherichia coli*, *Micrococcus lysodeikticus* and *Brevibacterium divaricatum* inhibited the reaction of 2E9 with the intestinal flora derived peptidoglycan [7, M.P. Hazenberg, personal communication]. The sections were subsequently rinsed in PBS/BSA and incubated for 30 min at room temperature with rabbit anti-mouse immunoglobulin (Z259, Dakopatts, Denmark) diluted 1:20 in PBS/BSA with 1% normal human serum. After rinsing in PBS/BSA, a 1:40 dilution of alkaline phosphatase-mouse-anti-alkaline phosphatase complex (APAAP) (D651, Dakopatts, Denmark) was applied for 30 minutes. The substrate used to develop the stain consisted of 0.012% (w/v) naphthol-ASMX phosphate, 0.025% (w/v) fast blue BB base, and 0.025% (w/v) levamisole (all from Sigma, St. Louis, MO). After 45 minutes incubation at room temperature in the dark, the sections were rinsed in PBS/BSA followed by distilled water and mounted in Kaiser's glycerol gelatin (Merck, Darmstadt, Germany).

Table 1 Monoclonal antibodies used in the study

code	specificity	concentration used	reference
2E9	peptidoglycan-polysaccharides	10 µg/ml	[7]
2-4	muramyl-dipeptide	10 µg/ml	[11]
NS7	sheep red blood cells	10 µg/ml	TIB 114, ATCC
RV202	vimentin	1:10	[12]
Mac	macrophages	1:100	M178, Dako
L25	dendritic cells	1:500	[13]
RFD1	dendritic cells	1:500	[14]
Leu4	CD3 (T cells)	1:50	347340 BD

Immuno double-staining

For double staining with acid phosphatase, the staining with the monoclonal antibody 2E9 was carried out as described above. After the incubation with the alkaline phosphatase substrate, acid phosphatase activity was demonstrated as described by Lojda et al [15].

To study how many 2E9-positive cells in the synovial tissue were mast cells, a double staining with toluidine blue was used. The staining with the monoclonal antibody 2E9 was carried out as described above. After the incubation with the alkaline phosphatase substrate the section were incubated for 1 minute with the 0.5% (w/v) toluidine blue (BHD, Gurr) and scored immediately.

For double staining with two monoclonal antibodies the synovial tissue cryostat sections

were acetone fixed. Endogenous peroxidase activity was blocked by 0.1% (w/v) phenylhydrazinium chloride (Sigma, St. Louis, MO) in PBS, for 1 hour at 37°C. After rinsing in PBS/BSA the sections were incubated with the peptidoglycan-polysaccharide-specific monoclonal antibody 2E9 for 1 hour at room temperature. As a second step a peroxidase conjugated rabbit anti-mouse immunoglobulin (P161, Dakopatts, Denmark) was used in a 1:250 dilution in PBS/BSA with 1% normal human serum. After 30 minutes incubation and rinsing with PBS/BSA, the sections were incubated with the second monoclonal antibody (RFD1, L25 or Leu4, Table 1) for 1 hour at room temperature. The staining was detected by the immuno alkaline phosphatase anti-alkaline phosphatase (APAAP) method as described above. After the incubation with the alkaline phosphatase substrate the sections were rinsed in PBS/BSA followed by 0.2 M sodium acetate buffer pH 4.6, and incubated with the peroxidase substrate 3-amino-9-ethylcarbazole for 30 minutes. Finally the sections were rinsed in PBS/BSA and distilled water and mounted in Kaiser's glycerol gelatin (Merck, Darmstadt, Germany).

Comparison of the results

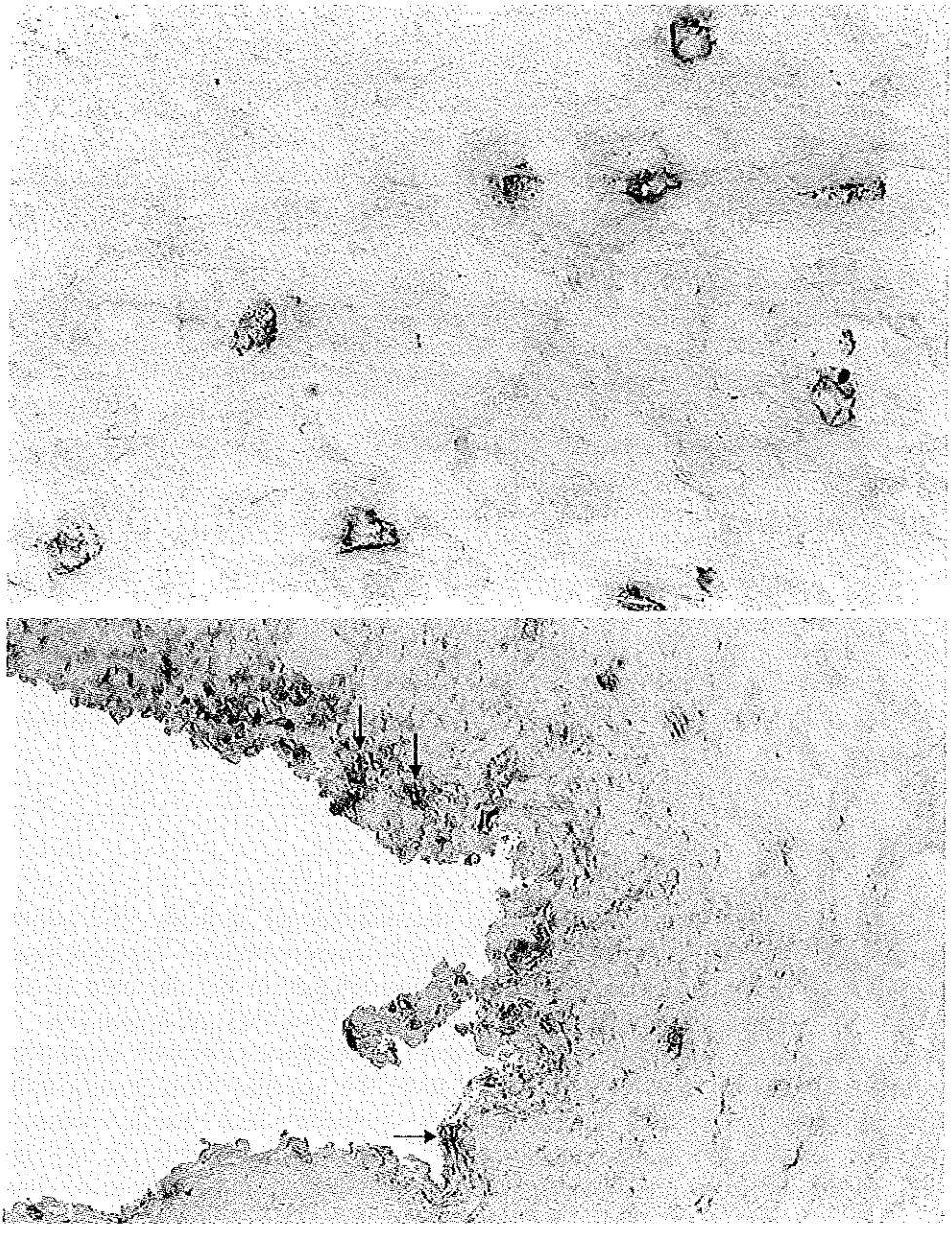
The total area of the synovial sections was measured using a calibrated measuring ocular (Kpl. 8x, Zeiss, Germany). Per section the total numbers of 2E9 positive cells was determined. To be able to compare the different patients, the number of positive cells per mm^2 was calculated. The Mann-Whitney U test was used to calculate the statistical differences.

RESULTS

Rheumatoid synovial tissue

Hematoxylin and eosin staining of the synovial tissues showed nodular infiltrates in 8 out of the 10 RA patients. Immunohistochemical staining with 2E9 specific for intestinal peptidoglycan polysaccharides showed that in the same 8 patients 8.3 (± 3.2) cells were positive per mm^2 synovial tissue. Two patient tissues were completely negative for 2E9. The staining was granular and restricted to the cytoplasm. The positive cells were found in the synovial lining as well as in the subsynovial layers (Fig. 1). Sometimes 2E9 positive cells were localized adjacent to small vessels. Cytochemical staining with acid phosphatase showed double positive cells in both areas (Fig. 2).

Figure 1. Immunohistologic staining of the subsynovial layer of a RA patient.
The 2E9 positive cells are stained (original magnification 80x).
Figure 2. Immunohistologic double staining of the synovium of a RA patient.
The 2E9 and acid phosphatase double positive cells are stained. Especially in the synovial lining there are cells present positive for both stainings (original magnification 50x).



In synovial tissue of two patients with the highest number of 2E9 positive cells, double staining with RFD1 and L25, both markers for dendritic cells, was performed and double staining cells were identified. Staining with 2E9 and toluidine blue showed that a minority of the 2E9 positive cells were mast cells. No clustering of the 2E9 positive cells in infiltrated areas was observed. However, in a staining of serial sections of a T cell infiltrate with numerous CD3 positive cells, we found also some 2E9 positive cells in the same area. Staining with 2-4 was negative in synovia of all RA patients.

Non-rheumatoid synovial tissue

Hematoxylin and eosin staining of the synovial tissues showed no infiltrations in the 20 control subjects. In 2 subjects 2E9 positive cells were found at the same frequency as in positive RA patients (6.2 and 8.7 cells per mm^2), in 5 subjects 0.3 to 2.8 positive cells per mm^2 were observed and in 13 subjects no positive cells were found. Staining with 2-4 was negative in all synovia.

Cartilage

The staining of the cartilage of 6 RA patients with 2E9 and 2-4 showed no positive staining. In one sample some synovial tissue was also present besides the cartilage. In this tissue 2E9 positive cells were found. A monoclonal antibody specific for vimentin, stained the cartilage sample heavily, showing that negative results with 2E9 were not due to the staining procedures, that were used.

DISCUSSION

In a previous paper we described the monoclonal antibody 2E9 which recognizes a human intestinal flora derived macromolecular peptidoglycan polysaccharide complex and which stains macrophages in the red pulp of rat spleens [7], antigen presenting cells in human bowel wall [8] and spleen [9].

In this study 2E9 positive macrophages and dendritic cells were present in synovial lining and subsynovial layers of most RA patients. Their significantly more frequent occurrence in RA patients compared with healthy subjects ($p=0.003$) should not lead to drastic conclusions. The groups differ in age and sex and the influence of these parameters is unknown. The differences are due to the fact that most of the reconstructive surgery in RA is done at elder age and the non-inflammatory knee injuries are the result of sport accidents. The

presence of 2E9 positive cells in non RA patients indicate that the presence of peptidoglycan polysaccharides in this tissue is not the only important factor. The development of RA seems to require more than the presence of 2E9 positive cells alone. Nevertheless in RA patients more synovia were positive for 2E9 and also more positive cells per mm^2 section were found. The serial sections stained with α-CD3 and 2E9, showed that in T cell infiltrations antigen presenting cells were present which are positive for peptidoglycan polysaccharides. Staining with monoclonal antibody 2-4 [12] directed to muramyl dipeptide was not observed in any of the synovial tissues. Obviously there is a difference between peptidoglycan antigens found in rat and human spleen were 2-4 and 2E9 staining always paralleled [8,10]. Perhaps this is due to a probably more prolonged stay of the antigen in the synovia.

Cartilage is considered to act as a sponge (or a filter) in which large antigens can be trapped [16]. No positive staining was found in RA cartilage while synovial tissue, present in two samples, did contain 2E9 positive cells. We realize us that we have no proof for a direct involvement of 2E9 positive cells in the immune reaction in RA, but the presence of these bacterial products in antigen presenting cells in the vicinity of T cell infiltration in the synovia of RA patients is intriguing and a novel finding.

Dendritic cells and macrophages may play an important role in the ongoing presentation of antigen to T cells in RA synovium as suggested by Thomas et al [17]. We propose that the antigen driven T cell response in RA is not necessarily autoreactive but could be directed to gut or other flora derived bacterial antigens (recognized by 2E9) in antigen presenting cells in the synovia.

Studies of Lehtonen et al [18] showed that in patients with acute inflammatory arthritis of unclear origin muramic acid (specific for peptidoglycan) was found in synovial fluid and could therefore also be present in synovial tissue. The presence of biochemically detectable muramic acid or peptidoglycan in RA synovia remains to be determined and is under current investigation by our group [9].

At this moment we are trying to isolate the 2E9 positive cells from the synovial tissues and are investigating whether these cells are responsible for the T cell autoreactivity so often reported in the literature [19] and also found in our investigations [20].

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Detection of muramic acid in a carbohydrate fraction of human spleen

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ABSTRACT

In previous studies we showed that peptidoglycan polysaccharides from anaerobic bacteria normally present in the human gut induced severe chronic joint inflammation in rats. Our hypothesis is that peptidoglycan from the gut flora is involved in perpetuation of idiopathic inflammation. However, in the literature the presence of peptidoglycan or subunits like muramyl peptides in blood or tissues is still a matter of debate. We were able to stain red pulp macrophages in all six available human spleens by immunohistochemical techniques using a monoclonal antibody against gut flora derived antigens. Therefore these human spleens were extracted and after removal of most of the protein the carbohydrate fraction was investigated for the presence of muramic acid, an aminosugar characteristic for peptidoglycan. Using three different methods for detection of muramic acid we found a mean of 3.3 µmol Muramic acid with HPLC, 1.9 µmol with a colorimetric method for detection of lactate and 0.8 µmol with an enzymatic method for detection of D-lactate per spleen (D-lactate is a specific group of the muramic acid molecule). It is concluded that peptidoglycan is present in human spleen, not as small muramyl peptides as was previously searched for by other investigators, but as larger macromolecules probably stored in spleen macrophages.

INTRODUCTION

Muramic acid (Mur) is one of the two aminosugars in the glycan backbone of peptidoglycan (murein). It is mostly present in an acetylated form i.e. N-acetylmuramic acid. Short peptides are linked to the lactyl groups of this molecule forming a large complex macromolecule which surrounds the cell as a basket around the cell membrane [29]. In this study Mur is used as a chemical marker for peptidoglycan polymers. Sen and Karnovsky [20] showed the qualitative detection of muramic acid in normal mammalian tissues. Fox and Fox [5] were not able to detect Mur in normal serum, which demonstrates the absence of appreciable amounts of circulating natural muramyl peptides. Our group is interested in the presence of peptidoglycan in tissues as we believe that such intestinal flora derived carbohydrates are important for physiological and immunological functions in the host. Johannsen et al. [11,12] showed that muramyl peptides have a variety of biological actions in mammals including the ability to enhance sleep and body temperature. In the last 15 years many authors described biological activities of peptidoglycan in vivo and in vitro, all

indicating that peptidoglycan can influence cells like lymphocytes and macrophages which are involved in inflammation. Lichtman et al. [15] showed that livers of rats injected intraperitoneally with peptidoglycan were able to reactivate arthritis after transplantation into a second rat with preinjured joints. They postulate that peptidoglycan present in the liver is redistributed to other tissues including the injured joints where it causes reactivation of arthritis. Following from the studies done by Lichtman et al. it would seem obvious to look for large bacterial fragments in human tissues. Our group is investigating the relationship between arthritis and intestinal bacteria [8]. Using a rat model the arthropathic properties of cell wall fragments of a number of obligate anaerobic bacteria belonging to the "major residents" of the intestinal flora were described [21,22,23,13]. Recently, we developed a monoclonal antibody against a bacterial flora derived peptidoglycan polysaccharide fraction from human faeces and found positive staining of rat spleen macrophages [14]. Therefore, we have strong support for the presence of bacterial flora derived products in the spleen. In this study we analysed 6 surgically removed human spleens. Using colorimetric Mur analysis, enzymatic determination of D-lactic acid and HPLC analysis, the presence of substantial amounts of Mur was demonstrated. We concluded that peptidoglycan polymers are present in human spleen.

MATERIALS AND METHODS

Spleen

Six sterile unfixed human spleens were obtained from the pathology department immediately after surgery [SSDZ, Delft, The Netherlands) and kept frozen until use. In 5 patients [1-5] with a gastric adenocarcinoma the spleen was removed for technical reasons and in patient 6 because of traumatic rupture. Spleen samples were aerobically and anaerobically cultured on bloodagarbase during 48 h at 37°C. No bacterial growth was observed. The anaerobic plates were cultured in a jar supplied with gaspack generator envelopes with palladium catalyst (Becton Dickinson).

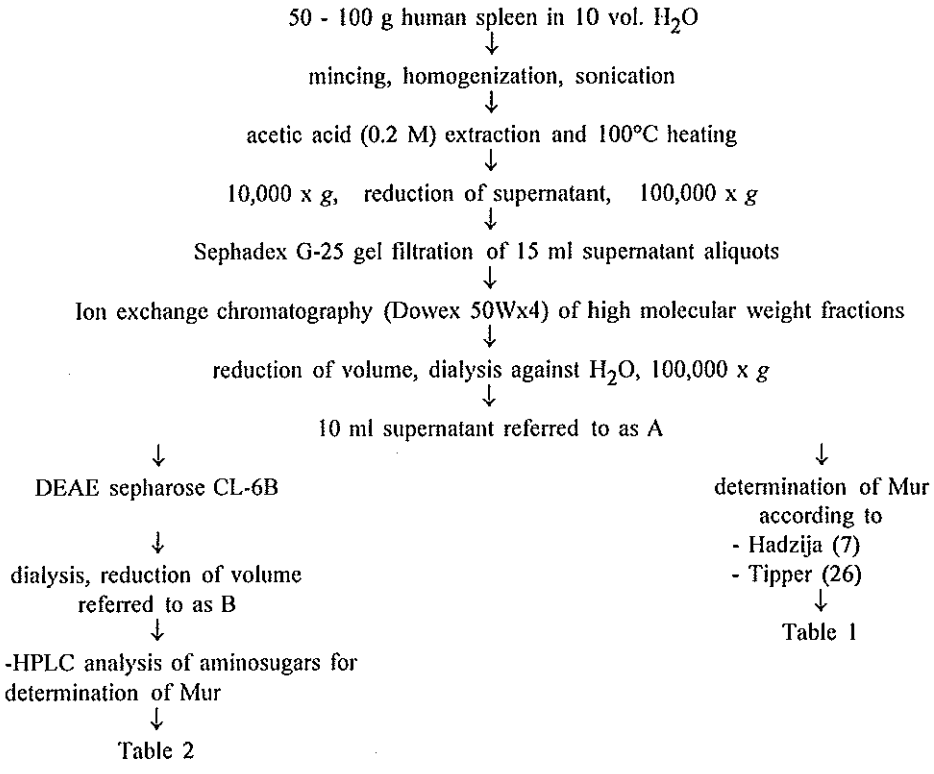
Immunochemical staining

Immunohistochemic staining of the human spleen was performed according to the method of Kool et al. used for the staining of rat spleen [14]. The same monoclonal antibody 2E9 (mouse IgG_3) recognizing intestinal flora derived peptidoglycan polysaccharides was used. Staining was also performed with 2-4, a monoclonal antibody recognizing muramyl dipeptide (MDP) as described by Bahr et al. [1].

Spleen extraction (see figure 1)

50-100 g minced spleen in 500-1000 ml H_2O was homogenized in portions using a Virtis homogenizer (Virtis company, U.S.A.) at 10,000 rpm for 30 s. The homogenate was sonicated 5 times 1 min at maximum amplitude (MSE soniprep 150, UK). Acetic acid (96%) was added to a

Figure 1. Flow diagram of the extraction and purification of a Mur containing fraction from human spleen.



final concentration of 0.2 M. The extract was incubated at room temperature for 2 h under rotation and was subsequently heated gradually in a waterbath to 100°C over a period of 30 min. The extract was centrifuged at 10,000 x *g*. The volume of the supernatant was reduced by lyophilization to 25-50 ml and the extract was then centrifuged at 100,000 x *g*. 15 ml portions the supernatants were separated by gel filtration on a 275 ml gel bed of Sephadex G-25 (Pharmacia, Sweden). Mur and protein was determined in all fractions according to Hadzija [7] and Bradford [2]. The high molecular weight fraction was collected and loaded onto a Dowex 50 W x 4 column (Fluka, Switzerland) equilibrated in 0.1 M acetic buffer (pH 4.6) for further protein reduction. The volume of the pooled fractions was reduced by lyophilization to 10 ml and this preparation was dialyzed against 1 dm^3 1 M NaCl and followed thereafter against 3 x 3 dm^3 H_2O (bidest). After centrifugation at 100,000 x *g*, the supernatant was stored and used for determination of Mur (see below). This preparation is further referred to as A. For HPLC analysis of Mur a further removal of protein was necessary. Therefore, a DEAE sepharose CL-6B (Pharmacia, Sweden) gel bed of 22 ml was washed with 0.5 M NaCl followed by a large volume of H_2O. Then 5 ml of A was loaded onto the column and eluted with H_2O, until no more protein was detected. A 15 ml Mur containing fraction was collected after elution with 0.5 M NaCl. This 15 ml sample was dialyzed against H_2O, lyophilized and dissolved in 2 ml H_2O. This preparation is further referred to as B. In B Mur, protein and total carbohydrates [17] were determined.

Determination of Mur

Preparation A: Mur was determined according to the method of Hadzija [7] with some modifications [9]. In short: samples were hydrolyzed by heating for 2 h at 90°C with an equal volume of 5 M H_2SO_4, then neutralized with 10 M NaOH. Hydrolyzed and unhydrolyzed samples (100 µl) were incubated with 50 µl 1 M NaOH at 36°C for 30 min. After the addition of 1 ml 18.8 M H_2SO_4 (concentrated), samples were heated for 3.5 min at 100°C, rapidly cooled in ice, then mixed with 10 µl 0.16 M $CuSO_45H_2O$ in H_2O and 20 µl 0.09 M *p*-hydroxydiphenyl in ethanol. The blue color developed to a maximum in 30 min at 30°C. Absorbance at 570 nm was determined using a Titertek Multiskan (Flow Laboratories, Irvine, Scotland). Solutions containing 0-100 µg muramic acid (Sigma, St. Louis, USA) per ml H_2O were used as standards. The data are given as the difference in concentration between the hydrolyzed and non-hydrolyzed samples. In this way only Mur linked to the peptides in peptidoglycan is determined and disturbing sugars like rhamnose and of course of lactic acid itself, which are determined in the non-hydrolyzed sample, can be excluded. The presence of Mur in A was also determined by measurement of D-lactic acid according to the method of Tipper [26]. 2 ml of A was hydrolyzed with 2 ml 12 M HCl at 90°C for 2 h. The sample was lyophilized and dissolved in 1 ml H_2O. D-lactate was determined by enzymatic bioanalysis of D-lactic acid/L-lactic acid (Boehringer Mannheim GmbH, Germany).

Preparation B: The presence of Mur was determined with an amino acid aminosugar determination using High Performance Liquid Chromatography (HPLC) reversed phase techniques according to Glauner [6]. In short: Dansylation of B: 1 ml was hydrolyzed with 500 µl 12 M HCL for three hours at 90°C. The sample was frozen, lyophilized and redissolved in 100 µl H_2O. This sample was added to 100µl 200 mM Borate buffer pH 8.8 and 100 µl 20 mM Dansyl chloride (5-Dimethylamino-naphthalene-1-sulfonyl chloride, Fluka Chemie AG, Swiss) in acetone and allowed to react for 2 h at 37°C. The reaction was stopped with 200 mM Phosphoric acid. Valine was used as an internal standard and all other standard aminoacids (Sigma) underwent the same treatment.

Separation of dansylated amino acids and aminosugars was accomplished by reversed phase HPLC. Samples were analyzed using a Pharmacia-LAB 2248 single pump solvent delivery system and VWM 2141 UV-VIS monitor both connected to a computer working with HPLC Manager software to control the pump, gradient mixer and UV-VIS detector operating at 330 nm. Integration and analysis of chromatograms was performed using the same software (Pharmacia, Sweden). Dansylated samples were separated using a Pharmacia SuperPac Sephasil C18, 5µm, 4x250mm column. The flowrate used was 1 ml/min and the buffers used were A: 20 mM Sodiumphosphate pH 5.25 and buffer B: 60% acetonitrile and 40% 50 mM Sodiumphosphate pH 4.0. At 0, 10, 16, 21.5, 22 and 27 min the % B was 30, 50, 100, 100, 30 and 30, respectively.

Eluted products were detected at 330 nm, the wavelength which is maximally absorbed by the dansylgroups. Internal standard Valine and external standards Valine and Mur were used for calculating muramic acid contents of the spleen samples.

RESULTS

Immunohistochemistry of the spleen

Red pulp macrophages in the 6 spleens were immunohistochemically stained. figure 2 shows an immunohistochemic staining of a human spleen with monoclonal antibody 2E9 recognizing intestinal flora derived peptidoglycan polysaccharides. Another monoclonal antibody 2-4, recognizing MDP also stained cells in these spleens (not shown). Negative control antibody NS7 of the same isotype, mouse IgG3, and with irrelevant specificity, did not stain the spleens. From these positive results we concluded that bacterial antigens were present in each of the spleens and that these could be used for isolation of a particular bacterial fraction.

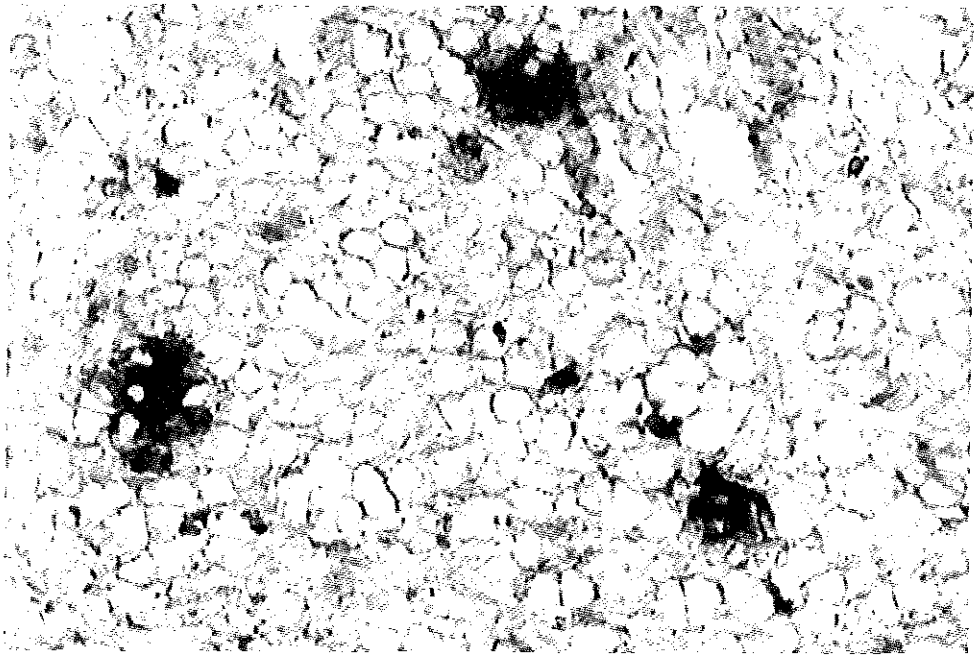


Figure 2. **Immunohistochemic staining with monoclonal 2E9 of human spleen.** Three large 2E9 positive macrophages are stained black. Bar corresponds with 21.8 μm.

Purification of Mur fraction from spleen

From all 6 spleens a preparation A was isolated according to the method described above in detail. The general outlines of the procedure are depicted in

figure 1. figure 3 shows the elution pattern after sephadex G25 gelfiltration of the extracted spleen from patient 1. Protein was found after an elution volume of 95 ml H_2O. Mur was detected after an elution volume of 85 ml H_2O and increased to a maximum of 32 µg Mur per ml. Then it decreased and finally it sharply increased to a level much higher than 100 µg/ml. We pooled the fractions between 85 and 130 ml which represented a relatively high molecular weight fraction and discarded the low molecular weight fraction, because it contained lactate which disturbed the colorimetric Mur assay. The pool was loaded on a strong cation exchange column Dowex 50W x 4 which bound 90% of the protein. After Dowex the volume was reduced and the preparation dialyzed. In a pilot experiment samples of lysozyme solubilized cell wall fragments of *Eubacterium aerofaciens* [22], a carbohydrate polymer with 20% (dry weight) Mur, were completely recovered in the corresponding fractions after the Sephadex G25 gelfiltration and for 80% after the Dowex chromatography.

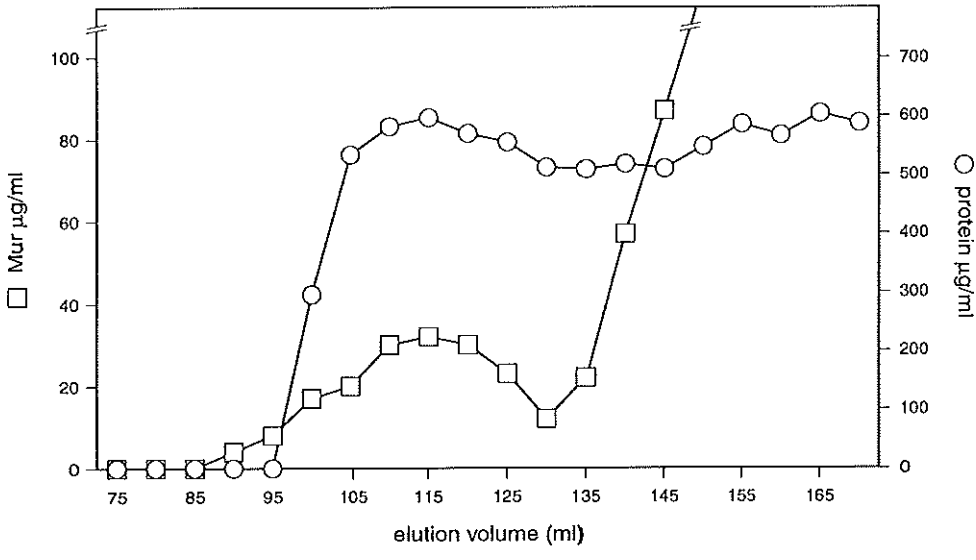


Figure 3. **Separation of high and low molecular weight fractions from human spleen extracts.** Sephadex G25 gelfiltration of an extract of human spleen of patient 1. In step 5 from fig. 1 15 ml spleen extract was loaded onto a 275 ml gelbed of a Sephadex G25 column and Mur and protein concentrations were determined in the fractions.

From all spleens 10 ml preparation A was prepared. In A the protein concentration was still 0.5 to 1.5 mg/ml. The concentration Mur was deter-

mined according to Hadzija with and without hydrolysis of the samples and the net values are given in Table 1. Mur was also determined using D-lactic acid dehydrogenase according to Tipper [26] and the table shows that in all preparations the concentrations determined with the colorimetric method were higher than found with the enzymatic method. Using *E. aerofaciens* cell wall fragments we found twice as much Mur with the colorimetric method as compared to the enzymatic method of Tipper. Because both determinations of Mur are based on the presence of the lactyl group in Mur we felt that a completely different assay for Mur should be used to confirm the results. Therefore, HPLC analysis of Mur was employed. Since the method is based on a separation of dansylated amino acids and amino sugars, the preparation must be as free as possible from disturbing protein aminoacids. Therefore, the remains of preparation A from spleen 1 to 5 were further purified by DEAE anion exchange. The *E. aerofaciens* cell wall polymers applied to the DEAE column showed that the protein part of the preparation was not bound. The Mur containing fraction, however, did bind and could be eluted from the column with 0.5 M NaCl. DEAE passage of preparation A reduced the protein contents considerably (<200 µg/ml).

Table 1. Recovered Mur determined according to Hadzija (modified) and Tipper in preparation A from 6 human spleen extractions

	1	2	3	4	5	6
Wet weight spleen (g)	91	70	55	93	57	73
µmol Mur						
According to Hadzija	2.82	2.27	2.29	2.66	1.79	2.56
According to Tipper	0.97	0.75	0.38	0.40	0.43	2.04

In this sample, called preparation B, Mur, protein and total carbohydrate concentrations were determined with colorimetric assays and the results are shown in table 2.

Samples of preparation B of spleen 1 to 5 were applied to HPLC. The strategy we used for Mur detection by HPLC was as follows: First, by adding a little dansylated muramic acid to the sample it was possible to spike a peak with a retention time of 11.3 min in the spleen sample as is depicted in figure 4A and B. There are many peaks in the chromatogram and therefore we decided to collect the peak with retention time 11.3 minutes and reanalyze this

peak to see if it really has the same retention time as the standard dansyl Mur. Indeed a major peak of the chromatogram of the collected peak had exactly the same retention time as dansyl Mur (see figure 4C and D). Finally we determined Mur in the collected peak using the colorimetric assay according to Hadzija to complete the circle and prove that the spleen samples contain Mur.

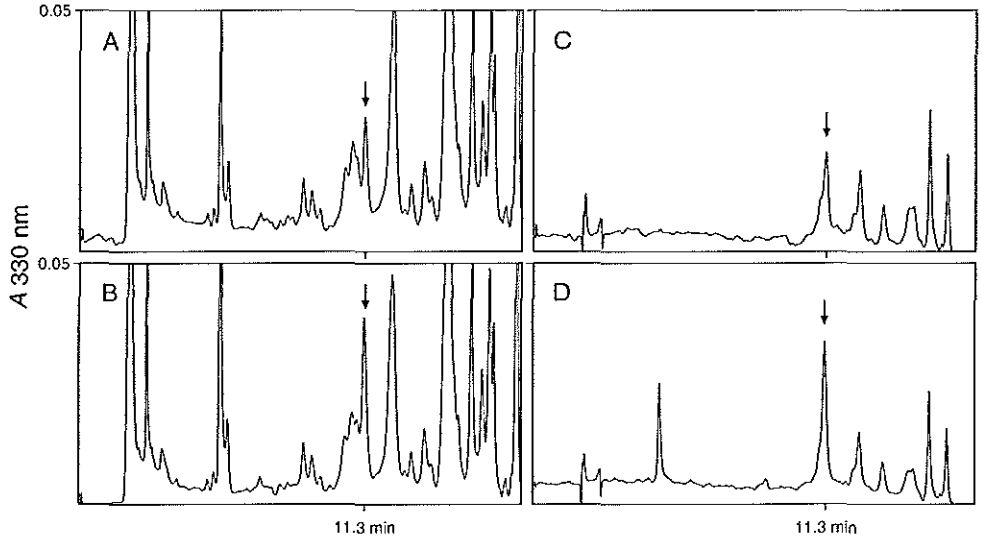


Figure 4. **Identification of Mur in preparation B from patient 1.** Reversed-phase HPLC on a C18 column (5µm, 4 x 250 mm) in a discontinuous gradient of 0-15% Methanol over 27 minutes at 1.0 ml/min of **A)** Hydrolysed and dansylated preparation B of spleen extract from patient 1. **B)** Idem as A but spiked with dansylated Mur. **C)** Collected peak with retention time 11.3 minutes from chromatogram A). **D)** Idem as C but spiked with dansylated Mur. Absorbance was measured at 330 nm. Peak with retention time 11.3 min identified as Mur, is indicated as ↓.

Calculations of Mur concentration using valine as an internal standard gave results in the same range as the other method but may have been hampered by contaminating protein amino acids (Table 2). If the results of Mur concentrations (Tables 1 and 2) are summarized as mean values they show that with HPLC 3.3 µmol, with Hadzija 1.9 µmol (B) and 2.4 µmol (A), and with Tipper 0.8 µmol per spleen (average 73 g) were found.

Table 2. Mur (μM), protein and total carbohydrates (μg/ml) in preparation B from 5 human spleen extractions

	1	2	3	4	5
Mur: Hadzija	2.64	1.40	1.04	3.60	0.76
Mur: HPLC	5.28	3.56	2.16	4.40	1.32
Protein	564	352	412	368	780
Carbohydrates	3320	2000	2000	5080	1240

DISCUSSION

Sen and Karnovsky found 100 pmol Mur per gram rat liver tissue and extracted muramyl compounds from brain and kidney [20]. A daily output of 1 μmol of free diaminopimelic acid, an amino acid specific for muramyl peptides, was found in human urine by Johannsen and Krueger [10]. Fox and Fox, however, could not detect Mur in human serum and therefore considered the presence of peptidoglycan or Mur containing subunits a matter of dispute [5]. In this study we determined Mur in spleen tissue by three different methods. The spleen was chosen because a previous immunohistochemical study with monoclonal antibodies showed the presence of bacterial antigen in macrophages in the rat spleen [14]. The six human spleens used in this study also contained macrophages positively stained by monoclonal antibody 2E9. Specificity of the monoclonal antibody was checked by inhibition ELISA's and by inhibition of immunohistochemic staining of the spleens. Peptidoglycan polysaccharides were used as antigen.

The isolation procedure was focussed on detecting Mur in polymeric peptidoglycan because that is the product which we think is present in the splenic macrophages based on our observations in rats injected with peptidoglycan-polysaccharides from *Eubacterium aerofaciens* [14]. Small muropeptide MDP administered in vivo in mice, however, is excreted unchanged in the urine within minutes [27]. The practical advantage of this assumption is that during the extraction and purification the low molecular weight fraction could be discarded. In this respect our study differed from the studies of Sen and Karnovsky [20] who were looking for low molecular weight muropeptides. Before and after homogenization of the spleen tissues, sterility was checked by cultivating the homogenate on a blood agar plate. No contamination could be detected. Acetic acid extraction was used to hydrolyse the lysosomes in the spleen homogenates in which the peptidoglycan might be located [19]. After this, the aim was to remove as much protein as possible while keeping the

carbohydrate fraction. In the end we succeeded in isolating such a fraction containing a considerable amount of Mur. Mur was used as a marker for the presence of polymers of peptidoglycan because Mur is considered to be unique for peptidoglycan.

We used three determination methods for Mur. 1) The method of Hadzija [7] (modified by Hazenberg et al. [9]) is based on the colorimetric determination of the lactyl group after acid hydrolyses to remove peptides in peptidoglycan and treatment with NaOH to hydrolyse the ether bond of the lactyl group in Mur.

2) The method of Tipper [26]; D-lactic acid is specific for bacteria [24] and the covalent ether bond of D-lactic acid in Mur is very specific for this molecule and therefore for peptidoglycan. We adopted the method of Tipper in which lactate dehydrogenase is used for the detection of D-lactic acid in peptidoglycan liberated after acid and alkali hydrolysis. Indeed D-lactate was found in our *Eubacterium aerofaciens* cell wall fragment preparation after consecutive acid and alkalic hydrolysis (data not shown).

3) Because both methods for determination of Mur are based on detection of the (D) lactyl group a third method not dependent on the presence of lactic acid was used. The HPLC analysis of amino sugars were carried out according to a modified procedure developed by Glauner [6] who studied the composition of peptidoglycan of *Escherichia coli* extensively. After collection of the peaks with a retention time of 11.3 min (figure 4), we were able to determine Mur in this fraction with the Hazija method. We conclude that in the human spleen Mur and therefore peptidoglycan is present, not as small muramyl-peptides as was previously looked for, but as larger molecules probably stored in spleen macrophages.

Peptidoglycan has many biological effects in vivo, like sleep induction [16], complement activation [30], adjuvant activity [4] and many other immunostimulatory effects like antibody production [28] and T cell mediated arthritis induction [8,21,22,23,13]. In vitro peptidoglycans are able to stimulate human monocytes to produce TNFα and Il-1 and inhibition or activation of monocyte migration [25,18]. From the results presented in this paper and results from other investigators it can be concluded that our body tissues are continuously exposed to these bacterial products which in some cases might lead to biological activities in vivo. If so, this in its turn might provide a clue to inflammation with unknown etiology, like rheumatoid arthritis and reactive arthritis. Presently we are investigating the biological properties of this peptidoglycan.

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Purification and characterization of N-acetylmuramyl-L-alanine amidase from human plasma using monoclonal antibodies

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ABSTRACT

N-Acetylmuramyl-L-alanine amidase (EC 3.5.1.28) cleaves the amide bond between N-acetyl muramic acid and L-alanine in the peptide side chain of different peptidoglycan products. The enzyme was purified from human plasma using a three step column chromatography procedure. Monoclonal antibodies were produced against the purified human enzyme. By coupling of a high affinity monoclonal antibody to sepharose beads an immunoadsorbent column was prepared. Using this second purification method it was possible to purify large amounts of the amidase from human plasma in a single step. SDS-PAGE showed one single band of 70 kDa and two-dimensional electrophoresis showed the presence of multiple isomeric forms of the protein with pI between 6.5 and 7.9. Two different methods were used for determination of substrate specificity, a HPLC method separating peptidoglycan monomers from the reaction products after incubation with amidase and a colorimetric method when high molecular weight peptidoglycan was used as a substrate for amidase. It is shown that the disaccharide tetra peptide, disaccharide penta peptide and the anhydro disaccharide tetrapeptide are good substrates for the amidase and that muramyl dipeptide and disaccharide dipeptide are not a substrate for the amidase. Using one of the monoclonal antibodies against the amidase it was shown in FACScan analysis that N-acetylmuramyl-L-alanine amidase is present in granulocytes but not in monocytes from unstimulated peripheral blood of a healthy donor. The presence of N-acetylmuramyl-L-alanine amidase in granulocytes is a novel finding and perhaps important for the inactivation of biologically active peptidoglycan products still present after hydrolysis by lysozyme.

INTRODUCTION

Peptidoglycan polymers, oligomers and monomers have potent biological effects. An important factor in the induction of the biological effects is the ability of the peptidoglycan products to persist in human tissues. Bacterial cell wall products can be degraded by 3 different human enzymes: lysozyme [1], β-N-acetylglucosaminidase [2] and the not well characterised N-acetylmuramyl-L-alanine amidase (NAMLAA). This NAMLAA hydrolyses peptidoglycan by cleaving the lactamide bond between N-acetyl muramic acid and L-alanine in the peptide side chain of the peptidoglycan molecule. *Bordetella pertussis* tracheal cytotoxin is an anhydro-disaccharide tetrapeptide peptidoglycan monomer which is capable of reproducing the respiratory cytopathology

observed during pertussis [3, 4]. NAMLAA is able to degrade this anhydro monomer very rapidly in vitro and might be the most important enzyme involved in the degradation of the tracheal cytotoxin in vivo. We hypothesize that this amidase, which we found in all human sera tested until now [5], plays an important role in the degradation and inactivation of biologically active peptidoglycan polymers and monomers in human tissue.

Amidase (NAMLAA) activity in human serum was first described by Ladesić et al. in 1981 [6] One year later Valinger et al. from the same group partially purified NAMLAA from human and mouse serum and defined its enzymatic activity [7]. In the present study we describe two methods for the purification of NAMLAA from human plasma. The first method is based on DEAE, heparin sepharose and hydroxylapatite column chromatography. The protein obtained by this three-step method was used to raise monoclonal antibodies against the enzyme. With these antibodies an immunoadsorbent affinity column was prepared with which amidase was purified in one single step with a 36% yield of the enzymatic activity.

MATERIAL AND METHODS

Purification of NAMLAA with three-step procedure

300 ml human plasma was dialyzed 3 times against 2 L 25 mM sodium phosphate buffer pH 7.5. After dialysis it was centrifuged for 15 min at 20,000 x *g*. The supernatant was then loaded onto a 500 ml DEAE sepharose 4B (Pharmacia) column. The column was washed with 8 L 25 mM sodium phosphate buffer pH 7.5 until no more protein could be eluted. Elution of the amidase containing fraction was performed by changing the buffer to a 25 mM sodium phosphate buffer with 0.5 M NaCl. Amidase-containing fractions were pooled and dialyzed 3 times against 25 mM sodium phosphate buffer pH 7.5 and loaded onto a 50 ml heparin sepharose Cl-4B column (Pharmacia). The column was washed with 10 column volumes of the sodium phosphate buffer until the eluate did not contain any protein. Elution was performed using a linear gradient of 300 ml sodium phosphate pH 7.5 with 300 ml sodium phosphate pH 7.5 with 0.25 M NaCl. Amidase-containing fractions were pooled and dialyzed 3 times against 2 L 25 mM sodium phosphate pH 6.8 and loaded onto a 15 ml hydroxylapatite (Biorad) column. Amidase does not bind to this column. The flow through was collected and dialyzed against 20 mM NH_4HCO_3 pH 8.0. All steps were performed in a cold room (4°C). The NAMLAA activity in the samples was determined using the colorimetric assay described below.

Peptidoglycan substrates

Peptidoglycan monomers from *Brevibacterium divaricatum* (ATCC 14020) GlcNAc-MurNAc-L-Ala-D-isoGln-m-Dpm]-(D)-amide-(L)-D-Ala-D-Ala pentapeptide, and GlcNAc-MurNAc-L-Ala-D-isoGln-m-Dpm-D-Ala tetrapeptide were prepared as described by Hazenberg and de Visser [5].

MurNAc-L-Ala-D-isoGlu (MDP) (Sigma), GlcNAc-MurNAc-L-Ala-D-isoGlu (GMDP)

(Calbiochem corp), GlcNAc-MurNAc-anhydro-L-Ala-D-Glu-m-Dpm-D-Ala (anhydro disaccharide-tetrapeptide) isolated from *Escherichia coli* was a kind gift from A. Dijkstra, Hoffman La Roche.

Escherichia coli, Brevibacterium divaricatum, Eubacterium aerofaciens, Bifidobacterium adolescentis and *Streptococcus pyogenes* peptidoglycans were prepared according to Severijnen et al. [8]. *Micrococcus lysodeikticus* was obtained from Sigma. The polymeric peptidoglycans were degraded by incubating 250 µg/ml overnight with a 25 µg/ml lysozyme solution in 10 mM sodium acetate buffer pH 5.6. Peptidoglycan concentrations vary between different experiments.

Detection of NAMLAA activity by a colorimetric method

Amidase activity was determined as described by Hazenberg and de Visser [5] with some modifications. The method is based on determination of the increase of a free lactyl group in the peptidoglycan substrate due to the removal of the peptide side chain. Peptidoglycan monomers from *Brevibacterium divaricatum* (ATCC 14020) were used as a substrate. 200 µl amidase sample and 200 µl substrate diluted in 20 mM NH_4HCO_3 pH 8.0 to a final concentration of 500 µg muramic acid/ml were incubated for 15 minutes at 37°C. The reaction was stopped by adding 200 µl 1M NaOH and incubating for 30 min. at 37°C. For determination of background, substrate was incubated with buffer for 15 min at 37°C. In these samples muramic acid was determined as follows. 100 µl sample + 1 ml (conc) H_2SO_4 was boiled for 3.5 min and rapidly cooled in icewater. Then 10 µl 0.16 M $CuSO_4.5H_2O$ and 20 µl 0.09 M p-hydroxydiphenyl in ethanol were added. After a 30 min incubation period at 30°C the absorbance at 570 nm was measured using a Titertek Multiskan (Flow Lab. Irvine, Scotland). 0-100 µg/ml muramic acid solutions were used as standards. For determining the specific activity of NAMLAA using polymeric peptidoglycan lower concentrations of peptidoglycan were used.

Detection of NAMLAA activity by HPLC

Substrate specificity was determined using *Brevibacterium divaricatum* GlucNAc-MurNAc-pentapeptide and tetrapeptide. Also MurNAc-L-Ala-D-isoGlu (MDP), GlcNAc-MurNAc-L-Ala-D-isoGlu (GMDP) (Calbiochem corp), GlucNAc-MurNAc[anhydro] tetrapeptide isolated from *E. coli* were used. 500 µg/ml samples were incubated with affinity-purified amidase in a final concentration of 1 µg/ml 25 mM NH_4HCO_3 buffer pH 8.0 at 37°C. For determination of the background the substrate was incubated with buffer. The reactions were stopped after 15 min by diluting the samples 10 times with 25 mM sodium phosphate pH 3.5 (HPLC buffer A). Peak areas were used to calculate the enzymatic activity. 1 Unit is defined as the amount of substrate (µmol) hydrolysed per minute at pH 8.0, 37°C.

Reversed phase HPLC was used for separation of the reaction products. 10 µl samples were analyzed using a Pharmacia-LKB 2248 single pump solvent delivery system and VWM 2141 UV-VIS monitor both connected to a computer working with HPLC manager software to control the pump, gradient mixer and UV-VIS detector operating at 205 nm. Integration and analysis of chromatograms was performed using the same software (Pharmacia, Sweden). The samples were separated using a Pharmacia Superfac Sephasil C18, 5µm, 4 x 250 mm column. The flowrate was 1 ml/min and the buffers were A: 25 mM sodium phosphate pH 3.50; B: 15% methanol in 25 mM sodium phosphate pH 4.70. At 0, 2, 10, 12.5, 13, 15 min the percentage B was 0, 0, 100, 100, 0, 0 respectively.

For determination of Km and maximal rate of catalysis (V max), substrate concentrations were used between 2 mM and 0.1 mM GlucNac-MurNAc-pentapeptide of *Brevibacterium divaricatum*. The aminoacid and aminosugar composition of the separated peaks before and after

amidase incubation was determined according to the method described in a previous paper [9].

Preparation of monoclonal antibodies

The purified amidase was used for preparing monoclonal antibodies. A male Balb/c mouse was injected intraperitoneally with 50 µg of NAMLAA in complete Freund's adjuvant. After 6 weeks the mouse received a booster of 10 µg NAMLAA in incomplete Freund's adjuvant. After another 6 weeks the mouse received a second booster of 10 µg amidase in incomplete Freund's adjuvant, intraperitoneally and 10 µg amidase in PBS, intravenously. Three days later, the mouse was sacrificed and cells isolated from popliteal, inguinal and axillary lymphnodes and the spleen were fused with the Sp2/0 plasmacytoma cell line in a ratio of 5:1 and 5:2 (spleen: Sp2/0) using standard procedures for the production of hybridomas [13]. Cells were seeded at a concentration of 8.10^4 cells/well in the presence of human growth factor (40 U/ml) [11]. The supernatants of 3000 wells were tested for the production of antibodies against amidase in an ELISA. The amidase-positive supernatants were tested in an isotype ELISA [12]. Only IgG-producing clones were used for further experiments. Monoclonal antibody AAA4 showed the highest affinity for NAMLAA and was used for preparing a FITC-labeled monoclonal antibody (α-NAMLAA-FITC) and for preparing an immunoadsorbent column.

Preparing immunoadsorbent column and purification of NAMLAA

Monoclonal antibodies were purified over a 10 ml Immunopure Immobilized Protein G column (Pierce) using the standard protocol of the supplier. 7.2 mg of monoclonal antibody AAA4 was dialyzed 3 times against 2 l 0.1 M $NaHCO_3$ + 0.5 M NaCl and coupled to 2 g CNBr-activated Sepharose 4B (Pharmacia LKB Biotechnology) according to the protocol of the supplier. Human plasma (obtained from the blood bank Rotterdam) was dialyzed against PBS and diluted 2 times in PBS before it was loaded onto the column. The column was washed with PBS until no more protein was detected in the eluate. The amidase was eluted by changing the buffer to 0.1 M Gly.HCl pH 3.0. The peak fractions were immediately dialyzed against 20 mM NH_4HCO_3 pH 8.0.

Immunofluorescence labeling, flow-cytometric analysis

Labeling of AAA4 with fluorescent isothiocyanate (FITC) was performed according to standard procedures [13]. Human peripheral blood cells were stained for intracellular antigens according to Syrjilä [14]. In short: 100 µl citrate blood was lysed and fixed by adding 2 ml of FACS lysing solution (Becton Dickinson) and incubating for 10 min at room temperature. The cells were centrifuged and washed 2 times with PBS + 0.5% BSA. Then the mouse α-NAML-AA-FITC or rabbit α-human lysozyme (DAKO, ITK Diagnostics) were added and the mixture was incubated for 10 min at room temperature. The cells were washed 2 times with 2 ml PBS-BSA. The rabbit α-lysozyme-treated cells were incubated with the second antibody goat-α-rabbit-FITC for 10 min, washed 2 times with PBS-BSA and finally resuspended in 200 µl FACS flow (Becton Dickinson). The analyses were performed with a FACScan cytofluorimeter (Becton Dickinson, Sunnyval, CA).

SDS-Polyacrylamide gel electrophoresis and immunoblot analysis

The amidase was subjected to 10% SDS-PAGE to check for purity. Affinity-purified amidase samples of 20 µl were boiled for 3 min together with 5 µl loading buffer (60 mM Tris. HCl pH 6.8; 23% glycerol 3% SDS; 0.06% bromophenol blue; 10% β-mercapto ethanol) 10 µl of these samples were analyzed on 10% SDS-PAGE (Mini Protein, BioRad). Samples were

visualized by Coomassie Blue staining or transferred to nitrocellulose in 25 mM Tris, 190 mM glycine and 20% methanol transfer buffer. Nitrocellulose sheets were blocked in low-fat milk for 30 minutes and subsequently washed three times with 0.5% Tween-20 in PBS. Nitrocellulose sheets were then incubated for 1 hour with monoclonal antibodies 500 times diluted in PBS-Tween at room temperature. Following three washes, goat anti-mouse IgG conjugated to alkaline phosphatase (TAGO) was added in a 1000 times dilution and incubated for 1 hour at room temperature. The blot was washed three times with PBS-Tween and then three times with PBS. For visualization of antibody-antigen complexes the alkaline phosphatase substrate, nitroblue tetrazolium/5-bromo-4-chloro indoxyl phosphate (NBT/BCIP), was used as described [20].

Two-dimensional electrophoresis

25 µl of affinity-purified NAMLAA was mixed with 25 µl sample buffer (0.3% SDS, 200 mM dithiothreitol, 28 mM Tris.HCl and 22 mM Tris-base) and heated for 4 min at 100°C. Two-dimensional electrophoresis was performed with the Millipore Investigator system. We used ampholytes with a pH range from 3-10 for the first dimension. For the second dimension we used 10% Duracryl gels. Glyceraldehyde-3-phosphate dehydrogenase carbamalytes (Pharmacia) were used as iso-electric-focussing markers. Proteins were visualized with a silver staining technique. All steps were performed according to the manufacturers' instructions.

Gel permeation chromatography

A superdex 200 HR 10/30 column with 10 mm internal bore diameter and 30 cm length (Vc=24 ml) was used (Pharmacia). The column was connected to a standard FPLC system (Pharmacia), consisting of a LCC chromatography controller, two P-500 pumps, a MV-7 injector with a 200 µl sample loop, a UV-1 monitor at 280 nM operating at 0.05 AUFS sensitivity and a FRAC 100 fraction collector. Routinely, isocratic elution with PBS was used. Column selectivity was determined by using mixtures of molecular weight marker proteins (Pharmacia) ranging from 670 kDa to 17 kDa. 200 µl purified
NAMLAA was injected on the column. Enzymatic activity was determined in the peak fractions.

Immunohistochemistry

Blood smears of a healthy person were fixed for 10 minutes in acetone. After rinsing in PBS with 0.2% bovine serum albumin (BSA), the smears were incubated for 1 hour at room temperature with monoclonal antibody AAA3 (diluted 40 µg/ml in PBS-BSA) or, for control staining, an irrelevant monoclonal antibody of the same isotype and concentration. Subsequently, the smears were rinsed in PBS-BSA and incubated for 30 minutes at room temperature with rabbit anti-mouse immunoglobulin (Z259, Dakopatts) diluted 1:20 in PBS-BSA with 1% normal human serum. After rinsing in PBS-BSA, a 1:40 dilution of alkaline phosphatase-mouse-anti-alkaline phosphatase complex (APAAP, D651, Dakopatts, Denmark) was applied for 30 minutes. To develop the stain the smears were incubated for 30 minutes at room temperature with new Fuchsin substrate (Chroma), which stained positive cells red. After rinsing in distilled water the nuclei of the cells were stained blue by incubation for 1 minute with hematoxylin and rinsing in fresh water for 10 minutes. Finally, the smears were mounted in Kaiser's glycerol gelatin (Merck).

RESULTS

Purification of NAMLAA from human plasma

Table 1 shows the purification scheme of the three step purification. The amidase activity in dialyzed and centrifuged plasma was 46 mU per mg protein. The final pure amidase had a specific activity of 6.7 U per mg protein. Most of the total activity was lost during this purification procedure (99%). Starting with 300 ml plasma it was possible to purify about 1 mg NAMLAA using this method.

Table 1. Purification of N-acetylmuramyl-L-alanine amidase from human plasma by three-step procedure

	protein conc (mg/ml)	total protein (mg)	specific activity (U/mg)	Yield (%)
plasma	48.7	15.6×10^{3}	4.6×10^{-2}	100
DEAE	13.6	2.7×10^{2}	0.11	41
Heparin	0.2	15.4	3.2	6.9
Hydroxylapatite	1.0×10^{-2}	1.0	6.7	0.9

Protein concentrations were determined according to the method of Bradford. Enzymatic activity was determined by the colorimetric assay.

Monoclonal antibodies and immunoaffinity purification

Six different monoclonal antibodies against NAMLAA were obtained. Monoclonal AAA4 showed the highest affinity for the human amidase in a competition ELISA (not shown) and was therefore used to prepare an immuno-adsorbent column. 7.2 mg purified monoclonal antibody was coupled to CNBr-activated sepharose beads. The capacity of the column was sufficient for the 50 ml plasma used. No amidase activity could be detected in the flow-through when diluted plasma was applied to the column. After elution with 0.1 M Gly.HCl pH 3.0, pure amidase was eluted from the column. From 50 ml plasma it was possible to obtain 0.6 mg pure amidase.

Table 2 shows the purification scheme. The amidase activity in this dialyzed and centrifugated plasma was 31 mU/mg protein, determined by the HPLC method. The purified amidase solution obtained by this method contained a specific activity of 46 U/mg protein and 36% of the enzymatic activity

was recovered. Therefore, by using the immunoadsorbent column it was possible to increase the yield 38-fold and the final product had a specific activity 6.9-fold higher compared with the three-step procedure.

Table 2. Purification of N-acetylmuramyl-L-alanine amidase from human plasma by immunoaffinity chromatography

	Protein conc (mg/ml)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
plasma	48.7	2435	3.1×10^{-2}	100
Immunoaffinity	0.1	0.6	46	34

Protein concentrations were determined according to the method of Bradford. Enzymatic activity was determined by the HPLC method.

SDS-Polyacrylamide gel electrophoresis and immunoblot analysis

Figure 1 shows a 10% SDS-PAGE. Purified amidase is electrophoresed as a single band with a molecular mass of 70 kDa. After deglycosylation by N-glycosidase-F digestion, the molecular mass decreased to 60 kDa (not shown).

Western blot analysis of purified NAMLAA before and after deglycosylation showed that four of the six monoclonal antibodies including AAA4, the antibody used for purification, were able to recognize NAMLAA in both forms.

Two-dimensional electrophoresis

Figure 2 shows affinity-purified NAMLAA subjected to iso-electrofocussing in the first dimension and 10% polyacrylamide gel electrophoresis in the second dimension. At least 8 different isomeric forms are visible of this glycoprotein. The pI values vary between 7.0 and 7.9 although the main spots are in the 7.5-7.8 range. No impurities are visible after silverstaining.

Detection of NAMLAA activity by HPLC

NAMLAA activity was tested with different peptidoglycan products. Muramyl dipeptide (MDP) was tested because this product is the smallest

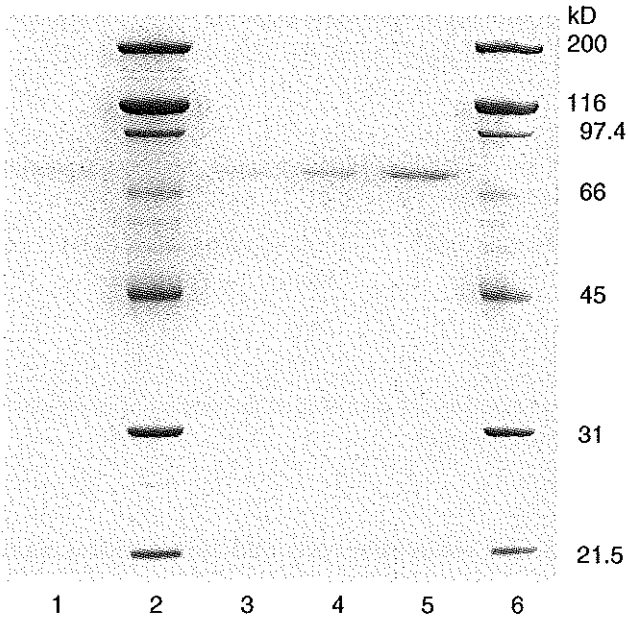

Fig 1. Polyacrylamide gel electrophoreses of purified N-acetylmuramyl-L-alanine-amidase. 10 µl samples of three different concentrations were analyzed on 10% PAGE-SDS. Lane 1 is amidase purified using the three-step procedure. Lane 2 and 6 are molecular weight markers and lane 3, 4 and 5 are 20, 40 and 100 µg/ml dilutions (resp.) of immunoaffinity-purified amidase. NAMLAA is clearly shown as a single band of 70 kDa. The gels were stained with Coomassie Blue.

component of peptidoglycan known to posses biological activity. Harrison and Fox [15] described that MDP was degraded by human serum. We did not find this activity for the purified amidase. N-acetylglucosamine-N-acetylmuramic acid di-peptide (GMDP) was not a substrate for NAMLAA either. Di-saccharide tetrapeptide and the anhydro-disaccharide tetrapeptide were good substrates for NAMLAA. The anhydro form was degraded four times slower than the non-anhydro disaccharide-tetrapeptide. The disaccharide-pentapeptide was degraded at the same velocity as the tetrapeptide, as determined by HPLC analysis (Figure 3).

Using the *Brevibacterium divaricatum* disaccharide pentapeptide as a substrate the maximal rate of catalysis is 0.10 mmol/min and the Km value is 2.5 mM.

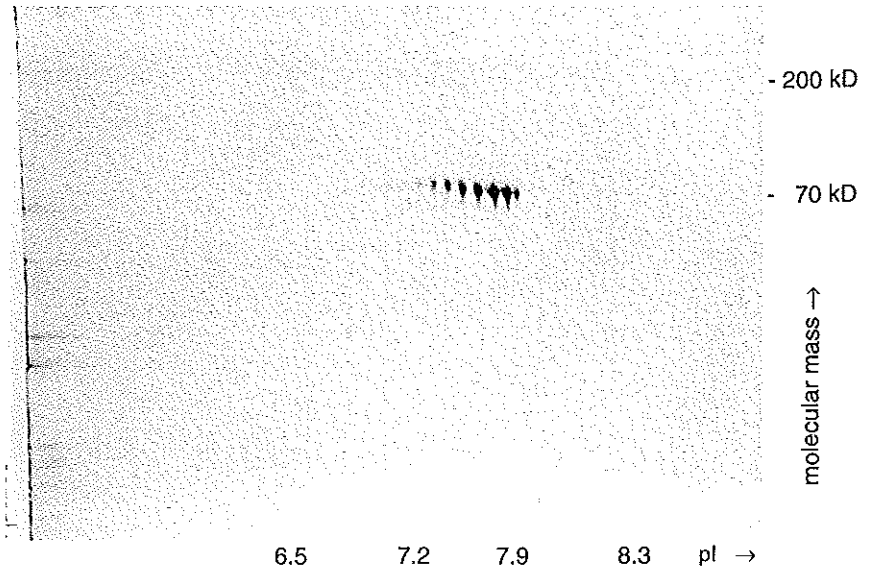

Fig 2. Two-dimensional electrophoresis of affinity-purified NAMLAA. First dimension isoelectrofocussing in a pH range of 3-10 and second dimension 10% Duracryl electrophoresis separation on molecular weight. Thirteen different isomeric forms of the glycoprotein are visible. The silverstained gel does not show any impurities.

Detection of NAMLAA activity by colorimetric assay

Six different lysozyme-solubilized peptidoglycan samples were incubated with NAMLAA. In Table 3 the differences in the peptide bridges of the peptidoglycans are shown with the corresponding specific activity of NAMLAA. *B. adolescentis, S. pyogenes* and *M. lysodeikticus* peptidoglycans are not degraded by NAMLAA. The substrate concentration was 350 µg/ml based on the concentration muramic acid in the reaction mixture.

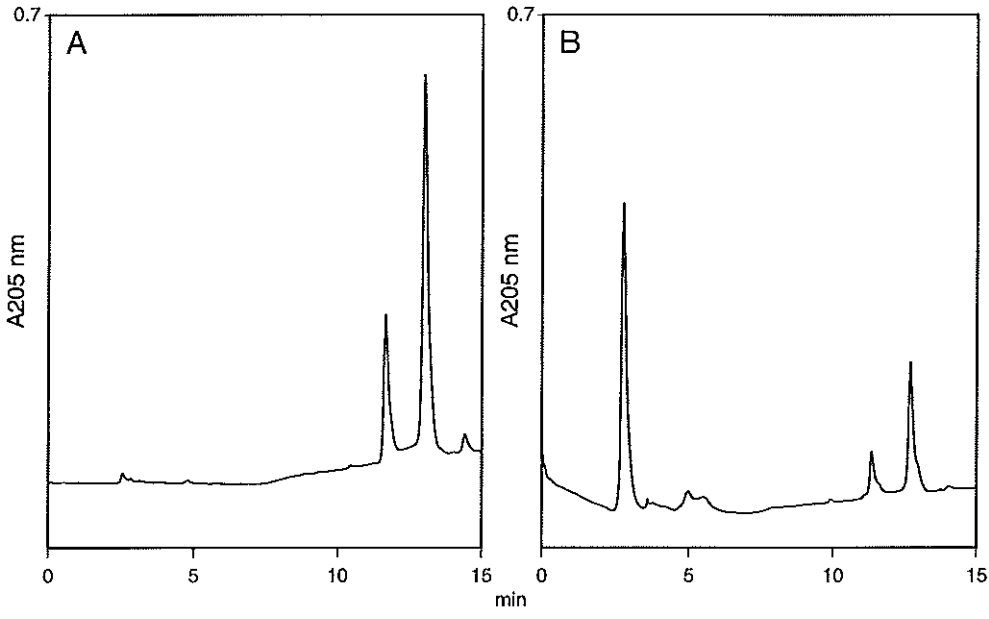

Fig 3. Reversed phase high performance liquid chromatography chromatogram of *Brevibacterium divaricatum* disaccharide pentapeptide before (A) and after (B) incubation with NAMLAA. The large peak with retention time 12.8 min in A represents the disaccharide pentapeptide. The small peak with retention time 11.8 min is the disaccharide tetrapeptide. The large peak in B with retention time 2.8 min (void volume) contains the peptide product released from the sugar moiety. The small peak at 5 min contains the disaccharide. The difference in area of the peaks in A with the corresponding peaks in B is a direct measure for the amount of substrate degraded by NAMLAA.

Flow-cytometric analysis

FACScan analysis of the nucleated cells from blood of a healthy donor were performed to determine if NAMLAA is produced by white blood cells. In Figure 4 it is shown that granulocytes contain NAMLAA but monocytes do not. Lysozyme could be detected in both monocytes and granulocytes (not shown). PBS and IgG-FITC were used as a negative control (Figure 4A and 4B).

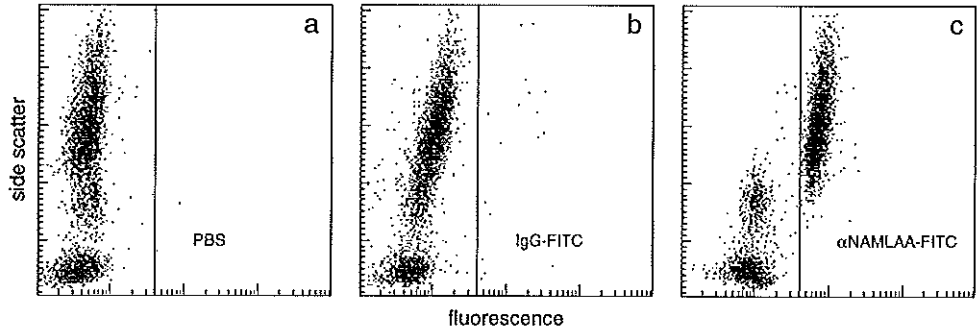

Fig 4. FACScan analysis of nucleated fraction from blood of a healthy donor. Based on sideward scatter human peripheral blood cell suspensions can be divided in three different populations. The lower population consists of lymphocytes, the middle population consists of monocytes and de upper population are the granulocytes. Granulocytes are positively stained by α-NAMLAA-FITC (C), monocytes and lymphocytes are not stained with this monoclonal antibody. PBS (A) and IgG-FITC (B) were used as negative controls.

Native molecular mass determination

The selectivity curve used was: $K_{AV} = 2.309 - 0.378 \log Mr$

$$K_{AV} = \frac{Ve - Vo}{Vt - Vo} = \frac{Ve - 8}{12.8}$$

where Ve is the elution volume
Vo is void volume (8.0 ml)
Vt is total volume (20.7 ml)

The elution volume of the NAMLAA activity containing peak was 14.0 ml. Therefore the calculated molecular mass is 69 kDa ± 10%. Which proves that the amidase is composed of one subunit.

Immunohistochemistry

The immunohistochemical staining of the blood smears of a healthy person with monoclonal antibody AAA3 showed a positive staining of the granulocytes. The mononuclear cells including monocytes were negative. No positive cells were found by using an irrelevant monoclonal antibody with the same isotype and concentration.

Table 3. NAMLAA activity related to differences in peptide bridges in peptidoglycan from 6 bacterial species

Peptidoglycan Source	Aminoacids in peptide bridge	Specific activity (U/mg)
E. coli	NAM-L-Ala-D-Glu-M-Dpm(D-Ala)–	57
B. divaricatum	NAM-L-Ala-D-isoGln-M-Dpm(D-Ala)–	111
E. aerofaciens	NAM-L-Ala-D-Glu-L-Lys(D-Ala)–	12.5
B. adolescentis	NAM-L-Ala-D-Glu-L-Orn(D-Ala)-Ala-D-Asp-D-Ser–	2
S. pyogenes	NAM-L-Ala-D-Glu-L-Lys(D-Ala)-D-Ala-L-Thr-L-Ala–	0
M. lysodeikticus	NAM-L-Ala-D-Glu(Gly)-L-Lys-D-Ala-L-Lys(Gly)-D-Glu-L-Ala–	0

Enzymatic activity was determined with the colorimetric method.

DISCUSSION

In 1990 Vanderwinkel et al published the purification of NAMLAA from human serum [16]. They found an enzyme of Mr 120.000-130.000 in native PAGE and two bands of 57 kDa and 70 kDa under denaturating conditions and considered amidase to be a dimeric protein with pI of 4.5-5.5. They also found the *E. coli*-derived MurNAc-tripeptide to be a good substrate for the enzyme as well as some polymeric peptidoglycans. Because it was only possible to purify very small amounts of amidase with their method and the properties of the enzyme did not always correspond with the protein we were purifying we started with the development of a large scale purification to make a good characterization of the enzyme possible.

This study describes two methods for the purification of NAMLAA from

human plasma. The purification with a NAMLAA specific immunoabsorbent column yielded a higher specific activity than the three-step column purification method. Apparently some of the amidase purified by the three-step method loses some of its activity during the different steps, because the specific activity is much lower compared to the immunoaffinity-purified NAMLAA. Purification of human NAMLAA can therefore best be carried out using the specific immunoabsorbent column. It is unknown if all the immunoaffinity-purified NAMLAA is still active. It is therefore not possible to determine exactly the concentration of NAMLAA in plasma, but it must be at least 10 μg/ml plasma. This is similar to normal serum concentrations of lysozyme which varies from 7 to 20 μg/ml [1].

The pattern of dots obtained after two-dimensional electrophoresis indicates that the NAMLAA contains variable amounts of charged groups. It was shown that NAMLAA is a glycoprotein because the molecular mass decreased after incubating the enzyme with N-glycosidase F. This was confirmed by experiments where to some extent NAMLAA activity bound to Sambuccus Nigra Agglutinin (SNA) coupled to sepharose beads (results not shown). SNA has a specific affinity for α-NeuNAc [2-6] GalNAc [17] which is a charged group.

Enzymatic activity of the enzyme is not restricted to peptidoglycan monomers. After lysozyme degradation, polymers with molecular mass greater than 10^6 Da from *E. coli* and *E. aerofaciens* were degraded for more than 80% depending on NAMLAA concentration and reaction time. The composition of the peptide side chains of these substrates differ widely starting from the third aminoacid, counted from MurNAc (Table 3). Therefore it seems obvious that the first three aminoacids together with N-acetylmuramic acid are the most important in determining substrate specificity. After alkali hydrolysis of the N-acetylgroup of N-acetylmuramic acid, the remaining glycopeptide is no longer degradable by NAMLAA.

The six purified monoclonal antibodies against NAMLAA all have different affinities for amidase. 3 out of 6 recognize NAMLAA in Western blot (data not shown). Flow-cytometric analysis of blood using a FITC-labeled monoclonal antibody against NAMLAA showed that granulocytes contain NAMLAA. This to our knowledge novel finding makes it very likely that the enzyme is involved in degrading peptidoglycan products in blood and tissues, which could be liberated during infections or absorbed from the gut. The monoclonal antibodies are currently being used to screen a human cDNA expression library in order to identify the gene coding for the enzyme.

Peptidoglycan is a polymer found in all bacterial cell walls. Biological activities of peptidoglycan are multifold. It has many biological effects in

common with LPS but it is also able to cause chronic inflammation [18]. Peptidoglycan monomer anhydromuramyl dipeptide possesses sleep-inducing capacity [19]. All these biological activities of peptidoglycan monomers/polymers are likely to be susceptible to degradation by amidase. Further research will be done to study the influence of amidase on the biological activities of peptidoglycan. The relatively simple method described in this paper for obtaining large amounts of pure NAMLAA will make it possible to study this human enzyme in greater detail.

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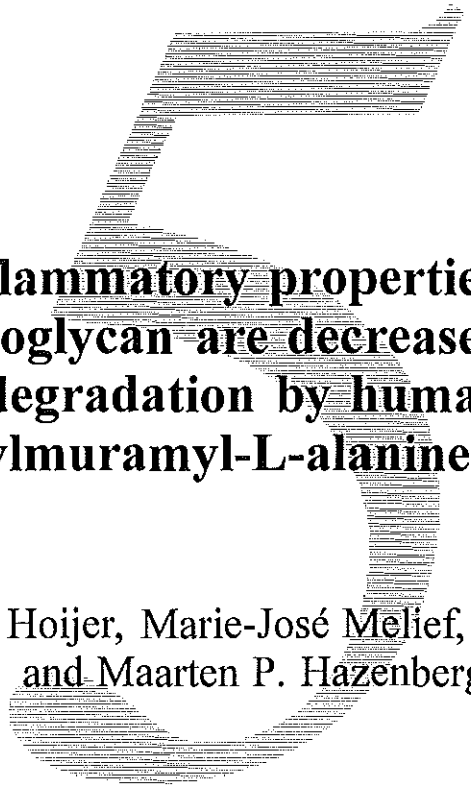

Inflammatory properties of peptidoglycan are decreased after degradation by human N-acetylmuramyl-L-alanine amidase

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ABSTRACT

N-Acetylmuramyl-L-alanine amidase (EC 3.5.1.28) purified from human plasma, cleaves the amide bond between N-acetyl muramic acid and L-alanine in the peptide side chain of peptidoglycan, an important component of bacterial cell walls. In humans, peptidoglycan can be degraded by lysozyme, N-acetyl-glucosaminidase and N-acetylmuramyl-L-alanine amidase. The biological activity of different peptidoglycan digests was investigated using two properties of peptidoglycan: the capability to induce arthritis in Lewis rats and the capability to induce the release of the inflammatory cytokines IL-1, IL-6 and TNF-α in vivo and in vitro. The results show that only after lysozyme and amidase treatment the peptidoglycan from *Eubacterium aerofaciens* was unable to induce arthritis in Lewis rats. IL-1, IL-6 and TNF-α production in mice after intravenous injection of cell wall fragments was lower after in vitro degradation of the cell wall fragments by amidase. These in vivo results were confirmed in human monocyte cultures and whole blood assays.

We postulate that degradation of peptidoglycan by N-acetylmuramyl-L-alanine amidase is an important mechanism for inactivation of inflammatory peptidoglycan products.

INTRODUCTION

The presence of N-acetylmuramyl-L-alanine amidase (NAMLAA) in human serum was first described by Ladešić et al. [25] who showed that the enzyme degrades peptidoglycan, a main component of bacterial cell walls, by hydrolyzing the lactyl bond connecting the glycan strands with the peptide side chain (see Fig. 1). Later, the presence of NAMLAA in human serum was reported by only 3 other groups [39,38,32]. We recently purified NAMLAA from human plasma and found it to be present in human granulocytes but not in monocytes [15]. The biological function of this enzyme was never investigated but might be important because of the potent biological and immunological effects of peptidoglycan in vivo, including sleep induction [17,18,29], complement activation [42], adjuvant activity [19], activation of macrophages [10] and T cell mediated arthritis induction [36,35,34,24].

When undergoing an infection our body is exposed to bacterial cell wall products including peptidoglycan. However, also under normal conditions peptidoglycan can be found in human tissues. In a previous study we were able to isolate macromolecular peptidoglycan from human spleen [14]. The results

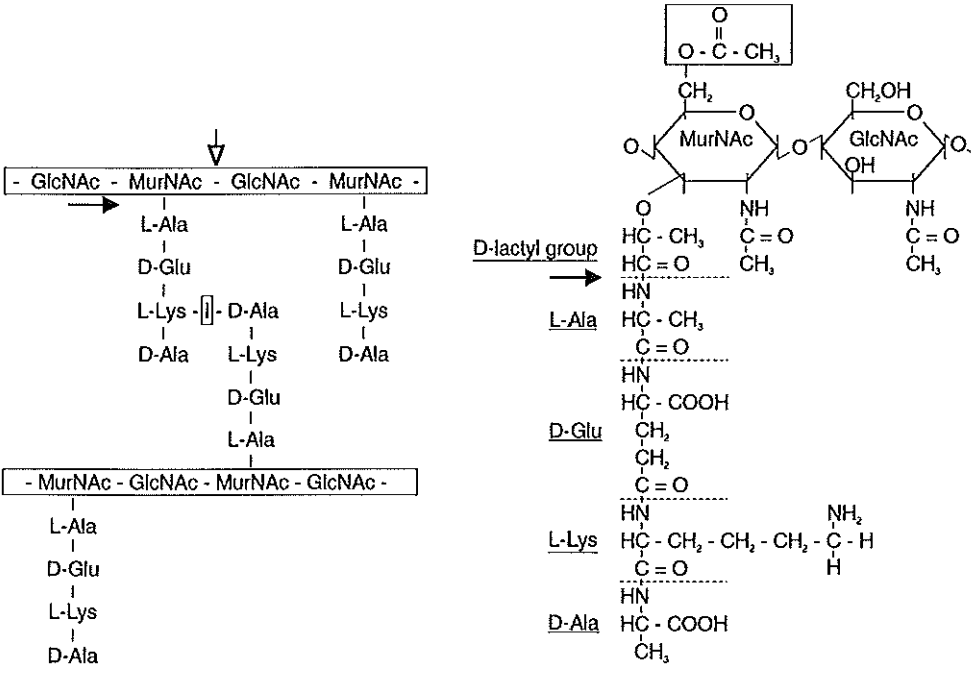

Figure 1. Simplified representation of the different peptidoglycan subunits. Alternating N-acetyl muramic acid (MurNAc) and N-acetyl glucosamine (GlcNAc) form long sugar chains interlinked by peptides, resulting in a large high molecular weight network. N-acetylmuramyl-L-alanine amidase (NAMLAA) hydrolyses the lactate bond between MurNAc and the first amino acid of the peptidoglycan chain L-Alanine (——>). Lysozyme hydrolyses the bond between MurNAc and GlucNAc (——▸). I = interpeptide bridge which may vary between microorganisms.

were confirmed by immunohistochemical studies, which also showed the presence of peptidoglycan in other tissues [31]. Lehtonen et al. [28] found muramic acid, a characteristic aminosugar of peptidoglycan, in human peripheral blood leukocytes. These results suggest that our body tissues are continuously exposed to these bacterial products. It is therefore important to investigate how these products are eliminated and loose their inflammatory properties.

In humans, peptidoglycan can be degraded by 3 different enzymes: lysozyme [20], β-N-acetylglucosaminidase [37,41] and the relatively unknown NAMLAA. We hypothesize that NAMLAA plays an important role in the degradation and inactivation of inflammatory peptidoglycan present in human tissues. To investigate this hypothesis we analyzed the effect of peptidoglycan degradation by NAMLAA in different in vivo and in vitro assays.

The source of peptidoglycan was a preparation of cell wall fragments from *Eubacterium aerofaciens* (ECWF) a major resident of the human intestinal

flora. In previous studies with Lewis rats we showed that ECWF induced severe chronic joint inflammation after a single intraperitoneal injection in PBS but also as an emulsion in oil (incomplete Freund adjuvant) [24]. The latter model was used in the present study to test the arthritogenicity of ECWF after solubilization with lysozyme and subsequent incubation with NAMLAA. The same preparations were also used for induction of TNF-α, IL-1 and IL-6 in vivo and in vitro. The inflammatory cytokines [2] were used as a measure for activity of peptidoglycan digests. The results show the effect of NAMLAA on the cytokine and arthritis inducing capacity of ECWF.

MATERIALS AND METHODS

Peptidoglycan degrading enzymes

N-acetylmuramyl-L-alanine amidase (46 U/mg) was purified from human plasma using an affinity chromatography column prepared with a monoclonal antibody against the enzyme, as was previously described [15]. In short: Monoclonal antibody AAA4 raised against NAMLAA, was coupled to CNBr-activated Sepharose 4B beads (Pharmacia LKB Biotechnology, Uppsala, Sweden) according to the protocol of the supplier. Human plasma (obtained from Red Cross Blood Center, Rotterdam) was dialyzed against PBS and diluted 2 times in PBS before it was loaded onto the column. The column was washed with PBS until no more protein was detected in the eluate. NAMLAA was eluted from the column by changing the buffer to 0.1 M Glycine·HCl, pH 3.0. The peak fractions were immediately dialyzed against 20 mM NH_4HCO_3 pH 8.0. Egg white lysozyme was obtained from Sigma, St Louis, MO)

Preparation of cell wall fragments (ECWF)

Eubacterium aerofaciens ATCC 25986 was obtained from the American Type Culture Collection (Rockville, MD) and was cultured overnight at 37°C on Schaedler broth (Oxoid Ltd., London, UK) under strict anaerobic conditions. ECWF were prepared according to Severijnen et al. [36] with slight modifications. Cells were harvested, washed and subsequently fragmented with glass beads in a Braun shaker (Melsungen, FRG). Cell walls were collected by 10,000 xg centrifugation, treated with ribonuclease and trypsin, washed and sonicated (MSE, Crawley, UK) for 75 minutes. The sonicated cell wall suspension was centrifuged 30 minutes at 1000 xg. The supernatant was centrifuged at 100,000 xg for 60 minutes. The pellet was collected, resuspended, dialyzed against water (milli-Q, Millpore) and subsequently lyophilized. All ECWF preparations were tested for endotoxin contamination by the Limulus Amoebocyte Lysate test at a sensitivity of 2 pg/ml. Even in the highest concentration used, the endotoxin concentration was lower than 2 pg/ml. Teichoic acid concentrations were measured by detecting phosphorus using the method of Chen et al. [4]. The cell wall preparation contained 0.5% phosphor (dry weight) corresponding with about 2 to 3% teichoic acids.

Lysozyme and NAMLAA digestion of ECWF

For the arthritis induction in rats and cytokine induction in mice the solubilized fraction of ECWF after lysozyme treatment was used. 10 mg ECWF and 1 mg lysozyme in 10 mM sodium acetate was incubated during 24 h at 37°C and centrifuged at 100,000 xg. The superna-

tant was dialyzed against milli-Q water and lyophilized. This preparation is further referred to as ECWF+L^{sol}. For in vitro cytokine induction 10 mg ECWF was incubated with 1 mg lysozyme in 5 mM sodium acetate for 24 h at 37°C. Without further centrifugation and dialysis this preparation is further referred to as ECWF+L.

NAMLAA digestion of ECWF was carried out by incubating 5 mg/ml ECWF+L^{sol} or ECWF+L with 25 µg/ml NAMLAA in 20 mM NH_4HCO_3 for 24 h at 37°C. These preparations are further referred to as ECWF+L^{sol}+A and ECWF+L+A.

Determination of N-acetyl muramic acid (MurNAc)

MurNAc was determined according to the method of Hadzija [11] with some modifications [12]. In short: samples were hydrolyzed by heating for 3 h at 90°C with an equal volume of 5 M H_2SO_4, then neutralized with 10 M NaOH. Hydrolyzed and unhydrolyzed samples (100 µl) were incubated with 50 µl 1 M NaOH at 36°C for 30 min. After the addition of 1 ml 18.8 M H_2SO_4 (concentrated), samples were heated for 3.5 min at 100°C, rapidly cooled in ice, then mixed with 10 µl 0.16 M $CuSO_4.5H_2O$ in H_2O and 20 µl 0.09 M *p*-hydroxydiphenyl in ethanol. The blue color developed to a maximum in 30 min at 30°C. Absorbance at 570 nm was determined using a Titertek Multiskan (Flow Laboratories, Irvine, Scotland). Solutions containing 0-100 µg MurNAc (Sigma) per ml H_2O were used as standards. The data are given as the concentration in the hydrolyzed and non-hydrolyzed samples. Subtraction of the values represent the amount of MurNAc linked to the peptides (net). MurNAc values were corrected for the presence of pentoses (rhamnose) using the method of Dische and Shettles [6]. In ECWF rhamnose is responsible for approximately 20% of the measured MurNAc concentration.

Animals and induction of arthritis

Female Lewis rats (Harlan Sprague Dawley, Bicester, Oxfordshire, UK) 7-8 weeks of age were injected subcutaneously in the base of the tail with 1 mg lyophilized ECWF digested with lysozyme, NAMLAA or both, adequately ground in 100 µl of Freund's incomplete adjuvant (Difco Laboratories, Detroit, MI) using a homogenizer (X 10/20, Ystral, ballrechten-Dottingen, FRG). Freund's incomplete adjuvant was used as a negative control. Rats were observed and paw diameters were measured for at least 100 days, twice a week. When the paw diameters increased compared to the diameters measured before the treatment and compared to the negative controle rats, the animal was counted positive. The arthritis was confirmed by histology as was described by Kool et al. [24].

Induction of cytokine release in BALB/c mice

ECWF after lysozyme solubilization (ECWF+L^{sol}) and lysozyme + NAMLAA (ECWF+L^{sol}+A) solubilization were diluted in sterile PBS to a final concentration of 0.2 mg/ml. 0.5 ml was injected in a vein of the tail. There were 4 groups: mice injected with ECWF+L^{sol}, ECWF+L^{sol}+A, LPS (0.2 µg/ml) and PBS. One hour and two hours after injection, 3 mice per group were killed by CO_2, the blood was recovered by heart puncture and collected in a tube containing sodium citrate. The blood samples were immediately put on ice and after centrifugation at 4°C the plasma was used for detection of cytokines.

Induction of cytokine release by human peripheral blood monocytes

Human peripheral blood mononuclear cells were isolated by the method of Böyum from blood freshly obtained from healthy donors [3]. The blood was collected in sodium-citrate containing tubes (Vacuette, Greiner, Kremsmuenster, Ostria). After 1:1 dilution in sterile PBS the

cells were separated by ficoll density centrifugation, 15 minutes, 1000 xg (Ficoll paque, 1.077 g/ml, Pharmacia). The interphase was diluted in PBS and centrifuged for 5 min. 400 xg. The pellet was then resuspended in Medium 199 containing glutamax (GIBCO, Breda, the Netherlands). Cells were counted and medium was added until a final concentration of 3 x 10^6 cells/ml.

The cells were transferred to flat-bottom tissue culture plates with 96 wells (Falcon, Lincoln Park, NY), 100 µl per well and then incubated for 1 hour at 37°C in a 5% CO_2 atmosphere in order to let the cells adhere. Non adherent cells were aspirated and 100 µl ECWF preparations or LPS (E. coli 026:B6, Difco Laboratories) dilutions were added to the remaining monocytes in triplicate. All ECWF dilutions were made in Medium 199 containing 10 µg/ml polymyxin B (Sigma). As a control the monocytes were incubated with medium alone. At maximum stimulation after 6 hours the supernatants were frozen to be tested later in bioassays for IL-1, IL-6 and TNF-α.

Induction of cytokine release in whole blood

Human peripheral blood was obtained from healthy donors and collected in sodium-heparin tubes (Vacutainer, Becton Dickinson). 12.5 µl of Medium 199 containing 100 µg/ml polymyxin B and the different ECWF preparations or LPS dilutions were transferred to a polypropylene tube (Falcon) containing 122.5 µl blood. The mixtures were incubated for 5 hours at 37°C in a 5% CO_2 atmosphere. Then 125 µl RPMI (1640 GIBCO) was added to each tube and the mixtures were centrifuged for 10 min, 400 xg. The obtained supernatants were tested in bioassays for IL-1, IL-6 and TNF-α activity.

Bioassays for cytokines

TNF-α bioactivity was measured using the murine fibroblast cell line WEHI 164.13 [8]. The cells were plated in flat-bottom tissue culture plates with 96 wells (Costar, Cambridge, MA) at a concentration of 1 x 10^5 cells/well. The cells were allowed to adhere by incubating them overnight at 37°C in a 5% CO_2 atmosphere. At day two 50 µl of Actinomycine-D (Sigma) 4 µg/ml and 50 µl diluted sample were added to the cells. Samples were tested in triplicate. After an overnight incubation (37°C, 5% CO_2) the MTT cytotoxicity test [33] was used to measure WEHI cell viability.

IL-1 bioactivity was measured using a sub line of the murine T cell line D10.G4.1, designated D10(N4)M using the method described by Hopkins and Humphreys [16] with some modifications [5].

IL-6 activity was detected by using the murine hybridoma cell line B9 according to Aarden et al. [1]. Proliferation of D10(N4)M and B9 cells was measured by ^{3}H-thymidine incorporation. Recombinant human IL-1ß (UBI, Lake placid, NY), IL-6 (Prof. Dr. L.A. Aarden, CLB, Amstedam, The Netherlands) and TNF-α (UBI) served as positive controls for the D10, B9 end WEHI assays. Cytokine activities of the samples were corrected for background activity of the culture medium and expressed as U/ml with 1 U/ml corresponding with half-maximal response.

RESULTS

N-acetylmuramic acid (MurNAc) in ECWF preparations after incubation with lysozyme and NAMLAA

After incubation of ECWF with lysozyme and NAMLAA the concentration of MurNAc was determined with and without hydrolysis (see M&M). The results are presented in Table 1. The difference found with and without hydrolysis corresponds to the amount of peptides still connected to muramic acid via the D-lactate group (see Fig. 1). The data show that incubation of ECWF with lysozyme did not change the concentration of MurNAc linked to the peptides in the ECWF+L nor in the centrifuged and subsequently dialyzed ECWF+L^{sol} preparations.

Further treatment with NAMLAA resulted in an increase of MurNAc found without hydrolysis due to the liberation of peptide from the D-lactate group of MurNAc. The amount of intact peptidoglycan was therefore reduced (expressed as net).

Table 1. N-acetyl muramic acid (MurNAc) in Eubacterium aerofaciens cell wall fragment (ECWF) preparations incubated with lysozyme (L) and NAMLAA (A) with and without hydrolysis

	µg MurNAc*		
	hydrolysis	no hydrolysis	net**
ECWF	130	22	108
ECWF+L^{sol}	155	45	110
ECWF+L^{sol}+A	157	106	51
ECWF+L	169	32	137
ECWF+L+A	168	112	56

* Expressed as µg MurNAc per mg dryweight. Corrected for the presence of pentoses.

** Net value is a measure of the amount of peptides still linked to MurNAc sol: supernatant after centrifugation.

Influence of lysozyme and NAMLAA digestion on arthritogenicity of ECWF

Severe chronic polyarthritis was induced in Lewis rats by subcutaneous injections of ECWF in oil. 32 out of 56 rats developed arthritis. The onset was 3 to 6 weeks post injection and the arthritis lasted for the entire observation period of 10 weeks. The arthritis was confirmed by histological examination of the affected joints. The lysozyme treated fraction of ECWF (ECWF+L^{sol}) was

also tested for arthritogenicity. 15 out of 50 rats developed arthritis. The onset, chronicity and histological characteristics were similar to those observed for rats injected with ECWF. The incidence was significantly lower than with untreated ECWF ($p<0.05$). ECWF+L was further treated with purified NAM-LAA resulting in a 50% decrease of peptides still linked to muramic acid. This preparation was not capable of inducing arthritis in 19 rats ($p<0.01$). This means that there was no increase in paw diameter measurable.

Table 2. The average cytokine production[#] in the blood of mice injected with lysozyme solubilized *Eubacterium aerofaciens* cell wall fragments before (ECWF+L^{sol}) and after (ECWF+L^{sol}+A) amidase treatment, 1 hour and 2 hours after iv injection

		1 hour		
	ECWF+L^{sol}	ECWF+L^{sol}+A	P[*]	LPS
TNF-α	310 ± 321	58.2 ± 15.5	0.29	1490 ± 733
IL-1	ND	ND	-	15.1 ± 6.4
IL-6	5.6 ± 1.2	2.6 ± 2.1	0.03[**]	4.7 ± 0.2
		2 hours		
TNF-α	44.7 ± 12.1	16.8 ± 11.0	0.06[**]	145 ± 125
IL-1	ND	ND	-	30.7 ± 8.1
IL-6	2.2 ± 0.8	0.7 ± 0.3	0.06[**]	3.5 ± 0.3

[#] U/ml ± SD; 3 mice per group.
[*] The two-tailed p value determined with paired t-test.
[**] Not significant according to two-tailed p value calculated with the paired wilcoxan signed rank test.
ND = Not detectable (< 0.02 U/ml).

Induction of TNF-α, IL-1 and IL-6 release in BALB/c mice

Table 2 shows the cytokines released in the blood of BALB/c mice upon injection of lysozyme and NAMLAA digested ECWF. After degradation of the lysozyme solubilized ECWF by purified NAMLAA (ECWF+L^{sol}+A), the capability of the preparation to induce TNF-α and IL-6 release was 2-3 times lower than after degradation by lysozyme alone. The decrease in TNF-α release 1 hr after injection was not statistically significant, so was the decrease measured after 2 hrs ($p=0.062$). The trend, however, was consistent and similar results were obtained with IL-6. The decrease in IL-6 release 1 hr after injection was significant ($p=0.033$), but at 2 hours after injection the decrease was no longer significant ($p=0.056$).

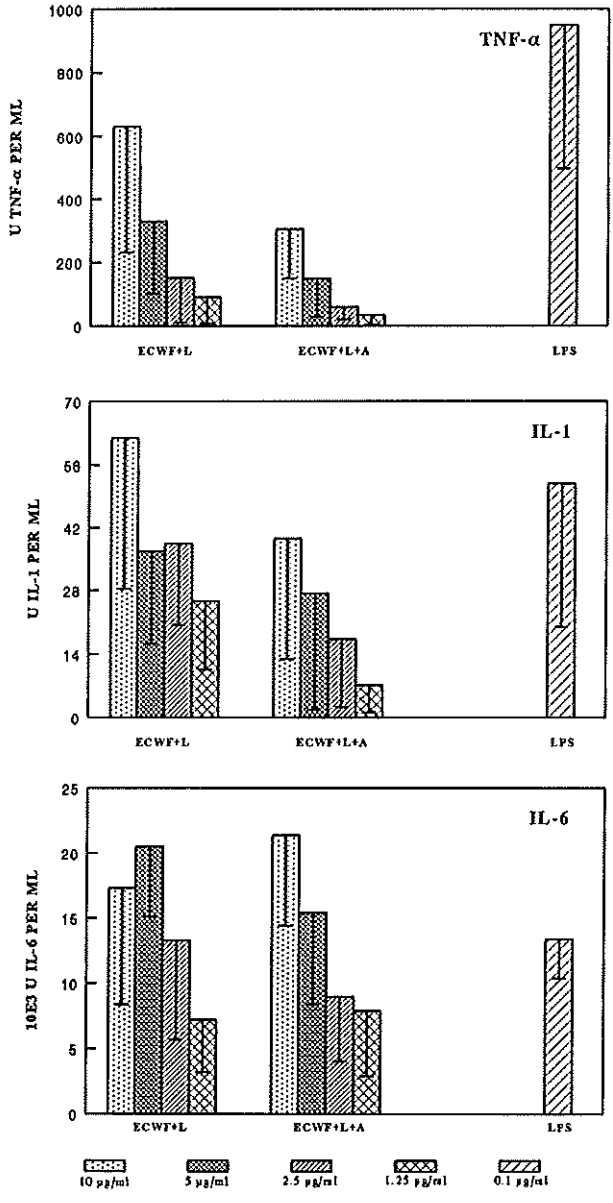

Figure 2. Induction of TNF-α, IL-1 and IL-6 ex vivo, after stimulation of whole blood with lysozyme treated and lysozyme plus NAMLAA treated (ECWF+L and ECWF+L+A) *Eubacterium aerofaciens* cell wall fragments in four different concentrations based on muramic acid contents. Values represent the mean of 10 independent experiments using 10 different blood donors (± SD).

Table 3. The average cytokine concentration[#] after stimulation of PBMC with 2.5 μg lysozyme treated *Eubacterium aerofaciens* cell wall fragments before (ECWF+L) and after (ECWF+L+A) NAMLAA treatment

	ECWF+L	ECWF+L+A	P*
TNF-α	6.3 ± 2.3	3.4 ± 1.7	0.06
IL-1	24.0 ± 48.6	9.1 ± 12.1	0.35
IL-6	729 ± 592	455 ± 520	0.12

[#] U/ml ± SD; 10 samples per group.
* The two-tailed p value determined with paired t-test.

The effect of NAMLAA treatment on the IL-1 levels was not clear. In the ECWF+L and ECWF+L+A stimulated mice, biologically active IL-1 was undetectable (<0.02 U/ml). However, the negative control and in the LPS stimulated samples contained detectable IL-1 levels. The results indicate that the peptidoglycan constituent of the cell wall fragments, the only moiety that is affected by NAMLAA, is responsible for the cytokine release. It further supports the hypothesis that NAMLAA degradation is an important mechanism for inactivating the inflammatory properties of peptidoglycan products.

Induction of TNF-α, IL-1 and IL-6 release in vitro using whole blood or peripheral blood mononuclear cells

After the hydrolysis of lysozyme treated ECWF by NAMLAA the capability of the product to induce cytokine production in whole blood was reduced. The values shown in Fig. 2 are the mean of 10 independent experiments using 10 different human blood samples from various healthy donors. Table 3 shows the results after stimulating with 5 μg/ml ECWF+L and ECWF+L+A. The two-tailed p values, calculated with the paired t-test, were significant for all cytokines ($p<0.01$)

After stimulation of the adherent peripheral blood monocytes, the values between 10 independent experiments differred a lot from each other due to the high variety between the donors. Therefore only one representative experiment is shown in Fig. 3. In table 4 the average cytokine production is given of PBMC stimulated with 2.5 μg/ml ECWF+L and ECWF+L+A. The differences in TNF-α, IL-1 and IL-6 production were not significant, although the trend was consistent in all experiments. In both assay systems all the blood samples from the 10 different healthy donors showed a reduced cytokine release when the ECWF preparation was hydrolysed by NAMLAA.

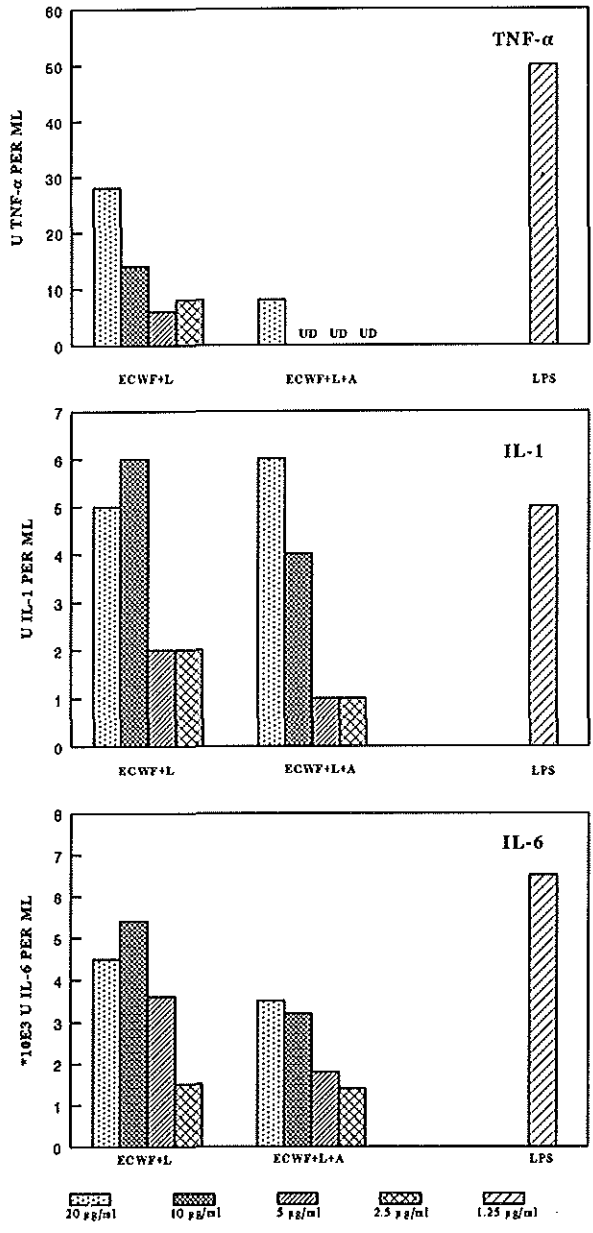

Figure 3. Induction of TNF-α, IL-1 and IL-6 release after stimulation of peripheral blood monocytes. The same stimuli were used as in Fig. 2. One representative experiment is shown from a total of 10 experiments.

Table 4. The average cytokine concentration[#] after stimulation of whole blood with 5 μg lysozyme treated *Eubacterium aerofaciens* cell wall fragments before (ECWF+L) and after (ECWF+L+A) NAMLAA treatment

	ECWF+L	ECWF+L+A	p*
TNF-α	330 ± 228	150 ± 121	0.0019
IL-1	36.4 ± 20.8	19.8 ± 12.8	0.0077
IL-6	20.5 ± 7.3	15.4 ± 5.4	0.0012

[#] U/ml ± SD; 10 samples per group.

DISCUSSION

To our knowledge these are the first results on the biological activity of NAMLAA showing that degradation of peptidoglycan by this enzyme is an important mechanism for inactivation of inflammatory peptidoglycan products.

NAMLAA purified from human serum hydrolysed 50% of the lactyl-peptide bonds from the peptidoglycan in ECWF after this had been incubated with lysozyme. Preincubation with lysozyme was necessary because NAMLAA was not able to use the non-soluble ECWF as a substrate (unpublished results). Previous studies showed that ECWF was arthropathic in Lewis rats using either the intraperitoneal route [36] or ground in oil and injected in the base of the tail [23]. However, Fox et al. [9] showed that the soluble fraction (after 100,000 xg centrifugation) of streptococcal cell wall fragments did not induce arthritis in rats after intraperitoneal injection. Therefore the adjuvant route was used in this study.

ECWF did not loose its arthropathic properties after solubilization by lysozyme although the incidence was lower. Additional NAMLAA degradation did abolish the arthritis inducing capacity of this preparation. Whether this is due to the decreased molecular weight or to the destruction of specific inflammatory epitopes like the muramyl-peptide moiety, needs to be further investigated. Studies of Kohashi et al. [21,22] showed that even very small subunits of peptidoglycan were able to induce arthritis in the adjuvant model provided that they contained intact muramyl-peptide subunits. This might indicate that it is a prerequisite for arthritis induction that the peptide is linked to the MurNAc-GlucNAc sugar chain.

The cooperative action of lysozyme and NAMLAA is important to completely abolish the arthitogenic capacity of the cell wall fragments in the arthritis model used. This is consistent with the finding that ECWF can only be

degraded by NAMLAA after it has been solubilized by lysozyme.

Intravenous injection of the solubilized fraction of ECWF in BALB/c mice resulted in an immediate cytokine release. LPS was a much more potent stimulator than the cell wall preparations. After ECWF stimulation no IL-1 was detectable although after stimulation with LPS a high IL-1 response was detected comparable to the responses described by Zanetti et al. [43]. They found that injection of living Gram-negative bacteria resulted in high levels of IL-1. We do not know why no biologically active IL-1 could be detected after stimulation with ECWF+L^{sol}. It might be due to the production of IL-1 soluble receptors and/or IL-1 receptor antagonist, interfering with the bioassay. This effect was not found when the whole blood assay was used.

Stimulation of PBMC or whole blood with lysozyme treated cell wall fragments from *E. aerofaciens* (ECWF +L) resulted in the induction of TNF-α, IL-1 and IL-6 comparable with the results described by Mattsson et al. [30], who stimulated peripheral blood monocytes with peptidoglycan from *Staphylococcus epidermidis*, and Heumann et al. [13] who used 10 different Gram-positive cell wall preparations for stimulation of TNF-α production by human monocytes. The concentrations of peptidoglycan used in the in vivo and in vitro studies were physiological since Lehtonen et al. [27] found in synovial fluids of patients with reactive arthritis 0.2 - 2 μg muramic acid per ml synovial fluid, corresponding with 1 -10 μg peptidoglycan per ml. This is in the same range as the MurNAc content of the preparations used for the experiments described in this paper.

NAMLAA acts specifically on peptidoglycan. Therefore the reduction in cytokine induction after degradation by NAMLAA demonstrates that the peptidoglycan in the preparations used is primarily responsible for the cytokine release and arthritis induction. The remaining activity of the NAMLAA degraded cell wall preparations may be explained by the fact that they still contain low concentrations of teichoic acids which are able to induce cytokine release [30] and/or because they contain undigested peptidoglycan, caused by modifications which make peptidoglycan partly undegradable by lysozyme [26] and NAMLAA [15,40].

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Localization of the human N-acetylmuramyl-L-alanine amidase; a bacterial cell wall degrading enzyme

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SUMMARY

N-acetylmuramyl-L-alanine amidase (NAMLAA) specifically hydrolyzes the lactamide bond that links N-acetylmuramic acid, in the polysaccharide chain of peptidoglycan, with L-alanine of the peptide side chain. Peptidoglycan is a bacterial cell wall product with strong inflammatory properties. For instance, peptidoglycan is capable of stimulating peripheral blood cells to release pro-inflammatory cytokines and is capable of inducing chronic arthritis in an animal model. In a previous study we found that degradation of peptidoglycan by purified NAMLAA reduced its inflammatory effects. To determine where NAMLAA is located in tissues, monoclonal antibodies against purified NAMLAA were produced for use in immunohistochemistry, immunoelectron microscopy, flow cytometric analysis and Western blotting.

The immunohistochemical studies revealed NAMLAA-positive cells in human spleen, liver, arthritic synovial tissues and in lymph nodes. In flow cytometric studies of blood and bone marrow, neutrophilic and eosinophilic granulocytes proved to be positive. Monocytes were negative although they do contain lysozyme, the other important peptidoglycan-degrading enzyme. However, mature macrophages obtained by bronchoalveolar lavage and subsequent selection based on auto-fluorescence did possess NAMLAA. In immunocytochemical staining of blood smears, thrombocytes were also positive for NAMLAA. Western blot analysis and immunoelectron microscopy of neutrophils and eosinophils showed that NAMLAA is located in azurophilic granules of neutrophils and in secretion vesicles and crystalloid-containing granules of eosinophils.

Flow cytometric analysis of blood and bone marrow from different FAB-classified acute myeloid leukemia (AML) patients showed that AML-M2 myeloblasts were the first in the granulocyte maturation lineage that were positive for NAMLAA. The more immature AML such as AML-M0 and AML-M1, did not express NAMLAA. CD15 and CD13-negative megakaryoblasts, corresponding to AML-M7, were also positive for NAMLAA.

INTRODUCTION

In human sera N-acetylmuramyl-L-alanine amidase (NAMLAA) activity was reported for the first time by Ladešić et al. [1]. This relatively unknown human enzyme degrades peptidoglycan, a major component of most bacterial cell walls, by hydrolysis of the lactyl bond that connects N-acetylmuramic acid in the glycan strands with L-alanine of the peptide side chain [2]. In a previous study we

described the purification of NAMLAA from human plasma, the preparation of monoclonal antibodies against it and the properties of this enzyme [3].

Peptidoglycan is present in the cell walls of almost all bacteria and is the major constituent of the cell walls of Gram-positive bacteria. The inflammatory properties of peptidoglycan in vivo include complement activation [4], arthritis induction [5], and activation of monocytes and macrophages resulting in the release of inflammatory cytokines [6,7].

Following the lysis of bacteria during an infection, cell wall fragments containing peptidoglycan are rapidly cleared through the action of phagocytic cells and their hydrolytic enzymes [8]. Lysozyme is thought to be the most important enzyme for the inactivation of peptidoglycan, degrades the sugar backbone of peptidoglycan by hydrolysing the bond between N-acetylglucosamine and N-acetylmuramic acid. However, in previous studies we and others showed that cell wall fragments from Gram-positive bacteria still possessed most of their inflammatory properties after degradation by hen egg-white lysozyme [9,10], but lost most of their cytokine and arthritis-inducing capacity after degradation by NAMLAA [11]. This indicates that the presence of lysozyme alone is not sufficient for complete inactivation of the inflammatory properties of peptidoglycan.

The presence of a peptidoglycan-degrading enzyme other than lysozyme in phagocytic cells is therefore required for the complete processing of peptidoglycan. Apart from lysozyme, N-acetylglucosaminidase was reported to be present in granulocytes and was shown to hydrolyze the N-acetylglucosamine group from the non-reducing end of the glycan chains [12], although its primary function is the degradation of oligosaccharides containing ß-glucosaminide linkages, present on many glycoproteins [13]. Whether the degradation by N-acetylglucosaminidase could have an effect on the inflammatory properties of peptidoglycan has not been investigated yet. It is, however, reasonable to assume that this enzyme has very little effect on these properties because of its limited enzymatic activity. Therefore the presence of NAMLAA in phagocytic cells might well complement the hydrolytic actions of lysozyme.

In the present study, monoclonal antibodies raised against human NAMLAA were used for immunohistochemistry, electron microscopy, flow cytometric analysis and Western blotting to determine the localization of NAMLAA in human tissues. Blood and bone marrow cells from healthy donors and from acute myeloid leukemic patients were analyzed to determine in which stages of myeloid differentiation NAMLAA is expressed. Bronchoalveolar lavage samples were used as a source of mature macrophages.

MATERIALS AND METHODS

Preparation of monoclonal antibody AAA4 against NAMLAA

Purified NAMLAA was used for preparing mouse monoclonal antibodies as described previously [3]. The IgG1 monoclonal antibody AAA4 showed the highest affinity for NAMLAA and was used for conjugation with FITC (AAA4-FITC) by standard procedures [14]. In a previous study, AAA4 proved to be highly specific for NAMLAA and was used for the purification of NAMLAA in one step from human plasma [3].

Bronchoalveolar lavage

Bronchoalveolar lavage samples, generously supplied by the Department of Pulmonary medicine, University Hospital Rotterdam, the Netherlands, were obtained as follows. After informed consent, bronchoalveolar lavage was performed on individuals during anesthesia for surgery. The lavage was performed with a flexible bronchoscope placed in wedge position in the right middle lobe. Four aliquots of 50 ml of saline were subsequently instilled and aspirated. The obtained fluid was collected in siliconized bottles.

The cells in the lavage fluid were kept at 4°C, washed twice in phosphate-buffered saline (PBS) containing 0.5% (w/v) bovine serum albumin (BSA) and 0.45% (w/v) glucose. The cells were then filtered through a 100-μm and a 30-μm gauze and subsequently sorted on a FACS-Vantage (Becton Dickinson, Erembodegem, Belgium) with a laser operating at 488 nm. The highly autofluorescent (530 nm) population was used to prepare cytospins and was shown to contain 100% alveolar macrophages. The cells were stained with AAA4 as described under immunocytochemistry (see below).

Immunocytochemistry

For the preparation of smears, blood and small bone marrow samples were obtained after informed consent from a healthy donor by the Department of Hematology, Erasmus University Rotterdam. The smears were fixed and permeabilized by incubation for 10 minutes in acetone. The smears were then rinsed in PBS containing 0.2% BSA and incubated for 1 hour at room temperature with 70 μl of AAA4 (diluted 40 μg/ml in PBS-BSA) or, for control staining, with an irrelevant monoclonal antibody of the same isotype and concentration. Subsequently, the smears were rinsed in PBS-BSA and incubated for 30 minutes at room temperature with rabbit-anti-mouse immunoglobulin (Z259; DAKO, Glostrup, Denmark) diluted 1:20 in PBS-BSA with 1% normal human serum. After rinsing in PBS-BSA, a 1:40 dilution of alkaline phosphatase-mouse-anti-alkaline phosphatase complex (APAAP, D651; DAKO) was applied for 30 minutes. To develop the stain, the smears were incubated for 30 minutes at room temperature with 0.012% (w/v) naphthol-ASMX phosphate, 0.025% (w/v) fast blue BB base and 0.025% (w/v) levamisol (all from Sigma, St. Louis, MO), which stains positive cells blue. Granulocytes were easily identified by the negatively-stained nucleus, rendering a counter stain unnecessary. Finally, the smears were mounted in Kaiser's glycerol gelatin (Merck, Darmstadt, FRG).

Immunohistochemistry

For the immunohistochemical studies, spleen tissue was obtained from the Department of Pathology immediately after surgery (SSDZ, Delft, The Netherlands). The spleen was removed for technical reasons from a patient with gastric adenocarcinoma. Liver biopsies obtained from the Department of Pathology, Erasmus University Rotterdam, were taken from an organ being prepared for transplantation. Lymph nodes were obtained from a patient undergoing surgery for tonsillitis.

Synovial tissues were obtained at the time of reconstructive surgery from the knees and hips of patients with definite or classical rheumatoid arthritis according to the American Rheumatism Association criteria.

For immunohistochemical staining with the monoclonal antibody AAA4, tissue cryostat sections were acetone-fixed as described previously [15]. Only the spleen cryostat sections were fixed with pararosaniline. The staining procedure was the same as described above for immunocytochemistry.

Immunohistochemical double staining

For double staining with two monoclonal antibodies, the tissue cryostat sections were fixed as described above. Endogenous peroxidase activity was blocked by a 10-min incubation in 0.5% H_2O_2 in PBS. The sections were washed in PBS/BSA and subsequently incubated with monoclonal AAA4 for 1 h at room temperature. As the second step, APAAP was used as described above. After incubation and washing in PBS/BSA, the sections were incubated with the second monoclonal antibody for 1 h at room temperature. The antibodies used are listed in table 1. The staining was detected with a peroxidase-conjugated rabbit anti-mouse-immunoglobulin (P161, DAKO) in a 1:250 dilution in PBS/BSA with 1% normal human serum. After a 30-min incubation and washing in PBS/BSA, the sections were incubated with the alkaline phosphatase substrate for 45 min in the dark,

Table 1. Immunohistochemical double labeling with AAA4 on human spleen

Code	Specificity	Concentration used	double labeling	Reference/source
RV202	Vimentin	1:10	-	[21]
5B5	Fibroblasts	1:20	-	DAKO
HLE-1	CD45, Leukocytes	1:100	+	BD
HLA-DR	MHC-II	1:250	-	BD
Leu4	CD3, T cells	1:100	-	BD
B4	CD19, B cells	1:100	-	Coulter Clone
Mac	Macrophages	1:500	-	DAKO
L25	Dendritic cells	1:500	-	[22]
LeuM3	CD14, Monocytes	1:100	-	BD
CD24	Granulocytes	1:100	+	Ortho Diagnostics
VIM-D5	CD15, Neutrophilic granulocytes	1:100	+	W. Knapp, Austria

followed by rinsing in PBS/BSA and 0.2 M sodium acetate buffer, pH 4.6. The peroxidase substrate 3-amino-9-ethylcarbazole was applied for 30 min. Finally, the sections were rinsed in PBS/BSA and distilled water and mounted in Kaiser's glycerol gelatin.

Flow cytometric analysis

Blood and bone marrow was sampled for diagnostic purposes from AML patients and were FAB classified [16,17]. As a control, peripheral blood and bone marrow, obtained for diagnostic purposes, from a healthy donor were used. The cells were stained for intracellular antigens according to Syrjalä [18]. In short, 100 µl of heparinized blood was incubated with the relevant conjugated antibodies for 10 minutes at room temperature. The antibodies used for flow cytometric analysis were: CD3-PerCP, CD4-FITC, CD8-PE (Becton Dickinson Immunocytometry systems, San José, CA), CD10-PE, CD14-PE (Coulter, San Fransisco, CA), CD15-PE (Immuno Quality Products, Groningen, The Netherlands) and CD19-TC (Sanbio, Uden, The Netherlands). The cells were then lysed and fixed by adding 2 ml of FACS Lysing Solution (Becton Dickinson) and incubating for 10 min at room temperature. The cells were centrifuged and washed twice with PBS + 0.5% BSA and incubated with AAA4-FITC for 10 min. Again the cells were washed twice with PBS-BSA and were finally resuspended in 200 µl of FACS Flow (Becton Dickinson). The analyses were performed with a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA).

Cryo ultramicrotomy and immunolabeling

Leukocytes from peripheral blood were fixed with a mixture of 0.5% (v/v) glutaraldehyde and 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 1 hour and were pelleted in 10% (w/v) gelatin in PBS. Ultrathin frozen sections were incubated at room temperature with AAA4 (60 µg/ml), followed by incubation with rabbit-anti-mouse-IgG (1/40) and 10-nm gold-conjugated goat-anti-rabbit-IgG (1/40). In double-labeling experiments, the cryosections were incubated first with AAA4, followed by incubation with rabbit-anti-mouse IgG and 5-nm gold-conjugated goat-anti-rabbit-IgG and then with 1% glutaraldehyde for 10 minutes. Subsequently, the sections were incubated with rabbit-anti-human myeloperoxidase (MPO, 1/1500) or rabbit anti-human lactoferrin (LF, 1/200) and anti-rabbit-IgG linked to 10-nm gold (MPO and LF were from Cappel Laboratories, Cocharanville, PA; anti-rabbit gold conjugates were from Amersham Nederland, 's-Hertogenbosch, The Netherlands). As a control, the primary antibody was replaced by a non-relevant murine or rabbit antibody. After immunolabeling, the cryosections were embedded in a mixture of methylcellulose and uranyl acetate and examined with a Philips CM10 electron microscope (Eindho-

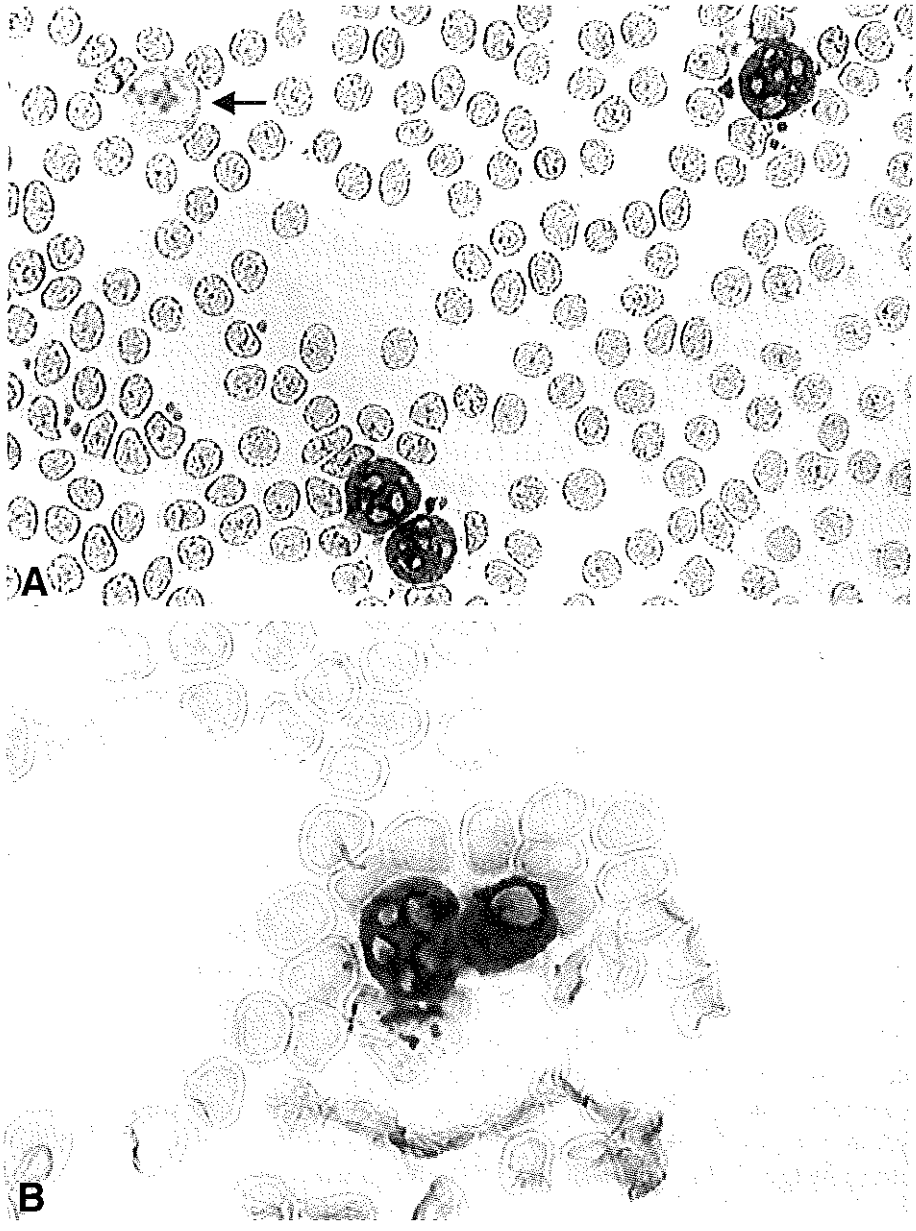


Figure 1. Immunocytochemistry of blood and bone marrow.
A). Blood smear from a healthy donor stained with MoAb AAA4, shows NAMLAA-positive granulocytes and a NAMLAA-negative monocyte. B). Bone marrow smear after staining with AAA4 shows a NAMLAA-positive promyelocyte and a mature granulocyte.

ven, The Netherlands).

Preparation of neutrophil fractions and Western blot analysis

Neutrophil fractions were isolated essentially as described by Bolscher et al. [19], except that a sucrose gradient of 15/40/52/60 % (w/v) was used to recover the plasma membrane, specific granule and azurophilic granule fractions.

The granulocyte fractions were subjected to 10% SDS-PAGE. Samples of 20 µl were boiled for 3 min with 5 µl of loading buffer (60 mM Tris·HCl pH 6.8; 23% glycerol; 3% SDS; 0.06% bromophenol blue; 10% 2-mercaptoethanol). Ten µl of these samples were analyzed on 10% SDS-PAGE (Mini Protein, BioRad, Richmond, CA). Samples were transferred to nitrocellulose in 25 mM Tris, 190 mM glycine and 20% methanol transfer buffer. Aspecific binding sites were blocked by incubating the nitrocellulose sheets in low-fat milk for 30 minutes and subsequently washing three times with 0.5% Tween-20 in PBS. The nitrocellulose sheets were then incubated for 1 hour at room temperature with monoclonal antibodies diluted 500 times in PBS-Tween. Following three washes, goat-anti-mouse-IgG conjugated to alkaline phosphatase (DAKO) was added in a 1000-times dilution, and the blots were incubated for 1 hour at room temperature. The blots were washed three times with PBS-Tween and then three times with PBS. For visualization of antibody-antigen complexes, the alkaline-phosphatase substrate nitroblue tetrazolium/5-bromo-4-chloro indoxyl phosphate (NBT/BCIP) was used as described [20].

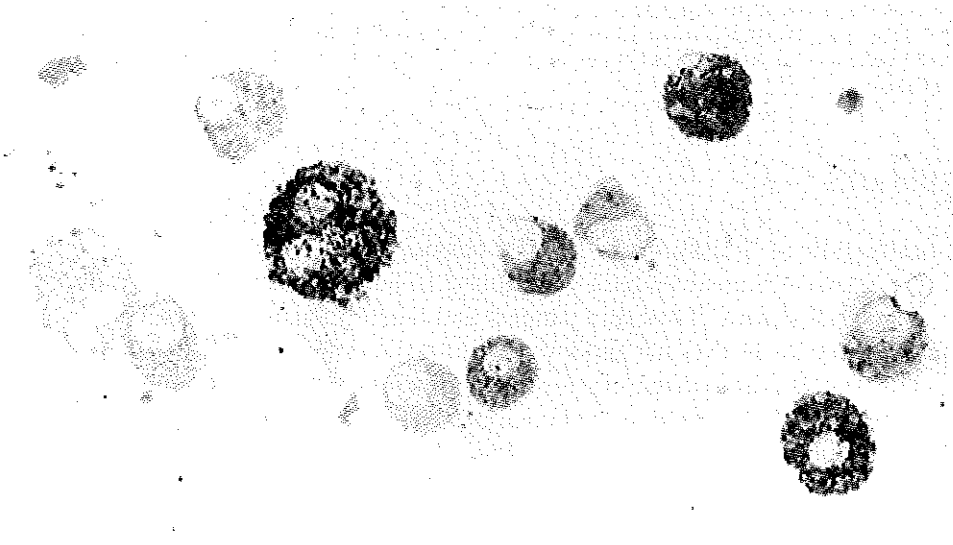


Figure 2. Bronchoalveolar macrophages, sorted for high auto-fluorescence, were stained for NAMLAA. Twenty percent of the macrophages stained positive.

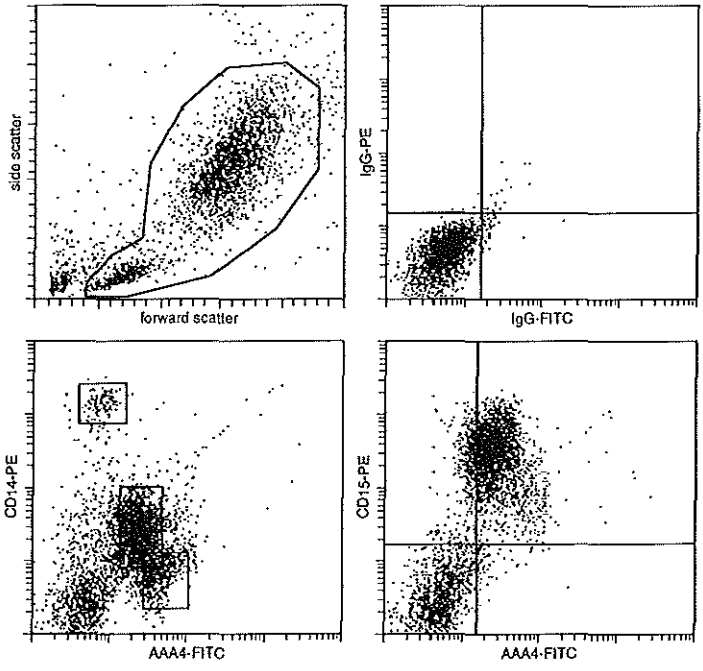


Figure 3. Flow cytometric analysis of bone marrow from a healthy donor.
Double labeling was performed with AAA4/CD14 and AAA4/CD15. The scatter morphology of the bone marrow sample and the control stainings with IgG-FITC and IgG-PE are given in respectively A) and B). Double-labeling of bone marrow cells with the NAMLAA-specific antibody AAA4-FITC and the monocyte marker CD14-PE (C) or the granulocyte marker CD15-PE (D) show that CD14-positive monocytes (Box 1 in fig. C) are negative for the NAMLAA marker AAA4, whereas the CD15-positive cells are positive for AAA4 (D). Boxes 2 and 3 in fig. C represent mature and immature granulocytes, respectively.

RESULTS

Localization of NAMLAA in human tissues

NAMLAA-positive cells were found in human spleen, liver, lymph nodes and synovial tissues of arthritis patients and healthy controls. Double staining of spleen tissue for NAMLAA and a variety of markers for fibroblasts, HLA class-II, T cells, B cells, monocytes, macrophages, dendritic cells and granulocytes (Table 1) showed double staining of granulocytes only (data not shown).

Immunocytochemical studies (Fig. 1) of blood and bone marrow showed that granulocytes, but not monocytes, stained positive for NAMLAA. Fig. 1B shows that myeloblasts were negative, while band-form granulocytes were positively stained for NAMLAA. Although monocytes and spleen macrophages did not express NAMLAA, a 20% subpopulation of highly fluorescent bronchoalveolar

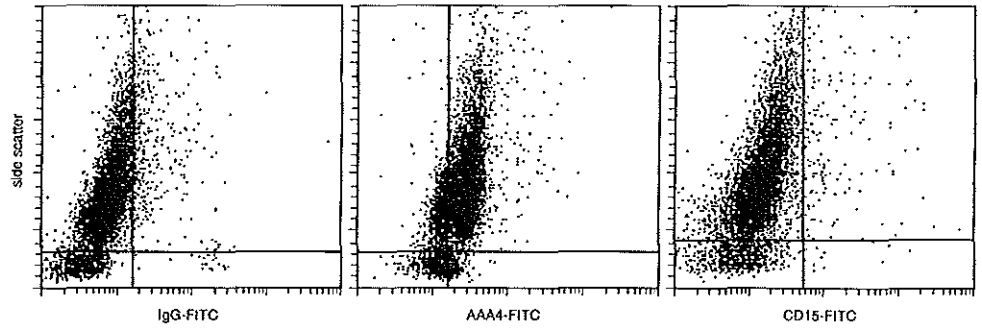


Figure 4. Flow cytometric analysis of bone marrow from an AML-M3 classified patient.
The mononuclear cells contain more than 80% NAMLAA (AAA4-FITC)-positive and CD15-negative promyelocytes.

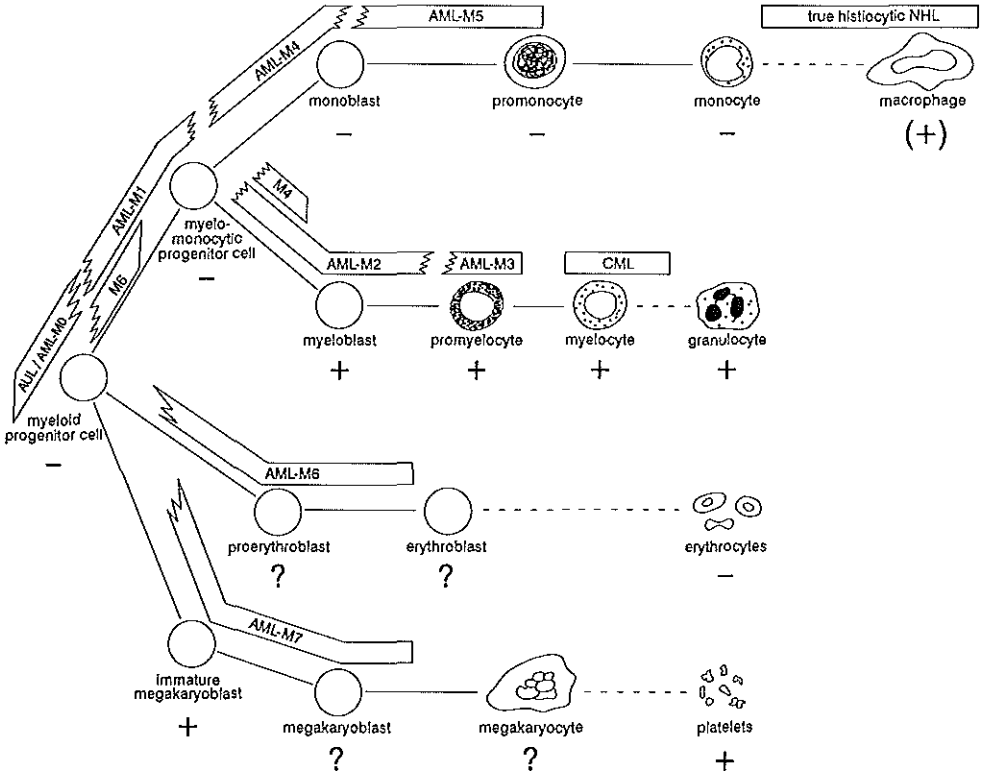


Figure 5. Hypothetical scheme of myeloid differentiation.
The expression of NAMLAA is indicated for each differentiation stage with + and -. The bars represent the various types of leukemias and non-Hodgkin lymphomas and indicate where these malignancies can be located according to their maturation arrest. To indicate the heterogeneous phenotype found in most AML patients, the bars fade into each other. CML=chronic myeloid leukemia, NHL=non-Hodgkin lymphoma.

macrophages was positive for NAMLAA (Fig. 2), indicating that differentiation of monocytes to macrophages induces NAMLAA expression.

The observation that granulocytes stain positive, and that monocytes and lymphocytes were negative, was confirmed by flow cytometric analysis of peripheral blood and bone marrow from a healthy donor. Double-labeling of bone marrow cells for NAMLAA and CD14 or CD15 showed that the CD14-positive monocytes were NAMLAA-negative while the CD15-positive cells, representing granulocytes, were NAMLAA-positive (Fig. 3). Immature granulocytes (CD15 low) showed a higher NAMLAA expression than the CD15 high, mature granulocytes (Fig. 3D). In double-labeling experiments, neither the B cell marker CD10 or CD19 nor the T cell marker CD3, CD4 or CD8 stained NAMLAA-positive cells. This indicates that in blood and bone marrow NAMLAA expression is restricted to the myeloid lineage.

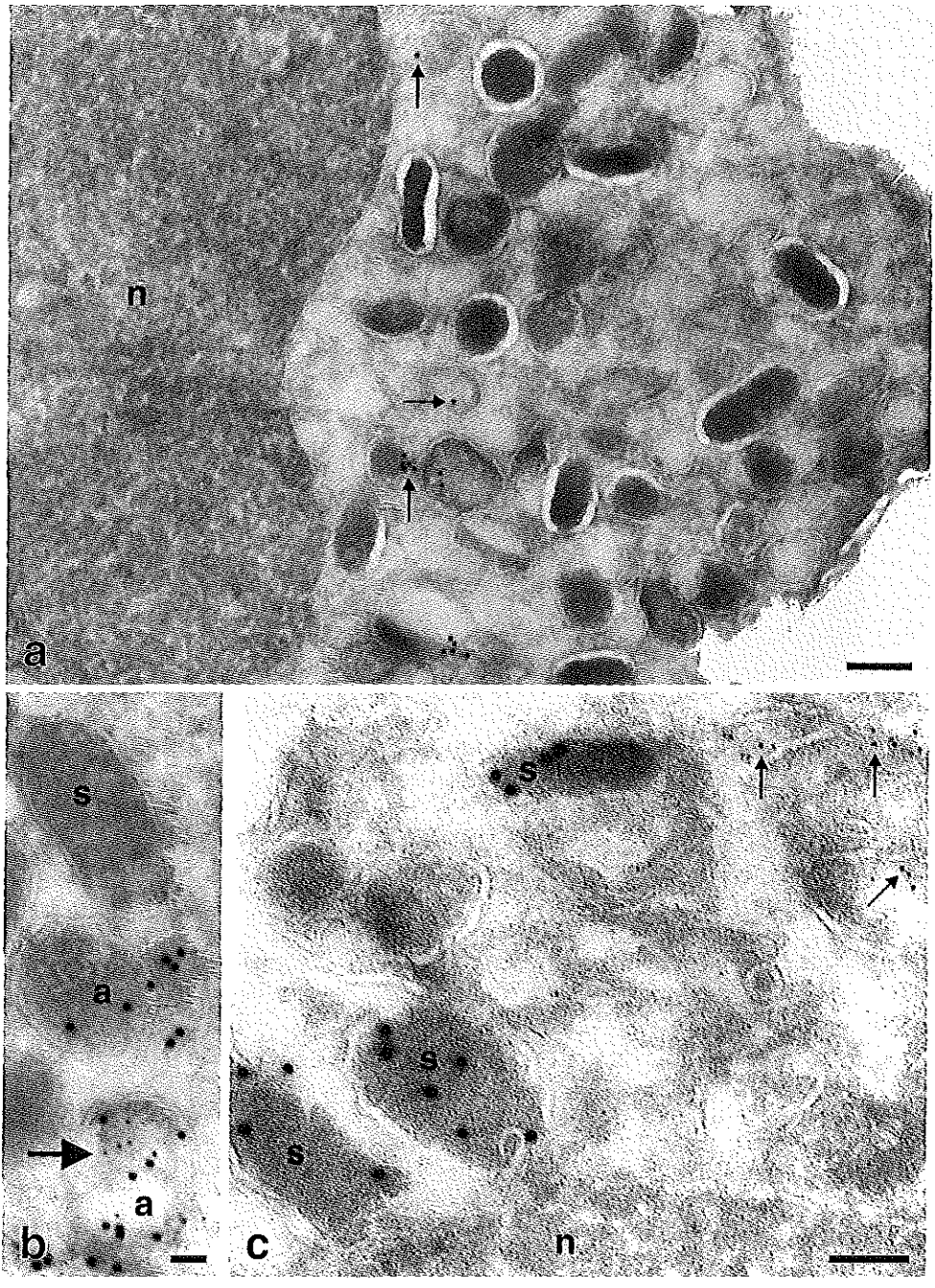
Expression of NAMLAA in various AML

Blood and bone marrow from FAB-classified AML patients were screened for NAMLAA-positive cells. M0 (myeloid precursor), M1 (myelo-monocytic precursor) and M4 (monoblast) leukemic cells were negative, whereas M3 (promyelocyte) leukemic cells were positive (Fig. 4). Also CML (myelocyte) and M7 (megakaryoblast) cells were positive (Fig. 5). In the myeloblast stage (M2), leukemic cells from one patient were NAMLAA-positive, while those of two other patients were negative, suggesting that expression of NAMLAA starts at this stage of granulocyte maturation.

Localization of NAMLAA in granulocytes

Immunoelectron microscopic studies were performed on ultrathin cryosections of neutrophils and eosinophils. The sections were incubated with the NAMLAA-specific monoclonal antibody AAA4 and were subsequently stained with gold-labeled antibodies. The gold label was found in vesicles and granules of neutrophils (Fig. 6a). No background staining was found on the nucleus. The granules were subsequently characterized in double-labeling experiments. Myeloid peroxidase, a marker of azurophilic granules, colocalized with NAMLAA in some but not all azurophilic granules (Fig. 6b). No colocalization was found with

Figure 6. Cryosections of neutrophils from peripheral blood.
A) Localization of NAMLAA after incubation with the NAMLAA-specific MAb AAA4. The gold label is shown in vesicles and granules (arrows). No background is shown on the nucleus (n); B). Micrograph showing double labeling for NAMLAA (5 nm gold) and myeloperoxidase (MPO), a marker for azurophilic granules (10 nm gold). Both labels were seen in some but not in all MPO-positive azurophilic granules. C) Localization of lactoferrin (10 nm gold) and NAMLAA (5 nm gold) showed that the lactoferrin- positive specific granules (s) were not labeled with AAA4, while other granules/vesicles were labeled with AAA4 (arrows). Bars, A) = 200 nm; B) = 50 nm; C) = 100 nm.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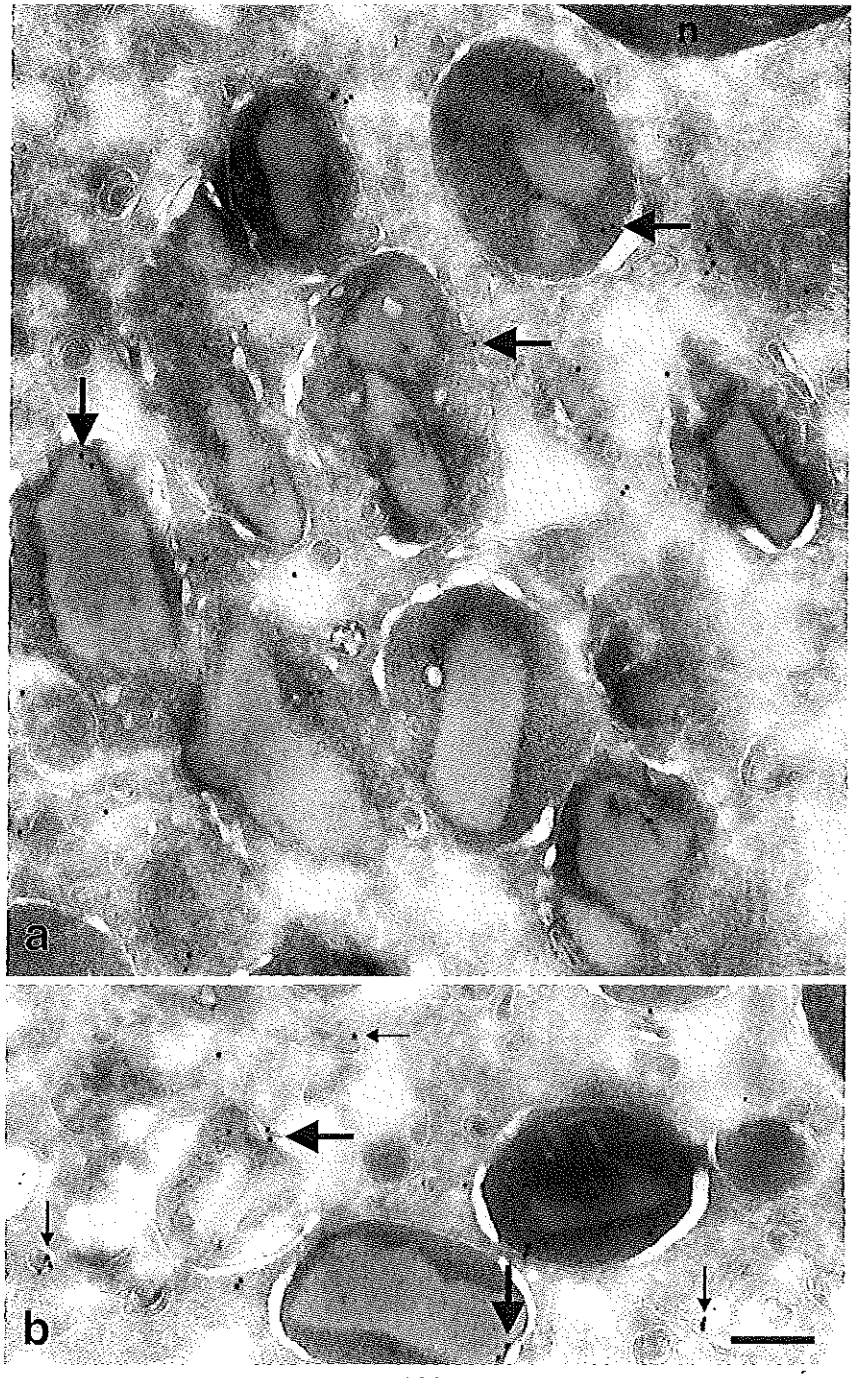
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lactoferrin (Fig. 6c), a marker for specific granules. NAMLAA was more abundant in the crystalloid containing granules and vesicles of eosinophils (Fig. 7). In the vesicles it seemed membrane bound (Fig. 7b). Monocytes present in the same sections were negative for NAMLAA.

To confirm the results obtained by immunoelectron microscopy the localization of NAMLAA in neutrophilic granulocytes was investigated also by Western blotting. The neutrophilic granulocytes were fractionated into a semi-purified membrane fraction, a cytosolic fraction without membranes, a fraction containing mainly specific granules and a fraction containing mainly azurophilic granules. The fractions were then analyzed by Western blot. As shown in Fig. 8, the membrane fraction (lane 5) and the azurophilic granule containing fraction

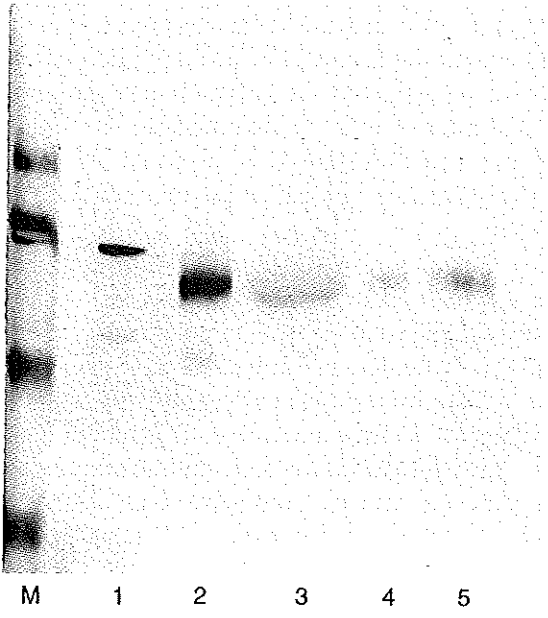

Figure 8. Western blot of neutrophil cell fractions.
Lane M corresponds to pre-stained molecular weight markers of 116, 80, 49 and 32 kDa. Lane 1 corresponds to purified NAMLAA from human plasma. Lanes 2, 3, 4 and 5 correspond to the azurophilic granule-containing fraction, the cytosol fraction, the specific granule-containing fraction and the purified plasma membrane fraction, respectively. Only the azurophilic granule-containing fraction and the membrane fraction were recognized by MoAb AAA4, producing a band with a lower molecular mass than the NAMLAA purified from plasma in lane 1.

Figure 7. Cryosections of eosinophils from peripheral blood incubated with MoAb AAA4.
A) area of an eosinophil showing labeling for NAMLAA on the crystalloid containing granules (arrows). B) Area of another eosinophil showing labeling on the same granules (thick arrows) and on the membrane of small vesicles (small arrows). Bar = 200 nm.

(lane 2) showed a band with a molecular mass of approximately 65 kDa. The glycosylated form of NAMLAA, purified from human plasma, had a molecular mass of 70 kDa (lane 1). The cytosol fraction and the specific granule containing fraction were stained very faintly.

DISCUSSION

The two most important enzymes capable of degrading peptidoglycan in humans are lysozyme and NAMLAA. Lysozyme is present in specific and azurophilic granules of neutrophils [23,24] and in the granules of monocytes [25], but its presence was not detectable in eosinophilic granulocytes [26,27]. NAMLAA is expressed differently. It is present in the azurophilic granules of neutrophils and in the crystalloid containing granules of eosinophilic granulocytes. In a previous study, in which we investigated the inflammatory properties of peptidoglycan products before and after NAMLAA degradation, we found that the cooperative action of lysozyme and NAMLAA is essential for optimal degradation of peptidoglycan. The difference in localization between NAMLAA and lysozyme is therefore remarkable.

Azurophilic granules store a number of digestive enzymes, which are essential for the inactivation of invading microorganisms. Using some of these enzymes as a marker, Egesten et al. [28] were able to classify heterogeneous forms of azurophilic granules in promyelocytes. By using antibodies against myeloperoxidase (MPO), bactericidal permeability increasing protein, cathepsin G, elastase and proteinase 3, they classified azurophilic granules into nucleated azurophils, large spherical azurophils and small azurophils. In Fig. 6b it is shown that NAMLAA did not always colocalize with MPO, which suggests that NAMLAA is also heterogeneously expressed between the different forms of azurophilic granules.

In previous studies it was shown that NAMLAA is a glycoprotein with a molecular mass of 70 kDa, and 60 kDa after deglycosylation [3]. Using two-dimensional gel electrophoresis, we detected multiple isoforms of NAMLAA with the main spots between pH 7.5 and 7.8. This proves that NAMLAA is a cationic protein, as are most of the proteins in the azurophilic granules [28]. The neutrophil fractions subjected to Western blot analysis showed a broad protein band with a molecular mass between the fully glycosylated 70 kDa band and the deglycosylated 60 kDa band. The molecular mass and the broadness of the band indicate that the enzyme may have been partly degraded during the isolation procedure. The presence of the band in the azurophil fraction but not in the fraction containing the specific granules confirms the results obtained by immunoelectron microscopy.

The fact that the membrane fraction also contained the same band indicates that NAMLAA possibly associates with membranes, perhaps as a result of fusion of secretory vesicles or azurophil granules with the plasma membrane.

The NAMLAA-positive cells in the tissues tested were granulocytes migrated into the tissues and granulocytes present in the blood vessels of the tissues. Surprisingly, the NAMLAA-containing cells in the liver were not Kupffer cells, which are known to be involved in the clearing of bacterial debris from the circulation. Why these cells do not express NAMLAA is unknown, but it is consistent with the findings of Daldorff et al. [29] and Lichtmann et al. [30] who showed in a rat model that bacterial cell walls can persist for long times in the liver.

The expression of NAMLAA during the maturation of the myeloid lineage shows that NAMLAA expression starts early in granulocyte maturation and late in monocyte/macrophage maturation. This difference is probably related to the effector function of these cell types. Monocytes are actually precursor cells of macrophages and may not need NAMLAA for their effector function in this stage, while granulocytes and macrophages are mature phagocytizing cells. The expression pattern of NAMLAA during myeloid differentiation shows that NAMLAA is also expressed in the megakaryocyte lineage, corresponding with the finding that thrombocytes in blood were also stained positive for NAMLAA.

In conclusion, the results presented in this paper demonstrate the presence of a novel enzyme, capable of degrading bacterial cell walls, in azurophylic granules of neutrophilic, in the cristalloid containing granules of eosinophilic granulocytes, thrombocytes and a sub-population of activated alveolar macrophages. The expression pattern of NAMLAA in the myeloid lineage suggest that AAA4 is useful for discrimination between AML in the monocyte lineage and in the granulocyte lineage. Therefore AAA4 might contribute to the diagnosis and classification of acute leukemias. The cloning of the gene coding for NAMLAA is in progress.

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N-acetylmuramyl-L-alanine amidase and lysozyme in serum and cerebrospinal fluid of patients with bacterial meningitis

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ABSTRACT

N-acetylmuramyl-L-alanine amidase (NAMLAA) is a relatively unknown human serum enzyme which cleaves the amide bond between N-acetylmuramic acid and L-alanine of the peptide side chain of bacterial peptidoglycan. Lysozyme degrades peptidoglycan differently by hydrolyzing the amino-sugar backbone of peptidoglycan and has been shown to act synergistically with NAMLAA. The presence of lysozyme and NAMLAA, which are both produced by granulocytes, was determined in serum and cerebrospinal fluid (CSF) of patients with bacterial meningitis. As could be expected based on the literature and cell counts, lysozyme was found in CSF while surprisingly, NAMLAA was not present. In previous studies it was shown that NAMLAA was able to reduce the inflammatory properties of peptidoglycan. The absence of NAMLAA in CSF could have important consequences for the pathogenesis of Gram-positive bacterial meningitis.

INTRODUCTION

N-acetylmuramyl-L-alanine amidase (NAMLAA) hydrolyses peptidoglycan by cleaving the lactamide bond between N-acetyl muramic acid and the peptide side chain. NAMLAA together with lysozyme, which hydrolyses the aminosugar backbone of peptidoglycan, can degrade peptidoglycan to less or non-inflammatory subunits as found in a recent study [1]. Although the presence and action of lysozyme in body fluids is described in an overwhelming number of papers, only 4 investigations on NAMLAA in human serum have been reported [2-5].

We previously described a colorimetric method for the determination of NAMLAA in serum and other body fluids [5], the purification of NAMLAA from human plasma, its localization in granulocytes and the biological significance of the enzyme [1,6]. Until now we were able to determine NAMLAA in every human serum tested (n>100).

It is well known that during bacterial meningitis granulocytes enter the cerebrospinal fluid (CSF). Determination of lysozyme in CSF was reported to be indicative for Gram-positive meningitis [7,8]. We assumed that, since both NAMLAA and lysozyme are present in granulocytes, amidase would be excreted and detected in CSF samples from patients with bacterial meningitis which contain lysozyme. To test this assumption we investigated serum and CSF of patients with bacterial meningitis due to *Streptococcus pneumoniae, Neisseria meningitidis* and *Haemophilus influenzae*. As controls we used CSF samples of patients with non-septic neurologic diseases.

METHODS

Patients and controls

Patients between the age of 3 months and 18 years who were diagnosed with bacterial meningitis between August 1992 and September 1994 were suitable for inclusion. The patients were admitted by the Departments of Pediatrics of the Sophia Children's Hospital and Zuiderziekenhuis, both in Rotterdam, Reinier de Graaf Gasthuis in Delft and Juliana Children's Hospital in The Hague. Bacterial meningitis was defined as the presence of a positive bacterial culture from CSF or the presence of a positive blood culture in combination with clinical evidence of meningitis and a CSF white blood cell (WBC) count above $10x10^6$/L. Patients with prior antibiotic treatment were excluded. Control samples of CSF were obtained from 10 patients from the Department of Neurology. Lumbar punctures in these patients were performed for diagnostic reasons, but infection was not present. CSF samples from patients and controls were examined for WBC count and levels of glucose and protein. Sera from 10 healthy subjects (20-45 years) were used as controls.

Collection of samples

Samples of blood and CSF in children with bacterial meningitis were obtained on admission prior to the initiation of antibiotic treatment and during treatment of the first 24 h at 6, 12 or 24 h. Blood was collected into sterile siliconized vacutainer glass tubes (Becton Dickinson, Meylan Cedex, France) and allowed to clot at room temperature. CSF samples were collected into pyrogen-free polystyrene tubes (Falcon^R, Becton Dickinson, Franklin Lakes, NJ). The samples were centrifuged at 2800 g at 4°C for 10 minutes. The supernatants were stored at -70°C until used.

Laboratory studies

Cultures of blood and CSF were processed according to standard procedures [9]. The CSF WBC count, glucose and protein concentration were determined by routine laboratory procedures in each of the participating hospitals.

Lysozyme

The lysozyme concentration in sera and CSF was determined with a turbidimetric assay according to the method of Klass and Neale [10] with some modifications. In short, a suspension of 5 mg *Micrococcus lysodeikticus* (Sigma, St. Louis, MO) per ml NH_4Ac (0.5 M) pH 6.5 was used as substrate for lysozyme activity. 12.5 µl Serum and CSF samples were diluted with 187.5 µl bidest and subsequently 50 µl substrate was added. Samples were incubated in 96 well microtiter plates at 37°C during 1 h on a Titertek shaker (Flow Lab, Scotland). The standard curve of chicken egg white lysozyme (Sigma) was linear up to 20 µg/ml. Samples above this limit were brought into the range by dilution. Absorption was determined in a Titertek Multiscan (Flow Lab, Irvine, Scotland) at 620 nm. The determination was carried out in duplicate for each sample. Data are expressed as µg lysozyme per ml. Determinations in 5 fold showed standard deviations of less than 10%.

NAMLAA

NAMLAA activity in sera and CSF was determined as described by Hazenberg and de Visser [5] and Hoijer et al [6] with some modifications. In short: Peptidoglycan monomers from *Brevibacterium divaricatum* (ATCC 14020) were used as a substrate. 50 µl 100 fold diluted serum or CSF in 0.02 M NH_4HCO_3 buffer (pH 8.0) and 50 µl substrate diluted in buffer to a final concentration of 400 µg N-acetylmuramic acid (the characteristic aminosugar of peptidoglycan) were incubated for 15 min at 37°C. The increase in the concentration of N-acetylmuramic acid

corresponds with peptide side chains released from the aminosugar due to NAMLAA activity. This was measured with a colorimetric method using a Titertek Multiscan. 0-100 µg N-acetylmuramic acid solutions served as a standard. The determination was carried out in duplicate for each sample and data are expressed as U/ml serum or CSF. One unit of enzyme activity was defined as the liberation of 1 µmol N-acetylmuramic acid from peptide side chains per minute. Determinations in 5-fold resulted in standard deviations of less than 5%.

Immunofluorescence labeling and flow-cytometric analysis

Labeling of AAA4, a human NAMLAA specific monoclonal antibody developed in our laboratory [6], with fluorescein isothiocyanate (FITC) was performed according to standard procedures [11]. Human peripheral blood cells were stained for intracellular antigens according to Syrjälä et al [12]. In short, 100 µl citrate blood was incubated with CD15-PE (Immuno Quality Products, Groningen, The Netherlands) in PBS + 0.5% BSA for 10 min. The cells were then lysed and fixed by adding 2 ml of FACS lysing solution (Becton Dickinson) and incubating for 10 min at room temperature. The cells were centrifuged and washed twice with PBS + 0.5% BSA. Then the mouse AAA4-FITC was added and the mixture was incubated for 10 min at room temperature. The cells were washed twice with 2 ml PBS-BSA and finally resuspended in 200 µl FACS flow (Becton Dickinson). The analyses were performed using a FACScan cytofluorometer (Becton Dickinson, Sunnyvale, CA).

Table 1. Clinical and laboratory characteristics of 18 children with bacterial meningitis and 10 control subjects with non-infectious neurologic diseases

	Meningitis patients (n=18)	Control patients (n=10)
Age (years)	3.8 (0.3 - 13.0)	57.5 (24 - 83)
Sex (No.)		
male	6	5
female	12	5
Micro-organisms (No.)		
H. influenzae	8	-
N. meningitidis	5	-
S. pneumoniae	5	-
Others	-	-
Cerebrospinal fluid		
WBC count (/mm^3)	6438 (96 - 34000)	3 (0 - 50)
glucose (mmol/L)	1.6 (<0.1 - 4.2)	3.8 (1.8 - 4.7)
protein (g/L)	1.9 (0.5 - 8.0)	0.46 (0.11 - 5.4)

Except as indicated, data are expressed as median (range). WBC - white blood cells.

RESULTS

Patients and controls

The clinical and laboratory characteristics of patients and control subjects are shown in Table 1. Eighteen patients with a proven bacterial meningitis were enrolled. The CSF samples showed lower glucose and high protein concentrations indicative of bacterial meningitis. CSF samples from the neurologic non-infectious control patients were sterile. The median age (range) of the control groups (neurologic patients as well as healthy subjects) was significantly higher than that of the meningitis patients.

Table 2. NAMLAA activity and lysozyme levels in sera and cerebrospinal fluid of patients with *S. pneumoniae*, *H. influenzae* and *N. meningitidis* meningitis and in control groups

	NAMLAA*)		lysozyme**)	
patients	serum	CSF	serum	CSF
S. pneumoniae (n=5)	2.1 (1.4-2.8) (n=13)	0.0 (0.0-1.1) (n=6)	7.5 (6.4-9.1) (n=13)	4.9 (1.3-7.0) (n=6)
N. meningitidis (n=5)	2.7 (2.3-3.3) (n=11)	0.0 (0.0-0.3) (n=8)	7.0 (4.8-8.4) (n=11)	7.0 (3.5-8.6) (n=8)
H. influenzae (n=8)	2.2 (1.6-3.2) (n=20)	0.0 (0.0-0.2) (n=13)	7.3 (4.3-8.8) (n=20)	7.1 (5.1-10.0) (n=13)
Neurologic non-infectious (n=10)	- -	0.1 (0.0-0.3) (n=10)	- -	0.0 (0.0-5.2) (n=10)
Healthy subjects (n=10)	2.8 (1.6-4.0) (n=10)	- -	6.7 (4.4-8.2) (n=10)	- -

*) Concentration in U/ml, median and range, and number of samples
**) Concentration in µg/ml, median and range, and number of samples.

NAMLAA and lysozyme in serum and CSF

NAMLAA and lysozyme were determined in serum and CSF. The results are presented in Table 2. Lysozyme was found in similar concentrations in serum of bacterial meningitis patients and healthy controls. Significant differences in concentrations on admission (n=6) after 6 h (n=11), after 12 h (n=13) and after 24 h (n=14) were not observed. Lysozyme levels in CSF of meningitis patients

were high and not different from those found in the sera. Differences in concentrations in the observed time period 0 to 24 h were not seen. In the non-meningitis control group lysozyme was not found in CSF.

NAMLAA activity was not or barely detectable in CSF of the meningitis patients. Addition of purified NAMLAA [6] to samples showed that activity was not inhibited by CSF (data not shown). In several samples the enzyme activity was determined more than once and the results were always consistent.

DISCUSSION

Normal values of lysozyme and NAMLAA activity were found in the sera of patients with a bacterial meningitis. The presence of lysozyme in the CSF of the meningitis patients was expected on the basis of leukocyte counts in the samples and the confirmation of meningitis. Porstmann et al. [7] and Ribero et al. [8] using an ELISA and lysoplate method, respectively, (the latter is comparable with our method) both found lysozyme concentrations in CSF samples from patients with bacterial meningitis as high as serum levels. In both studies the control CSF from multiple sclerosis patients (n=9), patients with "disturbances in CSF circulation" (n=11) and control subjects without bacterial meningitis (n=54) contained concentrations of lysozyme less than 5% of serum or CSF values in patients with bacterial meningitis.

Although no information on the presence of NAMLAA in CSF is available, the complete absence in lysozyme positive CSF specimens was surprising. Two possible explanations for the phenomenon are: (1) passage of the smaller lysozyme (14 kDa) via an impaired blood brain barrier to the CSF (molecular mass of NAMLAA is 70 kDa [6]) or (2) active excretion of lysozyme by granulocytes in the CSF compartment but not of NAMLAA or other proteolytic enzymes.

In a rabbit model of meningitis it has been shown that PG can provoke inflammation resulting in brain edema, leukocytosis and protein accumulation in the CSF. Solubilized PG was 10-fold more active in this model than intact cell walls [13,14]. Previous studies showed that after lysozyme treatment bacterial cell wall fragments still possessed inflammatory properties, such as the induction of IL-1, IL-6 and TNF-α release by macrophages and arthritis induction in a rat model, while after NAMLAA treatment the inflammatory properties were significantly reduced [1]. This indicates that the synergistic activity of NAMLAA and lysozyme is essential for the degradation and inactivation of peptidoglycan products. It is therefore possible that during Gram-positive bacterial meningitis cell wall fragments are not sufficiently degraded in CSF because of the absence of

NAMLAA. This very intriguing hypothesis needs further investigation to reveal whether NAMLAA in combination with antibiotics could prevent the symptoms seen during Gram-positive bacterial meningitis.

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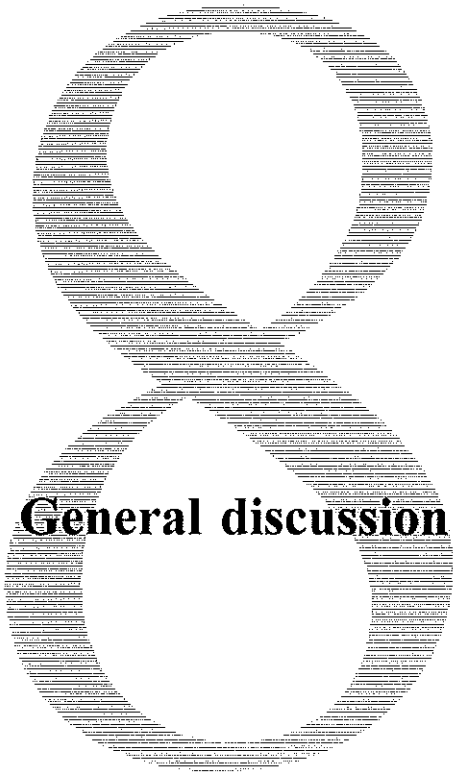

General discussion

GENERAL DISCUSSION

Rheumatoid arthritis [1] and inflammatory bowel diseases like Crohn's disease [2,3] and Ulcerative Colitis [2,3] are described in the literature as inflammatory diseases with unknown etiology. By a number of authors it has been suggested that peptidoglycan-polysaccharides (PG-PS) originating from bacterial cell walls, might be the triggering antigen [4-6]. This hypothesis is supported by several reports which show that PG-PS are able to induce inflammation. An overview on the inflammatory properties of PG-PS is given in chapter 1 of this thesis.

A prerequisite for the hypothesis that PG-PS is involved in inflammatory diseases is that these bacterial cell wall products can occur in relevant human tissues. However, hardly any evidence for the presence of PG-PS in human tissues can be found in the literature. This is probably due to technical difficulties in detection and purification of PG-PS. By employing sophisticated methods, Lethonen et al. [7] recently showed the presence of muramic acid (Mur), the characteristic aminosugar of PG (See introduction, Fig. 1), in synovial fluid of patients with an acute inflammatory arthritis of unclear origin but who had a history of bacterial disease. In chapter 2 of this thesis a monoclonal antibody recognizing PG-PS was used for the detection of these antigens in synovial tissues of arthritis patients and healthy controls. PG-PS was found to be present in tissue macrophages and dendritic cells that PG can cause inflammation. Both our results as well as the results of Lethonen et al. [7,8] suggest an involvement of inflammatory PG-PS products in the pathogenesis of human chronic polyarthritis. This suggestion is further strengthened by the results obtained from experiments using animal models.

In animal models PG-PS was shown to be present in synovial tissue macrophages [9] suggesting that these cells play a major role in transport of PG-PS to the synovial tissues. The capacity of PG to induce arthritis has been extensively investigated in animal models for arthritis [10]. In these models it was shown that PG-PS persisted in tissue macrophages for more than 90 days after the injection of PG-PS [11]. Lichtman et al. [12] investigated the role of macrophages in this respect. They supported the hypothesis that PG can cause inflammation by showing that PG-PS containing liver macrophages of rats were able to induce inflammation. The results strongly suggest that the presence and persistence of PG-PS in tissues is likely to be important in the pathogenesis of idiopathic arthritis.

In our immunohistochemical studies and the studies of Lethonen et al. [7,8] it was not necessary to purify the PG-PS from tissues. The purification of

these PG products, however, can give information on the molecular structure in which PG is present in tissues. The purified products could also be used in studies to test their inflammatory capacity, which would be very important in the elucidation of the role of PG in the pathogenesis of inflammatory diseases with unknown etiology. For this reason the purification of PG from human spleen was performed, as described in chapter 3. A macromolecular carbohydrate-rich fraction was obtained after several purification steps. We confirmed the presence of PG in this fraction by the detection of Mur using an aminosugar/aminoacid analysis method. Further investigations on the nature of the purified PG products will most likely elucidate whether these products possess inflammatory properties.

The presence of PG in the human bowel wall [13], synovia [14], and spleen [15], suggests that human tissues are continuously exposed to PG. It is clear that these inflammatory bacterial cell wall products, which can be translocated across the bowel wall [16] or released during an bacterial infection, need to be removed from the circulation.

Bacterial cell walls are rapidly cleared by the action of phagocytic cells and their hydrolytic enzymes. Since Fleming discovered lysozyme in human tissues in 1922 [17], this enzyme was thought to be responsible for lysing invading bacteria and subsequent degradation of PG. Later it was found that lysozyme only partly degrades PG from several bacteria [18,19]. The degradation of PG by lysozyme was shown to be dependent on the degree of O-acetylation of MurNAc in the aminosugar backbone of PG [20] (see Fig. 2, chapter 1). The studies described in chapter 5 of this thesis show that after lysozyme treatment cell wall fragments still possess inflammatory properties in an animal model for arthritis.

Therefore, the presence of another potent PG degrading enzyme N-acetylmuramyl-L-alanine amidase (NAMLAA) in human serum, described for the first time by Ladesic et al. [21], drew our attention. The possibility that this enzyme could degrade PG in such a way that it would loose its inflammatory properties, pushed our interest in the direction of this enzyme.

In chapter 4 the purification and characterization of NAMLAA from human plasma is described. The generation of monoclonal antibodies against NAMLAA made it possible to prepare an immuno-affinity column with which it was relatively simple to purify NAMLAA from plasma. The concentration of NAMLAA in serum was estimated to be around 10 µg/ml. NAMLAA was characterized as a glycoprotein of 70 kDa with an iso-electric point between 7.5 and 7.9 and a specific enzymatic activity of 46 µg PG monomer.min^{-1}.mg^{-1}. The purified enzyme was further used to investigate its biological function. We

felt that degradation of PG by NAMLAA could result in the inactivation of the inflammatory properties of PG. To test this idea, the in vivo adjuvant arthritis model was used as a measure for the inflammatory capacity of a bacterial cell wall preparation from *Eubacterium aerofaciens* (ECWF). As is described in detail in chapter 5, degradation of ECWF by lysozyme results in a preparation which was still arthritogenic. However, after lysozyme and subsequent NAMLAA degradation the ECWF had completely lost the arthritis inducing capacity.

Inflammation processes have a relation with the induction of IL-1, IL-6, IL-8 and TNF-α [22]. These pro-inflammatory cytokines are reported to play an important role in the pathogenesis of septic shock [23], pertussis [24,25], arthritis [26] and possibly in all bacterially induced inflammatory diseases. PG was described to be able of inducing cytokine release by human monocytes [27]. Therefore this property was also used to investigate the influence of lysozyme and NAMLAA degradation.

Mice were intravenously injected with ECWF digested with either lysozyme, NAMLAA or both to test the cytokines released in the blood. In vitro, the same ECWF digests were tested by stimulating whole blood or isolated human peripheral blood monocytes. We found in both test systems that the cytokine inducing capacity of NAMLAA degraded ECWF was much lower than after treatment with lysozyme alone.

These results are the first to show that NAMLAA is capable of decreasing the inflammatory properties of bacterial cell wall fragments. Because NAMLAA acts specifically on the PG part of the ECWF, the results also show now in a completely different way that the inflammatory properties of ECWF are for an important part related to the PG component.

Due to the rapid increase in bacterial species and strains which are multiresistant for antibiotics, it is plausible that treatment of the symptoms, induced by bacterial infections, will become more and more important. Excessive release of the inflammatory cytokines IL-1, IL-6, IL-8 and TNF-α have become synonymous with various clinical pathologies due to bacterial infections. Knowledge about the mechanisms responsible for the release of inflammatory cytokines is therefore important for future treatment of infections. To prevent the symptoms caused by these cytokines one can either block the production of cytokines or the activity of the produced cytokines. The latter possibility has been investigated using TNF-α inactivating monoclonal antibodies. The administration of these monoclonal antibodies has been shown to be beneficial for rheumatoid arthritis patients as well as for patients with Crohn's disease [25,27]. Whether NAMLAA administration may be used to reduce the production of pro-inflammatory cytokines is a challenging question which will

be investigated in the future, especially for bacterial meningitis (see chapter 7).

The localization of NAMLAA in tissues might give further evidence for its biological role in humans. We used the monoclonal antibody AAA4 for immunohistochemistry, immuno electron microscopy, FACScan and Western blot studies to investigate the localization of NAMLAA. The results described in chapter 6 of this thesis show the localization of NAMLAA in several human tissues which finally led to the discovery that NAMLAA is present in granulocytes. More detailed studies using immuno electron microscopy revealed the presence of NAMLAA in the azurophilic granules of neutrophilic granulocytes and in the crystalloid containing granules of eosinophilic granulocytes. The azurophilic granules also contain other bactericidal enzymes [29]. An interesting observation is that MPO-positive azurophilic granules were not all positive for NAMLAA, suggesting that the azurophilic granules do not form a homogenous population. This was also reported by Egesten et al. [30], who defined 3 different granule populations in neutrophilic granulocytes, based on differences in protein contents.

The localization of NAMLAA in granulocytes is not very surprising because the phagocytic cells are involved in the first line defence against invading bacteria or bacterial products. The absence of NAMLAA in monocytes, however, did come as a surprise because these cells are capable of phagocytizing bacteria and are well equipped for the killing of bacteria.

The absence of NAMLAA in monocytes possibly explains the observed presence of PG-PS in macrophages [9] as well as the finding that cultured monocytes release biologically active PG products into the medium after phagocytosis of *Staphylococcus aureus* cells [31]. The combined results suggest that due to the absence of NAMLAA, the cell wall fragments can persist and remain biologically active in monocytes and macrophages. It can be hypothesized that the PG products in these cells are structurally different from the products which can be degraded by lysozyme and NAMLAA and are subsequently excreted in the urine [32].

The expression of NAMLAA during the maturation of granulocytes is described in chapter 6. To investigate the maturation stage in which NAMLAA is expressed for the first time, cells from various acute myeloid leukemic (AML) patients were used. Fig. 5 of chapter 6 shows the myeloid differentiation lineages together with the FAB classification. NAMLAA is expressed in the myelocyte lineage from AML-M2 to mature granulocytes and in AML-M7 classified cells which are immature megakaryoblast cells. From the mature cells, platelets and granulocytes expressed NAMLAA. If NAMLAA can be used as a differentiation marker for AML patients needs further investigation

with higher numbers of patients. The preliminary results show that the AML-M2 classified cells can be subdivided into two groups based on NAMLAA expression. More M2 samples should be investigated to confirm this subdivision.

As is described in chapter 7, NAMLAA is absent in cerebrospinal fluid (CSF) of bacterial meningitis patients. In a rabbit model of bacterial meningitis it has been shown that PG can provoke inflammation resulting in brain edema, leukocytosis and protein accumulation in the CSF. Solubilized PG was 10 fold more active in this model than intact cell walls [33]. If during a Gram-positive bacterial infection, PG is released into the CSF due to the action of antibiotics on the bacterial cell walls, the PG fragments cannot be further degraded because NAMLAA is absent in this compartment. This very intriguing observation needs further investigation to reveal whether NAMLAA in combination with antibiotics may prevent the symptoms seen during Gram-positive bacterial meningitis.

Apart from the already mentioned suggestions for future research, more basic questions in NAMLAA research are mentionable as well. For example cloning of the gene encoding for human NAMLAA would provide great possibilities for further research. The generation of knock-out mice, assuming homology in the human and mouse gene, is one of the possibilities. In these animals the biological function of NAMLAA in the non-specific immune system could be established in vivo. The ability of granulocytes lacking NAMLAA, to digest phagocytized bacteria or bacterial products, would be interesting as well. The cloning of the gene would also allow the in vitro production of NAMLAA which would make the purification of NAMLAA independent of human plasma.

The tools required for the detection of the NAMLAA gene have been prepared. DNA expression libraries from human granulocytes and normal human bone marrow have been prepared recently, and the screening of these libraries can be performed, using the monoclonal antibodies we prepared previously against human NAMLAA.

Another approach to find the NAMLAA gene is by PCR techniques. The N-terminal aminoacid sequence of NAMLAA is known and recently three other peptides obtained after CNBr digestion were sequenced. It is possible to prepare degenerated primers based on the amino acid sequences, which can be used in PCR experiments [34].

The results described and discussed in this thesis add a new and important human enzyme to the list of anti-bacterial enzymes known until now. The results will hopefully lead to more research on the role of NAMLAA in non-

specific inflammation, caused by (peptidoglycan containing) bacterial cell wall fragments.

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SUMMARY

The cell wall surrounding the bacterial cytoplasmic membrane, which gives the cell its strength, contains several components which can be involved in the pathogenesis of bacterial infectious diseases. One of these components is the macromolecular peptidoglycan (PG). Previous to the research described in this thesis, it was found that cell wall fragments of some bacteria from the normal human gut flora were arthritogenic in Lewis rats. This led to the hypothesis that PG plays a major role in the pathogenesis of inflammatory diseases with unknown etiology like rheumatoid arthritis (RA).

In order to be able to induce inflammation, PG must of necessity be present in tissues. However, the presence of PG in human tissues has not been shown before. Using immunohistochemical techniques, we found (chapter 2) that PG is present in macrophages and dendritic cells of the synovia (from knee joints) of patients with RA (80%) and to a lesser extent in the synovia of control patients (35%). The presence of PG in antigen presenting cells supports the hypothesis that PG is involved in the induction of inflammation in the affected synovia.

The purification of PG fragments from human tissues would make it possible to investigate the inflammatory properties of these PG products and to characterize their composition. Chapter 3 describes the purification of a macromolecular, PG-containing, polysaccharide fraction from human spleen which was biochemically analyzed. These studies showed for the first time that macromolecular PG products are present in human tissues.

The presence of PG in inflamed tissues is a prerequisite assuming that PG can trigger inflammation. On the other hand, the degradation of PG is probably needed to prevent excessive inflammatory reactions. Two human enzymes are capable of significant degradation of PG: lysozyme and N-acetylmuramyl-L-alanine amidase (NAMLAA). In order to elucidate the possible anti-inflammatory role of the latter, NAMLAA was purified from human plasma (chapter 4). The development of monoclonal antibodies directed against NAMLAA improved the purification procedure. The characteristics of the purified enzyme were then determined.

To investigate the effect of NAMLAA on PG, we used two properties of PG: the capability of PG to induce arthritis in Lewis rats and the capability to induce the inflammatory cytokines interleukin-1, interleukin-6 and tumor necrosis factor-α in vivo and in vitro (chapter 5). The results showed that after degradation of cell wall fragments by lysozyme and NAMLAA the arthritis-

inducing capacity was completely lost, whereas after degradation by NAMLAA or lysozyme alone the cell wall fragments were still arthritogenic. Upon injection of PG in mice and after incubation of human blood monocytes with PG, the induction of cytokine release was lower after the combined PG degradation by lysozyme and NAMLAA compared to the degradation of PG by lysozyme or NAMLAA alone. The results show that NAMLAA, together with lysozyme, inactivates inflammatory PG.

In chapter 6, the monoclonal antibodies directed against NAMLAA were used to detect which cells produce NAMLAA. Using immunohistochemistry and flow cytometry, it was found that NAMLAA was expressed by neutrophilic and eosinophilic granulocytes. Thrombocytes were also positively stained for NAMLAA, but monocytes were negative. The localization of NAMLAA and lysozyme showed remarkable differences. Eosinophilic granulocytes expressed NAMLAA but not lysozyme, and monocytes expressed lysozyme but not NAMLAA. This is surprising because the experiments described in chapter 5 showed that lysozyme and NAMLAA work synergistically.

Chapter 7 describes another striking difference in the localization of NAMLAA and lysozyme. Cerebrospinal fluid normally does not contain lysozyme or NAMLAA activity. Patients suffering from a bacterial meningitis are known to possess elevated levels of lysozyme activity in the cerebrospinal fluid, sometimes even higher than in serum. We confirmed these findings, but NAMLAA activity was not detected in the same samples! The absence of NAMLAA might have important consequences for the pathogenesis of the disease. It is likely that the absence of NAMLAA gives rise to an increase in insufficiently degraded cell wall fragments, which might cause excessive cytokine production. Whether the administration of NAMLAA into the cerebrospinal fluid can suppress the symptoms should be investigated.

SAMENVATTING

De celwand die om de cytoplasmamembraan van de bacterie zit en de cel zijn stevigheid geeft, bevat een aantal componenten die een belangrijke rol kunnen spelen in de pathogenese van sommige infectieziekten. Peptidoglycaan (PG), een zeer groot molecuul dat als een gevlochten netwerk om de bacterie heen zit, is een dergelijke celwandcomponent. Uit voorgaand proefdieronderzoek is gebleken dat PG van sommige bacteriën van de normale humane darmflora gewrichtsontstekingen kan induceren bij Lewis ratten na eenmalige intraperitoneale of subcutane injectie. Dit onderzoek heeft geleid tot de hypothese dat PG een belangrijke rol speelt in de pathogenese van ontstekingen met een onbekende oorzaak zoals reumatoïde artritis (RA).

De aanwezigheid van PG in de relevante weefsels is een voorwaarde voor de juistheid van de hypothese. PG was echter nog niet eerder aangetoond in humane weefsels. Met immunohistochemische technieken werd gevonden (Hoofdstuk 2) dat PG aanwezig is in macrofagen en dendritische cellen van synovia (van knie gewrichten) van patiënten met RA (80 %) en in mindere mate (35%) in synovia van "gezonde" controle patiënten. De aanwezigheid van PG in deze voor de immuunreactie zo belangrijke antigeen presenterende cellen ondersteunt de hypothese dat PG betrokken kan zijn bij de inductie van ontstekingen in de synovia.

Nadat ook in de humane milt immunohistochemisch PG was aangetoond, werd daaruit een PG-bevattende fractie geïsoleerd, die het mogelijk maakt om deze PG produkten te karakteriseren en de invloed ervan op het immuunsysteem te onderzoeken. Hoofdstuk 3 beschrijft de zuivering van een macromoleculaire PG fractie uit humane milt, waarbij de fractie op biochemische wijze werd geanalyseerd. Met de resultaten beschreven in de hoofdstukken 2 en 3 is de aanwezigheid van macromoleculaire PG produkten in humane weefsels voor het eerst komen vast te staan.

Als de aanwezigheid van PG in weefsels ontsteking kan induceren, dan is de afbraak van PG produkten van belang ter voorkoming van dergelijke ontstekingsreacties. Twee humane enzymen die PG kunnen afbreken zijn hiervoor van belang: het zeer bekende lysozym en het vrijwel onbekende N-acetylmuramyl-L-alanine amidase (NAMLAA). Om de betekenis van NAMLAA voor de PG-afbraak te onderzoeken, werd dit enzym gezuiverd uit bloedplasma (Hoofdstuk 4). Met door ons ontwikkelde monoklonale antistoffen kon het enzym efficiënt met immunoaffiniteitschromatografie worden geïsoleerd en konden de specifieke kenmerken van dit enzym worden bepaald.

In hoofdstuk 5 werd onderzocht wat de gevolgen zijn van PG-afbraak

door NAMLAA op het vermogen tot artritisinductie in Lewis ratten en inductie van de afgifte van de ontstekingsmediatoren interleukine-1, interleukine-6 en tumornecrosefactor-α *in vivo* en *in vitro*. Daartoe werd PG geïncubeerd met alleen NAMLAA en met zowel NAMLAA als lysozym. Na afbraak van celwandfragmenten door lysozym en NAMLAA was het vermogen om artritis te induceren in Lewis ratten volledig verloren gegaan, terwijl na afbraak door alléén lysozym of alléén NAMLAA het PG preparaat nog steeds artritogeen was. Ook de inductie van cytokine afgifte was in alle gebruikte testsystemen lager na afbraak door lysozym en NAMLAA samen. De resultaten tonen aan dat NAMLAA en lysozym synergistisch werken en samen in staat zijn om PG die ontstekingen en ontstekingsmediatoren induceren, af te breken.

De monoklonale antistoffen die door ons werden ontwikkeld, zijn gebruikt om te bepalen waar, in welke cellen en weefsels, NAMLAA aanwezig is (Hoofdstuk 6). Met behulp van immunohistochemie en flowcytometrie werd gevonden dat NAMLAA in neutrofiele en eosinofiele granulocyten tot expressie komt. Bloedplaatjes bleken eveneens positief aan te kleuren met de monoklonale antistoffen tegen NAMLAA. Monocyten waren echter niet positief voor NAMLAA. Opvallend is dat het verschillende celtypen zijn die aankleuren voor lysozym en NAMLAA: eosinofiele granulocyten brengen wel NAMLAA tot expressie maar geen lysozym, en voor monocyten geldt het omgekeerde. Deze cellen brengen wel lysozym, maar geen NAMLAA tot expressie. Dit is verrassend omdat in hoofdstuk 5 werd aangetoond dat lysozym en NAMLAA synergistisch werken.

Hoofdstuk 7 beschrijft nog een opmerkelijk verschil in voorkomen van NAMLAA en lysozym. Cerebrospinale vloeistof bevat normaal geen lysozym en ook geen NAMLAA activiteit. Het was al langer bekend dat bij patiënten met een bacteriële meningitis het gehalte aan lysozym in de cerebrospinale vloeistof sterk verhoogd kan zijn, soms zelfs hoger dan in het serum van de patiënt. Dit vonden wij ook, maar in dezelfde monsters was geen NAMLAA activiteit aantoonbaar! De afwezigheid van NAMLAA kan belangrijke gevolgen hebben: het is waarschijnlijk dat door de afwezigheid van NAMLAA de PG fragmenten in de cerbrospinale vloeistof onvoldoende worden afgebroken, waardoor zij inflammatoire cytokinen kunnen induceren. Of toediening van NAMLAA aan de cerebrospinaale vloeistof ontstekingsremmend werkt, zal onderzocht moeten worden.

ABBREVIATIONS

Ala	alanine
AML	acute myeloid leukemia
APAAP	alkaline phosphatase mouse-anti-alkaline phosphatase complex
BSA	bovine serum albumin
CSF	cerebrospinal fluid
CWF	cell wall fragments
Dpm	diamino pimelic acid
ECWF	*Eubacterium aerofaciens* cell wall fragments
ELISA	enzyme-linked immunosorbent assay
FITC	fluorescein isothiocyanate
FPLC	fast performance liquid chromatography
FAB	French-American-British
GlucNAc	N-acetylglucosamine
GMDP	N-acetylglucosamine-N-acetylmuramic acid dipeptide
HPLC	high performance liquid chromatography
i.p.	intraperitoneal
IsoGln	Iso-glutamine
IsoGlu	Iso-glutamate
kDa	kilo dalton
LF	lactoferrin
LPS	lipopolysaccharide
LTA	lipoteichoic acid
Lys	lysine
MDP	muramyl dipeptide
MoAb	monoclonal antibody
MPO	myeloperoxidase
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
Mur	muramic acid
MurNAc	N-acetyl muramic acid
NAM	N-acetyl muramic acid
NAMLAA	N-acetylmuramyl-L-alanine amidase
Orn	ornithine
PBMC	peripheral blood mononuclear cells
PG-PS	peptidoglycan polysaccharides
pI	isoelectric point
RA	rheumatoid arthritis

SDS-PAGE	sodium dodecylsulphate-polyacrylamide gel electrophoresis
Ser	serine
TCT	tracheal cytotoxin
Thr	threonine
WBC	white blood cells

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