IMMUNE RESPONSE TO INFECTION

Studies on the lysozyme independence of immune immobilisation of *Treponema pallidum* and the frequency of lysozyme autoantibodies in syphilitic sera

SUSANNE ZIELINSKI and HERTHA-LORE BORKHARDT

Institute for Medical Microbiology, Otto von Guericke University of Magdeburg, Leipziger Str. 44, D-39120 Magdeburg, Germany

The role of lysozyme in the immune immobilisation of *Treponema pallidum* is not yet fully understood. The *T. pallidum* immobilisation assay was used to demonstrate that the immobilisation and lysis of *T. pallidum* *in vitro* by antibodies (serum, IgG fraction or IgM fraction) and complement proceed in a lysozyme-independent mode. In the presence of lysozyme the rate of immobilisation increased. In contrast with its effect on *Escherichia coli*, the effect of lysozyme on *T. pallidum* was governed exclusively by its enzymic activity rather than by the cationic protein nature of the molecule. Lysozyme, released from stimulated phagocytes, induced formation of lysozyme antibodies in 59.6% of syphilis patients as determined by lysozyme antibody ELISA. The highest frequency was found in patients with untreated secondary syphilis, whereas untreated primary syphilis was only rarely accompanied by the presence of lysozyme antibodies. Cross-reactivities between lysozyme and treponemal antigens were excluded by immunoblotting. The autoantibodies did not influence the lysozyme activity. It was concluded that the formation of lysozyme antibodies is only an epiphenomenon in the host defence against treponemal infection.

Introduction

The enhancing effect of lysozyme on antibody- and complement-mediated immobilisation and lysis of *Treponema pallidum* has been accepted for > 30 years [1, 2]. However, whether there is an absolute requirement for lysozyme in order for irreversible immunologically induced damage of treponemes to occur is not clear. While Born and Bhakdi [3] provided evidence of a lysozyme-independent mechanism of complement-mediated bacteriolysis for *Escherichia coli*, Müller *et al.* [4] demonstrated that treponemes, which are also gram-negative bacteria, are not killed by antibodies and complement without lysozyme. Only in the presence of lysozyme did they observe irreversible damage of treponemes in the *T. pallidum* immobilisation assay (TPI). In keeping with the results of Radolf *et al.* [5] and Walker *et al.* [6] on the limited antigenicity of the *T. pallidum* outer membrane (there is a 100-fold lower concentration of epitopes as compared to the outer membrane of other gram-negative bacteria), the kinetics of antibody- and complement-mediated killing

of treponemes is different from the situation with *E. coli*. The process of damage of treponemes by antibodies and complement [7] requires much more time than the immune bacteriolysis of *E. coli* [8]. Therefore, it seems possible that the few complement-induced holes in the outer membrane of treponemes could be repaired and that the endoflagella could remain inaccessible to immobilising antibodies unless there is additional destruction of the cell wall as a result of the lysozyme influx. This suggests an important potential role for lysozyme in host defence against treponemal infection and, consequently, that a lack of lysozyme could have a direct influence on the course of infection. One possible cause for the deficiency of lysozyme could be an inactivating factor such as antibody. Lysozyme antibodies have not been demonstrated in the serum of patients with syphilis. Such antibodies might influence the enzymic activity of lysozyme and thus delay the immobilisation of treponemes, depending upon the concentration ratio of lysozyme to lysozyme antibody. However, the cationic charge of the enzyme may not be influenced by antibody. Pellegrini *et al.* [9] showed that the bactericidal effect of lysozyme on *E. coli* is based on its enzymic properties as well as on its cationic protein

nature. Therefore this study tested the influence of enzymically inactive lysozyme on *T. pallidum*.

Materials and methods

Animals

Adult male 'Russian' rabbits were obtained from the Moellegaard breeding centre (Denmark), housed individually at 18–20°C and given antibiotic-free food and water. The rabbits were examined by the VDRL test to exclude infection with *T. paraluiscuniculi*.

Bacteria

T. pallidum ssp. *pallidum* (Nichols strain) was maintained by intratesticular passage in rabbits without the use of cortisone acetate. Treponemal suspensions were prepared from the testes of rabbits 6–8 days after intratesticular infection; organisms were either extracted into sterile phosphate-buffered saline (PBS) or basal reduced medium for prolonged incubation *in vitro* (48 h) [10]. Suspensions were centrifuged twice for 10 min at 500 *g* to remove gross testicular debris. The number of motile treponemes in the supernate was counted by dark-field microscopy and adjusted with PBS to $(3-5) \times 10^7$ treponemes/ml for passage. One ml of treponemal suspension in PBS was injected into each testis of rabbits. For the immobilisation assay *in vitro*, the treponemal suspension was adjusted to 2×10^6 /ml of basal medium.

Lysozyme

Hen egg white lysozyme (Reanal, Hungary; 10 000 U/mg) and human milk lysozyme (Sigma; 7900 U/mg) were used throughout. The lysozyme activity in sera was measured by means of the lysoplate technique [11], with minor modifications. This method permits the detection of lysozyme at concentrations as low as 0.1 µg/ml. Lysozyme was removed from human and guinea-pig sera by absorption with bentonite [8]. The treponemes were washed three times with basal medium to remove lysozyme. Hen egg white lysozyme was inactivated with dithiothreitol (DTT) as described by Pellegrini *et al.* [9]. Briefly, 5 mg of hen egg white lysozyme was dissolved in 1 ml of 0.01 M NaH₂PO₄/Na₂HPO₄ buffer (pH 7.4) containing 0.01 M DTT and incubated at 37°C for 2 h until a precipitate was formed. The precipitate was sedimented by centrifugation at 2000 *g* for 15 min, washed 10 times in distilled water and freeze-dried. Bentonite-absorbed sera as well as DTT-inactivated hen egg white lysozyme preparations were tested for residual lysozyme activity in the lysoplate technique. Lysozyme activity was no longer detected.

Human sera

Ninety-nine sera were collected from patients at various stages of untreated and treated syphilis (10 primary,

untreated; eight primary, treated; 18 secondary, untreated; 24 secondary, treated; 13 latent; two tertiary; and 24 not classified). Diagnosis was based upon reactive non-treponemal and treponemal serological tests and upon the presence of typical clinical signs. A further 50 normal sera from blood donors were obtained for control purposes. Human syphilitic sera with known immobilisation titres were used for immobilisation experiments. All sera were stored in small volumes at –70°C; repeated freezing and thawing were avoided.

Fractionation of immune sera

Ammonium sulphate precipitated globulins (50% saturation) from human syphilitic sera were resuspended in PBS, dialysed against PBS overnight and applied to Protein G Sepharose for fast flow (Pharmacia, Sweden). Unbound proteins (IgM fraction) were washed through and 1-ml fractions were collected. Bound IgG antibodies were eluted with 0.1 M glycine/HCl buffer (pH 2.5). Fractions of 1 ml were collected and immediately neutralised with 1 N tris (hydroxymethyl) aminomethane (TRIS). Fractions were pooled and the IgG- fraction was dialysed against PBS before both preparations were concentrated.

Complement

Guinea-pig serum as the complement source was obtained from Harlan.

Lysozyme antibody ELISA

Microtitration plates (Greiner, Germany) were coated overnight with anti-human lysozyme antibodies (Dako, Denmark) diluted 1 in 400 in 0.1 M carbonate buffer (pH 9.6). After blocking for 2 h with low-fat milk powder 1% in PBS, 100 µl of human milk lysozyme 2.5 µg/ml in PBS was added to each well and incubated for 2 h at room temperature. The plates were washed three times, control and test sera were diluted in PBS (1 in 20, 1 in 40, 1 in 80) and 100 µl of each dilution was added to replicate wells and incubated for 2 h at room temperature. For control purposes, to exclude non-specific reactivities, buffer was used instead of human milk lysozyme and human serum. After washing the plates as described above, peroxidase-conjugated rabbit anti-human IgG (Dako) diluted 1 in 500 in PBS was added and incubated for 2 h at room temperature. The plates were washed three times followed by the addition of substrate which comprised 25 µl of H₂O₂ 30% and 1 mg of tetramethylbenzidine dissolved in 1 ml of ethanol in 9 ml of citrate buffer (pH 5.0). The substrate reaction was stopped after 15 min by adding 50 µl of 2 M H₂SO₄. The plates were read immediately with a spectrophotometer (Anthos) at 450 nm. The cut-off value was defined as the mean absorbance of 50 negative sera (blood donors) plus 2 SD.

Immobilisation of treponemes

The TPI assay was performed by the method of Nelson and Mayer [12]. Lysozyme-free *T. pallidum* suspension (200 μ l) containing 2×10^6 bacteria/ml was mixed with 50 μ l of bentonite-absorbed syphilitic serum, or serum fraction and 250 μ l of bentonite-absorbed guinea-pig serum. Hen egg white lysozyme was added to final concentrations of 0.5 and 5 μ g/ml. DTT-inactivated lysozyme was added at concentrations of 5 and 50 μ g/ml. To evaluate the influence of lysozyme on treponemes in the absence of antibodies or of both antibodies and complement, hen egg white lysozyme at concentrations of 5, 10 or 100 μ g/ml was added to treponemes and complement, or to treponemes only.

Aliquots of 0.5 ml of these mixtures were placed into small tubes which were loosely plugged with cotton wool and incubated in an oxygen-reduced atmosphere at 35°C. The percentage of immobile treponemes was determined after 0, 4, 8 and 18 h by examination of 100 treponemes by dark-field microscopy. Each test was run in duplicate on different days.

SDS-PAGE and immunoblotting

T. pallidum antigen and human milk lysozyme were electrophoresed on SDS-PAGE 12.5% gels in a discontinuous buffer system according to Laemmli [13]. For calibration of mol. wt, a low mol. wt calibration kit (Pharmacia) was used. The semi-dry blot system [14] was used to transfer separated proteins on to nitrocellulose membrane (0.2 μ m, Schleicher and Schuell Co., Germany). The membrane was incubated for 1 h in PBS containing low-fat milk powder 5% for blocking. The treponemal antigen and the lysozyme were detected with rabbit anti-treponemal and rabbit anti-lysozyme antibodies, respectively, each diluted 1 in 100 in PBS, followed by incubation with peroxidase-conjugated anti-rabbit antibodies and substrate-reaction with a mixture of 8 mg of 4-chloro-1-naphthol in 4 ml of methanol and 25 μ l of H_2O_2 30% in 6 ml PBS.

Rheumatoid factors

The presence of rheumatoid factors was determined with a latex agglutination assay ('Rapitex', Behring, Germany) according to the manufacturer's instructions. Results were confirmed by particle enhanced nephelometry ('N Latex RF', Behring) in the Behring Nephelometer system according to the manufacturer's instructions. The detection limit was 9.1 IU/ml. Sera > 20 IU/ml were considered as rheumatoid factor positive.

Removal of rheumatoid factors from sera

Patient serum (40 μ l) was mixed with 160 μ l of an aqueous 1% suspension of polystyrene particles coated with human Gamma-globulin ('RapiTex RF new', Behring) and incubation for 15 min at 37°C. Particles were sedimented by centrifugation at 10 000 g for

5 min. The supernate was tested again for rheumatoid factors.

Results

The rates of immobilisation of *T. pallidum* with a high TPI titre serum (50% TPI titre 1200) and a low titre serum (50% TPI titre 40) were dependent upon the lysozyme concentration and are shown in Fig. 1. Immobilisation of *T. pallidum* was observed under lysozyme-free conditions. However, the lack of lysozyme had a greater influence on the immobilisation rate with the low-titre serum. Fig. 2 shows the immobilisation rates obtained with an IgM-enriched IgG-free serum fraction and with purified IgG under lysozyme-free conditions and in the presence of hen egg white lysozyme 0.5 and 5 μ g/ml. Purified treponemal specific IgG as well as the IgM-enriched fraction caused immune immobilisation in the absence of lysozyme. Hen egg white lysozyme inactivated by treatment with DTT had no effect on the immobilisation of *T. pallidum* at concentrations up to 50 μ g/ml (Fig. 3). Therefore, the effect of lysozyme on the

Fig. 1. Immobilisation of *T. pallidum* versus time and concentration of hen egg white lysozyme (HEL: $\square$, < 0.1 μ g/ml; $\square$, 0.5 μ g/ml; $\square$, 5 μ g/ml) in the presence of complement and (a) high TPI titre serum (50% immobilisation titre 1200) and (b) low TPI titre serum (50% immobilisation titre 40) (mean and SD, n = 8).

Fig. 2. Immobilisation of *T. pallidum* versus time and concentration of hen egg white lysozyme (HEL: □, 0.1 µg/ml; ▨, 0.5 µg/ml; ▩, 5 µg/ml) in the presence of complement and (a) purified treponemal specific IgG and (b) purified treponemal specific IgM (mean and SD, $n = 6$).

Fig. 3. Effect of enzymically inactive lysozyme (DTT-lysozyme: ▩, 5 µg/ml; ▧, 50 µg/ml) on the immobilisation of *T. pallidum* by *T. pallidum* immune serum (50% immobilisation titre 1200) in comparison with the effect of enzymically active (▨, 5 µg/ml) and no lysozyme (□, <0.1 µg/ml) (mean and SD, $n = 6$).

immune immobilisation of *T. pallidum* was mediated exclusively through the enzymically active form. In the absence of treponemal antibodies and complement, enzymically active lysozyme at concentrations of 5, 10 and 100 µg/ml did not influence the mobility of

treponemes. After incubation for 18 h, 100% of treponemes were mobile with each lysozyme concentration compared to the control without lysozyme. Even in the presence of an excess of active complement but in the absence of treponemal specific antibodies, lysozyme 100 µg/ml failed to affect the mobility of *T. pallidum* and 100% of treponemes remained mobile after 18 h.

Fifty-nine of 99 sera from patients with syphilis and one of 50 sera from healthy blood donors were active in the lysozyme antibody ELISA. In the sera from patients with untreated secondary syphilis, 100% reactivity was observed, but only 30% reactivity was noted at the untreated primary stage. Twenty-eight of the 59 lysozyme antibody-positive sera of syphilis patients were tested for rheumatoid factors. A positive result was obtained with 39.3% (11 of 28) of the sera. None of 28 sera from 40 syphilis patients with a negative lysozyme antibody ELISA result had rheumatoid factors. False positive results in the lysozyme antibody ELISA caused by rheumatoid factors were excluded. The removal of rheumatoid factors from lysozyme antibody-positive sera did not lead to a loss of reactivity in the lysozyme antibody ELISA. Cross-reactions between lysozyme and *T. pallidum* antigen were excluded by immunoblotting. No bands were observed when *T. pallidum* antigen was incubated with lysozyme antibodies, and similarly when human lysozyme was incubated with treponemal specific antibodies. Lysozyme antibody-positive serum showed a similar course of immobilisation to lysozyme antibody-negative serum (Fig. 4). A concentration of

Fig. 4. Effect of human lysozyme (HuL: □, none; ▨, 0.1 µg/ml; ▩, 0.5 µg/ml; ▧, 1 µg/ml) on the rate of immobilisation of *T. pallidum* in the presence of lysozyme-antibody positive syphilitic serum (serum 1) and lysozyme-antibody negative syphilitic serum (serum 2). Both sera were rheumatoid factor negative.

human as lysozyme as low as 0.1 $\mu\text{g/ml}$ caused an increase in the immobilisation rate in the lysozyme antibody-containing serum as well as in the lysozyme antibody-free serum.

Discussion

The mechanism of antibacterial activity of lysozyme is only partly understood [15]. It is well established that lysozyme hydrolyses the 1,4 β -glycosidic bond between N-acetyl-glucosamine and N-acetylmuramic acid in the murein layer. Furthermore, Pellegrini and co-workers [9, 16] found bactericidal activity resulting from the cationic nature of the protein in the cytoplasm of *E. coli* independent of enzymic activity. The present study revealed that lysozyme exerted an exclusively enzymic effect on *T. pallidum*. DTT-treated enzymically inactive lysozyme did not influence the lysis of treponemes in the TPI assay. A further difference between *E. coli* and *T. pallidum* in the mechanism of lysozyme action is that lysozyme acts on treponemes only in the presence of antibodies and complement. In contrast to *T. pallidum* [2, 4], lysozyme can penetrate into *E. coli* through the outer membrane and cell wall independent of antibody- and complement-mediated damage [9]. This finding may be attributed to the lack of lipopolysaccharide (LPS) in the outer membrane of *T. pallidum* [17]. The bactericidal permeability-increasing protein (BPI) which is released from granulocytes binds to the LPS of gram-negative bacteria and causes destruction of the barrier function of the outer membrane [18] which, in turn, permits an antibody- and complement-independent lysozyme influx into the cell. This membrane-destabilising effect of BPI does not operate with *T. pallidum* and, hence, the preconditions for an antibody- and complement-independent lysozyme influx do not exist. An essential role for lysozyme in addition to treponemal specific antibodies and complement for immobilisation and lysis of *T. pallidum* was not detectable *in vitro*. Thus, the present results obtained with treponemicidal antibodies in sera from infected rabbits or patients with syphilis are consistent with the data reported by Engelkens *et al.* [19] for natural antibodies with immobilising activity in normal human serum. Hederstedt first described these antibodies in 80% of healthy individuals [20, 21]. In contrast to the treponemicidal antibodies from patients with syphilis, these natural antibodies, which are not yet well characterised, belong predominantly to the IgM class [21]. Müller *et al.* [4] used only the IgG fraction from human syphilitic sera, but did not demonstrate the role of lysozyme in the immobilisation of treponemes by treponemal specific IgM. Engelkens *et al.* [19] studied the IgG and IgM fractions with immobilising activity from normal human serum. The results of the present study clearly show the *in-vitro* independence of *T. pallidum* immobilisation by separated treponemicidal IgG and IgM from lysozyme. The possibility that antibodies,

complement and lysozyme might have predamaged the treponemes during their passage in the rabbit testes was ruled out by controls. The lower the concentration of immobilising antibodies, the more pronounced was the quantitative dependence of the kinetics of immobilisation on the lysozyme concentration. IgM-immobilising antibodies exhibited a longer delay before immobilisation in the absence or in the presence of low lysozyme concentrations than did IgG. Immobilising antibodies that attack the endoflagella of treponemes are known to enter the periplasmic space. The murein layer of the bacterium, which is connected to the outer membrane, undergoes lysozyme-enhanced destruction and this results in the destabilisation of the membrane. Under lysozyme-free conditions this process requires more time than it does in the presence of an excess of lysozyme. We propose that IgM antibodies, due to their size, require a more pronounced destruction of the outer membrane than IgG antibodies in order for penetration into the periplasmic space to take place. If this hypothesis is correct, lysozyme would be particularly important in the early stages of infection (high IgM levels), whereas in the ongoing immune response IgG becomes predominant and the importance of lysozyme would be diminished. The most effective killing of treponemes is likely to take place with a combination of treponemal outer-membrane-specific IgM and endoflagella-specific IgG. Engelkens *et al.* [18] have postulated that the course of treponemal immobilisation begins with the primary disintegration of the outer membrane by IgM and complement; this in turn is followed by the degradation of murein by lysozyme and immobilisation of endoflagella by IgG.

Lysozyme antibodies were studied to determine whether they influenced lysozyme activity. Schmitt *et al.* [22] recently described the occurrence of lysozyme antibodies in the sera of patients with inflammatory renal, bowel and rheumatological diseases. The present study demonstrated the existence of lysozyme antibodies in 59.6% of syphilis patients for the first time. The fact that lysozyme antibodies in syphilis sera did not influence the kinetics of the TPI assay even in the presence of low concentrations of lysozyme (0.1 $\mu\text{g/ml}$) excludes a detectable neutralising effect of these autoantibodies. Therefore, the likelihood that lysozyme is inhibited by lysozyme antibodies in the serum of syphilis patients is negligible. Hence, it is unlikely that the elimination of bacteria from the infected host is impaired in patients with lysozyme antibodies. The occurrence of rheumatoid factors in 39.3% of syphilis patients with lysozyme antibodies is a further example of an autoimmune component in syphilis and has been reported previously by Baughn *et al.* [23]. It remains unclear whether or not, and how, these autoantibodies are involved in the pathogenesis of *T. pallidum* infection.

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