Properties of equine anti-lipopolysaccharide hyperimmune plasma: binding to lipopolysaccharide and bactericidal activity against gram-negative bacteria

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Summary. Anti-lipopolysaccharide equine hyperimmune plasma (anti-LPS), which has been used successfully to treat LPS (endotoxin)-mediated disorders, has been further characterised. IgG present in anti-LPS had the highest affinity for LPS prepared from Salmonella typhimurium, S. typhi, S. abortus equi and Shigella flexneri and intermediate affinity for Escherichia coli O55:B5, E. coli O127:B8 and S. enteritidis. Anti-LPS destroyed by means of complement activation a wide range of gram-negative bacteria, including various species and strains of Klebsiella, Enterobacter, E. coli, Sh. flexneri, Providencia, Salmonella and Pseudomonas. Control plasmas or saline had little or no effect. Maximum killing occurred within seconds to minutes. Electron microscopy showed that anti-LPS treatment of K. pneumoniae caused extensive cell wall and cytoplasmic membrane disruption, followed by the appearance of spheroplasts and cell ghosts. Antibodies were required in 100 000-fold excess to inhibit the limulus amoebocyte lysate reaction with LPS from E. coli. Anti-LPS thus contains IgG that binds to a wide range of LPS, and can destroy a wide range of gram-negative bacteria by means of complement activation.

Introduction

A major component of the high mortality (30-80%) and morbidity in bacteraemia with gram-negative species has been attributed to endotoxin (LPS, lipopolysaccharide), an integral part of the cell wall of gram-negative bacteria (Duswald et al., 1982). LPS is chemically very stable and highly toxic, being lethal in man at concentrations as low as 1 ng/ml of plasma (Wardle, 1975). Conventional antibiotic therapy for gram-negative bacteraemia does not reduce the concentration or activity of the LPS. Moreover, large amounts of LPS may be released into the bloodstream by destruction of the bacteria by antibiotics (Shenep and Mogan, 1984). Immunotherapy directed specifically at LPS has enjoyed increased attention (Gaffin, 1983a). Preparations of human antibodies directed against core glycolipid or other regions of the LPS molecule reduced mortality and morbidity in human patients with shock (Ziegler et al., 1982; Lachman et al., 1984a and b) as well as in animal models of endotoxic shock caused by haemorrhage (Gaffin et al., 1981) and bacteraemia (Gaffin et al., 1985a).

An equine anti-LPS hyperimmune plasma (anti-LPS) has been produced by active immunisation of horses. Such a preparation has been shown to be effective in the therapy and prophylaxis of gram-negative bacteraemia and endotoxic shock caused by intestinal ischaemia (Zanotti and Gaffin, 1984; Gaffin et al., 1986), radiation sickness (Gaffin et al., 1985b) and superior mesenteric artery occlusion shock (Gathiram et al., 1986), and in various experimental animal models and in veterinary clinical practice (Gaffin et al., 1982b; Thomson, 1983; Welsh et al., 1984; Wessels and Gaffin, 1986; Wessels et al., 1986). In a preliminary report, anti-LPS antibodies were also shown to bind to the LPS present on the surface of living bacteria (Gaffin et al., 1982c) and, by so doing, activate complement and destroy klebsiella organisms. Such bactericidal properties of anti-LPS were exploited in treating pseudomonas keratitis (Welsh et al., 1984). Other studies in this laboratory have shown that human anti-LPS IgG also increased the phagocytosis of gram-negative bacteria by neutrophils (Pudifin et al., 1985).

This present study was undertaken to further characterise the bactericidal properties and spectrum of activity of antibodies in the equine anti-LPS preparation.
Materials and methods

Anti-LPS

Anti-LPS-rich equine plasma was collected by plasmapheresis under sterile conditions from horses immunised by a proprietary procedure ("ATOXIN", ATOX Pharmaceutical Co., 14 Old Main Road, Gilletts 3600, South Africa). Anti-LPS (ATOXIN), presented as a frozen solution of citrated plasma containing no preservative, was stored at −20°C until used within 1 week after collection. The final pooled plasma contained anti-LPS IgG at a concentration of 1200 μg/ml according to an enzyme linked immunosorbant assay (ELISA) (Gaffin et al., 1982a) calibrated by an immunoprecipitation procedure (Gaffin et al., 1981).

ELISA procedure

Anti-LPS plasma was centrifuged for 5 min at 23 000 g and then diluted in sterile pyrogen-free saline 1 in 2, 1 in 4, 1 in 6, 1 in 8 and 1 in 10. Triplicate samples of diluted anti-LPS plasma were diluted 1 in 200 in 0·05 M Tris, adjusted to pH 8·0, and 2% each of sheep and bovine plasma. The LPS-precipitable IgG of anti-LPS equine plasma was then assayed with plates coated with 14 endotoxins at a concentration of 10 μg/ml in a carbonate-bicarbonate buffer, pH 9·6. The 14 endotoxins were obtained from Difco (Detroit, USA); they had been extracted from the parent bacteria by the procedure of Westphal et al. (1952). They were from: S. typhimurium, S. minnesota, S. abortus equi, S. enteritidis, S. typhosa (S. typhi), Sh. flexneri, Serratia marcescens, E. coli O127:B8, E. coli O111:B4, E. coli O26:B6, E. coli O55:B5, E. coli O128:B12, K. pneumoniae and Ps. aeruginosa. The general ELISA microplate method used here has been previously described for alpha fetoprotein assay and other applications (Engvall and Perlmann, 1972; Conradie et al., 1980). Microtitration plates (Dynatech M129B) were coated with a mixture of endotoxins for 2 h at room temperature. Conjugate of antibody to equine IgG with horseradish peroxidase was made according to the method of Wilson and Nakane (1978). Results were measured by absorption at 495 nm and calibrated by an immunoprecipitin reaction (Gaffin et al., 1981) with an equine anti-LPS IgG standard. With the latter standard, anti-LPS IgG was quantitated as described.

Control plasmas

Non-immune plasma was collected from horses before immunisation and treated as described above. The pooled plasma contained anti-LPS IgG at a concentration of 14·9 μg/ml.

Absorption with whole bacteria. Anti-LPS was absorbed thrice with a mixture of gram-negative bacteria (untyped hospital isolates of S. typhimurium, Ps. aeruginosa, Proteus vulgaris, E. coli, K. pneumoniae, Sh. flexneri and Entrobacter spp.) as previously described (Gaffin et al., 1982c). The treated plasma was filtered through a 0·45-μm membrane filter (Millipore) into sterile tubes and stored at −20°C. After this treatment, the LPS-specific IgG concentration was reduced from 1200 μg/ml to 10·2 μg/ml.

Absorption with purified LPS. An LPS solution containing 10 different types of LPS, each at a concentration of 20 μg/ml was incubated with an equal volume of anti-LPS for 30 min at 37°C. The type of LPS (Difco, Detroit, USA) used included E. coli O55:B5, E. coli O127:B8, E. coli O128:B12, E. coli O111:B4, Sh. flexneri, S. abortus equi, S. typhimurium, S. enteritidis, S. typhi and K. pneumoniae. The plasma was clarified and filtered as described above. After this treatment, the LPS-specific IgG concentration was reduced to 7·5 μg/ml.

Complement inactivation

Anti-LPS plasma was heated at 56°C for 30 min. Thereafter it was clarified, filtered and stored as described above.

Binding of IgG to LPS

This was measured by the ELISA procedure previously described (Gaffin et al., 1982a). Microtitration plates were coated with one of each of the following types of LPS at a concentration of 10 μg/ml (as required): E. coli O111:B4, E. coli O55:B5, E. coli O127:B8, E. coli O128:B12, Sh. flexneri, S. abortus equi, S. typhimurium, S. typhi, S. enteritidis and K. pneumoniae. Each LPS tested had 247 individual readings. Since the appropriate standard for each individual LPS is, unfortunately, not available commercially, results are presented as the percentages of the total relative binding rather than as quantitative measurements in μg/ml. All experimental data were then pooled and analysed statistically.

Inhibition of limulus amoebocyte lysate (LAL) LPS assay

Inhibition reaction. Normal non-immune equine plasma was mixed with 4·0 ng/ml of LPS (E. coli O111:B4) in the presence of increasing amounts of anti-LPS, saline or non-immune control plasma and incubated for 30 min at room temperature. LPS concentrations were then determined by the LAL assay.

LAL assay. LPS concentrations were determined in plasma by the chromogenic substrate modification of the LAL method as previously described (Cohen and McConnell, 1984) (MA Bioproducts). Results were standardised with calibration curves employing a reference LPS (E. coli O111:B4). The calibration curves were sensitive to LPS from 0·02 ng/ml and were linear for LPS concentrations from 0·02 to 0·2 ng/ml.

Measurement of plasma LPS by the LAL technique has been criticised in the past (Elin, 1979). Some plasma samples are known to contain non-specific activators that may cause false-positive results in the LAL test. A possible cause of inhibition is preformed antibody to
lipid A as suggested by Young (1975). Others have suggested that serum complement is required for neutralisation (Johnson and Ward, 1972) but, again, this system cannot be operating in the heat-treated plasma used in the e studies. Several complicated methods have been prr posed for the removal of plasma inhibitors to the LAL reaction (Du Bose et al., 1980), generally requiring conditions scrupulously free of pyrogen. Methods involving a combination of heating and diluting the sample are most convenient and effective, and have been almost universally adopted. Recent studies have shown that when properly performed, the chromogenic substrate modification of the LAL test provides a quantitative measure of LPS in human blood (Cohen and McConnell, 1984). When this method was used to compare the recovery of LPS from spiked pyrogen free saline and pooled plasma (measured simultaneously over an appropriate range of concentrations), no discrepancies in the values obtained in the two solutions were found (unpublished results).

Bactericidal activity

Bacteria. Isolates of pathogenic multiresistant gram-negative bacteria were obtained from the Microbiology Department, King Edward VIII Hospital, Durban and the University of Natal, Pietermaritzburg. All cultures were grown in Brain Heart Infusion Broth (BHIB) for 16 h at 37°C. Culture homogeneity was verified by the API 20E (Path-Ident) system (Smith et al., 1972). Bacteria were centrifuged, washed twice with sterile 0.9% saline and resuspended in saline. Plasma bactericidal activity was assayed by standard plate-count techniques and verified by transmission electronmicroscopy. (TEM).

Plasma bactericidal activity by standard plate counts. Anti-LPS (2 ml) was incubated with 1 ml of bacterial suspension (106 organisms/ml) and 0.5 ml of BHIB at 37°C for 10 min. The same medium with saline or non-immune control plasma served as positive controls. Bactericidal activity was then assessed by standard plate-count techniques. Plates were incubated for 24 h at 37°C and the average colony counts were determined. The 0 min control in saline was taken to be 100% and the percentage increases or decreases with time were calculated for all treatment groups. All data were combined and analysed by Student's t test; results were considered to be significant at the 5% level, i.e., p < 0.05.

Transmission electronmicroscopy. The total bacterial count of K. pneumoniae was adjusted to 1 x 108/ml. A 2-ml sample of bacterial suspension was added to 4 ml of anti-LPS plasma, incubated at 37°C for 5 min and placed on ice. The mixture was then centrifuged at 2000 g for 5 min at 0°C. The bacterial cell pellet was resuspended in glutaraldehyde 1% in 2 M sodium cacodylate (SC) buffer at pH 7.4 for 20 min. Thereafter, the bacteria were washed in SC buffer, post fixed in osmium tetroxide 1% in SC buffer at 4°C for 20 min, before dehydration through ascending grades of ethyl alcohol. At each stage during the processing schedule, the suspension was centrifuged at 3000 g for 3 min to form a pellet. Finally the pellet was embedded in Araldite resin. Ultrathin sections (50–70 nm) were cut with glass knives on a Reichert “Ultracut” Ultramicrotome. The resulting sections were floated on distilled water and mounted on uncoated copper grids. Sections were double stained in uranyl acetate 1% and Reynolds lead citrate. Electronmicrographs were obtained with a Zeiss Em 10B electron microscope. Morphometric measurements of bacterial cells were made from electronmicrographs with a VIDS image analyser set.

Results

Specificity of LPS binding

The ELISA colour development and, hence, the binding of anti-LPS IgG to several different LPS preparations is shown in fig. 1. The relative binding was highest to LPS extracted from S. typhimurium (16.6%), S. typhi (13.6%), S. abortus equi (12.8%), Sh. flexneri (11.1%) and E. coli O55:B5 (10.5%). Relatively lower binding was observed to the other bacterial LPS preparations.

Inhibition of LAL assay

Anti-LPS reduced the apparent concentration of LPS in a “spiked” plasma solution containing E. coli O111:B4 LPS 4 ng/ml according to the LAL assay (fig. 2). This anti-toxic effect was directly related to the concentration of IgG present in the reaction mixture—50% reduction with anti-LPS IgG 150 µg/ml and 99% reduction with 540 µg/ml. In contrast, non-immune control plasma with a negligible anti-LPS IgG titre, effected only a 10% reduction in the level of LPS.

Bactericidal activity

The presence of anti-LPS significantly reduced the number of viable organisms detected by plate counts for 24 different strains of gram-negative bacteria after 10 min (fig. 3); i.e., percentage count in anti-LPS groups at 10 min (n = 8) was significantly lower than the percentage count at 0 min (n = 8) for each strain (Student's t test; t = >8; p = 0.001). This effect was not seen when these strains were tested in the saline control and in the control plasmas absorbed with either whole bacteria or LPS. Many of these bacteria showed a marked resistance to a wide range of antibiotics (table I). Variation in the sensitivity of species to anti-LPS was observed. Amongst Pseudomonas species, a mean value of 94% of cfu were destroyed after anti-LPS treatment. Anti-LPS had a similar bactericidal
Fig. 1. The relative binding of anti-LPS equine hyperimmune plasma to LPS preparations from different strains of bacteria measured by ELISA. The height of each bar represents the reactions of the antibodies present in the plasma with each endotoxin. Westphal LPS preparations were made from: *E. coli* O55:B5, (1), *E. coli* O127:B8 (2), *E. coli* O128:B12 (3), *E. coli* O111:B4 (4), *Sh. flexneri* (5), *S. abortus equi* (6), *S. typhimurium* (7), *S. typhi* (8), *S. enteritidis* (9), *K. pneumoniae* (10).

Effect against *Proteus* spp. and *Providencia* spp. For one *Enterobacter* strain (fig. 3; no. 5) 63% of cfu were destroyed by anti-LPS. Whilst anti-LPS treatment resulted in a 99% reduction in cfu of a multiresistant *Ent. cloacae* (fig. 3, no. 7). Three species of *Salmonella* showed a mean reduction of 75% in cfu after anti-LPS treatment (fig. 3, nos. 22-24). A strain of *Klebsiella* resistant to seven of the antibiotics tested (table I), and which was most resistant to anti-LPS (49% destruction; fig. 3; no. 4), was later shown by electronmicroscopy to possess an extensive capsular slime region (Wells and Gaffin, 1986).

When anti-LPS was mixed with the bacteria for various periods the reaction between them occurred in a matter of seconds (fig. 4). Within the first minute of mixing, the number of viable *Prov. alcalifaciens* in the anti-LPS group had been reduced by 90.5%; i.e., from $100 \pm 4.057\%$ (n = 4) at 0 min to $9.5 \pm 0.26\%$ (n = 4) at 1 min (t = 22-245; $p < 0.001$). After 60 min the count was reduced only slightly more. By contrast, when treated with saline and control absorbed plasma samples, the number
Fig. 3. Bactericidal activity of anti-LPS against gram-negative bacteria (multiresistant strains indicated by *). Bacteria were incubated for 10 min at 37°C with control plasma (□), or anti-LPS (■), or saline (defined as 100% growth) and then plated on to nutrient agar. Control plasma was prepared by absorbing anti-LPS plasma with various gram-negative bacteria. Key: *Klebsiella* spp. (1), *K. oxytoca* (2), *K. pneumoniae*, (3), *Klebsiella* spp.* (4), *Enterobacter* spp. (5), *Ent. aerogenes* (6), *Ent. cloacae* (7), *E. coli* (8), *E. coli* (9), *P. vulgaris* (10), *P. mirabilis* (11), *Sh. flexneri* (12), *C. freundii* (13), *Pro. alcalifaciens* (14), *Pro. stuartii* (15), *P. rettgeri* (16) *Ps. aeruginosa* (17), *Ps. aeruginosa* (burn wound) (18), *Ps. aeruginosa* 322 (19), *Ps. syringae* (20), *Ps. fluorescens* (21), *S. typhi* (22), *S. typhimurium* (23), *S. anatum* (24).

of viable organisms had increased significantly by a factor of two during this 60-min interval. Percentage viable counts in saline increased from 110-91 ± 4-02% (n = 4) at 0 min to 275-05 ± 5-96% (n = 4) at 60 min (t = 7-0248; p < 0-001). This bacterium was sensitive to only four of the 12 antibiotics assayed (disk diffusion method) namely, kanamycin, amikacin, cefamandole and cefuroxime (table I).

Complement inactivated or decomplemented anti-LPS showed a complete loss of bactericidal activity as evident by increased bacterial numbers in six of seven strains following treatment (fig. 5). A multiresistant strain of *K. pneumoniae* was the only exception (fig. 5, no. 6). When complement was added to decomplemented anti-LPS, the mixture was again able to destroy bacteria to the same extent as the original anti-LPS (data not shown). Non-immune horse plasma alone reduced bacterial cell counts of all seven isolates from 99-99 ± 3-24% (n = 21) at 0 min to an average of 67-9 ± 3-6% after 40 min (t = 6-52; p < 0-001) of initial values (fig. 5). However anti-LPS reduced counts of all seven isolates from 99-99 ± 3-24% (n = 21) at 0 min to an average of 11-93 ± 2-0% after 40 min (t = 23-24; p < 0-001).

Electronmicroscopy

Microscopy of control *Klebsiella* spp. at low magnification showed a population of densely staining cells (fig. 6a), observed as typical cylindrical bacilli with well defined nuclear and cytoplasmic areas and two tri-laminar unit membranes (fig. 6b); several capsulated cells were also seen. The average volume of the control cells was 0-55µm³.
Table. Antibiotic susceptibility* of 11 strains of gram-negative bacteria

<table>
<thead>
<tr>
<th>Bacterium strain</th>
<th>Susceptibility to antibiotic†</th>
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<tr>
<td></td>
<td>1 2 3 4 5 6 7 8 9 10 11 12</td>
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<tr>
<td>K. oxytoca</td>
<td>R R R S R R ... ... ... ...</td>
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<tr>
<td>K. pneumoniae</td>
<td>R R R R R S R S S ... I</td>
</tr>
<tr>
<td>Klebsiella spp.</td>
<td>R R R R R S R S S I S</td>
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<tr>
<td>Ent. aerogenes</td>
<td>R R R R R S S R S S S</td>
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<tr>
<td>Ent. cloacae</td>
<td>R R R R R S S R S S S</td>
</tr>
<tr>
<td>E. coli</td>
<td>R R R R R R S S S S ...</td>
</tr>
<tr>
<td>C. freundii</td>
<td>R R R R R R R R R R</td>
</tr>
<tr>
<td>Prov. alcalifaciens</td>
<td>R S R R R R R R S I S</td>
</tr>
<tr>
<td>Prov. stuarti</td>
<td>R S R R R R ... ... ... ...</td>
</tr>
<tr>
<td>P. rettgeri</td>
<td>R R R R R R ... ... ... ...</td>
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<tr>
<td>Ps. aeruginosa</td>
<td>... ... ... R ... S R ... R ...</td>
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* Disk diffusion method.
† Key 1 = ampicillin 10 μg, 2 = cotrimoxazole 25 μg, 3 = chloramphenicol 10 μg, 4 = cephalothin 30 μg, 5 = gentamicin 10 μg, 6 = kanamycin 30 μg, 7 = amikacin 30 μg, 8 = carbenicillin 100 μg, 9 = cefoxitin 30 μg, 10 = cefamandole 30 μg, 11 = netilmicin 30 μg, 12 = cefuroxime 30 μg. S = sensitive; R = resistant; I = intermediate; ... = not tested.

After incubation for 5 min with anti-LPS equine plasma, the bacteria exhibited various morphological abnormalities. Lower power micrographs showed fewer cells than the controls with far greater intercellular distances (figs. 6c and d) and the presence of extracellular debris. Various stages of the antibody-complement-bacteria interaction were observed. There was early separation of the cell wall and the cytoplasmic membrane evidenced by electron-lucent areas separating the outer LPS layer and the cytoplasmic membrane, increasing the total cell volume to 0.92 μm³. There were spheroplasts with an average volume of 1.08 μm³ and ghost bodies resulting from partial or total disruption of the cytoplasmic membrane. Rupture of the cytoplasmic membrane caused discharge of the cytoplasm into the extracellular space.

Discussion

LPS is toxic to man and many animals at a plasma concentration of only 1 ng/ml (Wardle, 1975). The failure of antimicrobials in some patients with gram-negative bacteraemia is due, in part, to the inability of such agents to reduce the concentration or activity of the LPS endotoxin. This has led to an interest in immunotherapy as a possible alternative or complementary form of treatment (Ziegler et al., 1982; Gaffin, 1983a; Lachman et al., 1984a and b).

Fig. 4. Time dependence of the bactericidal activity of anti-LPS against Prov. alcalifaciens. Bacteria were allowed to react with saline (■, 1), control plasma which had been absorbed with several gram-negative bacterial isolates (▲, 2), and anti-LPS (●, 3). Each point was a mean of four readings.

A major problem in developing practical immunotherapy had been the large number of possible antigens on bacteria (Williams et al., 1983). One approach has been to seek protective antigens common to more than one species of gram-negative bacteria and thus reduce the number of vaccines required. The E. coli J5 mutant produces an incomplete LPS consisting of the central LPS region, called core glycolipid, which is similar amongst a range of different gram-negative bacteria. This led to the important study of the treatment of patients in shock with passive anti-J5 human antiserum by Ziegler et al. (1982). While undoubtedly effective, practical application of this therapy is limited because stocks of hyperimmune antibodies are expensive and difficult to prepare in large amounts (Stiehm, 1979; Young, 1984). The devel-
KILLING OF GRAM-NEGATIVE BACTERIA BY ANTI-LPS

Fig. 5. Role of antibody and complement on percentage survival of gram-negative bacteria after plasma treatment as determined by cfu plate counts. E. coli (1), Ent. cloacae (2), P. mirabilis (3), K. oxytoca (4), Prov. alcalifaciens (5), K. pneumoniae (6), Ps. aeruginosa (7). Incubation was 37°C for 40 min with 0.9% NaCl (■), anti-LPS plasma decomplemented by heating at 56°C for 30 min (▲), normal plasma obtained before immunisation (□), or anti-LPS (□).

Development of human monoclonal antibodies may become practical in the future (Young, 1984).

Some problems of producing clinically useful amounts of human anti-LPS antibodies were partially solved by the recognition that certain people have high concentrations of natural anti-LPS IgG in their blood (Gaffin et al., 1982a). An ELISA was developed and a simple routine procedure was established to screen donated plasma units in a blood bank for the presence of anti-LPS antibodies. Approximately 7% of units contained high concentrations (>40 µg/ml) of these antibodies (Gaffin, 1983b). These units were either lyophilised or pooled and fractionated into a γ globulin (Gaffin et al., 1985a).

Anti-endotoxin antibodies may have two main beneficial properties. By binding to the lipid A region they may neutralise the toxic region of endotoxin and, by binding to the outer O saccharide region, they may speed the uptake or clearance of LPS by the reticuloendothelial system (opsonisation). Whereas serum killing of bacteria is a well known phenomenon, there are few reports to suggest that the killing of gram-negative bacteria by autologous anti-LPS antibody is clinically important.

In agreement with the findings of other studies (Gaffin et al., 1982b, 1985a), our anti-LPS contained IgGs that could bind to LPS prepared from a wide range of gram-negative bacteria (fig. 1). Studies are currently in progress to determine the degree of specificity of equine anti-LPS IgG antibodies. IgG antibodies reduced the LAL reactivity towards LPS (fig. 2) and probably also the toxicity of LPS, since LAL reactivity is believed to be in the toxic regions of lipid A (Wildfeuer et al., 1974). Complete neutralisation in the LAL assay of E. coli O111: B4 LPS 4 ng/ml required the addition of anti-LPS IgG 540 µg/ml. This represents a mass ratio of 1:135 000 of LPS: anti-LPS and, assuming molecular masses of 400 000 and 150 000 for LPS and IgG, respectively, a molar ratio of approximately 1:360 000. The great excess of antibody required probably reflects both the low concentration of anti-lipid A IgG present in anti-LPS, as well as the much greater affinity of LPS for LPS-binding enzyme in LAL than for specific antibody (Wildfeuer et al., 1974) i.e. the anti-LPS antibodies appear to be directed mainly against the outer O antigenic sites. Other studies have shown that plasma treatment caused LPS to lose its ability to induce fever (Ng et al., 1976; Johnson et al., 1977).
Fig. 6. TEM observations of the bactericidal effect of anti-LPS and control non-immune plasma. (A) *K. pneumoniae* with control non-immune plasma. (B) Identical field at higher magnification showing the composite nature of the cell wall. (C) *K. pneumoniae* after treatment with anti-LPS; the intercellular distance is increased, the cell wall is damaged and the cytoplasmic membrane is separated (arrow); ghost bodies (G), lysed bacterial cells (L) and spheroplasts (S) are evident. (D) Identical field of study showing bacilli with increased cell volume and spheroplast formation with total disruption of the cytoplasmic membrane.
Our in-vitro observations are supported by results obtained in anti-LPS equine plasma treatment of canine endotoxaemia associated with haemorrhagic enteritis (Wessels et al., 1986). These dogs had significantly elevated circulating plasma LPS concentrations on admission to hospital of 0.368 ± 0.082 ng/ml. A subcutaneous dose of polyvalent equine anti-LPS hyperimmune plasma 0.5 ml/kg significantly reduced circulating plasma LPS concentrations to normal levels of 0.058 ng/ml within 24 h (p < 0.001) (Wessels et al., 1986). Similar results have been recorded in dogs with canine parvovirus disease (Wessels and Gaffin, 1986).

It is possible that, in our anti-LPS hyperimmune equine plasma, there are anti-gram-negative bacterial antibodies other than those specific to LPS which also contribute to the observed bactericidal effect of anti-LPS; currently, this possibility cannot be excluded.

Of special interest was the destruction of *Ps. aeruginosa* by anti-LPS, because pseudomonas infections are serious problems in war-related injuries (Walker et al., 1982) and in burns (Collins and Roby, 1982). Anti-LPS had generally similar activities against five strains of *Pseudomonas* including one that was multiresistant (fig. 3). Related studies showed that mice which received prophylactic human anti-LPS and were then infected with an LD80 dose of *Ps. aeruginosa* had a mortality of only 8% (Gaffin et al., 1985a). Antibiotic therapy is not always effective in the treatment of pseudomonas infections (Collins and Roby, 1982; Pegg et al., 1982).

As shown in the microbiological and ultrastructural studies, IgG not only bound to extracted LPS but also to that LPS which was still an integral part of the cell wall of living bacteria (fig. 3). The resulting complement activation caused a 50–94% reduction in bacterial counts after 10 min. When the anti-LPS had been previously absorbed with a mixture of gram-negative bacteria or pure LPS (fig. 3), or had been heat treated (fig. 5), its bactericidal effect was lost. Normal plasma containing low antibody titres, obtained from horses before immunisation, did show significant bactericidal properties (p < 0.001). However, anti-LPS hyperimmune antibodies had significantly greater bactericidal properties. Percentage cfu determinations were reduced to 67.9 ± 3.6%, and 11.9 ± 2.0% of 0-h values (fig. 5) after treatment for 40 min with normal non-immune plasma and anti-LPS plasma respectively; the counts in control non-immune plasma were significantly higher (t = 13.377; p < 0.001).

This finding is in agreement with previous studies reporting that normal serum, which is now known to contain small amounts of anti-LPS IgG, injures the peripheral structures of *E. coli* (Roantree and Pappas, 1960; Wilson and Spitznagel, 1968). The latter report showed that antibody and complement acted on the cell surface or LPS layer of gram-negative bacteria and resulted in a large release of phosphatides and a physical rearrangement of LPS or membrane lipoprotein, and caused the formation of pits or holes that might then allow further action on the cytoplasmic membrane. Selective permeability of the membrane would be destroyed and, in the absence of osmotic stabilisers, water would enter the cells, momentarily increasing intracellular pressure (Wilson and Spitznagel, 1968).

Anti-LPS thus has a combined effect: it destroys a wide range of gram-negative bacteria and binds to, and apparently neutralises, the LPS released.

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