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. Electronmicroscopy 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 $1200\text{ }\mu\text{g/ml}$ according to an enzyme linked immunosorbent 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 $23\,000\text{ }g$ 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 0.1 M NaCl containing 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 $10\text{ }\mu\text{g/ml}$ 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 $14.9\text{ }\mu\text{g/ml}$.

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 *Enterobacter* spp.) as previously described (Gaffin *et al.*, 1982c).

The treated plasma was filtered through a $0.45\text{-}\mu\text{m}$ membrane filter (Millipore) into sterile tubes and stored at -20°C . After this treatment, the LPS-specific IgG concentration was reduced from $1200\text{ }\mu\text{g/ml}$ to $10.2\text{ }\mu\text{g/ml}$.

Absorption with purified LPS. An LPS solution containing 10 different types of LPS, each at a concentration of $20\text{ }\mu\text{g/ml}$ 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 $7.5\text{ }\mu\text{g/ml}$.

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 $10\text{ }\mu\text{g/ml}$ (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 $\mu\text{g/ml}$. 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 studies. Several complicated methods have been proposed 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 (10^7 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 1×10^8 /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 0.2 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).

Fig. 2. Neutralisation of the limulus amoebocyte lysate assay by anti-LPS (●—●), and results with saline (■—■) and control non-immune plasma (◆—◆).

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 1), 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. freundt** (13), *Prov. alcalifaciens** (14), *Prov. stuarti** (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 $110.91 \pm 4.02\%$ ($n = 4$) at 0 min to $275.05 \pm 5.96\%$ ($n = 4$) 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 de complemented 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 de complemented 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 $99.99 \pm 3.24\%$ ($n = 21$) at 0 min to an average of $67.9 \pm 3.6\%$ after 40 min ($t = 6.52$; $p < 0.001$) of initial values (fig. 5). However anti-LPS reduced counts of all seven isolates from $99.99 \pm 3.24\%$ ($n = 21$) at 0 min to an average of $11.93 \pm 2.0\%$ 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 $0.53 \mu\text{m}^3$.

Table. Antibiotic susceptibility* of 11 strains of gram-negative bacteria

Bacterium strain	Susceptibility to antibiotic†											
	1	2	3	4	5	6	7	8	9	10	11	12
<i>K. oxytoca</i>	R	R	R	S	R	R	...	...	...	...	...	...
<i>K. pneumoniae</i>	R	R	R	R	R	R	S	R	S	S	...	I
<i>Klebsiella</i> spp.	R	R	R	R	R	R	S	R	S	S	S	I
<i>Ent. aerogenes</i>	R	R	R	R	R	R	S	R	R	I	I	I
<i>Ent. cloacae</i>	R	R	R	R	R	S	S	R	S	S	S	...
<i>E. coli</i>	R	R	R	R	R	R	S	R	S	S	...	...
<i>C. freundii</i>	R	R	R	R	R	R	...	...	...	...	...	...
<i>Prov. alcalifaciens</i>	R	R	R	R	R	S	S	R	R	S	I	S
<i>Prov. stuarti</i>	R	S	R	R	R	R	...	...	...	...	...	...
<i>P. rettgeri</i>	R	R	R	R	R	R	...	...	...	...	...	...
<i>Ps. aeruginosa</i>	...	...	...	...	R	...	S	R	...	...	R	...

* 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 $0.92 \mu\text{m}^3$. There were spheroplasts with an average volume of $1.08 \mu\text{m}^3$ 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-

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 decompounded by heating at 56°C for 30 min (■), normal plasma obtained before immunisation (▤), or anti-LPS (□).

opment 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 ($>40 \mu\text{g/ml}$) 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 $\mu\text{g/ml}$. 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 $67.9 \pm 3.6\%$ and $11.9 \pm 2.0\%$ 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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REFERENCES

- Cohen J, McConnell J S 1984 Observations on the measurement and evaluation of endotoxemia by a quantitative limulus lysate microassay. *Journal of Infectious Diseases* **150**: 916–924.
- Collins M S, Roby R E 1983 Anti-*Pseudomonas aeruginosa* activity of an intravenous human IgG preparation in burned mice. *Journal of Trauma* **23**: 530–534.
- Conradie J D, Gray R, Mbhele B E 1980 Serum alphafetoprotein determination by enzyme linked immunosorbent assay. *South African Medical Journal* **58**: 169–171.
- Du Bose D A, Lemaire M, Basamania K, Rowlands J 1980. Comparison of plasma extraction techniques in preparation of samples for endotoxin testing by the limulus amoebocyte lysate test. *Journal of Clinical Microbiology* **11**: 68–72.
- Duswald K H, Jochum M, Fritz H 1982 Pathophysiology and pathobiochemistry of sepsis and septic shock. *Intensive Care* **2**: 1–6.
- Elin R J 1979 Clinical utility of the limulus test with blood CSF and synovial fluid. In: Cohen E (ed) *Biomedical applications of the horseshoe crab*. (Progress in clinical and biological research vol. 24). Alan R Liss, New York, pp 279–292.
- Engvall E, Perlmann P 1972 Enzyme linked immunosorbent assay ELISA. III. Quantitation of specific antibodies by enzyme-labelled anti-immunoglobulin in antigen-coated tubes. *Journal of Immunology* **109**: 129–135.
- Gaffin S L 1983a Control of septic shock—present day concept. *South African Journal of Hospital Medicine* **8**: 4–11.
- Gaffin S L 1983b Large scale production of anti-gram-negative bacterial antibodies. *Lancet* **2**: 1420–1421.
- Gaffin S L, Badsha N, Vorster B J 1985a Properties of human

- antilipopolysaccharide gamma globulin: specificity and protective effects. *Vox Sanguinis* **48**: 276-283.
- Gaffin S L, Badsha N, Brock-Utne J G, Vorster B J, Conradie J D, 1982a An ELISA procedure for detecting human anti-endotoxin antibodies in serum. *Annals of Clinical Biochemistry* **19**: 191-194.
- Gaffin S L, Baker B, Du Preez J, Katzwinkel J, Fleming J, Brock-Utne J G 1982b Prophylaxis and therapy with anti-endotoxin hyperimmune serum against gastroenteritis and endotoxemia in horses. In: Milne J (ed) Proceedings of the 28th Annual convention of the American Association of Equine Practitioners, Atlanta, pp 335-340.
- Gaffin S, Brock-Utne J G, Zanolli A, Wells M T 1986. Hypoxia-induced endotoxaemia in primates: role of reticuloendothelial system function and anti-lipopolysaccharide plasma. *Aviation Space and Environmental Medicine* **57**: 1044-1049.
- Gaffin S L, Grinberg Z, Abraham C, Birkhan J, Schechter Y 1981 Protection against hemorrhagic shock in the cat by human plasma containing endotoxin specific antibodies. *Journal of Surgical Research* **31**: 18-21.
- Gaffin S L *et al.*, 1982c Antibiotic effect of human and equine anti-endotoxin antibody rich serum on *Klebsiella pneumoniae*. *South African Journal of Science* **78**: 91-92.
- Gaffin S L, Wells M, Jordaan J P 1985b Anti-lipopolysaccharide toxin therapy for whole body X-irradiation overdose. *British Journal of Radiology* **58**: 881-884.
- Gathiram P, Gaffin S L, Wells M T, Brock-Utne J G 1986 Superior mesenteric artery occlusion shock in cats. Modification of the endotoxemia by anti-lipopolysaccharide antibodies (Anti-LPS). *Circulatory Shock* **19**: 231-237.
- Johnson K J, Ward P A, Goralnick S, Osborn M J 1977 Isolation from human serum of an inactivator of bacterial lipopolysaccharide. *American Journal of Pathology* **88**: 559-574.
- Johnson K J, Ward P A 1972 The requirement for serum complement in the detoxification of bacterial endotoxin. *Journal of Immunology* **108**: 611-616.
- Lachman E, Pitsoe S. B., Gaffin S L 1984a Anti-lipopolysaccharide immunotherapy in management of septic shock of obstetric and gynaecological origin. *Lancet* **1**: 981-983.
- Lachman E, Pitsoe S B, Gaffin S L 1984b Anti-lipopolysaccharide antibodies: update. *Lancet* **2**: 875-876.
- Ng A K, Chen C H, Chang C M, Nowotny A 1976 Relationship of structure to function in bacterial endotoxins: serologically cross-reactive components and their effect on protection of mice against some gram negative infections. *Journal of General Microbiology* **94**: 107-116.
- Pegg S P, Amerena R, Tahir H M 1982 Changing patterns of *Pseudomonas aeruginosa* antibiotic sensitivity. *Burns including Thermal Injury* **8**: 151-155.
- Pudifin D, L'Hoste I, Duursma J, Gaffin S L 1985 Opsonisation of gram-negative bacteria by anti-lipopolysaccharide antibodies. *Lancet* **2**: 1009-1010.
- Roantree R J, Pappas N C 1960 The survival of strains of enteric bacilli in the blood stream as related to their sensitivity to the bactericidal effect of serum. *Journal of Clinical Investigation* **39**: 82-88.
- Shenep J L, Mogan K A 1984 Kinetics of endotoxin release during antibiotic therapy for experimental gram-negative bacterial sepsis. *Journal of Infectious Diseases* **150**: 380-388.
- Smith P B, Tomfohrde K M, Rhoden D L, Balows A 1972 API System: a multitude micromethod for identification of Enterobacteriaceae. *Applied Microbiology* **24**: 449-452.
- Stiehm E R 1979 Standard and special human immune serum globulins as therapeutic agents. *Pediatrics* **63**: 301-319.
- Thomson M 1983 The use of equine anti-endotoxin hyperimmune serum in the treatment of septic arthritis in foals. *Journal of South African Veterinary Association* **54**: 279-281.
- Walker R I, Daily O P, Krasner R, Merrell B R, Porvaznik M 1982 The relationship of enteric micro-organisms to causes of morbidity and mortality in operational situations. *Military Medicine* **147**: 15-22.
- Wardle E N 1975 Endotoxin and acute renal failure. *Nephron* **14**: 321-332.
- Wells M T, Gaffin S L 1986 A morphological study of the action of equine anti-lipopolysaccharide plasma on gram-negative bacteria. *Journal of Medical Microbiology* **24**: 165-168.
- Welsh N H, Rauch A J, Gaffin S L 1984 Topical immunotherapy for *Pseudomonas* keratitis in rabbits: use of antilipopolysaccharide plasma. *British Journal of Ophthalmology* **68**: 828-832.
- Wessels B C, Gaffin S L 1986 Anti-endotoxin immunotherapy for canine parvovirus endotoxaemia. *Journal of Small Animal Practice* **27**: 609-615.
- Wessels B C, Gaffin S L, Wells M T 1986 Circulating plasma endotoxin (LPS) concentrations in healthy and hemorrhagic enteric dogs: anti-endotoxin immunotherapy in hemorrhagic enteric endotoxaemia. *Journal of American Association of Small Animals* (in press).
- Westphal O, Lüderitz O, Bister F 1952 Über die extraktion von bakterien mit phenol/wasser. *Zeitschrift für Naturforschung* **7B** 148-155.
- Wildfeuer A, Heymer B, Schleifer K, Haferkamp O 1974 Investigations on the specificity of the Limulus Test for the detection of endotoxin. *Applied Microbiology* **28**: 867-871.
- Williams P, Lambert P A, Brown M R W, Jones R J 1983 The role of the O and K antigens in determining the resistance of *Klebsiella aerogenes* to serum killing and phagocytosis. *Journal of General Microbiology* **129**: 2181-2191.
- Wilson M B, Nakane P K 1978 Recent developments in the periodate method of conjugating horseradish peroxidase (HRPO) to antibodies. In: Knapp W *et al* (eds) Immunofluorescence and related staining techniques. Elsevier, Amsterdam. pp 215-226.
- Wilson L A, Spitznagel J K 1968 Molecular and structural damage to *Escherichia coli* produced by antibody, complement, and lysozyme systems. *Journal of Bacteriology* **96**: 1339-1348.
- Young L S 1984 Monoclonal antibodies: technology and application to gram-negative infections. *Infection* **12**: 303-308.
- Young L S 1975 Opsonizing antibodies, host factors and the limulus assay for endotoxin. *Infection and Immunity* **12**: 88-92.
- Zanolli A M, Gaffin S L 1985 Prophylaxis of superior mesenteric artery occlusion shock in rabbits by antilipopolysaccharide (Anti-LPS) antibodies. *Journal of Surgical Research* **38**: 113-115.
- Ziegler E J. *et al* 1982 Treatment of gram-negative bacteremia and shock with human anti serum to a mutant *Escherichia coli*. *New England Journal of Medicine* **307**: 1225-1230.