Comparison of the opsonic activity of polyclonal and monoclonal antibodies raised against *Salmonella minnesota* strain R595

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Summary. Murine monoclonal antibodies and immune rabbit serum were raised against the rough mutant *Salmonella minnesota* strain R595. These antibodies were tested for their opsonic activity against the homologous strain and the smooth wild type *S. minnesota* by luminol-dependent chemiluminescence and a microscopic assessment of phagocytosis. Immune rabbit serum opsonised both strains. Treatment with normal rabbit serum inhibited the phagocytic uptake of *S. minnesota* R595. None of the monoclonal antibodies RE01 (anti-KDO), RE12 (anti-KDO) and RE23 (anti-lipid A) were opsonic. Unopsonised *S. minnesota* R595 stimulated marked chemiluminescence possibly because of its hydrophobic surface, but this was not reflected in increased uptake by phagocytic cells. Results obtained with luminol-dependent chemiluminescence should be interpreted with caution when the opsonisation of rough bacterial strains or those with high surface hydrophobicity is being investigated.

Introduction

Despite potent antimicrobial agents and aggressive supportive therapy, the morbidity and mortality of sepsis and shock caused by gram-negative bacteria remains considerable. The severity of underlying disease and resistance to antimicrobial agents among bacteria such as *Pseudomonas aeruginosa* may, in part, account for this (Kreger *et al.* 1980a, b). The poor outcome of sepsis with gram-negative bacteria has also been attributed to the systemic effects of lipopolysaccharide (LPS) against which antibiotics are ineffective (Kirkland and Ziegler, 1984).

One approach to this problem has been to use antisera raised against strains deficient in O antigens, i.e., rough (R) mutants, in particular the rough mutants of *Escherichia coli* O111: B4 (*E. coli* J5) and *Salmonella minnesota* (*S. minnesota* R595), which only express lipid A and a portion of the core oligosaccharide as their LPS. Experimental work in animals (Ziegler *et al.*, 1973; Braude *et al.*, 1977; McCabe *et al.*, 1977) and clinical studies (Ziegler *et al.*, 1982; Baumgartner *et al.*, 1985) have shown the protective efficacy of such antisera. Although the results of these studies are encouraging, not all

investigators have found antisera to core glycolipid to be protective (Ng *et al.*, 1976; Greisman *et al.*, 1978; Pennington and Menkes, 1981; Van Dijk *et al.*, 1981). One problem in resolving these difficulties is that neither the precise molecular specificity nor the protective mechanism(s) of the antisera have been defined. Now that monoclonal antibodies to core glycolipid have been produced and are being investigated (Dunn *et al.*, 1985; Teng *et al.*, 1985), it is possible to address both these questions. One possible protective mechanism of core glycolipid antibody is by opsonisation leading to phagocytosis and intracellular killing. Ziegler *et al.* (1973) demonstrated that antiserum to *E. coli* J5 caused increased clearance of *E. coli* in agranulocytic rabbits. In this study we have investigated and compared the opsonic activity of polyclonal rabbit antiserum raised against formalin-killed *S. minnesota* R595 mutant, with three murine monoclonal antibodies directed against common core-glycolipid determinants from LPS of the same rough strain.

Materials and methods

Bacterial strains

S. minnesota wild type strain CN 1091 was obtained from the National Collection of Type Cultures, Colindale,

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London. *S. minnesota* R595 (Re), R4(Rd2), R5(Rc), R345(Rb2) and R60(Ra) rough mutants were kindly provided by Dr C. Galanos, Freiburg, West Germany. Clinical isolates of gram-negative bacteria were obtained from the diagnostic bacteriology laboratory, St Mary's Hospital Medical School. These isolates were identified by either the API 20E (API Laboratory Products, Basingstoke) or N/F (Flow Laboratories Irvine, Scotland) system.

Culture conditions

Bacteria, apart from *Neisseria meningitidis*, *Haemophilus influenzae* and *Bacteroides fragilis*, were grown in Brain Heart Infusion Broth (Difco) at 37° C for 18 h, harvested, washed and heat-killed at 100° C for 1 h in phosphate-buffered saline, pH 7.2 (PBS). *N. meningitidis* and *H. influenzae* were grown on chocolate agar plates and *B. fragilis* in Fastidious Anaerobe Broth (Lab M Ltd Bury). Heat-killed bacteria were resuspended in PBS at a concentration of 10⁹ cfu/ml.

Extraction and chemical treatment of LPS and lipid A

LPS from smooth wild type strains was extracted by hot phenol-water (Westphal and Jann, 1965). LPS from rough mutant strains was extracted by phenol: chloroform: petroleum ether (PCP) (Galanos *et al.* 1969). Free lipid A was precipitated from R595 LPS with acetic acid 1% v/v (Galanos *et al.*, 1971). Base-hydrolysed R595 LPS (BH-R595 LPS) and lipid A (BH-lipid A) were prepared by hydrolysing R595 LPS or lipid A with 0.2 M sodium hydroxide at 100° C for 1 h (Rietschel *et al.*, 1983).

Reduction of the carboxyl groups in LPS

Carbodiimide-activated carboxyl groups present on the 2-keto-3-deoxymannooctonate (KDO) moiety of R595 LPS were reduced with sodium-borohydride (Taylor and Conrad, 1972). Briefly, 25 mg of 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide (Sigma) was added to a 15-ml suspension containing 10 mg of R595 LPS. The mixture was agitated at room temperature and the pH of the suspension was maintained at 4.75 by the addition of 0.01 M HCl. The reaction was allowed to proceed until the hydrogen ion uptake ceased at about 2 h. The mixture was then transferred to a 50° C water bath and 5 ml of 2 M sodium-borohydride solution was added drop by drop. The pH of the mixture was maintained at pH 7 with 0.01 M HCl. After 3 h the mixture was transferred into dialysis tubing and extensively dialysed against distilled water (four 5-L changes over 24 h). Finally the mixture was rotary evaporated to a small volume (10 ml) and freeze dried. The material was designated reduced-R595 LPS.

Preparation of BSA complexes

The following antigens were complexed to bovine serum albumin (BSA; Sigma No. A7511): all rough and smooth forms of LPS, BH-R595 LPS, BH-lipid A, free lipid A and reduced-R595 LPS. The antigens were first dissolved in distilled water containing triethylamine 0.5% v/v to a concentration of 1 mg/ml by sonication for 30 s. An equal volume of BSA solution (10 mg/ml) was added and complexes produced by rotary evaporation (Galanos *et al.* 1972). The dried residue was dissolved and evaporated again. Finally, the complexes were stored at 4° C in pyrogen-free distilled water at an LPS antigen concentration of 1 mg/ml (or BSA 10 mg/ml) with sodium azide 0.01%.

Antiserum

Four female New Zealand white rabbits (2–2.5 kg) were immunised intravenously with formalin-killed *S. minnesota* R595 cells (4 mg/ml) (Johns *et al.*, 1983). Equal volumes of serum from each rabbit were pooled and stored at –80° C in 0.5-ml volumes. These were thawed just before use and never re-frozen.

Production of monoclonal antibodies (MABs)

Female, 8-week-old BALB/c mice were immunised intraperitoneally with 20 µg of R595 LPS on day 0 and given a booster injection of a similar dose on day 7. Three days later, spleen cells from mice with the highest anti-R595 LPS titres, measured by a solid-phase radioimmunoassay, were fused with NSO myeloma cells (Galfre and Milstein, 1982). Supernates were screened for specific anti-R595 LPS antibodies and antibody secreting hybridomas subsequently cloned by limiting dilution. Cloned cells were grown up as ascites in BALB/c mice primed with pristane (2,6,10,14-tetramethylpentadecane; Aldrich) 14 days earlier. Ascitic fluids were tested for antibody activity either directly or after precipitation of immunoglobulin with sodium sulphate 27% w/v (Ivanyi and Davies, 1980). MAB RE23 was kindly produced and provided by Mr B. J. Appelmek of the Free University, Amsterdam. RE23 was prepared by immunising mice with acetic acid-treated R595 cells.

Radioimmunoassay (RIA)

An RIA was used for the initial screening and subsequent characterisation of monoclonal and rabbit polyclonal antibodies. Briefly, flexible polyvinylchloride 96-well microtitration plates (Dynatech Laboratories, Inc., Alexandria, VA, USA) were coated with 50-µl volumes of R595 LPS at a concentration of 15 µg/ml in PBS and allowed to stand at 37° C for 2 h followed by overnight incubation at 4° C. The plates were washed six times with PBS, and available sites blocked with gelatin

(Difco) 0.1% w/v in PBS. Control plates without antigen were similarly blocked with gelatin 0.1%. After washing, 50- μ l samples of five 10-fold dilutions of antibody in gelatin 0.1% were added to the plate in duplicate and incubated for 8 h at room temperature. Bound antibody was detected by adding 50 μ l of radio-iodinated globulin fraction of rabbit anti-mouse or goat anti-rabbit immunoglobulin (20 μ Ci/ μ g; Wellcome Laboratories, Kent) diluted in BSA 3% w/v in PBS to give 5×10^4 cpm/well, and incubated overnight at 4° C. After washing with PBS, the plates were cut and the radioactivity retained in the wells counted for 1 min in a gamma-counter (NE/1600 Nuclear Enterprise, Edinburgh). Background counts were subtracted from the test counts and the results were expressed as 50% antibody titres (ABT50). The ABT50 was the titre that gave 50% specific binding of antibody to R595 LPS. A direct binding RIA was performed by adding 50 μ l of MAB or polyclonal antiserum at ABT50 to plates coated as above with 50- μ l samples of various antigens (LPS antigens, 15 μ g/ml, or a suspension of heat-killed organisms; see above). The samples were always assayed in quadruplicate and the plates developed as described above. Background counts were subtracted and the data presented as mean cpm $\pm$ SEM.

Inhibition studies were performed by pre-incubating 50 μ l of MAB or polyclonal antiserum at ABT50, with 50 μ l of either an LPS antigen (10^1 – 10^6 ng/ml) or heat-killed organisms (10^3 – 10^8 cfu/ml) at various concentrations for 4 h at 37° C before transferring the mixture (100 μ l) to microtitration wells coated with R595 cells or LPS. The assay was performed as described above and the data presented as mean percentage inhibition $\pm$ SEM (at an inhibitor concentration of 15 μ g/ml and at a 1 in 10 dilution of bacterial suspension). The percentage inhibition of binding was determined as: (cpm bound in absence of inhibitor – cpm bound with inhibitor/cpm bound in absence of inhibitor) $\times$ 100. Inhibition was considered to be positive only when $\geq 20\%$.

Growth and opsonisation of bacteria

For luminol-dependent chemiluminescence and phagocytosis experiments, washed overnight cultures (10^9 cfu/ml) of wild type *S. minnesota* or R595 mutant were resuspended at 10^9 cfu/ml in Hanks's Balanced Salt Solution without phenol red (HBSS: Gibco Ltd), pH 7.3, buffered with 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid; Gibco Ltd). To tubes containing 500 μ l of bacterial suspension (10^9 cfu/ml), 100 μ l of one of the following was added: normal rabbit serum (NRS), immune rabbit serum (IRS), heat-inactivated (56° C, 30 min) NRS or heat-inactivated IRS, each diluted to 10% in HBSS. Alternatively, 10 μ g of the globulin fraction of MAB, with or without 100 μ l of baby rabbit serum (10% in HBSS) as a source of complement, was added. After incubation for 15 min at 37° C, opsonised bacteria were washed twice in PBS, resuspended in 500 μ l of HBSS and kept on ice until needed.

Chemiluminescence (CL)

Human neutrophil polymorphonuclear leukocytes (PMNL) were separated by dextran sedimentation (45 min at room temperature) from defibrinated venous blood taken from healthy adult volunteers. Residual red blood cells were lysed by treatment with tris-buffered ammonium chloride 0.83% w/v and, after washing, PMNL were resuspended in HBSS and counted, and the suspension adjusted to 2×10^6 cells/ml.

Luminol-dependent CL was performed as described previously (Easmon *et al.* 1980). The bacteria:PMN ratio was 100:1 and Luminol (Sigma) was used at a concentration of 2×10^{-5} mol/l. Maximum CL produced was measured at 37° C with a Luminometer 1250 (LKB Instruments). Each experiment was repeated on five different occasions and data is presented as mean $\pm$ SEM.

Microscopical assay of phagocytosis

Bacteria were mixed with PMN at a ratio of 10:1 and incubated at 37° C for 15 min. Smears were prepared on glass slides by use of a cytocentrifuge (Shandon), fixed with methanol and stained with acridine orange (0.3 mg/ml) in distilled water. At least 300 PMNL were counted and the percentage of PMNLs with ingested bacteria was determined.

Bacterial cell-surface hydrophobicity

Bacterial cell-surface hydrophobicity was determined by measuring the affinity of bacteria toward octan-1-ol (Sigma) in an octan-1-ol/HBSS two-phase system (Rosenberg *et al.*, 1980). Equal volumes of bacterial suspension and octan-1-ol were vortex mixed for 2 min and the phases allowed to separate for 30 min at room temperature. Absorbance of the bacterial suspension in the aqueous phase was determined at 540 nm (A_{540}). Bacterial suspensions without octan-1-ol were used to measure initial absorbance. The percentage hydrophobicity was calculated as follows: (A_{540} of initial suspension – A_{540} of bacterial suspension after separation/ A_{540} of initial bacterial suspension) $\times$ 100.

Results

Cross-reactivity of rabbit antiserum and MABs

To assess cross-reactivity, a direct binding RIA with heat-killed whole organisms was used. Immune rabbit serum (IRS) raised against the R595 mutant of *S. minnesota* cross-reacted with most of the gram-negative bacilli tested (table 1). Pre-immune serum (NRS) showed some reactivity with the R4(Rd2), R5(Rc) and R345(Rb2) salmonella mutants, and with *Citrobacter freundii*, *E. coli*, *Klebsiella pneumoniae* and *Serratia marcescens*.

However, apart from the R595 mutant, the level of direct binding of NRS was significantly lower than that of IRS.

Three MABs were tested against the same gram-negative organisms (table II). RE01 (IgM) was broadly reactive, RE12 (IgM) showed a more limited cross-reactivity, whereas RE23 (IgG2a) reacted with all clinical isolates tested except *B. fragilis* and *H. influenzae*. RE23 displayed a different pattern of reactivity with the salmonella mutants R595 (Re) to R60 (Ra) when compared with RE01 and RE12. As expected, a decrease in the level of binding and reactivity was seen along the series from the R595(Re) mutant to the parent wild type strain, presumably due to the core glycolipid immunodeterminant(s) becoming less accessible. In contrast, MABs RE01 and RE12 showed strong binding to the R595(Re), R4(Rd2) and R345(Rb2) mutants but poor binding to R5(Rc), R60(Ra) and the parent wild type strain. An inhibition assay was also used to assess cross-reactivity of polyclonal serum and MABs. In this assay various concentrations of heat-killed organisms were pre-incubated with antibody (polyclonal or monoclonal) before transferring the mixture to RIA microtitration wells adsorbed with R595 cells. The results of a typical inhibition assay with RE12 is shown in fig. 1a. When the cross-reactivity of polyclonal serum

and MABs by RIA was compared with the inhibition assay, good correlation was found between the two assays employed (presented in tables I and II). In all assays the homologous rough parent strain was an effective inhibitor, as was *Ser. marcescens*.

Determination of epitope specificity

Table III shows direct binding RIA results for MABs and polyclonal serum screened against various LPS and chemically-treated LPS preparations. All three MABs bound strongly to R595(Re), R345(Rb2) and BH-R595 LPS. RE23 reacted with all rough LPSs tested, while RE12 exhibited activity towards R4(Rd2) and R5(Rc). In comparison, IRS reacted with the rough LPSs in order of decreasing intensity from R595(Re) to R60(Ra). Neither this antiserum nor the MABs showed any binding to the homologous wild type LPS. RE23 bound strongly to free lipid A and all the chemically-treated LPS preparations, suggesting that it recognised one or more immunodeterminants in the N-acetyl-D-glucosamine backbone of lipid A. In contrast, RE01 and RE12 did not bind to the reduced-R595 LPS. This suggests that the immunodeterminant recognised is the KDO moiety. However, RE01 also showed a slight reactivity with

Table I. Taxonomic specificity of rabbit polyclonal antiserum raised against *S. minnesota* R595 in a direct binding and inhibition RIA with heat-killed bacteria as antigen and inhibitor

Bacteria	Direct cpm (mean $\pm$ SEM) and percentage inhibition ($\pm$ SEM) with			
	immune rabbit serum		normal rabbit serum	
<i>S. minnesota</i> R595 (Re)	7 249 $\pm$ 60	75($\pm$ 5)%	1 143 $\pm$ 15	70($\pm$ 20)%
<i>S. minnesota</i> R4 (Rd2)	3 560 $\pm$ 30	56($\pm$ 3)%	766 $\pm$ 10	20($\pm$ 2)%
<i>S. minnesota</i> R5 (Rc)	1 213 $\pm$ 54	50($\pm$ 5)%	548 $\pm$ 32	20($\pm$ 4)%
<i>S. minnesota</i> R345 (Rb2)	1 064 $\pm$ 20	50($\pm$ 14)%	327 $\pm$ 18	25($\pm$ 5)%
<i>S. minnesota</i> R60 (Ra)	1 099 $\pm$ 60	50($\pm$ 4)%	299 $\pm$ 7	10($\pm$ 1)%
<i>S. minnesota</i> wild type	1 337 $\pm$ 44	42($\pm$ 7)%	145 $\pm$ 17	7($\pm$ 2)%
<i>Ser. marcescens</i>	4 430 $\pm$ 50	55($\pm$ 5)%	585 $\pm$ 10	32($\pm$ 12)%
<i>C. freundii</i>	1 512 $\pm$ 38	30($\pm$ 5)%	340 $\pm$ 19	27($\pm$ 9)%
<i>N. meningitidis</i>	1 289 $\pm$ 25	19($\pm$ 3)%	240 $\pm$ 5	12($\pm$ 3)%
<i>Enterobacter cloacae</i>	1 150 $\pm$ 40	27($\pm$ 9)%	379 $\pm$ 17	10($\pm$ 5)%
<i>E. coli</i>	1 100 $\pm$ 10	27($\pm$ 2)%	679 $\pm$ 30	30($\pm$ 21)%
<i>Ps. aeruginosa</i>	1 000 $\pm$ 14	20($\pm$ 8)%	100 $\pm$ 26	4($\pm$ 1)%
<i>Proteus mirabilis</i>	890 $\pm$ 20	12($\pm$ 2)%	227 $\pm$ 40	4($\pm$ 1)%
<i>K. pneumoniae</i>	800 $\pm$ 56	20($\pm$ 3)%	390 $\pm$ 4	22($\pm$ 15)%
<i>H. influenzae</i> type b	580 $\pm$ 40	10($\pm$ 4)%	212 $\pm$ 30	4($\pm$ 1)%
<i>B. fragilis</i>	217 $\pm$ 6	7($\pm$ 1)%	96 $\pm$ 12	0
<i>Streptococcus faecalis</i>	400 $\pm$ 56	0	160 $\pm$ 27	0
<i>Staphylococcus aureus</i>	169 $\pm$ 12	0	159 $\pm$ 23	0

Table II. Taxonomic specificity of MAB RE01, RE12 and RE23 in direct binding and inhibition RIA with heat-killed bacteria as antigen and inhibitor

Bacteria	Direct cpm (mean $\pm$ SEM) and percentage inhibition ($\pm$ SEM) with					
	RE01(IgM)		RE12(IgM)		RE23(IgG2a)	
<i>S. minnesota</i> R595 (Re)	7 064 $\pm$ 120	56($\pm$ 2)%	6 503 $\pm$ 60	62($\pm$ 8)%	7 450 $\pm$ 121	64($\pm$ 18)%
<i>S. minnesota</i> R4 (Rd2)	760 $\pm$ 20	27($\pm$ 2)%	2 000 $\pm$ 10	30($\pm$ 5)%	2 065 $\pm$ 19	37($\pm$ 5)%
<i>S. minnesota</i> R5 (Rc)	340 $\pm$ 17	21($\pm$ 1)%	658 $\pm$ 12	14($\pm$ 4)%	1 760 $\pm$ 25	32($\pm$ 4)%
<i>S. minnesota</i> R345 (Rb2)	4 207 $\pm$ 100	50($\pm$ 4)%	3 986 $\pm$ 60	30($\pm$ 5)%	1 295 $\pm$ 40	24($\pm$ 2)%
<i>S. minnesota</i> R60 (Ra)	310 $\pm$ 9	10($\pm$ 3)%	350 $\pm$ 15	9($\pm$ 1)%	1 200 $\pm$ 10	17($\pm$ 2)%
<i>S. minnesota</i> wild type	334 $\pm$ 10	5($\pm$ 1)%	216 $\pm$ 10	7($\pm$ 1)%	1 215 $\pm$ 15	15($\pm$ 3)%
<i>Ser. marcescens</i>	4 056 $\pm$ 58	40($\pm$ 10)%	1 363 $\pm$ 20	30($\pm$ 5)%	2 000 $\pm$ 10	34($\pm$ 2)%
<i>C. freundii</i>	3 060 $\pm$ 100	35($\pm$ 16)%	2 110 $\pm$ 30	28($\pm$ 2)%	1 226 $\pm$ 25	18($\pm$ 2)%
<i>N. meningitidis</i>	816 $\pm$ 12	15($\pm$ 4)%	1 059 $\pm$ 25	18($\pm$ 7)%	2 060 $\pm$ 42	20($\pm$ 5)%
<i>E. cloacae</i>	1 000 $\pm$ 14	27($\pm$ 6)%	1 419 $\pm$ 28	24($\pm$ 5)%	1 406 $\pm$ 30	15($\pm$ 4)%
<i>E. coli</i>	1 756 $\pm$ 160	27($\pm$ 4)%	578 $\pm$ 20	18($\pm$ 7)%	1 819 $\pm$ 60	10($\pm$ 2)%
<i>Ps. aeruginosa</i>	600 $\pm$ 18	15($\pm$ 4)%	273 $\pm$ 5	10($\pm$ 4)%	1 260 $\pm$ 12	12($\pm$ 2)%
<i>P. mirabilis</i>	1 151 $\pm$ 40	10($\pm$ 2)%	529 $\pm$ 10	12($\pm$ 2)%	1 159 $\pm$ 26	20($\pm$ 1)%
<i>K. pneumoniae</i>	1 010 $\pm$ 12	25($\pm$ 1)%	1 005 $\pm$ 10	26($\pm$ 5)%	2 120 $\pm$ 18	24($\pm$ 6)%
<i>H. influenzae</i> type b	210 $\pm$ 20	5($\pm$ 1)%	570 $\pm$ 32	6($\pm$ 2)%	180 $\pm$ 10	5($\pm$ 2)%
<i>B. fragilis</i>	159 $\pm$ 20	6($\pm$ 1)%	113 $\pm$ 20	5($\pm$ 1)%	90 $\pm$ 10	3($\pm$ 1)%
<i>Str. faecalis</i>	60 $\pm$ 15	0	120 $\pm$ 12	0	189 $\pm$ 47	0
<i>Staph. aureus</i>	100 $\pm$ 30	0	90 $\pm$ 10	0	200 $\pm$ 70	0

free lipid A and BH-lipid A, indicating that lipid A may be structurally important for recognition of the immunodeterminant. IRS also had activity against free lipid A and most of the chemically-treated LPS preparations. However, treatment of LPS and free lipid A with alkali led to a dramatic reduction in binding of IRS. The predominant antibody activity of IRS may, therefore, be directed against the KDO moiety and the ester-linked fatty acids. Again to confirm direct binding results, inhibition RIAs were performed with LPS and chemically-treated LPS preparations as inhibitors.

Typical inhibition curves obtained with RE12 are presented in fig. 1b.

Opsonisation of rough and smooth strains of S. minnesota with polyclonal antiserum

Table IV shows the results obtained before and after treatment of *S. minnesota* wild type and the R595 mutant with heat-inactivated and unheated NRS and IRS. As expected, the rough mutant was very hydrophobic. This probably accounts for the discrepancy between the high level of chemilumi-

Table III. Molecular specificity of immune rabbit serum (IRS) and MABs RE01, RE12 and RE23 in a direct binding and inhibition RIA with LPS and chemically-treated LPS as antigen and inhibitor

Bacteria	Direct cpm (mean $\pm$ SEM) and percentage inhibition ($\pm$ SEM) with							
	MAB RE01 (IgM)		MAB RE12 (IgM)		MAB RE23 (IgG2a)		Polyclonal IRS	
<i>S. minnesota</i> R595 (Re) LPS	4 596 $\pm$ 120	57($\pm$ 5)%	5 670 $\pm$ 57	60($\pm$ 9)%	6 450 $\pm$ 90	60($\pm$ 10)%	3 340 $\pm$ 10	88($\pm$ 5)%
<i>S. minnesota</i> R4 (Rd2) LPS	507 $\pm$ 40	8($\pm$ 0.7)%	1 200 $\pm$ 20	24($\pm$ 2)%	3 610 $\pm$ 50	42($\pm$ 5)%	1 206 $\pm$ 18	52($\pm$ 5)%
<i>S. minnesota</i> R5 (Rc) LPS	670 $\pm$ 20	12($\pm$ 4)%	470 $\pm$ 17	22($\pm$ 7)%	2 000 $\pm$ 130	22($\pm$ 2)%	649 $\pm$ 12	32($\pm$ 2)%
<i>S. minnesota</i> R345 (Rb2) LPS	2 200 $\pm$ 60	40($\pm$ 5)%	2 400 $\pm$ 40	30($\pm$ 2)%	4 350 $\pm$ 100	45($\pm$ 7)%	990 $\pm$ 10	20($\pm$ 8)%
<i>S. minnesota</i> R60 (Ra) LPS	200 $\pm$ 10	10($\pm$ 0.1)%	410 $\pm$ 10	10($\pm$ 1)%	1 100 $\pm$ 50	23($\pm$ 2)%	712 $\pm$ 20	17($\pm$ 1)%
<i>S. minnesota</i> Wild Type LPS	170 $\pm$ 15	7($\pm$ 0.5)%	200 $\pm$ 12	8($\pm$ 0.4)%	990 $\pm$ 20	17($\pm$ 6)%	547 $\pm$ 17	8($\pm$ 2)%
BH-R595 LPS	3 259 $\pm$ 24	47($\pm$ 10)%	5 610 $\pm$ 80	52($\pm$ 10)%	4 272 $\pm$ 60	37($\pm$ 4)%	689 $\pm$ 10	27($\pm$ 5)%
Reduced-R595 LPS	90 $\pm$ 12	2($\pm$ 0.4)%	100 $\pm$ 20	0	6 328 $\pm$ 112	50($\pm$ 8)%	1 237 $\pm$ 20	38($\pm$ 7)%
Free Lipid A	996 $\pm$ 30	25($\pm$ 7)%	130 $\pm$ 10	1($\pm$ 0.1)%	5 812 $\pm$ 48	50($\pm$ 10)%	1 127 $\pm$ 10	35($\pm$ 5)%
BH-Lipid A	840 $\pm$ 10	20($\pm$ 2)%	97 $\pm$ 5	2($\pm$ 0.4)%	4 169 $\pm$ 35	34($\pm$ 5)%	713 $\pm$ 9	24($\pm$ 5)%

Figure. Inhibition assay, the percentage inhibition of binding of MAB RE12 by (a) *S. minnesota* R595 (●); *Ser. marcescens* (○); *C. freundii* (■); *E. coli* (□); *Ps. aeruginosa* (▲) and *S. minnesota* wild type (△) bacterial suspensions, and (b) *S. minnesota* R595 LPS (●); BH-R595 LPS (○); *S. minnesota* R345 LPS (▲) and free lipid A (△).

nescence and the moderate degree of ingestion of the untreated organisms. Microscopy showed that many bacteria were adherent to but not ingested by neutrophils. This adherence may well have provided the membrane stimulus for chemiluminescence. In comparison with untreated organisms, treatment with NRS reduced chemiluminescence and phagocytic uptake. Again this may be linked to the reduction in hydrophobicity of the unopson-

ised organisms. Heat-labile factors would appear to be involved because the effect was lost with heated NRS.

IRS was opsonic. The microscopic phagocytic uptake assay showed this to be largely complement-independent while enhancement of chemiluminescence was complement-dependent. With the smooth (non-hydrophobic) wild type *S. minnesota*, unopsonised organisms were poorly ingested as were those treated with NRS. However, treatment with NRS triggered an apparently complement-dependent enhancement of chemiluminescence. IRS did not opsonise wild type *S. minnesota* in the absence of complement. This effect was not as marked as with the rough mutant.

Opsonisation of *S. minnesota* with MABs

None of the MABs showed any significant opsonic activity against the homologous rough parent strain in the presence or absence of complement (table V). However, with RE01 there was a significant enhancement of chemiluminescence, but without any corresponding increase in bacterial ingestion. None of the MABs were opsonic for the *S. minnesota* wild type strain (results not shown).

Discussion

Antisera raised against the rough mutants of *E. coli* O111: B4 (*E. coli* J5) and *S. minnesota* (*S. minnesota* R595) protect animals and man against bacteraemia with gram-negative bacilli (Nelles and Niswander, 1984), although results have not always been consistent (McCutchan *et al.*, 1983; Zinner and Peter, 1983). Neither the mechanism by which these antisera exert their protective effects nor the immunodeterminant(s) against which they are directed have been resolved (Wolff, 1982). Knowledge of both is important for the logical development of protective monoclonal reagents. Two likely protective mechanisms have been suggested—antitoxic immunity directed against the pathophysiological effects of LPS (Ziegler *et al.*, 1982; Johns *et al.*, 1983) and antibacterial immunity, e.g., opsonisation and phagocytosis (Crowley *et al.*, 1982). Current clinical evidence favours the former because protection has been directed mainly against fatal bacteriogenic shock (Baumgartner *et al.*, 1985).

Our results show that rabbit antiserum against *S. minnesota* R595 opsonises both the homologous strain and the wild type parent strain. This antiserum reacts with many other gram-negative

Table IV. Phagocytic uptake PMNL chemiluminescence induction and hydrophobicity of *S. minnesota* wild type and the R595 (Re) mutant after treatment with normal rabbit serum (NRS) and immune rabbit serum (IRS)

Opsonin source	<i>S. minnesota</i> wild type			<i>S. minnesota</i> R595 (Re)		
	Phagocytosis (%±SEM)	CL (mV±SEM)	Hydrophobicity (%±SEM)	Phagocytosis (%±SEM)	CL (mV±SEM)	Hydrophobicity (%±SEM)
Unopsonised	13±0.3	29±3	0.2±0.1	39±0.8	200±20	80±0.6
NRS 10%	18±0.7	186±17	38±0.6	25±0.4	20±3	65±1.0
H. NRS 10%	14±0.4	52±5	0.7±0.2	34±0.6	80±10	50±0.8
IRS 10%	40±1.0	151±20	67±1.0	78±1.0	180±25	45±0.8
H. IRS 10%	22±0.4	80±9	80±0.8	66±0.5	80±12	63±0.9

H.NRS and H. IRS = heat-inactivated serum (56° C for 30 min).

Table V. Phagocytic uptake and PMNL chemiluminescence induction of *S. minnesota* R595 (Re) after treatment with MABs RE01, RE12 and RE23

Opsonin	MAB RE01		MAB RE12		MAB RE23	
	Phagocytosis (%±SEM)	CL (mV±SEM)	Phagocytosis (%±SEM)	CL (mV±SEM)	Phagocytosis (%±SEM)	CL (mV±SEM)
Unopsonised	39±0.6	220±25	30±0.5	142±27	28±0.6	220±31
MAB	48±0.4	150±10	35±0.4	40±9	30±0.3	~170±15
MAB + baby rabbit serum 10%	26±0.2	440±37	27±0.7	52±4	32±1	210±28
Baby rabbit serum 10%	23±0.1	55±14	20±0.4	38±5	20±0.9	53±10
Baby rabbit serum heated at 56°C for 30 min	29±1	52±12	17±0.2	40±5	30±2	50±9

bacilli. It also reacts with LPS from a range of gram-negative bacilli and LPS preparations which have been treated chemically. We do not know the fine molecular specificity, although our results suggest that the predominant activity is directed against the core polysaccharide and the ester-linked fatty acids of lipid A.

The three monoclonal reagents RE01, RE12 and RE23 did not opsonise the homologous R595 mutant or the smooth *S. minnesota*. Whereas RE23 recognises an epitope(s) within the N-acetyl-D-glucosamine backbone of lipid A, RE01 and RE12 bind principally to the KDO moiety. These three antibodies, which represent the most likely isotypes relevant to opsonisation, all have targets within the core glycolipid. Their failure to opsonise the R595 mutant, which clearly has these structures exposed on the surface, could mean that the specific epitope recognised is either inappropriate or insufficient in density for efficient opsonisation. A cocktail of MABs with different isotypes and core glycolipid specificity has also been investigated and it also failed to opsonise (data not shown). Another

explanation could be that the antibodies may simply not be opsonic, although with an IgM this is perhaps unlikely (Polin and Harris, 1985). One key factor in resolving this dilemma is to determine whether the opsonic activity of the rabbit antiserum is related to targets within the core glycolipid or to other common determinants within the cell wall of gram-negative bacteria. This is currently being investigated by absorption studies.

In measuring opsonisation we found significant discrepancies between results with luminol-dependent chemiluminescence and the microscopic assay of phagocytosis. With the rough R595 mutant, unopsonised organisms stimulated marked chemiluminescence, but only moderate phagocytic uptake. Microscopy showed that most of the organisms were adhering to the surfaces of neutrophils, with relatively few ingested. This adherence is probably related to the extreme hydrophobicity of the rough mutants. Treatment of the R595 mutant with non-immune serum depressed chemiluminescence and phagocytic uptake. This surprising observation appeared to depend, at least partly,

on the reduction in surface hydrophobicity following incubation with normal serum, and partly on the interaction of heat-labile serum components with the bacterial surface, because the effect was lost when serum was heat-inactivated. Another discrepancy between chemiluminescence and phagocytosis was seen with immune serum. Opsonisation of the R595 mutant appeared to be complement dependent when measured by chemiluminescence, but complement-independent when measured by phagocytosis. Clark and Easmon (1986) found complement to be necessary for the stimulation of luminol-dependent chemiluminescence by opsonised bacteria. They found that high concentrations of opsonic immunoglobulin alone failed to stimulate

chemiluminescence despite microscopical evidence of bacterial uptake.

Chemiluminescence has been used frequently to measure opsonic function. Our results show that care should be taken in interpreting results obtained with hydrophobic organisms or with antibody driven systems in the absence of complement. We recommend that an independent assay of phagocytosis should always be used in conjunction with chemiluminescence.

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