Production and characterisation of mouse monoclonal antibodies reacting with the lipopolysaccharide core region of gram-negative bacilli

B. J. APPELMELK, A. M. J. J. VERWEIJ-VAN VUGHT, J. J. MAASKANT, W. F. SCHOUTEN,
A. J. R. DE JONGE*, L. G. THIJST† and D. M. MACLAREN

*Research Group for Commensal Infections, *Department of Pharmacology and †Medical Intensive Care Unit, School of Medicine, Vrije Universiteit, v.d. Boechorststraat 7, P.O.B. 7161, 1007 MC Amsterdam, The Netherlands*

Summary. Monoclonal antibodies to the lipopolysaccharide (LPS) core region were produced by immunising mice with *Escherichia coli* strain J5 (chemotype Rc). One of these bound to the deepest part of the core, i.e., Lipid A, and reacted with other heat-killed but not live gram-negative bacilli, including *E. coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Eight other monoclonal antibodies, binding to the terminal glucose residue of Rc LPS, reacted with live cells of *E. coli* strains only. Thus, the O antigen does not necessarily render the core inaccessible to antibody. However, despite binding to live bacteria, these monoclonal antibodies neither enhanced phagocytic killing, nor protected mice from dying from gram-negative infection or endotoxaemia. It is concluded that antibodies reacting with the most immunodominant parts of the J5 core are not protective.

Introduction

Antibodies reacting with the lipopolysaccharide (LPS) core have been shown to prevent death from gram-negative bacteraemia and endotoxaemia (Zinner and Peter, 1983; Telzak and Wolff, 1985). In these experiments, most investigators used polyclonal antisera to rough mutants of *Escherichia coli* strain J5, chemotype Rc or *Salmonella minnesota* strain R595, chemotype Re to immunise laboratory animals passively. In particular, Ziegler *et al.* (1982) demonstrated strikingly that human anti-J5 antiserum given to patients in severe shock reduced mortality significantly. It is difficult to prepare such an antiserum on a large scale; several investigators have, therefore, produced anti-core monoclonal antibodies from mouse cells (Dunn *et al.*, 1983, 1985, 1986; Bogard *et al.*, 1984; Mutharia *et al.*, 1984; Nelles and Niswander, 1984; Young, 1984; Elkins and Metcalf, 1985; Kasai *et al.*, 1985; Miner *et al.*, 1986) or human cells (Raubitschek *et al.*, 1985; Teng *et al.*, 1985). The protection afforded by such monoclonal antibodies has been described by Dunn *et al.* (1983, 1985, 1986), Bogard *et al.* (1984), Young (1984) and Teng *et al.* (1985). Compared with some years ago (Peter *et al.*, 1982), it is now

firmly established that anti-core antibodies protect, but several major questions have yet to be answered—are all antibodies to the core region protective, or, as a corollary, must a specific epitope be recognised in order to confer protection; do protective anti-core antibodies act primarily by promoting Fc-mediated opsonisation, so increasing phagocytosis and the killing of bacteria, or do they detoxify endotoxin by simple binding at or near its toxic groups? To answer these questions monoclonal antibodies reacting with different core epitopes must be produced and tested. This paper describes the production and characterisation of 12 such monoclonal antibodies.

Materials and methods

Bacteria and growth conditions

E. coli strain J5 and its parent strain *E. coli* serovar O111:B4 were provided by E. J. Ziegler, San Diego. *E. coli* strain ATCC 33985, serovar O125:B15 and *Pseudomonas aeruginosa* strain ATCC 27853 were bought from the American Type Culture Collection, Rockville, MD. *E. coli* strain AD 36 K⁻ is a capsule-less mutant derived from *E. coli* strain O6K2H1 (Verweij-van Vught *et al.*, 1983). *S. minnesota* strain R595 was obtained from G. Schmidt, Borstel, FRG, and *Haemophilus influenzae*

(strain 760705) from A. J. W. van Alphen, Amsterdam. Strains of *Klebsiella pneumoniae*, *Proteus mirabilis*, *P. vulgaris*, *Staphylococcus epidermidis* and *Bacteroides fragilis* strain BE1 were isolated from clinical specimens. The strain of *H. influenzae* was grown in Brain Heart Infusion Broth (Difco) containing NAD 1 µg/ml and haemin 10 µg/ml incubated in air plus CO₂ 5%. *B. fragilis* strain BE1 was grown anaerobically as described by Vel *et al.* (1985). The other strains were grown in Trypticase Soy Broth (BBL, Cockeysville, MD, USA).

Vaccines and polyclonal antisera

A vaccine of *E. coli* strain J5 was prepared as described by Appelmelk *et al.* (1985). Briefly, an overnight culture of strain J5 in trypticase soy broth was centrifuged, washed three times with NaCl 0.9% w/v, and resuspended in NaCl to a concentration of 7×10^9 cfu/ml; the suspension was heated in a boiling water bath for 2.5 h. A vaccine of *E. coli* serovar O111:B4 containing 5×10^9 cfu/ml was prepared similarly and used to immunise Swiss albino mice by injecting 0.1 ml intravenously (i.v.) on days 0, 14 and 28; antiserum was obtained on day 42 by cardiac puncture.

Rough strain lipopolysaccharides and Lipid A

Lipid A from *E. coli* strain F515 was a gift from H. Brade, Borstel. The following lipopolysaccharides, and Lipid A from *S. minnesota* strain R595, were bought from List Biological Laboratories, Campbell, CA: LPS from *E. coli* strain J5, chemotype Rc; and from *S. minnesota* strain R60, chemotype Ra; strain R345, chemotype Rb₂; strain R5, chemotype Rc; strain R7, chemotype Rd₁; and strain R595, chemotype Re. These LPS preparations from rough strains and Lipid A are referred to subsequently as core antigens.

Production of monoclonal antibodies

A BALB/c mouse was inoculated i.v. with 0.1 ml of J5 vaccine on days 0, 21, 42 and 45. On day 48, half of the spleen was fused with 8×10^7 myeloma cells of line no. X63-Ag8.653 as described by De Haan *et al.* (1985). Clones producing antibody were identified by enzyme-linked immunosorbent assay (ELISA) (Appelmelk *et al.*, 1985); twelve cell lines were stabilised by subcloning 2–4 times, and ascitic fluids produced with them in mice by injecting 5×10^6 hybridoma cells intraperitoneally (i.p.) and removing ascitic fluids after 2–3 weeks.

Reaction of anti-core monoclonal antibodies with Lipid A and LPS from rough strains and determination of their immunoglobulin class and subclass

The reaction of the anti-core monoclonal antibodies with core antigens was determined by ELISA (Appelmelk *et al.*, 1985). In short, individual core antigens complexed

to bovine serum albumin (BSA) were used to coat the solid phase. Serial dilutions of antibody-producing cell-culture supernates or ascitic fluids, prepared in phosphate-buffered saline containing Tween 80 0.05% v/v, pH 7.5 (PBST), were added and incubated. Rabbit anti-mouse immunoglobulin (Ig) labelled with horseradish peroxidase (HRP) was used to detect reacting antibody. Plates coated with BSA alone served as controls. Optical densities (OD) were measured at 492 nm in a Dynatech MR 600 microtitration plate reader (Dynatech Laboratories, Billingham, Sussex).

The titre of each antibody was defined as the highest dilution producing an OD value 0.1 above the control value.

A similar ELISA was used to determine the Ig class and subclass with HRP-labelled goat anti-mouse IgG (H+L); anti-IgG1; anti-IgG2a; anti-IgG2b and anti-IgG3; and rabbit anti-mouse IgM. These were obtained from Nordic, Tilburg, The Netherlands.

Binding of anti-core monoclonal antibodies to heat-killed bacteria

Bacteria grown to a concentration of 5×10^8 cfu/ml were washed three times in PBS, concentrated tenfold and boiled for 2.5 h. Binding of antibody to bacteria thus treated was measured in a competitive assay in which 5×10^9 bacteria were mixed with an equal volume of ascitic fluid diluted 1 in 50 000 in PBST, incubated for 16 h at 22°C and centrifuged. The antibody content of the supernatant fluids was titrated by ELISA in which the appropriate core antigen, i.e., either LPS from strain J5 or Lipid A was used to coat the plates. Diluted ascitic fluid, incubated overnight without bacteria, served as a control. When monoclonal antibody bound to bacteria, its titre fell. To ensure that binding was specific, two further controls were included: each monoclonal antibody was incubated in the conditions described above with gram-positive organisms (*S. epidermidis*) before titration. All heat-killed bacteria were incubated with diluted Pen 7 ascitic fluid (De Haan *et al.*, 1985). This monoclonal antibody binds to benzylpenicillin but not to LPS. In this case, non-specific binding was sought by titrating the supernatant fluids in an ELISA in which benzylpenicillin conjugated with transferrin was used to coat the solid phase (De Haan *et al.*, 1985).

Binding of anti-core monoclonal antibodies to live cells of E. coli serovars O111:B4 and O125:B15

The method described above for heat-killed cells was modified as follows: to ensure maximum viability, an overnight bacterial culture was diluted 1 in 100 in fresh trypticase soy broth and grown at 37°C to the mid-log phase; the bacteria were washed and concentrated, and 5×10^9 cfu were incubated with diluted ascitic fluid for 1 h at 37°C.

The binding of monoclonal antibodies, or polyclonal mouse antiserum to live *E. coli* cells was also investigated

more directly by immunofluorescence: cells of *E. coli* serovar O111:B4 in the exponential phase were washed and suspended in PBST to 5×10^8 cfu/ml, and incubated for 1 h at 37°C with an equal volume of ascitic pool diluted 1 in 10 in PBST, or with similarly diluted mouse antiserum to this strain. Cells were washed in PBST, and incubated with FITC-labelled porcine anti-immunoglobulin (Nordic), diluted 1 in 50 in PBST. After washing the cells in distilled water, they were air-dried on microscope slides and immersed in PBS-buffered glycerol. Fluorescence was examined with a Leitz Orthoplan microscope (Wetzlar, GDR) equipped with an HBO 200 lamp and a 12 filter block.

Effect of anti-core monoclonal antibodies on the Limulus assay

Lipid A from *S. minnesota* strain R595 complexed with BSA was used to activate *Limulus* amoebocyte lysate (Coatest, Kabi Vitrum, Sweden) in accordance with the procedure recommended by the manufacturers. A standard dose-response curve was first obtained before the ability of anti-core monoclonal antibodies to inhibit the assay was measured as follows: the Ig fraction of a pool of equal volumes of all ascitic fluids was separated by precipitation with 50% saturated ammonium sulphate and reconstituted to the original volume in PBS. Ten-fold dilutions, from 1 in 10 to 1 in 10^6 were made; samples (10 μ l) of each dilution corresponding to amounts of undiluted Ig of $1-10^{-5}$ μ l were incubated with 50 pg of Lipid A for 45 min at 37°C; thereafter the effect of these mixtures on the standard *Limulus* assay was measured. Ig from Pen 7 ascitic fluid, prepared in the same way, served as a control.

Effect of anti-core monoclonal antibodies on the killing of cells of E. coli in the mouse peritoneal cavity

This was measured by the method of Galanos *et al.* (1971), modified as follows: cells of *E. coli* serovar O111:B4 in the exponential phase were washed, and resuspended in saline to a concentration of 5×10^5 cfu/ml. Equal volumes of the bacterial suspension and of each of the following antiserum preparations were mixed and incubated for 45 min at 37°C: a pool of all ascitic fluids containing equal volumes of anti-core antibodies; Pen 7 ascitic fluid; mouse polyclonal antiserum to *E. coli* serovar O111:B4 diluted 1 in 10. After incubation, 0.2 ml of each mixture was injected i.p. into twelve 10-week-old female Swiss albino mice. At zero time and at 30, 60 and 90 min thereafter, three mice from each group were killed by spinal dislocation and the abdominal skin disinfected and removed. Bacteria were recovered from the peritoneal cavity by injecting 2 ml of PBS into the peritoneal cavity, massaging the abdominal wall and withdrawing 1 ml of fluid which was incubated for 5 min at 22°C with 1 ml of lysis buffer (Zierdt, 1982) pH 7.5,

consisting of 0.01M Na_2HPO_4 , Tween 80 1% v/v and trypsin 0.025% w/v. Immediately thereafter the fluid was spread on agar plates to determine the number of cfu present, with a spiral plating system (Spiral System Instruments, Inc., Md, USA). The results were analysed by Student's *t* test.

Effect of anti-core monoclonal antibodies in protecting mice against fatal bacteraemia caused by E. coli serovar O111:B4

A suspension of cells of *E. coli* serovar O111:B4 in the exponential phase was serially diluted in tenfold steps in PBS and added to a suspension in PBS of mucin (type II, Sigma, St Louis, MO, USA) and bovine haemoglobin (BBL, Cockeysville, MD, USA) (Marks *et al.*, 1982). Samples (0.5 ml) containing 50–50 000 cfu and mucin 6% w/v, haemoglobin 1.6% w/v were injected i.p. into four groups of six mice each. Two batches of mucin-haemoglobin, differing in potency, were used. Test mice were inoculated i.p. with 0.3 ml of pooled ascitic fluids 24 h before challenge. The LD50 was calculated by the method of Spearman-Kärber (Finney, 1971) from the number of deaths recorded after 1 week and the significance of the results calculated by the χ^2 test.

Effect of anti-core monoclonal antibodies in protecting against fatal endotoxaemia

The ability of anti-core monoclonal antibodies to prevent death from endotoxaemia was investigated with the actinomycin D-sensitised mouse model (Johns *et al.*, 1983; Appelmek *et al.*, 1985). Groups of six mice were challenged i.p. with J5 LPS (0.1, 1, 10 or 100 ng per mouse) or lipid A (10, 100 or 1000 ng per mouse). A volume of 0.1 ml was injected, consisting of endotoxin suspended in pyrogen-free PBS with 20 μ g of actinomycin D.

Results

In-vitro characterisation of anti-core monoclonal antibodies

Forty of the 720 wells into which the fusion mixture was distributed contained anti-core antibody. Table I shows the reaction of these with the following core antigens: LPS from *E. coli* strain J5 and *S. minnesota* strains R5 and R595, and Lipid A from *S. minnesota* strain R595. Thirteen of these cell lines were selected for further growth. One (line no. 13, producing IgG3 binding to LPS from strains J5 and R5) stopped producing antibody. The titres of antibody in ascitic fluids prepared with the remaining 12 lines, measured by ELISA against each of the core antigens, are shown in table II.

Table I. Reaction with core antigens and the immunoglobulin subclass of 40 anti-core monoclonal antibodies

Number of monoclonal antibodies	Reaction with core antigen				Ig subclass (number of antibodies)
	J5 LPS	R5 LPS	R595 LPS	R595 Lipid A	
36	+	+	—	—	IgG1 (1) IgG2a (1) IgG3 (34)
2	+	—	—	—	IgG3 (2)
2	—	—	—	+	IgG2b (1) IgG3 (1)

+ = binding; — = no binding.

Table II. Titres measured by ELISA of 12 anti-core monoclonal antibodies

Cell line no.*	Titre against†								IgG subclass
	<i>E. coli</i> Lipid A	R595 Lipid A	R595 (Re)	R7 (Rd ₁)	<i>E. coli</i> J5 (Rc)	R5 (Rc)	R345 (Rb ₂)	R60 (Ra)	
1	10 ⁵	10 ⁶	<	<	<	<	<	<	2b
6	10 ⁶	10 ⁶	<	<	<	<	<	<	3
7	<	<	<	<	10 ⁶	<	<	<	3
10	<	<	<	<	10 ⁶	<	<	<	3
2	<	<	<	<	10 ⁶	10 ⁴	<	<	1
4	<	<	<	<	10 ⁷	10 ⁶	<	<	2a
5	<	<	<	<	10 ⁷	10 ⁶	<	<	1
3, 8, 9, 11	<	<	<	<	10 ⁶	10 ⁶	<	<	3
13	<	<	<	<	10 ⁵	10 ⁵	<	<	3

*Line no. 12 lost production.

† Unless otherwise stated, the LPS core antigens used were isolated from *S. minnesota* rough strains.< = titre < 10³.*Binding of anti-core monoclonal antibodies to heat-killed bacteria and to live E. coli*

The ability of antibody in ascitic fluid produced with cell line 3 (representative of antibody from all 12 cell lines) to bind to heat-killed bacteria is shown in fig. 1. The ELISA results formed two separate zones, the first representing binding to cells of the strains of *E. coli* (antibody from cell-line 3 bound to cells of the four strains of *E. coli* tested), and the second showing that this antibody did not bind to cells of the other gram-negative species tested. Readings given by antibody incubated without bacteria fell in the second zone. Antibody from the other cell lines was tested in the same manner, except those from cell lines 1 and 6, which were titrated for their ability to bind to Lipid A after being incubated with bacteria. The results are shown in table III; this table also contains the results obtained in experiments with live cells of *E. coli* serovars O111:B4 and O125:B15. Despite

repeated testing, antibody from cell lines 1, 2 and 7 gave very inconsistent results; these have been omitted from table III. None of the 12 antibodies tested bound to *S. epidermidis* cells. Pen 7 antibody did not bind to any bacterial strain tested.

Live *E. coli* cells (serovar O111:B4) treated with homologous mouse antiserum exhibited a very bright fluorescence; in contrast, only a faint fluorescence was seen after treatment with the ascitic pool. Thus, in comparison with the polyclonal antiserum, the monoclonal antibodies bound very much less well to the bacterial surface.

Effects of anti-core monoclonal antibodies on the Limulus assay

A representative dose-response curve, with lipid A-BSA used for activation, is shown in fig. 2. No effect of anti-core antibodies on this assay was detected.

Fig. 1. Titration of antibody from cell line no. 3 against LPS from *E. coli* strain J5 after incubation with heat-killed bacteria. Zone I: response after incubation with *E. coli* strains J5, serovars O111:B4, O125:B15 and 36 K⁻. Zone II: response after incubation with *Ps. aeruginosa*, *S. minnesota* strain R 595, *H. influenzae*, *K. pneumoniae*, *B. fragilis*, *P. mirabilis*, *P. vulgaris* and *S. epidermidis*.

Influence of anti-core antibodies on the clearing of live cells of E. coli serovar O111:B4 from the mouse peritoneal cavity

The results of these experiments are shown in fig. 3. Treatment with anti-core monoclonal antibody did not result in significantly greater killing of *E. coli* after i.p. injection than in controls ($p=0.31$). However, treatment with polyclonal mouse antiserum to *E. coli* serovar O111:B4 did significantly enhance killing of bacteria, compared to treatment with anti-core antibodies ($p<0.01$) or with Pen 7 antibody ($p<0.001$)

Effect of monoclonal anti-core antibodies in preventing lethal gram-negative infection and endotoxaemia

Monoclonal anti-core antibodies given to mice before challenge with live bacteria or endotoxin did not protect them (table IV).

Discussion

Antibodies to the core of LPS from *E. coli* or *S. minnesota* have been shown to prevent the lethal effects of gram-negative bacteraemia and endotoxaemia, but neither the target site of these antibodies

Fig. 2. Activation of *Limulus* lysate with Lipid A-BSA.

nor the way in which they protect is clear. A better understanding of the protective effect requires the characterisation of the binding pattern of a large number of monoclonal antibodies, recognising different epitopes.

Most (75%) of the monoclonal antibodies produced in the present study were of the IgG3 subclass; few antibodies from other subclasses were present. Restriction of the antibody response to the

Fig. 3. Killing by mouse peritoneal cells of *E. coli* serovar O111:B4 after treatment with: Pen 7 antibody (▲), anti-core ascitic pool (△), or polyclonal mouse anti-*E. coli* O111:B4 antiserum (○). Bars represent 95% confidence limits.

Table III. Binding of anti-core monoclonal antibodies to heat-killed gram-negative bacteria and to live *E. coli* O111:B4 and O125:B15

Cell line no.	Binding to			Live <i>E. coli</i> strains (O111:B4 and O125:B15)
	Heat-killed bacteria			
	<i>E. coli</i> J5	Other <i>E. coli</i> strains*	Non- <i>E. coli</i> strains†	
6	+	+	+	—
10	+	—	—	ND
3, 4, 5, 8, 9, 11, 13	+	+	—	+

+ = binding; — = no binding.

ND = not determined.

* *E. coli* O111:B4, O125:B15 and AD 36K⁻.† *Ps. aeruginosa*, *S. minnesota* R595, *H. influenzae*, *K. pneumoniae*, *B. fragilis*, *P. mirabilis*, *P. vulgaris*.**Table IV.** The effect of anti-core monoclonal antibodies in protecting mice from fatal gram-negative infection and endotoxic shock

Experiment no.	Treatment before challenge	Challenge	LD50
I	anti-core ascitic pool	live bacteria	123 cfu NS
	none	live bacteria	140 cfu
II	Pen 7 ascitic fluid (control)	live bacteria	1285 cfu
	line 2 ascitic fluid	live bacteria	2312 cfu NS ^a
	line 4 ascitic fluid	live bacteria	724 cfu NS ^a
III	line 6 ascitic* fluid	Lipid A-BSA	220 ng NS
	none	Lipid A-BSA	330 ng
IV	anti-core ascitic pool	J5 LPS-BSA	1 ng NS
	none	J5 LPS-BSA	0.5 ng
V	Pen 7 ascitic fluid (control)	J5 LPS-BSA	0.7 ng
	line 2 ascitic fluid	J5 LPS-BSA	3.1 ng NS ^a
	line 3 ascitic fluid	J5 LPS-BSA	0.7 ng NS ^a
	line 4 ascitic fluid	J5 LPS-BSA	0.3 ng NS ^a
	line 5 ascitic fluid	J5 LPS-BSA	2.2 ng NS ^a
	line 7 ascitic fluid	J5 LPS-BSA	3.2 ng NS ^a

NS = not significant from untreated controls; NS^a = LD50 compared to Pen 7 controls.

* Antibody from cell line 6 binds to Lipid A.

IgG3 subclass has already been described by Perlmutter *et al.* (1978). From binding assay data (table II), the binding site of most of the antibodies examined in the present study could be localised precisely: antibody from cell lines 2, 3, 4, 5, 8, 9, 11 and 13 bound to LPS from strains J5 and R5 (both Rc chemotypes), but not to LPS from *S. minnesota* strains R595 (chemotype Re), R7 (chemotype Rd₁), R345 (chemotype Rb₂) or R60 (chemotype Ra), nor to Lipid A. From a review of the molecular structures of these core antigens (Galanos *et al.*, 1984) it can be stated that all these antibodies bind

to the terminal glucose residue present in the Rc structure, i.e., this glucose residue is an immunodominant group. Immunodominance of the terminal, non-reducing sugar is common when rough strains are used as immunogens (Galanos *et al.*, 1984). Considering the ratio of their titres to LPS from strains J5 and R5, the antibodies can be divided into three groups, each recognising a different epitope on the terminal glucose residue: (i) a group characterised by a ratio of 1:1, exemplified by antibody from cell lines 3, 8, 9, 11 and 13; (ii) a group with a ratio of 10:1, including

antibody from cell lines 4 and 5; and (iii) a group with a ratio of 100:1, containing antibody from cell line 2. Furthermore, although the prime target of antibody from cell lines 2, 4 and 5 is the terminal glucose residue, binding of these three antibodies is influenced by molecular groups in the cores of *E. coli* and *S. minnesota* LPS, most likely from the nearby heptose regions, which are not identical. Antibody from cell lines 7 and 10 did not bind to LPS from rough *Salmonella* strains and must, therefore, bind to an epitope unique to *E. coli*. Antibody from cell lines 1 and 6 did bind to free Lipid A from *E. coli* and *S. minnesota*, but not when covalently linked (i.e., there was no binding to LPS from strains of chemotype Re) and in so doing behaved like polyclonal antibodies to Lipid A (Galanos *et al.*, 1984). Several investigators have produced monoclonal antibodies to Lipid A with similar binding characteristics (Elkins and Metcalf, 1985; Kasai *et al.*, 1985), but monoclonal antibodies reacting with both free and covalently bound Lipid A have also been described (Bogard *et al.*, 1984; Young, 1984; Dunn *et al.*, 1985, 1986; Kasai *et al.*, 1985; Raubitschek *et al.*, 1985; Teng *et al.*, 1985).

The monoclonal antibody from cell line 6 which bound to Lipid A reacted with a panel of gram-negative strains, including the anaerobe *B. fragilis* (table III); evidently the site it recognises is present in all these species. In contrast, the three epitopes recognised on the terminal glucose of chemotype Rc were present in *E. coli* strains only.

Antibody from cell line 6 did not bind to live bacteria, and so also behaved like polyclonal antisera to Lipid A (Mullan *et al.*, 1974). Antibody from cell lines 3, 4, 5, 8, 9, 11 and 13 reacted with live cells of *E. coli* serovars O111:B4 and O125:B15. Thus, at least some epitopes of the LPS core can react with antibody on the surface of intact, smooth bacteria. In contrast, Gigliotti and Shenep (1985) were unable to demonstrate such binding to live bacteria. Differences both in the kind of anti-core monoclonal antibodies used to study binding and in experimental conditions may explain these conflicting results.

The monoclonal antibodies examined in the present study did not inhibit the *Limulus* assay. It has been calculated that the lack of inhibition was probably not due to a lack of competing antibody, but to the antibodies' intrinsic inability to inhibit.

Live *E. coli* cells, incubated with anti-core antiserum, were not better cleared by mouse peritoneal cells than were controls incubated with Pen 7 antibody although anti-core antibodies bound to live bacteria. In contrast, polyclonal antibody to *E. coli* serovar O111:B4 significantly enhanced

bacterial killing. With immunofluorescence techniques it was found, in the latter case, that much more antibody was bound to the bacterial surface than in the case of bacteria treated with anti-core antibody. We hold this minimal binding responsible for the lack of effect of anti-core monoclonal antibodies in this assay.

The anti-core monoclonal antibodies examined in the present study did not protect mice against lethal endotoxaemia, nor against experimental gram-negative sepsis. Protective monoclonal antibodies described so far belong to the IgM class (Young, 1984; Teng *et al.*, 1985), the IgG1 subclass (Bogard *et al.*, 1984; Dunn *et al.*, 1985, 1986) and the IgG class (subclass not specified) (Dunn *et al.*, 1983).

It is possible that the absence of IgM antibodies from the set tested in the present study explains their failure to protect, but it is more likely, however, that they fail to protect because they do not recognise the right epitope, search for which has so far met with limited success. Because Lipid A is the toxic part of LPS, antibodies to Lipid A are, theoretically, most likely to be those that protect. However, rabbit antibodies to Lipid A that bind solely to free Lipid A do not protect except in very special experimental conditions (Rietschel and Galanos, 1977), in contrast to antibodies to *S. minnesota* R595 (Mullan *et al.*, 1974; Bruins *et al.*, 1977). The absence of free Lipid A from the surface of intact bacteria has been put forward to explain this lack of protection (Mullan *et al.*, 1974), but this conflicts with the present finding that monoclonal antibody to Lipid A (from cell line 6) did not protect mice challenged with free Lipid A (table IV). It can be calculated that although the amount of antibody injected was sufficient to bind to at least 99.5% of the Lipid A present in the challenge dose, clearly, antibody binding did not block the toxic groups.

It is noteworthy that two protective anti-core monoclonal antibodies described recently (Bogard *et al.*, 1984; Teng *et al.*, 1985; Dunn *et al.*, 1986) bound to both free and covalently linked Lipid A, i.e., to an epitope on Lipid A other than that recognised by non-protective polyclonal antibody to Lipid A or by antibody from cell line 6.

To investigate this further, monoclonal antibodies to the ketodesoxyoctonate region of LPS have been prepared and are being studied.

This work was supported in part by the Praeventiefonds. We thank E. J. Ziegler (San Diego) for giving advice and A. J. van Winkelhoff and E. Claassen for critically reading the manuscript. Ms E. Seinen provided excellent secretarial assistance. The artwork was skilfully done by J. G. Meijer and J. M. Verbeek.

REFERENCES

- Appelmelk B J, Verweij-van Vught A M J J, MacLaren D M, Thijs L G 1985 An enzyme-linked immunosorbent assay (ELISA) for the measurement of antibodies to different parts of the Gram-negative lipopolysaccharide core region. *Journal of Immunological Methods* **82**: 199–207.
- Bogard W C, Abernethy K, Dunn D L, Kung P C 1984 Murine monoclonal antibodies against gram-negative bacterial core-glycolipid: criteria for cross-genera reactivity. *Federation Proceedings* **43**: 1682 abstract 1553.
- Bruins S C, Stumacher R, Johns M A, McCabe W R 1977 Immunization with R mutants of *Salmonella minnesota* III. Comparison of the protective effect of immunization with Lipid A and the Re mutant. *Infection and Immunity* **17**: 16–20.
- De Haan P, De Jonge A J R, Verbrugge T, Boersma D M 1985 Three epitope-specific monoclonal antibodies against the hapten penicillin. *International Archives of Allergy and Applied Immunology* **76**: 42–46.
- Dunn D L, Mach P A, Cerra F B 1983 Monoclonal antibodies protect against lethal effects of Gram-negative bacterial sepsis. *Surgical Forum* **44**: 142–143.
- Dunn D L, Bogard W C, Cerra F B 1985 Efficacy of type-specific and cross-reactive murine monoclonal antibodies directed against endotoxin during experimental sepsis. *Surgery* **98**: 283–290.
- Dunn D L, Ewald D C, Chandan N, Cerra F B 1986 Immunotherapy of gram-negative bacterial sepsis. A single murine monoclonal antibody provides cross-genera protection. *Archives of Surgery* **121**: 58–62.
- Elkins K, Metcalf E S 1985 Binding activity of a murine anti-Lipid A monoclonal antibody. *Infection and Immunity* **48**: 597–600.
- Finney D J 1971 Statistical method in biological assay, 2nd edn. Griffin, London, p 524.
- Galanos C, Luderitz O, Westphal O 1971 Preparation and properties of antisera against the Lipid A component of bacterial lipopolysaccharides. *European Journal of Biochemistry* **24**: 116–122.
- Galanos C *et al.* 1984 Immunogenic properties of Lipid A. *Reviews of Infectious Diseases* **6**: 546–552.
- Gigliotti F, Shenep J L 1985 Failure of monoclonal antibodies to core glycolipid to bind intact smooth strains of *Escherichia coli*. *Journal of Infectious Diseases* **151**: 1005–1011.
- Johns M, Skehill A, McCabe W R 1983 Immunization with rough mutants of *Salmonella minnesota*. IV. Protection by antisera to O and rough antigens against endotoxin. *Journal of Infectious Diseases* **147**: 57–67.
- Kasai N *et al.* 1985 In-vitro antigenic reactivity of synthetic Lipid A analogues as determined by monoclonal and conventional antibodies. *Biochemical and Biophysical Research Communications* **128**: 607–612.
- Marks M I, Ziegler E J, Douglas H, Corbeil L B, Braude A I 1982 Induction of immunity against lethal *Haemophilus influenzae* type b infection by *Escherichia coli* core lipopolysaccharide. *Journal of Clinical Investigation* **69**: 742–749.
- Miner K M *et al.* 1986 Characterization of murine monoclonal antibodies to *Escherichia coli* J5. *Infection and Immunity* **52**: 56–62.
- Mullan N A, Newsome P M, Cunningham P G, Palmer G H, Wilson M E 1974 Protection against gram-negative infections with antiserum to Lipid A from *Salmonella minnesota* R595. *Infection and Immunity* **10**: 1195–1201.
- Mutharia L M, Crockford G, Bogard W C, Hancock R E W 1984 Monoclonal antibodies specific for *Escherichia coli* J5 lipopolysaccharide: cross-reaction with other gram-negative bacterial species. *Infection and Immunity* **45**: 631–636.
- Nelles M J, Niswander C A 1984 Mouse monoclonal antibodies reactive with J5 lipopolysaccharide exhibit extensive serological cross-reactivity with a variety of gram-negative bacteria. *Infection and Immunity* **46**: 677–681.
- Perlmutter R M, Hansburg D, Briles D E, Nicolotti R A, Davie J M 1978 Subclass restriction of murine anti-carbohydrate antibodies. *Journal of Immunology* **121**: 566–572.
- Peter G, Chernow M, Keating M H, Ryff J C, Zinner S H 1982 Limited protective effect of rough mutant antisera in murine *Escherichia coli* bacteremia. *Infection* **10**: 228–232.
- Raubitschek A A, Pollack M, Larrick J W 1985 Preparation of human monoclonal antibodies (mAbs) that react with the conserved core region of endotoxin (LPS) *Clinical Research* **33**: 416 A.
- Rietschel E T, Galanos C 1977 Lipid antiserum-mediated protection against lipopolysaccharide- and Lipid A-induced fever and skin necrosis. *Infection and Immunity* **15**: 34–49.
- Telzak E E, Wolff S M 1985 Immunotherapy and immunoprophylaxis of Gram-negative rod bacteremia. In: Root R K, Sande M A (eds) Septic shock. Churchill Livingstone, New York, pp 257–276.
- Teng N N H *et al.* 1985 Protection against gram-negative bacteremia and endotoxemia with human monoclonal IgM antibodies. *Proceedings of the National Academy of Sciences of the USA* **82**: 1790–1794.
- Vel W A C, Namavar F, Verweij-van Vught A M J J, Pubben A N B, MacLaren D M 1985 Killing of *Escherichia coli* by human polymorphonuclear leucocytes in the presence of *Bacteroides fragilis*. *Journal of Clinical Pathology* **38**: 86–91.
- Verweij-van Vught A M J J, Van den Bosch J F, Namavar F, Sparrius M, MacLaren D M 1983 K antigens of *Escherichia coli* and virulence in urinary tract infection: studies in a mouse model. *Journal of Medical Microbiology* **16**: 147–155.
- Young L S 1984 Monoclonal antibodies: technology and application to gram-negative infections. *Infection* **12**: 303–308.
- Ziegler E J *et al.* 1982 Treatment of gram-negative bacteremia and shock with human antiserum to a mutant of *Escherichia coli*. *New England Journal of Medicine* **307**: 1225–1230.
- Zierdt C H 1982 Blood-lysing solution nontoxic to pathogenic bacteria. *Journal of Clinical Microbiology* **15**: 172–174.
- Zinner S H, Peter G 1983 The potential role of cell wall core glycolipids in the immunoprophylaxis and immunotherapy of gram-negative rod bacteriaemia. In: Easmon C S F, Jeljaszewicz J (eds) Medical microbiology, vol. 2, Immunization against bacterial disease. Academic Press, London, pp 71–85.