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Bacterial lipopolysaccharide (LPS)-specific antibodies in commercial human immunoglobulin preparations: superior antibody content of an IgM-enriched product

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SUMMARY

The anti-LPS antibody content of commercial intravenous immunoglobulins was examined by quantitative ELISA using LPS preparations from *Escherichia coli*, *Klebsiella* and *Pseudomonas aeruginosa* O serotypes occurring most frequently in Gram-negative septicaemia. Three IgG products from different manufacturers and one IgM-enriched product were tested. Mean antibody levels were significantly higher in the IgM fraction of the IgM-enriched product compared with 'pure' IgG products, indicating that natural antibodies against bacterial LPS belong primarily to the IgM class. Immunoblotting studies showed that antibody specificities were directed mainly against O side chain epitopes. Antibodies against rough mutant LPS representing various chemotypes were detected in IgG but not in IgM products. The virtual absence of antibodies against *Vibrio cholerae* LPS indicated that human anti-LPS antibodies result from continuous environmental exposure to Gram-negative pathogens. These data support the further development of IgM-enriched preparations for prophylaxis and treatment of Gram-negative nosocomial infections.

Keywords immunoglobulin(s) lipopolysaccharide endotoxin

INTRODUCTION

Gram-negative bacterial infections continue to be a major cause of morbidity in hospitalized patients [1,2]. Despite the availability of new-generation antimicrobials with enhanced activity against Gram-negative bacilli, antibiotic treatment alone is often unable to halt the progression of such infections to septic shock and death. Various adjunctive therapeutic approaches have been evaluated during the last decade, but most of these studies yielded controversial results (review in [3]).

The preventive use of standard intravenous immunoglobulins (IVIG) has proved effective in several studies involving patients with a high risk of infection, in particular septicaemia [4–9]. There are few studies in which immunoglobulins have been used for treatment of overt systemic infection [10,11], but two studies in which an IgM-enriched product was used showed a significant reduction in mortality from septicaemia in newborns [12] and Gram-negative septic shock [13]. Both of these studies involved small numbers of patients, however. An additional study demonstrated that this same preparation, when given prophylactically to patients undergoing bone marrow transplantation, significantly reduced the level of endotoxaemia and fever [14]. Since the preparation used in these studies is manufactured differently from standard IVIG products made from Cohn fraction II, it is important to compare the levels of specific antibacterial antibodies in it with levels of antibody in conventional IgG products used in previous sepsis trials.

Since bacterial LPS (endotoxins) are known to play a key role in the pathogenesis of Gram-negative septic shock [15], we measured the concentration of specific anti-LPS antibodies in the IgM-enriched product and compared them with those of four commercial IgG preparations. As antigens, we selected LPS from those *Escherichia coli*, *Klebsiella*, and *Pseudomonas* O antigen serotypes that are known from seroepidemiological studies to be involved most frequently in septicaemic infections. LPS antibodies were quantified by means of a standardized ELISA method, and the epitope specificity of the antibodies was roughly determined by immunoblotting. Antibodies in all products were found to be directed mainly against LPS side chain determinants and were concentrated significantly in the IgM class.

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MATERIALS AND METHODS

Bacteria and LPS preparation

The organisms used for LPS preparation are listed in Table 1. LPS

from these strains was extracted by the hot phenol water method [16]. The following LPS preparations were obtained commercially: *Vibrio cholerae* serotype Inaba strain 569B, *E. coli* J5 (Rc mutant), *Salmonella minnesota* wild-type, *S. typhimurium* TV119 (Ra chemotype), *S. minnesota* R5 (Rc chemotype), *S. minnesota* R7 (Rd chemotype), *S. minnesota* Re595 (Re chemotype) (all from Sigma, Deisenhofen, Germany).

IVIG preparations

Two batches each of Sandoglobulin (Sandoz, Basel, Switzerland), Polyglobin N (Tropon-Cutter, Köln, Germany), Intraglobin F (Biotest Pharma, Dreieich, Germany), and four batches of Pentaglobin (Biotest-Pharma) were included in the study. The three first-mentioned products are human polyclonal IgG preparations made compatible for i.v. use by treatment at pH 4 with traces of pepsin (Sandoglobulin), reduction and alkylation (Polyglobin N), and β-propiolactone treatment (Intraglobin F). Polyglobin N and Intraglobin F are supplied as liquid formulations containing a declared concentration of 50 g/*l* IgG. Sandoglobulin is supplied as a lyophilized preparation to which physiological sodium chloride solution has to be added to yield a final IgG concentration of 30 g/*l* or 60 g/*l*. For the sake of comparability, we added only 60 ml of sodium chloride solution to a 3-g bottle of Sandoglobulin to obtain a calculated IgG concentration of 50 g/*l*.

The IgM-enriched product Pentaglobin is made from Cohn fraction III by treatment with octanoic acid, Sephadex absorption and incubation with β-propiolactone. The final product contains approximately 38 g/*l* IgG, 6 g/*l* IgM and 6 g/*l* IgA. In preliminary experiments, we found that the IgG antibodies contained in Pentaglobin may compete with IgM antibodies for LPS binding. Therefore, for ELISA and immunoblot studies, the IgG and IgM fractions of the product were separated by gel filtration using the HiLoad 26/60 Superdex 200 column (Pharmacia, Freiburg, Germany), followed by affinity chromatography on the HiTrap protein G column (Pharmacia). Purity of the separated IgM was 98–99%. Total IgG and IgM concentrations of all products were determined nephelometrically (Beckman Array, Starnberg, Germany).

Table 1. Bacterial strains used for LPS preparation

Strain designation	Antigen formula	Source
E. coli E 864	O1:K1:H7	WRAIR*
E. coli E 518	O2:K-:H46	WRAIR
E. coli E 863	O4:K12:H1	WRAIR
E. coli E 701	O6:K5:H1	WRAIR
E. coli E 650/568	O15:K52:H1	WRAIR
E. coli E 794	O16:K1:H86	WRAIR
E. coli E 833	O18:K1:H7	WRAIR
P. aeruginosa	FD1†	WRAIR
P. aeruginosa	FD2	WRAIR
P. aeruginosa	FD3	WRAIR
P. aeruginosa	FD5	WRAIR
Klebsiella Friedländer 201	O1:K-	SSI‡
Klebsiella 7380	O2ab:K-	SSI
Klebsiella 390	O3:K11	SSI

* Walter Reed Army Institute of Research, Washington, DC.
† Fisher-Devlin immunotype.
‡ Statens Seruminstitut, Copenhagen, Denmark.

Quantitative ELISA

The concentration of specific IgG and, if applicable, IgM antibodies against individual LPS serotypes was determined as previously described [17,18]. In short, test LPS and purified human IgG- (or IgM-) specific capture antibodies (Sigma) were coated on separate segments of microtitre ELISA plates. Known dilutions of the immunoglobulins were added to both types of wells, and bound human IgG (or IgM) was traced with appropriate, alkaline phosphatase-conjugated secondary antibodies. Standard curves were generated by plotting optical density (OD) values against the corresponding concentrations of pure IgG or IgM added to anti-immunoglobulin-coated wells. OD values measured on LPS-coated segments (usually at dilutions of 1:32–1:64 for IgG and 1:2–1:16 for IgM) were used to calculate the concentration of LPS-specific antibody by comparison with the linear part of the standard curve. In these experiments, each immunoglobulin was used as its own standard, e.g. known concentrations of IgG from Polyglobin N were used to construct the standard curve for determining LPS-specific antibody in Polyglobin N. IgM antibody levels were measured using purified IgM from Pentaglobin.

Competitive ELISA

In order to test for competition between IgG and IgM antibodies for the LPS target, plates were coated with LPS as described above. A dilution series of IgG purified from Pentaglobin (batch 1461073) was added to duplicate wells, after premixing of each dilution with either buffer (control) or with purified IgM from the same batch at a constant final concentration of 1 mg/ml IgM. After incubation, the plate was labelled with IgG-specific secondary antibody and developed as described above. Alternatively, a dilution series of purified IgM was added to the wells, after premixing with buffer or 1 mg/ml (final concentration) purified IgG. The percentage inhibition by the alternative immunoglobulin class was calculated by the reduction of OD values compared with the buffer control in the linear part of the ELISA curve.

Determination of antibody avidity

Plates were coated with LPS as described above, and primary antibodies were incubated on the wells for 5 min, 15 min, 30 min, 1 h, 2 h and 4 h, whereafter they were washed off and the remainder of the ELISA reaction developed as described.

Electrophoresis and immunoblotting of LPS

Purified LPS preparations were separated by PAGE as described by Sidberry *et al*. [19], using the buffer system of Laemmli [20]. Electrophoresed LPS were either directly visualized by the silver-stain procedure [21], or transblotted to a 0·45-μm nitrocellulose membrane (Millipore, Molsheim, France) using the transfer buffer of Towbin *et al*. [22]. Blot membranes were reacted overnight with the immunoglobulin preparations diluted to a concentration of 5 g/*l* of total IgG or IgM, respectively, or other concentrations as indicated in Fig. 2. After washing, bound IgG or IgM was visualized by sequential addition of class-specific alkaline phophatase-conjugated antibodies and developing substrate as described [19].

Statistical analysis

IgG and IgM antibody concentrations against different O antigen serotype LPS preparations were compared by the Mann–Whitney *U*-test using a software package (Statistics for Windows, version 4.5, StatSoft, Tulsa, OK). $P \leq 0{\cdot}05$ was considered significant. All comparisons were two-tailed.

Table 2. LPS-specific IgG antibody content in commercial intravenous immunoglobulin preparations

Product	Batch no.	Total IgG content (mg/ml)	Specific IgG antibody content (μg/ml (μg/g total IgG)) against LPS derived from														
			E. coli O1	*E. coli* O2	*E. coli* O4	*E. coli* O6	*E. coli* O15	*E. coli* O16	*E. coli* O18	*E. coli* J5	Kleb.* O1	Kleb. O2ab	Kleb. O3	Ps. FD1†	Ps. FD2	Ps. FD3	Ps. FD5	*Vibrio cholerae*
Pentaglobin	1461102	38·2	14·7 (385)	0·7 (18)	2·4 (63)	3·9 (102)	3·1 (81)	2·2 (58)	11·0 (288)	<0·2 (<5)	8·0 (209)	7·0 (183)	24·3 (636)	4·8 (126)	21·1 (552)	1·4 (37)	0·9 (24)	<0·2 (<5)
Pentaglobin	1467112	37·6	19·8 (527)	0·5 (13)	8·1 (215)	6·7 (178)	5·7 (152)	5·8 (154)	13·6 (362)	<0·2 (<5)	9·5 (253)	6·4 (170)	9·0 (239)	2·7 (73)	15·5 (412)	0·3 (8)	0·4 (11)	<0·2 (<5)
Pentaglobin	1461073	41·9	9·6 (229)	1·1 (26)	4·0 (95)	5·0 (119)	2·9 (69)	6·7 (160)	8·9 (212)	<0·2 (<5)	10·2 (243)	5·7 (136)	10·3 (246)	9·6 (229)	35·7 (852)	1·4 (33)	1·0 (24)	<0·2 (<5)
Pentaglobin	1461083	40·9	16·0 (391)	2·3 (56)	3·6 (88)	5·7 (139)	5·0 (122)	3·0 (73)	5·1 (125)	<0·2 (<5)	9·9 (242)	7·8 (191)	12·2 (298)	10·3 (252)	26·0 (636)	1·4 (34)	3·3 (81)	<0·2 (<5)
Intraglobin F	1412101	44·2	9·9 (224)	0·3 (7)	2·0 (45)	2·0 (45)	2·3 (52)	2·5 (57)	9·6 (217)	<0·2 (<5)	6·1 (138)	4·8 (109)	3·0 (68)	2·6 (59)	7·0 (158)	0·7 (16)	0·4 (9)	<0·2 (<5)
Intraglobin F	1413092	48·2	17·9 (371)	0·2 (4)	5·8 (120)	3·8 (79)	6·7 (139)	9·0 (204)	15·4 (320)	<0·2 (<5)	16·3 (338)	16·2 (336)	8·0 (166)	0·6 (12)	1·9 (39)	0·9 (19)	0·8 (17)	<0·2 (<5)
Sandoglobulin	12001	41·0	27·2 (663)	0·6 (15)	4·8 (117)	8·8 (183)	7·0 (171)	12·6 (307)	20·6 (502)	<0·2 (<5)	16·6 (405)	22·1 (539)	16·3 (398)	8·3 (202)	25·2 (615)	3·0 (73)	1·9 (46)	<0·2 (<5)
Sandoglobulin	5201	43·0	15·5 (360)	0·5 (12)	4·3 (100)	4·5 (105)	6·1 (142)	5·4 (126)	18·5 (430)	<0·2 (<5)	6·9 (160)	8·0 (186)	12·4 (288)	6·1 (142)	14·3 (333)	1·9 (44)	0·8 (19)	<0·2 (<5)
Polyglobin N	1702792	51·4	26·1 (508)	2·5 (49)	8·3 (161)	8·9 (173)	6·3 (123)	9·3 (181)	24·3 (473)	<0·2 (<4)	20·5 (399)	18·5 (360)	16·6 (323)	12·6 (245)	26·1 (508)	5·3 (103)	1·7 (33)	0·5 (10)
Polyglobin N	AADU 1	50·4	32·6 (647)	2·6 (52)	9·3 (185)	9·4 (187)	8·6 (171)	15·4 (306)	33·3 (661)	0·2 (4)	23·7 (470)	26·9 (534)	19·8 (393)	12·2 (242)	26·1 (518)	3·8 (75)	1·8 (36)	0·3 (6)

* Klebsiella.

† *Pseudomonas aeruginosa* Fisher-Devlin immunotypes.

Table 3. LPS-specific IgM antibody content in a commercial IgG/A/M preparation

Pentaglobin batch no.	Total IgM content (mg/ml)	Specific IgM antibody content (μg/ml (μg/g total IgM)) against LPS derived from: *E. coli* O1	*E. coli* O2	*E. coli* O4	*E. coli* O6	*E. coli* O15	*E. coli* O16	*E. coli* O18	*E. coli* J5	Kleb. O1	Kleb. O2ab	Kleb. O3	Ps. FD1	Ps. FD2	Ps. FD3	Ps. FD5	*Vibrio cholerae*
1461102	7·35	13·0 (1769)	6·7 (917)	7·0 (952)	7·7 (1048)	9·1 (1238)	8·4 (1143)	31·9 (4333)	<1·1 (<143)	15·4 (2095)	28·4 (3864)	18·7 (2544)	7·0 (952)	39·6 (5388)	2·9 (395)	4·2 (571)	<1·1 (<143)
1467112	7·57	7·8 (1030)	3·7 (489)	5·5 (727)	8·7 (1149)	14·7 (1939)	7·8 (1030)	25·7 (3395)	<1·4 (<181)	8·7 (1149)	21·6 (2853)	26·6 (3515)	6·9 (912)	29·8 (3937)	3·3 (436)	4·8 (634)	<1·4 (<181)
1461073	6·47	10·1 (1560)	3·4 (526)	4·4 (678)	10·5 (1628)	4·0 (619)	4·8 (746)	16·7 (2577)	<0·7 (<102)	9·2 (1424)	8·2 (1272)	8·9 (1373)	3·6 (551)	20·2 (3120)	1·7 (255)	2·7 (417)	<0·7 (<102)
1461083	6·14	9·6 (1564)	2·7 (446)	5·7 (928)	14·7 (2394)	8·5 (1384)	11·5 (1873)	34·6 (5635)	<0·7 (<107)	12·5 (2034)	14·0 (2274)	12·1 (1971)	6·7 (1091)	25·4 (4137)	2·6 (424)	5·7 (928)	<0·7 (<107)

For abbreviations, see Table 2.

Table 4. Mean total LPS-specific antibody levels in the products tested

Product	Mean total immunoglobulin content (mg/ml)	Specific total antibody content (μg/ml) against LPS derived from: *E. coli* O1	*E. coli* O2	*E. coli* O4	*E. coli* O6	*E. coli* O15	*E. coli* O16	*E. coli* O18	*E. coli* J5	Kleb. O1	Kleb. O2ab	Kleb. O3	Ps. FD1	Ps. FD2	Ps. FD3	Ps. FD5	*Vibrio cholerae*
Pentaglobin	46·5	25·2	5·3	10.1	15.7	13.3	12.6	36·9	<1·6	18·4	24·8	30.5	12.9	53.3	3·8	5·8	<1.6
Intraglobin F	46·2	13·9	0·3	3.9	2.9	4.5	5.8	12·5	<0·2	11·2	10·5	5.5	1.6	4.5	0·8	0·6	<0.2
Sandoglobulin	42·0	21·4	0·6	4.6	6.7	6.6	9.0	19·6	<0·2	11·8	15·1	14.4	7.2	19.8	2·5	1·4	<0.2
Polyglobin N	50·9	29·4	2·6	8.9	9.2	7.5	12.4	28·8	<0·2	22·1	22·7	18.2	12.4	26.1	4·6	1·8	0.4

For abbreviations, see Table 2.

RESULTS

Quantification of LPS antibodies by direct ELISA

The results of these determinations are summarized in Tables 2 and 3. The concentration of total IgG measured nephelometrically was slightly lower in some of the products compared with the concentration declared by the manufacturer. Also, the IgM-enriched product contained a lower concentration of total IgG antibodies compared with the 'pure' IgG products. Therefore, corrected specific IgG concentrations were calculated by dividing the amount of specific IgG antibody (in μg) by the amount of total IgG (in g). These figures (shown in parentheses) allow a more direct comparison between individual products.

All 10 batches tested contained IgG antibodies against the enterobacterial LPS and against LPS derived from *P. aeruginosa* (Table 2). A statistical comparison of weight-corrected values could be made only for the IgG levels in Pentaglobin because values from four batches were available for Wilcoxon analysis. Specific IgG concentrations against *E. coli* O1 and O18 were significantly higher compared with those against all other *E. coli* O serotypes ($P < 0{\cdot}05$). The IgG antibody content against *Pseudomonas* Fisher-Devlin serotype 3 and Fisher-Devlin serotype 5 LPS was significantly lower compared with the two other Fisher-Devlin serotypes tested ($P < 0{\cdot}05$). The same trend was seen with the other commercial preparations, although statistical comparisons could not be made since only two batches were tested. With the exception of a weak reactivity in one preparation (Polyglobin N, Table 2, lower 2 rows), no antibody activity could be detected against *Vibrio cholerae* serotype Inaba LPS.

ELISA experiments were also performed using Salmonella Ra, Rc, Rd and Re as well as *E. coli* J5 (Rc) LPS as coating antigens. IgG levels against the Salmonella rough mutant LPS were $<0{\cdot}5\,\mu$g/ml in all of the IgG products, with the exception of the two batches of Polyglobin N, which contained variable concentrations of antibody to individual rough mutant antigens ranging from 0·2 to $2{\cdot}9\,\mu$g/ml (data not shown). Table 2 shows the data for *E. coli* J5 LPS, which were representative for the other chemotypes studied.

Specific IgM antibody determinations in Pentaglobin are summarized in Table 3. Preliminary experiments showed that LPS-specific IgG (and possibly IgA) antibodies present in this product competed with specific IgM for LPS binding in the direct ELISA (see below). Therefore, we removed both the IgG and IgA fractions by column chromatography and protein G trapping of IgG. IgM antibody levels determined in this purified material and recalculated to μg per ml of the original product are given in Table 3, with weight-corrected values in parentheses.

By adding LPS-specific IgG and IgM antibody levels of Pentaglobin, we calculated the mean concentration of total LPS-specific antibody per ml of infused product and compared these values with the mean anti-LPS levels found in the other products (Table 4). Pentaglobin contained more LPS-specific antibody than Intraglobin F and Sandoglobulin for all antigens tested. Polyglobin N contained the highest IgG anti-LPS levels, but even when compared with this product, Pentaglobin had higher levels against 11 out of the 14 LPS types studied. Table 5 shows the extent to which LPS-specific antibody was enriched in the IgM fraction of Pentaglobin. The proportion of specific antibody in this fraction was 3·9–21·3-fold higher than that in the IgG fraction of the same product.

Specificity of LPS-reactive antibodies

Western blot studies performed with all 10 immunoglobulin batches showed a uniform pattern of reactivity with the various smooth LPS. At equivalent total immunoglobulin concentrations, LPS reactivity appeared to be more intense with the IgM fraction of Pentaglobin compared with the IgG products, in particular with respect to staining of the side chains of *E. coli* O2, *E. coli* O15, and *Klebsiella* LPS (Fig. 1). The stepladder-like reaction pattern observed with all of the products showed that the reactivity of the LPS-specific antibodies was directed mainly against side chain epitopes. The low molecular weight bands corresponding to the core oligosaccharide regions were stained only faintly in blotting experiments performed with the purified IgM fractions (Fig. 1b), while they were seen more clearly in blots reacted with IgG products (Fig. 1c, e.g. lanes 1–4, lane 12). In accordance with the ELISA results, neither IgG nor IgM reactivity was seen with *V. cholerae* LPS (Fig. 1). For *E. coli* O18 LPS, stepwise dilutions of IgG and IgM purified from Pentaglobin were tested. The higher concentration of LPS side chain-specific antibodies in the IgM fraction again became apparent (Fig. 2).

Additional immunoblotting experiments were performed to examine the binding pattern for various rough mutant LPS. In contrast to the ELISA experiments, antibodies reactive with the fast-migrating bands corresponding to these LPS were detected in experiments with all of the IgG products, but not in blots reacted with two batches of purified IgM. A representative example of these findings is depicted in Fig. 3.

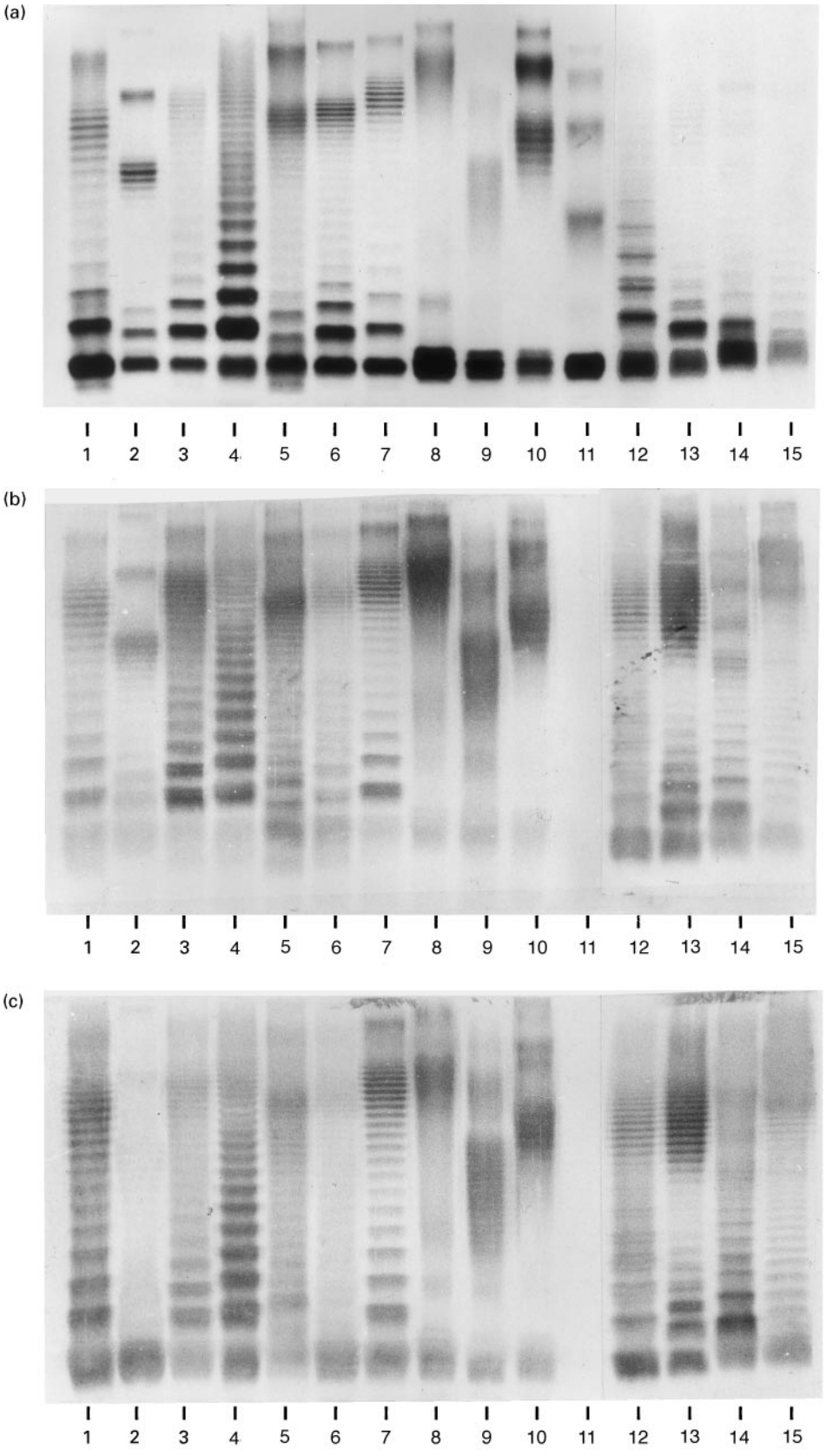
Competition between LPS-specific IgM and IgG

One concern when infusing a product containing both IgG and IgM may be competition for the target antigens *in vivo*, which could result in a disturbance of immune recognition. When we tested Pentaglobin solution as it comes from the manufacturer, we measured lower anti-LPS IgM antibody levels compared with tests in which we used the purified IgM fractions from the same batches. For instance, mean (± 1 s.d.) ELISA-reactive IgM antibody levels to Klebsiella O1 LPS in the four batches of Pentaglobin were $6{\cdot}3 \pm 1{\cdot}6\,\mu$g/ml (test with

Table 5. LPS-specific IgG and IgM antibody content in four batches of a commercial IgG/A/M preparation

LPS antigen	IgG (mean ± s.d.)*	IgM (mean ± s.d.)*	Fold difference†
E. coli O1	383 ± 122	1481 ± 274	3.9
E. coli O2	28 ± 19	595 ± 217	21.3
E. coli O4	115 ± 68	821 ± 139	7.1
E. coli O6	135 ± 33	1555 ± 614	11.5
E. coli O15	106 ± 38	1295 ± 543	12.2
E. coli O16	111 ± 53	1198 ± 480	10.8
E. coli O18	247 ± 102	3985 ± 1313	16.1
Kleb. 01	237 ± 19	1676 ± 464	7.1
Kleb. 02ab	170 ± 24	2566 ± 1084	15.1
Kleb. 03	355 ± 190	2351 ± 912	6.6
Ps. FD1	170 ± 85	877 ± 230	5.2
Ps. FD2	613 ± 184	4146 ± 938	6.8
Ps. FD3	28 ± 13	378 ± 84	13.5
Ps. FD5	35 ± 31	638 ± 214	18.2

* Values are μg/g total IgG or IgM, respectively.
† Ratios were calculated for mean values.
For abbreviations, see Table 1.


(a)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
(b)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
(c)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

native product) *versus* 11·5 ± 3·1 μg/ml (test with purified IgM). Thus, IgM antigen binding was approximately halved by the presence of 38 mg/ml IgG in the native product.

Since this IgG concentration is higher than the levels of exogenous IgG found in patients after infusion of IVIG [23], we performed additional experiments in which we added 1 mg/ml of either purified IgG or IgM as a competitor to a dilution series of the alternate immunoglobulin class. LPS from *E. coli* O1, O6, O18 and *Klebsiella* O1 were selected as representative antigens for these experiments. At this lower concentration, IgG did not inhibit IgM antigen binding (data not shown). However, IgM reduced the antigen binding of IgG by 15·9% (*E. coli* O1), 4·7% (*E. coli* O6), 17·2% (*E. coli* O18), or 7·8% (*Klebsiella* O1). Thus, both immunoglobulin classes were able to compete with each other, and IgM did so at a lower concentration than IgG. This is probably due to the higher concentration of specific LPS antibodies in the IgM fraction, since ELISA experiments in which we studied the avidity of anti-LPS antibodies did not show significant differences between the two immunoglobulin classes (Fig. 4).

DISCUSSION

Beneficial effects of polyvalent immunoglobulins have been demonstrated in animal models of sepsis. However, most of these studies have also shown that such effects are most pronounced when IVIG is given either before or very early after bacterial challenge [17,24,25]. In humans, treatment of established sepsis with IVIG has yielded controversial results, a fact that may be due to the heterogeneity of causative organisms and the variation in underlying diseases and clinical status of the patients included in such studies (review in [26,27]). Recently, it has been suggested that a careful stratification of patients with respect to their Acute Physiology and Chronic Health Evaluation (APACHE) and sepsis scores or the demonstration of endotoxaemia may lead to more conclusive results [27,28]. At least in Europe, variations of antibody levels in the raw material of IVIG may play a lesser role in the outcome of clinical studies, because the European Pharmacopoeia prescribes the inclusion of plasma units from ≥1000 donors per batch [29].

The potential effect of IVIG in established sepsis may be related to its recently discovered ability to inhibit the release of proinflammatory mediators [30]. Inhibition by IVIG of tumour necrosis factor-alpha (TNF-α) and IL-1 release from monocytes has been linked to the presence of anti-LPS antibodies in polyclonal immunoglobulin [30]. Our own data show that this inhibitory effect was stronger for an IgM-enriched IVIG preparation compared with 'pure' IgG products, which may point to a higher concentration (or avidity) of endotoxin-neutralizing antibodies in the IgM class [31]. We therefore decided to measure quantitatively the level of LPS-specific antibodies in IgM-containing *versus* conventional IVIG products.

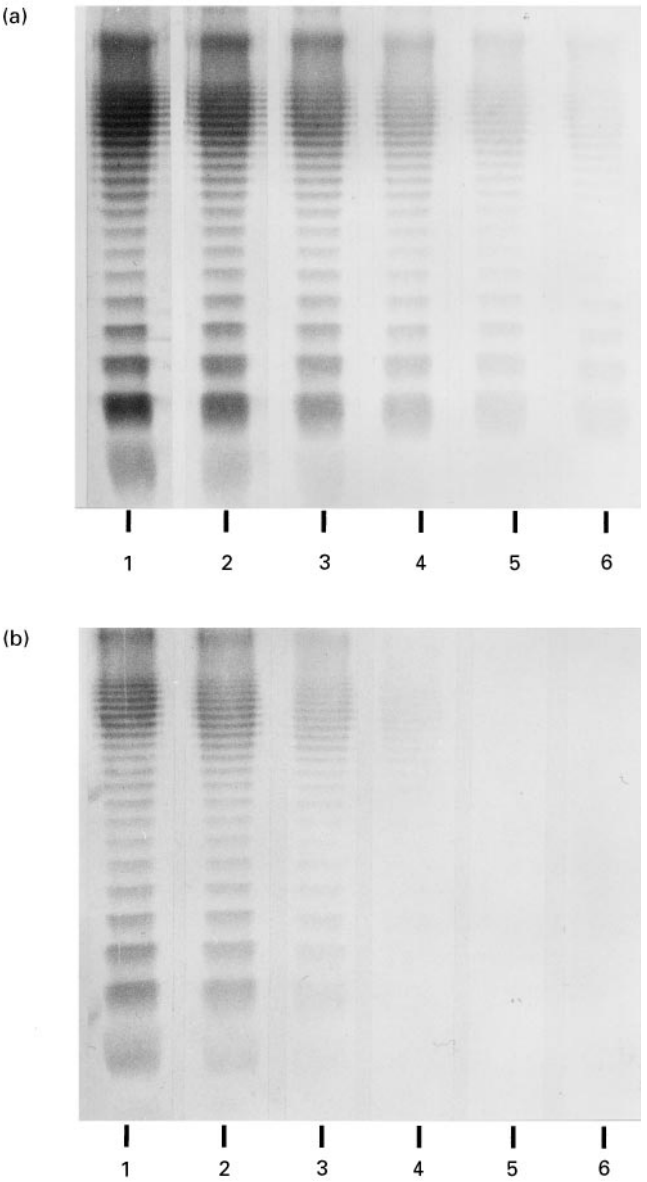
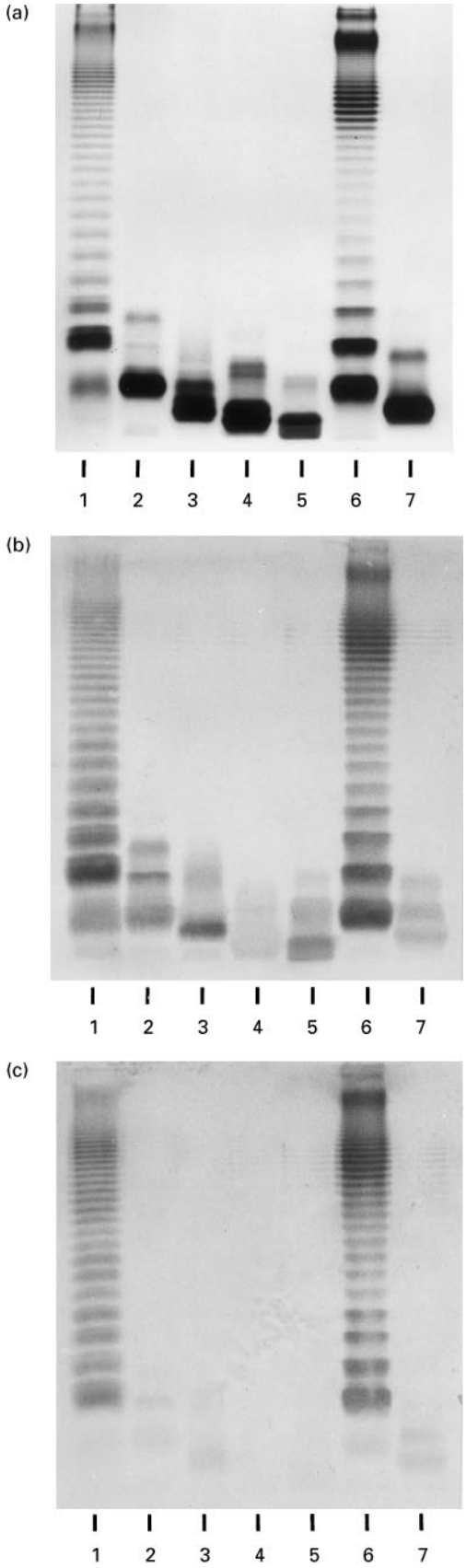


Fig. 2. Reactivity of IgM and IgG from Pentaglobin at various dilutions with LPS from *Escherichia coli* O18. LPS (20 μg) was electrophoresed as in Fig. 1 and blotted onto nitrocellulose. (a) Blot strips reacted with IgM purified from Pentaglobin and developed with anti-human IgM alkaline-phosphatase conjugate. (b) Blot strips reacted with Pentaglobin and developed with anti-human IgG alkaline-phosphatase conjugate. Class-specific immunoglobulin concentrations were as follows: lane 1, 5 g/*l*; lane 2, 1 g/*l*; lane 3, 0·2 g/*l*; lane 4, 0·04 g/*l*; lane 5, 0·008 g/*l*; lane 6, 0·0016 g/*l*. Side chain-specific staining by IgM is seen at concentrations as low as 0·0016 g/*l*.

Fig. 1. LPS silver stain (a) and corresponding immunoblots reacted with Pentaglobin-IgM (b) and Pentaglobin-IgG (c). LPS preparations (12–20 μg per lane) were subjected to electrophoresis on 14·5% polyacrylamide gels. Lane 1, LPS *Escherichia coli* O1; lane 2, LPS *E. coli* O2; lane 3, LPS *E. coli* O4; lane 4, LPS *E. coli* O6; lane 5, LPS *E. coli* O15; lane 6, LPS *E. coli* O16; lane 7, LPS *E. coli* O18; lane 8, LPS Klebsiella O1; lane 9, LPS Klebsiella O2ab; lane 10, LPS Klebsiella O3; lane 11, LPS *Vibrio cholerae* serotype Inaba; lane 12, LPS Pseudomonas FD1; lane 13, LPS Pseudomonas FD2; lane 14, LPS Pseudomonas FD3; lane 15, LPS Pseudomonas FD5. (a) Silver-stained gel. (b) Identical companion gel blotted onto nitrocellulose, reacted with IgM purified from Pentaglobin at 5 g/*l* and developed with anti-human IgM alkaline-phosphatase conjugate. (c) Additional companion gel reacted with Pentaglobin at an IgG concentration of 5 g/*l* and developed with anti-human IgG alkaline-phosphatase conjugate.



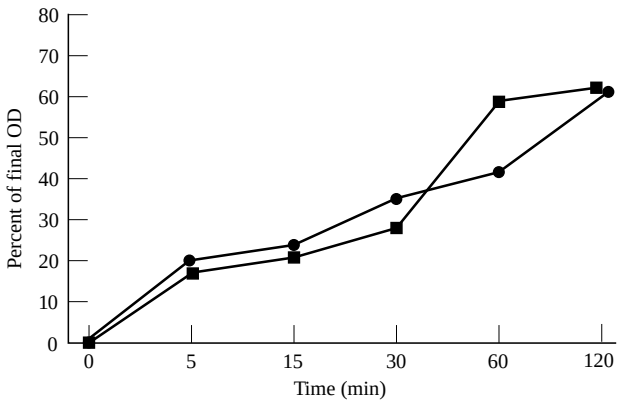


Fig. 4. Avidity for Klebsiella O1 LPS of IgG (■) and IgM (●) purified from Pentaglobin (batch no. 1461073). The purified immunoglobulins were added to the wells at dilutions yielding optimal ELISA reactivity (2 mg/ml for IgG and 0·5 mg/ml for IgM) and left on the plates for the time periods indicated. Optical density (OD) values reached after each period of incubation were expressed as percentage of the final OD measured after 4 h. The steepness of the curves can be regarded as a measure of antibody avidity. Similar results were obtained for LPS from *Escherichia coli* O6 and *Pseudomonas aeruginosa* FD1.

A drawback of previous studies examining the anti-LPS antibody content of IVIG was that either the O antigens examined were poorly defined, or that antibody concentrations were reported in arbitrary units, which precluded a comparison with antibody levels known to be protective from animal models of sepsis [17,32–35]. In the present study, we used a previously described quantitative ELISA system [17,18] to quantify the level of O antigen-specific IgG and IgM antibodies in four commercial IVIG preparations. Purified LPS preparations were obtained from those O antigen serotypes that have been identified most frequently among isolates causing Gram-negative septicemia. For *E. coli*, various seroepidemiological studies were available which showed that, of the more than 150 known O serotypes, the O antigens O1, O2, O4, O6, O8, O15, O16, O18 and O75 account for >80% of the antigenic types causing septicaemia [36–40]. When the data from the last-mentioned studies were pooled, the O6 antigen of *E. coli* occurred most frequently (11·9% of septicaemia cases), while the other indicated antigens each accounted for 6–8% of cases [36–40]. Studies of the seroepidemiology of bacteraemic *P. aeruginosa* isolates showed that Fisher-Devlin serotypes 1–5 were predominant in this setting [41,42]. In the study of Vásquez *et al*., Fisher-Devlin serotype 1 (corresponding to international type (IATS) type 6) was found in 50%, Fisher-Devlin type 2 (IATS 11) in 24·3%, and Fisher-Devlin type 3 (IATS 2) in 11·4% of bacteraemic isolates [42]. Recently, we examined the distribution of *Klebsiella* O serotypes in clinical material and found that the O serotypes O1, O2ab and O3 accounted for 36·7%, 12·7% and 30·4% (together,

Fig. 3. Reactivity of immunoglobulins with rough mutant LPS in a representative immunoblot experiment performed with Sandoglobulin and Pentaglobin-IgM. Gels were loaded with 15–20 μg of smooth LPS, and 3–4 μg of rough LPS. Lane 1, *Salmonella minnesota* wild-type LPS; lanes 2–5, Salmonella Ra, Rc, Rd and Re chemotype LPS, respectively; lane 6, *Escherichia coli* O18 smooth LPS; lane 7, *E. coli* J5 (Rc) LPS. (a) Silver-stained gel. (b) Immunoblot reacted with Sandoglobulin (5 g/*l*). (c) Immunoblot reacted with IgM purified from Pentaglobin (5 g/*l*).

79·8%) of bacteraemic isolates, respectively [43]. Thus, the panel of enterobacterial and *Pseudomonas* LPS preparations used in the present study comprises a significant portion of the O antigen serotypes associated with septicaemia.

All products tested contained specific IgG antibodies against the LPS antigens included in the study (Table 2). The differences of antibody levels against specific LPS antigens may be explained by different frequencies in which strains of individual O serotypes occur in the gut flora of the plasma donor populations. Furthermore, after having colonized the gut, strains carrying different O antigens may also differ in their ability to translocate to the mesenteric lymph nodes and stimulate an LPS-specific humoral immune response [44,45]. The view that the presence of natural antibodies in human serum is related to (transient or stable) intestinal colonization is substantiated by our finding that none of the immunoglobulin preparations contained detectable antibody against LPS derived from *V. cholerae*, an organism that does not usually colonize the human gut, at least in the Mid-European and North-American donor populations from which the plasma units used for commercial immunoglobulin production are obtained.

The main goal of the present study was to elucidate whether an enrichment of IgM antibodies, which is part of the specific production process of Pentaglobin, correlates with an enrichment of LPS-specific antibody. This was suggested by the results of Jackson *et al*., who tested a single batch of Pentaglobin against a pool of four LPS antigens and found that the anti-LPS IgM titre was 1/1024, compared with an IgG titre of 1/32 [14]. Also, after infusion of this product in bone marrow transplant patients, LPS-specific IgM levels rose significantly, in contrast to an insignificant rise of anti-LPS IgG levels [14]. Our data confirmed and extended these findings for a total of 14 LPS serotypes. Although Pentaglobin contains no more than 12% IgM, compared with 75% IgG, antibody levels measured in the IgM fraction of the various batches were as high or even higher compared with those found in the corresponding IgG fraction (Table 3). When values were corrected for total immunoglobulin content in grams, LPS-specific antibodies appeared to be enriched by a factor of 3·9–21·3 in the IgM fraction (Table 5). The calculation of the ratios given in this Table was performed on a weight basis and the results expressed as μg/g of total immunoglobulin; however, identical figures would result if ratios were expressed as micromoles of antibody per moles of total immunoglobulin.

In view of these findings, it seems noteworthy that early studies by Rosen and coworkers showed that 'natural' antibodies against Gram-negative bacteria were associated with the 19S (IgM) fraction of normal human sera [46–48]. In the study of Michael & Rosen, an IgM preparation obtained from Cohn fraction III had significantly higher opsonic and protective activity in an animal model of Gram-negative sepsis compared with the corresponding IgG fraction [46]. These early studies did not examine antigenically well defined strains, but our data show that antibodies belonging to the IgM fraction comprise specificities directed against many clinically relevant O antigen serotypes.

A novel finding of this study was the observation that antibodies in both IgG and IgM products were directed against O side chain determinants. All preparations produced stepladder-like reaction patterns on immunoblots typical of O side chain-reactive antibodies. Our ELISA experiments showed little or no antibody activity against LPS prepared from rough mutant strains such as *E. coli* J5 in most of the batches (Table 2). However, since subsequent immunoblotting studies revealed the presence of such antibodies in the IgG products (Fig. 3), it is possible that the direct ELISA system is not optimally suited to detect such antibodies. In future studies, the use of capture molecules such as polymyxin B [49] or poly-L-lysine [50] may enhance the detection of rough mutant antibodies.

The question whether IgG and IgM, when infused together, will compete for LPS recognition *in vivo* cannot be answered by this study. Our ELISA experiments show that both immunoglobulin classes are able to compete with each other *in vitro*. Both classes contain specific antibody which appears to have the same avidity for LPS (Fig. 4). When Pentaglobin is infused to septic patients, competition effects are less likely to occur because LPS is distributed widely throughout the vascular system. IgG and IgM antigen contacts may therefore occur at separate sites in the bloodstream compared with the situation on the ELISA plate, where both classes of antibody compete for antigen concentrated on a minimal area of contact. To our knowledge, clinical deterioration of septic patients related to the infusion of Pentaglobin has never been described.

Taken together, the present data show that IVIG preparations contain significant levels of LPS-specific antibodies directed against various clinically relevant O antigen serogroups of Gram-negative species. Such antibodies are concentrated in the IgM fraction of the IgM-enriched product by a factor of 3·9–21·3. Since antibodies of the IgM class have been suggested to be the most important in the neutralization and clearance of endotoxin [51], further experimental and clinical studies employing IgM-enriched preparations of IVIG are clearly warranted.

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