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A Broadly Cross-Protective Monoclonal Antibody Binding to *Escherichia coli* and *Salmonella* Lipopolysaccharides

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During the last decade, episodes of sepsis have increased and *Escherichia coli* has remained the most frequent clinical isolate. Lipopolysaccharides (LPS; endotoxin) are the major toxic and antigenic components of gram-negative bacteria and qualify as targets for therapeutic interventions. Molecules that neutralize the toxic effects of LPS are actively investigated. In this paper, we describe a murine monoclonal antibody (MAb; WN1 222-5), broadly cross-reactive and cross-protective for smooth (S)-form and rough (R)-form LPS. As shown in enzyme-linked immunosorbent assay and the passive hemolysis assay, WN1 222-5 binds to the five known *E. coli* core chemotypes, to *Salmonella* core, and to S-form LPS having these core structures. In immunoblots, it is shown to react with both the nonsubstituted core LPS and with LPS carrying O-side chains, indicating the exposure of the epitope in both S-form and R-form LPS. This MAb of the immunoglobulin G2a class is not lipid A reactive but binds to *E. coli* J5, an RcP$^+$ mutant which carries an inner core structure common to many members of the family *Enterobacteriaceae*. Phosphate groups present in the inner core contribute to the epitope but are not essential for the binding of WN1 222-5 to complete core LPS. Cross-reactivity for clinical bacterial isolates is broad. WN1 222-5 binds to all *E. coli* clinical isolates tested so far (79 blood isolates, 80 urinary isolates, and 21 fecal isolates) and to some *Citrobacter*, *Enterobacter*, and *Klebsiella* isolates. This pattern of reactivity indicates that its binding epitope is widespread among members of the *Enterobacteriaceae*. WN1 222-5 exhibits biologically relevant activities. In vitro, it inhibits the *Limulus* amoebocyte lysate assay activity of S-form and R-form LPS in a dose-dependent manner and it neutralizes the LPS-induced release of clinically relevant monokines (interleukin 6 and tumor necrosis factor). In vivo, WN1 222-5 blocks endotoxin-induced pyrogenicity in rabbits and lethality in galactosamine-sensitized mice. The discovery of WN1 222-5 settles the long-lasting controversy over the existence of anti-core LPS MAbs with both cross-reactive and cross-protective activity, opening new possibilities for the immunotherapy of sepsis caused by gram-negative bacteria.

In spite of significant improvements in antibiotic therapy and in intensive care, sepsis remains a leading cause of morbidity and mortality among hospitalized patients. In the United States, septicemia is the 14th most common cause of death and ranks 9th in the age group of 1 to 4 years (11). The sepsis syndrome is triggered by gram-negative and gram-positive bacteria, fungi, and other pathogenic microorganisms. Gram-negative bacteria are responsible for a large number of episodes (200,000 to 300,000 in hospitalized patients per year in the United States) which are associated with a high mortality rate (20 to 40%) (11). In patients developing septic shock caused by gram-negative bacteria, the fatality rate may reach 50% or more (8). *Escherichia coli* remains the leading causative organism, accounting for 40 to 52% of gram-negative blood isolates (12, 34, 58, 61).

Lipopolysaccharides (LPS) are major constituents of the outer membrane and major antigenic (O-antigens) and toxic (endotoxins) components. They thus qualify as targets for therapeutic intervention. LPS are structurally complex, amphipathic, microheterogeneous macromolecules which consist of three regions: the O-specific polysaccharide chain, the core oligosaccharide, and lipid A. These regions are genetically, biochemically, and antigenically distinct, but the core region and lipid A retain common structural features (47). In members of the family *Enterobacteriaceae*, the O-specific side chains are composed of repeating oligosaccharide units and are highly variable in structure and composition, giving rise to the high number of serotypes known. The core comprises an outer and inner region, the latter being phylogenetically more conserved. Five different chemotypes (R1, R2, R3, R4, and K-12) are known among *E. coli* strains, but only one core structure accounts for all *Salmonella* spp. (28, 47). These six core types differ in the structural makeup of the outer (hexose) region. The inner core is structurally conserved among *E. coli*, *Salmonella* spp., and *Shigella* spp. It consists of the uncommon sugars L-*glycero*-D-*manno*-heptose (Hep) and 3-deoxy-D-*manno*-octulosonic acid (Kdo). Microheterogeneity in the core structure is due to nonstoichiometric substitutions with phosphate and ethanolamine groups (27). The lipid A component constitutes the most structurally conserved LPS region and the endotoxic principle (47).

For the treatment of septic patients, novel therapies are

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needed, and anti-LPS antisera and monoclonal antibodies (MAbs) are obvious candidates for therapeutic interventions (13). Anti-O-side chain antibodies (Abs) offer effective protection, but, being specific for only one O-serotype, have limited clinical application (32, 44). In the past decade, the notion that a common inner core region is present in LPS of the UDP-Gal-4-epimerase-deficient J5 mutant of *E. coli* O111:B4 has led to many attempts to generate broadly cross-reactive MAbs (1, 2, 23, 40–43, 45, 49). However, the occurrence of broadly cross-reactive and cross-protective MAbs has remained controversial. Only a few among the many MAbs produced exhibited any limited cross-reactivity, and these only occasionally neutralized LPS (43, 49). Anti-lipid A MAbs have also been described (7, 31, 35, 53). However, the specificity of anti-lipid A MAbs of the immunoglobulin M (IgM) class remains uncertain. In vivo studies did not show LPS neutralizing activity (4, 57), and passive immunotherapy has generated controversial results (5, 24, 61).

In this study, we show that a broadly cross-reacting and cross-neutralizing anti-core LPS MAb can be identified. WN1 222-5 is a murine MAb of the IgG2a class which recognizes a common and exposed epitope in the core region of *E. coli*, *Salmonella* spp., and some other members of the *Enterobacteriaceae* as defined by different assays such as enzyme-linked immunosorbent assay (ELISA) with isolated LPS and heat-killed bacteria, inhibition ELISA, immunoblots, and passive hemolysis assay (PHA). Moreover, in relevant biological tests in vitro and in vivo, it shows relevant and reproducible neutralizing activity against the endotoxic effects of smooth (S)-form and rough (R)-form LPS.

(Some of the data were presented at the Second International Conference on Shock, Vienna, Austria, 2 to 6 June 1991, and at the Keystone Colloquium on Recognition of Endotoxin in Biological Systems, Lake Tahoe, Calif., 1 to 7 March 1992, and published in abstract form [15a, 15b].)

MATERIALS AND METHODS

Bacterial strains, LPS, and lipid A. *E. coli* O4 (E394), O6 (126), O12 (E253), O15 (E568), O16 (E449), O18 (Bort), and O18rf (rough mutant) were kindly provided by A. S. Cross (Walter Reed Army Institute of Research, Washington, D.C.). Blood culture and urine isolates from *E. coli*, *Salmonella* spp., *Klebsiella* spp., *Proteus mirabilis*, *Enterobacter* spp., *Citrobacter* spp., *Serratia* spp., and *Pseudomonas aeruginosa* were obtained from A. P. Gibb (Clinical Bacteriology Laboratory, Department of Medical Microbiology, University of Edinburgh, Scotland), from routine clinical specimens. The blood culture and urine isolates were from gram-negative bacteria collected in a 1-year period. Twenty-one fecal isolates of *E. coli* were obtained, each from a separate healthy volunteer (22).

LPS from *Salmonella abortus equi* (Freiburg strain collection) and *E. coli* O4, O6, O12, O15, O16, O18, O86, and O111 were isolated by phenol-water extraction (20). *E. coli* O111, used for in vivo studies, was obtained from C. Galanos (Max-Planck Institut für Immunbiologie, Freiburg, Germany). LPS from *E. coli* W3100 (K-12), F470 (R1 core), F576 (R2 core), F653 (R3 core), F2513 (R4 core), and F515 (Re); *Salmonella minnesota* R60 (Ra), R345 (Rb2), R5 (RcP^-), R7 ($Rd1P^-$), R4 ($Rd2P^-$), and R595 (Re); *Salmonella typhimurium* 878 (Rc); and *Acinetobacter calcoaceticus* (NCTC 10305; National Collection of Type Cultures, Central Public Health Laboratory, London, England) were obtained by extraction with phenol-chloroform-petroleum ether (59). LPS from *E. coli* O26:B6, *E. coli* O111:B4, and *S. typhimurium* TV119 (Ra), SL684 (Rc), and SL1181 (Re) were obtained from Sigma Chemical (St. Louis, Mo.). LPS from *E. coli* K235, *E. coli* J5 (Rc), and *S. minnesota* wild type and free lipid A from *E. coli* K-12 (ex-D31m4) and from *S. minnesota* R595 were from List Biological Laboratories Inc. (Campbell, Calif.). LPS from *S. typhimurium* SH 4305, SH 4809, and SL 3622; *Salmonella enteritidis* SH 1262; *Salmonella newport*; *Salmonella thompson*; and *Salmonella typhi* 253 Ty were obtained from BioCarb Chemicals (Lund, Sweden).

MAbs. WN1 222-5 was generated and selected by standard methods (18). Briefly, 22-week-old NZB mice were immunized intravenously (i.v.) with 10^8 heat-killed bacteria in 0.1 ml. Four immunizations, 1 week apart, were carried out (weeks 1 and 3: *E. coli* F576, *E. coli* F653, and *S. minnesota* R60; weeks 2 and 4: *E. coli* F470, *E. coli* F2513, and *E. coli* O18rf). After 1 month, two injections, 1 day apart, of a mixture of the six strains (10^8 heat-killed bacteria) were given, the first injection i.v. and the second intraperitoneally. On the fourth day, spleen cells were recovered and fused with a nonsecreting murine B-cell lymphoma cell line, by standard procedures (51). Supernatants were screened overnight in ELISA with seven different LPS mixtures (first: *E. coli* O4, *E. coli* O6, *E. coli* O16, and *E. coli* O18; second: *E. coli* O12, *E. coli* O15, and *E. coli* O86; third: *E. coli* F470 and *E. coli* F2513; fourth: *E. coli* F576, *E. coli* F653, *E. coli* W3100, and *S. minnesota* R60; fifth: *E. coli* J5 and *S. typhimurium* 878; sixth: *S. minnesota* R5, *S. minnesota* R7, *S. minnesota* R4, *S. minnesota* R595, and *E. coli* F515; seventh: free lipid A from *E. coli* K-12 and *S. minnesota* R595) and a bovine serum albumin (BSA) control. Microtiter plates were coated with LPS at the concentration of 2 μg/ml. Specific binding and cross-reactivity of MAbs in culture supernatants were revealed by the subsequent addition of biotin-labelled affinity-purified goat anti-mouse Ig Abs (Southern Biotechnology Associates; 3 h at room temperature [RT]), streptavidin-alkaline phosphatase conjugate (Jackson Immunoresearch Laboratories, Inc., West Grove, Pa.; 1 h at RT), and substrate (paranitrophenol phosphate [PNPP]) in diethanolamine buffer (pH 9.8). A_{405} was read after 20 min with a Titertek Multiskan ELISA reader (MCC/340, Flow Laboratories).

In most in vitro and in vivo experiments, samples of WN1 222-5 purified on a protein A column from serum- and pyrogen-free cultures were used. Supernatants of hybridoma cell cultures grown in serum-free medium (50) were employed in some in vitro tests. Ab concentration in culture supernatants was measured by ELISA and was in the range of 10 to 50 μg/ml (30). All samples of WN1 222-5 used for biologically relevant experiments in vitro and in vivo were *Limulus* amoebocyte lysate (LAL) negative and pyrogen free as assessed by the rabbit pyrogen test.

The MAb F32-15C8-3 is a murine MAb of the IgG2a subclass. It reacts with transforming growth factor 2 and was kindly provided by G. Zenke (Sandoz Pharma Ltd., Basel, Switzerland).

ELISA on isolated LPS. LPS or lipid A (2 μg/ml; 50 μl) was applied as a coating to 96-well Microtest III flexible plates (Becton Dickinson, Oxnard, Calif.). Plates were blocked with 250 μl of 2% BSA in phosphate-buffered saline (PBS) per well; 50 μl of purified WN1 222-5 (100 ng/ml) diluted in PBS–2% BSA was added in duplicate overnight. The reaction was revealed by the subsequent addition of 50 μl of biotin-labelled affinity-purified goat anti-mouse IgG2a per

well (Southern Biotechnology Associates; 3 h at RT) and of 50 μl of streptavidin-alkaline phosphatase conjugate per well (Jackson Immunoresearch Laboratories; 1 h at RT). Substrate (PNPP) in diethanolamine buffer was added, and A_{405} was read after 20 min with a Titertek Multiskan ELISA reader (MCC/340, Flow Laboratories). In all ELISA experiments, the interassay coefficient of variation was less than 5%.

Inhibition ELISA. Microtiter plates were coated with *E. coli* R3 LPS (100 ng/ml; 50 μl per well). To the plates, blocked with PBS–2% BSA, a mixture of biotin-labelled WN1 222-5 (30 ng/ml) and of increasing amounts of the different LPS in PBS–2% BSA (range, 0.003 to 10 μg/ml) was added in a volume of 50 μl per well for 3 h at RT. The plates were developed with subsequent addition of streptavidin-alkaline phosphatase (Jackson Immunoresearch Laboratories, Inc.; 45 min; dilution, 1/10,000) and of the substrate (PNPP in diethanolamine buffer, pH 9.8). Results are reported as optical density (OD) readings (405 nm) after 30 min and are the means of three values. The interassay coefficient of variation was less than 5%.

Gel electrophoresis and blotting. A total of 10 μl of different LPS solutions (50 μg/ml) was mixed with 10 μl of 0.1 M Tris-HCl buffer (0.1 M, pH 6.8) containing 1% (wt/vol) sodium deoxycholate, 20% (wt/vol) glycerol, and 0.001% bromophenol blue. The samples were loaded onto electrophoresis gels (4% stacking gel; 14% running gel). A modified Laemmli system (sodium deoxycholate-polyacrylamide gel electrophoresis) (33) and a Mini Protean II dual slab cell apparatus (Bio-Rad Laboratories) were used. The gels were blotted onto a 0.45-μm nitrocellulose membrane at 60 V for 20 min (Mini Transblot electrophoretic transfer cell apparatus, Bio-Rad Laboratories). The blots were soaked in Tris-buffered saline (20 nM Tris-HCl, 0.1 mM NaCl; pH 7.5)–1% BSA for 1 h at RT. The immunoblots were developed for 2 h at RT with WN1 222-5 (10 ng/ml in Tris-buffered saline-0.05% Tween 20–1% BSA), washed twice in the same buffer, and developed for 45 min at RT with biotin-labelled goat anti-mouse IgG2a Ab (Southern Biotechnology Associates) and subsequently with streptavidin-alkaline phosphatase conjugate (Jackson Immunoresearch Laboratories) and the 5-bromo-4-chloro-3-indolylphosphate toluidinium–Nitro Blue Tetrazolium alkaline phosphatase color development solution (Bio-Rad Laboratories). Companion gels were fixed by overnight incubation in a solution containing 40% ethanol and 5% acetic acid and were silver stained according to the method of Tsai and Frasch (54).

PHA. PHA was performed in 96-well microdilution plates as previously described (9). Briefly, sheep erythrocytes (SRBC) (0.2 ml in 5 ml of PBS) were mixed with different amounts of LPS and incubated at 37°C for 30 min. The LPS-coated SRBC were finally suspended in Veronal-buffered saline to give a 0.5% suspension. Serial dilutions of WN1 222-5 (50 μl) were mixed with 50 μl of LPS-coated SRBC and guinea pig serum (25 μl, 1/20) as a source of complement, and mixing was followed by incubation at 37°C for 1 h. After centrifugation, titers giving 50% hemolysis were determined. When titers of duplicate determinations differed by more than one dilution step, an additional determination was performed.

ELISA on heat-killed bacteria. ELISA on heat-killed bacteria was performed as previously described (22). Overnight cultures of bacteria were heat killed (30 min at 100°C) and applied as a coating to a Polysorb strip (Nunc, Roskilde, Denmark). Culture supernatants of WN1 222-5 were diluted 1/250 and added (100 μl; 90 min) to wells. The reaction was revealed with alkaline phosphatase-conjugated anti-mouse Ig (Zymed, Cambridge United Kingdom; final dilution, 1/500; 90 min) and substrate (PNPP in diethanolamine buffer, pH 9.8; 90 min). Plates were read at 405 nm. For each sample, triplicate determinations were performed. The interassay coefficient of variation was always less than 5%. Samples were stratified according to OD into three groups: negative (OD < 0.1), weak positive (OD from 0.1 to 0.8), and strong positive (OD > 0.8).

LAL assay. Equal volumes (25 μl) of LPS (1 μg/ml) and of WN1 222-5 (concentration range, 0 to 100 μg/ml) were mixed aseptically in the wells of microtiter plates and incubated for 3 h at 37°C. Chromogenic LAL reagent (Coatest Endotoxin; Chromogenix, Molndal, Sweden) was added to each well through a transfer plate to ensure that each well received that LAL reagent at the same time. The plate was read kinetically every 17 s at 405 nm (reference background, 650 nm) for 60 min in a Maxline Vmax plate reader (Molecular Devices Corporation, Menlo Park, Calif.) at 37°C. The replicate mean onset time for test samples was standardized against an endotoxin of known potency: *E. coli* O111:B4 (Coatest kit endotoxin standard, Chromogenix). The percent inhibition, found as a delay in onset time compared with those for uninhibited LPS controls, was calculated by expressing the potency value of the inhibited sample as a percentage of the potency value of the uninhibited sample and subtracting this from 100%. The inter- and intra-assay coefficients of variation of the LAL assays were less than 5% when applied to purified LPS or heat-killed bacterium preparation.

LPS-induced monokine secretion. Freshly collected peritoneal cells (5×10^5 cells per ml) from BALB/c mice (Bethesda Research Laboratories, Fullingsdorf, Switzerland) were cultured in 0.2 ml of serum and pyrogen-free medium (50) in the presence of *E. coli* R3 LPS (40 pg/ml) and in the presence or absence of increasing concentrations of WN1 222-5 or of a control MAb (F32-15C8-3). Culture supernatants were collected after 4 h for interleukin 6 (IL-6) and tumor necrosis factor (TNF) determinations. Specific biological assays for IL-6 (16) and TNF (17) were used. The content of IL-6 and TNF in culture supernatants was calculated relative to standard curves.

Pyrogenic response in rabbits. The pyrogenic response of Chinchilla bastard rabbits (2 to 3 kg; three to four animals per group) to i.v. administered LPS was measured rectally at RT with thermocouples and an automatic recording system (46). The minimal pyrogenic dose giving rise to a 0.6°C change of temperature 3 h after injection was determined. In protective assays, WN1 222-5 was administered to groups of four rabbits and followed 1 h later by a dose of LPS equal to 100 times the minimal pyrogenic dose giving rise to a 0.6°C change of temperature 3 h after injection. All animal experiments were performed in accordance with institutional guidelines.

Mouse lethality: galactosamine (D-GalN) model. Endotoxin shock was induced by i.v. injection of a 95% lethal dose of *S. abortus equi* LPS (50 ng/kg of body weight) or of *E. coli* O16 LPS (100 ng/kg) in groups of six female C57BL/6 mice, 6 to 8 weeks old (Charles River, Sulzfeld, Germany). D-GalN (800 mg/kg) was administered intraperitoneally at the time of LPS (19). WN1 222-5 (range, 10 to 1,000 μg per mouse), diluted in pyrogen-free NaCl solution, was administered i.v. 2 h prior to LPS. Survival was recorded up to 24 h. Animal experiments were performed in accordance with institutional guidelines.

TABLE 1. Binding of WN1 222-5 to isolated LPS and free lipid A in ELISA

Type of LPS or lipid A[a]	Serotype or chemotype	OD value for WN1 222-5 (0.1 μg/ml)[b]
E. coli smooth LPS		
E394	O4	1.006
126	O6	1.964
E253	O12	1.899
E568	O15	1.376
E449	O16	1.634
Bort	O18	1.447
	O26:B6	1.565
	O111:B4	1.204
	K235	2.121
E. coli rough LPS		
F470	R1	2.264
F576	R2	2.116
F653	R3	2.082
F2513	R4	2.066
W3100	K-12	2.329
J5	RcP^+	2.357
F515	Re	0.239
E. coli free lipid A	K-12	0.183
S. minnesota smooth LPS	Wild type	1.514
S. minnesota rough LPS		
R60	Ra	2.192
R345	Rb2	2.139
R5	RcP^-	0.835
R7	$Rd1P^-$	0.617
R4	$Rd2P^-$	0.369
R595	Re	0.042
S. minnesota free lipid A	Re	0.091
S. typhimurium smooth LPS		
SH 4305		1.251
SH 4809		1.407
SL 3622		2.117
S. typhimurium rough LPS		
TV119	Ra	1.933
SL684	Rc	2.208
SL1101	Rd2	0.062
Other smooth LPS		
S. enteritidis (SH 1262)		1.474
S. newport		2.158
S. thompson (ls40)		1.992
S. typhi (253 Ty)		1.974
Shigella flexneri	1A	2.072
A. calcoaceticus		0.056

[a] Purified LPS and free lipid A (2 μg/ml) were used to coat the plates.
[b] The OD reading was done at 405 nm 20 min after the addition of the substrate.

RESULTS

ELISA reactivity pattern of WN1 222-5. The reactivity of WN1 222-5 for isolated, purified S-form and R-form LPS and free lipid A is shown in Table 1. WN1 222-5 at the concentration of 100 ng/ml bound to S-form and R-form LPS of *E. coli*, *Salmonella* spp., and a *Shigella* sp. Reactivity with the five known core types of *E. coli* (R1 to R4 and K-12), with *Salmonella* core type (Ra), and with *E. coli* J5 (RcP^+) was evident. The broad cross-reactivity of WN1 222-5 for LPS indicates the existence of a common epitope. With relatively high LPS doses for coating (2 μg/ml) and an overnight incubation, WN1 222-5 showed binding to more defective core structures (*S. minnesota* RcP^- and $Rd1P^-$). However, because reactivity with Re structures from *E. coli* or *Salmonella* spp. and with free mono- and bis-phosphoryl lipid A was absent, a nonspecific interaction can be excluded. As $Rd1P^-$ mutant LPS contains the Kdo and Hep regions, the data suggest the participation of parts of the Hep region in the antigen binding site. An alternative explanation could be offered by the presence in the LPS preparation of longer core structure due to leakiness of the mutant, but chemical analysis of the strain excludes this possibility (26).

As the coating of LPS might alter its conformation, the ability of WN1 222-5 to bind to LPS in solution was analyzed in inhibition ELISA. In these experiments, short incubation times and nanogram amounts of biotin-labelled WN1 222-5 and of LPS were used. As shown in Fig. 1, the binding of WN1 222-5 to *E. coli* R3 (solid-phase antigen) was inhibited by nanogram amounts of soluble S-form and R-form LPS of *E. coli* and *Salmonella* spp. having a complete core. As LPS from *E. coli* J5 (Rc) and *S. minnesota* R345 (Rb2) were less inhibitory, it is likely that the oligosaccharides of the outer core contribute to the epitope. In these more stringent experimental conditions, no inhibition was seen with R-form LPS having an RcP^- or an RdP^- chemotype. The lack of inhibition with free lipid A or with distantly related S-form LPS, such as that of *A. calcoaceticus*, confirms the specificity of the reaction.

Reactivity of WN1 222-5 in immunoblots. Immunoblots confirmed the cross-reactivity of WN1 222-5 for S-form and R-form LPS. In silver-stained gels, S-form LPS are resolved into a series of components, with each band representing at least one individual molecular species (Fig. 2A) (54). The more slowly moving bands (top) have longer O-specific chains (i.e., more repeating units), and the faster-moving bands possess small or no O-side chains like R-form LPS. The ladderlike pattern seen in immunoblots of S-form LPS (Fig. 2B) demonstrates that WN1 222-5 in nanogram amounts reacts with all O-side chain-substituted (slowly migrating bands at the top), as well as with unsubstituted, LPS species (fast-migrating bands at the bottom) of smooth *E. coli* and *Salmonella* spp. WN1 222-5 also binds to complete core structures of rough *E. coli* and *Salmonella* spp. These results demonstrate that WN1 222-5 binds to an LPS-core epitope which remains accessible on O-side chain carrying LPS.

Reactivity of WN1 222-5 in PHA. Further analysis of the binding specificity and of the requirement for phosphate groups was conducted by PHA (Table 2). Significant differences between ELISA and PHA results with respect to particular MAb specificities have been reported (35, 45). In comparison with ELISA, in PHA, possible nonspecific interactions between Ab and LPS are minimized because the lipid A-associated fatty acids are likely to be intercalated into the SRBC membrane and because LPS orientation and conformation are closer to physiological conditions such as those present in the bacterial outer membrane (21). Moreover, the PHA reflects specificity and functional activity, i.e., the ability of an antibody to bind and activate complement. The results of PHA agreed with those of inhibition ELISA. WN1 222-5 caused hemolysis of SRBC coated with LPS of the RcP^+ chemotype (J5-LPS), Rb2, and the complete cores of R1 to R4, K-12, and Ra. Hemolysis was not observed when free lipid A or R-form LPS of the Re, RdP^-, or RcP^- chemotype were applied as a coating to the SRBC. When dephosphorylated LPS was used, only LPS with the complete core structure exhibited binding of the MAb whereas reactivity to *E. coli* J5 (a shorter core structure) was lost. This finding demonstrates that phosphate groups are involved in the interaction between LPS and WN1 222-5 but are nonessential in the case of LPS with a complete core. As the totality of LPS from clinical isolates is likely to possess

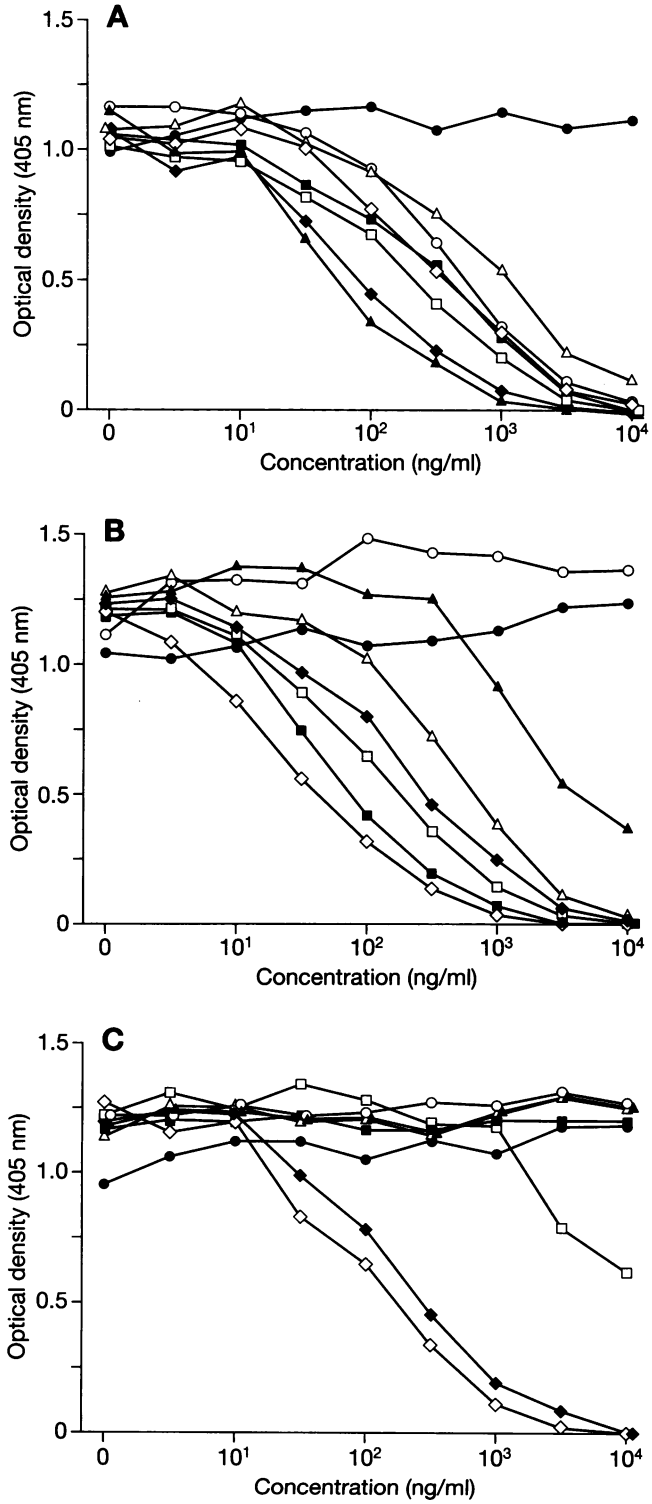

FIG. 1. Inhibition of WN1 222-5 binding to *E. coli* R3 LPS by different S-form and R-form LPS. Residual binding is shown as OD (405 nm). The following LPS were used: (A) *E. coli* O4 (◇), O6 (◆), O12 (□), O15 (■), O16 (△), O18 (▲), and O111 (○) and *A. calcoaceticus* (●); (B) *E. coli* R1 (◇), R2 (◆), R3 (□), R4 (■), K-12 (△), J5 (▲), F515 (○), and K-12 free lipid A (●); (C) *S. abortus equi* (◇) and *S. minnesota* Ra (◆), Rb2 (□), RcP$^-$ (■), Rd1P$^-$ (△), Rd2P$^-$ (▲), R595 (○), and R595 free lipid A (●).

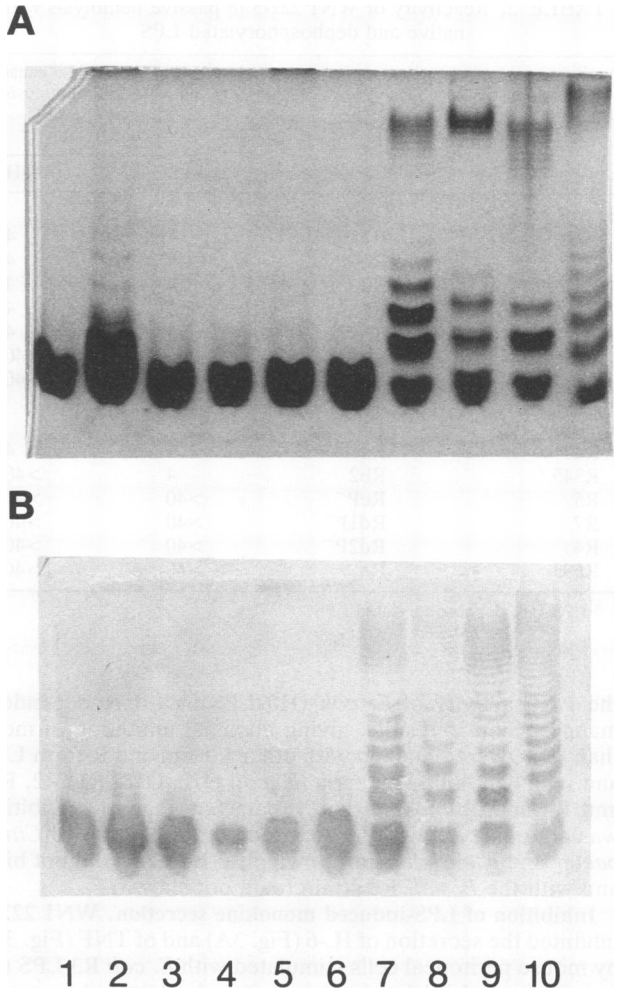

FIG. 2. MAb WN1 222-5 recognition pattern of S-form and R-form *E. coli* and *S. minnesota* LPS after sodium deoxycholate electrophoresis (A) and immunoblot (B). Lanes 1 to 10: *E. coli* K-12, R1, R2, R3, and R4; *S. minnesota* Ra; *E. coli* O4, O6, and O111; and *S. abortus equi*, respectively.

a complete core, our data show that lack of phosphate groups in the core will not affect the binding of WN1 222-5.

WN1 222-5 binding to heat-killed bacteria in ELISA. WN1 222-5 was also tested in ELISA against a large collection of heat-killed bacteria, isolated from clinical samples (Table 3). The strains of *E. coli* included 79 isolates from blood cultures, 80 from urine, and 21 from feces. Clinical isolates of *Salmonella* spp., *Citrobacter* spp., *Enterobacter* spp., *Klebsiella* spp., *Serratia* spp., *P. mirabilis*, and *P. aeruginosa* were also included. WN1 222-5 reacted strongly with all blood, urinary, and fecal isolates of *E. coli* and *Salmonella* spp. and with some *Citrobacter* spp. and weakly with some *Enterobacter* and some *Klebsiella* spp., showing that the epitope recognized by WN1 222-5 is widely distributed among members of the *Enterobacteriaceae*.

Inhibition of LAL assay activity. All preparations of the MAbs used for in vitro and in vivo biological assays were pyrogen free as shown by lack of LAL assay activity or pyrogenicity in rabbits at the highest tested dose (5 mg/kg). The results of a representative LAL assay inhibition experiments are shown in Table 4, in which WN1 222-5 inhibited

TABLE 2. Reactivity of WN1 222-5 in passive hemolysis with native and dephosphorylated LPS

Type of LPS	Chemotype	Amt of WN1 222-5 causing at least 50% hemolysis (μg/ml) of SRBC coated with:	
		LPS	LPS-HF[a]
E. coli			
F470	R1	4	4
F576	R2	4	4
F653	R3	4	4
F2513	R4	4	4
W3100	K-12	4	4
J5	RcP^+	4	>40
F515	Re	>40	>40
S. minnesota			
R60	Ra	4	4
R345	Rb2	4	>40
R5	RcP^-	>40	>40
R7	$Rd1P^-$	>40	>40
R4	$Rd2P^-$	>40	>40
R595	Re	>40	>40

[a] LPS-HF, dephosphorylated LPS.

the LAL activity of *E. coli* O15 LPS in a dose-dependent manner. Similar results, giving maximal inhibition of more than 80%, were obtained with other S-form and R-form LPS and with heat-killed bacteria (*E. coli* O12, O18, R1, R2, R3, and R4 and *Salmonella* spp.) (data not shown). Inhibition was not observed with LPS extracted from strains of *Citrobacter* and *Klebsiella* spp. to which WN1 222-5 did not bind and with the *E. coli* Re strain (data not shown).

Inhibition of LPS-induced monokine secretion. WN1 222-5 inhibited the secretion of IL-6 (Fig. 3A) and of TNF (Fig. 3B) by mouse peritoneal cells stimulated with *E. coli* R3 LPS (40 pg/ml). Such LPS levels are commonly detected in septic patients with endotoxemia (56). At a concentration of 75 ng/ml (about 1 nM), WN1 222-5 suppressed almost completely the release of IL-6, whereas higher concentrations (7.5 μg/ml) were required to inhibit TNF secretion. A similar neutralizing activity was obtained with other S-form (*E. coli* O4 and *E. coli* O111) and R-form (*E. coli* R1) LPS as well as with higher amounts of LPS (data not shown). A murine MAb of the IgG2a isotype (F32-15C8-3), used as a control, did not show any inhibiting effect. Modest increases in IL-6 and TNF secretion were, in fact, noted at certain dilutions of the control MAb (Fig. 3).

In the absence of LPS, both MAbs, even at the highest concentration used (75 μg/ml), did not cause any significant release of IL-6 or TNF, confirming the absence of contaminating LPS in MAb preparations.

Neutralization of pyrogen response of rabbits to LPS. In Table 5, the change in rectal temperature 3 h after i.v. LPS challenge in groups of rabbits having or not having received WN1 222-5 (1 h before LPS) is shown. The Ab inhibited fever induced by a dose of *E. coli* R-form and S-form LPS equal to 100 times the minimal pyrogenic dose giving rise to a 0.6°C change of temperature 3 h after injection and provided cross-protection to *Salmonella* LPS. In addition, WN1 222-5 exhibited antipyrogenic activity for many other R-form and S-form *E. coli* LPS including LPS extracted from *E. coli* R1, R2, O6, O12, and O15 (data not shown).

Inhibition of LPS-induced mouse lethality (D-GalN model). Protection by WN1 222-5 against LPS was also observed in D-GalN-sensitized mice. Representative experiments are shown in Table 6. WN1 222-5 blocked, in a dose-dependent manner, the lethality induced by *S. abortus equi* LPS (50 ng/kg i.v.), with 100 μg of MAb per mouse providing 100% survival. Protection was also obtained for *E. coli* O16 (100 ng/kg) (Table 6) and *E. coli* O111 (data not shown). Protective activity was completely abolished by heat treatment, again confirming that the MAb preparation was not contaminated with LPS and that protection was not due to Ab-independent phenomena such as endotoxin tolerance.

TABLE 3. Binding of WN1 222-5 to heat-killed gram-negative bacteria in ELISA[a]

Isolate source and species	No. of samples[b]			
	Tested	Negative	Weak positive	Strong positive
Blood				
Enterobacteriaceae				
Escherichia coli	79	0	0	79
Salmonella spp.	2	0	0	2
Citrobacter spp.	5	3	0	2
Enterobacter spp.	18	16	2	0
Klebsiella spp.	14	11	3	0
Serratia spp.	5	5	0	0
Proteus mirabilis	9	9	0	0
Pseudomonas aeruginosa	7	7	0	0
Urine (*Escherichia coli*)	80	0	0	80
Feces (*Escherichia coli*)	21	0	0	21

[a] Culture supernatants were diluted 1/250.
[b] Samples were stratified according to OD as negative (<0.1), slightly positive (0.1 to 0.8), and positive (>0.8).

TABLE 4. Inhibition of LAL activity of *E. coli* O15 LPS (1 μg/ml) by WN1 222-5

WN1 222-5 (μg/ml)	Inhibition (%)
100	92
10	77
1	59
0.1	53
0.01	22
0.001	16
0.0001	0

DISCUSSION

The LPS core region of members of the *Enterobacteriaceae* has a conserved structure with an inner Kdo-Hep region and an outer hexose region. There has been a long-standing interest in common surface determinants which might confer a broad spectrum of protection against gram-negative bacteria (13). However, most anti-core LPS MAbs described in the literature were not cross-reactive, had a narrow specificity recognizing only a few core structures, and did not bind to S-form LPS (1, 2, 23, 40–42, 45). They recognized mainly terminal oligosaccharides, and their Ab binding site was masked by substitution by more distal sugars of the core or by the O-specific chain. Only recently, a MAb of the IgM class cross-reactive for two complete core types (*Salmonella* spp. and *E. coli* R2) and recognizing S-form as well as R-form LPS was described (43). Moreover, cross-neutralization of LPS has been shown in vivo in only a

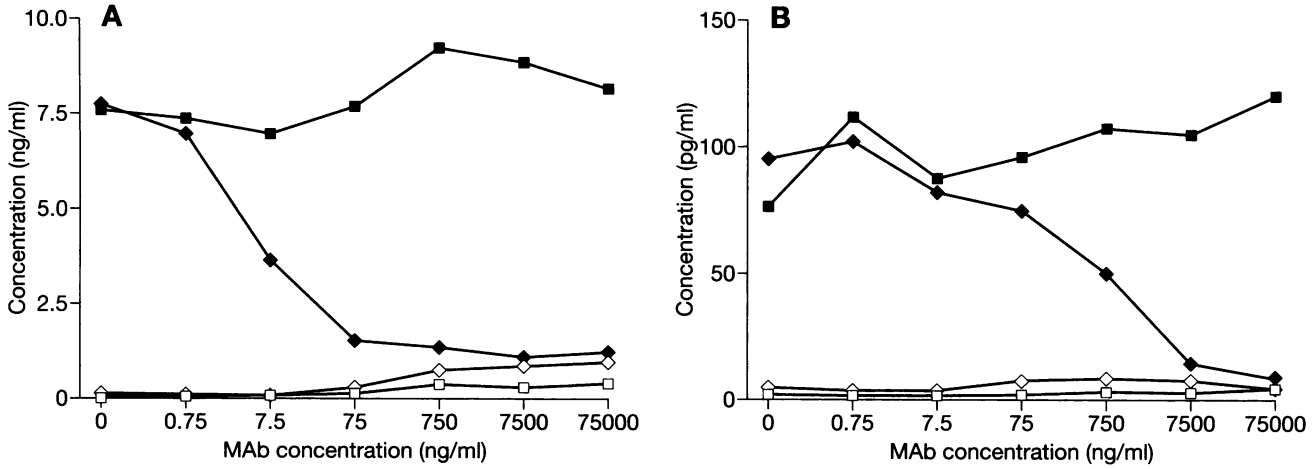

FIG. 3. IL-6 (A) and TNF (B) secretion by mouse peritoneal cells stimulated with *E. coli* R3 LPS. (A and B) ◇, WN1 222-5; ◆, WN1 222-5 plus *E. coli* R3 LPS; □, F32-15C8-3; ■, F32-15C8-3 plus *E. coli* R3 LPS.

few cases (49). Furthermore, it was suggested that cross-reactive MAbs are of the IgM class and lipid A specific (7, 61).

We now describe an anti-core LPS MAb of the IgG2a subclass (WN1 222-5) whose cross-reactivity and neutralizing activity cover many members of the *Enterobacteriaceae* including all known *E. coli* and *Salmonella* core types. This core epitope remains accessible in S-form LPS, indicating that it is not masked by additional oligosaccharides in the outer core and/or by the O-specific chain. Both the inner and the outer core appear to contribute to the Ab binding site, since in solid-phase ELISA the MAb binds to Rd1P mutants, while in inhibition ELISA the best competition is from LPS with a complete core structure. As shown by PHA, phosphate groups in the core region are nonessential for the binding to complete core structures but are essential for binding to *E. coli* J5 (RcP^+ chemotype). These data suggest a partial involvement of phosphate groups in the Ab combining site. The binding of WN1 222-5 to LPS fulfills all requirements of a high-affinity interaction as it shows low background binding, significant binding at low Ab and Ag concentrations, and specific and reproducible activity in more than one assay system. Therefore, our findings confirm at the immunochemical level the similarity in the inner core region of LPS predicted by genetic and chemical analysis. Moreover, conformational similarities in the outer core (hexose region) of *Salmonella* spp. and of *E. coli* R1 to R4 and K-12 LPS have been predicted by semiempirical calculations (29). These results are in line with our data which suggest that the outer core may contribute to the Ab binding surface.

As shown in the present paper, this common surface is widely distributed among members of the *Enterobacteriaceae*. WN1 222-5 binds to many clinical strains covering *Salmonella* spp. and *E. coli* strains carrying different core types and belonging to different O-serotypes (22). The presence of this epitope on some *Citrobacter* spp. is not unexpected as structural identity between the inner-outer core region of *Citrobacter* strain PCM 1487 and *E. coli* core type R3 was reported (48). Some reactivity was noted for some strains of *Klebsiella* and *Enterobacter*. It is interesting to note that the pattern of cross-reactivity of WN1 222-5 for the members of the *Enterobacteriaceae* appears to reflect the evolutionary relationship among these families, since *E. coli*, *Salmonella* spp., and *Citrobacter* spp. are the most closely related groups (36).

The endotoxin-neutralizing properties of WN1 222-5 were evaluated in vitro and in vivo. This MAb was able to block

TABLE 5. Protective activity of WN1 222-5 against LPS fever in rabbits

LPS source	LPS dose (μg/kg)	MAb[a]	MAb dose (mg/kg)	Change in temp 3 h post-LPS injection (°C ± SD)
E. coli R3	0.01			2.1 ± 0.3
	0.01	WN1 222-5	1	1.0 ± 0.3
	0.025			2.2 ± 0.2
	0.025	WN1 222-5	2	0.7 ± 0.3
E. coli R4	0.01			1.9 ± 0.3
	0.01	WN1 222-5	1	0.5 ± 0.2
E. coli O111	0.05			1.9 ± 0.3
	0.05	WN1 222-5	1	1.0 ± 0.4
S. abortus equi	0.05			1.6 ± 0.3
	0.05	WN1 222-5	1	0.8 ± 0.2

[a] MAbs were injected i.v. 1 h prior to i.v. administration of LPS.

TABLE 6. Protective activity of WN1 222-5 against LPS lethality in D-GalN-sensitized mice

WN1 222-5 dose (mg/kg)[a]	No. of survivors/no. of treated animals after administration of LPS from:	
	S. abortus equi (50 ng/kg)	*E. coli* O16 (100 ng/kg)
50	6/6	5/6
25	6/6	4/6
12.5	6/6	3/6
5	6/6	
2.5	2/6	
0.5	1/6	
Control buffer	0/12	1/6

[a] WN1 222-5 was injected i.v. 2 h prior to i.v. administration of LPS and intraperitoneal administration of D-GalN (800 mg/kg).

the LAL activity and the LPS-induced release of IL-6 and TNF by freshly explanted mouse peritoneal cells. Monokines such as TNF and IL-6 represent critical pathophysiological mediators in the initiation of sepsis and septic shock caused by gram-negative bacteria (6). Moreover, a strict correlation between Ab binding and in vitro neutralizing activity was observed. Structure-activity relationship studies have shown that lipid A is the active component in the activation of the LAL assay (52), and it is mainly responsible for the induction of monokines by host cells (37, 60). As WN1 222-5 does not bind to free lipid A, its inhibitory activity in the LAL assay and on monokine release could be explained by some form of steric hindrance, disaggregation of supramolecular LPS structures, or a modification of the conformation of the LPS molecule after Ab binding. Previous studies have evaluated the LAL neutralization and TNF release by anti-LPS MAb in vitro. McConnell et al. found that murine anti-core LPS MAbs were able to inhibit the LAL assay (39). Chia et al. could not demonstrate any inhibitory effect on TNF release by mouse-derived RAW 264-7 macrophages with several murine MAbs reactive with the O-side chain or the core oligosaccharide of LPS (14), whereas Vacheron et al. were able to inhibit TNF production by LPS-stimulated mouse macrophages with anti-LPS MAbs (55). Differences in culture conditions (cell type, culture medium), in specificity of the MAbs, and in amounts of LPS used could account for these discrepant results.

The ability of WN1 222-5 to suppress endotoxic activity in vivo was established with two animal models exploring important pathological effects of LPS, i.e., fever and lethality. The fact that WN1 222-5 exhibits neutralizing activity not only in vitro but also in vivo is evidence of the accessibility of the core-located epitope on the LPS molecule in vivo. For these assays to be meaningful, special precautions were taken to exclude contamination of WN1 222-5 samples by LPS (15). All MAb preparations were LAL negative and nonpyrogenic in rabbits at the highest dose tested (5 mg/kg). Moreover, heat treatment of the preparation abolished protection, and WN1 222-5 was not active against LPS to which it did not bind, indicating that induction of cross-tolerance was not responsible for inhibition of fever or protection against lethality (data not shown). The protective effect of WN1 222-5 might be due to direct neutralization of LPS with prevention of monokine induction (TNF or IL-6) as shown by in vitro results or to a less direct mechanism such as clearance of LPS-antibody complexes through the Fc receptor or the complement receptor or some other mechanism.

The concept of cross-protection afforded by core LPS antibodies was supported mainly by experimental studies of passive immunotherapy with antisera from rabbits immunized with *E. coli* J5 and *S. minnesota* R595 (Re chemotype) rough mutants (10, 13, 38) and by clinical trials which have suggested that human *E. coli* J5 antiserum increased the survival of patients with bacteremia and septic shock caused by gram-negative organisms for surgical patients at high risk of infection (62). However, direct experimental proof to support this hypothesis was lacking, the precise epitope involved in cross-reactivity was not identified, and importantly, contradictory results were obtained (3, 25). On the other hand, studies on the chemical structure of the LPS core unequivocally demonstrated the presence of shared structural regions among members of the *Enterobacteriaceae* and, in particular, among *E. coli* and *Salmonella* and *Shigella* species (27, 47).

In this study, we show that these predictions were correct, that cross-reactive MAbs can be produced, and that at least one cross-protective epitope can be defined in the core region of LPS. Moreover, the discovery of WN1 222-5 demonstrates that this conserved epitope is not only exposed and accessible in LPS of different strains of the *Enterobacteriaceae* but is also immunogenic. This MAb shows biological activities since it is able to activate complement (PHA) and to inhibit the LAL assay. As it neutralizes monokine release by mouse peritoneal cells, stimulated with various S-form and R-form LPS, WN1 222-5 is able to block a fundamental pathogenic step in the cascade of events which lead to multiple organ failure and death in septic patients. Our data also show that lipid A binding and IgM class are not essential for neutralizing endotoxin. The data, furthermore, resolve a long-lasting controversy regarding the existence of cross-reactive and cross-protective antibodies against the core region of LPS. The discrepant results obtained with polyclonal antisera (10, 25, 38) might be explained by differences in specificity, affinity, biological activity, and titer of the Abs obtained after immunization. We believe that the targeting of the toxic products of the infecting organism, specifically endotoxin, with the MAb has potential advantages originating from the long half-life and from the conserved biological activity of the Ab.

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