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Similarities and Disparities between Core-Specific and O-Side-Chain-Specific Antilipopolysaccharide Monoclonal Antibodies in Models of Endotoxemia and Bacteremia in Mice

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We have previously described cross-reactive antilipopolysaccharide (anti-LPS), or anti-endotoxin, monoclonal antibodies (MAbs) which provide cross-protection in several systems of endotoxin bioactivity. The protective effects of the murine cross-reactive MAb WN1 222-5 (immunoglobulin G2a(κ) [IgG/2a(κ)]) and of its chimerized version, SDZ 219-800 [human IgG1(κ)], have now been evaluated in lethality models against LPS from three different serotypes and in bacterial infection models. We confirmed the protective activity of the two MAbs in D-galactosamine-sensitized mice challenged with LPS of other *E. coli* serotypes (O18, O127, and O111). The protective effect correlated with the suppression of tumor necrosis factor formation. Furthermore, WN1 222-5 enhanced bacterial clearance of intravenously administered *E. coli* O111 bacteria, thus protecting mice from death. However, the MAbs were unable to provide protection in a peritonitis model (intraperitoneal inoculation). Our study, therefore, shows that LPS cross-reactive antibodies are capable of mediating cross-protection against LPS and bacteria but that the selected models have a clear influence on the results.

In recent years, several new types of therapeutic intervention have been evaluated in patients with septic shock. Results have been disappointing, and the search for additional forms of treatment remains a challenge. Among the new therapies, monoclonal and polyclonal antibodies to endotoxin have been investigated in patients with septic shock and in animal models of septic shock caused by gram-negative organisms or of endotoxemia (2). Polyclonal antibodies and monoclonal antibodies (MAbs) directed against the O side chain of lipopolysaccharides (LPS) are highly protective in animal models of endotoxemia and of sepsis caused by gram-negative organisms. However, their exclusive specificity for a given O serotype precludes their use in a large series of patients. In the past, many attempts have been made to generate broadly cross-reactive antibodies against the relatively conserved inner core region (1, 14–16) and against the lipid A component, which is structurally similar in LPS of many pathogenic bacteria (20). Cross-reactive antibodies directed against lipid A have been postulated to be adequate reagents for therapy of sepsis caused by gram-negative organisms. However, experimental data and clinical studies with so-called anti-lipid A antibodies, such as HA-1A and E5 MAbs, have not been conclusive (4, 8, 13, 22) and are the subject of controversy (2, 18).

Recently, cross-reactive and cross-protective anti-endotoxin core MAbs recognizing the core region of LPS of *Enterobacteriaceae* have been described (5, 6). This LPS portion is made up of an outer (hexose) region and an inner part consisting of the unusual sugars L-glycero-D-manno-heptose and 3-deoxy-D-manno-octulosonic acid (10, 11). A cross-reacting antibody recognizing the LPS core has been developed, namely, WN1 222-5, a murine MAb of the immunoglobulin G2a(κ) [IgG2a(κ)] class (5). In vitro, this antibody was shown to inhibit in a dose-dependent manner the *Limulus* amebocyte lysate assay activity of LPS and the LPS-induced release by macrophages of cytokines such as tumor necrosis factor (TNF) and interleukin 6 (5). In vivo, WN1 222-5 was found to block endotoxin-induced pyrogenicity in rabbits and lethality in D-galactosamine-sensitized mice (5). WN1 222-5 has been chimerized into a human IgG1 antibody [SDZ 219-800, IgG1(κ)] which expresses specificities identical to those of WN1 222-5 (6). These two antibodies do not react with free lipid A as HA-1A and E5 do but react with LPS and with whole bacteria of *Escherichia coli*, *Salmonella enterica*, and *Shigella* species. In this study we have extensively compared the properties of these cross-reactive antibodies with those of O-side-chain-specific MAbs in several murine models of endotoxemia and bacteremia.

D-Galactosamine model. The endotoxin-neutralizing properties of WN1 222-5 and SDZ 219-800 were first investigated in vivo by using D-galactosamine (D-GalN)-sensitized mice challenged with LPS (7). We selected three LPS derived from different *E. coli* serotypes (O111, O127, and O18). LPS of *E. coli* O111:B4 and *E. coli* O127 were from Sigma (St. Louis, Mo.). LPS from *E. coli* O18 was prepared by the hot-phenol-water extraction procedure (19). WN1 222-5 and SDZ 219-800 were found to recognize LPS derived from these strains (5). Antibodies purified from hybridomas growing in serum-free media by protein A chromatography were WN1 222-5, SDZ 219-800, J8N2 80-12 (a murine IgG2a directed against porin OmpA), and 184-10-2 (a murine IgG2a MAb specific for O18, kindly provided by R. Barclay, Scottish National Blood Transfusion Service, Edinburgh, Scotland). D6B3, a murine IgG1 MAb specific for LPS from *E. coli* O111 (3), was a gift from Cassenne Laboratories (Osny, France). It was purified from hybridoma supernatant by using protein G. All antibodies were endotoxin free (<10 pg/mg of protein), as determined by the *Limulus* amebocyte lysate test (Kabi Vitrum, Uppsala, Sweden).

In the first experiment (Table 1), 5-week-old female C57BL/6J mice (IFFA Credo, Lyon, France) were sensitized intraperitoneally (i.p.) with 15 mg of D-GalN in 500 μl of phosphate-

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TABLE 1. Effects of anti-LPS antibodies on lethality and TNF levels in blood in D-GalN-sensitized C57BL/6J mice injected with LPS of *E. coli* O111, O127, and O18

Expt and treatment	Mouse group (*n*)	Peak TNF level (ng/ml)[a]
I. Challenge with LPS of *E. coli* O111		
Saline	Survivors (2)	0 (0–0.4)
	Nonsurvivors (6)	5.1 (0.8–11.0)
Type-specific MAb	Survivors (8)	0[b]
	Nonsurvivors (0)	
WN1 222-5	Survivors (7)	0[b]
	Nonsurvivors (1)	0.5
SDZ 219-800	Survivors (8)	0[b]
	Nonsurvivors (0)	
II. Challenge with LPS of *E. coli* O127		
Isotype-matched antibody	Survivors (3)	0 (0–0.05)
	Nonsurvivors (9)	2.1 (0.2–6.3)
WN1 222-5	Survivors (12)	0 (0–0.05)[b]
	Nonsurvivors (0)	
III. Challenge with LPS of *E. coli* O18		
Saline	Survivors (0)	
	Nonsurvivors (5)	1.4 (0.3–10.2)
Type-specific MAb	Survivors (5)	0[b]
	Nonsurvivors (0)	
WN1 222-5	Survivors (4)	0.1 (0–0.1)[b]
	Nonsurvivors (1)	5.1
SDZ 219-800	Survivors (5)	0[b]
	Nonsurvivors (0)	

[a] Median (range) measured in plasma 1.5 h after LPS challenge by bioassay (3).
[b] $P < 0.01$ using the Mann-Whitney–Wilcoxon test comparing nonsurvivors in the control group (saline or isotype-matched antibody) with survivors among anti-LPS-treated mice.

buffered saline and challenged i.p. with 100% lethal dose (LD_{100}) of *E. coli* O111 LPS (50 ng) given 1 h after an intravenous (i.v.) injection of saline (500 μl) alone or saline (500 μl) containing SDZ 219-800 (500 μg), WN1 222-5 (500 μg), or D6B3 (100 μg) (type-specific anti-O111 LPS). In this setting, there was 100% protection ($P = 0.003$ by the Fisher exact test) with the type-specific MAb, 88% protection ($P = 0.01$) with WN1 222-5, and 100% protection ($P = 0.003$) with SDZ 219-800. Survival was associated with a suppression of detectable TNF levels (measured by bioassay in plasma) in survivors.

In the second experiment (Table 1), a similar protective effect of WN1 222-5 was observed if 1 LD_{100} of LPS derived from *E. coli* O127 (200 ng) was used as the challenging agent. Of 12 animals, 12 survived the LPS challenge ($P < 0.0001$ over control), while 9 of 12 died when preinjected with an irrelevant and isotype-matched IgG (J8N2 80-12, directed against porin). Here again, protection was associated with a complete suppression of blood TNF levels.

In the third experiment (Table 1), we further investigated the efficacy of the cross-reactive antibodies using LPS from *E. coli* O18 (100 ng). WN1 222-5 and SDZ 219-800 as well as a type-specific antibody directed against LPS from *E. coli* O18 (184-10-2; 100 μg) were effective in providing protection ($P < 0.001$) and in decreasing levels of TNF in blood.

There is general agreement that in the D-GalN model mice die from toxicity mediated by TNF (12, 21). Both type-specific MAbs and cross-reactive antibodies were actually able to suppress blood TNF and thus protected mice from lethality upon challenge with LPS of *E. coli* O111, O127, and O18, extending previous observations made with LPS of *E. coli* O16 and of *Salmonella abortus-equi* (5). In the present study, we found that WN1 222-5 and SDZ 219-800 as well as type-specific MAbs recognizing the O-specific chain of LPS were protective. The protective efficacy of WN1 222-5 and of SDZ 219-800 in this model was remarkable because there is only one epitope located in the inner region of the LPS molecule, which is quite distinct from the repeating epitopes recognized by O-side-chain-specific MAbs.

Bacterial models. Given the observation that WN1 222-5 and SDZ 219-800 were fully protective in endotoxemic models using LPS, we next investigated the potential protective effect of these antibodies in models of sepsis caused by gram-negative organisms. These are more complex models in which the relative contribution of bacterial load and toxicity from LPS and/or other bacterial compounds is not clearly established (9). For these experiments, we focussed on *E. coli* O111, which was previously shown to be able to bind to WN1 222-5 and SDZ 219-800 (5). Female mice (strain OF1; IFFA Credo) were injected i.v. with a bacterial challenge representing about 1 LD_{100} of *E. coli* (about 10^9 CFU) in 500 μl of saline. Mice were treated with WN1 222-5 (500 μg) or D6B3 (100 μg) in 250 μl of saline or with 250 μl of saline alone as a control, given 1 h before bacterial challenge. The survival rate was 100% in mice treated with MAb D6B3, while all animals in the control group injected with saline died (Table 2). Treatment with WN1 222-5 was protective, since six of eight animals survived ($P < 0.001$; Fisher's exact test). Protection with either D6B3 or WN1 222-5 was associated with lower blood bacterial counts. However, TNF levels determined at the peak of the response were not significantly different in the three treatment groups, although there was a trend towards lower levels in animals receiving D6B3. A second experiment was performed to compare the protective effects of WN1 222-5 and of SDZ 219-800. In this experiment, the inoculum was five times greater (5×10^9 CFU/mouse). This inoculum killed mice injected with saline in 6 h. WN1 222-5 and SDZ 219-800, however, delayed the death of animals to more than 24 h. Although neither antibody reduced lethality, blood bacterial counts were reduced from 4×10^8 CFU/ml 1 h after challenge in mice receiving saline to $3 \times$

TABLE 2. Effects of anti-LPS antibodies on survival, blood bacterial counts, and TNF levels in OF1 mice challenged i.v. with *E. coli* O111[a]

Treatment	No. of survivors/total	Blood bacterial counts (log CFU/ml) at[e]:		Blood TNF levels (ng/ml) at 1.5 h[e]
		1.5 h	5 h	
Saline	0/8	7.3 ± 0.2	6.8 ± 0.3	38 ± 21
D6B3[b]	8/8	4.9 ± 0.2[d]	3.7 ± 0.3[d]	27 ± 17
WN1 222-5[c]	6/8	6.4 ± 0.3[d]	4.8 ± 0.4[d]	41 ± 12

[a] 10^9 bacteria per mouse.
[b] 100 μg per mouse.
[c] 500 μg per mouse.
[d] $P < 0.001$ versus the saline group by the Mann-Whitney test (two-tailed).
[e] Mean ± standard deviation.

TABLE 3. Effects of anti-LPS antibodies on survival, blood bacterial counts, and cytokine levels in OF1 mice challenged i.p. with *E. coli* O111

Expt	Inoculum and treatment[a]	No. of survivors/ total	Blood bacterial count (log CFU/ml)[b]	Blood TNF level (pg/ml)[b]
I	10^4 CFU, no antibiotics			
	Saline	0/7	6.14 ± 0.14	510 ± 360
	D6B3	7/7	<2[c]	230 ± 310[d]
	WN1 222-5	1/7	6.05 ± 0.34	1,055 ± 720
	SDZ 219-800	1/7	6.03 ± 0.14	920 ± 500
II	10^5 CFU, no antibiotics			
	Saline	0/10	7.83 ± 0.16	810 ± 1,310
	D6B3	8/10	<3[c]	50 ± 10[c]
	WN1 222-5	0/10	7.59 ± 0.27[d]	910 ± 8,250
	SDZ 219-800	0/10	7.61 ± 0.10[d]	710 ± 390
III	10^7 CFU, antibiotics at 4 h[e]			
	Saline	1/8	7.82 ± 0.37	390 ± 210
	D6B3	4/8	4.71 ± 1.03[c]	340 ± 200
	WN1 222-5	0/8	7.61 ± 0.18	710 ± 475[d]
	SDZ 219-800	0/8	7.61 ± 0.18	1,540 ± 120[d]

[a] D6B3, 100 μg/mouse; WN1 222-5, and SDZ 219-800, 500 μg/mouse.
[b] Means ± standard deviations determined at 8, 6, and 4 h for experiments I, II, and III, respectively.
[c] $P < 0.001$ versus saline group by the Mann-Whitney test (two-tailed).
[d] $P < 0.05$ versus saline group by the Mann-Whitney test (two-tailed).
[e] Bacterial counts and cytokine levels in samples collected just before administration of cephalosporin Fortam 12.5 mg subcutaneously.

10^7 CFU/ml by WN1 222-5 and to 2×10^7 CFU/ml by SDZ 219-800 ($P < 0.01$).

The infection model with *E. coli* O111 without antibiotics is highly dependent on effective reduction of bacterial counts to afford protection (21). In the experiment reported in Table 2, elevated TNF levels were not associated with mortality and were not affected by MAb treatment; this is in agreement with a previous study that showed that the determinant factor in the *E. coli* O111 model is bacterial counts, not TNF levels (21). Indeed, mice injected with 10^8 CFU synthesize levels of TNF similar to those of mice injected with 10^9 CFU. However, they naturally clear bacteria and survive (21). Thus, any reduction of the bacterial load from about 1 log unit will induce protection. In the present experiment, protection with the type-specific antibody D6B3 in the infection model was correlated with decreased bacterial counts (2 to 3 log units 1.5 h after challenge). The two cross-reactive antibodies also protected but were less efficient in reducing bacterial counts (<1 log unit).

Since only large bacterial inocula induce lethality in models of i.v. bacterial infections, we finally investigated a peritonitis model, in which lower inocula, of about 10^3 CFU (LD_{50} for *E. coli* O111), were shown to induce lethality (21). This model can be set up with or without use of antibiotics. In the absence of antibiotics, reduction of bacterial count is a prerequisite for survival. OF1 mice were injected with 10^4 or 10^5 CFU of *E. coli* O111/mouse mixed with mucin-hemoglobin as previously described (21). Antibodies (500 μg of WN1 222-5 or SDZ 219-800; 100 μg of D6B3) were given i.v. 1 h before challenge. Table 3 shows that both these inocula induced 100% lethality in control mice. TNF levels were lower than corresponding levels observed with i.v. injections of bacteria but were sustained during the course of the disease. A treatment with the type-specific MAb D6B3 was protective ($P < 0.01$ by the Fisher test) and was clearly associated with a striking difference in bacterial counts compared with those measured in saline controls. Due to the lower number of bacteria in mice receiving D6B3, TNF levels were lower than in control mice. The two cross-reactive antibodies showed a trend (not significant in experiment I, significant in experiment II) towards reduced bacterial counts, but this very modest reduction was totally inefficient in mediating protection in this model.

The peritonitis model can also be set up by injecting mice with a higher inoculum (Table 3, experiment III) and treating mice with antibiotics during the course of infection. Control mice died despite antibiotic treatment (bacteria were undetectable 1 h after antibiotic treatment). Type-specific MAbs remained protective (50% protection), while cross-reactive antibodies did not. Observations regarding bacterial counts and TNF levels were similar to those in the model without antibiotics.

Conclusion. Our observations showed that cross-reactive antibodies were highly efficient in the D-galactosamine model with different LPS and in a model of i.v. injection of bacteria but ineffective in a peritonitis model. In the i.v. model of infection, these antibodies were able to reduce bacterial counts sufficiently to induce protection but failed to reduce them sufficiently in the peritonitis model. It is likely that a different distribution of both bacteria and antibodies accounts for this discrepancy. In the i.v. model, antibodies are directly in contact with bacteria and react rapidly with them. In the i.p. model, bacteria first have to divide before invading the blood stream, so that antibody reactions are thought to be prolonged.

The opsonic efficacy of antibodies (for killing or phagocytosis) also appears to be of paramount importance in explaining this discrepancy in the magnitude of bacterial clearance, as shown in the different activities of type-specific and cross-reactive MAbs. At least three explanations come to mind. First, the isotype is likely to be important, and these experiments were done with antibodies of different isotypes. It is well known that antibodies differ in their ability to fix complement depending on the isotype or origin (mouse or human). Experiments should be planned to carefully investigate killing and opsonophagocytosis of bacteria with these antibodies. Second, a different accessibility or distribution of the recognized epitope in live bacteria compared to free LPS, or a different tissue distribution of the bacteria, may account for the present results. Third, and perhaps more importantly, a type-specific antibody can bind several exposed epitopes on the same LPS molecule and be more efficient in activating mechanisms such as complement and Fc receptors on different cell types (17). The epitope recognized by WN1 222-5 and SDZ 219-800 is present only once in individual LPS molecules and is possibly located in a relatively cryptic position. A lower number of MAb molecules per bacterium may limit the action of complement-associated mechanisms of clearance.

In conclusion, even if mouse models probably do not have a direct relevance for the clinical situation, they remain tools to understand the interaction in vivo among LPS, bacteria, and antibodies and can help define the mode of action of the cross-reactive antibodies.

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