INFECTION AND IMMUNITY, May 1997, p. 1972–1979 Vol. 65, No. 5
0019-9567/97/$04.00+0

Modulation of the Biological Activities of Meningococcal Endotoxins by Association with Outer Membrane Proteins Is Not Inevitably Linked to Toxicity

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Received 2 May 1996/Returned for modification 7 August 1996/Accepted 18 February 1997

Meningococcal sepsis results partly from overproduction of host cytokines after macrophages interact with endotoxin. To obtain less toxic and highly immunomodulatory meningococcal endotoxins for prophylactic purposes, we investigated the relationship between endotoxicity and immunomodulatory activity of several endotoxin preparations from *Neisseria meningitidis* group B. Using the D-galactosamine-sensitized mouse model to determine endotoxin lethality, we found that the toxicity of purified lipooligosaccharide (LOS) from M986, a group B disease strain, was three to four times higher than those of purified LOSs from the noncapsulated strains M986-NCV-1 and OP$^-$, the truncated-LOS mutant. The LOSs of outer membrane vesicles (OMVs) and detergent-treated OMVs (D-OMVs) from the three strains were 2 to 3 and over 300 times less toxic than the purified LOSs, respectively. Intraperitoneal administration of these preparations induced production of tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) in serum 2 h after injections. However, repeated doses of low- and high-toxicity preparations induced lower amounts of TNF-α and IL-6, i.e., LOS tolerance. Injection of mice with low doses of LOS was as effective as injection with high doses in inducing tolerance. Peritoneal macrophages from tolerant mice pretreated with either high- or low-toxicity LOS preparations produced only a fraction of the amounts of TNF-α and IL-6 produced by control groups in response to LOS ex vivo. Despite tolerance to LOS induced by pretreatment with reduced-toxicity preparations, killing of *N. meningitidis* M986 by macrophages from these animals was enhanced. Protection was achieved when mice treated with LOS, and especially that of D-OMVs, were challenged with live *N. meningitidis*. The least toxic LOS, that in D-OMVs, was most effective in inducing hyporesponsiveness to endotoxin in mice but protected them against challenge with *N. meningitidis*. No inevitable link between toxicity and host immune modulation and responses was shown. Our results show that LOS is responsible for both toxicity and immunomodulation. When LOS is tightly associated with outer membrane proteins in D-OMV, it reduces toxicity but enhances beneficial effects compared to results with its purified form. Thus, systematic and critical evaluation of D-OMVs as adjuvants or as portions of group B meningococcal vaccines may help improve survival and outcome in meningococcal sepsis.

Endotoxin shock as a result of group B meningococcal infection continues to pose a major clinical problem worldwide. Despite intensive efforts at prophylactic intervention, mortality resulting from profound shock remains unacceptably high (22, 43). Effective vaccines for *Neisseria meningitidis* groups A, C, Y, and W135 have been developed, but there is no vaccine for serogroup B, which is responsible for most meningococcal diseases in the United States and Europe (2, 8, 17, 21, 31, 44, 45). Numerous in vitro and in vivo studies implicate bacterial endotoxin as a major contributing factor to the pathogenesis of septic shock (11, 28).

The severity of meningococcal disease is related to the level of endotoxin in plasma, which in turn is related to the intensity of the host immune response (6). The lethality of the infection is independent of bacterial viability but is induced by an overproduction of host cytokines after interaction of macrophages with lipooligosaccharide (LOS) and other cell wall components released from the cell envelopes of living and dead organisms (5, 29). In humans, levels of tumor necrosis factor (TNF) in serum correlate with survival from meningococcal septicemia and have also been associated with a poor outcome in critically ill septic patients (7, 16, 40). During acute bacterial infections, levels of interleukin 6 (IL-6) in plasma correlate with the severity of meningococcal septic shock (18, 19, 37, 39). Therefore, a successful strategy for preventing this condition should be to control the production of proinflammatory mediators to amounts that provide only beneficial immunostimulatory effects (10, 25).

The inability of conventional medical management to substantially improve the mortality associated with meningococcal sepsis has stimulated interest in the development of approaches aimed directly at endotoxins. The development of prophylactic preparations based on LOS may play a decisive role in the prophylaxis of group B meningococcal diseases accompanied by endotoxemia. However, the toxicity of lipid A, contained in LOS, makes it impossible to prepare a vaccine based on purified meningococcal LOS and removal of this toxic component leads to a decrease in the immunomodulatory potency of the preparation (20). In this study we investigated the relationship between the toxicities of purified endotoxins and endotoxin-associated preparations from group B meningococci and their biological activities.

The encapsulated group B meningococcal strain M986 (serotype 2a), the noncapsulated strain M986-NCV-1 (serotype 2a) (12), and the truncated M986 LOS mutant strain OP$^-$ (provided by C. E. Frasch, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Md.) were used. The strains were grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.) at 37°C for 2 days. To dissociate LOS from outer membrane vesicles (OMVs), 2 mM disodium EDTA was added to an opalescent OMV suspension in water,

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TABLE 1. Lethal toxicities of endotoxins associated with different preparations in galactosamine-loaded mice

Endotoxin and strain	$B_6D_2F_1$ mice		NIH/Swiss mice	
	LD_{50} (ng/kg)[a]	Confidence limits	LD_{50} (ng/kg)	Confidence limits
LOS				
M986	0.62	0.36–0.89	1.8	1.7–1.98
M986-NCV-1	2.75	2.35–3.75	6.9	5.5–8.23
OP^-	2.2	2.14–2.38	6.3	6.0–6.72
LOS in OMVs				
M986	1.95	1.8–2.34	4.0	3.1–5.25
M986-NCV-1	3.62	2.4–4.83	9.7	8.4–10.7
OP^-	3.43	3.1–4.12	9.2	8.0–10.6
LOS in D-OMVs				
M986	188.0	169–226	388.0	272–466
M986-NCV-1	1014.0	922–1,156	1,520.0	1,014–2,027
OP^-	791.3	594–888	1,180.0	788–1,577

[a] LD_{50}s are based on nanograms of LOS.

adjusted to pH 8.5 with 1 M Tris base, and then solubilized in 2% deoxycholate (DOC) at 37°C for 10 min. LOS and detergent-treated OMV (D-OMV) fractions were prepared simultaneously by Sephacryl S-300 chromatography (35). Retreatment of D-OMVs with a 2% or higher concentration of DOC removed little LOS (about 1%) from the remaining LOS in D-OMVs. Similar elution profiles were obtained for strains M986 (protein type 2), M986-NCV-1, and OP^-. LOS content was measured by determination of 2-keto-3-deoxyoctonate (Kdo) by the thiobarbituric acid (TBA) method (30). Purified LOSs (99% purity) from the various strains were used as standards to determine LOS concentrations in OMVs and D-OMVs. Nucleic acid content was determined by spectrometry. Purified LOS contained 1% (wt/wt) protein and less than 1% nucleic acid (33, 41). *Limulus* amebocyte lysate activities for purified LOSs were determined to be 1.4×10^8, 4.6×10^7, and 4.0×10^7 endotoxin units (EU)/mg; for LOSs in OMV preparations, they were determined to be 8.0×10^7, 5.2×10^7, and 4.2×10^7 EU/mg; and for LOSs in D-OMV preparations, they were determined to be 6.4×10^5, 2.5×10^5, and 3.2×10^5 EU/mg for strains M986, M986-NCV-1, and OP^-, respectively. Thus, the endotoxin associated with D-OMVs was 100 to 200 times less reactive than purified LOSs by chromogenic *Limulus* amebocyte assay.

Endotoxicities of the preparations. To investigate the endotoxicities of the different preparations, we examined their potential to induce shock in D-galactosamine-loaded mice. Female $B_6D_2F_1$ and NIH/Swiss mice, 6 to 8 weeks old and weighing 18 to 20 g, were obtained from Charles River Laboratories (Raleigh, N.C.). To determine the endotoxicity potentials of the preparations, groups of six mice ($B_6D_2F_1$ or NIH/Swiss) were simultaneously treated with D-galactosamine (400 mg/kg of body weight) intraperitoneally (i.p.) and then challenged within 30 min with one of the antigens intravenously (14). Survival of the animals was observed for 4 days, and the 50% lethal dose (LD_{50}) was calculated by probit analysis (26). Administration of galactosamine (400 mg/kg) rendered both mouse strains (inbred $B_6D_2F_1$ and outbred NIH/Swiss) more sensitive to endotoxins (data not shown). Purified LOS from the wild-type group B strain M986 was three to four times more toxic than those from the mutants M986-NCV-1 and OP^- (Table 1). The purified LOSs from the three strains were 1.5 to 3 times more toxic than LOSs in OMVs. In this model, endotoxin associated with DOC-treated OMVs was 200 to 400 times less toxic than the purified LOSs. Treatment of M986 LOS with neuraminidase rendered the preparation about three times less toxic than the untreated sample (data not shown). The sialic acid (SA) released from M986 LOS was assayed by the TBA assay (42). Pyrogenicity studies were performed with New Zealand White rabbits (36). Doses of purified LOS were 0.2 to 0.4 ng/kg and doses of LOS in OMVs were 0.5 to 1 ng/kg when preparations were administered intravenously. Under similar conditions, doses of LOS in D-OMVs were 0.4 to 1 μg/kg. The results indicate that LOS in D-OMVs is at least 1,000 times less pyrogenic than purified LOS.

Cytokine induction. We evaluated in vivo host-endotoxin interplay by measuring levels of circulating cytokines and by measuring in vitro cytokine production in macrophages from LOS-tolerant animals. Mice were treated with different concentrations of the preparations. The lowest concentration of purified LOS that could show measurable amounts of TNF in plasma after injection was 50 ng/mouse. Concentrations of LOS for the OMVs and D-OMVs were selected based on the results from the toxicity studies. The initial doses were therefore 150 ng of LOS in OMVs and 15 μg of LOS in D-OMVs per mouse, respectively. Increases in doses were also based on toxicity; all doses of LOSs, LOSs in OMVs, and LOSs in D-OMVs were based on LOS contents determined chemically. Cytokine-specific enzyme-linked immunosorbent assays (ELISAs; Endogen, Inc., Cambridge, Mass.) were used for quantitation. The TNF alpha (TNF-α) and IL-6 concentrations in serum and culture supernatants were determined by sandwich ELISAs for TNF (9) and IL-6 (23). Blood was taken from the retro-orbital plexus 2 h after the last dose of single or multiple i.p. injections. Sera were removed aseptically, pooled, and assayed for TNF-α and IL-6. Pooled sera from six mice per group were assayed in triplicate, and the data were presented as geometric means. One day after the last treatment, the thioglycolate-elicited peritoneal macrophages were also harvested. Cytospin preparations of the exudate cells showed that cells from all groups consisted predominantly of macrophages. Viability was assessed by the trypan blue exclusion method (34). Typically, more than 90% of the exudative cells were intact viable macrophages. Macrophages were incubated with different concentrations of purified LOS (1 to 1,000 μg) in serum-free medium. After 4 h, the supernatants were harvested and assayed for TNF-α and IL-6. Standard errors of the geometric means (SEM) were used as measures of variance.

Decrease in TNF-α in serum after multiple administrations of endotoxin-associated preparations. TNF-α was not detectable in the sera of the control mice (values were below 10 pg/ml) throughout the investigation. Two hours after i.p. injection with the different preparations of endotoxin, concentrations of TNF-α ranged from 34 to 130 pg/ml in the blood of mice after the first dose of either purified LOS, LOS in OMVs, or LOS in D-OMVs (Table 2). Purified LOS from strain M986 was the most potent inducer of TNF-α. Mice became tolerant after two consecutive i.p. injections. When these tolerant mice were injected for a third time, their serum TNF-α concentrations were much lower, specifically in response to strain M986. For strain M986, the TNF level obtained after injections with purified LOS was higher than that obtained after injections with LOS in D-OMVs. Differences for the two other mutant strains were less or not obvious. Our results indicate the down-regulation of TNF production as described previously (10). Tolerance was induced at low as well as at higher endotoxin doses.

Effect of LOS tolerance on IL-6 production in serum. Table 3 shows that mice injected once with LOS, LOS in OMVs, or LOS in D-OMVs secreted considerable IL-6. Peak IL-6 levels

TABLE 2. Serum TNF concentrations measured after administration of endotoxin-associated preparations

Bacterial strains	TNF concn (pg/ml) in pooled sera of mice injected with the indicated amt (μg/mouse) of[a]:								
	Purified LOS			LOS in OMVs			LOS in D-OMVs		
	0.05[b]	0.05[c]	0.25[c]	0.15[b]	0.15[c]	0.75[c]	15[b]	15[c]	75[c]
M986	130	25.9	31.5	110.0	26.7	27.7	74.0	22.9	24.8
M986-NCV-1	55.7	24.1	29.3	39.0	24.3	25.9	35.3	23.5	25.0
OP^-	33.8	24.7	28.0	35.6	24.1	26.6	38.0	25.3	23.3

[a] TNF was measured 2 h after the last dose of each preparation. Untreated control animals had serum TNF concentrations of <10 pg/ml, the lowest level of detection.
[b] Groups of six mice were injected i.p. once; mouse sera from each group were pooled.
[c] Groups of six mice were injected i.p. three times on three consecutive days; mouse sera from each group were pooled.

were 2,900 to 5,800 pg/ml at 2 h after a single injection and were lower after multiple treatments with low-dose preparations. Interestingly, increased levels of IL-6 were observed in the high-dose groups even after three treatments with LOS or LOS in OMVs. Nevertheless, IL-6 levels remained low in mice made tolerant with increasing doses of LOS in D-OMVs. Induced hyporesponsiveness was partially reversed by high doses of LOS or LOS in OMVs after three treatments, whereas mice treated with LOS in D-OMVs, which has reduced toxicity, maintained tolerance even at high doses.

Reduced production of TNF-α in vitro by endotoxin-induced desensitized macrophages. To confirm the above-described in vivo data, we measured in three experiments (six mice per group) the in vitro responses of macrophages from controls and animals treated with the endotoxin-associated preparations. The results shown are corrected for spontaneous TNF-α release by unstimulated cells (12 ± 10 pg/ml). As shown in Fig. 1, macrophages from placebo groups responded to LOS by releasing larger amounts of TNF-α (peak levels ranged from 849.9 ± 254.6 [geometric mean ± SEM] to 815.2 ± 336.4 pg/ml) compared to the macrophages from mice treated with endotoxin-associated preparations. Reduced amounts of TNF-α were detected when mice were treated once with a low dose of LOS, LOS in OMVs, or LOS in D-OMVs; levels ranged from 424.8 ± 113.4 to 384.7 ± 121.9, 437.3 ± 82.4 to 299.8 ± 70.3, and 461.6 ± 61.2 to 362.4 ± 92.3 pg/ml, respectively (Fig. 1A). All three treatment groups showed a much greater reduction when they were injected on three consecutive days than when they were injected one day before peritoneal lavage. Macrophages from animals similarly pretreated with 50 or 250 ng of LOS per mouse produced levels of 287.8 ± 83.9 to 227.1 ± 66.6 and 153.4 ± 58.1 to 88.6 ± 24.3 pg/ml, respectively. Mice given 150 or 750 ng of LOS in OMVs per mouse three times also produced less TNF-α in response to LOS (1 to 1,000 μg/ml); levels ranged from 389.8 ± 60.2 to 179.3 ± 58.9 and 137.8 ± 33.9 to 93.7 ± 16.9 pg/ml, respectively. Similar results were observed with LOS in D-OMVs at doses of 15 or 75 μg per mouse given three times; levels ranged from 386.3 ± 41.3 to 282.3 ± 63.4 and 73.4 ± 17.2 to 62.3 ± 15.7 pg/ml, respectively (Fig. 1B and C).

TABLE 3. Serum IL-6 concentrations measured after administration of endotoxin-associated preparations

Bacterial strain	IL-6 concn (pg/ml) in pooled sera of mice injected with the indicated amt (μg/mouse) of[a]:								
	Purified LOS			LOS in OMVs			LOS in D-OMVs		
	0.05[b]	0.05[c]	0.25[c]	0.15[b]	0.15[c]	0.75[c]	15[b]	15[c]	75[c]
M986	3,973	916	2,912	5,804	1,109	2,782	4,007	694	450
M986-NCV-1	2,916	961	1,659	4,190	966	2,031	3,332	570	314
OP^-	2,961	1,062	1,806	3,249	873	2,583	3,468	390	343

[a] IL-6 was measured 2 h after the last dose of each preparation. Untreated controls had IL-6 concentrations in sera below the lowest limit of detection (<10 pg/ml).
[b] Groups of six mice were injected i.p. once; mouse sera from each group were pooled.
[c] Groups of six mice were injected i.p. three times on three consecutive days; mouse sera from each group were pooled.

In vitro IL-6 production by macrophages from mice made tolerant. IL-6 levels in culture supernatants were also measured in three experiments (each group consisted of six mice). Peritoneal macrophages from placebo groups did not produce detectable levels of IL-6 when exposed to medium. However, when macrophages from the same groups were incubated with purified LOS, IL-6 peak levels of 2,356.8 ± 398.8 (geometric mean ± SEM) to 2,087.3 ± 463.6 pg/ml were detected in the incubation medium. Mouse peritoneal macrophages from mice treated once with endotoxin-associated preparations produced significantly less IL-6 than those from the placebo groups. For LOS, LOS in OMV, and LOS in D-OMV pretreatment, peak IL-6 values ranged from 1,464.4 ± 225.3 to 894.6 ± 113.5, 1,002.7 ± 198.8 to 715.9 ± 113.6, and 690.3 ± 62.9 to 578.6 ± 103.7 pg/ml, respectively (Fig. 2A). Macrophages from mice treated multiple times with the same low dose released less IL-6 than did mice treated once. Peak levels ranged from 387.8 ± 60.4 to 194.3 ± 51.2, 175.5 ± 80.4 to 105.3 ± 43.7, and 103.1 ± 16.2 to 94.3 ± 21.4 pg/ml in mice treated with LOS, LOS in OMVs, and LOS in D-OMVs, respectively (Fig. 2B). An increase in the amount of IL-6 production was detected regardless of multiple treatments with a high dose of LOS or LOS in OMVs. Levels ranged from 670.8 ± 105.4 to 495.6 ± 62.8 and 309.7 ± 57.2 to 216.4 ± 65.8 pg/ml, respectively. In contrast, induction of IL-6 by macrophages from the groups treated with the less toxic LOS in D-OMVs remained low even at the highest dose tested: 98.9 ± 15.4, 95.9 ± 21.7, and 60.9 ± 16.9 pg/ml for strains M986, M986-NCV-1, and OP^-, respectively (Fig. 2C).

Enhanced microbicidal capacity of peritoneal macrophages from mice made tolerant to endotoxin. The effect of immunostimulation by the meningococcal endotoxin preparations was investigated by measuring, ex vivo, the killing of M986 organisms by macrophages from mice treated with LOS preparations with high and low toxicities. Microbicidal activities of the macrophages were measured in incubation mixtures containing 0.2 ml (10^6) of macrophages from mice and 0.2 ml (10^6) of opsonized *N. meningitidis* M986 in Hanks balanced salt solution containing 10% fresh human serum incubated at 37°C for 4 h, and the lysates were plated. A timed study conducted earlier showed that organisms were best killed when mixtures were incubated for 4 h; therefore, incubation for this period was selected. Peritoneal macrophages from tolerant mice killed the test organism better than did cells from control animals. One treatment was not effective in enhancing phagocytic capacity against the organisms (data not shown), but three consecutive treatments with increasing doses enhanced the killing of organisms (Fig. 3). At the highest dose, the LOS in D-OMV group had the most activity, with a range of geometric mean values ± SEM of 91.6% ± 3.2% to 83.4% ± 4.8% compared with ranges of 4.5% ± 2.2% to 3.8% ± 2.0%, 76.3% ± 4.6% to 58.6% ± 6.8%, and 90.7% ± 1.5% to 38.4% ± 6.5% for the mice treated with the placebo, LOS in OMVs, and LOS, re-

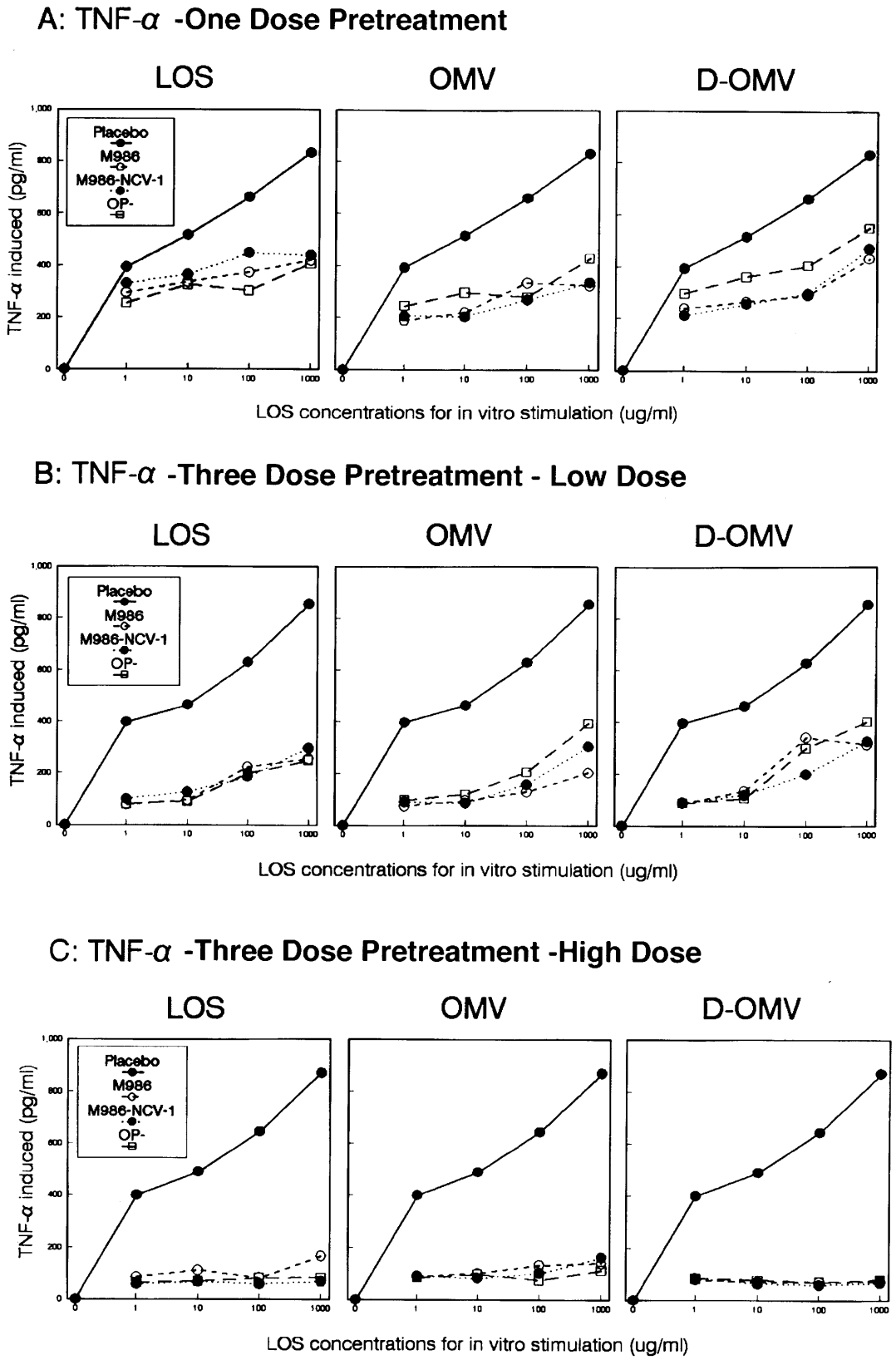

FIG. 1. In vitro production of TNF by macrophages, from mice made tolerant with endotoxin, in response to purified LOS. Groups of six mice were pretreated as follows: once with LOS (50 ng/mouse), LOS in OMVs (150 ng/mouse), LOS in D-OMVs (15 μg/mouse), or a placebo (A); three consecutive times with LOS (50 ng/mouse), LOS in OMVs (150 ng/mouse), LOS in D-OMVs (15 μg/mouse), or a placebo (B); and three consecutive times with LOS (250 ng/mouse), LOS in OMVs (750 ng/mouse), LOS in D-OMVs (75 μg/mouse), or a placebo (C). Macrophages from pretreated mice were stimulated with M986 LOS (1 to 1,000 μg) for 4 h at 37°C. TNF production was measured by ELISA.

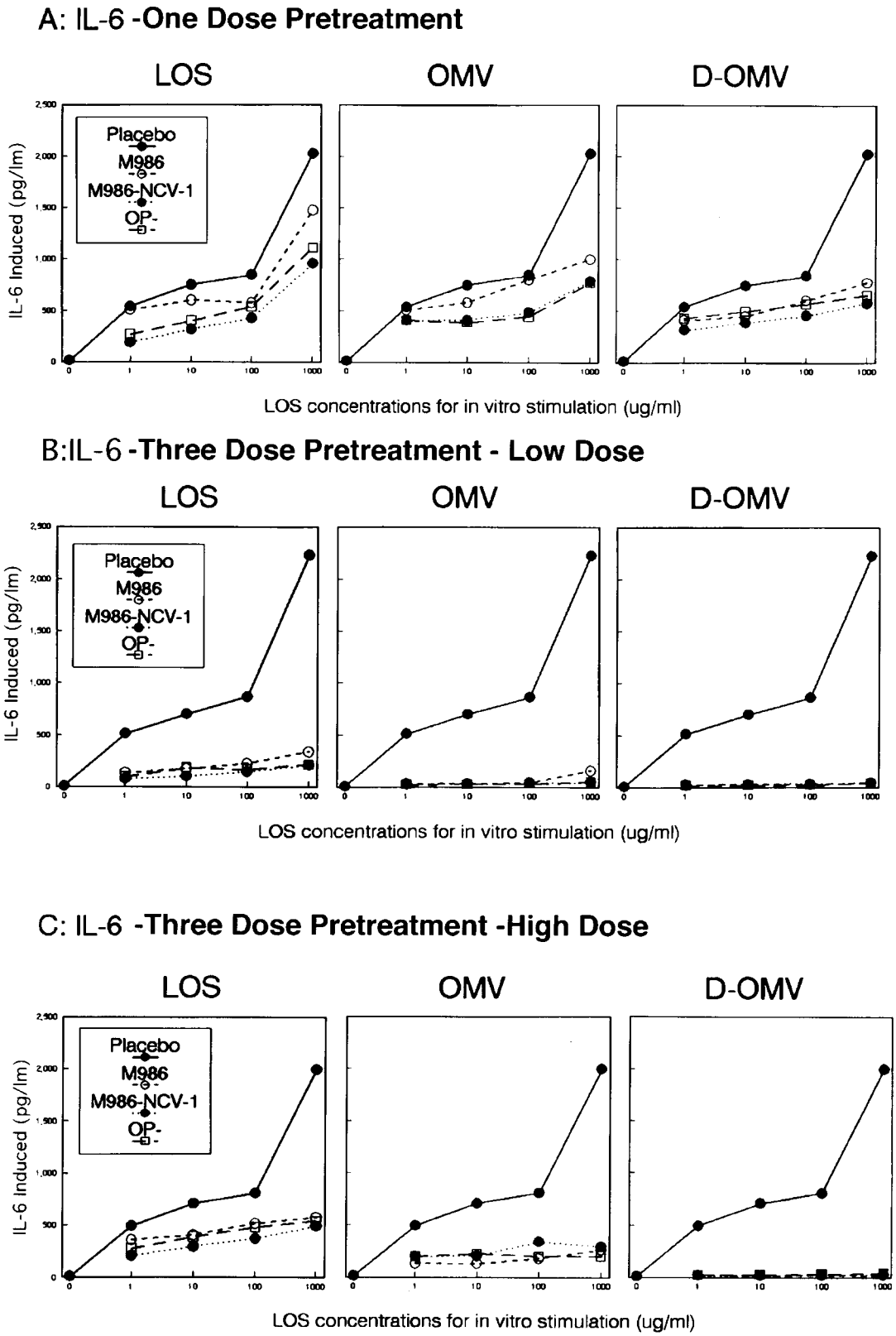

FIG. 2. In vitro production of IL-6 by macrophages, from mice made tolerant with endotoxin, in response to purified LOS. Groups of six mice were pretreated as follows: once with LOS (50 ng/mouse), LOS in OMVs (150 ng/mouse), LOS in D-OMVs (15 μg/mouse), or a placebo (A); three consecutive times with LOS (50 ng/mouse), LOS in OMVs (150 ng/mouse), LOS in D-OMVs (15 μg/mouse), or a placebo (B); and three consecutive times with LOS (250 ng/mouse), LOS in OMVs (750 ng/mouse), LOS in D-OMVs (75 μg/mouse), or a placebo (C). Macrophages from pretreated mice were stimulated with M986 LOS (1 to 1,000 μg) for 4 h at 37°C. IL-6 production was measured by ELISA.

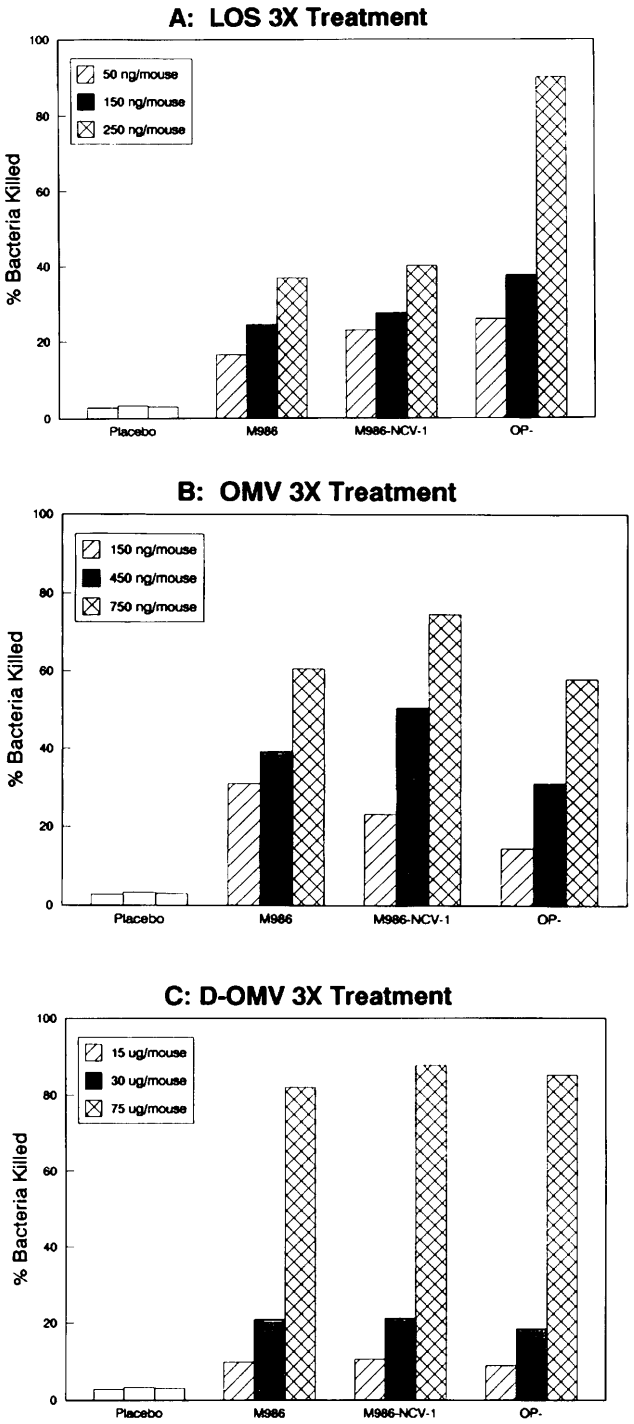

FIG. 3. Microbicidal capacity of peritoneal macrophages, harvested from mice pretreated on three consecutive days with either LOS (50, 150, or 250 ng/mouse) (A), LOS in OMVs (150, 450, or 750 ng/mouse) (B), or LOS in D-OMVs (15, 30, or 75 μg/mouse) (C). Placebo groups were always injected with pyrogen-free water, which was the vehicle used. M986 organisms were opsonized in 10% normal human serum. A mixture of opsonized bacteria and macrophages was incubated for 4 h at 37°C, and the number of surviving organisms was estimated by plating 10-fold dilutions of cellular lysate. Cumulative data from three separate experiments are shown.

spectively. Thus, macrophage hyporesponsiveness to LOS enhances rather than reduces the ability to phagocytose and kill, especially for the macrophages from the group treated with the least toxic LOS in D-OMVs.

TABLE 4. Survival of mice after intravenous inoculation of group B *N. meningitidis* 2 days after last endotoxin treatment

Endotoxin treatment[a]	No. of mice that survive/total no. of mice after inoculation of indicated amt (CFU/mouse) of *N. meningitidis* group B				
	10^4	10^5	10^6	10^7	10^8
Placebo	10/10	1/10	0/10	0/10	0/10
LOS					
M986	10/10	10/10	7/10	2/10	0/10
M986-NCV-1	10/10	9/10	8/10	0/10	0/10
OP^-	10/10	10/10	9/10	8/10	0/10
LOS in D-OMVs					
M986	10/10	10/10	10/10	8/10	0/10
M986-NCV-1	10/10	10/10	10/10	8/10	1/10
OP^-	10/10	10/10	10/10	10/10	3/10

[a] All groups of mice were treated three times with either a placebo (pyrogen-free water), purified LOS (1 μg/mouse), or LOS in D-OMVs (75 μg/mouse). Mice were then simultaneously given 8 mg of galactosamine per mouse and different doses of inoculum.

Protection against microbial infection in D-galactosamine-loaded mice. Mice were injected i.p. with either 1 μg of purified LOS or 75 μg of LOS in D-OMVs on three consecutive days. Two or 7 days after the last injection, mice were challenged intravenously with live meningococcal strain M986 organisms. The protective efficacies of LOS preparations were assessed in pretreated C57BL/6 mice challenged with different concentrations of living bacteria. Control mice that were challenged and administered D-galactosamine became moribund (i.e., exhibited extreme lethargy, tachypnea, piloerection, and closed eyes). More than 95% of the control mice given 10^5 CFU or higher doses of inoculum per mouse died within 24 h. No death was observed when galactosamine was omitted in the inoculum groups. Tables 4 and 5 present the survival of mice pretreated with purified LOS or LOS in D-OMVs. All mice treated with LOS in D-OMVs and 70 to 90% of mice treated with LOS were protected against bacterial challenge with an inoculum of 10^6 CFU/mouse 2 days after the last treatment. In contrast, when these animals were challenged 7 days after the last treat-

TABLE 5. Survival of mice after intravenous inoculation of group B *N. meningitidis* 7 days after the last endotoxin treatment

Endotoxin treatment[a]	No. of mice that survive/total no. of mice after inoculation of indicated amt (CFU/mouse) of *N. meningitidis* group B				
	10^4	10^5	10^6	10^7	10^8
Placebo	10/10	1/10	0/10	0/10	0/10
LOS					
M986	10/10	3/10	0/10	0/10	0/10
M986-NCV-1	10/10	1/10	0/10	0/10	0/10
OP^-	10/10	7/10	5/10	0/10	0/10
LOS in D-OMVs					
M986	10/10	10/10	9/10	7/10	0/10
M986-NCV-1	10/10	10/10	9/10	9/10	0/10
OP^-	10/10	10/10	10/10	9/10	2/10

[a] All groups of mice were treated three times with either a placebo (pyrogen-free water), purified LOS (1 μg/mouse), or D-OMV (75 μg/mouse). Mice were then simultaneously given 8 mg of galactosamine per mouse and different doses of inoculum.

ment, only mice pretreated with LOS in D-OMVs were protected.

Death occurs among patients infected with *N. meningitidis* because of the relentless progression of the shock state. The most important mediators that can be detected during the course of human septicemia are TNF-α and IL-6. In low concentrations, these mediators are necessary for host defense. Antimicrobial therapy alone to eliminate the organisms is often ineffective and may provoke the release of LOS from dying organisms (32). Interruption of the sequence of events that culminates in shock has been attempted because of the limited effectiveness of current treatments of meningococcal diseases. Incorporating membrane-bound endotoxin into vaccines remains a possibility; alteration of some immunobiological properties, in particular, decreasing an endotoxin's toxicity and enhancing its macrophage-stimulating activity, may be considered as an approach to preventing meningococcal sepsis.

In this study, LOSs in D-OMVs were 200 to 400 times less toxic than purified LOSs. LOSs in D-OMV preparations from the mutant strains were less toxic than LOS from the wild-type strain. In examining the LOSs of the wild-type and mutant strains, we found that the mutants have altered LOSs, as indicated by an increased electrophoretic mobility by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In our SDS-PAGE analysis, the molecular weight of the upper band of M986-NCV-1 was lower than that of M986. LOSs from *N. meningitidis* strains treated with *Clostridium perfringens* neuraminidase released SA, and SDS-PAGE analysis before and after treatment showed a reduction in the molecular weights of the upper bands that contain SA. It appears that sialylation of LOS affects induction of proinflammatory cytokines, and its presence might explain the differences in the toxicity levels of the three strains and the decrease in lethal toxicity after LOS from M986 was treated with neuraminidase. This observation may provide a means to reduce the toxicities of such preparations.

The LOS contents in the different preparations were determined by measuring Kdo (5% of LOS) by the TBA assay. The TBA assay detects Kdo as well as SA, because both produce the same chromophore, β-formylpyruvic acid. However, the assay conditions for Kdo and SA are quite different. The color yield for free SA was ≤10% and that for sialyllactose (bound SA) was ≤6% of that for Kdo under the assay conditions for Kdo. Based on calculated molecular weights (M_rs) of meningococcal LOSs (15), the Kdo content of the sialylated M986 LOS (M_r, 4,300) drops by about 8% compared to that of M986-NCV-1 LOS (M_r, 4,000). Thus, the overall error in the Kdo assay in determining the amount of purified M986 LOS is about 2%. For purified OP^- LOS with an estimated M_r of 3,600 (SDS-PAGE), a 10% adjustment (deduction) with respect to the amount of M986-NCV-1 LOS is required. With the three purified LOSs instead of Kdo as standards, the LOS contents in OMVs and D-OMVs were determined.

Development of LOS tolerance was independent of its toxic effect, as indicated by low doses of LOS preparations inducing tolerance to subsequent lethal doses. Further evidence of dissociation between tolerance induction and endotoxemic effects is that pretreatment of animals with low doses of endotoxin was as effective as treatment with high doses in inducing tolerance to the lethal effects of LOS. Furthermore, the LOSs in D-OMVs induced much lower levels of IL-6 but were more effective in inducing tolerance to cytokines induced by LOS. The mechanisms by which these preparations induce resistance to lethal effects of LOS involve a decreased responsiveness of host macrophages (13, 38). Hence, production of low amounts of putative mediators of endotoxin by macrophages from tolerant mice may occur because certain genes are apparently superinduced in the desensitized macrophages (27).

Although tolerance to the lethal effects of LOS may be relevant to the prophylactic control of overproduction of host cytokines after interaction of macrophages with endotoxin, there was a concern that the tolerance-inducing regimens may interfere with the clearance of meningococci by macrophages. No correlation between protection and toxicity was observed, because mice treated with either purified LOS or LOS in D-OMVs were protected. However, treatment with LOS in D-OMVs could protect the mice for a longer period after endotoxin treatment and also against higher doses of inoculum. Our results indicate that the association of LOS with OMVs after detergent treatment of OMVs drastically reduces endotoxicity while maintaining and even enhancing the immunomodulatory potency of the preparation. These findings suggest that effective control of meningococcal septicemia will require both down-regulation of proinflammatory cytokines and activation of residual immune systems.

The mechanism by which tolerant mice exhibit enhanced resistance to infections is not clear. However, because endotoxin-associated preparations are heterogeneous (3, 4), some of the nonendotoxin components, such as outer membrane proteins, may have beneficial effects or contribute to the beneficial reaction elicited by endotoxin, possibly by modulating the toxic effect of TNF-α. Our results support such a hypothesis. Our results suggest a way of dissociating toxicity from immune modulation in response to meningococcal endotoxin and of enhancing immunostimulation. They also indicate that there is no inevitable link between toxicity and the beneficial effects of meningococcal endotoxin.

The development of vaccines based on endotoxins obtained from *N. meningitidis* and other gram-negative bacteria is now attracting special attention. The main approach to solving the problem associated with these attempts consists of the elimination of lipid A and the conjugation of the carbohydrate component of endotoxin with a protein carrier (1). However, lipid A has an adjuvant effect in addition to its toxic action. For this reason, D-OMVs, which retain but simultaneously detoxify lipid A, are of great interest for the development of new vaccines. Such material (LOS-depleted outer membrane proteins) is used as the carrier protein for the Merck *Haemophilus influenzae* type b conjugate vaccine (24).

We are grateful to Pankaj Amin for his technical assistance and to Suzanne Epstein, Ira Berkower, Carolyn Deal, and Carl Frasch for critically reading the manuscript.

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Editor: R. E. McCallum