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Evidence for the Presence of Lipopolysaccharide in a Ribonuclease-Sensitive Ribosomal Vaccine of *Pseudomonas aeruginosa*

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To obtain information about the nature of the immunogens in the ribosomal vaccine (fraction II) of *Pseudomonas aeruginosa*, we studied the specificity of rabbit antibodies to fraction II. Crossed immunoelectrophoresis demonstrated the presence of antibodies which precipitated with ribosomal antigens, but not with lipopolysaccharide (LPS). By means of an enzyme-linked immunosorbent assay, antibodies to LPS were detected in antibodies to fraction II. Antibodies to fraction II could protect mice against a lethal challenge with *P. aeruginosa*. Absorption experiments demonstrated that the protective ability of antibodies to fraction II was due to antibodies to cell envelope antigens, whereas antibodies to ribosomal antigens did not contribute to the protection. Antibodies to LPS could be detected in mice 1 week after a single vaccination with fraction II. It was concluded that the protective activity of fraction II was due, at least in part, to the presence of LPS in the ribosomal vaccine. Treatment of fraction II with ribonuclease decreased the protective activity of the ribosomal vaccine. Addition of synthetic polyadenylic acid-polyuridylic acid restored the protective activity of ribonuclease-treated fraction II, indicating that RNA in the ribosomal vaccine might act as an adjuvant or a carrier in the presentation of the contaminating cell envelope antigens. The protective activity and the toxicity of fraction II were compared with the protective activity and the toxicity of fraction I, which contained cell envelope components, including LPS, and of purified LPS. The results indicated that protection by the ribosomal vaccine was associated with a slightly higher toxicity than was protection by fraction I, whereas purified LPS was the most toxic vaccine.

Since Youmans and Youmans showed the protective activity of ribosome-rich preparations from *Mycobacterium tuberculosis* (24), effective ribosomal vaccines have been prepared from various microorganisms (6). However, considerable controversy exists in literature concerning the immunogenic principle of these vaccines. Investigators working on ribosomal vaccines of salmonellae have variously concluded that the active moiety is ribonucleic acid (RNA) (21), protein isolated from the ribosomal fraction (14), O antigens (5), or cell surface proteins contaminating the ribosomal preparation (15). Recently, several authors concluded that the protective activity of the ribosomal vaccines was due to contaminating cell surface components; no evidence was presented showing that the ribosomes themselves contributed to the protection (7, 12, 15).

In a previous report, the isolation of a purified ribosomal vaccine (fraction II) and a fraction containing cell envelope components (fraction I) from *Pseudomonas aeruginosa* was described (8). Fraction I contained protein and lipopolysaccharide (LPS) and less than 4% RNA. Fraction II contained RNA and protein in a ratio of 1.94. The most remarkable property of fraction II was its sensitivity to ribonuclease (RNase), indicating that RNA was indispensable for the protective activity of this ribosomal vaccine. Treatment of fraction II with pronase and trypsin did not abolish the protection. The function of RNA in this ribosomal vaccine was not clarified. RNA might act as a carrier or an adjuvant for traces of an unidentified, protease-resistant component in the ribosomal vaccine. Alternatively, RNA might be the protective component. The former hypothesis was favored by the finding that the protection by fraction II was serotype specific. The serotype-specific O antigens of *P. aeruginosa* are part of the cell wall component LPS. Although no LPS could be detected in fraction II by the determination of 2-keto-3-deoxyoctonate (KDO), the presence of traces of LPS could not be excluded. The results presented in this paper demonstrated that, although

the ribosomal vaccine was sensitive to RNase treatment, it induced antibodies to LPS. Therefore, the hypothesis that RNA in the ribosomal vaccine might function as a carrier or an adjuvant in the presentation of the contaminating cell envelope antigens is supported. The protective capacity and the toxicity of fraction I, fraction II, and LPS have been compared.

MATERIALS AND METHODS

Bacteria. *P. aeruginosa* serotype 3 (strain RIV 76-5321) (8) was used throughout this study for the preparation of the vaccines and as the challenging organism.

Animals. Outbred male and female Cpb SE Swiss mice (body weight, 18 to 20 g) were purchased from TNO, Central Institute for the Breeding of Laboratory Animals, Zeist, The Netherlands. Male or female crossed Flemish giant rabbits, weighing 3 to 4 kg each, were used.

Vaccines. Fractions I and II were obtained from a crude ribosomal preparation by molecular sieve chromatography on Sepharose Cl-2B as described previously (8). Four batches of fraction I and four batches of fraction II were pooled. The determination of RNA and protein and the estimation of LPS by the determination of KDO were performed as described previously (8). Fraction I contained 34 μg of RNA, 285 μg of protein, and 98 μg of LPS per mg (dry weight). No LPS was detected in fraction II, which implies that fraction II contained less than 0.8 μg of LPS per mg (dry weight).

LPS was extracted from aqueous suspensions of freeze-dried bacteria with 45% (wt/vol) phenol at 70°C (9). The combined aqueous phases were dialyzed against water and subsequently centrifuged for 4 h at 100,000 × *g* at 4°C. The sediment was suspended in sterile water and freeze-dried. The dry material was suspended in sterile water and incubated at 37°C with deoxyribonuclease (0.5 mg/ml; E. Merck AG, Darmstadt, West Germany) for 30 min.

Pancreatic RNase (0.5 mg/ml) and T_1 RNase (200 U/ml) (Boehringer Mannheim Corp., Mannheim, West Germany) were added, and incubation was continued for 60 min. Subsequently, pronase E (0.5 mg/ml; Merck) was added, and incubation was continued for 1 h. The mixture was centrifuged for 4 h at 100,000 × *g* at 4°C, and the suspended pellet was freeze-dried. Treatment with RNase and pronase E was repeated, until the amounts of RNA and protein in the preparation were reduced to 2.4 and 8.4%, respectively. By the determination of KDO, the final preparation was estimated to contain 1,100 μg of LPS per mg (dry weight).

Rabbit antibodies against fraction I (fr. I-Ab) and fraction II (fr. II-Ab). All rabbits were bled from the ear vein before immunization to obtain preimmune sera. Rabbits were injected intracutaneously with 3 mg of fraction I (dialyzed overnight against distilled water) or with 20 mg of fraction II. The fractions were incorporated in 1 volume of Arlacel (1.5 and 2 ml, respectively) containing 200 μg of muramyl dipeptide (Institut Pasteur Production, Paris, France). Booster injections without adjuvant were given at 4 and 5 weeks after the first injection. At 4 days after the last injection, the rabbits were bled by cardiac puncture. Groups of three to four rabbits were used for each fraction, and the immune sera of the individual rabbits in each group were pooled.

The sera were cooled in ice and diluted with 1 volume of phosphate-buffered physiological saline (PBS). Two volumes of saturated $(NH_4)_2SO_4$ solution (pH 7.4) were added slowly. The mixture was stirred for 30 min at 0°C and centrifuged for 10 min at 1,200 × *g*. The sediment was suspended in the original volume of PBS. Subsequently, nine-tenths of this volume of saturated $(NH_4)_2SO_4$ solution was added. The mixture was stirred for 30 min at 4°C and centrifuged, and the sediment was suspended in one-third of the original volume of PBS. The antibody preparation was centrifuged for 15 min at 1,500 × *g* at 4°C to remove aggregated material. The supernatant was dialyzed against PBS for 48 h. The antibody preparation was filtered through a 0.2-μm Sartorius filter and stored in small portions at −20°C.

Decomplementation was performed by heating the antibody preparation at 56°C for 30 min. β-Mercaptoethanol treatment of decomplemented antibody preparations was performed as described by Lieberman et al. (11).

Antisera of mice. Sera were obtained from mice by orbital puncture at 6 days after vaccination. Sera from five mice were pooled for the determination of specific antibodies.

Absorption of rabbit antibodies. Absorption of fr. I-Ab and fr. II-Ab before use in crossed immunoelectrophoresis was performed by incubation for 30 min at 37°C with 1 volume of fraction I (10 mg/ml), fraction II (10 mg/ml), LPS (1 mg/ml), or living *P. aeruginosa* bacteria (2.5×10^9/ml). Precipitates were removed by centrifugation. After absorption with living bacteria, the antibody preparations were filtered through a 0.2-μm Sartorius filter.

Absorption of the antibody preparations before use in the enzyme-linked immunosorbent assay (ELISA) or in passive protection experiments was performed with a one-eighth dilution of decomplemented fr. I-Ab and with undiluted decomplemented fr. II-Ab. One volume of antibody preparation was incubated with 1 volume of fraction II (10 mg/ml), LPS (1, 2, or 4 mg/ml), or killed bacteria (2.5×10^{10}/ml). Bacteria were killed by incubating 19 volumes of bacteria with 1 volume of 35% (wt/wt) formaldehyde for 30 min at room temperature. The bacteria were washed twice with PBS before use.

Crossed immunoelectrophoresis. Crossed immunoelectrophoresis was carried out on glass plates (10 by 10 cm) with a 1.1% agarose gel (Merck) in 0.1 M barbital buffer (pH 8.6) containing 0.01% Merthiolate. The thickness of the gel was 1 mm. The volume of the sample was 10 μl. The first-dimension electrophoresis was carried out at 10 V/cm for 1 h at 12°C. The second-dimension electrophoresis was carried out at 2.5 V/cm for 16 h at 12°C in an agarose gel containing 0.1 ml of the antibody preparation per ml of gel. Each plate was divided in two parts, so that two samples could be run per plate. After the second-dimension electrophoresis, the gel was washed twice for 1.5 h in 0.9% NaCl and subsequently for 1 h in

distilled water. After drying, the plates were stained with 0.5% (wt/vol) Coomassie brilliant blue G (Merck) in ethanol-glacial acetic acid-water (45:10:45) for 15 min. The excess of dye was removed by repeated washings in dye-free solvent.

ELISA. ELISA for the detection of rabbit antibodies to LPS was performed as described by Vos et al. (22) with slight modifications. Coating of the microplates was performed overnight with a final concentration of 2.5 mg of LPS per ml at room temperature. The trays were incubated with serum dilutions for 1 h at 37°C. After washing, the trays were incubated with peroxidase-labeled goat anti-rabbit immunoglobulin G (Nordic Immunological Laboratories, Tilburg, The Netherlands) in a dilution of 1:500 for 1.5 h at 37°C. The substrate 5-aminosalicylic acid was purified by recrystallization after filtration over activated charcoal. The reaction was evaluated visually and expressed as $\log_2$ of the highest dilution giving a positive reaction. The specificity of ELISA was controlled by the use of *Escherichia coli* LPS for the coating of the microplates.

Mouse antibodies to LPS were determined in the same way with peroxidase-labeled sheep anti-mouse immunoglobulin G (Institut Pasteur Production) in a dilution of 1:500.

Passive protection of mice with rabbit antibodies. Mice were injected intraperitoneally with 0.2 ml of serial dilutions of rabbit antibodies in PBS. After 3 h, a lethal challenge with *P. aeruginosa* was given intraperitoneally.

Vaccination and challenge of mice. Mice were vaccinated and challenged as described previously (8) with the following modifications. Fraction II (0.1 ml) was mixed with an equal volume of adjuvant, i.e., a sonicated suspension containing 1.5 mg of dimethyl dioctadecyl ammonium bromide (DDA) per ml of PBS; vaccinations were given intraperitoneally. The challenge dose contained 3.5 50% lethal doses of *P. aeruginosa*. Deaths, which occurred principally within 2 days after challenge, were recorded 7 days after challenge.

PD_{50}. Vaccine doses that protected 50% of the mice against a challenge of 3.5 50% lethal doses (PD_{50}) were determined graphically as described by Nowotony (17).

Treatment of fraction II with RNase and addition of poly(A•U). Before enzymatic treatment, fraction II was dialyzed against 0.005 M ethylenediaminetetraacetic acid (pH 7.2) overnight at 4°C and then dialyzed against distilled water for 1 h. RNase A, ENZYGEL (20 mg; Boehringer Mannheim) was added to 2 mg of fraction II in a total volume of 4.5 ml. The mixture was incubated on a roller mixer for 4 h at 37°C. RNase A, ENZYGEL was removed from the mixture by centrifugation at 100 × *g* for 10 min and washed several times with a total volume of 15.5 ml of water. The wash fluid was added to RNase-treated fraction II. RNase-treated fraction II was subsequently filtered through a 0.2-μm Sartorius filter. Polyadenylic acid-polyuridylic acid [poly(A·U); Boehringer Mannheim] was added to RNase-treated fraction II; this preparation was mixed with DDA. Dialyzed fraction II which was not treated with RNase A, ENZYGEL was used as a control.

Toxicity of vaccines to lead acetate-sensitized mice. Vaccines were dialyzed against distilled water at 4°C overnight. To dilutions of the vaccine lead acetate was added to a final concentration of 20 mg/ml. A volume of 0.1 ml of the vaccine in lead acetate was injected intravenously in the tail vein. Deaths were recorded 7 days after injection. To inject 10 mg of fraction II per mouse, fraction II was concentrated by freeze-drying after dialysis. Lead acetate (0.1 ml) was injected 15 min before the injection of 0.1 ml of fraction II (100 mg/ml).

TD_{50}. Vaccine doses that were lethal to 50% of lead acetate-sensitized mice (TD_{50}) were determined graphically as described by Nowotony (17).

Statistical evaluation. Significance levels for protection were determined by the Fisher exact test, as described by Bradley (4).

RESULTS

Crossed immunoelectrophoresis. Crossed immunoelectrophoresis of fractions I and II against fr. I-Ab and fr. II-Ab, respectively, resulted in different precipitation patterns (Fig. 1A and B). Precipitation patterns of fraction I against fr. II-Ab and of fraction II against fr. I-Ab demonstrated the presence of cross-reacting material in fractions I and II (Fig. 1C and D). fr. I-Ab contained antibodies precipitating with LPS (Fig. 1E). No precipitation was observed when LPS was run against fr. II-Ab (Fig. 1F). Absorption of fr. I-Ab with fraction I (5 mg/ml) resulted in the complete disappearance of the precipitation pattern of fraction I against absorbed fr. I-Ab (Fig. 2A). After absorption of fr. II-Ab with fraction II (5 mg/ml), only one weak precipitation line was visible when fraction II was run against absorbed fr. II-Ab (Fig. 2B). Part of the precipitation pattern of fraction I against fr. I-Ab disappeared when fr. I-Ab were absorbed with LPS or with whole cells of *P. aeruginosa* (Fig. 2C and E). No loss of precipitation lines from the precipitation pattern of fraction II against fr. II-Ab was observed when fr. II-Ab were absorbed with LPS or with bacterial cells (Fig. 2D and F).

Detection of antibodies to LPS by ELISA. By means of ELISA, antibodies to LPS could be detected in fr. I-Ab and in fr. II-Ab (Table 1). The concentration of antibodies to LPS in fr. I-Ab was 8 to 16 times higher than that in fr. II-Ab. Absorption of fr. I-Ab and fr. II-Ab with increasing concentrations of LPS resulted in a gradual decrease in the titer of antibodies against LPS. Also, absorption of fr. I-Ab and fr. II-Ab with whole cells of *P. aeruginosa* reduced the titer of antibodies to LPS. Absorption of fr. II-Ab with fraction II did not reduce the titer of antibodies to LPS.

Passive protection of mice with fr. I-Ab and fr. II-Ab. fr. I-Ab and fr. II-Ab protected

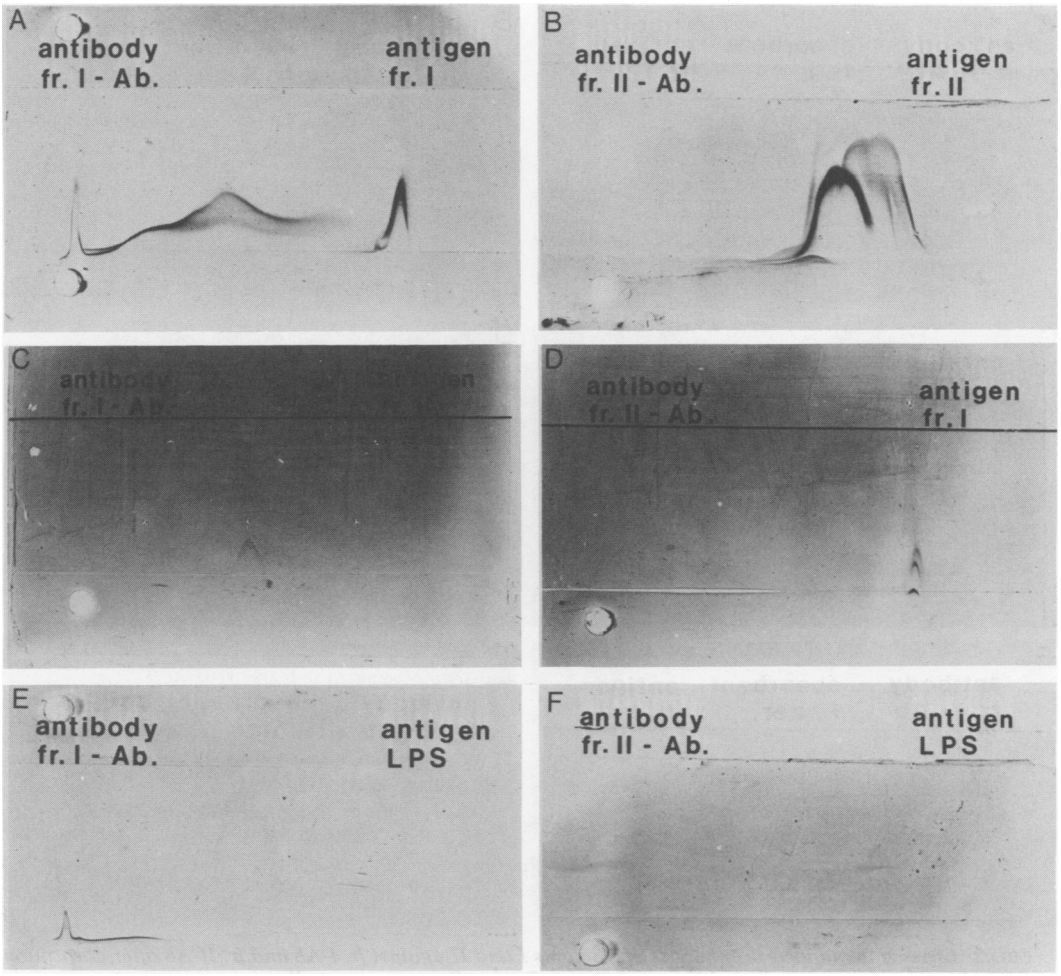

FIG. 1. *Crossed immunoelectrophoresis of fraction I, fraction II, and LPS against fr. I-Ab and fr. II-Ab.*

mice against a lethal challenge with *P. aeruginosa* (Table 2). To obtain similar percentages of survival, concentrations of fr. II-Ab about 10-fold higher than those of fr. I-Ab were required. Decomplementation of the antibody preparations by heating for 30 min at 56°C reduced the protection by the 1:10 dilution of fr. II-Ab (*P* value with respect to the corresponding dilution of untreated fr. II-Ab was 0.03). Treatment of decomplemented fr. I-Ab and fr. II-Ab with β-mercaptoethanol demonstrated that immunoglobulin M antibodies did not contribute to the protection.

Passive protection experiments with absorbed decomplemented antibodies were performed to study the specificity of the protective antibodies in fr. I-Ab and fr. II-Ab. Preliminary experiments indicated that LPS was impracticable for absorbing the antibody preparations which had to be used in passive protection experiments. Part of the LPS remained in the antibody preparation and influenced the outcome of the passive protection experiments due to its toxicity. Therefore, absorption of the antibodies was performed with whole cells of *P. aeruginosa*. The results demonstrated that protection by Fr. I-Ab and fr. II-Ab was due to antibodies against cell envelope components (Table 3). Absorption of fr. II-Ab with fraction II did not reduce the protection by fr. II-Ab.

Antibodies to LPS in mice induced by different doses of LPS, fraction I, and fraction II. The titers of antibodies to LPS in sera of mice and the percentages of survival of mice that were vaccinated with graded doses of LPS, fraction I, and fraction II are shown in Table 4. By means of ELISA, antibodies to LPS were detected at 6 days after a single vaccination with LPS, fraction I, or fraction II. Increasing doses of vaccine were associated with increasing titers

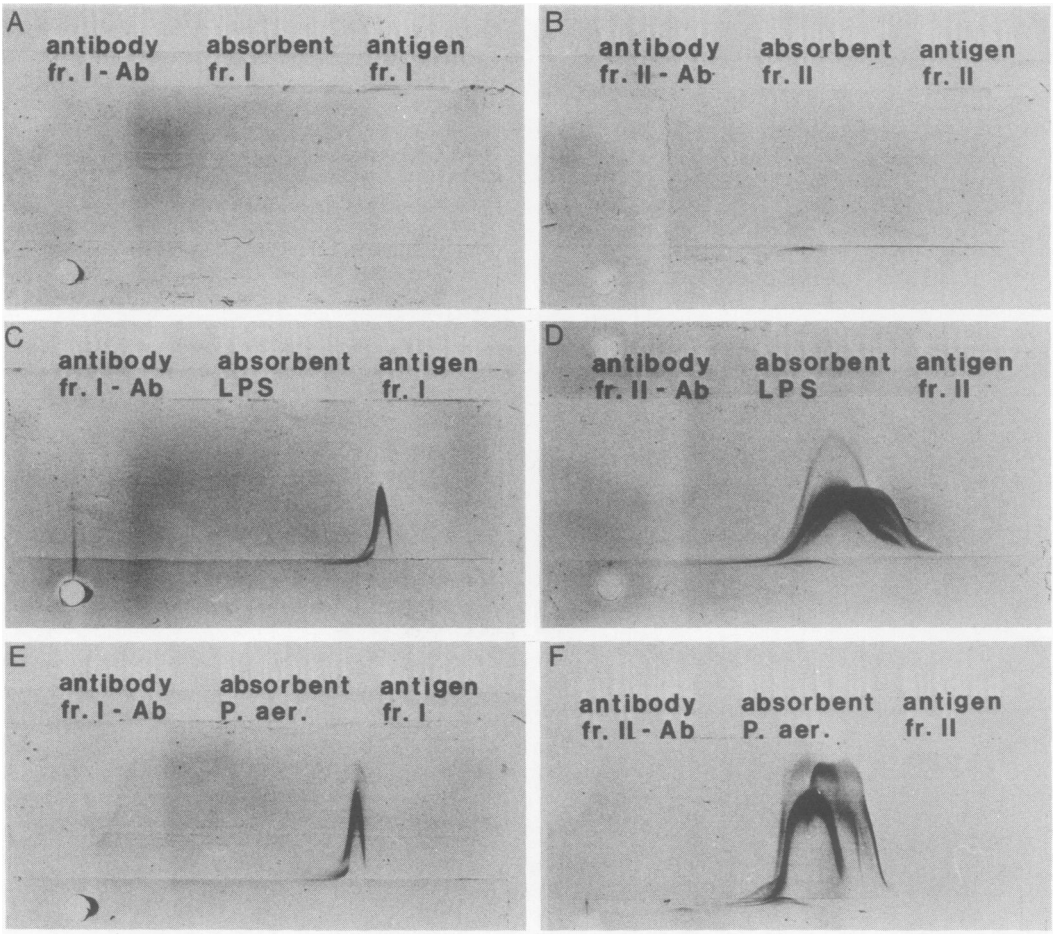

FIG. 2. *Crossed immunoelectrophoresis of fractions I and II against fr. I-Ab and fr. II-Ab after absorption of the antibody preparations with different antigens. P. aer., P. aeruginosa cells.*

of antibodies and increasing percentages of mouse survival.

When the percentages of survival were plotted as a function of the corresponding titers of antibodies to LPS, it appeared that a similar correlation existed between survival and titers of antibodies to LPS induced by fraction I or LPS (Fig. 3). The results suggested that protection by fraction II was associated with lower titers of antibodies to LPS than was protection by fraction I or LPS.

Since in fractions I and II the presence of LPS was demonstrated by the ability of the fractions to induce antibodies to LPS, it was of interest to determine whether the protective capacity of fraction I, fraction II, and LPS was correlated with the concentration of LPS in these vaccines. The PD_{50} values of the vaccines were calculated from the results shown in Table 4. LPS was estimated by the determination of KDO. It appeared that the PD_{50} of the purified LPS vaccine contained 24 times more LPS than did the PD_{50} of fraction I (Table 5). As LPS could not be detected in fraction II by the determination of KDO, the amount of LPS in the PD_{50} of fraction II could only be estimated to be less than 1.3 ng.

Restoration of the protective activity of RNase-treated fraction II by the addition of poly(A•U). Incubation of fraction II with RNase A, ENZYGEL reduced the protective activity of this vaccine (Table 6). Different amounts of poly(A·U) were added to RNase-treated fraction II in an attempt to restore the protective activity of this ribosomal vaccine. Addition of 50 or 300 μg of poly(A·U) to RNase-treated fraction II increased the protective activity significantly. Injection of 300 μg of poly(A·U) alone also induced protection.

Toxicity of LPS, fraction I, and fraction II for lead acetate-sensitized mice. Selye et

TABLE 1. *Quantification of rabbit antibodies to LPS by ELISA in fr. I-Ab and fr. II-Ab before and after absorption with LPS, P. aeruginosa, or fraction II*

Antibody prepn	Absorbent (per ml)	Titer (log_2) of antibodies to LPS
fr. I-Ab		14
fr. I-Ab[a]	0.5 mg of LPS	8.5
fr. I-Ab[a]	1.0 mg of LPS	7
fr. I-Ab[a]	2.0 mg of LPS	6
fr. I-Ab[a]	1.25×10^{10} *P. aeruginosa*	12
Preimmune antibodies		6.5
fr. II-Ab		10.5
fr. II-Ab	0.5 mg of LPS	8
fr. II-Ab	1.0 mg of LPS	7
fr. II-Ab	2.0 mg of LPS	6
fr. II-Ab	1.25×10^{10} *P. aeruginosa*	8.5
fr. II-Ab	5 mg of fraction II	10
Preimmune antibodies		6

[a] fr. I-Ab were diluted 1:8 before absorption.

TABLE 2. *Passive protection of mice by fr. I-Ab and fr. II-Ab*

Antibody prepn	Survival (%) after following treatment[a]:		
	None	56°C	56°C, β-ME
fr. I-Ab[b] at dilution of:			
1:20	75	85	75
1:50	60	55	60
1:100	40	50	15
Preimmune antibodies (1:20 dilution)	10	5	ND[d]
PBS	0		
fr. II-Ab[c] at dilution of:			
1:2	83	83	80
1:5	80	60	55
1:10	60	33	25
Preimmune antibodies (1:2 dilution)	20	17	ND[d]
PBS	7		

[a] fr. I-Ab and fr. II-Ab were applied without treatment and after heating for 30 min at 56°C or after heating and treatment with β-mercaptoethanol (β-ME).

[b] Groups of 20 mice were injected intraperitoneally with 0.2 ml of different dilutions of fr. I-Ab and challenged 3 h later with a lethal dose of *P. aeruginosa*.

[c] Groups of 30 mice were injected intraperitoneally with 0.2 ml of different dilutions of fr. II-Ab and challenged 3 h later with a lethal dose of *P. aeruginosa*. In the case of injection with fr. II-Ab which were heated at 56°C and treated with β-ME, only 20 mice per group were used.

[d] ND, Not determined.

al. (18) demonstrated that intravenous injection of lead acetate increased the susceptibility of rats to the toxicity of LPS. Misfeldt and Johnson (13) applied this method to mice. Graded doses of LPS, fraction I, and fraction II were injected into lead acetate-sensitized mice to determine the toxicity of these vaccines. This toxicity is likely to be due to the LPS in the vaccine. The TD_{50} of the vaccines was calculated, and the concentration of LPS in the TD_{50} was determined by an independent method (Table 7). The concentration of LPS in the TD_{50} of fraction I and that in the TD_{50} of the purified LPS preparation were about the same (2.9 and 2.1 μg, respectively). No LPS was detectable in fraction II, indicating that less than 1.6 μg of LPS is present in the TD_{50} of fraction II.

Ratio of the PD_{50} to the TD_{50} of LPS, fraction I, and fraction II. The ratio of the protective capacity of a vaccine to its toxicity has been used as a measure for its therapeutic quality (2). The lower the PD_{50}/TD_{50} ratio, the less toxic is the vaccine dose needed to achieve a defined level of protection. The ratio of the PD_{50} to the TD_{50} for lead acetate-sensitized mice of LPS, fraction I, and fraction II is shown in Table 8.

As judged by the PD_{50}/TD_{50} ratio, the therapeutic quality of LPS is inferior to that of fractions I and II. The PD_{50}/TD_{50} ratio of fraction II is somewhat higher than that of fraction I.

DISCUSSION

Conflicting results have been reported regarding the nature of the immunogen(s) in ribosomal vaccines. Recently, several authors concluded that the protection induced by their ribosomal vaccine was due to contaminating cell envelope components (7, 15); no evidence was found for a function of the ribosomes in these vaccines. In contrast, a purified ribosomal vaccine (fraction II) of *P. aeruginosa*, of which the protective activity was destroyed by treatment of the vaccine with RNase, has been described (8). Since the protective activity of this vaccine was dependent on the presence of intact RNA, RNA may have been the immunogen in the ribosomal vaccine. Alternatively, RNA could have acted as a carrier or an adjuvant for traces of contaminating cell surface antigens. No cell envelope components, in particular, no LPS, were detected in fraction II by means of chemical anal-

TABLE 3. *Passive protection of mice by fr. I-Ab and fr. II-Ab absorbed with P. aeruginosa or fraction II*

Antibody prepn		Survival (%)[a] of mice protected with antibody prepn[b, c] absorbed with:		
Type	Dilution	PBS	*P. aeruginosa* (1.25×10^{10}/ml)	Fraction II (5 mg/ml)
fr. I-Ab	1:20	85	15[d]	
fr. I-Ab	1:50	55	10[d]	
PBS		0		
fr. II-Ab	1:2	69	0[d]	100
fr. II-Ab	1:5	47	0[d]	55
PBS		3		

[a] Groups of 20 mice were injected intraperitoneally with 0.2 ml of different dilutions of fr. I-Ab and fr. II-Ab and challenged 3 h later with a lethal dose of *P. aeruginosa*.

[b] fr. I-Ab and fr. II-Ab were heated for 30 min at 56°C before use.

[c] fr. I-Ab were diluted 1:8 before absorption.

[d] P value is <0.01 with respect to corresponding dilutions of the antibody preparations that were not absorbed.

ysis (8). In this paper, an analysis of antisera to fraction II was presented which demonstrated the presence of LPS in the ribosomal vaccine.

Crossed immunoelectrophoresis with fr. II-Ab demonstrated the presence of many different antigens in the ribosomal fraction. Some ribosomal antigens cross-reacted with antibodies to fraction I, a fraction which contained cell envelope components. Antibodies to LPS could not be detected in fr. II-Ab by crossed immunoelectrophoresis. However, antibodies to LPS were detected in fr. II-Ab by ELISA. When fr. II-Ab were absorbed with fraction II, the antibodies which precipitated with the ribosomal antigens in crossed immunoelectrophoresis disappeared (Fig. 2B). In contrast, the titer of antibodies to LPS in fr. II-Ab was not reduced by this treatment (Table 1). Thus, LPS was present in the ribosomal vaccine, but the amount of LPS in fraction II was not sufficient to reduce the titer of antibodies to LPS.

Mice could be protected by fr. I-Ab and fr. II-Ab against a lethal challenge with *P. aeruginosa*. Absorption of fr. II-Ab with *P. aeruginosa* cells and with fraction II demonstrated that the protection by fr. II-Ab was due to antibodies to cell envelope antigens. Antibodies to ribosomal antigens did not contribute to the protection. Although it is likely that the protective activity of fr. I-Ab and fr. II-Ab is mainly due to the antibodies to LPS, it cannot be excluded that antibodies to other cell envelope components contributed to the protection. In this study, LPS was considered to be an indicator for the presence of cell envelope components.

TABLE 4. *Effect of vaccine dose on protection and $\log_2$ titers of antibodies to LPS induced in mice by LPS, fraction I, and fraction II*

Vaccine	Dose (ng)	Survival (%)	Titer ($\log_2$) of antibodies to LPS[a]
LPS	0.1	11 (27)[b]	2.8
	1.0	22 (27)	5.8
	10.0	41 (27)	6.8
	100.0	75 (20)	8.8
Fraction I	0.1	5 (20)	4.0
	1.0	19 (27)	5.4
	10.0	41 (27)	7.6
	100.0	85 (27)	9.3
	1,000.0	100 (20)	9.6
Fraction II, plus DDA	10.0	0 (27)	1.2
	100.0	4 (27)	2.5
	1,000.0	37 (27)	4.5
	10,000.0	90 (20)	7.3
Buffer		0 (28)	1.3
Buffer plus DDA		7 (28)	2.5

[a] Determined by ELISA. Each number represents the mean value of determinations of three pools of sera.

[b] Number of mice is given within parentheses.

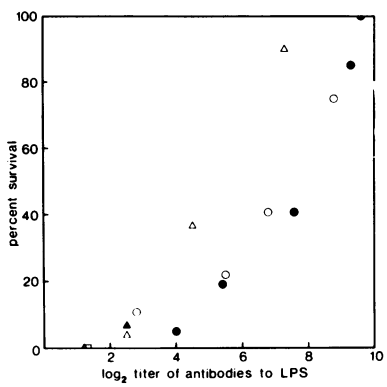

FIG. 3. *Protection of mice (percent survival) induced by LPS, fraction I, and fraction II as a function of the corresponding $\log_2$ titer of antibodies to LPS. Symbols: ○, LPS; ●, fraction I; △, fraction II plus DDA; □, buffer; ▲, buffer plus DDA.*

In mice, fraction I, fraction II, and LPS induced antibodies to LPS at 6 days after a single vaccination. Increasing doses of vaccine were

TABLE 5. *Comparison of the PD_{50}s of LPS, fraction I, and fraction II and the concentration of LPS in the PD_{50}*

Vaccine	PD_{50} (ng)[a]	LPS (ng) in PD_{50}[b]
LPS	22	24
Fraction I	10	1
Fraction II plus DDA	1,600	<1.3

[a] The values are calculated from the results shown in Table 4.

[b] The concentration of LPS was estimated by the determination of KDO.

TABLE 6. *Restoration of the protective activity of RNase-treated fraction II by the addition of poly(A·U)*

Vaccine	Dose of poly(A·U) added to the vaccine	Survival (%)[a]	*P* value
Fraction II (3 μg) plus DDA	0	50	
Fraction II (3 μg) treated with RNase[b] plus DDA	0	17	<0.01[c]
Fraction II (3 μg) treated with RNase plus DDA	5	17	0.63[d]
Fraction II (3 μg) treated with RNase plus DDA	50	67	<0.01[d]
Fraction II (3 μg) treated with RNase plus DDA	300	83	0.04[e]
Buffer plus DDA	5	3	
Buffer plus DDA	50	3	
Buffer plus DDA	300	60	
Buffer plus DDA	0	0	

[a] Each value was determined after challenging 30 mice.

[b] RNase, RNase A, ENZYGEL.

[c] Calculated with respect to fraction II plus DDA which was not treated with RNase.

[d] Calculated with respect to fraction II treated with RNase plus DDA without poly(A·U) added to the vaccine.

[e] Calculated with respect to buffer plus DDA to which 300 μg of poly(A·U) was added.

associated with increasing titers of antibodies to LPS and increasing percentages of mouse survival. It is concluded that traces of LPS which are, at least in part, responsible for the protective activity of fraction II by the capacity to induce antibodies to LPS are present in the ribosomal vaccine.

As treatment of fraction II with RNase reduced the protective activity of the ribosomal vaccine, RNA might function as an adjuvant or a carrier in the presentation of the contaminating cell surface antigens. The reduced protective activity of RNase-treated fraction II could be restored by the addition of poly(A·U). This result indicated that a synthetic polyribonucleotide could replace the adjuvant function of the ribosomal RNA and restore the capacity of cell envelope antigens to induce protective antibodies.

TABLE 7. *Toxicity of LPS, fraction I, and fraction II in lead acetate-sensitized mice*

Vaccine[a]	Dose (μg)	Survival (%)	TD_{50} (μg)	LPS (μg) in TD_{50}[b]
LPS	10	31 (35)[c]		
	1	57 (45)	1.9	2.1
	0.1	79 (34)		
Fraction I	100	38 (47)		
	10	60 (57)	29.5	2.9
	1	87 (32)		
Fraction II	10,000	10 (20)		
	1,000	71 (51)	1,995.3	<1.6
	100	82 (45)		
Control		95 (59)		

[a] Mice were injected intravenously with 2 mg of lead acetate mixed with the vaccine.

[b] The concentration of LPS was estimated by the determination of KDO.

[c] Number of mice is given within parentheses.

TABLE 8. *Ratio of PD_{50} to TD_{50} of LPS, fraction I, and fraction II*

Vaccine	PD_{50}/TD_{50}[a]
LPS	1.2×10^{-2}
Fraction I	3.4×10^{-4}
Fraction II	8.0×10^{-4}

[a] The values of PD_{50} and TD_{50} were presented in Tables 5 and 7, respectively.

Concerning the role of RNA in the ribosomal vaccine, two observations must be discussed. The results shown in Fig. 3 suggested that protection by fraction II was associated with lower titers of antibodies to LPS than was protection by fraction I and LPS. This might indicate that an additional protective mechanism contributed to the protective activity of fraction II. It was also observed that a high dose of poly(A·U) (300 μg) protected mice against a lethal challenge with *P. aeruginosa* (Table 6). Similar observations were made by Weinstein et al. (23), who found that injections of mice with polyinosinic acid-polycytidylic acid increased their resistance to bacterial infections. Preliminary experiments have demonstrated that RNA isolated from the

ribosomal vaccine protected mice against a *Pseudomonas* infection, whereas no antibodies to LPS were detected in the sera of these animals. Thus, there are several indications that the protective activity of the ribosomal vaccine is not due only to its capacity to induce antibodies to LPS.

As the serotype-specific protection by fraction I (8) was likely due to the LPS in this fraction, it was a surprise to find a discrepancy between the concentrations of LPS in the PD_{50} of fraction I and in the PD_{50} of the purified LPS preparation (1 and 24 ng of LPS, respectively). This discrepancy might be due to the presence of different immunogens in fraction I. However, the titers of antibodies to LPS induced by fraction I and the LPS vaccine were associated with similar percentages of survival (Fig. 3). Therefore, it is unlikely that protection induced by fraction I is primarily due to antibodies against non-LPS-like antigens. Another explanation could be that the immunogenicity of the purified LPS vaccine was decreased by the purification method. Skidmore et al. (19) and Morrison and Leive (16) have reported that several properties of LPS were affected when phenol extraction was used in comparison with milder extraction procedures.

Low toxicity has been mentioned as one of the attractive prospects of ribosomal vaccines, as these vaccines might contain low concentrations of toxic cell envelope components (10). However, only a few investigators have determined the toxicity of their ribosomal vaccine (2). Trial experiments have pointed out that toxic effects of the vaccines used in this study, including LPS, could be determined only when extremely high concentrations were applied. Therefore, the concentration of endotoxin in fractions I and II has been tested in lead acetate-sensitized mice since these were reported to have an increased sensitivity to the toxic effects of LPS (18). The toxicity of non-LPS-like materials in the vaccines was considered negligible. The TD_{50}s of the purified LPS vaccine, fraction I, and fraction II were estimated to contain 2.1, 2.9, and less than 1.6 μg of LPS, respectively, by an independent method for a determination of LPS. Thus, the toxicity of fraction I was in agreement with the concentration of LPS in this fraction. If the concentration of LPS in the TD_{50} of fraction II is near 1.6 μg, the toxicity of fraction II could be due to the LPS in this fraction. However, high quantities of material had to be injected intravenously to reach a toxic dose of fraction II, which might have influenced the determination of the TD_{50} of the ribosomal vaccine.

Whereas the concentration of LPS in the PD_{50} of the purified LPS vaccine was 24-fold higher than that in the PD_{50} of fraction I, the concentration of LPS in the TD_{50} of fraction I and that in the purified LPS vaccine were similar. If the former result is due to a decrease in the immunogenicity of purified LPS by the extraction method, the latter result implies that the toxicity of LPS is not decreased proportionally by the extraction. Since the toxicity of LPS is associated with the lipid A part of the molecule, whereas the carbohydrate side chains carry the serotype-specific O antigens, toxicity and immunogenicity may have been affected differently by the phenol extraction.

Calculation of the PD_{50}/TD_{50} ratio revealed that protection by fraction I was associated with the lowest toxicity for lead acetate-sensitized mice. This ratio was 2.4-fold higher for fraction II and 35-fold higher for the LPS vaccine. Thus, under these experimental conditions, protection by the ribosomal vaccine is associated with a slightly higher toxicity than is protection by fraction I. The purified LPS preparation is the most toxic vaccine.

Lieberman has prepared a purified ribosomal vaccine of *P. aeruginosa*, peak B, which was shown to be free of LPS by the use of labeled LPS and by chemical means (10). This vaccine induced mouse protective antibodies in rabbits (11). The nature of the antigens to which these protective antibodies were directed was not clarified. No evidence was found that antibodies to LPS were present in the antiserum to peak B by means of a passive hemagglutination assay. However, the protection by the ribosomal vaccine, as well as the passive protection of mice by the antiserum to the ribosomal vaccine, was reported to be serotype specific (10, 11). In view of our experience with the determination of antibodies to LPS, it is questionable whether the method used by Lieberman et al. for detecting antibodies to LPS in antiserum to peak B has been sensitive enough to exclude the presence of antibodies to LPS in this antiserum.

Different opinions have been expressed concerning the role of RNA in RNase-sensitive vaccines. Youmans and Youmans (25) concluded that RNA was the immunogenic principle in the ribosomal vaccine of *M. tuberculosis*. Araujo and Remington (3) found that protection against *Toxoplasma gondii* could be induced with RNA isolated from *Toxoplasma* cells, as well as with RNA from different sources, including synthetic polynucleotides. Thus, RNA might nonspecifically enhance resistance against *Toxoplasma*. Andron and Eigelsbach (1) demonstrated that an RNase-sensitive RNA vaccine of *Francisella tularensis* induced opsonizing antibodies in mice. Also, Thompson and Eisenstein (20) found that an RNase-sensitive subcellular preparation

of *Streptococcus pneumoniae* induced protective antibodies, which could be absorbed with the whole bacterial cells. These data suggested that RNA might play a role in the presentation of somatic antigens.

The results presented in this paper confirmed the latter observations. The purified ribosomal vaccine of *P. aeruginosa* has the capacity to induce antibodies to LPS, and the protective activity of this vaccine is sensitive to RNase, suggesting that the ribosomes have the function of a carrier or an adjuvant in the presentation of the cell surface antigens.

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