INFECTION AND IMMUNITY, July 1981, p. 178-185
0019-9567/81/070178-08$02.00/0

Vol. 33, No. 1

Serotype-Nonspecific Protection Induced by Ribonucleic Acid Isolated from the Ribosomal Vaccine of *Pseudomonas aeruginosa*

RINSKE GONGGRIJP,* WIL J. H. A. MULLERS, AND C. P. A. VAN BOVEN

Department of Medical Microbiology, State University of Limburg, Maastricht, The Netherlands

Received 27 January 1981/Accepted 17 April 1981

A ribosomal vaccine of *Pseudomonas aeruginosa* and a vaccine containing purified lipopolysaccharide (LPS) were compared with respect to their capacity to protect mice against a lethal challenge with *P. aeruginosa*. The route of vaccination appeared to be important for the protective activity of the ribosomal vaccine. Optimal protection was measured if both the immunizing and the challenge injection were given intraperitoneally. The ribosomal vaccine protected mice as early as 1 day after vaccination, and the protection lasted at least 6 days. LPS-specific antibodies were detectable 6 but not 2 days after vaccination. The ribosomal vaccine protected mice also against a heterologous serotype of *Pseudomonas*. Injection of purified LPS did not protect mice earlier than at day 3, and the protection induced by LPS was serotype specific. Ribonucleic acid (RNA) isolated from the ribosomal vaccine had the same protective properties as the ribosomes. RNA induced serotype-nonspecific protection as quickly as 1 day after injection, and the protection lasted at least 6 days. However, the capacity to induce antibodies to LPS was lost or reduced. It is concluded that the serotype-nonspecific protection induced by RNA and the serotype-specific protection induced by LPS are due to different mechanisms. Experiments with combined vaccines containing RNA and LPS demonstrated that the addition of RNA to LPS resulted in a slight increase in LPS-specific antibodies. The data presented indicate that both the serotype-specific protection induced by LPS and the serotype-nonspecific protection induced by RNA contribute to the protective activity of the ribosomal vaccine.

Protective vaccines have been prepared from the ribosomes or ribosomal extracts of many different microorganisms, including *Mycobacterium tuberculosis, Salmonella typhimurium, Streptococcus pneumoniae* (5), and *Pseudomonas aeruginosa* (9, 16). The diversity of the microorganisms from which protective ribosomal preparations could be prepared has raised the question of whether the ribosomes have a unique function in the induction of an immune response. However, several investigators recently concluded that the protective activity of their ribosomal vaccines was due to contaminating cell envelope antigens (6, 17, 18). Only a few experiments with ribosomal vaccines yielded evidence to support the idea that the ribosomes are required for the protective activity of the ribosomal vaccines (1, 2, 22).

In previous reports, a ribosomal vaccine of *P. aeruginosa* (fraction II) was described, the protective activity of which was decreased by treatment with ribonuclease (RNase) (9). This ribosomal vaccine also induced protective antibodies to lipopolysaccharide (LPS) (10), which pointed to the presence of contaminating cell envelope components. Since the RNase sensitivity of the ribosomal vaccine indicated that ribonucleic acid (RNA) was required for the protective activity of this vaccine, it was suggested that the ribosomes or RNA might act as a carrier or an adjuvant in the presentation of traces of the contaminating cell envelope antigens.

In this paper, experiments to elucidate the role of RNA are described. The ribosomal vaccine and a purified LPS vaccine were compared. The conditions for optimal protection and the specificity of the protection were determined. It is shown that the ribosomal vaccine induced serotype-nonspecific protection as early as 1 day after vaccination. Similar results were obtained with RNA isolated from the ribosomal vaccine. In addition, the protective activity of combined vaccines containing RNA and LPS were compared with the protective activity obtained with RNA or LPS alone.

MATERIALS AND METHODS

Bacteria. *P. aeruginosa* serotype 3 and serotype 8

(strain RIV 76-5321 and 76-5309, respectively [9]) were used throughout for the preparation of vaccines and as challenging organisms.

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.

Vaccines. The ribosomal vaccine (fraction II) was obtained from a crude ribosomal preparation by molecular sieve chromatography on Sepharose Cl-2B as described previously (9). Four batches were pooled and further used as the ribosomal vaccine. The amount of RNA was estimated by the orcinol method of Herbert et al. (13), using yeast RNA (Boehringer Mannheim Corp., Mannheim, West Germany) as the standard. The concentration of protein was determined by the Folin method modified by Hartree (12) with bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as the standard. LPS was estimated by determination of the 2-keto-3-deoxyoctonate (KDO) as follows. A sample (4 mg, dry weight) was hydrolyzed with 0.014 N H_2SO_4 for 20 min at 100°C. KDO was determined by the thiobarbituric acid method of Weissbach and Hurwitz (21) with KDO (Sigma Chemical Co.) as the standard. The amount of LPS was calculated by assuming that LPS contained 5% KDO (14). The lower limit of detection was 3 μg of LPS. The ribosomal vaccine contained 632 μg of RNA and 345 μg of protein per mg (dry weight). No LPS was detected in this preparation, which implies that the ribosomal vaccine contained less than 0.8 μg of LPS per mg (dry weight) (9). LPS of serotype 3 was extracted and purified as described previously (10). LPS of serotype 8 was extracted according to Galanos et al. (8) and purified by treatment with deoxyribonuclease, RNase, and pronase E as described for LPS of serotype 3 (10). The LPS of serotype 3 contained 24 μg of RNA, 80 μg of protein, and 1,100 μg of LPS per mg (dry weight), whereas the LPS of serotype 8 contained 47 μg of RNA, 100 μg of protein, and 840 μg of LPS per mg (dry weight).

Isolation of RNA and protein from the ribosomal vaccine. RNA was extracted from the ribosomal vaccine with phenol and sodium dodecyl sulfate and purified as described by Poulson (19). The final RNA preparation contained 5 mg of RNA per ml and 0.042 mg of protein per ml. The recovery of RNA was 71%. Protein was isolated from the ribosomal vaccine by extraction with acetic acid by the method of Hardy et al. (11). The protein supernatants were dialyzed against water overnight at 4°C. The final preparation contained 173 μg of protein per ml, and 76% of the protein was recovered. No RNA was detectable in this preparation.

Vaccination and challenge. *P. aeruginosa* serotype 3 was the source of the vaccines and the challenging organism, unless otherwise stated. Mice were vaccinated intraperitoneally. In the first experiment, the ribosomal vaccine was also injected subcutaneously. Where indicated, vaccines were mixed with an equal volume (0.1 ml) of the adjuvant dimethyl dioctadecyl ammonium bromide (DDA), which was sonicated in phosphate-buffered saline (PBS) before use (1.5 mg of DDA per ml of PBS). The preparation of the challenge and the determination of the 50% lethal dose (LD_{50}) of *P. aeruginosa* were described previously (9). Mice were challenged intraperitoneally with 3.5 LD_{50} of *P. aeruginosa* serotype 3. In case of a challenge with *P. aeruginosa* serotype 8, the challenge dose contained 3 LD_{50}. The LD_{50} of *P. aeruginosa* 5321 (serotype 3) was 2×10^7 bacteria. The LD_{50} of *P. aeruginosa* 5309 (serotype 8) was 1.5×10^7 bacteria. Whenever different challenging doses were applied, it is noted in Results. Deaths, which occurred principally within 2 days after challenge, were recorded 7 days after challenge.

Antibodies to LPS. Antibodies to LPS were determined by an enzyme-linked immunosorbent assay (ELISA) as described previously (10). The concentration of antibodies to LPS in a serum was expressed as the $\log_2$ of the highest dilution of the serum giving a positive reaction.

Statistical evaluation. The significance level (P) for protection was determined by the Fisher exact test, as described by Bradley (3). Determination of the one-side significance level (p) in the paired comparison of antibody titers induced by different vaccines was performed by the Wilcoxon signed-rank test (15). The data concerning the protection induced by combined vaccines containing RNA plus LPS and by vaccines containing only RNA or LPS were analyzed to test the hypothesis that the protection by LPS acted additively to the protection by RNA. The data were represented by a three-dimensional frequency table for the variables survival, LPS dose, and RNA dose, and were analyzed by the log-linear model with only first-order interactions present (7). The significance level of the Pearson chi-square statistic (goodness of fit for the model) is indicated as Q.

RESULTS

Effect of vaccination route on the protective activity of the ribosomal vaccine. Mice were vaccinated either subcutaneously or intraperitoneally with different doses of the ribosomal vaccine with or without the adjuvant DDA. Six days after vaccination, all mice were challenged intraperitoneally with a lethal dose of *P. aeruginosa*. Intraperitoneal vaccination with 5 or 20 μg of the ribosomal vaccine resulted in significantly higher percentages of survival than subcutaneous vaccination (Table 1). The adjuvant DDA enhanced the protective activity of subcutaneously as well as intraperitoneally injected ribosomes.

Effect of interval between vaccination and challenge on the protective activity of the ribosomal vaccine and of LPS. Mice were vaccinated with the ribosomal vaccine or with LPS at various intervals before the challenge was given (Fig. 1). The results indicated that the ribosomal vaccine protected mice as early as 1 day after vaccination, whereas with LPS the same level of protection was reached only 3 days after immunization. The protection by the ribosomal vaccine or by LPS lasted about 9 days.

Antibodies to LPS induced by the ribosomal vaccine or by LPS at 2 or 6 days after

TABLE 1. *Effect of vaccination route on the protective activity of the ribosomal vaccine*

Vaccine		Route of vaccination[a]	Survival[b] (%)	*P* value[c]
Type	Dose (μg)			
Ribosomes	5	s.c.	5	
		i.p.	45	<0.01
Ribosomes + DDA	5	s.c.	5	
		i.p.	85	<0.01
Ribosomes	20	s.c.	20	
		i.p.	55	0.02
Ribosomes + DDA	20	s.c.	45	
		i.p.	95	<0.01
Buffer + DDA		s.c.	3	
		i.p.	0	

[a] s.c., Subcutaneous; i.p., intraperitoneal.
[b] Groups of 20 mice were vaccinated as indicated and challenged 6 days later.
[c] *P* values with respect to subcutaneously injected vaccine were calculated by the Fisher test.

vaccination. The titer of LPS-specific antibodies was determined in the sera of mice injected intraperitoneally with the ribosomal vaccine or with LPS. Neither the ribosomal vaccine nor the LPS vaccine induced detectable antibodies to LPS 2 days after vaccination. After 6 days, both vaccines induced LPS-specific antibodies (Table 2).

Serotype specificity of the protection induced by the ribosomal vaccine or by LPS. Mice were vaccinated with the ribosomal vaccine or with LPS derived from *P. aeruginosa* serotypes 3 and 8. Two or six days after vaccination, all mice were challenged with serotype 8 *Pseudomonas* (Table 3). The ribosomal vaccines from both serotypes protected the mice to the same extent at 2 days after vaccination, whereas no protection was obtained with LPS. Six days after vaccination, only the LPS derived from the homologous serotype protected the mice, whereas both of the ribosomal vaccines induced significant protection. Thus, at 2 days as well as at 6 days after vaccination, the ribosomal vaccine derived from serotype 3 protected mice against a heterologous challenge. Six days after vaccination, the protection afforded by the homologous ribosomal vaccine may have been better than the protection by the heterologous ribosomal vaccine, although the difference between the percentages of survival was not statistically significant ($P = 0.1$).

Protective activity of RNA and protein isolated from the ribosomal vaccine. To obtain information about the nature of the protective components in the ribosomal vaccine, the protective activity of RNA and protein isolated from the vaccine was tested. RNA protected mice against a lethal challenge with *P. aeruginosa* (Table 4). The amount of RNA required for protection corresponded to the amount of RNA in protective doses of the ribosomal vaccine. Also, RNA protected mice as early as 1 day after vaccination (Fig. 1). Protein isolated from the ribosomal vaccine did not protect mice, whether it was combined with the adjuvant DDA or not. Protection by the ribosomal vaccine 6 days after vaccination was associated with the presence of antibodies to LPS (Table 2; 10). In contrast, RNA protected mice 6 days after vaccination, whereas no antibodies to LPS were detectable in their sera (Table 4). The protection by RNA was not serotype specific: RNA derived from seroptye 8 *Pseudomonas* protected mice against a challenge with serotype 3 *Pseudomonas* (Tables 5 and 6).

Protective activity and antibodies to LPS induced by combined vaccines containing RNA and LPS. To investigate whether RNA could potentiate the immune response to LPS, different amounts of LPS were added to RNA vaccines. The titers of antibodies to LPS and the protection induced by vaccines containing RNA plus LPS were compared with the titers of antibodies and the protection induced by vaccines containing LPS or RNA alone. All of the vaccines were incorporated in DDA.

The titers of LPS-specific antibodies which were induced by RNA plus LPS were slightly increased compared with those induced by LPS alone (Tables 5 and 6). Determination of the one-side significance level pointed out that a positive effect of RNA on the induction of LPS-specific antibodies cannot be neglected ($p < 0.06$ for titers presented in Table 5 and $p < 0.05$ for titers presented in Table 6).

The protection induced by combined vaccines containing LPS from the same serotype as the challenging organism in addition to RNA from either serotype was higher than the protection induced by RNA or LPS alone (Tables 5 and 6). In contrast, when the LPS in the combined vaccine was derived from a different serotype than the challenging strain, the presence of the heterologous LPS did not alter the protection by RNA (Table 5).

The survival induced by combined vaccines was associated with lower titers of LPS-specific antibodies than the survival induced by vaccines containing only LPS. For example, a combined vaccine induced 60% survival and an LPS-specific antibody titer of 1.4, whereas LPS induced 57% survival and an antibody titer of 2.3 (Table 5). Likewise, a combined vaccine induced 80% survival and an antibody titer of 4.7, whereas LPS induced 63% survival and an antibody titer

of 6.0 (Table 6). Thus, the higher protection induced by combined vaccines was not due only to the increased titers of LPS-specific antibodies.

The data on the survival induced by vaccines with different RNA and LPS doses were statistically analyzed to test the hypothesis that the protection induced by LPS acted only additively to the protection induced by RNA. The Q (significance level of the goodness of fit for the model) was 0.66 for the data presented in Table 5, supporting the hypothesis that LPS and RNA contributed additively to the survival. The Q was 0.09 for the percentages of survival presented in Table 6, indicating that the survival could not be explained by only additive protective effects of RNA and LPS.

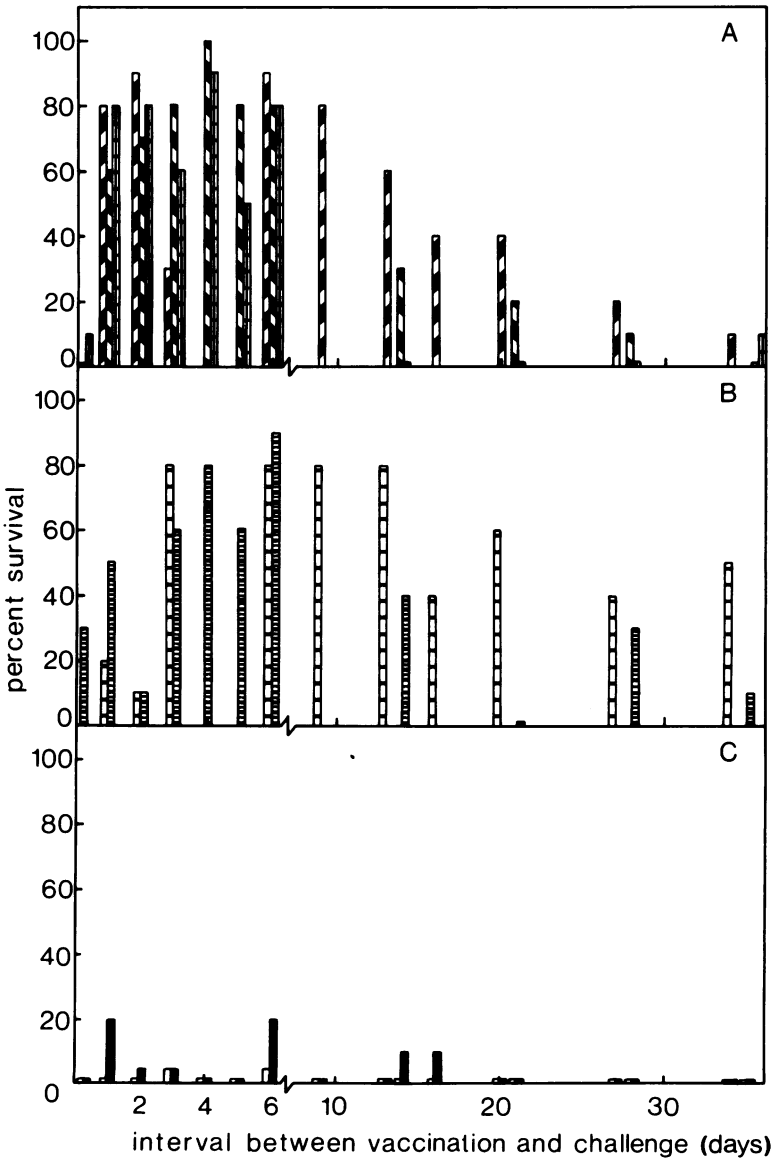

FIG. 1. *Effect of interval between vaccination and challenge on the protective activity of the ribosomal vaccine and of LPS. Groups of 10 mice each were immunized intraperitoneally on different days with the ribosomal vaccine plus DDA or RNA plus DDA (A), with LPS (B), or with buffer or buffer plus DDA (C). All mice were challenged at the same day with P. aeruginosa. Symbols: (A) ◪, 1 μg of ribosomal vaccine plus DDA; ▨, 5 μg of ribosomal vaccine plus DDA; ⊞, 3.3 μg of RNA plus DDA; (B) ⊟, 10 ng of LPS; ▤, 50 ng of LPS; (C) □, buffer; ■, buffer plus DDA.*

DISCUSSION

Although effective ribosomal vaccines have been prepared from many microorganisms, the nature of the immunogen(s) in these vaccines has not been clarified. In particular, the function of the ribosomes in the vaccines has been discussed, since contaminating cell envelope antigens were found in several of the ribosomal preparations (6, 17, 18). Previous studies of this laboratory (9) showed that a ribosomal vaccine of *P. aeruginosa* could be prepared which was sensitive to RNase, indicating that the ribosomal RNA was required for the protective activity of this vaccine. In addition, this vaccine could induce antibodies to the cell envelope component LPS (10). These results suggested that the RNA might act as a carrier or an adjuvant for cell envelope antigens. The purpose of the present study was to compare the properties of the ribosomal vaccine with those of an LPS vaccine in order to obtain more information about the function of RNA in the ribosomal vaccine.

The conditions for optimal protection by the ribosomal vaccine were determined first. The efficacy of the ribosomal vaccine was influenced by the route of vaccination. When the ribosomal vaccine was injected intraperitoneally, lower concentrations of the vaccine were required to protect mice against a lethal *Pseudomonas* challenge in comparison with subcutaneous injection. In subsequent experiments, all vaccines were injected intraperitoneally so that vaccination and challenge were given by the same route.

The ribosomal vaccine protected mice as soon as 1 day after injection. Two days after vaccination with either the ribosomal vaccine or LPS, no antibodies to LPS could be detected in the sera of the mice. At this time, the ribosomal vaccine protected mice also against a heterolo-

TABLE 2. *Antibodies to LPS induced by the ribosomal vaccine or by LPS at 2 or 6 days after intraperitoneal vaccination*

Vaccine		Log_2 titer of antibodies to LPS[a]	
Type	Dose (μg)	2 days	6 days
Ribosomes + DDA	10	0.9	7.3
Ribosomes + DDA	1	0.5	4.6
LPS	0.1	0.9	8.7
LPS	0.01	1.0	6.9
Buffer		1.6	1.3
Buffer + DDA		0.0	1.3

[a] Determined by ELISA. Each value represents the mean of two determinations on different pooled sera of five mice.

TABLE 4. *Protective activity of RNA and protein isolated from the ribosomal vaccine*

Vaccine[a]		Survival[b]			Log_2 titer[c] of antibodies to LPS
Type	Dose (μg)	%	No. challenged	P value[d]	
Ribosomes + DDA	5	85	20	<0.01	5.0
RNA + DDA	3.3	53	17	<0.01	0.1
RNA + DDA	5	65	20	<0.01	2.0
Protein + DDA	5	10	30	0.3	0.9
Protein + DDA	15	13	30	0.2	0.0
Protein	15	0	30	1.0	0.0
Buffer + DDA		3	30		1.0
Buffer		0	30		0.1

[a] All vaccines were derived from serotype 3 *Pseudomonas*.

[b] Mice were challenged with *P. aeruginosa* serotype 3 6 days after an intraperitoneal vaccination.

[c] Determined in pooled sera of five mice by ELISA. Each value represents the mean of two determinations.

[d] *P* values with respect to the corresponding controls (buffer or buffer plus DDA) were calculated by the Fisher test.

TABLE 3. *Serotype specificity of the protection induced by the ribosomal vaccine or by LPS*

Vaccine		Survival[a]					
		2 days			6 days		
Type	Dose (μg)	%	No. challenged	P value[b]	%	No. challenged	P value
Ribosomes (serotype 8) + DDA	5	77	30	<0.01	57	30	<0.01
LPS (serotype 8)	0.05	3	30	0.8	75	20	<0.01
Ribosomes (serotype 3) + DDA	5	70	30	<0.01	37	30	<0.01
LPS (serotype 3)	0.05	3	30	0.8	10	20	0.16
Buffer + DDA		27	30		3	30	
Buffer		3	30		0	30	

[a] Mice were challenged with *P. aeruginosa* serotype 8 at 2 or 6 days after intraperitoneal vaccination with homologous or heterologous vaccines.

[b] *P* values with respect to the corresponding controls (buffer or buffer plus DDA) were calculated by the Fisher test.

gous challenge. The protection by LPS was not evident for 3 days after vaccination, and this protection was serotype specific. It is concluded that the ribosomal vaccine could induce early, serotype-nonspecific protection which was likely to be different from the serotype-specific protection induced by LPS.

The ribosomal vaccine also induced serotype-nonspecific protection 6 days after vaccination. Although 2 days after vaccination the homologous and heterologous ribosomal vaccine protected mice to the same extent, the results suggested that 6 days after vaccination higher percentages of survival could be induced with the ribosomal vaccine derived from the challenging organism than with the heterologous ribosomal vaccine. This was best explained by the finding that 6 days after vaccination detectable amounts of antibodies to LPS were induced by the ribosomal vaccine.

In a previous report, it was demonstrated that the subcutaneously injected ribosomal vaccine derived from serotype 3 could not protect mice against a challenge with serotype 8 *Pseudomonas* (9). Preliminary experiments supported the explanation that this difference was due to the route of vaccination.

RNA and protein were isolated from the ribosomal vaccine to determine whether the protective activity of the ribosomes remained associated with one of these two components. No protection was obtained with protein isolated from the ribosomal vaccine. In contrast, RNA exhibited the same protective properties as the ribosomal vaccine. RNA induced protection as quickly as 1 day after vaccination, and the protection by RNA was not restricted to the homologous serotype. The concentration of RNA which was required to induce protection was similar to the concentration of RNA in protective doses of the ribosomal vaccine. However, in contrast to the ribosomal vaccine, RNA did not induce antibodies to LPS 6 days after vaccination. Probably the concentration of contaminating LPS in the ribosomal vaccine had been reduced during the isolation of RNA below the level that was required to induce antibodies.

Combined vaccines containing RNA plus LPS were used to investigate the function of RNA in the ribosomal vaccine. The addition of RNA to an LPS vaccine resulted in higher concentrations of LPS-specific antibodies. Also, the protection by combined vaccines containing RNA and LPS which was derived from the challenging serotype was increased in comparison with the protection induced by RNA or LPS alone. The higher protection induced by combined vaccines was in part serotype specific, since the protection by RNA was not affected by the addition of LPS from a different serotype than the challenging strain. The protection by combined vaccines is not due only to the increased titer of LPS-specific antibodies. The percentages of survival in-

TABLE 5. *Protective activity and antibodies to LPS induced by combined vaccines containing RNA and LPS from P. aeruginosa serotype 8*[a]

Dose		Log_2 titer[b] of antibodies to LPS	Survival (%) after challenge with:	
RNA (μg)	LPS (ng)		Serotype 8[c]	Serotype 3[d]
5		1.0	43	45
5	0.01	0.8		50
	0.01	0.9		
5	0.1	1.0		45
	0.1	0.4		
5	1.0	1.4	60	58
	1.0	0.9	23	
5	10	3.6	79	55
	10	2.3	57	
—[e]	—	1.6	10	0

[a] All vaccines were incorporated in DDA.

[b] The log_2 titer of antibodies to LPS derived from *P. aeruginosa* serotype 8 was determined in sera of mice 6 days after vaccination. Each value represents the mean of three determinations on different pooled sera of five mice.

[c] Each value was determined after challenging 30 mice with an inoculum containing 3 LD_{50} of *P. aeruginosa* serotype 8 6 days after vaccination.

[d] Each value was determined after challenging 20 mice with an inoculum containing 3.5 LD_{50} of *P. aeruginosa* serotype 3 6 days after vaccination.

[e] —, Controls injected with buffer in DDA.

TABLE 6. *Protective activity and antibodies to LPS induced by combined vaccines containing RNA from serotype 8 and LPS from serotype 3 of P. aeruginosa*[a]

Dose		Log_2 titer[b] of antibodies to LPS	Survival (%)[c] after challenge with serotype 3
RNA (μg)	LPS (ng)		
5		1.1	40
5	1	4.7	80
	1	4.0	13
5	10	6.8	87
	10	6.0	63
—[d]	—	0.8	3

[a] All vaccines were incorporated in DDA.

[b] The log_2 titer of antibodies to LPS derived from *P. aeruginosa* serotype 3 was determined in sera of mice 6 days after vaccination. Each value represents the mean of three determinations on different pooled sera of five mice.

[c] Each value was determined after challenging 30 mice with an inoculum containing 4 LD_{50} of *P. aeruginosa* serotype 3 6 days after vaccination.

[d] —, Controls were injected with buffer in DDA.

duced by combined vaccines were associated with lower titiers of LPS-specific antibodies than the survival induced by LPS alone. A previous report demonstrated that the protection induced by the ribosomal vaccine was associated with lower titers of LPS-specific antibodies than the protection induced by purified LPS (10). It is concluded that RNA contributed to the protective activity of the ribosomal vaccine in two ways. RNA induced serotype-nonspecific protection which was independent of the presence or absence of LPS-specific antibodies. In addition, RNA enhanced the production of antibodies to contaminating cell envelope antigens, i.e., LPS.

Preliminary experiments pointed out that the protection induced by RNA was not restricted to the *Pseudomonas* species; *Pseudomonas* RNA could also protect mice against a challenge with *Escherichia coli*. Thus, the protection induced by RNA might be nonspecific.

Coppel and Youmans (4) reported that the mycobacterial ribosomal fraction which was incorporated in Freund incomplete adjuvant protected mice against a lethal challenge with *Klebsiella pneumoniae* during 4 days after immunization. No protection was obtained against a challenge with *Listeria monocytogenes*. Weinstein et al. (20) demonstrated that nonspecific protection against several microorganisms was also obtained 1 day after injection of polyinosinic-polycytidylic acid without adjuvant. Araujo and Remmington (2) found that synthetic polynucleotides and RNA extracted from macrophages of mice protected mice against a challenge with *Toxoplasma gondii* at 30 days after the injection of RNA. Macrophages of mice which were immunized with RNA extracted from toxoplasma cells resisted a challenge with *L. monocytogenes* in vitro. Thus, although several investigators found that RNA could induce nonspecific protection in mice against different microorganisms, the results varied with respect to the duration of the protection, the degree of protection against particular microorganisms (i.e., *L. monocytogenes*), and several other aspects.

The present report demonstrates that serotype-nonspecific protection induced by RNA isolated from the ribosomal vaccine of *P. aeruginosa* could come to expression only under certain conditions, i.e., the use of an adjuvant, intraperitoneal injection of the vaccine (via the same route as the challenge was given), and injection of the challenge within 6 days after vaccination. These conditions might provide an explanation of why several investigators of ribosomal vaccines have not found any evidence that RNA contributes to the protective activity of their ribosomal preparations (6, 16, 17, 18). As the (serotype) nonspecific protection by RNA was induced as early as 1 day after vaccination, the protection may have been due to a nonspecific increase in the resistance of the host by an effect on the phagocytic system. The data in Table 5 suggested that the protective effects of RNA and LPS acted additively. Therefore, the protective activity of the ribosomal vaccine might be the result of an enhanced phagocytic capacity of the host induced by RNA and of the presence of serotype-specific antibodies which facilitated the phagocytosis of the organism from which the ribosomal vaccine was isolated.

ACKNOWLEDGMENTS

We thank Arno M. M. Muijtjens (Department of Biophysics, State University of Limburg, The Netherlands) for the statistical analysis. We also thank B. Davies (Department of Bacteriology, De Wever Hospital, Heerlen, The Netherlands) and J. M. N. Willers (Department of Immunology, Laboratory of Microbiology, State University of Utrecht, The Netherlands) for critically reading the manuscript.

LITERATURE CITED

1. **Andron, L. A., and H. T. Eigelsbach.** 1975. Biochemical and immunological properties of ribonucleic acid-rich extracts from *Francisella tularensis*. Infect. Immun. **12**:137-142.
2. **Araujo, F. G., and J. S. Remmington.** 1974. Protection against *Toxoplasma gondii* in mice immunized with Toxoplasma cell fractions, RNA, and synthetic polyribonucleotides. Immunology **27**:711-721.
3. **Bradley, J. V.** 1968. Distribution free statistical tests. Prentice-Hall, Inc., Englewood Cliffs, N. J.
4. **Coppel, S., and G. P. Youmans.** 1969. Specificity of acquired resistance produced by immunization with mycobacterial cells and mycobacterial fractions. J. Bacteriol. **97**:114-120.
5. **Eisenstein, T. K.** 1978. Ribosomal vaccines: a review, p. 211-222. *In* A. Voller and H. Friedman (ed.), New trends and developments in vaccines. MTP Press Limited, St. Leonard Gate, England.
6. **Field, L. H., C. D. Parker, C. R. Manclark, and L. J. Berry.** 1979. Evaluation of a ribosomal vaccine against pertussis. Infect. Immun. **24**:346-351.
7. **Fienberg, S. E.** 1979. The analysis of cross-classified categorial data. The MIT Press, London, England.
8. **Galanos, C., O. Luderitz, and O. Westphal.** 1969. A new method for the extraction of R. lipopolysaccharides. Eur. J. Biochem. **9**:245-249.
9. **Gonggrijp, R., W. J. H. A. Mullers, P. J. M. R. Lemmens, and C. P. A. van Boven.** 1980. Ribonuclease-sensitive ribosomal vaccine of *Pseudomonas aeruginosa*. Infect. Immun. **27**:204-210.
10. **Gonggrijp, R., M. P. W. Volleberg, P. J. M. R. Lemmens, and C. P. A. van Boven.** 1981. Evidence for the presence of lipopolysaccharide in a ribonuclease-sensitive ribosomal vaccine of *Pseudomonas aeruginosa*. Infect. Immun. **31**:896-905.
11. **Hardy, S. J. S., C. G. Kurland, P. Voynow, and G. Mora.** 1969. The ribosomal proteins of *Escherichia coli*. I. Purification of the 30 S ribosomal proteins. Biochemistry **8**:2897-2905.
12. **Hartree, E. F.** 1972. Determination of protein: a modification of the Lowry method that gives a linear photometric response. Anal. Biochem. **48**:422-427.
13. **Herbert, D., P. H. Phipps, and R. E. Strange.** 1971.

Chemical analysis of microbial cells, p. 308–328. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 5B. Academic Press, Inc., London.

14. **Horton, D., and G. Rodemeyer.** 1977. Analytical characterization of lipopolysaccharide antigens from seven strains of *Pseudomonas aeruginosa*. Carbohydr. Res. **55**:35–47.
15. **Lehmann, E. L.** 1975. Nonparametrics: statistical methods based on ranks. Holden-Day Inc., San Francisco.
16. **Lieberman, M. M.** 1978. *Pseudomonas* ribosomal vaccines: preparation, properties, and immunogenicity. Infect. Immun. **21**:76–86.
17. **Lin, J. H., and L. J. Berry.** 1978. The use of strain LT2-M1 in identifying the protective antigens in a *Salmonella typhimurium* derived ribosomal vaccine. RES J. Reticuloendothel. Soc. **23**:135–143.
18. **Misfeldt, M. L., and W. Johnson.** 1978. Identification of protective cell surface proteins in ribosomal fractions from *Salmonella typhimurium*. Infect. Immun. **24**:808–816.
19. **Poulson, R.** 1977. Isolation, purification and fractionation of RNA, p. 343–344. *In* P. R. Stewart and D. S. Letham (ed.), The ribonucleic acids, 2nd ed. Springer Verlag Inc., New York.
20. **Weinstein, M. J., J. A. Waitz, and P. E. Came.** 1970. Induction of resistance to bacterial infections of mice with poly I. poly C. Nature (London) **226**:170.
21. **Weissbach, A., and J. Hurwitz.** 1959. The formation of 2-keto-3-deoxyheptonic acid in extracts of *Escherichia coli* B. I. Identification. J. Biol. Chem. **234**:705–709.
22. **Youmans, A. S., and G. P. Youmans.** 1966. Effect of trypsin and ribonuclease on the immunogenic activity of ribosomes and ribonucleic acid isolated from *Mycobacterium tuberculosis*. J. Bacteriol. **91**:2146–2154.