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Response of Mice to Injection of Ribosomal Fraction from Group B *Neisseria meningitidis*

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Ribosomes of strain NOR-7 of group B *Neisseria meningitidis* were isolated by a procedure that included treatment of the cells with sodium dodecyl sulfate, disruption in a French pressure cell, and differential centrifugation. These preparations consisted of 66% ribonucleic acid and 24% protein and sedimented as a single component with a constant of approximately 66*S*. When used in immunodiffusion tests with homologous rabbit antiserum, untreated ribosomes formed two precipitin lines, when treated with ribonuclease three lines, and when Pronase-digested only one distinct line. Qualitatively indistinguishable reactions were obtained with the same antiserum and ribosomes from group A meningococci, but no precipitation occurred with those of *Escherichia coli*. When injected into mice, group B ribosomes elicited an increase in the number of antibody-producing spleen cells demonstrable by the hemolytic plaque technique using unsensitized sheep erythrocytes. Sensitization of the erythrocytes with increasing amounts of supernatant fluid of meningococcal cultures progressively reduced the number of demonstrable plaque-forming cells. Neuraminidase treatment of the erythrocytes increased immune hemolysis, whereas Pronase digestion reduced it. Injected mice were protected against homologous and heterologous meningococcal challenge. Both hemolysis and protection-inducing activities of the ribosomes were unimpaired by ribonuclease, but were reduced by Pronase. It is concluded that the immunological response elicited by the meningococcal ribosomes does not involve the group-specific carbohydrate antigen. The immunological mechanism by which the mice are protected against meningococcal challenge remains unknown.

The need for an immunogen from group B meningococci was summarized in a previous publication (14) which also described the properties of spheroplast membranes. A study of ribosomal preparations of group B meningococci as potential immunogens is discussed in this paper.

There is excellent evidence that homologous ribosomal antigens protect mice against infection with *Mycobacterium tuberculosis* (31). The immunogenicity of these preparations was not affected by trypsin, but optimal activity was dependent on the integrity of the ribonucleic acid (RNA). When injected with Freund's incomplete adjuvant, protection was demonstrated with as little as 0.5 μg of ribosomal preparation (29, 30). The ribosomes of *Salmonella typhimurium* were also shown to confer protection against homologous infection (21), and, as in this case also, the RNA was necessary for immunization (22). In contrast to infection with *M. tuberculosis*, immunization against *S. typhimurium* did not require Freund's adjuvant, and the immunogenicity was not destroyed by ribonuclease, suggesting that it was associated with double-stranded RNA. Immunity in both cases was cell-mediated (23, 24, 31). Ribosomal preparations were also found to be effective against infections of mice with *Staphylococcus aureus*, *Pseudomonas aeruginosa* (26, 27), and *Diplococcus pneumoniae* (20). The immunogenicity of these preparations appeared to be dependent in part on the protein moieties. In *Listeria monocytogenes* infection, ribosomal preparations proved to be ineffective (7).

In view of previous successes and failures with ribosomal immunogens, apparently it is not possible to assume that results obtained with one infection apply to another. The work described in this paper was prompted by the need to evaluate meningococcal ribosomes as potential immunogens.

MATERIALS AND METHODS

Microorganisms. The strains of *Neisseria meningitidis* group A (MK-204), group B (NOR-7), and

group C (NOR-20) employed in this study were described previously (9, 25). Frozen *Escherichia coli* cells (ATCC 11303, Kornberg medium, late log phase) were obtained from General Biochemicals.

Preparation of ribosomes. Ribosomes were prepared by the method of Youmans and Youmans (30). Disposable roller bottles, 2.2 liters, containing 1 liter of tryptic soy broth (TSB, Difco) were inoculated with 1 ml of cells. The inoculum was prepared by growing the organisms for 16 hr on Mueller-Hinton agar (Difco) slants and suspending the growth in 6 ml of broth. Following incubation for 10 to 12 hr at 37 C with shaking, cells from 6 liters of culture were harvested by centrifugation at 13,500 × *g* for 15 min and were washed once in phosphate-buffered saline (*p*H 7.2). The cells were resuspended in buffer containing 0.25% sodium dodecyl sulfate (SDS), mixed in a Sorvall Omni-Mixer (Ivan Sorvall, Inc.) operated at reduced speed, and passed through a French pressure cell (American Instrument Co.) at 15,000 to 19,000 psi. The supernatant fluids obtained following centrifugations at 27,000 × *g* for 10 min and 43,000 × *g* for 15 min were centrifuged at 105,000 × *g* for 3 hr. The pellet was resuspended in 0.01 M phosphate buffer (PB, *p*H 7.2) containing 10^{-4} M $MgCl_2$ (PB, Mg^{2+}) to a concentration of 50 mg (wet weight)/ml. SDS was added to a final concentration of 0.5%, and this suspension was gently rotated for 20 min. The SDS was allowed to precipitate overnight at 4 C, and the supernatant fluid was centrifuged at 105,000 × *g* for 4 hr. The sedimented ribosomes were suspended in the above PB, Mg^{2+} buffer and stored at −70 C until used. *E. coli* cells were thawed and washed once in PBS, and the ribosomes were obtained as above described.

Chemical and physical analyses. RNA and deoxyribonucleic acid (DNA) contents were determined by the orcinol (8) and diphenylamine (16) methods, respectively. Protein content was estimated by the method of Lowry et al. (18) using Dade Lab-Trol (American Hospital Supply Co.) as a standard, and *N*-acetylneuraminic acid content was assayed by the periodate-resorcinol procedure (15). Absorbance was determined in an Acta III spectrophotometer (Beckman Instrument Inc.).

For density gradient analysis, 1 ml of ribosomal preparation (containing 2 mg of RNA) was layered over a 5 to 20% continuous sucrose gradient prepared in 0.02 M tris(hydroxymethyl)aminomethane (Tris) buffer, *p*H 7.5, containing 10^{-4} M $MgCl_2$. The gradient was centrifuged in a SW25.1 rotor at 25,000 rev/min for 2 hr at 0 to 4 C. Fractions (0.7 ml) were drop collected by piercing the bottom of the tube. For absorbance determinations at 260 nm, the fractions were diluted to 2 ml with PB, Mg^{2+}.

Sedimentation velocity analysis was carried out at 20 C in 0.01 M PB, *p*H 7.2, containing 10^{-4} M $MgCl_2$ in a model E centrifuge (Beckman Instruments Inc.) operated at 35,600 rev/min for 28 min using Schlieren optics. A single moving boundary was obtained which was used for the determination of the sedimentation coefficient.

Immunodiffusion. Antiserum was produced in rabbits by three subcutaneous injections, given 1 week apart into the base of the neck, containing 1 mg of NOR-7 ribosomes incorporated in 2 ml of Freund's incomplete adjuvant (BBL). The rabbits were bled 10 days after the third injection. Immunodiffusion studies employed plastic templates mounted on microscope slides (25 by 75 mm) layered with 0.75% agarose by the method of Fink et al. (11). After incubation for 16 to 18 hr at room temperature in a humid chamber, the templates were removed, the slides were rinsed in 0.9% NaCl, and the precipitin lines were photographed with an immunodiffusion camera (Cordis Laboratories, Miami, Fla.).

Hemolytic plaque technique. Female Swiss white mice, inbred NIH-NMRI strain, weighing 16 to 20 g, were injected intraperitoneally with 1 ml of antigen, and the antibody-producing spleen cells were demonstrated by a modification of the hemolytic plaque assay described by Evans (9). Sheep red cells (Grand Island Biological Co.) were sensitized with NOR-7 carbohydrate antigen (25) and were sensitization-tested by either microhemagglutination or microhemolytic assays (9). Erythrocytes treated with neuraminidase (19) or Pronase (5) were also used in this procedure. The overlay medium of 1% Noble Agar (Difco) containing Eagle minimum essential medium (MEM, Grand Island Biological Co.) was kept at 56 C, the *p*H was adjusted to 7.0 with 4% $NaHCO_3$, and 2-ml samples were distributed in mixing tubes. The tubes were transferred to a 45 C water bath and 0.1 ml of 1% diethylaminoethyl (DEAE)-dextran in 0.15 M NaCl, 0.2 ml of 4% erythrocytes, and 0.1 ml of a mouse spleen suspension were added in this order. The tubes were quickly mixed and poured over a basal layer of 1.4% Noble Agar containing MEM, *p*H 7.2, maintained at 37 C. The plates were incubated at 37 C for 1 hr, and 3 ml of guinea pig complement (Grand Island Biological Co.), diluted 1:10 with 0.15 NaCl containing 0.001 M Ca^{2+} and 0.0019 M Mg^{2+}, was added. The plates were reincubated at 37 C for an additional hour and the plaques were enumerated at 15× magnification with a dissecting microscope. Viable spleen cells were enumerated by dye exclusion using 0.15% eosin Y, and the results were expressed as the number of plaque-forming cells (PFC) per 10^6 viable spleen cells.

Mouse protection. Mice were immunized intraperitoneally with 1 ml of antigen and challenged by the same route 7 days later with serial dilutions of organisms passed five times on TSB blood agar plates (BBL) and suspended in 4% gastric mucin as described by Branham and Pittman (2). The results are expressed as the number of organisms giving a median lethal dose.

Miscellaneous procedures. Enzymatic digestion of the ribosomal preparation was performed at 37 C for 30 min with pancreatic ribonuclease (Calbiochem), Pronase (Calbiochem), and neuraminidase (Sigma) at a concentration of 1 mg per mg of ribosomal RNA content.

Purified NOR-7 carbohydrate antigen was prepared as described by Weiss and Long (25).

RESULTS

Characterization of NOR-7 ribosomes. The ribosomal preparations, on the basis of dry weight, consisted of about 66% RNA, with little variation among the various preparations, 24% protein, and very small amounts of the other constituents tested (DNA, 0.3%; *N*-acetylneuraminic acid, 0.5%). The ratio between the optical density at 260 nm and at 280 nm was slightly greater than 2. When centrifuged over a sucrose density gradient, the ribosomal preparation sedimented as a single fraction (Fig. 1). The sedimentation coefficient, measured by ultracentrifugation at 35,600 rev/min in PB containing 10^{-4} M $MgCl_2$ using Schlieren optics, was approximately 66*S*. The reason why the ribosomes did not separate into subunits, despite the low Mg^{2+} concentration, is not known. However, following storage for several months at −70 C, fractions with sedimentation constants of 44, 30, 22, and 16*S* were obtained.

The ribosomal preparation was relatively toxic for rabbits. Intravenous injections of amounts containing 1 or 10 μg of RNA elicited a pyrogenic response of 6-hr duration, and 12.5 μg of RNA given intradermally plus 25 μg given intravenously the following day elicited a positive Shwartzman reaction.

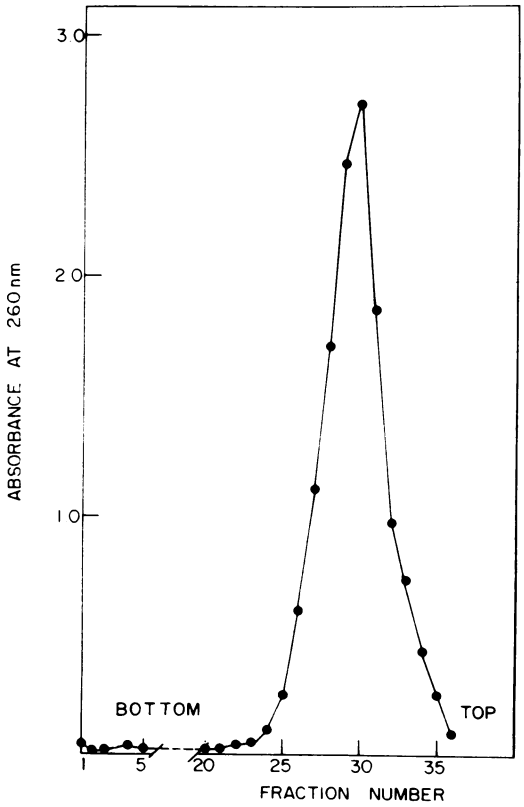

FIG. 1. *Absorbance pattern of 5 to 20% sucrose density gradient fractions of NOR-7 ribosome preparation following centrifugation at 25,000 rev/min for 2 hr at 0 to 4 C.*

In immunodiffusion tests done with a rabbit antiserum prepared against NOR-7 ribosomes, two precipitin lines were obtained with the homologous antigen (Fig. 2A). A ribosomal preparation from group A meningococci (strain MK-204) appeared to have the same two antigenic components and formed no spurs. No reaction was obtained with a high concentration of *E. coli* ribosomes. Solubilized NOR-7 spheroplast membranes reacted with the anti-ribosomal serum to produce two major precipitin lines which did not appear to be related to the two major ribosomal antigens (Fig. 2A). When the homologous ribosomal antigen was treated with ribonuclease, a third precipitin line appeared which was more highly diffusible than the other two. On the other hand, Pronase treatment resulted in reduction of intensity of the reaction with the less-diffusible antigenic component (Fig. 2B). The same anti-ribosomal rabbit serum failed to agglutinate sheep erythrocytes, unsensitized or sensitized with the supernatant fluid of a NOR-7 culture. The sensitized cells were agglutinated, however, by group B antiserum elicited with whole cells.

Stimulation of mouse spleen cells by NOR-7 ribosomes and other preparations. Mice stimulated by intraperitoneal injection of NOR-7 ribosomes responded with production of a moderate number of spleen cells that lysed sheep erythrocytes in the presence of complement. This reaction was measured by the number of PFC present at 3 days after injection. Although a response was elicited with lower amounts, most consistent results were produced by the injection of 50 μg (RNA content). Tables 1 and 2 portray representative experiments that investigated the nature of the specificity of the antibodies released by the spleen cells. Hemolysis was not dependent on the presence of meningococcal antigen on the surface of the erythrocytes. In fact, as shown in Table 1, sensitization of the erythrocytes with increasing amounts of supernatant fluid of NOR-7 cultures progressively reduced the number of demonstrable PFC. Attempts to sensitize red blood cells with purified ribosomal antigen were not successful because there was no evidence that this antigen was adsorbed onto untreated erythrocytes. Treatment with gluteraldehyde or other reagent that permitted sensitization (1) rendered the erythrocytes resistant to lysis. Since the antigen

conferring group B specificity is a polymer of *N*-acetylneuraminic acid (17), and sialic acid is a normal constituent of the erythrocyte surface (13), the effect of removal of this component was also investigated. Contrary to expectation, neuraminidase treatment greatly increased the sensitivity of the reaction, whereas Pronase treatment of the red blood cells reduced it (Table 2).

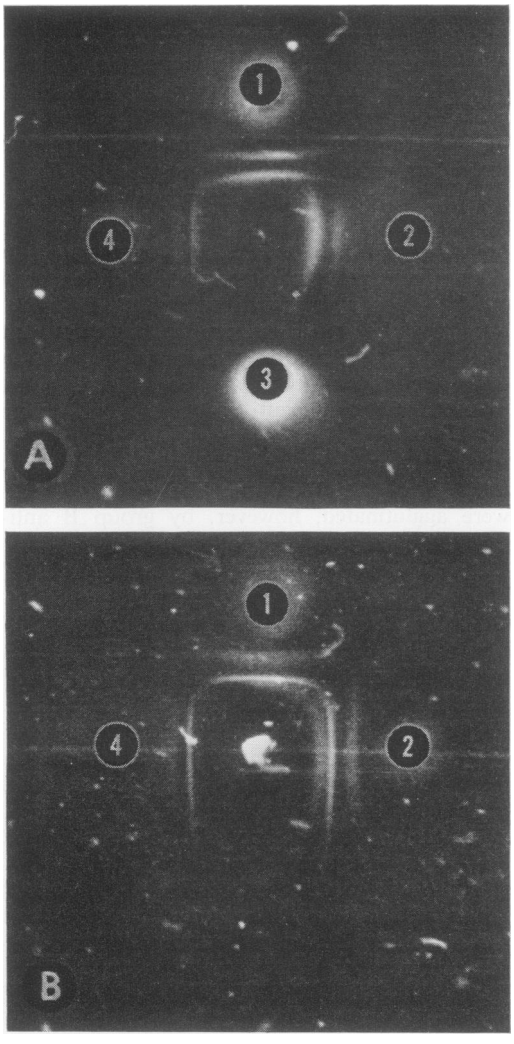

FIG. 2. *Immunodiffusion patterns of rabbit antiserum elicited by NOR-7 ribosomal fraction (in center well) and various cell fractions. A, well 1: NOR-7 ribosomal fraction (2 mg of RNA/ml); well 2: NOR-7 spheroplast membranes solubilized with 0.25% SDS (2.5 mg of protein/ml); well 3: E. coli ribosomal fraction (6 mg of RNA/ml); well 4: MK-204 ribosomal fraction (1 mg of RNA/ml). B, NOR-7 ribosomal fraction (2 mg of RNA/ml). Well 1: Untreated; well 2: treated with ribonuclease; well 3: not used; well 4: treated with Pronase.*

TABLE 1. *Effect of erythrocyte sensitization on demonstration of plaque-forming cell (PFC) response of mice injected with NOR-7 ribosomes*

Mouse no.	PFC/10^6 spleen cells[a]				
	0[b]	20	31	92	183
1	25	20	23	2	3
2	14	10	9	4	5
3	28	19	18	13	11

[a] Three days after intraperitoneal injection with 50 μg (RNA content).

[b] Micrograms (dry weight) per 0.1 ml of packed erythrocytes of crude antigen from NOR-7 culture supernatant fluid.

TABLE 2. *Effect of erythrocyte modification on demonstration of plaque-forming cell (PFC) response of mice injected with NOR-7 ribosomes*

Erythrocyte treatment	PFC/10^6 spleen cells[a]
None	17 ± 1.5[b]
Neuraminidase[c]	62 ± 8.8
Pronase[d]	9 ± 0.9

[a] Three days after intraperitoneal injection with 50 μg (RNA content).

[b] Mean ± standard error. Ten mice were used.

[c] From *Vibrio cholerae*. Erythrocytes (1%) were incubated at 37 C for 1 hr with an equal volume of enzyme (25 units/ml) and washed three times (18).

[d] Erythrocytes (50%) were incubated at 37 C for 1 hr with 0.25 mg of self-digested Pronase per ml and washed five times (4).

The effect of enzymatic treatment of the ribosomes on their efficacy as inducers of a PFC response is illustrated in Fig. 3. Neither neuraminidase or ribonuclease impaired the activity of the ribosomes. Treatment with Pronase, however, reduced the number of PFC obtained on day 3 to about one-half. Heating the ribosomal preparation at 100 C for 30 min did not reduce its plaque-inducing activity.

The kinetics of the mouse PFC response were studied with mice injected with a combination of NOR-7 ribosomal and purified carbohydrate antigens, but experiments conducted with ribosomal antigens alone indicated that the carbohydrate antigen contributed little, if at all, to the intensity and specificity of the response. Only the PFC response to unsensitized cells is shown in Fig. 4 and 5, since erythrocytes sensitized with moderate amounts of carbohydrate antigen yielded comparable results. A PFC response was first detectable at day 2, reached a peak at day 3 (ranging from 15 to 30 PFC/10^6

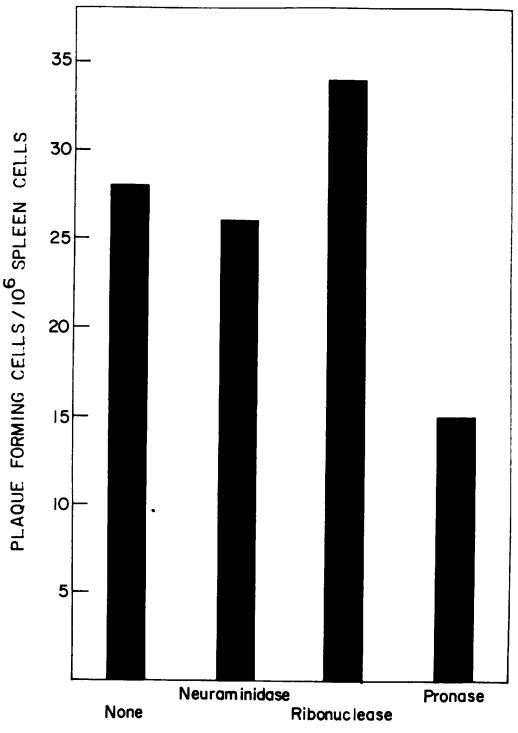

FIG. 3. *Effect of enzymatic treatment of the ribosomes on their PFC-enhancing activity in mice, 3 days after injection. Mean of two experiments, each involving three mice per group.*

spleen cells in most cases), and declined rapidly thereafter. The number of PFC remained slightly greater than in uninjected mice until day 8. The purified carbohydrate antigen injected by itself, on the other hand, elicited a weak reaction, barely measurable on day 4 (Fig. 4).

Some of the mice that received the combination of the two antigens were reinjected with the same combination on day 24 (Fig. 5). A large number of PFC were formed within 2 days (about 70/10^6 spleen cells). By the third day the number of PFC was greatly reduced, and by the fourth day it was only slightly greater than in the controls. Mice that were reinjected with purified carbohydrate antigen alone exhibited a more moderate, but otherwise very similar, response. About 20 PFC/10^6 spleen cells were demonstrated at 2 days after reinjection. An anamnestic type of reaction was also obtained when mice were reinjected 24 days after receiving purified carbohydrate antigen without ribosomes (Fig. 5). The peak was of the same magnitude at 2 days, but the decline in PFC activity on days 3 and 4 was somewhat less rapid. When the second injection was purified carbohydrate antigen alone, the response at 2 days was moderate (about 10 PFC/10^6 spleen cells), but significantly greater than on first injection.

Ribosomes derived from *N. meningitidis* strain MK-204 (group A) elicited a PFC response to unsensitized cells, comparable to the response to the NOR-7 preparation, but the peak of the reaction was reached on day 2, rather than on day 3. *E. coli* ribosomes elicited a similar effect, with peak PFC response on day 3, but this activity was greatly reduced by treating the ribosomes with either Pronase or ribonuclease. No PFC response resulted from the intraperitoneal injection into mice of 10 μg of purified *E. coli* lipopolysaccharide (Difco) or 50 μg of purified *E. coli* transfer RNA (General Biochemicals).

Mouse protection by NOR-7 ribosomes. Mice injected intraperitoneally with NOR-7 ribosomes became resistant to challenge with viable cells suspended in gastric mucin (Table 3). The smallest dose of ribosomes that conferred protection to mice was 5 μg (RNA content), but much better protection was afforded by 50 μg. The protective property of the ribosomes was not impaired by treatment with ribonuclease, but was destroyed in part by the action of Pronase. Acquired resistance of the mice was not confined to the homologous strain, but extended to challenge with group A and group C strains (Table 3).

Role of Forssman antigen and antibody. Since the mouse PFC response appeared to be directed against a Forssman-like antigen, tests were performed which yielded the following results. A PFC response was not demonstrated when human O cells were substituted for sheep erythrocytes. Neither human O nor sheep erythrocytes (10^8 cells/mouse) provided active protection of mice against subsequent challenge with NOR-7 meningococci. No reaction was demonstrated by immunodiffusion between rabbit anti-sheep red cell hemolysin and NOR-7 ribosomes. Rabbit anti-sheep red cell hemolysin did not provide passive protection of mice against meningococcal NOR-7 challenge.

DISCUSSION

Meningococcal ribosomes elicit a reaction in the mouse as revealed by PFC response and increased resistance to challenge with virulent meningococci. The two reactions appear to be expressions of the same stimulation, since they have similar dose responses and are affected in the same manner by procedures, such as Pronase treatment, which modify the ribosomes. The nature of the antigenicity and specificity of the ribosomes is not clear, however. Lack of ribonuclease sensitivity and heat stability, in them-

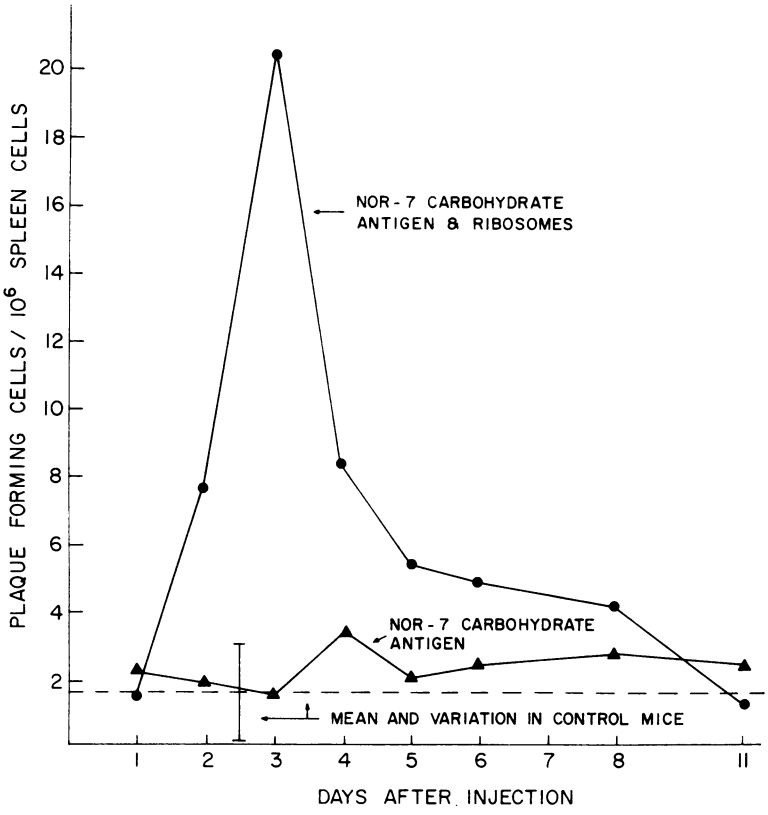

FIG. 4. *Kinetics of the PFC response of mice to a single injection of 25 μg of NOR-7 carbohydrate antigen or a combination of this antigen and NOR-7 ribosomes (RNA content, 50 μg). Each symbol represents the mean of three mice with each spleen suspension plated in duplicate. Control mice, a total of 14, were injected with the diluent used for the ribosomal preparation.*

selves, do not rule out RNA as an important moiety, since double-stranded sections of RNA may have withstood these treatments. It is quite possible that the RNA stimulated the response to other antigens (3). There is no evidence, however, that the response stimulated was directed against meningococcal sialic acid antigen contaminating the ribosomal preparation or purposely added to it, because erythrocytes sensitized with these antigens were less sensitive to hemolysis than untreated erythrocytes. The finding that the efficacy of the ribosomes as immunizing agent is reduced by treatment with Pronase suggests that the specificity is conferred by a polypeptide moiety or by a carbohydrate which is removed from the ribosomes by cleavage of a polypeptide. The neuraminidase and heat stability of the ribosomes favor the possibility that the moiety involved is a carbohydrate other than sialic acid, possibly a Forssman-like antigen.

It is possible that the ribosomal preparations were contaminated with endotoxin and this contamination was responsible in part for their toxicity for rabbits. It is unlikely, however, that endotoxin was a major constituent of the preparations or that it was solely responsible for the immunological stimulation. In sucrose density gradient centrifugation and sedimentation studies, fresh ribosomal preparations sedimented as single components, and other constituents, if present, must have been in relatively low concentrations or directly attached to the ribosomes. Purified *E. coli* lipopolysaccharide did not elicit a PFC response, and, although *E. coli* ribosomes had the capacity to stimulate a PFC response, they could be distinguished from meningococcal ribosomes by their susceptibility to ribonuclease.

Since sheep erythrocytes were lysed only in the presence of complement, there is little doubt that plaque formation measured antibody formation or release from mouse spleen cells, or both. The specificity of these antibodies remains unknown, however. The initial surprising ob-

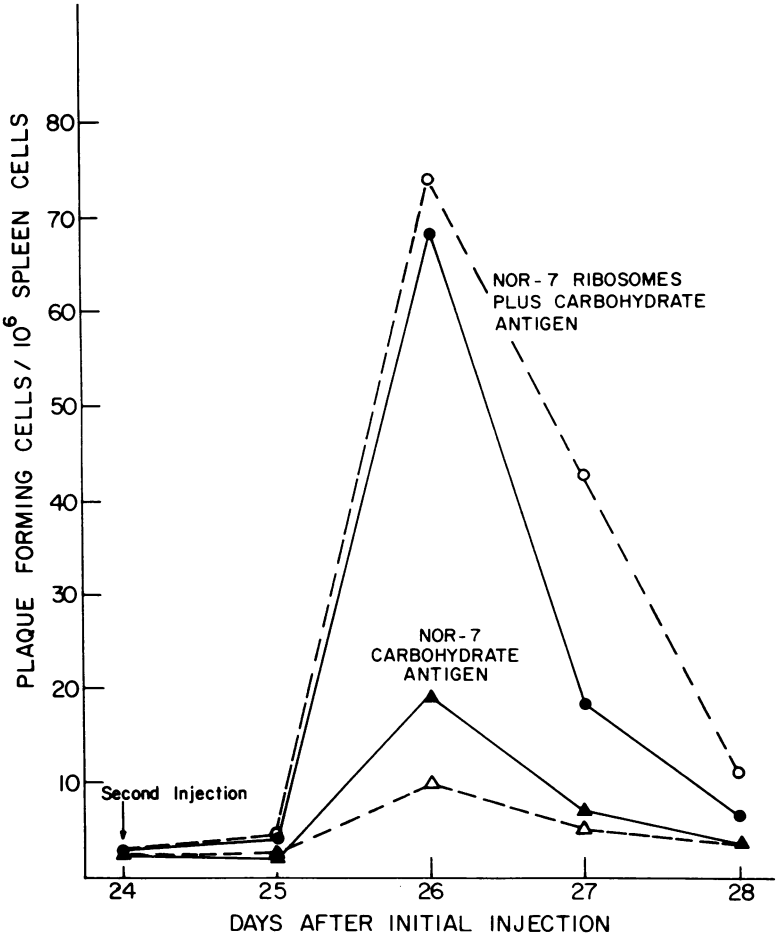

FIG. 5. *Secondary response of mice injected with carbohydrate plus ribosomal antigens (solid symbols and continuous lines) or carbohydrate antigen only (open symbols and dotted lines) and reinjected 24 days later with a combination of NOR-7 ribosomes and carbohydrate antigen or carbohydrate antigen alone. For other details, see legend of Fig. 4.*

servation that the control, unsensitized, erythrocytes were lysed at least as well as those that were treated with meningococcal antigen led to the study (Table 1) that demonstrated that sensitization with meningococcal antigen actually interfered with plaque formation. The surface of sheep erythrocytes contains sialic acids, 58% *N*-acetylneuraminic acid and 42% *N*-glycolylneuraminic acid (10). Neuraminidase releases the sialic acids from their attachment to surface carbohydrates (6, 10). Pronase, on the other hand, by its action on a polypeptide, removes most of the cell surface carbohydrate including the sialic acid (4). Thus, the experiment illustrated in Table 2 suggest the action of the antibody was directed towards a surface carbohydrate which was exposed by the action of neuraminidase. There is no evidence that it was directed against *N*-acetylneuraminic acid which is the monomer of the group B polysaccharide antigen (17). It is possible that sheep erythrocytes and meningococcal ribosomal antigens have identical or similar small carbohydrate components which stimulate an antibody response in the mouse. Despite this similarity, sheep erythrocytes, or corresponding rabbit antiserum, proved ineffective in protecting mice against meningococcal challenge.

Since the PFC response was determined directly, i.e., without the addition of antiserum against mouse immunoglobulin G, it measured, presumably, only immunoglobulin M antibody (28). In comparison with PFC responses to sheep erythrocytes (28) or other meningococcal antigen

TABLE 3. *Protective effect of NOR-7 ribosomes for mice*

Expt. no.	Ribosome treatment	Micrograms injected (RNA content)	LD_{50} dose[a] (-fold increase)
1	None	0	1[b]
	None	1.5	7
	None	5	17
	None	15	34
	None	50	>340[c]
2	None	0	1[b]
	None	50	100
	Ribonuclease	50	>100
	Pronase	50	10

[a] Mice were challenged a week later with four 10-fold dilutions of NOR-7. Forty mice were used for each median lethal dose (LD_{50}) titration.

[b] Four to six organisms.

[c] Less than 80 with MK-204 (group A), 85 with NOR-20 (group C).

(9), it is somewhat accelerated (Fig. 4), but it is not as rapid as the secondary response (Fig. 5).

The difficulty in assessing the value of a meningococcal antigen as a potential vaccine for man is illustrated in the experiments of Gotschlich et al. (12). Although the carbohydrate antigens tested proved to be immunogenic for man, no such evidence was obtained in mice or in 21 of 22 subhuman primates. Weiss and Long (25) protected mice with similar carbohydrate preparations, but the doses were very large (50 μg/mouse), and similar results were obtained with meningococcal polysaccharides of smaller molecular weight (Considine, Hammond, and Kingsbury, Bacteriol. Proc., p. 90, 1968). Thus, the protection of mice by large doses of ribosomal antigens, described in this paper, against homologous and heterologous meningococci offers encouragement that meningococcal ribosomes have immunogenic properties, but the mechanism of immunity requires further elucidation.

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