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Immunoprotective Activity of Ribosomes from *Haemophilus influenzae*

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Immunization with ribosomal preparations from *Haemophilus influenzae* type b elicited protective immunity in mice. Ribosomes from disrupted cells were isolated by differential centrifugation using sodium dodecyl sulfate. The washed ribosomes contained 25% protein and 75% ribonucleic acid and sedimented as a single peak on sucrose density gradient analysis with a sedimentation coefficient of 67*S*, using *Escherichia coli* ribosomes as a 70*S* marker. Immunodiffusion tests with antipolyribose phosphate serum showed that the ribosomes were free from capsular material. Mice immunized subcutaneously with ribosomes, with or without adjuvant, were challenged intraperitoneally with 100 to 1,000 mean lethal doses of *H. influenzae* type b suspended in gastric mucin. Significant protection was induced by ribosomes and was comparable to that obtained after sublethal infection with live cells. The protection was greatly enhanced after incorporation of ribosomes into adjuvants. Maximum protection (90 to 95%) was observed at 1 to 2 weeks after immunization. Ribosomes from a nonencapsulated strain of *H. influenzae* were as immunogenic as those from the encapsulated strain, demonstrating that the capsular material is not responsible for immunogenicity of *Haemophilus* ribosomes.

Haemophilus influenzae type b is the most common agent of bacterial meningitis in children between the ages of 3 months and 6 years (22, 29, 30, 42). Despite the availability of broad-spectrum antibiotics, the mortality rate has remained at 5 to 10% while 30 to 50% of the survivors develop chronic neurological complications such as blindness, deafness, convulsions, mental retardation, and hydrocephalus (19, 28, 29). There has been a significant rise in the incidence of *Haemophilus* meningitis during the past 25 years (18, 30, 39). Since present chemotherapy is not adequate to prevent the morbidity and mortality, immunoprophylaxis might provide an alternative way to control *Haemophilus* infections.

Polyribose phosphate, the capsular material of *H. influenzae* type b, has been utilized in a vaccine against *H. influenzae* infections (1, 2, 10, 22, 24, 26). Although antibody titers can be induced by immunization with capsular material, unequivocal evidence for immunoprotection has not been demonstrated. Immunization with killed whole-cell vaccine also has been unsuccessful in preventing respiratory infections (4, 8).

The pioneering work of Youmans and Youmans (45-47) has introduced a new concept in immunization by inducing significant protection in mice with ribosomal preparations from *Mycobacterium tuberculosis*. Similar ribosomal vaccines have been prepared from *Salmonella typhimurium* (13, 14, 31, 40, 41), *Staphylococcus aureus* (44), *Pseudomonas aeruginosa* (43), *Diplococcus pneumoniae* (33, 37), *Neisseria meningitidis* (35), *Vibrio cholerae* (12), *Listeria monocytogenes* (32), *Francisella tularensis* (3), *Streptococcus pyogenes* (25), *Histoplasma capsulatum* (9, 34), *Candida albicans* (E. S. Saunders, M. Solotorovsky, and R. P. Tewari, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, p. 89, F22), and *Leishmania enriettii* (20).

In the present investigation, the immunoprotective activity of ribosomes from encapsulated and nonencapsulated strains of *H. influenzae* was studied. Mice were immunized subcutaneously (s.c.) with different ribosomal preparations, and protection was assessed after intraperitoneal (i.p.) challenge. In addition, physical and chemical characteristics of ribosomes were studied.

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MATERIALS AND METHODS

Animals. White, male CF1 mice weighing 12 to 14 g were obtained from Charles River Co., Wilmington, Mass. The mice were divided randomly into groups of 10, housed in metal cages, and given feed and water ad lib. For each dose of vaccine, 20 mice were used and 20 nonvaccinated mice served as controls.

Organisms. *H. influenzae* type b, encapsulated (H-1), was obtained from a subculture of a primary isolate from the cerebrospinal fluid of a child with meningitis. The organism was classified as *H. influenzae* type b with typing serum (Difco) and was passaged through mice several times to maintain its virulence. The organism was reisolated from the brains of mice, subcultured once on Difco brain heart infusion (BHI) agar plates enriched with 5% Fildes supplement, and stored in a lyophilized state. The mean lethal dose (LD_{50}) of the strain for mice was 2×10^4 organisms suspended in gastric mucin given i.p. The avirulent strain of *H. influenzae* (H-U) was supplied by Grace Leidy, Columbia Presbyterian Hospital, New York. The avirulent strain did not possess the capsular material and was not typable with antiserum prepared from the virulent strain. *Escherichia coli* was obtained from our stock culture collection and was maintained in a lyophilized state.

Cultivation of organisms. *Haemophilus* cells were grown in BHI broth supplemented with 0.001% nicotinamide adenine dinucleotide (Sigma Chemical Co., St. Louis, Mo.), 0.002% hemoglobin, and 0.16% glucose in 1-liter Erlenmeyer flasks containing 250 ml of medium. The flasks were inoculated with 2.5 ml of a 10-h culture. The early-log-phase cells of *E. coli* were also grown in BHI broth. All flasks were incubated at 37°C in a gyratory shaker (New Brunswick Scientific Co., New Brunswick, N.J.) with a shaking speed of 150 rpm.

Preparation of ribosomes. Ribosomes were prepared by a modification of the procedure of Youmans and Youmans (47). The early-log-phase cells (8 h) of *H. influenzae* were harvested by centrifugation and washed twice in 0.01 M phosphate buffer (pH 7.0). All steps, unless otherwise specified, were performed at 4°C. Washed cells were resuspended in equal volumes of phosphate buffer (pH 7.0) containing 0.44 M sucrose, 0.25% sodium dodecyl sulfate (SDS), and 3×10^{-2} M $MgCl_2$, and were transferred to Braun (MSK) homogenizer glass bottles containing acid-washed glass beads (0.17 to 0.18 mm) equal to the wet weight of the cells. The cells were disrupted in the Braun homogenizer using three 2-min breaking periods, and the chamber was kept cool with liquid CO_2.

The homogenized mixture was centrifuged successively at 27,000 and 47,000 $\times g$ for 10 min each to sediment intact cells and cellular debris. The upper four-fifths of the supernatant was pipetted and centrifuged in a Beckman ultracentrifuge (L2-65B) at 270,000 $\times g$ for 65 min, using a 65 rotor. The pellet, designated as particulate fraction, was resuspended in 0.01 M phosphate buffer containing 10^{-2} M $MgCl_2$ to a concentration of 100 mg (wet weight) per ml. The particulate suspension was added to a flask containing an equal volume of 0.5% SDS in the phosphate buffer at room temperature. The flask was gently agitated by hand for 10 min. The suspension was transferred to a 25-ml Corex centrifuge tube and refrigerated at 4°C overnight in an ice bath to separate SDS by precipitation.

The following morning, SDS was sedimented by centrifugation at 37,000 $\times g$ for 15 min. The upper two-thirds of the supernatant was removed and centrifuged at 270,000 $\times g$ for 65 min to obtain the ribosomal fraction. The ribosomal fraction was washed twice and resuspended in the phosphate buffer. The ribosomal suspension was standardized on the basis of protein content. Inoculation of the ribosomal suspension on appropriate media did not reveal the presence of viable *Haemophilus* organisms.

Chemical and physical analyses. Protein was determined by the method of Lowry et al. (16), using bovine serum albumin fraction V as a standard. Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) were measured by the orcinol (27) and diaphenylamine (5) methods, respectively. Ribose and deoxyribose served as standards. Carbohydrate was assayed by the chromotropic acid (15) and the cysteine-H_2SO_4 (7) procedures, using glucose and ribose, respectively, as standards.

For density gradient analysis, 0.1 ml of ribosomal preparation (containing 400 μg of RNA) was layered over a 5-ml, 5 to 20% linear sucrose gradient prepared in 0.02 M tris(hydroxymethyl)aminomethane buffer (pH 7.8) containing 10^{-2} or 10^{-4} M $MgCl_2$. The gradient was centrifuged in an SW50L rotor at 45,000 rpm for 70 min at 4°C. The fractions were collected by upward displacement after piercing the bottom of the tube. The absorbance of the gradient at 260 nm was recorded by using a Gilford spectrophotometer equipped with a flow cell and a recorder. The optical density of each fraction at 235 and 280 nm was also determined.

The sedimentation coefficient of *H. influenzae* ribosomes was determined by the method of Martin and Ames (17), using ribosomes from *E. coli*, prepared by the method of Traub et al. (38), as a 70S marker.

Immunization of mice. Mice were immunized s.c. with ribosomal preparation with or without incorporation into Freund incomplete adjuvant (FIA). One part of the vaccine was mixed with three parts of the adjuvant. In some experiments, Hilleman adjuvant-65 (11) and *Bordetella pertussis* vaccine (Difco) were also used as adjuvants. Adjuvant-65 was prepared by emulsifying 0.4 g of aluminum monostearate in 8.6 g of peanut oil and 1 g of mannide monooleate (Arlacel A). These adjuvants were mixed with equal volumes of ribosomes. To obtain a stable suspension, the mixture of ribosomes and adjuvant was passed several times through a syringe. The final volume of vaccine for each mouse was 0.4 ml. For positive vaccine controls, formalin-killed and live-cell vaccines were also used. A formalin-killed cell vaccine was prepared by suspending a 6-h growth from Fildes-enriched BHI agar culture in 0.01 M phosphate buffer containing 0.85% NaCl and 0.5%

formaldehyde. The suspension was adjusted turbidimetrically to contain 15 × 10^8 cells/ml. Live-cell vaccine consisted of a sublethal dose of the organisms (5 × 10^3). Both live- and killed-cell vaccines were emulsified with FIA before immunization. Groups of 20 mice were used for each dose of vaccine, and 20 unvaccinated mice served as controls. Controls were injected s.c. with 0.4 ml of adjuvant or buffer.

Evaluation of immune response. At specified times after immunization, all mice were challenged i.p. with a lethal dose of *H. influenzae* type b suspension in 1 ml of 5% gastric mucin (pH 7.0) containing 1% glucose. The bacterial suspension for challenge was prepared from an 18-h Fildes agar plate culture. The growth was suspended in buffered saline and standardized turbidimetrically to contain the desired number of viable cells. The immune response was assessed by 7-day survival time and isolation of *Haemophilus* from internal organs of surviving animals. Animals were autopsied, and portions of spleen, liver, lung, brain, and heart blood were cultured on BHI-Fildes agar plates for the isolation of viable organisms. Statistical comparison between groups of mice was done by the chi-square test.

Chemicals. All chemicals used were of reagent grade or of the highest purity available commercially. Bovine pancreatic trypsin, Pronase, deoxyribonuclease, and bovine pancreatic ribonuclease were purchased from Worthington Biochemicals Corp., Freehold, N.J.; ribose, deoxyribose, and crystalline bovine serum albumin fraction V from Nutritional Biochemicals Corp., Cleveland, Ohio; mannide monooleate (Arlacel A) from Atlas Chemicals, Wilmington, Del.; and aluminum monostearate from Witco Chemical Co., New York, N.Y.

RESULTS

Characteristics of ribosomes. The ribosomal preparations of *H. influenzae* contained approximately 75% RNA and 25% protein but did not contain any detectable DNA or carbohydrate. When centrifuged over a sucrose density gradient, the ribosomal preparations sedimented as a single fraction (Fig. 1). The ratios between the absorbances at 260 and 280 nm and at 260 and 235 nm were 1.96 and 1.73, respectively. The sedimentation pattern was similar to that of *E. coli* ribosomes prepared under similar conditions (Fig. 2). The sedimentation coefficient of *Haemophilus* ribosomes was 67S as determined by the method of Martin and Ames (17), using *E. coli* ribosomes as the 70S marker. The ribosomes dissociated into 30S and 50S subunits after dialysis for 18 h against 0.01 M phosphate buffer containing 10^{-4} $MgCl_2$ (Fig. 3).

Immunogenicity of ribosomes. Mice were immunized with varying doses of ribosomes with or without incorporation into FIA. As positive controls, mice were immunized s.c. with

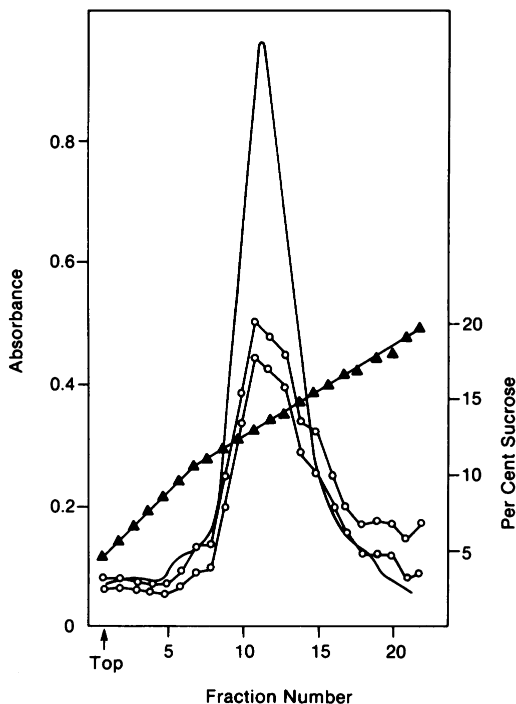

FIG. 1. *Absorbance pattern of 5 to 20% sucrose density gradient fractions of Haemophilus ribosomes after centrifugation at 45,000 rpm for 70 min at 4°C.*

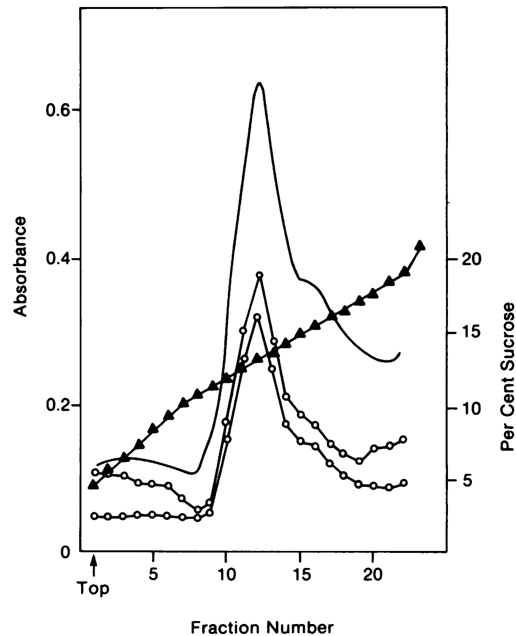

FIG. 2. *Absorbance pattern of 5 to 20% sucrose density gradient fractions of E. coli ribosomes after centrifugation at 45,000 rpm for 70 min at 4°C.*

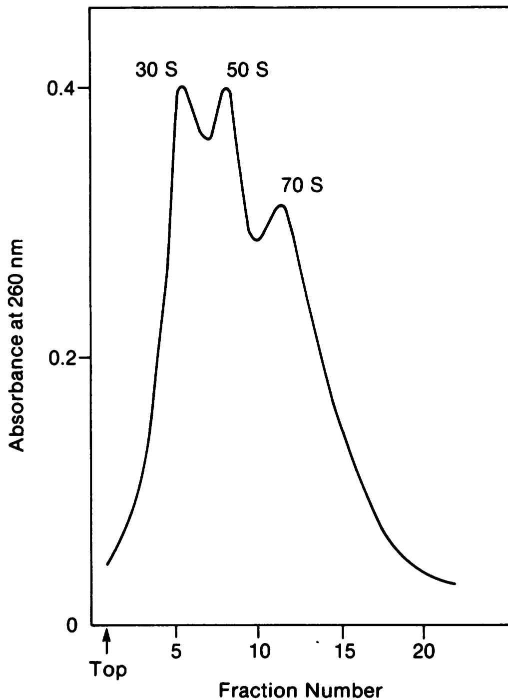

FIG. 3. *Absorbance pattern of 5 to 20% sucrose density gradient fractions of Haemophilus ribosomes (dialyzed against 10^{-4} M $MgCl_2$ buffer) after centrifugation at 45,000 rpm for 70 min at 4°C.*

either 5×10^3 live cells or 1.5×10^8 formalinized cells of *H. influenzae* (H-1) suspended in adjuvant. Nonimmunized controls received saline or saline plus adjuvant. Three weeks later, all animals were challenged i.p. with a lethal dose of *H. influenzae* type b suspended in gastric mucin. The results of such an experiment are shown in Table 1. Ribosomal preparations containing 100, 50, 25, 10, and 5 μg of protein, after incorporation into adjuvant, protected 60, 80, 60, 25, and 10% of the immunized mice, respectively. The same amounts of ribosomes without adjuvant provided lower levels of protection ($P < 0.01$). The degree of immunity was dose dependent from 5 to 50 μg of ribosomes. At the 100-μg level, however, the protection was lower than that with 50 μg of ribosomes ($P < 0.01$). The protection induced by immunization with ribosomes was comparable to that obtained with live cells and was superior to that elicited with killed cells ($P < 0.01$). Blood, spleen, liver, lungs, and brain of the surviving animals were free from viable cells of *H. influenzae*.

To determine the optimal interval between immunization and challenge, mice were inoculated with live cells or with ribosomal vaccines containing 50 μg of protein with or without incorporation into adjuvant. Twenty mice from each group were challenged at 1, 2, 3, 4, and 6 weeks after immunization (Table 2). Both live cells and ribosomal vaccines produced a high degree of protection against challenge with 100 LD_{50} of *H. influenzae* from 1 to 6 weeks after immunization. The live-cell vaccine elicited 90% protection at 1 week after immunization; this protection gradually declined to 70% after 6 weeks. Similarly, protection induced by ribosomal preparations reached a maximum of 95% at 1 week post-immunization; this declined to 60% after 4 weeks. No organism was isolated from the internal organs of the surviving animals. Similar results were obtained in two additional trials.

Effect of multiple immunization. The effect of multiple immunization was evaluated by s.c. inoculations of one, two, or three doses of ribosomes containing 50 μg of protein. The first dose of ribosomal preparation was incorporated into adjuvant; subsequent doses were given without adjuvant at weekly intervals. Controls included mice receiving 5×10^3 live cells of *H. influenzae*, saline, or adjuvant. Three weeks later all animals were challenged with 100 LD_{50}

TABLE 1. *Survival of immunized mice after challenge with 100 LD_{50} of H. influenzae type b*

Immunogen[a]	7-Day survival (%)	
	With adjuvant[b]	Without adjuvant
Live cells, 5×10^3		80
Killed cells, 1.5×10^8	45	
Ribosomes, 100 μg[c]	60	50
Ribosomes, 50 μg	80	65
Ribosomes, 25 μg	75	45
Ribosomes, 10 μg	25	10
Ribosomes, 5 μg	10	0
Controls	0	0

[a] Each group consisted of 20 mice.
[b] Freund incomplete adjuvant.
[c] Expressed as protein content of ribosomes.

TABLE 2. *Survival of immunized mice after challenge with 100 LD_{50} of H. influenzae type b at different intervals after immunizations*

Immunogen[a]	7-Day survival (%) after challenge at:				
	1 week	2 weeks	3 weeks	4 weeks	6 weeks
Live cells, 5×10^3	90	85	80	75	70
Ribosomes, 50 μg[b]	95	90	75	70	60
Controls	0	0	0	0	0

[a] Each group consisted of 20 mice.
[b] Expressed as protein content of ribosomes.

of *H. influenzae* (Table 3). Mice receiving one, two, and three doses of ribosomal vaccine showed 65, 70, and 85% protection, respectively. The internal organs of surviving mice were free from viable *H. influenzae*.

Effect of different challenge doses. Mice were inoculated s.c. with live cells, ribosomes, saline, or adjuvant. Immunized and nonimmunized mice were each challenged 7 days later with 100, 200, 500, or 1,000 LD_{50} of *H. influenzae* (Table 4). Immunization with live cells or ribosomes elicited maximal protection against challenge with 100 or 200 LD_{50} of *H. influenzae*. There was a significant reduction in the degree of protection when the challenge dose was increased to 500 or 1,000 LD_{50} ($P < 0.01$). No viable cells of *Haemophilus* were isolated from internal organs of surviving mice. Similar results were obtained in additional trials.

Comparative immunogenicity of ribosomes from nonencapsulated and encapsulated strains. Mice were immunized s.c. with 25 or 50 μg of ribosomes extracted from the encapsulated (H-1) and the nonencapsulated (H-U) strains of *H. influenzae*. Controls included mice receiving saline or adjuvant. Three weeks postimmunization, all animals were challenged with 100 LD_{50} of *H. influenzae* (Table 5). Ribosomal preparations from both nonencapsulated and encapsulated strains produced similar levels of protection: 70 to 75% with 50 μg and 50 to 55% with 25 μg of ribosomes. The internal organs of surviving mice were free from viable organisms. The immunity elicited by ribosomes was not type specific and was not dependent upon the presence of capsule.

Effect of different adjuvants on immunogenicity of ribosomes. Several experiments were performed to determine whether other adjuvants that produce a less inflammatory response could be substituted for FIA in immunization with ribosomal vaccine. Mice were inoculated with live cells, ribosomes, or ribosomes mixed with either FIA, *B. pertussis* antigen, or Hilleman adjuvant. All animals were challenged 7 days post-immunization (Table 6). Ribosomal fraction alone protected 60%, and ribosomes after incorporation into FIA raised the protection of immunized mice to 95% ($P < 0.01$). Incorporation of ribosomes into pertussis antigen or Hilleman adjuvant provided protection equivalent to that obtained with FIA. The internal organs of surviving animals were free from viable cells of *H. influenzae*.

DISCUSSION

These results demonstate that immunization of mice with ribosomal preparations from *H. influenzae* elicited significant protection against challenge with the homologous organism. The degree of immunity was dose dependent from 5 to 50 μg of ribosomes. However,

TABLE 3. *Effect of multiple immunizations on immunogenicity of ribosomal vaccine*

Immunogen[a]	No. of doses	7-Day survival (%)
Live cells, 5×10^3	1	75
Ribosomes, 50 μg[b]	1	65
	2	70
	3	85
Controls	3	0

[a] Each group consisted of 20 mice.
[b] Expressed as protein content of ribosomes.

TABLE 4. *Effect of different challenge doses on the immunogenicity of Haemophilus ribosomes*

Immunogen[a]	7-Day survival (%) after challenge with:			
	100 LD_{50}	200 LD_{50}	500 LD_{50}	1,000 LD_{50}
Live cells, 5×10^3	85	80	50	30
Ribosomes, 50 μg[b]	95	90	70	40
Controls	0	0	0	0

[a] Each group consisted of 20 mice.
[b] Expressed as protein content of ribosomes.

TABLE 5. *Survival of mice immunized with ribosomes from the nonencapsulated and encapsulated strains after challenge with 100 LD_{50} of H. influenzae type b*

Immunogen[a]	Source of ribosomes	7-Day survival (%)
Ribosomes, 50 μg[b]	Nonencapsulated	70
Ribosomes, 25 μg	Nonencapsulated	50
Ribosomes, 50 μg	Encapsulated	75
Ribosomes, 25 μg	Encapsulated	55
Controls		0

[a] Each group consisted of 20 mice.
[b] Expressed as protein content of ribosomes.

TABLE 6. *Effect of different adjuvants on immunogenicity of ribosomal vaccine*

Immunogen[a]	Adjuvant	7-Day survival (%)
Live cells, 5×10^3	Freund	85
Ribosomes, 50 μg[b]	None	60
	Freund	95
	Pertussis	85
	Hilleman	90
Controls		0

[a] Each group consisted of 20 mice.
[b] Expressed as protein content of ribosomes.

at the 100-μg level the protection was lower than that with 50 μg of ribosomes, suggesting an induction of tolerance due to an excess of antigens. The degree of protection elicited by immunization with ribosomes was comparable to that obtained with live cells and was far superior to the immunity induced by killed cells. The prolonged survival of live cells in the host tissues, providing continuing antigenic stimulus, has been proposed as a mechanism by which live cells induce better immunity (5, 46). The superiority of ribosomal vaccine may be due to the labile nature of ribosomal antigens, which are preserved by the procedure used in ribosomal extraction but destroyed in killed-cell vaccine.

The incorporation of *Haemophilus* ribosomes into adjuvant was necessary to obtain a high degree of protection. This is in conformity with the enhancing effect of adjuvant on immunogenicity of ribosomal preparations from *Mycobacterium* (47), *Staphylococcus* (44), *Pneumococcus* (33, 37), *Streptococcus* (25), and *Histoplasma* (9). In contrast, adjuvant was not required for immunization with ribosomal preparations from *Salmonella* (13, 31, 40), *N. meningitidis* (35), *V. cholerae* (12), and *F. tularensis* (3). The reason for the difference in the requirement of exogenous adjuvant by the ribosomes from different microorganisms is not known at the present time. FIA produces a severe inflammatory response and is unacceptable for human use. However, a number of other adjuvants (viz., pertussis vaccine, Hilleman adjuvant-65, sodium aliginate, and dextran) that produce less severe inflammatory response may be acceptable for human immunization. In the present study, two of these adjuvants, pertussis vaccine and Hilleman adjuvant-65, were highly effective in enhancing the immunogenicity of *Haemophilus* ribosomes. Pertussis vaccine is known to enhance the immunogenicity of tetanus and diphtheria toxoids in the DTP vaccine used for immunization of children.

The ribosomes from a nonencapsulated strain of *H. influenzae* were as immunogenic as that of the encapsulated strain, indicating that the capsular material (PRP) is not responsible for immunogenicity of *Haemophilus* ribosomes. This is further substantiated by the fact that ribosomal preparations from both encapsulated and nonencapsulated strains were free from PRP by chemical tests. The immunodiffusion and passive hemagglutination tests also failed to detect the capsular material in ribosomal preparations of *H. influenzae* (our unpublished data). These results are analogous to recent findings of two separate groups of investigators on immunogenicity of subcellular fractions from *Streptococcus pneumoniae*. Both a ribosomal preparation (33) and an undefined subcellular preparation (36) of *Pneumococcus* that were free from detectable capsular polysaccharide antigens elicited significant protection in mice. The protection was nonspecific since immunization with one serotype protected the immunized mice against challenge with other serotypes of *S. pneumoniae*.

The capsular material of *H. influenzae* type b is currently under investigation as a vaccine (2, 22–24). Although injection of the polysaccharide antigen to adults and children over 2 years old elicited a serum antibody response (22), unequivocal evidence for immunoprotection has not been demonstrated. Infants up to 6 months of age, the logical candidates for a *Haemophilus* vaccine, produced little or no antibody response after injection with PRP (22). The testing of immunoprotective activity of PRP is also hampered by the fact that the material acts as a hapten in experimental animals (22). To overcome this problem, Anderson and Smith isolated a high-molecular-weight polysaccharide from *H. influenzae* type b that induced serum antibody response in weanling rabbits (P. Anderson and D. H. Smith, Prog. Abstr. Intersci. Conf. Antimicrob. Agents Chemother., 14th, San Francisco, Calif., Abstr. 157, 1974). Robbins et al. (23) have introduced another concept by demonstrating the formation of antibody by feeding nonvirulent *E. coli* (K-100) that cross-reacts with the capsular material of *H. influenzae* type b. At present they are exploring the possibility of deliberate colonization with the nonpathogenic enteric bacteria as a method of immunization against encapsulated bacteria in children. The immunogenicity of the ribosomal preparations observed in the present study provides an alternative approach to immunoprophylaxis of *Haemophilus* infections.

At present, we are involved in the characterization of protective antigen in *Haemophilus* ribosomes and the mechanism of immunity elicited by immunization with the ribosomal preparations. Preliminary experiments have demonstrated that the ribosomal protein is the major immunogenic moiety in *Haemophilus* ribosomes. The details of these findings will be reported in a subsequent publication.

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