INFECTION AND IMMUNITY, May 1979, p. 346-351
0019-9567/79/05-0346/06$02.00/0

Vol. 24, No. 2

Evaluation of a Ribosomal Vaccine Against Pertussis

LEANNE H. FIELD,[1] CHARLOTTE D. PARKER,[1]* CHARLES R. MANCLARK,[2] AND L. JOE BERRY[1]

Department of Microbiology, The University of Texas at Austin, Austin, Texas 78712,[1] and Bureau of Biologics, Food and Drug Administration, Bethesda, Maryland 20014[2]

Received for publication 26 January 1979

A crude ribosomal vaccine derived from *Bordetella pertussis* administered to ICR and N:NIH (SW) strains of mice protected them effectively against a standardized intracranial challenge. The dose of vaccine that protected half the mice was less for N:NIH (SW) than for ICR mice and compared favorably with a killed reference vaccine. Ribosomes prepared from bacteria ground with washed sea sand were more immunogenic than those obtained by rupture with alumina or with a Braun homogenizer. The protective effect of the crude ribosomes was not an innate part of the organelle but was due to a substance or substances that could be removed from them by a 1 M NH_4Cl wash. The material in the wash was highly immunogenic and retained both the histamine-sensitizing and leukocytosis-promoting properties. It lost much of the dermonecrotic activity and was poorly pyrogenic in rabbits. The most potent pyrogen was present in the washed ribosomes, which, apparently, retained the endotoxic components of the cell wall. The best vaccines permitted acceptable weight gain in the immunized mice.

Since the introduction of a standardized pertussis vaccine in the 1940s, the incidence of this disease has steadily decreased. Pertussis vaccines remain, however, difficult to produce and assay. The vaccines currently in use may cause a variety of reactions, including pain, induration, and erythema at the injection site and fever. Serious sequelae such as convulsions, encephalopathy, or death are rare but have been associated with the administration of pertussis vaccine (7).

Bordetella pertussis produces a number of biologically active components, many of which are present in the vaccine. A partial list includes: protective antigen, histamine-sensitizing factor (HSF), lymphocytosis (leukocytosis)-promoting factor (LPF), dermonecrotic toxin (DNT), endotoxin, hemagglutinin, and hemolysin (9, 10). Attempts to isolate protective antigen free of the other biological components have been unsuccessful (9).

In recent years, subcellular vaccines derived from both procaryotic and eucaryotic pathogens have been isolated according to techniques first developed by Youmans and Youmans (14). These authors have summarized the work done with vaccines of this type and conclude that they are uniformly effective in experimental animals (15). Not only do the ribosomally derived vaccines afford high levels of protection at small dose levels, but the duration of the immunity they provide is often greater than that seen with many conventional killed whole-cell preparations.

The goal of the research summarized below has been to prepare a ribosomal vaccine from *B. pertussis* and to test its potency and toxicity in mice.

MATERIALS AND METHODS

Organisms. *B. pertussis* strains 114 and 18323 were used throughout these studies. Both strains were lyophilized, and new vials were opened for each experiment. Strain 114 was used to prepare ribosomal fractions; strain 18323 was used as the challenge organism in the pertussis vaccine potency assay.

Mice. Female ICR mice (Texas Inbred Mouse Co., Houston, Tex.) weighing 14 to 16 g were used for assaying the potency of vaccines and for the mouse weight gain test. CFW mice (Carworth Division, Charles River Farms, Wilmington, Mass.) raised in departmental animal facilities were used to assay for DNT, LPF, and HSF. Parallel pertussis vaccine potency assays and mouse weight gain tests were performed using N:NIH (SW) mice.

Cell growth and harvesting. Cells were grown in the liquid medium described by Stanier and Scholte (13), modified to contain 1.5 g of Trizma base (Sigma Chemical Co., St. Louis, Mo.) per liter. A lyophilized culture of strain 114 was reconstituted in sterile 1% Casamino Acids (Difco Laboratories, Detroit, Mich.) and cultured on Bordet Gengou medium (2) containing 1.5% proteose peptone (Difco). After 3 days of incubation at 35°C, the culture was transferred to several fresh Bordet Gengou plates and incubated for an additional 24 h. Growth was then harvested into 5 ml of liquid medium, adjusted to a standard turbidity, and used to inoculate a 500-ml flask containing 250 ml of liquid medium (seed culture). The flask was placed in a rotating shaker (180 rpm) at 35°C for 24 h. The growth from two such seed cultures was used to inoc-

ulate 24 similar flasks using a 5% (by volume) inoculum. Cells were harvested in mid- to late-log phase of growth at 24 to 28 h postinoculation by centrifugation at 8,000 × *g* for 15 min. Cell pellets from each harvest were pooled, weighed, frozen in a dry-ice–ethanol bath, and stored at −70°C until used for preparation of crude ribosomes.

Cell breakage. Three methods of cell breakage were used in the isolation of crude ribosomes: (i) disruption in a Braun MSK mechanical cell homogenizer (Bronwill Scientific, Rochester, N.Y.); (ii) grinding with alumina (type 305, Sigma); and (iii) grinding with acid-washed sea sand (Fisher Scientific Co., Fair Lawn, N.J.).

Cells to be broken in the Braun cell homogenizer were thawed and suspended in buffer A (0.02 M tris(hydroxymethyl)aminomethane-hydrochloride + 0.01 M $MgCl_2$, pH 7.4) to a concentration of 1,000 opacity units (U.S. opacity unit; 1 opacity unit corresponds to approximately 6×10^8 *B. pertussis* cells). Thirty milliliters of cell suspension was placed in a Duran sample flask (50-ml capacity) with 44 g of 0.17- to 0.18-mm glass beads (VWR Scientific Co., Houston, Tex). The cell suspension was alternatively shaken and rested for a total of 3 min, using a 15-s on/off cycle. The system was cooled throughout homogenization with liquid CO_2. The homogenate was centrifuged at 13,200 × *g* to remove the glass beads before further differential centrifugation for isolation of crude ribosomes.

Frozen cell pellets to be broken by grinding were placed in a cold mortar and mixed with alumina or sand in the ratio (by weight) of 1 part cells to 1.5 parts grinding agent. Approximately half the alumina or sand was added initially, and the cells were ground with a cold pestle until a smooth paste was formed. The remaining alumina or sand was added, and grinding was continued for 10 to 15 min. Ruptured cells were suspended in 1.5 ml of buffer A per g (wet weight) of cells. The suspension was centrifuged at 13,200 × *g* to remove the grinding agent.

Isolation of crude ribosomes. The supernatant from each preparation was incubated at 4°C for 15 min in the presence of 3 μg of deoxyribonuclease per ml (salt-free lyophilized powder from bovine pancreas, Sigma). This was followed by two centrifugations at 4°C for 30 min at 20,000 and 30,000 × *g* to remove unbroken bacterial cells and cellular debris. The resulting supernatant was then centrifuged at 4°C for 3 h at 170,000 × *g* to pellet the ribosomes. After the centrifugation, the supernatant from each preparation was frozen at −70°C and saved for comparative assays. The surface of the ribosomal pellet from each preparation was gently washed two to three times with buffer B (0.05 M tris(hydroxymethyl)aminomethane-hydrochloride + 0.02 M $MgCl_2$ + 0.05 M NH_4Cl, pH 7.7) to remove any loosely associated material. The washed pellet was then covered with 0.5 to 1.0 ml of buffer B and held for 12 to 18 h at 4°C.

The ribosomal pellet was resuspended in buffer B and adjusted to a concentration of approximately 10 mg of ribosomes per ml based on the assumption that 14.4 optical density units at 260 nm is equivalent to 1 mg of ribosomes per ml (12). This calculation measures only intact ribosomal particles, as it is based on absorbance of double-stranded ribonucleic acid in ribosomes. The crude ribosomes were divided into aliquots, quick frozen, and stored at −70°C.

Preparation of clarified ribosomes, high-salt wash, and washed ribosomes. Crude ribosomes were centrifuged at 13,200 × *g* for 10 min. The pellet from this centrifugation (insoluble material) was resuspended in buffer B to the original volume and stored at −70°C. The supernatant fluid (clarified ribosomes) was divided into two parts. One portion was stored at −70°C for further analysis. The remaining portion was brought to a concentration of 1 M with NH_4Cl, held overnight at 4°C, and then centrifuged at 170,000 × *g* for 3 h. The resulting supernatant fluid (high-salt wash) was retained and stored at −70° until used. The ribosomal pellet from this centrifugation was resuspended to the original volume in buffer B containing 0.5 M NH_4Cl and immediately recentrifuged to pellet the ribosomes. The final ribosomal pellet was resuspended to the original volume with buffer B (washed ribosomes) and stored at −70°C.

Chemical determination. Protein was measured by the method of Lowry et al. (6), using bovine serum albumin (fraction V, Sigma) or bovine plasma albumin (crystallized, Armour Pharmaceutical Co., Chicago, Ill.) as the standard. Ribonucleic acid concentration was determined by the orcinol procedure (3), using soluble *Escherichia coli* ribonucleic acid (General Biochemicals, Chagrin Falls, Ohio) as the standard. Deoxyribonucleic acid was measured by the diphenylamine assay (3), using calf thymus deoxyribonucleic acid (Calbiochem, La Jolla, Calif.) as a standard.

Malate dehydrogenase activity. The enzyme malate dehydrogenase, which has been shown to be intracellular in *B. pertussis* (4), was measured in 170,000 × *g* supernatants using a modification of the procedure described by Ochoa (11). The amount of enzyme present served as a means of comparing efficiency of cell rupture. The rate of formation of malate dehydrogenase from oxalacetate was measured by following the rate of oxidation of reduced nicotinamide adenine dinucleotide at 340 nm at 25°C. An enzyme unit was defined as that amount of enzyme required to cause a decrease in absorbance of 1.0 optical density unit per min.

Pertussis vaccine potency assay. Tests were performed in accordance with section 620.4, *Title 21 Code of Federal Regulations* (1), with the exception that in preliminary assays 0.1 ml of each graded vaccine dose was injected subcutaneously (s.c.) into the nuchal area of female ICR mice. Before injection, all experimental vaccine preparations were heated at 56°C for 30 min to inactivate DNT. Median immunizing doses were calculated by Wilson-Worcester (logit) analysis.

Mouse weight gain test. Groups of at least 10 female ICR mice were injected intraperitoneally (i.p.) or s.c. with crude ribosomal preparations diluted in buffer B in a volume of 0.5 or 0.1 ml, respectively. The weight of each group of mice was determined just before injection and at the end of 3 and 7 days after injection. The average weight gain (or loss) per mouse was calculated for each time interval.

DNT. Four-day-old CFW or N:NIH (SW) mice were injected s.c. in the nuchal region with dilutions of

vaccine contained in 0.05 ml. Diluent alone and corresponding doses of vaccines heated to 56°C for 30 min were used as controls. At the end of 18 to 24 h, skin reactions (necrosis) or death was recorded, using a scale of 0 to 4+.

HSF. HSF was assayed by injecting ribosomal fractions (heated to 56°C for 30 min) intravenously (i.v.) (in a volume of 0.1 ml) or i.p. (in a volume of 0.5 ml) into 20- to 25-g mice and challenging them i.p. 3 to 5 days later with 1 mg of histamine base (histamine diphosphate, Sigma).

LPF. Mice were injected i.v. (0.1 ml), i.p. (0.5 ml), or s.c. (0.1 ml) with various doses of ribosomal preparations (heated to 56°C, 30 min). On day 3 or 4 postinjection, blood samples were obtained from the tail vein, and leukocyte counts and differential counts were done. The percentage of lymphocytes was determined in smears stained with Camco Quick Stain (Scientific Products, Grand Prairie, Tex). One hundred cells were examined per slide.

Pyrogenicity. The *Limulus* amoebocyte lysate was used as a screening test for lipopolysaccharides, and rabbits were then used to test the pyrogenicity of the various fractions according to procedures previously described (8).

RESULTS

Comparison of cell breakage methods. In Table 1 the results obtained with each of the three methods for breaking pertussis cells are summarized. Grinding with alumina or sea sand resulted in the most efficient cell breakage (as measured by enzyme units of malate dehydrogenase released) and gave the highest yields of ribosomes. However, the ribosomes isolated from cells ground with sea sand were clearly more immunogenic than those isolated by the other methods. In subsequent experiments, therefore, cells were ruptured by grinding with sea sand.

TABLE 1. *Comparison of cell breakage techniques*

Method of cell breakage	Malate dehydrogenase[a] (U)	Crude ribosomes recovered (mg/g, wet wt)	Survival (%) of immunized mice[b] (survivors/total)
Braun homogenization	7.1	1.5	33 (3/9)
Grinding with alumina	12.4	2.4	33 (3/9)
Grinding with acid-washed sea sand	11.9	3.1	89 (8/9)

[a] Units per milligram of protein were measured in 170,000 × *g* supernatant fluids obtained during isolation of crude ribosomes.

[b] Percent survival of female ICR mice immunized s.c. with 100 μg of crude ribosomes (based on absorbancy at 260 nm). Mice were challenged intracerebrally with *B. pertussis* strain 18323 14 days postimmunization. Survivors/number infected was scored 14 days postchallenge.

Immunogenicity of crude ribosomes. The results of immunogenicity tests with three replicate preparations of crude *B. pertussis* ribosomes (heated at 56°C for 30 min) administered s.c. to ICR mice are presented in Table 2. The median effective dose did not vary significantly from one preparation to another, and the average was 46.9 μg. Two of the same preparations given i.p. were tested in N:NIH (SW) mice with the results shown in Table 3. For these animals, the average value was 9.3 μg. The protective potency of the crude ribosomal preparations was destroyed by heating at 80°C for 30 min before their use as a vaccine (data not shown).

Chemical characterization of crude ribosomes. The chemical composition of a representative crude ribosomal preparation adjusted to 10 mg/ml (as determined by optical density at 260) was found to be 12.8 mg of protein per ml, 6.1 mg ribonucleic acid per ml, and 0.740 mg of deoxyribonucleic acid per ml. The protein-ribonucleic acid ratio was 2:1 rather than the expected 1.5:1. This suggests that extraneous protein "contaminated" the ribosomes.

Biological activities of crude ribosomes. (i) Toxicity. A dose of 1 μg of unheated crude

TABLE 2. *Protective effect of crude ribosomal preparations in ICR mice*

Prepn no.	Survival/no. of mice immunized s.c. with heated crude ribosomes at the dosage shown[a]			ED_{50}[b] (μg)
	100 μg[c]	50 μg	25 μg	
8	6/8	6/11	3/12	47.6
9	8/10	5/10	2/11	50.8
10	7/7	5/10	3/12	42.2

[a] Mice were challenged intracerebrally with *B. pertussis* strain 18323 14 days postimmunization and scored 14 days postchallenge.

[b] ED_{50}, calculated median effective dose.

[c] Dosage of crude ribosomes based on absorbancy at 260 nm.

TABLE 3. *Protective effect of crude ribosomal preparations in N:NIH(SW) mice*

Prepn no.	Survival/no. of mice immunized i.p. with heated crude ribosomes at the dosage shown[a]			ED_{50}[b] (μg)
	40 μg[c]	10 μg	2.5 μg	
8	30/33	14/33	7/33	9.45
9	25/32	20/33	5/33	9.24

[a] Mice were challenged intracerebrally with *B. pertussis* strain 18323 14 days postimmunization and scored 14 days postchallenge.

[b] ED_{50}, calculated median effective dose.

[c] Dosage of crude ribosomes based on absorbancy at 260 nm.

ribosomes given s.c. to mice was sufficient to kill all mice within 24 h. Heating the preparations to 56°C for 30 min before injection destroyed DNT and prevented deaths. Preparations 8, 9, and 10 gave a strong 4+ dermonecrotic reaction with 0.5 μg, a 2 to 3+ response with 0.05 μg, and no response with 0.005 μg.

Toxicity in heated crude ribosomal vaccines was observed when high doses (50 μg or greater) were injected s.c. during immunization of mice used in potency assays. Two to 3 days postimmunization, an indurated lesion could be felt under the skin at the injection site. After 7 to 10 days the swelling had decreased, but a visible necrotic lesion that resulted in local hair loss appeared. Healing took place after several weeks. The size of the lesion was roughly proportional to the dose of vaccine injected, although size varied somewhat from one crude ribosomal preparation to another. The presence of skin lesions did not appear to affect, however, the health of the mice or to cause groups of mice given 50 to 100 μg of vaccine to fail the weight gain test (see Table 4).

(ii) The mouse weight gain test. Table 4 shows the results of a representative test performed with crude ribosomal preparation 10 in ICR mice. Administration i.p. of the ribosomal vaccine at 50- and 100-μg dose levels resulted in mortalities and in low weight gain by the survivors at 3 days postinjection. Weight gain was almost normal at 7 days. Subcutaneous administration of the same vaccine also afforded protection against challenge and was less toxic.

(iii) LPF. The presence of LPF in crude ribosomal preparation 10 was measured after i.p., s.c., and i.v. administration of the vaccine in adult mice. Figure 1 shows the average numbers of leukocytes and of lymphocytes per cubic millimeter in venous blood drawn from mice 3 days postinjection. The i.v. and i.p. routes of injection resulted in larger numbers of total circulating leukocytes than an s.c. injection. Only an i.p. dose of vaccine caused a sharp rise to more than 20,000 cells per mm^3 in lymphocytes. The same preparation given i.v. and s.c. increased the lymphocyte number to a maximum of about 13,000 per mm^3.

(iv) Histamine-sensitizing activity. The median histamine-sensitizing doses for crude ribosomal preparations 8 and 9 after i.v. administration to adult mice were 0.336 and 0.392 μg, respectively.

Fractionation of crude ribosomes. To determine whether the immunogenic activity associated with crude ribosomes was an integral part of the ribosomal complex or was only associated with it, crude ribosomes were clarified by differential centrifugation and then washed with 1 M NH_4Cl. The activity of the four fractions obtained by this process was assayed in a variety of tests and compared with crude ribosomes and whole cells from the same preparation. Each fraction was quantitated on the basis of its protein content rather than on its estimated total ribosomal content, since some of the fractions did not have intact ribosomes.

Table 5 summarizes an evaluation of the immunogenicity of the six preparations in N:NIH (SW) mice. On the basis of their protein content, by weight, twice the dose of crude ribosomes had to be given to achieve the same 50% protection as with whole cells. Clarification of the ribosomes by low-speed centrifugation reduced their potency nearly fourfold but did not cause a significant change in protein content. The insoluble material that sedimented in the clarification process was, however, low in protein, yet effective as an immunogen. The clarified ribosomes that were washed with 1 M NH_4Cl lost most of their immunogenic material, yet retained nearly half of the original amount of protein. The material present in the high-salt wash was low in protein but possessed potency.

TABLE 4. *Weight gain test in ICR mice*

Vaccine	Dose[a] (μg)	Wt gain (g) per mouse after:		Mortality (dead/total) after:	
		3 days	7 days	3 days	7 days
Control	Buffer B	+1.83	+4.0	0/12	0/12
Crude ribosomal prepn 10 (i.p.)	100	+0.69	+3.92	4/12	9/12
	50	+0.67	+1.79	0/12	3/12
	25	+0.70	+2.0	0/12	1/12
Crude ribosomal prepn 10 (s.c.)	100	+2.11	+2.73	0/12	0/12
	50	+2.54	+2.54	0/12	0/12
	25	+1.54	+3.17	0/12	0/12

[a] Dose of crude ribosomes based on absorbancy at 260 nm. Ribosomal preparation 10 was heated to 56°C for 30 min before injection. For further details, see text.

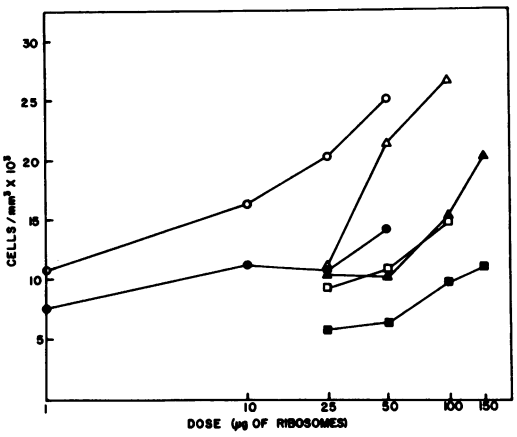

FIG. 1. *LPF activity at 3 days in ICR mice after i.v., i.p., and s.c. administration of different doses of a ribosomal vaccine (preparation 10). Open symbols indicate leukocytes per cubic millimeter × 10^3, and closed symbols indicate lymphocytes per cubic milliliter × 10^3: (○, ●) i.v. administration; (□, ■) s.c. administration; (△, ▲) i.p. administration. Doses were calculated on the basis of the optical density reading at 260 nm.*

TABLE 5. *Potency of cellular and subcellular preparations*

Immunogen	Protein content (mg/ml)	ED_{50}[a] (µg)
Whole cells	19.4	2.3
Crude ribosomes	20.0	4.6
Clarified ribosomes	18.9	19.5
Insoluble material	1.6	5.8
Washed ribosomes	8.9	204
High-salt wash	4.8	6

[a] The median effective dose (ED_{50}) was calculated from results obtained with mice immunized i.p. with fivefold dilutions of each preparation, challenged intracerebrally 14 days later with *B. pertussis* strain 18323, and scored 14 days postchallenge.

Whole cells, crude ribosomes, washed ribosomes, and high-salt wash were assayed, on the basis of their protein content, for HSF, LPF, and DNT in mice and for pyrogenicity in rabbits. Table 6 summarizes the results. Only the washed ribosomes showed a decrease in HSF. The other three preparations were active within a range of about twofold. All were similarly effective in LPF activity, whereas only whole cells and crude ribosomes had equivalent amounts of DNT activity. The other two were one-tenth as active. Washed ribosomes were unexpectedly pyrogenic since only 1.5 ng (expressed as protein) gave a significant febrile response. Ten times the dose of crude ribosomes and 100 times the dose of whole cells and high-salt wash were necessary to elicit the same temperature elevation.

DISCUSSION

Crude ribosomes prepared from *B. pertussis* provide an effective vaccine. When the median effective dose is based on the protein content of the vaccine, whole cells, crude ribosomes, an insoluble material obtained from crude ribosomes, and the high-salt wash of clarified ribosomes are all effective within a range of about 2.5-fold. The absolute quantity of active immunogen involved is not known, however, at this time.

The comparative effectiveness of the different techniques used to rupture the bacteria is of some interest. Apparently, the Braun homogenizer was less effective, possibly because of the specific conditions used. Both grinding methods seemed to yield closely similar results except that the material from sea sand was more immunogenic. It can only be surmised that the alumina adsorbed the protective antigen.

The toxicity of the vaccines was not eliminated by any of the methods used in their preparation. The question as to whether animals can be protected against pertussis by a prophylactic that contains no HSF nor LPF activity remains unresolved. It is possible, however, to greatly reduce the dermonecrotic activity and pyrogenicity of preparations (see Tables 5 and 6) without loss of immunogenicity.

The immunogenicity of crude *B. pertussis* ribosomes is largely eliminated by washing with 1 M NH_4Cl. This treatment has been found to remove the protective antigen from a ribosomal vaccine derived from *Salmonella typhimurium* without altering the ability of the ribosomes to act in an in vitro protein-synthesizing system (5). Thus, the integrity of the ribosomes remains,

TABLE 6. *Biological activities of different pertussis vaccines administered on the basis of their protein content*

Prepn tested	HSD_{50}[a] (µg)	LPF activity[b] (µg)	DNT activity[c] (µg)	Pyrogenicity[d] (ng)
Whole cells	23.5	100	0.25	150
Crude ribosomes	53	137.6	0.25	15
Washed ribosomes	250	169.2	2.50	1.5
High-salt wash	35	127.1	2.50	150

[a] HSD_{50}, median histamine-sensitizing dose.
[b] Amount (micrograms) required for a twofold increase in leukocytes per cubic millimeter.
[c] Amount (micrograms) required for a 2+ necrotic response.
[d] Amount (nanograms) required to raise the temperature of three rabbits an average of 0.5°C per animal.

but these organelles no longer possess an associated antigenicity. Since the material present in the NH_4Cl wash seems to contain the substance or substances responsible for protecting mice against infectious challenge, the ribosomes themselves can hardly be implicated in this process. In future work, it will be important to identify the antigens in the high-salt wash that are responsible for the protective immunity.

ACKNOWLEDGMENT

This research was supported by Public Health Service grant AI-12955 from the National Institute of Allergy and Infectious Diseases to C. Parker.

LITERATURE CITED

1. **Code of Federal Regulations.** 1978. Title 21. U.S. Government Printing Office, Washington, D.C.
2. **Field, L. H., and C. D. Parker.** 1977. Pertussis outbreak in Austin and Travis County, Texas, 1975. J. Clin. Microbiol. **6**:154–160.
3. **Herbert, D., P. J. Phipps, and R. E. Strange.** 1971. Chemical analysis of microbial cells, p. 308–320. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 5B. Academic Press Inc., New York.
4. **Hewlett, E. L., M. A. Urban, C. R. Manclark, and J. Wolff.** 1976. Extracytoplasmic adenylate cyclase of *Bordetella pertussis*. Proc. Natl. Acad. Sci. U.S.A. **73**: 1926–1930.
5. **Hoops, P., N. E. Prather, L. J. Berry, and J. M. Ravel.** 1976. Evidence for an extrinsic immunogen in effective ribosomal vaccines from *Salmonella typhimurium*. Infect. Immun. **13**:1184–1192.
6. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
7. **Manclark, C. R.** 1976. The current status of pertussis vaccine: an overview. Adv. Appl. Microbiol. **20**:1–7.
8. **Manclark, C. R., M. A. Urban, E. P. Summers, and J. H. Vickers.** 1977. Assay of pertussis vaccine toxicity by a rat-paw-oedema method. J. Med. Microbiol. **10**:115–120.
9. **Morse, S. I.** 1976. Biologically active components and properties of *Bordetella pertussis*. Adv. Appl. Microbiol. **20**:9–26.
10. **Munoz, J. J., and R. K. Bergman.** 1977. *Bordetella pertussis*—immunological and other biological activities. Marcel Dekker, Inc., New York.
11. **Ochoa, S.** 1955. Malic dehydrogenase from pig heart. Methods Enzymol. **1**:735–739.
12. **Ravel, J. M., and R. L. Shorey.** 1971. GTP-dependent binding of aminoacyl-tRNA to *Escherichia coli* ribosomes. Methods Enzymol. **20**:306–316.
13. **Stanier, D. W., and M. J. Scholte.** 1971. A simple chemically defined medium for the production of phase I *Bordetella pertussis*. J. Gen. Microbiol. **63**:211–220.
14. **Youmans, A. S., and G. P. Youmans.** 1965. Immunogenic activity of a ribosomal fraction obtained from *Mycobacterium tuberculosis*. J. Bacteriol. **89**:1291–1298.
15. **Youmans, G. P., and A. S. Youmans.** 1975. Implications of immunization against infectious diseases with ribosomal and RNA vaccines, p. 453–468. *In* E. Neter and F. Milgrom (ed.), The immune system and infectious diseases. Fourth International Convocation on Immunology, Buffalo, N.Y., 1974. S. Karger, Basel.