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Preparation and Effect of Different Adjuvants on the Immunogenic Activity of Mycobacterial Ribosomal Fraction

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Several emulsified and two nonemulsified incomplete adjuvants were examined for their adjuvant activity by use of mycobacterial ribosomal fractions as a substrate. A good adjuvant is defined as one which produces a high immunological response with the ribosomal fraction in mice to infection with virulent tubercle bacilli. Freund's incomplete adjuvant, consisting of Aquaphor and heavy mineral oil, and Arlacel A plus hexadecane were the best adjuvants tested. Aquaphor plus light mineral oil and Arlacel A plus 7-*n*-hexyloctadecane were not quite as effective. Peanut oil was not satisfactory when emulsified with either Aquaphor or Arlacel A. A moderate degree of immunity was produced in mice vaccinated with ribosomal fraction mixed with aluminum hydroxide gel. Sodium alginate mixed with ribosomal fraction produced a low degree of immunity only with the highest vaccinating dose. It was found that the effectiveness of the emulsified type of adjuvant depended upon the method of preparation. Careful standardization of technique to produce uniform and complete emulsification was essential for maximal adjuvant activity using minimal vaccinating doses. A rapid and practical method of preparing emulsified adjuvants is given. The mode of action of incomplete adjuvants as employed in these experiments is discussed, and it is thought that they acted primarily by protecting the ribosomes from being inactivated by host ribonuclease before they were engulfed by the macrophages.

Previous studies in this laboratory have shown that a ribosomal fraction obtained from ruptured viable attenuated mycobacterial cells is highly immunogenic in mice (18-20). This high degree of activity occurred only when the ribosomes were emulsified with Freund's incomplete adjuvant (FIA), or when the ribosomes were attached to a 'natural adjuvant" found in the particulate fraction from which the ribosomal fraction was prepared (18).

Variabilty in early experiments with the same preparation of the ribosomal fraction suggested that the methods used in preparing the adjuvant emulsions played an important role in their subsequent immunogenicity and prompted the present study.

Although the mode of action of incomplete adjuvants is still not entirely clear, widely held views suggest that adjuvants protect the antigen or immunogen from rapid degradation in the tissues of the animal, that they allow only slow release of the immunogen, and that they stimulate cellular proliferation essential for the immunological response. In view of these considerations, it was felt that the ribosomal fraction of *Mycobacterium tuberculosis* would be an excellent substrate to use to determine the effectiveness of adjuvants. It alone will not immunize mice against tuberculosis, and ribosomes alone are rapidly degraded or destroyed in vivo by the host's ribonuclease (1, 6, 10, 13, 14, 16; Youmans and Youmans, *unpublished data*). The use of the ribosomal fraction, therefore, should give information not only regarding mode of action of adjuvants, but also on the effectiveness of different adjuvants and on the best method of preparing emulsified ribosomal-adjuvant vaccines. In addition, it was hoped that another adjuvant might be found which would be tolerated better by the host.

MATERIALS AND METHODS

Preparation of the ribosomal fraction. The ribosomal fractions were prepared from viable cells of the H37Ra strain of *M. tuberculosis* as previously described (19). Briefly, the cells were harvested from 2 weeks of pellicle growth on a modified Proskauer and Beck medium (21), and were broken in a French pressure cell. Succeeding differential centrifugations

at 26,390 × *g*, 46,900 × *g*, and 144,000 × *g* resulted in the particulate fraction described previously (19). Freshly prepared particulate fraction was added slowly to a 1% solution of sodium dodecyl sulfate (SDS) dissolved in 0.01 M phosphate buffer (*p*H 7.0) containing 10^{-4} M $MgCl_2$ at room temperature, and was gently mixed by hand for 20 min. The clear fluid was poured into Spinco centrifuge tubes, placed in chopped ice, and allowed to stand over night in the refrigerator. The resulting precipitate was separated by decantation and centrifuged at 56,500 × *g* for 15 min to pack most of the remaining SDS. Again the supernatant fluid was carefully decanted to prevent SDS from being carried over into the next centrifuge tube. In spite of this care, some SDS usually was present. The supernatant fluid then was centrifuged at 144,000 × *g* for 3 hr to sediment the ribosomal fraction.

This fraction was characterized chemically as consisting of approximately 66% ribonucleic acid (RNA) and 34% protein. The RNA was measured by ultraviolet absorption at 260 mμ and by Dische's modified orcinol method (2), which we changed further by employing mycobacterial RNA as a standard instead of yeast RNA. Protein was measured by the method of Lowry et al. (9) with crystalline bovine albumin as the standard.

Measurement of immunological response. Male mice of the CF-1 strain were vaccinated intraperitoneally with various ribosomal-adjuvant preparations and viable H37Ra cells, and were challenged 4 weeks later intravenously with 1.0 mg of the virulent H37Rv strain of *M. tuberculosis*. Nonvaccinated mice were included in each experiment, and, as another control, mice were vaccinated with a portion of the living H37Ra cells from which the ribosomal fraction was prepared. All mice which survived 30 days were considered to be immune, since, by that time, all or most of the control nonvaccinated mice were dead. The method of evaluating the immunological response has been given in detail in a previous publication (22). Statistical evaluation between different groups of mice was made by use of the chi-square test.

Compounds used in the adjuvants. The substances used to prepare the adjuvants included two emulsifying agents: one, a cholesterolized absorbent ointment base which can absorb an aqueous solution several times its own weight, called by the trade name Aquaphor (Duke Laboratories, Inc., South Norwalk, Conn.); second, a nonionic partial ester surface active emulsifier, mannide mono-oleate, known by the trade name, Arlacel A (kindly supplied by Parke, Davis & Co., Detroit, Mich.; however, Arlacel A can also be purchased from Hilltop Laboratories, St. Paul, Minn.).

Three oils were tested: an extra heavy mineral oil (E. R. Squibb & Sons, New York, N.Y.), a light mineral oil, Klearol (L. Sonneborn Sons, Dallas, Tex.), and peanut oil, USP (Magnus, Mabee & Reynard Inc., New York, N.Y.). Two chemically defined oils were used; the straight-chained saturated hydrocarbon hexadecane, and a branch-chained hydrocarbon, 7-*n*-hexyloctadecane (both oils kindly supplied by Parke, Davis & Co.). Two adjuvants were used which were not of the emulsified type; one, an alumina hydroxide gel (kindly supplied by Lederle Laboratories, Pearl River, N.Y.), and sodium alginate (Consolidated Laboratories, Chicago Heights, Ill.).

Preparation of FIA. In these experiments, FIA was the standard adjuvant and always consisted of 1 part Aquaphor and 2 parts extra heavy mineral oil. The Aquaphor was warmed in a beaker until it melted, and the amount needed was measured, by use of a pipette, and was placed in another small glass beaker to cool. Originally, at the time it became translucent, the Aquaphor was added to the mineral oil which had been placed in a large mortar, and the two compounds were blended with a pestle. More recently, however, we have found that a small Waring Blendor mixed them much more quickly and easily. The mineral oil was poured into the blender first to prevent the Aquaphor from sticking to the blades. The two compounds were blended together at high speed for 2 to 3 min, or until the mixture became cream-colored and smooth. This mixture was scraped into a glass beaker with a wooden spatula while still warm, and a portion was poured into a 20-ml syringe to distribute, in the desired amounts, into small mortars and was allowed to cool to room temperature. It was found that better emulsions were obtained when Aquaphor from 1-lb(0.45-kg) tins was used rather than from 5-lb(2.3-kg) tins; more water from the air probably was absorbed by the Aquaphor in the larger container.

Standardization of FIA. To standardize the preparation of the water-in-oil emulsions through use of several methods of emulsification, and to determine some of the factors involved in the preparation, phosphate buffer alone was used as the aqueous phase, and FIA was the adjuvant. Three parts of the adjuvant were used to one part of the aqueous phase.

The quality of each emulsion was tested by placing a portion of the emulsion between a slide and cover slip and examining it under the microscope to determine the size of the aqueous droplets and the degree of absorption of the aqueous phase into the adjuvant. Visualization of this process was aided by adding a small amount of a water-soluble dye, acridine orange, to the phosphate buffer. A good emulsion consisted of very small droplets of uniform size in the oil.

Each emulsion was tested also by knocking or dropping a portion of the emulsion onto the surface of cool water. When the emulsion was good, the material remained firm on the water with no oil spreading over the surface. In contrast, when the emulsion was poor, the drop collapsed on the water and spread over the surface. There was a good correlation between the microscopic examination and the type of drop obtained. Routinely, now each emulsion which is prepared is tested by placing a portion on cool water.

Methods of preparing emulsions by use of FIA. Six different methods of preparing emulsions were tried. The first method of mixing was that used originally; all of the aqueous (vaccine) phase was added to the adjuvant in a small mortar, or, conversely, the adjuvant was added to the vaccine and then emulsified with a pestle. These preparations were examined microscopically, and it was found that the emulsions varied according to the person mixing and the amount

of time spent. The time in mixing varied, but was done until by gross observation all the aqueous phase had been adsorbed into the adjuvant, about 5 to 10 min. In a poor emulsion, microscopically, the aqueous droplets in the oil were irregular in size with many large drops, and nonemulsified fluid around the edge of the cover slip frequently was present. Oil from a drop from such an emulsion would spread over the surface of cool water, and the drop would not remain firm. Although better emulsions could be obtained with more stirring, this method obviously produced emulsions which were not uniform, and this lack of uniformity could be correlated with variation in the immunological response obtained in the vaccinated mice.

A second method of emulsifying the vaccine with the adjuvant was tried, as suggested by Brandon (*personal communication*). The vaccine was placed in a 10- or 20-ml Luer-lok syringe, and the adjuvant, in another syringe of the same size. The two syringes were joined by a double-hubbed, 18-gauge hypodermic needle, and the material was pushed back and forth between the two syringes until a good emulsion was obtained. It was suggested that the vaccine-adjuvant should be pushed back and forth several hundred times; after 12 to 18 times a good emulsion appeared to be obtained, as indicated by the water test method, and it was white and very hard. However, 12 to 18 times apparently was not enough, as the emulsion partly broke in the syringes while vaccinating. This process of emulsification was very laborious, time-consuming, and tedious, and so was not continued. Physically, it would be almost impossible to emulsify a vaccine-adjuvant in this manner if the material had to be pushed back and forth many more than 20 times by hand. An "emulsion-making" machine is available on the market for this purpose. It is expensive, however, and laboratories doing limited work with adjuvants might not wish to purchase it.

A third method, suggested by Freund and Thomson (5), involved addition of the adjuvant to the vaccine preparation in a rubber-stoppered vial and emulsification by repeatedly sucking up and pushing out through use of a syringe and 18-gauge needle. We could not get any type of an emulsion in this manner, even though the process was repeated many times. Prigal (12) emulsified a light mineral oil, Arlacel A, and glycerol mono-oleate (S1097) in a syringe by use of an amalgamator (Wig L Bug; Crescent Dental Mfg. Co., Chicago, Ill.), and he obtained stable emulsions within a matter of minutes. We did not try this method because the rapid shaking produced by this machine might have had an adverse effect on the ribosomes.

In a fourth method, the vaccine and adjuvant were placed together in a mortar or a beaker, and the two were emulsified, through use of a syringe only, by pulling the material back and forth rapidly. This produced a good emulsion, but it was time-consuming and messy.

A fifth method makes use of a small Waring Blendor. With certain antigens, this procedure should not only produce a good emulsion, but it should be the fastest and best. However, with ribosomal fraction as the vaccine, it was not used because of the possible adverse effect of the shearing action of the blades on the ribosomes and thus upon their immunogenic activity.

The sixth method was a return to our old system of emulsifying the ribosomal fraction with the adjuvant by the use of a small mortar and pestle. The use of a mortar with a small deep bowl produced an excellent emulsion in a shorter time than a mortar with a shallow bowl. The procedure was as follows: three parts of the adjuvant were added to the mortar and were allowed to cool. Then one part of the phosphate buffer solution, or cold ribosomal fraction, was added to the adjuvant drop-by-drop from a pipette by use of one hand, and each drop was absorbed by stirring constantly with the pestle with the other hand before the next drop was added. The manner in which the adjuvant was stirred to blend in the drops apparently made no difference. After the last of the aqueous phase was added, the mixture was stirred vigorously for another 1 to 3 min. The time seemed to depend somewhat on the person and on the manner in which the aqueous vaccine phase was added. The more slowly the vaccine was added, the faster an emulsion was obtained during the vigorous stirring period. As the emulsion was stirred, it became white, smooth, and very hard. When it became almost impossible to stir, a bit of it was placed on the surface of cool water, and the quality of the emulsion examined. FIA made such a thick emulsion that a drop would not fall on the water; the material had to be thrown onto the water from the pestle, and the emulsions remained firm with no oil spreading over the surface of the water. When, however, the drop tended to spread, the emulsion was more vigorously stirred until the drop became firm. Such an emulsion, when examined under the microscope, showed very small uniform droplets which remained the same in size, even with more mixing. When the emulsion was allowed to stand at room temperature, even for a few minutes, it had a tendency to become less stiff; therefore, before filling the syringes for vaccination, it was beaten again three or four times.

This technique appeared to produce emulsions that were reproducible. The time involved was shortened to not more than 5 min per vaccine preparation. In addition, mice vaccinated with such emulsions and the same ribosomal fraction now responded in the same way.

Dispensing the emulsified vaccines. By trial, it was found that the easiest and fastest manner in which to fill the syringes for vaccination was by scraping the vaccine-emulsion from the mortar into a 20-ml Luer-lok syringe and knocking the syringe against the table top to remove all the air pockets. A double-hubbed 16- or 18-gauge needle was attached, and to the other end of the needle was connected a 2-ml Luer-lok syringe which was filled from the larger syringe by pushing the barrel of the larger syringe, while held in a vertical position, against the table top and holding the larger syringe at the tip. Air pockets could be avoided by pressing on the barrel of the smaller syringe during filling. Since we used 30 mice per vaccination dose, six syringes were filled in this

manner for vaccination, and they were labeled and placed on a sheet of aluminum foil covering chopped ice in a pan, until time of vaccination 1 or 2 hr later. Use of syringes larger than 2 ml was impractical owing to difficulty in injecting the hard emulsion.

RESULTS

Amount of FIA injected. The mice were injected with 0.4 ml, and this amount was determined by several factors. When less than 0.4 ml was given, error in the vaccinating dose was introduced since there is a little oozing of the material from the needle or the syringe, and occasionally from the site of the injection. When 0.8 ml of FIA was given, with phosphate buffer instead of the ribosomal fraction as a control, a low grade nonspecific immunological reaction was produced against tuberculosis infection. As many as 33% of the injected mice could be protected, a significant difference from the noninjected controls ($P = <0.001$). The 0.4-ml injection dose through use of the control FIA and phosphate buffer produced no resistance in the mice. Thus, the volume of adjuvant used is important in order not to obtain a nonspecific immunological response.

Immunological response obtained with emulsified adjuvants. In comparing the effectiveness of the following adjuvants, FIA was included as the standard adjuvant in each experiment, and all the emulsions were made in the same manner as described under the sixth method.

(*i*) *Heavy and light mineral oil.* Because of the stiffness of FIA and the resulting difficulty in injecting this adjuvant into the mice, the first variation made in the adjuvant was to substitute light mineral oil, Klearol, for the heavy mineral oil. The results of pooled data from five experiments are shown in Table 1. There was no significant difference between the mice vaccinated with either type of adjuvant in the 1.0- and 0.1-mg vaccinating doses. However, there was a significant difference ($P = < 0.02, > 0.01$) between the two groups vaccinated with the 0.01-mg vaccinating dose. There was no significant difference between the mice vaccinated with the ribosomal fraction plus Aquaphor-light mineral oil and the nonvaccinated mice; however, there was a significant difference ($P = < 0.005, > 0.001$) between the control mice and those vaccinated with the ribosomal fraction plus FIA. The light oil, therefore, appears to make as good an adjuvant as the heavy oil when larger doses of vaccine are used. When small amounts of vaccine are tested for activity, the heavy mineral oil appears to be better. Thus, sensitivity appears to be increased by use of the heavier oil.

(*ii*) *Aquaphor and Arlacel A with both a light and heavy mineral oil.* Arlacel A has been used by investigators (11, 12, 23) as an emulsifying agent rather than Aquaphor; therefore, several experiments were conducted using Arlacel A and both light and heavy mineral oil. Emulsions were made as follows: 1 part of Arlacel A was added drop-by-drop to 9 parts of the oil while stirring in a small mortar with a pestle. After thorough mixing, an equal volume of the ribosomal preparation was added drop-by-drop, as with FIA, and was mixed vigorously until a good emulsion was obtained.

The Arlacel A plus heavy mineral oil produced a very heavy stiff emulsion. Arlacel A plus light oil produced an emulsion which resembled FIA, but was a little thinner. The quality of each emulsion was tested on the surface of cool water; the heavy oil emulsion remained very firm, and the light oil emulsion drop was less firm and formed a round smooth drop on the water.

Table 2 gives the pooled data from two experiments in which Arlacel A was compared with Aquaphor by use of both the heavy and light mineral oils. When heavy mineral oil was used, the ribosomal Aquaphor emulsion protected the mice to a far greater degree than did the ribosomal Arlacel A emulsion. These results were unexpected because this emulsion was the thickest and hardest of all the emulsions prepared. This adjuvant, therefore, might release the ribosomes so slowly that an immunological response would be detected only if the mice were challenged later than the standard 4-week period after vaccination. This possibility was not investigated as this adjuvant could not be used routinely because of its extreme thickness.

TABLE 1. *Immunogenic activity of the ribosomal fraction incorporated in FIA and in Aquaphor plus a light mineral oil*

Adjuvant	Amt injected (mg of wet wt)	No. of mice	No. of S-30 mice[a]	Per cent S-30 mice
Aquaphor plus heavy mineral oil	1.0	150	105	70
	0.1	149	57	38
	0.01	146	43	30
Aquaphor plus light mineral oil	1.0	148	105	71
	0.1	148	45	30
	0.01	142	25	18
H37Ra cells (no adjuvant)	1.0	145	106	73
Controls		145	21	15

[a] S-30 mice = number of mice which survived > 30 days.

TABLE 2. *Immunogenic activity of ribosomal fraction incorporated into adjuvants containing Aquaphor or Arlacel A and heavy or light mineral oil*

Adjuvant	Amt injected (mg of wet wt)	No. of mice	No. of S-30 mice[a]	Per cent S-30 mice
Aquaphor plus heavy mineral oil	1.0	60	46	77
	0.1	60	17	28
	0.01	58	17	29
Arlacel A plus heavy mineral oil	1.0	59	5	9
	0.1	59	13	22
	0.01	60	5	8
Aquaphor plus light mineral oil	1.0	59	43	73
	0.1	60	13	22
	0.01	57	6	11
Arlacel A plus light mineral oil	1.0	58	27	47
	0.1	57	9	16
	0.01	59	7	12
H37Ra cells (no adjuvant)	1.0	60	33	55
Controls		58	4	7

[a] S-30 mice = number of mice which survived > 30 days.

TABLE 3. *Immunogenic activity of the ribosomal fraction incorporated into an adjuvant containing Aquaphor and peanut oil*

Adjuvant	Amt injected (mg of wet wt)	No. of mice	No. of S-30 mice[a]	Per cent S-30 mice
Aquaphor plus heavy mineral oil	1.0	55	29	53
	0.01	59	20	34
Aquaphor plus peanut oil	1.0	60	14	23
	0.01	60	10	17
Arlacel A plus peanut oil did not produce a good emulsion				
H37Ra cells (no adjuvant)	1.0	60	37	62
Controls		59	7	12

[a] S-30 mice = number of mice which survived > 30 days.

With light mineral oil, the Aquaphor-oil adjuvant was significantly better (P = <0.005) with the 1.0-mg vaccinating dose than the Arlacel A adjuvant. Mice vaccinated with the 0.1-mg and the 0.01-mg vaccinating doses by use of the Arlacel A adjuvant did not differ significantly in immunological response from the control nonvaccinated mice.

(iii) Effectiveness of peanut oil with Aquaphor and Arlacel A. Peanut oil, a vegetable oil, also was tried with these emulsifying agents, since in contrast to the mineral oils it is absorbed and metabolized by the tissues. It is also a light oil and is more suitable for routine use than FIA. The quality of the emulsion obtained with Aquaphor was tested on water, and a thin round drop formed which spread rapidly to the side of the beaker. Although more vigorous mixing was done as compared with FIA, a good emulsion was never obtained. This is reflected in a pooled data of two experiments shown in Table 3 in which mice vaccinated with ribosomal fraction mixed with this adjuvant did not survive significantly longer than the nonvaccinated mice.

Arlacel A was added to the peanut oil in the same manner as described with mineral oils. After the vaccine preparation was added, the mixture was stirred vigorously for approximately 10 to 15 min. Only a poor emulsion could be obtained, and this separated within a few minutes on standing. Because of the very unstable nature of this adjuvant, mice were not vaccinated.

(iv) Effectiveness of two synthetic oils. Two synthetic oils, hexadecane and 7-*n*-hexyloctadecane were tested. These were emulsified only with Arlacel A as suggested by the supplier (Brandon, *personal communication*) by mixing 3 parts of Arlacel A to 17 parts of the oil in a small mortar with a pestle; an equal volume of the ribosomal fraction then was added slowly. Again vigorous mixing was done until a good emulsion was obtained as determined by a drop on cool water. In early experiments, the double syringe method was used for emulsification; however, the pooled data given in Table 4 are from two experiments in which the emulsions were made by use of a mortar and pestle.

There was no significant difference between the ribosomal preparations emulsified with FIA and Arlacel A plus hexadecane in the 1.0-mg and 0.01-mg vaccinating dose. In the 1.0-mg vaccinating dose, there was no difference between these two adjuvants and Arlacel A plus 7-*n*-hexyloctadecane, but at the 0.01-mg dose level there was a significant difference (P = < 0.05, > 0.025) between these two adjuvants and Arlacel A plus 7-*n*-hexyloctadecane. The lower degree of activity obtained with this dose through use of Arlacel A plus 7-*n*-hexyloctadecane, however, was real (P = <0.02, >0.01).

Immunological response obtained with nonemulsified adjuvants. Two adjuvants of the non-

TABLE 4. *Immunogenic activity of the ribosomal fraction incorporated into adjuvants containing Arlacel A and hexadecane or 7-N-hexyloctadecane*

Adjuvant	Amt injected (mg of wet wt)	No. of mice	No. of S-30 mice[a]	Per cent S-30 mice
Aquaphor plus heavy mineral oil	1.0	56	47	84
	0.01	57	30	53
Arlacel A plus 1% hexadecane	1.0	39	32	82
	0.01	48	22	46
Arlacel A plus 7-*N*-hexyloctadecane	1.0	48	39	81
	0.01	50	13	26
H37Ra cells (no adjuvant)	1.0	57	53	93
Controls		60	5	8

[a] S-30 mice = number of mice which survived > 30 days.

TABLE 5. *Immunogenic activity of the ribosomal fraction incorporated into aluminum hydroxide gel or sodium alginate*

Adjuvant	Amt injected (mg of wet wt)	No. of mice	No. of S-30 mice[a]	Per cent S-30 mice
Aquaphor plus heavy mineral oil	1.0	84	68	81
	0.01	57	30	53
Diluted in aluminum hydroxide gel	1.0	89	40	45
	0.01	51	16	31
Diluted in 4% Na alginate	1.0	30	10	33
	0.01	29	2	7
H37Ra cells (no adjuvant)	1.0	86	81	94
Controls		90	8	9

[a] S-30 mice = number of mice which survived > 30 days.

emulsified type were included in this series: 1% aluminum hydroxide gel, and 4% sodium alginate. One part of the ribosomal fraction was mixed with 20 parts of each of these adjuvants, and 0.2 ml was injected intraperitoneally into the mice. The results of these experiments are given in Table 5. The FIA vaccine was significantly better than either of these adjuvants, although the aluminum gel mixture in both doses protected the mice significantly ($P = <0.001$) when compared with the nonvaccinated control mice. The sodium alginate mixture protected the mice only in the 1.0-mg dose and then only to a slight degree.

Cellular reaction. Separate groups of mice were injected intraperitoneally with 0.4 ml of FIA containing phosphate buffer instead of the ribosomal fraction, FIA containing the ribosomal fraction, and ribosomal fraction alone. At weekly intervals thereafter for 28 days, five mice in each group and five nonvaccinated mice were sacrificed and underwent autopsy. The amount of peritoneal cellular exudate was estimated visually. The control mice and mice injected with the ribosomal fraction alone did not show at any time any evidence of a peritoneal cellular response. On the other hand, the mice given FIA alone and those given FIA-ribosomal fraction showed an appreciable amount of peritoneal cellular exudate 7 days after vaccination. The amount of exudate was greater at each subsequent inspection. Eventually, most of the visceral and parietal peritoneal surfaces were covered with a patchy, thick, and somewhat adherent white exudate. Visually, no difference in the amount of exudate could be detected between those mice given FIA alone and those given FIA-ribosomal fraction.

DISCUSSION

The results presented in this paper indicate, with the ribosomal fraction as a substrate, that of all the adjuvants tested, FIA and Arlacel A plus hexadecane were the best and were equally good. This was shown not only by the high degree of immunogenic activity obtained in the mice vaccinated with these preparations, but by the fact that the lowest vaccinating dose used, 0.01 mg, produced an immunological response. If the response of the host to minimal amounts of the antigen is of no great importance, Aquaphor plus light mineral oil and Arlacel A plus 7-*n*-hexyloctadecane also would be good adjuvants, because the larger vaccinating doses immunized the mice as well as FIA. Hoyt and co-workers (8) have shown also that hexadecane was better than 7-*n*-hexyloctadecane, and they ascribed the better response to greater tissue irritation obtained with hexadecane.

The adjuvants which could be handled more easily and perhaps tolerated better by the host were not as effective. One example was aluminum hydroxide gel. This adjuvant when mixed with ribosomal fraction not only produced a lower degree of immunological response in the vaccinated mice, but also some variation in the results between experiments. The variation indicated a lack of stability of the adjuvant ribosomal fraction mixture, which would result in some of

the ribosomes not being protected. It might be possible to use this adjuvant if the vaccinating dose of the ribosomal fraction were increased sufficiently to compensate for the loss of ribosomes by the host.

A second example was peanut oil since it did not produce a good or lasting emulsion with the vaccines, nor did it produce an immunological response in the mice vaccinated with the ribosomal fraction emulsified with it and Aquaphor. Freund and Bonanto (3) reported that peanut oil had "scant or no effect when compared with liquid petrolatum." However, Hilleman (7) has used peanut oil combined with aluminum monostearate plus Arlacel A (named adjuvant 65). He indicates that this adjuvant is as good as the mineral oil adjuvants in producing an antibody response. However, he compared adjuvant 65 with a light mineral oil plus Arlacel A; with this latter adjuvant we obtained only a moderate immunological response and only when used with the highest vaccinating dose. Since we were unable to obtain stable emulsions through use of peanut oil and Arlacel A, the addition of aluminum monostearate to the oil may help stabilize the emulsion. Adjuvant 65 (Merck and Co., Inc. Rahway, N.J.) was not available to us for testing

The mode of action of incomplete adjuvants has been well reviewed by McKinney and Davenport (11), Hilleman (7), and Hoyt and coworkers (8). Recently, Uchitel and Khasman (17) found that adjuvant stimulated protein synthesis in the host, perhap influencing the amount of antibody formation.

The mode of action of the adjuvants reported in this paper appeared to be primarily one of protection. When an excellent stable emulsion was obtained, a high immunological response was obtained with the ribosomes. This is expected since unprotected ribosomes would be destroyed by the host's ribonuclease. Since the two best adjuvants produced an immunological response with very small vaccinating doses of the ribosomal fraction, it would appear that they released the ribosomes very slowly. These results would support the opinion of Freund and co-workers (3, 4), who felt that adjuvants acted by protecting the antigen against destruction by the host, and prevented the antigen from being rapidly eliminated by the host.

Freund and Bonanto (3) felt also that adjuvants might act by increasing the monocytic response, and thereby promote antibody formation. In our experiments, a marked cellular response was obtained with FIA; however, antibody has not been shown to be involved in immunity against tuberculosis. Macrophages, in contrast, have been shown to be involved (15). Thus, the ribosomal-adjuvant could act by changing the macrophages both quantitatively and qualitatively. The adjuvant would increase the number of macrophages in the peritoneal cavity, and also would protect the ribosomes until they were engulfed by the macrophages. The ribosomes then could change the quality of the macrophage, in as yet an unknown way, to produce the immunological state.

Finally, an aspect of adjuvant activity which is especially interesting is the "natural adjuvant" which we have shown to be present in the immunogenic particulate fraction obtained from mycobacterial cells (18). This natural adjuvant was as good as FIA, unless the particulate fraction had been damaged either intentionally or during preparation. It appeared to consist of lipoprotein membranes, because the adjuvant activity was lost when the particulate fraction was treated with SDS or deoxycholate, or sonic vibration, or repeated freezing and thawing. Particulate fractions treated in these ways will be immunogenic only when FIA is added.

Two experiments were done to determine whether it might be possible to reunite in vitro mycobacterial cell membranes with ribosomes and restore the adjuvant activity. The results were negative, which suggests that for adjuvant activity the ribosomes must be held to the membranes by firm bonds.

In this connection, Youngner and Axelrod (23) have tested several lipids for adjuvant activity and found that, when used alone (i.e., without an emulsifying agent), the lipid, hexadecylamine, was the best of those they examined. It absorbed influenza virus and acted as a good adjuvant, although higher antibody titers were obtained with the virus emulsified with light mineral oil and Arlacel A. They found that, when small amounts of the antigen were absorbed onto the lipid and then added to the mineral oil emulsion, higher titers were obtained more rapidly than when using either of the adjuvants singly. In contrast, in our work, the addition of FIA to carefully prepared particulate fraction did not increase the immunological response in the vaccinated mice against tuberculosis. In the future, a lipid or lipoprotein may be found which will be as good an adjuvant as FIA and which will be tolerated better by human beings.

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ADDENDUM

Freund's incomplete adjuvant (Arlacel A plus Bayol F mineral oil) purchased from Difco Laboratories, Detroit, Mich., recently has been used in this laboratory. A relatively poor emulsion was obtained when the ribosomal fraction was emulsified with it, both by the double syringe and mortar and pestle techniques. In texture and droplet formation on the surface of cool water, it resembled the emulsion obtained with Arlacel A plus light mineral oil (Table 2).

LITERATURE CITED

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