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Effects of Oil-treated Mycobacterial Cell Walls on the Organs of Mice

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Intravenous vaccination of mice with oil-treated mycobacterial cell walls resulted in a marked macrophage accumulation in the lungs and spleens of vaccinated animals. Injection of oil emulsion alone or of cell walls alone failed to elicit the macrophage response. Although a correlation existed between the magnitude of the macrophage response and the degree of immunity against aerosol challenge with $H_{37}Rv$ organisms, the findings presented here do not rule out the possibility that qualitative differences may be present in the macrophages of animals vaccinated against tuberculosis. The ability of oil-treated cell walls to elicit an immune response appeared to be a function of the physical association of cell wall fragments and the surface of oil droplets.

An oil-in-water emulsion of mycobacterial cell walls injected intravenously into mice stimulates a high degree of resistance against subsequent aerosol challenge with *Mycobacterium tuberculosis* $H_{37}Rv$ (2, 4, 5). This emulsion is prepared by mixing lyophilized cell walls with a small quantity of light mineral oil or a synthetic hydrocarbon to form a paste which is then dispersed in saline with the aid of an emulsifier. The immune response is greater to such a cell wall vaccine than to infection with *M. bovis* strain BCG, persists for at least 6 months (1), and appears to be specific in character (3). The degree of immunity achieved has been studied with respect to the nature of the oil used, source of cell walls, effects of protoplasmic contamination, and dose of vaccine employed (2). The observations reported here deal with the alterations that occur in the lungs and to some extent in the livers and spleens of vaccinated mice, the relationship between cytopathology and immunity, the physical state of the oil-cell wall emulsion, and the distribution of intravenously administered vaccine in the tissues of the mouse.

MATERIALS AND METHODS

Preparation of cell walls. M. bovis strain BCG (Pasteur Institute strain 1173P2) was maintained on Sauton's potato medium, subcultured, grown as a pellicle on Sauton's liquid medium, and harvested by filtration through cotton gauze. The cells were washed twice with distilled water, dispersed in distilled water in a Sorvall omnimixer, and disrupted in a Sorvall refrigerated pressure cell at 35,000 psi at 5 to 10 C. As previously described (2), cell walls were separated from the pressure cell effluent by centrifugation and were washed twice by resuspension in distilled water and centrifugation at the same temperature. The clean cell walls were then lyophilized and stored at room temperature in tightly stoppered glass bottles (lot BCG CW 88/89).

Preparation of vaccines. The standard procedure for preparing vaccines was as follows. Light mineral oil or the synthetic hydrocarbon, 7-*n*-hexyloctadecane (supplied through the courtesy of Parke, Davis & Co., Detroit, Mich.), was added to lyophilized cell walls (0.12 ml oil per 25 mg of cell walls unless stated otherwise) in a Teflon tissue grinder, and the mixture was ground to a smooth paste. Physiological saline containing 0.2% Tween 80 (polyoxyethylene derivative of sorbitan mono-oleate; Atlas Powder Co., Wilmington, Del.) was gradually added to the paste, and grinding was continued until a well-dispersed oil-in-water emulsion containing a minimum of 1.5 mg of cell walls per ml was obtained. This emulsion was heated at 65 C for 30 min in a water bath. Further dilutions were prepared in saline containing 0.2% Tween. In some instances, preparation of the vaccines was modified in one or several respects; the details of these modifications are presented in Tables 3 and 4.

Vaccines in which silicone oil (Dow Corning 360 Medical Fluid, viscosity grade 50), olive oil, or ^3H-labeled 7-*n*-hexyloctadecane was substituted for light mineral oil (liquid petrolatum N.F. or Drakeol 6VR) were also prepared in an identical fashion. The ^3H-labeled 7-*n*-hexyloctadecane was diluted from an original specific activity of 12 mc/ml to 120 μc/ml with carrier 7-*n*-hexyloctadecane.

Mice. Three-week-old female Swiss albino mice from the colony maintained at the Rocky Mountain

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Laboratory were inoculated intravenously with 0.2 ml of the oil-treated cell wall vaccine. Mice used as controls received 0.2 ml of 0.2% Tween-saline intravenously. Mice were also inoculated with 0.2 ml of an emulsion of 7-*n*-hexyloctadecane in 0.2% Tween-saline or with a suspension of mycobacterial cell walls without oil. Ten mice from each group were killed with chloroform, and their lungs, spleens, and livers were removed, dipped in saline, trimmed free of adhering fat or connective tissue, blotted dry on paper toweling, and weighed individually.

Histology. Organs were fixed in Helly's modification of Zenker's Formalin solution for 18 hr, washed for 24 hr with distilled water, stored in 70% alcohol, and embedded in paraffin. Paraffin sections were stained with azure eosin.

Protection test. The method of performing tests for immunity was essentially that previously described (2).

Measurement of ^{3}H oil in mouse organs. Mice which had been inoculated intravenously either with ^{3}H-labeled 7-*n*-hexyloctadecane emulsified in Tween-saline or with the cell wall vaccine prepared with ^{3}H-labeled 7-*n*-hexyloctadecane were sacrificed, in groups of five, at 24 hr, 48 hr, and 5 days postinjection. Lungs, spleens, and livers from each group were placed in separate pools, weighed, and homogenized in a Teflon homogenizer with three volumes of a mixture of methanol and water (2:1). Each homogenate was then shaken with 16 volumes of dioxane in a glass-stoppered vial, the solids were allowed to settle, and 1 ml of the supernatant fluid was pipetted into 10 ml of toluene-methanol scintillation fluid (8) and was counted in a Packard Tri-Carb scintillation counter.

RESULTS

After vaccination with any of the preparations used, none of the mice appeared ill or showed decrease in physical activity, and all mice gained weight at the same rate as those in the control group. The body weight of the animals shown in Table 1, therefore, is the average for animals of all groups. Mice which received light mineral oil emulsified in 0.2% Tween-saline without cell walls and mice which received 300 μg of cell walls suspended in 0.2% Tween-saline without oil exhibited no increase in either lung or spleen weight as compared to control mice which received 0.2% Tween-saline alone. Animals inoculated with 300 μg of oil-treated cell walls developed a marked increase in lung and spleen weight and to a lesser extent in liver weight (Table 1). Of the three organs, the spleen showed the greatest percentage increase in weight, but the lung sustained the increase for a longer period of time. Although livers of vaccinated animals eventually regressed to a normal weight, lungs and spleens were significantly heavier than normal 6 months postvaccination.

A second intravenous inoculation of 300 μg of oil-treated cell walls, 1 month after the first vaccination, resulted in a 10% mortality within 24 hr. The organs of the revaccinated animals did not show a significant increase in weight above that of the organs from mice vaccinated only once when examined 1, 2, and 3 weeks after the second vaccination.

Histological examination revealed a pronounced cellular proliferation in the lungs and spleens of the vaccinated animals. Hypertrophy of the spleen was not apparent early but was easily discernible 4 weeks after vaccination. The histological appearance of the spleen from a mouse vaccinated 4 weeks previously (Fig. 1b) contrasts sharply with the spleen from a normal mouse (Fig. 1a).

Cellular proliferation or infiltration in the lung

TABLE 1. *Organ weights of control and vaccinated mice*[a]

Age (weeks)	Body wt (g)	Lungs (mg)		Spleen (mg)		Liver (g)	
		Normal[b]	Vaccinated[c]	Normal[b]	Vaccinated[c]	Normal[b]	Vaccinated[c]
3	12.1 ± 1.4[d]	149 ± 16[d]		77 ± 14[d]		0.9 ± .12[d]	
4	19.0	153 ± 19	251 ± 82	102 ± 20	178 ± 45	1.43	1.27
5	21.8	166 ± 22	258 ± 44	107 ± 26	198 ± 42	1.78	1.80
6	24.5	197 ± 27	230 ± 38	120 ± 19	240 ± 82		
7	26.9	220 ± 29	425 ± 70	131 ± 24	510 ± 143	1.99	3.00
9	27.5	240	464	159	438	2.03	2.76
12	28.1	218 ± 54	635 ± 117	154 ± 33	348 ± 49		
14	30.7	229 ± 19	702 ± 117	142 ± 25	350 ± 99		
18	35.7	230 ± 25	528 ± 96	135 ± 21	227 ± 66	1.82 ± .21	1.95 ± .38
22	34.1	268 ± 29	603 ± 103	117 ± 29	213 ± 67	1.81 ± .25	1.96 ± .35

[a] Mice vaccinated at 3 weeks.
[b] Inoculated intravenously with 0.2% Tween-saline.
[c] Inoculated intravenously with 300 μg of oil-treated BCG cell walls (lot 88/89) suspended in 0.2% Tween-saline.
[d] Standard deviation from mean.

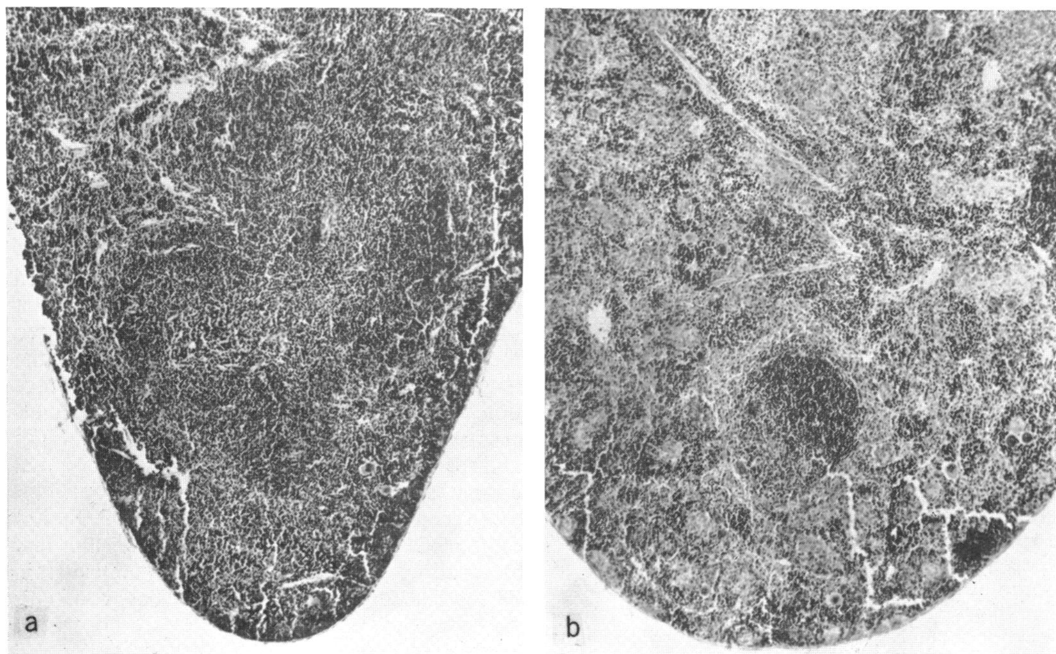

FIG. 1. *(a) Section of normal mouse spleen. × 90. (b) Section of mouse spleen 4 weeks after vaccination with 300 μg of oil-treated BCG cell walls (lot 88/89). × 90.*

was apparent within 24 hr of vaccination and progressed in degree for 4 to 6 weeks after vaccination. Figure 2 shows the appearance of the lung from a normal 7-week-old mouse. The alveolar septa were slender, delicate structures composed of alveolar epithelial cells, basement membranes, and capillaries. They did not contain any pronounced collection of cells. At 48 hr postvaccination (Fig. 3), thickening of interalveolar septa with generalized cellular infiltration was apparent. At the end of 2 weeks (Fig. 4), the alveolar spaces were smaller than those in normal mouse lung, the alveolar septa were thickened, and cellular infiltration was generalized. However, the reaction appeared more pronounced toward the periphery of the lung than in the more central portions. At 4 weeks postvaccination (Fig. 5), there was further encroachment on alveolar spaces, and the cellular infiltrate was developing a nodular pattern, especially toward the outer surface of the lung. Under a higher magnification (Fig. 6), the majority of the cells appeared to be macrophages, with a well-defined nuclear membrane, coarsely clumped chromatin, and a clear or foamy cytoplasm. Lymphocytes, small cells with deeply staining round nuclei and a scanty cytoplasm, surrounded the collections of macrophages. Six

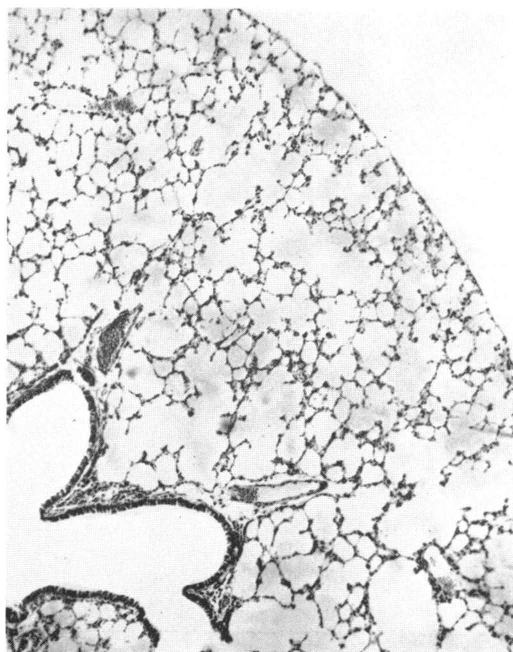

FIG. 2. *Section of normal mouse lung. × 90.*

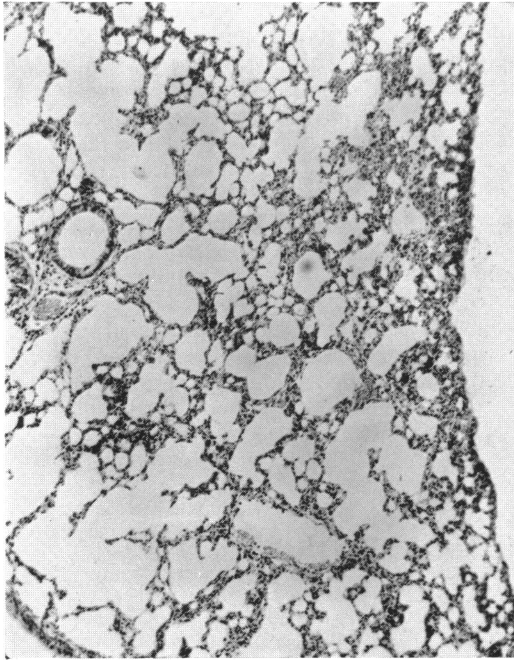

FIG. 3. *Section of mouse lung 48 hr after vaccination with 300 μg of oil-treated BCG cell walls (lot 88/89). × 90.*

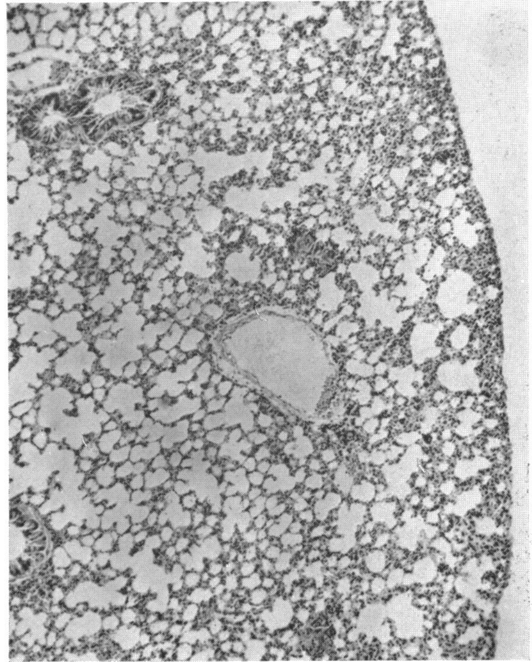

FIG. 4. *Section of mouse lung 2 weeks after vaccination with 300 μg of oil-treated BCG cell walls (lot 88/89). × 90.*

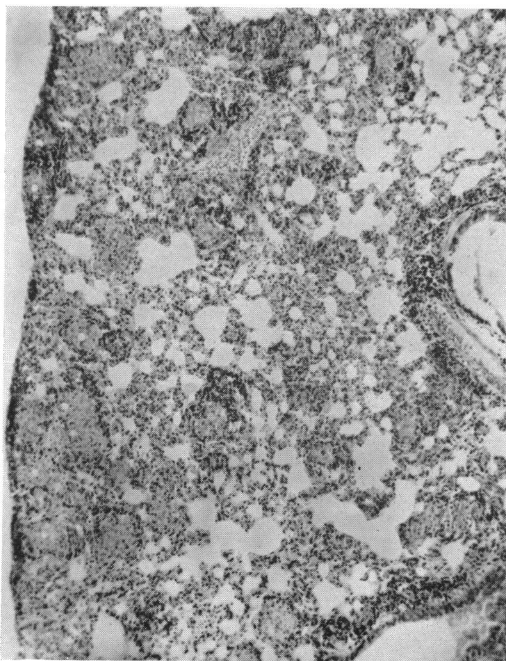

FIG. 5. *Section of a mouse lung 4 weeks after vaccination with 300 μg of oil-treated BCG cell walls (lot 88/89). × 90.*

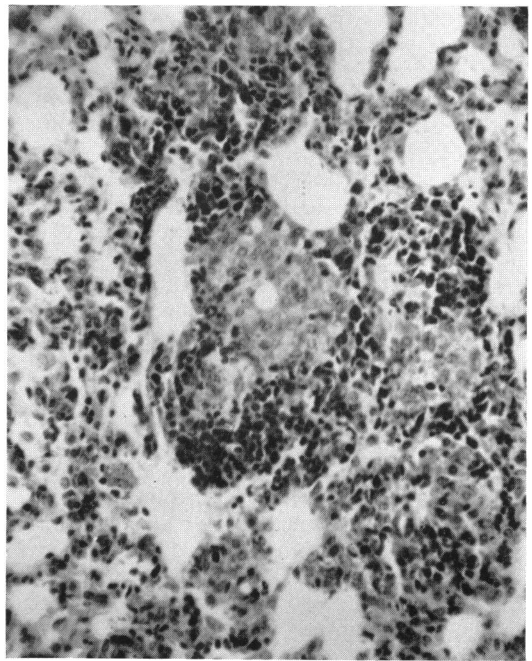

FIG. 6. *Section of mouse lung 4 weeks after vaccination with 300 μg of oil-treated cell walls (BCG lot 88/89). × 150.*

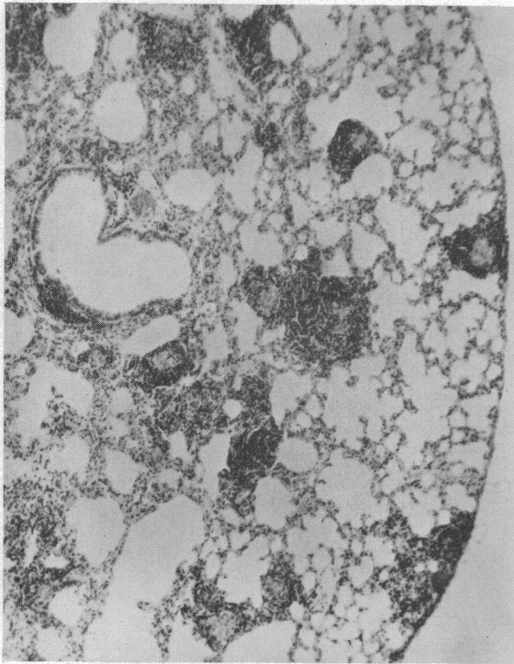

FIG. 7. *Section of mouse lung 6 months after vaccination with 300 μg of oil-treated BCG cell walls (lot 88/89). × 90.*

months after vaccination (Fig. 7 and 8), the lung contained well-organized nodules with a central zone of macrophages surrounded by a wide cuff of lymphocytes. Although the interalveolar septa were less thick at 6 months than at 1 month after vaccination, they were not restored to a completely normal appearance. The histological appearance suggested that, to some extent, this increase of cellular elements occurred at the expense of the alveolar gas space.

Distribution in mouse tissues of oil emulsified in 0.2% Tween-saline and of oil combined with mycobacterial cell walls as a vaccine was measured by use of ^{3}H-labeled 7-*n*-hexyloctadecane. The oil emulsion alone was found to have the same distribution as that of the oil-treated walls. The lung retained 31%, the spleen 3%, and the liver 58% of the administered dose of 300 μg of cell walls with oil 24 hr later. From the data in Table 2, it is apparent that there was no significant redistribution of the tritium label between 1 and 5 days after inoculation. Although the liver absorbed the largest absolute amount of the dose given, the lung absorbed the largest amount per gram of tissue.

Since the oil emulsion alone had the same general distribution as the vaccine and since the oil alone or cell walls alone did not stimulate a cell response, the nature of the oil-cell wall combination and its relation to cell stimulation were examined. The appearance of 7-*n*-hexyloctadecane emulsified with 0.2% Tween-saline is shown in Fig. 9. The oil droplets appeared as clear spheres varying in size from less than 1 μ to 15 μ or more in diameter. The vaccine prepared with BCG cell walls and 7-*n*-hexyloctadecane is shown in Fig. 10. The oil droplets were associated with cell wall particles. This particular vaccine produced a high degree of immunity to aerosol challenge and stimulated a marked increase in the weight of the lungs in vaccinated animals. Figure 11 shows the appearance of the vaccine on an electron-microscope grid. After evaporation of

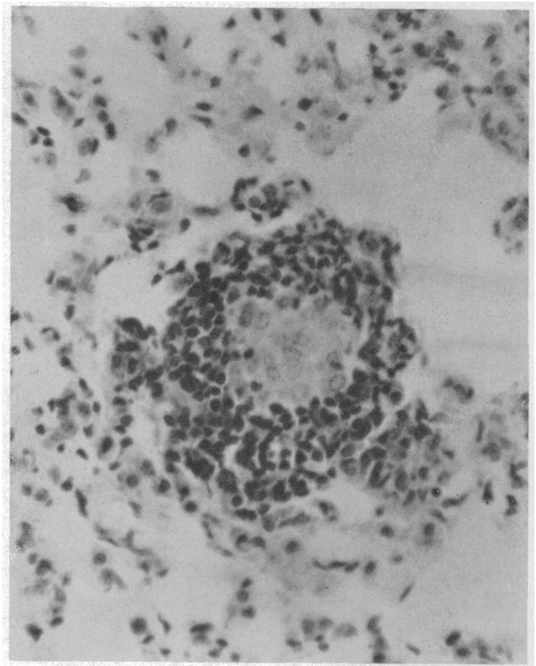

FIG. 8. *Section of mouse lung 6 months after vaccination with 300 μg of oil-treated BCG cell walls (lot 88/89). × 290.*

TABLE 2. *Percentage of ^{3}H-labeled 7-n-hexyloctadecane retained by mouse organs*

Time	Oil-cell wall vaccine		
	Lung	Spleen	Liver
24 hr	31	3	58
48 hr	26	6	58
5 days	32	7	54

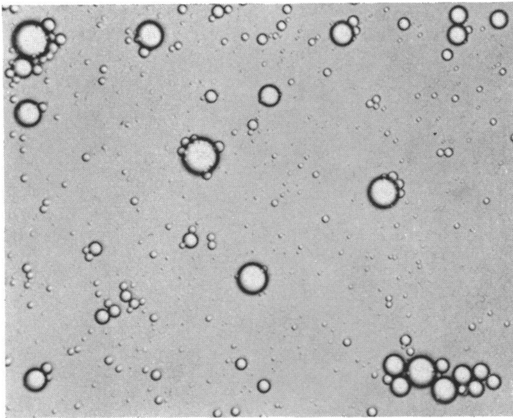

FIG. 9. *7-n-Hexyloctadecane emulsified in 0.2% Tween 80-saline.* × *480.*

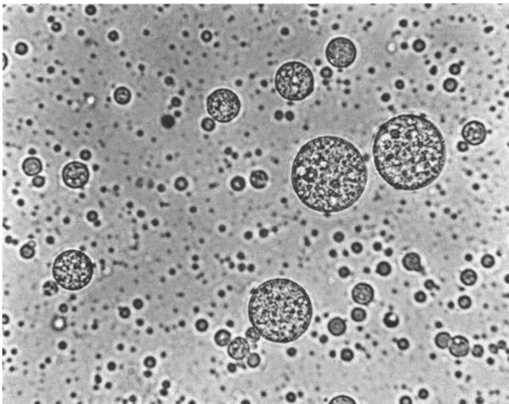

FIG. 10. *BCG cell wall vaccine prepared with 7-n-hexyloctadecane as described under Materials and Methods.* × *800.*

most of the oil under vacuum, extraction of the remainder with petroleum ether, and removal of Tween-saline by washing with water, the cell walls had the appearance shown in Fig. 12. They appeared as an interlaced network of cell walls. Light microscopic study showed that these walls could be distributed within the oil droplet, on the surface of the droplet, or both within the droplet and on the surface. However, three observations suggest an accumulation of cell wall particles at the oil-water interface. First, cell walls act as an emulsifying agent for the oil without the addition of Tween, but this emulsion is not as stable as that produced with Tween. Second, when vaccine is allowed to stand for several days, some of the cell walls separate from the oil droplets and pass into the water phase of the emulsion. Shaking such a vaccine produces a

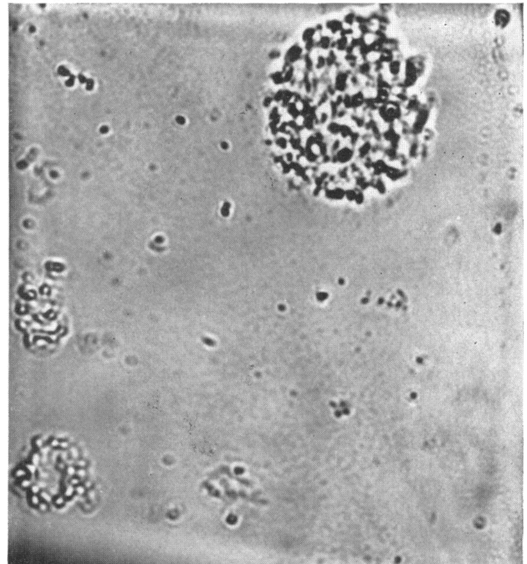

FIG. 11. *Low-power electron micrograph of BCG cell wall vaccine prepared with 7-n-hexyloctadecane as described under Materials and Methods. Most of the oil had been evaporated under high vacuum after the vaccine had been deposited on the specimen screen.* × *450.*

large percentage of clear oil droplets and masses of aggregated cell walls. Finally, when a high concentration of Tween is used, a very stable oil emulsion is produced, but it shows a limited association of cell walls with oil droplets, suggesting that the Tween covered the oil-water interface to the partial exclusion of the cell walls. Such emulsions generally have reduced immunizing potency which was particularly evident at low dose levels.

When silicone or olive oil was used in place of light mineral oil or the synthetic hydrocarbon, negligible association occurred between cell walls and oil (Fig. 13 and 14); these preparations did not stimulate immunity. In fact, anything which decreased the adherence of cell walls to the oil droplets diminished or abolished the potency of the preparation as an immunizing agent. For example, the presence of bacterial protoplasm with excessively high concentrations of Tween also prevented adherence of cell wall particles to the oil droplets (Fig. 15).

The factors which appeared to affect the immunizing potency of an oil-treated mycobacterial cell wall preparation were: source of cell walls, nature of the oil, concentration of emulsifier, presence of protoplasmic material, duration of storage, and freezing and thawing. The influence of some of these variables is shown in Tables 3 and 4. Inspection of Table 3 reveals that preparations

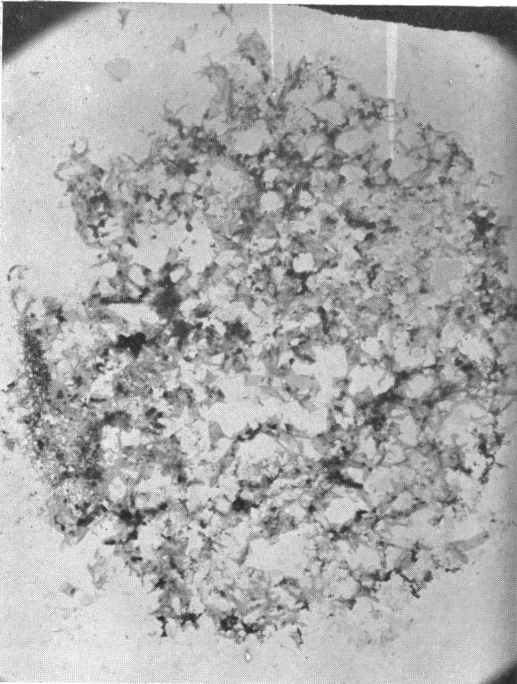

FIG. 12. *Electron microscopic appearance of cell walls from the same field shown in Fig. 11 after further treatment of the specimen screen with petroleum ether and distilled water to remove the remaining oil and salts.* × *1,400.*

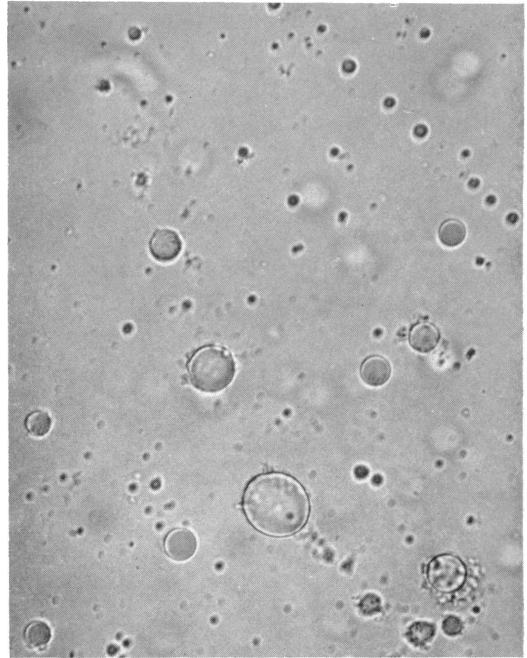

FIG. 13. *BCG cell wall vaccine prepared with silicone oil as described under Materials and Methods.* × *610.*

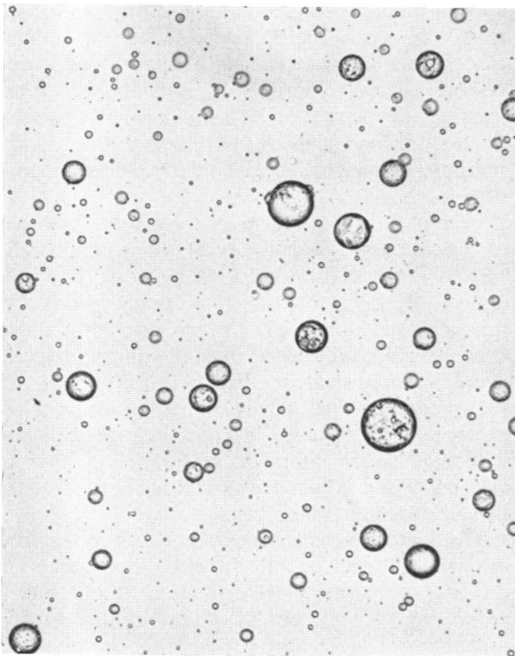

FIG. 14. *BCG cell wall vaccine prepared with olive oil as described under Materials and Methods.* × *610.*

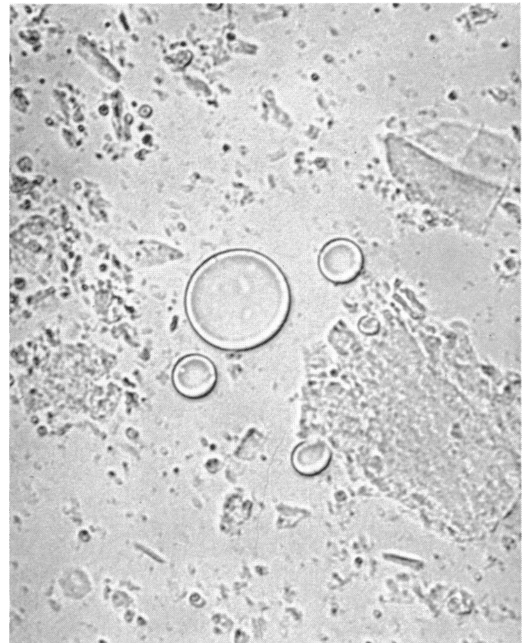

FIG. 15. *Vaccine made by grinding whole disruption product from BCG (cell walls and protoplasm) with 7-n-hexyloctadecane, admixing undiluted Tween 80, and dispersing the resulting paste in saline to a concentration of 0.2% Tween 80 as described for preparation 6, Table 3.*

TABLE 3. *Protection of mice against aerosol challenge with Mycobacterium tuberculosis $H_{37}Rv$: effects of Tween 80, protoplasm, freezing, and storage on oil-treated cell wall vaccines*

No.	Preparation[a]	Immunizing dose (μg of dry wt)	Results 30 days after challenge	
			No. with lung lesions/no. of mice tested	Viable-cell count[b]
1	BCG cell walls, light mineral oil, 0.2% Tween 80 in saline	450 150 50	0/20 2/19 4/20	7.5×10^1 9.0×10^2 1.6×10^4
2	BCG cell walls, light mineral oil, undiluted Tween 80	450 150 50	1/19 9/20 18/20	<10 5.8×10^4 6.1×10^5
3	BCG cell walls, 7-*n*-hexyloctadecane, undiluted Tween 80	400 200 100	7/20 11/17 18/20	7.0×10^3 1.8×10^4 3.2×10^6
4	Preparation 3, frozen and thawed	400 200 100	6/20 12/18 20/20	9.2×10^3 1.4×10^5 2.6×10^6
5	BCG whole water disruption product, light mineral oil, 0.2% Tween 80	250	5/20	6.1×10^4
6	BCG whole water disruption product, light mineral oil, undiluted Tween 80	250	19/20	7.5×10^5
7	BCG cell walls 88/89, light mineral oil, 0.2% Tween 80; freshly prepared	300	0/20	1.7×10^2
8	BCG cell walls 88/89, light mineral oil, 0.2% Tween 80; freshly prepared from cell walls stored at room temperature for 1 year	250 125	0/20 4/20	8.1×10^3 1.2×10^4
9	Preparation 7 stored for 6 months in refrigerator	250 125	8/20 12/20	3.2×10^5 2.6×10^6
10	Controls	None	170/170	3.3×10^6

[a] To prepare vaccines 1, 7, 8, and 9, lyophilized cell walls were ground with oil in the proportion of 0.12 ml of oil per 25 mg of walls and were suspended in saline containing 0.2% Tween 80 to a concentration of 1.5 to 2.25 mg/ml. For preparations 2, 3, and 4, lyophilized cell walls were ground with oil in the same proportion, and then an equal amount of undiluted Tween 80 was admixed, after which the paste was suspended in saline. To prepare vaccines 5 and 6, the lyophilized pressure cell effluent, containing both cell walls and protoplasm, was ground with oil in the proportion of 0.12 ml per 25 mg of material. Preparation 5 was suspended in saline containing 0.2% Tween 80 to a concentration of 6.25 mg/ml; for preparation 6, 0.12 ml of undiluted Tween 80 was admixed with the oil-disruption product mixture before dilution in saline to a concentration of 6.25 mg/ml. All vaccines were heated at 65 C for 30 min. Dilutions for inoculating mice with 0.2-ml volumes of vaccines were made with saline containing 0.02% Tween 80.

[b] Median number of viable *M. tuberculosis* $H_{37}Rv$ per 100 mg of lung tissue; 10 mice per group were sampled.

1 and 2 appeared to be equally efficacious at a dose level of 450 μg, and that only when the lower doses of 50 and 150 μg were employed did a difference between the preparations reveal itself. Only by a dose-response assay was it possible to show decreased immunogenicity for some preparations. With respect to silicone oil, olive oil, and kerosene, the differences in immunogenical response were of such magnitude (Table 4) that they were easily apparent at all dose levels used.

TABLE 4. *Protection of mice against aerosol challenge with Mycobacterium tuberculosis $H_{37}Rv$ by intravenous inoculation of BCG cell walls treated with different kinds of oil*

No.	Preparation[a] of BCG cell walls made into a paste with	Immunizing dose (μg of dry wt)	Results 30 days after challenge	
			No. with lung lesions/no. of mice tested	Viable-cell count[b]
1	Light mineral oil	300	0/20	2.3×10^2
		150	0/20	6.5×10^2
2	7-*n*-hexyloctadecane	300	0/20	1.0×10^2
		150	1/20	2.4×10^3
3	Silicone oil	300	20/20	Not cultured
		150	20/20	Not cultured
4	Kerosene	250	20/20	6.5×10^6
5	Olive oil	250	20/20	4.4×10^6
6	No oil used, cell walls only	300	20/20	2.0×10^6
7	Viable BCG standard vaccine	300[c]	2/20	7.5×10^3
		150[d]	4/20	1.0×10^4
8	Controls	None	40/40	4.4×10^6

[a] Lyophilized cell walls were ground with oil in the proportion of 0.06 ml of oil per 25 mg of cell walls to form a paste which was suspended in saline containing 0.2% Tween 80 to a concentration of 1.5 mg/ml for preparations 1, 2, and 3 and 6.25 mg/ml for preparations 4, 5, and 6 and was heated at 65 C for 30 min. Dilutions for inoculating mice with 0.2-ml volumes of vaccines were made with saline containing 0.02% Tween 80.

[b] Median number of viable *M. tuberculosis* $H_{37}Rv$ cells per 100 mg of lung tissue; 10 mice per group were sampled.

[c] Moist weight, equivalent to 7.0×10^6 cells.

[d] Moist weight, equivalent to 3.5×10^6 cells.

DISCUSSION

The mycobacterial cell wall vaccine prepared in this laboratory consistently protects mice against aerosol challenge with *M. tuberculosis* better than does BCG (1, 2); however, it also produces a more marked granulomatous reaction in the lung than does BCG. In this laboratory, mice vaccinated intravenously with 300 μg of living BCG had a lung weight of 250 ± 33 mg and a spleen weight of 391 ± 48 mg 4 weeks postvaccination, in contrast to a lung weight of 425 ± 70 mg and a spleen weight of 510 ± 43 mg in animals vaccinated with the oil-treated cell wall preparation.

Youmans and Youmans have described in detail the granulomatous response produced in mice by mycobacterial cells (7). They observed that the degree of resistance to subsequent challenge was approximately proportional to the granulomatous response as measured by mean lung weights. Although the degree of granulomatous response to heat-killed cells was the same as to living cells, there was a significantly higher resistance produced by living cells. These authors concluded that increased resistance to challenge infection depends more on the quality than upon the number of macrophages. The granulomatous response in mice used in this laboratory is similar to that described by Youmans and Youmans. However, we have observed that the response to oil-treated cell walls is more intense and persistent than that to BCG, and that resistance to pulmonary infection of intravenously vaccinated mice is correlated with the granulomatous response (1). Our present data do not conclusively establish that a granulomatous response in the lung is necessary for protection against aerosol challenge; experiments employing chemically modified cell walls are in progress to determine whether the granulomatous response is essential for protection.

It is apparent that the oil-cell wall complex is an essential ingredient of the vaccine, since any factor which interferes with the association of oil and cell walls reduces or eliminates immunogenicity, but the exact role of the oil in this complex is unknown. The oil could function primarily as a carrier to deposit the cell walls in the lung. When living mycobacteria are injected intravenously in mice, only 2% of the dose is phagocytized in the lung (6), and, presumably, the same small fraction of a mycobacterial cell wall suspension would deposit in lung macrophages. This would be only one-tenth to one-fifteenth of the dose deposited when an oil emulsion of cell walls is given intravenously. In addition, the oil might serve either to protect the cell walls against destruction by macrophages or

to orient the cell walls at the oil-water interface in a fashion that exposes a large surface area of immunologically active groups on the cell walls. Experiments to test these hypotheses are currently being conducted.

ACKNOWLEDGMENTS

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