JOURNAL OF BACTERIOLOGY, Oct. 1969, p. 51-57
Vol. 100, No. 1

Relationship of Footpad Sensitivity to Purified Protein Derivatives and Resistance to Airborne Infection with *Mycobacterium tuberculosis* of Mice Vaccinated with Mycobacterial Cell Walls

R. L. ANACKER, E. RIBI, D. F. TARMINA[1], L. FADNESS, AND R. E. MANN
Rocky Mountain Laboratory, National Institute of Allergy and Infectious Diseases, Hamilton, Montana 59840

Received for publication 16 May 1969

Footpads of mice sensitized by oil-treated Bacillus Calmette-Guérin (BCG) cell walls given either intravenously, subcutaneously, intradermally, intraperitoneally, or intramuscularly became swollen and reddened after injection of purified protein derivative (PPD). This reaction, greatest after intradermal and subcutaneous sensitization, generally reached a maximum about 24 hr after challenge and was still marked at 48 hr. The histological response was characterized by infiltration with both polymorphonuclear and mononuclear cells. The proportion of mononuclear cells increased with time and they predominated at 48 hr. The footpad reaction could be detected as early as 1 week after sensitization and persisted for at least 37 weeks. Footpad sensitivity to PPD and acquired resistance to airborne infection with *Mycobacterium tuberculosis* H37Rv were correlated in that (i) both reached a peak approximately 1 month after sensitization of the mouse, and (ii) cell walls treated with NaOH or given without oil neither protected mice against challenge infection nor sensitized them to PPD. Although, as we reported previously, mice vaccinated subcutaneously or intradermally exhibited little or no enhanced resistance to experimental infection, mice given oil-treated cell walls by these routes were highly sensitive to footpad inoculation of PPD. Therefore, the footpad test cannot be used to determine immunity of the mouse to pulmonary infection with tubercle bacilli.

Despite intensive efforts by many investigators, the nature of the relationship between delayed hypersensitivity and immunity in tuberculosis is still controversial (11). Recently, we reported that mice can be made resistant to airborne infection with virulent mycobacteria by intravenous (iv) injection of oil-treated mycobacterial cell walls (5, 13). Guinea pigs and rabbits inoculated subcutaneously with mycobacterial cell walls exhibit delayed dermal sensitivity to purified protein derivative (PPD) (10, 13), but the tuberculin sensitivity of cell wall-vaccinated mice has not been reported.

Although attempts to induce delayed reactions in the skin of mice have often failed (12), delayed reactions to PPD or old tuberculin have been elicited in the footpads of mice sensitized with either living or dead cells of *Mycobacterium tuberculosis* strains H37Rv or H37Ra or with Bacillus Calmette-Guérin (BCG) (6, 7, 9). Results of our experiments to determine (i) the footpad sensitivity to PPD and (ii) the relationship between footpad sensitivity and acquired resistance to airborne infection of mice vaccinated with mycobacterial fractions or viable BCG are reported here.

[1] Present address: Research Laboratories of the Variety Club Heart Hospital, Department of Pediatrics, University of Minnesota School of Medicine, Minneapolis, Minn.

MATERIALS AND METHODS

Cell walls. Cell walls were obtained as described previously (13) from BCG (Pasteur Institute strain 1173P2 or the Glaxo strain obtained from the Lederle Laboratories) disrupted in a Sorvall refrigerated pressure cell.

Extracts. An extract containing waxes C and D was obtained from BCG by the method of Aebi et al. (1). BCG cells and cell walls were also extracted with dimethyl sulfoxide or treated with ethanolic NaOH as described previously (3).

A crude sodium dodecyl sulfate (SDS) extract of BCG was prepared by stirring BCG cells [1 g (wet weight)/10 ml of 0.02% Tween 80 saline] with an equal volume of 0.5% SDS in water for 3 hr at 37 C.

The supernatant fluid obtained after centrifugation at 18,000 × *g* for 1 hr at 22 C was dialyzed exhaustively against distilled water and lyophilized.

Vaccines. Vaccines were prepared in the following ways: (i) 25 mg of the given lyophilized preparation were blended with 0.12 ml of Drakeol 6VR (Pennsylvania Refining Co., Butler, Pa.) in a Teflon homogenizer. This mixture was then suspended in 0.2% Tween 80 in saline to the appropriate concentration, and the resulting suspension was heated at 65 C for 0.5 hr. (ii) BCG cell walls were suspended in 0.2% Tween 80 in saline (1.5 mg/ml) and heated at 65 C for 0.5 hr. (iii) BCG cell walls were suspended in 0.2% Tween 80 in saline at a concentration of 6 mg/ml, and the suspension was heated at 65 C for 0.5 hr and emulsified in an equal volume of Freund's incomplete adjuvant (Difco). The absence of viable mycobacteria in these vaccines was verified by plating a portion of each vaccine on Dubos Tween-albumin medium.

Viable BCG vaccine was generously supplied by Sol Roy Rosenthal, Research Foundation, University of Illinois, Chicago.

Mice in groups of 10 were given 0.05 to 0.2 ml of the vaccines either iv, intraperitoneally (ip), subcutaneously (sc) in the nuchal region, intradermally (id) on the shaved flanks, or intramuscularly (im) in each thigh.

Mice. All mice were, at the time of vaccination, 3- to 4-week-old females reared at the Rocky Mountain Laboratory.

Footpad test. Sensitivity of mice to PPD (Parke, Davis, and Co., Detroit, Mich.) was determined essentially by the procedure of Gray and Jennings (7). PPD (0.03 ml), dissolved in the buffered diluent supplied with it, was injected into the pad of either rear foot, and 0.03 ml of the diluent alone was injected into that of the other. Usually at 4 hr and always at 24 and 48 hr after challenge with PPD, the thickness of the feet was measured with calipers. Significance of the differences in thickness of buffer- and PPD-inoculated feet in each group was evaluated by the *t* test.

Protection test. Protective potency of various vaccines was assayed in mice by the test described previously (13), except that the challenge organisms were obtained from a frozen suspension of *M. tuberculosis* H37Rv (8). Briefly, 3-week-old mice were inoculated with the vaccine by the appropriate route and 1 month later were infected in a Middlebrook apparatus with *M. tuberculosis* H37Rv by the pulmonary route. At 1 month after challenge, the animals were killed with chloroform vapor, and the lungs were removed for enumeration of virulent mycobacteria by culture on Dubos Tween-albumin medium.

Histology. Mice to be examined histologically were vaccinated with either 300 μg of oil-treated BCG cell walls or 300 μg of BCG cell walls suspended in 0.2% Tween 80 in saline. Six weeks later they were challenged in the left rear footpad with 5 μg of PPD. Diluent was injected into the other rear footpad. Thickness of the feet was measured and groups of mice were sacrificed with chloroform vapor at 4-, 24-, and 48-hr intervals after injection of PPD. Hind feet were amputated, fixed in 10% Formalin in 0.075 M phosphate buffer (*p*H 7.0), and decalcified in 5% formic acid. Two blocks of tissue from each hind foot of every animal were embedded in paraffin, sectioned, and stained with azure-eosinate. Sections through at least two sagittal planes from both a control and PPD-inoculated foot were examined.

RESULTS

Effect of challenge dose of PPD. The size of a satisfactory challenge dose of PPD was determined by injecting 2.5, 5, or 10 μg of PPD into the footpads of mice sensitized 4 weeks earlier by iv administration of 300 μg of oil-treated BCG cell wals (Table 1). At 4, 24, and 48 hr after challenge, feet of vaccinated mice inoculated with 2.5 μg of PPD and those of unvaccinated control mice inoculated with 10 μg of PPD were not significantly thicker than the feet inoculated with diluent only. However, both the 5- and 10-μg doses caused significant footpad enlargement in vaccinated mice at 4, 24, and 48 hr. Maximal enlargement in this and later studies was observed at 24 hr. From this experiment, 5 μg of PPD was chosen as a suitable dose.

TABLE 1. *Footpad sensitivity of mice to varying doses of PPD at 4 weeks after intravenous inoculation of oil-treated BCG cell walls*

Sensitizing dose of BCG cell walls	Challenge dose of PPD	Mean foot thickness[a] (mm)								
		4 hr			24 hr			48 hr		
		Left	Right	*P*[b]	Left	Right	*P*	Left	Right	*P*
μg	μg									
300	2.5	1.81	1.78	>0.1	1.87	1.82	>0.1	1.87	1.88	>0.1
300	5	1.81	1.75	<0.01	1.93	1.75	<0.01	1.92	1.83	<0.01
300	10	1.89	1.81	<0.01	2.00	1.80	<0.001	1.95	1.86	<0.05
None	10	1.96	1.91	>0.05	1.95	1.90	>0.1	1.90	1.91	>0.1

[a] Left footpad was inoculated with PPD in buffer; right footpad was inoculated with buffer only.
[b] Comparison of right and left feet in each group of 10 mice.

Effect of sensitizing dose of cell walls. Mice sensitized 4 weeks earlier by iv injection of 37.5, 75, 150, or 300 μg of oil-treated cell walls were challenged with 5 μg of PPD (Table 2). In this and several subsequent experiments, the PPD-inoculated feet of the unvaccinated control mice were slightly but significantly thicker than the buffer-inoculated feet at 4 hr but never at 24 hr. Early nonspecific and transient responses to old tuberculin were also noted by Gray and Jennings (7). Because of this nonspecific response of some of the control animals, the significance of differences in thickness of feet of PPD- and buffer-inoculated vaccinated mice at 4 hr is impossible to assess and therefore will not be considered further in this presentation. At 24 and 48 hr after challenge, PPD did not stimulate a significant increase in thickness of feet of mice sensitized with 37.5 or 75 μg of cell walls. However, 150- and 300-μg doses of oil-treated cell walls did induce hyperreactivity to PPD.

Effect of route of administration of cell walls on degree of footpad sensitivity. Mice were challenged with PPD 5 weeks after sensitization with 300 μg of oil-treated cell walls given id, iv, im, ip, or sc (Table 3). Cell walls given by any of these routes stimulated hypersensitivity to PPD, but footpads of the id and sc inoculated mice were far more sensitive to PPD than were those of the iv, im, and ip inoculated mice.

Development of footpad sensitivity. Mice vaccinated iv with 300 μg of oil-treated cell walls were challenged with 5 μg of PPD after 1, 2, 3, 4, 10, 20, or 37 weeks (Table 4). During the first 3 weeks minimal sensitivity was detected 24 hr after challenge but none 48 hr after. At 4 and 10 weeks after inoculation there was considerable footpad enlargement 24 and 48 hr after challenge with PPD. Reactions were less intense after 20 weeks than after 4 and 10 weeks, but were still significant both at 24 and 48 hr after challenge. At 37 weeks significant footpad enlargement was observed at the 48-hr reading only.

Induction of footpad sensitivity and acquired resistance. Mice were inoculated with a variety of vaccines, known to vary considerably in protective potency, and challenged with PPD 4 to 6 weeks later. Three footpad sensitivity experiments and protection tests were conducted separately with the same kind of preparations (Table 5). Preparations such as oil-treated BCG cell walls and viable BCG, which stimulated high resistance to airborne infection, also induced a high order of sensitivity to PPD. On the other hand, preparations of low protective potency, such as NaOH-treated BCG cell walls and BCG cell walls suspended in Tween 80 in saline (without oil), did not stimulate hypersensitivity detectable by the footpad test.

TABLE 3. *Footpad sensitivity of mice to PPD five weeks after injection of 300 μg of oil-treated BCG cell walls by various routes*

Route of sensitizing injection	Mean foot thickness[a] (mm)					
	24 hr			48 hr		
	Left	Right	P[b]	Left	Right	P
Intradermal	2.61	1.81	<0.001	2.35	1.88	<0.01
Intravenous	2.00	1.82	<0.05	2.00	1.87	<0.05
Intramuscular	2.01	1.80	<0.05	2.04	1.87	<0.001
Intraperitoneal	2.05	1.86	<0.05	2.07	1.91	<0.05
Subcutaneous	2.41	1.85	<0.001	2.32	1.97	<0.05
None	1.86	1.91	>0.1	1.82	1.87	>0.1

[a] Left footpad inoculated with 5 μg of PPD in buffer; right footpad inoculated with buffer only.
[b] Comparison of right and left feet in each group of 10 mice.

TABLE 2. *Footpad sensitivity of mice to PPD four weeks after intravenous injection of varying doses of oil-treated BCG cell walls*

Sensitizing dose of BCG cell walls	Mean foot thickness[a] (mm)								
	4 hr			24 hr			48 hr		
	Left	Right	P[b]	Left	Right	P	Left	Right	P
μg									
37.5	1.95	1.91	>0.05	2.03	1.97	>0.1	2.02	1.97	>0.1
75	1.94	1.86	<0.05	1.95	1.92	>0.1	1.93	1.91	>0.1
150	1.95	1.91	>0.1	2.11	1.97	<0.01	2.04	1.97	<0.05
300	1.81	1.75	<0.01	1.93	1.75	<0.01	1.92	1.83	<0.01
None	1.96	1.91	<0.05	1.98	1.98	>0.05	2.00	1.98	>0.1

[a] Left footpad inoculated with 5 μg of PPD in buffer; right footpad inoculated with buffer only.
[b] Comparison of right and left feet in each group of 10 mice.

TABLE 4. *Footpad sensitivity of mice to PPD at various intervals after intravenous injection (300 μg) of oil-treated BCG cell walls*

Interval between sensitization and footpad challenge (weeks)	Mean foot thickness[a] (mm)					
	24 hr			48 hr		
	Left	Right	P[b]	Left	Right	P
1	1.73	1.68	<0.05	1.69	1.72	>0.1
	1.76	1.75	>0.1	1.72	1.78	>0.05
2	1.90	1.82	<0.01	1.68	1.69	>0.1
	1.81	1.85	>0.05	1.69	1.71	>0.1
3	1.85	1.79	<0.05	1.80	1.76	0.1
	1.92	1.90	>0.1	1.84	1.81	<0.05
4	1.93	1.75	<0.05	1.92	1.83	<0.01
	1.93	1.98	>0.05	2.00	1.98	>0.1
10	2.21	1.98	<0.001	2.21	2.04	<0.05
	1.98	1.99	>0.1	2.01	2.04	>0.1
20	2.04	1.96	<0.01	2.03	1.94	<0.05
	1.95	1.99	>0.1	2.02	1.97	>0.1
37	2.02	2.10	>0.1	2.07	2.26	<0.05
	2.02	2.01	>0.1	2.03	2.02	>0.1

[a] Left footpad inoculated with 5 μg of PPD in buffer and right footpad inoculated with buffer only, except at the 37-week interval when these materials were injected into the opposite footpads. The second row of values for each time interval represent controls.

[b] Comparison of right and left feet in each group of 10 mice.

Development of acquired resistance. Mice were inoculated iv with 300 μg of oil-treated BCG cell walls and challenged with H37Rv by aerosol 1 day, 1, 2, 4, or 8 weeks later. From the data presented in Table 6, it is clear that maximal resistance to airborne infection was attained between 2 and 4 weeks after vaccination with cell walls.

Histology of footpad reaction. Normal animals exhibited a mild subcutaneous infiltrate in footpads inoculated 4 hr earlier with PPD. Most of the cells were neutrophils, but a few eosinophils and mononuclear cells were also present. By 24 hr this reaction had subsided and most sections taken from footpads of normal animals 24 and 48 hr after inoculation with PPD were considered to be within normal limits (Fig. 1a). Footpads injected with buffer only served as controls in vaccinated mice. They exhibited either no abnormal cellular response or only a mild subcutaneous infiltration of polymorphonuclear cells when examined 24 or 48 hr after injection.

In footpads of animals inoculated iv with oil-treated BCG cell walls, the reaction 24 hr after injection of PPD was located in both the subcutaneous tissue and dermis. Circumscribed areas of subcutaneous tissue were heavily infiltrated with polymorphonuclear cells, many of which were in various stages of disintegration. Only an occasional eosinophil could be identified with certainty. Scattered throughout both the subcutaneous tissue and deeper portions of the dermis were numerous mononuclear cells. By 48 hr the infiltrate was confined almost exclusively to the subcutaneous region and was composed primarily of mononuclear cells (Fig. 1b). Eosinophils and a few neutrophils appeared to comprise less than 10% of the cells in most sections.

In contrast with findings in animals vaccinated with oil-treated cell walls, footpads from animals inoculated iv with cell walls suspended in 0.2% Tween 80 in saline exhibited only mild reactions 24 and 48 hr after injection of PPD. The reaction consisted of only a few mononuclear cells, neutrophils, and eosinophils in the subcutaneous region.

The cellular reaction to PPD in footpads of animals inoculated id with oil-treated BCG cell walls was more intense than reactions observed in animals vaccinated by any of the other routes employed. The reaction extended through the entire dermis and subcutaneous area as well as between underlying bundles of skeletal muscle. At 24 hr after inoculation of PPD, the infiltrate was composed of approximately equal numbers of mononuclear and polymorphonuclear cells. By 48 hr mononuclear cells predominated but many eosinophils and a few neutrophils were also present (Fig. 1c).

DISCUSSION

Basically, results of the footpad tests with mice sensitized with oil-treated mycobacterial cell walls are in agreement with results obtained by others with mice sensitized with living or dead intact mycobacteria (6, 7, 9). PPD injected into the footpads of cell wall-sensitized mice induces a reaction characterized by swelling and reddening and which generally reaches a maximum at about 24 hr after challenge. In mice sensitized for 4 to at least 37 weeks, the reaction is still marked 48 hr after challenge. Histologically, elements of both immediate and delayed type reactions were observed in the lesions at various intervals after challenge. Large numbers of both neutrophils and mononuclear cells were present 24 hr after challenge, and mononuclear cells clearly predominated at 48 hr.

Our results differ from those of others in several minor respects. We detected a weak footpad reaction as early as 1 week after sensitization with cell walls, whereas Gray and Jennings (7) did not record positive reactions to old tuberculin until 2.5 weeks or later in mice infected with a small number of tubercle bacilli by the intranasal route. Presumably, we were able to detect positive reactions earlier than were Gray and Jennings,

TABLE 5. *Footpad sensitivity of mice to PPD four to six weeks after injection of various mycobacterial preparations*

Expt	Sensitizing preparation	Protective index[b]	Mean foot thickness[a] (mm)					
			24 hr			48 hr		
			Left	Right	P[c]	Left	Right	P
A	300 μg of oil-treated BCG cell walls iv	3.21	2.17	1.95	<0.01	2.00	1.94	>0.1
	300 μg of NaOH- and oil-treated BCG cell walls iv	0.48	1.98	1.96	>0.1	1.96	1.94	>0.1
	None		2.00	1.96	>0.1	1.97	1.97	>0.1
B	300 μg of oil-treated BCG cell walls iv	3.11	2.09	1.93	<0.001	2.01	1.90	<0.01
	300 μg of BCG cell walls in 0.2% Tween 80 saline iv	−0.28	2.01	1.96	>0.05	1.98	1.97	>0.1
	300 μg of BCG cell walls in Freund's incomplete adjuvant sc	0.05	2.01	1.99	>0.1	1.98	1.96	>0.1
	300 μg (moist weight) of viable BCG iv	3.00	2.17	1.99	<0.001	2.06	1.98	0.01
	None		1.99	2.00	>0.1	2.06	1.97	>0.1
C	300 μg of oil-treated BCG cell walls iv	3.59	2.22	1.91	<0.05	2.13	1.93	<0.001
	300 μg of oil-treated DMSO extract of BCG iv	1.22	2.20	1.92	<0.01	2.13	2.02	>0.05
	300 μg of oil-treated SDS extract of BCG iv	1.15	1.98	1.92	<0.01	2.02	2.00	>0.1
	300 μg of oil-treated wax C and D from BCG iv	0.12	1.94	1.97	>0.1	2.08	2.01	>0.1
	None		1.94	1.94	>0.1	1.99	2.02	>0.1

[a] Left footpad was inoculated with 5 μg of PPD in buffer; right footpad was inoculated with buffer only.

[b] $\log_{10}$ median count of H37Rv in lungs of control mice − $\log_{10}$ median count in lungs of vaccinated mice.

[c] Comparison of right and left feet in each group of 10 mice.

TABLE 6. *Development of resistance in mice vaccinated intravenously with 300 μg of oil-treated BCG cell walls against airborne infection with M. tuberculosis H37Rv*

Interval between vaccination and challenge	Median count H37Rv/lung[a]	
	Cell wall-vaccinated mice	Nonvaccinated mice
1 day	8.1×10^5	4.0×10^7
1 week	7.4×10^2	1.2×10^7
2 weeks	1.2×10^2	1.2×10^7
4 weeks	$<8.7 \times 10^1$	8.7×10^6
8 weeks	$<9.7 \times 10^1$	1.6×10^7

[a] Median count of viable units of H37Rv for 10 lungs in each group.

because (i) our mice were sensitized more rapidly by the larger quantities of mycobacterial antigen administered and (ii) the use of calipers to measure the response to PPD enabled us to recognize the early weak, but statistically significant, footpad response.

More difficult to explain is the fact that cell wall-sensitized mice responded to footpad injection of PPD, whereas Kong et al (9) reported that mice sensitized with phenol-killed BCG cells reacted to old tuberculin but not to PPD. Perhaps differences in the experimental procedures, such as the nature and preparation of the sensitizing material and the strain of mouse, are responsible for the differences in results.

In several of our experiments there was a definite correlation between footpad sensitivity and acquired resistance to airborne infection with tubercle bacilli. First, as shown in Table 5, the degree of footpad sensitivity varies directly with the potency of the vaccine. Oil-treated BCG cell walls and viable BCG stimulated high levels of resistance to infection and sensitivity to PPD, whereas NaOH-treated cell walls and cell walls administered without oil stimulated little or no

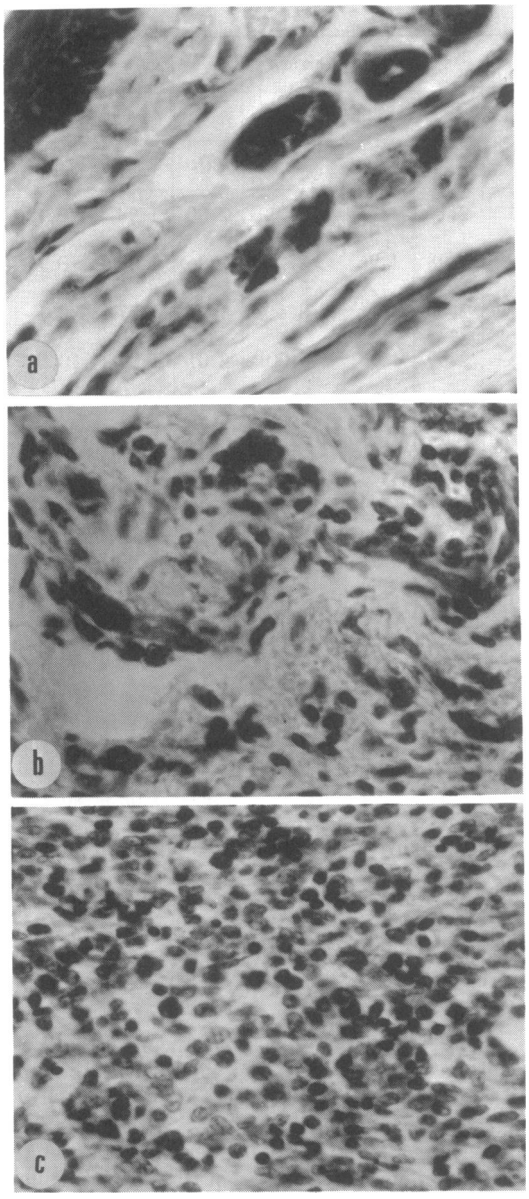

FIG. 1. *Skin and subcutaneous tissue from the footpads of mice inoculated with PPD 48 hr before sacrificing the animal. (a) A nonimmunized mouse. (b) A mouse immunized with 300 μg of oil-treated BCG cell walls administered intravenously. The infiltrate is composed primarily of mononuclear cells and is located in the subcutaneous tissue and deeper portions of the dermis. (c) A mouse immunized with 300 μg of oil-treated BCG cell walls administered intradermally. The intense infiltrate extending into the superficial portion of the dermis is composed primarily of mononuclear cells but eosinophils and a few neutrophils are also present. × 400.*

resistance or sensitivity. Secondly, footpad sensitivity and acquired resistance developed in mice at roughly the same rate after inoculation with the cell wall vaccine; both reached their highest levels at approximately 4 weeks (Tables 4 and 6).

At variance, at least superficially, with the concept of the absolute relationship of delayed sensitivity and acquired resistance is our finding that mice can develop sensitivity to PPD without concomitantly developing acquired resistance to airborne infection. Footpads of mice vaccinated id or sc react far more strongly to PPD than do footpads of mice vaccinated iv (Table 3), although mice given cell walls iv are far more resistant to airborne infection than are mice inoculated by other routes (2). These results conclusively demonstrate that footpad sensitivity is not correlated with resistance to pulmonary infection.

A similar conclusion was reported recently by Youmans and Youmans (14). They found that mice vaccinated with ribosomal and RNA fractions of *M. tuberculosis* H37Ra incorporated into Freund's incomplete adjuvant were resistant to intravenous challenge with *M. tuberculosis* H37Rv but failed to exhibit a delayed reaction to PPD inoculated into the footpad. Footpad swelling observed 24 hr after challenge was considered by them to be a waning Arthus-type reaction.

Despite the negative correlation between the footpad test and immunity to infection, the hypothesis that delayed hypersensitivity is related in some way to resistance to pulmonary infection has not necessarily been invalidated. The test employed in this study measured the hypersensitivity reaction in the footpad; the ability of the mouse to mount a delayed hypersensitivity reaction in the lung, the organ first exposed to the airborne bacteria, has not been determined. Although we have no a priori reason for suggesting that a delayed reaction cannot be readily demonstrated in the lungs of mice vaccinated by routes other than the iv, we cannot assume that the response would be the same in the lung as in the footpad, since delayed reactions at a third site, the skin, are notoriously difficult to produce.

Another factor which must be considered in the evaluation of the relationship of delayed hypersensitivity to immunity is the quantitative change which occurs after vaccination. We reported earlier that there is a considerable granulomatous response in the lungs (and other organs) of mice vaccinated iv but not by other routes (2, 4). This cellular response is presumably induced by the cell walls which are sequestered in

the lung after iv immunization only (2). Conceivably, resistance to airborne infection depends upon the presence of a critical number of sensitized cells in the lung; the threshold number may be achieved only after a specific quantity of antigenic material is deposited in the lung. Therefore, clarification of the relationship of hypersensitivity and immunity in experimental tuberculosis of mice must await qualitative and quantitative assessment of the hypersensitivity reaction in the lung.

LITERATURE CITED

1. Aebi, A., J. Asselineau, and E. Lederer. 1953. Sur les lipides de la souche humaine "Brévannes" de *Mycobacterium tuberculosis*. Bull. Soc. Chim. Biol. **35**:661–684.
2. Anacker, R. L., W. R. Barclay, W. Brehmer, G. Goode, R. H. List, E. Ribi, and D. F. Tarmina. 1969. Effectiveness of cell walls of *Mycobacterium bovis* strain BCG administered by various routes and in different adjuvants in protecting mice against airborne infection with *Mycobacterium tuberculosis* strain H37Rv. Amer. Rev. Resp. Dis. **99**:242–248.
3. Anacker, R. L., W. D. Bickel, W. Brehmer, M. Niwa, E. Ribi, and D. F. Tarmina. 1969. Immunization of mice by combinations of inactive fractions of *Mycobacterium bovis* strain BCG. Proc. Soc. Expt. Biol. Med. **130**:723–730.
4. Barclay, W. R., R. Anacker, W. Brehmer, and E. Ribi. 1967. Effects of oil-treated mycobacterial cell walls on the organs of mice. J. Bacteriol. **94**:1736–1745.
5. Brehmer, W., R. L. Anacker, and E. Ribi. 1968. Immunogenicity of cell walls from various mycobacteria against airborne tuberculosis in mice. J. Bacteriol. **95**:2000–2004.
6. Dietrich, F. M., A. A. Nordin, and H. Bloch. 1962. Delayed hypersensitivity in tuberculous mice. II. Generalized tuberculin reaction and footpad tests. Int. Arch. Allergy Appl. Immunol. **20**:129–142.
7. Gray, D. F., and P. A. Jennings. 1955. Allergy in experimental mouse tuberculosis. Amer. Rev. Tuberc. **72**:171–195.
8. Grover, A. A., H. K. Kim, E. H. Wiegeshaus, and D. W. Smith. 1967. Host-parasite relationships in experimental airborne tuberculosis. II. Reproducible infection by means of an inoculum preserved at −70 C. J. Bacteriol. **94**:832–835.
9. Kong, Y. M., D. C. Savage, and L. N. L. Kong. 1966. Delayed dermal hypersensitivity in mice to spherule and mycelial extracts of *Coccidioides immitis*. J. Bacteriol. **91**:876–883.
10. Larson, C. L., E. Ribi, W. C. Wicht, and R. List. 1961. Skin reactions produced in rabbits by cell walls and protoplasm of *Mycobacterium tuberculosis* and *M. butyricum*. Amer. Rev. Resp. Dis. **83**:184–193.
11. Mackaness, G. B. 1968. The immunology of antituberculous immunity. Amer. Rev. Resp. Dis. **97**:337–344.
12. Munoz, J. 1967. Immediate hypersensitivity reactions induced in mice by active and passive means. J. Immunol. **98**:638–647.
13. Ribi, E., R. L. Anacker, W. Brehmer, G. Goode, C. L. Larson, R. H. List, K. C. Milner, and W. C. Wicht. 1966. Factors influencing protection against experimental tuberculosis in mice by heat-stable cell wall vaccines. J. Bacteriol. **92**:869–879.
14. Youmans, G. P., and A. S. Youmans. 1969. Allergenicity of mycobacterial ribosomal and ribonucleic acid preparations in mice and guinea pigs. J. Bacteriol. **97**:134–139.