Specificity of Resistance to Tuberculosis and to Salmonellosis Stimulated in Mice by Oil-Treated Cell Walls. (31752)

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As reported previously(1-4), nonviable vaccines prepared from disrupted tubercle bacilli by suitable treatment with oil protected mice against air-borne challenge with virulent *Mycobacterium tuberculosis*. The effective immunogen was contained in the clean cell walls and was not found in the protoplasm (3). Because neither pertussis vaccine nor endotoxin but only products of tubercle bacilli were effective against such a challenge(5), it would appear that oil enhanced or protected the activity of a specific cell-wall immunogen. Additional evidence regarding the specificity of this system has, however, been sought experimentally. The effects of light mineral oil have also been studied in a variety of test systems to determine whether or not application of oil to dried cell walls would similarly enhance the effectiveness of vaccines against other bacterial pathogens.

Materials and methods. Mycobacteria were cultivated in Sauton's liquid medium(6). Heavy pellicles were harvested on sterile gauze supported by a stainless steel screen, transferred onto Whatman No. 1 filter paper in a Buchner funnel and washed by pouring distilled water over the cell mass. The cells were then dispersed in distilled water with a high-speed mixer to a concentration of 20% (moist weight/vol) and disrupted in a Sorvall cell fractionator at a pressure of 35,000 psi. The cell walls were collected, washed, and dried from the frozen state as described previously(3,4). Enterobacteriaceae were grown in the (slightly modified) liquid synthetic "ammonium" medium of Anderson(7), disrupted and processed for collection of clean cell walls as described by Fukushi *et al*(8). Other bacteria were grown in appropriate liquid media and processed similarly.

Five distinct types of mouse-protection tests were employed:

1. *Specific protection against paratyphoid challenge.* Male white mice of the Rocky Mountain Laboratory (RML) strain, 28 days old, were vaccinated subcutaneously (SC) in the nuchal region with graded doses (based on dry weight) of materials contained in 0.25 ml saline. They were challenged intraperitoneally (IP), 14 days later, with 1.0×10^7 plate-count units of *Salmonella enteritidis*, strain S-795(9). Survivors were determined after 4 days, and ED_{50} values were read from straight lines fitted to plots of probits-percent survivors *vs* log dose. In this test system, all mice die eventually, but life is prolonged by vaccination with appropriate antigens, and, within the effective range of doses, survival is lineally related to log dose at any interval from 2-4 days after challenge(10,11). It is referred to as a "specific" protection test because, with the 2-week interval between vaccination and challenge, only materials containing multiple O-antigenic determinants in common with *S. enteritidis* confer significant protection(12). 2. *Nonspecific protection against typhoid bacilli.* The test for "nonspecific" protection of mice against challenge with *Salmonelli typhi*, strain Ty 2, was conducted as described by Haskins *et al*(13). This test has been extensively employed as an assay for endotoxin, and any endotoxin is protective, whether serologically related or not. 3. *Early massive challenge test.* Mice vaccinated IP with various toxic and/or antigenic materials were challenged intravenously (IV) 24 hours later with 2×10^8 *M. tuberculosis*, strain H37Rv in 0.2 ml (a dose large enough to kill control mice within 7 days). Tests were evaluated by the proportion of survivors at intervals from 7 to 14 days post-challenge. 4. *Delayed massive challenge test.* This test was essentially that described by Youmans and Youmans(14). Mice were vaccinated IP and challenged IV, 30 days later, with 4×10^7 *M. tuberculosis*

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TABLE I. "Specific" Protection Against *S. enteritidis* by Homologous Antigens. Effect of treatment with oil.*

Preparation	Dose, μg	Survivors /Total	ED_{50}, μg
S. enteritidis cell walls, oil-treated	.4	15/48	
	2.0	23/49	2.4
	10.0	31/46	
S. enteritidis cell walls, untreated	.4	11/50	
	2.0	26/50	2.0
	10.0	36/50	
Citrobacter freundii endotoxin†	.5	10/50	No protection
	5.0	4/49	
	50.0	9/49	
Saline controls	—	5/48	—

* 28-day-old male mice vaccinated subcutaneously and challenged intraperitoneally, 14 days later, with 1×10^7 *S. enteritidis*, Strain S-795. Test read 4 days after challenge.
† Mouse $LD_{50} = 130$ μg.

H37Rv. Tests were evaluated by the proportion of survivors at intervals from 14-30 days after challenge. 5. *Pulmonary challenge test.* Mice were vaccinated IV and challenged, 30 days later, by aerosol in the Middlebrook apparatus(15) as described earlier(5). Tests were evaluated by median counts of viable tubercle bacilli (H37Rv) per 100 mg lung tissue in samples of 10 mice per group of 20 sacrificed 30 days after challenge(2,4). Evaluations were also made by the proportions of mice having grossly visible tubercles in the lungs after the same interval.

The strain of BCG employed was No. 1173 P2 of the Pasteur Institute, Paris. Other bacteria from the RML stock culture collection included: *M. tuberculosis* H37Ra, *Salmonella typhimurium* 5609B, *Citrobacter freundii* 5396/38, *Escherichia coli* 0113 (Braude), *Brucella abortus* strain 19, and *Listeria monocytogenes* 11-373 (Smith).

Wherever it appears, the word "oil" refers to light mineral oil, N.F. When used, it was applied to dry material with a Teflon grinder in the proportion of 0.48 ml oil per 100 mg cell walls. The resulting paste of oil-coated cell walls was dispersed to the desired concentrations in saline containing 0.2% Tween 80. Stock suspensions of all preparations, both oil-treated and untreated, were heated at 65°C for 30 minutes, and sterility was tested by culture before use.

Results. The effectiveness of cell walls from *S. enteritidis*, with and without oil treatment, in protecting mice specifically against the homologous bacterium was examined first. Vaccination of mice with graded doses of preparations and challenge 14 days later with a number of viable *S. enteritidis* less than 1/1,000 the lethal dose of killed bacteria gave the results shown in Table I. Four days after challenge, when 90% of the unvaccinated mice were dead, mice given homologous cell walls were protected in a manner that showed a graded response to dose over the range tested. Mice receiving much greater amounts of potent but antigenically unrelated endotoxin (from *Citrobacter freundii*) were not significantly protected at any tolerable dose. But whether the specific cell walls were or were not treated with oil was, in this system, quite without effect.

In another experiment, a test that measures "nonspecific" protection against challenge with *S. typhi* was employed. When RML mice are given doses of preparations IV and challenged IP 24 hours later with about 8×10^7 cells of strain Ty 2, 75-100% of the saline diluent controls die within 3 days, but any endotoxin-containing material confers measurable protection. Mice that do not die within 3 days will survive indefinitely and eliminate the typhoid bacilli. As seen in Table II, the toxic, but antigenically unrelated, cell walls from *Escherichia coli* conferred good,

TABLE II. Nonspecific Protection of Mice *vs* Challenge with *S. typhi*. Effect of treatment of antigens with oil.*

Preparation	Dose, μg	Survivors /Total	ED_{50}, μg
E. coli cell walls	4	5/32	
	20	21/32	12
	100	31/32	
E. coli cell walls, oil-treated	4	7/32	
	20	19/32	13
	100	30/32	
Controls	(saline)	6/32	—
BCG cell walls	16	7/32	No protection
	80	6/32	
	400	5/32	
BCG cell walls, oil-treated	16	8/32	
	80	3/32	"
	400	6/32	

* 28-day-old male mice vaccinated intravenously and challenged 24 hr later with 80 million *S. typhi*, Strain Ty-2.

TABLE III. Nonspecific Resistance to Early Intravenous Challenge with Virulent Tubercle Bacilli Stimulated in Mice by Oil-Treated Cell Walls.*

Preparation (cell walls)	Lethality for normal mice (LD_{50}), μg	Dose, μg	% Survivors at days after challenge 7	11	14
S. typhimurium	410	100	100	95	65
Br. abortus	5,400	1,000	100	90	20
		100	75	30	5
L. monocytogenes	>1,000	1,000	95	20	5
		100	50	10	0
BCG	>10,000	500	70	30	5
		100	30	5	0
Controls	—	—	0	0	0

* Groups of 20 mice vaccinated intraperitoneally and challenged intravenously, 24 hr later, with 200 × 10^6 *M. tuberculosis* H37Rv. All mice dead at 18 days post-challenge.

and equal, protection, with or without oil treatment. The cell walls of BCG, oiled or not, were without value, a finding consistent with our failure to detect endotoxin in BCG.

The question arose as to whether a test system patterned after the foregoing would measure, predominantly, nonspecific resistance of mice to challenge with virulent tubercle bacilli. For this purpose, mice vaccinated with a variety of materials were given early IV challenge with very large doses of H37Rv. The toxicity data at the left of Table III show that the brucella cell walls contained less than one-tenth the endotoxin of the salmonella cell walls per unit weight. Neither listeria nor BCG cell walls are known to contain endotoxin. It is evident from the percentages of mice surviving, particularly after 11 and 14 days, that endotoxin-containing cell walls from Gram-negative bacteria were considerably more protective than the other two varieties of oil-treated cell walls and that their protective value correlated roughly with the content of endotoxin. It is also apparent that listeria and BCG protected to approximately the same low degree—a further indication of the nonspecific nature of protection in such a test.

Table IV lists results obtained when the same cell-wall preparations were examined for ability to stimulate resistance to late massive challenge, that is, to IV challenge with about 4 × 10^7 cells of H37Rv 30 days after IP vaccination. This procedure, sometimes with the use of IV vaccination, has been widely employed to evaluate the efficacy of living and nonliving vaccines against experimental tuberculosis in mice. Here all of the materials led to some increase in survival times, but BCG was, under these conditions, at least as effective as the endotoxin-containing vaccines and much more so than the cell walls of listeria. From this one may conclude that both specific and nonspecific effects are operative in such a test, and that it may not always be easy to differentiate between them.

In an unrelated experiment, oil-treated and untreated cell walls from *M. tuberculosis*, strain H37Ra, were compared for ability to protect against a similar IV challenge 30 days after vaccination. The results (Table V) illustrate the inherent variability of the test but are not believed to indicate a significant enhancing effect of oil in this system.

Oil-treated cell walls from several species of bacteria also were tested for ability to protect against pulmonary challenge by the method described earlier. Table VI shows the specific nature of this test. Mice vaccinated with BCG cell walls were protected to the extent expected from previous experience. Few or no lung lesions developed. Not one mouse showed any degree of protection by any of the heterologous vaccines. The possible effect of endotoxin has here been tested to the limit. A dose of *S. typhimurium* cell walls slightly in excess of the LD_{50} was given

TABLE IV. Resistance to Delayed Intravenous Challenge with Virulent Tubercle Bacilli Stimulated in Mice by Oil-Treated Cell Walls.*

Preparation (cell walls)	Immunizing dose, μg	Survivors at days after challenge 14	17	21	30
		%			
S. typhimurium	100	100	90	60	50
Br. abortus	1,000	95	90	80	40
	100	100	60	25	20
L. monocytogenes	1,000	100	70	25	20
	100	95	65	20	10
BCG	100	100	95	80	55
Controls	—	80	35	5	0

* Groups of 20 mice vaccinated intraperitoneally and challenged intravenously, 30 days later, with 40 × 10^6 *M. tuberculosis* H37Rv.

TABLE V. Comparison of Oil-Treated and Untreated Cell Walls in Protecting Mice Against Delayed Intravenous Challenge with Virulent Tubercle Bacilli.

Preparation	Dose, μg*	Route	30-day survivors /No. challenged	% Survivors
A. Cell walls in 0.2% Tween-saline	250	IP	9/20	45
	1,000	"	19/20	95
	4,000	"	16/20	80
B. Oil-treated cell walls in 0.2% Tween-saline	250	"	7/20	35
	1,000	"	7/20	35
	4,000	"	15/19	79
C. Live BCG	200† (moist)	"	11/20	55
D. (Unvaccinated)	—	—	0/20	0
A. Cell walls in 0.2% Tween-saline	200	IV	12/19	63
	400	"	18/20	90
	800	"	18/20	90
B. Oil-treated cell walls in 0.2% Tween-saline	200	"	20/20	100
	400	"	19/20	95
	800	"	19/20	95
C. Live BCG	200† (moist)	"	16/20	80
D. (Unvaccinated)	—	—	2/20	10

* Dry weight, cell walls of *M. tuberculosis* H37Ra except where moist BCG is specified.
† 7.7×10^6 viable cells.

to 20 mice. Nine of these survived to be challenged, forming the test group on the top line of the table. None was protected.

From these results, together with those in the preceding tables, it is concluded that protection of mice against pulmonary infection with virulent *M. tuberculosis* depends upon a specific immunogen which is not found in any of the other species tested.

TABLE VI. Resistance to Pulmonary Challenge with Virulent Tubercle Bacilli Stimulated in Mice by Oil-Treated Cell Walls.*

Preparation (cell walls)	Immunizing dose (dry wt), μg	Results 30 days after challenge, No. with lung lesions /No. of mice tested
S. typhimurium	500	9/9
	100	20/20
Br. abortus	1,000	19/19
	100	20/20
L. monocytogenes	1,000	20/20
	100	20/20
BCG	500	0/20
	100	2/20
Controls	—	20/20

* Groups of 20 mice vaccinated intravenously and challenged by aerosol, 4 wk later, with a few virulent tubercle bacilli (H37Rv).

Whether or not it may be shared by other mycobacteria has only recently been examined. As will be reported elsewhere (Brehmer *et al.*, unpublished observations), oil-treated cell walls of certain other species of mycobacteria can, in higher doses than are required for BCG cell walls, protect against pulmonary challenge approximately as well as viable BCG.

Discussion. The major issue to be decided by the foregoing experiments is whether or not treatment of dried cell walls with oil, in the manner described, increases their capacity to stimulate nonspecific resistance to infection in mice. The evidence is strong that it does not in either experimental tuberculosis or salmonellosis.

In a test in which the measurable effect was known to be produced nonspecifically by endotoxin (Table II), the use of oil did not enhance protection. The well-known ability of mycobacteria or fractions thereof to protect against heterologous infections was not manifested in this test, probably because of the short interval between vaccination and challenge(16,17). The findings add to the evidence that the nonspecific protective ef-

fect of mycobacteria probably is not dependent upon endotoxin or endotoxin-like substances.

The test described in Table III employed a rigorous challenge with virulent tubercle bacilli 24 hours after vaccination, with the thought that any protective effects might depend chiefly upon the content of endotoxin in vaccines, as in the preceding test. In the main, results can be interpreted on this basis, although the moderate prolongation of life by cell walls of *L. monocytogenes* and BCG indicates that additional substances may protect mice similarly against tuberculosis, even after so short an interval. All of the preparations contained oil-treated cell walls, and it is not yet known if untreated cell walls would protect similarly in such a test.

When 30 days were allowed to elapse between IP vaccination and IV challenge (Table IV), the relative efficacy of BCG cell walls increased, but the protection from unrelated endotoxic cell walls remained striking. Again, all of the preparations were oil-treated; however, in the experiment reported in Table V, plain and oil-treated cell walls of *M. tuberculosis* H37Ra were compared in the same type of test and were judged not to differ significantly in effectiveness.

Thus, the data in Tables I-V provide no evidence that treatment with oil in any way affects the specificity of protection by whole cell walls, although a general lack of specificity has been demonstrated in the conventional "massive" IV-challenge test, as conducted in RML mice. It has been reported elsewhere(3) that treatment with oil was necessary to render cell walls of BCG or H37Ra protective against pulmonary tuberculosis in mice challenged by aerosol. The mechanism by which such resistance is conferred remains speculative, but data in Table VI show that it has a remarkable specificity. Oil-coated cell walls of additional unrelated species (*Corynebacterium pseudotuberculosis* and *C. parvum*) have since been tested and found to be as valueless in this test as those of *S. typhimurium* or *L. monocytogenes*.

On the basis of these and previously reported results, it might be concluded that the pulmonary challenge test should be preferred to other mouse-protection tests for evaluation of experimental prophylactics against tuberculosis. How results obtained in mice are related to effects in other animals remains, however, largely unknown. Furthermore, evaluation of the pulmonary challenge test in mice should be made more critical through longer term experiments in which both the interval between vaccination and challenge and the period of observation after challenge are increased. Experiments along each of these lines are in progress. Data already obtained (Anacker *et al,* unpublished) showed that mice vaccinated with oil-treated cell walls were protected at least as well as mice vaccinated with viable BCG when challenge was delayed as much as 4 months and when observed as much as 6 months after challenge.

Summary. Coating with oil, which was essential to render cell walls of BCG protective to mice against challenge with tubercle bacilli by aerosol, has been found not to affect the specificity of reactions conditioned by cell walls in this and other systems. When the interval between vaccination and challenge was short (24 hours), mice were protected against heavy intravenous challenge with *M. tuberculosis* principally by nonspecific factors; after a 30-day interval, protection appeared to depend upon a mixture of specific and nonspecific effects.

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Distribution of H-2 Specificities Within the LM Mouse Cell Line and Derived Lines.* (31753)

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Histocompatibility antigens on mouse cells, and antibodies to them, have been demonstrated by a variety of techniques. These include haemagglutination, absorption, cytotoxicity and stimulation of antibody production(1), measurement of colony formation *in vitro*(2), and staining with fluorescent antibody(3).

Gangal, Merchant and Shreffler(1) demonstrated that after 25 years in culture LM cells grown in modified medium 199(4), supplemented with 0.5% peptone (called 199P), still possessed all the specificities of the $H\text{-}2^k$ allele of the C3H mouse from which the L-cell line originally was derived. They demonstrated specificities 1, 3, 5, and 8 by absorption and haemagglutination, and specificity 11 (which was only weakly absorbed) by stimulation of antibody production. As specificity 32 is not readily detected by haemagglutination, they demonstrated it by cytotoxicity.

In the present study an indirect fluorescent antibody test was used to demonstrate all the specificities. The fluorescent antibody test also enables one to check for the presence of antigenic specificities at the cellular level. A proportion of antigen deficient cells in a population would not be observed by absorption tests, but would be detected by the fluorescent antibody method. It was proposed to determine whether all the cells in a population of LM cells carried each of the $H\text{-}2^k$ specificities.

In addition to confirming and enlarging upon the previous results with LM (199P) cells, it was considered interesting to know whether LM cells grown in 2× Eagle basal medium also possessed the known specificities of the $H\text{-}2^k$ allele. Eagle medium is simpler than 199P and is chemically defined(5). LM (2×E) cells are known to differ from LM (199P) cells in their alkaline phosphatase activity as well as in specific chromosome markers and karyotype(6). LM (2×E) cells have no alkaline phosphatase activity but LM (199P) cell populations are composed of a mixture of alkaline phosphatase positive and alkaline phosphatase negative cells(7). It has been suggested that alkaline phosphatase activity is closely associated with H-2 antigen specificity(8). If this were the case, LM (2 × E) cells might be expected to be lacking in one or more of the H-2 specificities.

It was also proposed to check for the presence of H-2 specificities on some other variants of the LM cells, of different karyotype, alkaline phosphatase activity, and colonial and morphological appearance to see whether possible deficiency of antigenic specificities can be related to any of these characteristics.

Materials and methods. Cell lines. The

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