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Induction of resistance to tuberculosis in mice with defined components of mycobacteria and with some unrelated materials

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Summary. Factors contributing to protection against experimental tuberculosis have been studied with refined and well characterized fractions from mycobacteria and with certain unrelated antigens. Mice were vaccinated intravenously with various combinations of materials presented on minute oil droplets in saline emulsion and were later challenged by aerosol. The minimal composition of an effective vaccine was P3 (a trehalose mycolate similar to cord factor) plus an antigen, which could be tuberculoprotein, or a low-molecular-weight tuberculin-active peptide, or unrelated antigen such as bovine serum albumin or bacterial endotoxin. Development of a hypersensitivity granuloma in the lungs appeared to be essential to protection in this laboratory model.

INTRODUCTION

An experimental model in which mice are vaccinated intravenously (i.v.) and later challenged by aerosol with virulent tubercle bacilli has been described previously (Anacker, Barclay, Brehmer, Goode, List, Ribi & Tarmina, 1969; Ribi, Anacker, Brehmer, Goode, Larson, List, Milner & Wicht, 1966). In this system, relatively small doses of non-viable BCG, administered in the form of cell walls attached to minute oil droplets, protected to approximately the same degree as the optimal quantity of viable BCG.

Intravenous injection of oiled cell wall vaccine also led to extensive granulomatous changes in the lungs, reflected by increased lung weight; and there was an obvious correlation between the presence of granuloma and resistance to challenge. However, certain observations permitted us to hold open the possibility that this relationship might be coincidental and avoidable (Meyer, Anacker & Ribi, 1974). In any case, the complex nature of cell walls and whole cells made it impossible to seperate these two responses. Therefore, further fractionation of the materials was attempted to determine whether individual components or chemical constituents could be associated with particular effects and, if possible, to define the minimal composition of a protective vaccine.

In earlier parts of this investigation, cell walls of BCG were treated with proteolytic enzymes and were then exhaustively extracted with organic solvents. The residual cell wall, designated as cell wall skeleton (CWS), was a polymeric mycolic acid-arabinogalactan-mucopeptide, containing remnants of undigested

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tuberculoprotein (Azuma, Ribi, Meyer & Zbar, 1974). A chromatographic fraction, P3, was a trehalose mycolate, normally isolated from wax D, but bearing most of the activities associated with cord factor (Azuma *et al.*, 1974; Lederer, Adam, Ciorbaru, Petit & Wietzerbin, 1975; Noll, 1956). Neither CWS nor P3 was appreciably active alone, but a combination of the two, presented at the surfaces of oil droplets, produced significant granuloma and protection (Ribi, Meyer, Azuma, Parker & Brehmer, 1975). As detailed below, these and several other materials, both related and unrelated to tubercle bacilli, have been used in the current studies.

The results presented here, considered in the light of other work, favour the interpretation that the pulmonary granuloma observed is an allergic response related to cell-mediated immunity (CMI) and delayed hypersensitivity (DH), and that resistance to the proliferation of inhaled tubercle bacilli probably does not occur in the absence of this response. Hypersensitivity to antigens other than tuberculoprotein can, however, produce resistance.

MATERIALS AND METHODS

Animals

Male mice of the random bred Rocky Mountain Laboratory strain were vaccinated i.v. at 21 days of age and killed or challenged 1 month later, except when stated otherwise.

Vaccines

Materials to be tested for prophylactic value were all dry powders initially. These were combined with light mineral oil in Teflon grinders and suspended in Tween-saline (Ribi *et al.*, 1975). Fractions described previously are listed with appropriate references: BCG cell walls (Ribi *et al.*, 1975), cell walls (CW) from *Escherichia coli* (Fukushi, Anacker, Haskins, Landy, Milner & Ribi, 1964), CW from *Francisella tularensis* (Shepard, Ribi & Larson, 1955). Endotoxins from re-mutant (heptoseless) strains of *Salmonella typhimurium* and *Salmonella minnesota* were furnished by J.A. Rudbach, University of Montana, Missoula. Other endotoxins (LPS) used were prepared in our laboratory. Purified protein derivative (PPD) was a product of Parke, Davis and Company, Detroit, Mich., lot 974562C. Tuberculin-active peptide (TAP) was prepared by hydrolysis of tuberculoprotein with mineral acid (Azuma, Yamamura, Tahara, Onoue & Fukushi, 1969). The fraction used was one that passed through a dialysis membrane (i.e. mol. wt < 10,000). Crystalline bovine serum albumin (BSA) was lot 47561 from General Biochemicals, Chagrin Falls, Ohio.

Challenge procedure

The general technique for exposing mice to *Mycobacterium tuberculosis* strain H37Rv in a Middlebrook aerosol chamber (Middlebrook, 1952) has been given previously (Ribi *et al.*, 1966), but a summary of current practice is included here for clarity and accuracy. The stock culture was kept on glycerol-potato or glycerol-egg medium (Hohn or Löwenstein-Jensen). For challenge, a transfer to Dubos liquid medium with albumin (Difco) was made and incubated at 37° for 7 days (Dubos & Middlebrook, 1947). A second subculture in Dubos medium was treated similarly and used to inoculate six flasks, containing 125 ml each of the same medium. After 7 days at 37°, growth in the flasks was harvested by centrifugation. The cells were resuspended in fresh Dubos medium and passed through a Cox M-780 media (filter), grade AA1000 (Cox Instrument Division of Lynch Corporation, Detroit, Mich.) to remove any large clumps. The suspension was then adjusted to a scale reading of 20 on a Klett-Summerson photoelectric colourimeter with blue filter number 42, divided into 2 ml quantities, and frozen at −60°. Vials of this stock suspension have been used for up to 1 year with very little change in the viable count, which was about 2×10^7/ml. (Because this type of turbidity adjustment is not exactly reproducible with different machines in different laboratories, it would be necessary for others to devise appropriate means for standardizing such suspensions.) For use, a vial was thawed and diluted 1:7 in Dubos medium; then a further 1:20 dilution was made in 0·15 M NaCl containing 0·02% Tween 80. Lots of ten to a maximum of 120 mice were placed in the chamber. The diluted suspension (about $1{\cdot}4 \times 10^5$ viable uml) was introduced into the nebulizer, and the air flow was adjusted to deliver 6 ml of the material over a 30-min exposure period. Our estimate that from 30 to 50 viable units are deposited in the lungs of each mouse is supported both by calculation and by observation of the number of primary lesions developing in unvaccinated mice. Mice were held in the chamber another 30 min while the device was flushed with air and germicidal lamps were operated.

Granuloma, protection and statistical analyses

The degree of pulmonary granuloma developing in

vaccinated mice was judged by increase in lung weight of samples killed for this purpose, usually 1 month after vaccination and immediately before the remainder of the mice were challenged. Protection was evaluated by decreased proliferation of tubercle bacilli in effectively vaccinated animals. One month after challenge, the lungs were removed, emulsified, and serially diluted. For each animal, at least three dilutions were plated in triplicate and, where possible, calculations were based on plates containing 30–300 colonies. Table 1, which contains results of a single experiment, has been analysed by Dr Martin Hamilton, then of Bozeman, Montana. The different experimental groups were compared with the control group by the method of Dunnett (1964) and compared with each other by the Tukey Studentized Range method (Keselman, 1975). Other tables of this type contain pooled data and have not been similarly analysed statistically. We believe the data can stand on their own to support any conclusion drawn.

Macrophage migration inhibition

This was tested for as described by Yamamoto & Anacker (1970).

RESULTS

The analysis of cell wall skeleton revealed small amounts of tuberculoprotein, which had not been removed by the extraction process. The proportion of this residue could be estimated from the quantities of amino acids not accounted for by the mucopeptide moiety, and was found to vary from batch to batch. Because there was also variability in the protective effect of different lots of CWS, when combined with P3 in an oil-treated vaccine, the role of tuberculoprotein in protection was investigated.

As reported previously (Ribi *et al.*, 1975), a preparation of CWS (lot 'Paris') with good protective effect contained about 1% of amino acids not accounted for by the mucopeptide moiety. Another preparation (lot 182) contained only 0·3% of such amino acids and was less active in the protection test. Table 1 presents results obtained in one typical experiment with lot 182, used with and without added PPD and/or P3. These show again that it is necessary to add P3 to CWS in order to get significant protection (compare groups B, D, and E). Although PPD alone, combined with CWS (lot 182) was not sufficient to restore protection (group

Table 1. The contribution of tuberculoprotein (PPD) to protection of mice against aerosol challenge with *Mycobacterium tuberculosis*

			Results*	
			Granuloma	Protection
Group	Material combined with oil droplets	Dose (μg)	Mean lung weight† (mg)	Median lung count‡
A	BCG whole cell walls	300	390±29	$2{\cdot}4 \times 10^3$
B	BCG cell wall skeleton lot 182	300	240±6	$1{\cdot}5 \times 10^6$
C	Trehalose mycolate (P3)	150	268±9	-§
D	CWS+P3	150±150	381±29	$4{\cdot}2 \times 10^4$
E	CWS+PPD	150±150	225±9	$2{\cdot}4 \times 10^6$
F	CWS+P3+PPD	150+150+150	550±26	$<2{\cdot}3 \times 10^2$
G	P3+PPD	150±150	429±6	$9{\cdot}2 \times 10^4$
H	Nothing (controls)	—	233±6	$4{\cdot}5 \times 10^6$

* Twenty mice were vaccinated i.v. in each group; ten were killed after 1 month, and the lungs were removed and weighed; means are given ±1SEM. The remaining mice were then challenged and killed after an additional month. The lungs were removed, emulsified, and evaluated by plate counts.

† Statistical analysis of mean lung weights indicates that Groups B, C, and E are not significantly different from controls (H). Comparisons among test groups can be described by F E B C D A G where any two symbols underlined by the same line indicate that the associated two means are not significantly different at a 0·05 simultaneous level.

‡Statistical analysis indicates that, with regard to median lung counts, Groups B and E were not significantly different from the controls (H); others were. Comparisons among test groups are described by F A G D B E.

§Plates overgrown by a contaminant already present in the medium.

E), the addition of PPD to CWS + P3 (group F) further improved protection. The high degree of protection achieved by an oil-treated vaccine composed of P3 + PPD (group G), without any CWS, indicated that these two fractions might be the most important components of cell walls for protective effect. It is notable that in all cases, including immunization with P3 + PPD, the degree of pulmonary granuloma paralleled the suppression of bacterial growth (protection).

The foregoing results are augmented by the data of Table 2, where the essential findings of several additional experiments are pooled. These show that the combination P3 + PPD consistently produced granuloma and protection, whereas neither P3 nor PPD did so alone. This suggests that tuberculoprotein is (or at least contains) the antigen to which the host becomes immunized through the adjuvant action of trehalose mycolate.

A further simplification of the vaccine could be achieved by substituting a small-molecular-weight component of tuberculoprotein for PPD. Azuma *et al.* (1969) had isolated tuberculin-active peptide from virulent *M. tuberculosis*, strain Aoyama B. The results of one experiment in which TAP was used are reported in Table 3, where it is seen that neither P3 nor TAP alone protected significantly but a combination of the two, even in rather small amounts gave marked protection. Essentially the same findings were obtained in additional experiments, where it also appeared that the combination P3 + TAP was slightly more effective than P3 + PPD. One noteworthy control showed the importance of the method of presentation of these materials. When P3 and TAP were ground with oil and suspended in Tween-saline separately, and then combined, the resulting vaccine produced neither granuloma nor protection.

Because the pulmonary granuloma observed in these studies is thought to be related to delayed-type hypersensitivity or cell-mediated immunity (Salvin & Neta, 1975; Unanue & Benacerraf, 1973; Warren, Domingo & Cowen, 1967), we explored the possibility that sensitization of the mice with antigens unrelated to mycobacteria might also protect against aerosol challenge with tubercle bacilli. Bovine serum albumin has been studied extensively in this regard. It was at once apparent, in experiments conducted as described thus far, that i.v. administration of BSA with P3 on oil droplets produced significant granuloma and some protection, but not at the significance levels observed with materials from mycobacteria. That DH specific for BSA indeed developed was also attested by macrophage migration inhibition tests, as seen in the experiment detailed in Table 4. This experiment indicates further that immunization with P3 + BSA did not lead to tuberculin hypersensitivity.

Table 2. Granulomatous inflammation and protection induced in mice by trehalose mycolate (P3) combined with PPD

No. of separate tests	Material associated with oil droplets administered i.v.	Dose (μg)	Results: Granuloma One month after vaccination mean lung weight ± SD (mg)*	Results: Protection One month after challenge mean H37Rv count per lung
			253 ± 16	$1{\cdot}0 \times 10^6$
			216 ± 19	$3{\cdot}0 \times 10^6$
5	P3	150	233 ± 52	$3{\cdot}6 \times 10^6$
			253 ± 22	$2{\cdot}3 \times 10^6$
			224 ± 20	$5{\cdot}8 \times 10^6$
			200 ± 14	$3{\cdot}8 \times 10^6$
3	PPD	300	225 ± 24	$3{\cdot}2 \times 10^6$
			215 ± 25	$2{\cdot}8 \times 10^6$
3	P3 + PPD	150 ± 150	359 ± 70	$3{\cdot}5 \times 10^3$
			311 ± 63	$3{\cdot}2 \times 10^5$
			303 ± 21	$7{\cdot}3 \times 10^4$
11	Controls	—	207–244	$3{\cdot}4$–$7{\cdot}5 \times 10^6$

*Ten mice per group. At this time the remaining ten mice in each group were challenged by aerosol with 30–50 viable units of H37Rv.

Table 3. Protection of mice against aerosol challenge with virulent tubercle bacilli: efficacy of tuberculin-active peptide (TAP) combined with trehalose mycolate (P3)

Material associated with oil droplets and administered i.v.	Dose (μg)	Results 1 month after challenge: No. with lung lesions/ no. of mice treated	Results 1 month after challenge: Median h37Rv count per lung
P3	300	9/9	$8{\cdot}4 \times 10^6$
TAP	300	10/10	$1{\cdot}0 \times 10^7$
P3+TAP	300+300	0/10	$4{\cdot}0 \times 10^3$
	150+150	0/10	$4{\cdot}4 \times 10^4$
	75+75	2/10	$3{\cdot}0 \times 10^5$
	37·5+37·5	3/10	$8{\cdot}9 \times 10^4$
BCG whole cell walls (Lot 180)	300	0/10	$1{\cdot}2 \times 10^3$
Controls (oil−Tween saline)	—	10/10	$5{\cdot}2 \times 10^6$

Table 4. Macrophage migration inhibition with cells from mice vaccinated with trehalose mycolate (P3) combined with PPD or BSA

Fractions associated with oil droplets and administered i.v.	Dose (μg)	Percentage migration of mixture of normal peritoneal cells and lung cells from vaccinated mice in the presence of antigen: PPD 30 μg/ml	BSA 100 μg/ml
P3+PPD	300+300	58	90
P3+BSA	300+300	95	45
P3	300	83	—
PPD	300	112	—
BSA	300	—	84
None	—	100	100

It occurred to us that the temporal sequence of immune responses might vary with different antigens, and this was found to be true. In the case of antigens from BCG, granulomatous inflammation was slow to develop, but it persisted for a long time. Following the same type of immunization with BSA, pulmonary granuloma, with attendant protective effect, developed rapidly and then declined. For the experiments presented in Table 5, groups of forty mice each were vaccinated as indicated. Two weeks thereafter, ten mice of each group were killed for determination of mean lung weight, and ten were challenged by aerosol. After an additional 2 weeks, ten more mice of each group were killed and the remaining mice were challenged. The mice in each challenged group were killed 4 weeks after challenge, and mean counts of tubercle bacilli in the lungs were determined. As expected from previous studies, mice vaccinated with whole BCG cell walls had substantially greater pulmonary granuloma after 4 weeks and were much better protected when challenged after that interval than when examination and challenge were performed after only 2 weeks. The reverse was true for mice vaccinated with the BSA + P3 combination. Their pulmonary granuloma was extreme after 2 weeks and, when challenged then, they were protected comparably to mice vaccinated with specific antigen and challenged after 4 weeks.

Other non-specific antigens will produce granuloma and protect when given to mice i.v. with P3 on oil droplets. Cell walls and endotoxic extracts of gram-negative bacteria have been shown to do so. The experiments outlined in Table 6 were conducted on the former time schedule, with challenge 1 month after vaccination, and it may be that adjusting this schedule will make further differences.

Table 5. Granulomatous inflammation and protection induced in mice by trehalose mycolate (P3) combined with BSA

Material associated with oil droplets and administered i.v.	Dose (μg)	Two-week interval*		Four-week interval†	
		Mean lung weight (mg)	Median H37Rv count/lung	Mean lung weight (mg)	Median H37Rv count/lung
BSA+P3	150+150	559	$8{\cdot}0\times10^{1}$	400	$4{\cdot}6\times10^{3}$
BSA	150	218	$1{\cdot}6\times10^{7}$	222	$8{\cdot}5\times10^{6}$
BCG cell walls	300	325	$2{\cdot}2\times10^{4}$	448	$6{\cdot}0\times10^{1}$
Vehicle controls	—	231	$2{\cdot}1\times10^{7}$	241	$4{\cdot}7\times10^{6}$
BSA+P3	300+150	599	$2{\cdot}2\times10^{4}$	374	$1{\cdot}8\times10^{5}$
P3	150	359	$2{\cdot}4\times10^{6}$	328	$1{\cdot}6\times10^{6}$
KLH+P3	300+150	525	$4{\cdot}0\times10^{3}$	449	$4{\cdot}6\times10^{5}$
BCG cell walls	300	282	$1{\cdot}1\times10^{6}$	432	$1{\cdot}4\times10^{3}$
Vehicle controls	—	235	$1{\cdot}9\times10^{7}$	291	$9{\cdot}1\times10^{6}$

*Two weeks after vaccination, ten mice per group were killed for determination of lung weight, and ten were challenged by aerosol. Plate counts on emulsified lungs were made 4 weeks after challenge.

†Four weeks after vaccination, ten mice per group were killed for determination of lung weight, and ten were challenged. Plate counts on emulsified lungs were made 4 weeks after challenge.

Table 6. Granulomatous inflammation and protection induced in mice by trehalose mycolate (P3) combined with cell walls and endotoxins from Gram-negative bacteria

Experiment number	Material association with oil droplets and given intravenously	Dose (μg)	Granuloma Mean lung weight (mg)	Protection Median H37Rv count/lung
13174	*E. coli* CW	300	284	$1{\cdot}0\times10^{7}$
	E. coli+P3	150+150	454	$3{\cdot}0\times10^{3}$
	P3	150	253	$2{\cdot}3\times10^{6}$
	BCG CW	300	533	$1{\cdot}7\times10^{2}$
	Controls	—	244	$7{\cdot}5\times10^{6}$
80974	*E. coli* LPS	150	233	$8{\cdot}2\times10^{6}$
	E. coli LPS+P3	150+150	428	$2{\cdot}1\times10^{4}$
	P3	300	328	$1{\cdot}7\times10^{6}$
	Salmonella enteritidis LPS	150	237	$8{\cdot}3\times10^{6}$
	S. enteritidis LPS+P3	150+300	356	$6{\cdot}0\times10^{5}$
	BCG CW	300	542	$<3{\cdot}0\times10^{2}$
	Controls	—	226	$3{\cdot}6\times10^{6}$
92074	*E. coli* LPS	150	234	$4{\cdot}5\times10^{6}$
	E. coli LPS+P3	150+150	398	$3{\cdot}0\times10^{5}$
	S. typhimurium Re LPS	150	253	$8{\cdot}0\times10^{6}$
	S. typhimurium Re LPS+P3	150+150	361	$1{\cdot}0\times10^{6}$
	E. tularensis CW	150	255	$7{\cdot}2\times10^{6}$
	F. tularensis CW+P3	150+150	446	$2{\cdot}2\times10^{4}$
	P3	150	268	$1{\cdot}7\times10^{6}$
	BCG CW	300	331	$4{\cdot}3\times10^{3}$
	Controls	—	235	$9{\cdot}8\times10^{6}$

Mean lung weights determined on samples of ten at 1 month after vaccination; median H37Rv counts per lung determined 1 month after aerosol challenge.

DISCUSSION

It is understood that this work is performed in an artificial experimental model; therefore, generalization of the conclusions to the whole problem of immunity to tuberculosis is not intended. Within this laboratory system, however, the two components of tubercle bacilli essential to stimulation of resistance have been identified as tuberculoprotein and a non-antigenic mycolic acid ester (P3). For the antigenic component, even a degraded tuberculin-active peptide will serve. P3 contains more than two different mycolic acids (Azuma *et al.*, 1974; Strain, Toubiana, Ribi & Parker, 1977) hence it is not a simple dimycolate of trehalose, although it is chromatographically more homogeneous than any sample of cord factor we have been able to obtain (Azuma *et al.*, 1974).

We refer to P3 as an adjuvant, but its mode of action, which is only partly understood, is not the same as that of other common adjuvants. When injected intradermally into foot-pads of guinea-pigs, P3 alone caused a transient inflammation but no granuloma (Granger, Yamamoto & Ribi, 1976). Combined with BSA in an oil-droplet preparation, however, it led to strong, persistent granuloma, and it greatly enhanced reactions of delayed hypersensitivity to BSA. Because no reactions of immediate hypersensitivity occurred, there was, evidently, no enhancement of antibody formation (Granger *et al.*, 1976). Ordinarily P3 had to be combined with some antigenic material, in oil-droplet vaccines, in order to produce appreciable granulomata in mouse lungs and protection against aerosol challenge with strain H37Rv. The same was true for cure of line 10 tumours in syngeneic strain 2 guinea-pigs (Ribi, McLaughlin, Cantrell, Brehmer, Azuma, Yamamura, Strain, Hwang & Toubiana, 1978). These seem to be enhancement phenomena, but they relate to delayed hypersensitivity or cell-mediated immunity. The conditions that most favoured the production of lymphokines in mice were the same as those that protected best against aerosol challenge (Salvin, Ribi, Granger & Youngner, 1975). It should be noted, on the one hand, that treatment with P3 plus BSA did not cure line 10 tumours in guinea-pigs. We favour the view that this was because the resulting granuloma was not sufficiently persistent. On the other hand, certain bacterial endotoxins, in combination with P3, formed the most potent immunotherapeutic agents; whereas these were only partially effective in protecting mice. The reason for this discrepancy is unknown.

In order to compare our findings with others in the literature that are probably relevant, the relationship between P3 and cord factor needs to be clarified. There seems little reason to doubt that P3, or some as yet inseparable fraction of it, is responsible for most, but not necessarily all, of the major activities attributed to cord factor. Different species and strains of animals, as well as different routes of administration, have been used in various laboratories. Bekierkunst, Levij, Yarkoni, Vilkas, Adam & Lederer (1969) found that cord factor alone produced tubercles in mouse lungs and protected against i.v. challenge with H37Rv, that it produced lasting granuloma in mouse foot pads (Bekiekunst, Levij, Yarkoni, Vilkas & Lederer, 1971a), and that it enhanced antibody formation to sheep red blood cells in these animals (Bekiekunst, Yarkony, Flechner, Monedi, Vilkas & Lederer, 1971b). Granger *et al.* (1976) found that P3 alone did not produce granuloma in guinea-pig foot-pads, and that it enhanced delayed-type, but not Arthus-type, hypersensitivity to BSA. In the current studies, we show that P3 alone, as administered here, produced only questionable granuloma and rarely, if at all, protected significantly against aerosol challenge with H37Rv. These discrepancies might be attributed to the differences in animals and preparations, but a study from this laboratory showed that factors such as the concentration of oil and the timing of inoculations seem to control the presence or absence of granuloma in particular cases (McLaughlin, Parker, Hadlow, Toubiana & Ribi, 1978). When strong and persistent pulmonary granuloma ensues, the mice are resistant to challenge.

We set out to study the relationship of pulmonary granuloma to resistance with the aid of better defined materials. The granuloma observed is thought to be a manifestation of DH resulting from intravenous administration of sensitizing components adherent to non-metabolizable particles. Foreign body granuloma probably is not involved because the vehicle controls do not show increased lung weight. Although proof that protection depends upon granuloma is still lacking, the data presented here are representative of many experiments in which correlation between granuloma and protection in this system is nearly perfect.

It has been shown here that sensitivity to tuberculin is not a requirement for resistance: although some form of local hypersensitivity appears to be necessary, it can be directed toward antigens thought to be wholly unrelated to tubercle bacilli. In this sense,

immunity to tuberculosis may be non-specific. The question of specificity is not thereby disposed of, however. Data to be presented elsewhere suggest that there may be a specific component of immunity that, when present, leads to a higher order of resistance, which is longer lasting (cf. Kallos, 1976). Also, as noted previously (Meyer *et al.*, 1974), there have been occasions when significant resistance, without massive pulmonary granuloma, has been stimulated by vaccination with live BCG. This could be attributable to the predominance of a specific factor, and it seems to involve a rapid onset of granuloma following challenge, probably brought on by restimulation with specific antigens in the challenge dose (Yamamoto, Granger, Brehmer, Azuma & Ribi, 1981). In the latter connection, it should be recalled that the vehicle for the aerosol used in challenge contained a small amount of BSA; thus restimulation could play a part in the protective effect of BSA. We have, however, obtained similar results with keyhole limpet haemocyanin where there was no secondary exposure (Table 5).

It will be noted in the tables that P3 alone, on oil droplets, sometimes led to a significant increase in lung weight, although this was not of the magnitude found in effectively vaccinated animals, and it rarely led to significant protection against challenge. Also, the phenomenon was not associated with particular batches of P3. Because P3 is incapable of eliciting reactions of DH (macrophage migration inhibition, foot-pad swelling, skin test) in animals previously exposed to it or to any other materials tested, it is possible that minute contamination of an occasional day's preparation with extraneous antigen was responsible. The grinding procedure in current use would be the most likely stage for contamination to occur. However, as noted earlier, there are as yet unreported complexities in the effects of P3 and oil droplets administered without antigen.

P3, even though apparently non-immunogenic in itself, is isolated from mycobacteria. To prove that resistance to challenge in this system can be a wholly non-specific effect of delayed hypersensitivity or cell-mediated immunity, it will be necessary to protect the animals with materials entirely unrelated to *M. tuberculosis*. Up to this point, it had not been possible to produce the degree of pulmonary granuloma associated with protection in the absence of P3 or cell walls of mycobacteria, although recent results indicate that this may be reported in the near future.

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