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The dissociation of adjuvant properties of mycobacterial components from mitogenicity, and from the ability to induce the release of mediators from macrophages

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Summary. Twelve preparations from mycobacterial cell walls and culture supernatant fluids were tested for their ability to activate lymphocytes from Balb/c or Nu/Nu mice, and to increase the release of mediators from macrophages *in vitro*. The peptidoglycolipids (wax D) were B-cell mitogens and induced plaque-forming cells. These properties were lost if the glycopeptide component was removed, leaving the pure lipid, mycolic acid. Neither the glycopeptide fractions, with well-documented adjuvant properties, nor mycobacterial polysaccharide II-activated B cells.

Only intact peptidoglycolipid showed synergy with the mitogenic effect of phytohaemagglutinin (PHA) on thymocytes from Balb/c mice. The effect was much smaller than with *E. coli* lipopolysaccharide (LPS) or dextran sulphate. The peptidoglycolipid also enhanced the release of factors from macrophages able to modify the response of Balb/c thymus cells to PHA. In this respect it resembled *E. coli* LPS. Adjuvant active glycopeptides did not share this property.

INTRODUCTION

There are two main reasons for interest in the immunological properties of mycobacterial components. First, there are the therapeutic implications of the adjuvant effects both of lipid-rich peptidoglycolipid preparations (White, Bernstock, Johns & Lederer, 1958; White, Jolles, Samour & Lederer, 1964; Stewart-Tull & White, 1967; Stewart-Tull, 1974) and of lipid-free glycopeptides (Adam, Ciorbaru, Petit & Lederer, 1972; Hiu, 1972; Migliore-Samour & Jolles, 1972; Stewart-Tull *et al*, 1975). These glycopeptides, which probably share common chemical constituents, may be present as components of peptidoglycolipid on the cell surface (Stewart-Tull *et al.*, 1975) and are active *in vivo* and *in vitro* (Bona, Heuclin & Chedid, 1974; Modolell, Luckenbach, Parant & Munder, 1974).

Secondly, it is necessary to discover why, in certain human mycobacterial diseases, such as leprosy (Myrvang, Godal, Ridley, Fröland & Song, 1973) and tuberculosis, or *M. ulcerans* infections or in several experimental infections of mice, e.g. *M. lepraemurium*, *M. ulcerans* and BCG (Rook, 1975a) a state in which *in vivo* and *in vitro* correlates of cell-mediated immunity to the organisms are positive can be followed by a state of specific, and eventually non-specific anergy.

There is suggestive evidence that like the adjuvant effects referred to above, this suppression of cell-mediated immunity may also be in part attributable to pharmacological effects of mycobacterial components. Thus the rate of DNA synthesis *in vivo* in

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the nodes of mice in this late anergic phase returns to normal levels. However, *in vitro*, lymph-node cells from such mice undergo mitosis spontaneously. This effect is mediated by macrophages, or factors released from macrophages (Rook, 1975b). The spontaneous mitosis is eliminated by removal of phagocytic cells. The addition of levels of mycobacterial antigen previously shown to be optimal for *in vitro* lymphocyte transformation decreases, rather than increases the mitosis, as does the addition of mitogens. It was suggested that similar effects might be responsible for the lack of lymph-node activity *in vivo* (Rook, 1975b).

Thus mycobacteria appear to exert both stimulatory and suppressive effects, which may or may not be related to the same structural components of the organism, and which probably act via macrophages or lymphocytes. Therefore, we have studied the ability of a range of well-defined mycobacterial components to activate lymphocytes *in vitro* and to modify the release of mediators by macrophages (Gery & Waksman, 1972; Calderon & Unanue, 1975). Mycobacterial interactions with the lymphoid system are discussed in relation to what is already known about the adjuvant properties of the same fractions (Stewart-Tull, 1974; Stewart-Tull *et al.*, 1975), and in relation to the abnormalities of lymph-node cell function which occur late in experimental mycobacterioses.

MATERIALS AND METHODS

Preparation of mycobacterial fractions

Glycopeptides (ST208, 216, 217, 221), polysaccharide II (ST215, 219) and proteins (ST214, 218) were prepared from large volumes of culture filtrates obtained from the Ministry of Agriculture, Central Veterinary Laboratories, Weybridge, Surrey, by the method of Stewart-Tull *et al.* (1975). The tuberculin PPD preparation (Batch 288, ST213) was also obtained from the Ministry of Agriculture.

The peptidoglycolipids were isolated from whole cells of *M. tuberculosis* strain DT (ST225) and *M. bovis* AN5 (ST228) according to the method described by Stewart-Tull & White (1967). Peptidoglycolipid (0·5 g) from *M. tuberculosis* strain C was dissolved in 2·5-ml boiling benzene, 2·5-ml 5 per cent methanolic KOH was added and the mixture was boiled for 2 min. The precipitate formed was removed by filtration and the supernate was placed over a boiling-water bath to remove methanol. The oily deposit was extracted with ether and after evaporation of the extract, mycolic acid (ST174) was obtained as a white powder.

Heat-killed *M. tuberculosis* strain C whole cells (30 g) were extracted with pyridine:water (50:50 v/v) solution for 48 h at 37°. The cells were removed by centrifugation and the supernate was dialysed against running tap-water to remove pyridine. An insoluble residue (ST223) after dialysis and the dialysate (ST224) were freeze dried.

Lymphocyte culture

Spleens or thymuses from Balb/c mice of either sex, 6–8 weeks old, or spleens from Nu/Nu mice (the original Edinburgh strain), were finely minced with scissors. Lumps of cells were removed by spinning for a few seconds at 1000 r.p.m. and the remaining cells were washed three times in Eagle's medium at room temperature, and resuspended in medium RPMI 1640 (Gibco-Biocult, Paisley, Renfrewshire, Scotland) containing penicillin 100 μ/ml and streptomycin, 100 μg/ml, and in some experiments, 5 per cent inactivated foetal calf serum. The cells were adjusted to 10^7 cells/ml and 0·1 ml of the suspension was added to each well of flat-bottomed microtitre trays. A further 0·1 ml of medium or of medium containing the preparations under study was added to each culture. Tests were performed in duplicate or triplicate. Tritiated thymidine (0·1 μCi, 18 Ci/mmol) was added to each culture 16 h before harvesting, and cultures were terminated at the times indicated in the text.

Preparation of reagents

Mitogens and mycobacterial components were dissolved in RPMI 1640, or when insoluble treated in an ultrasonic disintegrator with the wave peak distance set at 8–9 μm for 1 min. Lipopolysaccharide B (LPS) from *E. coli* 055:B5, was obtained from Difco, Detroit, dextran sulphate from Pharmacia, Uppsala, PHA (reagent grade) from Wellcome Research Laboratories, Beckenham, Kent.

Plaque assay

Spleen cells were cultured for 48 h with optimally mitogenic concentrations of the preparations, without the addition of antigen and direct plaque-forming cells (PFC) were assayed by the technique of Cunningham and Szenberg (1968) using sheep red blood cells (SRBC) or SRBC conjugated with 2,4,6-trinitrobenzene sulphonic acid as described by

Rittenberg & Pratt (1969) (TNP-SRBC). Guinea-pig serum absorbed at +4° with both agarose and TNP-SRBC was used as a source of complement.

When mitogenicity and PFC were to be compared, the two assays were performed simultaneously on replicate cultures from the same microtitre tray, and FCS was omitted from the medium (Coutinho, Moller, Andersson & Bullock, 1973).

Preparation and assay of peritoneal cell supernatants

Balb/c peritoneal cells were obtained by washing out unstimulated peritoneal cavities with cold RPMI 1640. These cells were washed twice, and cultured as described above for spleen cells with optimally mitogenic concentrations of the mycobacterial components, or of control mitogens. After 24 h, supernatants from six to twelve wells were pooled, and centrifuged at 3000 r.p.m. for 15 min to remove cells. The supernatant was then immediately assayed for lymphocyte activating factor (LAF) by a modification of the method of Gery & Waksman (1972). Balb/c thymus cells were cultured in 10 per cent FCS in RPMI 1640 in microtitre trays in the presence of doubling dilutions of the supernatants. A maximally mitogenic dose of PHA was also added to each well. This had little mitogenic effect on normal Balb/c thymus, so the enhancing effect of the supernatants could be assayed at 72 h. Control titrations were performed using supernatants from unstimulated cultures, or the B-mitogen itself, in the absence of supernatant. The latter control was essential to allow discrimination between the enhancing effect of some of the B-mitogens, and the enhancing effect of LAF, since both were present in the supernatants. In some experiments the supernatants were dialysed for 48 h against 250 volumes of RMPI 1640 to remove low molecular weight inhibitors of tritiated thymidine incorporation (Calderon & Unanue 1975; Opitz, 1975).

In order to test the roles of adherent and non-adherent cell populations, peritoneal cells were cultured for 3 h in microtitre trays. The non-adherent cells were depleted from half of the wells by repeatedly squirting RMPI into the wells from Pasteur pipettes, before changing the medium. Then the mycobacterial component to be tested was added to washed and unwashed wells. After 24 h LAF assays were performed on the supernatants as already described.

Depletion of macrophages

Partial depletion of macrophages from spleen cell cultures was achieved using carbonyl iron as previously described (Rook 1975b)

RESULTS

Mitogenicity for lymphoid cells

All the peptidoglycolipid preparations were mitogenic for spleen cells from Balb/c or Nu/Nu mice. This effect was enhanced by removal of some of the macrophages with carbonyl iron, and was seen in the presence or absence of foetal calf serum. Their potency was comparable to that of LPS, and appeared to be more dependent on physical characteristics of the suspension such as the particle size, than on the species from which the preparation was derived. Typical dose-response curves of moderately active preparations are shown in Fig. 1.

The mitogenic effect was almost totally eliminated by removing the glycopeptide from the peptidoglycolipid, leaving pure mycolic acid (ST174) (Fig. 1).

Both soluble (ST224) and insoluble (ST223) components of the pyridine/water extract were active. Since all the above preparations were active on Nu/Nu spleen cells, and not on thymus cells, they appear to be mitogenic only for B cells.

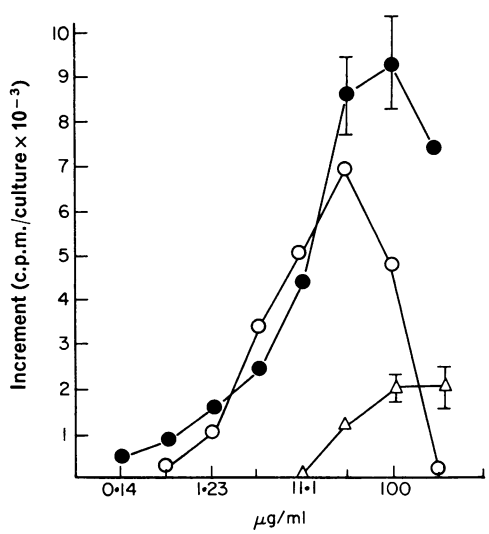

Figure 1. Mitogenicity for Balb/c spleen cells, 48-h culture. Dose-response curves for Wax-D (ST 225) (●); mycolic acid (ST 174) (△); phenol/water extract (ST 223) (○). Expressed as increment in ct/min/culture ± s.d. Background in unstimulated cultures was 2471 ± 23.

None of the other preparations (polysaccharide II (ST215, 219), protein material (ST214, 218) or the adjuvant-active glycopeptides (ST208), 216, 217, 221) were mitogenic. The glycopeptides were assayed at various concentrations from 3000 μg/ml to 0·1 μg/ml on lymph-node, spleen and thymus cells. There was no mitogenic activity in cultures harvested after 2, 3, 4, 5 or 7 days. However, these preparations did not impair cell viability even at 300 μg/ml.

The feature common to all the mitogenic preparations, including the pyridine/water extracts, was a significant lipid content. Therefore attempts were made to simulate the effect of intact mitogenic peptidoglycolipid by mixing glycopeptide (ST208) and mycolic acid (ST174) in various ratios. However the mitogenic effect was never greater than that of the mycolic acid alone.

Induction of plaque-forming cells

The mycobacterial preparations were tested for their ability to generate plaque-forming cells, and this effect was compared with their mitogenicity. These experiments were performed in the absence of foetal calf serum, in order to limit the numbers of background plaques. This resulted in some instability of the peptidoglycolipid suspensions which were therefore less mitogenic than in previous experiments, although a maximally mitogenic concentration was used. Non-mitogenic preparations were assayed at 100 μg/ml and 10 μg/ml.

The peptidoglycolipid preparations induced anti-SRBC and anti TNP-PFC (Table 1). Relative to their mitogenic effects, assayed simultaneously, they were usually more efficient inducers of PFC than LPS, but less efficient than PPD. On the other hand, the

Table 1. Polyclonal activation of B cells in cultures of normal spleen by mycobacterial products*

Preparation	Increment c.p.m./culture ± s.d.	PFC/10^6 cells cultured ± s.d.		Ratio c.p.m./TNP plaques
		SRBC	TNP	
Dextran sulphate	13165 ± 943	0	2 ± 3	—
E. coli LPS	12135 ± 1059	58 ± 9	292 ± 17	42
Medium alone	—	4 ± 2	20 ± 6	—
ST174 Mycolic acid	677 ± 445	4	24 ± 17	—
ST225 Peptidoglycolipid from *M. tuberculosis* DT	3319 ± 843	16 ± 5	88 ± 11	37
ST228 Peptidoglycolipid from *M. bovis* AN5	8545 ± 672	43 ± 7	224 ± 20	35
ST223 Pyridine:water extract of *M. tuberculosis* C, DT, PN cells—insoluble material after dialysis	2178 ± 371	16 ± 6	106 ± 14	20
ST224 Pyridine:water extract of *M. tuberculosis* C, DT, PN cells—soluble material after dialysis	1648 ± 339	10 ± 3	48 ± 11	34
ST208 Glycopeptide from *M. tuberculosis* strain DT	0			
ST216 Glycopeptide from *M. tuberculosis* strains C, DT & PN	0	3	18 ± 7	—
ST217 Glycopeptide from *M. tuberculosis* strains C, DT & PN	0			
ST221 Glycopeptide from *M. bovis* AN5	0			
ST215 Polysaccharide II from *M. tuberculosis* C, DT & PN	0	4	27 ± 8	—
ST219 Polysaccharide II from *M. tuberculosis* AN5				
ST213 Water-soluble PPD	7507 ± 679	40 ± 6	264 ± 23	28

* PFC and tritiated thymidine incorporation (added at 32 h) were estimated at 48 h in replicate cultures from the same microtitre tray. Cultures contained optimally mitogenic concentrations of the preparations. Results are expressed as means of triplicates ± s. d.

pyridine/water extract ST223 proved to be an exception in this respect. The mycolic acid preparation and all the non-mitogenic preparations failed to induce PFC, as did dextran sulphate (Gronowicz & Coutinho, 1974).

Synergy between B-cell mitogens and PHA

Although the mechanism remains uncertain, Diamantstein, Vogt, Riihl & Bochert (1973) have demonstrated that dextran sulphate can act synergistically with PHA on thymus cells, and Forbes, Nakao & Smith (1975), have made a similar observation in relation to LPS used with Con A. Our own preliminary studies showed that B mitogens vary widely in the extent to which they possess this property, which is strongest with dextran sulphate, absent using PPD, and intermediate using LPS or capsular polysaccharide of *Klebsiella aerogenes* (NCTC 5055). This provided another way to 'classify' the mitogenic properties of mycobacterial peptidoglycolipid relative to the other well-established mitogens. Therefore Balb/c thymus cells were cultured for 72 h in the presence of a constant, maximally mitogenic dose of PHA. Dilutions of B mitogens or mycobacterial preparations were added to give complete dose–response curves. None of the preparations produced any significant effect on thymus cells in the absence of PHA.

Dextran sulphate and LPS both showed the expected degree of activity. The peptidoglycolipid preparations showed weak activity, equal to approximately half that of LPS, but all the other mycobacterial preparations including PPD were inactive Fig. (2).

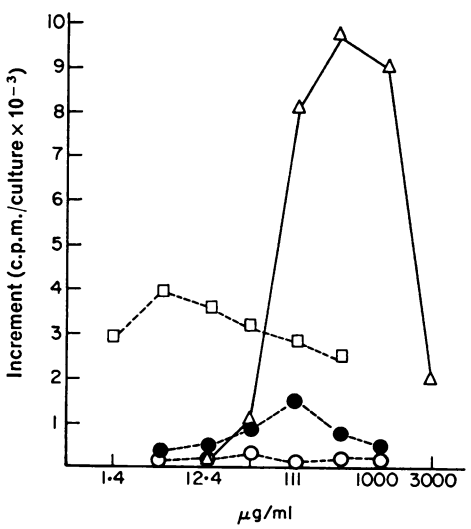

Figure 2. Synergy with the mitogenic effect of PHA on Balb/c thymus, 48-h cultures. All cultures contained PHA 1/150. Dose-response curves for Dextran sulphate (△); *E. coli* lipopolysaccharide (□); Wax-D, (ST 225) (●); PPD (○). Expressed as increment in ct/min/culture. Background in the presence of PHA 1/150 alone was 3491 ± 297.

The effect of mycobacterial components and other B mitogens on the release of mediators by normal Balb/c peritoneal cells

The adjuvant active glycopeptides (ST 208, 216, 217, 221) and the polysaccharide II preparations (ST 215, 219) had no effect on release of either the inhibitory or enhancing activities when supernatants of peritoneal cells incubated in their presence were assayed as described by Gery *et al.*, 1972. Concentrations of 1 μg/ml to 100 μg/ml were tried. (Data are not shown.) However, the peptidoglycolipid fractions which were active as polyclonal B-cell activators, also enhanced release of both activities as shown in Table 2. A complete titration of such a supernatant is shown in Fig. 3. In this respect they again resembled LPS and differed from the other B mitogens studied. Thus PPD increased output of

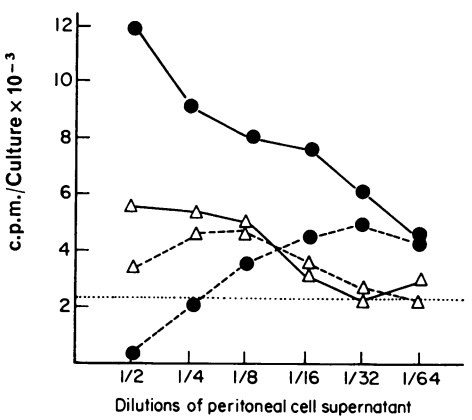

Figure 3. Assay of the effect of mediators in the supernatants of 24-h peritoneal cell cultures on PHA responsiveness of thymus cells (Gery & Waksman, 1972). Supernatant induced by peptidoglycolipid (ST 225), not dialysed (●- - -●); dialysed (●——●). Supernatants from unstimulated peritoneal cells, not dialysed, (△- - -△); dialysed, (△——△). Background stimulation of Balb/c thymus cells by PHA in the absence of supernatants (. . . . .).

Table 2. Assays of the response of Balb/c thymus cells to PHA in the presence of supernatants from 24-h peritoneal cell cultures (Gery & Waksman, 1972). The effect of four B mitogens on the release of enhancing and inhibitory activity into the supernatants

Source of supernatant		Assay of supernatants* c.p.m./culture†		
		Mitogen or medium alone	Supernatants (& mitogen)	
			Not dialysed	Dialysed
Medium alone	Whole PC	2416	1305	3706
	Adherent PC		1986	3800
Dextran sulphate 1000 μg/ml	Whole PC	13119	14512	14009
E. coli LPS 25 μg/ml	Whole PC	5013	468	11347
	Adherent PC		1500	8032
PPD 250 μg/ml	Whole PC	2324	320	2175
Peptidoglycolipid 225. 100 μg/ml	Whole PC	3764	289	7853
	Adherent PC		426	7146

* Assayed at a final dilution of 1/2.
† All cultures contained PHA 1/150.

dialysable inhibitors, without any detectable increase in lymphocyte activating factor (LAF) levels (Table 2). Dextran sulphate appeared not to cause mediator release. However, it showed such powerful synergy with PHA on the thymus cells that any superimposed effects of mediator release into the supernatant may have been masked. They did not reach statistical significance.

Removal of non-adherent cells from the peritoneal cell populations decreased, but did not eliminate the release by LPS and peptidoglycolipid, of both activities. Thus it remains unclear whether they act on macrophages directly, or indirectly via residual adherent B cells.

DISCUSSION

From the results of numerous workers the structure of the water-insoluble mycobacterial cell-wall peptidoglycolipid (wax D) has been postulated (reviews, Stewart-Tull, 1974; Lederer *et al.*, 1975). The glycolipid component of the molecule consists of mycolic acid ester-linked to a polysaccharide of arabinose, galactose and possibly mannose. This is bound to a glycopeptide structure composed of glucosamine and muramic acid together with the amino acids alanine, glutamic acid and meso-DAP.

The results of this investigation demonstrate that the lipid-rich peptidolycolipid fractions of mycobacteria are B-cell mitogens and polyclonal B-cell activators able to generate PFC to TNP, and SRBC *in vitro*, whereas the other fractions are not. Parallel studies with the same fractions (Stewart-Tull, Davies & Jackson, 1976) have shown that these mitogenic peptidoglycolipids increase the permeability of artificial lipid bilayers to potassium chromate, whereas the non-mitogenic glycopeptide component has the reverse effect. The significance of the membrane activity of the lipid-rich fractions in the triggering of B-cell mitogenicity or mediator release by macrophages has not been explored. It seems likely that only the arabinogalactan and mycolic acid components are essential for the mitogenic activity of the peptidoglycolipid, and that the peptide-containing moiety described above is not needed. It has recently been demonstrated that the simple glycolipid structure, *N*-palmitoyl D-glucosamine is a B-cell mitogen (Rosenstreich *et al.*, 1974). That the carbohydrate (arabino-galactan) and lipid (mycolic acid) components are essential is illustrated by the greatly reduced activity of the pure lipid, mycolic acid ST174, and the lack of mitogenicity of the lipid-poor glycopeptides (2·0–0·39 per cent lipid; Stewart-Tull *et al.*, 1975).

It is worth noting that the PPD (Weybridge, batch 288) contained 3·4 per cent lipid. Thus it is possible that the mitogenic effect of PPD is due to contaminating glycolipid. However this would not explain why its properties as a B-mitogen differ from those

of the peptidoglycolipid preparations, particularly with reference to LAF release.

The failure of the glycopeptides to act as mitogens demonstrates that B-cell mitogenicity is not an essential prerequisite for adjuvant activity since samples of the same batches of the non-mitogenic water soluble glycopeptides have been shown to possess adjuvant activity (Stewart-Tull *et al.*, 1975) as have similar preparations prepared by other workers (Hiu, 1972; Migliore-Samour & Jollès, 1972; Adam *et al.*, 1972). The water-soluble glycopeptide adjuvant of Adam *et al.*, (1972) is effective *in vivo*, and also enhances mixed lymphocyte cultures (Bona *et al.*, 1974) and antibody formation (Modolell *et al.*, 1974) *in vitro*, and yet it is non-mitogenic. Nevertheless, it remains a possibility that the mitogenic component of the peptidoglycolipid molecule has adjuvant properties of its own or can enhance the adjuvant effect of the glycopeptide. Mycobacteria may exert their adjuvant effect in more than one way.

The properties of the mycobacterial peptidoglycolipid resembled those of LPS more closely than those of the other B mitogens studied. However the analogy was not complete. First, peptidoglycolipid possessed a weaker ability than LPS, DS or *Klebsiella aerogenes* (NCTC 5055) capsular polysaccharide (Rook, unpublished data) to act synergistically with PHA on Balb/c thymus cells. Secondly, it induced larger numbers of PFC than LPS relative to its mitogenic effect and in this respect it resembled PPD. This implies, according to the hypothesis of Gronowicz & Coutinho (1974) that, like PPD, it acts on relatively well-differentiated B cells, which rapidly become antibody-secreting end-cells. These two sets of data considered together suggest an inverse relationship between the ability of B-cell mitogens to act synergistically with PHA, and their ability to induce PFC. If confirmed with a more comprehensive range of B mitogens, this relationship would have considerable theoretical implications.

However the peptidoglycolipid was similar to LPS in its ability to enhance the formation by macrophages of both dialysable proliferation inhibitory factor and non-dialysable lymphocyte activating factor (LAF) (Calderon & Unanue, 1975; Gery *et al.*, 1972). It was not clear from the data whether this was due to a direct effect on macrophages, or an indirect effect secondary to the B-cell mitogenicity, which may lead to the release of macrophage activating factors (Wilton, Rosenstreich & Oppenheim, 1975). There is not yet any evidence for an *in vivo* role for these mediators and recently doubt has been cast on the significance of the inhibitory activity (Opitz *et al.*, 1975). However LAF has a direct mitogenic effect on Balb/c lymphnode cells *in vitro* (Rook, unpublished data) so excessive release of this mediator by peptidoglycolipid-stimulated macrophages in the nodes of mice 'overloaded' with mycobacterial components may explain the spontaneous mitosis and abnormal responses *in vitro* of such node cells (Rook, 1975a, b) and perhaps also contribute to the initial enhancement, and subsequent suppression of cell-mediated responses seen during mycobacterial infections *in vivo*.

The work reported here illustrates the bewildering complexity of the interaction between mycobacteria, and the immune system of the host.

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