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Role of antigen-presenting cells in variation in immunogenicity of mycobacteria

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SUMMARY

The route of immunization was observed to play a significant role in deciding the outcome of immunization with killed mycobacterial vaccines. Whereas the slow growers were immunogenic by both intraperitoneal and intradermal routes, the rapid growers were immunogenic only by intradermal route. The non-responder state of mice to *Mycobacterium vaccae* by i.p. route of immunization could be corrected by prior treatment with poly I:poly C, an interferon inducer, or indomethacin, a prostaglandin inhibitor. Antigen-presenting efficiency of peritoneal and spleen cells were compared employing *M. vaccae* and *M. tuberculosis* H37Rv primed T cells and corresponding sonicates as antigens in an *in vitro* lymphocyte transformation test. Irradiated spleen cells presented both the antigens efficiently. However, with peritoneal cells as antigen-presenting cells, proliferative response against only *M. tuberculosis* was observed; proliferation of *M. vaccae* primed T cells was very poor. Peritoneal cells of poly I:poly C treated mice showed distinct improvement in their efficiency of presentation; even paraformaldehyde-fixed peritoneal cells gave an efficient stimulation with *M. vaccae*. The percentage of Ia-positive fraction in peritoneal cells was very low (5·95%) in comparison with spleen cells (38·37%). Poly I:poly C treatment resulted in increase in the Ia-positive cell fraction of the peritoneal cells to 24·5%.

Keywords antigen-presenting cells immunogenicity *Mycobacteria*

INTRODUCTION

Mycobacterial diseases such as leprosy and tuberculosis are characterized by phases of illness associated with immunological non-responder states, particularly so in lepromatous leprosy (LL) (Ridley & Jopling, 1966; Lenzini, Rottoli & Rottoli, 1977). There is also some evidence to suggest that the non-responder states are associated with immune suppression, particularly in LL (Mehra *et al.*, 1982). However, the pathogenesis of this phenomenon is still not clear. Some investigators have suggested a genetic defect in antigen presentation, linked to major histocompatibility complex (MHC) encoded DR antigens (van Eden & de Vries, 1984; Eden *et al.*, 1984). Based on the outcome of immunization of mice with *Mycobacterium leprae* by different routes, others have disputed this, suggesting instead that the pathogenesis of non-responder state in LL is related to route of infection (Shepard *et al.*, 1982). Route-related differences in the outcome of an infection with a protozoan such as *Leishmania tropica* have also been observed; these differences have been ascribed to the differences in generation of cell-mediated immune responses (Howard, Hale & Liew 1980; Poulter & Pandolph, 1982). The differences in immunogenicity of *M. leprae* by different routes could be related to the efficiency of antigen-presenting cells (APC) encountering the antigen. APC at different sites are known to differ in their MHC class II composition (Beller, Kiely & Unanue, 1980; Katz, 1984). Hence even the observation of Shepard *et al.* (1982) cannot rule out the role of MHC class II glycoproteins in the reported non-immunogenicity of *M. leprae* by some routes.

Using cultivable mycobacteria we have studied the role of route of immunization in their immunogenicity and related it to the presenting efficiency of APC encountering the antigen.

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MATERIALS AND METHODS

Organisms

M. tuberculosis H37Rv (NCTC 7416), *M. kansasii* (photochrogen, NCTC 10268), *M. avium* (non-chromogen NCTC 8559) and *M. phlei* (rapid grower NCTC 8151) were purchased from National Collection of Type Cultures (London, UK). *M. vaccae* (rapid grower) and *M. scrofulaceum* (scotochromogen) were kindly supplied by Dr J. L. Stanford of Middlesex Hospital, London.

Mice
BALB/c and Swiss white mice (aged 4–6 weeks) obtained from National Institutes of Health (Bethesda, MD) and bred at Cancer Research Institute and Haffkine Biopharmaceutical Corporation, Bombay, were used in the study.

Preparation of sonic extract
Four- to six-week-old cultures of slow-growing mycobacteria and 1-week-old cultures of rapid growing *M. vaccae*, on the medium of Doub & Youmans (1950), were killed by 2·4 megarad gamma radiation in a ^{60}Co source. The killed cells were washed with phosphate-buffered saline (PBS) disrupted in a sonicator (Branson Sonifier, B-30) and spun at 50 000 *g* for 1 h. The supernatant, i.e. the sonic extract was employed as 'test antigen'. The protein content of the test antigen was estimated by the method of Lowry *et al.* (1951).

Immunization
Different mycobacterial cultures were killed by irradiation, washed bacterial cells were pelleted by centrifugation at 2200 *g* for 1 h, and washed cell pellets were suspended in PBS to give 1% (v/v, about 10^9 bacilli/ml) suspension. The required number of bacilli were suspended in 0·5 ml for i.p. immunization and 0·05 ml for intradermal (i.d.) immunization.

Delayed-type hypersensitivity (DTH) test
This was done by the method of Gray & Jennings (1955). Sonicates containing 50 μg protein in 0·03 ml volume were injected into the hind foot-pad and measurements of foot-pad thickness were taken before and 24 and 48 h after injection. All sonicates were also tested in non-immune mice as controls. The results were expressed as corrected foot-pad enlargement (CPE), which is the difference between the foot-pad enlargements caused by a test sonicate in the immunized and control mice, simultaneously tested. The differences between the foot-pad enlargements of the test and the non-immune control mice were analysed by Student's *t*-test.

Interferon (IFN) assay
IFN assay on the sera of mice treated with poly I:poly C was carried out employing the technique described by Merigan (1971) and the results were expressed as international units (IU)/ml, employing commercial mouse IFN (Sigma; I, 1258) as standard.

Lymphocyte transformation test (LTT)
This was carried out employing standard microculture technique (Oppenheim & Schechter, 1976). Briefly, each well of a microtitre plate (Nunc, Roskilde, Denmark) received 2×10^5 immune T cells along with 2×10^4 (10%) or 5×10^4 (25%) syngeneic irradiated normal non-immune spleen or peritoneal cells (as APC), in 0·2 ml of culture medium—RPMI 1640 containing 2·0 mM glutamine, 10% fetal calf serum (FCS), 1% antibiotic antimycotic (AA; GIBCO, Grand Island, NY) and 10 μg/ml of sonicate of the mycobacterial culture under test. Cultures were pulsed 120 h later with 0·5 μCi of ^{3}H-thymidine (BARC, India; Specific activity 2 Ci/mmol) for a period of 16 h, harvested and incorporation of the label in cellular DNA was assayed. All tests were run in triplicates. Whenever required the source of APC was subjected to 1200 rad irradiation in a ^{60}Co source to eliminate T and B cell response to antigen completely.

Immune T cells
BALB/c mice were immunized intradermally with 10^8 killed cells of either *M. vaccae* or *M. tuberculosis* H37Rv. One week later, successful immunization was confirmed by tests for DTH. Single cell suspensions of the spleens of these mice were made in the wash medium, Hanks' balanced salt solution (HBSS) containing 10% FCS and 1% AA, red cells in the suspensions lysed by 0·83% NH_4Cl treatment, and T cells purified on a nylon wool column by the technique of Julius, Simpson & Herzenberg (1973).

Peritoneal cells
Peritoneal cells were collected from normal BALB/c mice and those treated intravenously with poly I:poly C. The mice were killed and within 5 min an i.p. injection of 4·0 ml wash medium was given, their abdomen massaged gently and the contents aspirated by a sterile syringe. Aspirates of several mice were pooled, the cells washed thrice and viable cell number estimated.

Antigen pulsing of peritoneal cells
Peritoneal (4×10^5) cells were suspended in 2·0 ml of the culture medium containing 200 μg of sonicate of *M. tuberculosis* H37Rv or *M. vaccae*, and incubated at 37°C for 60 min. They were then washed three times and the final pellet suspended in the culture medium.

Paraformaldehyde fixation of peritoneal cells
This was done by the method described by Allen & Unanue (1984). Briefly, antigen pulsed or non-pulsed 4×10^4 peritoneal cells were suspended in 2·0 ml HBSS containing 1% (w/v) paraformaldehyde (Analytical grade; BDH, Poole, UK) and incubated at 20°C for 15 min. At the end of the incubation period they were washed three times, incubated again at 37°C in the culture medium and washed again three times.

Enumeration of MHC class II or Ia expressing cells
Peritoneal and spleen cell suspensions of BALB/c mice were stained for MHC class II or Ia antigens employing indirect immunofluorescence technique (Hudson & Hay, 1980). Cells (10^6–10^7) were suspended in 0·1 ml of rat monoclonal antibody of I-$A^{b,d,q}$ I-$E^{d,k}$ specificity and kept on ice for 30 min. The cells were washed three times and suspended in 50 μl of commercial FITC-coupled rabbit anti-rat globulin (Miles Yeda, Rehovot, Israel) and incubated on ice for 30 min; suspensions were washed, pellets suspended in a drop of FCS, smears prepared and examined on a fluorescent microscope (Fluoval, Carl Zeiss Jena). As controls, the cells were incubated with normal rat IgG having the same concentration as the monoclonal anti-Ia antibody preparation.

Antibody to MHC class II
Supernatants of cultures of rat IgG2b clone of antibody specificity I-$A^{b,d,q}$ I-$E^{d,k}$ (Bhattacharya, Dorf & Springer, 1981), were pooled. Antibody was precipitated with 33% $(NH_4)_2SO_4$, dissolved in PBS and dialysed extensively. For the staining purposes protein concentration was adjusted to 500 μg/ml. The clone was kindly supplied by Dr M. E. Dorf (Harvard Medical School, Boston, MA).

Table 1. DTH responses of mice immunized with mycobacteria

Immunogen 10^8 cells/mouse	Group (10 mice/group)	Route	CPE ± s.e.m. (mm) after 48 h	*P*
M. tuberculosis H37Rv	Slow grower	i.p.	0·21 ± 0·04	< 0·01
M. avium	Slow grower	i.p.	0·25 ± 0·02	< 0·01
M. kansasii	Slow grower	i.p.	0·18 ± 0·02	< 0·01
M. scrofulaceum	Slow grower	i.p.	0·22 ± 0·02	< 0·01
M. vaccae	Rapid grower	i.p.	0·08 ± 0·03	NS
M. phlei	Rapid grower	i.p.	0·05 ± 0·02	NS
M. tuberculosis H37Rv	Slow grower	i.d.	0·30 ± 0·01	< 0·01
M. avium	Slow grower	i.d.	0·44 ± 0·03	< 0·01
M. vaccae	Rapid grower	i.d.	0·36 ± 0·01	< 0·01
M. phlei	Rapid grower	i.d.	0·36 ± 0·01	< 0·01

One week after immunization. delayed-type hypersensitivity responses of mice to the sonicates of respective immunizing mycobacteria were tested by foot-pad swelling technique.

CPE, corrected foot-pad enlargement; i.p., intraperitoneal; i.d. intradermal; NS, not significant.

Table 2. effect of poly I:poly C and indomethacin pre-treatment on i.p. immunization with *Mycobacterium vaccae*

Pre-treatment	Intraperitoneal immunization dose of *M. vaccae* (no. bacilli/mouse)	Test ssonicate	CPE ± s.e.m. (mm) after 48 h
None	10^8	*M. vaccae*	0·02 ± 0·01
Poly I:poly C*	10^8	*M. vaccae*	0·18 ± 0·01
Indomethacin†	10^8	*M. vaccae*	0·18 ± 0·02
Poly I:poly C*	—	*M. vaccae*	0·05 ± 0·01
Indomethacin†	—	*M. vaccae*	0·02 ± 0·01

Ten mice/group.

* Swiss white mice were given intravenously 100 μg of poly I:poly C 3 h prior to i.p. immunization with the indicated dose of *M. vaccae* and the delayed-type hypersensitivity (DTH) responses to the mycobacterial sonicate was tested 1 week later.

† Swiss white mice were given six i.p. injections of 50 μg indomethacin at 12-h intervals. Twelve hours after the last dose they were immunized by i.p. route with the indicated dose of *M. vaccae* and the DTH response was tested 1 week later.

RESULTS

Immunogenicity of mycobacteria by i.p. and i.d. routes of immunization (Table 1)

Swiss white mice were immunized with vaccines of different killed mycobacteria in doses varying from 10^5 to 10^9 bacilli per mouse by different routes and their DTH response against the sonicate of the immunizing mycobacterium assayed.

Slow-growing mycobacteria, namely *M. tuberculosis* H37Rv, *M. avium*, *M. kansasii* and *M. scrofulaceum* generated a DTH response on immunization by both i.p. and i.d. routes. The minimum immunogenic dose per mouse was 10^6 bacilli, by both routes. The rapid growers, such as *M. vaccae* and *M. phlei* failed to generate a DTH response on i.p. immunization, even at a dose of 10^9 bacilli/mouse. However, both the organisms were immunogenic by i.d. route and the minimum immunogenic dose again was 10^6 bacilli/mouse.

Effect of IFN inducer and indomethacin on i.p. immunization with rapid growers (Table 2)

Swiss white mice were treated with an i.v. injection of 100 μg of poly I:poly C, 3 h before receiving an i.p. immunization of 10^8 *M. vaccae*. One week later the DTH response to its sonicate was tested. A separate batch of 10 mice, similarly treated with poly I:poly C, showed a mean IFN level of 906 ± 371 IU/ml. It was observed that poly I:poly C treated mice mounted a definite DTH response after an i.p. immunization with *M. vaccae*, whereas the non-treated mice did not.

Effect of indomethacin, a prostaglandin inhibitor, on i.p. immunization with *M. vaccae* was also investigated. Swiss white mice were given six i.p. injections of indomethacin (50 μg/mouse) at 12-h intervals. Twelve hours after the last dose, they were immunized with 10^8 *M. vaccae* by i.p. route and their DTH response tested. It was observed that the indomethacin-treated mice showed a DTH response, whereas the non-treated mice did not. Effects of poly I:poly C and indomethacin treatment on i.p. immunization with the other rapid growers were also tested and found to be identical.

In vitro *LTT to compare presentation efficiency of peritoneal cells and splenic APC*

BALB/c mice were immunized with 10^8 killed *M. vaccae* or *M. tuberculosis*, H37Rv by i.d. route, their spleen cells were harvested 1 week later and immune T cells separated.

Primed splenic T cells (2×10^5) were co-cultured with irradiated non-immune normal spleen and peritoneal cells as APC, to compare the efficiency of the two types of APC to present *M. vaccae* and *M. tuberculosis* H37Rv antigens, and a standard 6-day LTT was performed.

M. tuberculosis-primed T cells could mount a proliferative response when antigen was presented by either spleen or peritoneal cells. In contrast, the *M. vaccae*-primed T cells mounted a response only when antigen was presented by spleen cells; *M. vaccae*-specific stimulation induced by peritoneal cells was poor (Fig. 1).

From these observations it was concluded that the irradiated spleen cells presented both the *M. vaccae* and *M. tuberculosis* antigens efficiently, whereas the peritoneal cells presented only *M. tuberculosis* antigen efficiently. Peritoneal cell presentation of *M. vaccae* antigens was poor.

Effect of poly I:poly C treatment on the efficiency of presentation of M. vaccae *by peritoneal cells*

BALB/c mice were given one, two, three and five i.v. injections of 100 μg of poly I:poly C, at 24-h intervals and their peritoneal cells harvested 3 h after the last injection, cultured with *M. vaccae*-primed splenic T cells and LTT performed. This treatment resulted in distinct improvement in the presentation efficiency and maximal stimulation was given by peritoneal cells of mice receiving two and three doses. Further treatment with five doses caused a reduction in the stimulatory capacity of peritoneal cells (Fig. 2).

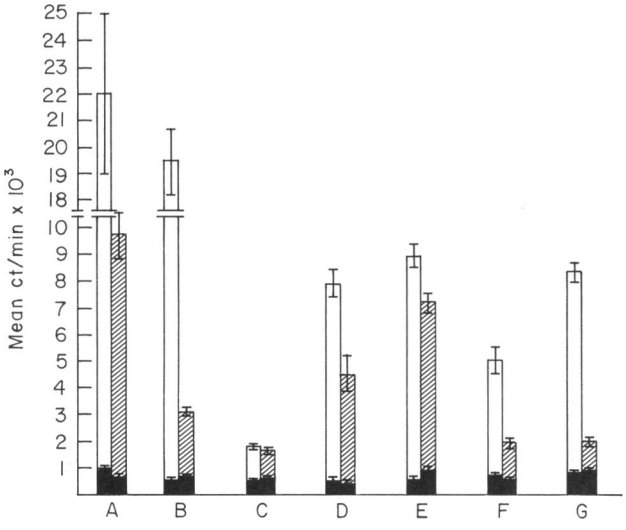

Fig. 1. Comparison of efficiency of presentation of *Mycobacterium tuberculosis* and *M. vaccae* antigens by irradiated spleen and peritoneal cells in an *in vitro* lymphoproliferative response. Proliferation of 2×10^5 *M. tuberculosis* H37Rv-primed (□) and *M. vaccae*-primed (■) splenic T cells co-cultured with different antigen-presenting cells and the corresponding bacterial sonicate antigen. A, proliferation of intradermally primed whole unfractionated spleen cells; B, proliferation of intraperitoneally primed whole unfractionated spleen cells; C, proliferation of unprimed whole spleen cells; D–G proliferation of intradermally primed T cells stimulated by 10% and 25% irradiated spleen cells and antigen (D, E), and 10% and 25% irradiated peritoneal cells and antigen (F, G). (■), without antigen. Bars are ± s.e.m.

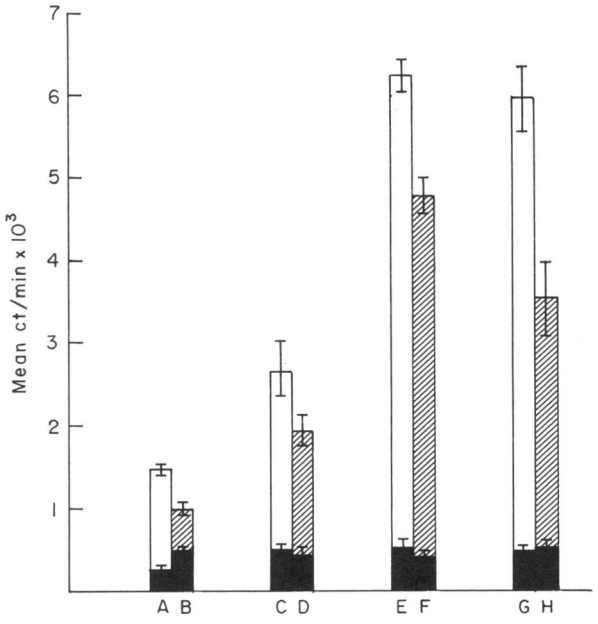

Fig. 3. Presentation of *Mycobacterium vaccae* antigens by paraformaldehyde-fixed peritoneal cells. Proliferation of 2×10^5 *M. vaccae* primed T cells co-cultured with 5×10^4 (25%) *M. vaccae* sonicate-pulsed and paraformaldehyde-fixed (■) or unfixed (□) peritoneal cells as antigen-presenting cells. A, B, peritoneal cells from normal mice; C, D, E, F; and G, H, peritoneal cells from mice receiving respectively one, two or three doses of poly I:poly C, 24 h apart. (■), Not pulsed with antigen. Bars are ± s.e.m.

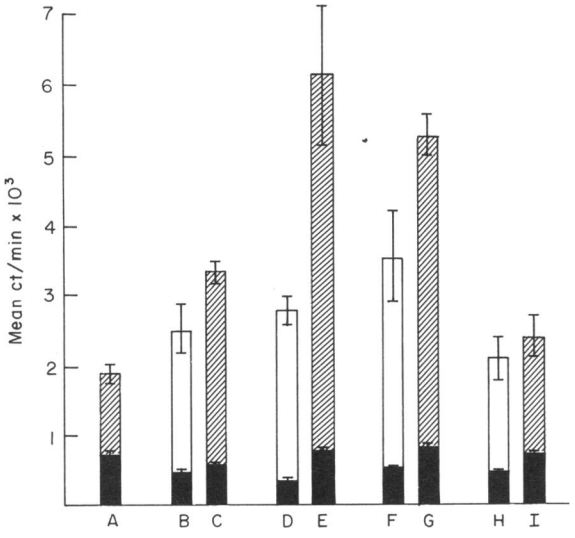

Fig. 2. Effect of poly I:poly C treatment on the efficiency of presentation of *Mycobacterium vaccae* antigens by peritoneal cells. Proliferation of 2×10^5 *M. vaccae* primed splenic T cells co-cultured with peritoneal cells from non-immune normal mice (A), or mice treated with one (B, C), two (D, E), three (F, G) and five (H, I) doses of poly I:poly C, 24 h apart. (■), No antigen; (□), 2×10^4 peritoneal cells and *M. vaccae* sonicate antigen; and (■), 5×10^4 peritoneal cells and *M. vaccae* sonicate antigen. Bars are ± s.e.m.

Presentation of M. vaccae *antigen by paraformaldehyde-fixed peritoneal cells from naive and poly I:poly C treated mice*

Peritoneal cells from normal and poly I:poly C treated BALB/c mice were harvested and pulsed with *M. vaccae* sonicate. These cells were then used as APC in a LTT either directly or after paraformaldehyde fixation. Proliferative response was observed even with fixed peritoneal cells from poly I:poly C treated mice whereas the peritoneal cells from normal mice generated a poor response. As before, maximal stimulation was seen with peritoneal cells from mice treated with two and three doses of poly I:poly C (Fig. 3).

Effect of poly I:poly C treatment on the MHC class II glycoprotein expression by peritoneal cells

Spleen cells from normal BALB/c and peritoneal cells from normal and poly I:poly C treated BALB/c were stained for Ia antigens. Only 5·95% of normal peritoneal cells were Ia positive whereas 38·3% of spleen cells were Ia positive. Poly I:poly C treatment of mice resulted into rapid increase in Ia positive peritoneal cells, rising from 5·95% to 18% in just 3 h. Peak rise to 24·5% was seen after three doses of poly I:poly C. When the spleen and the peritoneal cells were incubated with normal rat IgG in place of the monoclonal anti-Ia antibody, less than 0·1% cells were stained, indicating that the staining was not due to Fc receptor binding of the monoclonal antibody to the macrophages and other cells.

DISCUSSION

Data presented here show distinct differences in the ability to generate DTH response of rapid- and slow-grower mycobacteria. Whereas the slow growers were immunogenic by i.p. route, the rapid growers were not. Both the groups of mycobacteria were immunogenic by i.d. route. Non-responder state of mice to i.p. immunization with rapid growers and particularly *M. vaccae* is significant because of reported antigenic similarity of the organism with *M. leprae* (Stanford, Rook & Convit, 1975; Watson, Morrison & Collins, 1979; Sinha *et al.*, 1987). It may be noted that immunogenicity of *M. leprae* is also very similar, i.e. it is immunogenic by i.d. route and non-immunogenic by i.p. route (Shepard *et al.*, 1982).

It has been suggested that non-responder state in LL is the result of an HLA-linked effect in antigen presentation, thus implicating Ir genes in its pathogenesis (van Eden & de Vries, 1984; van Eden *et al.*, 1984; Ottenhoff & de Vries, 1987). Shepard *et al.* (1982) dispute this, based on their observation that the same animal behaves as a responder or a non-responder depending upon the route of immunization. Although mechanism of non-responder state after i.p. immunization is not clear, the phenomenon is implied to be universal and applicable to all the immunogens. Our results of i.p. immunization with *M. tuberculosis* H37Rv and other slow growers contradict this.

This antigen-related selective difference in the immunogenicity by the two routes could be due to differences in the APC involved in the generation of immune response at the two sites, namely the peritoneal cells and the Langerhans cells. Distinct differences in the density of MHC class II glycoproteins or Ia antigens of the two types of APC have been reported (Beller *et al.*, 1980; Katz, 1984), they being especially low on peritoneal cells, as shown also by us. Hence effects of drugs that enhance expression of Ia antigens, such as poly I:poly C (in IFN inducer) and indomethacin (a prostaglandin inhibitor) on the immunogenicity of rapid growers by i.p. route were investigated. These were compared with changes in the levels of Ia expression and changes in antigen-presentation efficiency of peritoneal cells.

Indeed, as expected, the non-immunogenicity of rapid growers by i.p. route could be corrected by prior treatment with poly I:poly C or indomethacin. However, effects of these agents seen *in vivo* may be quite complex and not restricted to peritoneal cells behaving as APC.

When efficiency of presentation of *M. vaccae* and *M. tuberculosis* antigens by peritoneal cells and irradiated spleen cells were compared in an *in vitro* LTT response, the results showed excellent correlation with *in vivo* immunogenicity of the two organisms. The peritoneal cell induced T cell stimulation against *M. tuberculosis* H37Rv was as efficient as that induced by irradiated spleen cells. However, with *M. vaccae* sonicate only the irradiated spleen cells could provide adequate antigenic stimulation to the primed T cells, the stimulation induced by peritoneal cells was very poor. The latter showed distinct improvement in their ability to provide stimulation with *M. vaccae* antigen even within 3 h after a single dose of the IFN inducer poly I:poly C, given prior to harvesting peritoneal cells. Peak response was seen after two and three doses given at 24-h intervals; any further treatment did not result in improvement.

It may be argued that effects of poly I:poly C on the peritoneal cells are not restricted to antigen presentation. It is also true that the peritoneal cells contain up to 30% lymphocytes (Stuart, Habeshaw & Davidson, 1978); observed effects of poly I:poly C may be related to these cells. Hence the *in vitro* T cell proliferative responses were also tested with antigen-pulsed paraformaldehyde-fixed peritoneal cells. For comparison, antigen-pulsed non-fixed peritoneal cells were used as APC. As expected, even the fixed peritoneal cells could provide adequate antigenic signal (with *M. vaccae* sonicate), only when they were obtained from poly I:poly C treated mice. Further, it was observed that along with improvement in antigen presentation, poly I:poly C treatment brought about distinct enhancement of Ia expression of the peritoneal cells.

On the basis of this evidence, we concluded that differences in the immunogenicity of rapid and slow growers by i.p. route and improvement in the immunogenicity of the former by prior poly I:poly C treatment of the mice was the result of poor antigen-presentation efficiency of peritoneal cells for rapid-grower antigens.

It is possible that antigens which associate with MHC class II glycoproteins with marginal efficiency may show the phenomenon of route-related variation in their immunogenicity. This is likely to be so, because the different APC processing the antigens introduced by different routes have gross differences in the density of MHC class II glycoproteins, which is so important in antigen presentation (Katz, 1984). Indeed it has been clearly demonstrated by Matis *et al.* (1983) that the magnitude of MHC-restricted T cell response is a function of concentration of the antigen as well as MHC class II glycoproteins. Thus, the selective *in vivo* and *in vitro* non-responder state of mice to *M. vaccae* and rapid growers when their antigens are presented by peritoneal cells and the reversal of this defect by prior treatment with IFN inducer can also be considered as an expression of an MHC-restricted phenomenon. These findings are significant because *M. leprae* has been reported to be antigenically similar to *M. vaccae* and its immunogenicity to mice by i.p. and i.d. routes is also very similar (Shepard *et al.*, 1982). Several cell types which have the potential of antigen presentation but lack adequate density of MHC-class II glycoproteins, such as capillary endothelium, tissue macrophages and alveolar lining cells may behave like peritoneal cells and thus play an important role in immunopathology of mycobacterial infections.

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