Cross-reactions in cell mediated Immunity induced by atypical mycobacteria

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Summary. Cross-reactivity in the delayed hypersensitivity response to mycobacteria of different Runyon groups was tested in Swiss white mice immunised with live mycobacteria. All the strains tested gave cross-reactions and, generally, slow growers gave stronger cross-reactions with other slow growers than with rapid growers and *vice versa*. Sonicates of cross-reacting mycobacteria were also tested for their ability to generate activated macrophages in mice immunised with *Mycobacterium avium*. All the mycobacterial sonicates generated activated macrophages but a sonicate of *Salmonella typhi* did not. The sonicate of *M. tuberculosis* H37Rv also generated activated macrophages, which indicates that there might be protective cross-reactions between *M. tuberculosis* and atypical mycobacteria.

Introduction

Atypical mycobacteria are common in the tropical environment. The majority of them are of either low or no virulence for man but many cause inapparent infections. Theoretically, such infections could be expected to alter the immune status of the host not only against the causative organism but also against the classically pathogenic mycobacteria, if the two were antigenically related. Classifications based on diverse metabolic characters need not necessarily reflect immunological relationships, particularly with reference to cell-mediated immunity which is considered to be important in mycobacterial infections. Palmer and Long (1966) and Shield *et al.* (1979) have proposed that a modification of the host's immune status by atypical mycobacterial infection is a possible explanation for the reported failure of BCG vaccination in certain studies. Magnusson (1961) and Chaparas and Maloney (1978) have reported extensive cross-reactivity between sensitins prepared from different mycobacteria. However, because the delayed hypersensitivity skin test is not considered to be necessarily an index of protective cell-mediated immunity, the reported cross-reactions between mycobacteria have often been ignored. In this paper, we present evidence to show that the delayed hypersensitivity cross-reactions of atypical mycobacteria both amongst themselves and with classical pathogenic mycobacteria correlate closely with an in-vitro index of protective cell-mediated immunity.

Materials and Methods

Bacteria

Mycobacterium tuberculosis H37Rv (NCTC7416), *M. kansasii* NCTC10268, *M. marinum* NCTC2275, *M. flavescens* NCTC10271, *M. avium* NCTC8559, *M. intracellulare* NCTC10425, *M. phlei* NCTC8151 and *Salmonella typhi* (0:9, 12, Vi, H:d) NCTC8385 were obtained from the National Collection Of Type Cultures, Colindale Avenue, London NW9 5HT. *M. vaccae* and *M. scrofulaceum* were kindly supplied by Dr J. L. Stanford, Middlesex Hospital, London. *S. enteritidis* (O:1,9,12 H:g,m) was isolated from naturally infected mice in the animal colony of the Haffkine Institute.

Animals

Swiss white mice were obtained from the National Institute of Health, USA and maintained in the animal colony of Haffkine Bio-Pharmaceutical Corporation Limited.

Immunisation

Four to six-week-old mice weighing 18–20 g were immunised intraperitoneally with 2–4 mg of cells of *M. avium* (nonchromogen), *M. scrofulaceum* (scotochromogen) or *M. kansasii* (photochromogen), suspended in 0.5 ml of phosphate buffered saline (PBS) pH 7.2, or intradermally with 10⁷ live cells of *M. vaccae* in 0.01 ml of PBS.

Sonic extracts of bacteria

Strains of mycobacteria were grown on the medium of Doub and Youmans (1950) for 4–6 weeks for the slow growers and for one week for the rapid growers. They were killed by exposure to 2.4 megarad of γ -irradiation from a ⁶⁰Co source. The killed cells were washed with PBS, disrupted by sonication (Branson B-30 Sonicator)

and centrifuged at 50 000 g for 1 h. The supernates were used as the test antigens after standardising the protein content by the method of Lowry *et al.* (1951). The sonic extract of *S. typhi* was prepared in a similar manner from an overnight culture on nutrient agar. The sonicate was then centrifuged at 100 000 g for 1 h to remove lipopolysaccharides and the supernate was used as antigen after standardisation of the protein content.

Footpad swelling test for delayed hypersensitivity

This test was performed by the method of Gray and Jennings (1955). An 0.03-ml volume of the sonicate of the immunising strain containing 50 µg of protein was injected into one hind footpad and the same volume of the sonicate of one of the other mycobacterial species in the other to detect cross-reactivity. The footpad thicknesses were measured before, and 24 and 48 h after injection. Non-immune mice served as controls and all sonicates were tested in these. Each sonicate was tested in at least five mice that gave a positive delayed hypersensitivity response to the sonicate of the immunising species. The results were expressed as "corrected footpad enlargement" which is the difference between the footpad enlargement caused by a test sonicate in the immunised and in the control mice.

Generation of activated macrophages

Mice immunised with *M. avium* 3 months earlier were given intraperitoneal injections of the sonicate of either *M. avium* or *S. typhi*; both sonicates contained 100 µg of protein in 0.5 ml PBS. The microbicidal activity of the cells in the peritoneal exudate was assayed 36 h later (see below). Similarly immunised mice were given intraperitoneal injections of 100 µg of a sonicate of one of the three mycobacteria that had produced cross-reactions in the delayed hypersensitivity test—*M. tuberculosis* H37Rv, *M. kansasii* and *M. vaccae*. The microbicidal activity of the cells in the peritoneal exudate was assayed 36 h later (see below).

Microbicidal activity of peritoneal exudate cells

The assay described by Nair and Kamat (1982) was used. Each mouse was given an intraperitoneal injection of $c. 2 \times 10^4$ cfu of smooth *S. enteritidis* preopsonised with heat-inactivated rabbit antibody to *S. enteritidis*. They were killed by cervical dislocation exactly 5 min later. Chilled Hanks's Balanced Salts Solution (HBSS) 2 ml, containing heat-inactivated rabbit antiserum against heat-killed *S. enteritidis* 2% v/v, new-born calf serum 10% v/v and heparin 20 IU, was immediately injected intraperitoneally into each mouse; the abdomen was massaged and the contents aspirated. The peritoneal exudate cells from several mice were pooled and washed and the number of cfu of unphagocytosed bacteria in the washings assayed. The pellet of washed peritoneal exudate cells was suspended ($c. 6 \times 10^6$ cells/ml) in warm HBSS containing newborn calf serum 12% v/v; 1-ml portions were incubated at 37°C for various periods and one portion was chilled immediately. After incubation, 0.1 ml of saponin was added to give a final concentration of 2.5%

v/v to lyse the peritoneal exudate cells. Lysates were kept on ice until dilution and plating for viable bacterial counts. The percentage of the number of cfu injected killed at each time was calculated.

Results

Cross-reactions in delayed hypersensitivity tests

The table shows the extent of the cross-reactions that occurred when mice immunised with *M. kansasii*, *M. scrofulaceum*, *M. avium* or *M. vaccae* were challenged with various mycobacterial antigens. There were extensive cross-reactions with all the mycobacteria, but in general slow growing strains gave stronger cross reactions with other slow growing strains than with rapid growers, and rapid growers gave stronger cross reactions with other rapid growers. *M. tuberculosis* H37Rv cross-reacted with both slow and rapid growers.

Generation of activated macrophages by sonicates of mycobacteria

Peritoneal exudate cells of mice immunised 3 months earlier with *M. avium*, but not stimulated with a sonicate before the assay, phagocytosed and killed 15% of challenge salmonellae in 5 min and 21% in 25 min. After stimulation with the *M. avium* sonicate, the microbicidal activity of the peritoneal exudate cells was enhanced and 60% of challenge salmonellae were phagocytosed and killed in 5 min and 72% in 35 mins. When the mice were stimulated with the *S. typhi* sonicate instead of the *M. avium* sonicate, there was no enhancement of the microbicidal activity of the peritoneal exudate cells. The peritoneal exudate cells of non-immune normal mice stimulated with the *M. avium* sonicate killed 35% of challenge salmonellae in 5 min (fig 1a). The differences between the percentages of salmonellae killed by mycobacterium-stimulated macrophages from immunised mice and the percentages killed by non-immune or unstimulated or *S. typhi*-stimulated macrophages at each time were highly significant ($p < 0.01$; Student's *t* test).

The microbicidal activity of the peritoneal exudate cells of mice immunised with *M. avium* was also enhanced by stimulation with the sonicates of *M. tuberculosis*, *M. kansasii* or *M. vaccae*. For example, the peritoneal exudate cells of mice stimulated with the sonicate of *M. tuberculosis* killed 53% of challenge salmonellae in 5 min. By comparison, cells from non-immune normal mice stimulated with the sonicate killed only 25% of challenge salmonellae in 5 min (fig. 1b). The differences in the percentages of salmonellae killed in the various assays were highly significant ($p < 0.01$, Student's *t* test). The microbicidal profiles of the peritoneal exudate cells from mice stimulated with the other

Table Delayed hypersensitivity cross-reactivity of mycobacteria

Test antigen	Corrected foot pad enlargement at 48 h in mice immunised with			
	<i>M. kansasii</i>	<i>M. scrofulaceum</i>	<i>M. avium</i>	<i>M. vaccae</i>
<i>M. tuberculosis</i> H37Rv	0.1312 ± 0.02*	0.0700 ± 0.03	0.0937 ± 0.03	0.0714 ± 0.01
<i>M. kansasii</i>	0.2051 ± 0.01	0.1042 ± 0.03	0.1375 ± 0.03	0.1300 ± 0.04
<i>M. marinum</i>	0.0862 ± 0.02	NT	NT	NT
<i>M. scrofulaceum</i>	0.1104 ± 0.02	0.1984 ± 0.01	0.1083 ± 0.02	0.1117 ± 0.05
<i>M. flavescens</i>	NT	0.1112 ± 0.03	NT	NT
<i>M. avium</i>	0.0762 ± 0.03	0.1275 ± 0.02	0.2319 ± 0.02	0.0881 ± 0.04
<i>M. intracellulare</i>	NT	NT	0.1770 ± 0.02	NT
<i>M. vaccae</i>	0.0675 ± 0.03	0.0750 ± 0.03	†0.0187 ± 0.02	0.1958 ± 0.04
<i>M. phlei</i>	0.0763 ± 0.02	0.0767 ± 0.02	0.0542 ± 0.01	0.1100 ± 0.01

NT = Not tested

*95% confidence limits. All the corrected foot pad enlargement responses are statistically significant ($p < 0.05$) except the one marked†. **Fig.** Microbicidal activity of pooled peritoneal exudate cells of mice: not immunised but stimulated with the sonicate of *M. avium* (○) or *M. tuberculosis* H37Rv (□); immunised with live *M. avium* and not stimulated (▲) or stimulated with the sonicate of either *M. avium* (●) or *M. tuberculosis* H37Rv (■) or *S. typhi* (△).

mycobacterial sonicates showed similar enhancement.

Discussion

Extensive cross-reactions between mycobacteria belonging to different groups have been reported in delayed hypersensitivity tests with mycobacterial extracts or 'sensitins' (Magnusson, 1961; Chaparas and Maloney, 1978). Such cross-reactions have been considered to be non-specific (Daniel and Janicki, 1978) and not a true recognition by effector T cells generated by cross-reacting mycobacterial immunisation. Recently, Hattikudur and Kamat

(1984) have characterised an antigen of *M. tuberculosis* H37Rv that elicited a delayed hypersensitivity response and also generated activated macrophages by interaction with effector T cells. In a separate report the same authors (Hattikudur and Kamat, 1985) showed that this antigen is highly polymorphous and has group-specific and genus-specific determinants on the same molecule. In view of this, cross-reactivity amongst mycobacteria as measured by delayed hypersensitivity may be due to recognition by T cells of cross-reactive determinants on a shared antigen or antigens. Hence it was logical to investigate if these antigens would also generate activated macrophages by interaction with effector

T cells generated in animals immunised with cross-reacting mycobacteria. Because activated macrophages are essential for controlling infections with facultative intracellular bacteria (Collins, 1974), their generation by cross-reactive T-cell-antigen interaction can be considered as an index of true protective cross-immunity.

As expected, sonicates of all the mycobacteria that gave cross-reactions with *M. avium* generated activated macrophages in mice immunised with *M. avium*. It could be argued that the observed enhancement of microbicidal activity of peritoneal exudate cells is due to the well known non-specific adjuvant effect of mycobacterial antigens. This was refuted by showing that the enhancement of microbicidal activity of peritoneal exudate cells from non-immune mice by stimulation with the various mycobacterial sonicates was not comparable with that obtained when cells from mice immunised with *M. avium* were stimulated (figs 1a and b).

Immunological specificity of these cross-reactions was proved by showing that after stimulation of mice immunised with *M. avium* with *S. typhi* sonicate there was no enhancement of microbicidal activity of peritoneal exudate cells.

Because of the practical difficulty of enumerating surviving mycobacteria as cfu, it was not possible to use them in an assay of bacterial killing by the macrophages. Activated macrophages are non-specific in their killing potential (Mackaness, 1964), and salmonellae, being facultative intracellular parasites (Suter, 1956), can be killed efficiently by

activated macrophages only. Therefore, challenge with *S. enteritidis* was used to assay the generation of activated macrophages. The specificity of cell-mediated immunity was demonstrated in our experiment by the interaction of effector T cells with antigens of the sonicates that was essential for generation of activated macrophages. However, caution is necessary in interpreting these data because activation to kill salmonellae may not necessarily imply enhanced mycobactericidal activity.

Several investigators have reported in-vivo studies of cross-protective immunisation with mycobacteria. Collins (1971) reported variable efficacy in mice of intravenous vaccines of live cells of several mycobacterial species in affording protection against challenge with *M. tuberculosis*. Shepard *et al.* (1980) have shown induction of cross-protection against *M. leprae* by BCG. However, in such studies, where the challenge was given to the vaccinated animal, it is difficult to rule out the non-specific protection induced by activated macrophages generated by vaccination itself. On the other hand, Lefford and Logie (1981) showed cross-protection against *M. lepraemurium* in mice by adoptive transfer of immune T cells generated by BCG vaccination and *vice versa*. Our findings lend further support to the hypothesis that natural infections with atypical mycobacteria modify the immune status towards classically virulent mycobacteria or BCG vaccination or both, as proposed by Palmer and Long (1966) and Shield *et al.* (1979).

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