In Vitro Characterization of T Cells from Mycobacterium w-Vaccinated Mice

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Tuberculosis caused by the intracellular bacterial pathogen Mycobacterium tuberculosis still represents a major health problem, and its effective control would best be accomplished by active vaccination. Although vaccination with M. bovis BCG has proven highly effective in certain parts of the world, in several developing countries, it has been found to confer only marginal protection. Hence, novel vaccination strategies are warranted. Mycobacterium w is a saprophytic cultivable mycobacterium which shares several antigens with M. tuberculosis. In the murine system, vaccination with killed M. w was found to protect against subsequent tuberculosis. In order to characterize the responsible immune mechanisms more precisely, mice were vaccinated with killed M. w and T cells restimulated in vitro with mycobacterial antigens. These T cells produced interleukin 2 and gamma interferon but no detectable interleukin 4 and interleukin 5. Killed M. w induced significantly stronger T-cell responses than killed M. tuberculosis, and both vaccination regimes were markedly improved by administration in a mild adjuvant, i.e., the Ribi adjuvant containing trehalose dimycolate, monophosphoryl lipid A, and mycobacterial cell wall skeleton. Our data suggest that M. w-induced immunity against M. tuberculosis rests primarily on TγΔ cells, which are thought to be of major relevance for acquired antituberculosis resistance. Our study therefore provides a further step toward the identification of a novel tuberculosis vaccine

Globally, tuberculosis is still a major health problem. It has been estimated that annually 10 million people become diseased and 3 million people die (17, 43). Furthermore, more than one-third of the total world population is thought to be infected with Mycobacterium tuberculosis (17). Tuberculosis can be treated chemotherapeutically, and in several countries, vaccination with Mycobacterium bovis BCG has been remarkably successful, particularly in preventing child hood tuberculosis. Nevertheless, several field studies indicate that BCG is of low to no effectiveness in preventing tuberculosis in adults (44, 49–51). The development of novel vaccination strategies is therefore urgently needed. This development, at the moment, follows two lines, namely, (i) construction of recombinant vaccines composed of defined antigens and carrier systems and (ii) application of killed atypical mycobacterial strains of high immunogenicity.

Mycobacterium w is a saprophytic cultivable mycobacterium which has been found to improve immunity against leprosy when given in killed form (45). A vaccine against leprosy based on M. w is approved for human use and is currently in phase III immunotherapeutic trials, where it has resulted in clinical improvement, accelerated bacterial clearance, and increased immune responses to Mycobacterium leprae antigens (46–48). M. w shares antigens not only with M. leprae but also with M. tuberculosis (9, 27), and it has been found recently that vaccination with killed M. w induces protection against tuberculosis in a mouse (39) and in a guinea pig model (38a). Here we report in vitro experiments aimed at characterizing the mechanisms underlying protective immunity caused by vaccination with killed M. w. We compared in vitro activities of T cells after vaccination with killed M. w, killed M. tuberculosis, and viable BCG with or without a mild adjuvant (21).

MATERIALS AND METHODS

Mice. C57BL/6 mice of both sexes were bred under specific-pathogen-free conditions and used at 8 to 12 weeks of age.

MAb. Rat monoclonal antibodies (MAb) against mouse gamma interferon (IFN-γ) from hybridomas R4-6A2 (42) and AN-18-17-24 (33) and rat MAb against interleukin 5 (IL-5), TRFK-4, and TRFK-5 (35) were purified from culture supernatants by 50% precipitations with NH4(SO4)2 followed by affinity chromatography on protein G-Sepharose (Pharmacia, Uppsala, Sweden). R4-6A2 and TRFK-4 were extensively dialyzed against phosphate-buffered saline (PBS; pH 7.4) and stored at 4°C with 0.1% sodium azide. AN-18-17-24 and TRFK-5 MAb were dialyzed against borate-buffered saline (pH 8.9) and conjugated with long-arm biotin. MAb were further dialyzed against PBS and stored at 4°C with 0.1% sodium azide.

Bacterial antigens. M. w was grown in Middlebrook 7H9 broth (Difco Laboratories, Detroit, Mich.) for 7 to 10 days; M. tuberculosis H37Rv and M. bovis BCG strain Phipps were cultured in Middlebrook 7H9 broth with 0.05% Tween 80 to log phase. Mycobacteria were harvested and washed three times with PBS, and the number of CFU of appropriate dilutions were determined on Middlebrook 7H10 agar plates. Bacteria were killed by autoclaving them at 121°C at a pressure of 15 lb/in2 for 15 min. Lyophilized M. tuberculosis H37Ra (Difco); Ribi adjuvant system containing trehalose dimycolate, monophosphoryl lipid A, and mycobacterial cell wall skeleton (Ribi, Immunochemical Research, Hamilton, Mont.); purified protein derivative (PPD; Statens Serum Institute, Copenhagen, Denmark); and con-

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canavalin A (ConA; Pharmacia) were purchased. *M. w* and *M. tuberculosis* H37Rv were solubilized by sonication and glass mill treatment. Protein concentrations were measured by Pierce bichinchoninic acid Protein Assay Reagent (Pierce Product Information, Pierce Chemical Co., Rockford, Ill. [41]).

**Immunization of mice.** The following four immunizations were employed: (i) 5 × 10^7 heat-killed *M. w*, (ii) 1 mg of killed *M. tuberculosis* H37Ra, (iii) *M. w* in Ribi adjuvant (5 × 10^7 organisms per 0.2 ml), and (iv) *M. tuberculosis* H37Ra in Ribi adjuvant (1 mg/0.2 ml). Injections were given subcutaneously (s.c.) at the base of the tail. In another set of experiments, animals were immunized s.c. with 5 × 10^6 viable *M. bovis* BCG strain Phipps organisms.

**T-cell stimulation.** At 2 and 3 weeks postimmunization, draining lymph nodes were removed and single-cell suspensions were prepared. Erythrocytes were lysed by NH_4Cl treatment, and T cells were enriched by two passages over nylon wool columns. Spleen cells from normal syngeneic mice were irradiated with 3,000 rads and used as accessory cells (AC). T cells (4 × 10^5) were cultured together with 10^5 AC in triplicate in 0.2 ml of Click’s RPMI medium supplemented with 10% fetal calf serum, HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid), penicillin, streptomycin (both from Biochrom, Berlin, Germany), 1-glutamine (2 mM), and 2 × 10^{-3} M 2-mercaptoethanol with or without antigen in 96-well plates. The following antigens were used for in vitro stimulations: (i) *M. w* (5 × 10^6, 1 × 10^6, and 5 × 10^5 organisms), (ii) *M. tuberculosis* H37Rv (5 × 10^6, 1 × 10^6, and 5 × 10^5 organisms), (iii) *M. tuberculosis* H37Ra (0.1, 1, and 3 μg), (iv) *M. w* lysate (0.6, 1.2, 2.5, and 5 μg), (v) *M. tuberculosis* H37Rv lysate (0.6, 1.2, 2.5, and 5 μg), (vi) PPD (1 and 3 μg), and (vii) ConA (1 μg). Cultures were incubated at 37°C with 7% CO_2 in air. After 24 h, supernatants were removed and frozen at −20°C until needed. For proliferation assays, cultures were incubated for 5 days and pulsed with 1 μCi of [3H]thymidine for the last 18 h. Cells were harvested onto glass filters, and [3H]thymidine incorporation was quantitated by liquid scintillation counting.

**IL-2.** IL-2 activities were determined with a modified MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay (12) using a CTLL line which responds to IL-2 but not to IL-4. In short, CTLL cells (1.5 × 10^5/50 μl) were added to individual supernatants in flat-bottomed microtiter plates (96 wells) at a final volume of 100 μl and incubated at 37°C. After 18 h, 20 μl of MTT solution was added. Four to 5 h later, 100 μl of sodium dodecyl sulfate (10%) was added, and the plates were incubated overnight at 37°C. The plates were read at 570 nm with a reference filter of 690 nm.

**IL-4.** IL-4 activities were determined by using CT4S cells (14), which respond to <5 U of IL-4 and >100 U of IL-2 per ml. Culture supernatants were transferred into 96-well tissue culture plates (50 μl per well). CT4S cells were seeded into supernatants at 5 × 10^5 cells in 150 μl of solution per well and incubated at 37°C. After 48 h, the cultures were pulsed with 1 μCi of [3H]thymidine for 16 h. A standard curve was drawn with twofold dilutions of recombinant IL-4 (rIL-4; gift from J. Langhorne, Max-Planck-Institute for Immunobiology, Freiburg, Germany) from 400 to 0.1 U/ml.

**IFN-γ.** IFN-γ activities in the culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) (40). ELISA microplates (96 well) (Immunoplate, Maxisorb; Nunc) were coated with anti-IFN-γ MAb R4-6A2 (6 μg/ml) in PBS (pH 7.4) overnight at 4°C. Unadsorbed sites were blocked with 1% PBS-bovine serum albumin (BSA; Sigma) for 1 h at 37°C. Plates were washed three times with buffer (PBS, 0.05% Tween 20), and 50-μl aliquots of supernatants from individual cultures were added to each microwell plate. After an overnight incubation at 4°C in a humid box, plates were washed three times and rinsed with PBS-0.1% BSA per well and incubated for 1 h at 37°C. The plates were washed again and incubated with a conjugate of streptavidin-alkaline phosphatase (Jackson Immunoresearch Laboratories Inc.) in PBS-0.1% BSA at a dilution of 1:1,000 for 1 h at 37°C. After three washes, the reaction was developed with p-nitrophenyl phosphate diosodium as substrate (1 mg/ml; Sigma) in diethanolamine buffer (pH 9.8) for 20 min at room temperature in the dark. Optical density was measured at 405 nm with a reference filter of 490 nm by using the Inter-med NJ-2000 Immunoreader. The detection threshold was 1 U/ml, as determined by a standard titteration of recombinant IFN-γ (rIFN-γ) starting at a concentration of 250 U/ml and serial twofold dilutions to 1 U/ml.

**IL-5.** For detection of IL-5 activity, an ELISA was used
TABLE 2. IL-2 secretion by T cells from M. w- and M. tuberculosis-vaccinated mice

<table>
<thead>
<tr>
<th>Immunization schedule (wk)</th>
<th>Amt (U/ml) of IL-2 after in vitro restimulation with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M. w (×10^5)</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>M. w</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.5</td>
</tr>
<tr>
<td>3</td>
<td>ND</td>
</tr>
<tr>
<td>M. w in Ribi</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3.3</td>
</tr>
<tr>
<td>3</td>
<td>ND</td>
</tr>
<tr>
<td>M. tuberculosis</td>
<td></td>
</tr>
<tr>
<td>in Ribi</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>ND</td>
</tr>
</tbody>
</table>

a T cells (4 × 10^5 per well) from immunized mice were restimulated with M. w or M. tuberculosis antigens in the presence of AC. Twenty-four hours later, culture supernatants were collected and IL-2 activities were assessed in CTLL as described in Materials and Methods. In the absence of antigen, IL-2 activities were either negative or less than 0.5 U/ml. Values are means for triplicate cultures; the standard deviation was <10%. ND, not detectable.

RESULTS

Proliferative responses of T cells from mice vaccinated with killed M. w or killed M. tuberculosis. Mice were immunized s.c. with killed M. w or with killed M. tuberculosis H37Ra with or without Ribi adjuvant. T cells from draining lymph nodes were restimulated in vitro with increasing doses of M. w and M. tuberculosis in the presence of AC, and after 5 days of culture, proliferative responses were determined. Vaccination with M. w alone induced small but significant proliferation, as did vaccination with M. tuberculosis (Table 1). Incorporation into Ribi adjuvant markedly increased proliferative responses in both instances. Lysates of M. w and M. tuberculosis induced stronger in vitro responses, indicating that this kind of antigen preparation facilitated the processing of these organisms. Optimum T-cell responses were observed 3 weeks after vaccination (Table 1). Two weeks after immunization, comparable but slightly lower responses were obtained (data not shown).

Interleukin secretion by T cells from mice vaccinated with killed M. w or killed M. tuberculosis. CD4+ T cells play a crucial role in antimycobacterial immunity. For the mouse, evidence has been presented that CD4 T cells segregate into \( T_{\text{H1}} \) and \( T_{\text{H2}} \) subpopulations (23). It is generally assumed that \( T_{\text{H1}} \) products, mainly IL-2 and IFN-γ, are important for antimicrobial protection (20), whereas the role of \( T_{\text{H2}} \) products, i.e., IL-4 and IL-5, is equivocal in antituberculous immunity (6). We therefore wanted to assess which interleukins are produced after vaccination with M. w and M. tuberculosis. Mice were vaccinated and T cells were stimulated in vitro with antigens or ConA in the presence of AC. Culture supernatants were collected after 24 h and tested for IL-2, IL-4, IL-5, and IFN-γ activities. T cells from mice vaccinated with M. w alone produced small but significant IL-2 activities after restimulation with M. w organisms and stronger responses after restimulation with M. w and M. tuberculosis lysates (Table 2). Ribi adjuvant did not further increase IL-2 production by T cells from M. w-immunized mice (Table 2). In the M. tuberculosis-vaccinated group, measurable responses were observed only after restimulation with lysates. Immunization with M. tuberculosis in Ribi adjuvant somewhat improved IL-2 production. IL-2 production was significantly lower in all groups 3 weeks after immunization.

Vaccination with M. w and restimulation with M. w, M. tuberculosis H37Ra, or M. tuberculosis H37Rv caused marked IFN-γ production which was further increased after administration of M. w in Ribi adjuvant (Fig. 1). Stronger responses were observed after restimulation with lysates than after restimulation with whole organisms. T cells from M. tuberculosis-immune mice failed to produce detectable amounts of IFN-γ after restimulation with whole organisms, while small but significant IFN-γ activities were observed after restimulation with lysates. Following immunization with M. tuberculosis in Ribi adjuvant, IFN-γ production was increased and responses to whole organisms were detectable. IFN-γ production in all four immunization groups was higher 3 weeks after vaccination, in contrast to levels of IL-2, which were significantly lower at that time. Production of IL-4 and IL-5, indicating the activation of \( T_{\text{H2}} \) cells, was also studied. There was no evidence for IL-4 or IL-5 activities in the culture supernatants after in vitro

ELISA microtiter plates (96 wells) were coated with anti-IL-5 MAb TRFK-4 (7 μg/ml) in PBS (pH 7.4) overnight at 4°C. Unlabeled sites were blocked as for IFN-γ ELISA, and the plates were washed and incubated with the culture supernatants at 37°C for 1 h. After the plates were washed, 50 μl of biotin-labeled TRFK-5 MAb (0.5 μg/ml in PBS-0.1% BSA) was added and incubated at 37°C for 1 h. The plates were washed and incubated with streptavidin-alkaline phosphatase, the reaction was developed, and the optical density was measured as for the IFN-γ ELISA. Murine rIL-5 from X63/Ag8-63 transfectants (16) kindly provided by Jean Langhorne (Max-Planck-Institute for Immunobiology) in RPMI 1640 at a starting concentration of 1:4 was used as the standard.
FIG. 1. IFN-γ secretion by T cells from M. w- and M. tuberculosis-vaccinated mice after in vitro stimulation with various mycobacterial antigen preparations. Mice were immunized s.c. with M. w in Ribi adjuvant (C), M. tuberculosis H37Ra (C), or M. tuberculosis H37Ra in Ribi adjuvant (Δ), and lymph nodes were collected at 3 weeks postinfection. T cells (4 × 10⁶ per well) were restimulated with increasing doses of heat-killed M. w (A), heat-killed M. tuberculosis H37Rv (B), killed M. tuberculosis H37Ra (C), M. w lysate (D), M. tuberculosis H37Rv lysate (E), or PPD (F) in the presence of AC. After 24 h of culture, culture supernatants were tested for IFN-γ activity by an ELISA. Values are means for triplicate cultures; the standard deviation was <10%. The closed symbols indicate IFN-γ activity in the absence of antigen. Values on the x axis are triplicate cultures; the standard deviation was <10%. The closed symbols indicate IFN-γ activity in the absence of antigen.

TABLE 3. IL-2 secretion by T-cell-enriched and unseparated lymph node cells from M. w- and M. bovis BCG-vaccinated mice

<table>
<thead>
<tr>
<th>Microorganisms for immunization</th>
<th>No. of cells in culture*</th>
<th>Amt (U/ml) of IL-2 after in vitro stimulation with:</th>
<th>M. w lysate (µg)</th>
<th>M. tuberculosis lysate (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1.2</td>
<td>2.5</td>
<td>5.0</td>
</tr>
<tr>
<td>M. w (heat killed)</td>
<td>4 × 10⁵ T cells</td>
<td>3</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>1.2 × 10⁵ LNC</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>BCG (viable)</td>
<td>4 × 10⁵ T cells</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1.2 × 10⁵ LNC</td>
<td>4</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Mice were immunized s.c. with killed M. w or viable BCG. Three weeks after immunization, nylon wool-passaged lymph node cells (LNC) (T cells) and unseparated lymph node cells were restimulated in vitro with increasing doses of M. w and M. tuberculosis H37Rv lysates in the presence of AC. Culture supernatants were collected after 24 h and tested for IL-2 activity. Values are means for triplicate cultures; the standard deviation was <10%. ND, not detectable.

** All cells were cultured with AC.
TABLE 4. IFN-γ secretion by T cells from M. w- and M. bovis BCG-vaccinated micea

<table>
<thead>
<tr>
<th>Microorganisms for immunization</th>
<th>No. of cells in culturea</th>
<th>M. w lystate (µg)</th>
<th>M. tuberculosis lystate (µg)</th>
<th>No antigen</th>
<th>ConA (1 µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1.2</td>
<td>2.5</td>
<td>5.0</td>
<td>1.2</td>
</tr>
<tr>
<td>M. w (heat killed)</td>
<td>4 × 10^5 T cells</td>
<td>84</td>
<td>250</td>
<td>250</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>1.2 × 10^5 LNC</td>
<td>3.2</td>
<td>6.2</td>
<td>2.8</td>
<td>40</td>
</tr>
<tr>
<td>BCG (viable)</td>
<td>4 × 10^5 T cells</td>
<td>3.1</td>
<td>2.5</td>
<td>2.7</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>1.2 × 10^5 LNC</td>
<td>80</td>
<td>250</td>
<td>250</td>
<td>44</td>
</tr>
</tbody>
</table>

a Mice were immunized s.c. with heat-killed M. w or viable BCG. Three weeks later, T-cell-enriched lymph node cells (LNC) and unseparated lymph node cells were restimulated in vitro with M. w and M. tuberculosis H37Rv lysates. The supernatants were collected after 24 h and tested for IFN-γ activity by an ELISA. Values are means for triplicate cultures; the standard deviation was <10%.

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