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T-Cell Proliferative Response to Antigens Secreted by *Mycobacterium tuberculosis*

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An infection model of human tuberculosis was established with C57BL/6J mice. The lymphocyte proliferative responses to antigens from *Mycobacterium tuberculosis* were investigated during the course of infection and compared with results obtained with a group of mice immunized with large amounts of killed bacteria. The two groups responded similarly to a number of mycobacterial antigens, but marked differences in responses against secreted antigens were found; only infected mice responded vigorously to these. The responding lymphocyte subpopulation was made up of L3T4$^+$ T lymphocytes under restriction of the Ia molecule.

It is established that resistance to infection with *Mycobacterium tuberculosis* is efficiently evoked only by the living organism (8, 17, 24). Killed bacteria are able to activate the immune system but are not able to evoke a long-term specific cellular immunity (24, 25). Furthermore, major differences in the T-lymphocyte repertoire stimulated by live and dead mycobacteria have been reported (27). On the basis of these results, a possible role in protection for proteins secreted from the actively replicating mycobacteria has been suggested (25, 27).

These observations provide the basis for the present study of mice injected with live and killed mycobacteria. An infection model with C57BL/6J mice which enabled us to investigate the immune responses through the course of infection has been established. By comparing the results from infected mice with those from animals receiving killed bacteria, we have discovered major differences which may be of importance for protection against infection.

MATERIALS AND METHODS

Animals. These studies were performed with C57BL/6J mice bred at Statens Seruminstitut, Copenhagen, Denmark. Female mice, 8 to 12 weeks old, were used in all experiments.

Bacteria. *M. tuberculosis* H37Rv, recently passed through mice to ensure optimal growth, was grown on Löwenstein-Jensen medium at 37°C. Sauton medium without albumin additive was enriched with 0.5% sodium pyruvate, 0.5% glucose, and 0.05% Tween 80 (modified Sauton) (9). A 250-ml volume was inoculated with 2×10^6 CFU/ml and was grown on an orbital shaker at 37°C for 10 to 12 days. These cultures were either aliquoted and stored at −80°C for use in experimental infections or used as inoculum for the production of short-term filtrate.

Bacteria were killed by incubation in 2% glutaraldehyde overnight. The suspension was washed three times in phosphate-buffered saline (PBS) and adjusted to the same optical density as the frozen aliquots of live bacteria described above. The suspension was aliquoted and frozen at −80°C.

Experimental infections. Mice were infected intravenously via a lateral tail vein with an inoculum of 2.5×10^3 bacteria suspended in 0.1 ml of PBS. Mice receiving killed bacteria were similarly injected with 10^5 killed bacteria suspended in PBS.

Bacterial enumeration. The numbers of viable bacteria in spleens of mice were determined by plating serial 10-fold dilutions of spleen homogenates on Löwenstein-Jensen medium. The colonies were counted after three weeks of incubation. The data were expressed as the $\log_{10}$ values of the geometric means of counts obtained with five mice.

Bacterial antigens. The short-term-filtrate procedures were as follows. The *M. tuberculosis* cultures described above were washed once and used for inoculation (4×10^6 CFU/ml) of modified Sauton medium without Tween 80. The bacteria were grown on an orbital shaker for 4 days at 37°C. This short growth period ensures minimal autolysis (4). The culture supernatants were sterile filtered and concentrated (×100) on an Amicon PM10 membrane (Amicon, Danvers, Mass.). The product was desalted by extensive washing in the ultrafiltration chamber with distilled water.

Our panel of monoclonal antibodies (MAbs), generated against a mixture of long-term culture filtrate and Freund's complete adjuvant (HBT3, HBT4, HBT7, HBT11, HBT12, HYT6, and HYT27), were used for affinity purification. The reactivity of MAbs and the schedule used for affinity purification have been described previously (3, 30). Briefly, long-term culture filtrate was passed through immunosorbent columns coupled with MAbs. Eluates of bound material were dialyzed against PBS and used as antigen in lymphocyte stimulation assays. A large pool of each antigen preparation was aliquoted in small vials and kept frozen (−20°C). Each vial contained the quantity of antigen to be used in one experiment. Control preparations for stimulation of lymphocytes were prepared by passing the eluting buffers through the columns which had not been loaded with culture filtrate prior to the elution. The eluting buffers were either 3 M KSCN (pH 7.4) or 0.1 M glycine hydrochloride (pH 2.8). These mock eluates were dialyzed against PBS and processed in the same manner as the antigen preparations.

The recombinant 65-kDa mycobacterial antigen was purified by ammonium sulfate precipitation followed by trisacryl ion-exchange chromatography. This preparation was a kind gift of R. van der Zee, RIVM, Bilthoven, The Netherlands.

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Sodium dodecyl sulfate (SDS) extraction of the bacteria was performed as described by Hugo et al. (12). Briefly, bacteria were extracted in a 10% SDS solution for 1 h. After centrifugation, the supernatant was collected and analyzed by immunoblotting with the MAbs corresponding to our affinity-purified antigens.

SDS-PAGE and immunoblotting of mycobacterial proteins. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out in a discontinuous system by using a 10 to 20% acrylamide separation gel (15). The gels were silver stained by the method of Morrissey (20). Alternatively, the proteins were transferred onto nitrocellulose paper and incubated with polyclonal mouse sera. The strips were screened for antibody binding by peroxidase-labeled rabbit anti-mouse (P261; Dakopatts, Tåstrup, Denmark) and subsequently stained with 5,5',3,3'-tetramethyl benzidine.

Lymphocyte proliferation assay. The spleens were removed, and single cell suspensions were prepared in RPMI 1640 supplemented with 5×10^{-5} M 2-mercaptoethanol, 100 IU of penicillin per ml, 50 μg of streptomycin per ml, nonessential amino acids, 1 mM glutamine, and 0.5% (vol/vol) fresh mouse serum. The erythrocytes were lysed by incubation in a solution of 0.84% ammonium chloride in distilled water. Cell cultures were established in 96-well round-bottomed microtiter plates (Nunc, Roskilde, Denmark). Each well contained 2×10^5 cells in a volume of 200 μl. On the basis of dose response studies (data not shown), the concentrations of antigens in the final cultures were chosen as follows: short-term filtrate, 1 μg/ml; 17 kDa (HBT 11), 3 μg/ml; 17 to 19 kDa (HYT 6), 2 μg/ml; 25 kDa (HBT 4), 3 μg/ml; 32 kDa (HBT 7), 3 μg/ml; 32 to 33 kDa (HYT 27), 1 μg/ml; 38 kDa (HBT 12), 4 μg/ml; 56 kDa (HBT 3), 3 μg/ml; and 65 kDa, 15 μg/ml. Concentrations which stimulated less than maximally were chosen in order to avoid high-dose inhibition. Some cells were left unstimulated or were stimulated with mock eluates from the affinity columns. The mock eluates were used at a 1/15 dilution, which approximately corresponds to the dilutions used for the antigens. Concanavalin A (Sigma) was used at a concentration of 1 μg/ml as a positive control for cell reactivity. All tests were carried out in triplicate. Cultures were incubated for 4 days at 37°C, the last 22 h in the presence of 1 μCi [^{3}H]thymidine (TRA 120; Radiochemical Centre, Amersham) in an atmosphere of 5% CO_2 in humidified air. The cells were harvested on fiberglass paper, and the incorporated radioactivity was measured in a liquid scintillation counter.

Selection of lymphocyte subsets. Spleen lymphocytes were depleted of T lymphocytes by treatment with the MAb anti-Thy 1.2 (Tib 107) and Low-Tox-M rabbit complement (Accurate Chemical & Scientific Corp, Westbury, N.Y.). The MAb was used as a hybridoma supernatant diluted 1/2, and the complement was diluted 1/8. The cells (2×10^7/ml) were incubated for 1 h at 37°C in the diluted antibody-complement mixture and were subsequently washed three times in medium.

Isolation of T lymphocytes was obtained by passing the cells through a nylon wool column as described previously (14).

The purities of both suspensions were assessed by flow cytometry.

The T-cell receptor and the Ia molecule on the leukocytes in cultures were blocked by diluting the MAbs anti-L3T4 (Tib 207), anti-Lyt2 (Tib 105), and anti-Ia (Tib 120) directly in the cultures (7). The MAbs were diluted 1/5 to 1/10 (on the basis of previous titrations).

Statistical methods. The statistical analysis was performed by using logarithmically transformed counts per minute. The standard deviations are estimated for each antigen separately as the square root of the average variance estimate. The standard errors obtained are presented in Fig. 3, where a logarithmic scale is used. For comparisons of the different groups of mice unimmunized ($n = 5$), infected ($n = 3$), and immunized with killed *M. tuberculosis* ($n = 5$), the same method was used. From the standard errors obtained, it was determined that the ratios of the geometrical mean responses of two different groups should exceed 2.4 (unimmunized/infected), 1.7 (unimmunized/immunized with killed *M. tuberculosis*), and 2.3 (infected/immunized with killed *M. tuberculosis*) to be significant at the 5% level.

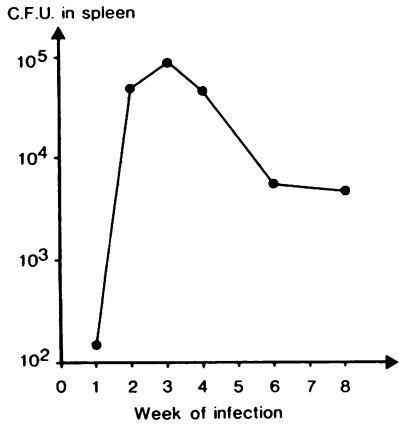

FIG. 1. Course of infection in C57BL/6J mice infected with *M. tuberculosis*. Mice were infected intravenously with 2.5×10^3 viable units, and the course of infection was monitored by plating whole organ homogenates on nutrient agar. CFU indicated are the geometric means of results from five individuals.

RESULTS

Course of infection. Mice were inoculated with 2.5×10^3 viable units of *M. tuberculosis* H37Rv intravenously. This inoculum was chosen to avoid the nonspecific anergy found in relation to higher doses (26). As seen in Fig. 1, a number of bacilli equivalent to 5% of this inoculum could be detected in the spleen after 1 week.

The mycobacteria multiplied logarithmically during the following 2 weeks and reached a maximal level at about 10^5 viable units per spleen at the third week. After this time, the bacteria were gradually eliminated.

The secreted protein pattern of *M. tuberculosis* H37Rv. Culture filtrate harvested from logarithmically growing *M. tuberculosis* contains primarily proteins released or secreted from the live bacteria (2, 9). A short-term filtrate harvested in the late log phase of growth was separated in a 10 to 20% polyacrylamide gel and visualized by silver staining (Fig. 2).

The short-term filtrate was found to be dominated by major proteins with molecular masses of 12, 22, 23, 24, 32, 33, and 45 kDa. None of the proteins were medium components as shown by a similar investigation of concentrated Sauton medium (results not shown).

The antigens present in this filtrate have been compared with the antigens present in an SDS extract of whole bacilli, reported to represent primarily cell wall antigens (12).

The two preparations were SDS-PAGE separated and immunoblotted with MAbs corresponding to our panel of affinity-purified antigens. It was found that although most

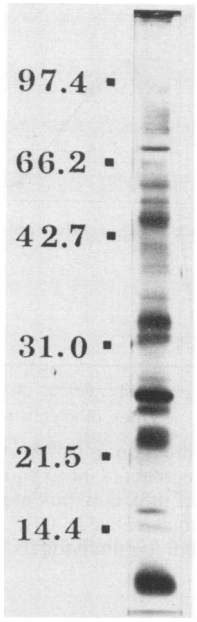

FIG. 2. Concentrated short-term filtrate of *M. tuberculosis* separated by SDS-PAGE and visualized by silver staining.

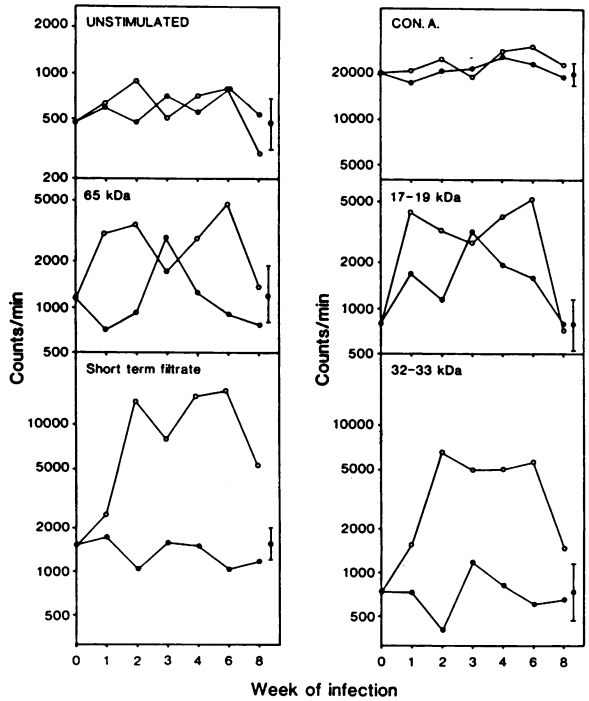

FIG. 3. The proliferative response of spleen lymphocytes from mice infected with 2.5×10^3 mycobacteria (○) and from mice injected with 1×10^5 killed bacteria (●). The responses depicted are the geometrical means of results from five individuals. The animals were killed and investigated at various times through the 8 weeks of infection. The vertical bar indicates a range of ± twice the standard error of the means plotted, and its center is placed in the same level as the value of week 0.

antigens were present in both preparations, their relative quantities markedly differed. The 65-kDa antigen which serves as a marker of autolysis (2) was present only in extremely small quantities in the short-term filtrate.

The proliferative response to mycobacterial antigens in infected mice and mice immunized with killed bacteria. Mice infected with 2.5×10^3 live mycobacteria were killed 1 to 8 weeks after infection. The spleen lymphocytes were used for lymphocyte proliferation assay, and the results were compared with those obtained with mice injected with large amounts of killed bacteria (Fig. 3).

The mice injected with killed bacteria received 10^5 bacteria. This inoculum was chosen to mimic the amount of bacterial protein present in an infected animal at the height of infection (Fig. 1).

During the 8 weeks of the study, the values for unstimulated controls of both groups of animals were low and at the same level as that of nonimmunized mice (week 0). This excludes the possibility of antigen stimulation due to mycobacteria carried over by the cell suspensions.

Concanavalin A gave similar stimulations in all experiments, demonstrating that no difference in the general response levels of the two groups of animals existed.

Short-term filtrate as well as a number of affinity-purified proteins of *M. tuberculosis* were used as antigens. The short-term filtrate and the 32- to 33-kDa antigen initiated a response exclusively in the infected animals. The animals responded weakly after 7 days, but as the infection became established 14 days after inoculation, a 10-fold increase in the proliferative response was obtained (short-term filtrate, 14,125 cpm; 32- to 33-kDa antigen, 6,025 cpm).

The mice receiving killed bacteria did not respond to short-term filtrate or the 32- to 33-kDa antigen at any time. These animals, however, exhibited marked responses against the 17- to 19-kDa and the 65-kDa antigen, which peaked after 3 weeks.

In the experiment which followed, the proliferative responses to a larger panel of antigens were investigated 16 days after infection or immunization with killed bacteria (Fig. 4). Both infection and immunization with killed bacteria were found to induce a significant antigen-specific proliferation to all antigens with the exception of the 17-kDa antigen, which was found to evoke a powerful proliferation in unimmunized mice, too.

The responses of the two treated groups were found to differ significantly (see "Statistical methods" above) only by the powerful response of infected mice to short-term filtrate and to the 32- to 33-kDa antigen.

Some variations between the two experiments were found. A higher response to the 32- to 33-kDa antigen was found in the latter experiment (Fig. 4), but no change in the general response pattern was observed.

The proliferation found in response to short-term filtrate is caused by L3T4$^+$ T cells under restriction of the Ia molecule. The lymphocyte population proliferating in response to the short-term filtrate has been identified by a combination of cytotoxic depletion, nylon wool separation, and receptor blocking.

T lymphocytes were removed by treating the cell suspension with anti-Thy 1.2 antibody and complement. The remaining cells were unresponsive to the short-term filtrate.

A nylon wool passage of the spleen suspension cell T cells by removing isolated B cells and macrophages (14). The response of this population returned after the addition of irradiated spleen cells as antigen-presenting cells (results not shown). This experiment identified T lymphocytes as the responding cell population.

The responding T-lymphocyte subpopulation and its re-

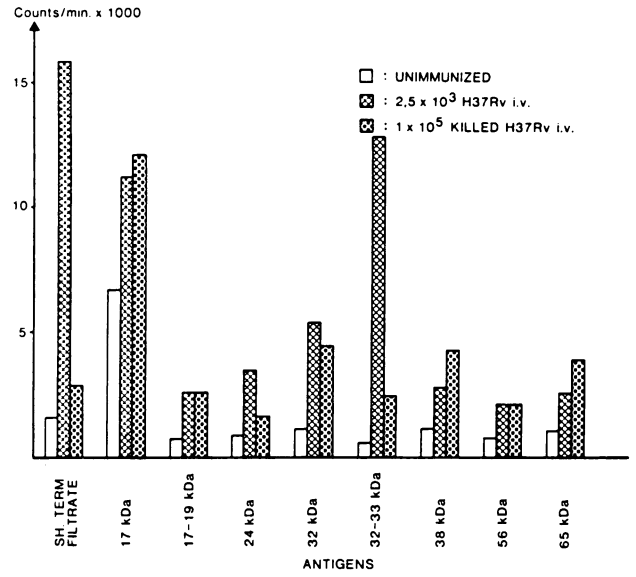

FIG. 4. The proliferative response of spleen lymphocytes from C57BL/6J mice infected or immunized for 16 days. The responses depicted are the geometrical means of results from three to five individuals. The proliferation of unstimulated lymphocytes or lymphocytes stimulated with control buffers was always below 1,000 cpm. The ratios between the responses of the two immunized groups were found to be 5.32 (short-term filtrate) and 5.04 (the 32- to 33-kDa antigen). These responses differ significantly (see "Statistical methods").

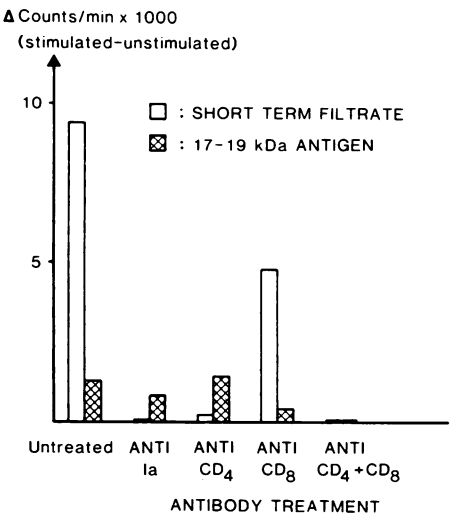

FIG. 5. The proliferative response of spleen lymphocytes to short-term filtrate and the 17- to 19-kDa antigen. The mice were infected 14 days previously with 2.5×10^3 *M. tuberculosis*. In some of the cultures, the T-cell receptor or Ia molecule was blocked by antibodies. The responses depicted are the geometrical means of results from four individuals.

striction element have been identified by MAbs capable of blocking either the antigen presentation or the T-cell receptor (Fig. 5).

Short-term filtrate was found to stimulate almost exclusively T lymphocytes of the helper ($L3T4^+$) phenotype under restriction of the Ia molecule. In contrast, the limited response to the 17- to 19-kDa antigen was not influenced by antibodies against $L3T4^+$ or the Ia molecule.

The antibody response to short-term-filtrate components. Short-term filtrate was separated by SDS-PAGE and immunoblotted with sera from the two groups of animals killed at various times through the period of 8 weeks.

None of the animals were found to produce antibodies to antigens in short-term culture filtrate. Low levels of background reactivity against a protein in the 30- to 32-kDa region were found with both treated and unimmunized mice (results not shown).

DISCUSSION

This study shows that mice infected with virulent *M. tuberculosis* acquire specific T lymphocytes during the period of progressive bacterial growth which proliferate vigorously in response to antigens secreted from *M. tuberculosis*. The lymphocytes responding are $L3T4^+$ T lymphocytes under restriction of the Ia molecule. Antibodies to the secreted antigens were, on the contrary, not detected during infection, a finding which might indicate that only small T-cell-stimulatory fragments of mycobacterial antigens escape the interior of the macrophage.

Differences in the T-lymphocyte repertoire stimulated by live and killed mycobacteria have previously been reported. T-cell clones reacting with sonicated mycobacteria were found not to recognize live ones (27). In the present study, it was demonstrated that specific T lymphocytes directed against immunodominant secreted proteins are acquired preferentially in response to live bacilli. Antigens which reside mainly in the cell wall (Table 1) were, on the contrary, responded to equally well by mice injected with killed bacteria.

Because only live bacilli efficiently generate protection to tuberculosis infection (8, 17, 24), it may be suggested that a T-lymphocyte population involved in protection is the $L3T4^+$ T lymphocyte subpopulation directed against certain antigens released from the metabolizing bacteria. The $L3T4^+$ subpopulation has previously been described as being of importance for protection against *M. tuberculosis* (16, 21, 25) and *Listeria monocytogenes* (10, 18). Furthermore, this population was reported to be solely responsible for protection during the first phase of tuberculosis infection

TABLE 1. Presence of identified and affinity-purified antigens in two *M. tuberculosis* antigen preparations[a]

Antigen preparation	Amount of the following antigens:							
	17 kDa	17–19 kDa	24 kDa	32 kDa	32–33 kDa	38 kDa	56 kDa	65 kDa
SDS extract	+[a]	+++	−	+++	++	+++	−	+++
Short-term filtrate	++	+	+	++	+++	+	+	+

[a] The preparations were SDS-PAGE separated and immunoblotted. Estimates of the relative concentrations are based on the intensity of the reactions. +, Small amounts; ++, intermediate amounts; +++, large amounts; −, not detectable.

(23), a phase characterized by rapid multiplication and, presumably, the release of bacterial metabolites.

Recently, secreted proteins have been described as part of a highly immunogenic protein peptidoglycan complex isolated from the *M. tuberculosis* cell wall (5). This finding and the observations in the present study call attention to the need for a thorough characterization of short-term filtrate and for the separation of components secreted or released during growth of the bacteria from the autolytic products inevitably present in small amounts in the filtrate.

Of approximately 30 major proteins present in short-term filtrate, only a few have been purified and characterized (6, 19, 22).

The 32- to 33-kDa antigen is a well-characterized secreted antigen known to be fibronectin binding and recently shown to be identical to the antigen recognized by the antibody HYT 27 (1). This antigen has previously been shown to elicit delayed-type hypersensitivity in guinea pigs (29) and to initiate production of gamma interferon in human patients (13). This antigen was interestingly found in the present study with infected mice to possess marked T-cell-stimulatory properties. Further investigations to identify other potent T-cell-stimulatory antigens from short-term filtrate are needed.

Proteins secreted by other pathogens have been shown to induce protective immunity (11, 28), and it is possible that the components of a future vaccine against tuberculosis should be sought among the components of short-term filtrate.

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