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Specificity of a Protective Memory Immune Response against *Mycobacterium tuberculosis*

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We have investigated the memory T-cell immune response to *Mycobacterium tuberculosis* infection. C57BL/6J mice infected with *M. tuberculosis* were found to generate long-lived memory immunity which provided a heightened state of acquired resistance to a secondary infection. The T-cell response of memory immune mice was directed to all parts of the bacilli, i.e., both secreted and somatic proteins. Major parts of the memory T-cell repertoire were maintained in a highly responsive state by cross-reactive restimulation with antigens present in the normal microbiological environment of the animals. A resting non-cross-reactive part of the memory repertoire was restimulated early during a secondary infection to expand and produce large amounts of gamma interferon. The molecular target of these T cells was identified as a secreted mycobacterial protein with a molecular mass of 3 to 9 kDa.

The ultimate goal of any rational vaccination procedure is the generation of a long-term protective memory immune response. In animals infected with *Mycobacterium tuberculosis*, it is established that protective memory immunity is mediated by a long-lived population of T lymphocytes (14, 29), presently of unknown specificity. Protective cell-mediated immunity seems to be generated efficiently only when live vaccines are used (22, 28), and it has recently been demonstrated that live *M. tuberculosis* bacteria predominantly provoke T-cell responses to antigens secreted by the bacteria during growth (5). However, these observations in mice at the height of an infection with *M. tuberculosis* may not correlate with the specificity of memory T cells because mycobacterial proteins released during growth change in a sequential way (4), which possibly causes changes in the antigenic repertoire presented to the immune system during the course of infection. Memory T cells present after infection have been found to differ markedly from actively dividing T cells with respect to localization in the body (17), cyclophosphamide sensitivity (27), and interleukin-2 uptake (24). The main purpose, therefore, was to compare these populations and investigate the specificity of the memory T-cell response in vitro.

It is possible that the rapid recognition of infected macrophages by a protective subset of memory T cells is an event of crucial importance in acquired resistance towards a secondary infection. T-cell specificities triggered during the initial phase of a secondary infection may therefore be involved in protective immunity. This rationale provided the basis for the second part of the study, the purpose of which was to investigate changes in T-cell responses as memory immune mice were provoked by a rechallenge infection.

Our study provides evidence that memory cells differ from T cells during primary infection by delayed proliferation, lymphokine production, and a markedly different specificity. Interestingly, only a very restricted part of the memory T-cell repertoire was triggered during a rechallenge infection. This finding suggests that the target for T cells involved in the recall of protective immunity against tuberculosis is secreted mycobacterial proteins with low molecular masses.

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MATERIALS AND METHODS

Animals. Female C57BL/6J mice bred at Statens Serum Institute, Copenhagen, Denmark, were used throughout this study. The mice were 6 to 8 weeks old when the infection studies were initiated.

Bacteria. *M. tuberculosis* H37Rv was grown at 37°C on Löwenstein-Jensen medium or in suspension in modified Sauton medium (12).

Experimental infections. Mice were infected intravenously via the lateral tail vein with an inoculum of 2.5×10^3 bacteria suspended in phosphate-buffered saline (PBS) as described previously (5).

Antibiotic therapy. Thirty days after intravenous infection with *M. tuberculosis*, the memory immune group of mice received isoniazid (Merck) at 100 mg/liter and rifabutin (Farmatalia Carlo Erba) at 100 mg/liter in its drinking water. The treatment was continued for 2 months, after which no viable *M. tuberculosis* bacteria could be recovered from the spleens of the mice, a finding in agreement with previous reports (14, 29).

Bacterial enumeration. The number of viable bacteria in the spleens of infected mice was determined by plating double serial 10-fold dilutions of organ homogenates on Löwenstein-Jensen medium. Colonies were counted after 3 to 4 weeks of incubation, and the data were expressed as the $\log_{10}$ values of the geometric means of counts obtained with five mice.

Mycobacterial antigens. Short-term culture filtrate (ST-CF) was produced as described previously (4). In brief, *M. tuberculosis* bacteria (8×10^6 CFU/ml) were grown in modified Sauton medium without Tween 80 on an orbital shaker for 4 to 7 days. The culture supernatants were sterile filtered and concentrated on an Amicon PM 10 membrane (Amicon, Danvers, Mass.).

Bacteria were killed by incubation overnight in 2% glutaraldehyde. The suspension was washed three times in PBS, and a rough estimate of bacterial numbers based on the optical density of the disperse suspension was obtained.

The recombinant 65-kDa mycobacterial antigen, provided by R. Van der Zee, RIVM, Bilthoven, The Netherlands, was purified by ammonium sulfate precipitation; this was followed by trisacryl ion-exchange chromatography.

Preparative SDS-PAGE. ST-CF was divided into 10 frac-

tions by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (3). Briefly, ST-CF was separated simultaneously in four gels, the gels were cut into fractions, and the protein was eluted overnight with 0.1% (wt/vol) SDS in PBS. Corresponding fractions were pooled and passed through Extractigel columns (Pierce Europe B.V., Oud-Beijerland, The Netherlands) to remove the SDS. After this treatment, no toxicity in cell cultures was present (2). The protein concentration of the fractions was estimated by the Coomassie brilliant blue method (11) with bovine serum albumin as the standard, and all fractions were adjusted and used in the same concentration.

The fractions were analyzed by nonreducing SDS-PAGE (21) by using a 10 to 20% acrylamide gel. The gel was fixed and silver stained (25). On the basis of the intensity of the silver staining (as can be seen in Fig. 4), the concentrations of the fractions were found to vary somewhat apparently because of differences in the dye-binding properties of the different proteins. All fractions were aliquoted and kept frozen at −20°C.

Lymphocyte proliferation assays. Lymphocytes were obtained either by washing the peritoneal cavity with PBS or by preparing single-cell suspensions from spleens. The cells were used in proliferation assays as described previously (5). Briefly, the bacterial antigens were added to microcultures containing 2×10^5 cells in a volume of 200 μl. All tests were carried out in triplicate. The antigens were used in the following concentrations: bacteria, 2×10^6/ml; ST-CF, 4 μg/ml; 65-kDa mycobacterial antigen, 15 μg/ml; and SDS-PAGE fractions, 2 μg/ml. Concanavalin A was used at a concentration of 1 μg/ml as a positive control for cell reactivity.

On the basis of kinetic studies (described in Results), active T cells (obtained 14 days after administration of the primary or secondary infection) were harvested at day 3 while memory T cells (obtained at day 140 of infection) and T cells from unimmunized mice were harvested at day 5 (see Fig. 2). The cultures were pulsed with 1 μCi of [^{3}H]thymidine per well (TRA 120 Radiochemical Centre, Amersham, United Kingdom) 22 h before harvest, and proliferation was quantified by conventional liquid scintillation counting. The proliferative responses are expressed as the geometric means of 5 to 10 individual animals.

Depletion of T lymphocytes and inhibition of antigen processing. Spleen lymphocytes were depleted of T cells by treatment with the monoclonal antibody anti-thy 1.2 (Tib 107) and Low Tox-M rabbit complement (Accurate Chemical & Scientific Corp., Westbury, N.Y.). Spleen cells (2×10^7/ml) were incubated in a mixture of hybridoma supernatant and complement (4:1, supernatant to complement) for 1 h at 37°C. The cell suspension was subsequently washed three times in medium.

To inhibit antigen processing and presentation, spleen cells were pretreated with 5 mM NH_4Cl (Sigma) (43) for 2 h at 37°C, after which antigen or mitogen was added and the cultures were incubated in the presence of 5 mM NH_4Cl. NH_4Cl was used at a concentration which did not inhibit mitogen-driven cellular proliferation.

IFN-γ ELISA. Supernatants were harvested from the active lymphocyte cultures at day 2 and from the memory T-cell cultures at day 4. The amount of gamma interferon (IFN-γ) present was quantitated by an IFN-γ enzyme-linked immunosorbent assay kit (Holland Biotechnology, Leiden, The Netherlands). Values below 0.5 U/ml were considered negative. All tests were carried out in duplicate.

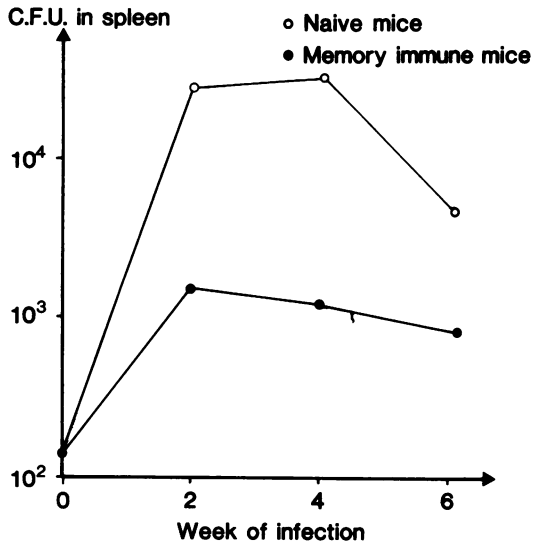

FIG. 1. Course of infection with *M. tuberculosis* in naive and memory immune mice. C57BL/6J mice were infected with 2.5×10^3 viable units of *M. tuberculosis*, and the growth of the organisms in the spleens was investigated for a period of 6 weeks. The counts of CFU indicated are geometric means, and the 95% confidence limits are 67 to 148%.

Statistical methods. With the exception of the results shown in Table 3, all results are the geometric means of results from 5 to 10 individual animals.

The statistical analysis of these data was performed on logarithmically transformed counts per minute and IFN-γ units. The 95% confidence limits have been expressed as percentages of the means and were calculated on the basis of the standard deviations pooled for each experiment, using a *t* value of 2.

RESULTS

Growth of *M. tuberculosis* in naive and memory immune mice. C57BL/6J mice were infected with *M. tuberculosis* for 30 days, after which the infection was cleared by antibiotic treatment for 60 days as described elsewhere (29). The memory immune mice obtained were given a rechallenge at day 140, and the course of infection was compared with that of a group of infected naive mice (Fig. 1). In agreement with previous findings (5), the bacteria grew rapidly in the spleen of naive animals for 4 weeks, after which the organisms were gradually eliminated. In contrast, only limited bacterial growth was observed in the memory immune mice, and the number of bacteria in the spleens of these mice was approximately 20 times less than that in the naive mice.

Differences in the active T-cell response and the memory T-cell response. Mice were infected with 2.5×10^3 *M. tuberculosis* bacteria, and the spleen lymphocytes were harvested after 14 days (active T lymphocytes). During this period, a powerful cellular immune response is generated (5). Memory T lymphocytes were harvested at day 140, 50 days after the termination of the antibiotic treatment. The spleen lymphocytes were stimulated with ST-CF in cultures for various periods of time. The cellular proliferation was investigated, and the supernatants were harvested to quantify the amount of IFN-γ present. Active lymphocytes were found to proliferate rapidly, and the maximal in vitro prolif-

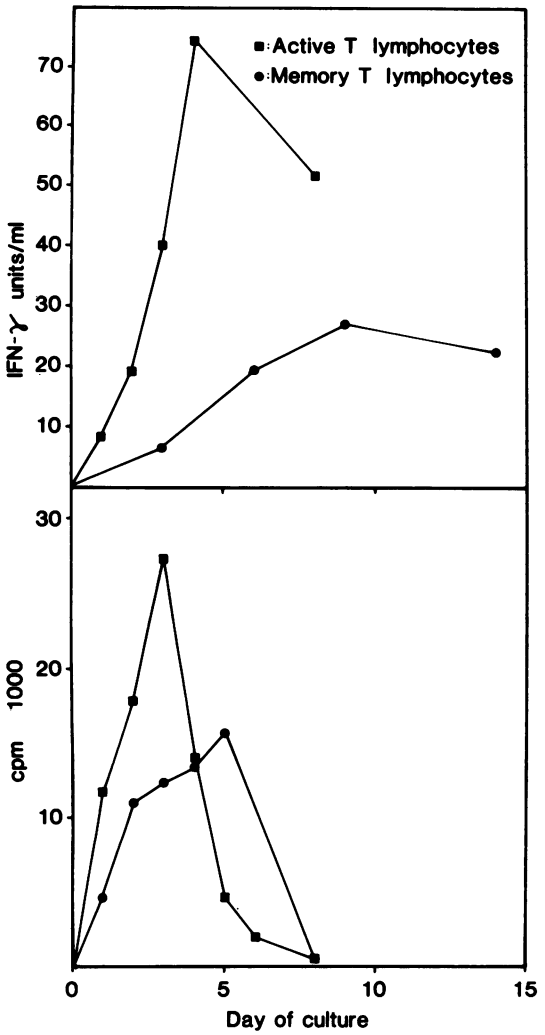

FIG. 2. Kinetics of T-cell responses. IFN-γ production and proliferation of spleen lymphocytes stimulated with ST-CF for various periods of time. Active T lymphocytes were isolated from mice infected with *M. tuberculosis* for 14 days, while memory T lymphocytes were isolated at day 140. The responses depicted are geometric means of results from five individual animals. The proliferation of unstimulated cultures was between 1,000 and 2,000 cpm. No IFN-γ production was found in unstimulated cultures.

eration was found at day 3 of culture (Fig. 2). Similarly, the concentration of IFN-γ was found to increase rapidly, reaching a maximum concentration at day 4.

In contrast, the kinetics of proliferation and IFN-γ release exhibited by memory lymphocytes were found to be slower, and only moderate response levels were reached. Thus, maximal proliferation was reached at day 5, while the IFN-γ concentration peaked at day 9. No difference in the general responsiveness of the two populations existed, as the proliferation of both populations induced by concanavalin A peaked simultaneously at day 2 (results not shown). This experiment was repeated three times with the same overall result.

Memory T cells have previously been described to reside predominantly in the extravascular tissues of the immune host (17). To determine the localization of the responsive cell population generated after infection with *M. tuberculosis*,

TABLE 1. Localization of cells responding to ST-CF

Mouse group[a]	Lymphocyte source	Stimulation index[b]
Active	Spleen	29.2 (22.27)
	Peritoneal cavity	3.5 (3.65)
Memory	Spleen	12.6 (7.89)
	Peritoneal cavity	1 (2.38)

[a] Mice were killed at either day 14 (active) or day 140 (memory), and lymphocytes were used in proliferation assays.

[b] Values are mean counts per minute with antigen divided by mean counts per minute without antigen. The corresponding counts per minute (10^3) are shown in parentheses. The 95% confidence limits are 50 to 201% of the index. All cultures were found to proliferate in response to concanavalin A.

we investigated the proliferative responses to ST-CF of lymphocytes obtained from either a vascular location (spleen) or an extravascular location (peritoneal cavity). Active lymphocytes responding to ST-CF were found in both locations, although the strong response of lymphocytes isolated from the spleen dominated (stimulation index, 29.2) (Table 1). In memory immune mice, however, responses were not detectable in the peritoneal cavity, and spleen lymphocytes were therefore chosen for the subsequent analysis of the memory T-cell repertoire.

Change of T-cell responses during the course of infection. Having established the localization and in vitro culture kinetics of T-cell responses in memory immune mice, we then investigated the specificity of these cells. ST-CF, killed bacilli, and the 65-kDa antigen represent three different classes of antigens. The 65-kDa antigen is believed to be located in the cytoplasm of *M. tuberculosis*, while ST-CF contains primarily proteins secreted to the surroundings by the actively metabolizing bacilli (1, 4). Killed bacilli contain large amounts of cell-wall-bound antigens, while secreted antigens are represented in only low quantities in this preparation (5, 40). Responses to these preparations were used to monitor major shifts in the T-cell responses at different stages of the infection.

ST-CF and the 65-kDa antigen both initiated a limited proliferation of lymphocytes isolated from naive mice (Fig. 3), and this marginal response was not stimulated further as the unimmunized mice became older (2). The T-cell responses at the height of infection were, in agreement with previous findings (5), dominated by the very strong response to ST-CF (24,121 cpm). A moderate response to killed bacilli was found (8,272 cpm), while the 65-kDa antigen was found to be very weakly stimulatory in these mice. In sharp contrast, memory lymphocytes responded equally well to all three antigen preparations, yielding proliferative responses of 6,000 to 9,000 cpm.

T-cell responses during rechallenge of memory immune mice. Memory immune mice were characterized by a broadly responding T-cell repertoire responding equally well to all classes of mycobacterial antigens. However, there is increasing interest in the possibility that protective T cells should be sought among the specificities which recognize the infected macrophage early in infection, thereby ensuring a rapid onset of bacteriostatic effector mechanisms. Thus, our next experiments were designed to compare the T-cell responses of memory immune mice with responses obtained 14 days after the mice had received a rechallenge infection with *M. tuberculosis*. As found in the previous experiment (Fig. 3), all preparations elicited a cellular response in memory immune mice, although the response towards the

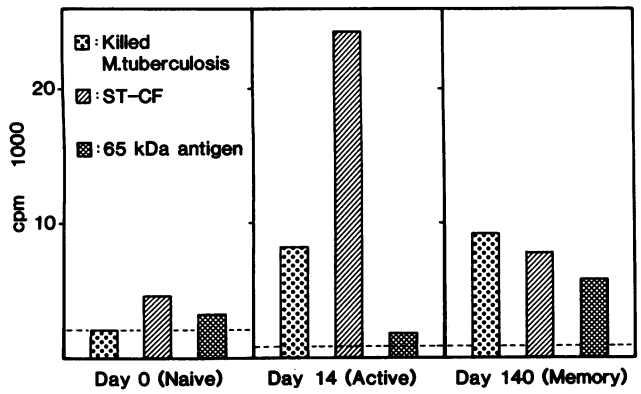

FIG. 3. Shifts in T-cell responses during the course of infection. The proliferative responses of spleen lymphocytes isolated from naive mice, mice infected with *M. tuberculosis* for 14 days, or memory immune mice are shown. The lymphocytes were stimulated with different mycobacterial preparations. The response of unstimulated cultures is indicated by the dotted line. The response depicted for naive mice (day 0) is also representative of mice left unimmunized and tested at day 140. The responses depicted are geometric means, and the 95% confidence limits are 75 to 133%.

65-kDa antigen was found to dominate somewhat in this experiment (Table 2).

A rechallenge infection markedly changed this T-cell response into a response exclusively to ST-CF antigens. The proliferative response to ST-CF was boosted more than four times, while the response to bacteria was unchanged and the response to 65-kDa antigen decreased. IFN-γ production by Th1 cells represents an important effector function in tuberculosis because this cytokine activates macrophages to inhibit mycobacterial growth (13, 32). Memory cells were found to produce only negligible amounts of IFN-γ, whereas a secondary infection resulted in a pronounced activation of IFN-γ-producing T cells directed towards secreted mycobacterial antigens present in ST-CF (Table 2).

Molecular targets of the T cells reactivated during a secondary infection. To further characterize the antigens responsible for reactivation of T cells during a secondary infection, ST-CF was divided into 10 fractions by preparative SDS-PAGE (Fig. 4). Each fraction was used to stimulate lymphocytes from memory immune or rechallenged mice, and the proliferation and release of IFN-γ were investigated. Cells from memory immune mice proliferated and produced moderate amounts of IFN-γ (5 to 10 U/ml) when stimulated with molecules in the molecular mass region of 9 to 14 (fraction 2) and 25 to 33 (fractions 5 and 6) kDa (Fig. 5).

TABLE 2. The cellular response of memory immune or memory rechallenge mice

Antigen	Response of memory immune mice		Response of memory rechallenge mice[a]		Relative size of rechallenge response[b]	
	Prolif.[c]	IFN-γ[d]	Prolif.[c]	IFN-γ[d]	Prolif.[c]	IFN-γ[d]
Medium alone	1.06	0	1.09	0	1.0	
Killed bacteria	3.20	0.9	3.63	0.6	1.1	0.7
ST-CF	3.18	0.3	13.43	24.5	4.2	81.7
65-kDa antigen	6.07	1.1	3.89	0.2	0.6	0.2

[a] Lymphocytes were isolated 14 days after the memory immune mice had received an intravenous challenge of *M. tuberculosis*.

[b] The relative size of the response induced by rechallenge was calculated by using the following formula: memory rechallenge response/memory immune response.

[c] Proliferation is expressed as counts per minute (10^3). The 95% confidence limits are 73 to 137% of the means.

[d] IFN-γ production is expressed in units per milliliter of lymphocyte culture supernatant. The 95% confidence limits are 50 to 202% of the means.

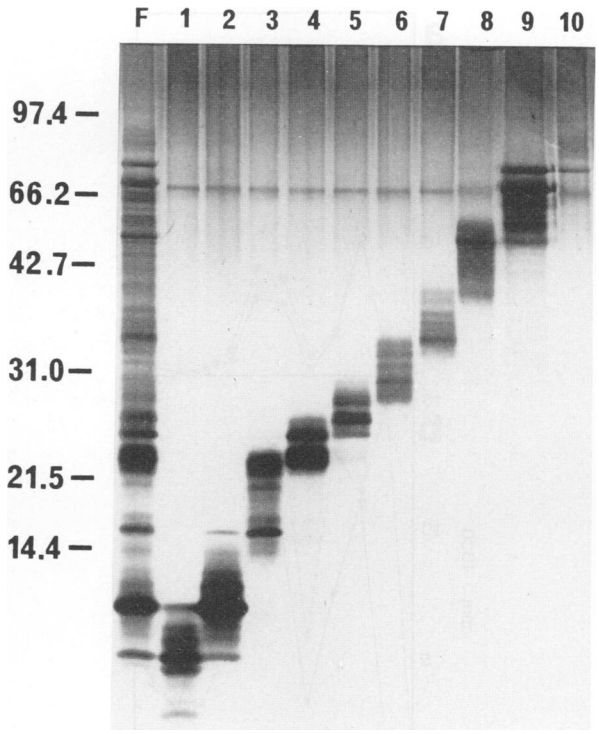

FIG. 4. Preparative SDS-PAGE fractionation of ST-CF. ST-CF was divided into 10 fractions by preparative SDS-PAGE. The products obtained were adjusted to the same protein concentration and analyzed in SDS-PAGE, and the gel was silver stained. Lanes: F, ST-CF; 1 to 10, fractions 1 to 10.

When memory immune mice received a secondary infection, major parts of the response pattern were unchanged, but, interestingly, a highly significant boosting of the cellular response towards the low-molecular-mass region of 3 to 9 kDa (fraction 1) was found (Fig. 5). Antigens within this narrow region were exceedingly potent inducers of IFN-γ and solely responsible for the majority of IFN-γ provoked by *M. tuberculosis* during a rechallenge.

Are the memory T-cell responses to *M. tuberculosis* maintained by cross-reactive stimulation? Memory immune mice exhibited a distinct T-cell repertoire in which a number of the specificities stimulated during the course of infection were maintained in a highly responsive state, as judged by strong T-cell proliferative responses and a substantial release of IFN-γ. To test whether these T cells are driven by cross-reactive epitopes present on other immunogens, we investigated the response of unimmunized mice to ST-CF fractions. These mice were kept in similar animal facilities and tested at the same age as the memory immune mice were, and they were found to exhibit a response pattern that had a striking similarity to the pattern of memory immune mice (Fig. 6), i.e., fractions to which memory immune mice responded strongly all initiated a significant proliferative response in lymphocyte cultures from unimmunized mice. However, the responses of unimmunized mice were at a

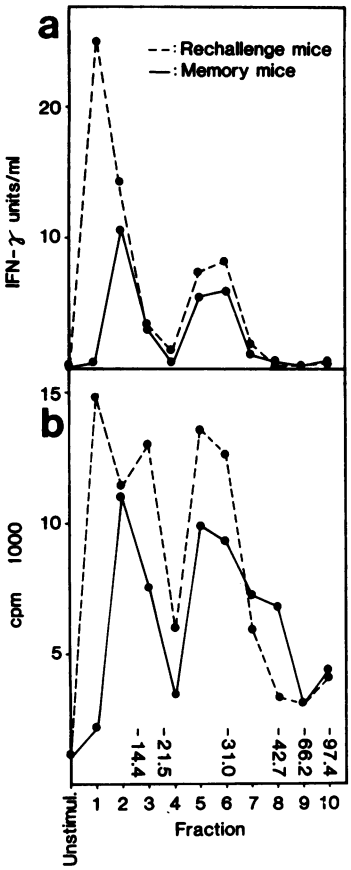

FIG. 5. T-cell reactivation during a secondary infection. IFN-γ production by (a) and proliferation of (b) spleen lymphocytes isolated either directly from memory immune mice or 14 days after the mice had received a rechallenge infection with *M. tuberculosis* are shown. The lymphocytes were stimulated with ST-CF fractions in cultures. All responses depicted are geometric means, and the 95% confidence limits are 50 to 202% (IFN-γ units) and 73 to 137% (counts per minute). The migration of molecular mass markers (as shown in Fig. 4) are indicated in the bottom of panel b.

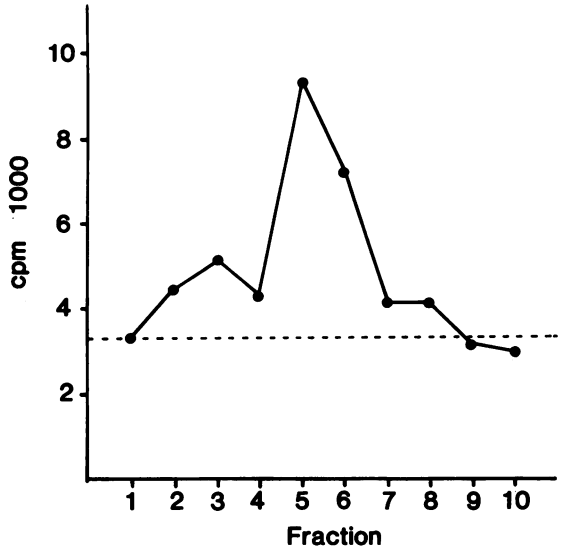

FIG. 6. Response of unimmunized mice to ST-CF fractions. Proliferative responses obtained with spleen lymphocytes isolated from 5-month-old unimmunized mice. The lymphocytes were stimulated with ST-CF fractions. The responses depicted are geometric means of results from 10 individuals. Responses above the dotted line are significantly higher than the proliferation found in unstimulated cultures (2,120 cpm).

lower level, and no IFN-γ was released in these cultures (results not shown).

To compare a specific T-cell proliferative response with a response to mitogens or superantigens, T-cell depletion and the lysomotrophic agent NH_4Cl were used. The depletion of T cells by complement-mediated cytotoxicity was found to reduce the proliferative responses to fractions 1 to 4 and 7 to 10 to the levels of the unstimulated culture responses, a result which emphasizes that T cells are solely responsible for the proliferation observed (Table 3). Fractions 5 and 6, in contrast, provoked a significant proliferation in the T-cell-depleted cultures, suggesting that B cells participated in the marked proliferative responses provoked by molecules with molecular masses of 25 to 33 kDa. The proliferative responses were further characterized by the use of NH_4Cl, which inhibits antigen processing and presentation (43). This agent was present throughout the culture period and resulted in a reduction of proliferation to all fractions and an increase in response to concanavalin A, a mitogen which does not require processing (Table 3).

These experiments were repeated three times with the same overall results.

DISCUSSION

Proteins secreted by growing mycobacteria are major T-cell antigens in the host during live infection. This has been demonstrated by data from this laboratory comparing T-cell responses to a variety of mycobacterial antigens in mice inoculated with either live or killed *M. tuberculosis* (5). Similarly, Orme et al. (30) recently reported a predominant recognition of bacterial filtrate proteins by murine T lymphocytes during the early course of infection. Of importance, however, is the demonstration by the present study that memory cells which reside in the animal after the termination of a primary infection respond to all classes of mycobacterial antigens, including constitutive cell wall and stress

TABLE 3. Characterization of the proliferative response of nonimmune cells towards ST-CF fractions

Antigen[a]	Proliferation (cpm, 10^3)		
	Spleen cells[b]	T cells depleted[c]	NH_4Cl incubation[d]
Unstimulated	2.42 (0.09)[e]	0.56 (0.14)	0.67 (0.27)
ConA	7.25 (0.77)	1.10 (0.47)	13.01 (7.13)
F1	3.00 (0.92)	0.09 (0.04)	0.48 (0.26)
F2	4.56 (0.79)	0.80 (0.37)	1.30 (0.19)
F3	5.71 (0.24)	0.90 (0.31)	1.17 (0.16)
F4	4.35 (0.61)	0.47 (0.05)	0.33 (0.21)
F5	8.61 (0.32)	5.41 (1.26)	2.31 (0.60)
F6	7.19 (0.54)	2.20 (1.01)	1.18 (0.20)
F7	4.07 (0.72)	0.44 (0.08)	0.52 (0.09)
F8	4.30 (0.45)	0.93 (0.52)	0.61 (0.05)
F9	3.18 (0.37)	1.05 (0.62)	1.06 (0.57)
F10	3.25 (0.26)	0.55 (0.22)	0.39 (0.04)

[a] F1 to F10 are ST-CF fractions.
[b] Spleen cells were obtained from 5-month-old unimmunized mice.
[c] T cells were depleted by complement-mediated cytotoxicity.
[d] NH_4Cl was present throughout the culture period.
[e] Values in parentheses represent standard deviations of triplicate wells.

proteins. These data suggest a shift in the repertoire of mycobacterial antigens during the course of infection, leading to a gradual sensitization of T cells to a wide spectrum of antigens.

Memory cells were demonstrated in the present study to have delayed proliferation and lymphokine production, results which support earlier findings that memory cells exist in a resting state, insensitive to the effects of cyclophosphamide (14, 29) and expressing only low amounts of interleukin-2 R (24). Memory T cells have been described as long-lived resting progeny of T cells stimulated during the primary response (29). However, since memory cells express low but definite amounts of several activation molecules (interleukin-2 R, intracellular adhesion molecule 1, major histocompatibility complex class II), it has recently been hypothesized that long-term memory immunity is maintained by frequent cross-reactive restimulation, leading to a continuously slow rate of cell division in the memory pool (10).

In the present study, evidence is provided to support both hypotheses. It is demonstrated that some antigenic fractions found to be dominating targets of memory immune T cells are also responded to by T cells isolated from mice never exposed to *M. tuberculosis*. This response is not mitogenic since it requires antigen processing and is presumably, therefore, directed against cross-reactive epitopes shared by *M. tuberculosis* and the normal microbiological environment of the animal. In addition, a markedly stimulatory ST-CF fraction with a molecular mass of 25 to 30 kDa was found to stimulate the proliferation of T-cell-depleted cultures. This finding might indicate the presence of B-cell-stimulatory compounds in ST-CF, in agreement with the previously reported existence of B-cell mitogens among mycobacterial products (36). T cells from naive animals have previously been demonstrated to respond to mycobacterial antigens (5, 6), and, recently, memory T cells directed to *Plasmodium falciparum* were detected in human donors who had never been exposed to malaria (16). Together, these findings imply the existence of several common epitopes in addition to the previously demonstrated conserved group of heat shock proteins (33).

However, although restimulation with cross-reactive antigens maintains part of the memory T-cell repertoire in a highly responsive state, our study provides data which indicate that other parts of the repertoire reside as resting, long-lived memory T cells. We have recently demonstrated that dominating T-cell responses at the height of infection are directed towards secreted mycobacterial proteins with molecular masses of 4 to 11 and 26 to 35 kDa (3). However, as no T-cell response towards the low-mass region below 10 kDa was found in memory immune mice, we suggest that these specificities reside in a resting state undetectable during conventional in vitro culturing. The present study demonstrates that these T cells are triggered by a reencounter with the pathogen, an event which causes an accelerated reemergence of strong reactivity and lymphokine production. The triggering of a very restricted part of the T-cell repertoire during a secondary infection contrasts with the multiple T-cell targets found during a primary infection (3), and the factors controlling this modulation of the T-cell repertoire are the subject of work presently in progress in our laboratory.

Both mycobacterial cell walls (8, 26) and heat shock proteins (18, 31, 37, 39) have been reported to contain immunodominant T-cell epitopes. Several studies have therefore focused attention on the purification and characterization of these components (15, 33), and both the cell wall (8) and the 65-kDa antigen (18) have been suggested as potential candidates for an improved antituberculosis vaccine. In the present study, memory immune animals responded to a variety of mycobacterial antigens, including killed bacilli and the 65-kDa heat shock protein. However, of importance was the finding that T cells directed towards these somatic antigens were not reactivated when the mice mounted a protective cellular immune response. In view of this, it is reasonable to hypothesize that these T cells are not involved in protective immune reactions but possibly mediate delayed-type hypersensitivity reactions if triggered by the appropriate antigen. T cells directed to ST-CF, in contrast, were rapidly reactivated, a finding which indicates that the infected macrophages are recognized early during the secondary infection by T cells directed to antigens secreted by the live, metabolizing bacteria. Our hypothesis is supported by the finding that protective T cells are efficiently generated only in response to live (22, 28) and dividing mycobacteria (35) and especially by the finding that the protective memory T-cell population is triggered only when the live organism is inoculated (29). Similar results have been obtained in investigations of the protective efficacy of *Listeria monocytogenes* (19, 38, 41), indicating that the present observation may represent a general feature of intracellular infections.

IFN-γ is one of the most important lymphokines in the activation of the macrophage and efficiently prevents the multiplication of phagocytosed mycobacteria (9, 13, 32, 34). The results of the present study provide the surprising finding that a secondary infection with *M. tuberculosis* triggers significant IFN-γ production only by the narrow part of the T-cell repertoire directed against secreted mycobacterial proteins with molecular masses of 3 to 9 kDa. Some of these low-molecular-mass polypeptides may represent partially degraded proteins, but fraction 1 contains, in addition, unique immunodominant antigens to which a specific response can be generated by immunization with the purified fraction (2).

Only a few low-molecular-mass mycobacterial proteins have been purified and characterized to date (7, 20, 42). However, both human leprosy and tuberculosis patients have been demonstrated to possess pronounced T-cell reactivity against antigens with molecular masses of 7 to 10 kDa (8, 23), and, in *M. tuberculosis*, these antigens were demonstrated in both cell walls and ST-CF (8). Together, these findings emphasize the need for further purification and characterization of secreted mycobacterial proteins and especially of the poorly defined low-molecular-mass components.

The findings presented in this article stimulate two lines of research: (i) propagation of mycobacterium-reactive T-cell lines obtained from mice during a secondary infection and study of their protective capacity by adoptive transfer experiments, and (ii) evaluation of the protective efficacy of ST-CF fractions in regular vaccination studies. These studies may elucidate the relationship between results obtained in vitro and the actual protection observed in vivo, a correlation of utmost importance in future work on a novel vaccine against tuberculosis.

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