Activation of CD8 T Cells with Specificity for Mycobacterial Heat Shock Protein 60 in Mycobacterium bovis Bacillus Calmette-Guérin-Vaccinated Mice

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Received 9 April 1997/Returned for modification 12 May 1997/Accepted 12 June 1997

Heat shock protein 60 (hsp60)-specific CD8 T cells lysed Mycobacterium bovis BCG-infected macrophages in vitro and adoptively transferred protection against mycobacterial infection. Moreover, CD8 T cells with this hsp60 specificity were activated in vivo by BCG vaccination. Our studies suggest there is participation of hsp60-specific CD8 T cells in BCG-induced immunity.

Heat shock proteins (hsps) are frequent antigens for antiinfectious T- and B-cell responses (6). Their ubiquitous distribution and high levels of conservation suggest that the focus of the immune system on these antigens promotes rapid and efficient identification of infection in the host (6). Several lines of evidence indicate a role of hsp-specific immunity not only in defense against pathogens, but also in autoimmunity. Notably, vaccination with naked DNA encoding mycobacterial hsp60 induced significant protection against tuberculosis (20). Recently, we have generated CD8 cytolytic T lymphocytes (CTLs) with specificity for hsp60 by in vitro stimulation with mycobacterial hsp60 peptides or by in vivo immunization with hsp60 in the adjuvant, immunostimulating complex (8, 16, 22). Cloned CD8 T cells are specific for an H-2Dd-restricted mycobacterial hsp60 peptide and cross-react with a peptide of the murine hsp60. Accordingly, these CTLs respond to stressed host cells and so are autoreactive. Although these experiments prove the existence of hsp60-cross-reactive CD8 CTLs, the question remains whether such cells are stimulated during infection with mycobacteria and thus participate in the antimycobacterial immune response.

Recent evidence suggests that the intracellular localization of bacterial pathogens has a critical influence on the type of T-cell response evoked. Antigens from cytosolic bacteria such as Listeria monocytogenes, which escape from the phagosome into the cytosol, are primarily presented through the major histocompatibility complex (MHC) class I processing pathway, leading to activation of CD8 T cells (1). In contrast, antigens from endosomal bacteria, such as Mycobacterium bovis BCG, which persist in the phagosome, preferentially stimulate CD4 T cells because the peptides are processed and presented by the MHC class II pathway (12, 13). Recent data, however, suggest that the division of these two antigen delivery pathways is less strict. These studies favor an alternative pathway capable of translocating antigens from pathogenic microbes which stay in the endosome (14). Hence, such endosomal antigens can also be loaded onto MHC class I molecules and, as a consequence, stimulate CD8 T cells (9, 19). The experiments described herein demonstrate activation of hsp60-specific CD8 T cells after M. bovis BCG vaccination and provide evidence for their participation in the BCG vaccine-induced immune response.

The generation, propagation, H-2Dd restriction, fine specificity for hsp60499–508 peptide, and autoreactivity of the CD8 CTL clone have been described previously (16, 18, 22). For determination of cytolytic activities, bone marrow-derived macrophages (BMMφ) were generated from femoral bone marrow cells as described elsewhere (3). These BMMφ were infected overnight at a multiplicity of infection (MOI) of 10:1 with BCG strain Chicago (ATCC 27289) or with L. monocytogenes EGD organisms. Infected cells were used as targets in a standard 51Cr release assay as described previously (19). To determine the in vivo activity of the hsp60-specific CD8 CTLs, cloned T cells were harvested 4 days after restimulation and washed with phosphate-buffered saline (PBS). C57BL/6 mice received 2 × 10⁶ CTLs in 0.2 ml of intravenous (i.v.) PBS. Thirty minutes later, the mice were infected with 3.5 × 10⁶

FIG. 1. Lysis of M. bovis BCG-infected BMMφ by cloned CD8 CTLs with specificity for hsp60499–508. BMMφ (10⁷) were infected at an MOI of 10:1 with M. bovis BCG (●) or L. monocytogenes (▲) or were left untreated (▲) and subsequently used as targets for hsp60499–508-specific CD8 CTLs in a standard ⁵¹Cr release assay. One group of uninfected target cells was pulsed with 1 μg of hsp60499–508 (■). E:T-ratio, effector-to-target cell ratio.
BCG organisms in 0.2 ml of i.v. PBS. On days 7 and 15 postinfection (p.i.), 2 × 10^6 CTLs were transferred i.v. Spleens and livers were harvested at 30 or 35 days p.i. and homogenized with a laboratory blender (Seward Medical, Oxford, United Kingdom). Aliquots of organ homogenates were plated on Middlebrook agar (Difco, Detroit, Mich.), and CFU were determined after incubation at 37°C for 3 to 4 weeks. Data are expressed as mean CFU per organ ± standard deviation of four or five mice per group. In order to generate hsp60-specific CD8 CTLs by BCG vaccination, C57BL/6 mice were infected with 5 × 10^6 viable M. bovis BCG organisms, and 2 weeks p.i., spleens were removed. Alternatively, CD8 T cells were generated in vivo by immunization with denatured mycobacterial hsp60 in the absence of adjuvant. One hundred micrograms of hsp60 was incubated with or without 1% (wt/vol) sodium dodecyl sulfate (SDS) and 2% (vol/vol) 2-mercaptoethanol (Gibco, Paisley, United Kingdom) in 1 ml of PBS. Nylon wool-enriched T cells (2 × 10^6 per ml) from infected or uninfected mice were cultured in the presence of irradiated (3,000 rads) syngeneic spleen cells at 2 × 10^6 per ml in RPMI 1640 medium supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany). T cells were restimulated weekly with 5% interleukin 2-containing concanavalin A (Sigma, St. Louis, Mo.), rat spleen cell supernatant (7), and irradiated (20,000 rads) peptide-pulsed RMA-S cells (H-2b; 10^4 per ml). The RMA-S cells have a defective peptide transport system for MHC class I processing and therefore fail to present peptides from endogenous proteins (17). However, they effectively present exogenously added peptides in the context of MHC class I (21). RMA-S cells (10^6 per ml) were incubated overnight with 50 mg of hsp60 499–508 per ml at 26°C, followed by irradiation and washing, and were used as stimulator cells as described above. We have found previously that this in vitro stimulation does not result in the activation of hsp60-specific CD8 CTLs with spleen cells from naive mice (22). Phenotypic characterization by microfluorimetry after 3 weeks of culture revealed that >95% of the T cells expressed the T-cell receptor αβ CD3+ CD44+ CD8+ phenotype (results not shown).

The data depicted in Fig. 1 demonstrate lysis of M. bovis BCG-infected target cells by the H-2D^b-restricted hsp60 499–508-specific CD8 CTL clone. Because stressed host cells are recognized by this T-cell clone (18, 22), the relevant epitope could have been derived from mycobacterial or from self hsps. Therefore, BMMφs were infected with M. bovis BCG or with L. monocytogenes and used as target cells. Only M. bovis BCG-

![Figure 2](https://example.com/figure2.png)

**FIG. 2.** In vivo activation of hsp60 499–508-specific CD8 CTLs by BCG vaccination. Spleen cells from mice infected with M. bovis BCG (A) or from uninfected mice (B) were tested 3 weeks after in vitro restimulation with a standard ^51Cr release assay. BMMφs were used as target cells either untreated ( ), pulsed with 5 μg of tryptic digest of mycobacterial hsp60 per ml ( ■), or pulsed with 1 μg of hsp60 499–508 per ml ( ●). E:T-ratio, effector-to-target cell ratio.

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**TABLE 1.** Adoptive transfer of antimycobacterial immunity by cloned CD8 CTLs with specificity for hsp60 499–508

<table>
<thead>
<tr>
<th>Organ</th>
<th>Control mice</th>
<th>T-cell-recipient mice</th>
<th>Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Log_{10} M. bovis BCG organisms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expt 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>4.68 ± 0.30</td>
<td>3.97 ± 0.20</td>
<td>0.71</td>
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<tr>
<td>Liver</td>
<td>4.95 ± 0.08</td>
<td>4.32 ± 0.13</td>
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</tr>
<tr>
<td>Expt 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>4.68 ± 0.26</td>
<td>3.88 ± 0.09</td>
<td>0.80</td>
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<tr>
<td>Liver</td>
<td>4.98 ± 0.05</td>
<td>4.44 ± 0.28</td>
<td>0.54</td>
</tr>
</tbody>
</table>

a T-cell recipients and control mice were infected with 3.5 × 10^6 M. bovis organisms, and their spleens and livers were harvested after 30 days (experiment 1) or 35 days (experiment 2).

b Data are expressed as mean log_{10} CFU per organ ± standard deviation of four or five mice per group.

c Significant protection in spleens and livers of T-cell-recipient mice versus that in controls at P < 0.01 (Student's t-test).
infected, not *L. monocytogenes*-infected, BMMφ were recognized, suggesting that the peptides originated from *M. bovis* BCG. Next, we wondered whether the hsp60_{499–508}-specific CD8 CTLs contributed to the immune response to mycobacterial infection. A small, although definite, amount of protection was adoptively transferred by cloned T cells (Table 1), suggesting participation of hsp60-specific CD8 T cells in protective immunity to BCG. For control purposes, another CD8 T-cell clone with distinct specificity for hsp60 was used in adoptive transfer experiments (data not shown). Although both T-cell clones were derived from mice which had been vaccinated with mycobacterial hsp60 in immunostimulating complex, they showed differential hsp60 fine specificity. The second T-cell clone expresses exclusive specificity for mycobacterial hsp60, with no cross-reactivity for peptides from self hsp60 or for stressed macrophages (22). This T-cell clone failed to transfer protective activity against *M. bovis* under similar conditions, as shown in Table 1. We therefore assume that the epitopes recognized by this nonprotective CD8 T-cell clone were not processed or presented by infected host cells. At the same time, failure to transfer protection with this T-cell clone supports the notion that specific protective activity was conferred by the hsp60_{499–508}-specific CD8 CTL clone. This latter T-cell clone secreted only minute concentrations of gamma interferon (IFN-γ), and only by PCR could IFN-γ mRNA be detected after peptide stimulation (data not shown). In contrast, target cells labeled with picomolar concentrations of hsp60_{499–508} were lysed efficiently by this CTL clone in vitro (22). Although we do not have any data about IFN-γ release or target cell lysis by this T-cell clone in the in vivo setting, these in vitro data could mean that lysis rather than IFN-γ release contributed to the protective response. Next, we wondered whether hsp60-specific CD8 T cells are indeed induced in vivo after BCG vaccination. As shown in Fig. 2, CD8 CTLs derived from *M. bovis* BCG-infected mice were capable of lysing target cells pulsed with the hsp60_{499–508} peptide. In contrast, CD8 T cells from uninfected animals failed to show any hsp60_{499–508}-specific target cell lysis (Fig. 2B). The relevant peptide, hsp60_{499–508}, could be titrated down to a concentration of 1 to 10 nmol, suggesting that it encompassed the relevant epitope (results not shown). These findings strongly suggest that hsp60 from *M. bovis* BCG has access to the MHC class I processing pathway in vivo, thus activating hsp60-specific CD8 CTLs. Recent experiments have demonstrated that protein denaturation provides a convenient method for CD8 T-cell stimulation by pure proteins in the absence of adjuvant (15). To verify whether hsp60-specific CD8 CTLs can be induced by this vaccination protocol, mice were immunized with SDS-denatured mycobacterial hsp60, and then CD8 CTL lines were established. The data depicted in Fig. 3A show that hsp60-specific CD8 T cells from mice immunized with denatured hsp60, but not those from mice immunized with native hsp60, expressed hsp60-specific CTL activity. Hence, hsp60-specific CD8 CTLs were induced by vaccination with adjuvant-free denatured protein.

Several lines of evidence emphasize a central role of CD8 T cells in protective immunity against tuberculosis (2, 4, 5, 11). However, immunization with BCG is considered to preferentially stimulate CD4 T cells (12, 13). This discrepancy between dependency on and activation of CD8 T cells has been implicated in the failure of various vaccination strategies to induce satisfactory protection against tuberculosis (4). However, the data presented here reveal that hsp60-specific CD8 CTLs are induced by BCG and by denatured protein. Thus, endosomal antigens from *M. bovis* BCG also apparently have access to the MHC class I processing pathway. Our observations are in line with recent findings from our laboratory, which demonstrate an active role of both CD4 and CD8 T cells in immunity to *M. bovis* BCG (10). Vaccination with naked DNA comprising the mycobacterial hsp60 gene not only stimulates hsp60-specific CD8 T cells but also provides protection against tuberculosis (20). Our experiment in which denatured hsp60 also stimulated CD8 CTLs suggests that this immunization schedule should be further exploited for vaccination against mycobacterial infection. At the same time, our findings demonstrate that vaccination with BCG or mycobacterial hsp60 stimulates CTLs with specificity for peptide hsp60_{499–508}, which is known to cross-react with a self epitope. Hence, our data also underline the possibility that autoimmunity may be induced by vaccines of this type.

Financial support from Sonderforschungsbereich 322 is gratefully acknowledged.

We thank R. Mahmoudi for secretarial help.
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Editor: R. E. McCallum