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Functional Maturation of an Antiviral Cytotoxic T-Cell Response

MARTIN F. BACHMANN,* DANIEL E. SPEISER, AND PAMELA S. OHASHI

Departments of Medical Biophysics and Immunology, Ontario Cancer Institute, Toronto, Ontario M5G 2M9, Canada

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Lymphocytic choriomeningitis virus (LCMV) is known to induce strong, polyclonal cytotoxic T-lymphocyte (CTL) responses. Using a set of variant peptides derived from the major CTL epitope of LCMV, we analyzed the functional fine specificity of the LCMV-specific CTL response. During the primary response, almost all the tested peptides were recognized. In contrast, the secondary response was purged of all minor cross-reactivities and very few peptides were significantly recognized. This study is the first demonstration of the functional maturation of a T-cell response and has important clinical and biological implications.

T-cell responses are characterized by rapid increases in the numbers of specific lymphocytes. After the peak of the primary response, most activated T cells die. The surviving T cells persist at elevated frequencies for a long time and constitute an important component of T-cell memory (11, 15, 38). In contrast to B-cell responses, in which selection of specific B cells and generation of memory B cells occurs in germinal centers (3, 18, 33), no specialized structure has been identified for T-cell responses. In addition, although specialization of the T-cell repertoire of the primary immune response has been described in many instances (7, 12, 16, 19, 34, 35), few details are known about the selective pressure exerted on T cells during the decline of the primary response and during the establishment of T-cell memory.

Structural analysis of the T-cell receptor (TCR) variable regions involved in the primary and secondary pigeon cytochrome *c*-specific $CD4^+$ T-cell responses revealed an enhanced presence of certain amino acids in complementarity-determining region 3 (CDR3) of memory T cells (19) and showed that selective pressure was exerted on germinal-center T cells (37). In another study, in which the TCR variable regions used during a tumor-specific $CD8^+$ T-cell response were analyzed, the researchers concluded that the same variable regions characterized TCRs of the primary and secondary immune responses (16). In each of these studies, T cells expressing one particular $V_\alpha V_\beta$ combination were analyzed.

In the present study, we analyzed the functional response of the polyclonal (7) lymphocytic choriomeningitis virus (LCMV)-specific cytotoxic T-lymphocyte (CTL) response in mice. The results presented indicate that the T-cell repertoire used during the secondary T-cell response is dramatically smaller than the repertoire of the primary response, indicating functional maturation of the T-cell response.

MATERIALS AND METHODS

Mice and viruses. C57BL/6 (*H-2*^{b}) mice were obtained from Jackson Laboratory (Bar Harbor, Maine). Transgenic mice expressing a TCR specific for LCMV (26) and RAG2-deficient mice (32) have been described previously. The LCMV isolate WE was originally provided by R. M. Zinkernagel, Zürich, Switzerland, and grown on L cells at a low multiplicity of infection.

Peptides. Peptides were generated at the Amgen Institute (Boulder, Colo.) by a solid-phase method using the Fmoc/tBu-based protocol on an ABI-431 instrument. The crude product was purified by high-performance liquid chromatography. Peptide p33 defines the major CTL epitope on the LCMV glycoprotein in the *H-2*^{b} haplotype (27). For stability reasons, the C-terminal C was replaced by M (25).

Detection of LCMV-specific cytotoxic T cells in vitro. Mice were immunized and spleen cell suspensions were prepared and tested directly in a ^{51}Cr release, with EL-4 cells pulsed with the peptides indicated in Fig. 1 as target cells. All peptides were used at a concentration of 10^{-5} M. Primary CTL responses were assessed 8 days after infection with 200 PFU of LCMV WE. To assess memory CTL responses directly ex vivo, LCMV-immune mice (infected 30 days previously with 200 PFU of LCMV) were challenged with 10^6 PFU of LCMV. Such a high dose of LCMV was necessary to induce a measurable response because LCMV is quickly eliminated in the immune host. Alternatively, cells of LCMV-immune mice were restimulated for 5 days with peptide-pulsed spleen cells (10^6 cells/well) at a density of 4×10^6 spleen cells in 2 ml of Iscove modified Dulbecco medium supplemented with 10% fetal calf serum. Restimulated spleen cells were resuspended in 0.5 ml of medium per culture well, and serial threefold dilutions of effectors were performed (referred to as dilution of standard culture) and tested in a conventional ^{51}Cr release assay, with peptide-pulsed EL-4 cells as targets.

Anti-CD8 blocking experiments. Lysis of peptide-pulsed EL-4 cells was inhibited as described previously with monoclonal anti-CD8 antibody YTS169 (4).

Determination of lytic units. Lytic units were determined for 30% specific lysis. Thus, the number of arbitrary units per spleen able to specifically lyse 30% of the target cells was calculated. The slightly lower number of lytic units in LCMV-immune mice on peptide p33 is explained by the fact that spleen cells were analyzed 3 days after infection with LCMV to exclude the contribution of T cells of the primary CTL response.

RESULTS AND DISCUSSION

Specificity of a monoclonal versus polyclonal LCMV-specific T-cell response. The primary LCMV-specific CTL response peaks 8 days after infection, and lifelong CTL memory is subsequently established during the following weeks (8, 15, 38). Mice were immunized with LCMV, and the fine specificity of the response was analyzed on day 8 with a set of peptides derived from peptide p33, the major CTL epitope of LCMV in the *H-2*^{b} haplotype (27) (Fig. 1). The peptides exhibited random mutations outside the major histocompatibility complex (MHC)-binding anchor residues; mutations were dominantly introduced at positions 4 and 6, which have been predicted to point towards the TCR (30) (Fig. 1A). Surprisingly, the polyclonal CTL response recognized almost all variant peptides (Fig. 1B). To compare the polyclonal CTL response with a monoclonal response, TCR-transgenic mice expressing a TCR specific for LCMV were also infected with LCMV. To ensure the presence of T cells with a single specificity, the TCR-transgenic mice were crossed with RAG2-deficient mice (32). Since no rearrangement of endogenous α-chains occurs in the absence of RAG2 (2), these mice have T-cell populations with monoclonal specificity. Eight days after infection, spleen cells were tested on EL-4 cells pulsed with the different peptides

* Corresponding author. Mailing address: Ontario Cancer Institute, Departments of Medical Biophysics and Immunology, 610 University Ave., Toronto, Ontario M5G 2M9, Canada. Phone: (416) 946-2000, ext. 5471. Fax: (416) 946-2086. E-mail: bachmann@oci.utoronto.ca.

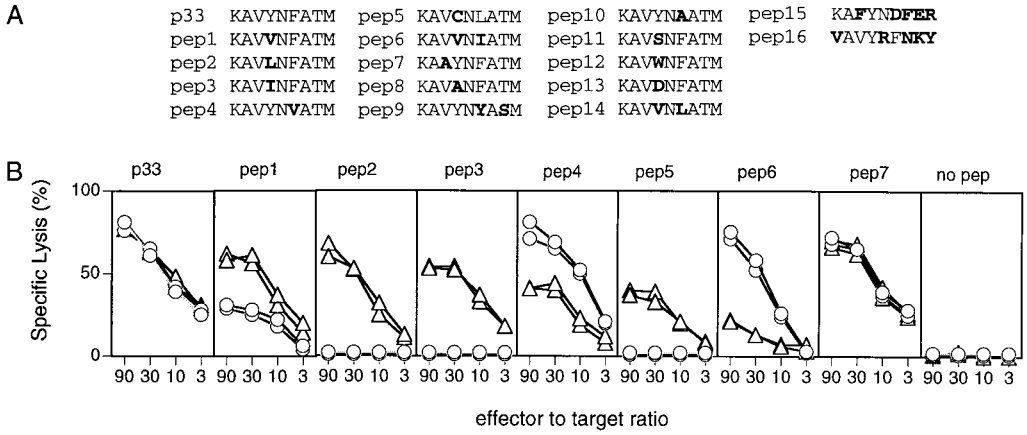

FIG. 1. Fine specificity of a monoclonal versus polyclonal LCMV-specific CTL response. (A) Amino acid sequences of peptides (pep) derived from peptide p33, which defines the major CTL epitope of LCMV. The C-terminal C was changed to M for stability reasons. This mutation does not affect the antigenicity. Letters in boldface type indicate amino acid substitutions. (B) C57BL/6 mice (triangles) and RAG2-deficient TCR-transgenic mice (circles) were immunized with LCMV (200 PFU); 8 days later, lytic activity of spleen cells was assessed on EL-4 cells pulsed with the indicated peptides (10^{-5} M). Each line represents one mouse. The results of one representative experiment (of three) are shown. Eight representative peptides (of 17 tested) are shown.

(Fig. 1B). To quantify the responses better, lytic units were determined for the different mice and peptides (Fig. 2). Although some peptides (e.g., peptides 2 and 3) were recognized only by the polyclonal T cells, other peptides, surprisingly, were recognized better by the monoclonal T cells. Peptide 6 was particularly interesting, because it was almost exclusively recognized by the transgene-encoded TCR but not by the polyclonal T cells. Thus, each TCR has its own specificity "fingerprint"; some of the specificities of a single TCR are apparently unique to it and are not necessarily easily detected in a polyclonal response.

Low-avidity recognition of variant peptides by polyclonal CTLs. To assess whether the variant peptides were recognized by the polyclonal T cells with lower avidity than that of the p33 peptide, anti-CD8 blocking experiments were performed (Fig. 3). Susceptibility of T-cell responses toward anti-CD8 antibodies has previously been correlated with the avidity of the TCR for the MHC-peptide complex (10, 29, 31). The results indicated that the variant peptides were in fact recognized with lower avidity (Fig. 3).

Drastically enhanced specificity of secondary CTLs. To compare the fine specificity of the primary LCMV-specific response with the secondary memory response, LCMV immune mice (day 30) were challenged with LCMV and ex vivo CTL activity was assessed 3 days later. A high dose of LCMV was necessary to induce a secondary response because of the presence of a memory CTL response that quickly eliminates the virus. At this early time point, no ex vivo CTL activity can be detected in naive mice (data not shown); this assay, therefore, exclusively detects memory CTL activity. Also, no LCMV-neutralizing antibodies can be detected in normal mice 1 month after infection (5); antibodies, therefore, do not interfere with the memory CTL response. The results of a representative experiment are shown in Fig. 4A. It is evident that very few peptides are recognized during the secondary response. In fact, only those peptides optimally recognized during the primary response were also recognized during the secondary response. Thus, T cells of the secondary response exhibited drastically less cross-reactivity, indicating that they recognized the original peptide, p33, with high specificity and probably high avidity.

To further evaluate the response, lytic units were determined for all peptides (Fig. 4B). These data confirm the dramatic increase in specificity for the secondary response. To reveal whether the CTL precursors recognizing the variant peptides during the primary response were reduced in frequency before secondary restimulation or, alternatively, could not compete with the highly specific CTLs during the secondary in vivo restimulation, CTLs of LCMV-immune mice were restimulated in vitro with the variant peptides and tested on

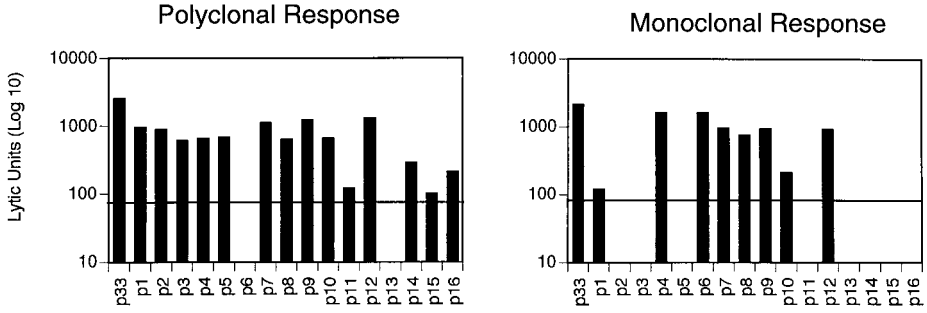

FIG. 2. Polyclonal CTLs have greater flexibility in peptide recognition than monoclonal CTLs. C57BL/6 mice and RAG2-deficient TCR-transgenic mice were immunized with LCMV (200 PFU); 8 days later, lytic activity of spleen cells was assessed on EL-4 cells pulsed with the indicated peptides (p) (10^{-5} M). Lytic units, which are proportional to the CTL precursor frequency per spleen, were determined for all peptides tested. The horizontal line indicates the detection limit. The results of one representative experiment (of two) are shown.

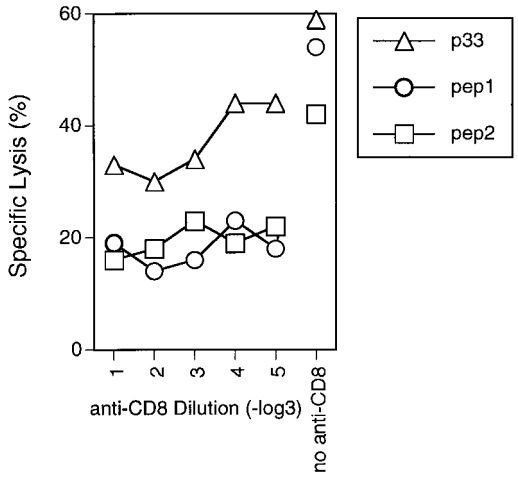

FIG. 3. Variant peptides (pep) are recognized with lower avidity than p33. C57BL/6 mice were immunized with LCMV (200 PFU); 8 days later, spleen cells were tested on p33-, pep1-, and pep2-pulsed target cells (peptide concentration, 10^{-5} M) in the presence of various concentrations of anti-CD8 antibody. Three representative peptides from one experiment (of three) are shown.

either p33 or the peptide used for restimulation (Fig. 5). Most variant peptides failed to restimulate CTLs significantly. Nevertheless, more cross-reactivity was observed after in vitro stimulation than after in vivo challenge. This finding is consistent with the notions that (i) many T cells cross-reacting with low-affinity ligands are eliminated from the repertoire during the decline of the primary response and the establishment of T-cell memory and (ii) during the secondary T-cell response, additional selective pressure is apparently exerted on T cells, allowing the proliferation of very-high-affinity T cells only. However, it also remains possible that conditions during the in vitro culture do not favor the proliferation of low-affinity T cells and that, therefore, the frequency of low-affinity memory T cells may be underestimated in these assays. Hence, although it is evident from our results that the T cells of the secondary response exhibit greater specificity than T cells of the primary response in vivo, further experiments are required to definitively assess the specificity repertoire of the memory T-cell population before secondary challenge.

Interestingly, the lysis of variant peptide-pulsed target cells was never higher than lysis of the p33-pulsed cells, even after restimulation with the variant peptide (Fig. 5). This indicates that a T-cell population highly specific for the variant peptide (and which could be amplified specifically by stimulation with the variant peptide) was not present in the LCMV-immune mice. The absence of T cells specific exclusively for a variant peptide suggests that individual T cells are not specific for single ligands but cross-react with multiple ligands with various affinities. This observation is also consistent with the finding that polyclonal T-cell responses remain primarily specific for the antigen used for in vivo immunization even after repetitive in vitro stimulation with a cross-reactive antigen (14).

Surprisingly, the T-cell population of the secondary immune response exhibited less cross-reactivity than the monoclonal T-cell population (Fig. 1). One explanation for this observation is that the TCR expressed in the particular transgenic mouse line used to generate the monoclonal T-cell population may exhibit lower specificity for p33 than the specificities of the effector T cells of the secondary in vivo immune response. Interestingly, memory T cells restimulated with p33 and tested on the various peptides exhibited a broader reactivity spectrum than the secondary T cells generated in vivo after challenge virus infection (data not shown). During such in vitro stimulations, T cells are confronted with large amounts of antigen

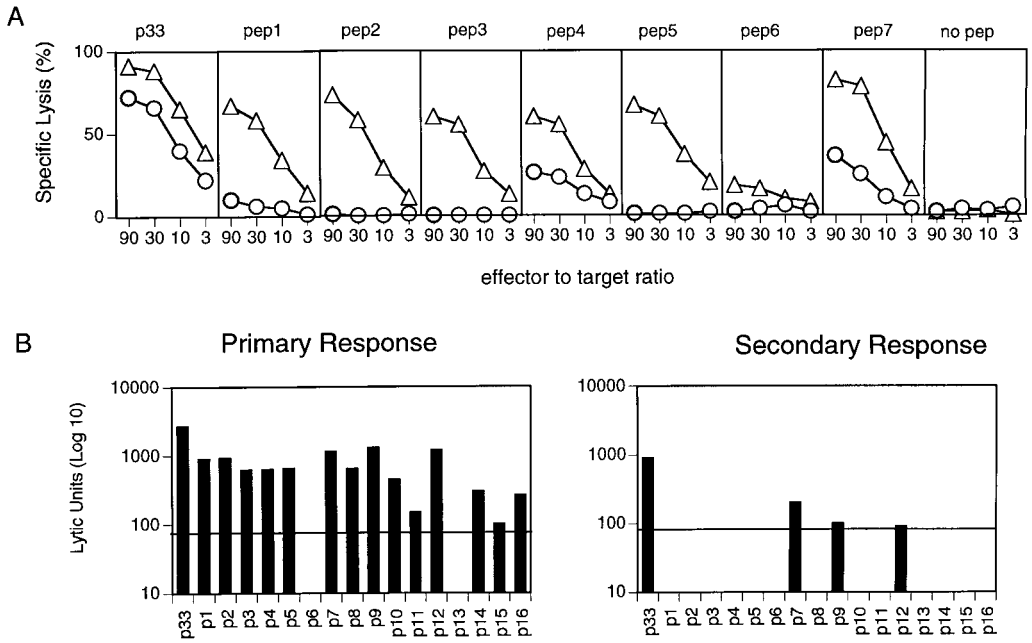

FIG. 4. Fine specificity of primary versus secondary in vivo-restimulated LCMV-specific CTLs. (A) Naive C57BL/6 mice (triangles) were immunized with LCMV; 8 days later, lytic activity of spleen cells was tested on EL-4 cells pulsed with the indicated peptides (pep). Alternatively, LCMV-immune (200 PFU, day 30) memory mice (circles) were challenged with LCMV (10^6 PFU), and lytic activity of spleen cells was tested 3 days later. Under these conditions, naive mice did not respond (data not shown). Eight representative peptides (of 17 tested) are shown. (B) Lytic units, which are proportional to the CTL precursor frequency per spleen, were determined for all peptides (p) tested. The horizontal line indicates the detection limit. The results of one representative experiment (of two) are shown.

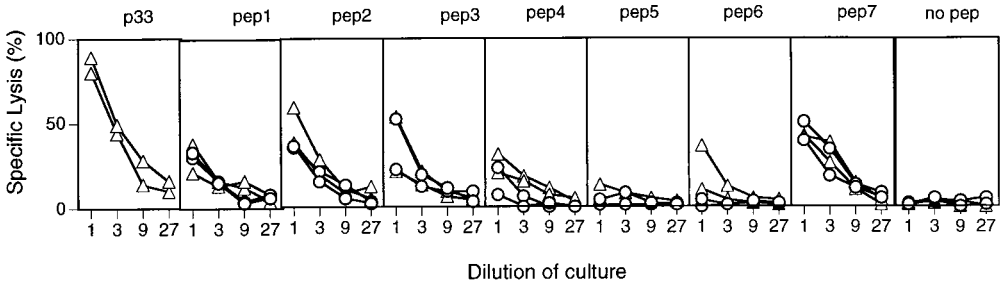

FIG. 5. Fine specificity of secondary in vitro-restimulated LCMV-specific CTLs. C57BL/6 mice were immunized with LCMV (200 PFU, day 30), and spleen cells were restimulated with different variant peptides (pep) and tested on p33 (triangles) or the variant peptide used for restimulation (circles). Each line represents one mouse. The results of one representative experiment (of three) are shown.

presented on many antigen-presenting cells. Competition between T cells of different fine specificity, therefore, is small. In contrast, during secondary antiviral in vivo responses, amounts of antigen are limiting because the virus is eliminated quickly from the host, creating strong competition between individual T cells. Thus, as opposed to the in vivo situation, in vitro culture of memory T cells and the generation of T-cell clones may favor the outgrowth of T cells exhibiting average, rather than high, affinity. This view is supported by the finding that most LCMV-specific T-cell clones are not specific for the immunodominant peptide p33 but instead recognize peptide p275 (1, 24, 36).

An alternative explanation for the relatively broad reactivity spectrum of the monoclonal T-cell population may be that positions 4 and 6 of p33 were predominantly mutated. The T-cell receptor expressed by the monoclonal T cells may be particularly flexible for recognition of peptides mutated at these two sites.

It may also be possible that memory T cells are intrinsically less responsive to altered peptide ligands than are primary T cells. Although there is at present no evidence for this hypothesis, such a hyporesponsiveness of memory T cells would also explain our results.

In summary, we found a surprisingly strong increase in specificity between primary and secondary T-cell responses after in vivo restimulation, indicating that the minor specificities present during the primary response were largely purged from the repertoire during the establishment of CTL memory and could not efficiently compete with highly specific memory CTLs during in vivo restimulation with virus. The much more specialized secondary response is surprising in light of recent studies, in which structural determination of TCR sequences suggested a high degree of conservation of TCR usage between primary and secondary responses (16, 19). Such conclusions were drawn from CDR3 sequence homologies and from spectratyping, revealing the size of CDR3s (17, 21). Despite the described homologies, however, there were always multiple nonconserved amino acid positions in CDR3s of the responding TCR populations. Furthermore, sequence analysis allowed the assessment of only a limited number of TCR samples. Thus, the structural approach to characterizing a specific TCR repertoire has technical limitations which seem to preclude the detection of the subtle differences between the quite broad primary T-cell repertoire and the much more specialized secondary TCR repertoire. These considerations suggest that for both basic and clinical research, a functional approach may be better suited to detecting subtle differences in T-cell responses and that structural analysis alone may not be sufficiently discriminative to characterize a highly antigen-specific secondary TCR repertoire.

Extensive maturation of the pigeon cytochrome *c*-specific T-cell response has recently been reported (37). Although no functional data were provided in this study, selection of specific T cells could be observed by analysis of TCR sequences of single T cells picked from histological sections. Interestingly, T-cell selection was confined to germinal centers. This particular mechanism is therefore very unlikely to operate during the LCMV-specific CTL response, because CTLs have not been reported to be present in germinal centers in high numbers. Thus it seems likely that extensive T-cell selection can occur outside germinal centers, possibly in the T regions of lymphoid organs.

Implications for virus CTL escape mutants. The data may also offer an explanation for the frequent occurrence of viral CTL escape mutants during human immunodeficiency virus, hepatitis B virus, or mouse hepatitis virus infections (6, 13, 20, 22, 23). In contrast to B-cell responses in which the B-cell repertoire is constantly broadened in germinal centers by hypermutation of the VDJ regions of immunoglobulins (9, 28), the CTL repertoire becomes heavily restricted during the course of the immune response, as shown in this study. Thus, the hypermutated immunoglobulins may constantly catch up with newly generated virus variants, whereas the highly selected CTLs fail to control the generation of long-term viral mutants, particularly during chronic infections. Interestingly, at the population level, the situation is different: no CTL escape mutants occur due to the MHC polymorphism, and only antibody escape mutants (serotypes) are observed.

Taken together, the results of this functional analysis of an antiviral CTL response reveal a surprisingly strong selective pressure exerted on CTLs during the decline of the primary T-cell response, and in particular during the generation of a secondary immune response, suggesting a crucial role for antigen in the selection of a highly specific memory T-cell population.

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