Epitope spreading upon P815 tumor rejection triggered by vaccination with the single class I MHC-restricted peptide P1A

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Abstract
Epitope spreading has been best characterized as an exacerbating factor in CD4+ T cell-dependent autoimmune disease models and is believed to occur via presentation of antigens liberated by tissue destruction initiated by CD4+ T cells specific for a primary epitope. The growing evidence that exogenous antigens can also be processed and presented by class I MHC molecules has suggested that epitope spreading could occur for CD8+ cytotoxic T lymphocyte (CTL) responses as well. In the context of anti-tumor immunity, expansion of a CTL response to include secondary epitopes could improve the efficacy of therapeutic vaccines. To determine directly whether epitope spreading can occur during an anti-tumor immune response, two defined class I MHC-binding peptides in the P815 tumor model were utilized. We observed that immunization against the single tumor peptide, P1A, followed by rejection of a P1A+ tumor, subsequently yielded CTL activity and tumor protection against a P1A− tumor variant. P1A immunized mice that subsequently rejected tumor challenge developed CTL against a second defined epitope, P1E. These results indicate that, as for class II-restricted peptides in autoimmune disease, epitope spreading can occur for class I-restricted peptides during tumor rejection. A broadened CTL response may help eliminate outgrowth of antigen-negative tumor variants.

Introduction
Epitope spreading is an extension of focus during the course of a specific immune response from one dominant antigenic peptide, or epitope, to include secondary epitopes distinct from and non-cross-reactive with the first peptide. Epitope spreading is a recognized phenomenon of autoimmune responses and is believed to be an exacerbating factor in CD4+ T cell-mediated autoimmune diseases. The phenomenon has been demonstrated in murine relapsing-remitting experimental autoimmune encephalomyelitis (EAE), Thielier’s murine encephalomyelitis virus-induced demyelinating disease and diabetes in the non-obese diabetic (NOD) mouse (1,2). A model has been suggested for how epitope spreading in autoimmune diseases mediated by CD4+ T cells occurs (2). This model is supported by direct evidence that tissue damage, TCR ligation on CD4+ T cells by MHC class II peptide complexes, CD40-CD40 ligand interactions and CD28-mediated co-stimulation are required for epitope spreading to become manifest (3–11). It is thought that an initiating self-antigen or a persistent viral epitope, presented in MHC class II molecules on the surface of professional antigen-presenting cells (APC) residing in the target tissue, causes the activation of CD4+ T cells specific for that antigen. This T cell activation results in chronic inflammation, leading to damage of the target tissue. Tissue debris is subsequently taken up by APC which have up-regulated expression of MHC class II and co-stimulatory molecules in response to inflammatory cytokines. These APC are then capable of activating CD4+ T cells specific for secondary tissue epitopes presented by the APC. The newly activated T cells then aid in destruction of the target tissue.

Due to the requirement for presentation by APC of exogenous antigen, epitope spreading has historically been thought of as a phenomenon unique to CD4+ T cell responses. However, recent data have indicated that cross-priming by
APC can participate in the induction of CD8\(^+\) cytotoxic T lymphocyte (CTL) responses as well (12–14). In particular, bone marrow chimera studies in murine tumor models have shown that tumor-specific CTL are predominately restricted to the MHC of the host rather than that of the tumor, suggesting that indirect presentation by host APC is involved in the generation of tumor-specific CTL (12,13). Moreover, there is increasing evidence that a pathway exists whereby exogenous antigen can be presented for eventual peptide loading onto class I MHC molecules. This phenomenon is best described for dendritic cells (DC) and provides a cellular mechanism to explain the process of cross-priming (15–17). Collectively, these data suggest that it may be possible for epitope spreading to occur during a class I MHC-restricted CTL response.

Because re-presentation of MHC class I-restricted tumor antigens is known to occur, we postulated that if tumor-bearing hosts could initiate a CTL response against a single tumor antigen, that following tumor cell damage caused by the CTL, epitope spreading might occur via a mechanism analogous to that described in CD4\(^+\) T cell-mediated autoimmune diseases. Unlike during an autoimmune response, however, CTL epitope spreading during an anti-tumor response could be beneficial to the host by possibly allowing for elimination of antigen-loss variant tumor cells. To address this question, we have utilized the well-defined murine P815 tumor model. At least five tumor antigens have been identified on P815 according to recognition by CTL clones, and the genes encoding two of these antigens have been cloned and characterized. The first of these genes, P1A, is expressed by several mastocytoma cell lines but not in normal tissues except testis and placenta (18). The dominant P1A peptide, LPYLGWLVF, when presented by H-2L\(^d\), is recognized by a specific CTL clone and an effective vaccination protocol using the P1A peptide has been established to induce the generation of P1A-specific CTL in DBA/2 mice that protects mice against a P815 tumor challenge (19). The second antigen cloned, known as P815E (P1E), results from a mutation in a ubiquitously expressed gene encoding methionine sulfoxide reductase (20). A peptide from the protein of this mutated gene, GYCGLRGTV, presented by H-2K\(^d\), is also recognized by a specific CTL clone.

In the present study, we observed that immunization against P1A, followed by rejection of a P1A\(^+\) tumor, subsequently yielded CTL activity against P1E and protection against a P1A\(^-\) tumor variant. These results indicate, that similarly to class II-restricted peptides in autoimmune diseases, epitope spreading can occur for class I-restricted peptides during tumor rejection.

Methods

Mice

DBA/2J mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and housed in a specific pathogen-free facility at the University of Chicago. Female mice 8–10 weeks of age were used for experiments.

Cell lines

All cells were cultured in DMEM supplemented with 10% FCS and incubated at 37°C in a 5% CO\(_2\) atmosphere. The following clones of P815 were used in this study: P1.HTR, a highly transfectable variant of P815; P1.HTR.B7-1, a P1.HTR transfectant expressing murine B7-1 along with a neomycin resistance gene (21); P511 an azaguanine-resistant variant of P815 expressing high levels of P1A and P1E; and P1.204, a P815AB\(^+\) variant carrying a deletion of the gene P1A (22). In addition, the following cell lines were used: L1210, a DBA/2 syngeneic leukemia cell line lacking expression of the known P815 antigens; YAC-1, a murine NK-sensitive target; transfectants of RMA-S expressing either the H-2L\(^d\) gene (RMA-S/L\(^d\)) or H-2K\(^d\) gene (RMA-S/K\(^d\)) along with a neomycin resistance gene. All transfectants were maintained in selection drug at least every second passage. Prior to use in experiments, the cells were always cultured in the absence of the selection drug.

Tumor peptides

Synthetic peptides, purified by reverse-phase HPLC to >95% purity, were purchased from Research Genetics (Huntsville, AL). The letter code sequences of the peptides used are as follows: H-2L\(^d\)-restricted P1A, LPYLGWLVF; H-2L\(^d\)-restricted P91A, QNHRALDLVA; H-2K\(^d\)-restricted P1E, GYCGLRGTVG; H-2K\(^d\)-restricted P198, KYQAVTTTL.

Recombinant murine IL-12

Highly purified rmIL-12 was kindly provided by Dr S. Wolf (Genetics Institute, Cambridge, MA).

P1A peptide immunization

Female DBA/2 mice were immunized following the protocol of Fallarino et al. (19). Briefly, DBA/2 peripheral blood mononuclear cells (PBMC) or spleen cells recovered after Ficoll-Hypaque gradient purification were pulsed with 20 μM P1A peptide in the absence of FCS or any other proteins in DPBS (Gibco, Grand Island, NY) for 1–2 h. The cells were washed, irradiated (2000 rad) and resuspended so the cells could be delivered in 50 μl DPBS per hind footpad. Mice were immunized weekly for 3 consecutive weeks with 2×10⁶ PBMC or 2×10⁷ spleen cells loaded with the P1A peptide. rmIL-12 (20 ng/mouse in the hind footpads) was given the day of each immunization and the subsequent 2 days. Control mice were injected with DPBS only.

Mixed lymphocyte-tumor culture (MLTC)

MLTC were performed using spleen cells as a source of responding lymphocytes. Spleen cells (5×10⁶/well) were stimulated with irradiated (10,000 rad) P1.HTR.B7-1 cells (2×10⁶/well) in 2 ml in 24-well plates. Cytolytic activity was measured by Cr release 5 days later.

Cr-release assays

Effector cells were diluted in duplicate in V-bottomed microtiter plates and mixed with 1000 or 2000 ⁵¹Cr-labeled target cells in a final volume of 200 μl of complete medium. Either 10⁵ or 2×10⁵ cold competitor cells (either L1210 or YAC-1) were mixed with the labeled target cells at a ratio of 30:1 before
These results suggest that the rejection of P1.204 is the result of a P1A-specific CTL response and specific immunity against a variant of P1. Immunization with P1A-loaded PBMC plus IL-12 generates a CTL response and specific immunity against P1.HTR in mice that were not depleted of T cells and had rejected P1.HTR. Tumor challenges

Between 2 and 4 weeks following the last immunization, mice were challenged by s.c. injection in the left flank with 10⁶ P1.HTR or P1.204 cells in 100 µl DPBS. In some experiments, following complete rejection of P1.HTR, mice were challenged with a secondary s.c. injection in the left flank with 10⁶ P1.204 cells in 100 µl DPBS. Tumor growth was assessed twice per week by measuring the largest and smallest tumor diameter of each mouse with calipers, and a mean was calculated. Results are expressed as the mean ± SEM of the tumor diameters for the entire group of mice at each time point.

mAb

The Thy1.2-specific 30-H12 hybridoma was purchased from the ATCC (Rockville, MD). mAb were purified from the hybridoma by using Protein G–Sepharose 4 Fast Flow (Amersham Pharmacia Biotech, Arlington Heights, IL) under sterile conditions.

T cell depletion in vivo

Purified 30-H12 mAb was administered in DPBS by i.p. injection: 1 mg was given the day before and the day of tumor challenge, and 0.5 mg was then given every 3 days following tumor challenge.

Results

Immunization with P1A-loaded PBMC plus IL-12 generates a P1A-specific CTL response and specific immunity against a P1A⁺ tumor challenge

Female DBA/2 mice were immunized weekly in the hind footpads for 3 consecutive weeks with irradiated P1A peptide-loaded PBMC plus rmIL-12 as described in Methods. rmIL-12 was included in the vaccination because it had been shown previously that co-administration of IL-12 was required for the induction of P1A-specific CTL via P1A-pulsed cells in DBA/2 mice (19). Two weeks after the third immunization, spleens from individual mice were stimulated in vitro in a MLTC for 5 days with irradiated P1.HTR.B7-1 cells, and the resulting effector cells were tested for lytic activity against P1.HTR.P511 (P1A⁺) and P1.204 (P1A⁻). To eliminate non-specific lytic activity, L1210 cells were used as cold targets. As shown in Fig. 1(A), effector cells generated from P1A peptide immunized mice expressed high lytic activity against P1.HTR.P511 but little against P1.204, whereas effector cells generated from non-immunized mice demonstrated little lytic activity against either target cell. These results demonstrate that the specific CTL response in immunized mice was limited to P1A.

To determine if induction of P1A-specific CTL conferred specific protection against a P1A⁺ tumor, immunized mice were challenged s.c. in the left flank with P1A⁺ P1.HTR cells or the P1A⁻ variant P1.204. Tumor growth was then assessed over time. As shown in Fig. 1(B), the P1A peptide-immunized mice rejected challenge with P1.HTR tumor cells, while the tumor grew progressively in non-immunized controls. In contrast, the P1A⁺ variant P1.204 grew progressively in both immunized mice and in non-immunized controls, requiring sacrifice of the animals due to a large tumor burden. These results demonstrate that the P1A-specific CTL response induced by specific immunization is associated with protection against a P1A⁺ but not a P1A⁻ tumor.

Rejection of P1.HTR following P1A peptide immunization generates protective immunity against P1.204 tumor challenge

It was conceivable that rejection of P1.HTR cells promoted by P1A immunization might have led to T cell responses against additional antigens via secondary antigen processing. To begin addressing this question, immunized mice that had rejected P1.HTR were given a secondary tumor challenge by s.c. injection of P1.204 cells in the left flank and tumor growth assessed over time. In contrast to P1A peptide-immunized mice that were able to reject P1.HTR but not P1.204, immunized mice that had subsequently rejected P1.HTR became capable of rejecting a secondary P1.204 challenge (Fig. 2). Inasmuch as P1.204 cells lack expression of P1A but share expression of at least three additional defined antigens with P1.HTR cells, the immune response generated during the rejection of P1.HTR appeared to become broadened to include T cells recognizing additional shared determinants.

It was conceivable that the subsequent rejection of P1.204 cells by mice that had rejected P1.HTR was due to hyperactivation of a non-antigen-specific effector mechanism, such as NK activity. In order to determine whether P1.204 rejection was in fact dependent on T cells, mice were depleted of T cells by treatment with a Thy1.2-specific mAb prior to and during P1.204 challenge (Fig. 3). In fact, P1.204 grew progressively in these T cell-depleted mice, while the tumor was rejected by control mice that were not depleted of T cells and had rejected P1.HTR. These results suggest that the rejection of P1.204 is the result of an expanded tumor-specific T cell response generated following primary rejection of P1.HTR.

Tumor-specific CTL activity is diversified following tumor rejection

To determine directly if the specificity of the tumor-reactive T cell response was expanded in immunized mice that had subsequently rejected tumors, antigen-specific lytic activity of cells from these mice was measured. Spleens were harvested from P1A peptide-immunized mice, immunized mice that had rejected P1.HTR, and immunized mice that had rejected P1.HTR and P1.204. The spleen cells were cultured in an MLTC with P1.HTR.B7-1 cells, and cytolytic activity was measured against P1.HTR.P511 and P1.204. As had been seen previously, effector cells generated from P1A peptide-immunized mice expressed high lytic activity against P1.HTR.P511 and P1.204 but little against P1.204 (Fig. 2), demonstrating that the P815-specific CTL response in these mice was limited to P1A specificity. Effector cells generated from immunized mice that had rejected P1.HTR or from immunized mice that had rejected both P1.HTR and P1.204 also expressed high lytic activity against P1.HTR.P511 (Fig. 4). However, unlike the immunized mice that had not received a tumor challenge, effector cells from mice that had rejected P1.HTR also expressed detect-
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Fig. 1. Immunization with P1A peptide-loaded APC plus IL-12 generates a P1A-specific CTL response and specific immunity against a P1A+ tumor challenge. (A) DBA/2 mice were immunized with P1A-loaded PBMC + mIL-12 (○) or DPBS (□) as described in Methods. Two weeks after the last immunization, spleen cells were stimulated in vitro in a MLTC for 5 days with irradiated P1.HTR.B7-1 cells. Effector cells were examined for lysis against 51Cr-labeled P511 (left panel) or P1.204 (right panel) cells, in the presence of unlabeled L1210 cells at a ratio of 30:1 with the labeled target cells. Each curve represents lysis by effectors generated from an individual mouse, with the lysis of the effectors at each E:T ratio measured in duplicate. (B) In a separate experiment, 2 weeks after the last immunization, P1A peptide-immunized mice (○) and DPBS-injected controls (□) were challenged with either P1.HTR (left panel) or P1.204 (right panel) cells s.c. in the left flank. The mean tumor diameter (± SEM) over time for each group of mice is shown, with five mice per group. Results are representative of at least three experiments.

able lytic activity against P1.204 (Fig. 4A), and this activity was further increased in mice that had rejected both P1.HTR and P1.204 (Fig. 4B). Further, effector cells from mice that had rejected both tumors showed decreased lytic activity against P511. The decreased P1A-specific response measured following rejection of both the P1A+ and P1A- tumors suggests that a shift in the tumor-specific CTL repertoire was required for elimination of the P1A- tumor cells. These results suggest that during the course of tumor rejection initiated by P1A-specific CTL, new CTL activity was generated against other antigens shared with the P1A+ variant.

It was desirable to examine whether CTL recognizing a second defined tumor antigen epitope were induced in P1A peptide-immunized mice that subsequently rejected P1.HTR and P1.204. To this end, we took advantage of the recently identified P1E gene cloned from P815 that encodes a mutated peptide antigen presented by H-2Kd (20). Spleen cells harvested from P1A peptide-immunized mice, and immunized mice that had rejected P1.HTR and P1.204, were cultured in an MLTC with P1.HTR.B7-1 and cytolytic activity was measured against RMA-S/Ld or RMA-S/Kd pulsed with the P1A or P1E peptide respectively. To control for peptide specificity, lytic activity was also tested against RMA-S/Ld pulsed with the irrelevant P91A peptide or RMA-S/Kd pulsed with the irrelevant P198 peptide. To eliminate the contribution of NK activity, assays were performed in the presence of unlabeled YAC-1 cells as cold target competitors. As shown in Fig. 5, effector cells generated from non-immunized mice exhibited little P1A- or P1E-specific lytic activity. As expected, effector cells generated from P1A peptide-immunized mice lysed P1A-pulsed RMA-S/Ld cells but not P1E-pulsed RMA-S/Kd cells, corresponding with the exclusive P1A-specific lysis seen previously with P511 and P1.204 cells as targets. However, effector cells generated from P1A peptide-immunized mice that had rejected both P1.HTR and P1.204 tumor challenges gained new lytic activity specific for the P1E peptide (Fig. 5). In addition, the magnitude of the P1A-specific lysis of these effectors was modestly but reproducibly lower than that of effectors generated from P1A-immunized mice that had not been challenged with tumor. This diminution of the P1A-specific response suggests a quantitative shift towards new CTL specificities as tumor rejection occurs. These results directly demonstrate acquisition of CTL specific for a secondary epitope following tumor rejection induced by effective immunization with a primary tumor antigen epitope.
Class I MHC-restricted epitope spreading upon tumor rejection

Fig. 2. Rejection of P1.HTR following P1A peptide immunization generates protective immunity against subsequent P1.204 tumor challenge. Groups of five DBA/2 mice were immunized with DPBS (left column) or P1A peptide-loaded PBMC + rmIL-12 (middle column). Two weeks after the last immunization, these immunized mice were challenged with P1.HTR cells (top row) or P1.204 cells (bottom row) by s.c. injection in the left flank. Following complete rejection of P1.HTR by P1A peptide-immunized mice, some mice were then challenged with P1.204 cells by s.c. injection in the left flank (right column). The mean tumor diameter (± SEM) over time for each group of mice is shown. Results are representative of at least three similar experiments.

Fig. 3. Rejection of secondary P1.204 challenge is T cell dependent. DBA/2 mice were immunized with P1A peptide-loaded spleen cells + rmIL-12 as described in Methods. Two weeks after the last immunization, 10 immunized mice were challenged with P1.HTR cells by s.c. injection in the left flank. Following complete rejection of P1.HTR, half of these mice were depleted of T cells by i.p. injection of anti-Thy1.2 antibody (●) and half were not (○). All mice were then challenged with P1.204 cells by s.c. injection in the left flank. For T cell depletion, 1 mg of anti-Thy1.2 antibody was administered the day before and the day of P1.204 challenge, with 0.5 mg given every 3 days following tumor challenge. The mean tumor diameter (± SEM) over time for each group of mice is shown. Results are representative of three experiments.

Discussion

Epitope spreading was originally described in murine models of autoimmune disease. The expansion of T cell specificities to include multiple antigens expressed in the target tissue exacerbates autoimmune aggression and poses a substantial barrier to treatment aimed at antigen-specific tolerance (23). The process of and the ramifications of epitope spreading have been best characterized in murine models of R-EAE and diabetes mellitus. In these models, disease development is dependent upon a CD4+ T cell response and it is within these CD4+ T cell populations that epitope spreading has been documented (2). The focus on CD4+ T cells in the study of epitope spreading is largely related to the requirement for exogenous cellular antigen released by destroyed tissue to be presented by APC for the phenomenon to occur. Historically, exogenous antigen was thought to be processed and presented in MHC class II, but not class I molecules, disallowing activation of antigen-specific CD8+ T cells through this pathway. However, accumulating evidence suggests that certain APC, most notably DC, are able to take up exogenous antigen and deliver peptides to MHC class I molecules, an event termed ‘cross-priming’ (15–17). Furthermore, data is now emerging that cross-priming may contribute substantially to the induction of CD8+ effector CTL in vivo (12–14). These observations suggest that epitope spreading could theoretically occur to expand the specificities of a developing CD8+ CTL response.
We hypothesized that, following tumor cell damage inflicted by CTL specific for a single tumor antigen peptide, released tumor antigen could be taken up by activated host APC that could then present additional tumor antigen peptides to CD8+ T cells specific for these secondary tumor antigens. The subsequent expansion of a tumor-specific CTL response could be beneficial to the host by providing a mechanism for eliminating antigen-loss tumor cell variants that have been shown to emerge under immune pressure (24,25).

Our present study suggests that the phenomenon of epitope spreading is not limited to CD4+ T cell responses. Immunization with P1A peptide followed by tumor rejection clearly led to CTL activity against a P1A- tumor. This population of CTL included cells that recognized the unrelated antigen P1E. Since this epitope was not included in the vaccine and is a mutated peptide not presented in normal tissues, the source of P1E antigen must have been the tumor cell challenge.

Epitope spreading has been characterized both in autoimmune responses generated spontaneously as well as in those generated by active immunization. One of the best characterized autoimmune disease models which has been used to study epitope spreading is murine EAE. In this model, the disease is induced by immunizing mice with a proteolipid protein epitope or myelin basic protein epitope, and as disease progresses, immune responses spread to other central nervous system antigen epitopes (1,3). We liken the broadened CTL response observed following P815 tumor rejection induced by immunization with the single P1A peptide to the broadened immune responses observed in EAE induced by active immunization.

Although the evidence that cross-priming through host APC is a dominant pathway for induction of class I MHC-restricted anti-tumor CTL suggests that epitope spreading is likely to occur via host APC as well, this has yet to be demonstrated directly. It is conceivable that the presence of P1A-specific CTL generates a microenvironment that facilitates direct antigen presentation by tumor cells. Further experiments will be required to distinguish whether the epitope spreading we observe is the result of increased direct antigen presentation by tumor cells or increased presentation of released tumor antigens by host APC. In addition, it will be of interest to determine the requirement for co-stimulatory molecules and other factors for priming against secondary epitopes recognized by CD8+ T cells.
Previous studies had suggested that selection of antigen-loss variants of P815 cells under immune pressure in vivo was not an uncommon event (22). P1A expression was commonly lost in progressively growing tumors. However, in our current studies using vaccination with P1A peptide-pulsed APC + rmIL-12, selection for P1A− variants has not been seen. This is likely explained by the epitope spreading process that follows immunization. It is conceivable that the IL-12 included during vaccination promotes a particularly effective immune response that favors re-presentation of antigens from destroyed tumor tissue.

The occurrence of epitope spreading following CTL-dependent tumor damage suggests a method for the generation of a CTL response against, and the elimination of, tumor cell variants that have lost expression of antigen. However, it is unlikely that this mechanism will be sufficient to eliminate large numbers of antigen-negative tumor cells. The time required for epitope spreading to occur may be too prolonged to keep up with the growth rate of abundant tumor cells lacking expression of the tumor antigen. Indeed, preliminary experiments have revealed that a 1:1 mixture of P1A+ and P1A− tumor cells grows progressively in P1A-immunized mice (M. Markiewicz, unpublished observation). However, the potential for CTL epitope spreading suggests that if tumor-bearing individuals can be induced to generate a CTL response against antigen expressed by the majority of their
tumor cells, that a secondary CTL response may then be generated against rare tumor cells lacking expression of the immunizing antigens.

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Abbreviations

APC antigen-presenting cells  
CTL cytotoxic T lymphocyte  
EAE experimental autoimmune encephalomyelitis  
DC dendritic cells  
MLTC mixed lymphocyte tumor culture  
NOD non-obese diabetic  
PBMC peripheral blood mononuclear cells

References