Identification of a Second Major Tumor-Specific Antigen Recognized by CTLs on Mouse Mastocytoma P815

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Murine mastocytoma P815 induces CTL responses against at least four distinct Ags (AB, C, D, and E). Recent studies have shown that the main component of the CTL response against the P815 tumor is targeted against Ags P815AB and P815E. The gene PIA has been well characterized. It encodes the P815AB Ag in the form of a nonameric peptide containing two epitopes, P815A and P815B, which are recognized by different CTLs. Here, we report the identification of the P815E Ag. Using a cDNA library derived from tumor P815, we identified the gene coding for P815E. We also characterized the antigenic peptide that anti-P815E CTLs recognize on the MHC class I molecule H-2K\(^d\). The P815E Ag results from a mutation within an ubiquitously expressed gene encoding methionine sulfoxide reductase, an enzyme that is believed to be important in the protection of proteins against the by-products of aerobic metabolism. Surprisingly, immunizing mice i.p. with syngeneic tumor cells (L1210) that were constructed to express B7-1 and P815E did not induce resistance against live P815, even though a strong anti-P815E CTL response was observed with splenocytes from immunized animals. The Journal of Immunology, 1999, 162: 3534–3540.

Materials and Methods

**Mice**

Syngeneic DBA/2 mice were raised in specific pathogen-free conditions. All mice used in these experiments were between 10 and 12 wk old.

**Peptides**

All peptides were synthesized in house on solid phase using fluorenylmethoxycarbonyl for transient NH\(_2\)-terminal protection (14). Peptides were characterized by mass spectrometry and stored at 20 mg/ml in DMSO at −20°C.

**Cell lines**

P1 is a clonal line isolated from a permanent cell line derived from the transplantable P815-X2 subline (15). P198, P89, and P35 are immunogenic tumor cell lines (tum-') produced by treatment of P1 with the mutagen N\(-\)-methyl-N\(^\prime\)-nitro-N\(^\prime\)-nitrosoguanidine (15). The cell lines P1.1stA B (P1.204), P1.1stA B 1st C D (15), L1210, L1210.P1A, and L1210.P1A.B7-1 have been described previously (3, 16). V\(_d\)D\(_m\) is a DBA/2 mastocytoma cell line produced by infection of an IL-3-dependent mast cell line with a retrovirus containing the v-Ha-ras gene (17). The L1210.P1E.B7-1 tumor cell line was obtained by transfecting L1210 with the B7-1 cDNA cloned into plasmid pEFBOS (18), into which the puromycin resistance gene had been introduced. Transfected cells were selected using 1.25 μg/ml of puromycin, cloned by limiting dilution, and transfected with the gene P815E that was cloned into pcDNA3.1 (Invitrogen, San Diego, CA). Transfected cells were selected with 2.5 mg/ml G418 and cloned by limiting dilution. The L1210.P1E cell line was produced by transfecting L1210 with the gene P815E cloned in pcDNA3.1, and positive clones were selected for as described above. All transfections were done by electroporation. All cell lines except V\(_d\)D\(_m\) were cultured in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% FCS in a humidified, 8% CO\(_2\) atmosphere at 37°C. V\(_d\)D\(_m\) was cultured in Iscove’s medium supplemented with 10% FCS.
CTL clones

To obtain CTL clones 89/62 and 351.1.E5, spleen cells (3 × 10^6) from mice immunized 4–15 wk previously with living P815 tum^−^ variant P89 or P35 cells were cultured in a mixed lymphocyte-tumor cell culture (MLTC) with 3 × 10^5 stimulator cells (P1.istA^2^B^2^iscC^2^D^2^Ag-loss variant). The MLTC was established in 15 ml of DMEM supplemented with L-arginine HCl (116 mg/l), L-asparagine (36 mg/l), L-glutamine (216 mg/l), glucose (4.5 g/l final concentration), 10 mM HEPES, 5 × 10^−5 M 2-ME, and 10% FCS. Cultures were maintained at 37°C in a humidified atmosphere containing 8% CO2. After 7 days, MLTC cells were cloned by limiting dilution in 96-well round-bottom plates as follows: Limiting numbers of cells were plated with 7 × 10^5 irradiated (2000 rads) DBA/2 spleen cells and 3 × 10^3 irradiated (5000 rads) stimulator cells in a final volume of 150 μl of MLTC medium supplemented with supernatant from secondary MLC. The plates were incubated for 7 days at 37°C in a humidified atmosphere containing 8% CO2; subsequently aliquots from each microculture were tested for cytolytic activity against the appropriate target cells.

CTL clones were maintained in culture by in vitro stimulation twice per week in 2 ml of MLTC medium supplemented with HAT (10^−5 M hypoxanthine, 3.8 × 10^−7 M aminopterin, and 1.6 × 10^−5 M 2-deoxythymidine) as well as 50% secondary MLC supernatant containing 10^5 P1.aza^−^ hypoxanthine aminopterin and 2-deoxythymidine-sensitive cells and 5 × 10^3 irradiated (3000 rads) splenocytes.

Chromium release assay

Chromium release assays were performed as described previously (19). Briefly, CTLs were incubated with 2000 ^51^Cr-labeled cells at various E:T ratios in 96-well conical microplates in a final volume of 200 μl. The supernatants were collected after a 4-h incubation at 37°C for the measurement of the chromium released from lysed cells.

Lytic units (LU)

One LU is defined as the number of effector cells that lyse 50% of 10^4 target cells in 4 h. This was estimated by the means of the regression (1-e^−kx ) from the specific chromium release obtained at different E:T ratios chosen in the linear range of the lysis curve. Results are expressed as LU/10^6 cells.

Peptide-pulsed assay

For peptide-pulsed assays, chromium-labeled V4 D6 cells were incubated for 30 min at 37°C with various concentrations of peptide in X-vivo 10 medium (Whittaker Bioproducts, Walkersville, MD) before the addition of CTLs. For peptide inhibition assays, chromium-labeled V4 D6 cells were

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3 Abbreviations used in this paper: MLTC, mixed lymphocyte-tumor cell culture; LU, lytic unit(s); MsrA, methionine sulfoxide reductase.
incubated for 30 min at 37°C in X-vivo 10 medium with various concentrations of competitor peptides followed by incubation with P815E peptide GYCGLRGTVGV at a concentration known to induce maximal lysis by anti-P815E CTLs. The concentration of P815E peptide inducing maximum lysis was determined on the same day of the competition assay. The cells were incubated with peptide for 30 min at 37°C, washed, and resuspended in 100 μl of fresh X-vivo medium; next, CTLs were added to achieve an E:T ratio of 5:1. Chromium release was measured after 2 h.

Inhibition by class I-specific Abs

A total of 15,000 P198 tum− cells were incubated together with a 1/10 dilution of hybridoma supernatant containing class I Abs (SF1-1.1.1 for Kd (ATCC HB159) and 28.14.8 S for Ld (ATCC HB27)) for 4 h at 37°C before the addition of 200 CTLs (clone 89/62 or clone 351.1.E5) per well. Cultures were incubated overnight, and supernatant was collected for an assessment of TNF production using the TNF-sensitive WEHI-164 clone 13 cells (20) in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide colorimetric assay (21).

cDNA library

Total cellular RNA was prepared from P1 cells by the guanidine-isothiocyanate procedure (22). Poly(A)+ mRNA was purified by chromatography on an oligo(dT) cellulose column (Pharmacia, Uppsala, Sweden) as described previously (22). cDNA was synthesized with the Superscript Choice System (Life Technologies) using oligo(dT)18 primers containing a NotI restriction site followed by ligation to BstXI adapters (Stratage, La Jolla, CA), phosphorylation, and digestion with NotI. Size-fractionated cDNA were ligated into the BstXI and NotI sites of pcDNA3.1 downstream from the CMV promoter. Recombinant plasmids were electroporated into Escherichia coli Top10F+ and selected by ampicillin (100 μg/ml). Plasmid cDNA from pools of 100 transformed colonies were extracted using the QIAprep 8 plasmid kit (Qiagen, Hilden, Germany).

Screening of transfected COS cells

A total of 15,000–20,000 COS cells were seeded in 96-well flat-bottom microwells in a total volume of 100 μl of DMEM supplemented with 10% FCS on the day before transfection. The gene encoding the murine H-2Kd (23) was cloned into plasmid pcDNA1.1/Amp, and COS cells were transiently transfected by the DEAE-dextran method (24) with 70 ng of DNA from the H-2Kd construct alone or together with competitor peptides followed by ligation to BstXI adapters (Stratage, La Jolla, CA), phosphorylation, and digestion with NotI. Size-fractionated cDNA were ligated into the BstXI and NotI sites of pcDNA3.1 downstream from the CMV promoter. Recombinant plasmids were electroporated into Escherichia coli Top10F+ and selected by ampicillin (100 μg/ml). Plasmid cDNA from pools of 100 transformed colonies were extracted using the QIAprep 8 plasmid kit (Qiagen, Hilden, Germany).

DNA sequencing and homology search

DNA sequencing was performed by the dideoxy-chain termination method either manually (Thermo Sequenase cycle sequencing kit, Amersham, Cleveland, OH) or on an Applied Biosystems Prism 310 Genetic Analyzer (Perkin Elmer Applied Biosystems, Norwalk, CT) using specific oligonucleotides as primers. The computer search for sequence homology was performed with BLAST (blast@ncbi.nlm.nih.gov) (National Library of Medicine, National Institutes of Health, Bethesda, MD).

Results

Characterization of P815E-specific CTLs

P815-specific CTLs were obtained previously by in vitro stimulation of splenocytes from mice immunized with P815. Some CTL clones showed lytic activity against an Ag-loss variant that had lost the expression of the Ags P815A, P815B, P815C, and P815D (Fig. 1A and Ref. 3). The additional Ag was named P815E.

To determine the MHC restriction element for these CTL clones, we studied the inhibitory effect of class I-specific mAbs on the production of TNF following antigenic stimulation (Fig. 1B). The anti-H-2Kd Ab SF1-1.1.1 was found to inhibit P815-induced TNF production by both CTL clones 89/62 and 351.1.E5, indicating that P815E was recognized on the MHC class I molecule H-2Kd.

Identification of a cDNA encoding P815E Ag

A directional cDNA library using poly(A)+ mRNA derived from the P815 subline P1 was prepared as described in Materials and Methods. We divided the library into 2300 pools of 100 recombinant clones and prepared DNA for transfection into COS cells together with the cDNA coding for H-2Kd as described above. After 24 h of incubation with the CTL clone 89/62, the level of TNF in the culture supernatant was assessed for each pool. One pool was shown to induce TNF production by the CTLs. This pool was subcloned, and DNA from single colonies were transfected as described above and tested for TNF induction. We found only one cDNA (B9.2) out of 1600 single colonies tested that induced the production of TNF from CTL 89/62 when transfected together with the class I restriction element H-2Kd (Fig. 2). The very low frequency of clones that were positive for the cDNA of interest appeared to result from the relatively slower growth rate of bacteria containing this cDNA.

Sequence and expression of the cDNA coding for P815E

The cDNA encoding P815E was 1326 bp long and was found to be 79% homologous with the bovine gene coding for methionine sulfone reductase (MrA), an enzyme involved in the reduction of protein methionine sulfone residues and methyl sulfone compounds (GenBank accession number U37150) (25). The full murine MrA sequence has not yet been published; however, a search within the expressed sequence tag bank produced almost the entire murine MrA sequence from normal mouse tissue. Comparison of the compiled murine MrA gene and the gene encoding P815E showed complete identity except for a single discrepancy at nucleotide position 704. This point mutation was confirmed by direct sequence analysis of the PCR product from normal mouse liver cDNA, showing that a guanine nucleotide in the normal gene was replaced by an adenine in the gene encoding P815E. The mutation resulted in an amino acid change at position 221 of the protein sequence, where a glycine was replaced by an arginine (Fig. 3). It appears thus far that the mutation is unique to mastocytoma P815, as sequence analyses of the PCR products from six other mastocytomas and three mouse leukemias were all negative for the P815E mutation.

We tested the expression of this gene in normal mouse adrenals, brain, bone marrow, colon, kidney, liver, lung, ovary, pancreas,
placenta, spleen, stomach, testes, and thymus. Reverse transcription products of mRNA were amplified by PCR using primers located at positions 264–281 and 1071–1094 of the P815E cDNA. A PCR product of 830 bp was obtained for each tissue tested, indicating ubiquitous expression of the MsrA gene (data not shown).

Identification of the P815E antigenic peptide
To determine whether the mutation was responsible for the recognition by CTL 89/62, we synthesized peptides that contained the modified residue and corresponded to the binding motif of the class I restriction element H-2K\(^d\). This motif is characterized by a tyrosine at position 2 and by a leucine, isoleucine, or valine at positions 9 or 10 (26). A decamer fulfilling the above criteria was identified at position 216–225 (Fig. 3) of the P815 murine MsrA protein sequence (GYCGLRGTGV). This peptide and a peptide corresponding to the normal protein sequence (GYCGLGGTGV) were synthesized and tested for recognition by CTL 89/62 (Fig. 4A). Only the peptide containing the changed residue was recognized by the CTL. However, the normal peptide was able to bind

![Nucleotide sequence of cDNA encoding the P815E Ag with the deduced amino acid sequence.](image-url)
to H-2K\(^d\), as increasing concentrations of both this peptide and a peptide known to bind to H-2K\(^d\) inhibited lysis of P815E-pulsed target cells (Fig. 4B). Therefore, we conclude that a nucleotide mutation in the murine MsrA gene of P815 gives rise to a new antigenic epitope recognized by CTLs generated in vivo.

**Lack of protection following immunization against P815E**

We have observed previously that a proportion of mice immunized i.p. with living cells of leukemia line L1210 transfected with both the gene P1A and the cDNA coding for the costimulatory molecule B7-1 (L1210.P1A.B7-1 cells) were protected against challenge with live P815 tumor cells (27). Following the identification of P815E, we were interested in testing both the relevance of P815E as a tumor-rejection Ag and the effectiveness of immunizing mice against two Ags expressed on the same tumor. Therefore, we transfected L1210 with the cDNAs coding for P815E and B7-1. Having established that P815E-specific CTLs could be generated in mice by immunization i.p. with 10\(^6\) live L1210.P1E.B7-1 cells (Fig. 5), we challenged immunized mice 3 wk later with 4 \times 10\(^5\) live P815 cells. Surprisingly, we found that the survival rate of mice immunized against P815E was similar to the nonimmunized controls (Fig. 6). Thus, contrary to P815AB, immunization against P815E did not appear to protect mice against P815 challenge, even though immunization with the Ags P815AB and P815E appears to produce CTL responses of similar magnitudes (Fig. 5). Moreover, we observed that immunization against both the P815AB and P815E Ags did not produce better protection than that induced by immunization against P815AB alone (Fig. 6).

**Discussion**

Tumor Ags can be grouped into several categories based on the pattern of expression of the protein from which they are derived (28). P815AB belongs to the category of tumor Ags that are shared between various tumors but not expressed on normal tissues (4). The expression of the gene coding for the Ag P815AB, P1A, is similar to that of the human MAGE-type genes, which are also silent in normal tissues except male germline cells and which are active in a significant proportion of tumors of various histological types (29–32). P815E belongs to the category of tumor Ags that result from tumor-specific mutations of genes that are ubiquitously expressed. Ags arising from mutations often appear to be unique to the tumor in which they were found. This seems to be the case for the P815E mutation, which was not detected in other tumors that we examined. Some antigenic mutations, however, are shared by different tumors and are usually mutations that cause oncogenesis, either favoring the uncontrolled growth of tumor cells or inhibiting apoptosis (33–35).
The P815E gene codes for MsrA, an enzyme that is capable of reducing methionine sulfoxide residues in proteins and is therefore thought to be important in controlling the oxidative stress induced by aerobic metabolism (36, 37). Enzymatic and gene expression studies of normal mouse and rat tissues has shown ubiquitous expression of this gene, although immunohistochemistry with MsrA-specific Abs has shown the specific localization of this protein to the retinal pigmented epithelium, alveolar macrophages, renal medulla, neurons, and central nervous system (36, 38). It is thought that these regions and cell lineages are subjected to higher metabolic rates, leading to oxidative environments and thereby requiring higher levels of MsrA (38). There is no evidence to suggest that the MsrA mutation we have found in P815 has an oncogenic function. This is in line with the unique occurrence of this mutation in P815.

Recently, immunization against P815AB was shown to protect mice against challenge with live P815 cells, but resistance to tumor growth was evident only in ~50% of the immunized animals (27). This result prompted us to ask whether immunizing mice against additional P815 Ags could improve this protection, and the identification and characterization of P815E Ag presented us with an opportunity to address this question. However, we found immunization against P815E alone to be completely ineffective in protecting mice against live tumor challenge, despite the fact that Ag-specific CTL activity was of a similar magnitude to that seen in mice immunized with P815AB. Immunizing mice against both the P815E and P815AB Ags did not improve resistance to tumor growth compared with P815AB alone.

The lack of protection observed after immunization with P815E Ag has relevance to a recent proposal that unique Ags resulting from mutations are likely to be more useful for cancer immunotherapy than tumor-specific shared Ags (39, 40). This proposal is based on two main arguments. The first is that shared Ags, being encoded by normal genes expressed in normal male germline cells, may generate some level of tolerance that prevents the most effective CTLs from coming into play. We agree that this possibility cannot be excluded a priori and believe that it is necessary to test it experimentally. The second argument is based on the past observation that immunizing animals with methylcholanthrene-induced tumors confers protection against the immunizing tumor but not against other methylcholanthrene-induced tumors (41). However, it is likely that, like the methylcholanthrene-induced tumor P815, these tumors carry several Ags, some of which may be unique and some of which may be shared. As a result, every tumor probably carries a unique set of Ags, and this alone could explain why protective immunization was effective on the same tumor and not on others.

An experiment directly addressing the protective ability of a shared Ag was performed by Ramarathnam et al., who reported that immunization with P815 failed to protect mice against tumors J558 and MethA, both of which share the P815AB Ag (42). However, they report that there is a certain degree of protection against J558 and, here again, the better protection against P815 may be due to several Ags not shared by MethA and J558. We have observed that immunization with tumor L1210 expressing P815AB can induce prolonged resistance to challenge with tumor P815. This protection can be attributed solely to the P815AB Ag, because P1A-transgenic mice, which are tolerant to P815AB, are not protected by immunization. Protection has also been observed after immunization with DNA encoding the P815AB Ag (13). Further, contrary to the notion that unique Ags may be more useful for immunotherapy, we found that immunization with the unique Ag P815E did not induce resistance to live P815 tumor challenge, even though it can induce a specific CTL response.

The reason why no protection was observed after immunization with P815E despite a good induction of CTLs is not clear. One explanation might be that the Ag is present on P815 cells at a low level or in an unstable form, which could prevent effective lysis in vivo. In support of this possibility, we have observed that anti-P815E CTLs lyse P815 targets in vitro with less efficiency than P815AB-specific CTLs (Fig. 1; C. Uyttenhove, unpublished observations). The fact that earlier studies detected significant levels of anti-P815E CTLs in P815-bearing animals implies that a low level of Ag expression on P815 cells would still be sufficient to stimulate the CTLs to proliferate (3, 6).

We do not wish to suggest that unique Ags will be ineffective for tumor immunotherapy, but our results indicate that each Ag needs to be assessed individually, not only for its ability to induce lymphocyte responses, but also for its ability to induce tumor rejection.

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