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TNF-Mediated Toxicity After Massive Induction of Specific CD8$^+$ T Cells Following Immunization of Mice with a Tumor-Specific Peptide

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We immunized mice with antigenic peptide P815E, which is presented by H-2K$^d$ and recognized by tumor-specific CTL raised against P815 tumor cells. This peptide is encoded by the ubiquitously expressed gene MsrA and carries a mutated residue conferring tumor specificity. Unexpectedly, we observed a severe toxicity occurring in the early hours after the third injection, resulting in the death of most mice within 24 h. The toxic syndrome was reminiscent of TNF-induced shock, and the sera of ill mice contained high levels of TNF. Toxicity was prevented by injection of neutralizing anti-TNF Abs, confirming the involvement of TNF. Depletion of CD8$^+$ T cells could also prevent toxicity, and ex vivo experiments confirmed that CD8$^+$ lymphocytes were the major cellular source of TNF in immunized mice. Tetramer analysis of the lymphocytes of immunized mice indicated a massive expansion of P815E-specific T cells, up to >60% of circulating CD8$^+$ lymphocytes. A similar toxicity was observed after massive expansion of specific CD8$^+$ T cells following immunization with another P815 peptide, which is encoded by gene P1A and was injected in a form covalently linked to an immunostimulatory peptide derived from IL-1. We conclude that the toxicity is caused by specific CD8$^+$ lymphocytes, which are extensively amplified by peptide immunization in a QS21-based adjuvant and produce toxic levels of TNF upon further stimulation with the peptide. Our results suggest that immunotherapy trials involving new peptides should be pursued with caution and should include a careful monitoring of the T cell response. The Journal of Immunology, 2002, 169: 3053–3060.

To better understand the processes required to induce antitumor immunity, we have used a murine mastocytoma tumor model that has been widely used for the study of antitumor immune responses. Murine mastocytoma P815 can induce CTL responses to at least four distinct Ags (AB, C, D, and E). P815AB was the first of these Ags to be identified. It belongs to the category of tumor-specific common Ags and is encoded by gene P1A, a gene silent in normal tissues except in spermatogonia, which do not express MHC class I molecules (1, 2). P815E, the second Ag to be identified on P815, is a tumor-specific individual Ag that arises as a result of a mutation within an ubiquitously expressed gene known as methionine sulfoxide reductase (MsrA) (3).

In previous years, Ag P815AB has been the focus of a number of studies as a model for designing and optimizing antitumor vaccines, including vaccines based on naked DNA, synthetic peptide, purified protein, pulsed dendritic cells, and recombinant viruses (4–9). CTL responses against Ag P815AB were achieved with naked DNA (6), viral vectors (10), or peptide-pulsed APC injected with IL-12 (8). The strongest CTL responses were obtained after injection of living cells of the MHC-matched leukemia line L1210, previously transfected with genes P1A and B7-1 (7). Such responses provided protection against a challenge with a lethal dose of P815 tumor cells, indicating that P815AB is a major tumor rejection Ag on P815 (6–8, 10). In contrast, protection against P815 challenge could not be achieved using L1210 leukemia cells transfected with P815E and B7-1 (L1210.P1E.B7-1), even though Ag-specific CTL were generated as a result of immunization (3). We have previously demonstrated that strong CTL responses against tumor-specific Ags could also be induced with synthetic peptides combined with IL-12 and an adjuvant containing QS21 and monophosphoryl lipid A (MPL) (9). Used with the P1A peptide, this protocol was well tolerated and induced strong CTL responses against Ag P815AB. We therefore used this protocol to immunize mice with peptide P815E for the purpose of assessing the induction of P815E-specific CTL in vivo and evaluating protection against tumor challenge. We report here that considerable toxicity was observed.

Materials and Methods

Mice

Syngeneic DBA/2 mice were raised in specific pathogen-free conditions. All mice used in these experiments were between 8 and 12 wk old.

Peptides

All peptides were synthesized in-house on solid phase using F-moc for transient NH$_2$-terminal protection according to the study of Atherton et al. 5

Abbreviations used in this paper: MPL, monophosphoryl lipid A; m, murine.
and were characterized using mass spectrometry. All peptides used in this study were purified by HPLC to >99% homogeneity. They were stored at −20°C at 20 mg/ml in DMSO. The single-letter code sequences of the peptides used are as follows: H-2Kd-restricted P815E, GYGCGLRTTGV; (3); H-2Ld-restricted P815A (positions 35–43 of the P1A protein), LPYLGLVLVFQGEGSNDK (13); and H-2Kd-restricted P198, KYQAVTTLT (14).

Cell lines

All of the experiments using cells referred to as P815 were performed with an azaguanine-resistant clone of cell line P815, named P511. Cell line L1210.P16.B7-1 has been described previously (3). All cell lines were cultured in DMEM (Life Technologies, Grand Island, NY) supplemented with 20% FCS in a humidified, 8% CO2 atmosphere at 37°C.

Adjuvant

The SBAS-1c adjuvant, comprising MPL and liposomal DQS21 (Quil A, GSK) was used in all experiments. Following this treatment and tested for the presence of CD4+ and CD8+ lymphocytes were isolated. The cell lines were cultured in DMEM supplemented with 20 U/ml recombinant human IL-2. CTL clones appeared 2–3 wk after the first stimulation.

Collection of plasma for kinetic studies

Mice were heated in an incubator at 42°C for 30 min before collection of blood samples. A small sample of blood was collected via the tail vein in a 75-mm/75-μl hematocrit-capillary tube containing sodium-heparin (Hirschmann Laboratories, Eberstadt, Germany). The hematocrit tube was centrifuged using a capillary tube rotor at 8000 rpm for 5 min. Separated plasma was collected into small Eppendorf tubes and stored at −20°C before testing. In the experiment reported in Fig. 6, mice were bled at the retro-orbital sinus. Blood samples were incubated 30 min at 37°C to allow clotting before centrifugation at 10,000 rpm for 5 min. Sera samples were stored at −20°C in flat-bottom microplates before testing.

Tetramer staining and FACS analysis

Cells were stained (105 cells/ml) for 15 min at room temperature in FACS buffer containing 1% FCS, 5 μg/ml anti-mouse CD16/CD32 (BD Pharmingen, San Diego, CA) and the appropriate PE-tetramer at 50–100 nM H-2 molecules. Then anti-CD3-FITC (BD Pharmingen) and anti-CD8-PerCP (BD Pharmingen) labeled Abs were added and cells were incubated for 15 additional min at room temperature. Cells were washed in FACS buffer and analyzed on a FACSScan flow cytometer (BD Biosciences, San Jose, CA).

Cloning of H-2Kd-P815E-tetramer-positive cells

PBL were isolated from mice 15 days after immunization with 105 living L1210.P16.B7-1 cells i.p. or 10 days after the second injection of peptide P815E with adjuvant and IL-12. Single CD8+ cells were isolated by HPLC to >99% homogeneity. They were stored as the concentration that stimulated half-maximal proliferation was determined as the dilution of the antiserum needed to inhibit 1 IU/ml TNF. Fifty microliters of the antiserum completely protected mice from a lethal challenge of LPS in combination with the sensitizing D-(+)-galactosamine. The antiserum was injected i.p. 2 h before the third immunization with peptide P815E.

Thalidomide treatment

Mice were injected i.p. with 100 mg/kg thalidomide (Sigma-Aldrich) dissolved in PBS.

TNF assay

Plasma samples from mice were serially diluted before addition to wells of flat-bottom 96-well plates. The level of TNF present in each plasma sample was assessed using the TNF-sensitive WEHI-164 clone 13 (21) in a MTT colorimetric assay (22). TNF quantification was made possible by the addition of a LT standard curve and all samples were tested at the same time.

Results

Immunization of mice with peptide P815E induces a lethal toxicity

DBA/2 mice were injected i.p. in the footpad at weekly intervals with 50 μg of purified synthetic peptide P815E combined with a human-use adjuvant and 300 U of IL-12. Surprisingly, we observed that after the third immunization the majority of mice suffered from a toxic shock-type syndrome resulting in death within 24 h after the injection. The mice that survived the third immunization succumbed after a fourth injection (Fig. 1A). The first signs of peptide-induced toxicity usually appeared within 5 h of peptide
immunization. The symptoms included loss of temperature (~4°C below normal), diarrhea, piloerection, lethargy, and loss of balance.

This toxicity was not caused directly by the adjuvant/IL-12 combination, as mice receiving two complete immunizations (peptide/adjuvant/IL-12) followed by two injections of adjuvant/IL-12 without peptide did not show any signs of toxicity (Fig. 1B). Moreover, a third injection with peptide P815E alone was sufficient to induce toxicity in mice having received two complete immunizations, further demonstrating that the toxicity was not caused directly by the adjuvant and/or IL-12 (Fig. 1C–E). This, along with our previous observation that the same immunization mode used with other peptides does not cause such toxicity, indicated that the toxicity was related to the response to the P815E peptide (9).

We previously observed that the peptide corresponding to the nonmutated sequence of the MsrA gene (wtMsrA) was capable of binding to H-2K^d (3). It was possible therefore that the toxicity resulted from the induction of an immune response cross-reactive with the wild-type peptide, thereby resulting in autoimmunity. However, we observed no toxicity when mice received two complete immunizations with peptide P815E followed by multiple boost injections with the wild-type MsrA peptide combined with adjuvant and IL-12 (Fig. 1F).

Role of TNF in the peptide-induced toxicity

Histological examination of tissues taken from animals before death showed evidence of hepatic panlobular necrosis due to microthrombi formation, acute tubular necrosis, “ischemic” colitis, and enteritis. Moreover, a large number of circulating cells comprised mostly of blastic CD3^+ cells with a small number of CD45R^+ cells (presumably B cells) and F4/80^+ cells (macrophages) were present in the blood vessels of peptide-immunized animals but not in samples from normal mice (data not shown). The symptoms listed above were similar to those reported for anti-CD3-mediated toxicity and LPS-induced shock, which are both known to induce a rapid increase in circulating TNF-α levels (23, 24).

We therefore tested TNF levels in the plasma of animals collected at four time points (30 min, 4 h, 8 h, and 24 h) following the last peptide immunization and compared these levels with those in plasma collected the day before immunization. Groups of animals were immunized with adjuvant plus IL-12 along with peptide P815E, PBS, or peptide P198, which also results from a mutation and is expressed by an immunogenic variant derived from P815 tumor cells (14). The latter peptide does not cause toxicity when injected in those conditions (9). The results showed a peak in TNF production 30 min after immunization for all animals, including those given PBS plus adjuvant and IL-12 (Fig. 2). TNF levels in animals immunized with PBS or P198 dropped quickly to 221 and 280 pg/ml, respectively, at the 4-h time point (means of 4 and 9 animals, respectively). However, TNF levels in plasma of P815E-peptide-immunized mice remained elevated, with a level of 9379 pg/ml at the 4-h time point (mean of eight animals) (Fig. 2). Sustained high levels of circulating TNF were also observed in mice receiving peptide without adjuvant and IL-12 at the third injection, with a level of 19,526 pg/ml at the 4-h time point (mean of six animals).

To determine whether those sustained levels of TNF were responsible for the lethal toxicity observed, we injected mice i.p. with neutralizing anti-TNF-α Abs 2 h before a third immunization with peptide P815E with adjuvant and IL-12. As shown in Table I, the highest dose of anti-TNF Abs provided complete protection against the lethal toxicity. This was also true with mice receiving peptide P815E alone at the third injection, in which 10 of 10 mice were protected with 100 μl of Abs as opposed to 9 of 11 dead mice in the control group (data not shown). The role of TNF in toxicity was further confirmed when we observed that treatment of mice with thalidomide, which is known to inhibit TNF-α production, allowed all mice to survive a third, a fourth, and even a fifth immunization with peptide P815E (Table I) (25, 26).

**CD8^+ lymphocytes are the major source of TNF**

Soluble TNF has been shown to play a significant role in bacterial endotoxin- and exotoxin-induced toxic shock (27–29). Lymphocytes appear to be the major source of TNF in the shock induced by bacterial exotoxins such as staphylococcal enterotoxin B, which...
have superantigenic properties resulting in massive T cell proliferation. SCID mice are not sensitive to staphylococcal enterotoxin B and only become sensitive upon T cell repopulation (30). On the other hand, SCID mice are sensitive to the shock induced by endotoxins such as LPS, which appear to trigger TNF production by macrophages (30).

We observed that SCID mice were not sensitive to the toxicity induced by immunization with peptide P815E, indicating that lymphocytes were most likely required for lethality (data not shown). The toxic response was observed in various strains of immuno-competent H-2d animals, although BALB/c mice were less sensitive than B10.D2 or DBA/2 animals (data not shown).

To confirm the involvement of lymphocytes and to determine which T cell subset may be directly implicated in TNF production, we isolated CD8+ T cells, CD4+ T cells, and B lymphocytes (B220+) from the spleen of animals 1 h after the third immunization with peptide P815E. Cell subsets were collected by magnetic separation and incubated in culture medium for 2 h at 37°C. Comparison of the TNF levels in the supernatant showed that the CD8+ lymphocyte population secreted most of the TNF (Fig. 3A). In agreement with those results, we observed that mice depleted of CD8+ lymphocytes were completely protected against toxic shock, whereas CD4+ -depleted mice were not (Fig. 3B). TNF levels in the plasma of CD8+ -depleted animals were lower than in the plasma of intact mice or CD4+ -depleted mice, which is in line with the notion that TNF is the main mediator of toxicity (Fig. 3B).

Massive expansion of anti-P815E CD8+ T cells in peptide-immunized mice

The development of toxicity was dependent on the presence of the P815E peptide in the immunization mixture and CD8+ lymphocytes in the recipient mouse, which suggested the involvement of P815E-specific CD8+ lymphocytes. We used H-2K4/P815E tetramers to estimate the number of P815E-specific CD8+ T cells among blood lymphocytes (PBL) after the second peptide immunization by ex vivo FACS analysis. Between 45 and 75% of CD8+ blood lymphocytes were labeled with the tetramer, indicating a massive expansion of Ag-specific CTL (Fig. 4). In contrast, the expansion of tetramer-positive cells in the PBL of mice immunized with L1210.P1E.B7-1 cells was much lower, which is in line with the lack of toxicity after immunization with those cells (Fig. 4) (3).

These data therefore suggest that development of toxicity is determined by the absolute number of specific T cells induced by the immunization.

To determine whether the massive expansion of P815E-specific CTL was the sole factor determining toxicity or whether the CTL induced after peptide immunization were also qualitatively different, we compared the activity of P815E-specific CTL clones isolated from mice immunized either with peptide P815E/adjuvant/IL-12 or with L1210.P1E.B7-1 cells. CD3+CD8+H-2K4/P815E-positive CTL were isolated and cloned directly from the blood of immunized animals using FACS. A total of 36 growing clones were obtained, 25 from mice immunized with peptide and 11 from...
mice immunized with L1210.P1E.B7-1 cells. They were tested for lytic activity and TNF production against P815 cells pulsed or not with peptide P815E. All clones were equally able to lyse P815 cells (data not shown) and produce TNF upon stimulation by P815 cells (Fig. 5). Although it is not impossible that the in vitro amplification has modified the activity of the clones, these results suggest that there is no qualitative difference in the effector function of the CD8$^+$ cells induced by the two types of immunization, and therefore the absolute number of CD8$^+$ cells appears to be the sole factor responsible for toxicity after peptide immunization. Irrespective of their origin, all clones also produced much more TNF after stimulation with peptide-pulsed P815 cells than with unpulsed cells (Fig. 5). This could explain why no toxicity was observed when L1210.P1E.B7-1 cells were injected into mice immunized twice with peptide/adjuvant/IL-12 (Fig. 1G).

Immunization with another tumor-derived peptide can also induce toxicity

To determine whether the toxicity we observed after massive expansion of specific CD8$^+$ T cells was peculiar to Ag P815E or
Death of the mouse. 

represents the percentage of tetramer-positive cells among CD8 F

Expansion of speci
P815A, the other de
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would also occur with other antigenic peptides, we used Ag
P815A, the other defined Ag of tumor P815, which is encoded by
gene P1A and presented by H-2L d (12). A number of reports have
described various immunization protocols against this Ag, some of
them leading to antitumor effects, without detectable toxicity (5–7,
9). To induce a greater expansion of CD8 + T cells against Ag
P815A, we immunized mice with a chimeric peptide (P1A-VIL1)
composed of the P1A antigenic peptide of nine amino acids co-
valently linked at its C terminus to a nine-residue peptide derived
from human IL-1 B, which has been reported to have the potent
immunostimulatory effects of IL-1 without its toxicity (13). We
observed the sudden death of 9 of 10 mice after the fourth immu-
nization with the chimeric peptide, whereas mice receiving the
P1A antigenic peptide alone or the IL-1 peptide alone were nor-
mal. In repeated experiments, we observed varying proportions of
mice dying after the fourth injection of P1A-VIL1 (3/10, 9/10,
9/20, and 9/20). Mice that survived the fourth injection often suc-
cumbed after a fifth injection. Because this toxicity looked very
similar to the toxicity following immunization with the P815E
peptide, we measured TNF levels in the serum of mice immunized
either with chimeric peptide P1A-VIL1 or with peptide P1A. We
observed higher and sustained levels of TNF in the mice immu-
nized with P1A-VIL1, suggesting the involvement of TNF in the
toxicity (Fig. 6). To see whether the toxicity was related to a mas-
tive expansion of specific CD8 + T cells, we used H-2L d-P1A tet-
ramers to stain PBL of immunized mice ex vivo. We observed an
approximate 8-fold increase in the percentage of H-2L d-P1A-pos-
tive cells among CD8 + cells in the blood of mice immunized with
P1A-VIL1 as compared with mice immunized with P1A (Fig. 7).
These results suggest that the linkage of the IL-1 peptide to the
P1A peptide allowed a great expansion of specific CD8 + T cells,
which results in TNF-mediated toxicity after repetitive immu-
nizations. Our data also indicate that toxicity can occur with different
antigenic peptides when massive CD8 + T cell responses are
induced.

Discussion

The toxicity reported here appears to result from TNF produced by
specific CD8 + T cells which were enormously expanded in mice
immunized with peptide P815E. The immunizations were per-
formed by footpad injections of peptide mixed with a QS21-based
adjuvant and IL-12 according to a protocol that we had optimized
to induce good CTL responses with various tumor antigenic pep-
tides (9). Mice receiving only the adjuvant and IL-12 at the third
injection showed a transient peak of circulating TNF (Fig. 2),
which might be due to MPL, one of the components of the adju-
vant, which is known to trigger TNF production by macrophages
(31). However, circulating TNF in those mice quickly dropped to
basal levels and did not cause toxicity. Therefore, neither the
adjuvant nor the IL-12 was directly responsible for the toxicity,
which was also triggered when the third injection was performed
with peptide alone. The presence of the adjuvant and IL-12 during
the first two injections did not cause immediate toxicity, but
allowed the induction of very large numbers of specific CD8 + T
cells.

Toxicity was also observed in mice immunized with other pep-
tides provided a strong expansion of specific CD8 + T cells was
achieved. For the P1A peptide, this required the adjuvant of an
IL-1 sequence to the peptide (Figs. 6 and 7). For peptide P198, in
experiments not reported here, we observed toxicity in some mice
after numerous (more than six) repetitive immunizations with pep-
tide/adjuvant/IL-12 which induced strong CTL responses. Never-
theless, it appears that an expansion of specific CD8 + lymphocytes
occurs much more readily after immunization with peptide P815E
than with other peptides. The reason for this is unclear. It could be
speculated that in a normal immune response, a number of regu-
latory mechanisms are responsible for maintaining the CD8 + re-
sponse within certain limits and that, for some reason, such mech-
anism would be less active or even ineffective to control the
response to peptide P815E. In this respect, it is interesting to note
that the CD8 + response to peptide P815E is CD4 + independent
(Fig. 3B) as opposed, for example, to the response to the P1A
peptide, which is known to be CD4 + dependent (Ref. 32 and data
not shown). Involvement of regulatory mechanisms could also ex-
plain why a large expansion of CD8 + T cells was not observed
when mice were immunized with L1210.P1.E.B7-1 cells instead of peptide P815E. The L1210.P1.E.B7-1 cells may trigger such regulatory mechanisms more efficiently than the peptide, e.g., through B7-1-CTLA4 interactions.

Toxicity after peptide immunization has not been reported before. The only undesired phenomenon described is the induction of T cell unresponsiveness after injection of mice with high doses of peptide (500 μg) (33, 34). Injected at this dose into naive mice, a lymphocytic choriomeningitis virus peptide was found to induce specific T cell tolerance, presumably resulting from presentation of the peptide to naive T cells by nonprofessional APC (35). When the same peptide was injected at the same dose into lymphocytic choriomeningitis virus-immune mice, general immunosuppression associated with spleen damage was observed. This nonfatal spleen immunopathology was believed to result from the destruction of peptide-loaded spleen cells by Ag-specific CTL in vivo and was shown to be dependent on cell-cell contact and therefore not mediated by cytokines (35). The toxicity report we here is different from this immunopathology, because it is lethal and clearly mediated by a cytokine.

Our results indicate that peptide immunization can be dangerous in certain circumstances, since very strong CTL responses can be induced and can result in cytokine-mediated toxicity. Caution should be taken however in extrapolating our observations to the human situation in view of the very large difference in the peptide dose used relative to the size of the organism. We have used 50 μg of peptide for each injection in mice. In human peptide vaccination trials, the dose of peptide injected is typically between 30 μg and 1 mg (36, 37), whereas the human body volume is ~3000 times larger than that of a mouse. We have tried to reduce the peptide dose in our mouse immunizations, and this clearly resulted in reduced incidence of toxicity. But it also reduced the induction of CTL (data not shown). This suggests that there is a minimal amount of peptide required for efficient induction of T cells locally, either at the vaccine site or in the draining lymph node. Because there is no reason to believe that this “local” threshold is different in mice and humans, it is possible that a dose of 50 μg of a putative human peptide with properties similar to peptide P815E would induce good T cell responses in humans. Yet toxicity could be avoided because an expansion of CD8+ T cells to the same absolute numbers would result in lower proportions of specific CD8+ as they would be diluted among 3000 times more lymphocytes than in a mouse. Similarly, the same amount of TNF secreted by those expanded CD8+ T cells would be diluted in a much larger volume of body fluid. On the other hand, it is not impossible that humans are more sensitive to the toxicity of TNF than mice. For all of these reasons, it is difficult to extrapolate our observations to the human situation, but our results nevertheless suggest that new peptide immunization trials should be applied with caution in humans, using escalating dose regimens of the different vaccine components combined with a careful clinical surveillance and evaluation of the T cell response, including a monitoring of the expansion of specific CD8+ T cells with tetramers or similar reagents.

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