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Protection against Lethal Vaccinia Virus Challenge in HLA-A2 Transgenic Mice by Immunization with a Single CD8$^+$ T-Cell Peptide Epitope of Vaccinia and Variola Viruses

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CD8$^+$ T lymphocytes have been shown to be involved in controlling poxvirus infection, but no protective cytotoxic T-lymphocyte (CTL) epitopes are defined for variola virus, the causative agent of smallpox, or for vaccinia virus. Of several peptides in vaccinia virus predicted to bind HLA-A2.1, three, VETFsm(498-506), A26L(6-14), and HRP2(74-82), were found to bind HLA-A2.1. Splenocytes from HLA-A2.1 transgenic mice immunized with vaccinia virus responded only to HRP2(74-82) at 1 week and to all three epitopes by ex vivo enzyme-linked immunosorbent spot (ELISPOT) assay at 4 weeks postimmunization. To determine if these epitopes could elicit a protective CD8$^+$ T-cell response, we challenged peptide-immunized HLA-A2.1 transgenic mice intranasally with a lethal dose of the WR strain of vaccinia virus. HRP2(74-82) peptide-immunized mice recovered from infection, while naïve mice died. Depletion of CD8$^+$ T cells eliminated protection. Protection of HHD-2 mice, lacking mouse class I major histocompatibility complex molecules, implicates CTLs restricted by human HLA-A2.1 as mediators of protection. These results suggest that HRP2(74-82), which is shared between vaccinia and variola viruses, may be a CD8$^+$ T-cell epitope of vaccinia virus that will provide cross-protection against smallpox in HLA-A2.1-positive individuals, representing almost half the population.

Smallpox vaccination was discontinued in the United States in 1972, and the disease was declared eradicated worldwide in the 1980s. However, recent events have raised concerns about the possible use of smallpox as a bioterror agent (32, 49). In addition, with more widespread travel and encroachment of human populations into new areas, there have been new emerging infections, such as monkeypox, which could threaten the population (20, 34, 54). As a consequence, there has been a renewed interest in immunization against smallpox and other poxvirus infections. However, there are concerns about the safety of the present vaccine (33). In addition, with the advent of AIDS, as well as the widespread use of immunosuppressive drugs for transplantation and chemotherapy for cancer, there is greater concern about possible side effects of the present vaccine. It is therefore of great importance to develop new types of vaccines that can protect against smallpox but which do not have the risk of side effects or the potential for spread of infection that the present vaccine carries.

The immune correlates of protection against poxviruses are still largely unknown. Early studies carried out in the 1970s (22, 28–30), before smallpox was fully eradicated, indicated that cytolytic T cells played a role in protection against poxvirus infection, although CD8 as a marker of cytolytic T cells was not known at the time. Recent studies indicate an important role for antibodies as well as T cells and gamma interferon (IFN-γ) (9, 26). Smallpox was virtually eradicated at the time that T lymphocytes were being discovered, so little is known about the importance of CD8$^+$ T cells in controlling the infection. Moreover, poxviruses are large viruses with a large number of potential CD8$^+$ T-cell epitopes. Until recently (18, 58), no CD8$^+$ T-cell epitopes had been defined for variola virus, the causative agent of smallpox, or for vaccinia virus, which has been used for vaccination against smallpox, and the role of these newly discovered CD8$^+$ T-cell epitopes in protection is unknown. In light of the need for a new generation of vaccines against poxviruses, it is now of interest to define more thoroughly the immune response to these viruses.

Some recent studies have demonstrated the efficacy of replication-incompetent vaccinia viruses, such as modified vaccinia virus Ankara (MVA) (9, 18, 37, 55, 56) or NYVAC (9, 57), in conferring immunity. Some of these vectors are in human trials. However, the protective efficacy of these vaccines cannot ethically be assessed in humans. Recent studies from our group and others (9, 18) have made use of a mouse model of protection against poxviruses. This involved immunization with a replication-deficient strain of vaccinia virus, followed by intranasal challenge with wild-type vaccinia virus. In these previous studies, vaccinia virus was used for both immunization and challenge.

In the present study, we identified new class I HLA-A2-restricted CD8$^+$ T-cell epitopes of vaccinia and variola viruses. We screened for a response to these epitopes in HLA-A2 transgenic mice that had been immunized with an attenuated, thymidine kinase-deficient vaccinia virus (vSC8). In addition,

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we immunized mice expressing the human HLA-A2 molecule with a peptide vaccine containing one of the epitopes, which gave a strong response following vaccinia virus immunization, and found that peptide immunization to this single epitope could confer a survival advantage for a lethal poxvirus challenge in the immunized HLA-A2 transgenic mice. Moreover, we demonstrate that the immune response following challenge is skewed toward this protective epitope in immunized HLA-A2 transgenic mice. This study is important for understanding the role of $CD8^+$ T cells in survival of a poxvirus infection and for development of newer, safer vaccines against smallpox.

MATERIALS AND METHODS

Peptides. Vaccinia and variola virus peptide epitopes were predicted using the BIMAS program (43, 44). Peptides were synthesized on an automated peptide synthesizer (catalog no. 430A; Applied Biosystems, Foster City, Calif.) or obtained from Multiple Peptide Systems (San Diego, Calif.).

Mice. HHD-2 mice were developed in the Lemonnier lab (21, 45). These are knocked out for β2 microglobulin and $H\text{-}2D^b$ and transgenic for a chimeric HLA-A2.1 with the α3 domain derived from $H\text{-}2D^b$ to allow interaction with murine CD8 and a covalently attached human β2 microglobulin chain. Therefore, they express no mouse class I molecules, only human HLA-A2.1. The AAD mice (17, 38), a gift of Victor Engelhard (University of Virginia, Charlottesville), and A2Kb mice (60), a gift of Linda Sherman (Scripps Research Institute, La Jolla, Calif.), similarly express chimeric HLA-A2.1 with an α3 domain from $H\text{-}2D^d$ or $H\text{-}2K^b$, respectively, but retain expression of murine class I $H\text{-}2K^b$ and $H\text{-}2D^b$. All three strains are on the C57BL/6 background. All mice were bred at the National Cancer Institute or BioCon, Inc. (Rockville, Md.), and used at 8 to 36 weeks of age.

Cells. The T2 cell line, a gift of Peter Cresswell, is deficient in TAP1 and TAP2 transporter proteins and expresses low levels of HLA-A2.1 (51, 53). Cell line C1R.AAD (HMYC1R transfected with the HLA chimeric molecule containing α1 and α2 domains from human HLA-A2.1 and α3 from mouse $H\text{-}2D^d$) has been previously described (38). Cell lines were maintained in complete medium (RPMI 1640 containing 10% fetal bovine serum [FBS], 4 mM glutamine, 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 50 μM 2-mercaptoethanol). For T-cell lines, we added 10% T-STIM (rat T-STIM from BD Discovery Labware, Inc., Bedford, Mass.).

Viruses. The Western Reserve (WR) strain of vaccinia virus, utilized for intranasal challenge, was obtained from the laboratory of Steve Feinstone (Food and Drug Administration-Center for Biologics Evaluation and Research). The vSC8 vaccinia virus strain, used for intraperitoneal (i.p.) immunization as a positive control, is a recombinant thymidine kinase-negative WR strain-based vaccinia virus expressing β-galactosidase (16) (a gift of Patricia Earl and Bernard Moss, National Institute of Allergy and Infectious Diseases).

T2 peptide binding assays. Peptide binding to HLA molecules was measured using the T2 mutant cell line according to a protocol previously described (39). Briefly, T2 cells (2×10^5 to 3×10^5/well) were incubated overnight in 96-well plates with culture medium (RPMI 1640 containing 2.5% FBS, 100 U of penicillin/ml, 100 μg of streptomycin/ml, 50 μM 2-mercaptoethanol) with 10 μg of β2-microglobulin (Sigma Chemical Co., St. Louis, Mo.)/ml and different peptide concentrations. The next day, cells were washed twice with cold phosphate-buffered saline containing 2% FBS and incubated for 30 min at 4°C with anti-HLA-A2.1 BB7.2 monoclonal antibody (1/100 dilution from hybridoma supernatant) followed by 2.5 μg of fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin (PharMingen, San Diego, Calif.)/ml for 30 min. Cells were washed twice after each incubation, and HLA-A2.1 expression was measured by flow cytometry (FACSCaliber; Becton Dickinson, Mountain View, Calif.). HLA-A2.1 expression was quantified according to the formula [(mean fluorescence with peptide − mean fluorescence without peptide)/mean fluorescence without peptide] × 100.

Immunizations and challenges. HHD-2 or A2Kb mice were immunized either once i.p. with 1×10^7 to 2×10^7 PFU of vSC8 in phosphate-buffered saline or three times subcutaneously (s.c.) in the base of the tail (4) at 2-week intervals with an emulsion of the individual vaccinia virus peptides (50 nmol per mouse) with 50 nmol of a hepatitis B virus core helper peptide, 5 μg of granulocyte-macrophage colony-stimulating factor (GM-CSF), and 800 IU of interleukin 2 (IL-2), 1:1 with incomplete Freund's adjuvant. One month after immunization, mice were lightly anesthetized with methoxyfluorane and challenged intranasally with 10^6 PFU of the WR strain of virulent vaccinia virus (10 μl of a 5×10^7-PFU/ml stock was administered in each nostril with a Pipetman) (12), and individual body weight was measured daily. Mice with weight loss of >25% were euthanized according to National Institutes of Health (NIH) guidelines.

CD8 depletions. Some of the peptide-immunized mice were depleted of $CD8^+$ T cells by 3× i.p. injections of anti-CD8 antibodies (prepared by GTC Washington Labs) at 8, 7, and 3 days prior to intranasal challenge with wild-type WR vaccinia virus. CD8 depletion was measured by flow cytometry analysis of peripheral blood lymphocytes. In addition, we screened the spleens and lymph nodes of treated mice compared with untreated mice to confirm that the CD8 depletion was complete following this treatment.

IFN-γ ELISPOT assay. Enzyme-linked immunorsorbent spot (ELISPOT) plates (Millipore) were precoated overnight with anti-IFN-γ antibody (MABTECH, Stockholm, Sweden). Target cells (C1R.AAD) were either infected overnight with vSC8 vaccinia virus (16) and UV-irradiated for 20 min or pulsed overnight with vaccinia virus peptides. Splenic effector cells were mixed with infected or peptide-pulsed target cells and centrifuged together in conical tubes or 96-well round-bottomed plates for 5 min at 200 × *g*. Cells were cocultivated for 1 h at 37°C and then transferred to the ELISPOT plate in a volume of 100 μl/well. After 18 to 20 h of cocultivation, IFN-γ spot-forming cells were developed by secondary anti-IFN-γ antibody (MABTECH), a Vectastain ABC kit (Vector Laboratories), and a 3-amino-9-ethylcarbazole substrate kit (Vector Laboratories).

Cytotoxicity assay. Cytotoxic T-lymphocyte (CTL) activity was measured using a 5-h assay with ^{51}Cr-labeled target cells. Target cells (5×10^5 C1R.AAD cells) were either unpulsed (control targets), pulsed overnight with 50 μM peptide in 200 μl of complete medium, or infected overnight with vaccinia virus strain vSC8 (100:1 virus/target ratio in medium without serum, followed by addition of complete medium and 20 min of UV treatment to kill free virus particles) and then incubated with 200 μCi of ^{51}Cr for 2 h, washed three times, and added to the plates containing different numbers of effector cells in a final volume of 200 μl. Free peptide (1 μM) was added back during the incubation with CTLs. In peptide titration assays, target cells were pulsed with different peptide concentrations along with ^{51}Cr for 2 h, washed three times, and added to plates with effectors at a 50:1 effector/target cell ratio. Supernatants were harvested and counted after 5 h of incubation. The percentage of specific ^{51}Cr release was calculated as: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). Spontaneous release was determined from target cells incubated without effector cells, and maximum release was determined in the presence of 5% Triton X-100 (no. 3786; Research Products International, Mount Prospect, Ill.) (13, 52).

Statistical analysis. Statistical analysis was carried out using a paired Student *t* test comparing weight loss and survival in groups of immunized and unimmunized mice following vaccinia virus challenge.

RESULTS

Identification of peptide epitopes. The immunological mechanisms of protection against poxviruses are largely unknown, although recent studies suggest that both antibodies and T cells may play different but important roles (9, 26). The cell-mediated (T-cell) response may be important in protection, but the $CD8^+$ T-cell response in humans to poxviruses has not been well characterized. We identified potential HLA-A2 binding epitopes of vaccinia virus, since this HLA type accounts for nearly half the U.S. population. We obtained the entire vaccinia virus sequence from the Swiss-Prot database and searched for HLA-A2 binding epitopes using the BIMAS program (43, 44). We narrowed our search to epitopes that are identical in all the known strains of vaccinia virus and either identical or highly homologous in variola virus (smallpox virus). Three of these peptides, from the vaccinia virus early transcription factor (VETF) small subunit [VETFsm(498-506)], from a putative A-type inclusion protein [A26L(6-14)], and from host range protein 2 [HRP2(74-82)], were studied further and are described in Table 1. These peptides were tested for HLA-A2 binding by the standard T2 binding assay (39, 52). As shown in Fig. 1, A26L(6-14) was the highest-

TABLE 1. Sequences of vaccinia and variola virus peptide epitopes

Peptide name	Sequence in vaccinia virus	MHC restriction	Protein	Sequence in variola virus
VETFsm(498–506)	VLPFDIKKL (9-mer); residues 498–506	HLA-A2 and H2-Db	VETF small subunit	Identical
A26L(6–14)	NLWNGIVPT (9-mer); residues 6–14	HLA-A2 and H2-Db	A-type inclusion protein	Homology = NLWNGIVPM
HRP2(74–82)	KVDDTFYYV (9-mer); residues 74–82	HLA-A2	HRP2	Identical

affinity HLA-A2 binding peptide, followed by VETFsm(498-506), while HRP2(74-82) was the weakest binder of the three.

We tested the immune response to these HLA-A2-restricted peptides by immunizing the AAD strain of HLA-A2 transgenic mice i.p. with the attenuated, replication-competent vaccinia virus strain vSC8. Mice were screened by IFN-γ ELISPOT at 1 week of infection (effector phase) or 1 month postinfection (memory phase) for responses on C1R.AAD targets either pulsed with VETFsm(498-506), A26L(6-14), or HRP2(74-82) peptide or infected with vaccinia virus. Surprisingly, at 1 week of infection, only responses on vaccinia virus-infected targets or targets pulsed with HRP2(74-82) were detectable (Fig. 2). By contrast, at 1 month postinfection, we were again able to detect responses on infected targets and HRP2(74-82)-pulsed targets, but we also detected responses on A26L(6-14)- and VETFsm(498-506)-pulsed targets (Fig. 2). When we looked at other HLA-A2.1 transgenic strains of mice, we observed differences in the magnitude of the response between the strains, such that HHD-2 mice had the strongest detectable response to these epitopes, while A2Kb mice had a small to undetectable response (data not shown). These differences may reflect the fact that the A2Kb strain also has the mouse MHC molecules, which may be dominant in response to the virus, while the HHD-2 strain of mice does not express any mouse MHC class I alleles and contains only the human HLA-A2 for presentation of antigen.

Natural processing of epitope. We wanted to determine if immunization with the HRP2(74-82) peptide could induce $CD8^+$ T-cell activity toward this HLA-A2-restricted epitope that would recognize naturally processed antigen in infected cells. This epitope has a detectable response during the acute phase of the infection (at 1 week) compared with the other two peptides, as well as the strongest response of the three at 1 month postinfection. We therefore immunized the A2Kb strain of mice either once i.p. with the attenuated vSC8 strain of vaccinia virus or three times s.c. in the base of the tail at 2-week intervals with an emulsion containing the HRP2(74-82) peptide. Following immunization with HRP2(74-82) peptide, the response to HRP2(74-82) becomes detectable by ELISPOT. It was important to determine whether $CD8^+$ T cells from mice immunized with HRP2(74-82) peptide would cross-react on vSC8-infected targets. Following 1 or 2 weeks of in vitro restimulation on HRP2(74-82) peptide, a response on infected C1R.AAD cells is highly detectable by ELISPOT (Fig. 3). Thus, $CD8^+$ T cells raised by immunization and restimulation only with peptide will recognize cells infected with virus, indicating that the epitope is naturally endogenously processed and presented on HLA-A2 molecules of infected cells.

Protection against lethal virus challenge. The mechanisms of protection against poxviruses are not well understood. Whether a $CD8^+$ T-cell response alone can be sufficient to protect against death, especially $CD8^+$ T cells directed to a single HLA-A2-restricted epitope (although it was shown in a previous study by our group [9] that the antibody response may be more important for protection against disease), is knowledge that is valuable for development of a new generation of vaccines against poxviruses. Thus, we sought to determine if the $CD8^+$ T-cell response to HRP2(74-82) peptide was protective against a lethal mucosal infection with vaccinia virus.

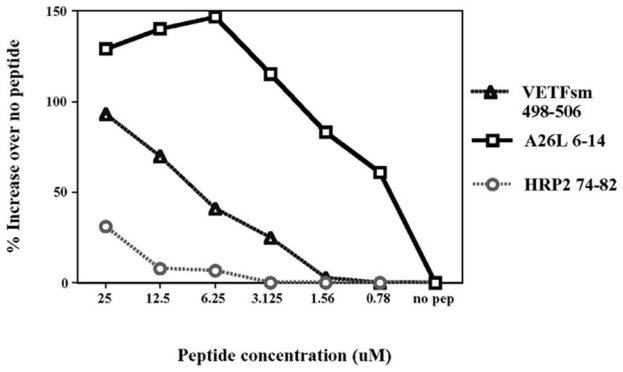

FIG. 1. Binding of vaccinia virus peptide to HLA-A2. The 9-mer peptides were tested for binding by the standard T2 binding assay. HLA-A2 expression was measured with the antibody BB7.2 and compared with expression on T2 cells unpulsed with peptide. Each data point represents a single peptide concentration on the titration curve. This result was reproduced in three independent experiments.

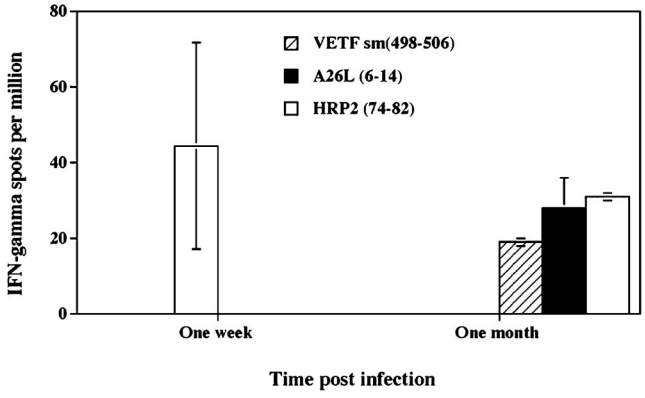

FIG. 2. Ex vivo ELISPOT on splenocytes from vaccinia virus-infected HLA-A2 transgenic mice. Mice from the AAD strain of HLA-A2 transgenic mice were injected i.p. with the replication-competent vaccinia virus vSC8. Spleens were removed at 1 week of infection or 4 to 6 weeks postinfection (1 month) and screened ex vivo by ELISPOT for IFN-γ-secreting T lymphocytes recognizing each of the three predicted vaccinia virus epitopes on peptide-pulsed C1R.AAD targets. The data are representative averages from the AAD strain of mice in three or four independent experiments with three to nine mice in each experiment. Error bars are based on standard errors of the means.

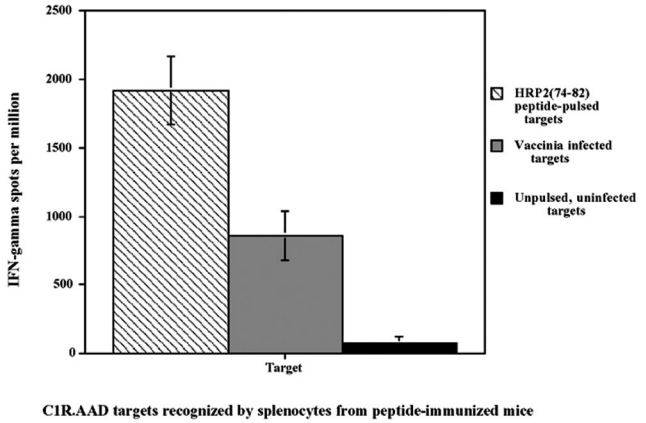

FIG. 3. Natural processing and recognition of HRP2(74-82) in vaccinia virus-infected C1R.AAD targets. HLA-A2 transgenic mice were immunized twice s.c. in the base of the tail with the vaccinia virus peptide HRP2(74-82). Spleens were removed and restimulated twice in vitro on HRP2(74-82)-pulsed antigen-presenting cells and then screened by ELISPOT for IFN-γ-secreting T lymphocytes recognizing either peptide-pulsed or vaccinia virus (vSC8)-infected C1R.AAD targets. [Error bars represent standard errors of the means; $P = 0.02$ for recognition of virally infected versus uninfected cells by T cells raised on HRP2(74-82) peptide.]

HLA-A2 transgenic A2Kb mice immunized with vSC8 or HRP2(74-82) peptide, or left unimmunized, were challenged intranasally with 10^6 PFU of the wild-type pathogenic vaccinia virus strain WR. This amount of virus is lethal for these mice. Two parameters were used to assess the protective effect of each vaccine: survival and weight loss as a measure of disease course. Mice that lost more than 25% of their body weight were euthanized to prevent suffering, as required by NIH guidelines. Mice generally cannot recover from a weight loss this substantial and eventually go on to succumb to the infection (9, 18). Thus, weight was monitored on a daily basis. As a positive control for protection, all mice that were immunized i.p. with vSC8 survived, showing no more than 3% loss of body weight (Fig. 4). By day 9, two-thirds of the unimmunized mice either had succumbed to the infection or had lost sufficient weight to require euthanasia (Fig. 4). The surviving mice in this group never fully recovered from the infection or regained their full body weight. Upon necropsy, there was evidence of continued, chronic infection (data not shown).

By contrast, 82% of the group that was immunized s.c. with HRP2(74-82) peptide survived the infection (Fig. 4A, $P = 0.004$). The mean weight loss for this group was greater than that of whole-virus-immunized mice, suggesting that they were not protected from disease, but all surviving mice recovered their full body weight and showed no evidence of chronic infection (Fig. 4B, $P = 0.008$ compared with unimmunized mice).Thus, the mice that were immunized with HRP2(74-82) peptide were not protected from disease, but immunization with this peptide conferred a survival advantage against a lethal virus challenge compared to unimmunized mice, suggesting that the $CD8^+$ T-cell response alone can have a beneficial role in survival of a lethal virus infection, since no neutralizing antibody response would be expected to be elicited to this

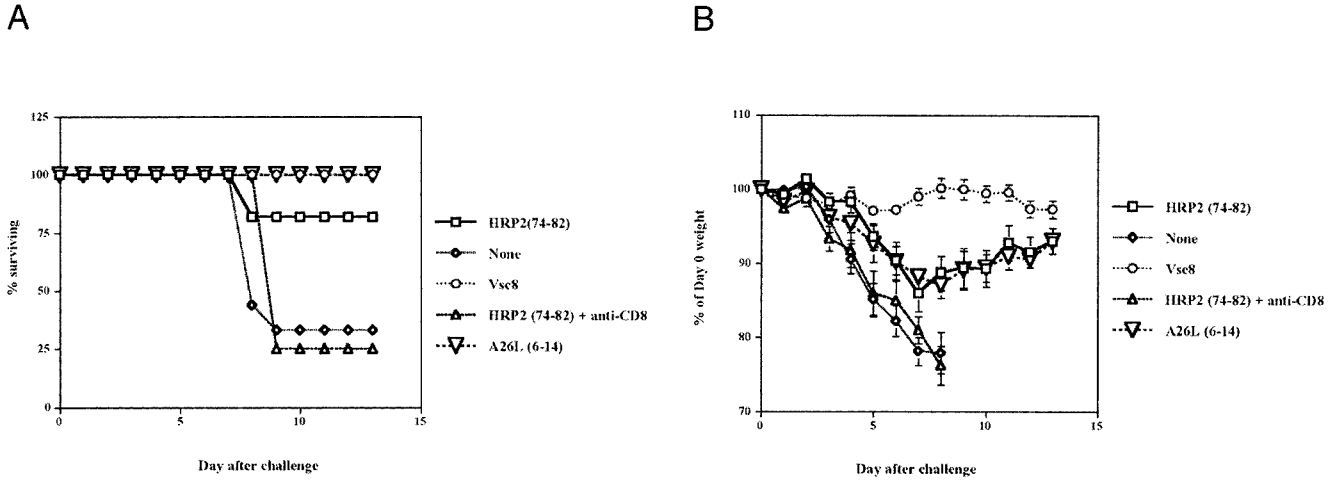

FIG. 4. (A) Survival of wild-type vaccinia virus challenge. HLA-A2 transgenic mice (strain A2Kb, five or six mice per group) were immunized once i.p. with vSC8 vaccinia virus or three times s.c. with peptide HRP2(74-82) or A26L(6-14) or left unimmunized. Some of the immunized mice were depleted of $CD8^+$ T cells. One month after the third immunization with HRP2(74-82) or A26L(6-14), the mice were challenged intranasally with a lethal amount (10^6 PFU) of wild-type vaccinia virus (strain WR). Survival was monitored out to 14 days postchallenge (where day 0 is the day of challenge) [$P = 0.004$ for unimmunized versus HRP2(74-82)-, vSC8-, or A26L(6-14)-immunized mice; $P = 0.40$ for CD8-depleted versus unimmunized mice; $P = 0.02$ for CD8-depleted versus HRP2(74-82)-immunized mice]. The results shown are from an experiment representative of two with similar results. (B) Disease course following vaccinia virus challenge. Disease course was tracked by monitoring mouse weight loss daily over the same 14-day period as survival. Mice that lost more than 25% of body weight were sacrificed according to NIH guidelines. Numbers represent pooled average weights of 11 mice [HRP2(74-82)-immunized group], 4 mice [A26L(6-14)-immunized group and CD8-depleted group], 7 mice (vSC8-immunized group), and 9 mice (unimmunized group), from two independent experiments [$P = 0.008$ for unimmunized mice versus HRP2(74-82)-immunized mice, $P = 0.02$ for vSC8-immunized mice versus unimmunized mice, $P = 0.03$ for A26L(6-14)-immunized mice versus unimmunized mice, $P = 0.001$ for CD8-depleted mice versus HRP2(74-82)-immunized mice, and $P = 0.45$ for unimmunized mice versus CD8-depleted mice]. Error bars represent standard errors of the means. Mean weights were not plotted beyond the point at which most or all of the mice in the group were dead. The results shown are from an experiment representative of two with similar results.

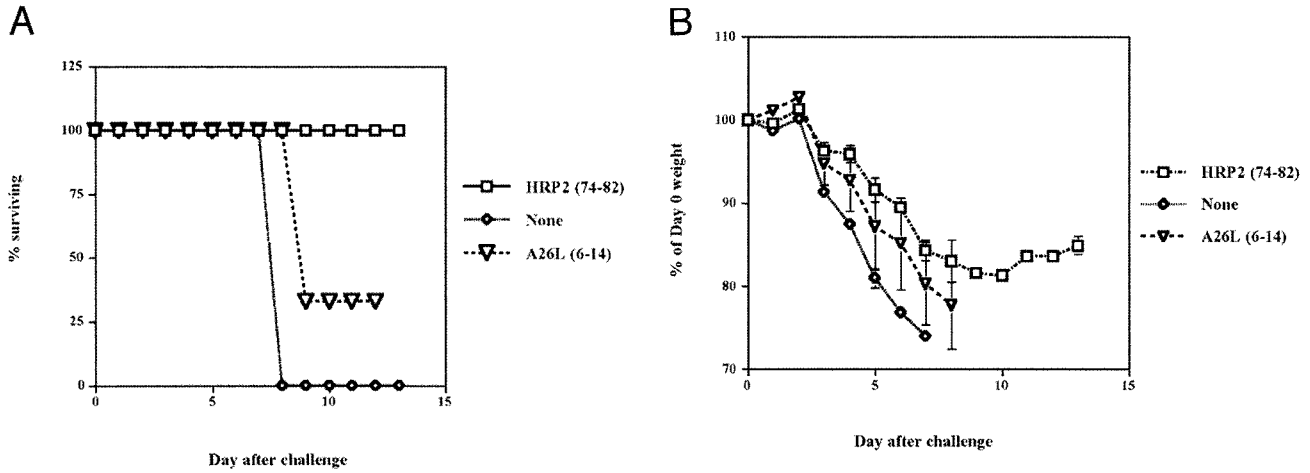

FIG. 5. (A) Survival of HHD-2 mice following vaccinia virus challenge. HHD-2 mice, which have only human HLA-A2 and no mouse class I MHC molecules, were immunized three times s.c. with either HRP2(74-82) (five mice) or A26L(6-14) (three mice) or left unimmunized (four mice). Two weeks after the second peptide immunization, mice were challenged intranasally with 10^6 PFU of wild-type vaccinia virus (strain WR). Survival was monitored to 14 days postchallenge (where day 0 is the day of challenge) [$P = 0.01$ for HRP2(74-82)-immunized mice versus unimmunized mice]. The results shown are from an experiment representative of two with similar results. (B) Disease course in HHD-2 mice following vaccinia virus challenge. As with A2Kb mice, weight loss was monitored in the HHD-2 mice over the same 14-day period as survival. Mice that lost more than 25% of body weight were sacrificed according to NIH guidelines. Numbers represent average weights of three to five mice in each group (as in panel A) (or average weight of surviving mice) [$P = 0.005$ for HRP2(74-82)-immunized mice versus unimmunized mice]. Error bars represent standard errors of the means. Mean weights were not plotted beyond the point at which most or all of the mice in the group were dead. The results shown are from an experiment representative of two with similar results.

small (9-mer) peptide. The protection against death afforded by immunization with the single epitope HRP2(74-82) was dependent on $CD8^+$ T cells, as depletion of these with anti-CD8 prior to challenge resulted in weight loss and death indistinguishable from that in unimmunized mice (Fig. 4, $P = 0.4$).

In addition to enhanced survival and lessened severity of disease, the onset of disease following viral challenge was delayed for 2 days in peptide-immunized mice compared with unimmunized mice (median day of onset is day 5 for immunized mice versus day 3 for unimmunized mice [data not shown]), suggesting that the response to this epitope may delay disease until a response to other virus epitopes (or an antibody response) can be mounted to enable complete clearance of the infection and recovery from disease.

We further examined protection following HRP2(74-82) immunization of the HHD-2 mice, which have only human HLA-A2 and no mouse class I MHC molecules. As shown in Fig. 5A, all mice immunized with the peptide survived a lethal intranasal challenge with wild-type vaccinia virus strain WR ($P = 0.01$). By contrast, none of the unimmunized mice survived this challenge. Weight loss was also monitored in these mice. As with the A2Kb mice, peptide-immunized mice became ill and lost body weight, but all mice recovered (Fig. 5B, $P = 0.005$ compared with unimmunized mice). By contrast, all of the naïve mice either succumbed to the infection within 7 to 8 days or lost sufficient body weight to require euthanasia.

As with the A2Kb mice, disease onset was delayed in peptide-immunized compared with unimmunized HHD-2 mice (median day of onset is day 4 in immunized mice versus day 3 in unimmunized mice [data not shown]). In addition, the disease course at onset was less severe, as measured by initial weight loss in peptide-immunized versus unimmunized HHD-2 mice (3.7% ± 0.5% weight loss in immunized mice versus 8.7% ± 0.7% weight loss in unimmunized mice, $P = 0.001$ [data not shown]). Thus, the HRP2(74-82) peptide is protective against a lethal virus challenge even in mice with only the human HLA-A2, excluding any response restricted to a murine class I MHC molecule as a mediator of protection.

In other experiments (Fig. 4 and 5 and data not shown), we found that the A26L(6-14) peptide also demonstrated some protection ($P = 0.004$ for survival, $P = 0.03$ for disease course) against a lethal virus challenge in A2Kb but not HHD-2 mice. This epitope also binds to the mouse D^b, so part of the protection in the A2Kb mice may be conferred by the mouse MHC, which is absent in HHD-2 mice. The VETFsm(498-506) peptide was found not to be protective in either strain (data not shown). Further experiments are being conducted on the A26L(6-14) peptide to determine the level of protection conferred, as well as whether $CD8^+$ T cells raised to this vaccinia virus epitope can cross-react with the corresponding epitope in variola virus, which differs by one amino acid residue in the anchor position. We are also conducting experiments to determine the natural processing of this epitope. Results of other preliminary experiments suggest that immunization with combinations of these peptides may be more protective than immunization with a single epitope. We have also conducted preliminary experiments that suggest that, in some conditions, $CD8^+$ T cells alone can protect from disease. Thus, a cocktail of these peptides may give not only protection against lethality but also better protection against disease.

Increased dominance of peptide epitope. If this protection was attributable to the HRP2(74-82) response, we reasoned that the response postchallenge to HRP2(74-82) should be more dominant in peptide-immunized mice and that $CD8^+$ T cells recognizing this epitope should expand following a chal-

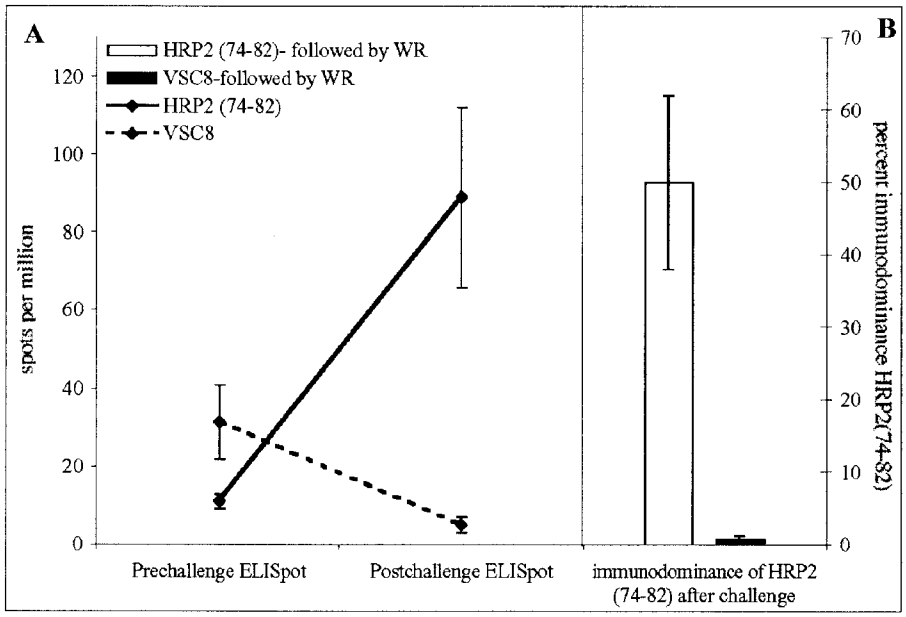

FIG. 6. Immunodominance of response to HRP2(74-82) in peptide-immunized versus whole-virus-immunized mice. (A) Average precursor frequency of HRP2(74-82) peptide-specific $CD8^+$ T cells as measured by IFN-γ ELISPOT in pre- and post-virus-challenge mice immunized with either peptide or whole virus. (B) Relative dominance of HRP2(74-82) epitope following virus challenge in each group of immunized mice. Percent immunodominance is defined as [spots per million on HRP2(74-82)-pulsed targets/spots per million on whole-virus-infected targets] × 100. In both panels, error bars represent the standard errors of the means. (For both expansion pre- to postchallenge in panel A and relative dominance in panel B, $P = 0.01$.)

lenge. We therefore screened the response by ELISPOT following the challenge. As predicted (Fig. 6), a larger portion of the response to the whole virus was due to HRP2(74-82), in comparison with mice immunized with the whole virus vSC8. In addition, this population of $CD8^+$ T cells expanded following challenge from the precursor frequency before challenge (Fig. 6), as would be expected if they were involved in protection. Thus, immunization with HRP2(74-82) peptide resulted in survival of a lethal virus challenge and a more dominant postchallenge response toward HRP2(74-82) in peptide-immunized mice, suggesting that this epitope was substantially boosted by the infection and may have contributed to survival of the lethal virus challenge.

DISCUSSION

The principal correlates of immunity to poxviruses in humans and other species are largely undefined. However, an understanding of the key components of the immune response to this class of viruses is critical to development of new-generation vaccines, against both smallpox and other emerging poxvirus infections, such as monkeypox, as well as to development of recombinant vaccines against cancer and human immunodeficiency virus, where poxviruses are frequently used as vectors. In addition, because of AIDS, as well as the widespread use of immunosuppressive drugs for transplantation and in some autoimmune syndromes, and the immunosuppressive effects of some anticancer drugs, the possibility of deleterious side effects from the present generation of smallpox vaccines is increased. Thus, development of more attenuated viral vaccines, such as MVA for smallpox (9, 18, 36, 37, 55, 56), and the possibility of priming with peptides before immunization with whole-virus vectors should be considered.

In this study, we searched for possible $CD8^+$ T-cell epitopes of vaccinia virus and tested the binding of several of these predicted peptides to HLA-A2. Of the peptides that were determined experimentally to bind HLA-A2, we found that one, HRP2(74-82), showed a consistent $CD8^+$ T-cell response, detectable by ELISPOT as well as by a chromium release assay (data not shown), on peptide-pulsed HLA-A2 targets, by splenocytes from HLA-A2 transgenic mice at both 1 week of infection and 1 month postinfection. Although another group also recently identified this epitope (58), we describe here several novel findings about its function. Firstly, this peptide is naturally processed and presented on a human cell line, C1R.AAD, infected with vaccinia virus. Secondly, we show for the first time that this epitope alone is protective. Other groups have identified immunodominant epitopes that were not protective by themselves (18). HLA-A2 transgenic mice immunized only with the HRP2(74-82) peptide were protected from death due to a challenge with a lethal amount of wild-type vaccinia virus (strain WR) by a $CD8^+$ T-cell-dependent mechanism. This represents the first observation of protection from a lethal vaccinia virus challenge by a single peptide epitope. Finally, we show that the T-cell response in these mice postchallenge was skewed toward a response to this epitope, HRP2(74-82). Because HHD-2 mice that express only human HLA-A2 without any mouse class I MHC molecules are protected by a short peptide binding HLA-A2 but unlikely to induce antibody, we conclude that the protection is mediated by $CD8^+$ T cells recognizing the peptide presented by the human HLA molecule. Taken together, these results suggest

that HRP2(74-82) is a protective epitope of vaccinia virus as presented by human HLA-A2 and, more generally, that a $CD8^+$ T-cell response to vaccinia virus can lead to survival of a lethal infection in immunized animals and that peptide immunization could be incorporated into a smallpox vaccine.

Although treatment with an anti-CD8 monoclonal antibody may also result in depletion of NK cells that also have CD8 (59), we feel that the principal effect is on the $CD8^+$ T cells, because CD8 NK cells represent only a small proportion of the total CD8 cells in mice and only a small portion of NK cells are CD8 positive in mice. Thus, CD8 depletion should not have a major impact on NK cell activity in this model. Moreover, we see expansion of a CD8 population that specifically recognizes the HRP2(74-82) peptide epitope, suggesting that this is the critical population involved in protection.

Protection against orthopoxviruses by $CD8^+$ T cells may involve more than one effector mechanism. Although lysis of infected cells has long been assumed to be the primary protective mechanism of antiviral $CD8^+$ T cells, as supported by loss of protection against lymphocytic choriomeningitis virus in perforin-deficient mice (27), Harris et al. (26) have shown a major role for IFN-γ and nitric oxide induced by this cytokine in the control of vaccinia virus infection. Likewise, Chisari and coworkers (24) have shown that $CD8^+$ T cells can inactivate hepatitis B virus in infected cells by a nonlytic mechanism involving cytokines such as IFN-γ. In the present study, the vaccinia and variola virus epitopes that we describe clearly induce IFN-γ production as measured by ELISPOT (Fig. 2). We were also able to measure lytic activity (by chromium release) against HRP2(74-82) peptide-pulsed A2 targets following 1 week of in vitro restimulation (data not shown).

Importantly, the HRP2(74-82) epitope is conserved in a wide range of poxviruses, including variola virus, the causative agent of smallpox, and differs by only two residues from a similar epitope in monkeypox virus (5, 23, 31, 58). It is part of a critical protein that determines the host range of these viruses (40, 46) and thus is important for viral infectivity. As the manuscript was in preparation, a study was published by Terajima et al. (58) in which it was shown that this peptide is also recognized by $CD8^+$ T cells from human subjects recently immunized with vaccinia virus, but protective efficacy, endogenous expression, and induction of an immune response by a peptide vaccine were not examined. Combining this finding with ours, we conclude that this peptide could serve as a protective $CD8^+$ T-cell epitope for vaccinia virus and smallpox not only in HLA-A2 transgenic mice but also in humans. We also have preliminary data (not shown) that suggest that $CD8^+$ T cells generated to this epitope may cross-react with the homologous epitope in monkeypox virus. Inasmuch as smallpox vaccination was discontinued more than 30 years ago, one possible use for this epitope would be to detect a long-term memory response, or the response to the epitope could be used as a criterion of effective immunization. This could be utilized in addition to other methods for detection of effective poxvirus immunity or vaccine "take," such as the recently developed vaccinia virus neutralization assay (35), or it could be used to look for long-term immunity to poxviruses in human subjects (25).

Of note, immunization with a single $CD8^+$ T-cell epitope was sufficient to protect mice against death from a lethal virus challenge. However, these mice did become ill and lose weight, unlike the mice that were immunized with whole virus, which showed only a small weight fluctuation. Moreover, the peptide is less effective, as two of the peptide-immunized mice did succumb to the viral challenge, while all mice immunized with the whole attenuated virus survived. Protection by the whole-virus vaccine is likely due not only to a $CD8^+$ T-cell response to other epitopes but also to an antibody response in these mice induced by the vaccine. In this regard, a recent study by our group (9) demonstrated that the antibody response to vaccinia virus is sufficient for protection, even in the absence of CD8 or CD4 effector T cells. Nevertheless, it is relevant that a $CD8^+$ T-cell response to this single epitope may delay disease until a response to other $CD8^+$ T-cell epitopes, or an antibody response, can be raised to effect complete clearance of the poxvirus. Inasmuch as the cellular response to this epitope still represents just one component of the overall response to the viral infection, as demonstrated by the postchallenge ELISPOT data, and insofar as this does not appear to be a major component of the cellular response following whole-virus immunization, it is likely that there are other, more dominant $CD8^+$ T-cell epitopes that have not yet been identified that could confer greater protection.

In this regard, it is possible that a combination of peptides may be more effective than immunization with a single peptide. Our preliminary data (not shown) with combination peptide vaccines suggests that incorporation of multiple epitopes may be more protective against disease. However, as in the case of mice that received an immunization with HRP2(74-82) alone, we observed an expansion of the HRP(74-82)-specific precursors in the postchallenge immune response, as measured by ELISPOT, but not of those specific for the other epitopes (data not shown), suggesting that the HRP2(74-82) epitope becomes more dominant in these mice and that HRP2(74-82)-specific precursors preferentially expand in response to the viral challenge.

The algorithms used to predict HLA-A2 binding are not completely perfect predictors, as the HRP2(74-82) epitope was predicted by the algorithm that we used (BIMAS) to be a strong binder but experimentally proved to be a weak binder. We also crosschecked this in two other commonly used algorithms, which also predicted it to be a strong HLA-A2 binder. Different algorithms may rank peptide binding differently, but they are usually consistent in identifying similar peptide epitopes. In addition, although this peptide was experimentally the weakest HLA-A2 binding peptide of the three described here, it was nonetheless the most protective of the ones which we tested, especially in the HHD-2 mice that lack mouse MHC. This may reflect, among other possibilities, a greater abundance of the protein from which this peptide is derived or better natural processing of this epitope in a viral infection. Thus, HLA-A2 binding is only one variable that should be considered when searching for a protective, immunogenic $CD8^+$ T-cell epitope.

The priming regimen utilized here, incorporating helper peptide, GM-CSF, and IL-2 in incomplete Freund's adjuvant, could be a very effective protocol for peptide immunization, as other studies by our group have shown (unpublished observations). In addition, a vaccine regimen incorporating peptides may also be utilized in a prime-and-boost strategy to reduce

side effects from the present vaccines, especially in immunocompromised individuals. Some recent studies have utilized attenuated viruses for this purpose, either alone or as part of a prime-boost regimen, in monkeys (19) and humans (61).

Other approaches incorporating peptides into a vaccine against poxviruses could involve addition of other cytokines, such as IL-12 (4) or IL-15 (41), into the peptide vaccine mixture, which may synergize with other cytokines in the mixture to improve efficacy of the vaccine (1, 2, 4, 14, 15). In addition, enhancement of peptide epitopes to improve binding to HLA-A2 or interaction with the T-cell receptor has also been shown to improve the efficacy of peptide vaccines (3, 42, 47, 48, 50, 52). Because orthopoxviruses are transmitted mucosally, another approach to improve the efficacy of vaccination may be delivering the peptide in the presence of an appropriate adjuvant to a mucosal site, such as by intranasal immunization (6–8, 11). Thus, it may be possible to find a combination of peptides and cytokines, or an alternative route of vaccine administration (10), that could effectively protect against disease as well as improve survival and which would not have the deleterious side effects associated with whole-virus vaccines.

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