Cross-Protection against Lymphocytic Choriomeningitis Virus Mediated by a CD4⁺ T-Cell Clone Specific for an Envelope Glycoprotein Epitope of Lassa Virus

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Recombinant vaccinia virus expressing the Lassa virus (LV) envelope glycoprotein precursor, V-LSGPC, was used to study the basis of LV-induced cross-protective immunity against the closely related arenavirus lymphocytic choriomeningitis virus (LCMV). C3H/HeJ mice primed with V-LSGPC developed neither circulating antibodies nor CD8⁺ cytotoxic T cells specific for LCMV, yet they resisted a normally lethal LCMV challenge. Spleen cells from such mice gave a proliferative response to LCMV *in vitro* that was inhibitable by anti-CD4 antibody. Synthetic peptides corresponding to predicted T-cell sites common to the envelope glycoprotein precursor (GP-C) of LV and that of LCMV were used to map the specificity of the proliferative response to an epitope located between amino acids 403 and 417 of LV GP-C. Several CD4⁺ T-cell clones specific for the 403–417 peptide were isolated and found to produce gamma interferon in response to both the peptide and LCMV. One of these clones, C9, was selected for further study. C9 lysed I-A^K-bearing target cells, and when adoptively transferred to C3H/HeJ mice, it was capable of mediating both a peptide-specific delayed hypersensitivity reaction and resistance to lethal LCMV challenge. These collective findings demonstrate, for the first time, that CD4⁺ T cells can play a major role in arenavirus-specific cross-protective immunity.

Infections produced by Lassa virus (LV), an Old World arenavirus (7), although usually benign, can lead to fatal hemorrhagic disease which, in humans, is known as Lassa fever (13). Usually, neutralizing antibodies appear in survivors of the disease after virus has been cleared (36, 37). Such antibodies are therefore of questionable value in eliminating LV infection, and a role for cellular immune mechanisms is suggested.

Current knowledge of arenavirus-specific cell-mediated immunity and its role in disease is based largely on studies of experimentally produced murine infections. Although the laboratory mouse is relatively insensitive to LV (36, 37), it is a highly suitable host for studying lymphocytic choriomeningitis (LCM) virus (LCMV), a close relative of LV (7, 40). When injected peripherally with LCMV, mice of most strains typically undergo a benign self-limiting infection (reviewed in reference 9). However, when administered intracranially (*i.c.*), LCMV causes a fatal, T-cell-mediated central nervous system (CNS) disease (LCM) (9). Consistent with the immunopathologic basis of LCM is its failure to occur following infection of neonatal mice or immunosuppressed adult mice (9); such animals, although incapable of clearing virus, survive with a persisting noncytopathic infection. The role of CD8⁺ cytotoxic T lymphocytes (CTL) in mediating both virus clearance and CNS disease is exemplified by the capacity of cloned LCMV-specific CTL to adoptively mediate protection when administered at the time

of *i.c.* challenge or to induce disease when given to persistently infected mice (3).

Immunization of guinea pigs or monkeys with an avirulent strain of LCMV or with the related nonpathogenic Mopeia virus protects them against a normally lethal LV challenge (24, 35), indicating that these arenaviruses share one or more protective epitopes. The cellular immune basis of this protection is suggested by studies in which donor lymphocytes from strain 13 guinea pigs that had recovered from LV infection could adoptively protect normal syngeneic recipients against lethal challenge with either LV or LCMV (37). This same result was obtained if the donor animals had been immunized with avirulent LCMV. In both cases, donor lymphocytes had the capacity to specifically lyse, *in vitro*, syngeneic cells infected with either LV or LCMV (37). These studies suggested that immunity to arenaviruses in the guinea pig, like that in the mouse, is T cell mediated (1, 3, 31).

In this report, we describe the use of the murine model of LCMV infection to identify cross-protective epitopes on LV and to characterize the T cells that mediate protection. Recombinant vaccinia virus (V-LSGPC) expressing the LV envelope glycoprotein precursor, GP-C (2), was chosen as a surrogate LV immunogen because it had already been shown to induce protective immunity in guinea pigs (2) and primates (12) against a lethal LV challenge. We found that in C3H/HeJ mice, V-LSGPC also engendered resistance to a lethal challenge dose of LCMV. Attempts to identify the basis of this resistance led to the unexpected finding that it could be mediated by a CD4⁺ T-cell clone having specificity for an epitope located between residues 403 and 417 of LV GP-C. To our knowledge, this is the first demonstration of a role for CD4⁺ T cells in arenavirus-specific, cross-protective immunity.

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MATERIALS AND METHODS

Mice. Female C3H/HeJ (*H-2^k*) mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and used between 6 and 12 weeks of age.

Viruses. Recombinant vaccinia viruses carrying either the gene for GP-C (V-LSGPC) or the nucleoprotein (V-LSN) of LV have been described in detail elsewhere (2). Briefly, the LV genes were inserted into the thymidine kinase gene of vaccinia virus under the control of the vaccinia virus P7.5 promoter. As in arenaviral morphogenesis, GP-C expressed by V-LSGPC is posttranslationally cleaved to form the mature envelope glycoproteins GP-1 and GP-2 (2). Gradient-purified stocks of recombinant virus and parental vaccinia virus (21) were prepared from infected BSC-40 cells by R. Bablanian (State University of New York, Brooklyn). In BSC-40 cells, each preparation had a titer of 2×10^8 PFU/ml, with a particle-to-PFU ratio of 100:1.

Stocks of BHK cell-derived UBC strain (3) and mouse brain-derived Armstrong strain of LCMV (15) were prepared as described previously. Except when noted, the UBC strain of LCMV was used in these studies. All viruses were stored at -70°C .

Cell lines and media. Several continuous murine cell lines were used as target cells in assays of T-cell-mediated cytolytic activity: L929 (L), a C3H-derived (*H-2^k*) fibroblastic line; M12.C3-F6, a B-cell line stably transfected with, and expressing, the α - and β -chain genes for I-A^k; and the parental line (M12.C3), which does not express class II major histocompatibility complex (MHC) antigens (16, 34). These cell lines were a gift from Suzanne Ostrand-Rosenberg (University of Maryland, Baltimore County).

The standard medium used for propagating cell lines, virus, and T-cell clones and for performing lymphoproliferative and cytotoxicity assays was RPMI 1640. It was supplemented with 10% fetal bovine serum (HyClone, Logan, Utah), sodium bicarbonate (0.2% [wt/vol]), glutamine (2 mM), gentamicin (40 $\mu\text{g}/\text{ml}$), penicillin (100 U/ml), streptomycin (100 $\mu\text{g}/\text{ml}$), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (10 mM, pH 7.5), and, except for propagation of virus, 2-mercaptoethanol (5×10^{-5} M). When used for culturing T-cell clones, standard medium contained 10 U of recombinant human interleukin-2 (Genzyme, Cambridge, Mass.) per ml.

Antibody reagents. LCMV infection of cells serving as targets in CTL assays was verified by indirect immunofluorescence assay (IFA), using rat anti-LCMV antibodies (30). To detect LV antigens expressed by V-LSGPC-infected target cells, heat-inactivated serum from convalescent, LV-infected rhesus monkeys and monoclonal antibodies to the nucleoprotein (LS2-93-4 BA01) and envelope glycoprotein, GP-2 (LS2-85-6 BG), of LV were obtained from P. Jahrling (U.S. Army Medical Research Institute for Infectious Diseases [USAMRIID], Frederick, Md.). An anti-vaccinia virus monoclonal antibody was provided by Alan Schmaljohn (USAMRIID). These antibodies were used in conjunction with fluorescein isothiocyanate (FITC)-conjugated goat antibodies (Sigma, St. Louis, Mo.) specific for either mouse or human immunoglobulin.

Hybridomas secreting monoclonal antibodies to murine CD4 (GK1.5) (45), murine CD8 (116-13.1) (39), and murine gamma interferon (IFN- γ) (R4-6A2) (41) were purchased from the American Type Culture Collection (Rockville, Md.). Culture supernatant containing the XMG1.2 antibody to IFN- γ (9) was provided by Barbara Fox (University of Maryland at Baltimore). Anti-CD4, anti-CD8, and anti-

IFN- γ antibodies were purified from spent culture supernatants by affinity chromatography on protein G columns (Pierce, Rockford, Ill.) according to the manufacturer's directions.

Peptides. The deduced amino acid sequences of LV GP-C and LCMV (Armstrong strain) GP-C (40) were analyzed for sites of $\geq 80\%$ homology that contained putative T-cell determinants as defined by previously published algorithms (29, 38). Peptides corresponding to eight such sites were synthesized in an Applied Biosystems 403A peptide synthesizer (Applied Biosystems, Foster City, Calif.), using the *t*-butoxycarbonyl method (19). Peptides were subsequently purified by high-pressure liquid chromatography. In procedures described below, reference is made to one of these peptides (GP-C403-417) representing amino acid residues 403 to 417 of LV GP-C.

Immunizations. For immunization with infectious virus, mice were inoculated intraperitoneally (i.p.) with 10^6 or 10^7 PFU of either V-LSGPC or vaccinia virus (as a control) or with 10^3 PFU of LCMV and used in experiments at least 1 month later. When primed with synthetic peptides, mice were injected in the rear footpads with a total of 100 μg of peptide emulsified in 50 μl of complete Freund's adjuvant. Lymph node cells from such mice were used 7 to 12 days later. To assess the efficacy of immunization, mice were challenged i.c. with lethal doses (20 or 200 PFU) of LCMV.

Assay of antibodies to LCMV and LV. Sera obtained by tail vein puncture were tested for anti-LCMV and anti-LV antibodies, using an IFA as described previously (30). Acetone-fixed spot slides of LCMV-infected L cells or of LV-infected Vero cells served as sources of viral antigens. (Fixed LV-infected cells were provided by P. Jahrling, USAMRIID.) Cells were overlaid first with dilutions of sera for 1 h at 37°C and, after washing, with an FITC-conjugated goat anti-mouse immunoglobulin (Sigma) together with Evans blue (0.05% [wt/vol]) as a counterstain (30). Antibodies bound to infected cells and similarly treated uninfected (control) cells were visualized by fluorescence microscopy.

Cytotoxicity assays. Target L cells were infected with LCMV (multiplicity of infection [MOI] = 0.1) for 2 days, V-LSGPC (MOI = 5) for 6 h, or, as a control, vaccinia virus (MOI = 5) for 6 h and labeled with 100 to 200 μCi of $\text{Na}_2^{51}\text{CrO}_4$ (^{51}Cr). These targets were used for assays of cytotoxic activity mediated by classical CD8⁺ CTL. Graded numbers of effector cells were cultured for 6 h at 37°C with 1×10^4 to 2×10^4 labeled target cells in triplicate round-bottom wells of 96-well plates (Costar, Cambridge, Mass.) containing 200 μl of medium per well. The cytotoxic activity of GP-C403-417-specific CD4⁺ T-cell clones against peptide-sensitized B-cell lines was measured by using labeled M12.C3 and M12.C3-F6 cells as targets in the presence or absence of 50 μg of peptide per ml. In these cases, effector-target cell mixtures were incubated for 18 to 20 h on the basis of a predetermined optimal time for obtaining maximal class II MHC-restricted cytotoxic activity. At the end of each assay, 100 μl of culture supernatant was harvested from each well, and the released ^{51}Cr was measured in a gamma 5500B spectrometer (Beckman, Irvine, Calif.). Percentages of virus-specific target cell lysis were calculated as previously described (35). When peptides were used to prepare target cells, the spontaneous release of ^{51}Cr was determined in both the presence and absence of peptide.

To facilitate comparisons of individual animals as to total virus-specific cytolytic activity per spleen, lytic units (LU) were calculated as described elsewhere (35). In these stud-

ies, one LU equaled the number of effector cells required to lyse 30% of target cells.

Secondary CTL. Virus-specific secondary CTL and vaccinia virus-specific secondary CTL were induced by culturing, in upright 25-cm² plastic culture flasks (Costar), 10⁷ primed splenic lymphocytes with 10⁶ PFU of the appropriate virus in 10 ml of standard medium (3). Five days later, cells were harvested, washed, assessed for viability by trypan blue exclusion, resuspended to the desired viable cell concentration, and tested for virus-specific CTL activity.

Peptide-specific T-cell clones. A T-cell line was established by culturing splenic lymphocytes from V-LSGPC-primed C3H/HeJ mice (3 × 10⁶ cells in 2 ml) in standard medium containing 1 µg of the GP-C403-417 peptide per ml for 7 days. The cells were then rested for 3 days in medium without peptide and subsequently subjected to five cycles of stimulation and resting in 24-well culture plates (Costar) during which 10⁵ cells, together with 3 × 10⁶ fresh γ-irradiated (3,000 R) spleen cells (stimulators) and 1 µg of GP-C403-417 per ml, were cultured per well. The resulting T-cell line was then cloned by limiting dilution (27) by culturing, in wells of flat-bottom 96-well trays (Costar), either 3, 1, 0.3, or 0.1 cells together with 5 × 10⁵ stimulators, 1 µg of peptide per ml, and 10 U of interleukin-2 per ml. Two weeks later, cultures containing proliferating cells were transferred to 24-well trays and restimulated, as described above, with peptide. Stable clones were restimulated every 10 to 14 days in this manner.

Phenotyping of T-cell clones. T-cell clones were purified on Lympholyte M (Cedarlane, Hornby, Ontario, Canada) and placed for 30 min on ice after mixing with the appropriate marker-specific antibodies diluted in Dulbecco's phosphate-buffered saline (PBS) supplemented with 2% fetal bovine serum and 0.05% sodium azide. The cells were washed twice, mixed with FITC-conjugated goat anti-mouse immunoglobulin for an additional 30 min on ice, washed twice, and fixed with 2% paraformaldehyde in PBS. At least 10⁵ cells from each mixture were analyzed in an EPICS C flow cytometer (Coulter, Hialeah, Fla.).

T-cell proliferative assays. Assays were performed in flat-bottom 96-well plates (Costar). For spleen or lymph node cells, triplicate cultures of 4 × 10⁵ cells per well in 200 µl of standard medium were stimulated with peptide or virus for 4 days and then pulsed with [³H]thymidine ([³H]TdR; 1 µCi per well) for 4 h. For cloned T cells, between 5 × 10⁴ and 1 × 10⁵ cells per well were cultured for 2 days with 5 × 10⁵ stimulators in the presence or absence of peptide and then pulsed with [³H]TdR. The cells were collected with a semi-automated cell harvester (Skatron, Sterling, Va.), and incorporated radioactivity was determined in a liquid scintillation spectrometer (Beckman). The results are expressed either as counts per minute or as a stimulation index calculated by dividing counts per minute of cells cultured with peptide or virus by counts per minute of cells cultured in medium alone. In some experiments, the phenotype of the responding T-cell subset was analyzed by adding antibodies to CD4 or CD8 to the cultures in order to inhibit proliferation. Percent inhibition was calculated as $100 \times (\Delta \text{cpm}_{\text{with antibody}} - \Delta \text{cpm}_{\text{without antibody}}) / \Delta \text{cpm}_{\text{without antibody}}$, where $\Delta \text{cpm} = \text{cpm}_{\text{with antigen}} - \text{cpm}_{\text{with medium}}$.

Induction and detection of IFN-γ. The culture conditions used for inducing IFN-γ from splenocytes (10⁶ per well) or cloned T-cell lines (5 × 10⁴ to 1 × 10⁵ per well) were the same as those described above for T-cell proliferation assays. When LCMV was used to induce IFN-γ production from T-cell clones, 1 × 10⁵ to 2 × 10⁵ nonactivated perito-

neal cells per well were cultured with 10⁴ PFU of LCMV in 200 µl of medium for 2 days. The culture plates were then irradiated (1,500 R), half of the medium was removed, and 10⁵ responder cells in 100 µl of fresh medium were added. When splenocytes were tested, 100 µl of supernatant was harvested from each of triplicate cultures 3 days later or, in the case of cloned T cells, 2 days later. Supernatants were kept at -70°C until assayed for IFN-γ in a capture enzyme-linked immunosorbent assay (ELISA) using anti-IFN-γ monoclonal antibodies XMG1.2 and R4-6A2 as previously described (8, 10).

Unless otherwise stated, reagents and samples were added to wells in 50-µl volumes and incubated for 1 h at room temperature, and all washing steps in the assay were with 50 mM Tris-buffered saline (TBS; pH 7.5) containing 0.05% Tween 20 (TBS-Tween). Wells of 96-well Serocluster enzyme immunoassay plates (Costar) were first coated overnight at 4°C with XMG1.2 at 1 µg/ml in PBS and then filled for 2 h at room temperature with a mixture of 10% fetal bovine serum and 10% nonfat milk in TBS (blocking solution) to prevent nonspecific binding. Culture supernatants to be assayed for IFN-γ were serially diluted in blocking solution and added to wells, and after incubation and washing, biotinylated R4-6A2 (1 µg/ml in blocking mixture) was added. Subsequently, streptavidin-alkaline phosphatase (Amersham, Arlington Heights, Ill.) diluted in TBS-Tween was added to washed wells for 30 min. After the cells were washed 10 times to remove unbound enzyme, that which was bound was detected in a two-step amplification procedure described elsewhere (42). The optical density was measured at 490 nm in a Vmax ELISA reader (Molecular Devices Corp., Menlo Park, Calif.). Natural murine IFN-γ (10,000 U/ml), kindly provided by M. Sarzotti-Kelsoe (VA Hospital, Baltimore, Md.), was used to generate a standard curve in all experiments. Supernatants were titrated in duplicate fivefold serial dilutions. Dilutions whose optical density fell within the linear portion of the standard curve were used to calculate IFN-γ content (expressed as units per milliliter).

DTH reactions. The capacity of a GP-C403-417-specific T-cell clone to mediate a delayed-type hypersensitivity (DTH) reaction was assessed by injecting, into each rear footpad, a mixture of T cells, peptide, and irradiated normal spleen cells in 30 µl of Hanks' balanced salt solution. Footpad thickness was measured with a Mitutoyo dial micrometer immediately prior to injection of the mixture and again 24 and 48 h later. DTH was scored as the percent increase in footpad thickness relative to the thickness prior to injection. Each test group contained at least four mice.

Adoptive protection assays. Cloned T cells were collected after being cultured for 10 to 14 days in the presence of GP-C403-417. The cells were washed and diluted in Hanks' balanced salt solution together with LCMV to give cell-virus mixtures containing 1 × 10⁵ to 2 × 10⁶ viable cells and 20 or 200 PFU of LCMV per 30 µl, the amount given i.c. per mouse. Inoculated mice were observed twice daily for disease and death for 15 days.

RESULTS

Resistance to LCMV induced by immunization with V-LSGPC. Of C3H/HeJ mice immunized with 10⁶ PFU of V-LSGPC, 89% survived i.c. challenge with 200 PFU of LCMV (Table 1), whereas control mice immunized with vaccinia virus and those that were normal (not immunized) had survival rates of only 12 and 5%, respectively. Interest-

TABLE 1. Protection of C3H/HeJ mice against lethal LCMV challenge by immunization with V-LSGPC^a

Immunizing virus	No. of survivors/total	% Survivors
V-LSGPC	24/27	89
Vaccinia virus	2/17	12
None	1/21	5
LCMV	10/10	100

^a C3H/HeJ mice were immunized with 10⁶ PFU of either V-LSGPC or vaccinia virus or with 10³ PFU of LCMV. They were challenged i.c. with 2 × 10² PFU of LCMV (UBC) 30 days later, and mortality was recorded for 21 days. Results were pooled from four independent experiments. None versus V-LSGPC, *P* < 0.0005; V-LSGPC versus vaccinia virus, *P* < 0.0005.

ingly, all V-LSGPC-immunized mice surviving challenge developed, coincident with normal mice, CNS symptoms typical of early LCM. These symptoms, which were not prevented by increasing the immunizing dose of V-LSGPC to 10⁷ PFU (data not shown), disappeared after several days. As expected, 100% of mice immunized with LCMV survived challenge in the absence of symptoms.

Cross-reactive antibodies. To verify that cross-reactive antibodies were not involved in cross-protection, several days before mice were challenged with LCMV, their sera were tested individually for virus-specific antibodies by IFA. Mice immunized once i.p. with either 10⁶ or 10⁷ PFU of V-LSGPC lacked detectable anti-LV or anti-LCMV antibodies, whereas those receiving 10⁷ PFU twice produced antibodies to LV but not to LCMV (data not shown). Antibodies reactive to both LCMV and LV could be detected in mice immunized i.p. with 10³ PFU of LCMV (data not shown).

Cross-reactive CD8⁺ T cells. Given the sizeable body of evidence (1, 3, 31) that both resistance to and recovery from murine LCMV infections are mediated by CD8⁺ T cells, we presumed that the same mechanism would underlie V-LSGPC-induced cross-protection against LCMV challenge.

C3H/HeJ mice failed to mount a detectable primary CTL response to LCMV during the 2-week period following i.p. inoculation of 10⁶ PFU of V-LSGPC (data not shown). Furthermore, when splenocytes from such mice were stimulated with LCMV in vitro and then assayed for secondary CTL activity against LCMV- or V-LSGPC-infected L-cell targets, none could be demonstrated (Table 2). Identically stimulated splenocytes from mice immunized with LCMV exhibited secondary CTL activity against L cells infected with LCMV but not against those infected with V-LSGPC. Conversely, secondary LV-cross-reactive CTL could not be induced by stimulating splenocytes of LCMV-immunized mice with V-LSGPC (Table 2).

From the foregoing results, it was reasoned that if class I MHC-restricted, cross-reactive CTL were mediating resistance to LCMV, their frequency in spleens of V-LSGPC-immunized mice was too low to be detected following secondary stimulation in vitro. Therefore, we next determined whether immunization of mice with V-LSGPC might possibly accelerate and/or enhance their CTL response to subsequent challenge with LCMV, thereby converting a lethal infection to one that was abortive. The CTL responses to challenge with LCMV were compared between mice immunized with either V-LSGPC or vaccinia virus and normal mice. Responses of individual mice in each category were measured on days 5 and 7 postchallenge, which, from previous studies (20), were known to be time points within the linear portion of the primary CTL response of normal mice to LCMV infection. As shown in Fig. 1, no significant

TABLE 2. Absence of arenavirus-specific, cross-reactive CTL in C3H/HeJ mice immunized with either V-LSGPC or LCMV^a

Immunizing virus	Stimulator virus	E:T	Mean % lysis of L cells infected with:			
			No virus	LCMV	V-LSGPC	Vaccinia virus
V-LSGPC	LCMV	15	1	1	22	15
		5	–1	0	8	5
	V-LSGPC	5	18	15	87	58
		2	2	1	50	36
	Vaccinia virus	5	17	14	86	75
		2	4	4	64	57
	None (medium)	15	1	2	13	10
		5	0	1	5	4
	LCMV	2	0	0	3	2
		15	17	62	20	19
LCMV	LCMV	5	3	20	3	5
		2	2	9	2	1
	V-LSGPC	15	6	6	6	8
		5	2	10	6	7
	Vaccinia virus	15	15	22	15	23
		5	2	6	5	5
	None (medium)	2	–1	1	1	1

^a Splenocytes from C3H/HeJ mice immunized twice with V-LSGPC (10⁷ PFU 1 month apart) or once with LCMV were stimulated in vitro with either LCMV, V-LSGPC, vaccinia virus, or medium alone and then tested in a 5 h ⁵¹Cr release assay for cytotoxic activity against normal L cells or L cells infected with either LCMV, V-LSGPC, or vaccinia virus. The standard errors are less than 5% of the mean and are not indicated. Lysis of virus-infected target cells is considered to be virus specific and is italicized if it is at least threefold higher than that of uninfected target cells at the same effector/target cell ratio (E:T). The target cells were shown by IFA, using the corresponding antiviral antibodies, to be greater than 90% infected with LCMV (rat anti-LCMV serum), 70% infected with V-LSGPC (rhesus monkey anti-LV serum and anti-vaccinia virus monoclonal antibody), and 90% infected with vaccinia virus (monoclonal anti-vaccinia virus antibody).

differences in the kinetics and magnitude of LCMV-specific CTL responses between immunized and normal mice were observed over a range of effector/target ratios. LCMV-specific cytotoxic activity in mice immunized with V-LSGPC (1,750 LU per spleen) or with vaccinia virus (1,500 LU per spleen) was comparable and only marginally higher than that of normal mice (1,000 LU per spleen).

Cross-reactive CD4⁺ T cells and their specificity. Having found no evidence that CD8⁺ T cells mediated cross-protection against LCMV, we next tested the capacity of splenocytes from V-LSGPC-immunized mice to proliferate in vitro upon stimulation with LCMV. Low but reproducible proliferative responses were observed (stimulation indices of 2.6 to 4.0) (Table 3) that were inhibitable (>90%) by anti-CD4 antibody (Table 4).

The specificity of these responses was mapped by using synthetic peptides corresponding to regions of LV GP-C having partial homology with LCMV GP-C (40) and containing potential T-cell sites (29, 38). These peptides consisted of residues 308 to 320, 354 to 368, 383 to 397, 393 to 407, 403 to 417, 410 to 425, 446 to 460, and 456 to 470 of LV GP-C (40), of which only GP-C403-417 (IEQQADNMITEMLQK) was found to be stimulatory. Proliferative responses to this peptide were given by splenocytes of mice immunized with either V-LSGPC or LCMV (UBC and Armstrong strains) but not by those of normal mice or mice immunized with the recombinant vaccinia virus expressing LV nucleoprotein (Table 4). These responses were reduced by at least 93% by anti-CD4 antibody but were unaffected by anti-CD8 antibody (<23% reduction) (Table 4).

Isolation of cross-reactive CD4⁺ T-cell clones. Five T-cell

FIG. 1. Kinetics of LCMV-specific CTL induction in V-LSGPC-primed mice. Groups of C3H/HeJ mice immunized with 10^6 PFU of either V-LSGPC or vaccinia virus, as well as normal mice, were challenged with 200 PFU of LCMV i.p. on either day 28 or day 30 after immunization. On day 35 after immunization (days 5 and 7 after LCMV challenge), spleen cell suspensions pooled from two mice per group were tested for LCMV-specific CTL in a ^{51}Cr release assay using LCMV-infected L cells as targets. The data points are represented as percent mean virus-specific lysis per mouse pair. The standard error of the mean is less than 10% (not shown). On day 7 after challenge, the LCMV-specific cytotoxic activity in mice immunized with V-LSGPC or with vaccinia virus was 1,750 or 1,500 LU per spleen, respectively, and that of normal mice was 1,000 LU per spleen.

clones specific for GP-C403-417 were derived from splenocytes of V-LSGPC-immunized mice. All were Thy 1⁺ CD4⁺ CD8⁻, as shown by flow cytometric analyses (data not shown). Three of these clones, C1, C3, and C9, were cross-reactive; i.e., each produced IFN- γ when stimulated with either GP-C403-417 or LCMV (Fig. 2). Another clone, C11, synthesized IFN- γ only in response to peptide (Fig. 2). Clones 9 and 11 are peptide specific because they do not respond to a synthetic peptide corresponding to amino acid residues 456 to 470 of LV GP-C (GP-C456-470) (Fig. 2). Clone 2, which proliferated in response to GP-C403-417 (data not shown), produced little or no detectable IFN- γ (<100 U/ml) in response to either peptide or virus (Fig. 2). None of the T-cell clones proliferated or produced IFN- γ in the absence of peptide or virus (data not shown). A single cross-reactive T-cell clone, C9, was selected for further study on the basis of its seemingly better growth characteristics in vitro.

Cytotoxic and DTH functions of cross-reactive C9 T cells. C9 T cells were found to be cytotoxic and I-A^k restricted. They lysed an I-A^k-expressing B-cell line (M12.C3-F6) in the presence of GP-C403-417 but not the untransfected parental cell line (M12.C3) under the same assay conditions (Fig. 3). In other experiments, clone 11 was also shown to be cytotoxic and I-A^k restricted (data not shown). In addition, C9 T cells mediated a peptide-specific DTH reaction (Fig. 4). Normal mice injected in the footpads with a mixture of C9 T cells, GP-C403-417, and irradiated normal spleen cells (added to ensure that antigen-presenting cells were not limiting) showed footpad swelling 24 h later. Little or no swelling was seen when either GP-C403-417 or C9 T cells were omitted

from the inoculum or when the nonstimulatory peptide, GP-C456-470, was used.

Adoptive transfer of protection with C9 T cells. Mice were inoculated i.c. with a challenge dose of 20 or 200 PFU of LCMV admixed with C9 T-cell numbers ranging from 2×10^6 to 1×10^5 . The cumulative survival rate from three experiments was 92% following challenge with 20 PFU of LCMV (Table 5). Protection was MHC restricted; only 1 of 3 BALB/c (*H-2^d*) mice and none of 18 CD-1 (*H-2^q*) mice that received identical T cell-virus mixtures survived. If given to mice 1 week prior to challenge with 20 PFU of LCMV, C9 T cells were not protective (data not shown), and they were without effect against 200 PFU of LCMV or after three cycles of freezing and thawing (Table 5).

DISCUSSION

The long-held view of protective immunity against the prototype of the arenavirus family, LCMV, is that it is mediated primarily by CD8⁺ (formerly Ly-2⁺) CTL having potent virus-specific cytolytic activity in vitro (1, 3, 31). Given the close antigenic relationship between LCMV and LV (7, 40), it seemed reasonable to expect that reciprocal cross-protective immunity induced by these viruses, as documented in other animal species (24, 37), would also apply in the mouse. Our initial observation, that C3H/HeJ mice immunized with V-LSGPC were resistant to LCMV challenge, conformed with this expectation. That resistance was induced in the absence of antibodies reactive specifically with LV- or LCMV-infected cells led to another expectation, which was that the mechanism of resistance would be found to be CTL mediated. Indeed, others had already concluded that in mice, virus-specific CTL accounted for the resistance to LCMV challenge following immunization with similar recombinant vaccinia viruses expressing either the glycoprotein or the nucleoprotein of LCMV (18, 25). However, in our studies, immunization with V-LSGPC did not induce CTL that were cross-reactive with LCMV, and it was without effect on the LCMV-specific CTL response following LCMV challenge.

Since it has been reported that only a very minor portion of the primary CTL response made by *H-2^k* mice to LCMV is directed against GP-C (18), it could be argued that C3H/HeJ (*H-2^k*) mice, as used in our studies, may have produced a weak CTL response to LV GP-C. However, even if present at a low frequency in V-LSGPC-immunized mice, any CTL with cross-reactivity against LCMV should have been detectable after repeated stimulation of splenocytes with LCMV in vitro. Using this approach, we still were unable to induce secondary CTL capable of lysing LCMV-infected targets (26). It is possible that immunization with V-LSGPC induced CTL with specificities for epitopes unique to LV GP-C. Such CTL would not have lysed LCMV-infected cells which were used as targets in our assay.

Continued efforts to identify the immunologic basis of resistance to LCMV challenge revealed that V-LSGPC immunization primed CD4⁺ T cells of C3H/HeJ mice for a proliferative response in vitro to both LCMV and a synthetic LV peptide, GP-C403-417 (IEQQADNMITEMLQK). The relevance of this epitope to cross-protection was confirmed by using a peptide-specific CD4⁺ T-cell clone (C9) derived from V-LSGPC-immunized mice to adoptively protect normal mice against LCMV challenge. GP-C403-417 corresponds to a highly homologous sequence (IEQEADN MITEMLRK) on the GP-C of LCMV (Armstrong strain)

TABLE 3. Proliferative responses of splenocytes from V-LSGPC-primed and LCMV-primed mice to LCMV and the LV GP-C synthetic peptide GP-C403-417^a

Expt	LCMV (PFU)	Peptide ^b concn (μg/ml)	Normal	[³ H]TdR incorporation (mean cpm [10 ²] ± SD)				
				V-LSGPC			LCMV (UBC)	LCMV (Armstrong)
				Mouse 1	Mouse 2	Mouse 3		
1 ^c	10 ⁵	None	17.2 ± 2.2 (1.5)	67.7 ± 0.8 (3.0)	26.4 ± 6.1 (2.7)	83.7 ± 1.0 (3.7)		301.8 ± 60.0 (31.2)
	5 × 10 ³	None	16.7 ± 5.2 (1.4)	60.4 ± 9.9 (2.7)	39.9 ± 2.9 (4.0)	59.6 ± 1.8 (2.6)		350.8 ± 9.4 (36.3)
	None	10	9.6 ± 1.4 (0.8)	77.7 ± 2.7 (3.5)	39.8 ± 6.4 (4.0)	64.6 ± 6.0 (2.8)		314.4 ± 54.0 (32.5)
	None	3	11.0 ± 2.6 (0.9)	67.0 ± 15.6 (3.0)	32.5 ± 0.7 (3.3)	52.8 ± 7.5 (2.3)		279.5 ± 40.8 (28.9)
	None	1	6.1 ± 0.5 (0.5)	38.5 ± 4.3 (1.7)	36.9 ± 8.9 (3.7)	40.2 ± 6.4 (1.8)		173.2 ± 49.6 (17.9)
2 ^c	None	None	11.7 ± 4.5	22.2 ± 6.8	9.9 ± 1.7	22.6 ± 8.8		9.7 ± 1.6
	10 ⁵	None	10.7 ± 1.7 (0.5)	48.9 ± 7.1 (2.3)	44.4 ± 12.1 (2.1)		953.2 ± 62.2 (44.4)	
	3 × 10 ⁴	None	16.2 ± 1.9 (0.8)	54.4 ± 5.9 (2.5)	44.7 ± 11.4 (2.2)		438.3 ± 46.1 (20.4)	
	None	10	18.6 ± 4.1 (0.9)	75.2 ± 6.4 (2.5)	59.7 ± 4.2 (2.9)		125 ± 18.8 (11.4)	
	None	3	16.6 ± 2.7 (0.8)	78.1 ± 9.1 (2.5)	52.6 ± 4.2 (2.5)		84.3 ± 20.4 (7.7)	
3 ^d	None	1	19.1 ± 5.9 (0.9)	57.7 ± 9.8 (1.9)	48.1 ± 5.1 (2.3)		41.9 ± 9.3 (3.8)	
	None	0.3	18.8 ± 6.4 (0.9)	58.9 ± 4.4 (1.9)	35.9 ± 4.6 (1.7)		39.8 ± 4.7 (3.6)	
	None	None	21.5 ± 4.1	30.5 ± 4.2	20.8 ± 7.7		10.9 ± 1.9	
	10 ⁵	None					108.4 ± 26 (25.2)	24.1 ± 4.9 (0.9)
	5 × 10 ³	None					ND	ND
	None	10					110.4 ± 22.0 (25.8)	28.0 ± 2.6 (1.0)
	None	3					94.8 ± 8.87 (22.2)	27.5 ± 4.3 (1.0)
	None	1					79.9 ± 3.8 (18.7)	26.5 ± 3.1 (0.9)
	None	None					4.3 ± 0.6	27.8 ± 7.6

^a Splenocytes from normal mice and those immunized with either LCMV, V-LSGPC, or V-LSN were stimulated in vitro with LCMV or the LV GP-C synthetic peptide GP-C403-417, and [³H]TdR incorporation was measured after 4 days. Stimulation indices are in parentheses. ND, not determined.

^b LV GP-C synthetic peptide GP-C403-417.

^c Splenocytes from V-LSGPC-primed mice were tested individually. Normal and LCMV-immune splenocytes were pooled from two animals each.

^d V-LSN-immune and LCMV-immune splenocytes were pooled from two animals each.

TABLE 4. Responses of CD4⁺ T cells from V-LSGPC-immune mice to LCMV and a synthetic peptide corresponding to residues 403 to 417 of LV GP-C^a

Antigen	Antibody ^b	Expt 1 ^c				Expt 2 ^d	
		Mouse 1		Mouse 2		³ H]TdR incorporation (mean cpm [10 ²] ± SD)	% Inhibition
		³ H]TdR incorporation (mean cpm [10 ²] ± SD)	% Inhibition ^e	³ H]TdR incorporation (mean cpm [10 ²] ± SD)	% Inhibition		
LCMV	None	117.0 ± 18.4 (3.2)		264.3 ± 31.4 (3.7)		ND	
GP-C403-417	None	131.3 ± 33.1 (3.6)		188.9 ± 10.8 (2.7)		122.5 ± 12.1 (9.8)	
Medium	None	36.9 ± 3.0		71.0 ± 22.0		12.5 ± 3.9	
LCMV	αCD4	10.9 ± 6.8 (1.0)	100	26.2 ± 11.2 (1.3)		ND	
GP-C403-417	αCD4	17.9 ± 4.2 (1.6)	93	15.5 ± 3.9 (0.8)	100	6.7 ± 1.1 (1.6)	98
Medium	αCD4	11.3 ± 5.4		19.5 ± 8.8		4.2 ± 0.6	
LCMV	αCD8	136.1 ± 54.2 (3.8)	0	236.6 ± 41.2 (4.2)	6	ND	
GP-C403-417	αCD8	108.4 ± 5.1 (3.1)	22	151.5 ± 12.6 (2.7)	23	120.7 ± 29.4 (7.9)	4
Medium	αCD8	35.1 ± 7.6		55.7 ± 4.2		15.3 ± 6.5	

^a Splenocytes from mice immunized with either V-LSGPC (experiment 1) or LCMV (experiment 2) were stimulated with either LCMV (10⁵ PFU per well) or the LV GP-C synthetic peptide GP-C403-417 (10 μg/ml) in the presence or absence of antibodies to CD4 and CD8. [³H]TdR incorporation was measured after 4 days. Stimulation indices are in parentheses. ND, not determined.

^b Antibodies GK1.5 and 116-13.1, specific for CD4 (αCD4) and CD8 (αCD8), respectively, were used at a final concentration of 5 μg/ml.

^c Splenocytes from two V-LSGPC-immune mice tested individually.

^d Splenocytes pooled from two mice immunized with LCMV.

^e Percent inhibition by antibodies of the proliferative response to LCMV or GP-C403-417, calculated as defined in Materials and Methods.

(40). Cleavage of LV GP-C occurs between residues 258 and 259 to form GP-1 and GP-2 (6); therefore, the epitope recognized by cross-reactive CD4⁺ T cells must be located on GP-2. The reciprocal nature of the cross-reactive immunity induced by V-LSGPC is illustrated by the fact that CD4⁺ T cells of mice immunized with either the UBC or Armstrong strain of LCMV also responded specifically to the GP-C403-417 peptide. In addition, three CD4⁺ T-cell clones raised against GP-C403-417 were responsive to LCMV (UBC)-infected peritoneal cells. The relevant epitope may also be present on Mopeia virus, whose corresponding GP-C sequence shares 10 of its 15 amino acids with

LV (Mopeia sequence, IEQEANNMFTDMLRK) (47). It is to be noted that GP-C403-417 is distinct from epitopes on LCMV GP-C recognized by *H-2^b* class I MHC-restricted CD8⁺ CTL, i.e., residues 34 to 40 (AVYNFAT) (25) and residues 278 to 286 (VENPGGYCL) (22).

Recent findings suggest that protective immunity to LCMV can be induced by immunization with a peptide containing an epitope recognized by CD8⁺ T cells (4). In a similar fashion, we immunized C3H/HeJ mice with GP-C403-417 in complete Freund's adjuvant, but they still succumbed to LCMV challenge (26). The basis for this lack

FIG. 2. Production of IFN-γ by GP-C403-417-specific T-cell clones in response to stimulation with peptide GP-C403-417 and LCMV. T-cell clones were stimulated with peptide GP-C403-417 (5 to 10 μg/ml) in the presence of irradiated syngeneic splenocytes (experiments 1, 2, and 3) or with LCMV-infected syngeneic peritoneal cells (experiments 2 and 3). No detectable IFN-γ (i.e., <100 U/ml) was produced by T-cell clones in the absence of peptide or in the presence of uninfected peritoneal cells. Splenocytes, with or without peptide, and peritoneal cells alone did not produce IFN-γ (data not shown).

FIG. 3. Peptide GP-C403-417-dependent lysis of class II MHC-bearing cells by C9 T cells. ⁵¹Cr-labeled target cells were cultured for 20 h with C9 T cells (effector/target cell ratio of 10) in the presence of GP-C403-417 (50 μg/ml) or medium alone. M12.C3-F6 is transfected with the *I-A^k* α and β genes and expresses *I-A^k* on its cell surface. The parental cell line M12.C3 does not express cell surface class II MHC molecules. The spontaneous release of ⁵¹Cr from each target cell line was <25% in the presence or absence of peptide.

FIG. 4. Evidence that C9 T cells mediate the peptide GP-C403-417-specific DTH reaction. Footpads of normal C3H/HeJ mice were inoculated with combinations of 10^6 C9 T cells, 4 μ g of peptide (GP-C403-417 or GP-C456-470), and 2×10^6 irradiated (1,500 R) normal splenocytes (as antigen-presenting cells [APC]) in a volume of 30 μ l. Footpad thickness was measured immediately before inoculation and 24 h later. Swelling was expressed as the mean percent increase ($\pm$ standard deviation) in footpad thickness at 24 h. Shown are the results from two experiments.

of protection was not investigated in any detail, but an important clue was noted. When stimulated *in vitro* with either GP-C403-417 or LCMV, T cells from these mice did not produce IFN- γ as did those from mice immunized with V-LSGPC (26), indicating that the two populations differ functionally and, by inference, in protective capacity.

Although CD4 $^+$ T cells have been reported to play a protective role in mice infected with vaccinia virus (5), hepatitis virus (43, 46), cytomegalovirus (23), and influenza virus (11), our studies appear to be the first to describe a protective effect of CD4 $^+$ T cells specific for an epitope that is conserved among arenaviruses differing in host range and biology. Generally, it has been difficult to demonstrate a life-or-death role for CD4 $^+$ T cells in the outcome of murine LCMV infections. Inactivation of CD4 $^+$ T-cell function, while reducing LCMV-specific CD8 $^+$ T-cell responsiveness (28), did not prevent the development of classical immune-

mediated LCM (28), and it had little (32) or no (1, 31) effect on the ability of mice to clear LCMV from infected extra-neural tissues. In contrast, LCMV-infected mice that had been depleted of functional CD8 $^+$ T cells by treatment with anti-CD8 antibodies, or those genetically deficient in CD8 $^+$ T-cell function, mounted no class I MHC-restricted LCMV-specific CTL response (1, 28, 31, 33), exhibited an impaired (31) or prolonged (14) process of virus clearance, and either failed to develop immunopathologic CNS disease pathognomonic of LCM (1, 28) or exhibited a more chronic disease process (14, 33).

There is a possibility that protection induced by immunization with V-LSGPC and protection conferred adoptively with C9 T cells differ in mechanism. In future studies, perhaps one way to strengthen our argument for the role of CD4 $^+$ T cells in cross-protection would be to demonstrate that treatment with anti-CD4 antibodies prior to challenge abrogates protection. However, equivocal results, e.g., as a result of incomplete elimination or inhibition of antigen-primed CD4 $^+$ T cells (17), would not negate the argument. Alternatively, recombinant congenic mouse strains could be used to show that the presence of *I-A^k* and *I-E^k* genes is necessary for V-LSGPC-induced protection. Such strains would require the C3H/HeJ background to control for the effects of non-*H-2* genes on susceptibility to LCMV (44) as well as *H-2^k* class I MHC genes to demonstrate protection by LV GP-C-specific, *I-A^k/I-E^k*-restricted CD4 $^+$ T cells.

In view of the incomplete protection afforded by immunization with V-LSGPC, the success of cloned CD4 $^+$ T cells in protecting mice against LCM might be attributed to their delivery directly to the challenge site and to the use of low LCMV challenge doses. C9 T cells protected against 20, but not 200, PFU of LCMV, a much less impressive effect than that produced by cloned CD8 $^+$ T cells, which, in comparable numbers, not only protect against 500 PFU of LCMV but also prevent the symptoms of LCM (3).

The mechanism by which C9 T cells protected is unclear. Virus was not simply inactivated during preparation of the C9 T cell-LCMV mixtures for adoptive transfer because it could be recovered from the mixture after 1 h in amounts

TABLE 5. Protection against LCMV challenge with adoptive transfer of C9 T cells^a

Expt	Cell dose	LCMV challenge dose (PFU)	No. of survivors/total		
			C3H/HeJ	BALB/c (<i>H-2^d</i>)	CD-1 (<i>H-2^d</i>)
1	1×10^6	20	5/5		
	None	20	1/5		
2	2×10^6	20	5/5	1/3	
	None	20	0/5	0/3	
	2×10^6	200	0/5	ND ^b	
	None	200	0/5	ND	
3	1×10^6	20	4/5		0/9
	3×10^5	20	4/5		0/9
	1×10^5	20	4/4		0/9
	None	20	0/5		0/9
	10^6 killed ^c	20	0/5		0/9

^a The indicated doses of LCMV and C9 T cells were injected i.c. into mice, which were monitored for disease and death for 15 days. The T cells were used 10 to 14 days after stimulation.

^b ND, not done.

^c Frozen and thawed three times.

that still killed mice (26). In addition, C9 T cells mixed with LCMV after being subjected to three successive freeze-thaw cycles did not confer protection. That mice protected by C9 T cells still developed typical, albeit transient, CNS symptoms indicated that the classical CD8⁺ T-cell-mediated immunopathology underlying LCM induction had occurred. We can only speculate as to how C9 T cells affected this process. Since the kinetics and magnitude of the LCMV-specific CTL response did not differ between V-LSGPC-immunized and normal mice after LCMV challenge, it seems unlikely that cross-reactive CD4⁺ T cells would have protected through a helper effect which accelerated or enhanced this response. On the other hand, CD4⁺ T cells have been shown to contribute to LCMV clearance from an extraneural DTH site (32), and in mice lacking CD8⁺ T cells, they will express class II MHC-restricted LCMV-specific cytotoxic activity (33). From the cumulative evidence, we envisage a scenario in which C9 T cells limit replication of LCMV exclusively at the site of their deposition within the CNS by mediating inflammation and producing IFN- γ . This would result in (i) fewer numbers of infected cells becoming potential targets of an unmodified LCMV-specific CTL response and (ii) less severe, nonlethal immunopathology.

Although our findings do not warrant any change in the traditional view of arenaviral immunopathogenesis, they illustrate a previously undescribed protective function of cross-reactive, virus-specific CD4⁺ T cells that could have relevance to the design of a vaccine against multiple Old World arenaviruses.

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