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Murine Cell-Mediated Immune Response Recognizes an Enterovirus Group-Specific Antigen(s)

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Splenocytes taken from mice inoculated with coxsackievirus B3 (CVB3) (Nancy) developed an in vitro proliferative response against CVB3 antigen. This response could not be detected earlier than 8 days postinoculation but could be detected up to 28 days after exposure to CB3. CVB3-sensitized splenocytes responded not only to the CVB3 antigen but to other enteroviruses as well. This response was found to be enterovirus specific in that no response was detected to a non-enteroviral picornavirus, encephalomyocarditis virus, or to an unrelated influenza virus. The generation of a splenocyte population capable of responding to an enterovirus group antigen(s) was not limited to inoculation of mice with CVB3, as similar responses were generated when mice were inoculated with CVB2. Cell subset depletions revealed that the major cell type responding to the enterovirus group antigen(s) was the $CD4^+$ T cell. Current evidence suggests that the group antigen(s) resides in the structural proteins of the virus, since spleen cells from mice inoculated with a UV-inactivated, highly purified preparation of CVB3 virions also responded in vitro against enteroviral antigens.

Human enteroviruses comprise 69 serotypically distinct viruses within the *Picornaviridae*, of which the best characterized are the three serotypes of poliovirus (PV1 to -3). Enteroviruses are common serious human pathogens and are confirmed or suspected agents of a number of diseases, including aseptic meningitis (10), poliomyelitis (39), myocarditis (49), congenital heart defects (3), and possibly diabetes mellitus (29). The genomes of PV1 to -3 (22, 24, 35, 40, 41) and the coxsackieviruses B1 (CVB1), CVB3, and CVB4 (18–20, 25, 43) have been cloned and sequenced. Comparison of the predicted amino acid sequences with data from the crystallographic solution of the three-dimensional structures of PV1 (14) and human rhinovirus 14 (37) strongly suggests a general unanimity of structure and function among enterovirion proteins.

The recognition of viral antigens by T cells occurs in the context of either class 1 or class 2 self-antigens in the major histocompatibility complex (51). Although the T-cell response is generally virus specific, closely related but serologically distinct viruses can induce a cross-reactive response (1, 6, 31, 36, 47). The influenza viruses are among the best-characterized examples of this type of cross-reactivity (1). Infection of humans or mice with one serotype of influenza A virus leads to a T-cell response that recognizes not only the infecting virus but also other, serologically distinct influenza A viruses.

Thus, by analogy to T-cell cross-reactivity observed in other virus groups and on the basis of predicted protein structures of enteroviruses, we postulated the existence of an enteroviral epitope(s) that could be recognized by enterovirus-immune T cells. The well-characterized CVB3 induction of myocarditis in mice (12) was chosen as a model system to study the ability of enterovirus-immune T cells to react to enteroviral antigens in vitro. Coxsackievirus B-induced myocarditis in mice is considered to produce pathological changes similar to those seen in human cardiomyopathies (5, 28), and the cell-mediated immune response has been reported to contribute significantly to myocarditis in the mouse model (11, 17, 50). Inoculation of male C3H/HeJ mice with CVB3 produces a predictable pattern of myocarditis 7 to 8 days postinfection (p.i.), with complete recovery by 28 days p.i. (21). We report here the development of an enterovirus antigen-driven, in vitro murine T-cell proliferation assay and the first evidence for a cross-reacting viral epitope among enteroviruses recognized by CVB3-induced immune T cells.

(A preliminary report of these data was presented at the American Society for Virology meeting, 12 to 16 June 1988, Austin, Tex.).

MATERIALS AND METHODS

Mice. Four-week-old male C3H/HeJ mice were obtained from Jackson Laboratory. Mice were housed five per cage and given chow and water ad libitum. All mice were maintained in the University of Nebraska Medical Center Animal Facility, which is fully accredited by the American Association of Laboratory Animal Care.

Viruses and antigens. Encephalomyocarditis virus (EMCV) and a myocarditic variant (in mice) of CVB3 (Nancy) (CVB3-M) were the gifts of C. Gauntt, University of Texas Health Science Center, San Antonio. CVB2 (Ohio-1), CVB6 (Schmitt), and PV1 (Chat) were obtained from the American Type Culture Collection. A naturally occurring isolate of coxsackievirus A16 (CVA16) was the gift of T. Smith, Section of Clinical Microbiology, Mayo Clinic, Rochester, Minn. Influenza virus A/Bangkok/1/79 was obtained from Flow Laboratories, Inc. Picornaviruses were propagated in HeLa cell monolayers in minimal essential medium–10% fetal bovine serum (GIBCO Laboratories)–50 μg of gentamicin per ml at 37°C in a humidified 5% CO_2 atmosphere. All picornavirus stocks were titered by plaque assay (45). Influenza virus was propagated in 10-day-old embryonated hen eggs and titered by hemagglutination (13).

Picornaviral antigens were prepared by two methods. Membrane antigen preparations were prepared from infected cells (multiplicity of infection, 50 PFU per cell) when the

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monolayer culture exhibited approximately 3+ cytopathic effect (i.e., 75 to 95% of cells showed classic enterovirus cytopathology). Cells were freeze-thawed three times and centrifuged at 3,000 × *g* for 15 min, after which the supernatant was collected and centrifuged at 10,000 × *g* for 30 min at 4°C. The pellet, consisting of viral antigen and cellular membranes, was suspended in RPMI 1640 medium (GIBCO), and small samples were stored at −75°C. For some experiments, highly purified virus was prepared by concentrating the virus by centrifugation at 90,000 × *g* for 15 h at 4°C through a cushion of 30% (wt/vol) sucrose–1 M NaCl–10 mM Tris hydrochloride (pH 7.5). The pelleted virus was suspended in 100 mM NaCl, ether extracted three times at room temperature, and then repelleted as described above through sucrose. The pellet was resuspended in 100 mM NaCl, and the virus was banded by isopycnic CsCl gradient centrifugation (30). Banded virus was collected from a puncture in the side of the centrifuge tube and then dialyzed extensively against 100 mM NaCl at room temperature to remove CsCl. Virus was titered and stored at −75°C. Protein concentrations of antigen preparations were determined by the method of Bradford (2).

UV irradiation or Formalin treatment was used to inactivate viral infectivity for some experiments. For UV inactivation, antigen preparations (both membrane-associated and CsCl-banded virus) were exposed in 6-ml quantities in 60-mm-diameter petri dishes to UV irradiation at 17 cm from a Philips TUV 30-W light source for 30 min. Formalin-inactivated viral antigen preparations were made 0.4% (vol/vol) Formalin (Sigma Chemical Co.), incubated at room temperature overnight, and then dialyzed extensively against 100 mM NaCl at room temperature. Prolonged exposure of HeLa cells to these inactivated preparations did not reveal any virus replication. Total protein was determined as described above, and the antigens were divided into equal portions for storage at −75°C. Influenza virus was Formalin inactivated as described above and used as antigen at a concentration of 1.0 hemagglutinating units per 10^5 spleen cells.

Infection of mice. Mice were inoculated intraperitoneally (i.p.) with 10^5 PFU of either viable CVB3 or CBV2 in 0.1 ml of RPMI 1640 medium. For some experiments, 10^5 PFU of UV-irradiated, highly purified CVB3 was inoculated i.p. Ether-anesthesized mice were bled by retro-orbital puncture at various times p.i. to obtain serum samples for determination of antibody titers.

Determination of antibody titers. Neutralizing antibody titers were measured by inhibition of viral cytopathic effects as described in detail elsewhere (8). Binding antibody titers were determined by a modification of the enzyme-linked immunosorbent assay (ELISA) procedure of Voller et al. (46). Briefly, 0.05 ml of highly purified CVB3 (20 μg/ml) diluted in a carbonate buffer (pH 9.6) was absorbed to flat-bottom microdilution plates (Costar) overnight at 4°C. Control wells consisted of mock-infected HeLa cell supernatant at an identical protein concentration. Nonabsorbed protein was removed the next day by washing the plates with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (Sigma) (PBS-t). PBS-t containing 5% ovalbumin (Sigma) was added to each well (0.05 ml per well) and incubated for 2 h at 37°C to inhibit nonspecific absorbance of assay components. Plates were washed with PBS-t, and fourfold serum dilutions (lowest dilution tested was 1:32) were added to the wells. After 2 h of incubation at 37°C and washing, goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Pel-Freeze) was added to the wells, and the plates were incubated for an additional 2 h at 37°C. After incubation and washing, 0.05 ml of the substrate, *O*-phenylenediamine (Pel-Freeze) in 0.3% hydrogen peroxide, was added to each well. The amount of color produced by the reaction of the enzyme on the substrate was measured in an eight-channel spectrophotometer (Biotek) at a wavelength of 492 nm. A net absorbance (A_{492}) was calculated by subtracting the A_{492} of the control well from the virus well A_{492}. Antibody titers were expressed as the reciprocal of the highest serum dilution that yielded a net absorbance greater than 2 standard deviations over the mean background values (wells that contained all components except test serum).

In vitro proliferation assay. Lymphocyte stimulation assays in each experiment were performed with cells from a pool of five mice. At various times p.i., mice were killed by cervical dislocation and the spleens were removed. Spleens were pressed through wire screens to obtain a suspension of single cells and then washed three times in RPMI 1640 medium. Washed cells were diluted to 2×10^6 cells per ml in RPMI 1640 medium supplemented with penicillin and streptomycin (10,000 U/ml), L-glutamine (29.2 mg/ml), 5×10^{-8} M 2-mercaptoethanol, and 10% decomplemented fetal calf serum (GIBCO). Cells were added to the wells of 96-well round-bottom Falcon plates in 100-μl volumes (2×10^5 cells per well). Antigen preparations were added in various concentrations in triplicate wells, and the plates were incubated for 1, 3, 5, or 7 days in a humidified atmosphere of 5% CO_2 at 37°C. On the appropriate day of in vitro culture, cells were incubated with 1.0 μCi of [^{3}H]thymidine (6.7 Ci/mmol; Amersham Corp.) and harvested 4 h later onto glass fiber filters, using a cell harvester (Cambridge Technology, Inc.). Filters were immersed in a scintillation cocktail (Ready-Safe; Beckman Instruments, Inc.), and radioactivity was determined by using a liquid scintillation counter. Student's *t* test (9) was used to evaluate significance of results.

Depletion of lymphocyte subsets by complement-mediated lysis. Specific cell subsets in the splenocyte mixture were depleted by using two cycles of complement-mediated lysis with monoclonal antibody TIB 99 (anti-Thy-1.2), TIB 150 anti-Lyt-2 (CD8), GK-1.5 anti-L3T4 (CD4), or 19G (anti-B cell) (American Type Culture Collection). Optimal concentrations of monoclonal antibodies and complement for depletion were determined in control experiments. Briefly, 4×10^6 spleen cells were suspended in 1 ml of a 1:5 dilution of monoclonal antibody and incubated for 30 min on ice. Guinea pig complement (GIBCO) was then added (final concentration, 1:5), and the cells were incubated further at 37°C for 45 min. After washing of the cells in calcium- and magnesium-free PBS, the cycle was repeated. Depletions were performed after 5 days of in vitro incubation with the appropriate antigens and just before the addition of [^{3}H]thymidine. Efficiency of depletion was monitored by indirect immunofluorescent staining. After monoclonal antibody-complement treatments, cells were stained with the appropriate monoclonal antibody for 30 min. Cells were washed, and fluorescein-conjugated goat anti-mouse immunoglobulin M (Bionetics) was added. Cells were incubated for an additional 45 min, pelleted by centrifugation, and suspended in mounting media (90% PBS, 10% glycerol). Fluorescence was observed under UV microscopy (Zeiss). Greater than 95% of the targeted cell subset was removed in each experiment.

RESULTS

Reactivity of mouse splenocytes to CVB3 antigen after CVB3 infection. Splenocytes from CVB3-infected C3H/HeJ

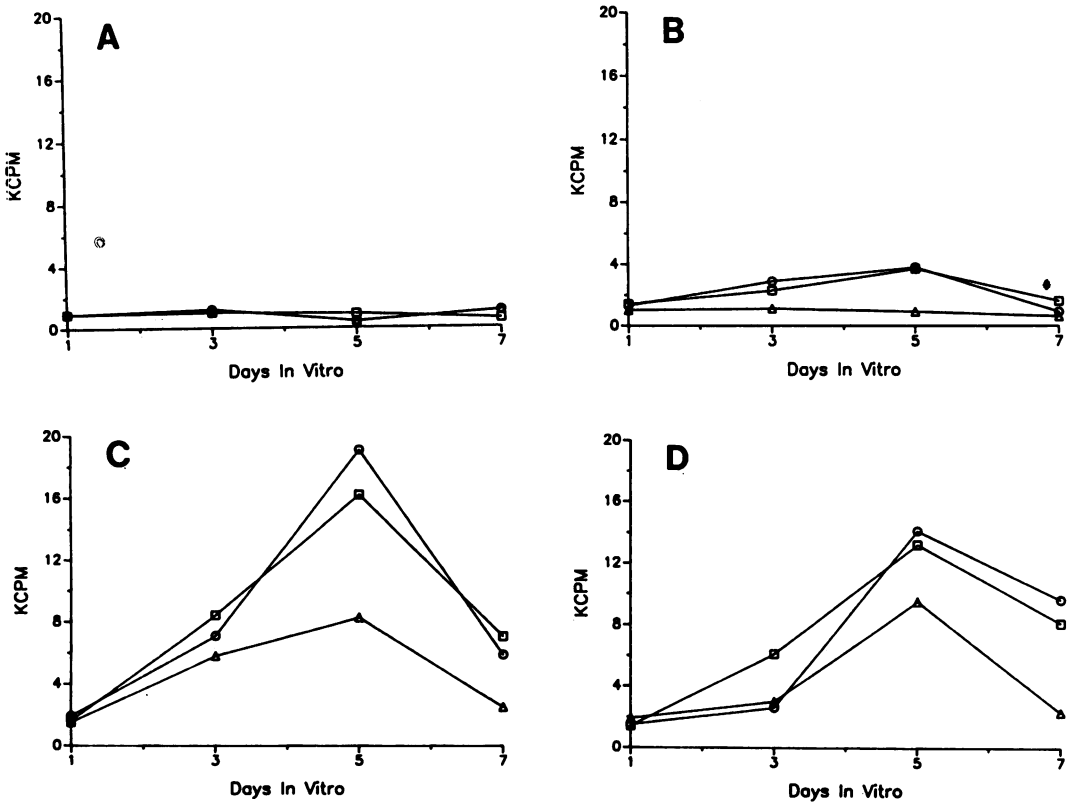

FIG. 1. Proliferative and antigen dose responses of splenocytes obtained from mice at various times after inoculation with CVB3. Mice were inoculated i.p. with 10^5 PFU and sacrificed at day 8 (A), 10 (B), 14 (C), or 28 (D) p.i. Splenocytes were exposed in vitro for 1, 3, 5, and 7 days to 10 (○), 1.0 (□), or 0.1 (△) μg of CVB3-infected HeLa cell membrane preparation. The background level of incorporation in cells exposed to the control uninfected HeLa cell membrane antigen preparation has been subtracted from each point. Proliferative responses against the uninfected HeLa cell membrane preparation never exceeded 1,956 ± 496 cpm (data not shown). KCPM, 10^3 cpm.

mice were tested for the ability to respond in vitro to a membrane fraction derived from CVB3-infected HeLa cells (see Materials and Methods). Spleens were obtained from mice infected i.p. with 10^5 PFU of CVB3 3, 8, 10, 14, or 28 days earlier. Splenocytes from the infected mice were cultured with three concentrations of infected HeLa cell membrane antigens. On days 1, 3, 5, and 7 of in vitro culture, [^{3}H]thymidine incorporation was monitored as a measure of cellular proliferation. Cellular proliferation was not detected in lymphocytes taken at 8 days p.i. (Fig. 1A) but was detectable in lymphocytes taken at 10 days p.i. (Fig. 1B). The peak response occurred at 14 days p.i. (Fig. 1C), with proliferative values still high (10 to 14 times background) at 28 days p.i. (Fig. 1D). Splenocytes taken from mice 3 days after infection with CVB3 or from uninoculated mice did not respond to the antigens at any dose tested (data not presented).

A clear antigen concentration-dependent response was observed at 14 and 28 days p.i. A concentration of >1 μg of total protein per well did not significantly improve proliferation ($P < 0.5$), whereas 0.1 μg per well was suboptimal. Thus, 1.0 μg per well was chosen as a working concentration of antigen for all other experiments. These data demonstrated that splenocytes from a CVB3-infected mouse were capable of responding to CVB3 antigen in vitro in a dose-dependent fashion as early as 10 days p.i. but with a peak response occurring at 14 days p.i.

Cross-reactivity between enteroviral antigens detected by lymphocyte proliferation, using splenocytes from CVB3-inoculated mice. Splenocytes from mice inoculated with CVB3 were tested for the ability to recognize and respond in vitro to enteroviral antigens other than CVB3. At 8, 10, 14, and 28 days after CVB3 infection, spleens were removed from infected mice and the splenocytes were exposed to membrane antigen preparations from HeLa cells infected with picornavirus CVB2, CVB6, CVA16, CVB3, PV1, or EMCV or with influenza virus A/Bangkok/1/79.

No cellular proliferation in response to any antigen was seen at 8 days p.i. (Fig. 2A), similar to results obtained with the CVB3 antigen (Fig. 1). At 10 days p.i., the PV1 antigen stimulated a significant proliferative response (Fig. 2B); by day 14 p.i., all enteroviral antigens had significantly stimulated the CVB3-immune T cells to proliferate (Fig. 2C). The proliferative response had decreased by day 28 p.i. but was still significant over background levels (Fig. 2D). On each day p.i., the PV1 antigen was a more potent stimulator of splenocyte proliferation than the other enteroviral antigens. No response was generated by EMCV, a murine cardiovirus with significant structural differences from the prototypical enterovirus, PV1 (22, 34, 41), and little nucleotide or predicted amino acid homology to other characterized enteroviruses (33, 34). In addition, the CVB3-immune splenocytes did not respond to the negative control, influenza virus A/Bangkok/1/79.

To determine whether the ability of the CVB3-immune murine splenocytes to proliferate in response to other enteroviral antigens might have been due to infection of the mice with more than one enterovirus, sera obtained from the

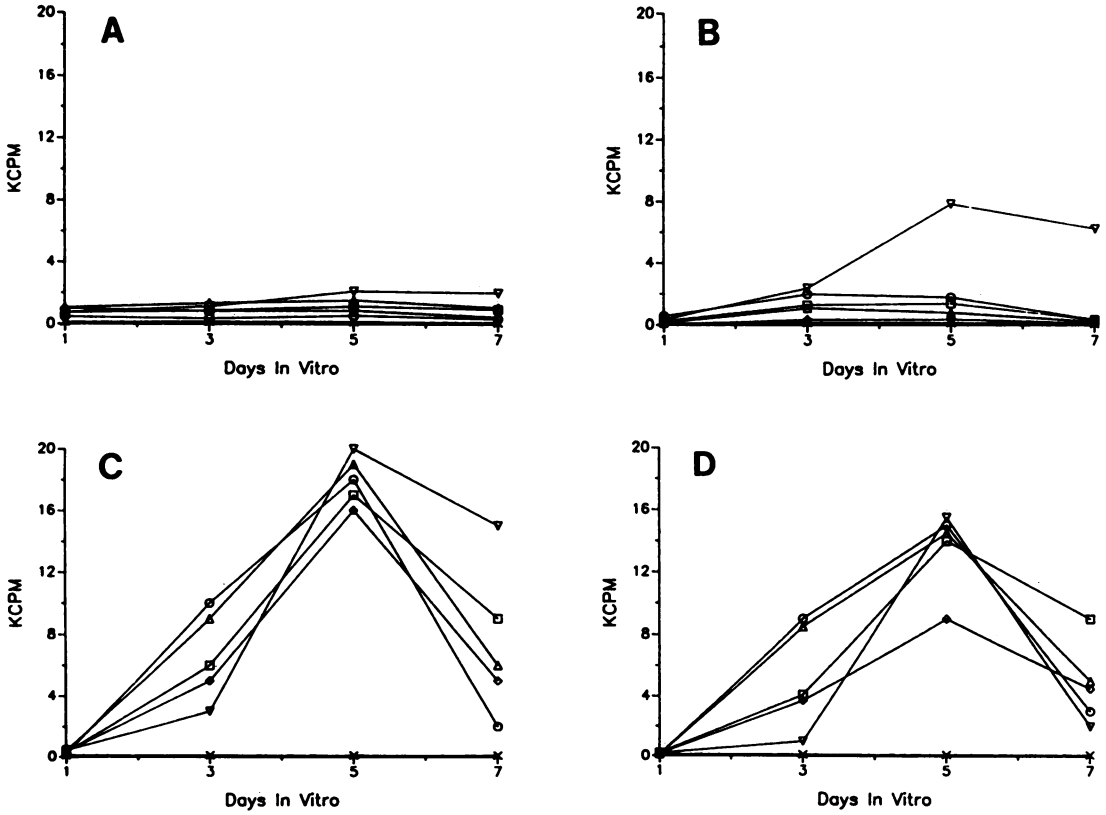

FIG. 2. Proliferative responses of splenocytes obtained from mice at various times after inoculation with CVB3. Mice were inoculated i.p. with 10^5 PFU and sacrificed at day 8 (A), 10 (B), 14 (C), or 28 (D) p.i. Splenocytes were exposed in vitro for 1, 3, 5, and 7 days to 1 μg of cell membranes derived from HeLa cells infected with CVB3 (○), CVB2 (□), CVB6 (△), CVA16 (◇), PV1 (▽), EMCV (×), or influenza virus A/Bangkok/1/79 (×). (Because the differences between the EMCV and influenza virus responses cannot be distinguished on the figure scale, the datum points are presented together.) The background level of incorporation in cells exposed to the control uninfected HeLa cell membrane antigen preparation has been subtracted from each point. Proliferative responses against the uninfected HeLa cell membrane preparation never exceeded 1,836 ± 350 cpm (data not shown). KCPM, 10^3 cpm.

mice before and after inoculation with CVB3 were tested for the presence of antibodies against the enteroviruses. Sera obtained before inoculation did not contain antibody against any of the enteroviruses tested, either by neutralization assay or by ELISA (Table 1). In addition, splenocytes from uninfected mice did not proliferate in response to enteroviral antigens (Table 1). Murine sera obtained 14 days after CVB3 infection contained neutralizing antibody against CVB3 at a titer of >1,280. No other virus in the panel was neutralized by the murine sera, although the splenocyte response was cross-reactive to all enteroviruses tested (Table 1). By ELISA, serum titers from mice before inoculation with CVB3 were negative to all enteroviruses tested. At 14 days p.i., ELISA titers to coxsackieviruses in addition to CVB3 were elevated, representing humoral cross-reactivity between the viruses (Table 1). Together, the antibody and proliferation data demonstrate that the mice did not experience an exogenous infection with any of the enteroviruses used in this study. Thus, splenocytes from mice infected with one enterovirus could respond in vitro not only to the infecting virus but also to other, serologically distinct enteroviruses. That this response was apparently enterovirus specific was demonstrated by a lack of a measurable response to EMCV, a murine cardiovirus, or to an unrelated influenza virus.

Removal of CD4$^+$ T cells diminishes the proliferative response. A typical murine spleen contains approximately 50 to 70% T cells and 30 to 50% B cells (26). To determine which major subset of splenocytes was responsible for the proliferative response, cell subset depletions were performed after 5 days of in vitro culture with antigen. Removal of T cells by the monoclonal antibody TIB 99 (Thy-1.2 specific) resulted in a marked decrease in proliferation relative to that of

TABLE 1. Neutralization and ELISA titers versus stimulation indices before and 14 days after CVB3 infection

Viral antigen[a]	Preinfection			Postinfection		
	Titer[b]			Titer		
	Neutralization	ELISA	SI[c]	Neutralization	ELISA	SI
CVB2	<10	<32	1.2	<10	512	10.7
CVB3	<10	<32	0.8	>1,280	128	10.5
CVB6	<10	<32	0.8	<10	128	9.9
CVA16	<10	<32	1.1	<10	64	8.5
PV1	<10	<32	1.3	<10	<32	11.9
EMCV	<10	<32	0.9	<10	ND[d]	0.9

[a] Used in vitro as HeLa cell-infected membranes (see Materials and Methods) at 1.0 μg of protein per 10^5 cells.

[b] Reciprocals of highest serum dilution that showed reactivity.

[c] SI, Stimulation index, calculated as the ratio of counts per minute in the presence of antigen over the counts per minute in the presence of uninfected HeLa membranes (background). A stimulation index of ≥3 is considered significant.

[d] ND, Not done.

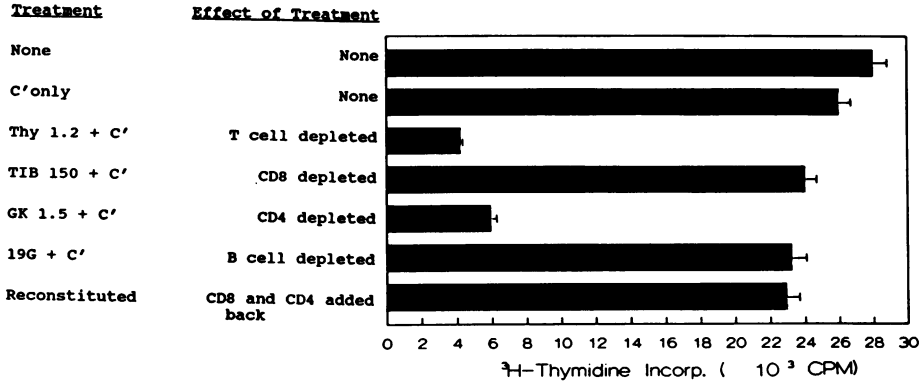

FIG. 3. CVB3 antigen-specific proliferative responses after cell subset depletion. Mice were inoculated with CVB3, and 28 days later their spleen cells were cultured with either CVB3 antigen or HeLa cell control antigen. After 5 days of in vitro culture, cells were depleted of specific subsets by exposure to monoclonal antibody and complement (C'). Proliferation in response to HeLa cell antigen (background), which never exceeded 1,721 ± 487 cpm, has been subtracted from each group. Each bar represents the mean and standard deviation of triplicate cultures.

control cultures treated with complement alone (Fig. 3). In contrast, removal of B cells with the monoclonal antibody 19G resulted in only a slight decrease in proliferation. These data suggest that T cells are the primary component of the enterovirus antigen-driven proliferative response and are consistent with histopathological observations that the primary cells found in inflammatory infiltrates of the CVB3-infected murine heart are T cells (4). To determine which subset of T cells was responsible for the antigen-specific proliferation, cultures were depleted of either $CD4^+$ or $CD8^+$ cells after 5 days of in vitro culture with either CVB3 viral or HeLa cell control antigen. The predominant T-cell population responding to viral antigen was the $CD4^+$ cells (Fig. 3). Similar results were obtained when CVB2 or PV1 was used as the viral antigen (data not presented). To demonstrate that monoclonal antibody depletion was specific, $CD4^+$- and $CD8^+$-depleted cultures were combined and cultured with [^{3}H]thymidine. The proliferative response was restored after reconstitution.

Effect of antigen preparation on proliferation levels. To determine whether nonreplicating virus could serve as antigen as well as to examine the effect of structural modification of the antigen on the proliferative response, antigen was prepared by different methods. Virus-infected HeLa cell membranes were treated with either Formalin or UV irradiation. In addition, highly purified, CsCl-banded virus was used to test for the effect of using viral antigen not associated with cellular membranes. The CsCl-banded virus preparations were assayed as either infectious, Formalin treated, or UV irradiated.

Splenocytes from mice inoculated with CVB3 14 days earlier were placed in culture with various viral antigen preparations. Regardless of the method of preparation, all antigen preparations induced in vitro proliferation of CVB3-immune splenocytes (Table 2). However, the magnitudes of the proliferative responses varied as a function of the method of antigen preparation. Infected cell membranes were 1.3- to 2.1-fold more potent than CsCl-banded virus as a stimulator of in vitro proliferation (Table 2). Formalin treatment or UV irradiation of either membrane preparations or purified virus increased proliferation slightly (1.1- to 1.7-fold) over that of untreated preparations; Formalin treatment of viral antigens provided the greater enhancement of T-cell proliferation. Clearly, virus particles incapable of replication could act efficiently as antigen in vitro. Although either membrane preparations or purified virus could stimulate in vitro proliferation of CVB3-immune splenocytes, association of the viral antigens with cellular membranes appeared to be a reproducibly and significantly stronger inducer of proliferation regardless of whether the antigen was Formalin or UV treated.

Infection of mice with CVB2 also generates T cells that respond to enteroviral antigens. To determine whether the induction of murine T cells capable of recognizing a common enteroviral antigen(s) could be promoted by infection with another enterovirus, mice were inoculated i.p. with 10^5 PFU of CVB2. Infection with CVB2 resulted in seroconversion of the mice, with neutralizing antibody titers ranging between 640 and 1,280 (data not presented), similar to values found after CVB3 infection (Table 1).

At 3, 8, 10, or 14 days after CVB2 infection, the in vitro proliferative response of splenocytes was compared with that of a panel of enteroviral antigens. Whereas no discernible proliferation occurred against any of the antigens tested at 3 days p.i. (Fig. 4A), a low-level proliferative response of splenocytes in vitro was observed at 8 days p.i. (Fig. 4B). Similar to the responses observed with splenocytes from CVB3-inoculated mice (Fig. 2), splenocytes from CVB2-inoculated mice at 10 days p.i. (Fig. 4C) proliferated signif-

TABLE 2. Effect of antigen preparation on splenocyte proliferation[a]

Viral antigen	Antigen prepn[b]					
	M	F-M	U-M	C	F-C	U-C
CVB3	15.6	21.2	17.4	7.5	9.5	8.9
CVB2	12.0	20.7	15.9	8.4	9.7	9.2
CVB6	13.1	15.2	14.9	10.1	11.7	11.0
CVA16	9.9	12.2	10.1	7.8	12.1	9.4
PV1	20.1	28.6	27.5	12.1	14.2	12.9

[a] Mice were inoculated i.p. with 10^5 PFU of CVB3, and spleen cells were set in culture 14 days p.i. Cells were assayed for proliferation against specific antigens on day 5 of in vitro culture.

[b] M, Infected HeLa cell membranes; F-M, Formalin-treated infected HeLa cell membranes; U-M, UV-irradiated, infected HeLa cell membranes; C, CsCl-banded virus; F-C, Formalin-treated, CsCl-banded virus; U-C, UV-irradiated, CsCl-banded virus. All membrane preparations were used at a concentration of 1.0 μg of protein per 10^5 cells; all CsCl-banded virus preparations were used at 1 PFU per cell. Data are presented as stimulation indices (counts per minute [virus]/counts per minute [uninfected HeLa cells]). A stimulation index of ≥3 is considered significant.

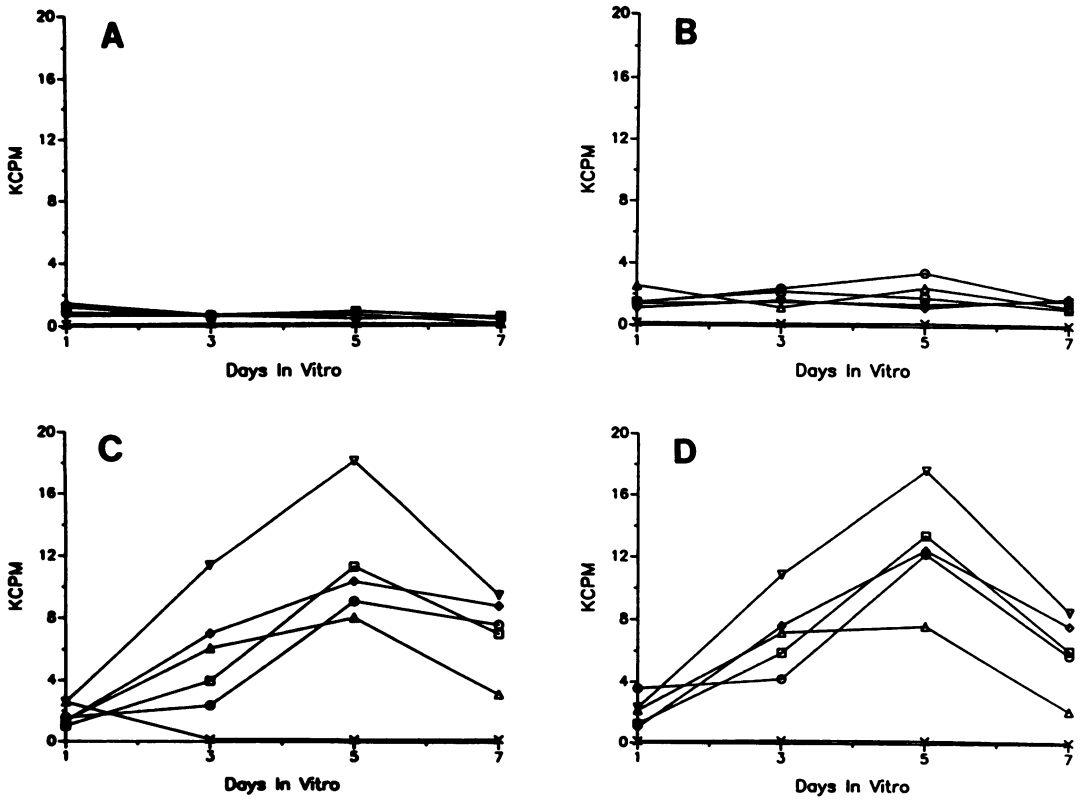

FIG. 4. Proliferative responses of splenocytes obtained from mice inoculated with CVB2. Mice were inoculated i.p. with 10^5 PFU of CVB2 and sacrificed on day 8 (A), 10 (B), 14 (C), or 28 (D) p.i. Cells (10^5 per well) were exposed in vitro to 1.0 μg of cell membranes derived from HeLa cells infected with CVB3 (○), CVB2 (□), CVB6 (△), CVA16 (◇), PV1 (▽), EMCV (×), or influenza virus A/Bangkok/1/79 (×) for 1, 3, 5, and 7 days of culture. (Because the differences between the EMCV and influenza virus responses cannot be distinguished on the figure scale, the datum points are presented together.) The background level of incorporation in cells exposed to the control uninfected HeLa cell membrane antigen preparation has been subtracted from each point. Proliferative responses against the uninfected HeLa cell membrane preparation never exceeded 1,492 ± 595 cpm (data not shown). KCPM, 10^3 cpm.

icantly in response to exposure to all enteroviral antigens tested, with an increased response observed at 14 days p.i. (Fig. 4D). Consistent with previous results obtained with CVB3-inoculated mice, neither EMCV nor influenza virus A antigens induced a proliferative response in splenocytes from CVB2-inoculated mice. These data demonstrate that inoculation of mice with CVB2 induced the production of T cells that also recognized an epitope shared by the enteroviruses tested.

Immunization of mice with UV-inactivated CVB3 generates T cells that respond to enteroviral antigens. The experiments described above involved the use of mice inoculated with live CVB2 or CVB3. In addition, it was demonstrated that virus incapable of replication could serve as a potent antigen for in vitro stimulation of enterovirus-immune splenocytes. To investigate whether in vivo replication of virus was required for the generation of T cells that recognize enteroviral cross-reacting antigen(s) in vitro, mice were inoculated i.p. with 10^5 PFU of highly purified (i.e., predominantly capsid protein and VPg), UV-inactivated CVB3. Neutralizing antibody titers (between 640 and 1,280) demonstrated that all mice seroconverted to CVB3 but to no other enterovirus used (data not shown). At 8, 10, 14, or 28 days p.i., spleens were removed and splenocytes were set in culture with CVB2, CVB3, CVB6, CVA16, PV1, EMCV, and influenza A virus antigens. Cultures were measured for proliferative responses on days 1, 3, 5, and 7 of in vitro culture.

Similar to the responses of splenocytes from mice inoculated with infectious CVB3 (Fig. 2), splenocytes from mice inoculated with inactivated CVB3 showed stimulation of proliferation against all enteroviral antigens tested (Fig. 5). The EMCV and influenza virus antigens did not induce a response, again indicating that the response was specific for enteroviruses. Similarly, the PV1 antigen induced the greatest proliferative response, as was observed for splenocytes from mice inoculated with infectious CVB3 or CVB2. These data suggest that exposure of mice to a replicating virus is not required for generation of T cells that recognize cross-reacting enteroviral antigen(s).

DISCUSSION

In light of the predicted amino acid homologies among numerous enteroviruses for which complete or partial genomic nucleotide sequences are known (18–20, 22, 24, 25, 35, 40, 41, 44) and because the T-cell response has been shown to recognize closely related but serologically distinct viruses in other families (1, 6, 31, 36, 47), we postulated that cross-reacting antigens might exist among the serologically defined enteroviruses. On the basis of extensive nucleotide and predicted amino acid sequence dissimilarities between enteroviruses and cardioviruses (33, 34), we also predicted that the cardiovirus EMCV might not share such a pan-enteroviral antigen. The results presented here establish that

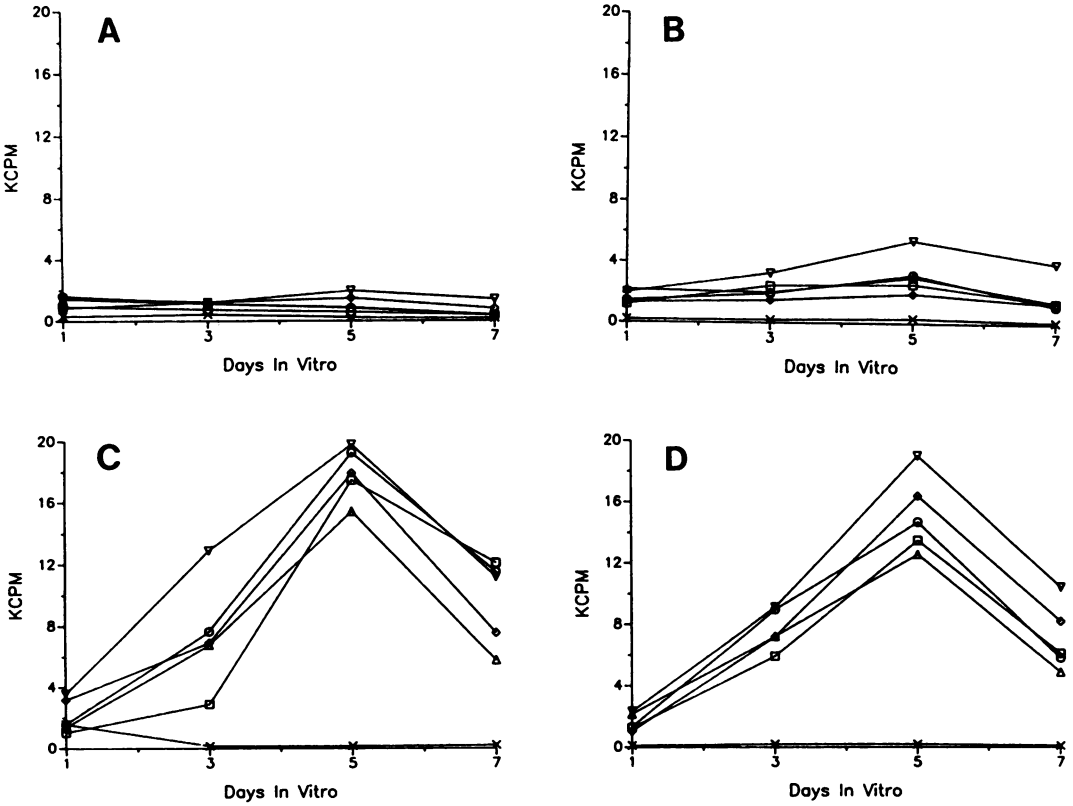

FIG. 5. Proliferative responses of splenocytes obtained from mice inoculated with UV-inactivated, highly purified CVB3. Mice were inoculated i.p. with 10^5 PFU of UV-inactivated CVB3. Mice were sacrificed and splenocytes were prepared at 8 (A), 10 (B), 14 (C), or 28 (D) days p.i. and exposed in vitro for 1, 3, 5, and 7 days to 1 μg of cell membranes derived from HeLa cells infected with CVB3 (○), CVB2 (□), CVB6 (△), CVA16 (◇), PV1 (▽), EMCV (×), or influenza virus A/Bangkok/1/79 (×). (Because the differences between the EMCV and influenza virus responses cannot be distinguished on the figure scale, the datum points are presented together.) HeLa cell membrane antigen background has been subtracted from each point. Proliferative responses against the uninfected HeLa cell membrane preparation never exceeded 1,832 ± 572 cpm (data not shown). KCPM, 10^3 cpm.

sensitized T cells taken from CVB3- or CVB2-inoculated mice at 8 to 14 days p.i. undergo proliferation to homologous and heterologous enterovirus antigens. That EMCV did not promote proliferation of enterovirus-sensitized T cells suggests that the recognized viral antigenic epitope(s) is unique to the enteroviruses. The results of these studies confirm and extend previous observations made with several other virus families (1, 6, 31, 36, 47) that specificity of viral antigen recognition by immune T cells cannot be based on serological dissimilarities among viruses within genera. In addition, as has been demonstrated for proliferative responses against other viral antigens (38, 48), the predominant T-cell subset that proliferated in response to the viral antigen was found to be $CD4^+$ (Fig. 3).

Previous attempts in this and other laboratories failed to generate in vitro virus-specific proliferation of splenocytes taken from CVB3-inoculated mice at 3 to 8 days p.i. This failure to obtain CVB antigen-reactive T cells from the spleen could be due to localization of virus-reactive T cells at the site of inflammation, the heart. Localization of T cells to the site of injury has been demonstrated in the heart after CVB3 inoculation (4) and at the site of an allograft (32). Myocardial lesion formation in mice peaks 8 days p.i. (7), and T cells are the predominant cell type infiltrating the lesions (4). Because T cells migrate between the bloodstream and lymphoid tissues from the sites of antigen deposition (15), it is possible that the inability to detect enterovirus antigen-reactive T cells in the spleen early after CVB3 infection may reflect patterns of cell trafficking.

The possibility existed that limited replication of the virus in the lymphoid cells in vitro might act to generate viral proteins with cross-reacting potential. Matteucci et al. (27) demonstrated that group B coxsackieviruses could establish persistent infections in selected human lymphoid cell lines. To prevent the virus from replicating in vitro, antigen preparations were treated with either Formalin or UV irradiation before testing in vitro. Both treatments inactivated the virus. Antigens prepared by either method stimulated T cells in vitro and were, in fact, somewhat better antigens than either native membrane or CsCl-banded virus preparations (Table 2). Thus, neither replication of the virus in vitro nor intact viral structure is required for the antigen to stimulate T-cell proliferation.

The generation of T cells that respond to an enteroviral group antigen(s) does not require an active infection in vivo. Mice were inoculated with UV-inactivated CVB3, and their splenocytes were tested against enteroviral antigens. As we had observed when mice were challenged with infectious CVB3, the in vitro proliferative response occurred at 10 days p.i. (Fig. 5). Together, these data suggest that replication of virus is required neither in vitro nor in vivo in order to generate T cells capable of recognizing an enterovirus group-specific antigen.

Inoculation of mice with CVB2 also results in generation

of T cells that recognize an enterovirus group antigen(s). In contrast to the severe inflammation in the heart that occurs after infection with CVB3, infection of mice with CVB2 results in only a mild inflammation of the heart (M. Beck and S. Tracy, unpublished observations). We found that splenocytes from mice infected with CVB2 responded to the CVB3, CVB6, CVA16, and PV1 antigen preparations in vitro in a manner consistent with our data for splenocytes obtained from CVB3-infected mice (Fig. 4). The response was found to be enterovirus specific; no proliferation was seen against EMCV or influenza A virus. Thus, the generation of T cells that respond to an enterovirus group antigen is not unique to infection with a specific serotype.

Conclusions about the antigenic nature of various enteroviruses should not be based on different reactivity patterns observed for the antigenic preparations used in this study. For example, T cells taken at 10 days p.i. from mice inoculated with CVB3 proliferated in response to the PV1 antigen, whereas the response to the other viral antigens was still minimal (Fig. 2). Although membrane antigen preparations were incubated with lymphocytes on the basis of equivalent concentrations of proteins and the CsCl-banded viruses were added on an equal-PFU basis, the titers of virus in the membrane preparations were all within 10-fold of each other (data not shown). Infected cells used to prepare the antigens were harvested when approximately 75 to 95% of the cells exhibited typical enterovirus-induced cytopathic effect (cell rounding), which cannot be quantitatively related to levels of viral proteins produced in these cultures. The antigenic stimulation potentials of these preparations could differ significantly, and we have as yet no way of measuring this difference.

Which enteroviral protein(s) contains the epitope(s) recognized by the T-cell response? Our results are compatible with the existence of one or more epitopes shared by enteroviruses within one or more of the four capsid polypeptides. The CsCl-banded CVB3 was highly enriched for the capsid proteins and the small VPg protein that is covalently linked to the 5′-terminal nucleotide of the RNA genome. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (23) revealed that this preparation did not contain significant contamination with nonviral or nonstructural proteins (data not presented). Therefore, mice inoculated with UV-inactivated, CsCl-banded CVB3 would be expected to mount an immune response only to the capsid proteins, VPg, or both. Indeed, splenocytes taken from UV-inactivated, CVB3-inoculated mice 14 days p.i. were capable of responding to all of the enteroviruses tested in vitro. The smallest and most internal of the four capsid proteins of CVB3, protein 1A (VP4), is the most highly conserved at the predicted amino acid level, with 45 of the 69 amino acids (65%) in this protein held in common with those of PV1 (42). The other three capsid proteins, 1B to 1D, share significant but lesser homology among different enteroviruses (42).

Although these data are consistent with the existence of the common epitope(s) in the structural proteins, they do not conclusively identify the viral protein or proteins that contain the cross-reacting epitope, and at this time we cannot rigorously exclude the involvement of nonstructural proteins. Testing of polypeptides corresponding to specific enterovirus coding regions is required to precisely locate the site(s) of antigenic cross-reactivity. We are now testing several such polypeptides derived from in vitro translation of the P1-, P2, and P3-coding regions of CVB3 RNA for antigenic potential in our in vitro proliferation assay.

In summary, we have presented data documenting the existence of an epitope(s) that appears to be conserved among enteroviruses and is recognized by CVB3- or CVB2-immune T cells. In other experiments (Beck and Tracy, unpublished observations), we have also shown that human peripheral T cells recognize the enteroviral group antigen in a manner similar to that described here for mice. Because T cells are implicated in the pathology of myocarditis (16, 17) and because they can recognize cross-reacting enteroviral antigens, the discovery of an enterovirus group-specific antigen recognized by CVB2- or CVB3-sensitized T cells may have implications for human enteroviral infections and inflammatory diseases.

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