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Evidence for a Group-Specific Enteroviral Antigen(s) Recognized by Human T Cells

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Human peripheral blood mononuclear cells from 15 normal, healthy adult volunteers proliferated in vitro against a panel of enteroviral antigens, including coxsackievirus B3, coxsackievirus B2, coxsackievirus B6, coxsackievirus A16, and poliovirus 1. No proliferation against the cardiovirus encephalomyocarditis virus occurred. Lymphocytes obtained from cord blood drawn from seven neonates were uniformly nonresponsive to enteroviral antigens. Although serum neutralization antibody titers indicated different exposure histories of the volunteers, only one had a titer against coxsackievirus B6, a rare isolate in the United States. The peripheral blood mononuclear cells from each volunteer responded in vitro to each enterovirus tested even though not all individuals had serum neutralizing antibody against each virus. The predominant cell type responding in vitro was the $CD4^+$ T cell. Denaturation of viral antigen by Formalin did not prevent the recognition of the common group antigen by the T cells, indicating that noninfectious virus can also serve as antigen. These data demonstrate that human T cells recognize a common enterovirus group antigen(s).

Enteroviruses (*Picornaviridae*) are etiologic in a wide spectrum of human disease (4, 14, 25). However, other than the well-documented destruction of neuronal cells in poliomyelitis (33), pathogenic enteroviral disease mechanisms are relatively ill defined. The coxsackieviruses of the A and B serogroups are myotropic in mice (19) and induce inflammatory infiltrates in both heart and skeletal muscle when inoculated into mice (11, 24, 36; S. Tracy, V. Wiegand, B. McManus, C. Gauntt, M. Pallansch, M. Beck, and N. Chapman, J. Am. Coll. Cardiol., in press). These infiltrates are composed primarily of cells of the T lineage (9).

The hypothesis that enteroviruses, especially the coxsackieviruses, are etiologic in the inflammatory human diseases myocarditis (44) and idiopathic dilated cardiomyopathy (24) is supported by repeated observations that elevated coxsackievirus-specific serum immunoglobulin M levels are more often observed in patients with myocarditis than in control groups (4, 18, 28, 30, 34, 39), by isolations of enteroviruses from diseased cardiac tissue (21–23, 37), and by demonstrations of enteroviral RNA in inflamed heart and skeletal muscle samples (1, 5, 10, 19, 20; S. Tracy, N. M. Chapman, B. M. McManus, M. J. Pallansch, M. A. Beck, and J. Carstens, J. Mol. Cell. Cardiol., in press). However, following the damage due to replication of enteroviruses in the muscle, it is unclear whether pathologic changes which occur in the human heart are due to a cell-mediated immune response (19, 44) or are sequelae of viral replication (15).

We examined the ability of human T cells to proliferate in vitro to enteroviral antigens to determine whether human T cells recognized the enterovirus group antigen described in earlier murine studies (2, 3) and to better understand the pathogenic mechanisms involved in human enterovirus-induced myocarditis. Serum neutralizing antibody titers, an indicator of previous enteroviral exposure, demonstrated that each volunteer had experienced infection with two or more of the enteroviruses we tested. Peripheral blood T cells from all volunteers proliferated in response to each of the five serologically distinct enteroviruses tested, regardless of infection history. The finding that human T cells recognize an enterovirus group antigen(s) may have implications for enterovirus-induced inflammatory disease in the adult population.

MATERIALS AND METHODS

Subject population. The study group consisted of normal, healthy adult volunteers. The group consisted of seven women and eight men, and the mean age of the population was 31.4 ± 5.8 years (mean ± standard deviation). All of the subjects had been vaccinated against poliovirus (either Sabin or Salk vaccine). Informed consent was obtained from the volunteers prior to drawing of venous blood (50 ml). As a control, cord blood was obtained from seven neonates at the time of parturition.

The guidelines for human studies of the U.S. Department of Health and Human Services and of our institution were followed in the conduct of this study.

Antigens. Coxsackievirus B3 (Nancy) (CVB3-M) and encephalomyocarditis virus (EMCV) were obtained from C. Gauntt, University of Texas Health Science Center, San Antonio. Coxsackieviruses B2 (Ohio-1) (CVB2) and B6 (Schmitt) (CVB6) and poliovirus 1 (Chat) (PV1) were obtained from the American Type Culture Collection, Rockville, Md. Coxsackievirus A16 (CVA16), a naturally occurring isolate, was obtained from T. Smith, Section of Clinical Microbiology, Mayo Clinic, Rochester, Minn. CVB2 and CVB3 are common human enteroviruses, while CVB6 is a rare isolate in the United States (8). CVA16 was chosen as a representative of the A group of coxsackieviruses. As a nonenteroviral control, we chose EMCV, a cardiovirus. Human exposure to EMCV is rare and is associated with contamination of foodstuffs with rodent feces (27).

Viral antigens were prepared as described elsewhere (3). Briefly, HeLa cell monolayers were infected with 50 virus 50% tissue infective doses per cell. When typical enteroviral cytopathic effect was observed to be approximately 3+, infected cells were harvested and lysed by three cycles of freezing and thawing. Uninfected HeLa cells were harvested at the same time, and control antigen was prepared identi-

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cally. The cell preparation was cleared of major cell debris by centrifugation at 3,000 × *g* for 15 min, following which the supernatants were collected and further centrifuged at 10,000 × *g* for 30 min. The pellets were suspended in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) to a final concentration of 500 μg of total protein per ml, as determined by the method of Bradford (7). For some experiments, viral antigen was exposed to 0.4% Formalin (vol/vol) overnight at room temperature and then extensively dialyzed against phosphate-buffered saline to remove the Formalin. All Formalin preparations were demonstrated to be noninfectious by passage onto HeLa cell monolayers.

PMBC culture. Peripheral blood mononuclear cells (PBMC) (>98% viability as determined by trypan blue exclusion) were isolated from 50 ml of heparinized venous blood or 3 to 7 ml of cord blood (10 U/ml; Lyphomed, Rosemont, Ill.) with a Ficoll-metrizoate gradient, as previously described (35). Cells were cultured in RPMI 1640 medium supplemented with penicillin-streptomycin (10,000 U/ml), 2.5% *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, and 10% heat-inactivated fetal calf serum (GIBCO). PBMC (2×10^5) were dispensed in 0.1-ml volumes per well in triplicate to 96-well round-bottom plates (Falcon; Becton Dickinson Labware, Oxnard, Calif.) in the presence of viral or mock antigen and incubated at 37°C under 5% CO_2 for 1, 3, 5, or 7 days. On the appropriate day of in vitro culture, 1.0 μCi of [^{3}H]thymidine ([^{3}H]TdR; 6.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.) was added to each well, and the cells were harvested 4 h later onto glass fiber filters by using a cell harvester (Cambridge Technology, Watertown, Mass.). Each filter was placed in fluor (Readi-Solve; Beckman Instruments, Inc., Palo Alto, Calif.), and the amount of incorporated [^{3}H]TdR in each sample was measured with a liquid scintillation counter. For some experiments, a stimulation index was calculated by dividing the mean counts per minute of triplicate wells of antigen-stimulated cultures by the mean counts per minute in triplicate wells of mock-stimulated cultures.

Neutralization assay. Neutralizing antibody was measured by inhibition of viral cytopathic effect, as described in detail elsewhere (13). Briefly, serum samples were diluted in twofold increments with minimal essential medium supplemented with penicillin-streptomycin and 10% heat-inactivated fetal calf serum to a maximum dilution of 1:1,280. The appropriate virus was added to each dilution at 4×10^4 50% tissue culture infective doses per dilution and incubated at 37°C for 30 min. Following the incubation period, the serum-and-virus mixture was added in triplicate in 0.1-ml volumes to confluent HeLa cell monolayers in 96-well flat-bottom plates and incubated for 24 h. Cells were fixed with the addition of 75% ethanol–25% acetic acid (vol/vol) and stained with 1% (wt/vol) crystal violet. The neutralizing titer was defined as the reciprocal dilution of the endpoint at which the majority of the wells showed ≥50% inhibition of cytopathic effect compared with the HeLa cell monolayers exposed to virus alone.

Depletion of cell subsets by complement-mediated lysis. PBMC were depleted of specific cell subsets by two cycles of complement-mediated lysis with monoclonal antibodies CD19 (pan B cells), CD3 (pan T lymphocytes), CD4 (helper T cells), and CD8 (cytotoxic/suppressor T cells) (Boehringer Mannheim, Indianapolis, Ind.). All depletions were done following 5 days of in vitro culture just prior to the addition of [^{3}H]TdR. Briefly, 4×10^6 cells were suspended in 0.2 ml of monoclonal antibody (1:10 dilution) and incubated on ice for 30 min. Following incubation, guinea pig complement (GIBCO) was added (final concentration, 1:4) and the cells were incubated for 30 min at room temperature. The cells were washed in calcium- and magnesium-free phosphate-buffered saline, and the antibody-complement cycle was repeated. Following the second depletion, equal volumes from each treated sample were added to 3 wells of a 96-well round-bottom plate and [^{3}H]TdR was added to each well. Cells were harvested following 6 h of incubation, and incorporation of radioactivity was measured as described above. Efficiency of depletion was monitored by incubating the cells with the appropriate antibody and then staining them with rabbit anti-mouse immunoglobulin labeled with fluorescein isothiocyanate (Cappel, West Chester, Pa.). Fluorescence was monitored by UV microscopy. In each experiment, more than 97% of the targeted cell population was removed.

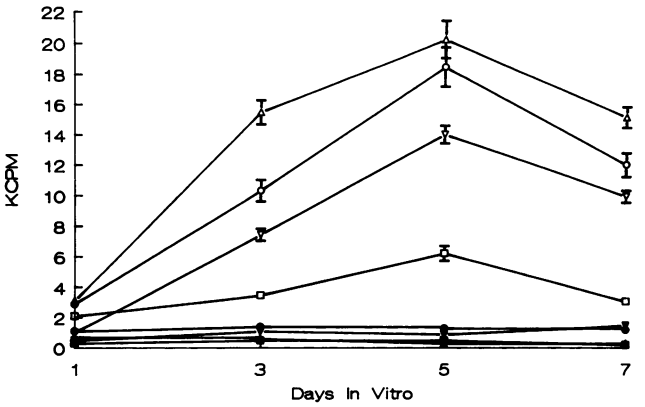

FIG. 1. Proliferative and antigen dose responses of human PBMC against CVB3 antigen. PBMC (2×10^5) were exposed in vitro for 1, 3, 5, and 7 days to 20 (△), 10 (○), 1.0 (▽), or 0.1 (□) μg of CVB3-infected HeLa cell membrane preparations or to the control uninfected HeLa cell membrane preparation at 20 (▲), 10 (●), 1.0 (▼), or 0.1 (■) μg. Each point represents the mean ± standard deviation of triplicate cultures.

Statistical analysis. Student's *t* test (one tailed) was used to determine the significance of [^{3}H]TdR incorporation between groups. Statistical significance was assessed at the $P < 0.05$ level.

RESULTS

Human PBMC proliferate in response to CVB3 antigen. PBMC from a volunteer previously determined to be CVB3 seropositive by neutralizing antibody titer were added in triplicate wells to four dilutions of CVB3 antigen. At 1, 3, 5, and 7 days of incubation, the cells were exposed to [^{3}H]TdR for 4 h and monitored for incorporation of radioactivity. Peak responses were seen after 5 days of in vitro culture at an antigen concentration of 20 μg/ml, although this result was not statistically different from the response with an antigen dose of 10 μg/ml (Fig. 1). Cells which were exposed to 1.0 μg/ml did not proliferate as strongly, although the response was significantly higher than background. An antigen dose of 0.1 μg/ml was clearly suboptimal; cells exposed to this concentration responded weakly. PBMC from three other CVB3-seropositive individuals responded to the viral antigen dilutions in a similar fashion (data not presented). Based on these results, a working concentration of 1.0 μg of viral antigen per ml was chosen for all other experiments.

Human PBMC proliferate against enteroviral antigens in vitro. Our previous work (2, 3) demonstrated that CVB2- or

TABLE 1. Stimulation indices of human PBMC in response to viral antigen[a]

Subject	Stimulation index in response to the following viral antigen[b]:					
	CVB2	CVB3	CVB6	CVA16	PV1	EMCV
1	8.1	12.3	10.5	11.3	24.3	0.9
2	12.7	13.6	15.4	14.4	14.5	0.7
3	11.6	8.2	9.4	10.7	18.2	1.1
4	18.2	17.6	8.6	7.5	7.9	0.7
5	7.3	8.6	8.3	7.1	10.4	0.8
6	4.3	4.2	5.3	5.7	9.4	0.9
7	7.8	4.3	4.9	6.7	10.6	1.1
8	12.8	7.5	6.7	4.4	7.9	1.2
9	11.1	14.9	18.0	16.7	20.8	0.6
10	16.6	8.7	15.4	12.6	12.6	0.5
11	10.2	9.4	9.8	14.3	ND[c]	0.7
12	3.6	3.7	5.6	4.9	ND	0.8
13	8.8	5.6	4.8	10.4	14.5	1.0
14	9.2	9.9	6.5	7.4	11.2	1.1
15	7.4	11.9	8.0	5.5	9.9	0.7

[a] Viral antigen used in vitro was as HeLa cell-infected membranes (see Materials and Methods) at 1.0 μg of protein per 10^5 cells. PBMC were placed in culture with the appropriate viral antigen and harvested 5 days later following a 4-h exposure to [^{3}H]TdR.

[b] The stimulation index is calculated as the ratio of the 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.

[c] ND, Not done.

CVB3-immune murine T cells recognize and respond to a variety of different enteroviral antigens in an in vitro proliferation assay. To determine whether human PBMC would respond to serologically distinct enteroviruses other than CVB3 in vitro, cells from 15 donors were exposed to CVB2, CVB6, CVA16, and PV1 antigens. Cells from each individual responded to every enteroviral antigen, but none proliferated against the control cardiovirus EMCV (Table 1). None of the subjects' PBMC responded to EMCV even when the concentration of antigen was increased to 75 μg/ml (data not shown), suggesting that the recognized epitope(s) is not present in this cardiovirus.

Lymphocytes obtained from cord blood samples were exposed in vitro to the enteroviral antigens for 1, 3, 5, and 7 days. None of the samples responded to the antigens, although all isolates proliferated in response to the mitogen concanavalin A (Table 2). Titers of neutralizating antibody in the cord blood plasma indicated that the mothers of the neonates had experienced exposure to various enteroviruses (Table 3). These data indicate that lymphocytes which respond to the enteroviral antigens in vitro are memory cells generated by exposure of the host to an enterovirus. Naive cells are not capable of responding to enteroviral antigens in vitro, as demonstrated by the cord blood lymphocytes.

TABLE 2. Stimulation indices of cord blood lymphocytes in response to viral antigen[a]

Sample no.	Stimulation index in response to the following viral antigen[b]:						
	CVB2	CVB3	CVB6	CVA16	PV1	EMCV	ConA
1	0.9	0.8	0.7	0.9	0.9	0.8	49.6
2	0.7	0.9	0.5	0.9	0.9	0.8	55.8
3	0.8	0.6	0.9	1.1	1.2	0.8	62.4
4	0.7	0.8	0.8	0.9	0.6	0.9	55.9
5	0.7	0.9	1.3	1.2	0.8	0.9	63.6
6	0.8	0.7	1.1	0.8	0.7	1.2	60.8
7	0.6	0.9	0.9	0.5	1.1	1.3	66.8

[a] Viral antigen used in vitro was as HeLa cell-infected membranes (see Materials and Methods) at 1.0 μg of protein per 10^5 cells. The mitogen concanavalin A (ConA) was used at a concentration of 2 μg/10^5 cells. Isolated lymphocytes were placed in culture with the appropriate viral antigen or mitogen and harvested at 48 h for mitogen and 120 h for antigen following a 4-h exposure to [^{3}H]TdR.

[b] The stimulation index is calculated as the ratio of the 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.

TABLE 3. Neutralization titers from cord blood plasma

Sample no.	Titer of neutralizing antibody to the following virus[a]:				
	CVB2	CVB3	CVB6	CVA16	PV1
1	<10	80	<10	80	20
2	20	320	<10	80	40
3	40	160	<10	160	40
4	<10	320	<10	640	20
5	40	80	<10	160	40
6	20	<10	<10	80	320
7	<10	640	<10	320	20

[a] Neutralization titers are presented as the reciprocals of the highest serum dilutions which showed reactivity.

The viral antigen preparations were treated with Formalin prior to use in the assay to determine whether denaturation of viral proteins would alter T-cell recognition of viral epitopes. Formalin treatment slightly enhanced the proliferative responses against each of the viral antigens (approximately 1.2- to 1.3-fold) (Table 4). Human PBMC did not proliferate to the Formalin-treated negative control cardiovirus antigen, EMCV. The lack of proliferation to denatured EMCV proteins is consistent with other data that cardioviruses (EMCV, Mengo virus, and Theiler's virus) do not contain the enterovirus group epitope(s) (3; M. Beck and S. Tracy, unpublished data).

Titers of human serum neutralizing antibody against enteroviruses demonstrate different infection histories. The histories of infectious exposure of the donors to the viruses used in the in vitro experiments were determined by testing serum samples for the presence of neutralizing antibody. The neutralizing antibody titers varied with each individual (Table 5). Only one patient (no. 9) demonstrated a titer of neutralizing antibody against CVB6, a rare isolate in the United States (8, 25); however, PBMC from all individuals proliferated in vitro against this antigen (Table 1). Similarly, six individuals (1 to 4, 11, and 12) had no detectable

TABLE 4. Stimulation indices of human PBMC in response to Formalin-treated viral antigen[a]

Subject	Stimulation index in response to the following Formalin-treated viral antigen[b]:					
	CVB2	CVB3	CVB6	CVA16	PV1	EMCV
1	10.2	15.2	12.4	14.4	28.3	0.7
2	15.6	16.3	18.3	17.4	16.9	0.8
3	15.9	11.7	12.2	13.8	21.3	0.9
4	21.3	19.9	10.3	11.2	9.9	0.7

[a] Viral antigen used in vitro was as Formalin-treated HeLa cell-infected membranes (see Materials and Methods) or Formalin-treated mock antigen at 1.0 μg of protein per 10^5 cells.

[b] The stimulation index is calculated as the ratio of the 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. Stimulation indices in the presence of nondenatured antigen are shown in Table 1.

TABLE 5. Serum neutralization titers of volunteers

Subject	Titer of neutralizing antibody to the following virus[a]:					
	CVB2	CVB3	CVB6	CVA16	PV1	EMCV
1	<10	640		320	80	<10
2	<10	640	<10	640	80	<10
3	<10	640	<10	160	40	<10
4	<10	>1,280	<10	<10	80	<10
5	320	<10	<10	<10	10	<10
6	320	640	<10	160	160	<10
7	320	640	<10	160	40	<10
8	40	160	<10	<10	40	<10
9	640	40	40	320	80	<10
10	640	80	<10	640	160	<10
11	<10	80	<10	320	40	<10
12	<10	80	<10	160	160	<10
13	40	80	<10	320	80	<10
14	80	80	<10	320	80	<10
15	320	80	<10	160	160	<10

[a] Neutralization titers are presented as the reciprocals of the highest serum dilutions which showed reactivity.

neutralizing antibody against CVB2, yet PBMC from all individuals proliferated in response to CVB2 antigen (Table 1). While no individual had neutralizing antibodies to the cardiovirus EMCV, neither did any of the PBMC samples proliferate significantly against this antigen.

Predominant PBMC subset responding to enteroviral antigens is the CD4$^+$ T cell. PBMC were depleted of specific cell populations following 5 days of antigen exposure and just prior to the addition of [^{3}H]TdR to determine which cell type was responsible for the in vitro proliferative response. When normalized against the complement-only treatment, removal of B cells with the CD19 monoclonal antibody decreased proliferation by about 12% (Fig. 2). However, removal of T cells with the CD3 antibody decreased proliferation by 80%. These results suggested that the primary proliferating cell type is of the T lineage. To better define the T-cell subset responsible for proliferation, we depleted both CD4$^+$ and CD8$^+$ cells in separate cultures (Fig. 2). Normalized to the proliferation which occurred in the presence of complement only, removal of CD4$^+$ cells lowered proliferation by 66% whereas depletion of CD8$^+$ cells had a far smaller effect on overall proliferative ability.

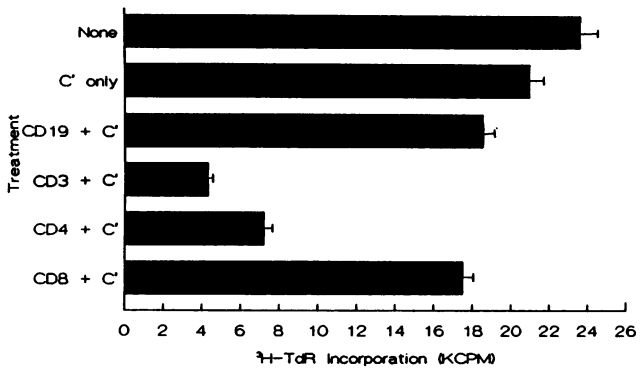

FIG. 2. Antigen-specific proliferative responses following cell subset depletions. PBMC were exposed in vitro to CVB3 antigen (1.0 μg/2 × 10^5 cells) or HeLa cell control antigen for 5 days, at which time specific cellular subset depletions were performed prior to the addition of [^{3}H]TdR. Proliferative responses against the control HeLa cell antigen never exceeded 1,542 ± 521 cpm. Each bar represents the mean ± standard deviation of triplicate cultures.

DISCUSSION

Proliferation against specific antigenic stimulation is one measure of a cell-mediated immune response, the quantitation of which can be used as a measure of the responsiveness of the cell population (16). We demonstrated in previous work that coxsackievirus-immune murine T cells from CVB2- or CVB3-inoculated mice proliferate in vitro to various serologically distinct enteroviruses even though the mice from which the T cells were isolated had been exposed to only one serotype of CVB (3). These data demonstrated the existence of an enterovirus group antigen which is recognized by murine enterovirus-immune T cells. Similar results should also be demonstrable in humans in order for a murine enterovirus-induced model of myocarditis to have validity for studies of human enterovirus-induced inflammatory heart disease.

We isolated serum and PBMC from 15 normal adult volunteers. The donors' histories of enterovirus infections were assessed by assaying for the presence of neutralizing antibody in serum. Consistent with the general population (8, 25), titers of serum neutralizing antibody against CVB6 were rare, all of the individuals had titers of antibody against PV1, and no titers of antibody to the negative control virus, EMCV, were detected (Table 5). However, regardless of the donors' infectious histories, PBMC from all volunteers proliferated well in vitro against all of the enteroviruses tested (Table 1). That the proliferative response was due to enterovirus-immune memory cells was demonstrated by the fact that cord blood lymphocytes did not respond to any of the enteroviruses tested (Table 2). Denaturation of viral proteins by Formalin did not prevent the recognition of the group antigen(s) by the PBMC (Table 4). The proliferating human immune cells were demonstrated to be primarily of the T-cell lineage (Fig. 2), with the predominant proliferating T-cell subset being the CD4$^+$ or helper subset. These data demonstrate that enteroviruses share a common group antigen which is recognized by human enterovirus-immune T cells and are consistent with our previous murine data (3).

Data from other laboratories have demonstrated that the T-cell response is not as discriminating as the humoral (B-cell) response to a viral antigen; T-cell cross-reactivity has been observed for polioviruses (42), flaviviruses (32), influenza A virus (6), reoviruses (38), alphaviruses (26), rhabdoviruses (31), and human retroviruses (41). The PBMC of several individuals who lacked neutralizing antibody to the specific enteroviruses in our panel proliferated as well as PBMC from individuals who had detectable titers of neutralizing antibody to the same viruses (Tables 1 and 5). Thus, human PBMC recognize a shared enterovirus group epitope(s) and this recognition is dependent only upon prior exposure to any enterovirus. The in vitro proliferative response was enterovirus specific in that no donor's PBMCs proliferated to the negative control virus, EMCV, a nonenterovirus picornavirus which rarely infects humans (27).

We have not yet established the identify of the antigenic group epitope(s) or determined whether the group enteroviral antigen resides as a single epitope or whether multiple epitopes are held in common by all enteroviruses. Murine studies, using both Formalin and UV-inactivated viral antigen (3), are consistent with those described here for human PBMC and suggest that the viral epitope(s) has no significant secondary structural component. In fact, T-cell epitopes have been shown to be linear amino acid sequences, approximately four to eight residues in length, which interact with the major histocompatibility complex proteins on the surface

of antigen-presenting cells (29, 40). An alternative possibility is that another epitope which is held commonly among enteroviruses and recognized by enterovirus-immune T cells may be exposed upon Formalin treatment. Formalin treatment can alter viral antigens through the alteration or destruction of epitopes or by exposing new epitopes. Experiments to locate the epitope(s) in the polyprotein of CVB3 and to distinguish between these possibilities are in progress.

Models of enterovirus-induced inflammatory disease in humans must now consider the role of memory T cells which readily recognize and proliferate in response to an enterovirus group antigen. As one example, most children in the United States are now vaccinated against poliovirus at 6 weeks of age. Thus, any enterovirus exposure after that time will stimulate the cell-mediated immune response via recognition of the group antigen. Although all murine models of enterovirus-induced inflammatory muscle disease have employed a single infectious event (11–13, 17, 24, 28, 30, 43, 44), the relevant approach should consider any enterovirus infection as at least a secondary infection. We recently examined the effect of exposing enterovirus-immune mice to a second enterovirus (2). We found that enterovirus-induced inflammation in the heart following secondary infection could be enhanced due to prior enteroviral exposure. Further investigation is required to identify the enterovirus group antigen in order to determine its role in the pathogenesis of enterovirus-induced inflammatory disease.

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