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In Vitro Culture of Coxsackievirus Group B, Type 3 Immune Spleen Cells on Infected Endothelial Cells and Biological Activity of the Cultured Cells In Vivo

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Spleen cells from male BALB/c mice infected 7 days earlier by an intraperitoneal injection of 3×10^4 PFU of a myocarditic strain of coxsackievirus B-3 lysed virus-infected endothelial cells in a ^{51}Cr release assay. Cytotoxic activity in the in vivo sensitized spleen cell population could be further increased by culturing the immune spleen cells from infected mice on virus-infected or uninfected endothelial cells for 6 to 7 days in vitro. Cytotoxicity of in vitro cultured spleen cells to infected targets was mediated by T lymphocytes since reactivity was abolished by treatment of the spleen cells with anti-thy 1.2 serum and complement. Reciprocal assays with BALB/c and C57BL cells indicated that maximum cytotoxicity occurred when spleen cells were sensitized on syngeneic endothelial cells. Other experiments showed that spleen cells sensitized to coxsackievirus B-3 or encephalomyocarditis virus were selectively cytolytic to targets infected with the homologous virus. Adoptive transfer of T cells cultured in vitro on infected endothelial cells retained their ability to induce myocarditis in T-lymphocyte-deficient mice.

Group B coxsackieviruses have been associated with a number of diseases depending upon the tissue infected (2, 12, 14, 15, 19, 21). In animal models, one of the better-studied diseases is myocarditis mediated by coxsackievirus B, type 3 (CVB3) (9, 10, 18–20). Several possible mechanisms of tissue damage exist, including direct virus-induced lysis of infected cells and immune-mediated damage occurring as a reaction to viral infection. Evidence to date in both human and animal CVB3 disease fails to implicate direct virus-mediated damage as the more important factor in the disease. This is based on the frequent inability to identify virus in individuals during peak myocarditis (19) and on the observation that T-lymphocyte-deficient animals infected with the virus fail to develope significant cardiac damage even though virus titers found in the hearts of these animals equal or exceed those found in T-lymphocyte-sufficient animals (20). In fact, it is this last observation which strongly implicates an immune etiology to the disease. The present communication furthers this concept by demonstrating that immune T cells obtained from mice after in vivo infection with CVB3 can be cultured in vitro on infected target cells to enhance their cytolytic activity. These in vitro cultured cells are also highly efficient in adoptively transferring myocarditis to T-lymphocyte-deficient animals.

MATERIALS AND METHODS

Mice. BALB/c mice were originally obtained from Cumberland Farms, Clinton, Tenn., and C57BL mice were from Jackson Laboratories, Bar Harbor, Maine. Neonates and 6- to 8-week-old adult males were obtained from colonies of these mice maintained at the University of Vermont.

Virus preparation and purification. A cardiotropic strain of CVB3 (Nancy strain) and encephalomyocarditis-M (EMC) virus were used. The procedures for growth and purification of the viruses on HeLa and L-cell cultures have been reported in detail earlier (4, 10). Virus was quantitated by determining PFU per milliliter. The titers of the purified viruses were 6×10^8 PFU/ml and 5×10^7/ml for CVB3 and EMC virus, respectively.

Virus titration. Virus titers in the heart were determined by homogenizing the tissue, removing cellular debris by centrifugation, and assaying the supernatant on HeLa cell monolayers in a plaque-forming assay described in detail previously (10).

Infection of mice. Animals were infected by an intraperitoneal injection of 3×10^4 PFU of CVB3 or 5×10^2 EMC virus in 0.5 ml of phosphate-buffered saline. Spleens were removed either 7 or 14 days after infection.

Preparation of endothelial cells and myofibers. Myofibers and endothelial cells were prepared by a modification of the procedure of Bollon et al. (2) which has been described in detail (10). Briefly, hearts were aseptically removed from neonatal mice within 72 h of birth, minced, subjected to stepwise enzymatic digestion with 0.25% pancreatin (GIBCO Laboratories, Grand Island, N.Y.), and washed in basal medium Eagle (BME) containing 100 U of penicillin and 100 μg of streptomycin per ml, 5% fetal calf serum (FCS), 10% horse serum, 0.2 mg of crystalline insulin, and 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (complete BME). Endothelial cells were separated from the myofibers by two 1-h adsorptions of the single-cell suspensions to 25-cm^2 plastic flasks (BD Labware, Oxnard, Calif.) at 37°C. Endothelial cells were detached by treatment with 0.25% trypsin (GIBCO) for 10 min at 37°C. The cells were washed, and resuspended in complete BME, and 10^6, 10^4, or 5×10^5 cells were dispensed into 25-cm^2 plastic flasks, 6-mm tissue culture wells (Linbro Chemical Co., Hamden, Conn.), or Leighton tubes (16 by 100 mm; W. C. Wheaton Co., Millville, N.J.), respectively.

Immunofluorescence and microscopie examination of myofibers and endothelial cells. After culture for 48 h in the Leighton tubes, the plated cells were fixed with acetone, incubated at 24°C for 20 min with a rabbit anti-human factor VIII-associated protein (Calbiochem-Behring, La Jolla, Calif.), washed, and subsequently incubated for 30 min at 24°C with fluoresceinated goat anti-rabbit immunoglobulin G

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(Cappel Laboratories, Cochranville, Pa.), which had been adsorbed with mouse thymocytes and liver and spleen cells. The cells were washed three times and examined with a Leitz-Ortholux fluorescence microscope equipped with a vertical illuminator.

Infectious-center assay. The procedure for the infectious-center assay has been described in detail elsewhere (10).

Spleen cell suspensions. Spleens were teased into cold BME with 5% FCS (BME-FCS), washed once (300 × *g* for 10 min), resuspended in NH_4Cl-Tris (9 volumes of 0.83% NH_4Cl and 1 volume of Tris, pH 7.2) to remove erythrocytes, and then rewashed with medium. Spleen cells used directly in the cell-mediated cytotoxicity (CMC) assay were depleted of adherent cells by adsorption to plastic petri dishes (60 by 15 mm; BD Labware) for 1 h at 37°C. Nonadherent cells were washed with medium and resuspended in BME-FCS.

In vitro augmentation of cytotoxicity. The procedure used for augmentation of cytotoxicity is a modification of that of Dunlop and Blanden (5). Briefly, 10^6 endothelial cells in 25-cm^2 tissue culture flasks were infected for 1 h at 37°C with 100 multiplicity of infectious units (virus PFU per cell), washed three times with medium, and fixed for 30 s with a 0.3% solution of glutaraldehyde (Sigma Chemical Co., St. Louis, Mo.) in BME (pH 7.2). Uninfected endothelial cells were similarlly treated. The fixed cells were washed four times and overlaid with 5×10^7 spleen cells in 10 ml of BME-FCS containing 5×10^{-5} M 2-mercaptoethanol (Sigma Chemical Co.). The flasks were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 6 days as stated in the text. At this time, the cells were removed, washed three times with medium, and resuspended in BME-FCS for use in the CMC assay.

CMC assay. The technique used for CMC assay has been described in detail (10). Briefly, 10^4 endothelial cells were plated into 6-mm plastic tissue culture wells (Linbro Chemical Co.) in 0.2 ml of complete BME and incubated for 24 to 48 h at 37°C in a humidified 5% CO_2 atmosphere. The target cells were then infected with virus (100 PFU per cell) for 1 h; excess virus was removed by washing the monolayers with medium, and the cells were labeled by the addition of 5 μCi of ^{51}Cr to each well ($Na_2{}^{51}CrO_4$; Amersham Corp., Arlington Heights, Ill.). After 45 to 60 min at 37°C, the monolayers were washed three times and overlaid with 0.2 ml of immune or nonimmune spleen cells in BME-FCS or with BME-FCS alone. Uninfected target cells were processed in an identical manner. The assays were incubated for 18 h at 37°C at an effector/target cell (E/T) ratio of 50:1 unless otherwise noted. ^{51}Cr in the supernatant and cells was determined with an Intertechnique CG4000 gamma counter. ^{51}Cr release was calculated by using the formula: [counts per minute in supernatant/(counts per minute in supernatant + counts per minute in cells)] × 100.

Cytotoxicity was expressed as the percentage of lysis calculated from the formula: [(average percent ^{51}Cr released from test group − average percent ^{51}Cr released from medium-treated group)/(average percent ^{51}Cr released after freeze-thaw − average percent ^{51}Cr released from medium-treated group)] × 100.

The percent specific lysis was calculated by subtracting the percentage of lysis by nonsensitized lymphocytes. Spontaneous ^{51}Cr release refers to the average percentage of ^{51}Cr released from the medium-treated group. A negative percentage of lysis occasionally occurred when ^{51}Cr released in the presence of lymphocytes was less than that of spontaneous ^{51}Cr release.

T-lymphocyte depletion of mice. The technique used for T-lymphocyte depletion of male BALB/c mice has been described earlier (20). Mice were thymectomized at 3 weeks of age by aspiration of the thymus through an opening in the anterior mediastinum. Two weeks later, the mice received 800 R of whole-body irradiation, using a Sethertron Jr. Cobalt X-ray machine, and 3×10^6 to 5×10^6 syngeneic sex-matched bone marrow cells intravenously through the tail vein (TXBM mice). The animals were rested for 5 weeks. Sham TXBM mice were opened through the anterior mediastinum but the thymus was not removed. Sham TXBM animals were subjected to irradiation and bone marrow reconstitution as above.

Anti-thy 1.2 and anti-immunoglobulin serum treatment of spleen cells. Monoclonal anti-thy 1.2 serum (Olac Ltd., Oxnard, England) used in these experiments had a cytotoxic titer of 1.5×10^5 against BALB/c thymocytes (1.25×10^6). Lysis of T lymphocytes by anti-thy 1.2 serum and immunoglobulin$^+$ cells by rabbit anti-mouse immunoglobulin serum (Cappel Laboratories) was performed as described earlier with the use of a 1:5 dilution of Low-Tox-M rabbit complement (Cedarlane Laboratories, London, Ont., Canada) (10).

Histology. Sections of Formalin-fixed hearts were stained with hematoxylin and eosin. One heart section from each animal was subjected to image analysis (Optomax image analyzer) to determine the percentage of total myocardial muscle area affected by inflammatory cells and necrosis (1). Only one section was analyzed since previous unpublished investigations by S.A.H. had shown that serial sections of infected BALB/c ventricles did not vary substantially in inflammation.

Statistical analysis. The Student *t* test was used to analyze the significance of difference between mean ^{51}Cr release values of the various experimental groups. The Wilcoxon ranked score test was used to evaluate differences between percent areas of myocardium inflamed.

RESULTS

Characterization of endothelial cells. Endothelial cells can be identified by the presence of intracellular factor VIII antigen, using immunofluorescence techniques (11). After 48 h in culture, 77% of the endothelial cell-enriched population used in these experiments stained with anti-factor VIII antiserum, compared with 13.5% of myofiber-enriched cells and 0% of skin fibroblasts (not shown). The remaining contaminating cells in the endothelial cell preparation were identified as myofibers (14%) and probably fibroblasts (9%) by staining with phosphotungstic acid-hematoxylin. Endothelial cells were susceptible to infection with CVB3. The results of infectious-center assays performed in triplicate demonstrate that 60% of the cells contained infectious virus at 16 h.

Cytotoxic activity of CVB3-immune spleen cells against infected endothelial cells. BALB/c mice infected with virus generate cytolytic spleen cells to virus-infected cells with peak activity 5 to 7 days postinfection. Most of the cytotoxic activity on days 5 to 7 was sensitive to treatment of the spleen cells with anti-thy 1.2 serum and complement, indicating that the later effector cell is a T lymphocyte. Significant cytotoxicity is not T-cell mediated, however, and is presumably due to natural killer cells generated after CVB3 infection (9).

The cytotoxicity assay with spleen cells sensitized in vivo requires a high E/T ratio (150:1) and a long incubation period (18 h) (10). To determine whether cytolytic activity could be

enhanced, spleen cells from BALB/c mice immunized 14 days earlier with 3×10^4 PFU of CVB3 were cultured on virus-infected, glutaraldehyde-fixed endothelial cell monolayers for 6 days. The viable lymphocytes (in vitro generated cells) were then assayed for cytotoxicity to virus-infected and uninfected endothelial cells at E/T ratios of 25:1, 50:1, or 100:1 (Fig. 1A) and for incubation periods of 6 to 24 h (Fig. 1B and C). In the same experiment, cytotoxicity was determined for spleen cells from 5 days after infection at the time of peak cytolytic activity (in vivo sensitized cells). Significant reactivity by in vitro generated spleen cells was demonstrated at an E/T ratio of 25:1, whereas in vivo sensitized spleen cells required a ratio of 100:1. Both in vitro generated and in vivo sensitized cells required approximately equal incubation times for maximum cytolysis and lysed uninfected as well as infected target cells, although lysis of infected targets was greater.

In the next experiment, nonimmune and CVB3-immune spleen cells obtained 14 days after in vivo infection were cultured on infected and uninfected endothelial cells for 4 to 7 days to determine the optimal culture time in vitro for generation of cytotoxicity (Fig. 2). Spleen cells from mice obtained 14 days after in vivo infection were not directly lytic to endothelial cells before in vitro culture, nor did the cells have infectious virus. Incubation of day 14 spleen cells on infected endothelial cells generated detectable cytolytic activity by days 5 to 6 of culture, with peak activity on days 6 to 7. Immune spleen cells generated in vitro on infected cells were also quite lytic to uninfected endothelial cells in the CMC assay, although cytotoxicity to infected targets was always greater. Sensitization of nonimmune cells on either infected or uninfected endothelial cells induced cytolytic activity, but the level of cytolytic activity never reached levels obtained by immune cells sensitized on infected endothelial cells.

Characteristics of effector cells. To characterize the precursor cell involved in in vitro generation of cytolytic activity, spleen cells were obtained from BALB/c mice 14 days after infection with 3×10^4 PFU of CVB3. One-half of the cells were directly cultured on CVB3-infected, glutaraldehyde-fixed endothelial cells for 6 days. The other half were further divided, with one group being treated with anti-thy 1.2 (group B; Table 1) and complement and the other group being treated with anti-mouse immunoglobulin plus complement (group D) to selectively deplete T and B cells, respectively. The residual viable cells from these treated spleen populations were cultured for 6 days on infected endothelial cells as above. After 6 days in culture, the recovered cells from the previously untreated spleen cell population were divided into three groups. One group (group A) remained untreated, whereas the other two groups were treated with anti-thy 1.2 (group C) and anti-mouse immunoglobulin (group E) serum and complement, respectively. All five groups were then assayed for cytolytic activity to uninfected and CVB3-infected endothelial cells.

Treatment of the spleen cells with anti-immunoglobulin and complement before in vitro culture enhanced subsequent cytotoxicity of the cultured cells to infected and uninfected targets in the CMC assay compared with untreated cells (Table 1). Treatment of the immune cells with anti-

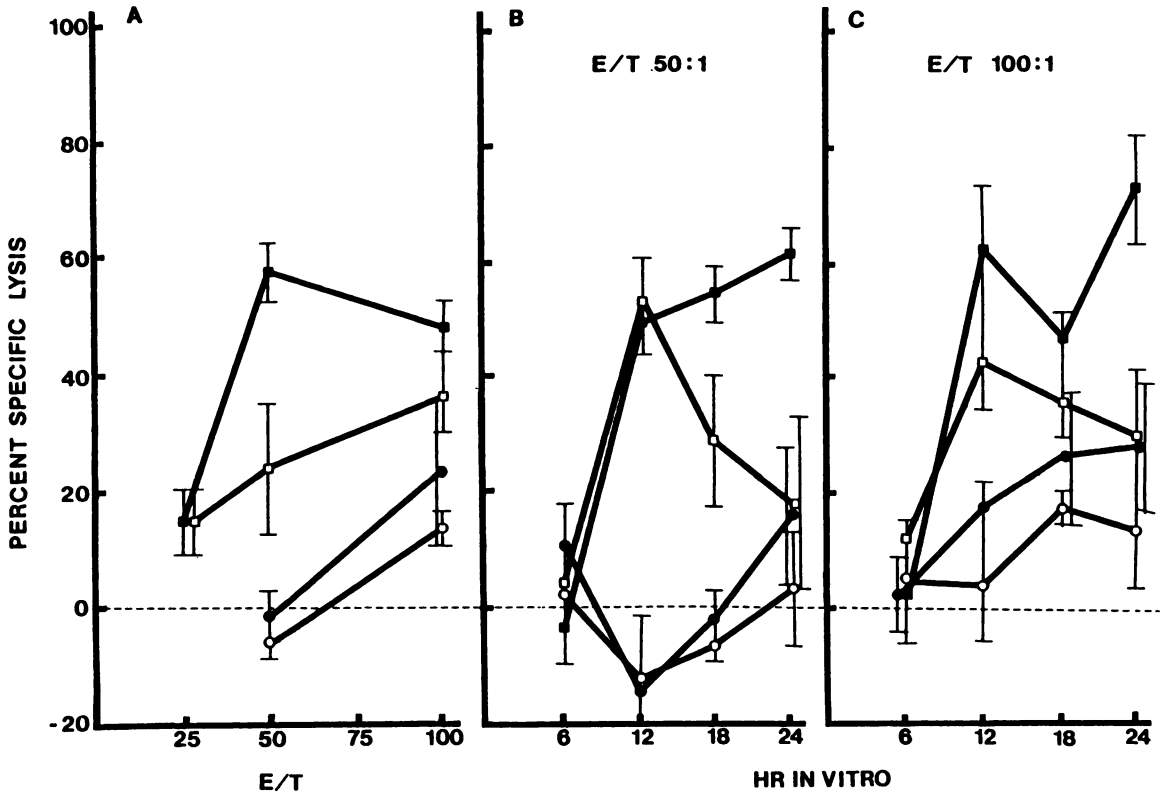

FIG. 1. Experiment illustrating comparative cytotoxicity of spleen cells sensitized to CVB3 in vivo (●, ○) or in vitro (■, □) and assayed to infected (■, ●) or uninfected (□, ○) endothelial cells. In vivo sensitized cells were obtained 6 days after infection of mice with 3.0×10^4 PFU of virus on virus-infected, glutaraldehyde-treated endothelial cells for 6 days. (A) Incubation time of 18 h. Spontaneous ^{51}Cr release at 6, 12, 18, and 24 h was 28.6, 38.9, 53.6, and 41.7% for uninfected and 39.1, 40.7, 50.4, and 47.5% for infected targets. Lysis by nonimmune spleen cells ranged from −4.5 to 9.3% and from −10.7 to 11.2% on uninfected and infected targets. Results are given as mean ± standard error.

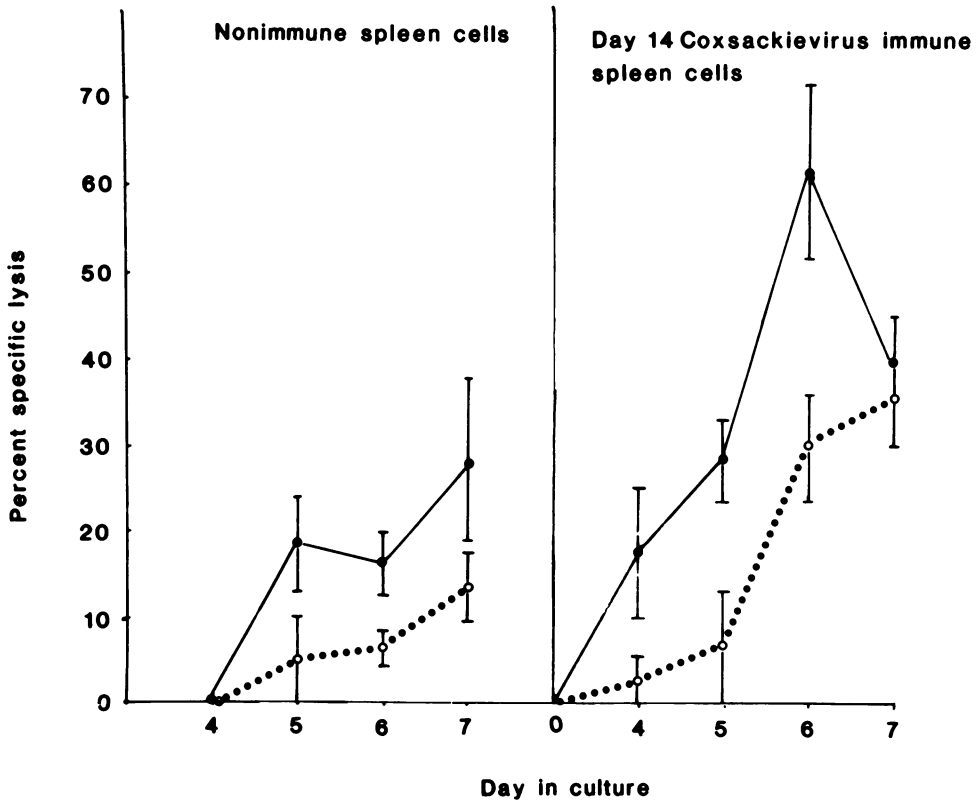

FIG. 2. Percent lysis of infected (●) and uninfected (○) endothelial cell targets by spleen cells from nonimmune (left) or day 14 CVB3-immune (right) animals which were cultured for 4 to 7 days on infected, glutaraldehyde-treated endothelial cells. The E/T ratio was 50:1 and the incubation time was 18 h. Spontaneous release values were 39.7 and 41.0% for uninfected and infected targets, respectively. Lysis by nonimmune spleen cells ranged from −5.1 to 7.8%.

thy 1.2 serum and complement, however, both prevented generation of cytolytic activity to infected targets when the spleen cells were treated before in vitro culture and reduced cytolytic activity of in vitro cultured cells when treated after culture, indicating that T cells are the cytolytic effector cells.

In another series of experiments, reciprocal sensitizations were done with either C57BL or BALB/c spleen cells from mice infected 14 days earlier with CVB3 and incubated on infected C57BL and BALB/c endothelial cells (respectively) for 6 days. The viable cells were subsequently assayed on infected and uninfected syngeneic and allogeneic endothelial cells (Table 2). Maximal cytotoxicity was noted only when spleen cells were sensitized and assayed for cytolytic activity on syngeneic endothelial cells.

Finally, studies were performed demonstrating the viral specificity of the generation of cytotoxicity. BALB/c mice were infected with either CVB3 or EMC virus 14 days earlier. The spleen cells were then cultured for 6 days on endothelial cells infected with either virus. The resulting spleen cells were assayed for cytotoxicity to uninfected endothelial cells and cells infected with EMC virus and CVB3 (Table 3). Maximum cytotoxicity was obtained when CVB3-immune cells were sensitized and then assayed on CVB3-infected cells. When the immune cells were sensitized on EMC virus-infected cells, significant cytotoxicity was only observed in the CMC assay, using EMC virus-infected targets.

Demonstration of retained ability of in vitro generated

TABLE 1. Mediation by T lymphocytes of cytolytic activity generated by in vitro culture

Group/treatment of spleen cells[a]	Time of treatment[a]	% Specific lysis[b]	
		Uninfected endothelial cells	CVB3-infected endothelial cells
(A) Untreated		14.7 ± 2.7	34.6 ± 3.0
(B) Anti-thy 1.2 + complement	Before culture	4.5 ± 0.2[c]	0.4 ± 0.3[c]
(C) Anti-thy 1.2 + complement	After culture	7.8 ± 2.2[c]	4.9 ± 3.7[c]
(D) Anti-immunoglobulin + complement	Before culture	17.7 ± 4.8	52.7 ± 3.0
(E) Anti-immunoglobulin + complement	After culture	11.5 ± 2.3	28.8 ± 3.2

[a] A total of 5×10^7 spleen cells obtained 14 days after infection of mice with 3×10^4 PFU of CVB3 were cultured on 10^6 CVB3-infected endothelial cells for 6 days. The spleen cells were depleted of T and B cells either before or after the 6-day culture on glutaraldehyde-fixed endothelial cells, using anti-thy 1.2 and anti-mouse immunoglobulin and complement.

[b] The E/T ratio was 50:1 with an 18-h incubation time. Spontaneous ^{51}Cr release and percent lysis by nonimmune spleen cells were 30.7 and 5.3% on uninfected targets and 38.9 and 2.3% on infected targets, respectively. Results represent mean ± standard error of two experiments.

[c] Percent specific lysis was significantly less than that of untreated spleen cells at $P < 0.05$.

TABLE 2. Viral specificity of in vitro cultured spleen cells on uninfected or CVB3- or EMC virus-infected endothelial target cells

Spleen cells immune to[a]:	Cultured 6 days on monolayers infected with:	% Lysis of target cells (mean ± SEM)[b]		
		Uninfected	EMC infected	CVB3 infected
Nonimmune	EMC virus	18.4 ± 3.0	32.2 ± 7.5[c]	17.4 ± 6.6
	CVB3	16.3 ± 5.1	21.7 ± 7.5	40.1 ± 1.9[c]
CVB3	CVB3	11.2 ± 6.7	3.7 ± 4.3	45.4 ± 7.6[c]
EMC virus	EMC virus	19.5 ± 15.2	44.1 ± 0.8[c]	18.4 ± 3.8

[a] Spleen cells, obtained from nonimmune BALB/c mice or mice infected 14 days earlier intraperitoneally with 3.0×10^4 PFU of CVB3 or 5×10^2 PFU of EMC virus, were cultured on uninfected endothelial cells or endothelial cells infected with 100 multiplicity of infection virus units for 6 days at an E/T ratio of 50:1. Recovered cells were assayed for cytotoxicity to uninfected and infected targets at an E/T ratio of 50:1 and an incubation time of 18 h.

[b] Mean ± standard error of three or four wells. Spontaneous ^{51}Cr release from uninfected or EMC virus- or CVB3-infected targets was 42.4, 45.1, or 35.1%, respectively.

[c] Lysis was significantly greater than on uninfected targets at $P \leq 0.05$.

cytolytic T lymphocytes to induce myocarditis. To determine whether the in vitro generated T cells still retain the capacity to induce myocarditis, adoptive transfer experiments were done. Mice were made T-lymphocyte deficient by thymectomy, whole-body irradiation, and bone marrow reconstitution (TXBM). Five weeks later, the TXBM animals and T-lymphocyte-sufficient control animals were infected with 3×10^4 PFU of CVB3. On day 3 after infection, 5×10^6 day 6 in vivo sensitized CVB3-immune spleen cells (Table 4, group C) and 5×10^5 cells from cultures of CVB3-immune spleen cells (group F) or nonimmune spleen cells (group E) which had been incubated for 6 days on CVB3-infected endothelial cells were injected intravenously through the tail vein. On day 7 after infection, the surviving animals were sacrificed and the hearts were examined for myocarditis. Even though only 1/10 the number of immune cells were injected in the group receiving the in vitro generated CVB3-immune cells compared with animals receiving in vivo sensitized cells, more myocarditis was induced (mean, 8.4 ± 1.2 versus 3.8 ± 1.4%; Table 4, Fig. 3). Culture of nonimmune spleen cells on infected endothelial cells for 6 days did result in a minimally sensitized lymphocyte population, since 1.2 ± 0.2% of the ventricles of TXBM animals receiving these cells had myocarditis compared with only 0.9 ± 0.5% for infected TXBM animals not given lymphocytes and 0.7 ± 0.3% for TXBM animals given fresh uncultured nonimmune spleen cells (not shown). The transferred cells responsible for myocarditis induction were T cells. Treatment of in vivo and in vitro sensitized immune cells with anti-thy 1.2 and complement into TXBM recipients before transfusion effectively eliminated their ability to induce myocarditis. Whereas T lymphocytes are not required for elimination of virus from the heart (19), T-cell-deficient animals consistently have more virus than T-cell-sufficient animals (Table 4); the differences, however, are slight and are not considered to be important in the disease. Therefore, in vitro culture of immune spleen cells enhances not only the cell populations' in vitro cytolytic activity, but also their pathogenic capacity.

DISCUSSION

Our results show that infection of male BALB/c mice with CVB3 induces cytolytic T and non-T lymphocytes which effectively destroy both virus-infected and uninfected target cells in vitro (10). Cytolytic activity is also generated after secondary exposure of the in vivo sensitized lymphocytes to infected or uninfected target cell surface antigen, although significantly greater stimulation occurs after exposure to infected cells. The greatest amount of cytotoxicity is obtained when the target cell is homologous to the lymphocyte and specific for the stimulating virus. The subpopulation of spleen cells involved in the secondary response are T cells, based on the failure to generate cytolytic activity in vitro if the spleen cells are first treated with anti-thy 1.2 serum and complement. The cytolytic activity induced after 6 days in culture is also majorly mediated by cells expressing thy 1.2 antigen, although occasional non-thy 1.2 cytolytic cells are also observed. This may indicate that the culture system used can induce natural killer cells or activated macrophage which are known to increase after infection in vivo with a wide range of viruses (6, 17).

Endothelial cells rather than myofibers were chosen for this work for two reasons. First, antigen-induced T-cell proliferation is known to be optimal with antigen presentation by Ia^+ cells (8). Whereas the Ia expression of myofibers

TABLE 3. Cytotoxicity to syngeneic and allogeneic targets by in vitro sensitized lymphocytes

Spleen cells[a]	CVB3-infected sensitizing cell monolayer	% Specific lysis (mean ± SEM)[b]			
		BALB/c		C57BL	
		Uninfected targets	Infected targets	Uninfected targets	Infected targets
BALB/c	BALB/c	2.0 ± 1.4	27.0 ± 8.6[c]	−3.4 ± 0.7	0.7 ± 7.3
C57BL	C57BL	−10.3 ± 4.1	4.8 ± 3.6	−3.3 ± 5.0	21.4 ± 5.9[c]

[a] Spleen cells from mice infected 14 days earlier with CVB3 were cultures for 6 days on syngeneic or allogeneic infected, glutaraldehyde-fixed endothelial cells at an E/T ratio of 50:1. Recovered cells were assayed for cytotoxicity to infected or uninfected syngeneic and allogeneic endothelial target cells at an E/T ratio of 50:1 and an incubation time of 18 h.

[b] Mean ± standard error of three or four wells per group. Spontaneous ^{51}Cr release for infected and uninfected BALB/c targets was 27.1 and 24.5% and that for C57BL targets was 47.1 and 34.7%, respectively. Lysis by nonimmune spleen cells ranged from 7.1 to 9.1%. Results represent the combined data of two experiments.

[c] Lysis was significantly greater than on uninfected targets at $P \leq 0.05$.

TABLE 4. Demonstration of retained capacity of in vitro generated cytolytic cells to induce myocarditis

Group[a]	Adoptively transferred spleen cells[b]	Virus titer/heart (10^3 PFU)	% Area of myocardium affected (mean ± SEM)
(A) Normal	None	7.9 ± 1.5	4.3 ± 1.9[c]
(B) TXBM	None	10.3 ± 0.9	0.9 ± 0.5
(C) TXBM	5×10^6 CVB3 immune, not cultured	6.5 ± 0.4	3.8 ± 1.4[c]
(D) TXBM	5×10^6 CVB3 immune, not cultured, treated with anti-thy 1.2 + complement	7.5 ± 0.5	1.2 ± 0.2
(E) TXBM	5×10^5 nonimmune, cultured	8.9 ± 1.1	0.9 ± 0.1
(F) TXBM	5×10^5 CVB3 immune, cultured	6.4 ± 1.2	8.4 ± 1.2[c]
(G) TXBM	Anti-thy 1.2 + complement	8.8 ± 1.2	0.9 ± 0.5[c]
(H) Sham TXBM	None	8.2 ± 0.7	4.6 ± 1.7[c]

[a] Normal male BALB/c, TXBM, and sham TXBM mice were infected intraperitoneally with 3×10^4 PFU of CVB3; 7 days after infection, the mice were sacrificed and the hearts were examined for inflammation, using image analysis to determine the percent area of the myocardium undergoing inflammation and necrosis.

[b] Three days after virus infection, mice were adoptively transferred intravenously with one of three spleen cell populations. Group C received CVB3-immune spleen cells obtained from mice 6 days after in vivo infection with the virus. Group E received nonimmune spleen cells cultured for 6 days at a 50:1 lymphocyte/target cell ratio on CVB3-infected, glutaraldehyde-treated endothelial cells. Group F received in vitro generated CVB3-immune cells obtained by culturing day 14 in vivo sensitized CVB3-immune cells for 6 days in vitro at a 50:1 lymphocyte/target cell ratio on virus-infected, glutaraldehyde-fixed endothelial cells.

[c] Values are significantly different than TXBM animals at $P \leq 0.05$.

is not known, endothelial cells lining blood vessels and capillaries are rich in Ia antigen (8). In fact, infected endothelial cells may play an important role in inducing CVB3 immunity in vivo since their distribution would undoubtedly ensure cytolytic lymphocyte precursor exposure to these cells before exposure to myofibers. The second reason was that endothelial cells tend to form confluent single-cell monolayers more readily than myocardial cells. Therefore, in the in vitro cultures, the endothelial cells will be more uniformly fixed by the glutaraldehyde and fewer empty spaces will exist where setting lymphocytes would not be exposed to antigen.

Although this study is mainly directed toward CVB3-induced myocarditis, a second hypothetical aspect may be that virus-induced injury to the arterial wall could become a focus for the deposition of lipid in the blood, resulting in the

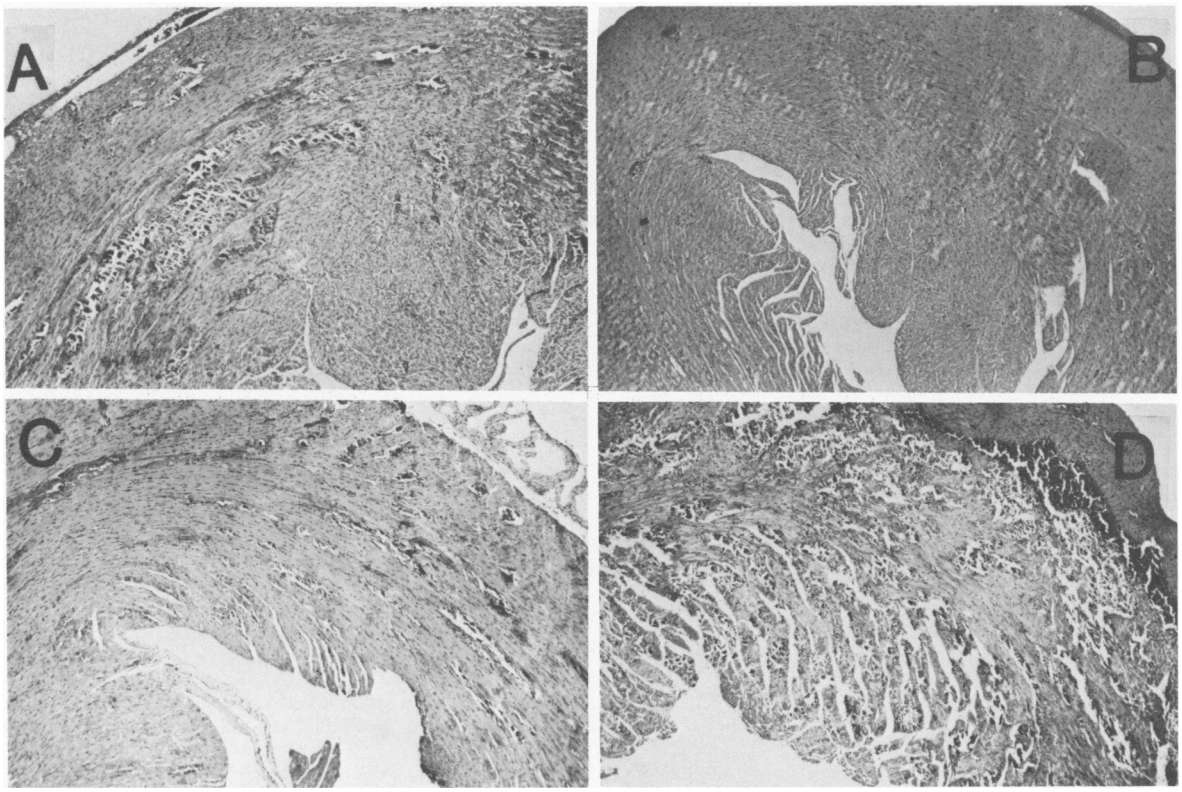

FIG. 3. Histology of myocarditis in CVB3-infected (A) normal mice, (B) TXBM mice, (C) TXBM mice transferred with day 7 in vivo sensitized lymphocytes, and (D) TXBM mice transfused with in vitro sensitized lymphocytes. (Hematoxylin and eosin; × 190.)

production, with time, of intimal thickening and atherosclerotic plaques. An excellent study of the role of virus infections associated with the production of atheroarteriosclerosis in chickens, using Marek's herpesvirus, showed markedly increased atherosclerosis-like changes in animals fed a high-cholesterol diet, although less extensive or severe arterial lesions were also seen in chickens administered either virus or cholesterol alone (13). These results strongly indicate a synergistic effect between viral infections which presumably induce arterial injury and the presence of dietary fat.

The mechanism by which the virus induces arterial injury is not clear. However, three possibilities have been suggested: (i) cellular proliferation of vascular smooth muscle cells (7); (ii) direct viral destruction or alteration of one or more cell types in the arterial wall; or (iii) immunological destruction or alteration of infected endothelial and muscle cells by lymphoid cells or cell products (i.e., lymphotoxin, antiviral antibody) (16). In this report we give evidence that cytolytic cells induced during CVB3 infection in mice can lyse infected endothelial cells in vitro, lending support to the role of the immune system in atheroarteriosclerosis. In addition, enhanced cytotoxicity can be induced by secondary exposure of sensitized spleen cells to either infected or uninfected endothelial cells. This may suggest that once the lymphocytes become sensitized to the cell surface antigens expressed on virus-infected cells, they can subsequently recognize, respond to, and kill cells by membrane surface antigens without the participation of virus antigens.

Therefore, a single virus infection may actually result in a chronic systemic disease involving arterial injury continuing long after the virus has been cleared. In this case, the disease could be divided into the initial virus-specific phase and a second "autoimmune" phase. Whereas no athrosclerotic lesions were observed in the mice used in this study, the failure to detect such changes may reflect more on the short time period used between infection and sacrifice of the mice (7 days), or on the fact that mice have very low cholesterol levels which would not be conducive to atherosclerosis development, than on the inability of similar CVB3 infections to cause atherosclerosis in humans. One potential future direction of this work, therefore, could be to determine whether CVB3 infection can induce atherosclerotic plaques in mice if the animals are followed for longer time periods (6 months) or after administered exogenous cholesterol.

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