Lysis of Infected Myofibers by Coxsackievirus B-3–Immune T Lymphocytes

Sally A. Huber, PhD, Lillian P. Job, PhD, and Jack F. Woodruff, MD

Spleen cells from male adult BALB/c mice given intraperitoneal injections of purified coxsackievirus B-3 were examined for the ability to lyse syngeneic neonatal myofibers in culture. Cytotoxicity against infected and uninfected targets was measured with the use of an *in vitro* ^{51}Cr release assay. Immune spleen cells obtained 4–7 days after infection were cytotoxic for viral-infected myofibers. Peak reactivity was observed 5 days after infection. At this time immune spleen cells showed significantly less reactivity against uninfected myofibers. Cytotoxicity against infected targets was mediated by T lymphocytes, since reactivity was abolished by treatment with anti-thy 1.2 and complement. Treatment with anti-Ig and complement caused no loss of activity. Reciprocal assays performed with BALB/c and CBA cells showed that maximal cytotoxicity occurred against infected syngeneic myofibers, providing further evidence that viral-specific effector cells were T lymphocytes. In addition, hyperimmune rabbit anti-coxsackievirus B-3 antiserum could not block immune spleen cell lysis of infected targets, suggesting that coxsackievirus-infected myofibers expressed surface membrane antigens not recognized by specific neutralizing antibody. (Am J Pathol 1980, 98:681–694)

COXSACKIE B VIRUSES are the most commonly identified cause of viral myocarditis and pericarditis in humans.[1–2] Characteristically the inflammatory infiltrate in the heart is composed of mononuclear cells that usually surround foci of necrotic myofibers. Mice are also susceptible to infection with Coxsackie B viruses, and these animals provide a model particularly useful in the study of the mechanisms involved in viral heart disease, since they develop cardiac lesions that closely resemble those observed in patients.[3–6]

The events underlying coxsackievirus-induced myocarditis are not completely understood, but experiments using the mouse model strongly indicate that immunologic reactions are involved in the pathogenesis of the disease. Several distinct immune mechanisms could be responsible for tissue destruction; these include killing exerted by effector cells, ie, thymus-derived (T) cells,[7] macrophages,[8] and natural killer (NK) cells,[9] and that mediated via antibody molecules, ie, antibody-dependent cell cytotoxicity [10] and complement-dependent lysis.[11]

From the Department of Pathology, Cornell University Medical College, New York, New York.

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Address reprint requests to Dr. Jack F. Woodruff, Department of Pathology, Cornell University Medical College, 1300 York Avenue, New York, NY 10021.

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Studies from this laboratory have provided evidence that T cell stimulation by coxsackievirus B-3 infection is a decisive factor in the production of myocardial lesions. Thus, in T-cell–depleted mice there is a marked decrease in the severity of cardiac inflammation and necrosis, even though the virus growth curve in the heart is the same as that observed in normal, immunologically intact animals.[12] Moreover, the spleens of coxsackievirus-infected mice contain cytotoxic lymphocytes that can be detected *in vitro* with the use of a radiochromium release assay. In experiments in which fibroblasts were used as the target cells, the cytotoxic cells have been identified as T lymphocytes and exhibit specific activity against coxsackievirus-infected syngeneic, but not allogeneic, targets.[13–15]

Cultured, virus-infected myofibers are also susceptible to killing by coxsackievirus-immune spleen cells,[14] but in this case nothing is known about the nature of the effector cells mediating the reaction. The present report deals with this subject and extends our earlier observations on the characteristics of the interaction between virus-immune spleen cells and myofibers *in vitro*.

Materials and Methods

Animals

BALB/c mice were originally purchased from Cumberland Farms, Clinton, Tennessee, and CBA mice from Jackson Laboratories, Bar Harbor, Maine. Neonates and 6–8-week-old adult males were obtained from colonies of these mice maintained at Cornell University Medical College.

Virus Preparation and Purification

A cardiotropic Coxsackie B-3 virus (Nancy strain) which was originally grown in human epidermoid carcinoma of the larynx cells (H. Ep. No. 2) was passaged three times in HeLa cell monolayers (Gey strain, Flow Laboratories, McLean, Va) and thereafter grown in HeLa cell suspensions (Mandel strain, courtesy of Dr. Richard Crowell, Hahnemann Medical College, Philadelphia, Pa) according to the established technique.[16] The maintenance medium was Joklik's modified minimum essential medium (MEM) (Flow Laboratories), with double strength essential amino acids and vitamins in Hanks' basic salt solution (HBSS), fetal calf serum (FCS), and antibiotics (100 μg streptomycin and 100 units penicillin per milliliter). HeLa cells were infected by the use of 100 virus plaque-forming units (PFUs) per cell. After incubation at 37 C for 9 hours the cells were harvested, washed, and disrupted by a freeze–thaw technique. Virus purification was carried out with the use of CsCl gradients according to the method of Oberg and Phillipson.[17] The titer of purified virus ranged from 9×10^9 to 2×10^{10} PFUs/ml; aliquots were stored at −70 C in phosphate-buffered saline (PBS) without Ca^{++} and Mg^{++}. Purified virus was used in all experiments.

Virus Titrations

Virus was quantitated by determination of tissue culture $dose_{50}$ (TCD_{50})/ml with the use of H.Ep. No. 2 cells[5] and PFUs/ml with the use of HeLa cells.[16]

Preparation of Coxsackievirus B-3 Antiserum

Hyperimmune 10^9 coxsackievirus B-3 antiserum was obtained from a rabbit 15 days after injection of 9×10^6 PFUs purified virus intravenously. The rabbit had received 3.7×10^9 PFUs of virus intraperitoneally and 10^9 PFUs of virus intramuscularly 67 and 55 days before the last injection. This antiserum had a neutralizing titer of 1:15,360/ml when tested on H.Ep. No. 2 cells challenged with 100 TCD_{50} of coxsackievirus B-3 as previously described.[18]

Infection of Mice

Animals were infected by an intraperitoneal inoculation of 0.5 ml PBS containing 2×10^5 PFUs of purified coxsackievirus B-3. Spleens were routinely removed 5 days after infection except as indicated in the text.

Spleen Cell Suspensions

Usually spleens from 3–5 mice were pooled and teased in cold basal medium Eagle (BME) with 5% FCS supplemented with 100 μg streptomycin and 100 units penicillin per milliliter. In one experiment individual spleens were processed. In each case cells were washed twice (300 g for 10 minutes), resuspended in NH_4Cl–tris (9 volumes 0.83% NH_4Cl and 1 volume 0.17 M tris hydroxymethyl aminomethane, pH 7.2) in order to lyse erythrocytes, and then rewashed with medium. Adherent cells were removed by plating spleen cells in 60 × 15-mm plastic Petri dishes (Falcon Plastics, Oxnard, Calif) at a concentration of 20×10^6/ml for 1 hour at 37 C. The nonadherent cells were harvested, washed once, and resuspended in BME–FCS at a concentration of 10^7 viable nucleated cells per milliliter; they were kept on ice until used in cytotoxic assays. Cell viability was at least 90% as assayed by trypan blue dye exclusion.

Preparation and Culture of Myofibers

Hearts were removed aseptically from BALB/c mice within 48 hours of birth. Single cell suspensions of myofibers were prepared by a modification of the procedure of Bollon et al.[19] Briefly, hearts were minced and then subjected to stepwise enzymatic digestion with 0.25% pancreatin (Gibco, Grand Island, NY). The isolated cells were washed with BME containing penicillin and streptomycin, 5% FCS, 10% horse serum (HS), 0.2 mg crystalline insulin, and 20 mM Hepes buffer (complete BME) and then depleted of endothelial cells and fibroblasts by two sequential 1-hour adsorptions in 25 sq cm plastic flasks (Falcon Plastics) at 37 C.[20,21] The nonadherent myofibers were recovered, washed once, resuspended in complete BME, and then dispensed into 6-mm tissue culture wells (Linbro Chemical Co, Hamden, Conn) at a concentration of 7×10^4/0.2 ml for cytotoxic and infectious center assays. Preliminary experiments using cell doses ranging from 2 to 10×10^4 myofibers/0.2 ml showed that optimal recovery of viable cells in 6-mm wells at 48 hours in complete BME was obtained with the use of an initial seeding of 7×10^4 myocardial cells. A 2-day incubation period was needed in order to allow the myofibers time to adhere firmly to plastic. In a separate experiment 2×10^6 myocardial cells were seeded onto 9 × 22 mm coverslips and maintained in 16 × 100-mm Leighton tubes (T. C. Wheaton Co., Millville, NJ) with 1.5 ml complete BME for light-microscopic examination. At 48 hours the plated cells were fixed with 10% formalin, and the percentage of myofibers was determined after staining with phosphotungstic-acid–hematoxylin (PTAH).

Infectious Center Assay

Myocardial cells in 6-mm wells were infected with virus (100 PFUs/cell) for 1 hour at 37 C in 5% CO_2 atmosphere. The monolayers were then washed with media, treated with

30 units of rabbit anticoxsackievirus B-3 antiserum for 1 hour, rewashed twice, and incubated in complete BME for 4 or 14 hours at 37 C. Thereafter, the monolayers were removed by trypsinization (0.25%), the cells were washed once with 30 units of rabbit anticoxsackievirus B-3 antiserum, and 0.2-ml aliquots of serial ten-fold dilutions were added to HeLa cell (Gey strain) monolayers in 60-mm Petri dishes. After 90 minutes, MEM containing 0.6% Bactoagar and 5% heat-inactivated FCS was added, and the dishes were kept at 37 C in a humidified atmosphere containing 5% CO_2. Plaque number was determined 48 hours later. In some experiments nucleated spleen cells were also examined for infectious virus.

Cytotoxic Assay

The technique used was similar to that described in previous reports.[13,14] Briefly, 7×10^4 neonatal myofibers in 0.2 ml complete BME were dispensed into 6-mm plastic tissue culture wells (Limbro Chemical Co) and incubated at 37 C in a humidified atmosphere containing 5% CO_2. Forty-eight hours later the cells were infected with virus (100 PFUs/cell). After 1 hour we removed unabsorbed virus by washing the monolayers with complete BME. Target cells not exposed to virus were processed in an identical fashion. Myofibers were then labeled by the addition of 5 μCi ^{51}Cr to each well ($Na_2\ ^{51}CrO_4$; Amersham-Searle, Arlington Heights, Ill). After 45–60 minutes at 37 C, the monolayers were washed 3 times and overlaid with 0.2 ml immune or nonimmune spleen cells in BME–FCS or with BME–FCS alone. The assays were incubated at 37 C for 15–21 hours at a spleen effector cell to target cell ratio (E:T) of 100:1 unless otherwise noted. ^{51}Cr in the supernatant and in cells was determined as described previously[12]; radioactivity was measured with the use of Intertechnique CG4000 gamma counter. ^{51}Cr release was calculated using the formula:

$$\frac{\text{cpm in supernatant}}{\text{cpm in supernatant + cpm in cells}} \times 100$$

Cytotoxicity was expressed as the percentage of lysis calculated from the formula:

$$\frac{\text{Av\% } ^{51}Cr \text{ released from test group} - \text{Av\% } ^{51}Cr \text{ released from media group}}{\text{Av\% released after freeze-thaw} - \text{Av\% released from media group}}$$

The percentage of specific lysis represented the percentage of lysis by sensitized lymphocytes minus the percentage by lysis by normal lymphocytes. Spontaneous ^{51}Cr release refers to the average percentage of radiolabel released by the media-treated group. A negative percentage of lysis occasionally occurred when ^{51}Cr release in the presence of nonimmune spleen cells was less than that of spontaneous ^{51}Cr release.

Immunofluorescence

A direct immunofluorescence technique was used to detect spleen cells with membrane immunoglobulin (Ig). Fluoresceinated rabbit IgG antimouse IgG (Cappel Laboratories, Downington, Pa), which had been absorbed with mouse thymus cells, was diluted 1:2, and 0.1 ml was added to an equal volume of BME-FCS containing 5×10^6 cells in 0.01 M NaN_3. After incubation at 24 C for 30 minutes, the cells were washed three times, resuspended in BME, and examined with a Leitz-Ortholux fluorescence microscope equipped with a vertical illuminator.

Anti-thy 1.2 Serum Treatment of Spleen Cells

Anti-thy 1.2 serum (kindly provided by Dr. Judith J. Woodruff, Downstate Medical Center, Brooklyn, NY) was raised and titrated as described previously.[12] The antiserum

used in these experiments had a cytotoxicity titer against BALB/c thymocytes (5×10^6 ml) of 1:1600 per 0.25 ml. Lysis of thy 1.2-positive cells was performed as described,[14] with the use of 1:5 dilution of Low-Tox-M rabbit complement (C) (Cedarlane Laboratories, London, Ontario, Canada). This treatment increased the percentage of Ig+ spleen cells from 46% to 87%.

Anti-Ig Treatment of Spleen Cells

Anti-Ig treatment was performed as described previously,[14] using rabbit antimouse IgG (Cappel Laboratories) that had been absorbed with mouse liver cells and thymocytes. Low-Tox-M rabbit complement was used. The percentage of Ig+ spleen cells decreased after treatment from 46% to 7%.

Statistical Analysis

The Student *t* test was used to analyze the significance of differences between mean ^{51}Cr release values found in the various experimental groups.

Results

Infection of Neonatal Myofibers with Purified Coxsackievirus B-3

When cultured in 6-mm wells for 24–48 hours, BALB/c myofibers were noted to contract rhythmically (50–80 beats/min). At 48 hours, 90% or more of the cells were identified as muscle fibers after staining with PTAH; sarcomeres were readily observed under light microscopy.

As shown in Table 1, at 48 hours of cultivation the myocardial cells readily incorporated radiolabel. ^{51}Cr-labeled, uninfected cells and myofibers infected 1 hour previously with a high multiplicity (100 PFUs/cell) of purified coxsackievirus B-3 had comparable levels of spontaneous radioactive release over a period of 21 hours. Moreover, a similar degree of cell survival was found in the two populations during the same time interval.

Neonatal BALB/c myofibers were susceptible to infection by coxsackievirus. The results of infectious center assays performed using three wells per time interval demonstrated that by 6 hours an average of 20% of the cells were infected; by 16 hours 75% of the myofibers contained infectious virus (data not shown).

Cytotoxic Activity of Coxsackievirus B-3–Immune Spleen Cells Against Infected Myofibers

Young adult male BALB/c mice infected with virus produced spleen cells that lysed virus-infected myocardial cells. Activity peaked on Day 5 with maximal lysis evident with the use of an 18-hour incubation period and an E:T of 100:1 (Text-figure 1). Some specific lysis of infected targets also occurred with the use of an E:T of 50:1, 150:1, and 200:1, but larger variations in ^{51}Cr release were noted at the higher ratios.

The results of several experiments using conditions optimal for detecting cytotoxicity showed that Day 5 immune spleen cells were virus-specific, in that reactivity against infected targets was always greater than ac-

Table 1—Characteristics of Uninfected and Coxsackievirus-B-3–Infected Neonatal BALB/c Myofibers*

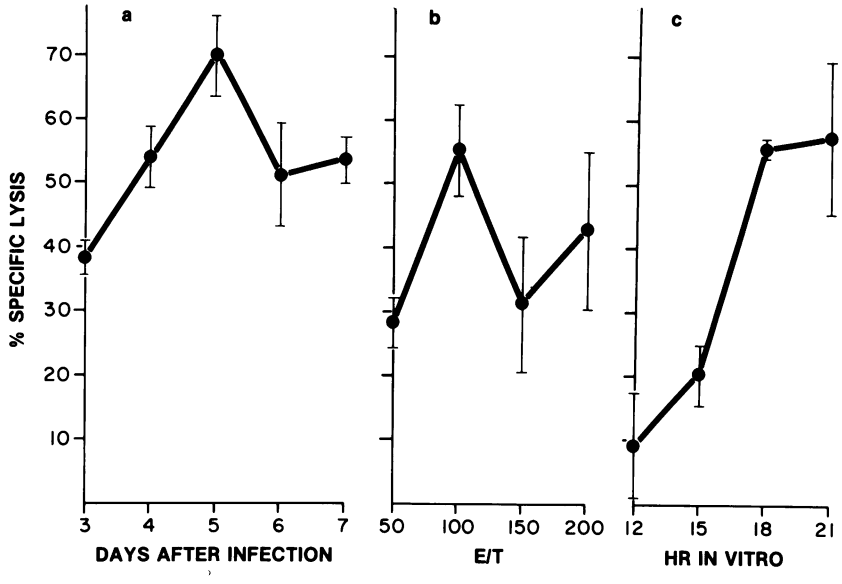
Myocardial cells	^{51}Cr Incorporation (CPM/well)	No. cells/well × 10^4			% Spontaneous ^{51}Cr release		
		0 hrs†	18 hrs	21 hrs	0 hrs†	18 hrs	21 hrs
Infected	1579.7 ± 124.2‡	1.4 ± 0.1	1.0 ± 0.1	0.7 ± 0.1	5.4 ± 2.8	36.6 ± 2.6	42.2 ± 3.8
Uninfected	1585.3 ± 45.9	1.5 ± 0.1	1.1 ± 0.2	0.7 ± 0.1	5.2 ± 2.0	36.4 ± 2.4	44.8 ± 5.4

* Neonatal myofibers cultivated for 48 hours in microtiter wells before infection.
† Beginning 1 hour after cells labeled with ^{51}Cr. Infected myofibers challenged with 100 MOI purified virus 1 hour prior to radiolabeling.
‡ Average value from 6 samples ± SEM.

tivity against uninfected myofibers (Table 2). These studies also showed that the magnitude of virus-specific lysis varied from experiment to experiment and demonstrated that immune spleen cells were capable of lysing uninfected myofibers.

Evidence That Lysis of Infected Myofibers Is Not Mediated by Infectious Virus

Experiments were performed in order to determine whether the lysis of myofibers was mediated by effector cells directly or was due to virus or other factors in the spleen cell preparations. As shown in Table 3, Day 5 immune spleen cells that were untreated exerted strong cytotoxicity, while disruption of effector cells by freeze–thaw treatment or sonication resulted in a complete loss of activity. In addition, virus was only occasionally detected in Day 5 immune spleen cells when an infectious center assay was used.



TEXT-FIGURE 1—Experiments illustrating optimal conditions for demonstration of cytotoxicity of male immune spleen cells against viral-infected myofibers. Spontaneous ^{51}Cr release in **a** and **b** averaged 28.6% and 32.2%, respectively. Similar release in **c** ranged from 27.1% to 32.2% over the first 18 hours and was 42.0% by 21 hours. Figures **b** and **c** are from the same experiment and represent values derived when immune cells obtained 5 days after infection were used. The incubation time in **a** and **b** was 18 hours, and the effector-to-target-cell ratio in **a** and **c** was 100:1. Vertical bars enclose SEM of the percentage of lysis observed with immune cells; SEM of the percentage of lysis observed with nonimmune cells ranged from 3.0% to 12.0%. The average percentage of lysis by nonimmune cells was 5.5% in **a** and ranged from −2.5% to −10.7% in **b** and −0.5 to 14.3% in **c**.

Table 2—Cytotoxicity of 5-Day Viral-Immune and Nonimmune Spleen Cells Against Infected and Uninfected Myofibers

Experiment	Spleen cell donor	% Lysis of target cells	
		Uninfected	Infected
1	Nonimmune	6.0 ± 3.0*	5.5 ± 12.5
	Immune	48.3 ± 4.7†	75.0 ± 5.1‡
2	Nonimmune	−13.0 ± 1.0	−3.5 ± 0.5
	Immune	21.7 ± 6.4†	66.0 ± 8.6‡
3	Nonimmune	−2.3 ± 6.4	−2.3 ± 4.8
	Immune	−8.7 ± 3.2	21.5 ± 3.5‡

* Mean ± SEM of three wells. Average spontaneous ^{51}Cr release from infected targets in Experiments 1, 2, and 3 was 28.6%, 38.4%, and 36.5%, respectively. Similar values for uninfected myofibers were 30.0%, 38.0%, and 38.9%.

† Significantly greater ($P < .05$–$.01$) than percentage of lysis by nonimmune spleen cells in the same experiment.

‡ Significantly greater ($P < .05$) than all other values in the same experiment.

Several experiments were then carried out in an effort to block virus-specific cytotoxicity by incubating infected targets with hyperimmune rabbit coxsackievirus B-3 antiserum. A typical experiment is summarized in Table 4. Although the concentration of antibody used (384 U/well) was capable of neutralizing 38,400 TCD_{50} infectious virus, it was incapable of inhibiting the activity of immune spleen cells against myofibers challenged with 100 MOI virus 2 hours previously.

Characterization of Effector Cells

In order to characterize the cells responsible for lysis of infected myofibers, selected lymphocyte populations were eliminated by appropriate antiserums and complement. Since immune spleen preparations were rou-

Table 3—Effect of Cell Disruption on Lysis of Virus-Infected Myofibers by Immune and Nonimmune Spleen Cells

Spleen cell donors	Treatment of spleen cells	Percentage of lysis*
Nonimmune	None	7.0 ± 1.0
	Frozen and thawed	−10.0 ± 0.5
	Sonicated	6.5 ± 1.5
Immune†	None	62.3 ± 4.2‡
	Frozen and thawed	5.0 ± 0.0
	Sonicated	2.0 ± 0.0

* Mean ± SEM of 3 wells. Average spontaneous ^{51}Cr release from infected targets was 27.1%.

† Obtained 5 days after infection.

‡ Significantly greater ($P < .01$) than all other values.

tinely depleted of adherent cells, macrophages would not be expected to play a major role in this reaction.

The results presented in Table 5 show the effects of various treatments on Day 5 effector cells. Immune cells treated with anti-thy 1.2 and complement lost all cytotoxic activity. In contrast, there was no reduction in lysis when anti-Ig plus C-treated spleen cells was used. The results indicate that the cytotoxic cells were thy 1.2 positive and Ig negative. Further evidence that lysis was mediated by T cells was obtained through reciprocal assays with BALB/c (H-2^d) and CBA (H-2^k) effectors and targets. BALB/c and CBA mice were infected with virus, and 5 days later their spleen cells were assayed on syngeneic and allogeneic myofibers. As shown in Table 6, BALB/c immune cells showed significantly greater activity against infected syngeneic than allogeneic myofibers. Similarly, CBA immune spleen cells were lytic for infected CBA myofibers; significantly less lysis was observed on the BALB/c targets.

In a separate but similar experiment in which Day 5 immune spleen cells were highly active, the percentage specific lysis of BALB/c effectors against infected syngeneic and allogeneic targets was 80 and −10, respectively. The reactivity of CBA immune cells against syngeneic and allogeneic infected myofibers was 91 and 0 (% lysis).

Discussion

Our results clearly show that during infection of young adult mice with a purified cardiotropic strain of coxsackievirus B-3, cytotoxic T lymphocytes are generated that can lyse virus-infected myofibers *in vitro*. The lysis of infected myofibers by immune cells was mediated by T lymphocytes, since cytotoxicity was abolished by incubating 5-day effector cells with anti-thy 1.2 and complement but not by treatment with anti-Ig serum. Moreover, H-2 restriction of male effector cell activity was readily demonstrated. Presumably effector T cells recognize both viral and H-2-determined components on myocardial cell membranes, a dual specificity phenomenon known to be characteristic of the murine cytotoxic T cell system.[22]

Coxsackie B viruses are small, nonbudding RNA viruses that lack envelopes and lipid and are preassembled in the cytoplasm before release.[3,23] These viruses may produce lytic infections, but the results presented here showed that infection of cultured myofibers did not result in degenerative changes by 18 hours. The finding that coxackievirus injection of mice induced cytotoxic T cells that specifically lysed infected myocardial cells showed that the membranes of these cells were altered. Nevertheless, it has not been possible to detect viral antigens, by immunofluorescence

Table 4—Effect of Coxsackievirus B-3 Antiserum on Lysis of Virus-Infected Myofibers by Immune and Nonimmune Spleen Cells

Spleen cell donors	Treatment of target cells*	Percentage of lysis†
Nonimmune	None	−15.0 ± 1.0
	NRS	−9.3 ± 2.9
	RAS	4.0 ± 4.0
Immune	None	15.7 ± 2.7‡
	NRS	18.5 ± 3.5‡
	RAS	19.0 ± 3.0‡

* ^{51}Cr-labeled target cells were overlaid with 384 units hyperimmune rabbit anti-coxsackievirus B-3 serum 2 hours after infection of myofibers with 100 MOI virus and immediately before addition of effector cells.

† Mean ± SEM of 3 wells. Spontaneous ^{51}Cr release from infected targets was 35.3%.

‡ Significantly greater than lysis elicited by nonimmune effector cells ($P < .05$).

NRS = normal rabbit serum; RAS = rabbit anti-coxsackievirus B-3 serum.

Table 5—Cytotoxic Activity of Immune Spleen Cells After Treatment With Anti-Ig and Anti-Thy 1.2 Serum and Complement*

Animal	% Specific lysis of infected myofibers after treatment of effector cells with:			
	Medium	C	Anti-Ig + C	Anti-thy 1.2 + C
1	14.0†	21.3	13.3	1.0‡
2	24.8	13.3	17.0	−7.7‡
3	18.3	16.3	20.6	−1.5‡
4	17.3	24.0	23.3	−8.7‡

* All spleen cell preparations depleted of adherent cells as described in Materials and Methods.

† Mean of three wells. With normal lymphocytes SEM ranged from 1.0% to 3.5%, and with immune spleen cells from 0.5% to 5.0%.

‡ Significantly less ($P < .01$) than all other values for same animal.

techniques, on the surface of infected cultured myofibers with the use of neutralizing antiserum [14] known to contain antibodies directed against structural components of the virus capsid.[24] Nor was it possible to block T-cell–mediated killing of coxsackievirus-infected myocardial cells or fibroblasts [13] by pretreating targets with antiviral serum. Taken together, these findings make it unlikely that T cell reactivity was directed against intact virus particles adsorbed to the membranes of target cells. These results do not necessarily support the idea, however, that structural virion antigens are excluded from the target cell membrane determinants recognized by T cells.[25] Further work is needed to clarify this issue, particularly in view of the recent findings that have emerged from studies on the cytotoxic T lymphocytes generated during reovirus infection of mice. Like

Table 6—Preferential Lysis of Syngeneic Coxsackievirus-Infected Myofibers by Immune Spleen Cells

Spleen cells	% Specific lysis*			
	BALB/c		CBA	
	Uninfected	Infected	Uninfected	Infected
BALB/c	9.0	32.3†	1.7	5.7
CBA	−0.6	7.3	−4.5	28.0‡

* Mean values. With normal lymphocytes SEM ranged from 2.5% to 6.0% and with immune spleen cells from 1.0% to 7.5%. Spontaneous ^{51}Cr release from infected and uninfected BALB/c targets was 28.6% and 28.5%, respectively, and that from infected and uninfected CBA targets 27.8% and 32.8%, respectively.

† Significantly greater than lysis of all other targets by BALB/c immune cells and lysis of infected and uninfected BALB/c targets by CBA cells ($P < .05$).

‡ Significantly greater than lysis of all other targets by CBA immune cells and lysis of infected and uninfected CBA targets by BALB/c immune cells ($P < .05$).

Coxsackie, this virus is also nonbudding. In this system the viral gene that codes for hemagglutinin protein was shown to be the predominant gene determining specificity of the effector cells.[26] Coxsackieviruses also hemagglutinate,[3] and it is possible that the structural component responsible for this function or some other capsid subunit not reactive with neutralizing antibody is recognized by cytotoxic T cells.

Since coxsackievirus B-3 is grown in continuous cell cultures, host cell proteins and/or contaminating noncytopathic infectious agents present in crude virus preparations might have played a role in the cytotoxic T cell production observed in our earlier studies. This idea seems very unlikely in view of the evidence presented in this report showing that infection with purified virus elicits a strong cytotoxic T cell response.

In coxsackievirus-infected mice, mononuclear inflammatory cells are first detected in the heart about 5 days after intraperitoneal infection, and the extent of inflammation increases during the next 2–3 days. Hypereosinophilic myofibers are occasionally noted at the time of peak viral replication in this organ (ie, Days 3 and 4 of infection), but destruction of myocardial cells does not become prominent until after viral titers begin to decline. Parenchymal necrosis is temporally related to the development of inflammation, suggesting that the cellular infiltrate plays a role in the production of tissue injury.[15,27] The finding that T cell deficiency protects mice against the development of severe cardiac lesions indicates that these cells play an important role in the myocardial damage induced by coxsackieviral infection.[12] Additional evidence supporting this view comes from the demonstration that the hearts of coxsackievirus-infected mice contain a KCl-extractable antigen that stimulates the production of migration inhibitory factor by immune peritoneal exudate cells.[28] The ability of coxsackievirus to elicit T cells that show selective cytotoxicity against infected myofibers, as shown in the present study, provides a system wherein cell-mediated reactions involved in myocarditis are accessible to *in vitro* analysis.

In the present work we also found that coxsackieviral immune spleen cells were capable of lysing uninfected myofibers. "Autoimmune" reactivity has also been seen with the use of skin fibroblasts as targets.[13,29] Concurrent studies in this laboratory have demonstrated that immune cells reactive against uninfected myofibers are seen early (by Day 3) after viral infection and at that time do not have the classical characteristics of T cells, B cells, or macrophages and are presumed to be natural killers (manuscript in preparation). It is possible that these "autoreactive" cells also play a role in the production of inflammatory lesions during coxsackieviral disease.

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