HUMAN CYTOTOXIC T LYMPHOCYTES EVOKED BY GROUP A STREPTOCOCCAL M PROTEINS

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It has long been known that acute rheumatic fever and rheumatic heart disease are triggered by antecedent infections due to group A streptococci. Nevertheless, the molecular mechanisms of the pathogenesis of the cardiac disease have remained a mystery. A number of studies have shown that group A streptococci contain antigens that crossreact with human myocardium (1–8), including the surface M protein of some serotypes (9–14). In addition, sera from patients with acute rheumatic fever contain heart-crossreactive antibodies (1, 4, 8) that react with streptococcal antigens (1, 8). However, heart-crossreactive antibodies are also present in the sera of patients after uncomplicated streptococcal pharyngitis (4). Thus, the role of the tissue-crossreactive antibodies in the pathogenesis of rheumatic carditis remains unclear.

We have previously shown that normal human adult and neonatal lymphocytes, when cultured in the presence of highly purified peptic extracts of M protein (pep M) undergo brisk blastogenic responses, as measured by [^{3}H]thymidine uptake (15). The proliferative responses were T cell specific, dependent on adherent cells, and did not correlate with serum antibody titers (15). Lymphocytes from nonimmune laboratory animals were unresponsive to any of the M proteins, but after immunization with one serotype of M protein, T cell blastogenesis was observed in response to all serotypes of M protein tested. Moreover, in a later study we showed that lymphocytes from rabbits immunized with a synthetic dodecapeptide of type 24 M protein were primed to respond to subsequent in vitro challenge with type 24 and type 5 M proteins (16).

Because the T lymphocyte responses to M proteins appeared to lack the serotype-specificity associated with humoral immunity to these antigens, and because all human adult and neonatal T cells tested underwent blastogenesis in response to purified M proteins, we undertook studies to determine the effector function of M protein–stimulated T lymphocytes. In the present study we show that highly purified peptic extracts of types 5, 6, and 19 streptococcal M proteins (pep M) induce human cytotoxic T lymphocytes (CTL) against several different human target cells, including cultured myocardial cells, whereas minimal cytotoxicity was observed against target cells of animal origin. We show in addition that the M protein–stimulated human CTL are $CD3^+$ and are distinct from

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classic natural killer (NK) cells, and that a subset of these cells has specificity for M protein–crossreactive antigens on the surface of myocardial cells.

Materials and Methods

Extraction and Purification of M Proteins. M protein was purified from limited peptic digests of types 5, 6, 19, and 24 streptococci as previously described (17). The purified proteins (pep M) were judged to be homogeneous by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (17).

Purification of Lymphocytes. Mononuclear cells (MNC)[1] were isolated by Ficoll-Hypaque gradient centrifugation (18) from heparinized (100 U/ml) whole blood obtained from normal adults. Cells were washed three times, counted, and resuspended in RPMI-1640 (Gibco, Grand Island, NY) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), and Hepes buffer (25 mM). Viability was assessed by trypan blue exclusion. Mononuclear cells were adherent cell–depleted by incubating overnight in 150-mm petri dishes (1013, Falcon Labware, Oxnard, CA) in complete medium with 10% FCS (Hyclone Laboratories, Logan, UT) at 37°C.

Mononuclear cells were further purified according to cell surface phenotype on a flow cytometer (Epics 753 Coulter Electronics, Hialeah, FL) using the monoclonal antibodies anti-CD3, anti-CD4, and anti-CD8 (Coulter Electronics). Nonadherent cells were washed once in RPMI-1640, incubated with the monoclonal antibodies according to the manufacturer's instructions, and then sorted using sterile reagents. Purity of the sorted cell populations ranged from 98.9 to 99.6%.

Target Cells. The following cell lines were obtained from the American Type Culture Collection (Rockville, MD): Girardi heart cells (CCL 27), Chang liver cells (CCL 13), and BHK-21 (C-13) (CCL 10). Human, rat, and mouse fibroblasts were obtained from primary explants of skin by methods previously reported (19). Cells were cultured in MEM (Gibco) supplemented with penicillin, streptomycin, L-glutamine, Hepes buffer in the concentrations stated above, and 10% FCS. K562 cells were kindly donated by Dr. Chris Bever of this institution, and were cultured in RPMI supplemented as above.

Lymphocyte Cultures. 2×10^6 MNC/ml were cultured in the presence of pep M proteins at a concentration of 5 μg/ml unless otherwise stated in complete RPMI with 10% FCS in 17 × 100-mm tubes (2057; Falcon Labware) at 37°C in an atmosphere of 5% CO_2 (15). In some experiments cells were cultured in the presence of either 10 U/ml IL-2 (Collaborative Research, Inc., Bedford, MA) 10 μg/ml Con A (Sigma Chemcial Co., St. Louis, MO) or pep M protein plus 250 U/ml rabbit anti–human IFN-γ (Ventrex, Portland, ME). Control MNC were cultured in complete medium alone. $CD3^+$, $CD4^+$, and $CD8^+$ cells (10^6 cells/ml) were cultured in complete medium plus 10% FCS, to which 5×10^5 cells/ml irradiated (3,000 rad) autologous MNC had been added as antigen-presenting cells.

Cytotoxicity Assays. Target cells grown in monolayers were plated into 96-well flat-bottom culture dishes (3596; Costar, Cambridge, MA) at 10^4 cells/well 16 h before performing cytotoxicity assays. Cells were labeled with ^{51}Cr by aspirating the culture medium and adding 0.1 ml Ca^{2+}/Mg^{2+}-free HBSS (Gibco) containing 2% FCS and 2 μCi $Na^{51}Cr$ (sp act 500 mCi/mg; New England Nuclear, Boston, MA). The cells were incubated for 3 h at 37°C, the supernatants were aspirated, and the cells were washed twice with complete medium. 10^6 K562 cells were labeled in 1 ml RPMI containing 10% FCS and 100 μCi ^{51}Cr for 3 h at 37°C, washed twice in complete medium, and plated into round-bottom 96-well culture plates (3799; Costar) at 10^4 cells/well.

Lymphocytes were washed twice in complete RPMI and added to target cells at the ratios stated. All assays were performed at 37°C for 3 h, after which supernatants were removed by using a supernatant collection system (Skatron, Inc., Sterling, VA), and counted for 1 min each in a gamma counter. Nonspecific ^{51}Cr release was consistently <10% of the total for all target cells. Percent specific ^{51}Cr release (percent cytotoxicity)

[1] *Abbreviation used in this paper:* MNC, mononuclear cell.

was calculated as follows: 100 × [(cpm experimental) − (nonspecific release)]/[(cpm total) − (nonspecific release)].

Cytotoxicity inhibition experiments were performed by incubating the target and effector cells in the presence of 10% rabbit antisera. Percent inhibition of cytotoxicity was calculated based on controls incubated with normal rabbit serum (NRS). The antisera were assayed for the presence of crossreactive antibodies against target cells by indirect immunofluorescence by methods previously reported (11). Heart-crossreactive pep M5 antibodies were affinity purified from sarcolemmal membranes of human myocardium, as described (11).

Generation of Cytotoxic T Cell Clones. MNC were isolated from normal human blood and grown in the presence of 2.5 μg/ml pep M5 for 5 d (15). Cells were cloned by limiting dilution in 96-well plates containing complete RPMI supplemented with 10% FCS, 2.5 μg/ml pep M5, 10 U/ml IL-2, and 10^5 irradiated (3,000 rad) MNC per well. Growing cells were expanded in the presence of pep M5 and IL-2, and initially assayed for cytotoxicity against Girardi heart cells and K562 cells. Stimulation indices were based on [^{3}H]thymidine incorporation in the presence and absence of pep M5. Cell surface phenotype was determined using the CD4 and CD8 monoclonal antibodies and analyzing on a flow cytometer only viable cells, as determined by forward angle and 90° light scatter.

Results

Induction of Human CTL by Streptococcal M Proteins. Because previous studies have shown that normal adult human lymphocytes proliferate in response to purified pep M proteins, initial experiments were performed to detect cytotoxic T lymphocytes that might be induced in vitro by the tissue-crossreactive M proteins. Girardi heart cells were chosen as target cells because preliminary studies have shown that heart-crossreactive antibodies evoked by pep M5 also crossreacted with membrane antigens on the surface of these cultured cells. Mononuclear cells cultured for 5 d in the presence of pep M5, M6, or M19 displayed significant cell-mediated cytotoxicity against Girardi heart cells at E/T ratios as low as 5:1 (Table I). Pep M24, which we have previously shown stimulates lymphocyte blastogenesis (15), was not effective in inducing cytotoxic activity. Control cells incubated in medium alone displayed little to no spontaneous cytotoxic activity against the cultured heart cells.

The stimulation of CTL was dependent on the concentration of M protein,

TABLE I

In Vitro Stimulation of Human Cytotoxic T Lymphocytes against Cultured Myocardial Cells with Purified Streptococcal M Proteins

E/T ratio	Percent specific ^{51}Cr release after lymphocytes were cultured with:				
	pep M5	pep M6	pep M19	pep M24	Medium control
60:1	72	74	ND	15	15
30:1	58	55	68	11	4
10:1	40	34	ND	7	2
5:1	25	26	ND	5	1

Lymphocytes were cultured with 5 μg/ml of M protein or in medium alone for 5 d. The cells were washed, counted, resuspended in fresh medium, and added to ^{51}Cr-labeled heart cells at the ratios indicated. After 3 h incubation, the amount of ^{51}Cr released was measured using a Skatron harvesting device.

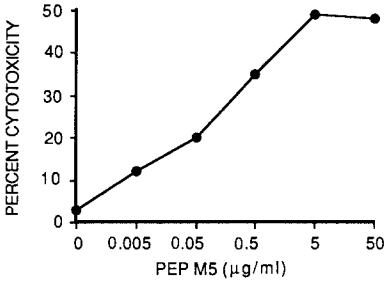

FIGURE 1. Dose-dependent pep M5 stimulation of cytotoxic lymphocytes against Girardi heart cells. Mononuclear cells (10^6 cells/ml) were cultured for 5 d in the presence of various concentrations of pep M5. The effector cells were washed and added to ^{51}Cr-labeled target cells at an E/T ratio of 30:1. After incubating for 3 h, the amount of ^{51}Cr released was assayed and percent cytotoxicity was calculated as stated in Materials and Methods.

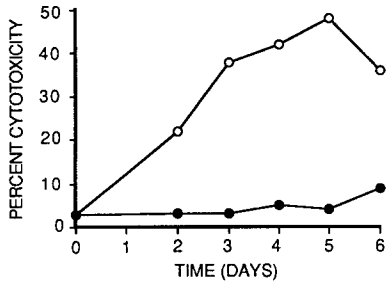

FIGURE 2. Kinetics of pep M5 induction of cytotoxic lymphocytes against Girardi heart cells. Mononuclear cells were cultured with 10 μg/ml pep M5 (○) or medium alone (●) for the times specified, and then assayed for cytotoxicity at an E/T cell ratio of 30:1.

TABLE II

Target Cell Specificity of Human Cytotoxic Lymphocytes Evoked by Type 5 Streptococcal M Protein

Target cells	Percent specific ^{51}Cr release*	
	Control lymphocytes	pep M5–stimulated lymphocytes
Human myocardial cells (Girardi)	3.7	71.5
Human fibroblasts	7.2	69.7
Human liver cells (Chang)	2.6	79.8
K562 cells	55.3	62.6
Mouse fibroblasts	0	14.5
Rat fibroblasts	5.0	19.5
Hamster kidney cells (BHK)	0	5.7

* E/T ratio 30:1.

and cytotoxicity against heart cells reached a maximum when cells were cultured with 5 μg/ml (Fig. 1). Studies of the kinetics of CTL induction showed that cytotoxicity was maximal after 5 d exposure to pep M5 (Fig. 2), which parallels previous data for peak blastogenic responses to M protein (15).

Target Cell Specificity of CTL Induced by M Protein. The target cell specificity of M protein–stimulated cytotoxic lymphocytes was assayed using cell lines and primary cultures of various human and animal tissues (Table II). In addition to cytotoxic activity against Girardi heart cells, pep M5 also induced cytotoxicity against primary cultures of human skin fibroblasts and Chang liver cells, a continuous cell line. Spontaneous cytoxicity in control cultures was present only against K562 cells, and pep M5 did not significantly enhance this activity. When

TABLE III
Inhibition of Cytotoxicity against Human Heart Cells with Heart-crossreactive pep M5 antibodies

Exp.	Inhibitor	Immunofluorescence reaction of antisera with Girardi heart cells	Percent specific ^{51}Cr release:*		Percent inhibition of cytotoxicity
			Control lymphocytes	pep M5–Stimulated lymphocytes	
1	Normal rabbit serum	0	18.1	78.3	—
	Rabbit anti–pep M5	2+	11.3	40.3	48.5
	Rabbit anti–human sarcolemmal membranes	3+	15.3	82.1	0
2	Normal rabbit serum	0	6.4	32.8	—
	Rabbit anti–pep M5	2+	2.1	15.1	54.0
	Anti–pep M5 eluted from human sarcolemmal membranes	2+	2.8	19.0	42.1

* E/T ratio 30:1.

pep M5–stimulated human lymphocytes were incubated with primary cultures of rat or mouse fibroblasts or a continuous cell line of hamster kidney cells, minimal cytotoxicity was observed over that seen with control lymphocytes (Table II). These data indicate that M protein stimulates cytotoxic cells with specificity for multiple human target cells, and that the reactivity with animal target cells is minimal to absent.

Inhibition of Cytotoxicity by Anti–pep M5 Heart-crossreactive Antiserum. To determine whether pep M5–stimulated cytotoxic lymphocytes were recognizing M protein crossreactive antigens on the surface of cultured heart cells, we performed inhibition experiments with heart-crossreactive pep M5 rabbit antisera (11, 12) (Table III). Preincubation of the target cells with rabbit antiserum against pep M5 inhibited pep M5–stimulated cytotoxicity by 48%, as compared with normal rabbit serum, whereas antiserum against human sarcolemmal membranes had no inhibitory effect. This latter antiserum, however, crossreacted strongly with the surface of Girardi heart cells, as determined by indirect immunofluorescence tests, but did not react at all with pep M5, as determined by ELISA (data not shown). In the second experiment (Table III), the anti–pep M5 antiserum again partially inhibited cytotoxicity against heart cells. The pep M5 antibodies that were affinity purified by eluting them from human sarcolemmal membranes also inhibited cytotoxicity, suggesting that antigens shared between sarcolemmal membranes of human heart and Girardi heart cells may be involved in the cytotoxic activity of pep M5–stimulated human lymphocytes. The pep M5 rabbit antisera did not crossreact by immunofluorescence tests with fibroblasts or liver cells, and had no effect on cytotoxic activity against these cells (data not shown).

Characteristics of Effector Cells Stimulated by pep M5. To characterize the populations of cytotoxic effector cells induced by pep M5, we fractionated human peripheral blood mononuclear cells according to cell surface markers by using a fluorescence-activated cell sorter. The sorted cells were cultured with irradiated

TABLE IV
Surface Phenotype of Human Cytotoxic T Lymphocytes Induced by pep M5

Effector cells E/T ratio	Culture conditions	Percent specific ^{51}Cr release with:	
		Girardi heart cells	K562
Unsorted 30:1	Medium control	7.4	48.0
	pep M5	69.0	53.8
$CD3^+$ 30:1	Medium control	2.0	4.9
	pep M5	45.2	37.1
$CD4^+$ 30:1	Medium control	0.5	0.5
	pep M5	18.9	19.0
$CD8^+$ 15:1	Medium control	0	1.5
	pep M5	37.4	46.9

feeder cells and M protein, and then each population was tested for cytotoxic activity against heart and K562 cells (Table IV). As shown previously, unsorted control cells demonstrated spontaneous cytotoxic activity against K562 cells, which presumably represented NK activity (20) present in the mixed mononuclear cell preparation. pep M5 induced cytotoxicity against heart cells in the unsorted populations. The $CD3^+$ lymphocytes cultured in the absence of pep M5 were devoid of cytotoxic activity against either target, indicating that cells with spontaneous cytotoxic activity had been removed. However, pep M5 induced cytotoxicity in the $CD3^+$ cells directed against both target cells. The majority of the cytotoxic activity of $CD3^+$ cells was recovered in $CD8^+$ lymphocytes; however, $CD4^+$ cells also showed some activity (Table IV). Although these results were obtained with effector cells that were sorted before stimulation with M protein, similar results were obtained when the effector populations were sorted after in vitro activation (data not shown), suggesting that the cytotoxic cells were derived from $CD4^+$ and $CD8^+$ precursor lymphocytes that retained their phenotypic expression of these antigens.

Because previous studies have shown that pep M proteins induce the production of IFN-γ but not IFN-α or -β from human mononuclear cells (21), and that IFN may augment or induce (22) certain cytotoxic effector cells, we cultured human lymphocytes in the presence of neutralizing antibody against IFN-γ and tested the effector cells for cytotoxic activity (Table V). Anti-IFN-γ completely abolished spontaneous cytotoxicity against K562 cells in unstimulated control cultures. However, the antibody had no effect on the cytotoxicity of lymphocytes cultured in the presence of pep M5 (Table V).

To determine whether cytotoxic lymphocytes against heart cells were simply the result of activation of $CD3^+$ cells by IL-2 (23), we cultured mononuclear cells in the presence of 10 U/ml purified IL-2 or 10 μg/ml Con A, and assayed for cytotoxic responses against heart cells, liver cells, and fibroblasts. None of the target cells was lysed by the activated effector cells (data not shown), suggesting that lymphocyte blastogenesis was necessary, but not sufficient, to induce cytotoxic effector cells with activity against the target cells tested.

Characteristics and Target Cell Specificity of M Protein–reactive Cytotoxic T Cell

TABLE V
Effect of Anti-IFN-γ on Cytotoxic Lymphocytes Induced by pep M5

Culture condition	Anti-IFN-γ	Percent specific ^{51}Cr release with:	
		Girardi heart cells	K562
Control	−	5	48
	+	6	1
pep M5	−	22	41
	+	29	67

TABLE VI
Target Cell Specific M Protein–responsive Human Cytotoxic T Cell Clones

Clone	Surface phenotype	Stimulation index in response to pep M5	E/T ratio	Percent specific ^{51}Cr release with:	
				Girardi heart cells	K562 cells
T15	$CD8^+$	2.5	15:1	38.1	2.5
IIIB2	$CD4^+$	10.5	22:1	57.1	47.9
T16	$CD4^+$	5.0	15:1	13.8	74.4
T20	$CD4^+$	2.2	10:1	0	59.0

Clones. The data obtained with mixed populations of mononuclear cells or $CD3^+$ cells suggested that M protein induced cytotoxic lymphocytes with multiple target cell specificities (Table II). To assess whether individual effector cells might have specificity for heart cells, as suggested by antibody inhibition studies, we generated M protein–responsive T cell clones and tested each for cytotoxicity against heart cells and K562 cells (Table VI). All of the T cell clones underwent blastogenesis in response to pep M5, as determined by [^{3}H]thymidine incorporation in the presence and absence of pep M5. None of the cells responded to streptokinase, whereas the donor lymphocytes showed brisk blastogenic responses to this control antigen (data not shown). One of the four clones chosen for study (T15) was cytotoxic against heart cells and not K562 cells. Two of the T cell clones were cytotoxic against both target cells, and one was cytotoxic only against K562 cells. Although the majority of the T cell clones were of the $CD4^+$ phenotype, the heart cell–specific clone (T15) was $CD8^+$. Taken together, these data suggest that M protein induces cytotoxic T cells with multiple target cell specificities; some of the clones have specific effector activity against heart cells and not NK targets, whereas others are cytotoxic against more than one type of target cell.

Discussion

In the present study, we have shown that purified pepsin-extracted fragments of streptococcal M proteins induce CTL from normal individuals with activity against several different target cells, including cultured myocardial cells. The

cytotoxic effector cells were derived from a $CD3^+$ population that was devoid of spontaneous cytotoxic activity against the NK-susceptible K562 cell line. Antibody inhibition experiments suggested that a portion of the cytotoxic cells recognized cell surface antigens of Girardi heart cells that were M protein–crossreactive, and initial experiments with M protein–reactive T cell clones demonstrated cytotoxic activity against heart cells alone, K562 cells alone or against both cell types.

The exact nature of the M protein–stimulated effector cells is not clear. The target cell specificity of the cytotoxic cells induced in mixed populations by M protein suggests that they are NK-like (24, 25), and the finding that lymphocytes from all humans tested were cytotoxic against target cells from multiple origins suggests that they are not MHC restricted. However, we showed that activation alone, either by IL-2 or Con A, was not sufficient to induce cytotoxicity against the target cells used in this study. The cytotoxicity against human but not xenogeneic cells is similar to the activity of "anti–human effectors" reported by Sugamura, et al. (26). Because the cytotoxic lymphocytes were induced in $CD3^+$ cells that showed no spontaneous cytotoxicity against K562 cells, yet were not MHC-restricted, we believe they represent non–MHC-restricted CTL, a term recently proposed by Lanier, et al. (27). It is not yet known whether the M protein–induced effector cells express the T cell receptor γ polypeptide, which has recently been described on the surface of non–MHC-restricted CTL from normal individuals (28).

The role of M protein–activated cytotoxic cells in the pathogenesis of streptococcal infections or acute rheumatic fever is as yet unknown. The ability to induce cytotoxic lymphocytes from all normal adults tested suggests that these effectors may not be involved in the pathogenesis of rheumatic fever, since only a small percentage of the population appears to be genetically predisposed to develop the disease. However, previous studies have shown that patients with acute rheumatic carditis have circulating cytotoxic lymphocytes with activity against myocardial cells (30), and guinea pigs immunized with group A streptococci develop cytotoxic lymphocytes against primary cultures of heart cells (31). Our data provide evidence that M proteins that contain tissue-crossreactive epitopes induce cytotoxic effectors with broad target cell specificity, but a portion of the cells appears to be specific for cultured myocardial cells. A detailed analysis of the target cell specificity of cytotoxic lymphocytes from individuals with rheumatic carditis or normal individuals that express the recently described alloantigen 883 that predicts rheumatic fever susceptibility (32) may show a role for tissue-specific cytotoxic cells induced by streptococcal M protein. The pathologic lesions of rheumatic carditis are most likely the result of complex interactions between humoral and cellular autoimmune mechanisms. Of interest is our finding that heart-crossreactive streptococcal antibodies inhibited cytotoxicity against cultured heart cells but not other target cells, suggesting that under some circumstances humoral autoimmune responses may be protective against cell-mediated myocardial damage.

Perhaps relevant to these studies is a recent study (29) showing induction of human cytotoxic lymphocytes by Salmonella species. The authors were unable to correlate cytotoxicity with antibacterial activity. Whether or not M protein–

induced CTL are bactericidal against group A streptococci is currently under investigation in our laboratory.

Our findings that M protein–induced CTL against several different target cells may have direct bearing on previous studies of OK-432, a killed group A streptococcal preparation that potentiates cytotoxic lymphocyte activity against a variety of tumor cells in humans and animals (33–35). Although OK-432 has been shown to augment NK activity (34), there is also evidence that the substance induces NK-like cells with multiple target cell specificities (33). Previous studies have shown that at least a portion of the immunopotentiating activity resides in the M protein fraction of streptococcal extracts (35).

In summary, we have shown that purified M proteins from three different serotypes of group A streptococci evoke cytotoxic T lymphocytes against several different cultured target cells; some of the cytotoxic cells appear to have specificity for myocardial cells. The exact role of M protein–stimulated cell-mediated cytotoxicity in the pathogenesis of rheumatic carditis is not yet clear; however, further studies to define the target cell specificity and mechanisms of cytotoxicity using lymphocytes from patients with acute rheumatic fever may provide more definitive answers.

Summary

Purified group A streptococcal M proteins were used to stimulate peripheral blood lymphocytes from normal adult volunteers. The activated lymphocytes were cytotoxic against cultured human heart cells, as well as liver cells, fibroblasts, and K562 cells, but showed only minimal cytotoxicity against several animal cell types. The cytotoxic activity evoked by type 5 M protein was dose and time dependent. Rabbit antisera against pep M5 that contained heart-crossreactive antibodies partially inhibited cytotoxicity against heart cells, but had no effect on other target cells, suggesting that a fraction of the effector lymphocytes may be recognizing M protein–crossreactive cell surface antigens. All of the cytotoxic activity was recovered from a $CD3^+$ population of lymphocytes obtained from a fluorescence-activated cell sorter, and $CD4^+$ and $CD8^+$ cells were also cytotoxic. M protein–responsive T cell clones were generated that showed specificity for heart and K562 cells, in addition to clones that were cytotoxic against both cell lines. Our data show that streptococcal M protein evokes cytotoxic T lymphocytes against multiple human but not animal target cells. Some of the effector cells may be specific for cultured myocardial cells, but their role in the pathogenesis of rheumatic carditis will require further studies of lymphocytes from patients with acute rheumatic fever and carditis.

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