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Diagnostic relevance of humoral and cell-mediated immune reactions in patients with acute viral myocarditis

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SUMMARY

Sera of 177 patients with acute myocarditis (10 coxsackie B 3/4, four influenza, four mumps, 15 cytomegalovirus, 144 undefined) were tested by indirect immunofluorescence for autoantibodies against heart and skeletal muscle and vital or air-dried adult cardiocytes. Antibody-dependent cytolysis, lymphocytotoxicity and antibody-dependent cellular lymphocytotoxicity were assessed using vital adult rat cardiocytes as target cells. Muscle-specific anti-sarcolemmal antibodies of the anti-myolemmal type—often associated with non-organ-specific anti-endothelial antibodies—were demonstrated in nine out of 10 patients with coxsackie B, in all patients with influenza and mumps and in 65 out of 144 patients with undefined myocarditis. In contrast, 13 out of 15 patients with cytomegalovirus myocarditis lacked anti-sarcolemmal antibodies but had low titre anti-inter fibrillary antibodies instead. In the presence of complement, anti-myolemmal antibodies induced cytolysis of vital cardiocytes, whereas hepatocytes remained unaffected. Titres of anti-myolemmal antibodies correlated with the degree of cardiocytolysis. The anti-myolemmal immunofluorescent pattern and the cytolytic serum activity could be absorbed with the respective viral antigens suggesting that these antibodies cross-react with moieties of the virus itself and may be both diagnostic and aetiological markers in acute viral myocarditis. Lymphocyte-mediated cytotoxicity against heterologous cardiac target cells could not be observed in our patients with myocarditis of proven viral aetiology. However, lymphocyte-mediated cytotoxicity was demonstrated in 10

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Abbreviations: ADCC = antibody-dependent cellular cytotoxicity; AEA = anti-endothelial antibodies; AFA = anti-fibrillary antibodies; AHA = anti-heart antibodies; ALK = activated lymphocyte killing; AMA = anti-mitochondrial antibodies; AMC = antibody-mediated cytolysis of target cells in the presence of complement; AMLA = anti-myolemmal antibodies; ANA = anti-nuclear antibodies; ASA = anti-sarcolemmal antibodies; BSA = bovine serum albumin; CI_{AMC} = cardiocyte index of antibody-mediated cytolysis; CI_{LC} = cardiocyte index of natural killer cell activity; CI_{ADCC} = cardiocyte index of natural killer cell activity in the presence of antibody; CFT = complement fixation test; CMV = cytomegalovirus; CTL = cytolytic T lymphocytes; DC = direct cytotoxicity; DNA = deoxyribonucleic acid; ECG = electrocardiogram; FCS = fetal calf serum; HI_{AMC} = hepatocyte index of antibody-mediated cytolysis; HI_{LC} = hepatocyte index of natural killer cell activity; HI_{ADCC} = hepatocyte index of natural killer cell activity in the presence of antibody; HLA = human leucocyte antigen; IFA = anti-interfibrillary antibodies; LC = lymphocytotoxicity; NK = natural killing; PBL = peripheral blood cells; SMA = smooth muscle antibodies; t_{50} = half-time of survival.

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ASA-positive and one ASA-negative patient with myocarditis of unknown origin. ASA-positive sera blocked lymphocytotoxicity in three of these patients.

INTRODUCTION

The aetiology of myocarditis is often difficult to ascertain. Complement-fixing antibodies against cardiotropic viruses can only be detected in 10–20% of patients. Alcoholic cardiomyopathy or other microbial infections excluded, viral aetiology has to be considered in most cases, especially in the light of published epidemiological evidence for viral infections (Burch & Giles, 1972). It was one purpose of this study to determine whether characteristic patterns of humoral and cellular immune reactions exist in different types of viral cardiomyopathies. These patterns could help to distinguish viral from non-viral myocarditis and might help to differentiate certain types of viruses involved.

Little is known about the occurrence of autoantibodies in acute myocarditis in men (Dragatakis *et al.*, 1979; Grèžlikowskí, 1972; Maisch, Berg, Kochsiek, 1980a; Slàdkovà, Stefan & Bìcovb̀, 1979), although anti-heart muscle immunoglobulins are established diagnostic markers in rheumatic carditis (Kaplan & Meyeserian, 1960), in post-pericardiotomy (Kaplan & Frengley, 1969; Maisch, Berg & Kochsiek, 1979a; Maisch *et al.*, 1979c) and post-infarction syndromes (Kuch, 1973; Maisch, Berg & Kochsiek, 1979b). There are no reports about cellular immune reactions during the course of myocarditis in men. Several possible pathogenic mechanisms are discussed to be operative in viral myocarditis:

(1) a viral infection of the myocardial cell;
(2) the induction of humoral and cellular autoimmunity against myocardial cells by neoantigens, by structurally altered antigens or by heterologous equivalent antigens with related antigenic determinants (cross-reaction) or
(3) a combination of both.

It was therefore of interest to investigate the pathogenic role of humoral and cellular immunological effector mechanisms in humans.

MATERIALS AND METHODS

Patients. Sera from 177 patients (98 male, 79 female) and lymphocytes of 34 patients presenting clinical signs of myocarditis were analysed in the acute phase of the disease (first to third week) and in the recovery period, when acute symptoms had disappeared.

The patients were divided into two groups: Group 1 included 33 patients with proven viral myocarditis. At least two complement fixation tests (CFT) were carried out against major cardiotropic viruses (coxsackie B_{1-5}, cytomegalovirus, mumps, influenza A/B) over a period of several weeks. Only changes in titre were accepted as proof of recent viral infection. CFT was positive in 15 patients for cytomegalovirus, in six patients for coxsackie B_4, in four patients for coxsackie B_3, in four patients each for influenza and mumps. Group 2 included 144 patients with acute myocarditis of unknown aetiology. Patients with alcoholic cardiomyopathy were excluded. For serological studies sera of 98 normal subjects of all blood groups and of patients having a viral infection without myocarditis (e.g. influenza ($n=4$), mumps ($n=4$), cytomegalovirus ($n=20$), coxsackie B_4 ($n=2$) served as controls.* Controls for lymphocyte cytotoxicity studies (LC; $n=32$) and antibody-dependent cytotoxicity (ADCC; $n=18$) were lymphocytes of HLA/DR-typed donors comprising all major HLA/DR specificities.

Immunological studies

Storage. All studies were performed in sera stored at −25°C for up to 5 years. Peripheral blood

*Some sera patients with viral myocarditis and of control subjects (viral diseases without myocarditis) were kindly donated by Professor Gerth, Department of Virology, University of Tübingen and by Dr Seuffer, Clinical Laboratories, Tübingen.

lymphocytes of patients separated by centrifugation on Ficoll-Amino trizoe acid were either used immediately or stored at −170°C in liquid nitrogen after they had been frozen by a cell freezer (Planer, Messer-Griesheim).

Indirect immunofluorescence on cryostat sections and cardiocytes. A modification of the indirect technique (Coons & Kaplan, 1950) was performed using operatively resected myocardium from patients with congenital heart disease (blood group O). Also skeletal muscle, kidney, homologous thyroid gland, uterus and skin, heterologous (rat) liver,oesophagus and stomach were used. After testing with polyspecific FITC-labelled antiserum ($F(ab)_2$ fragments, H- and L-chain specific: F/P molar ratio $2{\cdot}5 \pm 1{\cdot}5$, total protein content 23–25 mg/ml, dilution 1:10, Behring) immunoglobulin class specificity was analysed with monospecific FITC-labelled antisera ($F(ab)_2$ fragments, H- and L-chain specific, Behring) and anti-C_3 antiserum (Behring). The anti-myolemmal subtype (AMLA) of ASA was determined in isolated cardiocytes, deprived of their connective tissue and their basement membrane by treatment with collagenase (Maisch, 1981). According to electronmicroscopy and freeze-fracture studies (Liu & Spitzer, 1978; Masson-Pévet, Jongsma & Bruijne, 1976) after collagenase treatment connective tissue, reticulin, collagenous parts of the basement membrane and the glycocalix of the sarcolemma are removed and only the plasmalemma or myolemma is left in these preparations. Vital cardiocytes were allowed to sediment in a cylindrical chamber and air dried. Indirect immunofluorescence was carried out as described above.

Antibodies were classified either as muscle-specific or non-tissue-specific. Subtypes of the muscle-specific antibodies included those directed against sarcolemmal (ASA) or myolemmal (AMLA) membrane, against muscle fibrils (AFA) and the contractile proteins myosin and actin or interfibrillary substance (IFA). The latter are characterized by a homogenous interfibrillary fluorescence. Tissue-unspecific antibodies found in this study were anti-nuclear antibodies (ANA), smooth muscle antibodies (SMA), anti-mitochondrial antibodies (AMA) and antibodies directed against capillaries and the endothelium of small vessels (AEA). A heterologous renal tubular immunofluorescent pattern associated with a positive anti-sarcolemmal and anti-endothelial reaction was attributed to heterophil antibodies (Hawkins, McDonald & Dawkins, 1977) and was therefore excluded. Antibodies against double-stranded DNA were tested radioimmunologically (Amersham).

Sera were also tested for cytoplasmic complement-fixing antibodies using the CF test with rat kidney homogenate (Berg *et al.*, 1975).

Indirect immunofluorescence of vital cardiocytes was carried out with enriched myocardial cells as described in detail previously (Maisch *et al.*, 1981). $F(ab)_2$ fragments of FITC-antigammaglobulin (poly- and monospecific; Behring) were used to avoid non-specific fluorescence at a dilution of 1:10.

Assays of cytotoxicity

Preparation of cardiac target cells. After thoracotomy, isolation of cardiocytes from female Sprague Dawley rats (190–230 g) was carried out according to Powell & Twist (1976). In addition a one- or two-step continuous gradient centrifugation with Percoll™ (Pharmacia) was carried out to obtain more than 90% vital cells as described previously (Maisch, 1981).

Criteria for vitality were a typical microscopical morphology (cylindrical cells = vital, spherical cells = non-vital) of cardiocytes, their ability to contract, to exclude trypan blue and to be stained with fluoresceindiacetate (0·05%). Cardiocytes could be stored in sterile flasks (5×10^4/ml) at 4°C for 12 hr without a significant loss of viability.

Preparation of rat hepatocytes. After laparatomy and incision of the inferior v. cava, the portal vein was perfused at 21°C with solution II (calcium free MEM-solution containing Earle's Spinner BSS with glutamin, 20 mMol HEPES pH 7·2 [Serva]). After 15 min, the liver was explanted and placed on a perforated collector disc. The perfusion of the v. porta was discontinued and the superior v. cava (37°C, 40 ml/min) was perfused instead. The perfusion medium (II) was supplemented by 50 mg/100 ml collagenase (from clostridium histolyticum, 0·15 units/mg, Boehringer, Mannheim) and oxygenated. After perfusion the liver was placed in a petri dish, minced carefully and resuspended in 50 ml solution II supplemented with collagenase in the same concentration. The suspension was then filtered through a 150 μm nylon mesh. The filtrate was

diluted in 100 ml Medium II and washed three times (2 min, 50g, 21°C). Vitality assessed by trypan blue exclusion was between 75% and 85%.

Cytotoxicity assays. Assays of antibody-mediated cytolysis (AMC):

(1) Two hundred microlitres of cardiocytes in Krebs–Ringer and 0·1% BSA (Pentax Fraction V-type F (sigma) solution (Maisch, 1981) (5×10^4 cells/ml) + 50ml decomplemented serum were placed into wells of microtitre plates (Falcon multiwell 3008), 50μl of human complement (fresh normal serum) were added. The mixture was shaken for 10 sec and 200–400 cells/well were counted. Cells were incubated at 37°C (5% CO_2) for 24 hr. The percentage of vital cells was assessed after 3, 9, 18, and 24 hr by light microscopy. The assay was done in duplicates for each serum with a 5% limit of tolerance in counting between both samples. For each duplicate the half-life of the cardiocytes in the presence of the patients serum ($t_{50\,i}$) was compared with the half-life of cardiocytes in a identical, representative control serum ($t_{50\,c}$). The cardiocyte index of antibody-mediated cytolysis was calculated using the formula

$$CI_{AMC} = \frac{t_{50\,i}}{t_{50\,c}};\ \text{normals } (n=70):\ CI_{AMC} = 0{\cdot}92 \pm 0{\cdot}07\ (x \pm 1\ \text{s.d.})$$

An effective cytolysis was assumed at a $CI_{AMC} < 0{\cdot}75$.

(2) The corresponding assay of hepatocytolysis was carried out in buffer solution II with identical concentrations of vital hepatocytes. The hepatocyte index of antibody-mediated cytolysis (HI_{AMC}) was $0{\cdot}95 \pm 0{\cdot}12$ ($n=12$) in normals.

Assays of lymphocytotoxicity (LC):

(1) Two hundred microlitres of cardiocytes in solution I at a cell density of 5×10^4/ml and 1×10^4/ml were placed into the well of microtitre plates. Further additions were 50 μl RPMI and 70 μl of the patient's lymphocytes suspended in RPMI and 70 μl of the patient's lymphocytes suspended in RPMI (+20% FCS + 50 IE penicillin/streptomycin/ml). The concentration of the lymphocytes was adjusted to $1{\cdot}5 \times 10^6$/ml for all assays. The ratios target cell/lymphocyte were 1:10, 1:50 and 1:100. Prior to incubation (37°C, 5% CO_2) the suspension was shaken for 10 sec. The percentage of vital cells was assessed at 0, 3, 9 and 24 hr. The assays were set up in duplicate. Assays which varied in their densities of vital and non-vital cells more than 5% were excluded. For each duplicate the half-life of cardiocytes in the presence of the patient's serum and lymphocytes ($t_{50\,i}$) was compared with the half-life of cardiocytes in the presence of serum and lymphocytes of a representative panel of HLA-typed donors ($n=32$) comprising all known HLA- and DR-specifities.

The index of cellular cytotoxicity was calculated using the formula

$$CI_{LC} = \frac{t_{50\,i}}{t_{50\,c}};\ \text{Normals: } 1{\cdot}06 \pm 0{\cdot}14\ (n=32);\ \text{cytotoxic reaction effective, if } CI_{LC} < 0{\cdot}75.$$

(2) The corresponding assay of hepatocytotoxicity was carried out accordingly with vital hepatocytes suspended in buffer solution II. Target to lymphocyte ratios were identical.

The hepatocyte index of lymphocytotoxicity in normals ($n=32$) was $HI_{LC} = 0{\cdot}94 \pm 0{\cdot}14$.

Assays of antibody-dependent cellular cytotoxicity (ADCC)

(1) Parallel to the LC assays an additional test for the assessment of antibody-dependent cytotoxicity was set up with the same target cell to lymphocyte ratios. However, 50μl of decomplemented (56°C) autologous serum were added instead of the 50μl RPMI (+20% FCS).

The indices of ADCC against cardiocytes were calculated using the formula

$$CI_{ADCC} = \frac{t_{50\,i}}{t_{50\,c}}$$

Normals ($n=8$): $1{\cdot}11 \pm 0{\cdot}08$ cytotoxic reaction effective, if $CI_{ADCC} < 0{\cdot}75$.

(2) The corresponding indices of hepatocytotoxicity in normals ($n=18$) were $HI_{ADCC} = 1{\cdot}0 \pm 0{\cdot}16$.

Since the spontaneous cytotoxicity, as assessed in the previous assays, was low, it was included in the total ADCC activity.

Absorption studies. Sera of patients with coxsackie B, influenza A, and mumps myocarditis were absorbed with increasing quantities (up to 1000 ml of the original antigen suspension) of viral antigens (influenza A (Behring)), coxsackie B (Dynatech), mumps (Seromed) and in other experiments with a pellet of vital cardiocytes. The absorbed sera were compared with non-absorbed

sera using identical dilutions with respect to AMLA titres and cytolytic serum activity. Cross-absorptions (e.g. sera of patients with coxsackie B myocarditis were absorbed with influenza antigen and vice versa) were carried out as above and antibody pattern and cytolytic serum activity were also evaluated.

Statistics. Results are given as mean ($\bar{x}$) $\pm$ 1 standard deviation (s.d.). Standard *t*-test was used to evaluate the significance of difference between the groups of patients, after testing the homogeneity of variances with the Fisher-test. For paired samples the corresponding *t*-test was used. All tests were two-tailed. In the case of inhomogeneity a Welch-test was employed. The significance of correlations was tested by regression analysis. The regression was tested for linearity by variance analysis.

RESULTS

Clinical features of myocarditis

The clinical features found in 177 patients with viral (group 1) and undefined (group 2) myocarditis were comparable (Table 1). No significant differences between group 1 and 2 could be established in respect to symptoms, ECG alterations, age or sex.

Antibody patterns in viral and undefined myocarditis

The characteristic immunofluorescent feature in coxsackie B, influenza A and mumps myocarditis was the presence of AMLA in titres ranging from 1:40 to 1:320. They could be demonstrated almost immediately after admittance to hospital (Table 2). The time span that elapsed between the onset of

Table 1. Clinical features (percentage incidence) of myocarditis in 177 patients with defined and undefined myocarditis

	Aetiology	
	Group 1 viral ($n=33$)	Group 2 unknown ($n=144$)
Fatigue	100	69
Acuteness of sympton onset (within 4–8 weeks)	100	100
Association with pharyngeal inflammation or pleuro-pneumonia	75	63
Fever (> 38°C)	88	69
Precordial discomfort	82	74
Dyspnoea on effort	67	63
Arrhythmia	27	19
ST-T segment alterations	67	52
Tachycardia	67	48
Heart block	42	44
Ventricular extrasystole	58	33
Conduction disturbance	33	15
Transient cardiomegaly increased erythrocyte sedimentation rate > 12 in first hour	73	69
Male	75	63
Female	58	52
Age (years)	32 $\pm$ 6	31 $\pm$ 7

symptoms and admittance to hospital was between 1 and 60 days. AMLA could be demonstrated with sedimented air-dried cardiocytes of intact morphology and equally well in vital contracting cardiocytes.

Examination of cryostat sections revealed that AMLA were associated in 50–80% of cases with non-organ-specific anti-endothelial antibodies (Table 2). ASA, AMLA and AEA fixed complement in 16 out of 18 patients. The antibodies belonged to the IgG class in 16 out of 18 and to the IgM class in 10 out of 18 cases. AMLA were seen neither in 98 healthy blood donors nor in patients suffering from viral infections without myocardial involvement. None out of four patients with mumps orchitis were demonstrated to have AMLA, in three cases anti-basement membrane fluorescence of the sarcolemma was associated with AEA, however. The same staining pattern (AMLA−, ASA+/AEA+) was found with sera of all four control patients with influenza and one of two sera of patients with coxsackie B infections without myocardial involvement (heterophil antibodies).

In undefined myocarditis ASA were seen in 45% of patients. Only 39% of patients demonstrated muscle-specific AMLA. An association of muscle-specific AMLA and non-organ-specific AEA was found in 75% of AMLA-positive sera (Table 2).

To elucidate the specificity of AMLA and their relationship to viral antigens absorption studies were carried out. In a first series 18 AMLA-positive sera with titres of 1:40 to 1:320 were absorbed with a pellet of vital cardiocytes. After absorption AMLA titres fell to 0 or 1:20, demonstrating the specificity of this antibody to the myolemma. In a second series AMLA-positive sera of patients with viral myocarditis were absorbed with the causative viral antigens (Fig. 1, left panel): Whereas preabsorption AMLA titres ranged from 1:20 to 1:320, titres were significantly reduced to 1:20 and to 0 ($P < 0{\cdot}05$) after absorption. In contrast absorptions with equal amounts of hepatocytes did not alter the AMLA titres ($n = 10$). Cross-absorptions of sera from patients with coxsackie B myocarditis with influenza antigens ($n = 8$) or mumps antigen ($n = 8$) and vice versa ($n = 4$ respectively) could not abolish the anti-myolemmal fluorescence.

The prominent feature in sera of patients with CMV-myocarditis was the presence of IFA of low

Table 2. Autoantibodies in myocarditis

	% Positive				
	Group 1				Group 2
Antibody type	Coxsackie B ($n = 10$)	Influenza ($n = 4$)	Mumps ($n = 4$)	Cytomegalovirus ($n = 15$)	Undefined ($n = 144$)
Muscle-specific					
Anti-sarcolemmal (ASA)	100	100	100	7	45
Anti-myolemmal (AMLA)	90	100	100	0	39
Anti-fibrillary (AFA)	10	0	50	7	32
Anti-myosin	10	0	50	0	23
Anti-actin	0	0	0	7	9
Anti-interfibrillary (IFA)	20	0	0	87	29
Anti-intercalated disc (AIDA)	0	0	0	0	1
Anti-conducting tissue (OFT†)	0	0	0	14	13
Non-organ-specific					
Anti-endothelial (AEA)	80	75	50	7	36
Anti-smooth muscle (SMA)	10	0	0	7	15
Anti-nuclear (ANA)	0	0	25	0	23
Anti-mitochondrial (AMA)*	10	25	0	0	8
Heterophil	10	0	0	7	3

Normals ($n = 98$): muscle-specific antibodies not present; non-organ-specific antibodies < 8%.
*IFL-pattern similar to AMA, but negative in complement fixation test.
†OFT = Ox false tendon.

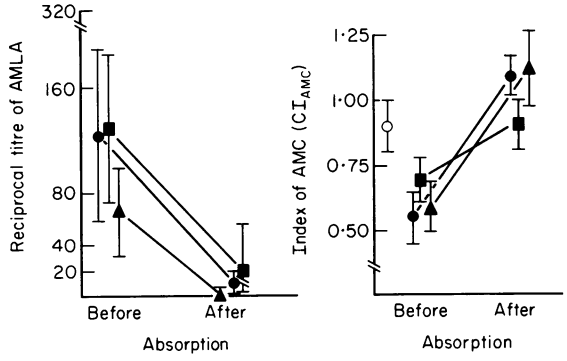

Fig. 1 Absorption of antimyolemmal antibodies (left panel) and of cytolytic serum activity (right panel) by the respective viral antigens. (● = Coxsackie, $n = 10$; ▲ = Influenza, $n = 4$; ■ = Mumps, $n = 4$; ○ = Normals, $n = 50$).

titres (1:10–1:40). They were of the IgG and IgM class in 13 out of 15 cases (Table 2). AMLA, ASA or AEA were not detected. Other muscle-specific and non-organ-specific antibodies were occasionally found and can be seen from Table 2.

Whereas in patients with myocarditis of proven viral origin at least one of the subtypes of anti-myocardial antibodies was found, in 53 out of 144 patients of the undefined group no anti-myocardial or non-organ-specific antibodies were detected.

Demonstration of antibody-mediated cytolysis (AMC)

Cytolytic serum activity against vital cardiocytes in acute viral myocarditis was closely related to the presence of muscle-specific AMLA. All AMLA-positive sera of the viral and undefined group with titres > 1:20 induced cytolysis of vital cardiocytes in the presence of complement (Fig. 2). The titres of anti-myolemmal antibodies correlated with the index of cytolysis (CI_{AMC}) in patients with AMLA-positive viral carditis ($y = 0{\cdot}75$–$0{\cdot}001x$, $r = 0{\cdot}501$). Among patients with viral myocarditis correlation was best in mumps myocarditis ($r = 0{\cdot}75$; $y = 0{\cdot}94$–$1{\cdot}9 \times 10^{-3}x$), followed by coxsackie B ($r = 0{\cdot}61$; $y = 0{\cdot}8$–$1{\cdot}2 \times 10^{-3}$), and influenza myocarditis ($r = 0{\cdot}42$; $y = 0{\cdot}55$–$0{\cdot}7 \times 10^{-3}x$). AMLA-positive sera of patients with undefined myocarditis also induced cytolysis of cardiac cells particularly in titres > 1:40 (Fig. 3). The correlation between reciprocal AMLA-titres and cytolytic serum activity was similar in patients with viral myocarditis ($r = 0{\cdot}55$; $y = 0{\cdot}78$–$1{\cdot}955 \times 10^{-3}x$). Only one ASA- and AMLA-negative serum and no ASA-positive but AMLA-negative serum ($n = 6$) was cytolytic.

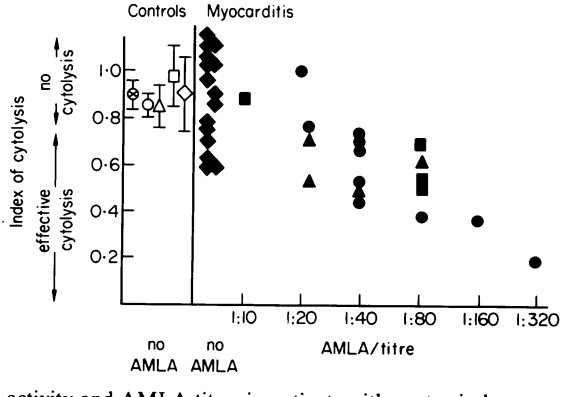

Fig. 2. Cytolytic serum activity and AMLA titres in patients with acute viral myocarditis. (● = Coxsackie B, $n = 10$; ▲ = Influenza A/B, $n = 4$; ■ = Mumps, $n = 4$; ♦ = Cytomegalovirus, $n = 15$; ○ = Coxsackie pleuritis, $n = 2$; △ = Influenza pneumonia, $n = 4$; □ = Mumpsorchitis, $n = 4$; ◊ = Cytomegalovirus, $n = 20$; ⊗ = Controls, $n = 70$).

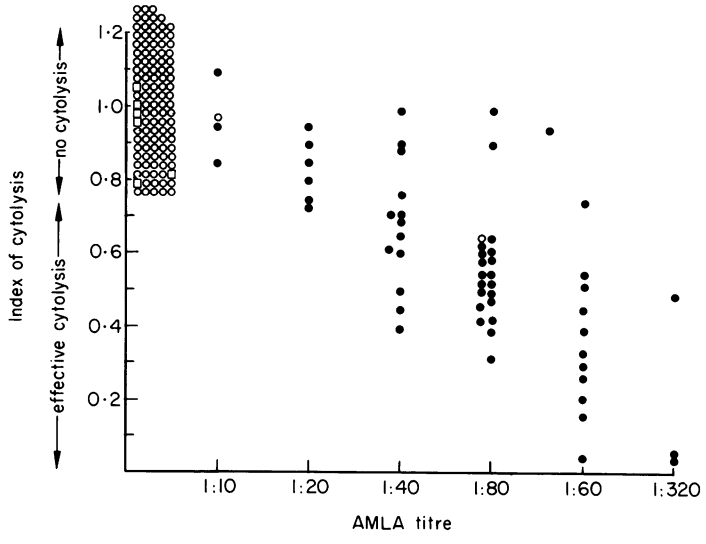

Fig. 3. Cytolytic serum activity and AMLA-titre in patients with acute undefined myocarditis, $n=144$. (● = AMLA positive; □ = AMLA negative, ASA positive; ○-AMLA negative, ASA negative).

The following experimental data suggest that cytolysis is an antibody-mediated process:
(1) AMLA-positive sera could be absorbed with the pellet of vital cardiocytes ($n=18$): Before absorption cytolytic serum activity was present with an index (CI_{AMC}) of $0{\cdot}58 \pm 0{\cdot}06$. After absorption sera became AMLA-negative and their cytolytic activity was reduced to control levels ($CI_{AMC}=0{\cdot}89 \pm 0{\cdot}07$, $P<0{\cdot}01$). Absorptions with identical amounts of hepatocytes did not significantly alter the cytolytic serum activity ($n=10$): The index of hepatocytolysis before absorption was $HI_{AMC}=0{\cdot}59 \pm 0{\cdot}10$. After absorption it was $0{\cdot}62 \pm 0{\cdot}14$ ($P<0{\cdot}05$).
(2) When sera patients with myocarditis of proven viral origin were absorbed with the corresponding viral antigens cytolytic serum activity was abolished. Before absorption the CI_{AMC} ranged from 0·26 to 0·76. After absorption no cardiocytolysis was present ($CI_{AMC}>0{\cdot}75$, Fig. 1, right panel).
(3) Cross-absorptions of sera from patients with coxsackie B myocarditis with influenza ($n=8$) or mumps antigens ($n=8$) and vice versa ($n=4$, respectively) could not change the cytolytic activity for more than 0·1 of the respective index of cytolysis.
(4) Patients having viral infections such as pleuritis (two coxsackie B, four influenza, 20 cytomegalovirus) or orchitis (four mumps) without myocarditis had no significant cytolytic serum activity. CI_{AMC} was $>0{\cdot}75$ in all cases.
(5) Furthermore, there was no cytolytic activity in the absence of complement indicating that complement had to be bound to the antibody for the cytolytic reaction ($n=10$). The CI_{AMC} in the presence of complement was $0{\cdot}61 \pm 0{\cdot}12$. Without complement no cardiocytolysis was observed ($CI_{AMC}=0{\cdot}83 \pm 0{\cdot}15$; $P<0{\cdot}01$).

Lymphocyte mediated cytotoxicity against heterologous target cells
Lymphocytotoxicity was measured in the absence (LC) and the presence (ADCC) of serum in 44 patients and in controls. Three different types of cytotoxic reactions were demonstrated:
(1) Demonstration of lymphocytotoxicity with (ADCC) and without antibody (LC)
(2) Demonstration of LC alone
(3) Absence of LC and ADCC

LC and ADCC against cardiocytes were found in seven ASA-positive patients with undefined myocarditis (Fig. 4) using a ratio of target cell/lymphocytes of 1:10 and 1:50 (enhancement or no blocking). Lymphocytes of five of these patients were also tested against rat hepatocytes. No significant lysis of hepatocytes was present ($HI_{LC}=0{\cdot}85 \pm 0{\cdot}14$; $HI_{ADCC}=0{\cdot}8 \pm 0{\cdot}13$) as was the case

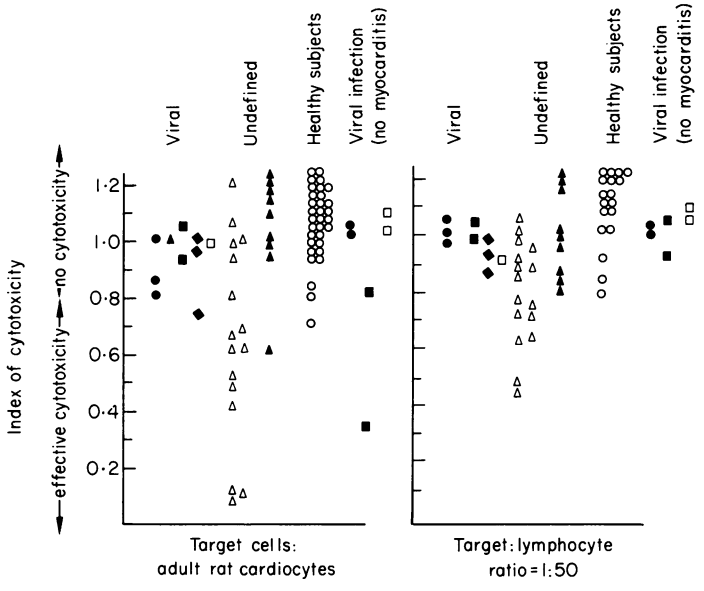

Fig. 4. Cellular cytotoxicity against vital cardiocytes with (ADCC) or without (LC) presence of autologous serum. Left panel: lymphocytotoxicity (LC) in patients with viral and undefined myocarditis as compared to viral infection without myocarditis and HLA-typed healthy donors. Right panel: antibody-dependent cellular cytotoxicity (ADCC) in the same groups of patients. LC and ADCC against adult cardiocytes were not detected in patients with viral myocarditis, viral infection without myocarditis and in healthy controls. In 10 ASA positive patients with undefined myocarditis LC was present. After addition of autologous ASA-positive serum (ADCC) lymphocytotoxicity was blocked completely in three patients. (● = Coxsackie B, $n = 3$; ■ = Influenza, $n = 2$; □ = Mumps, $n = 1$; ♦ = Cytomegalovirus, $n = 3$; △ = Undefined ASA positive, $n = 16$; ▲ = Undefined ASA negative, $n = 9$; ○ = Controls, HLA/DR typed donors).

in controls of 14 HLA-DR-typed donors ($HI_{LC} = 0{\cdot}94 \pm 0{\cdot}14$; $HI_{ADCC} = 1{\cdot}02 \pm 0{\cdot}16$). The clinical courses of patients with significant LC and ADCC against rat cardiocytes differed from those of patients without lymphocyte mediated cytotoxicity. Two died from heart failure. The courses of the other patients were protracted.

In four patients with undefined myocarditis LC activity could be demonstrated in the absence of serum only. After the addition of the autologous serum to the assay (ADCC) lymphocytotoxicity was abolished (blocking). All four sera were AMLA-positive.

Neither LC or ADCC could be detected in patients with myocarditis of proven viral aetiology, in the patients with viral infections without myocardial involvement, in 133 of 144 patients with myocarditis of unknown aetiology, and in a panel of HLA- and DR-typed donors, which comprised most of the known HLA- and DR-specificities.

DISCUSSION

The association of muscle-specific AMLA with non-organ-specific AEA was a recurring feature in sera of patients with coxsackie B, influenza and mumps myocarditis. In 30% of patients with myocarditis of unknown aetiology this pattern also prevailed. Muscle-specific AMLA most likely indicate the organotropicity of the viral assault, their absorption with the respective virus also identified the viral pathogen. It may be therefore concluded that a heterogeneity of AMLA exists. The association of muscle-specific AMLA with non-organ-specific AEA also indicates viral aetiology: non-organ-specific antibodies such as AEA are often found in viral diseases (Berg, Brand & Märker, 1973), whereas in classical autoimmune diseases such as lupus erythematodes these antibodies are not found. ASA, however, are not restricted to viral myocarditis. They have been described in rheumatic carditis (Kaplan & Meyeserian, 1960), in post-pericardiotomy (Kaplan &

Frengley, 1969; Maisch *et al.*, 1979a, c) and post-infarction syndromes (Kuch, 1973; Maisch *et al.*, 1979b).

Specificity of AMLA can be postulated only if heterophil antibodies (Hawkins *et al.*, 1977), isoantibodies against blood group substances (Duheille & Petitier, 1967), non-organ-specific autoantibodies against collagen (Berg *et al.*, 1973) or the collagenous part of the anti-basement membrane or anti-reticulin antibodies are excluded. This was accomplished by the use of homologous (human) and heterologous myocardium (rat/bovine) and by the exclusion of heterophil antibodies. Isoantibodies against blood group subtances were avoided by the use of tissues of blood group O donors only. A specific anti-myolemmal antibody pattern was attained by depriving cardiac muscle cells of reticulin, basement membrane and the glycocalix using cardiac cells isolated by treatment with collagenase. By electron microscopy and freeze-fracture studies (Liu & Spitzer, 1978; Masson-Pévet *et al.*, 1976) it was established that the myocyte loses almost all of the basement membrane after treatment with collagenase. The surface morphology of the myolemma, however, is not altered significantly.

A non-specific adsorption of the Fc fragment of antibodies to the myolemmal membrane, which is known to occur in cerebral tissues, could be excluded by the use of $F(ab)_2$ fragments of FITC-labelled antibodies. In addition, antibodies against DNA or mitochondria of patients with lupus erythematodes or primary biliary cirrhosis and immune complexes such as aggregated IgG did not bind to the myolemmal membrane (unpublished). Another argument against a non-specific binding of antibodies to the myolemma is the fact that these AMLA were absorbed selectively by the respective viral antigens. It is likely that out of the heterogeneity of ASA the heterophil, the iso-, the anti-basement membrane and anti-reticulin antibodies AMLA are muscle-specific.

In contrast IFA were sensitive indicators of cytomegalovirus myocarditis. However, IFA were also found in some patients with congestive cardiomyopathy (Maisch, Berg & Kochsiek, 1980b). Although they were of low titre in CMV myocarditis, it could be excluded that this immunofluorescent staining was due to a structural alteration of myocardial tissue (Duheille & Petitier, 1967), since it was also observed using morphologically intact air-dried myocytes. IFA could not be readily attributed to complement fixing anti-mitochondrial antibodies, since in CFT with purified mitochondrial antigens complement fixation was not demonstrated.

Antibodies were not found in one third of patients with myocarditis of unknown aetiology. In these cases a different aetiology of the patients' symptoms has to be considered, e.g. perimyocarditis in collagen diseases, in which anti-myocardial antibodies are not observed regularly, or mitral valve prolapse.

Our data favour the idea that AMLA induce cytolysis of vital cardiocytes, since cardiocytolysis (AMC) was almost exclusively present in AMLA-positive sera and correlated with the AMLA titres. Although AMLA titres correlated with the cytolytic serum activity the relationship is not linear. Reasons for non-linearity may be a heterogeneity of AMLA with different affinities for the antigenic sites of the myolemma or the attribution of the cytolytic activity to a certain IgG-subclass (e.g. IgG1) as postulated for complement activation in viral infections (Oldstone & Lampert, 1979). Differences may also occur by an additional activation of the alternate pathway. This could result in a more effective cytolysis without an increase in AMLA titre. An additive effect of cytotoxic serum factors (Bengmark, Frisen & Helander, 1963) which do not belong to the immunoglobulins may be another possibility. Moreover, AMC was effective only in the presence of antibody and complement.

The specificity of binding of AMLA to the myolemma could be examined by using cardiocytes treated with collagenase. Antigenic sites are better preserved in cardiocytes isolated by using collagenase rather than trypsin, since they are diminished substantially after trypsinization. The use of adult cardiocytes instead of fetal myocardial cells might explain why others using fetal cells with incomplete surface morphology could not find a cytolytic serum activity of AHA-positive sera (Friedman & Laufer, 1976; Ghose & Mammen, 1977; Thompson & Halbert, 1971).

Two views have been expressed about the pathogenesis of viral myocarditis: one that the heart is damaged by a cytopathic effect of the virus (Burch & Giles, 1972; Longson, Cole & Davies, 1969), the other that myocardial damage may also be the result of an immunological process (Laufer, 1975).

The observation that the AMLA fluorescence and the cytolytic serum activity could be absorbed with the respective viral antigens supports the hypothesis that cross-reacting antibodies directed against the myolemmal membrane and the virus are operative in coxsackie B, influenza A/B and mumps myocarditis.

Our studies suggest that antibody-mediated cytolysis *in vitro* may represent a model of an immune process that could be operative *in vivo* after the cytopathic assault of cardiotropic viruses has occurred. Similarly cross-reacting antibodies were demonstrated in rheumatic carditis (Kaplan & Meyeserian, 1960) and account for different manifestations of rheumatic fever.

Originally virus immunity was equated with antibody production (Rager-Zisman & Allison, 1973). Recently evidence for cell-mediated immune reactions in acute viral infections and murine coxsackie B_3 myocarditis was reported (Wong, Woodruff & Woodruff, 1977; Paque *et al.*, 1978). Experiments with virus infected or transformed target cells showed that specific lysis of target cells by T cells is related not only to the virus antigen or a neoantigen on the cell surface but also to the histocompatibility antigens (Biddison, Doherty & Webster, 1977). In murine, Coxsackie B_3 myocarditis it was demonstrated (Paque *et al.*, 1978; Woodruff & Woodruff, 1974; Wong *et al.*, 1977) that cytolytic T lymphocytes can be raised, which damage homologous infected and non-infected myocardial cells. In man, several reports have failed to find a requirement for histocompatibility between effector and target cells in the case of measles (Ault & Weiner, 1979; Ewan & Lachmann, 1977); mumps (Härfast, Andersson & Perlmann, 1978), CMV (Thong *et al.*, 1976), influenza (Greenberg, Criswell & Couch, 1975) and HSV (Steele *et al.*, 1975) infections. In some cases, as in our experiments, cytotoxicity still occurred using target cells of other species. Because of the nature of our cellular cytotoxicity test our studies are restricted to spontaneous or natural cytotoxicity in myocarditis in men. Our short-term assay does not involve pre-incubation or treatment of lymphocytes with lectins. This also indicates that alloreactive lymphocytes (Wilson, Blyth & Powell, 1968) are not of major importance in the cytotoxic cellular response, since it is unlikely that patients were previously exposed to epitopes of rat myocytes. Our data are compatible with the observation of Yang *et al.* (1972), who found allogeneic cytotoxicity only after 2–3 days of lymphocyte target contact in culture.

Lymphocytotoxicity was not demonstrated in patients with myocarditis of defined viral origin. This contrasts with the finding that in 11 patients with undefined myocarditis LC was present. The cytolytic activity of peripheral lymphocytes could therefore either be due to non-specific activated lymphocytes with efficient killing capacities (activated lymphocyte killing according to Masucci, Klein & Argor, 1980) or to target specific killing mechanisms as in murine coxsackie B_3 myocarditis (Wong *et al.*, 1977). The cardiocyte-specific cytolytic attack appears to rule out the possibility that the cardiocytotoxic activity is due to alloreactive lymphocytes. However, the cardiospecificity does not necessarily exclude that this cardiocytotoxic lymphocyte activity is due to natural killer cell activity since recently not only target specificity of killing has been made likely by the work of Warner (1980) but also different lysability of targets is obvious in natural killing (Singh, Sabbadini & Sehon, 1973; Welsh & Hallenbeck, 1980). In four patients, however, cytotoxic reactions did not occur in the presence of antibody containing autologous serum whereas cytotoxic reactions of lymphocytes without serum were strong. This may indicate the blocking of natural killing by serum factors, which may be attributed to AMLA or to circulating immune complexes. The latter possibility is known from the inhibition of cell-mediated cytolysis of tumour cells (Ewan & Lachmann, 1977).

Whether the lymphocyte reactivity results in myocardial damage—although an intriguing possibility—has to be established. This should be particularly rewarding in respect to prognosis, because letal or complicated courses of myocarditis were seen only when lymphocytotoxicity against heterologous cardiocytes occurred.

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