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Research Article

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Expression of Intercellular Adhesion Molecule-1 in Murine Hearts with Acute Myocarditis Caused by Coxsackievirus B3

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Abstract

A cell-mediated autoimmune mechanism has been strongly implicated in the pathogenesis of viral myocarditis. Using a murine model of myocarditis caused by coxsackievirus B3 (CVB3), we previously reported that the heart is infiltrated first by natural killer cells, which express a cytolytic factor, perforin, and then by activated T cells. This action may play an important role in the pathogenesis of the observed myocardial cell damage. Cell–cell contact and adhesion is required in immune responses, and intercellular adhesion molecule-1 (ICAM-1), which is a ligand for lymphocyte function–associated antigen-1 (LFA-1), plays an important role in this process. To investigate the essential role of the ICAM-1/LFA-1 pathway in the cell-mediated cytotoxicity involved in viral myocarditis, we examined by immunofluorescence the expression of ICAM-1 in murine hearts with acute myocarditis caused by CVB3. We also evaluated the induction of ICAM-1 in cultured cardiac myocytes treated with cytokines by immunofluorescence and Northern blot hybridization. Furthermore, we analyzed the effects of in vivo administration of anti-ICAM-1 mAbs on the inflammation associated with acute viral myocarditis. We found that CVB3-induced murine acute myocarditis resulted in enhanced expression of ICAM-1 in myocardial cells. The expression of ICAM-1 in myocardial cells could be induced in vitro by IFN-γ and TNF-α, which were shown to be synthesized by the infiltrating cells. In vivo treatment with $F(ab')_2$ fragments of an anti–ICAM-1 mAb significantly reduced the myocardial inflammation induced by CVB3. These data strongly suggest that the expression of ICAM-1 in myocardial cells plays a critical role in the cell-mediated cytotoxicity involved in acute viral myocarditis. (*J. Clin. Invest.* 1993. 91:1327–1336.) Key words: cell-mediated cytotoxicity • lymphocyte function–associated antigen-1 • natural killer cells • IFN-γ • TNF-α

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Introduction

Patients with viral myocarditis may develop acute heart failure, and some display dilated cardiomyopathy in the later course of the disease. This strongly suggests that the persistent myocardial cell damage involved in viral myocarditis may cause continuous destruction of contractile proteins and facilitate fibrosis, which may finally lead to dilated cardiomyopathy. Until now, many studies using murine models of viral myocarditis have shown that cell-mediated autoimmunity plays an important role in the pathogenesis of the observed myocardial cell damage. Studies (1–3) of nude mice or T cell–depleted mice indicated that T cells are one of the major effector cell types. We previously reported that MHC class I antigen was strongly induced on cardiac myocytes in murine acute myocarditis caused by coxsackievirus B3 (CVB3)[1] (4). Recently, we demonstrated in the same model of acute myocarditis that natural killer (NK) cells, which express a cytolytic factor, perforin (pore-forming protein), infiltrate the heart. Infiltration by T helper cells, then cytotoxic T lymphocytes (CTLs) subsequently occurs, suggesting that the expression of MHC class I antigen on cardiac myocytes facilitates the interaction between cardiac myocytes and CTLs, and that it leads to further myocardial cell damage (5). Both NK cells and CTLs, which were shown to express perforin, are known to be killer lymphocytes and to play a critical role in cell-mediated cytotoxicity in vivo (5–8).

The cell–cell interactions of immune responses are thought to be mediated by cell-adhesion molecules expressed on both the immune cells and target cells. Intercellular adhesion molecule-1 (ICAM-1), which is a ligand for lymphocyte function–associated antigen-1 (LFA-1), is thought to be induced on various target cells at the site of inflammation by cytokines and to play an important role in the recognition, adhesion, and cytotoxicity of killer lymphocytes (9–13).

The purpose of this study was to investigate in more detail the immunological mechanisms, and especially, to elucidate the essential role of the ICAM-1/LFA-1 pathway in the interaction between myocardial cells and killer lymphocytes in acute viral myocarditis. We first examined the expression of ICAM-1 in cardiac tissue and LFA-1 in the infiltrating cells in murine acute myocarditis caused by CVB3. Second, to confirm the expression of ICAM-1 in myocardial cells in vitro, we induced the expression of ICAM-1 in cultured murine myocar-

1. *Abbreviations used in this paper:* CTL, cytotoxic T lymphocytes; CVB3, coxsackievirus B3; ICAM-1, intercellular adhesion molecule-1; LFA-1, lymphocyte function–associated antigen-1; NK, natural killer (cells); VCAM-1, vascular cell adhesion molecule-1.

dial cells with IFN-γ and/or TNF-α, and evaluated the expression of ICAM-1 in these cells by immunofluorescence and Northern blot hybridization. Third, to examine cytokine synthesis in the infiltrating cells in the hearts, we analyzed the expression of IFN-γ and TNF-α mRNAs by in situ hybridization. Fourth, to estimate the contribution of the ICAM-1/LFA-1 pathway to the cell-mediated cytotoxicity involved in acute viral myocarditis, we analyzed the effects of in vivo treatment with anti-ICAM-1 mAbs on the inflammation associated with acute viral myocarditis.

We found that CVB3-induced murine acute myocarditis results in the enhanced expression of ICAM-1 on the surface of myocardial cells. This enhanced expression was shown to be induced mainly by cytokines synthesized by the infiltrating cells. Furthermore, in vivo treatment with $F(ab')_2$ fragments of an anti-ICAM-1 mAb significantly reduced myocardial inflammation, indicating that the expression of ICAM-1 plays a critical role in the cell-mediated cytotoxicity involved in acute viral myocarditis.

Methods

Animals

C3H/He mice, 5–7-wk-old male and pregnant female mice, were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan).

Virus

CVB3 (Nancy strain), a gift from Dr. Y. Kitaura (Osaka Medical College, Osaka, Japan), was grown in FL (human amnion) cells, which were supplied by Japanese Cancer Research Bank Cell Bank (National Hygienic Sciences, Tokyo, Japan). The virus preparation had a titer of 1×10^8 plaque-forming units/ml and was stored at −70°C. 5–7-wk-old male C3H/He mice were inoculated intraperitoneally with 5×10^6 PFU of CVB3 in 0.2 ml PBS.

mAbs

A rat anti-mouse ICAM-1 mAb (hybridoma YN1/1.7, IgG2b) was a gift from Dr. F. Takei (University of British Columbia, Vancouver, Canada) (14, 15). Another rat anti-mouse ICAM-1 mAb (KAT-1, IgG2a) was recently developed by K. Kato (Juntendo University, Tokyo, Japan) and Y. Hashimoto (Tohoku University, Miyagi, Japan). Sequential immunoprecipitation analysis showed that the antigen recognized by KAT-1 is identical to that recognized by YN1/1.7. Moreover, the inhibitory effect of KAT-1 on the homotypic aggregation of phorbol ester–stimulated mouse lymphoma cells was found to be much stronger than that of YN1/1.7 (data not shown, manuscript submitted for publication). A rat anti-mouse LFA-1 (CD11a) mAb (KBA, IgG2a) was a gift from Dr. T. Nishimura (Tokai University, Kanagawa, Japan) (16). A rat anti-mouse CD18 mAb (M18/2, IgG2a) was purchased from American Type Culture Collection (Rockville, MD) (17). The preparation of a mouse anti-cardiac myosin mAb (CMA19) was previously described (18). The reactivity of CMA19 for C3H/He mouse ventricular myosin heavy chain was confirmed by immunoblot analysis (data not shown).

Preparation of cultured cardiac myocytes

Heart ventricles were aseptically removed from 14–16-d-old fetal mice, minced in calcium-free PBS, and digested with 0.06% trypsin-EDTA in PBS. The isolated cardiac myocytes were washed in DME containing 10% FCS, dispersed into plastic dishes for 1 h to separate the fibroblasts, and removed to culture flasks and tissue culture chamber slides (Miles Inc., Diagnostics Div., Kankakee, IL). They were cultured overnight at 37°C in a humidified 5% CO_2/95% air incubator and were then divided into four groups designated as A, B, C, and D. After replacement with fresh culture media, recombinant murine IFN-γ (300 U/ml) (Shionogi & Co., Ltd., Osaka, Japan) (19) was added to group B, recombinant murine TNF-α (300 U/ml) (Genzyme Corp., Cambridge, MA) was added to group C, and both IFN-γ (300 U/ml) and TNF-α (300 U/ml) were added to group D. After 48 and 72 h under these conditions, the cardiac myocytes were subjected to Northern blot hybridization and immunocytochemical study, respectively.

Immunohistochemistry

Using spleen tissue samples as positive controls, we confirmed that the potential of the mAbs used in this study to recognize the antigens was not altered by acetone fixation (data not shown). Freshly dissected ventricles were frozen in optimal cutting temperature compound (Miles Inc.) in liquid nitrogen. Cryostat sections (6 μm thick) were prepared, air dried, and fixed in acetone for 5 min. They were blocked with 2% rabbit serum in PBS for 30 min at 37°C, then incubated with anti-mouse ICAM-1 (YN1/1.7), anti-mouse LFA-1 (CD11a), and anti-mouse CD18 mAbs for 60 min at 37°C. After washing in PBS, the sections were incubated with biotinylated rabbit anti-rat IgG antibody (Vector Laboratories, Inc., Burlingame, CA), which was preabsorbed with mouse serum for 60 min at 37°C. After washing in PBS, the sections were incubated with FITC-conjugated avidin D (Vector Laboratories, Inc.) for 30 min at 37°C. After washing in PBS, coverslips were mounted with glycerin. The sections were examined and photographed under a fluorescence microscope (MICROPHOT-FX; Nikon, Tokyo, Japan).

Immunocytochemistry

For immunocytochemical analysis, to distinguish cardiac myocytes from nonmuscle cells (mainly fibroblasts), we performed double staining for cardiac myosin heavy chain and ICAM-1. The cultured cells on the slides were washed in PBS and fixed in acetone for 5 min. They were incubated with CMA19 for 60 min at 37°C, washed in PBS, incubated with phycoerythrin-conjugated anti-mouse IgG antibody for 60 min at 37°C and washed again in PBS. The subsequent procedure for the staining of ICAM-1 was the same as that for the tissue samples.

For flow cytometric analysis, we removed the cultured cardiac myocytes from the flasks with trypsin-EDTA and stained them for ICAM-1 using FITC-conjugated anti-rat IgG antibody. Control samples without the first antibody (anti-mouse ICAM-1 mAb) were also analyzed by a flow cytometry (FACScan®; Becton-Dickinson Immunocytometry Systems, Mountain View, CA).

In vivo treatment of mice with Anti-ICAM-1 mAbs

As we previously reported (5), because the dominant population of infiltrating cells in hearts with acute myocarditis consists of NK cells, which are known to have Fc receptors, we used $F(ab')_2$ fragments of anti-mouse ICAM-1 mAbs in this study to avoid antibody-dependent cell-mediated cytotoxicity via Fc receptors. Two anti-mouse ICAM-1 mAbs, clones YN1/1.7 and KAT-1, were cut into $F(ab')_2$ fragments by papain digestion. 5-wk-old mice received each mAb (3 mg/kg i.p.) daily, beginning on the day of virus inoculation (day 0) until killed on day 7. Control mice received rat IgG $F(ab')_2$ fragments (Rockland, Inc., Gilbertsville, PA) in the same way (five mice were used for each group).

The hearts were aseptically removed and laterally sectioned approximately midway between the apex and the atria, which resulted in cross-sections of both ventricles. Half of each heart was fixed in 10% buffered formalin and used for histological study. The other half of each heart was frozen in liquid nitrogen and used for Northern blot analysis.

Preparation of labeled RNA probes

A mouse ICAM-1 cDNA clone (K4-1.1) was a generous gift from Dr. F. Takei (University of British Columbia, Canada) (20). An EcoRI fragment of ~ 2.5 kb from the insert DNA, which encodes the functional transmembrane protein, was subcloned into a pBluescript SK (+) vector (Stratagene Inc., La Jolla, CA). A mouse α-actin cDNA clone (plasmid 91), in which a PstI fragment of ~ 1.3 kb of α-actin

cDNA was subcloned into a pBR 322 vector, has been described in detail (21). A PstI fragment of ~ 1.1 kb from the insert DNA, which represents ~ 90% of the coding sequences for α-actin, was subcloned into a pBluescript SK (+) vector. A CVB3 cDNA clone (pCB3-M1) was a generous gift from Dr. R. Kandolf (Max Planck Institute for Biochemistry, Martinsried, Germany) (22). A BamHI fragment of 1.08 kb of the 3′ end from the insert DNA was subcloned into a pBluescript SK(+) vector. The ICAM-1, α-actin, and CVB3 cDNA clones were linearized by the appropriate enzymes, and antisense RNA probes were synthesized by T3 or T7 RNA polymerases with [^{32}P]UTP, an unlabeled mixture of ATP, GTP, and CTP, DTT, and human placental ribonuclease inhibitor. They were used for Northern blot hybridization.

A mouse IFN-γ cDNA clone was a generous gift from Shionogi & Co., Ltd. (23). A PstI fragment of ~ 0.68 kb from the insert DNA, which represents ~ 60% of the coding sequence for IFN-γ, was subcloned into pBluescript SK (+) vector. A mouse TNF-α cDNA clone (24) was a generous gift from Asahi Chemical Industry Co., Ltd. (Tokyo, Japan). A BamHI/EcoRI fragment including 1.1 kb of the 5′ end of the mouse TNF-α cDNA was subcloned into pBluescript SK (+) vector. After the linearization of these cDNA clones by the appropriate enzymes, ^{35}S-labeled antisense and sense RNA probes were synthesized by T3 and T7 RNA polymerases in the same way. They were used for in situ hybridization.

Northern blot hybridization

In vitro study. Total cytoplasmic RNA was prepared from the cultured cardiac myocytes by a previously described method (25). In each group, 8 μg of cytoplasmic RNA were subjected to formaldehyde-agarose gel electrophoresis and transferred to a nylon membrane. After drying in a vacuum for 1 h at 80°C, the nylon membrane was prehybridized in a solution containing salmon sperm DNA (200 μg/ml) for 2 h at 65°C, then hybridized with the ^{32}P-labeled mouse ICAM-1 antisense RNA probe overnight at 65°C. After washing, the nylon membrane was autoradiographed at −80°C. To confirm that equivalent amounts of RNA from each group were loaded onto the gel, hybridization with the α-actin probe was also performed in the same way.

In vivo mAb treatment study. Total cytoplasmic RNA was prepared from heart tissue from the control and KAT-1-treated groups by a method using RNA zol (Cinna/Biotecx Laboratories International, Inc., Friendswood, TX) according to the manufacturer's instructions. In both groups, 30 μg of cytoplasmic RNA from the heart was used for Northern blot hybridization with the ^{32}P-labeled CVB3 and α-actin antisense RNA probes. The procedures were the same as those followed in the in vitro study.

In situ hybridization

Cryostat sections (6 μm thick) of the ventricles of mice killed on day 7 after infection were prepared on slides, which were pretreated in 3× SSC (1× SSC; 0.15M sodium chloride, and 0.015M sodium citrate)–Denhart's solution containing 0.02% Ficoll-400, 0.02% polyvinylpyrrolidone-360, and 0.02% BSA, air dried, and fixed in 4% paraformaldehyde in PBS for 15 min at room temperature. After washing in PBS, the sections were dehydrated in ethanol and stored at −20°C until use. The sections were washed three times in 2× SSC for 15 min, incubated in 0.1M glycine, and 0.1M Tris-HCl (pH 7.0) for 30 min at room temperature, and washed in 2× SSC for 15 min. Prehybridization was carried out overnight at 50°C in a solution containing 50% deionized formamide, 2× SSC, 0.05M 2-mercaptoethanol, 1 mg/ml of tRNA, 2 mg/ml of methylated BSA, and 1 mg/ml of denatured salmon sperm DNA. The serial sections were hybridized overnight with ^{35}S-labeled antisense or sense RNA probe (of mouse IFN-γ and TNF-α) in the same solution at 50°C, then washed six times in 50% formamide and 2× SSC for 3 h at 50°C and twice in 2× SSC for 1 h at room temperature. They were then dipped into nuclear track emulsion (NTB-2; Eastman Kodak, Rochester, NY) that was diluted 1:2 with 6 M ammonium acetate. After exposure for 4 d at 4°C, the sections were developed and fixed (GBK developer and fixer; Eastman Kodak), counterstained with hematoxilin, dehydrated in ethanol, and mounted on coverslips in xylene with resin.

Histology

The cross-sections of formalin-fixed heart tissue from mice treated with anti–ICAM-1 mAbs were stained with hematoxylin and eosin and projected onto paper, where the total area of the myocardium and the areas of inflammation (consisting of cell infiltration and necrosis) were outlined. The percent area of the myocardium undergoing inflammation was determined with an image analysis system (Cardio 80; Kontron Elektronik, Eching, Germany).

Statistical analysis

One-way ANOVA (using *P* corrected by Bonferroni for multiple comparison) was used to evaluate differences between the groups.

Results

In vivo studies

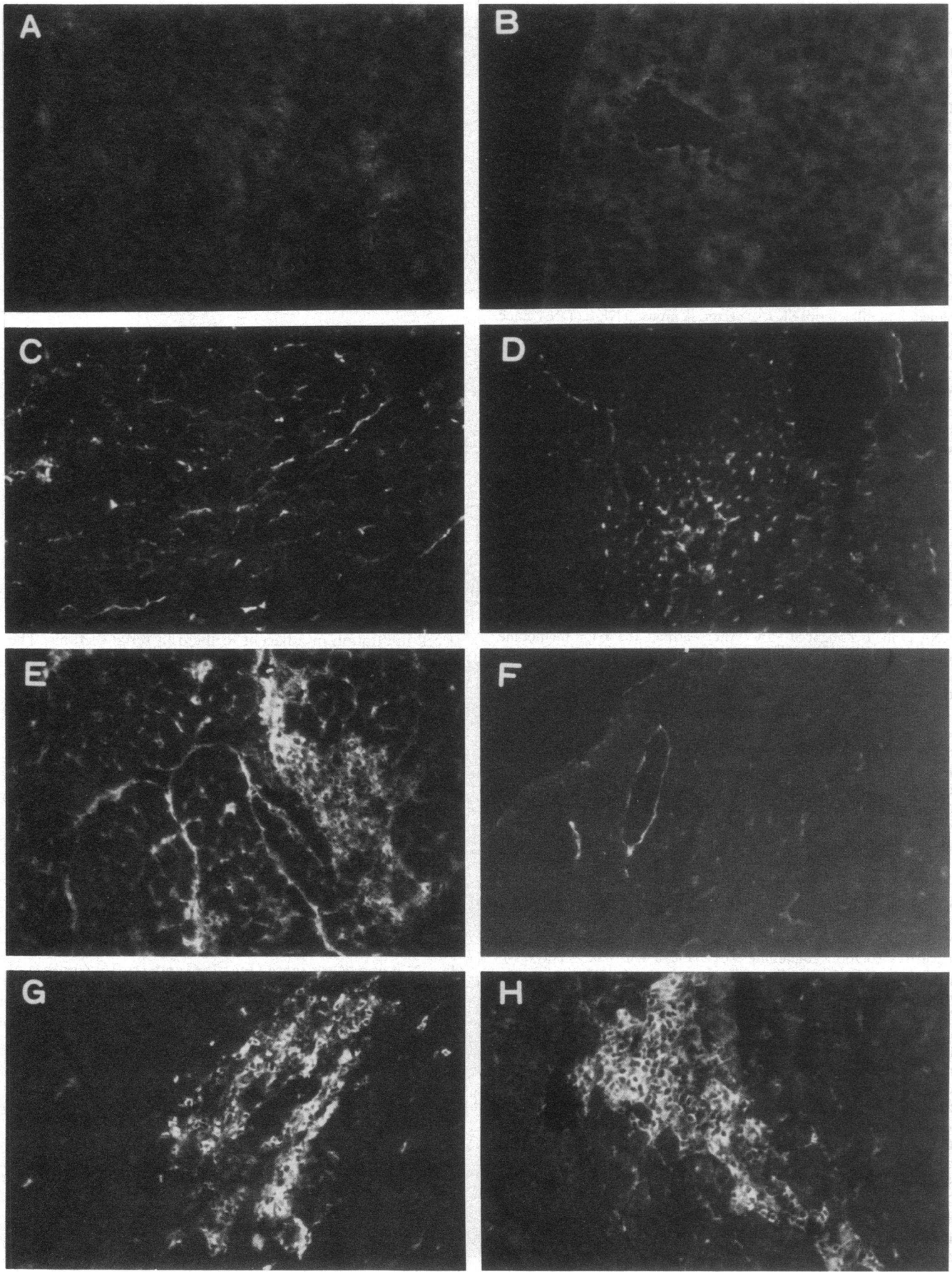
Expression of ICAM-1 in ventricular tissue and LFA-1 in the infiltrating cells. ICAM-1 expression was low or undetectable in normal ventricular tissue, including sarcolemma, fibroblasts, and capillary endothelial cells (Fig. 1, *A* and *B*). On day 5 after virus inoculation, just after massive cell infiltrations appeared, expression of ICAM-1 was clearly induced on the sarcolemma of cardiac myocytes, fibroblasts, endomyocardial cells, and capillary endothelial cells (Fig. 1, *C–F*, respectively). ICAM-1 expression, which reached a maximum level on about day 7 and continued for > 4 wk after virus inoculation, was seen nonuniformly over the myocardium and around the areas of cell infiltration or adjacent to them in serial sections. The time course of the expression of ICAM-1 in ventricular tissue is summarized in Table I.

Clear expression of ICAM-1, LFA-1 (CD11a), and CD18 was seen on most of the infiltrating cells (Fig. 1, *E–H*, respectively).

Detection of IFN-γ and TNF-α mRNAs in the infiltrating cells by in situ hybridization. Since both ICAM-1 and MHC antigen (4) were strongly induced on myocardial cells just after massive cell infiltrations appeared, we considered that the release of cytokines from the infiltrating cells is one of the mechanisms involved in this expression. To confirm the synthesis of cytokines in the infiltrating cells, we analyzed the expression of IFN-γ and TNF-α mRNAs in the infiltrating cells on day 7 after virus inoculation by in situ hybridization. Fig. 2 shows the results of in situ hybridization of serial sections with antisense (Fig. 2, *A* and *B*) or sense (Fig. 2, *C* and *D*) RNA probes of IFN-γ (Fig. 2, *A* and *C*) and TNF-α (Fig. 2, *B* and *D*). Strong signals of both IFN-γ and TNF-α gene transcripts were found in many of the infiltrating cells (Fig. 2, *A* and *B*), indicating the synthesis of these cytokines. Hybridization with sense RNA probes as negative controls revealed no significant levels of signals (Fig. 2, *C* and *D*), showing that the nonspecific background was low.

In vitro studies

Expression of ICAM-1 in cultured ventricular myocytes. Fig. 3 shows double-stained ventricular myocytes cultured in a medium with or without cytokines for 72 h. Fig. 3, *A* and *B* shows the staining pattern specific for ICAM-1. Fig. 3, *C* and *D*, which corresponds to Fig. 3, *A* and *B*, respectively, show the staining


A
B
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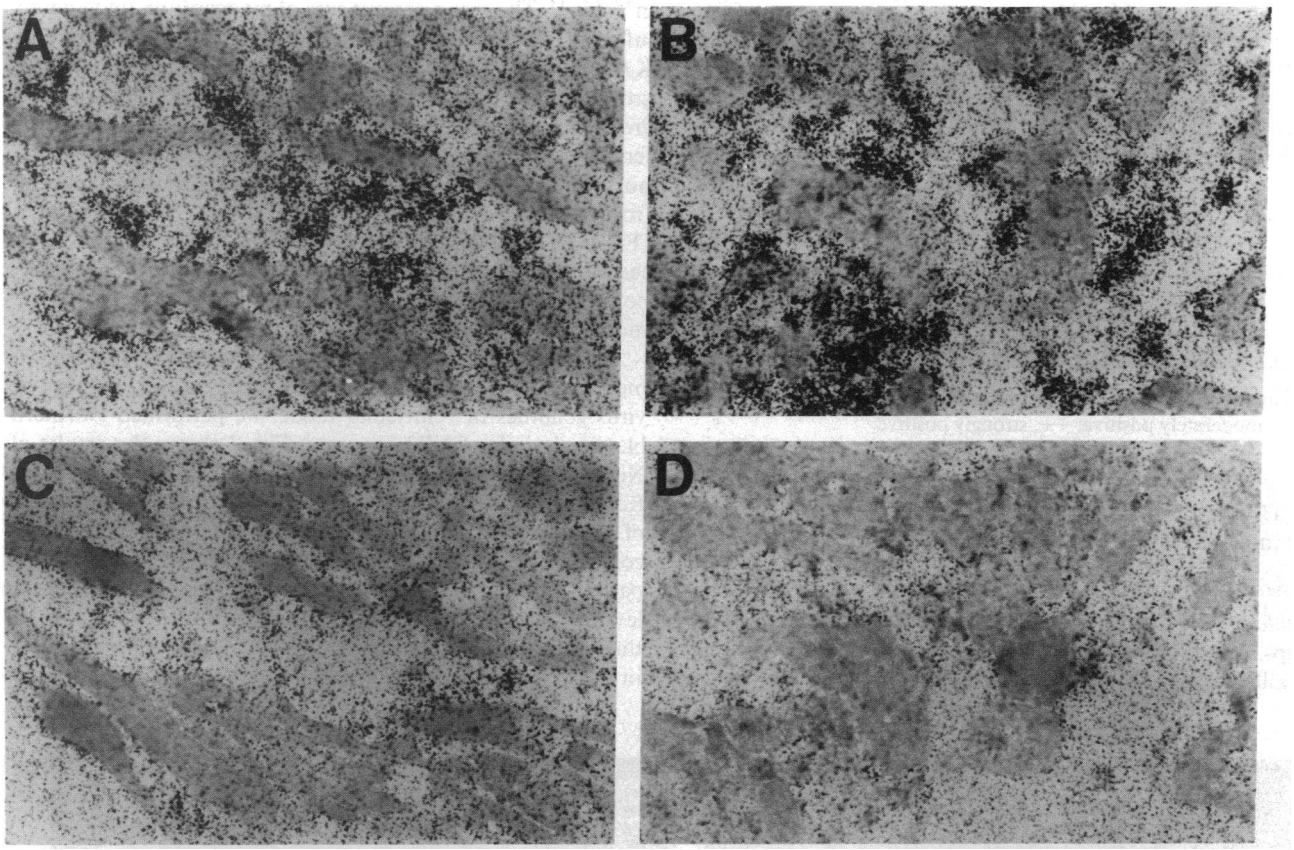

Figure 2. Detection of IFN-γ and TNF-α mRNA in the infiltrating cells by in situ hybridization. Serial sections of the hearts of CVB3-infected mice (day 7) were hybridized with ^{35}S-labeled antisense (*A* and *B*) or sense (*C* and *D*) RNA probes for IFN-γ (*A* and *C*) and TNF-α (*B* and *D*). (×400) Most of the infiltrating cells expressed both IFN-γ and TNF-α mRNA (*A* and *B*).

pattern specific for cardiac myosin heavy chain, and indicate that most of the cells (except a few) are cardiac myocytes. There was very slight or no expression of ICAM-1 in the ventricular myocytes (except a few) of the control group (Fig. 3 *A*). After treatment with IFN-γ plus TNF-α, many ventricular myocytes moderately to strongly expressed ICAM-1 on their surfaces (Fig. 3 *B*). Most of the nonmuscle cells, which mainly consisted of fibroblasts, also expressed ICAM-1 moderately to strongly on their surfaces (Fig. 3 *B, arrows*).

Flow cytometric analysis of the cultured ventricular myocytes after treatment with cytokines for 72 h is shown in Fig. 4. The ventricular myocytes of the control group stained for ICAM-1 did not show significant fluorescence above background levels. In contrast, a marked shift to the right was seen in the fluorescence profile of both the IFN-γ–and IFN-γ–plus TNF-α–treated groups. Only a slight shift to the right was seen in the fluorescence profile of the TNF-α-treated group. The effects of IFN-γ and TNF-α in order of potency was IFN-γ plus TNF-α > IFN-γ > TNF-α.

Modulation of the expression of ICAM-1 mRNA in cultured ventricular myocytes. Immunocytochemical study showed that the ventricular myocytes could express ICAM-1 at levels that were significantly increased by treatment with cytokines. To confirm the expression at the transcriptional level, we performed Northern blot analysis using a ^{32}P-labeled ICAM-1 antisense RNA probe.

Fig. 5 shows the results of Northern blot analysis of the cultured ventricular myocytes after treatment with cytokines for 48 h. Fig. 5 *B* shows the levels of α-actin mRNA for each group as an internal standard. This confirmed that equivalent amounts of RNA were prepared from each group. Fig. 5 *A* shows the levels of ICAM-1 mRNA. Very low levels of ICAM-1 mRNA were found in the control group, while significant or marked levels were induced by treatment with IFN-γ or IFN-γ

Figure 1. Immunohistochemical study for ICAM-1 and LFA-1. (*A* and *B*) Normal ventricular myocardium stained with anti–ICAM-1 mAb (×170). Only slight or undetectable levels of expression was observed on sarcolemma of cardiac myocytes, fibroblasts (*A*), and vascular endothelial cells (*B*). (*C–F*) Ventricular myocardium infected with CVB3 (day 7) stained with anti–ICAM-1 mAb (×170). Note the clear reactions of sarcolemma of cardiac myocytes (*C*), fibroblasts (*D*), endomyocardial cells and infiltrating cells (*F*). (*G* and *H*) Ventricular myocardium infected with CVB3 (day 7) stained with anti–LFA-1 (CD11a) mAb (*G*), and anti–CD18 mAb (*H*) (×170). Note clear reactions of the infiltrating cells for both mAbs.

*Table I. Expression of ICAM-1 in Ventricular Tissues Infected with CVB3**

Days after infection	Myocytes	Vascular endothelial cells	Fibroblasts	Endo-myocardial cells
0–4	−	±	− and ±	±
5	+	+	+	+
7	+	+	+ and ++	+ and ++
11–15	+	+	+	+
21	±	+ and ±	±	+
28	±	±	±	± and +

* The intensity of staining was shown as −, negative; ±, weakly positive; +, moderately positive; ++, strongly positive.

plus TNF-α, respectively. Only low levels of ICAM-1 mRNA were induced by treatment with TNF-α.

In vivo mAb treatment study

Histology. The incidence of myocarditis was 100% in all of the groups. One mouse in YN1/1.7-treated group died before getting killed. The results of the histological study are summarized in Table II. The mean percent area of myocardium undergoing inflammation was decreased in both the YN1/1.7-treated and KAT-1-treated groups as compared with the rat IgG–treated group. The difference between KAT-1–and Rat IgG–treated groups was statistically significant ($P < 0.01$), whereas the difference between the YN1/1.7–and rat IgG–treated groups was not significant. Fig. 6 shows the sections of the heart of a mouse from the rat IgG–treated (Fig. 6 *A*) and KAT-1–treated group (Fig. 6 *B*). Extensive cell infiltration and necrosis were seen in the mouse from the rat IgG–treated group (Fig. 6 *A*). However, both cell infiltration and necrosis were much less severe in the mouse from the KAT-1–treated group (Fig. 6 *B*).

Detection of CVB3 genomes in ventricular tissue. To examine the effects of in vivo mAb treatment on the existence of virus genomes in ventricular tissue, we performed Northern blot analysis for CVB3 genomes. Fig. 7 shows the results of Northern blot analysis of the ventricular tissue of mice in the Rat IgG–and KAT-1–treated groups. Fig. 7 *B* shows the levels of α-actin mRNA for each mouse as an internal standard. This confirmed that almost equivalent amounts of RNA were prepared from each mouse. Fig. 7 *A* shows the CVB3 genome levels. Low to moderate levels of CVB3 genomes were found in the rat IgG–treated group, whereas only low levels were found in the KAT-1-treated group. Since the sample size was rather

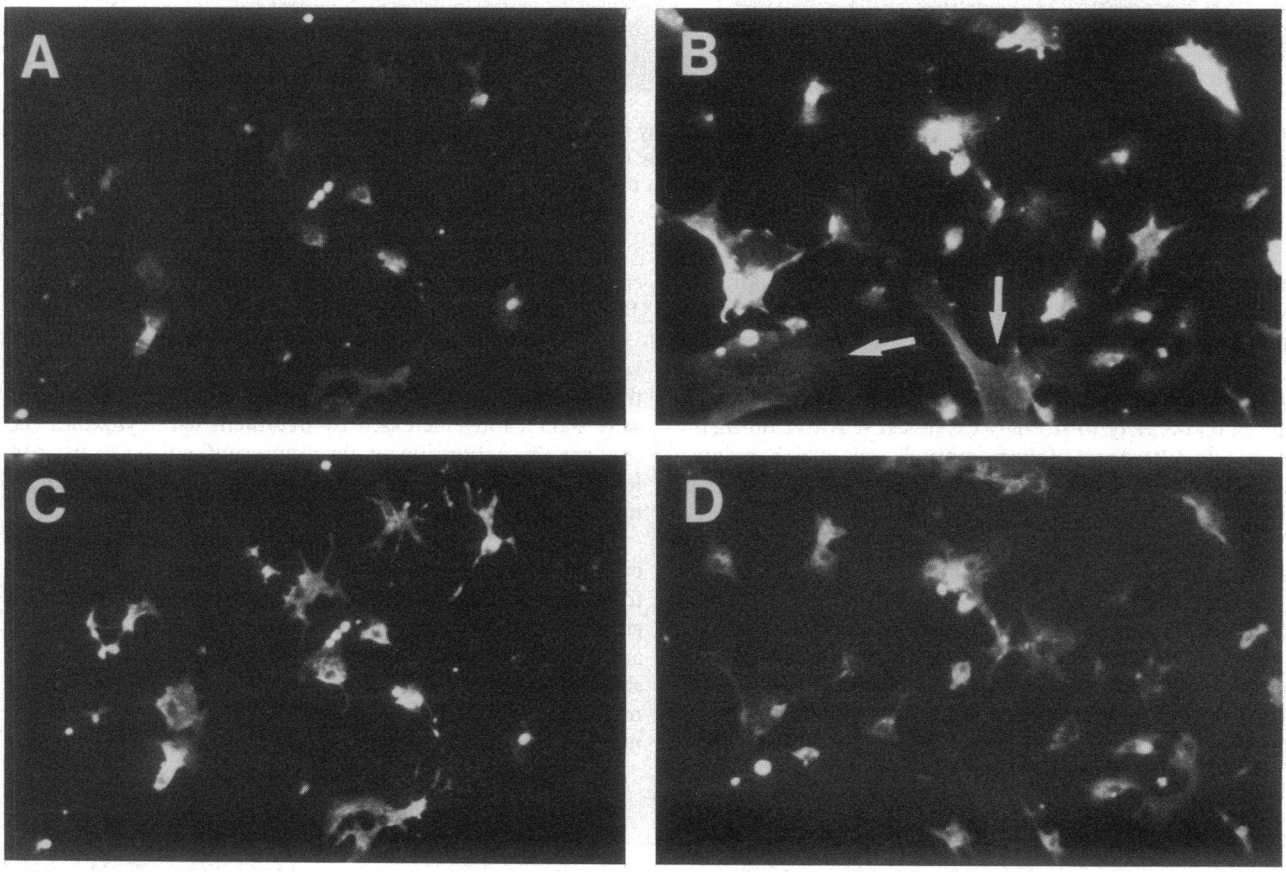

Figure 3. Immunocytochemical study of cultured ventricular myocytes for ICAM-1. Myocytes in control group (*A*) and cytokine-treated group (*B*), stained with anti–ICAM-1 mAb and labeled with FITC (×200). (*C* and *D*) Myocytes stained with an antibody for cardiac myosin heavy chain and labeled with phycoerythrin, corresponding to *A* and *B*, respectively (×200). Most of the cells reacted in both groups, indicating that they were cardiac myocytes. Note strong reactions in cardiac myocytes in cytokine-treated group (*B*). Slight or no reactions were seen in control group (*A*). Moderate reactions were also observed in nonmuscle cells (*B, arrows*).

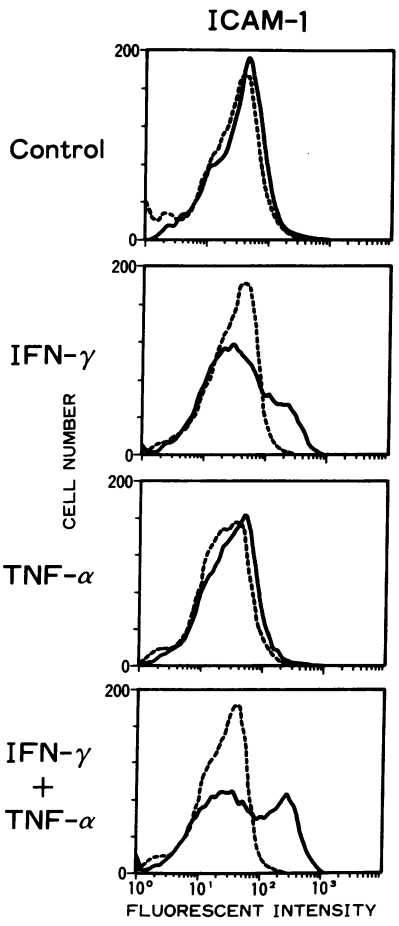

Figure 4. Induction of ICAM-1 expression on cardiac myocytes. Cardiac myocytes were cultured with no addition (*Control*), IFN-γ and/or TNF-α (each 300 U/ml) for 72 h. Dispersed cardiac myocytes were stained with anti-ICAM-1 mAb (*solid lines*) or without the first antibody (*dotted lines*).

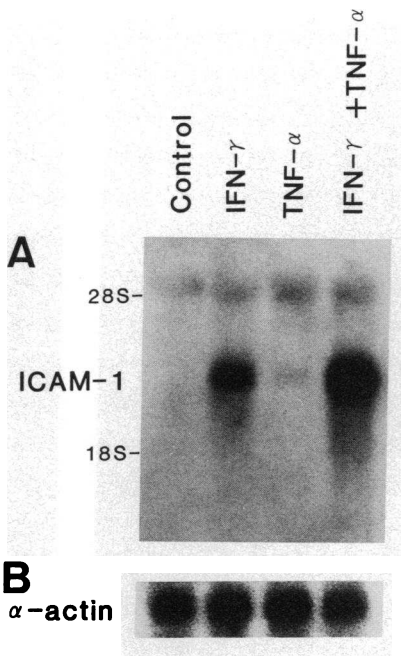

Figure 5. Northern blot analysis of cultured ventricular myocytes for ICAM-1. Cytoplasmic RNA (each 8 μg) from ventricular myocytes cultured with no addition (*Control*), IFN-γ and/or TNF-α (each 300 U/ml) for 48 h was hybridized with ^{32}P-labeled antisense RNA probes for ICAM-1 (*A*) and α-actin (*B*).

small and the expression of CVB3 genomes is variable, we can not conclude that KAT-1 treatment actually reduces viral titers.

Discussion

In this study, we have clearly demonstrated that in murine acute myocarditis caused by CVB3, ICAM-1 is strongly induced in myocardial cells. This finding suggests that the expression of ICAM-1 plays a critical role in myocardial cell damage mainly caused by cell-mediated autoimmune responses.

With regard to the therapy of acute viral myocarditis, several animal studies (2, 26) have reported that immunosuppressive agents such as corticosteroids or cyclosporine caused a significant increase in myocardial necrosis and mortality by enhancing virus titers in target organs, even though the animals were given the agents beginning on day 8 after virus inoculation. The mechanism by which cyclosporine interferes with normal viral clearance is unknown. In the present study, in vivo treatment with KAT-1 F(ab')$_2$ significantly reduced cell infiltration and myocardial necrosis without enhancing viral replication, indicating an improvement of myocarditis. Our results offer a useful immune therapy that can be initiated even at the time of virus infection to effectively improve the course of acute viral myocarditis.

Lymphocyte-mediated cytotoxicity as well as the proliferation and differentiation of lymphocytes are known to require intercellular adhesion. ICAM-1 is a glycoprotein and a member of the immunoglobulin superfamily, and is known to be expressed weakly on vascular endothelial cells, fibroblasts, and hematopoietic cells. Many studies have shown that ICAM-1 expression is strongly induced on various cells by cytokines in vitro (11, 12, 27, 28) and also on activated lymphocytes and nonhematopoietic cells at the site of inflammation (28–32). ICAM-1 was identified as a ligand for LFA-1, which is a heterodimeric glycoprotein (CD11a/CD18) and a member of the integrin superfamily. LFA-1 is known to be widely expressed on leukocytes, including T and B lymphocytes, NK cells, monocytes, and granulocytes, but not on nonhematopoietic cells (9, 33). From antibody-blocking studies in many cell types, it is evident that both homotypic and heterotypic adhesive interactions are at least in part mediated by the ICAM-1/LFA-1 pathway (14, 15, 34, 35). The results of inhibition by anti-ICAM-1 or anti-LFA-1 mAb of CTL target cell conjugation, as well as lymphocyte–lymphocyte adhesion and activation strongly suggest that ICAM-1/LFA-1 interaction plays a central role in the recruitment, activation, and especially target cell killing of lymphocytes (10, 33). Recently, Altmann et al. (36) demonstrated that L cells transfected with HLA class II acquired immune competence only after supertransfection with ICAM-1. This

Table II. Effect of In vivo Treatment with Anti-ICAM-1 mAb on Myocardial Inflammation

Treatment	Percentage of area of myocardium inflamed
Rat IgG F(ab')$_2$	18.07±2.57 (n = 5)
YN1/1.7 F(ab')$_2$	12.11±3.49 (n = 4)
KAT-1 F(ab')$_2$	4.96±1.03* (n = 5)

Values are mean±SE. *P < 0.01 compared with rat IgG F(ab')$_2$-treated group.

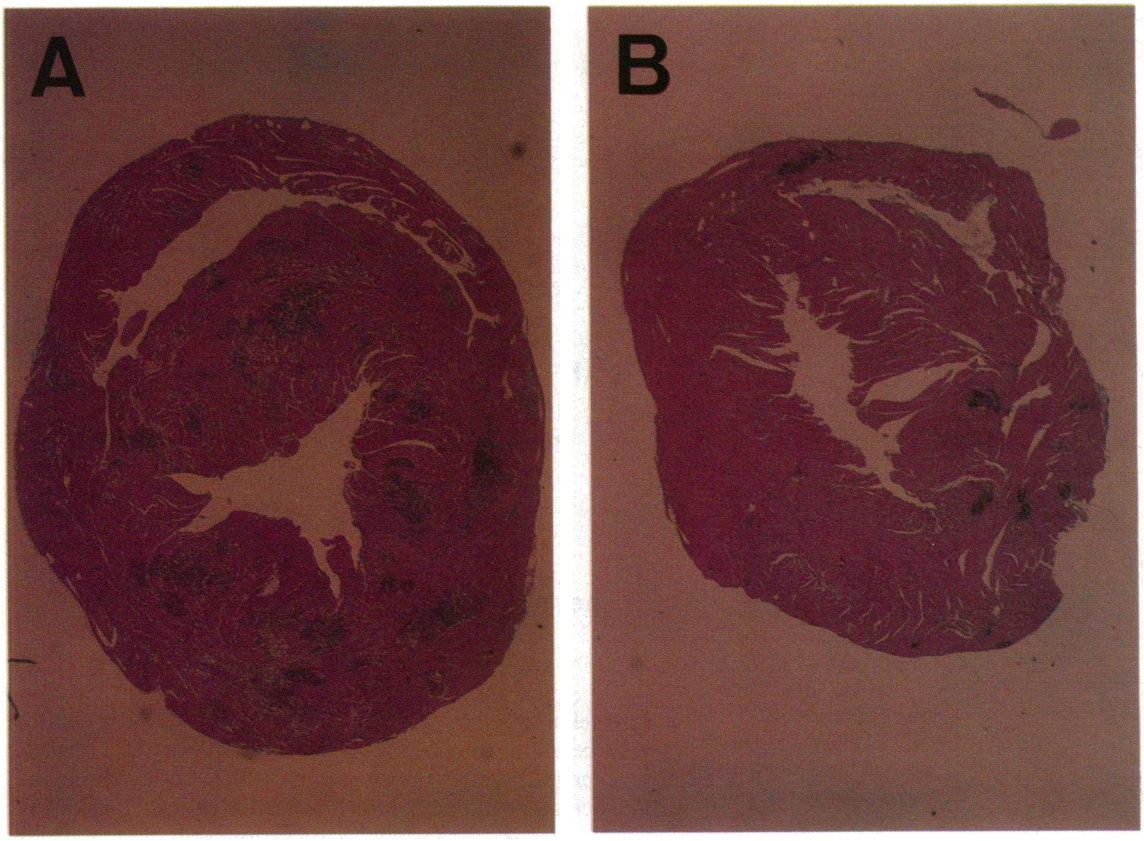

Figure 6. Histological analysis of the effects of in vivo mAb treatment. Cross-sections of the heart of a mouse (day 7) in rat IgG $F(ab')_2$–treated group (*A*) and in anti–ICAM-1 mAb (KAT-1) $F(ab')_2$–treated group (*B*) were stained with hematoxylin and eosin. (×15) Note the extensive cell infiltration and necrosis in the myocardium of rat IgG–treated group (*A*). Both cell infiltration and necrosis were much less in the myocardium of KAT-1-treated group (*B*).

indicates that coinduction of ICAM-1 and MHC antigens establishes an antigen-presenting cell, and suggests that ICAM-1/LFA-1 interaction plays an important role in the antigen-specific T cell recognition. This would be an advantageous mechanism in the situation of an acute viral infection such as viral myocarditis.

Several studies (37, 38) investigating ICAM-1 expression in carcinoma cells or their stromal cells suggested that the enhanced expression of ICAM-1 in these cells may contribute to tumor cell infiltration and metastasis. Adams et al. (39) studied ICAM-1 expression in liver tissue after transplantation. The authors found that there was greater ICAM-1 expression on bile ducts, endothelium, and hepatocytes in patients with acute rejection than in donor livers, patients with stable transplants, or patients with nonrejection complications. The authors also found that ICAM-1 expression was greatly reduced in patients with resolving rejection after high dose corticosteroid treatment. These data strongly suggest that the induction of ICAM-1 in tissue may be an important step in the development of the inflammatory response of rejection and in determining which cells are the targets of immune damage.

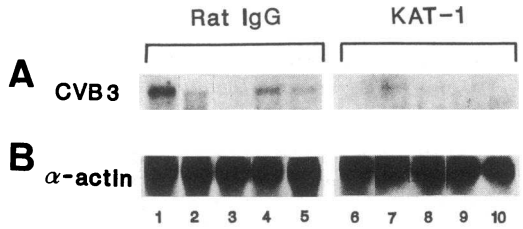

Figure 7. Northern blot analysis of the in vivo mAb-treated ventricular tissues for CVB3 genomes. Cytoplasmic RNA (each 30 μg) from ventricular tissues of mice in Rat IgG-treated group and KAT-1-treated group was hybridized with ^{32}P-labeled antisense RNA probes for CVB3 genome (*A*) and α-actin (*B*).

To our knowledge, no studies have examined the expression of ICAM-1 in cardiac tissue, especially cardiac myocytes. In addition, no previous reports have demonstrated the expression of ICAM-1 in acute viral myocarditis. The results of our study strongly suggest that the expression of ICAM-1 on myocardial cells, especially cardiac myocytes, along with the expression of MHC antigens, as we previously reported (4), establishes these cells as target cells for NK cells and T cells and may facilitate the recognition, adhesion, and cytotoxicity of these lymphocytes. As shown in the present study, cytokines such as IFN-γ and TNF-α, which are synthesized and released by the infiltrating cells, may play a major role in inducing the expression of ICAM-1 and MHC antigens in myocardial cells around the cell infiltrations. In this model of acute myocarditis, the expression of both ICAM-1 and MHC antigens on myocardial cells continued for about 4 wk after virus inoculation. We

also found by Northern blot analysis that CVB3 genomes had almost disappeared from the heart tissue on day 10 after virus inoculation (unpublished data). T cells, which mainly infiltrate the heart in a later phase when virus genomes have almost disappeared, are thought to recognize virus-derived antigens expressed with MHC antigens on myocardial cells. Therefore, the persistent expression of ICAM-1 and MHC antigens on myocardial cells enables them to be antigen-presenting cells for T cells and may cause further myocardial cell damage in a later phase of myocarditis.

In vitro study showed that the expression of ICAM-1 in myocardial cells was markedly enhanced by treatment with IFN-γ, but only slightly enhanced with TNF-α, and that IFN-γ and TNF-α synergistically enhanced ICAM-1 expression. Vascular cell adhesion molecule-1 (VCAM-1) is a member of the immunoglobulin superfamily and has been identified as another cell adhesion molecule for the interaction between lymphocytes and target cells, especially vascular endothelial cells. VCAM-1 was shown to be induced on endothelial cells treated with IL-1 or TNF-α but not with IFN-γ, and is thought to play a central role in the recruitment of lymphocytes into inflammatory sites in vivo (40, 41). Our in vivo mAb treatment study showed that KAT-1 F(ab')$_2$ significantly reduced cell infiltration and necrosis; however, there still some inflammation remained. This indicates that another pathway was involved in the mechanism of cell infiltration in acute viral myocarditis. VCAM-1 is one of the likeliest candidates because it is strongly induced by treatment with TNF-α, which was shown to be synthesized by the infiltrating cells. We also speculate that the release of some kind of chemotactic factors from virus-infected cells is the primary mechanism of cell infiltration into the heart.

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