**Immunohistochemical Study of the Myocardium in Murine Coxsackie B3
Virus Myocarditis Using Monoclonal Antibodies
—Significance of Lyt 1 Antigen-bearing Lymphocytes in Cell-mediated Immunity—**

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This light- and electron-microscopic study using monoclonal antibodies provides an immunohistochemical demonstration of lymphocytes *in situ* in the myocardium in murine coxsackie B3 virus myocarditis. On the 7th and 9th days after virus inoculation, we observed many necrotic cardiocyte foci infiltrated with numerous inflammatory cells including macrophages and T lymphocytes. There were only a few Lyt 1-bearing lymphocytes in this phase of inoculation. On the 14th day there were many Lyt 1-bearing cells among the T cells in the foci. By immuno-electron microscopy, some Lyt 1-bearing T lymphocytes were seen in close contact with macrophages and/or other lymphocytes. These Lyt 1-bearing lymphocytes appeared to represent helper T cells, although Lyt 2,3+ lymphocytes were not examined. It is well known that activation of helper T cells leads to stimulation of B cells and cytotoxic T cells. It is assumed, therefore, that Lyt 1-bearing T lymphocytes that infiltrated into the myocardial foci play an important role in immune responsiveness at this stage of viral myocarditis.

SEVERAL investigators postulate that cell-mediated immunity is involved in the pathogenesis of myocarditis¹⁻⁷. Most of these studies, however, have been carried out on peripheral blood or lymphocytes isolated from the spleen⁴⁻⁷. In our previous immuno-histochemical study on murine coxsackie B3 virus myocarditis^{9,10}, T lymphocytes and macrophages were the predominant elements of interstitial cellular infiltrates in the myocardial necrotic foci. We assume that these cellular elements play an important role in the immune mechanisms of myocarditis⁹⁻¹². In mice three functional T cell

subsets have been defined by monoclonal antibodies against lymphocyte surface antigens; Lyt 1, 2, 3+ precursor T cells, Lyt 1+ helper T cells and Lyt 2, 3+ cytotoxic/suppressor T cells. Identification of these subsets of T cells *in situ* in the myocardium, therefore, may shed light on understanding of the cell-mediated immune mechanisms involved in viral myocarditis.

The present study focused on *in situ* identification of Lyt 1+ bearing T cells and their interactions with macrophages and other cells infiltrated into the myocardial foci. The purpose of this study is to discuss the significance of the Lyt 1+ bearing T lymphocytes in the immune responsiveness of viral myocarditis.

Key Words:

Coxsackie B3 virus myocarditis
Monoclonal antibody
Helper T lymphocyte
Immunohistochemistry
Cell-mediated immunity

MATERIAL AND METHODS

The details of the material and methods used

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Fig.1. Light-microscopic features of the myocardium of an infected mouse on day 9. Many cardiocytes have undergone lysis and numerous mononuclear cells have infiltrated the necrotic foci. Hematoxylin-eosin stain; scale 20 μ m.

Fig.2. Electron micrograph of necrotic foci in the myocardium on day 14. A macrophage (Mac) is seen establishing membrane contact with a lymphocyte (Lym). Stained with uranyl acetate and lead citrate, scale 1 μ m.

Fig.3. Light-microscopic immunohistochemistry of the myocardium on day 14 after virus inoculation. Biotinylated anti lyt 1+ antibody and avidin-biotin-peroxidase complex (ABC) were used to identify mononuclear cell infiltrates in the necrotic foci. Lyt 1+ antigen-bearing lymphocytes can be identified by the red-brown reaction products on their membranes (arrow-heads). Counterstained with hematoxylin, scale 20 μ m.

in this study were described elsewhere.⁹ Eighty male C3H/He mice (3 weeks of age) were used. Forty of them were injected with $0.5 \text{ ml} \times 10^8$ TCID₅₀/ml of coxsackie B3 virus (Nancy Strain) intraperitoneally, and 40 control mice were injected with a virus-free culture medium. They were killed 5, 7, 9, 14, 21 and 30 days and 3 months after inoculation. The left ventricle of each heart was divided into three parts for observation by conventional light and electron microscopy and immunohistochemistry.

(1) Conventional Light- and Electron Microscopy

One part of each left ventricle was fixed in 10% neutral-buffered formalin and embedded in paraffin. The tissue sections were stained with hematoxylin-eosin and Mallory-azan stains and examined by light microscopy.

The second part of each left ventricle was fixed in 3% glutaraldehyde and postfixed with 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.2, for two hours. After dehydration in graded ethanols, the specimens were embedded in Epon. Ultrathin sections stained with uranyl acetate and lead citrate were examined by electron microscopy.

(2) Light- and Electron-microscopic Investigations of Immunohistochemistry

Each left ventricle was fixed in a periodate-lysine-paraformaldehyde fixative (PLP).¹³ These fixed tissues were then washed in phosphate-buffered saline (PBS) containing sucrose. The tissue samples were placed in OCT. They were immediately quick-frozen in a liquid nitrogen bath. Frozen sections of the specimens were cut and mounted on albumin glass slides. Next, the sections were incubated with biotinylated anti-mouse Thy 1.2 or Lyt 1+ antibody (Becton Dickinson, Sunnyvale, CA). After additional washing with PBS, the sections were incubated with avidin-biotin-peroxidase complexes (ABC) (Vector Lab., Burlingame, CA).¹⁴

For light microscopy, the sections were treated with diaminobenzidine (DAB)-peroxidase solution, dehydrated, and counterstained with Mayer's hematoxylin. They were viewed under a light microscope. In the sections stained with anti-Thy 1.2 or Lyt 1+ antibody, positive lymphocytes were identified by the red-brown reaction products in their surface or within their cytoplasm.

For immuno-electron microscopy, the incu-

Fig.4. Immuno-electron micrograph of the myocardium stained with anti Lyt 1+ antibody and ABC on day 14 after inoculation. DAB reaction products are uniformly distributed along the cell surface (arrowheads) and partly deposited in the endoplasmic reticulum. The Lyt 1 antigen-bearing T lymphocyte (Lym) contains a few small mitochondria and rough endoplasmic reticulum in the cytoplasm and shows a high nuclear cytoplasmic ratio. Unstained section, scale 1 μ m.

bated sections were postfixated with 2% glutaraldehyde, allowed to react with DAB peroxidase, and again fixed with osmium tetroxide. They were then dehydrated in graded ethanols and embedded in Epon. Ultrathin sections were examined without additional staining with an electron microscope. In the sections stained with each monoclonal antibody, positive lymphocytes were identified by the DAB reaction product that was usually present only focally along the membranes of the cell surface and cytoplasm. Occasionally, the reaction product was uniformly distributed along the entire cell membrane.

RESULTS

Conventional Light and Electron Microscopy

On the 5th day after virus inoculation, multiple small necrotic foci appeared in the myocardium. Under the electron microscope, necrotic cardiocytes showed myofibrillar lysis, mitochondrial inclusions, few glycogen granules and disrupted

plasma membranes. On days 7 and 9, macrophages and lymphocytes were the predominant elements of all the infiltrates in the necrotic foci. On day 9 necrotic areas became more prominent with numerous macrophages and lymphocytes and a few polymorphonuclear leukocytes, plasma cells, and eosinophils (Fig. 1). Some macrophages and polymorphonuclear leukocytes invaded the necrotic cardiocytes, and phagocytosed the necrotic cellular debris. Lymphocytes often appeared to be in close contact with macrophages or other lymphocytes in and around the necrotic foci (Fig. 2). Where lymphocytes were in contact with macrophages, a large portion of the surface membrane of each adjacent cell was usually separated by a distance of approximately 20 nm. The membrane contact between lymphocytes and macrophages was observed at all stages of this experiment, but appeared to be most prominent on day 14. On day 30 and in month 3, cell infiltrations were less prominent, but a few lymphocytes in contact with macrophages were

Fig.5. Immuno-electron micrograph of the myocardium stained with anti-Thy 1.2 antibody and ABC on day 14 after inoculation. The reaction product was uniformly distributed along the cell surface of a T lymphocyte (Lym). The surface membrane of the T lymphocyte is in close contact with a macrophage (Mac). Unstained section, scale 1 μ m.

Fig.6. Immuno-electron micrograph of the myocardium stained with anti-Lyt 1+ antibody and ABC on day 14 after inoculation. The reaction product was uniformly distributed along the cell surface (arrowheads) of a lymphocytes (Lym). The Lyt 1 antigen-bearing T lymphocyte is seen establishing membrane contact with a macrophage (Mac). is, interstitial space. Unstained section, scale 1 μ m.

still identifiable in and around the fibrotic foci.

Light- and Electron Microscopic Immunohistochemistry

On day 7, a few T cells including Lyt 1 antigen-bearing lymphocytes were seen in and around the necrotic foci. On day 9, T lymphocytes apparently increased in number, accounting for $10.5 \pm 5.0\%$ of all of the infiltrating cells a finding similar to that in our previous reports^{9,10}. In the foci several Lyt 1+ T lymphocytes were also identifiable. On day 14, T lymphocytes were the most predominant ($16.4 \pm 2.6\%$). Many Lyt 1+ lymphocytes also infiltrated in and around the foci (Fig. 3). By electron microscopy Lyt 1+ lymphocytes were often seen in the necrotic foci on day 14 (Fig. 4). Some Lyt 1 and Thy 1.2 antigen-bearing lymphocytes were seen establishing membrane contact with macrophages and other lymphocytes on day 14 (Fig. 4, 5 and 6). On the 30th day and in the 3rd month, a few T lymphocytes still remained in and around the fibrotic foci.

DISCUSSION

In our previous studies of viral myocarditis, we discussed the significance of infiltrating T lymphocytes in the cell-mediated immune response in the myocardium.^{8,9}

It has been well accepted that there are three T lymphocyte subsets defined through the use of antisera prepared against the murine Lyt allo-antigens: Lyt 1, 2, 3+, Lyt 1+ and Lyt 2, 3+ lymphocytes. Each subpopulation represents different T cell functions *in vivo*: precursor, helper and cytotoxic/suppressor functions, respectively.¹⁵ The authors^{9,10} and several other investigators^{2-5,8} have described that cytotoxic T lymphocytes are responsible for *in vivo* or *in vitro* cardiocyte destruction in myocarditis, but the significance of helper T cell in immune responsiveness is still obscure. The present study showed that many lymphocytes infiltrated in the foci showed Lyt 1 antigen on their surface. Although Lyt 2, 3+ T lymphocytes were not examined in this experiment, we assume that the Lyt 1+ T lymphocytes are helper in nature.

Macrophages often phagocytosed necrotic cellular debris. Some Thy 1.2 and Lyt 1 antigen-bearing lymphocytes were also in close contact with macrophages in and around the necrotic foci. In current immunology, much attention has been focused on the relationship between

antigen-presenting macrophages (APC) and helper T cells!¹⁵ APC bears surface Ia molecules that are encoded by genes of the major histocompatibility complex. The recognition of the Ia molecule-antigen complex on the APC by helper T cells is a very important step in immune responsiveness. When the T cells are in direct contact with APC and recognize Ia molecule-antigens, they are maximally stimulated. All through our experiment, direct contact between macrophages and lymphocytes was often seen, and these findings suggest the involvement of a cell-mediated immune response in viral myocarditis.

In general, helper T cells respond to immunogenic stimulation by proliferating and elaborating various factors that stimulate B-cells to differentiate into antibody-producing cells. In addition, helper T cells also release IL-2 that help in the development of cytotoxic T-cells!¹⁵ The present study indicates that a significant number of Lyt 1+ lymphocytes infiltrated into the foci, predominantly on the 14th day after inoculation. These Lyt 1+ cells may play a central role in providing positive signals for other lymphocytes that are involved in the cell-mediated and humoral immune reactions in viral myocarditis.

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