Immunological Study of Heart Diseases with Special Reference to the Cytotoxicity of the Heterophile Antibody against Cultured Myocardial Cells

SHINJI FUKUTA, M.D., KATSUTOSHI YAMAKAWA, M.D., YOSHIKO HAYASHI, M.D.
SETSUKO IWAMOTO, M.D., SEIJI UMEMOTO, M.D., REIZO KUSUKAWA, M.D.
AND KAZUNARI WADA, M.D.

In our previous study, a high rate of "heterophile antibody" was found in idiopathic cardiomyopathy. In the present investigation the cytotoxic activity of this antibody was examined in cultured myocardial cells. Trypsin treated cells cultured for 3 days were used in the two-step method of the complement dependent cytotoxicity test. The trypan blue dye-exclusion method was utilized to determine the percentage of surviving cells, and the cytotoxic effect was expressed by the cytotoxic index (CI). With fresh rabbit serum as complement, the mean CI value of the heterophile antibody positive sera was 20.7, which was in contrast to the value of anti-heart antibodies negative sera (p < 0.01). As the cytotoxic effect to the heterophile antibody was absent without the complement, it may be complement dependent. Thus, our results suggest that the heterophile antibody may play a role in the pathogenesis or be an incremental factor of idiopathic cardiomyopathy.

In our previous report we found circulating anti-heart antibodies in the sera of patients with heart disease using the indirect immunofluorescence method with cold ethanol fixed normal rat hearts. The immunofluorescent staining patterns were classified into four types: "heterophile," "intracellular," "intercalated disc" and "nuclear" patterns. The heterophile antibody has been frequently found in the sera of patients with idiopathic cardiomyopathy. However, the etiological role of circulating anti-heart antibodies in heart diseases is an unsettled question.

The purpose of the present study was to examine the cytolysis activity of the heterophile antibody and complement against cultured rat myocytes.

MATERIALS AND METHODS

Cell preparation. Myocytes derived from newborn rats were cultured according to the method of Harary and Farley (Fig. 1). Rats, 2–4 days old, were killed, and their hearts were removed rapidly under sterile conditions. After washing in Ca-free and Mg-free cold phosphate buffered saline, the ventricles were cut into about 1 mm³ fragments, and suspended in 0.125% trypsin dissolved EDTA solution. The supernatant was decanted, and the remaining pellet was resuspended in 0.125% trypsin. The ventricles were stirred gently for 10 min, at

Key Words:
Anti-heart antibodies
Heterophile antibody
Cultured myocardial cells
Cytotoxicity

Department of Internal Medicine, Yamaguchi University School of Medicine, Ube, Japan. *Onoda Red-cross Hospital
This work was supported in parts by Grants-in-Aid for Scientific Research (No.58570390) and for Encouragement of Young Scientists (No.57770582) from the Japanese Ministry of Education, Sciences and Cultures.
Mailing address: Shinti Fukuta, M.D., Department of Internal Medicine, Yamaguchi University School of Medicine, 1144 Kogushi Ube 755, Japan

1354 Japanese Circulation Journal Vol. 48, December 1984
REMOVING RAT HEART (2-4 DAY OLD)
WASHING THE HEART IN COLD PBS(Ca, Mg FREE) SEVERAL TIMES
DISSECTION OF RAT VENTRICLES AND CUTTING INTO FRAGMENTS
STIRRING IN 0.1% TRYPsin SOL, ADD EDTA, 10 MIN, 37 °C
FILTRATION BY MESH
ADDING 10% FCS/ MEM
KEEP IN 4 °C
FILTRATION BY MESH
CENTRIFUGATION AT 200XG, 5 MIN
ADDING 10% FCS/MEM TO THE PELLET
SEEDING AT CONC. OF 1.5-2 X 10⁵ CELLS/ML IN CULTURE DISH
ADHERENCE IN CO₂ INCUBATOR, 90 MIN, 37°C
CULTURING NONADHHERENT CELLS AS MYOCARDIAL CELLS IN
CO₂ INCUBATOR AT 37°C

Fig.1. Sequence used in primary culturing heart cells.

37°C, then filtrated through a mesh and suspended in Eagle’s minimum essential medium (MEM) containing 10% inactivated fetal calf serum (FCS), at 4°C. This procedure was repeated, and the collected supernatant was centrifuged at 800 rpm, for 5 min. The pellet was resuspended in Eagle’s MEM containing 10% FCS. Approximately 1.5-2 X 10⁵ cells/ml were placed in a Falcon plastic dish (35 mm diameter). After culturing for 90 min at 37°C in an incubator with 5% CO₂ and 95% O₂, non-adherent cells were collected and used as myocytes.

Assay of cardiolysis. The complement dependent cytotoxicity test with trypsin blue was performed according to the two-step method of Amos et al.³ (Fig. 2). A mixture of 3 x 10⁵ myocytes and 0.5 ml of serum at double dilution was incubated for 30 min at 4°C. After washing twice in cold Eagle’s MEM, myocytes were incubated for 45 min at 37°C with 0.5 ml selected rabbit serum at 1:10 dilution, as a complement source. The myocytes were incubated in 0.25% trypsin-added EDTA solution for 5 min at 37°C. The trypsin blue dye-exclusion method was used to determine the surviving cells (unstained). In the cytolytic assay system, negative control assays were conducted with serum or complement alone. When dead cells constituted more than 25% of the cells, the data in the assay system were excluded. Cytotoxicity was expressed by the cytotoxic index (CI) calculated as follows: Cytotoxic index = [%Living cells in control system - %Living cells in the patient serum system] / %Living cells in control system

Indirect immunofluorescence of cultured myocytes. Cultured myocytes were incubated with inactivated test serum (dilution 1:10) for 30 min. After washing three-times in the cultured medium, the specimens were incubated for 30 min with a 1:10 diluted FITC conjugated rabbit anti-human IgG-serum. The specimens were then washed 3 times for 45 min in the cultured medium. After fixing in 95% cold ethanol, the slides were mounted in 90% glycerol at pH 8.4, and viewed under a Leitz fluorescent microscope at x 400 magnification and photographed with 35 mm color film (Kodak Ektachrome 160).

Sera. Eight heterophile antibody positive sera
TABLE 1 RESULTS OF THE COMPLEMENT DEPENDENT CYTOTOXIC TEST

<table>
<thead>
<tr>
<th></th>
<th>No.</th>
<th>Cytotoxic index</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>Range</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HE(+) serum</td>
<td>3</td>
<td>-1.5</td>
<td>-4.8</td>
<td>5.1</td>
</tr>
<tr>
<td>HR(-) serum</td>
<td>2</td>
<td>0</td>
<td>-2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>MEM</td>
<td>1</td>
<td></td>
<td>-3.6</td>
<td></td>
</tr>
<tr>
<td>Complement and antibodies</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HE(+) serum</td>
<td>8</td>
<td>20.7*</td>
<td>10.8</td>
<td>34.6</td>
</tr>
<tr>
<td>HE(-) serum</td>
<td>4</td>
<td>-1.5</td>
<td>-6.0</td>
<td>3.6</td>
</tr>
</tbody>
</table>

**HE = heterophile antibody; ^p < 0.01**

and four circulating anti-heart antibodies negative sera were used.

RESULTS

Figure 3 shows an indirect immunofluorescence of the cultured myocytes with heterophile antibody positive serum. Fluorescence was found in the myolemmal membrane of the myocytes, and the fibroblasts were not stained. This finding indicates that the heterophile antibody was bound to the myolemmal membrane of the cultured myocytes.

Table I shows the results of the complement dependent cytotoxic test against cultured rat myocytes. In the assay system without complement, the CI values using heterophile antibody positive sera were from -4.8 to 5.1. The CI values using circulating anti-heart antibodies negative sera were from -2.4 to 2.4. In the experiment with complement, the CI values using the heterophile antibody positive sera were from 10.8 to 34.6 (mean 20.7), whereas those using the circulating anti-heart antibodies negative sera were from -6.0 to 3.6 (mean -1.5). The difference between these two means was statistically significant (p < 0.01).

DISCUSSION

Rat myocardial cells were used as target cells in this cytolytic assay, because circulating anti-heart antibodies were examined in alcohol fixed normal rat hearts. Many investigators have used cultured Girardi heart cells as target cells in assays of myocardial cell damage but these cells lose striation, cylindrical shapes and contractility, and thus they should not be used in the assay system.
This study demonstrated that the heterophile antibody bound to newborn rat myocardial cells and that the antibody induced cytolysis in the presence of a complement. The cytotoxic effect of the antibody and complement against these cells may be due to a defective control of the cell membrane osmolarity, that may be influenced by an interference in the Na-K ATPase activity as reported by Friedman and Laufer.

Antibodies similar to our heterophile antibody have been reported as anti-sarcolemmal antibody in viral myocarditis by Maisch et al. or endothelial-vascular-interstitial (EVI) autoantibody in Chagas’ disease by Cossio et al. Maisch and his coworkers demonstrated that the anti-sarcolemmal antibody has cytotoxic activity in the presence of a complement against adult rat cardiocytes. On the other hand, Sterin-Borda et al. have shown that the EVI antibody induces morphologic and functional alterations in rat myocardial cells in vitro. Further investigations are required to clarify the differences among these antibodies.

If the heterophile antibody plays a role in the pathogenesis of myocardial damage, a heterophile antigen must exist on the myocardial cell surface. A heterophile antigen has not been reported in the normal adult human heart. However, heterophile antigen and antibody have recently been demonstrated in pathologic sera and tissues by Nishimaki et al. They reported that this heterophile antigen may be produced as a novel antigen by pathologic tissue and that this antigen is composed of several antigenic molecules and some of them may exist as normal tissue components and possibly increase during a pathologic process. Furthermore, this antigen may be released from infectious agents containing the same antigenic molecules.

Whatever its ultimate significance, the heterophile antibody is found frequently in the sera of patients with idiopathic cardiomyopathy and the antibody has cytotoxic activity. Our results suggest that it may play a role in the pathogenesis or be an incremental factor of idiopathic cardiomyopathy.

REFERENCES