RNA TUMOR VIRUS-LIKE PARTICLES IN THYMUS CELLS OF PATIENTS WITH MYASTHENIA GRAVIS

AKIRA ONO*, TORU HIGASHINAKAGAWA†, SHUNZO KONDO‡,
TATSUYA NAKAMURA**, HIDETSUGU SAITO**, HIDERO SUZUKI‡,
HIROSHI YOSHIMATSU*, MASARU MURAKAMI‡
and MASAHARU TSUCHIYA**

*Department of Virology, The Kitasato Institute, Tokyo 108
†Department of Biology, Tokyo Metropolitan University, Tokyo 158
‡Mitsubishi-Kasei Institute of Life Sciences, Tokyo 194
#Department of Internal Medicine and Surgery, University of Occupational and Environmental Health, Kitakyushu 807
**Department of Internal Medicine, School of Medicine, Keio University, Tokyo 160, Japan

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ABSTRACT

We report here the appearance of retrovirus-like particles in cultured thymus cells from patients with myasthenia gravis. Cell from five patients with myasthenia gravis were examined. Histologically three were thymomas and two were thymus hyperplasias. The thymus cells were co-cultured for 7 days with allogenic B cells pretreated with mitomycin C. Retrovirus-like particles were demonstrated by electron microscopy in 4 cases of myasthenia gravis. These particles were detected in thymus epithelial cells. In 4 cases of myasthenia gravis examined, reverse transcriptase activity sedimenting at a density of 1.15-1.17 g/cm³ in sucrose density gradients was eluted in the culture fluids. These results suggest that in myasthenic thymuses, retroviruses that can be induced, following a proliferative stimulus, may be involved in the genesis of thymic disorders, and in the pathogenesis of myasthenia gravis.

The discovery of RNA tumor virus (retrovirus)¹ and of their involvement in the pathogenesis of autoimmunity of NZB mice²,³ have prompted many researchers to search for retroviruses in human autoimmune diseases. Although retroviral antigenic expression may be enhanced in some human diseases,⁴,⁵ retroviruses have so far not been found in tissues of human autoimmune patients.
except in the placentas of systemic lupus erythematosus (SLE) patients. Human myasthenia gravis is thought to be one of the autoimmune diseases, and is frequently accompanied with thymic changes. Recently some authors have reported the detection of acetylcholine receptors on thymus cells from myasthenic patients; and lymphoid follicle formation is frequently found in the myasthenic thymus. Moreover, thymectomy of myasthenic patients has been reported to have good therapeutic results. Thymic involvement has therefore been considered to be essential in the pathogenesis of human myasthenia gravis.

We reported elsewhere the detection of putative retroviruses in human fetal thymus cells. For the reasons mentioned above, we have turned our attention to the thymus of patients with myasthenia gravis and examined them for the presence of retrovirus-like particles. We report here the appearance of such particles in thymus cells of patients with myasthenia gravis when stimulated by B cells.

**MATERIALS AND METHODS**

Five cases from patients with myasthenia gravis (all female, with ages ranging from 22 to 51) were obtained at thymectomy. Histologically three were thymomas and two were thymus hyperplasias. These cases are summarized in Table 1. The birthplaces of these patients were located from north to south parts of Japan, and were not concentrated in southwestern parts of Japan, adult T cell leukemia (ATL) endemic areas. No antibodies to ATL-associated antigen

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Sex</th>
<th>Thymus histology</th>
<th>Activator</th>
<th>E.M.</th>
<th>RDDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 1</td>
<td>51</td>
<td>F.</td>
<td>hyperplasia</td>
<td>B cell (SB)</td>
<td></td>
<td>N.T.</td>
</tr>
<tr>
<td>No. 2</td>
<td>22</td>
<td>F.</td>
<td>hyperplasia</td>
<td>B cell (Raji)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>No. 3</td>
<td>42</td>
<td>F.</td>
<td>thymoma (epithelial type)</td>
<td>P.B.L.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>B cell (SB)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>No. 4</td>
<td>38</td>
<td>F.</td>
<td>thymoma (epithelial type)</td>
<td>P.B.L.</td>
<td>N.T.</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>B cell (SB)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>No. 5</td>
<td>45</td>
<td>F.</td>
<td>thymoma (mixed type)</td>
<td>B cell (SB)</td>
<td>N.T.</td>
<td>+</td>
</tr>
</tbody>
</table>

P.B.L.: peripheral blood lymphocyte. N.T.: not tested.
(ATLA)\(^{18}\) were detected in sera of these patients.

Briefly, for the recovery of virus-like particles, freshly isolated thymus cells\(^{19}\) were cultured for 7 days together with B cells (SB,\(^{20}\) Raji\(^{21}\)) pretreated with mitomycin C.\(^{17,22}\) In some cases peripheral blood lymphocytes (P.B.L.) from healthy donors were also used as stimulator cells. On day 3, the cells were harvested and processed for electron microscopy. The culture medium from day 3 to day 7 was collected and examined for RNA-directed DNA polymerase (RDDP) activity in 20–60% w/v sucrose density gradients (150,000×g, 18 hrs).\(^{22}\) Cultured thymus cells without stimulation were also examined. RDDP assays were carried out at 37°C for 80 min in a standard reaction mixture which contained in a final volume of 100 µl: 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM MnCl\(_2\), 5 mM dithiothreitol, 0.1% (v/v) Triton X-100, 4 µM \(^3\)H-dTTP (30 Ci/mM, 60,000 cpm/pmole), 2 µg (rA)\(_n\)·(dT)\(_{12-15}\) and 50 µl aliquot of each fraction.\(^{23}\) At the end of the reaction, 90 µl samples were applied to grass fibre discs (Whatman, GF/C), which were washed 5 times in 5% TCA/1% Na\(_2\)P\(_2\)O\(_5\) and twice in ethanol, dried and counted in a Liquid Scintillation Spectrometer using a toluene-base scintillation fluid. The endogenous polymerase reaction was performed similarly but the reaction mixture consisted of 50 mM Tris-HCl (pH 7.5), 5 mM dithiothreitol, 1 mM MnCl\(_2\) contained dATP, dGTP and dTTP at 0.1 mM each, 10 µCi of \(^3\)H-dCTP (30 Ci/mM), 0.03% Nonidet P-40, actinomycin D at 50 µg/ml and 50 µl aliquot of each fraction. For detecting viral RNA, B cell-stimulated thymus cells were incubated with 10 µCi of \(^3\)H-uridine (60 mCi/mmol)/ml and incubated for 3 days. The medium was collected, and fresh medium without \(^3\)H-uridine was added twice for 6 hour periods. The media were then pooled, and examined for radioactivity in sucrose density gradients described above. Radioactive fractions were collected and processed for extraction of RNA. RNA was extracted with sodium dodecyl sulfate (SDS)-phenol\(^{24}\) and the aqueous phase was then layered over a linear sucrose gradient (5–20%) and centrifuged at 150,000×g for 3 hrs at 4°C. 28S and 18S RNA prepared from mouse livers were used as external size markers. As a control viral RNA, the culture medium from murine leukemic virus (MuLV) producer cell lines\(^{25}\) was used.

RESULTS

Electron microscopic examination

Typical electronmicrographs of retrovirus-like particles in thymus cells stimulated by B cells are shown in Fig. 1. In Fig. 1-A and B a group of retrovirus-like particles was observed in the thymus cells of a patient with myasthenia gravis (histologically thymus hyperplasia with lymphoid follicle formation, case No. 1 in Table 1). These retrovirus-like particles were 95–115 nm in diameter.
Fig. 1  RNA tumor virus-like particles in thymus cells of patients with myasthenia gravis.
A, B, A group of retrovirus-like particles in the cytoplasm of cultured thymus epithelial cells. B is the higher magnification of the region marked in A. A (×4,800), B (×84,000). C, D, Two retrovirus-like particles in the thymus epithelial cells of a patient with myasthenia gravis. D is the higher magnification of the region marked in C. C. (×6,600), D (×84,000).

with a 60–75 nm electron dense core, indicating that they are mature particles. Such mature particles were detected only in this case. In other myasthenic cases, only budding particles and/or immature particles with electron luscent cores were found. The cells harbouring these particles appeared to be thymus epithelial cells with large irregular shapes and a large segmented nucleus. In the cytoplasm, many intracytoplasmic organelles were present, also indicating that these cells were of epithelial origin. In Fig. 1-C and D, two retrovirus-like particles were found in the thymus epithelial cells of a patient with myasthenia gravis (histologically epithelial type of thymoma, case No. 3 in Table 1). These particles were detected after P.B.L. stimulation. We also examined over 200 fresh thymus cells in 2 of cases of myasthenia gravis, but no retrovirus-like particles were detected (Table 1, cases No. 1 and 4). Retrovirus-like particles were detected in neither fresh B cells nor mitomycin C treated B cells.
Detection of RDDP activity and large RNA molecule in sucrose density gradients

Typical RDDP and large RNA molecule profiles in sucrose density gradients of the culture medium from B cell-stimulated thymus cells are presented in Fig. 2. Fig. 2-A shows RDDP activity in the culture medium from the myasthenic cases (case No. 3 in Table 1). A sharp peak of RDDP activity was detected at 1.158–1.165 g/cm³, the density associated with mammalian retroviruses. Incorporation activity of ³H-dCTP into polymer was detected at the same density without addition of the exogenous template. Similar RDDP profiles were also obtained in the other 3 cases of myasthenia gravis, where the culture medium from P.B.L.-stimulated thymus cells were also examined. RDDP activity at this density was not detected in the culture medium from unstimulated thymus cells, nor from mitomycin C treated B cells. ³H-uridine incorporation into polymer was also demonstrated in the same fractions as polymerase activity, indicating the presence of RNA in these fractions (Fig. 2-B). In these fractions large RNA molecule (35S RNA) was demonstrated after extraction of their RNA (Fig. 2-C).

Fig. 2  Detection of particulate RNA-directed DNA polymerase activity and large RNA molecule in a sucrose density gradient.
A, ○; RNA-directed DNA polymerase activity.
△; endogenous polymerase activity without addition of the exogenous template.
□; density.
B, RNA profile in a 20–60% w/v sucrose density gradient.
C, Profile of RNA extracted from the fractions 7–11 in B (5–20% w/v sucrose density gradient).
Summary of the experiments

Table 1 summarizes the series of experiments. In all 4 cases of myasthenic thymuses which were examined by electron microscopy, retrovirus-like particles were detected. Particulate RDDP activity was also detected in all 4 cases. In case No. 3 and No. 5, RDDP activity was demonstrated after both B cell and P.B.L. stimulation. Neither retrovirus-like particles nor RDDP activity was found in fresh thymus cells or in cultured thymus cells without stimulation.

DISCUSSION

We previously reported the detection of putative retrovirus-like particles in human fetal thymus cells. Using the same method for virus activation (B cell stimulation), we have demonstrated retrovirus-like particles in cultured thymus cells from patients with myasthenia gravis. The retrovirus-like particles detected here resemble retrovirus particles found in other species; and the fact that RDDP activity and large RNA molecule were found at 1.15-1.17 g/cm³ in sucrose density gradients strongly suggests that these virus-like particles really are retrovirus particles. These particles are unlikely to have been introduced by the B cell lines (SB or Raji) since the particles were also present after stimulation with peripheral B cells. The fact that the birthplaces of patients were not concentrated in ATL virus (ATLV) endemic areas of Japan and no antibodies to ATLA were detected in sera of these patients suggests that these particles are different from ATLV. To our knowledge this is the first demonstration of retrovirus-like particles in patients with myasthenia gravis in general and thymus cells in particular.

Recently it has been demonstrated that anti-acetylcholine receptor (Ach.R.) antibodies have been detected in sera of patients with myasthnia gravis and a correlation between the titer of these antibodies and severity of the disease has been reported. However, the mechanism of how these anti-Ach.R. antibodies are formed in myasthenia gravis is not clear, and the same is true for autoantibodies in other autoimmune diseases.

The recent finding that abnormal B cell proliferation is suggested in myasthenic thymuses together with our finding that retrovirus-like particles were detected only when thymus cells were co-cultured with B cells, lead us to the following hypothesis (see Fig. 3). It is generally accepted the existence of a blood-thymic barrier. Once this barrier is destroyed, B cells from the blood stream can enter the thymus and may stimulate cells of the thymus (immunological activation). Retroviruses which already exist in thymus epithelial cells in a latent form will then be activated. This may be accompanied by other changes in gene expression, such as Ach.R. on thymus epithelial cells of myasthenia
Retrovirus in Myasthenic Thymus

Fig. 3 The role of retrovirus in thymus of patients with myasthenia gravis (Hypothesis). Ach. R.: acetylcholine receptors.

gravia. Virus activation may cause proliferation of thymus cells themselves, leading to thymoma and thymus hyperplasia, and perhaps also expression of Ach.R. protein on the cell surface as a neoantigen. In support of this hypothesis of gene activation accompanying retrovirus activation, one can list recent reports concerning the role of retrovirus in the production of retrovirus protein p30 that is associated with the induction of differentiation in myeloid cell lines. Newly expressed Ach.R. on thymus epithelial cells may sensitize thymocytes, and through differentiation, sensitized T cells may then induce myasthenia gravis. One cannot conclude that retrovirus plays a direct role in the onset of myasthenia gravis and should therefore consider the involvement of other factors as well such as genetic factors including HLA. The association between H-2 and virus susceptibility of mice of different strains has been repeatedly demonstrated.

The further characterization of these retrovirus-like particles, the mechanism of gene activation in thymus cells and the search for antivirus antibodies in the sera of myasthenic patients should give us further information concerning the role of retroviruses in the myasthenic thymus.

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