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Individual germinal centres of myasthenia gravis human thymuses contain polyclonal activated B cells that express all the V_H and V_κ families

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SUMMARY

Using *in situ* hybridization, we analysed the immunoglobulin repertoire expressed by the B cells present in myasthenia gravis thymuses from four patients. B cells, mostly in activated state, were clustered in germinal centres, in which multiple isotypes were identified. A majority of cells expressed IgG as compared with IgM, with a roughly similar contribution of κ and λ chains. Hybridization with the six V_H and the 4 V_κ human family probes was observed in serial sections, providing additional evidence that individual germinal centres were polyclonal. The thymic B cell repertoire closely reflected the V_H and the V_κ family usage of normal peripheral blood lymphocytes with the preferential utilization of V_H3, $V_\kappa1$ and $V_\kappa3$.

Keywords germinal centres thymus myasthenia gravis B cell repertoire

INTRODUCTION

Myasthenia gravis is an autoimmune disease affecting neuromuscular transmission, due to the loss of acetylcholine receptors (AChR), which correlates with the occurrence of anti-AChR antibodies (Lindström *et al.*, 1976; Engel, 1986). Myasthenia gravis is frequently associated with thymic abnormalities consisting of either thymomas or thymus hyperplasia (Castelman, 1966; Compston *et al.*, 1980; Berrih-Aknin *et al.*, 1987). In the latter case, myasthenia gravis thymus is characterized by the presence of germinal centers containing numerous DR-expressing cells and B lymphocytes (Fujii *et al.*, 1983; Bofill *et al.*, 1985) producing immunoglobulin directed against AChR (Fujii *et al.*, 1986). The epitopes of this antigen recognized by myasthenia gravis serum antibodies have been studied (Tzartos, Seybold & Lindström, 1982: Heidenreich *et al.*, 1988) and a major immunodominant region (MIR) has been more precisely located (Tzartos *et al.*, 1988). We thus asked whether the autoimmune response occurring in hyperplastic myasthenia gravis thymus would involve a peculiar V_H or V_κ repertoire. We also investigated whether B lymphocytes present in a single germinal centre arise from a single clone. For these purposes, we performed *in situ* hybridization using V_H and V_κ subgroup-specific probes. The repertoires so identified were compared with that of normal activated peripheral B lymphocytes.

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MATERIALS AND METHODS

Patients

Four patients with myasthenia gravis presenting thymic hyperplasia (Levin & Rosai, 1978) were thymectomized at the Centre Chirurgical Marie Lannelongue (Le Plessis Robinson, France). Clinical data are summarized in Table 1.

Thymic sections

Immediately after surgical removal, myasthenia gravis thymic fragments were frozen in liquid nitrogen and stored. Sections (5-μm thick) were cut at -18°C on a cryomicrotome (Bright, Shandon, London, UK), and air-dried before hybridization.

DNA probes

V subgroup and C region specific probes have been subcloned in M13 mp18 or mp19 vector, leading to the isolation of negative strands that detect mRNAs and positive strands that were used as non-hybridizing controls. V_H1 ($V_{E3\text{-}D10}$: 0·3-kb *Eco*RI–*Bgl*II), V_H2 (VCE-1: 0·25-kb *Hha*I) were subcloned from plasmid kindly provided by T. Honjo (Kodaira *et al.*, 1986). V_H3 (F9: 0·32-kb *Eco*RI–*Hae*III) is derived from fetal cDNA clone given by Milili *et al.*, 1991, V_H4 (1-911: 0·33-kb *Pst*I), V_H5 (5-1R1: 0·38-kb *Hinc*II–*Pst*I) and V_H6 (6-1R1: 0·32-kb *Eco*RI–*Stu*I) originated from Berman *et al.* (1988). $V_\kappa1$ (pWV1: 0·55-kb *Hinc*II–*Pvu*II), $V_\kappa2$ (p607VJ: 0·29-kb *Bam*HI–*Sph*I), $V_\kappa3$ (m41v-7: 0·32kb *Acc*I–*Bam*HI) and $V_\kappa4$ (pJIJV: 0·38-kb *Bam*HI–*Pst*I) were derived

Table 1. Clinical characteristics of myasthenia gravis patients in this study

Patient no.	Sex	Age (years)	Thymic histology	Anti-AChR antibody titre (nM)	Disease duration	Disease severity
1	F	37	Severe hyperplasia	60·00	3 Mo	Severe
2	F	40	Mild hyperplasia	0·90	3 Mo	Mild
3	F	32	Mild hyperplasia	4·00	1 y	Severe
4	F	39	Mild hyperplasia	0·70	3 Mo	Severe

AChR, acetylcholine receptor.

from plasmid given by H. G. Zachau (Klobeck, Combriato & Zachau, 1984a; Klobeck, Solomon & Zachau, 1984b; Klobeck *et al.*, 1985a, 1985b). Cμ probe (mF2: 0·38-kb *Eco*RI) and C_γ (γR1: 0·38-kb *Hae*III) were subcloned from DNA given by Milili *et al.*, 1991. C_κ probe (00158: 0·38-kb *Sst*I) was from Hieter *et al.* (1980).

M13 DNA containing inserts were ^{3}H-labelled by primer extension technique as described by Berger (1986). DNA was digested with appropriate restriction enzymes (as indicated above) and the resulting labelled probes were purified on 6% polyacrylamide gel. Specific activity averaged $3–4 \cdot 10^8$ ct/min per μg.

In situ *hybridization*

Thymus sections were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde, for 15 min at room temperature and washed twice in PBS. In order to reduce the background, preparations were acetylated for 10 min at room temperature with a solution 0·25% acetic acid, 0·1 M triethanolamine, pH 8·0, 5 mM $MgCl_2$. After washing in 2× SSC, slides were immersed in 75% and 95% ethanol successively and air-dried. Pre-hybridization solution was made at 35°C for 3–5 h with 0·1 ml of a solution 50% formamid, 0·6 M NaCl, 2 mM EDTA, 10 mM Tris–HCl, pH 7·5, 1·6X Denhart's, 150 μg/ml sonicated salmon sperm DNA, 500 μg/ml tRNA. Hybridization was made with 9 μl of the same buffer containing 15 000 d/min per μl of the DNA probe. Slides were covered with a cover-slip and incubated for 20 h at 35°C in a humid chamber containing 50% formamid, 0·6 M NaCl. Washings were performed during 24 h first in 50% formamid, 1 mM EDTA, 10 mM Tris–HCl, pH 7·5, and various concentrations of NaCl (0·6, 1·2 or 2·4 for C, V_κ and V_H probes, respectively), and then in PBS. Dehydration was achieved by increasing concentrations of ethanol (30, 60, 80, 95 and 100%), each step for 5 min at room temperature. Slides were finally air-dried, and dipped in a Kodak nuclear track NTB2 emulsion (Eastman Kodak, Rochester, NY) melted at 43°C and diluted 1/1 with water. The emulsion coated slides were dried overnight at room temperature and were exposed for 3 weeks at 4°C. Development was performed in D19 (Kodak) solution for 4 min and fixed (Kodafix). Counter-staining was done with 20% Giemsa for 1 min.

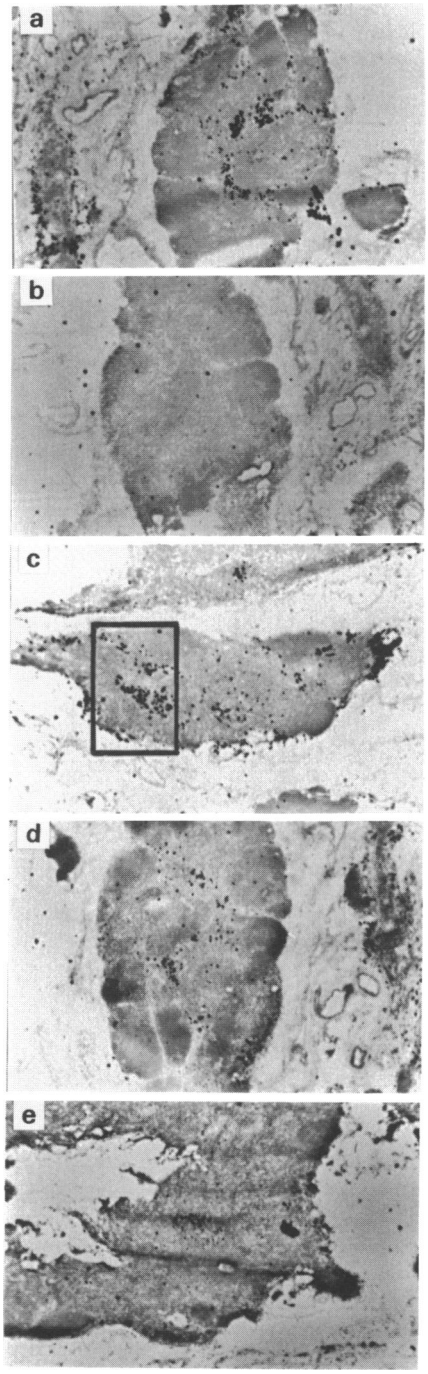

Fig. 1. *In situ* hybridization on myasthenic thymus sections (patient 1) with C_γ-negative (a); and C_γ-positive (b) stands; C_κ (c); C_λ (d); and C_μ (e) probes (magnification ×31). The area delineated in the insert (c) was serially analysed and is presented in Fig. 2 at a higher magnification.

RESULTS

Polyclonality of the immunoglobulin repertoire in myasthenia gravis thymuses

Four myasthenia gravis thymuses, analysed by *in situ* hybridization using immunoglobulin C_μ, C_γ, C_κ and C_λ probes, displayed a similar pattern of expression. A strong hybridization was observed with the four negative-strand probes, an example of

Table 2. Relative expression of the various V_κ and V_H families in four myasthenia gravis thymuses compared with that of non myasthenia gravis peripheral B lymphocytes (PBL)

Immunoglobulin probes	$V_\kappa 1$	$V_\kappa 2$	$V_\kappa 3$	$V_\kappa 4$	$V_H 1$	$V_H 2$	$V_H 3$	$V_H 4$	$V_H 5$	$V_H 6$
MG1	++	++	+++	++	++	+	+++	++	+	±
MG2	++	++	+++	++	+	±	++	++	±	±
MG3	++	+	++	+	±	±	+++	+	±	±
MG4	+++	±	++	++	+	±	++	+	+	±
Lymph node*	+++	+	+++	++	++	±	+++	++	+	±
PBL (%)†	43	9	29	19	11	3	52	19	10	5

Relative expression in myasthenia gravis thymuses (MG1–4) and in lymph node is indicated by an arbitrary scale (± to +++), which correlates with the labelling intensity of the different slides.

* From benign follicular hyperplasia.

† Average values from nine normal individuals.

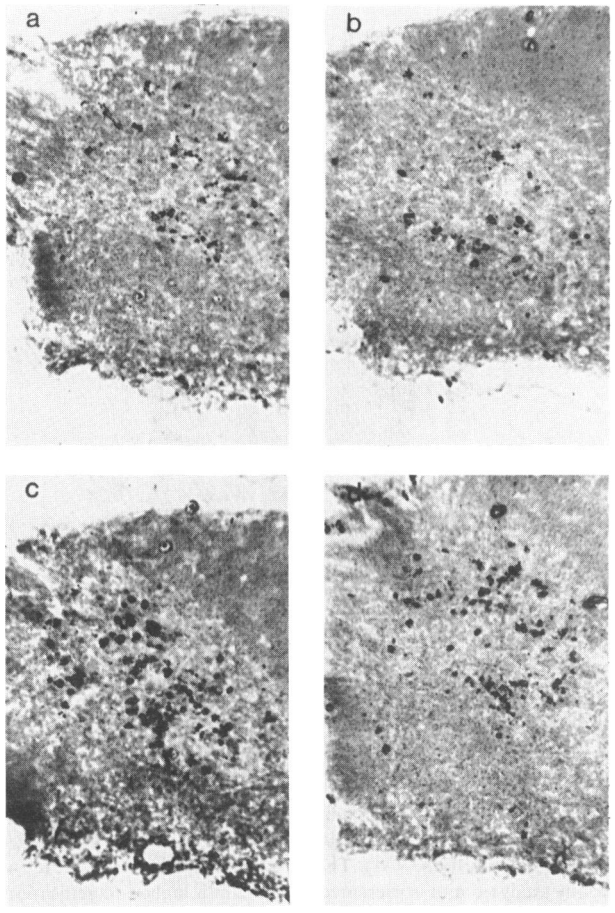

Fig. 2. *In situ* hybridization on serial myasthenic thymus sections (patient 1) with $V_{\kappa 1}$ to $V_{\kappa 4}$ probes (a to d, respectively; magnification ×80).

which is shown in Fig. 1 (a, c, d, e). In contrast, no signal was detected with the positive-strand probes, providing a negative control (data shown for C_γ in Fig. 1b). The number of IgG-producing B lymphocytes was clearly higher than that of IgM-producing cells. Interestingly, whereas IgM-producing B lymphocytes were mostly located within germinal centres, IgG producers were mainly found outside the follicle, in the connective tissue emerging from the septae and entering the lobules. Regarding the light chain expression, both κ and λ chains were found to be expressed. The κ/λ ratio was close to 1, suggesting the polyclonal origin of immunoglobulin-producing cells. This conclusion was confirmed by analysing the V repertoire used by myasthenia gravis thymic B lymphoctes. *In situ* hybridization was performed using $V_\kappa 1$ to $V_\kappa 4$ and $V_H 1$ to $V_H 6$ family-specific probes. Absence of cross-reactions between members of the different families was checked on a panel of Epstein–Barr virus (EBV) clones (Guigou *et al.*, 1990). Positive signals were identified for all V probes and the relative expression of the various V families is indicated in Table 2. The analysis of serial sections showed that several V_H and V_κ gene families were simultaneously expressed within individual germinal centres. An example of this analysis is given in Fig. 2 for the V_κ families. Therefore, B lymphocytes found in germinal centres of hyperplastic myasthenia gravis thymuses are of polyclonal origin and express a wide V_H and V_κ repertoire.

The immunoglobulin V gene utilization pattern in myasthenia gravis thymuses is close to that of peripheral B lymphocytes

To determine whether the B lymphocytes present in myasthenia gravis hyperplastic thymuses used a peculiar repertoire, we compared their V_κ and V_H gene expression pattern with that of peripheral B lymphocytes from control subjects (Table 2). Samples were taken either from a lymph node exhibiting a benign follicular hyperplasia or from peripheral blood lymphocytes from normal donors (Guigou *et al.*, 1990). In all cases we observed a similar expression pattern of the various V_H and V_κ families, with a preferential usage of $V_\kappa 1$, $V_\kappa 3$ and $V_H 3$, regardless of the origin. Therefore, activated B lymphocytes from myasthenia gravis hyperplasia thymuses do not use a preferentially restricted V gene repertoire.

DISCUSSION

We took advantage of the fact that about 10–20% of thymocytes present in hyperplastic myasthenia gravis thymuses are consti-

tuted of B lymphocytes, to study the immunoglobulin repertoire of these B cells in four patients. Using immunoglobulin C and V probes covering most of the known human immunoglobulin V_H (Kodaira *et al.*, 1986; Berman *et al.*, 1988) and V_κ (Klobeck *et al.*, 1984, 1984b, 1985a, 1985b) repertoire, we have shown that the immunoglobulin-producing cells present in myasthenia gravis thymuses are polyclonal and express different V_H and V_κ gene families. This wide usage of V genes is somewhat surprising in the view of the limited number of AChR epitopes recognized by autoreactive antibodies (Tzartos *et al.*, 1982; Heidenreich *et al.*, 1988) with a major immunodominant region (MIR) identified (Tzartos *et al.*, 1988). Moreover, oligoclonal responses have been reported in other autoimmune diseases (Farrell *et al.*, 1985, Fishleder *et al.*, 1987).

However, the lack of restriction in the expressed repertoire of the myasthenia gravis thymus B lymphocytes that we observe is in agreement with recent reports (Tesch, Hohlfeld & Toyka, 1989; Vincent *et al.*, 1987). The polyclonality of immunoglobulin production in myasthenia gravis thymuses might be due to the fact that the antibody response occurring in these tissues is directed not only against AChR, but also against other autoantigens such as myosin, α-actinin and actin (Williams & Lennon 1986).

We also report that IgG-producing cells are preferentially found at the periphery of germinal centres. This location would favour interactions with dendritic cells, which are able to present a putative autoantigen, and with activated T cells and monocytes producing cytokines potentially involved in B lymphocyte differentiation (Emilie *et al.*, 1991). This micro-environment found in myasthenia gravis thymuses may play a major role in the development of the autoreactive B lymphocyte activation.

The origin of the B cells present in hyperplastic myasthenia gravis thymuses remains unknown. Thymic B cells have been described in normal human thymuses (Isaacson, Norton & Addis, 1987) where they represent only a very low proportion of thymocytes (i.e. 0·1–1%). They synthesize IgM and express the CD5 marker (Miyama-Inaba *et al.*, 1988). We observed that, in contrast to this normal thymic population, most B lymphocytes obtained after purification are activated, synthesize IgG (mostly anti-AChR antibodies) and do not express the CD5 marker (unpublished result). This population may result from an intrathymic differentiation from the normal thymus B cells. The recent report of Kimoto *et al.*, (1989), showing that B cell precursors, present in normal mice thymus may differentiate is in keeping with this hypothesis. Alternatively, myasthenia gravis thymus B lymphocytes may be derived from the periphery rather than from intrathymic resident B cells. The fact that they use a V_H and V_κ family-expression pattern similar to that of peripheral lymphocytes would argue in favour of an extrathymic origin. Their focusing in the thymus would thus be linked to the specific expression of autoantigens in this environment.

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