The Immunohistology of the Thymus in Myasthenia Gravis

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We have investigated cell subpopulations in frozen sections of thymus tissue obtained from myasthenic (MG) and control subjects. With the use of an avidin–biotin immunoperoxidase system with monoclonal antibodies, the following cell surface antigens were studied on frozen sections (12 MG and 3 control thymus); T11, T4, T6, T8, IgM, IgD, and Ia. The pattern of T cell phenotypes in MG thymus is similar to that of normal control thymus when examined by immunohistologic techniques. MG cortical thymocytes are virtually all T11+, T4+, T8+, and T6+. In the medulla, at least 45% of thymocytes are T11+, with T4+ cells predominating over T8+ cells. Approximately 10% of medullary thymocytes are T6+. Scattered medullary cells expressing surface IgM and IgD are identified in both MG and normal thymuses. However, unlike the normal thymus, the MG thymus has numerous secondary follicles containing IgM- and IgD-bearing cells. This finding supports the hypothesis that the MG thymus microenvironment is aberrant. The Ia antigen is found in similar tissue section localization patterns in MG and control thymus. Ultramicroscopic studies show the Ia antigen predominantly on epithelial and interdigitating dendritic cells. By immunoperoxidase techniques, numerous keratin-positive cells are demonstrated in MG and control thymus. This suggests that thymic epithelial cells, like epithelial cells elsewhere, contain keratin. Because these data differ in degree from our previous findings in suspensions of MG thymocytes, this study emphasizes the importance of examining tissue sections as well as cell suspensions when one is studying lymphocyte surface markers. (Am J Pathol 1984, 117:184–194)

MYASTHENIA GRAVIS (MG) is an autoimmune disorder of unknown etiology that affects neuromuscular transmission. The thymus has been implicated in the pathogenesis of MG on the basis of pathologic changes observed in the myasthenic thymus[1] and on the favorable response to thymectomy.[2] Functional studies of thymic cell suspensions have demonstrated altered immunologic activity.[3,4] We have used monoclonal antibodies to lymphocyte phenotypic markers[5] to analyze subpopulations of cells in tissue sections and in cell suspension from the thymus of myasthenic and cardiac surgery control patients. In addition, we have investigated the number, distribution, and identity of Ia-antigen-expressing cells in thymuses from these two groups of subjects.

Materials and Methods

Fresh tissue was obtained from 12 MG patients (age range, 20–40) undergoing thymectomy and from 3 control subjects (ages 4 months, 33 years, and 42 years). The diagnosis of MG was based on clinical findings, edrophonium testing, and/or electromyographic criteria. Thymectomy was performed 6 months to 5 years after the diagnosis of MG was made. Serum anti-acetylcholine receptor antibody levels, measured by a modified radioimmunoassay technique,[6] were increased in 6 patients, normal in 4 patients, and not tested in 2 patients. The thymic T-cell subsets were studied in suspension in 3 of these MG patients. Suspension results are reported elsewhere.[7] The control subjects underwent surgery for localized cardiac disease without evidence

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of systemic disease. None of the MG patients had a thymoma. Tissue was frozen in liquid nitrogen and stored at −70 C until use. Cryostat sections (5 μ) were cut, air-dried, fixed in acetone for 10 minutes, and allowed to dry.

Replicate frozen tissue sections were washed with phosphate-buffered saline (PBS, pH 7.4) for 3 minutes and then incubated for 15 minutes with a 1:50 dilution of one of the monoclonal antibodies: 1) T11 (reacting with all T-lymphocytes); 2) T4 (putative helper/inducer cells); 3) T8 (putative suppressor/cytotoxic cells); 4) T6 (found on cortical thymocytes and Langerhans cells); 5) Ia (found on some B lymphocytes, monocyte/macrophages, and activated T cells); 6) IgM (found on B cells); and 7) IgD (found on some B cells). All monoclonal antibodies were obtained from Ortho Pharmaceuticals, Raritan, NJ, except for anti-T11 and anti-T4 (Coulter Corp., Hialeah, Fla), anti-IgD (Cappel Labs, Cochranville, Pa), and anti-IgM (kindly provided by Dr. Mary Ellen Conley, Children's Hospital of Philadelphia). Rabbit anti-keratin antiserum was obtained from Dako Corporation (Santa Barbara, Calif) and used in a 1:1000 dilution.

After another 3-minute wash in PBS, biotinylated anti-mouse immunoglobulin (Ig) (Vector Laboratories, Burlingame, Calif) used as second antibody was applied in a 1:75 dilution for 15 minutes. (Biotinylated anti-rabbit Ig antibody [Vector Laboratories] was used in a 1:75 dilution as second antibody with anti-keratin.) Sections were then rinsed in PBS and incubated with the avidin–biotinylated peroxidase complex (Vector Laboratories) for 15 minutes.

After another wash in PBS, the sections were incubated with 1 of 2 chromogens for 10 minutes. The chromogen used was either: 1) 3-amino-9-ethylcarbozole (AEC) (Aldrich Chemical Co., Milwaukee, Wis); or 2) diaminobenzidine (DAB) (Sigma, St. Louis, Mo) (20 mg AEC in 5 ml N-N, dimethyl formamide plus 95 ml sodium acetate buffer, 0.10 M, pH 5.2, or 50 mg DAB in 100 ml PBS; 0.03% hydrogen peroxide was included in either chromogen solution). Equivalent results were obtained with AEC and DAB with the choice based on the color of the end product desired (AEC, red; DAB, brown).

Sections were then rinsed in tap water, counterstained in hematoxylin (45 seconds), and mounted in Elvanol (E. I. duPont Co., Wilmington, Del). All incubations were at room temperature. Included in each test run were: 1) negative controls in which PBS was substituted for the primary antibody; 2) positive controls in which the binding of the same monoclonal antibody was assayed on frozen sections of lymph nodes. For studies with anti-keratin antibody, negative controls were run in which normal rabbit serum (1:1000 dilution) was substituted for the primary antibody. For a second negative control, sections were incubated with rabbit anti-human chorionic gonadotropin (1:1000 dilution) (Dako Corp., Santa Barbara, Calif) instead of rabbit anti-keratin antibody. For positive controls, binding of anti-keratin antibody was assayed on sections of human skin.

Using a microscopic ocular grid, we determined the percentage of cells bearing such membrane phenotypic marker for the cortex and medulla in the replicate frozen sections. At least 100 cells were counted in each area of the thymus in each section.

Electron Microscopy – Tissue Sections

Small pieces (0.5 mm in diameter) of thymic tissue from three MG patients were fixed in 2% buffered glutaraldehyde (Karnovsky's fixative) for 4 hours, then washed in cacodylate buffer (0.1 M, pH 7.4) for 3 days. Tissue for immunoelectron microscopy was then incubated for 3 days at 4 C with either PBS or monoclonal anti-human Ia mouse monoclonal antibody (Ortho Pharmaceutical Corp., Raritan, NJ) diluted 1:50 in PBS.

Tissue was then washed in PBS for 3 days and incubated in biotinylated anti-mouse Ig antibody (1:75 dilution) (Vector Laboratories, Burlingame, Calif) for 3 days. After another 3-day wash in PBS, the tissue was then incubated with avidin–biotinylated peroxidase complex (Vector Laboratories, Burlingame, Calif) for 2 days. After a 3-day wash in PBS, tissue was incubated in DAB (6 mg/10ml) and 0.03% hydrogen peroxide for 4 hours. Tissue was then washed in PBS for 4 hours, dehydrated in graded ethyl alcohols, placed in propylene oxide with Epon, and polymerized. Sections were cut on an ultratome, and alternate sections were stained with uranyl acetate and then lead citrate. The remaining sections were unstained. Sections were observed and photographed with a Philips 200 electron microscope at 60 kV.

Electron Microscopy – Cell Suspensions

Thymic lymphocyte suspensions were processed for immunoelectron microscopy. One MG patient and one control were studied. Thymic lymphocyte suspensions obtained by mincing tissue and passing it through No. 50 stainless wire mesh were washed twice with RPMI 1640 (GIBCO, Grand Island, NY). Thirty × 10^6 cells were incubated with 1 ml of Ia antibody (1:50 dilution) or PBS for 1 hour. Cells were then washed three times in 10 ml and incubated with 1 ml of biotinylated anti-mouse Ig antibody in a 1:75 dilution for 1 hour. After another PBS wash (×3), cells were resuspended in 1

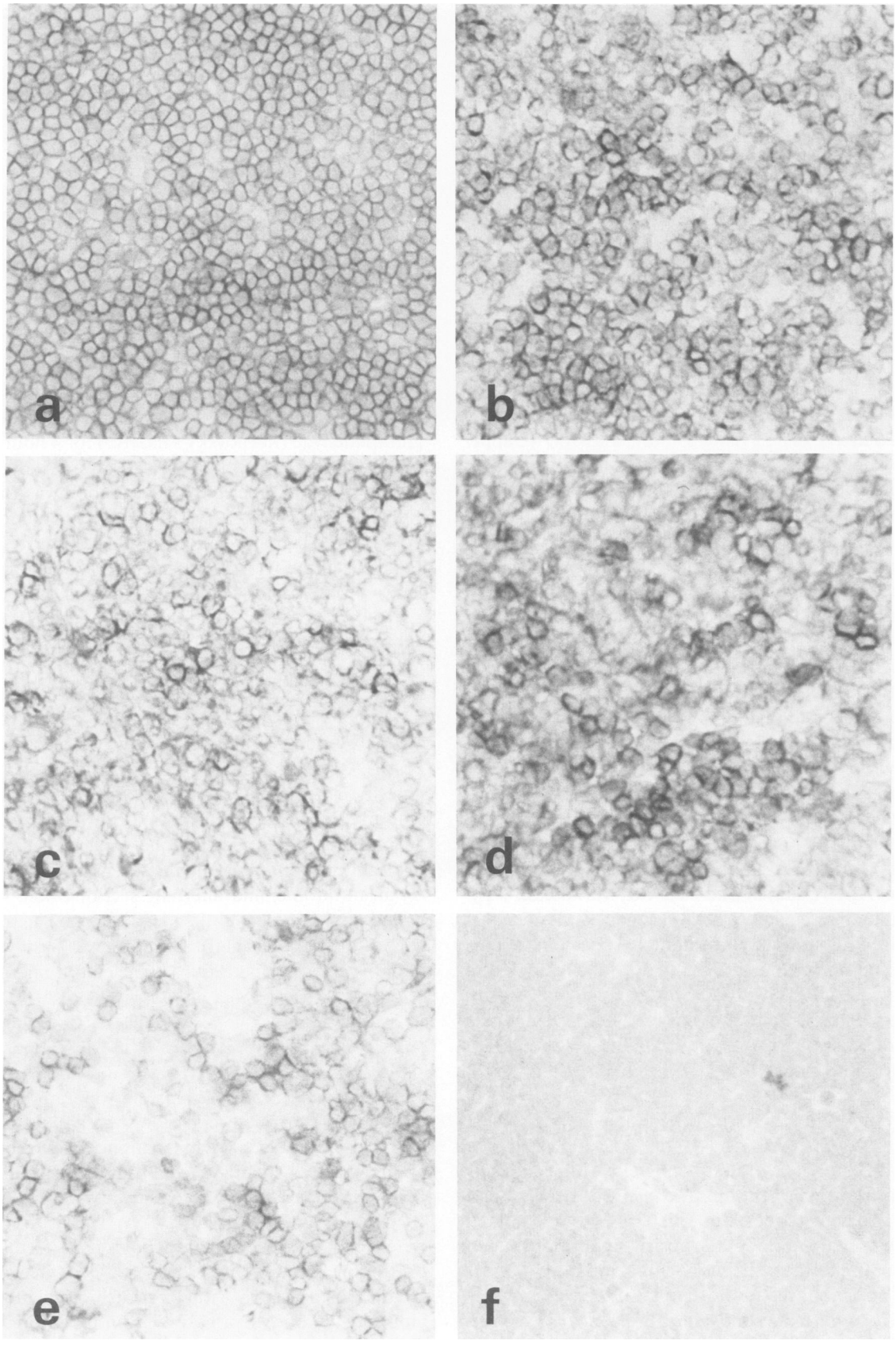
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Table 1—T-Cell Subsets in the Thymus

	Tissue section data (% positive cells)							
	T11		T4		T8		T6	
	Cortex	Medulla	Cortex	Medulla	Cortex	Medulla	Cortex	Medulla
MG patients								
1	99	60	99	50	99	32	99	10
2	99	45	95	50	95	20	ND	ND
3	99	45	85	45	85	30	90	8
4	99	45	99	60	99	25	99	3
5	99	62	ND*	ND*	99	44	99	10
6	99	62	99	35	99	37	99	8
7	99	50	99	45	99	28	99	5
8	99	68	99	60	99	20	99	1
9	99	65	99	71	99	36	99	9
10	99	55	99	61	99	41	99	10
11	99	75	99	75	99	22	99	9
12	99	62	99	77	99	27	99	5
Mean	99 ± 0†	58 ± 9.9	97 ± 13	57 ± 13.5	97 ± 1.3	30 ± 8.0	98 ± 0.8	7.1 ± 3.1
Control subjects								
1	99	90	99	50	99	30	99	11
2	99	70	99	75	99	30	99	10
3	99	52	99	60	99	22	99	4
	99 ± 0	71 ± 19.0	99 ± 0	62 ± 12.6	99 ± 0	27 ± 4.6	99 ± 0	8.3 ± 3.8

* Not done.
† Mean ± SD.

ml of avidin-biotinylated peroxidase complex for 1 hour.

The cells were then washed in PBS (×3) and incubated in 1 ml of DAB (0.05%) for 15 minutes. After another three washes, the cell pellet was suspended in 0.5 ml of Karnovsky's fixative. All incubations and centrifugations were at 4 C. The cells were then placed in agarose gel and processed for electron microscopy as described above.

Results

T Cells

In tissue sections of thymus from patients and controls, all the cells in the cortex with morphologic characteristics of lymphocytes were T11-positive. These cells also bore T4, T6, and T8 determinants (Figure 1). The few scattered cells in the cortex which were negative for those antigens did not have the morphologic appearance of lymphocytes. None of the cortical cells were IgM or IgD positive.

In the medulla, a different pattern was observed (Figure 1 and Table 1). Approximately half of the cells were T11-positive. A similar percentage bore T4 markers. About one-third were T8-positive, and 10% were T6-positive. A large number of cells had no apparent T-cell membrane determinants. The relative frequencies of these markers in the thymus (cortex and medulla) can be approximated as T11 = T4 with T4 > T8 > T6. There was no difference in the pattern of T-cell subsets when MG thymus was compared with control thymus. No relationship between the immunohistologic characteristics of MG thymus and the level of anti-acetylcholine receptor antibody could be discerned.

B Cells and Germinal Centers

There were very few, if any, IgM or IgD-positive cells in the cortex of either MG or control thymus tissue sections. Germinal centers were frequent and prominent in the medulla of the thymic tissue from 10 of the 12 MG specimens examined. A single germinal center was seen in one of the control thymus specimens. The cells in the mantle zone of the germinal center were gener-

Figure 1—MG thymus frozen sections demonstrating localization of lymphocyte subsets. **a**—Thymic cortex examined for T11. Virtually all cortical lymphocytes are T11-positive. T11-negative cells appear to be nonlymphoid. This same pattern is seen in the cortex for T11, T4, T6, and T8. **b-e**—Thymic medulla stained for T11 (**b**), T4 (**c**), T6 (**d**), and T8 (**e**). **f**—Thymic cortex stained for IgM (negative control). (Hematoxylin counterstain, ×400)

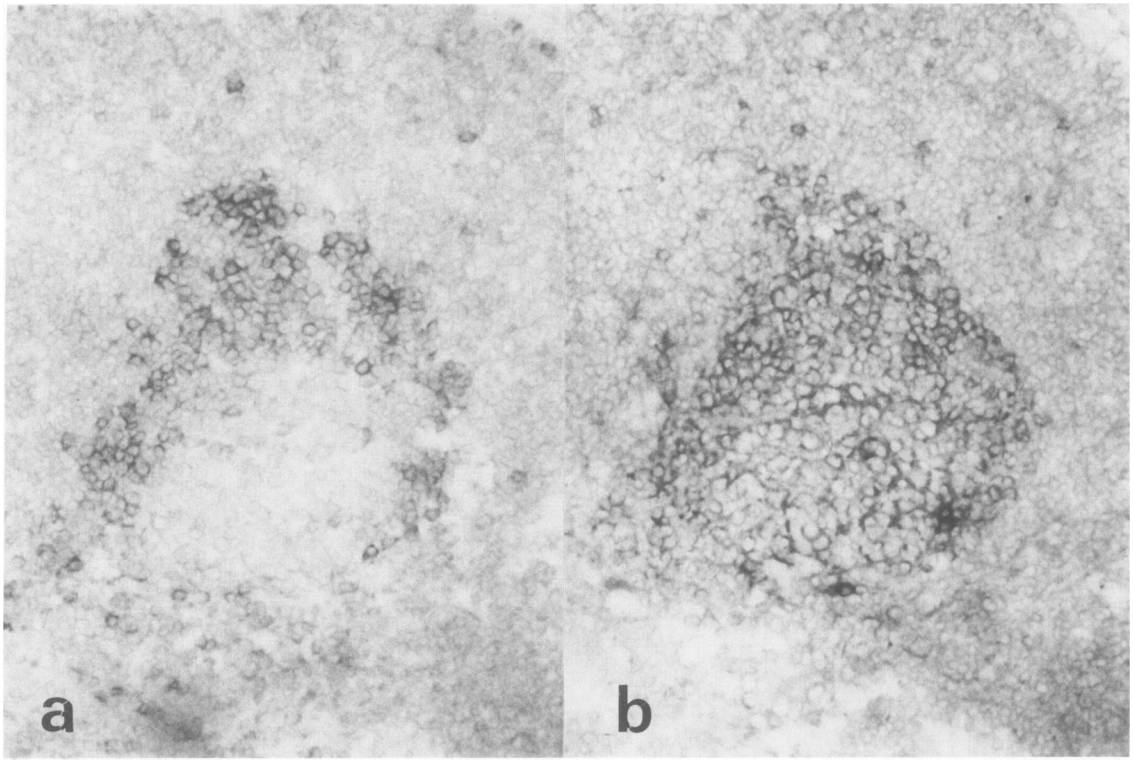

Figure 2 – Frozen section of MG thymus with germinal center. **a** – Immunoperoxidase staining demonstrates IgD-positive cells in the mantle zone. **b** – This section demonstrates IgM-positive cells in the mantle zone and follicular center. Scattered IgD- and IgM-positive cells are present in the medulla outside follicles. (Hematoxylin counterstain, ×400)

ally both IgM- and IgD-positive, whereas cells in the follicular center were usually positive only for IgM (Figure 2). This pattern was similar to that seen in the germinal centers of lymph node sections used by us as positive controls. A small number of IgM-positive and IgD-positive cells were scattered throughout the medulla apart from the germinal centers. It is of note that T11-positive cells were present in the area immediately surrounding the germinal centers with occasional T11- and T4-positive cells within the center. T8-positive cells were

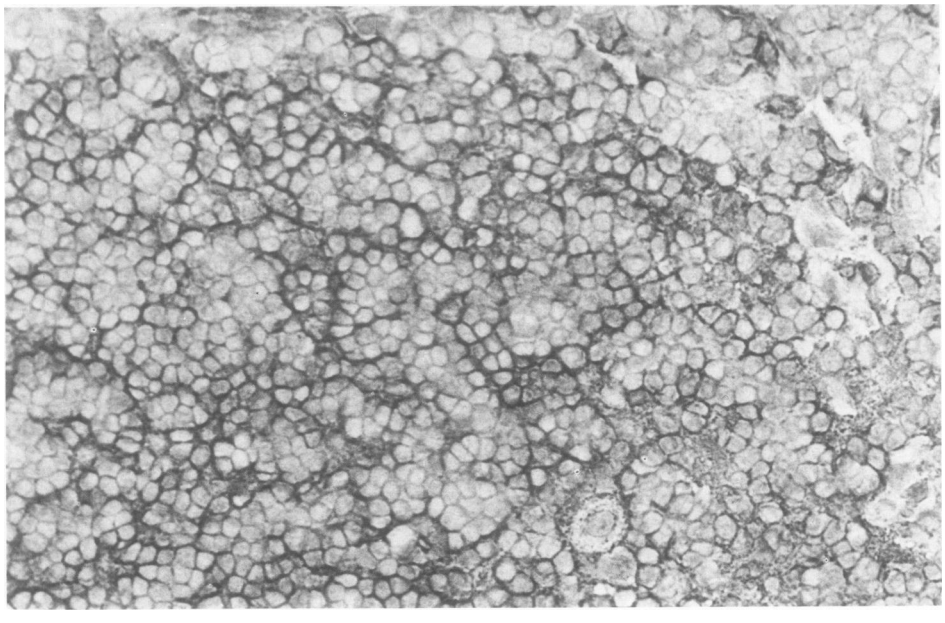

Figure 3 – Frozen section of MG thymic cortex demonstrating localization of Ia antigen. The dendritic pattern precludes enumeration of positive cells. (Hematoxylin counterstain, ×400)

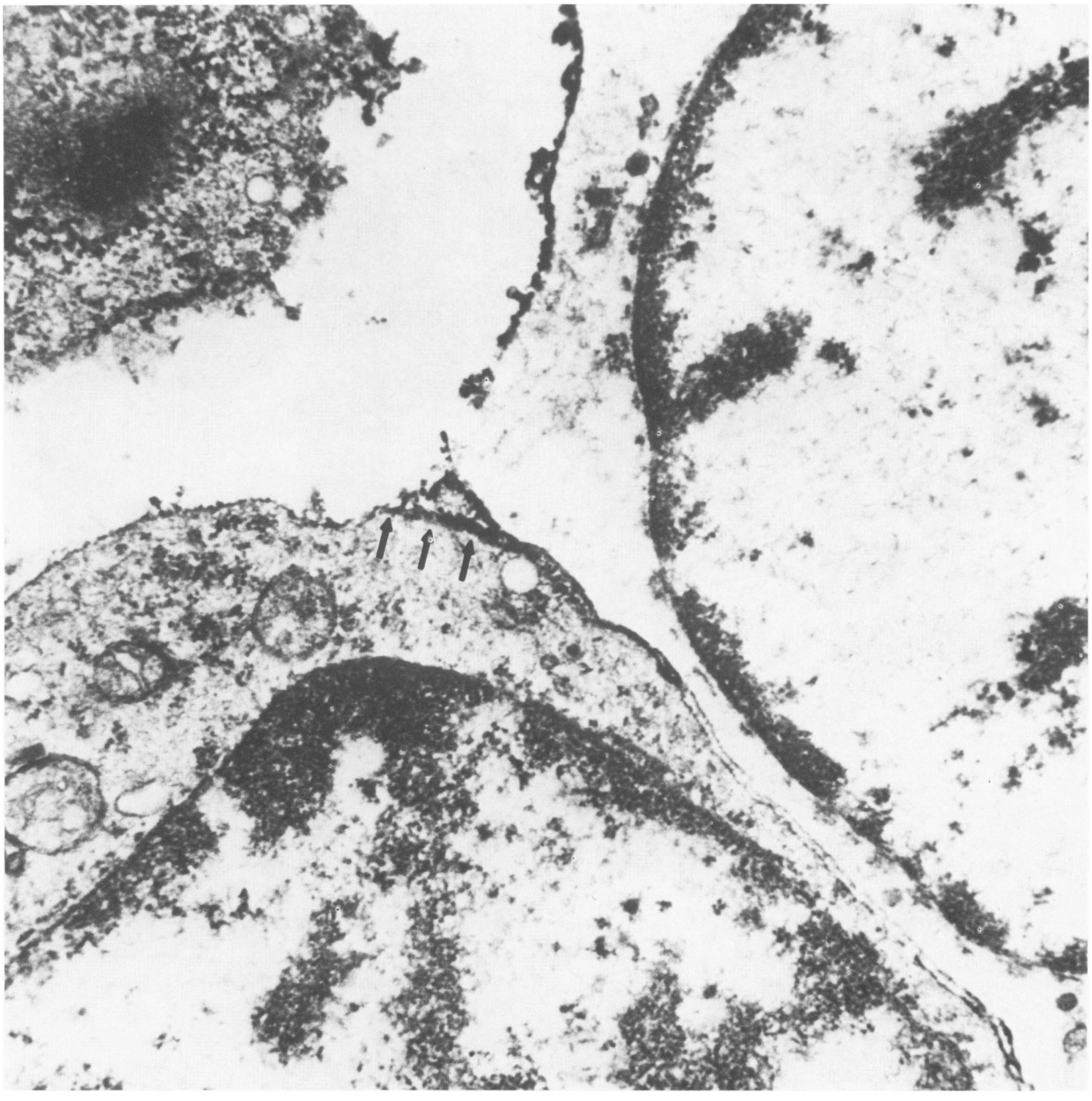

Figure 4 – Electron micrographs of MG thymus tissue sections demonstrating localization of Ia antigen. Ia-positive epithelial cell (upper right) and an adjacent thymic lymphocyte are shown. The lymphocyte has Ia positivity only on the membrane adjacent to the epithelial cell (*arrows*). (× 11,140)

rare, and T6-positive cells were nonexistent in germinal centers.

Ia Antigen

The staining patterns for Ia-positive cells in MG and control thymus were similar (Figure 3). Positive staining of long interdigitating processes of some cortical cells and the confluent pattern in the medulla precluded accurate identification and enumeration of positively stained cells. By electron microscopy, the Ia antigen was localized predominantly on the surface membrane of nonlymphoid cells (epithelial and interdigitating dendritic cells) of the thymus. However, Ia-positive portions of the surface membrane of thymic lymphocytes were observed adjacent to Ia-positive nonlymphoid cells in tissue (Figures 4 and 5). In thymus cell suspensions examined by electron microscopy, occasional lymphocytes were strongly Ia-positive on their entire surface (Figure 6).

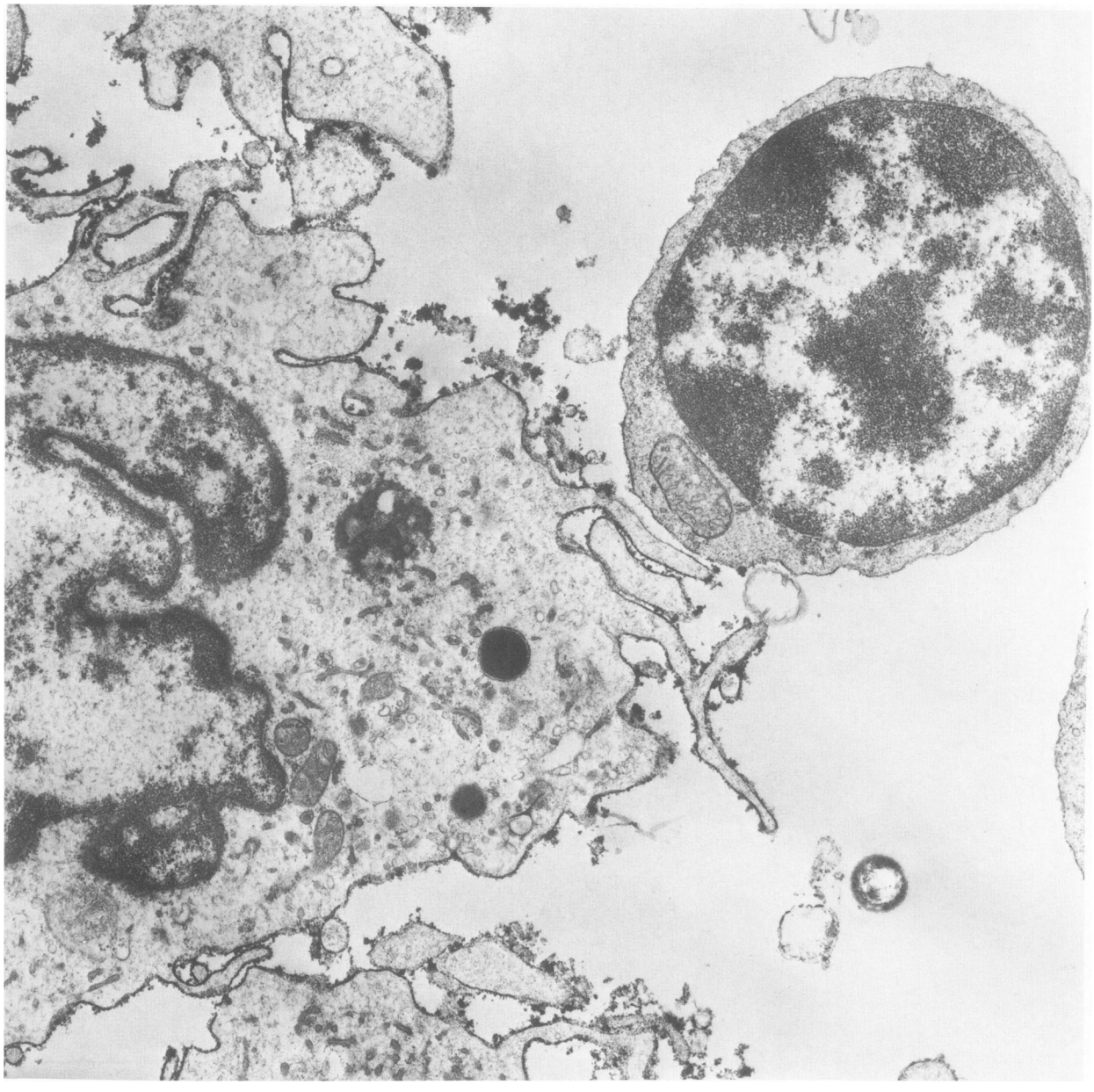

Figure 5— Electron micrograph of MG thymus tissue section demonstrating localization of Ia antigen. Dendritic cell (*left*) is Ia-positive. Lymphocyte (*upper right*) has weak Ia positivity on a portion of the membrane adjacent to the dendritic cell.

Keratin

A search for keratin-containing cells was made in frozen sections from 6 thymus specimens (5 MG and 1 control). No difference was noted between MG and control specimens. In the medulla, numerous keratin-positive large cells were present, and Hassall's corpuscles were strongly keratin-positive. In the cortex, scattered discrete large keratin-positive cells with long processes were noted (Figure 7). The outer borders of the cortex were sharply outlined by keratin-positive cells. In negative control sections, the only staining observed was due to endogenous peroxidase activity in occasional mast cells, macrophages, and neutrophils. The pattern produced by anti-keratin antibody is similar to that with anti-Ia antigen. However, staining for keratin results in more sharply defined borders of the outer cortex. The staining of the cortex itself appears to be somewhat less with keratin than with Ia, with some cortical areas appearing almost entirely keratin-negative.

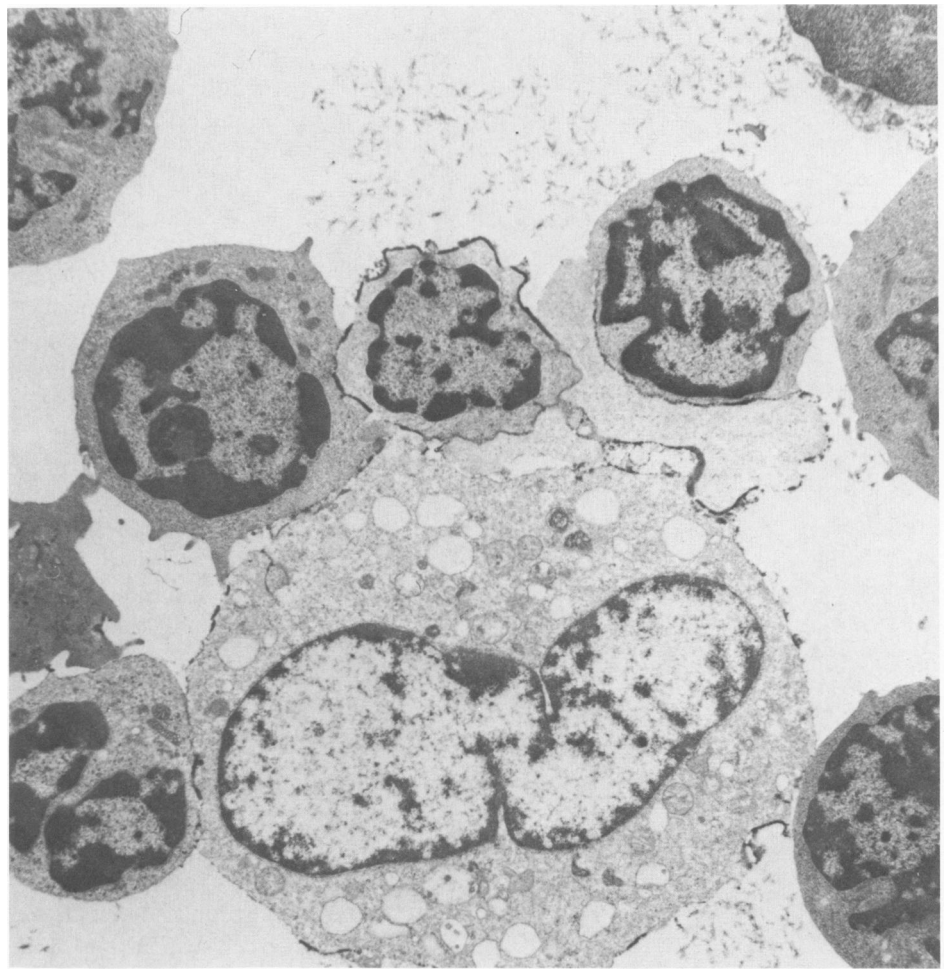

Figure 6 – Electron micrograph of MG thymus cell suspension demonstrating an Ia-positive epithelial cell (*large cell in center*) with Ia-positive and Ia-negative thymic lymphocytes. (×4080)

Discussion

Abnormalities of the thymus have long been implicated in the pathogenesis of MG. Castleman described germinal centers in the majority of MG thymuses.[1] Although germinal centers have also been observed in thymuses obtained at autopsy in nonmyasthenics, especially in accident victims (nonchronic disease controls),[8] the MG thymus has larger and more numerous germinal centers.[9] The favorable response of MG to thymectomy first noted by Blalock in 1939[2] further suggests a relationship between thymic abnormality and MG. Recently, MG has been shown to be an autoimmune disorder wherein the host's acetylcholine receptor is the target of autoantibody, as reviewed by Lisàk and Barchi.[10] The thymus has a central role in the immune system in the maturation of T cells to become self-restricted with respect to antigen–macrophage–helper cell priming responses or cytotoxic cell-target cell responses. Acetylcholine receptors, the apparent sensitizing antigen in MG, are expressed on myoid cells within the thymus.[2] Furthermore, the thymus is thought to be involved in maintenance of tolerance to self. One can hypothesize that a thymic abnormality may lead to altered immunoregulation with the production of autoantibodies.

In the studies reported here, we found no qualitative difference in the subset profiles and patterns for distribution of T cells in tissue sections of thymus from normals or MG patients. These patterns are similar to those observed by others in the normal thymus[11–13] and correlate with data from cell suspensions of MG thymic tissue as reported elsewhere by us[7,14] and by others.[15]

With regard to B cells, the germinal center staining pattern, with IgD-positive cells in the mantle zone and IgM-positive cells in the mantle and follicular center, is similar to the pattern found in lymph node germinal centers. In addition, scattered IgD- and IgM-positive cells are present in the medulla outside germinal centers. These findings suggest the presence of circulating B cells

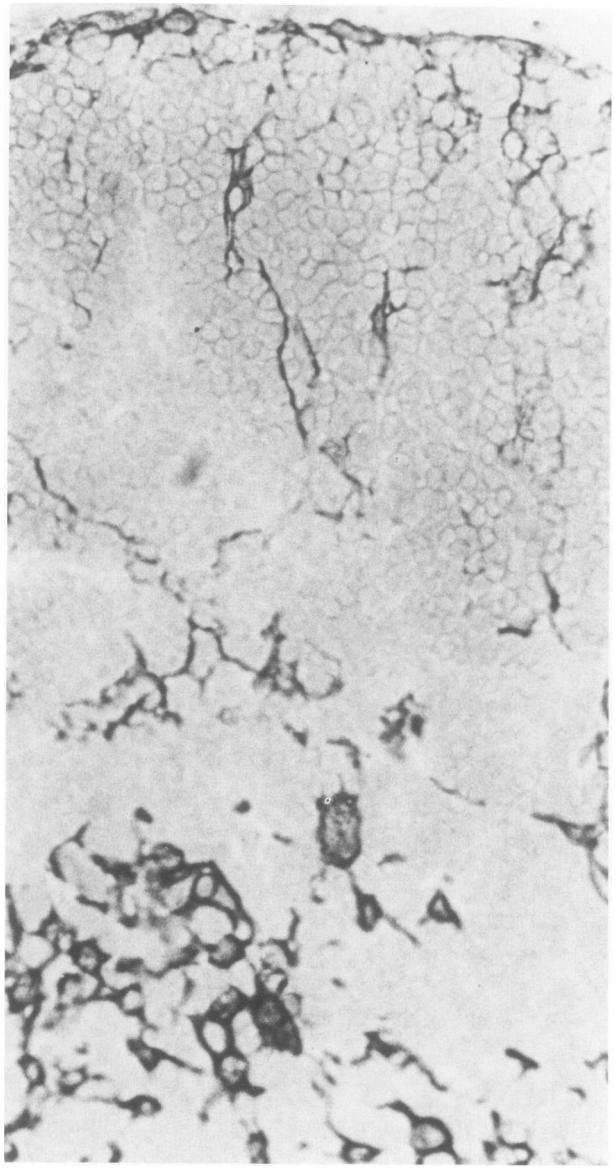

Figure 7 – Frozen section of MG thymus demonstrating localization of keratin. The dendritic pattern is similar to the pattern found for Ia antigen (see Figure 3). (Hematoxylin counterstain, ×400)

(surface IgD- and IgM-positive cells) within the thymic medulla, although the possibility of at least medullary B cells actually being located in "extrathymic" perivascular spaces, as suggested by Levine and Rosai, must still be considered.[16]

It is of interest to compare the data obtained from cell suspensions[7] and tissue sections. The prominence of B cells in the germinal centers of MG thymus in tissue sections was not reflected in the cell suspension data because the percentage of surface immunoglobulin (sIg)-positive cells was not significantly greater in the MG thymus (0.5%), compared with non-MG thymus (0.1%). Similarly, the predominance of T4-positive cells over T8- and T6-positive cells in tissue sections in medulla of both MG thymus and non-MG thymus was not reflected in the suspension data: T6, 64% ± 4.0% for MG and 70% ± 5.7% for control; T4, 51% ± 4.0% for MG and 48% ± 10% for control; T8, 59% ± 3.8% for MG and 53% ± 9.7% for control.[7,14]

Several possibilities may account for these observations. First, immunofluorescence, which was used for detection of lymphocyte subsets in cell suspension, is less sensitive than immunoperoxidase, which was used for tissue sections. Indeed, Bahn et al noted T4-positive cells in the medulla of the human thymus when they used immunoperoxidase but not when they used immunofluorescence on tissue sections.[11] We have preliminary data using the avidin–biotin immunoperoxidase technique on cytospin preparations from MG thymus cell suspensions. T4-positive cells were significantly more numerous with this technique than with immunofluorescence. However, the percentages of sIg-positive cells were similar (<1%) with immunoperoxidase and immunofluorescence. Thus, the difference in sensitivity between immunofluorescence and immunoperoxidase appears to contribute to the increased percentage of T4-positive cells observed in tissue sections over cell suspensions. However, this does not appear to account for the lack of significant numbers of surface Ig-positive B cells in suspension data.

Second, the very large contribution of cortical T cells to cell suspensions of thymic tissue may obscure differences in the prevalence of T4 and T8 within the medulla and the more prominent germinal-center B-cell distribution seen in tissue section of the myasthenic thymus. Indeed, removal of cortical thymocytes from cell suspensions of thymuses from children undergoing cardiac surgery resulted in a predominance of T4- over T8-positive cells in the remaining thymocytes.[17] Third, it is possible that heterogeneity among patients may account for the lack of significant differences between MG and control groups observed for B cells regarding suspension data. Finally, the method of cell suspension may be less capable of recovering follicular B-lymphocytes, because they are more tightly surrounded by reticulin fibers[16] and may be more difficult to obtain in suspension. These differences between data obtained by cell suspension and by tissue section emphasize the importance of both techniques in analyzing surface markers.

In thymus-cell suspension studies, the only surface marker significantly different in MG patients and controls is Ia antigen.[7,14,15] This antigen, coded for by the major histocompatibility complex and involved in antigen recognition by T-lymphocytes, appears to be pres-

Table 2—Quantitation of B Cells in the Thymus (% Positive Cells)*

	No. cases	IgD (%) Cortex	IgD (%) Medulla	IgM (%) Cortex	IgM (%) Medulla
MG	12	0	9 ± 7.0†	0	6 ± 5.4
Controls	3	0	6 ± 3.5	0	7 ± 2.9

* Not including germinal centers.
† Mean ± SD.

ent on increased numbers of suspended thymic cells in MG thymus, compared with control thymus.

We attempted to identify by immunohistologic means Ia-positive cells present in increased numbers in MG thymus. Our studies of tissue sections by light microscopy showed Ia positivity predominantly on cortical cells with long interdigitating processes and in a confluent pattern in the medulla. Better resolution at the ultramicroscopic level showed the Ia antigen mainly on nonlymphoid cells. Lymphoid cells generally had Ia positivity only on that portion of the plasma membrane in close apposition to Ia-positive nonlymphoid cells. This observation on localization of Ia antigen is similar to that reported in murine thymus[18,19] and normal human thymus.[20] Occasional lymphocytes in thymic cell suspensions examined ultrastructurally had diffuse, intense Ia positivity. These may be either B cells or activated T cells. The vast majority of Ia-positive cells examined ultrastructurally were nonlymphoid. Specific identification of this population is important, because the single most significant difference between the control and MG thymus was the increased percentage of Ia-positive cells in suspension of the MG thymus.[7]

By electron microscopy, these Ia-positive nonlymphoid cells were demonstrated to be both thymic epithelial cells and interdigitating dendritic cells. The nonquantitative characteristics of ultramicroscopic examination preclude enumeration of Ia-positive cells of different types, as compared with those in cell suspension. Yet it appears that the Ia-positive population is heterogeneous in the thymus of both MG patients and normal subjects and includes epithelial cells, interdigitating dendritic cells, and, to a lesser extent, lymphocytes. One method of distinguishing epithelial cells from other Ia-positive cells involves the use of antikeratin antibody.[21] We have shown that keratin-positive cells are present in similar distributions in MG thymus and control thymus. These cells appear to be thymic epithelial cells. Further studies employing antibody markers for epithelial cells and interdigitating dendritic cells[22] using double-labeling techniques are in progress and will further establish the nature of the Ia-positive cells.

In general, our findings confirm and extend the study of Thomas et al.[23] Like them, we report no abnormality in the distribution of T-cell surface phenotypes. Using electron microscopy and immunoperoxidase techniques, we were unable to confirm their observation with immunofluorescence of increased numbers of interdigitating dendritic cells in the MG thymus, compared with control thymus. Ia antigen is involved in recognition of antigen by T cells, and therefore increased numbers of Ia-positive cells in the MG thymus may be related to the autoimmune process in this disorder.

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