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Proteins with Epitopes of the Acetylcholine Receptor in Epithelial Cell Cultures of Thymomas in Myasthenia Gravis

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Thymomas from 12 patients with myasthenia gravis (MG) were investigated for the presence of epitopes of the alpha-subunit of the nicotinic acetylcholine receptor (AchR) using monoclonal antibodies (MAb) reacting against the AchR. In all but two of the tumors epitopes corresponding to antigenic determinants located on the cytoplasmic side of the AchR were identified. From eight thymomas cell lines were established that have been kept in culture for up to 6 months. The cultured cells expressed the same AchR-epitopes as did the primary tumors. During early passages the percentage of epithelial cells positive for the AchR epitopes approximately mirrored the percentage of positive cells in the original tumors. With passaging the relative number of positive cells usually declined but in some cultures an increase was observed. Three cell lines that showed extensive staining with an MAb against the AchR were radiolabeled to characterize the antigen. From protein extracts of these three cell lines proteins of 45 kd and 156 kd molecular weight (MW) were precipitated. These proteins are different from other proteins described in the context of both thymomas and MG. The negative reactivity with MAb against other epitopes of the alpha-subunit, especially against the main immunogenic region (MIR), speaks in favor of membrane-associated proteins of only limited crossreactivity to the AchR. A previous study found an almost exclusive occurrence of these AchR-epitopes in thymomas associated with MG, but not in other thymomas of similar histologic type. The expression of the proteins described here could therefore play a role in the triggering of the autoimmune process against the AchR of the motor endplate in MG patients. (Am J Pathol 1989, 134:865–877)

Myasthenia gravis (MG) is one of the best known human autoimmune diseases. The direct cause of the muscle weakness in almost all patients is the functional impairment of the neuromuscular junction by autoantibodies against the nicotinic acetylcholine receptor (AchR). Most patients exhibit thymic hyperplasia or a thymoma. However, the pathophysiologic link between these thymic abnormalities and the occurrence of autoantibodies against the AchR is not completely known.

The AchR of skeletal muscle is a pentameric ion channel composed of four kinds of subunits (structure $\alpha\beta\alpha\delta\gamma$[1]). The alpha-subunit contains the binding sites for both acetylcholine and the snake venom α-bun garotoxin (α-Btx). On the alpha-subunit a "main immunogenic region" (MIR) has been identified against which most of the autoantibodies of MG patients are directed.[2,3] In addition, the proliferation of autoreactive T cells isolated from peripheral blood or thymus of MG patients is stimulated by components of the alpha-subunit of the AchR different from the binding sites of anti-MIR antibodies.[4,5] In the 1970s the discovery of α-Btx-binding sites on thymic myoid and epithelial cells[6,7] was taken as evidence for the occurrence of intrathymic AchR. These AchR, particularly in thymomas, were thought to cause an immune response leading to the production of auto-antibodies against the AchR. However, it is now known that there exist both acetylcholine receptors that do not bind α-Btx, and α-Btx-binding structures that are not acetylcholine receptors.[8–10] Whether the cobrotoxin-binding protein recently isolated from thymomas of MG patients[11] is an AchR-like structure has yet to be resolved. Using a panel of monoclonal antibodies (MAb) we demonstrated the occurrence of some but not all epitopes of the alpha-subunit of the nicotinic AchR in particular thymic epithelial cells,

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Table 1. *Antibodies Used in This Study*

Antibody	Concentration or dilution applied	Producer animal	Antigen labeled	Source
35βH11	1:100	Mouse	54 kd keratins (present on all thymic epithelial cells)	Ref. 39
Ki-M 1	1:400	Mouse	Antigens on IDCs of thymic medulla, macrophages in thymic cortex and in germinal centers, monocytes	Ref. 40
Anti-HLA-A, B, C	1:2000	Mouse	Class I antigens of the MHC-complex	Bethesda Research Laboratories, Neu-Fsenburg, FRG
Anti-HLA-DR	1:2000	Mouse	Class II antigens of the MHC-complex	New England Nuclear Dreieich, FRG
MAb 147	0.6 μg/ml	Rat	Nicotinic AchR, α-subunit, cytoplasmic site. Binding to Torpedo AchR	Ref. 37
MAb 152 153 155	0.3 μg/ml 0.3 μg/ml 1.0 μg/ml	Rat	Nicotinic AchR, α-subunit, cytoplasmic site. Binding to Torpedo, human, calf, rat and mouse AchR	Ref. 37
MAb 195	0.5 μg/ml	Rat	Nicotinic AchR, α-subunit, extracellular MIR. Binding to human and bovine AchR	Ref. 38
MAb 203	0.4 μg/ml	Rat	Nicotinic AchR, α-subunit, extracellular MIR. Binding to human, bovine and Torpedo AchR	Ref. 38

and in epithelial cells of some thymomas.[12,13] Furthermore, we demonstrated a significant correlation between the expression of these epitopes and the presence of MG in patients with thymomas.[12] In the present paper we described the establishment of thymoma cell lines that enabled us to isolate two proteins with AchR-related antigenic determinants.

Materials and Methods

Materials

Twelve thymomas of myasthenia gravis patients were studied using cyrostat sections from snap-frozen tissue. The classification of the tumors followed the classification proposed by Müller-Hermelink and coworkers.[14,46] The residual nonneoplastic thymus was evaluated for the presence of either thymitis with lymphoid follicular hyperplasia or of diffuse B cell infiltration.[15]

The diagnosis of myasthenia gravis was based on clinical findings including electrophysiologic investigations and the determination of anti-AchR antibodies in the serum of the patients.

Immunohistochemistry

The antibodies used in this study are described in Table 1. Cyrostat sections (5 μ) were air-dried, fixed in acetone for 10 minutes at room temperature, and incubated for 30 minutes with one of the antibodies given in Table 1. These antibodies were diluted in TRIS buffer (pH. 7.4) containing 0.5% bovine serum albumine (BSA). After washing in TRIS buffer, sections were incubated for another 30 minutes with peroxidase-conjugated rabbit anti-mouse or anti-rat antibodies (Dako, Kopenhagen, Denmark) diluted 1:50 with phosphate-buffered saline (PBS, pH 7.4) containing 30% AB-Rh-positive human serum. After another rinse in TRIS buffer, the sections were incubated for 30 minutes with a peroxidase-conjugated goat anti-rabbit IgG (Sigma, Munich, FRG) diluted 1:50 with PBS (pH 7.4) containing 50% AB-Rh-positive human serum. The peroxidase reaction was performed with 0.015% H_2O_2 in the presence of 0.6 mg/ml 3,3'-diaminobenzidine in TRIS buffer (pH 7.6) following the method of Graham and Karnovsky.[16] To exclude nonspecific staining we included control experiments in which the specific first antibody was replaced by PBS. For an immunocytochemical characterization cells were grown on sterile slides for 2 days, washed in PBS, air-dried, and subjected to the same procedure as the cryostat sections.

Electronmicroscopy

Washed monolayers of epithelial cells were fixed with 2.5% glutaraldehyde dissolved in Na-cacodylate buffer (pH 7.2) and postfixed with 2% OsO_4 in phosphate buffer. After dehydration by a graded series of ethanol the monolayers were embedded in the culture dishes in Epon for

Table 2. *Clinical and Pathologic Findings in MG Patients with Thymic Epithelial Tumors*

Case no.	Sex	Age (y)	Invasiveness/metastases	Histologic type	AchR-epitope (MAb 153, 155)	Nonneoplastic residual thymus
19964	F	34	No invasion of adjacent structures, no metastases	Mixed thymoma	–	LFH
28441	F	71	No invasion of adjacent structures, no metastases	Mixed thymoma	+	Normal
13814	M	56	No invasion of adjacent structures, no metastases	Mixed thymoma	–	LFH
14382	M	49	No invasion of adjacent structures, no metastases	Predominantly cortical thymoma	+	Normal
15977	F	76	No invasion of adjacent structures, no metastases	Predominantly cortical thymoma	+	Normal
27602	M	54	No invasion of adjacent structures, no metastases	Predominantly cortical thymoma	+	Normal
5697/88	F	52	Invasion of pericard, no metastases	Cortical thymoma	+	Normal
6942/88	M	50	Invasion of pleura, no metastases	Cortical thymoma	+	Normal
32546	M	62	No invasion of adjacent structures, no metastases	Cortical thymoma	+	Normal
18683	M	53	Invasion of pleura, metastases to pleura	Well differentiated carcinoma	+	Normal
27920	M	45	Invasion of pleura and pericard, no metastases	Well differentiated thymic carcinoma	+	Normal
1494/88	M	37	Invasion of pleura and pericard, no metastases	Well differentiated thymic carcinoma	+	LFH

LFH; lymphofollicular hyperplasia.

48 hours. Ultrathin sections were stained with lead citrate and investigated with a Zeiss EM 902.

Epithelial Cell Cultures

Primary cell cultures were established within 2 hours after surgery. Thymomas were minced and passed through a cell culture sieve. Particles that sedimented within 5 minutes were digested further for 30 minutes with collagenase and hyaluronidase (each 2 mg/ml PBS (pH 7.4), Sigma). Particles that did not settle were directly placed in 75 sq cm flasks after washing in culture medium (DMEM, 10% FCS, 1% glutamine, 1% penicilline/streptomycin, GIBCO, Neu-Isenburg, FRG). Medium was changed twice per week. Confluent cultures were split with trypsin/EDTA and diluted 1:2. As controls in all staining assays and in the labeling experiments we used the human medulloblastoma cell line TE 671,[17] which contains a functional human nicotinic AchR,[18,19] and the human carcinoma cell line A 431 (ATCC), which is devoid of any reactivity detectable with the anti-AchR antibodies. These cell lines were maintained in the same medium as were the thymoma cell lines.

Biochemistry

For *in vivo* labeling 10^7 cells of each tumor cell line were placed in culture dishes in presence of a methionine-free medium containing 3% FCS. Cells were labelled overnight with 0.15 mCi/ml ^{35}S-methionine (Amersham, Braunschweig, FRG). Cells were then washed and scraped off the culture dish with a rubber policeman. This step and the next were performed on ice and in the presence of 1 mM PMSF as an antiproteolytic agent.

Cells were lysed by freezing in 0.1 mM triethylamine buffer (pH 7.0) and nuclei were removed by 10 minutes centrifugation at 1200*g*. The supernatant was further centrifuged at 33000*g* to separate membranes from cytosolic proteins. The membrane fraction was extracted firstly with TRIS buffer containing 0.2% Triton X-100 (first lysate) to remove the majority of proteins that stick to the membranes. Integral membrane proteins were then extracted

with TRIS buffer containing 1% Triton X-100. These second lysates were then incubated for 5 hours with anti-actin and anti-vimentin antibodies (Merck, Darmstadt, FRG) in the presence of excess of formaldehyde fixed and heat killed *Staphylococcus aureus* (Precipitin, BRL, Neu-Isenburg, FRG) to remove actin and vimentin (preclear step). After centrifugation at 12000*g* for 10 minutes the supernatants were incubated with MAb 155 and MAb 203 overnight. Antigen-antibody complexes were precipitated by staphylococcus aureus (Precipitin, BRL) and the precipitates eluted and denatured under reducing conditions (0.050 M TRIS buffer containing 2% SDS and 5% [w/v] 2-mercaptoethanol) by heating for 5 minutes to 95 C. Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis according to Laemmli.[20] Radioactive bands were visualized by autoradiography using Kodak XR films. Exposure times were between 2 and 3 weeks. There were three controls in the biochemical experiments: 1) The MAb 203, which does not stain thymic epithelial cells in culture but precipitates the AchR from lysates of the TE 671 cell line (Figure 5b, lane 2) was used in parallel with the MAb 155 in the precipitation step with lysates from cases #15977 and #18693. 2) The MAb 155 was applied in an immunoprecipitation experiment with a lysate from cells that were not stained with the MAb 155; because the thymoma cell lines that were devoid of AchR-epitopes (cases #13814 and #19964) did not proliferate in culture, the carcinoma cell line A 431 was used in this control. Because no proteins were precipitated in this experiment (Figure 5, lane 4) a nonspecific binding of the MAb 155 to a common epithelial antigen is excluded. 3) The TE 671 cell line was used as a positive control. As shown in Figure 5c, lane 2, the four different subunits of the human AchR were precipitated with the MAb 155, demonstrating the effectiveness of this control.

Results

Clinical and Pathologic Findings in MG Patients with Thymomas

Some clinical findings and histologic features of the investigated neoplastic and nonneoplastic thymic tissue are given in Table 2. All but two of the thymomas in this group of patients with myasthenia gravis expressed epitopes of the AchR. These epitopes were defined by the MAbs 152, 153, and 155, which have binding sites on the amino acid sequence α371–378 of the α-subunit of the torpedo californica and human AchR[21,22] and their binding on the intact AchR has been localized on its cytoplasmic[23] or intramembrane part.[45] MAb 147, which binds to the amino acid sequence α-360-370 of the torpedo-AchR[21,22] but exhibits no crossreactivity with the human AchR, did not bind to any thymoma investigated. MAb 195 and 203, which recognize the MIR, also did not bind to the tumors. The relative number of thymoma epithelial cells that bind the MAb 155 (expressed as grade 0–IV) is given in Table 3. The relative expression of AchR-epitopes did not correlate with the concentration of autoantibodies against the AchR in the respective patients ($r = 0.026$). Likewise, there was no HLA-determinant that was obviously correlated with either a high or a low grade expression of AchR-epitopes (not shown). Although the number of tumors is low, there seems to be a higher expression of AchR-epitopes in "well differentiated thymic carcinomas," cortical thymomas, and predominantly cortical thymomas than in mixed thymomas. As reported elsewhere,[46] the first group of tumors is more often associated with MG than are mixed thymomas.

In Vitro *Investigations*

Immunocytochemistry and Electronmicroscopy of Thymic Epithelial Cell Cultures

Of the tumors listed above, primary cultures could be established in all cases. The morphology of these cells (Figure 1a) indicated that these cultures consisted predominantly of epithelial cells. This was verified by staining the cells after the first passage with MAb against a cytokeratin (35βH11, Figure 1b). After the first passage more than 90% of the cells were positive with the MAb 35βH11. Except for epithelial cells, a few (<10%) interdigitating cells and macrophages were identified after the first passage by immunocytochemistry using the MAb Ki-M1. In contrast to primary cultures from normal thymuses and nonneoplastic thymuses from myasthenia gravis patients, early thymoma cultures did not contain any cells expressing desmin. Epithelial cells in culture express vimentin as intensively as do fibroblasts *in vivo* and *in vitro.* Therefore, fibroblasts had to be defined by the lack of expression of keratins, desmin, and reticulum cell markers. By morphology alone epithelial cells and fibroblasts could not be differentiated. On further passaging, cultured cells lost the polygonal shape and the close cell-to-cell contact typical of early cultured epithelial cells. This change in shape is shown in Figure 1c. Nevertheless almost all cells continued to express keratin and were therefore of epithelial origin (Figure 1d). At an electronmicroscopic level, cells from early and late passages exhibited desmosomes and tonofilaments as the typical features of epithelial cells (Figures 2, 3).

Proliferation of Thymoma Epithelial Cells In Vitro

The proliferation rate of epithelial cells *in vitro* was semiquantitatively estimated by determining the time between

Table 3. *Serum Concentration of Autoantibodies Against the AchR and Grade of Expression of AchR-Epitopes in Thymic Epithelial Tumors from Myasthenia Gravis Patients and in Early (Passages 1 to 3) and Late (Passages 4 to 15) Cell Cultures Derived from the Respective Tumors*

Tumor type	Case no.	Autoantibodies against AchR (nmol/l)	Grade* of expression of AchR epitopes		
			In the tumor	In early cell cultures	In late cell cultures
Mixed thymomas	19964	12.2	0	No proliferation	No proliferation†
	28441	12.5	I	No proliferation	No proliferation†
	13814	8.2	0	0	No proliferation
Cortical and predominantly cortical thymomas	14382	2.3	IV	IV	II
	15977	>1.0	IV	IV	I
	27602	3.8	IV	IV	I
	5697	24.1	II	II	I
	6942	4.2	IV	IV	I
	32546	2.7	III	III	II
Well differentiated thymic carcinomas	18683	16.7	I	I	IV
	27920	6.6	III	III	I–III‡
	1494	42.1	IV	IV	II

* The grade gives the approximate percentage of epithelial cells which express AchR epitopes (MAb 155): Grade I: <25%; II: 25 <50%; III: 50 <75%; IV: 75 to 100%.

† No confluency was achieved in primary cultures of mixed thymomas.

‡ The percentage of cells positive with the MAb 155 was 10% in the fourth passage and 60% in the tenth passage.

successive 1:2 splittings of confluent cell cultures. Of the 12 tumors investigated, 10 have been passaged for a maximum of 35 times and the cell line from case #1494 has been in culture for 8 months. The main findings (Table 4) are that mixed thymomas did not proliferate in culture whereas thymic carcinomas required splitting every second or third day and that predominantly cortical thymomas and cortical thymomas showed a slower proliferation *in vitro* than well differentiated carcinomas. Also the number of passages that could be achieved was highest with the carcinomas (10 passages with case #18683, 35 passages with case #1494). With the exception of case #1494, the thymic carcinomas also exhibited a decline of the *in vitro* growth rate with the number of passage. The cessation of growth was often indicated by the occurrence of Hassal bodylike structures in the culture dishes (Figure 1e).

AchR-Epitopes in Cultured Thymoma Epithelial Cells

Cultured thymoma epithelial cells did express the same AchR epitopes as the tumors from which they were derived as far as can be determined by our monoclonal antibodies. In addition, the percentage of epithelial cells that stained with MAb 155 was roughly similar in the tumor sections and the respectively early cultures (first to third passage, Table 3). In particular, the two tumors completely devoid of AchR-epitopes (cases #13814 and 19964) also were negative in culture (and case #13814 remained so during early passages), whereas the one tumor with all epithelial cells staining with the MAb 155 (case #15977) also was 95% positive in culture. As shown in Table 3, epithelial cells showed a gradual decrease of AchR-epitope expression in almost all cultures. However, in one case (case #18683), 10% of the cultured cells were positive with MAb 155 in the first and fourth passage and even increased to about 80% at the ninth. A similar but fluctuating expression of AchR-epitopes also was observed in case #27920.

The typical staining pattern of thymoma epithelial cells in culture stained with the MAb 152, 153, and 155 is given in Figure 4a. Immunoreactivity seems to be arranged in an extended paranuclear network of filaments that resembles that of intermediate filaments (Figure 4b). In fact, the procedures used here to prepare cellular membranes do not prevent the copurification of cytoskeletal proteins with the AchR.[41–43] However, a staining pattern similar to that encountered in thymoma cells also was seen in TE 671 cells with either the MAb 152, 153 and 155 (Figure 4c) or the anti-MIR MAb 195 and 203 (not shown), although the epitopes defined by these MAb are parts of intact AchR and not of filamentous structures.[18] Therefore it cannot be decided by our staining technique and on the light microscopic level whether the AchR-epitopes in cultured thymoma epithelial cells are parts of the cytoskeleton or of integral membrane proteins. Using unfixed cultured thymoma epithelial cells, we found that MAb 152, 153 and 155 do not stain the cells. Therefore, the AchR-epitope defined by these antibodies is not expressed on the cell surface.

Biochemical Study of the AchR-Epitope Containing Proteins

The proteins of thymoma cell cultures that gave a strongly positive staining with the MAb 155 were la-

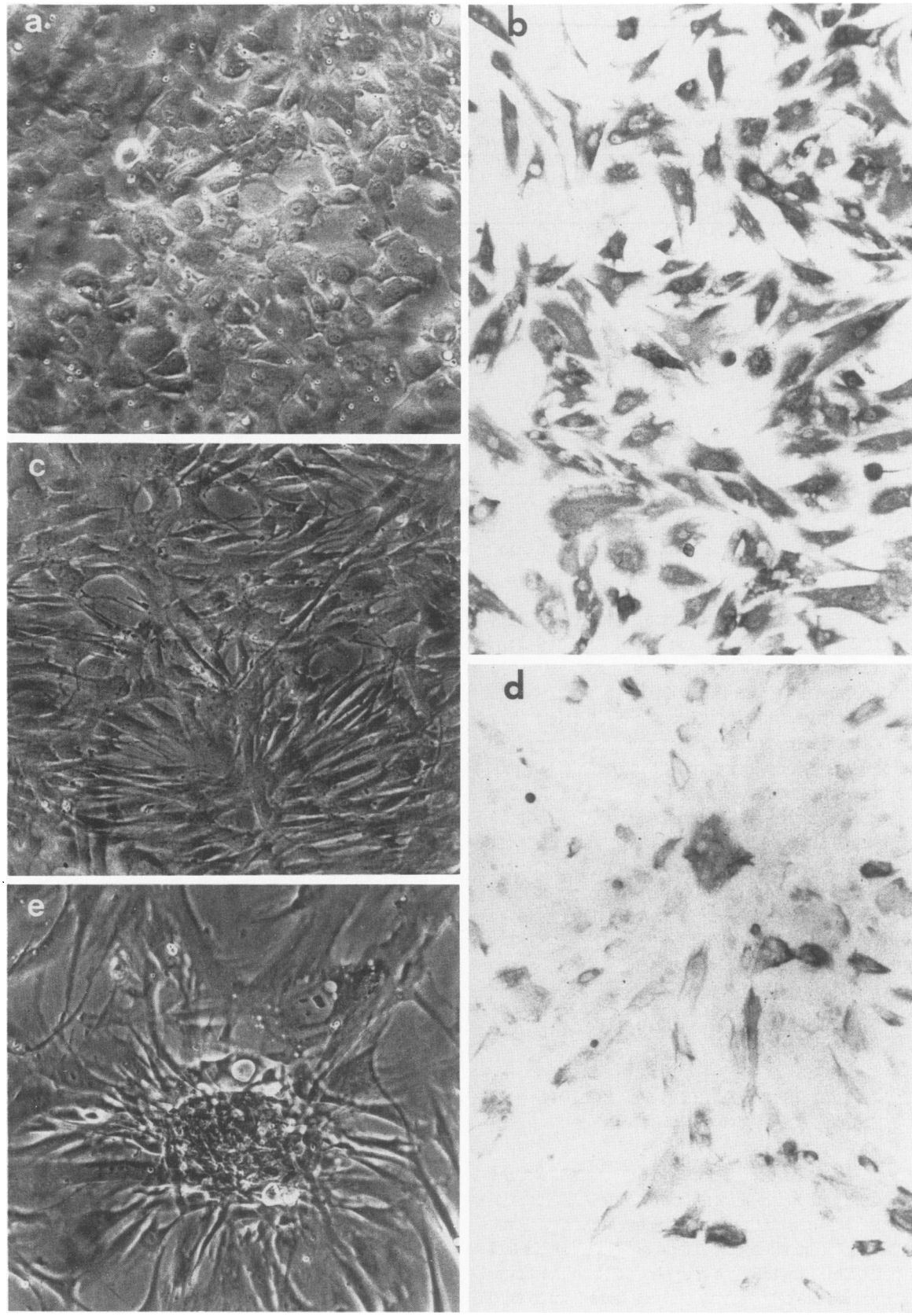

Figure 1. *Epithelial nature of cell lines derived from thymomas of MG patients.* **a:** *Phase contrast picture of a primary culture (P_0)* of polygonal cells derived from a thymic carcinoma (case #1494). **b:** *Almost all of these cells (case #1494) expressed keratin (35βH11) after passaging (P_1).* Grade of expression of AchR-epitopes in P_1 was Grade IV. **c:** *Cells of case #1494 became spindle shaped with further passaging (P_{10}).* **d:** *Cells in P_{10}* continued to express keratin, though to a lesser extent than in P_1. At the same time the grade of expression of AchR-epitopes was grade II. **e:** *Hassal bodylike structure which often indicates the cessation of growth of the cells in culture (case #6942, P_6).*

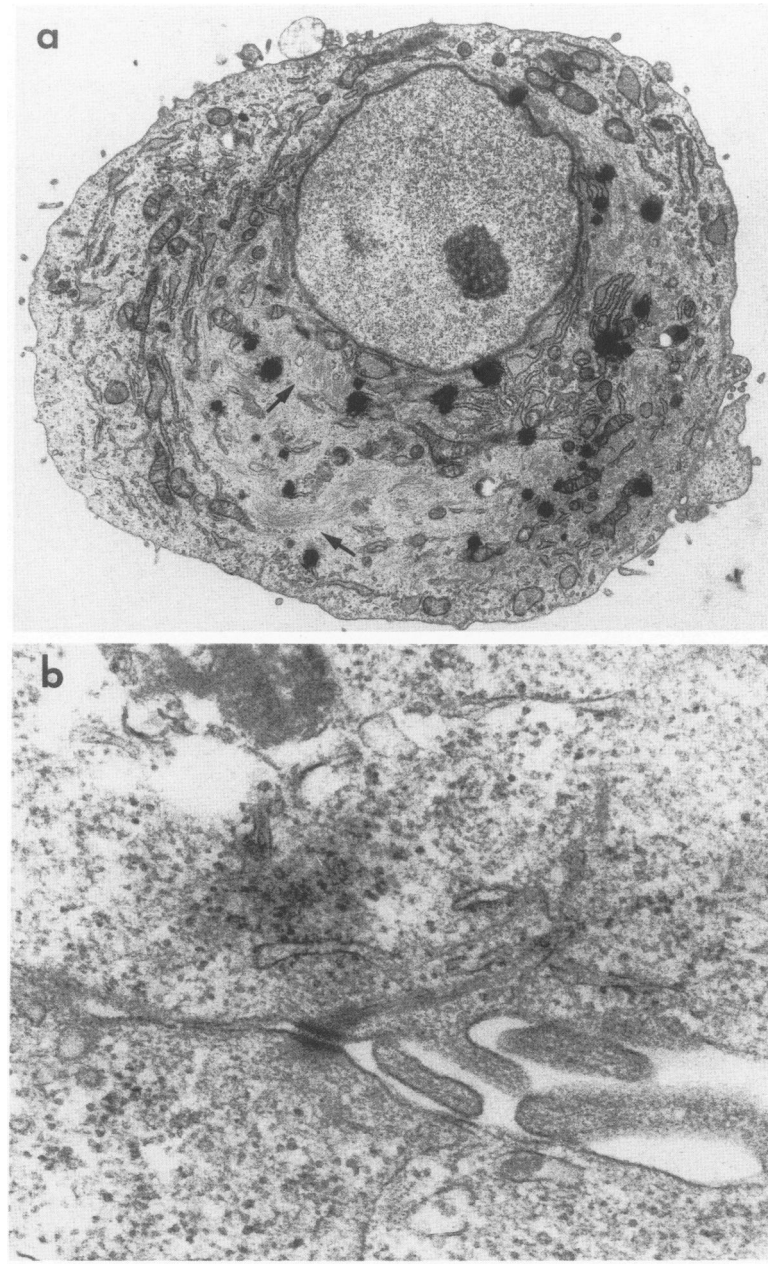

Figure 2. *Electronmicrograph of thymoma epithelial cells in early culture (P_1).* Tonofilaments (**a**, *arrows, ×1200) and desmosomes* (**b**, *×1200) as typical features of epithelial cells derived from a thymic carcinoma (case #1494).*

beled with ^{35}S-methionine. In case of thymoma #14382, the antigen was directly precipitated from a whole cell Triton extract by means of the MAb 155. In this tumor, a single antigen with a molecular weight of about 45 kd was detected. For the thymomas #15977 and #18683 cellular membranes were separated from cytosolic proteins and nuclei first. The separated membranes were used in the immunoprecipitation step. As shown in Figure 5a (lanes 3 and 6), a protein of molecular weight of about 45 kd was precipitated with MAb 155 in both cases. In addition a second protein of molecular weight of about 156 kd was found in these two lysates. The specificity of the precipitation of these proteins with the MAb 155 is demonstrated by the lack of these two bands in the control experiments applying the MAb 203 (Figure 5a, lanes 4 and 7).

Discussion

The present investigation confirms the previous finding by Kirchner et al[13] that the immunohistochemical properties of the AchR-epitopes on certain medullary epithelial cells of normal thymuses and on epithelial cells of thymomas from MG patients are qualitatively the same. However, in normal thymuses these epitopes never occur on cortical

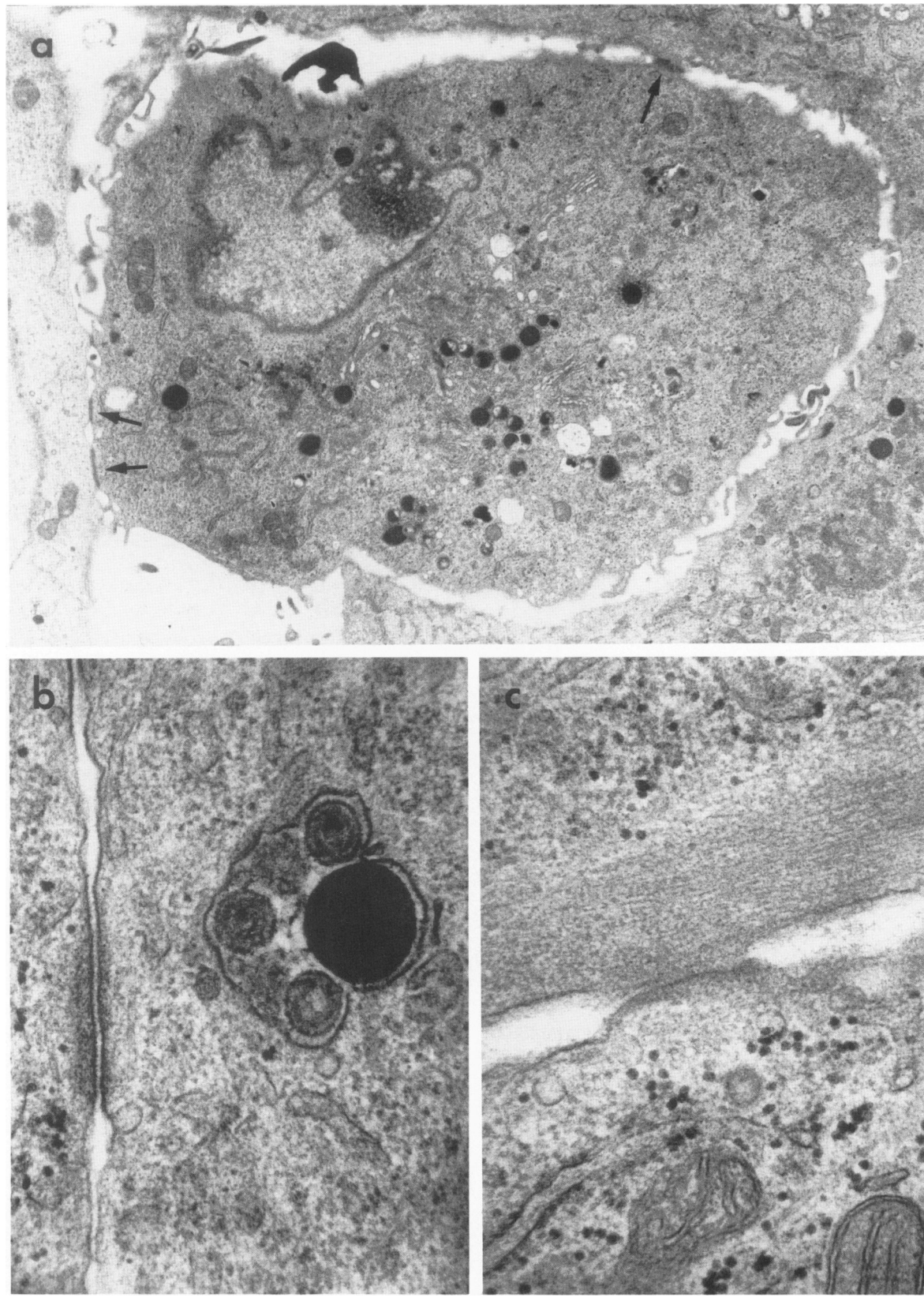

Figure 3. *Electronmicrograph of thymoma epithelial cells in long term culture (P_{15}).* Desmosomes (**a**, *arrows, ×1200;* **b**, *×30,000) and tonofilaments* (**c**, *×30,000) as typical features of epithelial cells derived from a thymic carcinoma (case # 1494).*

epithelial cells, whereas in thymomas the AchR-epitopes are most probably coexpressed with cortical epithelial cell markers. This atypical expression of a phenotypic marker of medullary epithelial cells in neoplastic cortical epithelium of thymomas also confirms previous investigations demonstrating the expression of cortical type and medullary type epithelial cell markers in epithelial cells of some thymomas.[24]

So far there have been no reports in the literature on the successful culture of neoplastic thymic epithelial cells from MG patients. In one investigation a failure of growth of epithelial cells from thymoma explants was reported whereas growth was achieved from nonneoplastic thymic explants of MG patients.[25] Interestingly, the morphology (Figure 1a) and ultrastructural features of the thymoma epithelial cells *in vitro* (Figures 2 and 3) were not significantly different from nonneoplastic epithelial cells derived from normal thymic explant cultures.[25–27] In addition we noted a stable expression of keratin (Figure 1b, d) and HLA class I molecules (not shown)[47] and the rapid loss of HLA class II molecules during primary culture. This finding is similar to nonneoplastic thymic epithelial cells.[28] The main difference between these former reports and the present investigation concerns the proliferative properties. With normal thymic epithelial cells an expansion of the number of cells beyond the primary culture could not be achieved whereas our thymomas could be propagated up to the 35th passage. This difference is probably not due to different culture conditions but due to different biologic properties of the epithelial cells. This idea is supported by the significant correlation observed between the proliferation rate *in vitro* and the tumor type (Table 4). In agreement with this, mixed thymomas that follow a slowly progressive clinical course[46] did not proliferate under our culture conditions. The well-differentiated thymic carcinomas, however, grew better than the cortical and the predominantly cortical thymomas. Therefore, these functional investigations lend support to the classification of thymomas that was originally based on histologic and clinical findings alone.[14,46]

It was of major interest that the approximate quantity and the phenotype of the AchR-epitope (as defined by our panel of MAbs) remained stable up to the third passage (Table 3) in cultured thymoma epithelial cells. We therefore used these cell cultures as tools to investigate the AchR-epitope expressing proteins. These experiments demonstrated that these proteins have molecular weights of 45 and 156 kd and that they are associated with cellular membranes. In addition, the AchR-epitope was found to be an intracellular antigen because our MAbs reacted only with fixed but not with live cells. It cannot be decided, however, whether the AchR-epitope is part of a transmembrane protein or of intracellular structures. Because we have not been able to culture enough

Table 4. *Proliferation of thymoma epithelial cells in vitro* *

Tumor type	Case no.	Time between two successive 1:2 splittings of: Early cultures	Late cultures
Mixed thymomas	19964	only P_0	–
	28441	only P_0	–
	13814	1–3 w	–
Cortical and predominantly cortical thymomas	14382	6 d	1–4 w
	15977	4 d	5 d–4 w
	27602	5 d	5 d–4 w
	5692	2 w	3 w
	6942	6 d	1–3 w
	32546	4 d	4 d–2 w
Well differentiated thymic carcinomas	18683	2 d	2 d–4 w
	27920	3 d	3 d–2 w
	1494	3 d	4 d

* Time (d = days, w = weeks) between two successive 1:2 splittings of confluent cell cultures is taken as a parameter of the proliferation rate. Early cultures are passages 1 to 3, late cultures are passages 4 to 15. P, primary culture.

epithelial cells from normal thymuses we do not know whether the AchR-epitope is a component of similar proteins in the neoplastic epithelial cells of thymomas and in the epithelial cells of the thymic medulla.

Both the 45 and 156 kd protein seem to be different from the proteins thus far described in the context of the pathogenesis of MG. Apart from the molecular weight,[29] the 45 kd protein described here does not share other characteristics with the α-subunit of the AchR. In contrast to the AchR found on myoid cells, the 45 kd protein does not express epitopes of the MIR. This is in agreement with previous immunohistochemical findings.[30,13] Unlike the AchR or other products of this gene family,[31–33] the 45 kd protein is probably not part of a compound consisting of different subunits; in all three thymoma cell lines, only one protein in the 40 to 70 kd range was precipitated, whereas all four subunits of the AchR were precipitated from our control cell line (Figure 5c). For the 156 kd protein described here, a relationship to the AchR apart from cross-reacting epitopes is even more unlikely. In addition, we are not aware of any protein with a similar molecular weight considered in the pathogenesis of MG.

As for the pathogenetic role of the two proteins described here, it is of interest that the MAb 155, which was used to isolate the proteins, shows a faint crossreactivity with striated muscle[13] but not with epithelial tumors other than thymomas. In fact, antigens shared by both muscle and thymoma epithelial cells have already been described and may play a critical part in the autoimmune process that leads to the production of anti-striated auto-antibodies in thymoma patients.[34–36] Most of these antigens, such as α-actinin, myosin, tropomyosin, and the proteins described by Dardenne and coworkers,[35] have molecular

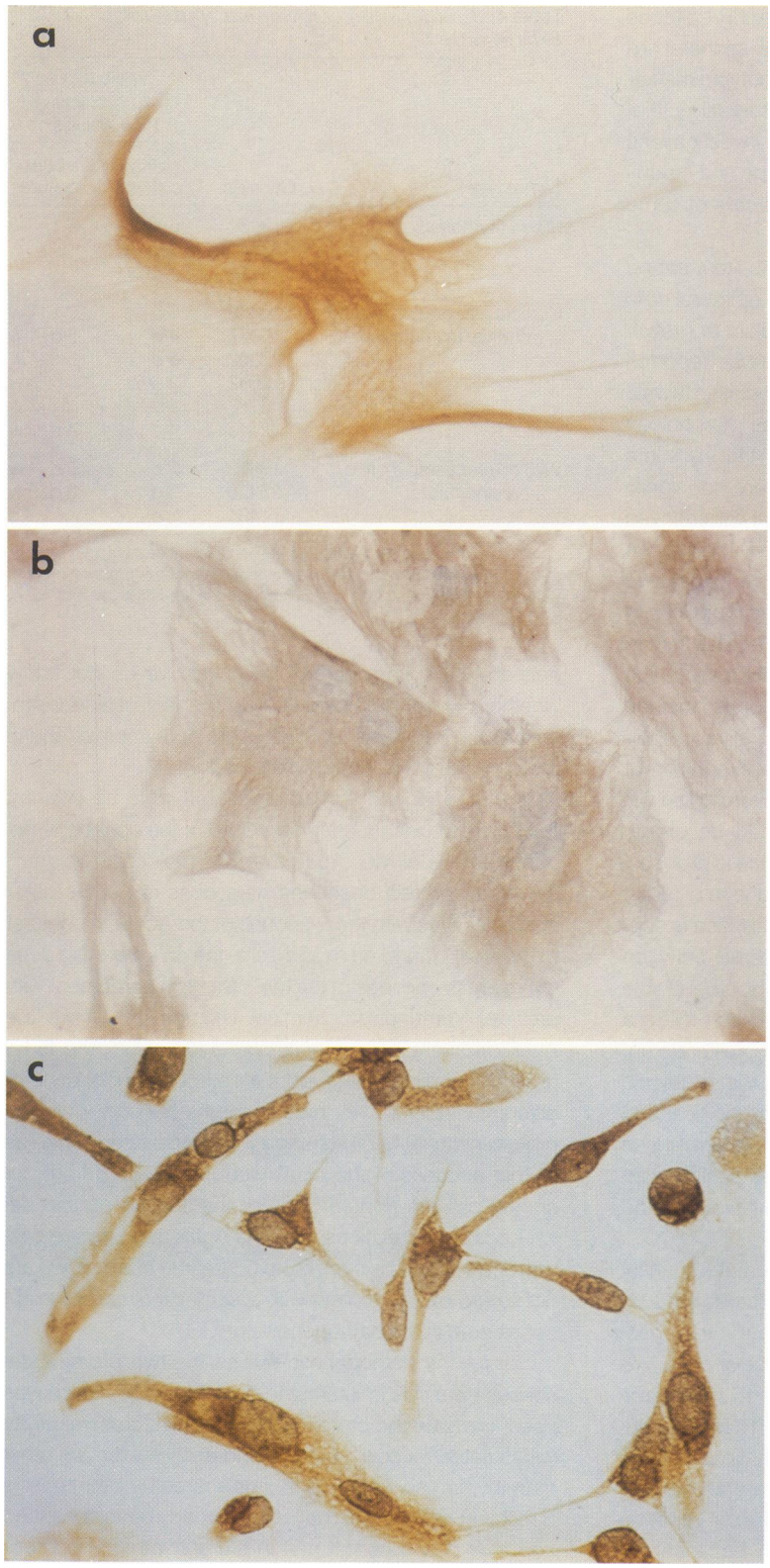

Figure 4. *Staining pattern of thymoma epithelial cells* (a) *and of cells of the TE 671 cell line* (c) *stained with the MAb 155. Arrangement of immunoreactivity in an extended filamentous network. A similar arrangement is observed when thymoma epithelial cells are stained by the antikeratin antibody 35βH11* (b).

weights different from 45 and 156 kd. Actin can have an apparent molecular weight of 45 kd depending on gel running conditions, but we have tried to exclude the unspecific precipitation of actin by a preclear step including anti-actin antibodies. In none of these have antigens shared by thymoma epithelial cells and muscle AchR-epitopes

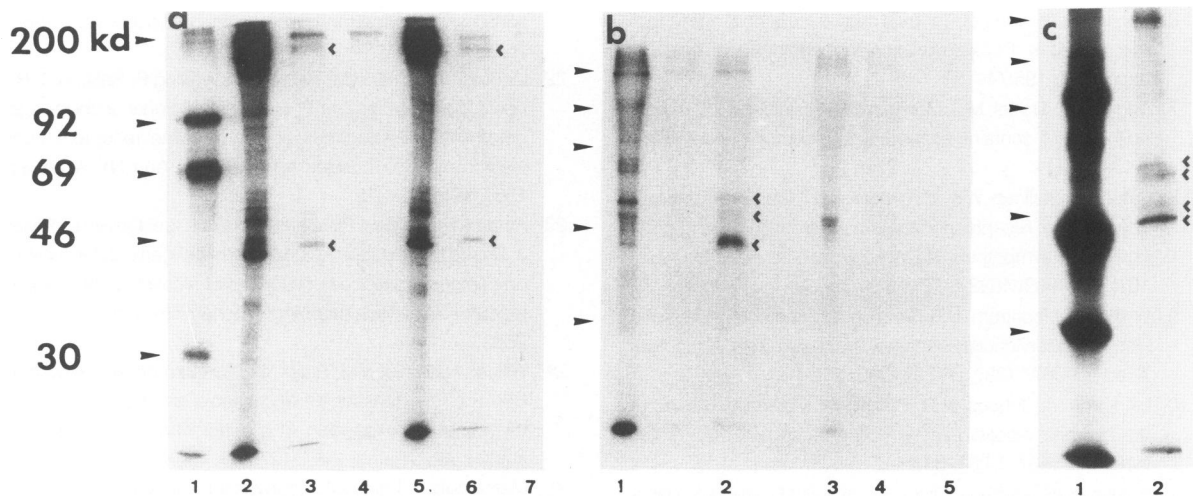

Figure 5. *Autoradiographs of ^{35}S-methionine labeled proteins immunoprecipitated from protein extracts of cellular membranes of thymoma epithelial cells and control cell lines. The cell lines of case #15977 and #18683 were labeled in P_1 and P_3, respectively. Separation of proteins was performed by 10%-SDS-PAGE. Arrows on the left side of each figure indicate the position of the molecular weight markers given in panel a.* a: *Immunoprecipitation experiment with protein extracts of thymoma epithelial cell lines from case #15977 (lanes 2 to 4) and case #18683 (lanes 5 to 7). Precipitation by the MAb 155 of a 45 and a 156 kd protein from protein extracts from case #15977 (lane 3) and case #18683 (lane 6). No precipitation is achieved with the MAb 203 (lanes 4 and 7). Lanes 2 and 5 are the proteins from cases #15977 and #18683 precipitated in the preclear steps. The molecular weight markers are in lane 1.* b: *Immunoprecipitation experiment with protein extracts of the A 431 cell line (negative control). No precipitation is achieved with the MAbs 155 and 203 (lane 4 and 5). In a parallel experiment the MAb 203 immunoprecipitates the α-, γ- and δ-subunit of the TE 671 cell line (positive control, lane 2). The lack of the β-subunit is probably due to the well known high susceptibility of the β-subunit to proteolytic degradation.*[44] In lanes 1 and 3 are the proteins from the TE 671 and A 431 cell lines precipitated in the preclear step. c: *Immunoprecipitation by the MAb 155 of the α-, β-, γ-, and δ-subunit of the AchR from protein extracts of the TE 671 cell line (positive control, lane 2). In lane 1 are the molecular weight markers.*

been demonstrated. These proteins are different from the new proteins described here in that the former both trigger the autoimmune process and express the epitopes to which the autoantibodies are directed,[35,36] which has not been found in our patients' sera so far (unpublished results). The 45 and 156 kd protein also lack the most important target epitope of the autoantibodies of MG patients—the MIR of the AchR. However, this peculiarity of the two proteins does not exclude their eventual role in the pathomechanism of MG. In fact, it has been demonstrated recently that the epitopes recognized by autoreactive T helper cells are different from the epitopes to which the respective autoantibodies are directed.[4,5] Our working hypothesis, therefore, is that the proteins described here trigger the autoimmune process by expressing recognition sites for autoreactive T cells but not binding sites for autoantibodies. With these two novel proteins available in sufficient quantities, this hypothesis should be a testable one.

Finally, we report for the first time that there are some MG patients with thymomas (cases #19964 and #13814) who do not exhibit any immunoreactivity with the MAb 152, 153, and 155 in their thymomas. Interestingly, these two patients exhibited lymphoid follicular hyperplasia in their nonneoplastic thymic remnants (Table 2), as does a particular group of MG patients without thymoma.[2] It is therefore tempting to speculate that MG in this minority of thymoma patients has another pathogenesis than MG in patients with thymomas expressing AchR-epitopes.

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