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Expression of Neurofilaments and of a Titin Epitope in Thymic Epithelial Tumors

Implications for the Pathogenesis of Myasthenia Gravis

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Autoantibodies against both striated muscle proteins, particularly titin, and the acetylcholine receptor are a hallmark of thymoma-associated myasthenia gravis. However, the stimulus for these responses remains enigmatic as whole titin is not detectable in these tumors. This study reports that in thymomas with cortical differentiation many of the neoplastic epithelial cells expressed low and medium molecular weight neurofilaments detected with several antibodies (on sections and blots) and at the RNA level (by reverse transcriptase polymerase chain reaction). Moreover, higher molecular weight forms sharing at least one epitope with titin were detectable slightly less frequently, as were the more strongly phosphorylated epitopes. In stark contrast, in medullary and mixed thymomas, and especially in the normal thymus, immunoreactivity with anti-neurofilament antibodies was rare. This aberrant overexpression of a titin epitope by epithelial cells with antigen-presenting phenotype in an inappropriate cortical microenvironment suggests that they might autosensitize maturing T cells there and so initiate anti-titin autoimmunity in these patients. (Am J Pathol 1996, 148:1839–1850)

Myasthenia gravis (MG) is an autoimmune disease caused by autoantibodies against the acetylcholine receptor (AChR) at the neuromuscular junction.[1–3] In approximately 10% of patients, there is an associated thymoma or well differentiated thymic carcinoma,[4–6] and they almost always have autoantibodies against striated muscle proteins,[7–11] particularly titin.[12,13]

Thymomas and well differentiated thymic carcinomas are epithelial tumors (TETs) that share morphological features with the normal thymus.[4–6] Moreover, they usually retain the uniquely thymic functions of attracting pre-T cells and promoting their maturation, although, in the process, they may induce T cell autoreactivity.[5,6,14,15] The most intriguing puzzle is its strong bias toward the AChR and striated muscle proteins as complete AChR[16–18] and titin[14,15] molecules are not expressed in TETs. However, the identification of epitopes from these proteins in neoplastic epithelial cells[9,10,16,19] is an exciting advance, although the molecular basis of these cross-reactivities has only partially been resolved.[10,19]

In the present investigation we identify neurofilaments (NFs) and a titin-like epitope that are aberrantly expressed in cortical-type TETs. Because these molecules share even more epitopes,[20,21] we suggest that their abnormal expression in thymomas might trigger anti-titin autoimmunity.

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Table 1. *Clinical and Pathological Findings in the Patients Whose Thymic Epithelial Tumors Were Investigated*

Case	Sex/age (years)	Diagnosis	Tumor stage*	MG†	Anti-AChR autoantibody titers‡	Immunosuppressive treatment[d]
31858- 91	NA	MDT	I	–	Negative	None
3028- 93	M/83	MDT	I	–	Negative	None
24998- 94	F/84	MDT	I	–	Negative	None
29117- 86	F/64	MXT	I	+	Low	None
19964- 87	F/34	MXT	I	+	Medium	None
11688- 89	F/69	MXT	I	+	Medium	None
8787- 90	M/32	MXT	I	+	Medium	None
16085- 90	F/43	MXT	I	+	NA	None
2306- 91	M/42	MXT	I	+	Medium	None
13578- 91	M/42	MXT	I	+	Low	None
14382- 87	M/49	PCT	I	+	Medium	None
27602- 87	M/54	PCT	I	+	Medium	None
32546- 87	M/87	CT	I	+	High	–
15977- 87	F/76	CT	II	+	Medium	Steroids
6942- 88	M/50	CT	III	+	High	None
20798- 89	M/38	CT	II	+	High	None
19490- 92	F/54	CT	III	+	High	None
1793- 93	M/45	CT	III	+	High	None
H674- 93	M/18	CT	II	+	Medium	None
1665- 94	F/54	CT	IV	+	Low	None
23169- 93	M/39	CT	III	–	Negative	None
18683- 87	M/53	WDTC	IV	+	High	None
27920- 87	M/45	WDTC	III	+	High	None
1494- 88	M/38	WDTC	III	+	High	None
22401- 88	F/63	WDTC	III	+	Medium	None
H1942- 91	F/78	WDTC	III	NA	NA	None
15904- 91	F/72	WDTC	III	+	Medium	Steroids
2080- 92	M/71	WDTC	III	+	High	None

NA, not available; MDT, medullary thymoma; MXT, mixed thymoma; PCT, predominantly cortical thymoma; CT, cortical thymoma; WDTC, well differentiated thymic carcinoma.

*Stage according to Masaoka.[23]

†Absence (–) or presence (+) of MG.

‡Low, 0.5 to 5 nmol/L; medium, <5 to 20 nmol/L; high, > 20 nmol/L.

Materials and Methods

Materials

Twenty-eight TETs were studied using cryostat sections from snap-frozen tissue obtained on ice within 0.5 to 4 hours of surgery. TETs were classified according to Müller-Hermelink and co-workers.[4–6,22] Tumors were staged according to invasiveness following the proposal of Masaoka et al.[23] Non-neoplastic thymuses (n = 12) were obtained from patients undergoing thoracic surgery (six with and six without MG; age range, 5 days to 68 years). The clinical diagnosis of MG was confirmed electromyographically in all cases and serologically in almost all (Table 1). To test the binding of anti-NF monoclonal antibodies (MAbs) on sections or blots, a ganglioneuroma was used as a positive control. The cervical carcinoma cell line A431 and a squamous cell carcinoma of the lung served as negative controls in blots. Furthermore, all MAbs were applied on frozen sections of skin from the neck and palm, esophagus, gastrointestinal mucosa, and cornea to exclude nonspecific binding to keratins. For the polymerase chain reaction (PCR) analysis of NF gene transcription, a sciatic nerve obtained at autopsy served as positive control and A431 cells served as negative control. Clinical and pathological findings of the patients investigated are given in Table 1.

Immunohistochemistry and Immunofluorescence

The antibodies are listed in Table 2. Apart from MAb63/15, a mouse MAb against fish titin, all of the MAbs had mouse anti-human NF specificity; we also used a guinea pig anti-human keratin antiserum (1:20; Sigma Chemical Co., Deisenhofen, Germany). The four-step immunoperoxidase labeling for single antigens in air-dried, acetone-fixed sections was described previously.[24] For double labeling, similar sections were incubated for 30 minutes with one of the MAbs given in Table 2, diluted in Tris buffer (pH 7.4) containing 0.5% bovine serum albumin (BSA). After washing in Tris buffer, sections were incubated for 30 minutes with fluorescein isothiocyanate (FITC)-

Table 2. *Antibodies Used in This Study*

Antibody	Working dilution	Specificity	Reference/ source
NR4	1:200	68-kd NF (P-I)	25
2F11	1:50	68- + 200-kd NF (P-D)	26
NN18	1:50	160-kd NF (P-I)	27
BF10	1:40	160-kd NF (P-I)	28
N52	1:50	200-kd NF (P-I)	27
RT97	1:100	200-kd NF (P-D)	28
NE14	1:20	200-kd NF (P-D)	25
63/15	1:100	160- + 200-kd NF (P-I) + Titin	21
T11	1:50	Titin	29
Leu6	1:800	$CD1^+$ T cells	Becton, Dickinson (Munich, Germany)

NR4, NN18, BF10, NF2, RT97, and NE14 are from Boehringer Mannheim (Mannheim, Germany); 2F11 is from Dako (Glostrup, Denmark); and T11 is from Sigma Chemical Co. (Deisenhofen, Germany).
P-I, phosphorylation-independent; P-D, phosphorylation-dependent.

labeled rabbit anti-mouse Ig (Dako, Glostrup, Denmark) diluted 1:100 in Tris buffer plus 30% AB/Rh-positive human serum. After washing in Tris buffer, sections were incubated for 30 minutes with a guinea pig anti-keratin serum (Sigma) diluted 1:20 in Tris buffer containing 10% each of rabbit and AB/Rh-positive human serum. After another wash, sections were incubated with Texas-Red-labeled goat anti-guinea-pig Ig (Dianova, Hamburg, Germany) diluted 1:50 in Tris buffer and 10% each of mouse and rabbit serum. As controls, the respective first antibodies in each of the two steps were omitted, excluding cross-reactivities of the FITC- or Texas-red-labeled sera. Specimens were investigated under a Leitz immunofluorescence microscope.

Immunoelectron Microscopy

Tumor pieces of 1 mm diameter were fixed for 4 hours at 8°C in a phosphate-buffered 4% paraformaldehyde/0.05% glutaraldehyde/15% saturated aqueous picric acid solution (pH 7.4). After successive dehydration in ethanol (up to 100%), the tissue was embedded in LR-White resin (hard grade; Science Services, Munich, Germany) for 18 hours at 20°C followed by polymerization for 24 hours at 50°C. Ultra-thin sections were incubated with MAb N52 in 0.5% BSA in 0.01 mol/L Tris buffer (pH 7.6) overnight, washed with 0.01 mol/L Tris buffer and then incubated with a gold-labeled (20-nm) goat anti-mouse IgG (Aurion, Wageningen, The Netherlands) in 0.5% BSA/0.1% gelatin in a 0.01 mol/L Tris buffer (pH 7.6). After extensive washing, sections were postfixed for 10 minutes in 2% glutaraldehyde in 0.01 mol/L Tris buffer, counterstained with uranyl acetate and lead citrate, and examined with a Zeiss EM 902 electron microscope.

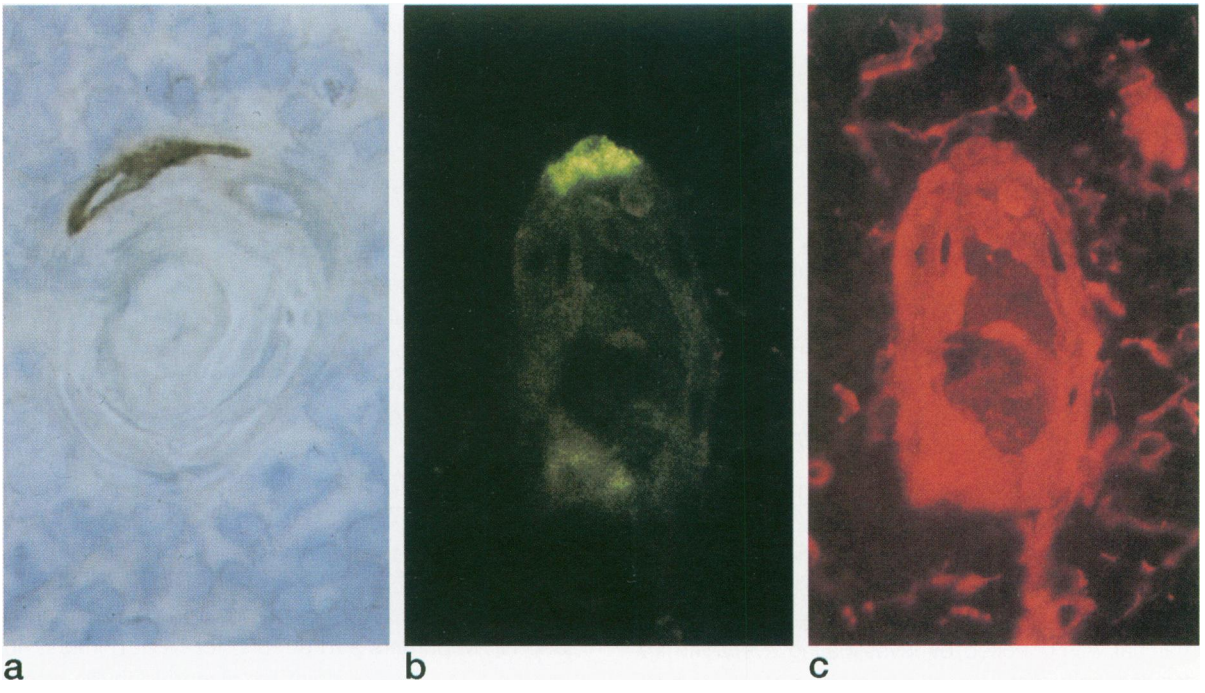

Figure 1. *Labeling of normal thymic medullary epithelial cells with anti-neurofilament MAb N52.* **a**: *Immunoperoxidase staining of a spindle-shaped cell close to a Hassall's corpuscle.* **b** *and* **c**: *Double immunofluorescence staining of medullary epithelial cells with MAb N52* (**b**) *and an anti-keratin serum* (**c**). *Magnification,* ×250.

Table 3. *Immunoreactivity of Thymomas for NF and/or Titin Epitopes and Comparison with NF RT-PCR*

Tumor type and case number	NF-L (68 kd)			NF-M (160 kd)			NF-H (200 kd)			
	NR4 P-I	2F11* P-D	RT-PCR	NN18 P-I	BF10 P-D	RT-PCR	N52 P-I	RT97 P-D	RT-PCR	63/15† P-I
MDT										
91-31858	−	−	ND	−	−	ND	−	−	ND	−
3028-93	−	−	ND	−	−	ND	−	−	ND	−
24998-94	−	−	−	−	−	+	−	−	+	−
MXT										
29117-86	−	−	ND	−	−	ND	+[f]	−	ND	+[f]
19964-87	−	−	ND	−	−	ND	−	−	ND	−
11688-89	−		ND	−	−	ND	+[f]	−	ND	+[f]
8787-90	−	−	ND	−	−	ND	+[f]	−	ND	−
16085-90	−	−	−	−	−	+	−	−	−	−
2306-91	−	−	−	−	−	+	+[f]	−	+	+[f]
13573-91	−	−	−	−	−	++	−	−	+	−
PCT										
14382-87	−	−	ND	−	−	ND	+[f]	−	ND	+[f]
27602-87	+[f]	+[f]	ND	+	+	ND	+	−	ND	+[f]
CT										
32546-87	+++	+[f]	ND	++	++	ND	+++	−	ND	++
15977-87	++[w]	−	+	++[w]	++	++	−	−	−	+[f]
6942-88	++[w]	−	+	++[w]	++	++	+[f]	−	+	+[f]
20798-89	+	−	ND	+	+	ND	−	−	ND	−
19490-92	+++	−	ND	+	+	ND	+	−	ND	+
1793-93	+++	−	ND	+	+	ND	+[f]	−	ND	+
23169-93	+++	−	+	+	−	++	−	−	−	−
1665-94	++	−	ND	−	−	ND	−	−	ND	−
WDTC										
18683-87	+++	−	ND	+	+	ND	−	−	ND	+
27920-87	++	−	ND	+	+	ND	−	−	ND	−
1494-88	++	+[f]	ND	++	++	ND	+[f]	−	ND	+
22401-88	++	−	ND	++	+	ND	−	−	ND	−
H1942-91	+	−	ND	++	+	ND	−	−	ND	+
15904-91	++	−	ND	++	+	ND	+[f]	−	ND	−
2080-92	+++[w]	+[f]	+	++	++[w]	++	+[w]	−	+	+

Immunoreactivity was graded according to the estimated percentage of immunoreactive epithelial cells[16,24] as +, <25%; ++, 25 to <50%; +++, 50 to <75%; ++++, 75 to 100%. PCR signals were graded as follows : −, no signal; +, strong signal; ++, very strong signal. MDT, medullary thymoma; MXT, mixed thymoma; PCT, predominantly cortical thymoma; CT, cortical thymoma; WDTC, well differentiated thymic carcinoma. f, focal positivity; ND, not determined; w, positive Western blot with the indicated MAb.

*68- plus 200-kd NF-L.

†160- plus 200-kd NF; titin.

Epithelial Cell Culture

Primary stromal cell cultures were established as described previously.[24] For double immunofluorescence, they were trypsinized, grown on histological slides for 3 days, fixed, and stained as described above.

Reverse Transcriptase (RT)-PCR

Total RNA was prepared from 0.1 g of snap-frozen tissue cut into 10-μm sections on a cryostat or from 10^6 cells using Trizol reagent (Gibco Life Technologies, Eggenstein, Germany) according to the manufacturer's description. The absence of nerves from the frozen material was checked by investigating every fifth frozen section by immunohistochemistry applying anti-NF MAb N52. After cDNA synthesis with oligo-dT primers and MMLV reverse transcriptase, 1/20 of the reaction mixture was amplified using Taq polymerase and sequence-specific primers.

The oligonucleotide primers, derived from the published NF sequences[30–32] of the high, medium, and low molecular weight NF proteins (NF-H, NF-M, and NF-L, respectively) were as follows: NF-H, 5′ AGATGCAGGCCGAGACGCGCG 3′ (bp 761 to 781) and 5′ CGAGAAAGGAATTGGGCCAAA 3′ (bp 1243 to 1263); NF-M, 5′ CAGTGGCTTCCGCTCGCAGTC 3′ (bp 96 to 116) and 5′ TCTCCGCCTCAATCTCCTTAT 3′ (bp 377 to 397); NF-L, 5′ GAGACGCCCCGGGTGCATATC 3′ (bp 55 to 75) and 5′ TTCTCGTTGGTGGTGGCATCT 3′ (bp 447 to 467).

Amplifications were carried out at 62°C for NF-H and NF-M primers and at 58°C for NF-L primers, 40 cycles each. Primer pairs for glyceraldehyde phosphate dehydrogenase were used as control (60°C, 25 cycles).[33]

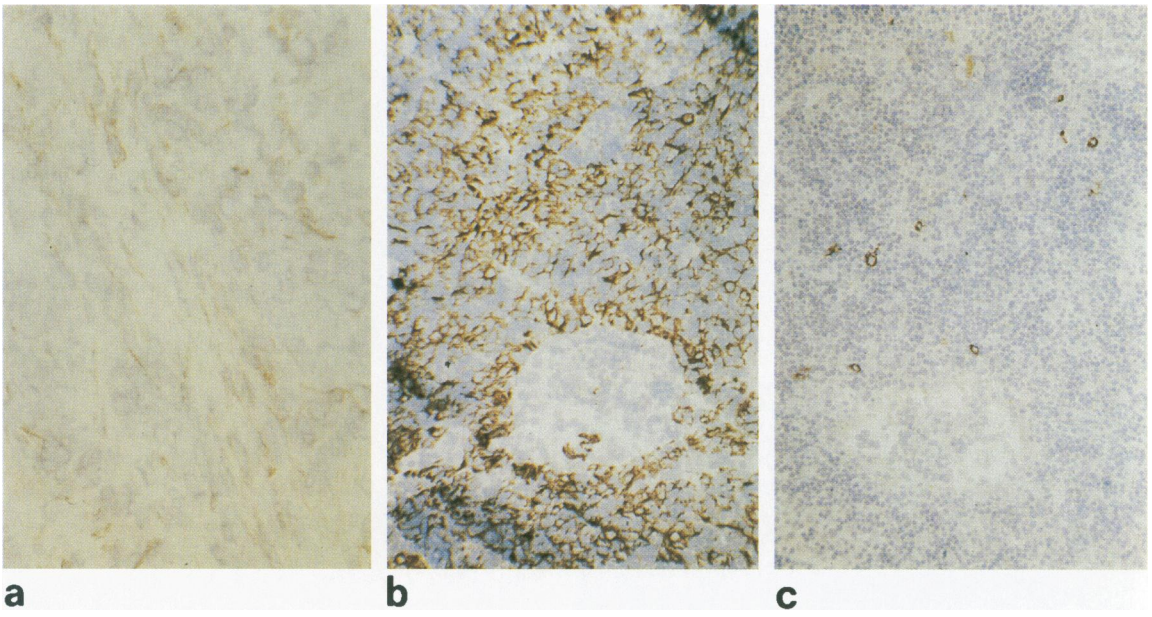

Figure 2. *Expression of neurofilament epitopes in thymomas.* a: *Faint immunoreactivity exclusively for one P-I epitope in NF-H (MAb N52) in the spindle cell area of a mixed thymoma (case 8787–90).* b: *Strong immunoreactivity for a P-I epitope of NF-M (MAb NN18) that was almost identical for a P-D epitope (MAb BF10) and for NF-H (MAb N52) (case 32546–87).* c: *In an adjacent section, only a few scattered cells labeled for P-D epitope shared by NF-L and NF-H (MAb 2F11). Two MAbs to P-D epitopes of NF-H gave completely negative results (not shown). Immunoperoxidase; magnification, ×160.*

One-fourth of the amplified products were separated by electrophoresis on a 2% agarose gel. After purification, PCR products were directly sequenced using the ABI 373 DNA sequencer.

Protein Extraction and Western Blotting

Detergent-insoluble fractions of TETs and of control tissues or cells were prepared from 0.5 g of snap-frozen tissue or 10^7 cells following the method of Bennett et al.[34] Triton-X-100-insoluble material was pelleted at 20,000 × *g* for 30 minutes, suspended in the reducing sample butter (50 mmol/L Tris, pH 8.0, 2% sodium dodecyl sulfate (SDS), and 5% (w/v) 2-mercaptoethnol), and heated for 5 minutes to 95°C. After centrifugation at 12,000 × *g* for 5 minutes, clear supernatants were used for electrophoresis in 7.5% SDS-polyacrylamide slab gels according to Laemmli.[35] Molecular weights were estimated using molecular weight standards and purified NFs from bovine spinal cord (Progen, Heidelberg, Germany). Proteins transferred to nitrocellulose membranes were probed with the anti-NF MAbs N52, NN18, and NR4 at dilutions of 1:100 as the first antibodies (for 1 hour at room temperature) and peroxidase-conjugated rabbit anti-mouse second antibodies (Dako, Hamburg, Germany) diluted 1:100 in a 50 mmol/L Tris buffer (pH 7.4; for 30 minutes). The absence of nerves from the frozen normal and neoplastic thymic tissue was checked as in the preceding section.

Results

Neurofilament Epitopes Are Restricted to the Medulla of Normal Thymic Tissue

In non-neoplastic human thymus, NF labeling was restricted to occasional single cells scattered throughout the medulla, especially around Hassall's corpuscles (Figure 1a), and the cortex was consistently negative. By double immunofluorescence, NF epitopes were detected only in epithelial cells (Figure 1, b and c). One of 6 MG and one of 6 control samples showed no staining, even in the medulla (ages 15 to 38 years and 5 days to 68 years, respectively). In all of the other 10 samples, there was expression of phosphorylation-independent (P-I) epitopes (MAbs NR4, NN18, and N52), but phosphorylation-dependent (P-D) epitopes of NF-M or NF-H were detected only in very rare medullary cells in 2 of these samples (MAbs 2F11 and BF10).

Neurofilament Epitopes Are Hyperexpressed in Cortical-Type Thymic Epithelial Tumors

In the thymomas, medullary differentiation correlated with minimal or undetectable labeling for NF-L and

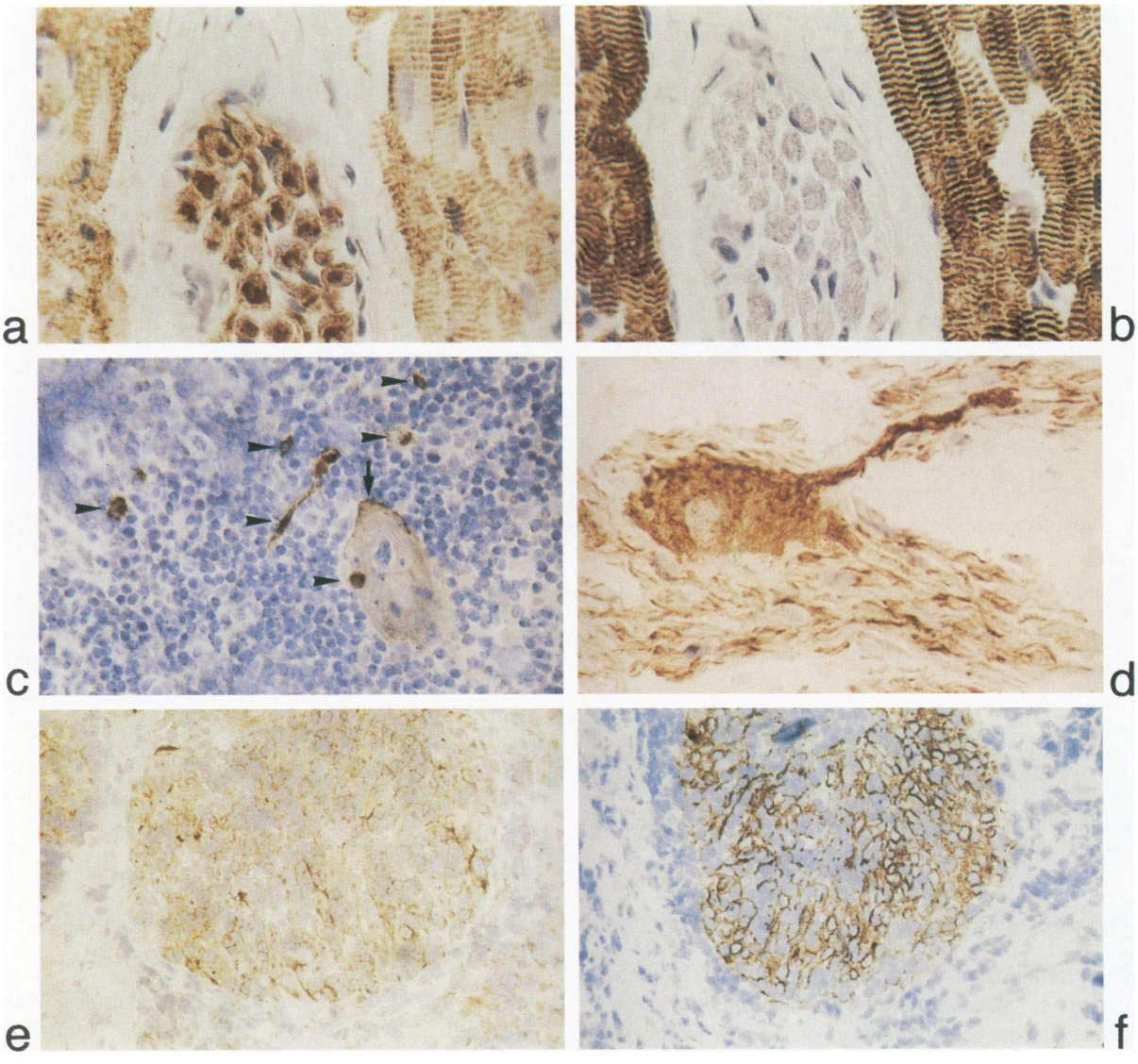

Figure 3. *An epitope shared by NF and titin (labeled by MAb 63/15) is expressed in both muscle and nerve* (a), *in both myoid cells* (arrowheads) *and a few epithelial cells* (arrow) *around a Hassall's corpuscle in thymus* (c), *in both axons and perikarya in a ganglioneuroma* (d), *and weakly in a cortical thymoma* (e; *case H 1793–93*) *that stains more intensely with MAb N52* (f). *By contrast, the anti-titin MAb T11 stains only striated muscle and not nerve* (b). *Immunoperoxidase; magnification,* ×*400* (a *to* d) *and* ×*160* (e *and* f).

NF-M in the mixed or medullary categories (Table 3). In 4 of 7 mixed thymomas, one P-I epitope of NF-H (MAb N52) was expressed weakly in a few scattered cells (Figure 2a), but no P-D epitopes were detected at all (Table 3). In sharp contrast, both the predominantly or purely cortical thymomas and the well differentiated thymic carcinomas showed much stronger and more widespread expression of both P-I and P-D epitopes especially in NF-L and NF-M (Table 3 and Figure 2b). This hyperexpression was seen in nearly all of the 17 cases, but for NF-H (MAb N52) it was much less striking and often very patchy (Table 3). In general, for NF-L and NF-H, expression was greater with the P-I epitopes, but for NF-M it was usually similar for P-I and P-D epitopes (Table 3).

Some Thymic Epithelial Tumors Express a Titin Epitope

As whole titin is not expressed in thymomas,[14] the stimulus for the autoantibody response against it in paraneoplastic MG[12,13,36] has remained enigmatic. Given the known cross-reactivities between titin and NFs,[20,21] we also tested MAb 63/15 that was raised against fish titin and cross-reacts with a titin epitope

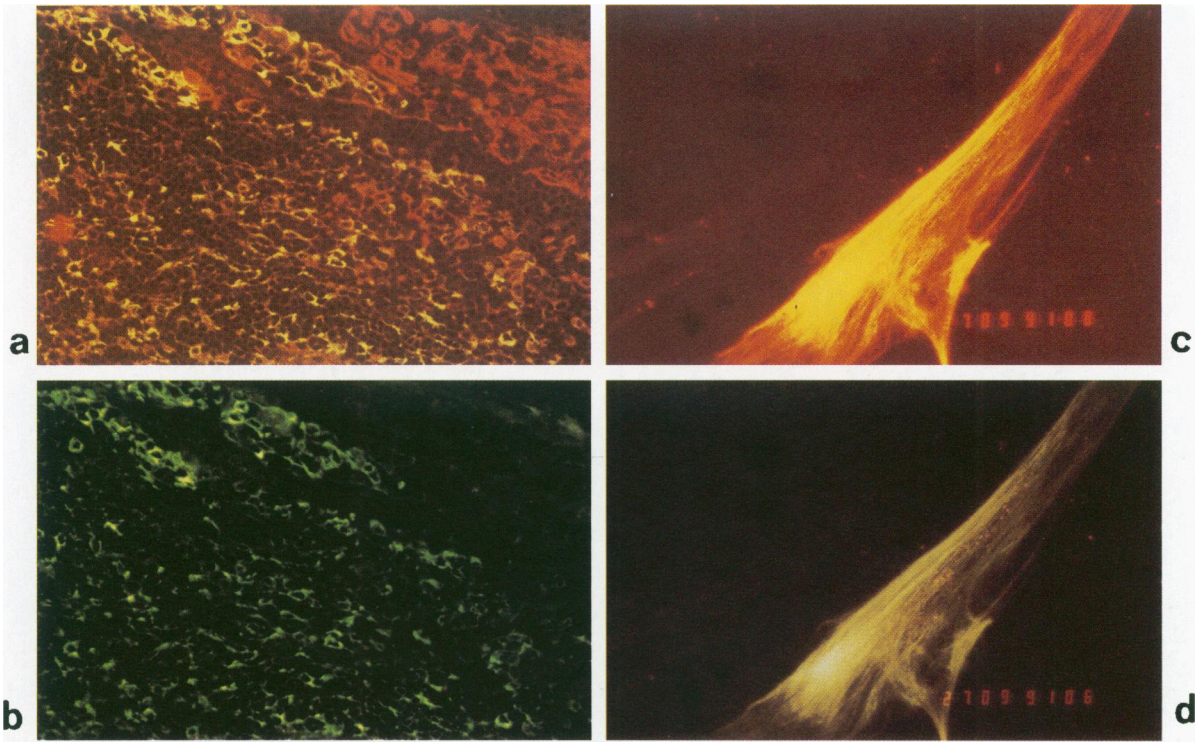

Figure 4. *Expression of NF epitopes in keratin-positive thymoma epithelial cells in sections* (a *and* b) *or monolayer cultures* (c *and* d); *double immunofluorescence for keratin* (a *and* c; *red*) *and with MAb N52 against NF-H* (b *and* d; *green; case 32546–87). After double exposure* (a *and* c), *a yellow fluorescence indicates double positivity. In* a, *one epithelial lobule* (*right upper corner*) *is clearly keratin*$^+$*/NF-H*$^-$ *only. Magnification,* ×*160* (a *and* b) *and* ×*600* (c *and* d).

in fish NFs.[21] As shown in Figure 3, the MAb 63/15 cross-reacts with human skeletal muscle, thymic myoid cells, and ganglion cells and binds to NF-M and NF-H of human spinal cord in Western blots (not shown). Interestingly, it also reacted with thymomas mainly of cortical and mixed types (Table 3). This expression correlated well with that of P-I epitopes in NF-M and NF-H (MAbs NN18 and N52, Table 3) and, as with the latter, was also noted in a few epithelial cells around Hassall's corpuscles (Figure 3c).

Neurofilament Epitopes Are Expressed in Thymoma Epithelial Cells

Double immunofluorescence confirmed that the NF epitopes were restricted to keratin-positive cells, both in tissue sections (Figure 4 a and b) and in primary epithelial cell cultures from the same thymomas (Figure 4, c and d; MAbs NR4, NN18, and N52). By immunoelectron microscopy, it proved to be the intracytoplasmic intermediate filaments that bound MAb N52 (Figure 5, a and b); the epitopes of MAbs NR4, NN18, and BF10 were lost during polymerization of the resin (LR-White).

Ectopic Expression of Neurofilament Epitopes in a Neoplastic Cortical Microenvironment of Thymomas

In the normal thymus, NF epitopes were never seen in the cortex, which is usually densely packed with immature CD1$^+$ thymocytes. However, in almost all cortical and some mixed thymomas, the epithelium typically formed a similar network enmeshing the abundant CD1$^+$ thymocytes, but here it often expressed these epitopes, too (Figure 6). They were therefore aberrantly hyperexpressed in an inappropriate microenvironment just as reported[16,37] for the AChR-α 373 to 380 epitope.

Evidence for Transcription of Neurofilament Genes in Thymomas

We next tested for expression of NF mRNA by RT-PCR. As shown in Figure 7, signals from NF-L, NF-M, and NF-H could be detected in a well differentiated thymic carcinoma (case 2080–92) that expressed epitopes of these three NFs at the protein level (Table 3). The results were similar with normal thymus

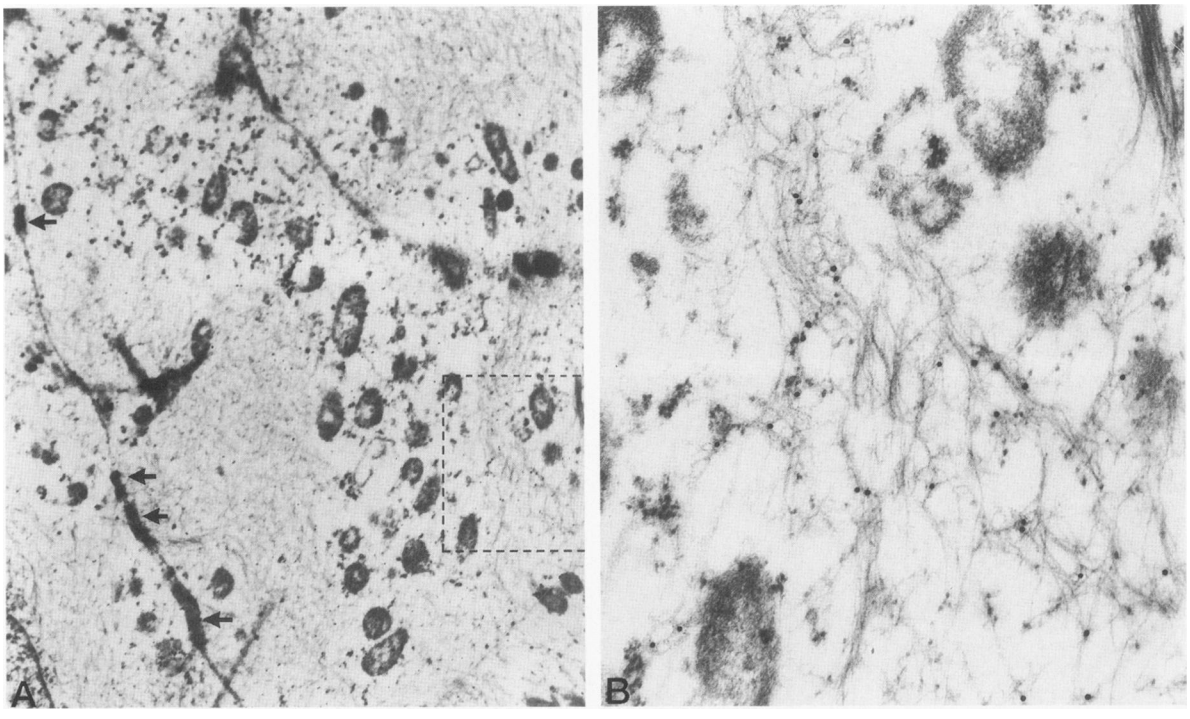

Figure 5. *Thymoma epithelial cells express a NF-H epitope (MAb N52) on their intermediate filaments.* A: *Epithelial cells of a well differentiated thymic carcinoma (case 2080–92) with desmosomes* (arrowheads). *Magnification, ×4400.* B: *High power view of the* inset *marked in* a, *showing filaments labeled with immunogold particles (20nm). LR-White; Magnification, ×32,000.*

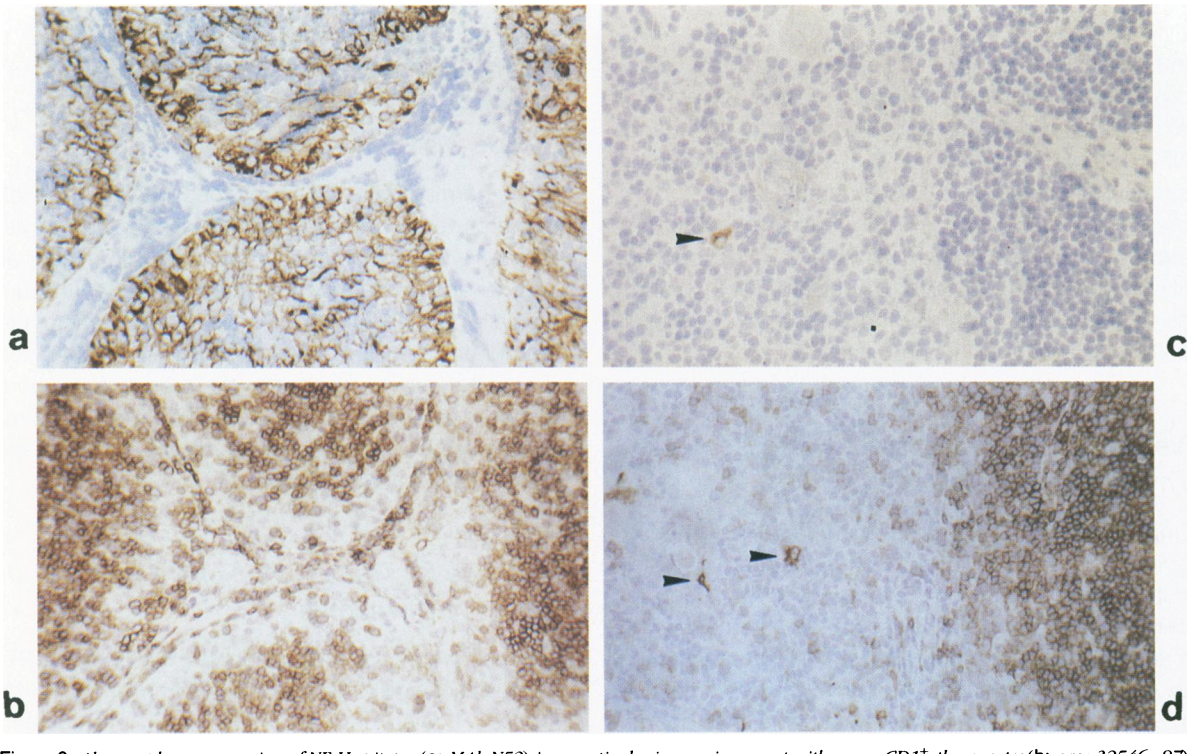

Figure 6. *Aberrant hyperexpression of NF-H epitopes* (a; *MAb N52*) *in a cortical microenvironment with many CD1$^+$ thymocytes* (b; *case 32546–87*), *whereas in the normal thymus* (c), *the rare MAb-N52-reactive cells are in the medulla* (arrowheads) *and are clearly separated from cortical (CD1$^+$) thymocytes* (d; *anti-CD1 MAb VIT6*). *The few medullary CD1$^+$ cells* (arrowheads) *are interdigitating dendritic cells. Immunoperoxidase on serial sections; magnification, ×160.*

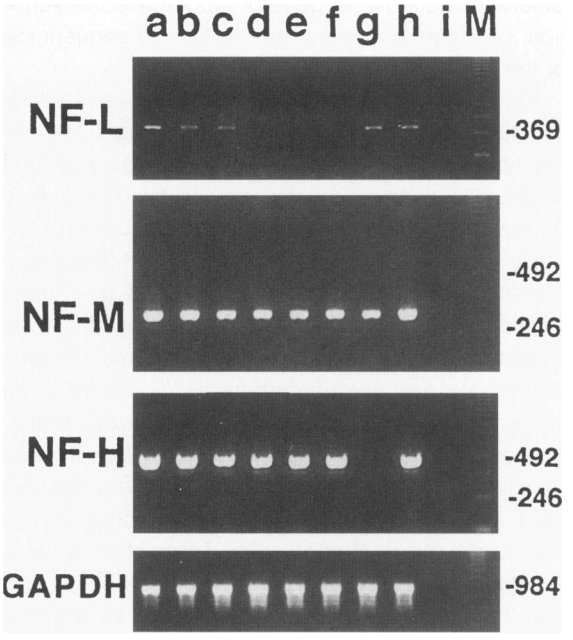

Figure 7. *Detection of NF RNA in thymomas by RT-PCR. Expression of all three NF genes and of glyceraldehyde phosphate dehydrogenase (as a control[33]) in a sciatic nerve* (lane a), *a normal thymus* (lane b) *and an epithelial cell line from it* (lane c), *and a well differentiated thymic carcinoma (case 2080–92,* lane h). *Selective expression of NF-H and NF-M both in a medullary thymoma (case 24998–94,* lane d) *and an epithelial cell line from it* (lane e), *a mixed thymoma (case 2306–91,* lane f), *and a cortical thymoma (case 23169–93,* lane g). *PCR amplification with cDNA derived from A431 carcinoma cells served as negative control* (lane i). *M, 123 bp marker (Gibco LIFE Technologies).*

and a sciatic nerve, both of which also expressed epitopes of all three NFs. In contrast, a thymoma (case 23169–93) that expressed epitopes only of NF-L and NF-M gave rise to PCR products of only these NFs (Figure 7). This finding was confirmed with another cortical thymoma from a MG patient. The absence of any PCR product for NF-H in these two tumors argues strongly against any contribution from mRNA in contaminating nerves as NF-H gave the strongest band in sciatic nerve (Figure 7, lane a), which is supported by the selective absence of NF-L signals in three mixed thymomas, which again paralleled the immunohistochemical findings. Furthermore, pure epithelial cultures from a normal thymus and a mixed thymoma both showed the same patterns as their tissues of origin (Figure 7).

Surprisingly, however, transcripts of the NF-M gene were found in all medullary and mixed thymomas investigated so far (Figure 7 and six additional cases not shown), although no NF-M epitopes were detectable by immunohistochemistry (Table 3). Likewise, NF-H transcripts were found in one medullary and one mixed thymoma although immunohistochemistry was negative. Future quantitative analyses should determine whether NF-M and NF-H transcription or translation is low. Sequencing of all PCR products from thymomas showed NF sequences that were 100% identical to the sequences of brain-derived NF cDNAs[30–32] as expected after this high stringency PCR.

Thymomas Express Hypophosphorylated Neurofilament Proteins

To test whether the epitopes were expressed on authentic NF polypeptides, we checked their size and antigenicity by immunoblotting. To provide enough material, we chose our largest and most purely epithelial thymoma (well differentiated thymic carcinoma) and compared it with a mature ganglioneuroma and a commercial bovine NF preparation. The anti-NF-L MAb NR4 identified doublet bands of approximately 68 and 65 kd in the well differentiated thymic carcinoma (case 2080–92) and ganglioneuroma extracts. The 68-kd bands correspond to the NF-L band in the bovine NF extract whereas the 65-kd band might be a degradation product, a synthetic intermediate, or cross-reacting protein (Figure 8).

The anti-NF-M MAb NN18 revealed the expected 160-kd band in the ganglioneuroma extract and the bovine NF and a 153-kd band in the thymoma that is typical of the hypophosphorylated NF-M[38] that this P-I MAb detects (Figure 8). Furthermore, MAb NN18 labeled a 130-kd band only in the TET extract, which is likewise compatible with a hypophosphorylated NF-M.[38,39] Results identical to those shown for case 2080–92 with MAb NR4 and NN18 were also obtained with two cortical thymomas (cases 6942–88 and 15977–87).

Similarly, with the anti-NF-H MAb N52, slightly smaller bands (approximately 165 and 170 kd) were detected in both the TET and ganglioneuroma extracts than in spinal cord NF (Figure 8); again, these are typically of hypophosphorylated NF-H.[38,40]

Discussion

Our main findings here are of expression at the RNA and protein levels of NF genes in normal and neoplastic thymus and of their hyperexpression in an inappropriate microenvironment in the more cortical thymomas.

In general, the expression at the RNA and protein levels correlated well in different tumors with few exceptions (see below). Together with the detection (by both immunohistochemistry and immunoblotting) of polypeptides with multiple epitopes (P-I and P-D)

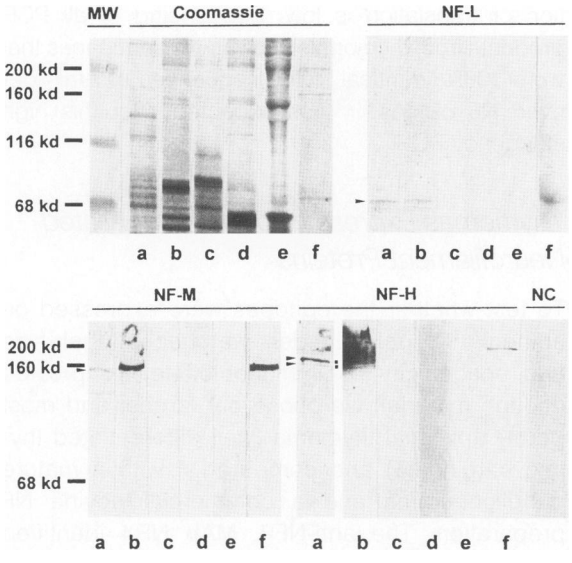

Figure 8. *Detection of NFs (NF-L, NF-M, and NF-H) in a TET by immunoblotting. Blots of cytoskeleton extracts of a well differentiated thymic carcinoma (case 2080–92,* lane a), *a mature ganglioneuroma* (lane b), *a squamous cell carcinoma of the lung* (lane c), *the cervical carcinoma cell line A431* (lane d), *normal human thymus* (lane e), *and a commercial preparation of bovine NFs* (lane f) *were developed with anti-NF-L MAb NR4, anti-NF-M MAb NN18, and anti-NF-H MAb N52. Bands in TET extracts that represent NFs are marked with* arrowheads. *The faint bands at approximately 65 and 130 kd in the NF-L and NF-M panel, respectively, may represent degradation products or hypophorylated NFs. In the NF-H panel there are minor hypophosphorylated 165- and 170-kd NF-H bands in both TET and ganglioneuroma extracts* (arrowheads *in* lane a, dots *in* lane f). *NC, anti-mouse negative control on the TET extract with no first antibody; MW, molecular weight markers (Bio-Rad, Munich, Germany). The Coomassie panel illustrates the protein content of the samples used in the immunoblots.*

and of multiple sizes, mostly showing a bias toward hypophosphorylated forms, this argues cogently that these tumors express authentic NF proteins rather than isolated cross-reacting epitopes. Moreover, their similarity in cultured thymoma epithelial cells conclusively demonstrates their presence in the neoplastic cells of origin.

The only exceptions were the medullary and mixed thymomas that all (n = 9) gave signals for the NF-M RNA (Figure 7) but did not express the NF-M epitopes of three distinct antibodies (Table 3). Likewise, NF-H transcripts but not NF-H proteins were detected in one medullary and one mixed thymoma (Table 3). These findings are most easily explained by the enormously greater sensitivity of RT-PCR than of immunohistochemistry. This will be tested further using more sensitive protein detection methods. However, as the amplified NF-M sequences from a mixed and a cortical thymoma gave comparable strong signals (Figure 7, lanes f and g) we suggest that medullary and mixed thymomas might translate their NF-M transcripts less efficiently. We are now also testing alternative explanations, such as partial deletions of the NF-M gene or incomplete transcription, by Northern blotting and complete sequencing of the NF-M cDNA.

Epitopes of NF-L and NF-M, and more rarely of NF-H, were hyperexpressed only in tumors of cortical type and were either absent or sparsely and focally distributed in thymomas with more medullary differentiation. All of this strongly echoes the previously reported expression of the AChR-epitope-bearing protein p153 that is restricted to cortical TETs.[6,16,19,24,37] Interestingly, p153, like NFs, was detected not only in TETs but also in normal nervous tissues and in neuronal tumors.[16,19,41] There is an interesting precedent for such neuronal differentiation; as Miettinen et al[42] reported, NFs are expressed *de novo* in parathyroid adenomas that stem from the third and fourth branchial pouch ectoderm as do cortical thymic epithelial cells. The potential for a neuronal-like differentiation in cortical TETs and parathyroid tumors may, therefore, be a legacy of a common epithelial ancestor.

Obviously, this neuronal differentiation of TETs is only abortive as indicated by the immaturity of the neurofilaments. For example, their NF-H was scarcely phosphorylated at all, perhaps because that requires a mature neuron-specific kinase.[43] By contrast, NF-L contains only few phosphate groups in mature neurons[38,40,44] and was also partially unphosphorylated in TETs (Table 3). Only NF-M was considerably phosphorylated, as implied by its near 160-kd molecular mass (Figure 8) and almost identical reactivity with P-I and P-D MAbs (Table 3); perhaps that reflects its phosphorylation by nonspecific kinases.[45–47]

We saw aberrant hyperexpression of NF in epithelial cells close to immature thymocytes in cortical TETs (Figure 6, a and b) that was not seen in the normal thymus (Figure 6, c and d). We had previously described a very similar hyperexpression of the AChR-epitope-bearing protein p153; as it correlated with autoimmunity to AChR,[5,16,37] we suggested that it might be the immunizing autoantigen in TETs.[18,37,48] By analogy, the titin-epitope-bearing NFs might play a similar role in autosensitization against titin, as this molecule is not expressed in complete form in TETs.[14] As these NF-positive epithelial cells are major histocompatibility complex class II positive and have appropriate accessory molecules (unpublished), they are well placed to either abnormally select or to stimulate precociously some of the adjacent developing thymocytes[18,37,48,49] or to activate recirculating mature peripheral T cells.[50–52] These possibilities can now be tested by comparing NF-positive and NF-negative

TETs with respect to their potential to select or stimulate T cells with anti-NF and anti-titin specificity.

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