Spontaneous production of anti-IFN-\(\alpha\) and anti-IL-12 autoantibodies by thymoma cells from myasthenia gravis patients suggests autoimmunization in the tumor

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Abstract

Myasthenia gravis (MG) is mediated by autoantibodies to the acetylcholine receptor (AChR), expressed in muscle and rare thymic myoid cells. Most early-onset cases show thymic lymph node-type infiltrates, including pre-activated plasma cells spontaneously producing anti-AChR antibodies. Since these are not evident in the associated thymomas found in another 10% of MG patients, AChR-specific B cells must be autosensitized elsewhere. Unexpectedly, at diagnosis, >70% of MG/thymoma patients also have high-titer neutralizing autoantibodies to IFN-\(\alpha\), and >50% to IL-12; moreover, titers increase strikingly if the thymomas recur, indicating a closer tumor relationship than for anti-AChR. To investigate this, we have measured autoantibody production by cells cultured from thymomas, any available thymic remnants and blood, with or without the B cell stimulant pokeweed mitogen (PWM). To check autoantibody specificity and clonal origins, we isolated Fabs from two combinatorial libraries from producer thymus/thymoma cells. Surprisingly, thymoma cells spontaneously produced antibodies to IFN-\(\alpha\) and/or IL-12 in >40% of seropositive cases, showing typical plasma cell behavior, whereas they produced anti-AChR only after PWM stimulation. We isolated 15 combinatorial Fabs to IFN-\(\alpha\) (versus only one to AChR). Their strong binding in radio-immunoprecipitation and Western blots implies high affinities. The four Fabs tested neutralized anti-viral actions of IFN-\(\alpha\). The diverse V genes clearly showed ongoing antigen-driven selection. These results imply pre-activation in situ by native IFN-\(\alpha\)/IL-12 expressed within a ‘dangerous’ tumor microenvironment. With these molecules, it should be easier to identify provoking cell type(s) that may give novel additional clues to autoimmunization against T-cell epitopes from the more complex AChR.
Introduction

The initiation of most autoimmune disorders is mysterious, but the well-established tumor associations in paraneoplastic conditions must hold vital clues. For instance, autoimmunization by the Ca^2+ channels in small cell lung cancers evokes pathogenic antibodies that inhibit the calcium-dependent release of acetylcholine (ACh) from nerve endings (1); if the tumors can be removed or destroyed, the antibody levels decline and the muscle strength improves (1).

Paraneoplastic autoimmunity is more common in patients with thymomas (2–5). These are epithelial neoplasms (4,5) found in ~10% of myasthenia gravis (MG) patients and can usually be removed surgically. They often resemble disorganized thymic cortex (5,6), typically generating abundant maturing thymocytes (6,7) and exporting many of their progeny to the periphery (7,8). Around 30% of all thymoma patients develop MG (2–5). In addition to the characteristic pathogenic autoantibodies to the muscle ACh receptor (AChR), they usually have others to various intracellular muscle proteins, including actin, myosin (9,10) and especially titin (11), although their pathogenicity is debatable. The association between MG and thymomas has long been a puzzle. It is not even clear whether they actively autoimmunize to the muscle-like myoid cells (20) that express AChR (16). In cases 6 and 9, there may have been some mechanical disruption and washing (16,24). Samples 14 and 15 were recurrent thymomas (with no residual thymus).

By contrast, in early-onset MG (EOMG) patients, lymph node-like infiltrates are regularly seen in the thymic medulla (3,4,14). They resemble those seen in the target organs in other autoimmune diseases (17–19) and are probably provoked by the muscle-like myoid cells (20) that express AChR (21,22). The co-localizing germinal centers and the T cell areas contain terminally differentiated (radio-resistant) plasma cells (23) that spontaneously synthesize anti-AChR (24,25) and are a rich source for cloning combinatorial antibodies (26,27). Thus, B cells reacting to AChR have clearly been selectively pre-activated in vivo, whereas those specific for extrinsic antigens produced antibodies only in the presence of pokeweed mitogen (PWM) (25,28), a T cell-dependent B cell stimulant that concomitantly reduces anti-AChR production by plasma cells (22,24).

Such lymph node-type infiltrates are often seen in the uninvolved thymic remnant adjacent to an MG patient’s thymoma (4), whereas, in the thymoma itself, myoid cells are rare (4,15) and infiltrates/anti-AChR synthesis are uncommon/undetectable (4–6,29).

Unexpectedly, high-titer neutralizing autoantibodies are found against IFN-α in >70% of MG/thymoma cases at diagnosis and against IL-12 in ~50% (30–32). These autoantibodies show an intriguingly close relationship with these tumors, also occurring in ~30% of thymoma cases without MG (31). Moreover, they often increase when thymomas recur (30–32), suggesting more direct autoimmunization in thymomas against the cytokines than against AChR.

Seeking footprints of such autoimmunization, we have compared production of autoantibodies against AChR, titin, IFN-α and IL-12 by cultured thymoma cells, and cloned combinatorial Fabs to check the specificity and clonal origins of the plasma cells detected.

Methods

Patients

Clinical details of the 23 thymoma patients with MG and the two without MG/serum anti-AChR are summarized in Table 1, and their autoantibody levels in Table 2. With informed consent and Ethical Committee approval, they were bled immediately before thymectomy. While separating their peripheral blood lymphocytes, we prepared cell suspensions from their thymoma, and any available uninvolved thymic remnant, by mechanical disruption and washing (16,24). Samples 14 and 15 were recurrent thymomas (with no residual thymus). From cases 5 and 11–13, we enriched low-density cells on Ficoll (16). In cases 6 and 9, there may have been some
contaminating thymoma cells in the suspension from the remnant (see Fig. 1, legend), but not vice versa. We tested all available new samples on the day of surgery (n = 13). To increase the numbers of cases (whether with thymic remnants or without anti-cytokine autoantibodies), we also thawed cells (n = 11) cryopreserved previously (24): sample 23 was used only for preparing combinatorial Fabs. Cells from remnant 6 or without anti-cytokine autoantibodies), we also thawed cells

### Table 2. Autoantibody productivity in culture and serum titer against each antigen

<table>
<thead>
<tr>
<th>Serum titer</th>
<th>Autoantibody production in culture from Thymoma</th>
<th>Thymic remnant</th>
<th>Peripheral blood lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>PWM stimulus</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>AChR &gt;10 nM</td>
<td>2/19</td>
<td>5/19</td>
<td>5/15</td>
</tr>
<tr>
<td>&lt;10 nM</td>
<td>0/ 5</td>
<td>0/ 5</td>
<td>0/ 2</td>
</tr>
<tr>
<td>0.5±550</td>
<td>7/17</td>
<td>2/17</td>
<td>8/13</td>
</tr>
<tr>
<td>&lt;0.5</td>
<td>0/ 7</td>
<td>0/ 7</td>
<td>0/ 2</td>
</tr>
<tr>
<td>IL-12 0.5±300</td>
<td>2/15</td>
<td>1/15</td>
<td>3/10</td>
</tr>
<tr>
<td>&lt;0.5</td>
<td>0/ 6</td>
<td>0/ 6</td>
<td>0/ 4</td>
</tr>
</tbody>
</table>

The serum anti-IFN-α and anti-IL-12 titers were determined by RIA (32). Only the two non-MG cases were seronegative versus AChR; their cells produced no detectable antibodies against AChR, IFN-α or IL-12. The threshold for a significant antibody level was 3% above the background of the cycloheximide control culture supernatants. A significant PWM response was at least twice the antibody level in the cultures without PWM.

In all the cases tested who were seropositive for anti-AChR or anti-IFN-α, spontaneous production was significantly more frequent for anti-IFN-α than anti-AChR antibodies [by Fisher’s exact test, P = 0.046 (two-tailed); by Mann–Whitney rank-sum test, P = 0.0047]. Neither spontaneous anti-AChR nor anti-IFN-α antibody production differed significantly between thymoma and remnants by either paired Student’s t-test or Mann–Whitney rank-sum tests.

**Cell cultures**

Cells were cultured at 5 × 10⁶/ml with and without PWM (Invitrogen, Paisley, UK) at 1:100 and 1:1000 or with cycloheximide (20 μg/ml; Sigma, Poole, UK) as a control to confirm antibody synthesis during the culture period (24); 75% of the supernatant was sampled and replaced on day 7 or 10 and again on day 15 or 20. We assayed both samples for autoantibodies (which confirmed that results were consistent); the results shown are from the earlier time points.

**Autoantibody assays**

The radio-immunoassays (RIA) for serum antibodies used [125I]α-bungarotoxin (α-BuTx)-human AChR (24–27), or [125I]IFN-α, [125I]IL-12p70 or [125I]titin (30-kDa recombinant fragment; DLD Diagnostika, Hamburg) (32). Culture supernatants (75 μl) were incubated overnight at 20°C with the labeled antigen (at least 5000 c.p.m.) before immunoprecipitation (plus normal serum as carrier) and meticulous washing [to ensure backgrounds below ~300 c.p.m. (24)]. With Fabs, we used an anti-human Fab as the precipitating antibody (26,27). Results were expressed as the percentage of the available c.p.m. precipitated (after subtraction of the background with cycloheximide).

For Western blotting, 20 μg of IFN-α plus 20 μg BSA was electrophoresed in acrylamide gels and blotted onto nitrocellulose strips, which were blocked overnight in 4% milk powder and then incubated in the various Fab or serum samples (all at 1:200 in 4% milk powder) for 1 h at 20°C. After extensive washing, the strips were probed with horseradish peroxidase-conjugated anti-Fab or anti-IgG antibodies (Dako, Ely, UK) at 1:10,000 or 1:2000 and processed for enhanced chemiluminescence (ECL; Amersham Pharmacia, Little Chalfont, UK).

Neutralization of the anti-viral activity of human IFN-α was assayed as described elsewhere (31). In brief, dilutions of Fabs or sera were pre-incubated with 25 Laboratory Units of each IFN-α preparation at 37°C for 1 h, before incubation with 2D9 cells for 24 h at 37°C. The antibody/IFN-α mixture was then replaced with a challenge dose of encephalomyocarditis virus; after 24 h, the wells were stained with amido blue black and absorbance read at 620 nm.

**Combinatorial Fabs**

Cases 11 and 23 were selected because of their high serum titers against IFN-α, IL-12, AChR and titin. Before starting the work shown in Fig. 1, we had already noted strong spontaneous anti-AChR production by cells from case 23’s remnant (but not his thymoma), so mRNA was isolated from frozen remnant cells; with case 11, we had already pooled equal numbers of cells from thymoma and remnant, thus using all the remnant cells to prepare RNA before any were cultured.

We prepared cDNA, amplified V₁–C₁ and V₅–C₅ segments, and co-ligated them to yield combinatorial libraries, which we then expressed in λ phage, as previously described (26,27). From each unamplified combinatorial library, we screened ~2.5 × 10⁵ plaques with [125I]-iodinated IFN-α or IL-12, titin or α-BuTx-labeled fetal AChR on colony lifts (see Fig. 2a), after optimizing conditions on dot-blots with the donors’ sera. Positive plaques were picked, cloned to homo-

Negative in all screens. The GenBank accession numbers of the positive Fabs are AY173081–AY173112.
have already been subtracted.

cycloheximide cultures gave similar results to medium alone which sampled after 7±10 days and assayed by RIA; those from thymomas were B2 and B3 (cases 15 and 14). Supernatants were B3 (sparser lymphocytes; see Table 1 footnote); the recurrent thymomas were WHO type B2 (with abundant lymphocytes) and two more that showed strong/unexpected PWM stimulation (Fig. 1a and Table 2). In contrast, spontaneous synthesis was more obvious with cells from the uninvolved thymic remnants (Fig. 1b and Table 2) (6,22,29); it was sometimes reduced by PWM, as is typical of terminal plasma cells (22,24). In two other remnants, there was clear stimulation by PWM; it was totally abolished by irradiation in case 6 (not shown).

Quiescent, but responsive, memory B cells may be located in the perivascular spaces in some thymomas (16); importantly, however, they had rarely been pre-activated in situ (Fig. 1a), as noted previously (6,22,29).

For both thymomas and thymic remnants, most of the anti-AChR antibody producers (with and without PWM) had been pre-treated with corticosteroids (Fig. 1a and b, solid symbols), which preferentially deplete immature thymocytes, and enrich B and plasma cells (6). In the producers, serum anti-AChR titers were sometimes modest. As expected (6,10,22,29), there were many non-producers despite high serum titers, including all peripheral blood lymphocyte samples (Table 2).

Production in culture of antibodies against titin

We found minimal anti-titin antibody production in 14 of 15 of the MG thymoma cultures and in all nine remnants tested (despite high serum titers in six cases). It reached 22% only with one thymoma (with PWM; case 8, who had low/medium serum titers against titin, IFN-α and IL-12). It was marginal in three other thymomas and three remnants (3–6.5%), again only with PWM (not shown).

Spontaneous anti-IFN-α and anti-IL-12 antibody production by thymoma cells

In surprising contrast with anti-AChR, we noted striking spontaneous antibody production against both cytokines in thymoma as well as remnant cultures (Fig. 1c and d; see Supplementary Data, available at International Immunology online); its frequent inhibition by PWM again implicates plasma cells (22,24). Furthermore, spontaneous anti-IFN-α production was seen in significantly more seropositive cases than for anti-AChR (Table 2, footnote a), including both recurrences (cases 14 and 15). The broadly similar binding levels and prevalences of antibodies against IFN-α and AChR in cultures from thymic remnants (and from thymomas with PWM) indicate comparable assay sensitivities.

Results

Production in culture of antibodies against AChR

We first confirmed that spontaneous anti-AChR antibody production was rare/near background in the 22 MG thymoma samples from anti-AChR-seropositive cases, even with the four that showed strong/unexpected PWM stimulation (Fig. 1a and Table 2). In contrast, spontaneous synthesis was more
thymomas than remnants and thus their total antibody productivities were ~20-fold greater (not shown).

In general, results were very similar for anti-IL-12p70 (Supplementary Data), although fewer cases were positive (Table 2). Again, autoantibody production in culture was detected only in cases seropositive against IL-12, supporting its specificity, as with the other antigens (Table 2).

Notably, two thymomas (thymomas 6 and 7) gave surprising PWM responses against IFN-\(\alpha\) despite low serum titers, as noted previously for anti-AChR in a patient whose serum titer later rose ~100-fold (10); similarly, one remnant (remnant 9) spontaneously produced anti-IL-12 unexpectedly well. All three had been pre-treated with corticosteroids.

Specificities of combinatorial Fabs in RIA, Western blotting and neutralization assays

We cloned Fabs from thymus/thymoma cells from two patients with high serum titers against all four antigens, \(\nu_c\)-C\(_{\mu}1\) and \(\nu_c\)-C\(_{\kappa}\) genes were recombined from cDNA libraries and expressed in \(\lambda\) phage (26,27). Even after careful screening of \(~2.5 \times 10^5\) \(\lambda\) phage plaques from each of these unamplified combinatorial libraries, none proved to be specific for IL-12 or titin and only one for AChR (‘D anti-AChR’), despite both donors’ high serum titers. In contrast, the same screening had yielded >20 distinct AChR-specific Fabs from three EOMG thymi (26,27).

Screening similarly with \([^{125}I]\)IFN-\(\alpha\) (Fig. 2a), we isolated nine specific Fabs from the thymoma-plus-remnant of case 11 (‘M1–M14’) and six from 23’s remnant (‘D1–D6’) (Fig. 2 and Table 3). All 15 Fabs bound IFN-\(\alpha\) significantly in RIA, but each immunoprecipitated only 15–70% of the available \([^{125}I]\)IFN-\(\alpha\), even with saturating amounts of Fab (Fig. 2b and Table 3); evidently, they recognized only a subset of the labeled IFN-\(\alpha\) molecules. Interestingly, however, both donors’ sera precipitated >90% of the total IFN-\(\alpha\). So did each donor’s Fabs when pooled; collectively, therefore, they represent the full range of reactivity. The completely negative RIA results with all of these Fabs against \([^{125}I]\)o-BuTx–AChR confirm their IFN-\(\alpha\) specificity (not shown).

Thirteen of the Fabs also bound IFN-\(\alpha\) in Western blots (Fig. 2c and Table 3). However, five gave relatively stronger signals in RIA, apparently preferring the native molecule (marked ‘>’ in the lower part of Table 3); only M11 did the reverse. The AChR-specific and pooled control Fabs showed...
no detectable IFN-\(\alpha\) binding in either RIA or Western blotting (Fig. 2b and c), despite the strong fetal AChR binding by the latter in RIA (not shown).

We tested four Fabs for neutralization of the anti-viral activity of the 12 human IFN-\(\alpha\) subtypes. The donors' sera had high titers against all subtypes, whereas the Fabs' titers were far lower and their profiles much more restricted (Fig. 3). Surprisingly, although originally screened against IFN-\(\alpha\)2, Fabs M4, D4 and D5 neutralized IFN-\(\alpha\)1 better, whereas M13 neutralized only IFN-\(\alpha\)2, 4, 5 and 21 (Fig. 3). When high- and medium- or low-titer Fabs were paired, we saw no sign of either synergy or interference in neutralization of IFN-\(\alpha\)1, 2, 4 or 21 (not shown).

\(\text{V} \text{h gene sequences of Fabs}\)

We found clear evidence of ongoing antigen-driven clonal diversification. All the \(\text{V} \text{h}\) and most of the \(\text{V} \text{k}\) genes were highly mutated, with at least 10 coding changes from the closest germline sequences (Table 3). In both \(\text{V} \text{h}\) and \(\text{V} \text{k}\), ~50% of substitutions were in the CDR1s and CDR2s (not shown), which cover <25% of the \(\text{V}\) regions.

Seven of the IFN-\(\alpha\)-specific Fabs—derived from either donor—had the commonly used \(\text{V} \text{h}3-23\), paired in six of them with the very prevalent \(\text{V} \text{k} \text{O}2/12\) gene, both being highly mutated in each Fab (Table 3, upper). This combination was seen in five of the six Fabs that bound IFN-\(\alpha\) most strongly, four of which derived from patient 23's thymic remnant ('D'). The \(\text{V} \text{k}\)s in these four were clearly related, with identical CDR3s and six shared coding mutations ('d' in Table 3), plus another three to five unique ones. Their \(\text{V} \text{h}\)s form two pairs, each again having identical CDR3s and 11±15 shared coding mutations ('b' and 'c' in Table 3) plus two to five unique ones. These results clearly indicate ongoing antigen-driven clonal evolution in this patient's thymic remnant.

A fifth strongly binding Fab (M4), from the other patient (patient 11), showed intriguing resemblances; all five share not only a Lys72→Asn replacement but also CDR3s with identical lengths and very similar amino acid compositions despite using different \(\text{D} \text{h}→\text{J} \text{h}\) genes (Table 3, lower). Moreover, in their \(\text{V} \text{h}\) CDR1s, M4 has a Ser31→Asn substitution, while the others have the same change at position 30.

Interestingly, patient 11's Fabs included another closely related pair of \(\text{V} \text{h}3-23\) sequences (M2 and M12), with four shared ('e' in Table 3) and two or three unique mutations; they had an identical 11-amino-acid insert in their CDR2 regions,

<table>
<thead>
<tr>
<th>Fab</th>
<th>RIA versus IFN-(\alpha)%</th>
<th>Western blot versus IFN-(\alpha)</th>
<th>(\text{V} \text{h}) gene usage</th>
<th>(\text{V} \text{h}) gene mutations</th>
<th>(\text{D} \text{h}) gene</th>
<th>(\text{J} \text{h}) gene</th>
<th>(\text{V} \text{k}) gene usage</th>
<th>(\text{V} \text{k}) mutations</th>
<th>(\text{J} \text{k}) gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>70</td>
<td>++++</td>
<td>(\text{V} \text{h}3.23)</td>
<td>DP 47 26 15 + 2(^{a}) 5–12(^{1})</td>
<td>4b</td>
<td>(\text{V} \text{k} \text{DPK9})</td>
<td>23 6 + 4(^{d}) 2</td>
<td></td>
<td></td>
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<tr>
<td>D6</td>
<td>70</td>
<td>++++</td>
<td>(\text{V} \text{h}3.23)</td>
<td>DP 47 33 15 + 5(^{a}) 5–12(^{1})</td>
<td>4b</td>
<td>(\text{V} \text{k} \text{DPK9})</td>
<td>22 6 + 5(^{d}) 2</td>
<td></td>
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</tr>
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<td>70</td>
<td>++</td>
<td>(\text{V} \text{h}3.23)</td>
<td>DP 47 31 11 + 4(^{a}) ? 4b</td>
<td>(\text{V} \text{k} \text{DPK9})</td>
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<td></td>
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<tr>
<td>M4</td>
<td>70</td>
<td>++</td>
<td>(\text{V} \text{h}3.23)</td>
<td>DP 47 36 11 + 4(^{a}) ? 4b</td>
<td>(\text{V} \text{k} \text{DPK9})</td>
<td>25 6 + 4(^{d}) 2</td>
<td></td>
<td></td>
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<tr>
<td>M2</td>
<td>15</td>
<td>+</td>
<td>(\text{V} \text{h}3.23)</td>
<td>DP 47 15 4 + 2(^{a}) 4–17/23(^{a})</td>
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<td>M13</td>
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<tr>
<td>M1</td>
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<td>++</td>
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<td>(\text{V} \text{k} \text{DPK9})</td>
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<tr>
<td>M3</td>
<td>35</td>
<td>+</td>
<td>(\text{V} \text{h}4.30–1)</td>
<td>DP 47 22 14 ? 4b</td>
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<tr>
<td>M7</td>
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<td>+</td>
<td>(\text{V} \text{h}1.03)</td>
<td>DP 47 25 10 1–26(^{b}) 6b</td>
<td>(\text{V} \text{k} \text{DPK9})</td>
<td>18 8 3</td>
<td></td>
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<td></td>
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<tr>
<td>M11</td>
<td>20</td>
<td>++++</td>
<td>(\text{V} \text{h}1.03)</td>
<td>DP 47 25 16 2–02/08(^{a}) 6b</td>
<td>(\text{V} \text{k} \text{DPK15})</td>
<td>2 0 4</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>D2</td>
<td>30</td>
<td>+</td>
<td>(\text{V} \text{h}1.69)</td>
<td>DP 47 33 17 ? 4b</td>
<td>(\text{V} \text{k} \text{DPK24})</td>
<td>0 0 2</td>
<td></td>
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</table>

\(^{a}\)Percent of total c.p.m. bound at maximum.

\(^{b}\)Numbers of shared + unique mutations. D\(\text{h}5–12\) uses reading frame 1. ? = DH gene could not be identified.

\(^{c}\)The 11-amino-acid insert in the \(\text{V} \text{h}\) is described in the text.

\(^{d}\)\(\text{V} \text{k} \text{DPK9}\) is also known as O2/12.
lengthening it to 28 residues, that might result from duplication and mutation of part of the CDR2. They were weak binders, although M2 also used a mutated 02/12 V<sub>k</sub>. Of the remaining eight Fabs, only M13 bound and neutralized strongly; five of them used V<sub>H</sub>4 and three used V<sub>H</sub>1 genes. The frequently recurring V<sub>k</sub> 02/12 showed very weak binding.
variable numbers of mutations that did not correlate clearly with binding (as in M12 and M13); however, it included numerous coding substitutions in the one anti-AChR Fab of case 23, as did the V\textsubscript{H}3-48.

Discussion

The present results show that specific B cells in thymomas had clearly been pre-activated in situ against IFN-\(\alpha\) and/or IL-12, resulting in an ongoing plasma cell response. In stark contrast, even when stimulable AChR-specific B cells were present, they were evidently quiescent in thymomas, but must often have been activated in vivo in the adjacent thymic remnant.

As reviewed in the Introduction, there is very similar pre-activation of a mature plasma cell response against AChR in the hyperplastic EOMG thymus and it correlates well with the presence of native AChR-expressing myoid cells. The closely analogous responses to the cytokines shown here likewise demonstrate prior activation, but now in the thymoma itself. By extension, they strongly imply (i) the presence of native IFN-\(\alpha\) and/or IL-12 molecules recognizable by specific B cells, and thus (ii) that some cell type(s) are actively autoimmunizing against these cytokines in both primary and recurrent thymomas. Therefore, (iii) thymomas constitute a ‘dangerous’ autoimmunogenic microenvironment and do not simply fail to tolerate the T cells they generate. Not only can these antibodies affect T\textsubscript{H}1 polarization (33), they must also hold unique clues to autoimmunizing cell types in paraneoplastic MG (see below).

The resulting responses against these cytokines may well involve the germinal centers noted within ~20% of MG thymomas (4,6), which could readily explain the ongoing clonal diversification evident in some of the ‘M’ Fabs. The contrasting minimal spontaneous production of anti-AChR fits well with the absence of the native receptor and myoid cells in thymomas (15), and with the proposed priming there only of T\textsubscript{H} against linear AChR epitopes (3,16,20).

The autoantibodies produced

Our success in isolating combinatorial Fabs correlates well with the easier detection of plasma cells specific for IFN-\(\alpha\) than the other three antigens (Fig. 1 and Table 2). It also confirms the apparent specificity of the antibodies produced in culture (Fig. 1 and Supplementary Data), as the Fabs showed no detectable cross-reactivity between IFN-\(\alpha\) and AChR. Moreover, they must have high affinities for the native molecules; even as monomers, they bind these antigens at low molarities (~10\(^{-9}\) M) and neutralized the anti-viral activity of some IFN-\(\alpha\) subtypes (Fig. 3). Their preference for native IFN-\(\alpha\) is shown by the stronger binding by several Fabs in RIA than in Western blots (Table 3, lower).

When pooled, the IFN-\(\alpha\)-specific Fabs appeared representative of the repertoire in these donors’ sera. However, each Fab bound only a subset of the labeled IFN-\(\alpha\), or neutralized only certain subtypes, implying recognition of distinct epitopes, perhaps differing in their sensitivity to iodination or to neutralization. [Differences in binding kinetics are unlikely because of the almost constant binding by each Fab over ~10-fold concentration range (Fig. 2)]. These samples are clearly a valuable source for cloning a range of human anti-IFN-\(\alpha\) and anti-IL-12 antibodies, especially in view of their specificity for the native molecules, and of the available mix of memory B cells and pre-activated plasma cells. That might help to define IFN-\(\alpha\) epitopes in more detail.

The V\textsubscript{H}3-23 and V\textsubscript{\kappa} 02/12 genes are widely used [e.g. (23,26,27)]; their co-occurrence in both the present cases, especially in five of their six strongest-binding Fabs, suggests that they may be natural partners in situ. The clear evidence of ongoing clonal evolution—in both V\textsubscript{H} and V\textsubscript{\kappa}—in case 23’s thymic remnant strongly implies an antigen-driven response in the germinal centers previously noted in this sample (6). With the other donor, the evidence is less compelling because of the greater diversity among his (‘M’) Fabs, which include one clonally related pair. They could well derive from his thymoma, which strongly produced anti-IFN-\(\alpha\) in culture (Fig. 1c; 11); interestingly, four of the five unmutated V\textsubscript{\kappa} sequences are from this case, suggesting a higher proportion of naive or recently primed clones.

Clues to potential autoimmunizing cell types in thymomas

Our findings predict an autoimmunizing cell type in thymomas that is (i) potently immunogenic, (ii) expresses IL-12 and/or a broad range of IFN-\(\alpha\) subtypes in their native form, and (iii) can activate both T\textsubscript{H} and B cells, (iv) apparently leading to antigen-driven antibody diversification.

Normally, after appropriate stimulation, both IFN-\(\alpha\) and IL-12 are produced mainly by dendritic cell (DC) subsets (34–36). In the thymus, DC, NK cells and thymocytes can all be generated from a common progenitor (37). As thymomas often generate thymocytes in vast excess (6,7), there could be parallel aberrations in DC differentiation or behavior. Moreover, since certain DC can also prime naive B cells (38,39), DC subsets in thymomas seem particularly plausible autoreactive agents. They are further implicated both by the coincident responses to IL-12p70—and its inducible p40 chain—as well as to IFN-\(\alpha\) in its native form, and by the much more broadly reactive antibodies to most IFN-\(\alpha\) subtypes than seen after IFN-\(\alpha\) therapy (A. Meager et al., in preparation). By contrast, MG/thymoma cases rarely have autoantibodies against IFN-\(\beta\) (31), which is mainly produced by fibroblasts. The neoplastic epithelial cells may well contribute too, although their APC activity is weak (40); in addition, titin is expressed by thymoma epithelial cells (rather than DC) (41), but we failed to detect spontaneous antibody production against it. Furthermore, since the patients’ anti-cytokine autoantibodies do not obviously correlate with the very variable thymoma histology (30–32), the DC there may be a more plausible common factor, as we are now investigating. In view of their hemopoietic origins, they also seem likelier candidates for maintaining these responses (often for years) after thymomectomy (31), and possibly even for provoking the autoimmune bone marrow aplasias that associate with ~5% of thymomas (2–5). Finally, IFN-\(\alpha\)-DC interactions have recently also been implicated in immunopathogenesis in systemic lupus erythematosus (42).

With the AChR, many groups have reported expression of isolated subunits by thymic epithelial cells, whether in normal thymus (43), in EOMG hyperplasia (44) or in thymomas (45,46), but the intact receptor has not been detected in thymomas (15). We have therefore hypothesized that linear
AChR epitopes from the hyperplastic or neoplastic epithelial cells initially autoimmunize T<sub>n</sub>, (perhaps with help from DC), whereas the eventual priming of B cells against the native AChR conformation depends on thymic myoid cells in EOMG (20) and possibly also in thymic remnants (see below). With IFN-α and IL-12, by contrast, we now propose that both T<sub>n</sub> and B cells could be primed in the thymoma against these simpler molecules by a cell type such as DC. These suggestions can readily explain the observed pattern of prior activation in the thymomas of plasma cell responses to IFN-α and/or IL-12, but not to AChR. Identifying this cell type may give valuable clues to T<sub>n</sub> autoimmunization against AChR and possibly also to the even more puzzling mechanisms in some late-onset MG patients who show similar serology with no thymic tumor or hyperplasia, but only atrophy.

Contributions from thymic remnants versus thymomas
Whereas the response patterns in thymic remnants were broadly similar to AChR, IFN-α and IL-12, spontaneous antibody production was much more striking in the thymomas against the cytokines than against AChR (Fig. 1a and c and Table 2); even when AChR-specific B cells were present there, they showed little sign of prior activation (Fig. 1a) (10,29). Several other findings also implicate the thymomas in the responses to IFN-α and IL-12 even more deeply than the remnants—where total antibody productivity was often much lower. In general, these serum antibodies co-occur particularly with thymomas, above all after their recurrence (30–32); by then, there is usually no thymic remnant—as must also apply in many elderly cases with primary thymomas. In contrast, these antibodies are not found in EOMG, where the thymus is even more consistently hyperplastic than in the remnants. The prior activation of similar responses in the remnants might reflect either migration of DC from thymomas or the trapping of circulating IFN-α or IL-12 in germinal centers after forming complexes with autoantibodies, just as occurs normally with many extraneous antigens (47). The parallel responses in remnants to AChR are probably provoked by the AChR<sup>+</sup> myoid cells, as in EOMG (20); interestingly, the one AChR-specific Fab was derived from a remnant and preferred the fetal AChR isoform that is expressed by thymic myoid cells (21). We suspect that B cells must also be primed elsewhere—especially in elderly MG/thymoma cases with no remaining thymus or myoid cells. If they are mainly induced in muscle ‘lymphorrhages’, that could explain the minimal anti-titin responses in thymomas. Alternatively, that antibody assay may merely be less sensitive.

Recirculating memory B cells in thymomas
Previously, anti-AChR production was noted only with cells from remnants (6,22,29). The unexpected responses here (with PWM) to AChR in occasional thymomas might paradoxically reflect the more frequent pre-treatment with corticosteroids (13 of 24 cases) and so might the surprisingly high productivity of anti-cytokine antibodies despite low serum titers in some cases. As well as enriching such rare cell types as B and plasma cells (6), this treatment seems to reduce serum titers against AChR more than against the cytokines (31,32) and so may again favor cytokine-specific plasma cells.

Resting but PWM-responsive B cells specific for IFN-α or IL-12 were detected in some peripheral blood lymphocyte samples, as noted previously for AChR in cases with high serum titers (unpublished). However, we have never seen significant spontaneous antibody production by peripheral blood lymphocytes, consistently with the general rarity of circulating plasma cells (48). Interestingly, one of the four thymomas with memory B cell responses to AChR (patient 8; corticosteroid pre-treated) also gave the only clear response to titin (22%, again with PWM, not shown); the same sample had previously also shown a very striking T<sub>n</sub> response to tuberculin (49). That indicates immigration of memory cells from the periphery, possibly into the perivascular spaces so characteristic of thymomas in MG (4,5), in which B cell clusters have also been observed (16). If they were very receptive to activated cells, that could explain their anti-AChR (Fig. 1a) and anti-striatal muscle antigen responses (9,10).

In conclusion, our results suggest active autoimmunization against IFN-α and/or IL-12 in a ‘dangerous’ thymoma microenvironment, and offer novel clues to the autoimmunizing cell type(s), which may be relevant in other MG subsets or other diseases.

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Abbreviations

- α-BuTX: α-bungarotoxin
- ACh: acetylcholine
- AChR: acetylcholine receptor
- APC: antigen-presenting cell(s)
- DC: dendritic cell
- EOMG: early-onset myasthenia gravis
- MG: myasthenia gravis
- PWM: pokeweed mitogen
- RIA: radioimmunoassay

References

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