Neutralizing antibodies against endogenous interferon in myasthenia gravis patients

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ABSTRACT. Anti-cytokine antibodies (Abs) play an important role in the regulation of the immune response, both under normal conditions and in several autoimmune and neoplastic disorders. In the present study, we have investigated the occurrence and the clinical significance of natural neutralizing Abs (NAbs) against interferons (IFNs) alpha (α), beta (β) and gamma (γ), as detected by bioassay, in 52 patients with myasthenia gravis (MG), and 43 sex- and age-matched healthy individuals. Patients showing titres ≥ 1.3 Log 1/10, confirmed in 2 consecutive samples collected two months apart, were considered positive. NAbs against any of the IFNs were not detected in healthy subjects. Of the 52 MG patients, 11 (21.1%) had NAbs against IFNα, and three (5.8%) had NAbs against IFNβ. None of these patients was found to be positive for NAbs against IFNγ. Of the patients positive for NAbs against IFNα, eight (15.4%) had NAbs at titres ≥ 2 Log t1/10. A positive association was observed between high titres of NAbs and the presence of thymoma. These data suggest the presence of a generalized activation of the humoral response in MG.

Keywords: Neutralizing antibodies, interferon, myasthenia gravis, thymus tumor, cytokines, anti-cytokines antibodies

Myasthenia gravis (MG) is a relatively uncommon disorder caused by antibody-mediated attack directed against acetylcholine receptors (AchRs) [1]. Autoreactive T cells have been shown to play a crucial role in stimulating B-lymphocytes to produce antibodies (Abs) against AchRs [2]. How autoreactive T cells originate and become capable of eliciting this autoimmune response is still a matter of debate.

MG is the most common disease associated with thymoma: approximately 10-15% of MG patients have a thymic tumour and 40% of patients with thymoma will develop MG [1]. Thymomas are tumours with the highest incidence of associated autoimmune disorders [3]. It is thought that neoplastic epithelial cells in thymoma express auto-antigenic epitopes, which either positively select helper T cells already present in the tumour or sensitise re-circulating peripheral T cells [4, 5]. Also, a recent study has shown that natural killer (NK) cells control autoreactive T cells, suggesting that alteration of this surveillance mechanism may determine the autoimmune response against endogenous antigens such as AchRs [6].

Cytokines are important contributors to the immune response, and deregulation of their production may be observed in autoimmunity and cancer. Furthermore, cytokines such as interferons (IFNs), have been widely used to treat neoplasm [7-9] and a number of autoimmune disorders [10-13]. Two types of IFNs are known: type I (alpha [α] and beta [β]) and type II (gamma [γ]). These two groups of molecules have different immunomodulatory effects [14]. For instance, IFNα and IFNβ have been shown to increase MHC class II antigen expression, but not as effectively as IFNγ [15, 16]. Also, IFNγ favours the secretion of pro-inflammatory cytokines including tumour necrosis factors and interleukins 1 and 6, whereas IFNα and IFNβ have been shown to down-regulate this activity [17, 18]. IFNγ specifically modulates the synthesis and/or expression of a number of antigens and complement proteins [19] and affects the function of B and T cells [20].

Endogenous anti-cytokine Abs may occur naturally, representing an autoregulatory mechanism of the immune system for controlling cytokine expression [21]. They can be detected in normal conditions and in many infectious, autoimmune and neoplastic diseases, but their role is poorly understood [21, 22]. Spontaneous production of neutralizing Abs (NAbs) to IFNα has been reported in patients with MG [23], and has been found to be associated with either the presence or recurrence of thymoma [23, 24]. However, there is little information regarding the presence and potential clinical significance of NAbs against IFNβ and IFNγ in patients with MG [25]. In this
study, we investigate whether serum NAbs to both types of IFNs, could represent a surrogate marker of disease activity in MG patients. To test this hypothesis, we assayed serum NAbs to either type I or type II IFN in a cohort of MG patients naive to IFN treatment, and evaluated whether the presence of NAbs was associated with any demographic or clinical features of the MG patients studied.

MATERIALS AND METHODS

Patients and study design

Fifty-two MG patients and 43 sex- and age-matched, healthy individuals were enrolled in the present study. The local ethics committee approved the study and informed written consent was obtained from each patient. Demographic and relevant clinical data are reported in Table 1. Patients were excluded from the study if they had been previously treated with IFNs. Patients who were pregnant or had psychiatric or other neurological disturbances, infectious, autoimmune, neoplastic or other clinically evident inflammatory processes were also excluded. Blood samples were collected in vials, not containing anticoagulants, from patients at the beginning of the study (baseline) and after two months, and were collected once from healthy individuals. Serum samples were then separated by centrifugation and stored at -20°C until testing.

Bioassay for NAbs

NAbs were measured by a previously described bioassay [26]. Briefly, to avoid interference by complement during the assay, sera were incubated at 56°C for 30 minutes, after which 60 µl of twofold serial dilutions (starting from 1:10) of the samples were incubated at 37°C with 20 IU/ml of each type of IFN. The following IFNs were used: human recombinant (r) IFN-α2a (Roferon, Roche); leucocyte IFNα (Alfaferone, Alfa Wassermann) indicated as natural (n) IFNα; rIFNβ-1a (Avonex, Biogen); rIFNγ (Gamma Interferon, Boehringer Ingelheim). After 1 hour, 100 µl of each serum-IFN mixture was added to duplicate monolayers of A549 cells in 96-well, microtitre plates. After culture for 18-24 hours and extensive washing, the cells were challenged with encephalomyocarditis virus and incubated at 37°C for 24 hours. Antiviral activity and its neutralization were assessed on the basis of the virus-induced, cytopathic effect (CPE). To quantify this, the cells were stained with crystal violet in 20% ethanol. The dye taken up by the cells was eluted with 33% acetic acid, and its absorbance was measured in a microdensitometer at 540 nm. The extent of virus-induced CPE, its inhibition by nIFNα, rIFNα or rIFNβ1a and IFNγ, and their inhibition by NAbs were shown by the amount of dye eluted from each well. Titres were calculated using the Kawade method [27], and were expressed as t1/10, namely the dilution of serum that reduces the laboratory units (LU/ml of IFN to 1 LU/ml) [28]. Controls included a titration of both the IFN used in the assay and a reference standard antibody to IFN (National Institute of Health, Bethesda, MD USA, code G038-501-572). The limit of quantification of the bioassay was 1:20 and serum samples found to contain ≥ 1.3 Log t1/10 in two consecutive samples were considered as positive (+) for neutralizing activity (NA) to IFNs.

The NA of the high titre (≥ 2 Log t1/10) samples was characterized as specifically due to the presence of NAbs to IFN by means of affinity chromatography on protein-A sepharose (Pierce, Milan, Italy). Conversely, it was not possible, due to the need for larger volumes, to associate NA and Nab in low titre sera (< 2 Log t1/10). These sera were examined using a previously described ELISA [29], which briefly was performed as follows: 96-well flat-bottomed plates (Greiner GmbH, Frickenhausen, Germany) were coated with either IFNγ or IFNβ diluted in PBS containing 1% bovine serum albumin (Sigma Chemicals, Milan, Italy), at concentrations of 10 ng/well. After overnight incubation at 4°C, the plates were rinsed 3 times, and a total of 100 µ of the patients’ serum, diluted in PBS, was then added to the wells, in duplicate, in two-fold serial dilutions starting from 1:20. After 1 h of incubation at 37°C, the plates were washed, and a total of 100 µl of anti-human immunoglobulin alkaline phosphatase (Sigma Chemicals, Milan, Italy), diluted to a concentration of 1:6,000 in PBS was added to each well and left to incubate at 37°C for 1 h. The ELISA plate was then washed three times and a colour reaction was observed by adding 100 µl OPD (Dako chemicals, Milan, Italy) for 30 min at room temperature, after which optical density (OD) was read at 480/589 nm. The sample was considered positive when the serum produced an OD higher than the mean of OD values.

Table 1

Demographic and clinical features of patients

<table>
<thead>
<tr>
<th></th>
<th>MG with thymoma (all AchR Abs +)</th>
<th>MG without thymic tumor (37/52)</th>
<th>AchR Abs + age ≤ 45 years at onset of disease</th>
<th>AchR Abs + age &gt; 45 years at onset of disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n° (%)</td>
<td>15/29</td>
<td>8/15</td>
<td>19/37</td>
<td>10/19</td>
</tr>
<tr>
<td>Male-female</td>
<td>9/6</td>
<td>5/3</td>
<td>5/14</td>
<td>6/4</td>
</tr>
<tr>
<td>Age (years): median (range)</td>
<td>47 (22-60)</td>
<td>47.5 (37-69)</td>
<td>42 (25-70)</td>
<td>70 (61-84)</td>
</tr>
<tr>
<td>Years of disease: median (range)</td>
<td>9 (2-45)</td>
<td>5.5 (2-21)</td>
<td>17 (3-40)</td>
<td>3.5 (1-14)</td>
</tr>
<tr>
<td>Ocular MG: n° (%)</td>
<td>–</td>
<td>3 (6)</td>
<td>2 (4)</td>
<td>3 (6)</td>
</tr>
<tr>
<td>Generalized MG: n° (%)</td>
<td>15 (25)</td>
<td>5 (10)</td>
<td>17 (33)</td>
<td>7 (13)</td>
</tr>
<tr>
<td>Immunosuppressive therapy: n° (%)</td>
<td>12 (23)</td>
<td>7 (13)</td>
<td>12 (23)</td>
<td>7 (13)</td>
</tr>
</tbody>
</table>
recorded with a pool of serum samples derived from ten healthy subjects, plus two standard deviations. All low titre sera reacted with IFN molecules, although to different extents thus indirectly indicating that low titre sera contain Abs to IFN. Therefore, all NA + sera were assumed to be NAb + as well.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Science (SPSS inc., Chicago, version 10.0). Mean values of NAb were expressed using the geometric mean. Student’s t-test and the Chi-squared test were used when appropriate. A p value based on two-tailed statistical tests ≤ 0.05 was considered significant.

RESULTS

Serum from normal individuals was negative for NAb to each type of IFN. Serum from 12 (23.1%) patients with MG was positive for NAb against nIFNα, and all but one of them were positive for NAb against rIFNα as well. Three patients (5.8%) were positive for NAb against IFNβ. These three patients were found to be positive for NAb against both IFNα preparations. None of the serum samples reacted against IFNγ. Of the 12 samples positive for anti-IFNα NAb, 8 (66.7%) (15.4% of the entire cohort) had NAb at high titres (≥ 2 Log t1/10). Table 2 indicates the levels of NAb against nIFNα and rIFNα at baseline and after two months for each of these 8 individuals. These high titre samples contained NAb against IFNα, either rIFNα or nIFNα. However, titres against rIFNα were significantly higher than those against nIFNα, when average values (i.e. geometric mean) were considered. The mean values of these titres were 3.1 ± 0.4 Log t1/10 for NAb against rIFNα and 2.5 ± 0.2 Log t1/10 for NAb against nIFNα (p = 0.001) at the time of the first sample collection and 3.1 ± 0.4 Log t1/10 for NAb to rIFNα and 2.6 ± 0.2 Log t1/10 for NAb to nIFNα (p = 0.003) at the time of the second evaluation, two months later.

No correlation was found between the presence of NAb and the demographic or clinical features of patients when running the statistical analysis by stratifying patients according to the presence or absence of NAbs, as determined by our assays, to each type of IFN. Nevertheless, a positive association was observed between high titres of NAb (≥ 2 Log t1/10) and the presence of thymoma (Table 3).

DISCUSSION

The presence of natural or therapy-induced Abs against an array of cytokines and growth factors has been reported [30]. As the use of recombinant human cytokines and growth factors for the treatment of different human diseases has become common practice, it is important to study the clinical relevance of these auto-antibodies. Production of anti-cytokine Abs may represent a common event in autoimmunity, and their occurrence can be postulated as either a specific attempt to counteract the production of potentially harmful cytokines [31] or as the result of a non-specific polyclonal autoimmune activation.

Anti-IFNα NAb have been detected in patients with autoimmune [32, 33] and malignant diseases [34, 35], red cell aplasia [35] and in bone marrow transplant recipients [36]. Evidence of sporadic, spontaneous formation of NAb against IFNβ in either normal or pathological conditions has also been reported [21, 25]. Finally, NAbs against IFN have been rarely reported in normal subjects [37] or in patients with infectious diseases [22] and Guillain Barre syndrome [38]. Interestingly, in the latter condition, it has been noted that upon clinical improvement, the levels of NAb increase while the number of IFNγ-secreting cells decrease [39]. However, NAb against IFNγ have not been found in patients with MG [25].

Here we analyse the presence of NAb against IFNα, IFNβ and IFNγ in a cohort of patients with MG and no other immunological, neoplastic or infective condition. Patients who were pregnant were also excluded for several reasons. Firstly, exacerbations may occur in up to 41% patients with MG during pregnancy [40], most likely because of an increase of B-cell activity [41]. Further, type 1 cytokines, such as IFNγ, TNFα, and IL-2 are down-regulated by the means of several immunosuppressive factors in the serum of pregnant women. Cytotoxic activity and IFNγ production by NK cells are suppressed as well [41]. As with patients affected by other immunological disorders also included from our study, the presence of concomitant immunological events occurring in pregnant women, would make it difficult to speculate on the probable mechanism(s) responsible for the possible presence or absence of NAb against IFN.

We demonstrate the presence of NAb against IFNα and β ranging from 5.8% to 23.1% in a group of MG patients never treated with IFN. In contrast, none of the patients were found to have NAb against IFNγ. In addition, high levels (i.e. ≥ 2 Log t1/10) of NAb were exclusively found

<table>
<thead>
<tr>
<th>Patient</th>
<th>First sample (baseline)</th>
<th>Second sample (2 months later)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NAb to IFNα2</td>
<td>NAb to nIFNα</td>
</tr>
<tr>
<td>1</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>2</td>
<td>3.4</td>
<td>2.5</td>
</tr>
<tr>
<td>3</td>
<td>3.4</td>
<td>2.8</td>
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<tr>
<td>4</td>
<td>3.4</td>
<td>2.8</td>
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<tr>
<td>5</td>
<td>3.4</td>
<td>2.5</td>
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<tr>
<td>6</td>
<td>3.4</td>
<td>2.2</td>
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<tr>
<td>7</td>
<td>2.8</td>
<td>2.2</td>
</tr>
<tr>
<td>8</td>
<td>2.8</td>
<td>2.5</td>
</tr>
</tbody>
</table>
against IFN

warranted to better clarify the pathogenic role of NAbs

ever, these data are insufficient to draw any definitive

effect on the natural history of MG in our patients. How-

to react against IFN

Antibodies, interferon and myasthenia

IFN

t1/10. From these results, it appears unlikely that the pres-

seriously higher than NAb titres against nIFN

ring lymphoblastoid- or leucocyte- derived IFN

administered in humans, compared to "naturally" occur-

and future MG patients should give us additional clues as

clarify whether the production of NAbs against IFN

may suppress disease activity by reducing the ex-

pression of the MHC class II molecules, thereby hindering

the production of anti-AchR Abs [51]. This would then

argue that NAbs against IFNα should reduce or hamper in

some way, autoregulation of immune stimulation thereby

worsening the clinical status of MG patients. We were

unable to ascribe a specific clinical role to NAbs against

IFNα in our cohort of patients since we could not identify

any differences in terms of clinical outcome or subtype of

MG patients negative or positive for NAbs. However, con-

sistent with previous reports [23-25], high titres of NAbs

against IFNα were associated with the presence of

thymoma underlining the pivotal role of this tumour in

triggering the production of theseAbs. Thymomas repre-

sent a particular autoimmunogenic microenvironment [3]

and affected patients may have Abs against either neuronal

or extra-neuronal antigens and against several cytokines

[23, 52, 53]. This phenomenon seems to be due to the fact

that specific T cells, sensitised against different autoanti-
gens, are generated in thymoma [5]. Under particular con-

ditions, these sensitised cells are capable of participating in

the generation of auto-Abs. Since IFNα has been shown to

possess anti-proliferative effects against normal and tu-
mour cells [15], one can speculate that the presence of

NAbs against IFNα may be a mechanism used by thym-

oma to create a more favourable environment for its

growth. In any case, a longitudinal follow-up study con-

sidering a larger sample size of MG patients at different

stages of disease (i.e. active versus non-active), would help

clarify whether the production of NAbs against IFNα is

meant to have a protective role or, does it aggravate the

clinical course of MG.

To conclude, our results confirm and extend previous find-

ings that have demonstrated the occurrence of NAbs

against IFNα in patients with MG, and particularly in those

patients also with thymoma. Also confirmed is the sporadic

occurrence of NAbs against IFNβ and the absence of NAbs

against IFNγ [25]. Data from follow-up studies of these

and future MG patients should give us additional clues as

to the biological mechanisms involved and the clinical

relevance of our aforementioned findings.

Table 3

Relationship between anti-nIFNα or anti-rIFNα NAbs at high titers (≥ 100 t 1/10) and the
demographic or clinical features of patients

<table>
<thead>
<tr>
<th></th>
<th>NAbs + patients</th>
<th>NAbs – patients</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean years)</td>
<td>28</td>
<td>26.2</td>
<td>Ns</td>
</tr>
<tr>
<td>Gender (male-female)</td>
<td>4-4</td>
<td>21-23</td>
<td>Ns</td>
</tr>
<tr>
<td>Type of disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(generalized-ocular)</td>
<td>8-0</td>
<td>36-8</td>
<td>Ns</td>
</tr>
<tr>
<td>Years of disease (mean)</td>
<td>22.5</td>
<td>27.2</td>
<td>Ns</td>
</tr>
<tr>
<td>Thymic tumor (yes-no)</td>
<td>5-3</td>
<td>10-34</td>
<td>0.02</td>
</tr>
<tr>
<td>Immunosuppressive therapy</td>
<td>8-0</td>
<td>30-44</td>
<td>Ns</td>
</tr>
<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td>MG without thymoma (37/52)</td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>Onset age ≤ 45</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AchR Abs + (29/52)</td>
<td>4/8</td>
<td>25/44</td>
<td>Ns</td>
</tr>
<tr>
<td>Onset age &gt; 45</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AchR Abs + (15/52)</td>
<td>4/8</td>
<td>12/44</td>
<td>Ns</td>
</tr>
<tr>
<td>AchR Abs – (8/52)</td>
<td>0/8</td>
<td>7/44</td>
<td>Ns</td>
</tr>
</tbody>
</table>

Ns: not significant

AchR Abs – (8/52) 0/8 7/44 Ns

Years of disease (mean) 22.5 27.2 Ns

Thymic tumor (yes-no) 5-3 10-34 0.02

Immunosuppressive therapy 8-0 30-44 Ns

MG without thymoma (37/52)

Years of disease (mean) 22.5 27.2 Ns

Thymic tumor (yes-no) 5-3 10-34 0.02

Immunosuppressive therapy 8-0 30-44 Ns

NS: not significant

As for the higher frequency of NAbs against IFNα compared
to other IFNs (i.e. β and γ), it has been suggested that
IFNα plays a key role in the pathogenesis and clinical
relapses of MG. It is well known that IFNα is capable of
stimulating the production of a number of cytokines in-
cluding IFNγ, which in turn can induce expression of
MHC II antigens and, as a consequence, generation of
antibodies. The latter is most likely responsible for the
development of MG and other autoimmune antibody-
mediated disorders in patients infected with hepatitis C
virus that are treated with IFNα [46-48]. In this respect, it
may be speculated that NAbs against IFNα would play a
protective role in MG patients. However, numerous data
have suggested a protective role of IFNα in MG, as well.
IFNα has been shown to activate cellular molecular path-
ways creating negative feedback mechanisms capable of
quenching initially stimulated, immune cell activity, either
directly [49] or indirectly through IL-2 production [50].
Also, data from experimental MG models suggest that
IFNα may suppress disease activity by reducing the ex-
REFERENCES


genetic role for the thymoma in myasthenia gravis. Autosensitiza-
tion of IL-4-producing T cell clones recognizing extracellular acetylcholine receptor epitopes presented by minority class II isotypes. J. Clin. Invest. 101: 2268.


cother. 4: 343.


neuropathy associated with monoclonal IgM. J. Neurol. Neuro-


alpha (TNF) in acute myeloid leukemia (AML) blasts. Leukemia 6: 1155.

19. Rothuizen LE, Buclin T, Spertini F, Trinchard I, Munafò A, Buch-
walder PA, Ythier A, Biollaz J. 1999. Influence of interferon beta-1a dose frequency on PBMC cytokine secretion and biologi-


neous neutralising antibodies to interferon–alpha and interleukin-

24. Buckley C, Newsom-Davis J, Wilcox N, Vincent A. 2001. Do titin and cytokine antibodies in MG patients predict thymoma or thy-

alpha, interferon-omega and interleukin-12 in patients with thy-


therapy 10: 93.


terferon Res. 9 S1: 67.


