Neutralizing antibodies against endogenous interferon in myasthenia gravis patients

Francesca Bagnato[1], Alessandro Clemenzi[1], Carolina Scagnolari[2], Luigi Strizzi[3], Antonella Di Pasquale[1], Francesca Bellomi[2], Paola Di Marco[2], Guido Antonelli[2], Giovanni Antonini[1]

[1] Department of Neurological Sciences, « La Sapienza » University, Rome, Italy
[2] Department of Experimental Medicine and Pathology, Virology Section, La Sapienza University, Rome, Italy
[3] Department of Oncology and Neurosciences, Gabriele d'Annunzio University, Chieti, Italy

Correspondence: Guido Antonelli, PhD, Department of Experimental Medicine, Virology Section "La Sapienza" University, Viale di Porta Tiburtina 28, 00185 Rome, Italy. Telephone: + 39-06-4474.1222-20. Fax: + 39-06-4474.1236. E-mail: guido.antonelli@uniroma1.it

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 $t_{1/10}$, 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 $t_{1/10}$. 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 autoantibody 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 naïve 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 enchephalomyocarditis 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 $\geq$ 1.3 Log $t_{1/10}$ in two consecutive samples were considered as positive (+) for neutralizing activity (NA) to IFNs.

The NA of the high titre ($\geq$ 2 Log $t_{1/10}$) 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 $t_{1/10}$). 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 IFNα 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

	MG with thymoma (all AchR Abs +)	MG without thymic tumor (37/52)		
		AchR Abs –	AchR Abs + age ≤ 45 years at onset of disease	AchR Abs + age > 45 years at onset of disease
Patients:				
n° (%):	15 (29)	8 (15)	19 (37)	10 (19)
Male-female	9-6	5-3	5/14	6/4
Age (years): median (range)	47 (22-60)	47.5 (37-69)	42 (25-70)	70 (61-84)
Years of disease: median (range)	9 (2-45)	5.5 (2-21)	17 (3-40)	3.5 (1-14)
Ocular MG: n° (%)	–	3 (6)	2 (4)	3 (6)
Generalized MG: n° (%)	15 (25)	5 (10)	17 (33)	7 (13)
Immunosuppressive therapy: n° (%)	12 (23)	7 (13)	12 (23)	7 (13)

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 NAbs 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 NAbs to each type of IFN. Serum from 12 (23.1%) patients with MG was positive for NAbs against nIFNα, and all but one of them were positive for NAbs against rIFNα as well. Three patients (5.8%) were positive for NAbs against IFNβ. These three patients were found to be positive for NAbs against both IFNα preparations. None of the serum samples reacted against IFNγ. Of the 12 samples positive for anti-IFNα NAbs, 8 (66.7%) (15.4% of the entire cohort) had NAbs at high titres (≥ 2 Log $t_{1/10}$). *Table 2* indicates the levels of NAbs against nIFNα and rIFNα at baseline and after two months for each of these 8 individuals. These high titre samples contained NAbs 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 $t_{1/10}$ for NAbs against rIFNα and 2.5 ± 0.2 Log $t_{1/10}$ for NAbs against nIFNα ($p = 0.001$) at the time of the first sample collection and 3.1 ± 0.4 Log $t_{1/10}$ for NAbs to rIFNα and 2.6 ± 0.2 Log $t_{1/10}$ for NAbs to nIFNα ($p = 0.003$) at the time of the second evaluation, two months later.

No correlation was found between the presence of NAbs 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 NAbs (≥ 2 Log $t_{1/10}$) 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 NAbs 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 NAbs 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 Guillan Barre syndrome [38]. Interestingly, in the latter condition, it has been noted that upon clinical improvement, the levels of NAbs increase while the number of IFNγ-secreting cells decrease [39]. However, NAbs against IFNγ have not been found in patients with MG [25].

Here we analyse the presence of NAbs 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 increases 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 excluded 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 NAbs against IFN.

We demonstrate the presence of NAbs 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 NAbs against IFNγ. In addition, high levels (i.e. ≥ 2 Log $t_{1/10}$) of NAbs were exclusively found

Table 2
Titer (Log $t_{1/10}$) of serum neutralizing antibodies (NAbs) to interferon (IFN) alpha2 and natural (n) IFN alpha preparation

Patient	First sample (baseline)		Second sample (2 months later)	
	NAbs to IFNα2	NAbs to nIFNα	NAbs to IFNα2	NAbs to nIFNα
1	2.5	2.5	2.5	2.5
2	3.4	2.5	3.4	2.8
3	3.4	2.8	3.1	2.5
4	3.4	2.8	3.4	2.8
5	3.4	2.5	3.4	2.8
6	3.4	2.2	3.4	2.5
7	2.8	2.2	2.5	2.2
8	2.8	2.5	2.8	2.5

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

	NAbs + patients 8/52 (15%)	NAbs – patients 44/52 (85%)	P value
Age (mean years)	28	26.2	Ns
Gender (male-female)	4-4	21-23	Ns
Type of disease (generalized-ocular)	8-0	36-8	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)			
	NAB + patients 8/52 (15%)	NAB – patients 44/52 (85%)	P value
Onset age ≤ 45			
AchR Abs + (29/52)	4/8	25/44	Ns
Onset age > 45			
AchR Abs + (15/52)	4/8	12/44	Ns
AchR Abs – (8/52)	0/8	7/44	Ns

Ns: not significant

to react against IFNα, whereas the levels of NAbs against IFNβ were consistenly below the threshold titre of 2 Log $t_{1/10}$. From these results, it appears unlikely that the presence of NAbs against IFNβ would have had any significant effect on the natural history of MG in our patients. However, these data are insufficient to draw any definitive conclusions, and a longitudinal, follow-up study would be warranted to better clarify the pathogenic role of NAbs against IFNβ. As mentioned above, we found higher levels of NAbs against IFNα. NAb titres against rIFNα were significantly higher than NAb titres against nIFNα in samples analysed at baseline and after two months. This result in our cohort of patients confirms previous data. It has been reported that rIFNα2 is more immunogenic when administered in humans, compared to "naturally" occurring lymphoblastoid- or leucocyte- derived IFNα mixtures [42, 43]. On the other hand, several other studies showed that IFNα subtypes are antigenically distinguishable, since antibodies to IFNα2 fail to react with all IFNα subtypes included in the IFNα mixture [42, 44, 45].

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 including 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 pathways 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 expression 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, consistent 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 these Abs. Thymomas represent 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 autoantigens, are generated in thymoma [5]. Under particular conditions, 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 tumour cells [15], one can speculate that the presence of NAbs against IFNα may be a mechanism used by thymoma to create a more favourable environment for its growth. In any case, a longitudinal follow-up study considering 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 findings 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.

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