Myasthenia Gravis: Reactivation of Clinical Disease and of Autoimmune Factors after Discontinuation of Long-Term Azathioprine

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In 15 patients with myasthenia gravis who were in stable clinical remission while receiving azathioprine, we monitored disease severity and serial autoantibody titers before and after discontinuation of azathioprine. Cellular immunoreactivity against tuberculin (PPD) and against *Torpedo* acetylcholine receptor (AChR) was measured serially in 11 patients. Eight of 15 patients (53%) had a clinical relapse after 3 to 11 months, necessitating the reinstitution of immunosuppressive treatment in 6 patients. Seven patients have remained clinically stable during an observation period of 20 to 40 months. Anti-AChR autoantibody titers correlated closely with the clinical course in the majority of patients, and rose markedly in 7 of the 8 patients who relapsed. Cellular stimulation indices correlated less closely with the clinical severity. Only in 3 patients did the clinical score, antibody titer, and cellular stimulation index rise concurrently. In 4 patients who had high cellular stimulation indices after the discontinuation of azathioprine, it was possible to isolate AChR-reactive inducer/helper T lymphocytes.

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Azathioprine (AZA) has only recently come into frequent use for the treatment of myasthenia gravis in the United States [9, 12, 21, 27]. In Europe, AZA has been employed for the treatment of severe generalized myasthenia gravis since the late 1960s [8, 23, 24]. Serious side effects such as severe hematopoietic depression, infection, and late neoplasms have been uncommon in these patients, and despite the lack of controlled studies, AZA has been increasingly accepted as an effective treatment [10]. We investigated the effects of AZA on disease activity in a selected but clearly defined subgroup of myasthenic patients before and after the discontinuation of long-term treatment. Half of our 15 patients experienced relapse but subsequently improved after reinstitution of immunosuppressive drugs. Clinical relapses were accompanied by reactivation of autoimmune factors on the B and T lymphocyte level.

Methods

Patients

Fifteen patients with previously diagnosed generalized myasthenia gravis who gave informed consent were included in the study provided they were in complete or almost complete stable remission for at least 6 months while taking AZA, 2 to 3 mg/kg body weight/day, alone (13 of 15 patients) or in combination with methylprednisolone, 10 mg/day or 40 mg every other day (2 of 15 patients). We administer immunosuppressive medication only to patients with severe generalized myasthenia gravis if their symptoms are not controlled satisfactorily with pyridostigmine bromide alone (up to 600 mg/day on a short-term basis). The response to anticholinesterase medication was judged satisfactory if patients were able to pursue their usual daily activities without subjectively unacceptable impairment (i.e., usually with clinical score values below 1). Ten of the patients had continued long-term anticholinesterase treatment with pyridostigmine, 120 to 480 mg/day. AZA was stopped abruptly at the onset of the study, whereas methylprednisolone was eliminated gradually over 1 month. Further details are given in the Table.

Patients were investigated at regular intervals. Clinical examination was performed with use of a standardized examination program including at least three of the following five test items: time of endurance for keeping arms outstretched (at 90 degrees, standing), leg outstretched (at 45 degrees, supine), and head lifted (at 45 degrees, supine); grip strength (decrement after 10 maximal closures measured with a dynamometer); and vital capacity, measured as described elsewhere [4, 5]. Clinical findings were expressed as a clinical score [4, 5], with values ranging from 0 (normal) to 3 (severe weakness). In this study a patient was considered in stable

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Comparison of Patients with and without Clinical Relapse after Withdrawal of Long-term AZA

Variables	No Relapse	Relapse
Patients[a]	7	8
Mean age (range)	36 years (21–58)	58.6 years (29–78)
Males/females	3/4	2/6
Mean age at onset (range)	31.4 years (20–52)	53.5 years (21–75)
Thymectomy	5[b]	3[c]
Mean duration of AZA treatment (range)	2.2 yr (1–4)	2.5 yr (1–5.5)
Mean maximum score (range)[d]	1.7 (0.9–3.0)	1.5 (0.8–2.0)
Mean maximum antibody titer (range)	44 nmol/L (2.9–156)	106 nmol/L (4.9–595)
Mean maximum AChR-induced stimulation index (range)[a]	8.2 (1.9–16.0)	7.7 (3.9–11.2)
Rise of antibody titer (> twofold)	0	7
Rise of stimulation index (> threefold/ > twofold)[a]	2/3	3/3

[a]Cellular stimulation indices were monitored in 5 of the patients who did not relapse and 6 of those who did.
[b]Benign thymic hyperplasia.
[c]Two malignant thymomas, 1 benign hyperplasia.
[d]See text for system of scoring.

AZA = azathioprine; AChR = acetylcholine receptor.

remission if the clinical score was less than 0.3 and did not change upon repeated examination during an observation period of more than 8 months (6 months in 1 patient). Clinical relapse was defined as an increase in the score (Δ score) of more than 0.3 in previously stable patients despite continued optimal anticholinesterase medication. When myasthenic signs recurred, the patients were observed at weekly intervals or were admitted before a decision on the reinstitution of immunosuppressive treatment was made. AZA, 2 to 3 mg/kg, was reinstituted alone (2 of 6 patients) or in combination with methylprednisolone in the more severely affected patients (4 of 6) to accelerate improvement (initial dose, 20 to 80 mg/day). Steroids were then gradually tapered over the ensuing months according to the clinical response. One patient with a fulminant relapse required plasma exchange.

Antibody Assay

Serial serum autoantibody concentrations were measured by the standard immunoprecipitation assay as described by Lindstrom and co-workers [20], with minor modifications [5, 31], using pooled Triton-X-100 extracts of human limb muscle labeled with ^{125}I-α-bungarotoxin (New England Nuclear, Boston, MA) as antigen.

Preparation of Acetylcholine Receptor for Microproliferation Assays

Purified acetylcholine receptor (AChR) was prepared from the electric organs of *Torpedo californica* by affinity chromatography on α-cobratoxin affinity columns as described elsewhere [17]. The specific activities of purified receptor preparations ranged from 5 to 7.5 nmol/mg protein. For use in tissue culture, it was essential to remove even trace amounts of detergent (Triton-X-100) by extensive dialysis in Ultra-Thimbles UH 100/25 (Schleicher & Schüll, FRG). Since large quantities of AChR were required, we searched for a simpler method to prepare AChR suitable for tissue culture. The following protocol yielded *Torpedo* AChR with a specific activity of 0.3 to 0.6 nmol/mg protein, which had the same stimulatory properties as the purified AChR if used in tissue culture at the same final concentration of approximately 25 pmol α-bungarotoxin binding sites per milliliter. Twenty milliliters of a 2% Triton-X-100 extract of *Torpedo* electric organ was dialyzed against phosphate-buffered saline (PBS), centrifuged at 48,000 *g* for 45 minutes, and mixed with 15 gm washed (PBS) Bio-Beads SM2 (Bio-Rad, Richmond, CA). The mixture was incubated for 2 hours under gentle agitation, and the beads were allowed to sediment. The supernatant was dialyzed against PBS and concentrated in Ultra-Thimbles UH 100/25 for 3 days. Receptor concentration was determined after labeling with an excess of ^{125}I-α-bungarotoxin and precipitation of the receptor–toxin complex with 33% ammonium sulfate [1]. Protein concentration was measured according to Lowry and associates [22]. The preparations had a receptor concentration of 1.6 to 1.8 nmol/ml and a protein concentration of 3 to 5 mg/ml. Receptor was stored at −80°C until use.

Microproliferation Assay of Cellular Immunoreactivity

Cellular immunoreactivity against a standard antigen (tuberculin, PPD) and against *Torpedo* AChR could be monitored serially in 11 patients. Twenty milliliters of heparinized venous blood was centrifuged over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) to isolate peripheral blood mononuclear cells: 2×10^5 were cultured in triplicate in round-bottom microtiter plates (NUNC, Roskilde, Denmark) in 0.2 ml of RPMI 1640 (GIBCO Europe, Karlsruhe, FRG) supplemented with 10% heat-inactivated (56°C, 30 minutes) pooled human serum, 2 mM L-glutamine, 100 U/ml penicillin, 50 μg/ml streptomycin (culture medium), and one of the following proteins: 25 pmol/ml *Torpedo* AChR, 1 μg/ml PPD (Behringwerke, Marburg, FRG), or 1 μg/ml phytohemagglutinin (PHA; Wellcome, England). After 72 hours the cultures were pulsed with ^{3}H-thymidine (1 μCi per well; specific activity, 5 Ci/mmol; Amersham, England). Sixteen hours later, the cells were harvested with a Titertek multiple harvester and thymidine incorporation was measured in a liquid scintillation counter.

Antigen-specific stimulation was expressed as stimulation index (SI):

$$SI = \frac{\text{mean counts in the presence of antigen}}{\text{mean counts without antigen}}$$

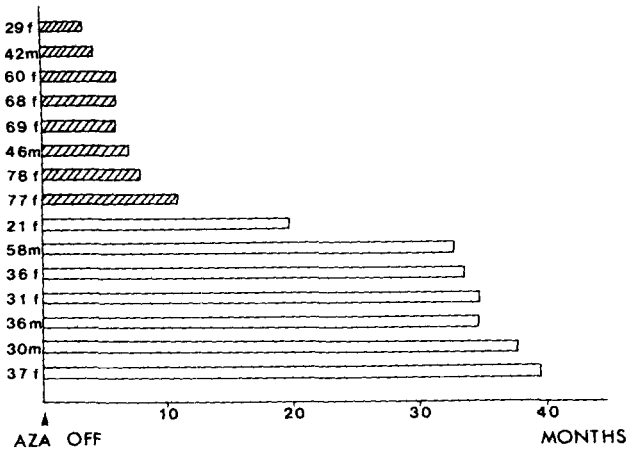

Fig 1. Clinical course in 15 patients after discontinuation of azathioprine (AZA) *at zero time. Before zero time, all patients had been in clinical remission as defined in the Methods section. Hatched columns indicate the time until relapse in 8 of the 15 patients. Open columns represent the observation time in the 7 patients who did not experience relapse. At left of the ordinate, age (years) and sex* (m,f) *are given for each patient.*

The mean stimulation index (*Torpedo* AChR; 25 pmol/ml) in 23 control patients with other neurological diseases was 1.0 (range, 0.4 to 2.0).

Results

Clinical Observations

Eight of the 15 patients (53%) had a clinical relapse within 3 to 11 months (mean, 6.5) after discontinuation of AZA (Fig 1). In each of the patients who relapsed, clinical deterioration occurred after a stable phase of variable duration, and myasthenic weakness usually developed rapidly within 2 to 3 weeks (Fig 2). Six of the 8 patients who relapsed were given a new course of AZA after an observation period of 1 to 2 weeks because of progressive weakness despite optimal anticholinesterase medication. In the 4 patients with the most rapid clinical deterioration, a short course of corticosteroids was added. In 1 patient a myasthenic crisis ensued that required a series of three plasma exchanges. In 4 of the 6 treated patients, clinical improvement by half of the respective peak score value occurred within 6 weeks. In 2 of the 6 patients relatively mild symptoms (score below 0.7) persisted for 4 and 8 months, respectively. In 1 patient the relapse followed a mild respiratory infection, and spontaneous improvement occurred 2 weeks later. In another patient the relapse included only mild generalized signs not warranting the reinstitution of immunosuppressive treatment.

Reactivation of Autoantibody Production

All 15 patients had elevated anti-AChR antibody titers (see Table). During the total observation period of 1 to 3 years before patients entered the study, individual titers correlated closely in a nonlinear fashion with the

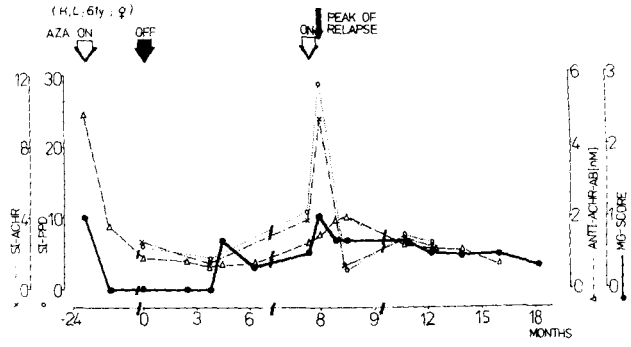

Fig 2. Synopsis of the clinical score, anti-acetylcholine receptor (AChR) antibody titer, and AChR- and PPD-induced cellular stimulation index before and after discontinuation of azathioprine (AZA) *at zero time in a patient who relapsed.* AZA ON (large open arrows) = *start of treatment with* AZA; AZA OFF (large black arrow) = *discontinuation of* AZA; SI-ACHR *and* SI-PPD = *stimulation index in microproliferation assay of peripheral blood lymphocytes with either* Torpedo *AChR or tuberculin as respective antigens;* ANTI-ACHR-AB = *antibodies to human solubilized acetylcholine receptor expressed in nanomoles of bungarotoxin binding sites per liter of serum;* MG-SCORE = *clinical score ranging from 0 (normal) to 3 (severe weakness). In this patient there was a sharp rise of the cellular immunoreactivity almost in synchrony with a rise of the clinical score. The autoantibody titer rose and decreased more slowly and remained elevated during the following months. A similar course was observed in 3 of the 6 patients who relapsed and in whom stimulation indices were monitored.*

clinical course. During the relapse, autoantibody titers rose more than twofold in 7 of the 8 patients; the onset of the rise usually occurred 2 to 3 months before the peak of relapse. One of the patients who relapsed continued to have only mild signs (score less than 0.4) and did not show a concomitant rise in serum autoantibody titer. None of the patients who remained clinically stable had a more than twofold increase in antibody concentrations. After AZA or AZA plus methylprednisolone was reinstituted, the titers decreased over the following months and usually approached the prestudy values within 4 to 7 months.

Reactivation of Cellular Immune Factors

During AZA treatment, the AChR-induced stimulation indices were elevated (greater than 2) in 6 of 11 patients (range, 2.6 to 13.8; mean, 5.8 in the patients with elevated stimulation indices). After AZA was stopped, the stimulation indices increased sixfold to ninefold with a variable latency of 1 to 6 months in only 3 of 6 patients who relapsed (see Fig 2). A similar rise occurred in 2 of the 5 patients who did not relapse and in whom stimulation indices were measured serially (Fig 3). After reinstitution of immunosuppressive drugs, the AChR-induced stimulation indices fell with a variable latency (1 to 8 weeks) to reach prestudy values. In the patients with sensitivity to tuberculin, the PPD-induced stimulation indices ran in parallel

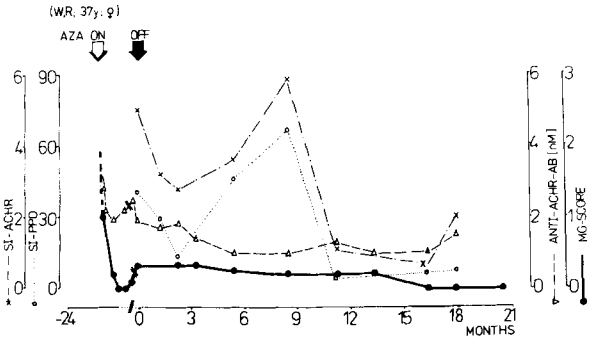

Fig 3. In this patient the cellular immunoreactivity rose intermittently without any parallel rise of the clinical score and antibody titer. This pattern of isolated reactivation of cellular reactivity was observed in 2 of the 5 patients who did not relapse and in whom stimulation indices were measured serially. The initial descent of the clinical score (dotted line) *10 months before discontinuation of azathioprine* (AZA) *(zero time of the present study) was induced by AZA in combination with plasmapheresis.* AZA ON (large open arrow) = *start of treatment with AZA;* AZA OFF (large black arrow) = *discontinuation of AZA;* SI-ACHR *and* SI-PPD = *stimulation index in microproliferation assay of peripheral blood lymphocytes with either* Torpedo *acetylcholine receptor or tuberculin as respective antigens;* ANTI-ACHR-AB = *antibodies to human solubilized acetylcholine receptor expressed in nanomoles of bungarotoxin binding sites per liter serum;* MG-SCORE = *clinical score ranging from 0 (normal) to 3 (severe weakness).*

with the myasthenia-specific AChR-induced stimulation indices (Figs 2, 3). This indicates that T cell responses to antigens other than the myasthenia-specific ones had also been reactivated. In the 2 patients with rising stimulation indices but with a stable clinical course, specific autoantibody titers also remained stable, indicating a dissociation between the B and T cell–derived immunoreactivity (Fig 3).

Discussion

In a recent clinical report, Mertens and associates [25] observed a relapse in 10 of 18 patients after withdrawal or reduction of AZA. Our prospective study confirms these findings in that half of our patients had a relapse after AZA was discontinued although they had been in stable remission during AZA treatment. At the time of this report, half of the patients have remained clinically stable for 20 to 40 months and therefore benefited from stopping a potentially dangerous medication. In the 6 patients who relapsed and who were given a new course of immunosuppressive medication, clinical improvement occurred with variable latency.

AZA and its biologically active metabolite 6-mercaptopurine appear to affect both B and T lymphocytes [6, 11, 13, 14]. We therefore monitored the humoral and cellular autoimmune activity before and after discontinuation of AZA. Anti-AChR autoantibody titers were elevated in all patients and correlated closely with the clinical course in individual patients. Moreover, in all but 1 patient the clinical relapse was preceded by a rise of the antibody titer. This further supports the notion that the intraindividual correlation among patients between the serum autoantibody concentration and the clinical severity of the disease is usually good [5, 28, 30–32]. After reinstitution of immunosuppressive treatment, the elevated titers declined slowly, approaching the pretreatment levels. This time course probably reflects the net effects of reduced antibody synthesis and rate of elimination of IgG.

Cellular stimulation indices correlated less closely with clinical course. After discontinuation of AZA, only in 3 of 6 patients did the cellular and humoral immunoreactivity and the clinical score rise in parallel. After reinstitution of immunosuppression, the elevated stimulation indices fell and had returned to their starting levels while the autoantibody titers were still elevated above pretreatment levels. The observed discrepancy between the humoral and cellular autoimmune activity may indicate that the cellular immunoreactivity (as reflected in the AChR-induced stimulation index) is linked only indirectly to the B cell–mediated (antibody-mediated) effector phase of myasthenia gravis. Elevated stimulation indices have been observed in only about 50 to 70% of patients with myasthenia gravis [7, 26]. There are conflicting reports of either no [7] interindividual correlation of the AChR-induced stimulation indices with disease severity or a positive [26] correlation. One reason for these conflicting results may be the source of AChR. The fish AChR that was used in most studies for proliferation assays may stimulate only subsets of the autosensitized human lymphocytes.

The significance of cellular autosensitization against AChR is still not clear. Theoretically, stimulation in vitro with soluble AChR could indicate proliferative activity of effector (cytotoxic, delayed-type hypersensitivity) or regulatory (inducer/helper, suppressor) T lymphocytes. In animal models of autoimmune disease it has been possible to isolate and characterize autoreactive T lymphocytes [2, 17]. In experimental autoimmune myasthenia gravis, which is induced by artificial immunization with purified AChR, it has been shown that lymph nodes and spleen cells contain a subpopulation of T lymphocytes that includes all of the AChR-induced proliferative activity and bears the surface markers of rat helper T cells. These purified helper T cells were found to exert helper function in vivo [16]. In human myasthenia gravis, analysis of unfractionated peripheral blood lymphocytes with monoclonal antibodies against surface differentiation antigens has shown either a modest decrease of suppressor T cells [3, 29] or no statistically significant changes [15]. To obtain defined populations of T lymphocytes for analysis (namely those lymphocytes carrying AChR-induced proliferative reactivity), we isolated

AChR-reactive T cells from 4 patients. For technical reasons, isolation was possible only in patients with high stimulation indices as seen after the discontinuation of AZA. These isolated AChR-specific T lymphocytes bear the OKT4 marker of inducer T cells [18].

We conclude that discontinuation of AZA in patients who are in stable clinical remission is associated with the risk of potentially serious relapses. Therefore, the experience with our small group of patients does not warrant a general recommendation to withdraw AZA from all patients in stable remission. At present, discontinuation should be limited to patients with hematopoietic or hepatic side effects or to very young patients in whom life-long treatment would carry an increasing risk of precipitating the formation of neoplasms [19]. After withdrawal of AZA, close follow-up is essential, with use of autoantibody titer measurements as a helpful indicator of the status of the disorder.

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