Influence of Azathioprine (Imuran) on In Vitro Immune Function in Multiple Sclerosis

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In vitro immune function was assessed in patients with multiple sclerosis (MS) who were receiving Imuran therapy, in untreated MS patients, and in controls. In untreated stable MS patients, concanavalin A (Con A)–driven mitogenic reactivity (T effector function) and Con A–induced suppressor activity were modestly reduced compared to controls; pokeweed mitogen–induced immunoglobulin G (IgG) secretion was increased. Untreated patients with active MS demonstrated high levels of IgG secretion and marked decreases in suppressor activity. In Imuran-treated patients, Con A mitogenic responses and suppressor activity were comparable to those observed in untreated stable patients, and IgG secretion was reduced. The results in the treated patients likely reflect a direct effect of Imuran on B cell function rather than an indirect effect mediated via suppressor cells.

Oger JJ-F, Antel JP, Kuo HH, Arnason BGW: Influence of azathioprine (Imuran) on in vitro immune function in multiple sclerosis. Ann Neurol 11:177–181, 1982

Multiple sclerosis (MS) is characterized by recurring relapses and remissions. In vitro studies indicate that immune activity fluctuates in MS patients depending on disease activity. Enumeration techniques have revealed decreased numbers of circulating T cells and of T cell subsets when disease is active [13, 18, 24, 26, 29, 32]. During attacks of MS, T_G cells [12] and T suppressor cells, defined by monoclonal antibodies [6, 30], are reduced in number from expected values while B cell number is suggestively increased [18, 26]. Functional assays of immunocyte function also detect abnormalities during relapses of disease. T suppressor cell function is markedly reduced [3, 11, 23], and in vitro response of lymphocytes to myelin basic protein has been reported to be increased [19, 33, 35].

Other aberrancies correlate less well or not at all with disease activity. Increased cerebrospinal fluid immunoglobulin G (IgG) and de novo IgG synthesis within the central nervous system are hallmarks of MS throughout its course. The numbers of avid T cells and active T cells are both reported to be lower in MS patients than in controls, but no convincing correlations with disease activity have been uncovered [14, 26, 34]. Finally, the proliferative response to concanavalin A (Con A) is subnormal in MS, but this abnormality is most readily discerned during remission [5, 20, 31]. Whether these changes in immune function contribute to the pathogenesis of the disease remains unresolved.

Given a suspected contribution by immunocytes to the pathogenesis of MS, numerous investigators have studied the effects of immunosuppression on the course of the disease. In several studies, azathioprine (Imuran) has been claimed to influence disease progression favorably, particularly in terms of reducing the frequency of relapses [2, 22, 27]. However, this opinion is not unanimous (reviewed in [8]).

The mechanisms responsible for the immunosuppressive effects of Imuran remain poorly understood, although Imuran does influence both effector and regulator lymphocyte function when added to lymphoid cells in vitro [9]. In the present study, we measured the effect of Imuran therapy in MS patients on in vitro T cell function (Con A mitogen response), B cell function (IgG response to pokeweed mitogen [PWM]), and T suppressor cell function (Con A–induced suppressor T cell activity).

Patients and Methods

All MS patients satisfied the usual clinical diagnostic criteria. Forty-eight untreated patients ranging in age from 16 to 52 years were studied in one or more assay systems. Patients were subdivided into three groups based on the state of their clinical disease—stable, active, or recovering—as defined previously [3]. Twelve additional patients ranging in age from 18 to 52 years were studied during Imuran therapy (0.3 mg/kg/day). One Imuran-treated patient had active disease at the time of study; the other patients had stable MS. Blood samples from 65 volunteers with an age range of 20 to 55 years were included in at least one assay.

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Received Feb 17, 1981, and in revised form Apr 30. Accepted for publication May 15, 1981.

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Fifty-five donors were normal controls and 10 had non-inflammatory neurological diseases.

Our methods for performing in vitro studies for Con A stimulation of mononuclear cells (MNCs) [4], for PWM-induced IgG secretion [21], and for Con A–induced suppressor cell activity have been reported [5].

MNCs were isolated from fresh peripheral blood by Ficoll-Hypaque density centrifugation (specific gravity, 1.078 gm/ml). Samples of MNCs from some individuals (8 MS treated and 4 MS untreated) were studied for their percentages of peroxidase-positive cells (monocytes), sheep erythrocyte rosetting cells (T cells), and sheep erythrocyte-antibody-complement binding cells (B cells), using standard techniques [4]. All results were within the normal limits for our laboratory. The remaining cells were divided into separate samples for use in either the Con A stimulation and suppressor assays or the PWM-induced IgG secretion assay.

For the Con A stimulation and suppressor assays, MNCs were washed three times in Hanks balanced salt solution without magnesium or calcium (HBSS), and 10^6 cells per milliliter were suspended in either RPMI or minimum essential medium (Microbiological Associates) with 20% fetal bovine serum plus glutamine (40 mM) and gentamicin (10 mg/dl). Five milliliter aliquots of cells were placed in flat-bottomed plastic flasks (Falcon; 25 cm^2 growth area) and cultured at 37°C for 96 hours with 3 μg/ml of Con A (Sigma Chemical Co.; Grade IV) (C_{con}) or without Con A (C_0).

After 96 hours, 100 μl aliquots of each culture were placed in microwells, pulsed with 25 μl (1 μCi) of ^{3}H-labeled thymidine (specific activity, 6.7 Ci/mol; New England Nuclear) for 5 hours, and harvested on a MASH II. The dried filters were counted in a liquid scintillation counter.

The remainder of the cells were decanted into 15 ml conical plastic tubes and treated with 25 μg/ml of mitomycin C for 30 minutes at 37°C. The treated cells (C^x) were washed four times in HBSS and resuspended at 10^6 per milliliter in culture media. Then 100 μl of C^x_{con} or C^x_0 cells were cocultured in microwells with 100 μl (10^5 cells) of freshly isolated autologous or heterologous responder (R) cells plus Con A (3 μg/ml) for 72 hours, and ^{3}H-thymidine uptake was determined after a 5-hour pulse. Percentage suppression was calculated as:

$$1 - \text{cpm}\,\frac{R + C^{x+}_{con} + \text{Con A}}{R + C^{x+}_0 + \text{Con A}} \times 100\%$$

Suppressor effect was comparable when minimum essential medium or RPMI was used or when autologous or heterologous responder cells were used; accordingly, data have been pooled.

For the assays of PWM-induced IgG secretion, MNCs were washed ten times at 4°C in HBSS and then suspended at 10^6 per milliliter in culture medium. One milliliter of cells were cultured in 12 × 75 mm plastic tubes either for one day without PWM or for seven days at 37°C with or without PWM (GIBCO; 1:100 final dilution from stock). Cultures were centrifuged and the supernatant collected and assayed for IgG content using a solid-phase radioimmunoassay as described previously [21, 25]. Data were compared among groups using Student's t test or, where noted, chi-square analysis.

Results

Con A Mitogenic Reactivity

The mean response (counts per minute ± SEM) of untreated stable MS patients (15,524 ± 2,775) was reduced compared to controls (24,578 ± 2,208; $p < 0.02$) (Table). The response of Imuran-treated patients (16,123 ± 5,331) was also low but not significantly different from that of controls. Wide variations in responses between individuals were observed. High responses (>15,000 cpm) were found in 36 of 49 controls (73%) but in only 9 of 24 stable untreated MS patients (38%) and in only 2 of 11 (18%) stable treated MS patients. The differences

Comparison of In Vitro Immune Function among Imuran-treated MS Patients, Untreated MS Patients, and Controls

Determination	Imuran-treated MS (a)	Untreated MS		Controls (d)	Significance†		
		Stable (b)	Active (c)		a–b	b–d	a–d
Response to Con A (cpm)	16,123 ± 5,331 (N = 11)	15,524 ± 2,775 (N = 24)	...	24,578 ± 2,208 (N = 49)	NS	$p < 0.02$	NS
PWM-induced IgG secretion (ng/ml)	1,248 ± 499 (N = 12)	2,372 ± 524 (N = 18)	9,008 ± 5,407 (N = 5)	1,377 ± 320 (N = 32)	NS	NS	NS
No. of high responders (>1,000 ng/ml)	3	13	5	13	$p < 0.02$	$p < 0.05$	$p < 0.02$
Con A suppressor activity (%)	23 ± 7% (N = 10)	30 ± 5% (N = 22)	9 ± 5% (N = 12)	40 ± 3% (N = 44)	NS	NS	$p < 0.05$

*Except for numbers of patients, values are means ± SEM. Number of subjects tested is given in parentheses.
†Means ± SEM are compared using Student t test; the numbers of high and low IgG responders are compared using chi-square analysis.

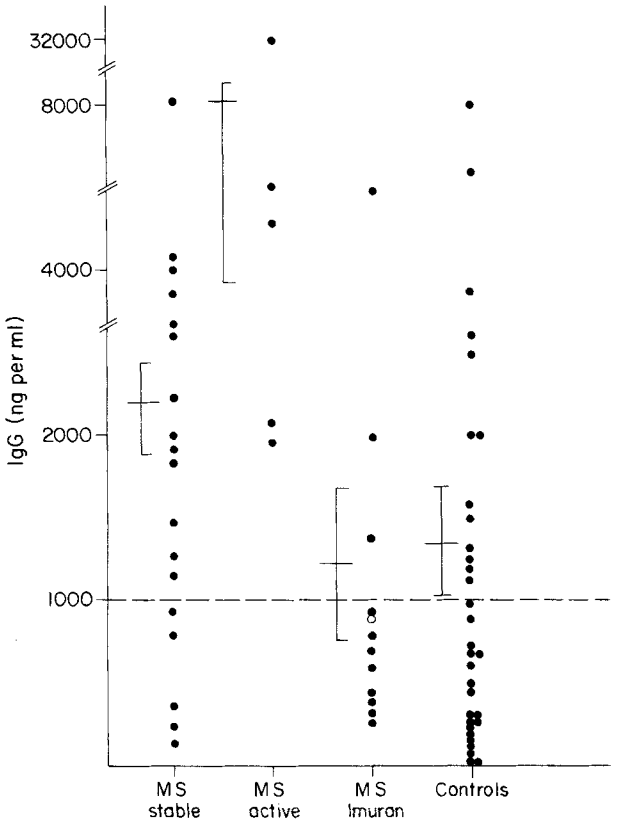

Fig 1. Pokeweed mitogen (PMW)–induced secretion of immunoglobulin G. Each point indicates the amount of IgG (in ng/ml) secreted by seven-day PWM-stimulated cultures (10^6 cells) from individual donors. The mean IgG secreted ± SEM is shown for each group. The open circle (○) indicates the patient with active disease.

were significant for untreated ($p < 0.005$) and for Imuran-treated patients ($p < 0.01$ by chi-square analysis) versus controls, but not for the two MS patient groups.

PWM-induced IgG Production

Data are presented as total IgG present in seven-day PWM-stimulated cultures (Fig 1). The mean IgG production in seven-day non-PWM-stimulated cultures for all donors was 398 ± 120 ng/ml and did not differ between groups. Mean IgG found in one-day controls was less than 200 ng/ml, indicating that the MNCs were washed sufficiently to remove serum IgG adsorbed to their surface and that IgG measured in seven-day cultures was produced in vitro. IgG secretion (mean ng/ml ± SEM) in seven-day PWM-stimulated cultures for Imuran-treated patients with stable MS was 1,248 ± 499 (N = 12) compared to 2,372 ± 524 (N = 18) for untreated patients with

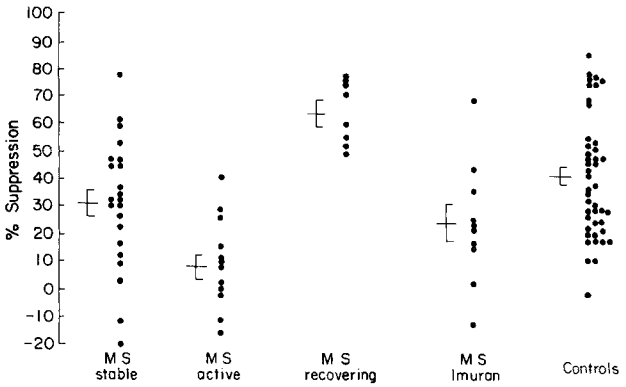

Fig 2. Concanavalin A (Con A)–induced suppressor cell activity. Each point indicates the percentage of con A–induced suppression for individual donors. Mean suppression ± SEM is shown for each group.

stable MS, 9,008 ± 5,407 (N = 5) for untreated patients with active MS, and 1,377 ± 320 (N = 32) for controls. Because individuals demonstrated wide variations (data points are given in Fig 1), patients and controls were subdivided into high responders (>1,000 ng/ml) and low responders (<1,000 ng/ml) (see the Table). Thirteen of the 32 controls (40%) were high responders compared to 13 of 18 (72%) untreated stable MS patients ($p < 0.05$ by chi-square) and 3 of 12 (25%) Imuran-treated patients ($p < 0.02$). The difference between untreated stable MS patients and treated MS patients is significant at $p < 0.02$ (chi-square analysis). The single Imuran-treated MS patient with active disease produced less than 1,000 ng/ml, whereas all 5 untreated active MS patients were high responders. Sequential IgG secretion studies done on 12 individuals (5 controls and 7 MS patients in the same disease or treatment state) revealed that 5 of 6 high responders remained so (>1,000 ng/ml), whereas all 6 low responders remained low responders.

Con A Suppressor Activity

The mean suppression ± SEM (see the Table) for the Imuran-treated patients with stable MS (23 ± 7%, N = 10) was reduced compared to the control group (40 ± 3%, N = 44, $p < 0.05$). Mean suppression was 30 ± 5% for stable untreated MS patients (N = 22) and 9 ± 5% (N = 12, $p < 0.005$) for untreated patients with active disease (individual data points are given in Fig 2). One untreated patient, stable at the time of the first study, showed low suppressor activity (8%); a flareup of disease occurred three weeks later. During recovery from this attack, suppression was 61%. When the patient was studied again while stable and taking Imuran, suppression was 13%.

Discussion

In the present study we compared in vitro immune function of Imuran-treated MS patients, untreated MS patients, and control donors. Among MS patients, immune function in vitro was found to correlate with the state of disease activity.

Untreated patients with stable MS differ from controls in T effector, B effector, and T regulator functions. MNCs of stable MS patients showed reduced proliferative response to Con A (T effector function) when compared to controls. In prior studies we found a similarly reduced Con A response in patients with stable MS [5]. Data on T cell mitogenic reactivity in MS have been reported previously by several groups [10, 15, 17, 29, 31]. Results between studies have differed substantially. This discrepancy may reflect differences in techniques used, patient age, disease activity, histocompatibility type, and autologous serum effects.

Mean PWM-induced IgG secretion (B effector function) was only suggestively increased in the untreated stable MS group compared to controls. The large standard errors in the results reflect the marked variations in amount of IgG secreted by those individuals who produced high levels of IgG. A similar trend can be found in the report of Kelley et al [15]. On the other hand, the proportion of stable untreated MS patients in our series who were high responders to PWM (>1,000 ng/ml) was increased significantly over controls ($p < 0.05$). Both the serial data in normal individuals reported by Fauci et al [9] and our sequential data in MS patients and controls suggest that a given individual will consistently show either a high or a low response to PWM. Family studies are needed to determine if increased B cell responsiveness in MS segregates with immunogenetic factors that are overrepresented in the MS population [28] or if it is characteristic of the disease itself. Our findings with regard to B cell function in patients with stable MS parallel those of Goust et al [11], who reported that MS patients' MNCs produced more plaque-forming cells in response to PWM than did MNCs from controls. Goust and associates attributed this finding to increased B cell reactivity in MS patients. Levitt et al [16] and Kelley and co-workers [15] have suggested that a defect in T control of B effector cells is present in MS. Levitt and associates attributed this defect to an imbalance in the T helper and T suppressor functions, whereas Kelley's group thought it represented a defect in the regulation of suppressor cells. Stable MS patients in our study had marginally low Con A–driven suppressor cell (T regulator) activity, in keeping with our earlier experience.

All 5 patients with active MS whom we tested showed very high levels of IgG secretion. Con A–induced suppressor cell activity was significantly decreased in untreated patients with active disease compared to both control individuals and stable MS patients. All 4 patients with active disease who were studied with both assays showed high IgG secretion and low Con A suppression. These and our earlier results on suppressor function [3] fit well with the data of Bach et al [6] and Reinherz and co-workers [30], who showed decreased T suppressor cell numbers using monoclonal antibodies (OKT5, OKT8), and of Huddlestone and Oldstone [12], who showed reduced T_G cells during active disease. The T_G subset contains suppressor cells.

In the Imuran-treated MS group, IgG secretion was markedly lower than in the stable untreated MS group whereas Con A–induced suppressor activity was comparable. Our study does not provide direct data on T helper cell function. The reduced B cell activity in the Imuran-treated MS patients is in agreement with the finding that B cells derived from Imuran-treated MS patients show decreased in vitro proliferation on exposure to PWM or antihuman immunoglobulin, as reported by Abdou et al [1].

Dimitriu and Fauci [7] reported that low concentrations of Imuran (0.01 μg/ml) added in vitro inhibited PWM-induced B cell response (measured in an assay of plaque-forming cells), indicating that B cells are highly sensitive to Imuran. At higher Imuran concentrations (1 to 10 μg/ml), Con A–induced suppressor cell activity was eliminated. Helper cells were resistant to Imuran. The finding in treated MS patients of reduced IgG secretion without change in suppressor cell function appears to be analogous to the effect of low concentrations of Imuran in vitro. Imuran in conventional dosages thus appears to be capable of correcting abnormal effector B cell function in MS without altering T cell regulation.

Supported by Grant MS:RG 1130-C-16 from the National Multiple Sclerosis Society.

We wish to acknowledge the skillful technical assistance of Mrs Martha Lutz and the fine secretarial assistance of Ms Ann Yarbrough and Mrs Jean Durden.

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