Peripheral blood lymphocyte phenotype and function in multiple sclerosis

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SUMMARY T suppressor cell function and phenotype are abnormal in patients with multiple sclerosis, especially during the chronic progressive phase but the sub-populations defined by mitogen stimulation and serological methods may not be identical. In this study, involving 45 patients with multiple sclerosis and 33 controls, there was no correlation between T suppressor function and CD8 cell phenotype in patients with multiple sclerosis or in controls. These phenotypic and functional studies cannot therefore be used interchangeably in the assessment of patients with multiple sclerosis since they provide different information about lymphocyte subpopulations.

Peripheral blood mononuclear (PBM) cell responses to Concanavalin A (Con A) and pokeweed mitogen (PWM) are abnormal in patients with multiple sclerosis; Con A preferentially stimulates T suppressor (Ts) cells whereas PWM is a T cell dependent polyclonal B cell activator. IgG synthesis by unstimulated peripheral blood B lymphocytes is increased in multiple sclerosis patients, especially during relapse and this response is enhanced by PWM stimulation. These observations have been extended by carrying out sequential assays in which the effect of Con A induced Ts cells on autologous Con A stimulation or PWM induced IgG secretion has been studied. The results are consistent with the interpretation that Ts cell function is impaired in multiple sclerosis patients especially during the chronic progressive phase; however, co-culture of autologous and allogeneic B and T cells from healthy controls and multiple sclerosis patients suggests that abnormal B cell responses exist in patients with multiple sclerosis which are independent of this putative Ts cell defect. Phenotypic studies provide additional evidence for a defect in Ts cells in chronic progressive multiple sclerosis. Before replacing time consuming and expensive Con A–PWM assays by T cell enumeration using fluorescent labelled monoclonal antibodies, as a method for investigating Ts cells in patients with multiple sclerosis, it is necessary first to demonstrate whether or not the subpopulations defined by these two methods are identical. In this study, PBM responses to Con A and PWM have been evaluated in 45 patients with multiple sclerosis and 33 unaffected controls; phenotypic and functional results were compared in aliquots of the same sample from 55/78 participants.

Methods

Preparation of peripheral blood mononuclear cells
PBMs were isolated from 60 ml venous blood, anticoagulated with calcium heparin, on a Ficoll-Hypaque density gradient in sterile 50 ml Falcon tubes by centrifugation at 1000 g for 35 minutes. PBMs were washed twice in incomplete medium (IM: RPMI 1640, Flow UK; to which 60 mg/l gentamicin, Roussel, had been added), assessed for cell viability and resuspended in complete medium (CM: 500 ml RPMI 1640 with 10 ml of 100 mM sodium pyruvate, and 10 ml of 200 mM L-glutamine, supplemented with 10% foetal calf serum, heat inactivated, all Flow, and 30 mg of gentamicin).

Two-stage PBM cultures
PBMs (5 x 10⁶) in 2 ml CM were incubated with Con A (10 μg/ml) or CM in Falcon culture tubes for 48 hours, washed with 0.3 M methyl α-D-mannopyranoside (Sigma, 5–68 g methyl α-D-mannopyranoside in 100 ml of IM) to inactive Con A, and resuspended in CM at a concentration of 5 x 10⁶/ml. Cell viability was assessed at this stage. 5 x 10⁵ Con A stimulated, and 5 x 10⁵ unstimulated cells were recultured with PWM (5 μl/ml) and made up to a final volume of 1 ml with CM in each tube. Triplicate cultures containing 1 x 10⁶ cells were incubated for ten days. The cells were then separated by centrifugation at 1000 g for 10 minutes and the supernatants stored at −70°C for later estimation of IgG concentration by enzyme linked immunoabsorbent assay (ELISA).

In order to compare Con A–PWM stimulated IgG synthesis with spontaneous and PWM responses, control cul-
tures were established in which $5 \times 10^5$ PBM, cultured without mitogen for 48 hours were recultured with and without addition of PWM (5 \mu l/ml), made up to a final volume of 1 ml in culture tubes.

These culture systems assess the interaction of Ts and B lymphocytes. The contribution of B cells to spontaneous IgG secretion is demonstrated by PWM stimulation and Ts activity indicated by sequential Con A–PWM incubation; decreased inducible Ts cell activity and B cell overactivity will each result in increased IgG synthesis during the second stage stimulation with PWM.

**ELISA**

Supernatant (200 \mu l) containing IgG was thawed, added to ELISA plates coated with goat antihuman IgG and incubated with goat antihuman IgG F(ab')2 fragment alkaline phosphatase conjugate (Sigma) for 2 hours at room temperature, followed by p-nitrophenyl phosphate disodium in substrate buffer; the reaction was stopped after approximately 10 minutes with 3 M NaOH and absorbance read. An IgG standard curve was used to calculate IgG concentration in test samples using dilutions between 1:2 and 1:20.

**Identification of CD8 positive cells**

$1 \times 10^6$ PBM, isolated from samples taken between 7.30 and 10 am, were incubated with 50 \mu l of OKT8 monoclonal antibody diluted 1:10 or phosphate buffered saline (PBS) at room temperature for 6 minutes. Cells were resuspended in medium with 50 \mu l of fluorescein isothiocyanate conjugated (FITC) goat anti-mouse immunoglobulin, incubated at room temperature for a further 6 minutes, resuspended in PBS with 4% paraformaldehyde and counted within 24 hours by flow cytometry using a fluorescein activated cell sorter (FACS III). Cells were detected and counted at 488 nm by forward scatter and any fluorescence detected longer than 520 nm was recorded.

**Statistics**

IgG synthesis in two-stage assays was calculated from triplicate samples, following subtraction of background absorbance, using standard curves and expressed in ng/ml as a mean and standard deviation. Clinical correlations were masked with respect to the laboratory results. Differences between IgG synthesis and OKT8 cell percentages in individuals from each group were compared by Student's unpaired t test; p values were not corrected for multiple comparisons. Individual results were used to derive the coefficient of correlation between functional and phenotypic results.

**Table 1  IgG synthesis in two stage Con A–PWM cultures**

<table>
<thead>
<tr>
<th></th>
<th>Spontaneous IgG synthesis (ng/ml, SD)</th>
<th>PWM stimulated IgG synthesis (ng/ml, SD)</th>
<th>Con A–PWM stimulated IgG synthesis (ng/ml, SD)</th>
<th>T8+ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All multiple sclerosis patients number</td>
<td>220, 361</td>
<td>4186, 5344</td>
<td>1525, 1969</td>
<td>17, 6</td>
</tr>
<tr>
<td>All controls number</td>
<td>76, 82</td>
<td>1324, 1818</td>
<td>990, 1193</td>
<td>21, 7</td>
</tr>
<tr>
<td>t</td>
<td>3.586</td>
<td>3.386</td>
<td>1.487</td>
<td>2.273</td>
</tr>
<tr>
<td>p</td>
<td>&lt; 0.02</td>
<td>&lt; 0.01</td>
<td>0.5 &gt; p &gt; 0.1</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>
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Table 2  IgG synthesis in two stage Con A–PWM stimulated cultures

<table>
<thead>
<tr>
<th></th>
<th>Spontaneous IgG synthesis (Mean, SD; ng/ml)</th>
<th>PWM stimulated IgG synthesis (Mean, SD; ng/ml)</th>
<th>Con A–PWM stimulated IgG synthesis (Mean, SD; ng/ml)</th>
<th>T8+ cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple sclerosis:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic progressive (n = 29)</td>
<td>210, 371</td>
<td>4953, 5940</td>
<td>1793, 2279</td>
<td></td>
</tr>
<tr>
<td>Neurological controls (n = 10)</td>
<td>75, 31</td>
<td>1505, 1746</td>
<td>1184, 1304</td>
<td>21, 8</td>
</tr>
<tr>
<td>Normal controls (n = 10)</td>
<td>50, 60</td>
<td>1500, 2502</td>
<td>649, 823</td>
<td>24, 7</td>
</tr>
<tr>
<td>Family studies:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients (n = 7)*</td>
<td>59, 48</td>
<td>2454, 2714</td>
<td>1810, 2293</td>
<td>13, 6</td>
</tr>
<tr>
<td>Cohabitant relatives (n = 7)</td>
<td>55, 42</td>
<td>528, 429</td>
<td>851, 855</td>
<td>15, 4</td>
</tr>
<tr>
<td>Non-cohabitant relatives (n = 6)</td>
<td>146, 159</td>
<td>1637, 1744</td>
<td>1395, 1850</td>
<td>22, 6</td>
</tr>
</tbody>
</table>

*Also included in all chronic progressive multiple sclerosis patients.
Statistical details are available in text.

PWM stimulated IgG synthesis was also significantly higher in these multiple sclerosis patients (4953, SD 5940 ng/ml) than normal (1500, SD 2502; p < 0.02) and other neurological disease controls (1505, SD 1746 ng/ml; p < 0.01). As expected the addition of Con A incubated autologous cells inhibited PWM stimulated IgG synthesis; there was a significant difference in IgG production between multiple sclerosis patients (1793, SD 2279 ng/ml) and normal (649, SD 823 ng/ml; p < 0.05) but not other neurological disease controls (1184, SD 1304; p NS) after sequential Con A–PWM stimulation.

Although spontaneous IgG secretion was not increased in the seven patients with chronic progressive multiple sclerosis (59, SD 48 ng/ml) in whom comparisons were made with cohabitant (55, SD 42 ng/ml) or non-cohabitant relatives (146, SD 159 ng/ml), these patients showed increased IgG synthesis in response to PWM stimulation (2454, SD 2714 ng/ml) compared with each group of relatives (528, SD 429 and 1657, SD 1744 ng/ml respectively) but these differences were not statistically significant.

Table 3  IgG synthesis in Con A–PWM stimulated cultures in azathioprine treated patients with multiple sclerosis

<table>
<thead>
<tr>
<th>Spontaneous IgG synthesis (Mean, SD; ng/ml)</th>
<th>Azathioprine (Mean, SD; ng/ml)</th>
<th>Placebo (Mean, SD; ng/ml)</th>
<th>All (Mean, SD; ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple sclerosis: Relapse</td>
<td>140, 99</td>
<td>205, 282</td>
<td>173, 177</td>
</tr>
<tr>
<td>Multiple sclerosis: Stable</td>
<td>188, 291</td>
<td>330, 500</td>
<td>259, 397</td>
</tr>
<tr>
<td>Multiple sclerosis: All</td>
<td>176, 250</td>
<td>299, 439</td>
<td>(n = 6)</td>
</tr>
<tr>
<td>Con A–PWM Stimulated IgG synthesis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple sclerosis: Relapse</td>
<td>2015, 2700</td>
<td>2832, 2358</td>
<td>2424, 2123</td>
</tr>
<tr>
<td>Multiple sclerosis: Stable</td>
<td>2934, 5236</td>
<td>2907, 3725</td>
<td>2920, 4332</td>
</tr>
<tr>
<td>Multiple sclerosis: All</td>
<td>2704, 4561</td>
<td>2889, 3272</td>
<td>(n = 8)</td>
</tr>
</tbody>
</table>

OKT8 Cells (percentages)

| OKT8 Cells | Multiple sclerosis: Relapse | 13-5, 4-9          | 15-0, 1-4          | 14-2, 3-1          |
|           | Multiple sclerosis: Stable | 18-2, 5-1          | 21-3, 6-0          | 19-7, 5-6          |
|           | Multiple sclerosis: All    | 17-0, 5-2          | 19-7, 5-9          | (n = 12)           |

Statistical details are available in text.
azathioprine was demonstrated under these culture conditions.

**CD8 positive phenotypes**

Mean CD8 positive cell percentage was lower in 23 multiple sclerosis patients (17, SD 6%) than 32 controls (21, SD 7%; p < 0.05: table 1). There was no correlation between T lymphocyte function in Con A–PWM 2 stage cultures and CD8 positive cell phenotype in patients or controls (R = -0.15: fig).

Mean CD8 positive cell percentages were significantly lower in seven untreated patients with chronic progressive multiple sclerosis (14, SD 6%) than nine normal (24, SD 7%; p < 0.01) or 10 neurological disease controls (22, SD 8%; p < 0.05), and six non-cohabiting siblings (22, SD 6%; p < 0.05: table 2). Mean CD8 positive cell percentages were also reduced in seven cohabiting family members (15, SD 4%) compared with non-cohabitants (p < 0.05) and unrelated normal (p < 0.01) or other neurological disease controls (p < 0.05). These results reproduce the findings of a previous study involving the same participants.17

Overall there was no difference in mean CD8 positive cell percentages in eight azathioprine (17, SD 5%) compared with eight placebo treated patients (20, SD 6%). Mean CD8 positive cell percentages were lower in four patients during relapse (14, SD 3%) than stable cases (20, SD 6%; p < 0.05) an effect which was seen in placebo (21, SD 6% vs 15, SD 1%) and azathioprine treated patients (18, SD 5% vs 13, SD 5%; table 3).

**Discussion**

In this study we found no overall correlation between CD8 cell phenotype (using the OKT8 marker) and Con A–PWM induced IgG synthesis in patients with multiple sclerosis or controls. The lack of even a non-causal relationship is at first surprising, since each method gave abnormal results in individuals with multiple sclerosis. But the outcome of cultures, containing an heterogenous population of cells, stimulated with one or more plant mitogens for up to 12 days is likely to depend on more than just the initial number of CD8 cells in the test sample. There was a statistically significant difference in IgG synthesis after PWM but not sequential Con A–PWM stimulation between groups consistent with the interpretation that increased spontaneous IgG synthesis in patients with multiple sclerosis, which reflects the balance of lymphocyte interaction in vivo, is more likely to depend on B cell overactivity than impaired Ts function.

Abnormalities of PWM stimulated IgG secretion and number of OKT8 positive cells or T cells bearing...
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receptors for IgG immune complexes (Tg), were originally correlated in groups of patients with multiple sclerosis recovering from relapse; later the reduction in OKT8 cells during relapse was associated with impaired control of EBV specific T cell mediated cytotoxicity. However, a number of other detailed studies in multiple sclerosis patients have shown no correlation between phenotypic and functional tests. Tjernlund et al using a two stage Con A-mixed lymphocyte reactivity culture, demonstrated an increase in OKT4/8 ratio and spontaneous or PWM stimulated IgG synthesis with reduced stimulation indices in patients with chronic progressive multiple sclerosis but not cases in remission; the results of neither functional test correlated with phenotype in individual patients. Oger et al demonstrated comparable Leu 2a/OKT8 cell phenotypes and two-stage Con A-Con A proliferative responses in normal individuals whereas in multiple sclerosis patients functional abnormalities correlated only with the number of Leu 2a positive cells. The authors considered that the population determining suppressor function could best be identified by double-labelling using OKT8 and Leu 7 monoclonal antibodies. In a series of papers, Antel and colleagues concluded that the Ts defect in multiple sclerosis patients is largely accounted for by a combined numerical and functional abnormality of the CD8 population, whereas T8 cell cytotoxic function is normal in an assay involving pooled allogeneic responder cells. Other cell types, including CD4 and all E rosette positive cells, contribute to suppressor function but the deficit in multiple sclerosis patients may selectively involve the CD8 population.Recently, a reduction in peripheral blood suppressor inducer cell phenotype, identified by dual labelling with OKT4 and 2H4 monoclonal antibodies, has been correlated with a two stage assay in which Ts cells were first expanded in a mixed allogeneic lymphocyte reaction, and then incubated with PWM stimulated mononuclear cells, followed by estimation of supernatant IgG concentration.

Patients involved in a controlled treatment trial of azathioprine were investigated in order to extend the comparison between T cell phenotype and function and to assess the role of antigen non-specific suppressor assays in the assessment of immunological treatment. In patients with active multiple sclerosis, azathioprine has been shown to decrease Ts cell numbers and return abnormal Con A mitogenic responses and suppressor activity in Con A-Con A assays to the normal values seen in untreated stable cases. We have demonstrated differences in two stage assays in untreated patients, which varied with disease activity, but these were uninfluenced by azathioprine given at a dose of 2.5 mg/kg; the variables on which disease activity depends remained a more potent influence on mitogen responsiveness. However, it remains to be shown whether improving Ts function or CD8 cell number is beneficial for multiple sclerosis patients and despite theoretical considerations there are sufficient gaps in understanding the pathogenesis of the disease to make the clinical consequences of any immunological treatment unpredictable. Better understanding of the in vitro effect of each drug on immune abnormalities in multiple sclerosis may accelerate progress in identifying the most promising candidates for future clinical trials.

The results of this investigation of peripheral blood T cells in patients with multiple sclerosis indicate that phenotypic and functional tests provide different information about cellular interactions and so cannot be used interchangeably in the assessment of peripheral blood lymphocytes in patients with multiple sclerosis.

References

15 Hauser SL, Reinherz EL, Hoban CJ, Schlossman SF, Weiner HL. Immunoregulatory T-cells and lymphocytotoxic antibodies in


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From the Archives of Surgery

Sir Jonathan Hutchinson’s (1829–1913) modern description of symmetrical temporal arteritis.

"The subject . . . was an old man . . . the father of a well-remembered beadle at the London Hospital College 30 years ago . . . I was asked to see him because he had red ‘streaks on his head’ which were painful and prevented his wearing his hat. The ‘red streaks’ proved . . . to be his temporal arteries which . . . were . . . inflamed and swollen. Pulsation could be feebly detected in the affected vessel, but it finally ceased; the redness then subsided, and the vessels were left impervious cords. The old gentleman lived, I believe, several years after this without any other manifestation of arterial disease."

RT Ross, MD

Reference

1 Hutchinson J. Diseases of the Arteries. *Arch Surg* 1890;1:323–33.