Multiple sclerosis: Comparison of copolymer-1-reactive T cell lines from treated and untreated subjects reveals cytokine shift from T helper 1 to T helper 2 cells

Oliver Neuhaus*, Cinthia Farina*, Alexander Yassouridis†, Heinz Wiendi*, Florian Then Bergh†, Tatjana Dose†, Hartmut Wekerle*, and Reinhard Hohlfeld*‡§

*Department of Neuroimmunology, Max Planck Institute of Neurobiology, Am Klopferspitz 18A, 82152 Martinsried, Germany; †Department of Statistics, Max Planck Institute of Psychiatry, Kraepelienstrasse 2-10, 80804 Munich, Germany; and §Institute for Clinical Neuroimmunology and Department of Neurology, Klinikum Grosshadern, Ludwig Maximilians University, Marchioninistrasse 15, 81366 Munich, Germany

Communicated by Michael Sela, Weizmann Institute of Science, Rehovot, Israel, April 13, 2000 (received for review February 10, 2000)

Copolymer 1 (COP), a standardized mixture of synthetic polypeptides consisting of L-glutamic acid, L-lysine, L-alanine, and L-tyrosine, has beneficial effects in multiple sclerosis and experimental autoimmune encephalomyelitis. We selected a panel of 721 COP-reactive T cell lines (TCL) from the blood of COP-treated and untreated multiple sclerosis patients and from healthy donors by using the split-well cloning technique. All TCL selected with COP proliferated in response to COP but not to myelin basic protein (MBP). Conversely, 31 control TCL selected with MBP proliferated in response to MBP but not to COP. We used intracellular double-immunofluorescence flow cytometry for quantitative analysis of cytokine production (IL-4, IFN-γ) by the TCL. The majority of the COP-reactive TCL from untreated multiple sclerosis patients and normal donors predominantly produced IFN-γ and, accordingly, were classified as T helper 1 cells (TH1). In contrast, the majority of the COP-reactive TCL from COP-treated patients predominantly (but not exclusively) produced IL-4—i.e., were TH2 (P < 0.05 as assessed by using a suitable preference intensity index). Longitudinal analyses revealed that the cytokine profile of COP-reactive TCL tends to shift from TH1 to TH2 during treatment. Interestingly, although there was no proliferative cross-reaction, about 10% of the tested COP-reactive TCL responded to MBP by secretion of small amounts of either IL-4 (TH2 or TH0 lines) or IFN-γ (TH1 or TH0 lines), although none of our COP-reactive TCL proliferated in the presence of MBP. The results indicate that the therapeutic effect of COP in MS may be related to a cytokine shift of COP-reactive T cells from TH1 to TH2, and to a cross-reaction with MBP at the level of cytokine production.

Materials and Methods

Patients and Control Subjects. Blood was drawn with informed consent from 26 MS patients and 4 healthy donors. At the time of first sampling, 15 patients were treated with COP (20 mg s.c. per day; Teva Pharma, Kirchzarten, Germany). Six of the untreated patients were later started on COP; from these patients, only the data before treatment were included in the statistical analysis. All donors were HLA-typed (Table 1).

Antigens. COP (batch 242992997, average molecular mass 7,000 Da, and batch 242992899, average molecular mass 6,400 Da) was obtained from Teva Pharmaceutical Industries, Petah Tiqva, Israel. The two batches were cross-reactive with each other as assessed in a proliferation assay. MBP was purified from human brain by standard methods (21). Overlapping peptides covering the entire human MBP molecule were synthesized by using an automatic peptide synthesizer (431A; Applied Biosystems). The extracellular Ig-like domain of human myelin-oligodendrocyte glycoprotein (MOG), amino acids 1–125, was expressed in an Escherichia coli system as described before (22). As a recombinant control, rat S100β protein was expressed and prepared in the same way (23). Tetanus toxoid (TT) was kindly provided by Chiron Behring, Marburg, Germany. Tuberculin purified pro-

Abbreviations: APC, antigen-presenting cells; COP, copolymer 1; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; MOG, myelin-oligodendrocyte glycoprotein; MS, multiple sclerosis; PE, phycoerythrin; PPD, purified protein derivative; SI, stimulation index; TCL, T cell line(s); TCR, T cell antigen receptor; TH0, TH1, and TH2, T helper type 0, 1, and 2 cells, respectively; TT, tetanus toxoid.

§To whom reprint requests should be addressed at: Institute for Clinical Neuroimmunology, Klinikum Grosshadern, Ludwig Maximilians University, Marchioninistrasse 15, 81366 Munich, Germany. E-mail: hohlfeld@neuro.mpg.de.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.
Cell Culture and Isolation of TCL. All cell cultures were performed in RPMI medium 1640 (GIBCO) supplemented with 5% pooled
and heat-inactivated human AB serum (German Red Cross,
in RPMI medium 1640 (GIBCO) supplemented with 5% pooled
methyl

Tein derivative (PPD; batch RT49) was purchased from Statens Serum Institut, Copenhagen.

Table 1. Basic characteristics of MS patients and healthy donors (HD)

<table>
<thead>
<tr>
<th>No.</th>
<th>Initials</th>
<th>Sex</th>
<th>Age, yr</th>
<th>Duration of disease, yr*</th>
<th>EDSS†</th>
<th>HLA-DR type</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS 1</td>
<td>Stt</td>
<td>F</td>
<td>35</td>
<td>9</td>
<td>4.0</td>
<td>2, 5</td>
</tr>
<tr>
<td>MS 2</td>
<td>HK</td>
<td>M</td>
<td>34</td>
<td>8</td>
<td>1.0</td>
<td>3, 12</td>
</tr>
<tr>
<td>MS 3</td>
<td>AZ</td>
<td>M</td>
<td>28</td>
<td>1</td>
<td>1.0</td>
<td>2, 7</td>
</tr>
<tr>
<td>MS 4</td>
<td>HM</td>
<td>F</td>
<td>25</td>
<td>2</td>
<td>1.0</td>
<td>2</td>
</tr>
<tr>
<td>MS 5</td>
<td>SW</td>
<td>F</td>
<td>40</td>
<td>11</td>
<td>3.5</td>
<td>2, 8</td>
</tr>
<tr>
<td>MS 6</td>
<td>CS</td>
<td>F</td>
<td>42</td>
<td>4</td>
<td>1.0</td>
<td>4, 8</td>
</tr>
<tr>
<td>MS 7</td>
<td>GJ</td>
<td>F</td>
<td>30</td>
<td>1</td>
<td>1.0</td>
<td>4, 6</td>
</tr>
<tr>
<td>MS 8</td>
<td>WHa</td>
<td>F</td>
<td>43</td>
<td>1</td>
<td>2.5</td>
<td>2, 11</td>
</tr>
<tr>
<td>MS 9</td>
<td>HC</td>
<td>F</td>
<td>32</td>
<td>2</td>
<td>2.5</td>
<td>11, 12</td>
</tr>
<tr>
<td>MS 10</td>
<td>SZ</td>
<td>F</td>
<td>34</td>
<td>4</td>
<td>2.5</td>
<td>2, 7</td>
</tr>
<tr>
<td>MS 11</td>
<td>LN</td>
<td>F</td>
<td>36</td>
<td>1</td>
<td>2.5</td>
<td>1, 2</td>
</tr>
<tr>
<td>MS 12</td>
<td>BG</td>
<td>F</td>
<td>33</td>
<td>4</td>
<td>1.5</td>
<td>4, 7</td>
</tr>
<tr>
<td>MS 13</td>
<td>UB</td>
<td>F</td>
<td>39</td>
<td>10</td>
<td>6.0</td>
<td>4, 7</td>
</tr>
<tr>
<td>MS 14</td>
<td>US</td>
<td>F</td>
<td>35</td>
<td>2</td>
<td>1.0</td>
<td>7, 10</td>
</tr>
<tr>
<td>MS 15</td>
<td>SH</td>
<td>F</td>
<td>31</td>
<td>1</td>
<td>1.0</td>
<td>3</td>
</tr>
<tr>
<td>MS 16</td>
<td>BS</td>
<td>M</td>
<td>21</td>
<td>1</td>
<td>1.5</td>
<td>13, 14</td>
</tr>
<tr>
<td>MS 17</td>
<td>KR</td>
<td>F</td>
<td>39</td>
<td>4</td>
<td>1.5</td>
<td>1, 7</td>
</tr>
<tr>
<td>MS 18</td>
<td>RD</td>
<td>M</td>
<td>40</td>
<td>17</td>
<td>2.5</td>
<td>2</td>
</tr>
<tr>
<td>MS 19</td>
<td>RM</td>
<td>F</td>
<td>34</td>
<td>7</td>
<td>1.5</td>
<td>7, 13</td>
</tr>
<tr>
<td>MS 20</td>
<td>IB</td>
<td>F</td>
<td>48</td>
<td>20</td>
<td>4.0</td>
<td>7, 11</td>
</tr>
<tr>
<td>MS 21</td>
<td>MB</td>
<td>F</td>
<td>34</td>
<td>1</td>
<td>1.0</td>
<td>2, 7</td>
</tr>
<tr>
<td>MS 22</td>
<td>MC</td>
<td>F</td>
<td>30</td>
<td>3</td>
<td>5.0</td>
<td>3, 7</td>
</tr>
<tr>
<td>MS 23</td>
<td>BK</td>
<td>F</td>
<td>43</td>
<td>5</td>
<td>1.0</td>
<td>2, 4</td>
</tr>
<tr>
<td>MS 24</td>
<td>RR</td>
<td>M</td>
<td>28</td>
<td>2</td>
<td>4.0</td>
<td>2, 11</td>
</tr>
<tr>
<td>MS 25</td>
<td>RO</td>
<td>M</td>
<td>28</td>
<td>2</td>
<td>3.5</td>
<td>7, 13</td>
</tr>
<tr>
<td>MS 26</td>
<td>AF</td>
<td>F</td>
<td>18</td>
<td>2</td>
<td>1.0</td>
<td>10, 12</td>
</tr>
<tr>
<td>HD 1</td>
<td>WH</td>
<td>M</td>
<td>30</td>
<td>—</td>
<td>—</td>
<td>2, 11</td>
</tr>
<tr>
<td>HD 2</td>
<td>CH</td>
<td>M</td>
<td>31</td>
<td>—</td>
<td>—</td>
<td>11, 13</td>
</tr>
<tr>
<td>HD 3</td>
<td>CB</td>
<td>F</td>
<td>29</td>
<td>—</td>
<td>—</td>
<td>4, 11</td>
</tr>
<tr>
<td>HD 4</td>
<td>VV</td>
<td>F</td>
<td>34</td>
<td>—</td>
<td>—</td>
<td>4, 15</td>
</tr>
</tbody>
</table>

*At the time of first sampling.
†EDSS, expanded disability status scale (31).

Medicinal Sciences

Characterization of the Cytokine Profile by Intracellular Double-Fluorescence Flow Cytometry. The cytokine profile of the TCL was analyzed 8–10 days after restimulation in the absence of viable APC. COP-reactive TCL cells were stimulated with phorbol 12-myristate 13-acetate (PMA, 2.5 μg/ml) and ionomycin (250 ng/ml; both from Sigma) for 3 h, the last 2 h in the presence of the glycophorin secretion blocker monensin (2 nmol/ml; Sigma). The T cells were then washed with PBS, fixed with 4% paraformaldehyde (Merck), and permeabilized with 0.1% saponin/PBS (Sigma). The T cells were stained by using appropriate concentrations of mAbs directed against IL-4 and IFN-γ. The cytokine profile of the TCL was analyzed by FACScan (Becton Dickinson).

Phenotypical Characterization of TCL by Flow Cytometry. TCL were stained with labeled mAbs directed against CD3 (UCHT1, FITC-labeled; DAKO), CD4 (RPA-T4, PE-labeled, PharMingen), and CD8 (DK25, FITC-labeled; DAKO) and the corresponding isotype controls (mouse IgG1 PE-labeled, Becton Dickinson; mouse IgG1 FITC-labeled, Immunotech). The cytokine profile was analyzed by FACScan (Becton Dickinson).

The cytokine profile was analyzed with a FacScan (Becton Dickinson). Data from 5,000 cells were accumulated and the results were analyzed as dot plots representing the relative fluorescence intensity (Fig. 1). On a dot plot showing forward and sideward scatter, lymphoid cells were gated for further analysis (Fig. 1). Note that for unknown reasons, dead cells stained positive with the anti-IL-4 mAb and had to be excluded by gating. To define the predominant cytokine profile of each TCL, the following algorithm was applied (Fig. 1): Only cells positive for IFN-γ, IL-4, or both were considered “activated.” If a single-positive fraction (i) exceeded 50% of all “activated” cells, and (ii) was at least 20% higher than the other single-positive fraction, the line was defined “TH1” (IFN-γ) or “TH2” (IL-4). All other TCL were designated as “TH0.” TCL with less than 100 “activated” events were not taken into account. Five or more TCL per donor were considered representative.
labeled goat anti-mouse IgG antibody (Jackson ImmunoResearch, West Grove, PA).

Statistical Analysis. Each line was assigned one of three numbers, namely −1 for the TH1 lines, 0 for the TH0 lines, and +1 for the TH2 lines. For each individual donor, a “preference intensity index” (I) was calculated for the TCL isolated from the donor according to the formula:

\[ I = \frac{(1 + n_{TH2}) + (0) \times n_{TH0} + (-1) \times n_{TH1}}{n_{TH2} + n_{TH0} + n_{TH1}}, \]

where \( n_{TH2}, n_{TH0}, \) and \( n_{TH1} \) denote the total number of TH2-, TH0-, and TH1-type TCL isolated from a given individual at a particular time. In this way, I is a metric variable expressing the proportion of TH1 TCL with respect to TH2 TCL independent of the absolute number of TCL obtained per donor. I varies between two extreme values, −1 (when all TCL are TH1) and +1 (when all TCL are TH2). For comparing the three groups (treated vs. untreated MS patients vs. healthy donors), a one-factorial analysis of covariance (ANCOVA) was applied with the age of the donors as covariate. If a significant group effect was observed, post hoc tests (tests with contrasts) were applied to identify pairs of groups with significant preference intensity differences. \( \alpha = 0.05 \) was accepted as nominal level of significance and corrected for the post hoc tests according to the Bonferroni procedure to keep the type I error \( \leq 0.05 \).

Results

Isolation and Characterization of COP-Responsive TCL. We plated a total of 3,031 wells and isolated a panel of 721 COP-responsive TCL (23.8%). One hundred and sixty TCL were isolated from 1,054 plated wells (15.2%, untreated MS patients), 300 TCL from 1,155 plated wells (26.0%, COP-treated patients), and 90 lines from 330 plated wells (27.3%, healthy donors). In addition, 171 TCL could be established after onset of COP treatment from 492 plated wells (34.8%, previously untreated patients). All COP-responsive TCL showed a proliferative response to COP (mean SI 78.2, median SI 44.7, range 3.0–1,826), but none of the tested TCL proliferated significantly in response to MBP, MBP peptides, or any other antigens tested (MOG, S100β, and TT) (Fig. 2).

Cross-Sectional Analysis: Effect of COP Treatment on the Cytokine Profile of COP-Responsive TCL. We analyzed the cytokine profile of 693 of our COP-responsive TCL (150 TCL from untreated patients, 284 TCL from COP-treated patients, 90 TCL from healthy donors, 169 TCL from previously untreated patients after onset of COP treatment) by double-fluorescence flow cytometry at various restimulation steps (mostly R2 and R3). To minimize the influence of culture conditions, we strictly kept all TCL under identical conditions. Longitudinal comparisons of the cytokine profile of individual TCL at different restimulation steps showed that despite small fluctuations, the predominant cytokine profile remained stable: Of nine TCL that were followed up to five times (R2–R16), seven TCL strictly kept their predominant cytokine profile, whereas two TCL shifted from TH1 (R3) to TH0 (R8–R14).

Fig. 3 shows the overall cytokine profiles of the complete panel of our COP-responsive TCL. Comparing the three groups (treated...
vs. untreated MS patients vs. healthy donors), the analysis of covariance revealed a significant effect \( F(2, 29) = 10.26 \), significance of \( F = 0.001 \). Although the donors were not age-matched, “age” as a covariate did not seem to play a role. The mean preference intensity index in COP-treated MS patients \( (I = 0.23) \) was significantly higher (indeed positive and thus skewed to TH2) than in untreated MS patients \( (I = 0.42) \) and untreated control subjects \( (I = -0.65) \) (tests with contrasts, \( P < 0.05 \)). By pooling untreated patients and healthy donors and subsequently comparing the mean preference intensity of the combined group \( (I = -0.48) \) with the treated patients’ group \( (I = +0.23) \) by ANCOVA, significant differences were observed \( F(1, 29) = 20.40 \), significance of \( F < 0.0001 \). The results suggest that treatment with COP induces a shift from TH1 (COP lines from untreated patients and healthy donors) toward TH2 (COP-treated MS patients). Clearly, the data do not allow us to decide whether this change occurs at the level of the cell population, individual cells, or both.

The TH2-inducing effect was specific for COP, as it was not seen with PPD- and TT-reactive TCL from two treated patients (one example is shown in Fig. 4). After 6 months of COP treatment, the COP-reactive TCL from the donor CS were either TH2 or TH0 (cf. Fig. 3), whereas 3 of 3 PPD- and 2 of 2 TT-reactive TCL were TH1 (Fig. 4). Moreover, while 14 of 15 COP-reactive TCL from the donor SH (after 15 months of treatment) were TH2 and 1 was TH0, all of this donor’s 4 PPD- and 3 TT-reactive TCL were TH1.

**Longitudinal Analysis: Change of the Cytokine Profile of COP-Reactive TCL During Treatment of Individual Patients.** To further corroborate the results obtained by cross-sectional analysis, we investigated some patients longitudinally, that is, before and after various time periods of COP treatment (Fig. 3).

**Cytokine profile before and after treatment.** TCL from one patient (GJ) had an unbiased cytokine profile before treatment (preference intensity \( I = -0.08 \)), which almost completely shifted toward TH2 after 1 month of treatment \( (I = +0.92) \). After 3 months of treatment, the cytokine profile shifted back to TH0 \( (I = -0.10) \) (Fig. 3). Another patient, CS, kept a TH1-biased cytokine profile from before treatment \( (I = -0.38) \).
production of IL-4 or IFN-γ (5.5 pg/ml vs. 0 pg/ml in the negative control). Furthermore, 2 of 12 tested COP-reactive TCL responded to MBP by secretion of low amounts of IFN-γ, depending on the predominant cytokine profile of the TCL. We tested 111 TCL for IL-4 secretion, and 53 TCL responded to MBP by cytokine secretion but not proliferation. Both observations are remarkably consistent with previously reported results in EAE (13, 15).

An important technical aspect of our study is that we analyzed the cytokine profiles of the COP-reactive TCL by intracellular double-fluorescence flow cytometry. This allowed us to precisely quantify the cytokine profile of each individual TCL and assign a preference intensity index to each patient, ranging on a continuous scale from −1 (100% TH1 lines) to +1 (100% TH2 lines). Parallel determinations with ELISA in a subset of TCL were consistent with the flow cytometry data. Although the observed cytokine shift was most obvious on cross-sectional analysis, our (limited) longitudinal data support the idea that a cytokine shift occurs during treatment. It is important to note, however, that the observed shift is a statistical phenomenon: individual patients showed a TH1- rather than TH2-biased cytokine profile despite prolonged treatment with COP. Furthermore, about 10% of our COP-reactive TCL responded to MBP by cytokine secretion but not proliferation. Both observations are remarkably consistent with previously reported results in EAE (13, 15).

### Cytokine Secretion of COP- Reactive TCL After Cross-Stimulation with MBP
As mentioned before, none of the tested human COP-reactive TCL showed a proliferative response when challenged with MBP, and vice versa. Previous observations in EAE demonstrated that COP-reactive TCL from mice treated with COP also did not proliferate in the presence of MBP, but some COP-reactive TCL produced IL-4 when stimulated with MBP (13). On the basis of these results, we tested whether human COP-reactive TCL can also be induced to cytokine secretion by stimulation with MBP. We found that, indeed, several COP-reactive TCL responded to MBP by secretion of low amounts of IL-4 or IFN-γ, depending on the predominant cytokine profile of the TCL. We tested 111 TCL for IL-4 secretion, and 53 TCL for IFN-γ secretion. Of these, 9/111 (8.1%) and 8/53 (15.1%) responded to MBP by significant (>2 SD above background) production of IL-4 or IFN-γ, irrespective of the source of the TCL (treated and untreated MS patients, healthy donors) (Fig. 5). Conversely, 1 of 7 tested MBP-specific TCL responded to COP by production of IL-4 (5.5 pg/ml vs. 0 pg/ml in the negative control). Furthermore, 2 of 12 tested COP-reactive TCL responded to MBP by production of IFN-γ (27.0 vs. 3.7 and 18.1 vs. 3.3 pg/ml, respectively).

### Discussion
Our analysis of a large panel of COP-reactive human TCL revealed that the cytokine profile of the COP-reactive TCL tends to shift from TH1 before treatment to TH2 during treatment. Furthermore, about 10% of our COP-reactive TCL responded to MBP by cytokine secretion but not proliferation. Both observations are remarkably consistent with previously reported results in EAE (13, 15).

As mentioned before, none of the tested human COP-reactive TCL showed a proliferative response when challenged with MBP, and vice versa. Previous observations in EAE demonstrated that COP-reactive TCL from mice treated with COP also did not proliferate in the presence of MBP, but some COP-reactive TCL produced IL-4 when stimulated with MBP (13). On the basis of these results, we tested whether human COP-reactive TCL can also be induced to cytokine secretion by stimulation with MBP. We found that, indeed, several COP-reactive TCL responded to MBP by secretion of low amounts of IL-4 or IFN-γ, depending on the predominant cytokine profile of the TCL. We tested 111 TCL for IL-4 secretion, and 53 TCL for IFN-γ secretion. Of these, 9/111 (8.1%) and 8/53 (15.1%) responded to MBP by significant (>2 SD above background) production of IL-4 or IFN-γ, irrespective of the source of the TCL (treated and untreated MS patients, healthy donors) (Fig. 5). Conversely, 1 of 7 tested MBP-specific TCL responded to COP by production of IL-4 (5.5 pg/ml vs. 0 pg/ml in the negative control). Furthermore, 2 of 12 tested COP-reactive TCL responded to MBP by production of IFN-γ (27.0 vs. 3.7 and 18.1 vs. 3.3 pg/ml, respectively).
such as IL-4, IL-5, IL-6, and IL-10 but not IL-2 or IFN-γ in response to COP (13, 15). As in our study, some of the mouse COP-reactive T cells cross-react to MBP by cytokine secretion but not proliferation (13). The observed cross-reaction between COP and MBP at the cytokine level is apparently not unique to MBP but could also be observed with another myelin autoantigen, MOG, in a few TCL.

Regarding the possible mechanism of COP in vivo, it is known that COP binds efficiently to MHC class II molecules (11–13), and it competes with MBP at both the MHC class II and TCR levels (14, 17, 18). However, because it seems unlikely that significant amounts of COP can reach the central nervous system, these in vitro effects probably do not explain the clinical effects observed in vivo.

The following hypothetical scenario would accommodate both the previously reported EAE results and our observations in COP-treated patients: Chronic s.c. application of COP induces COP-reactive TH2 cells, which are able to cross the blood–brain barrier because they are activated (28). Inside the central nervous system, the COP-reactive T cells are confronted with products of myelin turnover presented by local APC (29). Some of the COP-reactive T cells react to MBP by secretion of protective cytokines such as IL-4. This might exert suppressive bystander effects on other inflammatory cells (13, 16, 30).

We are grateful to Drs. J. Haas and U. Augustin (Jewish Hospital, Berlin), Dr. N. König (Marianne-Strauß-Hospital, Berg, Germany), and Dr. C. Zimmermann (Institute for Clinical Neuroimmunology, University of Munich, Germany) for providing clinical samples. We thank Drs. E. Albert and S. Scholz (Department of Immunogenetics, University of Munich) for the HLA-typing and Dr. L. Jiang and Ms. M. Solch for excellent technical assistance. This work was supported by Teva Pharma/Hochst Marion Roussel. O.N. and H. Wiendl are postdoctoral fellows supported by the Deutsche Forschungsgemeinschaft. The Institute for Clinical Neuroimmunology is supported by the Hermann and Lilly Schilling Foundation.


