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*J Immunol* 2000; 165:6858-6864;

doi: 10.4049/jimmunol.165.12.6858

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Reactivity and Regulatory Properties of Human Anti-Idiotypic Antibodies Induced by T Cell Vaccination

Jian Hong,* Ying C. Q. Zang,* Maria V. Tejada-Simon,* Sufang Li,* Victor M. Rivera,* James Killian,* and Jingwu Z. Zhang2*†

Immunization with irradiated autoreactive T cells (T cell vaccination) induces anti-idiotypic T cell responses that preferentially recognize complementarity-determining region 3 sequences, contributing to clonal depletion of autoreactive T cells. However, it remains unknown whether T cell vaccination elicits anti-idiotypic humoral responses and whether the anti-idiotypic Abs play a similar role in the regulatory mechanism induced by T cell vaccination. In this study we examined the occurrence, the reactivity pattern, and the regulatory role of anti-idiotypic Abs elicited by T cell vaccination in patients with multiple sclerosis. We demonstrated for the first time that B cells producing anti-idiotypic Abs could be isolated from vaccinated patients. These EBV-transformed B cell lines were selected for specific reactivity to a 20-mer TCR peptide incorporating a common complementarity-determining region 3 sequence of the immunizing T cell clones. The resulting anti-idiotypic Abs were found to react with the original immunizing T cell clones and exhibit an inhibitory effect on their proliferation. The findings suggest that anti-idiotypic Ab responses can be induced by T cell vaccination in humans and that their regulatory properties are likely to contribute to the suppression of myelin basic protein-reactive T cells in vaccinated patients. The study has important implications in our understanding of the regulatory role of the anti-idiotypic humoral responses induced by T cell vaccination. The Journal of Immunology, 2000, 165: 6858–6864.

Autoimmune T cells are not completely deleted in the thymus and circulate in the periphery. These T cells are controlled by various regulatory mechanisms, including the idiotypic network that forms the internal image through the recognition of the idiotypic determinants of specific Abs or T cells in regulating the immune responses to both foreign and self Ags (1, 2). Anti-idiotypic regulatory T cells and Abs are part of the normal T cell repertoire and can be identified in healthy human subjects (3, 4). It has been demonstrated that anti-idiotypic T cell responses can be activated in vivo by repeated immunization (T cell vaccination) with autologous T cells that are inactivated by irradiation or chemical treatment (5). T cell vaccination has been shown to effectively treat various experimental autoimmune diseases in animals, including experimental autoimmune encephalomyelitis, an animal model for multiple sclerosis (MS) (6, 7). Recently, T cell vaccination has been tested in patients with MS (8) in whom autoreactive T cell responses to myelin basic protein (MBP) may play an important role in the disease processes (9–13). In two clinical trials we demonstrated that s.c. inoculations with irradiated autologous MBP-reactive T cell clones induced substantial anti-idiotypic T cell responses in patients with MS, which correlated with progressive depletion of circulating MBP-reactive T cells used for vaccination (14) (J. Z. Zhang et al., manuscript in preparation). Although the treatment efficacy remains to be determined in controlled clinical trials, the preliminary clinical results suggest that depletion of MBP-reactive T cells altered favorably the clinical course of the disease (14) (J. Z. Zhang et al., manuscript in preparation).

It is increasingly clear that T cell vaccination induces the Id anti-idiotypic network in regulating selected T cells used for vaccination. We demonstrated previously that T cell vaccination elicited CD8+ cytotoxic T cell responses that specifically recognized and lysed the immunizing T cell clones (8, 15). These anti-idiotypic T cell lines isolated from MS patients immunized with irradiated MBP-reactive T cell clones were found to react preferentially with complementarity-determining region 3 (CDR3) sequences of the immunizing T cells (16). In contrast to anti-idiotypic T cell responses, little is known about the occurrence and regulatory role of anti-idiotypic humoral responses in vaccinated patients. It has been speculated that the same vaccination protocol may be equally effective in eliciting anti-idiotypic Ab responses in MS patients. However, previous attempts to identify anti-idiotypic Abs in vaccinated patients were largely unsuccessful, even though the presence of such Abs was occasionally detected (8, 17). In these studies nonfractionated sera obtained from vaccinated patients were used to stain the immunizing T cells or cell lysates using flow cytometry or immunoblot analysis (8, 17). It was unclear whether the failure to detect the anti-idiotypic Abs in vaccinated patients was due to the lack of the humoral responses or whether it was associated with technical difficulties in detecting them using the whole T cells or T cell lysates as the Ags. Resolution of these issues is important, as anti-idiotypic humoral responses may potentially contribute to immune regulation induced by T cell vaccination. Identification and characterization of such
anti-idiotypic Abs may help to understand the regulatory mechanism underlying T cell vaccination.

This study was undertaken to examine the occurrence and functional properties of anti-idiotypic Abs induced by T cell vaccination in patients with MS. Special considerations were given in the experimental design. First, in addition to TCR, the whole T cells or T cell lysates express numerous surface molecules that may interfere with the binding assay to detect anti-idiotypic Abs. Second, anti-idiotypic Abs of interest represent only a small fraction of serum Ig, which may account for the difficulties in detecting the specific Abs in sera. Based on these considerations, we synthesized a 20-mer TCR peptide incorporating the CDR3 sequence expressed by the immunizing MBP-reactive T cell clones and used it as a pure agent in the initial screening to identify anti-idiotypic Abs. Second, we employed a cell culture-based technique combining EBV transformation and limiting dilution to generate Ab-producing B cell lines. This technique was used successfully in previous studies to identify B cells producing autoantibodies, which are present at low frequencies in patient’s blood (18, 19). As a result, the precursor frequency of B cells producing specific Ab of interest can be estimated, and higher concentrations of relatively pure Abs can be obtained from individual B cell lines for detailed characterization. The study revealed for the first time that B cells producing anti-idiotypic Abs could be isolated from MS patients that received T cell vaccination. These Abs initially screened for their specific reactivity to the CDR3 peptide bound to and had an inhibitory effect on the immunizing MBP-reactive T cells expressing the CDR3 sequence. The findings have important implications in the understanding of the regulatory role of the B cell component of the Id anti-idiotypic network that can be modulated by T cell vaccination and in the development of a peptide-based vaccination approach.

Materials and Methods

Reagents and peptides

The medium used for cell culture was AIM-V serum-free medium (Life Technologies, Grand Island, NY). Recombinant human IL-2 was purchased from Roche (Indianapolis, IN). The immunodominant peptide (residues 83–99) of MBP and two TCR peptides of 20 amino acids were synthesized by Chiron Mimotope (San Diego, CA). The purity of the peptides was >95%.

Estimation of the precursor frequency of MBP-reactive T cells

PBMCs were plated at 200,000 cells/well (or for a total of 96 wells) in the presence of MBP (40 µg/ml). Seven days later, all cultures were restimulated with MBP in the presence of irradiated autologous PBMCs. After another week, each well was split into four aliquots (~106 cells/aliquot) and cultured in duplicate with 105 irradiated autologous PBMCs in the presence and the absence of MBP. Cultures were kept for 3 days and pulsed with [3H]thymidine (Nycomed Amersham, Arlington Heights, IL) at 1 μCi/well during the final 16 h of culture. Cells were then harvested using an automated cell harvester, and [3H]thymidine incorporation was measured.

A well/culture was defined as specific for MBP or the peptides of MBP when the counts per minute were >1000 and exceeded the reference counts per minute (in the absence of MBP) by at least 3-fold (8, 9, 11). The precursor frequency of MBP-reactive T cells was then estimated by dividing the number of specific wells by the total number of PBMCs (19.2 × 106 cells) seeded in the initial culture (8, 9, 11).

Myelin-reactive T cell clones

The positively identified T cell lines were cloned using limiting dilution assay in the presence of PHA-protein at 2 µg/ml. Cultures were fed with fresh medium every 3–4 days. Growth-positive wells were tested for specificity to the MBP83–99 peptide in proliferation assays. The resulting MBP83–99-specific T cell clones were further characterized and used for T cell vaccination.

TCR V gene analysis and DNA sequencing

TCR V gene rearrangements of the immunizing MBP-reactive T cell clones were analyzed using reverse transcribed PCR. TCR α- and β-chain transcripts were amplified and directly sequenced as previously described (20, 21). Briefly, total RNA was extracted from 105 cells of each MBP83–99-reactive T cell clone using the RNeasy mini kit (Qiagen, Santa Clarita, CA). First-strand cDNA was reverse transcribed from total RNA subjected to PCR amplification with a set of primers specific for TCR Vα and Vβ genes whose sequences were published previously (20, 21). The amplified PCR products were separated in a 1% agarose gel by electrophoresis and stained with ethidium bromide. The visualized PCR products were cut and purified subsequently using a QiAquick gel extraction kit (Qiagen) before sequence analysis. The purified PCR products were directly sequenced with the T7 sequencing kit (Pharmacia, Uppsala, Sweden). Template (1.5 µg) was sequenced with 2 pmol of the corresponding V gene primer using the method of dideoxy chain termination (20, 21).

Immunization of MS patients with irradiated autologous MBP-reactive T cell clones

Two patients with clinically definite MS confirmed by magnetic resonance imaging (22) were included in this study. They were diagnosed as having relapsing-remitting MS for >2 years. The patients had not taken any immunomodulatory drugs at least 3 mo before the study. Immunizations with irradiated autologous MBP83–99 reactive T cell clones was performed as previously described (8, 14). Briefly, MBP83–99-reactive T cell clones were activated and expanded in the presence of PHA 7 days before injection. T cells were then irradiated at 10,000 rad (a60 Co source) and thoroughly washed with sterile saline. A total of 4 × 106 cells of two autologous T cell clones was resuspended in 2 ml of sterile saline and was injected s.c. in the arms. Each patient received a total of four injections at a 2-mo interval to achieve adequate immune responses as defined by the proliferation of PBMC to the immunizing T cell clones. The protocol was approved by the institutional human subjects committee at Baylor College of Medicine. Consent forms were obtained from the patients after explaining the experimental procedures. The patients were evaluated for adverse events and disability score (Expanded Disability Scale Score) before and after each immunization. Gadolinium-enhanced magnetic resonance imaging scans were performed before and at different time points after immunization. The clinical and radiographic evaluation was part of a separate clinical study (J. Z. Zhang et al., manuscript in preparation).

Generation of Ab-producing B cell lines by EBV transformation

The method used was described previously (18, 19). Briefly, PBMC were plated out at 20,000 cells/well in microtiter plates (Costar, Cambridge, MA) in the presence of cell-free supernatant of a B95.8 line producing EBV (American Type Culture Collection, Manassas, VA) and 0.5 µg/ml cyclosporin A (Sandoz, Basel, Switzerland) to selectively inhibit T cell growth. Cells were cultured for 14 days with changes of medium every 3–4 days. On day 14, the growth-positive wells were transferred subsequently to 24-well plates (Costar) for expansion. As described previously, the B cell lines typically produced 2–10 µg/ml of relatively pure Abs.

Detection of anti-TCR Ab by ELISA

Culture supernatants were collected from individual B cell lines and tested in duplicate for the presence of anti-idiotypic Abs using ELISA. Briefly, microtiter plates were coated overnight at 4°C with the motif-positive TCR peptide or the control TCR peptide, respectively, at a concentration of 1 µg/well. Wells were then blocked at 37°C for 2 h with PBS containing 2% BSA (Sigma, St. Louis, MO) and washed four times with 0.02% Tween 20. Plates were then blocked at 37°C for 2 h with PBS containing 2% BSA (Sigma, St. Louis, MO) and washed four times with 0.02% Tween 20 in a 0.9% NaCl solution. Each sample and its control were added to the adjacent wells and incubated for 2 h. Plates were washed four times and incubated for 30 min with the goat anti-human IgG/Fc (Invitrogen, Carlsbad, CA) at a concentration of HRP at 1/1500 dilution (Sigma). Tetramethylbenzidine (0.0125%/0.008% H2O2 in citrate buffer (pH 5.0)) was used as a substrate, and color development was stopped using 2 N H2SO4. ODs were measured using an ELISA reader (Bio-Rad, Hercules, CA). Wells containing medium alone served as the background control. Supernatants were considered to contain specific anti-idiotypic Abs when mean OD in wells coated with the motif-positive peptide subtracted by background OD exceeded mean OD in wells coated with the control TCR peptide subtracted by background OD by at least three times (19).

The Journal of Immunology 6859

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Immunoblot analysis

Lysates were prepared from a representative MBP-reactive T cell clone (MS7-E2.6) expressing the common CDR3 sequence and a motif-negative T cell clone (MS7-D2.2), respectively, using a standard method described previously (23). Briefly, 5 × 10^6 T cells were lysed in 100 μl lysis buffer containing 150 mM NaCl, 50 mM Tris (pH 7.6), 0.5% Triton X-100, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Cell debris were spun down at 13,000 × g for 20 min at 4°C. The resulting lysates were electrophoresed using 10% SDS-PAGE. After blotting, nitrocellulose membranes were cut into strips and then blocked with 5% low fat milk powder in TBS containing 0.1% Tween 20. The strips were then incubated with undiluted supernatants in mini-incubation trays for 1 h at room temperature. A goat anti-human IgG and IgM (H+L chains) coupled to HRP were used as secondary Abs (5% low fat milk powder in TBS containing 0.1% Tween-20) and incubated with washed strips for 45 min, followed by ECL visualization of the proteins on membrane (Amersham, Arlington Heights, IL). Supernatant obtained from an EBV-transformed B cell line producing nonreactive Abs of the IgM type was used as a negative control. A rabbit polyclonal anti-human TCR-β chain Ab (Santa Cruz Biotechnology, Santa Cruz, CA) was included as a positive control.

Flow cytometry

A representative MBP-reactive T cell clone (MS7-E2.6) expressing the common CDR3 sequence was incubated with the anti-idiotypic Abs derived from individual B cell lines at 4°C for 30 min. Supernatant obtained from an EBV-transformed B cell line producing nonreactive Abs of IgM type was used as a control. A motif-negative T cell clone (MS7-D2.2) was stained with the same anti-idiotypic Abs and the control Ab under similar experimental conditions. After washing with FACS buffer (PBS containing 5% FCS and 0.01% sodium azide) by centrifugation at 2000 rpm for 2 min at 4°C, cells were resuspended and stained with a goat anti-human IgG/IgM Ab conjugated with FITC. After two washes the cells were resuspended in 300 μl of FACS buffer and analyzed by flow cytometry using a FACSscan (Becton Dickinson, San Jose, CA). FITC-conjugated anti-IgG1 was used to detect background staining (Becton Dickinson).

The inhibition assay

Twenty thousand cells of the immunizing MBP-reactive T cell clones (motif-positive and motif-negative T cell clones) were cultured in 150 μl with irradiated autologous PBMC (100,000 cells/well) in the presence and the absence of the 83–99 peptide of MBP (20 μg/ml). Fifty microliters of undiluted supernatants were added to each well. Cell proliferation was measured after 72 h in 3H-thymidine incorporation assays. Supernatant obtained from an EBV-transformed B cell line producing nonreactive Abs of the IgM type was used as a negative control. A rabbit polyclonal anti-human TCR-β chain Ab (Santa Cruz Biotechnology, Santa Cruz, CA) was included as a positive control.

Results

Functional and structural characteristics of the immunizing MBP-reactive T cell clones

A panel of four MBP-reactive T cell clones was generated from two patients with relapsing-remitting MS. These T cell clones expressed the CD4 phenotype and recognized the 83–99 immunodominant peptide of MBP in the context of DR4 or DR2 (DRB1*1501) molecules (Table I). They were analyzed for TCR V gene rearrangements by RT-PCR using VA and VB-specific primers and subsequently sequenced for the Vα-Jα and Vβ-Dβ-Jβ junctional regions. As shown in Table II, an independent T cell clone (E2.6) derived from patient MS7 shared the same TCR Vα17 and Vβ13.1 genes with another T cell clone (C3.1) obtained from a different patient (MS27). The two T cell clones had an identical sequence (LGRAGLTY) within the Vβ13.1-Dβ1-Jβ1 junctional region, while their Vα17 chains had two distinct Vα-Jα junctional region sequences. As demonstrated previously, the identified LGRAGLTY sequence represented a common CDR3 motif among Vβ13.1 T cells that recognize the 83–99 immunodominant region of MBP in different patients with MS (24).

Induction of the immune responses to the immunizing MBP-reactive T cell clones and a 20-mer TCR peptide by T cell vaccination in patients with MS

Each patient received a total of four s.c. inoculations with two irradiated autologous MBP-reactive T cell clones (2 × 10^7 cells/dose) at a 2-mo interval. The proliferative responses of PBMC to the autologous immunizing T cell clones were examined at two time points, corresponding to baseline and 2 mo after the last immunization. As shown in Fig. 1A, the proliferative responses to both irradiated immunizing T cell clones were increased in the patients after T cell vaccination and substantially exceeded the baseline value. Furthermore, the responses to the TCR peptide incorporating the common CDR3 sequence (motif-positive peptide), as opposed to a control CDR3 peptide (motif-negative peptide) derived from a nonimmunizing T cell clone, was apparent after vaccination. However, the magnitude of the specific proliferation in response to the motif-positive peptide was considerably lower than that induced by irradiated immunizing T cells (Fig. 1A). The proliferative response to the immunizing T cells correlated inversely with a decline in the frequency of circulating MBP-reactive T cells in the immunized patients (Fig. 1B), as demonstrated previously in other studies (8, 15, 16).

Generation of B cell lines producing specific Abs to the TCR peptide incorporating the common CDR3 sequence from immunized patients

We then examined whether immunization with irradiated T cells would elicit specific anti-idiotypic Ab responses in the patients. As the whole T cells expressed an array of surface molecules that could interfere with the detection of serum anti-idiotypic Abs, the TCR peptide incorporating the common CDR3 sequence (motif-positive peptide) was used as the Ag for screening. A 20-mer TCR peptide derived from a nonimmunizing MBP-reactive T cell clone (motif-negative peptide) was included in all experiments as a control. The CDR3 sequence of the control peptide was not detected in the immunizing T cell clones.

As we experienced previously, no specific Ab reactivity to either the TCR peptide or the original immunizing T cells could be detected using ELISA or flow cytometry when tested with sera derived from the two patients (data not shown). To further verify whether anti-idiotypic Abs were present in the vaccinated patients, we then examined whether immunization with irradiated T cells would elicit specific anti-idiotypic Ab responses in the patients. As the whole T cells expressed an array of surface molecules that could interfere with the detection of serum anti-idiotypic Abs, the TCR peptide incorporating the common CDR3 sequence (motif-positive peptide) was used as the Ag for screening. A 20-mer TCR peptide derived from a nonimmunizing MBP-reactive T cell clone (motif-negative peptide) was included in all experiments as a control. The CDR3 sequence of the control peptide was not detected in the immunizing T cell clones.

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Table I. Characteristics of the immunizing MBP-reactive T cell clones

<table>
<thead>
<tr>
<th>Patient</th>
<th>Clone</th>
<th>MBP&lt;sub&gt;83–99&lt;/sub&gt;</th>
<th>Medium Alone</th>
<th>MHC Restriction</th>
<th>TCR V Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS7</td>
<td>E2.6</td>
<td>31,701 ± 913</td>
<td>625 ± 28</td>
<td>DR4</td>
<td>AV17</td>
</tr>
<tr>
<td></td>
<td>D2.2</td>
<td>4,918 ± 333</td>
<td>399 ± 91</td>
<td>DR4</td>
<td>AV9</td>
</tr>
<tr>
<td></td>
<td>C3.1</td>
<td>5,492 ± 213</td>
<td>839 ± 102</td>
<td>DRB1*1501</td>
<td>AV17</td>
</tr>
<tr>
<td></td>
<td>D4.4</td>
<td>7,328 ± 128</td>
<td>827 ± 110</td>
<td>DR4</td>
<td>AV22</td>
</tr>
</tbody>
</table>

* MBP-reactive T cell clones recognizing the immunodominant 83–99 peptide (MBP<sub>83–99</sub>) were generated from the blood of two MS patients (MS7 and MS27). The reactivity of the clones to the 83–99 peptide is expressed in cpm and is compared with background cpm in the absence of the peptide. MHC restriction of the clones was tested in proliferation assays using mouse L cell lines transfected with DR4 or DR2 (DRB1*1501). All T cell clones were examined for TCR V gene usage by PCR analysis with oligonucleotide primers specific for VA and VB families.
we generated a panel of Ab-producing B cell lines from the post-vaccination blood specimens using a cell culture-based technique combining EBV transformation with limiting dilution (see Materials and Methods). As prevaccination PBMC were not available for the experiments, cells obtained from two randomly selected healthy individuals were used as control subjects and analyzed under the same experimental conditions. Supernatants of the resulting B cell lines (92 cell lines from each patient/individual) were tested for the presence of Abs to the motif-positive TCR peptide and the control TCR peptide, respectively, in ELISA. Abs were defined as anti-idotypic when they exhibited specific reactivity to the motif-positive TCR peptide but not the control TCR peptide. As shown in Fig. 2, B cells producing specific anti-idiotypic Abs occurred at the precursor frequency of $1.9 \times 10^{-8}$ (7 of 92) and $0.82 \times 10^{-6}$ (3 of 92) in patients MS7 and MS27, respectively, compared with $0.27 \times 10^{-6}$ (1 of 92) in both non-immunized control subjects. In contrast, no specific Ab reactivity to the control peptide was detected in the same supernatants.

Reactivity pattern and regulatory property of the anti-idiotypic Abs

Next, the resulting anti-idiotypic Abs were characterized in a series of experiments for the reactivity pattern and potential functional effect on the proliferation of the immunizing T cells in response to the 83–99 peptide of MBP. We first addressed whether the anti-idiotypic Abs reactive to the TCR peptide would bind to the whole immunizing T cells expressing the common CDR3 sequence. Supernatants collected from three representative B cell lines that produced relatively high concentrations of anti-idiotypic Abs were further characterized (Table III). These independent Abs derived from the two immunized patients were of the IgM isotype and were found to bind to original immunizing T cell clones but not to control T cell clones that did not express the common CDR3 sequence. Such a representative experiment using flow cytometry is shown in Fig. 3. Similarly, the selected Abs also exhibited the specific reactivity to the TCR of the immunizing T cell clones in immunoblot analysis (Fig. 4). Furthermore, the anti-idiotypic Abs were examined for an inhibitory effect on the proliferation of the original immunizing T cell clones. As shown in Fig. 5, all three Abs examined were found to inhibit substantially the proliferation of the immunizing T cell clones expressing the CDR3 motif (MS7-E2.6 and MS27-C3.1) but not the motif-negative immunizing T cell clones (MS7-D2.2 and MS27-D4.4).

Discussion

There is increasing evidence indicating that the Id anti-idiotypic regulatory network can be induced by T cell vaccination in experimental animals and humans (5, 8). Most of these studies described the functional properties and the regulatory role of anti-idiotypic T cell responses in vaccinated patients (8, 14–16). However, little is known regarding the potential role of the humoral component within the idiotypic regulatory circuitry. The study described here provides the first evidence indicating that anti-idiotypic Ab responses can be elicited by T cell vaccination in patients with MS. These anti-idiotypic Abs selected for the specific reactivity to the CDR3 peptide were found to bind to the original immunizing MBP-reactive T cells.

**TABLE II. TCR sequence of the immunizing MBP$_{83-99}$-reactive T cell clones**

<table>
<thead>
<tr>
<th>Clone</th>
<th>VJP</th>
<th>VδJP</th>
<th>n-JP</th>
<th>ACJ</th>
<th>BVJ</th>
<th>YFCAS</th>
<th>VDJ Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS7-E2.6</td>
<td>AV17</td>
<td>YFCA</td>
<td>SPGGSNKLTFKGTLTTVNP</td>
<td>NIQN</td>
<td>BV13.1</td>
<td>YFCAS</td>
<td>SLGRAGLTYEYQYFPGTRTLTVT EDLKN</td>
</tr>
<tr>
<td>MS7-D2.2</td>
<td>AV9</td>
<td>YYCA</td>
<td>KRNFGNEKLTFGTGTRLTI</td>
<td>NIQN</td>
<td>BV1</td>
<td>YFCAS</td>
<td>SLGRAGLTYEQYFPGTRTLTVT EDLKN</td>
</tr>
<tr>
<td>MS27-C3.1</td>
<td>AV17</td>
<td>YFCA</td>
<td>MGDFGNEKLTFGTGTRLTI</td>
<td>NIQN</td>
<td>BV13.1</td>
<td>YFCAS</td>
<td>SLGRAGLTYEQYFPGTRTLTVT EDLKN</td>
</tr>
<tr>
<td>MS27-D4.4</td>
<td>AV22</td>
<td>YFCAL</td>
<td>SVAGGTSYGKLFQGTILTVHP</td>
<td>NIQN</td>
<td>BV9</td>
<td>YFCAS</td>
<td>SQDRFVWGTYVNEAFFQGGTRTLTVV EDLKN</td>
</tr>
</tbody>
</table>

**FIGURE 1.** The proliferative responses of PBMCs to the immunizing MBP-reactive T cell clones and the TCR peptides in relationship to the frequency of MBP-reactive T cells in immunized MS patients. A. The proliferative responses of PBMCs obtained from two immunized patients are expressed as stimulation indexes, which were defined as follows: cpm of PBMC cultured with irradiated immunizing MBP-reactive T cell clones expressing the common CDR3 sequence (motif-positive T cell clones, MS7-E2.6 and MS27-C3.1) or control immunizing T cell clones (motif-negative T cell clones, MS7-D2.2 and MS27-D4.4)/the sum of cpm of PBMC cultured alone and cpm of irradiated T cells cultured alone. The proliferative responses to the motif-positive peptide (amino acid sequence: FCASSLGRAGLTYEYQYF-GPG) and a control TCR peptide (amino acid sequence: YLCASSTRQGPETQYFGP) were determined in proliferation assays in which PBMCs were cultured at 100,000 cells/well with the TCR peptides (20 μg/ml), respectively, for 5 days. All experiments were performed at two time points corresponding to baseline (before) and 2 mo after the fourth vaccination (after). B. The precursor frequency of T cells specific for MBP was estimated at the same time points. NS, normal subjects.
The estimated precursor frequency of B cells producing anti-idiotypic Abs to the TCR peptide in PBMCs of immunized patients. PBMCs were obtained from two immunized MS patients and two randomly selected healthy individuals who were not immunized. Cells were cultured in the presence of supernatants derived from an EBV-producing cell line and cyclosporin A. All growth-positive wells were screened for the presence of Abs reactive to the motif-positive TCR peptide and the control TCR peptide in ELISA. The precursor frequency of B cells producing anti-idiotypic Abs to the TCR peptide was estimated by dividing the number of positive wells by the total number of PBMC plated initially.

The successful detection of anti-idiotypic Abs as described here was attributable to the technical improvements made in the study, which enabled us to estimate the precursor frequency of B cells producing anti-idiotypic Abs in blood specimens and to obtain relatively high concentrations of specific Abs from the identified B cell lines for detailed characterization. There are several issues related to the interpretation of the results. 1) The general efficiency of EBV transformation of human B cells was reported to be <10% (25). Therefore, the precursor frequency of B cells producing Ab of interest is likely to be underestimated in the assays involving EBV transformation. The actual frequency of B cells producing anti-idiotypic Abs in the vaccinated patients was probably much higher than that described in the present study. 2) The preferential IgM expression of the obtained anti-idiotypic Abs is consistent with the recent immunization in the patients. However, it should be noted that EBV-transformed B cells usually produce Abs of predominantly the IgM isotype (26). Therefore, the observed preference of the resulting anti-idiotypic Abs for IgM is likely to result from the isotype selection by EBV transformation and may not represent the original isotype of the B cells. 3) EBV appears to transform preferentially recently activated B cells (26) that represent relevant subsets of B cells induced by T cell vaccination, providing a technical advantage for the purpose of this study. 4) Some of the B cell lines generated by EBV transformation as described here are likely to contain multiple B cell populations that may produce Abs to other Ags, which is indicated by the reactivity of Ab MS7-C8 to an additional Ag present in the T cell lysates (Fig. 4).

In the study described here only one TCR peptide incorporating the common CDR3 sequence motif was used to identify anti-idiotypic Abs induced by T cell vaccination. The selection of the 20-mer CDR3 peptide was based on the considerations that it contained the common Vbeta13.1 sequence motif among MBP_83-99-reactive T cells and expressed by the immunizing T cell clones used for both patients. It is conceivable that the LGRAGLTY sequence contains the idiotypic epitope(s) recognized by the obtained anti-idiotypic Abs, as the TCR peptide incorporating the LGRAGLTY

Table III. Ab isotype and reactivity of representative B cell lines derived from the two immunized patients

<table>
<thead>
<tr>
<th>B Cell Line</th>
<th>Isotype</th>
<th>TCR peptide</th>
<th>Control peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS27-C9</td>
<td>IgM</td>
<td>0.51 ± 0.05</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>MS27-B9</td>
<td>IgM</td>
<td>0.53 ± 0.05</td>
<td>0.01 ± 0.05</td>
</tr>
<tr>
<td>MS7-C8</td>
<td>IgM</td>
<td>1.25 ± 0.12</td>
<td>0.13 ± 0.01</td>
</tr>
</tbody>
</table>

* Supernatants derived from anti-idiotypic B cell cultures were tested for the Ab reactivity to the motif-positive TCR peptides or the control TCR peptide (motif-negative) in ELISA. Data are expressed as net OD calculated by subtracting background OD of the control wells containing culture medium.

ANTIDIOTYPIC AB INDUCED BY T CELL VACCINATION

FIGURE 2. The estimated precursor frequency of B cells producing anti-idiotypic Abs to the TCR peptide in PBMCs of immunized patients. PBMCs were obtained from two immunized MS patients and two randomly selected healthy individuals who were not immunized. Cells were cultured in the presence of supernatants derived from an EBV-producing cell line and cyclosporin A. All growth-positive wells were screened for the presence of Abs reactive to the motif-positive TCR peptide and the control TCR peptide in ELISA. The precursor frequency of B cells producing anti-idiotypic Abs to the TCR peptide was estimated by dividing the number of positive wells by the total number of PBMC plated initially.

FIGURE 3. The reactivity of anti-idiotypic Abs to the immunizing MBP-reactive T cells. A representative MBP-reactive T cell clone expressing the common CDR3 motif (MS7-E2.6, shown as solid curves) and a motif-negative T cell clone (MS7-D2.2, shown as open curves) were stained with supernatants of B cells producing anti-idiotypic Abs (C9, B9, and C8), respectively. Supernatant derived from an EBV-transformed B cell line producing nonreactive Abs of the IgM type was used as a control Ab (Control) to stain both T cell clones. The secondary Ab used was a goat-anti-human IgG/IgM Ab conjugated with FITC. Specific staining of T cells was determined by flow cytometry.

FIGURE 4. Immunoblot analysis. Three selected anti-idiotypic Abs were tested for their reactivity to the lysates prepared from a representative immunizing T cell clone (MS7-E2.6, shown in lanes 1–5) and a motif-negative T cell clone (MS7-D2.2, shown in lanes 6–10), respectively, in immunoblot analysis. Lanes 1 and 6, Supernatant derived from an EBV-transformed B cell line producing nonreactive Abs of IgM type. Lanes 2 and 7, A rabbit polyclonal Ab raised against human TCR-β chain. Lanes 3–5 and lanes 7–10 correspond to supernatants containing the three anti-idiotypic Abs: MS7-C8, MS27-B9, and MS27-C9.
The inhibition effect of anti-idiotypic Abs on the proliferation of immunizing MBP-reactive T cell clones. The immunizing motif-positive T cell clones and motif-negative T cell clones were cultured in the presence and the absence of the 83–99 peptide of MBP, respectively. Supernatants containing the anti-idiotypic Abs (~10 μg/ml) were added to the culture. Supernatant derived from an EBV-transformed B cell line producing nonreactive Abs of the IgM type was used as a control. Cells were cultured for 72 h, and proliferation was measured by thymidine uptake assays. The data are presented as counts per minute incorporated. The SD was <10%.

FIGURE 5. The inhibitory effect of anti-idiotypic Abs on the proliferation of immunizing MBP-reactive T cell clones. The immunizing motif-positive T cell clones and motif-negative T cell clones were cultured in the presence and the absence of the 83–99 peptide of MBP, respectively. Supernatants containing the anti-idiotypic Abs (~10 μg/ml) were added to the culture. Supernatant derived from an EBV-transformed B cell line producing nonreactive Abs of the IgM type was used as a control. Cells were cultured for 72 h, and proliferation was measured by thymidine uptake assays. The data are presented as counts per minute incorporated. The SD was <10%.
Human B cell lines secreting IgM antibody specific for myelin basic protein. *J. Neuroimmunol.* 23:249.


