S-antigen specific T cell clones from a patient with Behçet's disease

Joyce Hisae Yamamoto, Yujiro Fujino, Chiu Lin, Mic Nieda, Takeo Juji, Kaniro Masuda

Abstract
The isolation and characterisation of T cell clones or lines specific to retinal antigens are valuable tools to clarify the underlying mechanisms of autoimmune to retinal antigens as a contributing factor in ocular inflammation. Patients with Behçet's disease have been reported to be sensitised to S-antigen (S-Ag). In the present study, four T cell clones established from the peripheral blood of a patient with Behçet's disease were analysed. A CD4+ T cell clone (clone 2) and a CD8+ T cell clone (clone 10) proliferated specifically to bovine S-Ag. Although these S-Ag specific T cell clones proliferated vigorously to the intact antigen, their responses to S-Ag derived synthetic peptides M and G were weak, suggesting that the sites of human T cell recognition of S-Ag may be different from those established in the experimental model. The proliferative responses of both clones (2 and 10) were inhibited by anti-HLA-DR monoclonal antibody but not by anti-HLA-class 1 monoclonal antibody. The other two clones studied, clones 6 and 30, were CD3+, CD4+, CD8+ and they did not proliferate specifically to S-Ag. Clone 6 expressed γδ T cell receptors (TCR) and showed non-specific cytotoxic activity toward K562 and Daudi cell lines. Clone 30 expressed αβ TCR, and was devoid of cytotoxic activity. Human T cell lines and clones specific to retinal antigens will provide the framework necessary to examine the events that lead to ocular inflammation. (Br J Ophthalmol 1994; 78: 927-932)

So far, very little has been reported about the isolation of T cell clones or lines specific to retinal antigens from patients with uveitis. To our knowledge, there has been no report on the isolation of retinal antigen specific T cell clones from patients with BD. In the present study we report on the establishment of T cell clones specific to S-Ag from a patient with BD.

Subjects, materials and methods
PATIENT
Peripheral blood was obtained from a 38-year-old male patient with the complete form of BD (diagnostic criteria proposed by Behçet's Disease Research Committee of Japan, 1974), who had been followed for 5 years at the uveitis clinic of the department of ophthalmology of the University of Tokyo. He was taking cyclosporine (5 mg/kg/day) and colchicine (0-5 mg/day) therapy, with a frequency of five ocular attacks per year and recurrent oral aphtha. His cellular immune responses of peripheral blood lymphocytes, previously assayed by the lymphocyte proliferative method and represented as stimulation index (refer to proliferative studies), were 8-4 to bovine S-Ag, 4-7 to peptide M, and 4-6 to peptide G. The patient's HLA was A2, Bw46, Bw62, Bw6, CW9, DR9, DR8-1, DRw52, DRw53, DQw1, DQw3 as determined by the complement dependent microcytotoxicity method on the peripheral blood mononuclear lymphocytes. This study was conducted with the approval of the ethics committee of the University of Tokyo and with the patient's informed consent.

ANTIGENS
Bovine S-Ag was purified by the method of Dorey et al. Two peptides, M and G, derived from the sequence of human S-Ag, were synthesised by Bio Science Laboratory (Fuiyai Co Ltd, Kanagawa, Japan) according to the t-BOC method using a peptide synthesiser 430A (Applied Biosystems, Foster City, CA, USA). Peptide M corresponds to positions 306 to 323 (DTNLASTTIKEGIDRTV) while peptide G corresponds to positions 343 to 362 (GELTSSEVATEVFPRLMHPQ) of the human S-Ag. Purified protein derivative (PPD) of tubercle bacillus (donated by Dr Akira Aaway of Mitsui Pharmaceuticals, Inc, Tokyo, Japan) was used as a control antigen.

MONOCLONAL ANTIBODIES
Surface phenotyping of T cell clones was performed using the following monoclonal antibodies (mAbs), labelled with fluorescein
isothiocyanate (FITC): anti-human CD3 (Leu 4), CD4 (Leu 3a), CD8 (Leu 2a), TCRβ (TCR-1 α/β) and TCRγδ (TCR-γ/δ-1) (Immunocytochemistry System, Becton Dickinson, San Jose, CA, USA). FITC conjugated goat anti-mouse IgG (Becton Dickinson) was used for the indirect immunofluorescence analysis. Mouse IgG, conjugated to FITC (G1CL, Becton Dickinson) was used as control.

For determining the MHC molecules involved in the proliferative responses of the T cell clones, a panel of murine mAbs to HLA-DR (Becton Dickinson), HLA-DQ (Leu 16, Becton Dickinson) and HLA class I (Sera-Lab, Crawley Down, Sussex) was used. Mouse IgG2a (G2CL) and mouse IgG1 (G1CL, Becton Dickinson) were used as negative control.

**ESTABLISHMENT OF THE T CELL CLONES**

Peripheral blood mononuclear cells (PBMCs) from heparinised blood samples were separated by gradient centrifugation (Ficoll-Paque, Pharmacia, Uppsala, Sweden). Cells were cultured (3×10^5 cells/well) with bovine S-Ag (0.05 μM and 0.2 μM) in RPMI 1640 medium with anti-human CD3 (Becton Dickinson, San Jose, CA, USA). The MHC restricting pattern was determined with 0.05 μM and 0.2 μM of S-Ag. The system was cultivated for 72 hours at 37°C, 5% carbon dioxide with bovine S-Ag (0.05 μM and 0.2 μM) or medium only in the presence of 10^5 irradiated (2000R) autologous PBMCs in RPMI 1640 medium with HEPES supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (2 mM), and 10% human AB serum. Responses to the S-Ag derived peptides, M and G, were similarly assayed. For the analysis of the HLA restriction pattern, anti-HLA class I, anti-HLA-DR, or anti-DQ mAbs were added to culture plates at 0.1 μg/ml final concentration. Microcultures were then pulsed with 0.5 μCi of tritiated thymidine ('H-TdR, New England Nuclear, Boston, MA, USA; 2 Ci/mmol) for 16 hours before harvesting. Cell proliferation was evaluated by incorporated radioactivity counted by a liquid scintillation counter. The results were expressed as disintegrations per minute (dpm) or stimulation index (dpm in control wells with antigen/dpm in control wells without antigen). The inhibitory effect of anti-HLA mAb on the antigen induced proliferative responses was calculated with the following equation:

\[
\% \text{ of specific lysis} = \left( \frac{\text{dpm in culture wells with antigen and mAb}}{\text{dpm in culture wells without antigen or mAb}} \right) \times 100
\]

**PROLIFERATIVE STUDIES**

Assays for antigen specific responses of cloned cells were performed by culturing 2×10^5 resting T cells in 200 μl RPMI 1640 containing irradiated (1000R) autologous PBMCs in RPMI 1640 medium with HEPES supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (2 mM), and 10% human AB serum. Responses to the S-Ag derived peptides, M and G, were similarly assayed. For the analysis of the HLA restriction pattern, anti-HLA class I, anti-HLA-DR, or anti-DQ mAbs were added to culture plates at 0.1 μg/ml final concentration. Microcultures were then pulsed with 0.5 μCi of tritiated thymidine ('H-TdR, New England Nuclear, Boston, MA, USA; 2 Ci/mmol) for 16 hours before harvesting. Cell proliferation was evaluated by incorporated radioactivity counted by a liquid scintillation counter. The results were expressed as disintegrations per minute (dpm) or stimulation index (dpm in control wells with antigen/dpm in control wells without antigen). The inhibitory effect of anti-HLA mAb on the antigen induced proliferative responses was calculated with the following equation:

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\]

**CYTOTOXICITY ASSAYS**

Autologous Epstein-Barr virus (EBV) transformed B cells, allogeneic DR9 EBV transformed B cells, K562, and Daudi cells were used as target cells. Target cells harvested from fresh cultures were labelled with 100 μCi chromium-51 (Cr, CJS 11, Amersham Life Science, Tokyo, Japan) and subsequently washed three times with RPMI 1640 medium containing 10% fetal bovine serum (FBS). Target cells were then incubated in RPMI 1640 medium plus 10% FBS only or added with bovine S-Ag (1 mg/ml) for 1 hour at 37°C. Effector cells (T cell clones) were incubated in triplicate with 1×10^5 S-Ag pre-incubated or non-incubated labelled target cells at effector to target ratios of 1:1 and 20:1 in 96 well round bottomed microwells for 4 hours at 37°C. Supernatants were then harvested and measured for radioactivity in a gamma counter. Chromium release of the labelled target cells is termed as experimental release. Maximum release and spontaneous release of chromium were measured in wells containing target cells in the presence of detergent or medium alone, respectively. The percentage of specific release was calculated according to the following equation:

\[
\% \text{ of specific lysis} = \left( \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \right) \times 100
\]
S-antigen specific T cell clones from a patient with Behçet's disease

Results

PHENOTYPIC PROFILES OF THE T CELL CLONES
Eleven T cell clones could be isolated from the BD patient, whose CD4/CD8 ratio in the peripheral blood was 0.7 and the percentage of TCRγδ+ T cells was 9.2%. Their respective phenotypes were as follows: CD4+, TCRαβ+ (clones 2 and 9); CD8+, TCRαβ+ (clones 10, 17, 19, and 41); CD3+ double negative (DN CD4+, CD8+), TCRδ+ (clones 30 and 43); and, CD3+, DN, TCRγδ+ (clones 6, 18, and 44). Among these T cell clones, four long term (12 weeks) T cell clones; clones 2, 10, 30, and 6 were further analysed. Their phenotypes are shown in Figure 1. Clone 2 expressed CD3, CD4, and TCRγδ phenotype. Clone 10 expressed CD3, CD8, and TCRβ phenotype. Clones 30 and 6 were stained by anti-CD3, but not by anti-CD4 nor anti-CD8 (DN); clone 30 expressed TCRδ, while clone 6 expressed TCRγδ.

S-Ag SPECIFICITY OF THE T CELL CLONES
Clones 2 and 10 proliferated specifically to bovine S-Ag with a stimulation index varying from 4.4 to 6.8, as described in Table 1. These T cell clones did not proliferate when cultured with PPD. Clones 30 and 6 were not specific to S-Ag.

CYTOLYTIC ACTIVITY OF THE T CELL CLONES
All four T cell clones were tested as effectors in a 4-hour 51Cr release assay against various target cells, before and after incubation with S-Ag. Clone 30 (CD3+, DN, TCRαβ+) was devoid of cytotoxic activity towards all target cells tested. Clone 6 (CD3+, DN, TCRγδ+) lysed K562 and Daudi target cell lines independent of the presence of S-Ag (Table 2). Clones 2 and 10, although proliferating specifically to S-Ag, did not show any specific killing activity against the autologous EBV transformed B cells, nor to any of the other lines tested (allogeneic EBV transformed B cells, K562, and Daudi cells) (data not shown).

Discussion

Patients with BD were previously reported by us and others16-18 to be sensitised to the retinal specific antigens, S-Ag and IRBP. As cellular autoimmunity to retinal antigens may be involved in the initiation or in the perpetuation of the inflammatory processes in the eye, detailed studies of the T cell responses to the retinal antigens observed in patients with BD are warranted. For this purpose, T cell clones are valuable tools to analyse the cellular autoimmune responses found in patients with uveitis. In 1984, Nussenblatt et al. first described the isolation of T cell clones specific to S-Ag derived from the peripheral blood of a patient with presumed Eale's disease.19 In 1998, Hirose et al. described the isolation of T cell lines and clones specific to S-Ag from healthy donors.20 We report here the establishment of T cell clones obtained from the peripheral blood of a patient with BD. The phenotypes of the 11 CD3+ T cell clones isolated in this study were diverse: two clones were CD4+, four were CD8+, two were DN (CD4+, CD8+), TCRαβ+, and three were DN, TCRγδ+. Four of these T cell clones were further analysed: clone 2 (CD4+), clone 10 (CD8+), clone 30 (DN, TCRαβ+), and clone 6 (DN, TCRγδ+). Two clones, one CD4+ and the other CD8+, proliferated specifically to S-Ag. The T cell clones specific to S-Ag isolated from a synthetic peptides known to be uveitogenic in animal models, peptides M and G,19 20 were used as antigen in the proliferation assay. At least three antigen concentrations were tested for each peptide, and the highest lymphocyte proliferative responses obtained are shown in Figure 2. Even though these clones vigorously responded to intact S-Ag, their responses to peptide M and peptide G were weak.

HLA RESTRICTION MOLECULES
The mAbs to HLA-DR, HLA-DQ, and HLA-class I molecules were used to define which element restricts the observed T cell proliferative response to S-Ag in the CD4+ clone (clone 2) and CD8+ clone (clone 10). As seen in Figure 3, anti-DR antibody reduced proliferation by approximately 40%, even for the CD8+ clone, while the anti-class I antibody had no effect on either clone.

Table 1 Proliferative responses of the T cell clones to S-antigen (S-Ag)

<table>
<thead>
<tr>
<th>S-Ag</th>
<th>Clone</th>
<th>Medium</th>
<th>0-0.5 μM (2 μg/ml)</th>
<th>0.2 μM (8 μg/ml)</th>
<th>PPD (1 μg/ml)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>2</td>
<td>1957 (339)*</td>
<td>12598 (1822)</td>
<td>13410 (4163)</td>
<td>1248 (18)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2781 (897)</td>
<td>12380 (2200)</td>
<td>9179 (3654)</td>
<td>3618 (1186)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>2210 (91)</td>
<td>3300 (720)</td>
<td>NT</td>
<td>352 (139)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>659 (279)</td>
<td>228 (204)</td>
<td>669 (426)</td>
<td>NT</td>
</tr>
</tbody>
</table>

2×10^6 cells of each clone were stimulated with S-Ag (0.05 and 0.2 μM); PPD (purified protein derivative) (1 μg/ml) as a control antigen, or medium only, in the presence of 10^5 antigen presenting cells (autologous irradiated PBMCs). Cells were cultured for 3 days and pulsed with [3H]-Thy 16 hours before harvesting. Dpm of each clone alone ranged from 294 to 2790; APC alone ranged from 148 to 1437; APC + S-Ag ranged from 100 to 1803. *Disintegrations per minute (SE). NT = not tested.

Figure 1 Phenotype of T cell clones. T cell clones were stained by direct or indirect immunofluorescence, and analysed in an Ortho Spectrum III flow cytometric system. Histograms presented display the number of cells versus the intensity of fluorescence on a logarithmic scale.
Patient with Eale’s disease were also either OKT4+ or OKT8+. Hirose et al established S-Ag specific T cell lines and clones from a helper T cell enriched fraction of PBMCs from a healthy donor. In addition to the CD4+ and CD8+ T cell clones, we could also isolate DN T cell clones. A notable finding of the present study is the diverse range of phenotypes displayed by the isolated clones. This may reflect the proportions of T cells present in the original peripheral blood samples. Interestingly, the CD4/CD8 ratio was low, and the percentage of TCRγδ+ T cells of the peripheral blood, although within the normal range, was in the upper limit. This may represent a tendency to elevated TCRγδ+ T cells in BD, as described by others. However, it may be due to the cloning technique used where no fractionation of cell was done.

CD4+ (clone 2) and CD8+ (clone 10) T cell clones proliferated specifically to bovine S-Ag. Neither of them responded to PPD, substantiating the S-Ag specific recognition by the clones. The other T cell clones grew in an antigen independent manner. It may be due to the method of cloning in which exogenous IL-2 was included during the antigen pulse as well as during the expansion phase. CD4+ T cells internalise ligated high affinity IL-2r and become unresponsive until further high affinity receptors are expressed after restimulation with specific antigen. In contrast, CD8+ T cells are less able to regulate expression of the high affinity IL-2r and will continue to expand in response to IL-2 over a long period even in the absence of specific

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Cytotoxicity Acuity of T Cell Clones</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K562</td>
</tr>
<tr>
<td>Clone</td>
<td>S-Ag added</td>
</tr>
<tr>
<td>30</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
</tr>
</tbody>
</table>

Target cells were labelled with ⁶⁷Cr, and subsequently incubated with S-Ag. Target cells were S-Ag incubated or not and used in a standard 4-hour *Cr release assay.
*Effector to target cell ratio. Specific cytolysis was calculated as stated under Materials and methods. Spontaneous release less than 1000 counts per minute (cpm), maximum release ranged from 4319 to 6166 cpm.
antigen stimulation through the TCR. In addition, DN T cells in the periphery are generally TCRγδ T cells and although defective for signal transduction through p56, may express IL-2r (especially thymocytes) allowing antigen non-specific proliferation.

To determine the S-Ag epitope involved in the T cell response, peptides M and G from the sequence of human S-Ag were used. Both these peptides from the human sequence of S-Ag are reported to be strongly uveitogenic, and much emphasis has been given to peptide G as a dominant epitope in rat. However, these peptides evoked weak responses from both clones, not comparable with the strong responses to the intact antigen. Although the amino acid sequence of peptide M based on the sequence of human S-Ag differs in one amino acid from the sequence of bovine S-Ag (arginine instead of lysine at position 321), the amino acid at this position is not required for uveitogenicity. Further, both peptides (based on bovine and human sequences) were used as antigen in the lymphocyte proliferative assay in this patient and responses of similar magnitude were obtained (data not shown). The sequence of peptide G does not differ between bovine and human S-Ag. Thus, the low responsiveness to the peptides was not due to the difference found in peptides raised from human S-Ag sequence. Similar results were reported by Hirose et al. The cell line they obtained did not respond to peptide K or peptide M derived from the sequence of bovine S-Ag. Therefore, our results indicate the recognition by T cells from a BD patient to T cell epitopes distinct from those described to be uveitogenic in animal models. Further studies in patients using peptides that cover the entire sequence of S-Ag may clarify this issue.

The ability of anti-class II mAb to inhibit the S-Ag driven proliferation indicates that HLA-DR is the critical molecule involved in the proliferative response of the T cell clones obtained. The inhibition of a CD8+ T cell clone response to S-Ag with anti-class II mAb was rather unusual, since CD8 T cells are usually restricted by class I. However, a few exceptions to this association of CD8 with MHC antigens have been reported. In the murine system, class II restricted allogeneic Lyt 2+ cytolytic T cells have been found.

The analysis of the preferential usage of a specific T cell receptor variable region gene by the T cell clones has been pursued in various studies in animals and also in humans. There is a study on the TCR Vα of PBMCs stimulated with the IRBP of patients with Vogt-Koyanagi-Harada syndrome showing the restricted usage of TCR Vα 10 by the analysis of RNA-PCR amplification of TCR Vα. In rat EAU, some reports cited the preferential Vβ8.2 usage, similar to the findings in mice and rats undergoing experimental allergic encephalomyelitis after immunisation with myelin basic protein, the experimental model for multiple sclerosis. In the present study, the S-Ag specific T cell clones showed negative staining to mAbs against human TCR variable region genes; Vβ5.1, Vβ5.2, Vβ5.3, Vβ6.7, Vβ8, Vβ12, and Vα2 (Diversi-Tm αβ TCR Screening Panel 1A; T Cell Sciences Inc, Cambridge, MA, USA), indicating other usage of Vα and Vβ variable regions (data not shown).

The S-Ag specific T cell clones did not show any S-Ag specific killing activity to the autologous EBV transformed B cells in the chromium release assay. Since there are various types of cells that function as antigen presenting cells with varying capacity to process and present the antigens, an antigen specific killing function cannot be totally disregarded. Moreover, a hypothetical dominant peptide specific killing activity could also have been undetected in our assay using whole S-Ag.

The DN T cell clones 30 and 6 were not specific to S-Ag. The TCRαβ clone (clone 30) was devoid of killing activity contrary to the described cytotoxic function for DN, TCRαβ T cells. The TCRγδ clone showed cytotoxic activity against both target cells. TCRγδ T cells are thought to represent a separate lineage with a capacity of antigen independent killing and may contribute to the disease process. The data presented in Table 2 support this hypothesis. Recently, evidence has accumulated suggesting that TCRγδ T cells may participate in the immune response to several infectious organisms, and may function in surveillance against microbial agents and bacteria infected cells. Streptococcus sanguis have been isolated in a high frequency from the oral flora from patients with BD. Further, the isolation of TCRγδ T cell clones specific to KTH-I, an antigen derived from the Streptococcus sanguis, taken from the peripheral blood, as well as increased peripheral blood TCRγδ T cells have been reported in patients with BD. These TCRγδ T cells are suggested to play a role in the physiopathogenesis of BD. The speculation if there should be any correlation between the responsiveness to S-Ag and the presence of TCRγδ T cells in the physiopathogenesis of BD is an interesting topic for future research. Alternatively, non-S-Ag specific TCRγδ T cells could participate as a recruited cell population in the mechanisms of the ocular inflammation in BD. It should be stressed that retinal autoimmunity is not an exclusive finding in BD, but patients with retinal inflammatory or non-inflammatory diseases other than BD, such as birdshot retinochoroidopathy or retinitis pigmentosa are also sensitised to the retinal antigens. Cellular autoimmunity to retinal antigens cannot be excluded as a consequence of primary tissue damage by other causes, thus playing a secondary role in the physiopathogenesis of inflammatory retinal disorders. In order to better understand the contribution of autoimmunity to the pathogenesis of endogenous uveitis, further studies are needed involving patients with BD and other retinal disorders.

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Yamamoto JH, Fujino, Lin, Nieda, Fujii, Masuda


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