INFLAMMATORY DISORDERS

Retinal S-antigen Th1 cell epitope mapping in patients with Behcet's disease

Changlin Zhao · Peizeng Yang · Hao He · Xiaomin Lin · Liping Du · Hongyan Zhou · Aize Kijlstra

Abstract

Background Retinal S-antigen (S-Ag) is a most character- ized autoantigen of autoimmune uveitis. The recognized immunodominant epitope of human S-Ag in patients with uveitis has not been identified. In this study, we selected certain patients with active uveitis to map the Th1 cell epitope spectrum of human S-Ag in Behcet's disease(BD).

Methods Blood samples were taken from eight active BD patients who showed an immune response to 40 mixed overlapping peptides spanning the entire sequence of human S-Ag. Peripheral blood mononuclear cells were isolated and stimulated with single S-Ag peptide at 5 μg/ml or 20 μg/ml. Single-cell immune responses were measured by IFN-γ ELIspot assay.

Results BD patients heterogeneously responded to the S-Ag peptides at two concentrations. In general, the responses to 5 μg/ml peptides were slightly stronger than those to 20 μg/ml peptides, while the maximum SFC frequency to single peptide at the two concentrations was similar. Several peptides including P31, P35 and P40 induced a prominent response, with the frequency of S-Ag specific cells being about 0.007%. Significant reactivity pattern shift was noted in patients with different disease courses.

Conclusions Certain active BD patients have S-Ag specific Th1 cells with a low frequency. The S-Ag epitope specificity between patients is highly heterogeneous, and varies with the uveitis course.

Keywords Behcet's disease · Epitope mapping · Retinal S antigen · Uveitis

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Introduction

Uveitis is a group of clinically heterogeneous intraocular inflammatory diseases often leading to severe impairment of visual acuity. It has been presumed to be mediated by autoreactive $CD4^+$ T cells recognizing specific retinal antigens [1]. The most persuasive evidence stems from the observation that autoantigens such as retinal S-antigen (S-Ag) can induce experimental autoimmune uveitis (EAU), similar to human uveitis clinically and histologi- cally in susceptible animals [2]. Furthermore, oral admin- istration of bovine S-Ag or immunizing rats with human S-Ag-Ig fusion protein can induce immune tolerance, thereby preventing uveitis [3, 4]. Usually a typical immune response against a self protein is focused on one or two

epitopes within that protein [5]. Immunodominant epitopes of human S-Ag have been investigated in a variety of uveitis entities through lymphocyte proliferation assay [6–8]. However, no peptide was found to induce a predominant immune response in all or most responders.

Behcet's disease (BD) is one of the common uveitis entities in China [9], and lymphocytes in certain BD patients proliferated upon S-Ag stimulation [10]. Enzyme-linked immunospot assay (ELIspot) has been shown to be the most specific and sensitive technique for assessing low-frequency antigen specific T cells. In this study, we adopted the ELIspot assay to map the T cell epitope peptide spectrum of human S-Ag to identify the dominant peptides in BD patients.

Materials and methods

Synthetic peptides

Forty peptides spanning the entire length of human S-Ag sequence (pubmed locus P10523) were synthesized by conventional solid-phase chemistry and purified to at least 95% purity (Sangon Biological Engineering Technology & Services Company, Shanghai, China). Each peptide determinant measured 20 amino acids (aa) in length and overlapped the neighboring sequence by 10 aa, except for the last one, which measured 15 aa. The exact sequence and naming of the overlapping peptides are shown in Fig. 1.

Subjects

Eight male BD patients with active uveitis with an average age of 31 years (ranged from 20 to 43 years) were included

Fig. 1 The sequence of 40 overlapping peptides that spanned the entire amino acid sequence of human S-Ag

in this study. The patients met the diagnostic criteria established by the International Behcet's Disease Study Group [11]. All patients showed cellular responses to mixed S-Ag peptides and were positive for human leucocyte antigen (HLA)-B51 in our previous study [12]. Their mean durations of uveitis were 27 months (ranging from 13 to 35 months). All the patients relapsed into panuveitis, manifested by dust-like keratic precipitates, flare and cells in the anterior chamber, hypopyon, vitreous cells and retinal vasculitis as disclosed by fluorescein angiography. Five patients were receiving low-dose prednisone (20 mg/day or less) therapy, and the remaining three BD patients had no systemic treatment at the time of blood sampling. No healthy donors showed positive cellular responses to mixed S-Ag peptide in our previous study; therefore we did not use normal individuals as a control. All procedures followed the tenets of the Declaration of Helsinki and were approved by the Zhongshan Ophthalmic Center Ethics Committee, and blood samples were obtained from the patients after they had given their written informed consent.

IFN-γ ELIspot

Peripheral venous blood (18 ml or 34 ml) was drawn in a heparinized tube, and peripheral blood mononuclear cells (PBMCs) were isolated immediately by Ficoll–Hypaque density gradient centrifugation (Pharmacia Biotech, Shanghai, China). The ELIspot assay kit for IFN-γ was purchased from BD Pharmingen (BD Biosciences, San Diego, CA, USA) and utilised according to the manufacturer's instructions. Briefly, 96-well nitrocellulose plates (Millipore) were coated with 5 μg/ml anti-human ‡IFN-γ capture antibody at 4°C overnight. A total of 2×10^5 fresh PBMCs were added to microwells in duplicate, and incubated with 40 single S-Ag synthetic peptides (5 μg/ml or 20 μg/ml for each peptide) for 20 h in the presence of 1 μg/ml costimulatory anti-CD28 (BD Pharmingen, clone CD28.2) monoclonal antibody (mAb). PBMCs cultured without peptides acted as negative controls. PBMCs stimulated with 10 ng/ml anti-CD3 (BD Pharmingen, clone HIT3a) plus 1 μg/ml anti-CD28 mAbs were used as positive controls. Two μg/ml biotinylated anti-human IFN-γ, streptavidin–HRP at 1:100 dilution and AEC were used as detection antibody, biotinylated detection antibody and substrate reagent respectively. The spots were counted automatically using ImmunoSpot Image Analyzer (Cellular Technology Limited, Cleveland, OH, USA). Digitized images were analyzed for the presence of areas in which color density exceeds background by an amount set on the basis of the comparison of experimental wells and negative control wells. A spot called spot-forming cell (SFC) represented an IFN-γ-producing cell. The number of spots in negative control wells was less than two.

Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was performed using the Mann-Whitney U test and the linear mixed-effects model fit by the maximum likelihood method by statistical software SAS 9.1 (SAS Inc., Cary, NC, USA). A P value of less than 0.05 was considered statistically significant.

Results

### Assessment of cellular immune response to S-Ag peptides by IFN-γ ELIspot

Our preliminary study found that 5 μg/ml of mixed S-Ag peptides was an optimal concentration for ELIspot assay. Therefore, that concentration was used in this study. Since earlier studies showed that 20 μg/ml of S-Ag peptides induced an optimal lymphocyte proliferative responses in patients with uveitis[7, 8], we simultaneously adopted this concentration for ELIspot assay in five out of eight patients. The results revealed highly heterogeneous responses against individual peptides at either concentration in all the patients (Fig. 2a,b). In general, each patient responded to more than one of the peptides in the panel, and the

reactivity of the same peptide varied between patients. Both concentrations of antigens induced a similar SFC frequency with 0.007% ($14/2 \times 10^5$) vs 0.006% ($12/2 \times 10^5$) for the strongest reactive peptide at 5 μg/ml vs 20 μg/ml (Fig. 2c). The maximum frequency of T cell response against single peptide at the two concentrations was the same as that against mixed peptides at 5 μg/ml (0.0065% [$13/2 \times 10^5$]). However, generally speaking, the responses to 5 μg/ml of 40 single peptides were stronger than that to 20 μg/ml of peptides (Fig. 2d). Additionally, the dominant sites of S-Ag at 5 μg/ml obviously localized at the C-terminal region of this protein, whereas that at 20 μg/ml was noted at the N-terminal.

When PBMCs were stimulated with polyclonal anti-CD3 plus anti-CD28, a high frequency of IFN-γ-secreting cells (0.1525% [$305/2 \times 10^5$]) was observed in all patients. It was much higher than that induced by S-Ag peptide ($P<0.01$, $P<0.01$) (Fig. 2c).

Identification of immunodominant T cell epitopes of S-Ag

In view of the highly heterogeneous reactivity spectrum in different individuals, we applied the linear mixed-effects model fit by the maximum likelihood method with the number of cases as the random effect, to analyze the difference in SFC frequency between 40
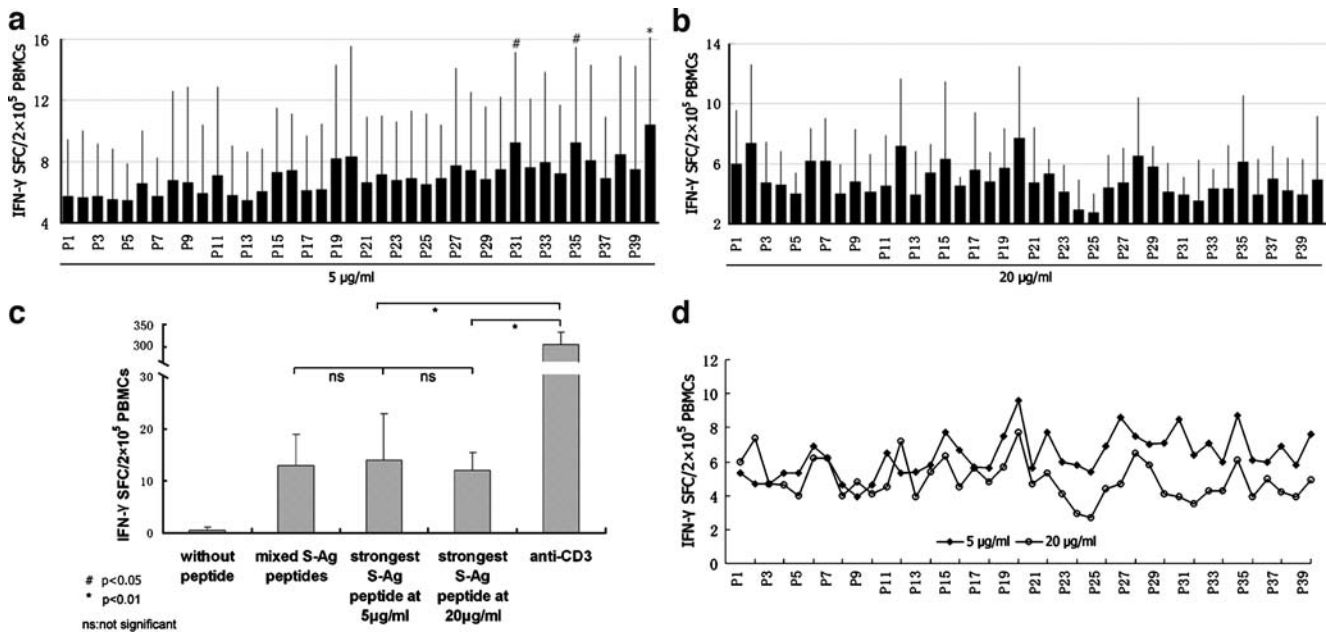
Fig. 2 Mapping of Th1 cell responses to individual S-Ag peptides. PBMCs were cultured with mixed S-Ag peptides at 5 μg/ml, single peptides at 5 or 20 μg/ml and anti-CD3 respectively. IFN-γ-producing cells were determined by ELIspot and expressed as SFC. Data are expressed as means ± SD and *bars* indicate mean values. A statistically significant difference was set at $P<0.05$ (#), $P<0.01$ (*), ns: not significant. **a** Shows the Th1 cell response spectrum of S-Ag peptides at 5 μg/ml ($n=8$). The SFC frequency was analyzed by the linear mixed-effects model fit by the maximum likelihood method, and the dominant responses were marked. **b** Shows the Th1 cell response spectrum of S-Ag peptides at 20 μg/ml ($n=5$). **c** The SFC frequency for stimulation with mixed peptides, strongest single peptide (5 and 20 μg/ml respectively) and anti-CD3 were compared. Statistical analysis was performed using Mann-Whitney U test. **d** The SFC frequency of the single peptides screening at the two concentrations was compared ($n=5$)

peptides at 5 μg/ml. The results showed that the responses to P31 (P=0.013), P35 (P=0.015) and P40 (P=0.001) were significantly stronger than to other peptides.

Shift in peptides recognition in patients with different disease course

To investigate whether the S-Ag peptides response spectrum was influenced by the disease course of uveitis, we divided the eight cases into two groups according to their different duration of uveitis. Group one included three patients with a uveitis course of no more than 2 years; the other included five patients having uveitis for more than 2 years. Their SFC frequency of response to the 40 peptides at 5 μg/ml was compared by linear mixed-effects model fit by the maximum likelihood method. In general, the response in patients with a disease course less than 2 years was stronger than that in patients with a disease course of more than 2 years. The patients with uveïtis duration less than 2 years dominantly recognized P40 (P=0.001). When the uveitis course extended to around 3 years in BD patients, the dominant response to S-Ag peptides varied to P27 (P=0.009), P29 (P=0.049), P30 (P=0.030), and P31 (P=0.001) (Fig. 3).

Discussion

In this study, we applied the ELIspot assay to map the S-Ag Th1 cell epitopes response in a series of BD patients with similar disease characteristics. The results show that the peptides induced a low frequency of S-Ag specific Th1 cells, and that the T cell response against peptides is highly heterogeneous in different individuals. Several dominant peptides including P31, P35 and P40 were observed

statistically in certain patients. The reactivity pattern to the peptides varied in patients with different disease duration.

It has been shown that a greatly heterogeneous T-cell proliferative response spectrum against S-Ag peptides in uveitis may be attributed to variation in disease entities and different stages of disease activity of the examined patients [6–8]. To exclude the above factors in the antigenic peptide mapping, we chose BD patients with a similar intensity of uveitis at the time of blood sampling. In view of the fact that an efficiently processed specific antigen epitope is restricted by major histocompatibility complex (MHC) on the surface of an antigen-presenting cell, and that BD was associated with HLA-B51 in many ethnic groups [13, 14], we chose the patients expressing HLA-B51 as subjects in this study. The result showed that these patients also heterogeneously recognized the S-Ag peptides, suggesting that the S-Ag epitopes may be not associated with HLA-B51.

Although lymphocyte proliferation assay is a classic technique to assess cellular immunity, a non-proliferation measurement to characterize low-frequency antigen-specific T cell in autoimmune disease (AID) is needed to avoid overall clonal expansion and promiscuous T cell populations. The cytokine-specific ELIspot assay is able to display one reactive cell among one million T cells at the single cell level [15], and has been extensively applied to research on autoimmunity in various AID [16, 17]. Because an IFN-γ mediated Th1 response has been implicated in the development of BD [18], we adopted the IFN-γ ELIspot assay to map the S-Ag Th1 cell epitopes in BD patients.

To identify the immunodominant epitope of human S-Ag in uveitis, we used 40 overlapping peptides that are identical to the peptides used in earlier studies in uveitis patients [6–8]. The immune responses in those studies were
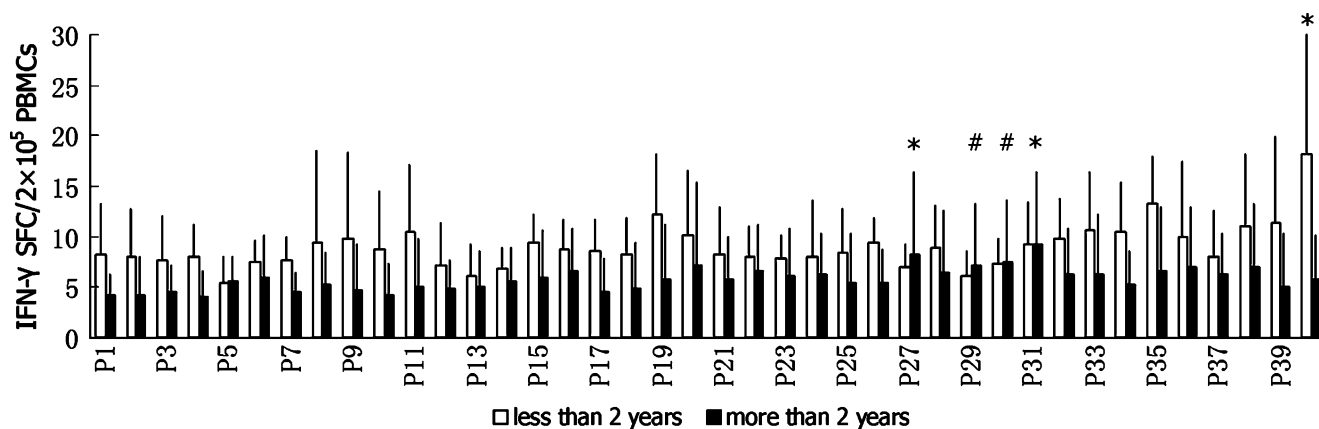
Fig. 3 Shift in S-Ag peptide recognition in patients with different disease courses. According to the different duration of uveitis, the subjects were divided into two groups. Group 1 included patients with a uveitis course of less than 2 years (n=3), group 2 included patients with a disease duration of more than 2 years (n=5). The SFC frequency was analyzed using the linear mixed-effects model fit by the maximum likelihood method

tested by lymphocyte proliferation assay. If a stimulation index (SI) ≥2 was considered a positive reaction, almost all of the S-Ag peptides except for P12 induced a positive response in different patients with various uveitis entities [6–8]. In BD patients, as shown in Table 2 of their paper, de Smet et al. [6] found that the responses to P18 and P40 were significantly higher than those in healthy controls. Similar peptides have been tested in the EAU model for their pathogenicity [19]. Ten peptides induced visible inflammation in the eye, and the most pathogenic sequences were P19, P35 and P36. Our ELIspot assay similarly showed a heterogeneous reactivity pattern in BD patients. The dominant peptide P35 coincided with the major pathogenic epitope in rat, and the prominent P40 was identical with the result of proliferation assays in BD. Therefore, P40 may be the most potential BD-associated S-Ag epitope peptide, and the immunodominant epitopes of S-Ag in uveitis may nest in the amino acid sequence of P35 and P40. However, it needs to be verified using smaller synthetic peptides in more uveitis patients.

The ELIspot assay quantified a low frequency of 0.007% ($14/2 \times 10^5$) of S-Ag specific T cells in the peripheral circulation of BD patients with active uveitis. It was notably lower than that seen in animals with EAU [20]. The low frequency of SFC observed in our study is consistent with the weak proliferative response to S-Ag in uveitis patients [6–8].

Epitope spreading has been considered to be one of the mechanisms contributing to relapses in AID [21]. Deeg et al. [22] found that the epitopes recognized by uveitic horses could spread to different peptides of S-Ag or to the peptides derived from other uveitogenic autoantigens during the uveitic episodes. A shift in the specific proliferative response of S-Ag peptides was observed in two BD patients by testing the responses on two separate occasions[6]. Interestingly, we found that the BD patients with shorter uveitis course reacted more strongly to S-Ag and intensively responded to one dominant peptide, while those with a long disease course responded to others. This phenomenon seem to suggest that autoantigenic epitope spreading may be present in human uveitis.

Our patients showed a stronger response to low-concentration peptides than high-concentration ones, which was different from de Smet's finding [6]. In addition, the dominant responses induces by the two concentration antigens were not coincident. The discrepancy between the two concentration peptides may be due to different antigen doses affecting the development of T-cell repertoire to a given antigen in vitro, in compliance with the different avidity of T-cell-receptor for specific MHC-peptide complex [23]. We speculated that the 5 μg/ml peptides preferred inducing a immune response mimicking the in vivo condition.

In summary, this is the first report concerning the analysis of T cell immunity against S-Ag by ELIspot assay wherein the S-Ag Th1 cell epitope response is measured in one uveitis entity. The peptides recognition pattern varied between BD patients with a mild reactive intensity. The responses to the dominant peptides and a possible shift reactivity pattern should be verified in more BD patients.

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