Experimental and Molecular Pathology **70,** 140–145 (2001)
doi:10.1006/exmp.2000.2338, available online at http://www.idealibrary.com on IDEAL®

Human Retinal S-Antigen: T Cell Epitope Mapping in Posterior Uveitis Patients

G. Rai, S. Saxena,* H. Kumar,† and V. K. Singh[1]

*Laboratory of Molecular Immunology, Department of Immunology, Sanjay Gandhi Post-Graduate Institute of Medical Sciences, Lucknow 226 014; *Department of Ophthalmology, King George's Medical College, Lucknow 226 003; and †Vivekanand Polyclinic, Lucknow 226 020, India*

Received August 4, 2000

Uveitis, an intraocular inflammatory disease which affects the uveal tract and the retina of the eye in humans, is one of the major causes of visual impairment. Posterior uveitis is often associated with inflammation of the retina and vitreous. Unfortunately, etiological diagnosis of the disease is not possible in the majority of patients. It is generally felt that an autoimmune mechanism may be involved in so-called idiopathic cases. The role of retinal S-antigen, its 20 linear peptides spanning the entire sequence, and 2 additional peptides, known to be uveitopathogenic in experimental animals, was studied in 26 patients with uveitis. Lymphocyte proliferative response was tested *in vitro* to identify the epitopes of S-antigen involved and to establish their role in the pathogenesis of uveitis. Of 26 uveitis patients tested, 11 showed a significant T cell proliferative response *in vitro* to at least 1 antigen used. None among the controls showed any response to the peptides or native S-antigen used in this study. We have found that uveitis patients respond most frequently to peptide 4 (61–80), peptide 5 (81–100), peptide 8 (141–160), peptide 9 (161–180), peptide 12 (221–240), and peptide 13 (241–260) of the human S-antigen. These results further confirm that autoimmunity to retinal S-antigen may play a role in the etiopathogenesis of a subset of patients with idiopathic uveitis.

Key Words: lymphocyte proliferation; retinal S-antigen; T cell epitope; uveitis.

INTRODUCTION

Inflammation of the uveal tract is termed uveitis. It predominantly affects the choroid, ciliary body, and iris. However, because of close proximity, adjoining retina, vitreous, and sclera may also be affected (Nussenblatt and Gery, 1996). The disease is further subdivided by the anatomic location of primary pathology into anterior uveitis, intermediate uveitis, posterior uveitis, or panuveitis (Bloch-Michel and Nussenblatt, 1987). Posterior uveitis is a common cause of ocular morbidity and may lead to loss of vision (Nussenblatt *et al.,* 1996). The etiological agent is not evident in the majority of patients with uveitis and is labeled idiopathic in nature (Suttorp-Schulten and Rothova, 1996). It has been proposed that the T cell-mediated autoimmune response to retinal antigens may play a role in the causation of idiopathic uveitis. This hypothesis is based on the observations that several retina-specific antigens induce uveal inflammatory changes in susceptible animal strains which resemble the disease in humans (Faure, 1980; Nussenblatt *et al.,* 1981; Singh *et al.,* 1998). The disease can be transferred through sensitized $CD4^+$ T cells to naive animals (Mochizuki *et al.,* 1985; Singh and Nagaraju, 1996).

Retinal S-antigen, the most characterized retinal autoantigen, is a 45-kDa glycoprotein (Wacker *et al.,* 1977). It is highly conserved among mammalian species (Shinohara *et al.,* 1992; Singh and Nagaraju, 1996). Experimental studies have identified major uveitopathogenic epitopes of S-antigen in animals (Donoso *et al.,* 1987; Singh *et al.,* 1988, 1989, 1998; Gregerson *et al.,* 1990; de Smet *et al.,* 1993). A lymphoproliferative response to retinal S-antigen has been reported in patients with uveitis (Nussenblatt *et al.,* 1980,

[1]To whom correspondence and reprint requests should be addressed. Fax: 91–522–440017/440973. E-mail: vksingh@sgpgi.ac.in.

0014-4800/01 $35.00

1982; Doekes *et al.,* 1987; de Smet *et al.,* 1990; Rajasingh *et al.,* 1996). Earlier investigators have shown a significant T cell response to few synthetic peptides of S-antigen as well (de Smet *et al.,* 1990; Hirose *et al.,* 1990; Nityanand *et al.,* 1993; Rajasingh *et al.,* 1996). But it is not known whether these are dominant epitopes in humans. In the present study, we have investigated the cellular immune response to native S-antigen, as well as to 20 linear peptides spanning the entire sequence of the S-antigen, and 2 additional peptides known to be uveitopathogenic in susceptible animal strains.

MATERIALS AND METHODS

Antigens. Antigens used in the assay included native bovine S-antigen, 20 linear synthetic peptides spanning the entire sequence, and 2 additional uveitopathogenic peptides (G and GL-11) derived from the human S-antigen sequence (Yamaki *et al.,* 1988, 1990). Bovine S-antigen was purified from retinal tissue as described earlier (Wacker *et al.,* 1977). The linear peptides were synthesized by Chiron Technologies (Clayton Victoria, Australia) by conventional solid phase chemistry on a benzhydrylamine resin. For these peptides the amino acid sequence and positions in human retinal S-antigen are given in Table 1. Peptides G and GL-11 were synthesized as described earlier (Sunil *et al.,* 1993).

TABLE 1

Peptide Sequences and Their Amino Acid Position in Human Retinal S-Antigen

Peptide No.	Sequence	Amino acid position
1	MAASGKTSKSEPNHVIFKKI	1–20
2	SRDKSVTIYLGNRDYIDHVS	21–40
3	QVQPVDGVVLVDPDLVKGKK	41–60
4	VYVTLTCAFRYGQEDVDVIG	61–80
5	LTFRRDLYFSRVQVYPPVGA	81–100
6	ASTPTKLQESLLKKLGSNTY	101–120
7	PFLLTFPDYLPCSVMLQPAP	121–140
8	QDSGKSCGVDFEVKAFATDS	141–160
9	TDAEEDKIPKKSSVRYLIRS	161–180
10	VQHAPLEMGPQPRAEATWQF	181–200
11	FMSDKPLHLAVSLNREIYFH	201–220
12	GEPIPVTVTVTNNTEKTVKK	221–240
13	IKACVEQVANVVLYSSDYYV	241–260
14	KPVAMEEAQEKVPPNSTLTK	261–280
15	TLTLLPLLANNRERRGIALD	281–300
16	GKIKHEDTNLASSTIIKEGI	301–320
17	DRTVLGILVSYQIKVKLTVS	321–340
18	GFLGELTSSEVATEVPFRLM	341–360
19	HPQPEDPAKESIQDANLVFE	361–380
20	EFARHNLKDAGEAEEGKRDKNDADE	381–405
21(G)	GELTSSEVATEVP	344–356
22(GL-11)	LTSSEVATEVP	346–356

Note. The single-letter amino acid code is used for the sequence. The amino acid position in the human retinal S-antigen has been given.

Subjects. Twenty-six uveitis patients (20 males and 6 females) were selected for the study (numbered 1 to 26). These patients were referred by various ophthalmologists in Lucknow. Informed consent was obtained from all patients prior to their inclusion in the study. There was no bias for inclusion with respect to their current medical therapy or degree of disease activity. The patients' ages ranged between 17 and 47 years with a mean of 31 $\pm$ 9.3 years (median 30 years). The classical signs of uveitis, viz, circumcilliary congestion, loss of luster and pattern of iris, irregularity of pupil, posterior synechiae, and pigment on lens surface, were present in most of the patients. In 3 patients the disease was of acute onset, in 3 subacute, and in 20 chronic. Ten patients had choroiditis, 1 had pars planitis, and 1 patient was diagnosed to be suffering from Behçet's disease. Three patients had ankylosing spondylitis and 1 had polyarticular juvenile chronic arthritis. In 12 patients fundus examination could not be done due to associated secondary cataract, vitreous haze, opacity, pigmented lens, and cells in the vitreous chamber. In 2 cases fine, old, keratic precipitates (Kps) were visible on slit lamp examination, and in 2 others large mutton fat Kps were seen. Of these 4 cases, 3 had cells in the anterior chamber and/or flare. One case had a history of ocular trauma preceding the onset of uveitis. One patient showed a broken synechial ring and 3 had posterior synechiae. Pupillary reaction was present in 7 patients. In one patient fundus examination showed deep excavation in the optic nerve region (glaucomatous cupping). One patient had been treated for pulmonary tuberculosis. Two cases showed delayed hypersensitivity response to purified protein derivative (PPD). One patient showed increased branchovascular markings on chest X rays and another showed signs of sacroilitis on both sides. The patients were included in the study irrespective of their current medical therapy. Six normal healthy volunteers from the laboratory who did not handle the retinal S-antigen or the peptides served as the control group.

Lymphocyte proliferation assay. Mononuclear cells were separated from peripheral blood on a lymphoprep (Nycomed As, Oslo, Norway) gradient. Cells were cultured in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 25 mM Hepes (Sigma), L-glutamine (2 mM), penicillin (100

U/ml), streptomycin (100 μg/ml), and 10% heat-inactivated fetal calf serum (Biological Industries, Kibbutz Beit Haemek, Israel). The cells were cultured as described earlier (Bajpai *et al.,* 1995). Briefly, 1×10^5 cells/well in a total volume of 200 μl in 96-well U-bottom plates (Tarson, Calcutta, India) were set up in triplicate with or without stimulants. The stimulants included PPD (Span Diagnostics, Bombay, India; 20 μg/ml), native S-antigen (20 μg/ml), and various peptides (20 μg/ml). Earlier dose–response studies with various retinal antigens/peptides had shown an optimal response to 20/40 μg/ml. Hence, in all experiments reported here, 20 μg/ml dose of the peptide was added to the test wells. The cultures were incubated at 37°C for a total of 5 days in a CO_2 incubator containing 5% CO_2 and 100% humidity. The cultures were pulsed with 0.5 μCi [^{3}H] thymidine (6 Ci/mmol, BARC, Bombay, India) per well during last 18 h of incubation and harvested on glass fiber filters (Advance Microdevice Pvt. Ltd., Ambala, India) using a multiharvester (Skatron, Lier, Norway).

Thymidine uptake was determined by liquid scintillation counter (LKB, Fullerton, CA). The mean count per minute (CPM) of the triplicate cultures was calculated for each set of replicate cultures. Standard deviation (SD) of the mean CPM of triplicates was routinely $<$20%. The stimulation index (SI) was derived by dividing the mean CPM for each of the antigen-stimulated cultures by the mean CPM of the control cultures to which no antigen was added. An SI of 2 and above was taken as positive in this study. Results are presented both as CPM $\pm$ SD and as SI.

Statistical analysis. Data were analyzed using statistical software SPSS 9.0. The significance of the T cell proliferation response was assessed by *Z* test for proportions. *P* values $\leq$0.05 were considered significant.

RESULT AND DISCUSSION

Eleven of 26 patients ($\sim$46%) with uveitis showed a significant lymphocyte proliferative response against S-antigen or at least one of its fragments (Tables 2 and 3). None among 6 healthy volunteers (data not presented) showed any significant proliferative response (SI more than 2) to retinal S-antigen or peptide. We have tested 49 healthy volunteers for a T cell proliferative response to native S-antigen and different synthetic peptides of S-antigen in previous studies. No normal individual has been found to show a significant T cell proliferative response (SI $>$2) to S-antigen or its synthetic peptides. Compared to controls, the frequency of responders in the patient group was again significant (P = 0.049). Important clinical features of 11 uveitis patients showing response to S-antigen peptides are given in Table 2. This table also shows peptides against which these patients have shown response.

Of the 11 responders, 10 were males and 1 was female. Among responders, none showed a response to native S-antigen. Two patients responded against peptide 1, 1 against peptide 3, 5 against peptide 4, and 4 against peptide 5. There was 1 responder against peptide 6, 2 against peptide 7, and 4 each against peptides 8 and 9. One patient showed a significant response to peptide 11 and four each showed a response to peptides 12 and 13, while 2 patients each responded to peptides 15 and 16. There was 1 responder to peptide 20 and 2 each to peptides G and GL-11.

Observations from experimental autoimmune uveitis as well as other models of autoimmune diseases have clearly demonstrated that the immune systems of different species respond to different epitopes of a given antigen. Cell-mediated and humoral responses are invariably directed against different parts of the molecule. Several previous studies demonstrated that cellular proliferative responses to S-antigen were present under inflammatory conditions affecting the posterior pole, and in particular the retina of patients with uveitis (Nussenblatt *et al.,* 1980,1982; Doekes *et al.,* 1987; de Smet *et al.,* 1990; Rajasingh *et al.,* 1996). We examined the cell-mediated responses of patients with uveitis to various epitopes of S-antigen. This is the first report where all 20 linear peptides spanning the entire sequence of human S-antigen have been used to map the epitope using lymphocytes of uveitis patients.

In earlier studies only a few peptides of S-antigen have been used (de Smet *et al.,* 1990; Hirose *et al.,* 1990; Nityanand *et al.,* 1993; Rajasingh *et al.,* 1996). de Smet et al. (1990) have studied the cellular response of uveitis patients from the United States and Japan to bovine S-antigen and peptides N and M *in vitro* and found that a significant number of patients respond to these peptides. In their study, of 47 U.S. patients, 17 (36%) showed a positive response to peptide M, compared to 6 of the 35 (17%) Japanese patients. Six of the 8 U.S. patients with Behçet's disease responded to peptide M, in comparison to 2 of the 16 Japanese patients. In the case of Vogt Koyanagi-Harada syndrome, 4 of 9 U.S. patients and only 1 of 10 Japanese patients responded to peptide M. Their results suggest that there is a wide variation between U.S. and Japanese uveitis patients as far as *in vitro* T cell proliferation response to S-antigen peptide is concerned. Hirose *et al.* (1990) studied 21 U.S. patients of uveitis, of which 4 had anterior, 2 intermediate, and 15 posterior disease. Five of 15 posterior uveitis patients showed a positive

TABLE 2
Important Features of Uveitis Patients Showing a Significant T Cell Response *in Vitro*

Sr. No.	Patient No.	Important clinical features	Peptides (No.) showing response
1	3	Acute, macular degeneration	3, 4, 5, 7, 12, GL-11
2	4	Chronic, ankolysing spondylitis, signs of sacroilitis, reduction of joint spaces	4
3	5	Chronic, pupillary reaction, cataract, secondary glaucoma	1, 5, 7, 8, 9, 11, 15, 16, 20, G
4	8	Subacute, pupillary reaction, superiotemporal blood vessel occlusion with macular hemorrhage, PPD positive	G
5	11	Chronic, festooned pupil, pars planitis, history of pulmonary tuberculosis	4, 5, 8, 9, 13, 15
6	12	Chronic, flare present, pigmented lens	8, 9, 12, 13
7	13	Chronic, mutton fat Kps, cells in vitreous chamber	4, 5, 8, 9, 12, 13, 16
8	16	Chronic, posterior synechiae, pupillary reaction, pigmented lens, PPD positive	13
9	17	Acute, PPD positive	1
10	19	Chronic, both anterior and posterior uveitis, Behçet's disease, ankolysing spondylitis, PPD positive	4, 6, 12
11	24	Chronic, fine Kps, pigmented lens, cells in vitreous chamber	GL-11

response to S-antigen, with 3 of them also responding to peptide M. Nityanand *et al.* (1993) evaluated the lymphoproliferative response of Indian uveitis patients against peptide M and observed that 12 of 39 patients had significant proliferation against peptide M. Rajasingh *et al.* (1996) evaluated 38 patients with peptides G and M and 7 of them responded to at least one retinal antigen used in that study.

Recently, $CD4^+$ T cell lines from uveitis patients with specificity toward native S-antigen was generated to identify S-antigen peptide determinants (Soylu *et al.,* 1998). Proliferative responses of the cell lines were tested against 39 overlapping peptides that span the entire sequence of human S-antigen using autologous EBV-transformed B cells as antigen-presenting cells. Their cell lines recognized a small number of peptides, with individual profiles of specificity being exhibited by each line. They have observed a response against peptides 2, 7, 8, 10, 14, 16, 17, and 18 and overlapping fragments of peptides 8 and 9. In our study also, we have found a response against peptides 7 (2 patients), 8 (4 patients), and 16 (2 patients). We have 4 patients showing a response to both peptides 8 and 9. This is important since the above study has also shown a response against overlapping fragments of 8 and 9 in addition to a response against peptide 8. Similarly, 3 patients have shown a response to both peptides 3 and 4. Although no common pattern of specificity was observed among the responders, several peptides were recognized in multiple individuals.

Furthermore, we have evaluated the S-antigen antibody by ELISA in sera samples of these patients, which show that two uveitis patients (Nos. 5 and 12) have very high levels of S-antigen antibody (data not shown). There was a significant difference between the mean OD of the control group compared to the mean OD of uveitis patients ($P < 0.01$).

We have also analyzed the frequency of T cell proliferative response *in vitro* with individual peptides in uveitis patients. Peptide 4 has shown a response with lymphocytes of five patients ($P < 0.01$). Peptides 5, 8, 9, 12, and 13 had shown a response in four uveitis patients each ($P < 0.037$). In our study it is apparent that one patient shows *in vitro* lymphocyte proliferation against more than one peptide of S-antigen. Patient No. 5 in our study has shown a response against 10 peptides. de Smet *et al.* (1990) have also pointed out in their study that there are major differences between the experimental model and naturally occurring human autoimmune diseases. Patients are able to respond to more than one fragment of S-antigen while the animals respond only to that antigen against which they have been immunized. Our result further confirms that retinal S-antigen may play a role in the etiopathogenesis of idiopathic uveitis and its different epitopes may be involved in different subsets of patients. Such studies may encourage the development of treatment strategies using these molecules and other fragments.

ACKNOWLEDGMENTS

We express our thanks to Drs. J. P. Gupta, Rajat Dhesi, Shobhit Chawla, and Deepak Kumar for providing uveitis patients and to Drs. Vikas Agarwal, Praveen Hissaria, and Satyawati Deswal for help in the clinical evaluation of patients. We are thankful to Dr. C. M. Pandey, Department of Biostatistics, for statistical analysis. The study was

TABLE 3
Lymphocyte Proliferative Response of Uveitis Patients to S-Antigen Peptides

Peptide No. ↓ / Patient No. →	Extent of proliferation for patients showing positive response										
	3	4	5	8	11	12	13	16	17	19	24
Control	1433 ± 265	3243 ± 110	2655 ± 276	406 ± 27	155 ± 11	148 ± 0	137 ± 7	4356 ± 898	268 ± 24	844 ± 101	2688 ± 191
1			7050 ± 618 (2.6)						620 ± 7 (2.3)		
3	3040 ± 43 (2.1)										
4	3698 ± 121 (2.5)	7980 ± 375 (2.4)			547 ± 27 (3.5)		328 ± 10 (2.4)			1847 ± 299 (2.1)	
5	3017 ± 793 (2.1)		7991 ± 1199 (3.0)		429 ± 29 (2.7)		326 ± 28 (2.4)				
6										1734 ± 171 (2.0)	
7	2894 ± 259 (2.0)		6209 ± 1445 (2.3)								
8			5380 ± 199 (2.0)		448 ± 19 (2.8)	1376 ± 165 (9.2)	427 ± 8 (3.1)				
9			6446 ± 514 (2.4)		360 ± 22 (2.3)	1568 ± 333 (10.5)	388 ± 14 (2.8)				
11			7896 ± 714 (2.9)								
12	3342 ± 83 (2.3)					1313 ± 94 (8.8)	365 ± 63 (2.7)			1720 ± 42 (2.0)	
13					436 ± 60 (2.8)	1772 ± 23 (11.9)	540 ± 78 (4.0)	8968 ± 535 (2.0)			
15			7595 ± 248 (2.8)		413 ± 162 (2.6)						
16			8780 ± 653 (3.3)				355 ± 7 (2.6)				
20			6968 ± 1039 (2.6)								
G			7434 ± 67 (2.8)	866 ± 173 (2.1)							
GL-11	2914 ± 432 (2.0)										6197 ± 427 (2.3)

Note. Values are given in CPM ± SD for patients showing a positive response (SI ≥ 2). Actual SI values are given in parentheses.

supported by a research grant of the Indian Council of Medical Research (9301100), New Delhi, to VKS. GR is a senior research fellow. The laboratory infrastructure was provided by a JICA grant-in-aid to the SGPGI project.

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