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The effect of retinal S-antigen-specific monoclonal antibody therapy on experimental autoimmune uveoretinitis (EAU) and experimental autoimmune pinealitis (EAP)

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SUMMARY

We induced an autoimmune uveitis and pinealitis in Lewis rats by inoculating them with bovine S-antigen. This type of uveitis forms a useful experimental model of human chronic intra-ocular inflammation. Induction of experimental autoimmune uveitis (EAU) but not of experimental autoimmune pinealitis (EAP) could be prevented by the administration of S-antigen-specific monoclonal antibody simultaneously with the S-antigen. Inhibition of EAU was associated with significantly raised levels of anti-S antibodies during the first 2 weeks post-immunisation. Immunocytochemical staining for lymphocyte subsets, monocytes and macrophages showed that eyes of monoclonal antibody treated animals contained no immunocompetent inflammatory cells unless they also had clinical signs of inflammation. In contrast, the inflammatory exudate in the pineal glands of both treated and untreated animals contained equal numbers of infiltrating lymphocytes and monocytes in the same relative proportions. These results indicate that the inhibitory effect of the monoclonal antibody S2.4.C5 may be directed towards the effector arm of the immune-mediated cytotoxic response.

Keywords autoimmune uveitis pinealitis S-antigen monoclonal antibody

INTRODUCTION

Posterior uveitis is an ill-defined recurrent or chronic, progressive inflammatory eye disease that frequently affects young adults and in many instances leads to permanent visual impairment. Experimental autoimmune uveitis (EAU) induced by retinal S-antigen (Wacker & Lipton, 1965; de Kozak *et al.*, 1976; Forrester & Borthwick, 1984) is a good animal model of such chronic intra-ocular inflammation. This model has been used to study the pathology and pathogenesis of chronic uveitis (de Kozak *et al.*, 1978, 1981; Forrester, Borthwick & McMenamin, 1985) and, more recently, attempts have been made to control the inflammation by using monoclonal antibodies specific for S-antigen (de Kozak *et al.*, 1985, 1987). Besides uveitis, S-antigen is also known to induce an inflammatory reaction in the pineal gland (Kalsow & Wacker 1977, 1978; Mochizuki *et al.*, 1983). De Kozak *et al.* (1985) showed that the induction of EAU could be inhibited by the administration of an S-antigen-specific monoclonal antibody, simultaneously with the S-antigen. They used a mouse anti-S monoclonal antibody in Lewis rats. Later, de Kozak *et al.* (1987) showed that EAU could also be prevented by active immunization with autoantigen-specific monoclonal antibodies prior to challenge with S-antigen. In this heterologous system, it was suggested that the immunogenic and uveitogenic effects of S-antigen were inhibited via anti-idiotypic network responses. In the present study we have used a homologous system wherein we inoculated Lewis rats with a rat anti-S monoclonal antibody, S2.4.C5 (Reid *et al.*, 1987). We have observed that S2.4.C5 is effective in inhibiting S-antigen-induced EAU but not EAP. This suggests that anti-idiotypic mechanisms are not implicated in inhibiting EAU. Alternative mechanisms acting at the level of the blood-retinal barrier may be important.

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MATERIALS AND METHODS

Bovine retinal S-antigen

Bovine retinal S-antigen was prepared as described previously (Al-Madhawi, Forrester & Lee, 1987) by ion-exchange chromatography on DEAE-Sephacryl. Later samples were prepared by HPLC on a TSK-DEAE column; the antigen was shown to be homogeneous by silver staining on SDS polyacrylamide gel using the Pharmacia Phast system according to manufacturer's instructions.

Table 1. Immunization protocol

Group	No. of Animals	S-antigen* (μg)	FCA† (ml)	Pertussis‡ (ml)	Anti-S§ Monoclonal (ml)	Rat IgG¶ (ml)
1	10	50	0·25	1	1**	0
2	6	50	0·25	1	0	0
3	6	50	0·25	1	0	1

* Bovine S-antigen (200 μg/ml in phosphate-buffered saline (PBS) pH 7·4) prepared by the method of Al-Mahdawi *et al.* (1987) using an HPLC system (Pharmacia).

† Freund's complete adjuvant, H37Ra (Difco). The S-antigen and adjuvant were mixed and injected into the foot pad.

‡ *B. pertussis* organisms, suspension of 10^{10} cells/ml in PBS injected intraperitoneally.

§ Rat anti-S (Bovine) monoclonal antibody, S2.4.C5., IgG_1, 1 mg/ml in PBS given by intraperitoneal injection. Prepared from a hybridoma cell line kindly supplied by D.M. Reid.

¶ Non-specific rat IgG (Sigma), 1 mg/ml in PBS given intraperitoneally.

** The monoclonal antibody was purified by passage through a QAE sephadex column. For three animals in this group it was further purified through a protein A column.

Table 2. Monoclonal markers used (Serotec)

W3-25	(CD4)	T-helper, macrophages, thymocytes
OX8	(CD8)	T-supressor/cytotoxic, NK cells
OX19	(CD5)	Pan-T cells
OX44		Pan-T cells
MARD3		B cells, (anti-IgD heavy chain)
OX39		Interleukin-2 receptor
OX6		DR/Ia Monomorphic, (MHC class II)
ED1		Monocytes and macrophages

Monoclonal antibody to bovine S-antigen

A rat cell-line, S2.4.C5, was used to produce monoclonal antibody to bovine S-antigen (Reid *et al.*, 1987). Antibody was collected as cell supernatant and purified using established methods on QEAE Sephadex. Some samples were further purified by affinity chromatography on protein-A Sepharose, and shown to contain over 99% IgG by SDS-PAGE (Pharmacia Phast system).

Immunization Schedule

Twenty-two female Lewis rats, 8–10 weeks old and weighing 180–250 g each, were divided into three groups (Table 1). The eyes of all animals were examined by slit lamp biomicroscopy to exclude any pathology. The animals were then immunized as described in Table 1. The immunizing agents were given in a single dose as indicated. All animals were tail bled 30 to 40 min after inoculation and approximately 0·5 ml of blood collected. Serum was obtained from these blood samples and stored at −70°C.

Clinicopathological assessment

After immunization the rats were observed daily and clinical examination of the eyes with the slit lamp and direct ophthalmoscopy was carried out using one drop of 1% cyclopentolate in each eye to produce mydriasis. The day of onset of uveitis, its severity and its course were noted. The severity of the inflammatory activity was graded on a scale of 0–4 as described by Forrester *et al.* (1985). All animals were tail bled at weekly intervals until killed. Serum was separated from the blood samples and stored at −70°C.

Table 3. Clinical severity of uveitis

	GROUP*		
	1	2	3
Total number of animals	10	6	6
Number with uveitis	2†	5	5
Severity (mean)	1†	3	2
Day of onset	19	14	19

* 1.S2.4.C5-treated; 2. Untreated; 3. Rat IgG-treated.

† $P = < 0{\cdot}01$. (Chi square test).

The animals were killed at various time intervals, mostly around the peak of the inflammatory activity or soon thereafter. Animals that did not show any clinical disease were killed 28 to 30 days post-immunization. Under ether anaesthesia blood was collected by cardiac puncture and the animal killed by cervical dislocation. Both eyes and the pineal gland were dissected and removed. One eye from each animal and 50% of the pineal glands from each group were snap-frozen in tissue-TEK, OCT compound (Miles Scientific) by immersing in liquid arcton, which in turn had been brought near its freezing point by liquid nitrogen. The other eye and the remaining 50% of the pineals were fixed in 3% phosphate-buffered glutaraldehyde and processed for electron microscopy.

Immunocytochemistry

Frozen sections (5–6 μm thick) of the snap-frozen tissues were cut and spread onto chrome-alum gelatin coated glass slides. Sections from each tissue were stained by haematoxylin and eosin and each of the eight monoclonals listed in Table 2 using the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique.

Rabbit anti-mouse immunoglobulins (Dakopatts) were used as the secondary antibody and APAAP (mouse) complexes (Dakopatts) as the tertiary antibody. Semi-thin sections of the glutaraldehyde fixed tissues, stained with touilidine blue and basic fuschin, were also used for histopathological study.

ELISA assay

The serum samples were tested for anti-S antibodies using an enzyme-linked immunosorbent assay (ELISA). Microtitre 96-well plates were coated with bovine S-antigen (1 μg/ml in 0·02 M Tris/HCl buffer, pH 9·0), blocked with 0·5% bovine serum albumin, then incubated with the test sera. Bound antibodies were detected using peroxidase-conjugated rabbit anti-rat immunoglobulins (Dakopatts); *o*-phenylenediamine (Sigma, 0·4 mg/ml) in citrate (0·1 M) phosphate (0·2 M, pH 6·0) buffer was used as substrate for the peroxidase enzyme. The intensity of the

colour reaction was read at A490 with the aid of the Minireader II (Dynatech). Tris (0·2 M)/NaCl (0·2 M)/Tween 20 (0·05%)/HCl buffer, pH 7·4, was used as the wash buffer.

RESULTS

Clinical Uveitis

Untreated rats immunized with 50 μg of bovine retinal S-antigen in Freund's complete adjuvant and intraperitoneal pertussis antigen developed a severe, early onset uveitis involving the entire globe (anterior and posterior segments) in five of six animals (Table 3, Group 2). Clinically this was manifest as a hypopyon uveitis with obscuration of fundal view at its peak of inflammation. Similarly, five of six rats treated with non-specific IgG developed a marked uveitic response, although this was slightly reduced in severity with a delayed onset. In contrast, only two of 10 rats treated with the rat monoclonal antibody S2.4.C5 developed uveitis, in each case it was also delayed in onset and of least severity.

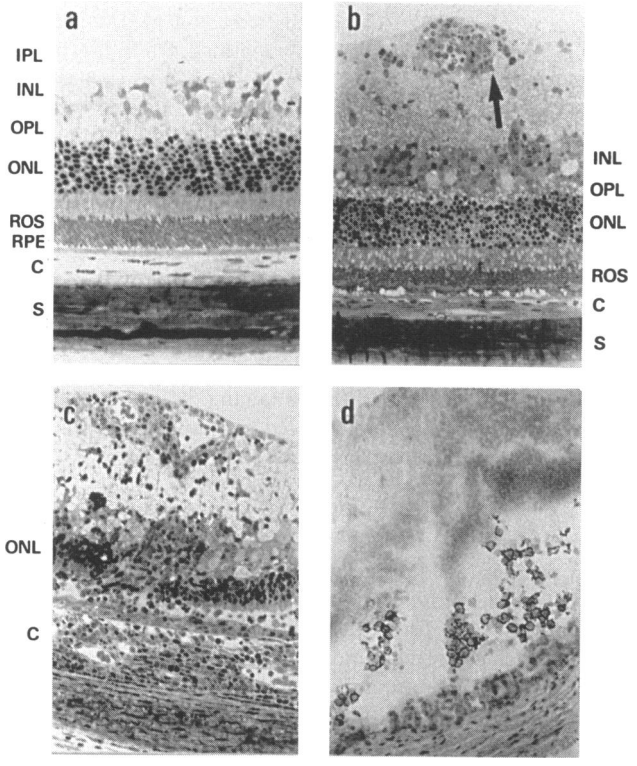

Fig. 1. Ocular changes. (a) Normal retina in an animal from Group 1 treated with anti-S monoclonal S2.4.C5. The rod outer segments (ROS) are well preserved. IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium; C, choroid; S, sclera. (Toluidine blue and basic fuschin, original magnification ×960). (b) Early lesion. Showing perivascular infiltration with inflammatory cells (arrow) and vacuolation and disruption of the photoreceptor layer. (Toluidine blue and basic fuschin, original magnification ×800). (c) Advanced lesion. Showing perivascular infiltration, focal granuloma in the outer nuclear layer (ONL), loss of photoreceptor layer with subretinal infiltration and a thickened choroid (C) packed with inflammatory cells. (Toluidine blue and basic fuschin, original magnification, ×640). (d) Subretinal exudate showing predominantly cells that are stained by the monocyte/macrophage monoclonal marker (ED1). Note absence of photoreceptor layer. (Alkaline phosphatase-anti-alkaline phosphatase (APAAP), original magnification ×400).

Histopathology

Ocular inflammation. Eyes from S-antigen-immunized rats treated with the monoclonal antibody S2.4.C5 which were clinically not inflamed (80%) showed no evidence of inflammatory cell infiltration (Fig. 1a). In contrast, eyes from untreated rats or rats treated with non-specific IgG demonstrated multiple focal inflammatory lesions which varied in severity from perivasculitis with early round cell infiltration in the photoreceptor layer (Fig. 1b), to extensive granulomatous infiltration of the retina and choroid with loss of photoreceptor cells (Fig. 1c) as described previously for Lewis rats (de Kozak *et al.* 1981). In most cases retinal damage was severe and was characterized by a cellular infiltrate consisting of monocytes, macrophages, lymphocytes and a few neutrophils. Similar cells infiltrated the choroidal layer. Immunocytochemical staining of the cell infiltrate indicated that a high proportion of the cells were monocytes/macrophages (Fig. 1d) and CD4 positive cells. CD8 positive cells were less common, while there were few B cells. The proportions of cells were the same irrespective of the site of the lesion eg. around retinal vessels, in the photoreceptor layer or in the choroid. Many cells in the focal lesions expressed Ia antigen but were negative for interleukin 2 receptor.

Pineal inflammation. Pinealitis was present in all three groups of S-antigen immunized rats whether untreated or treated with either rat IgG or the monoclonal antibody S2.4.C5 (Fig. 2b–c). Pinealitis in all groups occurred as a mild to moderate subcapsular focal granuloma, frequently close to the pineal stalk. Perivasculitis was a common feature and the predominant cells were lymphocytes and monocytes. Immunocytochemically, CD4 positive cells were scattered throughout the gland (Fig. 2d) with monocytes and CD8 positive cells next in frequency. A few B cells were also noted. The relative proportions of cells in inflammatory lesions was the same for all three groups of rats. Ia antigen expression was a common feature, as expected, but cells expressing interleukin-2 receptor were not observed.

Humoral response to S-antigen

Untreated and IgG-treated rats which were immunized with bovine S-antigen showed a progressive increase in anti-S antibodies by ELISA between 7 and 21 days after immunization (Fig. 3). Rats treated with monoclonal antibody S2.4.C5 consistently showed higher levels of anti-S antibodies in their serum than untreated or IgG treated controls (Fig. 3). After 21 days antibody levels were maximal for all three groups.

DISCUSSION

Modulation of the host immune response by the use of drugs such as steroids, azathioprine, cyclophosphamide and, more recently, cyclosporin, has proved to be effective in the supression of both clinical and experimental uveitis. Other regimes such as irradiation, anti-lymphocyte globulin and thoracic duct drainage have also been shown to facilitate the induction of immune tolerance (Shellam, 1969; Weigle, 1973). Presently the use of

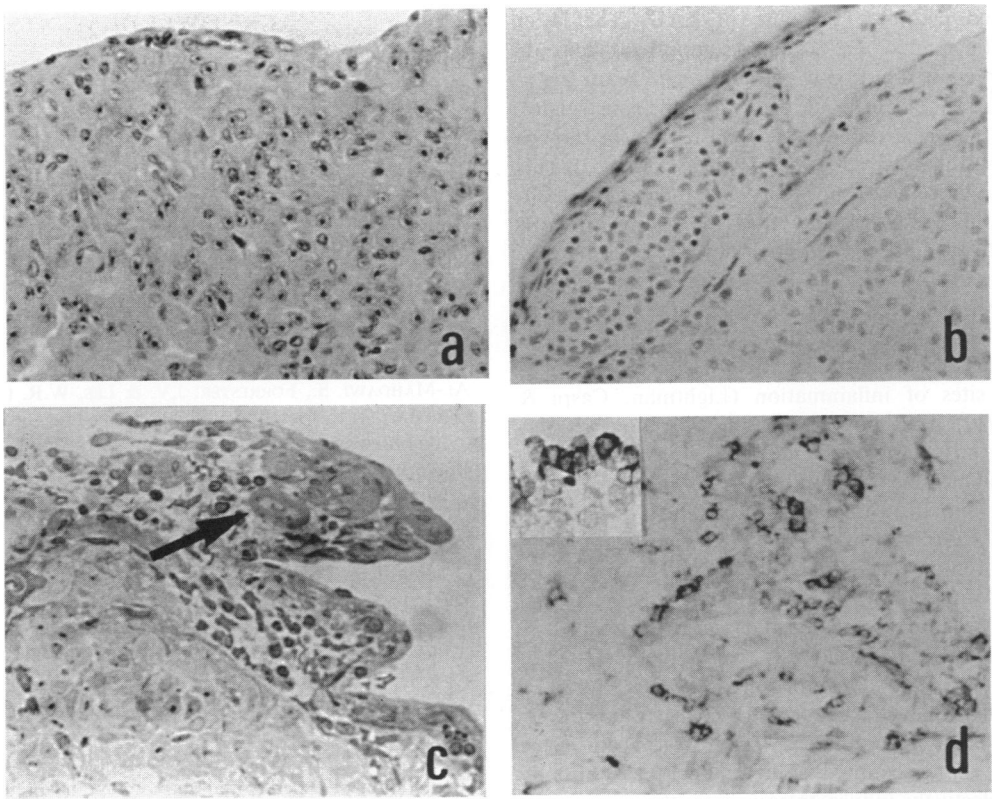

Fig. 2. Pineal changes. (a) Normal rat pineal. (Haematoxylin and eosin, original magnification × 500). (b) Subcapsular focal granulomatous infiltration. (Haematoxylin and eosin, original magnification × 450). (c) Perivasculitis (arrow) with subcapsular infiltration. (haematoxylin and eosin, original magnification × 575). (d) Inflammatory infiltration in the pineal stalk region showing predominantly cells that are stained by the monoclonal marker CD4 (W3-25). (APAAP, original magnification × 575). Insert: A row of CD4 (W3-25) positive cells on the pineal capsule. (APAAP, original magnification × 640).

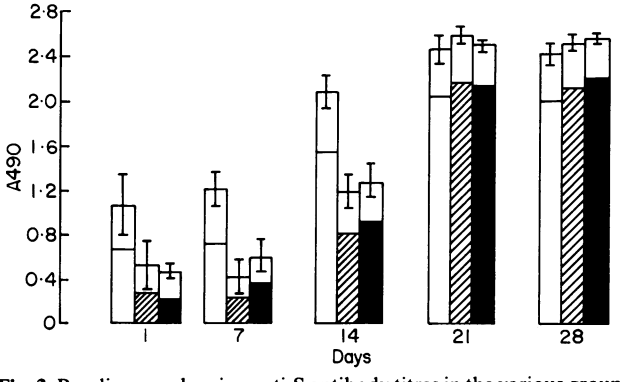

Fig. 3. Bar diagram showing anti-S antibody titres in the various groups measured by ELISA (A490) at weekly intervals from day 1 to day 28. □ Group 1, ■ Group 2, ■ Group 3.

monoclonal antibodies to modulate the immune response of the host is being investigated. Benjamin & Waldman (1986) were able to induce tolerance in mice to immunogenic antigens by treatment with rat monoclonal antibodies directed against the CD4 cells. The aim of this approach is to induce selective tolerance to certain antigens whilst retaining responsiveness to others, which would have great value in the management of autoimmune diseases.

De Kozak *et al.* (1985) succeeded in inhibiting EAU in rats by the administration of mouse S-antigen-specific monoclonal antibodies simultaneously with the S-antigen. They suggested that the heterologous monoclonal antibody assumed a protective role by inducing the formation of anti-idiotypic antibodies, acting via the idiotype-anti-idiotype network. We have used a homologous system of a rat monoclonal (S2.4.C5) in rats, which was extremely effective in inhibiting EAU. In contrast, S2.4.C5 was not effective in inhibiting the pineal inflammation. Considering the recent evidence that EAU- and EAP-inducing epitopes of antigen may be identical (see below) our results indicate that the idiotype-anti-idiotype network theory may not be an important mechanism in the prevention of EAU in this model.

There are several alternative explanations for the effects of S2.4.C5 in inhibiting EAU. The monoclonal antibody could have masked antigenic epitopes on the S-antigen molecule (Hutchinson, 1980) and thus interfered with its presentation to the lymphoid cells, or it might have provoked a peripheral blockade by complexing with S-antigen in the photoreceptor target organ. The former mechanism is unlikely because in this study the pineal glands showed an inflammatory response in all animals that had been treated with the S-antigen specific monoclonal antibody, even though the eyes of 80% of these animals remained protected and did not show a uveitic response. This may indicate that the inhibitory effect of the monoclonal S2.4.C5 is probably directed towards the effector arm of the

immune-mediated cytotoxic response in EAU. The latter suggestion that the monoclonal produces a peripheral blockade is unlikely too because if antibody combined with S-antigen in the target organ, it should do so more readily in the pineal gland, where a blood-pineal barrier does not exist, than in the eye where the blood-retinal barrier is well defined (Lee, 1982). This should render the pineal less prone to develop inflammation but the results of this study indicate that the reverse is true. Conversely, antigen masking may be relevant to homing of lymphocytes in sites where the endothelium constitutes a barrier. It has been shown that sensitized lymphocytes adhere to activated retinal vascular endothelium in EAU and it has been suggested that this mechanism is essential for directing lymphocyte traffic to sites of inflammation (Lightman, Caspi & Nussenblatt, 1987). It is possible that S2.4.C5 inhibits this interaction between lymphocytes and endothelium at the blood-retinal barrier but has no effect in the pineal gland, where there is no such barrier and the vascular endothelium is fenestrated (Milofsky, 1957; Wolfe, 1965). Possible mechanisms could include S-antigen-monoclonal anti-S complexes binding via Fc receptors to activate suppressor mechanisms, or monoclonal anti-S antibody V region recognizing and interacting with cross-reacting determinants on lymphocytes or endothelium (we are developing in-vitro assays to test these hypotheses).

The possibility that the uveitogenic epitopes on the S-antigen molecule may be different from the pinealitogenic epitopes cannot be discounted. The monoclonal antibody S2.4.C5 may selectively induce tolerance to the uveitogenic epitopes only. This seems an attractive hypothesis but is probably unlikely in view of the recent evidence presented by Donoso *et al.* (1987). They synthesized 23 peptides corresponding to the entire 404 amino acid sequence of the S-antigen molecule and demonstrated that one peptide, nominated peptide M (18 amino acids in length), was highly pathogenic and consistently induced EAU and EAP in Lewis rats. This would strongly indicate that the epitope(s) for uveitis and pinealitis on the S-antigen molecule is the same.

The presence of relatively higher levels of antibodies during the first 2 weeks of immunization in S2.4.C5-treated animals is of interest. Although the fate of the monoclonal antibody after inoculation in the rats is not known, some of this anti-S activity was probably due to the inoculated monoclonal antibody in the early stage. However, after 1 week it is difficult to predict how much of the injected monoclonal antibody would persist. In a heterologous system de Kozak *et al.* (1987) inoculated rats with a mouse anti-S monoclonal antibody and failed to detect its presence in the sera after 1 week. It is likely that in their model all the inoculated mouse monoclonal antibody was removed by the host immune system within the first week. However, in the present experiment some of the monoclonal antibody may have persisted for longer periods. In the subsequent weeks, particularly the third and fourth weeks post-immunization, anti-S titres were equally raised in all groups. At this stage the polyclonal host-response to S-antigen would have predominated and was clearly not related to induction of tolerance and disease prevention as it was similar in all groups of animals. De Kozak *et al.* (1987) also detected anti-S antibodies from day 7 to 14 after immunization with S-antigen and noted that the titres of antibodies levelled off at the end of the first month in all animals, irrespective of whether or not they had received anti-S monoclonal antibody.

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