Retinal S-antigen (S-ag) was purified by monoclonal antibody (MoAb) immunoaffinity chromatography from soluble protein extracts of bovine and human retina. Purification of S-ag was readily achieved by affinity chromatography using four different MoAb-Sepharose 4B columns. The four antibody columns gave different recoveries with material of comparable enrichment with greater than 95% purity as judged by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The use of two different MoAbs covalently bound to Sepharose 4B and known to be directed to disparate, spatially distant epitopes on S-ag led to at least a twofold increase in recovery, with the aforementioned purity. Immunoaffinity purified S-ag retained its serological and uveitogenic properties. The high recovery of S-ag associated with this one-step procedure is preferable to conventional preparatory techniques, and enables high antigen recovery when tissue availability is limited (eg human retina). Invest Ophthalmol Vis Sci 28:604-607, 1987

Retinal S-ag is a 50,000 daltons molecular weight glycoprotein found in the photoreceptors of the retina and in the pineal gland. Recent studies have shown that this protein is an important component of the photoreceptor enzyme cascade, where it specifically interacts with activated, phosphorylated rhodopsin, and is intimately involved in the visual biochemical pathway. The potent uveitogenic properties of this protein, when single doses are injected with adjuvants into susceptible species, and its possible pathogenic role in human autoimmune disease, have led to studies examining the cellular and molecular events of the immune response to this autoantigen.

A number of different biochemical preparative approaches have been used in the preparation of pure S-ag. However, all procedures described, either preparative or analytical, involve several chromatographic steps over a period of a few days and are not well-suited for S-ag preparations of high purity from retinas available in small numbers such as those from human eyes. In this report, we describe a single step immunoaffinity purification procedure for S-ag in which the use of a mixture of two different MoAbs immobilised on Sepharose 4B results in efficient recovery of pure S-ag. Materials and Methods. Monoclonal antibodies to porcine and bovine S-ag were prepared and characterised as described by Banga et al.* The MoAbs were purified from ascitic fluid by precipitation with 40% saturated ammonium sulphate followed by affinity purification on Protein A-Sepharose 4B. Either the purified or the ammonium sulphate precipitated MoAb was coupled to CNBr-activated Sepharose 4B (Pharmacia Ltd., Milton Keynes, United Kingdom) according to the manufacturers instructions, except that phosphate buffered saline (PBS) was substituted for the coupling buffer. When two different MoAbs were used on the immunoaffinity column, they were mixed in equal proportions before coupling to Sepharose 4B.

Retinal S-ag was purified on MoAb immunoaffinity columns from low ionic strength soluble protein extracts of human or bovine retinas. The protein extract was adjusted to physiological ionic strength with PBS and precleared at 4°C on 10 ml of 5 mg/ml ovalbumin-Sepharose 4B. This was passed through MoAb immunoaffinity columns at a flow rate of 12 ml/hr, and 1 ml fractions of the eluate collected. The columns were washed with cold PBS until protein measurement by protein-dye binding assay fell to 0.05 mg/ml, and then eluted with 50 mM diethylamine, pH 11.5. The eluates were immediately neutralised with solid glycine, dialysed against PBS at 4°C and freeze dried. For preparations of low S-ag concentration, the dialysis step was omitted to avoid losses on the dialysis tubing. After use, MoAb affinity columns were equilibrated in PBS containing 0.01% sodium azide and stored at 4°C. The S-ag in the eluted peaks was identified by SDS-PAGE and immunoblotting. This was performed by transferring the proteins to nitrocellulose sheets in a BioRad

Table 1. Retinal S-ag purification on different MoAb-sepharose 4B columns

<table>
<thead>
<tr>
<th>Monoclonal antibody-Sepharose 4B</th>
<th>Class of MoAb</th>
<th>mg MoAb/ml Sepharose 4B</th>
<th>Volume of MoAb-Sepharose 4B used</th>
<th>Amount of S-ag purified (µg)</th>
<th>Presence of S-ag in effluent following affinity chromatography</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDS-1</td>
<td>IgG1</td>
<td>2.2 mg</td>
<td>0.4 ml</td>
<td>11 µg</td>
<td>+</td>
</tr>
<tr>
<td>BDS-2</td>
<td>IgG2a</td>
<td>2.2 mg</td>
<td>0.4 ml</td>
<td>37 µg</td>
<td>+</td>
</tr>
<tr>
<td>BDS-3</td>
<td>IgG2a</td>
<td>1.7 mg</td>
<td>0.53 ml</td>
<td>9 µg</td>
<td>+</td>
</tr>
<tr>
<td>BDS-4</td>
<td>IgG2a</td>
<td>0.9 mg</td>
<td>1.0 ml</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>PDS-1/BDS-2</td>
<td>IgG1/IgG2a</td>
<td>5.4 mg</td>
<td>0.2 ml</td>
<td>80 µg</td>
<td>+</td>
</tr>
<tr>
<td>PDS-1/BDS-2</td>
<td>IgG1/IgG2a</td>
<td>5.4 mg</td>
<td>1.0 ml*</td>
<td>490 µg</td>
<td>-</td>
</tr>
</tbody>
</table>

1 Volume of MoAb-Sepharose 4B used to obtain a total of 0.9 mg MoAb in affinity column (except *).
2 20 mg retinal extract in 5 ml PBS was passed through each MoAb-affinity column. S-ag was measured by protein dye-binding assay and represents approximately 2.5% (w/w) of the soluble protein extract.
3 Presence of S-ag in effluent following passage through MoAb-Sepharose 4B column was ascertained by immunoblotting; (+) = present; (−) = absent.

( Watford, United Kingdom ) blot apparatus and immunoblotting with a polyclonal rabbit antibody to S-ag as described by Banga et al. Uveotogenicity was ascertained by immunization of albino Lewis rats with S-ag in Freund’s complete adjuvant into the hind footpads. Bordetella pertussis ( 1 x 10^{10} orgs ) was given intraperitoneally at the same time as immunization. The course of the disease was monitored by slit-lamp examination.

The treatment and handling of all animals in this investigation conformed to the ARVO Resolution on the Use of Animals in Research.

Results and Discussion. We first assessed the efficacy of the different MoAbs coupled to Sepharose 4B to bind and subsequently elute S-ag from soluble protein extracts of bovine retina. Antibody affinity columns containing 0.9 mg of different MoAbs were prepared. Soluble protein extracts of bovine retinas precleared on ovalbumin linked Sepharose 4B were divided into four equal aliquots (20 mg total protein containing approximately 2.5% S-ag) for passage through MoAb affinity columns. Following elution with 50 mM diethylamine, pH 11.5, varying amounts of purified S-ag were obtained from the different MoAb affinity columns as outlined in Table 1. The purity of S-ag preparations isolated from different MoAb columns was comparable to that assessed by SDS-PAGE, and all preparations contained a major contaminating polypeptide at 40,000 daltons. However, the recovery of S-ag was low, varying between 9 µg and 37 µg for BDS-3 and PDS-1 columns, respectively (Table 1). By immunoblotting, it was apparent that the effluent contained large amounts of S-ag, and recycling of this material recovered comparable amounts.

Higher yields of affinity purified S-ag were readily achieved when two different MoAbs, known to be directed to disparate epitopes of S-ag (Bang et al.* ) were immobilised onto Sepharose 4B. This important modification allowed the recovery to be increased by a factor of at least two, when compared with use of antibody affinity columns containing MoAbs of single specificity (Table 1). A 1 ml PDS-1/BDS-2 (total 5.4 mg MoAb) Sepharose 4B column was sufficient to recover approximately 500 µg pure S-ag from 5 ml of retinal extract containing a total of 20 mg protein. Analysis by immunoblotting showed that no S-ag remained in the effluent from the column (Fig 1, lanes d and e).

Optimal conditions for eluting S-ag from the MoAb affinity columns were also determined. The eluting capacities of 50 mM diethylamine, pH 11.5, 3 M potassium isothiocyanate (KCNS) and glycine-HCl, pH 2.8 were compared. The recovery of S-ag was highest with diethylamine, the chaotropic agent (KCNS) gave poor recovery, and glycine-HCl buffer eluted no S-ag (Fig 2, insert).

The species crossreacting MoAbs PDS-1 and BDS-2 have been used to purify human retinal S-ag with similar recovery values to those obtained from bovine preparation. The human S-ag obtained was of higher purity than the material purified by the Phenyl-Sepharose method (Fig 3, lanes c and d). The distinct advantage of antibody affinity purification for human S-ag is the rapid preparative procedure, coupled with virtually total recovery of highly enriched material from a small number of retinas.

The immunoabsorbent column of PDS-1/BDS-2 MoAb has been used to prepare several successive batches of S-ag with comparable yields in all the preparations. Retinal S-ag purified by MoAb affinity chromatography has all the biological properties of S-ag purified by conventional biochemical procedures. The material reacts with polyclonal and monoclonal antibodies by solid phase immunosassays, double diffusion in agarose, and immunoblotting following SDS-PAGE. A single dose of 10 µg affinity purified S-ag (purity of >95%) in Freund’s complete adjuvant was sufficient to induce uveoretinitis within 12–14 days in albino Lewis rats. Furthermore, chemical cleavage of this affinity purified material with cyanogen bromide gives rise to peptide fragments that exhibit the same mobility.
coomassie gel

immunoblot

Fig. 1. Immunoblotting following SDS-PAGE to show complete removal of S-ag from retinal extract by antibody affinity chromatography. The upper picture shows the gel stained with Coomassie brilliant blue after blotting; the lower picture is the autoradiograph of the immunoblot derived from the gel. Immunoblotting was performed with a polyclonal rabbit antiserum to bovine S-ag, followed by 125I-labelled affinity purified Donkey anti rabbit antibody. Lane a was loaded with 3 µg bovine S-ag purified by the Phenyl-Sepharose method. Lane c contained the same amount of affinity purified bovine S-ag. The bovine retinal extract (50 µg) applied to the antibody affinity adsorbant is shown in lane b, and the effluent from the column is shown in lane d (40 µg) and lane e (120 µg). The autoradiograph shows that no S-ag was present in the effluent following a single passage through the column. The arrows show the position of S-ag on SDS-PAGE urea gels with peptides generated from S-ag prepared by Phenyl-Sepharose chromatography.

S-ag eluted from MoAb affinity columns at pH 11.5 was able to induce experimental autoimmune disease, and retained its serological reactivity with several monoclonal and polyclonal antibodies. This suggests that a brief exposure to high pH conditions does not denature the S-ag to a significant extent. This is in contrast to heat or SDS denatured preparation of S-ag, which loose their serological reactivity to polyclonal and monoclonal antibodies while leaving the capacity to induce uveoretinitis unimpaired (Banga, unpublished observations).

The increase in yield of S-ag obtained by use of antibody linked on solid matrix support containing two MoAbs known to be directed to different spatially distant epitopes is most likely due to an increase in the avidity leading to enhanced binding efficiencies. The immunoafinity chromatography technique has been shown to be useful in achieving a rapid purification procedure for retinal S-ag, coupled with high purity of recovered material, and should be the method of choice.

Fig. 2. Elution of S-ag from the PDS-1/BDS-2 MoAb immunoaffinity column. Bovine retinal extract (20 mg) in 5 ml PBS was passed through a 1 ml PDS-1/BDS-2 (5.4 mg total MoAb)-Sepharose 4B column and eluted with 50 mM diethylamine, pH 11.5. Approximately 500 µg S-ag was recovered. Elution with diethylamine was essential for complete recovery of S-ag from the antibody affinity column, since the use of potassium isothiocyanate led to low yields. Glycine-HCl buffer did not elute any material from the column (inserts).
Fig. 3. SDS-PAGE to show purity of S-ag recovered from human retina by PDS-1/BDS-2 antibody affinity chromatography. Soluble protein extract from eight human retinas was applied to 1 ml (5.4 mg PDS-1/BDS-2 MoAb) Sepharose 4B column and the bound material eluted with diethylamine buffer. The protein extract of human retina is shown in lane a (60 µg). Human S-ag purified on the MoAb affinity column is shown in lane c (25 µg), and Phenyl-Sepharose purified human S-ag of highest purity obtainable is shown in lane d (25 µg). Lane b shows bovine S-ag purified by affinity chromatography. Human S-ag (arrowed) recovered by immunoaffinity method in lane c is of a higher purity than that obtained by the Phenyl-Sepharose method; the bands migrating below the arrow in this lane are not immunoreactive fragments of S-ag, as proven by immunoblotting (not shown).

References

Origin of Notches in CSF: Optical or Neural?

Grating contrast sensitivity was measured across a range of 1 to 32 cycles per degree (c/deg) in normal observers with a computer-automated method of ascending limits. Monocular contrast sensitivity functions (CSF) were obtained for vertical, oblique and horizontal orientations, with or without full refractive correction. Small amounts of astigmatic error resulted in loss of sensitivity at selective spatial frequencies. Coincident with these CSF “notches” was the presence of monocular diplopia induced, in this study, by the condition of astigmatic error. Experimental manipulation of the selective spatial frequency losses was possible by the introduction of slight cylindrical defocus and by changes in grating orientation. Determination of the angular displacement and orientation of the monocular double images allowed prediction of the spatial frequencies which would show reduced sensitivity due to partial cancellation of contrast. The close fit between the predicted and measured sensitivity loss supports the suggestion that refractive error can affect narrowly-tuned notches. These results indicate that before the presence of a notch in the CSF can be attributed to neural abnormality, an optical cause must be eliminated. Invest Ophthalmol Vis Sci 28: 607–612, 1987