The reactions of human and bovine retinal S-antigen (S-ag) with polyclonal rabbit antibodies were compared in ELISA, immunoblotting and inhibition ELISA. Titrations in ELISA with plastic-adsorbed S-ags revealed that the great majority (70-100%) of human S-ag epitopes and anti-human S-ag antibodies were cross-reactive. In contrast, the cross-reactivity of bovine S-ag (25%) and anti-bovine S-ag antibodies (15-20%) indicated an important contribution of species-specific epitopes to the antigenicity of bovine S-ag. Immunoblotting of S-ag fragments after treatment with chymotrypsin confirmed these differences and also demonstrated different chymotrypsin-induced cleavage patterns of human and bovine S-ag. Thus, in assays involving partial denaturation and/or degradation of the antigens, human S-ag showed little, and bovine S-ag a marked species-specific immunoreactivity. In inhibition ELISA however, in which S-ags in solution could be studied, species-specific reactions strongly predominated for both S-ags. Anti-human S-ag and anti-bovine S-ag antibodies could be absorbed with nM concentrations of fluid phase human and bovine S-ag, respectively, whereas in both cases the cross-reacting antigen had no detectable inhibitory potential. The epitopic structures of human and bovine S-ag in solution may thus be largely different. Invest Ophthalmol Vis Sci 30:1169-1173, 1989

The soluble retinal protein "S-antigen" (S-ag) is a regulatory component of photoreceptor cells, of which microgram amounts can induce a destructive autoimmune uveoretinitis (EAU) in experimental animals.1-3 Recent advances have provided detailed knowledge on its molecular structure, including the primary structures of the whole peptide chain4,5 and at least one uveitopathogenic epitope.6 These data are obtained with isolated bovine S-ag, which is very similar to and highly cross-reactive with all other S-aggs studied thus far.2,3 However, it is questionable whether such detailed data concerning bovine S-ag can be extrapolated to other S-ags. Since anti-S-ag autoimmune reactions are also supposed to be involved in clinical uveitis, the comparison with S-ag of human origin is of particular importance. Despite the very similar molecular properties and marked cross-reactivity of human and bovine S-ags, physico-chemical and immunochemical differences have been reported.7 We have studied these differences in more detail, in an attempt to assess the relative contribution of species-specific and cross-reacting epitopes to the immunoreactivity of both S-ags.

Materials and Methods. Antigens and Antibodies: Isolation of human and bovine S-ag, and production of specific antisera were described earlier.8,9 Immunoglobulins (Igs) were isolated from pooled rabbit sera (three animals per S-ag) by salt precipitation and dialysed against phosphate-buffered saline (PBS). Antigens (0.5-2.0 mg/ml) and anti-S-ag Igs (16-45 mg/ml) were stored in small aliquots at —20°C and thawed immediately before use.

Solid Phase ELISA: Polystyrene microwells (microtitre plates, no. 655101; Greiner, Nuertingen, FRG) were coated with serial dilutions of S-ags in carbonate/bicarbonate, pH 9.6, and, after extensive washing, incubated with anti-S-ag Igs diluted 1/1000 in PBS-0.1% Tween. Alternatively, S-ags were coated at 100 ng/well and the second step was performed with serial dilutions of anti-S-ag Igs. Bound Ig was measured by incubation with 1/500 diluted peroxidase-labelled goat anti-rabbit IgG (Nordic, Tilburg, The Netherlands) and 0.16 mM ABTS (Boehringer, Mannheim, FRG) as the peroxidase substrate.9

Fluid Phase Inhibition ELISA: The procedure was adapted from that of Friguet et al.10 Rabbit anti-S-ag Igs, diluted 1/4000 or 1/2000 were incubated (1 hr at 37°C and overnight at 4°C) with serial dilutions of human or bovine S-ag in PBS-Tween. The residual anti-S-ag activity of duplicate 0.1 ml samples was, without further dilution, measured in wells coated with 100 ng of either human or bovine S-ag as described above.

Chymotrypsin Treatment and Immunoblotting: Human and bovine S-ag (35 μg/ml) were incubated for 30 min at 37°C with 17 μg/ml of chymotrypsin (bovine pancreatic chymotrypsin type VII; Sigma, St. Louis, MO) in PBS-0.2% Tween. The incubation was stopped with an equal volume of 2% sodium dodecyl sulphate (SDS) and 5% mercaptoethanol in 0.0625 M Tris-HCl, pH 6.8, and heating for 10 min at 70°C. S-ag fragments were separated by SDS-PAGE (13%
acrylamide) under reducing conditions and electroblotted onto nitrocellulose. After saturation with bovine serum albumin, 0.5 × 10 cm strips were incubated with 0.1 mg/ml rabbit anti-S-ag Iggs, the binding of which was detected with 1/500 diluted goat anti-rabbit IgGs/PO and 0.4 mg/ml 4-chloro-naphtol-1 (Sigma).

Results. Cross-Reactivity in Solid Phase ELISA: Parallel semilogarithmic dose-response curves were found for plastic-adsorbed human and bovine S-ag titrated with fixed amounts of anti-human S-ag and anti-bovine S-ag Iggs (Fig. 1). Therefore, the relative reactivity of the coated antigens could be derived from the distance between the curves. Thus, human S-ag reacted approximately 1.5 times more avidly with anti-human S-ag Iggs than did bovine S-ag (Fig. 1a). The difference in reactivity with anti-bovine Iggs was more pronounced: the cross-reacting human S-ag reacted approximately four times less avidly than bovine S-ag (Fig. 1b). Similarly, the relative reactivities of the antibodies were derived from their titration curves on fixed amounts of both S-aggs. Anti-human S-ag Iggs were nearly completely cross-reactive (Fig. 2a), whereas the approximately 6-fold concentration difference between titration curves (Fig. 2b) indicated a much lower cross-reactivity of anti-bovine S-ag Iggs.

Immunoblotting of S-ag Fragments: Chymotryptsin treatment and SDS-PAGE, as reported by Kamada et al,9 were combined with immunoblotting. Relatively short incubation (30 min) with chymotryptsin resulted in a great number of various-sized, immunoreactive peptides (Fig. 3). The cleavage patterns of human and bovine S-ag were similar, but clearly different, presumably indicating small differences in amino acid sequence. Anti-human S-ag Iggs strongly cross-reacted with bovine S-ag fragments (compare strips 1 and 2), whereas anti-bovine S-ag Iggs reacted strongly with bovine (strip 4), but very poorly with fragments of human S-ag (strip 3).

Inhibition ELISA: In fluid phase inhibition ELISA, anti-S-ag Iggs could be absorbed out effectively with the S-ag against which they were raised, at concentrations of 5–500 nM (Fig. 4a, b), suggesting functional $K_a$ values of at least $10^8$ M$^{-1}$. As expected, the solid phase cross-reactions could be inhibited by both S-aggs, although in both cases the cross-reacting S-ag showed a 5–8-fold lower avidity (Fig. 4c, d). The lack of inhibition of the bovine S-ag/anti-bovine S-ag reaction by soluble human S-ag (Fig. 4b) was in agreement with its low level of cross-reactivity in solid phase ELISA (Figs. 1b, 2b). Surprisingly, however, fluid phase bovine S-ag appeared to be incapable of inhibiting the reaction of anti-human S-ag Iggs with coated human S-ag (Fig. 4a). From the dose-response curve of these antibodies (Fig. 2a) it could be derived that the maximum inhibition of 10–12% achieved with bovine S-ag indicated effective blockage of at most 30–40% of the anti-human S-ag antibodies. Thus, although the cross-reactivity of solid phase human and bovine S-ag with anti-human Iggs was nearly complete (Figs. 1a, 2a), in solution the same antibodies recognized them as clearly different structures.

Discussion. Like other retinal antigens, S-ag shows a marked interspecies cross-reactivity. In immunodiffusion studies, partial or even complete identity be-
Fig. 2. Titrations of rabbit anti-human (a) and anti-bovine (b) S-ag immunoglobulins in solid phase ELISA. Serial dilutions of antibodies were incubated in microwells coated with human (●) or bovine (○) S-ag at 100 ng/well.

Fig. 3. Immunoblotting of chymotrypsin-treated human (strips 1 and 3) and bovine (strips 2 and 4) S-ag. Blot strips were incubated with rabbit anti-human (strips 1, 2) or anti-bovine (strips 3, 4) S-ag immunoglobulins. Strip 5 was incubated with PBS-Tween instead of rabbit Igs. The figures on the left indicate the position of molecular weight markers after SDS-PAGE in a parallel lane of the gel.
these rabbit antibodies: in a number of mouse and rat anti-S-ag antisera we also found more than 70% cross-reactivity of anti-human S-ag, and less than 20% cross-reactivity of anti-bovine S-ag antibodies (not shown). Moreover, to exclude that these findings were merely due to the usually much longer post-mortem storage period of human eye tissue, we have isolated S-ags from bovine eyes after storage at 4°C for 2 and 4 days. Titration curves of such S-ags could not be distinguished from that of S-ags isolated from "fresh" bovine eyes, which indicates that we really compared human and bovine S-ag, and not S-ags that were more or less denatured (not shown).

The anti-S-ag Igs reacted strongly in immunoblotting with many of the peptides produced by limited chymotryptic cleavage of S-ags (Fig. 3). This seems to be at variance with the results of Kamada et al.\textsuperscript{11} who found immunoreactivity only on a limited number of such fragments. However, these authors used much longer incubation times which might have destroyed epitopes, and used an ELISA in which the lack of reactivity might have been due to insufficient coating, especially of the smaller fragments. The approach presented here might be very useful for further investigation of the epitopic structure of S-ags. For this study, the most relevant findings were the similar, but clearly different cleavage patterns of human and bovine S-ags and the strong and poor cross-reactions of anti-human S-ag Igs and anti-bovine S-ag Igs, respectively, confirming the ELISA results. In the cross-reaction of anti-bovine S-ag Igs with human S-ag fragments (Fig. 3, strip 3), even the reaction with the presumed native S-ag at 50 kD was not visible. In other experiments we have found a pronounced band at 50 kD when untreated human S-ag was immunoblotted with anti-bovine S-ag antibodies (Hagmeijer and Doekes, unpublished data). It therefore seems probable that the enzyme treatment had reduced the amount of native S-ag in the mixture to subdeteatable levels in this low-avidity reaction. Alternatively, the 50 kD band of the fragments seen on the other strips represents an enzymatically modified form with still the same size, but an altered epitopic structure.

Solid phase ELISA involves partial denaturation of the coated antigen, which might cause the loss of important conformational epitopes and/or the neoexpression of linear epitopes hidden in the (presumably more native) conformation of S-ags in solution. Fluid phase inhibition ELISA showed that the differences between human and bovine S-ag in solution were more pronounced (Fig. 4). Cross-reacting antibodies appeared to react with a higher avidity with the S-ag used for their production (Fig. 4c, d), while the solid phase reaction with the immunizing S-ag could not be inhibited at all with the cross-reacting S-ag in solution (Fig. 4a, b). The incapability of soluble bovine S-ag to inhibit the anti-human S-ag Igs is particularly noteworthy, since these antibodies strongly cross-reacted with coated bovine S-ag (Fig. 1a, 2a). This indicates differences between the expression of epitopes on solid phase-adsorbed, partially denatured S-ags, and S-ags in solution. Since partially denatured antigens presumably present mainly linear epitopes, the stronger cross-reactivity in the solid phase ELISA suggests that relatively small differences in the primary structure of human and bovine S-ag give rise to important conformational differences in solution. Since S-ag is in vivo probably a soluble protein, this may be of particular relevance for the interpretation of serological data obtained with homologous or heterologous S-ags in experimental and clinical studies. Furthermore, differences between solid phase and fluid phase behavior should also be accounted for in investigations on the detailed molecular and epitopic
structure of S-ags, their fragments, and corresponding synthetic peptides. The inhibition ELISA reported here might be a very simple but reliable method in such studies.

Key words: retinal S-antigen, human, bovine, cross-reactivity

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References

Ornithine Aminotransferase Distribution in Ocular Tissues and Retinas of Cat and Mouse

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Ornithine aminotransferase (OAT), a mitochondrial matrix enzyme, is genetically deficient in patients with gyrate atrophy of the choroid and retina. Histologically defined microsamples (10 ng–6.5 μg dry weight) were dissected out from freeze-dried tissue sections of eyeballs of cat and mouse, and the OAT activities were determined by a newly developed microassay method in the ocular tissues and retinal layers. Very high specific activities of OAT, expressed on a dry weight basis, were found in the feline ocular tissues of ectodermal origin, that is, neuroretina, retinal pigment epithelium, ciliary processes and epithelium of iris. In cat and mouse retinas, high OAT activities were distributed in the inner retina with an activity peak in the inner plexiform and ganglion cell layers. Very low activity was present in the outer nuclear layer. The inner segments of photoreceptor cells, which are very rich in mitochondria, contained the highest OAT activity. In contrast, the outer segments of photoreceptor cells contained the low activity resulting from contamination by small pieces of inner segments. Invest Ophthalmol Vis Sci 30:1173–1177, 1989

Ornithine aminotransferase (OAT, EC 2.6.1.13), which is a mitochondrial matrix enzyme, is known to be genetically deficient in patients with gyrate atrophy of the choroid and retina. This autosomal recessive choriotirenal degeneration is generally associated with hyperornithinemia as a result of OAT deficiency. Since hyperornithinemia is thought to adversely affect the cell and cell structures normally abundant in OAT, the distribution of OAT in the