Immunopathology of the lens

I. Humoral and cellular immune responses to heterologous lens antigens and their roles in ocular inflammation

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SUMMARY In experimental rabbits heterologous soluble lens proteins consisting of α -, β -, and γ -crystallins were found to be antigenic; they stimulated a marked antibody response compared to a rather weak T-cell response. The serum antibodies to α -crystallins appeared first, to be followed by antibodies to β - and γ -crystallins in that order. The rabbits did not respond to heterologous γ -crystallins unless these were injected with Freund's adjuvant containing mycobacteria. Incomplete Freund's adjuvant (i.e., without mycobacteria) was found to be an inferior immunoaccelerator so far as lens antigens are concerned. The response to lenticular antigens in both magnitude and duration varied in different rabbits, which suggested to us the important role played by a central control mechanism involving the immune response (Ir) genes.

Some of the antibodies in potent lens antisera cross-reacted with mitochondria, endoplasmic reticulum (i.e., microsomes), contractile organelles, and cell nuclei. This explains for the first time at least in part the reasons for the widely observed phenomenon of the reactivity of lens antisera with ocular and extraocular structures. Antibodies to soluble lens proteins as detected by immunofluorescence and immunoperoxidase techniques were shown to be of the IgG class. Systemic heterologous immunisation followed by dissection of the lens does not lead to the typical changes of phakoallergic endophthalmitis in the rabbit.

Interest in the antigenicity of the mammalian lens and its role in the production of autoimmune lesions in the eye appears to have originated with the study by Uhlenhuth (1903) in which rabbits were immunised with repeated doses of bovine lens material. Precipitating antibody was produced which reacted with lens extracts from many species. It did not react with bovine serum, however, which led to the general conclusion that the lens antigen, though not species-specific, was largely organ-specific.

This view prevailed for several decades among experimental ophthalmologists (Hektoen, 1922; Halbert *et al.*, 1957; Takata *et al.*, 1964), and even now in most textbooks on general medicine and immunology lenticular antigen is still regarded as a typical example of organ specificity. It is well established, however, that proteins resembling lens material are found not only in other ocular tissues but also in almost every organ of the body.

Antigenic specificity of the lens

It is believed that the lens proteins evolved in the most primitive vertebrates (i.e., Agnatha) about 450 million years ago and have been transmitted intact to all vertebrates (Manski *et al.*, 1960). In a study of the immunological relationships of the lens in different phyla Halbert and Manski (1963) showed that the earliest vertebrate antigens, as represented in the lamprey, have been transmitted unchanged throughout all the more recent phyla. New antigens added at a later period of evolution have similarly but less consistently been transmitted to subsequent phyla. This tendency for the original antigen to persist through the millions of years represented by the range of phyla involved is probably a reflection in part of the isolation of the lens from the vascular system (Glynn and Holborow, 1965).

A number of investigators have reported that the lens antigens are present not only in other ocular structures (e.g., the iris, the retina, the cornea, the vitreous, and the aqueous humour) but also in extraocular tissues such as the brain, the skin, the

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skeletal muscles, the liver, and the renal glomeruli (Hektoen, 1922; Rao *et al.*, 1955; Maisel and Harmison, 1963a, b; Nozaki *et al.*, 1963a, b; Perkins and Wood, 1963; Zwaan, 1963; Cate, 1964; Mehta *et al.*, 1964; Rathburn *et al.*, 1971; Bours and Doorenmaalen, 1972). It is not surprising, therefore, that Campbell *et al.* (1968) found about 2 dozen electrophoretically distinct antigenic components within the lens, that all other tissues examined contained some antigenic determinants similar to those found in lens proteins, and that all of the major classes of lens proteins contained cross-reacting groups.

It is clear, therefore, that the so-called organ specificity of the lens is the result of a unique selection of antigens genetically available, any one of which may be found in other tissues, rather than being due exclusively to the possession of some specific protein restricted to the lens (Clayton *et al.*, 1968). Why the lens should contain so many different antigens and why they should be so widely distributed in nature can be appreciated only if the embryology and the fine structure of this largely and apparently acellular organ is critically examined.

Development of the lens and the distribution of its antigens

As soon as the optic vesicle, which develops as an extension from the anterior region of the neural tube, reaches the surface ectoderm at about the 4 mm stage in the human embryo, changes begin to occur under the influence of an inducer, which involves a thickening of the epithelium to form the lens plate that later invaginates to form the lens vesicle. From this vesicle the lens is ultimately evolved by the growth of constituent cells which first fill the cavity and then increase the size of the embryonic lens by subsequent multiplication of the epithelium in the equatorial region. The hexagonal cells are laid down in layers, the old ones being pushed towards the centre in the embryo, and the process continues throughout postnatal life. The older cells, being elongated, are now called lens fibres. They collect in the centre of the lens to form the nucleus, which gradually grows in size from a small embryonic structure through fetal, infantile, and adult forms, but retains all the antigenic components synthesised during these periods because of its nonvascularity and a generally slow turn-over of lens protein.

The anterior surface of the lens is covered by epithelium, which has a somewhat granular cytoplasm and contains the usual organelles (albeit in smaller numbers), including rough endoplasmic reticulum, mitochondria, ribosomes, Golgi com-

plexes, microtubules, and microfilaments. The epithelium in the equatorial region, however, contains more fibrillar cytoplasm and chains of polyribosomes and elements resembling lysosomes. The cortical lens cells (i.e., the lens fibres) found immediately under the equatorial epithelium also contain all the cytoplasmic organelles, including microtubules; their number is greatly reduced, however, the cytoplasm becoming fibrillar and the nucleus markedly flattened. In the deeper cortex the morphology of the lens cells is totally altered. The organelles disappear progressively and the cytoplasm becomes amorphous owing to the accumulation of homogeneous granular material. The cell nucleus breaks up into small fragments, and the nuclear envelope is no longer discernible (Hogan *et al.*, 1971). The entire lens mass may be compared with a community graveyard containing the remains of several generations of lens fibres. It is not surprising, therefore, that it should contain a conglomeration of antigenic components associated with the cytoplasm and the nucleus.

The lens should not be simply regarded, however, as a graveyard, because all these changes have an evolutionary purpose and are essential for the transparency of the lens where active metabolism goes on throughout life (Heyningen, 1969) through gene repression and activation mechanisms directed towards the production of proteins, which do not oxidise readily to form insoluble components, thus maintaining the essential transparency of the lens.

Lens proteins: nature and classification

The lens proteins account for 35% of the lens mass, a higher concentration of protein than in any other tissue of the body.

Morner (1894) was the first to attempt fractionation of the various proteins of the lens, and he arbitrarily divided them into 4 types. After homogenisation and centrifugation the water-insoluble fraction was called the 'albuminoid'. The supernate was treated with tenth-normal acetic acid and the protein which precipitated at pH 5, he called α -antigen. What was left in solution was partially precipitated with a saturated solution of magnesium sulphate, and the precipitate was called β -antigen. The protein remaining in solution at the end of this procedure was mistakenly called 'albumin'.

This classification was modified 3 decades later by Burky and Woods (1928), who showed that the soluble proteins of the lens after initial precipitation of α -crystallin at pH 5 could be further precipitated by increasing the pH to 6 to obtain β -crystallin. What was then left in the solution was not 'albumin', as Morner thought, but contained another lens

protein which they obtained as γ -crystallin by precipitation with 50% saturated ammonium sulphate. These workers also found that Morner's 'albuminoid' was not an immunochemical entity but consisted of crystallins, mainly the α -fraction, which had become insoluble for some unknown reason (Woods and Burky, 1927).

After the introduction of electrophoretic techniques attempts were made to introduce a new classification for the lens antigens on the basis of their surface charge and therefore their electrical mobility. The fast moving fraction of the lens protein was called α -crystallin (Hesseltick, 1939; Viollier *et al.*, 1947; François *et al.*, 1954), the slow moving fraction at pH 7 or above was γ -crystallin (Resnik *et al.*, 1960), and the proteins of intermediate mobility were regarded as β -crystallins.

It was soon realised, however, that the lens homogenate contained several distinct proteins differing in their solubility at a different pH, in their electrical charge, and in their molecular size. It was Bjork (1961) who first attempted to separate and purify the various lens proteins on the basis of their molecular size using the new technique of gel filtration. This simple procedure is probably the best and most effective for the isolation of any single group of proteins from the complex mixture present in the vertebrate lens and has been widely used by subsequent investigators to purify the various α -, β -, and γ -crystallins.

The soluble lens proteins are now generally separated from each other by filtration on DEAE-cellulose by consecutive elution with buffers of increasing concentration; 0.002 M-phosphate elutes the γ -crystallin fractions, 0.03 M the β -crystallin fractions, 0.05 M a mixture of α - and β -crystallins, and finally 0.4 M-phosphate elutes α -crystallins.

α -CRYSTALLINS

The α -crystallins have the highest molecular weight and the greatest positive charge at alkaline pH and therefore move fastest on electrophoresis. Although they exist as variable polymers, α -crystallins consist of 4 different polypeptide chains. The A_1 chain (i.e., the acidic polypeptide) is derived from the A_2 chain, and the B_1 (i.e., the basic polypeptide) is derived from the B_2 chain. The molecular weight of A chains is approximately 19 500 and that of B chains 22 500 (Spector *et al.*, 1971). The α -crystallins have blocked terminal amino groups which are acetylated and contain methionine. It is of interest to note that there are few proteins which are N-acetylated, and the list includes tobacco mosaic virus, carp haemoglobin, histone 1V of calf thymus, basic protein from the human brain, high sulphur wool protein, and human heart cytochrome C

(Croft, 1973). Human α -crystallins have a much lower molecular weight, however, than calf α -crystallins and appear to increase in concentration with age; they represent about 40% of the total soluble lens protein in old persons (Spector *et al.*, 1973).

β -CRYSTALLINS

The β -crystallins are not as well defined, however, and apparently consist of groups of proteins with molecular weights ranging from 5×10^4 to 2×10^5 (Armand *et al.*, 1970). The β -crystallins contain many polypeptide chains, some of which appear to be present in aggregates. Shapiro (1968) has reported that this protein group contains 3 different-sized polypeptide chains of molecular weights 21 000, 23 000, and 29 000. These proteins have a relatively higher thiol content than α -crystallins. A high thiol content makes a protein susceptible to oxidation, which leads to the appearance of disulphide bonds and the formation of insoluble components. This process is normally kept under control in the lens by substances such as glutathione.

γ -CRYSTALLINS

The γ -crystallins are the least positively charged proteins at an alkaline pH and therefore move very slowly on electrophoresis. They consist of a number of closely related proteins, all containing single polypeptide chains with molecular weights of about 19 000 (Croft, 1971). They are rich in thiol groups (which means they become insoluble very easily) and unlike α -crystallins are not N-acetylated and have free N-terminal amino-acids. Human γ -crystallins behave like cryoproteins. The protein tends to precipitate from its solution at 4°C but readily redissolves on warming. These proteins are responsible for the phenomenon of 'cold cataract' (Zigman and Lerman, 1965). The γ -crystallins have an unusual amino-acid composition: alanine and lysine are rare, arginine and tyrosine abound. Furthermore, γ -crystallins appear to be relatively primitive proteins in terms of evolution and development, the trend being towards the synthesis of lens proteins of lower isoelectric point with improved oxidative stability.

It is now evident that the 4 lens antigens which Morner (1894) described as distinct chemical entities are in fact several families of proteins which are grouped together because of their solubility characteristics, molecular sizes, and surface electric charges at a particular pH. It is not surprising, therefore, that Manski (1973) now believes that the terms α -, β -, and γ -crystallins are as outdated as the term 'globulins'. There are now immunochemical data about the different β - and γ -crystallins which allow

us to classify each of the lens proteins in their β - and γ -crystallin families. In the bovine lens there are at least 7 different γ -crystallins, some of which are concentrated in the nucleus and others in the cortex (Croft, 1973).

In addition it is now well known that human as well as animal lenses contain pre- α -crystallins and crystallins which show electrophoretic mobility between β - and γ -crystallin; the latter are called β s-crystallin. The β s-crystallin, like γ -crystallin, is a low molecular weight protein, and in human cataractous lenses, when the γ -crystallins become insoluble, the β s still remain in solution (Croft, 1973; Vincentiis *et al.*, 1975). It should be realised, however, as Harding (1972) has recently pointed out, that most of the insoluble albuminoids in normal and cataractous lenses are in fact artefacts of oxidation. He has shown that, if lenses are homogenised in the absence of oxygen in an atmosphere of nitrogen alone, most of the proteins stay in solution.

In addition to the various crystallins the lens also contains other antigenic substances such as nucleoproteins, lipoproteins, glycoproteins, the various enzymes associated with cellular transport and metabolism, and the structural proteins associated with the lens capsule (Kuck, 1969).

The findings in the classical study of Uhlenhuth (1903) of a positive humoral immune response to heterologous lens antigens has been repeatedly confirmed by Hektoen (1922), Woods and Burky (1927), and many others by using simple liquid phase precipitation and complement fixation tests. The agar-gel diffusion technique of Oudin (1947) was first utilised by Rao *et al.* (1955), who were able to show that heterologous lens antisera contained antibodies to at least 6 different antigenic determinants. Several more antigenic determinants were detected when Manski *et al.* (1960) popularised the immunoelectrophoretic methods for the study of lenticular antigens.

In earlier studies the lenses were injected without any adjuvants, which meant higher doses and prolonged immunisation before a good antibody response was detected. Furthermore, the immune response in different animals was very variable and unpredictable, probably owing to innate unresponsiveness. Freund's complete adjuvant, which consists of water-in-oil emulsion with tubercle bacilli (Freund and McDermott, 1942) is a powerful immunoaccelerator. Apart from producing a depot effect leading to a prolonged antigenic exposure this adjuvant appears to break down the state of immune tolerance by a non-specific direct stimulation of T-lymphocytes. It can be recalled that T-lymphocytes are required not only for cellular immunity but also through the mechanism of cellular co-

operation for a good antibody response by the cells of the B-lymphocyte series. An incomplete Freund's adjuvant does not contain mycobacteria but acts as a good immunoaccelerator in many situations. Its role in lens antibody responses, however, has not been reported so far.

A review of the literature further suggested to us that the following aspects of humoral and cellular immune responses to heterologous lens antigens have also not been adequately investigated.

(1) Although agar-gel diffusion (i.e., Ouchterlony test), immunoelectrophoresis, complement fixation, and haemagglutination tests have been widely used, recent techniques such as immunofluorescence and immunoperoxidase methods have not been properly applied to the study of lens antibodies. Enough data are not, therefore, available to decide which techniques are best suited for the diagnosis of experimental or clinical phakoallergic* endophthalmitis. Immunofluorescence and immunoperoxidase techniques have the added advantage of localising the site of antigen-antibody reaction at microscopical levels.

(2) We are not aware of any study in which the kinetics of the humoral immune response to the same heterologous lens antigen in saline with incomplete and complete Freund's adjuvant have been examined and where the relative merits of agar precipitation, haemagglutination, immunofluorescence, and immunoperoxidase methods have been critically compared.

(3) It is now known that antibodies in man and most mammals belong to several distinct classes of immunoglobulins, and the various serological techniques which have been used in the past to detect lens antibodies demonstrate preferentially one class of immunoglobulin. For example, the agar precipitation test detects mainly high-affinity IgG, the haemagglutination test mainly IgM, and the complement fixation test only the complement-fixing IgG and IgM. Immunofluorescence and immunoperoxidase methods on the other hand can in addition detect IgD and IgE antibodies. With monospecific antisera it should now be possible to be certain which immunoglobulin is mainly involved in lens immunity.

(4) It has only recently become clear that delayed tissue allergy is produced by thymus-derived T-lymphocytes, and evidence for such a state can be obtained by a simple *in-vitro* technique in which thymus-dependent lymphocytes in tissue culture from a sensitised animal undergo blastic trans-

* The term phakoallergic endophthalmitis has been used throughout the text as a preferred alternative to such terms as lens-induced uveitis, endophthalmitis phakoanaphylactica, or phakogenetic endophthalmitis. The reasons for this are discussed in a separate communication.

formation when challenged with appropriate antigen. The transformation can be observed directly by examination of stained preparations or measured in terms of radioactive thymidine uptake (Ling and Kay, 1974). We are not aware of any study in which the lymphocytes of animals sensitised with heterologous lens antigens in saline, and with incomplete and complete adjuvants, have been examined and the response correlated with antibody levels.

(5) Although the cross-reactivity of lens antisera with ocular and extraocular tissues is well known, the microscopical localisation of these cross-reacting antigens has not so far been seriously attempted.

The present investigations were therefore carried out in an attempt to bridge some of the gaps in our knowledge concerning the antigenicity of the lens and the nature of the immune response to lens proteins and its role in ocular inflammation.

Material and methods

The experiments were performed on healthy New Zealand albino adult rabbits, litter mates being used where possible. Rat and guinea-pig soluble lens proteins in saline, or with incomplete and complete Freund's adjuvant, were injected separately into 6 groups of rabbits. The injections were given intramuscularly at different sites at 10-day intervals. Each injection contained 6 mg of soluble lens protein as measured by the technique of Lowry *et al.* (1951). The animals were bled on the day of initial immunisation and also at the time of subsequent injections in order to study the sequential antibody response. The sera were deep-frozen and stored in aliquots. After the thirteenth immunising dose the animals were skin-tested, and the site was biopsied 48 hours later. The animals were anaesthetised with intravenous sodium pentobarbitone and dissection of the right lens was performed with a Ziegler's knife using a dissecting microscope. They were killed a week later, and blood was collected from the heart into a preservative-free heparinised container for the lymphocyte transformation test. Both eyes were enucleated for microscopical examination. Two groups of control rabbits received intramuscular injections of complete or incomplete adjuvant only and blood was tested for evidence of humoral and cellular immunity to lens protein. In another group of rabbits lenses were needled without previous immunisation and the eyes examined histologically one week later for any evidence of inflammation.

IMMUNOLOGICAL INVESTIGATIONS

Agar-gel diffusion (Ouchterlony test) was performed at room temperature on glass plates coated with 1.5% agar in barbitone buffer (pH 8.2). The plates

were washed 5 days later, first in 3% saline and then in distilled water, and after drying at 37°C were stained with 0.1% nigrosine.

Immunoelectrophoresis and Osseman tests were performed on 10×10 cm agar plates coated with 1.5% agar in barbitone buffer (pH 8.2, I=0.05). The electrophoresis tank (Shandon U77) contained barbitone buffer of pH 8.6 (I=0.075). The electrophoresis was carried out at a constant current (10 mA/plate) for 3 hours according to the methods described previously (Rahi and Chignell, 1975) as a modification of the techniques of Grabar and Williams (1953) and Scheidegger (1955). The plates were washed, dried, and stained in a way similar to those described for the Ouchterlony test.

Passive haemagglutination test.—The technique used for coating the formalised sheep red cells with lens antigens was essentially the same as described by Cruickshank *et al.* (1975) for thyroid antigens. 2 mg of preabsorbed rabbit or human lens protein in phosphate buffer saline (pH 7.2, 0.15 M) was added to each millilitre of tanned red cell suspension and incubated for 30 minutes. The red cells thus sensitised were carefully washed with the buffer containing 1% normal rabbit serum, which was previously heat-inactivated and adsorbed. The fluid concentration of red cells was adjusted to 1.25%. Sera from immunised and control animals were inactivated and preadsorbed with unsensitised sheep red cells. The test was performed on the microtitre assay system (Cooke Laboratory). Proper controls were set up with sensitised cells in buffer alone and with non-immune serum obtained from the animals before initial immunisation. Controls were also set up using unsensitised cells with and without antiserum.

Immunofluorescence test.—The indirect immunofluorescence test was performed by a standard technique. A Zeiss epifluorescence microscope equipped with a fluorescein isothiocyanate (FITC) interference filter was used for the present study. Frozen sections of a composite block consisting of liver, kidney, stomach, parotid, and diaphragm from a rat and stomach from a mouse were used to detect cross-reacting antibodies in lens antisera. Rabbit, guinea-pig, and rat eye sections were used to detect an immune reaction not only with the lens but also with other ocular structures. The sera were tested at an initial dilution of 1:5, and both polyvalent (Wellcome) and IgG-specific (Dako) anti-rabbit immunoglobulin labelled with FITC were used for the present study.

Immunoperoxidase test.—Frozen sections of the composite blocks and of the eyes similar to those required for immunofluorescence studies were used for immunoperoxidase staining. Sections were first treated with 1:20 normal pig serum to block non-

specific reactions and then with 1:10 lens antiserum. After washing, peroxidase-labelled anti-rabbit IgG serum, raised in pigs (Dako) and diluted 1:50, was put on these sections which were incubated and later washed in a bath equipped with a magnetic stirrer. The slides were then incubated for 10 minutes in freshly-prepared diaminobenzidine tetrahydrochloride (DAB) reagent in a concentration of 6 mg/10 ml Tris saline and containing 2 drops of 10 vol. solution of hydrogen peroxide. The sections were washed well in distilled water and counterstained with haematoxylin. Fresh frozen sections were stained separately for endogenous peroxidase. Controls were also set up to detect any non-specific reaction with peroxidase-labelled antisera alone.

Lymphocyte transformation test.—The lymphocytes were separated from the rabbit blood by the Ficoll-Hypaque technique (Fotino *et al.*, 1971). 10^6 lymphocytes were cultured in 1 ml TC 199 prepared in HEPES buffer and enriched with 10% heat-inactivated fetal calf serum. Several such cultures were set up in Sterilin tubes. To each of the experimental tubes 0.1 ml of 1% lens solution passed through a millipore filter was added, and 48 hours later 2 μ Ci tritiated thymidine (30 000 mCi/mmol) was added to each tube and mixed. The control tubes were also treated with the same amount of radioactive isotope. After centrifugation, the proteins in the cellular deposits were precipitated with trichloroacetic acid and filtered on glassfibre filter papers, which were transferred to scintillation vials and air-

dried. The vials were counted after the addition of 10 ml toluene-based scintillation fluid, in a Packard tricarb liquid scintillation counter.

Results

Sera from all animals collected before immunisation were negative for lens antibodies as shown by simple agar diffusion, immunoelectrophoresis, passive haemagglutination, immunofluorescence, and immunoperoxidase tests. Antibodies to heterologous lens proteins appeared on the 10th day of immunisation when antigen was given either with complete or incomplete Freund's adjuvant, whereas when antigen was given in saline alone antibodies were not detectable until 30 days. The final haemagglutinin titre after 75 days of immunisation rose to 1:81 290 with complete adjuvant; when the antigen was injected with incomplete adjuvant the plateau was reached much earlier, and the haemagglutinin titre on the 75th day rose only to 1:20 840, a level which was the same as when saline homogenate only was used (Tables 1, 2, and 3).

In general, rabbits reacted better against guinea-pig lens antigens than against rat lens antigens, the number of precipitin lines being higher with the antisera produced against the former species.

At least 7 distinct antigenic substances were found in heterologous lens homogenates both by the Ouchterlony and immunoelectrophoretic methods (Fig. 1). At least 2 precipitin lines were seen in the

Table 1 Antibody response in a rabbit injected with heterologous soluble lens proteins in Freund's complete adjuvant

Methods of antibody detection	Days after initial immunisation						
	10	20	30	40	50	60	75
Gel diffusion (Ouchterlony)	—	+	+	+	+	+	+
Immunoelectrophoresis	—	(α)	(α , β)	(α , β)	(α , β)	(α , β , γ)	(α , β , γ)
Passive haemagglutination	+	+	+	+	+	+	+
	(1:10)	(1:640)	(1:5120)	(1:10 240)	(1:20 240)	(1:20 480)	(1:81 290)
Indirect immunofluorescence	$\pm$	+	+	+	+	+	+
Immunoperoxidase	*	+	+	+	+	+	+

+ Positive; $\pm$ weak positive; — negative; * not tested; (α , β , γ) = antibodies to α -, β -, and γ -crystallins

Table 2 Antibody response in a rabbit injected with heterologous soluble lens protein in Freund's incomplete adjuvant

Methods of antibody detection	Days after initial immunisation						
	10	20	30	40	50	60	75
Gel diffusion (Ouchterlony)	—	+	+	+	+	+	+
Immunoelectrophoresis	—	(α)	(α , β)	(α , β)	(α , β)	(α , β)	(α , β)
Passive haemagglutination	+	+	+	+	+	*	+
	(1:40)	(1:640)	(1:1280)	(1:2560)	(1:5020)		(1:20 480)
Indirect immunofluorescence	$\pm$	+	+	+	+	*	+
Immunoperoxidase	*	+	+	+	+	*	+

+ Positive; $\pm$ weak positive; — negative; * not tested; (α , β) = antibodies to α - and β -crystallins

Table 3 *Antibody response in a rabbit injected with heterologous soluble lens protein without adjuvant*

Methods of antibody detection	Days after initial immunisation						
	10	20	30	40	50	60	75
Gel diffusion (Ouchterlony)	-	-	-	-	++	+	+
Immunoelectrophoresis	*	*	*	*	(α)	(α, β)	(α, β)
Passive haemagglutination	-	-	++	++	(1:1280)	*	+
Indirect immunofluorescence	*	*	(1:10)	(1:640)	(1:1280)	*	(1:20 480)
Immunoperoxidase	*	*	±	+	+	*	+

+ Positive; ± weak positive; - negative; * not tested; (α, β) = antibodies to α - and β -crystallins

Fig. 1 *Ouchterlony test: The central well (LP) contains soluble lens proteins from a guinea-pig. The peripheral wells (AS) contain sera from 4 rabbits immunised with heterologous soluble lens proteins in Freund's complete adjuvant. There are a varying number of precipitin lines*

α -region, 3 in the β -region, and 2 in the γ -region when guinea-pig lens was injected with complete Freund's adjuvant. The antibodies to α -crystallins appeared several days before those to β -crystallins; antibodies to γ -crystallins developed 60 days after immunisation and then only when the antigen was injected with complete adjuvant. When the antigen was given in saline or with incomplete adjuvant (i.e., without mycobacteria), antibodies to γ -crystallins were not detectable even 75 days after continuous immunisation (Fig. 2).

The level of the immune response in the three groups of rabbits varied in a manner depending on the use of Freund's adjuvant. The kinetics of the antibody response to heterologous (mammalian) lens antigen in saline and with incomplete and complete Freund's adjuvant are shown in Fig. 3. Immunofluorescence and immunoperoxidase tests showed that antilens antibodies belong to the IgG

class. The possibility that they also belong to the IgM class, however, and also to other classes of immunoglobulins exists. Agar-gel diffusion, immunoelectrophoresis, and the Osseman test showed that extracts of iris, liver, and kidney from rabbits, guinea-pigs, and rats contained several antigenic substances which cross-reacted with antilens antibodies, mainly those against α -crystallins.

Fig. 2 *Agar-gel immunoelectrophoresis. The wells contain soluble lens proteins from a guinea-pig. The troughs are filled with heterologous lens antisera produced in rabbits with complete (CF) and incomplete (IF) Freund's adjuvant. Sera collected 20 days after immunisation contain antibodies to α -crystallins only. Although antibodies to β -crystallins are present on the 30th day of immunisation, antibodies to γ -crystallins are not even detectable 60 days after immunisation when the antigen was injected with Freund's incomplete adjuvant. They are present, however, when antigen and complete adjuvant are injected*

Fig. 3 Kinetics of average antibody response in 3 groups of rabbits injected with heterologous lens in saline (A), in incomplete (B), and in complete (C) Freund's adjuvant. The arrows represent immunising injections

The immunofluorescence test was positive with low-titre haemagglutinating sera, but the Ouchterlony test was found to be positive only when the haemagglutinin titre was 1:640 and more.

The immunofluorescence and immunoperoxidase tests showed that the antilens sera reacted strongly with the lens epithelium and the cortex. In addition, a positive reaction was seen with the iris, the ciliary body, the corneal epithelium, and the inner retina. Diffuse as well as coarse granular reactions were noted with gastric parietal cells and chief cells, hepatocytes, and renal tubular epithelium, suggesting the presence of antimitochondrial and antiribosomal antibodies in lens antisera. Moreover, some of the stronger antisera reacted with skeletal muscles in the rat diaphragm and smooth muscle fibres in the rat stomach and glomeruli, suggesting the presence of antibodies to contractile proteins. Some of the antisera also contained weak antinuclear antibodies (Figs. 4, 5, 6, 7, and 8).

The lymphocyte transformation test gave variable results. The transformation index was obtained by dividing the average radioactive count in the experimental tube by the average count in the control tube. The transformation index was highest (i.e., 2.5) when guinea-pig lens with Freund's adjuvant was used for immunisation. With incomplete adjuvant the index was only 1.5, whereas with saline homogenate very little change was observed. Animals immunised with complete and

Fig. 4 Indirect immunofluorescence test with unfixed sections of rat liver. The antilens serum reacts with cytoplasmic organelles (e.g., mitochondria and ribosomes) to produce a granular fluorescence. $\times 400$

Fig. 5 Parietal and chief cells of a mouse stomach, showing bright cytoplasmic fluorescence in the presence of lens antibody. $\times 315$

incomplete Freund's adjuvant but without lens protein showed no evidence of sensitisation to lens crystallins.

The changes in the skin in immunised rabbits after lens injection were minimal and showed a mixture of polymorphonuclear and lymphocytic infiltration. The needled eyes appeared inflamed clinically, as evidenced by circumcorneal congestion, aqueous flare, miosis, vasodilatation in the iris, and some cloudiness of the cornea, but the inflammation

persisted for only a few days, and histological examination a week later showed little evidence of a typical phakoallergic endophthalmitis. The needled lenses showed only mild cellular infiltration, consisting mainly of macrophages, and the iris appeared haemorrhagic and congested; some inflammatory cells were present in the anterior chamber (Figs. 9 and 10).

Discussion

The present study confirms earlier observations that soluble lens material consists of a large number of closely related but antigenically distinct components (Rao *et al.*, 1955; Manski, 1973) instead of only 4 proteins as previously suggested (Mörner, 1894; Burky and Woods, 1928). It also confirms the findings of Manski *et al.* (1965) that heterologous immunisation as opposed to homologous immunisation leads to antibody production against the whole range of α -, β -, and γ -crystallins. We have shown for the first time, however, that the levels of both humoral and cellular immune responses to heterologous lens proteins injected with incomplete adjuvant are higher than with simple saline homogenates but much weaker than with complete adjuvant. This suggests that for a good antibody response, especially to low-molecular-weight lens proteins such as γ -crystallins, the presence of mycobacteria in the adjuvant mixture is essential. This is in contrast to the response to retinal antigens, in

Fig. 6 Lens antiserum containing antibodies to ribosomes. Note the patchy fluorescence of the renal tubules. $\times 285$

Fig. 7 Positive indirect immunofluorescence test for antibodies to smooth muscle proteins. Two glomeruli and a blood vessel in a rat kidney show bright fluorescence. $\times 380$

which mycobacteria may sometimes suppress the immune response (Wacker and Lipton, 1971). Furthermore, mycobacterial proteins, as evidenced by lymphocyte transformation studies, appear to be necessary also for an effective stimulation of T-lymphocytes, which are required not only for delayed hypersensitivity reactions but also for a good antibody response by B-lymphocytes through the well-known mechanism of cellular co-operation.

Although in most textbooks lens proteins are still considered to be sequestered antigens, which means that animals should be intolerant to them and should be able to produce antibodies without the addition of adjuvant, our study showed that animals are in fact tolerant to at least γ -crystallins and that antibodies and cellular immune responses cannot be evoked without the use of an adjuvant enriched with mycobacterial proteins. Furthermore, the kinetics and the variability of the immune response studied

in several groups of animals clearly shows the role of immunological tolerance. It is now known that the immune responsiveness to a variety of antigens is controlled by the immune response genes (i.e., Ir gene) which are associated with the major histocompatibility region of the chromosome (Ellman *et al.*, 1970). The present study clearly emphasises the role of mycobacteria and the immune response gene in humoral and cellular immunity to lens antigens in general and γ -crystallins in particular. It is known that γ -crystallins are of low molecular weight and that lens antigens are detectable in normal aqueous although in small amounts (Clayton *et al.*, 1968), so that it is possible that γ -crystallin acts in a similar way to serum thyroglobulin (Bankhurst *et al.*, 1973), leading to a state of immunological tolerance involving mainly the T-lymphocytes. This aspect of lens immunology has not been examined before and demands further critical study.

Fig. 8 Hyperimmune serum from a rabbit injected with heterologous lens proteins in Freund's complete adjuvant. Antibodies react with the corneal epithelium and the keratocytes to produce nuclear fluorescence. $\times 400$

Fig. 9 Phagocytic mononuclear cells surround the needle-shaped cleft of an immune rabbit with circulating heterologous lens antibody. H and E. $\times 835$

A comparative study of the 5 different methods for the detection of specific antibodies which have been performed for the first time on the same serum samples shows clearly that the best method for detecting antibodies to lens proteins is the passive haemagglutination technique which becomes positive before the other tests. Immunofluorescence and immunoperoxidase tests are slightly less sensitive and perhaps more subjective. Agar diffusion and immunoelectrophoresis are least sensitive, being positive only when the lens haemagglutinin titres are 1:640 or more. The one advantage of the agar

diffusion technique, however, lies in its capacity to detect and differentiate a large number of antigen-antibody systems in a heterogeneous mixture of proteins.

The immunofluorescence and immunoperoxidase tests have the one advantage over the haemagglutination and agar diffusion tests of being able to detect the chemical class of the immunoglobulins involved in an immune reaction. It has been possible, therefore, to state with certainty that IgG is involved in lens antibody reactions. This does not exclude, however, the role of other immunoglobulins.

Fig. 10 Mildly inflamed iris, showing mainly congestion and haemorrhage 1 week after discission of the lens in a rabbit immunised with heterologous lens in Freund's complete adjuvant. $\times 200$

Our study of cross-reactivity has shown for the first time that potent lens antisera contain antibodies to mitochondria, ribosomes, contractile proteins, and cell nuclei. As previously noted in this paper, lens epithelium and the superficial cortex contain all or most of these organelles, which are themselves complex structures made up of a variety of proteins both structural and enzymatic. Furthermore these organelles, though not discernible electron microscopically in the deeper regions of the lens (Hogan *et al.*, 1971), might be present as inspissated structureless masses. That this may be so is supported by the evidence that ribonucleoproteins (RNA) can be extracted in a large amount not only from the lens cortex but also from the lens nucleus (Maraini *et al.*, 1964).

By means of differential centrifugation and equilibrium centrifugation in density gradients it has been shown that antibodies which react with the chief cells of the stomach, the hepatocytes, and the renal tubular epithelium to give diffuse fluorescence are antiribosomal (antimicrosomal) antibodies, whereas those which react with gastric parietal cells, hepatocytes, and the tubular epithelium to give a rather granular fluorescence are antimitochondrial antibodies (Taylor *et al.*, 1962; Anderson *et al.*, 1967; Wright, 1975). It is of interest that both these antibodies are non-species and non-organ specific. It is now known that antibodies to contractile proteins such as myosin and actin, which are common in autoimmune disorders including uveitis, react well with smooth muscle fibres in the rat stomach, the blood vessels, and the glomeruli (Rahi *et al.*, 1976). A similar fluorescence observed with antilens sera clearly shows that these sera contain antibodies to cell mitochondria, smooth muscle contractile proteins, and ribosomes. The exact nature of the antigens associated with these organelles is unknown but it is possible that it is composed of a mixture of structural and enzymatic proteins. In this context it is of interest that microsomal fractions from non-mammalian species (e.g., chick liver) contain antigens resembling important soluble crystallin (FISC) and long-line crystallins (Vincentiis *et al.*, 1975) of the avian lens.

The modern concept that lens antigens, which were once believed to be organ-specific are neither tissue- nor species-specific (Perkins and Wood, 1963; Clayton *et al.*, 1968; Bours and Doorenmaalen, 1972) is compatible with the findings presented in this paper, which provide for the first time a rational explanation, at least in part, for the cross-reactivity of antilens sera. Almost every tissue in the body contains mitochondria, endoplasmic reticulum (i.e., the microsomes of the cell biochemist), nuclei, and contractile filaments and would

be expected to cross-react with potent antisera produced against the lens homogenates, which for developmental reasons contain all these structures entrapped among the specific lens crystallins.

In our experiments systemic immunisation with heterologous lens protein followed by discission of the lens did not lead to a typical phakoallergic endophthalmitis. This is in keeping with the concept that heterologous antibodies are less pathogenetic than autoantibodies and that previous attempts to produce such inflammation resulted in variable and often unpredictable changes, and never typical granulomatous inflammation around the lens (Verhoeff and Lemoine, 1922; Burky, 1934; Scobee and Slaughter, 1944; Muller, 1952). It is known that the insoluble albuminoid is more potent than soluble lens crystallins in inducing T-cell sensitisation and therefore delayed tissue allergy (Manski, 1973). Since in the present study only soluble lens proteins were used, it is likely that the rabbits were not sufficiently sensitised to manifest a granulomatous reaction, which is the common histological feature of delayed hypersensitivity. That this is the case is supported by the fact that the animals in the present series did not show a marked lymphoblastic change when challenged *in vitro* with lens antigens. The histological changes in the needled eyes were reminiscent of a Schwartzman reaction.

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