Immunopathology of the lens

III. Humoral and cellular immune responses to autologous lens antigens and their roles in ocular inflammation

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SUMMARY In experimental rabbits it has been shown for the first time that autologous lens protein is antigenic when injected with Freund's complete adjuvant. Although lens haemagglutinins were detected in 6 out of 7 rabbits, in only 3 of the 6 animals did the titre reach a maximum of 1:640. A weak agar precipitation reaction was obtained with only 1 of the 3 sera. It would appear, therefore, that the passive haemagglutination test is superior for the detection of autologous lens antibodies.

The response to autologous lens antigens both in magnitude as well as in duration varied in different rabbits, which suggested to us the possible role played by a central control mechanism involving the immune-response (Ir) and immune-associated (Ia) genes which are part of the major histocompatibility complex. Alternatively, this variation may be the result of an antigenic competition between various autologous crystallins.

Antibodies to autologous lens protein as detected by immunofluorescence and immunoperoxidase techniques were shown to be of the IgG class. Systemic autologous immunisation produces only a mild uveitis and does not lead to a granulomatous intraocular inflammation. Intravitreal injections of autologous lens protein in pre-immunised animals, however, produced an Arthus type of acute endophthalmitis. Autologous lens antisera showed limited cross-reactivity with ocular and extraocular tissues, which could be detected only by such sensitive techniques as immunofluorescence and immunoperoxidase methods.

Uhlenhuth (1903) showed that rabbits could be immunised by repeated intravenous injections of bovine lens and that their sera showed positive precipitation reactions with lens proteins from other animals. It was well known at that time that injury to the lens in human beings might in some cases be followed by an inflammatory reaction. Verhoeff and Lemoine (1922), encouraged by these findings of Uhlenhuth, proposed that this 'endophthalmitis phako-anaphylactica' (the original term used by Straub (1919) was endophthalmitis phakogenetica) may depend upon some form of hypersensitivity to lens proteins. They strengthened this hypothesis by experiments in guinea-pigs and rabbits in which pre-immunisation with heterologous lens protein followed by dissection of the lens led to an intraocular inflammation. This reaction was produced, however, after the combination of autologous antigens from the ruptured lens with antibodies produced against

heterologous lens protein. It cannot be regarded, therefore, as a true autoimmune reaction, in which it is essential that the autologous lens should also act as the immunising antigen. In a later study Manski *et al.* (1965) claimed to have induced lens autoantibody production in rabbits by heterologous and homologous immunisation, because in their study the antiserum thus produced reacted *in vitro* with lenses obtained from the immunised animals. Clearly, this is a well-known phenomenon of cross-reactivity, and unless an antiserum is raised against autologous lens antigen the antibodies reactive with these proteins *in vitro* cannot be regarded as true autoantibodies.

In a recent study Behrens and Manski (1973) immunised inbred rats with homologous lens proteins from litter mates and obtained an antibody response to α -crystallins. Since the animals used were inbred, the authors argue that this may be regarded as equivalent to autoimmunisation. Although there may not be any appreciable differences

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in the nature of immune responses, the results of such experiments also cannot be regarded as true autoimmunity.

The present work was undertaken, therefore, to study both humoral and cellular immune responses to autologous lens proteins in rabbits. Although agar gel diffusion, immunoelectrophoresis, and passive haemagglutination techniques have been used widely to test homologous and heterologous lens antibody responses, and although data are now available on other sophisticated techniques such as immunofluorescence and immunoperoxidase methods (Misra *et al.*, 1977; Rahi *et al.*, 1977), it is not known which technique is best suited for the diagnosis of phakoallergic endophthalmitis* in man and experimental animals. Moreover, the class of antibody involved in this autoimmune reaction is unknown. Although it has been suggested that lens-induced uveitis is primarily an immune-complex disease (Marak *et al.*, 1976), it has also been claimed that a cellular hypersensitivity reaction plays an important pathogenetic role (Kincses and Szabo, 1976). Since studies have not been performed on experimental animals to examine critically the role of both B and T lymphocytes in an immune response to autologous lens protein, the nature of the lesion in clinical practice remains in dispute. Furthermore, although the cross-reactivity of heterologous lens antisera with various other ocular and extraocular tissues is well documented, it is still unknown if lens autoantibodies similarly show a degree of non-specificity.

Methods and materials

The experiments were performed on 7 New Zealand albino adult rabbits using litter mates where possible. Extracapsular lens extraction was performed on 1 eye under sodium pentobarbitone anaesthesia using a standard technique and a dissecting microscope, after which some lens matter remained *in situ*. The lenses were homogenised separately, divided into aliquots, and stored frozen. Each rabbit received 4 intramuscular injections of autologous lens in Freund's complete adjuvant at 10-day intervals. They were bled on the day of initial immunisation and also at the time of subsequent injections to study the sequential antibody response. The serum specimens were also deep frozen in aliquots. After the fourth immunising dose (i.e., 30 days) the animals were skin-tested and the sites biopsied 48 hours later. In 2 preimmunised animals 0.05 ml of autologous lens material was

injected intravitreally into the operated eye. All the animals were killed 10 days after the last immunising dose, and blood was collected from the heart in preservative-free heparinised containers for lymphocyte transformation tests. Both eyes were enucleated for microscopical examination. One control group of rabbits received intramuscular injections of Freund's complete adjuvant only, and blood was tested for evidence of humoral and cellular immunity to lens protein. In another group of rabbits not previously immunised 0.05 ml of autologous lens material was injected into the vitreous of 1 eye, and both eyes were examined histologically 1 week later for any evidence of inflammation.

Immunological investigations

The *Ouchterlony* test was performed on glass plates coated with 1.5% agar in barbitone buffer (pH 8.2). The plates were kept at 4°C for 1 week and then washed in 3% saline and later in distilled water. After air-drying they were stained with 1% nigrosine.

Immunoelectrophoresis and *Osserman* tests were performed on similar glass plates coated with 1.5% agar in barbitone buffer (pH 8.2; I=0.05). The electrode compartments of the electrophoresis tank were filled with barbitone buffer pH 8.6 (I=0.075). The electrophoresis was carried out at a constant current (10 mA/plate) for 3 hours according to the method described previously (Rahi and Chignell, 1975).

Passive haemagglutination test.—The technique used was essentially the same as described previously (Rahi *et al.*, 1977). The volume of tanned red cells coated with antigen was modified according to the availability of autologous lens protein. The test was performed on a microtitre assay system (Cooke Laboratory). Sera from immunised and control animals were heat-inactivated and preadsorbed with unsensitised sheep red cells. 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 anti-serum.

The indirect immunofluorescence test. was performed using a standard technique. A Zeiss epifluorescence microscope equipped with an 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 autologous antilens sera. Rabbit, guinea-pig, and rat eye sections were used to detect immune reactions not only with the lens

*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.

but also with other ocular structures. The sera were tested at an initial dilution of 1:2 and both polyvalent (Wellcome) and IgG specific (Dako) anti-rabbit immunoglobulin labelled with fluorescein were used for the present study.

The immunoperoxidase test.—Frozen sections of the composite block and of the eyes, similar to those required for immunofluorescence study, were used for immunoperoxidase staining. The sera were tested at the initial dilution of 1:2 and the technique was essentially the same as described previously (Rahi *et al.*, 1977).

The lymphocyte transformation test.— 10^6 lymphocytes separated from the blood of the immunised animals by the Ficoll-Hypaque technique were cultured in sterilised tubes containing 1 ml of medium 199, which was prepared in HEPES buffer. Antibiotics in the usual concentration were added to the medium, which was enriched by the addition of 10% fetal calf serum. 1 mg of autologous or homologous lens protein was added to each of the experimental tubes, and 48 hours later $2 \mu\text{Ci}$ of tritiated thymidine (30 000 mCi/mmol) were added to both the control and the experimental tubes. After further incubation overnight at 37°C the tubes were centrifuged, and trichloroacetic acid was added to the sediment to precipitate the proteins. These were filtered on glassfibre filter-paper and washed in methanol. The filter-papers were then transferred to scintillation vials and air-dried. The radioactivity was measured in a Packard tricarb liquid scintillation counter after the addition of 10 ml of toluene-based scintillation fluid.

Results

Blood collected from rabbits before immunisation was negative for lens antibodies.

Antibodies to autologous lens protein injected with Freund's complete adjuvant were detectable by the

passive haemagglutination test in 2 rabbits on the 10th day of immunisation. In 4 others it was delayed for several more days and in 1 rabbit no antibody was detected even after 40 days (Fig. 1; Tables 1 and 2).

Fig. 1 Biological variations in antibody response in a group of 7 rabbits injected with autologous whole-lens protein in Freund's complete adjuvant, 40 days after regular immunisation at 10-day intervals

Table 1 Antibody response in a rabbit injected with autologous whole-lens homogenate in Freund's complete adjuvant

Methods of antibody detection	Days after initial immunisation			
	10	20	30	40
Gel diffusion (Ouchterlony)	—	—	—	±
Immuno-electrophoresis	—	—	—	—
Passive haemagglutination	+	+	+	+
	(1:10)	(1:40)	(1:160)	(1:640)
Indirect immunofluorescence	—	±	+	+
Immunoperoxidase	*	*	*	+

+ Positive, ± weak positive, — negative, * not tested

Table 2 Antibody response in a group of rabbits injected with autologous lens protein in Freund's complete adjuvant; 40 days after regular immunisation at 10-day intervals

Methods of antibody detection	Rabbits studied						
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7
Gel diffusion (Ouchterlony)	—	±	—	—	—	—	—
Immuno-electrophoresis	—	—	—	—	—	—	—
Passive haemagglutination	+	+	+	+	+	—	+
	(1:640)	(1:640)	(1:10)	(1:40)	(1:640)	—	(1:10)
Indirect immunofluorescence	+	+	—	±	+	—	—
Immunoperoxidase	+	+	*	*	*	—	*

+ Positive; ± weak positive; — negative; * not tested

Fig. 2 *Passive haemagglutination test: Lens antibody titre in a rabbit before immunisation (i.e., day 0) and at 10, 20, 30, and 40 days after immunisation with autologous lens protein in Freund's complete adjuvant*

Fig. 3 *Kinetics of antibody response in 4 rabbits injected with autologous lens protein in Freund's complete adjuvant. The arrows represent immunising injections*

The final haemagglutinin titre 40 days after immunisation rose to 1:640 in 3 of the 7 rabbits.

A faint precipitin line was observed in the Ouchterlony plate with the serum from 1 rabbit and only after 40 days of regular immunisation. The haemagglutination titre in this rabbit at that time was 1:640 (Fig. 2).

The level of the immune response was subject to individual variation, and the kinetics of the antibody response to the autologous lens antigens are shown in Fig. 3.

The immunofluorescence and immunoperoxidase tests showed that the antilens sera reacted strongly with the lens epithelium and the outer cortex (Fig. 4). In addition sera with high lens haemagglutinin titre showed cross-reactivity with the iris (Fig. 5) and the corneal epithelium. A similar cross-reactivity, although of a relatively weak nature, was also observed with extraocular tissues such as smooth

Fig. 4 *Indirect immunofluorescence test: The anti-lens serum contains IgG antibodies which react strongly with unfixed sections of rabbit lens. $\times 480$*

Fig. 5 The iris of a rat showing bright fluorescence in the presence of lens antibody. $\times 480$

muscle fibres in the stomach, the hepatocytes and the renal tubules (Figs. 6 and 7) as previously described for heterologous and homologous antilens sera (Rahi *et al.*, 1977; Misra *et al.*, 1977).

The immunofluorescence and immunoperoxidase tests also showed that autologous lens antibodies belong to the IgG class. The possibility that they also belong to other classes of immunoglobulins, however, cannot be excluded.

Although the immunofluorescence and immunoperoxidase tests were positive with all those sera which contained haemagglutinating antibodies ($> 1:$

40), the immunofluorescence test being strongly positive when the lens haemagglutinin titres were 1:160 and above, the agar diffusion technique of Ouchterlony, in contrast, showed a weak positive result in 1 case when the haemagglutination titre was 1:640. The relative sensitivity of these *in vitro* techniques are shown in Table 2.

The lymphocyte transformation test was positive in only 3 of the 7 rabbits tested. The level of cellular hypersensitivity, however, was low (mean lymphocyte transformation index = 2.15) but correlated well with the lens haemagglutinin titre in the serum.

Fig. 6 Indirect immunoperoxidase test: The lens antiserum reacts with the cytoplasm (arrows) of the tubular epithelium of a rat kidney, suggesting the presence of mitochondrial and ribosomal antibodies. The nuclei have been counterstained with haematoxylin. $\times 900$

Fig. 7 Sections of a rat liver showing a positive immunoperoxidase test. The lens antiserum seems to contain antibodies which react with the cytoplasmic organelles (e.g., mitochondria and ribosomes) of the hepatocytes. The nuclei have been counterstained with haematoxylin. $\times 900$

Fig. 8 The iris and ciliary body of an immune rabbit show infiltration by a small number of lymphocytes and plasma cells. H and E. $\times 900$

Fig. 9 The choroid from the same rabbit immunised with autologous lens in complete Freund's adjuvant shows lymphocytic and plasma cell infiltration. H and E. $\times 900$

Histological examination of the eyes from immunised rabbits showed occasional lymphocytes and plasma cells in the iris, the ciliary body and the choroid (Figs. 8 and 9). There was evidence of subcapsular fibrosis in the lens remnants (Fig. 10), which were surrounded by lens-protein-containing macrophages (Fig. 11). In 2 preimmunised rabbits in which autologous lens extract was injected into the vitreous a hypopyon was present in the anterior chamber, and the lens remnants were surrounded by neutrophils, which were also seen in the vitreous and the retina. There was no evidence of a granulomatous reaction in any of the eyes, but there were a few large macrophages which contained nuclear debris in the anterior chamber (Fig. 12).

Discussion

The present study has shown for the first time that animals can be immunised against their own lens proteins, thus supporting the idea that auto-immunisation may lead to intraocular inflammation. The antibody response on the 40th day (i.e., after the fourth and final injection), however, was much weaker than that to heterologous lens antigens for the same period of immunisation (Rahi *et al.*, 1977). A similar weak response to homologous lens

proteins was observed during the early weeks of immunisation, the antibody titre reaching a peak only after 3 months (Misra *et al.*, 1977).

In 1 rabbit only a precipitating antibody was present which was probably against the more strongly antigenic α -crystallin. In the remaining animals antibodies were detected only by the much more sensitive haemagglutination technique (concentration of the serum or separation of the globulins from the other serum proteins was not attempted). It is possible that if this is done in future experiments the agar-gel immunoelectrophoretic methods may provide a better insight into the antigenic components of the autologous lens proteins.

Since it has been shown that the maximum haemagglutinin titre on the 40th day of immunisation using heterologous lens in Freund's complete adjuvant is 1:10 240 (Rahi *et al.*, 1977) as compared with a 1:640 titre obtained with either homologous (Misra *et al.*, 1977) or autologous lenses, it now seems certain that heterologous lens proteins are much more strongly antigenic than either homologous or autologous lens antigens. Furthermore, the haemagglutinin titre to autologous lens proteins, even when injected with Freund's complete adjuvant, never went higher (and, in fact, in some animals remained much lower) than the antibody response

Fig. 10 The lens remnants of a rabbit immunised with autologous lens in complete Freund's adjuvant show lymphocytic infiltration and subcapsular fibrosis. *H and E.* $\times 900$

to heterologous lens protein when injected without any adjuvant.

It is possible, however, that there are no real differences in the antigenicity of the various lenses but that the rabbits show a degree of immune unresponsiveness to lens proteins which can be overcome only with the addition of strong adjuvant when autologous antigen is used. In contrast, this tolerance seems to break down much more easily even when small doses of heterologous lens proteins are injected. In this situation adjuvant is required only when it is desirable to produce an accelerated immune response with a high antibody titre.

Since the antibody titre in 4 out of 7 rabbits remained very low even after 40 days of immunisation, it seems unlikely that animals will respond to autologous lens proteins when given without adjuvant unless injected in large doses, which is not possible for obvious reasons. This aspect of lens immunology is being investigated and will be reported at a later date.

In contrast to humoral responses, however, a complete Freund's adjuvant is required for a good cell-mediated immune response to lens protein irrespective of whether the antigens are auto-, homo-, or heterologous in nature.

The level of the antibody response to autologous lens protein varied in different animals, suggesting the possible role played by the immune-response (Ir) and immune-associated (Ia) genes, which are believed to control the immune reactivity of the lymphocytes of an individual to a variety of different antigens (Ellman *et al.*, 1970; Festenstein and Pena-Martinez, 1975; Munro and Taussig, 1975). Since in our study whole-lens homogenate rather than purified proteins was used for immunisation, it is possible that the variation in the immune response was due to the well-documented phenomenon of antigenic competition (Taussig *et al.*, 1973) between the molecules of the various crystallins. Furthermore, it is also possible that the variability of the

Fig. 11 Macrophages (arrows) and lymphocytes surround lens remnants in a rabbit immunised with autologous lens proteins in Freund's complete adjuvant. H and E. $\times 900$

Fig. 12 The anterior chamber of an immunised rabbit 24 hours after an intraocular injection of autologous lens homogenate, showing a large number of neutrophils and a few macrophages which contain nuclear debris. $\times 900$

response to lens antigens is not due to tolerance or antigenic competition but to another phenomenon in which T lymphocytes act as suppressor cells (Weber and Kolsch, 1973).

The immunofluorescence and immunoperoxidase tests used in this study have the one advantage over the haemagglutination test in 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 autologous lens antibody reactions. This does not exclude, however, the possible role of other immunoglobulins.

Systemic immunisation with autologous lens protein did not lead to a granulomatous inflammatory reaction either in the uvea or in and around

the lens remnants. There was some evidence, however, of a mild immune reaction as evidenced by the infiltration of the anterior uvea by a small number of lymphocytes and plasma cells and the presence of macrophages in the region of lens remnants. Intravitreal injections of autologous lens proteins in preimmunised animals led to marked polymorphonuclear infiltration suggesting an Arthus type of reaction. In addition there were a few large histiocytes which contained nuclear debris.

Since in the present study only soluble lens proteins were used and in relatively small doses, it is possible that the rabbits were not sufficiently sensitised to manifest a granulomatous reaction, which is a common histological feature of a delayed hypersensitivity reaction. 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 autologous lens antigen.

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