Ocular antigens VIII: assay of delayed hypersensitivity to corneal epithelium in the rabbit

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SUMMARY Rabbits were sensitised with complete bovine corneal epithelium. The lymphocyte stimulation test was performed with the lymphocytes of these rabbits using the soluble and sonicated insoluble fraction of the corneal epithelium as the antigens. A striking difference existed in the optimal test conditions for these antigen fractions.

By comparing the results of the lymphocyte stimulation test with other immunological parameters, namely, skin test reaction, antibody titre, and phytohaemagglutinin stimulation of the lymphocytes, we concluded that both antigen fractions stimulate predominantly the T-lymphocyte system, although boosting augmented the humoral immune response. Stimulation of the cultured lymphocytes by both the separate and mixed antigen fractions is evidence for the existence of cross-reacting antigens between the soluble and insoluble epithelial fractions.

It is generally accepted that T-lymphocyte cytotoxicity is the principal effector of corneal graft rejection. By means of the leucocyte migration inhibition test as a detector of delayed hypersensitivity cellular immunity to corneal antigens has been established in several disorders of the cornea and after keratoplasty (Henley *et al.*, 1971; Shore *et al.*, 1972; Henley and Okas, 1974; Szabó *et al.*, 1975). Moreover, it has been shown that antigens extracted from the whole cornea can elicit cellular immunity in guinea pigs (Ugrinski and Kirkpatrick, 1974).

Most of these studies were performed with soluble antigens extracted from the whole cornea. In addition to the intrinsic antigens of the cornea there are appreciable amounts of serum proteins especially localised in the stroma (Broekhuysen, 1972). These serum proteins, with the exception of an albumin-like protein, are absent in the epithelium of the cornea (Berger, 1969), which makes the epithelium suitable for a study of the immunological properties of corneal tissues. The possible role of the insoluble fraction of the corneal tissues, including cell membranes and connective tissue, needs further investigation. This fraction contains various antigens, including the membrane-associated histocompatibility antigens and structural glycoproteins. The latter appeared to display transplanta-

tion antigen activity (Treffers and Broekhuysen, 1977). Whether sensitisation with xenogenic mixtures supplies adequate information about allogeneic transplantation is doubtful, because this transplantation largely depends on histocompatibility antigens. We have, however, carried out such sensitisation in the present study as a preparatory investigation into the nature of the antigenicity of soluble and insoluble (membranous) epithelial constituents of the cornea.

After determining the optimal conditions for the lymphocyte stimulation test we compared the antigenicity of both the soluble and the particulate antigens of the corneal epithelium and correlated these results with other immunological parameters, namely, the antibody titre, the skin test, and phytohaemagglutinin stimulation.

Material and methods

ISOLATION OF THE EPITHELIAL ANTIGENS

Fresh calf eyes were washed with saline, dipped in 0.25% chloramphenicol, and transported on ice to the laboratory. All further procedures were carried out at 0 to 4°C unless indicated otherwise. After rinsing the cornea with saline only the central part of the epithelium was scraped off to avoid contamination with other tissue. Homogenisation was carried out in a Potter-Elvehjem homogeniser in 0.01 M ammonium acetate buffer (pH 7.2); the

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mixture was sonicated until a homogeneous suspension was obtained. For separating the soluble and insoluble fraction the homogenate was centrifuged at 15 000 *g* for 30 min. The supernatant was lyophilised, and the soluble epithelial fraction (EpSo) was stored at -20°C until used. The sediment was washed once with 0.01 M ammonium acetate buffer and several times with water. The sediment was then lyophilised and the product, the insoluble epithelial fraction (EpIn), was stored at -20°C until used.

SENSITISATION

Seventeen adult New Zealand rabbits of both sexes were injected subcutaneously with sonicated complete epithelium (100 mg wet weight dispersed in 1 ml phosphate buffered saline (PBS) and emulsified with 1 ml complete Freund's adjuvant (FCA)). After 1 week 8 rabbits received another identically prepared injection subcutaneously in order to stimulate further antibody response. Three rabbits were injected subcutaneously with 15 mg EpSo which had been solubilised in 1 ml PBS and emulsified with 1 ml FCA. Skin tests were carried out by injecting 0.05 ml EpSo solution in PBS (5 mg dry weight per ml). The reaction was recorded at 24 hours. Unsensitised rabbits were used as controls.

LYMPHOCYTE CULTURE

Blood was withdrawn from an ear vein or from the heart and defibrinated with glass beads. Lymphocytes were isolated from the diluted blood as described by Du Bois *et al.* (1973). 10⁶ lymphocytes were suspended in 1 ml Eagle's minimal essential medium (MEM) buffered with 0.025 M Tris (pH 7.4); this solution was supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 15% homologous inactivated rabbit serum. The lymphocytes were stimulated with 50 µg PHA-P (Difco), and with varying amounts of EpSo and EpIn. The EpSo and EpIn solutions were sterilised by ultrasonic treatment. Sterility was checked by incubating EpSo and EpIn alone in the culture medium.

The cells stimulated with PHA-P were harvested after 50 hours and those stimulated with EpSo or EpIn as indicated below under 'Results'. Twenty-four hours before harvesting, 0.5 µCi ³H-thymidine (Radiochemical Centre, Amersham, England; SA 23.3 Ci/mmol) was added in 0.02 ml MEM. The cells were collected on Millipore glass fibre filters and washed with saline. Radioactivity was determined by incubating the filters (after drying by heat) in 6 ml scintillation fluid containing 100 mg dimethyl-POPOP and 5 g PPO in 1 litre toluene. Counting was performed in an Isocap 300 scintillation counter (Nuclear Chicago). The results are reported as the stimulation index (SI), which is

expressed as: $SI = (\text{cpm of stimulated cells}) / (\text{cpm of unstimulated cells})$. A $SI \geq 2$ is considered to be positive.

ANTIBODY TITRE

The antibody titre was determined by the haemagglutination test as described by Herbert (1973) using 1 ml 2% (w/v) tanned erythrocytes incubated with 1 mg antigen (Treffers and Broekhuysen, 1977).

Results

CULTURE PERIOD

Table 1 shows the results of ³H-thymidine incorporation into lymphocytes stimulated with 500 µg EpSo and 250 µg EpIn using different culture periods. Maximal stimulation indices were achieved between 4 and 6 days for EpSo and between 3 and 4 days for EpIn. A period of 5 days for EpSo and a period of 4 days for EpIn were used for further investigations.

DOSE RESPONSE

Table 2 shows the results of the ³H-thymidine incorporation into lymphocytes cultured in the presence of various amounts of EpSo and EpIn. Mean values of maximal stimulation indices by these fractions occurred at concentrations between 490 µg and 780 µg for EpSo, and between 50 µg and 240 µg for EpIn. For EpSo a concentration of 500 µg and for EpIn a concentration of 100 µg were considered to be optimal for further investigations.

Table 1 1×10^6 rabbit lymphocytes sensitised with complete corneal epithelium were stimulated with 500 µg EpSo or 250 µg EpIn during varying periods of culture. The average period yielding the maximal stimulation index is given

Average culture period (days) $\pm$ SD	
EpSo	EpIn
5.0 $\pm$ 1.0	3.7 $\pm$ 0.75
(n=9)	(n=7)
(range 4-6)	(range 3-5)

Table 2 1×10^6 rabbit lymphocytes sensitised with complete corneal epithelium were stimulated with various amounts of EpSo and EpIn for 5 days and 4 days respectively. Range of dry weight material giving the maximal stimulation index is given

Range of dry weight material (µg) for maximal SI	
EpSo	EpIn
490-780	50-240
(n=9)	(n=6)

Table 3 Stimulation of corneal-epithelium-sensitised lymphocytes with EpSo (5 days' cultivation with 500 µg), EpIn (4 days' cultivation with 100 µg), EpSo plus EpIn (4 days' cultivation with 500 µg EpSo plus 100 µg EpIn), and PHA-P (50 h cultivation with 50 µg) is compared with the antibody titre of EpSo and EpIn and the skin test intensity recorded after 24 h (mm). Stimulation is given as the stimulation index

Rabbit no.	SI				Antibody titre		Skin reaction
	EpSo	EpIn	EpSo + EpIn	PHA-P	EpSo	EpIn	EpSo (ϕ in mm)*
8677	75.3	61.9	73.9	82.9	2 000	4 000	17
8678	19.4	15.0	15.6	30.2	500	100	17
8679	65.7	20.7	22.5	48.6	1 000	2 000	28
8680	81.6	23.9	38.9	152.2	2 000	4 000	26
8681	43.9	24.3	37.5	251.9	4 000	4 000	20
8683	68.0	28.0	18.0	46.2	1 000	500	20
8684	44.0	15.3	36.4	45.1	2 000	4 000	20
8685	69.7	28.5	34.5	52.8	4 000	8 000	26
8686	16.8	16.3	18.1	73.9	2 000	2 000	25
Mean	53.8	26.0	32.8	87.1	2 000	3 000	22

* ϕ represents diameter of erythema.

Table 4 As Table 3. The rabbits received a second dose of antigen emulsified in FCA 1 week after first injection

Rabbit no.	SI				Antibody titre		Skin reaction
	EpSo	EpIn	EpSo + EpIn	PHA-P	EpSo	EpIn	EpSo (ϕ in mm)*
8	4.5	1.1	2.6	6.7	8 000	16 000	9
9	ND	ND	ND	23.4	2 000	8 000	12
10	10.8	3.8	5.5	50.6	4 000	8 000	10
11	11.1	6.0	7.9	9.3	8 000	8 000	20
13	43.3	10.2	ND	60.4	8 000	4 000	18
14	81.8	9.2	ND	45.1	8 000	16 000	19
15	ND	ND	ND	38.2	4 000	8 000	18
16	17.6	12.2	ND	4.8	4 000	8 000	21
Mean	28.2	7.1	—	29.8	6 000	9 500	16

* ϕ represents diameter of erythema. ND = not done.

STIMULATION UNDER OPTIMAL CONDITIONS

Table 3 gives the results of experiments in which the lymphocytes from corneal-epithelium-sensitised rabbits were tested under optimal conditions. A high SI found with EpSo, EpIn, and PHA-P and an intense skin reaction were accompanied by a low antibody production. The data in Table 4 are from rabbits which had received a second antigen injection 1 week after the first. These rabbits had on the average an antibody titre three times higher than those of Table 3 ($P < 0.01$; Mann-Whitney U test). This high antibody titre was accompanied by a less intense skin reaction ($P < 0.05$) and a much lower SI with EpSo ($P < 0.09$), EpIn ($P < 0.0005$), and

PHA-P ($P < 0.02$) than the rabbits shown in Table 3. Statistical analysis of the ratios of SI (EpSo)/antibody titre (EpSo), SI (EpIn)/antibody titre (EpIn), and skin reaction (EpSo)/antibody titre (EpSo) in Tables 3 and 4 revealed highly significant differences ($P < 0.0009$, $P < 0.0005$, and $P < 0.0005$, respectively).

The lymphocytes of normal rabbits could not be stimulated by EpIn ($SI = 1.0 \pm 0.1$; $n = 4$) and sometimes weakly by EpSo ($SI = 1.5 \pm 1.2$; $n = 6$). The SI of normal rabbit lymphocytes cultured in the presence of 50 µg PHA-P for 50 hours (optimal circumstances) amounted to 44.0 ± 16.5 ($n = 10$). Normal rabbits showed negative skin reactions to EpSo and negative antibody titres to EpSo and EpIn.

Because Tables 3 and 4 show that stimulation of the lymphocytes with EpSo plus EpIn never exceeds the stimulation with EpSo added separately, we investigated the possibility of common antigens in both fractions. Table 5 shows that lymphocytes sensitised to EpSo are also stimulated by the EpIn fraction, which means that both fractions contain cross-reacting or possibly identical antigens.

Discussion

This investigation shows that it is possible to stimulate corneal-epithelium-sensitised lymphocytes with ultrasonically dispersed cell membranes isolated from the corneal epithelium. The particles obtained are vesicles consisting of membrane lipids and proteins. The amount of stimulation with the insoluble epithelial fraction was always less than with the soluble epithelial fraction when both were measured under optimal circumstances. Two explanations are possible for this phenomenon. It is possible that antigens hidden in the vesicle membrane or included in the vesicle lumen cannot reach the receptor of the lymphocyte membrane and therefore cannot trigger the stimulation. Another possibility is the limited variability of the antigens in the insoluble fraction as contrasted with the soluble fraction.

If EpSo and EpIn stimulated different lymphocyte populations, one would expect that incubation of the lymphocytes with EpSo plus EpIn would give additive stimulation indices. From the finding that stimulation of the lymphocytes with EpSo plus EpIn never exceeds the stimulation found by EpSo alone (Tables 3 and 4) we believe that the antigens of the EpIn fraction stimulate the same lymphocyte population as some or all antigens of the EpSo fraction. This suggests that both epithelial fractions possess common antigenicity. This is confirmed by the fact that EpSo-sensitised lymphocytes are also stimulated after incubation with the EpIn fraction (Table 5). The seemingly suppressive effect of EpIn on the SI of EpSo is caused by a suboptimal incubation period for EpSo of 4 days.

The present study shows the importance of having correct conditions for the lymphocyte stimulation

test. These conditions have to be determined accurately before any conclusion from the outcome of the test can be made. This is especially true for clinical application because the degree of stimulation may be very low. The lack of optimal conditions when using human lymphocytes for detecting cellular immunity in ocular diseases may be one of the causes of the discrepancies found among the tests of various authors (Henley *et al.*, 1971; Shore *et al.*, 1972; Henley and Okas, 1974; Szabo *et al.*, 1975; Freedman and Smit, 1974; Hammer, 1971; Hammer and Olah, 1975; Wong *et al.*, 1971).

Another possibility for low or negative results in the lymphocyte stimulation test may be the type of antigen. In this report it is shown that the insoluble epithelial fraction can elicit cellular immunity in rabbits. Marak *et al.* (1971) did not find stimulation of lymphocytes from patients with uveitis on incubation with sedimented antigens from several parts of the eye; nevertheless, they suggested that the major part of the active antigens was present in the insoluble fraction even though they were unable to demonstrate these antigens. Antibodies to the HL-A antigens can be provoked in keratoplasty patients, which demonstrates the antigenic properties of this insoluble part of the membrane (Stark *et al.*, 1973).

The lymphocyte stimulation test is believed to be an accurate test for detecting delayed-type hypersensitivity. However, we must be aware of the possible stimulation of B-lymphocytes in addition to or instead of T-lymphocytes (Greaves *et al.*, 1974). From Tables 3 and 4 it appears that a high lymphocyte stimulation index and an intense skin reaction correlate with a low antibody titre. The converse is also true. This is in agreement with the results of Jevitz and Ekstedt (1971), who showed that, when cultured *in vitro*, lymphocytes of rabbits favouring delayed hypersensitivity provoked a greater response to antigen than the B-lymphocytes. It also appears that a high stimulation found with PHA-P correlated with a relatively high stimulation with EpSo and EpIn and a more intense skin reaction. The transformation of lymphocytes by PHA is now well recognised as a measure of T-lymphocyte function; this also points to a dominating stimulation of the T-lymphocyte by EpSo and EpIn. From this point of view one could imagine that the activity of the T-lymphocytes could be triggered by the soluble antigens. This could be followed by graft rejection as a result of increasing cytotoxicity of the T-lymphocytes by recognising the cross-reacting insoluble antigens.

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Table 5 Stimulation indices of EpSo-sensitised lymphocytes after incubation with EpSo and EpIn under optimal circumstances

Rabbit no.	SI	
	EpSo	EpIn
34	53.0	17.3
35	144.1	44.7
36	53.3	19.7

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