Immunological activity to different corneal antigens in patients with corneal diseases

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SUMMARY Patients suffering from various corneal diseases and waiting for keratoplasty have been immunologically investigated in order to establish sensitisation to corneal antigens. The presence of lymphocytes sensitised to the soluble fractions from human corneas, bovine corneal epithelium, and bovine corneal stroma, which all possess common antigenicity, could be demonstrated in 30%, 50%, and 23%, respectively, of all patients. In none of these patients could a positive plasma antibody titre to human corneal antigens be detected. The results suggest the dominance of T-lymphocyte activity. No correlation was found between the degree of corneal vascularisation and the presence of sensitised lymphocytes to human corneal antigens. Arrangement of the patients according to diagnosis showed that especially those suffering from herpes simplex virus keratitis reacted positively to human corneal antigens. A possible explanation is given. Lymphocytes of controls showed no or only very low stimulation with the soluble fractions of human corneas or bovine corneal stromas. The soluble fraction of bovine corneal epithelium stimulated the lymphocytes of 6 out of 19 controls. The elimination of the donor corneal epithelium before transplantation may be beneficial in view of the involvement of histocompatibility antigens.

Corneal grafts may be subject to immunological rejection. It is stated that an allograft reaction develops in about 12% of cases with good prognosis (Polack, 1973), which may extend to 65% in patients with preoperatively heavily vascularised corneas (Khodadoust, 1973). The physiological state of the recipient cornea might influence the immune state of the patient.

The role of cellular immunity in corneal grafting has been demonstrated in animal studies by passive transfer of sensitised lymphocytes (Khodadoust and Silverstein, 1976) and by the detection of macrophage migration inhibition factor in the aqueous humour during corneal graft rejection (Sher *et al.*, 1976). This factor has been shown to be an in-vitro correlate of cell-mediated immune reactions (George and Vaughan, 1962; David *et al.*, 1964).

The present investigation has used the lymphocyte stimulation test to estimate the state of presensitisation to corneal antigens in a group of patients awaiting keratoplasty. Furthermore this technique was used to compare the antigenicity of corneal

stroma and epithelium to study a possibly beneficial effect of removing the donor corneal epithelium before transplantation.

Materials and methods

SUBJECTS

The investigation included 43 patients suffering from various corneal diseases for at least 6 weeks. All patients were evaluated for keratoplasty. Some patients received topical steroid therapy. Twenty-two healthy volunteers served as controls. Details are given with the results.

ANTIGEN PREPARATIONS

Human corneas, excised from fresh donor eyes, were stored at -70°C until used. All further procedures were carried out at $0-4^{\circ}\text{C}$ unless indicated otherwise. The corneas were cut in small pieces and homogenised in excess 0.01 M ammonium acetate buffer, pH 7.2, with an Ultra Turrax mixer in a nitrogen atmosphere. Homogenisation was continued by sonication, and the soluble corneal fraction was isolated by centrifugation for 30 minutes at 15 000 g. The supernatant was lyophilised, and the product (Hu-CoSo) was stored at -20°C until

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used. Protein content, based on dry weight, was determined according to Lowry *et al.* (1951) and amounted to 50% when bovine serum albumin was used as standard. Corneal epithelium soluble antigens (Bo-EpSo) were isolated from fresh bovine eyes as previously described (Brinkman *et al.*, 1978). After removing epithelium and endothelium the corneal stromas were treated for isolation of the soluble antigens (Bo-SaSo) as described above for Hu-CoSo. The protein content, based on dry weight, of Bo-EpSo and Bo-SaSo amounted to 65%.

LYMPHOCYTE STIMULATION

A sample of heparinised blood was used for plasma preparation and the remainder was diluted with an equal volume of Eagle's minimum essential medium (MEM; Gibco). The mononuclear cells were isolated from the diluted blood by Ficoll-Isopaque centrifugation (Du Bois *et al.*, 1973). Lymphocytes, 3×10^5 , were suspended in 1 ml MEM supplemented with 100 units of penicillin and 0.1 mg of streptomycin per ml and 20% heat-inactivated A-rh positive serum. Cultures of lymphocytes were incubated in triplicate with 1 mg Hu-CoSo, 1 mg Bo-EpSo, or 0.5 mg Bo-SaSo (optimal antigen concentrations) for 6–7 days. All antigens had been solubilised in MEM to a concentration of 10 mg/ml and sterilised by sonication. Sterility was checked by incubating the antigens in culture medium. Because some patients were topically treated with corticosteroids (usually dexamethasone), the stimulation activity of the lymphocytes was controlled by incubation with 5 μ g phytohaemagglutinin-P (Difco) for 3 days. Control cultures contained neither antigen nor mitogen. Twenty-four hours before harvesting 0.5 μ Ci tritiated thymidine (specific activity 25 curie/millimole) was added. Harvesting of the cells was done by filtration under reduced pressure through Millipore glass fibre filters. The filters were incubated in 0.5 ml Nuclear Chicago Solubiliser (NCS), diluted 1 to 3 with toluene-based scintillation fluid containing 100 mg di-methyl-POPOP (2,2'-p-phenylen-bis-(5-phenyloxazol)) and 5 g PPO (2,5-diphenyloxazol) per litre, for 30 minutes at 20°C. After addition of 9.5 ml scintillation fluid, containing 1 ml/l glacial acetic acid, the activity was counted in a liquid scintillation counter. The degree of stimulation is reported as the stimulation index (SI), which is expressed as: $SI = \text{mean counts per minute (c.p.m.) of cultures in the presence of antigen} / \text{mean c.p.m. of cultures in the absence of antigen}$.

An $SI \geq 2$ with Hu-CoSo and an $SI \geq 3$ with Bo-EpSo or Bo-SaSo was considered to be positive.

ANTIBODY TITRE

The plasma antibody titre was estimated by the

haemagglutination test (Herbert, 1973). 1 ml 2% (v/v) tanned sheep erythrocytes was incubated with 1 mg Hu-CoSo.

IMMUNODIFFUSION

Immunodiffusion was performed in 1% agarose in 0.1 M Tris-Veronal buffer, pH 8.8. Bo-EpSo and Bo-SaSo were solubilised in saline to a concentration of 10 mg/ml and 20 mg/ml respectively. In order to absorb antibodies to serum proteins the central well was filled with bovine serum and incubated for 1 hour at 37°C before applying the anti-total human cornea antiserum. This antiserum was prepared by injecting rabbits subcutaneously at multiple sites in the back with 5 mg human corneal antigens (protein based) solubilised in 1 ml phosphate buffered saline (PBS), pH 7.4, and emulsified with 1 ml Freund's complete adjuvant. Boosting was performed 4 and 6 weeks later with 5 mg human corneal antigens solubilised in 1 ml PBS. One week after the last injection the rabbits were bled.

Results

LYMPHOCYTE STIMULATION

A stimulation index of 10 or more was found after incubating the lymphocytes of patients and controls with PHA-P. This suggests that, despite the fact that some patients received corticosteroid therapy, their lymphocytes still possessed the potentiality to be stimulated. The stimulation activity of the different corneal antigen fractions on the lymphocytes of the patients with corneal disease and the healthy persons is shown in Fig. 1. A significant difference (Mann–Whitney U-test) was found between the stimulation indices of the patients with corneal disease and the healthy subjects after incubation of their lymphocytes with human corneal antigens ($P < 0.002$). No significant difference could be observed between both groups of subjects when tested with bovine corneal stroma antigens or bovine corneal epithelium antigens ($P > 0.09$, for both antigen fractions). The relation between the diagnosis of the patients and the presence of sensitised lymphocytes is also demonstrated in Fig. 1.

In comparison with control subjects patients suffering from keratitis caused by herpes simplex virus (HSV) infection showed a statistically highly significant difference in stimulation activity after incubation of their lymphocytes with human corneal antigens ($P < 0.001$). No difference was found with bovine corneal epithelium antigens ($P > 0.06$) and bovine corneal stroma antigens ($P > 0.06$). The lymphocytes of patients suffering from bullous keratopathy also reacted clearly positively with human corneal antigens ($P < 0.005$). With bovine

Fig. 1 Stimulation of lymphocytes of healthy subjects and patients with corneal disease with human corneal antigens, bovine corneal epithelium antigens, and bovine corneal stroma antigens expressed as stimulation index (SI). The results of the patients suffering from HSV keratitis, bullous keratopathy, and other corneal diseases have been taken together in the group indicated as cornea patients.

corneal epithelium and bovine corneal stroma antigens no statistically significant difference between these patients and healthy subjects could be detected ($P > 0.1$, for both antigen fractions). Patients suffering from keratoconus (1), scrofulosa (1), herpes zoster virus keratitis (1), Fuchs's dystrophy (1), pterygium (1), Peters's anomaly (2), physically or chemically induced keratitis (7), or idiopathic stromal keratitis (3) (classification according to Moore and Aronson, 1971) showed no statistically significant difference in lymphocyte activation with either antigen as compared to controls ($P > 0.05$ for each antigen).

An $SI \geq 2$ with human corneal antigens and an $SI \geq 3$ with bovine corneal antigens being considered a positive lymphocyte response, lymphocytes sensitised to the soluble fractions from human corneas, bovine corneal epithelium, and bovine corneal stroma could be demonstrated in 30%, 50%, and 23% respectively of all patients. According to this criterion significant differences (Fisher's one-sided exact test) in lymphocyte activity with human corneal antigens ($P < 0.0005$) and bovine corneal stroma antigens ($P < 0.05$) were found only between patients suffering from herpes simplex virus keratitis and healthy persons, as shown in Table 1. Six control subjects (31%) reacted positively with bovine corneal epithelium antigens.

Corneal vascularisation might influence the state of immunity of patients. However, as shown in Table 2, no relation could be detected between the degree of corneal vascularisation and the presence of lymphocytes sensitised (i.e., $SI \geq 2$) to human corneal antigens. Of 25 patients with heavily or mildly vascularised corneas 6 (24%) reacted positively with human corneal antigens. The patients with avascular corneas reacted in 7 (39%) of the 18 cases positively with this antigen fraction.

Table 1 Stimulation of lymphocytes of patients, suffering from corneal diseases and of healthy persons by Hu-CoSo, Bo-EpSo and Bo-SaSo

Diagnosis	Number of total subjects (with percentage) with positive SI to		
	Hu-CoSo	Bo-EpSo	Bo-SaSo
Bullous keratopathy	3/14 (21%)	7/14 (50%)	4/14 (29%)
Herpes simplex virus keratitis	8/12 (67%)*	7/11 (64%)	4/10 (40%)*
Other corneal diseases**	2/17 (12%)	6/15 (40%)	1/15 (7%)
Healthy***	1/22 (5%)	6/19 (32%)	1/18 (6%)

*Significant at $P < 0.05$ (Fisher's one-sided exact test). ***Eighteen healthy subjects were tested with Bo-SaSo, 19 with Bo-EpSo, and 22 with Hu-CoSo. **See text (Results).

Table 2 *Relation between corneal vascularisation and lymphocyte stimulation with Hu-CoSo*

Degree of vascularisation	Stimulation index	
	Positive	Negative
Heavy	4/13 (31)*	9/13 (69)
Mild	2/12 (17)	10/12 (83)
No	7/18 (39)	11/18 (61)

*Number of total patients (with percentage).

ANTIBODY TITRE

The plasma antibody titre to human corneal antigens of all patients and controls was zero.

IMMUNODIFFUSION

Fig. 2 shows the formation of 1 precipitin line between rabbit antihuman cornea antiserum and Bo-EpSo and 2 precipitin lines between rabbit antihuman corneal antiserum and Bo-SaSo. With bovine serum no precipitin line was found.

Discussion

Sensitisation of animals by tissue transplantation or antigen injection followed by keratoplasty leads to enhancement of the rejection of the corneal graft (Maumenee, 1951; Payrau, 1966; Treffers and Broekhuysse, 1977). Corneal graft rejection may be partly related to cell-mediated immunity (Polack, 1972; Khodadoust and Silverstein, 1976; Sher *et al.*, 1976), which may be detected by the lymphocyte stimulation test (Fleer *et al.*, 1976). Our results are

Fig. 2 *Immunodiffusion of antihuman corneal antiserum (A) versus bovine serum (1), bovine corneal stroma antigens (2), and bovine corneal epithelium antigens (3). The central well was prefilled with bovine serum for 1 hour at 37°C in order to absorb antiserum protein antibodies*

consistent with this hypothesis by showing the presence of corneal antigen sensitised lymphocytes in the blood of patients suffering from corneal diseases. The observed difference in lymphocyte responses due to different corneal antigens suggests some kind of antigen specificity of this immune activity.

The use of bovine corneal epithelium antigens and bovine corneal stroma antigens for investigation of a possible sensitisation of human beings to these antigens has been partly justified by immunodiffusion. Clear precipitin lines could be detected between antihuman corneal antiserum and the soluble antigens of these bovine tissues, indicating the presence of common antigenic determinants. The relatively high percentage of the controls reacting positively with the soluble antigens from bovine corneal epithelium as compared to stroma is a drawback and may be due to histoincompatibility. Incubation of human lymphocytes with these antigens may lead to a stimulation by histocompatibility antigens (or soluble precursors), probably more being present in the soluble fraction of the epithelium than in the soluble fraction of the stroma. The results described in this report may therefore suggest that separation of the epithelium from the donor cornea before keratoplasty eliminates most of the histocompatibility antigens and consequently might increase the chance of success.

Arrangement of the patients into groups according to the diagnosis of the corneal disease showed that bullous keratopathy sometimes caused an increased lymphocyte stimulation. The oedematous state of the cornea might result in a loss and alteration of corneal antigens in these patients, which might explain this increased response to human corneal antigens as compared to that of controls. Further evaluation of the results shows that especially those patients suffering from herpes simplex virus keratitis reacted positively with human corneal antigens. This group of patients appeared also to be more sensitised to bovine corneal epithelium and bovine corneal stroma antigens than the other groups of patients (Fig. 1). For bovine corneal epithelium antigens, however, this difference was statistically significant only at the $P=0.1$ level.

Several explanations are possible for the high number of herpes simplex virus keratitis patients showing lymphocyte stimulation. The intense vascularisation of the cornea often encountered in this type of patients might create the possibility of lymphocytes coming into contact with corneal antigens. However, the influence of another or additional factor is indicated by the absence of a correlation between corneal vascularisation and a positive lymphocyte response (Table 2). This is

Fig. 3 Corneal cells exposing membrane associated viral antigens are recognised by peripheral viral antigen-sensitized lymphocytes. The consequent cytolysis results in release of corneal specific antigens which activate the immune system of the patient. This is measured by the in-vitro lymphocyte stimulation

supported by the fact that corneal vascularisation is also seen in other forms of keratitis without eliciting an immune response. It is likely that the change of cell-surface antigens of corneal cells by incorporation of viral antigens, demonstrated after infection of the cornea with the herpes simplex virus (Henson *et al.*, 1974; Metcalf and Helmsen, 1977), might be one of the primary causes of the immune response. Cell-mediated immune activity to viral antigens has been demonstrated after herpes simplex virus infection in animals (Swyers *et al.*, 1967; Metcalf and Kaufman, 1976) and in man (Russell *et al.*, 1976). A second contact between systemic sensitised lymphocytes and viral antigens present on the corneal cell surfaces may lead to corneal opacification, probably by destruction of the corneal cells by direct contact or by the release of cytolytic lymphokines. The consequent liberation of soluble corneal specific antigens together with high vascularisation might explain the large number of patients with herpes simplex virus keratitis who are sensitive to corneal antigens, resulting in poor prognosis for successful keratoplasty. This hypothesis is illustrated in Fig. 3. The absence of detectable plasma antibodies to human corneal soluble antigens suggests also in this particular case that especially T-lymphocytes are responsible for this immune activity.

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