Secretory IgA specific for herpes simplex virus in lacrimal fluid from patients with herpes keratitis —a possible diagnostic parameter

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SUMMARY In the present study a solid-phase radioimmune assay was used for the demonstration of herpes simplex virus-specific IgG and secretory IgA antibodies in the lacrimal fluid from patients with active recurrent herpes keratitis. The method was quantitative and made it possible to test specifically for the production of secretory IgA antibodies produced during an active herpes simplex virus infection. The production of secretory IgA was followed in 2 patients with fresh recurrent lesions. The HSV-specific secretory IgA could be demonstrated during the first 10 days of infection, where the maximal concentration was reached 3-5 days after the first symptoms occurred. The secretory antibodies were locally produced, and it is shown for the first time that herpes virus-specific secretory antibodies were of diagnostic value.

Herpes keratitis is the most frequent cause of corneal opacities in developed countries, and because of its tendency to recurrence the infection often causes irreversible damage, followed in severe cases by the development of blindness.¹ Usually the diagnosis is based mainly on clinical evidence. However, herpes keratitis has various atypical manifestations which often makes it difficult to give an exact diagnosis.

The detection of antibodies reactive to HSV in the lacrimal fluid has mainly been done by the passive haemagglutination test (PHA), which allows the detection of antibodies of the IgG class.² HSV-specific antibodies were found in 14.2% of the tear samples from patients with inflammatory diseases of non-herpetic aetiology. The PHA test did not allow a differentiation between locally produced antibodies reactive to HSV and serum antibodies transmitted to the lacrimal fluid by impairment of the blood vessels caused by inflammation.

Secretory IgA (SIgA) differs from IgA in serum both chemically and immunologically by the presence of a secretory component that is coiled around the Fc fragment of both IgA molecules of the dimer

structure.³ The secretory antibodies are present mainly in external secretions.^{4,5}

In the present study we used a radioimmune assay developed for the detection of SIgA reactive specifically with HSV. The presence of the virus-specific SIgA in lacrimal fluid from a limited number of patients with active herpes keratitis and from control persons was studied. The HSV-specific SIgA was found only in the lacrimal fluid from the patients, and the possible role of the test for the diagnosis of herpes simplex infections of the eye is discussed.

Materials and methods

PATIENTS AND CONTROLS

Seven patients and 7 controls were selected as follows. The 7 patients (nos. 1 to 7) suffered from different types of herpetic keratitis. In the case of recurrent herpetic keratitis tears were collected in the active phase—so-called fresh recurrency—when the epithelial defects were present. Between herpetic attacks these patients are inactive from a clinical point of view. Patient no. 4 had suffered from a first-time herpetic keratitis, but it had shown a prolonged

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Table 1A Patients

Patient no., sex, age (years)	Number of previous attacks of herpetic keratitis	Duration of symptoms (days)	Extraocular herpetic infections	Presence of accompanying stromal reaction and/or iritis
1 M 66	1	30	—	Stromal reaction Iritis
2 M 67	1	70	—	Stromal reaction Iritis
3 F 49	2 (in both eyes)	20	—	Stromal reaction Iritis
4 M 57	—	90	+	Stromal reaction Iritis
5 M 82	1	4-5	—	Stromal reaction Iritis
6 F 71	1	2	+	—
7 F 26	4-5	6	+	—

course. The clinical information on the patients is listed in Table 1A.

Two patients who suffered from fresh recurrent herpetic keratitis were followed and tears collected on days 2, 5, 9 or 1, 3, 5, and 15 after the first symptoms occurred. Five of the patients (nos. 1, 3, 4, 5, and 6) had severely inflamed, red eyes, while 2 of them (nos. 2 and 7) had slightly inflamed eyes.

The group of controls was heterogeneous. Two healthy persons (nos. 9 and 10) with no signs of eye disease, and 5 persons (nos. 8, 11, 12, 13, and 14) with lesions of nonherpetic origin in the eye, were selected (Table 1B). The reason for the heterogeneous control group was that we wanted to include controls with lesions that allowed leakage of serum proteins into the lacrimal fluid in order to test for the specificity of locally produced antibodies induced during infection with HSV-1.

COLLECTION OF TEARS AND ESTIMATION OF SERUM ALBUMIN

Approximately 200 μ l tear fluid from both eyes of each patient was collected in 50 μ l capillary tubes. Tear secretion was insufficient in many patients, and

consequently the tear production was stimulated in order to collect enough lacrimal fluid.

Stimulation was performed by directing a jet of ice-cold air to the centre of the unaffected cornea. The air jet was made by a glass cone with an exit orifice of 1 mm and a flow of 1½ l air per second. The cone was placed at a distance of 5 cm from the cornea. The rapid cooling of the cornea provoked sufficient reflex tearing in both eyes.

The tear fluid was drawn into capillary tubes from the lacrimal river at the lateral canthus by capillary action, and when filled, the tubes were sealed with wax and stored at -70°C until use.

The content of serum albumin in tear fluid was measured in a micromodification of the electro-immunoassay of Prause.⁶⁷

PREPARATION OF HSV-1 AND MOCK COATED DISCS
VERO cells grown as monolayer cells in 75 cm² tissue culture flasks with minimum essential medium (MEM) supplemented with 10% fetal calf serum, were infected with HSV-1 (F) at a multiplicity of 10 plaque-forming units per cell in a volume of 1.0 ml MEM with 1% fetal calf serum. After 1 h of

Table 1B Controls

Control no., sex, age (years)	Clinical diagnosis	Presence of accompanying stromal reaction and/or iritis	Presence of corneal ulcer	Extraocular herpetic infections
8 F 25	Keratitis punctata (caused by contact lens)	—	Epithelial lesions	—
9 F 40	Healthy person	—	—	—
10 F 52	Healthy person	—	—	—
11 F 65	Adenovirus keratoconjunctivitis	—	Epithelial lesions	—
12 F 80	Corneal ulcer (due to traumatic affection)	+	+	—
13 F 73	Absolute glaucoma	+	Epithelial lesions	—
14 M 44	Dislocation of the lens with glaucoma	+	Epithelial lesions	—

adsorption at 37°C the inoculum was aspirated and 10 ml MEM with 1% fetal calf serum was added. 18 h after infection, HSV-1 and uninfected (MOCK) cell extracts were prepared and bound to activated diazobenzyl-oxymethyl paper discs.⁸⁻¹⁰

DETECTION OF HSV-1 SPECIFIC SECRETORY IgA IN LACRIMAL FLUID

The radioimmune assay in use has been detailed elsewhere.⁹ In order to measure secretory IgA, the IgG present in lacrimal fluid and in serum was removed by binding of the Fc-region of IgG to formalin-fixed staphylococcus carrying protein A on its surface (Cowan I strain).¹¹ The preparation of fixed staphylococcus was made as described elsewhere and stored in a 5% (v/v) suspension in PBS and 1% (w/v) sodium azide.¹²

50 µl of lacrimal fluid was diluted with 50 µl of a 0.05 M Tris-HCl buffer pH 7.4 (with 0.15 M NaCl, 0.05 M EDTA, 0.25% (w/v) gelatin, 0.05% (v/v) Triton X-100, and 2% (w/v) (BSA) and incubated with staphylococcus pelleted from 0.66 ml of a 5% suspension. After incubation for ½ h at 4°C the staphylococcus was removed by centrifugation for 1 min at 12000 rpm in a microcentrifuge (Rune, Germany).

100 µl of the IgG-depleted lacrimal fluid was incubated with 2% (v/v) of rabbit antibodies (IgG) to the secretory component of human IgA (antiselectory IgA) (DAKO, Copenhagen, Denmark). The incubation was done for ½ h at 4°C. 50 µl of the sample was incubated in microtitre plates (Nunc, Roskilde, Denmark) with either an HSV-1 or a MOCK disc for 4-5 at 37°C. The discs were washed extensively in the buffer detailed above, and then incubated with ¹²⁵I-labelled protein A (specific activity 30 mCi/mg; Amersham, England).^{9,10}

The amount of ¹²⁵I-labelled protein A bound to the discs was measured after extensive wash. The discs

were counted in a γ-counter (Selectronic), and the specific binding was calculated as the difference between the binding to an HSV-1 disc and that to a MOCK disc. The specific binding of secretory IgA present in lacrimal fluid from patients and controls was measured relative to the maximum binding obtained by incubation of the paper discs with the saturating amount of HSV-specific serum IgG. This was obtained from a strongly reacting, seropositive donor, and the same reference serum was used throughout the study.

DETECTION OF HSV-1 SPECIFIC IgG IN LACRIMAL FLUID AND SERUM

The content of HSV-1 specific IgG was measured in paired samples of lacrimal fluid and serum for all the persons selected for the present pilot study. The radioimmune assay with the discs was used as follows:

Lacrimal fluid was used unabsorbed and diluted 1:2 in the buffer. 50 µl of this dilution was incubated with either an HSV-1 disc or with a MOCK disc. The serum samples were diluted 1:10 before incubation with the HSV-1 and the MOCK discs, and 50 µl samples were applied as described above. After incubation for 4-5 h at 37°C the discs were washed extensively and incubated with ¹²⁵I-labelled protein A.^{9,10}

The quantitation of the amount of specifically bound, iodinated protein A was done after extensive wash as described in the previous section. The specific binding of IgG was calculated as a percentage of the maximum binding obtained with the reference serum IgG from the HSV-1 seropositive donor.

Results

QUANTITATIVE ANALYSIS OF HSV-SPECIFIC SECRETORY IgA (Siga) IN LACRIMAL FLUID

Samples of lacrimal fluid from all patients and

Table 2 *Herpes simplex virus specific secretory IgA in lacrimal fluid*

Patient no.	¹²⁵ I-labelled protein-A-bound (counts per minute)				Control no.	¹²⁵ I-labelled protein-A-bound (counts per minute)			
	HSV-1 protein	MOCK protein	Specific binding	Percentage* binding		HSV-1 protein	MOCK protein	Specific binding	Percentage* binding
0†	4550	469	4081	100					
1	2633	223	2410	59.5	8	438	284	154	3.8
2	2056	148	1908	46.8	9	379	268	111	2.7
3	1871	67	1804	44.2	10	304	199	105	2.6
4	1193	249	944	23.1	11	154	188	-34	0
5	2891	290	2601	63.7	12	238	256	-18	0
6	1553	249	1304	32.0	13	316	162	154	3.8
7	870	199	671	16.4	14	153	77	76	1.9

*Percentage binding was calculated as specific binding of the sample/specific binding of the reference × 100%.

†Maximum binding obtained by incubation of the paper discs with saturating amount of HSV specific IgG.

Fig. 1 HSV-specific secretory IgA present in lacrimal fluid from 2 patients with fresh recurrent herpes keratitis. The patients were followed up for 9 days ●—● and for 15 days ×—× respectively. Days post lesion were counted from when the first symptom of a corneal lesion appeared.

controls were analysed for the amount of HSV-specific SIgA, and the results are listed in Table 2.

The quantity of HSV-specific SIgA in lacrimal fluid from herpes keratitis patients varied between 16.4% and 63.7%, whereas the amount of HSV-specific SIgA in the control group (nos. 8 to 14) was maximally 3.8% (Table 2). All the controls were negative for the specific secretory IgA in lacrimal fluid, though the control persons with eye lesions of nonherpetic origin (nos. 11 to 14) showed leakage of serum protein into the lacrimal fluid.

The specific SIgA was followed in 2 patients during the active phase of the keratitis. The patient followed up on days 3, 5, 9 and the one followed on days 1, 3, 5, and 15 had the maximal amount of SIgA in their tears between days 3 and 5 (Fig. 1).

QUANTITATIVE ANALYSIS OF HSV-SPECIFIC IgG IN LACRIMAL FLUID AND IN SERUM

Paired samples of lacrimal fluid and serum from patients as well as from control persons were tested

for the quantity of HSV-specific IgG measured in the radioimmune test.

The quantity of IgG in the lacrimal fluid from the affected eye from patients nos. 1-7 ranged from 14.8% to 36.8% of the maximal amount of IgG bound under saturating conditions. Among the controls only the lacrimal fluid from nos. 12, 13, and 14 was positive for IgG, and the amount was from 24.2% to 55.3%. The lacrimal fluid from controls nos. 8-11 was negative for HSV-specific IgG (the maximum value was 2.6%). The controls nos. 12, 13, and 14, who were positive for HSV-specific IgG in lacrimal fluid, had severely inflamed eyes, so that leakage of serum IgG into the lacrimal fluid could not be excluded. That leakage occurred was confirmed by the measurement of albumin in the lacrimal fluid as described below.

It should be mentioned that all persons selected for the present study except control no. 10 were positive for HSV-specific IgG in serum, and the quantity ranged from 10% to 100%.

ALBUMIN MEASURED IN PAIRED SAMPLES OF LACRIMAL FLUID AND SERUM

Detection of increased amounts of albumin in lacrimal fluid is an indicator of the impairment of blood vessels, which leads to passive diffusion of serum proteins into the lacrimal fluid. The amount of albumin in the lacrimal fluid of patients as well as controls is listed in Table 3. The normal value of albumin measured in lacrimal fluid from unaffected eyes is approximately 1 to 25 μg per ml.¹³ The patients with severely inflamed, red eyes (nos. 3, 4, and 5) had increased amounts of albumin in the lacrimal fluid, with values ranging between 102 and 364 μg per ml. (SI conversion: $\mu\text{g}/\text{ml} \times 1 = \text{mg}/\text{l}$.) Patients nos. 2 and 7 did not have increased albumin (23.6 and 12.5 $\mu\text{g}/\text{ml}$ respectively), which was in agreement with the clinical findings, as these 2 patients had slightly inflamed eyes. The albumin concentration in tears from patients nos. 1 and 6 was not measured because of shortage of lacrimal fluid obtained.

Table 3 Albumin concentration in lacrimal fluid and in serum

Patient no.	Albumin		Control no.	Albumin	
	Lacrimal ($\mu\text{g}/\text{ml}$)	Serum (mmol/l)		Lacrimal ($\mu\text{g}/\text{ml}$)	Serum (mmol/l)
1	ND	682	8	16.6	699
2	23.6	600	9	45.6	675
3	142.5	673	10	9.9	575
4	364.0	668	11	16.0	610
5	102.0	611	12	160	ND
6	ND	549	13	ND	576
7	12.5	663	14	86.9	662

Normal range in lacrimal fluid: 1.4-21.6 $\mu\text{g}/\text{ml}$. (SI conversion: $\mu\text{g}/\text{ml} \times 1 = \text{mg}/\text{l}$.) Normal range in serum: 532-813 mmol/l. ND=not determined.

Among the control persons, one with no sign of clinical eye disease (no. 10) also had albumin concentrations within the normal range. The other (control no. 9) showed an increase of albumin in the lacrimal fluid. This cannot be explained from the information available on this person. The controls with eye lesions of nonherpetic origin (nos. 11 to 14) had different degrees of leakage. The patients with ulcer cornea and with glaucoma showed increased levels of albumin in the lacrimal fluid (albumin was not measured for control no. 13 because of shortage of fluid), but the control with adenokertoconjunctivitis maintained a normal concentration of albumin in the eye.

The concentration of serum albumin for all the patients and controls was within the normal range, from 532 to 813 mmol per ml (Table 3).

Discussion

Secretory IgA plays a significant role for resistance to a variety of viral infections. Protection against infections with for instance entero-, myxo-, or paramyxovirus is directly correlated to the presence of secretory immunoglobulins, largely of the IgA class.^{4,14}

Herpes simplex virus infections of the eye have previously been studied in both man and the rabbit. The production of herpes simplex-specific IgG and IgA was demonstrated in the lacrimal fluid during human eye infections.^{2,15} The possible role of IgA for the protection against herpes simplex virus infections of the eye has received some attention, and the possibility that the effective neutralisation of infectious virus is dependent on the co-operative effect of both IgG and IgA has been discussed.¹⁶ The methods used have not allowed the detection of HSV-specific secretory IgA, although this might represent the majority of the total IgA.

In the present study herpes-specific antibodies of the secretory IgA class were found in human tears during an active herpes keratitis. The quantity was increased up to 20 times above the background value present in lacrimal fluid from controls (Table 2).

It should be specifically noted that persons with severely inflamed eyes due to affections of non-herpetic origin (controls nos. 12, 13, and 14) were completely negative for HSV-specific SIgA in the lacrimal fluid, although leakage of serum proteins into the lacrimal fluid did occur (Table 3). Passive transmission of HSV-specific IgG and IgA from serum into the lacrimal fluid was therefore possible.¹³ The finding that all patients and controls except no. 10 were positive for HSV-specific IgG in serum made the demonstration of HSV-specific IgG in the lacrimal fluid of no diagnostic value.

The conclusion that the immunoglobulin measured in the test was secretory IgA was possible only when the second antibody used in the test was directed specifically towards the secretory component of human IgA.¹⁷ That this was the case was demonstrated by the inability of the reagent to bind to the α -chains of serum IgA.⁹

In conclusion, the radioimmune assay allows the demonstration and quantitation of HSV-specific SIgA in lacrimal fluid from patients with active herpes keratitis. SIgA apparently reached a maximal amount within the first week of the disease (Fig. 1). Our results indicate that the SIgA represents locally produced antibodies made only during the active phase of the disease. The local production is supported by a few observations where tears from the healthy eye of herpes keratitis patients were collected and shown to be negative for HSV-specific SIgA (data not shown). The secretory IgA might thus be of diagnostic value for the herpes keratitis, but this has to be evaluated in a double-blind study where lacrimal fluid from a significant number of patients and controls is collected.

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