Antigen presentation of herpes simplex virus by corneal epithelium – an in vitro and in vivo study

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Abstract

Ia antigen (class II antigen) is a histocompatibility antigen that foreign peptides associate with, before antigen presentation to T cells and subsequent triggering of the CD4 T cells. Although corneal epithelium is normally Ia negative it may become Ia positive under abnormal circumstances but the functional significance of this is uncertain. In this study the expression of Ia antigen on corneal epithelium of mice during in vivo primary and secondary herpes simplex keratitis and the in vitro accessory function of corneal epithelium in the presentation of herpes simplex virus (HSV) antigen to in vivo HSV primed T cells were evaluated. Whole mount preparations of corneal epithelium were found to express Ia antigen on days 3, 5, and 7 following corneal inoculation with live HSV. The intensity of the Ia expression was greater in non-immune mice on day 7 after corneal inoculation compared with immune mice. A cellular suspension of corneal epithelium induced HSV primed T cells to proliferate in the presence of HSV antigen. Induction of Ia antigen on corneal epithelium during herpes simplex keratitis may functionally expand the population of antigen presenting cells in the cornea and contribute to T cell activation.

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Specialised antigen presenting cells (APC) are required to present antigen to specific T helper cells.¹ T helper cell activity is central to the generation of an immune response since these cells provide lymphokines and other signals which support the expansion, development, and function of effector cells such as cytotoxic T cells and B cells. Processed foreign antigen is presented as a complex with self Ia antigen (class II major histocompatibility antigen, MHC) on the surface of the APC. This antigen/Ia complex docks with the T cell receptor and contributes to T cell activation. Langerhans cells (LC), which normally reside in the conjunctiva and at the limbus, are traditionally thought to be the APCs of the external ocular surface^{2,3} and it is therefore believed that they are the important APCs in the presentation of external ocular antigens, such as herpes simplex virus (HSV), to T cells.

Central corneal epithelium of the mouse does not normally contain LC nor does it express Ia antigens.² Normal mouse corneal epithelium is therefore an unusually pure population of epithelial cells and, unlike some other epithelial surfaces such as skin, does not require negative selection techniques with anti-Ia/class II monoclonal antibodies and complement to yield a pure

population of cells free from dendritic cells. Corneal epithelium can express class II MHC antigen during herpes simplex keratitis (HSK),³ corneal graft rejection,⁴ and following incubation in interferon γ .⁵ It has not been established whether the aberrant expression of Ia antigen is correlated with the ability of corneal cells to present antigen to T cells.

HSK is a relatively common cause of corneal blindness and may present clinically as a dendritic or amoeboid epithelial ulcer, or as stromal disease manifested by stromal oedema and corneal opacification, or as a combination of epithelial and stromal disease. Immunopathological mechanisms are important in the genesis of clinical stromal HSK and it has been shown that the immune response and clinical disease varies with the strain of virus and the strain of mouse.^{6,7} Local immune mechanisms are also involved in the control of epithelial disease since the use of local immunosuppression delays healing of dendritic ulcers and increases the size of the ulcer. HSK, in animal models, is known to induce LC infiltration of the cornea.^{2,8,9} In the various animal models of primary HSK studied, LCs infiltrate the cornea during the first 2 weeks following infection with HSV. Since the majority of the infiltrate occurs when HSV antigen can no longer be isolated from the cornea, the role of LC infiltration in presentation of HSV is unclear.

Using a well described mouse model of HSK we studied the expression of Ia antigen on mouse corneal epithelium during HSK over a defined period of time and furthermore we have assessed the ability of corneal epithelium to stimulate a T cell proliferative response to HSV in vitro.

Materials and methods

ANIMALS

Six to eight week old inbred male NIH mice (haplotype H-2q), bred in the departmental animal facility, were used throughout the study.

ANTIGENS

Herpes simplex virus type 1 strain KOS (HSV1KOS, the kind gift of Dr C Shimeld) was used throughout to immunise animals. HSV1 strain SC16 was used to produce clinical HSK and in the lymphocyte proliferation assays (LPA). HSV1 was obtained from a stock pool of HSV1 originally grown on a Vero cell monolayer and stored in 0.1 to 0.5 ml aliquots at -70°C .

Mock virus was used as a control antigen for immunisation and in the LPA, having been diluted in a similar manner to the virus preparation. It was prepared and stored in the same

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manner as the stock preparations of HSV, using Vero cells and culture medium, but no HSV was added.

Keyhole limpet haemocyanin (KLH, Calbiochem-Berking, La Jolla, USA), a commonly employed antigen, with a stock concentration of 10 to 20 mg/ml, was prepared and dialysed extensively in phosphate buffered saline (PBS) and pH adjusted to 7.2. It was used at a concentration of 100 µg/ml, as a control for antigen specificity.

IMMUNISATION

Mice were inoculated on the hind foot pad by direct scarification, under general anaesthesia, with 5 µl of live HSV1 strain KOS (2×10^8 plaque-forming units (pfu)/ml). HSV1KOS was chosen as the immunising virus because it is known to give good immunity and has minimal neurotoxicity. Control mice were similarly inoculated on the foot pad with mock virus.

CORNEAL INOCULATION

Fourteen days following foot pad inoculation, immune and non-immune mice were inoculated on the left cornea by direct scarification through 5 µl live HSV1 strain SC16 (4×10^8 pfu/ml and 10^5 pfu/ml respectively).¹⁰ HSV1SC16 was chosen as the corneal inoculating agent because considerable data exist in this laboratory on its associated ocular infection.^{8 11 12} A larger dose of virus was required to cause significant keratitis in immune compared with non-immune mice.

DEMONSTRATION OF Ia ANTIGEN ON CORNEAL EPITHELIUM

On days 3, 5, and 7 following corneal inoculation, mice were sacrificed, the left eye enucleated, incubated in a solution of 4 mM ethylenediamine tetra-acetic acid (tetra-sodium salt, Sigma Chemical Co, Poole, Dorset) and the epithelial sheet removed.¹³ The isolated epithelial sheets were rinsed in PBS and fixed with a 50:50 mixture of acetone and methanol.

Endogenous peroxidase was blocked with 0.06% hydrogen peroxide, the sheets immersed in PBS, and subsequently in normal goat serum (Sera Lab Ltd, Crawley Down, Sussex) (to block non-specific binding sites). The sheets were then immersed in the primary monoclonal antibody, rat anti-mouse immunoglobulin supernatant to Ia antigen, subregion H2, IA at a dilution of 1:10 (Sera Lab Ltd, Crawley Down, Sussex), incubated for 12–24 hours at 4°C and following rinsing, immersed in the horseradish peroxidase linked whole antibody (sheep anti-rat immunoglobulin, dilution 1:100) (Amersham, Bucks) for 2 hours and rinsed again. They were then immersed in a solution made from a diaminobenzidine Isopac (Sigma Chemical Co, Poole, Dorset) for 10 minutes, rinsed, laid out on glass slides, and mounted in Apathys aqueous mount.

CELL PREPARATION

Aseptically excised spleens were minced through a stainless steel mesh into PBS. Spleens from

immune mice were removed 9 days following foot pad inoculation with live virus. After the debris was allowed to settle, the cell preparation was decanted off and washed twice in PBS by centrifugation at 1000 rpm. T cells were obtained from the spleen cell preparation by passage of the spleen cells through rabbit anti-mouse immunoglobulin (Ig)-mouse Ig-coated glass bead affinity columns.¹⁴ The column passed cells were treated with anti-Ia^p monoclonal antibody (Cederlane Labs Ltd, Ontario, Canada) at a dilution of 1:1000 and complement (rabbit complement-low Tox-M, Cederlane Labs Ltd) at a dilution 1:10, as part of a one stage procedure for 30 minutes at 37°C. (The p in anti-Ia^p refers to the fact that this antibody detects a determinant shared by various Ia haplotypes). The resulting cell population was shown to be at least 92% T cells when the cell population was incubated with anti-Thy 1.2 antiserum (NEN Research Products, Boston, USA) and complement (rabbit complement-low Tox-M, Cederlane Labs Ltd) and did not respond to the B cell mitogen lipopolysaccharide (data not shown).

Control antigen presenting cells were unselected spleen cells from non-immune mice that were treated with γ irradiation (1500 Gy from a caesium source, Gravatam Industries Ltd, Gosport, Hampshire).

Corneal cells were obtained by incubating central corneal epithelial sheets from uninfected clinically normal mouse eyes (examined using slit-lamp biomicroscopy) in trypsin 1% (Difco, Detroit, USA) for 30 minutes. These cells were washed three times in PBS and were then γ irradiated (1500 Gy from a caesium source, Gravatam Industries Ltd, Gosport, Hampshire). Isolation of the central corneal epithelial sheet was performed by isolating whole corneal epithelial sheets following incubation of normal mouse eyes in a solution of 4 mM ethylenediamine tetra-acetic acid (tetra-sodium salt, Sigma).¹³ Using binocular microscopy for magnification, the limbal and peripheral corneal epithelium was dissected from the central corneal epithelial sheet leaving a central corneal sheet of approximately 1 mm diameter.

All cell preparations were assessed for cell viability in their ability to exclude trypan blue vital stain. Cell preparations with less than 90% cell viability were discarded.

HSV1SC16, inactivated by prior treatment with ultraviolet light, was used in the lymphoproliferative studies at a concentration of 10^7 pfu/ml. Mock virus as HSV1SC16 diluted in the same manner or KLH at a dilution of 100 µg/ml were used in control cultures.

CULTURES

The culture medium employed was the α modification of Eagles' minimal essential medium (Flow Labs, Irvine, Renfrewshire UK; Gibco Europe Ltd, Paisley, Ayrshire) supplemented with 4 mM L-glutamine (Gibco) 5×10^{-5} M 2-mercaptoethanol (Sigma), 100 U/ml benzyl penicillin (Glaxo Ltd, Greenford, Middlesex), 100 µg/ml streptomycin sulphate (Evans Medical Ltd, Greenford, Middlesex), 20 mM HEPES (Sigma), and 0.05% fresh autologous

Figure 1 Three days after corneal inoculation with $5 \mu\text{l}$ of 1×10^8 plaque forming units per ml of herpes simplex virus type 1 strain SC16 this corneal epithelial sheet (stained by an indirect immunoperoxidase method using a primary anti-Ia monoclonal antibody) has Ia antigen on the surface of peripheral corneal epithelial cells ($\blacktriangleright$). There is less intense surface Ia antigen on more central corneal epithelium ($\blacktriangleright$) (magnification $\times 275$).

normal serum. No heterologous serum was added.

Cultures were carried out in 2 ml volumes in 24 well plates (Nunc A/S, Roskilde, Denmark). In vivo HSV primed T cells and unprimed spleen cells were each cultured at 1×10^6 cells/ml. Corneal cells were used at 5×10^4 cells/ml.

All cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. At daily intervals from days 2 to 7 three replicate 0.1 ml samples were taken from each of three replicate cultures and pulsed with 1 μCi per volume of ^3H -thymidine (TdR; 73–85 Ci/mM; NEN Research Products, Boston, USA). After a further 6 hour incubation, as above, the samples were harvested on to glass fibre mats (Whatman Labsales Ltd, Maidstone, Kent), using a multi-sample harvester (Skatron AS, Lier, Norway).

Figure 2 A normal corneal epithelial sheet including limbus and conjunctiva (stained by an indirect immunoperoxidase method using a primary anti-Ia monoclonal antibody) has only Ia antigen on the surface of Langerhans cells ($\blacktriangleright$). There is no Ia antigen on corneal ($\blacktriangleright$) or conjunctival ($\blacktriangleright$) epithelium (magnification $\times 275$).

Table 1 Intensity and extent of Ia expression on corneal epithelium during the first week after corneal infection with HSV

Days	3	5	7
Immune mice	++ (8)	++ (8)	++ (8)
Non-immune mice	++ (8)	++ (8)	+++ (7)
Non-immune mice (mock virus inoculation)	– (3)	– (3)	– (3)
Normal corneal epithelium	– (2)	– (2)	– (2)
Negative control (no primary monoclonal antibody)	– (2)	– (2)	– (2)

++ = Ia antigen present on corneal epithelium.

– = No Ia antigen detected on corneal epithelium.

Numbers of epithelial sheets in parentheses.

The fibre mats were allowed to dry and following application of 100 μl of scintillation fluid (Unisolve E) to each mat the ^3H -thymidine incorporated into newly synthesised DNA was measured using an LKB rackbeta (LKB-Wallac, Turku, Finland). Results are expressed as the mean counts per minute (cpm) of radioactivity detected and the standard error of the mean of the replicate cultures. Stimulation index refers to the proliferative response measured in cpm divided by the background activity in cpm.

Results

EXPRESSION OF Ia ANTIGEN ON CORNEAL EPITHELIAL SHEETS DURING HERPES SIMPLEX KERATITIS

During herpes simplex keratitis, patchy expression of Ia antigen was present on corneal epithelium on the third day following corneal inoculation of HSV in non-immune and immune mice (Fig 1). This occurred mainly at the limbus and the peripheral corneal epithelium and in areas with and without infiltrating LCs. LCs were recognised by their dendritic morphology and epithelial cells by their 'paving stone' appearance.

The intensity of the Ia antigen staining on the corneal epithelium was greater on day 5 compared with day 3 in both herpetically infected groups but was more intense and diffuse on day 7 in the non-immune mice compared with mice immunised to HSV before corneal inoculation (Table 1).

No detectable levels of Ia antigen were apparent on corneal epithelium of normal mice (Fig 2), mice inoculated on the cornea through mock virus, or negative controls where no primary anti-Ia monoclonal antibody was used.

LYMPHOCYTE PROLIFERATIVE RESPONSES

There was a significant increase in the proliferative response of T cells on days 2–7 in culture when corneal epithelium (without LC) was used as the test antigen presenting cell population (Table 2). On day 4 in vivo HSV primed T cells proliferated in response to HSV, with a stimulation index of 172, when corneal epithelium was added to the cultures. This compared with a stimulation index of 7 when T cells and HSV alone were in culture. The proliferative response was specific to viral components since mock virus, prepared in a similar fashion to the

Table 2 Proliferative response of HSV primed T cells to antigens on days 2 to 7 in culture

	Day*	Proliferative response (SE)		Mock virus	KLH	St†
		No antigen	HSV			
T cells	2	276 (21)	248 (30)	202 (17)	147 (28)	1
T cells and spleen cells	2	288 (29)	20 200 (2 000)	187 (22)	214 (21)	70
T cells and corneal epithelium	2	195 (52)	3 323 (546)	263 (28)	107 (23)	17
T cells	3	383 (56)	1 532 (314)	517 (34)	389 (41)	4
T cells and spleen cells	3	295 (74)	50 187 (2 540)	297 (24)	335 (29)	170
T cells and corneal epithelium	3	202 (48)	13 147 (1 524)	473 (48)	512 (28)	65
T cells	4	364 (51)	2 590 (160)	246 (26)	376 (43)	7
T cells and spleen cells	4	305 (43)	65 109 (3 522)	255 (17)	403 (17)	213
T cells and corneal epithelium	4	107 (18)	18 448 (1 515)	625 (97)	523 (16)	172
T cells	5	382 (57)	3 062 (417)	176 (12)	396 (26)	8
T cells and spleen cells	5	299 (41)	27 520 (3 120)	246 (29)	463 (44)	92
T cells and corneal epithelium	5	200 (38)	26 245 (1 073)	448 (52)	276 (31)	131
T cells	6	765 (56)	3 826 (156)	376 (39)	417 (16)	5
T cells and spleen cells	6	302 (28)	30 210 (3 976)	336 (28)	209 (24)	100
T cells and corneal epithelium	6	252 (47)	26 221 (2 108)	219 (16)	109 (26)	104
T cells	7	357 (33)	3 218 (685)	188 (26)	165 (23)	9
T cells and spleen cells	7	358 (25)	1 075 (82)	314 (34)	193 (16)	3
T cells and corneal epithelium	7	300 (15)	17 714 (752)	276 (18)	207 (28)	59

Cultures were performed in 2 ml volumes with 2×10^6 in vivo HSV primed T cells, 2×10^6 unprimed γ irradiated spleen cells 10^6 γ irradiated corneal cells, 1×10^7 pfu/ml of ultraviolet inactivated HSV1 strain SC16, a similar dilution of mock virus, or KLH at a dilution of 100 μ g/ml. On days 2 to 7 in culture, three replicate 100 μ l samples were taken from each culture well and T cell proliferation assayed by measuring 3 H-thymidine incorporation into newly synthesised cell DNA.

* Number of days in culture; † Stimulation index = proliferative response/background proliferative response.

stimulatory virus but containing no virus, and the unrelated antigen, KLH, failed to stimulate a secondary response. It should be noted that the culture system employed did not contain any foreign stimulatory substances such as fetal calf serum because autologous serum was used as a protein supplement. This is reflected in the low background responses of cultures lacking antigen.

Discussion

Ia antigen has been shown to be inducible on corneal epithelium but its appearance has been attributed to the probable presence of T cell factors like interferon γ .¹⁵ In this work it was possible to investigate mouse corneal epithelium for expression of Ia antigen over a defined period from the onset of HSK in non-immune and immune mice. It is of interest in this investigation that the Ia antigen became apparent on the corneal epithelium of immune and non-immune mice on day 3 after corneal inoculation. Although T cells are known to reside at the normal limbus and in the conjunctiva^{16,17} we consider it unlikely that this Ia expression, 2 to 3 days following exposure to HSK, could be entirely the result of local T cell activity in an unprimed animal because it was too early in the primary immune response. The specific factors involved in the stimulation of Ia expression have not been investigated here but other workers have shown that increased expression of Ia antigen occurs on cultured LC in the absence of T cell conditioned media,^{18,19} and expression of class II antigen on corneal epithelium has been shown to occur within 24 hours of corneal transplantation in non-rejecting rat corneal transplants.²⁰ This evidence therefore supports the concept that increased expression of Ia antigen may not always be related to T cell factors, like interferon γ . Although low levels of Ia antigen have been detected on normal rabbit corneal epithelium,²¹ Ia antigen was never detected on the normal mouse corneal epithelium in this study and

therefore there is no evidence in this work to suggest that the Ia antigen expression on the corneal epithelium during HSK was an upregulation of low levels of Ia antigen rather than de novo expression. Differing staining methods have however different sensitivities for corneal antigens and this may explain the variability of reported expression of corneal class I and II antigens in the literature and possibly the apparent absence of Ia antigen on the normal corneal epithelium of the mouse. The more intense and extensive staining of Ia antigen on the corneal epithelium of the non-immune mice on day 7 after infection may be related to the continued presence of virus and the more severe corneal inflammation which occurs in non-immune mice compared with immune mice.¹⁰ It is acceptable to implicate a role for activated T cells and interferon γ at this stage of the keratitis.

The finding that corneal epithelium can either support or enhance the proliferation of HSV specific T cells in vitro indicates that the corneal epithelium has immunostimulatory or immunoenhancing properties and is more than just a physical barrier or target for immune responses. The normal central corneal epithelium of the mouse used in this study provides an unusually pure population of epithelial cells without passenger cells, such as LCs. In agreement with our workers,²⁸ specific staining for Ia antigen and ATP-ase bearing cells in clinically normal corneas of the mice used in this study and others never revealed LCs in the central corneal epithelium. It is therefore not likely that any LCs were contaminating the central corneal epithelial cellular preparations used in these proliferative assay studies.

It is probable that small numbers of Ia negative cells with the potential of becoming APCs were present in the Ia depleted T cell preparations but it is clear that these were unable to stimulate a strong T cell proliferative response since the addition of corneal epithelium to the cultures increased the stimulation index from 7 (for T cells with HSV) to 172 (for cultures with T cells, HSV, and corneal epithelium) after 4 days in culture. For T cell proliferation of this magnitude to occur antigen presenting cells and a cytokine co-factor, like IL1, along with antigen is necessary. Since corneal epithelium can produce corneal epithelial cell derived thymocyte activating factor (CETAF) which possesses IL1-like properties²² and we have shown in this study that corneal epithelium can express Ia antigen, these two factors along with HSV antigen are likely to have contributed to the T cell proliferation.

The function of Ia antigen expression on corneal epithelium and of the lymphocyte stimulatory properties of corneal epithelium is unknown but it may be speculated that this property is a manifestation of one component of the overall in vivo immune response involved in T cell activation and therefore subsequent control of a herpetic infection. The potential ability of corneal epithelium to behave as an antigen presenting cell may result in more rapid activation of T cells. Ia antigen on corneal epithelium may act as a target for class II restricted cytotoxic T cells, as has been suggested

for HSV infections in humans²³⁻²⁵ and mice.²⁶ This may decrease the need for corneal infiltration and may therefore reduce the overall corneal infiltration. Such a function would have beneficial effects on preserving visual acuity, since the infiltrative response during stromal HSK is thought to contribute significantly to corneal scarring. It is also possible that, under certain undefined circumstances, persistence of Ia antigen in the cornea following HSK could contribute to persistent immunopathology, occasionally associated with HSK, as has been suggested for some autoimmune disorders.

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