EXPRESSIÓN DE MOLÉCULAS B7 Y TLR:9 EN CÉLULAS EPITELIALES CORNEALES INFECTADAS CON ADENOVIRUS: IMPLICACIONES CLÍNICO-PATOLÓGICAS EN LA QUERATOCONJUNTIVITIS VIRAL

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ABSTRACT

Purpose: B7 molecules are a family of proteins that co-stimulate T cells during immune activation. Normally the corneal epithelial cells (CEC) do not express these molecules on their cell surface. Toll-like receptors play an important role in the innate immune response to invading pathogens and recently have been demonstrated to be expressed on mice cornea. The objective of this study was to determine whether adenoviral infection induces B7 molecules and TLR9 on human CEC.

Methods: CEC were isolated from human corneas treated with dispase-II, and grown in the presence of supplemented hormonal epithelial medium until confluence. Then CEC were then infected with adenovirus 5 (Ad5) and cultured for different times.

RESUMEN

Objetivo: Las moléculas B7 son una familia de proteínas que coestimulan al linfocito T durante la activación inmunitaria, normalmente las células epiteliales corneales (CEC) no expresan estas moléculas en superficie. Los receptores tipo Toll juegan un papel importante en la respuesta inmune innata hacia patógenos invasores y recientemente se demostró su expresión en córneas de ratón. El objetivo del presente estudio fue determinar si la infección viral induce moléculas B7 y TLR9 en CEC humanas.

Métodos: Las CEC fueron obtenidas de corneas humanas tratadas con dispasa II y crecidas en presencia de medio hormonal epitelial suplementado hasta su confluencia. Posteriormente las células
The CEC were then recovered and stained against human CD80, CD86, TLR-9 and cytokeratin. All cells were analyzed by flow cytometry.

**Results:** Ad5 infection of CEC induced the expression of B7 molecules and TLR-9 after 24 hours in culture, rising to maximum levels at 72 hours. B7 expression at 72 hours was as follows: CD80 expression on infected CEC was 62% (standard error [SE] 2.6) versus 3% (SE 1.2) on non-infected CEC (p<0.001); CD86 expression on infected CEC was 95% (SE 2.1) versus 5% (SE 1.2) on non-infected CEC (p<0.001). TLR-9 expression at 72 hours was 80% (SE 1.2) on infected CEC versus 5% (SE 1) on non-infected CEC (p<0.001).

**Conclusions:** Ad5 infection induced the expression of B7 molecules and TLR-9 on CEC (Arch Soc Esp Oftalmol 2006; 81: 391-400).

**Key words:** B7, TLR-9, adenoviral keratoconjunctivitis, corneal, epithelial cells.

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**INTRODUCTION**

The eye is an immunoprivileged spot due to its complex network of mechanisms maintaining immunological tolerance to its own antigens (Ag) (1). Among the elements regulating immune responses we find immune ignorance, where corneal epithelium cells (CEC) prevent the development of surface blood and lymph vessels, thus blocking access to activated lymphocytes (2); were this to happen, cells on the corneal endothelium could induce self-reactive lymphocytes to programmed cell death (3). Another mechanism of immune tolerance is the lack of molecules in the Major Histocompatibility Complex (MHC) and of molecules that favor specific immune responses by stimulating T Lymphocytes (4).

The role of accessory molecules is to increase signaling during lymphocyte activation (5); usually, corneal cells do not present accessory molecules, although it has been shown that in corneal stromas, dendritic and Langerhans cells (6) both can express accessory molecules of the B7, CD80 or CD86 families before an antigen stimulus and operate as antigen cells during cornea rejection, which suggests that its functional maturity is linked to the development of immune responses (7). This same model showed that corneal dendritic cells produce a beta-transforming growth factor (TGF-β) (8) that negatively regulates the expression of accessory molecules and T Lymphocite activation, inducing an immunosuppressive microenvironment (9,10).

Any infection may alter the eye’s microenvironment and cause damage to its surface; among the most frequent infections, we find those caused by adenovirus (Ad) (11). It has been shown that during Ad infection, infected CEC cells are capable of producing interleukin-1 (IL-1) (12). Some authors have pointed that IL-1 is capable of inducing B7 molecules in epithelial cells in other spots such as the skin (13) or the respiratory epithelium (14). Furthermore, in animal models for viral infection there is an increase of TGF-β in the microenvironment that would operate as anti-inflammatory agents with antiviral properties (15). On the other hand, we observed that during the immune response to viruses, some epithelium cells express molecules which recognize preserved molecular patterns; this type of structures are known as Toll-like receptors (TLR) and it has been shown that TLR-9 can interact with non-methylated CpG present in bacterial or viral DNA (16). The main purpose of the present paper is to determine whether adenovirus infection is capable of inducing in CECs the expression of molecules that contribute to specific immune responses (B7 molecules) or innate immune responses (TLR-9).
SUBJECTS, MATERIAL AND METHODOLOGY

Obtaining Corneal Epithelium Cells

CECs were obtained from human corneal buttons free of viral infection coming from individuals subjected to a secondary cornea transplant for keratoconus. The data submitted come from CECs expanded from 3 corneal buttons from unrelated individuals; each cell expansion was subjected to three tests. CECs were separated with dispasa II (Roche, Germany) from the stroma during 15 minutes at 37°C, CECs being separated from stroma cells via a nylon net. Once obtained, they were washed with DMEM (Dulbecco Modified Eagle Medium) (Gibco, Grand Island, NY, USA), subsequently assessing its viability without using trypan blue. Cells expanded in the presence of a supplemented hormonal epidermal medium (SHEM) in boxes with 24 slots (Costar, NY, USA). SHEM consists of DMEM/F12 v/v (Gibco, Grand Island, NY, USA), an epidermal growth factor (Sigma, St. Louis Missouri, USA), insulin, transferrin, selenium (Roche, Mannheim, Germany), hydrocortisone, gentamicin, streptomycin, pencilcline, anfotericine B (Sigma, St. Louis Missouri, USA) and 10% of fetal bovine serum (Gibco, Grand Island, NY, USA) (17). When cells achieved 80% confluence, approximately 15-20 days later, they were detached from the slots with 0.25% tripsine solution (Gibco, Grand Island, NY, USA) and were transferred to a 25 mm² box (Costar, NY, USA). There they were cultivated during 17-20 additional days in identical culture conditions until achieving 90% confluence, being used in later tests.

DNA Extraction and Polymerase Chain Reaction (PCR)

In order to extract DNA from tissue, we used the methodology followed by the manufacturer of the QIAamp minikit (QIAGEN Sciences, Maryland, USA). To guarantee that corneal buttons were free from prior viral infection, a PCR was performed to identify adenovirus or herpesvirus. Primers for adenovirus were ADRJC1, 5’-GACATGACTTTTGCAGGTGCATCCCATGGA-3’; antisense sequence ADRJC2, 5’-CGCGCT-GAGGGTGTGCAGGTA-3’, magnifying a 140pb product; for herpesvirus the sequence was ALE1 5’-TTATTGCCTAGCATCGCAGGG-3’; antisense sequence ALE2 5’-GGCGACCTGTATAACGTGTT-3’, magnifying a 278 pb product. PCR was performed using the Omniscript Reverse Transcription kit (QIAGEN Sciences, Maryland, USA), magnification in a Gene-Amp PCR System 2400 thermocycler (Perkin Elmer Co. Nor-walk, Connecticut, USA). Corneal buttons which yielded positive results for adenovirus and/or herpesvirus by PCR were not used in our study.

Ad5 Identification

Ad5 was identified through Eco-RI, Hind-III, Bgl-II and Sma-I restriction enzymes following manufacturer’s instructions (Sigma Chemical Co, Saint Louis Mo, USA). Restriction products were observed on 1% Agarose gel electrophoresis, run during 9 hours at 40 volts. DNA bands were stained with ethidium bromide at 0.1%, using molecular weight markers λ and φ (Promega, Madison, WI, USA). The restriction pattern was compared to profiles previously published for adenovirus (18).

Viral Particles Titulation

Vero cells (American Type Culture Collection, CRL-1586) were infected with serial Ad5 dilutions until cytopathic effects were observed. Infective particles were determined using the Reed-Muench method (19); the titulation of inoculated viruses was 6.08 X10⁸ /100 ml.

Cell Cultures

Cell cultures were performed being Ad5 both present and absent. CEC infection was achieved using a viral titulation obtained from vero cells. CECs were recovered at 24 hour intervals until the third day. Culture supernatant (SN) was frozen at -70°C until the time of use.

Immunofluorescence

Recovered culture CECs were processed for a flow cytometry using monoclonal antibodies com-
combined with fluorochrome and directed against CD80, CD86 (BD Biosciences Pharmingen, CA, USA), TLR-9 (e-Biosciences, CA, USA) and cytokeratine, clone MNF116 (DAKO, Carpintería, CA, USA).

**Determining Cytocins**

Culture SN was used to determine TGF-β via a quantitative ELISA sandwich following the methodology suggested by the manufacturer (R&D Systems, MN, USA). Results were interpreted with an ELISA reader (Termo-Labsystems, Helsinki, Finland) at 450nm wave length and 540 nm correction.

**Statistical Analysis**

We used U-Mann Withney to compare two groups, since when performing the normalcy analysis with a Kolmogorov-Sminov test it was found that our data did not show a normal distribution. p<0.05 was considered statistically significant. Statistical analysis was undertaken using SigmaStat (Systat Software, Inc. Point Richmond, CA, USA. Version 10) and graphics were obtained via SigmaPlot (Systat Software, Inc. Point Richmond, CA, USA. Version 10).

**FINDINGS**

**Adenovirus 5 Infection on Corneal Epithelium Cells**

The first goal of our study was to assess Ad5 infection in CECs. Viral infection was proven observing the characteristic cytopathic effect (fig. 1a) via direct immunofluorescence (fig. 1b) by PCR (fig. 1c); figure 1d shows the characteristic cross pattern of Ad5 restriction enzyme.

**Morphological Changes in Cultured Cells**

Cultured CECs gradually lost their classic epithelium morphological characteristics, acquiring instead a fibroblastic shape, whether Ad5 infected or not. It was thus necessary to determine the expression of cytokeratine, a classic phenotypic characteristic in epithelium cells (20), using anticytokeratin antibodies combined with fluorochrome.

Figure 2 (a) shows size (FSC-H) and granularity (SSC-H) for CECs in a flow cytometry. Figure 2 (b) shows that approximately 70% of CECs maintain their epithelium phenotypic characteristics, with positive Cytokeratins (CK) (+); whereas 30% of those cells which based on granularity and size correspond to CECs lose this marker’s expression [30% standard error (ES) 5.2]. CEC CK+ percentages were similar in infected (70% SE 6.4) and non-infected (72% SE 4.3) cells; no significant statistical differences were found between them.

**B7 Molecule Expression in Ad5 infected CECs**

B7 molecules (CD80 and CD86) kinetic expression was assessed in CECs CK+, both Ad5 infected and non-infected. The data obtained show that percentage for CEC CK+ CD80+ was 54% at 24 hours, 52.3% at 48 hours, 62% at 72 hours (fig. 3); in contrast, percentages for non-infected CEC CK+ CD80+ were < 5% during the three days of culture (fig. 3, table I). Kinetic expression for CD86 in CEC CK+ showed a similar behaviour, 62.3% at 24 hours, 86% at 48 hours and 95% at 72 hours (fig. 3, table I).

**TLR-9 Expression in Ad5 Infected CECs**

Kinetic expression for TLR-9 was assessed in CEC CK+, both Ad5 infected and non-infected. The data obtained show that percentages for CEC CK+ TLR-9+ were 62% at 24 hours, 63.3% at 48 hours and 80% at 72 hours (fig. 4); in contrast, percentages for non-infected CEC CK+ TLR-9+ stood at approximately 5% during the three days of culture (fig. 4, table II).

**TGF-β in Cultured CECs Supernatant**

TGF-β production was assessed with ELISA during the different days of culture. Results showed an increase in TGF-β production found in the culture supernatant of Ad5-infected CECs in comparison with non-infected CECs (fig. 5). Nonetheless, quantitative differences were not statistically significant.
Fig. 1: Assessment of Ad5 infection in CECs. (a) shows the cytopathic effect of the virus (white arrows) in cultured cells, 10x magnification; (b) shows positive immunofluorescence to tinction with antiadenovirus antibodies combined with FITC (Adenovirus DFA kit, CHEMICON International, USA) in infected cells (green arrow) and to nuclear tinction with propidium iodide in non-infected cells (red arrow); use of both tintions results in a yellow color (yellow arrow), 40x magnification. (c) shows PCR for adenovirus; track 1 corresponds to molecular weights, track 2 to non-infected cells, track 3 to infected cells. The latter presents a 140 bp product corresponding to magnified ADRCJ1, ADRCJ2 primers used for viral identification, tinction with ethidium bromide. (d) shows the restriction pattern characterizing Ad5; track 1 corresponds to uncut DNA, track 2 corresponds to DNA cut with Eco-RI, track 2 corresponds to DNA cut with Hind-III, track 4 corresponds to DNA cut with Bgl-II, track 5 corresponds to DNA cut with Sma-I; λ y φ correspond to molecular markers.
DISCUSSION

In the absence of infection, the eye uses several devices to maintain tolerance to its own Ag and thus avoid activating immune responses (1-3,8-10). In such context, where inflammatory responses in any other spots would be protective, it would cause more damage than good to the eye, thus the interest in studying certain immune mechanisms available to CECs whenever infected with a virus.

In order to achieve our goal, human CECs were infected with Ad5. Ad5 infection has been widely used as model adenovirus infection in lab animals (21,22) but there are few studies on human CECs; Trousdale MD et al. (23) showed that rabbit and human CECs could be infected with Ad5, achieving the maximum amount of viral particles between 48 and 72 hours. This is the reason why cell cultures in

![Image](https://example.com/image1.png)

**Fig. 2:** Cytometric characteristics for CECs. (a) show size (FSC) and granularity (SSC) characteristics for cultured cells. (b) shows a dot chart from region I «R1» shown in (a). R1 includes exclusively those cells whose size and granularity correspond to CECs. R2 represents CK+ cells. FSC in the «y» axis, tintion with anticytoke- ratin antibody combined with fluorochrome on the «x» axis.

![Image](https://example.com/image2.png)

**Fig. 3:** Expression of CD80 and CD86 in CECs. Hystograms show the intensity of fluorescence for CD80 (a) and CD86 (b) in non-infected CECs (thin line) and infected CECs (thick line) at 72 hours after culture. Hystograms were performed on R1+R2, so that subsequent data correspond only to CEC CK+. Data presented here come from CECs expanded in 3 corneal buttons belonging to three unrelated individuals (See section on material and methodology), and three tests were performed on each expanded cell.

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<th>Table I. Kinetic Expression of CD80 and CD86 in CEC CK+ during the different days of culture</th>
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<td>24 h</td>
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<tr>
<td>Infected CECs</td>
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<td>Non-Infected CECs</td>
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Results are expressed in percentages ± Standard error. *: p= 0.017; **: p < 0.001.
our study were conducted up until 72 hours. During the time of culture, CECs gradually lost their epithelial morphology and acquired instead a fibroblastic one. This phenomenon is frequently observed in primary cultures of corneal cells and is affected by local growth factors such as fibroblastic growth (FGDP) and TGF-β, which can induce, depending on their concentration, the expression of molecules that allow making phenotypic differences among cells in fibroblasts or microfibroblasts (24). Our study determined the concentration of TGF-β in cell cultures and it is likely that this cytokine could be involved in the observed morphological changes such as the fibroblastic shape, or in phenotypic changes such as the loss of cytokeratin expression, since Ogawa E. et al. (25) already reported similar data by using a latent adenoviral infection model in respiratory epithelium cells. The same authors observed that underlying the Ad-infected cells there is a stroma remodelation at the subepithelial level, suggesting that such remodelation could be mediated by TGF-beta (24).

Interaction of B7 with its natural ligand (CD28) expressed in T lymphocytes is critical during activation of specific Ag (26). Nevertheless, we know that CECs do not express accessory molecules (1,4) and therefore we studied whether Ad5-infected CECs could induce B7 molecules on its surface. Our experiments showed that CECs infected with Ad5 in vitro can express both CD80 and CD86. Prior to our work, it was reported that the only cells capable of expressing accessory molecules in the cornea were dendritic cells and Langerhans cells in response to a stimulus (6-7, 28). Proinflammatory cytokines [IL-1, IL-6 and tumor necrosis factor alpha (TNF-a)] are involved in the expression of B7 molecules in dendritic cells (29) in epithelium cells during chronic stimuli such as atopic dermatitis (13, 30) or viral infections in the respiratory epithelium (14). Our study did not determine concentrations of these cytokines in the supernatant, although they were possibly present in the microenvironment, since Chang CH et al. have proven that adenovirus infections could induce production of large amounts of IL-1 in infected CECs (12).

Table II. Kinetic Expression of TLR-9 in CEC CK+ during the different days of culture

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<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
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<tr>
<td>Infected CECs</td>
<td>62 ± 8.1*</td>
<td>63.3 ± 7.2**</td>
<td>80 ± 1.2#</td>
</tr>
<tr>
<td>Non-infected CECs</td>
<td>5 ± 1.2**</td>
<td>4 ± 1**</td>
<td>5 ± 1#</td>
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Results are expressed in percentages ± standard error; *: p= 0.002; **: p= 0.001; #: p< 0.001.
Lastly, the study assessed the expression of TLR-9 in CECs. Toll receptors belong to a family of receptors involved with innate immune responses and their ligands are molecular structures preserved, among others, as lipopolysaccharide, flagellin (16). Recently, the expression of TLR-2, TLR-4 and TLR-9 was reported for mice cornea (31). TLR-9 recognizes the non-methylated CpG present in viral DNA (16) and activation via TLR-9 could induce the production of proinflammatory cytokines (32,33).

The study’s findings could have significant clinical-pathological implications for viral keratoconjunctivitis. It is well known that adenovirus keratoconjunctivitis is the most frequent viral infection of the ocular surface (11). It is characterized by conjunctival hyperemia, foreign body sensation, epiphora, palpibral conjunctival follicular reaction, palpebral edema and lymphadenopathy. Observing the cornea, we find a thin diffuse epithelial keratitis found using fluoresceine that may persist for 2 to 3 weeks. These injuries may evolve, depending on viral activity, into a focal epithelial keratitis characterized by white and focal subepithelial opacities just underneath the epithelium injuries; this type of keratitis may persist for 1 to 2 weeks and in some cases could be associated to a moderate transitional anterior uveitis. Approximately 2 weeks after the onset of symptoms, an anterior stromal infiltrate which may persist for weeks or years (34).

The molecular mechanism involved in the production of clinical data could have the following hypothetical sequence: A. Ad infection in CECs interacts with its intracellular ligand (TLR-9); this molecule interacts with the virus non-methylated CpG and induces cell activation. Activation via TLR-9 induces production of proinflammatory cytokines such as IL-1. This cytokine is the main

Fig. 6: Hypothetical sequence of molecular events during adenovirus infection.
mediator for inflammatory reactions on the ocular surface since it governs the activation of endothelial cells, in which it induces the expression of intercellular adhesion molecules -1 (ICAM-1), which in turns favors leucocyte recruitment to the place were the viral infection is acting (12); B. IL-1 together with IL-6 and TNF-α trigger the expression of B7 molecules in dendritic cells (29-30); it is likely that these cytosines contribute to inducing B7 molecules in Ad-infected CECs. C. The expression of CD80 and CD86 in CECs may have two consequences: first, favoring the activation of T Lymphocytes that are specific to this virus, which entails the coexpression of MHC-I, although this same mechanism could activate self-reactive clones as those found in herpetic keratitis (35), resulting in moderate transitional uveitis. Secondly, the virus may use B7 molecules as ligands in order to spread infection among additional activated CECs, as proven at least in the case of Ad3 (36). D. Microenvironments play a significant role in terms of antiviral immune responses; TGF-α typically produced by CECs induces the expression of both TLR-9 and TGF-β. (37). At the same time TGF-β induces the expression of FGF by stromal fibroblasts (38), both cytosines TGF-β and FGF may be able to favor renovation (25) at the cornea level, which could correspond to subepithelial injuries (26) and to the corneal stroma opacities (fig. 6).

In brief, our findings suggest that CECs can express CD80, CD86 and TLR-9 during viral infections. Clinical-pathological implications suggested herein may be used as a target for future research or therapeutic interventions in viral keratitis.

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