Viruses are a common cause of eye infection. The local mucosal response, with production of antibodies released into tears, is believed to provide an important immune defense against these agents. However very little information exists on the viral specificity of normal tear immunoglobulins. In this study we obtained tears, parotid saliva and serum from 40 normal subjects without eye disease. Samples were examined by enzyme linked immunosorbent assay (ELISA) for antibodies to seven common viruses which invade mucosa: cytomegalovirus (CMV), Epstein Barr (EBV), herpes simplex type I (HSV1), measles, mumps, rubella and varicella zoster virus (VZV). The majority of normal tears contained antibodies to HSV1 (73%) and EBV (65%), occasionally to mumps (30%), rubella (30%), and VZV (20%), and rarely to CMV (5%). Tear viral antibodies were mainly IgA class, but it was not unusual to find IgG antibodies to HSV1, VZV, rubella and measles. Tear and parotid saliva immunoglobulins from the same individual had entirely different viral reactivity. In most cases tear viral antibodies were reflected in serum viral antibodies, although the immunoglobulin class might differ. However, 15% of normal tears had antibodies to HSV1 without detectable serum antibodies. From this study we conclude that normal tear immunoglobulins contain antibodies to common viruses, in particular to HSV1 and EBV. These tear antibodies are mainly IgA, but can consist of IgG. Viral antibodies in tears are independent of the antibodies present in parotid saliva, suggesting that there is preferential homing of committed B lymphocytes to different mucosal surfaces. Finally, the finding of tear antibodies to HSV1 in the absence of detectable serum antibodies suggests that the ocular surface may be the initial infection site for this virus in some healthy normal subjects. Invest Ophthalmol Vis Sci 29:1552-1558, 1988
mantic ammonia and 150 μl collected using glass capillary tubes gently inserted into the tear pool of the lower lid margin, as previously reported. To obtain this volume some subjects were stimulated two or three times. Care was taken not to touch or irritate the eye directly. Parotid saliva was stimulated with a sour lemon ball and 500 μl collected over 2 min directly from Stenson’s duct using a modified Lashley cup. This avoids the contamination present in a whole saliva sample. Blood was obtained by routine venipuncture at the time of tear and saliva sampling. After clotting, the blood was centrifuged and the serum collected. All samples were frozen at −20°C or −70°C until use.

**Immunoglobulin Determination**

IgG, IgA and IgM were determined in samples using ELISA as previously reported. Secretory IgA was determined in tears and parotid saliva using a modification of the IgA ELISA. Samples and all reagents were diluted in phosphate buffered saline (PBS)-0.5% Tween to prevent nonspecific binding and plates were washed three times with PBS-Tween between each step. Affinity purified goat anti-human IgA antibodies (Tago, Inc., Burlingame, CA) at 10 μg/ml were used to coat microtiter wells (Costar 96-well flat bottom plates, Data Packaging Corp, Cambridge, MA), and samples were incubated in triplicate wells as in the IgA assay. Tears were diluted 1:10,000 and 1:50,000 for this assay. Known amounts of purified human secretory IgA (Miles Scientific, Naperville, IL) were included in each assay to generate a standard curve. After three washes with PBS-Tween, 100 μl of mouse monoclonal antibodies to secretory component (Accurate Chemical and Scientific Corp, Westbury, NY) diluted 1:200 was added to each well and incubated for one hour at room temperature. After washings, 100 μl of goat antimouse IgG antibodies conjugated to horseradish peroxidase (HRP) (Hyclone Labs, Inc., Logan, UT) diluted 1:2000 was added to each well for 2 hours at room temperature. Tears were diluted 1:20, parotid saliva 1:5, and sera 1:400 and 1:700 in PBS-Tween. These dilutions were selected by standardizing the relative concentration of mean total immunoglobulins in each body fluid (3:1:250; tears:saliva:serum) and the minimum volume of sample required to run all the assays. The 1:400 serum dilution was included to assure detection of low levels of specific antibodies in serum. Certain sera were subsequently run at 1:100 to exclude the presence of any specific antibodies. Known positive and negative viral antisera were included on each plate as controls. HRP-conjugated anti-human immunoglobulin antibodies were diluted 1:2000 in PBS-Tween and used to screen for viral antibodies; class-specific antibodies were measured by substituting a 1:2000 dilution of HRP-anti-human IgG, IgA or IgM antibodies as the enzyme conjugate. In selected cases, tears were serially diluted to titrate specific antibody levels. When sufficient sample was available, tears which showed HSVI-IgA activity were subsequently screened for secretory IgA directed against HSVI. Reagents were used as outlined in the secretory IgA assay above, except on a viral microtiter plate. In this study the term “detectable antibodies” is defined as samples having a mean optical density (virus wells minus control wells) greater than the mean plus three standard deviations of at least five negative control antisera run simultaneously (commercial antisera or sera which were consistently negative in our laboratory for specific viral antibodies). All positive tear specimens were confirmed on two separate assays.

**Results**

**Immunoglobulin Levels**

All three body fluids showed a broad range in the concentration of immunoglobulins (Fig. 1). In tears secretory IgA (geometric mean level 192 μg/ml) was present in the greatest concentration, with low levels of IgG (mean 9.7 μg/ml) and IgM (mean 1.7 μg/ml) detected in all samples. Parotid saliva showed even lower levels of secretory IgA (mean 72 μg/ml), IgG (mean 1.4 μg/ml), and IgM (mean 0.7 μg/ml). Serum levels were 10 to 1500-fold higher than in tears: mean IgG was 1,539 mg/dl, mean IgA was 211 mg/dl, and mean IgM was 798 mg/dl.
Fig. 1. Immunoglobulin concentrations of 40 normal subjects were measured by ELISA and expressed in μg/ml for tears and parotid saliva, and in mg/dl for serum. IgG and IgM were measured in all three body fluids, secretory IgA (SIgA) in tears and saliva, and IgA in serum. The geometric mean value ± one standard deviation is indicated by the bar and dashed lines, respectively.
Prevalence of Viral Antibodies in Tears Compared to Saliva and Serum

Detectable tear antibodies to HSVI and EBV were found in 73% and 65% of normal subjects respectively (Table 1). Antibodies to mumps (30%), rubella (30%), VZV (20%), measles (10%) and CMV (5%) were distinctly less prevalent. Parotid saliva showed a different profile of detectable antibodies; mumps antibodies were the most prevalent, found in 70%. As expected, sera showed a high prevalence of detectable antibodies to all the viruses tested; only CMV reactivity was confined to a minority of these normal adults.

Antibodies to Multiple Viruses

Nearly half of our subjects had detectable tear antibodies to three or more of the viruses examined (Table 2), whereas only 13% of saliva samples showed detectable antiviral activity to multiple agents. All sera were positive for antibodies to three or more of the viruses looked at.

Compartmentalization of Viral Antibodies

The humoral response to each virus showed a distinct pattern with regard to the appearance of antibodies in various body fluids (Table 3). EBV and HSVI antibodies were most commonly detected in both tears and serum. Mumps antibodies were most commonly detected in both parotid saliva and serum. CMV, measles, rubella and VZV antibodies were confined to serum in the majority of subjects.

Class Specificity of the Tear Viral Antibodies

Detectable tear antibodies to EBV, HSVI, mumps and rubella consisted largely of IgA (Table 4). With VZV and measles however, it was more likely to find IgG than IgA antibodies. For all viruses tested there were certain normal subjects who had tear IgG antibodies. A few individuals even showed IgM (to mumps, rubella, EBV and measles). Of 22 tear samples with IgA directed against HSVI, there was sufficient volume to test 12 for viral specific secretory IgA. Five of 12 tear samples showed HSVI specific secretory IgA. Parotid saliva antibodies were exclusively IgA for almost all the viruses looked at; the only exception was CMV antibodies which were often IgG. Serum antibodies to all viruses were most commonly IgG. However, IgA was also frequently noted directed against mumps, measles, rubella and VZV.

Titration of Viral Tear Antibodies

Several tear samples with IgA viral antibodies were serially diluted and retested. Two samples with EBV antibodies were positive to dilutions of 1:640 and 1:1280; two samples with HSVI antibodies were positive out to 1:640 and 1:2560; and two samples with mumps antibodies were positive out to 1:40 and 1:10,240. One tear sample with measles antibodies titrated out to 1:80, and another sample with VZV antibodies titrated out to 1:40.

Viral Antibodies Confined to Tears

In initial screening dilutions (1:20 for tears, 1:5 for saliva, 1:400 and 700 for serum) several subjects showed anti-viral activity against EBV, HSVI, mumps and rubella confined exclusively to tears (Table 3). To exclude any antibodies in their sera, we retested serum at 1:100 dilution. Antibodies were then detected to EBV (two subjects), HSVI (four), mumps (one) and rubella (one). However, normal subjects remained with tear antibodies to HSVI, EBV and rubella in the absence of any detectable serum titers (Table 5). These tear antibodies were not confined to IgA, but included three subjects with IgG directed against HSVI and one with IgM directed against rubella.

Discussion

There is increasing evidence to support a common mucosal immune system in man.13-15 Studies both in

<table>
<thead>
<tr>
<th>No. of positive viruses</th>
<th>Tears</th>
<th>Saliva</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td>2 (2.5%)</td>
<td>2 (5%)</td>
<td>15 (37.5%)</td>
</tr>
<tr>
<td>4</td>
<td>6 (15%)</td>
<td>4 (10%)</td>
<td>10 (25%)</td>
</tr>
<tr>
<td>3</td>
<td>10 (25%)</td>
<td>2 (5%)</td>
<td>4 (10%)</td>
</tr>
<tr>
<td>2</td>
<td>15 (37.5%)</td>
<td>6 (15%)</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>2 (5%)</td>
<td>20 (50%)</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>6 (15%)</td>
<td>9 (22.5%)</td>
<td>0</td>
</tr>
</tbody>
</table>

* Data are expressed as the number (%) of 40 normal subjects who had antibodies by ELISA to the seven viruses tested. Viruses looked at included CMV, EBV, HSVI, measles, mumps, rubella and VZV. Simultaneous samples of tears, parotid saliva, and serum were examined.

Prevalence of antibodies to multiple viruses*
animals and humans have indicated that oral feeding or aerosol exposure of antigen will induce antigen-specific antibodies in external secretions not only of the local gastrointestinal or respiratory tract, but also in secretions at distant mucosal sites. This is apparently due to homing of committed B cells in a controlled migration pattern that is poorly understood. The local presence of antigen at a mucosal site results in greater clonal expansion of specific antibodies than at antigen-free, distal sites. This system helps explain why tears or parotid saliva might well contain antibodies to some of the viruses we studied, since all are mainly respiratory agents normally spread by aerosol droplets or saliva. Some of these viruses are even capable of directly infecting ocular tissues, including CMV, EBV, HSVI, measles, rubella and VZV. External secretions can harbor infectious agents; a variety of viruses have been isolated from tears or saliva, often in the setting of subclinical infection. Viruses in secretions would be expected to bind to specific antibody, forming immune complexes and resulting in lower free antibody levels. Although this theoretically could result in a false-negative assay for viral antibody, this would only occur very early in the immune response when antigen predominated over antibody. It is unlikely that this was a significant factor in the present study.

The role of mucosal immunity in preventing viral infections has been strongly suggested in a number of studies. The mucosal defense system involves non-specific mechanisms, such as the anatomic barrier offered by intact cells; mucosal colonization by non-pathogenic flora; the presence of a gelatinous mucin coating which interferes with organism attachment; and normal components of the neutral pH external secretions which have antimicrobial activity such as lactoferrin, lysozyme, lactate and long-chain fatty acids. Specific defense mechanisms include both cell-mediated and humoral mucosal immune reactions. However, the best correlate of effective immunity to many viruses appears to be the production of local antibodies by plasma cells within the conjunctiva and lacrimal gland, with their subsequent release into secretions.

Surprisingly little is known about the antiviral specificity of normal secretions. Most studies on tear viral antibodies have focused on patients with ocular infections. For example secretory IgA, IgG and IgA tear antibodies to HSVI have been reported in patients with ocular herpetic infections. The controls included subjects with a variety of ocular disorders; only two were normal. Tear antibodies after oral vaccination with influenza have been reported in order; only two were normal. The paucity of information on normal subjects has been due in part to the lack of sensitive assays capable of analyzing microliter sample volumes. In this study we used ELISA, which is known to be a sensitive and specific technique to detect tear viral antibodies.

The prevalence rates of detectable serum antibodies to the seven viruses are consistent with previous studies, confirming the validity of our assay.

Table 3. Compartimentalization of viral antibodies

<table>
<thead>
<tr>
<th>Virus</th>
<th>Tears &amp; serum</th>
<th>Tears &amp; saliva</th>
<th>Saliva &amp; serum</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>EBV</td>
<td>4</td>
<td>19</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>HSVI</td>
<td>10</td>
<td>18</td>
<td>—</td>
<td>4</td>
</tr>
<tr>
<td>Measles</td>
<td>3</td>
<td>1</td>
<td>23</td>
<td>1</td>
</tr>
<tr>
<td>Mumps</td>
<td>1</td>
<td>2</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>Rubella</td>
<td>3</td>
<td>8</td>
<td>2</td>
<td>19</td>
</tr>
<tr>
<td>VZV</td>
<td>1</td>
<td>6</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

* Antibodies to seven different viruses were examined by ELISA in 40 normal subjects. Data are expressed as the number of subjects positive for viral antibodies according to the body fluid compartments (tears, parotid saliva and serum) in which antibodies were detected.

Table 4. Class specificity of tear viral antibodies

<table>
<thead>
<tr>
<th>Virus</th>
<th>No. antibody positive</th>
<th>IgG</th>
<th>IgA</th>
<th>IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV</td>
<td>2†</td>
<td>1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>EBV</td>
<td>26</td>
<td>2</td>
<td>26</td>
<td>3</td>
</tr>
<tr>
<td>HSVI</td>
<td>29</td>
<td>18</td>
<td>22</td>
<td>—</td>
</tr>
<tr>
<td>Measles</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Mumps</td>
<td>12</td>
<td>2</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>Rubella</td>
<td>12</td>
<td>6</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>VZV</td>
<td>8</td>
<td>7</td>
<td>5</td>
<td>—</td>
</tr>
</tbody>
</table>

* Tear samples from 40 normal subjects which had tested positive for anti-viral immunoglobulin by ELISA were then examined by ELISA for class-specific IgG, IgA or IgM viral reactivity. Data are expressed as the number of subjects positive for viral antibodies.

† One tear sample with antibodies to CMV could not be tested for class specificity.
Using only 5 μl of tears per viral assay, we found tear antibodies to all seven viruses. The relatively low dilution used to screen for antibodies made it unlikely that positive tear specimens were missed. Viral reactivity was not a nonspecific background binding, since positive samples showed activity even when diluted several hundred to thousand-fold. Viral antibodies in tears and parotid saliva differed among individuals, suggesting that these antibodies were not simply the result of programmed B cell migration to all mucosal compartments. Rather, it suggests that there was selection of B cells migrating to different mucosal sites, or that local exposure to the virus differed at these sites.

While the detection of antibodies in tears was generally mirrored by serum, there were several subjects with detectable viral antibodies in tears not detectable in serum. There were also a number of examples of different classes of viral antibodies in tears and serum. Thus the detection of tear antibodies cannot be explained solely by simple transudation from serum. It is possible that a selective enrichment of committed B cells occurs within the ocular mucosa. Normal tears showed a high prevalence of antibodies to HSV and EBV. In fact, HSV antibodies were more frequently detected in tears than in sera in this normal population. Six subjects had tear HSV antibodies without detectable serum antibodies. This is an interesting and unexplained observation. It is known that primary HSV infection can involve ocular surfaces as well as perioral or more rarely genital areas. Virus can become latent in the trigeminal or superior cervical ganglia, and with reactivation can appear in tears. Viral transmission is probably via infected secretions. Yet none of these individuals had any history or signs of ocular infection. Could the eye serve as a portal of entry for HSV in certain individuals, thus generating a local immune reaction in the absence of a systemic reaction? This would require subclinical viral infection, which certainly occurs with HSV. Is there preferential organ-selective homing of HSV-committed B cells to ocular tissues, perhaps based on lymphocyte recognition of specific venule homing receptors, and does this serve to protect the eye from clinical disease? We also found that positive tear specimens were missed. Viral reactivity was not a nonspecific background binding, since positive samples showed activity even when diluted several hundred to thousand-fold. Viral antibodies in tears and parotid saliva differed among individuals, suggesting that these antibodies were not simply the result of programmed B cell migration to all mucosal compartments. Rather, it suggests that there was selection of B cells migrating to different mucosal sites, or that local exposure to the virus differed at these sites.

Table 5. Class specificity of viral antibodies confined to tears*

<table>
<thead>
<tr>
<th>Virus</th>
<th>No. antibody positive</th>
<th>IgG</th>
<th>IgA</th>
<th>IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBV</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSVI</td>
<td>6</td>
<td>3</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Rubella</td>
<td>2</td>
<td></td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

* Several of the 40 normal subjects showed anti-viral antibodies detected by ELISA in tears only, in the absence of serum or saliva antibodies. Sera from these subjects were tested to exclude any detectable levels of antibodies (1:100 dilution) and remained negative. The class specificity of the tear-specific viral antibodies in this group was then examined by ELISA. Data are reported as the number positive for IgG, IgA or IgM viral antibodies.

home to ocular tissue as a protective mechanism. Alternatively lacrimal or conjunctival tissue might act as a reservoir for this virus.

We observed that antiviral activity in normal tears is not limited to IgA, the major immunoglobulin in secretions. There are experimental data to suggest that the interplay of different classes of mucosal antibodies can contribute to virus-induced mucosal disease. In our normal subjects IgG antibodies were frequent, particularly to HSVI, and tear IgM antibodies were noted in a few cases to mumps, rubella and EBV. Most of the subjects with tear IgM antibodies showed evidence in serum of recent viral infection.

In summary, this study has shown that a variety of specific anti-viral antibodies are detectable in normal tears and that these antibodies do not necessarily correlate with serum or parotid saliva antibodies in the same individual. Tear anti-viral antibodies are mainly of the IgA class, but can consist of IgG and in the setting of recent systemic viral infection even IgM. Antibodies to HSVI were particularly common in tears, were frequently IgG, and in 15% of individuals were confined to tears in the absence of a systemic humoral response. The significance of an anti-viral humoral response confined to tears, and the relationship between different classes of tear viral antibodies, remains to be determined. ELISA now offers the ability to probe the ocular immune response to viral infection. Future studies on selected patient populations should help clarify the role of tear antibodies in determining susceptibility to viral eye invasion.

Key words: tears, viral antibodies, saliva, herpes simplex virus, mucosal immune system

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