Inhibition of Hydroxyl Radical Formation by Human Tears

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The effect of human tears on oxygen radical formation was investigated using xanthine-xanthine oxidase as the oxygen radical generating system. Superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) were measured using ferricytochrome c as indicator. OH$^*$ formation was monitored by measuring the hydroxylation of salicylate. Addition of traces of iron (Fe$^{3+}$) and chelator (EDTA) was a prerequisite for OH$^*$ formation in this system. Human tears did not detectably affect O$_2^-$ or H$_2$O$_2$ formation but markedly inhibited OH$^*$ formation. Tears obtained from eight different individuals all showed a marked inhibitory effect on OH$^*$ formation, whereby only a small individual variation was observed. During separation of human tears by gel filtration on a Sephadex G75 column, three protein peaks eluted from the column. The first contained lactoferrin, the second as yet unidentified material, and the third lysozyme. Inhibitory activity on OH$^*$ formation coincided with the first protein peak and also with fractions eluting after the protein peak containing lysozyme. The major inhibition on OH$^*$ formation was seen in these latter fractions, which contain small organic and anorganic substances. The fact that ascorbic acid could not be detected in human tears and that it did not affect formation of OH$^*$ in this investigation's assay system indicates that this compound was not involved in the observed low molecular weight inhibitory effect. Analysis of various cations suggested that the low molecular weight inhibitory effect could largely be ascribed to tear calcium. Tear calcium binds to EDTA and thus possibly prevents formation of the essential catalytic iron-EDTA complex. Experiments using purified human milk lactoferrin showed that this protein, which is abundantly present in human tears, can inhibit OH$^*$ formation in the model used here. The inhibitory effect of lactoferrin was counteracted by increasing the iron concentration in the reaction mixtures. These findings suggest that tear lactoferrin may play an important role in the protection of the ocular surface against OH$^*$ induced damage. Invest Ophthalmol Vis Sci 28:305–313, 1987

During lifetime the human eye is exposed to light every day. Furthermore, it is known that several parts of the eye need oxygen for their energy supply. Oxygen and light can be toxic to ocular tissues. Formation of reactive oxygen radicals eg, superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and the hydroxyl radical (OH$^*$), can be induced by photochemical oxidations in transparent ocular tissues. For example, the lens contains photosensitizers such as kynurenine derivatives and aged lens proteins. Since the lens is exposed to light, these photosensitizers are a potential source of oxidative stress possibly resulting in cataract formation. Oxygen radicals can also be formed during metabolic oxidations and inflammatory conditions.

Several parts of the eye such as the lens, cornea, retina, vitreous and aqueous humor possess so-called scavengers of oxygen radicals including superoxide dismutase, catalase, glutathione, glutathione peroxidase and glutathione reductase, α-tocopherol and ascorbic acid.

Little is known, however, about the mechanism whereby the outer parts of the eye are protected against potential damage caused by oxygen radicals. The tearfilm, which is known to play an important role in maintaining the integrity of the ocular surface, could play a role in the elimination of reactive oxygen species. Until now, no reports have been published concerning the presence in the tearfilm of known antioxidants such as superoxide dismutase, catalase, ascorbic acid, or glutathione/glutathione peroxidase. The tearfluid is a complex mixture consisting of proteins, enzymes, lipids, metabolites, and electrolytes. Of the known proteins present in human tears, lactoferrin is the only one that has been shown to play a role in the regulation of hydroxyl radical formation, albeit that the experiments were performed with milk lactoferrin and were dependent on the degree of iron saturation of the protein.

To investigate the role of tears in the protection of the outer eye against oxygen radicals, the effect of hu-
man tears on $O_2^{-}$, $H_2O_2$, and $OH^-$ generated by the xanthine-xanthine oxidase system was studied. The findings obtained showed that tears did not detectably affect $O_2^{-}$ or $H_2O_2$ formation but markedly inhibited $OH^-$ formation, as measured by the hydroxylation of salicylate.10

Materials and Methods

Reagents

Xanthine, xanthine oxidase, human milk lactoferrin (approximately 10% iron-saturated), bovine serum albumin, superoxide dismutase, and catalase were obtained from Sigma Chemical Company (St. Louis, MO). Ferricytochrome c was obtained from Boehringer-Mannheim (Indianapolis, IN), Dowex 50W (X4, 200–400 mesh) from Fluka AG (Buchs, Switzerland) and Sephadex G75 from Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals used were of analytical grade.

Tears

Tears were obtained from healthy colleagues (n = 8) at the Institute after obtaining informed consent. Tear production was stimulated by a jet of pressurized air or by cutting onions. Each volunteer collected his/her own tears using a mirror and a glass capillary. Approximately 200–1000 µl of tears were obtained from a trained donor during approximately 20 min. After collection, the tear samples were pooled, used immediately, or kept frozen at −20°C until use.

Tears were denatured by heating at 80°C for 10 min. The turbid solution was centrifuged for 10 min at 16000× g, and the supernatant was aspirated.

Detection of Hydroxyl Radicals

The formation of hydroxyl radicals was measured using the method described by Richmond et al10 with some modifications. All reagents were made in 150 mM KH2PO4/KOH buffer pH 7.45. Stock solutions of xanthine were made in 0.1 M KOH to a final concentration of 8 mM. Reagents were pipetted into glass tubes (1.5 × 8.5 cm) in the following order and with a final concentration in the reaction mixture as indicated: xanthine (1 mM), sodium salicylate (5 mM), FeCl3 (0–50 µM). Subsequently, crude tears (0–200 µl), G75-tear-fractions (720 µl), human milk lactoferrin (0–2 mg), or bovine serum albumin (0–2 mg) was added. Tubes were incubated for 30 min at room temperature, to allow equilibration of the reaction mixture. Then EDTA (30 µM) was added, and the tubes were incubated for another 30 min at room temperature. The reaction was initiated by adding 60 µl xanthine oxidase (20 mU/ml). Reaction mixtures (final volume: 1.2 ml) were incubated at 37°C for 90 min.

Reactions were terminated by adding 50 µl 70% perchloric acid to precipitate the proteins, followed by mixing and immersion in ice. The reaction product (hydroxylated salicylate) was extracted with 2.5 ml chilled ether by vigorous mixing for 30 sec. After centrifugation (10 min, 0°C, 1600× g), 1.5 ml of the upper ether-layer was pipetted off and evaporated to dryness in a water-bath at 40°C. The residue was dissolved in 250 µl of cold distilled water, followed by the addition of 125 µl of 10% trichloroacetic acid (30 µl in 0.5 M HCl), 250 µl of 10% sodium tungstate (30 µl in H2O2), and 250 µl of a fresh solution of 0.5% sodium nitrite (30 µl in H2O2). After incubation for 5 min, 500 µl of 0.5 M KOH was added and the optical density was measured at 510 nm using a Gilford spectrophotometer (Gilford, Oberlin, OH), equipped with a flow cuvette (1.0 cm) and using H2O as a blank. Values were used as such without further corrections. The background of the assay ranged between an ODS10 of 0.05–0.10. Performance of the assay for hydroxyl radicals in quadruplicate resulted in a standard deviation not exceeding 7% of the mean value. Separate control experiments included reaction mixtures in which the substrate (xanthine) or the enzyme (xanthine oxidase) were omitted (100% inhibition of OH⁻ formation; this is taken as the background value). Complete incubation mixtures (complete system) contained xanthine (1 mM), sodium salicylate (5 mM), FeCl3 (5 µM), EDTA (30 µM) and xanthine oxidase (20 µU/ml) with no further additions and were assayed for OH⁻ as described above (0% inhibition of OH⁻ formation). The inhibition percentage of OH⁻ formation for the various test samples was calculated as follows: % inhibition = 100 − [(OD510-test sample–OD510-background)/(OD510-complete system–OD510-background)] × 100.

The effect of superoxide dismutase and catalase on OH⁻ formation was measured, using incubation mixtures containing xanthine (1 mM), sodium salicylate (5 mM), FeCl3 (5 µM), and EDTA (30 µM). Subsequently, superoxide dismutase (10 U/ml) or catalase (10 U/ml) was added to each tube. After initiation with xanthine oxidase (20 µU/ml) (final volume: 1.2 ml), the reaction was terminated at t = 15, 30, 45, 60, and 90 min by adding perchloric acid and assayed for the salicylate hydroxylation product as described above.

Detection of Superoxide and Hydrogen Peroxide

Using the xanthine-xanthine oxidase system, the formation of $O_2^-$ and $H_2O_2$ was measured by monitoring the reduction of ferricytochrome c by $O_2^-$ and subsequent oxidation of ferrocytochrome c by $H_2O_2$.11,12 In order to prevent OH⁻ formation, no cat-

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alytic iron was added. Cuvettes contained the following substances: xanthine (1 mM), EDTA (30 μM), and ferricytochrome c (40 μM). After addition of 200 μl of human tears, the reaction was initiated by xanthine oxidase (20 mU/ml) (total reaction volume: 1.2 ml), and the reaction process was monitored in a spectrophotometer at 550 nm during a period of 20 min. As a positive control, superoxide dismutase (1.6 U/ml) or catalase (1.6 U/ml) was added. Negative controls contained bovine serum albumin (1.6 mg/ml) in 150 mM KH₂PO₄/KOH buffer pH 7.45 or buffer alone.

Gelfiltration of Human Tears

1.0 ml of human tears was applied onto a Sephadex G75 column (1.5 × 20 cm). The column was eluted with 150 mM KH₂PO₄/KOH buffer pH 7.45. Fractions of approximately 1 ml were collected and assayed for protein, lactoferrin, lysozyme and the presence of factors affecting the formation of OH⁻ radicals.

Cation Exchange Chromatography of Human Tears

One milliliter of human tears was diluted with 150 mM KH₂PO₄/KOH buffer pH 7.45 to 4.0 ml and incubated with 0.5 g Dowex 50W for 30 min. at 20°C while being gently shaken. As controls, a 0.5 mM CaCl₂-solution and a BSA solution (2.5 mg/ml) were treated in the same way. After incubation, the mixtures were centrifuged (5 min, 20°C, 1000×g) and 200 μl of each supernatant was tested for its effect on OH⁻ formation. Also 200 μl of each sample was tested, prior to treatment with Dowex 50W.

Lactoferrin

Lactoferrin was measured qualitatively using a modification of a method described earlier. Immunoassay tubes (Greiner, Labortechnik, Nürtingen, W. Germany) were coated with 5 μg purified human milk lactoferrin in sodium carbonate-bicarbonate buffer (pH 9.6) during 1 hr at 20°C. After washing three times with water, 5 μl of crude tear sample or 25 μl of G75 tear fraction was added in buffered saline solution, pH 7.4, containing 0.1% Tween and 1% bovine serum albumin, in a total volume of 0.5 ml. Subsequently, 0.5 ml of a peroxidase-labeled mouse monoclonal anti-human lactoferrin antibody solution was added (final dilution 1/100). After a further incubation of 30 min at room temperature and subsequent washing with water, 1 ml 0.05 M citric acid pH 4.0, containing 0.12% H₂O₂ and 0.16 mM ABTS (2',2'-azino-di-(3-ethyl-benzthiazoline-sulphonate) was added. After 30 min the reaction was stopped with 50 μl of 10% Sodium Dodecyl Sulphate and absorptions were read at 405 nm.

Results

Formation of O₂⁻, H₂O₂, and OH⁻ by Xanthine-Xanthine Oxidase

OH⁻ radicals were generated in vitro using the xanthine-xanthine oxidase system. This system is known to generate O₂⁻ and H₂O₂ and in the presence of catalytic iron OH⁻ formation can occur according to a Fenton-type Haber-Weiss reaction.

$$\text{O}_2^- + \text{Fe}^{3+} \rightarrow \text{Fe}^{2+} + \text{O}_2$$

$$\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^-$$

OH⁻ formation (in the presence of iron and EDTA) was monitored by measuring the amount of hydroxylated salicylate at different time intervals (Fig. 1). After
Effect of EDTA and FeCl₃ on OH· formation. Reaction mixtures (final volume: 1.2 ml) contained xanthine (1 mM), Na-salicylate (5 mM), and various amounts of iron with a constant amount of EDTA (●-●) or iron without EDTA (○-○). Mixtures were preincubated for 30 min at 20°C before initiation by xanthine oxidase (20 mU/ml). After incubation for 90 min at 37°C, hydroxylated salicylate was measured at 510 nm. Background values (OD510) in test tubes, incubated in the absence of xanthine oxidase, were 0.061.

75 min, a maximal amount of hydroxylated product is formed under these conditions. Addition of scavengers of H₂O₂ (catalase) or O₂⁻⁻ (superoxide dismutase) inhibited OH· formation showing that H₂O₂ and O₂⁻ are essential intermediate products. The effect of iron and EDTA on OH· formation (Fig. 2) shows that both compounds are necessary for OH· formation. Omission of either EDTA or iron resulted in very low OH· formation. In all further experiments, when measuring the effect of various compounds on OH· formation, concentrations of iron and EDTA of 5 μM and 30 μM were used respectively.

Effect of Tears on O₂⁻⁻, H₂O₂ and OH·

Addition of various amounts of tears to the oxygen radical generating incubation mixtures resulted in a dose dependent decrease of OH·-induced hydroxylation of salicylate (Fig. 3). Detectable inhibition was seen when 40 μl of human tears (3.3% of total volume) were added and complete inhibition occurred, when more than 160 μl of human tears (13.3% of total volume) were added to the system. As control, an albumin solution containing a similar protein concentration as in human tears did not affect OH·. In the absence of EDTA no OH· could be detected, and neither tears nor albumin could induce an OH· dependent hydroxylation of salicylate.

An inhibitory effect of human tears on OH· was found on all occasions (n = 30), when such an experiment was performed and only minor day-to-day variations were encountered over an 8-month period. Testing a 50-μl sample of human tears on OH· always resulted in an inhibition of at least 40%. Addition of human tears to test tubes, in which xanthine or xanthine oxidase was omitted, did not result in a detectable OH· formation, indicating that human tears lack both the substrate and enzyme of the oxygen radical generating system. No differences were seen between the effect of stimulated tears on OH· whether they were obtained by air or onion stimulation. Analysis of the inhibitory effect of air stream stimulated tears (50 μl), obtained from eight different individuals (male: 6, female: 2; age: 26–58 yr) and tested on the same day, showed a mean percentage of 44% inhibition, values ranging from 40.8–48.4%.

To investigate whether the decrease of OH· by tears was caused by an inhibition of the enzymatic activity of xanthine oxidase or due to the presence of O₂⁻⁻ or H₂O₂ scavengers, the following experiment was performed.

Evidence for O₂⁻⁻ and H₂O₂ production in the incubation mixtures was obtained by monitoring the reduction of added ferricytochrome c by O₂⁻⁻. In the first 4 min, ferricytochrome c is reduced to ferrocytochrome c by O₂⁻⁻, present in the incubation mixture, leading to an increase in OD550 (Fig. 4). Addition of tears had no effect on this phase of the reaction, whereas...
the addition of superoxide dismutase (O$_2^-$-scavenger) markedly inhibited the reduction of ferricytochrome c. In the second phase of the reaction, the formed ferrocytochrome c is oxidized back to ferricytochrome c, due to the formation of H$_2$O$_2$ in the reaction mixture. Addition of tears did not affect this phase of the reaction either, whereas the addition of catalase (H$_2$O$_2$-scavenger) markedly inhibited the oxidation of ferrocytochrome c.

To investigate whether tears could interact with the hydroxylated salicylate, thus explaining the observed inhibitory effect of tears on OH', tears were incubated for 30 min in a reaction mixture, in which hydroxylation of salicylate had already taken place. No effect of tears on the detection of hydroxylation product was observed, indicating that tears exerted their inhibitory action at an earlier phase in the reaction sequence (data not shown).

Gelfiltration of Human Tears on G75 Sephadex

To investigate which tear constituents caused the observed decrease of OH', human tears were subjected to gelfiltration on Sephadex G75. Three protein peaks eluted from the column (Fig. 5). The first peak, which also contains lactoferrin, had an inhibitory effect on OH'. The second peak consists of as yet unidentified proteins; the third peak was shown to contain lysozyme. These latter two protein peaks did not act inhibitory on OH'. A strong inhibitory activity eluted in fractions 32–40. These fractions did not stain in the protein assay used.

Evidence for a role of tear calcium in the inhibition of OH' formation was obtained in separate experiments whereby 700 µl of a human tear sample was diluted with a 2 mM CaCl$_2$ solution or with elution buffer to a final volume of 1400 µl, applied onto the same column and eluted in the same way. Fractions eluting after the tear proteins were tested on their effect on OH' formation. When extra calcium was added to tears and then eluted, the second peak of OH' inhibitory activity was markedly increased (see Table 1) compared to the same tear sample without added calcium.
Table 1. Inhibition of OH* formation by G-75 fractions of tear samples diluted before gel filtration with CaCl2 or elution buffer

<table>
<thead>
<tr>
<th>G-75 fractions eluting after the tear proteins</th>
<th>Tear sample diluted with CaCl2</th>
<th>Tear sample diluted with elution buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>32</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>33</td>
<td>11</td>
<td>15</td>
</tr>
<tr>
<td>34</td>
<td>67</td>
<td>27</td>
</tr>
<tr>
<td>35</td>
<td>96</td>
<td>51</td>
</tr>
<tr>
<td>36</td>
<td>91</td>
<td>57</td>
</tr>
<tr>
<td>37</td>
<td>96</td>
<td>87</td>
</tr>
<tr>
<td>38</td>
<td>95</td>
<td>29</td>
</tr>
<tr>
<td>39</td>
<td>51</td>
<td>7</td>
</tr>
<tr>
<td>40</td>
<td>7</td>
<td>0</td>
</tr>
</tbody>
</table>

To investigate whether known low molecular weight components in human tears could influence the assay system, we investigated various cations such as Ca2+, Mg2+, Na+, Mn2+, and Zn2+, known to be present in human tears6-21 on their effect on OH*. As can be seen in Table 2, calcium already causes a marked inhibition at a concentration that is normally present in human tears. Although other cations such as Mn2+ and Zn2+ may also affect OH*, their contribution in tears is such that they are probably not involved in the inhibitory effects observed.

Cation Exchange Chromatography of Human Tears

Further evidence of tear calcium as the main contributor to the observed inhibition on OH* formation by human tears was obtained by incubating human tear samples and CaCl2 solutions with Dowex 50W. As can be seen in Table 3, CaCl2 solutions (500 μM/L) lost their inhibitory effect, when subjected to cation exchange chromatography. Similarly, the diluted tear pool (estimated Ca2+-concentration: 145 μM/L) lost a great part of its inhibitory effect when treated in the same way.

Table 2. Effect of cations on formation of OH* radicals, generated by the Fe3+-EDTA catalysed xanthine-xanthine oxidase reaction, as detected by the hydroxylation of salicylate

<table>
<thead>
<tr>
<th>Cation*</th>
<th>μM/L, causing 50% inhibition of OH* formation</th>
<th>Estimated tear cation content (μM/L) in reaction mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca2+</td>
<td>30</td>
<td>15-100</td>
</tr>
<tr>
<td>Mg2+</td>
<td>145</td>
<td>15-30</td>
</tr>
<tr>
<td>Mn2+</td>
<td>10</td>
<td>0.010-0.045</td>
</tr>
<tr>
<td>Zn2+</td>
<td>20</td>
<td>2.5-5.0</td>
</tr>
<tr>
<td>Na+</td>
<td>&gt;160.101</td>
<td>6.103-8.5.101</td>
</tr>
</tbody>
</table>

* Cation content was estimated from literature data6-21 and calculated for a total reaction mixture of 1.2 ml containing 60 μl of human tears assuming a 50% inhibition of OH* formation under these conditions (Fig. 3).

Table 3. The inhibitory effect on OH* formation of human tears or CaCl2, pretreated with Dowex 50W

<table>
<thead>
<tr>
<th>Sample</th>
<th>No treatment</th>
<th>After Dowex 50W treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 μl tearpool (diluted 4 times)</td>
<td>48</td>
<td>8</td>
</tr>
<tr>
<td>200 μl 0.5 mM CaCl2 solution</td>
<td>71</td>
<td>0</td>
</tr>
<tr>
<td>200 μl BSA solution (2.5 mg/ml in incubation buffer)</td>
<td>9</td>
<td>4</td>
</tr>
</tbody>
</table>

Effect of Human Milk Lactoferrin on OH* Formation

Due to the fact that part of the inhibitory activity on OH* formation, obtained after gel filtration of human tears, coincided with a protein fraction containing lactoferrin, the effect of purified 10% iron-saturated milk lactoferrin on this system was investigated.

Incubation of the OH*-generating system in the presence of various amounts of lactoferrin resulted in a dose dependent inhibition of OH* formation, whereas similar amounts of bovine serum albumin had no effect (Fig. 6).

Effect of Iron on OH* Formation in the Presence of Lactoferrin or Human Tears

To investigate whether the observed inhibitory effects were due to a depletion of catalytic iron from the reaction mixtures, experiments were performed with a constant amount of tears, purified unsaturated human milk lactoferrin, or bovine serum albumin to which variable amounts of iron were added. As already shown earlier (Fig. 2), the amount of OH* formation is dependent on the iron concentration. Addition of lactoferrin results in an inhibition of OH* formation at low iron concentrations, which is counteracted, however, by increasing the amount of iron (Fig. 7). The inhibitory effect of human tears on OH* formation was only weakly affected by increasing iron.

Effect of Heat Denatured Tears on OH* Formation

Incubation of human tears for 10 min at 80°C resulted in a marked precipitation of tear constituents. This treatment resulted in the precipitation of 75% of the tear proteins as measured by the Lowry method. When the supernatant of heat denatured tears was tested in the OH*-generating system and compared with untreated tears, the following result was obtained. Normal tears (200 μl) caused 100% inhibition, whereas heat-treated tears (200 μl) still resulted in 73% inhibi-
tion on OH\(^{•}\) formation. Ouchterlony analysis of the supernatant of denatured human tears showed that heat treatment of human tears had resulted in the precipitation of tear lactoferrin.

**Effect of Ascorbic Acid on OH\(^{•}\) Formation**

OH\(^{•}\) formation in the xanthine-xanthine oxidase system was hardly affected by the use of ascorbic acid concentrations up to 50 \(\mu\)M. Concentrations higher than 50 \(\mu\)M were able to stimulate OH\(^{•}\) formation (data not shown).

Assaying human tear samples (50 \(\mu\)l) for ascorbic acid, by use of a colorimetric method with a sensitivity of 60 \(\mu\)M/L, no activity could be detected.

**Discussion**

This paper shows that stimulated human tears, under the in-vitro conditions used in this study, do not contain detectable scavengers of \(O_2^{•}\^-\) or \(H_2O_2\) but can inhibit OH\(^{•}\) formation. To our knowledge this is the first report in which inhibition of OH\(^{•}\) formation by human tears is described. This finding suggests a possible important role of human tears in protecting the outer parts of the eye against OH\(^{•}\)-induced damage. Oxygen radicals can be produced in several aerobic reactions. During inflammation, granulocytes are known to produce large amounts of \(O_2^{•}\^-\) and \(H_2O_2\) and in the presence of trace amounts of iron the extremely toxic OH\(^{•}\) radical can be formed,\(^{1,18}\) causing severe tissue damage. Our experiments and those of others\(^{18,19}\) have shown that OH\(^{•}\) formation in vitro occurs when catalytic iron is presented as an iron-EDTA complex. In the xanthine-xanthine oxidase system, no OH\(^{•}\) formation occurs when EDTA is omitted, even in the presence of iron, suggesting that iron is most effective in promoting OH\(^{•}\) formation in this system, when it is present in a chelated form. It is of interest that a large number of eye drops contain EDTA as one of the preservatives, which, as shown here, is very effective in presenting free iron as a catalyst of the Fenton-type Haber Weiss reaction.

Besides the induction of oxygen radicals during inflammatory processes, these radicals can also be induced in the presence of photosensitizers and light. In the lens, for instance, the combination of photosensitizers and light results in the generation of oxygen radicals, causing photochemical damage to this part of the eye.\(^{1-3,22}\)

In the retina, light is focused on a group of cells that are metabolically very active. Extended exposure of the retina to short wavelength light can induce retinal injury,\(^{23}\) possibly caused by free oxygen radicals, generated by light and oxygen.
Several parts of the eye possess protective means, which can handle oxidative insults very effectively.\textsuperscript{5,24-28} In the lens, cornea and retina superoxide dismutase, catalase and glutathione peroxidase can convert $O_2^{-\cdot}$ and $H_2O_2$ to less reactive species, thus preventing OH$^\cdot$ formation. On the ocular surface, however, and especially in the tear film, little is known about mechanisms by which oxygen radicals are being dealt with.

Our experiments indicate that human tears can inhibit the OH$^\cdot$-mediated hydroxylation of salicylate quite effectively. Various explanations can be given for these observations. Tears could contain constituents that eliminate the reactants, involved in the Fenton-type Haber-Weiss reaction ($O_2^{-\cdot}$, $H_2O_2$, catalytic iron-EDTA complex) or it is possible that tears contain factors, which scavenge or trap OH$^\cdot$, thus resulting in a decrease in OH$^\cdot$-hydroxylated salicylate.

We used the salicylate method to detect OH$^\cdot$ radicals, because it is a well-documented method that can be performed with simple laboratory equipment. The findings reported here concerning the effect of human tears on OH$^\cdot$ obviously need confirmation using other sensitive methods to detect OH$^\cdot$ radicals such as conversion of methional to ethylene by OH$^\cdot$ or electron-spin resonance techniques.\textsuperscript{29} The effect of human tears on OH$^\cdot$ is not mediated by superoxide dismutase or catalase activity. Peroxidase activity, which can scavenge $H_2O_2$ in the presence of a suitable substrate, has been detected in human tears.\textsuperscript{30} A possible $H_2O_2$-scavenging activity of peroxidase was not detectable, however, by use of the ferricytochrome c method as employed in our studies. Ascorbate, a compound that can serve as a water soluble oxygen radical scavenger,\textsuperscript{16} is abundantly present in the aqueous humor, lens, and cornea.\textsuperscript{3} However, in our oxygen-radical-generating system, OH$^\cdot$ formation was hardly affected by ascorbic acid levels up to 50 $\mu$M. Furthermore, no detectable amount of ascorbic acid was found in human tears, suggesting that in this fluid a role of ascorbate as a scavenger of oxygen radicals seems unlikely. Metabolites such as urea are weak OH$^\cdot$ scavengers in the salicylate detection method, whereas thiourea has been shown to be an extremely strong scavenger.\textsuperscript{10} Although urea has been detected in human tears,\textsuperscript{33} the presence of thiourea has not yet been reported. The average concentration of urea in human tears (5 $\mu$M) cannot account for the observed inhibition of crude human tears on OH$^\cdot$, since only 12% inhibition was reported earlier by Richmond et al\textsuperscript{10} using 10 mM urea.

Another possibility to explain the observed inhibition is that human tears contain substances that can withdraw catalytic iron. It is known that in human tears the iron-binding protein lactoferrin is present in large amounts.\textsuperscript{14,32} Under normal conditions, over 90% of the protein is present in its iron unsaturated form (A Kijlstra, unpublished observations).

Gelfiltration of human tears, as performed in the study described here, resulted in the isolation of lactoferrin containing protein fractions, which inhibited OH$^\cdot$ formation, possibly due to the binding of catalytic iron by this protein in the reaction mixtures. This result suggests a possible role for this protein in OH$^\cdot$ formation. Further evidence supporting this hypothesis was obtained by experiments using purified human milk lactoferrin, not saturated with iron. This purified protein also inhibited OH$^\cdot$ formation, especially at low iron concentrations, an effect that was counteracted by increasing the iron.

Reports on the role of lactoferrin on OH$^\cdot$ formation have been controversial until now.\textsuperscript{7-9,33} It seems that this protein can prevent OH$^\cdot$ formation when it is not saturated with iron,\textsuperscript{8} but when saturated, can promote OH$^\cdot$ formation.\textsuperscript{7-9,33} In various bodyfluids, however, lactoferrin is not saturated with iron, which suggests that this protein may play a local role in preventing OH$^\cdot$ radical-induced tissue damage.

In experiments in which tears were added to the xanthine-xanthine oxidase system, a 100% inhibition on OH$^\cdot$ formation was seen at a final tear concentration of 16.6% in the mixture, which corresponds with a tear lactoferrin concentration of approximately 200 $\mu$g/ml. In experiments using a similar concentration of purified human milk lactoferrin, only about 26% inhibition was observed. These findings show that the inhibitory effect on OH$^\cdot$ formation in human tears resides in factors other than tear lactoferrin alone. This is strengthened by the observation that heat treatment of tears, which resulted in precipitation of 75% of the proteins, including lactoferrin, did not cause a great loss of OH$^\cdot$ inhibitory factors.

Our experiments indicate that, concerning anorganic tear constituents, tear calcium mainly contributes to a great part in the observed inhibition by human tears. Tear calcium most likely exhibits its inhibitory effect by competing with iron in binding to EDTA, thus resulting in a lower amount of the catalytic iron-EDTA complex. Whether tear calcium can also block the interaction of iron with naturally occurring chelators remains to be investigated.

The combined action of tear lactoferrin and tear calcium may account for the largest part of the observed inhibition of the OH$^\cdot$ mediated hydroxylation of salicylate. The role of the as yet unidentified OH$^\cdot$-scavenging or trapping factors in human tears cannot, however, be completely excluded. Furthermore, it is also possible that OH$^\cdot$ reactive compounds in tears are underesti-
mated, because excessive H2O2 formed during the xanthine oxidation under the circumstances used here may have oxidized potential OH• reactive compounds.

So far the experiments described here show that stimulated human tears can inhibit the Fenton-type Haber-Weiss reaction by a dual action on the catalytic iron complex and thus may play an important role in protecting the ocular surface against OH•-induced damage.

Key words: tears, oxygen radicals, hydroxyl radical, xanthine oxidase, lactoferrin

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