Penetrating the Conjunctival Barrier

The Role of Molecular Weight

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Dinitrophenyl (DNP) derivatives of various molecular weights were tested for their ability to elicit ocular anaphylaxis after topical application to the eye of immunized animals. Adult male Sprague-Dawley rats were immunized by intraperitoneal injection of DNP-Ascaris suum extracts and alum and were then skin-tested with DNP-bovine serum albumin on day 13 post-immunization to assess their sensitivity to the DNP hapten. On day 14, animals were challenged topically with DNP derivatives in one eye; PBS was applied to the contralateral, control eye. Animals were evaluated clinically, and ocular tissues were processed for histologic evaluation. The compounds used for topical ocular challenge included the DNP derivative of egg albumin (MW 43,500 D), soybean trypsin inhibitor (MW 20,080 D), insulin (MW 5733 D), B-chain insulin (MW 3496 D), and lysine (MW 478 D). Only di-DNP-lysine elicited clinical signs of redness, edema, and tearing and histologic evidence of mast cell degranulation. None of the other compounds, tested in solutions of either equal numbers of milligram per milliliter or equimolar concentrations, elicited ocular anaphylaxis after topical application. A compound of low molecular weight, less than 3496, is needed to elicit ocular anaphylaxis when applied topically. Invest Ophthalmol Vis Sci 31:258-261, 1990

In previous studies we showed that ocular anaphylaxis could be elicited by intravenous administration of egg albumin (EA) or worm extract1 and by injection of EA into ocular structures of immunized rats.2 In an effort to more closely simulate the ocular component of human hay fever in which the allergic reaction is presumably elicited by topicaly applied antigen, we developed a hapten model of topically induced ocular anaphylaxis in the rat.3 Rats were immunized with dinitrophenylated Ascaris suum extract (DNP-Asc), a known potent immunogen,4,5 to stimulate the production of dinitrophenyl (DNP)-specific IgE antibodies.6 Di-DNP-lysine, a bivalent hapten of low molecular weight, was used to elicit ocular anaphylaxis. Previously, we had found that EA7 and Nippostrongylus brasiliensis extract8 did not elicit ocular anaphylaxis unless the conjunctiva was pretreated with dithiothreitol (DTT), a mucolytic agent. In the current study, we topically applied DNP derivatives of various molecular weights to investigate the role of molecular weight of the antigen in the elicitation of ocular anaphylaxis.

Materials and Methods

Di-DNP-lysine (N,N-di-[2,4-DNP]-1-lysine) (MW 478 D) was purchased from Sigma (no. D-0255; St. Louis, MO). The DNP conjugates of Ascaris suum extract, EA (MW 43,500 D) (Sigma no. A-5505), soybean trypsin inhibitor (SBTI) (MW 20,080 D) (Sigma no. T-9003), insulin (INS) (MW 5733 D) (Sigma no. I-3505), and B-chain of INS (MW 3496 D) (Sigma no. I-6383), were prepared according to the method of Eisen et al.9 The Ascaris suum extracts were prepared according to the method of Strejan and Campbell.5 Sixty-two adult male Sprague-Dawley rats were immunized by a 1.0-ml intraperitoneal injection of a suspension containing DNP-Asc (200 μg) and alum (20 mg). On day 13 post-immunization, active cutaneous anaphylaxis (ACA) was elicited by injecting each animal intradermally with 0.1 ml of 100, 10, and 1 μg/ml dinitrophenyalted bovine serum albumin (DNP-BSA) in phosphate-buffered saline (PBS), followed immediately by an intravenous injection of 1% Evans blue dye solution. Reactions were noted 15 min later, and only animals with at least a 5-mm blue skin lesion at all three test sites were included in the topical challenge study.

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Table 1. Characteristics of DNP derivatives

<table>
<thead>
<tr>
<th>DNP-derivative</th>
<th>Molecular weight (Da)</th>
<th>Moles DNP/mole carrier molecule</th>
<th>No. animals used</th>
<th>Weight of conjugate (mg/ml) to make equimolar solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>di-DNP-lysine</td>
<td>478</td>
<td>2.0</td>
<td>6</td>
<td>0.05</td>
</tr>
<tr>
<td>B-chain of INS</td>
<td>3496</td>
<td>0.4</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>INS</td>
<td>5733</td>
<td>0.5</td>
<td>6</td>
<td>3.29</td>
</tr>
<tr>
<td>SBTI</td>
<td>20,080</td>
<td>4.0</td>
<td>7</td>
<td>1.89</td>
</tr>
<tr>
<td>EA</td>
<td>43,500</td>
<td>6.6</td>
<td>5</td>
<td>2.78</td>
</tr>
</tbody>
</table>

Rats were assigned to groups that were topically challenged in one eye with either 10 μl of solution containing the various DNP conjugates at 1.0 mg/ml or 10 μl of equimolar solutions of the DNP derivatives (Table 1). Derivatives were dissolved in PBS, pH 7.4, immediately prior to use. The control eye of each rat received 10 μl PBS. For the equimolar experiment, solutions were adjusted so that each 10 μl contained approximately 2.9 × 10⁹ moles DNP.

If no clinical reaction was noted in a group of animals, an additional group was injected subconjunctivally to assess the ability of the derivatives to elicit an anaphylactic reaction by this route. Under a dissecting microscope, the upper lid was everted, and with a 30-gauge needle and a Hamilton syringe, the upper tarsal conjunctiva of one eye was injected with 10 μl of an equimolar solution. Control eyes were injected subconjunctivally with 10 μl PBS.

Thirty minutes after topical challenge (or injection), animals were evaluated clinically in room light. Conjunctival redness, tearing, and conjunctival edema were scored from 0 to 3+ in both the treated and control eyes. The investigators were not masked in making these assessments.

Immediately after the clinical evaluation, rats were deeply anesthetized with ether and exsanguinated from the cervical vessels. Orbits were exenterated, and tissues were processed for 1.0 μm Epon-embedded sections stained with alkaline Giemsa dye. The subepithelial area of the conjunctiva was examined. Granulated and degranulated mast cells were recognized by their oval nucleus and metachromatic granules. Mast cells in 30 consecutive subepithelial high-power fields were enumerated (10 fields on each of three sections separated by at least 15 μm to avoid counting the same cell twice). Neutrophils, eosinophils, macrophages, plasma cells, and lymphocytes were enumerated in 10 nonconsecutive high-power fields, as previously described.

The studies adhered to the ARVO Resolution on the Use of Animals in Research.

Within each group, the clinical and histologic findings in the treated eyes were compared statistically with those of the control eyes by a single-tailed
Mann-Whitney U test. A P value of ≤0.05 was taken to be significant.

Results

Clinical

Among the rats challenged topically in one eye with solutions containing 1.0 mg conjugate/ml, only those treated with di-DNP-lysine showed a significant clinical reaction compared with the control eyes (P < 0.01). Rats that received a single topical application of DNP-INS, DNP-SBTI, or DNP-EA to one eye did not show a significant clinical reaction (Fig. 1a).

Similar results were obtained in rats challenged with equimolar solutions of the DNP conjugates; only those receiving di-DNP-lysine showed a significant clinical reaction compared with the control eye (P < 0.01). None of the other rats, including those treated with DNP-B-chain of INS, showed a significant clinical reaction (Fig. 1b).

All DNP conjugates injected into the conjunctiva elicited a marked clinical reaction; injection of PBS produced no clinical response. With the DNP conjugates, the reaction was characterized by swelling of lid or conjunctiva beginning within 5 min of injection and reaching its peak in ~30 min–1 hr. The DNP
conjugates produced no clinical reaction upon injection into the ocular tissues of nonimmunized rats.

Histologic

The percentage of degranulated mast cells in the conjunctiva of the conjugate-challenged eye was significantly increased over that of the PBS-treated control eye only in tissues from rats challenged with di-DNP-lysine ($P \leq 0.01$ in both the mg/ml and the equimolar groups) (Figs. 2a, b). No other DNP derivative elicited a significant increase in the percentage of degranulated mast cells.

The number of infiltrating neutrophils was significantly increased over that in controls only in topically treated tissues from di-DNP-lysine-treated rats ($P < 0.01$ in the mg/ml group; $P < 0.05$ in the equimolar group). No other DNP derivative elicited a significant increase in the number of infiltrating neutrophils (Figs. 3a, b).

With one exception, the median number of total mast cells, eosinophils, macrophages, plasma cells, and lymphocytes in tissues from the treated eyes did not differ from the control eyes for each group. The exception was the number of eosinophils in the treatment versus control eyes from rats challenged with the equimolar concentration of INS ($P < 0.05$, single-tailed test only).

Discussion

Although it is generally recognized that the conjunctiva has a role in limiting the access of topically applied substances to the interior of the eye, we were unable to find confirmation of a formal test of this barrier function in the ophthalmic literature. In the current experiments, we investigated whether DNP derivatives varying in molecular weight from 478 to ~43,000 D were capable of reaching mast cells in the subepithelium of the rat conjunctiva. Of the molecules tested, only di-DNP-lysine induced clinical and morphologic evidence of an ocular anaphylactic response; larger DNP derivatives failed to elicit a significant response regardless of whether they were applied in solution of equal weight of conjugate per volume or as equimolar (for DNP) solutions. Because DNP derivatives of the B-chain of INS did not elicit a response, it appears that molecules of molecular weight less than ~3500 D are required to penetrate the conjunctiva.

The present study was not designed to determine the sites at which di-DNP-lysine traverses the epithelium and superficial stroma of the conjunctiva, nor did it test whether the same barrier exists in other species. Setzer et al. showed that the rat conjunctiva was unique among the species examined. All of the superficial epithelial cells were squamous, rather than columnar or polyhedral. Squamous cells are present in several layers. This unusual feature of the rat conjunctiva may be important in limiting access of antigen to the subconjunctival region of the eye. If so, a different molecular-size profile of conjunctival penetration may be found in other species.

Key words: molecular weight, conjunctival barrier, mast cell, anaphylaxis, dinitrophenylated *Ascaris suum*

References