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The Effect of Platelet-Activating Factor (PAF), Histamine, and Ethanol on Vascular Permeability of the Guinea Pig Conjunctiva

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Increased vascular permeability, one of the characteristic features of immediate hypersensitivity (Type I), is mediated through a variety of compounds, including histamine and platelet-activating factor (PAF), a phospholipid inflammatory mediator. The effects on vascular permeability of histamine, PAF, and ethanol, the solvent for PAF, were compared in the guinea pig conjunctiva. Permeability at 30 min was investigated by evaluation of conjunctival edema and Evans blue extravasation (clinically estimated and colorimetrically measured). Doses of PAF from 1 to 10 nmol produced an increase in vascular permeability, with a peak effect at 10 nmol. Ethanol had no effect on vascular permeability below 40 × 10⁻³ nmol; above this concentration, however, permeability increased, reaching a maximum at 175 × 10⁻³ nmol. At low doses of PAF and ethanol, the effects were additive, whereas at 20–80 nmol of PAF with high concentrations of ethanol there was no additive effect of PAF, producing a decrease in the net effect of PAF. Histamine increased vascular permeability, with a minimum effect at 10 nmol and a maximum effect at 450 nmol. The slopes of the dose–response curves for all three compounds were linear and parallel, with statistically different potencies. The potencies for each compound were identical by all three methods of evaluation. Therefore, we conclude that PAF is a potential mediator in hypersensitivity reaction in the guinea pig conjunctiva, and that its effect is similar to but much more potent than that of histamine or ethanol. Since ethanol alone has a significant effect on vascular permeability, studies on PAF effects using control solutions without ethanol may be difficult to interpret. Invest Ophthalmol Vis Sci 31:987–992, 1990

Immediate (type I) hypersensitivity (IH) is an element of the pathogenesis of both vernal keratoconjunctivitis and giant papillary conjunctivitis.⁴ Increased vascular permeability and conjunctival edema are prominent clinical findings in IH and in models of IH in the conjunctiva.⁵ In IH reactions, IgE-sensitized basophils and mast cells release several mediators (autacoids⁶), including histamine, bradykinin, lipoygenase pathway products (prostaglandins), and platelet-activating factor (PAF). PAF has been shown to induce a wide variety of biologic actions, including increased vascular permeability. It is a polar phospholipid (1-O-alkyl-2-O-acetyl-sn-glycero-3-phosphocholine) which has been isolated from basophils,⁷ and also liberated from platelets, monocytes, endothelial cells, neutrophils, and smooth muscle cells.⁸-⁹ To further understand the role of PAF in conjunctival hypersensitivity reactions, we examined and compared the influence of topically applied PAF, histamine, and ethanol on vascular permeability and edema in the guinea pig conjunctiva.

Materials and Methods

Animals

Fifty-eight female Hartley guinea pigs (Hartley Sprague-Dawley, Indianapolis, IN) weighing 300–400 g were anesthetized with a solution of ketamine HCl (Ketalar; Parke-Davis, Morris Plains, NJ), 100 mg/kg, and Xylazine (Rompun; Haver-Lockhart), 15 mg/kg, intramuscularly. One hundred milligrams per milliliter Evans blue dye (Eastman Kodak, Rochester, NY), which quantitatively binds to plasma albumin, was diluted in Dulbecco’s phosphate buffered saline (DPBS; Sigma, St. Louis, MO), and given intravenously (20–25 mg/kg) via the dorsal ear vein 5 min prior to application of the mediators. Animals were sacrificed with intracardiac sodium...
pentobarbital (0.5 ml) 30 min after application of mediator. All animals were maintained and used in conformance with the ARVO Resolution on the Use of Animals in Research.

Mediators

After evaporation of the original solvent, chloroform (5 ml), 10 mg PAF-acether (Mr ≈ 530 D; Sigma), which is insoluble in aqueous solutions, was resuspended in 5 ml absolute ethanol, providing a 2-mg PAF/ml ethanol solution (40 nmol/10 μl). The control solutions, containing an equivalent amount of absolute ethanol, and the PAF-containing solutions were diluted in DPBS. Histamine (Mr = 111.1 D; Sigma) was prepared in DPBS solution with DPBS serving as the control. For more comparable evaluation, all of the dosages were expressed in nanomoles.

Conjunctival Testing and Measurement Procedures

Mediators were applied topically in 10-μl aliquots to the conjunctiva using a Pipet-man 200® (Rainin, Woburn, MA). The doses* of PAF were 0.02 (4), 0.2 (4), 1 (4), 2 (4), 5 (4), 10 (4), 20 (4), 40 (6), and 80* (3) nmol. These PAF solutions had 0.087, 0.87, 4.35, 8.7, 21.8, 43.5, 87, 175, and 175 X 10^3 nmoles, respectively, of ethanol in the 10 μl. For each PAF dose, the control animals received the corresponding ethanol dose.

The doses of histamine were 0.9 (3), 9 (4), 90 (4), 300 (2), 450 (2), 675 (2), and 900 (3) nmol. Ten microliters of the control solution, ethanol (vide supra) in DPBS for PAF, and DPBS alone for histamine, were placed simultaneously in the opposite eye. Thirty minutes after application of the mediators, the degree of edema and extravasated Evans blue was clinically evaluated in five different areas of the conjunctiva: 1) upper bulbar; 2) upper palpebral; 3) caruncle; 4) lower bulbar; and 5) lower palpebral. The intensity of edema was graded: 0 = no change from untested conjunctiva; 1 = mild, diffuse swelling; 2 = moderate swelling; and 3 = maximal swelling. For clinical Evans blue evaluation the degree of extravasation was graded: 0 = dye limited to vessels with no extravasation; 1 = faint blueing with clear outlines of vessels; 2 = moderate blueing with no trace of white sclera; and 3 = intense blueing without identifiable vessel outlines. The final score for edema and clinical Evans blue extravasation was the summation of the scores in the five areas (maximum score = 15 each).

The entire conjunctivas were excised and weighed fresh on a Sartorius balance. The specimens were dried for 12 hr in a vacuum with dehydrating silica gel, and reweighed. Evans blue dye was extracted by incubating the specimens in 2 ml formamide (Sigma) for 24 hr in a water bath at 50°C. Formamide-extracted Evans blue solutions were read in a spectrophotometer at 620 nm. The machine was blanked with formamide, and a standard curve of Evans blue concentration was developed by dissolving known quantities of Evans blue in formamide. Values for Evans blue extraction were obtained by dividing the amount of extracted dye by the dry mass of formamide-extracted dried conjunctiva, and expressed as micrograms Evans blue per gram dry conjunctiva.

Analysis of Data

Doses are plotted as the logarithm to the base 10 (Log). Data for histamine and PAF are given as the difference between the test and control eyes of individual animals (net effects). Since DPBS alone was shown to have an insignificant effect in the histamine experiments, the ethanol data are the actual values obtained.

The fit of the treatment effect-log(dose) curves was based on ordinary least squares regression, assuming no two consecutive 0 or 100% effects, and using only the portion of the PAF curve with a positive slope. The lines were analyzed by two different methods for parallelism. Using normal distribution theory, the hypothesis of equality of slopes was tested at the 0.05 significance level. The second method of equality of slopes was after Litchfield and Wilcoxon. Two or more 0 or 100% values were eliminated. The "slope function ratio" (SR), where S is a function of the
doses producing a 16% (ED₁₆), 50% (ED₅₀), and 84% (ED₈₄) effect, and where R is the ratio of the largest to the smallest dose, was calculated. The f₅₀, a factor for determining the confidence limits of the SR, was determined. If SR < f₅₀, then with an experimental error of 0.05, one can assume the lines are parallel. The tests were performed on the three possible pairs of lines for each type of measurement. Wilcoxin's test for difference in potency was then applied to each set of data. In this test, the “potency ratio” (PR) was tested against a fₕₐ₀, similar to the f₅₀ to determine if the three agents had different potencies.

Results

PAF increased conjunctival edema (Fig. 1) and Evans blue extravasation (Figs. 2, 3) at a dose of 1.0 nmol and reached a maximum at 10 nmol. The ED₅₀ for clinical edema and Evans blue extraction was 4.76 nmol, and 6.25 nmol for clinical Evans blue extravasation (Table 1). PAF produced maximum scores of 8, clinical edema; 12, clinical Evans blue; and 2800 μg extracted Evans blue per g conjunctiva. The log dose–response curves over the region showing increasing effect were linear (Table 1). The slopes, intercepts, and statistical data are given in Table 1. Concentrations above 10 nmol PAF and ethanol produced a continued increase in Evans blue extravasation when measured by extraction (Fig. 4), whereas both clinical Evans blue extravasation (Fig. 5) and conjunctival edema (Fig. 6) leveled off. At concentrations above 10 nmol PAF, the net effect of PAF decreased to zero.

Histamine produced identifiable clinical edema at 10 nmol and a maximal effect (score = 15) at 450 nmol (Fig. 1). Similar findings were seen on Evans blue extravasation, estimated clinically (Fig. 2), maximum score of 15, or by extraction (Fig. 3), maximum 7000–9000 μg/g conjunctiva. The ED₅₀ was

Table 1. Analysis of data

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Treatment</th>
<th>m ± SE (slope)</th>
<th>b</th>
<th>R² (%)</th>
<th>P</th>
<th>ED₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Edema</td>
<td>Net PAF</td>
<td>2.8 ± 0.66</td>
<td>3.6</td>
<td>82.1</td>
<td>0.0129*</td>
<td>4.76</td>
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<tr>
<td></td>
<td>Histamine</td>
<td>5.6 ± 1.0</td>
<td>-2.1</td>
<td>85.8</td>
<td>0.0027*</td>
<td>229.6</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>5.9 ± 3.0</td>
<td>-26.5</td>
<td>66.5</td>
<td>0.18</td>
<td>76.0 x 10³</td>
</tr>
<tr>
<td>Clinical Evans blue</td>
<td>Net PAF</td>
<td>5.2 ± 0.99</td>
<td>6.5</td>
<td>83.2</td>
<td>0.0307*</td>
<td>6.25</td>
</tr>
<tr>
<td></td>
<td>Histamine</td>
<td>5.6 ± 0.52</td>
<td>-1.1</td>
<td>95.9</td>
<td>0.0001*</td>
<td>225.0</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>12.1 ± 2.66</td>
<td>-53.4</td>
<td>91.2</td>
<td>0.045*</td>
<td>76.0 x 10³</td>
</tr>
<tr>
<td>Evans blue extraction</td>
<td>Net PAF</td>
<td>971 ± 334</td>
<td>1287</td>
<td>67.8</td>
<td>0.0440*</td>
<td>4.76</td>
</tr>
<tr>
<td></td>
<td>Histamine</td>
<td>2341 ± 548</td>
<td>-1342</td>
<td>78.5</td>
<td>0.0080*</td>
<td>149.6</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>2438 ± 797</td>
<td>-10,767</td>
<td>78.5</td>
<td>0.092</td>
<td>76.0 x 10³</td>
</tr>
</tbody>
</table>

* Curves are not significantly different from linear.
Fig. 4. The effect of PAF, PAF combined with ethanol (PAF + ETOH), and ethanol on Evans blue extravasation measured by extraction from the conjunctiva (microgram Evans blue per gram dry conjunctiva).

229.6 nmol for edema, 225 nmol for Evans blue, clinically evaluated, and 149.6 nmol for Evans blue extraction (Table 1). The portion of the curve showing increasing log dose–response effect was linear (Table 1, which gives the intercepts, slopes, and statistical parameters). There was no significant effect of DPBS on any of the three parameters.

Ethanol (the control solution for PAF) also produced similar effects on all three parameters (Figs. 1–6). At low doses (below $40 \times 10^3$ nmol, there was no effect on any of the three parameters. Beginning at $40 \times 10^3$ nmol there was a linear response to the log dose of alcohol, producing a maximum effect at the highest dose ($175 \times 10^3$ nmol). The ED$_{50}$ was $76 \times 10^3$ nmol for clinical edema, for clinical Evans blue extravasation, and for extracted Evans blue (Table 1). Only the effect on clinical Evans blue was statistically linear (Table 1). The slopes, intercepts, and statistical analyses of the log dose–response curves are given in Table 1.

Statistical Analysis

Using both normal theory$^{13}$ (Table 2) and Litchfield and Wilcoxon's$^{12}$ evaluation (Table 3), the regression lines for the dose–response curves for all three compounds, for each of the three measurements (clinical edema, clinical Evans blue extravasation, and Evans blue extraction) were parallel. In addition, the potencies of each of the three compounds (Table 3) were significantly different by all of the measurements.

Discussion

Edema and increased vascular permeability are significant clinical features of IH (type I) reactions, and have been used as clinical and experimental indica-

Table 2. Tests for parallel slopes of dose–response curves for PAF, histamine, and ethanol, using normal theory

<table>
<thead>
<tr>
<th>Measurement</th>
<th>$T_0^*$</th>
<th>$F_{k-1,n-k}$ (df.05)$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical Evans blue</td>
<td>0.367</td>
<td>2.12 = 3.89</td>
</tr>
<tr>
<td>Evans blue extraction</td>
<td>0.974</td>
<td>2.13 = 3.74</td>
</tr>
<tr>
<td>Edema</td>
<td>0.695</td>
<td>2.14 = 3.81</td>
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</tbody>
</table>

$^*$ Test statistic for the assumption that the three slopes are equal.

† Table value corresponding to the probability of 0.05 with degrees of freedom $k - 1$ for the numerator, and $n - k$ for the denominator. $k$ is the number of experimental groups, and $n$ is the total number of animals on the linear portion of the curve.

Since $T < F$, the hypothesis that the slopes are identical is not rejected.
Table 3. Tests for parallel slopes and potency.\(^\text{12}\)

<table>
<thead>
<tr>
<th>Compared curves</th>
<th>SR*</th>
<th>$f_{st}$†</th>
<th>PR‡</th>
<th>$f_{sc}$§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical Evans blue</td>
<td>PAF/histamine</td>
<td>1</td>
<td>3.195</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>PAF/ethanol</td>
<td>1</td>
<td>11.72</td>
<td>12152.2</td>
</tr>
<tr>
<td></td>
<td>Histamine/ethanol</td>
<td>1</td>
<td>11.31</td>
<td>337.56</td>
</tr>
<tr>
<td>Evans blue extraction</td>
<td>PAF/Histamine</td>
<td>1</td>
<td>4.8</td>
<td>31.385</td>
</tr>
<tr>
<td></td>
<td>PAF/Ethanol</td>
<td>1</td>
<td>31.40</td>
<td>15937.4</td>
</tr>
<tr>
<td></td>
<td>Histamine/ethanol</td>
<td>1</td>
<td>30.90</td>
<td>507.87</td>
</tr>
<tr>
<td>Edema</td>
<td>PAF/Histamine</td>
<td>1</td>
<td>3.547</td>
<td>47.125</td>
</tr>
<tr>
<td></td>
<td>PAF/ethanol</td>
<td>1</td>
<td>30.87</td>
<td>15939.4</td>
</tr>
<tr>
<td></td>
<td>Histamine/ethanol</td>
<td>1</td>
<td>27.65</td>
<td>338.24</td>
</tr>
</tbody>
</table>

* Slope ratio of the regression curves.
† Since SR is $\leq 6_{SR}$, then the slopes are parallel within the experimental error.
‡ Potency ratio of the values of ED\(_{50}\) (effective dose producing a 50% effect).
§ Since PR is $\geq f_{PR}$ for each pair of substances, each has a significantly different potency.

tors of the severity of the reaction.\(^\text{3}\) In the presence of low doses of ethanol, which by itself did not increase vascular permeability, PAF, a mediator of IH, applied topically to the conjunctiva caused a significant linear increase in vascular permeability as measured by clinically evaluated edema, Evans blue extravasation, or chemically measured Evans blue content of the conjunctiva. PAF was 30–50 times more potent than histamine in increasing vascular permeability. Our findings were in qualitative agreement with those of Humphrey et al\(^\text{14}\) and Hwang et al,\(^\text{15}\) who found PAF 9000 and 1000 times, respectively, more potent than histamine in increasing vascular permeability in the skin. The quantitative differences between these studies\(^\text{14,15}\) and ours may be due to anatomic and physiologic differences between the vascular beds of the skin and conjunctiva. Alternatively, the differences may be the result of intradermal versus topical conjunctival application, resulting in dissimilar dilution in tears and the interstitial fluid, or due to differential penetration to the receptor site. A similar quantitative difference in potency was found by Handley et al,\(^\text{16}\) who demonstrated in guinea pigs that intravenous PAF was 17,000-fold more potent than histamine in producing hemoconcentration.

The colorimetric method of measurement of Evans blue evaluated total conjunctival content, including both intravascular and extravasated dye, and thus could be affected by vascular dilatation and increased blood volume within the conjunctiva. However, the potency of PAF, as well as that of histamine and of ethanol, was similar with each of the three methods of evaluation—clinical edema, clinical evaluation of Evans blue extraction, or colorimetric measurement of the total Evans blue content of the conjunctiva. The ratios of the potencies of each of the agents also was similar. Thus, in the conjunctival system, the clinical evaluation of conjunctival edema was as good an experimental estimate of mediator effect (dependent variable) as either the clinical or colorimetric measurement of Evans blue extravasation for all three agents. Therefore, clinical scoring is a reliable system and may be useful when repeated readings need to be done in the same animal. This study will serve as a control comparison for the three types of measurement.

We also established that ethanol (our control solution of PAF), which is known in high concentrations to liberate histamine from other mucosae,\(^\text{17}\) alone causes an increase in vascular permeability. However, ethanol was less potent than histamine by a factor of approximately 300–500. At the largest ethanol dose (10 $\mu$L absolute ethanol), maximum clinical edema or colorimetrically measured Evans blue extravasation was not manifest compared to the maximum response obtained with histamine. This ethanol dose did give the same maximum response as did histamine when Evans blue extravasation was measured clinically.

In the presence of high doses of ethanol, PAF had no additional additive effect. This may be due to maximization of the effects of the measurement scale, or due to high-dose stimulation of arteriole smooth muscle, causing vasoconstriction of the arterioles. Björk and Smegdegård\(^\text{18}\) documented by in vitro microscopy a terminal arteriolar vasoconstriction due to PAF. A vasoconstrictive effect has been shown by skin blanching with intradermal injection of PAF at 1 pmol and with histamine at 900 pmol in the guinea pig.\(^\text{14,18}\) Hwang et al\(^\text{19}\) did not find a decrease in vascular permeability due to PAF or histamine in guinea pig skin. These studies\(^\text{14,18,19}\) used only phosphate buffered saline as a control solution, which makes interpretation difficult since, as we have demonstrated, there is an effect on vascular permeability similar to but less potent than that of PAF and histamine by ethanol alone. Thus it is necessary to use ethanol in DPBS as a control solution for evaluation.
of PAF effects, since pure DPBS does not have an effect on vascular permeability.

Key words: platelet-activating factor, histamine, vascular permeability, conjunctiva, edema

References