STUDIES ON CAPILLARY PERMEABILITY USING COOMASSIE BLUE AS INDICATOR*

FREDERICK KALZ, M.D. AND ZOLTAN FEKETE, M.D.

We recently reported on the use of a protein dye, Coomassie blue (1), as an indicator of leakage of plasma proteins from the capillaries. This dye is useful for studying alterations in capillary permeability and for a more accurate characterization of whealing responses in the human skin. We found an excellent correlation between the area and degree of bluing, and the varying concentrations of wheal producing test substances. Consistent and reproducible results were obtained in any individual test subject, thus providing a method for investigating the influence of drugs and other agents on the capillary permeability of the human skin.

The present paper reports the results of such an investigation; experiments were conducted to study the effects of histamine release and blocking substances and of some other drugs on the bluing response elicited by several test agents. It was felt that additional insight in the mechanism of capillary permeability could be gained and a skin test could be developed for measuring or comparing the efficacy of anti-inflammatory drugs.

MATERIALS AND METHODS

The following wheal producing substances in the amounts shown were used to produce an increase in capillary permeability:

<table>
<thead>
<tr>
<th>Substance</th>
<th>µg per inj.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine Acid Phosphate (British Drug House)</td>
<td>100 10 1 0.1 0.01</td>
</tr>
<tr>
<td>Compound 48-80 (Burroughs Wellcome)</td>
<td>100 10 1 0.1 0.01</td>
</tr>
<tr>
<td>Polymyxin B. sulphate (Burroughs Wellcome)</td>
<td>500 50 5 0.5 0.05</td>
</tr>
<tr>
<td>5-HT (Serotonin) (Mann Research Laboratories)</td>
<td>1000 500 100 50 10</td>
</tr>
<tr>
<td>Trypsin (Frank W. Horner)</td>
<td>500 50 5 0.5 0.05</td>
</tr>
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</table>

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The test substances were dissolved in physiological saline and a volume of 0.1 ml was injected intradermally with a 25 gauge needle. The range of concentration was chosen after preliminary testing to establish the minimal amount of each test substance which still produced a bluing response. The lowest concentration of each compound represents the minimal amount and the other concentrations were arbitrarily selected, to give comparable intensity of whealing with all test substances. 125 volunteers served as test subjects, most of them were healthy individuals or had trivial disorders. A demarcated area of the back was chosen for all experiments, the right side served as test site and the corresponding left side was used as control area in the series of electrophoretic applications. The 5 injections of each substance representing the indicated amounts were spaced approximately 5 cm. apart, starting with the lowest concentration on the bottom of the field followed by the others in increasing order. Preliminary experiments had established that the responses did not vary significantly within this circumscribed area of the skin. Immediately before the intradermal injections, 400 mgms of Coomassie blue were given intravenously; 5 minutes after the intradermal injections the size of the wheals and flares was measured and the intensity of the bluing was recorded by color photography under standard conditions. (Kodak Pony camera for medical photography, 2 one thousand Watt lamps, 20 inches distance, No. 5 adaptation lens, 5.6 aperture, 3/50 sec. exposure. Kodachrome Type A prof. negative).

Drugs Administered by Mouth Prior to Repeat Testing

Cypseroheptadine (Prep. MK 141 Merck) 1 mgm 4 times daily.
This substance has a marked anti-serotonin and anti-histamine effect (2).

Promethazine Hydrochloride (Phenergan, Poulenc) 25 mgms 6 times daily (10 experiments).
A phenothiazine derivative with anti-histaminic and anti-serotonin properties as well as with local anaesthetic action (20 experiments).

Tripeptennamine hydrochloride (Pyribenzamin Ciba) 50 mgms 4 times daily.

A drug with anti-histaminic properties (3, 4) (10 experiments).

**Corticosteroids.** Dexamethasone (Decadron Merck) 0.75 mgms 4 times daily. Triamcinolone (Aristocort, Lederle) 4 mgms 4 times daily. 3.6-Methyl prednisolone (Medrol, Upjohn) 4 mgms 4 times daily (8 experiments).

In addition 2 patients were tested who had received high dosage of corticosteroids for a prolonged period of time. (One suffering from pemphigus, one from an erythroderma.)

**Drugs Introduced Locally into the Skin by Means of Iontophoresis**

A. **Blocking substances.**

Cyproheptadine (see above). 0.2% aqueous solution (10 cases).

Promethazine (see above). 1% aqueous solution (10 cases).

B. **Release substances.**

Compound 48-80, a condensation product of p-, ethoxyphenethylmethylamine and formaldehyde, a substance causing depletion of mast cells, liberating histamine and heparin and, in most animal species, serotonin. 0.1% aqueous solution (5, 6) (20 cases).

Polymyxin B. Sulphate, an antibiotic which is also a potent histamine liberator. 0.2% aqueous solution (7) (10 cases).

C. **Other Compounds**

Histamine phosphate. 0.1% aqueous solution (10 cases).

Procaine hydrochloride. 4% aqueous solution (8 cases).

Metanium (Leeming Miles) a mixture of titanium salts, (oxyde, peroxyde, sulicylate and tannate) a substance reputed to have anti-inflammatory properties. Saturated aqueous solution (10 cases).

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**D. Control**

Sodium chloride, 2% solution (8 cases).

The iontophoresis was carried out by means of a 7.5 x 12.5 cm. electrode, using 0.32 mA electric current for 15 minutes, except for histamine when only 0.16 mA was used for 10 minutes. Metanium and sodium chloride were applied from both the cathode and the anode, while in all other instances the effective component of the drug, or releasing substance, could be introduced only from the anode. In one series of experiments the iontophoretic applications were done only once and the intradermal test injections were given 30 minutes after the completion of the ionization. In a second
TABLE 1

The effect of the single and/or repeated iontophoresis of histamine, compound 48-80, polymixine B sulphate, cyproheptadine, promethazine, sodium chloride on the wheals and bluing provoked by intradermal injections of histamine phosphate, serotonin, compound 48-80, polymixine B sulphate and trypsin.

<table>
<thead>
<tr>
<th>INTRADERMALLY INJECTED SUBSTANCE</th>
<th>Histamine</th>
<th>Compound 48/80</th>
<th>Polymixine</th>
<th>Cyproheptadine</th>
<th>Promethazine</th>
<th>Na</th>
<th>Cl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Serotonin</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Compound 48/80</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polymixine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trypsine</td>
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</tr>
</tbody>
</table>

- Complete Abolition
- Marked Inhibition
- Moderate Inhibition
- No Effect on Subsequent Reactions

Physiological saline was used as control injection and never showed bluing in normal skin.

2. Effect of oral administration of drugs on the bluing reaction

Cyproheptadine, in the dosage used, prevented wheal formation and bluing of all test substances in all amounts used, except for the reactions to trypsin which remained unaltered. The axon reflex flares caused by histamine, serotonin and the release substances were not changed. (Figs. 2 and 3)

Promethazine, in the dosage used, had no effect on the size of the wheals and flares or on the intensity of bluing following any of the test injections.

Tripelemamine hydrochloride produced the following results: The reactions to the release substances were not altered. Serotonin responses were markedly suppressed while whealing and bluing after histamine testing was partially but not uniformly inhibited.

The treatment with corticosteroids in no way
3. Effect of iontophoretic application of drugs

Table I shows the effect of iontophoretic application of the various substances on the bluing response produced by the intradermal injections of the test substances. Not listed in the table is the effect of procaine hydrochloride and of metanium. The iontophoresis of the former caused erythema and local anaesthesia, but the intensity of the bluing remained the same. Iontophoresis of metanium from the cathode had no effect; however applied from the anode, it caused a very marked suppression of the serotonin reactions, while the histamine responses remained unaffected.

After one single iontophoretic application of the histamine release substances the reactions to the intradermal test injections remained inhibited for 24 hours. Forty-eight hours after completion of the iontophoresis the bluing responses began to reappear gradually. Triple iontophoresis of these substances did not prolong substantially the duration of the above mentioned inhibition. Single iontophoresis of the histamine releasers, however, did not result in an even and uniform depletion of histamine in the treated

decreased the intensity of the skin responses to the test substances.

3. Effect of iontophoretic application of drugs

Table I shows the effect of iontophoretic application of the various substances on the bluing response produced by the intradermal

Fig. 4. Compound 48-80 iontophoresis (right side of the picture) abolished the reactions to intradermal injections of compound 48-80, and moderately inhibited the reactions to histamine phosphate.

Fig. 5. Polymixine B sulphate iontophoresis (right side of the picture): marked inhibition of the reactions to compound 48-80, no influence on the reactions to histamine phosphate.

Fig. 6. Polymixine B sulphate iontophoresis (right side of the picture) did not alter the reactions to intradermal trypsin, but completely abolished those to serotonin.

Fig. 7. Histamine phosphate iontophoresis (right side of the picture) resulted in complete elimination of the reactions provoked by intradermal injections of polymixine B sulphate, and in a very marked inhibition of the reactions to intradermal injections of histamine phosphate.

Fig. 8. Histamine phosphate iontophoresis (right side of the picture) did not alter the flares, wheals, bluing provoked by intradermal serotonin.
area, as evidenced by the patchy bluing shown in pictures No. 16 and 17. Triple iontophoresis made the depletion more complete and uniform.

Histamine phosphate, given in one single iontophoretic application, reduced the intensity of the skin reactions for a short period (less than 24 hours).

The degree of the inhibition produced by the iontophoresis of the anti-histaminics and of metanium was the same whether a single or a triple application was used, but the inhibition after the former lasted less than 24 hours, while the effect after triple application lasted over 48 hours.

**Bluing Responses to Iontophoresis at the Site of the Electrodes**

In one series of experiments a specially shaped electrode (cross and half cross) was selected and the coomassie blue was given at the start of the iontophoresis. The electrode site showed immediate intensive bluing when histamine was iontophorsed. Compound 48-80 gave rise to bluing of a lesser degree, and least bluing occurred with polymyxin.

These results were obtained when these compounds were applied from the anode, but not when applied from the cathode. No bluing of the electrode site occurred with iontophoresis of histamine phosphate, given in one single iontophoretic application, reduced the intensity of the skin reactions for a short period (less than 24 hours).

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promethazine, cyproheptadine, metanium, sodium chloride (from either anode or cathode). (Figs. 13, 14, 15, 16)

An additional observation on a patient with marked cold urticaria is recorded. A piece of ice was applied for 2 minutes to the left forearm and coomassie blue was injected intravenously into the right arm, resulting in marked bluing of the wheal provoked by the ice application. The patient was then put on cyproheptadine and the experiment was repeated. Both whealing and bluing were markedly suppressed. (Figs. 17, 18)

**DISCUSSION**

Intradermal injections of histamine and of histamine liberators, of serotonin and of trypsin cause an increase in the permeability of the capillary wall, which within the limits of our experiments, appeared to be proportionate to the type and to the amount of the injected substance. If the increase in vascular permeability is of a degree sufficient to permit leakage of plasma proteins, it can be demonstrated by the indicator dye. If water and electrolytes only escape, wheal formation still occurs but the discoloration is absent. The onset of protein leakage immediately follows the trauma, in our experiments the intradermal test injections; it is of short duration, varying between 5 and 30 minutes, depending on the type and amount of test material injected. (8)

The use of serial dilutions of the test substances permitted a more accurate quantitation and repeated tests gave identical results in the same persons. Reproducibility of results made this method suitable for measuring and comparing the effects of drugs with antihistaminic properties. Two well known antihistaminic drugs, promethazine and trypelennamine were chosen as well as cyproheptadine, an experimental substance with anti-serotonin and anti-histaminic activity. Given orally in moderate therapeutic doses, only the last drug led to a suppression of the whealing responses and the capillary damage demonstrated by the bluing reaction, following the intradermal injections of histamine serotonin and the release substances. Promethazine did not influence any of the reactions and trypelennamine showed only minor alterations. This was a surprising finding since both trypelennamine and promethazine have a potent antihistaminic effect, established in animal experiments and by clinical trials. The failure of these two drugs, when taken orally, to protect the capillaries against the action of locally injected histamine, is probably due to an insufficient tissue concentration at the site of injury. This supposition is borne out of the fact that the iontophoretic application of promethazine resulted in a complete suppression of the reactions to histamine and the release substances, presumably because a higher tissue concentration was achieved by this method. As was to be expected from its oral effectiveness, iontophoretic introduction of cyproheptadine showed a similar inhibition. It is of interest to note that the usual therapeutic dose of promethazine taken by mouth, is 100 to 150 mgms per day, while cyproheptadine is effective in a dosage of 4 mgms per day. Thus, given by mouth, cyproheptadine is effective in a dosage 30 times smaller than that of promethazine. When however these two drugs were introduced by iontophoresis, the dose relation was only 1:5 which may be due to either a better penetration of promethazine by ionization, or due to a selective uptake of cyproheptadine by the skin, if given by mouth. Further experiments will be necessary to elucidate this point. It should be emphasized that neither oral nor electrophoretic application of any of the drugs tested influenced the size of the flares surrounding the wheals, demonstrating that these drugs do not affect the action of histamine on the axon reflex.

The failure of the steroids even if given in high dosage and over prolonged periods of time, to prevent the capillary damage caused by histamine was not unexpected since it is known that these compounds do not block the whealing effect of injected histamine. On the other hand, it is thought that steroids inhibit histamine synthesis, yet in our experiments no altered responses to the histamine releasers were noted; perhaps the duration of steroid therapy had been too short to produce a lowering of the tissue histamine content.

Iontophoretic application was also chosen for the introduction of the histamine release substances into the skin, because this method ensures a high and even tissue concentration of all substances carrying an electric charge without causing a trauma, as injections are prone to do. The successful penetration of these histamine liberators and of histamine phosphate, was demonstrated by the intravenous injection of coomassie blue while the iontophoresis was in progress. Intensive bluing of the electrode site occurred
when histamine was iontophoresed, while compound 48-80 and polymyxin showed bluing of a lesser degree. There was good agreement of these results with the degree of bluing observed after intradermal injection of the same substances. The same experiment was also done with 2% sodium chloride, as control, and with cyproheptadine and promethazine, because some antihistaminic substances are known to liberate histamine if injected into the skin. None of these substances caused any increase in capillary permeability and no trace of bluing was seen.

It is known that repeated trauma depletes the skin of histamine and that urticarial dermographism cannot be elicited in the depleted areas. Restoration of histamine content of the skin after depletion requires several days. A large number of chemicals also release histamine from the tissues and 48-80 and polymyxin are among the most potent release substances.

Iontophoresis of compound 48-80 markedly diminished the effect of subsequent intradermal injections of the same compound as well as that of serotonin, polymyxin and also of histamine phosphate. Iontophoretic application of polymyxin B gave comparable though weaker inhibitions. While it was expected that depletion of histamine would abolish the effect of subsequently introduced histamine liberating substances, the observation that the reactions to histamine injections into these areas were also markedly reduced as compared with the control areas, requires additional explanation. Wilhelm et al. (9) observed a diminished response to histamine in rats pretreated with high doses of compound 48-80 given intraperitoneally. These authors attributed this result to an non-specific toxic or debilitating effect of the 48-80 therapy. This, however, does not apply to our experiments where only minimal doses of 48-80 were given locally. Also, the testing with trypsin injections showed no diminished responses in the iontophoresed area. It is suggested that the iontophoresic application of histamine phosphate caused release of histamine from the tissues and the commonly seen whealing effect of intradermally introduced histamine may be due not only to the direct action of the injected material, but also to liberation of tissue histamine, or in other words, histamine phosphate acted in our experiments similar to the histamine release substances used (14). The cross-experiment, namely iontophoresis of histamine phosphate and subsequent injection of histamine release substances also showed inhibition of whealing and bluing responses, though to a lesser extent than that seen after iontophores with compound 48-80 and with polymyxin. The outcome of this experiment seems to support the thesis that histamine itself acts as a weak histamine releaser.

Compound 48-80 is known to release serotonin from the mast cells of some animal species, (besides histamine and heparin) but the serotonin content of human mast cells and of the human skin is probably negligible. Iontophoresis of compound 48-80 and of polymyxin B resulted in a diminished reaction of subsequently injected serotonin, suggesting that bluing after serotonin injections into human skin is probably also due to histamine release (13). We can offer no explanation, however, why intradermal serotonin injections remained unaltered after histamine iontophoresis. Further experiments will have to be done to investigate in more detail the role of serotonin and the action of serotonin inhibitors and the effect of other vaso-active substances and their inhibitors. Clendenning and Stoughton (10) observed no whealing response after serotonin injections in human skin and Demis and Davies (11) using Evans blue, saw no staining of the serotonin injections, while Møller and Rorsman (12), using sodium fluorescein, observed fluorescence of serotonin wheals in ultraviolet light. They state that the exudatory effect of serotonin is about 1000 times weaker than that of histamine; precisely the same relation was found in our experiments, comparing the minimal effective doses by weight.

The reactions of the intradermally injected trypsin were in no way affected by any of the drugs used either orally or locally, thus confirming that its action is different from that of any other test substances in our experiments, and that it does not involve histamine or its release in any significant degree. We included trypsin to investigate whether injections or iontophoresis of histamine and other permeability increasing substances may cause a state of fatigue or lack of responsiveness of the capillaries. No evidence of such a mechanism was suggested by our trials.

Most antihistaminic drugs exhibit some local anesthetic action. Iontophoresis with procain hydrochloride was therefore used, to see whether a local anesthetic agent without any significant effect on the histamine mechanism may alter skin responses. No such effect was observed.
Metanium was included in our series to investigate whether the “bluing” method would permit a differentiation between the efficacy of positively and negatively charged parts of a compound preparation. The alleged anti-inflammatory effect of this preparation appears to be due to the titanium ion since application from the anode markedly inhibited serotonin reactions. Mill (15) has demonstrated that salicylates inhibit responses to histamine and other vasoactive agents but in our experiments the salicylates contained in this product were not responsible for the inhibitions noted. Sodium chloride controls were made to rule out the possible influence of non-specific irritation produced by the iontophoretic procedure itself. Applied either from the anode or the cathode, no alterations occurred.

SUMMARY

A new (monazo dye) mono azo, Coomassie Blue, was used to demonstrate the leakage of plasma proteins from the capillaries after standardized trauma induced by intradermal injections of varying amounts of histamine, histamine release substances, serotonin and trypsin. The influence of iontophoretic applications of antihistaminic drugs, of histamine releasing substances and of histamine itself on the degree of plasma protein extravasation was measured and the effect of systemic therapy with antihistaminic and other drugs on the intensity of bluing was evaluated.

ACKNOWLEDGMENT

We are indebted to Ayerst, McKenna and Harrison Ltd. for the generous supply of Coomassie Blue. The Polymixine B Sulphate and the Compound 48-80 were donated by Burroughs Wellcome & Co. (Canada) Ltd., the Cyproheptadine in the form of MK 141 by Merek Sharp & Dohme, Division of Merek & Co. Ltd., the Promethazine in the form of Phenergan by Poulene Ltd., the Metanium by Leeming Miles Ltd.

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REFERENCES