THE EFFECT OF ANCROD ON THE DELAYED HYPERSENSITIVITY RESPONSE IN RATS

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Summary.—Delayed hypersensitivity was induced in rats by means of sheep erythrocytes and bovine serum albumin–lipid conjugate. Administration of heparin to rats sensitized to either antigen resulted in diminution of the delayed hypersensitivity reaction. Administration of ancrod, however, failed to inhibit the delayed cellular reaction to either antigen. Granuloma formation remained unaffected when rats were injected with either heparin or ancrod. The lack of ancrod effect, in contrast to heparin effect, on delayed hypersensitivity is discussed.

Several lines of evidence now suggest that fibrin deposition plays a vital part in various types of inflammatory processes, including the tissue reaction mediated by delayed hypersensitivity (Colvin et al., 1973). Administration of heparin or warfarin to sensitized guinea-pigs resulted in diminution or prevention of the delayed hypersensitivity reaction (Cohen et al., 1967). Ancrod, the purified fraction prepared from the venom of the Malayan pit viper, has shown effective anticoagulant properties (Bell, Pitney and Goodwin, 1968). Other studies, however, have provided evidence that ancrod, through its defibrinating action, interferes with the normal process of wound healing in rabbits (Holt et al., 1970). Silberman and Kwaan (1971) have likewise shown that ancrod causes delayed formation of granulating tissue in implanted clots in rats. Studies on the cellular response of the delayed hypersensitivity reaction have shown that the inflammatory cell response is no different in immune inflammations from that in nonspecific inflammatory reactions (Spector, 1967). These observations suggested studies to determine whether hypofibrinogenaemia induced by ancrod will influence the development of the delayed hypersensitivity reaction. The present study seeks to evaluate the effects of ancrod and heparin on the cellular reaction of the rat purposely immunized in a manner favouring the development of delayed hypersensitivity upon skin testing, with sheep red blood cells (Axelrad, 1968) and bovine serum albumin–lipid conjugate (Coon and Hunter, 1973) used as immunogens.

MATERIALS AND METHODS

Sprague–Dawley female rats weighing 250–300 g were used. Two in vivo models of the delayed cellular response were utilized in this study: sensitization to a particulate antigen, sheep red blood cells (SRBC) and to a soluble antigen, bovine serum albumin–lipid conjugate (D–BSA).

Sheep red blood cell sensitization (SRBC).—The preparation of SRBC and the sensitization programme were carried out as described by Axelrad (1968). Animals were subdivided into 3 groups: Groups A, B, and C.

Group A consisted of 10 rats which served as control animals. The group was subdivided equally into Groups A1 and A2. Group A1 rats were sensitized with a solution of equal volumes of Freund’s Complete Adjuvant (FCA) and saline, then skin tested with SRBC. Group A2 rats received no prior sensitization but were skin tested with SRBC as described below.
Group B was subdivided into 2 groups, B₁ and B₂, of 15 rats each. Both groups were sensitized and challenged with SRBC. In addition, Group B₂ rats were injected with ancrord (kindly supplied by Abbott Laboratories, North Chicago). Ancrod was injected through the tail vein, 57 u/kg body weight every 12 h beginning 24 h before challenge and throughout the skin testing period. Adequate defibrination was ensured by measuring a citrated sample of plasma for fibrinogen at the termination of the experiment by means of a thrombin clotting assay. All animals were sensitized intradermally with a 5% saline suspension of fresh SRBC mixed with an equal volume of FCA. A total of 0.5 ml of emulsion was injected over 3 sites along the nipple line.

Group C consisted of 10 rats sensitized and challenged with SRBC, in whom heparin was substituted for ancrord as anticoagulant. Heparin (obtained from Upjohn Co., Kalamazoo, Michigan (1000 u/ml)) was administered intraperitoneally 1000 u/kg body weight twice daily 24 h before skin testing with SRBC and throughout the experimental procedure. Adequate anticoagulation was ensured by measuring the whole blood clotting time.

For evaluation of skin test sensitivity to sheep erythrocytes, increased paw size was measured following injection of SRBC. Animals were injected intradermally on the dorsal surface of the left rear hind paw with 0.1 ml of a 7.5% suspension of SRBC in saline on the ninth day following sensitization. Saline in equal volume was injected in the other hind paw. Twenty-one h later the animals were anaesthetized with Nembutal (pentobarbitone sodium) intraperitoneally and the skin test reaction to SRBC was evaluated. The cellular response was measured by weighing each hind paw into a pre-weighed beaker containing mercury. The difference in weight between the challenged left hind paw and the saline injected paw was expressed as the percent increase of paw volume subsequent to challenge with the antigen. To test for significant difference in weights between right and left hind paws of untreated animals, 20 random rats were examined in similar fashion. The animals were anaesthetized and the weights of right and left rear hind paws were measured and the percent difference computed. Serum samples from 10 sensitized rats were obtained 4 days after the sensitizing injection and at the end of the experiment; SRBC agglutinin titres were determined on these samples. All animals were subsequently sacrificed and sections from both rear hind paws as well as from the initial site of sensitization were taken for histological examination.

Bovine serum albumin (BSA)-lipid sensitization (D-BSA).—BSA, 25 mg/ml was conjugated with a lipid, dodecanoic anhydride (Pfaltz and Bauer, Flushing, N.Y.) according to the method of Coon and Hunter (1973). The molar ratio of the anhydride to the BSA solution was 500 : 1. Animals were divided into Groups D, E and F.

The control Group D, containing 10 rats, was subdivided equally into Groups D₁ and D₂. Group D₁ rats were sensitized with equal volumes of FCA and saline, then skin tested with D-BSA. D₂ animals were skin tested without prior sensitization. Group E, containing a total of 30 animals, was subdivided into 2 groups of 15 rats each—E₁ and E₂. Both groups were sensitized with 50 mg of conjugate in 0.1 ml of FCA injected intradermally into the left rear paw and skin tested with BSA. Group E₂ rats in addition received ancrord, in similar manner to Group B₂ animals, 24 h before and during the challenge period. Group F consisted of 10 rats sensitized with D-BSA in FCA and challenged with BSA. This group received heparin in dose and manner similar to Group C rats.

Skin testing was carried out on all groups 12 days following sensitization with a solution of 50 µg BSA in 0.1 ml saline intradermally over the pre-shaven flank. The skin reactions were examined 24 h later for erythema and induration and the skin diameters measured in mm. At this time maximal swelling and erythema were observed, whereas at 48 and 72 h after injection of antigen, the swelling and erythema were decreased. The animals were bled for determination of fibrinogen, and serum samples from 10 animals were tested for the presence of antibodies to BSA by agar immunodiffusion. The animals were sacrificed and sections taken from sensitization and skin test sites for histological examination.

Statistical evaluation.—The results obtained in each sensitized group were compared with those in the respective control group. The results of experiments with heparin and ancrord also were compared with those of the corresponding untreated sensitized group. The significance of the differences was established by Student's “t” test.

RESULTS

Plasma fibrinogen levels were maintained below 50 mg/100 ml in all ancrord injected rats. Control values in normal animals ranged from 150 to 350 mg/100 ml. The heparinized animals exhibited clotting times greater than 30 min when measured 3 h after the last heparin injection. Values averaged 5 min in normal animals.
SRBC sensitization

Random untreated animals showed slight variations in foot pad size between the right and left hind paws, dependant on the animal size. The average percentile difference in weight computed in 20 normal rats by subtracting the weights of the right hind paws from the left paws was 3.2%, and by subtracting the weights of the left from that of the right hind paws was 2.9%. The difference in weights between the right and the left hind paws was negligible (P > 0.05) and subsequently considered not significant.

Saline injected right hind paws in each experimental group showed no swelling or erythema after 21 h. Paws of animals from Groups A\(_1\) and A\(_2\) likewise demonstrated minimal visible change and no significant difference in percent weight gain (Table I). B\(_1\) group animals manifested diffuse swelling of the left hind paw with erythema (12 rats) and moderate to minimal change (3 rats). The statistical response was significantly greater than the mean of the combined values of Groups A\(_1\) and A\(_2\) (A), at the 0.05 level of probability.

Animals injected with anerod (B\(_2\)) showed severe swelling and redness (5 rats), moderate swelling (7 rats) and minimal swelling (3 rats). The response, compared with that observed in Group A, was significant at the 0.01 level of probability. However, when compared with the response in Group B\(_1\) rats, the increase in paw swelling was not markedly significant at the 0.05 level.

In Group C animals, the left hind paws showed bluish discoloration due to subcutaneous bleeding in 4 rats, otherwise minimal swelling was observed. The percent increase in paw swelling in this group was not significant (Table I) compared with that observed for the control Group A (P > 0.1) but was statistically significant when compared with Group B\(_1\) untreated rats (P < 0.01).

**Histopathology**

Microscopic examination of sections from Group A\(_1\) and A\(_2\) animals showed minimal infiltration of the dermis and subcutaneous tissues with both mononuclear leucocytes and polymorphonuclear leucocytes (Fig. 1a).

Group B\(_1\) showed marked oedema and marked cellular infiltration consisting predominantly of mononuclear leucocytes, with few polymorphonuclear cells. focal areas of necrosis with accumulation of polymorphonuclear cells were visible in about half the sections examined. Phagocytosis of red blood cells by mononuclear leucocytes was prominent with up to 7 red cells per macrophage (Fig. 1b). Group B\(_2\) revealed moderate oedema and infiltration of the dermis and subcutaneous tissues with both mononuclear as well as polymorphonuclear leucocytes. In many areas

**Table I.** Percent Weight Gain and Standard Deviations Obtained 21 h following Challenge with Sheep Erythrocytes in Sensitized Untreated Rats (Group B\(_1\)), Sensitized Anerod Treated Rats (Group B\(_2\)), Sensitized Heparin Treated Rats (Group C) and Control Rats (Groups A\(_1\) and A\(_2\))

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of animals studied</th>
<th>Sensitization</th>
<th>Challenge</th>
<th>Treatment</th>
<th>Mean response (%) and standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A(_1)</td>
<td>5</td>
<td>CFA + saline</td>
<td>SRBC</td>
<td>—</td>
<td>2.8 ± 1.7</td>
</tr>
<tr>
<td>A(_2)</td>
<td>5</td>
<td>—</td>
<td>SRBC</td>
<td>—</td>
<td>3 ± 1.9</td>
</tr>
<tr>
<td>B(_1)</td>
<td>15</td>
<td>CFA + SRBC</td>
<td>SRBC</td>
<td>—</td>
<td>22* ± 6</td>
</tr>
<tr>
<td>B(_2)</td>
<td>15</td>
<td>CFA + SRBC</td>
<td>SRBC</td>
<td>—</td>
<td>14.8† ± 7.2</td>
</tr>
<tr>
<td>C</td>
<td>10</td>
<td>CFA + SRBC</td>
<td>SRBC</td>
<td>—</td>
<td>5.7‡ ± 2.7</td>
</tr>
</tbody>
</table>

* Differs significantly from control Group A (P < 0.01).
† Differs significantly from control Group A (P < 0.01).
‡ Does not differ significantly from control Group A (P > 0.01).
Fig. 1a.—Delayed hypersensitivity reaction in rat sensitized with a solution of equal volumes of FCA and saline, and skin tested with SRBC. There is minimal infiltration of the deep dermis and subdermal tissues with mononuclear cells and polymorphs. H. and E. × 60.

Fig. 1b.—Delayed hypersensitivity reaction in rat immunized then skin tested with sheep erythrocytes on Day 9. Animal killed 21 h later. There is marked infiltration of the deep dermis and subdermal tissues with mononuclear cells. H. and E. × 142.

Fig. 1c.—Delayed hypersensitivity reaction in ancrod treated rat. 21 h. There is infiltration of the deep dermis and subdermal tissues with mononuclear cells and polymorphs. H. and E. × 142.

Fig. 1d.—Delayed hypersensitivity reaction in heparin treated rat, 21 h. There is gross reduction in the cellular infiltration compared with the reaction in untreated rats. H. and E. × 142.

Fig. 1e.—Granuloma in nipple line of rat immunized with SRBC receiving heparin, 9 days after immunization: participation of foam cells, mononuclear cells, some polymorphs and epithelioid cells. H. and E. × 60.
the cellular infiltrate appeared to consist predominantly of polymorphonuclear leucocytes. Mononuclear cell phagocytosis of red blood cells was rarely observed (Fig. 1c).

Sections of animals from Group C showed marked haemorrhages within subcutaneous fatty tissues with a mild leucocytic infiltrate composed mainly of polymorphonuclear leucocytes (Fig. 1d).

All animals sensitized with SRBC and FCA, whether treated with anticoagulants or untreated, showed granuloma formation at the injection site. On histological examination the lesions were seen to extend from the dermis to the subcutaneous tissues, spreading between the muscle fibres. They were composed of oedematous areolar tissue containing inflammatory cells and foam cells. Numerous mononuclear cells and occasionally polymorphonuclear leucocytes and eosinophils were seen as well as epithelioid cells, but only rare giant cells (Fig. 1e). Animals that had received ancrod or heparin had formed granulomata that were indistinguishable from those seen in rats which did not receive those agents.

The reciprocal of agglutinin titres to SRBC on the 4th day after sensitization were < 2 (5 rats), 2 (1 rat), 4 (3 rats) and 8 (1 rat). On the 9th day after sensitization the titres were 32 (2 rats), 64 (6 rats), 128 (2 rats).

**D-BSA conjugate**

Animals from Groups D₁ and D₂ showed minimal induration or change in skin test diameter. Immunized animals challenged with BSA manifested the typical delayed hypersensitivity skin reaction of marked induration at 24 h; erythema, however, was absent. The distribution in skin test response in the various groups is shown in Table II. As can be seen from the Table, the skin reaction of ancrod treated rats was significantly increased compared with the control group, but did not differ significantly from the corresponding group of immunized rats (E₁) not receiving ancrod (P < 0.05). Heparin anticoagulation caused suppression of the delayed hypersensitivity skin test response. A significant difference in the skin test diameter was present compared with that seen in Group E₁ rats (P < 0.01), and the response was not markedly different from that of the control group, as shown in Table II.

**Histopathology**

The inflammatory infiltrate in the control groups D₁ and D₂ was minimal and

<table>
<thead>
<tr>
<th>Group</th>
<th>Sensitization</th>
<th>Challenge</th>
<th>Treatment</th>
<th>Reaction diameter (mm)</th>
<th>Average diameter (mm) and standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>D₁</td>
<td>CFA + saline</td>
<td>BSA</td>
<td>Ancrod</td>
<td>5(4), 10(1)</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>D₂</td>
<td>—</td>
<td>BSA</td>
<td>Heparin</td>
<td>5(3), 10(2)</td>
<td>7 ± 2.4</td>
</tr>
<tr>
<td>E₁</td>
<td>CFA + D-BSA</td>
<td>BSA</td>
<td>—</td>
<td>10(1), 15(2), 20(5), 25(6), 30(1)</td>
<td>21 ± 4.9</td>
</tr>
<tr>
<td>E₂</td>
<td>CFA + D-BSA</td>
<td>BSA</td>
<td>+</td>
<td>5(1), 10(3), 15(4), 20(4), 25(3)</td>
<td>16.6 ± 5.9</td>
</tr>
<tr>
<td>F</td>
<td>CFA + D-BSA</td>
<td>BSA</td>
<td>—</td>
<td>5(3), 10(4), 15(2), 20(1)</td>
<td>10.5 ± 4.7</td>
</tr>
</tbody>
</table>

* Number of animals
† Differs significantly from control Group D (P < 0.01).
‡ Differs significantly from control Group D (P < 0.01).
§ Does not differ significantly from control Group D (P > 0.1).
consisted of a mixture of mononuclear and polymorphonuclear leucocytes. Group E1 rats showed diffuse oedema of the dermis and subcutaneous tissues, with a marked mononuclear cellular reaction typical of the delayed hypersensitivity response (Fig. 2a). Following ancrod injections, marked oedema and cellular infiltration were present in 7 rats, and the remaining rats showed only mild to moderate cellular infiltration (Fig. 2b). Heparin treatment of sensitized rats caused a marked reduction of the mononuclear cellular reaction in the skin test sites (Fig. 2c). The granulomatous response in all groups of rats, whether sensitized with D-BSA or with FCA, was identical. Antibodies to BSA in immunodiffusion tests were present in 2 rats 12 days after sensitization; none were seen on the 6th day of immunization.

**DISCUSSION**

Several investigators have demonstrated the inhibitory effect of anticoagulants on the delayed hypersensitivity reaction. Cohen et al. (1967) reported that administration of heparin and warfarin to
guinea-pigs with allergic contact dermatitis exhibited decreased dermal hypersensitivity which lasted as long as the effect of those agents on blood clotting. Nelson (1963) induced delayed type sensitivity in guinea-pigs by vaccination with BCG and was able to inhibit the peritoneal cellular reaction to purified protein derivative (PPD) in animals treated with heparin and warfarin. A suppressive effect of heparin on the ocular reaction to tuberculin in sensitized rabbits has likewise been shown by Wood and Bick (1959).

In contrast, there have been several reports of the failure of ancord to affect certain sensitivity reactions. Slapack et al. (1971) studied the effect of ancord on the xenograft kidney rejection time of dogs receiving ancord compared with control transplanted animals. Ford et al. (1970) studied experimentally induced immune arthritis and were unable to demonstrate any inhibitory effect on the reduction of fibrin in joints of animals injected with ancord.

Commenting on the role of the coagulation system in cell mediated hypersensitivity reactions, Colvin et al. (1973) stressed the role of fibrin deposition in delayed hypersensitivity reactions in man. Others also have demonstrated the presence of fibrinogen in the vessels and adjacent connective tissues of affected skin lesions of guinea-pigs sensitized to tuberculin purified protein derivative (Paronetto et al., 1967).

The precise role of fibrin deposition in the inflammatory process is not clear. Menkin (1940) postulated that fibrin initiates oedema formation possibly by blocking the lymphatics or capillaries at the inflammatory site. Barnhart (1968) has attributed potent chemotactic and permeability properties to fibrin and its degradation products.

The different modes of action of heparin and ancord on plasma fibrinogen in vivo are of possible significance and may explain their different effects in the delayed hypersensitivity reaction. Whereas heparin by its antithrombin action prevents deposition of fibrin in vivo, ancord by its thrombin-like action converts fibrinogen to fibrin (Chan, Rizza and Henderson, 1965). The fibrin microclots induced by ancord are, however, of transient duration being rapidly cleared by the plasmin system (Kwaan, Barlow and Suwanwela, 1973) with the appearance of fibrin degradation products early in the course of ancord defibrination (Pitney, Bell and Bolton, 1969). This transient appearance of fibrin and its degradation products following injections with ancord could stimulate the inflammatory component of the delayed reaction by their angiostaxis and chemotactic properties.

In an attempt to further explain the difference between heparin and ancord on the delayed cellular reaction, it is important to consider other biological properties of each agent which could influence the inflammatory response. Heparin is known to possess anticomplementary activity (Ecker and Pillemér, 1941), although measurement of complement levels was not performed in our studies. Ancord on the other hand has been shown to inhibit the alternate pathway of the complement sequence but to possess no effect on the classic sequence of complement activation (Tesar and George, 1973).

Heparin has also been stated to possess anti-inflammatory properties and to be able to inhibit the mononuclear component of the inflammatory reaction (Miller and Page, 1963). Our results support this contention in that sensitized rats receiving heparin showed fewer mononuclear cells in their cellular reaction at skin test sites compared with those of ancord injected rats, when challenged with antigen. Finally, heparin has been shown to influence the movement of lymphocytes, the latter becoming trapped in the circulation of heparinized animals with a tendency for the lymph nodes to become depleted of small lymphocytes (Matous–Malbohan and Arnason, 1974). The work of Ford et al. (1970) failed to demonstrate an inhibitory effect by ancord on lymphocyte migration in joints of animals with
experimentally induced immune arthritis, but the draining lymph nodes were not studied.

Our experimental findings failed to show a significant difference in the cellular response to SRBC or D-BSA in sensitized rats treated with ancrod compared with a group of untreated sensitized rats whereas sensitized rats treated with heparin showed significant suppression in their skin test response when challenged with the corresponding antigen. They indicate, therefore, that adequate defibrination with ancrod does not adversely affect cell mediated immunity.

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REFERENCES


