ALTERED CUTANEOUS CONDITIONS IN THE SKIN OF TUBERCULOUS GUINEA PIGS AS DEMONSTRATED WITH A VITAL DYE

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PLATE 10

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The questions that arise concerning allergic reactions in the skin consequent on infection illustrate the inadequacy of our knowledge of the mechanisms involved. A recent study of reactions in guinea pigs after dermal inoculations with tubercle bacilli, carried out in connection with the preparation for publication of some experiments of the late Henry Sewall (1), prompted us to a further study of the skin. By the use of a dye relatively inert in the normal animal, we have found that in the tuberculous guinea pig there is a restriction to the spread of this material after intradermal injection. There is reason to suppose that this property of restriction of spread in a state known to be allergic may be a factor in the reaction of the skin to tuberculin.

Tuberculosis is the disease in which the study of dermal reactions has been most comprehensive and a proper understanding of the nature of such reactions is of increasing significance in connection with recent clinical surveys. The guinea pig has been the animal most used in the experimental work, since it reacts readily to small amounts of tuberculin after infection. This reaction to tuberculin is generally conceded to be specific. The type of the reaction is characterized by a delayed erythema and edema and, in more highly reactive states, by induration and necrosis. It differs from the Arthus reaction induced experimentally, in being more delayed in appearance and less transient, in that hemorrhage is not prominent, in the lack of correlation between the intensity of the dermal reaction and the antibody titer of the blood, and in that passive transfer has not been accomplished.
Dienes (2) has shown that the tuberculous guinea pig, when sensitized to a non-bacterial protein, such as egg white, reacts differently from a normal animal so sensitized. The skin reaction in the tuberculous-sensitized animal becomes more like a tuberculin reaction and after an intravenous injection of egg white the animal passes into a condition like tuberculin shock. In tuberculin shock an animal shows a gradual and profound fall in temperature and, as Dienes has found, a hypoglycemia leading in severe sensitization to death after several hours. Lurie (3) has shown that tuberculous animals, as illustrative of a phase of immune reactions, vary from the normal in their power to restrict the spread of foreign materials, such as agar and India ink. A study of the Arthus phenomenon by Opie (4) has demonstrated that when horse serum is injected intradermally into rabbits sensitized to it the protein remains localized at the point of injection, and the degree of the dermal reaction to the protein is correlated with the antibody titer in the serum. Freund, Laidlaw, and Mansfield (5) have shown that the sensitivity in the tuberculous animal differs from the normal in their power to restrict the spread of foreign materials. These observations justify the supposition that tuberculous animals have an altered condition of the skin with respect to specific and non-specific foreign material.

Hudack and McMaster (8) have made an extensive study of diffusion in the skin by means of the use of non-toxic "vital" dyes. They showed that practically every true dermal injection is both interstitial and intralymphatic. The mesh of the dermal lymphatic plexus is so close that the needle cuts through some of the vessels and the dye enters the open ends under pressure. By making a minute hole in the skin and then introducing extremely small amounts of dye into this space, Parsons and McMaster (9) have been able to make pure interstitial injections and thus have obtained material in which to study both the physiologic factors of diffusion and of normal lymphatic drainage.

In the present study, we have found that there is a restriction both to the diffusion of dye in the connective tissue spaces and to the drainage of the same dye into the lymphatics in guinea pigs in two different infections, namely, tuberculosis and epizootic lymphadenitis.

Materials and Methods

Guinea pigs inoculated with stock cultures of tubercle bacilli, strains H-37 and A-14, by Dr. K. C. Smithburn were employed as the tuberculous animals. The strain A-14 was received from Dr. E. R. Long of The Henry Phipps Institute, Philadelphia. Normal controls were stock guinea pigs apparently free from disease. For comparison with the reactions in tuberculosis, guinea pigs with epizootic lymphadenitis were used, the disease having been diagnosed by palpa-
tion of the enlarged lymph nodes and by a skin test with a bacterial extract furnished us by Dr. J. K. Moen (10). He has shown that animals reacting to this bacterial extract either have at the time, or have had recently, an infection with a hemolytic Streptococcus, Lancefield type C.

Areas of skin to be studied were prepared by several different methods. The hair was clipped and then shaved with a razor, or the hair was removed with an epilator or by close clipping with an electric animal clipper. The majority of the animals were prepared by the latter method 24 hours before the injections. Studies were made of the spread of dye after these different methods of preparation of the skin and no differences were observed. The material used for injection was a diazo dye, pontamine sky blue, which we received from Dr. McMaster, to whom we are indebted for assistance in our methods as well. The dye had been dialyzed in running water until free from salts and then made into a 2.5 per cent, approximately isotonic solution. A Dewitt and Herz syringe was used, equipped with a threaded plunger with an adjustable nut which can be set so as to limit the amount of fluid injected to amounts as small as 0.001 cc. with reasonable accuracy. It had a Luer tip for the needles which were No. 29 and were ¾ inch in length. A new syringe was used which had never been filled with any products from tubercle bacilli. The amount of dye injected was as close to 0.025 cc. as possible. Injections were performed under a dissecting microscope under a bright light. The needle was inserted just beneath the epidermis and the syringe was held parallel to the body of the animal. By exerting gentle pressure the point of the needle was introduced about a distance of 2 mm. The skin over the point of the needle was then covered with mineral oil to facilitate observation of the dye. The dye was then injected into the skin over a period of about 30 seconds. When the plunger had been pressed up to the limit allowed by the adjusted stop, the needle was held an instant and then slowly and carefully removed from the skin. In this manner no appreciable amount of dye leaked from the needle hole which was sealed by the mineral oil. After a moderate amount of experience had been gained by the use of the dissecting microscope it was possible to make the injections as well without it. However, in all other details the procedures described above were strictly adhered to throughout the experiments.

RESULTS

It was soon found that injections into the skin, in order to be comparable, must be made in similar areas dependent on the thickness of the fibrous layers of the dermis. In the guinea pig the three zones of the body, dorsal, lateral, and ventral, vary in thickness in about the proportion of 2:1.5:1 mm. and injections must be limited to one of these three zones. In fact, in a given normal animal the spread of dye in dorsal and ventral zones may vary by as much as 100 per cent. This difference measures a physiological variation in diffusion correlated with a difference in structure, that is, in thickness of the fibrous layer. In general we have limited the injections to either the dorsal or the ventral zones.
dorsal zone has been used, two injections have been made in the midline, spaced about 3 or 4 cm. apart; when the ventral zone has been used, we have made four injections in each of the four quadrants.

With the amount of dye used (0.025 cc.) a small bleb is formed at the site of injection from which the superficial lymphatics are filled at once. These lymphatics can be seen quite clearly through the mineral oil which makes the skin slightly translucent. From this superficial plexus the larger lymphatics of the subcutaneous plexus are filled almost immediately and the dye can then be seen extending in long streamers at this level. In the guinea pig the lymphatic trunks from the subcutaneous plexus penetrate the panniculus carnosus muscle only opposite the regional lymph nodes.

Besides this drainage of the injected dye into the lymphatics, there is a slow diffusion of dye from the bleb. In the area immediately surrounding the bleb, which usually measured originally about 2 mm. in diameter, the dye could then be seen to extravasate rapidly into the tissues so that by the end of 20 minutes the dark area measured about 5 or 6 mm. in diameter. From this period on the dye spread rather uniformly around the dark bleb and stained the skin a light blue, the limits of which could be seen and measured. It was found by repeated experiments that for purposes of comparison measurements taken at 1 hour, 4 hours, and 24 hours were sufficient to establish the rate of spread of the dye. The spread of dye in the skin of normal and tuberculous guinea pigs after 1 and 4 hours is illustrated in Figs. 1 to 4. When the dye has been injected for as long as 4 hours the limits of the spread in the normal animal become more difficult to ascertain and to measure on account of the hazy edge of the dye area, in contrast to the sharp, distinct border of the area in the skin of the tuberculous animal.

The measurements of the areas of the spread of dye in the different experiments are shown in Table I. They are classified according to whether the dye was injected into the dorsal or the ventral zones. In all instances there was a greater spread of dye in the thinner skin of the ventral zone. The first three groups of animals, namely, tuberculous guinea pigs in the allergic state, normal guinea pigs, and tuberculous in the hypoergic or anergic state, are arranged in the order of increasing spread of dye. The differences in the rate of spread were in all instances mathematically significant. In addition to the animals shown in the tables, ten normal and ten tuberculous guinea pigs have been studied more recently in the same manner with results showing the same ratio between tuberculous and normal. For measurements made after 24 hours, it will be noted in Table I that only eight measurements were made for the normal group. This was due to the difficulty in determining the outline of the area of the
primary injection, because the entire animal was stained, even to the foot pads, the tongue, and the conjunctivae. This extensive dis-

**TABLE I**
**Areas of Spread of Dye in the Skin of Normal and Infected Guinea Pigs**

<table>
<thead>
<tr>
<th>Groups of animals</th>
<th>Ventral</th>
<th>Dorsal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hour</td>
<td>4 hours</td>
</tr>
<tr>
<td></td>
<td>Mean sq. mm.</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>Tuberculous, allergic state</td>
<td>192 ± 27.0</td>
<td>77.8</td>
</tr>
<tr>
<td>40 injections</td>
<td>10 animals</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>218 ± 8.18</td>
<td>69.0</td>
</tr>
<tr>
<td>32 injections</td>
<td>8 animals</td>
<td></td>
</tr>
<tr>
<td>Advanced tuberculosis</td>
<td>461 ± 30.17</td>
<td>155.0</td>
</tr>
<tr>
<td>12 injections</td>
<td>3 animals</td>
<td></td>
</tr>
<tr>
<td>Epizootic lymphadenitis</td>
<td>140 ± 4.96</td>
<td>25.5</td>
</tr>
<tr>
<td>12 injections</td>
<td>3 animals</td>
<td></td>
</tr>
<tr>
<td></td>
<td>103 ± 4.7</td>
<td>29.4</td>
</tr>
<tr>
<td>Tuberculous, allergic state</td>
<td>18 injections</td>
<td></td>
</tr>
<tr>
<td>9 animals</td>
<td>121 ± 5.6</td>
<td>35.6</td>
</tr>
<tr>
<td>Normal</td>
<td>18 injections</td>
<td></td>
</tr>
<tr>
<td>6 animals</td>
<td>181 ± 8.4</td>
<td>30.7</td>
</tr>
<tr>
<td>Advanced tuberculosis</td>
<td>6 injections</td>
<td></td>
</tr>
<tr>
<td>3 animals</td>
<td>116 ± 5.7</td>
<td>20.6</td>
</tr>
</tbody>
</table>

* Eight measurements.

...tribution of the dye was due not only to a greater diffusion in the dermis but also to a far greater drainage of the dye into the lymphatics
and hence into the blood stream. On this account some of the primary areas could not be discriminated sharply enough to be measured.

The tuberculous animals whose records are shown in Table I have been divided into two groups according to the duration of the disease. In the first group the period after inoculation varied from 1 to 6 months and covered the time during which the animals showed a positive tuberculin reaction. An effort was made to see if the amount of restriction to spread of the dye was correlated with the degree of allergy but this was found not to be true, as demonstrated in Table II, from which it is clear that there was no significant difference in rate of spread of dye between guinea pigs that showed two plus and four plus tuberculin reactions. The second group comprised those guinea pigs in which the loss of weight had been so great that it was estimated that resistance was failing rapidly. They were tested with the usual amount of tuberculo-protein, 0.1 mg. of MA-100, and were either entirely negative or gave a one plus reaction. When these animals were injected with the dye it spread faster than in the normal animal (Table I).
It is of interest to know whether such a restriction of spread of injected materials is present in any other infection besides tuberculosis. Three guinea pigs which had a spontaneous infection diagnosed as the epizootic lymphadenitis due to a Streptococcus infection, Lancefield type C, both by palpation of the enlarged lymph nodes and by four plus reactions with the bacterial extract prepared by Dr. Moen, were injected with dye. The results are shown in Table I. A comparison with the corresponding injections in normal guinea pigs shows a significant restriction of the dye but not as great as in tuberculous animals.

DISCUSSION

We have found that a vital dye injected into the skin of guinea pigs made allergic by two different infections, namely, tuberculosis and epizootic lymphadenitis, has a significantly slower rate of spread than in normal animals. The skin of animals in the hypoergic and anergic state of advanced tuberculosis, on the other hand, allows a significantly faster rate of spread of dye than the skin of normal guinea pigs.

It is clear from our experiments that at least two different processes are involved in this restriction to the spread of dye. They are a change in the rate of diffusion in the spaces of the connective tissues and a decrease in the permeability of the endothelium of the lymphatic capillaries. That there is a difference in the rate of diffusion can be seen by comparing the photographs of the spread of dye in the normal and in the tuberculous guinea pigs 4 hours after injection. In the normal skin the edge of the area showing dye is hazy, while in the tuberculous animal the corresponding area is as sharp as a knife's edge. The difference in drainage through the lymphatics into the blood stream is shown in the fact that when as much as 0.1 cc. of dye was injected, as in the four injections in the ventral region, the dye had spread throughout the normal animal in 24 hours enough to stain all the mucous membranes, whereas in the tuberculous animal not enough had drained into the blood vessels to be seen away from the place of the injection. This observation indicates one factor in the changed reactions of an animal in the allergic state has to do with permeability of endothelium. It is interesting to note that the work of Duran-Reynals (11) and of Hoffman and Duran-Reynals...
(12) shows an involvement, but in reversed direction, of these same two factors in response to the injection of a spreading factor obtained from tissue extracts. They found that the injection of the spreading factor with dye was followed by an increased diffusion through the tissues, which could be demonstrated even in the skin which had been removed from the body. When they injected the spreading factor into the skin and the dye into the vessels, it was clear that the permeability of the vessels had increased because dye was concentrated into the area which had received the spreading factor. Thus this tissue extract, that is, spreading factor, brings about a condition which is the reverse of the situation which exists in the allergic state of tuberculous infection. These reactions of the skin in this allergic state are measured by a non-specific dye and hence cannot be related to an antigen-antibody reaction, though, as Opie (4) has shown, the antigen with which an animal has been sensitized is also restricted at the point of injection.

Our understanding of this complex phenomenon is far from satisfactory. There is a slight tendency toward edema when the dye is injected into the skin of the tuberculous animal. This is barely perceptible and can only be seen when closely observed and compared directly with the corresponding reaction in the normal animal. Parsons and McMaster (9) have shown that in a non-sensitized animal the spread of dye is increased while edema is forming. In the tuberculous animal, on the other hand, the development of edema does not counteract the tendency toward restriction. Thomas and Duran-Reynals (13) have shown that when the extent of the dermal reaction of a tuberculous animal to a test with a given amount of tuberculin is known, the addition of spreading factor to the same amount of protein markedly diminishes the reaction. When dye is added to the spreading factor, it can be noted that the spreading factor has created a condition which results in dilution and spread of the injected material. Guinea pigs in the advanced stages of tuberculosis, when they have become anergic to tuberculin, have lost the power of restricting the spread of dye in the skin and react rather as does the normal animal after receiving spreading factor.

From these observations it seems very probable that this phenomenon of restriction of spread of materials injected into the skin
in the allergic state may be one factor in the demonstration of the characteristic skin reactions. It may be that the increased irritability of the tissues and the changed permeability of the endothelium of the vessels have some share in concentrating the material injected, whether it is a specific, i.e., antigen, or a non-specific irritant at the site of injection. Any concentration of an antigen around sensitized cells would enhance their reaction. The demonstration of a changed reaction toward diffusion in the tissues and a changed amount of drainage into the vessels by the use of a wholly non-specific dye material, combined with the fact that such changes can be induced, even though in the reverse direction, with tissue extracts (Duran-Reynals), indicates the possibility of the presence of some general as well as specific factors in the allergic state. It has thus been shown that infection with tuberculosis in guinea pigs induces changes which are manifested as an altering of the rate of diffusion in the connective tissues and of the rate of absorption through the vessels; these changes can be demonstrated not only by specific antigens but also by non-specific materials.

CONCLUSIONS

1. In the skin of the tuberculous guinea pig while it is allergic, the spread of a vital dye, pontamine sky blue, and the drainage of the dye into the vascular system take place much more slowly than in the normal animal.

2. In the skin of moribund tuberculous guinea pigs, animals no longer allergic, dye spreads more rapidly than in the normal animal.

3. The spread of dye is somewhat restricted in the skin of guinea pigs infected with a hemolytic Streptococcus. The animals were allergic.

4. The findings suggest that the dye method may disclose altered tissue conditions in the allergic state.

BIBLIOGRAPHY


EXPLANATION OF PLATE 10

Fig. 1. Spread of pontamine sky blue in skin of normal guinea pig, No. R 5324, 1 hour after injection of 0.025 cc.
Fig. 2. Spread of dye in skin of tuberculous guinea pig, No. R 5266, 1 hour after injection as in Fig. 1.
Fig. 3. Spread of dye in skin of normal guinea pig, No. R 5378, 4 hours after injection as in Fig. 1.
Fig. 4. Spread of dye in skin of tuberculous guinea pig, No. R 5266, 4 hours after injection as in Fig. 1.
Photographed by Joseph B. Haulenbeck

Joyner and Sabin: Vital dye in skin of tuberculous guinea pigs