# Protease Changes in Transplanted Skin.* (30976)

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Graft rejection is recognized as the result of an antigen-antibody reaction. The precise mechanism by which the reaction brings about rejection of homologous or heterologous tissue remains, however, unknown. There is a growing tendency to assume that the antigen-antibody reaction elicits the allergic response through a sequence of biochemical events among which the activation of cellular and extracellular proteases may occupy an important place(1,2,3). This paper reports some changes observed in the protease content of skin homografts and autografts during a period of 16 days after transplantation.

*Methods and material.* Adult male rats received fitted whole skin grafts either from individuals belonging to genetically distinct strains or from themselves. Transplantation was carried out according to the procedure described by Billingham(4). After anesthesia with Nembutal (40 mg/kg), the hair of the back of the recipients and donors was removed with electric clippers and the skin washed with mild antiseptics. Pinch grafts, 10 to 15 mm in diameter, were removed from the donors, freed from their muscular layer and placed on the beds prepared in the thoraco-dorsal region of the recipients. The grafted area was then covered with sterile tulle gras, a compressive gauze bandage and a plaster cast.

In a first series of experiments, each animal received 2 autografts and 2 homografts. After intervals varying from 2 to 16 days, they were killed by decapitation. The grafts were removed, as well as a 3 to 4 mm ring of surrounding tissue and a sample of skin outside the grafted area.

In the second group of experiments, each rat received 6 homografts or 5 homografts and 1 autograft. At intervals of 2 or 3 days, one graft was removed from each animal under anesthesia. On the 15th or 16th day, all animals were sacrificed.

After their removal, the grafts and other tissue samples were placed into saline, freed of dead tissue, dried between filter paper and minced with fine scissors into fragments of 1 mm square or less.

Protease activity of the tissue was determined by the method previously described (2) using synthetic amino acid ester substrates. On hydrolysis, the $CO_2$ displaced by the carboxyl from bicarbonate is measured manometrically. The weighed amounts of minced skin are suspended in Warburg flasks containing saline and appropriate amounts of an $NaHCO_3$ buffer. The substrates, 0.3 ml of a 0.05 M solution, are placed in the side arm of the flask. The flasks are attached to the manometers, placed in a 37°C water bath and gassed with a mixture of 95% $N_2$ and 5% $CO_2$. The reaction starts with the tipping in of the substrate and readings are made for 60 minutes. Results are expressed in terms of $\mu$moles of substrates hydrolyzed per hour per g of fresh weight of tissue.

Preliminary experiments showed that within the pH range used (6.0 to 8.2), two substrates were hydrolyzed by the skin preparations: N-acetyl-L-tyrosine ethyl ester (ATyEe) and tosyl-L-arginine methyl ester (TAMe), purchased from Mann Research Laboratories, Inc., New York. The optimum pH for both substrates was found to be 7.7.

The following strains of rats were used: Sprague-Dawley (from Cheek-Jones, Houston, Texas), pure bred Wistar, F344 and F18 (from Microbiological Associates, Washington, D. C.).

*Results.* Protease activity of the normal skin of the thoraco-dorsal region of the strains of rats used is shown in Table I. It is seen that the enzyme acting on ATyEe has a considerably higher activity than the protease which hydrolyzes TAMe. The differences between the values obtained with dif-

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TABLE I. Hydrolysis of Synthetic Substrates by Normal Rat Skin.

| Strains | ($\mu$moles/h/g) TAMe ± S.D. | ATyEe ± S.D. | N |
|---|---|---|---|
| Wistar | 5.4 ± 1.25 | 183.9 ± 34.8 | 9 |
| Sprague-Dawley | 5.7 ± 1.2 | 165.7 ± 23.8 | 9 |
| F 344 | 5.8 ± 1.6 | 191.5 ± 19.6 | 3 |
| F 18, brown | 5.8 ± 1.6 | 166.2 ± 40.6 | 3 |
| Mean | 5.5 ± 1.2 | 175.5 ± 29.4 | 24 |

ferent strains were not greater than the individual variations.

In the first series of experiments, skin from Wistar donors was transplanted to Sprague-Dawley recipients. When the animals were killed, at the stated intervals, the 2 homografts were pooled and their protease activity, determined on both substrates, was compared with that of the 2 pooled autografts, as well as with the action of the skin surrounding the grafts.

The results are shown in Table II. It is seen that enzymic activity on both substrates exhibited marked deviations from that measured in normal skin. Hydrolysis of ATyEe by both homografts and autografts decreased by about 50% at an early stage and later tended to return towards the normal level. Enzymic action on TAMe increased up to 4- or 5-fold in the homografts and slightly but significantly in the autografts. All these modifications were strictly limited to the grafts; the ring of skin immediately surrounding them remained within the normal range.

In a second series of experiments (Table III) each recipient received 6 homografts or 5 homografts and 1 autograft. The recipients were Sprague-Dawley or Wistar and the donors Wistar, F344 or F18 brown rats. By taking several transplants at varying intervals from each rat, it was hoped to reduce individual variations but, in this respect, the experiment was not entirely successful probably because of local differences in enzyme content. The results of the first experiment were, however, confirmed: the TAMe-hydrolyzing enzyme increased in the homografts, particularly after the 5th day and the protease acting on ATyEe showed low values both in homografts and autografts as early as 2 days after transplantation.

Table IV shows the mean values of all the experiments grouped by intervals of 4 days. It is seen that the TAMe-hydrolyzing enzyme increased by almost 300% with a maximum rise between 5 and 8 days. In the autografts, the rise was slight but consistent. The protease acting on ATyEe was reduced in both groups to approximately the same extent.

*Discussion.* Although the role of a proteolytic process in homograft rejection has been assumed(5), the experiments just described probably represent the first demonstration that proteases do actually undergo a change in transplanted tissue.

Before speculating on the significance of these changes it had to be ascertained that they were not the result of some experimental artifact. For example, a change in water content of the tissues could be responsible for the observed protease values. In 22 normal rats the skin of the thoraco-dorsal region contained 68.7 ± 1.2% water. In autografts, the water content was increased from the earliest observations (2 days) throughout the duration of the experiment to 72.5 ± 1.4%. In homografts, the level was even higher for the first 10 days (74.2 ± 1.4%) but afterwards it declined rapidly and in the 16-day samples was below the normal level (65.2%). These differences are consistent and statistically significant but they are too small to account for the changes in enzyme activity.

It was also conceivable that these changes were due simply to the removal of the skin with all the subsequent changes taking place in non-irrigated tissues. Samples of skin were therefore kept under sterile conditions in Petri dishes on filter paper moistened with saline at room temperature (25°C) and in the refrigerator (2°C). One sample was taken every day for 10 days for protease determinations. In spite of small fluctuations, no significant changes occurred. At 25°C, the mean value of TAMe hydrolysis was 5.3 ± 1.5 $\mu$mole/h/g, for ATyEe it was 171.0 ± 39.5. At 2°C, the values were respectively 4.2 ± 1.4 and 164.4 ± 37.8.

It seems, therefore, probable that the modi-

TABLE II. Protease Activity ($\mu$moles/h/g) of Grafts and Surroundings at Various Intervals After Transplantation (1st Series).

| Days | TAMe | | | | ATyEe | | | |
|---|---|---|---|---|---|---|---|---|
| | Homografts | | Autografts | | Homografts | | Autografts | |
| | **Graft** | Surr.skin | Graft | Surr.skin | Graft | Surr.skin | Graft | Surr.skin |
| 2 | - | - | - | - | 96.2 | 157.0 | 109.8 | 148.0 |
| 3 | 9.15 | 4.9 | 5.8 | 5.45 | - | - | - | - |
| 4 | 10.0 | 5.0 | 9.4 | 5.1 | 61.3 | 122.5 | 91.2 | 105.0 |
| 5 | - | - | - | - | 65.0 | 138.5 | 84.8 | 100.5 |
| 5 | - | - | - | - | 68.8 | 143.5 | 48.3 | - |
| 6 | 22.5 | 9.5 | 8.5 | 6.0 | 55.0 | 137.5 | 51.5 | 136.0 |
| 7 | - | - | - | - | 50.3 | 171.5 | - | 179.0 |
| 7 | - | - | - | - | 54.0 | - | 110.5 | 198.5 |
| 7 | - | - | - | - | 56.2 | 105.5 | - | 196.0 |
| 8 | 15.6 | 7.0 | 11.3 | 6.7 | - | - | - | - |
| 8 | 29.8 | - | 9.4 | 5.8 | 93.0 | 120.0 | 57.0 | 160.0 |
| 9 | 23.8 | 7.2 | 10.9 | 6.9 | 147.5 | 260.0 | 69.4 | 202.0 |
| 10 | 10.25 | 6.4 | 9.15 | 6.2 | 44.3 | 187.0 | 59.3 | 165.0 |
| 11 | 9.1 | 7.0 | 8.6 | 4.35 | 40.2 | 164.0 | 78.2 | 175.0 |
| 11 | 11.8 | 7.3 | 7.3 | 4.8 | 46.7 | 215.0 | 123.0 | 172.5 |
| 12 | 14.5 | 5.8 | 6.6 | 4.5 | 134.0 | 172.0 | 126.5 | 136.0 |
| 13 | 12.2 | 6.8 | 7.9 | 3.9 | 77.7 | 195.5 | 107.0 | 211.0 |
| 14 | 8.3 | 6.3 | 8.1 | 5.8 | 64.0 | 201.0 | 111.5 | 164.0 |
| 16 | 8.3 | 6.3 | 6.0 | 8.4 | 57.7 | 101.0 | 159.0 | 125.0 |
| 16 | 27.8 | 8.4 | 8.6 | 8.8 | 47.0 | 170.5 | 106.0 | 126.5 |
| Mean | 15.2 | 6.8 | 8.4 | 5.9 | 70.0 | 162.5 | 93.2 | 158.8 |
| ±S.D. | 7.0 | 2.8 | 1.6 | 1.4 | 24.9 | 41.6 | 28.9 | 32.2 |
| N | 14 | 13 | 14 | 14 | 18 | 17 | 16 | 17 |
| P | | <.001 | <.01 | <.01 | | <.001 | | <.001 |

TABLE III. Protease Activity ($\mu$moles/h/g) of Homografts at Various Intervals After Transplantation (2nd series).

| Donor | Rcpt | Days | TAMe | ATyEe | Donor | Rcpt | Days | TAMe | ATyEe | Donor | Rcpt | Days | TAMe | ATyEe |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| W | S.D. | 4 | 4.45 | 70.0 | F344 | W | 4 | 10.3 | 92.0 | F18 | W | 3 | 7.1 | 68.6 |
| | | 6 | 36.0 | 106.0 | | | 7 | 18.5 | 107.3 | | | 5 | 11.2 | 114.4 |
| | | 8 | 17.2 | 63.7 | | | 10 | 17.1 | 107.4 | | | 7 | 9.4 | 122.6 |
| | | 10 | 11.5 | 118.5 | | | 14 | 10.2 | 82.1 | | | 12 | 12.4 | 171.5 |
| | | 12 | 12.8 | 103.8 | | | 16 | 20.3 | 173.5 | | | 14 | 12.7 | 119.5 |
| | | 14 | 6.6 | 63.7 | F344* | W | 2 | 8.1 | 92.0 | | | 16 | 10.1 | 158.0 |
| W | S.D. | 4 | 5.4 | 46.0 | | | 5 | 11.4 | 105.0 | F18 | W | 4 | 12.0 | 104.0 |
| | | 6 | 16.1 | 106.0 | | | 8 | 13.2 | 132.5 | | | 6 | 37.9 | 82.8 |
| | | 8 | 15.6 | 80.2 | | | 12 | 16.0 | 97.2 | | | 8 | 19.5 | 60.2 |
| | | 10 | 11.6 | 94.4 | | | 15 | 7.5 | 88.4 | | | 12 | 42.5 | 177.0 |
| | | 12 | 10.8 | 76.3 | F344* | W | 2 | 7.3 | 116.0 | F18 | W | 4 | 12.6 | 129.0 |
| | | 14 | 12.0 | 87.0 | | | 5 | 9.4 | 202.5 | | | 6 | 19.7 | 77.0 |
| F344 | W | 5 | 85.7 | 95.7 | | | 8 | 16.4 | 133.8 | | | 8 | 17.3 | 64.7 |
| | | 7 | 45.5 | 46.0 | | | 12 | 12.9 | 104.2 | F18 | W | 4 | 13.1 | 103.5 |
| | | 9 | 13.6 | 152.0 | | | 15 | 17.9 | 111.3 | | | 6 | 11.8 | 65.3 |
| | | 11 | 10.2 | 71.5 | F344* | W | 2 | 3.7 | 106.3 | | | 8 | 12.1 | 63.8 |
| | | 13 | 13.2 | 113.6 | | | 5 | 5.6 | 142.0 | | | 12 | 16.6 | 132.0 |
| F344 | W | 5 | 10.6 | 70.4 | | | 8 | 12.2 | 87.0 | F18 | W | 4 | 10.2 | 105.0 |
| | | 7 | 64.2 | 52.7 | | | 12 | 11.6 | 136.8 | | | 6 | 10.9 | 91.0 |
| | | 9 | 56.0 | 126.2 | | | 15 | 21.1 | 80.0 | | | 8 | 20.7 | 176.0 |
| | | 11 | 33.3 | 57.3 | F344* | W | 2 | 4.8 | 53.0 | | | 12 | 17.1 | 182.5 |
| | | 13 | 17.1 | 113.8 | | | 5 | 5.7 | 113.0 | F18 | W | 4 | 6.1 | 92.0 |
| F344 | W | 5 | 4.6 | 59.2 | | | 8 | 14.9 | 131.2 | | | 7 | 20.4 | 154.5 |
| | | 7 | 14.7 | 138.6 | | | 12 | 12.1 | 87.2 | | | 10 | 16.9 | 186.3 |
| | | 9 | 21.4 | 90.3 | | | 15 | 15.9 | 92.6 | | | 14 | 24.8 | 156.8 |
| | | 11 | 21.8 | 139.2 | F18 | W | 3 | 10.6 | 124.2 | | | 16 | 25.6 | 186.0 |
| | | 13 | 33.0 | 190.0 | | | 5 | 14.6 | 119.6 | F18 | W | 4 | 9.8 | 90.0 |
| | | 15 | 27.7 | 179.0 | | | 7 | 38.6 | 134.0 | | | 7 | 22.4 | 116.5 |
| F344 | W | 5 | 38.0 | 89.3 | F18 | W | 3 | 3.45 | 65.1 | | | 14 | 23.7 | 108.2 |
| | | 7 | 14.1 | 123.5 | | | 5 | 11.3 | 102.3 | | | 16 | 21.0 | 179.0 |
| | | 9 | 22.7 | 176.0 | | | 7 | 18.2 | 91.2 | | | | | |
| | | 11 | 26.9 | 116.5 | | | 12 | 18.3 | 139.5 | W = Wistar; S.D. = Sprague-Dawley; | | | | |
| | | 13 | 18.3 | 156.0 | | | 14 | 12.3 | 89.5 | F344 = Fisher 344 (white); F18 = Fisher | | | | |
| | | 15 | 15.1 | 216.0 | | | 16 | 7.8 | 127.5 | 18 (brown). | | | | |

* These animals also received one autograft, tested on day 15 and incorporated in the mean values shown in Table 4.

TABLE IV. Mean Protease Activity in Grafts at 4-Day Intervals.

| Interval (days) | TAMe | | | | | | ATyEe | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Homografts | ±S.D. | N | Autografts | ±S.D. | N | Homografts | ±S.D. | N | Autografts | ±S.D. | N |
| 1- 4 | 8.2 | 2.2 | 18 | 7.6 | — | 2 | 87.3 | 23.8 | 18 | 100.5 | — | 2 |
| 5- 8 | 20.8 | 15.8 | 40 | 9.7 | 1.4 | 3 | 92.1 | 36.7 | 44 | 70.4 | 26.7 | 5 |
| 9-12 | 18.2* | 10.5 | 28 | 8.5 | 1.7 | 5 | 116.3 | 42.9 | 28 | 91.3 | 31.3 | 5 |
| 13-16 | 16.5* | 7.1 | 26 | 7.5 | 2.0 | 8 | 119.8 | 46.4 | 26 | 109.7 | 29.1 | 8 |

All values are significantly different from normal skin.
* Significantly different from autografts.

fications observed in protease activity are due to the contact with a host organism. We have no explanation for the decrease in activity of the ATyEe-hydrolyzing enzyme. Since it takes place to the same extent and at the same time in both homografts and autografts, it is unlikely to have anything to do with the mechanism of rejection. It may have some role in the initial establishment of vascular connections between the host and the graft but this remains to be proved.

The increase in TAMe-hydrolyzing enzyme is considerably more marked in homografts than in autografts. Its augmentation precedes the first macroscopic signs of rejection which were observed 9.0 ± 2.2 days after transplantation. It seems, therefore, possible that this protease is somehow involved in the rejection process. A similar enzyme was shown to be activated by the antigen-antibody reaction in guinea pig lung and was found in the urine of the animals in anaphylactic shock(2).

There are many cellular and extracellular proteases which act more or less selectively on TAMe (trypsin, plasmin, thrombin, plasma kallikrein, C'1 "esterase," neutral cathepsins extracted from various tissues, etc.) so that this substrate specificity does not allow the identification of the enzyme. It is not certain that the additional protease activity observed in grafts represents an increase in the enzyme which is normally present in skin. It may result from the appearance of a new protease having the same substrate affinity.

There are two main possibilities for the origin of the TAMe-hydrolyzing enzyme: a) it may result from the activation of a precursor present in the graft either as a proenzyme or as an enzyme-inhibitor complex; b) it may come from the host when the graft is revascularized. In the latter case, it is probably brought in with the leukocytes of the host. If it were in the plasma, it would probably diffuse outside the limits of the graft.

Some of the questions just raised can probably be answered when the enzyme is extracted and isolated from the tissue. The main problem, that of the role of the protease in graft rejection, is being approached by measuring graft survival in animals treated with protease inhibitors.

*Summary.* Protease activity of rat skin homografts and autografts was estimated at varying intervals for 16 days after transplantation. An enzyme hydrolyzing ATyEe was considerably decreased throughout the period in the autografts as well as in the homografts. Another enzyme, acting on TAMe, showed a marked increase in the homografts beginning on the 5th or 6th day; it was slightly elevated in the autografts. The possibility of this enzyme taking part in homograft rejection is being further investigated.

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