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BLOOD FLOW, HISTAMINE CONTENT AND HISTIDINE DECARBOXYLASE ACTIVITY IN RAT SKIN GRAFTS AND THEIR MODIFICATION BY CYCLOSPORIN-A

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1 An attempt has been made to monitor simultaneously the changes in blood flow, histamine content, histidine decarboxylase (HDC) activity and mast cell population in rat skin isografts and allografts.

2 The histamine content in rat skin allografts during rejection was decreased in contrast with our earlier observation in rabbits.

3 Although no definite correlation has been found between blood flow changes, histamine content or HDC activity, a reciprocal relationship appeared to exist between histamine content and HDC activity in the skin grafts, which might be a reflection of the immunosuppressive activity of this amine.

4 The immunosuppressive agent, cyclosporin-A (20 mg/kg daily) was able to prolong skin allograft survival and prevent the changes in histamine and HDC activity in allografts.

5 Possible implications of these findings are discussed in relation to current knowledge concerning the interactions between endothelial cells, lymphocytes and mast cells/basophils in graft rejection.

Introduction

Graft rejection is a delayed hypersensitivity reaction in which a number of mediators and cell types are involved. Furthermore, distinct vascular changes occur during the normal course of skin graft establishment and rejection. The sequence of these vascular events can be summarized in three phases: (1) initial restoration of blood flow from the host into the donor graft, (2) increased blood flow, and (3) return of normal blood flow in autografts/isografts or a vascular shutdown in allografts. We have been investigating the possibility that these vascular changes are mediated by vasoactive agents formed and released at the site of the graft. Subsequently it might be possible to devise specific antagonists or inhibitors to prevent the vascular shutdown and help to create a host environment capable of immunological adaptation to an allograft. Previously we studied the blood flow changes and the concomitant changes of certain chemical mediators in rabbit skin grafts (Lewis & Mangham, 1978). In the present series of experiments we have extended those original findings further by using the rat. With inbred rats it was possible to obtain a consistent pattern of rejection of allografts and to use isografts to monitor the non-immune components of the reaction. In addition, groups of rats receiving allografts were treated with an immunosuppressive agent, cyclosporin-A (CSA), so that comparisons could also be made between isografts and untreated allografts as well as allografts treated with CSA.

Methods

Skin graft procedure

In the previous experiments, outbred rabbits were used as inbred rabbits were not available. In the present experiments outbred rats were studied initially in order to complement the work in rabbits. However, considerable variability was encountered and in subsequent experiments male inbred rats of WAG ($RT1^u$) and DA ($RT1^a$) strains were used. The rats weighing 150 – 200 g were anaesthetized with 0.25 ml/kg Hypnorm (0.315 mg/ml fentamyl citrate and 10 mg/ml fluanisone, Janssen). Full thickness skin grafts (2 cm × 2 cm) were removed from the abdomen of donor rats and transplanted onto dorsal graft beds of the recipients by means of interrupted 5/0 silk suture. Each rat received either two isografts (WAG→WAG) or two allografts (DA→WAG). Since these rats differ at their major histocompatibility complexes, allografts were always rejected while isografts were invariably accepted.

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Blood flow measurement

Blood flow measurements were carried out on conscious rats restrained in a modified Bollman cage by using a modified ^{133}Xe clearance technique (Fan & Lewis, 1981). The room temperature was kept constant at $20 \pm 1°C$. To minimize damage to the grafts during blood flow measurements, a micro-injection of $10\,\mu l$ ^{133}Xe in saline was made intradermally to each graft on alternate days. Instead of deriving the clearance constants from the washout curves of ^{133}Xe (Lewis, Peck, Williams & Young, 1976) which would involve continuous monitoring for up to 45 min, the percentage clearance after 6 min was taken as an estimate of blood flow. As previously described, the correlation coefficient between the values obtained by the two techniques, was $r = 0.982$ (Fan & Lewis, 1981). Therefore, the data obtained from the simplified technique provided rapid and reliable measurements.

Preparation of samples for histamine and histidine decarboxylase activity

At various times after grafting, rats were killed by cervical dislocation and the graft with subcutaneous tissue was excised and immediately frozen on dry ice. During the first few days after transplantation, the graft could readily be lifted from its wound bed. At later stages, it had to be dissected free. Therefore grafts were always carefully removed with the subcutaneous tissue so that meaningful comparisons could be made. The grafts were weighed after removal and stored at −20°C until they were extracted. Samples of normal ungrafted abdominal skin were stored and treated in the same manner as the grafts. In order to prepare extracts, the tissues were thawed, suspended in 5 ml of ice-cold phosphate-buffered saline (PBS) and homogenized at 4°C with a pestle and mortar, a small amount of cleaned sand being added to facilitate the procedure. The pestle and mortar were washed with a further 2 ml of PBS and the washings added to the homogenate. The homogenates were centrifuged at 30,000 *g* at 4°C for 20 min. The clear supernatant fluids thus obtained were divided into 1 ml aliquots in plastic tubes, capped and stored at −20°C until they were assayed. These samples were assayed direct, with no further purification.

Assay of histamine content

Each sample was assayed for histamine on strips of guinea-pig ileum smooth muscle with mepyramine (14.1 mg/l) being added to the perfusate in the later part of the bioassay to confirm the identity of histamine. Histamine content was further quantified fluorometrically with an Aminco-Bowman spectrophotofluorometer set at the wavelengths of 350 nm (excitation) and 440 nm (emission), using the method of Shore, Burkhalter & Cohn (1959).

Assay of histidine decarboxylase (HDC) activity

The HDC activity was determined by a method modified from Beaven, Wilcox & Terpstra (1978). The reagent mixture consisted of the following L-[carboxyl-^{14}C]-histidine (2 μCi/ml; specific activity: 51 mCi/mmol, Radiochemical Centre, Amersham), L-histidine (5×10^{-4} M) and pyridoxal phosphate (10^{-5} M) in PBS. A small volume (250 μl) of the samples was preincubated at 37°C in an Eppendorf tube placed inside a Packard scintillation counting vial. The reaction was started by adding 50 μl reagent mixture to the sample and the vial tightly closed and kept at 37°C for one hour. The reaction was terminated by injecting 100 μl of 2 M perchloric acid into the Eppendorf tube. The radioactive CO_2 thus released was absorbed in the 2 ml of hyamine hydroxide (Sigma) inside the counting vial. Finally, the Eppendorf tube was removed and discarded. Toluene (10 ml) was added to each vial and the radioactivity counted with a Packard Tri-Carb B2450 liquid scintillation spectrometer. Reagent blanks were included in each assay and the background counts subtracted from those of the samples. Values in ct/min were converted to d/min so that the HDC activity could be expressed in terms of pmol $^{14}CO_2$ released per g tissue and per hour (pmol $g^{-1}\,h^{-1}$).

Estimation of mast cell number in grafts and normal skin

Representative samples (0.5 cm × 0.5 cm) of skin grafts were taken from the centre of excised grafts and fixed in 8% formal saline. Transverse sections (7 μm thick) were cut so that a semi-serial series at 100 μm distance apart was obtained. The histological sections were stained with haemotoxylin and eosin (H & E) or toluidine blue. Since mast cells are not randomly distributed and it is difficult to distinguish between degranulating and regranulating mast cells, for practical purposes, cells showing metachromasia when stained with toluidine blue were counted in the whole section. For each sample of grafts at different days, at least fifty sections were taken and the mast cells counted.

Drug treatment

Cyclosporin-A (CSA, Sandoz) was dissolved in olive oil at 60°C for 2 h. Initial experiments established that a drug regimen (starting one day after grafting) of 20 mg/kg per day for 14 days was able to prolong allograft survival to about 30 days. In further experiments to study the time course of mediator changes

modified by CSA, this dose was used. CSA or the vehicle was administered intramuscularly to each leg on alternate days. Drug treatment was stopped on day 3, 5, 7, 9 and 12 so that comparisons could be made with isografts and untreated allografts.

Results

Blood flow changes

When the percentage ^{133}Xe clearance after 6 min was measured in the normal skin of all rats used in this study, the results were found to lie within the range of 65–72% as shown in Figure 1. In both isografts and allografts, blood flow was established between day 2 to 4 during which time the ^{133}Xe clearance increased from near zero to the normal range. After day 5 the changes in isografts and allografts were different. In isografts the blood flow increased to a plateau above the normal level and gradually returned to normal around day 12. In allografts the blood flow increased to a peak around day 6–7 after which it fell and ceased altogether around day 9. At this time there were no visible signs of rejection and even later, colour changes were not detectable in the dark skin allografts of DA rats.

A group of rats ($n = 6$) receiving allografts and treated with CSA (20 mg/kg) for 14 days showed no sign of rejection for more than 30 days. When the blood flow was examined in this group, the changes resembled those observed in isografts rather than untreated allografts (Figure 1).

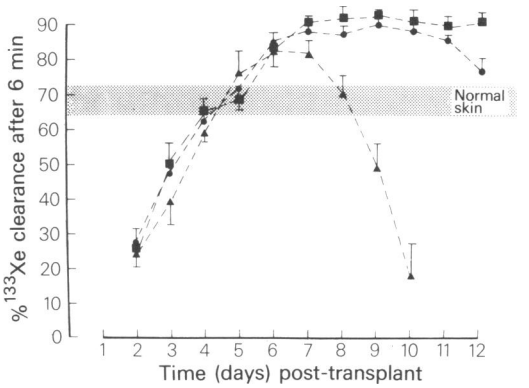

Figure 1 Blood flow changes in rat skin isografts (●), allografts (▲) and allografts treated with cyclosporin-A (20 mg/kg daily, ■) plotted against number of days after grafting. Each point is the mean of $n = 6$; vertical lines show s.e.mean. No blood flow could be detected before day 2. Normal blood flow was established in all grafts by day 5. In isografts the blood flow increased forming a plateau between day 7 to 11 and subsequently decreased to normal skin blood flow. In allografts, the plateau of active blood flow was maintained for 48 h only after which it fell and ceased altogether around day 9. The blood flow pattern of the CSA-treated group resembled that observed in isografts rather than untreated allografts.

Histamine content of grafts

Histamine content of the grafts has been expressed as a percentage of that of normal ungrafted abdominal skin (DA = 48 ± 5 μg/g; WAG = 39 ± 2 μg/g, $n = 6$). The changes in histamine content in both isografts and allografts, shown in Figure 2, decreased to about 60% two days after grafting. In isografts the histamine content returned to normal 7 to 9 days after grafting. In the untreated allografts the histamine content continued to fall until at the time of rejection it was only 30% of normal, whereas in the CSA-treated animals, the histamine content gradually returned to levels found in normal animals over the same period. Therefore, the changes in histamine content in allografts of the CSA-treated group closely resembled those of isografts.

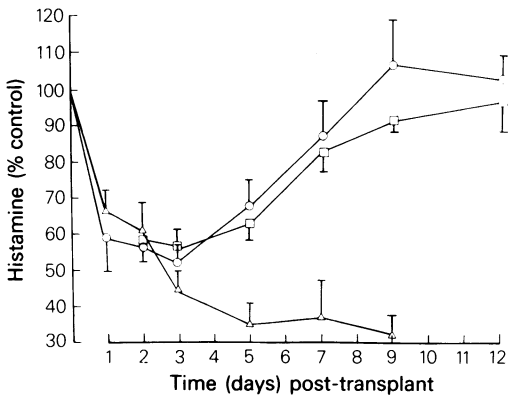

Figure 2 Histamine content of rat skin isografts (○), allografts (△) and allografts treated with cyclosporin-A (CSA, □), as % of that of normal ungrafted abdominal skin. (Isograft control = 39 ± 2 μg/g; allograft control = 48 ± 5 μg/g; $n = 6$). Each point represents the mean of at least 6 samples; vertical lines show s.e.mean. Initial fall of histamine content in all types of grafts was observed during the first three days after grafting and corresponds with massive mast cell degranulation. In isografts, histamine content recovered to that of normal in 7 to 9 days. In allografts, histamine content remained low throughout the rejection process. The changes of histamine content in the CSA-treated allografts resembled those in isografts.

Histidine decarboxylase (HDC) activity of grafts

As histamine is formed by the decarboxylation of L-histidine, we have assayed the HDC activity in the graft homogenates. The results are expressed in terms of pmol $^{14}CO_2$ released g tissue^{-1} h^{-1} as shown in Figure 3. In isografts the HDC activity increased from approx. 90 pmol before grafting to 420–460 pmol by day 3–5 when the healing-in pro-

cess was in progress. After day 5, the HDC activity in isografts gradually decreased to normal by day 12. The HDC activity in allografts also increased from 106 pmol before grafting to 398–420 pmol during the healing-in phase. This was followed by a further rise to a maximum HDC activity of about 700 pmol at the time when the allograft blood flow ceased (onset of rejection). In the CSA-treated rats, the HDC activity increased from 106 pmol before grafting to 150–390 pmol by day 3–5. However, the late rise in HDC activity observed in untreated allografts was completely prevented by CSA (Figure 3).

Histology and mast cell population of grafts

The skin grafts removed from the abdomen of donor rats comprised the epidermis, dermis and subcutaneous tissue including the panniculus carnosus. During the healing-in phase (day 2–4) the histological changes in both isografts and allografts were similar. By day 8–9, allografts exhibited perivascular inflammatory cell infiltration, haemostasis and interstitial haemorrhage, thus corresponding with the vascular shutdown as determined by the ^{133}Xe clearance technique. In isografts, retrograde differentiation of the tissue and healthy vasculature were observed at this time.

The distribution of mast cells in the rat skin is not uniform; they are more numerous in the subcutaneous tissue than in the dermis and tend to concentrate perivascularly along the panniculus carnosus, as was found by Riley (1959). The changes in mast cell number following transplantation is illustrated in Figure 4. Within 24 h after grafting, massive degranulation of mast cells was observed in both isografts and allografts, and the number of identifiable mast cells fell drastically to a minimum by day 5. In isografts, mast cells appeared to have repopulated by day 9–12 although they were well distributed in the dermis and not concentrated in the panniculus carnosus particularly. On the other hand, there were only a few identifiable mast cells in allografts by day 9; a substantial number of mast cells apparently of host origin were nevertheless located at the junction between the graft and the graft bed. In CSA-treated rats, the histology and the changes in mast cell population were very similar to those of isografts.

Discussion

Previously we have shown that the vasodilatation which occurred during the healing-in of rabbit skin autografts and allografts was associated with the concomitant increase of histamine content in the grafts. Furthermore, the onset of allograft rejection (as indicated by cessation of allograft blood flow)

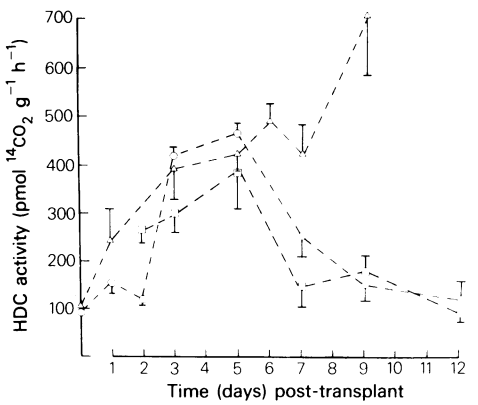

Figure 3 Alterations in the histidine decarboxylase (HDC) activity of rat skin isografts (○), allografts (△) and allografts treated with cyclosporin-A (CSA, □). During the healing-in of all grafts the HDC activity was increased 4 fold above that of normal ungrafted skin. In isografts and CSA-treated allografts, this was followed by a return to basal levels in the next 4–7 days. Allograft HDC activity continued to rise and reached a peak 7 fold elevation by day 9 when the blood flow was diminished.

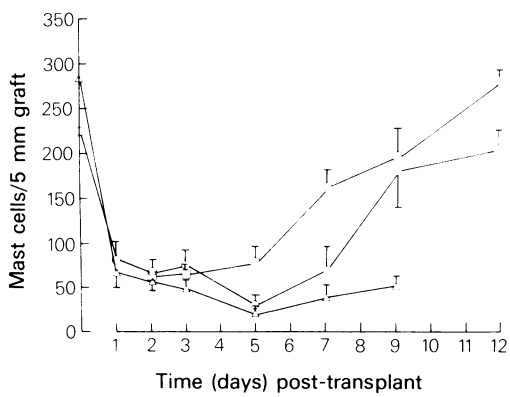

Figure 4 Number of mast cells in rat skin isografts (○), allografts (△) and allografts treated with cyclosporin-A (CSA, □). Number of mast cells per section (5 mm long, 7 μm thick) are plotted against the day after transplantation. Blocks were prepared from at least three animals on each day and at least 50 sections examined from each. Although there are different numbers of mast cells in the two types of skin before transplantation, in all the grafts, there was a significant degranulation and decrease of identifiable mast cells during the initial three days. Isograft control = 225 ± 3; allograft control = 281 ± 14. In isografts and allografts there was a continuous fall in the number of mast cells by day 5. In the next 4–7 days, the number of mast cells gradually recovered in isografts and even somewhat earlier in CSA-treated allografts, and these cells were well distributed in the dermis.

coincided with a peak histamine content (15 μg/g tissue) which was more than three times that in autografts. This concentration of histamine causes vasoconstriction in rabbits. In addition, mepyramine delayed the cessation of allograft blood flow thus suggesting that in rabbits the vasoconstriction was probably mediated by histamine (Lewis & Mangham, 1978).

In the present investigation we have examined this view further using the rat, a species in which histamine does not cause vasoconstriction. When the blood flow changes and histamine content of skin grafts are considered together it appears that there could possibly be a correlation between isograft blood flow and histamine content, as both increased during the healing-in phase, but this is certainly not the case for allografts. Therefore, mediators other than histamine appear to be responsible for the reaction of the microcirculation in allografts. This finding might have been predicted from the known characteristics of the peripheral vascular histamine receptors in the rat. Powell & Shamel (1979) have shown in this species that histamine causes vasodilatation mediated by both H_1- and H_2-receptors. This is in contrast to the situation in rabbits where histamine causes vasodilatation via H_2-receptors and vasoconstriction via H_1-receptors (Parsons & Owen, 1973; Brimblecombe, Owen & Parsons, 1974).

Results from these experiments demonstrate clearly that the changes in histamine content of skin grafts in rats were quite different from those in rabbits, although the vascular events remain basically the same. This can probably be explained by the different distribution of histamine-containing mast cells and basophils in the two species. In rabbits, mast cells are infrequent in connective tissue, while basophils account for about 30% of the circulating leucocytes. Extensive basophil infiltration has been reported in skin allografts in guinea-pigs (Dvorak, 1971). If the same histological changes occur in rabbit skin allografts, they could well account for the increase in the histamine content in skin allografts in rabbits. On the other hand, tissue mast cells are degranulated when the skin is removed from the donor so that in the rat where the histamine is contained in these cells, there is an initial reduction of histamine content in the grafts. In the surviving isografts, but not the untreated allografts, histamine content does return to normal. Similar results had been observed in rat skin autografts by Marckmann & Zachariae (1964). In the untreated allografts, histamine content remained low throughout the rejection process. This was in contrast to the CSA-treated allografts where histamine content returned to normal levels.

When we consider histamine content and HDC activity together, it appears that the level of HDC activity is inversely related to the histamine content, the maximum HDC activity corresponding with the lowest histamine content. This finding raises several questions: Is the elevated HDC activity as measured *in vitro*, a true reflection of increased histamine synthesis *in vivo*? If this is so, where is the histamine formed? Could it be the endothelial cells which are known to have the ability to produce histamine and which might release the amine as it is formed?

The changes in HDC activity are in agreement with the findings of Kahlson, Nilsson, Rosengren & Zederfeldt (1960) that growing tissues in skin wounds in the rat, produced histamine at a high rate as a result of increased HDC activity. The present results are also consistent with an earlier observation of Moore & Schayer (1969) of divergent patterns of HDC activity in rat skin autografts and allografts although no mention was made of the histamine content.

Based on studies on the histidine-histamine relationship *in vivo*, Schayer (1962) proposed that the endothelium is the major site of histamine synthesis within the vascular system under essentially physiological conditions. This premise was supported experimentally by Hollis & Rosen (1972) when it was shown that endothelial histamine-forming capacity was approximately 15 times greater than that of the intima-media. On the other hand, recent studies by Robinson-White & Beaven (1981) have revealed high activity of two histamine catabolizing enzymes, viz., histamine methyltransferase and diamine oxidase, in rat microvascular endothelial cells. Taken together it appears that while the induced histamine may be synthesized in or near vascular endothelial cells, these very cells may also be important sites of inactivation of circulating and newly formed histamine. The high histamine degrading activity of these cells would be consistent with the short half-life of histamine in the circulation (< 30 s, Ferreira, Ng & Vane, 1973) and its rapid clearance during perfusion through various vascular beds in rats and other species (Halpern, Neveu & Wilson, 1959; Ferreira *et al.*, 1973).

Histological studies in both isografts and allografts indicated that there is a parallelism between the histamine content and mast cell population. Consequently in isografts there is recovery of mast cells concomitant with histamine content. Since the divergent changes in the histamine content in isografts and allografts are not directly related to the local blood flow changes, perhaps a clue might be obtained from this model about a possible nonvascular role played by histamine in immunologically-induced inflammatory reactions. Jarosková, Viklicky, Holub, Trebichavsky & Stepánková (1979) in studying the participation of mast cells in murine skin allograft rejection have suggested that an inverse relationship

exists between the presence of mast cells and the extent or efficiency of the immune response.

A recent report has suggested that endothelial cells of the microvasculature are the critical target of immune response in first-set vascularized skin allograft rejection in man (Dvorak, Mihm, Dvorak, Barnes, Manseau & Galli, 1979). Any relationship between this possibility and the high HDC/histamine turnover as observed in our rat skin allograft model remains to be elucidated.

In addition there are several lines of evidence for the interaction of lymphocytes and mast cells/basophils via soluble mediators (Thueson, Speck, Lett-Brown & Grant, 1979a, b; Dy, Lebel, Kamoun & Hamburger, 1981; Nabel, Galli, Dvorak, Dvorak & Cantor, 1981; Shrader, Lewis, Clark-Lewis & Culvenor, 1981). The finding that CSA was able to modify the changes in allografts to those in isografts would lead to the possibility that this drug might interfere with the formation/release of other mediators *in vivo*. Therefore, CSA might well be a useful tool with which to study the role of pharmacological mediators in graft rejection.

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