The Antigenic Structure of Blood Platelets.
II. Histocompatibility Antigens in Rabbit Blood Platelets

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IN PREVIOUS STUDIES it was demonstrated that transplantation of skin homografts in rabbits elicited a significant degree of immunity towards homologous blood platelets later infused.1 2 This demonstration that blood platelets and skin share antigens in common could not, however, lead directly to the conclusion that blood platelets contain histocompatibility antigens. It is known that transplantation immunity is a complex phenomenon during which cellular and humoral reactions are produced and that the humoral factors cannot be clearly identified with the factor(s) which produce accelerated rejection of a tissue subsequently transplanted.3 There is at present no method available by which transplantation immunity can be tested with certainty, other than by acceleration of homograft rejection.

The question as to whether blood platelets contain histocompatibility antigens was the object of the present investigation. The results obtained indicated that the previous intradermal injection of homologous blood platelets in the rabbit could induce shortening of the rejection time of a skin homograft in a significant number of animals.

MATERIALS AND METHODS

In this group of experiments conducted in outbred rabbits, blood platelets were collected from donor rabbits and injected intradermally, on one occasion, in recipient animals in which the rejection time of homologous skin grafts was then determined.

Preparation of Platelet Suspensions Free of Leukocytes

Eighteen ml. of cardiac blood were drawn from the donor animal into 2 ml. of EDTA (1.5 per cent disodium ethylenediamine-tetraacetate in 0.7 per cent sodium chloride) and transferred to test tubes. The blood was centrifuged at 1000 rpm (170 x g) for 20 minutes at 4 C. The supernatant platelet rich plasma was pipetted into another test tube and centrifuged at 1000 rpm for 10 minutes. The supernatant was again transferred to another tube, discarding the sediment, and again centrifuged at 1000 rpm for 10 minutes. This platelet-rich plasma was then centrifuged at 3000 rpm (1650 x g) for 30 minutes. The plasma was poured off, and the platelet button thus obtained was resuspended in 1–2 ml.
0.85 per cent NaCl. Platelet counts were done on this suspension by phase contrast microscopy. The suspension was carefully examined for the presence of leukocytes by two methods: (1) 1.8 mm.², either undiluted or diluted 1:10 with EDTA, were examined directly under phase contrast microscope at 430 x magnification and (2) a drop of the suspension was mixed with a drop of rabbit globulin on a slide, dried, stained with Wright's stain, and examined microscopically for leukocytes. If any leukocytes were seen by either method, the platelet suspension was discarded. Only if no leukocytes were seen was it injected into the recipient animal. At the beginning of these experiments it was necessary to discard approximately one-half of the platelet preparations because of contamination with leukocytes, but later, with increased experience, the percentage of contaminated samples decreased considerably.

Injection of Platelets

(1) Without adjuvant: The saline suspension of platelets was injected intradermally into multiple sites on the shaved skin of the lower back of the recipient rabbits.

(2) With adjuvant: The saline suspension of platelets was mixed with an equal volume of complete Freund's adjuvant, then injected subcutaneously into multiple sites over the posterior thorax of the recipient animals.

All glassware used for collection, separation and injection of the platelets was siliconized.

Skin Homografting

Two weeks after the platelet injection, the recipient animals were challenged with a skin homograft from the platelet donor ("specific" skin) on one ear and one from another rabbit ("nonspecific" skin) on the other ear. Each graft, of full skin thickness, was ovoid in shape and measured 2-3 x 1 cm. in size. In these experiments the onset of graft rejection was taken as basic parameter for measuring tissue sensitization. The onset of rejection was determined by daily gross inspection and was judged by the onset of cyanosis, edema, brown discoloration, dryness, scaling, or partial (usually peripheral) necrosis which progressed to complete necrosis of the graft. Each of these changes was recorded and quantitated on a 0-4 plus basis. Minor changes (1 plus or 2 plus) in any one of these criteria were not considered significant. Two plus changes involving two or more of the above criteria were considered significant of initial graft breakdown, and 1 plus changes in three or more of the parameters were considered significant. Signs of graft breakdown were not considered significant if they were later reversed. The onset of homograft rejection was, thus, determined retrospectively, and as "rejection time" the time was taken at which there appeared definite, significant signs of graft breakdown which then progressed without reversal to complete necrosis of the graft.

RESULTS

By these criteria, the rejection times of skin homografts in 34 non-immunized recipient rabbits ranged from 3 to 8 days with an average of 5.8 days and a standard deviation of 1.1 (fig. 1). In these animals, the onset of rejection as defined above, was usually followed by complete necrosis in one day. By this technic, skin autografts regularly survived for an indefinite period of time.

The rejection times of 12 second-set skin homografts, i.e., in animals previously immunized by a skin homograft, ranged from 1 to 6 days with an average of 2.6 days (fig. 1).

The rejection times of "specific" and "nonspecific" skin homografts in animals previously injected with platelets are presented in table 1 and figure 1. Nineteen animals were so studied. Of these, 11 rejected both grafts within the time limits imposed by two standard deviations on either side of the mean for nonimmunized rabbits. Of the remaining eight rabbits: (1) five re-
REJECTION TIME OF SKIN HOMOGRAPTS IN PLATELET SENSITIZED RABBITS

Fig. 1.—In 42 per cent of the platelet sensitized rabbits, the “specific” skin homografts (2) were rejected significantly earlier than in nonsensitized animals (1) and as rapidly as in animals immunized by a previous skin homograft (4). The “non-specific” skin homografts were rejected sooner than normal in only 10 per cent of the experiments (3).

jected the “specific” homograft rapidly (1–3 days), while the “nonspecific” graft was rejected normally (5–6 days); (2) one rabbit rejected the “specific” graft rapidly, (1 day) while the “nonspecific” graft was maintained for a longer than normal time (13 days); and (3) two animals rejected both grafts rapidly (1–2 days).

The group of animals immunized to platelets also appeared to reject the grafts in a different fashion than nonimmunized controls. In these animals there was not infrequently a period of 2, 3 or 4 days during which the graft exhibited significant symptoms of breakdown before complete necrosis ensued. The mechanism of such changes will perhaps be revealed by studies in progress.

In summary, 42 per cent of the platelet-sensitized animals showed onset of rejection of the “specific” skin graft earlier than non-sensitized animals, with time values below two standard deviations of the mean value for the nonsensitized animals. On the contrary, only 10 per cent of the “nonspecific” skin homografts were rejected sooner than normal (fig. 1).
ANTIGENIC STRUCTURE OF BLOOD PLATELETS II

The addition of Freund's adjuvant to the platelet injection was applied in too small a number of experiments for evaluation at this time.

DISCUSSION

From the above results, the following is apparent:

Intradermal injection of blood platelets from one rabbit to another induced, in a significant number of animals, a degree of immunity towards skin later grafted from the same donor.

A clear, positive result of accelerated rejection of homologous skin grafts after platelet sensitization was obtained in only 42 per cent of the animals. It could be proposed that blood platelets carry transplantation antigens only in small doses. However, the reasons for this partial result may be multiple. In the above experiments, the onset of rejection of skin homografts in nonsensitized animals occurred earlier than in experiments by others. The larger size of skin grafts used may have been responsible for this. It is realized that the brief period of take of the homologous skin grafts in nonsensitized animals was probably an unfavorable condition for the demonstration of a significant shortening of the skin-rejection time in the platelet-immunized animals. Other technical reasons (the number of platelets injected, the single platelet injection given, the site of injection, the time schedule adopted, and the animal species used) may have also influenced the percentage of positive results. Further studies will be necessary to elucidate these points. However, as of now, we believe that the number of positive results obtained was such that they cannot be attributed to chance, and the conclusion is drawn that blood platelets contain histocompatibility antigens.

The occurrence of accelerated rejection of the "nonspecific" skin homograft in two of the recipients which had been injected with platelets was probably a reflection of antigenic overlap. A similar phenomenon of incomplete individual specificity of antigens common to platelets and skin was observed in our previous investigations in which platelet survival studies were done in animals which had been grafted with homologous skin. In one animal, the "nonspecific" skin homograft appeared viable for an unusually long period of time (13 days). Although this occurred in only one experiment, it may be thought that "enhancement" was produced in this animal, suggesting a high titer of humoral antibodies. The "specific" homograft, however, was rejected very rapidly in this animal (table 1).

Further studies on the histology of skin homograft rejection in platelet-sensitized animals are warranted. The possibility that blood platelets and vascular endothelium have antigens in common was repeatedly suggested in the past and it is possible that vascular damage in the skin homografts applied to the platelet-sensitized rabbits may be the prominent feature of the rejection phenomenon.

A relationship between isosensitization induced by blood transfusions and immunity elicited by tissue transplantation has long been suspected. While this relationship was clearly demonstrated for the white blood cells, the question is still debated for the red cells. Blood platelets have never been
Table 1.—Rejection Time of Skin Homografts in Platelet Sensitized Rabbits

<table>
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<tr>
<th>Animal Number</th>
<th>Number of Platelets Injected ($\times 10^9$)</th>
<th>Freund's Adjuvant</th>
<th>Rejection Time (Days)</th>
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<th>Nonspecific graft</th>
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Rejection time in 34 non-sensitized animals. Average value = 5.8 days ± 1.1*

*1 S.D.
†Not included in figure 1.

previously considered in this respect and have been even thought to be devoid of histocompatibility antigens.11

The experiments herein reported also demonstrate that antigens capable of inducing a state of immunity towards skin-homografts—that is, histocompatibility antigens—are not exclusively carried by cell nuclei as originally believed.6,12-14 In fact, blood platelets do not contain a nucleus, and presence of DNA in blood platelets could never be clearly demonstrated.15-20 The precise chemical nature of histocompatibility antigens is, at the present time, not completely defined.24 However, DNA, which was previously believed to be responsible for tissue antigenicity,14 has since been shown to lack such antigenicity25,26 Histocompatibility antigens have been demonstrated to consist of proteins with, possibly, carbohydrate and lipid components.21,25,26 Such substances are, of course, almost universally present in biological materials.

Although in previous experiments by others,26 accelerated homograft rejection was not obtained by sensitization with fractions of cell cytoplasm, the presence of histocompatibility antigens in the platelets demonstrated in this investigation lends support to the view that these antigens may also be present in cytoplasmic constituents. Evidence to this point has been recently given by Herzenberg and Herzenberg:21 most of the H-2 antigens were found to be contained in cell membranes, both nuclear and cellular, rather than in the nuclei themselves. It is conceivable that in earlier experiments, membrane fragments (nuclear and cellular) of disrupted cells may have contaminated nuclear, mitochondrial and microsomal fractions,22-24 all of which were be-
Antigenic Structure of Blood Platelets II

553

... believed to be themselves carriers of transplantation antigens. A cell membrane in platelets has been seen by electron microscopic observation, and mitochondria are known to be present in blood platelets. These various observations are summarized here to emphasize that current evidence concerning chemical structure and cellular location of histocompatibility antigens in no way excludes the possibility that blood platelets could contain such antigens. The finding by Basch and Stetson that tissues containing high doses of histocompatibility antigens regularly possess high contents of acid phosphatase is also consistent with this concept, as blood platelets are known to contain acid phosphatase abundantly.

Blood platelets possess, therefore, a complex antigenic structure. In addition to red cell blood-group antigens and platelet specific antigens already demonstrated by others in human platelets, our experiments indicate the presence of histocompatibility antigens. Classification of human blood platelets in "groups" and "types" on the basis of serologic tests in vitro may, therefore, have less meaning in the practice of platelet transfusions than previously prospected.

Summary

The rejection time of skin homografts was measured in rabbits previously sensitized by one intradermal injection of homologous blood platelets which had been carefully prepared free of leukocytes. The graft rejection time was determined by gross inspection and based on the onset of definite signs of graft breakdown.

In the platelet sensitized rabbits, the skin homografts from the platelet donors ("specific" grafts) were rejected significantly earlier than in nonsensitized animals in 42 per cent of the experiments. The homografts from animals other than the platelet donors ("nonspecific" grafts) were rejected sooner than normal in only 10 per cent of the experiments.

The results were interpreted as demonstration that blood platelets contain histocompatibility antigens.

Summario in Interlingua

Le tempore de rejection de homograffos cutanee esseva mesurate in conilios previemente sensibilisate per un injection intradermal de homologue plachettas de sanguine, le quales habeva essite liberate meticulosemente de leucocytos. Le tempore de rejection del graffo esseva determinate per inspection a oculo inerme e esseva basate super le declaration de definite signos de deterioration in le graffo.

In le conilios sensibilisate per medio de plachettas, le homograffos cutanee ab le donatores de plachettas (graffos "specific") esseva rejicite a un tempore significativamente plus precoce que in le animales nonsensibilisate in 42 pro cento del experimentos. Le homograffos ab animales altere que le donatores de plachettas (graffos "nonspecific") esseva rejicite post un tempore plus breve que normal in solmente 10 pro cento del experimentos.

Le resultatos esseva interpretate como un demonstration que le plachettas de sanguine contine antigeno a histocompatibilitate.
REFERENCES

ANTIGENIC STRUCTURE OF BLOOD PLATELETS II


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