Some aspects of the immunologic factors in corneal grafts

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The general basic problems of homotransplantation are briefly reviewed, and pertinent aspects of the factors involved are applied to the corneal graft problem. It is pointed out that one of the most important questions yet to be solved is the isolation and characterization of the antigens involved. The necessity for an in vitro technique to study this problem prompted the preparation of anticorneal antisera which were tested against freshly grown tissue culture cells. It is shown that such antisera are cytotoxic for corneal epithelial and endothelial cells, but not for fibroblasts of corneal stroma. These observations are in accord with the results of corneal heterotransplantation. For controls, antiheart sera were prepared, and were also found to be cytotoxic for corneal epithelial cells. However, the antiheart antibodies did not affect fibroblasts of corneal stroma, spleen, or conjunctiva, but did damage the fibroblast-like cells growing out of adult rabbit heart explants. The corneal antisera were examined for their content of antibodies against the soluble components of cornea by the immunoelectrophoretic technique. At least 6 or 7 corneal tissue antigens, unrelated to serum proteins, were detected. Recent important discoveries have been made in bringing about immunologic unresponsiveness, and the possible application of such information to the transplantation problem is discussed.

The great strides in surgical technology during recent years have raised high hopes of grafting normal tissues to replace diseased ones. However, transplantation of many tissues from one human being to another almost always results in rejection and sloughing of the graft after a short apparent survival period (homograft or homotransplantation reaction). It is now well established that the principal factors involved in these reactions are immunologic in nature.1 Each individual apparently possesses certain important antigens in his cells slightly different from those of almost any other individual. Upon transplantation into a recipient, these differences are detected by the new host, who then begins to form antibodies to them. The chemical nature, localization, and number of these antigens are essentially unknown at the present time.

Once the immune processes start, usually about 1 to 2 weeks after a homograft has been implanted, the destruction of the donor tissues begins. An appreciable amount of data are available about the chain of events which then occur. A number of careful studies, for example, that by Kidd and Toolan,2 have shown that lymphoid cell-mediated antibody is often essential for these cytotoxic effects on the donor tissues. If cell-bound antibodies were the only immunologic mechanism involved in graft rejection, analysis of the problem would be greatly complicated. However,

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recent evidence by several investigators has made it quite clear that the more usual humoral circulating antibodies may also be involved in these lethal attacks on the grafted donor cells. These lines of evidence include the following which are important because they significantly facilitate the detailed approach to the whole problem of transplantation antigens.

1. Stetson and Demopoulos immunized rabbits with spleen cells from a prospective rabbit skin donor. When this antidonor serum was injected into a normal rabbit simultaneously with a skin graft from the donor, the transplanted tissue apparently failed to survive for even a short period. It did not become vascularized in the first 1 to 2 weeks, as would be expected in an untreated rabbit, and remained chalky white. This “white graft” reaction developed rather rapidly, and these workers interpret it to be the result of cellular cytotoxic effects of high levels of the antibodies involved. Similar results were obtained with skin grafts between highly inbred incompatible mouse strains.

2. It has been known since the classic studies of Chase that antibody production can be transferred from one rabbit to another by the transfer of lymphoid cells from an immunized animal to a normal one. The antibody released in the recipient has been clearly shown to be synthesized by the donor lymphoid cells.

In a series of excellent studies by Harris and co-workers, it has been found that the antibody synthesis in the recipient can be prevented by rabbit antibody against the donor lymph node cells. In these experiments, rabbits were immunized with lymph node cells from a prospective rabbit donor. The donor was then immunized with a bacterial antigen. Donor lymph node cells were mixed with antidonor serum, or normal serum, and then transferred to normal recipients. Those animals given antidonor serum failed to develop antibodies caused by the cytotoxic effect on the lymphoid cells transferred.

3. Cell-impenetrable diffusion chambers have been developed which can be implanted in even small animals, such as mice. Tissues or cells from one animal can be placed within the chambers in an incompatible host, and they usually grow quite well on the nutritional factors which diffuse into the chambers from the host. However, when the recipient has been strongly immunized against donor tissues, the donor cells have been shown to be destroyed within the chamber by humoral factors, presumably antibodies. Recipient immune cells could not be involved in these reactions. Such observations have been made by Algire who developed the chambers, and by Amos and Wakefield.

4. Stetson and Jensen have examined the cellular effects of antibodies prepared by hyperimmunization of a recipient mouse strain with splenic tissue from another (donor) strain. With the use of an in vitro vital staining technique, they demonstrated striking cytotoxic effects on mouse tumor cells raised in the donor strain. Sera containing such antibodies would also cause necrosis of skin grafts from the donor in the recipient under certain conditions. Very recently, Terasaki and co-workers reported the development of cytotoxic antibodies active in vitro against donor cells following one or more skin homotransplants in rabbits.

Incidentally, it has been shown that recipients of grafts may produce several antibodies against different components of the transplanted tissue. Not all of these are necessarily involved in the graft-damaging effects, and, in some instances, it is clear that certain of the antibodies bear no relationship to the rejection mechanism. Fruitful analysis of the homotransplantation problem would thus be concerned primarily with those that are related, i.e., the cytotoxic antibodies.

It might also be pointed out that little or nothing is known about the details by which these homograft antibodies cause cell damage. It has been suggested that mechanisms similar to that operating in delayed hypersensitivity reactions are in-
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If, instead of homotransplantation, whole-thickness cornea is grafted from one species to another (heterotransplant or heterograft), the donor cornea almost always becomes turbid within several weeks. Heterotransplants of other tissues, i.e., skin, result in more vigorous rejection than homotransplants. The mechanisms are almost undoubtedly the same in both, but in the case of heterotransplants the antigens are more readily recognized by the recipient as being foreign and the antibody response is presumably more intense and frequent. Because of this, it was felt that an experimental attack at this level might hold out hope of eventually identifying the chemical nature and location of the transplantation antigens.

In order to obtain such heterologous antibodies, it would have been possible to carry out transplantation of rabbit corneas into a good antibody producer, such as ducks. Because the antigenic stimulus from a single corneal transplantation would almost certainly be inadequate for large amounts of circulating antibody to develop, we thought that more intensive and prolonged stimulation would be advisable. In a series of experiments, with this in mind, a group of ducks was immunized with a large pool of ground rabbit cornea in Freund’s adjuvant mixture. This vehicle has been demonstrated in numerous studies to increase and prolong the antibody response to many different types of antigens. As a control on the specificity of the reactions involved, other ducks were immunized with rabbit heart or rabbit serum.

The antisera were tested in two ways. In one assay, they were added to freshly grown rabbit corneal cells in tissue culture to see if damaging or killing effects could be observed. In the other tests, the anticernea and other sera were examined for their antibody content by the gel-precipitin technique against the soluble components of the corneal extracts.

The tissue culture results proved most interesting and were reported in detail.
earlier. When a sheet of rabbit corneal epithelium was exposed to cornea antisera, intense cytotoxic action was evident within a couple of hours. The cells in the sheet at first tended to separate from each other and become round, and often were held together by thin strands of cytoplasm. Clumps of these rounded cells accumulated and seemed to be enveloped in a refractile type of material. Examples of these effects are shown in Fig. 1, which also demonstrates the lack of effect of normal duck serum. When freshly grown corneal endothelium was similarly tested, it was also found to be susceptible to the toxic properties of these antibodies. It was surprising, however, to find that the corneal stromal fibroblasts appeared to be quite
resistant to the effects of these antisera. The results are shown in Fig. 2. It thus appears quite evident that certain antigens in the corneal epithelium and endothelium are distinct from or absent from the cells of the corneal stroma. In addition, these data suggest that the transplantation antigens of the stromal fibroblasts are extremely weak, in relation to those of the epithelium or endothelium, since whole corneal homogenates were used as the immunizing antigens. This latter possibility is supported by observations of other investigators carrying out corneal heterografts with and without epithelium. In the absence of epithelium, corneal transplants between different species may remain intact for substantial periods of time.

It may be added in passing that antisera, such as those used here, almost always contain antibodies to rabbit serum proteins which happened to be present in small amounts in the tissues used for immunization. In all of the present tissue-culture studies, these antibodies to serum proteins were removed by appropriate absorption. In addition, antiserum against normal rabbit serum revealed no cytotoxic properties.

In tests with the anti-rabbit heart sera used as controls, it was found that these also caused cytotoxic effects on the rabbit corneal epithelium in a manner apparently identical to those seen with the anticorneal antibodies. When corneal stromal fibroblasts were exposed to the antiheart sera, they proved to be also unaffected by these antisera. These data indicate that heart tissue possesses the same antigens stimulating cytotoxic antibodies, as does the cornea. In addition, however, it was found that the antiheart sera caused damaging effects on the fibroblast-like cells that grew out of rabbit heart explants although no effects were seen on spleen or conjunctival fibroblasts, or on fibroblasts of corneal stroma. The anticornea sera were without action on any of the fibroblasts. These results (Fig. 3) indicate that the heart tissue contains an additional “transplantation” antigen to that seen in the cornea, and that this antigen is lacking in fibroblasts of the spleen, conjunctiva, or cornea.

In order to determine whether the antigens involved might be traced by gel-precipitin techniques, the antisera were
tested against homogenates of the cornea and heart. As pointed out earlier, in order to study only the cellular or tissue antigens, all of the sera had to be thoroughly absorbed with lyophilized normal rabbit serum. An indication of the contribution of antiserum-protein antibodies to the precipitin patterns is shown in Fig. 4 in which typical reactions of anticornea and antihart sera are shown with and without such absorption.

It may be seen that this anticornea serum revealed at least 6 or 7 antitissue components, some of which were present in appreciably high concentrations. The antihart serum revealed even larger numbers of tissue-specific components. Since the antihart serum showed cytotoxic effects on the corneal epithelium, these sera were cross tested against both antigens in adjacent wells. Typical results are shown in Fig. 5, where these tissues, as well as others were examined. Of some importance was the apparent failure of the soluble corneal antigens to be found in appreciable quantities in the heart extracts, and vice versa. Since the antihart sera were cyto-

toxic for corneal epithelial cells, the seeming lack of sharing of soluble antigens in the gel-precipitin tests suggests that other, perhaps insoluble, antigens were involved in the cytotoxic reactions. It is also of some interest that the soluble corneal antigens do not appear to be shared with other tissues to a striking degree, although...
several of the cardiac antigens are present in abundance in a number of other organs (Figs. 5, A and 5, B).

The aforementioned observations on tissue culture could furnish a relatively simple tool for a concerted attack on the most important question—the isolation, identification, and characterization of the antigens involved in transplantation reactions. In the search for such corneal components, for example, corneal homogenates would be fractionated in a variety of ways including electrophoresis, differential centrifugation, chromatography, and other techniques. The various fractions would be added to a small challenge dose of cytotoxic antibody, and this would then be added to a freshly grown culture of corneal epithelium. The fractions containing the significant antigen would abolish the cytotoxic effect, and they would be further purified until substantial evidence of homogeneity and maximum absorption potency were found. The chemical nature of the components could then be determined as well as their localization in the cell, the latter being done with the fluorescent-antibody technique.

Other than for academic interest, why are the isolation and characterization of the transplantation antigens of such importance? The answer to this question is of the greatest practical significance. It may eventually enable us to surmount the immunologic barrier to free and widespread grafting of any tissue from one human being to another. This possibility has become apparent because a number of recent studies have shown that under certain circumstances an animal may become specifically unresponsive to an antigen which ordinarily evokes an immune response. This major breakthrough is still in its early stages of development, but several situations have been described where such unresponsiveness has been clearly demonstrated. Briefly, some of these are as follows:

1. Immunologic paralysis.\textsuperscript{29,30} This phenomenon occurs in normal adult animals, tends to be rather permanent, and has thus far been demonstrated principally with pneumococcal polysaccharides. A small dose (e.g., 0.05 \(\mu\)g) of an antigen stimulates abundant protective antibodies, but a large dose (500 \(\mu\)g) fails to produce any detectable response in mice. Furthermore, if animals which are given the larger dose are injected some months later with the small usually effective quantity, no antibody is produced. This state of affairs often persisted for almost the life-time of the animal and it proved to be specific. If an animal had received the large (paralyzing) dose of Type II pneumococcal polysaccharide, it produced antibodies normally in response to a small dose of Type I polysaccharide.

2. Protein-overloading paralysis.\textsuperscript{31} It has been shown that massive doses of protein in normal adult or x-irradiated adult rabbits can stifle the antibody response to these antigens for variable, but often long periods of time.

3. Immune tolerance.\textsuperscript{32-35} The studies by Medawar, Billingham, and others have revealed that a newborn animal or late fetus which receives living tissue from an incompatible donor of the same species, will freely tolerate a graft from the same donor after reaching adulthood. It has been shown that soluble antigens injected in utero will induce this same type of specific unresponsiveness, but larger doses are required, and the unresponsiveness tends to wane rather rapidly. The "tolerant" state to soluble antigens may apparently be prolonged by repeated small doses of the same antigen during adulthood.\textsuperscript{36,37}

The precise mechanisms by which these and other\textsuperscript{36,39} experimental states of immune unresponsiveness are brought about are not clear. A similar uniform basic process may be involved in all of them. The important fact is that such states exist, and they may eventually be applied to the problem of homotransplantation. One may hope that at some time in the future this may be accomplished in some what the following manner. Transplanta-
Fig. 4. Immunologic analysis of duck anti-rabbit cornea and anti-rabbit heart sera.

A. Double diffusion assay of anticornea serum against serial dilutions of soluble extract of rabbit cornea. The serum was completely absorbed free of antibodies directed against serum proteins by the use of normal lyophilized rabbit serum, at the rate of 40 mg. per milliliter. The numbers in the peripheral wells represent the concentrations of the rabbit corneal extract, in milligrams protein per milliliter.

B. Test of unabsorbed anticornea serum against rabbit serum proteins. Assay as in A, but peripheral wells contain normal rabbit serum at the stated concentrations.

C. Test of antihart serum against serial dilutions of soluble extract of rabbit heart (ventricles). The serum was absorbed free of antibodies directed against serum proteins, as in A. The peripheral wells contain the heart extract at the stated concentrations.

D. Test of unabsorbed antihart serum against rabbit serum proteins: assay as in B.

E. Immunoelectrophoresis of anticornea serum, absorbed with normal rabbit serum. Upper well, rabbit corneal extract, 60 mg. protein per milliliter; lower well, normal rabbit serum. Absorption can be seen to be complete.

F. Immunoelectrophoresis of unabsorbed anticornea serum. Upper well, rabbit corneal extract, 60 mg. per milliliter; lower well, normal rabbit serum.

G. Immunoelectrophoresis of antihart serum, absorbed with normal rabbit serum. Upper well, rabbit heart extract, 170 mg. per milliliter; lower well, normal rabbit serum. Absorption can be seen to be complete.

H. Immunoelectrophoresis of unabsorbed antihart serum. Upper well, rabbit heart extract; lower well, normal rabbit serum. In E to H, the anode was on the right, the cathode on the left.
Fig. 4, E, F, G, H. For legend see opposite page.

Fig. 5. Tests of duck anti-rabbit cornea serum, and duck anti-rabbit heart serum, against these and other tissues. Both sera were completely absorbed with lyophilized normal rabbit serum, as seen by the lack of reaction at the bottom wells: A, Anticornea serum, central well. B, Anticard serum, central well. The antigens in the peripheral wells were homogenates of the following rabbit tissues: C, cornea; H, heart; LI, liver; NRS, normal rabbit serum; BR, brain; KI, kidney.
tion antigens from a prospective donor would be purified, and then given to a recipient under conditions of dosage, route, etc., so that the recipient becomes unresponsive to these specific antigens. At such a time, the recipient will receive his graft from the donor, and his immune mechanism will henceforth be unable to produce antibodies that will damage the donor cells. The graft should survive indefinitely.

Alternatively, it might be feasible to purify transplantation antigens from very large pools of human tissues from many individuals. If the pools were large enough, they would conceivably contain all possible configurations of the transplantation components. This, in fact, appeared to be the case in the studies of Harris and co-workers on the transfer of rabbit lymphoid cells, where pools of tissue from sixty animals appeared to cover most possible antigenic configurations. Any prospective human recipient of a homograft would be given injections of the pool of purified transplantation antigens under suitable conditions. When he had become unresponsive, it should prove possible, with impunity, to give him grafts of almost any tissue from almost any other human being.

Although this may sound premature and somewhat fanciful, there are straws in the wind that medical knowledge may perhaps soon, achieve this state. One paper has recently been published which suggests that large doses of donor spleen cells given to adult mice may induce a specific state of unresponsiveness. These mice then accept and retain skin grafts from the same donor for many months. Another report has shown that massive skin homotransplants in adult rats often result in very prolonged survival, while small grafts are rejected in the usual period of about 2 weeks. The latter workers suggest that the large dose of antigen involved brought about a state of immunologic unresponsiveness and subsequent acceptance of the large grafts. At any rate, these and the above reports all bring hope that within the foreseeable future tissue grafts of many different kinds will be freely carried out among human beings.

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REFERENCES