Immune host response to corneal grafts sensitized to herpes simplex virus

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Stromal herpetic disease is of particular interest in view of its varied clinical picture and its tendency to reactivation. The inflammatory process is characterized by stromal edema, infiltration by leukocytes, and vascularization with or without ulceration. Another manifestation of stromal disease is characterized by circumscribed edema, a picture which has been called disciform keratitis.

Ultramicroscopic studies have demonstrated the presence of herpes virus (HSV) in the stroma of corneas with chronic or acute herpetic disease and in the stroma and keratocytes of experimental corneas. It has been proposed that the clinical picture of stromal keratitis is related to a hypersensitivity mechanism and that the viral infection modified the antigenic characteristics of host cells in such a way that they could be considered as foreign cells by the host. Ferritin labeling of herpes antigens has been demonstrated by electron microscopy on the infected cell surface in vitro as well as morphological changes in the cell membrane. These findings therefore support the idea that an immune response plays a most important role in herpetic keratitis.

The purpose of these experiments was to study the fate of corneas sensitized by intrastromal (convalescent) or subcutaneous injections of herpes simplex virus after their transplantation to HSV-sensitized hosts. The experiment showed that 100 per cent of convalescent grafts became opaque. While clinically the graft opacification was not typical of a graft rejection, in many cases the histological picture was indistinguishable from an allograft reaction. It also showed that a significant number (50 per cent) of those grafts obtained from systemically sensitized rabbits opacified in an accelerated manner when placed in sensitized hosts.

Materials and methods

Virus. All experiments were done with virus stock of the McKrae strain of HSV grown in human embryonic lung tissue and stored in 1
ml. vials at -70° C. Virus titer (6 x 10⁶ per milliliter) was determined in hamster embryonic fibroblast monolayers by absorbing 0.2 ml. of virus dilution for one hour at 37° C. with gentle rocking of the plastic containers every 15 minutes. At the end of the absorption period, 5 ml. of Earle’s basal minimal medium containing 10 per cent calf serum, 1 per cent glutamine, and 2 per cent methyl cellulose were added as the overlay. Bottles were incubated at 37° C. for 48 hours and stained with crystal violet for plaque counting.

Vials with the tissue culture media were thawed immediately before use. The same batch of McKrae virus was used throughout the experiment.

**Rabbit sensitization.** Adult albino rabbits (3.5 kilograms) received twelve injections of 0.1 ml. of HSV undiluted stock subcutaneously twice a week. Neutralizing tests for the presence of circulating antibodies were done in human embryonic kidney cells with an appropriate dilution of virus (10⁶ PFU). The titer of the antisera was 1/16 four weeks after the last injection. These animals became the recipients of corneal grafts which were done between four and six weeks after sensitization. They will be referred to as Systemically Sensitized (S) group.

**Corneal infection.** Adult albino rabbits of same weight were anesthetized with sodium pentobarbital (30 mg. per kilogram). A lid speculum was placed in one eye and with a 30 gauge needle, 0.06 ml. of HSV stock was injected intrastromally. Four to seven days later, all animals developed dendritic keratitis which resolved untreated in seven to ten days. Fifty per cent of the injected animals died with central nervous system (CNS) symptoms. This group of rabbits will be referred to as the Locally Sensitized (L) group.

**Keratoplasties.** Seventy-six penetrating keratoplasties were done by exchanging grafts between groups S and L. (S-L = 20 eyes; L-S = 20 eyes), between S and S (20 eyes) and between the groups S and L and a noninfected (Normal) group: S-Normal (8) and L-Normal (8). Thus, five groups were formed as follows: (1) locally sensitized graft in systemically sensitized host; (2) systemically sensitized graft in locally sensitized host; (3) normal graft in locally sensitized host; (4) normal graft in systemically sensitized host; and (5) systemically sensitized graft in systemically sensitized host.

One additional group of sixteen rabbits were grafted and served as controls. In one set (the recipients), whole tissue culture media (0.1 ml.) had been injected subcutaneously (12 times) and 0.06 ml. intracorneally in the donors. Animals were used four to six weeks after the last injection.

Rabbits were anesthetized with sodium pentobarbital (30 mg per kilogram). Penetrating grafts were 6.5 mm. in diameter. Animals were operated upon in pairs, so the grafts were rapidly exchanged. They were secured usually with 8-0 silk or, occasionally with 10-0 nylon. Sutures were removed between six and eight days later before any vessels reached the graft. The type of suture did not modify the results of keratoplasty or the fate of the graft. Transplants which were not perfectly clear, or those with wound defects were eliminated. Fourteen (14) good grafts remained in each of the first two groups, seven (7) or eight (8) in the smaller groups, and twenty (20) in the last group, and sixteen (16) in the control group. Donor corneas were clear and did not show epithelial defects at the time of keratoplasty. Four to six weeks had elapsed since the last subcutaneous injection and about the same period of time after corneal infection. Experiments were ended two months after keratoplasty.

**Virus culture of infected corneas.** These cultures were done with the purpose of determining the presence of infective virus in corneal grafts. At the end of the experiment, grafts from groups 1 and 2 were removed and cut in half. This half was then cut in small (2 by 2 mm.) pieces. They were gently crushed and teased apart with a scalpel in a small Petri dish with tissue culture media. The contents of the dish were placed in human embryonic kidney tube cultures.

**Histological study.** One-half of each corneal graft and host rim was fixed in 10 per cent formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin. A few samples from each group were embedded in Epon after fixation in 4 per cent glutaraldehyde; 1 μm thick sections were made and stained with Giemsa.

**Results**

Schema in Fig. 1 summarizes the results in the first four experimental groups.

**Group 1—locally sensitized transplants (L) in systemically sensitized recipients (S).** Fifteen rabbits received locally sensitized corneas (L). One graft became infected and 14 were clear five days after surgery. Subsequently, over two weeks all 14 grafts opacified (100 per cent). Vascular limbal engorgement and peripheral corneal vascularization became evident and progressed after sutures were removed on
Fig. 1. Schema summarizing results in four groups. (1) Locally sensitized grafts in systemically sensitized hosts (14 grafts). All grafts opacified (100 per cent). Cloudiness and vascularization predominated in ten grafts (a), and edema predominated in four of them (b). (2) Systemically sensitized grafts in locally sensitized hosts (14 grafts). Seven of the fourteen transplants opacified (50 per cent). (3) Normal grafts in locally sensitized hosts (7 grafts). One of seven grafts opacified. (4) No graft opacification occurred in this group. (L = Locally sensitized; S = Systemically sensitized.)

the seventh day. Similar behavior of vessels was observed in nylon-sutured grafts. Severe vascularization occurred in ten eyes (10 days) and advanced toward the center of the graft (Fig. 2) in a ten- to fourteen-day period.

Histological study of eight opaque grafts showed a pronounced lymphocytic and plasma cell infiltration at the limbus with a small number of polymorphonuclear leukocytes in most cases and none in others. Vascular channels were dilated in the limbal area. The host tissue showed the same type of cellular infiltration; however, the corneal transplant showed two types of reactions. One was characterized by a massive infiltration by lymphocytes, plasma cells, and polymorphonuclear leukocytes (Fig. 3). This infiltrate was more pronounced in the anterior half of the corneal stroma. The vascular reaction was very pronounced in these corneas; vessels were usually surrounded by a large number of leukocytes. Four grafts showed mostly stromal edema with a fewer number of round cells and few vessels. A large number of keratocytes were destroyed. Some showed cytoplasmic vacuolation and others were surrounded by several lymphocytes or plasma cells forming small nodules. Polymorphonuclear leukocytes were present in these corneas but in few numbers and grouped in certain areas of the stroma rather than being diffusely scattered. Lymphocytes and plasma cells were scattered between and under the endothelial
Table I. The relative degree of cellular infiltration and vascular response in grafted cornea and limbal area of rabbits from Groups 1, 2, and 3. Group 1 has been subdivided into sub-Groups A and B

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Legend: LY = lymphocytes; PC = plasma cells; POLY = polymorphonuclears; VASC = vascularization; E = edema; + = mild; ++ = moderate; +++ = severe.

Note: Groups 4 and 5 showed normal host and graft tissue.

layer (Fig. 4) which showed a proliferative reaction.

**Group 2**—transplants from systemically sensitized donors (S) in locally sensitized hosts (L). Recipients were 15 rabbits which had been donors for the systemically sensitized rabbits. One animal was lost to infection and the remaining 14 remained clear; however, at the end of seven days, most of them showed ciliary congestion and vascular engorgement with invasion of host cornea and scar of the graft. Sutures were removed at this point. At ten days, seven of these 14 rabbits (50 per cent) showed graft edema and vascularization and total opacification at fourteen days. Histological examination of eight specimens at two to three weeks (seven opaque grafts, one clear) showed a moderate lymphoplasmatic reaction with dilated vascular and lymphatic channels in the limbal area. Round cell infiltration and vascularization of the graft was moderate. The cellular infiltration was mostly by plasma cells with few polymorphonuclear leukocytes. Some round cells were present in the endothelium. This reaction was very similar to that seen in the second group of transplants done in systemically sensitized rabbits.

**Group 3**—normal transplants in locally sensitized (L) rabbits. In six of seven rabbits, vessels developed from one week to ten days after suture removal. These vessels reached the scar of the graft, and a few invaded the stroma of the transplant.
Only one of these seven rabbits' grafts became opaque. Histological examination of this cornea showed moderate lympho-plasmatic reaction at the limbus and a few cells infiltrating the stroma of the host tissue with lymphocytes and plasma cells grouping at the scar. The histological reaction in Groups 1, 2, and 3 is summarized in Table I.

*Group 4—normal transplants in system.*
ically sensitized (S) rabbits. All eight grafts remained clear throughout the experiment (two months).

Group 5—systemically sensitized (S) transplants in systemically sensitized (S) rabbits. Twenty transplants between this single group remained clear two months after keratoplasty.

Control group. Two of sixteen transplants become cloudy during the third to fourth week after keratoplasty. Histological examination of these corneal grafts suggested that the opacification was due to an immuno-allergic reaction since the grafts were invaded by lymphocytes and plasma cells. The donor tissue had been sensitized to tissue culture media and the recipients had received subcutaneous injections of the same tissue culture media.

Virus culture of infected corneas. Human embryonic cells inoculated with corneal tissue from groups 1 and 2 failed to show any cytopathic effect at the end of one week incubation; therefore they were considered negative for HSV growth. Fluorescein staining of some of these grafts was positive only in the stroma; however, no virus was found on electron microscopic examination. These studies will be the subject of a future report.

Comment

It has been shown\textsuperscript{11-14} that complete or incomplete virus particles can be found in the corneal stroma, stroma cells or nerves of eyes with chronic herpetic keratitis. It has also been shown\textsuperscript{10,11} that antigenic determinants can be incorporated into the cell membranes of infected cells making them permanent antigenic sources. In our experiments with previously infected grafts (Group 1), we can assume that viral antigens stimulated an immune reaction in the sensitized host. Histological study of the limbal area in two of these host animals systemically sensitized prior to transplantation showed that this area was free of cell infiltration. This is in contrast to local sensitization where one usually finds mononuclear response at the limbal area. The time lapse (7 to 10 days) for the immune response of delayed type to this sensitized graft in a sensitized host is shorter than the inflammatory response obtained after a single injection of herpes simplex virus. This accelerated response then would be similar to that seen in the second set of homografts or after the second injection of heterologous proteins. Since 100 per cent of these grafts opacified within ten days after transplantation, we can assume that HSV sensitization plays the important role, and not the sensitization to proteins contained in the tissue culture media. Most (14/16) of the control grafts remained clear, and the two that opacified did so three weeks after keratoplasty. We do not believe that these injections played an important role in the 100 per cent rejection of grafts of Group 1 especially if we consider that no rejections occurred in Groups 4 and 5, which received tissue culture media with cells. Even though no epithelial ulceration appeared and cultures were negative in-L-grafts of Group 1 at the time of opacification, it is possible that replicating virus was present. Endothelial cell destruction observed in four grafts (Group 1) with edema predominantly could be a manifestation of uveal response to HSV antigen localized in the corneal endothelium or Descemet's membrane; however, virus antigens were not determined in these tissues. It has been shown in electron microscopic pictures that virus is present in the anterior chamber of eyes with kerato-uveitis,\textsuperscript{14} and that the penetration of herpes virus from epithelium to endothelium can occur, as was shown by Maloney and Kaufman.\textsuperscript{15} In contrast to the first group, grafts from Group 4 (normal into S-sensitized) and Group 5 (S-sensitized into S-sensitized) remained clear. In previous experiments with rabbit corneal allografts and autografts infected with HSV postoperatively, graft rejection was not observed by clinical or histological standards and most of the initially opaque and vascularized transplants partially cleared within two months.\textsuperscript{13}
Opacification of 50 per cent of the grafts from systemically sensitized (S) rabbits placed in eyes previously infected (L) with HSV (Group 2) was an important and very significant finding because it suggests that virus antigens had reached the cornea of the donor animal. The sensitized grafts caused an accelerated reaction histologically similar to a severe graft rejection (See Table I). A strong local sensitization was apparently necessary since S-grafts in S-recipients did not opacify (Group 5).

The inflammatory reaction and vessels present in the limbal area of locally sensitized animals of Group 3 two to three weeks after keratoplasty may have been activated by the surgical injury and by the use of sutures. In this group in which the corneal graft had not been exposed to herpes antigens, a small number of lymphocytes and plasma cells stopped at the host-graft junction, except in one eye which became opaque as a result of rejection.

Normal corneal grafts in systemically sensitized rabbits remained clear during the follow-up period of two months.

Cultures of all corneal grafts which became opaque and were removed for histological study demonstrated no growth of HSV. It has been observed that cultures have been negative even in corneas with positive virus in electron microscopic sections. It has been suggested that modification of the cell membrane of corneal cells by the virus theoretically could make them so different from their homologous corneal cells that after transplantation in a sensitized host they could stimulate an accelerated homograft reaction. However, Roizman has indicated that HSV proteins present in the cell surface remains unchanged, so that the reactions observed could be a manifestation of viral hypersensitivity.

There is clinical as well as experimental evidence that nonspecific inflammation in the eye may cause cloudiness of the graft. However, this may be transient and not related to an immune response unless the inflammation is caused by the introduction of antigens which may be shared by cells of the graft, antigens not belonging but present in the graft, and by an exaggerated host response. As an example of the first situation, we have the experiments of Rappaport and Chase of accelerated skin graft rejections in animals sensitized to streptococcal and staphylococcal antigens. The other two situations could occur in corneal allografts or even autologous corneal tissue, which because of sensitization to viral antigens may be destroyed by the host in the same way as incompatible homografts, but not on the basis of reaction to transplantation antigens. This may have importance in the field of transplantation when donor and recipient have been sensitized to HSV, a likely possibility since a large number of the human population has been exposed to this virus.

Summary

Experiments were performed to study the fate of corneal grafts sensitized to herpes antigens when grafted to HSV-sensitized hosts. Nonsensitized grafts in systemically sensitized hosts remained clear, whereas all locally sensitized grafts in systemically sensitized hosts became opaque within ten days. The stroma showed severe lymphocytic and plasma cell infiltration and neovascularization. One-half of the corneas from systemically sensitized hosts grafted to eyes previously infected opacified in an accelerated manner. Round cells were found in the stroma and endothelium. The histological reaction in both groups was similar to that found in the immune graft reaction even though the clinical picture was not typical (peripheral and progressive edema with a rejection line). The accelerated reaction was probably related to the presence of virus antigens in graft stroma and subepithelial areas of the graft. The significance of HSV-sensitized donor tissue grafted onto HSV-sensitized hosts is discussed because of its possible implication in tissue transplantation.
REFERENCES