THE ROLE OF HUMORAL ANTIBODIES IN REJECTION OF SKIN HOMOGRAFTS IN RABBITS

II. Passive Transfer of Transplantation Immunity by Sensitized Lymph Node Cells within Diffusion Chambers

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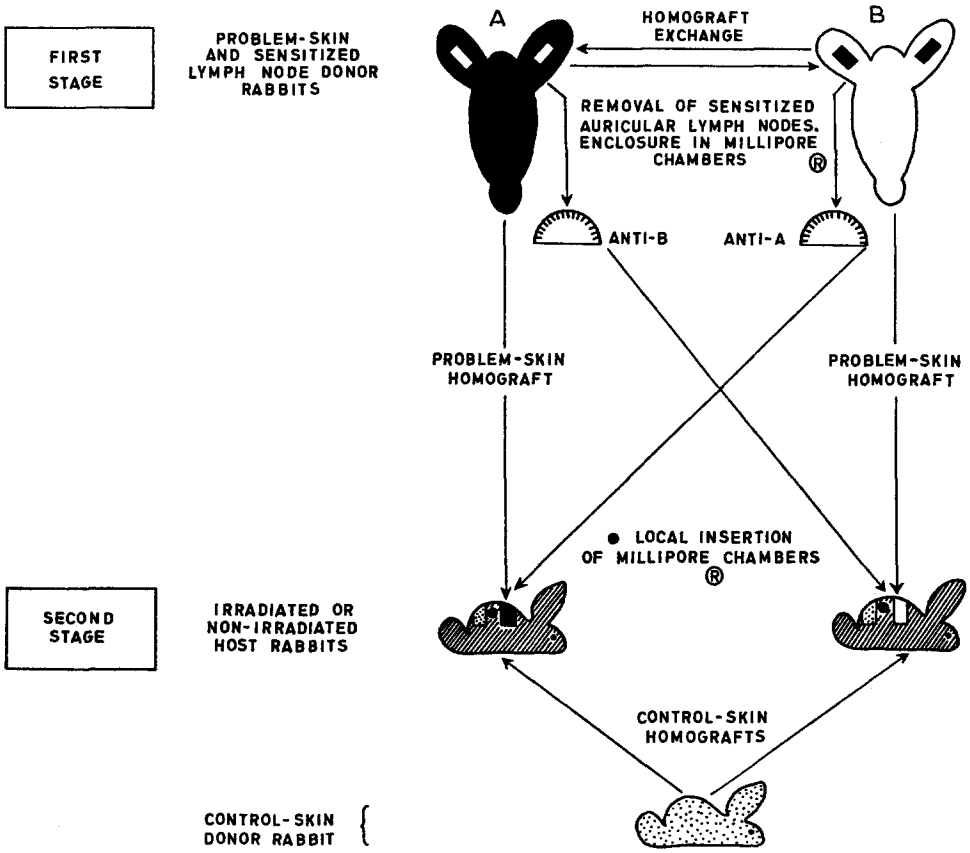
In a previous paper (1) evidence was presented against the exclusive interpretation of transplantation immunity as a form of delayed hypersensitivity. Local administration of isoimmune serum to animals bearing skin homografts resulted in a significantly earlier rejection of problem-skin graft with no change in the course of a neighboring control-skin graft. Similar results have been obtained by Stetson and Demopoulos (2), also with local passive transfer of immune serum, and by others (3–5) with systemic administration of immune serum and different homograft systems. Although passive transfer of sensitized lymphoid cells to animals with acquired tolerance will reestablish their capacity to reject otherwise tolerated grafts (6) such experiments fail to discriminate between humoral and cell-bound immunity since lymphocytes have been shown to be involved in the synthesis of humoral antibodies (7–9). Therefore, results of passive transfer of transplantation immunity by means of sensitized lymphoid cells do not constitute definite proof of the cell-bound nature of this phenomenon as long as the possibility remains that such cells are acting on the graft not directly but by means of humoral antibodies (10). In this paper we report a series of experiments in which transplantation immunity has been successfully transferred by sensitized lymphoid cells, held away from skin homografts by placing them within diffusion chambers.

Material and Methods

I. Antibody Synthesis within Diffusion Chambers.—The capacity of previously sensitized lymphoid cells to release humoral antibodies after transfer to homologous hosts has been amply documented (9, 11–14). On the other hand, only two brief reports exist (15, 16) on the ability of lymphoid cells to release humoral antibodies when placed within diffusion chambers. For that reason the following experiment was carried out. A series of rabbits weighing an average of 1500 gm was immunized against the "O" fraction of *Salmonella typhi*. The antigen was standardized to contain 1×10^9 bacteria per ml with a nephelometer (McFarland). In the first stage of the experiment 10 rabbits were immunized with 0.5 ml of antigen injected sub-

cutaneously in each ear every other day until a total dose of 6 ml was completed. Antibody titers were determined every other day by the plaque agglutination technique (17) in the serum obtained from the marginal ear vein. At the end of 15 days the auricular lymph nodes were removed, since according to Stark (18) and Vrubel (19) this is the site of maximal antibody synthesis under these experimental conditions. With careful aseptic precautions the



TEXT-FIG. 1. General design of experiments. See text for description

lymph nodes were cut into small fragments and approximately 25 per cent (70 mg) of each node was placed in each diffusion chamber. The chambers were prepared with Millipore®[1] (pore size 0.45 μ), following a modification of the technique described by Sturgis and Castellanos (20). This modification consisted in that each diffusion chamber was made with a single circular piece of Millipore® 25 mm in diameter, folded along the diameter, and sealed at the edges with adequate cement.[1] In order to avoid breaking the porous membrane it is convenient to wet it in sterile saline before folding it. Immediately after sealing the chambers

[1] Millipore Filter Corp., Bedford, Massachusetts.

were placed subcutaneously in the dorsum of non-sensitized rabbits, with antibody titers of 0. The time elapsed between removal of sensitized lymph nodes and completion of their transfer within diffusion chambers was never longer than 90 minutes. Antibody titers in these animals were measured following the same schedule and technique as with donors of sensitized lymphoid cells.

II. Transfer of Transplantation Immunity.—In view of the results of the previous experiment (see Results) the transfer of transplantation immunity by means of sensitized lymphoid cells within diffusion chambers was attempted as follows (Text-fig. 1):—

(*a*) *Animals.*—Rabbits were used throughout the experiments. They were obtained from different dealers to insure absence of inbreeding, and weighed an average of 1500 gm. In addition, this animal species was used because of its well known resistance to radiation (21).

(*b*) *Homografts.*—Skin homografts were always full thickness and were fixed in place by continuous cotton sutures; their size and site differed according to the phase of the experiment. Donor rabbits exchanged skin homotransplants measuring 2 × 1 cm on the outer aspect of their ears. Host rabbits received 2 × 2 cm skin grafts taken from the dorsum of the donor animals and placed on the same area in the hosts. In addition, host animals also received 2 × 2 cm skin grafts taken from the back of other donors which were not used in any other phase of these experiments; these grafts were also placed on the back of the host rabbits, separated from the previous homografts by a bridge of normal skin. The placing of both skin homografts (problem and control) was made simultaneously and with the host under general ether anesthesia.

(*c*) *Radiation.*—Host animals were separated into three groups: non-radiated, radiated with a whole body dose of 400 r, and radiated with a whole body dose of 800 r. All radiated animals received the full dose in a single session, 5 days before skin homografting (22) and following the technique described by Uhr and Scharff (23). A 220 kv and 15 ma Phillips apparatus was used, and half the dose was applied to each side of the rabbit at a source-skin distance of 60 cm. Radiation was used in order to depress the immunologic response of the host (24) and prolong survival of homografts (25, 26). The high doses were selected according to Uhr and Scharff (23) who, using a soluble antigen (diphtheria toxoid) and the same animal species, observed depression of humoral antibodies but preservation of delayed hypersensitivity with 400 r, and depression of both types of immune response with 800 r. Blood counts were carried out in one-half of the radiated animals every 3rd day, and the results were in agreement with other observations using similar doses (21). All radiated animals received 50,000 u of penicillin intramuscularly every other day starting from the day of radiation.

(*d*) *Diffusion Chambers.*—Ten days after the exchange of skin homografts on the ears of donor animals (27) the auricular lymph nodes of both sides were obtained through a small incision under local anesthesia. The lymph nodes were treated identically as in the previous experiment (see above). The diffusion chambers were placed subcutaneously in the dorsum of the hosts, through a 1 cm incision using local anesthesia, under the bridge of skin separating the two homografts. The grafts were placed 4 days after implantation of the diffusion chamber in order to allow sufficient time for diffusion of humoral substances.

(*e*) *Evaluation of Results.*—Differences in the time of rejection of problem-skin as compared with control-skin homografts would indicate active and individual-specific participation of humoral factors (antibodies) produced by sensitized lymphoid cells within the diffusion chambers in rejection of homografts. Acceleration or rejection of both homografts would signify active but non-specific participation of humoral factors (antibodies) in rejection.

Evaluation and tabulation of results were accomplished in a manner similar to the previous work (1). Microscopic examination was carried out in all dying animals and, in order to have a homogeneous series, those surviving were sacrificed 1, 2, or 3 days after both homografts had been rejected. All results were analyzed for statistical significance in a t table at a p level of 0.01.

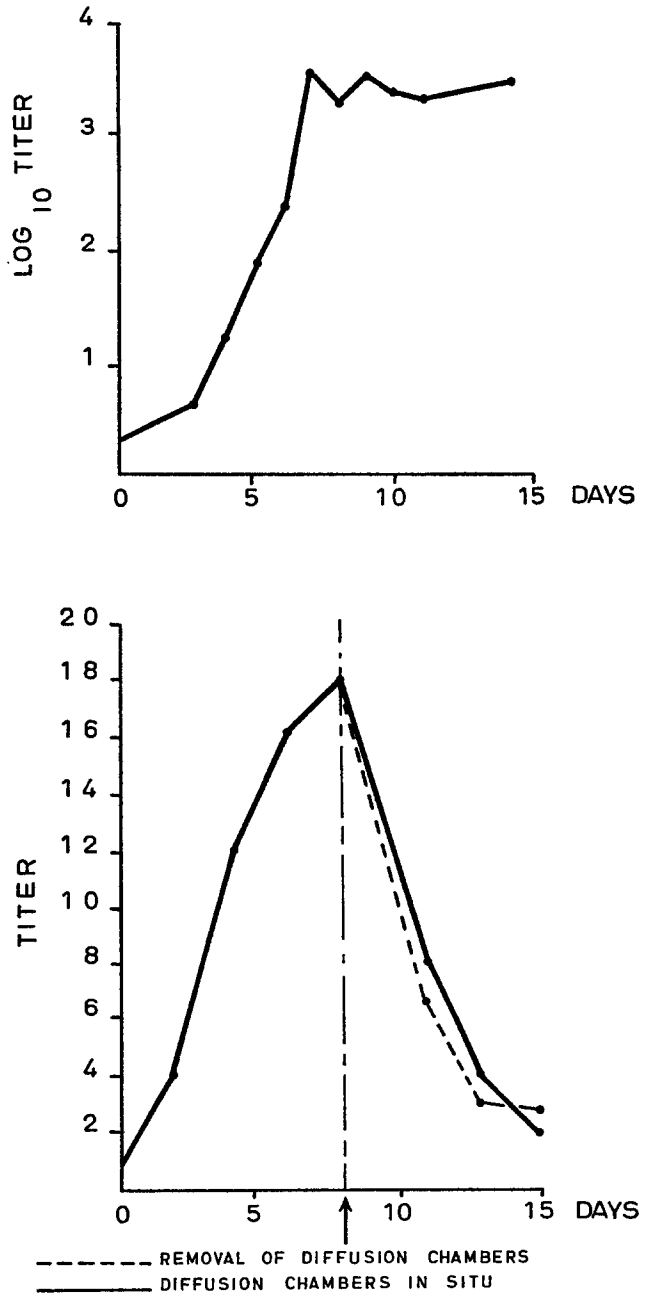
RESULTS

I. Antibody Synthesis within Diffusion Chamber.—The antibody titers obtained in the serum of animals sensitized with the *S. typhi* antigen, as well as those in the serum of rabbits with transferred sensitized lymphoid cells within chambers appear in Text-fig. 2. The appearance, day of maximum titer, and pattern of decrease in humoral antibodies in the latter group are comparable to those observed by Harris and Harris (13) with passive transfer of free sensitized lymph node cells. The titers, however, were lower, which might be due to differences in the antigen used and the duration of sensitization, as well as to the possible hindering effect of the diffusion chamber. It is also of interest that after 8 days of transfer humoral antibodies decreased rather sharply in the serum of the hosts. On this date, the diffusion chambers were removed in half of the animals for histologic study, but this removal introduced no differences in the decreasing titer of antibodies when compared with the rabbits in which the diffusion chamber remained intact. Histologic examination of the chambers revealed a capsule of loose connective tissue around them, and extensive signs of necrosis and degeneration in most of the lymphoid cells (Fig. 1 *a*). These histologic changes did not suggest inflammatory or immune damage, but rather appeared as ischemic or autolytic necrosis. Despite careful search, no evidence was found of passage of transferred lymphoid cells through the porous membrane, or of penetration of host leukocytes into the chamber.

II. Transfer of Transplantation Immunity.—The day of rejection for each one of the skin homografts in radiated and non-radiated hosts bearing diffusion chambers with previously sensitized lymphoid cells appears in Table I, and the average time of homograft rejection for each one of the experimental groups is shown in Text-fig. 3. In addition, in this figure a control group of intact animals bearing skin homografts identical in size and site has been included for comparison. This control group was prepared in our laboratory in the course of experiments previously published (1).

Control-skin homografts in non-radiated animals were rejected in an average of 7.5 days, a figure not significantly different from the general control result in our laboratory of 8.0 days. The same figure has been found by other authors (26, 28) with skin homografts in untreated rabbits. For that reason, the former figure is used in the remaining calculations as the time of homograft rejection in otherwise untreated rabbits. Problem-skin homografts were rejected in this same non-radiated group in an average of 4.7 days. The difference of 2.8 days between control and problem grafts is statistically significant.

Control-skin homografts in rabbits radiated with whole body doses of 400 r were rejected in an average of 10 days. (Fig. 2). The difference of 2.5 days with control-skin homografts in non-radiated animals is statistically significant, which confirms the effectiveness of radiation to prolong survival of homografts.



TEXT-FIG. 2. Anti-*Salmonella* agglutinin titers in animals sensitized with *S. typhi* antigen (above) and in animals with transferred sensitized lymph node cells within diffusion chambers (below).

Problem-skin homografts in this group of animals were rejected in an average of 6.7 days, which differs significantly from the time of rejection of control grafts in the same animals but not from the time of rejection of control grafts in untreated rabbits (Text-fig. 3). Control-skin homografts in the group of

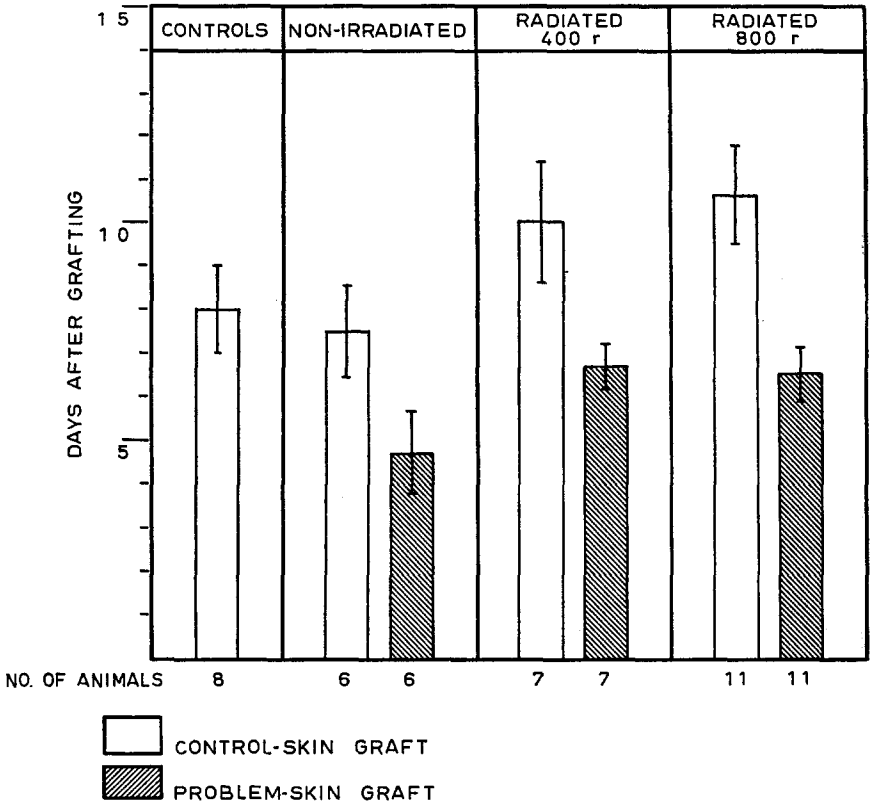
TABLE I

Critical Day of Homograft Rejection. Second Stage of the Experiment

Animal group	Animal No.	Critical day of rejection	
		Control graft	Problem graft
Non-radiated	1	9	4
	2	8	5
	3	7	3
	4	8	5
	5	7	5
	6	6	6
Radiated 400 r	7	10	7
	8	10	7
	9	11	6
	10	11	7
	11	10	7
	12	11	6
	13	7	7
Radiated 800 r	14	10	6.5
	15	11	6
	16	12	7
	17	12	8
	18	11	6
	19	10	7
	20	11	6.5
	21	8	6
	22	11	7
	23	10	6
	24	11	6

rabbits treated with 800 r of whole body radiation were rejected in 10.6 days which again is significantly different from the average of 7.5 days in which control grafts were rejected by untreated animals. Problem-skin homografts in this group of radiated rabbits were rejected in an average of 6.5 days, which differs significantly from the 10.6 average for control grafts in the same group but not from the 7.5 days for rejection of control grafts in untreated animals. A comparison of results between the two groups of radiated animals shows almost identical times of rejection for both types of skin homografts despite the 100 per cent difference in radiation doses.

Microscopic examination of the diffusion chambers placed in hosts radiated with 800 r, revealed adequate preservation of many lymphoid cells up to 10 days after skin grafting, which is 14 days after the diffusion chambers containing previously sensitized lymphoid cells had been placed subcutaneously in the hosts (Fig. 1 *b*). A comparison of the histologic picture of control and problem-



TEXT-FIG. 3. Average day of rejection of control- and problem-skin homografts in the various groups.

skin homografts in the same group 5 and 10 days after grafting showed the differences expected from their gross behavior. At 5 days both grafts appeared similar but the degree of infiltration by leukocytes and round cells, as well as the presence of degenerative changes in epithelium and collagen, was more marked in the problem-skin graft. This same difference prevailed throughout the experiment and at 10 days, although both grafts showed advanced histologic damage, this was more pronounced in the remnants of the problem-skin graft (see Figs. 3 *a* and 3 *b*).

DISCUSSION

The passive transfer of transplantation immunity by sensitized lymphoid cells within diffusion chambers was attempted on the basis of two assumptions. The first was that sensitized lymphoid cells will continue to release antibody when transplanted within the diffusion chamber, and the second was that this antibody will be effective in rejecting the corresponding skin homograft. The first of these two assumptions was put to test by sensitizing intact rabbits with a soluble antigen obtained from *S. typhi* and after antibody production had reached high titers (Text-fig. 1) the regional lymph nodes were removed from the animal, placed in diffusion chambers, and transferred to recipient rabbits. Antibodies against *S. typhi* antigen were detected in the serum of the recipient rabbit in small but increasing amounts from the 2nd to the 8th day after transfer; after this time they fell abruptly (Text-fig. 1). The possibility that such antibodies are not produced by the transferred lymphoid cells, but instead represent a response of the host to small amounts of antigen carried over with the transfer was considered unlikely because 72 hours had elapsed between the last injection of antigen and the removal of the lymph node from the donor. This period is more than enough for an almost complete elimination of antigen from a heavily sensitized animal (29). Furthermore, the curve of rising titer of antibody in the host lacks the characteristic lag phase of active immunization (13, 30). Other reasons for accepting the fact that sensitized lymphoid cells remain immunologically active after transfer have been summarized by Harris and Harris (13).

The results of this test were comparable to those obtained by others with the transfer of *free* sensitized lymphoid cells (11–13, 31) but differed in that the titers of antibody detected in the serum were lower. This difference may be due to any of the following possibilities, or combinations of them: (*a*) the free release of antibody by the lymphoid cells might have been hindered by the closely packed or "solid" nature of the transfer, by the diffusion membrane, or by the capsule of connective tissue which developed around the chamber; (*b*) the total number of transferred cells was only roughly quantified; (*c*) the rejection of the transfer, which acted as a homograft in the recipient; (*d*) the type of antigen, the number of injections, and the route of administration were also different; and (*e*) the technique for detection of antibodies was only roughly quantitative. The type of observations reported in this paper do not permit further analysis of the question. It was clearly shown, however, that sensitized lymphoid cells placed inside diffusion chambers will continue to release humoral antibodies for variable periods after subcutaneous transfer to intact hosts. Similar observations have been reported by Urso and Makinodan (15) and by Gengozian (16).

The following step was to attempt passive transfer of transplantation immunity by properly sensitized lymphoid cells within diffusion chambers.

The results of this experiment show that, under the conditions specified, problem-skin homografts placed in radiated and non-radiated hosts are rejected more rapidly than control-skin homografts when lymphoid cells previously sensitized against the problem-skin are introduced in their neighborhood within diffusion chambers. It is of interest that this rejection occurs earlier in non-radiated than in radiated rabbits. This result can be interpreted as the summation of two mechanisms of rejection: that primary in the host, which is intact, and that added by the passively transferred and previously sensitized lymphoid cells. This contention received further support from the fact that radiated hosts rejected their problem-skin homografts in the same average time as non-radiated animals rejected their control-skin homografts. Both groups of animals had essentially only one source of rejection: the former, the passively transferred lymphoid cells, and the latter, their own intact primary immune response. It is conceivable that when both rejecting forces are acting together the destruction of the homograft would appear earlier than when only one is functioning.

It is also of interest that both control- and problem-skin homografts were rejected in the same time in rabbits radiated with 400 r and with 800 r. This result is essentially the same as that obtained by Brooke (26) with the same animals species and identical homografts. Using diphtheria toxoid, Uhr and Scharff (23) observed that 400 r of whole body radiation in rabbits will depress only the immune response mediated by humoral antibodies, while the delayed form of hypersensitivity to the same antigen remains unaltered; on the other hand, 800 r will result in depression of both forms of the immune response. These data suggest that the effect of radiation on skin homografts in the rabbit is due to the suppression of humoral antibodies, since doubling the dose of x-rays with the consequent blocking of delayed hypersensitivity has no further influence on the average time of homograft survival.

The passive transfer of transplantation immunity achieved by means of previously sensitized lymphoid cells enclosed within diffusion chambers may be explained in different ways. First, it could be due to transplantation of a subcellular antigen carried over with the transplanted cells; such antigen would be able to leave the chamber and stimulate the immunologic mechanisms of the host. This possibility cannot be completely discarded but it is made unlikely by the results obtained in radiated animals, in which their own immune response was depressed, as shown by the prolongation of survival of control-skin homografts. The second possibility is that sensitized lymphoid cells are breaking down inside the chamber and that fragments small enough to pass through the pores of the membrane (0.45 μu) and bearing the cell-bound immunity against the graft, are responsible for rejection. In this regard it should be mentioned that, with the exception of man (32–35), transfer of delayed hypersensitivity or of transplantation immunity has never been achieved with dead cells or

cell fragments (36–39). The third possibility is that the sensitized lymphoid cells are releasing humoral antibodies against the isologous graft, and that this antibody is responsible for the rejection. Since this possibility is contrary to the generally held idea of the nature of the immune mechanism responsible for homograft rejection (40, 41), which is that it belongs to some form of delayed hypersensitivity, it might be worth while to briefly review the evidence for it.

Data favoring a form of delayed hypersensitivity as the basic mechanism in homograft rejection may be conveniently considered under two general headings: (*a*) evidence against participation of humoral antibodies, and (*b*) data in favor of cell-bound or delayed hypersensitivity.

(*a*) *Evidence against Participation of Humoral Antibodies.*—Many authors have reported failure to transfer transplantation immunity by means of serum (42–46). In addition, the studies of Algire *et al.* (47, 48) and others (49–51) showed prolonged survival of homografts in diffusion chambers, which are impervious to cells but presumably not to humoral factors. Finally, demonstration of circulating antibodies against homografts was technically difficult (52) and their role in rejection appeared dubious in the best case. More recent work, however, has considerably weakened the conclusions mentioned above. Thus, patients with agammaglobulinemia tolerate homografts permanently (53) despite the fact that they can develop adequate delayed hypersensitivity (54). Transfer of transplantation immunity has been achieved by several authors with local (1, 55) or systemic (3–5) applications of immune serum. By using heterologous (56) and tumor transplants (57, 58) or "hyperimmunized" recipients (59), several workers have observed rejection when homologous or heterologous tissue is protected from the cells of the host by diffusion chambers. Furthermore, Amos (60) has shown that complement will fail to penetrate inside some chambers, and it appears that cytotoxic antibodies require the presence of complement to damage cells (61–64). In the present study, one possible explanation for the death of lymphoid cells within diffusion chambers is a homograft rejection, and this possibility is now under study in our laboratory. Improvement in immunologic techniques (65–67) has resulted in wide demonstration of circulating antibodies against homografts (10, 67–70) and the cytotoxic activity of some of them can now be clearly demonstrated (66, 70, 71). The effect of humoral antibodies on homografts need not be only cytotoxic; Merrill (72) has shown in experiments *in vivo* that the effect can be mediated through vascular damage.

It may also be argued that both neonatal rabbits (73) and recently hatched chicks (74) are unable to form humoral antibodies adequately, and yet they show the same or even greater capacity to reject homografts as do adult rabbits and chicken (28, 74). The evidence in this respect is, however, controversial (75, 76), and Dixon and Weigle (77) have shown that lymphoid cells taken from neonatal rabbits are quite capable of antibody synthesis when transferred

to adult animals. Inability to demonstrate the presence of circulating antibodies should not be taken as definite proof of their absence, since, as in the case of delayed hypersensitivity (78), it might be the technique of demonstration that is at fault and not the immunologic competence of the cells. In summary, the evidence against the participation of humoral antibodies in homograft rejection is not very serious. On the contrary, many observations can be adequately explained if humoral antibodies are considered at least partly responsible for breakdown and rejection of homografts.

(*b*) *Data in Favor of Cell-Bound or Delayed Hypersensitivity.*—There are two main observations supporting the role of cell-bound immunity as the chief mechanism of homograft rejection. First, the demonstration by Mitchison (79, 80), others (11, 81–83), that transplantation immunity can be transferred from one animal to another by means of sensitized lymphoid cells. There is no doubt that transplantation immunity can be transferred by means of cells, but since the same cells have been shown to be capable of antibody synthesis such an experiment represents no proof of the ultimate mechanism of rejection. In fact, it has been suggested that the transferred cells are responsible for the breakdown of the graft, but the recent experiments of Najarian and Feldman (84) who transferred transplantation immunity by means of cells labeled with tritiated thymidine and failed to find the cells in the neighborhood of most of the sloughing grafts represents important evidence against such a mechanism. A similar observation had been made before by Mitchison and Dube (85) with sensitized cells labeled with acriflavin dye. Earlier, Dempster (86) and Darcy (87) called attention to the absence of infiltrating lymphoid cells during the initial periods of homograft rejection. But even if sensitized lymphoid cells were to be found in contact with the graft, the ultimate mechanism of tissue damage would remain unsolved, since the possibility would still exist that lymphoid cells were releasing humoral antibodies only when in close proximity to the corresponding antigen. Several authors have failed to observe damage to cells *in vitro* when they are placed in contact with sensitized homologous lymphoid cells (see reference 88). Exceptions to this have been published by Govaerts (89) and Rosenau and Moon (90) but their results are not free of the criticism made by Weaver (56) in relation to the diluting effect of the tissue culture media in the demonstration of minute amounts of humoral antibodies. There is also evidence that a critical concentration of antibodies may be required to cause tissue damage (1, 10, 84, 91).

The second observation supporting delayed hypersensitivity as the mechanism of homograft rejection is the development of a cutaneous reaction of the delayed type using donor cells as antigen (92). This is an interesting observation but there is no proof that the cutaneous reaction bears any relation to the rejection of homografts (93). Indeed, Billingham (94) found that the cutaneous reaction appears several days before the graft shows any signs of rejection, and

the same author (81) observed that the "second set" phenomenon persists longer than the ability of lymphoid cells of the recipient to cause accelerated rejection upon transfer to a recipient bearing a homograft of the isologous skin. Therefore, it appears that the evidence in favor of a form of cell-bound or delayed hypersensitivity as the main mechanism of homograft rejection is not conclusive.

The results of Najarian and Feldman (84) and those reported in this paper, which represent a confirmation of their findings in another animal species, together with the data summarized in the preceding paragraphs, suggest two final considerations. First, it is quite possible that homograft rejection may be the result of the contribution in variable proportions of the two major aspects (humoral and cell-bound) of the immune response, depending primarily on the age of the recipient (28, 95, 96), the type of homograft (1, 4, 56, 57), and the animal species studied. Second, the exclusion of one aspect or another of the immune response in a given instance of homograft rejection, if it is ever accomplished, will have to wait until more is known of the intimate mechanism by which hypersensitivity of any type produces cell damage, and also of the relation between delayed hypersensitivity and humoral antibody formation, which, according to Uhr (97), Pappenheimer (98), and Salvin (99), may show in some cases definite sequential relations, and which according to a hypothesis recently proposed by Karush and Eisen (100), may underlie a single phenomenon with different qualitative and quantitative expressions.

SUMMARY

Passive transfer of transplantation immunity by means of sensitized lymphoid cells enclosed within diffusion chambers has been accomplished in nonradiated and radiated rabbits. This result, together with other data available in the literature, suggests that humoral antibodies play an important role in rejection of skin homografts in the rabbit.

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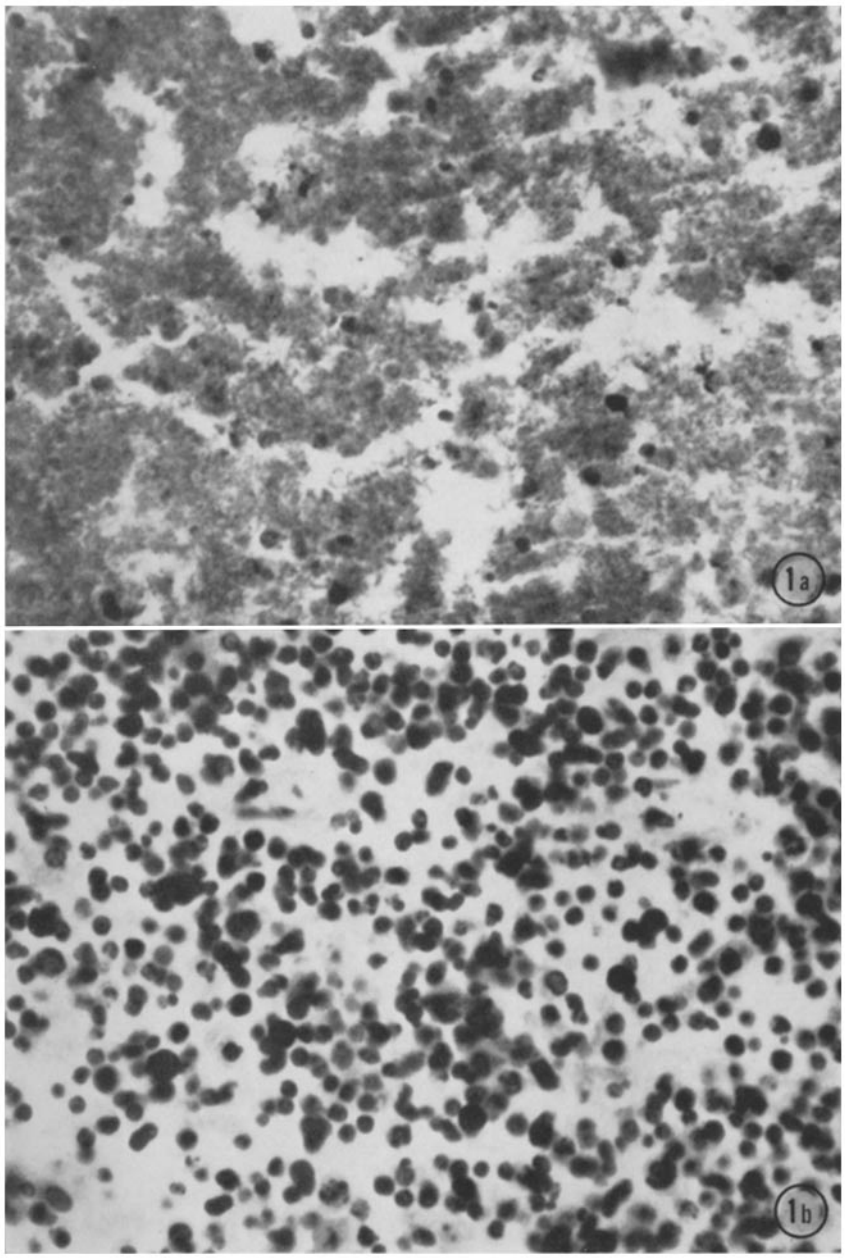
EXPLANATION OF PLATES

PLATE 117

FIG. 1 *a*. Microscopic aspect of lymphoid cells 8 days after being placed within a diffusion chamber in the subcutaneous tissue of an intact rabbit. There are only very few recognizable cellular elements. Most of the cells have undergone necrosis and disintegration. Hematoxylin and eosin. × 340.

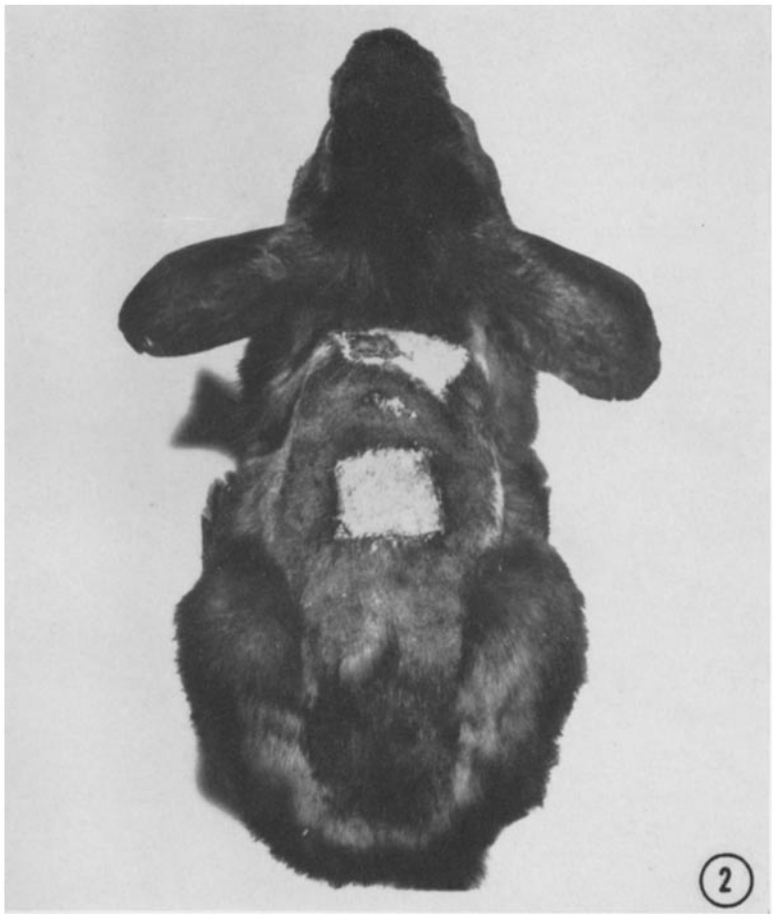
FIG. 1 *b*. Good preservation of many lymphoid cells 14 days after they were transferred within diffusion chambers to the subcutaneous tissue of rabbits given 800 r of total body radiation. There is pycnosis and nuclear hyperchromatism, but many cells still show normal morphologic features. Hematoxylin and eosin. × 340.



(Kretschmer and Pérez-Tamayo: Humoral antibodies. II)

PLATE 118

FIG. 2. Gross aspect of problem-skin homograft (above) and control-skin homograft (below) 10 days after grafting in a rabbit given 400 r of whole body radiation. The small lump visible in the bridge of skin separating the grafts is the diffusion chamber containing lymphoid cells previously sensitized against the problem-skin graft. The latter shows obvious signs of rejection while the control-skin graft is still well preserved.

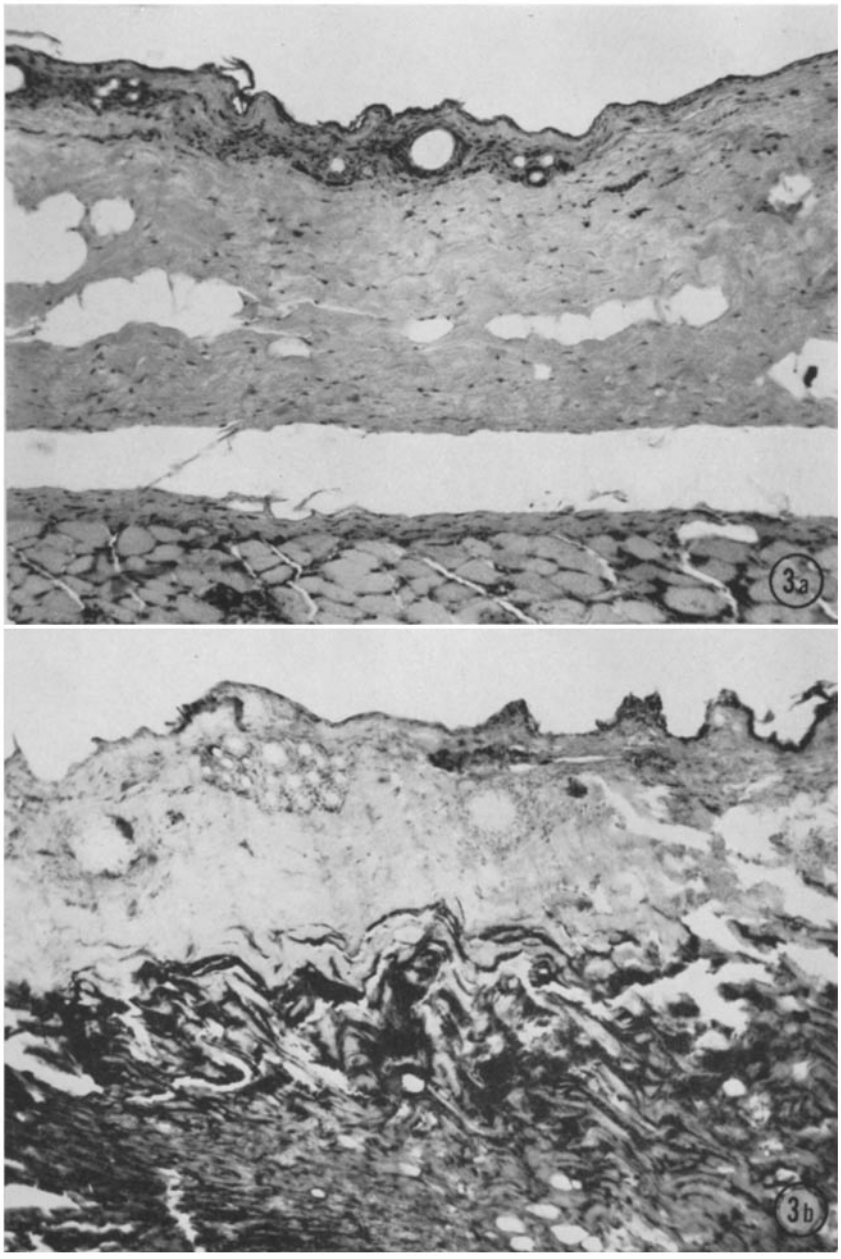


(Kretschmer and Pérez-Tamayo: Humoral antibodies. II)

PLATE 119

FIG. 3 *a* Microscopic aspect of control-skin homograft 10 days after grafting in a rabbit given 800 r of whole body radiation. There is some preservation of epithelial structures and both cellular and fibrillar elements in the dermis are visible. The muscle fibers below show infiltration by round cells. Hematoxylin and eosin. × 45.

FIG. 3 *b*. The problem-skin graft of the same animal bearing the control graft shown in Fig. 3 *a*. There is advanced destruction of the epithelium, fusion, and fragmentation of collagen fibers in the dermis, and cells are no longer visible. There is dense inflammatory infiltration in the deeper dermis and the muscle, primarily by round cells. Hematoxylin and eosin. × 45.



(Kretschmer and Pérez-Tamayo: Humoral antibodies. II)