Further Observations on Skin Homografts in Pyridoxine Deficient Animals *

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PREVIOUS reports from this laboratory [2, 5] indicated that pyridoxine (vitamin B_6) deficiency led to the prolonged survival of skin homografts in adult rats. To explain the continued survival of such grafts long after animals were returned to a pyridoxine containing diet, several possibilities were suggested. It was conjectured that perhaps during the deficient state of the animal a regression of the immunologic system to a *state of immaturity* occurred, resulting in a partial or total acceptance of the graft similar to that observed in the studies on actively acquired tolerance reported by Billingham and co-workers.[3] Another suggestion was that grafts were carried over the so-called *critical period* described by Woodruff [15] by suppression of the immune response through B_6 deficiency. He proposed that homotransplants become less vulnerable with the lapse of time and are eventually able to survive despite the development of immunity in the recipient. This would suggest that a change of antigenicity of the graft occurs or that it is replaced by host tissue.

To test these hypotheses and to learn more of the mechanisms involved in graft survival as a result of vitamin B_6 deficiency, studies were undertaken which included 1) the injection of pyridoxine deficient animals with donor spleen cells prior to B_6 re-feeding and subsequent skin grafting; 2) the application of a second graft from the same donor to animals with long standing intact first grafts; 3) the transferral of lymph node cells to animals with surviving grafts, such nodes being obtained from animals which had rejected skin taken from donors that had supplied the successful grafts; and 4) removal of prolonged successful skin grafts with their transferral back to the original donor and to non-donor animals. Presentation of the result of these studies is the purpose of this report.

Methods and Materials

All recipient animals in these experiments were Long-Evans male rats; ** all skin donors were Sprague-Dawley males,** each rat weighing 50 to 60 grams upon arrival at the laboratory. Donor animals were chow-fed *ad libitum* and recipients, with exceptions as noted, were given a prepared diet such as described in an earlier report.[5]

Desoxypyridoxine (DB_6), the specific pyridoxine antagonist, was prepared as a solution of 7.5 mg./ml. of DB_6 hydrochloride *** in normal saline neutralized to a pH of 6.8 to 7.0. Intraperitoneal injections

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** Long-Evans rats from Diablo Laboratory, Berkeley, Calif., and Simenson Laboratories, Gilroy, Calif.; Sprague-Dawley rats from Holtzman Farms, Madison, Wis.

*** Nutritional Biochemical Company, Cleveland, Ohio.

TABLE 1. *Survival of Skin Homografts in Animals Subjected to Prior Exposure with Donor Spleen Cells*

Group		No. Rats	No. of Surviving Grafts At 7 Days	21 Days	40 Days
A	Normal diet and graft	47	42 89%	1 2%	0 0%
B	Deficiency + recovery and graft	27	23 85%	1 4%	1 4%
C	Deficiency and graft	175	174 99%	116 66%	47 27%
D	Normal diet and spleen cells + graft	46	36 78%	3 7%	2 4%
E	Deficiency and spleen cells + recovery and graft	102	75 74%	13 12%	10 10%

of 0.75 mg./100 Gm. of body weight were given daily. Refrigerated stock solutions older than 30 days were not used.

All skin grafts were taken from the abdomen of Sprague-Dawley donors and placed on the backs of Long-Evans recipients as previously described.[5] They were covered for three or four weeks with gauze dressing which were changed at weekly intervals.

Spleen cell suspensions were prepared using the method of Woodruff and Simpson.[14] Approximately two-thirds of the spleen was removed from each Sprague-Dawley donor and each spleen fragment was dropped into a chilled solution of sterile physiological saline or buffered polyvinylpyrolidone (PVP) macrose.† It was minced, pressed gently through a 20 × 20 stainless steel mesh with a flat end glass pestle and allowed to filter through a 50 × 50 stainless steel mesh, using approximately 3.0 cc. of chilled diluent per spleen. This suspension was gently swirled and drawn into a tuberculin syringe. All operations were done in the cold immediately after excision of each spleen. No more than 30 minutes ensued from removal of the spleen until injection into recipient rats. Anesthetized rats were injected by way of femoral veins with 0.65 to 1.7 ml. of cell suspension containing 67–253 × 10^6 viable cells as determined by trypan blue staining and hemocytometer cell counts.[11]

Cell suspensions from lymph nodes were prepared in PVP-macrose with mechanical preparation as described above. Approximately 40–100 × 10^6 viable cells were obtained per lymph node harvest. Such suspensions were injected subcutaneously at several sites near the intact graft.

Observations concerning the gross state of each graft, i.e., reddening, hardening and/or ulceration, were noted at least weekly for the first six weeks and at less frequent intervals thereafter. The history of each graft was reviewed and the time of rejection was set as that time when the first evidence of it occurred. Uncontracted grafts with evident hair growth were considered successful.

Experimental Design and Results

Experiment I:

Effect of Prior Exposure to Donor Spleen Cells: Potential recipient animals were fed the basic synthetic diet plus a vitamin supplement containing 10 γ of vitamin B_6

† From Pathology Department, University of Pittsburgh.

until their body weights were greater than 100 Gm. (approximately 3 weeks). At this time, five groups were selected at random (Table 1).

Group A. Throughout the course of the experiment 47 rats were retained on the above diet. When body weights reached 200 to 250 Gm. and the rats were approximately three months old, they were skin homografted. These animals served as *normal* homograft controls. At 21 days only one of the skin grafts appeared intact, and at 40 days it had been rejected.

Group B. This group of 27 rats was placed on a B_6 free diet for three to four weeks, during which time they received a total of eight to 14 injections of DB_6. When an extreme state of deficiency was evident (body weights less than 100 Gm.), the animals were returned to the 10 γ B_6 diet and injections discontinued. After three to four weeks of recovery, animals appeared healthy and body weights approximated those in Group A at the time of skin grafting (200–250 Gm.). They were then skin grafted and continued on the same diet. One was still considered a fair take 21 days after grafting and persisted as such for more than 40 days.

Group C. These animals (135 in number) were maintained on a B_6 free diet and daily injections of DB_6 similar to the rats in Group B. Skin grafts were placed three to five days later when the first signs of deficiency were evident. Twenty-one days after grafting, 66 per cent demonstrated intact skin grafts, and at 40 days there was a 27 per cent survival.

Group D. Forty-six rats were injected intravenously with spleen cell suspensions and continued on a 10 γ B_6 diet. Thirty days later, each animal was skin grafted from the same donor that had earlier provided the spleen cells. Three, or 7.0 per cent, of the animals had surviving grafts at 21 days, and two persisted longer than 40 days.

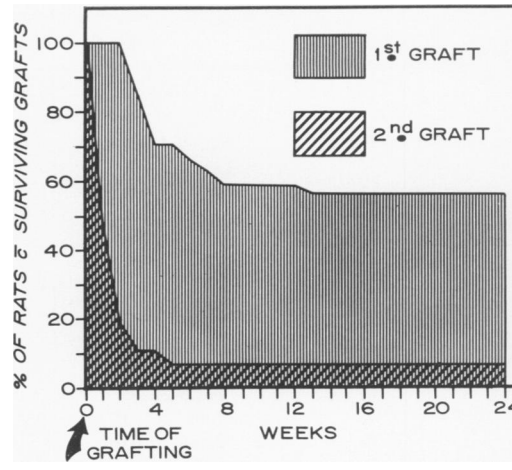

FIG. 1. Fate of first and second skin grafts in rats with long surviving first grafts prior to application of the second set.

Group E. In this group, as in Groups B and C, a severe deficiency was produced. At that time spleen cell suspensions prepared from individual animals were intravenously injected. Animals received no B_6 for several more days, after which they were returned to a 10 γ B_6 diet. Following a 30-day recovery period (when body weights had attained 200–250 Gm. and the animals appeared to be in good health similar to Group B), they were skin grafted. Spleen cells and skin were taken from the same donor for each recipient. Twenty-one days after grafting a 12 per cent survival of skin grafts was noted, and at 40 days 10 per cent of the grafts were still viable. None of the grafts at 40 days were total takes with an abundance of hair growth, but the latter was wispy, and while no contracture of grafts was observed, there was evidence of slow replacement by scar.

Experiment II:

Effect of Subsequent Grafting in Animals With Surviving First Grafts. Twenty-seven rats with excellent skin grafts 42 to 63 days old were regrafted from the same donor that supplied the first graft. Within seven days, 14 of the second grafts were

TABLE 2. *Effect of Injection of Lymph Node Cells from Skin Graft Rejected Animals into Skin Graft Tolerant Rats*

	No. of Viable Lymph Node Cells Injected Subcutaneously	Day Post-Inj. of Earliest Observation of Obvious Rejection of Graft	Remarks
1.	209.5×10^6	7	—
2.	188.4×10^6	—	No apparent effect
3.	178.3×10^6	11	Scabbing by 8th day
4.	175.1×10^6	14	—
5.	174.9×10^6	6	Reddening by 3rd day
6.	170.5×10^6	—	No apparent effect
7.	149.7×10^6	—	No apparent effect
8.	140.8×10^6	—	No apparent effect
9.	85.1×10^6	6	Scabbing during 1st week
10.	75.7×10^6	—	Reddened during 1st week
11.	70.8×10^6	—	No apparent effect
12.	43.7×10^6	—	Reddened during 1st week
13.	25.2×10^6	4	—

rejected and at two weeks only five (19%) could still be considered takes (Fig. 1). At 28 days three of these were still present, but two were only partial takes. One of the latter persisted for six months with a few wisps of hair, while another remained as an excellent second set graft. Thus, only one of 27 second set grafts was a completely successful take. By contrast, 28 days following regrafting, 19 of the 27 first grafts remained unaffected. Eight had become rejected between the third and fourth week after regrafting, four were considered non-takes between the fifth and eighth week, while 15 (56%) (Table 1) first set grafts remained unaffected for as long as six months.

The 10 rats with partially surviving grafts which had been injected with spleen cells at the time of deficiency (Experiment I-E) and skin grafted five to six weeks later were then regrafted after an additional 35 days. Partial survival of the second graft similar to that seen in the first was observed in nine of the 10 for longer than 70 days after the second graft. Hair growth was scant, and contracture did not occur.

Experiment III:

Effect of Subsequent Exposure to Injected Lymph Node Cells. Just as in Experiment I, potential recipients were maintained on a vitamin B_6 synthetic diet for approximately three weeks, at which time two groups were selected at random. Group A (59 rats) was placed on a B_6 free diet made deficient, and were grafted as described for animals in Experiment I-C. Twenty-eight days following skin grafting, each donor for animals with surviving grafts was used again to skin graft two more rats (Group B). The latter animals were continued on 10 γ B_6 diet throughout the balance of the experiment.

Two weeks later each of 13 rats from Group A with well-established skin grafts was injected with lymph node cells obtained from Group B animals which had by then rejected the grafts from the common donor. Table 2 summarizes the effects of the lymph node cell injections which affected eight of 13 animals so injected. Six grafts were rejected; two became reddened during the first week but survived. Animals are arranged in Table 2 according

to cell dosage administered, and it is suggested that the larger the number of cells inoculated the more readily rejection was effected.

Serving as control animals were 14 other rats with grafts as good as those of the rats injected with lymph node cells. The controls were left untreated and observed for a similar period, during which none of them rejected their skin grafts.

Experiment IV:

Transferral of Succesful Skin Grafts Back to Original Donor and to Non-Donor Animals: A group of 10 rats, having excellent grafts with abundant hair growth and no evidence of rejection 60 days after their initial application, were used in this experiment. These animals had long been returned to vitamin B_6 containing diet so that they were gaining weight, appeared healthy and showed no evidence of prior deficiency. The grafts were removed and transferred back to the original donor. Nine of the 10 demonstrated excellent takes with continued growth of hair which persisted for over four months. When grafts after four months back on the original donor were transferred to another Long-Evans rat, grafting promptly rejected. Excellent skin grafts of two to six months' duration were removed from 22 Long-Evans rats and were grafted to a Sprague-Dawley rat other than the original donor. All rejected by 21 days.

Discussion and Conclusions

The reports of Stoerk and Eisen [12, 13] and of Axelrod and associates [1, 10] that pyridoxine deficiency produces a severe impairment of antibody response to various antigens (human red blood cells and diphtheria toxoid) prompted an evaluation of such a deficiency in skin homografting. It was reported from this laboratory [5] that production of vitamin B_6 deficiency, either by dietary means or with the specific vitamin antagonist desoxypyridoxine (DB_6), resulted in a significant prolongation, and in many instances apparent permanent survival of skin homografts in rats long after their return to an adequate B_6 intake. Further studies by us during the last two years continued to confirm that under the experimental conditions originally described our prior observations are valid. Others have given support to these findings. Parkes [9] reported prolongation of ovarian graft survival in desoxypyridoxine deficient mice, and Hargis and associates [8] reported a significant increase in survival time of skin homografts in pyridoxine deficient mice.

It has been demonstrated that when animals with vitamin B_6 deficiency and depression of antibody synthesis are returned to a normal diet there is a gradual restoration of the capacity to produce antibodies so that after three or four weeks antibody titres become pronounced.[8] If the graft is a continuing source of antigens as suggested by Billingham and associates,[3] rejection of all grafts after several weeks, when animals return to a normal nutritional state, should be expected. Since this does not occur, it was believed worthwhile to gain more information as to why it does not.

The possibility was considered that graft persistence is due to a continued depression of antibody synthesis as a result of prolonged subclinical pyridoxine deficiency. This is extremely unlikely in view of the observations of Experiment I-B that 1) animals which are markedly vitamin B_6 deficient and not grafted at that time but permitted to return to a B_6 containing diet for three to four weeks and then grafted, fail to tolerate such grafts; and 2) the rejection of second skin grafts with persistence of first grafts for prolonged periods after loss of the former.

The almost universal failure of the second grafts would also suggest that no

significant degree of acquired tolerance had been achieved, although the results of Experiment I-E, where spleen cells were injected at the height of the deficiency, would suggest that this might have been achieved to a slight degree.

Meriting consideration is the observation that following transferral of viable lymph node cells from animals with rejected grafts to those with surviving grafts (skin having been from the same donor) a rejection of almost two-thirds of the grafts occurred, whereas grafts on controls not so treated continued to survive.

Using inbred strains of mice, Billingham, Brent and Medawar [3] reported that tolerance to a skin graft in A mice could be abolished by introduction of cells from regional lymph nodes of normal A mice which had been actively immunized by homografts of CBA-strain skin. They believed it essential that the lymph node donor be a member of the same strain as the tolerant mouse into which the lymph node cells are injected. Otherwise, a homograft reaction to the transferred cells would occur. From their results they concluded that immunity is the result of "incorporation of living lymph node cells into the mouse into which they are injected and not to passive transference of preformed antibody. . . ."

Dixon and associates [4] have demonstrated that adult lymph node cells transferred to adult x-irradiated recipients lose their ability to respond to antigens one to three days following transfer, probably as a result of their being damaged by the homotransplant reaction. Also, Harris and Harris [7] concluded from their results obtained when they injected lymph node cells taken from donors previously injected with dysentery bacilli into freshly irradiated animals of the same species and found agglutinins to dysentery organisms in the sera, that they did not passively transfer antibody in the cells. Rather, they believed the transferred lymph node cells continued to function in their new host and were the source of the antibody. However, they did concede that it was likely that these cells provided some material which made possible the synthesis of antibody by the new host.

Interpretation of our findings in the light of these data would suggest that the injected lymph node cells were tolerated by the host because of the hypo-active immunologic mechanism resulting from the prior deficiency state and that these *immune node* cells (Billingham) survived long enough to produce antibodies to the graft. Of course, that antibodies were not transferred with the inoculated cells is not disproved.

The observation that skin homografts (which had been well tolerated for several months) when transferred back to the original donor were completely accepted, whereas they were not when transferred to a third animal, would suggest that such skin had not been replaced by or incorporated into host tissue, but remained antigenically unchanged.

Thus, it would seem that the prolonged survival of skin grafts applied during a state of pyridoxine deficiency was not due to 1) a persistence of the deficiency state; 2) the production of acquired tolerance; 3) an inability of the skin to respond to antibodies; or 4) the incorporation of the skin into host tissue. Remaining is the explanation, as suggested by Woodruff,[15] that with protection over a *critical period* the graft becomes less antigenic, or even perhaps less responsive to antibody. That the latter may be so is suggested by the fact that second grafts rejected whereas the first graft continued to survive. Perhaps, also, injection of lymph node cells resulted in more antibody than through application of second skin grafts, thus provoking graft rejection. If this is indeed so, it is suggested that permanent blocking of antibody producing mechanisms is not necessary for continued homotransplant survival.

Summary and Conclusions

It has previously been reported from this laboratory that prolonged survival of skin homografts in a certain number of adult rats could be accomplished when such animals were made pyridoxine (vitamin B_6) deficient either by diet or through the administration of the specific vitamin B_6 antagonist desoxypyridoxine. *Takes* persisted even after animals had returned to a pyrodoxine containing diet and were no longer clinically deficient. Conjecture as to mechanisms involved in graft survival under such conditions prompted this study which revealed the following salient findings:

1. Graft persistence was not due to a continued depression of antibody syntheses as a result of prolonged subclinical pyridoxine deficiency. Animals which were skin grafted after receiving a B_6 containing diet for three to four weeks subsequent to the production of a severe deficiency of this vitamin failed to support such grafts.

2. Graft survival could not be ascribed to the achievement of a significant degree of acquired tolerance during the deficiency state. Almost universal failure of *second-set* grafts with continued survival of first grafts in animals once again on adequate B_6 containing diets mitigates against this possibility. Also, the injection at maximum deficiency of viable spleen cells taken from subsequent skin donors failed to confer tolerance for skin grafts applied when animals had returned to a normal nutritional state.

3. Excellent prolonged surviving grafts were affected by the injection in the regions of the graft of lymph node cells obtained from animals which had rejected a graft from the same donor. The larger the number of cells inoculated the more readily they rejected, suggesting that such grafts when confronted with sufficient antibody could be rejected.

4. That the skin graft had not become totally incorporated as a part of the new host was evident from the fact that transferral of such grafts back to the original donor resulted in successful *takes,* whereas their transferral to a third animal was unsuccessful.

5. Since the prolonged survival of skin grafts under such conditions does not seem to be due to 1) persistence of the deficiency; 2) a state of acquired tolerance; 3) a complete inability of the skin to respond to antibodies; or 4) to incorporation of skin into host tissue, it is suggested that with passage of time grafts become less antigenic or less responsive to antibody.

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BOOK REVIEWS

FELLOWSHIP OF SURGEONS, A HISTORY OF THE AMERICAN COLLEGE OF SURGEONS. By Loyal Davis, M.D., Charles C Thomas, 1960, 532 pages, $10.50.

It is fair to say that no progression has done more to keep its house in order and to constantly improve than medicine. As a result of these constant improvements brought about by the medical profession itself, the average life expectancy in the United States has been raised from 48 to 70 years and human suffering greatly reduced. The proper training and qualifications in the specialties of medicine has played an important part in this improvement. The book "Fellowship of Surgeons, A History of the American College of Surgeons" tells the story of the origin, development and progress of this great organization of surgeons. It is a factual story about the dedicated surgical pioneers who founded the American College of Surgeons. In the book are revealed for the first time the thoughts, motives and actions of these legendary figures in surgery in the United States. The book is an extremely well written, interesting saga of one of the great institutions in American medicine today.—HOWARD C. BARON, M.D.

APPRAISAL OF CURRENT CONCEPTS IN ANESTHESIOLOGY. Edited and assembled by John Adriani, M.D., The C. V. Mosby Co., 1961, $7.75.

This small pocket-sized volume is the outgrowth of a program in the Anesthesia Department of Charity Hospital in New Orleans, in which, during the past several years, the House Staff and trainees have been assigned for review topics of interest appearing in current medical journals on anesthesiology. With 30 young colleagues, Dr. Adriani has compiled the book for the medical profession, and for those specializing in anesthesiology. The book occupies an intermediate position between current medical journals and textbooks. The reviews are not exhaustive and are written simply in clinician's language. The book is not directed at the research worker nor to those preparing exhaustive reviews. From the standpoint of the aim and goal, it achieves its purpose well; that is, it serves as an excellent up-to-date review of the advances in anesthesiology made in the past several decades.—BLAIR O. ROGERS, M.D.

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