Effect of Methyl Bis (2-Chlorethyl) Amine Upon Survival of Skin Homografts in Rats and Rabbits.* (25488)

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Survival of homografted tissues has been shown to be possible where the immune response of host is congenitally impaired(1) or destroyed by X-irradiation(2,3). Chlorethylamine compounds, of which nitrogen mustard (HN_2) is representative, have a depressant and destructive effect upon bone marrow and lymphatic tissues. Hektoen and Corpor(4), studying sulfur mustards, first described an effect of this class of compounds upon immune mechanisms. More recently, Phillips and coworkers(5), Spurr(6), Bukantz *et al.*(7), and Schwab and associates(8) described a depression of titers of the antibody response to bacterial antigens and a delay in appearance of circulating antibodies after administration of nitrogen mustard. Recent studies by Green (9) question the degree of effect of nitrogen mustard upon antibody titers ultimately achieved. However, the profound change in lymphoid tissues after administering HN_2 suggest that there may be a hampering effect upon host organism's reactive capacity to homografted tissues. Furthermore, the role of circulating cells in homograft rejection response would indicate that a profound depression of circulating cellular elements should affect homograft survival. Infiltration of a homograft with cells, presumably from host circulation, is a prominent morphological accompaniment of rejection. On the other hand the classic experiments of Algire, Weaver, and Prehn(10), with millipore membranes interposed between host and graft, demonstrated that total exclusion of host cells allowed homograft survival. We were, therefore, attracted by the report of Levinson and Necheles(11), which suggested that a marked prolongation of homograft survival occurred in rats as a result of treatment with relatively low doses of nitrogen mustard. The following study was therefore carried out.

Methods. In 2 experiments, female rats (2 varieties of Sprague-Dawley origin) from Holtzman Co. and from Rolfsmeyer Co. were used. Female Long-Evans rats from Rockland Farms were a third strain used. Rats weighed 150-175 g each. Rats were equally distributed between treatment and control groups. In all instances reciprocal grafts were exchanged between treatment and control groups. The second group consisted of intrastrain grafts between Holtzman rats. Dosage schedules are summarized in Table I. In Group II both strain combination and dosage were selected to duplicate methods reported by Levinson and Necheles(11). All solutions of methyl bis (2-chlorethyl) amine (Mustargen‡) (HN2) were freshly prepared and injected into a tail vein immediately after dilution with appropriate volume of saline. In all grafting procedures, control and treatment group animals were selected by chance and lightly anesthetized with ether. 9 cm^2 full thickness skin grafts were removed from ventral thorax and abdomen by careful dissection in the fascial plane superficial to the panniculus carnosus and the recipient site was prepared in a similar fashion. Homografts were exchanged between pairs of animals by reciprocally exchanging skin from abdominal donor site of one animal and suturing it in the thoracic recipient site of the other animal. Skin from the thoracic donor site was sutured in the abdominal recipient bed of the same animal to serve as an autograft for evaluation of infection and technic. Care was taken to reverse the hair direction for future identification of the graft site. All grafts were sutured with interrupted 6-0 silk sutures at the corners and the continuous 6-0 silk suture

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TABLE I. Effects of Nitrogen Mustard on Rat Skin Homografts.

Group	Strains	No. treated	Dosage schedule	Wt loss	Leuko-penia	Effect on homograft
I	Rolfsmeyer Long-Evans	10	1 mg/kg on days 1, 3, 5, post-grafting only	++	±	Grafts rejected at same time as controls*
II	Holtzman intra-strain grafts	20	4 mg/kg on 1st day post-grafting and every 4 days for 7 doses	+		

* Sufficient quantitative data to make a statistically valid statement at 95% probability level.

around the margin. A thin layer of Bacitracin® ointment was applied to wound edges after which the graft was covered with a protective dressing consisting of a gauze pad held in place with several turns of waterproof adhesive tape. Dressings were changed every 2 days as long as grafts persisted. Daily examination of each graft was made possible by splitting the bandage along the back and retaping. In grafts between Long-Evans and Rolfsmeyer Sprague-Dawley rats, onset of rejection in untreated animals appeared abruptly and was a distinct endpoint revealed in cessation of capillary circulation as described by Taylor and Lehfeld(12). In Group II, intrastrain Holtzman-to-Holtzman grafts, onset of rejection typically was quite subtle and attenuated. Therefore, it was necessary to utilize another, less satisfactory standard for determining survival status in these sites of delayed rejection. We concluded that the grafts were no longer viable after a distinct follicular pattern became indiscernible because the donor epithelium was no longer identifiable under the microscope as revealed in serial biopsies made during this time sequence. Autopsies were performed and electrophoretic studies of serum were made on 5 additional rats treated according to dosage schedule used in Group II. Lymphatic tissues were grossly examined in representative animals of the other groups. In studies using rabbits, nitrogen mustard was given *via* ear vein. In all instances, grafting was reciprocally done between albino rabbits and black-and-white Dutch rabbits, both varieties obtained from commercial sources. During period of treatment with nitrogen mustard, daily white counts were done on all treated animals. The grafting procedure in rabbits differed from that in rats in the following ways: Pairs of animals were anesthetized with I.V. Nembutal, grafts were larger (12 cm^2), and protective dressing consisted of several turns of rapid-drying plaster of paris bandage over a gauze pad. These too were split down the back and retaped for daily inspection. Three studies were conducted in rabbits. *First,* 8 of the albino strain were intensively pretreated with 1 mg/kg of HN_2 for 4 days. On fifth day, when marked leukopenia was evident grafts were exchanged between treated animals and 8 Dutch rabbits. Additional doses of HN_2 were administered to all treated animals whenever the leukocyte count tended to rise. The days HN_2 was given are indicated by a solid bar at bottom of Fig. 1. *Second,* grafts were reciprocally exchanged between 13 albino rabbits and 13 Dutch rabbits. The 2 varieties were distributed equally as to treatment and control groups, one member of each pair being in the treatment group. HN_2 in doses of 1 mg/kg, was administered to the treatment group on day of grafting and for each of the next 3 days. Additional doses of 1 mg/kg were given on an individual basis, as needed to maintain leukocyte count below 2000 cells/mm^3. Relative height of solid bars on the baseline in Fig. 2 denotes number of animals receiving HN_2. *Third,* grafts were exchanged between 16 albino and 16 Dutch rabbits. One member of each reciprocally grafted pair was allocated to the treatment group, giving equal distribution of the 2 varieties of rabbits to treatment and control groups. Beginning 5 days following grafting, 2 mg/kg of HN_2 was administered to each rabbit daily for 3 days. Thereafter, the same dose was administered on an individual basis as required to maintain leukopenia. Relative height of bars in Fig. 3 indicates number of animals receiving HN_2. In all 3 treatment

groups, daily leukocyte counts were done on each animal. Differential leukocyte counts were done on treated rabbits in Group 1. Weights were taken serially. Penicillin and streptomycin were given every 2 days to combat development of respiratory infection.

Results. Dosage schedules, strain combinations, and effect of nitrogen mustard upon weight, total number of leukocytes, and homograft survival in rats are summarized in Table I. All rats in Group 1 had weight loss following HN_2 averaging 44 g/animal but the presence of leukopenia was variable from animal to animal. Grafts in the treatment group survived a mean of 9.2 days compared to a mean of 8 days in untreated controls. A "t" test confirms the lack of a significant difference in survival times. A 50% mortality resulted from HN_2 treatment whereas all 10 control animals survived well beyond the homograft slough time.

The experiment in "Group II rats" uses animals from the same source and repeats dosage schedule reported by Levinson and Necheles. No mortality resulted from this dosage schedule. In the treated group, total leukocyte count remained within pretreatment limits with the exception of 2 animals showing a transient leukopenia. In other experiments it was impossible to produce significant leukopenia with this dosage schedule, even though histologic study shows marked bone marrow suppression, lymph node and splenic atrophy. Weight loss averagd 43 g/animal in 8 animals, 2 animals lost no weight. Mean homograft survival in Group II was 27.3 days and 26.2 days in treatment and control groups respectively. This difference is not significant ($P > 0.50$). The longest graft survivals, over 100 days, occurred in 3 untreated control animals and 2 treated animals. The grafts had good hair regrowth and appeared normal except for altered hair direction. These prolongations were entered in the computations as 57 day survivals, the longest survival of any graft which was ultimately rejected. Animals treated according to the schedule of Group II had only a mild depression of the alpha-1 globulins on electrophoretic analysis.

Results of experiments in rabbits are summarized in Fig. 1, 2, and 3. In all 3 groups,

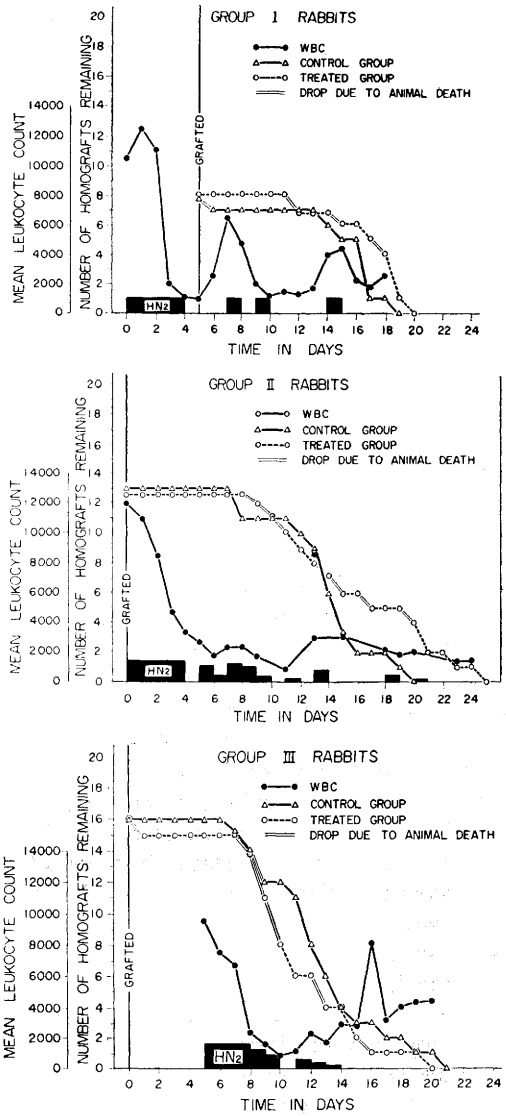

FIG. 1-3.

regardless of treatment used, certain general statements apply. *First,* a prolonged leukopenia could be produced. *Second,* treatment with HN_2 produced a significant mortality but survival was adequate to allow some quantitation of effect of treatment on skin homograft survival. *Third,* weight loss and anorexia were universal in treated rabbits.

The pretreated rabbits in Group 1 (Fig. 1) had a mean leukocyte count at time of placement of grafts of about 3000 cells/mm^3. This average value includes the 2 short periods of

WBC rebound. However, this preliminary depression of the reticular system resulting from intensive HN_2 treatment did not influence ultimate survival of grafts or alter time sequence of graft rejection. In Group 2 rabbits (Fig. 2), the more intense individualized treatment resulted in a more marked leukopenia and a significantly increased mortality. Although 2 grafts in the treated group survived longer than any of the control grafts, the prolongation was slight and not statistically significant (does not approach 90% probability). In Group 3 (Fig. 3) where HN_2 treatment was withheld until 5 days after grafting when vascular continuity between graft and host can be assumed to have been established, vigorous treatment produced a marked leukopenia, but was ineffectual in altering graft rejection since no graft in the treatment group survived longer than in controls.

In all 3 groups of rabbits, treated or controls, the endpoint of homograft rejection was clear-cut and abrupt. In 95% of all animals, the control autografts survived, appeared normal and in all animals surviving, the native transplant developed a regrowth of hair. In these rabbits, autopsies revealed lymph nodes which were small and atrophic. The differential leukocyte counts in Group I had that pattern essentially as described by Stetson and Good(13) for these dosages of HN_2 in rabbits.

Discussion. Our experiments emphasize that intensive HN_2 treatment does not significantly prolong survival of skin homografts when administered before, at the time of, or following grafting in rats and rabbits of these strains. This is true even with marked effect of treatment upon lymphoid tissues. This would seem to indicate that only a fraction of the antibody response mechanisms need to be functionally intact to prejudice survival of a homograft. Even HN_2 doses which proved fatal over 15-20 days produced, at most, but slight temporal delay in loss of homograft integrity. If one accepts the plasma cell system as being the principal center of antibody formation, the observations of Marshall and White(14) that the plasma cell system has proven relatively resistant to effects of nitrogen mustard would explain our failure to achieve depression of host immunity to the point of tolerance for a homograft. Furthermore, a recent publication by Green(9), appearing after this study was undertaken, described temporal relationships which condition the apparent effects of nitrogen mustard on antibody formation as revealed by timing of sampling technics following HN_2 administration. Green stimulated antibody production in rabbits by administering typhoid toxoid intravenously at varying intervals before, during and after 4 daily doses of HN_2 in 1 mg/kg doses. He then followed the titers of antibody in a serial manner. Maximum elevations in antibody titer were observed when the antigen was given after the second day of nitrogen mustard treatment. The antigen then elicited a delayed production of antibody, but titers ultimately equaled those of untreated control animals. When antibody appearance was maximally delayed, the peak occurred at about 15 days after toxoid stimulation rather than the 5 to 8 day peak titer noted in controls. Inherently the experimental design of earlier studies of effect of HN_2 on antibody production concealed the differing times for peak response in antibody formation.

These studies of homograft survival suggest existence of a lag in the host rejection mechanism. If the delay in transfer of antigenic material from the homograft to the host is about 24 to 48 hours, as suggested by data of Converse and co-workers(15), the work of Green (9) would indicate that the HN_2 therapy in Group II rabbits should have resulted in a maximal delay in rejection. If antibodies to histocompatibility factors are produced in the same time sequence as antibodies to a bacterial antigen, antibodies of histocompatibility antigens would occur soon enough to prevent significant prolongation of homograft survival. However, since animals receiving effective HN_2 doses all had weight loss and cachexia, any small prolongation in survival could represent a nutritional or metabolic effect rather than a primary effect of nitrogen mustard upon antibody producing cells.

The fact that marked, prolonged suppression of circulating leukocytes failed to prolong survival of skin homografts is of interest. This further supports the conclusion that if pro-

longation of survival of homografts is to be attempted by an attack on the cellular factors of rejection, exclusion of host cells must be virtually absolute either by chemically induced leukopenia or by interposing a suitable millipore filter between host and graft(10).

Finally, we must explain the conflict of our findings with those of Levinson and Necheles (11), who reported prolongations of graft survival up to 114 days by administering nitrogen mustard in doses of 0.4 mg/kg every 4 to 6 days for 40 days. The fact that employing their dosage schedule effect no change in outcome of intrastrain homografts (Group IV, Table I) when HN_2 treated animals were compared with simultaneous controls speaks against any profound effect of HN_2 on homograft survival. Certainly, this is the most favorable experimental design with a minimum of factors working against homograft acceptance. The key to the conflict appears to lie in the statement of Levinson and Necheles that "Sprague-Dawley Holtzman rats were utilized" presumably as both donor and recipient. We prevously reported the fact that frequently permanent survivals occur with intrastrain grafts in these inbred animals from this particular source(16). Since the prolongations reported by Levinson and Necheles do not exceed the survival found in reciprocal grafts between untreated Holtzman Sprague-Dawley rats, we can only conclude that prolongation of homograft survival reported by them was due to their selection of experimental animals and the absence of an adequate pairing between reciprocally grafted controls.

Summary. Intensive administration of methyl bis (2 chlorethyl) amine (Nitrogen mustard) to rats and rabbits in doses sufficient to produce and maintain marked leukopenia did not depress host response mechanisms in a degree sufficient to allow significant prolongation of homograft survival.

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Effect of L-3,3',5-Triiodothyronine on Epinephrine-Induced Contraction of Isolated Rabbit Aortic Strip. (25489)

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Thyroxine has been reported to increase sensitivity of isolated perfused swine arteries to epinephrine as evidenced by increase in degree and duration of constriction(1). Increased sensitivity to epinephrine was attributed to inhibition of amine oxidase in walls of the artery. Other experiments* indicated

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