decay time of proteins may thus be due to tyrosine-tryptophan energy transfer or to noncollisional quenching due to intramolecular bonding or to both. However, a small deviation from exponential decay due to energy transfer in this lifetime range would not be detected by our system. (ii) The decay times of about 4 nsec for reduced nicotinamide-adenine dinucleotide coenzymes (NADH) are considerably higher than that of 0.3 nsec calculated by Weber (*16*) if Eq. 2 applied. On the other hand, his calculations on the lifetimes of the flavin coenzymes are more or less correct (*17*). From his calculation of τ for flavin-adenine dinucleotide (FAD) he postulated an equilibrium between fluorescent and nonfluorescent coenzyme forms in solution; and we therefore suggest that such an equilibrium also exists for the NAD coenzymes. Our postulate is supported by evidence from chemical relaxation data (*18*). (iii) The fluorescence lifetimes of several compounds are relatively long; for example, 6-methoxyquinoline (the parent compound of quinine), the porphyrins, anthranilic and 3-hydroxyanthranilic acids, and the naphthalene sulfonates. Protein complexes containing these compounds may therefore be useful in hydrodynamic studies on proteins by fluorescence polarization, in that such studies require long fluorescence decay times of the order of the protein rotational relaxation time (*19*). In our study, γ-(1-pyrene)-butyric acid had the longest lifetime observed, 93.1 nsec; the compound has already been used in studies of fluorescence polarization in proteins (*20*). (iv) Several directly determined values of τ (Table 1) conflict with those predicted by calculations based on certain assumptions. The case of NADH and NADPH has been mentioned; and the decay times, 2.6 nsec and 2.7 nsec, for tyrosine and indole are much shorter than predicted (*21*). These discrepancies emphasize the importance of direct τ measurements.

The apparatus also detects gross deviations from exponential decay (Fig. 2). The fluorescence signal from $10^{-3}M$ acridine orange could not be matched by the curve simulator. However, by matching the simulated curve first to the beginning of the decay of the fluorescence signal and then to the end (Fig. 2, B and C), apparent decay times of 2.9 and 8.8 nsec were read from the computer dial. Such nonexponential behavior of acridine orange fluorescence in concentrated solutions is due to the fluorescence of aggregates and intermolecular energy transfer (*12*).

Finally, we expect that the availability of convenient and rapid apparatus for decay time measurement will have considerable impact on studies of fluorescence, which have too long depended on assumed and calculated τ values.

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3 February 1967

Drug-Induced Tolerance for Skin Allografts across the H-2 Barrier in Adult Mice

Abstract. *Mice treated with methylhydrazine derivatives for 3 to 4 weeks before the transplantation showed markedly prolonged survival times for allogeneic skin grafts differing at the H-2 histocompatibility locus. Presumably permanent tolerance was induced in about 20 to 30 percent of the mice when the drug treatment was combined with a single injection of additional donor antigen. The tolerance persists without further drug treatment and is specific for donor tissue.*

Immunologic tolerance for a variety of antigens has been achieved in adult animals with immunosuppressive chemicals, notably 6-mercaptopurine (*1*). Attempts to produce tolerance for allografts have been successful only with skin grafts involving identical H-2 histocompatibility genes (*2*, *3*) and with tumor grafts (*4*). It has also been shown that administration of massive doses of tissue antigen (*5*) and of tissue antigen combined with chemical immunosuppression (*6*) can make foreign grafts more acceptable. Efforts to induce pharmacologically tolerance for normal tissue grafts across the H-2 histocompatibility barrier in adult mice and other animals have not yet succeeded (*2*, *4*, *7*).

Long-term survival of canine and human renal allografts after cessation of immunosuppressive therapy may reflect a high degree of histocompatibility rather than tolerance; second transplants from the original donors are generally rejected (*8*).

Of the chemicals tested in my laboratory for prolongation of survival of first-set (*9–11*) and second-set allografts (*12*), methylhydrazine derivatives were the most effective. Because results were regularly better when administration of these drugs began 8 to 10 days before the transplantation (*9–11*), we explored the effects of longer pretreatment on transplantation immunity; I now report some preliminary results.

Two distinct strain combinations of donors with recipients (all fully grown mice), were used:

$A^{H-2^a} \leftarrow CBA^{H-2^k}$ and $C57Br^{H-2^k} \leftarrow A^{H-2^a}$

Both typified strong histocompatibility differences, with different alleles at the H-2 locus. With both combinations, grafts survived for 8 to 9 days in untreated mice; so the relatively less

Table 1. Survival of CBA-strain skin on A-strain mice pretreated with methylhydrazine derivatives. Drug treatment started 24 to 28 days before the graft. Numbers of mice grafted appear in parentheses.

Drug	Additional antigen ($\times 10^6$ cells), days before graft	Grafts			Fatalities
		Days of survival (mean ± S.D.)	No. surviving >30 Days	No. surviving >150 Days	
Saline (8)	20 CBA spleen, 23	8.5 ± 0.5	0	0	0
Saline (8)	None	9.6 ± 1.3	0	0	0
Ro 4-6824 (12)	None	32 ± 15	7	0	1
Ro 4-6824 (13)	45 CBA spleen, 12	>51 ± 70	4	2	2
Ro 4-6824 (14)	65 CBA spleen, 1	>91 ± 74	11	6	2
Ibenzmethyzine (5)	20 CBA spleen, 23	21 ± 16	1	0	21

extensive A ← CBA H-2 differences may be compensated by more important differences at minor histocompatibility loci. The transplantations, by a standard technique (*13*), were from the general body skin of the donors. Dressings were removed after 8 days; the grafts were inspected daily and considered as rejected when breakdown was complete.

During the experiments listed in Table 2, in addition to administration of the chemicals, the drinking water of all mice contained streptomycin sulfate at 50 mg/liter. Either of two drugs was administered subcutaneously daily (six times weekly) at 300 mg/kg for 24 to 28 days to the day preceding transplantation: Ro 4-6824 (1-methyl-2-*p*-allophanoylbenzylhydrazine hydrobromide) and ibenzmenthyzine [1-methyl-2-*p*-(isopropylcarbamoyl) benzylhydrazine hydrochloride; Natulan$_R$]. From the day following transplantation (D +1) to the day of the first graft inspection (D +8), the doses were reduced to 150 mg kg^{-1} day^{-1}; thereafter there was no treatment.

Supplementary tissue antigen was prepared from the donor strains. Suspensions of spleen cells were obtained by cutting the spleens into fragments and teasing them in saline with fine forceps. The suspension was then gently passed back and forth, with a syringe, through an 18-gauge hypodermic needle and centrifuged for 10 minutes at 1200*g*; the cells were then resuspended in the desired volume of saline. Nucleated spleen cells were administered intravenously at 20 to 65 × 10^6 per mouse.

Tissue antigen was prepared from kidneys and livers of the donor strain. The organs were passed through a fine, stainless-steel mesh, and the remaining cells in the *brei* were lysed in distilled water for 30 minutes. The homogenate was made normotonic again by addition of NaCl. Three cell-free preparations were used: (i) crude homogenate—about 200 mg (wet weight) per mouse, intraperitoneally; (ii) sediment from the homogenate—the crude homogenate was centrifuged for 10 minutes at 1500*g*, 20 percent of the acute LD_{50} (intravenous) of the sediment was then injected intravenously in 0.4 ml of saline; (iii) supernatant—from the clear supernatant of the centrifuged homogenate, 20 percent of the acute LD_{50} (intravenous) was injected intravenously with saline to 0.5 ml.

Second CBA grafts were applied to A mice, still bearing their CBA grafts, after 2 to 3 months and to other A mice that had already rejected grafts; they were applied to the contralateral thorax. Simultaneously with the second CBA grafts, the recipients received unrelated third-party ($C57B1^{H-2^b}$) skin; in some instances, two second CBA-strain grafts were made (see Fig. 1 and Table 3). No treatment accompanied the second transplantation.

In the set of experiments summarized in Table 1, CBA skin was grafted on A-strain mice conditioned with the Ro 4-6824 in the manner described. Their grafts survived markedly longer than did the control's—30 to 80 percent for 30 days. But most interesting was the finding that 20 to 30 percent of the mice remained fully tolerant for more than 150 days, and that no graft that persisted so long was lost subsequently. As I write, survival times extend to 208 days, so that permanent tolerance for allogeneic skin grafts may have been induced. Most of the surviving grafts have grown healthy crops of donor-type hair. Partial tolerance manifested itself by shorter survival times or by growth of rather thin and wispy hairs on the transplant.

Permanent tolerance resulted in 8 of the 27 mice treated with additional antigen (spleen cells) 12 or 1 days before the transplantation. Without administration of additional antigen, tolerance remained transient, the mean time of survival of the graft being extended to about 30 days. In the series of experiments summarized in Table 2, the initial results were confirmed with the A ← CBA host-donor combination and repeated with another: C57Br ← A.

Both drugs, Ro 4-6824 and ibenzmethyzine, proved potent in diminishing the immune response to skin allografts in these series, with Ro 4-6824 seeming to offer a slight but distinct advantage. My experience is that Ro 4-6824 is generally better tolerated in mice

Table 2. Survival of skin allografts (C57Br ← A and A ←CBA) in mice pretreated with methylhydrazine derivatives; treatment started 25 days before the graft. Numbers of mice grafted appear in parentheses; numbers of grafts still surviving (after 60 to 80 days) appear in square brackets. Abbreviations: i.v., intravenously; i.p., intraperitoneally; k + l, kidney and liver.

Drug	Additional antigen, 1 day before graft	Grafts		Fatalities
		Days of survival (mean±S.D.)	Surviving >30 days (No.)	
	C57Br ← A			
Saline (8)	None	8,8 ± 0,9	0	0
Saline (6)	20 × 10^6 A spleen cells, i.v.	10,3 ± 1,3	0	0
Saline (6)	A-strain k + l homogenate, i.p.	8,0 ± 0	0	0
Ro 4-6824 (7)	None	19 ± 14	1	1
Ro 4-6824 (7)	20 × 10^6 A spleen cells, i.v.	>49 ± 23	6 [2]	1
Ro 4-6824 (9)	A-strain k + l homogenate, i.p.	14 ± 4	0	0
	A ← CBA			
Saline (8)	None	8.7 ± 0.9	0	0
Saline (8)	25 × 10^6 CBA spleen cells, i.v.	9 ± 0,8	0	0
Ro 4-6824 (12)	None	26 ± 5	2	0
Ro 4-6824 (9)	30 × 10^6 CBA spleen cells, i.v.	>38 ± 21	6 [3]	2
Ro 4-6824 (5)	CBA-strain k + l supernatant, i.v.	>43 ± 12	5 [1]	0
Ro 4-6824 (4)	CBA-strain k + l sediment, i.v.	>34 ± 22	1 [1]	1
Ibenzmethyzine (7)	25 × 10^6 CBA spleen cells, i.v.	>38 ± 21	4 [2]	1
Ibenzmethyzine (4)	CBA-strain k + l supernatant, i.p.	25 ± 11	1	0
Ibenzmethyzine (4)	CBA-strain k + l sediment, i.v.	27 ± 13	3	0

(*10–12*) and rabbits (*14*). Variation in the kind of additional antigenic challenge (spleen cells versus tissue homogenate or extract; all intravenously) influenced the ratio of animals showing lasting tolerance. Especially with stronger H-2 differnces (C57Br ← A) intravenous administration of spleen cells led to definitely better graft survival than did no additional antigen or intraperitoneally delivered tissue homogenate.

Table 3 shows the survival of two simultaneously applied CBA and C57B1 skin grafts in retransplanted A-strain mice. All eight mice still bearing their initial CBA grafts (applied 70 to 80 days earlier) accepted the second graft while rejecting the third-party C57B1 graft; thus was indicated the specificity of their tolerant state (Fig. 1). On the other hand, in ten A-strain mice that had rejected their first CBA transplant before the second graft, normal breakdown of both the C57B1 and the second CBA grafts occurred.

Four main points emerge from this evidence: (i) Treatment of mice with methylhydrazine derivatives, for 3 to 4 weeks before transplantation, leads to severe depression of their immune response to foreign grafts; it prepares for permanent acceptance of allogeneic skin in a substantial proportion of the animals; (ii) for permanent tolerance, exposure to additional antigen is necessary; (iii) if tolerance is established, it persists without further help from drugs; and (iv) induced tolerance is specific for the donor strain.

It was earlier demonstrated that the efficacy of methylhydrazine derivatives as immunosuppressive agents was increased when treatment was initiated within 10 days prior to transplantation of xenogenous tumors in rats (*15*) or of skin allografts in mice (*9–11*). Treatment of the hosts only just before transplantation did not prolong survival of the grafts, treatment onward from the day of transplantation had a moderate effect, whereas pretreatment, with further administration after the transplantation, could double or triple the survival times (*9–11*).

The relatively specific clinical efficacy of the methylhydrazine derivatives (such as ibenzmethyzine) in malignant disturbances of the lymphoid system (Hodgkin's disease, lymphosarcoma) is a strong indication that they display some selectivity toward lymphoid cells. As the role of these elements in immune responses—particularly in mediating the homograft reaction—is established, the basically cytotoxic methylhydrazine derivatives seem to have a prevailing effect on immunologically active cell populations. One is tempted to speculate that after prolonged treatment the immune system of the animals is—maybe by virtue of a scarcity of immunologically competent cells—reverted to a state showing analogies to embryonic tolerance. To account for the effect of additional antigen, one would assume that, by this means, the balance of immunologically competent cells and total antigen mass can be further tipped toward tolerance.

Table 3. Survival of simultaneously applied C57B1 and second CBA skin grafts in CBA-tolerant and intolerant A-strain mice. Numbers of mice appear in parentheses.

Between first and second graft (days)	Days of graft survival (mean ± S.D.)		
	First CBA	C57B1	Second CBA
81 (4)	>208*	11 ± 2.1	>127*
81 (5)	42 ± 11†	8.5 ± 0.6	9.4 ± 1.1
71 (4)	>151*	8.0	>80*‡
71 (5)	22 ± 13†	8.2 ± 0.9	10 ± 2.7

* All grafts still survive. † All grafts rejected. ‡ Bearing two second-CBA grafts.

Another mechanism of action could be explained by a lack of macrophages, which would alter the conditions under which the organism first met the antigen, or impede the recognition and the processing of antigen possibly necessary to induce immunity (*16*).

Cell counts in mice after 4 weeks of daily treatment with Ro 4-6824 at 300 mg/kg have shown a decrease of the circulating lymphocytes to 30 percent of the initial values. In addition, lymphoid organs such as spleen, lymph nodes, and thymus became aplastic and markedly reduced in size. This chemically induced deficiency of the lymphoid system causes typical wasting of the mice if the treatment is pushed to toxic levels. With the strains and dosage used by me, this deficiency was only occasional, accounting for the overall rate of fatality in the animals, which ranged during and after treatment from 5 to 15 percent; it may be caused by enhanced susceptibility and probably could be reduced by keeping the animals under pathogen-free conditions.

The specificity of the tolerance was clearly apparent when the interval between the first and second grafts amounted to 70 to 80 days. I have some

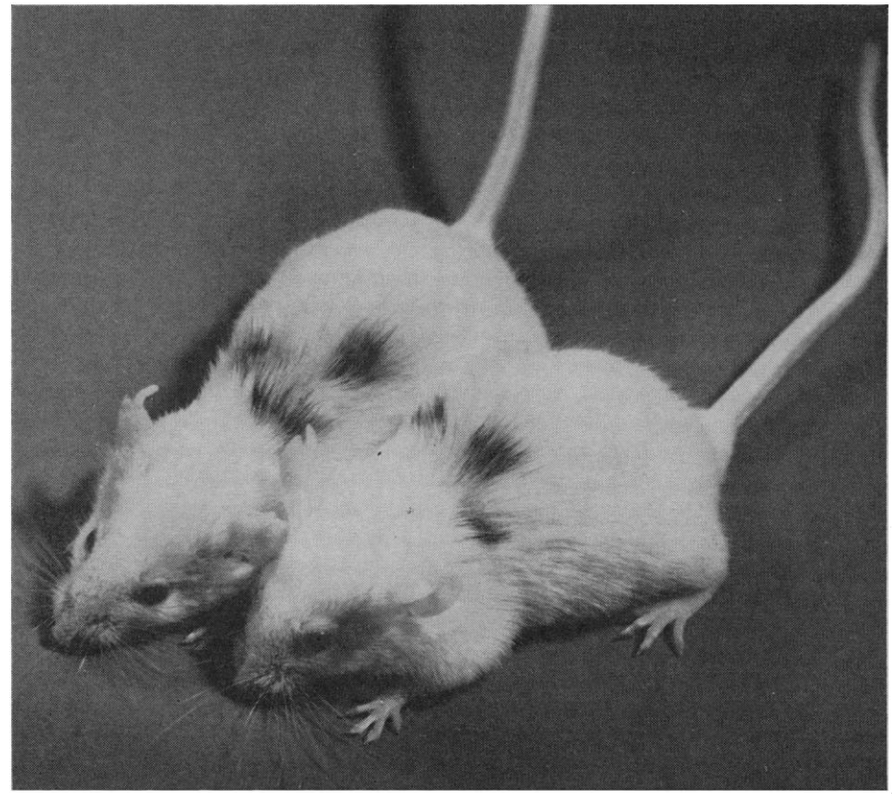

Fig. 1. Two A-strain mice conditioned with Ro 4-6824 and 65 × 10^6 CBA spleen cells on D −12. Each bears a CBA graft applied 139 days earlier (right thorax) and two CBA grafts planted 68 days earlier (left thorax).

indication that with shorter intervals the survival of third-party grafts also may be extended.

As to the relevance of the additional antigen, it may well be true that the antigen constantly released from the surviving grafts suffices to preserve the tolerant state. However, the administration of additional antigen was a prerequisite to lasting tolerance. Whereas the kind of additional antigen administered did seem to affect the outcome less conspicuously in the A ← CBA combination (Table 2), it may well be that still stronger antigenic differences, like the C57Br ← A pairing, are needed to demonstrate the superior efficacy of intravenously applied spleen cells. Both the possible occurrence of chimerism and the importance of the cell dose are now being investigated. The data relating to the role of additional donor antigen for the maintenance of tolerance need further amplification.

Analogous conditioning with other immunosuppressive drugs may produce equal tolerance to allografts. Earlier studies of the chemical suppression of transplantation immunity showed the methylhydrazine derivatives to be the most effective, with cyclophosphamide as the next best (*10, 11*). The described method of inducing tolerance seems to have the advantage over currently available alternatives (actively acquired tolerance, neonatal thymectomy, and lethal irradiation), that the possibility of clinical application should not be ruled out as impracticable.

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5 January 1967

Protection through Parabiosis against the Lethal Effects of Exposure to Large Doses of X-Rays

Abstract. *Parabiotic rat pairs with a skin-vascular anastomosis were used to test whether shielding of one member of the pair would protect the irradiated partner against exposure to very large doses of x-rays (1200 to 2400 roentgens). Except with the lowest dose, all unshielded, irradiated pairs or single irradiated animals died before 5 days had elapsed. In contrast, irradiated rats that had a shielded parabiont partner survived the 5-day period and many survived beyond 30 days. This is interpreted as protection against the acute intestinal death that normally occurs in the dose range investigated.*

During the course of a study aimed at clarifying some of the gastrointestinal disturbances (gastric stasis and decreased food intake) that occur after irradiation, we observed what appears to be a remarkable degree of survival for irradiated members of parabiotic rat pairs if the partners were shielded. This exceptional survival was observed in irradiated rats that had sustained radiation doses reported by others (*1–4*) to cause "acute intestinal radiation death" within 3 to 5 days of exposure in single animals.

These observations led us to formalize an investigation that was designed to test, in unrestrained parabiotic rat pairs, whether shielding of one member of the pair would lengthen the survival time of the irradiated partner exposed in the supralethal dose range.

Male littermates of the specific pathogen-free strain of Sprague-Dawley rats bred at the U.S. Naval Radiological Defense Laboratory were paired by weight on removal from their litter at 24 days of age. The parabiosis operation on certain pairs was usually performed 1 to 2 days later. A skin-to-skin anastomosis was made with the Bunster-Meyer technique (*5*), modified by deleting the abdominal muscle union. Losses among the anastomosed animals, amounting to about 35 percent, occurred during the first 3 weeks from parabiosis intoxication (*6*), with no additional losses following this period. The remaining pairs were maintained, with ad libitum access to food and water, up to the age of 100 days, before use in the experimental design.

At 100 days of age, the pairs were subdivided into the following three treatment groups: parabiont pairs with one animal x-irradiated and the partner shielded (X-C); parabiont pairs with both partners irradiated (X-X); and pairs of animals physically tied together by sutures (but with no anastomosis) with one member irradiated and the other shielded (X/C). Each treatment group contained 15 pairs of animals.

For irradiation purposes, the rats were placed in double-chambered radiation exposure units that were oriented radially on a radiation exposure table at isodose distances from the x-ray target. Members of each experimental group were represented in each exposure run. X-rays were generated by a 250-kv-peak unit operated at 250 kv peak, 25 ma, with 0.5 mm Cu and 1.0 mm Al filter (half-value layer = 1.49 mm Cu). The unshielded partners and the X-X pairs were irradiated with whole-body exposures to 1200, 1500, 1800, or 2400 r at a dose rate of approximately 25 r/min. During exposure, the shielded partner received less than 30 r at the highest exposure level used, primarily through scatter.

After irradiation, the animals were returned to the home cages and maintained with ad libitum access to food and water. All pairs were checked for deaths at 12-hour intervals for the first 10 days and at 24-hour intervals thereafter.

The criterion used in this study to assess protection against acute intestinal radiation death was the percent survival after the 3- to 5-day period of mortality. The percent survival for the period of intestinal death and also for the 30-day postirradiation survival period are shown in Table 1.

It can be observed that a dose of 1200 r does produce some acute (3- to 5-day) intestinal deaths among the X members of the X/C group and in the X-X group. Although not shown in the table, a bimodal distribution of death occurred at this dose level, which would