PRODUCTION OF IMMUNOLOGICAL TOLERANCE IN MICE AFTER REPEATED INJECTIONS OF DISRUPTED SPLEEN CELLS*

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It is now well established that tolerance of tissue homografts can be induced in adult mice of certain strains by performing a parabiotic union between allogenic individuals (1–14) or by the intravenous administration of either a single dose of large numbers of viable spleen cells (7, 15) or by repeated injections of spleen cells intravenously and intraperitoneally over a prolonged period.

The total number of cells injected and the length of time during which parabiosis had to be maintained to bring about a permanent state of immunological unresponsiveness was found to be dependent on the genetic disparity between the donor and the host (16). Tolerance obtained by these methods may be transferred to isologous mice by injecting these animals at birth with a single intravenous injection of approximately 5 million viable spleen cells derived from tolerant donors (17–19). More recently Brent and Gowland (20–22) confirmed and extended our observations that immunological tolerance of allogenic skin grafts could be obtained in adult mice by repeated injections of allogenic viable spleen cells. On the other hand, most examples of acquired immunological tolerance of allogenic tissue grafts brought about either during the immediate newborn period or later in adult life have involved the administration of intact cells obtained from lymphoid tissues. Linder (23, 24), however, has recently demonstrated that tolerance of allogenic skin grafts can be achieved in adult mice differing at weak histocompatibility loci by transplantation of ovarian tissue, and Billingham and Silvers (25) reported a significant prolongation of male skin survival in female mice of the C57B1 strain treated at early age by repeated injections of an antigenic extract prepared from lymphoid tissue of syngenic male mice. More recently Linder (26) induced tolerance of male skin isografts in adult (DBA × C57B1)F_1 female mice using a cell-free preparation taken from male donors. Spleen, kidney, and liver taken from male mice were homogenized and after centrifugation the supernate was used after being previously subjected to freezing and thawing. Of 6 adult females

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receiving 12 intraperitoneal injections of this material over a period of 3 to 4 months, 5 became tolerant and accepted the corresponding male skin isografts permanently. In other donor-host systems such as C57B1 male to C57B1 female in which the recipient females were conditoned by 16 intraperitoneal injections of cell-free material taken from male donors, only 2 out of 8 animals showed acceptance of the male skin isografts. Also, Medawar (27) reported use of cell free preparations of homologous lymphoid tissues to prolong homograft survival in adult mice receiving a single injection of the antigenic material combined with treatment by x-irradiation, amethopterin, or homologous isoantibody.

In this report studies will be presented demonstrating that specific immunological tolerance of tissue homografts can be induced in mice of several strains by treating these animals either at birth or during adult life with a series of injections of large amounts of non-cellular disrupted material prepared by initial mechanical homogenization and then by repeated freezing and thawing. Upon repeated injections of this material both weak and strong histocompatibility barriers in the mouse have been overcome, permitting transplantation of male skin to female mice and allogenic tumor or skin to mice conditioned in this way.

Material and Methods

Mice of the C57B1/1, C57B1/4, C57B1/6, C3H/Bi, A, DBA/2, and Balb/C strains as well as F_1 hybrids resulting from the cross between C3H and A strain were used.

Non-Viable Spleen Cell Preparation.—Adult mice (3 to 5 months of age) were used as spleen cell donors. Animals were killed by an overdose of ether and after cleaning the skin with alcohol the spleens were removed, freed of the surrounding fat, and placed into a tightly fitted glass homogenizer containing 2 to 4 ml of lactate–Ringer's saline solution. After reducing the tissue to a homogeneous paste, the suspension was subjected to rapid freezing and thawing employing dry ice in absolute alcohol for freezing and warm (37°C) tap water for thawing. Four cycles of freezing and thawing were employed. After the last thawing the spleen suspension was brought to a concentration of 1 spleen per ml and strained through 4 layers of surgical gauze. After this treatment the spleen suspension was checked under the microscope for the presence of intact cells following staining with trypan blue. In no instance could intact cells be detected in the spleen suspension prepared in this manner. Additional tests for presence of intact cells in the spleen suspensions consisted of ascertaining whether or not the injection of large quantities of this material into susceptible recipients would elicit graft *vs.* host reactions. Several trials of this kind, injecting spleen homogenates derived from A strain mice into (A × C57B1/1)F_1 hybrids, failed to induce runt disease in the latter animals, demonstrating the absence in the spleen inocula of significant members of immunologically competent cells.

Spleen Injection Schedule.—

(*a*) *Adult mice:* In most experiments, recipient mice received an initial intravenous injection of 1 spleen equivalent of the disrupted cell suspension into the tail vein. Prior to this treatment, mice received an intraperitoneal injection of heparin in dose of 50 U.S.P. units[1] per mouse. The single heparin treatment was found to be essential to prevent the occurrence of sudden death during or immediately after intravenous administration of tissue homogenates.

[1] Liquaemin sodium, Organon, Inc., West Orange, New Jersey.

Following the initial intravenous injection of spleen material, some experimental groups of mice were injected twice a week with the same amount of material by the intraperitoneal route. In other groups injections were performed also twice a week alternating the intravenous and the intraperitoneal routes.

The total number of injections of spleen material varied with the donor-host strain combinations used, and in all experiments treatment was continued after test-grafting for periods of time ranging from 3 to 6 weeks.

(*b*) *Newborn mice:* In all experiments using newborn mice, animals were injected intraperitoneally with the spleen suspension according to the following schedule: during the 1st week of life mice received 0.1 of a spleen equivalent of the disrupted cell suspension divided into 2 injections. One of these was given immediately after birth and the other 3 days later. During the 2nd week the dose of spleen material was increased to 0.2 of a spleen equivalent divided in 2 equal doses injected 3 days apart. During the 3rd week mice were injected with 0.4 of a spleen equivalent also divided in 2 equal doses, and finally, during the 4th week mice were injected with 1 spleen equivalent of tissue divided in 2 equal doses. Skin grafting to test for the tolerant state of treated mice was performed 1 week after weaning or when these animals were approximately 30 days of age.

Skin Grafting.—Skin grafting was performed by the technique routinely used in this laboratory (28) which essentially consists in placing a piece of full thickness abdominal skin taken from the donor onto a skin defect prepared in the back of the recipient. The graft was held in place with silk sutures.

Evaluation of the success or failure of the skin graft was based on gross inspection. Grafts considered successful and reflecting full immunological tolerance showed no signs of inflammation, did not become hard, and grew hair luxuriantly. Rejection of grafts was characterized by an initial take followed by inflammation, induration, hardening of the graft, and ultimate complete slough. Prolonged survival of the graft was considered to be present when the graft showed a delayed onset of the early evidence of rejection.

Tumor Grafting.—The tumor used in these studies was an adenocarcinoma arising spontaneously in a breeder mouse of the A strain. Grafting was performed by placing a piece of healthy tumor into the subcutaneous tissue of the groin. This was done under light ether anesthesia through a small incision of the skin which was closed with a single silk suture.

Grafted mice were inspected 3 times a week for the appearance of new tumors in the site of implantation. Criteria of tumor "take" were appearance of a distinct mass of at least 1 cm in diameter in the site of implantation, and progressive growth of the tumor leading to death of the recipient.

RESULTS

Male Skin Isograft Acceptance in Adult Female Mice of the C57B1/1, C57B1/4, and C57B1/6 Strains after Repeated Injections of Disrupted Male Spleen Cells.—Eichwald and Silmser (29) demonstrated in certain strains of mice, particularly in those of the A and C57B1 strains, a histoincompatibility determined by the sex of the animal. In animals of these strains, whereas skin isografts performed from male to male, female to male, or female to female were always successful, those performed from male to female resulted in a rejection of the male graft with all the characteristics of an allogenic graft-rejection reaction. This phenomenon was interpreted by Eichwald *et al.* (30), Hauschka (31), and Snell (32) as being due to the lack in the recipient female of the isoantigen dependent upon the Y chromosome which is present in the tissues of the donor male mouse. Stud-

ies from this and other laboratories support the concept that the Eichwald and Silmser phenomenon is of immunological nature since immunological tolerance of male syngenic grafts could be achieved in females injected at birth with male spleen cells (33, 34). Furthermore, studies from this laboratory using this system have demonstrated that tolerance can be induced in females even in adult life if male spleen cells are administered in sufficient large dosage in a single intravenous injection (7).

In the present experiments tolerance of male skin syngenic grafts has been produced in adult female mice of the C57B1/1, C57B1/4, and C57B1/6 strains subjected to repeated injections of disrupted spleen cells taken from male donors.

In 1 group of experiments female mice of the C57B1/1 strain 2 to 3 months old were injected intravenously with 1 spleen equivalent of the disrupted cell preparation. Seven days later, injected mice were submitted to grafting of skin from male donors of the same strain. In another group, similar female mice received an initial injection of 1 spleen equivalent of the disrupted spleen cell preparation by the intravenous route, and this was followed 3 days later by a series of intraperitoneal injections of the same amount of antigenic preparation given 3 times per week. Mice of this 2nd group were skin grafted after the 3rd intraperitoneal injection, and similar injections were continued for several additional weeks, the actual number being somewhat variable. Control groups of non-injected females of the C57B1/1 strain were prepared by grafting them with skin derived from male donors.

In another set of experiments adult C57B1/4 and C57B1/6 female mice received an initial intravenous injection of 1 spleen equivalent of the disrupted spleen cell preparation followed 3 days later by a series of intraperitoneal injections of the same amount of material. This treatment was then repeated 3 times a week for 2 weeks. In these 2 groups treated mice were skin-grafted after the 3rd intraperitoneal injection. Groups of non-treated adult female mice of both of these C57B1 sublines were grafted with their corresponding male skin and served as controls.

The results of these experiments are summarized in Table I. It will be seen in the table that in the group of adult female mice of the C57B1/1 strain receiving a single intravenous injection of frozen-thawed spleen suspension taken from male donors, most of the animals rejected the male skin graft as in the group of non-injected controls. By contrast, most of the females injected repeatedly with the disrupted spleen cell suspension accepted and maintained the male skin graft up to the time this report was written (9 months). For all practical purposes these grafts can be considered as permanent, and it can be postulated that the animals have been rendered permanently tolerant of the male skin graft by this method of treatment. Further, the adult female mice of the C57B1 (sublines 4 and 6) prepared by repeated injections of disrupted male spleen cells again showed a high incidence of prolonged survival of male skin grafts and were most certainly rendered tolerant during adult life by these manipulations.

Allogenic Skin Graft Acceptance across Non-H-2 Histocompatibility Barriers.—

Having succeeded in producing what appeared to be a tolerant state with respect to syngenic male-female skin transplantation by treatment of the females with disrupted male spleen cells during adult life, it seemed of interest to attempt to extend these observations to allogenic transplantation barriers. The initial efforts were designed to attempt to overcome the relatively weak non–H-2 barrier represented by the Balb/C and DBA/2 strain combination. Adult DBA/2 and Balb/C strain mice known to by syngenic at the H-2 histocompatibility genetic locus (H-2^d) but allogenic with respect to minor and

TABLE I

Male Skin Isograft Acceptance in Adult Female Mice of the C57B1 Strain (Sublines 1, 4, and 6) Repeatedly Injected with Disrupted Spleen Cells Derived from Male Donors

Host strain	Donor strain	No. of injections*	No. of female mice accepting male skin grafts	
				per cent
C57B1/1 females	C57B1/1 males	1‡	1/10	10
		24§	14/16	87.5
		None	2/19	10.5
C57B1/4 females	C57B1/4 males	6§	6/7	85.7
		None	1/10	10
C57B1/6 females	C57B1/6 males	5§	7/8	87.5
		None	0/8	0

* Each injection consisted in one spleen equivalent/mouse.

‡ Intravenously.

§ First injection, intravenously. Subsequent injection intraperitoneally.

weaker loci, were used. Mice of the DBA/2 were employed as recipients and mice of the Balb/C strain as donors.

In 1 group of experiments DBA/2 mice (2 to 3 months of age) were injected with spleen cell preparations taken from Balb/C donors. The dosage of material employed was 0.5 spleen equivalents per injection. A total of 13 injections was given alternating the intravenous and intraperitoneal routes. The interval between injections was 3 days. After the 5th injection these animals were test-grafted with Balb/C skin, and the treatment continued for 8 additional injections. In another group DBA/2 strain mice of the same age were given a total of 9 injections of spleen material twice a week by the intraperitoneal route at the dose of 1 spleen equivalent per injection. In this instance treated mice were skin-grafted with the allogenic skin after the 4th injection. A 3rd group of adult DBA/2 mice was given an initial intravenous injection of 1 spleen equivalent followed by a series of 7 intraperitoneal injections of the same amount of material twice a week. Also, in this group mice were grafted with Balb/C skin immediately after the 4th injection. Finally, a 4th group of DBA/2 mice was treated according to the same schedule as was used in the preceding group, but in this instance the number of intraperitoneal injections following skin grafting was increased to 14. Thus, in the last group a total of 18 injections of disrupted spleen cell suspension was used.

The results of these experiments summarized in Table II demonstrate that all groups with the exception of that in which the administration of spleen material was performed solely by the intraperitoneal route showed a significant prolongation of the survival time of the allogenic skin graft, as compared to the survival time observed in the group of non-treated controls. Furthermore, in the group receiving a total of 18 injections of the spleen material, 3 of 11 mice (27 per cent) maintained the Balb/C graft for at least 4 months. At this time each animal now apparently tolerant of the Balb/C skin was grafted with skin obtained from C3H donors which was rejected with a vigorous homograft-rejection reaction in

TABLE II

Induction of Tolerance of Skin Homografts by Repeated Injections of Disrupted Spleen Cells in Adult Mice Syngenic at the H-2 Histocompatibility Locus

Host strain	Donor strain	No. of injections	Dose per injection, spleen equivalents	Skin graft acceptance		Median rejection time
					per cent	*days*
DBA/2	Balb/C	13*	1/2	0/13	0	26.3
		9‡	1	0/9	0	15.5
		8§	1	0/10	0	30.0
		18§	1	3/11	27	29.8
		None	—	0/12	0	14.6

* Alternating intravenous and intraperitoneal injections twice a week.

‡ All injections intraperitoneally twice a week.

§ First injection intravenously. Subsequent injections intraperitoneally.

10 to 11 days. In no instance did the rejection of the third party allogenic skin affect the condition of the initial Balb/C graft toward which the tolerant state had been produced.

It is to be concluded from these observations that a series of intravenous and intraperitoneal injections of material obtained from completely disrupted spleen cells can be used to produce both partial and complete tolerance of skin grafts across allogenic barriers in mice. In these studies, to be sure, the barrier overcome was the somewhat weaker non–H-2 allogenic barrier represented by the genetic disparity between the Balb/C and DBA/2 strains. The specificity of the manipulation, however, was apparent since no takes or even prolongation of homograft survival was observed when transplants were made of skin from donors not employed in the original manipulation.

Allogenic Skin and Tumor Graft Acceptance across Strong H-2 Histocompatibility Barriers.—It next seemed pertinent to attempt with this technique to manipulate the strong H-2 histocompatibility barrier presented by the genetic difference at the H-2 genetic locus.

In these studies mice of the C3H, A, and F_1 hybrids resulting from the cross between C3H females and A males were used. Mice of the C3H and A strain differ at the strong H-2 genetic locus ($H\text{-}2^k$ and $H\text{-}2^a$, respectively). In one group of experiments adult C3H mice (2 to 3 months of age) received an initial intravenous injection of frozen-thawed spleen material derived from (C3H × A)F_1 hybrid donors followed by a series of intraperitoneal injections of the same amount of material given twice a week for 5½ weeks (11 injections). Immediately after the last injection in this series of mice, the animals were grafted with skin taken from (C3H × A)F_1 hybrid donors. After skin grafting, treatment was continued with disrupted spleen cells for an additional period of 3 weeks. Another group of C3H mice of this same age was injected twice a week for 8 weeks with disrupted spleen cell material derived from A strain donors, alternating the intravenous and intraperitoneal routes. In this instance animals were

TABLE III

Induction of Tolerance of Skin and Tumor Homografts in Mice Across the H-2 Barrier

Host strain	Donor strain	No. of injections	Incidence of homograft acceptance			
			Skin		Tumor	
				per cent		*per cent*
C3H (adults)	(C3H × A)F_1	18*	2/10	20	—	
	None	—	0/10	0	—	
	A	22‡	1/7	14	4/8	50
	None	—	0/10	0	0/8	0
C3H (newborns)	A	8§	4/7	57	—	

* First injection intravenously. Subsequent injections intraperitoneally.

‡ Alternating intravenous and intraperitoneal injections, twice a week.

§ First week, 10 million cells equivalent, intraperitoneally, 2nd week, 20 million cells equivalent, intraperitoneally, 3rd week, 40 million cells equivalent, intraperitoneally, 4th week, 80 million cells equivalent, intraperitoneally.

grafted in some instances with skin and in other instances with tumor tissue derived from A strain donors. Following transplantation, the disrupted spleen cell treatment was continued for an additional 4 weeks. Finally, a 3rd group of mice of the C3H strain was injected intraperitoneally with disrupted cell material derived from A strain donors beginning at birth. The disrupted spleen cell treatment was given every subsequent week in increasing doses until the time of weaning as was described in the section of Material and Methods. All experimental groups had a corresponding set of non-treated C3H controls grafted with skin from either (C3H × A)F_1 hybrids or A skin or tumor.

The results of this series of experiments are summarized in Table III. In this table it will be seen that a few C3H mice injected repeatedly with nonviable spleen material derived from either (C3H × A) F_1 hybrids or from A strain donors showed lasting tolerance and accepted the allogenic skin grafts. For example, 2 of 10 (20 per cent) of C3H mice prepared by repeated injection during adult life of disrupted spleen cell preparation from (C3H × A) F_1 mice subsequently accepted skin grafts from (C3H × A) F_1 donors. Similarly, 1 of

7 of C3H mice prepared during adult life with disrupted cell preparations from A strain donors became tolerant and accepted an A strain skin graft permanently. Fig. 1 illustrates the A strain skin growing with luxuriant white hair on the brown C3H recipient made tolerant by the repeated injection of completely disrupted spleen cells. Further, 50 per cent of C3H mice pretreated during adult life with disrupted spleen cell preparation accepted allogenic grafts of a mammary adenocarcinoma which had developed spontaneously in A strain hosts. In these instances the allogenic tumor grafts not only showed successful take, but grew progressively and metastasized ultimately causing the death of their tolerant allogenic recipients.

Finally, as will be observed in the table, treatment with disrupted spleen cell material beginning immediately after birth induced tolerance of allogenic skin grafts in 57 per cent of treated C3H mice. By contrast, in no instance were allogenic grafts of A strain skin or tumor accepted in unconditioned C3H mice.

Transfer of Tolerance Induced by Treatment with Disrupted Cells.—Previous studies from this laboratory have demonstrated that tolerance for allogenic tissue grafts induced in mice by the intravenous administration at birth of viable lymphoreticular cells can be transferred to syngenic individuals by injecting these animals during the newborn period with lymphoreticular cells derived from tolerant donors (17). Similar transfer of tolerance was achieved in newborn mice when the cells injected were derived from individuals made tolerant during adult life (13). This transfer of tolerance has been attributed to transfer of viable lymphoreticular cells replicating in the tolerant recipient and reflecting the chimeric state. Since repeated injections of non-viable spleen material into either adult or neonatal mice appeared from the experiments reported herein to result in tolerance of allogenic tissue grafts, it was considered desirable to ascertain whether or not this state of non-reactivity could also be transferred to syngenic mice by injecting the latter animals at birth with viable lymphoreticular cells derived from mice previously made tolerant by injection of disrupted spleen cells.

In 1 group of experiments neonatal female mice of the C57B1/1 strain were made tolerant of syngenic male skin grafts by a single intravenous injection of approximately 5 million viable spleen cells derived from male donors or by the repeated administration of disrupted non-viable male spleen cells following the regular procedure described in this report. Approximately 3 months after test skin grafting, tolerant females from each of these groups were sacrificed, the spleens were removed and made into a cell suspension containing 6 to 10 million viable cells in 0.1 ml of lactate–Ringer's saline solution. These suspensions were then injected intravenously into groups of newborn female mice of the same strain. When these injected mice were approximately 2 months of age they were grafted with skin grafts taken from syngenic male donors, and the incidence of tolerance obtained in each group was determined. Groups of non-injected controls were also prepared and similarly grafted with male skin.

The results of these experiments are shown in Table IV. These results demonstrate that C57B1/1 females made tolerant of male skin by injection at

birth of viable male spleen cells were able to transfer their tolerance to syngenic newborn female recipients. Indeed, such transfer resulted in tolerance of all female animals tested (8 of 8). By contrast, when tolerance of the male skin had been produced during adult life by injection of disrupted male spleen cells tolerance could not be transferred. Further, DBA/2 mice made tolerant at birth by the injection of viable spleen cells obtained from Balb/C donors were capable of transferring this tolerance to syngenic newborn mice by means of their spleen cells in 16 of 19 instances (85 per cent). Again in striking contrast, DBA/2 mice made tolerant during adult life by repeated injections of disrupted

TABLE IV

Attempt to Transfer Tolerance Induced by Disrupted Cells

Host strain	Donor strain cells	Incidence of skin graft acceptance	
			per cent
C57B1/1 females (newborns)	C57B1/1 females tolerant of male*	8/8	100
	C57B1/1 females tolerant of male‡	0/6‡	0
	None	2/19	10.5
DBA/2	DBA/2 tolerant of Balb/C*	16/19‖	84
	DBA/2 tolerant of Balb/C§	0/6‖	0
	None	0/12	0

* Tolerance induced by intact cells at birth.

‡ Grafted with skin from male donors of the same strain.

§ Tolerance induced by disrupted cells during adult life.

‖ Grafted with skin from Balb/C donors.

spleen cells obtained from Balb/C mice were unable to transfer tolerance *via* their intact spleen cells to syngenic newborn recipients in any instance.

These observations indicate clearly that whatever its basis, the specific immunologic negativity induced with intact cells in neonatal animals or in adult life is transferable to newborn syngenic recipients, while that induced during adult life with disrupted spleen cells cannot be transferred in this way.

DISCUSSION

The results of these experiments are of interest for a number of reasons. In the first place it is shown that in newborn as well as adult mice a tolerant state may be produced by repeated injections of large amounts of material derived from completely disrupted spleen cells. In this regard our observations confirm and extend those of Linder (26), and Billingham and Silvers (25). Billingham and Silvers found that a significant prolongation of male skin graft survival may be obtained in C57B1 mice treated in the neonatal period by repeated injections of material prepared from lymphoid tissue of male donors. Linder (26) showed that adult females of the (DBA × C57B1)F_1 strain may be made tolerant of male skin by repeated injections of

disrupted male spleen cells. In our experiments the histocompatibility barrier dependent on the Y chromosome in mice of the C57B1 strain (sublines 1, 4, and 6) was overcome regularly by injecting female mice during adult life with extracts of spleen tissue from syngenic male mice. The preparation of spleen tissue involved mechanical disruption and repeated freezing and thawing. In the experiments where the greatest tolerance-providing effect was obtained the injection of disrupted cell preparations was given by the intravenous route, both prior to and following transplantation of skin. Similar manipulations of recipient adult mice served regularly to prolong homograft survival in strains of mice differing at non–H-2 genetic loci as in the Balb/C and DBA/2 strains. In these strain combinations these manipulations regularly permitted prolonged survival of grafts and in some instances permanent survival of allogenic skin grafts. Even the histocompatibility barrier conditioned by the H-2 histocompatibility locus represented by (C3H × A)F_1 into C3H or A into C3H could be bridged by injections of the disrupted spleen cell preparations in the neonatal period and during adult life. When the tissue transplanted was tumor rather than skin the transplantation barrier was more readily overcome by this form of conditioning. Thus, the conditioning provided by repeated intravenous and intraperitoneal injections of preparations of complete disrupted spleen cells regularly led (*a*) to prolonged homograft acceptance across all genetic barriers studied; (*b*) regular acceptance of male skin by female mice in several C57B1 subline strains; (*c*) frequent acceptance of skin homografts across the weaker non–H-2 histocompatibility barriers; (*d*) regular acceptance of tumor homografts across the strong (H-2) genetic barrier; and (*e*) occasional acceptance of skin homografts across the strong (H-2) histocompatibility barrier. Further, long-lasting tolerance was quite regularly produced even across the strong H-2 genetic barrier by repeated intravenous and intraperitoneal injections of disrupted spleen cell preparations given in the neonatal period. Evidence is presented indicating that like the several other forms of immunological negativity the tolerant state produced during adult life by repeated injection of disrupted allogenic spleen cells is specific. In these experiments, for example, DBA/2 strain mice made tolerant of Balb/C skin by injection of disrupted Balb/C spleen cells were fully capable of rejecting allogenic skin grafts derived from a 3rd party strain (C3H).

The dose of material employed and the route of administration also were important in production of tolerance with disrupted spleen cell preparations. A single intravenous injection of one spleen equivalent of disrupted spleen cells in the C57B1 male-female combination did not induce tolerance of male skin, whereas 4 injections of the same amount of material given prior to grafting together with a series of injections provided following grafting yielded a high incidence of tolerance. Further, the studies employing DBA/2 mice injected with disrupted spleen cells from Balb/C donors indicated that at least 1 injection by the intravenous route is essential to production of a tolerant state with this material. When treatment was provided by the intraperitoneal route alone, complete tolerance was never observed and strikingly the survival of the allogenic skin was not even prolonged. The age of the recipient also appeared to play a role. When injection of disrupted spleen cells from A strain mice were made into C3H recipients the younger animals developed a higher incidence of complete tolerance than was the case when the recipient mice were older. This observation is in keeping with our earlier postulates (35), also stated by others (22), that there is nothing

mysterious about the relative ease of production of tolerant states in embryonic or neonatal life, but rather that this facility is a reflection of the relative cellular deficiencies of the lymphoreticular tissues during the early stages of development.

Comparison of efforts to transfer tolerance to syngenic newborn mice from mice made tolerant with viable spleen cells and with disrupted spleen cell preparations supports our previous contention that long-lasting tolerance produced by injection of viable spleen cells is associated with persistence and replication of cells from the original donor (10, 13, 17–19). The mice made tolerant either as adults or as neonates by injection of the disrupted spleen cell preparations revealed no capacity to transfer the tolerant state to syngenic newborn recipients.

Another point worthy of discussion in regard to these observations is the finding that the tolerant state induced either in neonatal or adult mice by injection of the disrupted spleen cell preparations appears to be very long-lasting and may be considered permanent for all practical purposes. Skin grafts from male to female in C57B1/1 strain have remained in place more than 9 months, and allogenic skin grafts to animals conditioned with disrupted spleen cells have remained in place more than 5 months without signs of deterioration or sloughing. These observations would suggest either that the antigens responsible for the tolerant state are only very slowly metabolized, like the pneumococcal polysaccharide in Felton's experiments (36–39), or that the skin graft itself is able to maintain the tolerant state once tolerance has been established by injection of the disrupted cell preparation.

The observations presented in this paper we consider to provide support for our contention repeatedly formulated that immunologic tolerance of allogenic skin or organ grafts is not a unique phenomenon but is essentially a function of the same central immunologic failure responsible for immunologic paralysis, protein antigen over-loading (40, 41), oral ingestion of haptenic materials (42), and immunological unresponsiveness of soluble protein antigens presented in the neonatal period (43–45). All of these phenomena represent a state of specific immunologic negativity, the basic mechanism of which has not yet been elucidated and continues to demand analysis. The observations, reported herein, that tolerance produced with completely disrupted spleen cells cannot be transferred might be taken as a rather telling argument against Gorman's (46) hypothesis that tolerant states represent positive cellular proliferation of immunologically incompetent clones of cells which possess receptors capable of competing with those of the immunologically competent clones for antigen.

The implications of these observations on approaches to tissue and organ transplantation in higher animals including man are meaningful. Certainly direct application of these findings to facilitate organ transplantation in man is not possible. First of all, the dosage of disrupted cell material needed is prohibitive in any system where virtually unlimited resources of syngenic lymphoid tissue are not available. However, it is most encouraging to note that a tolerant state can be produced with cell-free preparations during adult life. The relative ease of accomplishment of a tolerant state in strain combinations where the histocompatibility barriers are relatively weak portends favorable results in higher forms attributable to a degree of matching of donor and host.

Further, in none of these studies have efforts been made to reduce those lymphoreticular resources of the host capable of positive immunological response. Such manipulation could materially reduce the antigenic requirements and diminish the demand for prohibitively large donor contributions.

Study of the antigenic resources of the donor has in this report been limited to spleen cells, and studies currently in process may reveal additional resources in the potential donor that will narrow the gap between requirement and resources available. Finally, encouragement to pursue this direction of investigation is to be derived from observations of Dresser (47) and Battisto and Miller (48) that the condition of the antigen, and the route of exhibition of antigen may play a crucial role in determining whether tolerance or immunity is produced with even small amounts of antigenic material.

SUMMARY

1. Tolerance of male skin isografts has been regularly produced in female mice of the C57B1 strain sublines 1, 4, and 6 during adult life by repeated injection of completely disrupted spleen cells derived from male donors. The tolerant state is long-lasting since such grafts have remained in place more than 9 months.

2. Prolonged survival of homotransplants of skin has regularly been produced in DBA/2 mice during adult life by repeated injections of completely disrupted spleen cells from Balb/C donors. When injections of disrupted spleen cell material are continued over a sufficiently long period, permanent acceptance of the skin homografts may be obtained between these strains.

3. Immunological tolerance across even the strong H-2 histocompatibility barrier was obtained in the neonatal period and during adult life by repeated injection of disrupted spleen cell preparations. The tolerant state has been revealed by both mammary adenocarcinoma and skin homografting across this strong histocompatibility barrier.

4. In contradistinction to the tolerant state produced by injection of intact spleen cells in neonatal animals or during adult life or that produced by parabiotic union, the tolerance produced by repeated injection of disrupted spleen cell preparations cannot be transferred to syngenic neonatal mice with spleen cells of the tolerant animal.

5. The implications of these findings in transplantation biology and in consideration of the basic nature of tolerance are discussed.

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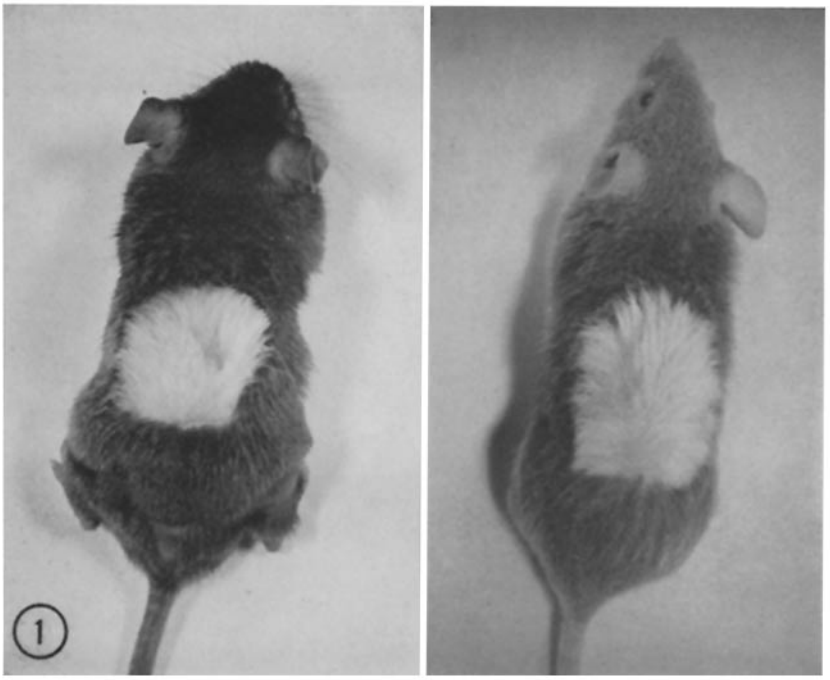
EXPLANATION OF PLATE 80

FIG. 1. Tolerance of allogenic skin grafts induced by repeated injections of disrupted spleen cells. Mouse at right is of the DBA/2 strain made tolerant of Balb/C and mouse at left is of the C3H strain made tolerant of A strain. Picture was taken approximately 3 months after grafting. × 1.



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