THE ABROGATION OF SHEEP ERYTHROCYTE TOLERANCE IN RATS BY MEANS OF THE TRANSFER OF ALLOGENEIC LYMPHOCYTES

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The transfer of thoracic duct lymphocytes from normal syngeneic donors fails to initiate a hemolysin response in rats tolerant of sheep erythrocytes. Although some hemolysin is formed in response to the challenge of sublethally irradiated tolerant recipients of thoracic duct lymphocytes from normal rats, this response is much weaker than that observed in irradiated normal rats similarly resuscitated with lymphocytes. It appears likely that such hemolysin formation in irradiated tolerant rats that have been resuscitated with lymphocytes is attributable to the transferred cells (1). The failure of transferred lymphocytes to abrogate tolerance in unirradiated tolerant rats cannot be dismissed as the inability of the normal cells to colonize the lymphoid tissues of the host. When "normal" lymphocytes are injected intravenously into tolerant rats, many of these cells localize in the host's spleen; they retain their capacity to differentiate for antibody formation provided they do not remain in the tolerant host for more than 3 days (2). After this period, cells with antibody-forming potentiality can no longer be recovered from the tolerant host's spleen. Despite this initial occurrence of antibody-forming cell precursors, differentiated plaque-forming cells cannot be detected in the spleen of the tolerant host at any time after the receipt of normal syngeneic lymphocytes.

In contrast to these previous experiments, the present investigation is concerned with the transfer of allogeneic lymphocytes to tolerant rats. In this situation, a strong hemolysin response may be observed in the tolerant host. This paper describes some of the features of this abrogation of erythrocyte tolerance following the transfer of allogeneic lymphocytes.

Materials and Methods

Rats.—The rats used in these experiments came from two inbred colonies of Lewis and DA strains. (Lewis × DA)F_1 hybrid rats were bred from members of these two strains. The breeding nuclei of both strains were obtained from the Walter and Eliza Hall Institute, Melbourne, Australia; the colonies were based on rats provided by Dr. W. K. Silvers.

Induction and Maintenance of Immunological Tolerance of Sheep Erythrocytes.—Performed as described in a previous paper (1).

Induction and Tolerance of (Lewis × DA)F_1 Hybrid Tissues in Lewis Rats.—Newborn rats of the Lewis strain received approximately 10^8 thoracic duct lymphocytes from a (Lewis × DA)F_1 hybrid donor by the intravenous route. The lymphocyte donor was reserved and used as a source of skin for grafting of the injected Lewis rats at 6 wk of age. If a skin graft was intact 30 days after grafting, its recipient was classified as "tolerant."

Skin Grafting.—6 wk old Lewis rats which had received (Lewis × DA)F_1 hybrid lymphocytes at birth were grafted with skin from the same donor. Full-thickness skin grafts were transferred, under sterile conditions, to the right flank of the host.

Collection of Cells.—Lymphocytes were collected from unanesthetized rats using Gowans' (3) modification of the method of thoracic duct cannulation of Bollman, Cain, and Grindlay (4). Thymus cells were prepared by gently teasing this organ apart in ice-cold Hanks' saline with fine forceps.

Hemolytic Plaque-Forming Cell Assays.—Performed using Cunningham and Szenberg's (5) modification of the technique of Jerne, Nordin, and Henry (6).

Isoantisera Incubation of Cells.—Rat isoantisera were prepared by injecting members of one strain of rat with lymphoid cells from the other strain. Each prospective antiserum donor received an initial intraperitoneal injection of approximately 10^8 thoracic duct lymphocytes from a rat of the other strain, suspended in complete Freund's adjuvant. Two or three subsequent intraperitoneal injections of thoracic duct lymphocytes or spleen cells were given at intervals ranging from 1 wk to 1 month. Antiserum was prepared from blood taken 7 days after the last injection.

When spleen cells were to be incubated with antisera prior to testing for plaque-forming cells the following procedure was followed, each cell suspension being incubated with both anti-DA and anti-Lewis sera. 2×10^7 cells suspended in 0.2 ml of medium 199 were mixed with 0.2 ml of a 1:3 dilution of the specific antiserum in medium 199. 0.2 ml of a 50% mixture of freshly reconstituted lyophilized guinea pig serum (Commonwealth Serum Laboratories, Melbourne, Australia) was added as a source of complement. After incubation at 37°C for 25 min, the cells were counted, washed in ice-cold Hanks' saline to remove antiserum, resuspended at a concentration of 10^7 cells/ml, and tested for plaque-forming cell content. As previously reported (7 and 8), there was a variable degree of nonspecific loss of plaque-forming cells during incubation. No allowance for this loss has been incorporated in the presentation of the results of incubation with isoantisera. Thus, the numbers given for survival of plaque-forming cells/10^6 spleen cells, after incubation of each specimen in Lewis and DA sera, are the results actually obtained.

Hemolysin Titrations.—Performed on sera as described in a previous paper (1).

RESULTS

Rats tolerant of sheep erythrocytes were injected with allogeneic lymphocytes together with sheep erythrocytes. The recipients' spleens were examined subsequently for the presence of plaque-forming cells.

The Effect of Transferring Lymphocytes from Normal Rats of Lewis Strain to Erythrocyte-Tolerant (Lewis × DA)F_1 Hybrid Rats

(Lewis × DA)F_1 hybrid rats, which were tolerant of sheep erythrocytes as a result of repeated injections of this antigen since birth, received thoracic duct lymphocytes from normal Lewis rats and were simultaneously challenged with 10^8 sheep erythrocytes. The spleens of the recipients were examined for hemolytic plaque-forming cells at intervals thereafter. Large numbers of such anti-

body-forming cells were regularly detected (Table I). There did not appear to be any correlation between the numbers of lymphocytes transferred and the yield of plaque-forming cells over the range of lymphocyte dosage tested (3×10^8–11.5×10^8). While larger numbers of lymphocytes were used initially, as few as 10^8 cells were subsequently found to be effective (Table V). When the origin of the plaque-forming cells was tested by means of specific isoantisera, the majority of these cells were found to be of host origin.

TABLE I

The Transfer of Lymphocytes from Lewis Rats to (Lewis × DA) F_1 Hybrid Rats Tolerant of Sheep Erythrocytes

No. of lymphocytes transferred ($\times 10^8$)	Interval before PFC determination	PFC (per spleen)	Origin of PFC as determined by incubation with isoantisera (PFC/10^6 spleen cells)		
			Preincubation	Incubated with anti-DA serum	Incubated with anti-Lewis serum
	hr				
3	89	170,000			
6.2	96	107,000			
7.2	94	340,000			
8	90	720,000			
9.2	94	158,000			
11.5	96	290,000			
2.5	92	166,000	130	1	4
2.5	92	1,625,000	1,260	17	180
4	92	802,000	590	104	102
4	92	434,000	350	13	43

Thoracic duct lymphocytes were administered intravenously, together with 10^8 sheep erythrocytes, to each tolerant rat. Technique of incubation of spleen cells with isoantisera is described in Materials and Methods. Numbers of plaque-forming cells (PFC)/10^6 spleen cells surviving incubation with isoantisera are expressed as determined without any modification for nonspecific loss.

Plaque-forming cells of host (hybrid) type would be expected to be susceptible to both isoantisera used. Any plaque-forming cells derived from the donor (Lewis) should be insusceptible to anti-DA serum. Thus, while incubation with anti-Lewis serum cannot indicate origin of plaque-forming cells, incubation with anti-DA serum will only decrease plaque-forming cells of host origin.

The Effect of Transferring Lymphocytes from Normal Rats of DA Strain to Erythrocyte-Tolerant Lewis Rats

The lymphocytes from parental strain rats, which were transferred to hybrid rats in the preceding experiments, were capable of initiating a graft-*versus*-host reaction directed against the host, but were themselves insusceptible to homograft rejection by the host. The effect of transferring allogeneic lymphocytes which would themselves be subject to homograft rejection by the erythro-

cyte-tolerant host has been tested (Table II). Although prolonged survival of the transferred cells in this situation is not possible, abrogation of host tolerance of sheep erythrocytes was again observed. A similar result was obtained when the direction of lymphocyte transfer was from Lewis to DA, i.e., the opposite direction to the experiments summarized in Table II. The majority of the plaque-forming cells in these experiments were again of host origin.

TABLE II

The Transfer of Lymphocytes from DA Rats to Lewis Rats Tolerant of Sheep Erythrocytes

No. of lymphocytes transferred ($\times 10^8$)	Interval before PFC determination	PFC (per spleen)	Origin of PFC as determined by incubation with isoantisera (PFC/10^6 spleen cells)		
			Preincubation	Incubated with anti-DA serum	Incubated with anti-Lewis serum
	hr				
3.6	92	83,000			
4	108	8,000			
6	89	310,000			
2.5	93	15,700	32	28	5
2.5	93	24,800	27	25	3
3.6	92	158,000	110	88	11
3.6	92	200,000	200	110	19
5.4	92	162,000	202	150	8
6.6	76	380,000	328	214	38
7	86	99,000	166	112	24

The experimental system, apart from the types of lymphocyte donor and host, was similar to that in Table I. Plaque-forming cells (PFC) of donor type should be susceptible to anti-DA serum only, whereas cells of host type should be susceptible only to anti-Lewis serum.

The Effect of Transferring Lymphocytes from Normal (Lewis × DA)F_1 Hybrid Rats to Erythrocyte-Tolerant Rats of the DA Strain

Lymphocytes derived from a hybrid rat are incapable of initiating a graft-*versus*-host reaction in a host of the parental strain. Furthermore, the transferred cells will be themselves subject to homograft rejection by the host. Nevertheless, such lymphocytes are capable of evoking the abrogation of erythrocyte tolerance in parental strain recipients (Table III).

The Effect of Transferring Thymus Cells from Normal DA Strain Rats to Erythrocyte-Tolerant Rats of Lewis Strain

The capacity of thymus-cell suspensions prepared from DA strain rats to evoke production of plaque-forming cells in erythrocyte-tolerant Lewis rats has been tested. Although abrogation of tolerance was effected, the number of plaque-forming cells produced tended to be less than the number produced

following the injection of allogeneic thoracic duct lymphocytes. This occurred even though the numbers of thymocytes transferred were larger (Table IV).

TABLE III

The Transfer of Lymphocytes from (Lewis × DA) F_1 Hybrid Rats to DA Rats Tolerant of Sheep Erythrocytes

No. of lymphocytes transferred (× 10^8)	Interval before PFC* determination	PFC (per spleen)
	hr	
5.7	90	12,000
6	95	450,000
6	110	240,000

The experimental system, apart from the types of lymphocyte donor and recipient, re sembled that of Table I.

* PFC, plaque-forming cells.

TABLE IV

The Transfer of Allogeneic Thymus or Bone Marrow Cells to Rats Tolerant of Sheep Erythrocytes

Tolerant recipient	No. of cells transferred (× 10^8)	Interval before PFC determination	PFC (per spleen)
		hr	
Lewis	10 DA thymus	88	nil
	10	90	3,000
	8.5	92	21,000
	8.5	94	125,000
	10	97	14,000
(Lewis × DA) F_1 hybrid	2 Lewis bone marrow	89	nil
	2 " "	92	1,300
	2 " "	96	4,100
	2 " "	99	1,500

The thymic or bone marrow cells were administered intravenously together with 10^8 sheep erythrocytes to each tolerant rat.

The Effect of Transferring Bone Marrow Cells from Normal Lewis Strain Rats to Erythrocyte-Tolerant (Lewis × DA)F_1 Hybrid Rats

Allogeneic bone marrow cells were of very low efficiency with regard to the abrogation of tolerance (Table IV).

The Effect of Transferring Lymphocytes from Lewis Rats Tolerant of (Lewis × DA)F_1 Hybrid Tissues to Erythrocyte-Tolerant Hybrid Rats

To determine whether genetic disparity between lymphocyte donor and tolerant host is sufficient to ensure the abrogation of tolerance of sheep erythrocytes or, alternatively, whether immunological interaction between two popu-

lations of unrelated cells is required, further experiments were performed. The procedure was identical with that described earlier except for the substitution as the lymphocyte donor of a Lewis rat tolerant of hybrid tissues for a normal rat. At the time of thoracic duct cannulation, the lymphocyte donor had been bearing a graft of hybrid strain skin for 6 wk. Although the skin graft was in good condition at this time, its subsequent contraction indicated that tolerance was not complete. Each of the erythrocyte-tolerant hybrids received from 10^8 to 2×10^8 Lewis strain lymphocytes. Control experiments in

TABLE V

The Effect of Transferring Thoracic Duct Lymphocytes or Spleen Cells from Lewis Rats Tolerant of (Lewis × DA) F_1 Hybrid Tissues to (Lewis × DA) F_1 Hybrid Rats Tolerant of Sheep Erythrocytes

Status of cell donor (with respect to hybrid tissues)	No. of cells transferred (× 10^8)	Interval before PFC determination	PFC (per spleen)
		hr	
normal	1 TDL*	91	85,000
normal	1.5 TDL	91	330,000
normal	2 TDL	91	21,000
tolerant	1.5 TDL	89	nil
tolerant	2 TDL	92	2,400
tolerant	1 TDL	94	900
tolerant	2 TDL	100	1,700
tolerant	1.5 TDL	100	200
tolerant	1.5 TDL	109	nil
normal	3.6 spleen cells	90	124,000
normal	3.6 spleen cells	97	653,000
tolerant	3.6 spleen cells	90	3,700
tolerant	3.6 spleen cells	97	1,200

* TDL, thoracic duct lymphocytes.

Each tolerant recipient received Lewis strain cells plus 10^8 sheep erythrocytes intravenously.

which similar numbers of lymphocytes from untreated Lewis rats were transferred to erythrocyte-tolerant hybrid rats had indicated that abrogation could be effectively achieved with this cell dosage (Table V). In contrast, when lymphocytes from a donor tolerant of hybrid tissues were transferred to erythrocyte-tolerant hybrid rats, very few plaque-forming cells appeared in the spleens of the recipients (Table V). Spleen cells from a Lewis rat tolerant of (Lewis × DA)F_1 hybrid tissues evoked very few plaque-forming cells in the spleens of erythrocyte-tolerant hybrid recipients in comparison with spleen cells from normal Lewis rats (Table V).

The Effect of Transferring Lymphocytes from Erythrocyte-Tolerant Lewis Rats to Erythrocyte-Tolerant (Lewis × DA)F_1 Hybrid Rats

This group of experiments was similar to those described earlier except for the substitution as lymphocyte donors of erythrocyte-tolerant for normal

Lewis rats. Thus, both lymphocyte donors and lymphocyte recipients were tolerant of sheep erythrocytes. If abrogation of tolerance were to require on the part of the transferred lymphocytes some factor specifically required for a hemolysin response that was absent from the cells of the tolerant rat, then the injection of cells from an allogeneic but erythrocyte-tolerant donor should not produce abrogation. However, abrogation of tolerance was found to be at least as marked as in similar tolerant hybrid rats receiving lymphocytes from normal (nontolerant) Lewis rats (Table VI).

TABLE VI

The Effect of Transferring Lymphocytes from Erythrocyte-Tolerant Lewis Rats to Erythrocyte-Tolerant (DA × Lewis) F_1 Hybrid Rats

No. of lymphocytes transferred ($\times 10^8$)	Interval before PFC determination	PFC (per spleen)
	hr	
1.7	93	440,000
2	93	360,000
3.7	94	100,000
6.3	92	87,000
13	96	1,380,000

Thoracic duct lymphocytes plus 10^8 sheep erythrocytes were administered intravenously to each tolerant rat.

DISCUSSION

Thoracic duct lymphocytes from normal rats are incapable of abrogating tolerance of sheep erythrocytes in syngeneic recipients. The possibility that syngeneic lymphocytes transferred to a tolerant host are subject to a specific suppression of the relevant immunological reactivity in the transferred cells has been suggested (2). When this failure of transferred syngeneic cells to break tolerance is considered, abrogation of tolerance by allogeneic cells is surprising. In an attempt to delineate the cellular basis of this abrogation, the origin of the plaque-forming cells was analyzed by means of isoantisera. This technique clearly established that the majority of the plaque-forming cells are derived from cells of the tolerant host. Nonspecific loss of plaque-forming cells during incubation with antisera impairs the quantitative accuracy of the determination. However, the incrimination that plaque-forming cells are of host origin regardless of the strain of host (Table I and II) excludes the possibility of a nonspecific bias towards survival of cells of one particular genotype during incubation.

The demonstration that the plaque-forming cells appearing after the transfer of allogeneic lymphocytes to tolerant rats are of host origin suggests that the mechanism of abrogation observed here differs fundamentally from that operating when syngeneic lymphocytes are transferred to irradiated tolerant

rats. In this latter situation, the hemolysin response appears to be attributable to lymphocytes transferred from the normal donor (1).

Consideration of the variety of strain combinations of lymphocyte donor and tolerant host which result in abrogation of tolerance gives some indication of the nature of this process. The observation that hybrid strain lymphocytes abrogate tolerance in DA rats and DA strain lymphocytes abrogate tolerance in Lewis rats implies that prolonged survival of the transferred cells is not a prerequisite for abrogation. Thus, in both of the preceding situations, rejection of the transferred cells by a homograft reaction would be expected to intervene, probably before the time at which abrogation of tolerance becomes apparent. Furthermore, it will be seen that neither a graft-*versus*-host attack mounted by the transferred lymphocytes, nor a homograft reaction directed against these cells by the host is a common feature of all of the donor–host combinations in which abrogation occurs. The only feature that is common to all of the effective combinations is that there exists the capacity for an interaction between the transferred lymphocytes and tolerant host. Either a graft-*versus*-host reaction or a homograft reaction will suffice.

The observations discussed above indicate that a difference in genotype between lymphocyte donor and tolerant host is required. They do not, however, distinguish between the requirement for a genetic disparity alone and the need for an immunological interaction to occur between lymphocytes and host cells. In most donor–host combinations, both of these requirements would be met simultaneously and, hence, it would not be possible to determine whether both were necessary. A distinction can be drawn between the requirement for genetic disparity alone or for the additional occurrence of immunological interaction by transferring allogeneic lymphocytes from a donor tolerant of the tissues of the erythrocyte-tolerant host. It is apparent that, when lymphocytes from a parental-strain rat tolerant of hybrid tissues are transferred to a hybrid rat, there will be a genetic difference between donor and host but no immunological reaction will occur. When this experiment was undertaken, the parental strain lymphocyte donor appeared fully tolerant of hybrid tissues at the time of thoracic duct cannulation, but subsequent slow cicatrization of its skin graft indicated that tolerance was incomplete. In view of this incompletion, the very marked discrepancy in abrogating capacity when transplantation-tolerant lymphocytes are compared with normal lymphocytes is striking. Cells from a homograft-tolerant donor have little capacity to evoke abrogation. It can be confidently inferred from this observation that the genetic disparity between the transferred lymphocytes and their tolerant host is not of itself sufficient for abrogation. An immunological interaction between graft and host is also mandatory.

The reduced ability of allogeneic thymus cells to abrogate tolerance is in accord with the generally reduced immunological capacity of these cells when compared with thoracic duct lymphocytes. The activity of bone marrow cells

in achieving abrogation correlates with the immunological capabilities of this cell population.

Investigation is being directed to the elucidation of the mechanism of abrogation of tolerance following immunological interaction between transferred lymphocytes and host cells. In one experiment, reported in this paper, the efficacy of lymphocytes transferred from erythrocyte-tolerant, allogeneic donors for abrogation of erythrocyte tolerance in the host was examined. This experiment was intended to test the possibility that, as a result of, and in the course of the immunological interaction between transfused lymphocytes and host cells, a factor specifically required for hemolysin formation could be transferred to the host cells. If the absence of such a factor was responsible for the specific incompetence of tolerant cells, it is evident that lymphocytes transferred from erythrocyte-tolerant donors would be unable to supply it; lymphocytes from such a source would be incapable of abrogating tolerance. That lymphocytes from erythrocyte-tolerant donors effectively abrogate tolerance in allogeneic recipients makes such a hypothesis untenable. It seems most unlikely that transferred allogeneic lymphocytes abrogate tolerance by providing a factor specifically required for a hemolysin response but which is lacking from the cells of the tolerant rat.

Although the mechanism of abrogation of tolerance with allogeneic lymphocytes remains unclear, some inferences can be drawn concerning the cellular basis of tolerance of erythrocytes. When a population of lymphocytes manifests immunological tolerance, this is most commonly considered to be a consequence of the death or irreversible inactivation of all of its cells capable of responding to the tolerated antigen. If this explanation is correct, the abrogation of tolerance will require the reappearance of cells with the relevant reactivity. Such cells may have been present previously in undetectably small numbers or may have arisen spontaneously by mutation. An alternative explanation has been proposed for sheep erythrocyte tolerance of rats (2). This explanation, namely that a population of cells is tolerant because it contains tolerant cells in which a particular reactivity has been repressed, is supported by the current experiments. If tolerance reflects the presence of tolerant cells, its abrogation is most likely to entail the reversal of this condition in individual cells, i.e., their "derepression." Under these circumstances, the sudden appearance of a large population of immune cells might be predicted as a result of derepression of a preexisting population of tolerant cells. The current observations would seem to be more satisfactorily accommodated in a scheme of this type than in one which requires a very small or newly generated population to have undergone rapid multiplication. As an example of the rapid appearance of a large number of plaque-forming cells in a rat previously tolerant, 380,000 such cells were present in the spleen of one Lewis rat 76 hr after stimulation (Table II). Any preexisting population of reactive cells, of which these plaque-forming cells are postulated to be the progeny, would be required to be extremely small

when one considers the consistent inability to detect any plaque-forming cells following antigenic challenge of tolerant rats (2). It is not possible to unequivocally exclude an extremely rapid multiplication of reactive host cells present in undetectably small numbers in the present experiments. However, it seems to be a most unlikely explanation. The conversion of tolerant cells to immune cells could explain the sporadic breakage of tolerance reported when a tolerant population of lymphocytes is maintained in vitro (9).

SUMMARY

Whereas the transfer of lymphocytes from normal syngeneic donors fails to abrogate tolerance of sheep erythrocytes in rats, lymphocytes from allogeneic donors are effective. When tolerance is abrogated in this situation, the hemolysin-forming cells are predominantly of host origin. Immunological interaction between transfused lymphocytes and host cells is a prerequisite for the abrogation of tolerance. From the time required for abrogation to occur after transfer of the allogeneic cells, it is suggested that tolerance of sheep erythrocytes in rats represents the repression of a specific reactivity in cells rather than the elimination or irreversible inactivation of reactive cells. This explanation implies the existence of specifically tolerant cells.

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