ROLE OF PRETHYMIC CELLS IN ACQUISITION OF SELF-TOLERANCE*

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Conceptual and experimental approaches have made it evident that self-tolerance is not laid down genetically but is instead acquired during development and is then actively maintained throughout adult life. The emergence of T-lymphocyte diversity and the acquisition of self-tolerance are presumed to be processes well-synchronized in ontogeny, in order to avoid reactivity against self.

Not only is there a lack of definitive evidence regarding mechanisms leading to self-tolerance, but at which stage of lymphocyte differentiation self-tolerance occurs also remains obscure.

Self-non-self discrimination by T lymphocytes could be achieved at any of three stages, i.e., prethymic, intrathymic, or postthymic. In the first of these stages, i.e., before cells enter the thymus, information about self would be obtained from antigens expressed at that stage by the host, provided that prethymic cells manifest sufficient diversity for recognition of these antigens. In the second stage, information about self would be derived from the H-2 haplotype displayed by the thymus. This proposition stems from views expressed by Burnet (1) and Jerne (2). After T-cell emigration from the thymus, information about self would be obtained from antigens being expressed elsewhere in the animal at that time.

As a model, the thymusless nude mouse provides an exceptional situation where precursors of T cells that would normally develop to mature T cells in the thymus do not find their natural site of homing. Thymus implantation in a nude mouse leads to reconstitution of the animal by host-derived precursor T lymphocytes (3, 4). By the choice of the appropriate time, thymus grafting permits better definition of the significance of the pre-, intra-, and postthymic stages for the acquisition of self-tolerance.

To study these issues, the temporal pattern of skin graft rejection in nude mice implanted with allogeneic or syngeneic thymus at various times before and after skin grafting, and in some cases in nude mice inoculated with F_1 hybrid spleen cells, was investigated. This report presents evidence strongly suggesting that (*a*) the H-2 haplotype of the thymus is not an essential consideration in the acquisition of self-tolerance and (*b*) self-tolerance begins to be programmed in T precursor cells early in ontogeny, i.e., well before these cells enter the thymus.

Materials and Methods

BALB/c mice and congenitally athymic nude mice with BALB/c genetic background (eight–nine backcrosses) were purchased from Bomhaltgaard, Ry, Denmark in 1977 and maintained

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under conventional conditions. Newborn nude mice were obtained by mating BALB/c nu/+ mice. C57BL/6J, BALB/c, and SJL/J mice served as donors of skin and thymus. C57BL/6J and SJL/J mice were obtained from the Institut für Biologisch-Medizinische Forschung, Füllinsdorf, Switzerland. Two or three thymuses from newborn BALB/c or C57BL/6J donors were grafted into the axillary region of nude mice. (BALB/c × C57BL/6J) hybrid mice used as spleen cell donors were bred by mating BALB/c females with C57BL/6J males.

Results

Acquisition of Self-tolerance Can Be Dissociated from the H-2 Complex of the Thymus. Experiment 1 was designed to determine whether thymusless BALB/c mice implanted with allogeneic thymus tolerate skin grafts expressing either their own haplotype or the haplotype of the implanted thymus. To assure the continuous presence of the antigens before and after thymus implantation, skin grafts were performed before thymus grafting. Thymusless nude mice were grafted with C57BL/6J and BALB/c skin simultaneously at 25 d of age. 8–14 d later, the animals were grafted with two or three thymuses of newborn C57BL/6J mice. The results are presented in Table I. In contrast to the short-lived thymusless mice, six out of seven of these thymus-allografted nude mice are still alive and well 1 yr later. None (zero out of six) of the recipients of allogeneic thymuses rejected the BALB/c skin and only two out of seven rejected the C57BL/6J skin 27–29 d after thymus grafting. SJL/J skin transplanted at 2–4 mo after thymus implantation was rejected in all animals between 13–19 d after thymus grafting.

The fact that BALB/c nu/nu animals with allogeneic thymus implants did not reject BALB/c skin, and their longevity as well, suggest that massive anti-BALB/c reactivity was not expressed. This lack of anti-self reactivity cannot be attributed to a mechanism in which the H-2 haplotype expressed by the thymus plays a major role. Thus, it follows that self-tolerance was induced either at the prethymic or postthymic cell stage.

The establishment of tolerance to C57BL/6J skin in the majority of the animals could have occurred before, during, or after the implantation of the C57BL/6J thymus. The rejection of C57BL/6J skin in two animals indicates that in this instance, postthymic competent T cells of these animals were not rendered tolerant despite the continuous presence of the antigen.

Recently Emigrated Postthymic Cells Can Discriminate between Self and Non-Self. It was conceivable that recently emigrated postthymic cells upon encountering antigens then present in the host would be rendered tolerant. Accordingly, two kinds of experiments were designed to explore this issue.

Experiment 2. 25-d-old thymusless BALB/c mice were grafted with C57BL/6J skin. 10–20 d later, the animals were grafted with two or three thymuses from newborn BALB/c animals. All of a total of six animals rejected the C57BL/6J skin graft between 14 and 47 d after thymus implantation (Table II).

Experiment 3. 25-d-old thymusless nude mice were injected intravenously with 100–120 × 10^6 (C57BL/6J × BALB/c)F_1 hybrid spleen cells. 8–15 d later, two to three thymuses from newborn BALB/c animals were implanted and 15–26 d later, C57BL/6J skin was grafted (Table III). 10 out of 12 animals rejected the C57BL/6J skin between 10 and 41 d after skin transplantation. The control group consisted of nude mice that had been implanted with two to three newborn BALB/c thymuses at 31–45 d of age and 18–21 d later, grafted with C57BL/6J skin. Nine out of nine animals rejected the allografted skin between 14 and 37 d after transplantation.

TABLE I

Host's Own Haplotype in the Thymus Is Not Required for Acquisition of Self-tolerance

Experiment number	Protocol: Age at which successive treatments were applied to BALB/c nu/nu	Protocol: Treatment	Results: C57BL/6J skin graft survival (d after thymus grafting)	Results: BALB/c skin graft survival* (d after thymus grafting)
1	Birth	—	27	ND‡
	↓		29	§
	24–26 d	C57BL/6J and BALB/c skin graft	327‖	327‖
	↓		336‖	336‖
			340‖	340‖
	32–44 d	C57BL/6J thymus graft	365‖	365‖
			413‖	413‖

* SJL/J skin grafted 2–4 mo after thymus implantation was rejected between 13 and 19 d.

‡ ND, not done.

§ This animal died 11 d after rejection of the C57BL/6J skin graft.

‖ Skin still not rejected.

The aforementioned results of experiments 2 and 3 show that even though the postthymic cells had the opportunity to encounter the C57BL/6J haplotype soon after leaving the thymus, they were not tolerant to this antigen. Clearly, early postthymic cells are already capable of discriminating self from non-self.

The results of experiments 2 and 3 can also be interpreted in the light of the outcome of experiment 1, where the majority of the adult nude mice implanted with newborn C57BL/6J thymus proved to be tolerant to C57BL/6J skin grafts. It seems therefore, that in this particular situation, the conditions of syngeneicity between the thymus and the skin graft were relevant. However, Kindred (5) has reported that a large proportion of BALB/c nude mice grafted with C57BL/6J skin syngeneic with the thymus implanted before, rejected the skin. Furthermore, in BALB/c nude mice implanted with AKR thymus, it had been found that AKR skin grafted later was rejected in 100% of the animals tested (6). In both cases, syngeneicity between thymus and the grafted skin reflected conditions insufficient for induction of tolerance. Related observations in neonatally thymectomized mice have also been reported (7). Thus, it is not a general rule that skin syngeneic with the thymus implanted before is tolerated. The difference in our model (experiment 1) where five out of seven BALB/c nude animals tolerated C57BL/6J skin grafted before thymus implantation, is that this tissue was presented at a time when only prethymic cells were present, which in addition contacted the C57BL/6J H-2 haplotype of the thymus during their intrathymic stage. Consequently, it seems that, in adult, thymus-grafted nude mice, tolerance is developed to antigens that were not present since early life, provided that cells of the T lineage contact them before and during the intrathymic stage.

Tolerance Induction in Newborn Nude Mice. From the results of experiment 1, it can be inferred that information about self is not obtained from the H-2 haplotype of the implanted thymus. From experiments 2 and 3, it can be concluded that whereas the nude's own haplotype is taken as self, introduction of new antigens into an adult nude mouse does not result in a situation in which the new antigens (C57BL/6J) are treated as self components by adult prethymic cells or recently emigrated postthymic

TABLE II

Adult Nude Mice Can Reject Allogeneic Skin Grafted before Thymus Implantation

Experiment number	Protocol		Results
	Age at which successive treatments were applied to BALB/c nu/nu	Treatment	C57BL/6J skin graft rejection (d after thymus grafting)
2	Birth	—	14
	↓		14
	23–26 d	C57BL/6J skin graft	32
	↓		41
	↓		41
	33–46 d	BALB/c thymus graft	47

cells. Consequently, nude mice perceive self before thymus implantation, that is, at a time when they have only immature precursors of T cells. The decisive point seems to be the presence of antigens early in ontogeny. Accordingly, it could be predicted that nude mice, even though they possess only prethymic T-cell precursors, pass through a stage in their development in which they manifest a distinctive susceptibility for induction of tolerance. The data of experiment 4 confirm this prediction.

Experiment 4. BALB/c thymusless mice were injected at birth intravenously with 8–21 × 10^6 (C57BL/6J × BALB/c)F_1 hybrid spleen cells. 9–16 d later, two to three thymuses from newborn BALB/c mice were implanted and 20–26 d later, C57BL/6J skin was grafted (experiment 4a, Table IV). Four out of four animals injected with 8 × 10^6 hybrid spleen cells have retained their grafts for >100 d, three of these four, for >200 d. Among other newborn nudes injected with 15–21 × 10^6 hybrid spleen cells and grafted with BALB/c thymuses, three of five animals have retained the C57BL/6J skin grafts for 400 d (two rejected them after 54 and 56 d, respectively). These results show that early exposure of thymusless nude mice to antigens of F_1 hybrid cells can result in long-term tolerance.

To verify immunocompetence in the grafted animals that had not rejected the C57BL/6J skin after 3–4 mo, these animals still carrying the C57BL/6J graft were additionally transplanted with SJL/J skin. Prompt rejection of the SJL/J skin grafts occurred in all animals between 13 and 22 d.

In the control group (experiment 4b, Table IV) not injected with hybrid spleen cells at birth, five out of five animals rejected the C57BL/6J skin between 11 and 20 d.

When normal thymus-bearing littermates of the nudes used in experiment 4a were injected with 8 × 10^6 cells from the same suspension, early rejection (9–13 d) of the C57BL/6J skin occurred in 10 out of 11 animals (experiment 4c, Table IV). With higher doses, there was moderate prolongation of skin graft survival (33–82 d); this was, however, still of much shorter duration than in the nudes in experiment 4a.

The fact that nude mice are more readily rendered tolerant than normal thymus-bearing mice provides further confirmation that, apart from the ability of prethymic cells to recognize antigen, their early contact with antigen results in the induction of

TABLE III

Adult Nude Mice Are Not Rendered Tolerant by F_1 Spleen Cells Injected before Thymus Implantation

Experiment number	Protocol		Results
	Age at which successive treatments were applied to BALB/c nu/nu	Treatment	C57BL/6J skin graft rejection (d after skin grafting)
3	Birth	—	10
			12
			12
			13
			15
	23–26 d	(C57BL/6J × BALB/c)F_1 cells*	15
			17
			25
	33–40 d	BALB/c thymus graft	28
			41
			146
	53–65 d	C57BL/6J skin graft	370‡
3 (control)	Birth	—	14
			14
			14
			15
			15
	31–45 d	BALB/c thymus graft	17
			17
			26
	53–65 d	C57BL/6J skin graft	37

* 100–120 × 10^6 hybrid spleen cells were injected intravenously.

‡ Skin still not rejected.

neonatal tolerance. A further conclusion emerges from comparing the data on neonatal tolerance induction in nude mice with those in experiment 3, where 23- to 26-d-old nude mice were injected with large amounts of F_1 hybrid cells. As these animals were not rendered tolerant, adult prethymic cells are therefore more refractory to tolerance induction.

Discussion

The findings emerging from this investigation point to the lack of an essential role for the thymus in the process by which T cells develop the capability of discriminating between self and non-self. It is also considered evident that this process begins early in ontogeny, at the stage of prethymic T cells in the situation of the nude mouse. However, it is conceivable that for acquisition of self-tolerance in a normal thymus-bearing animal, prethymic and intrathymic stages may overlap in ontogeny. Should such overlap actually occur, it is visualized that the thymus would participate, not because it provides an unique situation for establishment of self-tolerance, but rather because precursor T lymphocytes migrate naturally into this organ where they also

TABLE IV

Nude Mice Can Be Rendered Tolerant by F_1 Spleen Cells Injected at Birth; their Susceptibility to Tolerance Induction Is Higher than in Normal Thymus-bearing Littermates

Experiment number	Strain	Protocol		Results	
		Age at which successive treatments were applied to BALB/c nu/nu	Treatment	Number of F_1 cells received at birth*	C57BL/6J skin graft survival (d after skin grafting)‡
				× 10^6	
4a	BALB/c nu/nu	Birth	(C57BL/6J × BALB/c)F_1 cells	8	103
				8	229
				8	231
				8	285§
		9–16 d	BALB/c thymus graft	15	56
				15	384§
				21	54
		29–36 d	C57BL/6J skin graft	21	397§
				21	397§
4b (control)	BALB/c nu/nu	Birth	—	—	11
				—	12
		10–11 d	BALB/c thymus graft	—	12
				—	12
		30 d	C57BL/6J skin graft	—	20
4c	BALB/c	Birth	(C57BL/6J × BALB/c)F_1 cells	8	9
				8	9
				8	9
				8	9
				8	9
				8	11
				8	11
				8	12
				8	13
				8	13
				8	43
		29–34 d	C57BL/6J skin graft	15	56
				21	33
				21	33
				21	82

* (C57BL/6J × BALB/c)F_1 spleen cells were injected intravenously into newborn mice.

‡ SJL/J skin was grafted to those mice which had not rejected the C57BL/6J skin graft 2–4 mo after thymus implantation. SJL/J skin was rejected in all animals between 13 and 22 d.

§ Skin still not rejected.

happen to encounter self-antigens expressed there. In fact, because in the thymusless nude mice the prethymic cells have no opportunity to contact thymus antigens, they learn self elsewhere early in life as attested by our experiments.

Expression of self-antigens occurs to a high degree during early life. It is therefore a reasonable expectation that during the generation of diversity of the T-lymphocyte repertoire and at the time of maturation of effector cells, the processes leading to tolerance to self-antigens have to operate very efficiently. The proposal by Jerne (2) that generation of diversity by T cells and self-tolerance could be achieved by one

and the same process in the thymus is obviously relevant to the present work. His theory assumes that during their intrathymic stage, cells are able to recognize self-antigens displayed by the thymus and will be excluded from the future repertoire of T cells unless they mutate. By this means, self would be learned in the thymus. However, as experiment 1 (Table I) has demonstrated, thymusless BALB/c mice grafted with BALB/c skin and later implanted with the allogeneic C57BL/6J thymus tolerated the BALB/c skin and even after >1 yr continue to survive in a healthy state. Accordingly, acquisition of self-tolerance is quite separable from information expressed by thymic antigens. Apart from this dissociation, our experiments also indicate that discrimination between self and non-self is an event initiated as early as at the prethymic stage. The data thus imply that prethymic cells display ample diversity for such discrimination.

Even though anti-self reactivity starts to be filtered out as early as the prethymic stage, T cells generated subsequently still have to recognize self so they can cooperate specifically with cells of the same haplotype or be reactive against foreign antigen presented or expressed together with self H-2 antigens. Consequently, a mechanism is required by which cells that retain affinity for self and which have deviated from their normal developmental pathway or are actively suppressed (without loosing affinity for self) would be still available after contact with self-antigens early in life.

The still-controversial proposal (8–12) has been made that the thymus imposes allogeneic restriction on T cells and, as originally conceived by Jerne, acts as a mutant-breeding organ, generating T-cell diversity. Were that the case, these phenomena should have their basis in selection by thymus antigens of those cells which, although tolerant, can still recognize these antigens. To ascertain whether restriction and diversity is linked with the H-2 haplotype of the thymus, fetal liver cells or T-depleted bone marrow (A × B)F_1 cells have often been transferred to irradiated A hosts. It becomes important to define why in these chimeras the B haplotype, which is not present in the host, is tolerated just as is the A haplotype. Blanden and Ada (13) proposed that "a suppressive backlash must occur either in the thymus or in secondary lymphoid tissues, or in both." Our data, on the other hand, are in harmony with the view that the (A × B)F_1 cells of the inoculum were already educated to tolerate B as self, just as well as A.

Neonatally induced tolerance does not result in the generation of cells that can either cooperate with cells displaying the tolerated H-2 haplotype (14) or kill virally infected targets expressing this haplotype (15). It seems to us that these facts reflect a dissociation of the mechanisms leading to self-tolerance from those of allogeneic restriction and the generation of diversity.

A substantial body of contemporary work has ascertained that the thymus accounts for a number of basic immunobiologic functions, viz., production of immunocompetent T cells, self-tolerance, imposition of allogeneic restriction, and generation of diversity. This implies that prethymic cells would still have to learn, in the thymus, most of the functions a T cell can perform. However, our findings in nude mice show that at least one presumed thymic event, that of the acquisition of self-tolerance, begins to occur at the prethymic stage, quite independently of this organ.

Summary

The sequential character of T-lymphocyte development as it pertains to the stage at which self-tolerance is acquired was investigated. Three phases were studied,

defined here as prethymic, intrathymic, and postthymic as determined by the timing of thymus implantation. The model utilized was the temporal pattern of skin graft rejection in thymusless BALB/c nude mice implanted with allogeneic, C57BL/6J, or syngeneic thymuses before or after skin grafting; in some instances, F_1 hybrid spleen cells were also given to newborns or young adults. These experiments in nude mice showed that, (*a*) self-tolerance could be established despite the absence of the host's own haplotype in the implanted thymus; (*b*) recently emigrated postthymic cells could already discriminate self from non-self; (*c*) specific neonatal tolerance could be induced in nudes by inoculation of F_1 hybrid cells; (*d*) nudes showed a higher capacity for induction of neonatal tolerance than did normal littermates. These findings indicate that the process of self-tolerance in the T cell's lineage begins during the prethymic state early in ontogeny.

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