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Immunology

Functional clonal deletion in immunological tolerance to major histocompatibility complex antigens

(T lymphocyte clones/cytotoxic lymphocyte precursors/T cell growth factor/suppressor T cells)

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ABSTRACT **CBA ($H\text{-}2^k$) mice were rendered tolerant to $H\text{-}2^d$ antigens by injection of (CBA × BALB/c)F_1 spleen cells at birth. At intervals of 2 days to 12 weeks, the frequencies of anti-$H\text{-}2^d$ cytotoxic T lymphocyte precursor cells (CTL-P) in thymus and spleen were determined by using a limiting-dilution microculture assay system for CTL-P. This assay, utilizing irradiated $H\text{-}2^d$ stimulator cells and concanavalin A-induced spleen cell conditioned medium, was shown to be linear over the range 30 to 100,000 responder cells and uninfluenced by IJ-positive cells. A profound and long-lasting deficit in activatable CTL-P, first demonstrable by day 5 of life in the thymus and day 8–10 in the spleen, developed in mice rendered tolerant, reaching a >95% reduction by 6 weeks. Functional clonal deletion thus seems to be at least as important in the tolerant state as suppressor T cells. Repeated *in vivo* administration of anti-IJ^k serum partially inhibited clonal deletion, suggesting either that suppressor T cells are actively involved in producing clonal deletion or that IJ^k-bearing cells in the donor inoculum or the host represent an important factor.**

The experimental validation of the concept of immunological tolerance (1) involved induction of tolerance in immature mice to the antigens of the major histocompatibility complex (MHC). Originally, it seemed logical to ascribe tolerance to an elimination of potentially self-reactive lymphocyte clones through early contact with antigen, a view that has subsequently received substantial (2, 3) but not unchallenged (4, 5) experimental support. Since the discovery of suppressor T lymphocytes (6), it has been amply documented that these play a role in several different models of tolerance involving MHC antigens (7–13). Given that a lymphocyte population containing suppressor T cells may effectively inhibit the activation of normal immunocompetent cells ("infectious tolerance"), it becomes a matter of some difficulty to determine the presence or absence of functional T lymphocytes within a population whose phenotype is suppressor. In recent analyses of B lymphocyte tolerance (14–16), our approach has been to isolate the B cell from various regulatory influences of the immune network, including suppressor T cells and antibody-mediated feedback, by culturing B lymphocytes either individually or in very small numbers, employing limiting-dilution techniques. In recent years, elegant methods have been devised that allow the development of clones of cytotoxic lymphocytes (CTL) from single CTL precursor cells (CTL-P) (17–20). In this report, we have used this approach to investigate the early, inductive phase of tolerance to MHC antigens in newborn mice.

MATERIALS AND METHODS

Tolerance Induction. Mice used were CBA/CaHWehi, BALB/c AnBradleyWehi, C57BL/6J Wehi, and their hybrids. Cell suspensions were prepared as described (20, 21). In the standard tolerance protocol, CBA mice received 5×10^7 adult (CBA × BALB/c)F_1 spleen cells within 24 hr of birth. Control mice received (CBA × C57BL)F_1 or CBA spleen cells, or saline.

Preparation of Concanavalin A-Stimulated Spleen Cell Conditioned Medium (CAS). See ref. 22. Wistar rat spleen cells (10^7 per ml, 30 ml) were incubated (37°C, 2 hr) with serum-free RPMI 40 medium containing concanavalin A (Pharmacia, Sweden) at 5 μg/ml such that the bulk of the spleen cells settled and adhered. The supernatant was decanted, and the adherent cells were washed twice and then incubated (37°C, 18 hr) in mitogen-free RPMI 40 medium. The supernatant medium (CAS) containing T cell growth factor (TCGF) and other interleukins was centrifuged twice to remove cells and debris, stored at −70°C, and sterilized by filtration just prior to use. The final CAS concentration in microcultures (usually 10–15%, vol/vol) was predetermined for each batch as measured by optimal CTL clone development from normal adult splenic T cells. Preliminary studies showed that α-methyl mannoside addition in no way affected the final lysis procedure.

CTL-P Assays. Limiting-dilution microculture CTL-P assays were modifed from Ceredig (20). Briefly, 30–100,000 responder CBA ($H\text{-}2^k$) spleen or thymus cells were cultured (37°C, 7 or 8 days, 10% CO_2 in air) with 3×10^5 x-irradiated [3000 roentgens (0.77 coulombs/kg)] stimulator BALB/c ($H\text{-}2^d$) spleen cells in 0.2ml of medium supplemented with 10% (vol/vol) fetal calf serum and CAS. Each culture was individually assayed for its capacity to lyse 10^4 ^{51}Cr-labeled P815 × 2.1 ($H\text{-}2^d$) target cells. Minimal cytolytic activity was detected when cultures were assayed against ^{51}Cr-EL4.1 ($H\text{-}2^b$) target cells. The frequency of CTL-P was determined by Poisson analysis as described in detail by Lefkovits and Waldmann (23), using lysis exceeding background by three standard deviations to define a positive clone.

Anti-IJ^k Serum. This serum, prepared in B10.A(3R) mice against B10.A(5R) cells, was the generous gift of J. F. A. P. Miller and J. Gamble.

RESULTS

Validation of the CTL-P Assay. Antigen-presenting cells and helper T lymphocytes are required for the activation of anti-MHC CTL-P and the development of CTL clones. Recent evidence (18, 22, 24) indicates that these functions can be circumvented by addition of TCGF, so that in the presence of adequate numbers of irradiated stimulator cells, the presence of CTL-P of the requisite specificity becomes limiting. Cells from thymus, spleen, or both of CBA mice that had received 5×10^7 (CBA × BALB/c)F_1 spleen cells at birth (tolerant mice) or various

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Abbreviations: CAS, concanavalin A-stimulated spleen cell conditioned medium; CTL, cytotoxic T lymphocyte(s); CTL-P, cytotoxic T lymphocyte precursor cell(s); MHC, major histocompatibility complex; TCGF, T cell growth factor.

controls were used as a source of CTL-P. Usually, controls had received irrelevant (CBA × C57BL)F_1 spleen cells. To control for possible contributions by donor cells to host CTL-P numbers, some controls received no cell inoculum at birth, and for possible influence by neonatal host-versus-graft reactions, other controls received syngeneic CBA cells. No systematic differences were noted among the three sets of controls. Preliminary experiments using the fluorescence-activated cell sorter showed that an inoculum of 5×10^7 semiallogeneic directly fluoresceinated (25) spleen cells distributed in the newborn host such that, 24 hr later, 1 per 1000 thymus and 40 per 1000 spleen cells were of donor origin. Mice were killed 2 days to 12 weeks after putative tolerance induction, and the frequency of anti-H-2^d CTL-P was determined by limiting dilution, examples being shown in Fig. 1. Linearity was maintained even when responder cell numbers were reduced to 30 per culture (normal mice) or 300 per culture (tolerant mice) in experiments (data not shown) using very large numbers of replicates, indicating that only a single variable, namely presence or absence of a CTL-P, determined the development of a CTL clone.

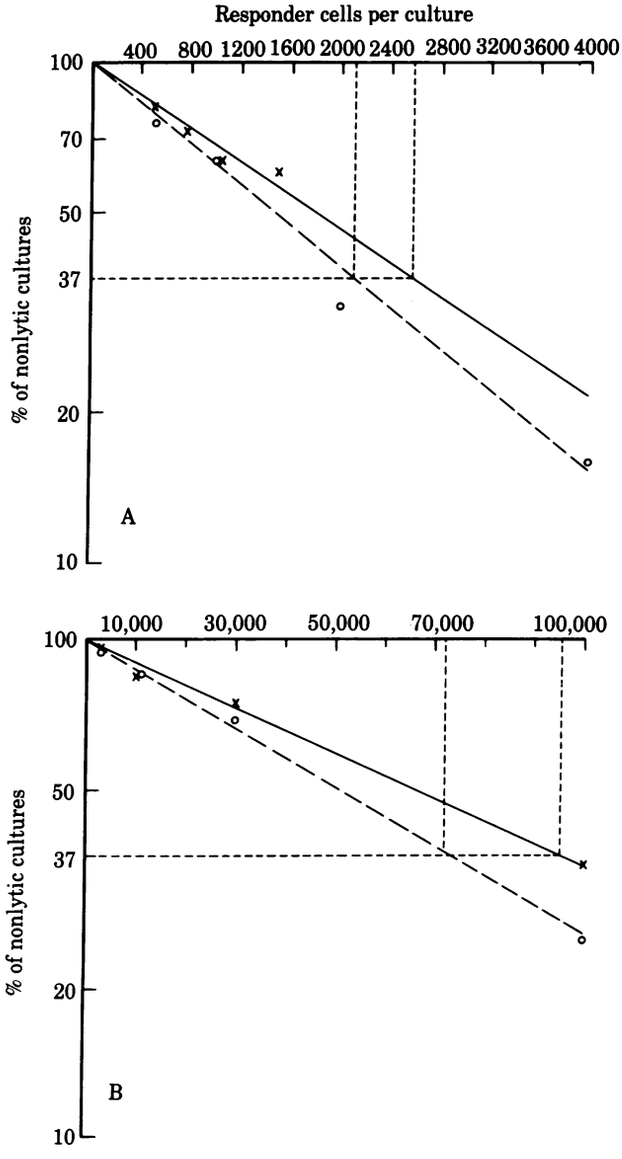

FIG. 1. Linear regression curves relating percent negative cultures to input responder cell numbers. (*A*) Control: ○, 4-week spleen (1 in 2100) and ×, 6-week thymus (1 in 2600); (*B*) tolerant: ○, 10-week thymus (1 in 95,000) and ×, 12-week thymus (1 in 72,000). The numbers in parentheses are the incidence of CTL-P.

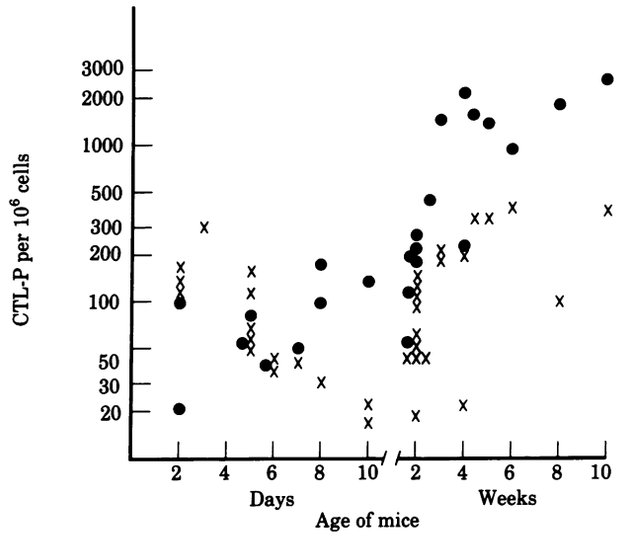

FIG. 2. Frequency of anti-H-2^d CTL-P in thymus (×) and spleen (●) of control mice of various ages.

Frequency of CTL-P in Control Mice of Various Ages. Fig. 2 gives the results of 34 limiting-dilution experiments with thymus and 24 with spleen cells from control mice. Despite substantial individual variation between experiments, a clear pattern emerged. The frequency of CTL-P in thymus decreased at 2–10 days of age but then increased to reach 100–400 CTL-P per 10^6 cells at around 4 weeks. The relatively high frequency near birth agrees with prior work on the graft-versus-host reaction (26). In spleen, the frequency of CTL-P was 20–100 per

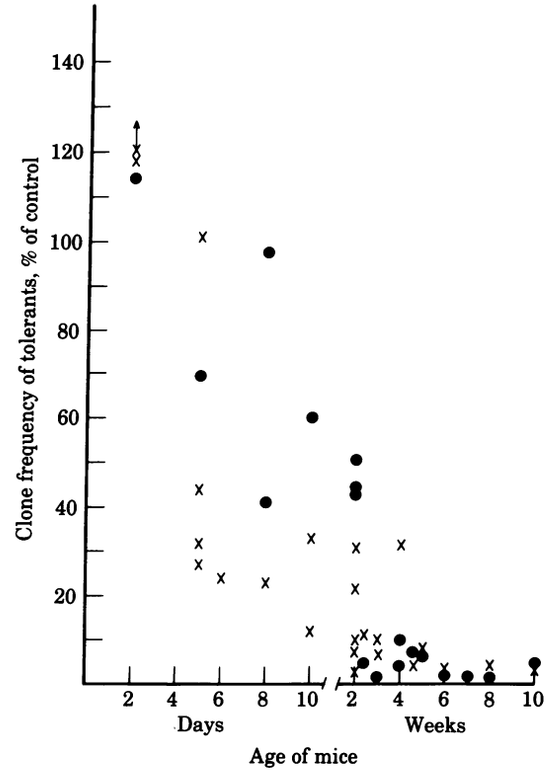

FIG. 3. Kinetics of reduction of CTL-P frequency in thymus (×) and spleen (●) of tolerant mice.

Table 1. Percent specific lysis induced by positive clones*

Exp.	Age, weeks	Organ	Control No. of clones	Control % lysis	Tolerant No. of clones	Tolerant % lysis	P value for difference†
1	2	Spleen	7	17.6 ± 7.5	16	4.3 ± 0.66	<0.05
2	2	Spleen	15	16.2 ± 4.3	20	5.1 ± 0.56	<0.005
3	2	Thymus	12	13.8 ± 3.3	8	12.6 ± 5.6	NS
4	4	Thymus	13	11.9 ± 2.9	13	10.0 ± 2.3	NS
5	6	Thymus	18	19.1 ± 4.5	25	9.1 ± 1.3	<0.01

Percent lysis is presented as mean ± SEM.

* Clones were from cultures in which the clone frequency was sufficiently low for negligible incidence of clonal overlap.

† There was no systematic effect of age or organ source on the significance of the differences between tolerant and control. The five experiments are broadly indicative of our total experience. NS, not significant.

10^6 over the first 2 weeks of life, and then it rose to reach adult levels of 1000–2500 per 10^6 by 4 weeks or shortly thereafter. These frequencies are higher than those previously reported from our laboratory (20) for systems without TCGF addition but are in broad agreement with recent reports (e.g., ref. 27).

Frequency of CTL-P in Tolerant Mice. In order to establish the optimal number of semi-allogeneic cells for tolerance induction, newborn mice received inocula of 10^6–10^8 cells and the thymic CTL-P numbers were determined 4 weeks later. Control mice showed a frequency of 193 CTL-P per 10^6 thymus cells. With the lowest cell dose (10^6), this was reduced to 83.6 per 10^6 (−57%). Inocula of 10^7 cells reduced thymic CTL-P numbers to 41.7 per 10^6 (−68%), 5×10^7 cells to 9.7 per 10^6 (−95%), and 10^8 cells to 8.4 per 10^6 (−96%). For subsequent experiments, 5×10^7 cells were used as the tolerizing inoculum. The frequency of functional, competent CTL-P in the thymus and spleen of tolerant mice is given in Fig. 3, expressed as a fraction of control value in order to minimize the variability in culture and assay conditions. No reduction in clone frequency is present at day 2. By day 5, the clone frequency for thymus is reduced in 3 out of 4 experiments. This deficit becomes more marked and profound thereafter, being down to 3–8% of control values at all points after 4 weeks. The reduction in spleen occurs somewhat later but is equally profound.

Lytic Capacity of CTL Clones in Tolerant Mice. CTL clones exhibit great variation in their lytic capacity, probably mirroring variability both in numbers of effector CTL and in their avidity for target cells (Table 1). Frequently, but not always, the CTL clones from tolerant mice were significantly less lytic than controls.

Effects of Anti-IJ^k Serum and Complement on CTL-P Frequency. Because the observed CTL clone numbers developing in cultures from tolerant mice did not depart from linearity, even in cultures containing as few as 300 responder cells, it seemed unlikely that suppressor T cells could be affecting the clonal readout system for CTL-P. However this possibility was addressed by treatment of control and tolerant cell populations with anti-IJ^k serum and complement prior to culture. Table 2 shows that such treatment did not affect CTL-P frequency.

Table 2. Effects of anti-IJ^k and complement treatment *in vitro* on CTL-P clone frequency analysis in tolerant mice*

Status of mice	*In vitro* treatment	CTL-P per 10^6 cells
Nontolerant	Control	76.4
	Anti-IJ and complement	81.8
Tolerant	Control	18.1
	Anti-IJ and complement	16.0

* Thymus cells from 2-week-old mice were used. The results are the mean of two experiments.

Table 3. Partial abrogation of clonal silencing through daily injections of anti-IJ^k serum

Age, days	Organ	% of control response* Saline-injected	% of control response* Anti-IJ^k-injected
5–8	Thymus	43.6	78.1
5–8	Spleen	69.1	135.4
14	Thymus	5.1	45.7

* Frequency of CTL-P in tolerant mice versus controls, means of two to four experiments.

Effects of Anti-IJ Serum *in Vivo* on Kinetics of Tolerance Induction. To assess any possible effects of suppressor cells acting *in vivo* in the process of apparent clonal silencing or elimination, control and tolerant mice were given daily injections of anti-IJ^k serum from the day of birth to the day before killing according to the regimen of Pierres *et al.* (28). The results (Table 3) show that this treatment markedly impeded the lowering in CTL-P frequency within the thymus and the spleen during early stages of tolerance induction. One experiment (not shown) in which mice were injected daily for 4 weeks showed a maintained impedence in thymus but a significantly lesser effect in spleen.

DISCUSSION

We have used a linear limiting-dilution clonal assay for CTL-P to MHC antigens that is dependent on TCGF and independent of helper and suppressor T cells to examine the cellular basis of tolerance induced at birth. The study has shown that a tolerizing cell inoculum causes a rapid, profound, and long-lasting silencing of CTL-P within the host mouse, detectable within the thymus at about 5 days and noted within the spleen a few days later, presumably as the "clonally purged" T cells colonize that organ. Whether the diminution in clonable CTL-P is due to their elimination through the process of clonal abortion (14) or to their functional silencing by some process akin to clonal anergy in B cells (16) is not revealed. It is clear that tolerance is not solely due to the presence of suppressor T cells within the tolerant population, because anti-IJ and complement treatment prior to culture or the process of extreme dilution in culture did not diminish the observed CTL-P deficit. In other words, suppressor T cells capable of inhibiting the differentiation of CTL-P into CTL clones may have been present as previous workers have claimed, but functional clonal elimination must be at least equally important in the final tolerant state.

Gorczynski and MacRae (12, 13) have claimed that two types of suppressor T cells determine tolerance in this model. The first, which develops very early during tolerance induction, inhibits conversion of CTL-P into CTL. The second, which develops only later, actually prevents the creation of CTL-P of the right specificity from more primitive, prethymic precursors. This second cell, which could possess anti-idiotypic specificity, would accomplish a purging of the CTL-P repertoire, leading to a deficit of CTL-P such as we have observed. To examine possible *in vivo* effects of this second suppressor population, we gave daily injections of anti-IJ^k serum according to a protocol known to counteract T cell suppression (28). Interestingly, this treatment appears to cause substantial partial reversal of clonal silencing in the thymus, but its effects are not absolute, especially with regard to splenic CTL-P numbers after long treatment. We intend to examine this effect more closely, with special reference to anti-IJ dosage, but the finding may offer some

support for the idea that clonal abortion is effected by suppressor T cells. An alternative possibility is raised by the work of Streilein (5). In the present experimental protocol, the antigen IJ^k is present on certain cells both of the recipient and of the donor, tolerizing cell inoculum. Streilein believes that IJ-bearing cells may be important as inducers of tolerance, because cells differing from host at $H\text{-}2^D$ and $H\text{-}2^K$ but not mid-*I* region loci are poor at establishing tolerance to skin grafts. This special property of IJ-bearing cells as inducers of a suppressor state that "spills over" to cells not bearing IJ has received recent support in two further systems (29, 30). It will be of interest to examine the capacity of cell inocula deprived of IJ-bearing cells, or lacking IJ disparity with the host, to induce tolerance using the present, highly quantitative readout system. A possible role of host IJ-bearing accessory cells in tolerance induction could be examined in suitable chimeras.

The kinetics of tolerance induction suggest that clonal silencing or elimination takes place in the thymus or at a prethymic stage of T cell differentiation. The splenic repertoire loses the relevant CTL-P more gradually, presumably as emigrant cells from the tolerant thymus build up within the spleen. Even within the thymus, the tolerant state supervenes somewhat less rapidly than in the case of immature B lymphocytes being negatively signalled by hapten-protein conjugates (31). Because only 0.1% of the tolerizing cell inoculum enters the thymus within 24 hr, this delay could reflect a need for antigen buildup, processing in the thymus, or both. Alternatively, it could mean that it takes some time for clone-eliminating, anti-idiotype suppressor T cells to become activated. Even when the tolerant state is fully established, a few reactive cells remain. It is possible, as in the case of the B lymphocyte (16), that these really represent pre-CTL-P that lack antigen receptors *in vivo* and therefore escape negative signaling but rapidly mature *in vitro* in the artificial cloning system under the influence of the various stimulatory factors present in CAS.

More precise analyses of the process of tolerance induction will demand that the tolerance be induced, as well as read out, *in vitro*. Whether it will prove possible to devise the necessary systems remains to be determined.

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