Neonatal Tolerance Revisited: Turning on Newborn T Cells with Dendritic Cells

John Paul Ridge,* Ephraim J. Fuchs,† Polly Matzinger

For some time it has been thought that antigenic challenge in neonatal life is a tolerogenic rather than immunogenic event. Reexamination of the classic neonatal tolerance experiments ofBillingham, Brent, and Medawar showed that tolerance is not an intrinsic property of the newborn immune system, but that the nature of the antigen-presenting cells determines whether the outcome is neonatal tolerance or immunization.

Nearly half a century ago, Burnet proposed that the function of the immune system is to distinguish self from nonself (1) and that self-tolerance is set early in life by the elimination of self-reactive lymphocytes (2, 3). Though Burnet’s group could not demonstrate such an early critical period (4, 5), the paradigm was established when Medawar and colleagues (6) found that rodents injected at birth with hematopoietic cells from a genetically different donor were later able to accept transplants from the same donor, thereby providing support for the idea that neonatal lymphocytes are uniquely susceptible to the induction of tolerance.

In the ensuing decades, inquiries into the mechanisms involved led to two main categories of interpretation. Passive models suggest that experimental neonatal tolerance occurs by negative selection in the same way as does natural self-tolerance. Neonatal mice, having so few mature T cells, would be unable to reject the donor cells, which would therefore take up residence and circulate to the thymus to impart tolerance by deletion in the same way as do the normal cells of the recipient (3, 7). Active models suggest that the newborn T cells generate predominantly suppressive, anti-idiotypic, or “deviated” T helper cell 2 (Th2) immune responses that protect from self-rejection (8, 9). However, newborn mice have occasionally been immunized to generate Th1 responses (10) and, though some viruses induce tolerance if given neonatally (11), others immunize (4). These examples of neonatally induced immunity are not easily explained by either the passive or the active models of neonatal tolerance.

We analyzed the possibility that the critical components in experimental neonatal tolerance are the donor cells, not the responding T cells. Our theoretical basis was the “Danger” model (12), which suggests that the immune system does not discriminate between self and nonself but between dangerous and harmless entities, and that the primary distinction is made by antigen-presenting cells (APCs), which are activated to up-regulate costimulatory molecules only when induced by alarm signals from their environment [for example, by tissues undergoing stress and abnormal death or by microbial products (12, 13)]. If, as suggested by “Two-Signal” models, lymphocytes are rendered tolerant by antigen recognition in the absence of costimulatory signals (14–16), then the absence of costimulation by normal, healthy peripheral tissues (17, 18) should continuously induce T cell tolerance in the periphery. From this perspective, there is no need for an early period of tolerizability, and newborn T cells should have the same options as adult virgin T cells, being activated in the presence of costimulatory signals and tolerized in their absence.

Why then are newborn T cells tolerized by an injection of large numbers of spleen or bone marrow cells? We speculated that the reason might lie with the mixture of cells in the donor inocula, which contain very few professional APCs (19) and a large percentage of T and B cells, which cannot costimulate virgin T cells (20–22). Thus the tiny number of virgin T cells in newborn mice might easily be overwhelmed by interactions with the tolerogenic cells in the inoculum before ever having an opportunity to meet an activating APC such as a dendritic cell. We expected, however, that if we isolated the critical components of the inoculum, the neonates should become primed by an injection of dendritic cells and tolerized by the B cells.

To test this view, we injected newborn female C57BL/6 (B6) mice with B6 male cells and tested their cytotoxic T lymphocyte (CTL) responses to the male antigen H-Y. In the classic studies (6), the donors and recipients differed by major histocompatibility complex (MHC) antigens, to which primary responses are strong and for which the window of tolerizability is short and the induction of tolerance is a major accomplishment. To test for neonatal priming, we chose the in vitro CTL response to H-Y, which is completely dependent on prior in vivo immunization. In addition, the responses are less vigorous than those to

Section on T Cell Tolerance and Memory (Ghost Lab), Laboratory of Cellular and Molecular Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA.

*To whom correspondence should be addressed.
†Present address: Johns Hopkins Oncology Center, Baltimore, MD 21287, USA.
MHC antigens, the window of tolerizability is long (23), and tolerance induction requires fewer donor cells (23). This is a combination of donor and recipient in which tolerance is easy to induce and in which priming should be difficult.

Because the classic experiments were done using skin grafts as tests, we first established that CTL killing was also an appropriate assay for neonatal tolerance. FollowingBillingham and Silvers (23), we injected B6 females with increasing doses of male spleen cells at various times after birth, immunized them at adulthood with a known priming dose of B6 male spleen cells, and tested them 2 weeks later for the generation of H-Y–specific killers. Figure 1A shows that they were still tolerizable at day 24 of life although, as noted before (23), the induction of tolerance required ever larger inocula. This decrease in sensitivity correlated with the rising number of T cells found in the spleens of the recipients (Fig. 1B). On average, we found that tolerance induction was achieved with an injection of about five male spleen cells for every resident female splenic T cell.

We next asked if tolerance was the only option available to newborn females or if they could be primed by an inoculum of professional APCs. To test this, we injected them at birth with highly enriched male dendritic cells and tested their CTL responses when they reached maturity. In a representative experiment, eight out of eight adult females that had been injected at birth with male dendritic cells were able to generate good CTL killing (Fig. 2). However, before concluding that neonatal T cells had been primed, we needed to explore the possibility that they had ignored the dendritic cells, becoming either primed but not tolerized, until they matured enough to react. If the male dendritic cells were long-lived, the CTL reactivity that we measured at 8 weeks might actually have been the result of efficient priming of these adult T cells.

We therefore asked whether neonatally primed females could be tolerized on day 7 with injections of whole male spleen cells.
or purified B cells (20). If the neonatally primed T cells had become typical memory T cells, they should now respond to a dose of male cells that would be tolerogenic for their immunized littermates. We found that 66% of mice (35 of 53) that were given dendritic cells on day 1 were resistant to tolerance induction (Fig. 3, column e) and were primed to the same extent as were the adult female controls (compare Fig. 3, columns d and e, with Fig. 3, column f), whereas unprimed littermates were tolerantized by the same injections (Fig. 3, column c). Thus, the dendritic cells injected on day 1 did not need to wait for a mature immune system—they were fully able to activate neonatal T cells. From these data, we conclude that neonatal T cells are neither innately tolerizable nor able to make only T_{H}2 responses. When offered the individual components of a classic tolerizing inoculum, they are activated by antigens presented by professional APCs, such as dendritic cells, and tolerized by nonprofessional APCs [Fig. 3 and (24)]. In this respect they do not differ from virgin T cells in adults (18–20, 25).

Why then does an injection of 5 × 10^6 mixed male spleen cells tolerize the neonatal mouse and immunize the adult? The difference may lie with the dose. Although immunization can result from the activation of only a few T cells, the induction of tolerance requires that virtually all of the potentially responsive cells be turned off (26). Thus, the tolerizing dose for an adult is likely to be much higher than for a neonate. To the neonate, containing only a few thousand (virgin) T cells, an injection of 5 × 10^6 spleen cells contains about 100 non-APCs for every T cell; however, to an adult containing 2000 times more T cells, the ratio is reversed and the dose of non-APCs in the inoculum is less than 1 for every 10^7 T cells. Consequently, because adult T cells are also susceptible to tolerance induction in the absence of second signals (16, 20, 27), we predicted that they should be rendered tolerant by a correspondingly large dose of mixed male spleen cells, and Fig. 4 shows that they were. For example, adult females primed with an injection of 500 × 10^6 male spleen cells (about five non-APCs for each circulating T cell) did not generate efficient CTLs (Fig. 4), nor can they subsequently reject male skin grafts (28).

Thus, neonatal and adult T cells have similar options. Both can be activated by professional APCs and tolerized by non-costimulatory cells, and in both cases the dose is important. This offers a simple alternative explanation for the classic experiments in which injections of bone marrow or spleen cells induced neonatal tolerance. We suggest that tolerance occurred not because the neonate is inherently tolerizable, but because an inoculum of spleen or bone marrow cells contains a great many cells that are unable to deliver costimulation to virgin T cells. Both neonates and adults can be tolerized by such an overdose. It simply takes far fewer cells to generate tolerance in a newborn because there are far fewer T cells to turn off.

This interpretation also explains cases in which neonates clear virus infections (4) or give other types of T_{H}1, T_{H}2 (9, 10) [or T_{H}3? (29)] responses. If tolerance versus activation is governed by the dose and form of antigen presentation (12, 14, 16), then we might expect that newborn mice could be appropriately immunized to generate all the classes of response normally found in adults. Together with our data, the studies in two accompanying papers in this issue show that mature virgin T cells, even as recent thymic immigrants, can be immunized, tolerized, or switched to T_{H}1 or T_{H}2 responses according to such simple para-

![Fig. 3](image-url)  
*Fig. 3.* Neonatal T cells first primed with dendritic cells (DCs) are resistant to tolerance induction. To test whether dendritic cells have their effect on the newborn or, later, on the adult immune system, we immunized newborn mice with 1 × 10^6 dendritic cells and then injected them on day 7 with a tolerizing dose of 3 × 10^6 purified B cells (open circles) (19) or 5 × 10^6 spleen cells (solid circles) (column e). As controls, we also treated littermates with nothing (column a); with a tolerizing dose alone (column b); with a tolerizing dose at day 7 plus an immunization with 2 × 10^6 spleen cells at 6 weeks of age (column c, to test that the tolerizing dose had been effective); with dendritic cells at day 1 (column d, a positive control that the dendritic cells were able to prime); or with dendritic cells at day 1, followed by a tolerizing dose of spleen cells at day 7 (column e, the experimental group). We also injected a control group of adult mice with the same batch of dendritic cells as those used in columns d and e (column f, to test whether the dendritic cell immunizations were as efficient in newborns as in adults), and we injected a control group of untolerized littermates at 6 weeks of age with the same batch of spleen cells as those used to immunize in column c (column g, to test the efficacy of the spleen cells as immunogens). All the mice were tested for anti-H-Y CTLs when the experimental groups were 8 to 12 weeks old. Each point represents the specific killing generated by the spleen cells from an individual mouse at a responder-to-target ratio at which the specific killing drops off the plateau. Horizontal lines are the group averages. Background killing on female targets was subtracted.

![Fig. 4](image-url)  
*Fig. 4.* Tolerance to H-Y in adult female mice. Adult female B6 mice were injected ip with increasing numbers of male spleen cells in 500 μl of sterile PBS and tested 3 to 4 weeks later for the ability to generate H-Y-specific killers. Values represent CTL killing from individual animals. Solid symbols show killing on male targets, and open symbols show killing on allogeneic targets. Lines connect the means for each group.
meters as the dose of antigen (30), the type of adjuvant (31), and the type of APC (Figs. 2 to 4). They are also compatible with the view that tolerance or activation to a peripheral antigen is not determined by the self or nonself origin of the antigen but rather by the conditions under which it is introduced.

REFERENCES AND NOTES

1. F. M. Burnet, The Clonal Selection Theory of Acquired Immunity (Vanderbilt Univ. Press, Nashville, TN, 1959).
2. M. T. and F. Fenner, The Production of Antibodies (Macmillan, Melbourne/London, 1949).
3. J. Ledderose, Science 129, 1649 (1959).
4. G. J. V. Nossal, Aust. J. Exp. Biol. 35, 549 (1957).
5. F. M. Burnet, J. D. Stone, M. Edney, ibid. 28, 291 (1950).
6. R. E. Billingham, L. Brent, P. B. Medawar, Nature 172, 603 (1953); R. E. Billingham, Proc. R. Soc. London Ser. B 229, 44 (1956).
7. P. J. Morrissey, D. Bradley, S. O. Sharrow, A. Singer, J. Exp. Med. 158, 365 (1983); M. Inaba et al., ibid. 173, 549 (1991).
8. A. Bandera, A. Coutinho, C. Cannaudi, F. Jacquemart, L. Foron, Proc. Natl. Acad. Sci. U.S.A. 86, 272 (1989); B. J. Roser, Immunol. Rev. 107, 179 (1989); D. Belgrand, D. Smilke, D. B. Wilson, J. Exp. Med. 151, 163 (1980); R. K. Gershon and K. Kondo, Immunology 21, 903 (1971); C. Y. Lu et al., Cell 50, 265 (1992).
9. M. Schumans et al., J. Immunol. 145, 2460 (1990); T. J. Powell and W. J. Streiten, ibid. 144, 854 (1990); N. Chen and E. H. Field, Transplantation 59, 933 (1995).
10. A. Miller, L. Oster, A. Oded, H. Weiner, Eur. J. Immunol. 24, 1026 (1994); S. Strobel and A. Ferguson, Pediatr. Res. 39, 589 (1996).
11. J. Chak and F. Lehmehr-Grube, Immunology 34, 265 (1978); P. M. Hoffman, D. S. Robbins, H. C. Morse, J. Virol. 52, 734 (1984).
12. P. R. L. Bissell et al., Eur. J. Immunol. 12, 991 (1992).
13. A. J. Arney, Acta. Immunol. Today 13, 11 (1992).
14. P. Bretsch and M. Cohen, Science 169, 1042 (1970); K. J. Lafferty and A. Cunningham, Aust. J. Exp. Med. 16, 216 (1978).
15. F. Finkelstein, A. Lees, R. Bimbam, W. C. Gaus, S. C. Morris, in preparation.
16. J. R. Lamb et al., J. Exp. Med. 157, 1434 (1983); M. K. Jenkins and R. H. Schwartz, ibid. 165, 302 (1987).
17. R. I. Lechler and J. R. Batchelor, ibid. 156, 1835 (1982); N. N. Shehadeh, R. G. Gill, K. J. Lafferty, Science 181, 1274 (1973); R. K. Gershon and K. Kondo, Immunology 21, 903 (1971); C. Y. Lu et al., Cell 50, 265 (1992).
18. V. Fial et al., Eur. J. Immunol. 20, 1893 (1990).
19. O. Lissia, D. Vaino, P. Matzinger, Nature 304, 253 (1983).
20. E. J. Fuchs and P. Matzinger, Science 258, 1156 (1992).
21. E. E. Eynon and D. C. Parker, Transplant. Proc. 23, 729 (1991); J. Exp. Med. 175, 131 (1992).
22. W. Lauchart, B. J. Akins, D. A. Davies, Transplantation 29, 259 (1980).
23. P. E. Billingham and W. K. Silvers, J. Immunol. 85, 16 (1960).
24. T. Ishizaka, C. Cannaudi, O. Stutman, ibid. 137, 2093 (1980).
25. S. W. Urlich et al., Immunol. Rev. 133, 177 (1993); S. E. Macatonia, P. M. Taylor, S. C. Knight, B. A. Askonas, J. Exp. Med. 169, 1255 (1989); H. G. Ram- mensee, P. J. Fink, M. J. Bevan, J. Immunol. 133, 2390 (1984).
26. Maintaining tolerance is, of course, different from inducing it. Here we agree with the passive model described earlier. Once the neonatal or adult mouse has become tolerant, stem cells in the donor inocu-lum can set up residence and establish microchimeris-m to maintain both central and peripheral toler-

Induction of Protective CTL Responses in Newborn Mice by a Murine Retrovirus

Marcella Sarzotti,* Deanna S. Robbins, Paul M. Hoffman

The susceptibility of neonates to virus-induced disease is thought to reflect, in part, the immaturity of their immune systems. However, inoculation of newborn mice with low doses of Cas-Br-M murine leukemia virus induced a protective cytotoxic T lymphocyte (CTL) response. The inability of neonates to develop a CTL response to high doses of virus was not the result of immunological immaturity but correlated with the induction of a nonprotective type 2 cytokine response. Thus, the initial viral dose is critical in the development of protective immunity in newborns.

In neonates, B cell and T cell responses to antigen are impoverished compared to those in adults (1). In part, these reduced responses are the result of deficient accessory cell numbers or function (2). However, T cells from neonates express receptors for cytokines and co-stimulatory molecules in amounts similar to those expressed by adult T cells (3), and in vitro CTL responses to alloantigen can be detected by 4 to 6 days postpartum, gradually increasing to adult amounts by 11 to 20 days postpartum (4). Interaction of neonatal NFS/N mice (Fv-lm*1, H-2*m) with Cas-Br-M murine leuke-mia virus (Cas) [1000 plaque-forming units (PFU) per mouse] (5) results in rapid virus replication, detectable (6) in the spleen (104 to 106 PFU/g) and brain (102 to 104 PFU/g) within 2 weeks of infection (7). This perinatal infection does not elicit protective CTL and interferon-γ (IFN-γ) responses and results in virus-induced neurologic disease (8, 9). However, these mice do not exhibit a generalized suppression of T cell function and remain fully competent to generate alloreactive CTL responses (8). In contrast, Cas infection (1000 PFU) in 21-day-old mice leads to a protective CD8+ CTL response and no neurologic disease (8, 9). Thus, as in other viral systems, the ability to develop a CTL response influences the outcome of viral disease (10).

Because the number of T cells per spleen is 3 to 3.5 log units lower in neonates than in adult mice (11), we tested whether inoculation of newborn mice with a dose of Cas proportional to their splenic T cell number

33. J. P. Ridge, E. J. Fuchs, P. Matzinger, data not shown.
34. We obtained the enriched dendritic cell preparations by incubating B6 male spleen cells in Iscove’s modi-fied Dulbecco’s medium plus 10% fetal bovine se-rum in Falcon tissue culture dishes (number 3020) for 2 hours at 37°C, removing nonadherent cells, and then incubating the remaining adherent cells overnight at 37°C in medium containing mouse recombinant granulocyte-macrophage colony-stimulating factor (5 ng/ml) (Phar- mingen, San Diego, CA). Nonadher-

enceptent cells were then harvested and further purified over a 50% Percoll density gradient. By flow cytom-etry, the cell populations were typically bright for MHC class II staining and contained <2% T cells, <5% B cells, and >80 to 90% dendritic cells, as seen by staining for the dendritic cell markers CD301 and N418.
35. We thank L. Yuan for suggesting the day 7 toler-ance experiment; A. Bendelac for other useful sug-gestions and encouragement; and A. Bendelac, L. Chiordi, L. D’Adamo, M. Epstein, R. German, R. Schwartz, and A. Sher for reading the manuscript. We especially thank B. Tineo and C. Fenton for caring for the 351 baby mice and their mothers reported here, as well as several hundred mice that did not make it into these pages.
28 November 1995; accepted 28 February 1996