deletion (*24*), because clonal deletion of pCTL was not observed in mice infected at birth with a high dose of Cas (*25*). Our data indicate that the dose of virus encountered by the immune system of a newborn mouse determines the development of type 1 or type 2 responses and influences the generation of protective immunity. Thus, T cells in newborn mice, like those in adults, may be activated to either type of response by the appropriate antigen-presenting cells (*26*), costimulatory signals (*27*), and dose of antigen (Figs. 1 and 3) (*18*).

REFERENCES AND NOTES

1. P. G. Spear and G. Edelman, *J. Exp. Med.* **139**, 249 (1974); D. E. Mosier and B. M. Johnson, *ibid.* **141**, 216 (1975).
2. C. Y. Lu, D. I. Beller, E. Unanue, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1597 (1980).
3. B. Adkins, A. Ghanai, K. Hamilton, *J. Immunol.* **153**, 3378 (1994).
4. L. M. Pilarski, *J. Exp. Med.* **146**, 887 (1977).
5. M. B. Gardner *et al., J. Natl. Cancer Inst.* **51**, 1243 (1973).
6. W. P. Rowe, W. E. Pugh, J. W. Hartley, *Virology* **42**, 1136 (1970).
7. P. M. Hoffman, S. K. Ruscetti, H. C. Morse III, *J. Neuroimmunol.* **1**, 275 (1981).
8. P. M. Hoffman, D. S. Robbins, H. C. Morse III, *J. Virol.* **52**, 734 (1984); D. S. Robbins and P. M. Hoffman, *J. Neuroimmunol.* **31**, 9 (1991); P. M. Hoffman, E. F. Cimino, D. S. Robbins, *ibid.* **33**, 157 (1991).
9. M. Sarzotti, D. S. Robbins, P. M. Hoffman, *Viral Immunol.* **6**, 207 (1993).
10. J. A. Byrne and M. B. A. Oldstone, *J. Virol.* **51**, 682 (1984); A. E. Lukacher, V. L. Braciale, T. J. Braciale, *J. Exp. Med.* **160**, 814 (1984); B. D. Jamieson and R. Ahmed, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2265 (1988); D. Moskophidis, F. Lechner, H. Pircher, R. M. Zinkernagel, *Nature* **362**, 758 (1993).
11. P.-F. Piguet, C. Irle, E. Kollatte, P. Vassalli, *J. Exp. Med.* **154**, 581 (1981).
12. A. Oliff, S. Ruscetti, E. C. Douglass, E. Scolnick, *Blood* **58**, 244 (1981).
13. The cytotoxic response of mice primed with 0.3 PFU of Cas was high when tested against Cas-infected NS467 cells [85 ± 2% cytotoxicity at an effector/target (E/T) ratio of 20:1], but remained at background amounts when tested against the other targets (8 ± 2 against TP-3; 15 ± 1 against EL-4; 26 ± 4 against YAC-1; E/T ratio, 20:1) (mean ± SE of three to seven individually tested mice). Cytotoxic activity in splenocytes from sham-inoculated mice that were cultured in vitro with Cas-infected cells was uniformly low against all targets (14 ± 4 against NS467; 6 ± 2 against TP-3; 11 ± 3 against EL-4; 23 ± 2 against YAC-1). The specificity and efficacy of the CTL response from newborn mice were the same as in adult mice (*8, 17*).
14. D. S. Robbins, M. P. Remington, M. Sarzotti, D. St. Louis, P. M. Hoffman, *J. Virol.* **69**, 6847 (1995).
15. Splenocytes from adult mice infected at birth with 0.3 PFU of Cas were restimulated in vitro, enriched for T cells with the use of affinity columns (*17*), labeled with phycoerythrin-conjugated monoclonal antibodies to CD4 and fluorescein isothiocyanate–conjugated monoclonal antibodies to CD8, and positively sorted. Virtually the entire CTL response was recovered in the $CD8^+$ T cell population (>95% of the cytotoxic activity of unsorted cells), with no appreciable amount of cytotoxicity detected in the $CD4^+$ population.
16. M. Sarzotti, D. S. Robbins, P. M. Hoffman, unpublished results.
17. M. Sarzotti, T. A. Dean, M. Remington, P. M. Hoffman, *AIDS Res. Hum. Retroviruses* **10**, 1695 (1994).
18. C. R. Parish, *Transplant. Rev.* **13**, 35 (1972); P. A. Bretscher, G. Wei, J. N. Menon, H. Bielefeldt-Ohmann, *Science* **257**, 539 (1992); C. Pfeiffer, J. Murray, J. Madri, K. Bottomly, *Immunol. Rev.* **123**, 65 (1991); N. A. Hosken *et al., J. Exp. Med.* **182**, 1579 (1995).
19. T. R. Mosmann and R. L. Coffman, *Annu. Rev. Immunol.* **7**, 145 (1989).
20. T. J. Powell Jr. and J. W. Streilein, *J. Immunol.* **144**, 854 (1990); N. Chen and E. H. Field, *Transplantation* **59**, 933 (1995).
21. F. P. Heinzel, M. D. Sadick, B. J. Holaday, R. L. Coffman, R. M. Locksley, *J. Exp. Med.* **169**, 59 (1989); A. Sher *et al., Immunol. Rev.* **127**, 183 (1992); S. Schurmans *et al., J. Immunol.* **145**, 2465 (1990).
22. The IL-4 response of splenocytes to neonatal infection with a high dose of Cas was mediated by $CD4^+$ T cell populations (unfractionated splenocytes, 10,311 ± 1220 cpm; CD4 T cells, 15,368 ± 1247 cpm; $CD8^+$ T cells, 419 ± 98 cpm, as measured with the CT.4S proliferation assay).
23. J. K. Actor *et al., Proc. Natl. Acad. Sci. U.S.A.* **90**, 948 (1993).
24. R. Zamoyska, H. Waldmann, P. Matzinger, *Eur. J. Immunol.* **19**, 111 (1989).
25. M. Sarzotti and D. S. Robbins, in *Modern Approaches to New Vaccines Including Prevention of AIDS*, R. M. Chanock, H. S. Ginsberg, F. Brown, R. A. Lerner, Eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1991), pp. 355–358.
26. E. J. Fuchs and P. Matzinger, *Science* **258**, 1156 (1992); P. Matzinger, *Annu. Rev. Immunol.* **12**, 991 (1994); J. P. Ridge, E. J. Fuchs, P. Matzinger, *Science* **271**, 1723 (1996).
27. K. J. Lafferty and A. Cunningham, *Aust. J. Exp. Biol. Med. Sci.* **53**, 27 (1975); G. R. Otten and R. N. Germain, *Science* **251**, 1228 (1991); K. J. Lafferty, S. K. Babcock, R. G. Gill, *Prog. Clin. Biol. Res.* **224**, 87 (1986); M. K. Jenkins and R. H. Schwartz, *J. Exp. Med.* **165**, 302 (1987); V. K. Kuchroo *et al., Cell* **80**, 707 (1995).
28. Responder splenocytes (3×10^6) and irradiated, Cas-infected splenocytes (4×10^6) or irradiated, Cas-infected NS467 lymphoma cells (5×10^5) were cocultured for 5 days in each well of 24-well tissue culture plates in Eagle's minimal essential medium, 10% fetal bovine serum, antibiotics, 2-mercaptoethanol, and L-glutamine (*8, 9, 14, 17*). The cytotoxicity of the responder cells against uninfected syngeneic concanavalin A blasts was <15% and was omitted for clarity.

29. J. Hu-Li, J. Ohara, C. Watyson, W. Tsang, W. E. Paul, *J. Immunol.* **142**, 800 (1989).
30. We thank T. A. Dean and R. Germain for excellent assistance; M. Sztein for cell sorting; C. Via for the CT.4S cell line; P. Matzinger and A. O'Garra for sharing their unpublished data; and G. Kelsoe for critically reading the manuscript. Supported by grants from the Medical Research Service, Department of Veterans Affairs, and by the Bressler Research Fund, University of Maryland at Baltimore (to M.S.).

28 November 1995; accepted 27 February 1996

Induction of T_H1 and T_H2 Immunity in Neonatal Mice

Thomas Forsthuber, Hualin C. Yip, Paul V. Lehmann*

The neonatal period has been thought of as a window in ontogeny, during which the developing immune system is particularly susceptible to tolerization. In the present study, the classic system for induction of neonatal tolerance to protein antigens was reexamined in mice. The presumably tolerogenic protocol was found to trigger a vigorous T helper cell type 2 (T_H2) immune response. Thus, neonatal "tolerization" induces immune deviation, not tolerance in the immunological sense. Neonates are not immune privileged but generate T_H2 or T_H1 responses, depending on the mode of immunization.

Dizygotic twin cattle, which share a placental blood supply during gestation, have erythrocytes of both their own and their twin's genotype, that is, these cattle do not reject the allogenic cells to which they were exposed early in life (*1*). On the basis of this observation, reported in 1945, the neonatal period was postulated to represent a critical window in ontogeny, during which the developing immune system learns to discriminate self from nonself by developing a tolerance to antigens it encounters (*2*). This hypothesis has defined immunological thinking ever since and has been supported by subsequent experiments (*3–5*).

The mechanism underlying neonatal tolerance has remained controversial. Proposed mechanisms include suppressor cell development (*6*) and the clonal deletion of antigen-reactive T cells (*7*). Because these two models conflict, with suppression representing an active tolerance mechanism and clonal deletion a passive one, we revisited the neonatal tolerance paradigm.

We followed the classic protocol for inducing neonatal tolerance to protein antigens, injecting the protein into mice within 24 hours of birth in incomplete Freund's adjuvant (IFA) intraperitoneally (i.p.) (*5*), a regimen that is considered to be tolerogenic in adults as well (*8, 9*). When they reached adult age, the mice were reinjected with the antigen in complete Freund's adjuvant (CFA) subcutaneously (s.c.). Mice injected neonatally with hen egg lysozyme (HEL) displayed an impaired response in the lymph nodes (LN) that has been considered a hallmark of tolerization (*10*) (Fig. 1A). However, the spleen cells of these mice proliferated vigorously in response to HEL, even when HEL was not reinjected at the adult age (Fig. 1B). Because of technical limitations, earlier studies were confined to LN responses; splenic recall responses to protein antigens

Department of Pathology, Biomedical Research Building, Case Western Reserve University, Cleveland OH, 44106–4943, USA.

* To whom correspondence should be addressed.

have only recently become measurable (*11*). Thus, neonatal injection did not tolerize, as previous studies suggested, but induced functional memory cells that were detectable in the spleen but not in the LN.

The accumulation of memory cells in spleens rather than LN of neonatally injected mice might be attributed to normal migration patterns of lymphocytes. Memory T cells down-regulate their LN homing receptor, L-selectin (*12*), and subsequently do not home efficiently to the LN (*13*). In contrast, T cell migration to the spleen does not rely on L-selectin (*14*). We found that the recall response in the spleens of neonatally injected mice resided in the L-selectin–negative fraction of $CD4^+$ lymphocytes (*15*), which strongly supports the notion that the impaired LN response of these mice results from the redistribution of memory cells, and not from clonal deletion or suppression, as this finding has been interpreted (*16*).

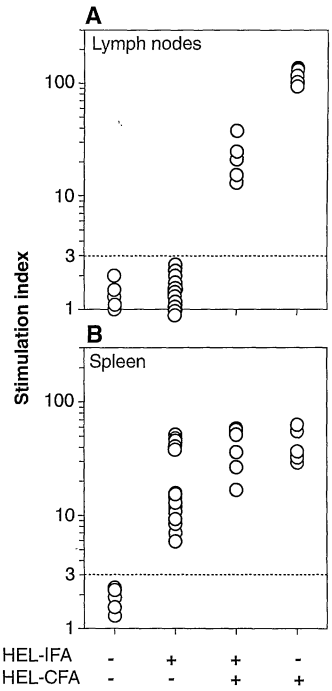

Fig. 1. Mice injected neonatally with HEL-IFA, when immunized with HEL-CFA as adults, display an impaired LN but increased spleen cell response. Mice injected neonatally with HEL-IFA (*24*) or left untreated were challenged at 6 weeks of age, s.c., with 50 μg of HEL-CFA or left untreated, as indicated. Nine days after the adult injection, a T cell proliferation assay was performed on draining LN (**A**) or spleens (**B**) (*24*). The proliferative response to HEL is shown, expressed as stimulation index (SI). Each dot represents data from an individual mouse. The medium background was between 200 and 3000 cpm in all measurements. Responses of SI > 3 are considered significant (indicated by a dashed line). The tolerized-immunized mice and the naïve group showed a comparable primary response to purified protein derivate of *Mycobacterium tuberculosis* (*15*). The results were reproduced in three independent experiments (*10, 11, 16*).

Neonatal injection of antigen not only primed T cells for a secondary in vitro response, it also resulted in the production of antibody in vivo. Eight-week-old, neonatally injected mice contained low but detectable concentrations of specific antibodies (Fig. 2A). These antibodies were of the immunoglobulin G1 (IgG1) subclass (Fig. 2, B to D). When reinjected with soluble HEL (a protocol that is not immunogenic to naïve mice, but that boosts an established immune response), these mice mounted a vigorous secondary antibody response that again consisted almost entirely of IgG1 (Fig. 2, A to C). IgG1 production is characteristic of a secondary, T cell–dependent response (*17*); therefore, neonatal injection primed the immune system for functional antigen-specific T and B cell memory.

Because the anti-HEL response of neonatally injected mice was dominated by T helper type 2 (T_H2)–dependent IgG1 (*17*), we looked for other evidence that the neonatally induced T cells are of a T_H2 phenotype. When freshly isolated spleen cells of mice injected neonatally with HEL-IFA or myelin basic protein (MBP)–IFA were tested with an enzyme-linked immunosorbent (ELI) spot assay for cytokine production to the recall antigen, interleukin-5 (IL-5) but no interferon γ (IFN-γ) was detected (Table 1) (*18*). In contrast, adult mice immunized with HEL or MBP in CFA produced IFN-γ, but virtually no IL-5 (Table 1), consistent with the induction of a T_H1 response. The unipolar T_H1 or T_H2 cytokine pattern was seen in both T_H2-biased BALB/c and in T_H1-biased B10.PL mice (Table 1). The ELI spot assay detects individual cytokine-producing cells; thus, the clonal size of antigen-specific memory T cells generated after neonatal injection of the antigen in IFA was comparable to that seen after adult injection of the antigen in CFA, yet entails T_H2 rather than T_H1 memory cells.

Neither the neonate nor the adult are limited to a particular class of response. Immunization of the T_H2-biased BALB/c mice with HEL in CFA induced unipolar T_H1 immunity, independent of the age of mice or the route of injection (Table 1). At either age, BALB/c and the T_H1-biased B10.PL mice developed unipolar T_H2 immunity in response to injection of antigen with IFA. Thus, not the age the of mice but the adjuvant determines whether T_H1 or T_H2 type immunity develops. Apparently, the polarizing properties of CFA and IFA override the genetic bias of mice for one or the other class of T cell response, as seen after parasitic infections (*19*).

Injection of the autoantigen MBP in IFA, into either neonates or adults, prevents the subsequent development of the T_H1 cell–mediated autoimmune disease experimental allergic encephalomyelitis (EAE) (*5, 20*). In light of our data, this IFA-induced neonatal or adult "tolerance" to EAE development might result from T_H2-type immune deviation (*21*), not from tolerance in the immunological sense. Neonatal transplantation tolerance has also been linked to alloantigen-specific T_H2 immunity (*22*). However, unlike IFA-induced "tolerance," induction of transplantation tolerance is confined to the neonatal period and to certain donor-recipient strain combinations. The two tolerance models might behave differently because of the dissimilar preimmune T cell repertoires involved (*23*).

The T_H2 model of IFA-induced neonatal (or adult) tolerance to protein antigens that we propose here incorporates the data on which the two previous models were based. First, induction of T_H2 type immunity is consistent with the observations that this state of "tolerance" is active, that is, adoptively transferable, which has substan-

Table 1. Neonates were injected within 24 hours of birth, then tested at 6 weeks of age. Adults were injected at 6 weeks of age, then tested 21 days later. All mice received a single injection of 100 μg of antigen.

Mouse strain and age	Injection	Route	Recall response* (cytokine ELI spots per 10^6 spleen cells)			
			IFN-γ		IL-5	
			HEL	MBP	HEL	MBP
BALB/c						
Neonate	HEL-IFA	i.p.	<5	<5	31.8 ± 7.9	<5
Neonate	HEL-CFA	i.p.	37.8 ± 15.1	<5	<5	<5
Adult	HEL-IFA	i.p.	<5	<5	38.3 ± 12.1	<5
Adult	HEL-CFA	i.p.	51.4 ± 19.9	<5	<5	<5
Adult	HEL-CFA	s.c.	49.3 ± 20.5	<5	<5	<5
B10.PL						
Neonate	MBP-IFA	i.p.	<5	<5	<5	35.6 ± 15.3
Adult	MBP-CFA	i.p.	<5	73.7 ± 28.5	<5	<5

*Data are expressed as an arithmetic mean ± SEM of 9 to 12 spleens independently tested in three experiments.

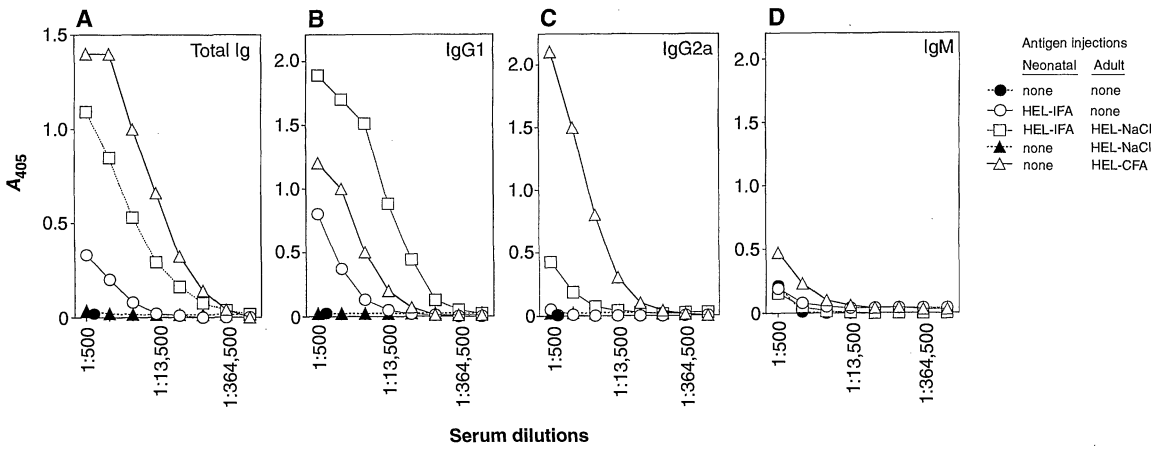

Fig. 2. (**A** to **D**) Mice injected neonatally with HEL in IFA produce specific antibody and mount a secondary antibody response when reinjected with HEL at adult age. The unboosted, neonatally injected group was tested at 8 weeks of age. The groups neonatally injected with HEL-IFA (*24*) and the naïve groups were injected i.p. at 6 weeks of age with 50 μg of HEL in saline as indicated. The HEL-CFA–immunized group was injected at 6 weeks, s.c. Two weeks after the adult injection, mice were bled, and the titer of antibodies to HEL was measured by enzyme-linked immunosorbent assay (*25*). Absorbance (*A*) was measured at 405 nm. The titrations shown started with 1:500 serum dilutions, progressing in steps of 1:3. Data obtained for one mouse of each group are shown, representative of groups of three to five mice tested in three experiments.

tiated the suppressor cell model. Second, by showing that the $CD4^+$ cells primed in neonates are deficient in their migration to the LN, we have accounted for the data (derived from studies involving only the LN response) that substantiated the clonal deletion model of IFA-induced tolerance.

Thus, neonates are immunocompetent, and their $CD4^+$ T cell response to prototypic antigens does not essentially differ from that of adult mice. Therefore, the neonatal period may not be an immunologically privileged window in ontogeny that is more essential to the establishment of self-tolerance than later periods of life, but an immunologically normal period that can be exploited both to vaccinate and to tolerize. Having identified an immunization regimen that induces vigorous, unipolar T_H2-type immunity, our data can potentially be applied to vaccine development and the therapy of T_H1 cell–mediated autoimmune disease.

REFERENCES AND NOTES

1. R. D. Owen, *Science* **102**, 400 (1945); ———, H. P. Davies, R. F. Morgan, *J. Hered.* **37**, 291 (1946).
2. F. M. Burnet and F. Fenner, *The Production of Antibodies* (Macmillan, Melbourne, 1949), pp. 102–105.
3. R. E. Billingham, L. Brent, P. B. Medawar, *Nature* **172**, 603 (1953).
4. P. Y. Paterson, *Ann. N.Y. Acad. Sci.* **73**, 811 (1958).
5. J. P. Clayton *et al.*, *J. Exp. Med.* **169**, 1681 (1989); Y. F. Qin, D. M. Sun, M. Goto, R. Meyermann, H. Wekerle, *Eur. J. Immunol.* **19**, 373 (1989).
6. S. Dorsch and B. Roser, *Nature* **258**, 233 (1975); M. Rieger and I. Hilgert, *J. Immunogenet.* **4**, 61 (1977).
7. G. Gammon *et al.*, *Nature* **319**, 413 (1986); G. Gammon, A. Oki, N. Shastri, E. E. Sercarz, *J. Exp. Med.* **164**, 667 (1986).
8. E. R. Einstein, J. Csejtey, W. J. Davis, H. C. Rauch, *Immunochemistry* **5**, 567 (1968); J. E. Swierkosz and R. H. Swanborg, *Fed. Proc.* **33**, 814 (1974).
9. E. R. Kearney, K. A. Pape, D. Y. Loh, M. K. Jenkins, *Immunity* **1**, 327 (1994).
10. The ~80 to 90% reduction of the LN response that we measured has also been reported in the publications that used the deficient LN response as a readout for the efficiency of tolerization (*5, 7*).
11. Earlier studies on $CD4^+$ T cell responses to protein antigens in the mouse were confined to testing draining LN shortly after immunization. Spleen cells tested under the same conditions did not reveal recall responses because of high background proliferation in serum-containing media. Splenic recall responses became measurable with the introduction of serum-free assay conditions [P. V. Lehmann *et al.*, *Nature* **358**, 155 (1992)].
12. T. M. Jung, W. M. Gallatin, I. L. Weissman, I. L. Dailey, *J. Immunol.* **141**, 4110 (1988); L. M. Bradley, G. G. Atkins, S. L. Swain, *ibid.* **148**, 324 (1992).
13. C. R. Mackay, W. L. Marston, L. Dudler, *J. Exp. Med.* **171**, 801 (1990); C. R. Mackay *et al.*, *Eur. J. Immunol.* **22**, 887 (1992); C. R. Mackay, *Immunol. Today* **12**, 189 (1991).
14. R. Pabst, *Immunol. Today* **9**, 43 (1988); ——— and R. M. Binns, *Immunol. Rev.* **108**, 83 (1989).
15. T. Forsthuber, data not shown.
16. Impaired LN responses, reminiscent of the data shown in Fig.1, are also seen in mice preimmunized with CFA (*9*), suggesting that memory cells in general may not readily migrate to LN.
17. T. R. Mosmann and R. L. Coffman, *Annu. Rev. Immunol.* **7**, 145 (1989).
18. For the ELI spot assay, plates (T-Spot, Athersys, Cleveland, OH) were coated overnight with IFN-γ– or IL-5–specific capture antibody, R4-6A2 or TRFK5, respectively, each at 5 μg/ml. After the plates were washed, they were blocked with 1% bovine serum albumin in phosphate-buffered saline for 1 hour and washed again. Spleen cells were plated at 10^6 cells per well alone or with antigens or peptides (7 μM) in HL-1 medium and cultured for 36 hours. Subsequently, the cells were removed by washing, biotinylated detection antibody was added (XMG 1.2 or TRFK4, both at 2 μg/ml), and the plates were incubated overnight. The plate-bound secondary antibody was then visualized by adding horseradish peroxidase and 3-amino-9-ethylcarbazole. The number of spots were counted by a computerized image analysis system (T-Spot Image Analyzer, Athersys). Because 10^6 cells were plated per well, the number of spots reflects the frequency of cells per million that produce the cytokine after antigen challenge.
19. F. P. Heinzel, M. D. Sadick, B. J. Holaday, R. L. Coffman, R. M. Locksley, *J. Exp. Med.* **169**, 59 (1989).
20. G. A. Falk, M. W. Kies, E. C. Alford, *J. Immunol.* **103**, 1248 (1969); J. E. Swierkosz and R. H. Swanborg, *ibid.* **115**, 631 (1975); A. M. Welch and R. H. Swanborg, *Eur. J. Immunol.* **6**, 910 (1976); C. C. Bernard, *Clin. Exp. Immunol.* **29**, 100 (1977).
21. G. L. Asherson and S. H. Stone, *Immunology* **9**, 205 (1965); G. L. Asherson, *Br. Med. Bull.* **23**, 24 (1967).
22. T. J. Powell Jr. and J. W. Streilein, *J. Immunol.* **144**, 854 (1990); D. P. Abramowicz *et al.*, *Eur. J. Immunol.* **20**, 1647 (1990); H. Chen *et al.*, *Transplantation* **60**, 1182 (1995); N. Chen, Q. Gao, E. H. Field, *ibid.*, p. 1187.
23. The notion that the neonate is immune privileged originated from experiments on transplantation tolerance (*1, 3*). Because the specificity spectrum of the alloreactive T cell repertoire is very broad, ~50% of the preimmune repertoire will consist of memory cells in the adult. In neonates, where all T cells are naïve, the preimmune alloreactive repertoire will consist exclusively of naïve cells. Unlike naïve T cells, memory T cells are not readily tolerizable and are committed with respect to T_H1 or T_H2 lineage. Thus, unlike that of the neonate, the preimmune alloreactive repertoire of the adult entails a large number of precommitted T_H1 cells. A regimen that would induce a T_H2 alloresponse in a naïve T cell pool (neonate) will boost a substantial number of T_H1 cells in the adult. In contrast, the preimmune repertoire to protein antigens consists of naïve T cells in the adult and the neonate alike, which results in unbiased T_H1 or T_H2 development.
24. Mice (Jackson Laboratory) were bred under special pathogen-free conditions. Neonates were injected i.p. within 24 hours of birth with antigen (100 μg) in IFA. Mice were killed at the age specified and their LN or spleen cells were tested for the antigen- or peptide-induced proliferative response in a primary in vitro assay. Single cell suspensions were plated, 1×10^6 spleen cells or 5×10^5 LN cells per well, in flat bottom 96-well microtiter plates. Cells were cultured for 4 days with 7 μM antigen or peptide in 200 μl of serum-free HL-1 medium (Ventrex) supplemented with 1 mM L-glutamine. During the last 18 hours of culture, [^{3}H]thymidine was added (1 μCi per well), and the incorporation of label was measured by liquid scintillation counting.
25. Plates were coated with HEL (10 μg/ml) overnight at 4°C, and 0.1% gelatin in phosphate-buffered saline with Tween 20 was used for subsequent blocking. The test serum was added, and then the plates were incubated for 2 hours at room temperature. Plate-bound antibody was detected by alkaline phosphatase–coupled antibody to mouse Ig. For detection of total Ig, affinity-purified goat antibody to mouse IgG+A+M (H+L) from Zymed was used; the isotype-specific antibodies for detection of IgG1 and IgG2a were from Southern Biotech Associates and IgM was from Sigma. Para-nitrophenyl phosphate was used for the development of the colorimetric reaction.

26. We thank A. Karulin for developing the cytokine ELI spot assay, R. Trezza for developing cytokine ELI spot image analysis and performing the ELI spot assays, and C. Etling for technical assistance. Supported by research grant 2470 A-1/2 from the National Multiple Sclerosis Society, grant 194150 from the Juvenile Diabetes Foundation International, and developmental funds from the Center For AIDS Research at Case Western Reserve University (AI36219-02).

28 November 1995; accepted 28 February 1996