Neonatal Peptide Exposure Can Prime T Cells and, upon Subsequent Immunization, Induce Their Immune Deviation: Implications for Antibody *vs.* T Cell–mediated Autoimmunity

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Summary

Neonatal exposure to antigen is believed to result in T cell clonal inactivation or deletion. Here we report that, contrary to this notion, neonatal injection of BALB/c mice with a hen egg lysozyme peptide 106-116 in putative "tolerogenic" doses induced a T cell proliferative and an immunoglobulin G (IgG) antibody (Ab) response of both T helper cell 1 (Th1)- (IgG2a, IgG2b, and IgG3) and Th2-dependent (IgG1) isotypes. Upon subsequent challenge with the peptide in complete Freund's adjuvant in adult life, although this neonatal regimen suppressed proliferation and the production of Th1 cytokines (interleukin[IL]-2 and interferon γ), Th2 cytokine (IL-5, IL-4, and IL-10) secretion was increased, and the serum levels of Th1- and Th2-dependent isotypes of peptide-specific Ab remained elevated. The in vitro proliferative unresponsiveness in Th1 cells could be reversed by Abs to Th2 cytokines (IL-4 and IL-10). Thus, neonatal treatment with a peptide antigen induces T cell priming including production of IgG Abs of both Th1- and Th2-dependent isotypes. Upon subsequent peptide exposure, the peptide-specific T cell responses undergo an effective class switch in the direction of Th2, resulting in T cell proliferative unresponsiveness. Accordingly, this shift towards increased Ab production to autoantigen could be deleterious in individuals prone to autoantibody-mediated diseases. Indeed, neonatal treatment with a self-autoantigenic peptide from an anti-DNA monoclonal Ab (A6H 58-69) significantly increased the IgG anti–double-stranded DNA Ab levels in lupus-prone NZB/NZW F1 mice, despite suppressing peptide-specific T cell proliferation. This adverse clinical response is in sharp contrast to the beneficial outcome of neonatal treatment with autoantigens in Th1-mediated autoimmune diseases, such as autoimmune encephalomyelitis, as reported by others. A Th1 to Th2 immune deviation can explain the discordant biological responses after the presumed induction of neonatal tolerance in autoantibody- vs. Th1-mediated autoimmune diseases.

How does the immune system evade harmful self reactivity in early life? This important question relating to the establishment of self tolerance has been addressed in experimental models of tolerance, where neonatal injection of proteins or their peptide determinants has resulted in Ag-specific unresponsiveness in the adult animal (1–10). This approach of inducing neonatal tolerance to autoAg has been used in attempts to block the development of disease in experimental models of autoimmunity, such as experimental allergic encephalomyelitis (EAE)[1], collagen-induced arthritis (CIA), and diabetes (10–13).

Neonatally induced T cell unresponsiveness has been attributed to several mechanisms, including clonal deletion and/or anergy of Ag-reactive cells (6–10), or generation of specific suppressor or regulatory cells (2, 4, 5). Most work on neonatal tolerance suggesting clonal deletion or inactivation as its mechanism has been based on the inability to mount Ag-specific T cell responses, either proliferative, or proliferative and helper for Ab production, after subsequent antigenic challenge (1–3, 7–10). In contrast to these reports, we found that mice treated neonatally with a peptide underwent a state of "split tolerance," i.e., an increased IgG Ab response despite a marked decrease in T cell proliferation (14). To further understand the consequences of neo-

[1]*Abbreviations used in this paper:* BWF1, New Zealand black/New Zealand white F1; CIA, collagen-induced arthritis; ds, double stranded; EAE, experimental allergic encephalomyelitis; MBP, myelin basic protein; p106, hen lysozyme peptide 106-116; p58, peptide A6H 58-69; PPD, purified protein derivative.

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natal peptide exposure, we studied in vitro peptide-specific T cell proliferation, cytokine production (IL-2, IFN-γ, IL-4, IL-5, and IL-10), and in vivo IgG Ab responses in BALB/c mice treated neonatally with a hen egg lysozyme peptide 106-116 (p106) before and after subsequent peptide challenge.

CD4$^+$ T cells have been divided into Th1, Th2, and Th0 subsets, depending, on their cytokine secretion profile and their specific function (15). Th1 cells secrete IL-2, IFN-γ, and TNF-β, and Th2 cells secrete IL-4, IL-5, IL-6, and IL-10 in response to Ag (15); Th0 cells can secrete mixtures of both types. Th1 cells help in the Ig isotype switch from IgM to IgG2a, whereas Th2 cells help in the switch to IgG1 and IgE (16). The two types of T cells reciprocally regulate each other through the cytokines they secrete, with IL-4 and IL-10 inhibiting Th1 cells, and IFN-γ inhibiting Th2 cells (15). Selective cytokine production and the resultant immune response may be protective or pathogenic in autoimmune and other pathological states (17). For example, in EAE, autoAg-specific immune deviation from Th1 to Th2 has a profound effect on disease (18–20).

In this report, we demonstrate that a putative neonatal "tolerogenic" regimen (8) in itself can prime and activate T cell responses, and upon subsequent peptide challenge, induces immune deviation towards Th2, resulting in proliferative unresponsiveness. Control animals immunized with p106 in CFA produce a predominant Th1-like response. Although Ag exposure in neonatal life has been suggested to result in clonal inactivation, deletion, or suppressor cell induction (3–10), our data support "immune deviation" as a major mechanism of neonatal tolerance.

We tested the biological significance of our findings in a model in which peptides derived from the V region of heavy chains of syngeneic autoAb upregulate autoimmunity in the lupus-prone NZB/NZW F1 (BWF1) mice (21–23). We reasoned that the enhanced Ab responses resulting from neonatal split "tolerance" to autoAg may be deleterious in autoAb-mediated diseases, such as SLE. Indeed, neonatal treatment with a self autoAb-derived peptide (A6H 58-69 [p58]) accelerated autoAb production in BWF1 mice. Thus, while neonatal tolerance to autoAg confers resistance to the development of Th1-mediated autoimmune diseases such as EAE and CIA (10–13), it may accelerate disease in Ab-mediated autoimmunity, providing an applied test for our hypothesis that "neonatal tolerance" actually involves immune deviation from Th1 to Th2.

Materials and Methods

Animals. BALB/c and BWF1 mice were bred in the UCLA Rheumatology Vivarium (NZB and BALB/c stock were derived from mice purchased from Jackson Laboratory [Bar Harbor, ME]; NZW stock was derived from mice received from the National Institutes of Health [Bethesda, MD]). Animals were maintained in accordance with the guidelines of the UCLA Animal Research Committee.

Peptides. Peptides p106 (NAWVAWRNRCK) and p58 (FYNQKFKGKATL) were synthesized in the UCLA Peptide Synthesis Facility using F-*moc* chemistry. Peptide p106 is an I-E^d–binding dominant determinant in the H-2^d haplotype (24), and p58 is an I-E^d– and I-E^u–restricted immunodominant peptide derived from the VH region of an anti-DNA mAb (21, 22). The synthetic polypeptides were analyzed for purity by high-performance liquid chromatography and by mass spectroscopy. The peptides chromatographed essentially as a sharp single peak. The purified peptides were found to have the expected molecular mass.

Neonatal Treatment for the Induction of Tolerance. Newborn mice received two injections of 20 μg (14–15 nmol) of peptide in 50 μl of emulsion containing IFA (Difco Laboratories, Detroit, MI) i.p. at 24 and 72 h after birth, a regimen previously reported to induce T cell tolerance (8). Control littermates received 50 μl of PBS/IFA emulsion using the same time course.

Immunization. At 8–12 wk of age, mice that were peptide-treated and PBS-injected mice were immunized subcutaneously at the base of their tails and in the hind footpads with 5 μg (3.5 nmol) of peptide emulsified 1:1 in CFA (Difco Laboratories). Mice in each group were bled and killed after 9–10 d, and cells from the draining LN and spleen were collected to study proliferative responses. Sera were collected, frozen, and later tested for IgG Ab to the peptide. Age-matched untreated mice were bled to determine the background OD for peptide-reactive Ab.

T Cell Proliferation Assays. Lymphocyte suspensions were prepared from the draining LNs and spleen. The LN or splenic cells (5×10^5 cells/well) were incubated in 200 μl of serum-free medium (HL-1; Ventrex, Portland, ME) with 2 mM L-glutamine in 96-well microtiter plates without Ag, with different concentrations of each peptide, or with purified protein derivative (PPD) of *Mycobacterium tuberculosis* (700 U/ml; Evans Medical Ltd., Langhurst, UK) for 5 d. The cells were pulsed with 1 μCi [^{3}H]TdR during the last 18 h of the culture. The cells were collected onto glass fiber filters with a semiautomatic cell harvester (Skatron Instruments, Sterling, VA), and the radioactivity was measured in a beta counter (Beckman Instruments, Fullerton, CA). Positive control responses were those to PPD. Negative controls were cells but no Ag (background cpm), or cells cultured with an irrelevant peptide. Results are expressed as the mean triplicate Δcpm (cpm with Ag − background cpm).

Determination of Ab to Peptide. Peptide (p106 and p58)–specific Ig levels for IgG and isotypes (IgG1, IgG2a, IgG2b, and IgG3) were determined by ELISA. Peptide (0.3–0.6 μg) was incubated in 96-well high-binding microtiter plates (Costar Corp., Cambridge, MA) overnight at 4°C. After washing and blocking with 10% FCS in PBS, test sera were added at serial threefold dilutions beginning at 1:30, and were incubated for 1 h at 37°C. After three washes, alkaline phosphatase–conjugated goat anti–mouse IgG or isotypes (Fisher Biotech, Pittsburgh, PA) were added and incubated at 37°C for 1 h. After washing, the color reaction was developed by adding 1 mg/ml *p*-nitrophenyl phosphate (Sigma), and after 30 min, read at 405 nm using an ELISA reader (M600; Dynatech, Chantilly, VA). Results are expressed as the mean ± SEM of mean triplicate ΔOD (i.e., actual OD − OD in sera of age-matched unmanipulated mice).

Detection of Lymphokines in Culture Supernatants. Neonatally treated mice were immunized as described in the "Immunization" section above. 9–11 d later, LN and spleen cells were collected and cultured separately with medium alone, PPD, or peptide. Culture supernatants were collected after 24–30 h for IL-2, and after 48–60 h for detection of IFN-γ, IL-4, IL-5, and IL-10. IL-2 was detected by a standard bioassay using an IL-2 indicator cell line, a subclone of CTLL-2, which does not respond to murine rIL-4 (25). The proliferative responses of this cell line could be blocked by an anti–murine IL-2 mAb, S4B6 (26). IL-2 standard curves

were generated using IL-2 (PharMingen, San Diego, CA). IL-4 levels were detected by a standard bioassay using an IL-4–dependent cell line, CT.4S (27), kindly provided by Dr. William Paul (National Institutes of Health, Bethesda, MD). Proliferative responses of CT.4S were blocked by an anti–IL-4 mAb, 11B.11 (28), a kind gift from Dr. Craig Reynolds (National Cancer Institute, Frederick, MD). IL-4 was also detected by a capture ELISA using mAb pairs purchased from PharMingen. Standard curves were generated using IL-4 that was generously provided by Dr. Steven Gillis (Immunex Corp., Seattle, WA). IFN-γ, IL-5, and IL-10 levels were determined by a capture ELISA using purified mAb pairs, and murine IFN-γ, IL-5, and IL-10 as standards (PharMingen) (22, 29).

Measurements of Effect of Neonatal Treatment with p58 in Lupus-prone BWF1 Mice. Neonatal BWF1 mice were treated with p58/IFA in "tolerogenic" doses described above (8), or with PBS/IFA. Both groups of mice were monitored for serum IgG anti-p58 and anti-DNA Ab levels and clinical nephritis. Serum Ab to DNA were measured by a standard ELISA, as described previously (22, 30). Anti–double-stranded (ds) DNA Ab titers are expressed as units per milliliter, using a positive reference standard of pooled serum from 8-mo-old BWF1 mice. Proteinuria was estimated by urine examination using albustix (Ames, Elkhart, IN); azotemia was estimated using azostix (Ames) (22).

Statistical Analysis. Comparisons between peptide-treated and control groups were done using Student's t test and the rank sum Mann-Whitney U test.

Results

Neonatal Exposure to the Putative "Tolerizing" Peptide Regimen Itself Induced a T Cell Proliferative and Helper Response. 6–10-wk-old BALB/c mice treated neonatally with p106 in IFA had a peptide-specific splenic T cell proliferative (Fig. 1 *A*) and an IgG Ab response (Fig. 1 *B*), even before any immunization with the peptide. These peptide-reactive Ab were of both Th1 (IgG2a, IgG2b, and IgG3)– and Th2 (IgG1)–dependent IgG isotypes, IgG1 being predominant (Fig. 1, *C* and *D*, and data not shown). Splenic T cell culture supernatants from the tolerized mice contained very low levels of IL-4 and IL-5, but no detectable IFN-γ by ELISA (data not shown). T cell proliferation was detected in all "tolerized" animals tested, IgG Ab responses in 8 of 12 mice, and cytokine responses in 7 of 12 mice. A similar pattern of observations was observed in two experiments.

Control PBS/IFA-injected mice (Fig. 1 *A–D*), or mice treated neonatally with a control peptide, p58 (not shown), did not have detectable p106-reactive Ab or T cell proliferation. Also, the p106-treated mice did not have a nonspecific increase in IgG Ab (such as anti-DNA) or T cell proliferation to other Ags, such as p58 (data not shown).

This suggests that the putative tolerizing regimen itself initially induces either Th0 cells or it induces both Th1- and Th2-like cells capable of mediating production of all IgG isotypes.

Peptide-specific T Cell Proliferative Unresponsiveness but Primed IgG Ab Formation Occurred upon Subsequent Peptide Immunization. Animals treated with the peptide-tolerizing regimen showed a marked reduction of peptide-specific T cell proliferation in draining LN cells (Fig. 2 *A*). T cell proliferative responses to in vitro PPD stimulation were equivalent in peptide-treated vs. PBS-injected animals, demonstrating the specificity of unresponsiveness (Fig. 2 *A*). Peptide-specific proliferation was also decreased in the spleen cells of peptide-treated animals (Fig. 2 *B*), although this effect was less pronounced in splenic T cells than in draining LN cells. The decrease in splenic cell proliferation was only 2–3-fold (P = borderline, 0.05) compared to a 15–50-fold decrease in LN cell proliferation (P <0.01 to <0.05, Student's t test). Residual proliferative responses in splenic cells from treated animals were inhibitable by anti-CD4 or anti–I-E^d Ab (data not shown), suggesting that a significant number of p106-specific, I-E^d–restricted CD4$^+$ T cells had migrated to the spleen and/or had escaped the proliferative tolerance process in p106-tolerized mice.

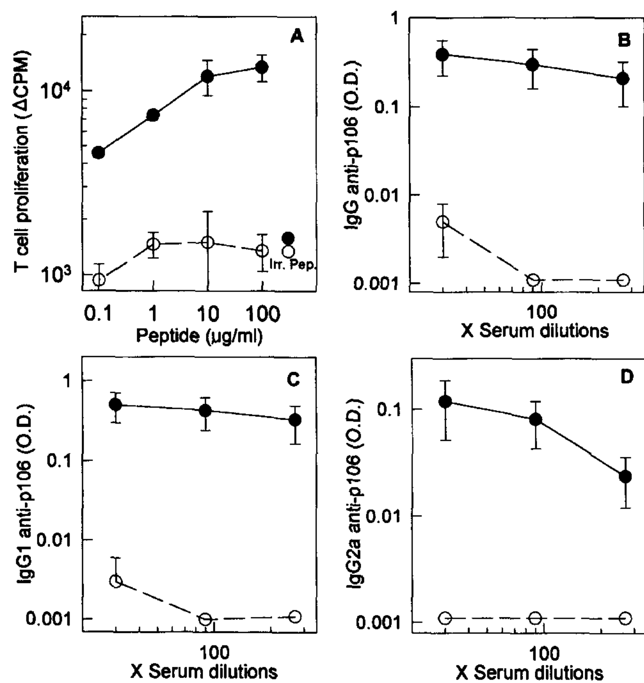

Figure 1. Neonatal peptide administration in a putative tolerogenic regimen (8) induces T cell proliferative and helper responses even before any immunogenic challenge. 19 BALB/c neonates received 20 μg each of two injections of p106 in IFA (●—) within 24 and 72 h of birth. Control neonates received PBS in IFA (○---). Both groups of mice were tested for splenic T cell proliferation and serum IgG Ab at the age of 6–10 wk. (*A*) Significant (more than threefold) peptide-specific T cell proliferation was present in all seven p106-treated mice tested, but not in controls (P <0.01 to <0.001 at different in vitro peptide concentrations, Student's t test). The mean ± SEM of mean triplicate Δcpm values at different in vitro peptide concentrations are shown. Neither group of mice made a proliferative response to an irrelevant peptide, p58 (*Irr. Pep.*). Background cpm values in the p106- and PBS-treated groups were 3,300 ± 410 and 3,099 ± 1,878, respectively. (*B*) 12 p106-treated and 6 control mice were tested for the presence of serum IgG anti-p106 Ab. Data shown here are the mean ± SEM of OD absorbance values at serial serum dilutions. Peptide-reactive IgG Ab titers were significantly increased in p106-treated mice (P <0.01 to <0.001, Mann-Whitney U test). (*C* and *D*) The IgG Ab formed in peptide-treated mice were of both IgG1 and IgG2a isotypes (P <0.05 to <0.001, Mann-Whitney U test). Results are from one of two similar experiments.

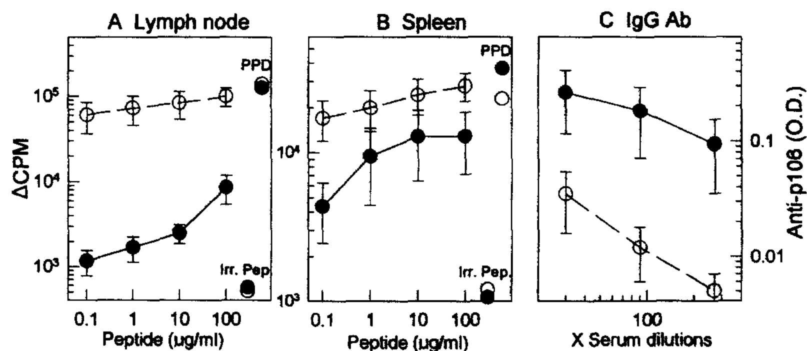

Figure 2. Upon subsequent exposure to the same peptide in CFA in adult life, neonatally tolerized mice develop T cell proliferative unresponsiveness, but priming for IgG Ab. Newborn mice received either PBS/IFA (○---) or p106/IFA (●—), and were then immunized with p106/CFA at the age of 8–12 wk. 9–10 d later, these mice were bled for detection of serum IgG Ab, and LN and spleen tissues were harvested. (*A*) Peptide-specific proliferative unresponsiveness in draining LN cells. LN and spleen cells from five mice in each group were cultured separately in the presence of medium alone, PPD, an irrelevant peptide (*Irr. Pep.*), and different concentrations of p106. Results shown are the mean ± SEM of mean Δcpm from triplicate culture wells. Background cpm values were 986 ± 267 and 1,686 ± 381 in p106 and PBS groups, respectively. (*B*) Splenic T cell proliferation was also decreased in p106-tolerized mice. Background cpm values were 1,853 ± 476 and 1,389 ± 257 in p106 and PBS groups, respectively. Note that T cell hyporesponsiveness was much less marked in spleen cells than in LN cells. (*C*) In contrast to decreased proliferative responses in *A* and *B* above, peptide-reactive IgG Ab were increased in neonatally treated mice. The mean ± SEM ΔOD values from 10 mice in each group are shown. The mean ΔOD values in peptide treated mice were 8–19-fold higher than in the control group. Similar results were obtained in another experiment.

In contrast to the reduced proliferative response, tolerized mice had an increase in p106-specific IgG Ab responses compared to PBS-injected animals (Fig. 2 *C*). The increase in IgG Ab to the peptide did not result from nonspecific hypergammaglobulinemia, since animals treated with p106 did not have IgG Ab to irrelevant Ags (data not shown). Similarly, mice that were treated neonatally and immunized subsequently with another peptide (p58) did not have p106-reactive Ab (data not shown).

In Vivo IgG Isotype Expression in Peptide-tolerized Mice Suggests Persistence of Both Th1- and Th2-dependent Ab. The enhanced peptide-reactive IgG Ab could be caused by selective priming of one $CD4^+$ T cell subset, Th2-like cells, as suggested in adult high dose tolerance models (31–33). We measured IgG isotypes of peptide-specific Ab in "tolerized" mice. Neonatally treated mice made a potent response of both Th1-dependent (IgG2a, IgG2b, and IgG3) and Th2-mediated (IgG1) IgG Ab to p106, IgG1 being predominant (Fig. 3, and data not shown).

This indicated clearly that the T helper function of both Th1 and Th2 cells was intact after neonatal administration of peptides in IFA.

In Vitro Lymphokine Secretion Suggests a Th1 to Th2 "Immune Deviation." To further understand the differential role of Th subsets in the outcome of neonatal tolerance induction, we studied lymphokines released by T cells in culture supernatants of draining LN or spleen cells from PBS- and peptide-treated animals after peptide immunization in adult life (Fig. 4). IFN-γ and IL-2 levels were significantly decreased in peptide-tolerized animals ($P < 0.05$ and borderline, respectively, Student's *t* test) (Fig. 4, *A* and *B*). In contrast, IL-5 and IL-10 were only detected in culture supernatants from such mice, but not from control mice ($P < 0.01$ and < 0.05, respectively, Student's *t* test) (Fig. 4, *C* and *D*). IL-4 levels detected by ELISA were only slightly increased in treated animals (Fig. 4 *E*). IL-4 levels detected by a bioassay using the CT.4S cell line also showed a similar pattern (data not shown). Control animals (PBS tolerized, p106/CFA immunized) mounted a predominant Th1 response (Fig. 4 *A*–*E*). Thus, in vitro lymphokine production favored the speculation that neonatal treatment deviates the subsequent immune response towards Th2.

In Vitro Manipulation of T Cells with Anti–IL-10 and –IL-4 Restores T Cell Proliferation in Tolerized Animals. To test the hypothesis that in vitro T cell proliferative tolerance was at least in part a result of induction of Th2 cytokines which then downregulate Th1 activities, we cultured LN and

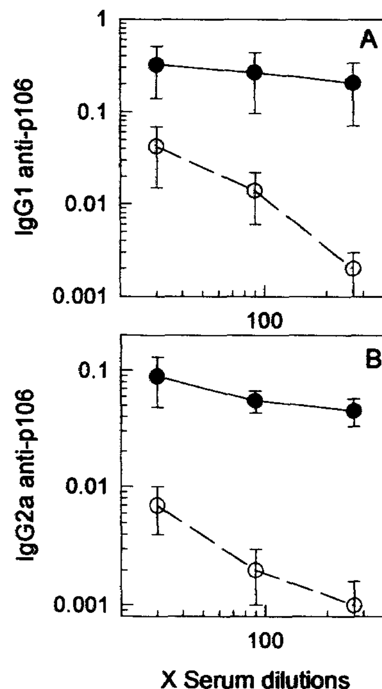

Figure 3. Increased IgG anti-p106 Ab in mice neonatally treated with p106 are of both Th1- and Th2-dependent isotypes. Sera from PBS- (○---) or p106 (●—)-treated mice described in Fig. 2 were tested for IgG anti-p106 Ab of different isotypes. Results are expressed as the mean ± SEM of mean triplicate ΔODs. IgG2b and IgG3 Ab were also increased in treated mice (not shown). The findings are from one of two similar experiments.

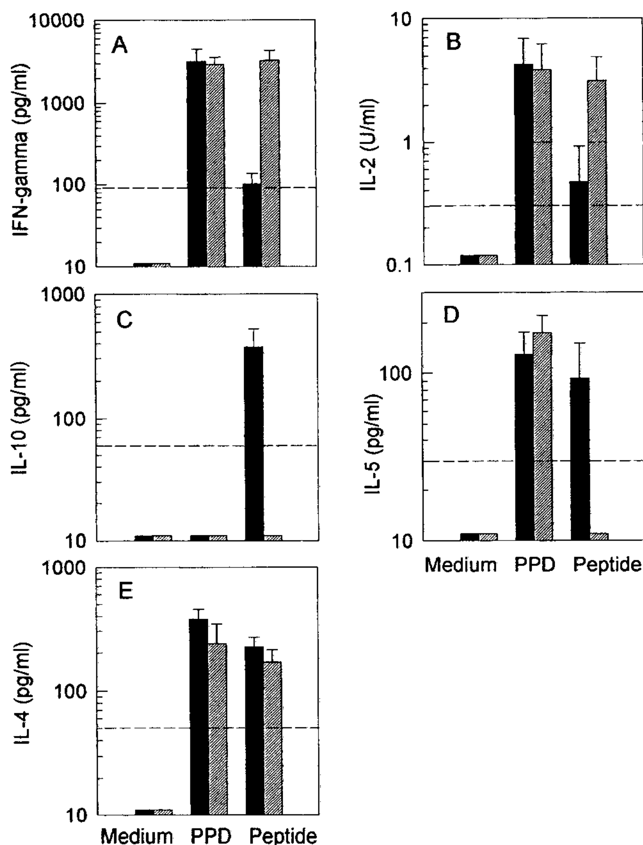

Figure 4. Neonatal treatment with p106 results in an in vitro decrease in Th1 cytokines but an increase in Th2 cytokines. Draining LN cells from PBS-treated (*hatched bar*) or p106-treated (*solid bar*) and subsequently immunized mice (n = 5 in each group), as described in the legend of Fig. 2 were cultured with medium alone, PPD, and peptide. Culture supernatants were assayed for IFN-γ (*A*), IL-10 (*C*), IL-5 (*D*), and IL-4 (*E*) by ELISA. The sensitivity of this assay was 100 pg/ml for IFN-γ, 60 pg/ml for IL-10, 30 pg/ml for IL-5, and 50 pg/ml for IL-4 shown by the horizontal lines crossing the figure completely (------). (*B*) For detection of IL-2 (U/ml), supernatants were cultured with an IL-2-sensitive subclone of CTLL-2. The background cpm value of this cell line cultured with medium alone was 78 ± 10; and in the presence of titered amounts of a murine IL-2 standard, 50 U/ml = 70,714 ± 2,766, 10 U/ml = 12,350 ± 1,494, 2 U/ml = 2,070 ± 46, 0.4 U/ml = 323 ± 47, and 0.08 U/ml = 115 ± 10 U/ml. The sensitivity of this assay was 0.3 U/ml. Results are expressed as the mean ± SEM from one of three similar experiments.

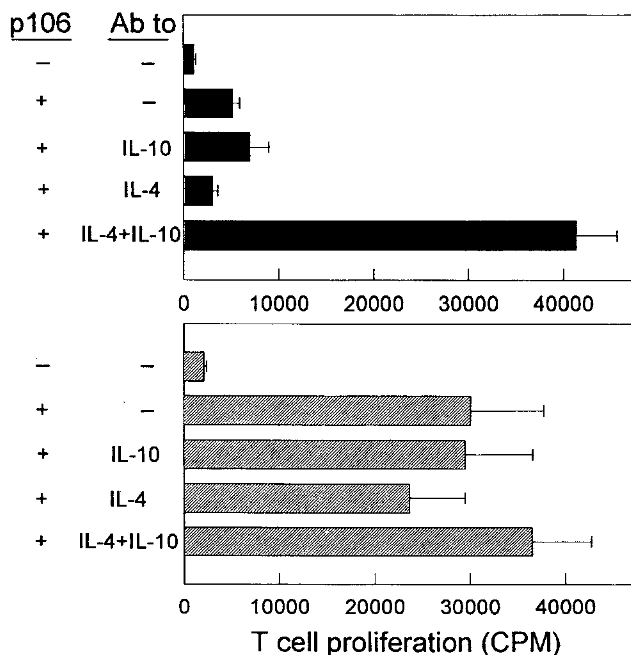

Figure 5. Antibodies to IL-10 and IL-4 can restore T cell–proliferative hyporesponsiveness in spleen cells of tolerized animals. BALB/c mice were treated neonatally with p106 (*upper panel, solid bars*) or PBS (*lower panel, hatched bars*), and were later immunized as described in the legend of Fig. 2. Spleen cells from four mice in each group were cultured in medium alone (−/−), in the presence of peptide (+/−), and peptide + anti–IL-4 and/or anti–IL-10. Results are shown as the mean ± SEM of T cell proliferation (cpm). These results are from one of two similar experiments.

splenic T cells from immunized, p106-tolerized mice with the peptide (1–10 μg/ml) and varying concentrations (0.005–50 μg/ml) of anti–IL-4 (11B.11) and/or anti–IL-10 (JES5-2A5). Peptide-specific splenic T cell proliferation in p106-tolerized mice was restored (equivalent to that in control immunized animals) in the presence of both anti–IL-4 and –IL-10, whereas these Ab did not significantly affect proliferative responses in PBS-treated controls (Fig. 5). Anti–IL-10 or –IL-4 alone had minimal or no effect. LN T cell proliferation was also increased, but to a lesser degree (data not shown), suggesting that Th1 precursors may have migrated away from the LN. Thus, reversal of T cell proliferative unresponsiveness by Ab to Th2 cytokines suggests that the presence of IL-4 and IL-10 may be responsible for proliferative Th1 tolerance.

Neonatal Tolerance Induction with p58 Increases IgG Anti-dsDNA AutoAb in BWF1 Mice. The outcome of neonatal treatment with p58 in BWF1 mice was similar to that achieved with p106 treatment in BALB/c mice. Similar to the results shown in Fig. 1 *B*, serum IgG anti-p58 Ab were significantly increased in p58-treated mice compared to PBS-injected mice before any further challenge immunization (Fig. 6 *A*). Upon subsequent challenge with p58/CFA in adult life, T cell proliferation was decreased in response to the peptide in culture, but peptide-specific IgG Ab were increased (data not shown).

We reported earlier that immunization of young BWF1 mice with p58 increases circulating IgG Ab to dsDNA and accelerates clinical nephritis (21). We reasoned that if T cell responses of Th1 and Th2 were implicated in such an autoAb-mediated disease, then neonatal treatment that leads to split tolerance to this peptide might accelerate autoAb production and clinical disease. To test this, we compared IgG anti-dsDNA levels in the sera of p58-treated animals with those in PBS/IFA-injected controls. In conformity with our hypothesis, serum IgG anti-DNA levels were significantly increased among p58-treated mice compared to controls (P <0.01 to <0.05, Student's t test) (Fig. 6 *B*). This increase in anti-DNA Ab was associated with an increase in proteinuria. The mean ± SEM proteinuria in p58-treated mice was 873 ± 309 mg/dl compared to 101 ± 36 mg/dl in control mice (P <0.05; Student's t test). Simi-

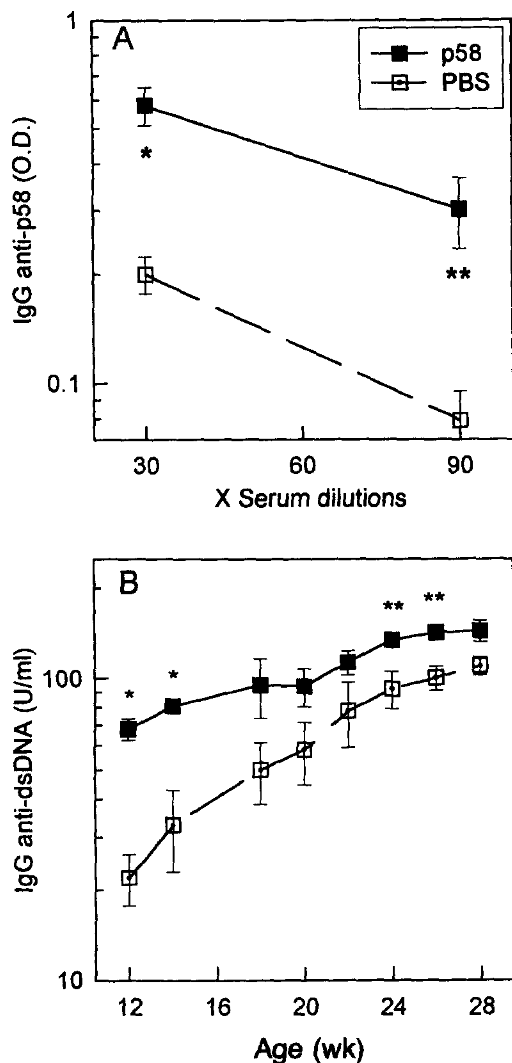

Figure 6. Neonatal administration of a self-autoAb-derived peptide, p58, increases serum levels of IgG anti-p58 and anti-dsDNA Ab in BWF1 mice. 10 newborn mice in each group were treated with two injections of p58/IFA or PBS/IFA within 72 h of birth. Both groups of mice were monitored for serum IgG anti-p58 and anti-dsDNA Ab. (*A*) Serum p58-reactive IgG Ab were significantly increased in peptide-treated mice compared to PBS-injected mice (*P <0.01, **P <0.001, Student's t test). Results are shown as the mean ± SEM of mean triplicate OD at serial serum dilutions in 10–12-wk-old mice. (*B*) Serum IgG anti-dsDNA Ab levels are expressed as the mean ± SEM U/ml at different age groups. BWF1 mice treated at birth with p58 had a significant increase in anti-DNA Ab than in PBS-injected controls (*P <0.01, **P <0.05; Student's t test).

lar results were obtained in a separate experiment, where 10 p58/IFA-treated mice had significant increases in anti-DNA Ab and proteinuria compared to 5 control peptide (p106)-treated and 11 unmanipulated mice.

Discussion

Ag Exposure in Neonatal Life Can Prime T Cells Rather Than Completely Eliminate/Inactivate Them. It is generally believed that Ag exposure in early life results in clonal deletion or anergy, thus maintaining self tolerance and preventing autoimmunity (1, 3, 6, 8–10). Contrary to this notion, we show that a T cell determinant peptide administered in neonatal life can induce T cell proliferative and IgG Ab responses. The neonatally treated mice had circulating IgG Ab of both Th1- and Th2-promoted isotypes. However, weak T cell proliferative response, predominant IgG1 Ab production, and detectable Th2 cytokine secretion would suggest a preferential priming of Th2-like cells by this peptide administered to newborns.

Functional Split Tolerance: Proliferative Tolerance but Primed Helper Responses. Previous attempts to address the mechanism of neonatal tolerance have used various protein (3, 7, 10) or peptide (8, 9) Ags as tolerogens, and have reported different outcomes at the level of T cell proliferation or Ab formation upon subsequent challenge with the same Ag in CFA. For example, neonatal injections of deaggregated human gamma globulin resulted in the induction of unresponsiveness in B as well as T proliferative and Th cells (3), whereas newborns tolerized with myelin basic protein (MBP) developed complete nonreactivity to MBP at the T cell proliferation level, but made some anti-MBP Ab (10). Most studies using minimal immunogenic peptides have reported T cell proliferative tolerance (7–9). In this study, we have shown that neonatal mice, treated with peptide doses corresponding to milligrams of a protein Ag such as IgG, develop tolerogen-specific proliferative unresponsiveness, but priming for a T helper response producing IgG Ab upon subsequent peptide challenge. This phenomenon of functional split tolerance has not been previously reported in a neonatal peptide tolerance model. The discrepancy between ours and previous reports on tolerance in Ab production could be caused by differences in the tolerogen used, or by the use of a single determinant in a peptide vs. multiple determinants in protein Ags. Tolerance induction to a protein Ag may involve many T cell determinants; some determinants may activate Th1 cells, others Th2 cells, or still others regulatory or suppressor cells, and the ultimate outcome may depend on the patterns of their reciprocal regulation. Differential sensitivity to in vivo adult high dose tolerance induction has been shown at the level of Th subsets (31–33). Parenteral administration of large quantities of Ag in adult mice results in a state of tolerance characterized by unresponsiveness in Th1- but not in Th2-type lymphocytes (31–33), although this view has been contested by others (34).

Immune Deviation as a Mechanism of Neonatal split T Cell Tolerance. In our experiments, control animals immunized with peptide in CFA elicited a predominant Th1 response, whereas animals neonatally treated with peptide and subsequently immunized with peptide/CFA had a predominant Th2 response. The sequence of events leading to this immune deviation may be visualized as follows: neonatal injection of p106/IFA initially induces peptide-specific Th0 or both Th1 and Th2. The in vivo peptide/CFA challenge in adult life followed by in vitro restimulation with

the peptide results in a switch towards Th2. This phenomenon may be similar to other in vitro and in vivo situations, where repeated Ag exposure can divert immune responses towards Th2 (35, 36). For example, mice immunized once with irradiated *Schistosoma mansoni* cercariae develop a predominantly Th1 response, while repeated immunization with the same parasite causes the response to become predominantly Th2 (35). In an in vitro system, Th1-like T cell clones can be converted into Th2-like after 3 wk of culture (36). Increased IL-10 and IL-4 secreted by Th2 cells in culture should lead to decreased development and proliferation of peptide-specific Th1-like cells, as well as a decrease in Th1 type lymphokines (15, 37). In this sense, neonatal peptide-induced tolerance actually would represent an effective class switch in the direction of Th2, and is brought about by repeated exposure of Th cells to the Ag, as well as by inhibition of Th1 cells mediated by IL-4 and IL-10.

In the experiments described here, decreased peptide-specific T cell proliferation in tolerized mice could be restored to normal by in vitro inhibition of Th2 cytokines (Fig. 5), supporting the idea of immune deviation and inhibition of Th1 function. The reversal of proliferative unresponsiveness was more pronounced in splenic than in LN cells. This suggests that some peptide-specific Th1 cells in treated mice may have migrated from the LN to the spleen and other organs during peptide immunization in adult life. In the spleen, preferential Th2 activation (turning off Th1) can be reversed with Abs to Th2 cytokines.

Thus, immune deviation, as well as partial migration of peptide-specific Th1 cells from the draining LN sites, may each contribute to our findings of neonatal split tolerance. Although clonal deletion or anergy of some peptide-specific T cell populations cannot be ruled out in our experiments, it does not appear to be a major element in this model, since unresponsiveness in Th1 cells could be reversed to the control level in the presence of anti-Th2 cytokines.

How to Explain Increased In Vivo Th1-dependent (IgG2a) Isotype Expression, but Decreased In Vitro Th1 Lymphokine Secretion, after Immunization of Neonatally Treated Mice in Adult Life. The findings described in this paper reveal an additional interesting phenomenon: peptide-tolerized mice actually make increased levels of peptide-reactive Ab of IgG isotypes that are considered to be Th1 mediated (16), while failing to show another Th1 property of Ag-specific proliferation. We can put forth the following possibilities to explain this intriguing observation: (*a*) persistence of serum IgG2a Ab that was induced by neonatal peptide-primed Th1 or Th0, and was formed before peptide challenge and immune deviation; (*b*) differential effects of tolerance on separate Th1 effector functions, e.g., engaging the TCR of a $CD8^+$ T cell clone in the absence of costimulatory signals can result in decreased proliferation and IL-2 secretion, but enhanced cytolytic potential (38); and (*c*) anergy may be established only in high affinity Th1 cells, but the remaining low affinity Th1 may still display a partial IFN-γ response that is responsible for IgG2a Ab production.

It has been reported that tolerized Th1 retain their ability to deliver a signal that stimulates early B cell activation (39). The second signal for activation of resting B cells in our case may be delivered by activated Th2. It is known that the ability of Th1 to stimulate B cell proliferation and/or Ab synthesis increases significantly if the B cells have been preactivated or cultured with Th2 lymphokines (40). Thus, in our case, it is possible to imagine that B cells that have been activated before the adult peptide challenge can still make IgG2a Ab in the presence of partially tolerized Th1 and activated Th2.

Functional Relevance of Neonatal "split" Tolerance in Autoimmune Diseases: Implications in Pathogenesis and Immune Intervention. We show here that a functional dichotomy exists with apparently selective tolerance induction for Th1 proliferation, but activation of T helper function for both Th1- as well as Th2-dependent Ab isotypes. This observation could be relevant for our understanding of therapeutic intervention in autoimmune diseases such as EAE and SLE. In EAE, interventions that prevent, suppress, or deviate autoAg-specific Th1 responses have a beneficial influence on disease (18–20). In SLE, production of autoAb, including pathogenic IgG anti-DNA, is dependent on T cell help (22, 41, 42). IL-10 administration in BWF1 mice accelerates autoAb production and disease, while treatment with anti–IL-10 Ab delays onset of disease (43). Therefore, a shift towards Th2 and autoAb production within all isotypes could be deleterious in individuals prone to autoAb-mediated diseases. To address this, we studied the effect of neonatal treatment with a self–autoAb-derived peptide (p58) on the autoAb production in BWF1 mice. Immunization with p58 has been shown previously to accelerate autoimmunity in the BWF1 mice (21). In the present work, BWF1 mice neonatally treated with this peptide had a significant increase in IgG anti-DNA Ab, which was associated with functionally split tolerance: T cell proliferative unresponsiveness concomitant with a primed IgG Ab response. Thus, immunogenic regimens and traditional neonatal tolerogenic regimens both induce peptide-specific Th stimulating the secretion of IgG autoAb in this murine model of SLE. Since p58-reactive T cells exist in young unprimed BWF1 mice (23), the lack of tolerance at the helper level to this and similar peptides may be physiologically significant in the induction and/or maintenance of autoimmunity. Such Th presumably have both escaped tolerance and been concomitantly primed by this self peptide (22, 23). Thus, neonatal treatment with an autoantigenic peptide can accelerate autoimmunity in individuals prone to autoAb-mediated diseases. Nevertheless, we have recently shown that intravenous adult high dose tolerance to p58 can suppress both proliferative and Ab responses, delaying development of anti-DNA Ab in BWF1 mice (23), demonstrating that differences in dose and distribution of Ag and the development of the immune system play an important role in influencing the tolerance induction pathway.

Thus, neonatal treatment of T cells with Ags, as well as the subsequent induction of immune deviation and resultant split tolerance upon repeated exposure to self Ag, may be

deleterious in individuals that are genetically programmed to make responses to autoAg that result in pathogenic autoAb. On the other hand, this behavior may prevent widespread Th1-mediated autoimmunity. Clearly, the many parameters influencing responsiveness vs. tolerance must be taken into account to avert the undesired consequences that can follow the administration of Ag.

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