In Vivo Induction of Tolerance in Murine CD4+ Cell Subsets

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Summary

The induction of tolerance in mice to preparations of deaggregated human gamma globulin (DHGG) results in in vitro antigen-specific unresponsiveness in CD4+ T cells as well as in both the T helper 1 (Th1) and Th2-like subpopulations. Whereas both CD45RB^{hi} and CD45RB^{lo} cells from lymph nodes of HGG/complete Freund's adjuvant-immunized mice (control) proliferated in vitro to HGG, both subpopulations from mice previously tolerized with DHGG failed to respond. Furthermore, CD4+ T cells from control, but not from DHGG-injected mice, secreted high levels of interleukin 2 (IL-2) after in vitro stimulation with HGG. Although significant levels of IL-4 in supernatants of control CD4+ cells stimulated with HGG were detected in some, but not all, experiments, significant levels of IL-4 were never detected in supernatants of HGG-stimulated tolerant CD4+ cells. The demonstration that serum IgG1 anti-HGG is preferentially produced in a few tolerant mice that exhibit a leaky tolerant state suggests that tolerance induction may be more difficult to induce in IL-4– than in IL-2–producing cells.

A solid and long-lasting tolerant state can be induced in adult mice by a single injection of deaggregated human gamma globulin (DHGG)[1] (for a review see reference 1). Although tolerance is induced in both T and B cells, the dose of tolerogen required for B cell tolerance is 2–3 $\log_{10}$ greater than that for T cell tolerance (for a review see reference 1). The tolerant state in T cells is of long duration, whereas in B cells it is of relatively short duration (2). Induction of tolerance in T cells is independent of the thymus and is not controlled by regulating T cells (3). Although the in vivo induction of tolerance in the T cells can be interfered with by generators of IL-1 or IL-1 itself (4, 5), once induced, the tolerant state cannot be terminated by these reagents. In addition to the in vivo induction of tolerance, tolerance has been induced in HGG-specific T cell clones using either DHGG (6, 7) or inappropriate antigen presentation (8–10). Whereas tolerance in Th1 clones is induced in terms of both proliferation and T cell help, tolerance in Th2 clones is induced at only the T helper level (10, 11). Whether the in vivo induction of tolerance occurs in all CD4+ cell populations has recently been questioned. Data have been reported that imply that DHGG induces tolerance in IL-2–producing T cells, but induces responsiveness in IL-4–producing cells, resulting in IL-4 production and T helper activity (12).

In this report, the susceptibility of CD4+ cells and their subsets to the induction of tolerance to DHGG was assessed. Our results demonstrate that when mice are exposed to HGG as a tolerogen before immunization, such that the in vitro antigen-specific proliferation of CD4+ T cells is totally abrogated and serum antibody levels are dramatically reduced, antigen-specific proliferation of both Th1- and Th2-like subsets is abrogated. Furthermore, a decrease in IL-4 as well as IL-2 production following antigen stimulation can be demonstrated in CD4+ T cells from tolerant versus control mice. Thus, these results indicate that under appropriate conditions of tolerance induction, both Th1- and Th2-like CD4+ functions can be inhibited.

Materials and Methods

Animals. Female CBA/CaJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used between 8 and 12 wk of age.

Tolerization and Immunization. HGG was purified from Cohn Fraction II of human plasma (lot RC104; Hyland Laboratories, Glendale, CA, obtained through the courtesy of the American Red Cross National Fractionation Center, Bethesda, MD) by ion exchange chromatography (DEAE cellulose) as previously described (13). DHGG was prepared by ultracentrifugation of the HGG in a swinging bucket rotor (SW 50.1; Beckman Instruments, Inc., Palo Alto, CA) at 150,000 *g* for 150 min. After centrifugation, the upper quarter of the solution was carefully removed and diluted to the appropriate concentration with 0.15 M pyrogen-free NaCl. To induce tolerance, 2.5 mg DHGG was injected intraperitoneally. Mice

[1] *Abbreviations used in this paper:* DHGG, deaggregated human gamma globulin; HGG, human gamma globulin.

were challenged subcutaneously in both hind footpads with a total of 200 μg HGG emulsified in CFA. Mice challenged with KLH (Calbiochem, San Diego, CA) were similarly injected with a total of 100 μg KLH in CFA.

Cell Preparations. Inguinal and popliteal lymph nodes were aseptically removed from mice and dissociated into single-cell suspensions. Cells (pooled from four mice per group unless otherwise indicated) were then incubated with mAbs to B220 (RA3–6B2, rat IgG2a), CD8α (53-6.7, rat IgG2a), CD8β (53-5.8, rat IgGl), IA^k (11-5.2, mouse IgG2b), and the TCR-γ/δ (GL3, hamster IgG) (all mAbs from PharMingen, San Diego, CA) as well as mAbs to Mac-1α and the heat-stable antigen. Hybridoma cells producing antibody to Mac-1α (M1/70, rat IgG2b) were obtained from the American Type Culture Collection (Rockville, MD) and the IgG2b fraction was generously provided by Dr. D. N. Ernst (The Scripps Research Institute). Hybridoma cells secreting antibody to the heat-stable antigen (J11d.2, IgM) were a generous gift of Dr. S. R. Webb (The Scripps Research Institute), and the antibody was used as a hybridoma supernatant. $CD4^+$-enriched cells were negatively selected using magnetic beads coated with goat anti-rat IgG and goat anti-mouse IgM (Advanced Magnetics, Inc., Cambridge, MA). Splenic cells depleted of T cells were used as APC.

Proliferation Assay. $CD4^+$-enriched lymphocytes were cultured at 37°C in 5% CO_2 in RPMI-1640 media supplemented with 1% Nutridoma-SP (Boehringer Mannheim, Indianapolis, IN), 2% fetal bovine sera, 2 mM glutamine, 5×10^{-5} M 2-ME, vitamins, sodium pyruvate, 1 mM Hepes, 100 U penicillin/ml, and 100 μg streptomycin/ml. 2×10^5 $CD4^+$-enriched cells were incubated with 3×10^5 APC, with or without HGG at a final concentration of 2 mg/ml, in a final volume of 0.2 ml in 96-well flat-bottom tissue culture plates. Stimulation with plate-bound hamster anti-mouse CD3ε (145–2C11, PharMingen) was done as previously described (14). APC consisted of spleen cells from naive mice that were treated twice with anti-Thy 1.2 mAb (New England Nuclear, Boston, MA) and rabbit complement (Rabbit Low-Tox M; Accurate Chemical and Scientific Corp., Westbury, NY) and then passaged over Ficoll-Hypaque and irradiated 3,000 rad. Quadruplicate cultures were pulsed with 1 μCi [^{3}H]TdR (Amersham Corp., Arlington Heights, IL) before harvest. The incorporation of [^{3}H]TdR was measured by standard liquid scintillation counting.

Lymphokine Analysis. $CD4^+$-enriched cells were cultured as above for proliferation assays, except that 4×10^5 $CD4^+$-enriched cells were incubated with 6×10^5 APC in a final volume of 0.2 ml. Supernatants were harvested at 48 h and assayed for IL-2 levels by a two-site sandwich ELISA essentially as previously described (14), using JES6-1A12 anti-mIL-2 as a coating antibody and biotin-JES6-5H4 anti-mIL-2 as detecting antibody (PharMingen). Wells were then incubated with ExtrAvidin alkaline phosphatase before adding *p*-nitrophenylphosphate substrate (Sigma Chemical Co., St. Louis, MO). Absorbance was measured at 410 nm using a microplate reader (model MR600; Dynatech Labs., Inc., Chantilly, VA). The picograms per milliliter of IL-2 in supernatants was calculated from a log–log plot of absorbance versus concentration of a standard preparation of mIL-2 (PharMingen) with the lower limit of sensitivity at 10 pg/ml. For quantitation of IL-4, supernatants were harvested at 72 h and assayed using a murine IL-4 ELISA with protein G–purified anti-mIL-4 (11B11, generously provided by Dr. D. N. Ernst) used as a coating antibody and biotin-BVD6-24G2 anti-mIL-4 (PharMingen) as detecting antibody. The lower limit of sensitivity was 20 pg/ml, using a standard preparation of mIL-4 (PharMingen). In some experiments, a murine IL-4 ELISA with a sensitivity of 5 pg/ml was used (Endogen, Inc., Boston, MA).

Antibody Assays. Mice were bled 9 d after immunization with HGG/CFA and the serum anti-HGG antibody of individual mice was assayed by ELISA as previously described (5) with an alkaline phosphatase–conjugated sheep anti-mouse IgG (Cappel Laboratories, Cochranville, PA) with no cross-reaction to HGG as a developing antibody and *p*-nitrophenylphosphate (Sigma Chemical Co.) as substrate. Standard curves were generated using affinity-purified polyclonal mouse anti-human IgG (Jackson ImmunoResearch Laboratories, Inc., Avondale, PA). ELISA results are expressed as mean units anti-HGG/ml, with 1 unit equivalent to 1 μg of standard. To determine the relative contribution of IgG1 and IgG2a antibodies to the anti-HGG response, goat anti-mouse IgG1 and goat anti-mouse IgG2a (Southern Biotechnology Associates, Inc., Birmingham, AL) were used as developing antibodies with results expressed as the reciprocal of the dilution at a designated OD.

Flow Cytofluorometric Analysis. $CD4^+$-enriched lymph node cells (30×10^6/ml) were incubated with mAb to CD45RB (C363.16A, rat IgG2a, PharMingen) at a concentration of 0.34 μg/ml for 1 h at 4°C, washed, and then incubated with FL-mouse anti-rat IgG2a (Serotec Ltd., Oxford, UK) for 30 min at 4°C. An aliquot of cells was similarly stained with PE-rat anti-mouse CD4 (YTS 191.1, rat IgG2b, Caltag Laboratories, South San Francisco, CA) to determine cell purity. Cells were sorted for CD45RB using a FACStar® flow cytometer (Becton Dickinson & Co., Mountain View, CA) and then cultured in triplicate in 96-well half-area plates in a final volume of 0.1 ml, with half the number of T cells and APC as in the standard proliferation assay.

Results

Total Absence of HGG-specific Proliferation in $CD4^+$ T Cells from Tolerant Mice. The antigen-specific proliferative response of $CD4^+$ T cells from HGG-primed mice was assayed in mice injected with 200 μg HGG in CFA subcutaneously in the hind footpad (control animals) and in mice injected with 2.5 mg DHGG 15 d before injection with HGG/CFA (tolerant animals). A kinetic analysis of the HGG-specific proliferative response of lymph node $CD4^+$-enriched T cells from control and tolerant mice demonstrated that no detectable HGG-specific proliferation could be observed in HGG-tolerant $CD4^+$ T cells at any time point throughout a 5-d culture period (Fig. 1). The response of $CD4^+$ T cells from control mice has been shown in other experiments to reach a peak at either 96 or 120 h after culture initiation (data not shown).

Tolerance Induction in Both $CD45RB^{hi}$ and $CD45RB^{lo}$ Subsets of $CD4^+$ T Cells. Because the CD45 marker defines two $CD4^+$ subsets with Th1- and Th2-like properties, and HGG-specific T cells are found in each subset upon immunization (15), the effect of HGG tolerization on the proliferative response of each subset was evaluated. Lymph node $CD4^+$-enriched cells from control and tolerant mice were similarly heterogeneous (bimodal) after staining with 16A mAb (anti-CD45RB) (Fig. 2, *A* and *B*). Cells other than lymphocytes were excluded by gating based on forward and side scatter. The $CD4^+$-enriched cells (95% $CD4^+$, as indicated by staining with PE-rat anti-mouse CD4) were sorted for nonoverlapping subpopulations of $CD45RB^{hi}$ and $CD45RB^{lo}$ cells. Fig. 2 (*C* and *D*) shows a reanalysis of the sorted subpopulations in a representative experiment in which the $CD45RB^{hi}$ cells comprised 37% of the control and

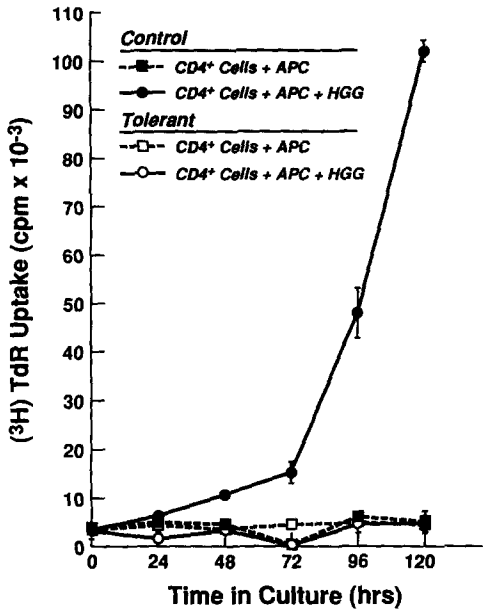

Figure 1. HGG-specific proliferative response in CD4+-enriched lymph node cells from control and tolerant mice. CD4+-enriched T cells were harvested from lymph nodes of mice injected with HGG in CFA 9 d previously (*control*) or in similarly injected mice that had received 2.5 mg DHGG i.p. 15 d before immunization (*tolerant*). Cultures were harvested at the times indicated after a 6-h pulse with 1 μCi [^{3}H]TdR.

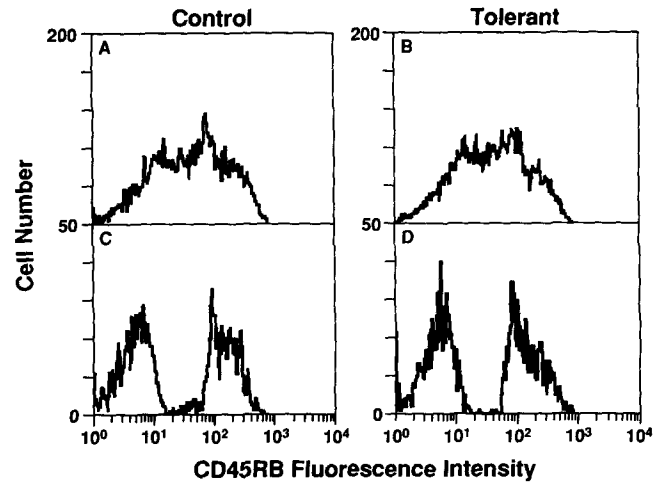

Figure 2. Expression of CD45RB on CD4+-enriched lymph node cells from control and tolerant mice. CD4+-enriched T cells were isolated from lymph nodes of mice injected with HGG in CFA 11 d previously (*control*) or in similarly injected mice that had received 2.5 mg DHGG i.p. 20 d before immunization (*tolerant*). Cells were stained with FITC-anti-CD45RB as described in Materials and Methods. Results are expressed as frequency distributions of CD45RB expression on gated cells (*A*) control cells; (*B*) tolerant cells. Cells were sorted for nonoverlapping populations of CD45RB^{hi} and CD45RB^{lo} cells. A composite of the reanalysis of sorted CD45RB^{hi} and CD45RB^{lo} cells is shown for control (*C*) and tolerant (*D*) cells.

tolerant CD4+-enriched T cells, whereas the CD45RB^{lo} cells were 16 and 14% of the sorted control and tolerant cells, respectively. The antigen-specific proliferative response of both of the subpopulations as well as the total gated population was assayed. Whereas both control CD45RB^{hi} and CD45RB^{lo} cells proliferated in response to HGG, neither CD45RB^{hi} cells, CD45RB^{lo} cells, nor the total gated CD4+-enriched cells from tolerant animals responded above background when stimulated with HGG (Fig. 3). Tolerant CD4+-enriched cells and the CD45RB^{hi} and CD45RB^{lo} subpopulations responded as well as control cells to stimulation with plate-bound anti-CD3 (data not shown).

Decreased Antigen-specific Induction of IL-2 and IL-4 Secretion in CD4+-enriched T Cells from Tolerant Mice. Because IL-2 and IL-4 secretion are functions of Th1- and Th2-like cells, respectively, the effect of tolerization on these functions was also evaluated. In preliminary experiments, IL-2 could readily be detected in supernatants of antigen-stimulated CD4+-enriched cells from lymph nodes of mice immunized with HGG in CFA, whereas IL-4 production was barely detectable or absent. Because substantial IL-4 production has been reported after antigen stimulation of KLH-primed CD4+ lymph node cells (16), we assayed IL-2 and IL-4 production in CD4+-enriched lymph node cells from HGG/CFA-immunized mice and similarly injected KLH/CFA-

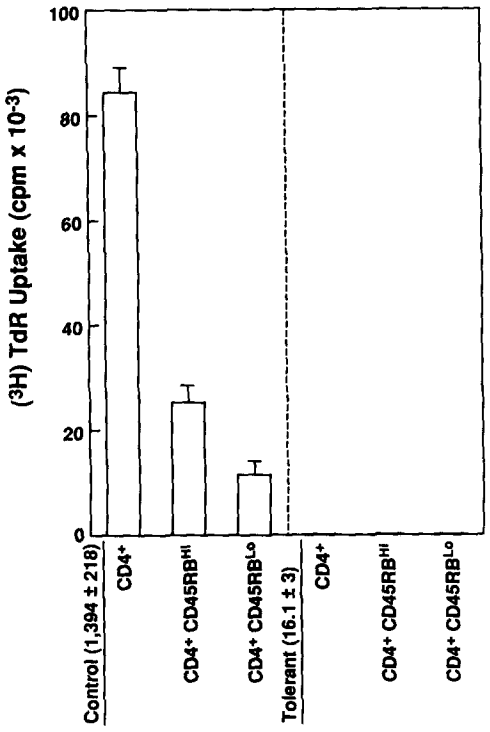

Figure 3. HGG-specific proliferation of CD45RB^{hi} and CD45RB^{lo} subsets of CD4+ lymph node cells from control and tolerant mice. Ag-specific proliferative responses (net cpm) of CD45RB^{hi} and CD45RB^{lo} subpopulations of CD4+-enriched T cells from control and tolerant mice are as described in Fig. 2. Cultures were harvested at 100 h after a 12-h pulse with [^{3}H]TdR. Numbers in parentheses indicate day 9 serum antibody levels (ELISA U/ml).

Table 1. *Comparison of the Antigen-specific Proliferative and IL-4 Responses of CD4⁺-enriched Cells from Lymph Nodes of HGG- and KLH-primed Mice*

Culture additions	Proliferation response: [³H]TdR uptake (cpm) HGG CD4⁺	Proliferation response: [³H]TdR uptake (cpm) KLH CD4⁺	IL-4 production HGG CD4⁺	IL-4 production KLH CD4⁺
			pg/ml	
CD4⁺ + APC	3,487 ± 1,133	3,987 ± 543	<20	<20
CD4⁺ + APC + Ag	32,510 ± 6,933	212,551 ± 8,674	<20	213 ± 6
CD4⁺ + APC + αCD3	103,458 ± 3,122	147,090 ± 15,331	<20	57 ± 1

CD4⁺-enriched T cells were harvested from lymph nodes of mice primed 5 d previously with either HGG/CFA or KLH/CFA. Proliferative responses were assayed at 115 h, after a 24-h pulse with [³H]TdR. IL-4 was assayed in supernatant from cells cultured for 70 h.

immunized mice. Using identical conditions for in vitro stimulation and lymphokine assay, CD4⁺-enriched cells from KLH-primed cells were shown to produce substantial amounts of IL-4 after KLH stimulation in vitro, whereas CD4⁺-enriched T cells from HGG-primed mice were markedly deficient in their ability to produce this lymphokine in response to antigen stimulation (Table 1). Fig. 4 details the response of control and tolerant cells in an experiment where low but significant levels of IL-4 were generated from control cells after in vitro stimulation with antigen. As shown previously, control CD4⁺-enriched lymph node cells proliferated to both antigen and plate-bound anti-CD3. In contrast, a total lack of antigen-specific proliferation was observed in CD4⁺-enriched cells from tolerant mice. However, these cells were capable of proliferating to plate-bound anti-CD3 (Fig. 4 *A*). Compared with control mice, serum antibody levels were reduced by 98% in tolerant mice (Fig. 4 *B*). Furthermore, a marked suppression of IL-2 levels was observed in supernatants of antigen-stimulated tolerant CD4⁺-enriched cells compared with control levels (Fig. 4 *C*). In contrast to the low but significant levels of IL-4 produced by CD4⁺-enriched cells from control mice, IL-4 levels from antigen-stimulated tolerant mice were reduced to below the limits of detection, as assessed by an assay sensitive to 5 pg/ml (Fig. 4 *D*). Plate-bound anti-CD3 induced high levels of IL-2 and IL-4 production in both control and tolerant CD4⁺-enriched cells.

Anti-HGG Antibody Production by Individual Control and Tolerant Mice and Its Relative Isotype Distribution. In occasional experiments, significant anti-HGG antibody can be detected in serum from a few of the tolerant mice after immunization with HGG in CFA. Thus, in these mice, at least some of both B and T cells must escape tolerance induction and, under stringent conditions of immunization, be capable of mounting an antibody response. Because IFN-γ has been shown to stimulate the production of antibody of the IgG2a isotype, whereas IL-4 enhances the production of antibody of the IgG1 isotype (17, 18), the relative contribution of these isotypes to the anti-HGG antibody response was analyzed by determining the reciprocal dilution of serum giving a selected OD reading at 410 nm after addition of alkaline phosphatase–conjugated IgG, IgG1, or IgG2a to HGG-coated plates incubated with dilutions of serum samples. The total anti-HGG response of control mice assayed in this manner averaged 20,625, whereas

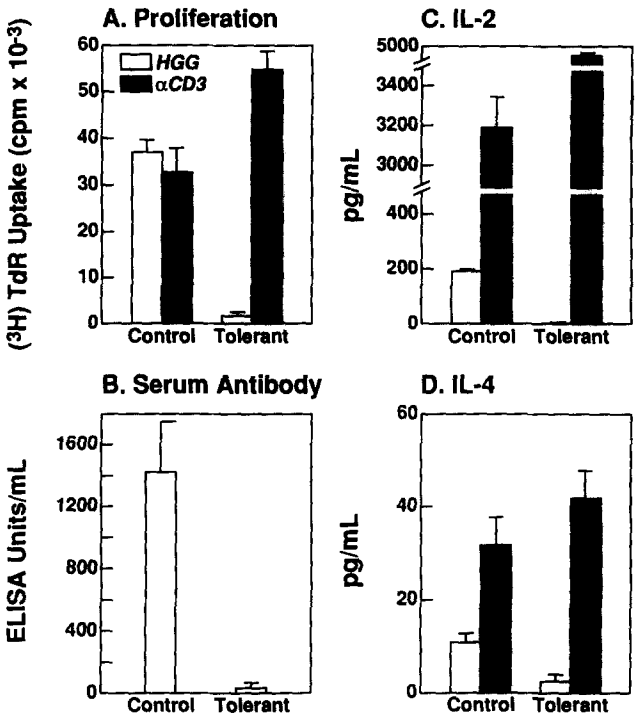

Figure 4. HGG-specific proliferative response, serum antibody, and lymphokine response from control and tolerant mice. CD4⁺-enriched T cells were harvested from lymph nodes of mice injected with HGG in CFA 11 d previously (*control*) or in similarly injected mice that had received 2.5 mg DHGG i.p. 25 d before immunization. Proliferative responses are net cpm of CD4⁺ cells incubated with APC and either HGG or anti-CD3. Cultures were harvested at 120 h after an 8-h pulse with [³H]TdR (*A*). Lymphokines were assayed in supernatants of cultures harvested at 48 h for IL-2 (*B*) or 72 h for IL-4 (*D*). Antibody was measured in serum from mice bled 9 d after immunization (*B*).

Table 2. *Anti-HGG Isotype Produced by HGG-tolerant CBA/CaJ Mice Immunized with HGG-CFA*

Tolerant mouse no.	Percent reduction from control response		
	IgG	IgG2a	IgG1
1	85	99	73
2	69	100	57
3	99	100	100
4	98	100	99
5	85	87	87
6	100	100	100
7	87	99	88
8	96	100	98
	Units anti-HGG/ml ± SD		
Control average (n = 8)	20,625 ± 4,517	6,575 ± 3,632	4,668 ± 1,606

Normal mice (control animals) or mice pretreated 10 d previously with DHGG (tolerant animals) were bled 9 d after immunization with HGG/CFA. Serial dilutions of serum were incubated on HGG-coated plates followed by alkaline-phosphatase conjugates of either anti-mouse IgG, anti-mouse IgG2a, or anti-mouse IgG1. Units of anti-HGG were determined as the reciprocal dilution of serum at a constant OD in the linear range.

the response of tolerant mice was 2,097. Although the total IgG anti-HGG response of eight tolerant mice showed a suppression from the average control response ranging from 60 to 100%, the suppression of the IgG2a anti-HGG response ranged from 87 to 100% (Table 2). On the other hand, suppression of the IgG1 anti-HGG response ranged from 57 to 100% of control responses. In several of these mice (nos. 1, 2, 5), although the IgG2a anti-HGG response was totally suppressed (99–100%), significant levels of IgG1 antibody to HGG were still produced. All three of these mice also showed a leaky tolerance at the IgG anti-HGG level. These results suggest that tolerance can be induced in both Th1- and Th2-like T cell subpopulations, although if tolerance is not complete, responsiveness is most readily observed in the Th2-like subpopulation.

Discussion

A solid tolerant state was induced in $CD4^+$ T cells after a single injection of DHGG in CBA/CaJ mice. This tolerant state was manifested by inhibition of T cell proliferation, lymphokine secretion, and antibody helper activity. In contrast to untreated immunized mice, $CD4^+$ cells from tolerized, immunized mice failed to proliferate upon in vitro stimulation with HGG, and tolerant mice showed a marked inhibition of serum anti-HGG antibody. Furthermore, antigen-specific proliferation was abrogated in both the $CD45RB^{hi}$ and $CD45RB^{lo}$ T cell subpopulations from tolerant mice. IL-2 production was dramatically decreased in antigen-stimulated $CD4^+$ cells from tolerant mice compared with control $CD4^+$ cells. Although HGG stimulation of control $CD4^+$ cells did not consistently induce significant levels of IL-4, in experiments where IL-4 levels could be quantitated, IL-4 levels in supernatants from antigen-stimulated tolerant $CD4^+$ cells were reduced.

These observations are in contrast to those of De Wit et al. (12), who reported that T cells from tolerized but not control mice immunized with HGG/CFA produce high levels of IL-4 but reduced levels of IL-2. These workers interpreted their data to mean that tolerance is induced in Th1-like cells, whereas Th2-like cells are activated and expanded. The difference in the latter observations from our data could be explained by impurities in their HGG preparation, differences in specificities of the assays used, or, most likely, by differences in the level of tolerance induced. Previous studies have shown that the presence of LPS or aggregates of HGG (as well as other inducers of IL-1 production) can interfere with the induction of tolerance to DHGG, resulting in priming to HGG (4). The significant amount of HGG-induced proliferative response in T cells from "tolerant" mice observed by De Wit et al. (12) at 72 h, and 24–48 h before the peak response (see Fig. 1), suggests that total T cell tolerance may not have been induced in their study. In our study, in all experiments to date (five), no IL-4 could be detected in antigen-stimulated $CD4^+$ cells from HGG-tolerant mice. Our failure to detect HGG-stimulated IL-4 production in $CD4^+$-enriched T cells from tolerant mice is not likely to be due to the lack of sensitivity of the IL-4 ELISA assay used (5 pg/ml) because low but significant levels of IL-4 could be detected in $CD4^+$-enriched cells from control mice stimulated with HGG in some experiments. Furthermore, this assay readily detected IL-4 secretion in $CD4^+$ cells from KLH-immunized mice stimulated in vitro with KLH.

The finding of enhanced T helper activity in tolerized, immunized mice by De Wit et al. is incompatible with established features of the HGG mouse model and with the in vitro data obtained with Th clones. Tolerance at the T cell helper compartment after injection of DHGG was previously shown by cell transfer techniques (2, 19) and by the failure to stimulate T cell helper activity in mice long after tolerance in the B cell compartment was lost (20). Using a similar model, Eynon and Parker (21) also demonstrated a solid tolerant state in T helper function. In these studies, tolerance was induced with ultracentrifuged Fab fragments of rabbit anti-mouse IgD. The tolerant state of T cell helper function in the latter study was readily demonstrated after transfer of the T cells to SCID mice. Furthermore, although HGG-specific T cell proliferation is not inhibited in Th2 clones exposed to tolerogen, T helper activity is readily tolerized (10). The ability of De Wit et al. (12) to generate anti-TNP antibody by injecting tolerant mice with hapten-conjugated HGG is not surprising because the ability of hapten-conjugated proteins to bypass tolerant T cells and activate competent B cells in vivo is well established (for reviews see references 1 and 22). The ability to inhibit the proliferation of IL-4–producing Th2-like cells in the present in vivo studies, in light of the

inability to inhibit the proliferation (but not help) in HGG-specific Th2 clones, suggests that, in the former, tolerance is induced in precursors of Th2-like cells rather than the Th2 cells themselves.

The ability of tolerance to suppress functions of subsets of $CD4^+$ cells further supports the contention that tolerance can be induced in two subsets that have, at least, some of the characteristics of Th1 and Th2 cell clones. The CD45 (leukocyte common antigen) surface marker has been shown to be differentially expressed on $CD4^+$ cells (23, 24). The extracellular domain of the CD45 molecule is encoded by three exons in mice (A, B, and C) and expression of various isoforms of this molecule is achieved by differential splicing of precursor mRNA (25). Lymphocytes that express high levels of CD45RB ($CD45RB^{hi}$) have been demonstrated to secrete IL-2 upon mitogen stimulation and were thought to be naive cells, whereas $CD45RB^{lo}$ cells secrete predominantly IL-4 (26) and have been associated with activated memory lymphocytes. However, both $CD45RB^{hi}$ and $CD45RB^{lo}$ T cells from HGG/CFA injected mice have been shown to proliferate to antigen in vitro (15), as has also recently been shown with other antigens (for a review see reference 27), suggesting that neither antigen-specific subset is naive. Furthermore, when HGG-primed cells are separated on the basis of the memory marker CD44, all the cells proliferating to antigen belong to the memory ($CD44^{hi}$) subset. Our finding that, as indicated by proliferation, a solid tolerant state is induced in both $CD45RB^{hi}$ and $CD45RB^{lo}$ subsets after tolerogen and subsequent immunization (Fig. 3) corroborates our data on the lack of both IL-2 and IL-4 lymphokine production by tolerized $CD4^+$ cells. Similar preliminary observations have been reported by us with CD45RB cell subsets from control and tolerant C57BL/6 mice (28).

Although a solid state of tolerance can be induced after the in vivo injection of DHGG, our data regarding the subclass of anti-HGG antibody produced by control and tolerant mice and data by others (12, 29) suggest that the IL-2–producing Th1-like cells are more susceptible to the induction of tolerance than are the IL-4–producing Th2-like cells. The failure to always completely inhibit antibody production in every mouse by pretreatment with DHGG suggests that tolerance is not always absolute in the HGG mouse model. The findings that when tolerance is not always complete as far as total antibody is concerned and that this leakage is reflected in the IgG1 anti-HGG response suggest that when tolerance is incomplete, the leakage is most likely at the IL-4–producing cells. IgG1 antibody has been associated with Th2-like IL-4–producing cells, whereas IgG2a has been associated with Th1-like IL-2–producing cells (17). Such incomplete tolerance may explain the recent findings of Burstein et al. (29), who reported that injection of TNP-OVA and TNP-KLH into mice induced tolerance in IL-2– but not IL-4–producing cells. It is unlikely that a solid tolerant state is induced with OVA conjugates because OVA, with a molecular weight of 39,000, is rapidly cleared from the circulation through the glomerular capillaries before it can adequately diffuse into the extravascular spaces (30–32) and is thus, at least in the rabbit, a poor tolerogen (30). Likewise, KLH, a large molecule that is rapidly removed from the circulation, does not equilibrate between the intra- and extravascular spaces and requires extremely high and repeated doses to significantly suppress antibody responses in rabbits (33). Furthermore, the response to KLH may be unique in that it appears to cross-react with mycobacterial antigens (34). Thus, the inability of TNP-OVA and TNP-KLH to induce tolerance in IL-4–producing Th2-like cells is most likely the result of the combination of a poor tolerogenic signal with the relative resistance of IL-4–producing (Th2-like) cells to be tolerized compared with IL-2–producing (Th1-like) cells. HGG, on the other hand, equilibrates between the intra- and extravascular fluid spaces at a ratio (50:50) predicted by a function of its diffusion coefficient (35) and persists in the body fluids of mice with a half-life of 7 d (36). Recently, Peterson et al. (37) have also suggested that IL-4–producing Th2-like cells are more resistant to tolerance induction than IL-2–producing Th1-like cells. They reported that injection of mice with inactivated Theiler's murine encephalomyelitis virus resulted in suppression of IL-2 secretion and IgG2a antibody production, but not of IL-4 secretion or IgG1 antibody production. Again, this particulate antigen would be rapidly cleared from circulation upon injection (for a review see reference 38); thus, it is not surprising that tolerance occurs only in the IL-2–producing cells. It is likely that the use of antigens or protocols that in the past have failed to show tolerance induction based on total T cell proliferation or total antibody production will now be shown to induce such "split" tolerance in $CD4^+$ subsets using lymphokine secretion as the assay. Such loss of self-tolerance at the IL-4–producing Th2-like cell level may be instrumental in antibody-mediated autoimmunity. The observed differences in the susceptibility of Th1- and Th2-like cells to tolerance induction is not surprising because antigen-specific Th1 and Th2 cells differ dramatically in lymphokine requirements (39), lymphokine secretion (39), early activation events (40), use of APC (39, 40), and pathway of tolerance induction (10, 41, 42).

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References

1. Weigle, W.O. 1980. Analysis of autoimmunity through experimental models of thyroiditis and allergic encephalomyelitis. *Adv. Immunol.* 30:159.
2. Chiller, J.M., G.S. Habicht, and W.O. Weigle. 1971. Kinetic differences in unresponsiveness of thymus and bone marrow cells. *Science (Wash. DC).* 171:813.
3. Gahring, L.C., and W.O. Weigle. 1989. The induction of peripheral T cell unresponsiveness in adult mice by monomeric human gamma-globulin. *J. Immunol.* 143:2094.
4. Weigle, W.O., W.V. Scheuer, M.V. Hobbs, E.L. Morgan, and D.E. Parks. 1987. Modulation of the induction and circumvention of immunological tolerance to human γ-globulin by interleukin 1. *J. Immunol.* 138:2069.
5. Gahring, L.C., and W.O. Weigle. 1990. The regulatory effects of cytokines on the induction of a peripheral immunologic tolerance in mice. *J. Immunol.* 145:1318.
6. Levich, J.D., D.E. Parks, and W.O. Weigle. 1987. Tolerance induction in antigen-specific helper T cell clones and lines in vitro. *J. Immunol.* 135:873.
7. Levich, J.D., A.P. Signorella, G. Wittenberg, and W.O. Weigle. 1987. Macrophage handling of a tolerogen and the role of IL 1 in tolerance induction in a helper T cell clone in vitro. *J. Immunol.* 138:3675.
8. Jenkins, M.K., D.M. Pardoll, J. Mizuguchi, T.M. Chused, and R.H. Schwartz. 1987. Molecular events in the induction of a nonresponsive state in interleukin 2–producing helper T-lymphocyte clones. *Proc. Natl. Acad. Sci. USA.* 84:5409.
9. Jenkins, M.K., and R.H. Schwartz. 1987. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. *J. Exp. Med.* 165:302.
10. Gilbert, K.M., K.D. Hoang, and W.O. Weigle. 1990. Th1 and Th2 clones differ in their response to a tolerogenic signal. *J. Immunol.* 144:2063.
11. Gilbert, K.M., A.L. Rothermel, D.N. Ernst, M.V. Hobbs, and W.O. Weigle. 1992. Ability of tolerized Th1 and Th2 clones to stimulate B cell activation and cell cycle progression. *Cell. Immunol.* 142:1.
12. De Wit, D., M. Van Mechelen, M. Ryelandt, A.C. Figueiredo, D. Abramowicz, M. Goldman, H. Bazin, J. Urbain, and O. Leo. 1992. The injection of deaggregated gamma globulins in adult mice induces antigen-specific unresponsiveness of T helper type 1 but not type 2 lymphocytes. *J. Exp. Med.* 175:9.
13. Doyle, M.V., D.E. Parks, and W.O. Weigle. 1976. Specific suppression of the immune response by HGG tolerant spleen cells. I. Parameters affecting the level of suppression. *J. Immunol.* 116:1640.
14. Ernst, D.N., M.V. Hobbs, B.E. Torbett, A.L. Glasebrook, M.A. Rehse, K. Bottomly, K. Hayakawa, R.R. Hardy, and W.O. Weigle. 1990. Differences in the expression profiles of CD45RB, Pgp-1, and 3G11 membrane antigens and in the patterns of lymphokine secretion by splenic $CD4^+$ T cells from young and aged mice. *J. Immunol.* 145:1295.
15. Gahring, L.C., D.N. Ernst, C.G. Romball, M.L. Thoman, B.E. Torbett, M.V. Hobbs, and W.O. Weigle. 1993. The expression of CD45RB on antigen-responsive $CD4^+$ lymphocytes: mouse strain polymorphism and different responses to distinct antigens. *Cell. Immunol.* 148:269.
16. Bradley, L.M., D.D. Duncan, S. Tonkonogy, and S.L. Swain. 1991. Characterization of antigen-specific $CD4^+$ effector T cells in vivo: immunization results in a transient population of $MEL\text{-}14^-$, $CD45RB^-$ helper cells that secrete interleukin 2 (IL-2), IL-3, IL-4, and interferon γ. *J. Exp. Med.* 174:547.
17. Snapper, C.M., and W.E. Paul. 1987. B cell stimulatory factor-1 (interleukin 4) prepares resting murine B cells to secrete IgG1 upon subsequent stimulation with bacterial lipopolysaccharide. *J. Immunol.* 139:10.
18. Snapper, C.M., and W.E. Paul. 1987. Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. *Science (Wash. DC).* 236:944.
19. Chiller, J.M., G.S. Habicht, and W.O. Weigle. 1970. Cellular sites of immunologic unresponsiveness. *Proc. Natl. Acad. Sci. USA.* 65:551.
20. Parks, D.E., S.M. Walker, and W.O. Weigle. 1981. Bacterial lipopolysaccharide (endotoxin) interferes with the induction of tolerance and primes thymus-derived lymphocytes. *J. Immunol.* 126:938.
21. Eynon, E.E., and D.C. Parker. 1992. Small B cells as antigen-presenting cells in the induction of tolerance to soluble protein antigens. *J. Exp. Med.* 175:131.
22. Paul, W.E., G.J. Thorbecke, G.W. Siskind, and B. Benacerraf. 1969. The effect of dose in tolerance induction on the subsequent response to a cross-reactive antigen. *Immunology.* 17:85.
23. Thomas, M.L. 1989. The leukocyte common antigen family. *Annu. Rev. Immunol.* 7:339.
24. Bottomly, K. 1989. Subsets of CD4 T cells and B cell activation. *Semin. Immunol.* 1:21.
25. Luqman, M., L. Greenbaum, D. Lu, and K. Bottomly. 1992. Differential effect of interleukin 1 on naive and memory $CD4^+$ T cells. *Eur. J. Immunol.* 22:95.
26. Bottomly, K., M. Luqman, L. Greenbaum, S. Carding, J. West, T. Pasqualini, and D.B. Murphy. 1989. A monoclonal antibody to murine CD45R distinguishes CD4 T cell populations that produce different cytokines. *Eur. J. Immunol.* 19:617.
27. Swain, S.L., L.M. Bradley, M. Croft, S. Tonkonogy, G.G. Atkins, A.D. Weinberg, D.D. Duncan, S.M. Hedrick, R.W. Dutton, and G. Huston. 1991. Helper T-cell subsets: phenotype, function and the role of lymphokines in regulating their development. *Immunol. Rev.* 123:115.
28. Gahring, L.C., D.N. Ernst, and W.O. Weigle. 1991. Activation and tolerization of $CD4^+$, $CD45RB^{hi}$ and $CD45RB^{lo}$ cells from human gamma globulin (HGG) primed mice. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 5:A1688. (Abstr.)
29. Burstein, H.J., C.M. Shea, and A.K. Abbas. 1992. Aqueous antigens induce in vivo tolerance selectively in IL-2- and IFNγ-producing (Th1) cells. *J. Immunol.* 148:3687.
30. Smith, R.T. 1961. Immunological tolerance of nonliving antigens. *Adv. Immunol.* 1:67.
31. Lippman, R.W. 1949. Mechanism of proteinuria. II. Identity of urinary proteins in the rat following parenteral protein injection. *Proc. Soc. Exp. Biol. Med.* 71:546.
32. Bott, P.A., and A.N. Richards. 1941. The passage of protein molecules through the glomerular membranes. *J. Biol. Chem.* 141:291.
33. Weigle, W.O. 1966. The induction of a hyporesponsive state to hemocyanin. *J. Immunol.* 96:319.
34. Bradley, L.M., G.G. Atkins, and S.L. Swain. 1992. Long-term $CD4^+$ memory T cells from the spleen lack Mel-14, the lymph node homing receptor. *J. Immunol.* 148:324.
35. Nakamura, R.M., H.L. Spiegelberg, S. Lee, and W.O. Weigle. 1968. Relationship between molecular size and intra- and extravascular distribution of protein antigens. *J. Immunol.* 100:376.

36. Weigle, W.O., L.C. Gahring, and K.M. Gilbert. 1990. Induction of peripheral tolerance to a serum protein antigen. *In* Tolerance: Du Soi Au Xenogenique. J.M. Dubenard, editor. Laboratories CILAG, Paris, France. 39–52.
37. Peterson, J.D., W.J. Karpus, R.J. Clatch, and S.D. Miller. 1993. Split tolerance of Th1 and Th2 cells in tolerance to Theiler's murine encephalomyelitis virus. *Eur. J. Immunol.* 23:46.
38. Weigle, W.O. 1973. Immunological unresponsiveness. *Adv. Immunol.* 16:61.
39. Mossman, T.R., and R.L. Coffman. 1989. Heterogeneity of cytokine secretion patterns and functions of helper T cells. *Adv. Immunol.* 46:111.
40. Gajewski, T.F., S.R. Schell, and F.W. Fitch. 1990. Evidence implicating utilization of different T cell receptor–associated signaling pathways by T_H1 and T_H2 clones. *J. Immunol.* 144:4110.
41. Gajewski, T.F., M. Pinnas, T. Wong, and F.W. Fitch. 1991. Murine Th1 and Th2 clones proliferate optimally in response to distinct antigen-presenting cell populations. *J. Immunol.* 146:1750.
42. Gilbert, K.M., and W.O. Weigle. 1992. B cell presentation of a tolerogenic signal to Th1 clones. *Cell. Immunol.* 139:58.