"The Injection of Deaggregated Gamma-globulins in Adult Mice Induces Antigen-specific Unresponsiveness of T-helper Type-1 But Not Type-2 Lymphocytes"

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Abstract
Injection of adult mice with high doses of monomeric human gamma globulins (dHGG) has been previously shown to produce a state of peripheral tolerance in both B and T cells. To gain insight into the mechanism of induction and maintenance of adult tolerance in this model, we have analyzed the pattern of lymphokines produced by control and tolerant animals in response to the tolerogen. The data presented indicate that HGG-specific, interleukin 2 (IL-2)- and interferon-gamma (IFN-gamma)-producing T cells (thus referred to as T helper type 1 [Th1] cells) are rendered unresponsive after in vivo administration of soluble HGG. In contrast, antigenic stimulation of T cells isolated from tolerant adult mice leads to increased production of IL-4 in vitro. In vivo challenge of dHGG-treated adult animals with hapten-coupled HGG (p-azophenylarsonate [ARS]-HGG) induced a significant ARS-specific antibody response, suggesting that tolerance induction in this model does not completely abrogate toler...

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The Injection of Deaggregated Gamma Globulins in Adult Mice Induces Antigen-specific Unresponsiveness of T Helper Type 1 but not Type 2 Lymphocytes

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Summary

Injection of adult mice with high doses of monomeric human gamma globulins (dHGG) has been previously shown to produce a state of peripheral tolerance in both B and T cells. To gain insight into the mechanism of induction and maintenance of adult tolerance in this model, we have analyzed the pattern of lymphokines produced by control and tolerant animals in response to the tolerogen. The data presented indicate that HGG-specific, interleukin 2 (IL-2)- and interferon γ (IFN-γ)-producing T cells (thus referred to as T helper type 1 [Th1] cells) are rendered unresponsive after in vivo administration of soluble HGG. In contrast, antigenic stimulation of T cells isolated from tolerant adult mice leads to increased production of IL-4 in vitro. In vivo challenge of dHGG-treated adult animals with hapten-coupled HGG (p-azophenylarsonate [ARS]-HGG) induced a significant ARS-specific antibody response, suggesting that tolerance induction in this model does not completely abrogate tolerogen-specific Th activity in vivo. In agreement with the in vitro data, hapten-specific antibody response of tolerant animals is characterized by a selective deficiency in the IFN-γ-dependent IgG2a subclass. Injection of immunogenic forms of HGG into tolerant animals also produced an IL-4-dependent increase in total serum IgE levels, indicative of an increased activity of HGG-specific Th2 cells in these animals. The finding that tolerance induction differentially affects Th subpopulations suggests that crossregulation among lymphocyte subsets may play a role in the induction and/or maintenance of acquired tolerance in adults.

A prime aim in the treatment of allograft rejection and autoimmune disorders is to convert antigen recognition by a mature immune system into a state of specific immune unresponsiveness. Although induction of immune tolerance in adults meets difficulties, antigen-specific unresponsiveness in mature lymphocytes has been induced in vivo by intravenous administration of allogeneic (1) or Mls-disparate cells (2), hapten-coupled syngeneic cells (3, 4), soluble antigen (5), and antigen-pulsed, chemically modified, syngeneic cells (6). In most cases described, induction of unresponsiveness does not require a thymic environment (7, 8), and appears to be mediated by clonal anergy rather than clonal deletion (2, 9).

Th unresponsiveness can be induced in vitro by confronting mature lymphocytes with antigen-pulsed, purified MHC molecules (10), chemically (6) or UV-treated APC (11), high doses of antigen (12), or IL-2 (13).

It is noteworthy that, in most systems described to date, different sensitivities to tolerance induction in vitro have been noticed when murine helper clones producing distinct patterns of lymphokines were studied. In particular, only IL-2-producing CD4⁺ clones (14, 15), and more recently CD8⁺, IL-2-producing clones (16), have been shown to be susceptible to anergy induction by chemically damaged APC. IL-4-producing clones (Th2 cells) appear to be resistant to in vitro tolerance induction under the same experimental conditions.

These findings, together with the recent evidence showing that selective activation of Th subsets can lead to strikingly different immune effector functions (17), prompted us to study whether CD4⁺ Th subsets from adult animals were equally sensitive to tolerogenic signals delivered by in vivo administration of soluble human gamma globulins (HGG).1

Data are presented showing that under identical experimental conditions, soluble antigen-induced Th1 clonal anergy/deletion, and Th2 clonal activation/expansion. These

1 Abbreviations used in this paper: ARS, p-azophenylarsonate; HGG, human gamma globulin.
findings suggest that crossregulation among Th subsets may play a role in the maintenance of acquired tolerance in adults.

Materials and Methods

Animals, Reagents, and Immunizations. A/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were used between 8 and 12 wk of age. To induce tolerance to HGG (CH-9470; Fluka Chemie AG, Buchs, Switzerland), 1 mg of monomeric, deaggregated HGG (dHGG; obtained by centrifugation at 150,000 g for 3 h) was injected into the peritoneal cavity (intraperitoneally) of adult mice on day 0. Animals were challenged intraperitoneally on day 15 with 100 µg of A/RS-HGG (18) or 300 µg of HGG emulsified in CFA (Difco Laboratories, Detroit, MI) subcutaneously at the tail base and in the footpads. Secondary responses to HGG: p-azophenylarsonate (ARS) were elicited by injecting intraperitoneally 100 µg of antigen in saline. In vivo treatment with anti-IL-4 antibodies was performed as follows (see reference 19). Mice received three intraperitoneal injections of 1 ml of ascitic fluid containing 5 mg of rat IgG1 mAbs specific for murine IL-4 (clone 11B11) or the hapten DNP (control group, clone LO-DNP-P1). Ascites were injected 1 d before and on the day of antigenic challenge, as well as 4 d later.

Assay for Serum Antibodies. Serum levels of antigen-specific antibodies were determined by RIA and ELISA according to standard procedure using anti-mouse Ig antibodies known to react with all murine isotypes. When indicated, enzyme-labeled rat mAbs specific for murine IgG1 (clone LO-MG1-D) and IgG2a (LO-MG2a-7) (20) were used as secondary reagents.

Total serum IgE levels were determined by ELISA using two rat mAbs specific for nonoverlapping epitopes on mouse IgE (20). Briefly, affinity-purified mAb LO-ME-13 was used as capture antibody and absorbed to microtiter plates. After addition of serial dilutions of serum sample, plates were revealed with peroxidase-coupled LO-ME-2 mAb. Standard curves were generated using a calibrated source of monoclonal murine IgE (clone LB4; 20).

T Cell Proliferation. All in vitro assays were performed in complete media as described (19). Responder cells were isolated from the draining lymph nodes of control and experimental animals and depleted of most B and accessory cells by negative selection on RA11-coated plates. 104 responder cells and 105 APC (Thy-1-depleted naive spleen cells) were incubated in 96-well culture plates (Falcon 3072; Becton Dickinson & Co., Oxnard, CA) in the presence or absence of serial dilution of antigen (HGG). Proliferation was assessed in these cultures by measuring [3H]thymidine incorporation during the last 18 h of a 72-h incubation period.

Lymphokine Assays. Antigen-driven IL-2 production was measured by a standard bioassay using a subclone of the CTLL cell line insensitive to murine IL-2 (21). Proliferation of this cell line induced by supernatants from antigen- or anti-CD3-stimulated T cell cultures is completely inhibitable by the anti-murine IL-2 mAb S4B6. Standard curves were generated using human IL-2 and supernatants from antigen-stimulated cultures were assayed after 40–48 h of incubation. IL-4 production was monitored by a bioassay based on the ability of IL-4 to increase la expression on B cells, as previously described (19). Briefly, responder T cells were incubated with antigen and B cells (Thy-1-depleted naive spleen cells). After 36–40 h of culture, cells were harvested and stained with fluorescein-labeled 14.4.4 mAb (anti-I-F4). The mean channel fluorescence of class II-positive cells was determined by FACScan® (Becton Dickinson & Co.). A linear standard curve was generated by incubating Thy-1+ spleen cells with serial dilutions of murine rIL-4 (range, 0.02–4 U/ml; mean channel FL1 range, 177–1752). Increased fluorescence in antigen-stimulated culture was shown to be mediated by IL-4 since inhibition by anti-IL-4 mAbs always exceeded 90%. The results are expressed as the mean of three independent determinations. IFN-γ production by antigen-stimulated lymph node cells was evaluated by a commercially available ELISA test kit (Holland Biotechnology, Leiden, Holland) using two mAbs specific for nonoverlapping epitopes on mouse IFN-γ. Briefly, lymph node cells isolated from control and tolerant immune animals were cultured at 5 × 106/ml in 1-ml cultures (Falcon 3047; Becton Dickinson & Co.) with or without antigen (HGG; 2 mg/ml). Supernatants were collected after 40 h of culture and assayed for IFN-γ content according to the manufacturer’s recommendations.

Results and Discussion

Experimental Design. Injection of dHGG has been widely used to induce unresponsiveness in adult animals. In this model, it has been shown that mice treated with dHGG failed to produce HGG-specific antibodies after challenge with immunogenic forms of HGG emulsified in CFA (5). More relevant to this study, peripheral T cells isolated from tolerant animals showed a diminished proliferative response to HGG in vitro (8). This state of peripheral tolerance could be induced in the absence of thymic environment and was independent of CD8+ suppressor activity (8, 22).

In this study, HGG tolerance was induced in adult A/J mice by injecting 1 mg of dHGG as described in Materials and Methods. Fig. 1 indicates that immune responses elicited by immunogenic doses of HGG were impaired in tolerant animals. In particular, no significant antibody production was detected in the serum of tolerant mice after injection of HGG in CFA as compared with control animals (Fig. 1 a). Antigen-specific T cell proliferation by tolerant (dHGG-treated) or control (untreated) mice was evaluated after in vivo challenge with immunogenic doses of HGG (in CFA) and in vivo restimulation with soluble HGG and APC. As shown in Fig. 1 b, HGG-specific T cell proliferation was reduced in tolerant animals, although a weak, but significant response was clearly demonstrable. No proliferation was observed when animals were treated with CFA alone (Fig. 1 b, open symbols). In all cases examined, reduction of proliferative capacities was only observed in response to HGG, since anti-CD3-induced proliferation was not affected by tolerance induction (data not shown).

In Vitro Lymphokine Production. To assess the immunocompetence of tolerant animals, the following experiments were performed. As for proliferation, control and tolerant animals were challenged in vivo with immunogenic doses of HGG in CFA. Responder cells isolated from draining lymph nodes 10–15 d later were stimulated in vitro with antigen and naive APCs and assayed for lymphokine production. Data obtained from two independent experiments are presented in Fig. 2. The top two panels represent the pattern of IL-2 and IL-4 production after in vitro restimulation of control and tolerant animals. Under identical experimental conditions, tolerant animals failed to produce significant levels of IL-2 in the presence of the tolerogen, while secreting high levels...
of IL-4 in response to the same stimulus (Fig. 2, top and middle). In contrast, control animals produce high levels of IL-2 and low amounts of IL-4 in response to antigen. No lymphokines were secreted by animals treated in vivo with CFA only (data not shown). All animals produced identical levels of lymphokines (IL-2 and IL-4) upon stimulation by anti-CD3 antibodies in vitro (data not shown).

![Figure 2](image_url)

**Figure 2.** Split tolerance in dHGG-treated mice. Lymph node cells from control (n = 3, hatched bars) and tolerant (n = 3, crosshatched bars) animals were cultured in the presence (+) or absence (−) of HGG (2 mg/ml) and assayed for lymphokine production as indicated. (Top and middle) Data from a single experiment in which each individual mouse was tested for both IL-2 and IL-4 production. (Bottom) A separate experiment performed according to the same general protocol except that IFN-γ production was assayed by ELISA.
In a separate experiment, cells isolated from antigen-boosted, control and tolerant animals were assayed for their production of IFN-γ in response to antigen restimulation in vitro. The results obtained (Fig. 2, bottom) are indicative of a profound suppression of tolerogen-specific, IFN-γ production in tolerant animals. As in the previous group, these animals displayed an increased production of IL-4 in response to HGG (data not shown).

Thus, in vivo exposure to tolerogenic forms of HGG results in priming of antigen-specific, IL-4-producing T cells, and functional inactivation of antigen-specific, IL-2- and IFN-γ secreting cells.

**In Vivo Isotype Expression.** We next wished to investigate whether the distinct in vitro response of Th1 and Th2 cells after tolerance induction was also observed in vivo. For this purpose, we designed an experiment that relies on the distinct function of Th subsets in the regulation of Ig isotype production. In particular, it has been demonstrated that Th1 clones induced antigen-specific B cells to secrete IgG2a Isgs, while Th2 clones favor the production of IgG1 and IgE (23). Since dHGG treatment induces B cell tolerance (5), the helper activity of HGG-specific T cells was studied in vivo after challenge with hapten-coupled HGG (ARS-HGG). In this situation, most of the hapten-specific antibody response is dependent upon help provided by carrier-reactive T cells.

The humoral secondary response to both HGG and ARS is shown in Fig. 3. As previously shown by others, tolerant animals displayed a profound impairment of the HGG-specific antibody response (Fig. 3, top left). Inhibition of the tolerogen-specific antibody production exceeded 99% in this experiment. In contrast, tolerant animals retained a significant antibody production to the ARS hapten (Fig. 3, top right). Although the presence of ARS-specific, Th cells cannot be formally excluded, this finding is consistent with the in vitro data and suggests that HGG-specific helper functions are not totally abrogated in dHGG-treated animals. Analysis of the subclass profile revealed a selective deficiency of the ARS-specific, IgG2a subclass antibody production in tolerant animals (>100-fold reduction), compatible with the selective deficiency in IFN-γ production observed in vitro. As shown in Fig. 3, production of ARS-specific, IgG1 was not affected to the same extent in tolerant animals (approximately fourfold reduction).

The possible effects of in vivo IL-4 production in dHGG-treated animals was then evaluated. As shown in Fig. 4, injection of ARS-HGG (or HGG alone; data not shown) caused a marked increase in total serum IgE levels in tolerant but not control animals. ELISAs failed to show any IgE reactivity to either ARS or HGG, suggesting that most IgE secreted were from polyclonal origin. Hyper-IgE production was completely abrogated by in vivo treatment with anti-IL-4 mAbs but not with DNP-specific, control antibodies, indicating that increased production of IL-4 in response to ARS-HGG was responsible for the elevated serum levels of IgE.

Of note, injection of ARS-KLH in dHGG-treated animals induced normal levels of ARS-specific antibodies of the IgG2a subclass and only minimal increase in total serum IgE level, indicating that tolerance induction in this system only affects HGG-specific T and B lymphocytes (data not shown). Thus, the antibody profile observed after immunization of tolerant animals with ARS-HGG is compatible with a selective deficiency in Th1 cells required for IgG2a production and increased activity of Th2 cells leading to IgE production.

**Conclusions.** The major observation from this study is that Th subsets are not equally sensitive to tolerance induction in vivo. Injection of adult animals with dHGG induced a state of immune tolerance characterized by a selective deficiency in IL-2 and IFN-γ antigen-specific production.

The finding that Th1 and Th2 clones differ in their requirement for costimulatory signals delivered by APC offers an explanation to the results reported in this study. The current hypothesis is that stimulation of Th1 clones, but not Th2 clones, requires both receptor triggering and a costimulatory signal delivered by APCs. Inappropriate APCs (lacking the costimulatory factor) not only fail to induce proliferation of Th1 clones, but also induce a state of unresponsiveness (14). Of interest, it has been demonstrated that antigen presented by B cells stimulates optimal proliferation of Th2 but not Th1 clones (24). It is therefore tempting to postulate that deggregated, monomeric HGG is preferentially presented in vivo by resting B cells, perhaps through Fc receptor-facilitated endocytosis. Antigen presented by B cells (lacking the putative costimulatory factor) may cause Th1 clonal anergy but Th2 clonal expansion/activation. As a consequence, immunogenic challenge with HGG will selectively amplify the Th2 subset and the production of lymphokines required for IgG1 and IgE synthesis.

Of interest, we and others (25-28) have recently reported that neonatally induced tolerance to alloantigens is similarly restricted to the Th1 subset. Donor-specific Th2 cells not

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**Figure 4.** Induction of in vivo IgE production in tolerant mice and role of IL-4. Control (open bars) and tolerant (hashed bars) mice (n = 3) were either left untreated (mean serum IgE, respectively, 170 and 370 ng/ml) or injected with ARS-HGG (mean serum IgE 7 d after in vivo challenge, 610 and 4,100 ng/ml, respectively). The mAb treatment consisted of a total dose of 15 mg of the 11B11 anti-IL-4 mAb or the LO-DNP-1 control mAb administered as indicated in Materials and Methods. Data are presented as the mean (± SD) of three individual determinations in each experimental group.
only escape tolerance in this system but are responsible for the development of a systemic autoimmune disorder associated with increased production of IgG1 and IgE isotypes (HVGD) (see reference 28). Thus, in both experimental systems (tolerance to alloantigens and to HGG), IL-4-producing Th2 cells appear to be resistant to peripheral tolerance as if immune stimuli that induce Th1 tolerance activate Th2 cells or induce multipotent precursors to differentiate in a Th2-type cell.

Crossregulation between Th subsets has been clearly documented although its physiological significance remains to be established. In particular, activated Th2 cells could produce IL-10, a cytokine that suppress the production of cytokines by Th1 cells responding to antigen and APC (29). Preferential activation of the Th2 subset could result in the production of soluble factors inhibiting Th1 functions and/or differentiation of thymic precursors into Th1 cells. The present study may therefore provide a useful model to explore the in vivo requirements of Th subset activation in response to a foreign antigen and the potential role of Th2 cells or Th2-derived factors in the maintenance of the tolerant state in adult animals.

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