TOLERANCE INDUCTION DURING ONTOGENY

I. Presence of Active Suppression in Mice Rendered Tolerant to Human \( \gamma \)-Globulin In Utero Correlates with the Breakdown of the Tolerant State*

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If, as first postulated by Burnet, tolerance to self-antigens is an acquired characteristic of the lymphoid system, then clones with anti-self recognition capability should arise during lymphopoiesis (1). In the absence of an efficient mechanism for inactivating such clones, autoimmunity would regularly be induced whenever any of these clonotypes achieved effector capacity. Three basic types of model for inactivating anti-self-reactive clones have been proposed to account for the relative infrequency of autoimmune events: clonal abortion, clonal deletion (2-12), and active suppression (13-22). All three models have found experimental support in the literature although the details of the intercellular and intracellular mechanisms by which they are presumed to operate still remain largely undefined.

The theory of clonal abortion proposes that a phase in lymphocyte maturation exists when contact with antigen can only result in immunological paralysis (23). The early and continuous presence of self-antigens in appropriate concentrations would therefore ensure that clones having anti-self receptors would be permanently inactivated. Recent studies on the sequential appearance of surface Ig isotypes during B-cell ontogeny have been interpreted in favor of this viewpoint (24, 25). Thus the heightened susceptibility of neonates to tolerance induction has been associated with the presence of \( \mu \)-chain-bearing antigen receptors on some B-cell precursor populations before their maturation into \( \mu \)- and \( \delta \)-chain-bearing immunocompetent cells. Another interpretation of self-tolerance, viz. clonal deletion, predicts that the outcome of an encounter between an immunocompetent cell and antigen depends on the capacity of the cell to qualitatively and quantitatively discriminate between tolerogenic and immunogenic signals (26). The observation that neonatal B cells are resistant to tolerance induction in the presence of specific T-cell help would favor this type of interpretation (12).

Active suppression, which appears to be particularly relevant in the general regulation of immune responsiveness, has also been proposed to account at least in part for regulation of responsiveness to self (27). Although it is possible to operationally reconcile suppressor cells with a clonal deletion mechanism, some proponents of this theory favor a type of homeostatic equilibrium between suppressors and a functionally

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inactivated though undeleted pool of target T and/or B lymphocytes (19).

Most in vivo self-tolerance models are based on extrapolations of data from autoimmune phenomena (27) or from experiments with adult immunocompetent animals in which the time of antigen exposure is not considered a critical developmental feature of the system (18). In view of these considerations, we felt it appropriate in the present study to utilize a model system for tolerance induction which permits access of the extrinsic test antigen to the fetus well before immunocompetent cells arise. The tolerogenic potency of deaggregated human \( \gamma \)-globulin (dHGG)\(^1\) administered in utero via the maternal circulation was assessed in BALB/cCr mice. When tested at various times during adult life, the offspring of these treated mothers were found to be completely and specifically unresponsive to an HGG challenge at the level of both T and B cells. Foster nursing experiments indicated that sufficient transplacental transfer of HGG occurred over a 4-d period to render the offspring unresponsive. Waning of unresponsiveness began to occur past the 12th wk of age unless additional dHGG was administered. It was during, but not before, the period when the unresponsive state was breaking that HGG-specific suppressor cells were found. In contrast, suppressor cells found in adult HGG tolerized animals appear to be associated with either the establishment and/or maintenance of the unresponsive state (14, 15, 28). The evidence presented in this paper is thereby consistent with the clonal deletion-abortion view of self-tolerance.

Materials and Methods

**Animals.** Inbred BALB/cCr mice of both sexes were obtained from pedigree lines maintained at the University of Alberta Animal Breeding Facility, Alberta, Canada. The age of animals is specified in the text.

**Antigens.** Human \( \gamma \)-globulin (HGG) and chicken \( \gamma \)-globulin (CGG) were obtained from Sigma Chemical Co., St. Louis, Mo., and Miles Laboratories, Inc., Kankakee, Ill., respectively, and used without further purification as Cohn fraction II. Mouse \( \gamma \)-globulin (MGG) was prepared from pooled normal BALB/cCr serum by ammonium sulfate precipitation and dialysis against phosphate-buffered saline (PBS) (29). Bovine serum albumin (BSA) was obtained from Sigma Chemical Co. as the crystalline fraction V powder. The antigen was trinitrophenylated (TNP) where indicated according to the procedure of Okuyama and Satake by using 2,4,6-trinitrobenzene sulfonic acid purchased from Sigma Chemical Co. (30). The molar conjugation ratio of TNP to protein was determined on the basis of dry weight and the extinction coefficient of TNP-lysine (\( \epsilon = 15,400 \) at 348 nm, pH 7.4). These ratios were 10–15 mol TNP per mole of serum protein for all haptenated preparations used in these experiments. Sheep erythrocytes (SRBC) and burro erythrocytes (BRBC) were obtained in Alsever's solution from animals maintained by the Provincial Laboratory of Public Health, Alberta, Canada, and from Colorado Serum Company Laboratories, Denver, Colo., respectively. Trinitrophenylated sheep erythrocytes (TNP-SRBC) were prepared according to the method of Rittenberg and Pratt (31).

**Induction of Unresponsiveness.** Unresponsiveness to HGG was induced by a single intravenous injection of HGG deaggregated by ultracentrifugation at 100,000 \( g \) for 180 min at 4°C in an SW 50.1 rotor on a Beckman model L3-50 Ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.). The upper one-third of the supernate was carefully removed for immediate use as tolerogen. BALB/cCr females gestating syngeneic fetuses were injected with 5 mg of dHGG or 0.05 mg dHGG as indicated in the text.

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\(^1\) Abbreviations used in this paper: BRBC, burro erythrocytes; BSA, bovine serum albumin; CGG, chicken \( \gamma \)-globulin; dHGG, deaggregated human \( \gamma \)-globulin; DNP, dinitrophenyl; GVH, graft-versus-host; HGG, human \( \gamma \)-globulin; MGG, mouse \( \gamma \)-globulin; MS, mannitol-saline solution; PBS, phosphate-buffered saline; PFC, plaque-forming cells; SRBC, sheep erythrocytes; TNP, trinitrophenyl.
Treated mothers were found to be unresponsive to HGG based on absence of passive hemagglutination titers and splenic plaque-forming cells (PFC) during the prenatal and postnatal periods, respectively, up to 6 wk (C. Waters, unpublished data). Experiments involving induction of unresponsiveness in adults utilized 8-wk-old mice of both sexes that had received a single intravenous injection of 5 mg dHGG 17 d previously.

**Immunizations.** To prepare a strongly immunogenic form of protein antigen, the protein was alum-precipitated according to the technique of Proom (32) and washed three times with saline. For assay of T-cell responsiveness, 0.5 mg of alum-aggregated protein and $1.5 \times 10^9$ killed *Bordetella pertussis* organisms (Connaught Laboratories, Willowdale, Ontario, Canada) were given intraperitoneally, followed 4 d later by 0.5 mg of alumn-aggregated haptenated protein and $1.5 \times 10^9$ *B. pertussis*. In a procedure found to give optimal PFC responses, an additional i.p. injection of 0.5 mg aggregated haptenated protein was given 2 d later. Spleens were assayed individually for anti-TNP PFC 10 d after the initial administration of alum-aggregated protein. Numbers of anti-TNP PFC per spleen were taken as a measure of carrier function in these animals. To determine B-cell responsiveness, animals were challenged intravenously with 0.2 ml of a 10% suspension (approximately $10^9$ cells) of HGG-conjugated BRBC. HGG used for erythrocyte conjugation was obtained from Miles Laboratories. The conjugation procedure used to prepare the antigen was the CrCl3 technique of Gold and Fudenberg (33); the extent of conjugation was determined by passive hemagglutination of coated cells with rabbit anti-human IgG antiserum obtained from Miles Laboratories, Inc. Little variation (~1 serial twofold dilution) was observed in the degree of HGG substitution among different HGG-BRBC preparations. Spleens of challenged mice were individually assayed for anti-HGG and anti-BRBC PFC 6 d later.

**Cell Suspensions.** Spleens were removed aseptically, minced, and gently pressed through a fine mesh stainless steel sieve into either cold Mishell-Dutton balanced salt solution or Leibowitz medium containing 5% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.). Cell aggregates were disrupted further by aspiration with a Pasteur pipette. After removing large cell clumps and debris by settling at 1 g for 8 min, the cells were washed three times with medium at 4°C and viability estimated by eosin dye exclusion.

**Hemolytic Plaque Assay.** The technique of Cunningham and Szenberg (34) was used to determine numbers of splenic PFC by using trinitrophenylated sheep erythrocytes as targets in the T-cell responsiveness assay and HGG-conjugated sheep erythrocytes as targets to assess B-cell responsiveness. The procedure used for conjugation of HGG (Miles Laboratories, Inc.) to erythrocytes was a modification by Dr. T. Mosmann of this department of the carbodiimide method of Golub et al. (35). Briefly, SRBC were washed twice with PBS at 4°C then once with mannitol-saline solution (0.35 M d-mannitol [Sigma Chemical Co.], 0.01 M NaCl). SRBC (0.4 ml packed cells) were resuspended at 10% in mannitol-saline (MS) for coupling. In a separate vessel, 200 #1 HGG (Miles Laboratories, Inc.) were dissolved in MS at 0.25 mg/ml and reacted for 2 min at 21°C with 200 #1 of 1-ethyl-3 (3-dimethyl-amino-propyl)-carbodiimide HCl at 1 mg/ml in MS. This solution was added to 4 ml of the 10% erythrocyte suspension, mixed, and the reaction allowed to proceed for 60 min at 21°C. HGG-conjugated cells were washed twice in PBS and the extent of coupling determined by passive hemagglutination and lysis in the presence of 4% guinea pig complement and rabbit anti-human IgG antiserum obtained from Miles Laboratories, Inc. Very little variation (~1 serial twofold dilution) was found among different preparations. This modified procedure requires far less protein for coupling than techniques previously described by others.

Guinea pig serum (Flow Laboratories, Inc., Rockville, Md.) was used as a source of complement. Rabbit anti-mouse γ-globulin (N. L. Cappel Laboratories, Inc., Cochranville, Pa.) was used to amplify indirect (IgG) PFC at a concentration found to be optimal. 19S (IgM) PFC reported here are those obtained by assay of individual spleens after the background PFC response to normal unconjugated erythrocytes had been subtracted. Numbers of cells secreting 7S (IgG) immunoglobulin are reported as the difference between assays done in the presence and absence of anti-globulin antiserum as described by Miller and Sprent (36).

**Serum Antibody Titers.** Anti-HGG titers in the serum of mothers receiving dHGG were determined by passive hemagglutination of HGG-coated SRBC (33) at a final concentration of 2.5%.

**Foster Nursing.** Females to be used as normal foster mothers were mated syngeneically 2 d
before the mating of those animals which were to be treated with dHGG during pregnancy. Treated mothers were given 5 mg dHGG on day 14 of pregnancy. After the normal foster mothers had delivered their own litters and 1 d before their natural day of birth, the offspring of dHGG-treated mothers were delivered by caesarean section and transferred to the normal lactating mothers. All neonates that were transferred survived.

Irradiation. 12- to 15-wk-old BALB/cCr recipients received a whole body dose of 750 rads from a $^{137}$Cs source in a Gammacell 40 irradiation chamber (Atomic Energy of Canada, Ltd., Ottawa, Canada) 24 h before cell reconstitution. Animals were housed two to three per cage and maintained on a diet of Purina pellets and acidified-chlorinated water containing tetracycline.

Cell Transfer. A cell-transfer system similar to that described by Doyle et al. was used (14). Briefly, spleen cell suspensions were made as described above in Leibowitz medium containing 5% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.) and 0.1 mg/ml gentamicin (Schering Corp., Kenilworth, N. J.). Mixtures of $5-7 \times 10^7$ normal 8- to 10-wk-old spleen cells and/or an equal number of spleen cells from in utero tolerized mice were injected intravenously into irradiated recipients. Immediately after cell reconstitution, recipients received intraperitoneally 0.5 mg of heat-aggregated HGG (37) and 0.1 ml of a 10% suspension of thrice-washed BRBC ($\approx 10^9$ cells) in saline. 7 d later, each animal received intravenously 0.1 ml of a 10% BRBC suspension as well as 0.2 ml of a 10% suspension of syngeneic erythrocytes coated with HGG by the CrCl3 method described above. This protocol consistently generated high anti-HGG 7S PFC responses and considerably reduced the variability inherent in other immunization regimens. 7 d later, the animals were sacrificed and splenic PFC individually enumerated on either BRBC target cells or on SRBC coated with HGG by the modified method by Golub et al. described above.

Another series of cell-transfer experiments utilized a system similar to that described by Basten and colleagues (16) in which $2 \times 10^7$ HGG primed spleen cells and $5 \times 10^6$ anti-Thy 1 + complement-treated DNP primed spleen cells were admixed with $8 \times 10^7$ normal or in utero tolerant cells and injected intravenously into irradiated recipients. Each recipient was challenged immediately with an intraperitoneal injection of 0.5 mg of alum-aggregated TNP-HGG which was repeated 2 d later. Animals were sacrificed 7 d after the initial challenge and splenic PFC enumerated as previously described.

Anti-Thy 1 Treatment. Hyperimmune anti-Thy 1 antibodies were raised in AKR/J mice by the technique of Reif and Allen (38); the antiserum was a gift of Dr. C. Shiozawa from this department. Both agarose-absorbed guinea pig serum and, in one experiment, rabbit serum absorbed as described by Boyse et al. (39) were used as a complement source. Spleen cells were resuspended at a cell concentration of $2 \times 10^7$/ml in anti-Thy 1 serum diluted 1:12. After incubation for 30 min at 4°C an equal volume of absorbed guinea pig complement at a final dilution of 1:12 was added. After incubation for 30 min at 4°C an equal volume of absorbed guinea pig complement at a final dilution of 1:6 was added and the mixture incubated for 45 min at 37°C. This treatment, which routinely reduced spleen cell viability by 30-35%, completely abolished (>98%) the adoptive 7S PFC response of normal spleen cells to SRBC and markedly reduced (>82%) the 19S SRBC response.

Nylon-Wool Cell Filtration. Spleen cells were passed through nylon-wool columns essentially as described by Julius et al. (40). Nylon-wool FT-242 was purchased from Fenwall Laboratories, Morton Grove, Ill.

Preparation of Iodinated Antigens. Carrier-free Na$^{125}$I purchased from Amersham Corp. (Oakville, Ontario, Canada) was used to iodinate HGG (Miles Laboratories, Inc.), according to the method of Greenwood et al. (41). Briefly, $^{125}$I was added to 100 µg protein dissolved in 1 ml of PBS; this was followed by addition of 65 µg (25 µl) of chloramine-T and incubation for 3 min at 21°C. The reaction was stopped by the addition of 1.5 mg (50 µl) of sodium metabisulfite and the product dialyzed free of excess $^{125}$I by several changes of saline at 4°C. For use in radioimmunoassay, $^{125}$I-HGG required chromatography on a calibrated 0.8 × 37 cm Sephacryl column (S-200 Fine, Pharmacia Fine Chemicals, Uppsala, Sweden) to remove aggregated material which contributed to an otherwise high background.

Radioimmunoassay. $^{125}$I-HGG (50 µl in saline; 5,000 cpm) was added after chromatography to mouse serum (20 µl) and 50 µl of rabbit anti-human IgG (IgG fraction, N. L. Cappel Laboratories) at a dilution which precipitated ≈70% of labeled material. The mixture was diluted with 0.5 ml of 50% normal rabbit serum (Grand Island Biological Co.) in 0.2 M Tris-
HCl buffer, pH 7.2, as described by Desbuquois and Aurbach (42). The solution was mixed thoroughly and incubated for 17 h at 4°C followed by addition of 0.5 ml of 7.5% polyethylene glycol-6000 (J. T. Baker Chemical Co., Phillipsburg, N. J.) in 0.2 M Tris·HCl, pH 7.2. The mixtures were centrifuged at 1,000 g for 60 min. Precipitates were washed once with 1 ml of 3.9% polyethylene glycol-6000 in 0.2 M Tris-HCl and the washing added to the previous supernate. The activity (cpm) in both the precipitate and combined supernates was determined on a Beckman Gamma 300 System (Beckman Instruments, Inc., Fullerton, Calif.).

Statistics. Data analysis was carried out by computer with either analysis of variance and the Newman-Keuls multiple range test or Student's t test where applicable. The Wilcoxon rank sum test was used in one application (primed cell adoptive transfer) where nonnormal distributions precluded the use of the t test.

Results

Both T- and B-Cell Populations are Rendered Specifically Unresponsive by Exposure to HGG In Utero. Offspring of BALB/cCr mothers that had received dHGG during pregnancy were challenged repeatedly with TNP-HGG to assess carrier responsiveness (Fig. 1 a). All litters tested generated <5% of the 7S PFC response of an age-matched (11 wk) control litter (P < 0.001). Furthermore, littermates of these HGG unresponsive animals challenged with TNP-CGG were completely responsive to the CGG carrier determinants. Similar results were obtained when TNP-BSA was used for challenge. Thus, in utero-treated animals displayed specific T-cell unresponsiveness to HGG.

Immune function in the HGG-specific B-cell population was assessed by immunizing offspring of dHGG-treated mothers with HGG-conjugated BRBC (Fig. 1 b). Because there is little or no cross-reactivity at the PFC level between BRBC and SRBC (C. Waters, unpublished data), the anti-HGG response was assayed on HGG-conjugated SRBC as target antigen. All treated mice gave a normal response to the BRBC determinants but gave no 7S PFC response to HGG. Thus, B cells were rendered completely and specifically unresponsive to the HGG hapten (P < 0.001) by treatment with dHGG in utero.

Duration of T- and B-Cell Unresponsiveness Is Correlated with Serum HGG Concentration. Individual mice treated with dHGG in utero had initially high but progressively declining levels of HGG in their serum as judged by radioimmunoassay (Fig. 2). At ≈12 wk old, the serum concentration of HGG in treated offspring fell below 4 μg/ml. Before this time, most animals tested in vivo for degree of T- and B-cell tolerance fell into response groups that were 5 SD removed from the response of a normal age-matched group (Table I). During the 11–13-wk period, some recovery of T- and B-cell responsiveness could be observed with more animals falling into response groups within 3–4 SD of the control. In the absence of additional dHGG waning of HGG tolerance progressively increased until by 40 wk of age, treated animals had recovered full responsiveness. Administration of small amounts of dHGG biweekly reversed this effect (Fig. 3). Specific tolerance in the T-cell population could be maintained until 50 wk of age, when animals were finally killed, with as little as 0.1 μg dHGG biweekly from 3 wk of age onward. B cells required 100-fold more dHGG (10 μg) for tolerance to remain virtually complete.

Diminished 19S Response to TNP-HGG In In Utero Tolerized Mice Does Not Indicate Incomplete Carrier Unresponsiveness. Other investigators studying HGG tolerance induced in adults reported significant reduction of the 7S response with less of an effect on the 19S response (16). Similarly, animals treated with dHGG in utero and
FIG. 1a. Specificity and degree of T-cell unresponsiveness induced in utero to dHGG. IgM and IgG PFC were enumerated in the individual spleens of 11-wk-old mice treated with dHGG in utero and challenged with an immunogenic preparation of TNP-HGG or TNP-CGG. Data are presented as the geometric mean $\pm$ SE. $P$ values between groups: IgG PFC to TNP-HGG; untreated cf. dHGG treated, $<0.001$; IgG PFC to TNP-CGG in littermates: untreated cf. dHGG treated, $>0.05$. $\square$, TNP-HGG, IgG response; $\diamond$, TNP-HGG, IgM response; $\blacksquare$, TNP-CGG, IgG response; $\bigcirc$, TNP-CGG, IgM response.

FIG. 1b. Specificity and degree of B-cell unresponsiveness induced in utero to dHGG. IgM and IgG PFC were enumerated in the individual spleens of 8-wk-old mice treated with dHGG in utero and challenged with HGG-coupled BRBC as described in Materials and Methods. Data are presented as the geometric mean $\pm$ SE for the immune response to HGG determinants and BRBC determinants. $P$ values between groups: IgG PFC to HGG; untreated cf. dHGG-treated, $<0.001$; IgG PFC to BRBC; untreated cf. dHGG-treated, $>0.05$. $\boxdot$, HGG, IgG response; $\blacklozenge$, HGG, IgM response; $\blacksquare$, BRBC, IgG response; $\bigcirc$, BRBC, IgM response.
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Fig. 2. Rate of disappearance of HGG in serum of in utero-treated mice. Serum samples were taken from the retro-orbital plexus of mice of various ages after tolerance induction in utero and HGG concentration (μg/ml) individually determined by radioimmunoassay. Data are presented as the arithmetic mean of three to four mice per group ± SD. 6-wk and 14-wk ages: serum sample from a single mouse.

TABLE I
Waning of In Utero-Induced HGG Tolerance

| Age* | n × SD† | T-cell response | B-cell response |
|------|---------|----------------|----------------|
| wk   |         |                |                |
| 6-8  | 1       | —              | —              |
|      | 2       | —              | —              |
|      | 3       | —              | —              |
|      | 4       | —              | 10             |
|      | 5       | 9              | 10             |
| 11-13| n = 1   | —              | —              |
|      | 2       | —              | —              |
|      | 3       | 6              | 20             |
|      | 4       | 18             | 30             |
|      | 5       | 31             | 50             |
| 15-18| n = 1   | —              | —              |
|      | 2       | 9              | 10             |
|      | 3       | 25             | 20             |
|      | 4       | 55             | 40             |
|      | 5       | 68             | 40             |

* 10–40 mice per age group.
† n, integer denoting multiple of standard deviation (SD) units from geometric mean of normal age-matched controls.
FIG. 3. Maintenance of in utero induced unresponsiveness in T and B cells with additional dHGG. Mice treated in utero with dHGG were given decreasing amounts (1 mg–1 ng per mouse) of dHGG beginning at 3 wk of age and continuing biweekly until testing at 50 wk of age. A control group received no additional dHGG. One group was challenged with an immunogenic preparation of TNP-HGG to assess T-cell unresponsiveness (○); the other group was immunized with HGG-coupled BRBC to assess B-cell unresponsiveness (●). IgG PFC to TNP or to HGG were enumerated in individual spleens.

challenged with TNP-HGG as described previously, produced diminished but not absent 19S responses (≤40% control). No significant 19S response to HGG was observed at the B-cell level. Because the 19S response is presumably less T-dependent than the 7S response, the presence of a residual response even at this time could indicate incomplete T-cell unresponsiveness. Alternatively, a diminished but not absent 19S response may represent the normal response of an animal to HGG determinants modified by haptenation, or to the hapten itself, to which the animal had therefore not been rendered unresponsive. To distinguish between these possibilities, we challenged both in utero dHGG-treated and adult dHGG-treated mice with trinitrophenylated autologous γ-globulin (TNP-MGG) using the same assay conditions as above (Fig. 4). Both treated groups generated indistinguishable (P > 0.05) 19S responses to TNP-MGG which were comparable to those made by normal groups (P > 0.05). Because normal individuals should be unresponsive to their own γ-globulins, the presence of a 19S TNP response in all three groups indicates the involvement of either TNP-specific help or chemical modification of the HGG carrier, or both. We therefore restricted our analysis of HGG tolerance induced in utero to the 7S PFC response in the following experiments.
Administration of HGG In Utero Is Sufficient for Tolerance Induction. Although dHGG-treated mothers made no demonstrable immune response to the antigen during either the pregnancy or the weaning period (C. Waters, unpublished data), it is necessary in considering the general case of natural self-tolerance to exclude the possibility that dHGG-treated mothers influenced the immune status of their offspring by factors other than 19S or 7S antibody in the colostrum or milk. Thus, we devised a foster nursing experiment in which normal lactating mothers were used to foster nurse the caesarean-delivered offspring of dHGG-treated mothers (Fig. 5). When challenged at 6 wk of age, dHGG-treated litters nursed either by their own or by normal foster mothers were completely unresponsive to TNP-HGG. At 11–12 and 17–18 wk of age, when waning of T-cell unresponsiveness can normally be observed, both treated groups made low but indistinguishable 7S PFC responses ($P > 0.05$). Thus we conclude that this brief period of in utero exposure to dHGG (4 d) is sufficient to induce carrier unresponsiveness.

Maternal HGG Concentration and In Utero Tolerance Induction. Females made pregnant 15 d earlier were given intravenously either 5 mg or 0.5 mg dHGG. The offspring of these treated females were challenged with TNP-HGG at 11 wk of age to determine the effect of the dose of dHGG on the degree of carrier-specific unresponsiveness (Fig. 6). Animals treated with a low dose (0.05 mg) gave 40–50% of a normal 7S PFC response while animals treated with a high dose (5 mg) gave a characteristic 5% normal response. Thus a 100-fold lower dose of dHGG was considerably less effective in inducing long-lived T-cell unresponsiveness.

Mechanism of In Utero Induced HGG Unresponsiveness. A number of investigators
studying unresponsiveness to HGG induced in adult animals have reported the existence of specific suppressor cells associated with at least some phases of the unresponsive state (14-16). We have endeavored to examine this question in the in utero system by using similar adoptive cell transfer regimens. Lethally irradiated animals were reconstituted with 5-7 x 10^7 normal adult spleen cells admixed with an equal number of either spleen cells from animals treated in utero with dHGG or untreated age-matched cells. Cell transfer was followed by immediate intraperitoneal challenge with 0.5 mg of heat-aggregated HGG. 7 d later, an additional challenge of 10^8 HGG-conjugated syngeneic erythrocytes was given intravenously. This assay procedure induced consistently good 7S PFC responses to HGG and considerably reduced the variability of the response inherent in other immunization regimens. Admixture of equal numbers of normal and 14-wk-old in utero dHGG-treated spleen cells and transfer to irradiated recipients resulted in a 70% reduction of the normal response (Fig. 7). Little additional suppression was observed if twice as many treated spleen cells were added (C. Waters, unpublished data). Furthermore, an equivalent number of normal age-matched spleen cells admixed with normal cells did not suppress. Challenge of animals receiving the cell mixtures above with BRBC revealed that suppression was specific; spleen cells from mice treated in utero with dHGG did not suppress the BRBC response. Spleen cells from older mice treated in utero with dHGG also specifically suppressed the HGG response, often to a greater extent.

Contrary to expectations, no suppression of this nature was found when spleen cells
**Fig. 6.** Degree of T-cell unresponsiveness in offspring of mice receiving different amounts of dHGG during pregnancy. At 11 and 15 wk of age, offspring of mice treated during pregnancy with 5 mg or 0.05 mg dHGG were challenged with an immunogenic preparation of TNP-HGG. IgG PFC were enumerated in individual spleens. Data are presented as the geometric mean ± SE. P values between groups: untreated cf. litter of mother receiving 0.05 mg dHGG; >0.05 at both ages of testing; untreated cf. litter of mother receiving 5.0 mg dHGG; <0.001 at both ages of testing. [], untreated; [, 0.05 mg dHGG; ■, 5 mg dHGG.

**Fig. 7.** Presence of active suppression in spleens of 14-wk-old mice treated in utero with dHGG. Adoptive cell transfer to irradiated recipients of $7 \times 10^7$ normal or tolerant spleen cells alone or a 1:1 mixture of both populations was followed by intraperitoneal challenge with both heat-aggregated HGG and BRBC. After an additional challenge as described in Materials and Methods, mice were sacrificed and IgG PFC to HGG and BRBC were enumerated in individual spleens. Data are presented as the geometric mean ± SE. P values between groups: IgG PFC to HGG: recipients of normal cells cf. recipients of 1:1 mixture of normal and tolerant cells, <0.02; cf. recipients of tolerant cells, <0.001; cf. recipients of 1:1 mixture of normal and of normal 14-wk-old cells, >0.05. IgG PFC to BRBC, normal cell recipients cf. all other groups >0.05. [], HGG; ■, BRBC.
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Fic, 8. Absence of active suppression in spleens of 5-wk-old mice treated in utero with dHGG. Adoptive cell transfer to irradiated recipients of $7 \times 10^7$ normal or tolerant spleen cells alone or a 1:1 mixture of both populations was followed by intraperitoneal challenge with both heat-aggregated HGG and BRBC. After a secondary challenge as described in Materials and Methods, mice were sacrificed and IgG PFC to HGG and BRBC were enumerated in individual spleens. Data are presented as the geometric mean ± SE. P values between groups: IgG PFC to HGG; recipients of normal cells cf. recipients of a 1:1 mixture of normal and tolerant cells, >0.05; cf. recipients of tolerant cells, <0.001; cf. recipients of 1:1 mixture of normal and normal 5-wk-old cells, >0.05. IgG PFC to BRBC: recipients of normal cells cf. all other groups >0.05. HGG; BRBC.

from younger in utero-treated mice were admixed with normal cells (Fig. 8). Cells from 4- to 6-wk-old mice treated in utero with dHGG consistently failed to suppress the HGG response even when twice as many tolerant cells were added (C. Waters, unpublished data). As before, tolerance was stable upon adoptive transfer. This lack of suppression by spleen cells from 4- to 6-wk-old tolerant mice would not have been predicted if the mechanism maintaining the tolerant state requires suppressor participation. HGG tolerance began to break down in in utero-treated mice at 14 wk of age or older although the tolerant state remained stable in 4- to 6-wk-old in utero-treated mice.

We have also utilized an adoptive cell-transfer system similar to that described by Basten and colleagues in which DNP-primed B cells and HGG-primed spleen as a T-cell source are admixed in an optimal ratio (16). Again, suppression was observed when older in utero-treated mice were used as tolerant cell donors at a 4:1 ratio of tolerant to normal cells. Spleen cells from younger in utero-treated mice did not suppress. The strength of suppression observed in this system was somewhat less dramatic, however, than with the system utilizing normal unprimed spleen cells (C. Waters, unpublished data).

The cell population responsible for active suppression in transfers of spleen cells from 14- to 33-wk-old in utero-treated mice was relatively resistant to a single treatment with anti-Thy 1 + complement. Partial restoration of unresponsiveness (20–60% of the normal control group) was achieved after treatment of the in utero tolerized cell population and subsequent admixture with normal cells ($P \leq 0.02$ with respect to the suppressed group). In two of our experiments with nylon-wool cell
filtration, restoration levels of 75–95% of the normal response were achieved \( P < 0.05 \) with respect to the suppressed group.

**Discussion**

In this paper, we describe experiments designed to elucidate the mechanism of natural self-tolerance by comparison with a model system in which antigen is administered in utero before immunocompetent cells arise. Insofar as any such model which relies on the use of extrinsic antigens can conform to possibly peculiar structural properties or modes of presentation germane to true self-antigens, this system at least rules out interference from regulatory effector functions in immunocompetent animals. The latter may render difficult the extrapolation of data obtained from adults or possibly even neonatally tolerized animals to self-tolerance. Furthermore, unresponsiveness in this system is not achieved or amplified by nursing or by serum factors, making it unlike several others which have been proposed as models for self-tolerance \((4, 43)\). There have been reports of the induction of unresponsiveness via the maternal-fetal route; the mechanisms of the unresponsive states, however, were not investigated \((44-46)\).

The basic experimental protocol involved the administration of deaggregated HGG to BALB/cCr mice during pregnancy and the subsequent assessment of T- and B-cell immunocompetence towards this antigen during the postnatal life of the offspring. The mechanism responsible for the unresponsive state so induced was then investigated in cell-transfer studies. For example, offspring of pregnant mice that had received a specified quantity of dHGG in utero were completely unresponsive at both the T- and B-cell level for a substantial portion (\(>12\) wk) of their adult lives. The specificity of the tolerant state in the case of T cells was established by showing that littermates of HGG unresponsive animals gave normal responses when immunized with one of two haptenated noncross-reacting carriers, TNP-CGG and TNP-BSA. Specificity of B-cell tolerance was demonstrated by showing that animals made unresponsive in utero to HGG generated normal PFC responses to BRBC. B-cell unresponsiveness began to slowly disappear in mice at 12 to 18 wk of age. This waning occurred in parallel with the breakdown of T-cell unresponsiveness. Although this observation may represent a departure from that of Chiller et al. \((37)\) who found that B-cell tolerance to HGG disappeared more rapidly than T-cell tolerance in adult tolerized animals, we wish to point out that the level of sensitivity of our in situ assay and that of the above investigators may not be readily comparable.

The gradual waning of unresponsiveness correlated with the disappearance of HGG from the serum of in utero-treated animals as detected by radioimmunoassay. This observation is consistent with Burnet's postulate on self-tolerance which predicts that presence of antigen is required to maintain antigen specific unresponsiveness. The small PFC response that did occur in both B- and T-cell classes upon challenge at 12 wk of age correlates with a serum HGG concentration of \(\leqslant 4\) \(\mu\)g/ml. In addition, we found that unresponsiveness to HGG could be maintained if small quantities \((0.1-10 \mu \text{g})\) of dHGG were administered biweekly to in utero-treated animals.

Although mice rendered tolerant in utero to HGG gave no detectable 19S PFC response when immunized with HGG coupled to BRBC in the B-cell assay, it should be noted that both littermates of animals treated with dHGG in utero and animals tolerized with dHGG as adults gave diminished but not absent 19S PFC responses to
the hapten when challenged with TNP-HGG to test for T-cell tolerance. The degree
to which in utero treatment actually reduced the 19S response was not immediately
apparent, however; animals tolerized either as adults or in utero as well as normal
controls made similar 19S responses when challenged with TNP-substituted autolo-
gous immunoglobulins. These results indicate that T-cell unresponsiveness in in utero-
treated animals was probably complete; either new antigenic determinants are created
by trinitrophenylation or TNP-specific helper-cell activity may have been generated
by the immunization regimen.

Foster nursing experiments showed that a maximum of 4 d in utero exposure to
HGG was sufficient to achieve a long-lived specific tolerance to HGG in the offspring.
Thus tolerance induction was independent of maternal factors which could be
transmitted to the fetus by nursing. In fact, no evidence was found for a maternal
immune response to dHGG at least at the level of agglutinating antibody during
pregnancy. These latter observations are in agreement with those of others who
investigated the antibody response of normal adults given a tolerogenic dose (5 mg)
of dHGG (16, 18).

The most significant finding of this study of tolerance induced in utero is that
suppressor cells do not appear to be associated with the maintenance of the tolerant
state. Rather, active suppression was routinely found in mixed spleen-cell transfers
when the tolerant cell population was derived from animals undergoing tolerance
breakdown (14-33 wk old). Suppression was specific for HGG because 19S and 7S
PFC responses to an unrelated antigen such as BRBC were normal in irradiated mice
reconstituted with a mixture of normal and HGG-treated spleen cells. No evidence
for active suppression was found in younger (4- to 6-wk-old) in utero-treated mice
where tolerance was intact, even when a 2:1 tolerant to normal-cell ratio was used in
attempts to inhibit an adoptively transferred immune response to HGG in irradiated
recipients. It would therefore appear that within the limits of detection defined by
cell-transfer assays, suppressor cells are not involved in maintaining the HGG unre-
 sponsive state induced in utero.

The suppressor-cell population in in utero dHGG-treated mice which were partially
tolerant at 14-33 wk of age was relatively resistant to a single treatment with anti-
Thy 1 antiserum and complement. It is of interest in this regard that other investigators
have described a suppressor cell which expresses Ly 1+2+3+ Qal+ but is also resistant
to a single anti-Thy 1+ complement treatment (47, 48). Furthermore, they showed
that Thy 1+ Ly 1+ 2,3+ Qal+ help is required in vitro for Ly 1+2+3+ Qal+ cells to exert
potent feedback suppressor effects. Because the data from our in situ assays indicate
that low levels of T-cell help are available during the period of partial tolerance, it
would not be unreasonable therefore to expect the appearance of suppressors at this
time.

The fact that the suppressor cell population in our experimental system was
partially retained by nylon-wool cell filtration suggests adherent properties similar to
those of B cells, macrophages or Ly 1+2+3+ amplifiers of suppression. These observa-
tions are somewhat reminiscent of those of other investigators who described the
presence of two suppressor-cell populations in mice rendered tolerant to dHGG as
adults (28, 49). One cell type was anti-Thy 1-sensitive whereas the other, though
resistant to anti-Ig treatment, had glass-wool adherent properties.

The results of the experiments reported here are at variance with those of other
investigators who have induced unresponsiveness in adult immunocompetent animals to various antigens including HGG. In the majority of cases, specific suppressor cells have been implicated in the induction and/or maintenance of the unresponsive state (14–16). It is of course difficult to establish causality in any case and at least one investigator has suggested that because HGG specific suppressor cells are only transiently associated with the HGG unresponsiveness in the adult model, they cannot be regarded as obligatory elements for maintaining it (14). Moreover, these investigators have recently demonstrated that the induction of suppressor cells along with unresponsiveness is a function of the source from which HGG was obtained: HGG isolated from the plasma of a normal individual or from an IgG1 λ-myeloma did not induce suppressors but did induce unresponsiveness (50). Our data are thus in agreement with those of others who failed to find suppressor cells associated with a state of tolerance even in adult animals (2–4).

The experiments described in this report link the appearance of suppressor cells to the breakdown of the in utero tolerant state and represent the first demonstration of its kind for soluble protein antigens. There is, however, some precedence for these observations in reports on transplantation tolerance. For example, rat leukocyte chimeras have been described in which specific suppressor cells were absent until tolerance was partially terminated by the adoptive transfer of normal syngeneic lymphocytes (7). These suppressors appeared to be associated with the homeostatic component of an active alloimmune response.

Current studies in this department support and extend these observations. When chimerism was induced in chickens in ovo, animals which presented a partial state of unresponsiveness (no graft-versus-host (GVH) reactivity but specific serum alloantibodies present) have inhibitory activity for GVH. Suppression, however, is absent in animals that are tolerant at both the cell-mediated and humoral level.²

The results of our experiments are consistent with the view that suppressor cells do not represent an obligatory feature of self-tolerance and that the absence of anti-self reactivity reflects a state of central immunological unresponsiveness.

Summary

A specific state of T- and B-cell tolerance to human γ-globulin (HGG) was induced in utero by intravenous administration of the deaggregated antigen to pregnant BALB/cCr mice. Tolerance persisted in the offspring until the 12th wk of age and then began to gradually disappear. Suppressor cells could only be found when responsiveness to HGG ultimately appeared in the in utero-treated animals but not when they were completely unresponsive. In contrast, HGG-specific suppressors found in animals made unresponsive to HGG as adults appear to be associated with either the establishment and/or maintenance of the unresponsive state. To the extent that these experiments are consistent with natural self-tolerance to a serum protein, we conclude that active suppression is not a prerequisite from maintenance of unresponsiveness to self.

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² Havele, C., B. M. Longenecker, and T. G. Wegmann. Manuscript in preparation.
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