

INDUCTION AND MODE OF ACTION OF SUPPRESSOR CELLS GENERATED AGAINST HUMAN GAMMA GLOBULIN

I. An Immunologic Unresponsive State Devoid of Demonstrable Suppressor Cells*

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Since the initial observations of the involvement of lymphocytes in the active suppression of immune processes, a variety of suppressor cells and substances have been associated with regulation of the immune response to a number of antigens (1, 2). Not only has such suppressor activity been associated with humoral and cellular mediated responses, but suppressor cells have also been implicated in various models of immunologic unresponsiveness (3, 4). The suppressor cells shown to be present in mice rendered tolerant to human γ -globulin (HGG)¹ (5-8) have been characterized as lymphocytes bearing T-cell antigens (6, 9), including theta (6) and Ly-2 (10), and Ia antigens (10, 11), including I-J (12). Suppressor T cells generated during the induction of specific tolerance to HGG can suppress HGG-specific T and B cells in vivo (9) and hapten-specific B cells in vitro when hapten-coupled HGG is present in the cultures (6, 11, 13). Additionally, soluble suppressor substances have been associated with spleen cells of HGG-tolerant mice (14-17). Adherent cells have also been implicated in the suppression of the immune response to HGG (6-9). Although suppressor cell activity is well documented in the tolerance to HGG, it appears to be only transiently associated with the unresponsive state (6, 8, 18).

Presently, it is unclear whether suppressor T cells, which are involved in the regulation of the induction and magnitude of immune responses, play a major role in the establishment and maintenance of tolerance to serum proteins resembling self-antigens. Delineation of the precise mechanisms of induction and maintenance of the immunologic unresponsive state to HGG awaits the clarification of the role of suppressor cells in these processes. The observation that suppressor T cells accompany the tolerant state is not universal. Specific suppressor cells have not been found in

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Abbreviations used in this paper: Δ -AHGG, heat-aggregated HGG; DHGG, deaggregated HGG; Doy, normal plasma from healthy donor; Fit, plasma from a myeloma patient; GRBC, goat erythrocytes; HGG, human γ -globulin; LPS, lipopolysaccharide; M, Cohn fraction II of human plasma obtained from Miles Laboratories, Inc.; PFC, plaque-forming cells; RC, Cohn fraction II of human plasma obtained from the American Red Cross; S, Cohn fraction II of human plasma from Sigma Chemical Co.; US, Cohn fraction II of human plasma from United States Biochemical Corp.

mice rendered tolerant to HGG as neonates (19), in tolerant mice given a second dose of tolerogenic HGG at a time when the original suppressor cells have disappeared (20), or during parabiosis of normal mice with HGG-tolerant mice (21). Suppressor cells have not been universally demonstrated even within adults of a single inbred strain of mice (22, 23). In light of the past variability in the induction of suppressor cells during the establishment of the HGG-tolerant state, it is of interest that nearly all of the investigators reporting suppressor cell activity appear to have utilized HGG from either of two commercial sources. The studies presented here demonstrate that the source of antigen is crucial to the degree of suppressor cell activity stimulated in tolerized mice and that this degree of suppression does not reflect the induction and maintenance of the tolerant state of these animals. These studies further emphasize the lack of an obligatory role of suppressor cells in these events.

Materials and Methods

Animals. Male A/J mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) at 4–5 wk of age. They were maintained on Wayne Lab Blox F6 (Allied Mills, Inc., Chicago, Ill.) and acidified water ad libitum. All mice were housed five to a cage. Mice ranging in age from 8 to 13 wk were used in these experiments.

Antigens. Cohn Fraction II of human plasma was obtained through the courtesy of the American Red Cross (RC) National Fractionation Center with the partial support of National Institutes of Health grant HE 13881 HEM, lot RC-104, and it was also purchased from Miles (M) Laboratories, Inc. (Elkhart, Ind.), lot 41; Sigma (S) Chemical Co. (St. Louis, Mo.), lot 46C-0198; and United States (US) Biochemical Corp. (Cleveland, Ohio), lot 10600. Human plasma from a myeloma patient (Fit) expressing an IgG₁ λ-myeloma protein was obtained by plasmaphoresis through the generosity of Dr. Hans Spiegelberg of this department. Normal plasma was obtained from one of us (Doy) by defibrination with glass beads. The Cohn fractions were dissolved in 0.01 M potassium-phosphate buffer, pH 8.0, before column chromatography, whereas the normal and myeloma plasma were dialyzed at 4°C against 0.01 and 0.005 M phosphate buffers, respectively, to precipitate euglobulins from the samples before further purification. A nondialyzed sample of the normal plasma was precipitated in 20% ethanol at –6°C (24), lyophilized, and dissolved in 0.01 M phosphate buffer before chromatography. HGG was purified from these materials by column chromatography on DEAE-cellulose in 0.01 M phosphate buffer, pH 8.0, except in the case of the myeloma protein which was chromatographed in 0.005 M phosphate buffer at pH 8.0.

Induction of Tolerance. Tolerogenic, deaggregated HGG (DHGG) was prepared by ultracentrifugation of DEAE-purified HGG to remove aggregates by a modification (8) of the previously described method (25). 5 ml of HGG was centrifuged for 150 min at 4°C and 41,000 rpm in polyallomer tubes (model 326819) in a swinging bucket SW 50.1 rotor (both from Beckman Instruments, Inc., Palo Alto, Calif.). The upper quarter of the centrifuged solution was carefully removed and diluted to a final concentration of 2.5 mg/ml in sterile, nonpyrogenic, 0.15 M NaCl (McGaw Laboratories, Irvine, Calif.). Mice received 1 ml of DHGG i.p. Protein concentration was determined by optical density of appropriate dilutions with an extinction coefficient of $E_{280\text{nm}, 1\text{cm}}^{1\%} = 15$.

Immunizations. Immunogenic, heat-aggregated HGG (Δ-AHGG) was prepared from the DEAE-purified Cohn fractions and from normal plasma as described previously (25), using a modification of Gamble's method (26). Chromatographed myeloma protein, which did not aggregate upon heating (27), was aggregated with *bis*-diazotized benzidine (27). Benzidine was obtained from Calbiochem (San Diego, Calif.). Mice received a primary injection of 400 μg of Δ-AHGG or *bis*-diazotized benzidine-aggregated HGG i.v. into a lateral caudal vein followed 10 days later by a secondary injection of the same amount of antigen i.p. Those mice also given bacterial lipopolysaccharide (LPS) were injected i.v. 3 h after primary antigenic challenge with 50 μg of a phenol-extracted preparation from *Escherichia coli* 0111:B4, lot 614378 (Difco Laboratories, Detroit, Mich.), diluted in pyrogen-free, 0.15 M sterile NaCl.

Irradiated Recipients. Recipients for adoptive transfer were irradiated 2–3 h before reconsti-

tution. Mice placed in an aluminum chamber in a Gamma Cell 40 small animal irradiator (Atomic Energy of Canada, Ltd., Ottawa, Canada) received 900 R of whole body irradiation from a cesium 137 source emitting a central dose of 109 R/min. Reconstituted recipients received 100 μ g of gentamicin (Schering-Pharmaceutical Corp., Kenilworth, N. J.) diluted in 2.7% glucose in saline i.p. on the day of irradiation and again 3 days later, and they were caged in groups of two to avoid early irradiation death, presumably due to bacterial infection.

Adoptive Cell Transfer. Cell transfers were accomplished as described previously (28). Briefly, irradiated recipients were reconstituted i.v. with washed spleen cells, and they were immunized immediately and again 10 days later. 5 days after the secondary challenge, the recipients' spleens were removed and individually assayed for plaque-forming cells (PFC).

Hemolytic Plaque Assay. PFC to HGG were assayed by a slide modification of the hemolytic plaque assay (29). Cohn fraction II HGG at a concentration of 20 mg/ml was covalently coupled to goat erythrocytes (GRBC) (Colorado Serum Co., Denver, Colo.) using water-soluble carbodiimide [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride] from Calbiochem (30). This HGG was previously absorbed against GRBC. Plaques were developed with guinea pig serum (Pel-Freez Biologicals Inc., Rogers, Ark.) as a source of complement, and indirect plaques were amplified with rabbit anti-mouse IgG serum previously absorbed against HGG and GRBC.

Statistical Methods. Results of the plaque assay are expressed as the mean number of PFC per spleen or per 10^6 spleen cells \pm the standard error corrected for the background response to the indicator erythrocytes. Data were analyzed statistically with the Student's *t* test. The percent suppression was determined as follows:

$$\% \text{ suppression} = \left(1 - \frac{\text{mean PFC}/10^6 \text{ for recipients of normal and tolerant spleen cells}}{\text{mean PFC}/10^6 \text{ for recipients of normal spleen cells alone}} \right) \times 100.$$

Results

Inability of DHGG from a Healthy Individual to Induce Suppressor Cells. Although suppressor T cells have been recently demonstrated in HGG-tolerant spleen cell populations (6-8), universal demonstration of these suppressor cells has not been reported (19-23). A majority of the successful attempts to generate suppression utilized HGG purified from one source (RC) of Cohn fraction II (7-9, 14, 15, 20, 31). Suppressive activity of HGG-tolerant spleen cells was assayed by adoptive transfer of 70×10^6 viable spleen cells from mice tolerized 10 days previously and 70×10^6 viable spleen cells from normal animals into lethally irradiated (900 R) syngeneic recipients (8, 9).

Table I depicts the results obtained when the spleen cells from A/J mice tolerized with DHGG from either a healthy individual (Doy) or from the most commonly used source (RC) were assayed for the presence of suppressor cells. The injection of tolerant spleen cells from mice given 2.5 mg of DHGG (RC) substantially suppressed (61%) the PFC response of normal spleen cells (group 4), as previously reported (8, 9). In contrast, 2.5 mg of tolerogen purified by DEAE chromatography of euglobulin-depleted normal plasma (Doy) did not generate detectable suppressor cell activity against normal spleen cells (group 2). The response in these recipients was increased to greater than twice the response of normal controls (group 3). The DHGG (Doy) preparation induced considerable unresponsiveness as demonstrated by adoptive transfer of tolerant spleen cells alone (group 1). Neither the suppression accomplished by RC-tolerant spleen cells nor the increased response associated with Doy-tolerant spleen cells can be attributed to the passive administration of antigen-nonreactive spleen cells, since recipients injected with 70×10^6 viable and 70×10^6 lethally irradiated (900 R) normal spleen cells (group 5) supported PFC responses equivalent

TABLE I
Lack of Suppression after Tolerization with HGG from Euglobulin-Depleted Normal Plasma

| Group | Spleen cells transferred* | | | Immunogen‡ | PFC/10 ⁶ HGG (RC)§ | Percent sup- pression |
|-------|---------------------------|----------------------|---|--------------|----------------------------------|--------------------------|
| | Tolerant | | Normal | | | |
| | DHGG (Doy) | DHGG (RC) | | | | |
| 1 | 70 × 10 ⁶ | — | — | Δ-AHGG (Doy) | 56 ± 22 | — |
| 2 | 70 × 10 ⁶ | — | 70 × 10 ⁶ | Δ-AHGG (Doy) | 847 ± 198 | 0 |
| 3 | — | — | 70 × 10 ⁶ | Δ-AHGG (Doy) | 356 ± 64 | — |
| 4 | — | 70 × 10 ⁶ | 70 × 10 ⁶ | Δ-AHGG (Doy) | 138 ± 77 | 61 |
| 5 | — | — | 70 × 10 ⁶ + 70 × 10 ⁶ (irradiated) | Δ-AHGG (Doy) | 343 ± 60 | — |

* 900 R irradiated recipients were reconstituted with either normal spleen cells or spleen cells from tolerant mice that had received 2.5 mg of DHGG 10 days earlier or both.

‡ Reconstituted mice received 400 µg of AHGG on the day of cell transfer and again 10 days later.

§ The mean ± SE of the number of indirect PFC to HGG from 5–8 mice assayed 5 days after secondary challenge.

|| See Materials and Methods.

to those of recipients reconstituted with 70 × 10⁶ viable normal spleen cells alone (group 3).

Lack of Suppression at Various Times after Tolerization. Although suppressor cells could not be demonstrated in DHGG (Doy)-tolerized spleen cells 10 days after tolerization, the possibilities that suppressor cell activity had arisen later or waned sooner were investigated. Table II illustrates investigations of splenic suppressor cell activity 3, 10, and 17 days after tolerization with DHGG from a normal individual (Doy). Suppressor cell activity was not detected at any of the times assayed post tolerization (groups 2, 3, and 4). Furthermore, as in the previous table, the addition of tolerant, but nonsuppressive, DHGG (Doy)-treated spleen cells to normal spleen cells increased the number of PFC to HGG in the recipients.

Lack of Suppression with Tolerogen Purified from Individual Plasma. Investigations were undertaken to determine whether the lack of induction of suppressor cells by DHGG purified from euglobulin-depleted normal plasma (Doy) was associated with the original source of plasma or with the method of purification of the HGG. An aliquot of normal plasma (Doy) was precipitated with 20% ethanol and lyophilized to approximate two of the major procedures to which the pooled, outdated human plasma is subjected during Cohn fractionation of the suppressive (RC) preparation. In addition, DHGG was purified from a human myeloma protein (see Materials and Methods) to assess the ability of HGG of a single subclass to induce tolerance and suppressor cell activity. This IgG₁ myeloma protein (Fit) is of particular interest since it has previously been shown to be refractory to spontaneous aggregation (27). Deaggregated HGG from either the ethanol-precipitated normal plasma (Doy) or the myeloma protein (Fit) failed to induce demonstrable suppressor cells (Fig. 1) when assayed 10 days after tolerization, at the peak of suppressor T-cell activity (8). However, both preparations established unresponsiveness in the spleen cells of treated mice. Mice reconstituted with both normal spleen cells and cells from mice tolerized

TABLE II
Lack of Suppression at Various Times after Tolerization with HGG from Euglobulin-Depleted Normal Plasma

| Group | Spleen cells transferred* | | | | PFC/10 ⁶ HGG (RC)‡ | Percent suppression |
|-------|---------------------------|----------------------|----------------------|----------------------|----------------------------------|---------------------|
| | Tolerant | | | Normal | | |
| | 3 Days | 10 Days | 17 Days | | | |
| 1 | — | — | 70 × 10 ⁶ | — | 38 ± 22 | — |
| 2 | 70 × 10 ⁶ | — | — | 70 × 10 ⁶ | 508 ± 84 | 0 |
| 3 | — | 70 × 10 ⁶ | — | 70 × 10 ⁶ | 1,928 ± 332 | 0 |
| 4 | — | — | 70 × 10 ⁶ | 70 × 10 ⁶ | 190 ± 28 | 0 |
| 5 | — | — | — | 70 × 10 ⁶ | 189 ± 100 | — |

* 900 R irradiated recipients were reconstituted with either normal spleen cells or spleen cells from tolerant mice that had received 2.5 mg of DHGG (Doy) 3, 10, or 17 days earlier or both. Reconstituted mice received 400 µg of Δ-AHGG (RC) on the day of cell transfer and again 10 days later.

‡ See Table I, footnote §.

with either nonsuppressive preparation of DHGG (Doy or Fit) responded with substantially greater PFC than recipients of normal spleen cells alone, as previously observed in Tables I and II. Therefore, unresponsiveness to HGG can be established in the absence of demonstrable suppressor cells by the injection of DHGG purified from the plasma of either a healthy donor or a myeloma patient.

Generation of Suppression by Several Commercially Acquired Cohn Fraction II Preparations. Although pooled, outdated human plasma varies in many respects from an individual sample of human plasma, the commercial Cohn fractionation procedure may be responsible for the generation of the suppressive effects associated with the RC preparation. Cohn fraction II obtained from three additional commercial sources (Materials and Methods) was used to investigate the ability of various commercial samples of HGG to induce suppressor cell activity. In these experiments, irradiated recipients received either spleen cells from mice tolerized with DHGG prepared from the Cohn fraction II from the commercial source under investigation, or normal spleen cells, or both normal and tolerant cells. These recipients were compared with the recipients of spleen cells from mice tolerized with DHGG prepared from the conventional (RC) source.

The induction of suppressor cell activity in the spleens of mice treated with one commercial HGG preparation (US) is presented in Table III. In the two separate experiments presented, DHGG purified from the commercial HGG preparation (US) generated considerably less suppressor cell activity (group 2) than did the conventional (RC) source (group 5). This was true regardless of the magnitude of the normal response in these recipients or the degree of suppression stimulated by the RC preparation. Additionally, these results were unchanged irrespective of the source of HGG with which the recipients were challenged or against which they were plaqued. Thus, there was serologic identity between these two HGG preparations at the levels of both antigenic challenge and antibody production. For this reason, recipients were challenged with and PFC were detected against the conventional (RC) preparation of HGG in several experiments.

Similar results were obtained in a series of experiments undertaken to assess the ability of DHGG purified from each of the four sources of commercially fractionated

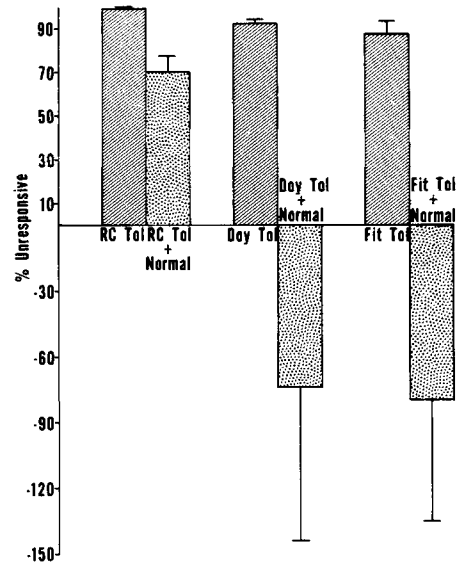


FIG. 1. Lack of suppression with DHGG purified from individual plasma. The percent of unresponsiveness or suppression was calculated for individual recipients of 70×10^6 tolerant (Tol) spleen cells alone or 70×10^6 tolerant and 70×10^6 normal spleen cells. Donors were tolerized 10 days before transfer with DHGG purified from Cohn fraction II (RC), ethanol-precipitated plasma from a healthy individual (Doy), or plasma from a myeloma patient (Fit). The mean \pm SE of 5-9 recipients is presented.

TABLE III
Suppression after Tolerization with HGG from Commercial (US) Cohn Fraction II

| Group | Spleen cells transferred* | | | Immunogen‡ | PFC/ 10^6 to HGG sources§ | | Percent suppression | |
|-------|---------------------------|------------------|------------------|---------------------|-----------------------------|---------------|---------------------|------|
| | Tolerant | | Normal | | (US) | (RC) | (US) | (RC) |
| | DHGG (US) | DHGG (RC) | | | | | | |
| 1 | 70×10^6 | — | — | Δ -AHGG (US) | 18 ± 13 | 11 ± 8 | — | — |
| 2 | 70×10^6 | — | 70×10^6 | Δ -AHGG (US) | 172 ± 46 | 163 ± 50 | 8 | 0 |
| 3 | — | — | 70×10^6 | Δ -AHGG (US) | 186 ± 46 | 161 ± 42 | — | — |
| 4 | — | 70×10^6 | — | Δ -AHGG (RC) | <1 | <1 | — | — |
| 5 | — | 70×10^6 | 70×10^6 | Δ -AHGG (RC) | 201 ± 73 | 173 ± 59 | 59 | 58 |
| 6 | — | — | 70×10^6 | Δ -AHGG (RC) | 495 ± 78 | 416 ± 76 | — | — |
| 1 | 70×10^6 | — | — | Δ -AHGG (US) | 19 ± 5 | 15 ± 4 | — | — |
| 2 | 70×10^6 | — | 70×10^6 | Δ -AHGG (US) | 587 ± 99 | 523 ± 94 | 28 | 22 |
| 3 | — | — | 70×10^6 | Δ -AHGG (US) | 810 ± 128 | 669 ± 117 | — | — |
| 4 | — | 70×10^6 | — | Δ -AHGG (US) | 1 ± 1 | <1 | — | — |
| 5 | — | 70×10^6 | 70×10^6 | Δ -AHGG (US) | 166 ± 23 | 135 ± 14 | 80 | 80 |

* See Table I, footnote *.

‡ Reconstituted mice received $400 \mu\text{g}$ of AHGG on the day of cell transfer and again 10 days later. The mean \pm SE of the number of indirect PFC from 5-8 mice per group plaqued 5 days after secondary challenge is presented.

§ Recipients were plaqued separately to HGG from either source.

|| % Suppression against either HGG source was determined.

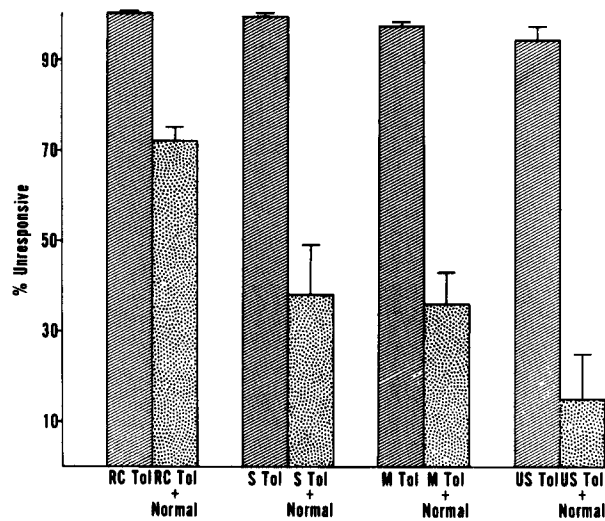


FIG. 2. Suppression and tolerance induced with DHGG from commercial Cohn fraction II. The percent of unresponsiveness or suppression was calculated for individual recipients of tolerant (Tol) spleen cells alone or tolerant and normal spleen cells (see Fig. 1). Donors were tolerized with DHGG purified from each of four sources (RC, S, M, or US). Recipients were separately challenged with and plaqued to the HGG source under investigation (S, M, or US) or the conventional source (RC). The mean \pm SE of two experiments with each source is presented.

plasma to induce suppression (Fig. 2). Although all preparations were capable of stimulating suppressor cells, the level of suppression generated by each preparation varied greatly. Nevertheless, DHGG purified from all sources established a solid unresponsive state which was stable upon adoptive transfer 10 days after tolerization.

Duration of Tolerance Established by Various HGG Preparations. The data in Table III and Fig. 2 suggest that the commercially acquired HGG preparations which were Cohn-fractionated from outdated, pooled human plasma were capable of stimulating suppressor cells to HGG, albeit with varying efficiencies. On the other hand, HGG purified from either the plasma of a single healthy donor (Doy) or a myeloma patient (Fit) was incapable of generating detectable suppressor cell activity. If suppressor T cells are responsible for the induction and maintenance of immunologic unresponsiveness, then the duration of unresponsiveness in the T and B lymphocytes in tolerized mice would be expected to reflect the level of suppressor cell activity in those mice. Therefore, mice were injected with 2.5 mg of DHGG from these various sources and assessed for the degree of immunocompetence of either the whole spleen or the splenic B cells at various times after treatment.

To assess the responsiveness of the total spleen cells, mice were injected twice, 10 days apart, and plaqued 4 days after the secondary challenge. As illustrated in Table IV, the conventional DHGG preparation (RC) induced a solid unresponsive state lasting at least 20 wk and reflecting a lack of responsive T cells as previously documented (22, 32). Similarly, DHGG from one of the commercial sources (US) and from a healthy donor (Doy) established unresponsive states in treated mice of up to 12 and 19 wk, respectively. However, mice treated with DHGG from the two remaining commercial sources (M and S) possessed responsive spleen cells as early as 3 wk after treatment. It can be assumed that the responsive spleen cells in the latter

TABLE IV
Duration of Tolerance after Injection of Various DHGG Preparations

| Days post tolerization | Source of tolerogen* | Antigenic challenge‡ | PFC/10 ⁶ to HGG sources§ | | | |
|------------------------|----------------------|----------------------|-------------------------------------|-----------|-----------|-----------|
| | | | (RC) | (M) | (S) | (US) |
| — | — | Δ-AHGG (RC) | 743 ± 350 | 863 ± 430 | 786 ± 409 | 658 ± 312 |
| 72 | (RC) | Δ-AHGG (RC) | 1 ± 1 | 2 ± 1 | 2 ± 1 | 2 ± 1 |
| 147 | (RC) | Δ-AHGG (RC) | 8 ± 3 | | | |
| 63 | (Doy) | Δ-AHGG (RC) | 4 ± 2 | | | |
| 137 | (Doy) | Δ-AHGG (RC) | 15 ± 9 | | | |
| 21 | (M) | Δ-AHGG (M) | 126 ± 86 | 55 ± 39 | | |
| 21 | (S) | Δ-AHGG (S) | 82 ± 64 | | 57 ± 42 | |
| 60 | (S) | Δ-AHGG (S) | 201 ± 44 | | 210 ± 51 | |
| 27 | (US) | Δ-AHGG (US) | 16 ± 5 | | | 11 ± 7 |
| 88 | (US) | Δ-AHGG (US) | 11 ± 2 | | | 6 ± 2 |

* 2.5 mg of DHGG was injected i.p.

‡ 400 μg of AHGG was injected i.v. at various times after the injection of DHGG as indicated. The mice were given a secondary challenge of 400 μg of AHGG i.p. 10 days later, and individual spleens were plaqued 4 days after the secondary challenge.

§ The mean ± SE of the number of indirect PFC to HGG from 4–6 mice plaqued separately to HGG from each source.

two groups reflect the presence of responsive T, as well as B, lymphocytes since the response to HGG is T-dependent.

To assess the responsiveness of splenic B cells in tolerized mice, advantage was made of the observation that the injection of LPS with antigen into HGG-tolerant mice stimulates any HGG-responsive splenic B cells present into PFC, even in the presence of unresponsive T lymphocytes (33). Using this protocol, it has been demonstrated that HGG-responsive splenic B cells reappear as early as 45 days after the induction of unresponsiveness established by DHGG (RC) and slowly repopulate the spleen (34, 35). Therefore, mice treated with DHGG from various sources were challenged with 400 μg of AHGG followed 3 h later with 50 μg of LPS and plaqued 6 days after this challenge.

Table V summarizes the results of experiments investigating: (a) the duration of unresponsiveness in splenic B cells as assessed by challenge with antigen and LPS; (b) the duration of unresponsiveness in splenic T cells as assessed by challenge with antigen alone (Table IV); and (c) the level of suppression generated by various DHGG preparations (Table III and Fig. 2). Of the three strongly tolerogenic HGG preparations (RC, Doy, and US), the capacity to generate suppressor cells encompassed both extremes. RC stimulated the strongest suppressor cell activity, US stimulated the weakest, and Doy stimulated no detectable suppression. On the other hand, of the three most strongly suppressive HGG preparations (RC, S, and M), RC induced a solid unresponsive state in both the T and B lymphocytes, whereas neither S nor M maintained unresponsiveness far beyond that demonstrated by adoptive transfer at 10 days post tolerization (Fig. 2). Thus, the degree of suppression generated by the various HGG preparations does not correlate with the ability of these preparations to induce and maintain unresponsiveness in either splenic T or B lymphocytes. These data suggest that suppressor T cells are not responsible for the establishment of tolerance to HGG.

TABLE V
Lack of Correlation between Establishment of Tolerance and Generation of Suppressor Cells to HGG

| Source of tolerogen* | Percent suppression‡ | Earliest response in: | |
|----------------------|----------------------|----------------------------|---------|
| | | B cells§ | T cells |
| | | <i>days post tolerogen</i> | |
| RC | 53-91 | 59 | >147 |
| S | 33-54 | 21 | 21 |
| M | 31-47 | 21 | 21 |
| US | 0-28 | 27 | >88 |
| Doy | 0 | 56 | >137 |

* See Table IV, footnote *.

‡ Range of percent suppression from nine experiments calculated as described in Materials and Methods.

§ 400 µg of AHGG was injected i.v. at various times after the injection of DHGG as indicated. 50 µg of LPS was injected i.v. 3 h after antigenic challenge, and individual spleens were plaqued 6 days later.

|| See Table IV, footnote ‡.

Discussion

The generation of suppressor cell activity in the spleens of HGG-tolerant mice is variable and dependent upon the source of the HGG utilized. The unresponsive state established by DHGG purified from each of the sources assessed was stable upon adoptive transfer into irradiated recipients. All four of the samples of Cohn-fractionated, pooled human plasma obtained commercially stimulated suppressor cells as assayed by the transfer of tolerant spleen cells and normal spleen cells into irradiated recipients. This suppression ranged from 0 to 54% with three of the commercial preparations (M, S, and US), whereas the preparation (RC) most widely used in previously published studies of HGG-specific suppressor cells (7-9, 14, 15, 20, 31) induced 53-91% suppression. On the other hand, samples prepared from individual donors, a myeloma patient and a healthy donor, did not generate demonstrable suppressor cell activity to HGG. These findings suggest that suppressor cells are not required for the induction or maintenance of tolerance to HGG, since tolerance was induced by each of the preparations. Conversely, the generation of suppressor cells does not appear to be a mandatory result of the establishment of an unresponsive state to HGG in adult mice. The concept that suppressor T cells are only incidental to the tolerant state is supported by previous observations in mice devoid of thymus-dependent lymphocytes. Congenitally athymic, nude mice can be rendered unresponsive to HGG by the injection of either aggregated HGG (36) or deaggregated HGG (28), as can adult thymectomized, irradiated, and bone marrow-reconstituted mice (23).

Additionally, evidence of the establishment of tolerance to HGG in the absence of suppressor T cells in adult mice bearing competent T lymphocytes has been provided using adoptive transfer (22, 23), parabiosis (21), and low-dose tolerization (20). The conditions under which HGG-specific suppressor cells can be demonstrated in the spleens of tolerant mice appear to be restricted. Suppressor cells are only transiently associated with the tolerant state to HGG (6, 8, 18), and they cannot be reinduced

with a second injection of tolerogen once they have been exhausted (20), despite the persistence to T-cell tolerance. Finally, suppressor cells cannot be detected in neonates tolerized to HGG through the colostral transmission of tolerogen (19). Although suppressor T cells may regulate immune responsiveness, an obligate role for these cells in the establishment of tolerance to HGG appears doubtful in light of these numerous examples of tolerance without suppression. The converse situation in which suppressor cells exist without tolerance is documented by the presence of suppressor cells in HGG-primed mice (18). In parallel to the data presented here, recent observations (37) suggest that suppressor cells are coincidental to, but not obligate for, tolerance to another soluble protein antigen human serum albumin in which suppressor T cells have been well documented (38).

The mechanism by which DHGG purified from certain preparations of HGG elicits suppressor cells whereas tolerogen from other preparations does not is unclear. The suppressive activity of the commercially acquired preparations may reflect their origin as outdated, pooled plasma or their handling, storage, or method of purification. Although the size of the various human plasma pools used in the commercial fractionation is unknown, their heterogeneity may account for the ability of these preparations to generate suppression. Variability in pool size and heterogeneity may be responsible for the varying efficiencies of suppressor cell induction among commercial sources. On the other hand, the observation that ethanol precipitation and lyophilization of a nonsuppressive preparation did not generate a suppressive preparation does not exclude the possibility that the HGG is altered during the more extensive commercial fractionation. A particular subclass of Ig or a denatured product of HGG present in small and varied quantity in the commercial preparations may be responsible for the varying levels of suppressor cell activity generated by these preparations. This concept would be consistent with the observation that suppressor cells are not stimulated in mice tolerized with a low dose of commercial HGG (18). There may be too little suppressive reagent at this dose of HGG to generate suppression. Further support for this concept comes from observations that fragments of β -galactosidase will activate T cells capable of suppressing the immune response to the whole protein (39). Experiments designed to investigate the active component(s) in the suppressive HGG preparations are in progress.

The level of suppressor cells to HGG detected in the present studies appears to be unrelated to: (a) the establishment of tolerance as assessed by the adoptive transfer of tolerized spleen cells into irradiated recipients; (b) the duration of tolerance in the B cells of these tolerized mice; or (c) the duration of tolerance in the T cells as reflected by the response of the total spleen at a time when splenic B cells are responsive. If the maintenance of tolerance were due to suppressor cells, then the level of unresponsiveness would be expected to parallel the degree and duration of suppression. This was not the case, underscoring the observation that no causal relationship between suppressor cells and tolerance could be detected to this soluble protein antigen HGG. Furthermore, the lack of suppressor cells in neonatally-induced tolerance to HGG (19) and the inability to induce new suppressors in unresponsive mice after the disappearance of the initial, transient suppressor cells (20) speak against a role for suppressor T cells in states of unresponsiveness to many self-antigens. The antigens contained in this category would be those that are present at birth and remain in contact with the immune system in tolerogenic concentrations throughout the life of the host.

Along with other models of tolerance devoid of or independent from suppressor cells, the model presented here suggests that the maintenance of tolerance to HGG in the B lymphocytes may be accomplished through an intrinsic unresponsiveness of these cells. The mechanisms responsible for the establishment and maintenance of unresponsiveness in B cells have been divided into two broad categories (40). During extrinsic (or peripheral) inhibition, competent B cells are present but inhibited by mechanisms that are independent of the B cells and that require perpetuation. These mechanisms may involve antibody, antigen-antibody complexes, suppressor cells, and soluble mediators. On the other hand, the mechanisms responsible for intrinsic (or central) unresponsiveness directly affect the B cells, resulting in the irreversible loss of competent B lymphocytes, and they do not require further external influences for their maintenance. The direct effect of antigen to clonally delete or abort B cells is possibly the clearest example of an intrinsic mechanism.

The concept of receptor blockade, advanced by Möller et al. (41), suggested that tolerant B cells are not irreversibly inactivated by antigen but rather are reversibly inhibited by the blockade of signal recognition at the antigen receptors on the cells. More recently, Fernandez and Möller (42) have reported that cells bearing both antigen receptors and receptors for a polyclonal B-cell activator are irreversibly inactivated when exposed to tolerogenic levels of both moieties at once. An extrapolation of this model to T-dependent antigens would postulate that cells bearing receptors for both antigen and T-cell help (analogous to polyclonal B-cell activation) could also be irreversibly inactivated. Such a model of inactivation would vary from other models of intrinsic unresponsiveness involving clonal deletion or abortion only in that helper T cells would be required to accomplish irreversible B-cell unresponsiveness.

If neither reversible receptor blockade nor T-cell suppression are fundamental in unresponsiveness to a T-dependent soluble protein antigen like HGG, then the tolerant state established to HGG may be a model for intrinsic unresponsiveness in which B lymphocytes are irreversibly inactivated. The only other extrinsic mechanism of note bearing on this unresponsive state is the network theory of anti-idiotypes (43), which has not been extensively investigated with soluble protein antigens to date.

Summary

A model of unresponsiveness to human γ -globulin (HGG) which is maintained in the absence of demonstrable suppressor cells has been described. A/J mice were tolerized with deaggregated HGG purified from a variety of sources. The spleen cells from these tolerized mice were assessed for their ability to suppress the response of normal spleen cells to HGG when transferred into lethally irradiated mice. All of the HGG preparations obtained from commercial sources as Cohn fraction II of pooled, outdated plasma induced suppressor cells to HGG, although not of equal magnitude. However, suppressor cells could not be demonstrated in the spleens of mice tolerized with deaggregated HGG purified from the plasma of a healthy individual. This inability to detect suppression was independent of the method of purification of the HGG and of the time of assessment of the putative suppressor cells after tolerization. Similarly, deaggregated HGG isolated from an IgG₁ λ -myeloma protein induced unresponsiveness to HGG but did not stimulate demonstrable suppressor cells. These data suggest that suppressor T cells are not involved in the maintenance of tolerance to this antigen, although they may play a regulatory role in the immune response to

HGG. Support for this concept was obtained by assessing the duration of unresponsiveness in the T and B lymphocytes of mice tolerized with the various HGG preparations. Mice tolerized with the HGG preparations that stimulated little or no suppression were among the last to recover responsiveness. Indeed, there was no consistent correlation between the level of suppressor cell activity and the degree of unresponsiveness in either the splenic T or B lymphocytes. Thus, although certain HGG preparations may provide a tool for the generation of antigen-specific suppressor T cells, the utilization of these suppressive preparations may be inappropriate for the investigation of the mechanisms of the induction and maintenance of the unresponsive state.

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