DEREGULATION OF IDIOTYPE EXPRESSION

Induction of Tolerance in an Anti-Idiotypic Response*

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Regulation of immune responsiveness has been attributed theoretically (1, 2) and experimentally (3, 4) to interactions between idiotypic and anti-idiotypic structures. Constituents of this idiotype network can be induced and triggered without the intervention of external antigens (5–10). For instance, anti-idiotypic antibodies can mimic specific antigen to promote the induction (9, 10) or suppression (5–7) of synthesis of the idiotypic antibody in the absence of antigen. In general, low doses of anti-idiotypic antibody directly induce idiotype or facilitate an enhancement of idiotype expression during subsequent antigenic stimulation. Higher doses of anti-idiotypic antibody promote suppression of idiotype expression (3, 11).

In the antigen-specific response to the *p*-azobenzenearsonate (ABA)[1] hapten group, a large proportion of the anti-ABA antibodies induced in A/J mice share a cross-reactive idiotype (CRI) (12) representing a nonidentical, but closely related, family of idiotypic determinants (13–16). Immunoregulation of this idiotype has been demonstrated by two approaches. First, anti-idiotypic antibodies have been passively administered, resulting in the suppression of the level of CRI^+ antibodies (6, 17, 18) and the induction of idiotype-specific suppressor T cells (T_s) (17–19). Second, CRI^+ serum antibodies (20, 21) or CRI^+ hybridoma proteins (HP) (22) covalently coupled to syngeneic lymphocytes have been used to induce idiotype-specific T_s and depress both the production of CRI^+ antibodies (20, 22) and delayed-type hypersensitivity to antigenic challenge (21). With both approaches, the production of total circulating anti-ABA antibodies is not substantially altered (6, 17, 18, 20, 22). Most attempts to investigate the regulation of idiotype expression have involved the creation of an artificial network by the passive administration of anti-idiotypic antibody or of the idiotype in adjuvant. However, in a few cases idiotypic modulation has been demonstrated during the development of an antibody response to specific antigen (8, 11, 23, 24).

In this paper, an attempt was made to investigate the regulation of idiotype by an

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[1] Abbreviations used in this report: ABA, *p*-azobenzenearsonate; CRI, cross-reactive idiotype; T_s, suppressor T cells; HP, hybridoma protein; R16.7 HP, immunoglobulin from the anti-ABA hybridoma cell line R16.7; BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin; CFA, complete Freund's adjuvant; RIA, radioimmunoassay.

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existing idiotype-anti-idiotype equilibrium through the induction of tolerance in the anti-idiotypic antibody response. Unresponsiveness was induced in anti-CRI^+ antibody-producing cells using the HP from the anti-ABA monoclonal hybridoma R16.7, a homogeneous representative of the family of CRI^+ antibodies possessing one or more idiotypes common to all CRI^+ serum antibodies (14, 25). Tolerance induction was accomplished in a manner previously demonstrated to establish a functional deletion in T and B cells specific for immunoglobulin antigen (26, 27). This type of unresponsiveness has been demonstrated to be independent of antigen-specific suppressor cells (28, 29). Mice tolerized to this idiotype produce little circulating anti-idiotypic antibody when challenged with either the antigen (ABA) or the idiotype (R16.7 HP). Unresponsiveness persists when tolerant spleen cells are transferred into irradiated recipients. Homeostatic regulation of idiotype expression is relaxed in unresponsive mice not producing anti-idiotypic antibody, although the level of total anti-ABA antibody is uneffected.

Materials and Methods

Mice. Male A/J and CAF_1 mice were obtained from The Jackson Laboratory, Bar Harbor, ME, at 4–5 wk of age. They were maintained ad lib on Wayne Lab Blox F6 (Allied Mills, Inc., Chicago, IL) in chlorinated water acidified to pH 3.0.

Monoclonal Idiotype$^+$ Anti-ABA Antibodies. The hybridoma cell line R16.7 secreting an IgG_1 immunoglobulin reactive to the ABA group was produced and characterized as described by Lamoyi et al. (14) and obtained through the generosity of Dr. Alfred Nisonoff, Brandeis University, Waltham, MA. Cells were maintained in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) supplemented with 20% fetal bovine serum (Microbiological Associates, Walkersville, MD), gentamycin (50 μg/ml) (Schering Corp., Kenilworth, NJ), glutamine, nonessential amino acids, and sodium pyruvate. Ascites fluid containing large amounts of antibody was produced by injecting $1–5 \times 10^6$ cells into Pristane-primed CAF_1 mice (30). The monoclonal R16.7 anti-ABA HP was purified by affinity chromatography on Sepharose 4B (Pharmacia Fine Chemicals, Pharmacia Inc., Piscataway, NJ) to which ABA-bovine serum albumin (BSA) had been conjugated as described elsewhere (14).

Antigens and Immunizations. ABA was conjugated to BSA or keyhole limpet hemocyanin (KLH) by procedures previously described by Nisonoff (31). To elicit primary responses to ABA, A/J mice were immunized intraperitoneally with 100 μg ABA-KLH in complete Freund's adjuvant (CFA). For secondary responses, mice received 400 μg ABA-KLH in alum intraperitoneally. Immunization of mice with R16.7 was accomplished by intraperitoneal injection of 400 μg R16.7 HP incorporated in CFA. The injected volume in all cases was 0.2 ml.

Anti-Idiotypic Antisera: Preparation and Assay. Anti-idiotypic antisera were prepared in rabbits by injecting specifically purified R16.7 HP in CFA. The antisera were adsorbed by passage twice over columns of Sepharose 4B to which a crude globulin fraction of normal A/J mouse serum had been conjugated. All precipitating activity against normal mouse immunoglobulin was lost after a single passage. Anti-R16.7 idiotype antibodies were further affinity-purified by elution from a R16.7 HP-coupled Sepharose 4B column. Anti-idiotypic activity was determined in the presence of an excess of A/J immunoglobulin, i.e., 20–25 μl of normal A/J serum. Binding tests were performed as described by Lamoyi et al. (14) with 10 ng of ^{125}I-labeled R16.7 HP as ligand and sufficient anti-idiotypic antibody to bind 50–70% of the ligand. Normal rabbit serum was added as a carrier and immune complexes were precipitated with a slight excess of goat anti-rabbit Fc (N. L. Cappel Laboratories Inc., Cochranville, PA). All proteins were radiolabeled by modification of the chlorimine-T method of protein iodination (32).

Anti-ABA Determination. Anti-ABA antibody titers were measured by solid phase radioimmunoassay (RIA) using ABA-BSA-coated polyvinyl microtiter plates (33). Residual protein-binding capacity of the plates was saturated with BSA. Reagents used in all RIA were diluted in PBS containing 1% BSA and 0.05% Tween-20. Plates were washed and 100 μl of ^{125}I-labeled,

purified goat anti-mouse Fab (N. L. Cappel Laboratories Inc.) was added to each well before overnight incubation, washing, and counting.

Idiotype Determination. Anti-ABA antibodies cross-reactive with the R16.7 idiotype used as a prototype of CRI were also quantitated with ABA-BSA-coated plates. After addition of 50-μl aliquots of experimental serum dilutions and subsequent washing, 100 μl of rabbit anti-R16.7 was added and incubated for 4 h at room temperature. After washing the plates, 100 μl of ^{125}I-goat anti-rabbit Ig was added to each well and incubated overnight. The plates were washed before counting individual wells.

Anti-Idiotype Determination. Anti-idiotypic antibody was quantitated by coating polyvinyl microtiter plates with F(ab')$_2$ R16.7 HP. Idiotype-coated plates were also blocked with BSA before addition of 50-μl aliquots of antisera to individual wells and incubation for 4 h at room temperature. After washing, 100 μl of ^{125}I-goat anti-mouse Fc antibody (N. L. Cappel Laboratories Inc.) was added to each well, incubated overnight, washed, and counted.

F(ab')$_2$ R16.7 HP Preparation. To obtain a F(ab')$_2$ fragment from the R16.7 idiotype$^+$ immunoglobulin, the latter was treated with pepsin as described by Spiegelberg (34). The digested material was purified by passage through a Sepharose 4B column to which goat anti-mouse Fc IgG had been conjugated. This column eliminated Fc fragments as well as undigested immunoglobulin. The F(ab')$_2$ fragment effluent was bound to ABA-BSA coated plates as described above.

Tolerance Induction to the R16.7 Idiotype. Tolerance to the R16.7 idiotype was attempted by intraperitoneal injection of the affinity-purified HP from R16.7 cells. When desired, the R16.7 HP was depleted of aggregates by ultracentrifugation at 150,000 *g* for 2.5 h (28). Treated mice were subsequently injected with either ABA-KLH in CFA and alum or R16.7 HP in CFA and bled at various times as indicated.

Adoptive Cell Transfer. For adoptive transfer experiments, a cell suspension from the spleens of tolerant or control mice was obtained (35). Lethally irradiated (900 rad) mice were injected with 7×10^7 spleen cells i.v. Reconstituted mice were immediately immunized with the appropriate antigen and bled at various times thereafter.

Half-Life Determinations of R16.7 HP. Quantitation of the half-life of R16.7 HP in A/J mice was performed by two methods. 200 μg of ^{125}I-labeled R16.7 HP (sp act 3 μCi/μg) were injected intraperitoneally, and the amounts of gamma radiation in both the blood and the whole body were measured at different time intervals. The half-life of injected HP was also determined in serum at various times after inoculation with 1 mg R16.7 HP by the solid phase RIA described above.

Results

In Vivo Catabolism of R16.7 Idiotype. Two experimental protocols were used to determine the rate of disappearance of the R16.7 HP from A/J mice after injection. First, the ^{125}I activity retained in both the whole body and the blood of mice injected with 200 μg of ^{125}I-labeled R16.7 HP was followed for 9 d. The results of whole body counts shown in panel A of Fig. 1 demonstrate that 50% of the HP was catabolized every 7.7 d. The persistance of TCA-percipatable counts in the blood of these animals, panel B of Fig. 1, indicated a very similar rate of elimination. The difference between the amount of radiolabeled R16.7 HP per ml of blood and the amount in the whole body suggests that, when administered passively, this immunoglobulin is distributed in equivalent proportions in intra- and extravascular fluid spaces. Similar rates and proportions have previously been reported for undenatured immunoglobulins in rabbits and mice (36, 37). These data indicate that this monoclonal murine immunoglobulin is catabolized at a normal rate and that all lymphoid cells are potentially exposed to the R16.7 idiotype.

Second, RIA determinations of R16.7 idiotype in the serum of mice receiving 1 mg of unlabeled R16.7 HP were also performed. Fig. 2 illustrates one such experiment. 7 d after injection, serum analysis indicated a mean of 106.3 μg/ml of R16.7 idiotype$^+$

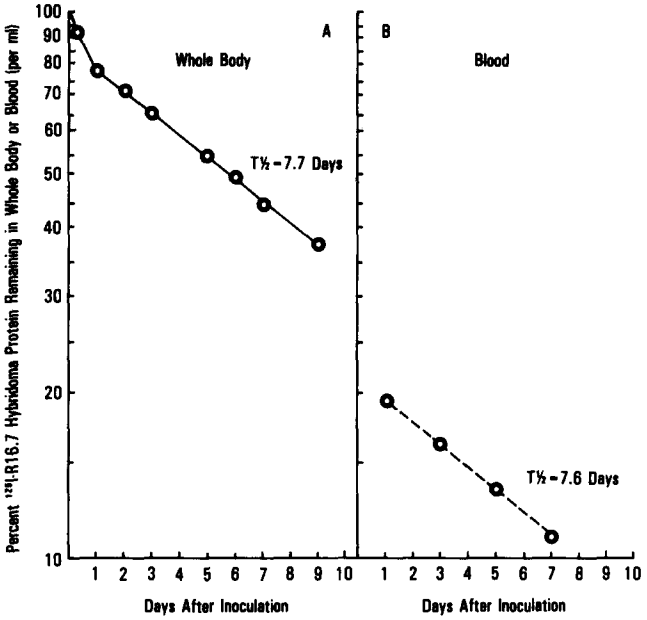

FIG. 1. In vivo catabolism of ^{125}I-R16.7 HP in A/J mice. The rate of disappearance of 200 μg of soluble ^{125}I-labeled R16.7 HP injected intraperitoneally was monitored by quantitating the ^{125}I activity in the whole body (panel A) and in the blood (panel B). The mean of five mice per group is presented.

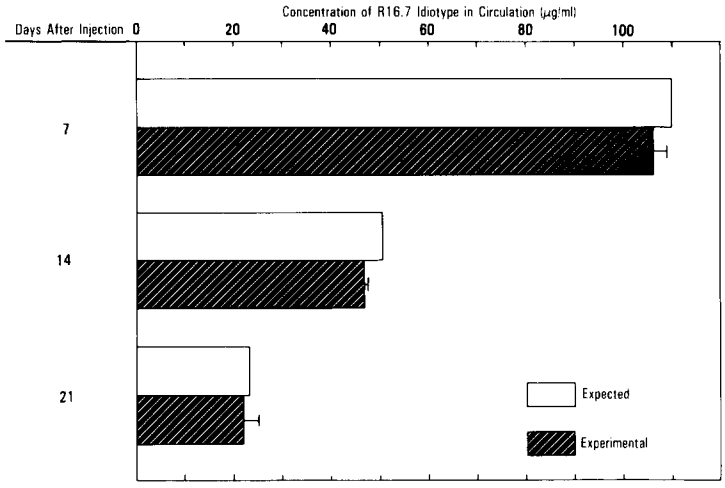

FIG. 2. Detection of passively administered R16.7 idiotype in serum by RIA. The concentration of circulating R16.7 idiotype was determined as described in Materials and Methods after the injection of 1.0 mg soluble R16.7 HP i.p. The mean plus 1 SE of three or four mice per group are presented. Theoretical, expected values were obtained from the rate of ^{125}I disappearance found in Fig. 1.

immunoglobulin. The amount of R16.7 idiotype expected from Fig. 1 is 110 μg/ml. After 14 and 21 d, the sera contained 46.8 and 21.0 μg/ml of R16.7 idiotype by RIA, whereas the theoretical values obtained from the rate of ^{125}I disappearance in Fig. 1

are 50.7 and 23.4 μg/ml, respectively. The experiments described here correlate with a half-life of 7.6 d for the R16.7 HP in serum and confirm that the RIA for R16.7 idiotype accurately quantitates this idiotype in circulation.

Induction of Immunologic Unresponsiveness to the R16.7 Idiotype. Experiments were performed to determine the ability to induce unresponsiveness in cells producing anti-idiotypic antibody by inoculating A/J mice with R16.7 idiotype. For this purpose, animals were injected intraperitoneally with increasing doses of R16.7 HP ranging from 0.1 to 2.0 mg. The affinity-purified, monoclonal immunoglobulin was deaggregated immediately before injection. 18 d after treatment, the mice were challenged with 400 μg of R16.7 HP incorporated in CFA. Fig. 3 illustrates the degree of responsiveness to the idiotype as monitored by anti-R16.7 idiotype antibody in circulation using the RIA described in Materials and Methods. In contrast to untreated mice (group 1) mice inoculated previously with deaggregated R16.7 HP (groups 2–5) produced no detectable anti-idiotypic responses 7 d after challenge with the idiotype in CFA. 14 d after challenge, only marginal responses were observed in tolerized mice. No differences could be detected in the hyporesponsiveness of animals given 500 μg or more of deaggregated R16.7 HP, with a maximum mean response of 15% of control values. These data demonstrate that it is possible to substantially restrict responsiveness to the R16.7 idiotype by prior injection of a tolerogenic dose of monoclonal immunoglobulin bearing the idiotype.

Duration of Unresponsiveness to the R16.7 Idiotype. Studies were undertaken to determine the duration of the unresponsive state induced to the R16.7 idiotype. For this purpose, mice were rendered unresponsive to the idiotype with a single, tolerogenic injection of 1.0 mg soluble R16.7 HP and were challenged at different intervals with immunogenic R16.7 HP in CFA. As illustrated in Fig. 4, tolerized mice challenged after 18 d (group 2) responded very poorly, if at all, either 7 or 14 d after antigenic

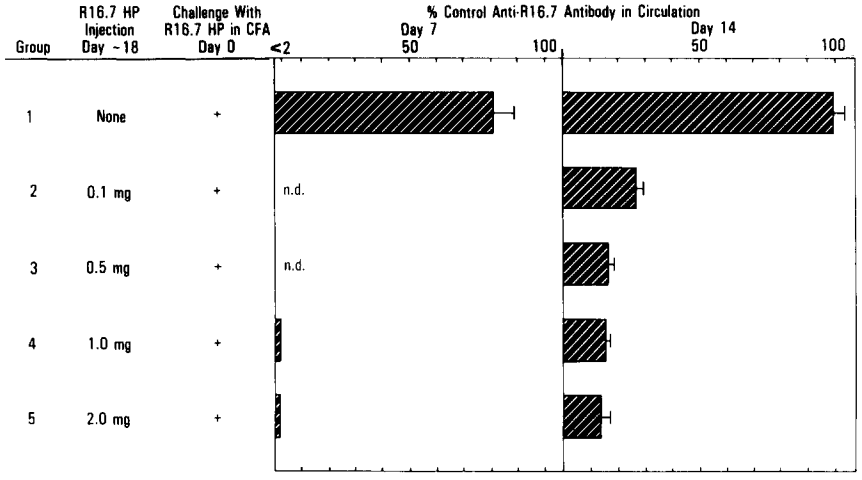

FIG. 3. Induction of immunologic unresponsiveness in the anti-idiotypic response with varying doses of R16.7 idiotype. 18 d after attempted tolerization with 0.1–2.0 mg deaggregated R16.7 HP i.p. mice were challenged with 400 μg R16.7 HP in CFA i.p. (groups 2–5). The concentration of circulating antibody to the R16.7 idiotype was determined 7 and 14 d after antigenic challenge as described in Materials and Methods. All results are compared with the control response of untreated mice 14 d after challenge (group 1). The mean plus 1 SE of four to six mice per group are presented. n.d., not done.

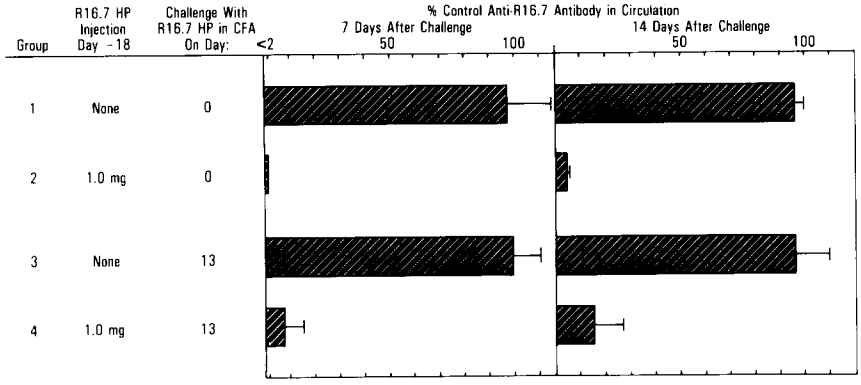

FIG. 4. Persistence of unresponsiveness to the R16.7 idiotype. The concentration of circulating antibody to the R16.7 idiotype was assessed by challenge with 400 μg of R16.7 HP in CFA i.p. 18 or 31 d after the induction of unresponsiveness with a single intraperitoneal injection of 1.0 mg soluble R16.7 HP. 7 and 14 d after antigenic challenge, determinations of the anti-idiotypic antibody were made. The results of tolerized mice (groups 2 and 4) are compared with the control responses of untreated mice. The mean plus 1 SE of three to five mice per group are presented.

challenge. In comparison, mice challenged 31 d after the induction of unresponsiveness (group 4) did mount a slight anti-idiotypic response. However, the mean response observed in these tolerized mice (group 4) was only 16% of untreated controls (group 3) even when assayed 45 d after tolerization. The responses of all tolerized groups are significantly different ($P < 0.001$) from their respective control groups. Thus, it appears that although the unresponsive state induced to this idiotype diminishes with time, profound unresponsiveness persists for more than a month after tolerization.

Primary and Secondary Anti-ABA Responses in Idiotype Unresponsive Mice. Anti-ABA responses were determined in A/J mice unresponsive to this CRI^+ R16.7 idiotype. Primary antibody responses to ABA were quantitated in sera of tolerized mice 14 d after immunization with 100 μg ABA-KLH in CFA. Similarly, secondary antibody responses to the ABA group were measured in the sera obtained from the same mice 4 d after a secondary injection of 400 μg ABA-KLH in alum. Fig. 5 represents the data obtained after both primary and secondary immunizations. The proportion of the total antibody produced to ABA determinants reactive with the rabbit anti-R16.7 idiotype antibodies was 64% and 59%, respectively, after primary or secondary immunization of normal mice with ABA-KLH. These results are in agreement with previously published percentages of CRI^+ anti-ABA antibodies in A/J mice (12, 38).

In contrast, the anti-ABA antibodies in circulation of tolerized mice are almost exclusively R16.7 $idiotype^+$ (96%) after a single immunization with ABA-KLH in CFA. After a second immunization with ABA-KLH, tolerized mice start to produce $idiotype^-$ anti-ABA antibodies, but the percent of $idiotype^+$ antibodies remains significantly higher (79% in group 2) than in normal, control mice (59% in group 1) $P < 0.01$. The amount of total anti-ABA antibody was the same in normal and tolerized mice after primary challenge with ABA-KLH and slightly increased in normal mice after a secondary antigenic challenge. These data indicate that regulation of anti-ABA antibodies possessing the R16.7 idiotype is relaxed in mice unresponsive to this idiotype. However, the regulation of total antibody appears unaffected.

Adoptive Transfer of Unresponsiveness to the R16.7 Idiotype. Spleen cells from mice

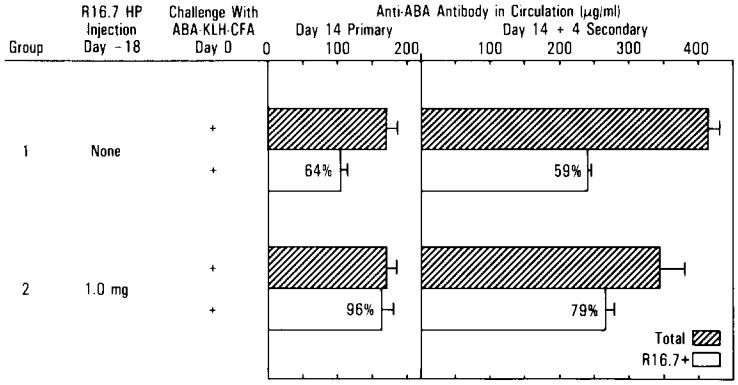

FIG. 5. Anti-ABA antibody responses in mice unresponsive to the R16.7 idiotype. The concentrations of total anti-ABA antibodies and R16.7 idiotype$^+$ anti-ABA antibodies were determined by solid-phase RIA as described in Materials and Methods. Mice were rendered unresponsive to the R16.7 idiotype by a single intraperitoneal injection of 1.0 mg soluble R16.7 HP. 18 d later, tolerized (group 2) and normal (group 1) mice received a primary injection of 100 μg ABA-KLH in CFA i.p. 14 d later, all mice were bled and their sera assayed. 4 h after bleeding, these mice were given a secondary challenge with 400 μg ABA-KLH in alum i.p., and they were bled again 4 d later. The means of the percentage of total anti-ABA antibody bearing the R16.7 idiotype in individual mice are indicated. The mean plus 1 SE of three or four mice per group are presented.

tolerized with R16.7 HP were transferred into lethally irradiated, normal recipients to remove persistent idiotype from the environment of the unresponsive cells. This procedure precluded the possible modulation of immune responsiveness or interference with serological determinations by passively administered R16.7 HP. Irradiated recipients were reconstituted by 7×10^7 viable spleen cells from normal or tolerized mice. One-half of these recipients were immunized within 1 h with 100 μg ABA-KLH in CFA, challenged 10 d later with 400 μg ABA-KLH in alum, and assayed for circulating anti-ABA antibody 5 d after secondary challenge. The data illustrated in Fig. 6 demonstrate that recipients of cells from tolerized mice (group 2A) produced slightly more anti-ABA antibody than did recipients of normal cells. However, both the amount and percent of anti-ABA antibodies that cross-reacts with the R16.7 idiotype were significantly higher ($P < 0.001$) in recipients of tolerized cells.

The other half of the reconstituted recipients were challenged with 400 μg R16.7 HP in CFA to assess the anti-idiotypic responsiveness of transferred cells. Fig. 6 illustrates that cells from mice tolerized to the R16.7 idiotype remain unresponsive to the idiotype after transfer. Therefore, unresponsiveness experimentally induced to the R16.7 idiotype is stable to adoptive transfer and cellular in nature. The observations that cells from mice unresponsive to the R16.7 idiotype respond in the reconstituted recipients as they do in the tolerized donor, both with respect to idiotype$^+$ anti-ABA and anti-idiotypic antibodies, confirm that the effects reported herein are the result of modulation of the idiotype-anti-idiotype network, not passively administered R16.7 HP.

Interference with Induction of Unresponsiveness to the Idiotype by Antigen. To determine the possible effect of antigen priming on the induction of unresponsiveness to the R16.7 idiotype, the hapten ABA was injected on the immunogenic carrier KLH before or during tolerization. 100 μg ABA-KLH was injected either at the same time as, or 2 mo before, 1.0 mg soluble R16.7 HP. Both groups plus control groups not

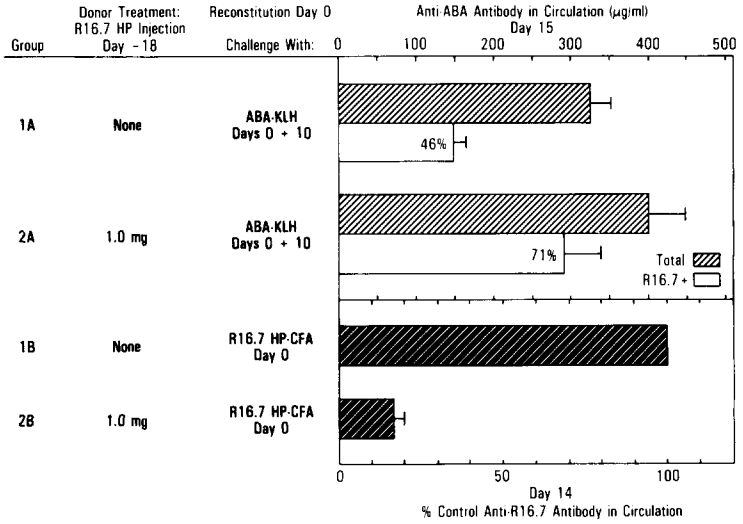

FIG. 6. Anti-ABA and anti-idiotypic antibodies of mice reconstituted with cells unresponsive to the R16.7 idiotype. The concentration of total anti-ABA, R16.7 idiotype$^+$, and anti-idiotypic antibodies were determined in lethally irradiated, syngeneic recipients reconstituted with 7×10^7 spleen cells from normal donors or donors tolerized 18 d previously with 1.0 mg soluble R16.7 HP i.p. Some recipients (groups 1A and 2A) were immunized intraperitoneally within 1 h of cell transfer with 100 μg ABA-KLH in CFA and 10 d later with 400 μg ABA-KLH in alum, and bled 15 d after reconstitution. The means of the percent of total anti-ABA antibody that is R16.7 idiotype$^+$ in individual mice is indicated. Other recipients (groups 1B and 2B) were immunized within 1 h of cell transfer with 400 μg R16.7 HP in CFA and bled 14 d later. All groups contained five mice each except group 1B, which contained three mice. The mean plus 1 SE are presented.

given ABA-KLH or the tolerogen were later challenged twice with ABA-KLH in CFA and alum. The results of one such experiment are presented in Fig. 7. Antigen priming with ABA at the time of tolerization does not interfere with the establishment of unresponsiveness to the idiotype as demonstrated by the lack of anti-idiotypic antibody in circulation (group 3). In contrast, mice primed to ABA 2 mo before attempted tolerization with R16.7 HP (group 4) had levels of anti-idiotypic antibody in their circulation indistinguishable from those of untreated control mice (group 1). Because none of the mice received R16.7 HP in the immunogenic form in CFA, the detected anti-idiotypic antibody was a consequence of immunization to the antigen ABA-KLH.

When the response to specific antigen challenge was examined, the levels of anti-ABA antibodies cross-reacting with the R16.7 idiotype were significantly higher ($P < 0.02$) in both the unresponsive groups (2 and 3) than in either of the groups (1 and 4) responsive to the idiotype. In previously primed mice in which the induction of unresponsiveness was abrogated (group 4), the percent of R16.7 idiotype$^+$ anti-ABA antibody was similar to that of normal mice, 60% and 66%, respectively. In contrast, in successfully tolerized mice not possessing detectable circulating anti-idiotypic antibodies, the anti-ABA antibodies secreted after secondary antigenic challenge are predominantly of the R16.7 idiotype, 88% and 83% for groups 2 and 3, respectively. These data indicate that unresponsiveness cannot be induced to this idiotype in mice previously primed to the specific antigen. However, prior stimulation of idiotype$^+$ anti-ABA antibody-producing cells, not the presence of antigen, appears

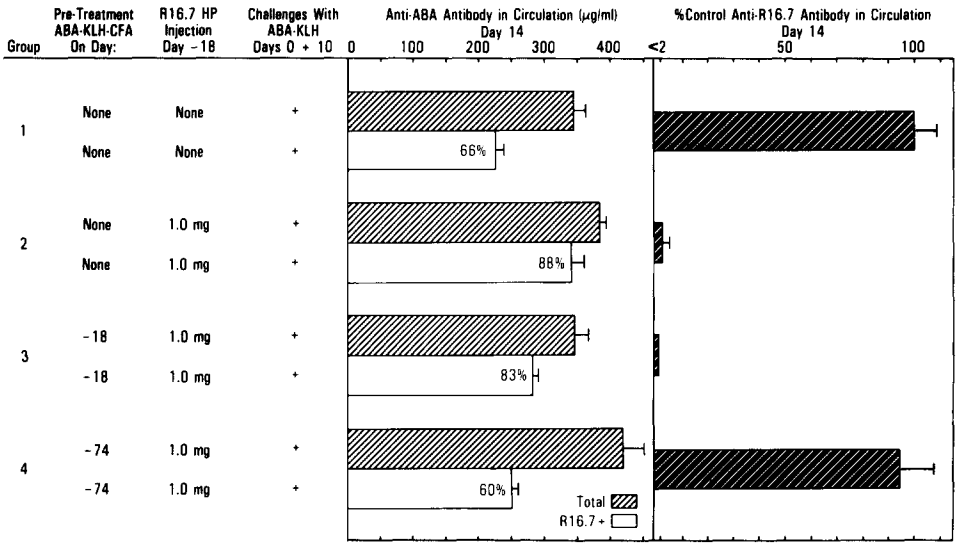

FIG. 7. Interference with the induction of unresponsiveness to idiotype by the antigen. Mice were injected with 100 μg ABA-KLH in CFA i.p. 2 mo before (group 4) or at the same time as (group 3) tolerization with 1.0 mg soluble R16.7 HP. 18 d after attempted tolerance induction, all groups were immunized with 100 μg ABA-KLH in CFA. 10 d later another injection of 400 μg ABA-KLH in alum was given. Concentrations of circulating total anti-ABA R16.7 idiotype$^+$ and anti-idiotypic antibodies were assessed 4 d after antigenic challenge in alum. The means of the percent of total anti-ABA antibody that bears the R16.7 idiotype in individual mice are indicated. The mean plus 1 SE of three to five mice per group are presented.

to be responsible for this interference.

Discussion

The studies presented herein document the feasibility of inducing tolerance in an anti-idiotypic antibody response by the administration of excess idiotype. The role of idiotype-specific cells and antibody in the regulation of total and idiotype$^+$ antibody are then assessed by virtue of the lesion created in the idiotype-anti-idiotype network following tolerization. This approach allows the examination of an existing network, not a network created or expanded by experimental manipulation.

Unresponsiveness was successfully induced in the anti-idiotypic antibody response by a single injection of monoclonal R16.7 immunoglobulin possessing conserved or public idiotypic determinants common to the closely related, but not identical, family of CRI$^+$ anti-ABA antibody molecules (14, 25). Tolerized mice were severely compromised in their ability to secrete circulating anti-idiotypic antibodies upon challenge with the R16.7 idiotype incorporated in CFA. This unresponsive state was stable upon adoptive cell transfer into irradiated syngeneic recipients, suggesting that unresponsiveness is not the consequence of passive inhibition by excess idiotype. Functional deletion in idiotype-specific B lymphocytes may have been accomplished as previously demonstrated during tolerization to other immunoglobulin antigens (26, 27, 39). Although the induction of tolerance to immunoglobulin antigens can be accomplished in the absence of antigen-specific suppressor cells (28, 29, 40, 41), the presence and role of suppressor cells specific for the anti-idiotypic antibody-producing cells remains unresolved in this work. Both idiotype (18) and anti-idiotype (17, 18, 20)

-bearing T_s have been described after the injection of anti-CRI$^+$ antibodies (17, 18, 19) or CRI$^+$ antibody coupled to syngeneic lymphocytes (20, 21). In the present work, anti-idiotypic T_s do not appear to predominate in tolerized animals because CRI$^+$ antibodies are elevated, not suppressed. However, the potential presence and contribution of anti-idiotype-specific T_s remains to be documented in this unresponsive state.

Tolerization of anti-idiotypic antibody-producing cells and the resulting reduction of circulating anti-R16.7 idiotype antibodies have profound effects on the regulation of idiotype expression. Deregulation of the idiotype produced upon challenge with the antigen ABA-KLH is observed in mice tolerized to the idiotype. Both the total amount and the percent of R16.7 idiotype$^+$ anti-ABA antibodies are increased during primary and secondary responses in tolerized mice. These findings unequivocally demonstrate that the expression of this idiotype is normally regulated by anti-idiotypic antibody or cells. In comparison, previous work has documented that CRI$^+$ antibodies could be regulated experimentally with passively administered anti-idiotypic antibody (6, 17), or T_s (17, 20), but left unresolved the question of whether this regulation occurs as a normal consequence of specific antigen responsiveness.

Elevation in the level of circulating idiotype$^+$ anti-ABA antibody after immunization of ABA-KLH can not be attributed to residual R16.7 HP remaining in circulation after tolerization. Injection of antigen would accelerate the clearance of tolerogen from circulation and adoptive transfer of cells from tolerized animals into normal recipients would remove all but cell-bound idiotype. Because low concentrations of passively administered anti-idiotypic antibody have been reported to stimulate the expression of idiotype (9–11), the possibility exists that the elevation of anti-R16.7 idiotype$^+$ antibodies in tolerized animals may not simply represent a lack of inhibition by anti-idiotype. Some of the tolerized mice continued to produce low levels of anti-R16.7 idiotype antibody, which may stimulate the production of idiotype$^+$ antibodies. However, correlation between the amount of anti-idiotypic antibodies and R16.7 idiotype$^+$ antibodies in individual tolerized mice was not obvious in these studies. Another explanation for the predominance of R16.7$^+$ antibody production in cells tolerant to this idiotype would be the presence of idiotype-specific helper T cells.

The level of total anti-ABA antibodies was substantially uneffected by elevated idiotype expression resulting from deregulation of R16.7 idiotype$^+$ B cells. In agreement with other approaches to experimental manipulation of idiotype expression (6, 7, 18, 20, 22), these findings confirm that homeostatic mechanisms other than idiotype-anti-idiotype equilibria are ultimately responsible for the regulation of the level of total antibody production. The observed maintenance of total antibody levels in the face of increased idiotype expression reveals that the synthesis of R16.7 idiotype$^-$ antibodies was selectively compromised in tolerized cells. This regulation of idiotype$^-$ antibody production may be a passive consequence of the expansion of deregulated idiotype$^+$ B cells or the result of an active process similar to indirect idiotype suppression (42).

The observations reported here indicate that a network of idiotypic and anti-idiotypic regulatory elements are normally present in the absence of overt antigen stimulation and can be perturbed without introducing antigen or idiotype in immunogenic form. Furthermore, compromise of a portion of this pre-existing network by tolerization with idiotype in a nonimmunogenic form has profound effects on the

components of the network in the absence of challenge with antigen (Figs. 3 and 4) or with the idiotype in CFA (Fig. 7).

Although prior exposure to antigen interfered with the induction of tolerance in idiotype-specific cells, the co-injection of ABA-KLH and tolerogenic R16.7 idiotype has no effect on induction of unresponsiveness. These data suggest that antigen alone does not influence the process of tolerance induction to the idiotype. It is more likely that the production of idiotypic and anti-idiotypic antibodies after antigen exposure modifies a subsequent tolerogenic signal to render it void or immunogenic. Although the induction of unresponsiveness to immunoglobulin antigens can be accomplished in previously activated B cells under certain conditions (43–45), mature, activated B lymphocytes are generally refractory to tolerance induction (43, 44, 46). This predisposition to activation would explain why tolerance to idiotypes, which are self antigens, would not be naturally established nor maintained. Low levels of circulating idiotype may activate anti-idiotypic cells precluding tolerization.

Documentation that tolerance to idiotype can be established experimentally provides a useful tool for the dissection of idiotype-anti-idiotype interactions and their role in the regulation of immune function. The modulation of immune responsiveness, which results from tolerization to idiotype, also provides a mechanism by which the subsequent production of that idiotype can be enhanced. In comparison to prior priming with antigen, which can mediate a decrease in the expression of a specific idiotype (47), tolerization with the idiotype appears to be more efficient in selecting for an antigen response enriched for that idiotype. Although the effects of tolerization upon network regulation have been addressed here, little evidence on the role of idiotypic networks in the induction and maintenance of tolerance has been forthcoming. Questions as to whether or not tolerance to antigen can be established in the presence of a compromised network lacking specific components remain to be resolved.

Summary

The induction of tolerance in an anti-idiotypic response was attempted by in vivo exposure to excess idiotype. Monoclonal immunoglobulin from the anti-*p*-azobenzenearsonate (ABA) hybridoma R16.7 was used as a representative of cross-reactive idiotype-positive (CRI^+) antibodies because this hybridoma protein (HP) shares one or more closely related public idiotypic determinants with the serum CRI in A/J mice. Immunologic unresponsiveness was established by a single injection of the R16.7 idiotype and persisted for at least 6 wk. The level of circulating anti-idiotypic antibodies in tolerized A/J mice was significantly depressed after immunogenic challenge with either antigen, ABA-keyhole limpet hemocyanin (KLH), or the idiotype R16.7 HP. Experimental depletion of anti-idiotypic antibodies by tolerization allowed assessment of immunoregulation within this altered idiotype-anti-idiotype network. Deregulation of idiotype expression in tolerized mice challenged with ABA-KLH was manifest with up to 96% of the anti-ABA antibodies cross-reacting with the R16.7 idiotype. This selective enhancement of a major idiotype was accomplished without substantial alteration of the level of the overall anti-hapten response. Both the unresponsiveness established in anti-idiotypic antibody-producing cells and the enhanced synthesis in idiotype-producing cells were stable upon adoptive transfer into lethally irradiated, syngeneic recipients. Finally, previous immunization with the antigen ABA-KLH interfered with the induction of unresponsiveness to the idiotype.

This interference is presumed to be mediated by prior activation of anti-idiotypic cells and/or antibody because injection of antigen with tolerogenic idiotype did not abrogate tolerance induction.

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