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

## II. Effects of Colchicine*

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It has become increasingly apparent that the regulation of immune responsiveness is accomplished by a variety of processes. Suppressor cells have recently emerged as a major element of this regulation. These cells may act either by directly suppressing responsive cells or by liberating soluble-suppressor factors. Not only have suppressor cells been found to be active in the regulation of ongoing immune responses, but they have also been found in association with the tolerant state to a number of T-dependent and T-independent antigens, including synthetic antigens (1, 2), polysaccharide antigens (3), and soluble protein antigens, human serum albumin (HSA)[1] (4) and human gamma globulin (HGG) (5–7). Although the role of suppressor cells in the establishment of tolerance has been convincingly demonstrated in some unresponsive states (8–11), this relationship remains to be proven in many systems. Several workers have reported that unresponsiveness to protein antigens can be established in the absence of detectable suppressor cells in normal animals (12–17), neonatal mice (18), adult thymectomized, X-irradiated, bone-marrow reconstituted mice (4, 13), and athymic nude mice (19–21). Although antigen-specific-suppressor cells may not be necessary for the establishment or maintenance of these tolerant states, their presence in antigen-primed animals (22, 23) suggests that they may play a regulatory role in immune responsiveness to these protein antigens.

The presence of suppressor cells and the degree of their activity can be modulated by a number of factors. Suppressor cells acting directly upon helper T cells can be induced by other T cells (24). In addition, HGG-specific-suppressor cells can be inhibited or inactivated by treatment with antisera specific for surface antigens present on suppressor cells, such as antigens coded for in the *I-J* region of *H-2* (25), and with antisera directed against cell-surface antigens on subsets of T lymphocytes such as Lyt-2 (25, 26) and Thy-1 (6, 25) antigens. Pharmacological agents known to serve as adjuvants may increase immune responsiveness by inhibiting the generation

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[1] *Abbreviations used in this paper*: AHGG, heat-aggregated HGG; Col, colchicine; DHGG, deaggregated HGG; GRBC, goat erythrocytes; HGG, human gamma globulin; HSA, human serum albumin; LPS, lipopolysaccharide; PFC, plaque-forming cells; SRBC, sheep erythrocytes.

or action of suppressor cells. Agents that have been demonstrated to interfere with suppressor cells affecting humoral or cellular-immune responses include cyclophosphamide (27–31), corticosteroids (32–35), indomethacin (36), and colchicine (Col) (37). Sublethal doses of irradiation can also increase immune responsiveness (38–41), possibly by disturbing cellular division of radiosensitive-suppressor cells.

Experimental abrogation of suppressor-cell activity allows investigation of the role of these cells in the regulation of the immune response and the establishment and maintenance of immunologic unresponsiveness in the absence of suppressor cells. The goals of this paper are to demonstrate pharmacological interference with both the induction and expression of antigen-specific-suppressor cells for the soluble protein antigen HGG by Col and to explore the resulting tolerant state to HGG. The effects of this agent are examined in hopes of elucidating the cellular and subcellular dynamics of the generation and action of suppressor cells and the induction and duration of immunologic unresponsiveness to a protein antigen in the absence of these cells.

## Materials and Methods

*Animals.* Male A/J mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) at 4- to 5-wk old. They were maintained on Wayne Lab Blox F6 (Allied Mills, Inc., Chicago, Ill.) and acidified water ad lib. All mice were housed five to a cage. Mice ranging in age from 8 to 9 wk were utilized in these experiments. These mice had a mean body weight of 24.4 ± 0.4 g.

*Chemicals.* Phenol-extracted bacterial lipopolysaccharides (LPS) from *Escherichia coli* 0111: B4 lot 614378 were purchased from Difco Laboratories, Detroit, Mich. When pharmacological agents were injected, the dosage per mouse was: 25 μg of LPS injected i.v. 3 h after antigenic challenge, 25 μg of colchicine (~ 1.0 mg/kg) (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) injected i.v. 2–3 h after exposure to tolerogen or antigen, or 2.5 mg of cyclophosphamide (~ 100 mg/kg) (Mead Johnson Laboratories, Evansville, Ind.) injected i.v. 2 d before antigen. These agents were diluted in pyrogen-free 0.15 M sterile NaCl.

*Antigens and Immunization.* Cohn fraction II lot RC-104 of human plasma was obtained through the courtesy of the American Red Cross National Fractionation Center with the partial support of the National Institutes of Health grant HE 13881 HEM. Human gamma globulin was purified from this material by column chromatography on DEAE-cellulose in 0.01 M phosphate buffer, pH 8.0. Immunogenic, heat-aggregated HGG (AHGG) was prepared from the DEAE-purified HGG as described previously (42) by using a modification of Gamble's method (43). Mice received a primary injection of 400 μg of AHGG i.v. into the lateral caudal vein followed in some cases by a secondary injection of the same amount of antigen intraperitoneally 10 d later. Sheep erythrocytes (SRBC) were purchased from Colorado Serum Co., Denver, Colo. Primary injection of $10^8$ SRBC was given 6 d before assessment of plaque-forming cells (PFC).

*Induction of Tolerance and Suppressor Cells.* Tolerogenic, deaggregated HGG (DHGG) was prepared by ultracentrifugation of DEAE-purified HGG for 150 min at 150,000 *g* to remove aggregated material as previously described (17). The upper quarter of the centrifuged solution was diluted and injected into mice i.p. Each mouse received 2.5 mg of DHGG. The spleens of mice tolerized 10 d previously served as the source of suppressor cells.

*Irradiated Recipients.* Recipients for adoptive transfer were irradiated 2–3 h before reconstitution. Mice placed in an aluminum chamber in a Gamma Cell 40 small animal irradiator (Atomic Energy of Canada, Ltd., Ottawa, Canada) received 900 roentgen (R) of whole body irradiation from a $^{137}$Ce source emitting a central dose of 106 R per min. Reconstituted recipients received 100 μg of gentamicin (Schering Pharmaceutical Corp., Kenilworth, N. J.) i.p. diluted in 2.7% glucose in saline on the day of irradiation and again 3 d later and were caged in groups of two to avoid the problem of early irradiation death, presumably due to bacterial infection.

*Adoptive Transfer Assay for Suppression.* The spleen cells of normal and tolerant mice that were

adoptively transferred into lethally irradiated animals were obtained as described previously (20). Briefly, spleens were aseptically removed and sterilely grated through 350 μm mesh stainless steel screens into balanced salt solution (BSS) supplemented with 100 U of penicillin and 100 μg of streptomycin per ml. The cell suspensions were washed three times in BSS, and $70 \times 10^6$ viable spleen cells were injected i.v. via a lateral caudal vein into irradiated recipients. Animals receiving more than one cell type received sequential injections. Primary antigenic challenge was delivered i.v. immediately after cellular reconstitution and was followed 10 d later by a secondary injection of AHGG i.p. 5 d after the secondary challenge, the recipients' spleens were removed and individually assayed for PFC.

*Hemolytic Plaque Assay.* PFC were assayed by a slide modification of the hemolytic plaque assay (44). Cohn Fraction II HGG at a concentration of 20 mg/ml was covalently coupled to goat erythrocytes (GRBC) (Colorado Serum Co.) by using water-soluble carbodiimide (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) (Calbiochem-Behring Corp.) (45). 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 $10^6$ spleen cells ± the standard error corrected for the background response to the indicator erythrocytes. Data were analyzed statistically with Student's *t* test. The percent suppression was determined as follows:

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

## Results

*Adjuvant Activity of Pharmacological Agents.* To assess the effectiveness of two pharmacological agents previously reported to mediate adjuvant activity in other immunologic systems, mice were injected with 2.5 mg of cyclophosphamide 2 d before, or 25 μg of Col at the time of, immunization with either 100 or 400 μg of antigenic AHGG. Some mice also received $10^8$ SRBC. The results of the PFC responses of these animals 6 d after antigenic exposure are presented in Table I. Both of these agents stimulated a marked increase in the number of PFC in spleens of animals given the lower dose of HGG. However, the response of mice to 400 μg of AHGG was less susceptible to augmentation by Col than was the response to 100 μg of antigen. The primary response to SRBC was also increased by the preadministration of cyclophosphamide. Thus, both cyclophosphamide and Col promoted an adjuvant increase in the primary PFC response in vivo to the soluble protein antigen HGG under appropriate conditions.

*Temporal Relationship between Col and Antigen Injection.* The possibility that the failure to detect an increase in PFC to HGG in animals given Col and the higher dose of 400 μg of AHGG was a result of the inappropriate timing of the Col injection was investigated. Mice immunized with either 100 or 400 μg of AHGG were injected with Col at various days ranging from 1 d before antigen through 5 d after antigen. As illustrated in Figs. 1 and 2, 400 μg of AHGG alone stimulated a greater PFC response at 6 d after immunization than did 100 μg of antigen. Furthermore, the injection of Col from 1 d before to 1 d after 100 μg of antigen stimulated a marked increase in the number of PFC detected to HGG (Fig. 1). On the contrary, Col was not able to exert an adjuvant effect in animals receiving the higher dose (400 μg) of antigen regardless

TABLE I

*Adjuvanticity of Col and Cyclophosphamide on Primary Immune Responses*

| Exp. | Treatment* | Antigenic‡ challenge | Mean PFC/$10^6$ ± SE to: | |
|---|---|---|---|---|
| | | | HGG§ | SRBC‖ |
| I | — | 100 μg AHGG | 581 ± 149 | |
| | Col | 100 μg AHGG | 1591 ± 334 | |
| | — | 400 μg AHGG | 954 ± 398 | |
| | Col | 400 μg AHGG | 946 ± 232 | |
| II | — | 100 μg AHGG + SRBC | 142 ± 25 | 43 ± 12 |
| | Cyclophosphamide | 100 μg AHGG + SRBC | 917 ± 440 | 241 ± 23 |

* 25 μg of Col was injected i.v. 2 h after antigen. 2.5 mg of cyclophosphamide was injected i.v. 2 d before antigen.

‡ 100 or 400 μg of AHGG was injected i.v., and individual spleens were plaqued 6 d later. In exp. II, both 100 μg of AHGG and $10^8$ SRBC were given in separate i.v. injections.

§ Indirect PFC.

‖ Direct PFC.

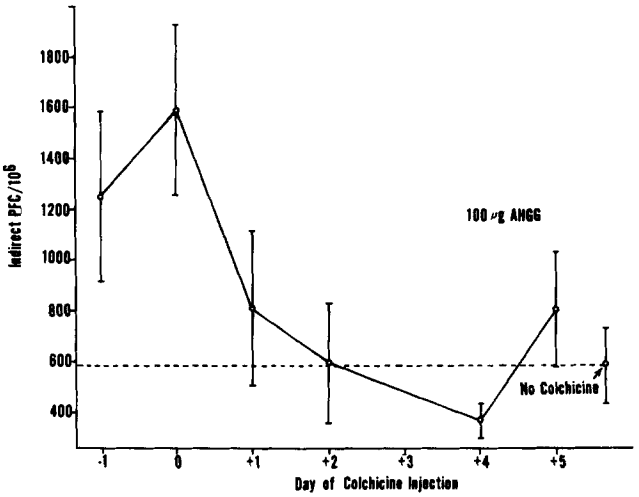


FIG. 1. Adjuvant effect of colchicine on the primary response to a low dose of HGG. 100 μg of AHGG was injected i.v. on day 0. 25 μg of Col was injected i.v. on various days before or after antigen, and PFC were assayed on day 6. The mean ± SE is presented.

of the temporal relationship between the injection of antigen and Col (Fig. 2). The data in Fig. 1 are in agreement with previous reports indicating that Col must be given in close proximity to antigenic challenge to serve as an effective adjuvant (46, 47). Additionally, these data suggest that the injection of Col within 1 d of the assessment of the immune response does not significantly decrease the number of antigen-specific PFC detected. Therefore, both the antigenic dose and the temporal relationship between the injection of antigen and Col appear to be crucial for the expression of colchicine-mediated adjuvanticity.

*Abrogation of the Generation of Suppressor Cells to HGG by Col.* To investigate the

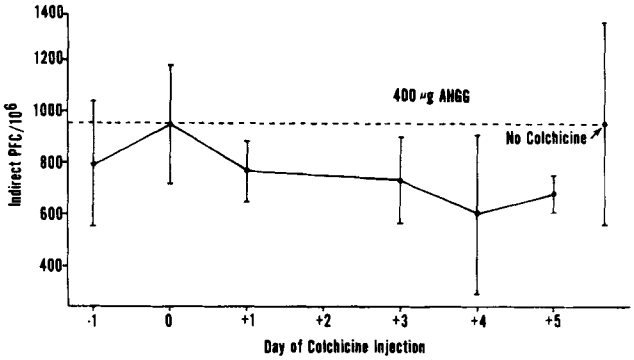


FIG. 2. Lack of adjuvanticity of colchicine with high antigen dose. The data were obtained as described in Fig. 1 except that 400 μg of AHGG was injected on day 0.

TABLE II

*Abrogation of the Generation of Suppressor Cells to HGG by Col*

| Group | Spleen cells transferred* | | Normal | Antigenic challenge§ | PFC/$10^6$ to HGG Mean ± SE | Percent suppression‖ |
|---|---|---|---|---|---|---|
| | Tolerant | | | | | |
| | DHGG + Col‡ | DHGG | | | | |
| 1 | $70 \times 10^6$ | — | — | AHGG | 8 ± 6 | — |
| 2 | $70 \times 10^6$ | — | $70 \times 10^6$ | AHGG | 192 ± 38 | 0 |
| 3 | — | — | $70 \times 10^6$ | AHGG | 191 ± 33 | — |
| 4 | — | $70 \times 10^6$ | $70 \times 10^6$ | AHGG | 80 ± 14 | 58 |
| 5 | — | $70 \times 10^6$ | — | AHGG | 4 ± 2 | — |

* 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 d earlier or both.

‡ 2.5 mg of DHGG was injected i.p. and followed 3 h later with 25 μg of Col, as indicated.

§ Reconstituted mice received 400 μg of AHGG on the day of cell transfer and again 10 d later and were assayed 5 d after secondary challenge. The mean ± SE of the number of indirect PFC to HGG from five to eight mice is presented.

‖ See Materials and Methods.

possibility that Col may act as an adjuvant for soluble protein antigens by interfering with the generation of antigen-specific suppressor cells as previously demonstrated for the synthetic copolymer L-glutamic $acid^{50}$-L-$tyrosine^{50}$ (37), the effects of Col upon HGG-specific suppressor cells were assessed. The basic protocol for measuring suppressor-cell activity to HGG involved the adoptive transfer of $70 \times 10^6$ viable spleen cells containing suppressor cells from mice tolerized 10 d previously with DHGG together with $70 \times 10^6$ viable spleen cells from normal animals into lethally irradiated, syngeneic recipients (17, 48). Control groups received either normal or tolerant spleen cells alone. In the experiments assessing interference with the induction of suppressor cells, all recipients were challenged with 400 μg of AHGG on the day of cell transfer and again 10 d later, and the PFC response was assessed 5 d after the secondary challenge.

To investigate the ability of Col to abrogate the generation of suppressor cells during the induction of tolerance to HGG, mice were injected with 2.5 mg of tolerogen (DHGG) followed 3 h later by 25 μg of Col injected i.v. (Table II and Fig. 3). As

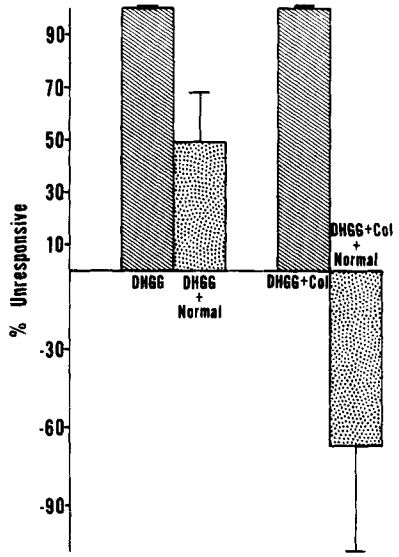


FIG. 3. Inhibition of induction of suppressor cells with Col. The suppressive activity of tolerant spleen cells from donors tolerized 10 d before transfer was assayed by adoptive transfer. As indicated, some donors were treated with 25 μg of Col 2 h after injection of DHGG. The percent of unresponsiveness (or suppression) was calculated as described in Materials and Methods. Suppression was calculated from experimental recipients receiving both normal and tolerant spleen cells. Unresponsiveness was calculated from experimental recipients of tolerant cells alone. The mean ± SE of five to six recipients are presented.

demonstrated in Table II, the injection of Col had no effect on the induction of tolerance to HGG. Spleen cells from mice treated with Col and DHGG (group 1) were unresponsive on adoptive transfer as were cells from animals treated with tolerogen alone (group 5). The injection of DHGG generated suppressor cells as evidenced by the depressed response of recipients receiving both tolerant (DHGG-treated) and normal spleen cells (group 4) when compared with the response of the recipients of normal spleen cells alone (group 3). However, mice injected with Col 3 h after the injection of DHGG possessed no detectable suppressor cells in their spleens as illustrated by the response of animals receiving spleen cells from both Col-treated tolerant and normal mice (group 2). On the contrary, the recipients of cells from both DHGG and Col-treated mice and normal mice showed responses equal to (Table II) or exceeding (Fig. 3) those of recipients of normal cells alone. The demonstration in Fig. 3 of heightened responsiveness in the recipients of cells from normal and tolerant mice not containing detectable suppressor cells compared to recipients of normal cells alone is in agreement with previously published data on the responsiveness of mixtures of tolerant but nonsuppressive spleen cells and normal cells (17).

*Interference with the Expression of Suppressor Cells by Col.* The demonstration that the injection of Col 3 h after tolerization inhibits the generation of HGG-specific suppressor cells was extended by assessing the ability of Col to interfere with the expression of suppressor cells. To investigate the effects of Col on the activities of suppressor cells previously generated by the injection of DHGG, spleen cells from animals tolerized 10 d previously were transferred together with spleen cells from normal mice into irradiated recipients. These recipients were injected with 400 μg of AHGG i.v., and 3 h after this antigenic challenge one-half of the recipients were

TABLE III

*Interference with the Expression of Suppressor Cells by Col*

| Group | Spleen cells transferred* | | Antigenic challenge‡ | PFC/$10^6$ to HGG§ | Percent suppression |
|---|---|---|---|---|---|
| | Tolerant | Normal | | | |
| 1 | $70 \times 10^6$ | — | AHGG + Col | 7 ± 4 | — |
| 2 | $70 \times 10^6$ | $70 \times 10^6$ | AHGG + Col | 298 ± 56 | 0 |
| 3 | — | $70 \times 10^6$ | AHGG + Col | 265 ± 31 | — |
| 4 | $70 \times 10^6$ | — | AHGG | 2 ± 1 | — |
| 5 | $70 \times 10^6$ | $70 \times 10^6$ | AHGG | 86 ± 21 | 47 |
| 6 | — | $70 \times 10^6$ | AHGG | 162 ± 37 | — |

* See Table II, footnote*.

‡ Recipients were challenged as described in Table II, footnote§, except that 25 μg of Col was injected i.v. 3 h after the primary antigenic challenge, as indicated.

§ The mean ± SE of indirect PFC to HGG from duplicate experiments containing 5–10 mice per group is presented.

injected with 25 μg of Col. The secondary response of these mice was assessed by injecting 400 μg of AHGG alone 10 d after the primary injection and plaquing 5 d after this secondary challenge. Table III demonstrates that the addition of Col to the primary antigenic challenge abolishes the activity of the suppressor cells present in the tolerant spleen-cell population (group 2). However, Col injected at this time does not terminate the unresponsive state in tolerant spleen cells transferred 10 d after tolerization (group 1). The adjuvant effect of Col can be detected by comparing the responsiveness of transferred normal spleen cells challenged with antigen and Col (group 3) or challenged with antigen alone (group 6). This adjuvant increase is due presumably to the inhibition of the generation of suppressor cells in the normal spleen-cell population. However, the increased responsiveness of normal spleen cells challenged with antigen and Col does not account for the abolition of suppressor-cell activity found in colchicine-treated recipients of normal and tolerant spleen cells.

*Inability of Col to Interfere with the Induction of Tolerance in T- or B-Spleen Cells.* The observations that Col can act as an adjuvant and can inhibit both the induction and the expression of suppressor cells raise the possibility that Col might interfere with the induction of immunologic unresponsiveness. However, the data presented above indicate that the injection of Col 3 h after DHGG does not interfere with the generation of unresponsiveness. In Fig. 3 and Table II, spleen cells from mice injected with DHGG and colchicine remained unresponsive when transferred into irradiated recipients and challenged twice with antigen. Similarly, the unresponsive state to HGG was not perturbed by the incorporation of Col into the antigenic challenge of tolerant mice (Table III). The effect of Col on the induction of tolerance was further examined in intact animals with particular emphasis on splenic B cells. Mice injected with DHGG and Col 10, 30, or 65 d before were challenged with 400 μg of AHGG i.v. followed 3 h later by 25 μg of LPS and were plaqued 6 d after antigenic challenge. The injection of LPS with antigen into mice possessing tolerant T cells but responsive B cells leads to the generation of PFC to the injected antigen (49). Therefore, this protocol would allow the demonstration of responsive splenic B cells at a time when splenic T cells are unresponsive to this antigen and would result in the stimulation of HGG-specific B cells if Col interferes with the induction of tolerance in these B cells. The responsiveness of the total spleen-cell population to HGG was also investigated

TABLE IV

*Inability of Col to Interfere with the Induction of Tolerance to HGG*

| Treatment* | Antigenic challenge | | Days posttreatment‡ | | |
|---|---|---|---|---|---|
| | Primary | Secondary | 10 | 30 | 65 |
| DHGG + Col | AHGG | AHGG§ | <1 | 12 ± 7 | 8 ± 5 |
| DHGG | AHGG | AHGG | 2 ± 2 | 3 ± 2 | 17 ± 14 |
| — | AHGG | AHGG | 211 ± 78 | 1,366 ± 264 | 229 ± 47 |
| DHGG + Col | AHGG + LPS‖ | — | <1 | 25 ± 10 | 233 ± 71 |
| DHGG | AHGG + LPS | — | 1 ± 1 | 40 ± 20 | 128 ± 76 |
| — | AHGG | — | 96 ± 14 | 180 ± 19 | 66 ± 10 |

* See Table II, footnote‡.

‡ The mean ± SE of indirect PFC/$10^6$ cells to HGG from duplicate experiments containing four to seven mice per group is presented.

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

‖ 400 μg of AHGG was injected i.v. at various times after treatment as indicated. 2.5 μg of LPS was injected i.v. 3 h after antigenic challenge, and individual spleens were plaqued 6 d later.

by injecting these mice with 400 μg of AHGG twice 10 d apart and plaquing 4 d after the second antigenic challenge. Table IV illustrates the inability of Col to interfere with the induction of tolerance in splenic T cells as illustrated by the unresponsiveness observed when mice tolerized with DHGG and Col are challenged with antigen alone. Furthermore, the addition of LPS to the antigenic challenge does not alter the reacquisition of HGG-responsive splenic B cells in mice tolerized with DHGG and Col as compared to mice tolerized with DHGG alone. By 65 d after tolerization, substantial numbers of HGG-responsive B cells are present in both animals tolerized with and without Col. Nevertheless, tolerance is still persistent in T cells as assayed by challenge with AHGG alone. Therefore, although Col interferes with both the generation and expression of suppressor cells specific for HGG, it does not appear to interfere with the induction or duration of unresponsiveness to this antigen in either the splenic T- or B-cell population assayed in situ (Table IV) or by adoptive transfer to irradiated recipients (Table II and Fig. 3).

*Inability of Col to Terminate Tolerance to HGG.* The unresponsive state to HGG is of much shorter duration in bone marrow (50) and splenic (12, 51) B cells than in T cells as illustrated by Table IV. It has been proposed that unresponsiveness of the whole animal is maintained in the face of responsive B cells by the absence of responsive-helper T cells and that these T cells are permanently inactivated or deleted by antigen (tolerogen) (50, 52). However, responsive-helper T cells might be present at such times but prevented from cooperating with responsive B cells by suppressor cells. If responsive-helper T cells are present but inhibited by suppressor cells in this latter stage of immunologic unresponsiveness to HGG and if Col interferes with both the induction and expression of HGG-specific suppressor cells as suggested by Tables II and III and Fig. 3, then the injection of AHGG and Col into such mice would be expected to terminate the tolerant state. Mice were injected with AHGG and Col or LPS 10 or 66 d after tolerization and plaqued 6 d after antigenic challenge to test this

TABLE V
*Inability of Col to Terminate Tolerance to HGG*

| Days post-tolerization | Immune status | Antigenic challenge* | PFC/$10^6$ to HGG‡ |
|---|---|---|---|
| 10 | Tolerant§ | AHGG + Col | 3 ± 1 |
| 10 | Tolerant | AHGG + LPS | <1 |
| 66 | Tolerant | AHGG | 2 ± 1 |
| 66 | Tolerant | AHGG + Col | 4 ± 1 |
| 66 | Tolerant | AHGG + LPS | 330 ± 52 |
| | Normal | AHGG | 62 ± 19 |

* 400 μg of AHGG was injected i.v. either 10 or 66 d after tolerization. 25 μg of Col or LPS was injected i.v. 3 h after antigenic challenge as indicated, and individual spleens were plaqued 6 d later.

‡ The mean ± SE of indirect PFC from five mice per group is presented.

§ 2.5 mg of DHGG was injected i.p. on day 0.

possibility. As demonstrated in Table V, 66 d after tolerization HGG-responsive B cells are detected in tolerant mice by the injection of LPS and AHGG. However, the injection of Col and antigen does not terminate tolerance in these mice. Neither Col nor LPS have any effect upon the unresponsive state in the cells of day-10 tolerant animals. These data suggest that interference with the induction or expression of suppressor cells is not sufficient to allow the termination of tolerance, even in mice possessing responsive B cells, and indicate that unresponsiveness to HGG in helper T cells is not maintained by Col-sensitive-suppressor cells.

## Discussion

The data presented in this report demonstrate that Col can interfere with suppressor cells specific for the soluble protein antigen HGG. Col prevents the generation of the antigen-specific-suppressor cells induced by the tolerogenic form of this antigen when the drug is injected within hours of tolerization. Furthermore, the expression of these suppressor cells is inhibited when Col is utilized during the assessment of the suppressor cells previously induced with tolerogen. This interference with the induction and expression of antigen-specific-suppressor cells may be the mechanism of the adjuvant activity previously attributed to Col. As early as 1954 (46, 53), Col was reported to enhance serum antibody of rabbits injected with diphtheria toxoid. Since these initial observations, several workers have reported similar enhancement of humoral antibody responses mediated by Col in rabbits (54), hamsters (55, 56), guinea pigs (57), and mice (37, 47) to a variety of antigens. Recently, Shek et al. (37) have demonstrated that Col can act as an adjuvant for the PFC response to the random, synthetic copolymer of L-glutamic $acid^{50}$-L-$tyrosine^{50}$ by interfering with the induction of suppressor cells to this synthetic peptide antigen. The data presented here confirm and extend these previous reports by demonstrating that the induction of suppressor cells specific for a naturally occurring protein antigen, HGG, can be abrogated with Col and that the expression of existing, mature suppressor cells can also be inhibited by this agent.

The ability of Col to block the activity of suppressor cells and thereby increase immune responsiveness may exemplify a class of immunopotentiating agents defined

by their mechanism of adjuvant action. In addition to Col, irradiation (38–41) and other pharmacological agents including corticosteroids (32–35), cyclophosphamide (27–31), and indomethacin (36) have been reported to serve as adjuvants for humoral and cellular-immune responses possibly by interfering with suppressor cells. Of these agents that may selectively inhibit suppressor cells, Col may have the greatest potential for use as an adjuvant. With the exception of Col, these agents possess adjuvant activity over a narrow dose range and are more widely recognized as potent immunodepressants (58). In contrast, in the present studies, Col did not depress immune responsiveness when injected up to 24 h before assessment of the PFC response (Figs. 1 and 2), and it has been reported to be immunodepressive in vivo only when injected repeatedly (59, 60) or at nearly toxic doses (61, 62). In the present work as in previous reports (46, 47), Col possessed adjuvant properties at concentrations approaching toxicity, and at concentrations that were toxic for individual animals, the surviving animals exhibited heightened immune responses.

The mechanism by which Col inhibits suppressor cells is unclear. However, it has been suggested that the selective inhibition of suppressor cells is due to the anti-mitotic activity of Col (37, 47). This postulate is applicable to the induction of suppressor cells that have been demonstrated to require cell division (63) and to be sensitive to irradiation (64). However, the expression of suppressor cells appears to be relatively more resistant to irradiation (64–67) or mitomycin C (33). If the effects of Col upon the expression of mature suppressor cells is limited to the anti-mitotic activity of this drug, then the cells directly affected by Col may be cells which amplify the action of the mature suppressor cells. These amplifier cells might require cell division before becoming fully competent. T lymphocytes that may augment the functions of mature suppressor cells have recently been described (24, 31, 68, 69). Alternatively, Col may interfere with subcellular processes associated with cell motility or communication, thus preventing cell-to-cell interaction between mature suppressor cells and their targets or intermediary lymphocytes.

Interference with the induction of tolerance has been proposed as the most stringent assay for adjuvant activity (70). However, this view can no longer be supported in light of the demonstration that Col promotes an adjuvant increase in both PFC (Table I and Fig. 1) (47) and circulating antibody (47) to HGG even though it does not interfere with the induction of tolerance to this antigen (Table II and Fig. 3). This segregation of the ability to interfere with the induction of tolerance from other mechanisms of adjuvant activity has been documented previously (71) in athymic nude mice (19, 20) and in the LPS-nonresponder C3H/HeJ mouse (72), with polymerized flagellin (19), LPS (20), and lipid A-associated protein (72).

A final area of interest arises from the inability of Col to interfere with the induction of tolerance although successfully interfering with the generation of suppressor cells. These data have direct implications on the putative role of suppressor cells in the tolerant state. Although the unresponsiveness established by the injection of DHGG and Col lacks demonstrable levels of suppressor cells, this tolerant state is: (*a*) as complete as in animals receiving tolerogen alone; (*b*) stable upon adoptive cell transfer; (*c*) persistent in splenic T lymphocytes for at least 66 d; (*d*) established in splenic B lymphocytes as assessed by challenge with antigen and LPS; and (*e*) maintained in B cells with the same kinetics as animals receiving only tolerogen. Furthermore, interference with suppressor cells by Col was unable to terminate

tolerance. This ability to induce and maintain immunologic unresponsiveness to HGG in the absence of antigen-specific suppressor cells is in agreement with previous reports (17, 73) and confirms the demonstration that suppressor cells are not necessary for this tolerant state. As suggested previously (52, 73), tolerance to HGG may exemplify a state of central or intrinsic unresponsiveness in which antigen-responsive B cells are irreversibly inactivated or permanently deleted. The data presented here support this postulate. Suppressor cells do not appear to be responsible for the induction of unresponsiveness in either T or B lymphocytes to HGG nor do they appear to influence the duration of tolerance in these cells. Similar conclusions have been drawn from systems in which tolerance is established in B cells in vitro (74, 75).

The tenuous association of suppressor cells with the unresponsive state to soluble protein antigens has been suggested by others (73). Suppressor cells are only transiently associated with the unresponsive state established to HGG (6, 48) and do not appear to be required for the maintenance of tolerance in either T or B lymphocytes (17). Furthermore, new suppressor cells cannot be induced in unresponsive mice after the disappearance of the initial, transient suppressor cells (16) speaking against a role for suppressor cells in states of unresponsiveness to persistent self-antigens. Unresponsiveness can be established in the absence of transient suppressor-cell activity (*a*) in adult animals by tolerizing with a low dose of commercially acquired HGG (16) or with HGG purified from either individual volunteers or myeloma patients (17); or (*b*) in neonatal animals (18). Unresponsiveness lacking suppressor cells has also been reported in adult animals (12–14) with the conventional DAGG tolerization protocol. The demonstration that tolerance can be induced in congenitally athymic nude mice devoid of competent T cells with both immunogenic (21) and tolerogenic (19, 20) forms of heterologous gamma globulins and in adult thymectomized, X-irradiated, bone marrow-reconstituted mice (4, 13) further support the postulate that tolerance can be induced in the absence of suppressor T cells. These observations coupled with the demonstration of the presence of antigen-specific-suppressor cells to HGG in primed mice (23) and the more efficient suppression of HGG-primed cells compared to normal cells (76) suggest that these suppressor cells may represent a normal regulatory mechanism operative during antigenic stimulation although their relevance to the unresponsive state to HGG must remain dubious. The existence of antigen-specific-suppressor cells in an animal immunologically unresponsive to that antigen does not in itself imply a causal relationship between the suppressor cells and the establishment and maintenance of the tolerant state. Any postulate that the suppressor cells present in a tolerant host represent the mechanism of unresponsiveness must be firmly established experimentally.

## Summary

The ability of colchicine (Col) to interfere with suppressor cells specific for the soluble protein antigen human gamma globulin (HGG) has been examined. This interference may be the mechanism of the adjuvanticity promoted by Col. When injected into A/J mice at the appropriate time and concentration, both Col and cyclophosphamide promoted an adjuvant increase in the plaque-forming cell response to 100 μg of immunogenic, aggregated HGG. Col abrogated both the induction of suppressor cells when injected within 3 h of tolerization with deaggregated (DHGG) and the expression of previously induced suppressor cells when injected with the

antigenic challenge. Interference with the generation and expression of antigen-specific-suppressor cells had no detectable effect on the immunologic unresponsive state to HGG. Col did not interfere with the induction of tolerance at a dose (1 mg/kg) that abolished the generation of suppressor cells. Furthermore, the absence of colchicine-sensitive-suppressor cells during the establishment of tolerance had no observable effect on the duration of unresponsiveness in either helper T- or B-lymphocyte populations. Finally, Col was not able to terminate the unresponsive state established by DHGG even when responsive splenic B cells could be demonstrated in tolerant animals. These data indicate that suppressor cells are not required for the establishment and maintenance of the unresponsive state to this antigen.

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