EFFECT OF COLCHICINE ON THE ANTIBODY RESPONSE
II. Demonstration of the Inactivation of Suppressor Cell Activities by Colchicine*

BY PANG N. SHEK,† CARL WALTENBAUGH,§ AND ALBERT H. COONS†

(From the Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115)

Experiments reported in the preceding paper established the experimental conditions for the optimal enhancement of the antibody formation by colchicine (CC)1 in mice (1). The effectiveness of CC in potentiating the antibody response raises the tantalizing question of how the drug acts to cause the enhancement. Since suppressor cells play an important role in the regulation of the antibody response (2–5), and the generation of these predominantly radiation-sensitive regulatory cells apparently requires mitosis (4, 6, 7), it is reasonable to assume that any interference with their proliferation or that of their precursors would reduce negative regulation. Considering the dose range (1.0–1.5 mg/kg) of CC which we find to be effective in enhancing antibody formation (1), one which has been shown to be anti-mitotic (8, 9), it is likely that CC exerts its mitosis-blocking effect on early dividing suppressor cells soon after their encounter with antigen (3, 7, 10, 11). Suppressor cells are thymus-derived (T) lymphocytes which suppress the antibody response in a number of systems (12–15). Thus it seems likely that in a system where T-cell regulation is absent, CC should be ineffective in enhancing the antibody response.

The random synthetic copolymer of L-glutamic acid5°-L-tyrosine5° (GT), which is not immunogenic in any of more than 20 inbred strains of mice, stimulates antibody responses when coupled to the immunogenic carrier, methylated bovine serum albumin (GT-MBSA) (16). Preimmunization with GT suppresses the plaque-forming cell (PFC) response to GT-MBSA in BALB/c mice. The specific suppressor activity has been shown to be mediated by T cells (17) and to be sensitive to cyclophosphamide (18). Therefore, in a system where specific suppressor T cells can be demonstrated, CC should be capable of preventing their activity.

Results obtained in the present study demonstrate that in the antibody response to a T-independent antigen, e.g. 2,4,6-trinitrophenyl—keyhole limpet hemocyanin (TNP-KLH)-Sepharose (19), CC effectively induces an elevated hapten-specific PFC response in intact animals, but not in congenitally athymic nude mice where T-cell regulation is absent. Furthermore, the generation of

* Supported by grant 20811 from the National Institutes of Health.
† Research Fellow of the American Heart Association. Present address: Connaught Laboratories, Willowdale, Ontario M2N 5T8, Canada.
§ Supported by grant 09130 from the U. S. Public Health Service.
‖ Career Investigator of the American Heart Association.
1 Abbreviations used in this paper: CC, colchicine; GAT, L-glutamic acid5°-L-alanine5°-L-tyrosine5°; GT, L-glutamic acid5°-L-tyrosine5°; KLH, keyhole limpet hemocyanin; MBSA, methylated bovine serum albumin; PFC, plaque-forming cells; SRBC, sheep erythrocytes; TKS, TNP-KLH-Sepharose; TNP, 2,4,6-trinitrophenyl.
GT-induced suppressor cells and the transfer of their suppressive activity to normal syngeneic recipients can be effectively prevented by the administration of CC.

Materials and Methods

Animals. BALB/c mice were purchased from the same suppliers and maintained under the conditions described in the preceding paper (1).

Antigens and Immunization. The TNP-KLH used in this study was the same as that described previously (1). The hapten-carrier was conjugated to Sepharose 2-B (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) using the method described by Feldmann et al. (19). The synthetic random copolymer, GT, with an average mol wt of 133,000, as well as the terpolymer of L-glutamic acid°°-L-alanine°°-L-tyrosine°° (GAT), mol wt 35,000, were obtained from Miles Laboratories Inc., Miles Research Products, Elkhart, Ind. MBSA was purchased from Worthington Biochemical Corp., Freehold, N. J. The GT-MBSA complex was prepared by the dropwise addition of MBSA (5 mg/ml) while stirring, to a solution of GT (1 mg/ml). After a 20-min interval during which maximum flocculation and aggregation occurred, the precipitate of GT-MBSA was washed three times in saline. For immunization, mice were injected intraperitoneally with 10 μg of GT as GT-MBSA in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). CC (Sigma Chemical Co., St. Louis, MO.) was freshly dissolved in physiological saline and administered intraperitoneally to animals at a dose of 1 mg/kg body weight.

Cell Transfers. BALB/c mice used as cell donors were injected intraperitoneally with Maalox, 100 μg GT in Maalox, or 100 μg GT in Maalox and 1.0 mg CC/kg. 3 days later, mice were sacrificed and their spleens were removed. Single cell suspensions were prepared, washed three times in Hanks' balanced salt solution, and 20 × 10⁶ lymphoid cells were adoptively transferred intravenously into normal syngeneic recipients. Mice were then immunized with GT-MBSA immediately after transfer.

Hemolytic Plaque Assay. The TNP-specific PFC response of immune animals was assessed as described previously (1). The PFC response to GT-MBSA was assayed on sheep erythrocytes (SRBC) coupled with the cross-reacting polymer GAT, GAT-SRBC (20). GT-specific plaques were determined by subtracting the number of PFC remaining after inhibition by a suitable dilution of GAT from the number of plaques detected on GAT-SRBC in the absence of the specific inhibitor (21).

Results

CC Enhances the PFC Response of Normal and Nude Mice to TNP-KLH-Sepharose. Although TNP-KLH is a thymus-dependent antigen, Feldmann and his colleagues have demonstrated that the conjugation of TNP-KLH to Sepharose (TKS) renders it thymus-independent (19). We performed experiments to test the effectiveness of CC in enhancing the antibody response to TKS in intact animals and in nude mice where T-cell regulation is absent. It can be seen from Fig. 1 that the simultaneous injection of CC with TKS into normal mice enhanced their hapten-specific PFC response. On the other hand, the administration of CC to congenitally athymic nude mice failed to increase their response (Table I).

CC Abrogates GT-Induced Suppression of the PFC Response to GT-MBSA. It has been established that preinjection of the copolymer GT suppresses the GT-specific PFC response of certain inbred mouse strains, e.g. BALB/c mice, to a challenge of the immunogenic GT-MBSA antigen (21). GT-induced suppression results from the stimulation of specific suppressor T cells (5, 17). We adopted this system to test the effect of CC on the generation of GT-stimulated suppressor cells. First, we examined the effectiveness of CC in blocking GT from inducing nonresponsiveness to GT-MBSA in BALB/c animals. It can be
Fig. 1. Kinetics of the TNP-specific primary IgM PFC response of BALB/c mice to TKS. Animals in the control group (○) were immunized intraperitoneally with 100μg of TKS on day 0, and animals in the experimental group (●) were similarly immunized, except that CC was also administered at a dose of 1 mg/kg body weight. On each day from day 5 to day 8 after the injection, spleens of the injected animals were assayed for their TNP-specific IgM PFC response. Each point represents the mean PFC ± SE of 5-10 animals. SC, spleen cells.

TABLE I

Effect of CC on the Primary Hapten-Specific PFC Response to TKS in BALB/c and Nude Mice

| Group | Strain     | Number of mice | Day 0 Treatment | TNP-specific IgM PFC per spleen | Day 7 | P |
|-------|------------|----------------|-----------------|---------------------------------|-------|---|
|       |            |                | Day 0 Treatment |                                  |       |   |
| I     | BALB/c     | 10             | TKS*            | 10,170 ± 1,310                  |       |   |
| II    | BALB/c     | 10             | TKS + CC$       | 25,980 ± 3,190                  | <0.003$ |   |
| III   | Nude (nu/nu)| 6              | TKS             | 8,500 ± 1,200                   |       |   |
| IV    | Nude (nu/nu)| 6              | TKS + CC       | 9,290 ± 1,020                   | n.s.$ |   |

* 100μg of TKS injected i.p.
$ CC administered at 1 mg/kg body weight, i.p.
§ P value in comparison to group I.
∥ n.s., not significant in comparison to group III.

seen from Table II that prior injection of GT effectively suppressed the GT-specific PFC responses of the injected animals in group II to a subsequent challenge with GT-MBSA. On the other hand, animals in group III, preinjected with GT as well as CC, developed a PFC response to GT-MBSA no different from that of control animals in group I.

Results of experiments described above strongly imply that CC may abolish the GT-induced suppression by inactivating the suppressor T cells. If this
TABLE II
Effect of CC on GT-Induced Suppression of the PFC Response to GT-MBSA in BALB/c Mice

| Group | Number of mice | Day -3 Treatment | Day 0 Treatment | Day 7 GT-specific PFC per spleen | P*  |
|-------|----------------|------------------|----------------|---------------------------------|-----|
| I     | 9              | Maalox GT-MBSA†  | 4,240 ± 770    |                                  |     |
| II    | 9              | GT§ GT-MBSA      | 180 ± 20       | <0.001                           |     |
| III   | 8              | GT + CC¶ GT-MBSA | 4,180 ± 400    | n.s.                            |     |

* P values in comparison to group I.
† 10 μg of GT as GT-MBSA in complete Freund's adjuvant, i.p.
§ 100 μg of GT in Maalox, i.p.
¶ CC administered at 1 mg/kg body weight, i.p.
+n.s., not significant.

TABLE III
Effect of CC on the Transfer of Suppression of GT-Specific PFC Response to Normal Syngeneic Recipients (BALB/c Mice)

| Group | Donors          | Normal syngeneic recipients | GT-specific IgG PFC per spleen | Number of mice | P*    |
|-------|-----------------|-----------------------------|--------------------------------|----------------|-------|
|       | Treatment       | Spleen cells transferred    | Immunization                   | Mean ± SE      |       |
| I     | Maalox         | 20 × 10⁶                   | GT-MBSA‡                       | 5,780 ± 930    | 12    |
| II    | GT§            | 20 × 10⁶                   | GT-MBSA                        | 500 ± 160      | 12    | <0.001|
| III   | GT + CC¶       | 20 × 10⁶                   | GT-MBSA                        | 6,150 ± 1,040  | 12    | n.s.‡ |

* P values in comparison to group I.
‡ 10 μg of GT as GT-MBSA in complete Freund's adjuvant, i.p.
§ 100 μg of GT in Maalox, i.p.
¶ CC administered at 1 mg/kg body weight, i.p.
+n.s., not significant.

If the implication were correct, one would predict that the administration of CC to GT-primed donors should prevent the transfer of suppressor activity to normal syngeneic recipients. It can be seen from Table III that the transfer of GT-primed spleen cells to normal syngeneic recipients immunized with GT-MBSA significantly suppressed the IgG PFC response to GT-MBSA (compare groups II and I). In contrast, the injection of CC to cell donors at the time of priming with GT abolished the transfer of suppressor activity to normal recipients challenged with GT-MBSA (compare groups III and II). These results suggested that CC abolished GT-specific suppression by the inactivation or elimination of GT-stimulated suppressor cells.

Discussion
Studies reported in the preceding paper clearly established the effect of CC in enhancing the antibody response in mice (1). Results of experiments presented
in this paper have demonstrated that (a) CC is ineffective in potentiating antibody formation in mice in which T-cell regulation is absent and (b) CC abolishes the expression of GT-induced suppressor cell activities.

It could be argued that CC induces an elevated antibody response by its direct effect on B cells. Hence, we tested its action on congenitally athymic mice to a T-independent antigen, TKS. Our results indicate that CC did not enhance antibody formation in homozygous nude (nu/nu) mice where T-cell regulation is absent (Table I). In contrast, the hapten-specific PFC response of intact animals to TKS can be enhanced by CC (Fig. 1). Although it has been shown by Feldmann et al. (19) that helper T cells and macrophages are not required to elicit a hapten-specific response to TKS, the magnitude of the response might still be under the control of regulatory T cells, e.g. suppressor cells. The effectiveness of CC in elevating the antibody response to TKS in intact animals where T-cell regulation is present, and the failure of the drug to enhance the response to the same T-independent antigen in T-cell-deficient mice strongly imply that under our experimental conditions, one of the target cells acted upon by CC is a regulatory T cell. Our observation is analogous to the report by Baker et al. (22, 23) that anti-lymphocyte serum enhanced the response to type III pneumococcal polysaccharide, a T-independent antigen, in intact animals but not in T-cell-deficient nude animals.

Although results up to this point suggested that CC acts on suppressor cells, direct evidence to support the view was lacking. To demonstrate that CC in fact inactivates suppressor cells, we used a well-established system in which specific suppressor T cells are induced by the injection of GT in BALB/c animals (5, 21). It is clear from these experiments that the simultaneous injection of CC with GT effectively prevented the induction of GT-specific suppression of PFC response to GT-MBSA (Table II). Furthermore, the administration of CC to GT-primed donor animals abolished the cell-mediated transfer of suppressive activity to normal syngeneic recipients challenged with GT-MBSA (Table III). This observation provided direct evidence that CC inactivated or eliminated suppressor T cells. It is noteworthy that GT-specific suppression in BALB/c mice can also be abolished by pretreatment with cyclophosphamide (18).

The selective action of CC on the inactivation of suppressor cells or their precursors appears to be a matter of the appropriate time of its administration. Recent studies by Eardley and Sercarz (10) demonstrated that a wave of suppression occurs as the initial event immediately following immunization in a hapten-carrier system, and that carrier-specific suppressor cells are generated as early as 24 h after the injection of antigen. Moreover, such suppression required cell division (7). Hence, the finding that simultaneous administration of CC with an antigen consistently enhances the antibody response (1), and that CC is capable of inactivating suppressor cell activities (Tables II and III), strongly substantiate the view that CC interferes with the generation of the initial wave of suppression, and this in turn leads to a subsequent augmentation of the antibody response.

Of course, the demonstration that CC acts on suppressor cells does not exclude the possibility that it may also be acting on other cell types. For example, we cannot rule out as yet its possible effect on macrophages, and its possible action on helper T cells in the enhancing process. A possible contribu-
tion by macrophages in enhancing the antibody response may be partly the result of increased efficiency of macrophages in processing the antigen. However, CC has been shown to inhibit phagocytosis (24-26) and to interfere with certain metabolic processes in particle ingestion (24, 27). Thus, these observations on the effect of CC upon neutrophil and macrophage functions suggest that the administration of CC with an antigen probably would not significantly facilitate macrophages in antigen processing. It also appears unlikely to us that any interference with the generation of helper T cells by an anti-mitotic dose of CC would facilitate rather than impair helper function. In view of our current finding that the early administration of CC in relation to antigen injection is a prerequisite for successful enhancement of the antibody response, we tend to favor the alternative possibility that suppressor cells or their precursors, which appear early after antigenic stimulation, are its principal targets.

Summary

The simultaneous administration of colchicine (CC) with a T-independent antigen, e.g. 2,4,6-trinitrophenyl—keyhole limpet hemocyanin-Sepharose, to intact animals effectively enhanced their hapten-specific plaque-forming cell (PFC) response. However, in congenitally athymic nude mice in which T-cell regulation was absent, CC was ineffective in producing enhancement. These observations suggest that the target cell acted upon by CC is most likely thymus-derived. Furthermore, the injection of CC with the co-polymer of L-glutamic acid30-L-tyrosine60 (GT) abolished GT-specific suppression of the PFC response to GT-methylated bovine serum albumin. Spleen cells from CC-treated and GT-primed hosts could no longer transfer suppressive activity to normal recipients. These results provide evidence that CC is capable of inactivating or eliminating suppressor cells or their precursors. Thus, CC-induced enhancement of the antibody response may be explained, at least in part, by its anti-mitotic, and hence lethal effect on dividing suppressor T cells.

The authors wish to thank Daniel Cargill and Linda Kapp for their excellent technical assistance.

Received for publication 28 October 1977.

References

1. Shek, P. N., and A. H. Coons. 1977. Effect of colchicine on the antibody response. I. Enhancement of antibody formation in mice. J. Exp. Med. 147:1213.
2. Gershon, R. K. 1974. T cell control of antibody production. Contemp. Top. Immunobiol. 3:1.
3. Tada, T., M. Taniguchi, and T. Takemori. 1975. Properties of primed suppressor T cells and their products. Transplant. Rev. 26:106.
4. Pierce, C. W., and J. A. Kapp. 1976. Regulation of immune responses by suppressor T cells. Contemp. Top. Immunobiol. 5:91.
5. Benacerraf, B., J. A. Kapp, P. Debre, C. W. Pierce, and F. De La Croix. 1975. The stimulation of specific suppressor T cells in genetic non-responder mice by linear random copolymers of L-amino acids. Transplant. Rev. 26:21.
6. Anderson, R. E., and N. L. Warner. 1976. Ionizing radiation and the immune response. Adv. Immunol. 24:215.
7. Eardley, D. D., and E. E. Sercarz. 1977. Recall of specific suppression: co-dominance
INACTIVATION OF SUPPRESSOR CELLS BY COLCHICINE

of suppression after primary or secondary antigen stimulation. J. Immunol. 118:1306.
8. Baney, R. N., J. J. Vasquez, and F. J. Dixon. 1962. Cellular proliferation in relation to antibody synthesis. Proc. Soc. Exp. Biol. Med. 109:1.
9. Rowley, D. A., F. W. Fitch, D. E. Mosier, S. Soliday, L. W. Copleson, and B. W. Brown. 1968. The rate of division of antibody-forming cells during the early primary immune response. J. Exp. Med. 127:983.
10. Eardley, D. D., and E. E. Sercarz. 1976. Modulation of help and suppression in a hapten-carrier system. J. Immunol. 116:600.
11. Yamamoto, H., T. Hamaoka, M. Yoshizawa, M. Kuroki, and M. Kitagawa. 1977. Regulatory functions of hapten-reactive helper and suppressor T lymphocytes. I. Detection and characterization of hapten-reactive suppressor T-cell activity in mice immunized with hapten-isologous protein conjugate. J. Exp. Med. 146:74.
12. Basten, A., J. F. A. P. Miller, and P. Johnson. 1975. T-cell-independent suppression of an anti-hapten antibody response. Transplant. Rev. 26:130.
13. Kapp, J. A., C. W. Pierce, S. Schlossman, and B. Benacerraf. 1974. Genetic control of immune responses in vitro. V. Stimulation of suppressor T cells in nonresponder mice by the terpolymer L-glutamic acid°-L-alanine°-L-tyrosine° (GAT). J. Exp. Med. 140:648.
14. Tada, T. 1974. The mode and sites of action of suppressor T cells in antigen induced differentiation of B cells. In Immunological Tolerance. D. H. Katz and B. Benacerraf, editors. Academic Press, Inc., New York. 471.
15. Rich, R. R., and C. W. Pierce. 1973. Biological expressions of lymphocyte activation. II. Generation of a population of thymus-derived suppressor lymphocytes. J. Exp. Med. 137:649.
16. Debré, P., C. Waltenbaugh, M. Dorf, and B. Benacerraf. 1976. Genetic control of specific immune suppression. III. Mapping of H-2 complex complementing genes controlling immune suppression by the random copolymer L-glutamic acid°-L-tyrosine° (GT). J. Exp. Med. 144:272.
17. Waltenbaugh, C., J. Théze, J. A. Kapp, and B. Benacerraf. 1977. Immunosuppressive factor(s) specific for L-glutamic acid°-L-tyrosine° (GT). III. Generation of suppressor T cells by a suppressive extract derived from GT-primed lymphoid cells. J. Exp. Med. 146:870.
18. Debré, P., C. Waltenbaugh, M. E. Dorf, and B. Benacerraf. 1976. Genetic control of specific immune suppression. IV. Responsiveness to the random copolymer L-glutamic acid°-L-tyrosine° induced in BALB/c mice by cyclophosphamide. J. Exp. Med. 144:277.
19. Feldmann, M., M. F. Greaves, D. C. Parker, and M. B. Rittenberg. 1974. Direct triggering of B lymphocytes by insolubilized antigen. Eur. J. Immunol. 4:591.
20. Kapp, J. A., C. W. Pierce, and B. Benacerraf. 1973. Genetic control of immune responses in vitro. I. Development of primary and secondary plaque-forming cell responses to the random terpolymer L-glutamic acid°-L-alanine°-L-tyrosine° (GAT) by mouse spleen cells in vitro. J. Exp. Med. 138:1107.
21. Debré, P., J. A. Kapp, and B. Benacerraf. 1975. Genetic control of specific immune suppression. I. Experimental conditions for the stimulation of suppressor cells by the copolymer L-glutamic acid°-L-tyrosine° (GT) in nonresponder BALB/c mice. J. Exp. Med. 142:1436.
22. Baker, P. J., P. W. Stashak, D. F. Amabaugh, B. Prescott, and R. F. Barth. 1970. Evidence for the existence of two functionally distinct types of cells which regulate the antibody response to type III pneumococcal polysaccharide. J. Immunol. 105:1581.
23. Baker, P. J., N. D. Reed, P. W. Stashak, D. F. Amabaugh, and B. Prescott. 1973.
Regulation of the antibody response to type III pneumococcal polysaccharide. I. Nature of regulatory cells. *J. Exp. Med.* 137:1431.

24. Lehrer, R. I. 1973. Effects of colchicine and chloramphenicol on the oxidative metabolism and phagocytic activity of human neutrophils. *J. Infect. Dis.* 127:40.

25. Pesanti, E. L., and S. G. Axline. 1975. Colchicine effects on lysosomal enzyme induction and intracellular degradation in the cultivated macrophage. *J. Exp. Med.* 141:1030.

26. Chang, Y. H. 1975. Mechanism of action of colchicine. II. Effects of colchicine and its analogs on phagocytosis and chemotaxis in vitro. *J. Pharmacol. Exp. Ther.* 194:159.

27. Goldfinger, S. E., R. R. Howell, and J. E. Seegmiller. 1965. Suppression of metabolic accompaniments of phagocytosis by colchicine. *Arthritis Rheum.* 8:1112.