Effect of Immunosuppressive Agents on Retention of Antigen in the Mouse Spleen[1] (34547)

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(Introduced by E. H. Perkins)

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Germinal centers are formed in lymphatic tissues in response to antigenic stimulation and are the sites of intense lymphoid cell proliferation (1, 2). A number of investigators have shown that antigen is concentrated and preferentially retained on the surface of dendritic reticular cells in these germinal centers (3–6). The close topographic relationship between antigen and proliferating lymphoid cells is thought to be of functional significance, particularly during the later phases of antibody response (7–9).

Recent evidence demonstrated that the mechanism of antigen trapping in germinal centers is sensitive to X-irradiation (10–12). We decided to determine whether other immunosuppressive agents would similarly interfere with antigen trapping and retention in lymphatic tissue. The present communication is an account of these studies.

Materials and Methods. Animals. Twelve-week-old male specific-pathogen-free BC3F1 (C57BL/6♀ × C3H/An ♂)F_1 mice were used. The mice were kept 8 to 10 in a cage and allowed free access to food and water.

Antigen. ^{125}I-labeled human gamma globulin was aggregated (AHGG) as described previously (13) and injected intravenously (iv) in amounts of 0.1 mg/mouse either before or after treatment with immunosuppressive agents.

Immunosuppressants. The following agents, known to suppress antibody formation, were utilized: whole-body X-irradiation (400 R), actinomycin D[2] (0.9 mg/kg); cyclophosphamide[2] (300 mg/kg); and cortisone acetate[3] (500 mg/kg). These dose levels were selected on the basis of preliminary experiments showing that they were nonlethal, caused comparable reduction in spleen weights, and suppressed the hemagglutinin response to sheep red blood cells. All chemicals were dissolved or suspended in sterile physiological saline and injected intraperitoneally.

Experimental Procedure. The immunosuppressive treatment was given either 3 days before or 2 days after administration of ^{125}I-AHGG (0.1 mg). Elimination of antigen from serum, liver, and spleen was determined with the use of an autogamma spectrometer. Histological preparations and autoradiograms of spleen and liver sections were prepared and exposed for 8 weeks as described previously (2).

Results. To study antigen retention in spleen germinal centers of mice previously exposed to immunosuppressive agents, ^{125}I-AHGG (2.5×10^6 cpm) was injected iv 3 days after the chemical or physical insult. This was the time of maximum spleen weight reduction. Groups of 5 mice were killed on days 1, 3, and 7 after antigen injection. Figure 1 summarizes the effects of the pretreatment on elimination of radioactive antigen from the serum. Also shown are the effects on its localization in and elimination from liver and spleen. On days 1 and 3 after antigen

[1] Research jointly sponsored by the National Cancer Institute and the U. S. Atomic Energy Commission, under contract with the Union Carbide Corporation.

[2] We are grateful to Merck, Sharp and Dohme Research Laboratories, Rahway, New Jersey, for generous supply of actinomycin D, and to Mead Johnson Research Center, Evansville, Indiana, for generous supply of cyclophosphamide.

[3] Purchased from Mann Research Laboratories, New York, New York.

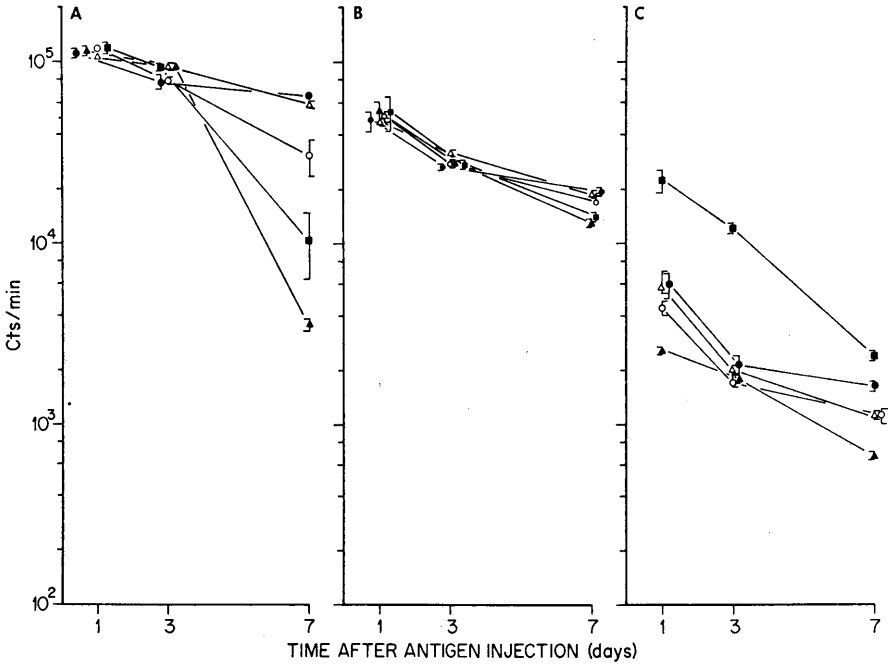

FIG. 1. Effect of immunosuppressive agents, given 3 days before injection of ^{125}I-AHGG, on elimination of the antigen from (A) serum (cpm/1 ml serum); (B) liver; and (C) spleen. Mice were treated with X-ray (△); actinomycin D(○); cortisone acetate (▲); cyclophosphamide (●); or they remained untreated (■). Each point represents 5 animals; horizontal bars represent one standard error of the mean.

injection, the level of radioactivity in the serum was approximately the same for all groups, including the untreated controls. Between days 3 and 7, reduction in serum antigen levels typical of immune elimination occurred in the untreated control group, the cortisone acetate group, and (although to a lesser extent) the actinomycin D-treated group, as evidenced by an 8-fold, 25-fold, and 2-fold reduction, respectively, in serum radioactivity. The reason for the enhanced elimination in the cortisone-treated group is presently unknown. No immune elimination was observed in the groups treated with X-irradiation or cyclophosphamide. In contrast to these marked differences in serum antigen levels observed in the various groups, the initial uptake and the subsequent retention of antigen by the liver was similar in all cases and unaffected by the immunosuppressive treatment. Conversely, the spleen's capacity to localize and retain antigen was markedly reduced by all four immunosuppressants tested. Part of this reduction in uptake and retention of antigen can be accounted for by the spleen weight loss caused by the various agents. However, calculation of the amount of radioactivity per milligram of spleen revealed that the capacity to localize antigen per unit of spleen tissue was also significantly diminished. It should be noted that retention of antigen in spleen and liver was independent of serum antigen levels. Autoradiograms prepared from the spleen sections showed a considerable decrease in the number of lymph follicles containing radioactive label, as well as in the amount of label retained per follicle in all treatment groups. With the doses applied in the present experiment, antigen localization in germinal centers decreased in the following order: controls $>$400 R $>$ actinomycin $\geq$cyclophosphamide $>$cortisone acetate. In Fig. 2, antigen localization in spleens from control and from cortisone acetate-treated mice on day 3 after injection is compared.

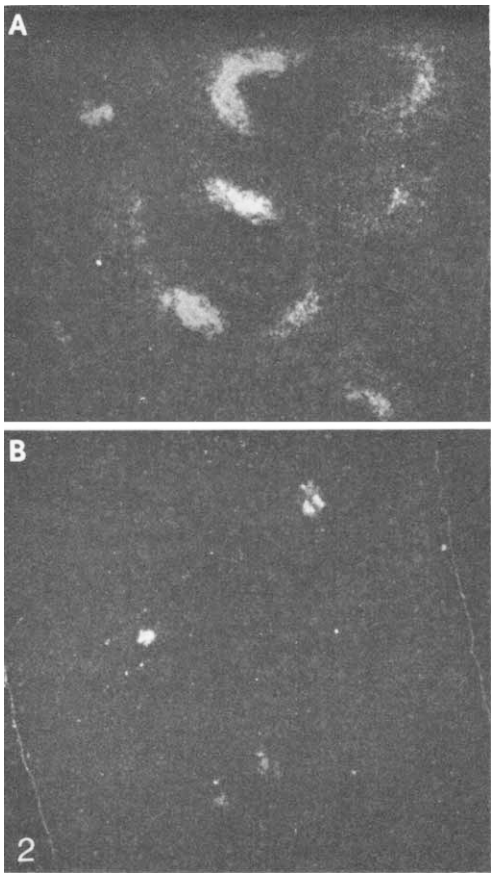

FIG. 2. Effect of cortisone acetate, given 3 days before injection of ^{125}I-AHGG, on antigen localization in spleen germinal centers, day 3 after injection of the test antigen. Darkfield photomicrographs of spleen lymphatic nodule (A) of untreated control mouse, and (b) of cortisone acetate-treated mouse; ~85×.

To determine the effect of the respective immunosuppressive agents on retention of antigen already localized in germinal centers, mice were injected with ^{125}I-AHGG (1.2×10^6 cpm) and 48 hr later the immunosuppressive agents were given (the doses were the same as in the previous experiment). The results are summarized in Fig. 3. As shown, no immune elimination occurred from the serum of animals that received actinomycin or cyclophosphamide; it did occur, however, in both the cortisone-treated and the 400-R-irradiated group, although the onset of immune elimination was clearly delayed in the latter. Retention of radioactive antigen by the liver (not shown in Fig. 3) did not show any particular change, regardless of treatment. The overall spleen capacity to retain antigen was not markedly affected under the present experimental conditions, though from day 6 after antigen injection on, which is day 4 after immunosuppressive treatment, the levels of antigen in the spleens of treated mice were consistently lower than in spleens of control mice. With the doses applied, cortisone acetate was the most effective in this regard. However, when the data were corrected for the spleen weight changes occurring after treatment, the amount of antigen retained in spleen tissue of treated animals was

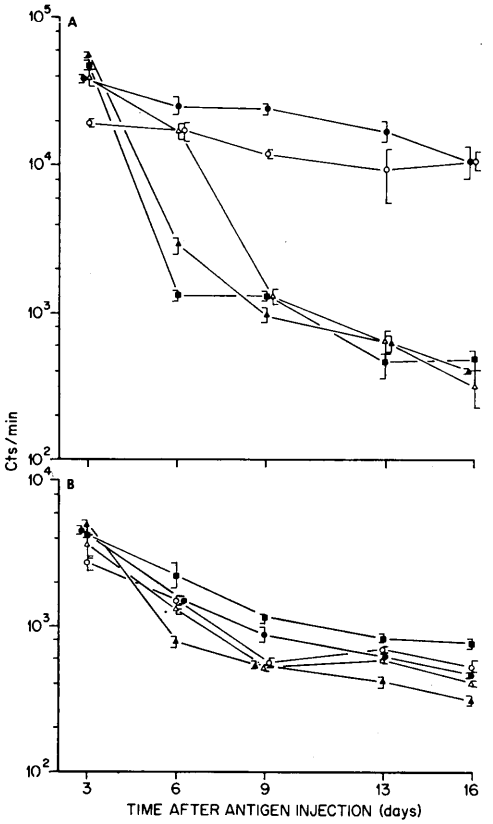

FIG. 3. Effect of immunosuppressive agents, given 2 days after injection of ^{125}I-AHGG, on elimination of the antigen from (A) serum (cpm/1 ml serum), and (B) spleen. Mice were treated with X-ray (Δ); actinomycin D(○); cortisone acetate (▲); cyclophosphamide (●); or remained untreated (■). Each point represents 5 animals; horizontal bars represent one standard error of the mean.

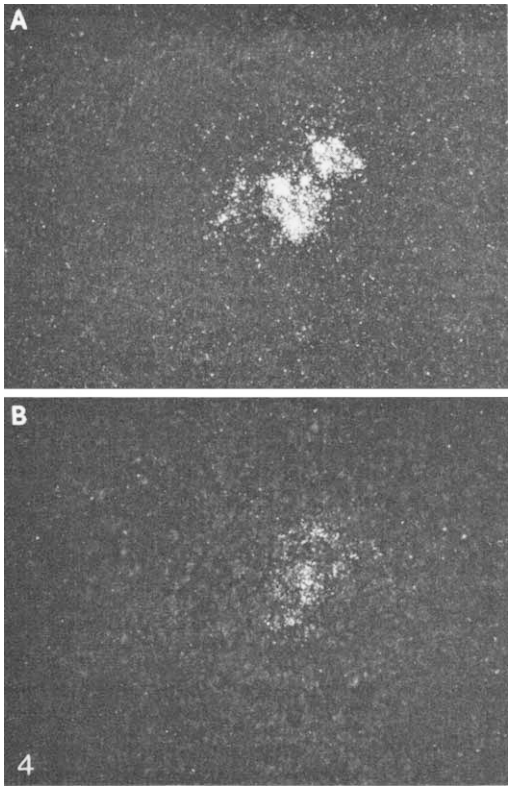

FIG. 4. Effect of actinomycin D, given 2 days after injection of ^{125}I-AHGG, on antigen localization in spleen germinal centers, day 9 after the injection of the test antigen. Darkfield photomicrograph of spleen lymphatic nodule (A) of untreated mouse; (B) of actinomycin D-treated mouse; ~310×.

actually equal to or higher than that in the control group on days 3, 6, and 9 after antigen (with the noted exception of the cortisone acetate group). Only at later time points, *i.e.*, days 13 and 16 after antigen injection, did the radioactivity counts per mg spleen weight of the treated groups fall below those of untreated controls. The retention of antigen in the spleen lymphatic nodules as visualized by the autoradiograms followed a similar pattern. No clear-cut difference between controls and treatment groups could be observed on days 3 and 6 after antigen; however, from day 9 after antigen injection (*i.e.*, day 7 after insult) on, the number of labeled follicles, as well as the amount of label per follicle, was markedly lower in all treatment groups, when compared to the controls (Fig. 4). Only in the cortisone-treated group did a defect in antigen retention become obvious before day 9 after antigen injection. At the presently employed dose levels, cortisone acetate was the most effective in reducing antigen retention in spleen germinal centers.

Discussion. The primary purpose of the presented experiments was to determine whether known immunosuppressive agents would affect antigen retention in spleen germinal centers. Since the agents were studied only at one particular dose, a quantitative comparison of their relative activity is not possible. We found that all four immunosuppressants tested, whether given before or after antigen, do impair the capacity of spleen tissue—and germinal centers in particular—to localize and retain antigen. With the doses applied, cortisone acetate and cyclophosphamide were most effective in this regard. Antigen localization was more easily impaired when the immunosuppressants were given before, rather than after the antigen.

The mechanism by which these agents interfere with antigen trapping and subsequent retention is presently unknown. One possibility is that the antigen-retaining cells themselves are damaged. Previous studies with higher X-ray exposures revealed morphologic changes that suggest cell injury to germinal center reticular cells (12). Another possibility is interference with some ancillary mechanisms such as destruction of lymphocytes elaborating opsonic factors (14, 15). A decrease in the capacity to retain antigen became visible only at approximately 1 week after treatment (with the noted exception of cortisone acetate), when the antigen injection preceded the injury. This delayed effect could be indicative of a rather firm binding of the antigen to the reticular cell surface, even in the presence of cellular injury. The experiments also suggest that the mechanism responsible for trapping of antigen on reticular cell surfaces is more sensitive to injury than is the mechanism responsible for continued retention. Other yet unpublished data show that depression of antigen retention is manifested within 24 hr after injury, when the injurious agent is given before antigen.

Of interest is the finding that the amount of radioactive antigen retained in liver and spleen was largely independent of the serum antigen levels. One might have expected that organs with great phagocytic activity, such as the liver and the spleen, would be instrumental in removing antigen–antibody complexes from the serum and that the level of radioactivity would therefore increase in these organs along with a drop in serum radioactivity in those groups in which antibody formation ensued. This was clearly not the case. If, however, the kidney is mainly responsible for clearance of antigen–antibody complexes, it would have been reasonable to predict that antigen would be "drained" out of the spleen and liver with the onset of immune elimination from the serum and that the antigen clearance from spleen and liver would parallel that of the serum. However, the data reveal that the rate of antigen clearance from liver and spleen was not appreciably affected by the onset of immune clearance from the serum. This suggests that these tissues bind the antigen rather firmly and retain it, at least for some time, even against a rather steep gradient.

Also of significance is the difference in response of liver and spleen to the immunosuppressive agents. Both organs contain a vast number of phagocytic elements (presumably responsible for handling particulate antigens); yet liver tissue of treated animals exhibited no apparent defect in antigen uptake and retention, while spleen tissue was significantly impaired in this respect (even when correction was made for the spleen weight changes). This suggests that the mechanism of antigen uptake and/or retention is *not* the same in these two organs. Inhibition of phagocytosis may not be the major factor in the X-ray- and drug-induced suppression of antigen retention in the spleen.

It was recently shown that cortisone acetate preferentially inhibits the late antibody response (16) and suppresses production of 7*S* antibody (17), particularly when given in close time relationship to the antigenic stimulus. In the present investigation cortisone acetate did not inhibit immune elimination, thus suggesting that the *early* antibody response was not markedly affected; however, this drug very strongly interfered with antigen retention in spleen germinal centers. This observation complements previous findings which indicate that the antigen-induced germinal center hyperplasia is more closely related to the late 7*S* than to the early 19*S* antibody response (7, 9). Our present findings would suggest that the functional significance of antigen retention by germinal center reticular cells might be to provide the continuous antigenic stimulus necessary for prolonged antibody production (particularly 7*S* antibody) during the late stages of the immune response. The effect of cortisone acetate on the later phases of antibody production (16) could therefore be a consequence of a rapid depletion of antigen depots in germinal centers. However, this interpretation is complicated by the fact that cortisone acetate (suspended in saline) persists in the tissues for several days after injection (whether given intraperitoneally or subcutaneously), as evidenced by a small pellet regularly found near the injection site (unpublished findings). Thus, the more "chronic" effects of this drug may be due, at least in part, to a continuous destruction of lymphocytes, particularly when given by repeated injections (16). Our findings also stress the fact that immunosuppressive agents not only damage and destroy immunologically competent cells, but also reduce uptake and retention of antigen by lymphatic tissues.

Summary. The effects of four immunosuppressive agents (X-ray, actinomycin D, cyclophosphamide, and cortisone acetate) on localization and retention of radioactive antigen in liver and spleen were studied. All four agents interfered with antigen retention by spleen and spleen germinal centers but *not* with that by the liver; this damage of splenic antigen retention was more easily induced when the insult was given before the test antigen. At the dose applied (500 mg/kg) cortisone acetate did not interfere with immune elimination—a sign of early antibody production—but most severely damaged retention of antigen in spleen germinal centers. The data are consistent with the hypothesis that the antigen depots in germinal centers are

of functional significance for the late (particularly 7*S*) antibody production. Our experiments also emphasize the dual effect of immunosuppressive agents on lymphatic organs: (i) reduction of their immunologically responsive cell population, and (ii) impairment of their capacity to capture and retain antigen.

1. Hanna, M. G., Jr., Int. Arch. Allergy Appl. Immunol. **26**, 230 (1965).

2. Hanna, M. G., Jr., Nettesheim, P., and Walburg, H. E., Jr., *in* "Germ-Free Biology" (E. A. Mirand and N. Back, eds.), p. 237. Plenum, New York (1969).

3. White, R. G., *in* Ciba Foundation Study Group No. 16, "The Immunologically Competent Cell: Its Nature and Origin" (G. E. Wolstenholme and J. Knight, eds.), p. 16. Churchill, London (1963).

4. Mitchell, J. and Abbot, A., Nature (London) **208**, 500 (1965).

5. Szakal, A. K. and Hanna, M. G., Jr., Exptl. Mol. Pathol. **8**, 75 (1968).

6. Nossal, G. J. V., Abbot, A., Mitchell, J., and Lummus, Z., J. Exptl. Med. **127**, 277 (1968).

7. Fitch, F. W., Stejskal, R., and Rowley, D. A., *in* "Lymphatic Tissue and Germinal Centers in Immune Response" (L. Fiore-Donati and M. G. Hanna, Jr., eds.), p. 223. Plenum, New York (1969).

8. Thorbecke, G. J. and Hurlimann, J., Nouv. Rev. Fr. Hematol. **5**(3), 385 (1965).

9. Hanna, M. G., Jr., Nettesheim, P., and Francis, M. W., J. Exptl. Med. **129**, 953 (1969).

10. Jaroslow, B. N. and Nossal, G. J. V., Aust. J. Exptl. Biol. Med. Sci. **44**, 609 (1966).

11. Hunter, R. L., Wissler, R. W., and Fitch, F. W., *in* "Lymphatic Tissue and Germinal Centers in Immune Response" (L. Fiore-Donati and M. G. Hanna, Jr., eds.), p. 101. Plenum, New York (1969).

12. Nettesheim, P. and Hanna, M. G., Jr., *in* "Lymphatic Tissue and Germinal Centers in Immune Response" (L. Fiore-Donati and M. G. Hanna, Jr., eds.), p. 167. Plenum, New York (1969).

13. Sassen, A., Perkins, E. H., and Brown, R. A., Immunology **14**, 247 (1968).

14. Williams, G. M., Immunology **11**, 467 (1966).

15. Williams, G. M., Immunology **11**, 475 (1966).

16. Dukor, P. and Dietrich, F. M., Int. Arch. Allergy Appl. Immunol. **32**, 131 (1967).

17. Elliot, E. V. and Sinclair, R. N. St. C., Immunology **15**, 643 (1968).

Received Sept. 29, 1969. P.S.E.B.M., 1970, Vol. 133.