Prevention of Cyclophosphamide-Induced Tolerance to Erythrocytes by Pretreatment with Cortisone (34456)

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According to current concepts of the mechanism of immunological paralysis, nonspecific suppression of the immune response is likely to facilitate the induction of specific tolerance (1). However, representatives of distinct classes of immunosuppressants have been shown to differ in their capacity to promote specific unresponsiveness. Thus, tolerance to heterologous red cells is promptly induced in adult mice by injecting large amounts of antigen together with a moderate dose of cyclophosphamide (2–4). By contrast, administration of cortisone is associated with a striking nonspecific inhibition of antibody formation, but fails to elicit specific unresponsiveness to strongly immunogenic foreign erythrocytes (5, 6).

Cyclophosphamide-induced tolerance can readily be explained in terms of the antiproliferative properties of akylating agents (6, 7). Steroids, on the other hand, are known to diminish the number of small lymphocytes and to interfere with antigen-handling by phagocytic elements (8). Elimination of immunologically competent lymphoid cells by cortisone would be expected to favor the development of specific tolerance. Impairment of phagocytic functions, however, could well have the opposite effect. Certain antigens may not only have to be processed in order to become immunogenic, but also to become tolerogenic: phagocytic dissociation of large particulate antigens into smaller fragments may be a prerequisite for their tolerogenicity. Steroids could conceivably abolish this step.

These studies were undertaken to determine whether pretreatment with cortisone acetate would either facilitate or prevent the establishment of cyclophosphamide-induced unresponsiveness to heterologous erythrocytes. The results presented in this communication indicate that cortisone blocks a pathway leading both to tolerance and to immunity.

Materials and Methods. Six-week-old colony bred male Swiss albino mice (Charles River CD-1, obtained from Charles River, France S.A.) weighing 18 to 22 g, in groups of seven to ten received at the beginning of the experiment (Day 0) a single subcutaneous (sc) injection of either 125, 250, or 500 mg/kg cortisone acetate (CIBA). The drug was suspended in phosphate-buffered saline (0.15 *M*, pH 7.4) (PBS) containing 0.5% carboxymethylcellulose.

On the next day (Day 1), washed red blood cells from sheep (SRBC) or guinea pig (GPRBC) were administered intraperitoneally (ip) or intravenously (iv) at five different dose levels as indicated below. Cell suspensions were standardized using an electronic particle counter (Coulter Model B).

Again 1 day later (Day 2), the mice were injected with a single dose of either 25, 50, or 100 mg/kg cyclophosphamide (Endoxan, Asta) in PBS sc. Groups receiving no antigen, antigen alone, only one of the immunosuppressive agents, or no pretreatment at all were also included. The timing for the administration of both cortisone acetate and cyclophosphamide relative to the injection of antigen was chosen according to the previously established conditions for maximum immunosuppressive activity of either drug (9).

One week after immunization (Day 8), the animals were bled by retroorbital puncture, re-injected with 6.3×10^8 SRBC, or $6.6 \times$

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10^8 GPRBC on the same day and bled again on Day 14. Serial twofold dilutions of individual sera were analyzed for hemagglutinins using a Takatsy microtiter outfit (10). The titers were expressed as logarithms to the base 2 of the reciprocal of the final dilution showing agglutination. Titer 0 was assigned to sera producing no hemagglutination at a dilution of 1:2.

The effect of a single component of the treatment schedule on antibody production before or after the antigenic challenge on Day 8 was determined by calculating a suppressive index (SI). It was defined as the ratio of average antibody titer of a treated group of animals to average titer obtained from appropriate controls, in which one of the components of the treatment schedule was omitted.

Results. The checkerboard depicted in Fig. 1 represents the mean hemagglutinin titers of unchallenged mice treated with 80 different combinations of cortisone acetate, SRBC, and cyclophosphamide, and bled 7 days after immunization. It is evident that both compounds exerted a considerable drug dose-dependent inhibition of antibody formation. The effect of treatment with cortisone alone (outer left vertical row in Fig. 1) was much enhanced when small numbers of erythrocytes were used for immunization. Contrariwise, immunosuppression by cyclophosphamide (top horizontal row in Fig. 1) did not seem to depend on the antigen dose employed. Combinations of the two drugs with each other produced complex patterns of mutual potentiation and inhibition. In order to visualize this more clearly, the effect of either compound on immunosuppression by the other was considered separately by calculating the appropriate suppressive indices. Values > 1.0 indicate abrogation, indices < 1.0 enhancement of immunosuppression by adding the second drug.

Figure 2 shows the influence of cyclophosphamide on antibody formation in cortisone-treated mice. Cyclophosphamide (25 mg/kg) antagonized immunosuppression by cortisone, while 50 mg/kg had the opposite effect. However, the extent of abolition or

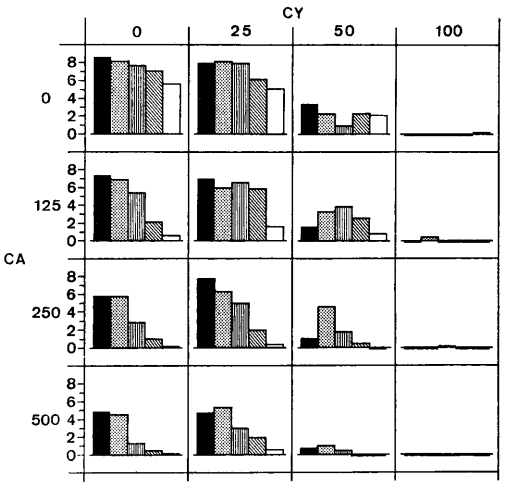

FIG. 1. Effect of cortisone acetate (CA) and cyclophosphamide (CY), given in combination, on hemagglutinin formation to various doses of sheep red blood cells (SRBC). Figures on the ordinate denote doses of CA (mg/kg) injected subcutaneously on Day 0, while those on the abscissa denote doses of CY (mg/kg) injected subcutaneously on Day 2; the five bars in each square represent, from left to right, mean titers on Day 8 in groups of eight animals injected intraperitoneally with 5.9×10^9, 1.1×10^9, 2.4×10^8, 4.7×10^7, or 9.4×10^6 SRBC on Day 1.

potentiation depended on the amount of immunizing antigen. Increasing additive immunosuppression by cyclophosphamide was seen in animals receiving large numbers of erythrocytes, whereas reversion of the cortisone effect was restricted to the lower range of antigen doses.

An altogether different pattern applied to the effect of pretreatment with cortisone on cyclophosphamide-induced inhibition of antibody formation (Fig. 3). Here, 125 and 250 mg/kg cortisone acetate blocked immunosuppression by 50 mg/kg cyclophosphamide, provided the mice were immunized with intermediate doses of antigen. All other drug combinations revealed an additive effect of cortisone which was most marked if small amounts of erythrocytes were used for immunization.

Agglutinin titers were reassessed 6 days after challenge with an optimal immunizing dose of SRBC on Day 8. As shown in Fig. 4, antibody responses were greatly impaired or

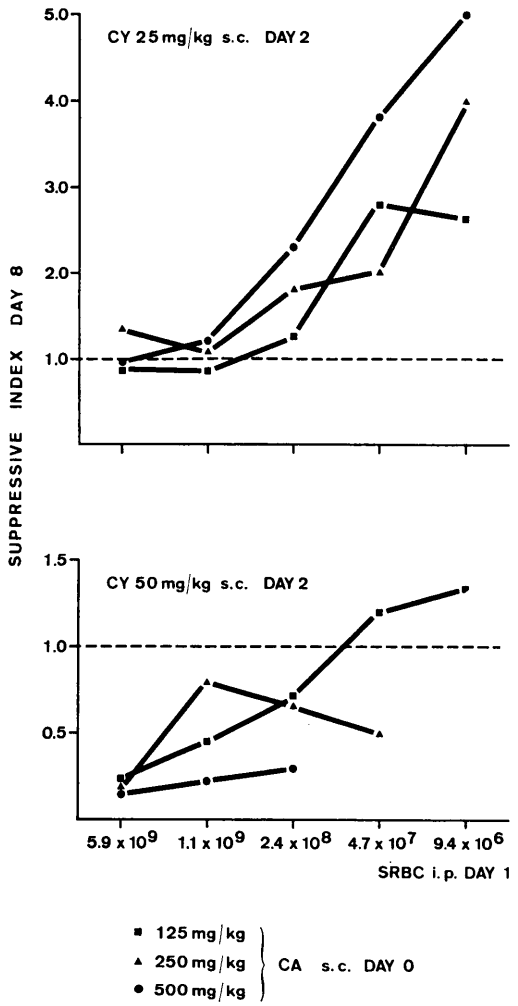

FIG. 2. Effect of cyclophosphamide (CY) on inhibition of hemagglutinin formation to various doses of sheep red blood cells (SRBC) by cortisone acetate (CA). Suppressive index: (Mean Day 8 titers from groups of eight mice injected with CA on Day 0 and CY on Day 2) / (Mean titers from groups receiving CA only).

altogether absent after pretreatment with 5.9 $\times$ 10^9 SRBC and 100 mg/kg cyclophosphamide. Lesser doses of cells or drug (data not included in Fig. 4) failed to induce tolerance. Intravenous administration of the antigen was slightly more effective than when it was given intraperitoneally. Almost the same results were obtained with GPRBC which are less immunogenic in the mouse than SRBC (11). Accordingly, smaller numbers of GPRBC were required in order to produce unresponsiveness. Specificity of the tolerant state was demonstrated by the normal or near-normal responses of tolerant animals to a challenge with noncrossreacting erythrocytes of comparable or weaker immunogenicity (3, 6).

In contrast to cyclophosphamide, cortisone acetate was unable to abolish reactivity to either type of cells irrespective of the dosage of both drug and antigen. As is further evi-

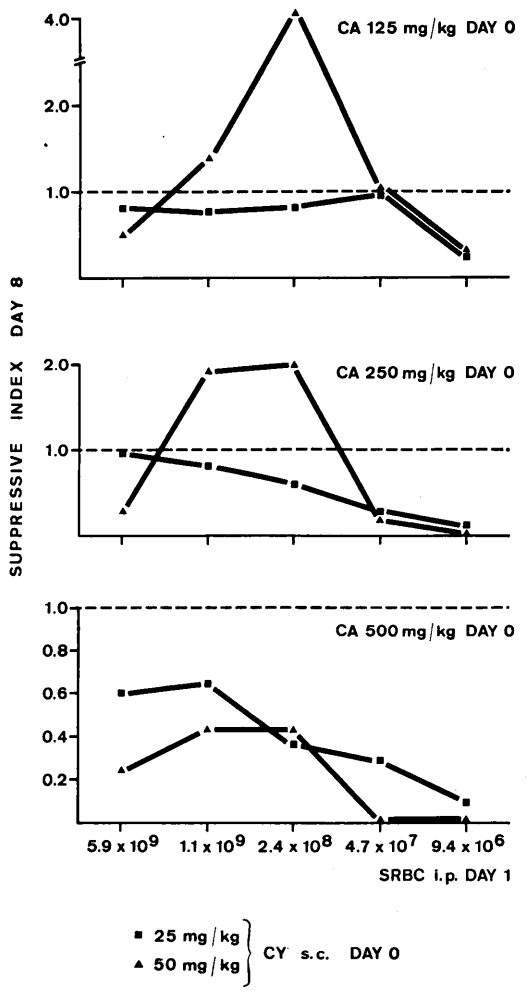

FIG. 3. Effect of cortisone acetate (CA) on inhibition of hemagglutinin formation to various doses of sheep red blood cells (SRBC) by cyclophosphamide (CY). Suppressive index: (Mean Day 8 titers from groups of eight mice injected with CA on Day 0 and CY on Day 2) / (Mean titers from groups receiving CY only).

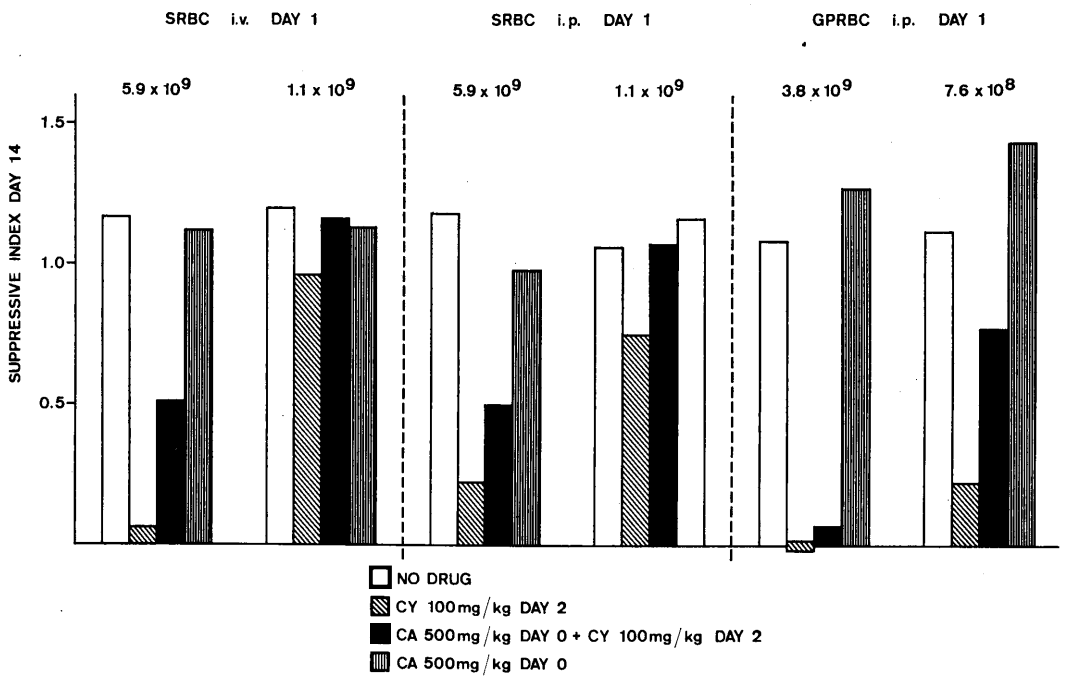

FIG. 4. Effect of red blood cells, cortisone acetate (CA), and cyclophosphamide (CY), given in combination, on responsiveness to a challenge injection of 6.3×10^8 sheep red blood cells (SRBC) or 6.6×10^8 guinea pig red blood cells (GPRBC) given 7 days after injection of the homologous antigen on Day 1. Suppressive index: (Mean Day 14 hemagglutinin titers from groups of 7 to 10 mice injected with red cells on Day 1) / (Mean titers from groups receiving no antigen on Day 1).

dent from Fig. 4, combination of the steroid with cyclophosphamide failed to reduce the amount of erythrocytes necessary for the establishment of tolerance. Moreover, cortisone seemed even to prevent paralysis. While eight of ten animals injected with the largest dose of SRBC (iv) and cyclophosphamide did not produce any detectable agglutinins at the time of sampling, antibody activity was found in all the sera of a group of seven mice which had been pretreated in addition with 500 mg/kg cortisone acetate. Similar results were obtained in the groups receiving SRBC or GPRBC by the intraperitoneal route. Still, tolerance induced with the highest dose of GPRBC could apparently not be prevented by cortisone.

Discussion. The combined administration of cortisone acetate and cyclophosphamide altered the degree of immunosuppression which was observed when either drug was given alone. Before reinjection of the antigen, addition of small doses of either compound tended to inhibit the suppressive effect of the other one. Contrariwise, addition of large doses led to an increased suppression of antibody formation.

Synergistic and antagonistic actions of methotrexate and cortisone given in combination with antilymphocytic serum in a homotransplantation system have been described (12). But in view of the great differences between the two experimental models it would seem difficult to draw any valid comparisons. On the other hand, several laboratories have clearly shown that immune responses may be enhanced by small doses of either cortisone (13–16) or antiproliferative agents (17). Such adjuvant effects may account for the apparent abolition of the immunosuppressive action of one drug by adding small doses of another.

Both mutual potentiation and inhibition depended on the amount of antigen used for immunization. In keeping with earlier findings (9, 18) there was an inverse relation

between the suppressive activity of the steroid and the initial antigen dosage. In the present experiments, the effectiveness of cyclophosphamide did not seem to be influenced by the amount of antigen injected, but a positive correlation between red cell numbers and the sensitivity of the hemagglutinin response to cyclophosphamide has been reported in slightly different systems (9, 19). Antigen dose-dependence of the additive drug effects described in this paper would thus seem to follow a simple pattern: enhancement of the immunosuppressive action of compound A by adding compound B is most pronounced where the suppressive effect of A alone is minimal and that of B alone is maximal.

While cyclophosphamide effectively fostered tolerance to SRBC and GPRBC, cortisone failed to do so. Pretreatment with cortisone was even found to prevent the establishment of cyclophosphamide-induced paralysis. This observation appears to be at variance with earlier reports that corticosteroids facilitate the development of specific unresponsiveness to bovine γ-globulin in mice (20), and skin allografts in newly hatched chicks (21), and that hydrocortisone may delay the spontaneous loss of tolerance to allogeneic red cells in chickens (22). Nevertheless, both sets of data can be reconciled with each other.

It was considered possible (see above) that cortisone might block or delay the disassembly in phagocytic cells of large particulate antigens into smaller tolerogenic fragments. Such a hypothesis is supported by the data reported in this paper. However, this mechanism may not operate in the case of soluble antigens, such as serum proteins, which are probably already tolerogenic before, but probably not if they have been processed by macrophages. Development of tolerance to skin homografts, on the other hand, is likely to involve different compartments of antigen-reactive cells and different kinetics of antigen-release than paralysis by antigenic components of dissociated cells which are injected by the intravenous or intraperitoneal route. Finally, maintenance of an already established state of unresponsiveness probably no longer depends on the rapid processing of very large amounts of antigenic material within a critical period of time. The seemingly contradictory effects of cortisone in a variety of systems may simply reflect the diversity of rate-limiting steps involved in induction and loss of tolerance to different types of antigen.

Summary. Inhibition of antibody formation to heterologous red cells in mice was studied using combinations of cortisone acetate with cyclophosphamide. The two drugs mutually potentiated or antagonized each other. Interaction between the compounds depended on the dose of antigen used for immunization. Pretreatment with cortisone prevented the development of cyclophosphamide-induced tolerance. It is suggested that in the case of large particulate antigens steroids may block a common pathway leading both to tolerance and to immunity.

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