Immunology, 1968, **15**, 643.

Effect of Cortisone Acetate on 19S and 7S Haemolysin Antibody

A TIME COURSE STUDY

E. V. ELLIOTT AND N. R. ST C. SINCLAIR

Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, Fulham Road, London, S.W.3; and Cancer Research Laboratory, The University of Western Ontario, London, Ontario, Canada

(*Received* 24*th February* 1968)

Summary. A single subcutaneous injection of cortisone acetate (400–500 mg/kg body weight) depressed the serum haemolysin response of adult Swiss mice to sheep erythrocytes when administered near the time of antigen injection. The greatest suppression of the early haemolysin response occurred when cortisone was injected 3–4 days prior to antigen, while the late antibody response (20–30 days after antigen injection) was markedly decreased when cortisone was given up to 4 days after antigen. Cortisone depressed both 19S and 7S haemolysin when given prior to antigen, but depressed only the 7S antibody when administered after antigen. An attempt was made to correlate the depression of the immune response with the decrease in lymphoid tissue following cortisone treatment and a striking correlation was observed between the number of circulating lymphocytes at the time of antigen injection and the 7 day titres of total and 19S antibody.

INTRODUCTION

The immunosuppressive effect of an agent depends on numerous factors including the nature of the agent, dose, route of administration, number of times the agent is given, timing of the administration of the agent in relation to the antigenic stimulus, nature of the antigenic stimulus, and the method of analysis of the antibody response attained. It is difficult to cover all these variables in any one study, and more information regarding the influence exerted by these variables is needed in the elucidation of the mechanism of action of immunosuppressive agents.

The haemolysin response to sheep erythrocytes is a process with many phases, consisting of the early production of 19S antibody followed by the production of 7S antibody (Adler, 1965; Dietrich, 1966; Sinclair, 1967a, b). In assessing the mode of action of an immunosuppressive agent, it is important to demonstrate the effect of the immunosuppressive agent on the 19S and 7S response separately (Sahiar and Schwartz, 1965), and to observe how this effect on 19S and 7S antibody is altered when the timing of a single exposure to the immunosuppressive agent is varied with respect to the time of antigen administration (Berenbaum, 1964). The present study demonstrates that cortisone acetate, administered prior to antigen, decreased both 19S and 7S haemolysin titres, while 7S

antibody was lowered to a far greater extent than 19S when cortisone acetate was injected after exposure to antigen.

Cortisone acetate has been shown to suppress the primary haemolysin response to sheep erythrocytes (Berglund, 1956a, b; Berglund and Fagraeus, 1956, 1961) and antibody responses to other antigens (Berglund, 1965c) in rats and mice. The powerful cytolytic effect of cortisone on lymphocytes in the circulation and in lymphoid tissues has been well documented (reviewed by Dougherty, 1952; Ishidate and Metcalf, 1963). The present report deals with a correlative study of the relationship between lymphoid depletion and the suppression of the primary haemolysin response. The most striking correlation observed was between the circulating lymphocyte levels at the time of antigen administration and the total and 19S haemolysin titres at 7 days following antigen injection.

MATERIALS AND METHODS

Mice

Colony bred male and female Swiss albino mice were used for all the experiments, and were maintained under conventional conditions. The animals were given water and commercial cubed food *ad libitum*. Mice were weaned and separated according to sex at 1 month of age.

Cortisone acetate injection

A single subcutaneous injection of cortisone acetate (Organon) was administered to adult mice, 8–12 weeks of age, weighing 27–32 g. The cortisone acetate was absorbed on carboxy-methyl cellulose, and 0·5 ml (400–500 mg/kg body weight) was injected. The experiment was controlled with uninjected mice. Cortisone acetate was administered from 32 days before to 10 days after antigen.

Immunization

Sheep erythrocytes in Alsever's solution (Wellcome Research Laboratories, Beckenham, England) were washed four times in 0·9 per cent saline. All mice received 0·1 ml of a 10 per cent suspension (2×10^8 erythrocytes) intraperitoneally.

Measurement of serum haemolysin activity

Blood was obtained from the retro-orbital sinus, and diluted 2:1 with 0·9 per cent saline to avoid gel formation in the serum. The serum was collected following centrifugation, and endogenous complement inactivated by incubating the serum at 56° for 30 minutes. Serum samples were serially diluted 1:1 with 0·9 per cent saline in Microtiter plates (Cooke Engineering Co., Arlington, Virginia, U.S.A.) (Sever, 1962). A standard amount of guinea-pig complement (Wellcome Research Laboratories) was added, and the serum and complement incubated for 30 minutes at 37°. The pre-incubation of serum and complement lowered the incidence of titrations which were negative at low dilutions but became positive at higher dilutions. Washed sheep erythrocytes (0·05 ml of a 0·5 per cent suspension) were added and the complete mixture incubated for 2 hours at 37° and then read, the end point being roughly half haemolysis on visual inspection. The plates were stored overnight at room temperature and read again the following morning. Titres were usually one $\log_2$ unit higher on the second reading. All titres are expressed as the $\log_2$ of the dilution.

Ultracentrifugation

Pooled serum, layered on a 10–40 per cent linear sucrose gradient made up to 0·9 per cent with respect to NaCl concentration, was centrifuged in a Spinco Model L ultracentrifuge at 35,000 rev/min for 18 hours at 5° (Kunkel, 1960). Most of the serum samples were spun in 5 ml nitrocellulose tubes in either the SW 39 or the SW 50 rotor. Other serum samples were centrifuged in the Type 50 titanium fixed-angle rotor, fitted with adapters to accommodate $\frac{1}{2} \times 2\frac{1}{2}$ in. polyallomer tubes. The separation of 19S and 7S

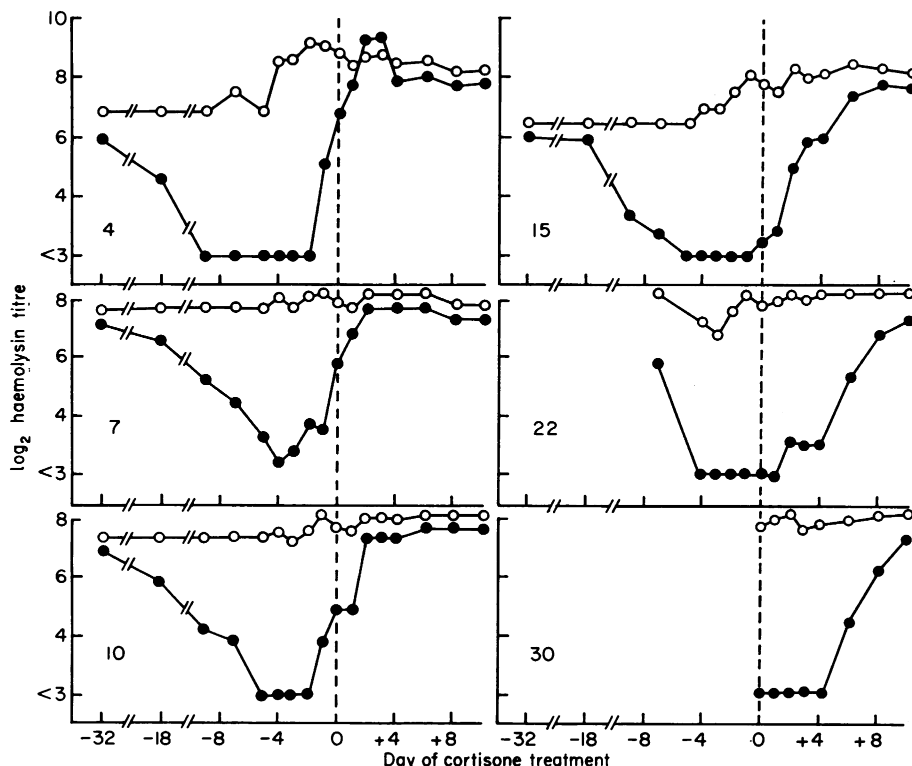

FIG. 1. Total haemolysin responses of normal (○) and cortisone-treated (●) Swiss mice. Cortisone was administered once from 32 days before antigen injection (−32) to 10 days after antigen (+10). The numbers 4, 7, 10, 15, 22 and 30 represent the days of bleeding for antibody containing sera after antigen injection. The *x*-axis represents the time of cortisone injection in relation to antigen injection, the time of antigen injection being at day 0.

haemolysins in these 6·5-ml polyallomer tubes in the fixed-angle rotor was slightly better than that obtained in the swinging bucket rotor. The fixed-angle rotor had the advantage of being able to accommodate twelve gradient separations in one ultracentrifugational run compared to three in the swinging bucket rotor. The change to the fixed-angle rotor was suggested by publications dealing with caesium chloride density gradient centrifugation in fixed-angle rotors (Fisher, Cline and Anderson, 1964; Flamm, Bond and Burr, 1966). Twenty-four fractions were collected by puncturing the centrifuge tube at the bottom with a No. 18 lumbar puncture needle and each fraction was tested for haemolysin activity.

Calculations

The calculations of total 19S and 7S haemolysin activity were carried out as previously described (Sinclair, 1967).

Organ weight and blood studies

Mice were injected with cortisone acetate as above, and controlled with uninjected animals. The thymus, spleen and lymph nodes (internal axillary, external axillary, inguinal and popliteal) were removed and weighed on various days (between 0 and 30

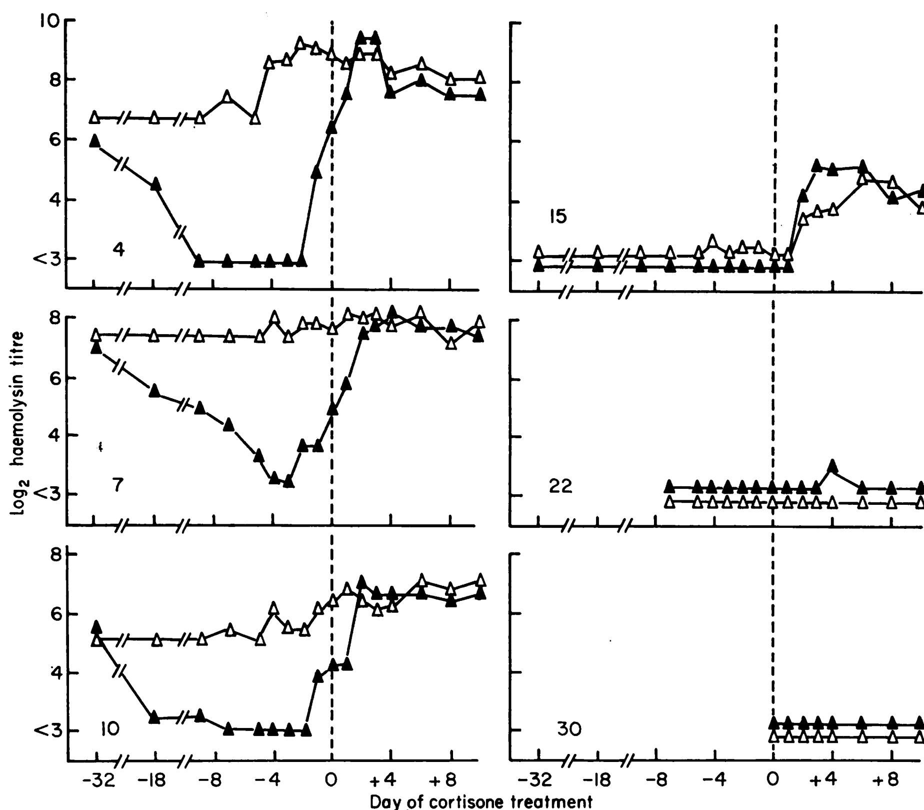

FIG. 2. The 19S haemolysin responses of normal (△) and cortisone-treated (▲) Swiss mice. Cortisone was administered once from 32 days before antigen injection (−32) to 10 days after antigen (+10). The numbers 4, 7, 10, 15, 22 and 30 represent the days of bleeding for antibody containing sera after antigen injection. The *x*-axis represents the time of cortisone injection in relation to antigen injection, the time of antigen injection being at day 0.

days) after cortisone treatment. Total white cell counts and differential blood smears (stained with Giemsa) were assessed, and the total numbers of polymorphonuclear and mononuclear leucocytes per cubic millimetre of blood were calculated.

RESULTS

Fig. 1 shows the total haemolysin responses of cortisone treated and normal mice, plotted in relation to time of cortisone treatment before and after antigen injection

(Berenbaum, 1964). The different graphs express the 4-, 7-, 10-, 15-, 22- and 30-day titres of animals injected with cortisone from 32 days before antigen to 10 days after antigen. Each point represents the average haemolysin titre in five to ten mice.

When cortisone was administered between 0 and 4 days after antigen, there was little or no depression of the early haemolysin response (4- and 7-day titres) but a very marked depression of the late haemolysin response (22- and 30-day titres). If cortisone was given at

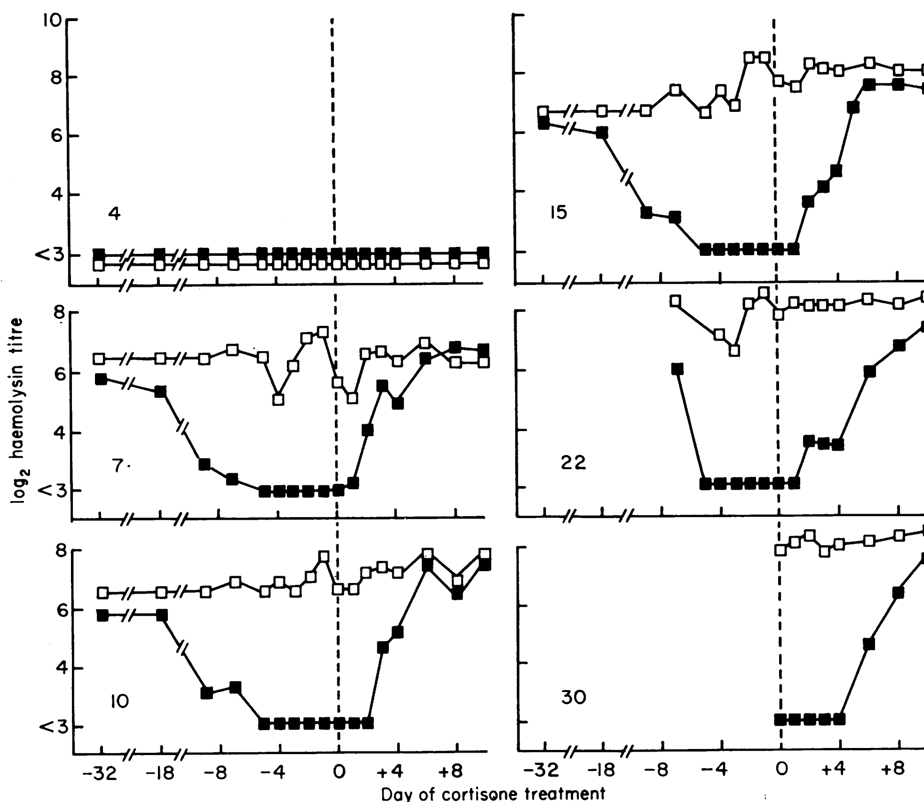

Fig. 3. The 7S haemolysin response of normal (□) and cortisone-treated (■) Swiss mice. Cortisone was administered once from 32 days before antigen injection (−32) to 10 days after antigen (+10). The numbers 4, 7, 10, 15, 22 and 30 represent the days of bleeding for antibody containing sera after antigen injection. The *x*-axis represents the time of cortisone injection in relation to antigen injection, the time of antigen injection being at day 0.

8 and 10 days after antigen, there was no marked depression of the haemolysin titres at any time. Day +6 showed an intermediate response.

Both the early and the late haemolysin responses were depressed when animals were injected with cortisone from 1 to 5 days (−1 to −5) before antigen injection, with the maximum depression at days −3 and −4. When cortisone was administered at days 7 or 9 (−7 or −9) before antigen, there was still a marked depression of the haemolysin response, but it was not as great as that on days −5 to −1, and eventually the circulating antibody returned to near normal levels by 22 days in the −7 group. When cortisone was injected 32 days before antigen there was no detectable depression of any phase of the immune response, whereas the −18 group showed a slight depression in titres. The +4 to

−4 day animals were bled subsequently at 112 days after antigen injection and shown to have no circulating haemolysin antibody at that time.

Figs. 2 and 3 show the haemolysin response as 19S and 7S antibodies in cortisone treated and normal mice, when cortisone was administered from 32 days before to 10 days after antigen injection.

When cortisone was administered between 0 and 4 days after antigen there was hardly any effect on 19S haemolysin activity, but a very marked effect on 7S haemolysin titres. When cortisone was given at days 8 and 10 after antigen there was hardly any effect on

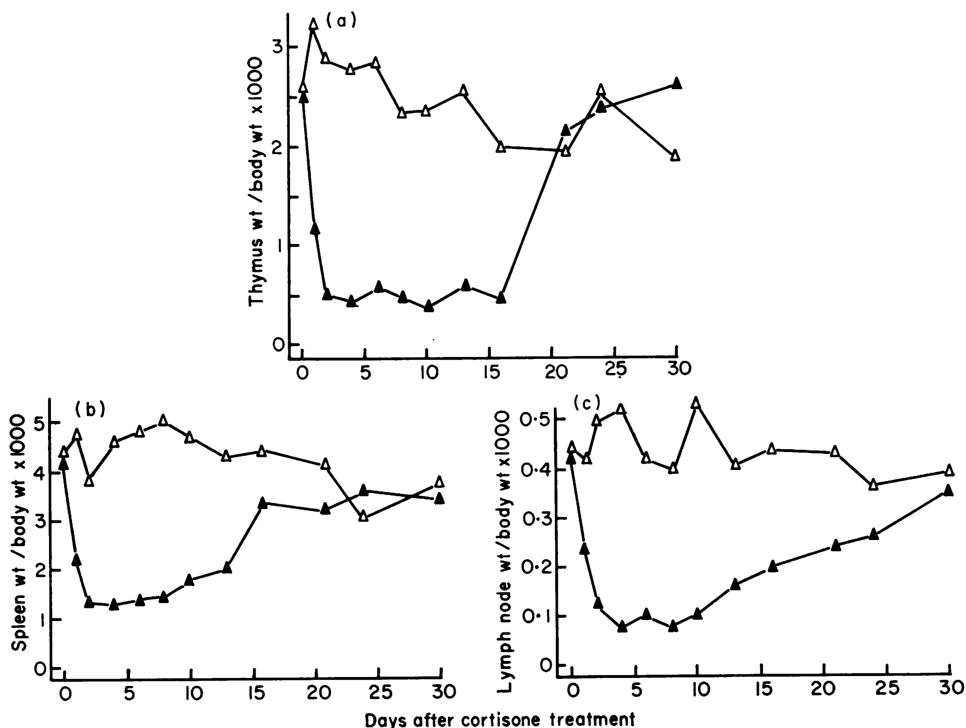

Fig. 4. Lymphoid organs to body weight ratio in normal (△) and cortisone-treated (▲) Swiss mice. Cortisone was injected once at day 0. (a) Thymus, (b) spleen, and (c) lymph nodes.

19S or 7S haemolysin activity. The +6 group showed an intermediate effect on 7S antibody. In mice treated with cortisone between 1 and 5 days before antigen there was a complete lack of 7S haemolysin and a very small amount of 19S haemolysin. Day −7 mice showed a marked depression of 19S and 7S antibody during the early haemolysin response, but eventually the 7S response returned to near normal levels by 22 days and remained normal when tested for haemolysin activity at 112 days. The −9 group showed the same pattern of the early haemolysin response as the −7 group, but the late response was not determined. When cortisone was injected 18 days prior to antigen the 19S and 7S serum haemolysin responses were both slightly depressed, but in the −32 day group both 19S and 7S antibody titres were normal. Thus, when cortisone was administered before antigen, there was a marked depression of 19S production, whereas, if cortisone was injected after antigen, there was hardly any effect on 19S production. The 7S haemolysin response in these mice was suppressed greatly when cortisone acetate was administered 5

days before to 4 days after antigen injection. The later 7S response had a longer period of cortisone sensitivity after the injection of antigen than did the early 7S response.

When the effect of a single subcutaneous injection of cortisone acetate on the lymphoid organ weights was studied (Fig. 4), it was found that there was a thymolytic effect at day 1, which reached maximum by day 2 and remained at maximum depression until day 16 (Fig. 4a). Between day 16 and 21 after cortisone injection, the thymus weights rose from minimum to normal values. Other experiments in this laboratory indicate that the rise in thymus weight could take place within a day or two. Spleen (Fig. 4b) and lymph nodes

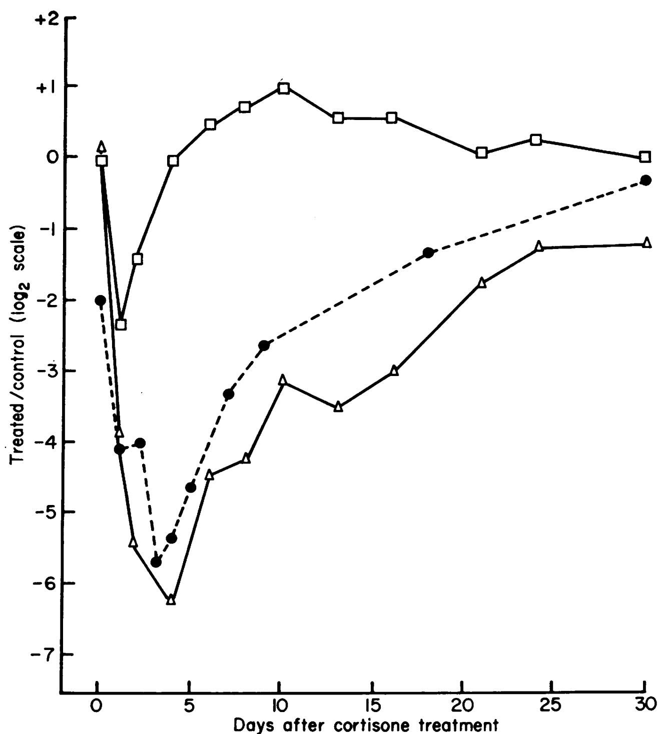

FIG. 5. Granulocytes (□), lymphocytes (△) and 7-day total haemolysin titres (●) in Swiss mice injected once with cortisone at day 0. The granulocytes and lymphocytes are plotted as treated over control on a log_2 scale. Since the antibody titres are on a log_2 scale, the relationship between treated and control group, comparable to that of the cells, is given by the difference in titres. The difference in 7-day titres is plotted as the day of antigen injection after cortisone treatment rather than the day of bleeding after cortisone treatment.

(Fig. 4c) gave a different pattern of regeneration from that observed in the thymus. The maximum depression of spleen and lymph node weights was observed between days 2 and 10. From day 10 onwards, there was a gradual increase in spleen and lymph node weight so that the spleen weight returned to control levels by day 24 and the lymph node weights by day 30 after cortisone administration.

Fig. 5 shows the effect of cortisone acetate on the total numbers of polymorphonuclear and mononuclear leucocytes when plotted as treated over control on a log_2 scale. The polymorphonuclear leucocyte level decreased to 25 per cent of controls on the 1st day after injection of cortisone acetate, and was back to control levels by the 4th day. The polymorphonuclear leucocyte level then rose gradually to twice the control level by 10 days

after injection of cortisone acetate, then gradually subsided to control levels over the next 10–20 days. The mononuclear leucocytes (mainly lymphocytes) decreased for the first 4 days after cortisone administration to 1 per cent of the control values, then increased slowly over the next 20 days to about 50 per cent of control values. The difference between treated and control haemolysin titres obtained 7 days after antigen injection was plotted (Fig. 5) against the day of antigen after cortisone treatment. The decrease in total haemolysin titre at 7 days following antigen injection followed closely the decrease in circulating mononuclear leucocytes present at the time of antigen administration. The behaviour of 19S haemolysin at 7 days after antigen injection (Fig. 2) also followed the level of circulating mononuclears at the time of antigen injection.

DISCUSSION

The immune response was depressed when a single large dose of cortisone acetate was administered to mice from 18 days before to 6 days after antigen exposure. The maximum depression of the early haemolysin response occurred when cortisone acetate was injected between 3 and 4 days before antigen. The maximum depression of the late haemolysin response occurred when cortisone acetate was injected from 4 days before to 4 days after antigen. When cortisone was given after antigen, the 19S haemolysin response showed only slight alterations, hence the normal early total haemolysin response. On the other hand, injection of cortisone up to 4 days after antigen induced maximal lowering of the late 7S haemolysin response. The early 7S haemolysin production showed some evidence of cortisone resistance between 1 and 4 days after antigen challenge. Although the difference in cortisone sensitivity between early and late total haemolysin response relates to the different classes of antibody formed at that time, there is some evidence for a shift in the period of cortisone sensitivity within one class of antibody (the 7S) depending on whether the antibody was formed early or late in the response. When cortisone was administered 2–4 days after antigen, the 19S response was somewhat prolonged. A similar but more pronounced prolongation of the 19S response was observed in 6-mercaptopurine treated rabbits (Sahiar and Schwartz, 1965) which may be due to the lack of suppression of 19S antibody by 7S antibody, which is itself suppressed under these conditions.

The importance of the circulating lymphocyte in antibody formation has been firmly established (reviewed by Gowans and McGregor, 1965), although the exact role played by the circulating lymphocyte is not clear. The correlation between the 7-day haemolysin titres and the number of circulating lymphocytes is quite striking. Such a finding implies that a major contributing factor involved in the suppression of the haemolysin response following a single injection of a large amount of cortisone is the depletion of the circulating lymphocyte pool. The defects which were observed in total, 19S and 7S haemolysin titres before and after the 7-day titres were more pronounced than the defects in 7-day titres. Such a finding implies that defects other than the loss of circulating lymphocytes may be added to the basic defect caused by the loss of the circulating lymphocytes. A defect in antigen trapping, processing and retention could be present and could be due to a direct action of cortisone or to an indirect effect through the lowering of the circulating lymphocyte and presumably the opsonin levels (Williams, 1966a, b; Jaroslow and Nossal, 1966). Some rough correlations between lymphoid organ weights and antibody titres could be made, but until the lymphocyte content of these organs is assessed, any correlation of the type made with circulating lymphocytes is not worth while.

The argument that the major defect in antibody formation in cortisone treated mice involves the depletion of the circulating lymphocyte pool is supported by the observation that restoration can be attained by administering thymic lymphocytes along with antigen (Berglund and Fagraeus, 1956; Fagraeus and Berglund, 1961). Although there were changes induced in the bone marrow of these cortisone treated mice (Elliott and Sinclair, unpublished observations), there was no marked depletion of cells similar to that which occurred in the lymphoid organs. These considerations indicate that the lymphoid tissue is the target tissue involved in cortisone immunosuppression.

In a recent paper, Dukor and Dietrich (1967) described the effects of multiple low doses of cortisone on a number of immunological responses, and found that the later antibody responses to sheep erythrocytes were low whereas the earlier responses were not markedly affected. They have recently shown that cortisone has a larger effect if given prior to antigen (Dukor, personal communication). In a number of experimental conditions, Dukor and Dietrich (1967) were not able to demonstrate that thymectomized mice were more susceptible than intact animals to the effects of cortisone. The major difference in experimental conditions between the present work and that of Dukor and Dietrich (1967) is in our use of a single large exposure to cortisone which produced a profound suppression in the early, as well as the late, haemolysin response. A lymphocyte-correlated reduction in the early haemolysin response after cortisone treatment was not apparent in the results of Dukor and Dietrich (1967), and they concluded that cortisone appears to have affected a population of cells other than the competent lymphocytes initially involved in the immune response. We have investigated experimental conditions in which thymectomized and intact mice have had the opportunity to show different rates of recovery from the lymphocyte-correlated depression of immunological responsiveness prior to testing with antigen, and we find that recovery from this more extensive damage is not dependent on the presence of the thymus (Elliott and Sinclair, unpublished observations). In the present results, the initiation of recovery in the immune response prior to the recovery in the thymus weights gives an indication of the thymus-independency in recovery of immunological responsiveness.

The cortisone sensitive phase of the 19S response extends into the period after antigen exposure to a limited extent, possibly as much as 1 day, whereas the 7S response remains maximally sensitive to cortisone up to 4 days after antigen and is still affected at 6 days. Since a major effect of cortisone is on the circulating lymphocytes and on lymphoid tissue generally, the difference in cortisone sensitivity between 19S and 7S response could reflect differences in the time at which the lymphocytes are activated and recruited for the synthesis of 19S and 7S antibody.

ACKNOWLEDGMENTS

The authors acknowledge with thanks the technical assistance of Miss R. K. Lees, Mr B. Forbes, and the members of the Animal Division at Pollards Wood Research Station, Nightingales Lane, Chalfont St Giles, Bucks. This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research, Royal Cancer Hospital) from the Medical Research Council and the British Empire Cancer Campaign for Research, by the Public Heath Service Research Grant No. CA-03188-89 from the National Cancer Institute, United States Public Health Service, by the Operating

Grant No. MA-2644 from the Medical Research Council of Canada awarded to one of us (N.R.St C.S.), and by the National Cancer Institute of Canada.

One of us (N.R.St C.S.) was a Fellow then Scholar of the Medical Research Council of Canada.

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