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Lack of Correlation Between the Effects of Cortisone on Mouse Spleen Plaque-forming Cells and Circulating Anti-sheep Red Blood Cell Haemolysins

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Summary. The effect of 5·5 mg of cortisone acetate given to adult mice 1 day prior to the injection of 4×10^8 sheep erythrocytes was studied. This drug is able to suppress more than 95 per cent of splenic plaque-forming cells (PFC) compared with immunized animals not treated with cortisone. However, the anti-sheep red cell haemolytic titre in the blood was not affected. Several hypotheses to explain this paradox were tested experimentally.

Plaque-forming cells in the bone-marrow of cortisone-treated mice, instead of being suppressed, increased to more than ten times their number compared with the controlled animals.

INTRODUCTION

The dynamics of the immune response in mice after injection of sheep red blood cells (SRBC) have been extensively studied at cellular and humoral levels. There is agreement that the peak of plaque-forming cells (PFC) is reached about 4 days after antigenic stimulation and the peak of serum haemolysins (SH) occurs a day later. The responses of PFC and SH follow a parallel course (Jerne, Nordin and Henry, 1963; Ingraham and Bussard, 1963; Dietrich, 1966; Wigzell, Möller and Andersson, 1966; Pantelouris and Flish, 1971; Weyer, Bourgarit and Bussard, 1972).

There are several studies dealing with the response of mice to SRBC when cortisone is used. PFC formation can be extensively suppressed (Wlodarski and Zaleski, 1970; Petrányi, Bénzcur and Alföldy, 1971). The optimum immunosuppressive effect of cortisone on circulating antibodies depends on the time of administration of cortisone and on the amount of both cortisone and antigen that is used. Characteristics of immunosuppression by cortisone depend also on the nature of the immunoglobulins being studied (Berglund, 1956; Elliot and Sinclair, 1968; Cohen and Claman, 1971). When the effects of cortisone upon both PFC and SH were examined, a lack of correlation was observed since SH were not suppressed (Petrányi *et al.*, 1971). On the other hand, a lack of correlation between cellular and humoral responses was not observed when immunosuppression was induced by passive administration of specific antibody (Möller and Wigzell, 1965; Möller, 1969; Ryder and Schwartz, 1969; Eisenberg and Weissmann, 1971; Sinclair and Chan, 1971). The purpose of the present communication was to study further the relationship between the PFC and SH responses.

MATERIALS AND METHODS

Mice

Experiments were performed with 14–16-week-old B10/C3H F_1 hybrid mice of both sexes. In all experimental groups, five to six animals were used of about the same body weight (20–22 g).

Immunosuppression

Groups of animals were injected i.m. with 5·5 mg cortisone acetate (Cortisone Merck) 24 hours before antigen stimulation.

Suppression with specific antibody was obtained by injection of 15 μl of an anti-SRBC antiserum. This dose was diluted in 0·2 ml of saline. The antiserum was injected i.v. 2 hours before antigen. This serum was obtained from B10/C3H F_1 mice hyperimmunized with 2×10^8 SRBC given i.v. once a week, for a month.

Immunization, bleeding and titration

Experimental animals were injected i.v. with 4×10^8 SRBC and were bled from the tail. Care was taken to obtain the same volume from each animal, avoiding excessive bleeding. Sera thus collected were quickly frozen and kept at $-20°$. All titrations were done simultaneously.

Haemolytic titre was expressed as $\log_2$ of two-fold serial dilutions, using the Takátsy microtitrator method (Takátsy, 1955). Dilutions were made in sodium chloride-barbital buffer, pH 7·3 (Wasserman and Levine, 1961), but bovine serum albumin was omitted. SRBC at a concentration of 0·5 per cent and 0·025 ml of guinea-pig complement, diluted 12·5 times in the same buffer, were added and the mixture incubated for 1 hour at 37°.

Complement

Pooled fresh guinea-pig sera from at least four animals were freeze-dried, sealed in vacuum and stored at $-20°$.

Mercaptoethanol treatment

Inactivation of haemolysins was done with 2-ME according to Uhr and Finkelstein (1963). Control samples were incubated under the same conditions except that 2-ME was omitted. Samples were dialysed overnight at 4° against barbital buffer. Volumes were equalized and the samples titrated.

Column fractionation

One millilitre of serum was passed through a $50 \times 3{\cdot}1$-cm Sephadex G-200 column. Fractionation was made at 4° using 0·85 per cent saline–0·01 M phosphate buffer, pH 7·2.

Immunodiffusion

Ouchterlony's plates with 1 per cent agar dissolved in saline were employed. Goat anti-mouse IgG serum from Hyland Division, Travenol Laboratories, was used.

Detection of PFC

Jerne's direct plaque technique (Jerne *et al.*, 1963) was used to test the presence of PFC in the spleen, marrow (BM) and blood. BM cells were collected from the femur and

were suspended in cold Tyrode's solution. Blood cells were obtained from the buffy coat of citrated whole blood and resuspended in Hanks's medium.

TABLE 1

INHIBITION OF PLAQUE-FORMING CELLS BY CORTISONE AND BY ANTI-SHEEP ERYTHROCYTE SERUM

Group	Days			PFC/10^6 spleen cells	Per cent inhibition
	−1	0	4		
1	Cortisone	SRBC	PFC	2·7	98·6
2		Anti-serum* +SRBC	PFC	0·8	99·6
3		SRBC	PFC	190	0

* Fifteen microlitres hyperimmune anti-SRBC (haemolytic titre 1/4096) 2 hours before antigen.

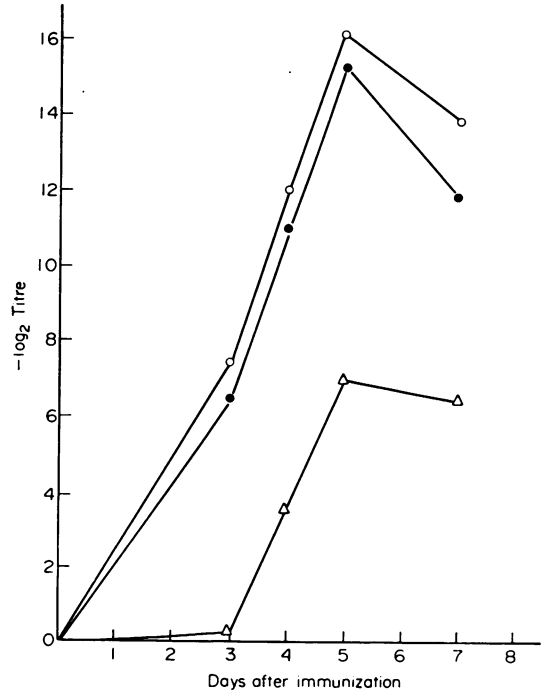

FIG. 1. Serum haemolytic titres in mice immunized with SRBC; (○) treated with cortisone and immunized, (△) treated with anti-SRBC serum and immunized and (●) untreated.

In vitro inhibition of PFC was carried out using 0·02 M KCN in the agar-plates. Cycloheximide (Sigma Chemicals) at a concentration of 10 μg/ml was included in both lower and upper agar layers.

The diameter of the haemolytic plaques was measured from photographs of plates stained with benzidine according to the method of Jerne *et al.* (1963).

RESULTS

EFFECT OF CORTISONE AND SPECIFIC ANTI-SRBC ANTIBODIES ON PFC AGAINST SHEEP ERYTHROCYTES

Table 1 shows that both cortisone (group 1) and specific anti-SRBC antiserum (group 2) inhibit spleen anti-sheep erythrocyte PFC to the same extent (>98 per cent) compared to immunized-untreated controls (group 3).

If a comparison of the same groups is made with respect to the titres of SH, a completely different picture is observed (Fig. 1)—groups 1 and 3 (cortisone-treated and untreated respectively) match each other at every single point along the curve, both groups reaching a peak titre of 15 and 16 $\log_2$ units respectively on the fifth day. In contrast, mice receiving anti-SRBC antibodies (group 2) show a significant decrease in titre (from 16 to 7/$\log_2$ units) in agreement with the marked inhibition of PFC.

CHARACTERIZATION OF CIRCULATING ANTIBODIES

Jerne's direct technique detects cells producing IgM haemolysins (Sterzl and Riha, 1965). It was necessary therefore, to examine the nature of SH in cortisone-treated animals. The 2-ME sensitivity of haemolytic anti-SRBC antibodies was tested. Sera from each group at the fifth day were pooled, treated with 2-ME and titrated against SRBC. The results showed that over 99 per cent of SH from both cortisone-treated and untreated control mice are sensitive to 2-ME. The same was true for the small amount of haemolytic antibody present in sera from mice suppressed by anti-SRBC antiserum (Table 2).

TABLE 2

2-MERCAPTOETHANOL SENSITIVITY OF SERUM HAEMOLYSINS FROM MICE TREATED WITH CORTISONE AND ANTI-SRBC SERUM

Group	Days			Haemolitic titre after treatment with:	
	−1	0	4	2-ME	Saline*
1	Cortisone	SRBC	Bled	< 1/6	1/2024
2		Anti-serum + SRBC	Bled	< 1/6	1/16
3		SRBC	Bled	< 1/6	1/6246

* 2-ME was omitted.

Another sample of SH from cortisone-treated animals was fractionated on a Sephadex G-200 column. Effluent volumes were analysed for protein concentration and haemolytic activity, and by immunodiffusion against anti-mouse IgG antiserum. Results depicted in Fig. 2 show a clear-cut relationship between haemolytic activity and the high molecular weight immunoglubulins, both present in the excluded volume of the column (tubes 20–30). The lack of correlation between IgG and haemolytic activity is also demonstrated, since IgG concentration reaches its peak in tubes 36–38. From these results we conclude that the SH of cortisone-treated animals are of the IgM class and therefore related to the PFC detected by Jerne's direct method.

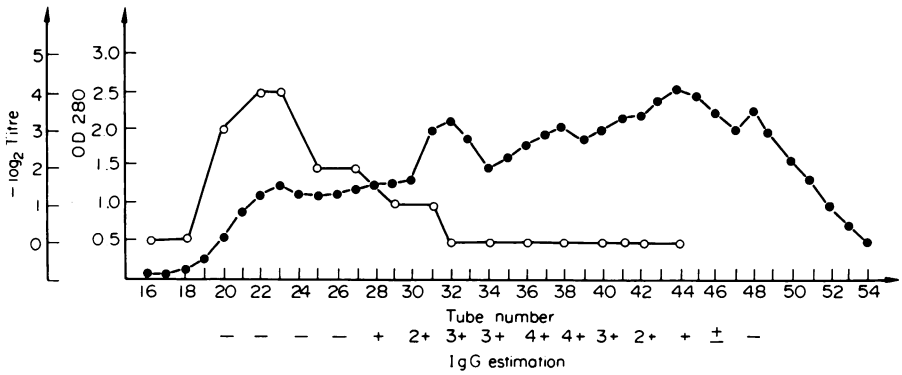

FIG. 2. Fractionation of a pool of anti-SRBC serum from cortisone-treated mice on a Sephadex G-200 column. IgG was estimated by immunodiffusion. ●, Optical density; ○, haemolitic activity.

EFFECT OF CORTISONE UPON SH SYNTHESIS AND DEGRADATION

The high titre of SH observed in cortisone-treated animals could be due to an increase in the efficiency of production of antibody by cortisone-resistant cells or to a decrease in catabolic rate of circulating antibodies.

The first hypothesis was tested measuring the diameters of haemolytic plaques from cortisone-treated and untreated animals. No significant difference was found between both groups—0·48±0·09 mm for cortisone-treated versus 0·46±0·11 mm for untreated cells.

With regard to the second possibility, the following experiment was performed: IgM anti-SRBC with haemolytic titre of 1/1024 was injected into non-immunized, cortisone-treated and untreated mice (0·7 ml given i.v. into each mouse in two injections 1 hour apart). SH was titrated at 17, 24, 42 and 138 hours after injection. Special care was taken to withdraw each time a constant, minimal amount of blood (about 0·15 ml). Results clearly show that the fall in titre is almost the same in cortisone-treated and untreated mice (Table 3).

TABLE 3

In vivo FALL OF IgM ANTI-SRBC ANTIBODY* INJECTED INTO NORMAL AND CORTISONE-TREATED B10 Sn/C3H MICE

Group	Cortisone†	Haemolytic titre‡ of serum at: 17 Hours	24 Hours	42 Hours	138 Hours
I	—	7	7	7	4
II	+	8	7	6	4

* Antibody: 0·7 ml IgM anti-SRBC (haemolysis titre 1/1024).
† Cortisone: 5·5 mg i.m. 1 day before the antibody injection.
‡ Titration in duplicate; titre shown as $-\log_2$.

EFFECT OF CORTISONE GIVEN 1 DAY BEFORE ANTIGEN STIMULATION ON PFC FROM SPLEEN, BLOOD AND BONE MARROW

It is known that the extent of cortisone depression varies depending on the immuno-

competent organ (Dougherty, 1952; Levine and Claman, 1970). The following experiments were done to find out if cortisone affects the formation of PFC from organs other than the spleen. The results in Table 4 show that the effect of cortisone on BM is quite opposite to that seen on the spleen: BM PFC increase in their relative and absolute numbers (fifteen times in experiment No. 1 and eleven times in experiment No. 2, when the total number from each femur were considered) while spleen PFC are more than 99 per cent suppressed.

TABLE 4

EFFECT OF CORTISONE ON THE NUMBER OF PFC. RESULTS OBTAINED FROM THE SPLEEN, BLOOD AND BONE MARROW

		PFC/10^6 nucleated cells					
Experiment	Cortisone	Spleen	BM	Blood	PFC/spleen	PFC/femur	PFC/total blood volume
1	+	5·1	6·5	1·3	200	50	26
	−	738	0·20	2·1	164000	3·3	42
2	+	2·8	2·1		200	18	
	−	755	0·1		166800	1·6	

In Table 4 we can also see that the number of blood-borne PFC does not make a major contribution to anti-SRBC SH in mice. The low numbers of PFC obtained do not allow us to attach any significance to differences observed between cortisone-treated and untreated animals.

EFFECT OF CORTISONE ON SPLEEN PFC WHEN GIVEN AFTER ANTIGEN STIMULATION

Cortisone, besides acting in the induction period of the antibody response, could also destroy cells after they had already produced and released a certain amount of antibody. This antibody could represent part of the SH antibody present in cortisone-treated mice.

The experiments shown in Table 5 were aimed at finding out if cortisone was able to destroy antibody-producing cells. For this purpose, the drug was given to mice already sensitized with SRBC 78 hours previously. Eighteen hours later, they were tested for spleen PFC and for anti-SRBC haemolytic titre. The results show that there were only 55 per cent of the number of PFC in the whole spleen of cortisone-treated animals compared to untreated ones. When these results are expressed per 10^6 cells, the number of

TABLE 5

EFFECTS OF CORTISONE ON PFC AND TITRE OF CIRCULATING HAEMOLYSINS, WHEN INJECTED WITH CORTISONE 18 HOURS BEFORE TESTING. RESULTS OBTAINED ON THE FOURTH DAY AFTER ANTIGEN STIMULATION

Group	Cortisone	PFC/spleen	PFC/10^6 cells	Titre
I	+	47020	471	13·0
II	—	82080	319	12·8

PFC in cortisone-treated animals is 45 per cent higher than the control value. Haemolytic titres were the same in both groups. These results will be discussed later but it is evident that the decrease in PFC of cortisone-treated animals is by no means comparable to that obtained when the drug was given 24 hours before antigen injection.

In vitro EFFECTS OF KCN AND CYCLOHEXIMIDE ON PFC FROM UNTREATED AND CORTISONE-TREATED IMMUNE ANIMALS

The numbers of PFC detected with Jerne's method could be an overestimation of the number of cells that actually contribute to maintain the level of SH. Our results using cortisone suggest that only a small number of PFC are sufficient to maintain the level of SH. As for the rest, one could assume that they synthesize antibody but do not release it, or that they secrete antibody only when incubated in the plate. If this were so, interference with antibody synthesis or antibody secretion during the incubation period should substantially decrease PFC in untreated immune animals but should not affect cortisone-treated mice. Two such agents have been used: KCN and cycloheximide.

The results in Table 6 refer to blocking of haemolysin synthesis and its secretion in PFC from cortisone-treated and untreated immune mice. It can be seen that KCN affects both groups in a similar way: cortisone-treated cells show 80 per cent and untreated cells show 70 per cent PFC suppression. Cycloheximide behaves differently; there was no significant difference in the number of PFC between plates incubated with and without the drug when cortisone-treated mice were examined. On the other hand, a 25 per cent decrease in PFC was found in cycloheximide-treated plates from untreated animals, a result which is on the borderline of significance.

TABLE 6

RELATIVE REDUCTION OF PFC FROM SPLEEN WHEN KCN AND CYCLOHEXIMIDE WERE PRESENT IN THE AGAR AT THE TIME OF INCUBATION. COMPARISON OF CORTISONE-TREATED AND UNTREATED ANIMALS

Group	Inhibitor present in the plate		
	None	KCN	Cycloheximide
I cortisone	100±24	19±13	130±30
II no cortisone	100±13	29±14	74±11

Results expressed as a percentage (±standard deviation) relative to controls in the same groups. PFC counts refer to the whole spleen.

DISCUSSION

The results in this paper show that cortisone, under the experimental conditions used here, suppresses the immune response as measured by the number of spleen PFC, although it does not affect the level of circulating haemolysins. These results are in agreement with the work of Petrányi *et al.* (1971). The suppression of PFC by cortisone has already been described (Wlodarski *et al.* 1970; Bennett, 1970) and therefore, from the experimental point of view, these observations seem well supported. We have attempted several explanations for these facts and they will be discussed in the light of our experimental evidence.

One could claim that the method of haemolysis is not sensitive enough to pick up a difference from 100 to 1 as shown in PFC. Nevertheless, such a decrease in PFC was actually found when passive antibody was administered to immunized animals, with a corresponding decrease in haemolytic titre. Here, the decreases in PFC and SH were of the same magnitude (Table 1, Fig. 1). From these results we conclude that if there was such a decrease in the concentration of SH from cortisone-treated animals, we should have detected it with the methods used here.

Characterization of SH by 2-ME sensitivity, gel filtration and the lack of association of SH with IgG (Table 2) show that the antibody is almost certainly IgM, and therefore related to that antibody detected by direct measurement of PFC. The same antibody injected to cortisone-treated and untreated mice showed no differences in the rate of fall of SH titre (Table 3), thus demonstrating that there is no accumulation of SH in cortisone-treated animals due to an increase in the half-life of the antibody in the circulation. Our results are in agreement with those of Gabrielsen and Good (1967), who found that cortisone had no influence on the half-life of passive antibody in rabbits.

Variation in the diameter of haemolytic plaques has been shown (Merchant and Petersen, 1968) thus suggesting that the total amount of antibodies synthesized by different cells is different (Weyer *et al.*, 1972). Cortisone-resistant PFC in the spleen, although in small number, could secrete more antibody. If one relates the diameter of the lytic plaques with the amount of antibody per cell, one concludes that PFC from cortisone-treated and untreated mice produce on the average the same amount of antibody.

The correlations observed by Jerne and by others between PFC and haemolytic titre refer to PFC from the spleen (Jerne *et al.*, 1963). The lack of correlation observed by us in cortisone-treated animals could result from a differential effect of cortisone on immunocompetent organs other than the spleen. Levine and Claman (1970) have described the resistance of the bone marrow (measured by PFC and blast transformation) to cortisone, in spite of the well documented general lymphoid cytotoxic effect of cortisone (Dougherty, 1952; Stevens, Bedke and Dougherty, 1967). Our results clearly show that PFC from BM are not only resistant to cortisone but that the numbers can increase by an order of magnitude in cortisone-treated animals compared to untreated mice (Table 4).

If one extrapolates the results obtained from femurs, estimating the number of PFC to be 3 per cent of the whole body BM, the numbers (about 2000–4000) are still insufficient to compensate for the decrease in spleen PFC observed in cortisone-treated animals. Nevertheless one should be cautious as there is no experimental support validating this extrapolation based on erythropoietic activity (Schofield, 1969). The assumption of an equal stimulation of PFC thoughout the whole BM compartment has to be taken with reservation. One could suggest that the titre in cortisone-treated mice results from the release into the circulation of antibody from PFC destroyed by cortisone. That this is not so is shown in the experiments carried out by injecting cortisone 18 hours before measuring PFC and SH titre: the amount of PFC actually decreases only to half the peak number (Table 5). This would be expected to produce only a minor difference in SH titre.

These results are best explained by assuming that cortisone interferes with the development of new PFC, rather than by destruction of already differentiated antibody-forming cells. According to the rate of production of PFC observed in untreated controls, the diminution in the number of PFC is that which one would expect to result from a blocking of differentiation 18 hours before testing took place. These results indicate that cortisone interferes with all phases of the antibody-induction period.

A rough estimate can be made concerning the efficiency of spleen PFC with reference to their contribution to SH, assuming that SH titre differences between 2 consecutive days (say the fourth and fifth days) correspond to the decrease in PFC between these days. If such a calculation is made, we arrive at the conclusion that spleen PFC have a rather low antibody-producing capacity *in vivo*, or that most of the synthesized antibody remains in the spleen and is not released into the circulation. If the capacity for antibody formation is low *in vivo*, it is possible that, in cortisone-treated animals, a compensatory mechanism increases their efficiency. That this is not so is indicated from the measurements of the diameter of the plaques and from the experiments on the inhibition of antibody synthesis or secretion with cycloheximide or KCN.

No substantial differences were observed between cortisone-treated and untreated mice which could be attributed to differences in synthesizing or secretory activity of the PFC of either group *in vivo*. Experiments with cycloheximide showed on untreated mice a small decrease in the number of PFC caused by the action of the drug *in vitro*, indicating that the antibody-producing mechanism was already active *in vivo* and not triggered during the incubation period *in vitro*.

We have drawn two conclusions from our results. First, that most of the antibody synthesized in the spleen in non-cortisone-treated animals is not released into the circulation; direct evidence for this assumption must come from further experiments. Secondly, that the decrease in spleen PFC in cortisone-treated animals is at least partially compensated for by an increase in the number of marrow PFC.

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