Onderstepoort J. vet. Res. 41, 29–38 (1974)

STUDIES ON THE EFFECT OF CORTISOL ON THE PRIMARY IMMUNE RESPONSE TO SHEEP ERYTHROCYTES *IN VIVO* BY MOUSE SPLEEN CELLS

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ABSTRACT

HELLIG, HERMIONE and WALDEK, J. F., 1974. Studies on the effect of cortisol on the primary immune response to sheep erythrocytes *in vivo* by mouse spleen cells. *Onderstepoort J. vet. Res.* 41, 29–38 (1974).

Female mice were injected with a suspension of 0,2 ml of 10 per cent v/v sheep red blood cells (SRBC) and sacrificed on the 5th day for recording of spleen mass and assay of plaque-forming cells (PFCs) in the spleens. All PFCs from a pool of five spleens and the individual spleen masses were subjected to rigid statistical analysis to verify whether differences from the control groups were significant ($P < 0,05$).

Three doses of 4 mg cortisol administered at 24-hourly intervals were given at various times relative to the time of immunization to determine the optimum stage at which to inhibit the formation of PFCs. The greatest inhibitory effect was obtained when the first dose was given at —6 h, with a lesser though still significant depression of PFCs with the —30 h dose regimen. Earlier and later injections had relatively little effect.

An attempt was made to determine the response to different dosage levels by injecting doubling doses of steroid starting at the —6 h period. A maximal effect was attained using either 1 to 2 mg cortisol suspension or 0,5 mg of the soluble pharmaceutical preparations, Efcortolan* and Betsolan*. Inhibition of PFCs appeared to be a more sensitive indicator of steroid action than loss of mass.

In addition, spleen mass was determined after injection of either 4 mg cortisol three times or SRBC. During steroid treatment the spleen mass decreased, but the masses started to increase again almost immediately this treatment was discontinued; the maximum antigenic response, as evinced by attainment of maximal mass, occurred at +4 days, when the PFC production reached its peak.

INTRODUCTION

Since the classical experiment of Selye (1936), it has been known that glucocorticoids secreted by the adrenal exert a marked effect on the reduction of lymphatic tissue mass. This effect has been measured quantitatively by numerous workers such as Ringertz, Fagreus & Berglund (1952), Frank, Kumagai & Dougherty (1953), Santisteban & Dougherty (1954), and Elliot & Sinclair (1968), and cortisol has been found to have the most potent action (Santisteban & Dougherty, 1954). However, conflicting results were obtained when attempts to correlate antibody levels with hormone treatment were made (Dougherty, 1952; White, 1958, 1963). Fagreus (1952) using rabbits as experimental animals and *Salmonella typhimurium* Ag H as antigen, showed conclusively that glucocorticoids also produced a marked reduction in humoral antibody response. Berglund (1952) confirmed this effect using rats.

Berglund (1956a) measured the effect of cortisol on the primary haemolysin response (fifth day) in rats and showed the vital importance of the time of hormone administration relative to the time of administration of antigen. Suppressive effects on haemolysin titres were observed only when cortisol was given from —2 to 0 days. He also showed (Berglund, 1956b) that the amount of cortisol needed for suppression was proportional to the dose of sheep red blood cells (SRBC) injected. Later he showed that the period of maximum sensitivity to corticoid in his system was at about —10 hours (Berglund, 1962), and that the effect of steroid could be reversed by injecting spleen or thymus cells shortly after SRBC (Berglund & Fagreus, 1956, 1961).

The effects of a single large dose of steroid varying from 2,5 to 15 mg per 20 mg mass on the haemolysin response to SRBC in mice was studied by Dietrich (1966), Elliott & Sinclair (1968) and Petranyi, Benczúr & Alfödy (1971); the latter authors were the first to demonstrate a drop in plaque-forming cells (PFCs) in the spleens of cortisol-treated mice. Haemolysin levels, at Day +5 at least, are mainly accounted for by splenic production (Rowley, 1950; Adler, 1965). Following the description by Jerne & Nordin (1963) of the simple assay method for splenic PFCs, which form IgM haemolysin, the more direct assay procedure can now be applied. PFC formation and the depression thereof caused by cortisol, have not previously been thoroughly investigated. Accordingly we have studied time and dose-response relationships using the dual criteria of PFCs/10^6 spleen cells and reduction of spleen mass to assess glucocorticoid effect. The mice used were of the strain maintained in the Onderstepoort colony.

MATERIALS AND METHODS

Animals

Eight-week old female white mice of the strain maintained in the Onderstepoort closed colony were used throughout. In some of the dosage experiments only selected mass-measured mice, $20,5 \pm 1,0$ g, were used. In the other experiments their mass ranged from 15,5 to 25,5 g.

Treatment of animals

The mice were fed a balanced pelleted ration and tapwater *ad lib.* They were subjected to normal seasonal fluctuations in temperature throughout the 18-month period during which the experiments were conducted, as the animal room is not air-conditioned. No stringent light and dark cycles were maintained. They were housed in batches of from five to seven to a box, the same numbers being used for both controls and for each of one or two test groups per experiment. The only exception was the case of the measurement of spleen mass variation with time after either steroid treatment or immunization, when 10 animals were used for each experimental measurement. Both controls and test groups received SRBC. Test groups were treated with steroids as well.

Steroids were injected intraperitoneally (i.p.) at the designated dosage and time relative to the time of

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Received on 8 February 1974.—Editor

immunization. Immunization was with 0,2 ml 10 per cent (v/v) SRBC in saline, by intravenous (i.v.)* injection, at 15h00 on day 0. The animals were sacrificed by cervical dislocation at 08h00 on day 5, i.e. four days and 17 h after immunization, unless otherwise specified.

Spleen mass

Immediately after sacrificing the animals, the spleens were excised and put into 5 ml cold balanced salt solution (BSS) (Mishell & Dutton, 1967). They were then mass-measured to 0,1 mg on wax paper and placed in a fresh solution of BSS. The spleens usually measured from 100 to 200 mg, though chronological "bunching" occurred giving occasional high mass group spleens, i.e. measuring from 200 to 300 mg. About 5 per cent measured more than 300 mg, a few as much as 600 to 1 500 mg. All the spleens that measured more than 300 mg were rejected as they probably indicated a pathological condition and moreover they lie outside the range of the experimentally determined normal population distribution. It should be noted that the mass accepted as normal was considerably higher than the mean of 70 mg reported for specific pathogen-free animals (Gisler, Bussard, Mazié & Hess, 1971).

Preparation of cell suspensions

For each experimental measurement of either control or test mice, the total cells from five to seven spleens were combined.

The maximum PFC response of these mice is at Day 4 (Oellermann, Veterinary Research Institute, Onderstepoort, personal communication, 1973). However, for convenience we adopted the regimen given above, resulting in PFC measurements a day after the maximum. The cell suspensions were obtained by teasing two to three spleens in 5 ml BSS with a forceps and a scalpel. The suspensions so obtained were then aspirated 10 times using a 10 ml syringe fitted with a 20 gauge needle. The 5 ml of suspension was then mixed with 5 ml BSS in a conical centrifuge tube and the debris allowed to settle for three minutes. The suspension was then decanted into a graduated conical centrifuge tube and centrifuged at 500 *g* for 10 minutes (1 000 rpm on a Christ centrifuge, model No. IV KS). The supernatant fluid was decanted, the packed cell volume (PCV) measured, and the precipitated cells taken into suspension in 80 volumes of BSS. All these operations were carried out at 4 °C. A differential count utilizing nigrosin dye exclusion for viability was then made in a haemocytometer and generally >99,0 per cent were found to be viable. An aliquot of the cell suspension was then made to contain 1×10^7 total spleen cells per ml.

Complement

Adult guinea-pigs were bled from the heart. After standing at room temperature for 4 h, the clotting blood was left in a refrigerator at 4 °C overnight and then centrifuged. The serum was frozen in 1 ml aliquots and stored at −20 °C for up to three weeks before use.

SRBC

Forty ml fresh sheep blood was mixed with 5 ml 10 per cent LiCl and stored at 4 °C for not longer than three days before use. The solution was centrifuged at 4 °C at 750 *g* for 10 min, the cells washed with 8,5 per cent NaCl, and subsequently centrifuged again. After three washes, the packed cell volume was measured and either a 7 per cent v/v solution made in BSS for PFC assay, or a 10 per cent v/v solution made in isotonic NaCl. For immunization 0,2 ml of the latter solution was injected i.v. This was the standard immunizing dose of SRBC used in our experiments. It has been reported to be maximally effective for IgM haemolysin production (Dietrich, 1966).

Assay of PFC

Five dilutions of 1×10^7; $7{,}5 \times 10^6$; $5{,}0 \times 10^6$; $2{,}5 \times 10^6$ and $1{,}0 \times 10^6$ cells/ml were made and 0,1 ml aliquots taken for PFC assay (in triplicate for each dilution) performed with 0,55 ml 0,5 per cent agarose gel containing 0,05 ml 7 per cent SRBC in BSS on microscope slides (Mishell & Dutton, 1967). Each slide was treated with 0,2 ml 5 per cent guinea-pig serum in BSS as the source of complement and incubated for 3 h in a humidified incubator at 37 °C before plaque counts were made. The elapsed time from sacrifice to incubation was about 1,5 h. The slide was scored into squares with a diamond pencil and plaque counts from 50 to 450 recorded with a strong background source of illumination. Background counts from unimmunized animals never exceeded 8 per 10^6 spleen cells and were therefore not routinely determined.

Cortisol preparations

A suspension of 20 mg/ml cortisol(1) was made by agitating the crystalline steroid powder in $\frac{1}{5}$ volume ethanol on a rotary vibrator, and then adding $\frac{4}{5}$ volume almond oil(2) and vibrating for two min. The oil was stored in a dark bottle at 4 °C. This preparation was used after toxicity testing had shown most commercial preparations to be unsuitable. Routinely a standard dose of 0,2 ml (4 mg) of this oil suspension of steroid was injected i.p. at 08h00 using a 26 gauge needle. The suspension was stable for at least 30 min. Later aqueous suspensions of the same and lower concentrations were prepared, but these were very unstable and reproducibility could only be attained by continuously vibrating the 1 ml syringe between injections. Betsolan Soluble is supplied as an aqueous solution of 2 mg/ml and was used as such (0,25 to 0,5 ml injected i.p.). Efcortolan is supplied as a powder of monosodium cortisol succinate soluble in 2 ml H_20. This was further diluted with saline to give the necessary concentrations for i.p. injection.

Statistical methods

Spleen mass and PFCs/10^6 spleen cells for control and test groups were analyzed by analysis of variance (Steel & Torrie, 1960). The assumptions underlying the analysis of variance were then checked by analysing the residuals (Draper & Smith, 1966). If the assumptions seemed to hold, the means were compared by (a) Tukey's method if the number of observations per treatment was the same and (b) Bonferroni's method if the numbers differed (Miller, 1966). If, however, the assumptions seemed unrealistic, a Kruskal-Wallis nonparametric analysis of variance was performed (Reinach, 1966). The different types of analysis were necessary because there were such large variations in the mass data. This was reflected by the high coefficient of variance (C.V.) found in most of these experiments. If a significant ($P<0,05$) result was obtained the treatments were compared using the method of Dunn (1964).

* Mice placed in a 37 °C oven for 10 minutes—humidity and ventilation controlled—to expose tail vein

(1) Merck

(2) Sweet almond oil manufactured by Harmon & Reinar, S.A. (Pty) Ltd, received as a gift from Dr W. A. Parsons of the firm

To plot the spleen mass after immunization or steroid treatment, the curves were fitted to the data by means of polynomials (Graybill, 1961). The adequacy of the model to describe the observed data was tested by means of a "lack of fit" test (Miller, 1966 In some cases some observations were discarded by the standard residual technique (Draper & Smith, 1966).

As stated previously, anomalous spleen mass data were rejected on both statistical and biological grounds. The statistical method consists essentially of checking the probability that a certain observation belongs to its group (Draper & Smith, 1966). It is worth noting that the discards obtained for the controls (SRBC-injected) by the statistical method of calculating residuals agreed perfectly with an empirical distribution study, made at the conclusion of the experiments, on all the control SRBC data.

Results

A chronological study of variation in spleen mass after three doses of 4 mg cortisol

For this experiment 100 mice mass measuring 20,5 $\pm$ 1,0 g were used. Ten animals were sacrificed every 24 h from Day 0 to Day nine. Cortisol was administered on Day 0, 1 and 2. No SRBC were given to these mice. The mean masses of the spleens of these 10 groups are plotted in Fig. 1. The curve obtained by using all the observations seemed to describe the data well, as the "lack of fit" test was non-significant. After some observations were discarded by the standardized residual technique the curve obtained was essentially the same, and indicates a rapid loss of spleen mass, progressive with each injection of cortisol, to about 45 per cent of normal. Recovery begins one day after the cessation of hormone treatment and masses are within the normal range four days later. However, the mass regained does not quite enable the spleens to attain pretreatment levels.

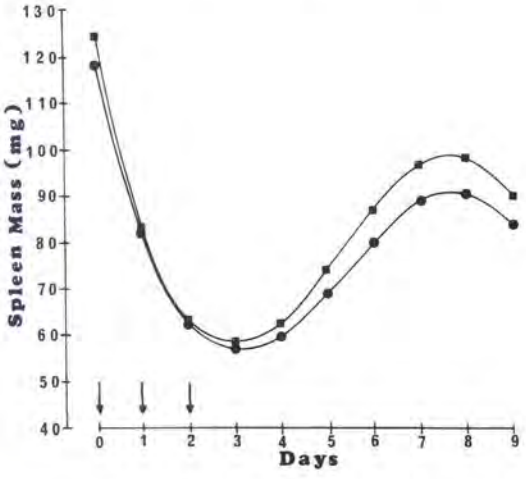

FIG. 1 Chronological variation in spleen mass after three injections of 4 mg cortisol. Time of steroid injection shown by the arrows. ■——■, all data with no discards; ●——●, data after discarding by means of the standardized residual technique. (Both curves show a non-significant "lack of fit" test)

A chronological study of variation of spleen mass after administration of SRBC

For this experiment also, 100 mass measured mice (20,5 $\pm$ 1,0 g) were used and were injected i.v. with 0,2 ml 10 per cent SRBC on Day 0. As in the previous experiment 10 animals were sacrificed every 24 h. The results are depicted in Fig. 2. The curve obtained by using all but four very high observations shows a maximum at Day 4, a result in accord with the maximum of PFC production. However, there was reason to doubt the mathematical validity of this curve as the "lack of fit" test was significant, indicating that the curve does not fit the data well. After abstracting data by the standardized residual technique a rather different curve was obtained. Although the "lack of fit" test for this curve was indeed non-significant, its shape is rather difficult to explain on biological grounds. Since we encountered such large variations in individual spleen masses and the rise is only of the order of 25 per cent it is possible that the time curve can only be validated by the use of a much larger number of mice.

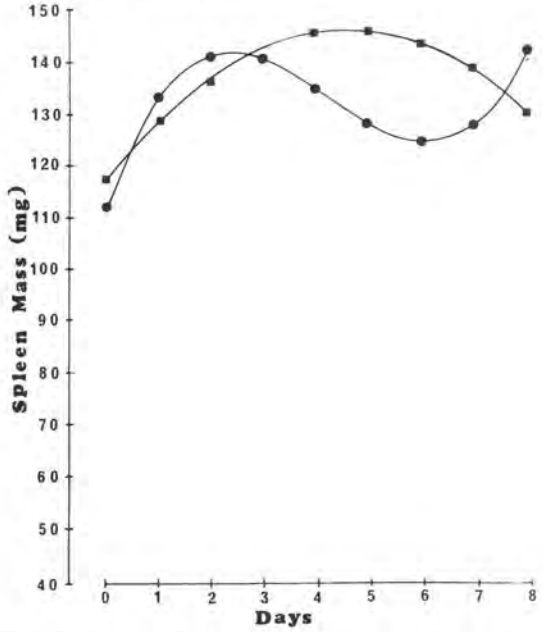

FIG. 2 Chronological variation in spleen mass after injection of 0,2 ml 10 per cent SRBC on day 0 at 08h00. ■——■ data minus 4 very high discards; ●——●, data after discarding by means of the standardized residual technique. (Only the latter curve shows a non-significant "lack of fit" test)

Effect of cortisol administration at various times before and after immunization with SRBC

Groups of mice were given three injections of 4 mg cortisol at 24 h intervals commencing -84, -30, or -6 h before or $+1$ or $+16$ h after immunization with SRBC. In addition groups of mice were given a single injection of 4 mg cortisol, at either -6 h before or $+1$ h after immunization (Fig. 3). Mass measured animals (20,5 $\pm$ 1,0 g) were used where noted in the tables.

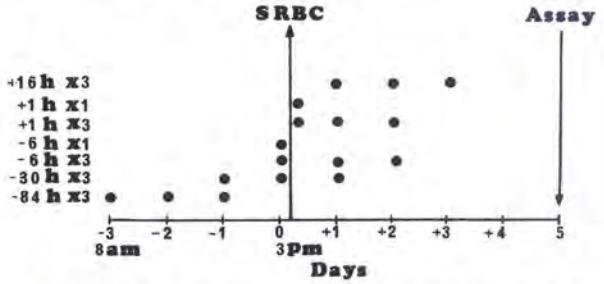

FIG. 3 Schedule of administration of one or 3 injections of 4 mg cortisol at various times before or after immunization with SRBC

The results of all these experiments are given in Tables 1 to 7. In each case the C.V. (%) is given for both spleen mass and PFCs/10^6 spleen cells. A significant difference ($P<0,05$) is indicated by the appropriate sign, whereas underlining of results indicates $P>0,05$, i.e. the difference is not considered significant.

The results obtained when 4 mg cortisol was administered three times, beginning at −84 h, are given in Table 1. As expected from the results depicted in Fig. 1, no significant difference is seen in spleen mass. The PFCs/10^6 spleen cells were greater than the controls in two cases and less in the other two experiments. The effect is therefore somewhat difficult to assess, but the variability of the response implies that there is no constant diminution or augmentation of PFCs/10^6 spleen cells with this regimen of steroid injections.

There is an invariable drop of spleen mass when the first injection was given at −30 h (Table 2), again as expected from the data of Fig. 1. In all but one case this decrease in mass was accompanied by a significant drop of PFCs/10^6 spleen cells. Similarly, as shown in Table 3, the regimen of three doses commencing at −30 h invariably results in a drop in spleen mass and PFCs/10^6 spleen cells were always considerably lower than in the controls (>50 per cent of control except in Experiment 10). These reductions in PFCs were greater on a percentage basis than those shown in Table 2, at −30 h. However, this apparently greater drop than at −30 h may be fortuitous. As shown in Table 4, in only two out of four experiments was the drop in PFCs/10^6 spleen cells greater at −6 h than at −30 h.

Starting the cortisol regimen at +1 h resulted in a lower percentage decrease in mass than with the previous regimens (Table 5). In four out of five experiments, however, this was accompanied by a significant rise in the PFCs/10^6 spleen cells, not a drop. Delaying the start of the series of cortisol injections to +16 h was rarely followed by any significant diminution of mass (Table 6). From a comparison of Fig. 1 and 2, it appears that the diminution in spleen mass brought about by the steroid is nullified, in this case, by the simultaneous maximal rise in mass in response to the SRBC. Similarly the numbers of PFCs/10^6 spleen cells were always either equal to or greater than they were in the controls. Thus delaying the first steroid injection till after immunization resulted in little or no effect on them. Similarly, a single injection of steroid failed to reduce the PFCs/10^6 spleen cells whether it was given before or after the antigen injection (Table 7).

The maximum effect is apparently produced by one or more injections of steroid administered before the SRBC, followed by at least one at +16 h. Furthermore, a reduction in spleen mass of less than 50 per cent is not necessarily accompanied by a drop in PFCs/10^6 spleen cells. However, if one considers the total production of PFCs per spleen, it is obvious that a markedly lower spleen mass will result in a decrease in the total number of cells, and therefore of PFCs. Since PFCs/10^6 total spleen cells are reported, it was also necessary to determine whether steroid treatment affected the percentage of nucleated cells present in the suspension. A number of individual mouse spleen suspensions was prepared, with and without SRBC and/or steroid treatment, and differential counts were performed in the usual manner. Although differences were found, analysis of variance for the different groups failed to reveal significance ascribable to the various treatment regimens and the ranges of variation encountered were also not significantly different. Differential counts were performed in all experiments, and nucleated cells generally comprised 50 to 70 per cent of the total. Differences between treated and control spleens were, at the most, five to 10 per cent and were ignored. This implies that had our results been presented as PFCs/10^6 nucleated spleen cells, the relative values would have been the same and all the figures would simply have been 1,5 to 2 times higher. It should be noted that only nucleated cells and not specifically lymphocytes were counted.

Another factor that was calculated in all experiments was the yield, i.e. the number of spleen cells obtained per 100 mg spleen mass. Although the method of preparation of spleen suspensions was not strictly quantitative, other factors being equal, this "yield" should be constant in all experiments. This was not found. Invariably steroid treatment, at whatever time, resulted in a lower yield. Using SRBC-treated spleens of 80 to 250 mg mass, there was no proportional relationship between the yield and the mass, the former being constant at approximately 2×10^8 cells/100 mg spleen. However, some steroid-treated spleens measuring 60 mg or less did contain proportionately fewer cells, as might be expected from the relatively greater mass of the capsule. Nonetheless the lower numbers of cells yielded by steroid-treated spleens could not be ascribed to this alone, because most such spleens were of more than 60 mg mass, and yet yielded relatively few cells. A transient oedematous condition of the spleen after steroid treatment has been described (Dougherty & White, 1945), but we obtained lower yields of cells even after steroid was given at −84 h. This reduced the yield of total PFCs per spleen, even when there was no significant mass loss or drop in PFCs/10^6 spleen cells. The mean results obtained from the various steroid regimens for total PFCs per spleen and the relevant yields of cells are given in Table 8. From this it can be seen that significant drops in total PFCs per spleen were obtained following three injections starting at −30 h, −6 h and +1 h, although the latter was due only to a reduction in spleen mass, and not to a drop in PFCs/10^6 spleen cells (cf Table 5). Some reduction was also obtained with a single injection at −6 h. The other apparent drops in PFCs per spleen are accounted for solely by the lower yields.

Response to different dosage levels

Having established that steroid treatment was most effective when it was commenced at −6 h and three doses were given, an attempt was made to determine the dosage required to produce a drop in mass and in PFCs/10^6 spleen cells. The results are given in Tables 9 and 10. For technical reasons it was possible to handle only three batches of mice at a time. However, in some cases (e.g. Experiment 1a, b and c) we tried to make more valid comparisons by using one batch of mice, SRBC from a single batch of blood, and performing experiments on consecutive days. The figures given in Table 9 show that 1 to 2 mg cortisol suspension was required to produce loss of mass, that increasing the dosage to 4 mg brought about no further reduction in mass, and that no difference was observed between aqueous and oil suspensions of the same concentration. The soluble preparations, Betsolan and Efcortolan, produced a maximal effect at a dosage of 0,5 mg.

TABLE 1 The effect of three injections of 4 mg cortisol starting at −84 h

Exp.* No.	Spleen mass (mg)			C.V.	PFCs/10^6 spleen cells					C.V.
	Control†		Test 1‡	Test 2‡	%	Control		Test 1	Test 2	%
1.....	143		111	154	26,8	422	>	350	236	15,1
2.....	139		103	111	19,0	253	<	317	474	15,0
3.....	137		99	127	24,5	441	>	360	254	11,0
4.....	194		110	173	32,0	322	<	651	659	10,6

C.V. Coefficient of variance.——, Difference not significant, i.e. $P > 0,05$

* Replicate experiments performed
† Batch of 5 mice-mean
‡ Replicate batches of 5 mice-mean

TABLE 2 The effect of three injections of 4 mg cortisol starting at −30 h

Exp. No.	Spleen mass (mg)				C.V.	PFCs/10^6 spleen cells				C.V.
	Control		Test 1	Test 2	%	Control		Test 1	Test 2	%
1.....	190		141	98	29,1	318	>	247	212	12,5
2.....	166	>	77	77	26,6	520		468	440	12,5
3.....	156	>	96		26,0	452	>	274		19,2
4[a].....	161	>	95		40,7	496	>	125		17,1
5.....	162	>	106		16,7	361		360		14,6
6[b].....	163	>	98		16,9	473	>	393		11,5

[a] Aqueous instead of oil suspension
[b] 1 mg cortisol dissolved in 50 per cent aq. propylene glycol

TABLE 3 The effect of three injections of 4 mg cortisol starting at −6 h

Exp. No.	Spleen mass (mg)				C.V.	PFCs/10^6 spleen cells				C.V.
	Control		Test 1	Test 2	%	Control		Test 1	Test 2	%
1.....	154	>	83		22,4	540	>	242		12,2
2.....	99	>	54		27,8	—		—		—
3.....	194		175	107	30,6	464	>	185	176	13,4
4.....	218	>	92		52,3	298	>	110		9,9
5.....	159	>	68		24,0	455	>	125		9,9
6[a].....	121	>	62		30,0	—		—		—
7[a].....	147	>	76		27,3	403	>	295		10,6
8.....	156		110		26,0	452	>	132		19,2
9.....	161	>	81		40,7	496	>	130		17,1
10.....	162	>	90		16,7	361	>	307		14,6
11[a].....	124	>	79		31,6	602	>	203		11,1
12.....	135	>	89	82	26,7	620	>	325	260	9,7

[a] Aqueous instead of oil suspension

TABLE 4 Comparison of the effect of three injections of 4 mg cortisol starting at −30 h and −6 h

Exp. No.	Spleen mass (mg)			C.V.	PFCs/10^6 spleen cells			C.V.
	Control	−30 h	−6 h	%	Control	−30 h	−6 h	%
1.....	156>	96	110	26,0	452>	274>	132	19,2
2[a].....	161>	95	81	40,7	496>	125	130	17,1
3.....	162>	106	90	16,7	361	360>	307	14,6
4[b].....	163>	98	92	16,9	473>	393	408	11,5

[a] Aqueous instead of oil suspension
[b] 1 mg cortisol dissolved in 50 per cent aq. polyethylene glycol

TABLE 5 The effect of three injections of 4 mg cortisol starting at +1 h

Exp. No.	Spleen mass (mg)			C.V.	PFCs/10^6 spleen cells			C.V.
	Control	Test 1	Test 2	%	Control	Test 1	Test 2	%
1.....	162 >	74	86	31,5	281 <	329		13,5
2.....	137	99	79	30,2	550 >	344	353	16,3
3.....	—	—		—	324 <	418		12,5
4.....	—	—		—	621 <	908		15,1
5[a].....	137 >	98	89	27,5	429 <	487	481	14,0

[a] Aqueous instead of oil suspension

TABLE 6 The effect of three injections of 4 mg cortisol starting at +16 h

Exp. No.	Spleen mass (mg)			C.V.	PFCs/10^6 spleen cells			C.V.
	Control	Test 1	Test 2	%	Control	Test 1	Test 2	%
1.....	155	131	114	29,3	302 <	374	397	14,1
2.....	132	115	92	37,2	492	467	—	9,7
3.....	207 >	118	103	32,2	290	306 <	424	16,7

TABLE 7 The effect of a single 4 mg injection of cortisol at either −6 h or +1 h

Exp. No.	Spleen mass (mg)			C.V.	PFCs/10^6 spleen cells			C.V.
	Control	Test 1	Test 2	%	Control	Test 1	Test 2	%
1[a].....	206 >	114	148	21,7	472	419 <	547	12,1
2[a].....	216 >	159	140	22,4	418	436 <	508	14,0
3[b].....	169	139	116	25,4	684	674 <	935	10,4

[a] −6 h; [b] +1 h

TABLE 8 Summary of the total PFCs per spleen obtained after various regimens of steroid treatment and relationship to yield of cells per 100 mg spleen mass

Injection schedule*	No. of exps.	PFCs per spleen				Cell yield (10^8 cells per 100 mg spleen)			
		Control mean	Test mean	Per cent control	+S.D. per cent	Control mean	Test mean	Per cent control	±95% C.i.R.
−84 h × 3.....	4	112 900	71 900	63,7	23,32	2,30	1,53	66,5	23,29
−30 h × 3.....	6	151 200	45 500	30,1	11,88	2,13	1,41	66,3	6,46
− 6 h × 3.....	12	144 500	20 380	14,1	7,44	1,96	1,22	62,2	8,23
− 6 h × 1.....	2	162 200	74 900	46,2	16,25	1,54	1,17	76,0	37,42
+ 1 h × 3.....	3	109 300	47 550	43,5	17,08	1,86	1,37	73,7	55,08
+16 h × 3.....	3	112 300	78 830	70,2	33,76	1,94	1,27	65,4	22,76
+ 1 h × 1.....	1	190 000	144 400	76,0	—	1,65	1,35	81,8	—

S.D. = The standard deviation of the differences between control and test
C.i.R. = The 95 per cent confidence interval for yield of cells calculated by a two-tailed Student's test at $P = 0,05$ and equal to the S.D. of the difference between control and test $X\ t\frac{0,05}{n}$ where n = number of degrees of freedom for the difference

* The usual 4 mg suspension of cortisol injected at each time interval

TABLE 9 Variation in spleen mass following three injections of either cortisol, Betsolan or Efcortolan starting at −6 h

Exp. No.	Control	Cortisol suspensions						Betsolan		Efcortolan		C.V. %
		0,5 mg aq.	1,0 mg aq.	2,0 mg aq.	2,0 mg oil	4,0 mg aq.	4,0 mg oil	0,5 mg	1,0 mg	0,5 mg	4,0 mg	
1a.....	134,7>						89,3	84,3				26,7
1b.....	124,2			101,7		78,5	81,5					31,6
1c.....	143,8	126,0	98,6									24,7
2a.....	162,9>		91,7*									16,9
2b.....	161,9>						90,4					16,7
3a.....	160,6>					81,1						40,7
3b.....	155,7>						110,1					26,0
4.....	147,4			116,6		76,0						27,3
5.....	161,7	134,1	92,6									22,4
6.....	202,5>			119,6	81,2							33,9
7.....	120,9>			70,3		62,4						30,0
8.....	198,1	171,0	136,4									26,9
9.....	158,7	124,6>		68,3								19,0
10.....	225,2>								89,7			35,9
11.....	187,3>									108,3	117,5	

a, b, c are the same batch of mice injected with a solution of the same blood on consecutive days
C.V. = coefficient of variance. —— = No significant difference at $P < 0,05$. In experiments 1 to 4 mice of 20,5 ± 1,0 g mass were used. In the remaining experiments unmeasured (mass varied from 16 to 25 g) 8-week-old mice were used

* 1 mg cortisol dissolved in 50 per cent aq. polyethylene glycol

TABLE 10 Variation in PFCs/10^6 spleen cells following three injections of either cortisol, Betsolan or Efcortolan starting at +6 h

Exp. No.	Control	Cortisol suspensions						Betsolan		Efcortolan		C.V. %
		0,5 mg aq.	1,0 mg aq.	2,0 mg aq.	2,0 mg oil	4,0 mg aq.	4,0 mg oil	0,5 mg	1,0 mg	0,5 mg	4,0 mg	
1a.....	620>						326	297				9,7
1b.....	602>			437>		203	260					11,1
1c.....	500>	370	<450									11,9
2a.....	473>		408*									11,5
2b.....	361>						307					14,6
3a.....	496>					130						17,1
3b.....	452>						132					19,2
4.....	403>			326		295						10,6
5.....	582>	399>	169									24,5
6.....	370>			289	241							11,2
8.....	508>	360	354									12,7
9.....	426	434				125						12,4
10.....	326>								205			11,3
11.....	464>									183	<270	20,7

a, b, c are the same batch of mice injected with a solution of the same blood on consecutive days
C.V. = Coefficient of variance. —— = No significant difference at $P<0,05$. In Experiments 1 to 4 mice of 20,5 ± 1,0 g mass were used. In the remaining experiments unmeasured (mass varied from 16 to 25 g) 8-week-old mice were used

* 1 mg cortisol dissolved in 50 per cent aq. polyethylene glycol

Apart from the fact that 0,5 mg cortisol suspension caused a reduction in PFCs/10^6 spleen cells, though it caused no diminution of mass, a similar pattern for this parameter is shown in Table 10. In Experiment 1, the 4,0 mg dosage produced a drop greater than with 2,0 mg. On these grounds it appears that PFCs/10^6 spleen cells are a more sensitive indicator of steroid action than loss of mass by the spleen when three injections of steroid are administered beginning at −6 h.

Discussion

In general our findings both confirm and extend those of previous investigators in the field. Thus, the maximum PFC production at Day +4 after SRBC injection has also been reported by Jerne, Nordin & Henry (1963), Miller & Mitchell (1968) and Pantelouris & Flisch (1972). Similarly, the apparent coincident maximum rise in spleen mass has been observed by Wissler, Fitch, La Via & Gunderson (1957), working with rats and by Petranyi *et al.* (1971), working with mice. In all these cases though, the control values were lower than those found in the mice used in these experiments. After a single dose of 7,5 mg per 20 g mouse, Elliott & Sinclair (1968) also observed a gradual recovery of spleen mass to normal. In our experiments the much more rapid return to normal was comparable to that found by Frank *et al.* (1953) after merely fasting their animals. These authors worked with mice of similar mass, but the control spleens measured only 65 ± 3 mg; the mean was less than half of that which we measured, and the standard deviation was considerably smaller. Although strain-dependent differences do exist in relative spleen mass (Gisler *et al.*, 1971), it should be noted that our mice were kept in an uncontrolled environment, and this would also contribute to the wide variation in mass we observed in many of our experiments.

This large variation in spleen mass in different groups of mice made the response to different dosage levels very difficult to assess, but three 2 mg doses starting at −6 h had a maximal effect on both loss of mass and reduction in PFCs/10^6 spleen cells, although the large 4 mg dose could sometimes cause a further drop in the PFCs. The cortisol dosage of 4 mg, which was similar in effect to 0,5 mg of the soluble preparations, Betsolan and Efcortolan, is equivalent to that reported by Berglund (1956b) as being necessary to cause a drop in haemolysin titres with a comparable antigen dose. Although Newsom & Darrach (1954) found that a single injection of 2,5 mg cortisol on Day −1 completely inhibited the haemolysin response after half the dose of SRBC, our experiments showed that a single dose of hormone given at this time, though causing some depression of both mass and PFCs/10^6 spleen cells, was inadequate for maximal inhibition.

Maximal reduction in both spleen mass and PFCs occurred following three injections starting at −6 h. Three treatments commencing at −30 h also caused quite a large drop in both parameters measured. This is in broad agreement with previous reports that the period of maximal steroid sensitivity occurs in the 24 hour period predecing antigen injection, the "pre-induction" period (Newsom & Darrach, 1954; Berglund, 1956a, 1962; Taliaferro, 1957; Elliot & Sinclair, 1968; Petranyi *et al.*, 1971). However from the experiments reported here, it appears that one or more steroid injections given immediately after the

antigen injection cause a further reduction of PFCs/10^6 spleen cells. Nonetheless, cortisol given as early as an hour after SRBC has no effect unless it is preceded by at least one steroid treatment given 6 h before the antigen.

It is known that three cell types contribute to the PFC response to SRBC in mice (Claman, Chaperon & Triplett, 1966; Mosier, 1967; Mosier & Coppleson, 1968; Miller & Mitchell, 1969; Katz & Benacerraf, 1972). Both B (bursa-derived) and T (thymus-derived) lymphocytes as well as macrophages occur in the spleen and it was impossible to determine from the present experiments which of these cell types are affected by the cortisol. Current evidence suggests that although many T cells are inactivated (Ringertz *et al.* 1952), those T cells with specific helper activity remain relatively unaffected (Moorhead & Claman, 1972). The defect is thought to be in the destruction and inactivation of B cells in the spleen which give rise to antibody-producing cells (PFCs) by differentiation and proliferation (Levine & Claman, 1970; Cohen & Claman, 1971).

Acknowledgement

The authors would like to express their grateful thanks to Mr P. Carter, upon whose willing and skilled technical assistance much of this project depended. We would also like to extend our thanks to Dr R. Oellermann for constant encouragement during the course of this work.

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Printed by and obtainable from The Government Printer, Private Bag X85, Pretoria, 0001.