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Cell-mediated immunological responsiveness in mice decomplemented with cobra venom factor

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Summary. *In vivo* administration of cobra factor (CoF), the C3-activating protein of cobra venom, had no suppressive effect on the *in vitro* response of lymphocytes to PHA, LPS or allogeneic cells; nor did it affect the generation of cells cytotoxic to allogeneic target cells. HVG reactivity was inhibited by commercially available but not purified CoF, and the latter also failed to prolong skin allograft survival. It is concluded that *in vivo* complement depletion does not interfere with T cell responses, and that previous reports of prolonged survival of skin allografts and inhibition of cutaneous delayed hypersensitivity reactions following treatment with CoF may have been due to impurities in the preparations used.

INTRODUCTION

In vivo administration of cobra factor (CoF) suppresses the humoral antibody responses to T-dependent but not T-independent antigens (Pepys, 1972, 1974; Pryjma & Humphrey, 1975), and in T-dependent responses IgG (Pepys, 1974; Nielsen & White, 1974), IgA (Pepys, Wansbrough-Jones & Mirjah, 1976) and IgE (Pepys, Brighton, Hewitt, Bryant & Pepys, 1977) antibody formation is inhibited to a greater extent than the relatively T-independent production of IgM (Pepys, 1974; Nielsen & White, 1974). These effects, which are apparently a result of *in vivo* complement depletion produced by CoF (Pepys, 1972, 1974, 1976), suggest that T cell function may be selectively affected. However, in animals in which a T-dependent antibody response is suppressed by CoF, T cells undergo normal antigen-induced blast transformation (Pepys, Mirjah, Dash & Wansbrough-Jones, 1976) and mitosis (V. Wallis, A. J. S. Davies & M. B. Pepys, unpublished observations).

Although antigen recognition and subsequent cellular proliferation as measured in a local graft-versus-host (GVH) reaction were substantially suppressed by CoF treatment, this has been shown to be largely due to a cytotoxic contaminant in the CoF preparations used (Ballow, Day & Good, 1973). Similarly, cutaneous delayed hypersensitivity skin reactions in guinea-pigs were abrogated only when crude CoF was used; purified CoF with unimpaired anti-complementary activity was ineffective in this assay (Schwartz & Naff, 1971). It has, however, been shown that CoF preparations prolong skin allograft survival in guinea-pigs if given shortly before the expected time of rejection (Glovsky, Ward & Fudenberg, 1973) and in mice if given just before grafting (Pepys, 1972).

In view of these conflicting data on the immuno-

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suppressive effect of *in vivo* decomplementation we have further investigated the effect of both commercially available and purified CoF on T cell function *in vivo* and *in vitro*.

MATERIALS AND METHODS

Mice

Inbred CBA/caf (H-2k), BALB/c (H-2d) and C57Bl/6 (H-2b) mice, and (CBA × C57Bl/6)F_1 and (CBA × BALB/c)F_1 hybrids, were used. All animals were 3–4-month-old females except in the experiments described in Table 4, in which males of comparable age were used.

Cobra factor (CoF)

Two preparations of CoF were used. (1) Commercially available CoF obtained from Cordis Laboratories, Miami, U.S.A. Mice were injected intraperitoneally (i.p.) with a total of 6 units, divided into four doses, given within a 24 h period. (2) Purified CoF was obtained by fractionation of *Naja naja* venom (Sigma Chemical Co.) by the method of Ballow & Cochrane (1969). Chromatography on DEAE cellulose and then G200 Sephadex (Pharmacia) yielded a preparation of CoF containing only one demonstrable component, lacking phospholipase-A activity and not contaminated with low molecular substances from the venom (Pepys, 1976). Mice were injected with a total of 4 units (Ballow & Cochrane, 1969), divided into four doses as above, and their plasma C3 fell in the usual way (Pepys, 1972).

Low molecular components of venom including phospholipase-A

Gel filtration on G200 Sephadex of the crude CoF, isolated from *Naja naja* venom on DEAE cellulose, separated a C3-activating protein (CoF) of mol. wt 150,000 from at least three lower molecular weight components detectable as distinct antigens, and from phospholipase-A activity (Pepys, 1976). These materials were pooled together and concentrated by ultrafiltration on a PM30 membrane (Amicon). The O.D. 280 of the concentrated pool was 0·68.

Cell culture

Spleens or lymph nodes were removed aseptically into aliquots of 1 ml neat foetal calf serum (FCS). The tissues were teased out into RPMI medium supplemented with 2 g/l sodium bicarbonate, 3·5 g/l HEPES and 10,000 U/l of the antibiotics cloxacillin and ampicillin. The cells were washed twice and resuspended in RPMI with 10% FCS, counted and adjusted to the required concentration.

(a) *Mitogenic stimulation.* 10 μg/ml PHA (phytohaemagglutinin, reagent grade, Wellcome Laboratories) or 10 μg/ml LPS (lipopolysaccharide *E. coli* 055 : B5, Difco Laboratories) were added to 1×10^6 spleen cells suspended in 1 ml. Control cultures received medium in place of mitogens. 200 μl aliquots were dispensed in quadruplicates into microtest plates (microplates, flat-bottomed wells, Nunc, Denmark) and incubated for 48 h at 37° in an atmosphere of 5% CO_2. 1 μCi of [^{3}H]-thymidine (^{3}H methyl thymidine, 5 Ci/mM, Radiochemical Centre, Amersham) was added per well 24 h before harvesting.

(b) *Mixed lymphocyte culture (MLC).* Mesenteric lymph node cells at a concentration of 10×10^6 cells/ml were used as a source of responder cells. Spleen cells at a concentration of 5×10^6 cells/ml were irradiated with 2000 rad and used as a source of stimulator cells; the reactions were therefore one-way. Equal volumes of responder and stimulator cells were mixed to give a 2:1 ratio. Control cultures contained responder cells and irradiated syngeneic spleen cells. 200 μl aliquots of mixed cells were dispensed into microtest plates and incubated for 4 days at 37° in an atmosphere of 5% CO_2. Twenty-four h before harvesting the cultures, 1 μCi of [^{3}H]-thymidine was added per well. Cultures were harvested onto filter paper discs and washed twice in cold 10% trichloroacetic acid and twice in cold methanol. Counting of radioactivity was done in a Packard liquid scintillation spectrometer.

Host-versus-graft reactions (HVG)

This method was based on the popliteal lymph node weight gain assay as described by Twist & Barnes (1973). Recipient mice were injected in one foot pad with 20 μl of a mesenteric lymph node cell suspension containing 5×10^6 F_1 cells. Host reactivity to the injected cells was assessed 5 days later by weighing the nodes or by measuring the incorporation of the thymidine analogue ^{125}IUdR. For the latter test the mice were injected intra-

venously (i.v.) with 1 μCi ^{125}IUdR 4 h before removal of their nodes. Results are expressed as a reactivity index, obtained by calculating the ratio of injected to uninjected nodes.

Induction of cytotoxic cells in vivo

Cytotoxic cells were induced in C57Bl/6 animals by an i.p. dose of 2×10^7 P815-X2 DBA/2 mastocytoma cells. Ten days later the spleens were removed and the cytotoxic test was carried out as described by Brunner, Mauel, Cerrottini & Chapius (1968).

Skin grafting

Skin grafting was performed using allogeneic tail skin according to the method of Billingham & Medawar (1951). Median survival times (MST) were calculated by determining the day on which 50% of grafts showed complete rejection as judged by total scabbing of the epithelial surface.

Statistical methods

Significant differences between the results in test groups treated with CoF and untreated controls were sought in each experiment by unpaired *t*-tests.

RESULTS

Effect of *in vivo* administration of CoF on the *in vitro* response of lymphoid cells to mitogens and allogeneic cells

Spleen cells obtained from CBA mice 1, 3 or 6 days after injection with Cordis CoF or purified CoF responded somewhat variably to the mitogens PHA and LPS (Table 1). With a single exception—purified CoF given on day 6 (exp. 3)—no suppression of responsiveness occurred. Indeed, the occasional statistically significant departures from the normal were in the direction of enhancement rather than suppression. This is essentially in line with the finding that the relative proportion of T and B cells is unaffected by CoF treatment (Pepys *et al.*, 1976a), and it indicates that their functional capacity to respond to mitogenic stimuli was unimpaired.

In further experiments the capacity of lymph node cells from CoF-treated mice to respond *in vitro* to allogeneic cells was examined, and this, too, was found to be unaffected (Table 2), with a single exception (exp. 1) which involved the use of Cordis CoF.

Table 1. Effect of *in vivo* administration of Cordis CoF and purified CoF on *in vitro* lymphocyte transformation induced by mitogens

Experiment	Treatment	Medium c.p.m. ± s.d.	PHA (10 μg/ml) c.p.m. ± s.d.	LPS (10 μg/ml) c.p.m. ± s.d.
1	Control	7492 ± 266	93,670 ± 6517	67,409 ± 3710
	6 u CoF‡ day −1	7649 ± 676	126,705 ± 8503†	79,454 ± 6153*
	6 u CoF‡ day −3	8790 ± 1797	112,908 ± 14,686	73,984 ± 988
	6 u CoF‡ day −6	7719 ± 948	116,484 ± 5693*	87,566 ± 1886†
2	Control	3457 ± 473	47,883 ± 1933	40,214 ± 1673
	6 u CoF‡ day −1	2931 ± 186	52,330 ± 6521	45,220 ± 3428
	6 u CoF‡ day −6	3755 ± 107	49,841 ± 2051	56,369 ± 5442*
3	Control	1960 ± 400	12,032 ± 1683	10,381 ± 891
	4 u purified CoF day −1	2253 ± 558	16,405 ± 2465*	11,978 ± 1438
	Control	1561 ± 174	18,483 ± 1043	10,325 ± 885
	4 u purified CoF day −6	1621 ± 279	14,589 ± 1986*	9423 ± 342*
4	Control	9788 ± 3241	45,754 ± 6572	—
	4 u purified CoF day −1	9035 ± 1997	45,053 ± 7018	—
	4 u purified CoF day −6	12,352 ± 2169	47,613 ± 10,852	—

Results are expressed as the mean ± s.d. per group of 4–5 CBA females, based on a minimum of four cultures/animal.

* Significant difference from control $P < 0.05$.

† Significant difference from control $P < 0.001$.

‡ Cordis CoF.

Table 2. Effect of *in vivo* administration of Cordis CoF and purified CoF on MLC responses by CBA lymph node cells against C57Bl target cells

Experiment	Treatment	Days after CoF injection	Control cultures CBA + CBA c.p.m. ± s.d.	Mixed cultures CBA + C57Bl c.p.m. ± s.d.
1	Control	—	26,305 ± 2063	74,112 ± 10,658
	6 u CoF†	1	24,547 ± 1873	56,284 ± 9144*
2	Control	—	10,491 ± 1922	19,976 ± 2246
	6 u CoF†	1	9676 ± 1221	19,248 ± 654
	Control	—	8328 ± 909	24,036 ± 2686
	6 u CoF†	6	9725 ± 386	25,949 ± 1888
3	Control	—	11,294 ± 2531	24,121 ± 7742
	4 u purified CoF	1	10,714 ± 1486	23,838 ± 2883
	4 u purified CoF	6	11,564 ± 1674	25,166 ± 3901

Results are expressed as the mean ± s.d. per group of 4–5 mice, based on a minimum of four cultures/animal.

* Significant difference from control $P < 0{\cdot}03$.

† Cordis CoF.

HVG reactions in CoF-treated mice

Because *in vivo* administration of CoF had little effect on *in vitro* lymphocyte responsiveness, *in vivo* activation of lymphocytes was studied in the HVG reaction. The Cordis CoF was found to be strongly inhibitory (Table 3, exp. 1), whereas purified CoF had no effect (Table 3, exp. 2).

Table 3. Effect of CoF on the HVG reaction

Experiment	Treatment*	Mean ratio, injected/uninjected ± s.d. ^{125}IUdR	Weight
1	5×10^6 (BALB/c × CBA)F_1 cells in normal CBA recipients	5·06 ± 1·18	3·40 ± 0·98
	5×10^6 (BALB/c × CBA)F_1 cells in CBA recipients treated with 6 u of CoF§	1·90 ± 0·42†	1·16 ± 0·28‡
2	5×10^6 (CBA × C57B1)F_1 cells in normal CBA recipients	3·60 ± 0·62	—
	5×10^6 (CBA × C57Bl)F_1 cells in CBA recipients treated with 4 u purified CoF	3·84 ± 0·82	—

* CoF was given during the 24–36 h before injection of F_1 cells into the right footpad and reactivity of the popliteal lymph nodes on both sides was measured on day 5 in terms of IUdR incorporation and weight gain. Controls received no pretreatment. There were five recipients in each group.

† Significant difference from control $P < 0{\cdot}001$.

‡ Significant difference from control $P < 0{\cdot}005$.

§ Cordis CoF.

Effect of CoF on the generation of cytotoxic cells *in vivo*

Generation of specific cytotoxic T cells *in vivo* was tested 10 days after injection of P815 mastocytoma cells by measuring the *in vitro* capacity of spleen cells to kill target cells. Administration of Cordis CoF on either day 1 or day 9 after the dose of mastocytoma cells had no effect on the *in vitro* assay when the effector : target cell ratio was 100 : 1, but there was significant inhibition at a ratio of 50 : 1. In contrast purified CoF given on day 1 had no effect at ratios of 50 : 1 or 25 : 1.

Skin allograft survival in mice treated with purified CoF

It has been reported that mice treated with CoF 2 days before grafting reject skin allografts more slowly than normal mice (Pepys, 1972), but a subsequent analysis of the batch of CoF used showed it to be contaminated with phospholipase A and other low molecular weight substances (Pepys, 1976). An identical protocol was used in the experiment des-

Table 4. Effect of *in vivo* administered CoF on the generation of cytotoxic cells in the spleens of C57Bl/6 mice*

Experiment	Cells injected on day 0	CoF treatment	Effector : target ratio	^{51}Cr release c.p.m. ± s.d.†	Cytotoxicity (%)
1†	None	None	100 : 1	115 ± 22	—
	P 815	None	100 : 1	202 ± 5	36
			50 : 1	181 ± 7	27
	P 815	6 u CoF‡ on day 1	100 : 1	198 ± 11	34
			50 : 1	152 ± 20§	15
	P 815	6 u CoF‡ on day 9	100 : 1	198 ± 7	34
			50 : 1	165 ± 12¶	20
2†	None	None	50 : 1	114 ± 10	—
	P 815	None	50 : 1	414 ± 12	46
			25 : 1	231 ± 5	25
	P 815	4 u purified CoF on day 1	50 : 1	402 ± 8	44
			25 : 1	243 ± 20	26

* Pooled spleen cells from five donors tested on day 10 after i.p. inoculation of tumour cells. Target cells were P815-X2 tumour cells.
† Mean of five cultures.
‡ Cordis CoF.
§ Significant difference from untreated control $P < 0{\cdot}02$.
¶ Significant difference from untreated control $P < 0{\cdot}05$.

Table 5. Effect of purified CoF and low molecular weight contaminants on skin allograft survival (ten mice/group)

Experiment	Strain combination	Treatment	Median survival time (days)
1	CBA→BALB/c	—	7
		CoF i.p. on days −1 and 0 (Total of 4 u)	7½
		CoF i.p. on days −3 and −2 (Total of 4 u)	7
2	CBA→BALB/c	—	7
		CoF i.p. on days −3 and −2 (Total of 4 u)	7
		Low molecular weight pool on days 3 and 2 (Total of 0·4ml)	7
3	A→CBA	—	11½
		Low molecular weight pool on days −3 and −2 (Total of 0·6 ml)	12

cribed in Table 5, except that a purified CoF preparation was used. No prolongation of skin allograft survival was observed.

Crude CoF preparations may be contaminated with several substances of lower molecular weight than the CoF itself, including phospholipase A and materials with lymphocytotoxic (Ballow *et al.*, 1973) and adjuvant (Morrison, Louis & Weigle, 1976) activity. The effect on allograft rejection of a pool of some of these components, which had been separated from CoF by gel filtration, was therefore studied. The same injection schedule was used as for CoF administration, and the total dose (in different experiments 0·4 ml and 0·6 ml of a 1/10 dilution of the pool) was estimated to correspond with the extent of contamination of the earlier batch of CoF (Pepys, 1972). Graft survival was unaffected (Table 5).

DISCUSSION

In vivo treatment with CoF failed to suppress T cell activity in both *in vivo* and *in vitro* assays. Cells from CoF-treated mice responded normally *in vitro* to mitogens or allogeneic cells. Purified CoF had

no effect on the *in vivo* HVG reaction, the generation of spleen cells specifically cytotoxic for mastocytoma cells *in vitro*, or the survival of skin allografts. However, treatment of mice with commercially available CoF (Cordis) impaired both HVG reactions and the generation of cytotoxic effector cells, suggesting that impurities in the CoF preparation may have been responsible. This is in agreement with Ballow *et al.* (1973), who demonstrated that inhibition by a CoF preparation of HVG reactions in the rat was attributable to a low molecular weight contaminant with lymphocytotoxic activity. The suppressive effects of CoF on antibody formation (Pepys, 1972) have all been substantiated with purified, uncontaminated CoF (Pepys, 1976), but it now seems likely that the effect on allograft survival found previously by Pepys (1972) was caused by an impurity present in the batch of CoF used at that time. Failure of the low molecular weight pool in the doses used in the present study to affect skin allograft survival does not exclude this interpretation, particularly as the pool was concentrated before use on a PM30 membrane (Amicon). Although this retained substantial phospholipase A activity, other smaller molecules would have been lost in the ultrafiltrate.

In the work on guinea-pig skin allograft rejection (Glovsky *et al.*, 1973), CoF was effective only when given shortly before the onset of rejection, suggesting that complement might participate in the effector phase. However, there is insufficient information about the purity of the CoF used. In mice, the terminal phase of complement activation is not necessary for allograft rejection as genetically C5-deficient strains reject allografts quite normally (Caren & Rosenberg, 1965; Crisler & Frank, 1965).

It has been claimed that cytotoxic T cells possess C3 receptors (Arnaiz-Villena, Jones & Roitt, 1975) but this has not subsequently been confirmed (D. Slovik, L. Heppel & I. M. Roitt, personal communication). In the present study, variations in C3 levels affected neither the generation of cytotoxic cells nor their effector function.

Our experiments indicate that T cells responding to PHA or in MLC and HVG reactions do not have their activity impaired by purified CoF, and the same is true for cytotoxic T cells. Helper T cells are a different population (Cantor & Boyse, 1975; Cantor, Shen & Boyse, 1976). Even so, the education by sheep erythrocytes, in irradiated mice, of either thymus cells or splenic T cells for co-operation in an adoptive anti-sheep cell response is also unimpaired by CoF (M. B. Pepys, unpublished observations). *In vivo* administration of purified CoF leading to complement activation and depletion does not therefore suppress any aspect of direct T cell function which has so far been examined.

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