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Delayed hypersensitivity reactions to *Listeria monocytogenes* in rats decomplemented with cobra factor and in C5-deficient mice

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Summary. The *in vivo* effect of cobra factor (CoF), the complement-activating protein of cobra (*Naja naja*) venom, was investigated, using quantifiable assays for localization of labelled donor lymphoblasts and of host macrophages in intraperitoneal and subcutaneous sites of injection of antigens from *Listeria monocytogenes*. Both commercially available (Cordis) and highly purified CoF impaired these inflammatory responses, suggesting that the complement-activating protein was itself responsible rather than lymphocytotoxic or other contaminants. CoF had no measurable effect on lymphoblast localization during the first 7 hr, and only a slight effect at 24 hr, whereas macrophage accumulation was reduced by about 50% at 24 hr. This suggests that CoF treatment affected non-specific components of the early inflammatory reaction but had little or no effect on the subsequent immunospecific reaction. The effect of CoF on macrophages may be direct, or via depletion of complement components acting on macrophages, such as factor B and/or C3 or fragments thereof. It does not seem to involve the terminal complement components, C5–C9, since neither delayed-type hypersensitivity (DTH) nor cellular resistance to *Listeria* was reduced in C5-deficient mice when compared with C5-sufficient congenic controls.

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INTRODUCTION

Delayed-type hypersensitivity (DTH) reactions to bacterial antigens are characterized by the co-operation of sensitized T cells and monocyte-derived macrophages (Mackaness, 1972). Following deposition of antigen in tissues, some sensitized T cells amplify and sustain an inflammatory event that otherwise would have waned promptly. They do so in part by trapping lymphoblasts in the tissues; some of these cells may recognize the relevant antigen and thereby participate actively in the reaction (McGregor & Logie, 1974; Jungi & McGregor, 1978a). However sensitized T cells also promote the focal deployment (Jungi & McGregor, 1977; McGregor & Logie, 1975) and functional activation (McGregor & Logie, 1975; Jungi & McGregor, 1978b) of monocyte-derived macrophages which are the critical effector cells in a variety of cell-mediated immune reactions (Mackaness, 1972; WHO Scientific Group, 1973).

The effects of antigen recognition by specific T cells on lymphoblasts and macrophages are mediated largely by soluble T-cell products, but, in addition, other humoral mediators may amplify the inflammatory process in parallel with cell-mediated phenomena (Hill, 1969). For example, it was recently shown (Jungi & McGregor, 1979a) that DTH reactions to *Listeria monocytogenes* antigen were suppressed in rats that had been pretreated with cobra venom factor (CoF), a potent complement-activating and depleting agent (Cochrane, Muller-Eberhard & Aikin, 1970). The

mechanism of this effect of CoF was not clear. Some preparations of the complement-activating protein have been reported to contain lymphocytotoxic and other contaminants (Ballow, Day & Good, 1973; Lachmann, Halbwachs, Gewurz & Gewurz, 1976; Morrison, Louis & Weigle, 1976; Rumjanek, Brent & Pepys, 1978) and it is therefore important to determine whether the suppression of DTH is due to these or genuinely reflects a role for the complement system in such reactions. We now report that highly purified CoF, free of detectable phospholipase A or other toxic contaminants (Pepys, Tompkins & Smith, 1979), suppressed DTH reactions to bacterial antigens. Whilst these observations suggest that complement may affect expression of DTH, experiments in C5-deficient mice indicated that the terminal components C5–C9 are not involved.

MATERIALS AND METHODS

Animals

LEW and DA rats, DA × LEW F_1 rats and BALB/c mice were obtained from the 'Institut fur Medizinisch-Biologische Forschung', Fullinsdorf, Switzerland. They were either used directly or bred for one more generation, at the 'Schweizerisches Forschungsinstitut', Davos. B10.D2/oSn (congenitally C5-deficient) and the congenic B10.D2/nSn were obtained from Jackson Laboratory, Bar Harbor, Maine.

Micro-organisms

L. monocytogenes, EGD strain, was grown and quantified as described (Jungi & McGregor, 1977). A particulate bacterial antigen preparation (LMA) was made from alcohol-killed lyophilized *L. monocytogenes* bacteria as reported (Jungi & McGregor, 1977). Soluble antigens (LMAs) were made following the protocol of Kostiala & McGregor (1975).

Immunization

Rats were immunized by subcutaneous (s.c.) infection with 5×10^6 live *L. monocytogenes* (LM). Mice received intravenously (i.v.) an immunizing inoculum of ca. 5×10^3 LM. In some experiments, animals were immunized adoptively by infusion of TDL (rats) or splenocytes (mice) from pre-infected donors.

CoF

CoF was either purchased from Cordis, Miami, Fla. (CoF-Cordis) or prepared as recently described (Pepys *et al.*, 1979). Rats received 0·2 units CoF per g body weight, either as one intravenous injection or divided into four equal doses. In some experiments CoF was heated at 72° for 1 hr to inactivate the complement-activating protein. The level of total haemolytic complement was determined as described (Jungi & McGregor, 1979a). C3 was measured by electroimmunoassay as outlined elsewhere (Pepys, 1975) except that monospecific rabbit anti-rat C3 serum was used.

Measurement of delayed inflammatory reactions

Two cellular parameters associated with DTH were determined in antigen-stimulated ear sites: localization of radiolabelled donor lymphoblasts and of labelled host macrophages. Lymphoblast-enriched populations were obtained from thoracic duct lymph 5–6 days after infection of rats or from spleen of mice infected 7 days previously. Cells were labelled with [^{14}C]-thymidine and transferred 1 hr after antigen stimulation of recipients (Jungi & McGregor, 1977). Monocytes were labelled by pulsing animals with [^{3}H]-thymidine (0·2 μCi/g for rats, 0·5 μCi/g for mice) 24 hr before antigen administration (Jungi & McGregor, 1977). Six micrograms LMAs, dissolved in 20 μl saline, were injected into the pinnae of the test ears, whereas control ears received 20 μl saline. Twenty-four hours later, ear sites, 6 mm in diameter, were punched out and processed for radiometry as described. The same cellular parameters were also determined in the antigen-stimulated peritoneal cavity. One millilitre (rats) or 0·2 ml (mice) of a LMA suspension (50 μg/ml) were injected intraperitoneally; 24 hr later, exudates were collected, and lymphoblast- and macrophage-associated radioactivity was determined. The procedure has been described more fully elsewhere (Jungi & McGregor, 1977; 1979a).

Acquired cellular resistance: Resistance to LM was determined in actively immunized, adoptively immunized and normal mice. They were challenged i.v. with 10^4 LM, and 48 hr later, the numbers of organisms in livers and spleens were determined by quantifiable bacteriology (Jungi & McGregor, 1978b, 1979a).

RESULTS

The effect of CoF treatment on the rat complement system

Injection of CoF into rats leads to rapid and almost

complete loss of complement haemolytic activity (Jungi & McGregor, 1979a) in association with profound C3 depletion (Cochrane *et al.*, 1970). Both preparations of CoF used here had similar effects (Table 1) and reduced haemolytic activity and circulating C3 to minimal levels during the period in which measurements of the expression of DTH were performed.

Highly purified CoF impairs expression of DTH to bacterial antigens

The effect of highly purified CoF on DTH was tested in a two-part experiment as follows. One set of animals was infected with LM leading to enhanced antimicrobial resistance and a state of delayed-type hypersensitivity. The other set was left uninfected. Each set was subdivided into three groups which received, respectively, commercial CoF (Cordis), highly purified CoF, and no treatment. Twelve hours after administration of CoF, all groups were pulsed with [^{3}H]-thymidine. DTH was then elicited by subcutaneous administration of soluble *Listeria*-derived antigens (LMAs) into one ear, the other ear serving as saline injection site control. At the same time, particulate LMA was instilled into the peritoneal cavity thereby evoking an intraperitoneal delayed inflammatory response. One hour later, all rats received ^{14}C-labelled TDL from pre-infected donors. This inoculum provided a means of adoptive immunization for the non-infected rats, and permitted estimation of the degree of lymphoblast localization in antigen injection sites. After 24 hr, peritoneal exudates and ear sites were collected and analysed radiometrically for content of donor lymphocyte-associated (^{14}C) and host macrophage-associated (^{3}H) activity. Two parameters of delayed inflammation, lymphoblast localization and macrophage accumulation, were thus determined in each animal in both subcutaneous and intraperitoneal sites of antigen deposition.

Table 2 shows the extent of lymphoblast and macrophage accumulation in 24 hr exudates of CoF recipients and appropriate control groups. These data and similar results obtained with antigen-stimulated ear sites are summarized in Table 3 where responses in CoF-treated groups were expressed as a percentage of the appropriate controls. Several findings emerge from these tables. Firstly, there was no significant difference between the inhibition of the inflammatory response caused by commercial and by highly purified CoF, suggesting that the C3-activating protein rather than toxic contaminants or phospholipase A was responsible for the suppression. Secondly, the inhibition was more conspicuous for macrophage accumulation than for lymphoblast accumulation. Thirdly, it was more pronounced in adoptively immunized animals, in which there is a relatively modest response, than in actively immunized rats expressing relatively high levels of DTH. These observations suggest that *in vivo* treatment with CoF affects some early, acute com-

Table 1. The effect of CoF on haemolytic complement and C3 levels in rat serum

Treatment	Time after last CoF injection (hr)	Haemolytic activity*	C3 level†
CoF Cordis‡	24	<0	1·7±0·0
Purified CoF§	12	<0	1·6±0·2
Saline‡	24	6·6	140 (pool)
CoF Cordis‡	36	<0	2·7±1·0
Purified CoF§	24	<0	3·7±0·6
Heat inactivated CoF Cordis	36	6·5	118·0±4·5
Saline	36	6·9	113 (pool)
CoF Cordis‡	60	<0	5·1±0·4
Purified CoF§	48	<0	4·7±0·5
Heat inactivated CoF Cordis	60	7·1	104·0±9·9
Saline‡	60	6·6	125 (pool)

* $\log_2$ of dilution at which 50% lysis of antibody-treated sheep red blood cells occurred.
† % of level in a pool of normal rat serum; means of 5–10 rats±SD.
‡ Given i.v. in a single dose.
§ Given i.v. in 4 equal doses over 24 hr.

Table 2. The effect of commercial and highly purified CoF on lymphoblast and macrophage accumulation in exudates collected 24 hr after induction with LMA

Treatment of animals				% of lymphocyte associated radioactivity transferred day 7	Macrophage-associated radioactivity in 24 hr exudate (corr. c.p.m.)
Active immunization day 0	CoF *ca.* day 5	Adoptive immunization day 6	Exudate induction day 6		
LM		LM_i-TDL§	LMA	5·44 ± 0·36	40,297 ± 10,664
LM	0·2 μ/g CoF Cordis*	LM_i-TDL	LMA	3·32 ± 1·17	25,061 ± 7833
LM	0·2 μ/g heat inactivated CoF Cordis*	LM_i-TDL	LMA	4·89 ± 1·62	44,118 ± 17,872
LM	4 × 0·05 μ/g purified CoF†	LM_i-TDL	LMA	3·20 ± 0·43	25,562 ± 3516
—	—	LM_i-TDL	LMA	3·09 ± 0·91	11,370 ± 7620 ($n=2$)
—	0·2 μ/g CoF Cordis*	LM_i-TDL	LMA	2·03 ± 0·50	5658 ± 1486
—	0·2 μ/g heat inactivated CoF Cordis*	LM_i-TDL	LMA	3·46 ± 0·42	19,344 ± 2062
—	4 × 0·05 μ/g purified CoF	LM_i-TDL	LMA	1·75 ± 0·97	7431 ± 2101
—	0·2 μ/g heat inactivated CoF Cordis‡	LM_i-TDL	No antigen	0·19 ± 0·02	Not done

* Given i.v. 24 hr before exudate induction.
† Given i.v. in four equal doses 48–24 hr before exudate induction.
‡ Given i.v. 12 hr before exudate induction.
§ $1·5 \times 10^8$ [^{14}C]-thymidine-labelled TDL from *Listeria*-immune donors were infused.

Table 3. The effect of commercial and highly purified CoF on delayed-type hypersensitivity to *Listeria* antigens

Test model	CoF Cordis	Purified CoF
Lymphoblast localization‡	(% of controls)*	
Actively immunized†‡		
Peritoneal cavity§	64·7±22·8	62·3±8·4
Ear site¶	102·1±56·4	83·5±7·7
Adoptively immunized‡		
Peritoneal cavity§	62·3±15·1	53·9±29·7
Ear site¶	52·1±16·9	53·8±31·0
Macrophage accumulation**	(% of controls)	
Actively immunized†‡		
Peritoneal cavity§	59·3±18·6	60·5± 8·3
Ear site¶	86·2±49·7	72·9±13·0
Adoptively immunized‡		
Peritoneal cavity§	29·9±7·8	39·3±11·1
Ear site¶	30·6±16·3	41·3±13·5

*Control group: data of untreated and heat-inactivated CoF-treated rats were pooled.

† Animals were pre-infected with LM 6 days before antigen stimulation.

‡ Animals received $1·5 \times 10^8$ LM-immune [^{14}C]-thymidine labelled TDL at time of antigen stimulation.

§ Corrected c.p.m. in exudates 24 hr after antigen injection were computed.

¶ Differences between c.p.m. of antigen injection and saline injection site were calculated.

** Animals were pulsed with [^{3}H]-thymidine in order to selectively label cells of the monocyte lineage.

ponent of the inflammatory reaction. For example activation *per se* or depletion of complement proteins might interfere directly with non-specific inflammation at the site of antigen deposition, or it might affect the initial accumulation there of immunospecific lymphoblasts in the lesions.

Impairment of delayed inflammatory response by CoF is not the consequence of a smaller number of lymphoblasts accumulating in the early phase of inflammation

In this experiment, various numbers of immune TDL were infused into ^{3}H-labelled rats in whom DTH and peritoneal exudates were induced with *Listeria*-derived antigens. One set of recipients was treated with highly purified CoF, the other served as control. Exudates and ear sites were collected 24 hr after stimulation and analysed radiometrically. Two groups of recipients were assessed at 7 hr, corresponding to the early phase of lymphoblast and macrophage localization.

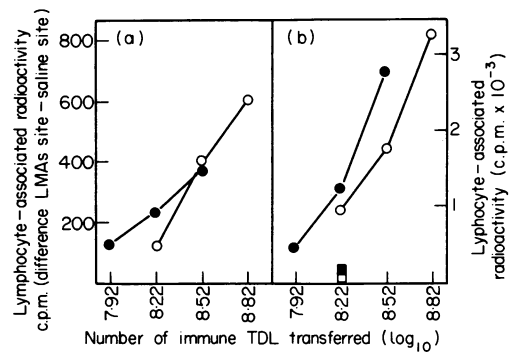

Figure 1. Accumulation of donor lymphoblasts in LMAs-induced ear sites (a) and LMA-induced peritoneal exudates (b) borne by normal (●) or CoF-treated (○) rats that had received various numbers of immune TDL. The slight defect in lymphoblast accumulation in inflammatory sites of CoF-treated rats could be compensated by injection of higher numbers of immune TDL. However, similar numbers of lymphocytes gathered in exudates of normal (■) or CoF-treated (□) rats during the first 7 hr.

The results (Figs 1 and 2) indicated a pronounced influence of CoF on accumulation of macrophages whereas the lymphoblast response was once again affected less. The suppression in DTH produced by CoF could be overcome by the transfer of a higher

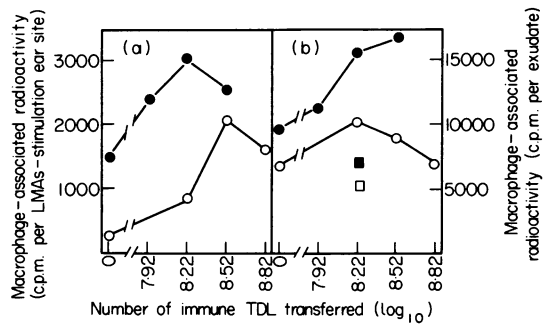

Figure 2. Accumulation of host macrophages in LMAs-induced ear sites (a) and LMA-induced peritoneal exudates (b) borne by normal (●) and CoF treated (○) rats that had received various numbers of immune TDL (same experiment as Fig. 1). Macrophage accumulation was slightly (but not significantly) impaired in 7 hr exudates of CoF recipients (□) when compared with control rats (■). In all exudates collected at 24 hr, macrophage accumulation in CoF recipients (○) was significantly reduced when compared with normal counterparts (●) ($P < 0·05$ for rats receiving no TDL, $P < 0·01$ for rats receiving $3·3 \times 10^8$ or $1·65 \times 10^8$ TDL). In ear sites, less macrophage-associated radioactivity was observed in CoF-treated rats (○) when compared with controls (●) ($P < 0·05$ for rats receiving no TDL, $P < 0·01$ for rats receiving $3·3 \times 10^8$ TDL).

Table 4. Normal recruitment of monocyte-derived macrophages into the antigen-stimulated peritoneal cavity in C5-deficient mice

Exp.	Mice*	Immunization	Day	Challenge (10 μg i.p.)	Day	Macrophage-accumulation in peritoneal exudates within 24 hr† (c.p.m.; means ± SD)
1	o	5×10^3 LM i.v.	0	LMA	7	22,453 ± 8781
	n	5×10^3 LM i.v.	0	LMA	7	19,389 ± 6082
	o	—		LMA	7	5105 ± 2860
	n	—		LMA	7	3685 ± 909
2	o	5×10^3 LM i.v.	0	LMA	8	9226 ± 5149
	n	5×10^3 LM i.v.	0	LMA	8	11,990 ± 2167
	o	SC‡ i.v.	0	LMA	8	3476 ± 1200
	n	SC‡ i.v.	0	LMA	8	3643 ± 1188

* o: B10D2/oSn; n: B10D2/nSn.
† Mean ± SD c.p.m. in groups of 5–6 mice.
‡ Spleen cells collected at day 8 after i.v. infection with 5×10^3 LM of BALB/c mice.

number of sensitized cells. On the other hand, accumulation of macrophages was also impaired in non-immune rats, indicating the effect of CoF on non-specific acute inflammation. There was, however, no evidence that this non-specific anti-inflammatory action of CoF reduced the early accumulation of specific lymphoblasts in the lesion. Although only relatively few lymphoblasts were present at 7 hr there was no difference between the CoF-treated and control groups. These results suggest that CoF has little effect on the immunospecific lymphoblast-mediated component of the DTH reaction but that, via its action on the complement system, it suppresses the non-specific inflammatory reaction elicited by *Listeria* antigens.

Expression of DTH to LMA is unrelated to C5 levels

A role for C5 and the terminal complement components C5–C9 in expression of DTH reactivity to LMA was sought using genetically C5-deficient B10.D2 old line mice and their congenic C5-sufficient counterparts. Actively immune mice of either strain were compared with normal mice or with adoptively immunized animals. The latter received splenocytes

Table 5. Comparison of innate and acquired resistance to LM in C5-deficient and C5-sufficient mice

Mice*	Immunization	Day	Challenge	Day	Log_{10} bacteria 48 hr after challenge†		Protection‡	
					Spleen	Liver	Spleen	Liver
o	5×10^3 LM i.v.	0	8×10^3 LM i.v.	6	1·68 ± 0·49†	2·07 ± 0·39	4·75	3·27
n	5×10^3 LM i.v.	0	8×10^3 LM i.v.	6	2·64 ± 0·64	2·23 ± 1·08	2·73	2·13
o	SC§ i.v.	6	8×10^3 LM i.v.	6	4·88 ± 0·49	5·25 ± 0·27	1·55	0·09
n	SC§ i.v.	6	8×10^3 LM i.v.	6	4·57 ± 0·14	4·78 ± 0·33	0·80	−0·42
o	—		8×10^3 LM i.v.	6	6·43 ± 0·14	5·34 ± 0·43	—	—
n	—		8×10^3 LM i.v.	6	5·37 ± 1·08	4·36 ± 1·03	—	—

* o: old line; n: new line.
† Mean ± SD of 5–6 mice per group.
‡ Difference in $\log_{10}$ bacterial counts between test group and non-immune controls.
§ Spleen cells from LM-infected BALB/c mice (suboptimal immunization).

from BALB/c donors 8 days after these had been infected with LM.

The two experiments depicted in Table 4 make clear that C5-deficient mice manifest no defect in marshalling monocyte-derived macrophages into LMA-induced exudates, regardless of the level of sensitization of the mice.

Finally, it was tested whether C5-deficient mice differed from C5-sufficient counterparts in their resistance to LM. Preinfected (actively immune) and normal mice of both congenic strains were challenged i.v. with LM, and 48 hr later, numbers of live LM per liver and spleen were assessed. Table 5 shows that C5-deficient normal mice were slightly inferior in combatting an LM infection over the first 48 hr. However, pre-infected mice of both strains eliminated the challenge organism with similar vigour, suggesting that acquired T cell-mediated defence operates without involvement of C5 or the terminal complement proteins.

DISCUSSION

We confirm here the earlier observation that *in vivo* pretreatment of rats with CoF preparations suppresses their expression of DTH reactions to LMA (Jungi & McGregor, 1979a). Since the CoF used in the present experiments was highly purified (Pepys *et al.*, 1979) and was known to be free of phospholipase A and low molecular contaminants the effect on DTH must be due to CoF, the complement-activating protein itself.

When CoF is injected *in vivo* it combines with factor B to form a complex, CoFBb, which is insusceptible to the control proteins of the alternative pathway and which circulates in the plasma with a relatively long half-life (Cochrane *et al.*, 1970; Pepys, 1975). The complex cleaves and activates C3 and leads thereby to massive depletion of C3 and to a lesser extent of the terminal complement proteins, C5–C9. Effects of CoF treatment on any *in vivo* biological process may therefore be due to either complement depletion *per se* or to the generation of active complement fragments during the activation and cleavage process which precedes and maintains complement depletion.

Suppression of the DTH reaction in CoF-treated rats was manifest predominantly as a diminution in the accumulation of macrophages whilst accumulation of specific lymphoblasts was only slightly affected. This is in agreement with our earlier work in which neither CoF itself nor complement activation nor complement depletion affected the direct recognition of antigens by T cells or their responses *in vivo* or *in vitro* (Pepys, 1972; 1974; 1976; Rumjanek *et al.*, 1978). The effect of CoF on macrophage accumulation observed here was seen in non-immune as well as in immune rats and this suggests involvement of complement in the non-specific inflammation which follows introduction of LMA into the tissues. LMA are known to be potent activators of the alternative pathway.

There is good evidence in mice that the mobility and 'activation' of mononuclear phagocytes is subject to regulation by complement proteins. Macrophages cultured *in vitro* are immobilized and stimulated to spread by exposure to Bb, whilst C5a is a potent chemotactic attractant which enhances their motility (Gotze, Bianco & Cohn, 1979; Bianco, Gotze & Cohn, 1979). It is not yet known what the relative *in vivo* roles of these products of complement activation may be in terms of attracting mononuclear cells to a local site and immobilizing them when they get there. However in mice decomplemented with CoF, exudation of macrophages following intraperitoneal injection of thioglycollate is inhibited (Gotze *et al.*, 1979), suggesting that complement probably does have important effects *in vivo*. In addition fixed C3b is the most important complement ligand for adherence reactions of macrophages. The suppression in CoF-treated rats of macrophage accumulation in sites of LMA injection is thus probably due either to depletion of complement proteins which, acted upon directly by the LMA, generate non-specific mediators, or to inhibition of normal macrophage responses by complement activation products generated by the CoF. The latter is perhaps less likely since in these experiments complement levels, and therefore the amounts of activation products, were minimal at the time of LMA injection. Also less likely, although not formally excluded, appears the possibility that CoF has effects on host components unrelated to the complement system which are involved in expression of DTH. However this may be, the terminal components C5–C9 or C5a do not have an important role in expression of DTH to *Listeria* antigens, as DTH reactions were normal in C5-deficient mice.

The finding that acquired cellular resistance to *Listeria* is manifested to a similar degree in C5-deficient and C5-sufficient mice (Table 4) deserves some comment. Lawrence & Schell (1978) showed that C5-sufficient mice survived a *Listeria* challenge better

than their congenic C5-deficient counterparts. Petit (1980) came to similar conclusions, using quantifiable bacteriology to assess the number of viable organisms. While we could confirm the inferiority of C5-deficient mice in the latter system, this holds only for animals that had not been pre-infected. Pre-infected (immune) mice of the two strains are similarly resistant to rechallenge. This leads us to conclude that the defect of C5-deficient mice is unrelated to the expression of acquired, T-cell-mediated defence, a notion compatible with the findings on DTH expression (Table 4). Indeed, the studies of Petit (1980) suggested that the difference between the two strains is unrelated to C5-levels, but may be related to a difference in macrophage performance. A macrophage defect in C5-deficient B10.D2 mice could well affect 'innate' resistance in naive mice as it is known that a number of non-T-cell-dependent cellular defence mechanisms operate in elimination of *Listeria* (Newborg & North, 1980; Chears & Waller, 1975; Skamene, Kongshavn & Sachs, 1979). It may even influence the rate of mortality when high infective doses are used (Lawrence & Schell, 1978). As regards T-cell-mediated defence, however, neither C depletion in the rat nor C5 deficiency in the mouse seems to alter expression of acquired resistance (Jungi & McGregor, 1979a and Table 5).

In conclusion we have shown here that *in vivo* treatment with preparations of CoF can suppress DTH reactions via effects of the complement-activating protein itself. The suppression of DTH is not mediated by effects on T cells or specific immunological recognition processes, but by decreasing the early non-specific inflammation elicited by injection of LMA. This decrease is probably due to the action of, or depletion of, fragments of factor B and/or C3 and predominantly involves macrophages. The normal DTH reactions mounted by C5-deficient mice exclude a necessary role for C5–C9 in expression of delayed hypersensitivity.

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