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Dissociation of anticomplementary and adjuvant properties of proteins derived from cobra venom*

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Summary. The ability of preparations of cobra factor (CoF) to convert C3 via the properdin pathway (anticomplementary activity) has been demonstrated to be unrelated to its ascribed ability to convert a tolerogenic signal into an immunogenic stimulus (adjuvant activity). It has been shown that CoF preparations which have become inactive with regard to anticomplementary activity still retain full adjuvant properties. Further, when both activities are present in preparations of CoF, they can be separated by gel filtration chromatography into a fraction with approximate molecular weight 150,000 which contains anticomplementary activity and a fraction with approximate molecular weight 20,000, which contains adjuvant properties.

INTRODUCTION

A role for complement in the initiation of the humoral immune response has been proposed by several investigators (Pepys, 1974; Feldmann & Pepys, 1973; Dukor, Schumann, Gisler, Dierich, Konig, Hadding and Bitter-Suermann, 1974); in part based on the observation that an anticomplementary protein present in cobra venom (CoF) has been shown to interfere with the induction of an unresponsive state in mice (Azar, Yunis, Pickering and Good, 1968). Recently, a model has been proposed (Dukor and Hartmann, 1973) in which activated C3 may, through its binding to the C3 receptor on bone marrow-derived lymphocytes (B cells) provide a necessary signal for triggering B cell response. It has been well documented that the anticomplementary CoF protein, when complexed with factor B of the properdin system (C3 proactivator), enzymatically converts C3 to its activated form (Cooper, 1973). Therefore, the demonstrated ability of CoF preparations to play a role in the modulation of the immune response may occur as a direct consequence of its ability to activate the third component of complement.

The capacity of a substance to transform the induction of immunological unresponsiveness into an immunogenic stimulus can be viewed as the most stringent manifestation of its adjuvant properties (Dresser, 1973). As a consequence, the ability of CoF to interfere with the development of an unresponsive state, would provide experimental evidence for its adjuvant properties. Evidence supporting such a concept has been suggested by the experiments of Azar *et al.* (1968) who demonstrated in mice that preparations of CoF were able to inhibit the development of immunological tolerance to

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human gamma-globulin (HGG). However, in more recent experiments, Pepys and Taussig (1974) have obtained contrasting data which demonstrated that the *in vivo* depletion of serum complement by purified preparations of CoF had no demonstrable effect on the induction of tolerance to heterologous gamma-globulins in mice.

Experiments in our laboratory indicated that a preparation of CoF which, apparently as the result of prolonged storage in solution had lost its anti-complementary activity (CoFi; inactive CoF) was still able to convert a tolerogenic regimen of HGG into an immunogenic stimulus. This preparation of CoFi, in addition to having lost its ability to interfere with complement-mediated haemolysis of sensitized erythrocytes *in vitro*, had no detectable effect on serum levels of C3 *in vivo*. These results, in addition to the conflicting reports described above on the adjuvant activity of CoF with regard to its ability to interfere with the induction of tolerance, prompted further investigation of the relationship between CoF anticomplementary and adjuvant activities. The results of the present study demonstrate that, whereas partially purified preparations of CoF manifest both anticomplementary and adjuvant activities, these properties can be differentiated by further purification procedures.

MATERIALS AND METHODS

Animals

A/J male mice, 6 weeks of age, were purchased from the Jackson Laboratory, Bar Harbor, Maine. They were maintained on Purina chow pellets and chlorinated water acidified to a pH of 3 with HCl (McPherson, 1963).

Antigen

HGG was obtained through the courtesy of the American Red Cross National Fractionation Center.* IgG was purified as described earlier (Chiller and Weigle, 1971) and was used to prepare de-aggregated HGG (DHGG, tolerogen) or heat-aggregated HGG (AHGG, immunogen).

Haemolytic plaque assay

Antibody-forming cells specific to HGG were enumerated using a modification for protein antigens (Golub, Mishell, Weigle and Dutton, 1968) of the Jerne plaque assay (Jerne and Nordin, 1963).

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Cobra venom preparation

Partially purified preparations of CoF were prepared by DEAE–Sephadex chromatography as described by Cochrane, Müller-Eberhard and Aikin (1970). The column fractions containing anticomplementary activity as described below were pooled and concentrated by ultrafiltration (Amicon Company, Lexington, Massachusetts) (UM2) to approximately 1·0 mg/ml and either stored frozen at − 20° or used directly. In some preparations further fractionation on Sephadex G-200 was performed using phosphate-buffered saline, pH 7·4 (PBS). Anticomplementary activity was assayed as described by Cochrane *et al.* (1970) and was expressed as units of activity (1 unit = 4 μg protein).

Radiolabelling of proteins

^{125}I-Na carrier-free (ICN, Irvine, California) and ^{131}I-Na carrier-free (Cambridge Nuclear, Boston, Massachusetts) were used to radiolabel protein preparations according to the chloramine T procedure as described by McConahey and Dixon (1966). Presence of radioactivity was determined using a gamma scintillation counter (Baird Atomic).

Sucrose gradient ultracentrifugation

Velocity sedimentation was carried out in sucrose using 5–20 per cent linear gradients in PBS. After centrifugation, gradient fractions were collected from the bottom of the tube.

Toxicity assay

Contamination of CoF preparations by LPS was determined by lethality to mice after intraperitoneal (i.p.) injection of 0·5-ml of samples. As it has been previously demonstrated that actinomycin substantially increases the sensitivity of mice to LPS (Mancini, Carbonara and Heremans, 1965) all samples were supplemented with 12·5 μg of actinomycin D. Deaths were recorded after 72 h.

Lipopolysaccharide

LPS from *Escherichia coli* O111:B4 (Lot B35527) was obtained from Difco Laboratories (Detroit, Michigan).

Determination of C3

To determine circulating levels of C3 after administration of preparations of CoF, mice were bled retro-orbitally and their sera tested by quantitative immunodiffusion according to the procedure of

Mancini *et al.* (1965). Rabbit antisera monospecific for mouse C3 was kindly provided by Dr James Clagett.

RESULTS

Effect of CoF on the induction of immunological unresponsiveness to HGG *in vivo*

To examine the participation of the anticomplementary protein on the modulation of tolerance induction *in vivo*, A/J mice were injected i.p. with 1·0 mg of DHGG in pyrogen-free saline. After 3 h, one group received an i.p. injection of 40 μg of CoF (a frozen preparation of CoF as prepared by DEAE–Sephadex chromatography); a second group received 50 μg of LPS, given intravenously (i.v.) and a third group received 0·2 ml pyrogen-free saline, given i.p. After 20 days, each group was challenged with 400 μg AHGG given i.v. As a further control, a previously untreated group of mice was similarly challenged with AHGG. Five days after challenge, spleen cells from each animal were assayed for the presence of plaque-forming cells (PFC) specific to HGG.

The results of this experiment are shown in Fig. 1 and confirm the earlier observations that mice injected with the tolerogen DHGG become unresponsive to a subsequent challenge with AHGG. In contrast, mice injected with tolerogen and CoF preparations showed an anamnestic response upon subsequent AHGG challenge. Similarly, as previously described (Louis, Chiller and Weigle, 1973), mice treated with tolerogen and LPS also became primed to the antigen.

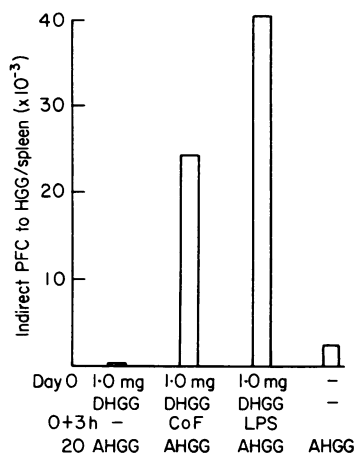

Figure 1. Anamnestic response of mice primed with DHGG (tolerogen) and CoF preparation. The treatment of various groups of mice (five mice per group) is indicated on the abscissa. The arithmetic mean of the indirect PFC response to HGG in the spleens of each group of mice is represented on the ordinate. The PFC were enumerated 5 days after challenge with 400 μg of AHGG.

Specificity of the CoF-mediated adjuvant effect

The above experiment demonstrates that the immune response of mice may be initiated by preparations of CoF in a manner comparable to that observed with LPS treatment. While this is suggestive of a specific CoF preparation-mediated response, contamination of CoF preparations by LPS (endotoxin) cannot be ruled out. Furthermore, although less likely, the possibility that CoF preparations can alter the physical form of the antigen and render it immunogenic must be considered. To exclude these two possible modes of action of CoF preparations in the induction of tolerance, the following experiments were performed.

The presence of endotoxin in the preparation was assessed by its lethality in actinomycin-treated mice. Groups of five mice were injected with 12·5 μg of actinomycin D and either 40 μg CoF, 0·05 μg LPS or 1 μg LPS; one group of mice was infected with actinomycin alone. As shown in Table 1, as little as 0·05 μg of LPS caused 50 per cent lethality in the actinomycin-treated mice. In contrast, no deaths were reported in mice treated with CoF, suggesting that the level of LPS contamination in the CoF preparation was probably not sufficient to account for the observed *in vivo* initiation of an immune response.

To rule out the possibility of aggregation of DHGG by CoF preparations, the former protein was radiolabelled with ^{131}I and the latter with ^{125}I. These preparations were then incubated either separately or

Table 1. Assay for the presence of LPS in the CoF preparation

Treatment on day 0	Percentage survival at day 3
Actinomycin D*+1 μg LPS†	0
Actinomycin D+50 ng LPS	50
Actinomycin D+40 μg CoF†	100
Actinomycin D	100

* Actinomycin 12·5 μg given i.p.
† Given i.p.

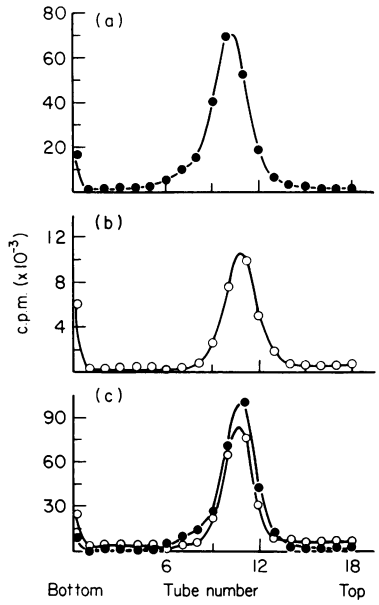

Figure 2. Velocity sedimentation of CoF and DHGG. Preparations of radiolabelled DHGG and/or CoF were incubated for 30 min at 37° and then sedimented for 18 h at 86,000 *g*. Fractions of approximately 0·25 ml were collected from the bottom of the tubes and assayed for radioactivity. (a) ^{131}I-labelled DHGG; (b) ^{125}I-labelled CoF; (c) ^{131}I-labelled DHGG+^{125}I-labelled CoF.

together at 37° for 60 s and subsequently examined by velocity sedimentation in sucrose density gradients. The results of this experiment (Fig. 2) demonstrate several important facts. First, there is no detectable aggregation of HGG by CoF. Secondly, as no detectable shift in the sedimentation profile of either protein occurs after incubation, there is very little interaction between these two proteins. Thirdly, the sedimentation profile of HGG confirms that the tolerogen is uniformly in the monomeric form. Finally, the uniformity of the sedimentation profile of CoF demonstrates that almost all of the protein in this preparation which may be radiolabelled sediments at about 7S (i.e. equal to that of DHGG).

Anticomplementary activity of CoF

In an attempt to correlate the loss of circulating C3 with the adjuvant activity found in the experiment described in the legend to Fig. 1, circulating levels of C3 were determined at various times after the injection of CoF. Surprisingly, no detectable consumption of C3 was observed in any of the mice tested. To

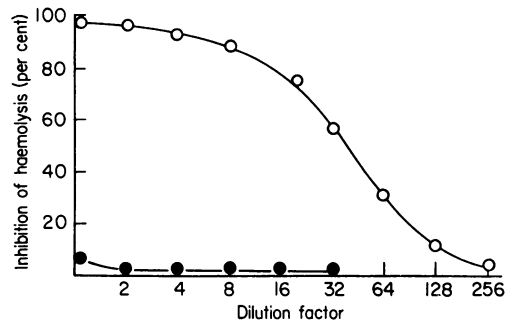

Figure 3. Inhibition of haemolysis of sensitized erythrocytes by preparations of CoF. Dilutions of preparations of CoF were incubated with a 1:20 dilution of human serum at 37° for 30 min prior to addition of sensitized sheep erythrocytes. (○) Active CoF (175 u/ml); (●) inactive CoF.

substantiate this observation, the *in vitro* anticomplementary activity of the CoF preparation was again assessed by the inhibition of haemolysis of sensitized erythrocytes. The results of this experiment (Fig. 3) (inactive CoF) demonstrated that virtually all of the original anticomplementary activity of this preparation which had been stored for more than 2 years at − 20°, had been lost.

The lack of detectable anticomplementary activity prompted our re-examination of the ability of CoF preparation with demonstrable anticomplementary activity to interfere with the induction of a tolerant state. To this end, a fresh preparation of CoF was prepared by DEAE–Sephadex chromatography as described in Materials and Methods section. After concentration by ultrafiltration, the anticomplementary and adjuvant activities were assessed. As shown in Fig. 3, the fresh CoF preparation contained potent anticomplementary activity. When tested *in vivo*, the adjuvant activity of this active preparation of CoF was found to be almost identical to that observed with the preparation shown in Fig. 1 which had no detectable anticomplementary activity. This lack of correlation between these two biological activities suggested that the anticomplementary activity of CoF may in fact be unrelated to its ability to act as an adjuvant.

Separation of anticomplementary and adjuvant properties

The observed dissociation of the two biological activities of CoF preparations could occur as a result of the CoF protein having two distinct unrelated

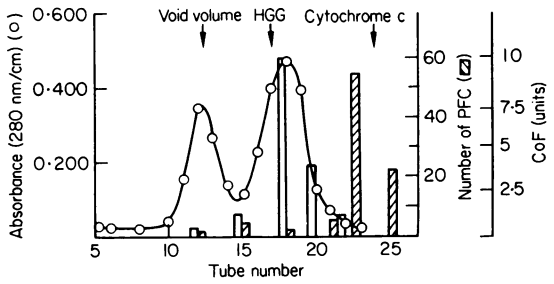

Figure 4. Gel filtration chromatography of CoF prepared by DEAE–Sephadex chromatography. A preparation of fresh CoF, active with respect to both anticomplementary and adjuvant activities, was chromatographed on a 22 × 2 cm column of Sephadex G-200 in PBS. Fractions of 2·5 ml were collected and absorbance at 280 nm determined. Aliquots of column fractions were titred for total anticomplementary activity and assessed for ability to act as an adjuvant *in vivo*.

functions. Conversely, the preparation could contain two or more distinct molecules, each with a defined biological activity. To distinguish between these two possibilities, preparations of active CoF were further fractionated by gel filtration on Sephadex G-200. Column eluates were assayed for both anticomplementary activity and ability to interfere with the induction of tolerance. The results of this experiment (Fig. 4) demonstrate that anticomplementary activity is associated with the major ultraviolet absorption peak eluting at an approximate molecular weight of 150,000. This activity is clearly dissociable from the column fractions containing adjuvant activity which has peak activity eluting near the inclusion volume at an approximate molecular weight of 20,000.

One possible alternative explanation for the lack of adjuvant activity in the fractions with maximal anticomplementary activity would be that excess anticomplementary CoF protein inhibits the adjuvant effect. This was found not to be the case, as dilution of the major anticomplementary activity fraction to a level of complement activity comparable to that seen in fractions containing adjuvant activity was also without adjuvant activity.

Thus, these two activities of CoF preparations occur as the result of the presence of more than one biologically active molecule in the preparations.

DISCUSSION

The present data demonstrate that preparations of CoF are indeed able to interfere with the induction of an unresponsive state to HGG by modulating the induction of tolerance to a state of immunity. This modulation of the immune response does not, however, result from the direct effect of complement activation initiated by the anticomplementary protein, chromatographing at approximately 150,000 molecular weight. Rather, such activity is correlated with a low molecular weight (approximately 20,000) contaminant present in CoF preparations. This conclusion is based upon the observations that preparations of CoF, inactive with respect to the well known anticomplementary activity, still retain adjuvant properties. A similar adjuvant effect was observed with fresh preparations of CoF which had demonstrated potent anticomplementary activity. Further purification of such active preparations of CoF showed that the fraction possessing anticomplementary activity (approximate molecular weight of 15,000) could be separated from the fraction displaying adjuvant activity (approximate molecular weight of 20,000). These results thus confirm the presence in our CoF preparation of more than one biologically active molecule.

These data are in agreement with the previous observation of Azar *et al.* (1968) who showed that preparations of CoF were able to inhibit the development of immunological tolerance to HGG. They do not, however, support the hypothetical mode of action of this adjuvant effect of CoF as it has been postulated by these authors. Azar *et al.* favoured the interpretation that an intact complement system would be necessary for the establishment of an unresponsive state. Our results would not sustain such an interpretation as the anticomplementary activity of CoF preparations was shown to be physically distinct from its adjuvant activity. This observation would thus rule out a direct correlation between the two biological activities.

Our data demonstrating a dissociation of adjuvant and anticomplementary activities of CoF preparations may also reconcile the conflicting observations of Azar *et al.* (1968) and Pepys and Taussig (1974), on the effect of CoF on the induction of immunological tolerance. The former investigators prepared CoF by chromatography on DEAE and CM cellulose whereas Pepys and Taussig utilized the procedure of Ballow and Cochrane (1969) which involves a Sephadex G-200 step in the purification of CoF. Although this latter procedure would probably remove the adjuvant activity of conventionally purified CoF preparations, the procedures employed

by Azar *et al.* may not have dissociated the adjuvant and anticomplementary activities we have demonstrated here to be distinct entities.

It should be further noted that the 20,000 molecular weight molecule with adjuvant properties may be present in very low quantities in the CoF preparations. This observation is based upon the fact that radiolabelled preparations of CoF sedimented in sucrose display essentially no slow-sedimenting fraction as estimated by radioactivity (see Fig. 2) and by the fact that there is little detectable absorption in the ultraviolet in the region of the G-200 column which contains maximal adjuvant activity.

The precise nature of this 20,000 molecular weight molecule is not, at present, clear. Ballow, Day and Good (1973) have described a cytotoxic factor for lymphocytes of approximately 13,000 molecular weight which is present in preparations of CoF after DEAE chromatography but which may be separated by gel filtration chromatography. More recently, Waldmann and Lachmann (1975) have demonstrated the presence of phospholipase A activity in preparations of CoF even after fractionation on Sephadex G-200. The presence of contaminating phospholipase A was found by these authors to markedly inhibit immune response *in vitro*. The relationship of our 20,000 molecular weight molecule to either the lymphocytotoxic factor of Ballow *et al.* on the contaminating phospholipase A described by Waldmann and Lachmann (1975) is currently under investigation in our laboratory.

The mechanism by which the active part of the CoF preparation in adjunct with a tolerogenic form of an antigen can lead to antibody formation is not understood. The temporal relationship between the injection of tolerogen and that of the 20,000 molecular weight from CoF is critical for the manifestation of the adjuvant effect, with a 24-h period after administration of tolerogen being the maximal allowable period which still allows a modulation of the induction of unresponsiveness to a state of immunity. When administered 4 days after the tolerogen, no interference with the establishment of an unresponsive state is observed (unpublished data). Previous observations (Louis, Chiller and Weigle, 1973; Chiller and Weigle, 1973) have suggested that in the state of tolerance presently studied it takes 2 to 4 days to induce an irreversible state of unresponsiveness in the B-cell population. Therefore, it would appear that the active component no longer has the capacity to manifest its adjuvant properties in animals whose B cells have already been made irreversibly tolerant.

It has been shown previously that LPS is also capable of modulating the induction of unresponsiveness to a state of immunity (Louis *et al.*, 1973). Consequently an alternative mechanism by which the 20,000 molecular weight fraction could mediate its activity would be that the preparations utilized are contaminated by LPS. This would appear unlikely for several reasons. No demonstrable toxicity initiated by LPS could be demonstrated with CoF preparations in mice made extremely sensitive to LPS by treatment with actinomycin D. In contrast, 50 ng of a control preparation of LPS produced a 50 per cent lethality. Although the type of LPS may partially influence these results, it may be estimated that there was less than 50 ng of LPS in the dose of CoF utilized throughout the present study. We have observed previously (unpublished data) that the minimal dose of LPS able to interfere with the induction of an unresponsive state in this system was 1·0 μg. It seems therefore that the described adjuvant effect of CoF cannot be explained by its contamination by LPS. Furthermore, as the active adjuvant component was demonstrated to have a molecular weight of about 20,000, it would appear extremely unlikely that such a preparation would contain LPS, as such molecules normally have a molecular weight of the order of 10^5–10^6. Another alternative mechanism by which CoF preparations could act as an adjuvant would be that *in vivo* it is capable of altering the physical form of the tolerogen transforming it to an immunogen. We have shown that monomeric preparations of HGG are not physically aggregated by *in vitro* incubation at 37° for 30 s with CoF preparations.

Our data would thus support the concept that activation of the complement system does not play a major role in the initiation of a host immune response or in the initiation of immunological tolerance. A similar conclusion excluding a necessary role for C3 in antibody production has also recently been demonstrated by Waldmann and Lachmann (1975).

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