

DETECTION OF THE COMPLEMENT FRAGMENT C5a IN
INFLAMMATORY EXUDATES FROM THE RABBIT
PERITONEAL CAVITY USING RADIOIMMUNOASSAY*

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Intradermal injection of boiled yeast cells (zymosan) or *Bordetella pertussis*-killed organisms in the nonsensitized rabbit induces local inflammatory edema thought to be mediated by two endogenous substances acting synergistically (1). One mediator is a vasodilator prostaglandin (PG); the other is a substance that increases venular permeability to plasma proteins. This report is concerned with the second. Incubation of blood plasma or lymph with zymosan (the standard inflammatory stimulus in these experiments) results in the generation of permeability-increasing activity. These plasma and lymph samples induce little edema when injected intradermally (measured by the 30-min accumulation of intravenously injected ¹²⁵I-albumin) but are highly active mixed with vasodilator prostaglandins (2). The permeability-increasing factor has been purified and characterized and its physico-chemical properties found to correspond to the complement fragment C5a (3). Although the chemical structure of rabbit C5a is not established, the activity is stable in plasma in spite of the presence of carboxypeptidase N, suggesting that rabbit C5a is active when devoid of its carboxyl terminal arginine (i.e., C5a des Arg). Human C5a, with a known structure (4), has also been shown to be a potent permeability-increasing substance with and without its carboxyl terminal arginine (5). Again the presence of a vasodilator is required to induce significant edema formation in rabbit skin. Independence from the carboxyl terminal arginine relates permeability-increasing activity in rabbit to leukotactic activity (6) rather than mast cell histamine release (7). Thus, it has been found that responses to C5a/prostaglandin mixtures cannot be abolished by antihistamines (3), but are abolished by depletion of circulating polymorphonuclear (PMN) leukocytes (5, 8). This indicates that increased permeability induced by the presence of C5a in the extravascular tissue fluid results from a rapid interaction (within 6 min) between circulating PMN leukocytes and venular endothelial cells (8).

From these observations it is possible to assemble the putative sequence of events that occurs after inflammatory stimulus and leads to changes in the microvasculature characteristic of the acute inflammatory reaction (3, 5, 8). Until now, we have had no direct evidence of local extravascular generation of C5a in response to an inflammatory (e.g., microbial) stimulus. Therefore, we have

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investigated a model in the peritoneal cavity because of the relative ease in collection of exudate compared with the skin. Earlier, we reported the appearance of permeability-increasing activity, prostaglandin, plasma proteins, and PMN leukocytes in the rabbit peritoneal cavity in response to a local injection of zymosan (9). The permeability-increasing activity exhibited characteristics similar to those of C5a. Here we describe a radioimmunoassay (RIA) for rabbit C5a and the detection of high levels of immunoreactive C5a (irC5a) in peritoneal exudate in response to zymosan. Because of the relevance of these observations to inflammatory reactions initiated by antigen-antibody complexes, a reversed passive Arthus-type reaction was also investigated in the peritoneal cavity, and again high levels of irC5a were detected.

Materials and Methods

Animals. Male New Zealand White rabbits (3.5–4 kg) were purchased from Buxted Farm, Sussex, United Kingdom; and male Dunkin Hartley guinea pigs from David Hall, Darley Oakes Farm, Burton-on-Trent, Staffordshire, U.K.

Materials. Saffan (Alphaxolone and Alphadolone) was from Glaxovet, Greenford, Middlesex, U.K.; Evans blue dye from Searle Diagnostics, High Wycombe, Buckinghamshire, U.K.; Arterioveine 11720, 14-gauge cannulae, from Vygon, 95440, Ecouen, France; Freund's complete and incomplete adjuvants (FCA, FIA) from Difco Laboratories, West Molesey, Surrey, U.K.; Na¹²⁵I from Amersham International, Amersham, Buckinghamshire, U.K.; Enzymobeads from Bio-Rad Laboratories, Watford, Hertfordshire, U.K.; Protein A Bacterial Adsorbent (PABA) from Miles Scientific, Stoke Poges, Slough, U.K.; carboxymethyl (CM)-Sephadex C-25, Sephadex G-25M, Sephadex G-100, Polybuffer (PB 96) and Polybuffer exchanger (PBE 94) from Pharmacia Fine Chemicals, Hounslow, Middlesex, U.K.; CM-cellulose CM32 from Whatman, Maidstone, Kent, U.K.; bovine gamma globulin (BGG) from British Drug Houses, Poole, Dorset, U.K. Zymosan was prepared by boiling bakers' yeast type II from *Saccharomyces cerevisiae* (Sigma Chemical Co., Poole, Dorset, U.K.) in water followed by extensive washing and lyophilization. Indomethacin was a gift from Merck, Sharp and Dohme, Hoddesdon, Hertfordshire, U.K.

Collection of Inflammatory Exudate from the Peritoneal Cavity. Rabbits were anesthetized with Saffan (0.5 ml/kg) and given maintenance doses when required. Evans blue dye (2.5% wt/vol, 0.5 ml/kg) was injected i.v. to enable plasma protein leakage into the peritoneal cavity to be monitored. A sterile 14-gauge concentric polythene cannula and steel needle were inserted i.p. in each rabbit. The needle was withdrawn and 50 ml of a zymosan suspension in saline (10 mg/ml), or saline as a control, injected via the cannula. The cannula was stoppered, the abdomen gently massaged to disperse the injection fluid, and a 3-ml sample withdrawn immediately by removing the stopper and collecting fluid into sodium EDTA pH 7.2 and indomethacin (10 mM and 5.6 μ M final concentrations, respectively) on ice. After removal of 100 μ l for a leukocyte count, the remainder was immediately centrifuged (7,800 g for 1 min) to ensure rapid separation of soluble factors from zymosan and leukocytes. Supernatants were stored at -20° C. This procedure was repeated at 1, 2, 4, and 6 h. The cannula was removed at 6 h and reinserted at 12 h for the final collection. Blood samples (1.5 ml, collected into EDTA and indomethacin) were taken from a small ear vein incision immediately before, and 1 h after, i.p. injection of zymosan. Blood samples were centrifuged at 7,800 g for 5 min and plasma samples stored at -20° C. To collect exudate from reversed passive Arthus-type reactions, the above procedure was used with the following differences. 50 ml of anti-BGG antiserum (diluted $\frac{1}{4}$) was injected i.p. followed 5 min later by an i.v. injection of BGG (5 mg/kg). Total available exudate was removed 2 h later. Antiserum was raised previously in rabbits by giving s.c. injections (4 \times 0.25 ml) of BGG (1 mg/ml emulsion) in FCA, followed by booster injections (4 \times 0.25 ml) of the same BGG concentration in FIA at 14 d and an injection of alum-precipitated BGG (300 μ g/0.1 ml) at 28 d. Blood was collected by carotid cannulation at 38 d. Serum from five rabbits was pooled, heat inactivated at 56° C

for 30 min, and stored in aliquots at -20°C . An IgG fraction made from the antiserum by ammonium sulphate precipitation at 50% saturation and dialysis against saline was used in some experiments.

Preparation of C5a. Rabbit C5a (presumed to be C5a des Arg because of the action of serum carboxypeptidase N) was prepared from zymosan-activated serum by step-wise elution from CM-Sephadex at pH 6.0 and gel filtration on Sephadex G-100 as described previously (3). This was followed by a second cation exchange step and chromatofocusing as described below. Sample was applied to a column (0.9 cm \times 20 cm) of microgranular CM-cellulose equilibrated in 0.1 M ammonium formate buffer, pH 5.0; the column was then eluted with a linear gradient ending in 0.5 M ammonium formate, pH 5.0 (C5a eluted at 0.3 M buffer). The chromatofocusing column (0.9 cm \times 19 cm) contained Polybuffer exchanger 94 equilibrated in 0.025 M ethanolamine/HCl buffer, pH 10.5; sample was applied in Polybuffer 96 (adjusted to pH 7.0 with HCl) and eluted with the same buffer (C5a eluted at pH 9.0). This preparation migrated as a single band (corresponding to 13,000 mol wt) in SDS-PAGE using an 8–20% acrylamide gradient gel.

Preparation of Antiserum to C5a. Rabbit C5a (640 $\mu\text{g}/\text{ml}$ phosphate-buffered saline, PBS) was emulsified with an equal volume of FCA and 0.2 ml was injected into each of the rear foot pads of three guinea pigs (day 0). A similar emulsion was made with FIA and 0.5 ml injected subcutaneously in the neck on day 19. Each guinea pig received $6 \times 100 \mu\text{l}$ intradermal injections of C5a (100 $\mu\text{g}/\text{ml}$ PBS) on days 26 and 34. Blood was obtained by cardiac puncture on day 41 and the antiserum frozen in small aliquots. A single precipitin band was obtained when this antiserum was reacted with C5a, zymosan-activated plasma or zymosan-induced peritoneal exudate fluid in double diffusion plates.

Preparation of ^{125}I -C5a. ^{125}I -C5a (3–4 $\mu\text{Ci}/\mu\text{g}$ protein) was prepared by incubation of rabbit C5a (10 μg) with Na ^{125}I (250 μCi), -D-glucose and solid phase glucose oxidase/lactoperoxidase "Enzymobeads" in sodium phosphate buffer, pH 7.2. The reaction was terminated after 45 min by adding sodium azide and centrifuging at 7,800 g for 1 min. The supernatant was mixed with carrier protein (bovine serum albumin, BSA) and sodium iodide and applied to a column (0.9 \times 16 cm) of Sephadex G-25M equilibrated in PBS containing BSA (2 mg/ml). Fractions containing iodinated protein were pooled and stored at 2°C in the presence of sodium azide (0.1%). Using the RIA procedure described below, binding of radioactivity in the presence of excess antiserum was 85%.

Radioimmunoassay for C5a. The buffer used for RIA was 0.1 M tricine-buffered saline, pH 8.0 containing 0.1% sodium azide (TBS). Protamine sulfate and gelatin were added where appropriate to ensure low nonspecific binding, as described (10) for RIA of human C5a. Samples (200 μl) were incubated for 1 h at 2°C with an equal volume of TBS containing polyethylene glycol 6000 (PEG, 22% wt/vol) and EDTA (10 mM) and centrifuged for 5 min at 7,800 g in a microcentrifuge. Supernatants (300 μl) were removed and mixed with TBS containing 7.5% protamine sulfate (150 μl). PEG removes C5, which cross-reacts with the anti-C5a serum, and IgG, which can interfere with the separation of free and antibody-bound tracer. The recovery of C5a from plasma was $84.8 \pm 1.0\%$ ($n = 14$), from zymosan-induced peritoneal exudate fluid $97.4 \pm 0.6\%$ ($n = 18$), and from saline-induced peritoneal exudate fluid $95.4 \pm 0.6\%$ ($n = 8$).

The concentrations of reagents in samples prepared as described above were: PEG (7.3%), protamine sulfate (2.5%) and EDTA (6.7 mM); dilutions of sample and standard solutions of C5a were performed using TBS containing these reagents. ^{125}I -C5a, guinea pig anti-rabbit C5a and PABA (a solid phase reagent that binds IgG) were diluted in TBS containing protamine sulfate (2.5%) and gelatin (0.6%). Duplicate micro-centrifuge tubes containing 100 μl volumes of sample or standard C5a were incubated for 16 h at room temperature (20 – 24°C) with 50 μl ^{125}I -C5a (5 ng \approx 20,000 cpm) and 50 μl antiserum (diluted 1/5,000). This was followed by a 30-min incubation with 50 μl of 2% PABA. After dilution with 1 ml TBS (0.6% gelatin) and immediate centrifugation (2 min at 7,800 g), supernatants were removed by suction and antibody-bound radioactivity in the pellets counted in an automatic gamma counter.

Control binding in the absence of added C5a was $41.2 \pm 0.6\%$ ($n = 5$), nonspecific binding was $1.8 \pm 0.2\%$ ($n = 4$) and the concentrations of C5a required to produce 10% and 50% inhibition of binding were 6.8 ± 0.4 ($n = 5$) and 66.4 ± 0.4 ($n = 5$) ng/ml

respectively. The limit of detection of the assay (assessed at 10% inhibition of binding and allowing for recovery and dilution of sample during preparation) was 24.0 ng/ml for plasma samples and 21.5 ng/ml for peritoneal exudate samples. The interassay coefficient of variation was 6.0% in five assays of the same sample. Results are given as the mean \pm SEM for n observations. Student's t test was used for statistical evaluation.

Results

Incubation (37°C for 30 min) of rabbit heparinized (10 U/ml) plasma with different concentrations of zymosan resulted in the dose-related generation of irC5a as previously observed for the generation of permeability-increasing activity (3). The highest dose of zymosan (10 mg/ml plasma) generated $5,820 \pm 590$ ng irC5a/ml ($n = 4$ rabbits). When zymosan-activated plasma was subjected to gel filtration on Sephadex G-100, a single peak of irC5a was observed, corresponding with the peak of permeability-increasing activity in rabbit skin (3). Plasma prepared from blood collected into sodium EDTA (10 mM) contained 36.6 ± 2.3 ng irC5a/ml ($n = 4$), whereas plasma from blood collected into heparin (10 U/ml) contained 53.5 ± 4.5 ng/ml ($n = 4$) and serum contained 78.5 ± 1.4 ng/ml ($n = 3$). For each rabbit, the values were always EDTA plasma < heparinized plasma < serum. We do not infer that we are detecting circulating C5a. These low levels may reflect slight activation during or after blood collection, activation which may be increased by heparin or clotting (10).

Fig. 1 shows the time course of generation of irC5a in rabbit peritoneal fluid. No irC5a was detected in fluid obtained after intraperitoneal injections of sterile saline (collection was terminated after 4 h because little fluid was present in the cavity at this time). In the rabbits given intraperitoneal injections of zymosan, irC5a concentrations rose from a low level at 0 h to high levels at 1, 2, and 4 h, with a maximum of 802 ± 184 ng/ml at 2 h. By 6 h the concentration was 302 ± 63 ng/ml, falling to a low level at 12 h. No significant differences in the levels of irC5a were found in blood plasma samples taken before (44.2 ± 3.9 ng/ml, $n = 5$) and 1 h after (41.9 ± 6.1 ng/ml, $n = 4$) intraperitoneal injection of zymosan.

Peritoneal exudate fluid recovered 2 h after initiation of a reversed passive Arthus-type reaction contained concentrations of 304 ± 60 ng irC5a/ml ($n = 6$) using unfractionated antiserum and 330 ± 30 ng irC5a/ml ($n = 3$) using the IgG

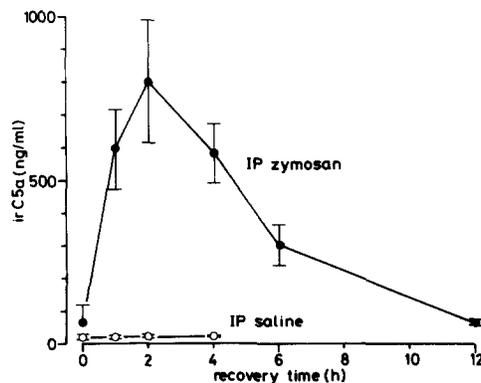


FIGURE 1. Time course of appearance of immunoreactive C5a (irC5a) in exudate fluid generated in response to an i.p. injection of zymosan (●; 500 mg suspended in 50 ml saline) in the rabbit. Control samples were taken from rabbits given intraperitoneal saline (○). The ordinate represents the irC5a level measured by RIA and the abscissa the time of collection following intraperitoneal injection. The values are given as mean \pm SEM for $n = 4$ rabbits.

fraction. The mean total recovery per animal in these 9 experiments was $10,300 \pm 2,300$ ng irC5a.

Discussion

Our previous studies suggest that certain inflammatory stimuli, e.g. some microbial cell walls, can initiate a nonallergic local edema response in rabbit skin that is dependent on extravascular complement activation (3). C5a is liberated as a byproduct of activation and this peptide causes circulating PMN leukocytes to attach to venular endothelial cells, followed by leukocyte emigration into the tissue. This cell-cell interaction, by an unknown mechanism, induces a rapid increase in venular permeability resulting in leakage of blood proteins leading to tissue edema (8). Protein leakage has been interpreted as functional in this response, as blood proteins will be supplied to the tissue in proportion to the size of the stimulus (3, 5, 8). Thus, C5a controls the supply of complement components in order to effect extravascular microbial lysis and opsonization, and also controls the accumulation of phagocytic leukocytes.

The rate of leakage of plasma proteins from venules is dependent on blood supply. In rabbit skin, with its low basal blood flow, the concomitant generation of a vasodilator prostaglandin is necessary for significant leakage to occur (3). In the peritoneal cavity, with higher basal blood flow, prostaglandins would be expected to enhance leakage without being obligatory. We have found that the vasodilator PGI₂ (prostacyclin) is released in the cavity in response to zymosan as evidenced by the high levels of its inactive metabolite 6-oxo-PGF_{1 α} in exudate (9). In the same study it was shown that the exudate was able to induce edema in the skin when mixed with a stable vasodilator, PGE₂, and injected intradermally. In this paper we describe a radioimmunoassay for rabbit C5a and the demonstration of high levels of irC5a in peritoneal inflammatory exudates induced by zymosan and in an Arthus-type reaction. The levels detected (i.e., up to 80 ng/100 μ l) are well above the minimum effective dose for responses in skin (i.e., less than 10 ng/100 μ l in the presence of PGE₂) (8).

Ward and Hill (11) found that extracts of Arthus lesions in rat skin contained, in addition to chemotactic activity, a C5 cleaving enzyme activity thought to be derived from PMN leukocytes. Depletion of circulating PMN leukocytes was found to suppress the level of C5-related chemotactic activity in Arthus lesions (11) and it has been postulated that >90% of the activity present in such lesions is derived from C5 by the action of leukocyte-derived enzymes, rather than from complement activation (12). The results presented here do not illuminate on this interesting hypothesis because the RIA will not distinguish between these two sources of C5a generation. Further, the antiserum used is likely to cross-react with heterogeneous C5 fragments, containing all or part of the C5a sequence, generated by leukocyte-derived enzymes. However, our findings lead us to suggest an alternative explanation for the observations of Ward and Hill (11). PMN leukocytes, in their interaction with the vessel wall, are effectively regulating the supply of complement components, including C5, to the tissue. Therefore, depletion of PMN leukocytes would be expected to reduce the extent of extravascular complement activation and consequently the level of C5a. The protease inhibitor Trasylol, which inhibits the cleavage of C5 by PMN leukocyte-derived enzymes *in vitro* (13), does not inhibit the generation of C5a during complement activation nor the production of edema induced by zymosan in

rabbit skin (3). This suggests that complement activation is more important in this model than cleavage of C5 by leukocyte-derived enzymes. It will be interesting to determine which of the mechanisms for generating C5-related chemotactic activity predominate in different inflammatory reactions.

Summary

We describe a radioimmunoassay for rabbit C5a and its use to obtain evidence of extravascular C5a generation in two inflammatory reactions in the peritoneal cavity. These observations, together with the potent activity of C5a in inducing increased microvascular permeability involving circulating PMN leukocytes, strengthen the case for considering C5a an important inflammatory mediator. These findings offer an explanation for the many different experimental inflammatory reactions where oedema formation can be suppressed either by systemic depletion of complement or by depletion of circulating PMN leukocytes.

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