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# Neutrophil Chemoattractants Generated in Two Phases during Reperfusion of Ischemic Myocardium in the Rabbit

## Evidence for a Role for C5a and Interleukin-8

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### Abstract

The neutrophil chemoattractants generated in a model of myocardial infarction in the anesthetized rabbit were investigated. Coronary artery occlusion was followed by reperfusion for periods from 5 min to 4.5 h. Extracts of myocardial tissue in normal and post-ischemic zones were tested for C5a and interleukin-8 (IL-8) using specific radioimmunoassays. In the post-ischemic zone, immunoreactive C5a was detected within 5 min and rose progressively to reach a plateau at 3–4.5 h. In contrast, immunoreactive IL-8 concentrations rose after a delay and were highest at the last time point tested, 4.5 h. Myeloperoxidase activity levels, an index of neutrophil accumulation, rose progressively as the concentrations of chemoattractants increased. Using cation exchange and reversed phase HPLC, immunoreactive C5a and IL-8 co-eluted with authentic standards. Fractions taken at the C5a and IL-8 peaks from reversed phase HPLC exhibited neutrophil aggregating activity which was neutralized by the respective antibody used in the radioimmunoassays. Depletion of circulating neutrophils virtually abolished immunoreactive IL-8 in the post-ischemic myocardial tissue. These observations suggest a sequential release of chemoattractants: the first, C5a is generated in interstitial fluid, followed by IL-8 generated by infiltrating neutrophils. Thus, over the time period studied, IL-8 generation would be expected to be indirectly dependent on C5a production. (*J. Clin. Invest.* 1995; 95:2720–2728.) Key words: myocardial infarction • complement • cytokine • leukocytes • radioimmunoassay

### Introduction

Interruption of the blood supply to a tissue for longer than a critical period results in irreversible cell death. The critical period depends on the metabolic rate of the tissue and the extent of collateral blood supply. After ischemia in the human heart, there is a correlation between the amount of tissue which becomes necrotic and the prognosis of the patient (1, 2). Salvage

of the myocardium depends on early reperfusion. However, there is evidence that reperfusion itself can accelerate damage (3) and result in the death of myocytes which are reversibly injured.

Ischemia induces all the characteristics of an acute inflammatory response, of which an early neutrophil accumulation is a prominent feature. Neutrophil accumulation is markedly accelerated during reperfusion (3, 4) and early studies implicated this cell in the associated tissue damage. Thus, systemic neutrophil-depletion (5) and antibodies blocking leukocyte adhesion molecules have been shown to limit infarction (6–8). This link between neutrophils and damage is controversial, as it has been reported in some models that agents affecting neutrophils do not influence infarction (9, 10). However, this relationship appears to be critically dependent on the duration of ischemia (8) for a given model.

The advent of clinical techniques which permit early reperfusion of ischemic myocardium has led to a renewed interest in the mechanisms involved in neutrophil accumulation and the potential of this cell as a therapeutic target. An important first step in neutrophil accumulation is the local generation of chemoattractants, chemical signals responsible for leukocyte recruitment. This paper is concerned with the identity of neutrophil chemoattractants generated in a model of myocardial infarction in the rabbit. The observations presented suggest a potentially important role for the complement fragment C5a and the cytokine, interleukin-8 (IL-8).

There is evidence to implicate the complement system in infarction. C1q and products of complement activation, e.g., C3b and C5b-9, have been detected in infarcted myocardium (11–13) and in the systemic circulation (14). Moreover, agents such as cobra venom factor or soluble complement receptor-1 which deplete or inactivate complement have been shown to reduce infarct size in animal models (15, 16). Activation of the complement system results in the liberation of several soluble products, amongst which is the cleavage product of the fifth component, C5a. This fragment is a potent neutrophil chemoattractant which has been detected in the systemic circulation in man after myocardial infarction (14). Chemotactic activity, neutralized by an anti-C5a antibody, has also been detected in cardiac lymph in a dog model (17). In a peritonitis model in the rabbit we detected high levels of C5a in exudate (18, 19) followed, after a delay, by high levels of interleukin-8 (20, 21) and a related cytokine, melanoma growth-stimulatory activity (MGSa) (22). The possibility of a similar relationship was investigated in the present study.

IL-8 is a potent neutrophil chemoattractant *in vitro* and *in vivo* (23–25). Its major effects are on neutrophils, whereas other members of the chemotactic cytokine (chemokine) supergene family exhibit selectivity for other cell types (25). A transient rise in circulating levels of IL-8 has been reported in

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patients during acute myocardial infarction (26) and in those undergoing cardiopulmonary bypass surgery (27–29). In addition, IL-8 has been detected on reperfusion of ischemic lung in the rabbit (30).

This paper provides evidence for the sequential generation of immunoreactive and functionally active C5a and IL-8 in a rabbit model of myocardial infarction. The observations suggest a scenario which could explain the relationship between these two mediators. These findings do not contribute to the controversy surrounding the importance of the neutrophil to reperfusion injury, set in context above, but they do throw light on some of the basic mechanisms involved in the process of neutrophil accumulation in the heart.

## Methods

**Surgical procedure.** Male and female New Zealand white rabbits (weighing 2.5–3 kg) were anesthetized using intravenous sodium pentobarbitone (45 mg/kg). The animals were artificially ventilated with room air via a tracheal cannula at positive pressure. Arterial BP was monitored continuously via a carotid artery cannula and recorded together with a lead I electrocardiogram. A left thoracotomy at the fifth intercostal space was performed on each animal, exposing the heart. A 5/0 silk ligature was placed around a branch of the left main coronary artery and occlusion achieved by passing both ends of the thread through a small length of polythene tubing and clamping under tension. Coronary artery occlusion (CAO)<sup>1</sup> was performed for 45 min in all animals, with differing reperfusion times (see below). The rabbits were killed by an overdose of sodium pentobarbitone and the hearts were immediately removed and perfused with ice-cold saline.

**Preparation of heart tissue.** The ligature used for occlusion was tied and monastral blue dye perfused through the ex-vivo heart allowing demarcation of the non-ischemic normal zone (NZ; dyed blue) and ischemic area at risk (AR). In some experiments a sample of tissue (0.11–0.57 grams) from each zone was immediately frozen in liquid nitrogen and stored at –20°C (for myeloperoxidase assay). The remaining AR and NZ tissues were immediately homogenized separately in ice-cold 0.1% tri-fluoroacetic acid (TFA, 20 ml/gram tissue). The homogenates were centrifuged at 5,400 g for 20 min at 4°C, the supernatant removed and dialyzed overnight in 5 liters of 0.1% TFA at 4°C. After dialysis the samples were frozen and then lyophilized, resuspended in 0.05 M ammonium acetate (10 ml/gram tissue; pH 5.5) and centrifuged at 3,470 g for 20 min to remove the majority of unwanted proteins. The supernatants were removed, filtered through a 0.45 µm filter and lyophilized. The samples were then resuspended in distilled water (5 ml/gram tissue), lyophilized again to remove remaining ammonium acetate, and stored at –20°C before assay.

**Assignment of animals to study groups.** CAO was confirmed by changes in the electrocardiogram (e.g., ST segment elevation, indicative of ischemia), and clear AR demarcation with monastral blue. On the basis of these criteria CAO was not achieved in two animals which were therefore excluded from the study together with seven animals that underwent irreversible ventricular fibrillation during occlusion. Of the remaining 41 animals, 12 were successfully defibrillated during CAO and were included in study groups as indicated below. For the time course studies, four groups of animals were reperfused for 5 min ( $n = 5$ , including 2 defibrillated), 1.5 h ( $n = 5$ , including 1 defibrillated), 3 h ( $n = 5$ , including 1 defibrillated) and 4.5 h ( $n = 6$ , none defibrillated). To obtain material for HPLC analysis, a further 4 animals (none defi-

brillated) were reperfused for 4.5 h. To study the effects of neutrophil depletion, a further 16 animals, 8 (including 4 defibrillated) of which were pretreated with intravenous mustine hydrochloride and 8 (including 4 defibrillated) with intravenous sterile saline, were also subjected to 4.5 h reperfusion.

**Depletion of circulating neutrophils.** Circulating neutrophils were depleted in eight rabbits (subsequently subjected to 45 min CAO and 4.5 h reperfusion) by a single intravenous injection of mustine hydrochloride in sterile saline (1.75 mg/kg) administered 3 d before the experiment. Previous studies of different blood cell types using depletion/repletion and bone marrow exclusion have shown that this procedure induces a selective depletion of neutrophils in the rabbit (31). As a control, eight rabbits were treated with sterile saline alone. Venous blood samples were taken before and after treatment for total and differential leukocyte counts.

Levels of myocardial tissue IL-8 and C5a were measured in five of the saline-treated animals and five of the mustine-treated animals. The remaining three animals from each group were used to measure myocardial tissue myeloperoxidase (MPO) levels (see below).

**Radioimmunoassays for C5a and IL-8.** Goat anti-rabbit C5a antiserum was generated as described previously (32). Anti-rabbit IL-8 antiserum was generated in a similar manner by immunization of guinea-pigs with emulsions of synthetic rabbit IL-8 in Freund's complete and incomplete adjuvants injected subcutaneously. Rabbit C5a and IL-8 (2.5–5 µg in 20 µl 0.1 M sodium borate buffer pH 8.3) were iodinated with Na<sup>125</sup>I (500 µCi) in tubes coated with Iodogen as described previously (21).

Lyophilized NZ and AR samples were dissolved in PBS at a concentration of 1 ml/gram tissue and mixed with an equal volume of 22% polyethylene glycol 6000 (PEG) containing 1% protamine sulphate (PS) to precipitate any remaining C5 which would otherwise cross-react in the C5a assay (33). Lyophilized HPLC fractions were dissolved in 300 µl PBS and 300 µl PEG/PS added. After 1 h incubation at 4°C, samples were centrifuged (5,400 g for 10 min at 4°C) and the supernatant used for assay.

The assay protocol was as described previously (18, 33). Briefly, 100 µl of the PEG/PS supernatant, or standard (19.5–20,000 pM in 11% PEG/0.5% PS), was incubated at room temperature for 24 h together with 50 µl <sup>125</sup>I-ligand (60 fmol) and 100 µl antiserum (goat anti-rabbit C5a diluted 1/1500 or guinea-pig anti-rabbit IL-8 1/6000). This was followed by the addition of 50 µl of a second antibody (donkey anti-goat IgG 1/30 or goat anti-guinea-pig IgG 1/30) and further incubation for 16 h. After addition of 1 ml PBS and immediate centrifugation (5,400 g for 10 min), the supernatants were removed and the antibody-bound radioactivity in the pellets counted. <sup>125</sup>I-ligands and antisera were dissolved in PBS containing 0.2% gelatin, 0.5% PS and 0.02% sodium azide. In addition, 0.3% BSA was added to the buffer for <sup>125</sup>I-IL-8 to reduce nonspecific binding (to 7.4%). The nonspecific binding for <sup>125</sup>I-C5a was 3.5%. IL-8 (up to 20,000 pM) did not cross-react in the C5a assay nor did C5a cross-react in the IL-8 assay. Rabbit MGSA, which has been found together with C5a and IL-8 in inflammatory exudates (21) and is structurally related to IL-8 (22), did not cross-react in these assays. C5a and C5a desArg cross-reacted 100% in the C5a RIA.

**Measurement of myeloperoxidase.** Myeloperoxidase (MPO) activity was measured as described previously (8). The frozen NZ and AR samples were homogenized in 0.02 M NaPO<sub>4</sub> buffer (pH 4.7) containing 0.1 M NaCl and 0.015 M Na<sub>2</sub> EDTA, and centrifuged at 20,000 g for 15 min at 4°C. The supernatant containing hemoglobin was discarded. The pellets were homogenized again in 0.05 M NaPO<sub>4</sub> (pH 5.4) with 0.5% hexadecyltrimethylammonium bromide and freeze-thawed in liquid nitrogen three times. Homogenates were centrifuged at 20,000 g for 15 min at 4°C and the MPO-rich supernatant taken for assay. The assay was carried out by measuring the change in absorbance at 690 nm using 1.6 mM tetramethylbenzidine, 0.3 mM H<sub>2</sub>O<sub>2</sub>, 12% dimethyl formamide, 40% Dulbecco's PBS and 0.08 M NaPO<sub>4</sub> (pH 5.4). One unit of MPO was defined as the quantity required to reduce 1 µmol of H<sub>2</sub>O<sub>2</sub>/min.

1. Abbreviations used in this paper: AR, area at risk; CAO, coronary artery occlusion; LTB<sub>4</sub>, leukotriene B<sub>4</sub>; MPO, myeloperoxidase; NZ, normal zone; PEG, polyethylene glycol; PS, protamine sulphate; RP, reversed phase; TFA, tri-fluoroacetic acid.

**Purification of immunoreactive rabbit IL-8 and C5a by Cation exchange HPLC.** Four paired samples of AR (total weight = 3.19 grams) and NZ (total weight = 2.83 grams) tissue from animals that underwent 45 min ischemia and 4.5 h reperfusion, were subjected to homogenization and dialysis as described above. The extracts were pooled by resuspension in a total volume of 2.5 ml 10 mM NaPO<sub>4</sub> (pH 5.5) and loaded onto Ultropac TSK HPLC columns (SWP guard, 7.5 × 75mm and 535 CM cation-exchanger, 7.5 × 150 mm in series). Proteins were eluted using a 0.15–2M NaCl gradient in 10 mM NaPO<sub>4</sub> (pH 5.5) run over 60 min at a flow rate of 0.5 ml/min. Sequential 2-min fractions were collected. An aliquot (5%) of each fraction was lyophilized in preparation for RIA and the remainder stored at –20°C.

**Reversed phase HPLC.** Cation exchange fractions containing immunoreactive IL-8 and C5a were loaded separately onto a wide pore (300 Å) C18 column (4 × 250mm, Vydac) in 0.1% TFA and proteins eluted using a 0–80% acetonitrile gradient in 0.1% TFA over 80 min at a flow rate of 1 ml/min. Sequential 30-s fractions were collected. An aliquot (5%) of each fraction was lyophilized and kept for RIA and the remainder stored at –20°C.

**Measurement of neutrophil aggregation.** Lyophilized reversed phase HPLC fractions were resuspended in PBS (containing 0.1 mg/ml BSA) and tested for functional activity using a homotypic neutrophil aggregation assay. Neutrophils were isolated from peripheral rabbit blood using the method of Haslett et al. (34). Briefly, blood was collected into 3.8% tri-sodium citrate and centrifuged at 300 g for 20 min producing platelet-rich plasma. This was removed and centrifuged for a further 20 min at 2,000 g to produce platelet-poor plasma. Erythrocytes were sedimented out of the remaining blood with 6% dextran for 30 min. The resulting leukocyte-rich supernatant was removed and centrifuged at 275 g for 6 min to produce a leukocyte pellet which was resuspended in platelet-poor plasma and layered onto a discontinuous Percoll-plasma gradient. After centrifuging at 260 g for 11 min the neutrophil-rich band was collected, washed and resuspended at 10<sup>7</sup> cells/ml in assay buffer (NaCl 138 mM; KCl 2.7 mM; Na<sub>2</sub>HPO<sub>4</sub> 8.1 mM; KH<sub>2</sub>PO<sub>4</sub> 1.5 mM; glucose 10 mM; Hepes 10 mM; CaCl<sub>2</sub> 1.4 mM, and MgCl<sub>2</sub> 0.7 mM; pH 7.4). The cell suspension (300 µl) was to siliconized glass cuvettes and continuously stirred at 700 rpm for 5 min at 37°C in a dual channel aggregometer (Chrono-log) before the addition of agonists in 10 µl volumes. Test substances used were human recombinant IL-8 (hrIL-8; this was used as insufficient bioactive rabbit IL-8 standard was available), rabbit C5a (prepared in the presence of a serum carboxypeptidase inhibitor to prevent loss of the COOH-terminal arginine) (35), and reversed phase HPLC fractions. Aggregation was monitored for at least 5 min and expressed as a percentage of the maximal aggregation induced by 10<sup>–6</sup> M phorbol 12-myristate 13-acetate (PMA). IgG-purified anti-rabbit IL-8 and anti-rabbit C5a antibodies (21) were mixed in 10 µl volumes with the relevant agonists immediately before addition to the cell suspension. Assay buffer alone was used as a reference. The assay was repeated using separate neutrophil preparations from 3–9 donor rabbits.

**Materials.** Monostral blue, hexadecyltrimethylammonium bromide, BSA (endotoxin < 0.1 ng/mg) and protamine sulphate (grade II) were purchased from Sigma chemical Co. Ltd. (Poole, UK). Iodogen and Spectra/por dialysis membranes (3.5 kD MWCO) were from Life Science Lab. Ltd. (Luton, UK). Sodium pentobarbitone was from May & Baker Ltd. (Dagenham, UK). Sephadex G 25M columns and Percoll were from Pharmacia (Milton Keynes, UK). The TSK HPLC columns were from LKB Ltd. (Milton Keynes, UK). The C18 HPLC column was from Hichrom Ltd. (Reading, UK). Na<sup>125</sup>I was from Amersham International PLC, Aylesbury, UK. Goat anti-guinea-pig IgG and donkey anti-goat IgG anti-sera were from Nordic Immunological Labs (Tilberg, The Netherlands). The following were generous gifts: the synthetic rabbit IL-8 used for raising antiserum from Dr. H. Showell (Pfizer Central Research, Groton, CT); and bioactive synthetic rabbit IL-8 used for all other purposes from Dr. I. Clark-Lewis (University of British Columbia, Vancouver, Canada); hrIL-8 from SmithKline

**Table I. Heart Rate and Mean Arterial Blood Pressure Monitored in Anesthetized Rabbits Undergoing 45 min CAO and 4.5 h Reperfusion (n = 10)**

Time	Heart rate (beats/min)	Blood pressure (mm/Hg)
Preocclusion	262±8	67.7±3.5
45 min post-occlusion	265±9	66.2±4.6
1.5 h reperfusion	259±9	65.6±4.3
3 h reperfusion	275±11	60.9±3.9
4.5 h reperfusion	259±10	59.0±3.9

Beecham (King of Prussia, PA). All other chemicals and reagents were purchased from BDH Chemicals Ltd. (Dagenham, UK).

**Statistical analysis.** All results are presented as the mean±SEM of n experiments. Paired Student's *t* tests were performed on data comparing AR and NZ from the same heart. An unpaired Student's *t* test was used when comparing data from mustine and saline-treated animals. All data were log<sub>10</sub>-transformed to normalize distribution before testing.

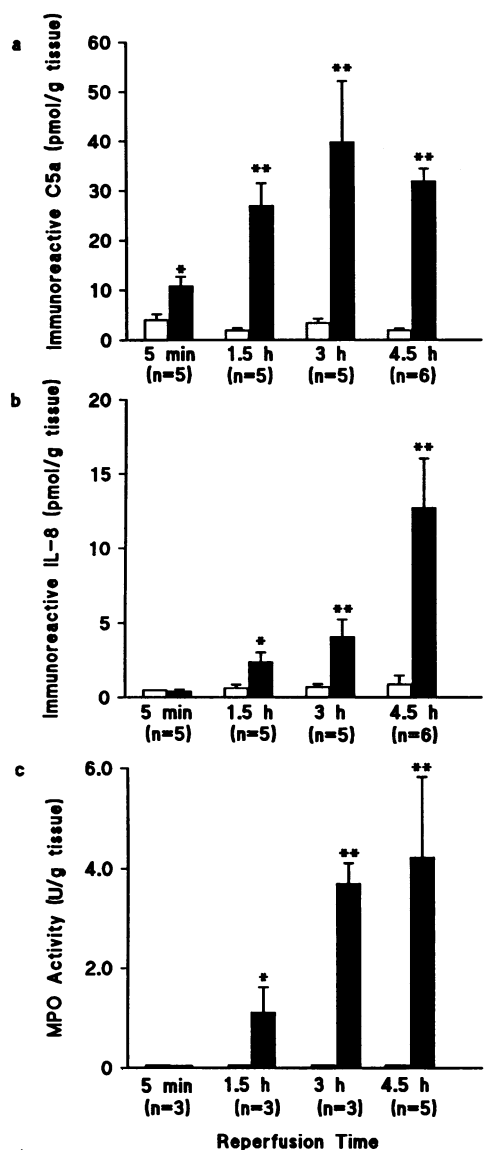
## Results

No significant changes in the heart rate or blood pressure occurred during the experiments within or between groups of animals except during fibrillation. After successful defibrillation (12 of 41 animals), these parameters rapidly returned to normal levels and remained normal for the remainder of the experiment. Table I shows the heart rate and blood pressure values in animals that underwent 45 min CAO and 4.5 h reperfusion (excluding those given intravenous pretreatments, n = 16); none of these animals required defibrillation.

**Generation of neutrophil chemoattractants during reperfusion.** Both IL-8 and C5a immunoreactivity was detected in the AR myocardium, while little could be found in the NZ. However, the time course for the generation of these two neutrophil chemoattractants differed (Fig. 1). Significantly elevated immunoreactive C5a in the AR was detected within 5 min of reperfusion and rose progressively, plateauing at 3–4.5 h. Immunoreactive IL-8 levels in AR rose slowly after a delay, with the highest level attained at the last measurement period of 4.5 h.

**Neutrophil accumulation during reperfusion.** Myocardial tissue MPO was measured as an index of neutrophil accumulation in a proportion of the animals from each of the groups subjected to varying reperfusion times (indicated in Fig. 1), in addition to determination of IL-8 and C5a generation. Fig. 1 c shows that MPO activity in the AR was not detectable after 5 min reperfusion but was significantly elevated at 1.5 h and further elevated at 3 h reperfusion. The MPO content in all NZ samples was below the detection limit for the assay (0.25U MPO/gram tissue).

**Purification of neutrophil chemoattractants using HPLC.** Since it was clear that the concentrations of C5a and IL-8 were greatest in animals subjected to 4.5 h reperfusion, cation-exchange HPLC was performed on lyophilized extracts of AR and NZ tissue pooled from a further four hearts taken at this reperfusion time. Using cation exchange chromatography with a NaCl gradient, immunoreactive C5a and IL-8 each separated into a major peak followed by a tail (Fig. 2). We do not know



**Figure 1.** The effect of duration of reperfusion on C5a and IL-8 concentrations and MPO activity in ischemic rabbit myocardium. CAO was performed in anesthetized rabbits for 45 min followed by increasing reperfusion times. After reperfusion, the hearts were removed and divided into post-ischemic AR (solid columns) and non-ischemic NZ (open columns). (a) C5a and (b) IL-8 concentrations were measured by RIA on lyophilized AR and NZ extracts from 5–6 animals per time point and (c) MPO activity was measured by enzyme assay (detection limit 0.25 U MPO/gram tissue) on frozen AR and NZ samples from a proportion ( $n = 3–5$ ) of the above animals (as indicated). The results are presented as the mean of  $n$  rabbits per time point; the error bars show the SEM. A significant increase in immunoreactive C5a, IL-8 and MPO using a paired Students  $t$  test is indicated by \*  $P < 0.05$ , \*\*  $P < 0.01$ .

the identity of the materials in these tails but they may represent enzymic degradation products of the chemoattractants. The C5a peak (fractions 22–23) was free of IL-8, but the IL-8 peak (fractions 25–26) was contaminated by the C5a tail.

The cation exchange peaks obtained from AR samples were further purified separately by reversed phase HPLC (RP-

HPLC). For comparison, rabbit C5a, C5a desArg and IL-8 standards were chromatographed after the relevant AR-derived sample. Immunoreactive C5a eluted at 33–34 min (33% acetonitrile) and IL-8, free from the immunoreactive C5a contaminant, eluted at 41–42.5 min (41% acetonitrile). Both eluted in the same fractions as their respective standards (Fig. 3). In the case of C5a, a following tail eluting in the same fraction as the desArg metabolite standard (34.5–35 min) was also observed.

**Induction of neutrophil aggregation with RP-HPLC fractions.** The amounts of active material available from RP-HPLC were limited, but it was possible to demonstrate functional activity in fractions containing immunoreactive C5a and IL-8 using rabbit neutrophil aggregation as the *in vitro* test system. Human recombinant IL-8 (hrIL-8) and rabbit C5a standards induced dose-dependent neutrophil aggregation (Fig. 4). The HPLC fractions corresponding to the two peaks of immunoreactive C5a activity were tested separately. The first peak, retention time 33–34 min corresponding to the C5a standard, induced aggregation (Fig. 4). In contrast, the second peak, retention time 34.5–35 min corresponding to the standard desArg metabolite, did not induce aggregation (data not shown). Fractions containing IL-8 activity (retention time 41–42.5 min) were pooled and at an estimated concentration of 1 nM consistently induced aggregation greater than that of an equal dose of the human IL-8 standard (Fig. 4).

Addition of a guinea-pig anti-rabbit IL-8 IgG-purified antibody (which binds to human IL-8 standard) to 10 nM hrIL-8 immediately before testing resulted in a 50% inhibition of aggregation (Fig. 5). When added with the RP-HPLC fractions containing IL-8, almost total inhibition was seen. The anti-rabbit C5a IgG-purified antibody completely blocked aggregation induced by both 30 nM rabbit C5a and the RP-HPLC C5a fractions.

**Effect of neutrophil depletion.** Preliminary experiments using immunocytochemistry of AR tissue after 45 min CAO and 4.5 h reperfusion showed immunoreactive IL-8 associated with infiltrating leukocytes. Hence, the effect of neutrophil depletion using mustine hydrochloride on IL-8 and C5a levels was investigated. Mustine pre-treatment resulted in a significant reduction in circulating neutrophil levels 3 days later (pre-treatment:  $2.45 \pm 0.60 \times 10^6$  cells/ml, post-treatment:  $0.09 \pm 0.01 \times 10^6$  cells/ml,  $n = 8$ ,  $P < 0.01$ ) while saline treatment had no effect. The levels of myocardial tissue IL-8 and C5a after 4.5 h reperfusion were measured in five of the saline-treated animals and five of the animals depleted of circulating neutrophils. Fig. 6 *b* shows that neutrophil depletion virtually abolished IL-8 levels ( $P < 0.05$ ) in the AR when compared to the control group. There was an apparent reduction, but no significant difference ( $P > 0.1$ ), between C5a levels in mustine-treated animals and the control group (Fig. 6 *a*).

The remaining three mustine-treated and saline-treated animals were used to measure myocardial tissue MPO levels. No MPO was detected (detection limit of the assay was 0.25 U MPO/gram tissue) in the AR or NZ from the neutrophil-depleted animals (Fig. 6 *c*).

**Effect of defibrillation.** There was no detectable effect of defibrillation during CAO (12 of 41 animals) on either C5a or IL-8 generation or MPO activity.

## Discussion

This study demonstrates that immunoreactive C5a and IL-8 are present in myocardial tissue after ischemia and reperfusion.

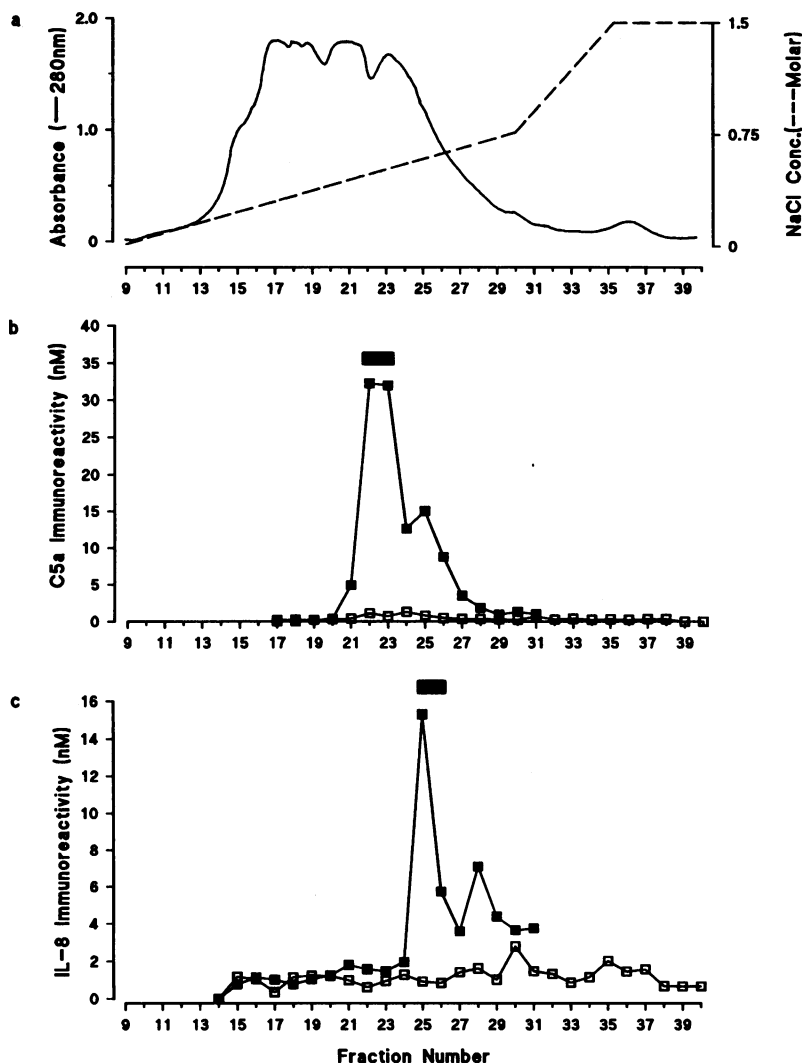


Figure 2. Cation exchange purification of C5a and IL-8 from post-ischemic AR and non-ischemic NZ rabbit myocardium, pooled from four animals, after 45 min CAO and 4.5 h reperfusion. Lyophilized AR and NZ extracts were dissolved in 10 mM NaPO<sub>4</sub> (pH 5.5) and purified using a 0.15–2 M NaCl gradient in 10 mM NaPO<sub>4</sub> (pH 5.5) run at a flow rate of 0.5 ml/min. (a) Absorbance profile of AR at 280 nm vs. NaCl gradient. (b) C5a and (c) IL-8 immunoreactivity detected by RIA in sequential 2-min fractions collected from AR (solid square) and NZ (open square) myocardium after HPLC purification. The bars in b and c indicate the fractions pooled for reversed phase HPLC.

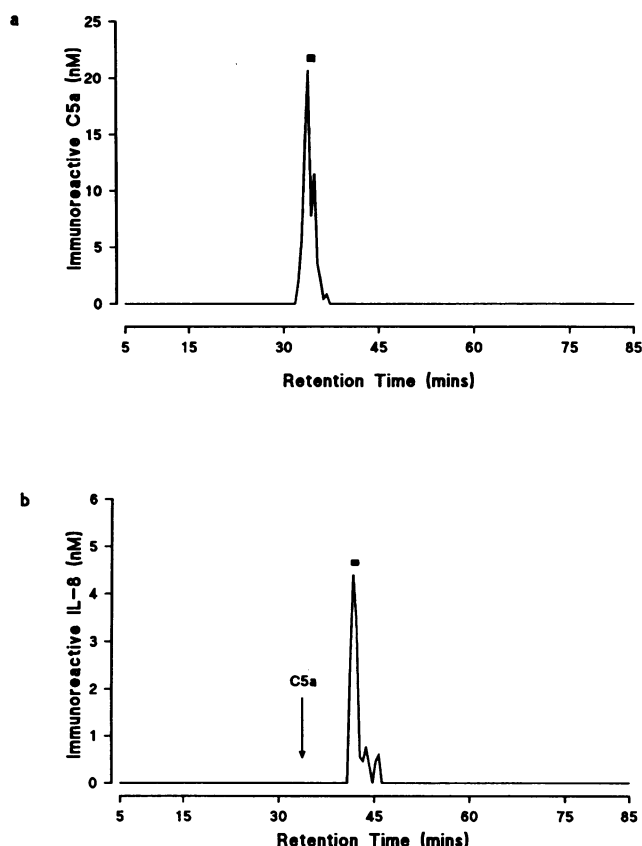
Both proteins co-eluted with authentic standards on HPLC separation and the active fractions induced rabbit neutrophil aggregation, the functional *in vitro* assay used in this investigation. The anti-C5a and anti-IL-8 antibodies employed in the radioimmunoassay neutralized the aggregating activity of the respective ligand standards and the co-eluting HPLC peaks.

The time-courses of appearance of C5a and IL-8 were quite distinct. Significantly elevated C5a concentrations were detected in the previously ischemic zone 5 min after the initiation of reperfusion, and reached a plateau at 3–4.5 h. In contrast, IL-8 concentrations rose slowly after a delay. IL-8 concentrations were significantly elevated at 1.5 h and were highest at the last measurement time point at 4.5 h. Interestingly, this is a similar pattern to that seen using an exogenous inflammatory stimulus, zymosan, in an experimental model of peritonitis (21).

There is long-standing evidence for complement activation in ischemia/reperfusion in the heart (11, 13, 17). Human heart mitochondrial membranes can bind C1q, leading to activation of complement (36). It has been proposed that during myocardial ischemia, injured cells release subcellular constituents rich in mitochondrial membranes, which bind to C1q and thus activate

complement (37). Products of complement activation have been detected on myocytes, so activation appears to be predominantly extravascular, although some intravascular activation may also occur on the surface of injured endothelial cells. Activation may occur to some extent during the occlusion phase, however, this will be limited by the availability of substrate (complement components). During reperfusion, coronary microvascular permeability to proteins is elevated and this will increase the supply of complement to the extravascular space, thus facilitating C5a production. As shown in this paper, the following IL-8 production appears to be indirectly related to the first phase of complement activation.

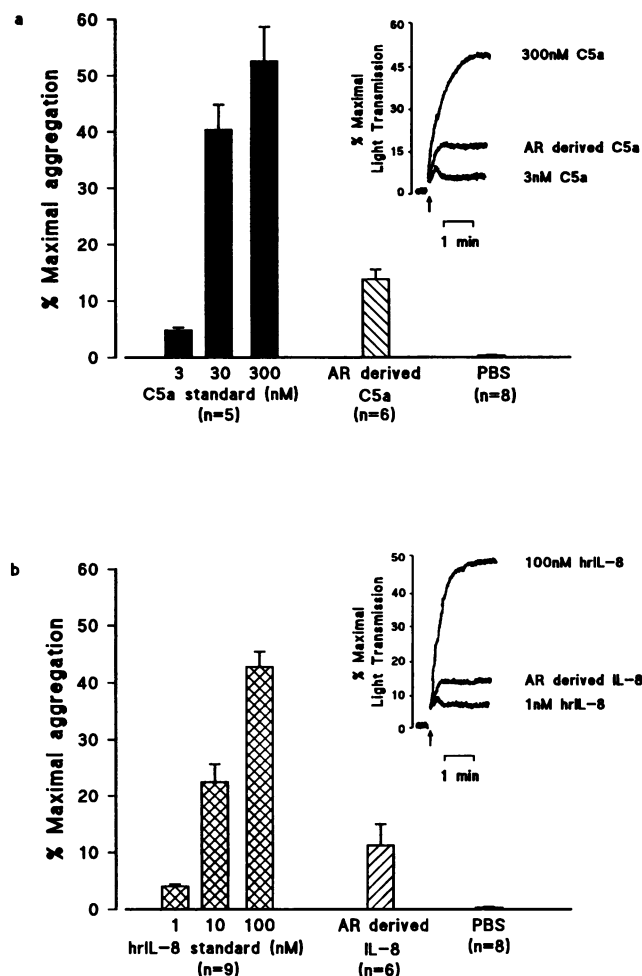
IL-8 was originally demonstrated as a secreted product from stimulated monocytes (24) and subsequently shown to be produced by numerous cells including fibroblasts (38), endothelial cells (39), lymphocytes (40) and neutrophils (41–43). In the model described here immunocytochemistry showed a close association of anti-IL-8 antibody with infiltrated leukocytes (C. L. Ivey et al., unpublished). Further experiments were therefore carried out in rabbits depleted of circulating neutrophils. Depletion virtually abolished IL-8 generation in the myocardium, but had no significant effect on C5a generation. These results sug-



**Figure 3.** Reversed phase HPLC purification of cation exchange fractions with C5a and IL-8 immunoreactivity. Cation exchange fractions that were shown to possess either C5a or IL-8 immunoreactivity by RIA (see Fig. 2) underwent further purification by RP-HPLC using a 0–80% acetonitrile gradient in 0.1% TFA at a flow rate of 1 ml/min. (a) C5a immunoreactivity in cation exchange fractions 22–23. The bar indicates where rabbit C5a standard eluted, as detected by RIA. (b) IL-8 immunoreactivity in cation exchange HPLC fractions 25–26. The bar indicates where synthetic rabbit IL-8 standard eluted, as detected by RIA. The small amount of immunoreactive C5a contaminant in the IL-8 fractions after cation exchange HPLC was clearly separated on the reversed phase column (arrow) from immunoreactive IL-8.

gest that infiltrating neutrophils are the source of IL-8 in the myocardium over the time period investigated. In this respect the results from the myocardial infarction and peritonitis models are different, as neutrophil depletion in the peritonitis model does not affect IL-8 generation (measured in exudate accumulated over 6 h) whereas macrophage depletion abolishes it (B. T. Au, P. D. Collins, and T. J. Williams, unpublished).

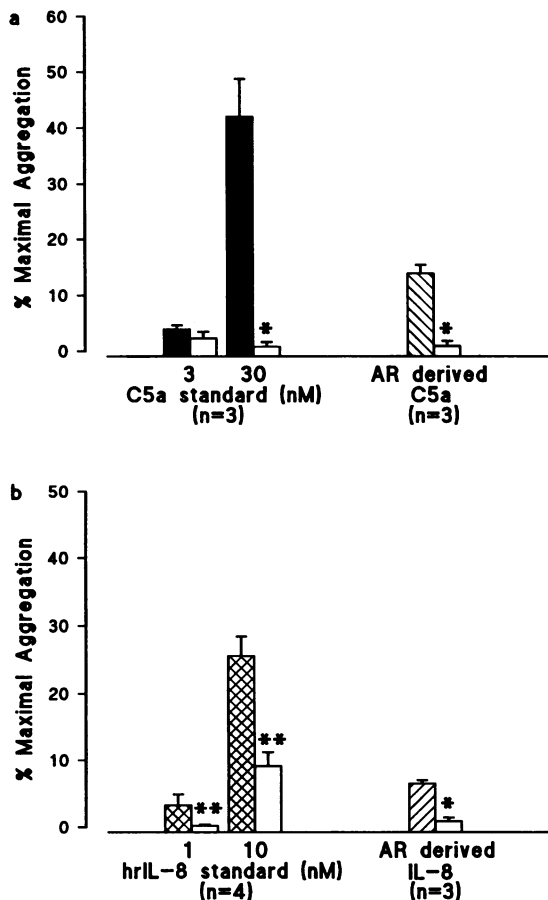
The situation with C5a is less clear cut; neutrophil depletion apparently induced some reduction in C5a levels in the myocardium, but this was not significant. C5a induces neutrophil-dependent oedema formation (31) and the continuation of the extravascular generation of C5a is dependent on a supply of complement components, including C5, from the intravascular compartment, as previously discussed (44). Neutrophil depletion may, therefore, also be expected to suppress C5a generation by suppressing increased coronary microvascular permeability and, as a consequence, limiting the supply of precursor C5 and other complement components. However, we have previously



**Figure 4.** Homotypic aggregation of rabbit neutrophils induced by rabbit C5a, hrIL-8 and immunoreactive RP-HPLC fractions. Peripheral rabbit neutrophils were suspended at a concentration of  $10^7$  cells/ml in assay buffer and stirred at 700 rpm at 37°C in a dual channel aggregometer. Aggregating agents were added in volumes of 10  $\mu$ l. Responses are shown to (a) Rabbit C5a (solid bars) and lyophilized RP-HPLC fractions with C5a activity (retention time 33–34 min; hatched column); (b) hrIL-8 (cross-hatched bars) and lyophilized RP-HPLC fractions with IL-8 immunoreactivity (retention time 41–42.5 min; hatched bar). Aggregation is expressed as a percentage of maximal aggregation induced by  $10^{-6}$  M PMA. Results are presented as the mean of 5–9 experiments using neutrophils from different donors; error bars show the SEM. Insets show typical aggregation traces plotted against percent maximal light transmission induced by  $10^{-6}$  M PMA.

demonstrated that coronary microvascular plasma protein leakage, measured using <sup>125</sup>I-albumin, is not neutrophil-dependent in the rabbit model (45) showing that additional mechanisms are responsible for increased microvascular permeability in this case (e.g., the permeability is elevated because of direct injury to the endothelium or because of chemical mediators acting on endothelial receptors).

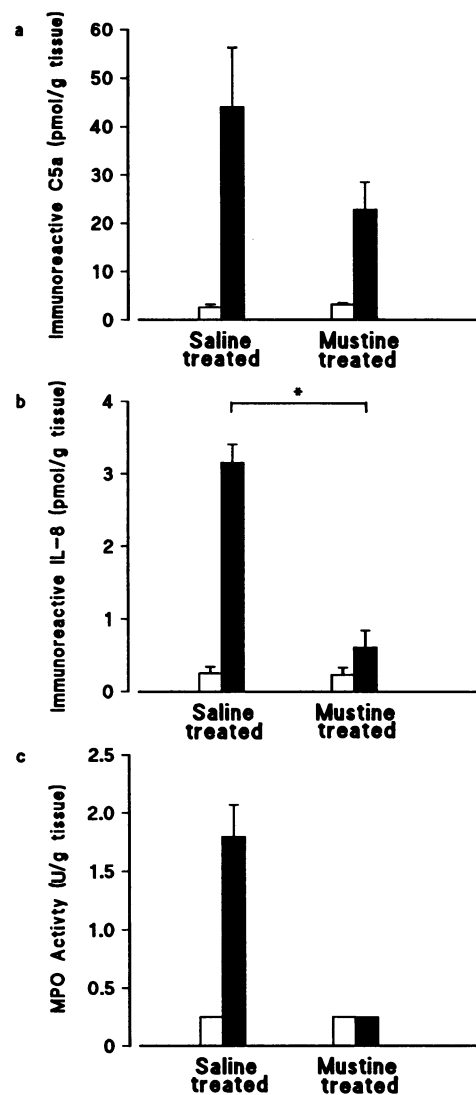
Other studies have demonstrated that drugs inhibiting arachidonic acid metabolism via the 5-lipoxygenase pathway are able to suppress neutrophil accumulation and limit infarct size (46). Thus, leukotriene B<sub>4</sub> (LTB<sub>4</sub>), a powerful neutrophil chemoattractant metabolized from arachidonic acid, may also



**Figure 5.** The effect of IgG-purified antibodies on homotypic rabbit neutrophil aggregation induced by rabbit C5a, hrIL-8 and immunoreactive RP-HPLC fractions. Peripheral rabbit neutrophils were suspended at a concentration of  $10^7$  cells/ml in assay buffer and stirred at 700 rpm at 37°C in a dual channel aggregometer. IgG-purified antibodies and aggregating agents were added in 10-ml volumes. In *a* rabbit C5a standard (solid bars) and reversed phase HPLC fractions containing C5a, retention time 33–34 min (hatched bar), were added alone or together with anti-rabbit C5a antibody (open bar). In *b* rabbit IL-8 standard (cross-hatched bars) and reversed phase HPLC fractions containing IL-8, retention time 41–42.5 min (hatched bar), were added alone or together with anti-rabbit IL-8 antibody (open bars). Aggregation is expressed as a percentage of maximal aggregation induced by  $10^{-6}$  M PMA. Results are presented as a mean of 3–4 experiments using neutrophils from different donors; the error bars indicate SEM. A significant reduction in neutrophil aggregation using a paired Student's *t* test is indicated as \*  $P < 0.05$ , \*\*  $P < 0.01$ .

have a role in addition to IL-8 and C5a. Indeed,  $LTB_4$  has been detected in the myocardium in a rat model of myocardial infarction (47). Another, less direct, explanation for the observed effect of 5-lipoxygenase inhibitors on neutrophil accumulation in the heart is that these compounds are able to inhibit cytokine synthesis in some cells (48). Relevant to the observation described here, we have recently obtained evidence that  $LTB_4$  (and other lipids) (43) produced by human neutrophils can act as an autocrine regulator of IL-8 synthesis (B.T. Au, T. J. Williams, and P. D. Collins, unpublished).

In conclusion, mechanisms have evolved to induce a rapid accumulation of neutrophils in the heart during reperfusion after



**Figure 6.** Effect of depleting circulating neutrophils on C5a and IL-8 concentrations and MPO activity in ischemic rabbit myocardium. Circulating neutrophils were depleted in 8 rabbits by intravenous injection of mustine hydrochloride (1.75 mg/kg) 3 d before 45 min CAO and 4.5h reperfusion. Control animals ( $n = 8$ ) were injected with sterile saline. After reperfusion the hearts were removed and divided into post-ischemic AR (solid bars) and non-ischemic NZ (open bars). (a) C5a and (b) IL-8 concentrations were measured using RIA on lyophilized AR and NZ samples from five animals, and (c) MPO activity was measured by enzyme assay (detection limit 0.25 U MPO/gram tissue) on frozen AR and NZ samples from the remaining three animals (see Methods). The results are presented as the mean of 5 (a and b) or 3 (c) experiments; the error bars show SEM. A significant reduction in immunoreactive IL-8 levels using an unpaired Student's *t* test is indicated as \*  $P < 0.05$ .

a period of ischemia. The following hypothetical scenario is proposed. A fast onset is ensured by liberating C5a from pre-formed substrate, C5, in interstitial fluid, which is supplemented by plasma leaking from coronary microvessels. C5a can then induce a first phase of neutrophil infiltration. Once in the tissue, these cells appear to be responsible for synthesizing and releasing the mediator responsible for subsequent neutrophil accumu-



lation, IL-8, although the endogenous stimulus for synthesis has not been established. Thus, as these are sequential events, suppression of C5a generation or action may be expected to be a more effective means of inhibiting neutrophil accumulation than more direct means of suppressing the generation or action of IL-8. These findings provide an insight into some of the mechanisms involved in neutrophil recruitment during reperfusion of the myocardium following a period of ischemia.

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## References

- Hamarayan, C., M. A. Bennett, B. L. Pentecost, and D. B. Brewer. 1970. Quantitative study of infarcted myocardium in cardiogenic shock. *Br. Heart J.* 32(6):728-732.
- Page, D. L., J. B. Caulfield, J. A. Kastor, R. W. DeSanctis, and C. A. Sanders. 1971. Myocardial changes associated with cardiogenic shock. *N. Engl. J. Med.* 285:133-137.
- Sommers, H. M., and R. B. Jennings. 1964. Experimental acute myocardial infarction. Histologic and histochemical studies of early myocardial infarcts induced by temporary or permanent occlusion of a coronary artery. *Lab. Invest.* 13:1491-1503.
- Engler, R. L., M. D. Dahlgren, M. A. Peterson, A. Dobbs, and G. W. Schmid-Schonbein. 1986. Accumulation of polymorphonuclear leukocytes during 3-h experimental myocardial ischemia. *Am. J. Physiol.* 251:H93-H100.
- Romson, J. L., B. G. Hook, S. L. Kunkel, G. D. Abrams, M. A. Schork, and B. R. Lucchesi. 1983. Reduction of the extent of ischemic myocardial injury by neutrophil depletion in the dog. *Circulation.* 67:1016-1023.
- Ma, X.-L., P. S. Tsao, and A. M. Lefer. 1991. Antibody to CD-18 exerts endothelial and cardiac protective effects in myocardial ischemia and reperfusion. *J. Clin. Invest.* 88:1237-1243.
- Simpson, P. J., R. F. Todd III, J. C. Fantone, J. K. Mickelson, J. D. Griffin, and B. R. Lucchesi. 1988. Reduction of experimental canine myocardial reperfusion injury by a monoclonal antibody (Anti-Mo1, Anti-CD11b) that inhibits leukocyte adhesion. *J. Clin. Invest.* 81:624-629.
- Williams, F. M., M. Kus, K. Tanda, and T. J. Williams. 1994. Effect of duration of ischaemia on reduction of myocardial infarct size by inhibition of neutrophil accumulation using an anti-CD18 monoclonal antibody. *Br. J. Pharmacol.* 111:1123-1128.
- Chatelain, P., J.-G. Latour, D. Tran, M. de Lorgeril, G. Dupras, and M. Bourassa. 1987. Neutrophil accumulation in experimental myocardial infarcts: relation with extent of injury and effect of reperfusion. *Circulation.* 75:1083-1090.
- Tanaka, M., S. E. Brooks, V. J. Richard, G. P. FitzHarris, R. C. Stoler, R. B. Jennings, K.-E. Arfors, and K. A. Reimer. 1993. Effect of anti-CD18 antibody on myocardial neutrophil accumulation and infarct size after ischemia and reperfusion in dogs. *Circulation.* 87:526-535.
- McManus, L. M., W. P. Kolb, M. H. Crawford, R. A. O'Rourke, F. L. Grover, and R. N. Pinckard. 1983. Complement localization in ischemic baboon myocardium. *Lab. Invest.* 48:436-447.
- Rossen, R. D., J. L. Swain, L. H. Michael, S. Weakley, E. Giannini, and M. L. Entman. 1985. Selective accumulation of the first component of complement and leukocytes in ischemic canine heart muscle: a possible initiator of an extra myocardial mechanism of ischemic injury. *Circ. Res.* 57:119-130.
- Schafer, H., D. Mathey, F. Hugo, and S. Bhakdi. 1986. Deposition of the terminal C5b-9 complement complex in infarcted areas of human myocardium. *J. Immunol.* 137:1945-1949.
- Langlois, P. F., and M. S. Gawryl. 1988. Detection of the terminal complement complex in patient plasma following acute myocardial infarction. *Atherosclerosis.* 70:95-105.
- Maclean, D., M. C. Fishbein, E. Braunwald, and P. R. Maroko. 1978. Long term preservation of ischaemic myocardium after experimental coronary artery occlusion. *J. Clin. Invest.* 61:541-551.
- Weisman, H. F., T. Bartow, M. K. Leppo, H. C. Marsh, G. R. Carson, M. F. Concino, M. P. Boyle, K. H. Roux, M. L. Weisfeldt, and D. T. Fearon. 1990. Soluble human complement receptor type 1: in vivo inhibitor of complement suppressing post-ischemic myocardial inflammation and necrosis. *Science (Wash. DC).* 249:146-151.
- Dreyer, W. J., L. H. Michael, T. Nguyen, C. W. Smith, D. C. Anderson, M. L. Entman, and R. D. Rossen. 1992. Kinetics of C5a release in cardiac lymph of dogs experiencing coronary artery ischemia-reperfusion injury. *Circ. Res.* 71:6:1518-1524.
- Jose, P. J., M. J. Forrest, and T. J. Williams. 1983. Detection of the complement fragment C5a in inflammatory exudates from the rabbit peritoneal cavity using radioimmunoassay. *J. Exp. Med.* 158:2177-2182.
- Forrest, M. J., P. J. Jose, and T. J. Williams. 1986. Kinetics of the generation and action of chemical mediators in zymosan-induced inflammation of the rabbit peritoneal cavity. *Br. J. Pharmacol.* 89:719-730.
- Beaubien, B. C., P. D. Collins, P. J. Jose, N. F. Totty, M. D. Waterfield, J. Hsuan, and T. J. Williams. 1990. A novel neutrophil chemoattractant generated during an inflammatory reaction in the rabbit peritoneal cavity in vivo: purification, partial amino acid sequence and structural relationship to interleukin 8. *Biochem. J.* 271:797-801.
- Collins, P. D., P. J. Jose, and T. J. Williams. 1991. The sequential generation of neutrophil chemoattractant proteins in acute inflammation in the rabbit in vivo: relationship between C5a and a protein with the characteristics of IL-8. *J. Immunol.* 146:677-684.
- Jose, P. J., P. D. Collins, J. A. Perkins, B. C. Beaubien, N. F. Totty, M. D. Waterfield, J. Hsuan, and T. J. Williams. 1991. Identification of a second neutrophil chemoattractant cytokine generated during an inflammatory reaction in the rabbit peritoneal cavity in vivo: purification, partial amino acid sequence and structural relationship to melanoma growth stimulatory activity. *Biochem. J.* 278:493-497.
- Schröder, J.-M., U. Mrowietz, E. Morita, and E. Christophers. 1987. Purification and partial biochemical characterization of a human monocyte-derived, neutrophil activating peptide that lacks interleukin 1 activity. *J. Immunol.* 139:3474-3483.
- Yoshimura, T., K. Matsushima, S. Tanaka, E. A. Robinson, E. Appella, J. J. Oppenheim, and E. J. Leonard. 1987. Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines. *Proc. Natl. Acad. Sci. USA* 84:9233-9237.
- Baggiolini, M., B. Dewald, and B. Moser. 1994. Interleukin-8 and related chemotactic cytokines - CXC and CC chemokines. *Adv. Immunol.* 55:97-179.
- Abe, Y., M. Kawakami, M. Kuroki, T. Yamamoto, M. Fajii, H. Kobayashi, T. Yaginuma, A. Kashii, M. Saito, and K. Matsushima. 1993. Transient rise in serum interleukin-8 concentration during acute myocardial infarction. *Br. Heart J.* 70:132-134.
- Finn, A., S. Naik, N. Klein, R. Levinsky, S. Strobel, and M. Elliott. 1993. Interleukin-8 release and neutrophil degranulation after pediatric cardiopulmonary bypass. *J. Thorac. Cardiovasc. Surg.* 105(2):234-241.
- Jorens, P., R. DeJongh, W. DeBacker, J. VanDamme, F. VanOverveld, L. Bossaert, P. Walter, A. Herman, and M. Rampart. 1993. Interleukin-8 production in patients undergoing cardiopulmonary bypass. *Am. Rev. Respir. Dis.* 148:890-895.
- Kalfin, R. E., M. D. Engelman, J. A. Rousou, J. E. Flack, D. W. Deaton, D. L. Kreutzer, and D. K. Das. 1993. Induction of Interleukin-8 Expression During Cardiopulmonary Bypass. *Circulation.* 88:II-401-II-406.
- Sekido, N., N. Mukaida, A. Harada, I. Nakanish, Y. Watanade, and K. Matsushima. 1993. Prevention of lung reperfusion injury in rabbits by a monoclonal antibody against interleukin-8. *Nature (Lond.).* 365:654-657.
- Wedmore, C. V., and T. J. Williams. 1981. Control of vascular permeability by polymorphonuclear leukocytes in inflammation. *Nature (Lond.).* 289:646-650.
- Hellewell, P. G., P. J. Jose, and T. J. Williams. 1992. Inflammatory mechanisms in the passive cutaneous anaphylactic reaction in the rabbit: evidence that novel mediators are involved. *Br. J. Pharmacol.* 107:1163-1172.
- Jose, P. J., I. K. Moss, R. N. Maini, and T. J. Williams. 1990. Measurement of the chemotactic complement fragment C5a in rheumatoid synovial fluids by radioimmunoassay: role of C5a in the acute inflammatory phase. *Ann. Rheum. Dis.* 49:747-752.
- Haslett, C., G. S. Worthen, P. C. Giclas, D. C. Morrison, J. E. Henson, and P. M. Henson. 1987. The pulmonary vascular sequestration of neutrophils in endotoxemia is initiated by an effect of endotoxin on the neutrophil in the rabbit. *Am. Rev. Respir. Dis.* 136:9-18.
- Nourshargh, S., M. Rampart, P. G. Hellewell, P. J. Jose, J. M. Harlan, A. J. Edwards, and T. J. Williams. 1989. Accumulation of <sup>111</sup>In-neutrophils in rabbit skin in allergic and non-allergic inflammatory reactions in vivo: inhibition by neutrophil pretreatment in vitro with a monoclonal antibody recognising the CD18 antigen. *J. Immunol.* 142:3193-3198.
- Pinckard, R. N., M. S. Olson, R. E. Kelley, D. H. DeHeer, J. D. Palmer, R. A. O'Rourke, and S. Goldfein. 1973. Antibody-independent activation of human C1 after interaction with heart subcellular membranes. *J. Immunol.* 110:1376-1379.
- Rossen, R. D., L. H. Michael, A. Kagiya, H. E. Savage, G. Hanson, M. A. Reisberg, J. N. Moake, S. H. Kim, D. Self, S. Weakley, E. Giannini, and M. L. Entman. 1988. Mechanism of complement activation after coronary artery occlusion: evidence that myocardial ischemia in dogs causes release of constit-

uents of myocardial subcellular origin that complex with human C1q in vivo. *Circ. Res.* 62:572-584.

38. Van Damme, J., B. Decock, R. Conings, J-P. Lenaerts, G. Opdenakker, and A. Billiau. 1989. The chemotactic activity for granulocytes produced by virally infected fibroblasts is identical to monocyte-derived interleukin 8. *Eur. J. Immunol.* 19:1189-1194.

39. Schröder, J.-M. and E. Christophers. 1989. Secretion of novel and homologous neutrophil-activating peptides by LPS-stimulated human endothelial cells. *J. Immunol.* 142:244-251.

40. Gregory, H., J. Young, J. -M. Schröder, U. Mrowietz, and E. Christophers. 1988. Structure determination of a human lymphocyte derived neutrophil activating peptide (LYNAP). *Biochem. Biophys. Res. Commun.* 151:883-890.

41. Strieter, R. M., K. Kasahara, R. Allen, H. J. Showell, T. J. Standiford, and S. L. Kunkel. 1990. Human neutrophils exhibit disparate chemotactic factor gene expression. *Biochem. Biophys. Res. Commun.* 173:725-730.

42. Bazzoni, F., M. A. Cassatella, F. Rossi, M. Ceska, B. Dewald, and M. Baggiolini. 1991. Phagocytosing neutrophils produce and release high amounts of the neutrophil-activating peptide 1/interleukin 8. *J. Exp. Med.* 173:771-774.

43. Au, B.-T., T. J. Williams, and P. D. Collins. 1994. Zymosan-induced interleukin-8 release from human neutrophils involves activation via the CD11b/

CD18 receptor and endogenous platelet activating factor as an autocrine modulator. *J. Immunol.* 152:5411-5419.

44. Williams, T. J., and P. J. Jose. 1981. Mediation of increased vascular permeability after complement activation: histamine-independent action of rabbit C5a. *J. Exp. Med.* 153:136-153.

45. Williams, F. M., P. D. Collins, M. Tanniere-Zeller, and T. J. Williams. 1990. The relationship between neutrophils and increased microvascular permeability in a model of myocardial ischaemia and reperfusion in the rabbit. *Br. J. Pharmacol.* 100:729-734.

46. Mullane, K., M. A. Hatala, R. Kraemer, W. Sessa, and W. Westlin. 1987. Myocardial salvage induced by REV-5901: An inhibitor and antagonist of the leukotrienes. *J. Cardiovasc. Pharmacol.* 10:398-406.

47. Sasaki, K., A. Ueno, M. Katori, and R. Kikawada. 1988. Detection of leukotriene B<sub>4</sub> in cardiac tissue and its role in infarct extension through leucocyte migration. *Cardiovasc. Res.* 22:142-148.

48. Kristensen, M., T. Jinquan, M. K. Thomsen, C. Zachariae, K. Paludan, I. Ahnfelt-Ronne, K. Matsushima, K. Thestrup-Pedersen, and C. G. Larsen. 1993. ETH615, a synthetic inhibitor of leukotriene biosynthesis and function, also inhibits the production of and biological responses towards interleukin-8. *Exp. Dermatol.* 2(4):165-170.