centration of D-Phe[7] SP, tissue sensitivity to acetylcholine and histamine was unaltered (Fig. 3). The results suggest that a specific tachyphylaxis or desensitization of the tissue to the effect of SP had been caused by D-Phe[7] SP. The same effect could be demonstrated for a supramaximal concentration of SP against SP, but not with EC50 or threshold concentrations. Moreover, these effects were observed using the 10 min incubation period as outlined by Yamaguchi et al (1979) with which desensitization occurred while it was not possible to demonstrate this phenomenon with the 2 min incubation period routinely used in this laboratory.

A similar claim for SP-antagonist activity has subsequently been made for Ile[8]-SP (Rackur et al 1979). Although this peptide has not been investigated using the present protocol, it is tempting to speculate that a similar desensitization may also be responsible for the apparent antagonist activity of this analogue.

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Aggregation of rat polymorphonuclear leucocytes in vitro

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The intravenous administration of either zymosan-activated serum (ZAS) or formylmethionyl-leucyl-phenylalanine (FMLP), a synthetic chemotactic peptide, causes a transient but profound neutropenia in the rat (Cunningham & Smith 1980). Complement derived peptides and FMLP may induce changes in the neutrophil membrane which in turn lead to increased margination and aggregation, the neutrophil aggregates being trapped in the pulmonary capillary bed. The neutropenia therefore results from a combination of margination and aggregation. Similar effects have been described in other species following the intravenous injection of cytotaxins, inulin-treated plasma, or agents known to activate complement in vivo. e.g. cobra venom factor (CVF) (O'Flaherty et al 1977; Craddock et al 1977a). These observations suggest that the neutropenia seen in man during haemodialysis (Craddock et al 1977a) and nylon fibre filtration leucopheresis (Hammerschmidt et al 1978) may be due to margination plus aggregation, these effects being caused by the release of complement-derived peptides after contact of blood with the Cellophane membrane or nylon fibres.

Although the ion dependency of aggregation and adherence vary, aggregation being both calcium and magnesium-dependent whereas adherence is dependent upon magnesium ions (O'Flaherty et al 1978b), it seems likely that the alterations in structure and function of the neutrophil (PMN) membrane occurring during aggregation and adherence both in vivo and in vitro are similar (Craddock et al 1979). During haemodialysis there is evidence that, having undergone these changes, altered PMNs are able to produce endothelial damage in the lungs, reflected by changes in arterial Po_2, diffusing capacity and an increased flow of protein-rich lymph occurring during the period of neutropenia (Craddock et al 1977b). There is, however, no penetration of PMNs across the vascular endothelium except in the presence of an extravascular source of activated complement, generated for example by alveolar macrophages, when neutrophils migrate into the interstitium and play an essential role in the inflammatory reaction (Henson et al 1979). It seems likely that in other circumstances a localized inflammatory process may be associated with intravascular activation of PMNs at the site of inflammation. This in turn may increase their tendency to adhere to the vascular endothelium and to each other and may lead to increased permeability of the vascular endothelium before PMN migration into the tissue. In the tangled web of processes comprising the acute inflammatory reaction it is not yet possible to ascertain the relative importance of such phenomena but a study of PMN aggregation may provide new insights into the response.

We have, therefore, studied the effect of FMLP and ZAS on the aggregation of PMNs in vitro. An in vitro model of aggregation could provide a useful method of investigating structure activity relationships in potential anti-inflammatory drugs with regard to their actions on polymorph function.

Methods and materials. Peritoneal exudates containing 84 $\pm$ 4% PMNs were elicited in female Wistar/OLA albino rats (150–200 g) by the injection of 6 ml of 12% sodium caseinate. Cells were harvested after 24 h by the intraperitoneal injection of 20 ml Hanks Balanced Salt Solution (HBSS) containing 5U ml^{-1} heparin (Cunningham et al 1979). The cells were then washed once in HBSS and resuspended at a concentration of $1{\cdot}0$–$1{\cdot}3 \times 10^7$ cells ml^{-1} in Eagle's Minimum Essential Medium (MEM) buffered to pH 7·4 with 30mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid). Cell suspensions were allowed to equilibrate at 37 °C for 30 min before use. In an experiment com-

* Correspondence.

paring peripheral and peritoneal cell aggregation, 250–300 g female Wistar/King's rats were used. Blood was withdrawn from the descending aorta into heparinized (10 U ml^{-1}) syringes and PMNs were then separated by dextran sedimentation followed by Hypaque/Ficoll centrifugation. Red cells were removed by hypotonic lysis and the leucocytes were then washed once and resuspended at a concentration of $1 \cdot 1 \times 10^7$ cells ml^{-1} in MEM/HEPES. Peritoneal cells were elicited as described above and one half of the sample was subsequently exposed to dextran and Hypaque/Ficoll. The chemotactic peptide (FMLP) was dissolved in dimethylsulphoxide (DMSO) at a concentration of 10^{-2} M and serial dilutions made in MEM. The final concentration of DMSO in each sample was never more than 0·1%. Freshly prepared rat serum was activated with 500 μg ml^{-1} washed, boiled zymosan, at 37 °C for 30 min followed by heat inactivation at 56 °C for 45 min. The zymosan was removed by centrifugation before use. Dilutions of activated serum were made with 0·9% NaCl.

Cell suspensions (450 μl) were stirred at 900 rev min^{-1} in siliconized glass cuvettes at 37 °C in a Payton Dual Channel Aggregation Module connected to a Rikadenki Dual channel recorder. After 5 min an arbitrary standard volume of 50 μl of an aggregating agent or control was added to the cuvette and aggregation was measured as a change in light transmittance (T) (Hammerschmidt et al 1979). Aggregation ratios were calculated as follows:

$$\text{Aggregation ratio} = \frac{\Delta T\ (\text{aggregating agent}) - \Delta T\ (\text{control})}{\Delta T\ (\text{control})}$$

At the beginning of each experiment the system was calibrated using the difference in transmittance between the standard cell suspension and a suspension diluted to 80% of the cell count with MEM.

Results. The change in transmittance which occurs when FMLP or ZAS is added to a stirred suspension of rat peritoneal PMNs is illustrated in Fig. 1. The change in transmittance after MEM or heat inactivated serum is due to the dilution of the suspension. Examination of the cells by light microscopy showed that aggregates predominantly of two and three cells had formed.

Fig. 2 shows the response of a standard suspension of PMNs ($1 \cdot 3 \times 10^7$ cells ml^{-1}) to FMLP over the range 10^{-8} to 10^{-5} M. There is a direct relationship between the $\log_{10}$ FMLP concentration and the size of the aggregation response over the range tested, the responses being expressed as the aggregation ratio. There is also a dose-related aggregation response of rat peritoneal PMNs to final concentrations of ZAS in the range 0·5–10% (Fig. 3) and a dose-related response to zymosan activated plasma (results not shown).

The results were reproducible with FMLP dilutions prepared from single stock solution. The coefficient of variation for aggregation ratios obtained on individual days using the same stock cell suspension was always less than 8·5%. However, when the results from several different days were pooled the coefficient of variation rose to between 11–14%. Over the range of initial cell concentrations $1 \cdot 5 \times 10^6$ to $2 \cdot 4 \times 10^7$ a direct relationship was found between the aggregation ratio and $\log_{10}$ cell concentration; the optimal response being obtained with an initial concentration of $1 \cdot 2 \times 10^7$ cells ml^{-1} (Fig. 4). The presence of 5% human serum albumin in the medium reduced the response of the cells to a fixed concentration of FMLP by 34% ($P < 0 \cdot 05$).

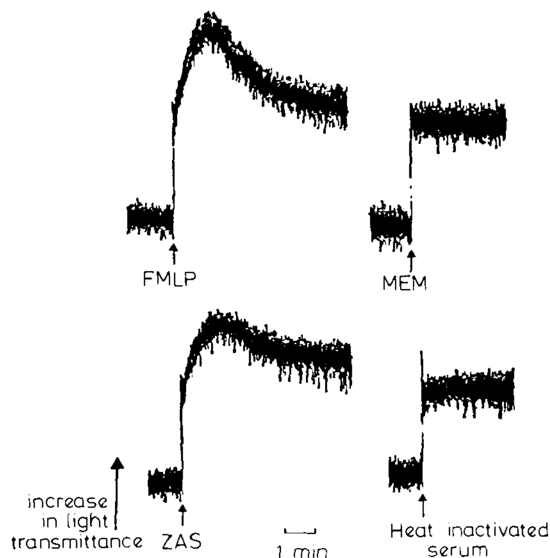

FIG. 1. Changes in transmittance of stirred suspensions ($1 \cdot 1 \times 10^7$ cells ml^{-1}) of rat peritoneal PMNs after the addition of 50 μl quantities of MEM, FMLP (final concentration 5×10^{-7} M), heat-inactivated serum and ZAS (final concentration 2·5% v/v).

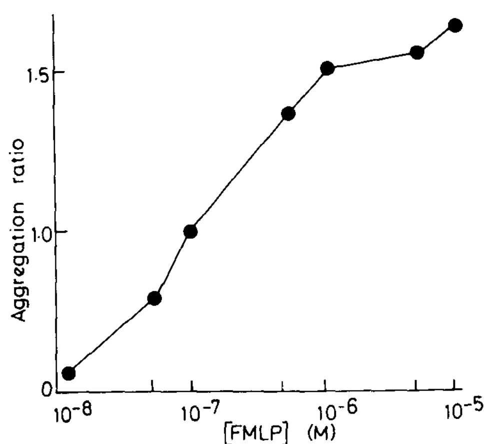

FIG. 2. Aggregation of rat peritoneal PMNs by FMLP. Each point is the mean of between 7 and 11 separate observations.

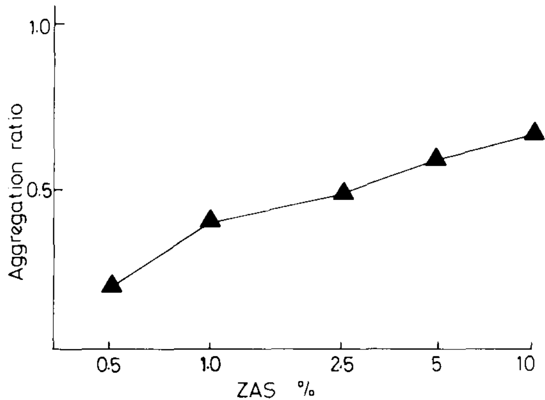

FIG. 3. Aggregation of rat peritoneal PMNs by ZAS. Results given as mean of 5 observations.

Table 1. Aggregation of PMN preparations in vitro by FMLP ($2\cdot5 \times 10^{-7}$M).

Group	Aggregation ratio*
Peritoneal PMNs	$1\cdot82 \pm 0\cdot05$
Peritoneal PMNs exposed to dextran and Hypaque/Ficoll	$1\cdot92 \pm 0\cdot07$
Peripheral cells	$1\cdot33 \pm 0\cdot06$**

* Results expressed as means ± standard errors (6 separate experiments per group).
** $P < 0\cdot001$ from results of peritoneal PMN groups.

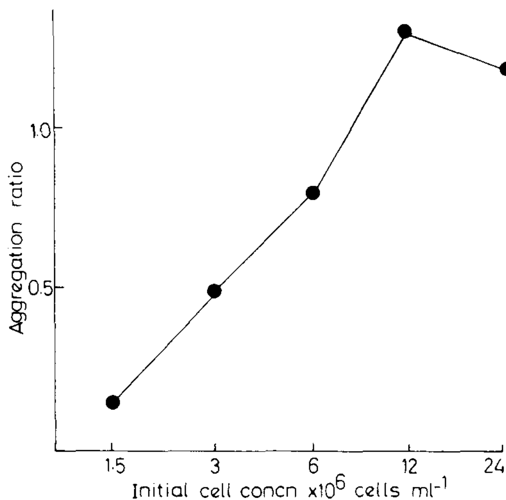

FIG. 4. Relation between cell concentrations and aggregating effects of 10^{-7} M FMLP on rat peritoneal PMNs. Results given as mean of 6 observations.

The aggregation ratios for the responses of peripheral PMNs, peritoneal PMNs, and peritoneal PMNs exposed to dextran and Hypaque/Ficoll are shown in Table 1. The aggregation of peripheral cells was significantly reduced when compared with that of peritoneal cells treated with dextran and Ficoll ($P < 0\cdot001$). There was no difference between the responses of peritoneal cells exposed to dextran and Hypaque/Ficoll compared with untreated peritoneal cells. Differences seen in the aggregation ratios in this work are probably attributable to the use of different stock solutions of FMLP.

Discussion. The intravenous injection of cytotaxins causes a rapid and reversible neutropenia in the rabbit (O'Flaherty et al 1977). A transient and dose-related fall in the neutrophil count also occurs in the rat after the administration of either synthetic cytotaxins (FMLP) or zymosan activated serum (ZAS) (Cunningham & Smith 1980). The extent of the neutropenia is considerable, the neutrophil count being reduced by over 95% within 1 min of injection. The mechanism of the effect results from activation of the leucocyte cell membranes leading to margination and aggregation of the PMNs in the venous circulation, the leucocyte aggregates becoming trapped in the pulmonary vascular bed. If the cytotaxins are given intra-arterially, however, the aggregates accumulate in the systematic microvasculature. This intravascular aggregation of PMNs in vivo in response to the intra-arterial injection of ZAS has been directly observed in the arterioles of exposed rat mesentery (Hammerschmidt et al 1979).

In the present work we have studied the aggregation of rat elicited PMNs in vitro in response to either FMLP or ZAS. Aggregation was measured by changes in the light transmittance of stirred suspensions of the leucocytes and both agents produced a dose-related effect. The magnitude of the response was proportional to the $\log_{10}$ of the initial cell concentration in the suspensions, could be detected within 5 to 10 s after addition of the cytotaxin, reached a maximum at 1 to 2 min and then partially reversed and stabilized by 3 to 5 min (Fig. 1). Aggregation was not accompanied by an increase in release of the cytoplasmic enzyme, lactate dehydrogenase.

The maximum response to FMLP was greater than that to ZAS. One possible explanation of this is that the ZAS preparation is a more complex mixture than the FMLP solution and may contain materials antagonistic to the aggregatory effect of C5a.

The response of elicited PMNs from the rat differed from that of human peripheral cells in that the aggregation of the latter, when exposed to complement-derived cytotaxins, is biphasic (Craddock et al 1977c). It has also been reported that human peripheral cells differ from PMNs elicited in the rabbit peritoneal cavity in their response to aggregating agents (O'Flaherty et al 1978a). To obviate the problem of species differences we studied the response of rat peripheral and elicited PMNs to FMLP in vitro. Although the relatively small

numbers of neutrophils in rat peripheral blood limited the range of concentrations of cytotaxin which could be compared directly, the results showed that the peripheral PMNs were significantly less responsive to the aggregation induced by FMLP. This change is not due to different separation processes for the leucocytes since exposure of the elicited cell suspension to dextran and Hypaque/Ficoll had no effect. One possible explanation is the presence in the suspensions of peripheral and elicited cells of variable proportions of sub-populations of PMNs with differing responsiveness to aggregating agents. It has been reported that there are at least two distinct sub-populations showing different adherent behaviour in human peripheral PMN preparations (Klempner & Gallin 1978).

The possible relationship between aggregation, adherence and chemotaxis of PMNs is of interest with request to the role of the leucocyte in the initiation and maintenance of inflammatory reactions. Adherence and aggregation are associated phenomena (Craddock et al 1979) and concentrations of cytotaxins, such as FMLP, which enhance the adherence of human PMNs in vitro also inhibit the directed migration of the leucocytes (Fehr & Dahinden 1979). We have observed that the concentrations of FMLP which induce maximum aggregation of human peripheral PMNs also seem to inhibit chemotaxis of the cells (unpublished observations). This may serve as a mechanism to trap PMNs at inflammatory sites.

Whereas rat peripheral PMNs show a lesser tendency to aggregate than do elicited cells, they show a greater chemotactic response (Cunningham et al 1979). This may be due to changes induced in the cells during the attraction and entry into the inflammatory site. If desensitization were the explanation of the reduced chemotactic ability of elicited PMNs it would be expected that aggregation and adherence would also be reduced. This does not occur. One possible explanation is that desensitization may differentially affect these functions. Cell swelling, increased adherence and aggregation are also likely to reduce the mobility of the elicited cells.

The formation of PMN aggregates in the vascular compartment with their subsequent embolization in capillary networks has been extensively studied in the context of haemodialysis and nylon fibre leucopheresis (Craddock et al 1977a; Hammerschmidt et al 1978). Other situations where similar phenomena occur are Purtscher's retinopathy, a rare complication of pancreatitis and severe trauma, endotoxin shock and the shock lung syndrome.

The relationship of the phenomena of PMN aggregation, adherence and chemotaxis and their relevance in vivo during the inflammatory process, however, remains unclear. Production of activated complement in, for example, systemic lupus erythematosus or other immune complex diseases, may result in localized intravascular activation of PMNs especially under conditions of relative blood stasis due to vasoconstriction and tissue oedema. Intravascular adherence to endothelium and aggregation may exacerbate such stasis and by bringing the activated PMNs into close contact with the endothelial cell may promote an increase in focal vascular damage. By studying such phenomena independently and by comparing their properties it may be possible to further elucidate the contribution of each to the inflammatory process.

It is suggested that the technique described could be used as one of the in vitro tests required to define the spectrum of activity of anti-inflammatory drugs. It is becoming clear that a greater understanding of any effects on leucocyte behaviour is of increasing importance in the design of improved anti-inflammatory and antirheumatic agents.

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