

Heat-induced aggregated human IgG modifies the adherence of human polymorphonuclear cells to cultured endothelium

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SUMMARY

The adherence of human blood polymorphonuclear cells (PMNC) to cultured porcine aortic endothelium was enhanced by high concentrations of heat-stable IgG aggregates (HAGG) when sera was omitted from the culture media. With 20% human serum present in the media, HAGG induced a dose-related inhibition of PMNC adhesions with concentrations as low as 10 µg/ml producing a significant effect. This inhibitory action of HAGG, which was optimally expressed after 30 min of incubation, seemed to be directed at the PMNC rather than the endothelium. Heat-inactivation of the sera resulted in a marked decline of the inhibitory activity of HAGG. Aggregates of size 15–21 s were demonstrated to be most effective in inhibiting PMNC attachment and it is complexes of this size which are commonly found in the circulation of patients with chronic inflammatory diseases. Immune complex modification of PMNC adherence may control leucocyte extravasation during inflammation.

Keywords polymorphonuclear cells adherence endothelium IgG aggregates

INTRODUCTION

Increased levels of circulating immune complexes are often associated with chronic inflammatory disorders, such as rheumatoid arthritis (RA) (Hay *et al.*, 1979) and systemic lupus erythematosus (SLE) (Nydegger *et al.*, 1974), yet their pathogenetic contribution remains to be defined. Soluble immune complexes were previously shown to increase the adherence of blood polymorphonuclear cells (PMNC) to cultured vascular endothelium (Hashimoto & Hurd, 1982), suggesting that an important action of these complexes is their enhancement of PMNC margination. In a recent study we demonstrated that sera and plasma from patients with RA inhibited the attachment of blood PMNC from normal subjects to cultured vascular endothelium and that the extent of this inhibition was directly related to the level of C1q-binding immune complexes in rheumatoid blood (Chasty *et al.*, 1987). The aim of the present investigation was to define the conditions under which soluble immune complexes modified PMNC adhesion. The adhesion of PMNC to cultured vascular endothelium was measured in a quantitative adhesion assay (Sheehan *et al.*, 1987) using heat-induced, stable human IgG aggregates (HAGG) as a model of circulating immune complexes.

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MATERIALS AND METHODS

Separation of PMNC

The separation procedure was a modification of that of Dioguardi *et al.* (1963). We have used this method on several occasions (Chasty *et al.*, 1987; Brown *et al.*, 1988a) and find it to be quicker, simpler and cheaper than others without compromising loss of PMNC purity and functional responsiveness. Thirty millilitres of heparinized blood (10 U/ml) were diluted with 210 ml of 0.83% NH₄Cl, left for 10 min at room temperature, and centrifuged at 450 g for 10 min. The supernatant containing the lysed erythrocytes was removed and the NH₄Cl lysis procedure repeated. The pellet containing the leucocytes was resuspended in calcium- and magnesium-free Hank's balanced salt solution (CMF-HMSS) and centrifuged at 55 g for 10 min. This washing procedure was repeated on two further occasions. During the slow centrifugation the more dense PMNC were pelleted, whereas the less dense ones were suspended in the supernatant which was discarded. The final cell pellet was resuspended in Eagle's minimum essential medium (EMEM) supplemented with 1 mM glutamine, 200 U/ml penicillin, and 100 U/ml streptomycin, buffered to pH 7.3 with 10 mM Hepes buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid). Cell viability was >96%, and purity was approximately 90%.

Preparation of monolayers of porcine aortic endothelial cells

The lumen of aortae from freshly killed pigs were washed with phosphate-buffered saline (PBS) and filled with 10 ml of collagenase solution (1 mg/ml PBS). After incubation for 10 min at 37°C the collagenase solution was aspirated and each aorta was washed through with medium (RPMI 1640 supplemented with 20% fetal calf serum (FCS), 2 mM glutamine, 200 U/ml penicillin, and 100 U/ml streptomycin). The washings and eluted collagenase solution obtained from each aorta were pooled and centrifuged at 600 g for 10 min. The endothelial cell pellet was resuspended in 10 ml medium and added to a tissue culture flask which was incubated in a humidified 5% CO₂ incubator at 37°C. On attainment of a confluent endothelial cell monolayer the medium was expelled and the cells treated with 2.5 ml of 0.5% trypsin and 0.02% EDTA in PBS for 3–5 min. Following gentle shaking of the flask, the trypsin digestion was stopped by the addition of 2.5 ml medium and contents, together with 5 ml of washing medium, centrifuged at 600 g for 10 min. Endothelial cells were resuspended in medium to 1 × 10⁵ cells/ml and 1 ml was placed in each well of a Linbro multi-well plate. Confluent monolayers of endothelial cells were obtained after culture for 2 days at 37°C in a humidified CO₂ incubator.

Radiolabelling of PMNC with ⁵¹Cr

⁵¹Cr (100 μCi, 3.7 MBq) was incubated with 1 × 10⁸ PMNC for 1 h at 37°C with agitation every 15 min. The radiolabelled PMNC were washed three times by suspending in 5 ml CMF-HBSS supplemented with 5% FCS and then centrifuging at 450 g for 10 min. The final cell pellet was resuspended in 5 ml EMEM and filtered through a 4-mm metal gauze to remove any large clumps. The viability of the PMNC was not modified by this procedure and the cells were adjusted to 1 × 10⁶ cells/ml.

Adherence assay

Prior to assay the medium was aspirated from each well and the endothelial monolayers were washed twice with PBS at 37°C. Each monolayer was treated with 100 μl of labelled cells (1 × 10⁶ cells/ml) and with, according to experimental design, either 200 μl of normal human serum with 700 μl of EMEM, or 900 μl EMEM alone. All tests were performed in triplicate in randomly allotted wells. The PMNC were incubated with endothelial cells for 1 h at 37°C. Thereafter, the non-adherent PMNC were aspirated and the endothelial monolayers were washed gently five times with PBS to remove loosely adherent PMNC. Each monolayer was osmotically disrupted by treatment with 0.5 ml 0.1 NaOH for 15 min; lysate was collected and counted in a Packard auto-gamma scintillation counter.

The percentage of PMNC adhering to endothelium was calculated as follows:

$$\% \text{adherence} = \frac{\text{ct/min in each well} - \text{ct/min background}}{\text{ct/min of original PMNC} - \text{ct/min background}} \times 100$$

Preparation of HAGG

HAGG were prepared as reported previously (McCarthy *et al.*, 1981a). Briefly, human IgG (Sigma) was dissolved in borate-buffered saline (BBS) at a concentration of 30 mg/ml, and clarified by centrifugation at 5000 rev/min for 10 min. The supernatant was heated at 60°C until the absorbance at 400 nm reached 0.25 units, and then allowed to stand at 4°C for 48 h. The aggregates were selectively concentrated by polyethylene

glycol (3.5%) precipitation, washed twice by centrifugation in BBS, resuspended in 5 ml BBS and centrifuged through sucrose (10–30%) density gradient, as described (McCarthy *et al.*, 1981b). Fractions containing IgG aggregates with sizes 15S–21S, 22S–45S, and 55S–70S were collected (here after referred to as fractions I, II and III, respectively) and dialysed against BBS to remove the sucrose. Protein concentrations were estimated by measuring the absorbance at 275 nm:

$$A \frac{1 \text{ mg/ml}^{-1}}{275 \text{ nm}} = 1.43$$

Statistical analysis

The Student's *t*-test was used to determine the significance of differences between the means of two groups.

RESULTS*Effect of unfractionated HAGG on PMNC adherence to endothelium*

Figure 1 shows the results of a typical experiment in which increasing concentrations of HAGG were added to PMNC that were co-cultured with endothelial cells for 1 h in serum-free medium. Over the concentration range 10–50 μg/ml PMNC adherence was not modified, whereas the addition of 100 μg/ml HAGG produced a significant enhancement of adhesion (47%; *P* < 0.001). From four experiments 100 μg/ml HAGG induced a mean increase of 38% ± 12. When 20% autologous serum was present in the medium HAGG produced a dose-related inhibition of PMNC attachment (Fig. 2) with 10 μg and 100 μg/ml generating a mean 17% and 43% inhibition, respectively (*P* < 0.001). As shown in Fig. 3, the impairment of PMNC

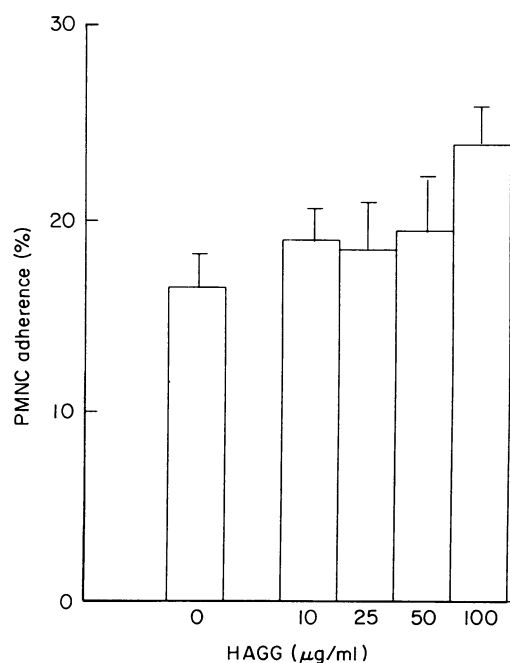


Fig. 1. Effect of HAGG on the adherence of PMNC to endothelium when serum is absent from the culture media. Mean of three experiments; vertical bars represent s.d.

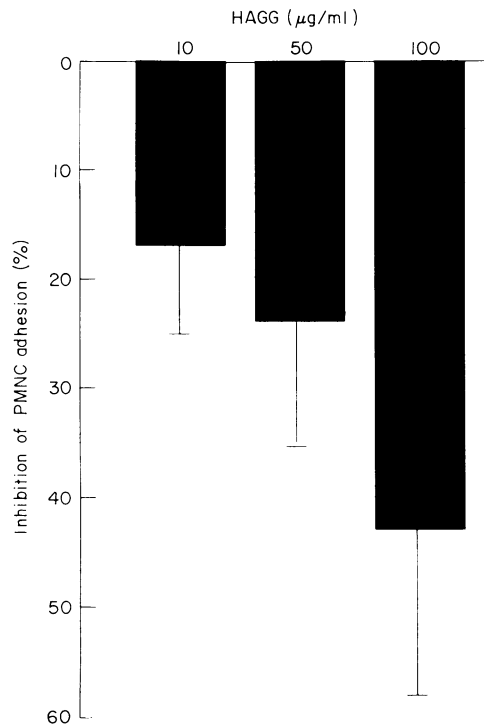


Fig. 2. Unfractionated HAGG inhibits the attachment of PMNC to cultured endothelium when 20% human serum is present in the culture media. Mean of four experiments; vertical bars represent s.d.

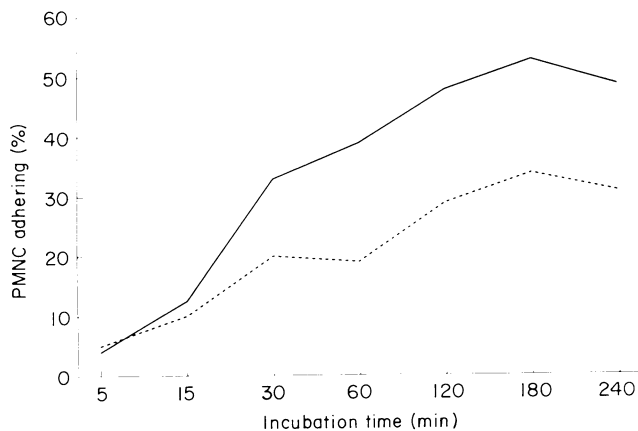


Fig. 3. Inhibitory activity of HAGG on PMNC adherence during a 4-h incubation with endothelium. The culture medium contained 20% human serum throughout the duration of the experiment. Continuous line represents the percentage of PMNC adhering in the absence of HAGG; broken line represents the percentage of adherent PMNC when co-cultured with 100 µg/ml unfractionated HAGG. Similar results were obtained in two other experiments.

attachment was apparent after a 30 min incubation; the maximum inhibition being seen after 1 h incubation. Thereafter, extending the incubation to 4 h did not modify the abrogatory activity of HAGG. This effect of HAGG seemed to be directed against the PMNC rather than the endothelium as a similar

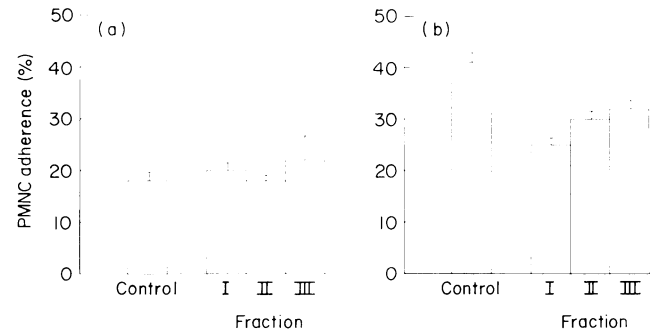


Fig. 4. Comparison of the action of fractionated IgG aggregates on PMNC adherence to endothelium in the absence (a) and presence (b) of human serum. Fraction I, 15–21s; fraction II, 22–45s; and fraction III, 55–70S. All fractions were used at 10 µg/ml. The results are the mean of three experiments; vertical bars denote s.d.

Table 1. Inhibition of PMNC adhesion by HAGG fractions of various sizes

Fraction	Inhibition of adhesion (%)
7s	11
10s	9
17s	46*
30s	30*
42s	28*
58s	33*

PMNC, polymorphonuclear cells; HAGG, heat-induced aggregated IgG.

All fractions were used at a concentration of 50 µg/ml. The results are the mean of three experiments.

* $P < 0.001$.

inhibition of PMNC adherence (mean 24% ± 6; three experiments) was seen when serum-coated plastic was used as the adherence substrate. In all experiments there was no evidence of endothelial cell detachment or change in viability as measured by trypan-blue exclusion.

Effect of fractionated HAGG on PMNC adherence to endothelium

The next stage of this investigation was to determine whether the inhibitory activity of HAGG resided in a fraction of a particular size. Thus, 10 µg/ml of HAGG fractions I, II and III were simultaneously added with PMNC to monolayers of endothelium. In the absence of serum none of the fractions modified PMNC adherence in contrast to the inhibitory activity expressed by all fractions when the culture media contained 20% serum. Fractions I, II and III produced 40, 27 and 22% inhibition of adherence, respectively ($P < 0.001$ for all samples).

Further experiments were undertaken using HAGG fractions of specific sizes. The addition of 50 µg/ml of the 10s fraction and monomeric IgG (7s) to the assay, with serum present, did not modify PMN adherence (Table 1). In contrast,

Table 2. Reduction of HAGG inhibitory activity following heat-inactivation of serum and plasma

	Percentage inhibition of adhesion	
	Fraction I (15–21s)	Fraction II (22–45s)
Serum	36	31
HI serum	24	16
Plasma	33	25
HI plasma	14	9

Polymorphonuclear cells and 50 µg/ml of either/fraction I or II were simultaneously added to endothelium and incubated for 1 h at 37°C in the presence of 20% untreated plasma or serum or 20% heat-inactivated (HI) plasma or serum. Results are typical of those obtained from two additional experiments.

all the remaining fractions produced a significant inhibition of adherence with the greatest effect being seen with the 17s fractions.

Previously, we found that rheumatoid plasma, containing high levels of immune complexes, was more inhibitory than rheumatoid sera in impeding the attachment of normal blood PMNC to cultured endothelium (Chasty *et al.*, 1987). Table 2 shows that the inhibitory activity of fractions I and II was also demonstrable when 20% human plasma was substituted for the serum in the culture media. However, when serum and plasma which had been heated at 56°C for 30 min were introduced into the adherence assay, there was a marked reduction in the inhibitory activity of fractions I and II (Table 2).

DISCUSSION

Immune complexes and HAGG, at high concentrations (100 µg/ml and 1 mg/ml), were previously shown to enhance PMNC adhesion to endothelium in an assay in which serum was absent (Hashimoto & Hurd, 1981). Using similar experimental conditions the results of the present study support those findings. However, when 20% human serum was introduced into the assay, low (10 µg/ml) and high (100 µg/ml) concentrations of the aggregates produced an inhibition of adhesion which was dose dependent.

This inhibitory activity of HAGG was diminished when the sera present in the culture media had been heated at 56°C for 30 min; this suggests that the effect may be partly mediated by the action of complement. From the demonstration that in the presence of serum the HAGG impeded the attachment of PMNC to serum-coated plastic also, it appears that the inhibitory activity of the aggregates is directed at the PMNC rather than the endothelium. This view is supported by experiments which show that Fc receptors do not appear to be present on the surface of endothelial cells isolated from normal (untreated) bovine pulmonary artery (Ryan *et al.*, 1980), human umbilical vein (Lyss *et al.*, 1980; Hashimoto & Hurd, 1981;

Hanssen *et al.*, 1984) and pig aortic endothelial cells (our unpublished findings) and that HAGG is not cytotoxic for cultured endothelial cells and does not modify their growth or morphology (Fillit *et al.*, 1982).

The attachment of PMNC to endothelial cells and artificial substrates is generated in part by the surface expression of Mo1 (Springer *et al.*, 1986) which is the complement receptor CR3 (Wright *et al.*, 1983). Occupation of the CR3 receptors by HAGG-generated C3bi may have masked these adhesion promoting determinants, resulting in a 'down-regulation' of adhesion, whereas in the absence of complement the binding of HAGG to Fc receptors may have induced the release of factors which augment PMNC adherence (Bockenstedt & Goetzl, 1980; Oseas *et al.*, 1981). Experiments are in progress to determine if binding of HAGG to the PMNC surface modifies the expression of the adhesion molecules.

Since all nucleated cells possess a net negative surface charge (Brown, 1983), and PMNC adhesiveness is enhanced by a decrease in this charge (Gallin, 1980), a lowering of the surface charge of the PMNC or the endothelial cells or both is likely to reduce their electrical repulsion and promote attachment. In a serum-free system we recently demonstrated that the surface charge of PMNC from the blood of healthy subjects was significantly reduced following treatment with HAGG for 30 min (Brown *et al.*, 1988a) and this effect may have some bearing on the adhesion-promoting activity of these aggregates.

Complexes with sedimentation coefficients of $\geq 22S$, and which generally consist of IgG-IgM rheumatoid factor and are commonly found in RA serum (Andreiss *et al.*, 1978), whereas intermediate complexes (9–15S), which are more likely to be composed of IgG rheumatoid factor complexes (Pope, Telker & Mannik, 1975), are associated with severe rheumatoid disease (Theofilopoulos *et al.*, 1974). In the present study fractions of 15–21S possessed the greatest inhibitory activity of PMNC adhesion. Previous studies have shown that IgG aggregates of size $< 20S$ (which are internalized and membrane-bound by PMNC) (Goddard *et al.*, 1984), are more effective at reducing PMNC chemotaxis than are larger complexes. Since many circulating PMNC in RA have immune complexes on their surface (Camusi, Tetta & Cappio, 1979), membrane-bound IgG aggregates, particularly of size 15–21S may inhibit their adhesion to vascular endothelium and hence their extravasation into tissue. In RA there is a marked infiltration of blood PMNC into the articular synovial fluid and an impediment of PMNC margination by circulating immune complexes may seem to be at variance with the pathology of this disease. However, by inhibiting PMNC adhesion to endothelium of the general vasculature, immune complexes could direct PMNC to endothelium whose phenotype is functional in the presence of circulating immune complexes as follows: Fc receptors are not present normally on endothelial cells (see above), but are induced by injury (Ryan, Schulz & Ryan, 1981) and viral infection (Cines *et al.*, 1982). Thus, at sites of inflammation, it is possible that the expression of Fc receptors on the local microvascular endothelium allows the attachment of immune complex-bearing PMNC with subsequent diapedesis. Experiments are in progress to investigate this possibility. This study has used endothelial cells derived from porcine aortae; preliminary experiments demonstrate that similar manoeuvres lead to a HAGG-induced enhancement and inhibition of PMNC adhesion to endothelial cells cultured from human umbilical vein. The present results

support the idea that circulating immune complexes have a role in controlling the attachment of PMNC to vascular endothelium.

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