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Inhibition of human neutrophil migration by aggregated gammaglobulin

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SUMMARY

Heat-aggregated human gammaglobulin has been shown to inhibit the random migration of human neutrophils in serum-containing medium. This inhibition was not due to metabolic exhaustion or deactivation of the cells, since migration in the presence of aggregated gammaglobulin and casein as a chemotactic stimulus was not inhibited. The inhibition of migration was not mediated by a negative chemotactic gradient produced as a result of complement activation, and could be demonstrated in complement-depleted serum. Sera obtained from patients with rheumatoid arthritis with evidence of circulating immune complexes were able to significantly inhibit neutrophil migration, indicating that this phenomenon may be a useful means for the detection of circulating immune complexes. It is suggested that aggregated gammaglobulin or immune complexes can inhibit the chemokinetic effect of serum on neutrophils by a reversible interaction with the neutrophil surface, and that this inhibition could contribute to the accumulation of neutrophils at sites of immune complex deposition *in vivo*.

INTRODUCTION

Immune complexes or heat-aggregated gammaglobulins can attract rabbit (Boyden, 1962; Keller & Sorkin, 1966) and human (Laster & Gleich, 1971; Wagner, Abraham & Baum, 1974) neutrophils in serum-containing medium. This activity of immune complexes plus serum has been shown to be due to complement activation with the release of the chemotactic fragment C5a (Snyderman, Phillips & Mergenhagen, 1970), and it has been suggested that this is a mechanism which promotes the accumulation of neutrophils at sites of immune complex deposition *in vivo* (Snyderman, Phillips & Mergenhagen, 1971).

Immune complexes or aggregated immunoglobulins can also interact directly with human neutrophils (Lawrence, Weigle & Spiegelberg, 1975) with resultant enzyme release (Henson, Johnson & Spiegelberg, 1972), activation of the hexose monophosphate shunt (Henson & Oades, 1975), release of superoxide anion (Johnston & Lehmeyer, 1976) and inhibition of bacterial phagocytosis (MacLennan *et al.*, 1973). However, the results of this interaction on neutrophil locomotion are not clear. Mowat & Baum (1971) observed that pre-incubation of human neutrophils with whole serum or purified immune complexes obtained from patients with rheumatoid arthritis reduced their subsequent response to a chemotactic stimulus and suggested this finding may be due to the phagocytosis of complexes. The effects of the immune complexes on random migration and the chemokinetic influence of serum were not examined. Neutrophil migration *in vitro*, and presumably *in vivo*, is the result of a complex interaction between chemokinetic and chemotactic environmental stimuli (Keller *et al.*, 1977). In this paper we examine the effect of heat-aggregated immunoglobulin and sera obtained from patients with rheumatoid arthritis on neutrophil locomotion *in vitro*.

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MATERIALS AND METHODS

Neutrophils. Blood was collected from normal healthy adults into preservative-free heparin and mononuclear cells were removed by centrifugation through a Ficoll–Hypaque gradient (Böyum, 1968) at 400 *g* for 30 min. Red cells were removed by sedimentation in 2% dextran in PBS at 4°C for 45 min. The neutrophils were washed three times in Dulbecco's phosphate buffered saline (PBS) and then suspended in Hanks's balanced salt solution (HBSS) for use in the assay. In assays where neutrophils were incubated in homologous sera, neutrophils from blood group O donors were used.

Preparation of heat-aggregated gammaglobulin (HAGG). Gammaglobulin, Cohn Fraction II (Commonwealth Serum Laboratories, Melbourne) at a concentration of 20 mg/ml was heated in a glass test tube at 63°C for 20 min. The aggregated IgG was then separated from the monomeric IgG by gel filtration on a Sepharose 6B column (2·5 × 90 cm, Pharmacia Fine Chemicals AB, Sweden). This column had previously been calibrated with dextran blue and with normal human serum, the position of IgG and IgM being determined by quantitative precipitation with specific antisera using laser nephelometry. Chromatography was performed at room temperature in PBS, pH 7·3, with an upward flow rate of 20 ml/hr. Eluates containing IgG larger than monomeric were pooled and concentrated by positive pressure dialysis using an Amicon XM-100A filter. The protein concentration was measured by absorbance at 225 and 215 nm (Waddell & Hill, 1956) and dialysed against HBSS. Monomer IgG was prepared in a similar fashion. Aggregated IgG preparations of differing molecular size were prepared by the collection of 29 × 5·4 ml fractions after chromatography on HAGG. Fractions were pooled to make the preparations, F1 (fractions 1–4), F2 (fractions 6–11) and F3 (fractions 13–17), and concentrated as above.

Neutrophil migration. The method was essentially as detailed by Wilkinson (1974) with the exception that HBSS was utilized as a suspending medium. Chemotactic chambers were sawn off tuberculin syringes with 3·0 μ pore size filters (Millipore Corporation, Bedford, Massachusetts) glued to the end. Triplicate chambers were suspended in a 10 ml glass beaker containing 4·75 ml of medium and incubated in a humidified atmosphere of 5% CO_2 and air, at 37°C. Filters were stained according to Wilkinson (1974) and stored in xylol. For determination of the leading front, filters were mounted under a cover-slip in immersion oil, and the leading front was measured by determination of the distance in microns through the filter to the furthest plane which contained at least three cells in focus on a Leitz Ortholux microscope with a × 40 objective. Three fields were read per filter. The migration in microns is expressed as the mean ± s.d. of the means obtained from each of the triplicate filters. Casein ('Hammarsten' from Merck, AG, Dormstadt) was used as a chemotactic stimulus at a concentration of 5·0 mg/ml.

To examine the effect of HAGG on the migration of neutrophils, neutrophils were suspended in 10% autologous serum plus HAGG at a final concentration of $2{\cdot}5 \times 10^6$ cells/ml and pre-incubated at 37°C for 30 min prior to transfer of 0·2 ml of the neutrophil suspension containing HAGG to the chemotactic chambers. For determination of the effects of normal sera, zymosan-treated sera, heated serum or sera obtained from patients with rheumatoid arthritis, neutrophils were suspended at a concentration of $2{\cdot}5 \times 10^6$/ml in 10% serum and pre-incubated for 30 min. 0·2 ml of the neutrophil suspension was then transferred to each chemotactic chamber.

In the experiment where suspensions of cells were placed on both sides of the filters, as it was necessary to reduce the volume on the attractant side of the filter, a different sized container for the attractant was used. In this experiment neutrophils ($2{\cdot}5 \times 10^6$/ml) were pre-incubated for 30 min at 37°C in 10% normal or zymosan-treated serum in the presence and absence of HAGG (50 μg/ml). 0·1 ml of neutrophil suspension was placed above the filter and 0·4 ml below the filter on the attractant side in a 2·0 ml autoanalyser sample cup (Technicon, Tarrytown, New York). There was one chemotactic chamber in each autoanalyser cup. Each assay was performed in triplicate.

All assays were incubated for 60 min, unless otherwise stated.

Serum. Autologous serum was obtained by allowing blood to clot in glass tubes at 25°C and used without freezing. Sera from control persons and patients with rheumatoid arthritis were stored at −80°C prior to use.

Zymosan. Zymosan was prepared as described by Lachmann, Hobart & Aston (1973). Sera were treated by incubation with zymosan (10 or 100 mg/ml) at 37°C for 1 hr with occasional shaking.

Alternate complement pathway assay. Alternate complement pathway activity was determined after the method of Platts-Mills & Ishizaka (1974).

Assay for immune complexes by monoclonal rheumatoid factor and anticomplementary activity. Sera from patients were assayed for immune complexes by the quantitation of precipitation with isolated monoclonal rheumatoid factor by means of a laser nephelometer (Roberts-Thomson, unpublished observations). Sera were assayed for the presence of anticomplementary activity after the method of Johnson & Mowbray (1977). A positive result was greater than 30% inhibition of lysis.

Rheumatoid factor. Rheumatoid factor was assayed by the Rose–Waaler test. Sera with titres of > 1:16 were regarded as positive.

TABLE 1. Migration of neutrophils in autologous serum

Percentage serum concentration*	Migration in microns (mean ± s.d.)
0	16 ± 3
1·25	41 ± 3
2·5	44 ± 8
5	59 ± 10
10	67 ± 12
20	112 ± 7

* Serum and neutrophils were placed on top of filter with HBSS below filter.

RESULTS

Migration of neutrophils suspended in autologous serum

Table 1 shows the migration of neutrophils suspended in varying concentrations of autologous serum with HBSS on the attractant side of the filter. The migration of neutrophils is markedly increased by the addition of normal autologous human serum as compared to the migration of cells suspended in HBSS alone.

Effect of heat-aggregated and monomeric immunoglobulin

The presence of HAGG inhibited neutrophil migration (Fig. 1). The inhibition curve was sigmoidal in shape, inhibition being observed from concentrations of 12·5 to 50 μg/ml of HAGG. Increasing HAGG concentrations over 50 μg/ml did not produce increased inhibition. Monomeric IgG could also produce a lesser degree of inhibition at higher concentrations.

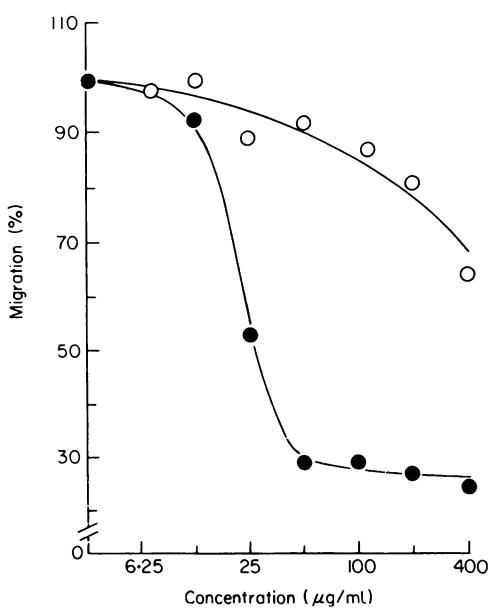

FIG. 1. Effect of aggregated and monomeric immunoglobulin on neutrophil migration. Neutrophils were pre-incubated for 30 min at 37°C in 10% autologous serum plus varying concentrations of heat-aggregated (●) or monomeric (○) immunoglobulin. The suspension was then transferred to chemotactic chambers for the determination of migration with HBSS below the filter. Migration is expressed as a percentage of that obtained in 10% serum alone.

Effect of aggregates of differing molecular size

Aggregated gammaglobulin preparations of three different sizes were obtained following chromatography of HAGG, and were tested for inhibitory activity at final concentrations of 10 and 100 μg/ml. Fig. 2 shows the elution profile of the column and the relative sizes of the fractions tested. The migration is expressed as a percentage of that observed in the absence of HAGG. All three fractions produced a similar degree of inhibition when compared at the same protein concentration.

Requirement for pre-incubation

Experiments were performed to examine the requirement for the pre-incubation of neutrophils and

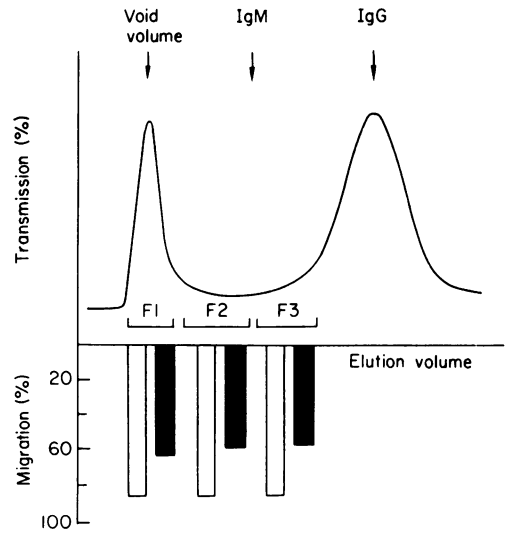

FIG. 2. Effect of aggregates of differing molecular size. HAGG preparations of differing molecular sizes (F_1, F_2 and F_3) were obtained. Migration of neutrophils was determined after 30 min pre-incubation with 10 μg/ml (□) or 100 μg/ml (■) of each fraction in HBSS and 10% autologous serum. Migration is expressed as a percentage of that obtained in 10% serum alone. In all cases, migration was determined with HBSS below filter.

TABLE 2. Effect of pre-incubation

	Percentage migration*	
	Expt. 1	Expt. 2
Pre-incubation at 37°C†	59	55
Pre-incubation at 4°C†	62	59
No pre-incubation‡	85	75
Pre-incubation at 37°C, then wash§	83	93

* Migration of neutrophils exposed to HAGG (50 μg/ml) expressed as a percentage of the migration of cells similarly treated in the absence of HAGG. In each case HBSS was placed below the filter and neutrophils in 10% serum above the filter.

† Pre-incubation for 30 min in presence of 10% serum and 50 μg/ml HAGG.

‡ HAGG added to cells to a final concentration of 50 μg/ml immediately before transfer to the chemotactic chambers.

§ Neutrophils were pre-incubated in 10% serum and 50 μg/ml HAGG and washed twice in HBSS prior to determination of migration in 10% autologous serum.

HAGG, and whether the persistence of HAGG after the pre-incubation period was required (Table 2). Neutrophils in the presence of HAGG were pre-incubated at 37°C or 4°C. HAGG was added to other neutrophil suspensions directly prior to being placed in the chemotactic chamber. Other neutrophil suspensions pre-incubated with HAGG at 37°C were washed twice to remove the HAGG, and migration was then determined in serum-containing medium alone. In each case, the migration observed is expressed as a percentage of the migration of a neutrophil preparation given an identical treatment in the absence of HAGG. Pre-incubation for 30 min at either 37°C or 4°C produced a similar degree of inhibition. However, with no pre-incubation or the removal of HAGG after the period of pre-incubation by washing, lesser degrees of inhibition were observed.

Effect of serum and a chemotactic stimulus

The random migration and chemotactic response of neutrophils to casein were compared in serum-containing and in serum-free media. The results of two separate experiments shown in Table 3 demonstrate that the random migration and the chemotactic response of neutrophils in serum-free medium was not inhibited by the presence of HAGG. Although migration was inhibited in the presence of serum and HAGG, the addition of the chemotactic stimulus casein was able to overcome this inhibitory effect.

TABLE 3. Effect of serum and casein

Medium below filter	Serum	Migration in microns* (mean ± s.d.) Expt. 1 −HAGG	Expt. 1 +HAGG	Expt. 2 −HAGG	Expt. 2 +HAGG
HBSS	−	18±5	20±3	30±9	28±2
Casein	−	64±4	61±10	81±9	75±6
HBSS	+	91±3	41±5	97±9	64±10
Casein	+	84±7	84±5	100±6	92±3

* The cells were pre-incubated in the absence or presence of 10% autologous serum plus or minus HAGG (50 μg/ml) for 30 min at 37°C. Migration was determined with HBSS or casein below the filter.

Effect of complement depletion

Serum was pre-treated with zymosan (10 or 100 mg/ml for 60 min) to deplete the alternate pathway, or heat-inactivated (56°C for 60 min) to deplete the classical pathway, and examined for its ability to support the inhibition of neutrophil migration in the presence of HAGG. The activity of the alternate pathway was reduced from 16 units in serum maintained at 37°C for 1 hr to 0 units in both zymosan-treated preparations (normal range in untreated serum, 20–37 units). The results in Table 4 show that zymosan-treated serum alone substantially inhibited migration in the absence of HAGG; however, it was possible to demonstrate some further inhibition on the addition of HAGG. Migration in heat-inactivated serum was also less than that observed in untreated serum. The addition of HAGG produced further inhibition to a degree similar to that observed in unheated serum plus HAGG.

Removal of chemotactic gradient

If the inhibition observed with HAGG or zymosan-treated serum is due solely to the establishment of a negative chemotactic gradient, the inhibition should be reversed if equal concentrations of zymosan-treated serum or HAGG plus serum are placed on both sides of the filter, thus removing the gradient. The results in Table 5 show that the inhibitory effect of zymosan-treated serum was removed when serum was placed on both sides of the filter. In contrast, the inhibitory effect of HAGG in either normal or zymosan-treated serum was not removed by the abolition of any potential chemotactic gradient.

TABLE 4. Effect of complement depletion

Serum treatment*	Migration in microns† (mean±s.d.) −HAGG	+HAGG
Heat 37°C alone	85±3	29±3
Zymosan 10 mg/ml	37±6	27±6
Zymosan 100 mg/ml	42±7	29±2
Heat 56°C	51±4	36±1

* Autologous serum was absorbed with zymosan at 37°C for 60 min or heated at 56°C for 60 min.

† Migration of neutrophils was determined after pre-incubation in serum treated as described plus or minus HAGG (50 μg/ml) at 37°C for 30 min. HBSS alone was below the filters.

TABLE 5. Removal of the chemotactic gradient in normal and zymosan-treated serum

Medium above filter	Medium below filter	Migration in microns* (mean±s.d.)
Serum	HBSS	56±3
Serum+HAGG	HBSS	24±3
Zymosan serum	HBSS	32±5
Zymosan serum+HAGG	HBSS	21±3
Serum	Serum	91±4
Serum+HAGG	Serum+HAGG	45±7
Zymosan serum	Zymosan serum	88±4
Zymosan serum+HAGG	Zymosan serum+HAGG	44±2

* Neutrophils were pre-incubated in normal or zymosan-treated (10 mg/ml for 60 min) autologous serum plus or minus HAGG (50 μg/ml) for 30 min at 37°C, then the whole suspension was placed above or on both sides of the filter and incubated for 45 min. In this experiment 0·1 ml volumes were above and 0·4 ml volumes below the filter.

Effect of serum from patients with rheumatoid arthritis

The migration of group 0 neutrophils suspended in 10% serum from healthy controls or patients with rheumatoid arthritis was compared with that of the cells suspended in 10% fresh autologous serum. All the patients' sera showed evidence of immune complexes by an assay for anti-complementary activity and the formation of significant precipitation with monoclonal rheumatoid factor (Table 6). The mean migration of neutrophils in twenty-four homologous control sera was 107%±14 (s.d.) giving a normal range of 79–135% (2 s.d.). The migration in all sera obtained from patients with rheumatoid arthritis was reduced. There was no obvious correlation between either rheumatoid factor titres or precipitation with monoclonal rheumatoid factor and the inhibition of neutrophil migration.

DISCUSSION

The experiments described in this paper demonstrate an inhibitory effect of aggregated gammaglobulin on neutrophil motility. The migration of neutrophils *in vitro* is influenced by the chemokinetic activity of serum. It has been demonstrated in the present system that serum placed on the same side of the filter

TABLE 6. Neutrophil migration in serum from patients with rheumatoid arthritis

Patient	Rose–Waaler	Monoclonal rheumatoid factor precipitation (μg/ml HAGG equivalent)	Anti-complementary activity	Percentage neutrophil migration*
1	1:256	114	+	55
2	1:8192	52	+	50
3	>1:16384	68	+	67
4	1:4	9	+	67
5	1:2048	13	+	62
Normal	<1:32	<5	−	>79

* Neutrophil migration in 10% serum from patients expressed as a percentage of migration of cells in fresh autologous serum. The mean percentage migration of neutrophils suspended in 10% control serum was 107% (s.d. 14; $n = 24$).

as the cells can increase the migration of the cells into the filter, i.e. it has a chemokinetic effect. Exposure of neutrophils to HAGG in the presence of autologous serum inhibits their migration; however, this inhibition was not due to the establishment of a negative chemotactic gradient as a result of complement activation (Keller & Sorkin, 1966). Thus, we have demonstrated an inhibition of migration when neutrophils are suspended in zymosan-treated serum which was removed when equal concentrations of zymosan-treated serum were placed on both sides of the filter. We interpret this inhibition to be due to a negative chemotactic gradient established as a result of complement activation by zymosan. In contrast, the inhibition of migration of HAGG-treated neutrophils was not removed by the abolition of any potential gradient.

The present findings suggest that interaction of HAGG and the neutrophil surface is required for the inhibition of migration. The requirement for a period of pre-incubation which can take place at 4°C or 37°C and the reversibility of the inhibition by washing the cells, suggests a reversible surface interaction as a factor in determining inhibition, rather than the phagocytosis of complexes as previously suggested by Mowat & Baum (1971). The results of the present study are also consistent with previous observations that the interaction of aggregated gammaglobulin with human neutrophils can take place at 4°C (Lawrence *et al.*, 1975). The sigmoidal slope of the inhibition curve with maximal inhibition occurring over a restricted range (12·5–50 μg/ml) of HAGG concentration, suggests that maximal inhibition is mediated once a certain level of HAGG–neutrophil interaction is attained. The inhibition curve is very similar to that obtained by MacLennan *et al.* (1973) who studied the inhibition of bacterial phagocytosis by HAGG. The similar effect of HAGG on migration and phagocytosis is in keeping with the concept of Henson (1976) that both these functions are surface phenomena with similar basic mechanisms.

Aggregated gammaglobulin can stimulate the metabolic activity of human neutrophils (Henson & Oades, 1975; Johnston & Lehmeyer, 1976). However, in this study it was clearly demonstrated that the inhibition of migration was not due to a metabolic exhaustion of the cells. After the application of the chemotactic stimulus casein, HAGG-treated cells were shown to migrate as well as untreated cells. This finding also suggests that the inhibition of migration is not by means of either neutrophil immobilising factor (Goetzel & Austen, 1972), or 'deactivation' by immune complexes (Ward & Becker, 1968), as in both these cases neutrophils could not respond to a subsequent chemotactic stimulus after treatment.

Although the presence of serum was required to demonstrate the inhibition of neutrophil migration by HAGG, that inhibition did not depend on the presence of a functioning complement pathway. Two possible explanations for the serum requirement are that serum is necessary for the interaction of HAGG and neutrophils, or that it is the chemokinetic influence of serum that is inhibited by HAGG. There is substantial evidence from other systems that serum is not required for the interaction of human neutrophils and immunoglobulin aggregates. Thus, adherence of aggregated immunoglobulins (Lawrence *et al.*, 1975), release of enzymes (Henson *et al.*, 1972), stimulation of hexose monophosphate shunt activity

(Henson & Oades, 1975) and production of superoxide anion (Johnston & Lehmeyer, 1976) can all occur when neutrophils are mixed with aggregates in serum-free medium. The action of HAGG cannot be a general interference with either motility or the cell's capacity to recognize exogenous stimuli as the response to casein is unimpaired. We suggest that HAGG may have a selective inhibitory effect on the mechanisms involved in the chemokinetic activity of serum.

The inhibitory effect observed provides an attractive mechanism which would contribute to the accumulation of neutrophils that is frequently observed at the site of immune complex deposition *in vivo* (Cochrane, 1968). A chemotactic stimulus is able to attract neutrophils to the site of immune complex deposition and interaction with immune complexes could then reduce the random mobility, thus favouring the accumulation of neutrophils. These neutrophils are not prevented from reacting to another exogenous chemotactic stimulus if presented and thus would retain the capacity to migrate elsewhere in the local environment. It is of interest that sera from patients with rheumatoid arthritis with evidence of circulating immune complexes inhibited the migration of neutrophils *in vitro*, as the accumulation of neutrophils is a feature of inflammation in this disease.

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