

Activation of inflammatory cells by immune complexes containing IgE in serum and synovial fluid of patients with rheumatoid arthritis: a study using flow cytometric analysis

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Abstract

Neutrophil and monocyte activation by immune complexes containing IgE from serum and synovial fluid of patients with rheumatoid arthritis is reported. Activation of the inflammatory cells was measured by stimulation of the respiratory burst with production of intracellular hydrogen peroxide. Generation of hydrogen peroxide was analysed by a flow cytometric method, using the fluorochrome dichlorofluorescein. The technique was modified to allow measurement of cell activation of both neutrophils and monocytes by immune complexes in suspension. Ten of 14 polyethylene glycol precipitates from serum of patients with rheumatoid arthritis and 10/16 synovial fluids of these patients could activate neutrophils. A positive relation was found between the activation of neutrophils and the total concentration of immune complexes, the presence of IgG, and the presence of IgE in the immune complexes. Activation of monocytes was also shown, but to a lesser extent (8/14 rheumatoid serum samples and 8/16 rheumatoid synovial fluids activated monocytes). There was a weak correlation between the concentration of IgE immune complexes and the intensity of fluorescence measured in the monocytes.

In an earlier study we demonstrated *in vitro* neutrophil activation by immune complexes containing IgE. To measure superoxide release in the supernatant immune complexes had to be coupled onto plastic wells. Moreover, activation of monocytes could not be shown.

Here the activation of both neutrophils and monocytes by immune complexes in suspension was studied by flow cytometric analysis. The technique used was based on the method of Bass *et al.*,² measuring the intracellular hydrogen peroxide production through oxidation of added 2',7'-dichlorofluorescein diacetate. The technique was modified to allow measurement of cell activation of both neutrophils and monocytes by immune complexes in suspension.

We used this technique to explore the role of immune complexes containing IgE in serum and synovial fluid of patients with rheumatoid arthritis in the activation of these inflammatory cells.

Patients and methods

PATIENTS AND CONTROLS

Polyethylene glycol (PEG) precipitates of 14 serum samples and of 16 synovial fluids of

patients with classical or definite rheumatoid arthritis were studied. Twenty of these 30 samples were positive for immune complexes containing IgE, 10 were negative. A total immune complex score was calculated; thus immune complexes containing IgG, IgA, and IgM were also determined. The total immune complex score was defined as the sum of the four different isotypes (IgG, IgA, IgM, and IgE) of immune complexes divided by their respective cut off values. Twelve samples had a high immune complex score (>8), 18 were <8. Only three samples were negative for immune complexes containing IgG, IgM, IgA, and IgE. Ten PEG precipitates from normal subjects, all negative for IgE and other isotypes of immune complexes, served as controls. All samples were kept at -20°C until used.

IMMUNE COMPLEX PRECIPITATION AND DETERMINATION

Immune complexes were precipitated from patient serum samples and synovial fluids with PEG 6000, (Merck, Darmstadt, FRG).³ IgE, IgA, and IgM immune complex determinations were made by an enzyme linked immunosorbent assay (ELISA).⁴ Briefly, microtitre plates (Costar, Cambridge, USA) were coated with rabbit antihuman IgE (Dako, Glostrup, Denmark), goat antihuman IgA (Tago, Burlingame, USA), or rabbit antihuman IgM. Polyethylene glycol precipitates diluted 1/1 (IgE) or 1/500 (IgA and IgM) were added to the wells and incubated overnight at 4°C. IgE was detected with biotinylated mouse monoclonal antihuman IgE (Diagnostics Pasteur, Marnes, France) and peroxidase labelled streptavidin (Amersham, Amersham, UK). IgA and IgM were detected with peroxidase conjugated F(ab')₂ goat antihuman IgA and IgM (Tago) respectively. Immune complexes containing IgE were calibrated with an IgE reference preparation (Behringwerke AG), IgA and IgM immune complex assays with N protein standard serum (Behringwerke AG, Marburg, FRG). Immune complexes containing IgG were determined by a radioimmunoassay using iodinated protein A (Amersham).

The 95th centile of the serum values of 75 normal subjects matched for age was taken as cut off value.

CELL FRACTIONATION

Heparinised blood was obtained from a normal donor. After centrifugation a buffy coat was collected and washed in Hanks's balanced salt

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solution without Mg^{2+} , Ca^{2+} , and phenol red (pH 7.4; Gibco, Paisley, UK). Cells were then fractionated on a discontinuous Percoll density gradient (Percoll, Pharmacia LKB, Uppsala, Sweden). Mononuclear and polymorphonuclear cells were washed twice in Hanks's balanced salt solution without Mg^{2+} , Ca^{2+} , and phenol red, and concentrated to 10^7 cells/ml in phosphate buffered saline with 0.1% gelatin and 0.1% glucose (pH 7.4).

CELL ACTIVATION

For the cell activation PEG precipitates were washed in 2.5% polyethylene glycol and concentrated twice in phosphate buffered saline with 0.1% gelatin and 0.1% glucose (pH 7.4). The activation obtained with the PEG precipitates was compared with the activation caused by heat aggregated human IgG³ or formyl-methionyl-leucyl-phenylalanine (fMLP, final concentration 10^{-6} mol/l; Sigma, St Louis, USA).

Cells were incubated with 2',7'-dichloro-fluorescein diacetate, a non-fluorescent compound that easily diffuses into the cells. This diacetate hydrolysed to 2',7'-dichlorofluorescein and afterwards oxidised to the highly fluorescent 2',7'-dichlorofluorescein by intracellular hydrogen peroxide. 2',7'-Dichlorofluorescein is a polar molecule and thus trapped in the cell. It is an indicator of the respiratory burst in stimulated inflammatory cells. Cells (0.1 ml) were incubated with 0.1 ml 2',7'-dichlorofluorescein diacetate (final concentration 0.01 mg/ml in phosphate buffered saline with 0.1% gelatin and 0.1% glucose; Eastman Kodak Company, Rochester, USA) for 15 minutes at 37°C. Thereafter, 0.1 ml of a Ca^{2+} and Mg^{2+} solution (0.1 mg/ml $CaCl_2 \cdot 2H_2O$ and 0.1 mg/ml $MgCl_2 \cdot 6H_2O$ in phosphate buffered saline with 0.1% gelatin and 0.1% glucose and 0.1 ml of the stimulus were added and incubation was prolonged for 30 minutes at 37°C. Reaction was stopped with 1 ml cold phosphate buffered saline with 0.1% gelatin and 0.1% glucose and intracellular 2',7'-dichlorofluorescein fluorescence was measured by flow cytometry (FACScan; Becton Dickinson, Sunnyvale, USA). Cells were gated by forward scatter and side scatter analysis methods, and results were expressed as the ln mean fluorescence per cell by conversion of logarithmic channel numbers into relative linear fluorescence intensity.⁵

STATISTICAL ANALYSIS

Statistical analysis was carried out by non-parametric analysis of variance (Kruskal-Wallis), the Wilcoxon matched pairs signed rank test, the Spearman rank correlation coefficient, and the χ^2 test, where appropriate. A p value <0.05 was considered significant.

Results

NORMAL SERUM SAMPLES

Neutrophils The mean ln fluorescence (SD) of polymorphonuclear cells caused by the 10 control PEG precipitates was 0.98 (0.19). The

mean +2 SD of the 10 control PEG precipitates was taken as the cut off point. All the control values were below this limit.

Monocytes The mean ln fluorescence (SD) was 1.67 (0.09). As for the polymorphonuclear cells, all the control values were below the mean +2 SD.

RHEUMATOID ARTHRITIS SAMPLES

Neutrophils Ten of 14 rheumatoid arthritis PEG precipitates from serum and 10/16 from synovial fluid led to enhanced fluorescence of polymorphonuclear cells (patients *v* controls: $p=0.001$, table). Of the 20 IgE immune complex positive samples (serum and synovial fluid taken together), 14 activated polymorphonuclear cell oxidative metabolism.

Polyethylene glycol precipitates of patients with rheumatoid arthritis added to polymorphonuclear cells labelled with 2',7'-dichlorofluorescein diacetate gave on average 65% of the fluorescence intensity of heat aggregated human IgG, whereas fMLP gave a lower activation (34% of the aggregated IgG value).

Figure 1A shows an example of a strongly stimulatory PEG precipitate of a patient with rheumatoid arthritis in comparison with heat aggregated human IgG and fMLP.

Monocytes Eight of PEG precipitated serum samples and 8/16 synovial fluids of patients with rheumatoid arthritis activated normal donor monocytes, though the activation was less than for polymorphonuclear cells. Ten of 20 IgE immune complex positive rheumatoid arthritis samples caused a significant activation of monocytes, whereas none of the 10 PEG precipitates of normal controls led to enhanced fluorescence of the cells. Immune complexes of patients with rheumatoid arthritis added to 2',7'-dichlorofluorescein diacetate labelled cells gave on average 82% of the intensity of heat aggregated IgG stimulated cells. The monocyte activation

Polymorphonuclear cell activation in relation to diagnosis and immune complexes

	Polymorphonuclear cell activation		p Value*
	Positive	Negative	
Diagnosis			
Controls	0	10	
RA† serum samples	10	4	
RA synovial fluids	10	6	0.001
Total CIC† score:			
>8	11	1	
<8	9	19	<0.002
IgG CIC:			
Positive	17	7	
Negative	3	13	<0.004
IgA CIC:			
Positive	8	4	
Negative	12	16	NS
IgM CIC:			
Positive	10	5	
Negative	10	15	NS
IgE CIC:			
Positive	14	6	
Negative	6	14	<0.05

*Patients *v* controls. Statistical analysis by χ^2 test.
†RA=rheumatoid arthritis; CIC=circulating immune complexes.

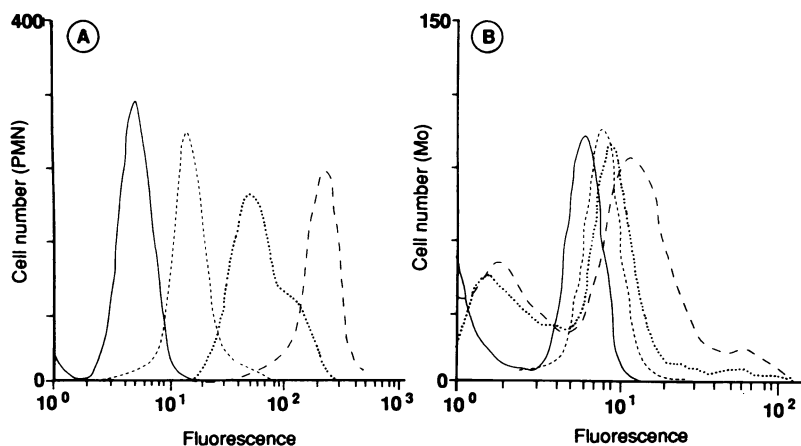


Figure 1 Fluorescence histograms of resting (—) and stimulated (A) polymorphonuclear cells (PMN) and (B) monocytes (Mo) after incubation with 2',7'-dichlorofluorescein diacetate. Cells were stimulated by formyl-methionyl-leucyl-phenylalanine (---), heat aggregated human IgG (.....), polyethylene glycol precipitate (-.-.).

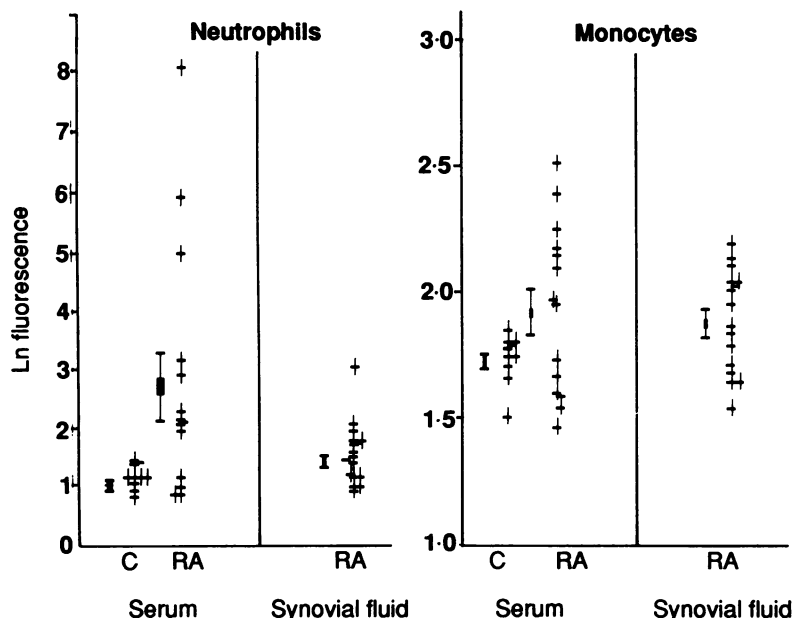


Figure 2 Mean fluorescence (Ln fluorescence) per cell of normal donor neutrophils and monocytes stimulated by polyethylene glycol precipitates from serum samples of patients with rheumatoid arthritis (RA) and from controls (C) or from RA synovial fluids.

induced by fMLP was 80% of the aggregated IgG value. Figure 1B shows an example of a stimulatory PEG precipitate.

COMPARISON OF RHEUMATOID ARTHRITIS SERA WITH SYNOVIAL FLUIDS

Figure 2 shows the results of polymorphonuclear cell and monocyte fluorescence for controls, rheumatoid arthritis serum samples and synovial fluids.

There was no statistically significant difference in stimulatory effect between PEG precipitates from serum and from synovial fluid.

RELATION BETWEEN INFLAMMATORY CELL ACTIVATION AND IMMUNE COMPLEX CONCENTRATIONS

A positive relation was found between the activation of polymorphonuclear cells and the presence of an increased total immune complex

score (table). When the different isotypes were considered a positive relation was found between IgG and IgE in the complexes and the activation of polymorphonuclear cells. For monocytes there was a weak but significant ($r=0.32$, $p=0.03$) correlation between the concentration of IgE immune complexes and the intensity of fluorescence measured.

Discussion

Flow cytometry is a useful technique for studying cells in suspensions; its sensitivity makes it possible to calculate cell per cell fluorescence. It is possible to apply this principle for the measurement of intracellular hydrogen peroxide produced in the respiratory burst: added dichlorofluorescein diacetate can readily be oxidised by intracellular hydrogen peroxide to highly fluorescent dichlorofluorescein. In this way activation of neutrophils can be studied.²⁻⁶

By this technique we have shown that immune complexes containing IgE from serum samples and synovial fluids of patients with rheumatoid arthritis can stimulate oxidative metabolism of polymorphonuclear cells. This makes the test system used more sensitive than assays formerly described which used the extracellular reduction of cytochrome *c*,⁷ and in which immune complexes had to be coupled onto plastic wells to release a measurable amount of superoxide.¹ In this study we found a positive relation between the activation of polymorphonuclear cells and complexes in suspension containing IgG and IgE.

It is well known that monocytes produce less hydrogen peroxide in the respiratory burst than neutrophils.⁸ Although less obvious, activation of monocytes could also be shown in this study. The technique used made it possible to study activation of monocytes by immune complexes without prior enriching procedures and thus without the danger of activation of cells by these manipulative procedures.

It has been shown earlier that immune complexes containing IgE are more prevalent in patients with severe arthritis and in patients with vasculitis,⁴⁻¹¹ and that these complexes can activate polymorphonuclear cells¹ and basophils.¹² This study provided further evidence for the inflammatory role of IgE immune complexes in rheumatoid arthritis, showing that mononuclear cells could also be activated by these complexes. This might be expected since it has been shown that some monocytes and macrophages possess IgE-type II receptors with a high affinity for aggregated IgE.¹³ Moreover, we found a weak but significant correlation between the concentration of circulating immune complexes containing IgE and the intensity of intracellular fluorescence measured in the monocytes.

In summary, the results of this study confirm our previous experiments showing the activation of neutrophils by IgE immune complexes fixed to plastic wells, and extend these findings to immune complexes in suspension—that is, serum and synovial fluid. Furthermore, the technique used allowed us to show activation of monocyte

respiratory burst by immune complexes containing IgE of patients with rheumatoid arthritis, which could not be shown by the former technique.

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