IN VIVO FIXATION OF IMMUNE COMPLEXES ON POLYMORPHONUCLEAR CELLS AND RELEASE OF NEUTROPHIL CATIONIC PROTEINS IN SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

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Summary

Neutrophils (PMN) appear to be involved in inflammatory phenomena as a result of direct interaction with immune complexes (IC).

In SLE glomerulonephritis IC are fixed in vivo on the PMN surface through the receptors for FC fragments of complexed immunoglobulins and complement. Phagocytic properties are lost and immunological lysosomal release in vitro is markedly reduced by virtue of receptor occupation.

The elimination of neutrophil cationic proteins (NCP) in urine is an expression of PMN lysosomal constituent release.

Introduction

Polymorphonuclear neutrophils (PMN) have been implicated in the mediation of tissue damage. Neutrophil lysosomes contain proteolytic enzymes and cationic proteins (NCP) which are known to increase vascular permeability and trigger the degranulation of mastocytes.

The release of lysosomal constituents has been demonstrated to occur during phagocytosis, and NCP have been obtained by either breakage of the cells or stimulation of PMN with several agents, including immune complexes (IC).

It has been shown in vitro that PMN have receptors for and are stimulated by the FC fragment of IC formed by IgG and IgA and by C3b.

On the one hand, we showed in vitro that in the presence of IC with or without complement PMN excrete not only lysosomal enzymes but also NCP.

On the other hand we demonstrated that NCP are capable of degranulating human basophils and mastocytes, resulting in histamine and platelet-activating factor (PAF) release.

PAF is a newly discovered mediator of anaphylaxis released by circulating basophils and tissue fragments by immunological mechanisms, that induces platelet aggregation and release of their permeabilising and phlogistic content.

IC and complement are able to initiate a cascade of interactions between
PMN, basophils/mastocytes and platelets.

The result of these cellular interactions, mediated by NCP release, is enhanced vascular permeability, causing IC trapping along the filtering basement membrane.

In this article we will show that in vivo PMN are involved in immune injuries through their surface receptor for IC in systemic lupus erythematosus (SLE).

Materials and Methods

Procedures for the Preparation and Purification of PMN

Human blood was drawn into 10 ml plastic tubes containing 5 x 10^{-3} M (final concentration) ethylene-diamine-tetra-acetate (EDTA) pH 7.2.

The blood was centrifuged for 20 min at 700 g; platelet-rich plasma and buffy-coat cells were removed and the neutrophil-rich layer collected and resuspended in a 1/2 proportion with 2.5% gelatin in tris-buffered physiological saline. Following erythrocyte sedimentation, the gelatin layer was removed, centrifuged at 770 g for 20 min and washed twice with Tris-buffered Tyrode without calcium and magnesium (TT no Ca^{++}, no Mg^{++}) containing 0.25% BSA and then resuspended in the same buffer with calcium and magnesium ions.

PMN preparations were between 95 and 99% pure.

Detection of Immunoglobulins and C3 Complement Fraction on PMN Surface

Preparations of patient PMN were incubated with anti-human IgG, IgA, IgM, C3 fluoresceinasted serum (Beringwerke) in tris-buffered Tyrode (no Ca^{++}, no Mg^{++}) (pH 7.4) containing 0.25% BSA, and colchicine 1 µg/mL as an inhibitor of phagocytosis, for 20 min at 20°C. PMN washed three times and stained with ethidium bromide (sigma) 20 µg/mL (final concentration) were examined by immunofluorescence (IF) and the result expressed as percentage of fluorescent PMN.

The nucleoprotein of PMN gives a red fluorescence in the presence of ethidium bromide allowing the total number of PMN to be counted; Ig and C3 were detectable as a green positivity on the cell-membrane.

Purification of Neutrophil Cationic Proteins (NCP) to Obtain Antisera

Human PMN (1 x 10^8) in TT BSA were sonicated for 5 min and cationic proteins were extracted by adding 5 volumes of 0.2 M pH 4 acetate buffer at 4°C and stirring continuously at 4°C for 3 hr.

After centrifugation at 40,000 g for 30 min, the supernatant was dialysed for 48 hr against 0.01 M phosphate buffer pH 8.5, then chromatographed on a 1.5 x 30 cm DEAE-Sephadex column equilibrated with the same buffer. The free eluate from the column was collected and concentrated to approximately 5 mL with an Amicon ultrafiltrating unit using an UM2 membrane.

Molecular sieve filtration was performed on a 2.6 x 100 cm Sephadex G100 column equilibrated with 0.1 M acetic acid-sodium acetate buffer, pH 3.7 (flow-rate 12 mL/hr and 3 mL fraction).
The molecular weight of NCP was estimated using $^{125}$I BSA, peroxidase, cytochrome C, and $^{125}$I glucagon as reference substances.

Anti-NCP serum was obtained in New Zealand White rabbits as described by Kabat and Mayer.  

**Preparation of Soluble Antigen-antibody Complexes**

BSA-anti-BSA soluble IC were prepared as follows: the precipitate obtained at equivalence was washed three times with saline at 4°C, the pH was brought to 2.8 with HCl and either Ab or Ag was added twenty times in excess of that used for precipitation at equivalence. The pH was then adjusted to 7.4.

**Enzyme Determinations**

Lactate dehydrogenase was assayed with reduced nicotinamide adenine dinucleotide; acid and alkaline phosphatase according to Kind and King and β galactosidase according to Brittinger et al. All protein determinations were done by the Folin-Lowry method.

**Immunologic Release of NCP**

Purified human neutrophils, usually 5 x 10⁶ in 1 mL were incubated at 37°C for 30 min with IC in twenty-times Ag excess (20 μg Ab P – 76 μg PBSA/mL) or in twenty-times Ab excess (20 μg Ab P – 0.2 μg PBSA/mL) with or without 10% of fresh serum or with complement-activated baker’s yeast spore to generate NCP.

After incubation the cell-free supernatant was obtained by centrifugation and tested for the presence of enzymes and NCP. Total amount of NCP were obtained by sonication of 2.5 x 10⁶ neutrophils per mL.

**NCP Assay**

The presence of NCP was assessed in rabbits as follows: 0.1 mL of a twofold serial dilution of supernatants were injected intradermally following the intravenous injection of 1.5 to 2 mL of 2.5% Evans blue in saline. Results were expressed as the highest dilution that gave a blueing reaction at least 5mm in diameter.

We also tested NCP action by degranulation of, and histamine release from, rat peritoneal mastocytes, or by the Ouchterlony technique, using an anti-NCP serum. NCP were also tested in concentrated (Amicon UM₂) urine, after DEAE sephadex chromatography as previously described.

**Evaluation of Phagocytosis**

Uptake by PMN of particles a x 10⁷/mL of baker’s yeast was quantified by light microscopy. Cells which took up one or more particles were considered phagocytic. PMN were stained by 30% alcoholic Toluidine blue solution pH 3.5.
TABLE I. Receptors for IC and Enzyme Release from Normal Human PMN Exposed to IC

<table>
<thead>
<tr>
<th>IC from rabbit*</th>
<th>% IF positivity for rabbit IgG</th>
<th>% phagocytosis inhibition †</th>
<th>% cells with eosin uptake</th>
<th>Percent enzyme activity released into supernatant**</th>
<th>Acid phosphatase</th>
<th>β glucuronidase</th>
<th>Alkaline phosphatase</th>
<th>LDH</th>
<th>NCP ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag Ex</td>
<td>10</td>
<td>5</td>
<td>1.0</td>
<td>0.5%</td>
<td>2.2</td>
<td>1.5</td>
<td>1.2</td>
<td>1/10</td>
<td></td>
</tr>
<tr>
<td>Ag Ex + s</td>
<td>32</td>
<td>42</td>
<td>1.2</td>
<td>10.0%</td>
<td>14.6</td>
<td>2.8</td>
<td>1.8</td>
<td>1/10</td>
<td></td>
</tr>
<tr>
<td>Ab Ex</td>
<td>28</td>
<td>18</td>
<td>1.4</td>
<td>6.2%</td>
<td>6.4</td>
<td>1.0</td>
<td>2.4</td>
<td>1/20</td>
<td></td>
</tr>
<tr>
<td>Ab Ex + s</td>
<td>88</td>
<td>68</td>
<td>2.0</td>
<td>28.0%</td>
<td>19.0</td>
<td>12.8</td>
<td>6.0</td>
<td>1/50</td>
<td></td>
</tr>
</tbody>
</table>

* IC in amount of Ag and Ab described in Materials and Methods were incubated at 37°C for 30 min with 2.5 x 10⁶, 99% pure human PMN.
† phagocytosis was evaluated after preincubation of PMN with IC as % inhibition of particle uptake, Normal uptake 89.9 ± 12.0.
‡ 100% activity was determined by sonication of cells (2.5 x 10⁶); acid phosphatase 45.10 ± 12.5 IU, alkaline phosphatase 38.7 ± 8.3 IU, β glucuronidase 14 ± 0.8 µg phenolphthalein/hr, Lactate dehydrogenase (LDH) 948 ± 52 AU
‡ NCP were determined by subcutaneous tests as described in Materials and Methods.

TABLE II. In Vivo Fixation of Ig and C₃, Phagocytosis and In Vitro Immunological Enzyme Release from SLE PMN

<table>
<thead>
<tr>
<th>Condition</th>
<th>No. of determinations</th>
<th>% IF positivity for†</th>
<th>% phagocytosis</th>
<th>NCP*</th>
<th>Percent enzyme activity**</th>
<th>Acid phosphatase</th>
<th>Alkaline phosphatase</th>
<th>LDH</th>
<th>Urinary NCP * excretion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>12</td>
<td>9±5.8</td>
<td>3.5±3.4</td>
<td>0.6±12</td>
<td>0.9±1.3</td>
<td>89.5±11.8</td>
<td>1/50</td>
<td></td>
<td>29.4±3.5</td>
</tr>
<tr>
<td>Acute SLE GN</td>
<td>12</td>
<td>70±26.6</td>
<td>14.5±9.7</td>
<td>27±20.3</td>
<td>38.7±30.1</td>
<td>7±8.5</td>
<td>1</td>
<td></td>
<td>3.5±1.2</td>
</tr>
<tr>
<td>Chron. SLE GN</td>
<td>21</td>
<td>30.5±13.5</td>
<td>10.9±3.5</td>
<td>19.8±72</td>
<td>4.8±4.7</td>
<td>51.7±16.7</td>
<td>1/20</td>
<td></td>
<td>5.9±3.2</td>
</tr>
<tr>
<td>SLE with mixed IgM- IgG cryoglobulins</td>
<td>6</td>
<td>40.5±20.3</td>
<td>11±7.3</td>
<td>38±18.5</td>
<td>7±6.1</td>
<td>8.3±2.0</td>
<td>ND†</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>SLE with positive rheumatoid factor</td>
<td>3</td>
<td>24±15.7</td>
<td>9.6±4.7</td>
<td>21±7.7</td>
<td>7±6.0</td>
<td>34±24</td>
<td>ND</td>
<td></td>
<td>ND</td>
</tr>
</tbody>
</table>

† PMN percentage with in vivo fixation of Ig and C₃ on their surface.
‡ NCP were determined as in Materials and Methods
** Enzyme release was obtained after exposure of PMN to IC in AB excess in presence of 10% of fresh serum. 100% activity was determined by sonication of cells (2.5 x 10⁶). Values of normal subjects as in Table I; values of SLE, acid phosphatase 32.1±14.3IU, alkaline phosphatase 30±6.8 IU. Lactate dehydrogenase 998±48 AU.
† ND = not done
Viability of Cells
The viability of PMN was assayed by exclusion of Y eosin and by LDH determination.

Results
In Vitro Effects of IC on Human Leucocytes
The in vitro effect of soluble IC in Ag or Ab excess, with or without serum was investigated by adding IC to purified neutrophils (Table I).

IC was detectable on the surface of PMN by immunofluorescent (IF) technique and triggered release of enzymes and cationic proteins. The pre-incubation of PMN with IC induced an inhibition of complement-activated baker’s yeast spore phagocytosis. These phenomena were enhanced in the presence of normal human serum.

Figure 1. Patient with SLE diffuse proliferative glomerulonephritis. Creatinine clearance ml/min = ——; Proteinuria g/24 hr = ——; PMN immunoglobulins % = • • • • • • • ; Phagocytosis % = ○ ○ ○ ○ ○; PEG OD A280 = ○ ○ ; Leucocytes/mm³ = * * * ; Anti-DNA Ab = ○ ○ ○ ; Platelets 10⁴ mm³ = ▲ ▲ ▲.
In Vivo Fixation of IC on PMN Surface in Systemic Lupus Erythematosus (SLE)

As shown in Table II immunoglobulins and C3 complement fraction were detected on the PMN surface in SLE and the amount was related to the clinical data (Figure 1) and the levels of circulating IC detected by the polyethylene-glycol method (PEG).

Urinary elimination of NCP was detectable in the acute phase of SLE (Table II). The percentage of PMN with Ig on the surface appeared to be correlated with in vitro inhibition of phagocytosis (Figure 2).

![Graph showing correlation between Ig and phagocytosis inhibition]

Figure 2. Correlation between immunoglobulins (Ig) in vivo fixed on PMN surface, and phagocytosis inhibition. Normal subjects ○ – ○; acute SLE △ – △; chronic SLE * – *; SLE with mixed cryoglobulins • – •; SLE with positive rheumatoid factor • – •

The immunological release of the PMN lysosomal constituents was reduced when the receptors for IC were involved in vivo (Table II).
Discussion

It has been demonstrated that PMN have receptors for and are stimulated by the FC fragment of IC (or aggregates) formed by IgG, IgA, IgD and by the C₃b fraction of complement.

Receptors for IgM-FC are not present on these cells and IgM-IC act on the PMN surface only through C₃b. Stimulated PMN release their lysosomal constituents, a process instrumental in the onset of inflammation.

Our in vitro results clearly indicate that IC and complement are capable of initiating an interaction between PMN, basophils/mastocytes and platelets. IC and complement act upon neutrophils triggering first adherence then phagocytosis and release of granule constituents.

PMN excrete not only lysosomal enzymes but also NCP. This effect was enhanced when PMN were incubated with IC in Ab excess in the presence of serum. On the other hand NCP are capable of degranulating human basophils and mastocytes resulting in the release of very potent mediators into the circulation and in the tissues. Platelets are involved through NCP induced PAF release.

The end result of these cellular interactions is increased vascular permeability.

In this article our results indicate by immunofluorescent technique, that the immune complexes can be detected on the cellular surface after incubation with PMN, and that the pre-incubation of IC-PMN induces an inhibition of the phagocytic capacity of PMN.

In SLE PMN show an in vivo fixation of IC on their surface correlated with the levels of detectable circulating IC and the activity of the disease. NCP present in the urine in the acute phase of SLE glomerulonephritis are an expression of PMN-lysosomal constituent release.

In vitro, PMN of these subjects show phagocytosis inhibition and reduction of immunological lysosomal enzyme release. Immunosuppressive therapy greatly reduces the amount of Ig on the PMN surface.

In conclusion the results of our studies suggest that PMN become involved in inflammatory phenomena as a result of a direct interaction with IC.

The NCP release from PMN may activate the cascade cell-to-cell interactions that we described involving basophils, mastocytes and platelets resulting in enhanced vascular permeability and perpetuating the deposition in, and the passage through, vessel walls of IC.

References


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Open Discussion

D'AMICO (Milan) What do you think is the mechanism of action of cationic proteins on basophils and mastocytes?

CAMUSSI Neutrophil cationic proteins act on the basophils and mastocytes in a manner comparable with that of C5a anaphylotoxins. In our experiment release of a PAF and histamine from basophils by cationic proteins and the C5a anaphylotoxin was higher at 22° than at 37°. That is an active process which was abolished in the absence of calcium ions and magnesium ions. Both anaphylotoxin and neutrophil cationic proteins are inhibited by carboxypeptidase B and we have some evidence for competition between neutrophil cationic proteins and C5a for the same cellular receptors. The time for the release is very short. The start of release is within 20 seconds and the release is complete in one minute.

SCHUTTERLE (Chairman, Giessen) Did you estimate the lysosomal enzymes of leucocytes?

CAMUSSI Beta glucuronidase, acid phosphatase and alkaline phosphatase and the lactic dehydrogenase. We do not find release of lactic dehydrogenase in the polymorphonuclear cell of normal and SLE glomerulonephritis patients. We have a good release of acid phosphatase and beta glucuronidase that are contained in the primary granules and a poor release of alkaline phosphatase contained in the secondary granules. Neutrophil cationic proteins are probably contained in primary granules.