A Role of Complement Receptors on Polymorphonuclear Leukocytes in the Adherence to Immune Deposit

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YAMAMOTO, T., HARA, M., YAMAMOTO, K. and KIHARA, I. A Role of Complement Receptors on Polymorphonuclear Leukocytes in the Adherence to Immune Deposit. Tohoku J. exp. Med., 1984, 143(2), 149–159 — Polymorphonuclear leukocytes (PMNs) adhered tightly to glomeruli with immune complex in vitro in the cryostat sections of nephritic kidneys. The sections were incubated with PMNs for 40 min at 37°C. The kidneys were obtained from rats with experimental glomerulonephritis induced by the prolonged administration of bovine serum albumin. This PMN adherence occurred when PMNs were suspended in fresh rat serum (one-step method) or the sections were treated with the fresh serum prior to the incubation with PMNs (two-step method). However, this adherence was inhibited by treatment of PMNs with trypsin to destroy their complement receptors. The inhibition was concomitant with the decrease in the percentage of rosette-formation by complement-coated zymosan particles. In addition, the adherence was markedly suppressed in both methods using decomplemented serum with zymosan. The aggregated rabbit gammaglobulin opsonized with fresh serum also inhibited the binding of PMNs to the glomeruli by the occupation of the complement receptors on PMNs. These findings indicated the important role of complement components on the glomeruli with immune deposits and complement receptors on PMNs in the PMN adherence. ——— PMNs; complement; receptor; immune complex; glomerulonephritis

The infiltration of polymorphonuclear leukocytes (PMNs) to the injured sites is one of the early cellular events seen in immunologically induced acute inflammation. It has been appreciated that PMNs play an important role in the mediation of acute inflammation (Cochrane 1968; Morita et al. 1971). Some types of glomerulonephritis in both humans and animals have been considered as immune complex mediated diseases (Germuth 1953; Dixon et al. 1958, 1961; Germuth and Rodriguez 1973). Immune complexes deposited in tissues including glomeruli were considered to have many biological activities on induction or promotion of inflammatory processes, for example, activation of the complement system (Burkholder 1961; Cochrane et al. 1965) or coagulation system (Vassalli and McCluskey 1964; Naish et al. 1972). To evaluate one of the biological

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activities of the immune complexes deposited in glomeruli, a PMN binding assay was devised (Yamamoto et al. 1979, 1980). In brief, PMNs adhered tightly to glomeruli with immune complex deposition in vitro and the number of PMNs was thought to reflect a biological activity of the deposits. The adherence was possibly mediated through the activation of complement components by the deposits, as PMNs hardly adhered to glomeruli with the deposits when they were suspended in decomplemented serum.

The present study was intended to disclose the role of complement receptors on PMNs in the PMN binding. PMNs were either treated with trypsin or they were incubated beforehand in a fluid containing aggregated gamma-globulin with or without fresh serum. In some experiments, fresh serum was treated with zymosan to consume complement.

**MATERIALS AND METHODS**

**Kidney sections**

Immune complex nephritis was induced, as previously described, in rats by bovine serum albumin (Yamamoto et al. 1978). Cryostat sections with 2 μm thickness were cut with a minotome (DAMON/IEC, USA) by setting the scale to 1, washed with 0.01 M phosphate buffered saline (PBS), pH 7.2 and stored in −20°C freezer until use.

**PMNs**

Approximately 20 ml of 0.1% glycogen in saline was injected into the peritoneal cavity of the rats. 4 hr later, the exudate was collected and centrifuged at 1000 rpm for 10 min (Cohn and Morse 1959). The cell pellet was suspended in 0.01 M Veronal buffered saline, pH 7.6 containing 0.15 mM CaCl₂ and 0.5 mM MgCl₂ (VBS++) or treated with various concentrations of trypsin.

**Trypsinization of PMNs**

Trypsin, purchased from Difco Laboratories (Detroit, USA), was dissolved in saline containing 0.01 M NaHCO₃ and adjusted to pH 7.6 with 1 N HCl. One ml of trypsin solution at the concentration of 1%, 0.4% and 0.2% was added to each of ml of PMN suspension (2 x 10⁶/ml) to yield final trypsin concentrations of 0.5%, 0.2% and 0.1%, respectively and incubated for 30, 60 or 120 min at 37°C. As a control, 1 ml of saline was added to the PMN suspension to incubate at 37°C. Then, the reaction was stopped by adding 0.5 ml of normal rat sera and cooling in a cold bath. After centrifugation at 1000 rpm for 10 min, PMNs were resuspended in 1/10 diluted rat sera with VBS++ or serum-free VBS++ to a final cell density of 2 x 10⁶/ml.

**PMN binding assay**

The one-step and two-step methods for PMN binding are summarized in Fig. 1. In brief, PMNs were suspended in diluted fresh serum, zymosan-treated, or warmed serum in the one-step method at a cell density of 2 x 10⁶/ml. In the two-step method, the cryostat sections were incubated beforehand with these sera at 37°C for 60 min and washed in cold PBS with 3 changes of bath. Then, the sections were incubated for 40 min at 37°C with PMN suspension (2 x 10⁶/ml of VBS++). In both methods, an empty glass slide was placed over the one bearing 6 sections. Two strips of electric insulating tape were used to hold the 2 slides together, leaving a space 0.2 mm apart. PMN suspension was filled into the space. The number of PMNs adhering to each of the 40–60 glomeruli could be counted easily under a light microscope. The average number( ± 1 s.d.) was used in figures and tables.
Treatment of fresh rat serum with zymosan

Zymosan A (Sigma Chemical Co., USA) was suspended in saline at a concentration of 2 mg/ml, boiled for 30 min and centrifuged to discard saline. Fresh rat serum was added to the zymosan pellet, yielding 2 mg/ml of zymosan concentration, and incubated for 30 or 60 min at 37°C. As a control, fresh serum was incubated at 37°C for the same periods (warmed serum).

Rosette formation by zymosan coated with complement

Zymosan-rosette of PMNs was performed by the modified method of Kajdacsy-Balla and Mendes (1976). Opsonized zymosan obtained by the incubation with fresh serum at 37°C for 60 min, was suspended at a density of $1 \times 10^6$/ml in VBS++. 0.5 ml of the zymosan suspension were mixed with 0.5 ml of PMN suspension (5 x 10^6/ml in VBS++) with gentle shaking for 60 min at 37°C. After centrifugation at 1000 rpm for 10 min, one drop of methylene blue (0.3% in VBS++) was added to aid visualization, and the cell pellet was gently resuspended with a Pasteur pipette. Two hundred PMNs were counted and rosettes were defined as the cells with three or more adherent zymosan particles. The viability of PMNs was examined by a dye exclusion test using trypan blue.

Aggregated rabbit gammaglobulin (ARG)

Rabbit gammaglobulin fraction was prepared from pooled normal rabbit sera by salting-out with 50% and 33% ammonium sulfate and dissolved in VBS++. The concentra-
Lion was adjusted spectrophotometrically at the wave length of 280 nm to 20 mg/ml in VBS++. The solution (normal rabbit gammaglobulin, NRG) was heated for 15 min at 63°C to prepare ARG, as described elsewhere (Ishizaka et al. 1961).

Fresh rat sera were serially diluted with ARG solution or VBS++ to suspend PMNs at the density of $2 \times 10^7$/ml for the one-step method of PMN binding assay. For the two-step method, PMNs were first preincubated with these serially diluted sera containing ARG, washed 3 times with VBS++, and then suspended in the same buffer at the density of $2 \times 10^7$/ml to react with serum-treated sections. PMNs were also suspended in 2-fold dilutions of ARG with VBS++ in the absence of serum at the density of $2 \times 10^7$/ml, and incubated with sections pretreated with fresh serum (two-step method). To evaluate the effect of temperature of incubation periods in the interaction between PMNs and ARG, PMNs were preincubated with ARG or NRG solution of 20 mg/ml for 30 or 120 min at 37°C or 4°C, washed three times with the buffer and resuspended in 1/2 dilution of serum at the cell density of $2 \times 10^7$/ml to examine by the one-step method. For the two-step method, these preincubated PMNs were resuspended in VBS++.

**RESULTS**

PMN binding assay of the one-step method was performed with PMNs, pretreated with trypsin at varying concentrations. The number of PMNs adhering to a glomerulus with immune deposits decreased significantly by the use of trypsin on PMNs (Table 1). Diminishing numbers, however, was not observed with the increase in concentration of trypsin. PMNs were not killed by the treatment with 0.5% trypsin, as far as examined by a dye exclusion test.

PMNs treated with 0.1% trypsin for 30 min at 37°C formed zymosan-rosettes with less percentage than non-trypsinized warmed PMN. The number of rosette-forming PMNs seemed to decrease with the prolongation of the trypsinization periods, however, not to zero (Table 2). Warmed PMNs did not lose their ability to form rosette with opsonized zymosan particles. PMN binding assay of the one-step method was done with PMNs immediately after trypsinization. PMNs warmed for varying periods showed no significant decrease in the number of PMNs adhering to a nephritic glomerulus. On the other hand, the number of trypsinized

<table>
<thead>
<tr>
<th>Trypsin</th>
<th>Number of PMNs adhering to a glomerulus</th>
<th>(% suppression)</th>
<th>Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5%</td>
<td>45.6±6.0*</td>
<td>(38.0%)</td>
<td>98.0%</td>
</tr>
<tr>
<td>0.2</td>
<td>46.6±5.8*</td>
<td>(36.3 )</td>
<td>99.5</td>
</tr>
<tr>
<td>0.1</td>
<td>42.2±8.3*</td>
<td>(42.6 )</td>
<td>100.0</td>
</tr>
<tr>
<td>(—)</td>
<td>73.5±8.7</td>
<td>—</td>
<td>100.0</td>
</tr>
</tbody>
</table>

PMNs ($2 \times 10^7$/ml) were incubated with trypsin at various final concentrations for 30 min at 37°C and resuspended in a 1/10 dilution of fresh rat serum to react with cryostat sections of nephritic kidney (one-step method).

* statistically significant (Student's test, p<0.001) in comparison to non-treated PMNs.
PMN Binding to Immune Deposit

**Table 2. Zymosan-rosette and adherence of trypsinized PMNs**

<table>
<thead>
<tr>
<th>Trypsin (0.1%)</th>
<th>Digestion period</th>
<th>Zymosan-rosette (%)</th>
<th>PMN binding assay (one-step method)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>30 min</td>
<td>30.5</td>
<td>59.1 ± 5.6</td>
</tr>
<tr>
<td>-</td>
<td>30</td>
<td>68.0</td>
<td>56.5 ± 5.0</td>
</tr>
<tr>
<td>+</td>
<td>60</td>
<td>24.5</td>
<td>30.2 ± 3.3*</td>
</tr>
<tr>
<td>-</td>
<td>60</td>
<td>73.5</td>
<td>65.8 ± 4.8</td>
</tr>
<tr>
<td>+</td>
<td>120</td>
<td>22.5</td>
<td>25.0 ± 3.5*</td>
</tr>
<tr>
<td>-</td>
<td>120</td>
<td>66.5</td>
<td>67.0 ± 4.8</td>
</tr>
</tbody>
</table>

PMNs treated with trypsin (0.1%) for varying periods were examined by the one-step method of PMN binding assay, or were mixed with serum-treated zymosan particles (1 × 10⁶/ml) of equal volume to the PMN suspension (2 × 10⁶/ml) and incubated for 60 min at 37°C to count rosette-forming PMNs by zymosan particles.

* Statistically significant (P < 0.001) in comparison to corresponding control of non-trypsinized PMNs (next line).

**Table 3. PMN binding assay with zymosan-treated sera**

<table>
<thead>
<tr>
<th>Sera treated with</th>
<th>One-step method</th>
<th>Two-step method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zymosan for 30 min</td>
<td>39.7 ± 5.7*</td>
<td>27.8 ± 3.9*</td>
</tr>
<tr>
<td>Zymosan for 60 min</td>
<td>28.4 ± 3.4*</td>
<td>26.1 ± 4.0*</td>
</tr>
<tr>
<td>Warmed for 30 min</td>
<td>80.3 ± 5.1</td>
<td>58.7 ± 8.6</td>
</tr>
<tr>
<td>Warmed for 60 min</td>
<td>77.6 ± 10.1</td>
<td>56.7 ± 9.6</td>
</tr>
<tr>
<td>Fresh rat serum</td>
<td>77.3 ± 8.5</td>
<td>62.2 ± 10.5</td>
</tr>
<tr>
<td>Serum-free VBS*</td>
<td>22.1 ± 2.1</td>
<td>20.0 ± 1.7</td>
</tr>
</tbody>
</table>

Zymosan was added to fresh rat serum at a concentration of 2 mg/ml, and incubated for 30 or 60 min at 37°C. As a control, an aliquot of fresh serum was incubated in the same bath without zymosan (warmed). These sera were used to suspend PMNs (one-step method) or to preincubate cryostat sections prior to the incubation of PMNs suspended in VBS* (two-step method).

* Statistical suppression (p < 0.001) in comparison to a control with fresh rat serum.

PMNs adhering to glomeruli decreased with the increase of the trypsinization period. When comparing results of the percentage of rosette-forming PMNs to the number of PMNs binding on a glomerulus, there was an apparent distinction on PMNs trypsinized for 30 min: although the percentage of zymosan-rosette forming PMNs decreased fairly, PMN binding was not affected at all.

The effect of zymosan on fresh serum was examined in PMN binding assay of the one- or two-step methods (Table 3). Zymosan reduced to a half or one third the activity of fresh serum to augment the number of PMN binding to a nephritic glomerulus; however, warmed serum had the same activity as fresh serum. In the two-step method, PMN binding also decreased by about a half when fresh serum was treated with zymosan. There was no significant decrease in the
activity whether fresh serum was treated with zymosan for 30 or 60 min. In either method using sera treated with zymosan for 60 min, the number of PMNs bound to a glomerulus neared to that obtained by the assay with serum-free medium but did not reach the same count.

PMN binding assay was performed by the one-step method with two-fold dilutions of sera treated with zymosan or warmed at 37°C for 60 min. The results are depicted in Fig. 2. The number of PMNs adhering to a glomerulus with glomerulonephritis tended to increase with dilution of warmed serum, reached a peak at 1/4 dilution, and then decreased. On the other hand, PMN binding assay with zymosan-treated serum revealed a gradual decrease in the PMN binding activity with dilution of the sera but kept a slightly higher level than with serum-free VBS++.

Adherence of PMNs suspended in ARG-containing serum to glomeruli was inhibited by the increase in concentration of ARG, and by decrease in serum concentration (Table 4). Although the number of PMNs bound to a glomerulus markedly diminished with the dilution of serum, a marked inhibition was achieved by addition of ARG to the PMN suspension of the one-step method. By the pretreatment of PMNs with ARG in the presence of serum, it was also apparent that PMN adherence in the two-step method was impaired.

Cryostat sections preincubated with fresh serum were covered with PMNs suspended in 2-fold dilutions of ARG solution with the buffer in the absence of serum (Fig. 3). This showed a gradual increase in the number of PMNs bound to nephritic glomeruli with a decrease in ARG concentration. At a 1/16 dilution, the number to approximately the same level as the VBS++ control.

Both methods investigated whether the reduction of the adhesive faculty by ARG depends upon the temperature or the incubation time of PMNs with ARG (Table 5). The binding was significantly impaired when the leukocytes were
Table 4. Effect of addition of ARG in fresh serum on PMN binding

<table>
<thead>
<tr>
<th>Serum dilution</th>
<th>One-step method</th>
<th>Tow-step method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ARG</td>
<td>VBS⁺</td>
</tr>
<tr>
<td>1 : 2</td>
<td>27.7±4.9*</td>
<td>83.5±9.1</td>
</tr>
<tr>
<td>1 : 4</td>
<td>11.2±2.1*</td>
<td>84.8±5.6</td>
</tr>
<tr>
<td>1 : 8</td>
<td>2.9±1.8*</td>
<td>68.8±7.1</td>
</tr>
</tbody>
</table>

PRNs (2×10⁷/ml) were suspended (one-step method) or preincubated (two-step method) in fresh sera diluted in 1 : 2, 1 : 4 or 1 : 8 with ARG solution (20 mg/ml in VBS⁺) or VBS⁺.

* *p<0.005 in comparison to each VBS⁺ control.

Fig. 3. Effect of preincubation of PMNs with ARG in two-step method of PMN binding assay.

PMNs were suspended in ARG (20 mg/ml) of 2-fold dilution in VBS⁺ and incubated with sections pretreated with fresh serum. As a control, PMNs were suspended in VBS⁺ prior to the incubation (■).

Preincubated with ARG for 120 min at 37°C, while slightly impaired for 30 min at 37°C; however, such impairment was not observed by the incubation for 120 or 30 min at 4°C in the one-step method. The two-step method also showed a remarkable reduction of PMN binding when they were preincubated with ARG for 120 min at 37°C, but not at 4°C for the same period. Although some reduction in binding was observed when PMNs were preincubated with NRG for 120 min at 37°C, there was no significant suppression by NRG under other conditions tested.

Discussion

PMNs recognize the antibody bound to bacteria or foreign materials and
dispose of them by the processes of phagocytosis, bacteriocidal activity, digestion, etc. The complement system acts as an intermediate amplifying system by coating the antigen-antibody complex with materials capable of being recognized by leukocytes (Wright and Douglas 1903; Müller-Eberhard et al. 1967). Recent concepts of cell biology have emphasized the role of cell surface receptors in such recognition phenomena, one of which is the receptor for Fc region of IgG (Berken and Benacerraf 1966; Henson 1969) and another for C3b (Gigli and Nelson 1968). PMN adherence to glomeruli with immune complexes in our system was considered to occur through these receptors, as reported previously (Yamamoto et al. 1979, 1980). To clarify further which of the two receptors is critical in the PMN binding, PMNs were trypsinized, as the Fe receptor is resistant to trypsin but the C3b receptor is sensitive (Lay and Nussenzweig 1968). For the degree of the impairment of the C3b receptor upon trypsinization, the procedure of rosette formation was carried out with opsonized zymosan. The results have clearly shown that the C3b receptor of PMNs was easily lost by the dose of trypsin used (Tables 1 and 2). In the present study, it was apparent that treatment of PMNs with trypsin decreased the number of PMNs bound to a glomerulus, which presumably resulted from the loss of complement receptors on PMNs by the trypsin treatment.

Zymosan is known to activate the alternative complement pathway and consume native C3 in fresh serum, resulting in the liberation of some biologically active fragments in the fluid phase (Pillemer and Ecker 1941). The reduction of PMN binding activity in Zymosan-treated serum was considered to be due to the depletion of C3, as intact C3 is presumed to play an important role in the PMN binding (Yamamoto et al. 1979). The binding of PMNs to serum-treated sections in a serum-free suspension was also mediated by the interaction of complement receptors on PMNs and activated complements components present on the immune deposits of the glomeruli which had been generated in vivo. However, depletion

<table>
<thead>
<tr>
<th>Temperature, Incubation period</th>
<th>One-step method</th>
<th>Two-step method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ARG</td>
<td>NRG</td>
</tr>
<tr>
<td>37°C, 30 min</td>
<td>70.2±12.8</td>
<td>74.8±7.3</td>
</tr>
<tr>
<td>37°C, 120 min</td>
<td>46.2±7.9*</td>
<td>71.5±10.8</td>
</tr>
<tr>
<td>4°C, 30 min</td>
<td>78.8±8.9</td>
<td>79.4±12.2</td>
</tr>
<tr>
<td>4°C, 120 min</td>
<td>96.5±12.0</td>
<td>86.2±11.0</td>
</tr>
</tbody>
</table>

PMNs (2×10^7/ml) were preincubated with ARG (20 mg/ml) or NRG and resuspended in a 1/2 dilution of fresh serum (one-step method) or VBS* (two-step method) to evaluate the adhesive faculty.

* significant (p<0.05) in comparison to NRG control

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**Table 5. Effect of temperature or incubation periods on suppression of PMN binding by ARG**
PMN Binding to Immune Deposit

of C3 with zymosan was probably incomplete, and the value of the binding activity in zymosan-treated serum was slightly higher than that in serum-free medium (Table 3). This may indicate mechanisms other than the interaction between complement receptors on PMNs and activated complement components on immune deposits. For one thing, the zymosan-treated serum might merely provide good conditions for PMN binding in comparison to the serum-free buffer. For another, a relatively large amount of the early components of a classical pathway in the complement system should be reserved in zymosan-treated serum. These components could fix to the immune deposits on glomeruli and PMNs could recognize these components to bind to the immune deposits. This possibility was supported by the evidence that the C4b receptor was completely or partially the same to the C3 receptor (Ross and Polley 1974). This interaction should be elevated the PMN binding activity in zymosan-treated serum. Furthermore, in the one-step method of the binding, the liberated fragments of the complement in the fluid phase, such as C5a, attract PMNs and increase their adhesive faculty (Craddock et al. 1977). Zymosan-treated serum should contain such fragments in their active forms. Indeed, the binding rate of PMNs in the one-step method is higher than that in the two-step method, presumably indicating some effect of these fragments. Although they were not main factors in the PMN binding, they may act supplementarily as the binding activity did not apparently increase whether PMNs were suspended in zymosan-treated serum.

Activation of the classical pathway occurs on aggregated IgG in fresh serum. Aggregated IgG consumes some complement components in fresh serum or generates some active products in the fluid and on the aggregated IgG, similar to that on zymosan particles. The present study showed that the PMN binding was suppressed by incubation of PMNs with ARG in the presence of fresh serum (Table 4). This result suggested that the complement receptors on PMNs were occupied by complement-fixed ARG and the occupation of receptors inhibited PMNs from binding further to nephritic glomeruli. However, it was not negligible that Fc receptors on PMNs might concern in the PMN binding because some ARG solutions in the absence of fresh serum inhibited PMNs from binding to the nephritic glomeruli, with an increase in the concentration of ARG. This Fc receptor-mediated inhibition seemed to result from the conjugation of ARG with Fc receptors on PMNs. This may indicate that the Fc receptor also plays a role in the PMN binding. Alternatively, it must be considered that the conjugation of ARG may trigger the capping phenomenon of Fc receptors and their internalization at 37°C. This process would consume some energy in PMNs and interfere with tight adhesion of PMNs which was initiated by the interaction of complement receptors and activated complement components on the immune deposits. Furthermore, the decrease of PMN binding by preincubation with ARG in the absence of serum was temperature-dependent and affected somewhat by the incubation periods (Table 5). As the rosette formation of PMNs with antibody-coated
erythrocytes occurs at 4°C (Spiegelberg et al. 1974), the decrease seems to be brought about by temperature-or energy-dependent processes, which follow the binding of Fe receptors on PMNs and ARG.

Acknowledgments

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References

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