Complement Potentiation of Phagocyte-Mediated ADCC of HSV-Infected Corneal Cells

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The authors have shown previously that rabbit alveolar macrophages, peritoneal exudate macrophages, and polymorphonuclear leukocytes are highly effective mediators of antibody-dependent cellular cytotoxicity (ADCC) against herpes simplex virus (HSV)-infected rabbit stromal keratocytes. Using a completely homologous system of nonimmune rabbit phagocytes, anti-HSV serum, complement, and HSV-infected stromal keratocyte targets, the authors have found significant potentiation of ADCC by complement. Lymphocyte-mediated ADCC, on the other hand, was not potentiated by complement. Using a 1:100 dilution of complement (noncytolytic in antibody-mediated lysis) and a 1:10 dilution of antisera obtained from rabbits undergoing stromal keratitis, ADCC mediated by the three phagocytic cell types was augmented by approximately 22%. For example, using alveolar macrophages as effector cells, the addition of complement increased the level of ADCC from 40% to 60%, a 20% increase. Percent ADCC was augmented less by complement at higher antisera dilutions. However, the percent of the total ADCC attributable to complement potentiation increased at these higher antisera dilutions. In some cases, ADCC was not detectable at these high antisera dilutions without the added complement. The potentiating effect was observed at complement dilutions as high as 1:400. Further dilution or heat-inactivation resulted in ADCC values comparable to those obtained without complement. Target cells exposed to complement alone were not killed by phagocytes. Invest Ophthalmol Vis Sci 25:440–446, 1984

Stromal keratitis caused by herpes simplex virus (HSV) is a common corneal disease in humans. Its importance stems from the frequency of initial attacks and from the likelihood that recurrences will lead to progressive scarring of the cornea. Most evidence suggests an immunologic basis for the corneal damage from recurrent disease. Histologic data has shown the presence of polymorphonuclear and mononuclear cell infiltrates in rabbit corneal stromas following induction of herpetic stromal keratitis. The presence of viral surface antigens on stromal keratocytes of HSV-infected rabbit corneas has also been documented.

Of the many complex immunologic processes that may operate during the course of herpetic stromal keratitis, antibody-dependent cellular cytotoxicity (ADCC) may play an important role. Data from this laboratory have established that rabbit phagocytes from various anatomical sites act as efficient effector cells in ADCC of HSV-infected rabbit corneal cells. Rabbit lymphocytes, on the other hand, were shown to mediate only moderate levels of ADCC against these same target cells. We have furthered our investigation in the rabbit model of herpetic stromal keratitis. This report describes the ability of complement to enhance ADCC in a completely homologous system of rabbit effector cells, antibody, complement, and stromal keratocyte target cells. The implications of this phenomenon as it relates to immunologic events in herpetic stromal keratitis are discussed.

Materials and Methods

Cells

Rabbit stromal keratocytes were isolated from the middle layer of corneas excised from New Zealand, adult, white rabbits. Four stromas were separated from epithelial and endothelial layers, minced, and dissociated in 4 ml of phosphate buffered saline (PBS), pH 7.2, which contained clostridial collagenase (260 units/ml, Sigma Chemical Co., St. Louis, MO), chicken serum (10%), and antibiotics, as modified from the method of Berman et al. Following 2 hr of incubation at 37°C with constant agitation, 10 ml of minimal essential medium with Earle's salts (MEM, GIBCO, Grand Island, NY), which contained 10% fetal calf serum (FCS), penicillin (100 units/ml), streptomycin (100 μg/ml), amphotericin B (1 μg/ml), and L-gluta-
mine (2 mM) was added to the suspension. The cells were pelleted by centrifugation at 400 \times g for 10 min and then suspended in 5 ml of MEM supplemented as described above. The suspension was transferred to a 25 cm\textsuperscript{2} plastic flask and incubated at 37\textdegree C until a complete monolayer was formed. Monolayers were dispersed and passaged using a solution containing 0.25% trypsin and 0.05% ethylene diamine tetraacetic acid (EDTA). Fibroblastic morphology was constant throughout serial passage, and cells were passaged no more than 20 times.

**Virus**

The RE strain of HSV-1 was used. This strain was isolated from a human corneal lesion and has been used extensively in studies of herpetic eye disease in rabbits without high mortality from encephalitis.\textsuperscript{10-12} Virus stock was prepared from supernatant fluids of infected VERO cells. Monolayers were infected at a multiplicity of infection (MOI) of 0.1, and virus was allowed to adsorb for 2 hr at 37\textdegree C. Unadsorbed virus was washed from the monolayers and a minimal amount of MEM with 2% FCS was added. Incubation continued at 34\textdegree C for 2–3 days until total cytopathic effect was observed. Virus was harvested from the supernatant fluid of the infected cells by two cycles of centrifugation (800 \times g, 10 min at 4\textdegree C) to clarify the fluid of cells and debris. The infectious titer of the cell-free supernatant was determined by plaque assay on VERO cell monolayers employing the agarose method of Smith et al.\textsuperscript{13} and expressed as plaque forming units per ml (PFU/ml). Stock virus preparations were stored at -70\textdegree C.

**Animals**

Male and female New Zealand white rabbits weighing 1 to 3 kg were used for all experiments.

**Antiserum**

Antiserum to HSV was obtained from rabbits inoculated intrastromally with $1 \times 10^6$ PFU of the RE strain of HSV-1 and killed at 28 days postinfection. The titer of this immune serum was 1280 as determined by an antibody-complement dependent cytolytic assay.\textsuperscript{14} Normal rabbit serum was pooled, commercial, rabbit serum (GIBCO) and was negative for antibody to HSV-1 by plaque neutralization.\textsuperscript{13}

**Complement**

Normal rabbit serum was used as the source of active as well as heat-inactivated (56\textdegree C for 30 min) complement.

**Effector Cells**

Polymorphonuclear leukocytes were isolated from rabbit peripheral blood following Ficoll-Hypaque centrifugation.\textsuperscript{15} The pellet containing erythrocytes and polymorphonuclear leukocytes was suspended in 20 ml of Hanks' balanced salt solution without Ca\textsuperscript{2+} and Mg\textsuperscript{2+} (HBSS, GIBCO) mixed with autologous plasma, and 8 ml of 4.5% dextran T-500 (Pharmacia Fine Chemicals, Upsala, Sweden) in PBS was added. The mixture was incubated 60 min at 37\textdegree C to allow erythrocytes to sediment. The polymorphonuclear leukocyte-enriched supernatant was collected and the cells pelleted by centrifugation at 250 \times g for 15 min. Contaminating erythrocytes were lysed by resuspending the pellet in 5 ml of ACK buffer (30 mM NH\textsubscript{4}Cl, 2 mM KHCO\textsubscript{3}, 0.02 mM disodium EDTA). The polymorphonuclear leukocytes were pelleted, washed twice with HBSS, and suspended in RPMI-1640 medium (RPMI, GIBCO) containing 10% FCS, 2 mM L-glutamine and antibiotics as described for MEM. Preparations were greater than 90% polymorphonuclear leukocytes as determined by morphologic evaluation of smears stained with Wright's reagents.

Alveolar macrophages were collected from excised rabbit lungs by lavage as described by Myrvik et al.\textsuperscript{16} Cells were pelleted and suspended in RPMI with 10% FCS. Preparations were greater than 98% macrophages as determined by cytochemical staining for alphonaphthyl acetate esterase activity (Kit #90-Al, Sigma). Peritoneal exudate macrophages were elicited by intraperitoneal injection of 35 ml of sterile paraffin oil (J. T. Baker Chemical Co., Phillipsburg, NJ) 72 hr before harvest. Cells were washed from the peritoneal cavity by infusion of 400 ml of cold HBSS through a mid-line incision, followed by suction of the fluid into polypropylene centrifuge bottles. Oil was removed by three cycles of centrifugation at 400 \times g for 15 min. The cells were suspended in RPMI with 10% FCS. Preparations contained greater than 90% macrophages based on cytochemical staining.

Peripheral blood monocytes were obtained from heparinized blood by Ficoll-Hypaque centrifugation.\textsuperscript{15} The mononuclear cell band was removed, washed, and suspended in RPMI with 10% FCS. Monocytes were allowed to adhere to plastic plates for 2 hr at 37\textdegree C. The nonadherent cells were washed from the adherent cell layer, and the adherent cells were removed by gentle scraping with siliconized rubber squares attached to a hemostat. The monocytes were suspended in RPMI with 10% FCS. The suspension was generally greater than 95% pure, as assessed by cytochemical staining.

Lymph node lymphocytes were obtained from the mesenteric lymph nodes. The lymph nodes were placed in a plastic dish containing RPMI, sliced with a scalpel.
blade, and teased with a forceps to loosen the lymphocytes. Clumps of tissue were removed by filtration through gauze. The cells were pelleted and suspended in RPMI with 10% FCS.

**Target Cells**

Monolayers of stromal keratocytes were dispersed with 0.25% trypsin and 0.05% EDTA. The cell concentration was adjusted to $5 \times 10^4$ cells/ml by addition of MEM with 10% FCS. One hundred $\mu$l of the cell suspension was dispensed into wells of 96-well flat-bottom polystyrene plates (Linbro, Hamden, CN). The plates were incubated overnight at 37°C to allow the cells to attach and return to monolayer morphology. Medium was then aspirated from the wells, and the cells were infected at an MOI of 10 by diluting the virus stock in MEM with 2% FCS so that 50 $\mu$l contained $5 \times 10^4$ PFU. Control cells received 50 $\mu$l of MEM with 2% FCS. Following 2 hr of adsorption, the unadsorbed virus was rinsed from the wells, and 1 $\mu$Ci of $^{51}$Cr (sodium chromate; New Engand Nuclear, Boston, MA) in 50 $\mu$l MEM with 5% FCS was added to each well. The plates were incubated 5 hr at 37°C followed by four washes to remove free $^{51}$Cr. Fifty microliters of MEM with 5% FCS was added to each well.

**ADCC Assays**

Potentiation of ADCC was assayed by adding 50 $\mu$l of anti-HSV serum diluted in MEM, 50 $\mu$l of rabbit complement diluted in MEM, and 50 $\mu$l of effector cells ($2.5 \times 10^5$ cells for an effector to target ratio of 50:1) to the target cell monolayers. Controls consisted of target cells with (1) medium (spontaneous release); (2) 0.5% sodium deoxycholate (maximum release); (3) normal rabbit serum, complement, and effector cells; (4) normal rabbit serum and effector cells; (5) antiserum and effector cells (nonpotentiated ADCC); (6) normal rabbit serum and effector cells; (7) medium and complement; (8) effector cells and complement; and (9) antiserum and complement. The plates were centrifuged at 50 x g for 3 min to induce contact between effector cells and target cells. The plates were incubated 6 hr at 37°C in a humidified CO$_2$ incubator, centrifuged at 400 x g for 5 min, and 100 $\mu$l of supernatant fluid was removed and analyzed for $^{51}$Cr activity. Activity was expressed as counts per minute (cpm) from the average of quadruplicate wells. The percent specific $^{51}$Cr release was calculated as follows:

$$\text{% specific }^{51}\text{Cr release} = 100 \times \frac{\text{sample cpm} - \text{spontaneous cpm}}{\text{maximum cpm} - \text{spontaneous cpm}}$$

The percent ADCC was calculated with the same formula modified by substituting the activity obtained from target cells exposed to effector cells and non-immune serum for the activity obtained from exposure of target cells to medium (spontaneous release). The percent potentiation of ADCC was calculated as follows:

$$\text{% potentiation} = \frac{\text{% ADCC with complement} - \text{% ADCC without complement}}{\text{% ADCC without complement}}.$$

**Results**

**Determination of End-point Dilution of Complement Cytolytic Activity**

The dilution at which rabbit complement would no longer mediate cytotoxicity of target cells in the presence of anti-HSV serum was determined. Results in Figure 1 indicate that the cytolytic activity of the complement was lost at a 1:80 dilution. Therefore, in subsequent assays, complement was used at a 1:100 dilution or greater. This precluded the possibility that ADCC would appear increased due to lysis of the target cells by antibody-mediated complement-dependent mechanisms.

**Complement enhancement of ADCC of HSV-infected stromal keratocytes**

Figure 2A shows that the ADCC activity of alveolar macrophages was increased in the presence of rabbit complement. The ADCC effectiveness of antiserum dilutions from 1:10 to 1:10,000 was elevated by the addition of complement diluted 1:100. At higher dilutions of complement, its enhancing effect decreased until the % ADCC returned to the level observed without complement. Complement alone did not mediate lysis, and heat-inactivated complement did not mediate enhancement (data not shown). Similar results were obtained using peritoneal exudate macrophages as effector cells (Fig. 2B). With polymorphonuclear leukocyte effector cells (Fig. 2C), ADCC activity occurred only with antiserum dilutions of 1:10 and 1:100 accompanied by minimal complement potentiation.

The ADCC activity of peripheral blood monocytes (Fig. 2D) was considerably less than that observed for alveolar and peritoneal exudate macrophages on even polymorphonuclear leukocytes. Most of the ADCC was apparently due to the enhancing effect of complement as little lysis was observed in the absence of complement. Complement failed to potentiate the modest amount of ADCC by lymph node lymphocytes (data not shown).

The data from Figure 2 are presented quantitatively in Table 1. A value of % potentiation was derived as...
Fig. 1. Effect of complement dilution on complement-dependent lysis by cytolytic antibody. Target cells were HSV-infected stromal keratocytes. Anti-HSV and normal rabbit sera were diluted 1:10. O --- O, anti-HSV serum; • --- • normal rabbit serum.

a measure of the ADCC-enhancing effect of complement. For example, with alveolar macrophages and a 1:10 dilution of antiserum, the addition of 1:100 complement increased ADCC from 43% to 60%, a 17% potentiation representing 28% of the total ADCC. The average % potentiation, calculated as the mean % potentiation of the four antiserum dilutions at 1:100 complement dilutions, were 19%, 18%, 11%, and 8% for alveolar macrophages, peritoneal exudate macrophages, polymorphonuclear leukocytes, and blood monocytes, respectively. The % of the total ADCC due to complement potentiation was greatest at the higher dilutions of antiserum. For blood monocyte effector cells, over 80% of the total ADCC activity with 1:100 and 1:1000 dilutions of antiserum was the result of complement potentiation.

Discussion

Our data concerning rabbit phagocyte-mediated ADCC have demonstrated marked complement potentiation of lysis of HSV-infected stromal keratocytes. Enhancement was observed at high antiserum dilutions, which alone failed to mediate significant levels of ADCC. Additionally, the potentiation apparently was dependent on the concentration of complement, as the degree of potentiation decreased with increasing dilutions of complement.

Our results extend the findings of Rouse et al. and Grewal and Rouse in the bovine system. We found that ADCC by polymorphonuclear leukocytes was likewise enhanced by complement in the homologous rabbit system. We extended the cell types examined to include peritoneal exudate macrophages, alveolar macrophages, and peripheral blood monocytes; the ADCC activity of all was found to be enhanced.

Some differences from results in the bovine system were noted. We were unable to show complement potentiation of lymphocyte-mediated ADCC. The bovine and rabbit systems may not be analogous with respect to which cells are capable of acting as effectors in ADCC. Rouse et al. found that bovine lymphocytes mediated ADCC only in the presence of complement. A percentage of the lymphocytes was shown to possess Fc and C3 receptors. Although we did not do Fc or C3 receptor analysis on our lymphocyte effectors in this study, we have reported that rabbit lymphocytes demonstrate moderate activity in ADCC of HSV-infected stromal keratocytes and that depletion of Fc receptor bearing lymphocytes removes all activity. Others have reported that rabbit lymphocytes failed to demonstrate ADCC activity against several nucleated cell lines and that activity was not enhanced in the presence of complement.

We did not observe complement-enhanced lysis of our targets by polymorphonuclear leukocytes in the absence of antibody. Grewal and Babiuk speculate that complement binds to the polymorphonuclear leukocyte via the complement receptor and also to the target cells, thereby bridging the effector cell to the
Fig. 2. Complement potentiation of phagocyte-mediated ADCC of HSV-infected stromal keratocytes: effect of antiserum and complement dilution on ADCC activity. A, Alveolar macrophages; B, Peritoneal exudate macrophages; C, Polymorphonuclear leukocytes; D, Peripheral blood monocytes. Dilutions of HSV antiserum: • •, 1:10; O O, 1:100; △ △, 1:1000; ▼ ▼, 1:10,000; ■ ■, complement alone.

target cell. This situation, similar to the antibody bridge in ADCC, may be sufficient for lysis. Alternatively, they speculate that complement could be activated by viral membrane antigens, which in turn activates the polymorphonuclear leukocyte to lyse the target cell. Since we used different target cells, HSV-infected stromal keratocytes, it is possible that they may not bind complement or may not express antigens capable
Complement potentiation of phagocyte-mediated ADCC expressed as percent potentiation and percent of total ADCC activity

Table 1

<table>
<thead>
<tr>
<th>Effector cells</th>
<th>HSV antiserum dilution</th>
<th>Complement dilutions</th>
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<tbody>
<tr>
<td></td>
<td>1:100</td>
<td>1:200</td>
</tr>
<tr>
<td></td>
<td>% Pot.* (% tot.)†</td>
<td>% Pot. (% tot.)</td>
</tr>
<tr>
<td>Alveolar macrophages</td>
<td>17 (28)</td>
<td>13 (30)</td>
</tr>
<tr>
<td></td>
<td>22 (42)</td>
<td>17 (37)</td>
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<tr>
<td></td>
<td>18 (42)</td>
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<td></td>
<td>17 (85)</td>
<td>13 (81)</td>
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<tr>
<td>Aver.</td>
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<td>15</td>
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<td>Peritoneal exudate</td>
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<td>17 (28)</td>
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<tr>
<td>Macrophages</td>
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<td></td>
<td>18 (51)</td>
<td>13 (39)</td>
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<td>6 (33)</td>
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<tr>
<td>Aver.</td>
<td>18</td>
<td>14</td>
</tr>
<tr>
<td>Polymorphonuclear</td>
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<td>10 (24)</td>
</tr>
<tr>
<td>Leukocytes</td>
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<td>9 (38)</td>
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</tr>
<tr>
<td></td>
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<td>4 (80)</td>
</tr>
<tr>
<td>Aver.</td>
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<td>8</td>
</tr>
<tr>
<td>Blood monocytes</td>
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<td>8 (33)</td>
</tr>
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<tr>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Aver.</td>
<td>8</td>
<td>6</td>
</tr>
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</table>

* Percent potentiation calculated by subtracting % ADCC activity when no complement was present from % ADCC activity when complement was added.
† Percent of total ADCC activity attributed to complement was calculated by dividing % potentiation by % ADCC in the presence of complement x 100.

of activating complement. Likewise, it is possible that the rabbit polymorphonuclear leukocytes function differently in ADCC, as has already been evidenced by the variations in ADCC activity found among the animal species examined. Continued experimentation will have to be performed before the final mechanism is established.

It is reasonable to speculate that ADCC of HSV-infected corneal cells and its enhancement by complement may contribute in vivo to the immunopathology of herpetic stromal keratitis. In studying the rabbit model of the disease, both macrophages and polymorphonuclear leukocytes have been shown to accumulate in HSV-infected cornneas. Additionally, both antibody and complement have been found to be present in the cornea. Our studies have shown both polymorphonuclear leukocytes and macrophages are highly effective in ADCC against HSV-infected stromal keratocytes and that ADCC mediated by these cells can be significantly enhanced by complement. These results, together with the findings that the components for phagocyte-mediated ADCC are present in HSV-infected cornneas, suggest a direct relationship to the disease process. Further studies are necessary to elucidate the mechanism of complement enhancement of ADCC and its possible in vivo significance.

Key words: herpes simplex virus, stromal keratocytes, antibody-dependent cellular cytotoxicity, complement, phagocytes

References