BIOLOGICAL ACTIVITY OF COMPLEMENT IN VIVO

ROLE OF C5 IN THE ACCUMULATION OF POLYMORPHONUCLEAR LEUKOCYTES IN INFLAMMATORY EXUDATES

BY RALPH SNYDERMAN, M.D., JEAN K. PHILLIPS, AND STEPHAN E. MERGENHAGEN, Ph.D.

(From the Immunology Section, Laboratory of Microbiology and Immunology, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20014)

(Received for publication 17 June 1971)

The development of quantitative in vitro methods for the analysis of various mediators of the inflammatory response has led to a greater understanding of the functions of such mediators. Indeed, recent investigations have suggested that the complement (C) system plays a central role as an effector of a number of events in the inflammatory process (1, 2). A point which has emerged from various in vitro studies, however, is that there are a multiplicity of mediators for any given biological function. For example, fragmentation products from each of the complement, clotting, and kinin systems have been reported to increase vascular permeability, contract smooth muscle, and chemotactically attract polymorphonuclear leukocytes (PMN) (3). Important questions for future investigations will be aimed at determining the relative quantitative and qualitative importance in vivo of each of the known biological effectors in mediating any given aspect of the inflammatory process.

A cardinal event in the acute inflammatory response is the accumulation of PMN at the inflammatory site. While we have previously discussed the importance of the fifth component of C (C5) in the generation of PMN, as well as mononuclear leukocyte chemotactic activity in vitro (4, 5), information on the role C5 plays in mediating the local accumulation of leukocytes in vivo is quite sparse. In this report, data will be presented which suggest an important role for C5 in the early accumulation of PMN in inflammatory exudates.

Materials and Methods

Animals.—Hartley-strain allino male guinea pigs were obtained from the Animal Resources Branch of the National Institutes of Health. Male mice of the lines B10D2/SN “new” (C5-normal) and B10D2/SN “old” (C5-deficient) (6) were purchased from Jackson Laboratories, Bar Harbor, Maine.

Inflammatory Stimulants.—Shellfish glycogen (Mann Research Labs. Inc., New York) was used at a final concentration of 0.5% (w/v) in pyrogen-free saline. Endotoxin derived from

1 Abbreviations used in this paper: BSA, bovine serum albumin; C, complement; C5-deficient, B10D2/SN “old”; C5-normal, B10D2/SN “new”; GVB, gelatin Veronal buffer; PBS, phosphate-buffered saline; PMN, polymorphonuclear leukocytes.
Salmonella typhosa 0901 (Difco Laboratories, Detroit, Mich.) was used in varying concentrations where indicated.

Antiserum.—Rabbit antiserum to highly purified guinea pig C5 (120 μg antibody N/ml) was generously provided by Dr. Hyun S. Shin.

Inflammatory Exudates.—
(a) Glycogen-induced guinea pig exudates: Guinea pigs were injected intraperitoneally with 30 ml of glycogen solution. At various times thereafter, the animals were sacrificed by cervical dislocation and the peritoneal cavity was exposed by an abdominal incision. The exudates were collected and immediately stored at 0°C. The peritoneal cavity was then washed with 0.005 M disodium ethylenediaminetetraacetate (EDTA) in saline (pH 7.4) to bring the total volume of recovered exudate to 40 ml. The exudates were centrifuged at 0°C for 10 min at 900 g. The supernatant was re-centrifuged at 0°C for 30 min at 28,000 g and the resultant supernatant was assayed for chemotactic activity. The sediment of the first centrifugation was resuspended in 1.0 ml Gey's medium (7) and the total and differential white blood cell counts were determined.
(b) Endotoxin-induced guinea pig exudates: Guinea pigs were injected intraperitoneally with 5.0 ml of pyrogen-free saline containing 500 μg endotoxin. The animals were sacrificed at various times thereafter and the exudates were processed in the same manner as the glycogen-induced exudates.
(c) Endotoxin-induced peritoneal exudates in mice: C5-normal and C5-deficient mice were injected intraperitoneally with 2.0 ml pyrogen-free saline containing varying quantities of endotoxin (50–500 μg). At varying times thereafter, mice were sacrificed by CO₂ asphyxiation. The peritoneal cavities were exposed by abdominal incision and the exudates were removed and immediately stored at 0°C. The peritoneal cavities were then washed with sufficient EDTA saline to bring the total volume recovered to 5.0 ml. The exudates were then processed in the same manner as the glycogen-induced exudates from guinea pigs.

Chemotaxis Assays.—Various materials were tested for in vitro chemotactic activity using a modification (7, 8) of the method developed by Boyden (9). Chemotaxis chambers were purchased from Neuroprobe, Inc. (Cabin John, Md.). In experiments where guinea pig exudates or fractions thereof were tested for chemotactic activity, rabbits served as the source of PMN and 1.2 μm nitrocellulose filters (Brinkman Instruments Inc., Westbury, N. Y.) were used in the chemotaxis chambers. In experiments where mouse peritoneal exudates, mouse serum, or mouse PMN were tested, PMN from peripheral blood were used and were obtained as follows. Mice of the strains BALB/c, B10D2/SN "old," or B10D2/SN "new" were bled by cardiac puncture into syringes containing heparin (final concentration of heparin approximately 250 units/ml blood). The mouse blood was mixed with an equal volume of 3% (w/v) dextran (250,000 mol wt) in normal saline. The erythrocytes were allowed to sediment for 30–60 min at room temperature and the leukocyte-rich plasma was withdrawn. The leukocyte-rich plasma was then centrifuged at 500 g for 10 min. Contaminating erythrocytes in the sediment were removed by hypotonic shock (0.2% NaCl for 20 sec followed by 1.6% NaCl). The leukocytes were washed in Gey's medium and resuspended in Gey's medium to a final concentration of 2.2 × 10⁶ PMN/ml.

Molecular Sieve Fractionation of Exudates.—Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden) was used as the chromatographic medium in columns of 2.5 × 30 cm. Phosphate (0.02 M)-buffered (pH 7.2) isotonic saline (PBS) was used as the eluent. Exudates were dialyzed against at least 5000 times their volume with distilled water at 4°C for at least 16 hr. The dialyzed exudates were then dried from the frozen state and resolubilized in an amount of pyrogen-free saline to give six times the original concentration of exudate. The concentrated exudate was then applied to the chromatographic medium.
RESULTS

Generation of Chemotactic Activity for Rabbit PMN in Guinea Pig Peritoneal Exudates.—Glycogen has long been known to induce sterile inflammatory exudates when instilled in the peritoneal cavities of a number of animal species (10). Indeed, this technique has commonly been used to obtain the PMN needed for in vitro chemotaxis assays (9). Peritoneal fluids were taken from guinea pigs at various times after intraperitoneal glycogen injection. The fluids were tested in vitro for the presence of chemotactic activity for PMN as well as for total number of PMN which had accumulated in the peritoneal cavity. For up to 2 hr after injection of glycogen into the peritoneal cavity there was no significant increase in the total number of accumulated PMN (Fig. 1). From the 2nd to the 24th hr, however, the number of PMN markedly increased and then slowly began decreasing in numbers over the next 48 hr. It should be noted that before the influx of PMN the appearance occurred in the peritoneal fluid of activity which was chemotactic for rabbit PMN in vitro (Fig. 1). Such activity could be detected within 30 min after glycogen injection and increased in amount until the numbers of accumulated PMN began to rise.
In another experiment, endotoxin, rather than glycogen, was used as the inflammatory stimulant and was injected into the peritoneal cavities of guinea pigs. Endotoxin, like glycogen, generated PMN chemotactic activity in peritoneal fluids followed by an increase in the number of accumulated PMN. In the case of endotoxin-induced exudates however, significant numbers of PMN did not accumulate until 6 hr after injection.

Characterization of Chemotactic Activity Derived from Guinea Pig Peritoneal Exudates.—Animals were injected intraperitoneally with endotoxin and the peritoneal fluids were collected at varying times thereafter. The pooled peritoneal fluids from three guinea pigs were collected 6 hr after injection, concentrated six times, and applied to a Sephadex G-100 column which had previously been calibrated with markers of known molecular weight (mol wt) (Fig. 2). Two peaks of chemotactic activity were found. The heavier molecular weight peak eluted just before a bovine serum albumin marker (BSA) (mol wt 68,000). The lighter molecular weight peak eluted slightly ahead of cytochrome c (mol wt 12,500) and contained the majority of chemotactic activity present in the exudate. In other chromatograms of exudates taken at times ranging from 1 to 6 hr after injection of endotoxin the heavy molecular weight peak of chemotactic activity was variable in amount. Its activity was usually only barely detectable but occasionally almost equalled the low molecular weight activity. On the other hand, the low molecular weight chemotactic activity was present in all cases and always accounted for the majority of
activity in each chromatographic separation. In addition to molecular sieve chromatography, concentrated exudates taken at varying times after endotoxin injection were fractionated by sucrose-density gradient ultracentrifugation (10–30% linear sucrose gradient at 150,000 g for 18 hr). Exudates were taken from zero to 6 hr after injection. In these experiments, marked chemotactic activity sedimenting with a cytochrome c marker appeared within 30 min after endotoxin injection and persisted for 6 hr. Chemotactic activity sedimenting just ahead of BSA was found only in a 3 hr exudate and accounted for less than 50% of the activity found in the lighter fractions.

To determine if the chemotactic activity generated in peritoneal exudates was antigenically related to C5 (11, 12), the low molecular weight activity

<table>
<thead>
<tr>
<th>Agents tested for chemotaxis</th>
<th>Cells per high power field</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp. 1</td>
</tr>
<tr>
<td>15,000 mol wt peak alone</td>
<td>156</td>
</tr>
<tr>
<td>15,000 mol wt peak + 0.025 ml anti-C5</td>
<td>13</td>
</tr>
<tr>
<td>15,000 mol wt peak + 0.025 ml anti-BSA</td>
<td>168</td>
</tr>
</tbody>
</table>

* The 15,000 mol wt peak of chemotactic activity was isolated by Sephadex chromatography. Fractions (0.7 ml) of the peak of activity were reacted (37°C for 15 min) alone or with the indicated volume of preheated (56°C for 30 min) rabbit antiserum to guinea pig C5 or to BSA. The reaction mixtures were then brought to 1.7 ml with Gey's medium and tested for chemotactic activity.

was isolated on a Sephadex G-100 column from a pooled 1 hr glycogen-induced exudate. Portions of the peak of this low molecular weight activity were reacted (37°C for 15 min) with rabbit antiserum to highly purified guinea pig C5 before testing for chemotactic activity (Table I). The data indicated that the low molecular weight chemotactic factor derived from the inflammatory exudates was similar to C5a not only in terms of molecular weight but also in antigenic determinants.

**Ability of PMN from C5-Deficient and C5-Normal Mice to Respond to Chemotactic Factor In Vitro.**—To study the role of C5 in the kinetics of PMN accumulation in vivo it was necessary to determine if the PMN from C5-deficient animals behaved normally in vitro in response to preformed chemotactic factor. PMN were obtained from the peripheral blood of C5-deficient and C5-normal mice and were tested for their ability to respond to the chemotactic activity generated in C5-normal mouse serum by endotoxin (Table II). The data indicated that PMN from both groups of mice responded equally well to chemotactic activity in vitro. Thus any differences in the kinetics of PMN accumula-
tion in vivo in either group of mice would not be expected to be a result of differences in the responsiveness of their PMN to chemotactic factor.

Inability to Generate Chemotactic Activity in C5-Deficient Mouse Serum.— Serum derived from C5-deficient or C5-normal mice was reacted (37°C for 60 min) with varying amounts of immune precipitates or endotoxin and then was tested for the presence of chemotactic activity in vitro for normal mouse PMN (Table III). The data indicated that mouse serum deficient in C5 was also deficient in its capability to generate chemotactic activity for mouse peripheral PMN. On the other hand, serum from mice with normal C titers, when interacted with immune precipitates or endotoxin, generated substantial

### TABLE II

**Chemotactic Response* In Vitro of PMN from C5-Deficient and C5-Normal Mice**

<table>
<thead>
<tr>
<th>Reagent Tested</th>
<th>CS-deficient PMN</th>
<th>CS-normal PMN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mouse serum (0.2 ml)</td>
<td>3</td>
<td>30</td>
</tr>
<tr>
<td>Normal mouse serum (0.1 ml) + endotoxin (200 µg)</td>
<td>108</td>
<td>128</td>
</tr>
<tr>
<td>Normal mouse serum (0.2 ml) + endotoxin (200 µg)</td>
<td>300</td>
<td>228</td>
</tr>
</tbody>
</table>

* Expressed as cells per high power field.
† The indicated amount of normal mouse serum was reacted (37°C for 60 min followed by 56°C for 30 min) with 0.1 ml normal saline or with the indicated amount of endotoxin contained in 0.1 ml normal saline. The reaction mixtures were brought to 1.7 ml with Gey's medium then tested for chemotactic activity using PMN from either C5-deficient or C5-normal mice.

### TABLE III

**Comparison of Chemotactic Activity* Generation in the Serum of C5-Deficient and C5-Normal Mice**

<table>
<thead>
<tr>
<th>Reagent tested</th>
<th>CS-deficient serum</th>
<th>CS-normal serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum alone</td>
<td>5</td>
<td>43</td>
</tr>
<tr>
<td>Serum + BSA anti-BSA (50 µg AbN)$</td>
<td>19</td>
<td>168</td>
</tr>
<tr>
<td>Serum + BSA anti-BSA (100 µg AbN)</td>
<td>12</td>
<td>273</td>
</tr>
<tr>
<td>Serum + BSA anti-BSA (400 µg AbN)</td>
<td>36</td>
<td>420</td>
</tr>
<tr>
<td>Serum + endotoxin (50 µg)</td>
<td>10</td>
<td>480</td>
</tr>
<tr>
<td>Serum + endotoxin (200 µg)</td>
<td>2</td>
<td>480</td>
</tr>
</tbody>
</table>

* Expressed as cells per high power field.
† Serum (0.05 ml) from either C5-deficient or C5-normal mice was treated (37°C for 60 min) with 0.95 ml gelatin Veronal buffer supplemented with calcium and magnesium (GVB⁺⁺), or GVB⁺⁺ containing the indicated amount of rabbit BSA anti-BSA immune precipitate made at equivalence, or endotoxin. The reaction mixtures were brought to 1.7 ml with Gey's medium and tested for chemotactic activity.
§ AbN, antibody nitrogen.
activity for mouse peripheral PMN. These data confirmed previous findings that C5-deficient mouse serum interacted with endotoxin or immune precipitates was deficient in generating chemotactic activity in vitro for rabbit PMN (7, 13). It is interesting to note that even "background" chemotactic activity in untreated C5-normal serum was higher than that of C5-deficient serum (Table III).

These findings, taken together with the observations that PMN from both C5-deficient and C5-normal mice responded normally to chemotactic stimuli in vitro, indicated that these two groups of mice would make a useful model for the in vivo study of the role of C5 in the kinetics of PMN accumulation in inflammatory exudates.

**Kinetics of PMN Accumulation in Inflammatory Exudates in C5-Deficient and C5-Normal Mice.**—C5-deficient and C5-normal mice were injected intraperitoneally with 125 or 250 μg of endotoxin. Neither of these doses resulted in significant mouse mortality for at least 24 hr after injection. Groups of six mice were sacrificed immediately or at 1, 3, 6, 12, and 24 hr after endotoxin injection. Peritoneal exudates were removed and the total number of PMN
in the exudates was determined (Fig. 3). A significant increase in the accumulated PMN was noted in C5-normal mice as early as 3 hr after injection of endotoxin and the number of PMN increased linearly during the 24 hr test period. In contrast, mice deficient in C5 did not have a significantly increased number of PMN until 12 hr after injection and at no time in the 24 hr period did the numbers of PMN approach those found in C5-normal mice.

In other experiments, however, exudates were collected for up to 48 hr after injection of endotoxin. In C5-normal mice, the numbers of PMN at 48 hr were approximately double the numbers found at 24 hr. In C5-deficient mice the numbers of cells also increased markedly in the period between 24 and 48 hr, and approached (approximately 75%) the numbers of PMN found in C5-normal mice. The differences in PMN in the inflammatory exudates of C5-deficient versus C5-normal mice was most marked ($P < 0.01$) from 3 to 24 hr after injection of endotoxin. At 48 hr the difference in the two groups of mice was less pronounced ($P < 0.5$).

**Chemotactic Activity for PMN in Peritoneal Exudate Fluid of Endotoxin-Treated Mice.**—Supernatants of peritoneal exudates from endotoxin-treated mice were tested in vitro for chemotactic activity at various times after injection of endotoxin. PMN derived from the peripheral blood of normal BALB/c mice served as the source of cells in these experiments. In C5-normal
mice, marked chemotactic activity for PMN was detected within 1 hr after endotoxin injection (Fig. 4). The activity diminished gradually over a 24 hr period but was detectable as late as 24 hr after endotoxin administration. In contrast, supernatants of C5-deficient mouse exudates contained no detectable chemotactic activity for at least 6 hr and at 12 and 24 hr contained barely detectable levels of activity.

**Characterization of Chemotactic Activity for PMN in the Peritoneal Fluid of**

![Graph](image)

**Endotoxin-Treated C5-Normal Mice.**—Endotoxin (125 μg) was injected into the peritoneal cavities of 10 C5-normal mice and the peritoneal fluid was removed 90 min later. The chemotactic activity in the supernatant fluid was heat stable (56°C for 30 min) and nondialyzable. A portion of the concentrated (6X) cell-free exudate was chromatographed on a Sephadex G-100 column (Fig. 5). A sharp peak of chemotactic activity eluted just before a cytochrome c marker (mol wt 12,500). In addition, a smaller peak of activity eluted just after the void volume (mol wt > 150,000). In repeated experiments using exudates taken at 60 min–2 hr, the heavier peak of activity was only variably found and never accounted for more than 50% of the chemotactic activity contained in the lower molecular weight peak.
DISCUSSION

It is generally accepted that the complement, kinin, and clotting systems are important humoral mediators of the acute inflammatory response (1-3, 14). However, evidence detailing a specific function related to the mediation of inflammation in vivo to any of these systems or component parts is fragmentary. While it is reasonable to expect that such humoral effectors of inflammation function in an interrelated and synergistic manner, it is equally reasonable to expect that specific events in the inflammatory response might be mediated predominately by specific components of one of the effectors. The accumulation of PMN in inflammatory sites is of major significance to the host for at least two reasons. First, the PMN is intimately involved in host defense against microbial invasion and second, in certain inflammatory disease states, the PMN appears to be responsible for a large degree of host-tissue destruction (15). Recognizing the importance of PMN accumulation in various pathophysiological states, studies were undertaken to elucidate the role of the complement system in vivo in mediating this aspect of the inflammatory response.

PMN used for in vitro studies of chemotaxis have characteristically been obtained from the peritoneal cavities of animals previously injected with glycogen (9). If chemotaxis was the mechanism by which PMN accumulated in the peritoneal cavity in response to glycogen, it was reasoned that a chemotactic factor for PMN might be detectable in the peritoneal fluid. Indeed, fluids removed from guinea pigs at various times after glycogen stimulation contained chemotactic activity for rabbit PMN in vitro. The appearance of this activity in vivo preceded the influx of PMN and diminished somewhat as the number of PMN increased. The reason for the decrease of chemotactic activity concurrently with PMN accumulation has not yet been resolved. There was substantial evidence to support the notion that the chemotactic activity derived from the peritoneal fluid was generated in vivo rather than after removal from the animal. First, fluids were collected in sufficient EDTA to block activation of the complement and clotting systems. Second, fluids were immediately brought to 0°C after collection and could be heated to 56°C without a substantial loss of chemotactic activity. Third, neither glycogen nor endotoxin alone was chemotactic for PMN in vitro. These findings indicated that the use of the peritoneal cavity as an in vivo model for the study of inflammation might prove useful in studying the kinetics and mediators of PMN infiltration. Injection of endotoxin into the peritoneal cavity of guinea pigs proved to be similar to glycogen in that, shortly after injection, peritoneal fluids contained chemotactic activity for PMN in vitro and this activity was followed by the accumulation of PMN.

The chemotactic activity derived from endotoxin-induced guinea pig peri-
toneal exudates was characterized in order to determine its relationship to previously described chemotactic factors. Chemotactically active exudate fluids were fractionated by molecular sieve chromatography and sucrose-density gradient ultracentrifugation. In all instances, the majority of the chemotactic activity behaved similar to the C5a chemotactic factor (11, 12). In some experiments, additional activity was detected and had a molecular weight >68,000. The relationship of this high molecular weight activity to the C system is unknown at present. The low molecular weight chemotactic factor was isolated by gel filtration and tested for antigenic determinants in common with C5. The inhibition of this activity by antibody to guinea pig C5 established its identity with C5a.

In recent years a number of investigators have found the C5-deficient mouse to be a useful tool for the study of the biological role of complement in vivo (16-19). Of particular interest were the findings that the C5-deficient animals were more susceptible to certain microbial infections than their C5-normal counterparts (17-19). Since the PMN is of major importance in host defense against microbial invasion, studies were undertaken to determine if animals lacking C5 were deficient in mobilizing PMN in response to an inflammatory stimulant. The data showed that the accumulation of PMN in response to a local endotoxin challenge was markedly deficient in mice lacking C5. The abnormality was most dramatically seen within the first 24 hr after endotoxin injection. The data also suggested that this abnormality found in the C5-deficient mice was because of their inability to mobilize a PMN chemotactic factor which was dependent upon the presence of C5. The evidence for this was that while C5-normal mice generated a PMN chemotactic factor in their peritoneal fluid before the accumulation of PMN, the C5-deficient mice generated no substantial PMN chemotactic activity for the first 12 hr after endotoxin injection. Moreover, the PMN from C5-deficient mice were normal in their response in vitro to preformed chemotactic factors. Finally, the elution profile on Sephadex G-100 of the vast majority of chemotactic activity derived from peritoneal exudate fluids of C5-normal mice was strikingly similar to that of guinea pig and human C5a (12, 20).

The present findings, taken in conjunction with other studies showing an increased susceptibility of C5-deficient mice to certain microbial infections, suggests an important role for C5-dependent chemotaxis of PMN. Since phagocytosis and intracellular killing of microorganisms is a primary protective function of the PMN, the early local accumulation of these leukocytes at sites of microbial invasion would be expected to aid in the rapid destruction of microorganisms. Alternatively, a delay in the accumulation of PMN would allow for proliferation and perhaps dissemination of microorganisms. The marked deficiency in the rate of local accumulation of PMN in C5-deficient mice in response to endotoxin was found to be related to an inability of such
mice to liberate a C5-dependent chemotactic factor. We suggest that the deficiency in releasing this C5-dependent chemotactic activity results in a deficient early accumulation of PMN locally and accounts, at least in part, for their increased susceptibility to infection.

SUMMARY

The importance of C5 in the generation of complement (C)-dependent chemotactic activity in vitro is well recognized. However, the actual role C5 may play in the accumulation of polymorphonuclear leukocytes (PMN) at inflammatory sites in vivo has not been established. Injection of glycogen or endotoxin into the peritoneal cavities of guinea pigs resulted, shortly thereafter, in the local accumulation of PMN. Preceding the influx of leukocytes, the peritoneal fluid became chemotactic for rabbit PMN in vitro. The majority of this activity could be attributed to a cleavage product of C5 (C5a). Similarly, injection of endotoxin into the peritoneal cavity of C5-normal mice resulted in the generation of a chemotactic factor for mouse PMN which was followed by the accumulation of PMN in the peritoneal fluid. In contrast, injection of endotoxin into the peritoneal cavity of C5-deficient mice resulted in the generation of virtually no detectable chemotactic activity and a markedly depressed accumulation of PMN during the first 24 hr after injection. The data suggest that C5 plays an important role in the early phases of PMN accumulation in response to inflammatory stimuli. The rapid accumulation of PMN in response to an inflammatory stimulus such as bacterial endotoxin would be expected to be a major factor in host defense against proliferation and dissemination of infectious agents.

BIBLIOGRAPHY

7. Snyderman, R., H. Gewurz, and S. E. Mergenhagen. 1968. Interactions of the


