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Opsonized Streptococcal Cell Walls Cross-Link Human Leukocytes and Erythrocytes by Complement Receptors

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Serum-opsonized group A streptococcal cell walls, consisting of peptidoglycan-polysaccharide polymers (PG-APS), induced monolayers of human neutrophils, monocytes, and eosinophils to aggregate. When erythrocytes were present in the incubation medium, they also were associated with the leukocyte aggregates. By immunofluorescence staining, PG-APS was localized at the site of cell-to-cell contact. By scanning electron microscopy the cells appeared to adhere to one another by surface contact; filopodia often acted as connectors, particularly in leukocyte-erythrocyte interaction. Cellular binding of PG-APS and aggregation were dependent upon C3 fixation. No aggregation was observed when heat-inactivated serum was used as an opsonin. In contrast to peptidoglycan, an activator of the alternative complement pathway, the group-specific polysaccharide moiety of PG-APS induced no cellular aggregation. Rosette formation was observed in suspensions when neutrophils were incubated with erythrocytes coated with C3b-opsonized PG-APS. Cell monolayers bound serum-opsonized PG-APS, but aggregation was observed only when serum was present in the incubation medium. Similar results were obtained with C5-deficient serum. No aggregation was observed with heat-inactivated serum or bovine serum albumin. A heat-labile serum component(s) appears to be required to elicit leukocyte aggregation. It is suggested that C3 fixed to PG-APS acts as a bridge to link cells together in clusters as a result of common recognition of C3 by leukocyte and erythrocyte complement receptors.

It is well established that peptidoglycan-polysaccharide polymers of group A streptococcal cell walls (PG-APS) produce a recurrent, inflammatory, erosive arthritis in experimental animals (2, 41). Many features of the animal model of arthritis are similar to those of human rheumatoid arthritis, including reactivation, severe synovitis, pannus formation, and erosion of cartilage and bone (41). The peptidoglycan moiety of PG-APS has been reported as the most active cell wall component for activation of the alternative complement pathway both in vitro (16) and in vivo (20). The alternative complement pathway serves as a primary recognition mechanism in a nonimmune host for a variety of microbial components such as peptidoglycan from staphylococci (46), lipopolysaccharides from gram-negative bacteria (12), teichoic acid from pneumococci (47), and zymosan from yeasts (32).

Complement activation results in cleavage of C3 to a major fragment C3b, which is fixed to the bacterial surface and serves as an opsonin for phagocytes. Human neutrophils, monocytes, and eosinophils express two types of membrane complement receptors designated CR1 and CR3, in contrast to erythrocytes, which express only CR1. These receptors have specificities for different sites on the C3 molecule (5, 10, 38, 39). The CR1 binds to C3b and iC3b, whereas CR3 binds only to iC3b. Lymphocytes express, in addition to CR1, another complement receptor designated CR2, which binds to the C3d or d region of iC3b and C3dg (10, 22, 38, 39). Specific membrane receptors for C3 present on neutrophils, monocytes, B-lymphocytes, and erythrocytes (5, 6, 9, 22, 37, 38) mediate participation of these cells in immune adherence (7, 27, 28, 36, 43, 44) and stimulation of antibody-secreting cells (25, 26, 31).

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To understand the pronounced inflammatory response to streptococcal components by the host, it is essential to investigate the early cellular response, especially those cells involved in antigen clearance. Our previous investigation has shown that opsonized PG-APS induces capping of CR1 and CR3 on the neutrophil membrane (33), and recently Leong and Cohen (24) have shown that serum-opsonized PG-APS inhibits neutrophil killing of *Staphylococcus aureus*. In the present study, sonicates of group A streptococcal cell walls, opsonized with either purified C3 or normal human serum, were used to investigate the in vitro interaction of bacterial debris with human leukocytes and erythrocytes. Aggregation of leukocytes and erythrocytes was observed within 30 min after incubation with serum-opsonized cell walls. It is suggested that C3b fixed to PG-APS acts as a bridge to link cells together in clusters as a result of common recognition by leukocytes and erythrocytes of this complement component by complement receptors.

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MATERIALS AND METHODS

The physical, chemical, and arthropathic properties of a 100P fraction of purified cell walls derived from group A streptococci have been described (11). This preparation has a molecular weight average of 5.0×10^7 and is composed of peptidoglycan-polysaccharide polymers. The polymers consist only of *N*-acetylglucosamine, *N*-acetylmuramic acid, and rhamnose as sugars and have less than 0.5% non-peptidoglycan-associated amino acids. *N*-Acetylmuramic acid, *N*-acetylglucosamine, rhamnose, and glucose were assayed as their alditol acetates by gas-liquid chromatography (40). Rhamnose was also assayed by the method of Dische and Shettles (4). Amino acid analysis was performed

by using a high pressure liquid chromatography system adapted to perform post-column derivatization amino acid analysis (18). This 100P fraction can be sedimented at 100,000 × *g* for 1 h in a T-865 rotor in a Sorvall OTD-2 ultracentrifuge. For these studies the pellet was suspended in phosphate-buffered saline (PBS) to a concentration of 2 mg of rhamnose per ml. These cell wall fragments are referred to as PG-APS.

Purified group A polysaccharide (APS) and group A peptidoglycan (PG) were prepared as described previously from PG-APS (16). PG was sonicated in a Raytheon 9KC sonic oscillator for 2 min. These cell wall preparations were opsonized at a concentration of 100 μg (dry weight) per ml with 10% human serum for 15 min at 37°C.

PG-APS was incubated with normal human serum as follows: 4 mg of PG-APS in a total volume of 2 ml of PBS was incubated for 15 min at 37°C with whole human type AB serum to achieve a final concentration of 10% serum. The pellet, referred to as serum-(PG-APS), was washed three times with cold PBS at 4°C by centrifugation at 100,000 × *g* and suspended in PBS to a final concentration of 2 mg of rhamnose per ml. A control fraction was prepared as described above, but incubated in PBS. These washed and opsonized PG-APS were used for the rosette assay.

In addition, C3b was fixed to PG-APS [C3b-(PG-APS] by using C3, which was purified from human plasma as described in detail elsewhere (21–23). C3 was judged to be >95% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunodiffusion analysis. Four milligrams of PG-APS in a total volume of 2 ml was incubated with 5 mg of C3 in the presence of 100 μg of trypsin for 2 min at 37°C. The reaction was stopped by the addition of a threefold molar excess of soybean trypsin inhibitor (300 μg) and put on ice. The C3b-(PG-APS) was washed three times by centrifugation at 100,000 × *g* with cold PBS at 4°C.

C5-deficient serum (100%) was also used as an opsonin. Serum was immunoabsorbed with agarose coupled to antibody specific for C5, and the serum was reconstituted to its original volume. The specificity of the anti-C5 has been reported (21).

Preparation of antiserum to group A streptococcus. Antiserum to group A streptococcus was raised in rabbits immunized with group A streptococcal vaccine. The antibody specific for the *N*-acetylglucosamine epitope of the group A polysaccharide (APS) was isolated by affinity chromatography (8) and conjugated with fluorescein isothiocyanate as previously described (35).

Preparation of cell monolayers and suspensions. Peripheral human venous blood was allowed to clot onto 8-mm glass cover slips as previously described (34). The cells were layered with Gey balanced salts plus 10% human serum, 10% heat-inactivated serum (serum heated for 30 min at 56°C), 10% C5-deficient serum, or 1% bovine serum albumin (BSA). Monolayers consisted of 90% neutrophils, 8 to 10% monocytes, and 1 to 2% eosinophils as determined by Wright staining. Approximately 5 to 10 erythrocytes per leukocyte were present.

Neutrophils for suspension studies were purified by Ficoll-Hypaque sedimentation of heparinized blood as previously described (35). Erythrocytes were removed by hypotonic lysis in distilled water. Isotonicity was restored with sodium chloride. Cells were suspended in Gey salts in 1% BSA at a concentration of 4×10^6 cells per ml. Cell viability was routinely assessed by trypan blue exclusion; in all cases, this was greater than 95%.

Incubation of cells with cell wall fragments. PG-APS was sonicated for 5 min in a Raytheon 9KC sonic oscillator to disperse the particles and opsonized with 10% serum for 15 min. Monolayers of cells were incubated for 30 min with 100 μg of serum-(PG-APS) per ml in the presence of 10% serum, 10% heat-inactivated serum, 10% C5-deficient serum, or 1% BSA in Gey balanced salts. Cells were also incubated with PG-APS that was not preopsonized. In this instance cells were incubated for 45 min to obtain equal exposure time of PG-APS to serum factors. The reaction was stopped by removing the medium and simultaneously fixing and washing the cells with 1% paraformaldehyde for 30 min (33, 34). Monolayers were also incubated for 30 min with PG or APS in the presence of 10% human serum, 10% heat-inactivated serum, or 1% BSA.

Cells were stained with fluorescein-labeled antibody to APS for 30 min at 4°C, washed in PBS, mounted, and viewed with a Leitz Orthoplan Fluorescent Microscope with incident light excitation with filter pack H and edge filter K480 to minimize quenching.

SEM. Cells were fixed with 1.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) and prepared for scanning electron microscopy (SEM) as previously described (34). The cells were examined with a JEOL model JSM-35 at 20 kV.

Rosette formation. The aggregation of human erythrocytes with neutrophils in the presence of opsonized PG-APS was tested with a rosette assay performed in 10- by 75-mm plastic tubes. One hundred microliters of erythrocytes (2×10^8/ml) was mixed with 100 μl of preparations (100 μg/ml) of serum-(PG-APS), C3b-(PG-APS), or PG-APS for 30 min at 37°C. The erythrocytes were washed twice with Veronal buffer (pH 7.2) containing 1% BSA, 3.2% dextrose, and 0.02% NaN_3, suspended in 100 μl of Veronal-BSA-dextrose-NaN_3, and mixed with 100 μl of neutrophils (4×10^6/ml). After incubation on a tube rotator for 15 min at 37°C, the rosettes were counted by phase microscopy.

Quantitation of cell aggregates. An aggregate is defined as a cluster of four or more cells. One hundred fields were scanned with a 50× phase objective, and the number of aggregates was recorded.

RESULTS

Immunofluorescence. Patches of PG-APS were observed with fluorescein isothiocyanate-labeled antibody to APS on the surface of neutrophils and monocytes during the first 5 min of incubation with PG-APS in the presence of serum. As previously reported (33) the cell walls were localized after 10 min of incubation as a cap on approximately 85% of the neutrophils. Patching of PG-APS, but not capping, was observed on monocytes. Aggregates of cells were observed within 30 min, and cell walls were localized at points of contact between cells (Fig. 1). Phase microscopy identified the cells in the clusters as monocytes (usually under the other cell types), neutrophils, and eosinophils. When erythrocytes were present in the incubation medium, they were also associated with the aggregate. No binding of PG-APS or aggregation was detected in the absence of serum or when PG-APS was opsonized with heat-inactivated serum.

SEM. The surface morphology of the cell-to-cell interaction was studied by SEM. Aggregates of cells incubated with PG-APS in the presence of serum consisted of neutrophils, monocytes, and erythrocytes. The aggregates varied in size and distribution of cell types. Erythrocytes frequently appeared to rosette around the leukocytes (Fig. 2, 3, and 4). The shape of the erythrocyte was often distorted, extended, or stretched. Erythrocytes displayed short processes extending toward other erythrocytes or leukocytes (Fig. 3).

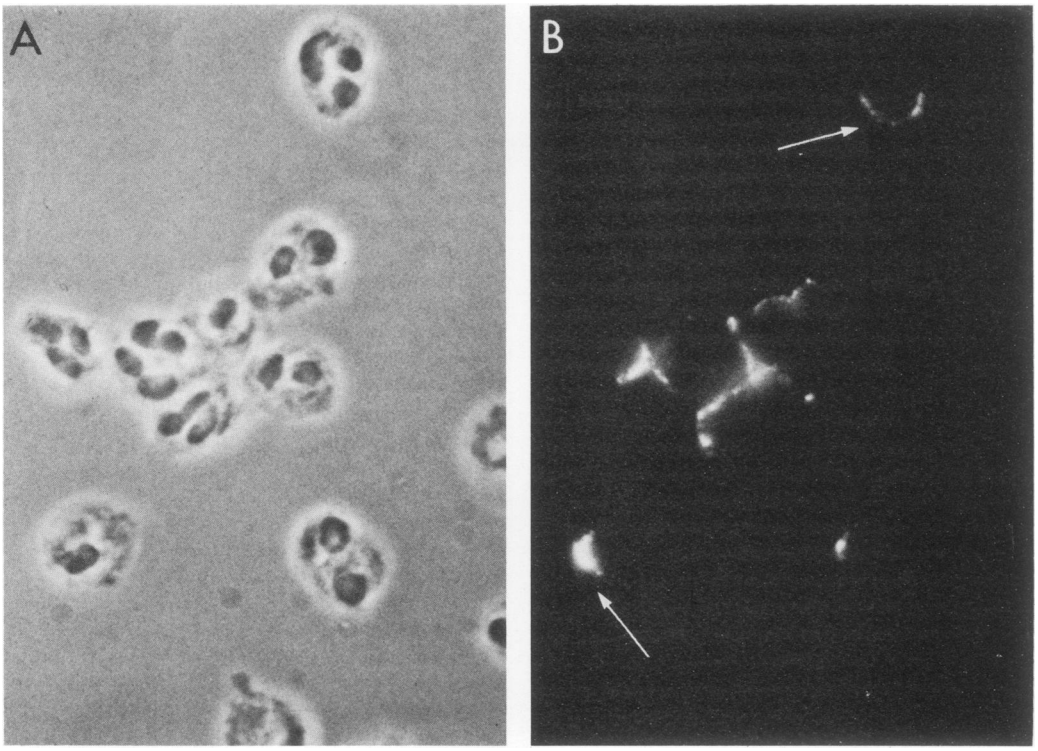

FIG. 1. Cell aggregation of leukocyte monolayers after 30 min of incubation with PG-APS in the presence of serum. Phase microscopy (A) reveals the cells as predominately neutrophils. Immunofluorescent staining with fluorescein isothiocyanate-labeled antiserum to APS of the same field shown by phase microscopy (B) shows localization of PG-APS at the site of cell-cell contact. Note that PG-APS is localized as a cap on individual neutrophils (arrows).

The number of cells in the aggregates increased with time. By 30 min some aggregates consisted of 10 or more cells which included several neutrophils and erythrocytes, one or two monocytes, and a rare eosinophil. Figure 4 shows an aggregate consisting of several neutrophils, erythrocytes, and a monocyte. The monocytes are usually well spread at the bottom of the aggregate. Contact areas of erythrocytes to neutrophils in the larger aggregates usually are limited to the ends of processes, which extend from the irregularly shaped erythrocyte surface to the neutrophil. In addition, neutrophils also extend filopodia to the erythrocyte surface. Surface distortions of erythrocytes vary from slight indentations to deep invaginations. No erythrophagocytosis was observed. Monolayers of cells incubated without cell walls

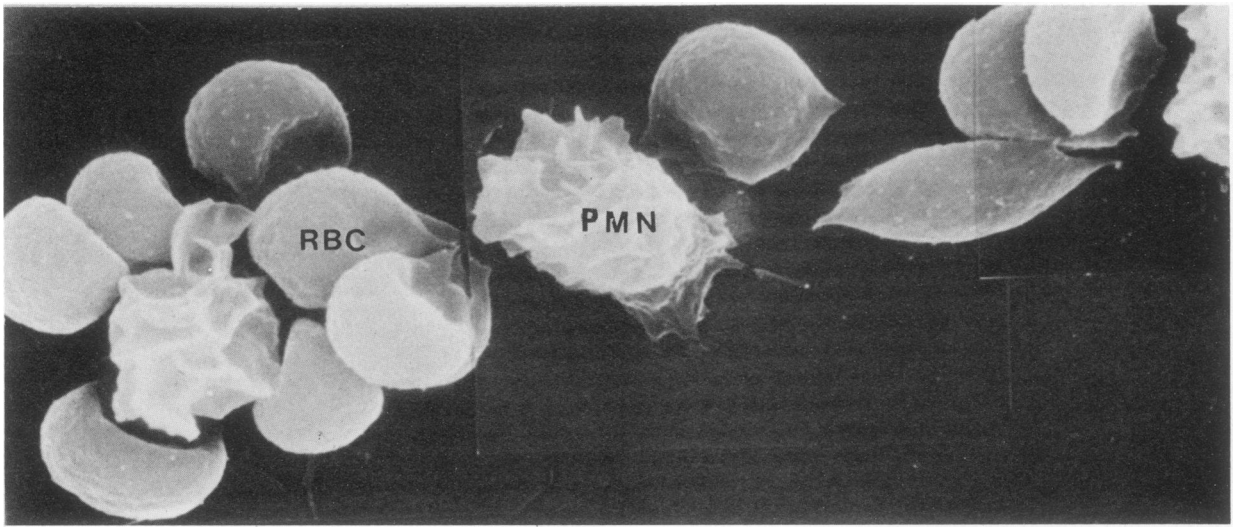

FIG. 2. SEM of leukocyte monolayer incubated for 10 min with PG-APS in the presence of serum. Erythrocytes (RBC) are seen in the process of forming aggregates with neutrophils (PMN). Several erythrocytes are distorted. 5,200×.

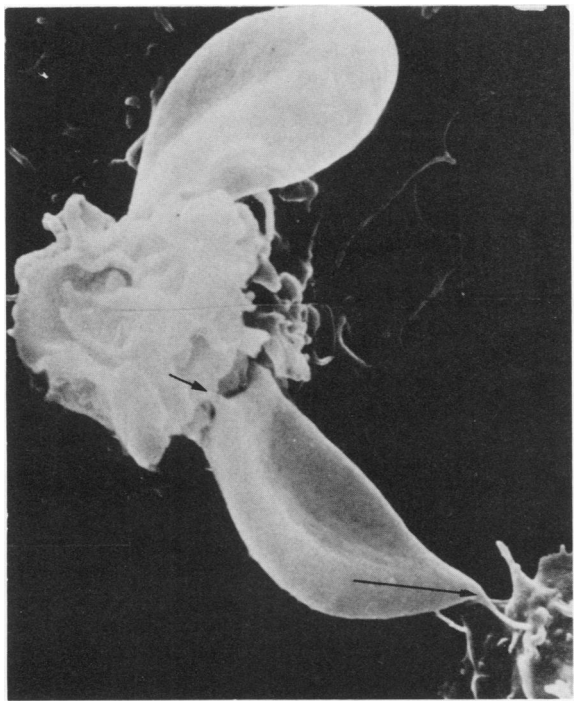

FIG. 3. SEM of erythrocytes in physical contact with two neutrophils after 30 min of incubation with PG-APS in the presence of serum. One erythrocyte has several short processes (short arrow) extending to a neutrophil; at its other end, one longer filament extends (long arrow) to a different neutrophil. 7,000×.

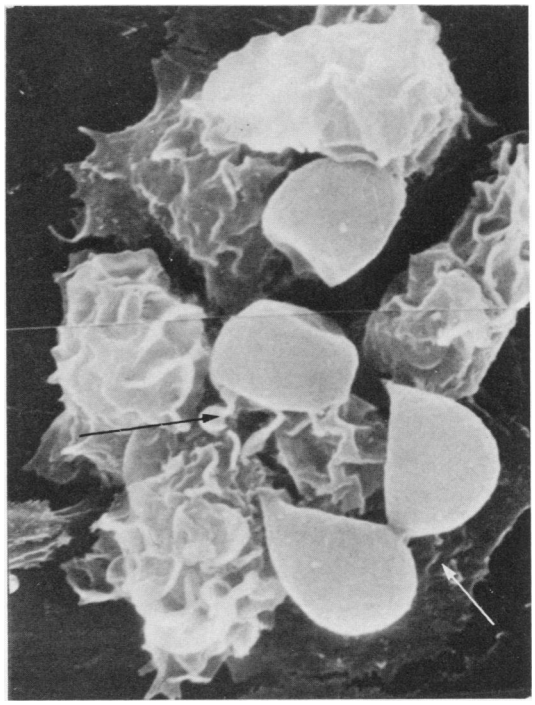

FIG. 4. SEM of an aggregate after 30 min of incubation of PG-APS in the presence of serum. The cluster consists of four erythrocytes, at least four neutrophils, and an underlying well spread monocyte (white arrow). Processes from the neutrophil extending toward an erythrocyte are seen in the area of the black arrow. 5,000×.

or with PG-APS opsonized with heat-inactivated serum did not form aggregates.

Aggregate formation with streptococcal cell wall components. When monolayers of cells were incubated in the presence of serum with 300 μg of purified PG, PG-APS, or crude cell walls per ml, more than 50 aggregates consisting of four or more cells were observed in 100 fields with a 50× objective (data not shown). No aggregates were observed with cells incubated with APS.

Aggregate formation with serum-opsonized PG-APS. To understand the serum factors involved in eliciting cell aggregation, serum-(PG-APS) (preopsonized) or PG-APS (not preopsonized) were incubated with monolayers of cells in the presence of fresh serum, heat-inactivated serum, C5-deficient serum, or BSA. Immunofluorescence microscopy confirmed that complement activation was required for binding of PG-APS to neutrophils (Table 1). No binding of PG-APS to cells was observed with heat-inactivated serum or BSA.

TABLE 1. Serum factors required for PG-APS induced leukocyte aggregation and leukocyte binding of PG-APS[a]

Incubation medium	Serum-(PG-APS)		PG-APS	
	Aggregation[b]	Binding[c]	Aggregation	Binding
Normal serum	+	+	+	+
Heat-inactivated serum	−	+	−	−
C5-deficient serum	+	+	+	+
BSA	−	+	−	−

[a] Monolayers of leukocytes were incubated with serum-opsonized or nonopsonized PG-APS for 30 or 45 min, respectively, with different serum factors.

[b] +, >40 aggregates consisting of four or more leukocytes observed in 100 fields with a 50X objective.

[c] +, >85% binding. Immunofluorescence microscopy with antibody to APS was used to determine binding of PG-APS to leukocytes.

Binding of PG-APS to leukocytes was insufficient to induce cell aggregation (Table 1). Although cells bound serum-(PG-APS), aggregation was not observed when cells were incubated with heat-inactivated serum or BSA, but aggregation was observed in normal serum or C5-deficient serum.

Rosette formation. We have previously shown that PG-APS binds to neutrophils by CR1 and CR3 and that binding of C3b-(PG-APS) can be blocked with neutrophils preincubated with anti-CR1 or fluid-phase C3b (33). Aggregation of human erythrocytes with neutrophils was tested by using C3b-(PG-APS) with a rosette assay. Rosette formation was positive for suspensions of neutrophils incubated with human erythrocytes coated with purified C3b-(PG-APS) (95% rosettes) or serum-(PG-APS) (75% rosettes). No rosettes were observed with heat-inactivated serum or PG-APS alone.

DISCUSSION

We have previously shown that binding of group A streptococcal cell walls to human neutrophils depends upon complement activation, fixation of C3 to the cell walls, and recognition of opsonized cell wall fragments by neutrophil CR1 and CR3 receptors (33). The present experimental data show that serum-opsonized PG-APS fragments induce aggregation of cells that are known to have C3 receptors, namely, neutrophils, eosinophils, monocytes, and erythrocytes. The cell walls are localized at the site of cell-to-cell

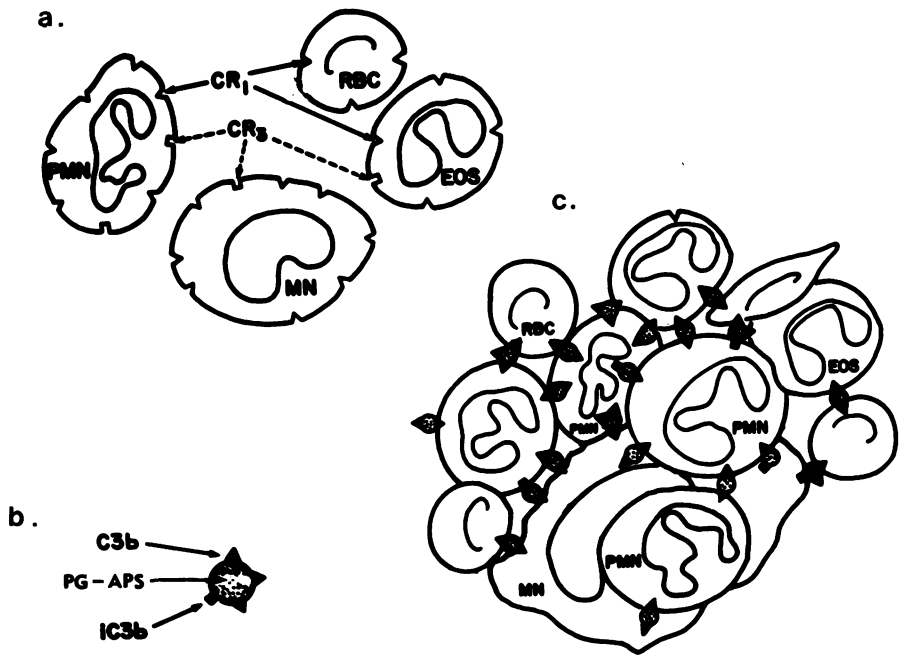

FIG. 5. Hypothetical model of blood cell interaction of leukocyte monolayers and erythrocytes before and after incubation with PG-APS. (A) Neutrophils (PMN), monocytes (MN), eosinophils (EOS), and erythrocytes (RBC), are represented with surface membrane receptors for C3b (CR1) or iC3b (CR3). (B) PG-APS is represented after opsonization with serum. PG-APS activates complement, and a product of C3 cleavage, C3b, is fixed on multiple sites on the PG-APS fragments. Action of serum factors I and H on the fixed C3b generates iC3b. (C) An aggregate is represented of neutrophils, erythrocytes, eosinophils, and monocytes after incubation with serum-opsonized PG-APS. It is hypothesized that C3b-(PG-APS) or iC3b-(PG-APS) cross-links cells with receptors for C3b and iC3b.

contact, and erythrocytes, if present, form rosettes with leukocytes. Erythrocyte rosetting with neutrophils is dependent upon coating erythrocytes with PG-APS opsonized with whole serum or purified C3. The rosetting assay provides evidence that C3b fixed to PG-APS binds to erythrocyte C3b receptors, and rosetting of erythrocytes to neutrophils is achieved by neutrophil recognition of excess molecules of C3b fixed to the PG-APS-coated erythrocytes.

Aggregation of leukocyte monolayers by PG-APS is dependent upon soluble factors generated during complement activation. Although monolayers bind PG-APS opsonized with serum, aggregation occurs only when serum is present in the incubation medium. Because C5-deficient serum also induces aggregation, it appears that chemotactic factor C5a is not essential. No cell aggregation is observed with heat-inactivated serum or BSA. Thus, it appears that activation of a heat-labile serum factor(s) is important to stimulate cell aggregation.

With cell suspensions, purified C3b is sufficient for rosette formation, and serum in the incubation medium is not required. Differences in these observations lie in the assay. The rosette assay requires repeated cell contact, which is achieved by rotating cell suspensions in a small volume of liquid. In contrast, cell contact is minimal during random motility of leukocyte monolayers. The data suggest that aggregation of cell monolayers exposed to PG-APS is elicited by C3 fixation to the cell walls and heat-labile serum factor(s). Since no aggregation is observed in cell suspensions, adherence also seems to be required. A model suggesting a mechanism for aggregate formation of leukocytes and erythrocytes induced by C3 opsonized PG-APS is shown in Fig. 5.

Because monolayers of leukocytes can be stimulated to cluster after response to chemotactic stimuli, e.g., migration toward necrotic cells or bacteria, we hypothesized that chemotactic factor C5a might be involved in the aggregation response. Therefore, C5-deficient serum was used in the assay to test this hypothesis. We were surprised to find that C5-deficient serum elicited results similar to those of normal serum. Experiments are in progress to determine the required serum factors.

PG is the moiety of the cell wall polymer required for cell aggregation. No aggregates are formed with group-specific carbohydrate APS. It has prevously been shown that PG of group A streptococcal cell walls is the most active component for activating the alternative complement pathway, and that APS does not activate complement (16). The normal serum used in these studies apparently has insufficient antibody to APS to activate the classical pathway and cause C3b fixation to the cell walls.

Aggregate composition and surface morphology were determined by phase microscopy and SEM. The monocyte, which has a greater surface area during adhesion than the neutrophil, is generally found at the bottom of the aggregate, with the other cell types located on top of or at the periphery of the monocyte. Filopodia, present on both neutrophils and monocytes, often appear to act as connectors between cells. This connection is particularly evident between leukocytes and erythrocytes. The erythrocytes, which form rosettes with leukocytes, often appear distorted, since they are pulled by adjacent leukocytes. With microcinematography we have observed neutrophils carrying erythrocytes during locomotion; aggregation appears to be transient, because cells begin to detach from the aggregate after 1 h of incubation. No erythrophagocytosis is observed.

Both clinical and experimental studies have shown that C3 plays an important role in host defense (7, 26, 27, 29). For example, in experimental animals complement appears to

influence the course of disease. PG-APS produces a recurrent, inflammatory erosive arthritis in rats (2, 41). The severity of the acute joint inflammation correlates with serum complement levels in complement-depleted rats injected with PG-APS (42). Similarly, granulocytopenia is one of the earliest responses after intravenous injection of pneumococcal cell walls in rabbits (13), and pneumococcal challenge of complement-depleted rabbits produces a significantly smaller reduction in circulating granulocytes and lack of pulmonary leukostasis (14).

Dierich and Landen (3) provide evidence that C3 and C5 can initiate the formation of bridges between leukocytes and erythrocytes that have been preincubated with complement components. They suggest that enzymes on one cell liberate labile binding groups of complement components on adjacent cells, thus inducing coupling of the two cells. C3b was unable to serve the same response, suggesting that cell interaction elicited by C3b-(PG-APS) is different.

Recently C3 has been shown to play a role in the formation of cell aggregates of concanavalin A-stimulated murine spleen cells (17). In the presence of autologous serum, C3 is deposited on the surface of T blast cells, which is followed by aggregation of cells expressing complement receptors. Based on the data presented in this paper, aggregation of cells by opsonized streptococcal cell walls represents another mechanism of cell bridging which may play a role in cell-cell interaction.

Numerous immunological phenomena are based upon cell-cell interactions involving immune adherence mediated by humoral factors (29). Some examples include induction of an antibody response by B cells (45), sensitization of T cells (15), macrophage-lymphocyte effects on target cells (30), and granulocyte-endothelium interactions (19). It can be hypothesized that binding of cell walls to leukocytes and erythrocytes with subsequent cell-to-cell interaction in vitro could also occur in vivo. It has recently been observed that immune complexes administered intraarterially to primates adhere to erythrocytes and are removed in the liver by Kupffer cells (1). Since 85% of the C3b receptors in peripheral blood are found on erythrocytes (10) and they bind PG-APS, it is likely that they play an important role in clearance of bacterial debris as well. Although the biological significance of attachment of complexes or cells bearing C3b to erythrocytes is not clear, it may act as an immobilizing mechanism important in dealing with pathogenic agents. We suggest that the immune adherence phenomenon of PG-APS to cells with C3 receptors may play a role in the transport of bacterial debris to various tissues and the subsequent pathology.

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