Effect of Pneumococci on Blood Clotting, Platelets, and Polymorphonuclear Leukocytes

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Infections due to *Streptococcus pneumoniae* and products from the organism have been associated with alterations in blood clotting and function of platelets. Pneumococci and pneumococcal polysaccharide shortened the clotting times of whole blood, platelet-rich plasma (PRP), and platelet-poor plasma (PPP) in vitro. Clotting times of PPP and PRP from C6-deficient animals were likewise decreased. The bacteria had no effect on the one-stage prothrombin time or the partial thromboplastin time when the organisms were used as activating agents. Platelets aggregated in the presence of pneumococci, but aggregation was prevented by the addition of cyclic adenosine 3',5'-monophosphate (cAMP). Furthermore, cAMP corrected the shortened clotting time of PRP in the presence of pneumococci. The clumping and release of polymorphonuclear coagulant that was induced by pneumococci was not prevented by cAMP. Thus, pneumococci exert several dose-dependent thromboplastic effects: (i) release of platelet thromboplastic substances; (ii) a direct thromboplastic effect; and (iii) release of polymorphonuclear coagulant.

In 1899, a "purpura producing principle" was found in filtrates from broth cultures of pneumococci (5, 16); the purpura was at least partially due to thrombocytopenia, and the "toxin" was lytic for platelets in vitro (25). Platelet aggregation, thrombocytopenia, and induction of adherence of platelets to leukocytes by several species of bacteria have also been demonstrated both in vivo (14, 21, 32) and in vitro (10).

Furthermore, pneumococcal bacteremia in humans (8, 12, 24, 31) and experimental animals (9) may be accompanied by intravascular coagulation. In humans, it most frequently follows splenectomy or hyposplenism (2, 17, 33, 35). Indeed bacteremias of any etiology probably are associated with intravascular coagulation, even though it may be subclinical (30). We have also demonstrated intravasular coagulation in rabbits with pneumococcal bacteremia (Guckian, unpublished observations). The pathogenesis of this process is unknown. This report expands our previous studies to the effects of virulent pneumococci on clotting factors, platelets and polymorphonulear leukocytes (PMNs and suggests some pathogenetic explanations for the events in lethal pneumococcemia.

MATERIALS AND METHODS

Preparation of pneumococci for in vitro studies. Virulent *Streptococcus pneumoniae* type I, SV-1 was stored anaerobically at 4 C in defibrinated rabbit blood under petroleum jelly and passed through rabbits every 4 weeks to maintain its virulence. Brain heart infusion broth (10 ml) was inoculated with a 0.01-ml loop of rabbit blood and incubated for 18 h at 36 C. The broth was centrifuged at 15,000 × g for 20 min. The button of pneumococci was washed twice with sterile saline and resuspended in 2 ml of saline. This suspension contained 6 × 10⁸ to 2 × 10⁹ pneumococci per ml. For some studies, pneumococci were incubated with hexadimethrine (polybrene, which inhibits activation of factor XII), 100 mg/ml, for 15 min at 37 C, recentrifuged, washed twice with saline, and resuspended in saline.

Clotting studies. Blood was drawn from New Zealand albino rabbits, or rabbits deficient in the sixth component of complement (C6) (Rancho de Conejo, Vista, Calif.), into plastic syringes containing 3.8% sodium citrate, 1 volume to 9 volumes of blood. Siliconized glassware or plastic tubes were used unless otherwise indicated. Platelet-poor plasma (PPP) was obtained by centrifuging blood at 2,000 × g for 10 min. Platelet-rich plasma (PRP) was obtained after centrifuging blood at 180 × g for 5 min. PPP and PRP clotting times in siliconized and unsiliconized glass tubes were determined by incubating 0.1 ml of plasma with either 0.1 ml of saline, kaolin (10 mg/ml), or pneumococci (10⁹/ml) for 60 min at 37 C and then recording the time required for clot formation after adding 0.1 ml of 0.02 M CaCl₂. Whole-blood clotting time was determined by adding 1.0 ml of blood to tubes containing either 0.1 ml of kaolin, pneumococci (10⁹/ml), pneumococcal polysaccharide (50 μg) (27), or saline. Prothrombin times (15) using PPP were determined in glass tubes after incubating with pneumococci or saline for 15 min at 37 C.

The partial thromboplastin time was done with PPP using either pneumococci (10^9/ml) or kaolin (10 mg/ml) as the activating substance (22).

Human gamma globulin, 165 mg/ml, was diluted 1:10 in saline and heated to 60 C for 10 min. PPP (0.9 ml) was incubated for 30 min at 37 C with 0.1 ml of heat-aggregated gamma globulin (HAGG). A 0.1-ml amount was then mixed with 0.1 ml of either pneumococci, kaolin, *E. coli* lipopolysaccharide (200 μg), or saline. A 0.1-ml amount of 0.02 M $CaCl_2$ was added, and the time required for clot formation was recorded. The control consisted of PPP to which no HAGG had been added.

PMNs were obtained from peritoneal exudate by the method of Cohn and Hirsch (6) and resuspended in citrated saline (1 part 3.8% sodium citrate and 5 parts 0.15 M saline) to give 5 $\times$ 10^7 PMN/ml in siliconized glass tubes. The release of PMN coagulant was determined by incubating 0.1 ml of PMN and 0.1 ml of PPP with either 0.1 ml of pneumococci (10^9/ml), *E. coli* lipopolysaccharide (200 μg), latex particles (0.81 mm in diameter; 1:20 dilution in saline of a stock solution), kaolin (10 mg/ml), or saline on a rotator at 37 C for 60 min. A 0.1-ml amount of 0.02 M $CaCl_2$ was then added, and the time required for clot formation was recorded.

In some studies dibutyryl cyclic adenosine 3′,5′-monophosphate (d-cAMP) was added to the incubation mixture to give a final concentration of 10^{-3} M. In other studies acetylsalicylic acid was dissolved in 0.1 M phosphate-buffered saline, pH 7.4, and added to the incubation mixture to give a final concentration of 200 μg/ml.

Platelet aggregation. One milliliter of PRP was incubated in siliconized glass tubes with either 0.1 ml of pneumococci (10^9/ml), pneumococcal polysaccharide (50 μg), kaolin (10 mg/ml), latex particles, or saline on a rotator at 37 C at 15 rpm. Aliquots were removed at 0, 60, 120, and 180 min, and the number of clumps containing more than five platelets per high-power field under phase microscopy was observed. Platelet counts were done by phase microscopy (4).

PMN clumping. Peritoneal PMNs were suspended in either balanced Hanks solution with 0.1% gelatin or autologous plasma to give 5 $\times$ 10^6/ml. A 0.9-ml amount of PMN and either 0.1 ml of pneumococci (10^9/ml), pneumococcal polysaccharide (50 μg), glycogen (0.01%), latex, or saline was added to

Sykes-Moore chambers (Bellco Glass, Inc., Vineland, N.J.). The cells were incubated at 37 C and observed for clumping by phase microscopy at 0, 5, 15, 30, and 120 min. At 0 and 120 min an aliquot was removed and stained with 1% trypan blue, and the percentage of viability was determined by dye exclusion.

Phagocytosis and killing of pneumococci were assayed by tumbling PMNs, autologous plasma, and pneumococci in siliconized tubes for 120 min at 37 C (23). Aliquots were removed at 0, 60, and 120 min, stained with methylene blue, and observed for intracellular organisms. Other aliquots were cultured quantitatively on sheep blood agar.

Chemicals. Chemicals used were: kaolin N.F. colloidal (Mallinckrodt Chemical Works, St. Louis, Mo.), dibutyryl cyclic adenosine monophosphoric acid (Sigma Chemical Co., St. Louis, Mo.), thromboplastin (Simplastin, Warner-Lambert, Morris Plains, N.J.), latex particles (0.81 μm in diameter; Difco, Detroit, Mich.), hexadimethrine (Abbott Laboratories, North Chicago, Ill.), human gamma globulin (Parke-Davis, Detroit, Mich.), *E. coli* O:127-B:8 lipopolysaccharide (Difco), and acetylsalicylic acid (Mallinckrodt Chemical Works).

RESULTS

Table 1 summarizes the effect of various agents on clotting parameters. Pneumococci and pneumococcal polysaccharide shortened normal whole-blood, PRP, and PPP clotting times in siliconized glass but had no effect on whole-blood or PPP clotting in glass. Pneumococci had no effect on the prothrombin time or the partial thromboplastin time. Likewise, the PPP and PRP clotting times of C6-deficient plasma was shortened by the presence of pneumococci. Thus, the microorganism manifests a direct thromboplastic activity that does not require activation of the entire complement cascade or platelets. Fewer than 10^8 pneumococci per ml had no effect. Incubation of pneumococci with hexadimethrine, which if adsorbed onto the surface of pneumococci might prevent activation of factor XII, did not alter the effect of pneumococci on clotting times. Kaolin, as expected, shortened the clotting times in all stud-

TABLE 1. *Effect of pneumococci on clotting* [a]

Clotting agent	Whole-blood clotting time (min)	PRP clotting time (s)	PPP clotting time (min)	PTT (s)
Saline	23.1 ± 2.4	10.0 ± 1.8	26.8 ± 2.5	267 ± 31
Pneumococci	10.9 ± 2.2	5.3 ± 1.2	13.0 ± 3.1	308 ± 38
Pneumococcal polysaccharide	3.5 ± 1.4	2.2 ± 0.7		
Kaolin	5.4 ± 1.0	2.4 ± 1.2	2.1 ± 0.9	114 ± 16
Pneumococci + cAMP[b]		12.5 ± 2.3	10.4 ± 2.5	

[a] Mean ± standard derivation in siliconized glass tubes. Abbreviations: PTT, Partial thromboplastin time; PRP, platelet-rich plasma; PPP, platelet-poor plasma.

[b] Dibutyryl cyclic adenosine monophosphoric acid.

TABLE 2. *Effect of activation of complement on clotting*

Complement activator[a]	PPP clotting time (min)[b]
Saline	26.8 ± 3.1
HAGG	16.0 ± 1.9
Endotoxin	10.1 ± 1.5
PNC	13.0 ± 2.0
HAGG + PNC	7.1 ± 1.8
Endotoxin + PNC	8.1 ± 1.6

[a] Abbreviations: HAGG = Heat-aggregated gamma globulin; PNC, pneumococci; PPP, platelet-poor plasma.

[b] Mean ± standard deviation.

TABLE 3. *Effect of pneumococci on polymorphonuclear coagulant[a]*

Clotting agent	Clotting time (min)[b]
Saline	8.4 ± 1.8
Pneumococci	4.2 ± 1.2
Endotoxin	5.8 ± 1.5
Latex	6.3 ± 1.8
Kaolin	2.4 ± 0.7
Pneumococci + cAMP[c]	4.1 ± 1.4

[a] Mixture included 0.1 ml (5 × 10^{6}) of PMNs, 0.1 ml of platelet-poor plasma, and 0.1 ml of agent to be studied incubated at 37 C for 60 min. Each value represents the mean of at least six determinations.

[b] Mean ± standard deviation.

[c] Dibutyryl cyclic adenosine monophosphoric acid.

ies. D-cAMP failed to correct the shortened PRP clotting time caused by kaolin but corrected the shortened time induced by pneumococci. D-cAMP, however, did not correct the shortened clotting time of PPP after addition of pneumococci, suggesting that d-cAMP inhibited release of platelet procoagulants that might have been induced by pneumococci. To determine whether the effect of pneumococci on plasma in which the complement had been activated was additive, the experiment illustrated in Table 2 was performed. HAGG and endotoxin, activators of complement, shortened the clotting time of PPP. Pneumococci shortened further the clotting time after complement was activated, again suggesting differing or at least additive mechanisms.

Pneumococci, latex particles, endotoxin, and kaolin released a coagulant from the PMNs (Table 3). D-cAMP did not prevent the release of the coagulant by pneumococci.

When PRP was incubated with 10[6] and 10[8] pneumococci or kaolin, clumping of the platelets occurred within 60 min and persisted for 180 min (Fig. 1). A total of 10[5] pneumococci/ml produced slight clumping, and 10[4]/ml failed to cause clumping. Pneumococcal polysaccharide and latex did not cause clumping of platelets. D-cAMP produced a definite decrease in number and size of the platelet aggregates caused by pneumococci but failed to inhibit that elicited by kaolin. This agrees with the prior observation on the effect of d-cAMP on the clotting of PRP in the presence of pneumococci. Acetylsalicylic acid failed to inhibit platelet aggregation caused by pneumococci.

PMNs suspended in Sykes-Moore chambers aggregated within 5 min in the presence of 10[7] or 10[8] pneumococci/ml and within 45 min with 10[2] to 10[6] pneumococci/ml (Fig. 2). In addition to the clumping, the PMNs became "sticky," adhering to the chamber walls so that vigorous agitation could not displace them. The cells became progressively smaller and opaque. After 240 min 70 to 95% of the cells were nonviable in the presence of 10[4] to 10[8] pneumococci/ml, whereas in the saline control 95% of the PMNs were viable. Although suspending PMNs in plasma did not prevent clumping with pneumococci, the PMNs remained viable and did not adhere to glass. Pneumococcal polysaccharide produced a more striking effect on PMNs than did pneumococci, but latex and glycogen elicited very little aggregation or death. D-cAMP and acetylsalicylic acid did not prevent clumping, adherence or death.

DISCUSSION

These studies support the hypothesis that pneumococci act as a thromboplastic substance, probably mediated through the polysaccharide capsule. Whole-blood, PRP, and PPP clotting times were shortened in the presence of virulent type I pneumococci, and d-cAMP corrected the shortened clotting time in PRP but not in PPP. The shortened clotting times occurred independent of cellular elements or the late-forming complement components and were dose dependent since fewer than 10[6]/ml penumococci had little effect. This level of bacteremia, and probably the level of circulating polysaccha-

FIG. 1. (A) *Control; platelet-rich plasma, 0.9 ml with 0.1 ml of saline incubated at 37 C on a rotator at 15 rpm for 60 min. (B) Platelet-rich plasma, 0.9 ml with 0.1 ml (10[8]/ml) of pneumococci after 60 min. Phase contrast microscopy, ×100.*
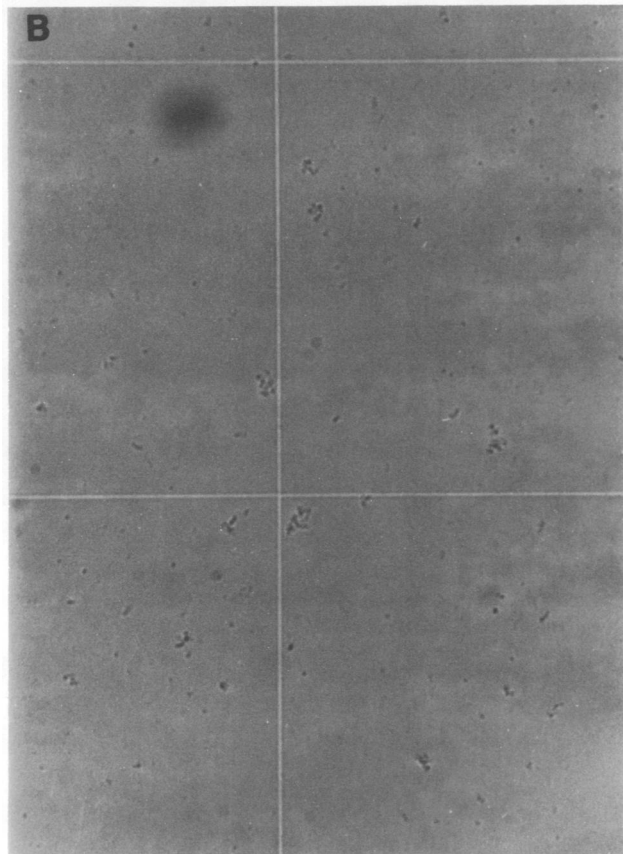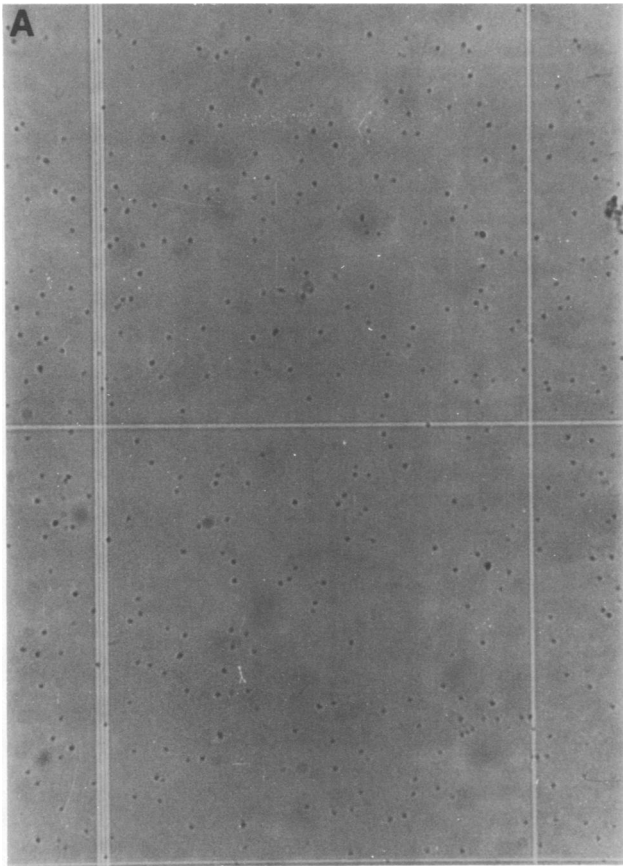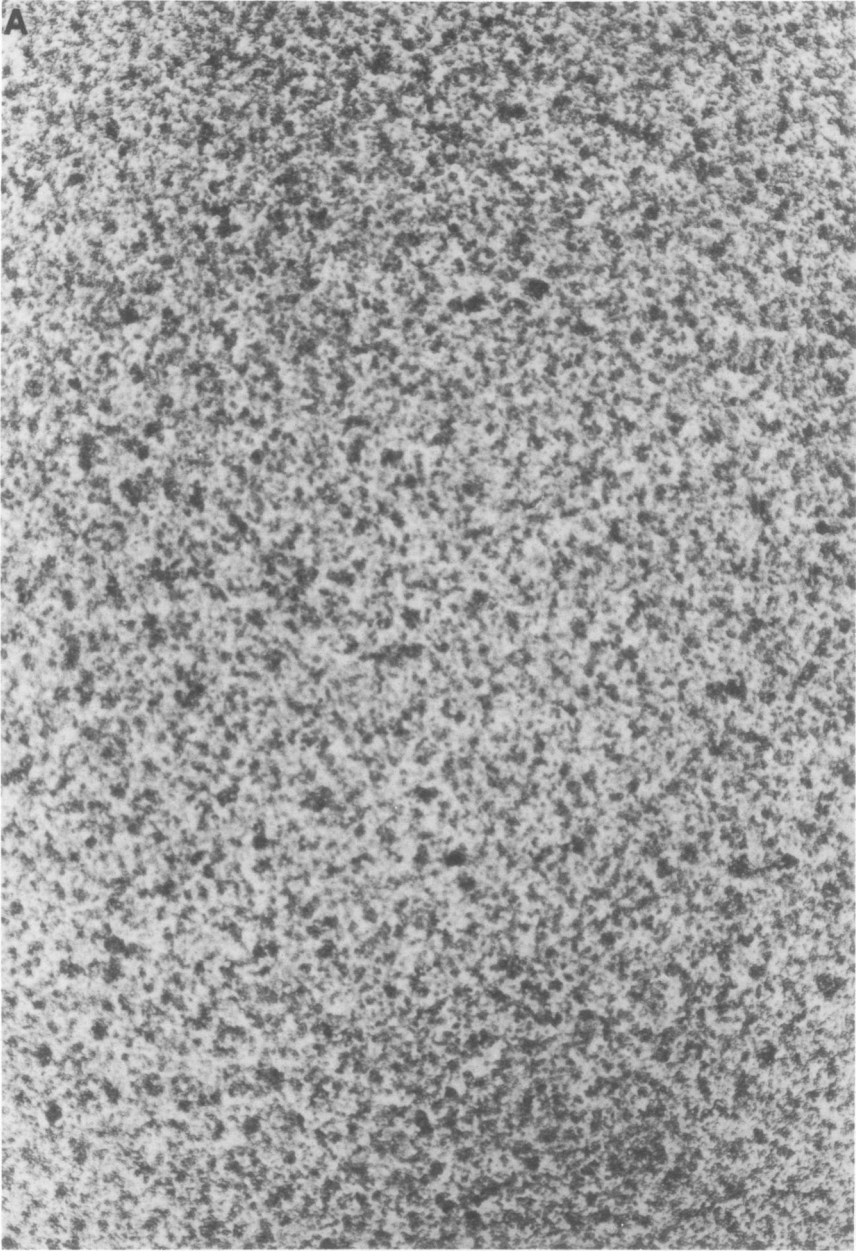
FIG. 2. (A) Control; polymorphonuclear leukocytes, 0.9 ml (5 × 10⁶/ml) with 0.1 ml of saline incubated at 37 C for 90 min. (B) Polymorphonuclear leukocytes with pneumococci (10⁸/ml) after 90 min. (C) Polymorphonuclear leukocytes with 50 µg of pneumococcal polysaccharide after 90 min. ×50.

ride, is obtained only in the final stages of the infection (7, 21).

The effect of bacterial endotoxin on clotting phenomena has been studied extensively. This substance, in relatively high concentrations, has been reported to decrease whole-blood and PRP clotting times (13, 18, 26, 28), cause platelet aggregation, and release platelet factor 3 (10, 13). Endotoxin also activated the complement system, predominantly by way of activation of C3–9 (11), which led to increased prothrombin consumption (36). The effect of bacte-
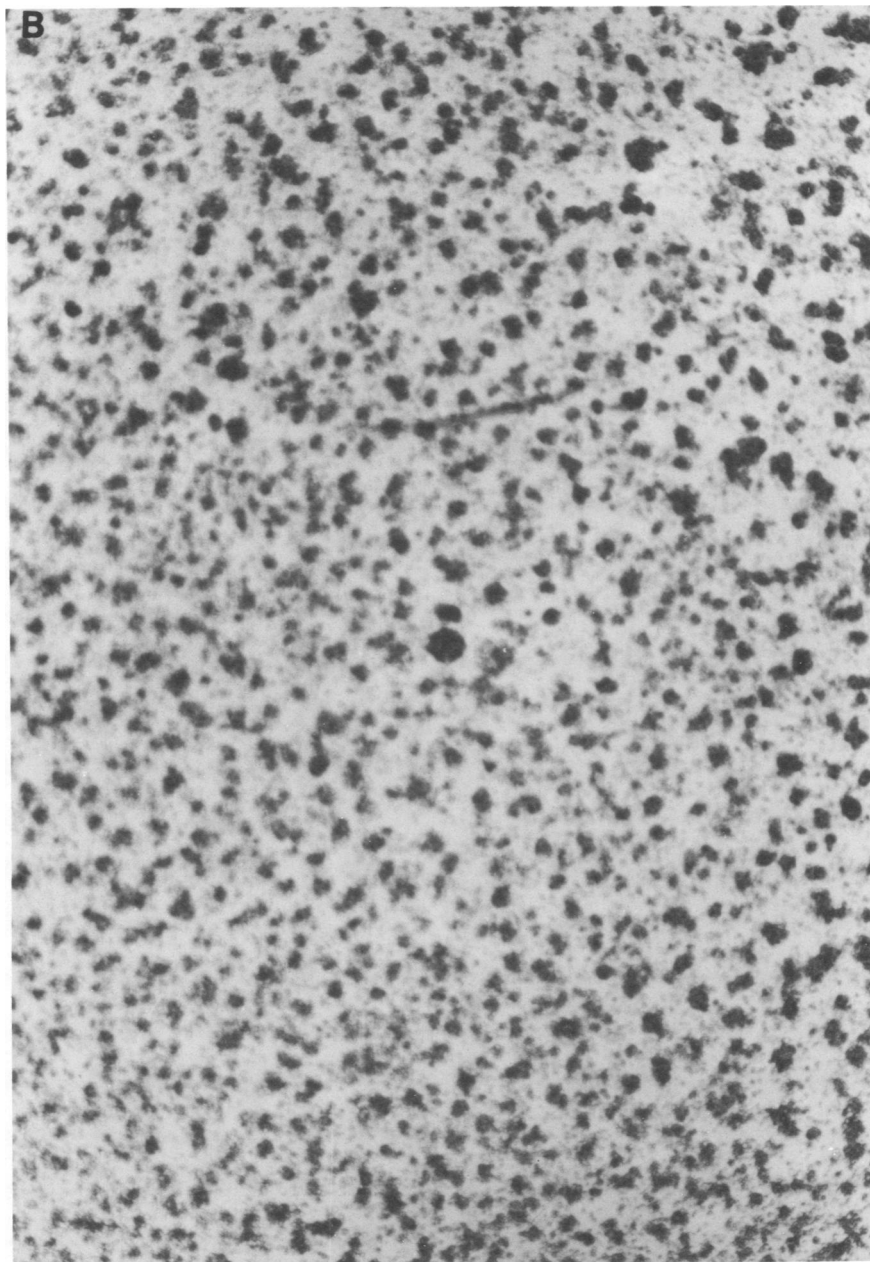
Fig. 2B

rial endotoxins on clotting factors and activating systems is unclear and has been attributed to activation of factor XII, release of platelet phospholipid, activation of complement, and release of PMN coagulant.

Platelet aggregation in the presence of pneumococci was dose dependent and was prevented by d-cAMP. However, pneumococcal polysac-charide failed to result in platelet aggregation. cAMP, when added to PRP, produces marked inhibition of platelet aggregation and clumping in response to many stimuli (29). It also blocks the release of platelet factor 3. Generally, those agents that stimulate platelet clumping are associated with a fall in platelet cAMP and either a fall in membrane-bound adenyl cyclase or a
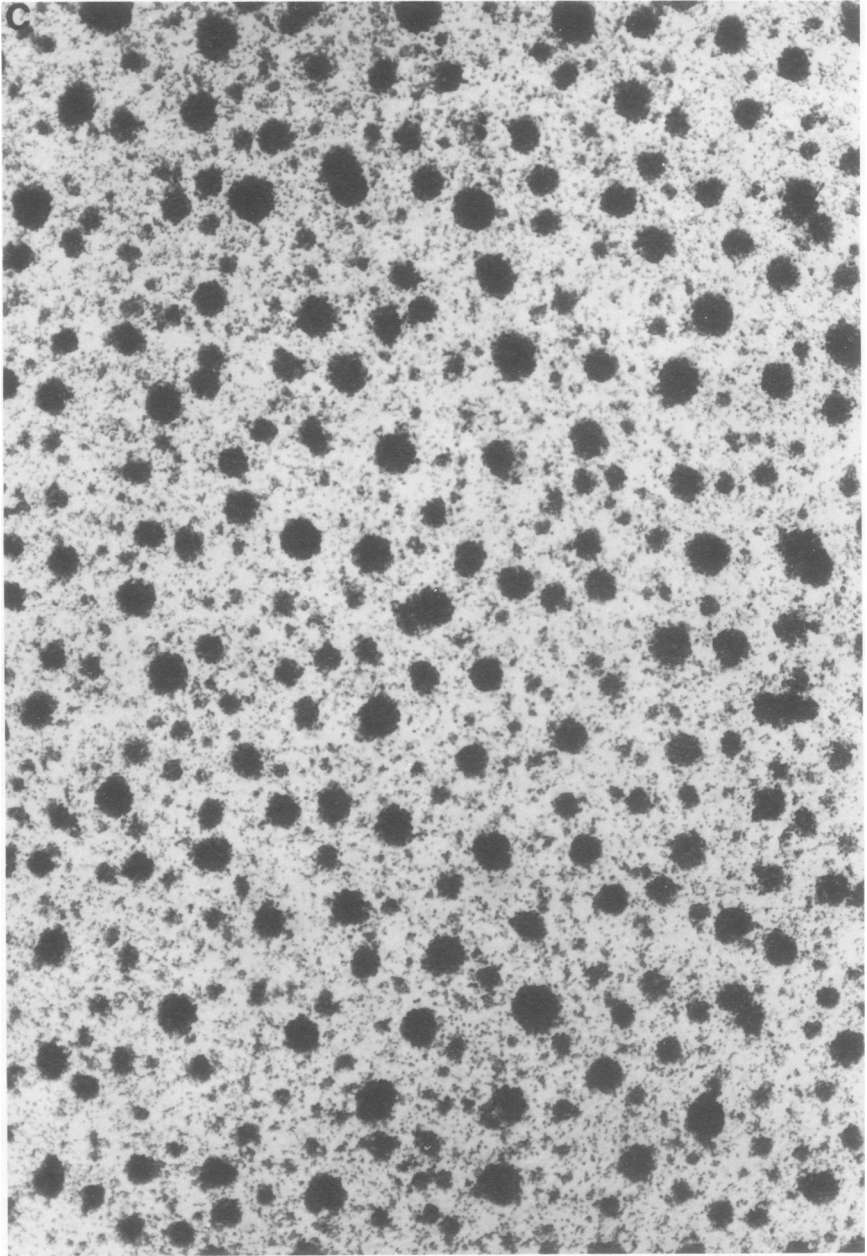
FIG. 2C

rise in phosphodiesterase. The shortened clot-ting time and clumping of PRP that was in-duced by pneumococci and corrected by d-cAMP indicates that the bacteria probably interact with this membrane-active nucleotide system and the platelet release reaction.

Pneumococci and pneumococcal polysaccha-ride caused the release of PMN coagulant. This was not prevented by d-cAMP. Release of coagu-lant was associated with dose-dependent clump-ing and eventual death of PMNs.

PMNs contain adenyl cyclase and phosphodi-esterase and can synthesize cAMP (3). Further-more, cAMP inhibits movement, candidacidal activity, and phagocytosis (34). Allison et al. demonstrated that PMNs aggregate during

phagocytosis of rough pneumococci (1). In our experiments, the PMNs did not phagocytose or kill virulent type I pneumococci, yet aggregated, and cAMP, which inhibits phagocytosis and candidacidal activity, inhibited neither release of coagulant nor aggregation. Furthermore, when pneumococci and PMNs were incubated together, neither cathepsin D nor beta-glucuronidase appeared in the cell-free supernatant (Guckian, unpublished observations). These data suggest that clumping may occur unassociated with phagocytosis.

Oram observed that filtrates from cultures of pneumococci and pneumococcal polysaccharide destroyed leukocytes (20). Previous studies documented profound neutropenia in pneumococcal bacteremia (21). Although margination and sequestration of PMNs may account for the neutropenia, direct toxic destruction is also a possibility. Since leukocytes contain coagulant activity (19), their alteration by pneumococci may contribute to the coagulopathy seen in pneumococcemia.

Previous studies demonstrated a progressive increase in fibrin(ogen) degradation products and partial thromboplastin time in rabbits with pneumococcal sepsis (Guckian, unpublished observations). This study demonstrates that pneumococci exert several effects on clotting parameters in vitro: (i) a direct thromboplastic effect; (ii) release of platelet thromboplastic substances; and (iii) release of polymorphonuclear coagulant. Our studies are compatible with the suggestion that pneumococci may act on the alternate pathway of activation of complement (Douglas P. Fine, personal communication), but the thromboplastic effect is not dependent on this pathway.

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