INFECTION AND IMMUNITY, Aug. 1980, p. 335-341
0019-9567/80/08-0335/07$02.00/0

Vol. 29, No. 2

Influence of Complement on the Chemiluminescent Response of Human Leukocytes to Immune Complex

STURE LÖFGREN,[1]* ARNE TÄRNVIK,[1] AND JAN CARLSSON[2]

Departments of Clinical Bacteriology[1] and Oral Microbiology,[2] University of Umeå, Umeå, Sweden

Vaccine bacteria of *Francisella tularensis* were mixed with human serum containing specific antibodies against the same organism, and the mixture was incubated at 37°C for 30 min. The mixture induced a two-peak chemiluminescent response in polymorphonuclear leukocytes. The initial peak was induced by a soluble agent, formed during incubation. The formation of this agent involved the activation of complement component C5. The second peak of the chemiluminescent response was induced by the opsonized bacteria; this peak was augmented by complement component C3, whereas C5 had no influence.

Phagocytes emit chemiluminescence when ingesting bacteria or other particles (1). Chemiluminescence probably includes membrane as well as intracellular events (11, 12, 19, 20) and is thought to depend on the generation of reactive products upon reduction of oxygen (1, 6, 11).

When polymorphonuclear (PMN) leukocytes have been exposed to opsonized particles, chemiluminescence has usually been traced as one peak of emittance. The time of maximal response has been found to be temperature dependent (4, 11) and to be reached within 10 to 20 min at ambient temperatures (3, 5, 6, 10, 14, 15, 25). Besides this peak, an initial peak has been reported to appear within 2 min (11, 24). In the present report, evidence is presented suggesting that the initial peak is induced by products formed by the activation of complement component C5.

MATERIALS AND METHODS

Medium. A culture medium containing 85% RPMI 1640 and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (RPMI-HEPES, GIBCO Diagnostics, Madison, Wis.) was used.

Serum products. Goat anti-human C5 antiserum and goat anti-human C3 antiserum were purchased from Meloy Laboratories (Springfield, Va.). Two preparations of complement component C5 were used. One was a gift from U. Johnsson, Lund, Sweden, prepared according to techniques described by Nilsson and Müller-Eberhard (20). It was more than 95% pure and appeared at a concentration of 1 mg/ml as one band in agarose electrophoresis and without any detectable C3. The other C5 preparation was purchased from Cordis Laboratories (Miami, Fla.). Both C5 preparations were washed by repeated dilution in saline (154 mM NaCl) and concentrated by ultrafiltration through immersible molecular separators (PE 809, Millipore Corp., Bedford, Mass.). Finally, they were concentrated to five times normal serum concentration as determined by radial immunodiffusion with human anti-C5 plates (Meloy Laboratories).

Chemicals. Epsilon-amino caproic acid (0.4 g/ml) was purchased from AB KABI (Stockholm, Sweden). Ethanolamine was purchased from BDH Chemicals Ltd. (Poole, England). *N*-formyl-L-methionyl-L-phenylalanine and zymosan A were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Bacteria. A viable, attenuated vaccine strain of *Francisella tularensis* LVS, was supplied by the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Md. (8). The bacteria were cultivated on McLeod agar and suspended in RPMI-HEPES at a density of 10^{11}/ml. They were then divided into 2-ml volumes in glass tubes and stored at a temperature below −70°C until used.

Zymosan. Twenty milligrams of zymosan was suspended in 5 ml of saline in a glass tube and gently shaken for 15 min in boiling water. The suspension was centrifuged at 1,500 × *g* for 10 min at 4°C, and the sedimented zymosan particles were suspended in saline at a density of 3 mg/ml.

Serum. Serum was prepared and pooled from five persons vaccinated 3 months previously with the tularemia vaccine (immune pool serum), as well as from five persons with no history of previous tularemia or tularemia vaccination (nonimmune pool serum). The serum pools were stored at −70°C. On the day of testing, 92 μl of epsilon-amino caproic acid was added per ml of serum, giving a concentration of 280 mM.

Removal of complement component C3 or C5 from serum. Affinity chromatography was used to remove C3 or C5 from serum. Cyanogen bromide-activated Sepharose 4B (Pharmacia AB, Uppsala, Sweden) particles (30 g) were treated as previously described (18), and three columns were prepared, containing goat anti-human C3 antiserum, goat anti-human C5 antiserum or normal goat serum, respectively.

Immune pool serum (3 ml) was applied to each column and eluted, and the eluates were concentrated to 2.5 ml and stored as previously described (18). The concentrated eluates were assayed for immunoglobulin G (IgG), IgM, IgA, C3, C4, and complement reactive protein by using Partigen plates (Behringwerke AG,

Marburg, West Germany) and for C5 by using human anti-C5 plates. There was no detectable C3 in the serum that had been chromatographed in the column containing C3 antiserum and no detectable C5 in the serum that had been chromatographed in the column containing C5 antiserum. Otherwise no quantitative difference was found among the three chromatographed sera. The hemolytic activity of serum was tested in twofold dilutions (17). No hemolytic activity was found in the sera that had been chromatographed on the columns containing C3 or C5 antiserum. When these two chromatographed sera were mixed (1:1), the hemolytic activity was one titer step lower than that of nonchromatographed serum. The hemolytic activity of the serum that had been chromatographed on the column containing normal goat serum was also one titer step lower than that of nonchromatographed serum. When 1 part of the concentrated preparation of complement component C5 was added to 2.5 parts of C5-depleted serum, the hemolytic activity was fully reconstituted. On the day of the chemiluminescence test, 92 μl of epsilon-amino caproic acid was added per ml of chromatographed serum, giving a concentration of 280 mM.

Preparation of human PMN leukocytes. Venous blood was obtained from healthy volunteers with no history of previous tularemia or tularemia vaccination and lacking demonstrable serum agglutinins against *F. tularensis*. The PMN leukocytes were prepared as previously described (18). Dextran sedimentation of the erythrocytes (23) was performed, and the remaining erythrocytes were lysed with 0.87% ammonium chloride (7). After preparation, the PMN leukocytes were suspended in RPMI-HEPES at a density of 11 $\times 10^6$ PMN leukocytes per ml.

Preparation of bacteria, zymosan, and serum for chemiluminescent tests. A suspension of vaccine bacteria (120 μl) at a density of 3.75×10^9 bacteria per ml of RPMI-HEPES, or a suspension of zymosan particles (120 μl) at a density of 3 mg per ml of saline, was mixed with 360 μl of serum and incubated at 37°C for 30 min. After incubation, the mixture was used immediately for initiation of chemiluminescence in PMN leukocytes or kept at 4°C until used.

In some experiments, the cell-serum mixture was centrifuged at 1,500 × *g* for 10 min at 4°C. The pellet was washed once in RPMI-HEPES and then suspended to 480 μl in RPMI-HEPES. The supernatant fluid of the cell-serum mixture was recentrifuged before use.

In two kinds of experiments, the effect of C5 supplementation was studied. In the first, 110 μl of a C5 preparation was added to 275 μl of C5-depleted serum to which 92 μl of epsilon-amino caproic acid had been added per ml of serum. A suspension of vaccine bacteria (120 μl) was then incubated with 360 μl of this solution at 37°C for 30 min. In the other kind of experiment, a suspension of vaccine bacteria (220 μl) was incubated with 660 μl of C5-depleted serum. The incubated mixture was then centrifuged at 1,500 × *g* for 10 min at 4°C. The pellet was washed once in RPMI-HEPES and suspended to 660 μl RPMI-HEPES. To this supension was then added 220 μl of a C5 preparation to which 92 μl of epsilon-amino caproic acid per ml had been added. After incubation at 37°C for 30 min, 400 μl of this bacterium-C5 mixture was used immediately for initiation of chemiluminescence in PMN leukocytes. The rest of the bacterium-C5 mixture (480 μl) was centrifuged at 1,500 × *g* for 10 min at 4°C. The pellet was washed once in RPMI-HEPES and suspended to 480 μl in RPMI-HEPES. The supernatant fluid of the bacterium-C5 mixture was recentrifuged before use.

Demonstration of chemiluminescent response. The chemiluminescent response was measured in a liquid scintillation counter (model LS 3133 T, Beckman Instruments, Inc., Fullerton, Calif.) kept at 25°C, and the coincidence circuit was turned off. The emission of chemiluminescence was measured in glass vials (no. 161698, Beckman) which had been kept in the dark for at least 24 h before use. They were filled under red illumination with 4.1 ml of RPMI-HEPES and 1.0 ml of the PMN leukocyte suspension and were allowed to stand until a stable background of chemiluminescence, fewer than 20,000 cpm, was obtained. To activate the PMN leukocytes, 0.4 ml of the preparation described in the previous paragraph was added, and the vials were stirred for 10 s. The light emission was recorded for 12 s every 16 s for at least 30 min.

RESULTS

Vaccine bacteria and immune pool serum were mixed and incubated at 37°C for 30 min. This incubated mixture induced a chemiluminescent response in PMN leukocytes. When the time course of chemiluminescent emission was studied, an initial peak was found within 2 min of initiation and a second peak was found within 10 to 20 min (Fig. 1a). The possible release of chemiluminescence-inducing agents into solution during incubation of the mixture was studied. The supernatant fluid of the incubated mixture did not induce any chemiluminescent activity. However, when epsilon-amino caproic acid was present during incubation, the supernatant fluid induced a peak of chemiluminescence within 2 min of initiation, coinciding with the initial peak of chemiluminescence induced by the mixture (Fig. 1b). Bacteria that had been washed after incubation did not induce any initial peak but did induce a peak coinciding with the second peak of chemiluminescence induced by the mixture (Fig. 1c). Epsilon-amino caproic acid did not affect the chemiluminescent response induced by the mixture and did not induce any chemiluminescent response by itself. Furthermore, an incubated mixture of bacteria and RPMI-HEPES induced no chemiluminescent response in PMN leukocytes, nor did the immune pool serum induce a chemiluminescent response by itself. An incubated mixture of bacteria and nonimmune pool serum induced no second peak of chemiluminescence in PMN leukocytes; but, in some experiments, there was a small initial peak within 2 min.

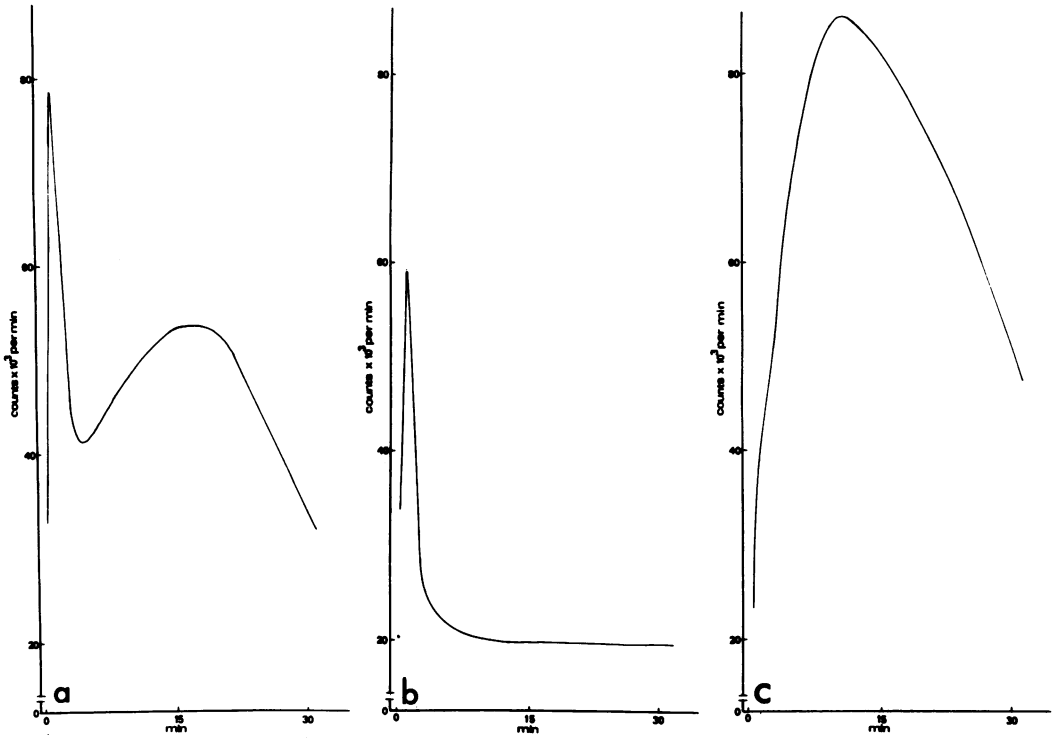

FIG. 1. *Time trace of chemiluminescence emitted from human PMN leukocytes when exposed to (a) an incubated mixture of vaccine bacteria and immune pool serum, (b) the supernatant fluid of this mixture, and (c) a washed pellet of this mixture obtained after centrifugation. The chemiluminescence was recorded for 12 s every 16 s. The three curves were obtained from the same experiment.*

The immune pool serum was heated at 56°C for 30 min; after that, 92 μl of epsilon-amino caproic acid was added per ml of serum. An incubated mixture of bacteria and the heat-treated immune pool serum induced no initial peak but did induce a second peak of chemiluminescence (Fig. 2). The second peak was of a lower magnitude than that induced when non-treated serum was used. Supernatant fluid from the incubated mixture of bacteria and the heat-treated immune pool serum induced no chemiluminescent activity (Fig. 2).

The supernatant fluid obtained after incubation of a mixture of bacteria and immune pool serum in the presence of 210 mM epsilon-amino caproic acid was treated at 56°C for 30 min. This fluid induced an initial peak of chemiluminescence, which was of the same order of magnitude as that induced by nontreated supernatant fluid. All of these results were reproduced when zymosan and normal serum were used instead of vaccine bacteria and immune pool serum.

The dependence on complement activation of the initial burst of chemiluminescence was studied. A mixture of bacteria and immune pool serum, from which complement component C3 had been absorbed, was incubated. The incubated mixture induced virtually no initial peak of chemiluminescence, and the second peak was very low (Fig. 3). When the mixture contained immune pool serum, from which C5 had been absorbed, no initial peak was induced, whereas the second peak was present. When C3-depleted serum and C5-depleted serum were pooled and incubated with the bacteria, the mixture induced an initial as well as a second peak of chemiluminescence into the leukocytes (Fig. 3). When a preparation of complement component C5 was added to C5-depleted immune pool serum and the serum was incubated together with the vaccine bacteria, the incubated mixture induced a two-peak response (Fig. 4). After incubation in the presence of C5-depleted serum, bacteria were centrifuged, washed, and suspended in RPMI-HEPES. The suspension induced no initial peak but did induce a second peak of chemiluminescence (Fig. 5). The pellet was also suspended in RPMI-HEPES, supplemented with a preparation of complement component C5. After incubation, the suspension was added to PMN leukocytes. The suspension induced an initial as well as a second peak of chemiluminescence into

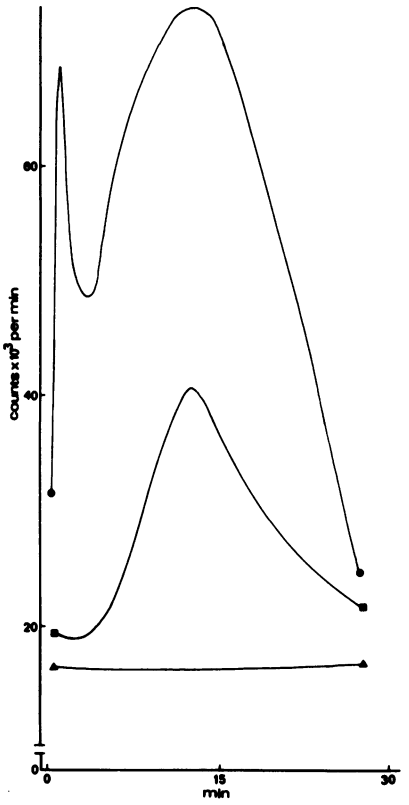

FIG. 2. *Chemiluminescence induced in human PMN leukocytes by an incubated mixture of vaccine bacteria and heat-treated (56°C, 30 min) immune pool serum containing 210 mM epsilon-amino caproic acid* (■) *and by the supernatant fluid of this mixture* (▲). *The chemiluminescence induced by an incubated mixture of vaccine bacteria and nontreated immune pool serum is also shown* (●).

the leukocytes (Fig. 5). The suspension was centrifuged, and the supernatant fluid was tested; it induced no chemiluminescence. These results were the same for both C5 preparations used. The C5 preparations, as well as the vaccine bacteria, did not induce any chemiluminescent activity by themselves, nor did the addition of saline, in the same concentration as used to dissolve the complement component C5, influence the chemiluminescent response. In addition, immune pool serum chromatographed on a column to which normal goat serum was coupled did not influence the ability of the serum to induce two peaks of chemiluminescence in PMN leukocytes.

The chemiluminescence induced in PMN leukocytes by the chemotactic oligopeptide *N*-formyl-L-methionyl-L-phenylalanine was studied. A peak of chemiluminescence was induced within 2 min of initiation, and this peak coincided with that induced by a mixture of bacteria and immune pool serum (Fig. 6).

DISCUSSION

Vaccine bacteria of *F. tularensis* were mixed with human serum containing specific antibodies against the vaccine bacteria, and the mixture was incubated at 37°C for 30 min. The incubated mixture induced a two-peak chemiluminescent response in PMN leukocytes. The initial peak of the response occurred within 2 min and depended on a soluble agent formed during opsonization. The second peak occurred within 10 to 20 min and was induced by the opsonized bacteria.

The second peak depended on the activation of complement component C3. This conclusion seems to be valid, since the second peak was markedly reduced by the depletion of C3 from immune pool serum, whereas it was virtually unaffected by the depletion of C5. However, a low chemiluminescent response was induced also by a mixture of C3-depleted or heat-treated immune pool serum and bacteria. This response was probably due to the presence on the bacteria of IgG antibodies. A mixture of specific IgG antibodies and vaccine bacteria has previously

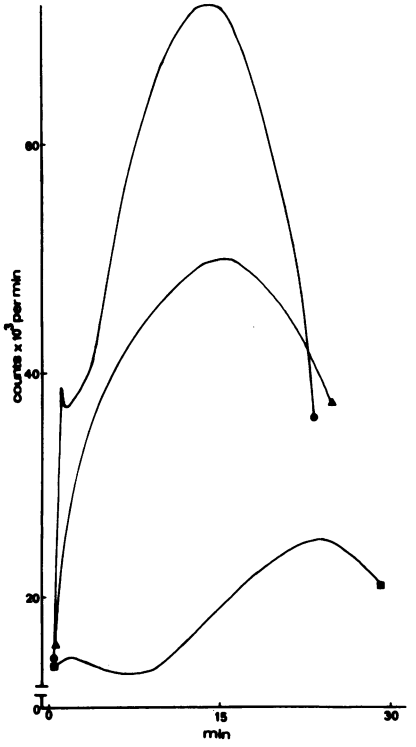

FIG. 3. *Chemiluminescence induced in human PMN leukocytes by an incubated mixture of vaccine bacteria and immune pool serum from which C3* (■) *or C5* (▲) *had been absorbed and by an incubated mixture of vaccine bacteria and a pool (1:1) of the C3-depleted and the C5-depleted serum* (●).

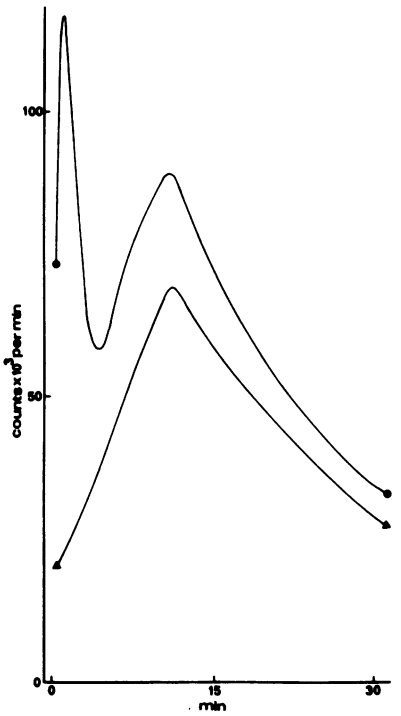

FIG. 4. *Chemiluminescence induced in human PMN leukocytes by an incubated mixture of vaccine bacteria and immune pool serum from which complement component C5 had been absorbed* (▲) *and by a mixture of vaccine bacteria and this C5-depleted immune pool serum to which complement component C5 had been added* (●).

been found to induce chemiluminescence in PMN leukocytes (18).

Inducement of the initial peak of chemiluminescence was found to depend on the activation of C5. An incubated mixture of bacteria and immune pool serum from which C5 had been depleted induced no initial but an intact second peak. When C5 was added to C5-depleted immune pool serum before incubation with bacteria, the incubation mixture induced a two-peak response. When, finally, bacteria were opsonized with C5-depleted immune pool serum, washed, and incubated in the presence of C5, the mixture induced a two-peak response. Bacteria or C5 did not induce any chemiluminescent response by themselves. These results strongly suggest that the agent inducing the initial peak of chemiluminescence was formed by the activation of C5. In one experiment, no chemiluminescence-inducing agent was recovered in the soluble fraction of an incubated mixture, although the incubated mixture as such induced a two-peak response. This happened when bacteria were opsonized with C5-depleted immune pool serum, washed, and incubated in the presence of C5. Neither the soluble nor the particulate fraction of the mixture induced any initial peak of chemiluminescence. The result does not exclude the presence of a soluble chemiluminescence-inducing agent in this incubated mixture. The concentration of the agent may have been too low. Possibly, the effect of the soluble agent may be augmented by particles present in the incubation mixture. Such a phenomenon has been reported by Allred and Hill (2). They determined the chemiluminescent response of PMN leukocytes to various chemotactic agents and found that these agents induced no response unless zymosan was simultaneously present.

The nature of the soluble agent is unknown. One obvious possibility is that the agent is identical to C5a. C5a is known to be a potent chemotactic agent for PMN leukocytes (16), and other chemotactic agents such as *N*-formylated oligopeptides (22) are known to induce chemiluminescence in PMN leukocytes with kinetics similar to those of the chemiluminescent response to the soluble agent used in the present experiments (13). This coincidence of kinetics was confirmed in the present study. Similar to C5a, the soluble agent was heat stable and protected by epsilon-amino caproic acid (9, 16).

Although the present results strongly indicated that elicitation of the initial peak of chemiluminescence depends on the activation of C5,

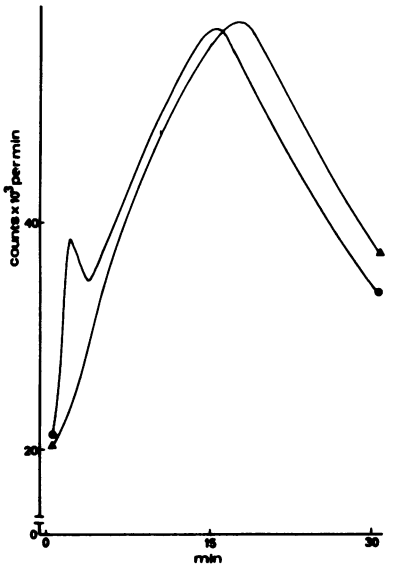

FIG. 5. *Chemiluminescence induced in human PMN leukocytes by a suspension of the pellet obtained by centrifuging an incubated mixture of vaccine bacteria and immune pool serum from which complement component C5 had been absorbed* (▲) *and by an incubated mixture of this suspension and complement component C5* (●).

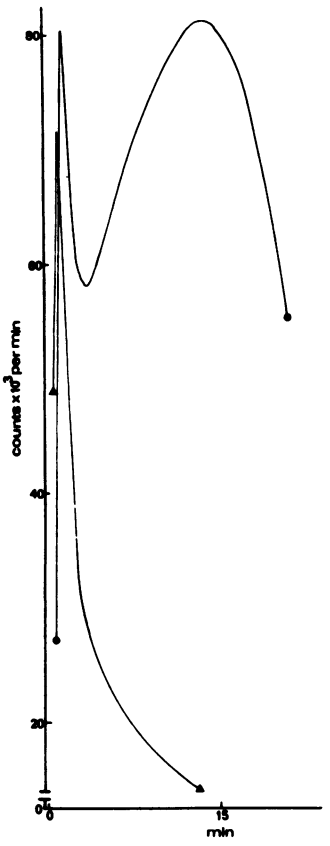

FIG. 6. *Chemiluminescence induced in human PMN leukocytes by 4 μM N-formyl-L-methionyl-L-phenylalanine (▲) and by an incubated mixture of vaccine bacteria and immune pool serum (●).*

the experimental conditions did not exclude the possibility that the peak was induced also by the activation of complement components later in the sequence than C5.

The data suggest that the two peaks of chemiluminescence found resulted from an initial response of the PMN leukocytes to a chemotactic stimulus followed by a response of the leukocytes to the opsonized particles. It is an interesting question whether these responses are due to different metabolic events of the leukocytes or to one specific metabolic activity, triggered by different stimuli.

Similar chemiluminescent responses of the PMN leukocytes were obtained whether vaccine bacteria and immune pool serum or zymosan and normal serum were used. These results indicated that the chemiluminescent responses were induced by products formed by either of the two principal ways of complement activation. *F. tularensis* has been found to activate complement by the classical (18), and zymosan by the alternative (10), pathway.

ACKNOWLEDGMENTS

This investigation was supplemented by grants from the National Defense Research Institute and from the Medical Faculty, University of Umeå, Umeå, Sweden.

We thank Wigert Sjöberg for skillful technical assistance.

LITERATURE CITED

1. **Allen, R. C., R. L. Stjernholm, and R. H. Steele.** 1972. Evidence for the generation of an electronic excitation state(s) in human polymorphonuclear leukocytes and its participation in bactericidal activity. Biochem. Biophys. Res. Commun. **47**:679–684.
2. **Allred, C. D., and H. R. Hill.** 1978. Effect of chemoattractants on chemiluminescence. Infect. Immun. **19**: 833–838.
3. **Allred, C. D., A. O. Shigeoka, and H. R. Hill.** 1979. Evaluation of group B streptococcal opsonins by radiolabeled bacterial uptake. J. Immunol. Methods **26**:355–363.
4. **Andersen, B. R., and A. M. Brendzel.** 1978. Use of a unique chemiluminescence spectrometer in a study of factors influencing granulocyte light emission. 1978. J. Immunol. Methods **19**:279–287.
5. **Andersen, B. R., A. M. Brendzel, and T. F. Lint.** 1977. Chemiluminescence spectra of human myeloperoxidase and polymorphonuclear leukocytes. Infect. Immun. **17**: 62–66.
6. **Cheson, B. D., R. L. Christensen, R. Sperling, B. E. Kohler, and B. M. Babior.** 1976. The origin of the chemiluminescence of phagocytosing granulocytes. J. Clin. Invest. **58**:789–796.
7. **Dioguardi, N., A. Agostoni, G. Fiorelli, and B. Lomanto.** 1963. Characterization of lactic dehydrogenase of normal human granulocytes. J. Lab. Clin. Med. **61**:713–723.
8. **Eigelsbach, H. T., and C. M. Downs.** 1961. Prophylactic effectiveness of live and killed tularemia vaccines. I. Production of vaccine and evaluation in the white mouse and guinea pig. J. Immunol. **87**:415–425.
9. **Goldstein, I. M., D. Roos, H. B. Kaplan, and G. Weissmann.** 1975. Complement and immunoglobulins stimulate superoxide production by human leukocytes independently of phagocytosis. J. Clin. Invest. **56**:1155–1163.
10. **Grebener, J. V., E. L. Mills, B. H. Gray, and P. G. Quie.** 1977. Comparison of phagocytic and chemiluminescence response of human polymorphonuclear neutrophils. J. Lab. Clin. Med. **89**:153–159.
11. **Harvath, L., H. J. Amirault, and B. R. Andersen.** 1978. Chemiluminescence of human and canine polymorphonuclear leukocytes in the absence of phagocytosis. J. Clin. Invest. **61**:1145–1154.
12. **Harvath, L., and B. R. Andersen.** 1979. Defective initiation of oxidative metabolism in polymorphonuclear leukocytes. N. Engl. J. Med. **300**:1130–1135.
13. **Hatch, G. E., D. E. Gardner, and D. B. Menzel.** 1978. Chemiluminescence of phagocytic cells caused by *N*-formyl-methionyl peptides. J. Exp. Med. **147**:182–195.
14. **Hemming, V. G., R. T. Hall, P. G. Rhodes, A. O. Shigeoka, and H. R. Hill.** 1976. Assessment of group B streptococcal opsonins in human and rabbit serum by neutrophil chemiluminescence. J. Clin. Invest. **58**:1379–1387.
15. **Hill, H. R., N. A. Hogan, J. F. Bale, and V. G. Hemming.** 1977. Evaluation of nonspecific (alternatively pathway) opsonic activity by neutrophil chemiluminescence. Int. Arch. Allergy Appl. Immunol. **53**:490–497.
16. **Hugli, T.E., and H. J. Müller-Eberhard.** 1978. Anaphylatoxins: C3a and C5a. Adv. Immunol. **26**:1–53.
17. **Lange, K.** 1960. Über den immunologischen Mechanismus der akuten und chronischen Glomerulonephritis, p. 232–245. *In* E. Wollheim (ed.), Glomeruläre und

Tubuläre Nierenerkrankungen. Internationales Nierensymposion Würzburg. Georg Thieme Verlang, Stuttgart.
18. **Löfgren, S., A. Tärnvik, and J. Carlsson.** 1980. Demonstration of opsonizing antibodies to *Francisella tularnesis* by leukocyte chemiluminescence. Infect. Immun. **29**:329–334.
19. **McPhail, L. C., L. R. DeChatelet, and R. B. Johnston, Jr.** 1979. Generation of chemiluminescence by a particulate fraction isolated from human neutrophils. Analysis of molecular events. J. Clin. Invest. **63**:648–655.
20. **Nilsson, U. R., and H. J. Müller-Eberhard.** 1965. Isolation of β1F-globulin from human serum and its characterization as the fifth component of complement. J. Exp. Med. **122**:277–298.
21. **Rosen, H., and S. J. Klebanoff.** 1976. Chemiluminescence and superoxide production by myeloperoxidase-deficient leukocytes. J. Clin. Invest. **58**:50–60.
22. **Schiffmann, E., B. A. Corcoran, and S. M. Wahl.** 1975. *N*-Formylmethionyl peptides as chemoattractants for leucocytes. Proc. Natl. Acad. Sci. U.S.A. **72**:1059–1062.
23. **Skoog, W. A., and W. S. Beck.** 1956. Studies on the fibrinogen, dextran and phytohemagglutinin methods of isolating leukocytes. Blood **11**:436–454.
24. **Stjernholm, R. L., R. C. Allen, R. H. Steele, W. W. Waring, and J. A. Harris.** 1973. Impaired chemiluminescence during phagocytosis of opsonized bacteria. Infect. Immun. **7**:313–314.
25. **Webb, L. S., B. B. Keele, Jr., and R. B. Johnston, Jr.** 1974. Inhibition of phagocytosis-associated chemiluminescence by superoxide dismutase. Infect. Immun. **9**: 1051–1056.