INFECTION AND IMMUNITY, Aug. 1980, p. 329-334
0019-9567/80/08-0329/06$02.00/0

Vol. 29, No. 2

Demonstration of Opsonizing Antibodies to *Francisella tularensis* by Leukocyte Chemiluminescence

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Twenty-three individuals were vaccinated with a viable attenuated strain of *Francisella tularensis,* and blood was collected at various time intervals during 4 weeks. To demonstrate opsonizing antibodies, a mixture of serum and vaccine bacteria was incubated, whereafter the chemiluminescent response of polymorphonuclear (PMN) leukocytes to this mixture was recorded. No opsonizing antibodies against *F. tularensis* were found in sera obtained before vaccination. Eleven days after vaccination, sera from nine individuals, and 21 days after vaccination, sera from all 23 individuals contained antibodies. Antibodies were demonstrated earlier with the chemiluminescent technique than with the agglutination reaction. Heat treatment (56°C, 30 min) or removal of complement component C3 from immune serum reduced the chemiluminescent response of the leukocytes. A high chemiluminescent response of the leukocytes was induced by immunoglobulin G (IgG)- and IgM-enriched fractions of immune serum in the presence of complement. In the absence of complement, the IgG fraction induced a low chemiluminescent response; the IgM fraction induced no response at all.

Tularemia is caused by *Francisella tularensis,* which is endemic among small rodents in the Northern Hemisphere and causes epidemics in humans. Depending upon the mode of transmission from the animal or arthropod vector, several clinical forms of tularemia, such as glandular, abdominal, septic, and pulmonary, may result. Mortality rates are less than 1 to 5% and the highest mortality is associated with the untreated septic and pulmonary forms of the disease. The length of convalescence and the mortality rates are reduced by early treatment with antibiotics (14). A viable attenuated vaccine exists (8), and vaccination is known to reduce the incidence of the disease (3). After infection or vaccination, circulating agglutinating antibodies (9) and a cell-mediated immune response (21) develop. Cell-mediated immunity seems to be important in defense, whereas the role of specific antibodies is unknown (11). For diagnosis of the disease, however, the antibodies are of major importance; demonstration of agglutinating antibodies (9) in the serum from a patient is the method used in most laboratories. Antibodies with biological functions other than agglutination have, however, not been studied in humans. Opsonizing antibodies against *F. tularensis* develop in the serum of monkeys after immunization (4). The appearance of such antibodies after natural infection or vaccination in humans has been little studied.

Opsonizing antibodies induce several activities into polymorphonuclear (PMN) leukocytes. A respiratory burst and release of lysosomal enzymes usually precede engulfment of the bacteria. The leukocytes may ultimately kill the bacteria. All these activities can be studied in vitro and used in detecting opsonizing antibodies (5, 16, 19, 22). One activity of leukocytes that is easily demonstrated is the emission of chemiluminescence (1, 2). In this report the chemiluminescent response of PMN leukocytes has been used in studying the appearance in humans of opsonizing antibodies against *F. tularensis* after vaccination with a viable vaccine strain.

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.

Bacteria. A viable attenuated strain of *F. 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.

Vaccination and collection of serum samples. Twenty-three healthy volunteers without a previous history of tularemia and without agglutinating serum antibodies against *F. tularensis* were vaccinated intracutaneously on the arm with the tularemia vaccine (8) by the multiple-pressure technique. Blood was collected before and on days 3, 7, 11, 16, 21, and 25 after vaccination. Serum was prepared and divided into 1-ml portions in glass vials, which were stored at a

temperature below −70°C.

Pooled serum from five persons vaccinated 3 months previously (immune pool serum) and from five persons without previous tularemia or tularemia vaccination (nonimmune pool serum) were similarly stored.

Removal of complement component C3 from serum. Affinity chromatography was used to remove C3 from serum. Cyanogen bromide-activated Sepharose 4B (Pharmacia AB, Uppsala, Sweden) particles (10 g) were swollen and washed in 1 mM hydrochloric acid and equilibrated in 0.1 M sodium bicarbonate buffer, pH 9.0, containing 0.5 M sodium chloride. Excess bicarbonate buffer solution was removed, and 4 ml of goat anti-human C3 antiserum (Meloy Laboratories, Springfield, Va.) in 36 ml of the bicarbonate buffer solution was added. The material was then treated according to the directions of the manufacturer with the exception that 0.67 M instead of 1 M ethanolamine (BDH Chemicals Ltd., Poole, England) was used to block remaining reactive groups. The material was then put into a column (15 by 300 mm, Pharmacia). Another column was prepared with normal goat serum instead of goat anti-human C2 antiserum.

Immune pool serum (3 ml) was applied to each column at room temperature and eluted with phosphate-buffered saline, pH 7.4 (12.6 mM KH_2PO_4, 54.0 mM Na_2HPO_4, and 85.5 mM NaCl) at a flow rate of about 12 drops/min. The eluate was kept in an ice bath and concentrated to 2.5 ml by ultrafiltration through immersible molecular separators (PE 809, Millipore Corp., Bedford, Mass.) and was then stored at −70°C. The concentrated eluates were assayed for immunoglobulin G (IgG), IgM, IgA, C3, C4, and complement reactive protein by radial immunodiffusion by using Partigen plates (Behringwerke AG, Marburg, West Germany). There was no detectable C3 in the serum that had been chromatographed in the column containing C3 antiserum. Otherwise no quantitative difference was found between the two chromatographed sera.

Separation of IgM and IgG antibodies. Fractionation by gel chromatography on Sephadex G-200 (Pharmacia) was performed. Immune pool serum (3 ml) was applied to a column (900 by 26 mm) and eluted at 4°C with phosphate-buffered saline, pH 7.4, at a flow rate of 9 ml/h. Fractions of 5 ml were collected and tested for the presence of IgM and IgG by radial immunodiffusion in Partigen plates. Fractions containing detectable IgM or IgG but not both were pooled. The pooled fractions were concentrated 20 to 40 times by ultrafiltration through immersible molecular separators at 4°C and were then stored at −70°C. On the day of testing, the material was thawed and heated to 56°C for 30 min.

Preparation of human PMN leukocytes. Venous blood was obtained from healthy volunteers who had not previously had tularemia or tularemia vaccination and lacked demonstrable serum agglutinins against *F. tularensis*. The blood was collected in heparinized glass tubes (Becton, Dickinson & Co., Rutherford, N. J.). PMN leukocytes were separated from erythrocytes by dextran sedimentation (20). Three parts of blood were mixed with 1 part of 6% dextran. After sedimentation for 1 h at room temperature, the leukocyte-rich supernatant fluid was removed and centrifuged at 350 × *g* for 10 min. The PMN leukocytes were resuspended and treated with 0.87% ammonium chloride to lyse the remaining erythrocytes (6). They were washed twice and suspended in RPMI-HEPES at a density of 11×10^6 PMN leukocytes per ml.

Opsonization. One part of vaccine bacteria at a density ranging from 3×10^8 to 1×10^{11} bacteria per ml of RPMI-HEPES was mixed with 3 parts of serum and incubated at 37°C for 30 min. The suspension was then kept at 4°C until used. When bacteria at an increasing density were opsonized with immune pool serum and added to 11×10^6 PMN leukocytes, there was an increase in the chemiluminescent response to a maximum, when 2×10^9 bacteria/ml was used. When the density of bacteria exceeded 5×10^{10}/ml a decrease in the chemiluminescent response followed. The optimal bacterial density was the same when varying dilutions of immune pool serum were used. A density of 3.75×10^9 bacteria per ml of RPMI-HEPES was chosen for all chemiluminescence experiments, giving a ratio of bacteria to PMN leukocytes of about 300:1.

Demonstration of the chemiluminescent response. The chemiluminescent response was measured at ambient temperature in a liquid scintillation counter (model LS 3133 T, Beckman Instruments, Inc., Fullerton, Calif.), with the coincidence circuit turned off. The emission of chemiluminescence was measured in glass vials (no. 161698, Beckman) which were 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 PMN leukocyte suspension and were allowed to stand until a stable background of chemiluminescence was obtained. To activate the PMN leukocytes, 0.4 ml of opsonized bacteria was added and the vials were stirred for 30 s. The light emission was recorded for 30 s every 10 min for 30 min, except in one experiment where the light emission was recorded for 12 s every 16 s. The total number of counts per 30 min was estimated, and this value was used when comparing the results of various tests (1).

Agglutination test. Suspensions of *F. tularensis* were purchased from Statens Bakteriologiska Laboratorium, Stockholm, Sweden. One part of serum and 4 parts of phosphate-buffered saline were mixed. Twofold dilutions of the mixture were made in phosphate-buffered saline, and 0.5 ml of each dilution was mixed with 0.5 ml of the bacterial suspension. The tubes were incubated for 20 h at 37°C, and the antibody titer was expressed as the reciprocal of the highest dilution giving agglutination with a clear supernatant. Agglutination tests could not be evaluated with a serum dilution lower than 1:20 because of opalescence.

RESULTS

Appearance of opsonizing and agglutinating antibodies after vaccination against tularemia. Sera obtained at various intervals after vaccination of 23 individuals were tested. Sera obtained before vaccination induced a chemiluminescent response just above the background values (~6×10^5 counts/30 min). The response was considered to be increased when counts were 3×10^5 or more above the response

induced by serum obtained before vaccination. An increased chemiluminescent response was demonstrated earlier than agglutinating antibodies in 20 of the 23 individuals; the response occurred at the same time as agglutinating antibodies in three (Fig. 1).

Chemiluminescent response induced by heat-treated, nontreated and C3-deficient immune pool serum. The chemiluminescent response induced by the immune pool serum was reduced when the serum was treated at 56°C for 30 min. The low chemiluminescent response induced by the nonimmune pool serum was not influenced by heat treatment (Fig. 2). The chemiluminescent response induced by the immune pool serum was also reduced when complement component C3 was removed from the serum by affinity chromatography (Fig. 3). As a control, immune pool serum was chromatographed on a column to which normal goat serum had been coupled. This procedure did not significantly influence the ability of the serum to induce chemiluminescence.

Chemiluminescent response induced by antibodies of IgM and IgG class. IgM- and IgG-enriched fractions from immune pool serum were supplemented with an equal volume of fresh or heat-treated nonimmune serum in order to study the chemiluminescent response to these fractions in the presence and in the absence of intact complement. In the presence of a fresh serum supplement, the IgM as well as the IgG fraction induced a strong chemiluminescent response. When the serum supplement was heat treated (56°C, 30 min), no chemiluminescent response was induced by the IgM fraction, whereas the response to the IgG fraction was only partly reduced (Fig. 4). Thus, the IgM-induced chemiluminescent response seemed to have an obligatory requirement for complement

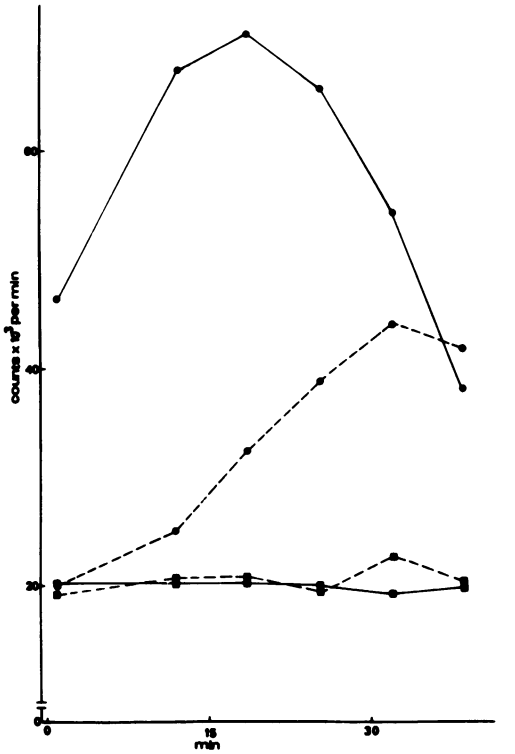

FIG. 2. *Chemiluminescent response induced by heat-treated (56°C, 30 min) (--●--) and nontreated (—●—) immune pool serum and by heat-treated (--■--) and nontreated (—■—) nonimmune pool serum.*

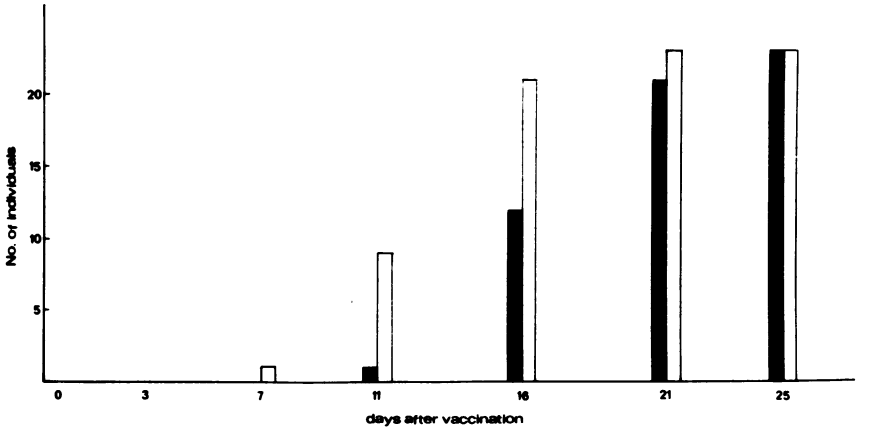

FIG. 1. *Appearance of specific antibodies in serum from 23 individuals after tularemia vaccination. Serum was obtained before and at various intervals after vaccination. At each interval, the number of individuals with an increased chemiluminescent response in PMN leukocytes (□) and with agglutinating antibodies in a titer of 20 or more (■) is indicated.*

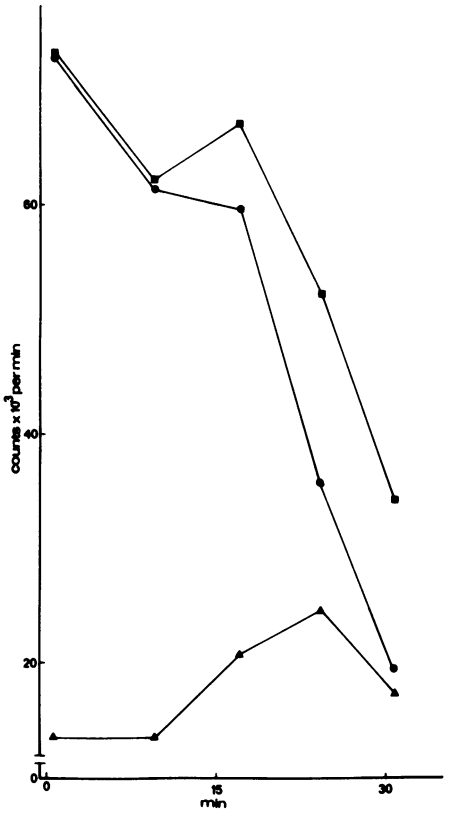

FIG. 3. *Chemiluminescent response induced by immune pool serum after chromatography on a cyanogen bromide-activated Sepharose 4B column coupled with normal goat serum (■) or with anti-C3 antiserum (▲). The response to nonchromatographed immune pool serum is also shown (●).*

activation, and the IgG-induced response seemed to be augmented by complement activation.

Effect of heat treatment of sera, obtained at various time intervals after vaccination, on the chemiluminescent response. The chemiluminescent response to heat-treated and nontreated serum obtained at various time intervals after vaccination was tested. A representative experiment with serum from one individual is shown in Fig. 5. The effect of heat treatment was most pronounced on serum obtained early after vaccination. For example, the chemiluminescent response induced by serum obtained on day 11 was completely abolished, whereas the response induced by serum obtained late after vaccination was only partly reduced.

In this investigation, the kinetics of the chemiluminescent response were not thoroughly studied, since the response was recorded at intervals of 10 min. The experiments indicated, however, that the chemiluminescent response was complex. For example, sera obtained early after vaccination induced a chemiluminescent response with a maximum after 10 to 20 min of exposure of the leukocytes to the opsonized bacteria, whereas sera obtained late after vaccination induced a maximum response with the first measurement within 1 min (Fig. 5). When the kinetics of the chemiluminescent response was studied by recording the light emission every 16 s after stimulation with bacteria and immune pool serum, a two-peak response was found (Fig. 6). The mechanisms responsible for these two peaks have been studied separately (13).

DISCUSSION

Intradermal vaccination of humans with a viable attenuated strain of *F. tularensis* (8) led to the development of specific opsonizing antibodies. These antibodies were easily detected by the use of the chemiluminescent reaction of PMN leukocytes (2). Determination of opsonizing antibodies has been used very little in the routine diagnosis of bacterial infections and not at all in the diagnosis of tularemia. According to an early Russian report, "opsono-phagocytic" antibodies may be demonstrated within 3 days of disease (17). Recent studies on the time of appearance of opsonizing anti-*F. tularensis* antibodies in monkeys have also suggested that these antibod-

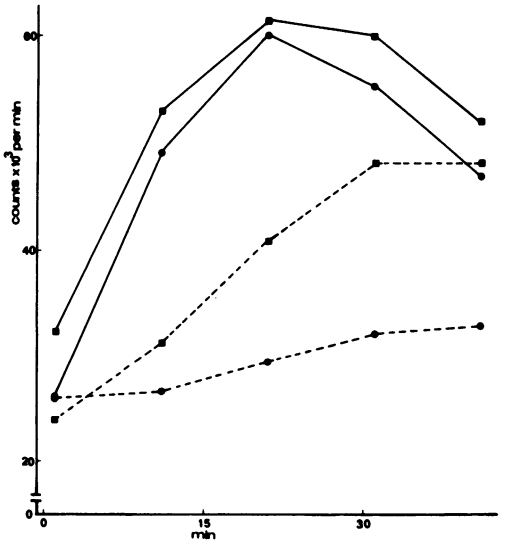

FIG. 4. *Chemiluminescent response induced by IgG and IgM fractions (Sephadex G-200) of immune pool serum. The IgG fraction was mixed with an equal volume of heat-treated (- -■- -) and nontreated (—■—), and the IgM fraction with an equal volume of heat-treated (- -●- -) and nontreated (—●—) nonimmune human serum.*

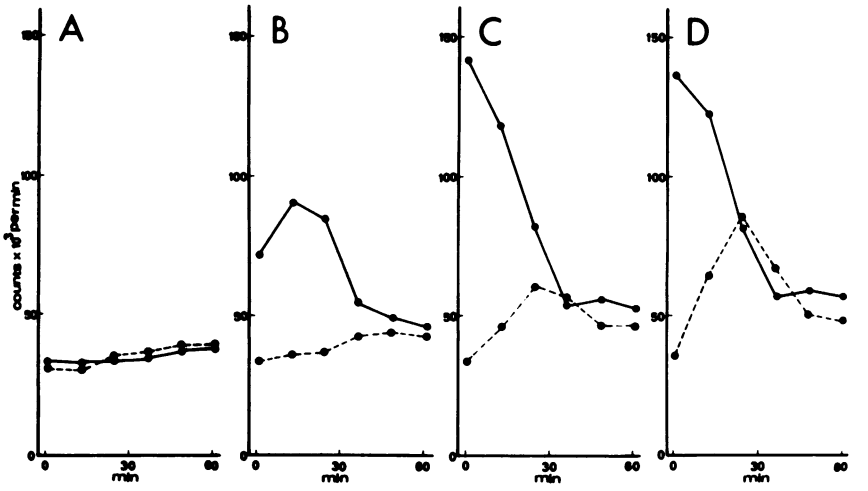

FIG. 5. *Chemiluminescent response induced by heat-treated* (- -●- -) *and nontreated* (—●—) *serum obtained before (A) and on days 11 (B), 16 (C), and 25 (D) after tularemia vaccination.*

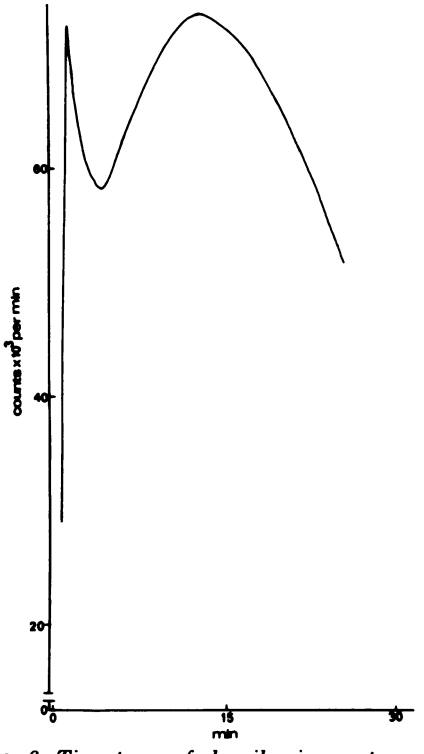

FIG. 6. *Time trace of chemiluminescent response induced in PMN leukocytes by a mixture of vaccine bacteria and immune pool serum. Light emittance was recorded during 12 s every 16 s.*

ies may appear earlier than the agglutinating ones (4). The present results indicate that human opsonizing antibodies may indeed be demonstrated earlier than agglutinating antibodies. Their development during illness remains to be investigated.

F. tularensis induced PMN leukocytes to emit chemiluminescence in the presence of homologous antibodies of the IgG class, and the response was augmented by complement activation. *F. tularensis* also induced chemiluminescence in the presence of antibodies of the IgM class, but the effect of IgM was obligatorily dependent on complement activation. These results agree with current concepts on opsonization with the Fc fragment of IgG on one hand and the complement component C3b on the other (for reviews, see references 7, 15). When sera obtained on various days after vaccination were heat inactivated, the reduction of the induced chemiluminescent response was most pronounced early after vaccination. This result is compatible with the appearance of IgM before IgG antibodies during immunization. Opsonization with IgG alone, IgG together with C3b, or IgM together with C3b may affect the leukocytes in different ways. By using erythrocytes as the antigen, it has been shown that PMN leukocytes ingest cells opsonized with IgG but not with IgM and C3b. The presence of C3b molecules on the erythrocyte surface, however, augmented the ingestion induced by IgG (7). It is, therefore, possible that the chemiluminescent responses of leukocytes to particles opsonized in different ways are due to different cellular events. The inducement of chemiluminescence by *F. tularensis* does not prove that the bacteria are ingested by the leukocytes, since chemiluminescence can be induced by soluble substances (10) and in the presence of cytochalasin B (12). Simian PMN leukocytes have been reported to

ingest and kill F. *tularensis* in the presence of homologous antibodies (18). The effect of opsonizing antibodies on the fate of the bacteria after interaction with human PMN leukocytes is unknown. Further studies should be done to relate the chemiluminescent responses of the leukocytes to the various phases of phagocytosis such as adherence, ingestion, and killing of bacteria.

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 Eva-Lena Hörnfeldt and Wigert Sjöberg for skillful technical assistance.

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