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Phagocytosis by Sheep Alveolar Macrophages: Relationship Between Opsonin Concentration and Light Emission in the Presence of Luminol

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The observations reported may be applied to determining the effects of various compounds, e.g., environmental pollutants and agricultural chemicals, upon the phagocytic activity of alveolar macrophages, and the method described will aid in detecting compounds which alter Fc receptor activity. A direct linear relationship existed between the concentration of antibody used to opsonize bacterial particles and the quantity of luminol-dependent light emitted by a population of sheep alveolar macrophages exposed to the opsonized particles. The relationship can be illustrated with a Lineweaver-Burk-style double-reciprocal plot. An analogy is suggested between the kinetics of enzyme-substrate reactions and the interaction of antibody-coated particles with Fc receptors on cell membranes.

I have been interested in identifying environmental pollutants which may find their way to the lung and diminish the defensive activities of the alveolar macrophages. A rapid, simple, and inexpensive screening test for the inhibition of phagocytosis is needed, particularly one which can detect compounds that act by binding and blocking Fc receptor activity.

The usual method for measuring Fc receptor activity, rosette formation between antibody-coated erythrocytes and macrophages (11), is somewhat cumbersome and requires a great deal of labor at the microscope. It is not suitable for a screening program. An alternative approach might be to measure the rapid metabolic changes that occur upon contact between an opsonized particle and the alveolar macrophage cell membrane. Under appropriate conditions, the rate of increase (or change) in metabolism within a population of macrophages exposed to opsonized particles should be a function of the kinetic interaction between the opsonized particles and the macrophage cell membranes. Thus, a rapid and simple method for screening numerous compounds and detecting those which might act upon Fc receptors may be devised by quantifying the metabolic changes which occur when opsonized particles interact with macrophages.

Reports (4, 8) indicate that blood monocytes and polymorphonuclear leukocytes will chemiluminesce when they are ingesting opsonized particles. The mechanism responsible for chemiluminescence by these cells is not understood, but the following events occur: (i) attachment of the target particle to the cell membrane's Fc receptors; (ii) change in metabolic mode from glycolysis to the hexose-monophosphate shunt; and (iii) the production of superoxide within the phagocytizing cell.

Allen and Loose (1) have shown that alveolar macrophages do not chemiluminesce during phagocytosis but that the addition of luminol to the reaction mixture results in light emission by the active macrophages. Measurement of luminol-dependent chemiluminescence is a rapid, simple, inexpensive procedure for evaluating phagocytic activity of a population of alveolar macrophages. Because the phagocytic activity is a function of Fc receptor activity when the macrophages are ingesting opsonized particles, chemiluminescence may be used as an indirect measurement of Fc receptor activity for screening purposes, provided that other examinations follow to verify the mechanism of inhibition. This report gives evidence that the quantity of light emitted by a population of alveolar macrophages is a direct function of the quantity of antibody used to opsonize the target particles. Hence, a Lineweaver-Burk-style double-reciprocal plot (2) of light emission versus opsonin concentration can be constructed. Such a graph can be used to evaluate the effects of various agents upon Fc receptor activity in a population of alveolar macrophages.

MATERIALS AND METHODS

Bacillus thuringiensis cultures were maintained on tryptic soy agar (Difco Laboratories, Detroit, Mich.)

slants. Viable cells for bacterin production and for use as target particles for alveolar macrophages were obtained by washing 24-h slant cultures with sterile physiological saline. The recovered cells were then washed twice with sterile physiological saline.

Antiserum to *B. thuringiensis* was prepared by inoculating a sheep with a saline suspension consisting of 0.5 ml of viable cells (5×10^8 cells) mixed with 0.5 ml of Freund adjuvant (complete) (Grand Island Biological Co., Grand Island, N.Y.). The sheep was reinoculated 21 days later with 10^9 viable cells without Freund adjuvant. Ten days later, blood was collected asceptically from the immunized sheep, and the serum fraction was obtained. The serum was heat inactivated at 56°C for 30 min and stored at 0°C. Immunoglobulins for use in these experiments were obtained by ammonium sulfate precipitation of the immune serum followed by dialysis and gel filtration on Sephadex G200 (Pharmacia Fine Chemicals, Piscataway, N.J.).

Alveolar macrophages were obtained daily from normal healthy sheep by bronchopulmonary lavage with 150 ml of sterile physiological saline. The recovered cells were washed once with Eagle minimum essential medium with Earle salts (Grand Island Biological Co.) containing 0.8% glucose and resuspended in Eagle minimum essential medium containing 0.8% glucose but no sodium bicarbonate. Final cell concentrations were adjusted to between 1×10^6 and 1×10^7/ml.

The basic demonstration of luminol-mediated chemiluminescence by sheep alveolar macrophages is conducted by adding the following components to plastic liquid scintillation counting vials (New England Nuclear Corp., Boston, Mass.): 7.5 ml of Ringer salt solution (Grand Island Biological Co.) containing 0.8% glucose; 1 ml of luminol (Eastman Kodak, Rochester, N.Y.)- saturated heat-inactivated horse serum (Grand Island Biological Co.) prepared as described by Allen and Loose (1) for fetal bovine serum; 0.1 ml of sheep anti-*B. thuringiensis* serum or immunoglobulins which have been diluted as appropriate to each experiment with Ringer salt solution; and 0.15 ml of a saline suspension of *B. thuringiensis* (minimum optical density at 540 nm = 0.33).

The vial containing this mixture was placed in a 37°C water bath, and the temperature within the scintillation vial was allowed to equilibrate. One milliliter of the alveolar macrophage suspension was then added to the vial, and the vial was transferred to a Beckman LS3133T scintillation counter exactly 5 min later. The average counts per minute was determined with the normal tritium channel for a 5-min interval.

RESULTS

Light emission above background levels occurred only when each of the following components was included in the scintillation vial: Ringer salt solution plus glucose, luminol-saturated horse serum, alveolar macrophages, and opsonized bacteria. Counts per minute attainable, and measurable, ranged from 75 to 250,000. When all experimental conditions except antibody concentration were kept constant, the investigator could easily show that light emission was a direct function of the antibody concentration used to opsonize the bacteria (Fig. 1). Thus, the kinetics of attachment of opsonized particles to the cell membranes of alveolar macrophages apparently determined the rate of light emission.

Figures 2 and 3 show the types of data obtainable with this system when inhibitors of phagocytosis or cell metabolism were added to the reaction mixture. These figures are included as illustrations of data obtainable, not as definitive proofs of the mechanisms of action of the two compounds used. Figure 2 shows the inhibition of Fc receptor activity achieved by adding dextran sulfate to the reaction mixture. This compound was probably interpositioned among the Fc receptors and thereby blocked the binding of opsonized particles to the cell surface (9). The results shown indicate that dextran sulfate was probably not a competitive inhibitor.

The sesquiterpene lactone helenalin is a toxic agent obtained from certain plants of the family *Compositae*. This plant poison is of economic importance to sheep and cattle raisers (3, 10). Figure 3 shows the inhibition of light emission (phagocytosis or Fc receptor activity) achieved by addition of 10 μg of helenalin to the reaction mixture. This compound is a cytotoxin that is

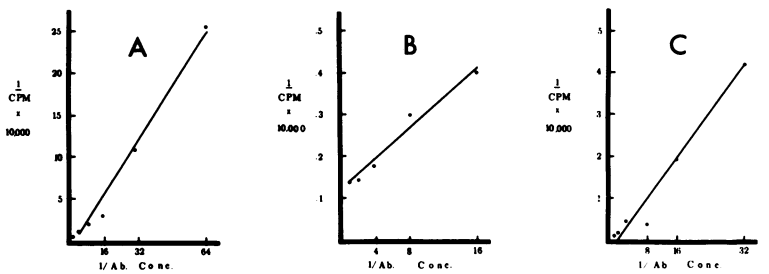

FIG. 1. *Three double-reciprocal plots of light emission in counts per minute versus the relative concentration of antibody used as opsonin. Maximum total dilution of immunoserum in the scintillation vials was 1/25,600. In (B), column-purified immunoglobulins were substituted for serum. The correlation coefficients are 0.986, 0.989, and 0.985, respectively. Ab. Conc., Serum or immunoglobulin dilution from which 0.1 ml was withdrawn and added to the scintillation vial.*

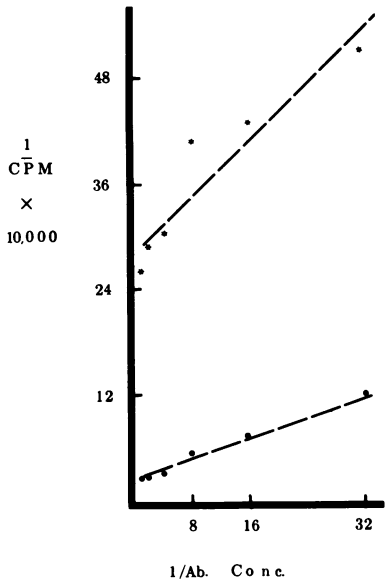

FIG. 2. *A double-reciprocal plot showing inhibition of the chemiluminescence response when dextran sulfate was added to the Ringer salt solution at a concentration of 0.05 mg/ml. Slope, intercept, and correlation coefficients are 0.285, 2.48, and 0.99, respectively, for control groups (●) and 0.813, 27.7, and 0.91, respectively, for experimental groups (*). Ab. Conc., Serum or immunoglobulin dilution from which 0.1 ml was withdrawn and added to the scintillation vial.*

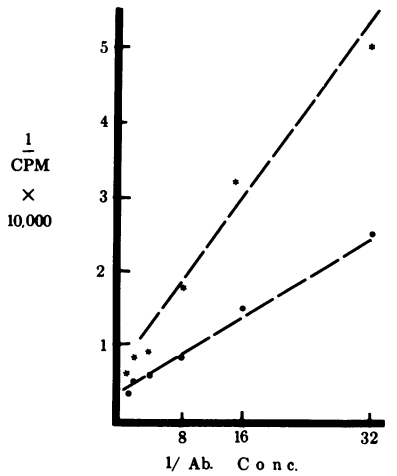

FIG. 3. *A double-reciprocal plot showing inhibition of the chemiluminescence response when helenalin (10 μg/ml) was added to the Ringer salt solution. Slope, intercept, and correlation coefficients are 0.068, 0.3, and 0.99, respectively, for control groups (●) and 0.149, 0.6, and 0.97, respectively, for experimental groups (*). Ab. Conc., Serum or immunoglobulin dilution from which 0.1 ml was withdrawn and added to the scintillation vial.*

known to bind to sulfhydryl groups in protein molecules (5, 7). Although at first glance these data appear to indicate competitive inhibition, close examination reveals that the intercepts on the *y* axis are different. (The biological activities of helenalin are probably too broad for it to be a specific competitive inhibitor.)

DISCUSSION

Enzymologists have long used the Lineweaver-Burk (2) double-reciprocal plot to analyze the behavior of inhibitors of enzyme-substrate reactions. The binding of opsonized particles to alveolar macrophage cell membrane through the Fc receptors on these membranes can be thought of as analogous to the binding of a substrate to an enzyme. Similarly, the double-reciprocal plot of light emission versus antibody concentration can be used (with caution, because the analogy is not exact) to analyze the effects of agents on the binding of opsonized particles to alveolar macrophages and the resultant metabolic changes in these cells.

Although several authors have reported chemiluminescence by phagocytizing cells and have alluded to a requirement for opsonization of the particles to be ingested, this study shows that the intensity of the chemiluminscence (counts per minute) is a direct function of the concentration of opsonins in the reaction mixture, provided that all other experimental conditions are held constant. Although the phenomenon of chemiluminescence by phagocytizing cells is a much more complex reaction than the reaction of a single enzyme and substrate to yield a product, the analogy that the interaction of Fc receptors and opsonized particles results in light emission proportional to the quantity of antibody (substrate by analogy) appears to be valid. The methods presented here are thus of practical significance for the rapid identification of inhibitors of phagocytosis, and these methods may be a tool for identifying specific competitive inhibitors of the binding of opsonized particles to Fc receptors. The identification of specific competitive inhibitors would be an important step in analyzing the chemical nature of Fc receptors. The rapid identification of other types of inhibitors is important as a screening tool for evaluating environmental pollutants that are potentially damaging to the alveolar macrophages and immunological defenses possessed by the lungs.

Caution is needed, however, in interpreting results obtained through the kinetic analysis described. Similar results may be obtained through the action of an inhibitor on any of the several mechanisms which, acting in concert, result in a chemiluminescent response. Suspect compounds should therefore be studied with additional procedures, e.g., rosette assay or flu-

orescence quenching by crystal violet (6), to verify the mechanism of actions.

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