Bactericidal Antibody Assay Using ^{14}C-Labeled *Neisseria meningitidis*[1] (36324)

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(Introduced by S. B. Formal)

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The bactericidal assay is an important test in the study of immunity to bacterial diseases and has been used to measure natural and vaccine induced antibodies in the meningococcal research field (1, 2).

Recently, Gold and Wyle (3) described a bactericidal antibody assay which produced a detailed serological classification of *Neisseria meningitidis* into serotypes. Their studies were predicated on the work of Roberts (4) and Goldschneider *et al.* (1) who independently described antigenic diversity of strains of organisms within a serogroup, based on bactericidal reactions. These serotypes are separate and distinct from the classical serogroups which are based on bacterial agglutinations (5). The uncovering of these serotypes led to the recognition of protein antigens which induce this serotype specific bactericidal antibody (6).

This report describes the development of a simplified and more efficient method of measuring bactericidal activity using ^{14}C-labeled organisms.

Materials and Methods.[3] *Culture methods.* All strains of meningococci were from the Walter Reed Army Institute of Research collection, stored in the lyophilized state within 3–4 passages from the initial isolation. Several group C and B strains were tested using this assay; however, the prototypes described are 138I (group C, serotype II), 99M (group B, serotypes II, III), and 60E (group C, serotype I).

After overnight growth on BYE agar (Baltimore Biological Laboratory) at 37° in a CO_2 incubator, a large loopful of organisms was inoculated into 25 ml of Mueller-Hinton broth (Difco Laboratories) in a 250 ml nephelometry flask and incubated aerobically at 37° in a rotary shaker water bath. Forty-five to 90 min after inoculation, the culture reached a concentration of 3×10^8 organisms/ml ($A_{650nm} = 0.30$; Spectronics 100, Bausch and Lomb). At this point, 4 ml was transferred to another nephelometry flask containing 100 μCi of ^{14}C-sodium acetate (New England Nuclear) in 21 ml of Mueller-Hinton broth. After growth to a concentration of 3×10^8 labeled organisms/ml, the culture was centrifuged at 3000*g* for 10 min at 4° and washed three times with Gey's salt solution at 4°. The washed radioactive organisms were then suspended in cold Gey's solution and placed in an ice bath until used. Meningococci could be maintained in Gey's solution at 0° with minimal release of radioactivity for at least 2 hr.

Antiserum preparation. Antiserum to the organisms was prepared in 2–3 lb New Zealand white rabbits by the iv administration of 3×10^8 viable meningococci, 3 times/week for 2 weeks. A booster injection was given 1 week after the last inoculation and the rabbits were exsanguinated 1 week later. Serum was obtained by centrifugation

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[3] In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

of clotted blood, sterilized by filtration through a 0.45 μ Millipore filter, and stored at $-70°$. All antisera were inactivated at 56° for 30 min prior to use. Serotype specific serum was prepared by absorption with group specific polysaccharide according to the method of Gold and Wyle (3).

Bactericidal assay. Reaction mixture composition:

a. Diluent. Gey's balanced salt solution (Microbiological Associates, Inc.) in quantity sufficient to bring the total volume of the reaction mixture to 1 ml.

b. Complement. Normal rabbit serum was included in all reaction mixtures as a source of complement.

To eliminate naturally occurring nonserotype, nonserogroup specific bactericidal antibody in our normal rabbit serum, complement was absorbed with a standard meningococcal strain (1381). This absorption was performed by transferring the organisms from an overnight growth on one BYE agar plate to 5 ml of fresh normal rabbit serum and incubating for 1 hr in an ice bath at 0°. The organisms were removed by centrifugation, followed by filtration through a 0.45 μ Millipore filter. The absorbed serum was stored in 1 or 2 ml samples at $-70°$ and thawed immediately prior to use. This absorption significantly reduced the content of natural antibody; but did not reduce the level of complement as determined by C'H50 (7).

c. Meningococci. Harvested as in Culture methods and suspended for use at 3×10^8 organisms/ml.

d. Reaction mixtures were set up in 12 $\times$ 75 mm tubes with gauze plugs. Test mixtures contained 0.1 ml of complement, 0.1 ml of antiserum and 0.1 ml of meningococci. Complement controls without antiserum and heat-inactivated (56° for 30 min) complement controls with antiserum were run. All reaction mixtures were brought to a volume of 1 ml with Gey's solution.

Reaction mixtures were incubated in a rotary shaker water bath at 37°. At various time intervals after the addition of radioactive organisms to the reaction mixture, samples were removed for testing. Filtration through a 0.45 μ Millipore membrane was used to separate the soluble radioactivity released from disrupted organisms from that remaining in live intact organisms.

Unfiltered controls were run concurrently. The filtered zero time counts were assumed to be background because no detectable immune reaction had yet occurred.

Upon completion of the sampling, 7 ml of scintillation fluid was added to each vial containing 0.1 ml of test sample. The scintillation fluid was prepared by mixing 130 ml of Liquefluor (New England Nuclear), 300 ml of NCS solubilizer (Amersham/Searle) and 3 liters of toluene (Matheson, Coleman and Bell). The organisms were labeled at a sufficiently high specific activity so that accurate counts could be obtained in 1 min in a Packard Tricarb liquid scintillation counter.

e. Analysis of results. Bactericidal activity is expressed in terms of the percentage of radioactivity released compared to the unfiltered control.

$$\frac{F_{60} - F_0}{(U_0 + U_{60})/2} \times 100 = \% \text{ release},$$

where F_{60} = filtered sample at 60 min; F_0 = filtered sample at 0 min; U_0 = Unfiltered sample at 0 min; U_{60} = Unfiltered sample at 60 min.

Duplicate samples were taken from the reaction mixture at each sampling time and their results were averaged.

The difference in the number of counts released during incubation is divided by the average counts of the unfiltered sample and multiplied by 100 to give the percentage of total radioactivity released. Results are given as the net percentage release, that is, the percentage release of the complement control is subtracted from the experimental sample.

Results. Incorporation of radioactivity into a growing culture. A study of the kinetics of ^{14}C-acetate incorporation into a growing log phase culture of meningococci revealed that near maximal uptake of radioactivity was achieved at an optical density of 0.3 at 650 nm which is midlog phase and is equivalent to 3×10^8 organisms/ml, therefore, organisms for the assay were harvested at this

TABLE I. Efficiency of Recovery of Released Radioactivity.

Method of recovering released radioactivity	% of total radioactivity recovered after bactericidal reaction
0.45 μ Millipore filter	44.6
3000*g*, supernate	92.3
15,000*g*, supernate	45.1
3000*g*, 0.45 μ filtrate	44.2

optical density. Organisms labelled in this manner had a sp act of approximately 5000 cpm/10^6 organisms. Roberts (4) has shown this midlog point to be a phase of growth at which meningococci were optimally susceptible to bactericidal action. This was also the point at which Gold and Wyle (3) harvested their cultures for the standard bactericidal test.

Efficiency of the filtration method. Filtration and differential centrifugation were compared as methods of recovering released radioactivity in a bactericidal reaction (Table I), where a maximum number of meningococci were killed.

Previous experiments had shown that centrifugation for 15 min at 3000*g* completely sediments intact organisms. Centrifugation of bactericidal reaction mixtures in this manner left 92% of the radioactivity in the supernatant. The Millipore filtrate of this supernatant fraction contained only 44.2% of the radioactivity.

The supernatant fraction of a reaction mixture centrifuged at 15,000*g* for 15 min contained 45.1% of the radioactivity, and the Millipore filtrate of a noncentrifuged reaction mixture contained 44.6%.

Filtration and high speed centrifugation removed labeled cell walls, but low speed centrifugation did not. In this system 45% release is at least fivefold higher than control values. Because of its simplicity, filtration is preferred to differential centrifugation.

Background controls (Fig. 1). In order to determine nonspecific release due to time, temperature, protein enrichment, and isotonicity, an experiment was run with ^{14}C-labeled 99M organisms and reaction mixtures of complement, complement plus heterologous serum (60E), inactive complement plus homologous, serotype specific serum (138I),

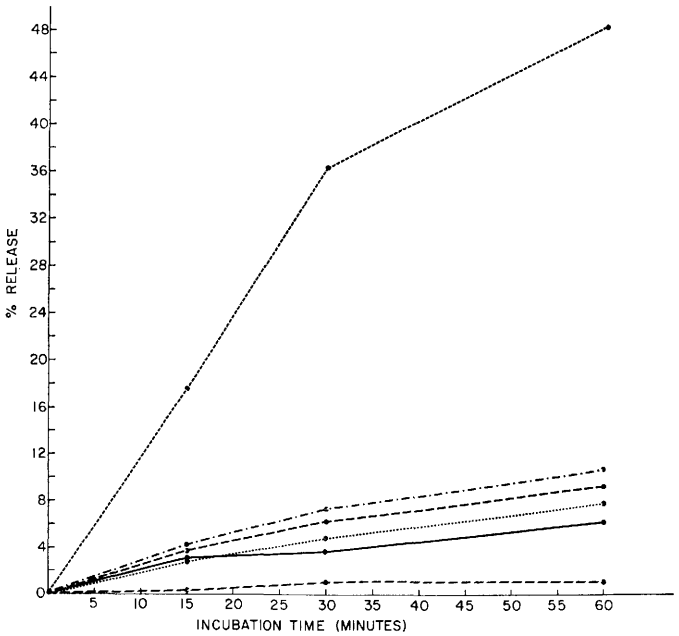

FIG. 1. Effect of varying reaction mixture components on percentage of radioctivity from 99M organisms: (- - -) complement + homologous serotype sera (138I) + Gey's buffer; (——) complement + heterologous serotype sera (60E) + Gey's buffer; (— - —) complement + Gey's buffer; (- - - -) 5% BSA + Gey's buffer; (—) heated-inactivated complement + homologous serotype sera (138I) + Gey's buffer; and (▬▬) Gey's buffer.

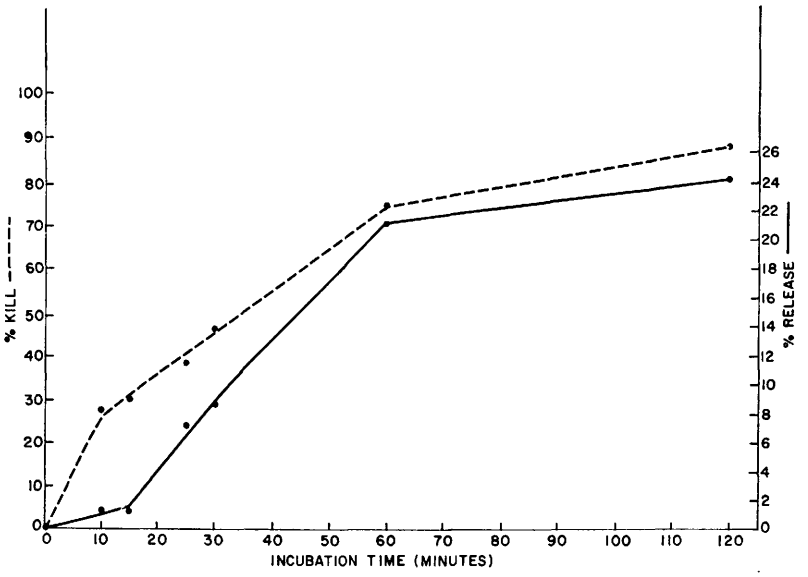

FIG. 2. Kinetics of percentage release and percentage kill vs time of incubation of a reaction mixture which contained complement + homologous serotype sera (138I) + 99M organisms: (— —) percentage of organisms killed, determined by 10-fold tube dilution platings; (—) percentage of radioactivity released, determined by methods described.

and 5% bovine serum albumin (BSA). All had essentially the same low percentage release when incubated as long as 60 min at 37°. A control with Gey's solution alone showed less release of radioactivity than the others. In an active complement plus immune serum test (138I), release began very early and continued to increase to the 60 min sampling.

Relationship of standard and radioactive bactericidal test. To study the relationship of the standard and radioactive bactericidal tests, these two systems were compared simultaneously using: (i) time of incubation; and (ii) dilution of antisera as variables. The two assays were run on the same reaction mixtures, one sample being plated in 10-fold tube dilutions for colony counts; the other being filtered and the supernatant assayed for radioactivity.

When the antiserum concentration was kept constant and the time of incubation was varied (Fig. 2), the initial rate of killing exceeded that of release. At the first sampling (10 min), release of radioactivity was barely detectable; whereas, 27% of the organisms were no longer viable. At 60 min, the percentage of maximal radioactivity released and percentage of killing closely approximated each other.

The relationship of percentage kill and percentage release is significant at the .001 level with a correlation coefficient of 0.9933.

Using a 60 min incubation period, an immune serum was titrated against a homologous serotype organism (Fig. 3). At each dilution, both radioactive counts and colony counts were done. The rates of decrease with increasing antiserum dilution approximated each other very closely in both assays.

Experimental reproducibility. The reproducibility of the radioactive bactericidal test was ±2% release under standard conditions of complement and antisera in 17 tests of 99M antiserum (group B, serotype II, III) against 138I organisms (group C, serotype II).

Discussion. This study describes a bactericidal assay system which is predicated on the assumption that complement mediated bactericidal activity results in disruption and lysis of *N. meningitidis* with release of radioactively labeled cellular material. The standard test measures percentage kill and the radioactive test measures percentage of re-

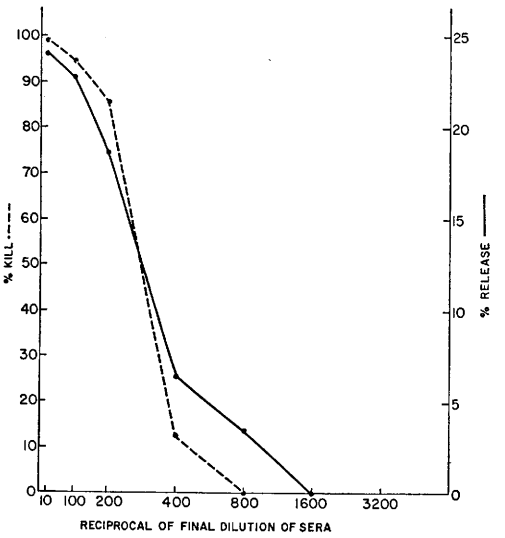

FIG. 3. Dose–response curve of percentage release of radioactivity and percentage kill vs dilutions of hyperimmune homologous serotype serum: (- -) percentage of organisms killed determined by 10-fold tube dilution platings; (—) percentage of radioactivity released determined by methods described.

leased radioactivity.

The present study has shown that the amount of radioactivity released correlates well with the killing of meningococci in the bactericidal system. This is not to say that the two tests are measuring the same cellular event. There is a lag of net percentage release of radioactivity behind percentage kill. The standard bactericidal test measures the viability of an organism after interaction with antibody and complement, which is probably an immediate event; whereas the radioactive test requires that some disruption of cellular integrity occur in order that radioactivity be released, which probably takes several minutes to occur.

Swanson and Goldschneider (8) showed that exposure of meningococci to the bactericidal system of normal rat serum initiates a series of ultrastructural changes that accompany death of the organism. Among these are complement dependent holes in the cell wall outer membrane, dissolution of the peptidoglycan layer, and rupture of the cytoplasmic membrane.

Spitznagel (9) and Spitznagel and Wilson (10) showed that ^{32}P loss from labeled *E. coli* was associated with gross disruption of the bacterial cell and accompanied by a loss in bacterial viability.

There are some distinct advantages to using the radioactive bactericidal test. In the standard bactericidal test the results are not available until the next day and, even then, not until colony counts have been performed, which may itself require several hours work. In the radioactive test the final results are available within 1 hr after completion of sampling, after which no more of the experimenter's time is required.

The standard assay has at least a 10% error associated with spreading and colony counting. The radioactive assay's sources of error are small and depend predominantly on the scintillation counter and pipetting errors.

In the standard test, strict sterile technique is required at all stages of the test including the colony count plates. In the radioactive assay, sterility is only necessary up to the point of sampling; hence later contamination of plates is no problem.

The radioactive bactericidal test has been shown to be a simple and precise assay with distinct advantages over the standard bactericidal test. It has been successfully used for epidemiological and immunochemical investigations of meningococcal cell surface antigens (6) in our laboratory.

Summary. Complement mediated bactericidal activity of sera produces cellular death and disruption of *Neisseria meningitidis* organisms. This has permitted development of a test measuring release of radioactivity from ^{14}C-labeled organisms exposed to the bactericidal effects of immune rabbit serum and complement. The percentage of radioactivity released from ^{14}C-labeled meningococci by the action of specific bactericidal rabbit antibody and complement was 45%, while normal rabbit sera control released only 8% of the radioactivity.

The overall correlation between this new test and the standard meningococcal bactericidal test is excellent.

Results are obtainable with excellent reproducibility within 1 hr. The need for

plating and colony counting is eliminated and the sources of error are small.

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