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TECHNICAL MANUSCRIPT 601

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AEROBIOLOGY & EVALUATION LABORATORIES

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streptomycin and kanamycin per ml. Cells were maintained in mixture 199 and 5% FCS. For agent assay, cells were cultivated on circular cover slips (diameter, 15 mm) inserted in flat-bottomed glass vials (18 by 100 mm). A 1-ml amount of cell suspension, containing from 10^5 to 3 x 10^5 cells, was introduced onto cover slips that were then incubated at 35 C for 24 hours, or until a complete cell monolayer was formed.

C. ANTISERUM

Psittacosis antiserum was prepared by injecting roosters intravenously with 1 ml of yolk sac suspension of the psittacosis agent containing 10^7 egg LD_{50}. Three weeks later, surviving fowls were bled. Human serum was obtained from individuals diagnosed as suspected subclinical or established psittacosis infections on the basis of clinical and serological findings. Goat anti-human and anti-chicken gamma globulin sera were obtained from Microbiological Associates, Bethesda, Maryland. Normal goat serum was purchased from Pentex Laboratories, Kankakee, Illinois. All sera were inactivated at 56 C for 30 min.

D. NEUTRALIZATION TESTS

The general procedure for the serum neutralization anti-IgG test consisted of mixing appropriate dilutions of antiserum or normal serum with equal volumes of a constant quantity of psittacosis agent. The concentration of agent used was such that an average of one to three infected cells per microscopic field was obtained with normal serum-agent mixtures after incubation. Both agent and serum dilutions were routinely prepared in phosphate-buffered saline (PBS) solution, pH 7.1. Test mixtures were incubated at 35 C for 2 hours. To each test mixture, undiluted goat anti-human IgG serum was added in a volume to give a final dilution of 1/3. After further incubation of mixtures at 35 C for 10 min, 0.2 ml of each mixture was introduced onto one of three cover slip cell monolayers. Centrifugation (500 x g for 15 min) was used to aid the attachment of unneutralized agent onto cell monolayers.[7] After residual inoculum was removed, cell monolayers were rinsed with maintenance medium and 1 ml of medium was then added to each vial containing a cover slip cell monolayer. Vials were incubated at 35 C for 20 to 22 hours. They were then rinsed twice with cold PBS, fixed with cold (-60 C) acetone, and prepared for staining with conjugated antiserum.

To determine the 50% serum neutralizing end point, the per cent reduction of fluorescent cell counts for each antiserum dilution was computed from normal serum (control) counts. Reduction percentages were then plotted against the logarithm of the corresponding final dilutions of antiserum on probability paper. A linear relationship was obtained over a critical range. By interpolation, the dilution of antiserum that neutralized 50% of psittacosis agent was determined.

In the mouse serum neutralization test, the protocol for preparing agent-serum mixtures and for incubating them was similar to that described for the fluorescent cell counting neutralization test. For each test mixture, ten Swiss mice, each weighing 10 to 14 g, were inoculated intracerebrally with 0.03 ml. Animals were observed daily for 15 days and the survivors were noted. The 50% serum neutralizing end point was calculated by the Reed and Muench formula.[9]

E. IMMUNOFLUORESCENCE TECHNIQUES

The direct fluorescent antibody technique was employed to demonstrate immunofluorescence of agent antigens in infected cells. The preparation of psittacosis antiserum and conjugation with fluorescein isothiocyanate have been described previously.[7] Unbound dye was removed from conjugated globulin by passing the globulin through a column of Sephadex G-50; nonspecific fluorescence was reduced by absorbing conjugated globulin with acetone-dried mouse liver powder.[10] Fixed cell monolayers were washed twice with PBS and stained with conjugated antiserum for 30 min at room temperature. Cover slip cell monolayers were then rinsed in two changes of PBS and mounted in a semipermanent medium.[11]

An American Optical microscope equipped with a Fluorolume illuminator (model 645), Corning No. 5840 and Schott BG-12 exciter filters, and an E.K. No. 2A barrier filter was used to examine stained cover slip cell monolayers. With this optical system at a magnification of 430X, the number of microscopic fields in the area of a 15-mm cover slip was 1,064. The number of CIU per milliliter of agent suspension was calculated by the method described elsewhere.[7]

III. RESULTS AND DISCUSSION

The neutralizing effect at equilibrium of different concentrations of antibody on a constant quantity of virus generally results in a multiplicity curve.[12] This curve initially resembled a kinetic curve but later decreases in slope and becomes horizontal. The latter portion of the curve indicates that there is a fraction of virus that is resistant to neutralization in the presence of high concentrations of antibody.

A multiplicity curve also resulted when different concentrations of rooster antiserum were mixed with constant amounts of psittacosis agent (1×10^6 CIU/ml), incubated at 35 C for 2 hours, and then assayed for surviving agent (Fig. 1). Agent survival's being constant at the latter portion of the curve indicates that there is a resistant fraction of approximately 20%. This is more than 1,000-fold higher than that reported with other virus-antibody systems.[13]

Evidence that virus resistant to neutralization is in the form of infectious virus-antibody complexes that may be neutralized by anti-IgG serum[6,14] prompted a test to determine whether these findings could be extended to the psittacosis-antibody system. Parallel neutralization tests were performed to determine the 50% neutralizing titer of psittacosis rooster antiserum in the presence of goat antiserum to chicken IgG or normal goat serum. Details of the neutralization tests have been described earlier. Results (Fig. 2) show that the 50% serum neutralizing end points in the presence of anti-IgG or normal goat sera were 1/3,400 and 1/5, respectively. The serum neutralizing titer of antiserum reacted with anti-IgG serum was approximately 700-fold higher. This indicates that the resistant fraction of the psittacosis agent was in the form of an infectious agent-antibody complex. That anti-IgG serum was inactive against the agent was confirmed from the comparable titration values that were obtained when the psittacosis agent alone was incubated in the presence of either anti-IgG or normal goat sera.

To estimate the precision of the serum neutralization anti-IgG test, eight determinations were made under the same test conditions. The standard deviation (±249), expressed as a percentage of the mean reciprocal 50% serum neutralizing end point (3,525), was 7.0.

The sensitivity of the serum neutralization anti-IgG test was compared with that of conventional serum neutralization tests and the complement-fixation test for estimating psittacosis antibodies of human serum samples. Results (Table 1) show that significantly higher neutralizing titers of sera were obtained with the serum neutralization anti-IgG test than with the other neutralization tests. Results obtained with the serum neutralization anti-IgG test correlated well with those of the complement-fixation test. Neutralizing antibodies were detected in all sera that contained complement-fixing antibodies and, in general, they were higher in titer than the latter.

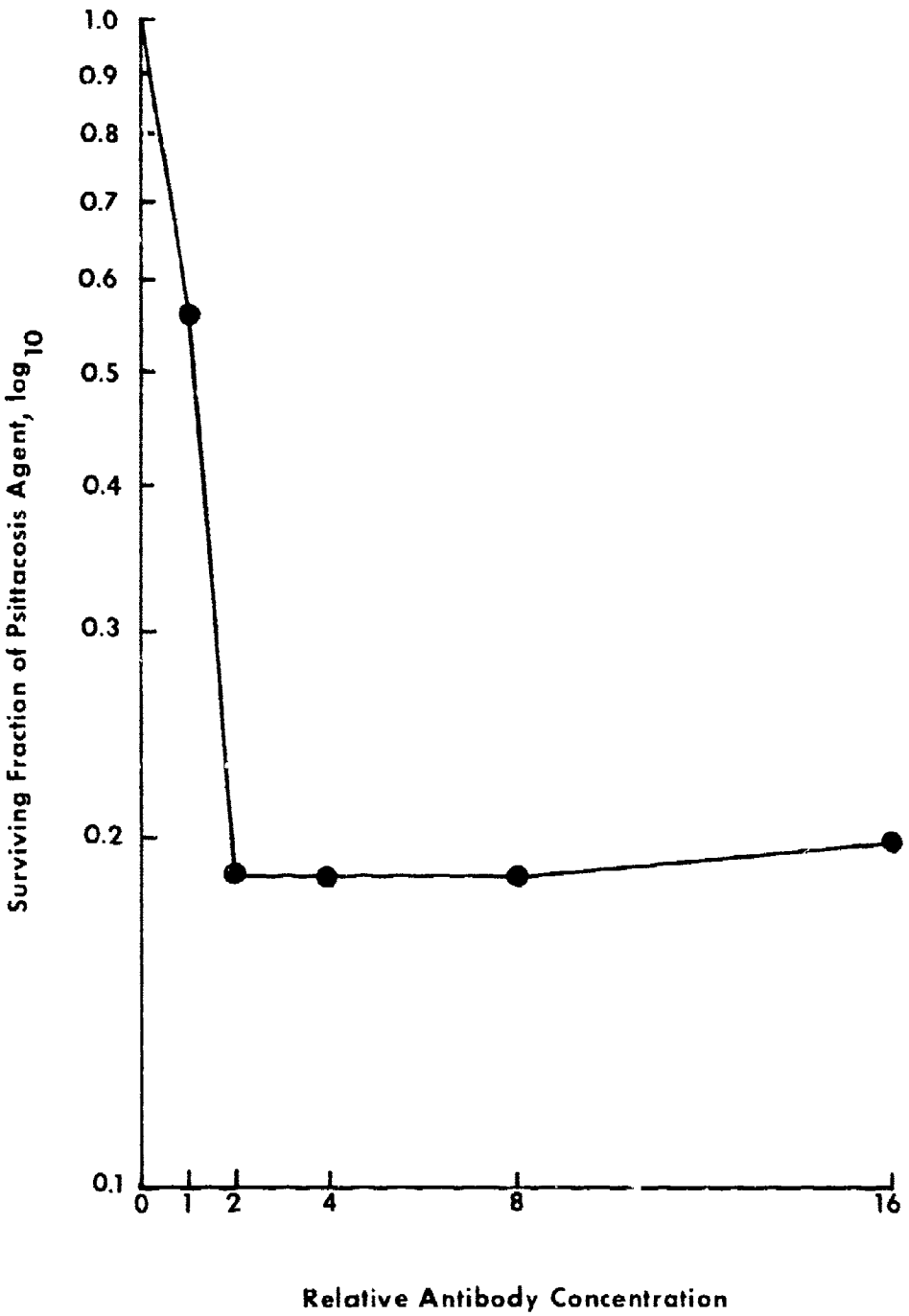

FIGURE 1. Multiplicity Curve of Neutralization of Psittacosis Agent by Antiserum Dilutions.

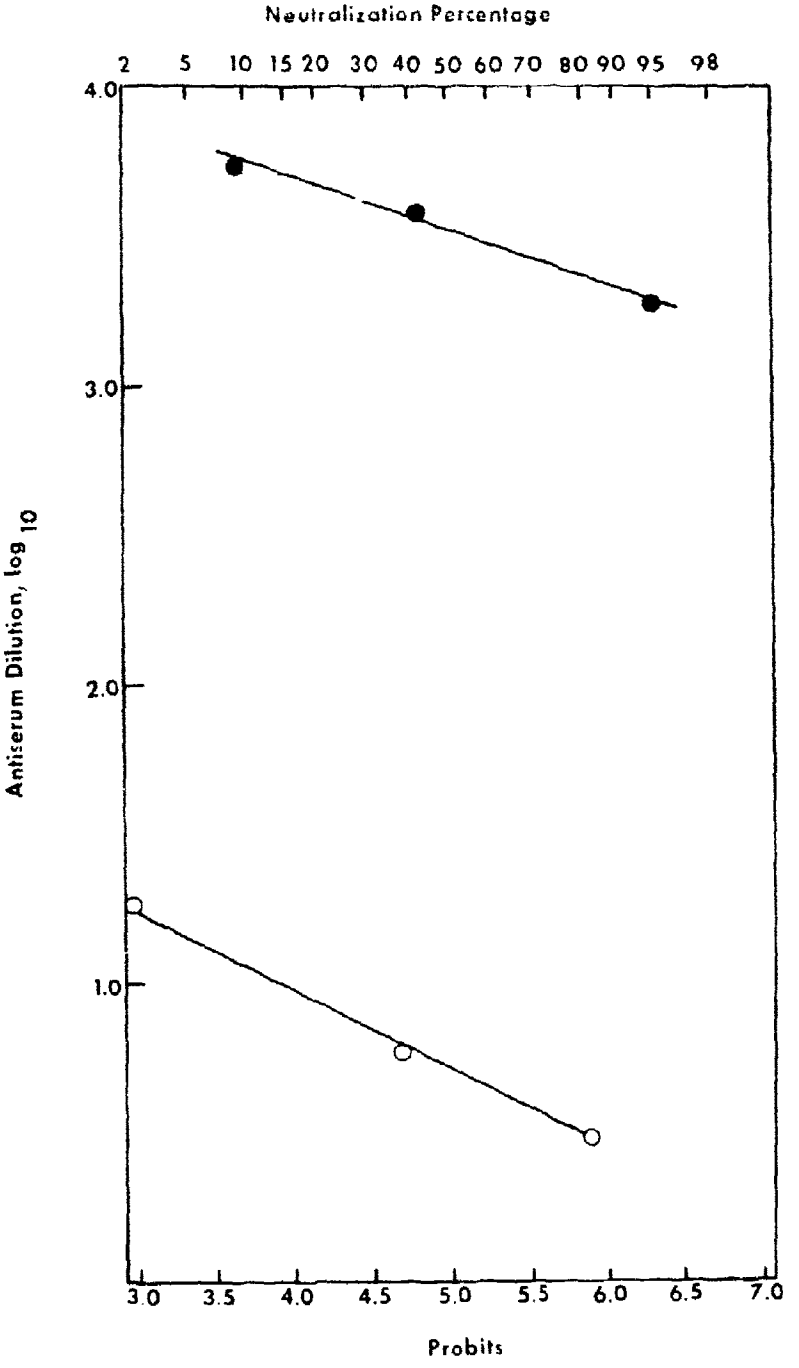

FIGURE 2. Comparative Determination Between the Serum Neutralization Anti-IgG Test (●) and a Conventional Serum Neutralization Test (○). Fifty per cent serum neutralizing end points were interpolated from the per cent reduction of psittacosis-infected cells by antiserum dilutions.

TABLE 1. SEROLOGICAL DETERMINATIONS ON HUMAN SERUM FROM PATIENTS WITH ESTABLISHED OR SUBCLINICAL PSITTACOSIS INFECTIONS

Patient	Serum Sample		CF Test[b]	Mouse SN Test[c]	SNFCC Test[d]	SNFCC Anti-IgG Test[e]	Comment
	Type[a]	Date					
1	A	12/5/61	≤5	≤2	≤2	≤3	Suspected subclinical psittacosis infection (Patients 1 through 4)
	C	12/17/66	10[f]	≤2	4	65	
2	A	7/1/63	≤5	≤2	≤2	≤3	
	C	12/19/63	10	≤2	2	70	
3	C	12/16/63	10	≤2	4	70	
4	A	7/8/63	≤5	≤2	≤2	≤3	
	C	12/28/63	5	≤2	3	78	
5	C	4/15/64	80	≤2	3	110	Subclinical psittacosis infection.
6	A	DNR[g]	≤4	≤2	≤2	≤3	Clinical and serological diagnosis of psittacosis (Patients 6 and 7).
	C	DNR	32	≤2	8	210	
7	A	DNR	≤4	≤2	≤2	≤3	
	C	DNR	128	≤2	2	110	

a. A, acute; C, convalescent.
b. Complement-fixation test.
c. Mouse serum neutralization test.
d. Serum neutralization fluorescent cell counting test.
e. Serum neutralization fluorescent cell counting anti-gamma globulin test.
f. Reciprocal of 50% serum neutralizing dilution.
g. Date not recorded.

The serum neutralization anti-IgG test described in this study for estimating psittacosis serum-neutralizing antibodies is dependent on the interaction of the psittacosis agent with antiserum and the neutralization of the resultant infectious complex with anti-IgG antibodies. It is the latter reaction that significantly enhances the sensitivity of the test. Although the mechanism by which neutralization of infectious agent-antibody complexes by anti-IgG serum occurs has not been resolved, it has been postulated that anti-IgG antibodies may form bridges that cover critical infective sites on agent particles or act by stabilizing the attachment of antibody.[14,15] The serum neutralization anti-IgG test for psittacosis in conjunction with fluorescent cell counting is highly sensitive, precise, and rapid. For diagnostic purposes, the ability to determine the neutralizing antibody content of serum within 24 hours is a singular advantage. The test may be applicable for retrospective epidemiological surveys, correlation between levels of neutralizing antibodies and development of resistance to infection, and studies to determine the immunological interrelationships among members of the psittacosis groups of microorganisms.

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13 ABSTRACT

Relatively high levels of serum-neutralizing antibodies were detected in tests employing anti-IgG and sera from patients with diagnoses of subclinical or established psittacosis infections where conventional tests disclosed only marginal levels of antibody. Use of this test in conjunction with the fluorescent cell counting technique permitted serum antibody determinations within 24 hours.

14. Key Words

Serum neutralization test
Psittacosis agent
Gamma globulin
Immunofluorescent technique

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