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Fluorescent Cell-Counting Neutralization Test for Psittacosis

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ABSTRACT

HAHON, NICHOLAS (Fort Detrick, Frederick, Md.), AND KENNETH O. COOKE. Fluorescent cell-counting neutralization test for psittacosis. J. Bacteriol. **89**:1465–1471. 1965.—A sensitive, precise, and specific serological procedure, the fluorescent cell-counting neutralization test, was developed to detect and to measure quantitatively psittacosis serum-neutralizing antibodies within 24 hr. The test is based on the reduction of fluorescent cells in McCoy cell monolayers resulting from the neutralization of infective agent particles by specific antiserum. Small but significant rises in neutralizing titers were measured in serum specimens from monkeys previously exposed to the psittacosis agent and from humans with diagnoses of subclinical or established psittacosis infections.

In comparison with the complement-fixation and toxin-neutralization tests and various modified forms of agglutination reactions, the serum-neutralization test has not been generally employed in serological studies with members of the psittacosis group of microorganisms. An exception to this statement is the use of the neutralization reaction in studies on the immunological relationships among this group of agents made feasible by the production of serum of high neutralizing potency in immunized fowls (Hilleman and Gordon, 1943, 1944; Hilleman, 1945; St. John and Gordon, 1947). An important factor predisposing to the limited usefulness of the neutralization test is the low levels or absence of neutralizing antibodies that have been encountered in serum derived from experimentally or naturally infected animals and humans (Hilleman, 1945). In the majority of studies in which neutralization tests have been attempted, only qualitative estimates of the neutralizing capacity of serum have been obtained. There is a need for a sensitive and precise system to detect effectively and to measure quantitatively small but significant differences in neutralizing potency between sera.

In the course of developing a quantitative assay for the psittacosis agent by use of the fluorescent cell-counting technique (Hahon and Nakamura, 1964), significant reductions of fluorescent cell counts were noted when psittacosis antiserum was mixed with the specific agent. A linear relationship was demonstrated between the quantity of agent neutralized and dilutions of antiserum. This finding, coupled with the high sensitivity, precision, and unusual rapidity of the assay (less than 24 hr), offered a system potentially capable of quantitatively determining serum-neutralizing antibodies against at least one member of the psittacosis group of agents. This report describes the standardization, application, and advantages of the fluorescent cell-counting neutralization test for psittacosis.

MATERIALS AND METHODS

Agent. The Borg strain of psittacosis agent was used throughout this study; its history has been recorded elsewhere (Officer and Brown, 1960). A stock suspension of agent was prepared by infecting monolayers of McCoy cells with psittacosis agent that had been passed once in this cell line. After incubation at 35 C for 48 hr, tissue culture fluids and cells were harvested, frozen and thawed once, clarified by low-speed centrifugation, dispensed into glass vials, and stored in an electric freezer at −60 C. The stock-agent suspension assayed by the fluorescent cell-counting technique contained 1.1×10^7 cell-infecting units (CIU) per ml.

Cell line and cultivation. The established cell line, McCoy, derived from human synovial tissue (Fernandes, 1959) was used for assay of unneutralized agent particles. Nutrient medium for the cell line consisted of mixture 199 containing 0.5% lactalbumin hydrolysate, 10% heat-inactivated calf serum, and 50 and 75 μg of streptomycin and kanamycin, respectively. Cells were maintained in mixture 199 and 5% calf serum. For agent assay, cells were cultivated on circular cover slips (di-

ameter, 15 mm) inserted in flat-bottomed glass vials (18 by 100 mm). A 1-ml amount of cell suspension, containing 10^5 to 3×10^5 cells, was introduced onto cover slips that were then incubated at 35 C for 24 hr, or until a complete cell monolayer was formed. Cover-slip cultures were washed twice with 2 ml of maintenance medium prior to the addition of agent-serum mixtures.

Antiserum. Psittacosis hyperimmune serum was prepared by injecting roosters intravenously with 1 ml of yolk-sac suspension of psittacosis agent containing $10^{7.0}$ egg LD_{50}. Three weeks later, surviving fowls were similarly injected and then bled 10 days after the last inoculation. An independent experimental study, in which *Macaca mulatta* were exposed to an aerosol of psittacosis (Borg) agent and bled periodically, constituted the source of paired monkey serum (McGavran et al., 1962). Human serum was obtained from individuals diagnosed as suspected subclinical or established psittacosis infections on the basis of clinical and serological findings. Serum was heated at 56 C for 30 min before use in serological tests.

Neutralization tests. For carrying out the fluorescent cell-counting neutralization test, serial twofold dilutions of paired serum in phosphate-buffered saline (PBS) were mixed with an equal volume of a constant quantity of psittacosis agent. The dilution of agent suspension employed contained a final concentration of 2×10^4 CIU in the inoculum. After mixtures were incubated at 35 C for 2 hr, 0.2 ml of each mixture was introduced onto three cover-slip cultures of McCoy cells. Adsorption of unneutralized agent was carried out by centrifugation at $500 \times g$ for 15 min at 21 to 23 C. For this procedure, vials containing cover-slip cultures were placed in slotted cups containing tube adapters; these were sealed with a screw-dome cover and mounted on a four-place, pin-type head. Centrifugation was performed in an International centrifuge (size 2, model V). Cover-slip cultures were rinsed twice with maintenance medium after the adsorption periods; 1 ml of the medium was then added to each vial. After incubation at 35 C for 20 to 22 hr, cover-slip cultures were rinsed twice with cold PBS, fixed with cold (−60 C) acetone, and either prepared immediately for immunofluorescence staining and cell counting or stored at −60 C for subsequent examination.

Fluorescent cell counts obtained from an examination of 50 microscopic fields for each cover-slip culture were totalled, and the per cent reduction in counts by convalescent serum of the acute serum or appropriate control was calculated. From a plot of the logarithm of serum dilutions against the per cent reduction of counts straddling the 50% value, the 50% serum-neutralizing end point was determined by interpolation.

In the mouse serum-neutralization test, a similar protocol for the preparation of agent-serum mixtures was followed. The dilution of agent suspension, however, was adjusted to give a final oncentration of CIU in the inoculum that was equivalent to that employed in the fluorescent cell-counting neutralization test. After incubation of agent-serum mixtures at 35 C for 2 hr, 0.03 ml of each mixture was then inoculated intracerebrally into Swiss mice weighing 10 to 14 g each. Ten mice were inoculated for each agent-serum mixture. Animals were observed daily for 14 days and the survivors were noted.

Complement-fixation test. The complement-fixation test was carried out by the procedure described in the Lederle brochure on "Rickettsial and Viral Diagnostic Antigens." Serum was titrated against 2 units of Lederle psittacosis antigen; titers were expressed as the highest dilution of serum exhibiting 100% inhibition of hemolysis.

Immunofluorescence techniques. The direct fluorescent-antibody technique was employed to obtain immunofluoresence of infected cells. The preparation of psittacosis antiserum and conjugation with fluorescein isothiocyanate have been described previously (Hahon and Nakamura, 1964). Infected cell monolayers, previously fixed with cold acetone, were washed three times with PBS and stained with serum conjugate for 30 min. Cover-slip cultures were then rinsed in three changes of PBS to remove excess conjugate and mounted in 10% glycerol in PBS. Cover slips were examined with an American Optical microscope equipped with a Fluorolume illuminator (model 645), Corning no. 5840 and Schott BG-13 exciter filters, and an E. K. no. 2A barrier filter. With this optical system, the number of microscopic fields contained in the area of a 15-mm cover slip was 1,280 at a magnification of 645×. The number of CIU per milliliter of agent suspension was calculated by the method described elsewhere (Hahon and Nakamura, 1964).

RESULTS

Agent adsorption. Preliminary experiments revealed that prolonged contact between inoculum from agent-serum mixtures and cell monolayers during the period of agent adsorption resulted in an effect that was deleterious to the viability of cells 24 hr later. The effect occurred with mixtures containing high concentrations of serum when the adsorption period was carried out with cell monolayers maintained in a stationary position at 23 or 35 C for 1 to 2 hr. Because attempts to estimate the quantity of unneutralized agent particles were precluded under these conditions, a rapid and efficient procedure for agent adsorption was imperative. An experiment was performed to determine the rate of agent adsorption onto cell monolayers during stationary incubation (35 C) and centrifugation ($500 \times g$). To vials containing cover-slip cultures, 0.5 ml of a 10^{-2} dilution of agent suspension was added. Vials were removed at designated intervals during the period of agent

adsorption, and 0.25 ml of residual inoculum from each vial was introduced onto additional cell monolayers to measure the quantity of unadsorbed agent. Residual inocula derived from cell monolayers that had been previously centrifuged or held at stationary incubation were adsorbed at 500 × *g* for 1 hr at 23 C and for 2 hr at 35 C. After the designated periods of agent adsorption for both initial and residual inocula, all cover-slip cultures were rinsed twice with maintenance medium, incubated, and fixed in the prescribed manner.

The per cent of agent adsorbed during each interval with each procedure is shown in Fig. 1. Within 15 to 30 min, more than 98% of agent was adsorbed during centrifugation, whereas approximately 55% was adsorbed during stationary incubation for 2 hr. Since the efficiency and rapidity of agent adsorption onto cell monolayers attained by the use of centrifugal force was clearly superior to results with stationary incubation, centrifugation at 500 × *g* for 15 min at room temperature was employed

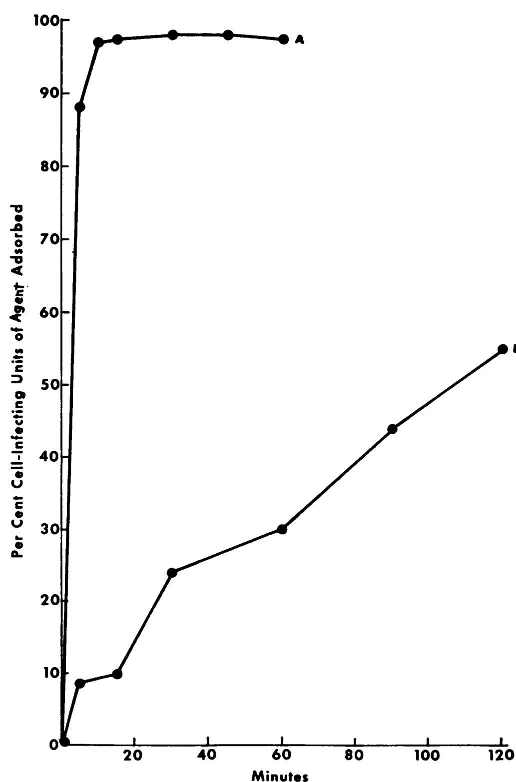

FIG. 1. *Adsorption of psittacosis agent onto cover slip cultures of McCoy cells by (A) centrifugation (500 × g, 23 C), and at (B) stationary incubation (35 C).*

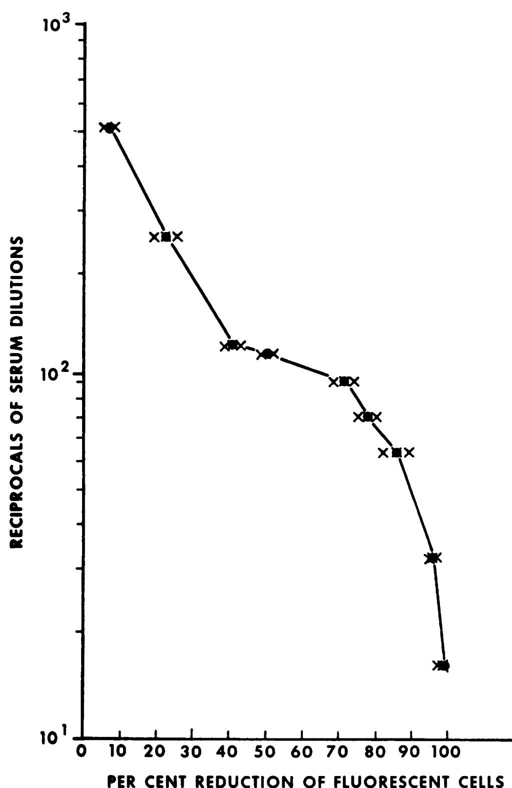

FIG. 2. *Curve relating per cent reduction in fluorescent cells by dilutions of hyperimmune serum in the fluorescent cell-counting neutralization test. Solid circles represent the mean of three determinations.*

as the procedure for adsorption of unneutralized agent in all subsequent tests. Cell monolayers were unaffected by high concentrations of serum in agent-serum mixtures when this procedure was used for the prescribed time.

Determination of 50% serum-neutralizing end point. Replicate twofold dilutions of hyperimmune rooster serum and an appropriate control were mixed with an equal quantity of agent suspension, incubated at 22 C for 1 hr, and introduced onto cover-slip cultures in the manner described previously to determine the quantity of unneutralized agent particles. By plotting the logarithm of serum dilutions against the per cent reduction of fluorescent cell counts for each dilution, a symmetrical curve is formed that is linear over a certain critical range on either side of the 50% reduction of cell counts (Fig. 2). By interpolation, the 50% serum-neutralizing end point can be determined accurately. The value of employing small dilution

increments of serum in the proximity of the 50% fluorescent cell-reduction point is obvious.

Nine determinations were made to estimate the precision of the fluorescent cell-counting neutralization test. The procedure of the test was similar to that described previously, except that agent-serum mixtures were incubated at 35 C for 2 hr prior to inoculation onto cell monolayers. The results in Table 1 reveal the unusual precision of the test. The standard deviation, expressed as a percentage of the mean 50% serum-neutralizing end point, was 3.3 with all end points ranging within one-half of this value.

Incubation of agent-serum mixtures. Because diverse opinions exist as to whether incubation of virus-serum mixtures is necessary (Lennette, 1959), varied conditions of incubation were tested to ascertain the effect on serum-neutralizing end points. Both time and temperature markedly influenced serum-neutralizing titers (Table 2). Neutralizing endpoints were approximately 10-fold higher after incubation of mixtures at 35 C for 2 hr compared with 24 C for 0.5 hr. Prolonged incubation, especially at higher temperatures, reduced the fluorescent cell count of controls, which was indicative of agent inactivation. Since higher neutralizing titers were associated with reduced agent concentration of controls, it was necessary to determine whether these events were related. Three different

TABLE 1. *Precision of the fluorescent cell-counting neutralization test*

Test	Serum dilution[a]				50% serum-neutralizing end point[d]
	1:512		1:1,024		
	Count[b]	Neutralization[c]	Count	Neutralization	
		%		%	
1	55	90.60	344	41.20	890
2	91	84.45	347	40.69	880
3	84	85.65	314	46.33	910
4	107	81.71	332	43.25	890
5	85	85.48	325	44.45	900
6	105	82.06	332	43.25	890
7	99	83.08	330	43.59	890
8	98	83.25	313	46.50	910
9	106	81.89	330	43.59	890

[a] Test carried out with hyperimmune psittacosis rooster serum.

[b] Total fluorescent cells in 50 microscopic fields.

[c] Reduction of the number of fluorescent cells expressed as a percentage of control cell count of 585.

[d] Reciprocal of serum dilution. The arithmetic mean was 896, with a SD of ±30.4 and a SE of ±10.1.

TABLE 2. *Effect of time and temperature on serum-neutralizing end points*

Time	Temp	Fluorescent cell count of control	Serum dilutions*						50% serum-neutralizing end point†
			1:32	1:64	1:128	1:256	1:512	1:1,024	
hr	C								
0.5	24	1,041	122	303	619	662	674	928	105
1	24	932	6	68	402	515			180
2	24	669	22	38	64	325	401	583	680
2	35	518	8	2	7	4	72	157	>1,024
2	4	873	152	223	213	331	492	539	400

* Tests carried out with psittacosis hyperimmune rooster serum.

† Reciprocal of serum dilution; mean of three determinations.

TABLE 3. *Serological determinations on serum from Macaca rhesus exposed to psittacosis agent*

Monkey	Serum	No. of days after virus exposure	CF test[a]	FCCNT[b]	Mouse SN test[c]
J 93	Pre-exposure	0	<5	<2	
	Postexposure	0	<5	<2	
J 84	Pre-exposure	0	<5	<2	
	Postexposure	7	<5	<2	
J 86	Pre-exposure	0	<5	<2	<2
	Postexposure	17	20[d]	<2	<2
J 16	Pre-exposure	0	<5	<2	<2
	Postexposure	19	640	<2	<2
J 22	Pre-exposure	0	<5	<2	<2
	Postexposure	26	320	21	<2
G 13	Pre-exposure	0	<5	<2	<2
	Postexposure	33	60	32	<2
J 90	Pre-exposure	0	<5	<2	<2
	Postexposure	43	20	8	<2
L 33	Pre-exposure	0	<5	<2	<2
	Postexposure	68	20	8	<2

[a] Complement-fixation test.

[b] Fluorescent cell-counting neutralization test.

[c] Mouse serum-neutralization test.

[d] Reciprocal of serum dilution.

quantities of agent were mixed with appropriate control fluid and a 1:128 dilution of antiserum, incubated at 23 C for 1 hr, and treated in the prescribed manner. Fluorescent cell counts from control mixtures containing varied quantities of agent were 1,427, 932, and 561; in the presence of antiserum, they were reduced 51.6, 58.0, and 52.1%, respectively. The reduction of a constant percentage of agent, irrespective of the quantity present, by a given strength of antiserum reaffirms the operation of the "percentage law" described by Andrewes and Elford (1933)

and negates, in this instance, any relationship between reduced agent concentration of controls during incubation and serum-neutralizing end points.

Comparative serological determinations. Complement-fixation, fluorescent cell-counting neutralization, and mouse serum-neutralization tests were employed to estimate the antibody content of paired serum from monkeys previously exposed to an aerosol of psittacosis agent. The results in Table 3 show the development of complement-fixing and serum-neutralizing antibodies over a period of approximately 2 months. The former was first detected on the 19th day; the latter was noted on the 26th day after exposure of animals. Their order of appearance was in agreement with previous reported findings (Meyer and Eddie, 1939). Complement-fixing titers were uniformly higher than serum-neutralizing end points. In animals that had been exposed to the agent approximately 1 or more months previously, however, an equivalent increase in the geometric ratio of titers of both types of antibodies was noted. It is significant that serum-neutralizing antibodies were only detected by the fluorescent cell-counting neutralization test.

The results obtained from testing paired human serum by the same serological procedures are shown in Table 4. Both serum-neutralizing and complement-fixing antibodies were found in serum from patients in which the diagnosis of psittacosis was made previously on the basis of clinical findings and serological tests (Table 4, patients 1 to 4). From suspected cases of psittacosis (Table 4, patients 5 to 8), the highest increase in complement-fixing and serum-neutralizing titers, between early and later serum

TABLE 4. *Serological determinations on human serum from patients with subclinical or established psittacosis infections*

Patient	Serum sample		CF test[b]	FCCNT[c]	Mouse SN test[d]	Comment
	Type[a]	Date				
1	A	DNR[e]	<4	<2	<2	Clinical and serological diagnosis of psittacosis (patients 1–4)
	C	DNR	32[f]	8	<2	
2	A	DNR	<4	<2	<2	
	C	DNR	128	2	<2	
3	A	DNR	<4	<2	<2	
	C	DNR	32	2	<2	
4	A	DNR	NT[g]	<2	<2	
	C	DNR	NT	2	<2	
5	A	1 July 1963	<5	<2	NT	Suspected subclinical psittacosis infection (patients 5–7)
	C	19 December 1963	10	2	NT	
6	A	19 December 1961	<5	<2	<2	
	C	16 December 1963	10	4	<2	
7	A	5 December 1961	<5	<2	<2	
	C	17 December 1963	10	4	<2	
8	A	8 July 1963	<5	<2	<2	Technician working with psittacosis agent
	C	18 December 1963	5	3	<2	
9	C[h]	15 April 1964	80	3	<2	Subclinical psittacosis infection
10	C	10 February 1964	<5	2	<2	Hospital case of psittacosis 5–10 years previously (patients 10–12)
11	C	8 May 1957	<5	2	<2	
12	C	18 May 1964	<5	2	<2	

[a] A, acute; C, convalescent.
[b] Complement-fixation test.
[c] Fluorescent cell-counting neutralization test.
[d] Mouse serum-neutralization test.
[e] Date not recorded.
[f] Reciprocal of serum dilution.
[g] Not tested.
[h] Acute serum sample not available in patients 9–12.

specimens, was only twofold. The possibility exists that higher serum-neutralizing titers may have been demonstrated if the specific strains of psittacosis agent responsible for each infection had been known and available for use in the neutralization tests. In cases of psittacosis infection experienced 5 to 10 years before (Table 4, patients 10 to 12), serum-neutralizing antibodies were detected in the absence of complement-fixing antibodies. Similar to the findings obtained with paired monkey serum, neutralizing antibodies were demonstrated only by the fluorescent cell-counting neutralization test.

No cross reactions were noted when the fluorescent cell-counting neutralization test was performed with monkey Q fever antiserum, rabbit variola, vaccinia, Coxsackie type A-9, Echo type 1, or poliovirus type 1 antiserum, or with human influenza type A soluble antiserum.

Discussion

The wide divergency of methodology employed in neutralization tests has not fully resolved the general difficulty experienced in demonstrating low titers of serum-neutralizing antibodies that occur as a result of psittacosis infection (Rivers and Berry, 1931; Bedson, 1933; Rivers and Schwentker, 1934; Hilleman, 1945; St. John and Gordon, 1947; Moulder et al., 1958; Piraino and Abel, 1964). Tests that were carried out employed different agent assays of varying sensitivity and accuracy, minimal or extended incubation periods for agent-serum mixtures, estimates of neutralization based on a variety of reactions and responses, and a protocol (constant serum-varying agent) for the preparation of agent-serum mixtures that required large changes in agent titer to reveal small differences in antibody concentration. In the fluorescent cell-counting neutralization test, the utilization of a protocol in which a constant quantity of agent is mixed with serial dilutions of serum provides a true measure of the amount of serum antibody present that can be expressed quantitatively as the 50% serum-neutralizing end point. In addition, by employing an assay for psittacosis agent of high accuracy (fluorescent cell-counting technique), an efficient procedure for adsorption of unneutralized agent onto cell monolayers (centrifugation), and an optimal incubation period for agent-serum mixtures (35 C for 2 hr), a sensitive, precise, and specific serological procedure is available that can detect and measure small but significant differences in neutralizing antibody titers between two serum specimens. The ability to determine the neutralizing antibody content of serum in less than 24 hr is a singular advantage of the test.

The fluorescent cell-counting neutralization test may be applicable not only for diagnostic purposes but for retrospective epidemiological surveys, evaluation of the efficacy of vaccines, correlation between levels of neutralizing antibodies and development of resistance to infection, identification of agent strains, and studies to determine the immunological interrelationships among members of the psittacosis group of microorganisms.

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