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A Simplified Tissue Culture Tube Neutralization Test for Rous Sarcoma Virus Antibodies. (28947)

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Various methods have been described for the assay of neutralizing antibodies to Rous sarcoma virus. Most commonly used are those based on prevention of tumors in young chickens or in chicken embryonic tissues (1-17) and prevention of a hemorrhagic disease in chicken embryos(18,19). Recently, Rubin(20) described an *in vitro* petri dish tissue culture test based on the neutralization of a discrete foci of altered cells in monolayer cultures of chicken embryo fibroblasts.

Recently we found that antibodies to Rous sarcoma virus could be assayed efficiently by means of a simple tissue culture tube neutralization test using the constant virus-serum dilution technique. This paper describes the test, its relative sensitivity and specificity, its value in distinguishing between different serotypes of Rous sarcoma virus, and preliminary observations on its application to surveys for antibody in various populations.

Materials and methods. Virus. Two strains of Rous sarcoma virus, the Bryan strain and the Schmidt-Ruppin strain(21) were used. The Bryan strain was kindly provided by Dr. W. Ray Bryan, Nat. Cancer Inst., as partially purified virus suspension CT 929(22) which was stored at —60°C and used directly in the experiments to be reported. The Schmidt-Ruppin strain (hereafter referred to as S-R strain) was kindly provided by Dr. C. G. Ahlstrom, Univ. of Lund, Sweden. The stock virus for this strain was made as follows: 11-day-old chick embryos were inoculated on the chorioallantoic membranes with a 20% clarified extract of wing web tumors induced by this virus. Membranes with confluent lesions 6 days later were made into a 20% suspension in Eagle's medium(23) and the suspension was clarified at 2500 rpm for 10 minutes in the International refrigerated centrifuge, distributed in screw-capped vials, and stored at —60°C until used.

Solutions and media. Chicken embryo fibroblasts were grown in a medium (EGM) consisting of 90 parts of modified Eagle's medium(23), 5 parts calf serum‡ (inactivated at 56°C for 30 minutes), 5 parts Bacto tryptose phosphate broth and one part stock antibiotic solution to give a final concentration of penicillin 250 units per ml and streptomycin 250 μg per ml. Tris buffer (0.05 M) in isotonic sodium and potassium chloride solution, pH 7.4 was used as wash fluid and as diluent for trypsin in preparation of tissue cultures. The trypsin solution used con-

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sisted of 0.25% Bacto trypsin in Tris buffered saline.

Tissue cultures. Embryos from chickens that had been tested and found to be free of Resistance Inducing Factor (RIF)(24) were used for preparation of tissue cultures.§ The method of preparation of primary cultures of chicken embryo fibroblasts (CEF) was essentially similar to that described by Rubin(25) with the following modifications: Tris buffered saline was substituted for phosphate buffered saline as wash fluid and trypsin diluent. The cells were suspended in EGM at 3 million cells per ml. Screw-capped 32-oz prescription glass bottles were inoculated with 40 ml per bottle and the cultures incubated at 37°C. Three to 4 days later, the cells were removed by trypsin treatment and resuspended in EGM at a concentration of 350,000 cells per ml. Screw-capped tubes of 16 × 125 mm size were inoculated with 1.5 ml per tube. The cultures were incubated stationary at 37°C and inoculated the following day.

Procedures—Titrations of virus. The titers of stock virus preparations were determined in CEF tubes as follows: Ten-fold dilutions of the virus were prepared in Eagle's medium (with antibiotics). Each dilution was inoculated in 0.2 ml amounts into each of 4 CEF tubes. The medium was replaced on the 3rd or 4th day and the tubes examined microscopically under low power for Rous foci(26) on the 7th day. The extent of positive involvement was graded by a system of + signs.‖ A tube was considered positive for Rous effects if it contained one or more Rous foci. The 50% endpoint infectious dose for tissue culture (TCD_{50}) was computed by the method of Reed and Muench(27).

Serum neutralization tests. These tests were conducted as follows: Serial 2-fold dilutions of sera (inactivated at 56°C for 30 minutes) were prepared in Eagle's medium containing antibiotics. The serum dilutions were mixed with equal volumes of a suitable virus dilution so as to contain approximately 100 TCD_{50} of the virus in 0.2 ml of the virus-serum mixture. The mixtures were incubated at room temperature for one hour and each inoculated into sets of 2 tubes using 0.2 ml per tube. The medium††† was replaced on the 3rd or 4th day and the tubes examined microscopically for Rous foci on the 7th day or on the day on which 2+ foci were seen in controls. The titer of the serum was taken to be the highest dilution¶ that completely neutralized the effects of the 100 doses of virus in both tubes.

Initial observations. Photomicrographs of tube cultures inoculated with virus-serum mixtures showing the effects of the virus and of neutralization are given in Figure 1. Antisera prepared in 3 chickens and a turkey** against Bryan strain gave titers of between 1:128 to 1:1024 vs homologous virus, whereas the pre-immune chicken sera were negative at 1:8 serum dilution (Figure 2). These sera were negative for antibodies to the S-R strain at a serum dilution of 1:8. On the other hand, a convalescent serum obtained from a moribund chicken 22 days after inoculation of the S-R strain had a titer of 1:128 against the S-R strain, but showed no neutralizing antibodies to the Bryan strain at a 1:8 serum dilution. An avian myeloblastosis antiserum prepared in chickens†† gave a titer of 1:1024 against the Bryan strain and a 1:16 titer against the S-R strain.

Antiserum from a chicken infected with the prototype RPL-12 virus and a serum from a case of natural infection with avian visceral lymphomatosis (AVL)‡‡ gave titers of 1:32 and 1:256, respectively, against the Bryan strain, but were negative when tested

§ Chicken embryos obtained from Kimber Farms, Inc., Fremont, Calif.

Cultures were furnished on contract by Dr. T. G. Ward and D. Mullinax, Microbiological Associates Inc., Bethesda, Md.

‖ F = sheet with very few Rous foci (between 1 and 10). + = Rous effects seen in approximately 25% of the sheet; similarly, ++ = 50%; +++ = 75%; ++++ = entire sheet involved.

††† Serum concentration reduced to 1%.

¶ Serum dilutions are expressed as dilutions after mixture with virus.

** Turkey antiserum kindly provided by Dr. W. Ray Bryan.

†† Kindly provided by Dr. M. Okuyan of this laboratory.

‡‡ Kindly provided by Dr. B. R. Burmester.

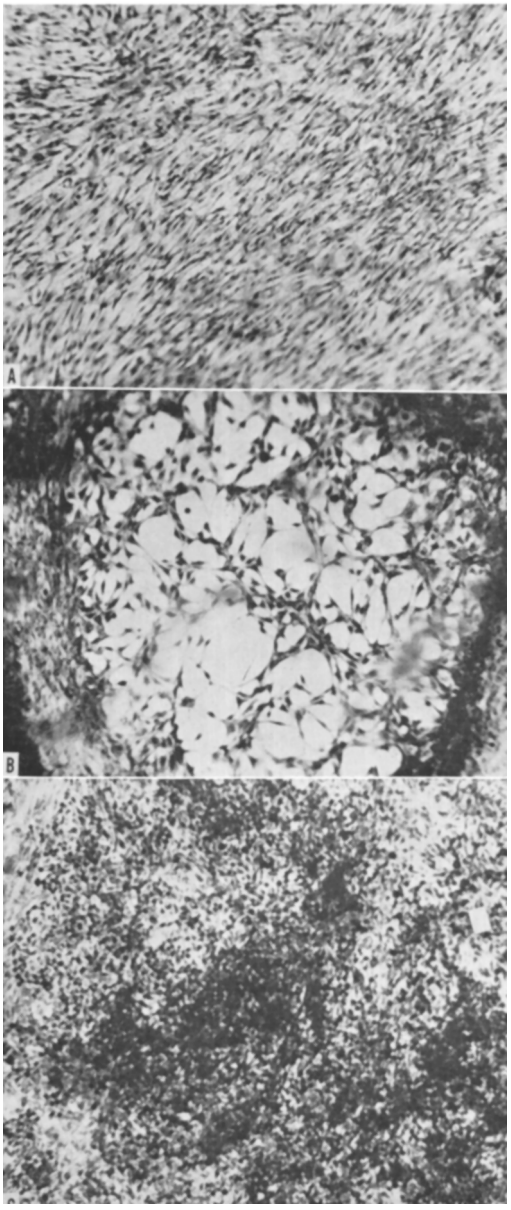

FIG. 1. Tissue culture tubes of chicken fibroblasts 7 days after inoculation of virus-serum mixtures. Stained with Wright-Giemsa; magnification 90 ×. A. Tube inoculated with a mixture of 100 TCD_{50} of Bryan strain of Rous sarcoma virus and chicken antiserum to this strain diluted 1:256 (titer 1:256). The entire sheet was normal. B. Tube inoculated with a mixture of 100 TCD_{50} of the Bryan strain and normal serum diluted 1:8 (serum of chicken prior to immunization with the virus). Most of the transformed cells of this Rous focus have floated off into the medium. C. Tube inoculated with a mixture of 100 TCD_{50} of the Schmidt-Ruppin strain of Rous sarcoma virus and a chicken convalescent serum against this virus diluted 1:256. The titer of this serum was 1:128. Note diffuse foci induced by this strain.

against the S-R strain at a 1:8 serum dilution (Fig. 2).

Reproducibility. The reproducibility of the test was determined in repeated tests of a standard Bryan strain chicken antiserum vs 100 TCD_{50} of the Bryan strain. In 15 tests,

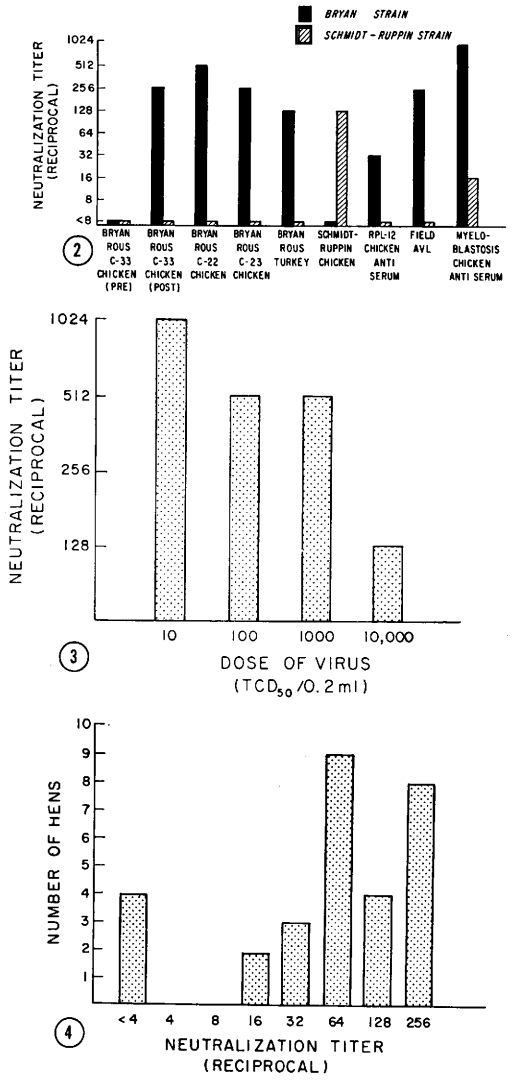

FIG. 2. Antibody titers of various chicken antisera against Bryan strain and the Schmidt-Ruppin strain of Rous sarcoma virus. Black bars indicate titers against the Bryan strain and lined bars titers against the Schmidt-Ruppin strain.

FIG. 3. Antibody titers of a chicken antiserum to the Rous sarcoma virus (Bryan strain) against increasing doses of the virus.

FIG. 4. Rous sarcoma virus (Bryan strain) antibody titers of sera of thirty 10- to 12-mo-old White Leghorn chickens of a commercial laying flock in Maryland.

TABLE I. Titer of a Rous Chicken Antiserum by the Tube Neutralization Test as Compared with the Petri Dish Test: Tubes and Petri Dishes Received Identical Inocula Containing 100 TCD_{50} of Rous Virus in 0.2 ml of Virus-Serum Mixture.

	Readings												
	Virus + antiserum dilutions									Virus + diluent			
Assay method	1:512			1:1024			1:2048						Titer†
RSV tube effects*	0	0	0	+	+	+	++	++	++	++	++	++	1:512
RSV foci count, petri dish	0	0	0	3	3	5	50	40	60	75	60	59	1:512

* See *Methods*.

† Highest dilution of serum that completely neutralized the effects of Rous virus in tubes or petri dishes.

a titer of 1:128 was obtained in 8, 1:256 in 6, and 1:64 in one.

Sensitivity. The titer of a standard Bryan strain chicken antiserum against increasing doses of Rous sarcoma virus (Bryan strain) is shown graphically in Fig. 3. Although the highest titer was obtained using 10 infectious units of the virus, these tubes could not be read on the seventh day because of insufficient development of foci. However, after a second medium replacement on this day, the tubes could be read on the 10th day. With the higher doses of virus, the foci developed early enough to enable a reading to be made on the seventh day.

Sensitivity as compared to petri dish neutralization test. The sensitivity of the tube test was compared to the petri dish tissue culture agar overlay method(20). Simultaneous titrations of the standard Bryan strain chicken antiserum vs the Bryan strain virus were done in both systems. The procedure followed for agar overlay and Rous foci counting was similar to that described by Rubin *et al*(20) with the following modifications: a) Each petri dish was inoculated with 0.2 ml of the virus-serum mixture containing 100 TCD_{50} of the virus; b) the agar overlay medium consisted of EGM with 0.8% agar; c) foci were counted on the seventh day.

The results of a representative experiment are presented in Table I. It will be seen that the titer of the serum was the same in both tests, if 100% neutralization of foci was taken as the criterion for determining endpoints. If 90% reduction of foci was used (20), the titer by the petri dish method was higher by one dilution.

Specificity. The specificity of the simplified test tube procedure is attested to in part by the differences revealed between the Bryan and S-R strains (Fig. 2) and the results obtained by the petri dish neutralization procedure which gave titers equivalent to those obtained in the tube test (Table I). The specific neutralization of homologous virus by antisera prepared for the Bryan and S-R strains was shown by absorption tests to be directed against the respective viruses and not against cells. Normal chicken embryo fibroblasts scraped from secondary tube cultures were sedimented in a centrifuge and resuspended in EGM as a 25% suspension (v/v). This suspension was subjected to 2 cycles of freezing and thawing and mixed with equal volumes of antiserum and incubated at 37°C for one hour. The cellular material was resedimented and the antibody titers of the supernatant and the original untreated serum were determined. Both antisera retained their original titers against their homologous viruses.

Surveys of chickens in commercial flocks. The Bryan strain of Rous sarcoma virus is immunologically closely related to naturally occurring avian visceral lymphomatosis virus and RPL-12 virus(20,28-31), and therefore has been used for antibody studies of various chicken populations(9,10,18,20,30). The test tube procedure described above offers a simple method applicable to large scale serological surveys for prevalence of antibodies to naturally occurring avian visceral lymphomatosis virus. Sera from 10- to 12-month-old White Leghorn laying hens in a commercial flock§§ were tested for antibodies to the

Bryan strain; 26 of 30 hens showed levels of antibodies from 1:16 to 1:256 while 4 were negative at a 1:4 serum dilution (Fig. 4). Subsequent sera taken at monthly intervals for 4 months from the 4 negative hens revealed that 3 of the 4 hens continued to be negative throughout this period; one hen (#11) developed 1:64 titer between the first and the second month after the original blood collection. The 2 subsequent monthly specimens from this chicken showed a titer of $>$ 1:128. Fourteen hens with initial titers of 1:32 or higher gave titers of $>$ 1:128 four months later. One hen which originally showed a titer of 1:16 was found to be negative at a 1:4 serum dilution at the fourth monthly bleeding.

Sera taken from one-day-old chicks derived from 2 antibody positive hens (titers, 1:128) showed titers of 1:32 and 1:64, respectively, whereas sera of 2 chicks from each of the 3 antibody negative hens (#12, 15 & 20) were negative at a 1:4 serum dilution. A one-day-old chick derived from hen #11 while she was still free of serum antibodies was also negative. However, a one-day-old chick derived one month after the mother became positive for antibody (titer $>$ 1:128) was also found to have antibody (titer 1:64). Tests for RIF(24,33) revealed a viremia in one of the 3 persistently antibody negative hens (*vide supra*). The sera of the other 2 were negative for RIF. Embryos and freshly collected sera from these hens are under test to determine their current antibody and RIF status(33).

Tests for antibodies to Rous virus (Bryan strain) in adult chickens from 2 other flocks gave the following results: From a flock in Eastern Maryland,‖‖ 17 of 20 were positive at serum dilutions greater than 1:64 as were 20 of 22 from a flock in Connecticut.¶¶ The others were negative at 1:16.

Surveys for antibodies in species other than chickens. Although our serum antibody surveys are not extensive, it is perhaps worthwhile to note that the sera of the following were negative for antibodies to both Rous strains at 1:4 dilution: Twenty-four 8-week-old turkeys,*** 24 Japanese quail,*** 6 barn pigeons, 2 ducks, and one guinea fowl. Sera from 4 groups of human subjects were also negative for neutralizing antibodies to both the Bryan and the S-R strains; included were: a) 7 laboratory workers intimately exposed to avian leucosis virus, b) 14 adults with leukemia, c) 7 adults without leukemia or other neoplasma and d) 11 children who had been inoculated one month before with live attenuated measles vaccine produced in chick embryos.

Discussion. The tissue culture neutralization test described here provides a simple, reproducible and economical method for *in vitro* assay of Rous sarcoma virus antibodies which should make possible larger scale sero-epidemiological studies of avian leucosis. It can be performed with commercially prepared tissue cultures.

The immunological differences disclosed in this test between the Bryan and the Schmidt-Ruppin strains of Rous sarcoma virus may permit better evaluation of the immunological patterns of wild strains of leucosis virus occurring in nature.

Our observations on neutralization of the Bryan strain by various chicken sera, including sera prepared by immunization with avian myeloblastosis and RPL-12 viruses and sera produced in nature presumably by wild strains of lymphomatosis virus, suggest that these viruses are serologically closely related to the Bryan strain, and are in agreement with previous reports(10,20,28,29,31). However, the possibility cannot be excluded that contamination of virus stocks with RIF or Rous-Associated Virus (RAV), antigenically similar to the Bryan strain(24,31,32), was responsible for the Bryan strain neutralizing antibodies present in the antisera. While the Schmidt-Ruppin strain does not appear to be closely related to this serological group, we have observed neutralization of this strain by low

§§ We are indebted to Truslow Farms, Inc., Chestertown, Md., for these sera.

‖‖ Courtesy Dr. S. B. Hitchner.

¶¶ Courtesy Dr. C. F. Helmboldt.

*** From 2 commercial chicken farms in Eastern Maryland.

dilutions of certain sera obtained from chickens in Maryland flocks (unpublished data). It is undetermined at present whether or not these low level reactions represent specific S-R antibodies, or a manifestation of low level antigenic relationship between these viruses as noted with the myeloblastosis serum (Fig. 2). It is also possible that these low level reactions indicate the presence of non-specific inhibitors of virus in some of the sera, although this is unlikely because several hens showed serum neutralizing titers of 1:256 or greater for both strains.

Rubin *et al*(20) found that adult chickens in a commercial flock could be divided into 2 categories: 1) Chickens with persistent RIF viremia and no serum neutralizing antibodies to Rous virus, which he attributed to a state of immunological tolerance; and 2) chickens with Rous antibody and usually free of demonstrable RIF virus. Combined with the RIF test(25), the tube neutralization test described herein should simplify the work of locating antibody-free highly infectious viremic chickens in flocks where most are antibody-positive, and also for locating individual chickens and flocks free of both RIF and neutralizing antibodies. Our preliminary surveys showed hens of 3 commercial flocks to be 85% or more positive for antibodies to Rous virus (Bryan strain).

Summary. A simplified tube neutralization test for assay of antibodies to Rous sarcoma virus and related avian leucosis viruses was described which appears to be suitable for differentiating serotypes and for surveys for naturally occurring antibodies against these viruses. The test was used in preliminary surveys for antibodies to Rous virus in sera of various species. Most hens in 3 commercial flocks of laying chickens were found to be positive for antibody. One of 3 hens without detectable antibodies was found to have RIF viremia. No antibodies were found in limited numbers of sera from turkeys, Japanese quail, pigeons or in sera from human subjects with or without leukemia.

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