Immunoelectrophoretic Analysis of Avian Ribonucleic Acid Tumor Virus Group-Specific Antigens

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Tumors induced in pigeons by inoculation with the Schmidt-Ruppin strain of Rous sarcoma virus regressed after about 6 weeks. Sera from these pigeons, taken 8 weeks after inoculation, had complement-fixing group-specific antibody titers of 1:2 to 1:256. In immunoelectrophoresis with the pigeon serum, disrupted BAI strain A (myeloblastosis) avian tumor virus showed at least five precipitin arcs. The pattern of precipitin lines was dependent in part on the means used for virus disruption, and ethyl ether and nonionic detergents appeared to be both effective and relatively mild reagents. Immunoelectrophoretic comparison of pigeon serum with serum from a tumor-bearing hamster and that from virus-inoculated rabbits yielded similar, though not identical, results.

Avian ribonucleic acid (RNA) tumor viruses contain two classes of antigenic components, the type-specific envelope antigens (15, 25), and the group-specific (gs) antigens which are shared by all members of this virus group (4, 13). Two antisera have been used for study of the gs antigens, that from rabbits immunized with disrupted BAI strain A (myeloblastosis) virus (10, 11) and that from hamsters (13, 20) bearing tumors induced by the Schmidt-Ruppin strain of Rous sarcoma virus (SR-RSV). The availability of both types of antisera is desirable for comprehensive studies. The rabbit serum is particularly advantageous when purified antigens can be used for inoculation. Sera from tumor-bearing animals, on the other hand, contain antibodies to native antigens, avoiding possible alterations resulting from reagent treatments during antigen extraction or virus disruption. Difficulties in producing sufficient quantities of high-titer gs antiserum in hamsters have aroused interest in the possible use of other tumor-bearing animals, particularly the pigeon. Tumors induced in these birds by inoculation with SR-RSV developed slowly and then regressed (22), while gs complement-fixing (CF) antibodies appeared in the serum (21) in titers as high as those in hamsters. These results were confirmed in later studies (19), and the pigeons were also found to produce virus-neutralizing antibodies and a low level of virus infectious for chickens. The pigeon serum functioned as well as hamster serum (19) in the complement fixation test for gs antigen of avian tumor virus complex (COFAL test) (20).

The gs antigens are multiple. Immunodiffusion (2, 18) and immunoelectrophoresis (18) studies of disrupted avian tumor viruses with serum from hamsters bearing SR-RSV-induced tumors revealed four antigenic components. Examination of RSV (RAV-1) virus proteins after fractionation by isoelectric focusing has revealed five components which were described as gs on the basis of reaction with the hamster serum. Two reacted strongly and three weakly in CF tests (14). In the present work, the gs antigenic component constitution as determined with serum from an SR-RSV tumor-bearing pigeon was compared with the findings obtained with sera from an SR-RSV tumor-bearing hamster and from rabbits inoculated with BAI strain A virus. The results obtained here have confirmed the development of gs CF antibodies in tumor-bearing pigeons and have demonstrated the applicability of such pigeon sera to immunoelectrophoretic study of the gs antigens of the BAI strain A virus. Because of the morphological and chemical complexity of the avian tumor virus particles, the method of disruption strongly affects the analytical results obtained. This report describes the findings in comparative studies with immune sera from different animals employed with products of virions disrupted by several detergents and organic solvents.

MATERIALS AND METHODS

Viruses and antigens. The SR-RSV was kindly provided by H. Sazawa who had obtained it from H. Rubin. Virus stocks were prepared from tumors obtained by two passages in the wing web of two-week-old line 15 white Leghorn chickens (Regional

Poultry Research Laboratory, East Lansing, Mich.). Tumors harvested at 9 to 10 days after virus inoculation were kept at −78 C until used. Virus extract was prepared, just before use, as a 20% tumor suspension in tris(hydroxymethyl)aminomethane (Tris)-buffered saline (pH 7.4) containing 1 unit of hyaluronidase per ml, with a model 45 VirTis homogenizer (VirTis Co., Gardiner, N.Y.) operated at medium speed. The suspension was centrifuged at 3,000 rev/min for 10 min, and the supernatant extract was recovered.

BAI strain A (avian myeloblastosis) virus was separated from plasma of leukemic chicks (6) by a sequence of alternate low-speed (5,000 rev/min, 10 min, Sorvall rotor SS34) and high-speed (35,000 rev/min, 15 min, Spinco rotor 40) centrifugation through three cycles. The suspending fluid was 7.5% sucrose in water. Virus particle concentrations were determined by sedimentation onto agar and electron microscopy (24).

BAI strain A virus in suspension in 7.5% sucrose in water was disrupted by treatment with Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.), Nonidet P-40 (a gift of the Shell Chemical Co., New York, N.Y.), Tween 20 (Atlas Chemical Industries, Wilmington, Del.), or digitonin (Sigma Chemical Co., St. Louis, Mo.) at a final concentration of 0.2%, and the mixtures were transferred to the immunoelectrophoresis sample wells after 15 to 30 min at room temperature. For ether extraction, four volumes of ethyl ether mixed with the virus suspension were allowed to stand for 3 min at about 2 C and centrifuged, and the ether phase was discarded. The treatment was repeated, and residual ether was removed with a stream of nitrogen. One extraction of virus suspension with four volumes of chloroform-methanol (3:1, v:v) was used at about 2 C, and the phases were separated by centrifugation.

Antigen for the CF test was prepared from tumors produced in chickens inoculated into the wing web with SR-RSV. Growths were excised, homogenized, and frozen and thawed three times, and the suspensions were treated with ether and centrifuged at 3,000 rev/min for 10 min. The water-soluble fraction was used as antigen.

Antisera. One-month-old pigeons (Palmetto Pigeon Plant, Sumter, S.C.) were inoculated with 0.5 ml of SR-RSV tumor extract in each wing web. After 8 weeks, when the tumors were regressing, the pigeons were bled by heart puncture. Harvested sera were stored at −20 C and inactivated for 30 min at 56 C before use.

Antiviral immune serum obtained from rabbits by inoculation of BAI strain A virus purified by two cycles of low- and high-speed centrifugation and banding in a potassium tartrate gradient was the same as that used in previous work (12).

Serum from a hamster bearing a tumor induced with SR-RSV, designated "A121 #36," was the generous gift of Robert M. Dougherty.

Immunological procedures. The micro CF tests (23) used 4 units of antigen (determined in preliminary checkerboard experiments), 2 units of hemolysin, and 2 units of complement. Positive tests were recorded as 3+ to 4+ beyond 50% hemolysis in-

hibition. Virus-neutralizing capacity of the serum was measured in 10-fold dilution steps by inhibition of focus formation by the SR-RSV on chick embryo cells (15).

For immunoelectrophoresis, microscope slides (1 by 3 inches) were coated with 2.0 ml of melted 1% agarose in 0.1 M Tris-acetate buffer, pH 9.0 (at 23 C). Troughs were 1.3 mm wide, and wells were punched with an 18-gauge needle attached to suction. After a prerun of 30 min, the wells were charged with antigen mixtures (0.5 to 1.0 μliter per well) and subjected to electrophoresis at 150 v (panel meter), about 1 ma per slide, at room temperature in a Buchler immunoelectrophoresis chamber, with Whatman 3MM paper wicks. The buffer was 0.1 M Tris-acetate with a pH of 9.0 at 23 C. The pH in the gel decreases to about 8.7 due to warming during electrophoresis. The diffusion step was at 2 C for 1 to 4 days. The slides were photographed with dark-field illumination, washed in 0.9% NaCl and in water, and stained with amido black or Ponceau S.

RESULTS

Pigeon sera. Fifteen pigeons inoculated with the SR-RSV preparation all developed tumors. Two birds died, but, in all others, the tumors began to regress about 6 weeks after inoculation. The 13 surviving birds bled by heart puncture at 2 months yielded about 10 ml of serum each. The CF antibody titer measured with an extract of SR-RSV-induced tumor (Fig. 1) ranged from 1:2 to 1:256. Neutralizing antibody titer against the SR-RSV preparation used for pigeon inoculation was determined in vitro. There was little or no correlation between CF and neutralizing antibody titers (Fig. 1). Serum 933, with CF titer of 1:256 and neutralizing antibody titer of 1:100, was used for immunoelectrophoresis.

Immunoelectrophoresis. When the pigeon serum was used, immunoelectrophoresis of BAI strain A virus broken in various ways revealed five precipitin arcs, two of which tended to separate into pairs (Fig. 2).
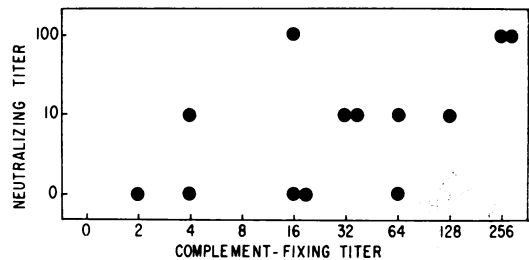

FIG. 1. Complement-fixing and neutralizing antibody titers of 13 sera from tumor-bearing pigeons. Complement fixation was measured with an extract of SR-RSV chicken tumor as antigen, and neutralization was measured as 50% or greater inhibition of focus formation by SR-RSV on chick embryo cells.

Most of the arcs were apparent with virus disrupted with 0.2% Triton X-100 (Fig. 3). The most prominent arc (A) was associated with an antigen which was apparently almost isoelectric at pH 8.7 and which moved about 7 mm toward the cathode. Dextran (uncharged) moved about 6 mm toward the cathode, while bovine albumin migrated about 27 mm toward the anode. Arc A often showed a tendency to separate into two concentric arcs. A second prominent arc (B) lay close to the origin, was closer to the well than to the trough, and was relatively short. Arcs A and B were partially fused. A long thin arc (C), also close to the origin, was located very near the trough. A very long arc (D) began with arc B but extended far toward the anode, usually with a slight double hump. A rather diffuse and only slightly curved line (E) blends with the anodic end of arc D in Fig. 3, but was sometimes more distinct.

The results of immunoelectrophoresis of similarly broken BAI strain A virus against serum from a tumor-bearing hamster are shown also in Fig. 3. The pattern was similar to that with pigeon serum, except that the thin arc (C) was absent in the hamster pattern, and the long double-humped arc (D) seen with pigeon serum was separated into two. This latter appearance may be related to the generally fainter lines seen with this hamster serum.
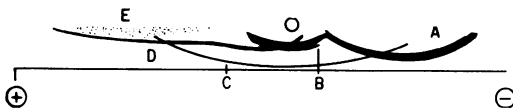

FIG. 2. *Diagrammatic representation of precipitin lines observed after electrophoresis of disrupted BAI strain A virus and diffusion against serum from a pigeon bearing an SR-RSV- induced tumor. The letters are used in the text to refer to the various precipitin arcs.*

Effect of virus disruption procedure. The immunoelectrophoresis pattern was related to the reagent used to disrupt the virions (Fig. 4). Treatment with the nonionic detergent Nonidet P-40 or extraction with ether (Fig. 4A) gave results similar to those with Triton X-100 (Fig. 3, 4C), as did the combined treatment with Tween 20 and ether (Fig. 4B). Virus disrupted with Tween 20 alone also gave a similar pattern, but this detergent was less effective than Triton X-100 or Nonidet P-40 at the same concentration. After virus disruption with digitonin, on the other hand, only one very faint precipitin line, possibly arc B, was detectable (Fig. 4B) and only after staining. Treatment with chloroform-methanol (3:1, v/v), an effective lipid extractant, resulted in only one line, arc C (Fig. 4C). Treatment of the virus with sodium dodecyl sulfate (SDS) gave the antigens a negative charge (Fig. 5) due to binding of the detergent, and the mobility was dependent on SDS concentration. With brief electrophoresis, three arcs could be distinguished

FIG. 4. *BAI strain A virus broken in various ways was reacted with pigeon serum after 90 min of electrophoresis. Virus disruption treatments were as follows: A, Nonidet P40 (upper well), and ether extraction (lower well); B, digitonin (upper well), Tween 20 followed by ether extraction (lower well); C, chloroform-methanol (3:1, v/v) extraction (upper well), and Triton X-100 (lower well). Stained with amido black 10B.*
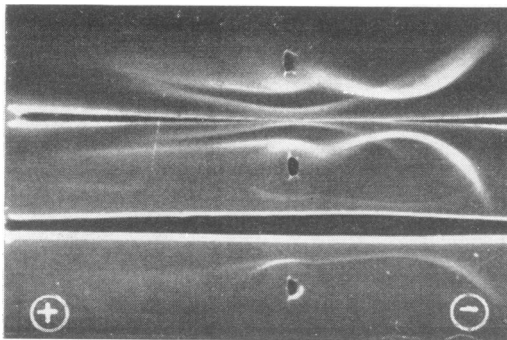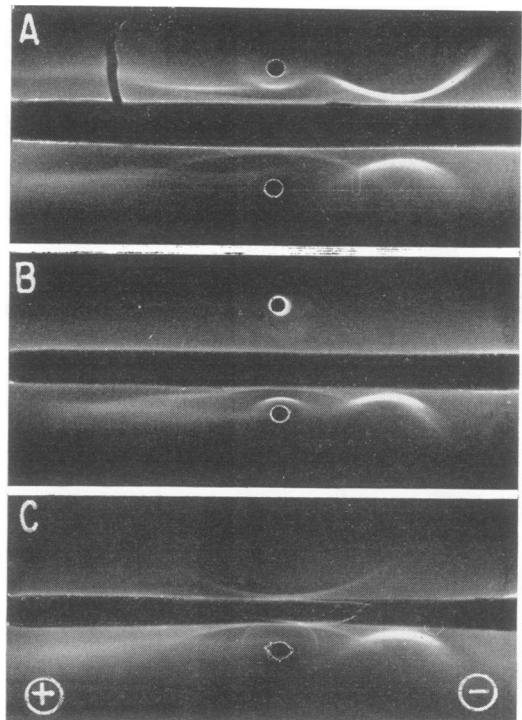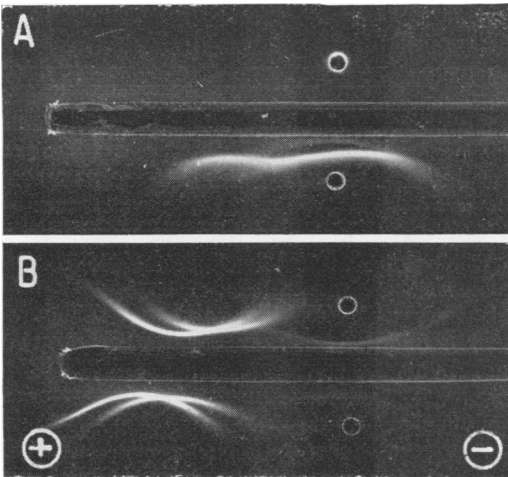

FIG. 3. *BAI strain A virus was disrupted with Triton X-100. After 90 min of electrophoresis, diffusion was against pigeon serum (upper trough) and hamster serum (lower trough). Dark-field photograph.*

FIG. 6. *Comparison of pigeon serum with rabbit serum in reaction with BAI strain A virus disrupted with Triton X-100 and in reaction with normal chicken serum. Upper trough contained pigeon serum, the lower trough, rabbit serum. Upper and lower wells were charged with normal chicken serum and the center well with virus treated with Triton X-100. Electrophoresis was for 90 min; amido black 10B stain.*

FIG. 5. *Effect of sodium dodecyl sulfate (SDS) treatment on BAI strain A virus with 45 min of electrophoresis followed by diffusion against pigeon serum. Virus concentration was 5.4 × 10^{12} particles/ml, containing about 2.6 mg of protein/ml. SDS concentrations and the corresponding ratios of grams of SDS/gram of protein were: A, upper well, no SDS; lower well, 0.1% SDS (0.4 g/g); B, upper well 0.2% SDS (0.8 g/g); lower well 0.5% SDS (1.9 g/g). Stained with Ponceau S.*

FIG. 7. *Effect of absorption of rabbit serum with normal chick tissue. The well was charged with virus disrupted with Triton X-100. Upper trough contained rabbit serum absorbed twice with 0.1 g of an acetone powder of normal chicken liver per ml. Lower trough had the same serum without absorption. Electrophoresis was for 90 min; stained with Ponceau S.*

after diffusion of the SDS-disrupted virus against either pigeon (Fig. 5) or rabbit serum. Two of these were strong, and the antigens showed increasing negative charge with increasing SDS concentration, as expected for this range of SDS: protein ratios (17). The third arc, in the position of C, showed little change in mobility with increasing SDS concentration but appeared to decrease in intensity. The higher SDS concentrations also solubilized more of the virus, as indicated by the smaller amounts of protein left in the well (Fig. 5). Immunoelectrophoresis of untreated virus concentrates yielded no detectable precipitin lines (Fig. 5).

Comparison of gs antisera from different species. Because of the simplicity and efficiency of the procedure, treatment with 0.2% Triton X-100 was used to disrupt the virions to provide antigen for comparison of immune sera from pigeon, hamster, and rabbit. A comparison of pigeon and hamster sera is illustrated in Fig. 3. Figure 6 shows the reaction of pigeon and rabbit antisera against Triton X-100-disrupted virus. As with the hamster serum, that from the rabbit did not reveal arc C, but E was somewhat stronger with rabbit than with pigeon serum. Figure 7 shows the lack of any marked effect on the pattern by absorption of the rabbit serum with an acetone powder of normal chicken liver which was gs-negative by COFAL test. With rabbit
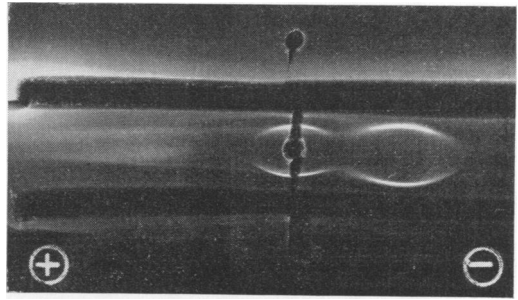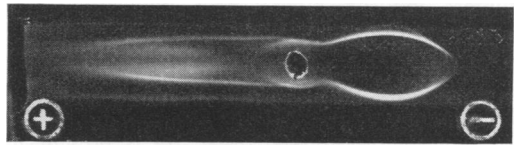
serum, the long arc (D) was sometimes replaced by two parallel arcs as seen in Fig. 8. Also in Fig. 8, a very faint C arc can be seen with the concentrated rabbit serum.

When normal chick serum was subjected to electrophoresis and then diffused against the various immune sera, faint arcs indicative of serum proteins were seen with the rabbit serum (Fig. 6) but not with the hamster or pigeon sera. There was no indication of the presence of serum protein in any of the disrupted virus preparations with any of the antisera.

DISCUSSION

The morphological complexity of the avian tumor viruses (7, 8) is clearly reflected in the large number of proteins in the virion. Eight principal components were separable from RSV (RAV-1) (14), and there may be a very large number of minor protein constituents (5). At least five precipitin arcs could be distinguished when BAI strain A virus broken in various ways was subjected to electrophoresis and then to
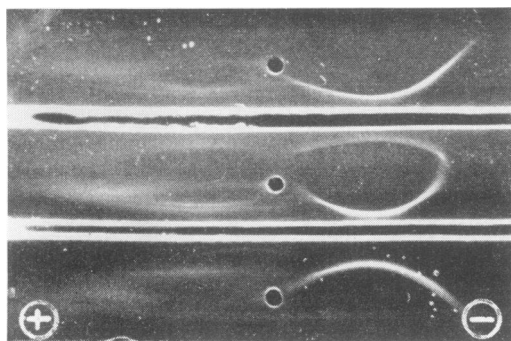
FIG. 8. *Effect of fivefold concentration of rabbit serum. All wells contained Triton X-100-disrupted virus. Lower trough had concentrated rabbit serum, upper trough, unconcentrated serum. Electrophoresis was for 90 min; dark-field photograph.*

diffusion against serum from pigeons bearing tumors induced by SR-RSV, indicating that these five antigens are common to the two virus strains. Two of the lines (A and B of Fig. 2) showed partial identity of determinants, and one long arc (D of Fig. 2) was double-humped. Apparently the antigen of arc D is present in forms or complexes of at least two different sizes. Mild treatment of an avian tumor virus liberated the glycoproteins as a large complex (14), and disulfide bonds may link some virus polypeptides (5). The degree of association of lipid with protein antigens would also affect their electrophoretic mobility. These factors all contribute to the complexity of the immunoelectrophoretic pattern and to its dependence on the means of virus disruption. In addition, carbohydrate and lipid may contribute directly to the viral immunogenicity and must be considered in analyses of the antigenic constitution of the virus. The absence of any precipitin lines after immunoelectrophoresis of untreated virus concentrates 'Fig. 5) indicates that the virions were stable under the conditions used, at least with respect to solubilization of internal antigens.

The procedure used to disrupt the virus affected the immunoelectrophoresis pattern. Two nonionic detergents, Triton X-100 (Fig. 3 and 4C) and Nonidet P-40 (Fig. 4A), a nonionic detergent, Tween 20, plus ethyl ether (Fig. 4B), and ether alone (Fig. 4A) all yielded similar results. Use of Tween 20 alone at the same weight concentration resulted in a qualitatively similar but weaker immunoelectrophoretic pattern. As observed before (17, 18), the proteins treated with the strong anionic detergent SDS were negatively charged (Fig. 5). Within the limits of this very simple experiment (Fig. 5), the

results were consistent with the expected degree of binding of SDS to protein (17). The observation that one antigen, apparently that corresponding to arc C, reacted to increasing SDS concentration with almost no change in mobility, but possibly with decreasing intensity, indicated that it must be substantially different chemically from the others. This is suggested also by its being the only one to survive chloroform-methanol treatment of the virus. Whether the loss of the other antigens after extraction by chloroform-methanol was due to its effectiveness as a lipid extractant or as a protein denaturant remains to be determined. Digitonin apparently blocked the immunoprecipitin reaction, perhaps through binding to the proteins.

The three or four arcs observed with the hamster serum appeared identical to three of those seen with pigeon serum: A, B, and D. Although arc C was not seen with this hamster serum, it does appear to have been present in an earlier study (18). The apparent qualitative differences between pigeon and hamster sera were due, at least in part, to difference in potency. Whether they were also related to species difference is not known. The behavior of rabbit serum closely resembled that of the pigeon (Fig. 6) except that it gave little or no arc C while E was more distinct.

Differences in procedures of extraction and analysis complicate the comparison of various studies of avian tumor virus proteins, but some conclusions seem reasonable. The most prominent precipitin arc (A) appears to be nearly isoelectric at the pH, about 8.7, used for electrophoresis. It is almost certainly the protein designated no. 4 (isoelectric focusing peak no. 11) by Hung et al. (14) which they found to have a molecular weight of 28,700 and an isoelectric point (pI) of 8.9, and to have a strong CF titer with anti-gs hamster serum. This appears to be the same protein as that designated as no. 4 by Bauer and Bolognesi (3, 5), no. 3 by Duesberg et al. (9), C-1 by Bauer and Schäfer (4), and probably the gs-a of Allen et al. (1). Additional correlations of the antigens observed here with those described by others, such as the three isolated from RSV-infected cells (16), must await further studies.

Because of the virus-neutralizing capacity of the serum from tumor-bearing pigeons (Fig. 1), it cannot be assumed that all of the antigens observed were of the gs category. They were shared by the SR-RSV used to induce the tumors and the BAI strain A virus, but these were not tested for subgroup specificity. Also, the rabbit serum used contains antibodies against virus envelope proteins as well as gs antibodies (12).

However, the immunoelectrophoretic patterns with pigeon, hamster, and rabbit sera were similar. Also, the envelope antigens were apparently destroyed by Triton X-100 or Nonidet P-40, but not by Tween 20, as judged by CF tests (R. Ishizaki, *unpublished data*) while all yielded the same kind of immunoelectrophoretic patterns. These findings suggest that the antigens giving arcs A, B, C, and D are group specific. The nature of the antigen giving arc E remains uncertain.

The rabbit immune serum contained a low level of antibody against chicken serum proteins (Fig. 6) even though the virus had been isolated by both differential and density gradient centrifugation. The absence of any albumin precipitin lines when virus preparations were reacted with the rabbit serum indicates that the virus concentrates used for rabbit inoculation contained a very low level of serum proteins, sufficient to stimulate a weak antibody response but probably not enough to be detectable by immune precipitation or, presumably, to affect physical or chemical analyses significantly. Absorption of the rabbit serum with gs-negative normal chick tissue powder caused no obvious change in the immunoelectrophoretic pattern (Fig. 7). It should be pointed out that the rabbit serum used here differed from that used by others (3, 4, 10, 11) in that the virus used for inoculation was not disrupted with ether or detergent, although it was probably damaged to some extent by the density gradient centrifugation step and the emulsification with adjuvant.

Serum from pigeons bearing SR-RSV-induced tumors offers a useful reagent for study of the avian tumor virus group-specific antigens. The virus particles may be disrupted by a variety of detergents and organic solvents, of which the nonionic detergents and ether appear to be both effective and mild.

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