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Radioimmunoassay for Avian C-Type Virus Group-Specific Antigen: Detection in Normal and Virus-Transformed Cells

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A radioimmunoassay has been developed for detection of avian C-type virus (30,000 mol wt) group-specific (gs) antigen. The method is 10- to 1,000-fold more sensitive than immunological methods previously available. By the radioimmunoassay technique, normal chicken embryo cells, which have previously been classified as gs negative or weakly gs positive, contain clearly detectable amounts of gs antigen. The assay has been used to study the effect of chemical induction and superinfection by mammalian C-type viruses on the expression of avian gs antigen in mammalian cells nonproductively transformed by avian sarcoma viruses.

C-type RNA viruses are known to be associated with tumors of a large number of species. Sensitive and specific methods for detection of virion proteins have helped in studies of the natural occurrence of these viruses (15) and of their interactions with the host cell (14, 18). Immunodiffusion techniques as well as more sensitive complement fixation (CF) methods (14, 31) have primarily been utilized for group-specific (gs) antigen detection. Recently, radioimmunoassay (RIA) methods that have been shown to be both rapid and extremely sensitive for detection of a variety of antigens have been adapted for measurement of the gs antigens of mouse and cat RNA C-type viruses (27, 32 S. R. Tronick, J. R. Stephenson, and S. A. Aaronson, Virology, in press).

In the present studies, a gs antigen of avian RNA tumor viruses has been isolated and purified. A RIA method has been developed which is both very sensitive and highly specific for detection of this protein. Avian gs antigen is readily detectable by RIA in normal chicken embryo cells previously classified as either gs negative or only weakly gs positive by other methods (7, 29). The avian gs RIA procedure has been used to study avian tumor viruses in mammalian cells transformed by them and to study the effects of chemical activators and infection with mammalian C-type viruses on the level of avian gs antigen expression in those cells.

MATERIALS AND METHODS

Media. Cells were grown in Dulbecco's modification of Eagle medium supplemented with 10% calf serum (Colorado Serum Co.) in 50-mm petri dishes (Falcon Plastics, Los Angeles).

Cells and viruses. The control cell lines used included BALB/3T3 (3) and normal rat kidney (NRK) cells (8). The Schmidt-Ruppin strain of Rous sarcoma virus (SR-RSV) subgroup D (5) was used to transform BALB/3T3 and NRK. Two weeks after infection with this avian tumor virus, focal areas of transformed cells were visible; these were selected by means of cloning cylinders. The resulting mixed cultures of normal and transformed cells were cloned in microtiter plates (34). The SR-RSV-transformed lines obtained were designated SR-BALB and SR-NRK. A B77 avian sarcoma virus (36) transformant of BALB/3T3, B77-BALB, was provided by P. Vogt. This line was also cloned prior to use. The Kirsten (Ki-) and Rauscher (R-) strains of murine leukemia virus (MuLV) were propagated as previously described (34). The Carr-Zilber strain of RSV and Rous-associated viruses, RAV-1 and RAV-2, were obtained from the Resources and Logistics Segment, National Cancer Institute (NCI).

Purification of avian gs antigen. Chicken serum containing high-titered avian myeloblastosis virus (AMV) was provided by J. W. Beard, Duke University, through the Resources and Logistics Segment, NCI. After clarification by centrifugation at 10,000 rpm for 15 min, the virus was pelleted at 30,000 rpm for 90 min, banded in a 15 to 60% sucrose gradient, and then repelleted. After resuspension and dialysis overnight against 0.01 M Tris (pH 7.8)-0.1 M

NaCl-0.001 M EDTA, the virus was disrupted with Triton X-100 and applied to a G-100 Sephadex column as described previously (32). Individual fractions were tested for avian gs antigens by CF with hamster anti-SR/RSV serum (31). Fractions containing the highest titers were pooled, dialyzed for 24 h at 4 C against 0.01 M Tris-hydrochloride (pH 7.8)-0.014 M β-mercaptoethanol, and concentrated by lyophylization. The material was suspended in water and applied to an isoelectric focus column (pH 7–10 range) as described by Oroszlan et al. (26). The major peak of gs activity as measured by CF occurred at pH 7.5. Fractions near this pH were pooled, dialyzed against 0.01 M Tris-hydrochloride (pH 7.8), lyophylized, and suspended in distilled water at a final concentration of approximately 100 μg/ml.

Polyacrylamide gel electrophoresis. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was carried out by the method of Laemmli (21). A series of protein standards of known molecular weight were included to calibrate gels (21).

Iodination. Purified gs proteins were iodinated by the method of Greenwood et al. (12). Iodinated gs antigens had specific activities of 2×10^6 to 5×10^6 counts per min per ng.

Antisera. High-titered antiserum against avian gs antigen was made in a pig by repeated immunization with Tween-ether-disrupted AMV. Antiserum to murine gs antigen was obtained from a goat immunized with ether-disrupted R-MuLV. For precipitating antibodies, anti-porcine gamma globulin was prepared in a goat and anti-caprine gamma globulin was obtained by immunization of a pig. Hamster antiserum to a SR-RSV-induced hamster tumor (COFAL serum; 31) was provided by R. Huebner, NCI. The methods for CF have been previously described (14, 31).

RIA procedure. Avian and murine gs antigens were assayed by double antibody competition RIA procedures. A limiting amount of antiserum against the appropriate gs antigen was incubated with an unknown quantity of unlabeled avian gs antigen for 1 h at 37 C in a reaction mixture consisting of 0.01 M potassium phosphate (pH 7.8), 0.01 M EDTA (pH 7.8), and 1% normal rabbit serum, in a final volume of 0.8 ml. ^{125}I-labeled gs antigen was added at a total of approximately 10,000 counts per min per reaction mixture, and samples were incubated a further 4 h at 37 C and 18 h at 4 C. A 25-μliter amount of undiluted precipitating antibody was then added to each reaction mixture, and samples were incubated a further 1 h at 37 C followed by 3 h at 4 C. Precipitates were removed by centrifugation at 2,500 rpm for 15 min, and supernatant fluid radioactivity was measured in a Beckman LS-355 liquid scintillation counter.

Preparation of embryos and tissue culture cells for RIA. For measurement of avian or murine gs antigen in tissue culture material, cells were washed three times with phosphate-buffered saline (PBS), scraped, and resuspended in PBS at a concentration of 5 mg/ml. Resistance-inducing factor (RIF)-negative (30), C/O White Leghorn chicken embryos were obtained from W. J. Jahmes, Food and Drug Administration, Rockville, Md. After 10 to 12 days of incubation, chicken embryos were finely minced and washed three times with PBS. Both tissue culture and whole embryo cell suspensions were sonically treated for 5 s with a Biosonik II sonic oscillator (Bronwill Scientific, Inc., Rochester, N.Y.) prior to immunoassay.

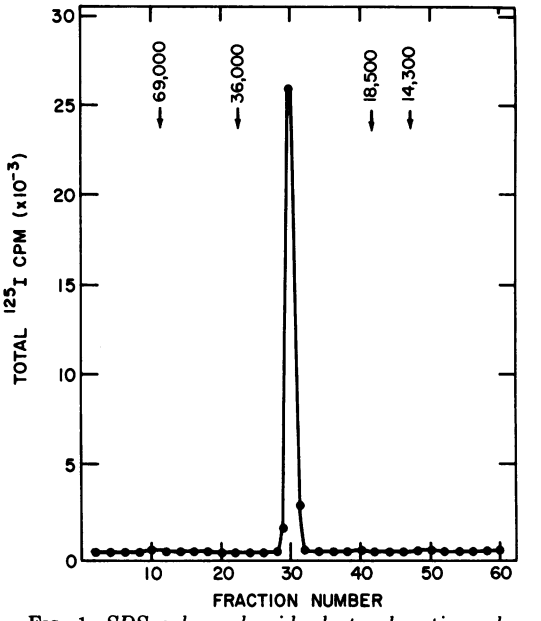

FIG. 1. *SDS-polyacrylamide electrophoretic analysis of ^{125}I-labeled avian gs antigen. A total of 30,000 counts/min of ^{125}I-gs antigen were applied to a gel, and electrophoresis carried out as described previously (21). Gels were fractionated with a Savant autogeldivider and counted in a Packard scintillation counter. The protein standards used to calibrate the gels were: bovine serum albumin (mol wt 69,000), glyceraldehyde-3-phosphate dehydrogenase (mol wt 36,000), β-lactoglobulin (mol wt 18,500), and lysozyme (mol wt 14,300). Positions of the stained bands are indicated.*

RESULTS

Characterization of avian gs antigen. Avian gs antigen, purified by Sephadex G-100 chromatography and isoelectric focusing and labeled with ^{125}I, was analyzed by SDS polyacrylamide gel electrophoresis. A single peak of radioactivity was found at a position in the gel corresponding to a mol wt of approximately 30,000 (Fig. 1). No other major peaks of radioactivity were found. Thus, the ^{125}I-labeled gs protein was at least 95% pure and corresponded to the 26,000 to 30,000 mol wt avian viral structural protein described in previous studies (6, 9, 11).

RIA for avian gs antigen. A double antibody RIA was used for quantitation of the avian gs protein. Porcine anti-AMV sera was titrated for its ability to precipitate ^{125}I-labeled avian gs antigen. Over 85% of the ^{125}I counts were precipitated at a serum dilution of 1:25,000; even at a dilution of 1:1,000,000 detectable

precipitation was obtained. The titration pattern was linear in the range of 20 to 80% precipitation. This is consistent with the results expected for a homogeneous antigen. Control sera from nonimmunized pigs produced no precipitation of the ^{125}I-labeled antigen at any concentration.

For measurement of unlabeled avian gs antigen, a radioimmunoprecipitation competition assay was used. Porcine anti-AMV serum was kept at a constant final dilution of 1:50,000; this resulted in precipitation of approximately 25% of the ^{125}I-gs antigen (Fig. 2). Purified non-radioactively-labeled avian gs antigen was titrated on the basis of its capacity to displace ^{125}I-gs antigen into the supernatant fluid by competing for the limiting antibody in the assay mixture. This method was sufficiently sensitive to detect avian gs antigen at concentrations as low as 3 to 4 ng/ml (Fig. 3). Thus the avian RIA was similar in sensitivity to RIAs previously described for murine and feline gs antigens (27, 32).

Comparison of CF and RIA for detection of avian gs antigen. The standard method for assaying avian gs antigen has been a CF assay with sera selected from hamsters bearing SR-RSV-induced tumors (31). The sensitivity of this method was compared with that of the RIA. A CF test using the porcine antiserum to AMV was also tested. It is clear that, with each of several strains of avian C-type viruses tested, the CF test with hamster anti-SR-RSV serum was at least four times as sensitive as the CF test with porcine anti-AMV serum (Table 1). However, the RIA method had over a 10-fold higher sensitivity than the more sensitive CF test. It is of interest that, for detection of the homologous AMV gs antigen, the RIA was approximately 1,000-fold more sensitive than the CF test with the same antiserum. The much greater sensitivity of the RIA for detection of AMV gs antigen compared to other avian gs antigens suggests the possibility of some degree of type specificity.

To establish the specificity of the RIA for detection of avian gs antigen, several different strains of avian and murine C-type viruses were tested in RIAs for both avian and for mouse gs-1 antigens. Each of the avian C-type viruses examined, while positive in the avian gs assay, was negative in the RIA for mouse gs antigen

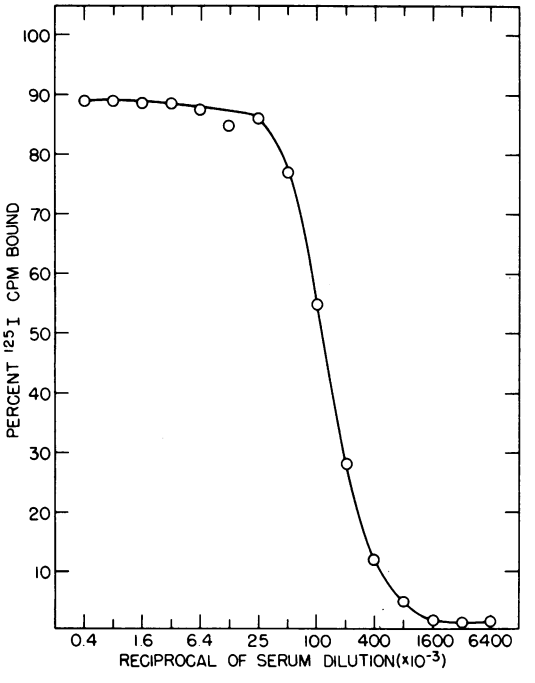

FIG. 2. *Tritration of porcine anti-AMV serum by precipitation of ^{125}I-avian gs antigen in the double antibody radioimmunoprecipitation assay. The procedure followed is described in detail in Materials and Methods.*

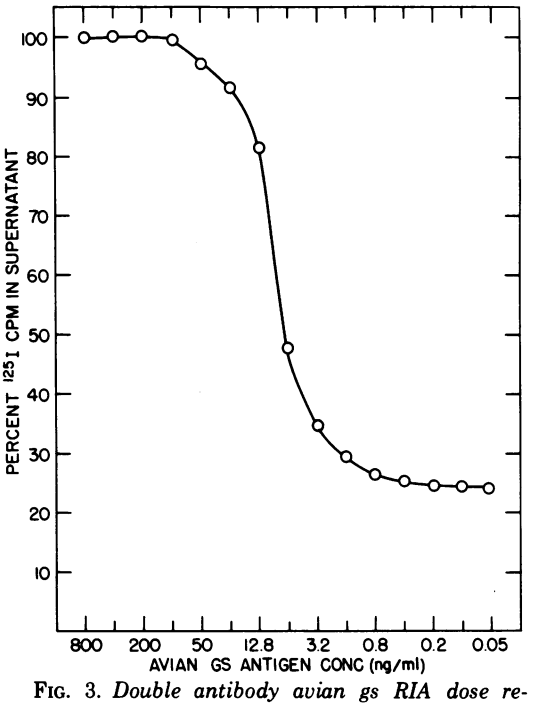

FIG. 3. *Double antibody avian gs RIA dose response curve for purified avian gs antigen. The unlabeled avian gs antigen was purified by G-100 Sephadex chromatography and isoelectric focusing, and the protein concentration was determined by the spectrophotometric method of Groves et al. (13). Porcine anti-AMV serum was used at a dilution of 1:50,000, and there was a total input radioactivity of 10,000 counts/min ^{125}I-avian gs antigen.*

(Table 1). Further, mouse leukemia viruses were completely unreactive in the assay for avian gs antigen despite the fact that they were positive at dilutions of 8,000 to 16,000 in an RIA for the mouse gs protein. Other mammalian C-type viruses, including isolates from the cat, rat, woolly monkey, and gibbon ape, were also completely unreactive in the avian gs RIA (unpublished observations). These findings demonstrate the high degree of specificity of the avian immunoassay.

Detection of avian gs antigen in normal chicken embryo cells and avian sarcoma virus-transformed mammalian cells. Normal chicken embryo cells have previously been classified as either gs^- or gs^+ (7, 29). A series of eight chicken embryos, which were either gs negative or positive at very low titer by CF (less than 1:8 with hamster anti-SR-RSV serum), were assayed by the RIA method. The range of titers was 1:32–1:128 (Table 2). That the positive reactions were specific for avian gs was indicated by the fact that the embryos were each negative in the RIA for mouse gs antigen. On the basis of these results, it is clear that the avian RIA is much more sensitive than CF for detecting gs antigen in normal chicken embryo cells and that cells which have been regarded as low-level positive or gs negative may contain detectable levels of avian gs antigen when tested by RIA.

Avian gs antigen has been demonstrated by CF in mammalian cell lines nonproductively transformed by avian sarcoma viruses (10, 16).

TABLE 1. *Comparison of the relative sensitivities of CF and RIA for detection of avian gs antigen*[a]

Antigen tested[b]	Complement fixation		Radioimmunoassay	
	Porcine anti-AMW	Hamster anti-SR-RSV	Porcine anti-AMV	Goat anti-MuLV
AMV gs	16	256	16,000	<2
RSV (Carr Zilber)	8	32	1,000	<2
RAV-1	4	16	256	<2
RAV-2	16	64	500	<2
Ki-MuLV	<2	<2	<2	8,000
R-MuLV	<2	<2	<2	16,000

[a] The results of the CF tests are expressed as the reciprocal of the highest antigen dilution which gave a positive reaction (14). The RIA titers are the reciprocal of the highest antigen dilution which resulted in displacement of a minimum of 10% of ^{125}I-labeled gs into the supernatant fluid. Results are expressed as the average, to the nearest twofold dilution, of three separate determinations.

[b] AMV gs antigen was purified as described in Materials and Methods. Each of the other virus isolates was disrupted by extraction with 5 vol of ether.

Studies were performed to test the generality of this phenomenon by examination of avian gs antigen expression in cells transformed by different strains of avian sarcoma virus, in different mammalian species and in different subclones of avian sarcoma virus-transformed clonal lines. A total of five subclones of clonal lines of SR- and B77-transformed BALB/3T3 and SR-transformed NRK were isolated, and each was assayed for both avian and mouse gs antigen. It is seen that, whereas there was some variation in the level of avian gs antigen among different subclones, there were no significant differences between the mean antigen levels in individual subclones of the three different transformants (Table 2).

Activation of avian gs antigen in mammalian cells nonproductively transformed by avian sarcoma virus. It has been shown that treatment of mouse (4, 23) and rat (1, 20) embryo cultures with chemicals such as 5-iododeoxyuridine (IdU) and 5-bromodeoxyuridine results in activation of endogenous C-type viruses. Except with cells obtained from high-incidence leukemia strains of mice (33), the production of virus after IdU treatment is generally transient, reaching a peak at 3 to 4 days and subsequently declining.

The RIA for avian gs antigen was used to determine whether the avian C-type virus genome in nonproductively transformed mammalian cells was activated by IdU. Avian sarcoma virus-transformed SR-BALB and SR-NRK, as well as control BALB/3T3 and NRK cell lines, were each treated with IdU at a dose of 20 μg/ml for 24 h. At the time of peak cell-associated gs antigen after chemical treatment (3 days), cellular levels of avian and mouse gs-1 antigens were compared in treated cells and controls. With both SR-BALB and BALB/3T3 cells there was a twofold increase in the level of mouse gs antigen in the IdU-treated cells (Table 3). Although this increase was small, it was reproducibly found in at least five separate experiments and is therefore considered significant. Under these experimental conditions, approximately 1% of the treated cells release infectious MuLV (unpublished observations). Similarly, with each of the avian sarcoma virus-transformed lines, IdU treatment in repeated experiments resulted in a two- to threefold increase in avian gs antigen. These results indicate that avian and mouse gs antigen expression is increased in avian sarcoma virus-transformed mouse cells exposed to chemical inducers.

It has been shown in previous studies with

TABLE 2. *Comparison of gs antigen expression in normal chicken embryo cells and avian sarcoma virus-transformed mammalian cell lines*

Cells tested	Titer of viral gs antigen[a]	
	Avian	Mouse
Chicken embryo cells	32-128	<2
BALB	<2	8
NRK	<2	<2
B77-BALB	16-32	4-16
SR-BALB	16-32	8-16
SR-NRK	16-32	<2

[a] The titer of gs antigen is expressed as the reciprocal of the highest dilution detectable by RIA as described in Table 1. A constant amount of cell protein (5 mg/ml) was used in all assays. The protein concentration was determined by the method of Lowry (22). For each of the three avian sarcoma virus-transformed lines, the results are presented as the range of mean values for five different subclones.

mammalian cells nonproductively transformed by murine sarcoma viruses that activation of the endogenous leukemia virus genome (1, 20) or superinfection with exogenous leukemia virus (2, 17) results secondarily in rescue of the sarcoma virus genome. Studies were performed to determine whether IdU acted directly to increase avian gs antigen expression in mammalian cells transformed by avian sarcoma viruses, or alternatively, whether the increase in avian gs antigen expression was an indirect effect of replication of the induced murine leukemia virus. To distinguish between these two possibilities, SR-BALB and B77-BALB cells were each superinfected with R-MuLV. Two weeks later the levels of cell-associated avian and mouse gs antigens were measured. Although the amount of mouse gs antigen per milligram of cell protein increased approximately 100-fold after superinfection with R-MuLV, there was no detectable increase in the expression of avian gs antigen (Table 4). These findings indicate that treatment of mammalian cells transformed by avian sarcoma viruses with IdU induces avian gs antigen expression independently of its effect on the endogenous mouse C-type virus.

DISCUSSION

In the present study, the development and some of the biological applications of a radioimmunoassay for avian C-type virus gs antigen are described. The use of RIAs for measurement of mammalian C-type virus gs antigens has been recently reported by other laboratories (27, 32). The sensitivity of the present method compares favorably with those for detection of gs antigens of mouse and cat C-type viruses and is at least 10- to 100-fold more sensitive than the standard CF tests for avian viruses. The specificity of the avian gs RIA was shown by its ability to react with each of several different strains of avian C-type virus but not with preparations of several different mammalian viruses.

It appears that regulatory factors, which normally control endogenous C-type virus expression in mouse cells, are impaired at least temporarily by exposure of cells to IdU (4, 23). Such factors may also be involved in the control of avian gs antigen expression in mammalian cells nonproductively transformed by avian sarcoma virus since IdU-treatment results in an increase in avian gs antigen expression. That this effect was not an indirect result of replication of induced murine leukemia virus was shown by the lack of increase in cellular levels of avian gs antigen after MuLV superinfection of

TABLE 3. *Alterations in gs antigen levels after activation with IdU*

Cell line	IdU treated[a]	Titer of viral gs antigen[b]	
		Avian	Mouse
BALB	-	<2	8
	+	<2	16
SR-BALB	-	32	8
	+	64-128	16
NRK	-	<2	<2
	+	<2	<2
SR-NRK	-	32	<2
	+	128	<2

[a] Subconfluent cell cultures were treated with IdU (20 μg/ml) for 24 h and assayed for gs antigen 48 h later.

[b] The titer of gs antigen is expressed as the reciprocal of the highest dilution detectable by RIA as described in the legend to Table 2.

TABLE 4. *Effect of superinfection by R-MuLV on gs antigen expression in avian sarcoma virus-transformed BALB/3T3 cells*

Cell clone	Titer of viral gs antigen[a]	
	Avian	Mouse
B77-BALB	16	8
B77-BALB(R-MuLV)	16	500-1,000
SR-BALB	16	8
SR-BALB(R-MuLV)	16	500-1,000

[a] Gs antigen titer is expressed as the reciprocal of the highest dilution detectable by RIA as described in Table 2.

avian sarcoma virus-transformed mouse or rat cells.

Normal chicken embryo cells which do not release detectable levels of virus have been classified as either gs positive or negative on the basis of their reactivity in a standard CF test (7, 29). The present results show that even some "gs-negative" cells contain detectable levels of avian gs antigen when tested by the more sensitive RIA. These findings are analogous to those in this report and in other studies (18) where mouse cells of many strains have been found to contain low, but detectable, levels of mouse leukemia virus gs antigen. Both mouse and chicken cells have been shown to be inducible by chemical or physical agents to produce C-type viruses (4, 23, 37). It will be of obvious importance to determine whether cells of other species, from which C-type viruses have been isolated, contain detectable levels of RNA tumor virus gs antigens when tested by RIA techniques.

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