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Isolation of a Second Avian Leukosis Group-Specific Antigen (gs-b) from Avian Myeloblastosis Virus

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Abstract. Gs-b, a second group-specific antigenic protein of the avian leukosis group, has been isolated from Tween 80–ether treated avian myeloblastosis virus by Sephadex and carboxymethylcellulose chromatography, and compared by immunological and chemical means with the antigen (gs-a) described previously.

Group-specific antigens of the avian leukosis viruses are useful indicators of infection by these viruses, which produce leukemia, sarcoma, and other neoplasms in chickens.[12] The antigens are also of interest in demonstrating at least a portion of the viral genome in cells transformed by these agents, but not actively producing infectious virus.[20] The group-specific antigens are apparently internal constituents of the virion[6,13]; they have been isolated from the virion by Tiselius[5], cellulose acetate[1], and polyacrylamide gel electrophoresis[1,9]. One antigen, gs-a, has been shown to be a basic protein,[1] with molecular weight about 20,000, with C-terminal alanine,[2] and with N-terminal proline.[3] Low molecular weight, basic, group-specific, antigenic proteins have also been isolated from murine leukemia viruses.[11,18]

In this paper, we wish to report the separation by column chromatography of an avian leukosis group-specific antigen, gs-b, which fixes complement with sera from hamsters and pigeons with the Schmidt-Ruppin (SR) strain of Rous sarcoma virus-induced tumors and is immunologically distinct from gs-a.

Gs-b is probably equivalent to the group-specific antigen, avian myeloblastosis virus$_1$ (AMV$_1$) first recognized by Duesberg *et al.*[9] Although we observed the protein gs-b,[1] we did not recognize its reactivity in the complement-fixation test at that time due to the failure of the rabbit antisera against Tween–ether treated AMV$_1$ to react with gs-b.

Comparison of the N-terminal and C-terminal amino acids indicates that gs-b is chemically, as well as immunologically, distinct from gs-a.

Materials and Methods. The AMV used for isolation of the protein antigens was obtained from the peripheral blood of leukemic White Leghorn chicks (Spafas, Inc. Norwich, Conn.), and purified by differential centrifugation essentially by the methods of Beard and co-workers.[7] The virus was solubilized by treatment with Tween 80 and ether by the method of Eckert *et al.*[10] but with two additional extractions of the fatty interface with 0.01 M phosphate buffer, pH 7.4, containing Tween 80 (0.5 mg/ml). The

aqueous phases were combined, clarified by centrifugation at 78,000 × *g* for 30 min and concentrated to a volume of 2–3 ml by ultrafiltration using a Diaflo UM 10 membrane.

Sucrose was added to a concentration of 10%; the sample was layered on a 2.6 × 26 cm column of Sephadex G-100 and eluted using 0.1 M phosphate buffer, pH 7.4, at the rate of 20 ml/hr. 5 ml fractions were collected, the absorbance was determined at 260 nm and 280 nm, and those fractions that contained the gs antigens were pooled and dialyzed against 0.015 M sodium citrate buffer, pH 4.3, containing 0.02% thiodiglycol. After it was concentrated to 5 ml by ultrafiltration, the solution of proteins was placed on a 0.9 × 9 cm column of carboxymethylcellulose (equilibrated with the citrate buffer) and the proteins were eluted by a linear gradient formed from 200 ml of this buffer and 200 ml of 0.02 M Na_2HPO_4.[11] 5 ml fractions were collected at the rate of 30 ml/hr. The absorbance of the eluted fractions at 260 nm and 280 nm and the pH were determined.

Polyacrylamide gel electrophoresis was by the method of Reisfeld *et al.*[15] at pH 4.3 and of Shapiro *et al.*,[19] as modified by Weber and Osborn,[21] at neutral pH with 0.2% sodium dodecyl sulfate (SDS). For molecular weight determinations, the SDS-containing gels were calibrated with pepsin (Worthington), ribonuclease A (Worthington), and cytochrome *c* (Calbiochem).

The micro complement fixation test employed hamster[12] and pigeon[18] and anti SR Rous sarcoma virus sera. Rabbit anti gs-a was prepared using gs-a purified by cellulose acetate electrophoresis.[3] Immunodiffusion (Ouchterlony) tests were carried out in 2% Noble agar (Hyland pattern C immunoplate) at room temperature in humidified Petri dishes.

The N-terminal amino acid of the protein was determined by reaction with [^{14}C]fluorodinitrobenzene, hydrolysis, chromatography on silica plates, and autoradiography, as described previously,[2,15] but with repeated development with chloroform–benzyl alcohol–glacial acetic acid 70:30:3 to separate leucine and isoleucine. The C-terminal amino acid residues were released by diisopropylphosphofluoridate-treated carboxypeptidase A (Worthington) from 5 nmol protein after incubation at room temperature as reported previously[2] except that the residues were analyzed using a Beckman Spinco 120C amino acid analyzer equipped for high sensitivity analysis.

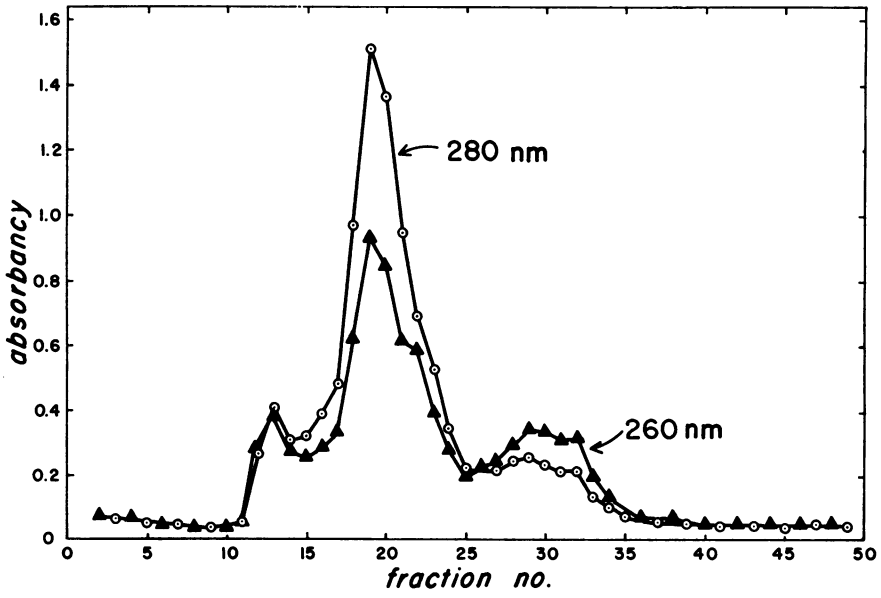

FIG. 1. Chromatography of avian myeloblastosis virus proteins, solubilized by treatment with Tween 80 and ether, on Sephadex G-100.

Results. Sephadex column chromatography of Tween–ether solubilized AMV is shown in Fig. 1, in which the absorbance at 280 nm and 260 nm is plotted against the fraction number. The peak fractions 18–21 eluted with the same effluent volume as proteins of molecular weight 10,000–25,000. These fractions had a ratio of absorbance at 280/260 nm of 1.72 and at 280/250 nm of 2.36, indicating little contamination of protein by nucleic acid. These fractions contained both gs-a and gs-b (identified by polyacrylamide gel electrophoresis). Since the peak consisted almost entirely of gs-a and gs-b, and is 50–60% of the total viral protein, it is evident that gs-a and gs-b are principal constituents of the viral protein (see also Fig. 3, and ref. 9).

Fig. 2 shows the elution pattern of these proteins from carboxymethylcellulose as measured by the absorbance at 280 nm, compared with the pH of the eluted fractions. A peak identifiable as gs-a emerges at pH 5.2 and gs-b at pH 6.1. Although about twice as much protein is obtained from the same amount of virus by column chromatography as by cellulose acetate electrophoresis, yields have still been poor. In one preparation, 360 ml of AMV-rich chick plasma (10^{12} particles per ml) that contained, theoretically, 162 mg of virus protein before differential centrifugation,[7] we obtained 40 mg of solubilized AMV protein, 21 mg of protein in the Sephadex peak (combining gs-a and gs-b) and 4 mg of gs-a and 2 mg of gs-b from the carboxymethylcellulose. Gs-a can be purified by repeated Sephadex G-100 chromatography alone, but recoveries of gs-b from Sephadex have been low and contaminated with gs-a.

Polyacrylamide gel electrophoresis, at pH 4.3, of the Tween–ether solubilized AMV, the peak fractions from Sephadex, and gs-a and gs-b from carboxymethylcellulose are shown in Fig. 3. The antigens have apparently been well separated on the carboxymethylcellulose column.

The results of SDS–gel electrophoresis of gs-b compared with three proteins of known molecular weight are shown in Fig. 4, in which the logarithm of the molecular weight is plotted against mobility. By this means it appears that gs-b has a molecular weight of about 11,000. Gs-a was found in a similar experiment to have a molecular weight of about 20,000, as reported previously.[2,3]

Complement fixation titers of gs-a and gs-b, at a concentration of 0.5 and 1.0 mg/ml, are shown with three different antisera in Table 1. Sera from pigeons and hamsters, bearing tumors induced by SR Rous Sarcoma Virus, fixed complement with both gs-a and gs-b to approximately the same extent, indicating that both these proteins were group-specific. Rabbit anti-sera against gs-a

TABLE 1. *Complement fixation titers of group-specific antigens.*

Antigens (0.5 mg/ml)	Anti-SR-RSV* pigeon serum	Anti-SR-RSV hamster serum	Anti-gs-a rabbit serum
gs-a	128†	512	512
gs-b	128	256	64
(1.0 mg/ml)			
gs-a	$\geqslant 1024$	$\geqslant 1024$	$\geqslant 1024$
gs-b	$\geqslant 512$	$\geqslant 512$	64

* SR-RSV, Schmidt-Ruppin strain of Rous sarcoma virus.
† Given as reciprocal of highest titer active.

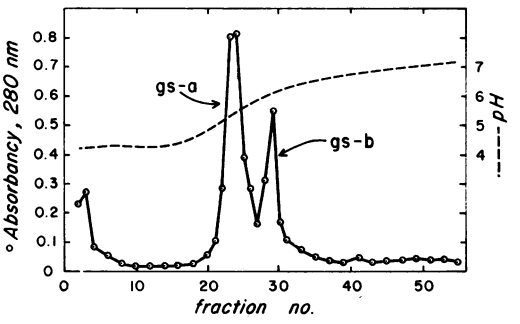

FIG. 2

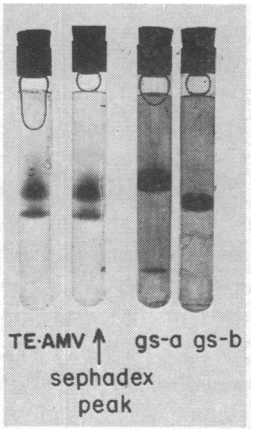

FIG. 3.

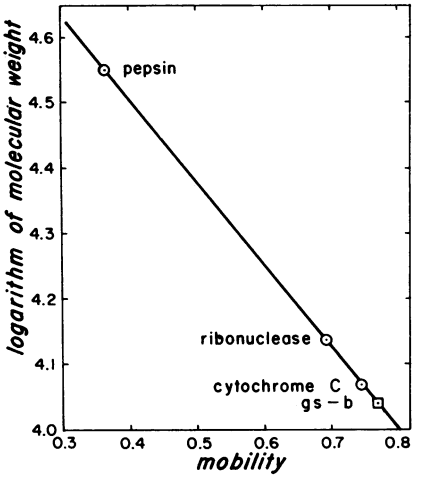

FIG. 4.

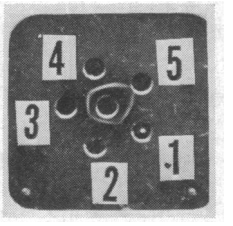

FIG. 5.

FIG. 2. Separation of the group-specific antigens of AMV on carboxymethylcellulose by a pH gradient.

FIG. 3. Polyacrylamide gel electrophoresis at pH 4.3. From left to right: AMV solubilized with Tween–ether (TE AMV), concentrated fractions from the Sephadex peak, gs-a, and gs-b.

FIG. 4. Results of neutral SDS–polyacrylamide gel electrophoresis, in which the logarithm of the molecular weight is plotted against the mobility.

FIG. 5. Immunodiffusion precipitation lines of various AMV derivatives against anti gs-a (*center well*). (*1*) Tween–ether interface; (*2*) Tween–ether aqueous layers; (*3*) Sephadex peak; (*4*) gs-a; (*5*) gs-b. Evidently, despite two extractions, the interface still contained some gs-a.

fixed complement at an at least 8 times greater titer with gs-a than gs-b. Ant gs-a also produced immune precipitation lines on Ouchterlony immunodiffusion plates with gs-a at various stages of purification, but not with purified gs-b (Fig. 5). The rabbit anti gs-a at 1:15 dilution used in these experiments had a titer equal to or more than 512 against standard SR Rous sarcoma virus wing web and hamster tumor antigens.

Leucine was the only N-terminal amino acid evident after reaction of gs-b with [^{14}C]dinitrofluorobenzene, whereas proline is the N-terminal residue of gs-a.[3] Carboxypeptidase A released leucine from gs-b and alanine from gs-a[2]

TABLE 2. *Results of treatment of antigens with carboxypeptidase A.*

Protein	Amino acid*	Incubation time: 15 min	30 min	1 hr	2 hr
		(moles amino acid released/mol protein)			
gs-a					
	alanine	0.38	...	0.57	0.65
	leucine	0.03	...	0.04	0.09
gs-b					
	alanine	...	0.08	...	0.05
	leucine	...	0.34	...	0.52

* No other amino acid exceeded 0.2 mol/mol protein after 2 hr.

indicating that these proteins differed in the C-terminal amino acid residue (Table 2).

Discussion. Evidence has been presented here for two avian leukosis group-specific antigens that may be distinguished by immunologic and chemical means. Gs-a (isolated by chromatography) has been identified with band a, and gs-b with the predominant component of band b, observed on cellulose acetate electrophoresis.[1]

Our inability to produce antibodies against gs-b rabbits, using complement fixation techniques, explains our tardiness in recognizing gs-b as a group-specific antigen.[1,2] However, Duesberg *et al.* had found two group-specific antigens using hamster anti SR Rous sarcoma virus to study Rous sarcoma virus and AMV proteins separated by gel electrophoresis.[9] Although these workers did not estimate the molecular weight of their proteins from the neutral SDS–polyacrylamide gels, their data do allow identification of our proteins by comparison of relative mobility. Thus it is likely that their AMV_1 corresponds with gs-b and their AMV_3 with gs-a. They also observe a third minor component, AMV_2, which we have detected in crude Tween–ether AMV preparations but not in effluent fractions from the carboxymethyl cellulose columns. Their AMV_3 and AMV_1 contained about one-tenth the complement fixation titer at the same concentration as gs-a and gs-b. Since the complement fixation test was done in the same laboratory, it is possible that the use of phenol extraction and alcohol precipitation of AMV_3 and AMV_1[9] may have decreased the antigenicity, as preparative gel electrophoresis should have provided them with proteins as pure as ours.

Several authors have identified multiple group-specific antigens by immunodiffusion[4,8,17] but it is difficult to correlate their data with ours. One possibility is that some of the multiplicity of antigens observed may be due to differences in the degree of aggregation or association with other molecules of the same polypeptide chain.

Some evidence for the chemical dissimilarity of gs-a and gs-b is presented here, and more detailed studies of the chemical structures will be discussed separately (in preparation). Although it might appear possible, judging from the molecular weight alone, that gs-a is a dimer of gs-b, this was found not to be the case, and it will be shown that there is very little if any indication of similarity between the two. The column chromatographic methods presented here, with their greater capacity and yield, allowed this structural study.

The excellent technical assistance of Mrs. Patricia Muldoon and Mrs. Cecile Willems is gratefully acknowledged.

Abbreviations: SDS, sodium dodecyl sulfate; AMV, avian myeloblastosis virus; SR, Schmidt-Ruppin strain (of Rous sarcoma virus); gs-a, group-specific antigen a; gs-b, group specific antigen b.

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