Wild-Type Gross Leukemia Virus: Classification of Soluble Antigens (GSA)

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Received for publication 7 August 1972

By inhibiting techniques using indirect immunofluorescence tests and indirect immunoelectron microscopy, the G(Gross) soluble antigens (GSA) in the body fluids of AKR and C58 mice, which have a high incidence of spontaneous leukemia, were classified according to the known specificity of G antigens in the murine Gross leukemia system. GSA existing in the plasma of nonleukemic and leukemic AKR mice and in the ascitic fluid of transplanted AKR spontaneous leukemia K36 showed the several specificities corresponding to G cell surface antigens, GCSAa, b, and c, and type-specific and group-specific viral envelope antigens, tsVEA and gsVEA, respectively. However, the plasma of nonleukemic C58 mice lacks GSAC, which can be recognized by the G-typing mouse serum. GSA corresponding to G_{IX} antigen was not detected in the body fluids.

It has been reported that a soluble antigen associated with Gross virus (G soluble antigen[s], GSA) occurs naturally in the plasma and tissue extracts of infected mice (3). The characteristics of GSA have been extensively studied by serology, immunogenetics, and experimental pathology (3-5, 10, 20, 21, 25). Initially, GSA was demonstrated by the indirect immunofluorescence method using the G-typing mouse serum C57BL/6 ♀ anti-transplanted AKR spontaneous leukemia K36, which recognized only GCSAa, a G cell surface antigen (6). Recently, a complex variety of Gross virus-associated antigens has been identified (see Table 1). Consequently, GSA detected with this G-typing mouse serum shares a common specificity with GCSAa. In other studies, however, the G-typing rat serum (W/Fu × BN)F₁ anti-W/Fu(C58NT)D and serum from aged auto-immune NZB mice also demonstrated GSA by using the same technique (20, 21; T. Aoki, *unpublished observations*). The G-typing rat serum contains antibodies to known G antigens of three groups: (i) G cell surface antigens, GCSAa and b and G_{IX}; (ii) G type-specific viral envelope antigen(s), tsVEA; and (iii) group-specific intraviral antigens (gsl, 2, 3, etc.) (1, 13, 30). The NZB serum contains antibody to a common VEA shared by C-type particles of murine leukemias and myelomas, group-specific VEA (gsVEA) (7). Therefore, it was likely that there were several different classes of GSA in nature.

In the present study, we have analyzed the

specificity of GSA, by utilizing mutual inhibiting effects of G antibodies and GSA (3), and have found that there are several classes in the plasma and ascitic fluid from mice of strains AKR and C58 with high incidence of spontaneous leukemia.

MATERIALS AND METHODS

Animals. AKR, C58, 129, NZB, and C57BL/6 strains of mice and W/Fu and (W/Fu × BN)F₁ rats were obtained from the Division of Immunology, Sloan-Kettering Institute for Cancer Research, New York, and the Animal Production Branch, Division of Research Services, National Institutes of Health.

Leukemias. The standard G⁺C57BL/6 mouse leukemia E♂G2 was originally induced by Passage A Gross virus. The G⁺ AKR spontaneous leukemia K36 was converted into transplantable ascitic form by intraperitoneal inoculation into AKR mice (23).

The W/Fu rat leukemia (C58NT)D was originally induced in a W/Fu male by neonatal inoculation with normal thymus from C58 mice, a G⁺ strain (13).

These three G⁺ leukemias were gifts from L. J. Old and E. A. Boyse, Sloan-Kettering Institute.

G-typing sera. (i) G-typing mouse serum C57BL/6 anti-K36 was prepared by immunization of C57BL/6 ♀ mice with K36 leukemia, and was tested exclusively against E♂G2 cells to obviate reactions with allo-antigens. In this system, only GCSA can be demonstrated by various serological methods (6, 23). (ii) G-typing rat serum (W/Fu × BN)F₁ anti-W/Fu-(C58NT)D was obtained from (W/Fu × BN)F₁ hybrid rats inoculated with (C58NT)D (for detailed information about this antiserum, see reference 13). To remove natural anti-mouse heteroantibody from the G-typing rat serum, *in vivo* absorption was always

TABLE 1. *Gross leukemia virus-associated antigen system*

Antigen	Abbreviation	Description	Techniques for direction used previously	References
Gross cell surface antigens	GCSA(a,b,—)	Type-specific nonvirion but viral genome-determined antigens on the surface of Gross virus-infected cells	Cytotoxic antibodies, immunoelectron microscopy	1, 6, 13, 23, 24, 29, 32
	G _{IX}	An alloantigen confined to the surface of lymphoid cells, sometimes occurring independently of productive Gross virus infection	Cytotoxic antibodies	30
Viral envelope antigens	tsVEA	Type-specific viral envelope antigen shared by various groups of murine leukemia virus (Gross, Friend, Moloney, and Rauscher) but not by murine myeloma extracellular C-type viruses	Virus neutralization, immunoelectron microscopy	1, 6, 7, 13, 16: (T. Aoki, Bibl. Haematol., <i>in press</i>)
	gsVEA	Group-specific viral envelope antigen shared by various murine leukemia viruses and murine myeloma extracellular C-type viruses		
Group-specific intraviral antigens	gs (1, 2, 3,—)	Intraviral components shared by various C-type viruses	Ouchterlony, C fixation	13-15, 17, 19, 22, 26, 27, 28
Gross soluble antigens	GSA(a, b,—) ^a	Gross virus-associated soluble antigens detected in the body fluids and extracts of the cell surface	Immunofluorescence and immunoelectron microscopy of indicator cells carrying adsorbed GSA	3-5, 10, 20, 21, 25; (T. Aoki, Bibl. Haematol., <i>in press</i>)

^a In this report, GSA have been classified further.

employed before use. One milliliter of undiluted antiserum was injected intraperitoneally into normal 2-month-old C57BL/6 mice. The serum was recovered 2.5 hr later by cardiac puncture under ether anesthesia. The absorbed serum was examined for its specificity by both indirect immunofluorescence tests and immunoelectron microscopy with C57BL leukemia EL4(G⁻) to confirm that natural heteroantibody against mouse cells had been absorbed out, and, with E σ G2, to show that antibodies against different classes of G antigens still remained.

GSA sources. Blood from three groups of mice, (i) 5- to 8-month-old nonleukemic AKR, (ii) 5- to 8-month-old leukemic AKR, and (iii) 3- to 5-month-old nonleukemic C58, was collected with heparinized pipets from retro-orbital venous plexes. Plasma from 8 to 10 mice was pooled for each group. Pooled ascitic fluid of AKR mice bearing K36 leukemia was used as a fourth GSA source. These four GSA sources were used before and after removing Gross leukemia viruses by ultracentrifugation at 100,000 $\times g$ for 60 min. Since they showed no difference with or without removal of Gross leukemia virus, the results will be described together.

Inhibition tests of the G-typing sera with various

GSA sources. Both G-typing mouse and rat sera were titrated by indirect immunofluorescence tests (2) with viable E σ G2 cells to detect various G antigens and with thymocytes of 129 strain mice to detect G_{IX} antigen. For inhibition tests, the sera were used at twice the final concentration of sera producing 100% fluorescent cells. G-typing mouse serum and G-typing rat serum were used with E σ G2 cells at 1/20 and 1/200 dilution, respectively, and the G-typing rat serum was used with 129 mouse thymocytes at 1/20 dilution. The diluted antiserum, 0.05 ml, was incubated at 4 C for 30 min with 0.1 ml of serial twofold dilutions of GSA sources in Earle balanced salt solution (EBSS). These GSA sources had been previously incubated at 4 C for 30 min with 0.05 ml of the appropriate G-typing serum at the same dilution as the test antiserum of EBSS. The antiserum was then incubated with 10⁶ to 2 $\times$ 10⁶ well-washed, viable E σ G2 cells at 4 C for 30 min. After washing twice in 2.0 ml of EBSS, the cells were resuspended in 0.05 ml of one-tenth diluted goat anti-mouse-immunoglobulin G (IgG) or goat anti-rat-IgG coupled to fluorescein isocyanate (Huntingdon Research Lab., Baltimore, Md.). The fluorescent reagents were analyzed by immunoelectrophoresis with whole mouse or rat serum

before use and gave a strong single IgG line. Approximately 100 cells were examined for fluorescence (Leitz fluorescence microscope, model 250, with immersion dark field condenser; exciter filter BG12; barrier filter 54; high-pressure mercury bulb HBO, 200 w, as fluorescence light source). In various tests, the following controls were included: (i) antiserum omitted (negative control)—test cells incubated with the fluorescent reagent; (ii) GSA sources omitted (positive control)—the G-typing mouse or rat serum demonstrated fluorescent G antigens on the surface of E σ G2 cells, indicating proper performance of immunofluorescence tests.

The concentrations of G-typing sera and GSA preparations most effective for inhibition in indirect immunofluorescence tests were used as a guide for the immunoelectron microscopy studies (7). Since immunoelectron microscopy requires twice the number of test cells as immunofluorescence tests, however, the amounts of G-typing sera and GSA preparation found by immunofluorescence tests to be most effective for inhibition were doubled to maintain the same experimental ratio in immunoelectron microscopy. The mixtures (the G-typing sera and GSA preparations) were then reacted with 3×10^6 to 5×10^6 well-washed, viable E σ G2 cells at 0 C for 30 min. After washing, the cells were resuspended in hybrid antibodies with dual specificity for (i) mouse- γ G or rat- γ G and (ii) southern bean mosaic virus (SBMV) or ferritin at 0 C for 30 min. After another washing, the cells were incubated with SBMV or ferritin at 0 C for 30 min. The final pellets of viable cells were fixed with glutaraldehyde and osmium tetroxide. After incubation

overnight in cold 0.5% uranyl acetate, they were dehydrated and embedded in Epon as usual. Thin sections were stained with uranyl acetate and lead citrate, and examined with a Siemens Elmiskop 1A electron microscope. Sections of samples labeled with ferritin were stained only with lead citrate to add contrast to the ferritin.

RESULTS

Indirect immunofluorescence method. Ascitic fluid of the AKR leukemia K36 and plasma of nonleukemic and leukemic AKR mice showed similar inhibiting effects on the reaction of G-typing mouse and rat sera with viable E σ G2 cells (Tables 2 and 3). These GSA sources themselves completely inhibited the G-typing mouse serum. When the ascitic fluid or plasma was pretreated with the G-typing mouse or rat serum, they lost their inhibiting effects on the G-typing mouse serum. The results indicate that all classes of GSA in these sources which are capable of reacting with the G-typing mouse serum were completely eliminated by the pretreatment. The GSA sources also completely inhibited the G-typing rat serum. When they are pretreated with the G-typing mouse serum, however, a partial inhibiting effect of these GSA sources on the G-typing rat serum remained, forming a plateau at the first several concentrations of the mixture (GSA sources and G-typing serum). The results suggest

TABLE 2. Inhibition of G-typing sera by ascitic fluid of AKR leukemia K36 by using indirect immunofluorescence tests with viable C57BL/6 Gross leukemia E σ G2 cells

G-typing serum	Materials used for inhibition		Percent E σ G2 cells showing fluorescence					
			Dilution of GSA sources					
	GSA source	Pretreated with ^a	2/1 ^b	1/1	1/2	1/4	1/8	1/16
Mouse serum C57BL/6 <i>anti-K36</i>	None	None	100	100	100	100	100	100
	Ascitic fluid	None	<5	<5	<10	82	95	100
	Ascitic fluid	NZB serum	<5	<10	36	95	100	100
	Ascitic fluid	G-typing mouse serum	100	100	100	100	100	100
	Ascitic fluid	G-typing rat serum	100	100	100	100	100	100
Rat serum (W/Fu $\times$ BN)F1 anti - W/Fu-(C58NT)D	None	None	100	100	100	100	100	100
	Ascitic fluid	None	<5	7	59	80	100	100
	Ascitic fluid	NZB serum	<5	<5	65	89	100	100
	Ascitic fluid	G-typing mouse serum	40	41	47	77	93	100
	Ascitic fluid	G-typing rat serum	100	100	100	100	100	100

^a These mixtures of K36 leukemia ascitic fluid with the G-typing serum had been initially incubated at 4 C for 30 min, were added again to the test G-typing serum, and then incubated at 4 C for 30 min (see Materials and Methods).

^b Double amount of a 1:1 dilution.

TABLE 3. Inhibition of G-typing sera by plasma of nonleukemic (NL) and leukemic (L) AKR mice by using indirect immunofluorescence tests with viable C57BL/6 Gross leukemia E σ G2 cells

G-typing serum	Materials used for inhibition			Percent E σ G2 cells showing fluorescence						
				Dilution of GSA sources						
	GSA source		Pretreated with ^a	2/1 ^b	1/1	1/2	1/4	1/8	1/16	1/32
Mouse serum C57BL/6 anti-K36	None		None	100	100	100	100	100	100	100
	AKR plasma	NL	None	<5	17	32	95	100	100	100
		L	None	<5	<5	<10	46	68	89	100
	AKR plasma	NL	NZB serum	<5	<10	23	94	100	100	100
		L	NZB serum	<5	<10	30	81	100	100	100
	AKR plasma	NL	G-typing mouse serum	100	100	100	100	100	100	100
		L	G-typing mouse serum	100	100	100	100	100	100	100
	AKR plasma	NL	G-typing rat serum	100	100	100	100	100	100	100
		L	G-typing rat serum	100	100	100	100	100	100	100
	None		None	100	100	100	100	100	100	100
Rat serum (W/Fu $\times$ BN)F ₁ anti-W/Fu-(C58NT)D	AKR plasma	NL	None	<5	20	37	95	100	100	100
		L	None	<5	<5	27	64	80	95	100
	AKR plasma	NL	NZB serum	<5	<5	51	100	100	100	100
		L	NZB serum	<5	<5	48	100	100	100	100
	AKR plasma	NL	G-typing mouse serum	47	50	82	95	100	100	100
		L	G-typing mouse serum	45	44	47	73	91	100	100
	AKR plasma	NL	G-typing rat serum	95	95	100	100	100	100	100
		L	G-typing rat serum	100	100	100	100	100	100	100
	None		None	100	100	100	100	100	100	100
	None		None	100	100	100	100	100	100	100

^a These mixtures of plasma from AKR mice with the G-typing serum had been initially incubated at 4 C for 30 min, were added again to the test G-typing serum, and then incubated at 4 C for 30 min (see Materials and Methods).

^b Double amount of a 1:1 dilution.

that the G-typing rat serum detected additional antigens in these GSA sources, which did not react with the G-typing mouse serum.

C58 plasma showed different results from the other GSA sources mentioned above (Table 4). C58 plasma was only able partially to inhibit the G-typing mouse serum. When AKR plasma was added to C58 plasma, however, the mixture was able to inhibit completely the G-typing mouse serum, suggesting that C58 plasma lacks at least one specific GSA necessary to inhibit completely the G-typing mouse serum. On the other hand, the G-typing rat serum was absolutely inhibited by the C58 plasma. This was also substantiated by results obtained when C58 plasma was pretreated with the G-typing rat serum. All inhibiting effects on the G-typing rat serum were eliminated.

Serum of aged autoimmune NZB mice was also inhibited completely by the GSA sources used (Table 5). Pretreatment of GSA sources with NZB serum, however, did not eliminate the inhibiting effects of the GSA on the G-typing mouse and rat sera within the sensitivity range of indirect immunofluorescence tests (Tables 2-4) (see below). Therefore, it seems most likely that only some class(es) of GSA inhibited the reaction of NZB serum with E σ G2 cells.

When thymocytes of 129 strain mice were used as target cells instead of E σ G2 cells, no GSA sources used inhibited the reaction of G-typing rat serum. Consequently, these GSA sources had no detectable GSA corresponding to G_{IX} antigen.

The results of all the inhibition experiments by

TABLE 4. *Inhibition of G-typing sera by plasma of nonleukemic C58 mice using indirect immunofluorescence tests with viable C57BL/6 Gross leukemia E♂G2 cells*

G-typing serum	Materials used for inhibition		Percent E♂G2 cells showing fluorescence					
			Dilution of GSA sources					
	GSA source	Pretreated with ^a	1/1	1/2	1/4	1/8	1/16	1/32
Mouse serum C57BL/6 anti-K36	None	None	100	100	100	100	100	100
	C58 plasma	None	45	44	66	85	95	100
	C58 plasma	NZB serum	39	40	48	61	86	100
	C58 plasma	G-typing mouse serum	95	95	95	100	100	100
	C58 plasma	G-typing rat serum	94	95	95	95	100	100
	C58 plasma	AKR plasma ^b	<5	47	73	95	100	100
Rat serum (W/Fu × BN)F1 anti - W/Fu-(C58NT)D	None	None	100	100	100	100	100	100
	C58 plasma	None	<5	20	44	69	93	100
	C58 plasma	NZB serum	<5	18	39	50	95	100
	C58 plasma	G-typing mouse serum	26	32	64	84	95	100
	C58 plasma	G-typing rat serum	95	100	100	100	100	100

^a These mixtures of plasma from C58 mice with the G-typing serum had been initially incubated at 4 C for 30 min, were added again to the test G-typing serum, and then incubated at 4 C for 30 min (see Materials and Methods).

^b Leukemic mice.

TABLE 5. *Inhibition of aged autoimmune NZB serum by various GSA sources by using indirect immunofluorescence tests with viable C57BL/6 Gross leukemia E♂G2 cells*

Materials used for inhibition		Percent E♂G2 cells showing fluorescence			
		Dilution of GSA sources			
GSA source	Pretreated with ^a	1/1	1/2	1/4	1/8
None	None	54	50	58	55
Ascitic fluid of AKR leukemia K36	None	<5	<10	20	48
AKR plasma { NL ^b	None	<10	20	28	38
{ L ^c	None	<5	<5	10	44
C58 plasma (NL)	None	<10	16	23	55
AKR plasma (L)	G-typing mouse serum	<5	<10	15	40
C58 plasma (NL)	G-typing mouse serum	<5	12	18	44
AKR plasma (L)	G-typing rat serum	<10	14	19	49
C58 plasma (NL)	G-typing rat serum	<10	18	25	53

^a These mixtures of various GSA sources with the G-typing serum had been initially incubated at 4 C for 30 min, were added again to the test NZB serum, and then incubated at 4 C for 30 min (see Materials and Methods).

^b Nonleukemic mice.

^c Leukemic mice.

immunofluorescence tests are summarized in Table 6.

Immunoelectron microscopy. Immunoelectron microscopy facilitates analysis of the detailed relationship among specificities of GSA, GCSA, and VEA.

As summarized in Fig. 1, these results correlate

with the results obtained by the indirect immunofluorescence method. The G-typing mouse serum recognized only GCSA (Fig. 2), and this activity was completely inhibited with GSA sources from AKR mice, K36 ascitic fluid, and plasma of both nonleukemic and leukemic mice (Fig. 3). GSA showing this inhibiting effect were eliminated by

TABLE 6. *Indirect immunofluorescence tests on inhibition effects of GSA from AKR and C58 mice on the reaction of G-typing sera and of NZB serum with viable E δ G2 cells*

GSA sources used for inhibition	Pretreated with	Fluorescence seen with test serum		
		G-typing mouse serum	G-typing rat serum	NZB serum
None	None	++	++	+
From AKR mice	None	—	—	—
	G-typing mouse serum	++	+	—
	G-typing rat serum	++	++	—
	NZB serum	—	—	+
From C58 mice	None	+	—	—
	G-typing mouse serum	++	+	—
	G-typing rat serum	++	++	—
	NZB serum	+	—	+

pretreatment with either the G-typing mouse or rat serum. In contrast, C58 plasma only partially inhibited the G-typing mouse serum reaction with GCSA (Fig. 4), indicating that the G-typing mouse serum recognized at least two different GSA. Pretreatment with either the G-typing mouse or rat serum completely eliminated the inhibiting effect of C58 plasma on the reaction of the G-typing mouse serum with GCSA.

GSA sources from AKR or C58 mice completely inactivated the reactions of the G-typing rat serum with both GCSA and VEA (Fig. 5 versus 6), suggesting that these GSA sources contained GSA corresponding to all classes of GCSA and VEA detectable by the rat antiserum. Pretreatment of GSA sources with the G-typing mouse serum partially eliminated the reaction of the G-typing rat serum with GCSA, but did not prevent the inhibiting effects of the GSA corresponding to VEA (Fig. 7). On the other hand, pretreatment with the G-typing rat serum eliminated all inhibiting effects of GSA sources, since positive reactions with GCSA and VEA were observed.

These results indicate that, although GSA sources from both AKR and C58 mice contain all classes of GSA recognized by the rat antiserum, at the dilution used, the mouse antiserum contains antibody(ies) against only some classes of GCSA.

These results indicate that, although GSA sources from both AKR and C58 mice contain all classes of GSA recognized by the rat antiserum, at the dilution used, the mouse antiserum contains antibody(ies) against only some classes of GCSA.

As previously reported, NZB serum recognized only gsVEA of C-type viruses (Fig. 8; references 1, 6, 7). All GSA sources inhibited the activity

of NZB serum with the envelope of Gross leukemia virus (Fig. 9). Moreover, the G-typing rat serum recognized tsVEA of murine leukemia and myeloma viruses (T. Aoki, *Bibl. Haematol., in press*) and was inhibited by all GSA sources used in this study. Therefore, both GSA corresponding to gsVEA and tsVEA were detected in the body fluids. Pretreatment of GSA sources with the G-typing rat serum did not eliminate inhibiting effects on the NZB serum. In other words, specificities of anti-VEA antibodies in the G-typing rat serum and NZB serum were different.

DISCUSSION

The present study demonstrates several classes of GSA with different antigenic specificities. This type of analysis is essential for the future isolation of individual classes of GSA.

It has become evident that previously reported GSA detectable with the G-typing mouse serum C57BL/6 anti-K36 correspond to some classes of GCSA (6). Inhibition tests of this typing serum with GSA sources from AKR mice and from C58 mice indicate that the G-typing mouse serum recognized at least two different specific GCSA: the previously described GCSAa (13) and another, which may be designated GCSAc. The C58 plasma contains only one GSA corresponding to one of these antigens, GCSAa, and both specificities exist in GSA sources of AKR mice; when AKR plasma was added to C58 plasma, the G-typing mouse serum was completely inhibited.

If the GCSAc specificity is absent from the GSA of C58 mice, why did GSA from both AKR and C58 mice have identical inhibiting effects on the G-typing rat serum? Two alternative explana-

Test serum	Combination of GSA sources and G-typing serum used at appropriate dilution for neutralization	GSA sources	
		AKR mice	C58 mice
<u>Mouse serum</u> <u>C57BL/6 anti-K36</u>	None
	GSA + EBSS or C57BL/6 serum
	GSA + G-typing <u>mouse</u> serum
	GSA + G-typing <u>rat</u> serum
	GSA + NZB serum
<u>Rat serum</u> <u>(W/Fu x BN) F₁</u> <u>anti-W/Fu (C58NT) D</u>	None
	GSA + EBSS or C57BL/6 serum
	GSA + G-typing <u>mouse</u> serum
	GSA + G-typing <u>rat</u> serum
	GSA + NZB serum
<u>NZB serum</u>	None
	GSA + EBSS or C57BL/6 serum
	GSA + G-typing <u>mouse</u> serum
	GSA + G-typing <u>rat</u> serum

FIG. 1. Summary analysis of inhibition of G-typing sera by various GSA sources using immunoelectron microscopy with viable C57BL/6 Gross leukemia E σ^3 -G2 cells. Inhibition is demonstrated by the degree of reaction of appropriately treated G-typing sera with the cell surface or viral envelope, or both. (Reference to the appropriate electron micrographs will aid in the interpretation of the figure.)

tions may be considered. (i) The rat serum may not contain antibody against GCSAc, since it was prepared by immunization with the syngeneic rat leukemia originally induced by C58 wild-type Gross virus. However, one must then account for the observation that pretreatment of GSA

sources from AKR mice with the rat serum completely eliminated the inhibiting effects of AKR-GSA on the mouse serum. It has been shown that the rat antiserum to (C58NT)D contains an antibody with broad specificity, reacting with an antigen found on leukemia cells induced both by

FIG. 2. E♂G2 cells were reacted with the G-typing mouse serum C57BL/6 anti-K36 and labeled with SBMV. Sectors on the cell surface are labeled with SBMV, but the viral envelope is not labeled. $\times 90,000$.

FIG. 3. E♂G2 cells were reacted with the G-typing mouse serum C57BL/6 anti-K36 pretreated with plasma of leukemic AKR mice and labeled with SBMV. No labeling with SBMV is observed, showing complete inhibition (compare with Fig. 2). $\times 12,000$.

FIG. 4. E♂G2 cells were reacted with the G-typing mouse serum C57BL/6 anti-K36 pretreated with plasma of nonleukemic C58 mice and labeled with SBMV. Small sectors on the cell surface are labeled with SBMV, showing partial inhibition. $\times 12,000$.

FIG. 5. $E\delta G2$ cells were reacted with the G-typing rat serum ($W/Fu \times BN$) F_1 anti- $W/Fu(C58NT)D$ and labeled with ferritin. The entire envelope of virion and sectors on the cell surface are labeled with ferritin. $\times 12,000$.

FIG. 6. $E\delta G2$ cells were reacted with the G-typing rat serum ($W/Fu \times BN$) F_1 anti- $W/Fu(C58NT)D$, pre-treated with plasma of nonleukemic C58 mice, and labeled with ferritin. No labeling with ferritin is observed, showing complete inhibition (compare with Fig. 5). $\times 12,000$.

FIG. 7. $E\delta G2$ cells were reacted with the G-typing rat serum ($W/Fu \times BN$) F_1 anti- $W/Fu(C58NT)D$, pre-treated with the mixture of plasma from leukemic AKR mice and the G-typing mouse serum, and labeled with ferritin. A small sector on the cell surface is labeled with ferritin, showing the inhibition of antibodies against viral envelope antigens and some G cell surface antigens with AKR plasma (compare with Fig. 5). $\times 14,400$.

FIG. 8. *E♂G2* cells were reacted with the serum of aged NZB mice and labeled with SBMV. The entire envelope of both complete and budding viruses is labeled with SBMV, but the cell surface is not labeled at all. $\times 12,000$.

FIG. 9. *E♂G2* cells were reacted with the NZB serum pretreated with plasma of leukemic AKR mice and labeled with SBMV. No labeling with SBMV is observed, showing complete inhibition (compare with Fig. 8). $\times 12,000$.

Gross virus and by other murine leukemia viruses. This antigen has been called GCSAb (18). It is possible that GCSAb and GCSAc have some common determinants and that the rat antibody can react with either antigen. By contrast, the G-typing mouse serum can recognize only GCSAc but not GCSAb. (ii) GSAc may exist in an incomplete or modified form in the plasma of C58 mice, and may be designated GSAc' to distinguish it from GSAc detected in AKR mice. The mouse serum cannot recognize this modified antigen, but the rat serum can, due to the difference in species. This incomplete form may have arisen in conjunction with solubilization, since viable cells of nonleukemic C58 mice absorbed all activity of the G-typing mouse serum against the

G antigens on the surface of viable *E♂G2* cells (23). This indicates that GCSAc is probably on the surface of C58 cells.

G_{IX} antigen may be coded by a cellular gene that can be switched on by Gross leukemia virus infection, although this hypothesis has not yet been finally confirmed (30). We were unable to detect G_{IX} soluble antigen in various GSA sources used. G_{IX} antigen may behave like other allo-antigens which are also coded by a cellular gene. For example, TL (Thymus-Leukemia) antigens act like G_{IX} antigen (8, 30), i.e., both of them convert from negative to positive states by malignant transformation of their carrier cells, and they have never been found in soluble form in the body fluids (T. Aoki, *unpublished observations*).

Only G antigens coded by the viral genome may exist in soluble form. This assumption is supported by the evidence that leukemic AKR mice contained larger amounts of GSA, as well as GCSA, than nonleukemic AKR mice because of higher activity of the viral genome or increased replication of Gross leukemia virus in leukemic mice.

It has been demonstrated by immunoelectron microscopy that the antibody(ies) in the rat serum against tsVEA was also completely inhibited by GSA. This rat serum contains inhibiting antibody against Gross leukemia virus which can be absorbed with Gross leukemia virus, resulting in no reaction with tsVEA by immunoelectron microscopy (T. Aoki, *unpublished observations*). Therefore, isolated GSA corresponding to tsVEA may be utilized for the production of Gross leukemia virus inhibiting antiserum without any risk of virus infection of normal host cells.

GSA corresponding to gsVEA was also detected in this study. Both GSA, corresponding to gsVEA and tsVEA, are circulating in soluble form in the body fluids (Table 7). This observation may correlate with the results obtained by P.J. Fischinger et al. (11) that gsVEA and tsVEA may coexist on a single molecule or as antigen complexes. The final solution of this problem could be provided by the isolation of these GSA. Studies in the isolation of individual classes of GSA are presently in progress.

What is the relationship between GSA and G antigens on the surface of G⁺ cells and viruses? From where are these GSA derived? It is a reasonable possibility that G antigens on the surface may be released into the body fluids during turnover of the cell membrane (including virus budding).

TABLE 7. *Comparison among specificities of G soluble antigens (GSA), G cell surface antigens (GCSA), and viral envelope antigens (VEA)*

Known G antigens on the surface of E σ G2 cells and viruses (detected with G-typing sera)	G soluble antigens (sources of mouse strains)
Cellular	
GCSAa (mouse and rat sera)	GSAA (AKR, C58)
GCSAb (rat serum)	GSAb (AKR, C58)
GCSAc (?) (mouse and rat sera)	GSAC (AKR), GSAC' (C58)
G1x (rat serum)	—
Viral	
tsVEA (rat serum)	tsGSA (AKR, C58)
gsVEA (NZB serum)	gsGSA (AKR, C58)

During release their molecular constitution may be modified but antigenicity itself remains intact. Conversely, GSA may be adsorbed from the body fluids onto the surface of cells and viruses as was previously observed in adsorption tests with GSA adsorbed on the G⁻ indicator cells (3). This is a kind of antigenic conversion in which originally murine leukemia virus-associated antigen-negative cells become positive by the adsorption of GSA on the cell surface (31).

Finally, the presence of group-specific intraviral antigens (gs1, 3) in the various GSA sources used has been examined by both complement fixation and Ouchterlony methods. Since conclusive results were not obtained in the present study, however, their description is omitted in this paper. A large amount of purified gs antigens will be required for this solution.

ACKNOWLEDGMENTS

We are indebted to R. J. Huebner for valuable advice; L. J. Old for supply of hybrid antibody; H. A. Wood for supply of SBMV; and R. Sims, K. Nagashima, and S. P. Chan for excellent technical assistance.

M. Liu and M. M. Sturm are supported by Public Health Service grants NIH-69-21-60 and NCI-72-2006 from the National Cancer Institute.

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