ANTIGENS OF LEUKEMIAS INDUCED BY NATURALLY OCCURRING MURINE LEUKEMIA VIRUS: THEIR RELATION TO THE ANTIGENS OF GROSS VIRUS AND OTHER MURINE LEUKEMIA VIRUSES*

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Study of the specific antigens of mouse leukemias has advanced considerably in recent years, largely as a result of the development of effective methods of serological analysis in vitro (1, 2). Of the various antigens that can be demonstrated in leukemia cells of the mouse, the G (Gross) antigen (3, 4) is of particular interest because of its widespread distribution among leukemias of natural occurrence (4–6). The G antigen is demonstrable by the cytotoxic test in vitro, and is found in: (*a*) the cells of leukemias induced by Passage A Gross virus; (*b*) the cells of leukemias arising in mice of strains with a high incidence of leukemia,—AKR, AKR.K, C58, PL, F, and C3Hf/Figge-Law; (*c*) the normal lymphoid tissues of these high-incidence strains (which we therefore refer to as G+ strains), but not of low-incidence strains (G− strains); and (*d*) some leukemias and solid tumors of low-incidence strains. The presence of G cellular antigen indicates infection by Gross virus and provides a means to identify the etiology of leukemias induced by Gross virus.

In a further investigation of the G antigenic system, we have made a study of leukemias induced in inbred rats by virus from nonleukemic C58 mice, which are naturally infected with leukemia virus. In contrast to leukemias induced by Gross virus in the mouse (either as a result of natural infection or by inoculation of Passage A virus) these rat leukemias are highly antigenic and are not transplantable to adult isogenic recipients. The serum of adult rats that have resisted transplants of isogenic leukemias contains cytotoxic and precipitating antibodies to antigens belonging to the G system. With these antisera it has been possible to conduct a more extensive analysis of the G (Gross) antigenic system, which is now seen to comprise a complex group of distinct antigens.

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Materials and Methods

Rats.—Our colony of W/Fu inbred rats (7) originated from a litter provided by Dr. J. Furth in 1961 and is now in the 10th generation of inbreeding in this laboratory. Thirteen spontaneous leukemias have been observed during this period, an incidence of the order of 5% in animals kept for 18 months or longer. According to the criteria of (*a*) acceptance of skin grafts, and (*b*) invariable transplantability of spontaneous leukemias, the strain is homogeneous. The colony of BN (Brown Norway) rats is descended from a litter provided by Dr. R. E. Billingham and Dr. W. K. Silvers in 1964.

Mouse Leukemias.—The spontaneous and induced mouse leukemias used for serological tests and for inoculation into newborn rats, arose in mice of our own colonies and were transplanted only in these mice. Methods of inducing leukemia by X-radiation, by chemical carcinogens, and by viruses [Gross Passage A (8), Friend (9), Moloney (10), and Rauscher (11)] are recorded in previous publications from this laboratory.

Inoculation of Newborn Rats.—Rats less than 24 hr old were inoculated either with viable cell suspensions of normal or leukemic mouse tissue or with filtrates of leukemic tissue. Cell suspensions were prepared from the thymus of normal C58 mice or from organs of leukemic mice (thymus, spleen, and lymph nodes) by mincing with curved scissors in Earle's balanced salt solution (EBSS). The released cells were washed and resuspended in medium 199; viability was determined by counting in the presence of trypan blue, which invariably showed that less than 10% of the cells were dead. Cell-free extracts were obtained by filtration (Selas 02) of a 20% (v/v) homogenate of leukemic tissue that had first been clarified by centrifugation at 2000 *g* for 1 hr. Newborn rats received 0.25 ml of these filtrates either subcutaneously or intraperitoneally.

Antisera.—Serum was obtained by bleeding from the tail under ether anesthesia and was stored at −70°C.

Cytotoxic Test (12, 13).—Serial dilutions (0.05 ml) of rat antiserum, (inactivated at 56°C for 30 min) were incubated with cells (0.05 ml of a suspension containing 5×10^6 cells/ml) and pooled guinea pig serum diluted ½ (0.05 ml) to provide C′. After incubation for 45 min at 37°C, trypan blue was added and a count of stained and unstained cells was made. Titers refer to the dilution of antiserum in which the proportion of dead cells (stained) was nearest to 50%. In every test, cells were incubated also in: (*a*) antiserum alone, (*b*) guinea pig serum alone; these showed no more than 10% stained cells. The diluent was medium 199.

Typing by Absorption.—The typing of tissues for G antigen was performed according to the absorption procedure outlined previously (4). With this technique, a typing antiserum of known titer against a selected standard G+ cell line is diluted to two 2-fold dilutions below the endpoint and absorbed with equal volumes of packed washed cells of the tissue to be tested. The mixture is incubated for 45 min at room temperature followed by 45 min at 4°C, with occasional resuspension, and the serum is recovered by centrifugation. The absorbed serum is then tested for residual activity on the standard G+ cells. Absence of cytotoxic activity shows that the cells used for absorption contain G antigen.

Immunodiffusion.—Double-diffusion (Ouchterlony) tests were performed on slides in 2% Noble agar (Immunoplate pattern C, Hyland Laboratories, Los Angeles). The slides were left at room temperature and inspected over a 4 day period. Optimal precipitation usually occurred by 18 to 24 hr.

Preparation of Antigen for Immunodiffusion.—

Soluble antigen from cells or plasma: Suspensions of normal cells or leukemia cells were prepared by mincing tissues with curved scissors in EBSS and discarding the larger fragments that settled rapidly. The cells were washed twice by slow centrifugation to obtain whole cells free of debris. They were then packed at 2000 *g* for 10 min, resuspended in 1 to 5 volumes of EBSS, and disrupted by 3 cycles of freezing and thawing or by means of a Vertis

"45" high speed homogenizer (medium speed for 3 min). This material was spun at 2000 *g* for 45 min and the sediment was discarded. The supernate, or heparinized plasma, was then spun at 100,000 *g* for 1 hr in a Beckman L-2 centrifuge (type 50 rotor). This supernate was reduced to $\frac{1}{15}$ to $\frac{1}{20}$ of its original volume either by vacuum dialysis (Carl Schleicher & Schuell, Keane, New Hampshire) or by placing it in dialysis tubing (Visking dialysis membrane, size 8, Union Carbide, New York) surrounded by Sephadex G-100. The protein content of the final extracts (280 mμ absorbance, ovalbumin standard) varied considerably according to the tissue extracted, ranging from 50 to 250 mg/ml (both G+ and G− preparations). In the case of plasma, the volume was reduced to approximately $\frac{1}{4}$ of its original volume. These procedures were carried out in the cold. Storage of antigen in the frozen state did not lead to loss of precipitating activity.

Viral antigens: Infective plasma was obtained from: (*a*) C3Hf/Bi mice with primary leukemia induced by Passage A Gross virus, (*b*) BALB/c mice with primary Rauscher leukemia, (*c*) W/Fu rats with primary leukemia induced by Moloney virus (lot No. 3042-70 Chas. Pfizer and Co., Maywood, New Jersey), and (*d*) DBA/2 mice infected with Friend leukemia virus (kindly provided by Dr. Charlotte Friend, Sloan-Kettering Institute for Cancer Research). Pellets of virus were obtained from 10 to 20 ml of fresh plasma by centrifugation at 100,000 *g* for 1 hr. Mammary tumor virus (MTV) prepared from infective milk of RIII mice by density-gradient centrifugation was kindly provided by Dr. D. Moore, Rockefeller University. Viral pellets from these various sources were dispersed in 0.1 to 0.15 ml EBSS and examined by immunodiffusion either untreated or after exposure to ether. Exposure to ether was effected by placing 0.05 to 0.1 ml of the resuspended pellet in a glass beaker (internal diameter, 27 mm) and adding 2 ml ether (Squibb, anesthesia grade). The mixture was left at room temperature and shaken frequently until the ether had evaporated (usually less than 10 min). The residue was placed on ice if it was to be used on the same day, or was stored at −70°C.

RESULTS

Induction of Leukemias in W/Fu Inbred Rats by Inoculation of Viable Mouse Cells or by Filtrates.—A variety of viable mouse cells, normal or leukemic, or filtrates, were inoculated into newborn W/Fu inbred rats. Table I and Table II summarize the results of these experiments. In the case of viable leukemia cells, three types of response were observed: (*a*) progressive growth of inoculated mouse leukemias, leading to death of the host 2 to 3 wk later; (*b*) inoculated cells did not grow progressively, but the recipients developed primary leukemia in adult life (identified histologically as lymphoblastic); and (*c*) no leukemia developed, of either mouse or rat origin, during observation periods of 1 yr or more.

Those cell types and filtrates that induced primary leukemia on inoculation into newborn rats are listed in Table I. Normal thymus cells from the G+ C58 strain were particularly effective; of 19 newborn rats inoculated with 30 to 50 × 10^6 washed thymus cells from C58 mice aged 6 to 8 wk, 15 developed leukemia. In subsequent experiments the incidence was as high as 100%. Leukemia cells, or filtrates of leukemia cells, from the high-incidence G+ AKR strain also were highly effective. Cells from 3 leukemias of mice of G− strains—DBA/2, BALB/c and (C57BL/6 × A)F_1—induced primary leukemias in W/Fu rats (last 3 entries in Table I). With these exceptions, the inoculation of viable cells

TABLE I

Induction of Leukemia in W/Fu Inbred Rats by Neonatal Inoculation of Viable Mouse Cells or Filtrates of Mouse Cells

Inoculum				Incidence of leukemia*	Latent period from time of inoculation to development of leukemia—	Transplantability of primary W/Fu leukemia‡			
								W/Fu rats	
Origin of cells (transplant generation)	G antigen type (reference 4)	No. of cells	Route			Leukemia tested	Mice	<7 days old	adult
		(× 10^6)			*days*				
AKR♀									
Spontaneous leukemia (0)	G+	140	s.c.	5/6	55 (♀A)	A	−	+	
					62 (♀B)	B		+	−
		140	i.p.		152 (♂C), 189 (♀D)	C, D			−, −
					189 (♀E)				
					1 NL at 12 months				
AKR♀									
Spontaneous leukemia (0)	G+	100	s.c.	1/5	88 (♀F) = *W/Fu(AKR-SL)F*	F		+	−
					4 NL at 6 months				
AKR♀									
Leukemia DMBA 2 (4)	G+	Filtrate	s.c.	2/5	132 (♂1), 138 (♀2)	1, 2		+, +	−, −
					3 NL at 12 months				
AKR♀									
Leukemia U7 (0)	G+	Filtrate	s.c.	4/10	127 (♀1), 144 (♂2)				
					223 (♂3)	3		+	
					223 (♀4)				
					6 NL at 12 months				
C58♀♀ (6 to 8 wk old)									
Thymus	G±	30	i.p.	5/8	142 (♀), 146 (♂A), 214 (♀B)	A, B	−, −		−, −
					225 (♂), 255 (♂C)	C		+	−
					3 NL at 117, 161 and 183 days				
	G±	50	i.p.	10/11	173 (♂D) = *W/Fu(C58NT)D*	D	−	+	−
					179 (♀E), 204 (♂F)	E, F,		+, +,	−, −,
					209 (♂G)	G		+	−
					204 (♀H)	H		+	−
					258 (♀I) = *W/Fu(C58NT)I*	I		+	−
					259 (♀J), 276 (♀K)	J		+	−
					276 (♂L)				
					290 (♀M)	M		+	−
					1 NL at 12 months				
DBA/2♀									
Leukemia DMBA 15 (5)	G+	32	i.p.	1/1	228 (♂A)	A	−	+	−
BALB/c♀									
Spontaneous leukemia (4)	Not tested	50	s.c.	4/8	213 (♂A), 220 (♂B)	A, B			−, −
					328 (♂C), 330 (♂D)	C, D		+, +	−, −
					4 NL at 20 months				
(C57BL/6 × A)F1♀									
Leukemia U11 (2)	Not tested	187	s.c.	3/7	190 (♀A), 203 (♀B)	A, B			−, −
					210 (♂C)	C	−		−
					4 NL at 12 months				

Abbreviations: NL, not leukemic; i.p., intraperitoneally; s.c., subcutaneously; (for abbreviations used in designating leukemias, see Table II).

* Excluding all rats in which the inoculated mouse cells grew progressively.

‡ +, progressive growth; −, no progressive growth: tested by inoculation of 5 to 50 × 10^7 cells i.p. into 1 to 4 adult rats (depending upon availability of cells and animals); approximately 5 × 10^7 cells i.p. or s.c. into 3 to 10 rats <7 days old; 5 to 25 × 10^7 cells i.p. into adult mice. One primary leukemia inoculated sc into adult rats was seen to grow temporarily and then regress.

TABLE II

Summary of Experiments in Which Newborn W/Fu Rats Inoculated with Viable Mouse Leukemia Cells or with Filtrates of Mouse Leukemia Cells, Failed to Develop Primary Leukemias

Leukemia* (transplant generation)	No. of cells	Route	No. survivors‡ / No. rats inoculated	Period of observation
	× 10^6			
Radiation-induced				
BALB♂RL1 (12)	30	s.c.	0/2	
	30	i.p.	0/2	
(BALB × C3H/An)F_1♂RAD1 (0)	40	s.c.	4/4	4 NL§ at >20 months
E♂RL9 (6 and 7)	110	s.c.	0/4	
	110	i.p.		
E♂RL9 (8)	50	i.p.	0/4	
ERLD (43)	30	s.c.	0/3	
ERLD (43)	16	s.c.	1/3	1 NL at 18 months
EARAD1 (69)	25	i.p.	0/3	
RADA1 (59)	20–40	s.c. or i.p.	0/14	
RADA2 (29)	16	s.c.	3/3	3 NL at >15 months
	16	i.p.	0/4	
RADA2 (29)	Filtrate	s.c.	17/17	17 NL at >15 months
Spontaneous				
E♀SL1 (3)	20	s.c.	9/9	1 NL at 13 months 8 NL at >22 months
E♀SL1 (3)	113	s.c.	6/6	6 NL at 20 months
	113	i.p.		
DBA 2♀SL1 (11)	37	s.c.	0/5	
	37	i.p.		
DBA 2♀SL1 (24)	Filtrate	i.p.	9/9	9 NL at >14 months

(*Table II continued on following page*)

TABLE II—*Concluded*

Leukemia* (transplant generation)	No. of cells	Route	No. survivors‡ / No. rats inoculated	Period of observation
Spontaneous, *cont'd*	$\times 10^6$			
DBA 2♂SL2 (4)	100	i.p.	0/10	
DBA/2♂SL2 (19)	Filtrate	s.c.	6/6	6 NL at >12 months
Chemically induced				
(BALB × C3H/An)F_1 ♀ U5 (3)	60	s.c.	10/10	10 NL at 21 months
EA♂U6 (1)	90	s.c.	11/11	1 NL at 13 months 10 NL at >20 months
EA♂U8 (2)	70	s.c.	8/9	8 NL at >21 months
DBA/2♂DMBA4 (27)	59	s.c.	0/5	
	59	i.p.		
DBA/2♂DMBA5 (11)	Filtrate	i.p.	12/12	12 NL at >12 months
DBA/2♀DMBA6 (13)	15	s.c.	3/3	3 NL at >12 months
	15	i.p.	0/5	
DBA/2♂DMBA6 (33)	Filtrate	s.c.	10/10	10 NL at >12 months
DBA 2♀DMBA15 (5)	120	i.p.	0/10	
DBA/2♀DMBA16 (14)	4	s.c.	4/4	4 NL at >17 months
DBA/2♀DMBA16 (14)	45	i.p.	0/2	
DBA/2♀DMBA16 (20)	Filtrate	i.p.	9/9	9 NL at >17 months

* The abbreviations used in designating leukemias are: A, A strain; E, C57BL/6; EA, (C57BL/6 × A)F_1; BN, Brown Norway rat; F, Friend; M, Moloney; R, Rauscher; SL, spontaneous leukemia; DMBA, induced by 9,10-dimethyl-1,2-benzanthracene; RL or RAD, induced by X-radiation; U, induced by urethan.

‡ Survivors: refers to those rats that did not succumb to progressive growth of the inoculated mouse leukemia cells.

§ NL, not leukemic.

or filtrates from leukemias of mice of G− strains (whether G− or G+) either failed to induce primary leukemias or, in the case of viable cells, gave rise to progressively growing transplants (Table II).

The induced primary leukemias had the following characteristics (Table I):

(*a*) failure to grow on transplantation to adult rats; this distinguishes them from spontaneous W/Fu leukemias, which grow invariably in W/Fu rats of all ages; (*b*) commonly, massive involvement of the thymus, which was not seen with the 13 spontaneous W/Fu leukemias we have observed; (*c*) failure to grow on transplantion to mice of the same strain that provided the inoculum of cells (in those instances where this was tested); and (*d*) progressive growth on transplantation to W/Fu rats less than 7 days old.

The leukemia used most extensively in these studies was W/Fu(C58NT)D. (The designation indicates a W/Fu rat leukemia induced by neonatal inoculation of C58 mouse normal thymus, "D" in the series.) It is identified as a rat leukemia not only by the transplantation criteria (Table I) but also by its lack of sensitivity to cytotoxic rat *anti*-mouse serum (*W/Fu anti-G- normal mouse tissue*) and by the karyotype, which was identified as rat by Dr. J. Biedler of this Institute.

The serum of rats with primary induced leukemias or transplants of these leukemias, or of adult rats that had resisted several inoculations of these leukemias, contained no HI polyoma antibody (Dr. B. A. Briody, New Jersey College of Medicine and Dentistry).

Production of Cytotoxic Antibody by Adult W/Fu Rats Immunized with Isogenic Induced W/Fu Leukemias Maintained in Passage in Newborn Hosts.—Several lines of induced leukemias were established by serial passage in newborn rats. Adult W/Fu rats, which rejected transplants of these leukemias, were further immunized by up to 12 intraperitoneal inoculations of 8×10^6 to 300×10^6 viable leukemia cells over periods of 3 to 8 months. These rats were found to develop antibody that was cytotoxic for cells of the leukemia used for immunization and for the cells of all other W/Fu leukemias induced by the same method, but not for W/Fu spontaneous leukemias or for normal cells from W/Fu thymus, spleen, or lymph nodes. In tests with a variety of mouse cells, normal and leukemic, it appeared that these sera were typing for G antigen. Representative cytotoxic tests are recorded in Table III.

Determination of the Specificity of the Cytotoxic Antibody in Immune Rat Serum by Absorption Tests.—For purposes of further identifying the specificity of the cytotoxic antibody, the serum *W/Fu anti-W/Fu(C58NT)D* (6 to 12 inoculations) was absorbed with the various tissues listed in Table IV, and the absorbed serum was tested for cytotoxic activity on W/Fu(C58NT)D or I cells, as described under Materials and Methods. The presence or absence of G antigen in the tissues selected for absorption was in most instances known from a previous study (4). (This is determined by absorption of the G typing serum *C57BL/6 anti-(AKR)K36* in tests with the transplanted C57BL/6 leukemia E ♂ G2 which was originally induced by Passage A Gross virus.) Table IV indicates that all G+ tissues, both leukemic and nonleukemic, removed activity from the immune rat serum. (Thymus cells of G+ strains, which absorb G antibody only very weakly in comparison with other lymphoid tissue (4),

TABLE III

Some Examples of Direct Cytotoxic Tests With the Serum of W/Fu Inbred Rats Immunized With Isogenic W/Fu Rat Leukemia Cells

Test Cells (transplant generation and antigenic type)*	Dilution of W/Fu anti-W/Fu(C58NT)D serum							
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
	Dead cells (stained by trypan blue)							
	%	%	%	%	%	%	%	%
Normal tissues								
Rat (W/Fu)								
Spleen (G−)								
Lymph nodes (G−)	<10	<10	<10	<10	<10			
Mouse (C57BL/6)								
Spleen (G−)								
Lymph nodes (G−)								
Mouse (AKR)								
Spleen (G+)	17	24	15	11	<10			
Lymph nodes (G+)	35	33	20	<10				
Leukemias								
Rat (W/Fu) spontaneous								
♀ SL1 (11: G−)	<10	<10	<10	<10				
♀ SL7 (50: G−)	<10	<10	<10	<10				
Rat (W/Fu) induced by mouse cells								
♂ (C58NT)D (57: G+)	>95	>95	>95	87	70	45	<10	
♀ (C58NT)I (10: G+)	>95	>95	>95	>95	>95	>95	53	<10
♀ (AKR-SL)F (1: G+)	>95	>95	>95	80	70	<10		
Mouse								
ERLD (95: G−, FMR−)	<10	<10	<10	<10				
E♀SL2 (48: G−, FMR−)	<10	<10	<10	<10				
EL4 (>100: G−, FMR−)	<10	<10	<10	<10				
E♀R2 (76: G−, FMR+)	<10	<10	<10	<10				
E♀G1 (52: G+, FMR−)	>95	>95	>95	>95	91	44	<10	
E♂G2 (57: G+, FMR−)	>95	>95	>95	>95	>95	93	47	<10

For abbreviations used in designating leukemias see footnote to Table II.

* G: G (Gross) leukemia antigen (4). FMR: antigen associated with leukemias induced by Friend, Moloney, and Rauscher viruses (14).

and so are recorded as G±, did not absorb activity from the rat serum.) In the case of G− tissues, however, a number of exceptions were noted. In several instances, leukemias that were repeatedly found to be G− in the mouse test system absorbed cytotoxic activity from the rat immune serum. Thus the rat

TABLE IV

Specificity of the Serum W/Fu Anti-W/Fu(C58NT)D Tested by Absorption With Various Tissues of Known Antigenic Type (G+ or G−)*

Cells used for absorption	Antigenic type‡ (transplant generation)	Result of absorption§ (transplant generation)
Normal tissue		
Spleen‖		
AKR, AKR.K and C58	G+	+
A, C57BL/6, BALB/c, I and C3H/An (mouse), W/Fu and BN (rat)	G−	−
Thymus‖		
AKR, AKR.K and C58	G±	−
A, C57BL/6, BALB/c and I (mouse), W/Fu and BN (rat)	G−	−
Rat leukemias		
Induced by inoculation of mouse cells or filtrates of AKR leukemia cells		
W/Fu(C58NT)D ascites (tested repeatedly)	G+	+
W/Fu(C58NT)I (tested repeatedly)	G+	+
W/Fu(C58NT)M	NT	+ (1)
W/Fu(AKR-D2-filtrate) No. 1	G+ (3)	+ (8)
W/Fu(AKR-SL)F	G+ (1)	+ (8)
W/Fu(AKR-U7-filtrate) No. 2	G+ (0)	NT
W/Fu(BALB-SL)D	G+ (5)	NT
BN(C58NT)1	NT	+ (2)
Spontaneous		
W/Fu ♀ SL1	G− (9)	− (11)
W/Fu ♀ SL4	G− (8)	NT
W/Fu ♀ SL5	G− (8)	− (9)
W/Fu ♀ SL7 (tested repeatedly)	G−	−
Mouse leukemias		
Spontaneous		
AKR ♂ SL	G+ (0)	+ (0)
AKR K36 ascites (tested repeatedly)	G+	+
AKR.K(H-2^a)SL3	G+ (3)	+ (10)
C58SL5	G+ (12)	+ (10)
DBA/2 ♀ SL1	G+ (44)	+ (54)
DBA/2 ♀ SL6	G− (0)	+ (4)
DBA/2 ♂ SL2	G− (30)	± (38) + (47)
DBA/2 ♀ SL4	G− (1)	− (3)
ASL1 (tested repeatedly)	G−	−
ASL6	G− (3)	− (11)
E ♀ SL2	G− (40)	− (46)
Radiation-induced		
E ♂ RL9	G+ (46)	+ (50)
E ♂ RAD '65S	G+ (3)	+ (7)

(*Table IV continued on following page*)

TABLE IV—*Concluded*

Cells used for absorption	Antigenic type‡ (transplant generation)	Result of absorption§ (transplant generation)
Radiation-induced, *cont'd*		
E♂RL4	G± (32)	+ (37)
RADA1	G− (121, 131)	+ (120, 131)
RADA2	G− (53, 79)	+ (79, 82)
E♂RAD '65A	G− (3)	± (2)
E♀RAD '65B	G− (1)	± (2)
E♀RAD '65D	G− (2)	± (2)
ERLD (tested repeatedly)	G−	−
E♀RL9	G− (27)	− (31)
E♀RAD '65C	G− (1)	− (1)
E♂RAD '65E	G− (2)	− (1)
E♀RAD '65J	G− (0)	− (0)
E♀RAD '65K	G− (0)	− (0)
E♀RAD '65L	G− (2)	− (1)
E♀RAD '65Q	G− (1)	− (1)
E♀RAD '65R	G− (1)	− (1)
EARAD1	G− (111)	− (126)
BALB♀RL1	G− (29)	− (32)
129♂RAD1	G− (2)	− (3)
Induced by inoculation of virus		
Gross Passage A		
C3H/Bi♂	G+ (0)	+ (0)
E♀G1	G+ (14)	+ (44)
E♂G2 (tested repeatedly)	G+	+
Rauscher		
E♀R2	G− (29, 52)	± (62, 65)
BALB/c	G± (0)	± (0)
Moloney		
E♂M2	G− (36)	+ (79)
Friend		
Swiss	G± (0)	± (0)
Chemically induced		
DBA/2♂DMBA4	G± (49)	+ (58)
DBA/2♀DMBA15	G+ (52)	+ (58)
DBA/2♀DMBA2	G± (23)	+ (36)
DBA/2♀DMBA6	G− (46)	+ (58)
DBA/2♀DMBA12	G± (53)	+ (63)
DBA/2♀ DMBA16	G− (37)	+ (42)
EL4 (tested repeatedly)	G− (>100)	− (>100)

For abbreviations used in designating leukemias see footnote to Table II.

* Antiserum diluted to two tubes below end point (doubling dilutions), absorbed with tissues listed, and titrated in cytotoxic test with W/Fu(C58NT)D or I cells.

‡ Determined by absorption of cytotoxic activity from the G typing serum (*C57BL/6 anti-(AKR)K36*) in tests with the C57BL/6 leukemia E♂G2 induced by Passage A Gross virus—see reference 4.

§ −, no absorption; ±, partial absorption; +, complete absorption; and NT, not tested.

‖ Separate absorption tests with each of the tissues listed.

system detects all G+ cells and also some cells that are G−, whereas the mouse G system detects no cells that are not detected in the rat system.

Thus it appears that not all the specificities indicated by the two systems are identical. All doubly positive cells must contain all specificities detected by both systems, but evidently some cells lack a determinant that is revealed when they are tested by absorption in the mouse typing system but not when tested by absorption in the rat typing system. The likely explanation of this difference is that at least two specificities are represented on the cells of leukemias induced by Gross virus—G(a), which is the component detected by absorption in the mouse system, and G(b), which is detected in the rat system. Leukemias induced by Passage A Gross virus, the lymphoid cells and leukemias of mice of G+ strains, and the leukemias induced in the rat by G+ mouse cells, have both antigens. Certain leukemias, such as the two radiation-induced strain A leukemias and some of the DBA/2 leukemias shown in Table IV, contain only G(b) and so are active in absorption in the rat system while typing G− in the mouse system. Thus the rat system extends the possibilities of analysis to a further cellular antigenic determinant that is associated with wild-type Gross virus, that was not revealed by the original analysis in the mouse (4), and that may point to an unsuspected connection between Gross virus and certain leukemias of the mouse whose etiology is uncertain.

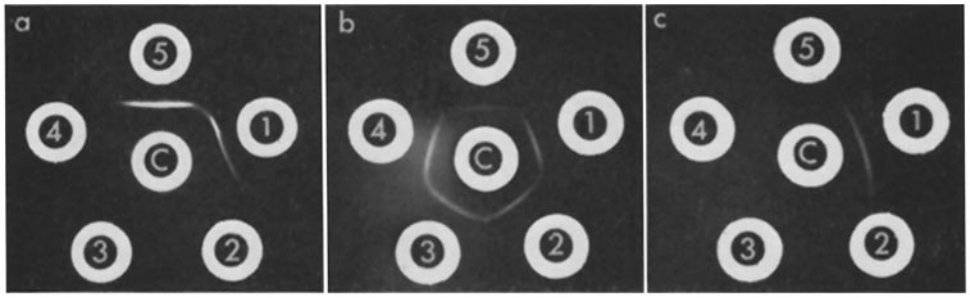

FIG. 1 *a*. Center (C), extract of G+ transplanted W/Fu leukemia W/Fu(C58NT)I.

Antisera: (1) *W/Fu anti-G+ leukemia W/Fu(C58NT)D;* (2) *W/Fu anti-G− normal mouse tissue;* (3) *C57BL/6 anti-(AKR)K36–mouse G typing serum* (reference 4); (4) *BN rat anti-G− spontaneous leukemia W/FuSL7;* and (5) *W/Fu anti-G+ leukemia W/Fu(AKR-SL)F.*

FIG. 1 *b*. Center (C), serum *W/Fu anti-G+ leukemia W/Fu(C58NT)D.*

Extracts: (1) G+ transplanted W/Fu leukemia W/Fu(C58NT)I; (2) G+ primary AKR leukemias (pooled); (3) G+ transplanted W/Fu leukemia W/Fu(AKR-SL)F; (4) G+ transplanted W/Fu leukemia W/Fu(C58NT)D; and (5) G+ transplanted C57BL/6 leukemia (induced by Passage A Gross virus) E♂G2.

FIG. 1 *c*. Center (C), Serum *W/Fu anti-G+ leukemia W/Fu(C58NT)D.*

Extracts: (1) G+ transplanted W/Fu leukemia W/Fu(C58NT)I; (2) G− transplanted C57BL/6 leukemia ERLD; (3) G− transplanted C57BL/6 leukemia EL4; (4) Two G− primary C57BL/6 radiation-induced leukemias (pooled); and (5) G− transplanted spontaneous W/Fu leukemia W/FuSL7.

Precipitating Antibody in the Serum of Adult Inbred W/Fu Rats Immunized with Isogenic Induced W/Fu Rat Leukemias.—Precipitating activity was first

detected in the sera of adult W/Fu rats that had received multiple inoculations of the isogenic leukemia W/Fu(C58NT)D and had provided the cytotoxic antisera described above. Fig. 1 *a* shows the reactions observed with 5 different antisera in an immunodiffusion test with antigen prepared from the induced rat leukemia W/Fu(C58NT)I. Precipitation occurred with the antisera *W/Fu anti-W/Fu(C58NT)D* and *W/Fu anti-W/Fu(AKR-SL)F*. A rat *iso*antiserum against a W/Fu spontaneous (G−) leukemia, *BN anti-W/Fu(SL)7*, and a W/Fu antiserum against G− normal mouse tissue, were negative. No precipitating activity was demonstrable in the mouse G typing serum *C57BL/6 anti-K35 (AKR leukemia)*.

Concentrated extracts for immunodiffusion tests (see Materials and Methods) were prepared from a variety of G+ and G− sources; a total of 127 extracts have been studied. The 77 G+ extracts comprised 34 prepared from the tissues or plasma of W/Fu or BN rats with leukemia induced by the inoculation of mouse cells, and 43 from mouse tissues or plasma (18 from AKR primary spontaneous leukemias and 12 from plasma or serum of these leukemic mice; 2 from lymphoid tissue of normal AKR mice; 2 from C58 primary leukemias and 1 from the serum of these leukemic mice; 5 from C3Hf/Bi leukemias induced by Passage A Gross virus and 3 from a transplanted C57BL leukemia induced by Passage A Gross virus, E ♂ G2). These 77 G+ extracts invariably gave positive reactions (and reactions of identity) in immunodiffusion tests with the precipitating rat antisera (see Fig. 1 *b*). The *unconcentrated* serum or plasma of primary leukemic animals may give positive reactions with precipitating rat antiserum; this is more common with the serum of leukemic rats than with the serum of leukemic mice.

The 50 concentrated G− extracts were prepared from different tissues of mice of G− strains (C57BL/6, DBA/2, C3Hf/Bi, C3H/An and I), and of BN and W/Fu rats. These included separate extracts of thymus, spleen, lymph nodes, kidney, liver, and brain of normal mice or rats, extracts of G− leukemias, and extracts of mammary tumors from mice of strains that carry MTV (Bittner virus). None gave a reaction with precipitating antisera; tests with 4 of these preparations are illustrated in Fig. 1 *c*.

There was no correlation between the cytotoxic and precipitating activities of the rat immune sera (Table V). Some precipitating sera had no cytotoxic activity and some cytotoxic sera had no demonstrable precipitating activity. Of the examples shown in Table V, the serum with the highest cytotoxic titer had no precipitating activity.

Reactions of Precipitating W/Fu Antisera with Intact and Ether-Treated Gross Virus (Fig. 2 a).—Seven different preparations of virus were made by resuspension of pellets obtained by centrifugation of plasma from *C3Hf/Bi* mice with primary leukemia induced by Passage A Gross virus (as described under Materials and Methods). No precipitation lines were observed when the fresh preparations were tested by immunodiffusion with the rat precipitating antisera.

However, faint lines were obtained with preparations that had been frozen and thawed. Samples of the same preparations were then treated with ether and were now found to give a strong precipitation line showing reactions of identity with G antigen prepared from both AKR spontaneous leukemia and from G+

TABLE V

Cytotoxic Titer and Precipitating Activity of Sera from Individual W/Fu Rats Immunized with the Isogenic Leukemia W/Fu(C58NT)D

W/Fu ♂ rat No.: (No. of inoculations)	Cytotoxic titer (reciprocal)*	Precipitating activity‡
3549 (12)	<2	+
3639 (11)	<2	−
3486 (9)	<2	+
3422 (11)	<2	+
3639 (9)	2	−
3547 (12)	4	−
3549 (8)	4	−
3486 (11)	4	−
3410 (11)	4	−
3420 (11)	4	−
3547 (8)	8	−
3485 (9)	8	−
3485 (11)	8	−
3545 (12)	8	+++
3548 (12)	8	++
3484 (11)	16	−
3440 (9)	16	−
3440 (11)	16	+
3430 (11)	16	−
3306 (11)	16	−
3484 (9)	32	−

* End point: 50% W/Fu(C58NT)D ascites cells stained by trypan blue; all sera inactivated at 56°C and all tested on the same day with the same suspension of W/Fu(C58NT)D cells.

‡ Ouchterlony technique; antigen prepared from W/Fu(C58NT)D leukemia cells (see text); all tests performed on same day with same preparation of antigen, and read at 48 hr. −, no precipitation; +, faint reaction; ++, strong reaction; +++, very strong reaction.

induced rat leukemias. Sediment obtained from the plasma of *normal* C3Hf/Bi mice by centrifugation at 100,000 *g* for 1 hr, resuspended and treated with ether as in the case of the pellets from leukemic C3Hf/Bi mice, gave no reaction. Precipitating activity was removed from positive sera by the addition of ether-treated virus, but not by the addition of equivalent quantities of untreated virus.

Reactions of W/Fu Precipitating G Antisera with Intact and Ether-Treated Friend, Moloney, Rauscher, and Bittner (Mammary Tumor) Viruses.—As with

Gross virus, no lines of precipitation occurred with pellets of *intact* virus prepared by centrifugation of plasma from mice with Friend or Rauscher leukemias or from W/Fu rats with primary Moloney leukemias. Intact virus (MTV) from the milk of RIII mice also gave no reaction. After treatment of the pellets with ether, however, lines of precipitation were observed with Friend,

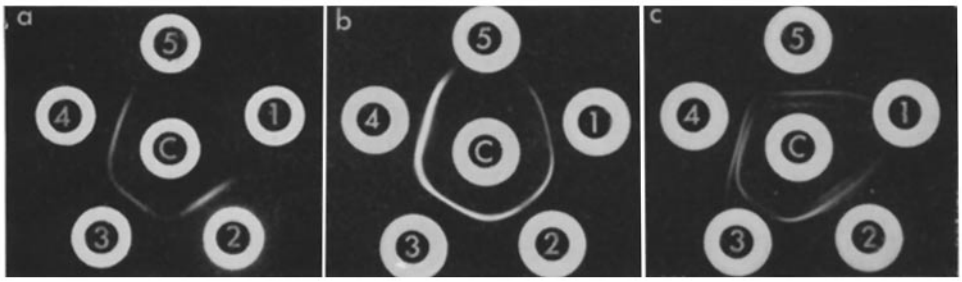

FIG. 2 *a*. Center (C), serum *W/Fu anti-G+ leukemia W/Fu(C58NT)D.*

(1) Freshly prepared untreated pellet from plasma of C3Hf/Bi mice with primary leukemia induced by Passage A Gross virus. (2) Same preparation after treatment with ether. (3) Extract of pooled primary G+ AKR leukemias. (4) Extract of G+ transplanted W/Fu leukemia W/Fu(C58NT)D. (5) Ether-treated pellet from plasma of normal C3Hf/Bi mice.

FIG. 2 *b*. Center (C), serum *W/Fu anti-G+ leukemia W/Fu(C58NT)D.*

Ether-treated pellet from: (1) plasma of C3Hf/Bi mice with primary leukemia induced by Passage A Gross virus; (2) plasma of DBA/2 mice with Friend virus leukemia; (3) plasma of W/Fu rats with primary Moloney virus leukemia; (4) plasma of BALB/c mice with Rauscher virus leukemia; and (5) MTV separated by density-gradient centrifugation from milk of MTV-infected mice (RIII strain).

FIG. 2 *c*. Center (C), serum of (W/Fu × BN)F_1 hybrid bearing large subcutaneous growth of G+ leukemia W/Fu(C58NT)D.

(1) Freshly prepared untreated pellet from plasma of primary G+ leukemia W/Fu(C58NST*)4. (2) Same preparation after treatment with ether. (3) Extract of transplanted G+ leukemia W/Fu(C58NST*)2. (4) Extract of transplanted G+ leukemia W/Fu (C58NT)D. (5) Concentrate of ascites fluid from the transplanted G+ W/Fu leukemia W/Fu(C58NST*)1.

* From a series of W/Fu leukemias induced by inoculation of C58 normal spleen and thymus cells (C58NST).

Moloney, and Rauscher viruses, but not with MTV. Fig. 2 *b* shows reactions of identity with W/Fu precipitating G antiserum and ether-treated pellets of Gross, Friend, Moloney, and Rauscher viruses.

Cytotoxic and Precipitating Antibodies in the Serum of Adult Rats Bearing Transplants of the Induced Leukemia W/Fu(C58NT)D.—Recently the induced W/Fu leukemia W/Fu(C58NT)D, that has been maintained by ascites passage in W/Fu hosts aged 10 to 15 days, acquired the ability to grow subcutaneously or intraperitoneally in adult W/Fu or W/Fu hybrid rats. Some transplants grow progressively and some ultimately regress. In both instances, but

particularly in the case of rats with progressively growing subcutaneous transplants, the serum contains very high levels of cytotoxic G antibody (titers ranging from 1/512 to 1/2048 against the C57BL/6 typing leukemia E ♂G2). In immunodiffusion tests with a variety of extracts from G+ sources these sera produce 5 distinct precipitin lines. Three of these are distinctly visible in Fig. 2 *c*. (Two other components were visible on this plate but cannot be seen in the figure.) The component that is common to preparations 2 to 5 in Fig. 2 *c* is the group-specific component of the leukemia viruses illustrated in Fig. 2 *b*. Fig. 2 *c* shows once again that ether-treatment is required to release this antigen from intact virus.

DISCUSSION

The rat is highly susceptible to mouse leukemia viruses, and the leukemias they induce are in general similar to those that occur in the mouse (see reference 15). The W/Fu rat has only a low incidence of spontaneous leukemias and in our hands procedures that are highly effective in inducing leukemia in mice—fractional X-radiation, percutaneous application of 9,10-dimethyl-1,2-benzanthracene, and injection of urethan during the neonatal period—do not raise this incidence appreciably. If these procedures are leukemogenic in the mouse because they activate latent virus, as is commonly believed, then their failure to be effective in W/Fu rats might be taken to indicate the absence of viruses of similar type. The absence of such virus, together with the considerable sensitivity of W/Fu rats to murine leukemia viruses, may suggest that they are favorable hosts for the isolation of leukemia viruses.[1]

Leukemias may be induced in rats not only by the inoculation of infective filtrates of mouse cells but also by the inoculation of viable cells. Kirsten and Platz (17) induced leukemias by inoculating cells of spontaneous leukemias of AKR mice, and those appearing 13 to 20 wk later were shown by transplantation tests to be composed of rat cells. We find that normal cells from the thymus of C58 mice, which carry Gross virus, are as effective as leukemia cells, the incidence of leukemia in W/Fu recipients being as high as 100% in some experiments. These primary leukemias occurring as a result of the inoculation of mouse leukemia cells or of mouse thymus cells presumably are induced by virus released from the inoculated cells before their rejection. The strong antigenicity of the induced leukemias, indicated by their failure to grow progressively in adult recipients,[2] is characteristic also of some leukemias induced in W/Fu rats by rat-adapted Passage A Gross virus (15). Antigenicity of this degree is not

[1] In this context, Huggins and Sugiyama recently demonstrated that a high incidence of leukemia can be induced in Long-Evans rats by multiple small intravenous doses of DMBA (16). The susceptibility of W/Fu rats to this procedure is not known.

[2] For unknown reasons, the leukemias induced by Kirsten and Platz were freely transplantable in adult recipients (17).

seen with leukemias induced by Gross virus in the mouse, whether they are induced by neonatal inoculation of Passage A Gross virus or arise spontaneously in mice of strains that carry Gross virus (the high-incidence G+ strains such as AKR and C58). This difference in behavior of leukemias induced by Gross virus in the rat and in the mouse may be ascribed to the fact that the rat is not the natural host of Gross virus; under natural conditions in the mouse it seems likely that a virus with minimal antigenicity has been selected, and that this is reflected in the induction of leukemias that are poorly antigenic.

Direct cytotoxic tests and absorption analysis with cytotoxic antisera prepared in adult rats by repeated inoculations of isogenic leukemia cells showed positive reactions with all cells previously typed G+ in the mouse G typing system (4). However, several G− leukemias absorbed cytotoxic activity from the rat immune serum. This points to the presence of more than one characteristic antigenic determinant on leukemia cells induced by Gross virus. The G− leukemias that are positive when tested with the rat immune system evidently lack one of the determinants detected by the mouse system and so fail to remove all cytotoxic activity from the mouse G typing serum. This determinant, however, is responsible for the major reaction in the rat cytotoxic system and consequently all cytotoxic activity is absorbed by these leukemias. According to this view the mouse G typing system recognizes predominantly the specificity G(a), whereas the rat typing system recognizes predominantly G(b). Although there is no certainty as to the nature of the G cellular antigens responsible for cytotoxicity it is probable that they are proteins of the viral coat that are incorporated in the cell membrane. The different specificities revealed by the use of mouse and rat antisera may thus be evidence of antigenic variants of Gross virus.

In addition to its cytotoxic activity, rat immune serum precipitates antigen prepared from G+ tissues. This antigen cannot be intact Gross virus, because it is prepared from material that had been centrifuged under conditions known to remove intact virus. Furthermore, Passage A Gross virus prepared from infective plasma does not react with precipitating antibody in immunodiffusion tests. The antigen is present in these preparations of virus, however, because treatment with ether yields an antigen which is precipitated by rat antiserum. As shown by reactions of identity, this antigen from ether-treated virus preparations is the same as that obtained from G+ cells. Precipitating antibody is absorbed by ether-treated preparations of virus, but not by equivalent quantities of untreated virus preparations. (This lessens the possibility that the failure of intact virus to react in immunodiffusion tests can be explained solely by its failure to penetrate the agar.) The conclusion is that the precipitating antibody is directed towards an internal component of Gross virus that is present also, in the free state, in cells infected with Gross virus. The same antigenic determinant occurs in ether-treated Friend, Moloney, and Rauscher viruses (but

not in mammary tumor virus); once again it is not demonstrable in untreated preparations of these leukemia viruses, but is released by treatment of these preparations with ether. The most direct explanation of the cross-reactions seen in immunodiffusion is that these viruses share an internal component comparable to the group antigens of other classes of virus.

Analysis of the surface antigens of the virus can be approached by the use of antisera in neutralization tests with the various leukemia viruses. For study of surface antigens of the virion the W/Fu rat antisera have a unique value because they are formed against isogenic W/Fu rat leukemia cells and so cannot contain antibodies against any normal constituents of the cell membrane that may be incorporated in the virion. Antisera prepared by repeated inoculation of isogenic leukemia cells into adult resistant rats have strong neutralizing activity against Passage A Gross virus;—in titrations of Passage A Gross virus in C3Hf/Bi newborn mice, pooled immune rat serum has completely neutralized the activity of a filtrate which had an ID_{50} (leukemia induction, observation period 240 days) of 3.5 log units. We are now studying the neutralizing activity of this antiserum against Friend, Moloney, and Rauscher viruses. Antisera of this sort had little or no neutralizing activity for Rauscher virus. However, the more recently available high-titer antisera obtained from rats with large growing transplants have appreciable neutralizing activity for Rauscher virus. These results are to be expected if these viruses are characterized by both shared and distinct surface antigens.

The leukemia viruses have been compared with myxoviruses, especially on the grounds of their similar modes of replication. It may be that further parallels can be drawn with regard to their antigenic structure. Myxovirus antigens include V antigen, which is present in the virus coat where the components responsible for hemagglutination and neuraminidase activity are situated, and the internal ribonucleoprotein g antigen. Both have their counterpart in the infected cell, which contains free s antigen, thought to be identical to viral g antigen, and also V antigen attached to the endoplasmic reticulum, in addition to virus forming at the cell surface (18). One of the main difficulties in effecting a comparable separation of components of the leukemia viruses is that these viruses lack readily demonstrable hemadsorption activity. Thus further description of the leukemia virus antigens will depend upon the morphological identification of antigenically distinct components separated by other methods.

The simplest formulation that accounts for the serological reactions of the rat antisera is that neutralizing and cytotoxic antibodies are reacting with the same viral antigens, which are situated in the coat of the virion and are incorporated in the surface membranes of infected cells; antibodies of different specificities are identified in immunodiffusion tests by precipitation with antigen which is present in soluble form in infected cells and which can be released by ether-treatment of the virion (Figs. 1 *a* to 1 *c* and 2 *a* to 2 *c*). However, there

are other findings that must be accommodated in any coherent account of the murine leukemia antigens.

1. The mouse G typing serum *C57BL/6 anti-(AKR)K36* is cytotoxic but has little neutralizing activity and no demonstrable precipitating activity. This may indicate that mice of G− strains are tolerant of some determinants, possibly as a result of endemic infection with related viruses, and so can be immunized against only those determinants that distinguish the inoculated virus or the inoculated histoincompatible leukemia cell from viruses indigenous in the strain.

2. The class of soluble antigens that is detected by adsorption to viable indicator cells (19) is divisible into FMR and G types (2), in parallel with the results of cytotoxic tests (14). This contrasts with the common antigen detected by immunodiffusion in ether-treated Gross, Friend, Moloney, and Rauscher viruses, and in leukemias induced by these viruses.

The former may be analogous to the V antigen of myxoviruses, displaying capacity to adsorb to cell surfaces and type-specificity. The antigen showing group-specificity in immunodiffusion may, as suggested above, be comparable with the s antigen (cellular g antigen) of myxoviruses.

3. Our recent experience with the serum of adult rats bearing growing transplants indicates that at least 5 antigens can be detected by immunodiffusion with concentrated extracts of G+ leukemias of mouse and rat. One of these is the antigen demonstrable with serum from rats immunized by repeated inoculations of isogenic leukemia cells and is the component found both in leukemia cells and in ether-treated preparations of the Gross, Friend, Moloney, and Rauscher viruses. Whether the other 4 are antigens of the virion or nonvirion antigens produced in the cell under the direction of the viral genome or the genome of the leukemia cell is not yet known.

SUMMARY

Leukemias can be induced in W/Fu inbred rats by neonatal inoculation of normal thymus cells of C58 mice. These leukemias are not transplantable to C58 mice or to adult W/Fu rats, but they can be kept in passage in W/Fu rats aged 0 to 7 days. Adult W/Fu rats inoculated repeatedly with these isogenic leukemias produce cytotoxic and precipitating antibodies. These antisera are of particular value in the analysis of the antigens of leukemia cells and of leukemia viruses because their mode of preparation precludes the formation of antibody against any normal constituents of the cell.

Analysis based on the cytotoxic test indicates the presence of 2 distinct cell surface antigens in leukemias induced by Passage A Gross virus or occurring spontaneously in mice of high-incidence strains. All leukemias and other tissues known to contain G (Gross) leukemia antigen have both determinants, but certain leukemias of low-incidence strains have only 1 of them and so were previously classified G−.

Immunoprecipitation with these antisera reveals the presence of a cellular antigen common to G+ cells and absent from G− cells; the same antigen can be demonstrated in ether-treated Gross virus, but not in intact virus. This antigen is present also in ether-treated preparations of the Friend, Moloney, and Rauscher leukemia viruses, but not in Bittner (mammary tumor) virus. Thus it may be regarded as a group-specific antigen of murine leukemia viruses, in contrast to the type-specific cellular antigens demonstrable by the cytotoxic test. Four additional antigens associated with leukemias induced by wild-type Gross virus have been demonstrated by immunoprecipitation, but their relation to viral and cellular antigens has not been determined.

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