A Study of Passive Immunization Against a Transplanted G+ Leukemia with Specific Antiserum.* (31667)

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Cytotoxic antibodies directed against leukemia-specific antigens can be prepared in mice and the use of such antisera has led to the description of 5 distinct antigenic systems associated with murine leukemias(1-review). These are: 1) G (Gross) antigen associated with leukemias induced by Gross virus, either naturally in mice of high-incidence strains or in other strains by inoculation of Passage A Gross virus(2,3)—this antigen is present also in the lymphoid tissue of strains with a high incidence of leukemia, which are therefore referred to as G+ strains; 2) FMR (Friend-Moloney-Rauscher) antigen, associated with leukemias induced by these viruses (4,5,6,7); 3) ML (Mammary Leukemia) antigen, found in leukemias of only one strain, DBA/2, but in the mammary tissue and mammary tumors of mice infected with the mammary tumor virus(8); 4) TL (Thymus Leukemia) antigen, the only leukemia specific antigen known to originate from a normally unexpressed gene of the leukemia cell(9); and 5) E antigen, found in spontaneous leukemias of the low-incidence C57BL/6 strain(1).

Recently we have been able to prepare antisera in the rat with very high titers against antigens of the G system(10). These antisera were prepared in W/Fu inbred or W/Fu F_1 hybrid rats immunized against histocompatible W/Fu leukemias induced by wild-type Gross virus from C58 mice. They contain in high titer 1) cytotoxic antibody active against G+ cells, 2) neutralizing antibody to Passage A Gross virus, and 3) precipitating antibody to the group-specific antigen of murine leukemia viruses. The fourth property of these antisera, which we report here, is their ability to protect mice against transplants of a histocompatible G+ leukemia.

Materials and methods. Animals. Inbred mice and inbred rats of the W/Fu and BN (Brown Norway) strains, were obtained from our own colonies.

Antiserum. This was obtained from (W/Fu $\times$ BN)F_1 hybrid rats inoculated with the W/Fu rat leukemia (C58NT)D(10). This leukemia was originally induced in a W/Fu male by neonatal inoculation of normal thymus cells from the C58 strain, a G+ strain with a high incidence of spontaneous leukemia. A characteristic of W/Fu leukemias induced by wild-type Gross virus in this manner is their strong antigenicity, making it necessary to pass them initially in immunologically immature hosts. After its 54th passage leukemia W/Fu(C58NT)D acquired the capacity to grow progressively in adult W/Fu or W/Fu hybrid hosts, if the inoculum of cells was sufficiently large. For the production of antisera, 5×10^7 viable leukemia cells (unstained by trypan blue) were inoculated subcutaneously into adult (W/Fu $\times$ BN)F_1 males. In a proportion of rats the leukemia grows to only a small size (average diameter 1 cm or less) and then regresses; serum from such rats has only low levels of cytotoxic activity. In the majority, the leukemia grows locally to a massive size, often with equally massive involvement of the regional lymph nodes, the animals dying with little evidence of generalized disease. The serum used in the experiments described here was a pool obtained from rats with growing tumors larger than 2 cm in average diameter. Blood was collected from the tail, twice weekly under ether anesthesia. A pool of non-immune rat serum was obtained from normal W/Fu, BN and (W/Fu $\times$ BN)F_1 hybrid rats of comparable age. Serum used for passive immunization was not heat-inactivated. Serum was stored at -70°C.

Cytotoxic test(11). Serial dilutions of heat-inactivated rat serum were incubated with viable leukemia cells (5×10^6/ml) and

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TABLE I. Cytotoxic Titer of Pooled Antiserum *(W/Fu × BN)F₁ anti W/Fu leukemia (C58NT)D* Against Normal Lymphocytes and Leukemia Cells.

Strain	Test cells: Designation (transplant generation)	Mode of induction of leukemia	G (Gross) antigen type*	Titer (reciprocal)
C57BL/6	Normal lymphocytes	—	G—	<2
	E♀SL1 (54)	Spontaneous	G—	<2
	EL4 (>500)	DMBA	G—	<2
	ERLD (101)	X-radiation	G—	<2
	E♂RL9 (20)	"	G+	512
	E♂G2 (79)	Passage A Gross virus	G+	2048
	E♀R2 (103)	Rauscher virus	G—	<2
	E♂M2 (110)	Moloney "	G±	16
BALB/c	Normal lymphocytes	—	G—	<2
	BALB♂RL2 (77)	X-radiation	G+	512
	BALB♀RL10 (7)	"	G—	<2
	BALB♂E4 (6)	Estradiol	G+	1024
AKR	Normal lymphocytes	—	G+	<2
	K36♀ (234)	Spontaneous	G+	32
	SL1♀ (primary)	"	G+	2048
	SL2♀ "	"	G+	1024
	SL3♀ "	"	G+	128
	SL4♀ "	"	G+	4096
C58	Normal lymphocytes	—	G+	<2
DBA/2	" "	—	G—	<2
A	" "	—	G—	<2

* Determined in previous studies(3,10) by absorption of cytotoxic activity from the G typing serum [*C57BL/6 anti (AKR)K36*] in tests with the C57BL/6 typing leukemia E♂G2 induced by Passage A Gross virus.

guinea pig serum diluted 1/3 (source of complement). The volume of each of these 3 constituents was 0.05 ml. After incubation of the cells at 37°C for 45 minutes, viability was assessed by adding trypan blue. The titer of the serum is taken as that dilution of serum in which the proportion of dead cells approximated 50%. The method is described in detail elsewhere(12).

Preparation of cell suspensions for cytotoxic tests and for inoculation into mice. Normal lymphoid cells were prepared by gently mincing the lymph nodes of male or female mice aged 6-10 weeks. Cells were similarly obtained from spontaneous leukemias of AKR mice and from transplanted leukemias maintained in this laboratory (origins given elsewhere)(3,10). All cells were washed twice before use. For inoculation IV or IP, leukemia cells were prepared from the spleen and the concentration of cells was adjusted to contain in 0.2 ml the number of cells specified in the text.

Results. Titration of cytotoxic antibody. Table I shows the results of cytotoxic tests with the pool of antiserum from (W/Fu × BN)F_1 rats bearing growing transplants of the W/Fu leukemia (C58NT)D. Positive reactions were obtained only with cells known from previous work to contain G (Gross) antigen (G+ cells)(3,10). Particularly high titers were seen with AKR spontaneous leukemias and with certain induced leukemias of strains with a low incidence of spontaneous leukemia. The latter finding accords with previous experience that G+ leukemias often occur in mice of strains that show no evidence of carrying Gross virus (G— strains)(3). All normal lymph node cells tested were negative, although those from G+ strains, such as AKR and C58, are known to be G+(3,13). However, this lack of sensitivity of G+ lymphocytes reflects the relatively low concentration of G antigen on these cells as compared with leukemia cells; the presence of G antigen in this case is shown by absorption tests in which AKR lymph node cells removed cytotoxic activity from rat G antisera whereas G— lymph node cells do not.

Passive immunization. The G+ leukemia

TABLE II. Effect of Antiserum *(W/Fu × BN)F, anti W/Fu leukemia (C58NT)D* on the G+ C57BL/6 Leukemia E♂G2 (Induced by Passage A Gross Virus) and the G— C57BL/6 Leukemia ERLD (Induced by X-Radiation) Inoculated IP into C57BL/6 Hosts.

Leukemia cells	Serum*	Survivors/total	Survival time (days)
E♂G2 (G+)	None	0/5	13, 13, 13, 15, 15
(10^5 cells IP)	Non-immune	0/5	15, 16, 16, 16, 19
	Immune	4/4	
ELRD (G—)	None	0/5	16, 16, 16, 17, 17
(10^4 cells IP)	Non-immune	0/5	16, 16, 16, 17, 17
	Immune	0/5	15, 16, 16, 19, 19

* Each treated mouse received 1 ml at the following times after inoculation of leukemia cells: IV at 27 hr, IP at 31 hr, IP at 48 hr, (total 3 ml).

selected for these experiments, E♂G2 (ref 3), was originally induced in a C57BL/6 mouse of our colony by neonatal inoculation of Passage A Gross virus and was in its 62nd to 80th transplant generation at the time of these experiments. The G— leukemia ERLD (ref 14) selected as a control for specificity also was induced in a C57BL/6 mouse of our colony, and was in its 101st to 106th transplant generation at the time of these experiments.

In the first experiment, 20 C57BL/6 male mice were inoculated with 10^6 E♂G2 cells IP (day 0). Five of these mice were not treated. Their average survival time was 13.8 days (range 12-15). Five others received non-immune rat serum, starting with 1 ml IV on day 5, followed by 0.5 ml IP daily subsequently until the time of death from leukemia. Their average survival time was 12 days (range 10-16). The remaining 10 mice received immune serum according to the same schedule until they were obviously leukemic (the total dose ranged from 7.5 ml to 11.5 ml per mouse). The average survival time of mice in this group was 28 days (range 27-31). Serum was obtained from each mouse of the last group and titrated individually by the cytotoxic test with E♂G2 cells on days 13 and 19, at which time the mice showed no evidence of leukemia. All mice had G antibody in the serum, the titers ranging from 1/128 to 1/512. On day 23, however, when signs of leukemia had developed, free antibody was no longer demonstrable even in those mice that had received antibody the day before. Another group of 20 C57BL/6 male mice was inoculated with 10^6 ERLD (G—) cells IP, and was divided into 3 groups that received precisely the same treatment as outlined above for E♂G2. The 2 groups treated with serum received a total of 5.5 ml. Average survival times were as follows: controls (no serum), 15.5 days; treated with non-immune rat serum, 15.8 days; treated with immune serum, 14.6 days. Thus the results *in vivo* paralleled the data obtained in the cytotoxic test *in vitro,* mice with the G+ leukemia showing a very marked increase in survival time as a result of treatment with immune serum, mice with the G— leukemia showing no response to treatment.

Complete protection can be achieved when the initial inoculum of leukemia cells is decreased and treatment started at an earlier time. Table II shows that C57BL/6 male mice inoculated with 10^5 E♂G2 cells IP can be completely protected by immune serum given for the first time 27 hours after inoculation of the leukemia cells. Once again, mice bearing the G— leukemia ERLD and receiving the same treatment showed no response.

As little as 0.1 ml of the antiserum produced complete suppression of E♂G2 when administered IP 4 hours after the mice had received 10^4 cells IV (Table III). Increased survival time was seen with doses of antiserum as low as 0.0125 ml.

After the inoculation of 10^4 E♂G2 IV, a single injection of 0.5 ml of immune serum IP produced complete suppression in 2/5 treated mice even when treatment was delayed for 3 days (Table IV).

In none of these experiments did treatment with immune serum or non-immune serum cause loss of weight and protected mice remained in good health.

Protected survivors from these experi-

TABLE III. Passive Immunization of C57BL/6 Mice Against Intravenous Transplants (10^4 Cells) of the G+ C57BL/6 Leukemia E♂G2 by Antiserum *(W/Fu × BN)F, anti W/Fu leukemia (C58NT)D* Administered (IP) 4 Hours After Inoculation of the Leukemia Cells.

Serum (IP)	Volume (ml)	Survivors/ total	Survival time (days)
None	—	0/11	11, 13, 14, 15, 16, 17, 17, 17, 17, 18, 18
Non-immune	1.6	0/6	11, 15, 16, 17, 18, 18
"	.4	0/5	15, 16, 16, 16, 18
Immune	1.6	4/4	
"	.8	4/4	
"	.4	7/8	42
"	.2	7/8	32
"	.1*	4/4	
"	.05*	0/4	16, 20, 22, 30
"	.025*	0/4	20, 21, 22, 22
"	.0125*	0/4	15, 20, 21, 22

* Brought to a volume of 0.2 ml by addition of saline.

ments were rechallenged with 10^3 E♂G2 cells IV 75 to 100 days after the initial inoculation. In all cases these mice died of leukemia with no significant delay in comparison with controls. Thus there was no indication that active immunization had occurred.

Discussion. For a variety of reasons it has previously been very difficult to assess the value of specific passive immunization as a means of suppressing tumors or leukemias, primary or transplanted. Antisera prepared by immunizing foreign species with tumors or leukemias contain antibodies to normal tissue constituents and so are often highly toxic to the recipient. Furthermore, it has never been satisfactorily demonstrated that sera used in studies of passive immunization in fact contain antibodies specific for the tumor or leukemia used for immunization. Antisera prepared within the same species by immunization with allogeneic tumor or leukemia cells (isoantisera) have not been investigated extensively from the standpoint of passive immunization. The presence of antibodies to components of normal tissues is a complicating factor here also, and it may be that protection against the growth of inoculated leukemia cells is produced by isoantibodies that are not efficiently absorbed by the normal tissues of the host. These problems are discussed more fully elsewhere(15).

The ideal sera for passive immunization against tumors or leukemias are those that are produced under conditions where isoantibodies and heteroantibodies cannot be formed. Such antisera can be obtained from inbred mice that have rejected transplants of highly antigenic leukemias induced by Rauscher, Moloney or Graffi viruses in mice of the same inbred strain(6,7,16). Klein and Klein showed that administration of antiserum of this type can protect against the subsequent inoculation of Moloney leukemia cells(6). The rat antiserum used in the experiments described here belongs to this category of antisera but differs from any previously described in having extremely high titers of specific cytotoxic antibody. This is undoubtedly a consequence of the strong antigenicity of leukemias induced in the rat by wild-type Gross virus. The specificity of the rat antiserum is best illustrated by cytotoxic tests with cells of leukemias induced by different methods in mice of the same inbred strain, G+ cells being highly susceptible and G— cells unaffected. This correlation extends, in the case of the 2 leukemias tested, to the capacity of the antiserum to convey passive immunity. Mice with the G+ leukemia E♂G2 were completely protected under vari-

TABLE IV. Passive Immunization of C57BL/6 Mice Against Intravenous Transplants of the G+ C57BL/6 Leukemia E♂G2 (10^4 Cells) by 0.5 ml Antiserum *(W/Fu × BN)F$_1$ anti W/Fu leukemia (C58NT)D* Administered (IP) at Various Times After Inoculation of the Leukemia Cells.

Interval between inoculation of leukemia cells and inj of serum	Serum (IP)	Survivors/ total	Survival time (days)
—	None	0/9	13, 13, 13, 14, 14, 15, 15, 16, 16
4 hr	Non-immune	0/4	12, 14, 16, 16
"	Immune	5/5	
24 hr	Non-immune	0/4	13, 15, 16, 16
"	Immune	4/5	26
3 days	Non-immune	0/4	14, 15, 15, 16
"	Immune	2/5	22, 23, 24
5 days	Non-immune	0/4	15, 15, 15, 16
"	Immune	0/5	17, 22, 22, 22, 25

ous conditions of testing, whereas mice with the G— leukemia ERLD were not protected.

The protection afforded by passive immunization with the rat antiserum is impressive, particularly when it is considered that some mice are protected when treatment is delayed as long as 3 days after intravenous inoculation of the leukemia cells. Nevertheless, there is a definite limit to the protection that can be achieved by passive immunization. In the first experiment described, in which daily treatment was initiated 5 days after inoculation of E♂G2, complete protection was not achieved even though free antibody was demonstrable in the serum up to the 20th day. Thus beyond a certain stage of the disease it is not simply the availability of serum antibody that determines the outcome.

Several factors are worth consideration in connection with the effectiveness of passive immunization as a means of controlling established leukemia, when a specific antiserum of the sort used in this study is available. These relate to: 1) *The antiserum.* Its titer will determine the volume that need be given and if the volume is excessive separation of globulins may be necessary. Immunoglobulins of different classes have different biological properties; γ_1 antibody of the guinea pig, for example, does not fix complement and is not cytotoxic(17). The relative proportions of the various immunoglobulins in a given antiserum, therefore, may influence its activity *in vivo*. With regard to the possibility of immunological enhancement by antiserum (18) this has been shown only with histoincompatible leukemias(19,20). Immunological enhancement cannot occur unless the leukemia is capable of actively immunizing the host in which it is growing. Therefore it need not be considered as a complication of passive immunization unless there is evidence that a particular leukemia is immunogenic. 2) *Extent of disease.* This is a question of the total number of leukemia cells and also of their accessibility to antibody. Cerebral deposits clearly are relatively inaccessible in comparison with leukemia cells in the blood. Treatment with chemotherapeutic agents would reduce the total number of leukemia cells and render passive immunization more effective. 3) *Disposition of antigen.* In some instances, notably in mice of G+ strains such as AKR, G antigen is present not only in leukemia cells but also (in lower concentration) in infected but non-leukemic cells throughout the lymphoid system. Competition for the antibody lowers the effectiveness of antiserum under these conditions; in fact we have observed that the rat antibody is clearly much faster in non-leukemic AKR (G+) mice than in C57BL (G—) mice. This would probably be the major adverse factor in passive immunization of mice with primary leukemias induced by virus that was congenitally acquired and so induced immunological tolerance and antigenic conversion(21) of normal lymphoid tissue. However, this may not be insuperable, as there is a considerable quantitative difference in the amount of antigen in G+ leukemia cells as compared with G+ non-leukemic lymphoid cells (as discussed elsewhere in this report). 4) *Degree of immunogenicity of leukemia cells.* Murine leukemias differ markedly in their immunogenicity according to the particular virus by which they are induced. Leukemias induced by the Friend, Moloney, Rauscher and Graffi viruses are in general highly immunogenic (see 1) and it may be anticipated that in these instances active immunization would contribute to the effects of passive immunization. Leukemias induced by Gross virus in the mouse, either naturally or by inoculation of Passage A virus, on the other hand, are only weakly antigenic and in most instances arise in tolerant hosts. No contribution from active immunization seems possible here. 5) *Availability of complement.* Complement is required for lysis of cells by antibody and for antibody-dependent phagocytosis of nucleated cells(22). Availability of complement may be particularly critical in the mouse, where complement activity is low and at least one component may be lacking (23,24). Little is known about raising the level of complement either actively or passively. However, we have observed in unpublished experiments that progressive growth of H-2-incompatible ascites sarcomas may occur in highly immunized mice despite the fact that the tumor cells are sensitized by iso-

antibody; injection of guinea pig serum to provide additional complement can bring about rapid and complete rejection of these otherwise fatal transplants. Thus exhaustion of serum components other than specific antibody may limit the success of passive immunization. This is a likely explanation of the finding that mice treated relatively late were not cured despite the presence of free serum antibody in these animals. 6) *Occurrence of immunoresistant cells.* The clearest example of this is found with TL+ leukemias where growth in actively or passively immunized mice invariably leads to disappearance of the antigen (antigenic modulation) (9,25). This is not the explanation for the outgrowth of E♂G2 in mice treated relatively late, as these cells have been found to retain their sensitivity to cytotoxic antibody *in vitro.*

Summary. Antiserum with high titers of cytotoxic antibody against G+ leukemia cells can be prepared in inbred rats by immunization with histocompatible rat leukemias induced in the same inbred strain by wild-type Gross virus. Under these conditions heteroantibody and isoantibody to normal tissue antigens are not formed. Passive immunization with this antiserum was successful against a G+ transplanted mouse leukemia but not against a G— mouse leukemia of the same inbred strain. These results conform to the specificity of the antiserum for G+ cells, as shown by the cytotoxic test *in vitro.* Complete protection can be achieved in some mice by serum given as late as 3 days after intravenous inoculation of leukemia cells. Protected mice are not immune to rechallenge with minimal numbers of G+ leukemia cells after the transferred G antibody has been cleared.

1. Old, L. J., Boyse, E. A., Fed. Proc., 1965, v24, 1009.

2. Slettenmark-Wahren, B., Klein, E., Cancer Res., 1962, v22, 947.

3. Old, L. J., Boyse, E. A., Stockert, E., ibid., 1965, v25, 813.

4. Old, L. J., Boyse, E. A., Lilly, F., ibid., 1963, v23, 1063.

5. Wahren, B., J. Nat. Cancer Inst., 1963, v31, 411.

6. Klein, E., Klein, G., ibid., 1964, v32, 547.

7. Old, L. J., Boyse, E. A., Stockert, E., Nature, 1964, v201, 777.

8. Stück, B., Boyse, E. A., Old, L. J., Carswell, E. A., ibid., 1964, v203, 1033.

9. Boyse, E. A., Old, L. J., Stockert, E., Immunopathology, IVth Int. Symp., Schwabe & Co., Basel, 1965.

10. Geering, G., Old, L. J., Boyse, E. A., J. Exp. Med., 1966, v124, 753.

11. Gorer, P. A., O'Gorman, P., Transplant. Bull., 1956, v3, 142.

12. Boyse, E. A., Old, L. J., Stockert, E., Ann. N. Y. Acad. Sci., 1962, v99, 574.

13. Wahren, B., Exp. Cell Res., 1966, v42, 230.

14. Old, L. J., Boyse, E. A., Stockert, E., J. Nat. Cancer Inst., 1963, v31, 977.

15. Old, L. J., Boyse, E. A., Ann. Rev. Med. 1964, v15, 167.

16. Pasternak, G., Graffi, A., Brit. J. Cancer, 1963, v17, 532.

17. Bloch, K. J., Fed. Proc., 1965, v24, 1030.

18. Kaliss, N., Cancer Res., 1958, v18, 992.

19. Boyse, E. A., Old, L. J., Stockert, E., Nature, 1962, v194, 1142.

20. Möller, G., J. Nat. Cancer Inst., 1963, v30, 1177.

21. Stück, B., Old, L. J., Boyse, E. A., Nature, 1964, v202, 1016.

22. Bennett, B., Old, L. J., Boyse, E. A., Transplantation, 1964, v2, 183.

23. Cinader, B., Dubiski, S., Wardlaw, A. C., J. Exp. Med., 1964, v120, 897.

24. Erickson, R. P., Tachibana, D. K., Herzenberg, L. A., Rosenberg, L. T., J. Immunol., 1964, v92, 611.

25. Boyse, E. A., Stockert, E., Old, L. J., Nature, in press.

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