THE AKR THYMIC ANTIGEN AND ITS DISTRIBUTION IN LEUKEMIAS AND NERVOUS TISSUES*

By ARNOLD E. REIF, D.Sc., AND JOAN M. V. ALLEN

(From the Biochemistry Section, Department of Surgery, Tufts University School of Medicine, Boston City Hospital, Boston)

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Gorer and Amos reported presence of the "X" antigen in certain mouse leukemias (1). On further study, three distinct X antigens, which appeared to be present specifically in C57BL leukemias, were found (2). Slettenmark and Klein showed that mice can develop specific cytolytic isoantibodies against isologous Gross virus leukemia cells; such isoantibodies could not be obtained by immunization with normal lymph node cells (3). None of these workers related the specificities shown to specificity against thymus.

Old, Boyse, and Stockert reported that isoantisera prepared in C57BL/6 mice against radiation-induced leukemias of strain A origin were cytolytic for certain leukemias of strain A and strain C57BL/6 origin. The cytolytic activity was absorbed by thymus of strain A or C58 origin. These data indicated presence of a common antigen, the LT antigen, in the tissues mentioned. The LT antigen was not found in any normal tissue other than thymus in A mice, nor in *any* normal tissue in C57BL/6 mice. Nor was it present in thymus or leukemias of AKR and certain other strains of mice (4).

Following a single observation of substantial cytolysis of mouse thymic lymphocytes by Gorer and Boyse (5), we investigated a system for immune cytolysis of mouse thymic lymphocytes (6–8). In this system, an isoantiserum prepared in C3HeB/Fe mice against AKR thymic lymphocytes showed strong cytolytic potencies against the latter cells, despite compatibility in H-2 allele (7). It was concluded that one or more strong antigens are present on the surface of AKR thymic lymphocytes (9). The present report extends our previous findings (7, 9). A preliminary report has been made (10).

Materials and Methods

Mouse Strains and Isoantisera.—Unless stated otherwise, all work was done with inbred mice of either sex, 5 to 12 weeks old, obtained from the Jackson Laboratory. Tissues from AKR mice were injected into C3HeB/Fe mice that possess the same allele H-2^k or H-$2^{k'}$, or into C57BL/6 mice that possess the allele H-2^b. A group of 9 to 27 mice was used for preparation of each isoantiserum.

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In general, six injections spaced at weekly intervals were given. The following quantities of AKR tissues were injected intraperitoneally each time: for thymocytes and marrow, 10 million viable cells; for red blood cells, 0.4 ml of a 30 per cent suspension; for spleen, lymph nodes, and liver, 0.4 ml of a 30 per cent homogenate. For the solid tumors BW5147, S775, and T283, 0.3 ml of a tissue mince was injected subcutaneously.

L4946 leukemia will take when injected intraperitoneally at high doses into C3HeB/Fe mice (9). Therefore 100,000 viable L4946 cells were injected intraperitoneally, followed 3 weeks later by 10 million cells aged for 1 week at 3°C, and 2 weeks later by 10 million viable cells. C57BL/6 mice, which reject L4946 leukemia (9), received 6 weekly intraperitoneal injections of 10 million viable cells. For red cells, thymocytes, spleen, and tumors BW5147, S775, and T283, an additional subcutaneous injection of tissue incorporated in Freund-McDermott adjuvant (11, 12) was given initially. Blood was obtained by cardiac puncture 10 days after the

TABLE I

Data on AKR Leukemias Used in This Study

Designation	Worker or laboratory	Date of first transplant	AKR tissue used for transplant	Transplant generation March 1, 1964	Approximate mean time to death*	Tissue of origin determined in this study
					days	
L4946	L.W.L.	1951	—	+200	11	Non-thymic
BW5147	Jackson	1954	Spleen and nodes	+200	12	Thymic
S775	G.D.S.	Feb. 15, 1952	—	+200	14	Thymic
RA1	Tufts	Sept. 20, 1963	Spleen	10	18	Non-thymic
RA2	Tufts	Oct. 9, 1963	Marrow	Lost	14	Thymic
RA3	Tufts	Nov. 5, 1963	Thymus	11	11	Thymic
RA4	Tufts	Nov. 12, 1963	Marrow	6	18	Non-thymic
RA5	Tufts	Dec. 9, 1963	Thymus	6	25	Thymic

* Peritoneal cavity of tumor-bearing mouse washed out with 2 ml sterile saline. Then 0.2 ml aliquots of fluid injected intraperitoneally into AKR mice.

last injection. For each group of mice, the sera were pooled, inactivated for 30 minutes at 56°C, and stored at −20°C without addition of preservative.

Tumors.—Eight leukemias (Table I) and osteogenic sarcoma T283 were used; all these tumors originated spontaneously in AKR mice. The leukemias BW5147 and S775 (the latter provided through the courtesy of Dr. G. D. Snell) were converted to ascites form in our laboratory. Sarcoma T283 resisted this conversion.

The five newly derived ascites tumors (Table I) were obtained by the transplantation of cells from spontaneously leukemic AKR mice 7 to 12 months of age. Sterile isotonic saline that contained 100 I.U. penicillin G and 100 μg streptomycin per ml was used to prepare minces or single cell suspensions (see below) of leukemic tissues for intraperitoneal injection into AKR mice. Tumor takes, certified by biological and histological evidence, were obtained in all transplants from leukemic mice.

Cell Suspensions.—For use in immunizations and cytolysis, suspensions of red blood cells (13), thymic lymphocytes (8), and tumor cells (12) of mice were prepared as previously described. Cells were kept at 0–5°C unless otherwise specified. In cytolysis and in absorption studies, cells were used within 2 hours after preparation.

Spleens were pressed through a 60-mesh stainless steel screen into modified Locke's solution (12), and a single cell suspension was prepared by the method of Möller (14). Intraperitoneal lymphocytes were washed from the peritoneal cavity with the same buffer, centrifuged at 5°C for 10 minutes at 90 *g*, and resuspended in fresh buffer. Lymph nodes were suspended in buffer and teased with needles; the lymphocytes that resulted were filtered through an 80-mesh stainless steel screen.

Bone marrow cells were expelled from femurs into modified Locke's solution. Cell clumps were disrupted by suction into a hypodermic syringe. The suspension was centrifuged at 5°C for 10 minutes at 135 *g*, redispersed in fresh buffer, and filtered through an 80-mesh stainless steel screen.

Complement.—Fresh guinea pig serum was absorbed twice for 30 minutes at 3°C with 1/20 volume of packed AKR erythrocytes. The serum was stored at −40°C in sealed 1.5 ml pyrex ampoules. Fresh hamster serum was absorbed twice with 1/20 volume of AKR erythrocytes and three times with 1/10 volume of packed AKR liver homogenate and stored frozen in like manner (8).

Cytolysis Assay System.—The cytolytic potency of isoantisera was determined in the small-scale assay system previously described in detail (12). The only change was use of 100,000 cells per assay tube; also, an incubation period of 1 hour rather than 1½ hours at 37°C was used for all cells other than the three long-transplanted leukemias. For cytolysis of thymocytes, absorbed hamster serum rather than absorbed guinea pig serum was employed as complement (8). Cytolytic titers were based on the stained cell count rather than on the deformed cell count, since thereby a closer proportionality between cytolytic titer and cell concentration in the assay system was obtained (12).

The results for each isoantiserum were plotted to give the cytolytic titer, defined as the final concentration of antiserum (per cent) that caused 50 per cent staining of intact cells present in the control tube. Factors were determined (see Results) to convert titers obtained in the small-scale assay system to the basis of the regular assay system, in which the cell concentration was 5 million cells/ml (8, 12). The potency of each isoantiserum was expressed at 100 divided by the cytolytic titer obtained in the regular assay system (13).

No isoantiserum was tested at a final concentration above 17 per cent. A titer obtained at this level in the small-scale assay system corresponded to a titer of 3.6 × 17 or 61 per cent in the regular assay system, since the latter was less sensitive by a factor of 3.6 (mean value obtained for different cell types, see Results). Thus, depending on the cell types assayed, the minimum cytolytic potency that could be quantified was approximately 100/61 or 1.6.

A potency of 1000 corresponded to a final isoantiserum concentration of 0.1 per cent in the regular assay system, or 0.03 per cent in the small-scale assay system (Fig. 1, top). Titers were generally sharp and had standard deviations of 20 to 30 per cent when determined in different experiments; deviations were lower when the relative potencies of several antisera were determined in a single experiment (8, 11, 12). Potencies below approximately 7 were subject to error because of the occasional presence of prozones (Fig. 1, bottom), and have therefore far less quantitative validity.

If an isoantiserum caused between 40 and 50 per cent cell staining and no prozone was evident, the cytolytic curve (Fig. 1, top) was extrapolated to 50 per cent and a titer was recorded. If staining was between 10 and 40 per cent or if a prozone was evident, the record shows the highest percentage of stained cells above control (per cent a.c.) obtained at any dilution that was tested. Stained cell counts under 10 per cent were not considered significant (compare with reference 15), and the potency of the isoantiserum is stated to lie below the minimum that could be quantified (<2).

When two different types of cells could be distinguished in the cytolytic system (Fig. 2, top), the titer was read as the final concentration of antiserum that caused 50 per cent staining of the intact cells of a specified type.

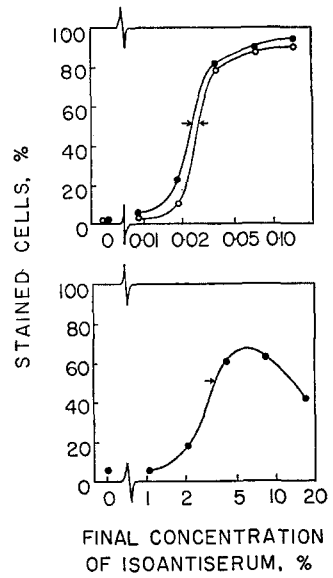

FIG. 1. *Top*: Example of a potent isoantiserum. Cytolytic titration of thymocytes from adult (●—●) and newborn (O—O) AKR mice by C3HeB/Fe isoantiserum to AKR thymocytes. Cytolytic titers were read at the final concentrations of isoantiserum corresponding to the arrows.

Bottom: Example of a strong prozone. Cytolytic titration of BW5147 leukemia by C3HeB/Fe isoantiserum to BW5147 leukemia.

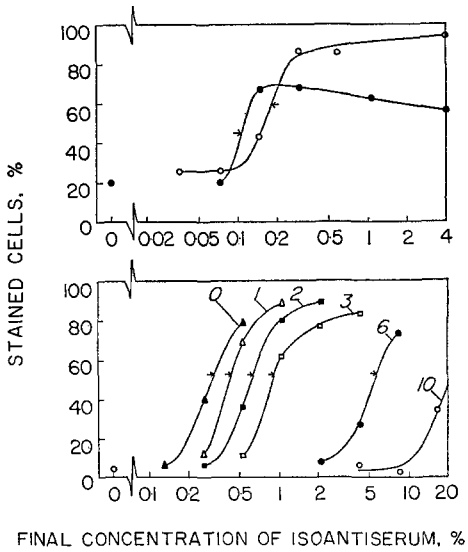

FIG. 2. *Top*: Differentiation between leukemic and normal lymphocytes in peritoneal cells from an AKR mouse that carried the 4th transplant generation of leukemia RA2. Cytolytic titration of peritoneal cells by C3HeB/Fe isoantiserum to AKR spleen, toxic only to the leukemic cells (●—●), and by C57BL/6 isoantiserum to L4946 leukemia (O—O), toxic both to the leukemic and to the normal peritoneal cells.

Bottom: Cytolytic titration of supernatants from an absorption experiment. Aliquots of 0.1 ml C3HeB/Fe isoantiserum to AKR thymocytes (in 1 to 10 dilution) were absorbed with the stated numbers (millions) of AKR thymocytes. The supernatants were diluted 1 to 4 and titrated as shown against AKR thymocytes.

Absorptions.—An adaptation of the method employed by Möller (16) and by Amos and coworkers (17) was used. Aliquots of suspensions that contained known numbers of single cells were centrifuged at 5°C for 10 minutes at 220 *g* and the supernatant was removed. Tissues that do not easily yield single cell suspensions were homogenized in modified Locke's solution to 10 per cent on a wet weight basis. After centrifugation at 5°C for 10 minutes at 2000 *g*, the supernatant was removed and the residue resuspended (or, if necessary, rehomogenized) in the original volume of buffer. Then suitable aliquots were recentrifuged to give packed residues derived from a known wet weight of intact tissue. These residues were stirred to a slurry-like consistency.

Absorptions were carried out at room temperature. One volume of isoantiserum dilution was added to the requisite quantities of single cells or tissue residues, and mixed every 5 minutes for 30 minutes. Then the tubes were placed in ice water, 3 volumes of cold buffer added, and the contents mixed. Tubes were centrifuged at 5°C for 10 minutes at 700 *g* or 2400 *g*, for contents of single cells or tissue residue, respectively. The supernatants were decanted and stored at −20°C. They were subsequently titered by cytolysis against the appropriate cell type.

RESULTS

Assay Systems.—

Cytolytic assays: Perhaps the most important criterion for validity of a cytolytic assay is the dependence of cytolytic titer on cell concentration. This criterion was satisfied over specified ranges of cell concentration in the assays previously investigated (8, 11, 12, 18) and in those assays that were tested in this study (Table II). Ideally, the dependence should be direct proportionality, but in practice this has been attained only in one instance, in work with heterologous antiserum (8).

Factors were determined to convert titers obtained with the small-scale assay system to titers based on the regular assay system (12). For each cell type, two isoantisera were titered simultaneously in both assay systems and the same experiment was repeated on another day. The small-scale assay system

TABLE II

*Relationship between Cell Concentration and Cytolytic Titer. Cytolysis of Various AKR Cell Types by C57BL/6 Isoantisera**

Final cell concentration in assay system, millions	Relative cytolytic titer at stated cell concentrations, compared to the cytolytic titer (100) at a final cell concentration of 5 million cells/ml				
	Thymocytes: data from reference 8	Splenic lymphocytes	Lymph node lymphocytes	S775 leukemia	BW5147 leukemia
10	174	141	147	148	155
5	100	100	100	100	100
2.5	67	60	40	72	65
1.25	47	35	26	56	51

* All results are the mean for two experiments, except for BW5147 leukemia.

was more sensitive than the regular assay system by a factor of 3.7, 2.7, 2.3, 2.7, 4.0, 5.3, 3.4, 3.7, 4.2, and 3.7, for thymic, lymph node, splenic, intraperitoneal, L4946, BW5147, S775, RA1, RA2, and RA3 cells, respectively.

No isoantiserum tested in this study cytolyzed more than 20 per cent of marrow cells. Even use of a rabbit antiserum against mouse marrow cells, prepared in the same manner as the antiserum against thymocytes (8), did not result in a satisfactory assay system: cytolytic titers were shallow, and the dependence of cytolytic titer on cell concentration was low, even though complementation was adequate. These data suggest that the surface properties of marrow cells, rather than the inadequacy of the assay system, are responsible for the present results with marrow cells.

Quantitative absorption experiments: A new quantitative method was used to calculate absorption of isoantisera by viable cells and packed tissue residues. The method is illustrated by Table III and Fig. 2, bottom, and Fig. 3. The final result for each tissue is a simple and direct estimate of absorptive capacity.

An isoantiserum was absorbed with a known number of intact cells or with a packed residue derived from a homogenate of a known wet weight of tissue (Table III). Control absorptions were done with tissues obtained from the strain of mouse (C3Heb/Fe) in which the isoantiserum had been prepared. The supernatant that remained after absorption was titered against AKR thymocytes (Fig. 2, bottom, and Table III). The potency of the supernatant was calculated as a percentage of the potency before absorption. When this potency was plotted on logarithmic probability paper against the number of thymocytes used for absorption, a straight line was obtained (Fig. 3). This line served as a calibration curve to express the reduction in potency obtained by absorption with any tissue (Table III) in terms of the equivalent number of AKR thymocytes required to produce the same reduction in potency. A slight correction was applied for absorption by control tissues. The absorptive capacity of intact single cells (or of tissue residues) was calculated as a percentage of the absorptive capacity of the same number of AKR thymocytes (or of thymic residue derived from the same wet weight of tissue). Thus in every experiment, AKR thymocytes (or AKR thymic residue) were included to provide a standard of absorption; in addition, a complete calibration curve was occasionally run.

Thymic residues could not be redispersed adequately by manual mixing with isoantiserum during the absorption period. When absorptions were performed with occasional mechanical mixing in a small pyrex homogenizer (Macalaster-Bicknell Company, Cambridge, Massachusetts, No. 2976), 38 per cent greater absorption of isoantiserum was obtained. Under the latter conditions, residue from a homogenate of 1 mg wet weight of thymus gland absorbed as much isoantiserum as 2.25 million fresh thymocytes (mean value of four determinations).

Positive results in absorption experiments were reproducible if the absorptive capacity exceeded 1.5 per cent. The sensitivity of the method varied with the

TABLE III

Results and Calculations for Two Typical Absorption Experiments

0.1 ml of 1 to 10 dilution of C3HeB/Fe isoantiserum against AKR thymocytes, absorbed with:			Cytolytic titer of supernatant against AKR thymocytes	Potency of supernatant*	Equivalent No. of AKR thymocytes, read from log-probability plot (Fig. 3), millions of cells	Correction for absorption by control tissue‡, millions of cells	Corrected equivalent No. of AKR thymocytes, millions of cells	Absorptive capacity relative to AKR thymocytes§
Strain	Tissue	Quantity, millions of cells						
			per cent	*per cent*				*per cent*
Absorptions with intact single cells								
Unabsorbed antiserum			0.30	100				
AKR	Thymocytes	3	0.92	32.6	2.6	0.1	2.5	100
C3HeB/Fe	Thymocytes	30	0.40	75.0	1.00			
AKR	Splenic lymphs	41	0.97	30.9	2.66	0.86	1.80	5.3
C3HeB/Fe	Splenic lymphs	41	0.38	78.9	0.86			
Absorptions with residues from tissue homogenates								
		mg‖						
Unabsorbed antiserum			0.21	100				
AKR	Thymus	2	3.4	6.2	6.4	0.1	6.3	100
C3HeB/Fe	Thymus	20	0.29	72.4	1.03			
AKR	Brain	1.6	1.91	11.0	4.9	0.1	4.8	95
C3HeB/Fe	Brain	16	0.31	67.8	1.17			

* The potency of the supernatant is $100 \times \frac{\text{titer of unabsorbed antiserum}}{\text{titer of supernatant from absorption}}$ = per cent.

For instance, after absorption with 3 million AKR thymocytes, the potency is

$$100 \times \frac{0.30}{0.92} = 32.6 \text{ per cent.}$$

‡ The correction is $\frac{\text{No. of cells (or residue weight) used in experimental absorption}}{\text{No. of cells (or residue weight) used in control absorption}} \times$ equivalent No. of thymocytes for control absorption.

For instance, for absorption by 3 million AKR thymocytes, the correction is $\frac{3}{30} \times 1.00 = 0.1$.

§ Absorptive capacity is

$$100 \times \frac{\text{equivalent No. of AKR thymocytes per million experimental cells}}{\text{equivalent No. of AKR thymocytes per million thymocytes used}} = \text{per cent.}$$

For instance, for absorption by AKR splenic lymphocytes, absorptive capacity is $100 \times \frac{1.80/41}{2.5/3} = 5.3$ per cent.

For tissue residues, absorptive capacity is

$$100 \times \frac{\text{equivalent No. of AKR thymocytes per mg experimental residue}}{\text{equivalent No. of AKR thymocytes per mg thymic residue}} = \text{per cent.}$$

For instance, for absorption by AKR brain, absorptive capacity is $100 \times \frac{4.8/1.6}{6.3/2} = 95$ per cent.

‖ Residue from homogenates, derived from stated wet weight of tissue.

quantity of tissue used for absorption. It was optimal when the largest quantity was employed that would still leave sufficient cytolytic potency in the supernatant to permit accurate determination of that potency.

Serological Data.—

Cytolysis of normal AKR lymphocytes by C3HeB/Fe isoantisera: C3HeB/Fe mice possess the same H-2 allele as AKR mice but presumably differ in other histocompatibility factors (17). Surprisingly, *all* C3HeB/Fe isoantisera cyto-

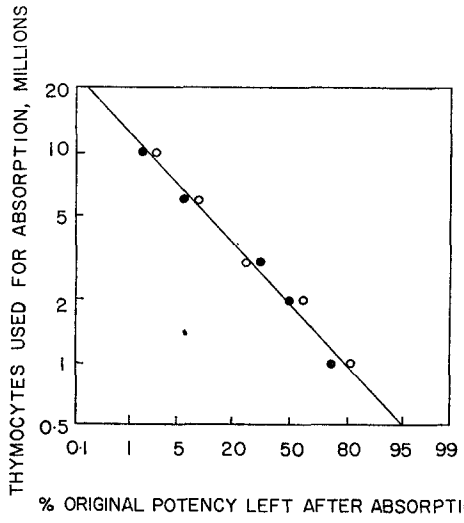

FIG. 3. Calibration curve for an absorption experiment, plotted on logarithmic probability paper. Percentage of original cytolytic potency that remains in the supernatant (abscissa) following absorption with a stated number of thymocytes (ordinate). Data taken from Fig. 2, bottom (●—●), and from an identical repeat experiment (O—O).

lyzed AKR thymocytes, while *none* cytolyzed other types of lymphocytes or marrow cells of normal AKR mice (Table IV). Very different results were obtained with isoantisera prepared in a strain that differed in H-2 allele (Table V).

Contrary to expectation, the C3HeB/Fe isoantiserum against AKR spleen was more potent in cytolysis of AKR thymocytes than the C3HeB/Fe isoantiserum against AKR thymocytes (Table IV). Isoantisera against AKR lymph node cells, washed blood cells, and marrow were approximately one-fifth, one-fifteenth, and one-fifteenth as potent, respectively (Table IV). These results are partially explained by absorption experiments (Table VI). Thus, splenic and lymph node lymphocytes did absorb C3HeB/Fe isoantiserum against AKR thymocytes, although not avidly. In general, there was some correlation between the potency of isoantisera prepared against normal tissues and

TABLE IV

*Cytolysis of Normal AKR Cells by Isoantisera Prepared in C3HeB/Fe Mice (Same H-2 Allele) Against AKR Cells**

C3HeB/Fe isoantisera against the following AKR cell types:	Potency‡ of C3HeB/Fe isoantisera for cytolysis of the following normal AKR cell types:				
	Thymocytes	Lymph node cells	Spleen	Intraperitoneal lymphocytes	Marrow
Washed blood cells	36	<2	<2	<2	<2
Thymocytes	450	<2	<2	<2	<2
Lymph nodes	101	<2	<2	<2	n.t.§
Spleen	524	<2	<2	<2	<2
Marrow	33	<2	<2	<2	<2
Liver	9	<2	<2	<2	<2
L4946 leukemia	340	<2	<2	<2	<2
BW5147 leukemia	261	<2	<2	<2	<2
S775 leukemia	30	<2	<2	<2	<2
T283 sarcoma	15	<2	<2	<2	<2

* Each value represents the mean result of two determinations performed on different days.

‡ Potency is expressed as 100/cytolytic titer.

§ n.t., not tested.

TABLE V

*Cytolysis of Normal AKR Cells by Isoantisera Prepared in C57BL/6 Mice (Differ in H-2 Allele) against AKR Cells**

C57BL/6 isoantisera against the following AKR cell types:	Potency of C57BL/6 isoantisera for cytolysis of the following normal AKR cell types:				
	Thymocytes	Lymph node cells	Spleen	Intraperitoneal lymphocytes	Marrow
Washed blood cells	290	36	62	23	2
Thymocytes	450	61	82	9	2
Lymph nodes	<2	30	77	(20 % a.c.)‡	2
Spleen	1.6	660	710	137	(21 % a.c.)
Marrow	<2	1.8	9§	(17 % a.c.)	2
Liver	<2	10.9	16.3§	(25 % a.c.)	2
L4946 leukemia	4	53	112	32	(21 % a.c.)
BW5147 leukemia	5.9	47	66	21	(12 % a.c.)
S775 leukemia	8.6	27	34	8.6	<2
T283 sarcoma	<2	3.0	4.4§	<2	<2

* Each value represents the mean results of two determinations performed on different days.

‡ (20 % a.c.) means that the highest stained cell count was 20 *per cent above* the *control* level.

§ Potency for cytolysis of 60 ± 5 per cent of the cells; the remaining 40 per cent remained viable even at high concentrations of isoantiserum.

the absorptive capacity of those tissues. No such correlation was found for leukemias (see Discussion).

Cytolysis of normal AKR lymphocytes by C57BL/6 isoantisera: This system involves a known difference in H-2 allele and presumably also differences in other histocompatibility factors (17). Isoantiserum prepared against spleen gave strong cytolysis of spleen cells but very weak cytolysis of thymocytes; the isoantiserum against thymocytes also gave strong cytolysis of thymocytes but in contrast gave potent cytolysis of spleen cells (Table V). These are examples

TABLE VI

Absorption of C3HeB/Fe Isoantiserum against AKR Thymocytes by Viable Single Cell Suspensions of AKR Mice

AKR tissue used for absorption	Absorptive capacity* relative to AKR thymocytes
	per cent
Thymocytes	100
Neonatal thymocytes	78
Lymph node lymphocytes	5.7
Splenic lymphocytes	5.0
Neonatal splenic lymphocytes	<1.5
Marrow cells	<1.5
Red blood cells	<0.3
L4946 leukemia	13.3
S775 leukemia	95
RA1 leukemia	4.4
RA3 leukemia	39
RA4 leukemia	7.4
RA5 leukemia	95

* Calculated relative to absorption by the same number of AKR thymocytes. All results are the mean of two experiments.

of cross-reactions. Nevertheless, the relative potencies of the isoantisera involved in these and other reactions (Table V) give evidence of wide *quantitative* differences in the surface antigens of thymocytes and of other normal lymphocytes.

C57BL/6 isoantisera failed to differentiate convincingly between lymph node and splenic lymphocytes (Table V). The lower potencies shown by isoantisera in their cytolysis of intraperitoneal lymphocytes may indicate a real difference in susceptibility to isoantibodies. Alternatively, lymph node and splenic lymphocytes may be more susceptible to immune cytolysis because their cell surfaces have been injured during physical disruption of the whole organs in preparation of single cell suspensions.

The above explanation cannot account for the resistance of marrow cells to

cytolysis by C57BL/6 isoantisera. The absorption datum (Table VI), the low potencies of isoantisera prepared against marrow cells (Tables IV and V), and the data obtained with heterologous antiserum (see above) suggest a different explanation. This is, that marrow cells possess a relatively low concentration of surface isoantigens.

Cytolysis of AKR leukemias by C3HeB/Fe isoantisera: In contrast to the selective cytolysis of normal AKR tissues by C3HeB/Fe isoantisera (Table IV),

TABLE VII

*Cytolysis of AKR Leukemia Cells by Isoantisera Prepared in C3HeB/Fe Mice (Same H-2 Allele) against AKR Cells**

C3HeB/Fe isoantisera against the following AKR cell types:	Potency of C3HeB/Fe isoantisera for cytolysis of the following AKR leukemias:						
	L4946 long transplanted	BW5147 long transplanted	S775 long transplanted	RA1 5th generation	RA2 4th generation	RA3 1st generation	RA3 5th generation
Washed blood cells	<2	<2	2.6	<2	<2‡	(30 % a.c.)	1.8
Thymocytes	(39 % a.c.)	24	103	<2	(20 % a.c.)‡	96§	73
Lymph nodes	<2	2.1	12.8‡	<2	n.t.	2.5	n.t.
Spleen	(10 % a.c.)	28	122	<2	174‡, §	97‖	58
Marrow	(23 % a.c.)	<2	3.3‡	<2	<2‡	n.t.	n.t.
Liver	<2	<2	<2	<2	<2‡	n.t.	n.t.
L4946 leukemia	(46 % a.c.)¶	30	127	<2	6.7‡	65	96
BW5147 leukemia	(19 % a.c.)	7.4¶	32‡	<2	<2‡	19‖	n.t.
S775 leukemia	<2	<2	4.7‡	<2	<2‡	4.7	n.t.
T283 sarcoma	<2	<2	2.3	<2	<2‡	1.8	n.t.

* Each value represents the mean of two determinations, unless otherwise stated.

‡ Single determination.

§ The stained cell count did not exceed 70 per cent at high isoantiserum concentrations.

‖ The stained cell count did not exceed 80 per cent at high isoantiserum concentrations.

¶ Strong prozone.

four out of six AKR leukemias were highly sensitive to C3HeB/Fe isoantisera (Table VII). Isoantisera to thymocytes, spleen, and L4946 leukemia were the most potent. The relative potencies of C3HeB/Fe isoantisera were similar not only for cytolysis of three of the four highly sensitive leukemias, but also for cytolysis of normal thymocytes (Table VIII). This raises the possibility that the various C3HeB/Fe isoantisera all possessed antibodies directed against the same antigen (or group of antigens). Absorption studies (Table VI) support the suggestion that the same antigen was present in different concentration or spacial arrangement on the surface of normal thymocytes and of leukemias with high absorptive capacities.

Cytolysis of AKR leukemias by C57BL/6 isoantisera: Cytolysis of *normal AKR* cells by C57BL/6 isoantisera revealed striking differences among three cell types: first, thymocytes; secondly, lymph node, splenic, and intraperitoneal lymphocytes; and thirdly, marrow cells (Table V). Cytolysis of AKR *leukemias* by the same isoantisera shows that certain leukemias reacted almost exactly like one of these normal cell types (Table IX).

For instance, L4946 leukemia (Table IX) reacted similarly to normal lymph

TABLE VIII

Relative Potencies of C3HeB/Fe Isoantisera Prepared against AKR Cells for Cytolysis of AKR Thymocytes and of Three AKR Leukemias

C3HeB/Fe isoantisera against the following AKR cell types:	Relative potencies* of C3HeB/Fe isoantisera for cytolysis of the following AKR cell types:			
	Thymocytes	BW5147	S775	RA3
Washed blood cells	0.08	—	0.03	—
Thymocytes	1.0	1.0	1.0	1.0
Lymph nodes	0.22	0.09	0.12	0.03
Spleen	1.2	1.2	1.2	1.0
Marrow	0.07	—	0.03	—
Liver	0.02	—	—	—
L4946 leukemia	0.8	1.2	1.2	0.7
BW5147 leukemia	0.6	0.3	0.3	0.2
S775 leukemia	0.07	—	0.05	0.05
T283 sarcoma	0.03	—	0.02	0.02

* Relative potency is expressed as the ratio of potencies of two isoantisera reacting with the same cell type. The potencies of all isoantisera have been compared with the potencies of the isoantiserum prepared against AKR thymocytes. Data taken from Tables IV and VII.

node, splenic, or intraperitoneal lymphocytes (Table V); the chief differences were somewhat stronger cytolysis of L4946 cells by isoantisera against washed blood cells and marrow. Absence of cytolytic potencies in excess of 2 for any C3HeB/Fe isoantiserum assayed against L4946 cells (Table VII) again indicates a strong similarity with non-thymic lymphocytes (Table IV). Further, when the absorptive capacity of L4946 cells for a C3HeB/Fe isoantiserum (Table VI) is calculated on the basis of unit cell surface (19), it becomes 4.8 per cent or essentially equal to that of splenic lymphocytes.[1] The above data strongly support the concept that L4946 leukemia is non-thymic in origin.

Similar considerations indicate that leukemia RA1 is non-thymic in origin (Tables VI, VII, and IX). In contrast, leukemias BW5147, S775, RA2, and RA3

[1] 200 fresh AKR lymphocytes and L4946 cells had mean diameters of 6.1 and 10.3 μ, which corresponded to mean spherical surface areas of 121 and 333 μ^2, respectively.

reacted similarly to thymocytes with C3HeB/Fe isoantisera (Tables IV, VII, and VIII), and their absorptive capacities for such isoantisera were high (Table VI). However, their cytolytic reactions with C57BL/6 isoantisera (Table IX) were more similar to those of splenic than of thymic lymphocytes (compare with Table V). The data suggest that these leukemias developed from the same stem cell as thymocytes, but possess higher concentrations of the antigens that correspond to the H-2^k and H-$2^{k'}$ allele, than do thymocytes.

TABLE IX

*Cytolysis of AKR Leukemia Cells by Isoantisera Prepared in C57BL/6 Mice (Differ in H-2 Allele) against AKR Cells**

C57BL/6 isoantisera against the following AKR cell types:	Potency of C57BL/6 isoantisera for cytolysis of the following AKR leukemias:						
	L4946 long transplanted	BW5147 long transplanted	S775 long transplanted	RA1 5th generation	RA2 4th generation	RA3 1st generation	RA3 5th generation
Washed blood cells	116	30	n.t.	<2	300‡	8.9	n.t.
Thymocytes	24	27	72	1.8	86‡	18.1	n.t.
Lymph nodes	28	3.0§	(19 % a.c.)	<2	46‡	<2	n.t.
Spleen	218	65	143	19.4	626‡	74	58
Marrow	14	(24 % a.c.)	5.8‡	<2	21‡	<2	n.t.
Liver	4.3§	<2	<2‡	<2	>10‡, ‖	<2	n.t.
L4946 leukemia	78	15.0	27	5.4	106‡	14.2	9.2
BW5147 leukemia	31	10.8	29	4.8	102‡	8.8	10.6
S775 leukemia	10.4	5.8	13.4	2.8	26‡	4.9	5.3
T283 sarcoma	1.5	<2	<2	<2	10.0‡	<2	<2

* Each value represents the mean of two determinations, unless otherwise stated.

‡ Single determination.

§ Strong prozone.

‖ Percentage of vitally stained cells did not exceed 63 per cent at high isoantiserum concentrations. Titer was not run out to low isoantiserum concentrations.

Detailed data on immune cytolysis of leukemias RA4 and RA5 are not presented here. C3HeB/Fe isoantisera cytolyzed only RA5, while C57BL/6 isoantisera cytolyzed both leukemias. Absorption experiments (Table VI) showed the same sharp quantitative distinction and the same correlation with cytolysis by C3HeB/Fe isoantisera that was found for the other leukemias. These data suggest that leukemias RA4 and RA5 are respectively non-thymic and thymic in origin, and further strengthen the close correlation between the results of cytolysis and absorption experiments.

Analysis of mixtures of normal and leukemic lymphocytes: Various C3HeB/Fe isoantisera caused strong cytolysis of thymus-derived leukemias (Table VII),

but had no effect on normal intraperitoneal lymphocytes (Table IV). Hence, C3HeB/Fe isoantisera could be used to determine the percentage of normal and thymus-derived leukemic cells in peritoneal exudates (Fig. 2, top). This method may prove to be a useful tool for study of the biology of recently derived AKR leukemias.

TABLE X

Absorption of C3HeB/Fe Isoantiserum against AKR Thymocytes by Packed Residues Derived from Homogenates of AKR and RF Tissues

Mouse strain	Tissue residue used for absorption	Absorptive capacity* relative to AKR thymus
		per cent
AKR	Thymus	100
	Brain	107
	Neonatal brain	1.6
	Appendix	3.3
	Lung	1.5
	Liver	0.9
	Skeletal muscle	0.8
	Kidney	0.6
	Testis	0.6
AKR	Hemisphere	152
	Pituitary	89
	Brain stem	76
	Cerebellum	57
	Spinal chord	54
	Olfactory bulb	41
	Sciatic nerve	29
RF	Thymus	71
	Brain	111
	Hemisphere	181
	Brain stem	79

* Calculated relative to absorption by an AKR thymic residue derived from the same wet weight of tissue. All results are the mean of two experiments.

Antigenic stability of newly derived leukemia: Leukemia RA3 was subjected to immune cytolysis experiments both in its first and in its fifth transplant generation. Isoantisera prepared against normal tissues showed lower cytolytic potencies for cells of the fifth transplant generation, while isoantiserum against L4946 tumor showed higher potencies (Tables VII, and IX). However, the changes were too small to constitute definite evidence for a change in content of antigens between transplant generations 1 and 5.

The original transplant of leukemia RA1 was made separately from thymus

and from spleen of the same AKR mouse, and the tumors that resulted were carried separately. Ten C57BL/6 isoantisera of varying cytolytic potencies, when tested against cells of the fifth transplant generation of both tumor lines, gave results that corresponded closely. These results suggest that the two leukemic tissues that were originally transplanted contained the same type of leukemic lymphocyte.

Presence of the AKR thymic antigen in normal AKR tissues: The AKR thymic antigen was present in high concentration not only in thymus but also in all adult nervous tissues that were examined (Table X). Low levels of the antigen were present in adult lymph node lymphocytes, splenic lymphocytes, appendix and lung (Tables VI and X). The antigen was almost fully developed in the thymus of newborn mice (Tables VI and XI); in contrast, neonatal brain contained less than 2 per cent of the antigen found in adult brain (Table X), and neonatal spleen contained less of the antigen than adult spleen (Table VI).

Presence of AKR thymic antigen in other strains of mice: RF thymocytes were cytolyzed by C3HeB/Fe isoantiserum against AKR thymocytes at a potency similar to that effective against the homologous cells (Table XI). RF splenic lymphocytes, in common with AKR splenic lymphocytes, were not cytolyzed by this isoantiserum. The thymocytes of other strains that were tested gave no indication of cytolysis (Table XI).

Absorption experiments with thymocytes of various strains were equally clear-cut. Thymocytes from RF mice absorbed almost as much of the C3HeB/Fe isoantiserum as did AKR thymocytes. In contrast, thymocytes from other strains of mice did not absorb significant quantities of isoantibody (Table XII).

The above results are consistent with the presence of the AKR thymic antigen on RF thymocytes in a quantity similar to that on the homologous cells. Thymocytes from the other strains tested either lack this antigen, or possess less than one-hundredth of the amount present on the surface of AKR or RF thymocytes (Tables XI and XII). In tissues of RF mice that were tested, the distribution of the thymic antigen was similar to that in tissues of AKR mice (Table X).

Distribution of C3HeB/Fe thymic antigen: Most experiments described above were done with C3HeB/Fe isoantisera to AKR tissues. Experiments described below were done with the inverse isoantisera. In particular, AKR isoantisera to C3HeB/Fe thymocytes and to C3HeB/Fe splenic lymphocytes were used, since isoantisera to these tissues were most potent in the inverse situation (Table IV).

The AKR isoantisera cytolyzed thymocytes from all mouse strains tested except AKR and RF (Table XI). The AKR isoantisera were inactive in cytolysis of splenic lymphocytes from adult mice of strains C3HeB/Fe, A, C57BL, and C57BL/6; of lymph node lymphocytes from adult mice of strains C3HeB/Fe, A, and C57BL/6; and of leukemia EL4 of C57BL mice. These results parallel those obtained with the inverse isoantisera (Table IV).

TABLE XI

*Cytolysis of Thymocytes of Various Mouse Strains by Isoantisera Prepared against AKR Thymocytes and against C3HeB/Fe Thymocytes**

Thymocytes of mouse strain	Cytolytic potencies shown against these thymocytes by isoantisera prepared in:	
	C3HeB/Fe mice *vs.* AKR thymocytes	AKR mice *vs.* C3HeB/Fe thymocytes
AKR	775	<2
AKR, neonatal	710	—
RF	626	<2
C3HeB/Fe	<2	14.2
C3H/DiSn	<2	15.9
C3H/St‡	<2	12.9
A	<2	12.3
A/He	<2	8.4
BALB/c	<2	13.9
C57BL	<2	16.0
C57BL, neonatal	<2	11.0
C57BL/6	<2	15.9
C57BL/10	<2	14.1
C57L	<2	10.8
C58	<2	5.1
DBA/1	<2	15.0
DBA 2	<2	13.2
ICR Swiss§	<2	15.4
SWR	<2	13.8
129	<2	12.3

* Mean results for either two or three experiments.

‡ Subline of C3H/He.

§ Non-inbred strain CD1 (Charles River Breeding Laboratories, Wilmington, Massachusetts).

TABLE XII

Absorption of C3HeB/Fe Isoantiserum against AKR Thymocytes by Viable Thymocytes from Various Strains of Mice

Source of thymocytes	Absorptive capacity relative to AKR thymocytes
	per cent
AKR	100
RF	87
C3HeB/Fe	<2.0
A	<2.0
BALB/c	<2.0
C57BL/6	<2.0
C57BL/10	<2.0
DBA/1	<2.0
DBA/2	<2.0
SWR	<2.0

Data obtained by absorption of an AKR isoantiserum with different numbers of intact C3HeB/Fe thymocytes gave a straight line parallel to that in Fig. 3. This line was used as caliberation curve for the quantitative determination of the absorptive capacities of intact cells or tissue residues.

An AKR isoantiserum to C3HeB/Fe splenic lymphocytes was absorbed with intact cells from strains that possessed the C3HeB/Fe thymic antigen. Relative to absorption by adult C3HeB/Fe thymocytes, the absorptive capacities of these cells were as follows: thymocytes from C57BL/6, C57BL, and neonatal C57BL mice, 88, 74, and 74 per cent respectively; lymph node lymphocytes from C3HeB/Fe, C57BL/6, and C57BL mice, 19, 11, and 16 per cent respectively; splenic lymphocytes from the same three strains, 12, 5.3, and 9.8 per cent respectively; neonatal splenic lymphocytes of C57BL and ICR Swiss mice, 2.7 and 2.3 per cent respectively; and EL4 leukemia of C57BL mice, 11.4 per cent, or 1.7 per cent if calculated on the basis of equal cell surface area rather than equal cell number.[2] These results are similar to those obtained with an inverse isoantiserum (Table VI).

The same AKR isoantiserum was absorbed with tissue residues from strains C3HeB/Fe and C57BL/6. Since results for the two strains were closely similar, they have been averaged: the absorptive capacity of thymus, brain, appendix, lung, liver, and kidney was 100, 122, 5.0, 1.5, 0.65, and 0.49 per cent respectively. Neonatal brain of C57BL, ICR Swiss, and A mice averaged 2.4 per cent of the absorptive capacity possessed by adult brain of these three strains. Again, these data resemble those obtained with an inverse isoantiserum (Table X).

DISCUSSION

Gorer and coworkers reported three X antigens present specifically in EL leukemias of C57BL mice: EL4 X, EL6 X, and EL8 X. Since some EL leukemias arose in thymus, they searched for X antigens in that tissue, but were not successful. EL leukemias could not be cytolyzed by isoantibodies to the X antigens (2).

In contrast, isoantibodies to the AKR or C3HeB/Fe thymic antigens cytolyzed thymocytes that possessed either of these antigens (Table XI). Further, EL4 leukemia did not absorb measurable quantities of an isoantiserum to AKR thymus, and absorbed less isoantiserum to C3HeB/Fe thymus than did normal splenic lymphocytes of strain C57BL. These data indicate that both the present thymic antigens are distinct from EL4 X and cast doubt on identity with EL6 X or EL8 X.

Old, Boyse, and Stockert reported presence of the LT antigen in some C57BL leukemias (4), reminiscent of Gorer's results (2, 20). However, the LT antigen was distinct from EL4 X. Their findings include absence of the LT antigen in

[2] The mean diameter of EL4 cells was 15.6 μ.

normal C57BL thymus, and presence of the LT antigen in A and C58 thymus (4).

These latter data clearly distinguish the LT antigen both from the AKR and from the C3HeB/Fe thymic antigen (Table XI). More recent work (21, 22) has confirmed the differentiation between the LT and AKR antigens. Since the LT antigen is present in A but not in C57BL thymus (4), while the C3HeB/Fe thymic antigen is present in both strains (Table XI), it appears that more than one thymic antigen can be present in thymus of a single inbred mouse strain.

The AKR and C3HeB/Fe thymic antigens could represent previously unrecognized expressions of known mouse histocompatibility (H) factors. Comparison of the present Table XI with Table 10 of Amos *et al.* (17) excludes association of either antigen with any of the six known mouse histocompatibility factors H-1 to H-6. Moreover, since thymocytes of female AKR or C3HeB/Fe mice possess a full complement of the respective thymic antigens, Y-linked histocompatibility antigens (23) are not implicated.

Amos *et al.* have suggested that apparently new serologically determined mouse antigens be first designated by Greek letters (17). In line with this suggestion, the names θ-AKR and θ-C3HeB/Fe are proposed for the two thymic antigens described here and (in the case of the AKR antigen) previously (9). When the precise relationship of these antigens to the new mouse antigens presently under investigation by Snell (24) and others (21) has been determined, perhaps a different nomenclature will be more appropriate.

The disproportionately high antibody response to viable L4946 cells (Table IV), relative to the absorption of isoantibody by these cells (Table VI), requires explanation. At low doses of antigen, antibody response is loosely correlated with the quantity of antigen injected (25). More important, the initial multiplication of viable tumor cells in an incompatible host provides a powerful stimulus to antibody response (24, 26–28).

Lymphocytes resist immune cytolysis, unless a minimum concentration of sites that can bind the relevant antibodies is present on their surfaces (14, 19, 29, 30). If this minimum is exceeded and the potencies of the isoantisera are sufficiently high to avoid prozone formation, then immune cytolysis provides clear-cut, quantitative evidence of an immune reaction. However, present results support the conclusion of Möller (30), that absorption experiments are more sensitive.

The LT antigen is present in certain C57BL leukemias but absent from thymus and other normal C57BL tissues (4); one possible explanation is that the LT antigen is latent in this strain. Therefore, the high content of the θ-AKR antigen in certain AKR leukemias (Table VI) does not necessarily prove that these leukemias originated in thymus; they may have originated in a cellular precursor of thymocytes. This latter possibility is supported by the difference in the spectrum of cytolytic reactions of C57BL/6 isoantisera with thymocytes

and with leukemias that possessed a high content of θ-AKR, such as S775 and RA3 (Tables V and IX). In contrast, the cytolytic reactions with C57BL/6 isoantisera strengthen the evidence that leukemias with low contents of the θ-AKR antigen were of non-thymic origin.

The θ-AKR and the θ-C3HeB/Fe antigens had an inverse distribution in eighteen mouse strains (Table XI). Both antigens had a similar distribution in tissues of the strains that were tested. There were no exceptions to these two rules.

It was unexpected to find the same antigen (either θ-AKR or θ-C3HeB/Fe) present in high amounts in thymus and in nervous tissue of each of the six strains tested. However, neonatal brain possessed only between 1.5 and 2.8 per cent of the content of antigen found in adult brain in the four strains studied. In contrast, the antigen was almost fully developed in neonatal thymus.

The above results suggest that θ-AKR and θ-C3HeB/Fe antigens originated either in the thymus or in closely related cellular precursors. The concept that an immunological maturation of mouse brain takes place after birth is new. Present indications of a close immunological relation among thymus, leukemias, and nervous tissues require further investigation.

The low but definite content of the θ-AKR or θ-C3HeB/Fe antigen in spleen, lymph nodes, appendix, and lung of the strains studied may have been due either to presence of the respective antigens on cells of these tissues or to migration of thymocytes into the tissues. It remains to be determined whether the development of θ-isoantigens in neonatal spleen parallels the development of H-2 isoantigens, which has been well documented by the Möllers (14, 16, 19, 30). The present data fit the concept that thymocytes migrate to neonatal spleen and there induce differentiation of lymphoblasts to immune competent cells (31–35).

SUMMARY

A clear-cut serological differentiation between AKR lymphocytes of thymic and non-thymic origin is reported: these two cell types are antigenically distinct.

In newborn mice, the AKR thymic antigen was found at a high concentration only in thymus. In adult mice, the antigen was present at a high level in thymus, all nervous tissues tested, and some leukemias. It was present at much lower levels in lymph node lymphocytes, splenic lymphocytes, appendix, lung, and certain other leukemias, which appeared to be of non-thymic origin. The AKR thymic antigen was present at a high level in thymus and nervous tissues of RF mice, but was absent from thymocytes of sixteen other mouse strains.

These sixteen strains possessed the C3HeB/Fe thymic antigen. The distribution of this antigen in neonatal and adult tissues of the strains tested was similar to that of the AKR thymic antigen in AKR mice. No exceptions were found.

These results were obtained by use of immune cytolysis, and of a new method for the quantitative treatment of data from absorption experiments.

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