Passive Immunity in Mice against C57BL Leukosis E.L.4 by Means of Iso-immune Serum

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The finding that serum from a hyperimmunized mouse would protect against leukotic cells after incubation *in vitro* (9) has since been confirmed by a number of workers, both in the case of malignant tissues (7, 14, 16) and with suspensions of normal epidermal cells (6). We may refer to this as a "neutralization" technic. Attempts to show that iso-immune antibodies could produce true passive immunity in mice have not so far been successful.

Following the development of a more sensitive method for the detection of antibody by the dextran method (12) and the demonstration that agglutinating antibody persisted for as long as 10 days and protective action for 3 days after intraperitoneal inoculation of 0.1 ml. of immune serum (2), it was decided to reopen the question, with the use of antibodies of known high hemagglutinin titer.

The disappointing results of attempts at passive immunity obtained by others have led many workers to question the significance of the circulating antibodies, although few would deny the importance of the H-2 system in transplantation immunity. The close correlation between H-2 factors demonstrated by tumor inoculation and circulating antibodies demonstrated by hemagglutination, leukocytic agglutination, or neutralization technic has been well substantiated (1, 11, 12, 13), and it is hard to believe that such antibodies must be dismissed as mere passengers. Some of the negative findings reported have almost certainly been due to the use of serum from inadequately immunized animals, to cell doses in excess of those which could be neutralized by the effective amount of antibody used, or to the use of cell clumps or fragments of tumor into which antibody could not penetrate. In other cases it is probable that mechanisms other than a simple antibody-antigen reaction, such as cellular participation, are also involved. Our experiments were designed to show whether antibody alone can be effective in preventing the growth of tumor cells.

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MATERIALS AND METHODS

The mice used were of four well known strains maintained by brother-sister mating; A, BALB/c, C3H, and C57BL; the F_1 cross between BALB/c and C57BL, and the backcross obtained by mating the F_1 (BALB/c × C57BL) to BALB/c. The origins of the mouse strains, of the C57BL leukosis E.L.4, and the dextran method of detecting hemagglutination have already been described (10, 12).

Antibody production.—The dosage scheme adopted for the purpose of these experiments differs somewhat from that followed for the identification of antibodies and has been found to give antisera of uniformly high titers and high avidity without loss of specificity.

The mice used were between 8 and 12 weeks old. The first inoculation usually consisted of 200,000–700,000 fresh E.L.4 ascites cells given subcutaneously. If larger doses were used, the primary nodules were often very extensive, and although the antibody finally obtained was of high titer it was unsuitable for genetic experiments when tested with backcross animals. The second inoculation was given after 1 month and was usually of the order of 10 million cells, often divided between the subcutaneous and intraperitoneal routes. The third inoculation was large, usually between 50 and 100 million cells, and was given 2 weeks after the second inoculation. The animals were bled from the heart 9–12 days later.

"Neutralization" technic.—The method described by Gorer (9) was used. Freshly collected ascites cells were diluted with normal unheated mouse serum (from various stocks) to the required dilution and then incubated for 20–30 minutes at room temperature with an equal volume of antibody. The mixture was inoculated into the mice subcutaneously in 0.2-ml. amounts.

Passive immunity.—Serum from hyperimmunized mice, either fresh or stored at −20° C. for periods of several weeks, or, in a few cases, lyophilized and reconstituted immediately before use, was injected into the peritoneal cavity. In a few experiments volumes ranging from 0.05 to 0.5 ml. were used, but the most frequently used dose was 0.1 ml. Mice were usually challenged with cells suspended in mammalian Ringer's solution, but if the dose was below 100,000 cells diluted mouse serum was used as a vehicle. The inoculations were made subcutaneously in the right pectoral region. In control animals the tumors began to appear after the 4th day, and measurements of two diameters were made daily until the tumors had regressed or until they had grown too large for useful measurements to be taken. In foreign strains the tumor is usually palpable about the 5th day and begins to regress at about the 9th day.

Titration of sera.—The hemagglutinin activity of all sera was measured by the dextran method. Except in a few special

cases, only sera of high titer were used (over 1/4000 for A strain cells or 1/1000 for cells of other strains). In some samples the activity of the serum was checked by the neutralization technic, but this proved too cumbersome for routine use.

RESULTS

a) Passive protection in strains A, BALB/c, and C3H.—The bulk of our work has been done with strains A and BALB/c, but sera obtained with any of the three strains were equally effective.

It is convenient to use a constant volume of serum (0.1 ml.) and to estimate protective activity in terms of cell dose, giving the antibody 24 hours before challenge. Measured in this way, it may be said that our sera gave protection against cell doses ranging from 400 thousand to 2 million cells, the latter figure being exceptional. Table 1 shows a result with a high-titer serum. It will be noticed that the results are perfectly clear-cut, no measurable tumors being seen in the protected animals at the cell doses shown. Controls with normal serum were sometimes used in our earlier experiments but were discarded later, since no inhibitory effect was found at doses of up to 0.3 ml.

In over-all experiments of this simple type, passive protection has been demonstrated in all but nine of 211 mice, whereas only eight of 135 controls failed to develop tumors. Furthermore, one of us[1] has succeeded in demonstrating the phenomenon with the A strain lymphoma #2, lymphosarcoma 6C3HED and with a DBA/2 thymoma.

That this is true passive protection is demonstrable by subsequent challenge of protected mice. Foreign strain animals, protected with antibody and injected with E.L.4, did not develop tumors. A further inoculum of E.L.4 cells was given 1 month later. The animals then developed tumors and gave the same type of antibody response as normal mice. The hemagglutinin titer following re-inoculation was about one in 64, while the response to a second stimulus in unprotected animals is usually well over one in 1000. This finding is in contrast to the adoptive immunity transferred by lymph node suspensions by Mitchison and Dube (17), using SA 1, and by Billingham, Brent, and Medawar (5), using skin. Adoptive immunity approximates to a transfer of active immunity.

Table 2 shows the results of an experiment to test the effect of varying the time of administering antibody. Protection was still demonstrable 7 days later, though less strongly than if antibody inoculation and challenge were performed on the same day. As was expected, the effectiveness was greatly diminished if antibody was given subsequent to E.L.4, although some effect was demonstrable with serum administered 48 hours after challenge. Once tumors had appeared, serum had no obvious effect. Indeed, relatively little of the antibody appears to reach an established tumor, as shown previously by Amos (2). We have performed relatively few experiments on the effect of timing, and the observed results vary in detail with different sera, but in general the results conform with previous knowledge concerning passive protection.

b) Protection of C57BL mice by immune sera.—Having shown that passive protection by means of hyperimmune sera was easily reproducible in incompatible mice, we wished to explore the effect of various H-2 antibodies that these sera were known to contain. Some details of the H-2 complex

[1] D. B. Amos, data to be published in Ann. New York Acad. Sc.

TABLE 1

THE DEMONSTRATION OF PASSIVE IMMUNITY AGAINST E.L.4 BY BALB/c ANTI-E.L.4 IN BALB/c MICE

Antibody volume	Approximate dose of E.L.4	No. mice	No. developing tumor	Mean tumor size (mean of two diameters in cm.) days 6	7	8	10
0.1 ml.	500,000	4	0	—	—	—	—
None	500,000	4	3	1.5	1.7	1.6	1.1
0.1 ml.	1,000,000	4	0	—	—	—	—
None	1,000,000	4	4	1.7	2.1	2.5	1.4
0.1 ml.	2,000,000	3	1*	—	—	—*	—
None	2,000,000	3	3	2.9	3.1	3.1	2.2

* Small transient mass, not measurable.

TABLE 2

DURATION OF PASSIVE IMMUNITY AGAINST E.L.4 IN RESISTANT STRAINS

A Anti-E.L.4 in A Strain Mice or BALB/c Anti-E.L.4 in BALB/c Mice (500,000–750,000 E.L.4 cells)

No antibody	0.1 ML. ANTIBODY INJECTED: Before E.L.4 days 7	3	2	1	With E.L.4 0	After E.L.4 days 1	2
12/12	1/4	0/4	0/13	0/13	0/14	9/3	3/5

Numerator = number of mice developing tumor.
Denominator = number of mice in group.

have already been published by Hoecker, Counce, and Smith, and by Amos, Gorer, and Mikulska (4, 13). E.L.4 has the H-2B complex, which has antigens B, E, and F. The A strain appears only to form anti-B, while BALB/c forms anti-B and anti-E, and C3H forms anti-B and anti-F. The H-2A complex contains antigens E and F; thus, if BALB/c anti-E.L.4 or C3H anti-E.L.4 is inoculated into A strain mice, anti-E and anti-F are absorbed, respectively, leaving anti-B. In experiments of this type the protective titer appeared unchanged. This might have been owing to residual anti-B acting alone. We therefore performed *in vitro* absorptions with E.L.4 and with C57BL liver (Table 3). A single absorption with an equal volume of packed E.L.4 ascitic cells greatly reduced the protective activity of a high-titer serum. However, five successive absorptions with equal volumes of C57BL liver left considerable residual activity in a sample of BALB/c anti-E.L.4, the drop in activity that was observed being about equal to that obtained by absorption with BALB/c liver. We therefore decided to absorb *in vivo* in C57BL.

Surprisingly, a definite protective effect was observable. Table 4 shows the result of a typical experiment. The degree of protection varies and is considerably greater with some of the sera tested. With a sufficient excess of antibody the tumors might be completely aborted. This was unusual. In unprotected mice nodules are usually palpable on the 4th or 5th day following a dose of the order of 500,000 cells, and the mice die about the 21st day or 16 days following the appearance of a palpable nodule. With 0.1 ml. of our hyperimmune sera, growths may become palpable between the 10th and 21st days, there being no overlaps between the time of onset in experimental and control groups. A significant delay has been demonstrated in over 50 C57BL. Once a tumor has become palpable the disease pursues its course unchecked, death occurring about 16 days thereafter. Thus, a mouse in which a tumor occurred on the 21st day died on the 39th day or 18 days after the appearance of the growth.

The protective effect in C57BL is more sensitive to variations in experimental procedure than is the case with the naturally resistant strains. The effect of the time of administration of antibody is shown in Table 5. Some effect was still seen with antibody administered a week previous to challenge, though less strongly than with the same serum in A strain mice (Table 2). Similarly, the effectiveness is more rapidly reduced if the serum is given after challenge, all effect being lost at 48 hours.

TABLE 3

ABSORPTION OF PROTECTIVE ANTIBODIES OF BALB/c ANTI-E.L.4 BY VARIOUS TISSUES. TESTED IN BALB/c MICE

No. mice	Un-absorbed	No antibody	Absorbed with tissue from: BALB/c fetus	C57BL fetus	BALB/c liver	C57BL liver	E.L.4
Developing tumors at 7th day	0	9	0	1	0	1	7
In group	10	10	5	10	6	6	10

TABLE 4

PASSIVE IMMUNITY AGAINST E.L.4 IN C57BL MICE

(900,000 E.L.4 Cells)

Tumor size (mean of 2 diameters in cm.) Days: 5	7	10	12	14	17	18	19	26	29	37
(0.1 ml. BALB/c anti-E.L.4)										
—	—	2.2	2.8	3.0	++	++	++	D	..	..
—	—	—	1.7	2.1	2.8	++	++	++	D	..
—	—	—	—	—	—	+	1.8	++	++	D
—	—	—	—	—	—	+	2.0	++	++	D
(Controls, no antibody)										
1.1	2.2	3.2	++	++	++	D	..	..	..	..
1.7	2.5	3.2	++	++	++	++	D	..	..	..
1.7	2.4	3.1	++	++	D	..	..	..	..	..
1.7	2.4	3.1	++	++	++	D	..	..	..	..
1.8	2.1	2.9	++	++	++	++	D	..	..	..

D = Died.
++ = Tumor too large for effective measurement.
+ = Tumor present but too small for effective measurement.

It is clear that the antibody conferring protection upon the C57BL is distinct from the H-2 antibodies. We thought it might react with an antigen present in the embryo. However, as seen in Table 3, minced embryos did not absorb the antibody. Thus far, only E.L.4 cells have given successful absorption. The antibody was not inactivated following exposure to 56° C. for 30 minutes. We have decided to refer to the antibody as anti-X and to the antigen as the X component.

X was clearly not present in demonstrable amounts in C57BL adult or fetal tissues. It is possible that it arose as a somatic mutation in an H-locus in the tumor, or by mutation in the C57BL. In this case it might be expected that antibodies against it would be developed in the C57BL. This was unlikely, since the tumor has never regressed in the C57BL strain of origin or in any F_1 hybrids between the parent strain and foreign strains in this or Dr. George Klein's laboratory (personal communication).

Alternatively, antigen X could be a tumor-specific substance. C57BL mice and the F_1 (BALB/c × C57BL) were given inoculations of E.L.4 subcutaneously, and the tumor was allowed to grow to a large size. The animals were bled and their pooled sera tested for passive immunizing activity. Injected amounts of up to 0.7 ml. had no effect on an inoculum of 360,000 E.L.4 in C57BL and 900,000 in BALB/c. Neither normal mouse serum nor serum from C57BL strongly immunized against tumors from foreign strains showed any immunizing effect in the blacks. The effect was not, therefore, specific for tumors in general.

A number of backcross mice of the constitution (BALB/c × C57BL)F_1 × BALB/c were typed for their H-2 antigens and placed in two groups, the H-2 positive group heterozygous for H-2B, and the H-2 negative group homozygous for H-2D. The H-2B positive group was given a preliminary immunizing dose of citrated C57BL blood (3). Both groups were then given progressively increasing doses of E.L.4 to produce good hemagglutinin titers in the H-2B negative group. Passive immunity experiments were performed with sera from these two groups. The passive immunizing power of both was lower than that obtained with antibodies produced in a similar manner in foreign strains. When BALB/c mice were used as indicators, complete immunity was given by 0.1 ml. of serum from the H-2B negative group against 620,000 leukotic cells. Serum from the H-2 positive group protected only half the animals. This difference could be explained if the protection in the first group was given largely by antibodies against H-2. Neither antibody would protect against 440,000 E.L.4 cells in C57BL, but the antibody from the H-2 negative group gave complete immunity against 150,000 cells. Although only two samples of antiserum were available, it appeared that the serum produced in the backcross H-2 negative animals was considerably less active than antibodies produced in a foreign strain.

Pursuing the hypothesis that there might be antigenic incompatibility between the tumor and the C57BL host that we had not been able to detect, we injected the tails of F_1 mice with varying doses of E.L.4. The tumor did not take readily in this site, in some animals local lesions appearing only after repeated inoculations. Ovarian and renal metastases were common, even after amputation of the tail or without the development of a tumor at the site of inoculation. This test provided no evidence of the development of immunity.

TABLE 5

THE DURATION OF PASSIVE IMMUNITY AGAINST E.L.4 IN C57BL MICE

A Anti-E.L.4 in C57BL Mice (500,000–750,000 E.L.4 Cells)

	0.1 ML. ANTIBODY INJECTED					
	No	Before E.L.4 days			After E.L.4 days	
No. MICE	antibody	7	2	With E.L.4 0	1	2
Developing tumor by 10th day	10	2	0	0	0	5
In group	10	4	8	5	5	5

An attempt to induce immunity by the ligation method described by Foley (8) was also unsuccessful. The tumor appeared to spread too rapidly to be isolated by this technic.

Antibodies against antigens other than E.L.4.— Antibodies were prepared in BALB/c against a suspension of minced C57BL liver and spleen suspended in streptomycin solution containing 500 μg/ml, and also against a C57BL mammary carcinoma that arose some years later than E.L.4. Both antibodies produced passive immunity against E.L.4 in BALB/c but not in C57BL, even when cell doses as small as 14,000 were employed. Evidently in this case the protection was by antibodies against H-2 and not against antigen X, which must have been absent from the mammary tumor as well as from the liver.

Neutralization tests were performed with the antibody produced in BALB/c against an A strain tumor. This should protect against E.L.4, since the C57BL and A strain share the antigen H-2E. Complete protection was given against 17,000 and 60,000 E.L.4 cells. An attempt to produce passive immunity with a similar system was unsuccessful. Experiments of this type are difficult to assess,

since the shared antigen between the three strains is not necessarily the most potent for antibody production and there is often no direct means of determining the amount of each antibody available. The further development of this problem, to attempt to assess the relative importance of the five known H-2 components and their alleles, would be a major piece of research that cannot be attempted at this time.

DISCUSSION

The difference between the immunized and nonimmunized groups in a typical experiment of the type depicted in Table 1 shows how effective circulating antibodies alone may be in preventing the growth of a tumor. This type of immunity is similar to that which can be produced against various bacteria and viruses and resembles this "classical" type of passive immunity in its failure to abort an established lesion.

With the tumor E.L.4, and probably with other leukoses, antibodies appear to have a direct effect on tumor cell growth. C57BL and F_1 animals usually develop a progressively growing tumor after the effect of the serum has worn off. BALB/c and other foreign strains do not develop a nodule at any time, presumably because the effect of the antibody is augmented by the natural defense mechanisms of the host.

The situation with respect to many other tumors (especially sarcomas) and normal tissue homografts is more complex. The vascular changes of the type recently summarized by Taylor and Lehrfeld (19) or vascular and infiltrating changes (15) are not usually met within a regressing leukotic nodule, which may be destroyed without any leukocytic infiltration (18).

The nature of the X component remains to be considered. The most obvious explanation would be to attribute X to a mutation at some H locus in E.L.4 or in C57BL. While this possibility cannot be eliminated, there are reasons for doubting that this is so. It has been found impossible to produce active immunity in C57BL or in two F_1 hybrids derived from this strain (by crossing with strains A and BALB/c, respectively). This is in marked contrast to results obtained in genetic experiments performed by us (3). In backcrosses to strains A and BALB/c, E.L.4 was highly virulent when inoculated by the intraperitoneal route, killing a large number of mice incompatible for the H-2 antigens as well as all those compatible. However, if the mice were given a small dose of C57BL blood 7–10 days prior to challenge, all the incompatible mice survived, as did the majority of those compatible for H-2. Indeed, many of the latter withstood large and repeated challenges. This shows that a relatively weak active immunization enables mice to respond effectively to minor antigenic components, the genetic picture in immunized hybrids being similar to that which might be anticipated with normal tissue transplants (3). As judged by the effectiveness of its antibody, X cannot be regarded as a minor component, and our inability to demonstrate any sign of an active response to it in C57BL or in the two F_1 generations is the more surprising. Furthermore, if X is an ordinary H antigen, it is difficult to see why actively immunized backcross animals should not form anti-X, regardless of H-2 constitution. Our data, as far as they go, show that this is not so, anti-X being demonstrable only in the sera of mice that have formed H-2 antibodies. If, for reasons just given, X is not to be regarded as an H antigen, it seems likely that X acts synergistically with the H-2 antigens.

If X is not to be regarded as an H antigen, there are numerous possibilities regarding its nature. Rather than enter into detailed speculations it seems to us desirable to attempt to ascertain whether components of this nature are frequent in leukoses, particularly in their earliest transfers. Should this prove to be so, we should have to explore the possibility that X has some etiologic significance.

SUMMARY

Published evidence suggests that circulating antibodies play a simpler and more direct role in leukotic homografts than appears to be the case with other types of tissue.

Antibodies were produced against the C57BL leukosis E.L.4 following intensive immunization of strains A, BALB/c, and C3H. An empirical measure of the efficacy of the response may be obtained from the hemagglutinin titer. With the systems described, sera giving a hemagglutinin titer of over 4,000 for A cells or over 1,000 for cells of other strains were found satisfactory. These high-titered sera injected into mice of the three resistant strains gave passive immunity to E.L.4 over a wide range of cell doses.

Injection of such sera into C57BL mice resulted in the rapid removal of all hemagglutinating activity, but a considerable degree of protection was obtainable in mice of this strain. The antibody concerned in this case was termed anti-X.

Sera produced against antigens other than E.L.4 contained hemagglutinins but no demonstrable anti-X. Such antibodies would protect mice of

heterologous strains but less efficiently than sera in which anti-X was present and failed to protect C57BL mice.

The nature of the X component is discussed. It is doubtful if it is to be regarded as the product of a mutation at an H locus. Further speculation must await data on the frequency of such components in leukoses, particularly in those of recent origin.

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