Specificity of Antileukemia Sera Prepared by Immunization with Leukemia Cells Admixed with Normal Antigen-blocking Sera

Peter J. Smith, Cynthia M. Robinson, and Arnold E. Reif

SUMMARY

Two types of antisera were tested, namely, conventional antileukemia serum (ALKS), prepared by immunization of rabbits with mouse leukemia cells, and antileukemia normal antigen-blocked serum (ALK-NABS), prepared by immunization of rabbits with a mixture of mouse leukemia cells plus normal antigen-blocking serum (NABS). The latter was raised in rabbits by immunization with normal mouse lymphoid cells. Two types of mouse leukemias were used: Gardner leukemia transplanted in C3HeB/FeJ mice and L1210-MTX murine leukemia cells transplanted in DBA/2J mice.

Compared with ALKS reagents, ALK-NABS reagents had cytolytic potencies against leukemia cells that were depressed 3- or 4-fold. Long-continued immunization was necessary to elicit increased specificity of ALK-NABS for immune cytolysis of leukemia cells relative to normal splenic lymphocytes. If only small amounts of NABS were used to prepare ALK-NABS, no increase in specificity was obtained. Small amounts of allogeneic NABS prepared against H-2 and B incompatibilities were also ineffective. Absorption of ALKS or ALK-NABS with normal mouse lymphoid cells increased their specificity against leukemia cells. However, this increase was greater for ALK-NABS, even after compensation for its lower initial cytolytic potency. Thus, ALK-NABS reagents appear to contain a higher proportion of antibodies that react with leukemia cells. In therapy experiments, ALK-NABS were less toxic, required less normal mouse tissue for absorption, and gave slightly better results than ALKS.

INTRODUCTION

For preparation of antileukemia sera with high specificity, allantoisera are far preferable to heterologous antisera, since the need to remove anti-species antibodies is avoided. However, only with heterologous antisera would the eventual extension of this work from mouse to humans be convenient (1). Happily, preparation of potent heterologous antisera to tumors is relatively simple (7, 12, 26). Use of such antisera for passive therapy must be restricted to types of tumors for which inhibition rather than enhancement of growth is usually obtained. The conditions to be avoided, which may result in enhancement, are (a) for tumor cells, a relatively low concentration of surface antigens that are recognized as foreign by the host, and (b) for antibody, that it should block most of these antigens and be administered in low concentration (13, 14, 35). As a class, leukemia cells contain a relatively large number of surface antigen sites and are therefore more likely to be inhibited than enhanced on passive therapy; certain long-transplanted and marrow-derived mouse leukemias constitute exceptions (25). Furthermore, leukemia cells are more readily accessible to antibodies than most solid tumors, even after they have disseminated.

Past studies of conventionally prepared heterologous ALKS in mouse model systems have shown the presence of modest in vitro specificity for leukemia cells relative to normal lymphoid cells (1, 12, 26). In vivo therapy of mouse leukemias by ALKS is effective only if the leukemia grows slowly or if a small inoculum of a malignant leukemia is given and therapy is begun early after its inoculation (12, 17, 24, 25). Thus, results obtained so far with ALKS have not been sufficiently positive to warrant high expectations for its use in humans, unless it can be improved substantially.

Several methods for increasing the specificity of ALKS against leukemia cells have been investigated. Induction of active tolerance toward normal tissue antigens in newborn animals has been unsuccessful when confined to H-2 antigens by ALKS (13, 14, 35). As a class, leukemia cells contain a relatively large number of surface antigens that are recognized as foreign by the host, and (b) for antibody, that it should block most of these antigens and be administered in low concentration (13, 14, 35). As a class, leukemia cells contain a relatively large number of surface antigen sites and are therefore more likely to be inhibited than enhanced on passive therapy; certain long-transplanted and marrow-derived mouse leukemias constitute exceptions (25). Furthermore, leukemia cells are more readily accessible to antibodies than most solid tumors, even after they have disseminated.

For preparation of antileukemia sera with high specificity, allantoisera are far preferable to heterologous antisera, since the need to remove anti-species antibodies is avoided. However, only with heterologous antisera would the eventual extension of this work from mouse to humans be convenient (1). Happily, preparation of potent heterologous antisera to tumors is relatively simple (7, 12, 26). Use of such antisera for passive therapy must be restricted to types of tumors for which inhibition rather than enhancement of growth is usually obtained. The conditions to be avoided, which may result in enhancement, are (a) for tumor cells, a relatively low concentration of surface antigens that are recognized as foreign by the host, and (b) for antibody, that it should block most of these antigens and be administered in low concentration (13, 14, 35). As a class, leukemia cells contain a relatively large number of surface antigen sites and are therefore more likely to be inhibited than enhanced on passive therapy; certain long-transplanted and marrow-derived mouse leukemias constitute exceptions (25). Furthermore, leukemia cells are more readily accessible to antibodies than most solid tumors, even after they have disseminated.

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In basic work on antibody formation, passive administration of antibody directed against one of several antigenic determinants of a complex antigen at the time of immunization with this antigen has been tested; antibody formation against that determinant is reduced, and antibody formation against nonsuppressed determinants is increased (3, 11, 19, 20). Such an approach has been applied to the preparation of antileu-

[1] This investigation was supported by USPHS Research Grant CA-04469 from the National Cancer Institute.

[2] Summer Research Fellow in Surgery. Present address: University of Connecticut School of Medicine, Farmington, Conn.

[Received June 21, 1973; accepted September 28, 1973.

The abbreviations used are: ALKS, antileukemia serum; NABS, normal antigen-blocking serum; ALK-NABS, antileukemia normal antigen-blocked serum; NRS, normal rabbit serum; L1210-MTX, murine leukemia cells, a methotrexate-resistant form of lymphocytic leukemia L1210.
kemia sera (15-17, 31-34). In this case, antisera to normal lymphoid cells have been injected together with the complex antigen, leukemia cells.

This study extends this work to further define optimal conditions for immunization and to determine the in vitro specificity and in vivo effectiveness of such antisera. Initial trials of additional methods of immune suppression of antibody formation against normal lymphoid cell antigens have been made. These include the use of alloantisera against H-2 and θ antigens, as well as production of low-dose tolerance by preimmunization with repeated minute doses of normal lymphoid cells. A preliminary report has been made (29).

MATERIALS AND METHODS

**Animals and Cells.** Rabbits were obtained from the Gloucester Rabbitry (Gloucester, R. 1.). Two inbred mouse strains, C3HeB/FeJ and DBA/2J (The Jackson Laboratory, Bar Harbor, Maine), were used in separate systems. Mice were of either sex and aged 5 to 12 weeks.

Gardner lymphosarcoma 6C3HED was derived in 1941 in a C3H mouse given injections of estradiol benzoate (5), and was transplanted i.p. by us in the low-mammary-tumor substrain C3HeB/FeJ (5, 24). The L1210-MTX line (8, 18) was derived in 1961 by I. Wodinsky of Arthur D. Little, Inc. (Cambridge, Mass.) and transplanted i.p. in DBA/2J mice.

Single cell suspensions of mouse thymic, lymph node, splenic, and leukemic lymphocytes were prepared and maintained at 4° (22) in dilute Locke's solution (28) and used within 2 hr. The thymus was placed in buffer, minced, and filtered through a 60 mesh stainless screen. Lymph nodes were teased apart in buffer with needles and similarly filtered. Splenics were cut, then pressed and washed through a 60 mesh screen; cell clumps were allowed to settle in a centrifuge tube for 5 min, then the top one-half of the suspension of splenic lymphocytes was collected. Leukemic cells were washed from the peritoneal cavity.

**Preparation of Heterologous NABS.** In the Gardner system, NABS was prepared by multiple immunizations of rabbits with H-2k thymocytes from C3HeB/FeJ and AKR/J mice (1). The potency of this antiserum for cytolyis of C3HeB/FeJ splenic lymphocytes was 320.

In the L1210-MTX system, each rabbit received a s.c. injection of complete Freund-McDermott adjuvant that contained approximately 40 million thymocytes and 10 million lymph node lymphocytes from DBA/2J mice. Three weeks later, 3 daily injections, each composed of the same cell inoculum suspended in buffer, were given i.v. (6). Two weeks later, the rabbits received a series of 3 weekly i.v. injections. They were bled 1 week later, and the complement content of the sera was inactivated by being heated for 30 min at 56°.

**Preparation of Allogeneic NABS.** In the Gardner system, allogeneic NABS was prepared by 6 weekly i.p. injections of AKR/J erythrocytes into C57BL/6J mice that were bled 1 week after the last injection. This antiserum reacted with all but 1 (Antigen 5) of the H-2k specificities of C3HeB/FeJ lymphocytes (30).

In the L1210-MTX system, the allogeneic NABS was prepared by a similar schedule of immunizations of C3HeB/FeJ mice with approximately 20 million DBA/2J lymphocytes (from spleen and lymph nodes) per injection. This antiserum had a cytolytic potency of 698 against DBA/2J splenic lymphocytes, and reacted with all H-2 antigens other than 3 and 8 (30). Alloantisera reacting with Antigen 3 (obtained from The Jackson Laboratory through the courtesy of Dr. George Snell and the Transplantation Immunology Branch, NIH), was added in equal volume. An equal volume of AKR/J anti-C3HeB/FeJ alloantiserum to θ-C3H (22) of potency 700 was also added (Table 3, Footnote d).

**Preparation of ALKS and ALK-NABS.** In the Gardner system, ALKS and ALK-NABS were raised in 4 groups of 3 rabbits (Tables 1 and 2). Immediately before injection, the Gardner cells were incubated with the appropriate antiserum for 30 min at room temperature. Following the immunizations, antisera were stored frozen.

When absorptions of sera were done, the divalent cations required as cofactors for the lytic activity of complement were removed by addition of 0.5 M disodium EDTA (3% of the volume of the serum). For absorptions (Tables 1 and 2), antisera and absorbing tissues were incubated for 30 min at room temperature, then were centrifuged at 2875 rpm. for 15 min at 5° in an International Model PR-2 centrifuge. Specified sera were fractionated by the addition of an equal volume of 8% saturated ammonium sulfate and then were reabsorbed (Table 2). After the absorption of all antisera, disodium EDTA was neutralized, and divalent cations were restored by the addition of a mixture containing 1.0 M MgCl2•6H2O and 0.3 M CaCl2 (6% of the volume of the serum).

In the L1210-MTX system, similar schedules of injections and absorptions were followed (Table 3).

**Low-Dose Tolerance Injections.** For attempted production of low-dose tolerance, spleen, thymus, and lymph node lymphocytes were homogenized in a Potter homogenizer (60 strokes), then were disrupted for 3 min in a DF 101 Raytheon sonic oscillator at 70% power. The supernatant obtained after centrifugation for 30 min at 3,000 X g was used. Each rabbit received material that resulted from disruption of 500,000 cells in each of 9 i.v. injections spaced over 3 weeks, extrapolating from mice to rabbits (9).

**Assay Systems.** The in vitro assay was performed in 0.3-ml volume (22). Each assay tube contained 10⁵ viable lymphocytes or leukemia cells, absorbed 10% guinea pig complement (21), and serial doubling dilutions of antisera. After incubation for 1 hr at 37°, 100 cells/tube were classified as stained or unstained. Results are expressed in terms of the "cytolytic potency," defined as the final dilution at which an antiserum produces 50% cell lysis in the assay system; for instance, an antiserum that reaches this end point at a final dilution of 1:100 has a cytolytic potency of 100 (22).

For in vivo testing, 9 to 12 mice were inoculated with the appropriate reagent (0.9% NaCl solution, ALKS, or ALK-NABS) on the 5 consecutive days following the injection of 105 leukemia cells. The statistical significance of the survival data was determined by Student's t test.

**Specificity Ratio.** We previously named a measure of relative specificity, which was already in use by Ehrlich in 1906, the "specificity ratio." We defined it as the ratio of potencies (a) of a single antiserum reacting against 2 antigens...
Table 1

Cytolytic potencies and relative specificities of ALKS produced in rabbits by immunization with G° with or without NABS

Eight i.v. injections were given over 7 weeks. Each rabbit was given 20 million G cells each time, admixed with the indicated serum. All values are the mean of 2 determinations.

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum</th>
<th>Unabsorbed</th>
<th></th>
<th>Absorbed&lt;sup&gt;b&lt;/sup&gt;</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Cytolytic potency vs.</td>
<td>Specificity ratio&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Cytolytic potency vs.</td>
<td>Specificity ratio&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>N</td>
<td>G/N</td>
<td>G</td>
<td>N</td>
</tr>
<tr>
<td>ALKS-1</td>
<td>2.0 ml normal rabbit serum</td>
<td>1220</td>
<td>1400</td>
<td>0.87</td>
<td>1030</td>
</tr>
<tr>
<td>ALK-NABS-1</td>
<td>0.2 ml rabbit anti-normal C3HeB/FeJ lymphocytes</td>
<td>1080</td>
<td>570</td>
<td>1.90</td>
<td>756</td>
</tr>
<tr>
<td>ALK-NABS-2</td>
<td>2.0 ml rabbit anti-normal C3HeB/FeJ lymphocytes</td>
<td>304</td>
<td>176</td>
<td>1.73</td>
<td>284</td>
</tr>
<tr>
<td>ALK-NABS-3</td>
<td>0.2 ml C57BL/6J anti-AKR/J erythrocyte alloantibody</td>
<td>2250</td>
<td>1840</td>
<td>1.22</td>
<td>2300</td>
</tr>
</tbody>
</table>

<sup>a</sup> G, Gardner murine leukemia cells; N, normal splenic lymphocytes.
<sup>b</sup> Absorbed with 50 million C3HeB/FeJ splenic lymphocytes per ml antiserum.
<sup>c</sup> Ratio of cytolytic potencies against 2 cell types.

Table 2

Effect of multiple absorptions on the cytolytic potencies and relative specificities of ALKS and ALK-NABS

<table>
<thead>
<tr>
<th>Absorption procedure</th>
<th>ALKS&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ALK-NABS&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytolytic potency vs.</td>
<td>Specificity ratio</td>
</tr>
<tr>
<td></td>
<td>G&lt;sup&gt;c&lt;/sup&gt;</td>
<td>N</td>
</tr>
<tr>
<td>A None</td>
<td>9630&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3660</td>
</tr>
<tr>
<td>B 1/15 volume C3HeB/FeJ packed RBC + 50 x 10&lt;sup&gt;6&lt;/sup&gt; C3HeB/FeJ lymphocytes&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2200</td>
<td>3660</td>
</tr>
<tr>
<td>C None: B was fractionated with 40% ammonium sulfate&lt;sup&gt;f&lt;/sup&gt;</td>
<td>3370</td>
<td>6160</td>
</tr>
<tr>
<td>D Procedure of C with 1/3 volume C3HeB/FeJ RBC + 80 x 10&lt;sup&gt;6&lt;/sup&gt; C3HeB/FeJ lymphocytes&lt;sup&gt;e&lt;/sup&gt;</td>
<td>3440</td>
<td>1490</td>
</tr>
<tr>
<td>E Procedure of D with 80 x 10&lt;sup&gt;6&lt;/sup&gt; C3HeB/FeJ lymphocytes&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2740</td>
<td>580</td>
</tr>
</tbody>
</table>

<sup>a</sup> Prepared like ALKS of Table 1, plus 7 additional i.v. injections.
<sup>b</sup> ALK-NABS Groups 1 and 2 of Table 1, plus 7 more i.v. injections like ALK-NABS-1.
<sup>c</sup> G, Gardner murine leukemia cells; N, normal splenic lymphocytes.
<sup>d</sup> Single values.
<sup>e</sup> Lymphoid cells contained thymic and splenic lymphs in 2:1 proportion.
<sup>f</sup> Volume of fractionated ALKS and ALK-NABS: 23 and 22% of initial volume, respectively.

or (b) of 2 antisera reacting against 1 antigen (23). In the present instance, specificity ratios have been used in Tables 1, 2, and 3 to determine the specificity of an antiserum against leukemia cells relative to its reaction against normal lymphocytes. This ratio is only a relative value that depends upon the individual susceptibility of each of the 2 cells for immune cytolysis.

**Specificity Index.** We first suggested use of the specificity index (23) as a single dimensionless measure of the specificity of 2 antisera (for instance, ALK-NABS and ALKS) each reacting against the same 2 antigen complexes (in this case, leukemia cells and normal lymphocytes). The specificity index is independent of the potency of the antisera and of the methods used to determine potency, provided 2 conditions are fulfilled. These are that the assays are proportional in the sense that doubling the concentration of antiserum doubles the reaction observed, and that the same assay is used to determine the potency of both antisera against any 1 antigen complex (23).

**RESULTS**

**Experiments with Gardner Leukemia.** The objective of all the immunizations reported (Tables 1 to 3) was to obtain antisera with high cytolytic potencies and high specificities.
Table 3

Cytolytic potencies and relative specificity of rabbit antisera to L1210-MTX prepared by different regimens with or without various NABS

Eight i.v. injections were given over 4 weeks. Each rabbit was given 25 million L1210-MTX cells each time, admixed with the indicated serum.

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum</th>
<th>Unabsorbed</th>
<th></th>
<th></th>
<th>Absorbed&lt;sup&gt;a&lt;/sup&gt;</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cytolitic</td>
<td>Specificity</td>
<td>Cytolitic</td>
<td>Specificity</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>potency</td>
<td>ratio</td>
<td>potency</td>
<td>ratio</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>L</td>
<td>N</td>
<td>L/N</td>
<td>L</td>
<td>N</td>
</tr>
<tr>
<td>ALK-NBS-a</td>
<td>0.3 ml NRS</td>
<td>1190</td>
<td>1350</td>
<td>0.88</td>
<td>868</td>
<td>1040</td>
</tr>
<tr>
<td>ALK-NBS-a</td>
<td>0.3 ml rabbit anti-DBA/2J lymphocyte serum&lt;sup&gt;b&lt;/sup&gt;</td>
<td>390</td>
<td>528</td>
<td>0.74</td>
<td>302</td>
<td>336</td>
</tr>
<tr>
<td>ALK-NBS-b</td>
<td>0.3 ml rabbit anti-DBA/2J lymphocyte serum&lt;sup&gt;c&lt;/sup&gt;</td>
<td>454</td>
<td>655</td>
<td>0.69</td>
<td>362</td>
<td>524</td>
</tr>
<tr>
<td>ALK-NBS-c</td>
<td>1.5 ml rabbit anti-DBA/2J lymphocyte serum</td>
<td>390</td>
<td>622</td>
<td>0.63</td>
<td>236</td>
<td>92</td>
</tr>
<tr>
<td>ALK-NBS-d</td>
<td>0.3 ml normal DBA/2J mouse serum</td>
<td>1290</td>
<td>4040</td>
<td>0.29</td>
<td>1060</td>
<td>2880</td>
</tr>
<tr>
<td>ALK-NBS-d</td>
<td>0.3 ml mouse antigens antiserum to H-2 and θ antigens&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1020</td>
<td>2160</td>
<td>0.47</td>
<td>720</td>
<td>1560</td>
</tr>
</tbody>
</table>

<sup>a</sup> Thirty million splenic and thymic lymphocytes were used per ml antiserum for all absorptions, plus 1/15 volume of whole DBA/2J blood.

<sup>b</sup> Hyperimmune rabbit antiserum to DBA/2J thymocytes and lymph node lymphocytes; the cytolytic potency against DBA/2J splenic lymphocytes was 10,590.

<sup>c</sup> Preinjection with 0.3 ml of the same antiserum given 1 day before each injection.

<sup>d</sup> A mixture of alloantisera reacting with all DBA/2J H-2 specificities except Antigen 8, and with θ-C3H.

against leukemia cells. The cytolytic potency (defined in "Assay Systems") denotes the strength of an antiserum for immune cytolysis of a specified cell type. The specificity of an antiserum against leukemia cells relative to its reaction against normal splenic lymphocytes is expressed by the specificity ratio (Table 1, Footnote c); a comparison of the values of this ratio for 2 different antisera gives an even more meaningful measure of their relative specificities (see "Materials and Methods").

Contrasted with antiserum for the 3 ALK-NBS groups harvested after 8 immunizations (Table 1), those obtained after 4 immunizations had relatively low cytolytic potencies. There was no increase in the specificities of these sera compared with that of the conventional ALKS. Additional immunizations were necessary to elicit such an increase (Table 1).

Before absorption, the ALK-NBS-2 group, prepared with a high dose (2.0 ml) of NABS had an approximately 4-fold lower cytolytic potency against Gardner leukemia cells than antisera from the other groups (Table 1, Column 1). However, the specificity ratios for this and for ALK-NBS-1 were twice as high as for the conventionally prepared ALKS (Table 1, Column 3). After absorption with splenic lymphocytes, the cytolytic potencies against Gardner leukemia were only slightly depressed (Table 1, Column 4), but the specificities of all 3 ALK-NBS reagents were approximately doubled (Table 1, Column 6).

Antisera prepared as described in Table 1 (ALKS and ALK-NBS-1) and absorbed with 66 million C3HeB/FeJ splenic lymphocytes per ml antiserum were tested for therapy of Gardner leukemia (Chart 1). The 2 control groups, 0.9% NaCl solution and NRS, had identical survivals, 15.0 days after inoculation. The 2 experimental groups, ALKS and ALK-NABS, had greatly extended survivals, 23.8 and 25.4 days, respectively, that were not significantly different from each other (0.1 < p < 0.2). However, in the ALKS group there were 3 toxicity deaths without presence of widespread leukemia (as substantiated by autopsy) that are not included in this survival figure. In contrast, in the ALK-NBS group, there were 2 long-term (over 100 days) survivors. These data are suggestive of lower toxicity and higher effectiveness for ALK-NBS.

There were no deaths in toxicity control experiments, in which the same doses of antiserum (Table 1) were injected

![Chart 1. Therapy trial of conventional ALKS and ALK-NABS to Gardner leukemia (G. LEUK.) in C3HeB/FeJ mice. There were 9 or more mice in all groups. Therapy injections of 0.4 ml absorbed antiserum with known cytolytic potencies against Gardner leukemia and normal splenic lymphocytes (N. SP. LY.) (see inset) were given i.p. on Days 1, 2, 3, 4, and 5 following the i.p. inoculation of 10⁶ Gardner cells; saline, 0.9% NaCl solution.](chart1.png)
without prior injection of leukemia cells. Therefore, it appears that the toxicity of antisera can be higher in the presence of leukemia cells.

Antisera harvested after further immunizations (Table 2, Footnotes a and b) were retested. Again, the cytolytic potency of unabsorbed ALK-NABS was more than 3-fold lower than that of the conventional ALKS (Table 2, Line A). Absorption (Table 2, Line B) decreased the cytolytic potencies of both antisera against Gardner leukemia cells approximately 4-fold. These absorbed antisera were then fractionated with ammonium sulfate and reconstituted to equal cytolytic potencies against Gardner leukemia cells (Table 2, Line C). More thorough absorption (Line D) had little effect on the cytolytic potencies of both antisera against leukemia cells, but disproportionately decreased the potency of the ALK-NABS against normal lymphocytes to the point at which this serum had shown no toxicity in the assay. Additional absorption (Line E) increased the specificity of the conventional ALKS.

A 2nd therapy experiment (Chart 2), performed with such fractionated and absorbed antisera (Table 2, Line C), showed that ALKS reagents were far more toxic than ALK-NABS reagents that had essentially identical cytolytic potencies against Gardner leukemia cells. Compared with a mean survival of 15.8 days for the 0.9% NaCl solution control group, the 2 ALKS groups had very significantly curtailed mean survivals of 7.1 and 5.3 days ($p < 0.001$ in each case). The deaths in these groups, as substantiated by autopsy, were unrelated to dissemination of leukemia cells. For mice in the ALK-NABS groups, the mean survival times were very significantly prolonged, namely 21.1, 20.9, and 21.3 days in order of increasing dosage ($p < 0.001$ in each case); in addition, there were, respectively, 0, 1, and 2 long-term survivors (over 100 days) in these 3 groups, and there was 1 death from either toxicity or anesthesia at the highest dosage of ALK-NABS (Chart 2). A further increase in dosage of ALK-NABS (Chart 3) decreased the survival time of the experimental group to a value (15.5 days) not significantly higher ($p > 0.80$) than that of the control group (14.2 days).

**Experiments with L1210-MTX.** To validate the above results, similar experiments (Table 3) were done with L1210-MTX. As for Gardner leukemia, the cytolytic potencies of ALK-NABS reagents (Table 3, ALK-NABS-a, -b, and -c) against L1210-MTX were approximately 3-fold lower than that of the conventional ALKS-a. Substantial specificity against leukemia cells appeared only after absorption of the antiserum prepared with the highest dose of NABS (ALK-NABS-c). ALK-NABS prepared with the use of allologic NABS showed decreased rather than increased specificity against leukemia cells, possibly due to increased reactivity against immunoglobulin molecules on the surface of normal splenic lymphocytes (Table 3, ALKS-b and ALK-NABS-d).

**Low-Dose Tolerance.** An attempt was made to use low-dose tolerance for the present purpose. To produce tolerance, rabbits were given preinjections of very small quantities of lyses of normal DBA/2J lymphocytes (see "Materials and Methods"). The rabbits were then immunized as for production of ALKS or of ALK-NABS (exactly as for ALKS-a and ALK-NABS-a of Table 3, except 0.9% NaCl solution was substituted for NRS). The resultant ALKS had potencies of 2550 against leukemia cells, and 1620 against normal splenic lymphocytes, which dropped to respectively 1250 and 1490 after absorption (Table 3, Footnote a); these potencies are approximately 50% higher than for the conventional ALKS (Table 3, ALKS-a). In contrast, ALK-NABS had a potency of 29.9 against L1210-MTX and 34.8 against splenic lymphocytes, which values dropped too low (less than 3.0) for measurement after identical absorption; these potencies are less than one-tenth those for the conventional ALKS (Table 3, ALK-NABS-a). Rabbits given preinjections but not immunized with leukemia cells had potencies of 35.0 and 41.9, respectively, against the same 2 cell types; these potencies dropped to less than 3.0 and to 4.0, respectively, after absorption.

**DISCUSSION**

This study continues the work of Motta (15–17), Drake et
al. (4), Ungaro et al. (31–33), and Weiner et al. (34). To facilitate comparisons, we shall retain use of the present terminology and abbreviations. Thus, conventional antileukemia sera are called ALKS, and antisera produced by immunization with leukemia cells plus normal antigen-blocking serum (NABS) are called ALK-NABS.

Use of the specificity index permits incisive comparison of the results obtained in this and previous studies. It is the quotient of the specificity ratios of 2 antisera (in our case, ALK-NABS and ALKS) in their reaction against leukemia cells as compared to their reaction against normal lymphocytes (see "Materials and Methods"). For instance, for work with L1210-MTX, the specificity index was 0.63/0.88 or 0.72 before absorption, and 2.4/0.84 or 2.9 thereafter (Table 3, quotients of specificity ratios for ALK-NABS-c and ALKS-a).

Motta (15), in work with a rat anti-mouse leukemia model (he used Friend virus-induced splenic leukemia) found that the cytolytic potency of his ALK-NABS was depressed 4-fold, compared with ALKS, and that there was no difference in specificity between his 2 antisera (specificity index, 0.85). When he used rabbit rather than rat antisera, Motta (15) obtained similar cytolytic potencies and a specificity index of 3.1 for the 2 antisera. While the data of Motta are similar to those obtained in this study with L1210-MTX, our results suggest that extended immunizations might have increased the specificity index of his rat antisera.

Weiner et al. (34), who worked with a rabbit anti-mouse leukemia EL4 system, found 4-fold higher cytolytic potencies and a specificity index above 50 for ALK-NABS as compared to ALKS. The reason for these highly positive results may be that, compared to our study, 40-fold higher cell dosages and 10-fold higher quantities of NABS were used for each immunization. It is also possible that their good results are due in part to a relatively high antigenic disparity between EL4 leukemia and splenic lymphocytes, since this long-transplanted leukemia was carcinogen induced, and since splenic lymphocytes may not (or may no longer) be the exact normal counterpart of EL4 cells. Analogous reasons may explain why we obtained far higher specificities with Gardner leukemia than with L1210-MTX in this study.

When burro antisera to EL4 leukemia were tested (31), the results closely resembled those reported here. The cytolytic potency of unabsorbed ALK-NABS was over 4-fold depressed compared with that of conventional ALKS, and the specificity index was only 3.8. Production of a new type of ALK-NABS, by formalin treatment of tumor cells after incubation with NABS, has also been attempted (32). The cytolytic potency of such new ALK-NABS reagents was only 1.8-fold higher than that of ALK-NABS produced without use of formalin fixation. Compared with conventional ALKS, the new ALK-NABS had almost 4-fold lower cytolytic potencies and a specificity index of only 0.8. Thus, the present analysis raises doubts as to the efficacy of such formalin treatment.

An additional report (33) that in vivo therapy results are improved by use of antisera of high cytolytic potencies confirms the conclusions of previous work (17, 24). However, use of ammonium sulfate precipitation to concentrate antibody (33) always entails some loss in total cytolytic antibody activity; in our experience, recovery usually runs between 30 and 60%. Furthermore, the present study shows that the dose-response curve for administration of a partially toxic reagent such as ALK-NABS may have a maximum beyond which therapeutic benefit is reduced (Charts 2 and 3).

The attempt to utilize low-dose tolerance for preparation of ALK-NABS was flawed by our failure to show directly that low-dose tolerance was attained; this would have required a challenge with the cell type used for the preinjections. Nevertheless, the ALK-NABS raised following the tolerogenic preinjections had 10-fold lower cytolytic potencies than the conventional ALK-NABS; this constitutes indirect evidence that tolerance was indeed attained. In contrast, the ALKS raised after tolerogenic preinjections had 50% higher cytolytic potencies than the conventionally raised ALKS; however, the 2 antisera were not exactly comparable, since NRS rather than 0.9% NaCl solution was admixed with leukemia cells in preparation of the latter. Since this lack of parallel seems insufficient to explain the conflicting result obtained for tolerogenically prepared ALKS, the preliminary experiment reported here requires confirmation.

There is general agreement that the antileukemia specificity of ALK-NABS reagents is improved by their absorption with tissue cells that are the normal counterparts of the leukemia cells under investigation. Previous (15, 31, 34) and present (Table 2) results suggest that production of active ALK-NABS that are almost nontoxic is a possibility. However, neither here nor previously has the toxicity of such sera been tested against a spectrum of normal lymphoid cell populations and subpopulations. Neither have we tested whether repeated absorption with normal cells will remove all reactivity against normal cells, yet leave residual potency against leukemia cells; this has been possible in additional experiments (to be reported later), with the caution that "removal" of reactivity means only that reactivity cannot be detected at the level of sensitivity provided by the assay. Furthermore, absorption with tissue homogenates rather than with single cell suspensions can introduce toxic factors (possibly including unesterified fatty acids) into the antisera. Experiments on this topic are in progress.

There is an indication that NABS might be effective through the masking of normal cellular antigenic determinants, rather than due to a central depressive effect (32). There is also a suggestion that NABS should contain enhancing antibodies (2) for production of optimal ALK-NABS. In addition, clarification of the complexities of the different roles (4) played by both autologous and passively administered heterologous complement in mediating the action of passively administered heterologous antibody (17, 35) seems in order. Finally, for present data on the specificity of various antisera against leukemia cells relative to normal splenic lymphocytes, we have no information whether the antigens on the leukemia cells that give rise to specificity are tumor associated, or whether they are present because of differences in cell type not associated with oncogenesis or due to minor histocompatibility differences.

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Specificity of Antileukemia Sera


Specificity of Antileukemia Sera Prepared by Immunization with Leukemia Cells Admixed with Normal Antigen-blocking Sera

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