Immunization Schedules for Potent Rabbit Antisera to Leukemia L1210

Chung-Ai H. Kim and Arnold E. Reif

Department of Surgery, Tufts University School of Medicine, and the First (Tufts) Surgical Service, Boston City Hospital, Boston, Massachusetts 02118

SUMMARY

The immunization of rabbits with cells of mouse leukemia L1210 has been studied. The immune process was dissected into its γM and γG components. The main objective was the preparation of antisera with high cytolitic potencies.

A single i.v. injection of viable L1210 cells produced a classical immune response in rabbits, with γM antibody activity first detected on Day 4, maximal on Day 6, and diminishing thereafter; in contrast, γG activity was detected on Day 8, was maximal around Day 14, and diminished thereafter. Interestingly, additional immunizations gave new peaks of γM activity, each smaller than the previous one. As expected, γG activity rose sharply after secondary immunization and more gradually with further immunizations.

For production of highly potent cytolytic antisera, repeated i.v. injection of large numbers of viable leukemia cells was the most effective procedure tested. Immunization with homogenized or sonically disrupted cells was significantly inferior. Evidence for immune exhaustion after long-continued immunization was obtained.

INTRODUCTION

Attempts to treat leukemia by passive administration of heterologous antisera have been made both in experimental animals and in man (3-5, 7, 10, 12, 13, 15-17, 22-25, 29). With one exception (3), mild but significant therapeutic benefits have been obtained in experimental animals (7, 12, 16, 17, 22, 23, 25). While clinical trials with antisera to leukemia cells or with antilymphocyte globulin are currently proceeding at a number of laboratories (5, 15, 24, 29), it remains questionable whether such antisera have substantial therapeutic potentials in man, especially since antilymphocyte globulin possesses strong immunosuppressive properties (26).

We recently found that when a rabbit antiserum prepared against mouse leukemia cells is injected into mice carrying that leukemia there is a direct relationship above a certain threshold between the number of cytolytic potency units injected and therapeutic effectiveness (22). This finding suggested that passive therapy with antiserum can give positive results only when the antiserum has sufficiently high cytolytic strength and is administered in sufficient amount (22); similarly, Mohos and Kidd stressed the need for high cytolytic potency in antisera which are to be used for passive immunotherapy of tumors (17). Unfortunately, much of the more recent experience (9, 18, 30) in production of the closely related ALS 3 is irrelevant, since ALS is raised to be effective for allograft prolongation in vivo, rather than to be strongly cytolytic, and there is poor correlation between these two properties (2, 8, 9).

The in vivo therapeutic potential of rabbit antiserum to L1210 leukemia cells has already been demonstrated (16, 22, 23), and the in vitro specificity of such antiserum to thymic, splenic, and leukemic lymphocytes has been investigated (1). The present study was therefore confined to attempts at production of highly cytolytic rabbit antiserum to Leukemia L1210. For this purpose, immunization schedules that differed in amount of tissue, route of administration, and disruption of cells were studied; where pertinent, the immune response was dissected into its γG and γM antibody components.

MATERIALS AND METHODS

Mice and Leukemia. All mice were obtained from the Jackson Laboratory. The lymphocytic Leukemia L1210 (11) was transplanted i.p. in DBA/2 mice. Leukemia cells were harvested 6 days after i.p. transplantation of approximately 300,000 cells. Erythrocytes present were destroyed by hypotonic lysis; 1.5 volumes distilled water were added slowly with stirring at room temperature. After stirring further for 10 min, concentrated buffer was added to restore isotonicity. Cells were washed in isotonic Locke's buffer (20), resuspended in buffer, and standardized by hemacytometer count.

Cytolysis Assay System. A small scale assay system (21) was used. In outline, each assay tube contained 100,000 viable lymphocytes, suitably absorbed 10% complement (19), and various dilutions of antiserum. Tubes were incubated for 1 hr at 37° and placed in ice water. For each tube in turn, the supernatant was sucked off the cells that had settled, 0.02 ml of vital dye was added, and 200 cells were classified under the microscope as stained or unstained. Results, in percentage of stained cells, were plotted against final antiserum concentration (%), and the cytolytic titer was read at 50% stained cells, with a correction for stained cells in the control tube (19). The potency of an antiserum was expressed as 100 divided by the cytolytic titer obtained in this assay system (19, 21).

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2 Fellow in Surgery.

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The abbreviation used is: ALS, antilymphocyte serum.
Separation of $\gamma G$ and $\gamma M$ Antibodies. The 7 S and 19 S antibodies were separated by centrifugation for approximately 20 hr at 112,000 \( \times g \) (28) in a horizontal rotor (SB-283) of a Model B-60 ultracentrifuge (International Equipment Co., Boston, Mass.). The rabbit antiserum to be separated was diluted with 4 parts 10% sucrose, and 1.0 ml was layered on 11.0 ml of a continuous gradient of 10 to 40% (w/v) sucrose in 0.9% NaCl solution. The gradient was formed from the bottom upward with a finger pump (Model 600-1200, Harvard Apparatus Co., Dover, Mass.). After centrifugation, the bottom of the tube was punctured with a 20-gauge hypodermic needle, and 0.5 ml fractions were collected with the finger pump. The fractions were tested for light absorption at 280 nm in microcuvets (Chart 1) to determine the point of separation between $\gamma M$ and $\gamma G$ antibodies (28).

RESULTS

Immunization Experiment A. Three rabbits were each given i.v. injections of 30 million L1210 cells in a suspension of 0.9% NaCl solution. The same quantity of cells was injected again on Days 50, 87, 89, 99, 101, 106, and 108. The rabbits were bled at various times during this course of immunization. At each time point, equal volumes of sera from each of the 3 rabbits were pooled. Sera were separated into $\gamma G$ and $\gamma M$ fractions by ultracentrifugation in a continuous sucrose gradient (see "Materials and Methods"). These fractions contained all proteins present in the whole antiserum, except those contained in the single tube discarded at the minimum of absorbance (Chart 1). Each fraction could be tested directly for cytolytic antibody activity against L1210 cells without removing sucrose by dialysis.

The results obtained with a single injection of L1210 cells (Chart 2) show a classical primary immune response. The $\gamma M$ antibody was first detected on Day 4, was maximal on Day 6, and diminished thereafter; in contrast, $\gamma G$ antibody was first detected on Day 8, was maximal on or after Day 14, and then declined. Secondary immunization on Day 50 produced a new peak of $\gamma M$ antibody activity, but this was smaller than after primary immunization; in contrast, $\gamma G$ antibody activity was raised to a very much higher peak as early as 5 days after the secondary immunization. Further hyperimmunization resulted in new peaks in $\gamma M$ activity, but each peak was lower than the previous one; in contrast, $\gamma G$ activity continued to increase and was maximal after the last (8th) immunization (Chart 2).

Immunization Experiment B. Twenty-four rabbits were divided into 6 groups of 4, designated A to F, and immunized with L1210 cells by different procedures (Table 1). For the i.v. immunizations, each rabbit received the number of viable L1210 cells recorded in Footnote a of Table 1, or their equivalent in homogenized cells (3 min in a cooled semimicro container of a Waring Blendor), or sonically disrupted cells (3 min at 70% power in a water-cooled Raytheon Model DF101 sonic oscillator).

The potencies of the resulting antisera were determined by immune cytolysis against L1210 cells. On Day 25, 1 week after the 2nd immunization with L1210 cells, the antibody response of rabbits in Group C, injected with homogenized cells, was much lower than that of other groups. However, after immunization with 6 further i.v. injections and/or 1 further i.m. injection of adjuvant-dispersed cells, the cytolytic potencies of sera from all groups of rabbits converged (Table 1).

The antibody activity of sera from rabbits given injections of leukemia cells incorporated in Freund-McDermott adjuvant (Groups E and F) was dissected into its $\gamma M$ and $\gamma G$ components. For rabbits in Group E, all the cytolytic antibody activity of serum taken on Day 6 was present in the $\gamma M$ fraction. For the serum of Day 18, 52% of the antibody activity was in the $\gamma M$ fraction, and the balance was in the $\gamma G$ fraction;
Table 1

<table>
<thead>
<tr>
<th>No. of rabbits at end of experiment</th>
<th>State of L1210 cells injected</th>
<th>Route</th>
<th>Immuonization 1</th>
<th>Immuonization 2</th>
<th>Immumizations 3–8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Day 11&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Day 18&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>A 3</td>
<td>Whole cells, 1 dose/day</td>
<td>i.v.</td>
<td>46</td>
<td>47</td>
<td>23</td>
</tr>
<tr>
<td>B 3</td>
<td>Whole cells, 3 divided doses&lt;sup&gt;d&lt;/sup&gt;</td>
<td>i.v.</td>
<td>47</td>
<td>64</td>
<td>24</td>
</tr>
<tr>
<td>C 4</td>
<td>Homogenized whole cells</td>
<td>i.v.</td>
<td>8</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td>D 3</td>
<td>Sonically disrupted whole cells</td>
<td>i.v.</td>
<td>34</td>
<td>136</td>
<td>42</td>
</tr>
<tr>
<td>E 2</td>
<td>Freund-McDermott adjuvant only</td>
<td>i.m.</td>
<td>30</td>
<td>78</td>
<td>64</td>
</tr>
<tr>
<td>F 4</td>
<td>Whole cells + Freund-McDermott adjuvant</td>
<td>i.v. + m.p.</td>
<td>60</td>
<td>103</td>
<td>84</td>
</tr>
</tbody>
</table>

<sup>a</sup> Immunizations i.v., each with approximately 40 million L1210 cells, were given on Days 0 (initial injection), 19, 26, 28, 30, 33, 35, and 37. Injections i.m. of approximately 40 million L1210 cells incorporated in complete Freund-McDermott adjuvant (Groups E and F) were given on Days 0, 19, and 35. For multiportal injections (m.p.), the total dose of approximately 40 million L1210 cells incorporated in adjuvant was divided between i.m., s.c., i.p., and foot pad sites.

<sup>b</sup> Mean of two determinations made on different days on sera pooled for each group.

<sup>c</sup> After first immunization with L1210 cells.

<sup>d</sup> The same dose of L1210 cells used for Group A was divided into 3 equal parts and injected separately, spaced over 24 hr.

Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>State of injected L1210 cells</th>
<th>No. of L1210 cells injected each time&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Rabbits alive at end of experiment</th>
<th>Cytolytic potencies&lt;sup&gt;b&lt;/sup&gt; of antisera</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>Whole cells i.v.</td>
<td>20 million</td>
<td>a</td>
<td>2426 1933</td>
</tr>
<tr>
<td>H</td>
<td>Whole cells i.v.</td>
<td>60 million</td>
<td>b</td>
<td>1440 3352</td>
</tr>
<tr>
<td>I</td>
<td>Whole cells i.v.</td>
<td>180 million</td>
<td>c</td>
<td>5380 2280</td>
</tr>
<tr>
<td>J</td>
<td>Whole sonically disrupted cells</td>
<td>60 million</td>
<td>d</td>
<td>2395 8210</td>
</tr>
<tr>
<td>K</td>
<td>Supernatant after sonic disruption, centrifugation at 1000 x g</td>
<td>60 million</td>
<td>e</td>
<td>7190 8720</td>
</tr>
<tr>
<td>L</td>
<td>Homogenized, then treated as for K</td>
<td>60 million</td>
<td>f</td>
<td>8210 2085</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>g</td>
<td>8710 2540</td>
</tr>
<tr>
<td></td>
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<td>h</td>
<td>8210 2540</td>
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<td>j</td>
<td>8720 2540</td>
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<td>k</td>
<td>2085 2540</td>
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<td>m</td>
<td>2655 2253</td>
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<td>o</td>
<td>1020 1344</td>
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<td></td>
<td>p</td>
<td>1610 1403</td>
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<tr>
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<td></td>
<td></td>
<td>q</td>
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</tbody>
</table>

<sup>a</sup> All injections were given i.v. twice weekly for 3 weeks.

<sup>b</sup> Mean of two separate determinations. Rabbits were bled 10 days after the last injection.

for the serum of Day 25, only 14% was in the γM fraction. Since similar results were obtained for Group F, it appears that the γM response of rabbits is similar, whether immunized i.v. with viable cells or i.m. with adjuvant-dispersed cells.

Immunization Experiment C. Further experiments were done to test whether injection of rabbits with large numbers of leukemia cells increased the degree of immunization obtained. Also, the initially depressed cytolytic potencies that were obtained by injection of homogenized cells (Group C, Table 1) required investigation. As an aid to statistical evaluation, the cytolytic potencies of sera from individual rabbits were determined.

Six groups of rabbits were immunized with varying numbers of viable L1210 cells or with a constant number of L1210 cells disrupted in different ways. The results obtained (Table 2) were tested for statistical significance by Student's t test. With regard to cell dose, 6 injections of 180 million viable L1210 cells gave a significantly greater cytolytic potency than injection of 20 million cells (p = 0.05) or 60 million cells (p < 0.001). Immunization with disrupted cells (Groups J, K, and
were retested. When the results obtained (Table 3) were evaluated by the analysis of variance, the overall findings were nonsignificant. However, the progression of mean results obtained for the 6 groups of rabbits appeared to be geometric upon repetition of an analysis of variance following such transformation, the potencies of antisera from rabbits initially immunized with viable cells (Groups G, H, and I) were significantly greater (0.01 < p < 0.025) than for rabbits immunized with disrupted cells (Groups J, K, and L). These results further confirm the findings (Table 2) that viable cells are more immunogenic than disrupted cells.

The 7 rabbits with the highest cytolytic potencies (Table 3) were further immunized on Days 5, 7, 19, 21, 33, 35, 47, 49, 61, 63, 75, 77, 89, 91, 117, and 119 after this bleeding. Between 40 and 282 million viable L1210 cells were injected i.v. each time. Approximately 40 to 50 ml of whole blood were taken from each rabbit by cardiac puncture on days 13, 18, 42, 47, 56, 69, 89, and 130. The sera of Rabbits a, d, f, g, h, j, and l, obtained on Day 130, had mean cytolytic potencies (determined in duplicate experiments) that were, respectively, 24, 24, 51, 56, 76, 57, and 35% of the potencies on Day 0 (Table 3); the mean reduction in cytolytic potency for all rabbits was 46%.

The above experiments suggested the presence of “immune exhaustion.” To check this further, the same rabbits were immunized with 150 ± 30 million viable L1210 cells, given i.v. on each of 6 occasions spaced evenly over 3 weeks, without intermediate bleedings. Sera were collected 7 days after the last injection or 158 days after harvest of the sera evaluated in 18, 42, 47, 56, 69, and 89. All rabbits that survived were given 2 further i.v. injections of 140 million viable L1210 cells twice in 1 week and were bled 7 days after the last injection.

Table 3. Compared to the latter, the cytolytic potencies of sera from the surviving Rabbits a, d, f, g, h, and j were changed by -64, -40, +70, -48, +6, and +14%, respectively (mean of duplicate experiments). Thus, following intensive hyperimmunization, antibody activity decreased in 3 rabbits, increased in 1 rabbit, and was substantially unchanged in 2 rabbits. Taken together with the data in the previous paragraph, these results substantiate a mild and individually variable degree of immune exhaustion in rabbits subjected to courses of long-continued immunizations with intermittent bleedings.

**DISCUSSION**

This study has illustrated that the immune response of rabbits given i.v. injections of mouse leukemia cells follows a classic pattern (27). There is an early γM response, followed quickly by a γG response, which shuts off γM production (28). In addition, a far less widely known facet of the immune response has been illustrated: repeated i.v. injections cause new but gradually diminishing peaks of γM antibody activity (Chart 2).

We previously found that a strong correlation exists between the in vitro cytolytic potency of heterologous anti-leukemia sera and their in vivo therapeutic effectiveness against mouse leukemias (22). Since the cytolytic activity of γM antibody produced by i.v. injection was highest after the very first immunization, and even then was only about 3 to 5% of the maximal cytolytic activity of γG antibody produced after hyperimmunization, it seems unlikely that rabbit γM antibody can be used successfully for in vivo therapy of mouse leukemias. Further, the distribution of γM and γG antibody activity was similar after i.m. immunization with leukemia cells incorporated in complete Freund-McDermott adjuvant. Hence, we know of no method of immunization which would produce a high level of γM cytolytic antibody activity in rabbits. Similarly, γG, rather than γM, appears to be the active antibody in ALS (14, 30).

The most effective procedure for preparation of ALS, as evaluated in vivo by skin allograft survival, is i.v. injection of viable lymphoid cells, plus foot pad injection of cells incorporated in complete Freund's adjuvant (30). In contrast, the in vitro leukagglutination titer of rabbit antisera was essentially unchanged when injection of adjuvant-suspended cells was omitted (30). Similarly, in the present study, we failed to obtain statistically significant evidence for increased cytolytic potency for sera from rabbits that received adjuvant-suspended cells in addition to their immunization by the i.v. route. With regard to future work, use of leukemic lymphocytes cross-linked with 1,5-difluoro-2,4-dinitrobenzene may produce further increases in the potency of the resulting antisera (6). Most desirable would be the chemical purification of leukemia-specific cell surface antigens for use in immunizations.

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