Antibody-producing cells: Virus-induced alteration of response to antigen

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** ABSTRACT **
Spleen antibody-forming cells of mice yield a 3- to 10-fold increase in their response to sheep erythrocyte antigen if they are acutely infected by lactate dehydrogenase-elevating virus. This early stimulation is replaced by a long-term inhibition of the antibody-forming cells as the viremia goes into its persisting chronic stage. These contrasting immunological phenomena are examined as contributing factors responsible for the enhancement by this virus of asparaginase (EC 3.5.1.1; L-asparagine amidohydrolase) therapy against leukemia in mice, and for the alteration of the susceptibility of mice to various neoplastic processes.

This report describes the modifications by a benign virus infection (1-5) of certain elements of the immune apparatus of mice. The data demonstrate that a persisting benign virus infection is capable of impairing the reproduction, or activation, of antibody-forming cells (AFC). In contrast, a significant but ephemeral stimulation of AFC was associated with the early acute phase of infection by the lactate dehydrogenase-elevating virus (LDH-virus).

These influences on AFC are coupled, by inference, to the more general effects of the LDH-virus in potentiating certain types of oncogenesis (6). The short-term stimulation of AFC by the LDH-virus in its acute stage may explain the immunological adjuvant effects ascribed to the virus under some circumstances (6-8). Two other effects induced by the virus are relevant to oncogenic and immunological studies. One involves the potentiation of cancer therapy when certain enzymes are used; the other promotes malignant processes.

The presence of the LDH-virus greatly enhances asparaginase (EC 3.5.1.1; L-asparagine amidohydrolase) therapy in mice (9-12, 36). Although several contributing factors are involved in this therapeutic phenomenon (13-15), an important effect appears to be the delayed appearance of effective antibodies against the administered asparaginase. This enzyme has potent antileukemic properties but generates an undesirable neutralizing immunological response in the recipient, either mouse or patient, as expected for any foreign protein (14, 15). The data reported here provide a reasonable explanation for an impairment of antibody production by a long-term suppression of B-cell activity by the chronic viral infection, and thus by inference, a delay or reduction in humoral antibody production against asparaginase.

This capability, in addition to its demonstrated suppression of T-cells and the induction of thymus involution through the elevation of plasma adrenal corticoids (16-18), identifies the LDH-virus as a useful tool in the study of immunological competence and surveillance processes, and in the search for subtle infectious and physiological factors capable of modifying oncogenic and host processes. Because of the ubiquitous distribution of the LDH-virus, and its frequent contamination of mouse tumors and murine virus preparations, the hazard exists that spurious interpretations may be made from data obtained through experimental systems that unknowingly harbor this inconstant agent. Either direct or indirect effects of the virus may thus be erroneously assigned to the action of implanted tumors or to administered oncogenic viruses or other infectious agents (22, 23).

** MATERIALS AND METHODS **

Localized Hemolysis in Gel Assay. Spleen antibody-forming cells were detected and enumerated by standard Jerne plaque techniques (19) carried out essentially as described (20). Only direct IgM (19 S) antibody plaques were assayed.

Antigen. Fresh sheep erythrocytes (sRBC) were suspended in Alsever's solution (Totum Lab Products, or Colorado Serum Co.) and were washed three times by centrifugation in 0.9% saline. They were diluted to a standard concentration of 10⁶ cells per ml, as determined by the Coulter Counter, and 0.1 ml, or 10⁶ sRBC were injected intraperitoneally into mice at appropriate times. The data of Table 1 were obtained from mice that received the sRBC intravenously. Similar sRBC were used in the Jerne agar plates.

Virus. The LDH-virus (1) was introduced by intraperitoneal inoculation of 0.1 ml of infected mouse plasma diluted 1:100 in saline. The undiluted plasma had an original titer of 10¹⁰ infectious units/ml; thus the total virus injected was 10⁷ infectious units per mouse.

Mice. Hybrid BAF (C57Bl×A)F₁ female mice, 6-10 weeks old, weighing 18-22 g, were used throughout unless otherwise noted. A few short-term experiments were done with 5-week-old CFW female mice, which gave results similar to those obtained with the BAF mice.

L-Asparaginase. Commercially prepared Squibb Escherichia coli L-asparaginase, having approximately 300 IU/mg of protein, was used for comparative tumor regression studies.

Asparaginase was injected intraperitoneally in 0.2 ml of solution per 20 g of mouse body weight. In the text and figures, enzyme concentration is expressed as IU/kg of body weight. Asparaginase assays were performed as described below.

Sensitivity of Asparaginase Assays. The sensitivity of the conventional assay is limited, with about one International Unit (IU) of asparaginase being the lowest concentration that can be measured with confidence. This limited sensitivity is not adequate to determine the low levels of circulating asparaginase in mice. To solve this problem, we have developed an asparaginase assay method that increases the sensitivity by approximately 1000-fold (38, 44). The principle involves the quantitative measurement of aspartic acid, which is the other product formed during the enzymatic deamination of asparagine and

Abbreviations: LDH-virus, lactate dehydrogenase-elevating virus; AFC, antibody-forming cells; sRBC, sheep erythrocytes; T and B cells, thymus- and bone marrow-dependent lymphocytes, respectively.
Table 1. Stimulating influence of the LDH-virus on spleen AFC during early viremia

<table>
<thead>
<tr>
<th>Treatment of spleen donors</th>
<th>Spleen AFCd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated controlsa</td>
<td>28 (+4.5)</td>
</tr>
<tr>
<td>sRBC onlyb</td>
<td>347 (+32)</td>
</tr>
<tr>
<td>sRBC + LDH-virusc (−24 hr)</td>
<td>2128 (+140)</td>
</tr>
<tr>
<td>sRBC + LDH-virus (0 hr)</td>
<td>1800 (+379)</td>
</tr>
<tr>
<td>LDH-virus only (−24 hr)</td>
<td>172 (+32)</td>
</tr>
<tr>
<td>LDH-virus only (0 hr)</td>
<td>39 (+4.6)</td>
</tr>
</tbody>
</table>

a Mice: CFW females 5 weeks old, five mice/group.
b sRBC were injected intravenously, 1 × 10⁸ per 0.1 ml, per mouse.
c LDH-virus: 1 × 10⁷ infectious units per 0.1 ml, injected intraperitoneally at the times shown relative to sRBC injections (1).
d Spleens were harvested and the Jerne plaque assay was done 48 hr after sRBC inoculation. The plaque counts shown are the means of five mice assayed individually for each group. The number of AFC is based upon the number of plaques observed per 10⁹ spleen cells added to the agar plates (20). The numbers in parentheses are the standard deviations. The differences induced by the virus are highly significant statistically (P < 0.001).

which can be measured with precision in the automatic amino acid analyzer (39, 40).

Amino Acid Determinations. Amino acids were assayed with a Beckman model 120B amino acid analyzer, modified to provide accelerated, semi-automatic runs (39). Improved methods of analysis were used (40), with sodium citrate buffers for the basic amino acids and lithium citrate buffers for the acidic and neutral amino acids. The latter buffer system is essential for the satisfactory separation of asparagine, glutamine, and glutamic acid.

Protective Animal Facilities. The importance of using animal housing and handling techniques that minimize stress and prevent inadvertent contamination of mice used for immunological, viral, or cancer studies has been documented (6, 21–23). Since experiments, such as those reported here, are undoubtedly sensitive to the physiological consequences of acute, chronic, or intermittent stress, use of appropriate animal holding facilities capable of controlling such undesirable disturbances are essential for obtaining reliable data. Such protective facilities have been briefly described (21, 24). We used such stress-free facilities in all of the long-term experiments described in this report. This is significant because psychosocial and other forms of anxiety-induced stress cause rapid and significant increases in plasma corticosterone, which in turn causes involution and weight loss of the thymus, spleen, and lymph nodes, as well as a decrease in circulating thymus-dependent lymphocytes (T cells) (6, 16–18). These physiological consequences of stressful stimuli have adverse influences on the immunological apparatus (25, 26), and therefore should be adequately controlled, especially in studies that involve spleen antibody-producing cells, or related immunological cooperative elements.

RESULTS

Stimulation of Bone Marrow-Dependent Lymphocytes (B Cells) by LDH-Virus. Fig. 1 illustrates both stimulating and inhibitory influences of the LDH-virus on the numbers of active spleen AFC that are detectable 48 hr after inoculation of sRBC. The chart on the left (A) depicts the 10-fold stimulating influence of the virus on AFC when the virus was inoculated 24 hr before intraperitoneal administration of the sRBC antigen. The LDH-virus reaches its peak titer of 10¹⁰ infectious units/ml of plasma by about 12 hr after the injection, and maintains this concentration for 72–96 hr. This period is designated as the “acute” phase (1).

Splenomegaly. The data of Fig. 1 are expressed as the total number of AFC plaques per spleen, whereas the data in Table 1 are based on AFC per 10⁸ spleen cells. It is appropriate to examine plaque production based upon both criteria, inasmuch as the LDH-viremia induces a mild splenomegaly (16). It has been determined that the spleen enlargement is due to hyperplasia with an increase in the number of cells per spleen, but not in the number of cells per mg of spleen (Table 2).

Effect of Acute Viremia. The data of Table 1 reflect the results of a typical experiment showing the relative number of AFC activated by sRBC antigen only, compared with those of similar animals that were also injected with the LDH-virus at two separate time periods in relation to inoculation of the antigen; namely, both 24 hr before and at the same time as the injection of the sRBC. In these experiments, the numbers of plaques or spleen antibody-producing cells were increased 3- to 6-fold when the virus was injected 24 hr before the sRBC antigen. When the virus was injected simultaneously with the sRBC, the stimulation was somewhat less, ranging from 2.5 to 5.2 times that of the mice receiving sRBC only. The data of Table 1 also show a “nonspecific” production of AFC capable of reacting to sRBC when the LDH-virus was introduced, in the absence of sRBC, 72 hr before the spleens were harvested. Although the absolute numbers of AFC in both groups were smaller, the increase was nevertheless 4- to 6-fold over the numbers in untreated control animals. When the LDH-virus was injected 48 hr before spleen harvesting, no appreciable increase in AFC over those of the untreated controls was observed.

Influence of Virus on AFC Lag Period. Fig. 2 shows the long-term interrelationship between the response of AFC of acutely as compared with chronically infected mice. Acute infection reduces the normal 48- to 72-hr delay in the response of AFC observed in noninfected controls by about 50%.

Effect of Chronic Viremia. The AFC response of mice
Table 2. Influence of LDH-virus on weight of the spleen and on relative number of spleen cells in suspensions

<table>
<thead>
<tr>
<th>Determinations</th>
<th>Control mice</th>
<th>Infected mice</th>
<th>% Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen wt. (a) (mg)</td>
<td>87</td>
<td>126</td>
<td>45</td>
</tr>
<tr>
<td>Mouse wt. (b) (g)</td>
<td>23.6</td>
<td>24.2</td>
<td>2.5</td>
</tr>
<tr>
<td>Rel. spleen wt. (c)</td>
<td>3.7</td>
<td>5.2</td>
<td>41</td>
</tr>
<tr>
<td>Spleen cells (d) (\times 10^{-7})</td>
<td>5.3</td>
<td>8.6</td>
<td>62</td>
</tr>
<tr>
<td>Spleen cells (e) (\times 10^{-7})/mg</td>
<td>6.1</td>
<td>6.8</td>
<td>11</td>
</tr>
</tbody>
</table>

\(a\) Average number of 28 harvested spleens from each group. Two spleens were harvested per day per group on each of the following days after infection: 1, 2, 3, 4, 6, 8, 10, 14, 21, 28, 35, 42, 49, and 56.

\(b\) Average mouse weights before spleen removal.

\(c\) Relative spleen weight, obtained by dividing the average spleen weights by the average mouse weights. Values expressed as mg of spleen per g of mouse.

\(d\) The number of spleen cells obtained from the two groups of homogenized spleens, as determined by standard Coulter counts. The spleens were harvested on day 5 after LDH-virus injection. No sheep erythrocytes were administered to these groups. The differences between both the spleen weights and the cell numbers in the two groups are highly significant statistically (\(P < 0.001\)).

\(e\) Average number of spleen cells obtained in suspension per mg of intact spleen. The small differences shown are not statistically significant.

burdened with the chronic LDH-virus infection demonstrates both an extended lag period and a reduced response to the rRBC antigen, showing only a modest temporary increase on days 4 and 5, which corresponds in time to the peak period of the

![Fig. 2. Comparison of the numbers of AFC detected in the spleens of mice with and without LDH-virus infection. Also demonstrated are the opposite influences of acute and chronic LDH-virus infection on the initial stages of AFC production. The number of Jerne plaques is plotted as a function of time after inoculation of rRBC. BAF female mice, 6-10 weeks of age, were injected intraperitoneally on day zero with \(10^6\) rRBC. The acutely infected mice were injected intraperitoneally with \(10^7\) infectious units of LDH-virus 24 hr before rRBC inoculation; the chronically infected mice were injected with the same dose of virus 23 days before receiving rRBC antigen. The number of AFC was determined by counting the number of direct IgM plaques developing per \(10^6\) spleen cells introduced onto the agar plates.](image1)

![Fig. 3. Delay in the appearance of asparaginase antibodies in mice infected with the LDH-virus (B), as compared with noninfected controls (A). The various curves illustrate the consequences of the virus infection on the duration of plasma asparaginase depletions by the enzyme, and thus of the effectiveness of asparaginase therapy against the asparagine-dependent EARAD-1 leukemia-lymphoma. The tumor regression, followed by recurrence in experiment (A), is shown by the solid line with open circles. Note the early return of plasma asparaginase (broken line) in the absence of the virus (A), as compared with the virus-infected mice in experiment (B), despite the continued daily dosage of equivalent amounts of asparaginase, as indicated by the black bars. The LDH-virus has no direct influence on the growth of this neoplasm (12); however, its indirect effects are illustrated in these therapeutic experiments.](image2)

noninfected controls. By day 6, the number of AFC returned to a level considerably below that of the control spleens. It appears that chronic infection has a more profound long-term inhibitory influence on spleen AFC than the striking but ephemeral stimulation seen with acute infection, and thus, consequentially, on the long-term immunological competence of the host.

Impairment of Asparaginase Antibody. Although multiple forces are involved, a significant aspect of the LDH-virus potentiation of asparaginase therapy in mouse leukemia and lymphoma may be related to this immunological defect. It is the early appearance of asparaginase antibody that impairs the effectiveness of the therapeutic enzyme and thus permits the return of asparaginase to the blood in noninfected mice. When this occurs, the asparaginase-requiring malignancy regrows with lethal consequences.

Fig. 3 reveals the delayed appearance of circulating asparaginase antibodies in infected mice treated with the enzyme. This delay is in contrast to the conventional appearance of neutralizing antibodies about 1 week after the initiation of asparaginase therapy in the absence of LDH-virus infection. The presence of neutralizing antibodies against asparaginase destroys the effectiveness of the enzyme therapy, which is indicated in Fig. 3 by the return to the plasma of the earlier depleted asparaginase. This is followed by regrowth of the leukemia-lymphoma. Similar circumstances also occur with leukemia patients undergoing asparaginase therapy (14, 15, 41-43). For a summary of additional physiological and biological effects of the LDH-virus that relate to immunological competence, see Table 3.

Suppression of Asparaginase Antibodies by the LDH-Virus In Vivo. The data of Fig. 4 depict the behavior of administered asparaginase in the plasma of normal mice, as compared with those infected with the LDH-virus, during 14 days of asparaginase administration in which both groups of animals received the enzyme on a daily basis. The two curves are plots of the concentrations of asparaginase found in the blood plasma of the two experimental groups as a function of time.

After day 6, in the noninfected animals, despite a continued
Table 3. Various physiological and biological effects of LDH-virus which relate to immunological competence

<table>
<thead>
<tr>
<th>Determinations</th>
<th>Virus effects</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymus wt.</td>
<td>50% wt. loss&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(16,1)</td>
</tr>
<tr>
<td>Thymus-dependent tissue and cells</td>
<td>Cell lysis</td>
<td>(1,16,29,30)</td>
</tr>
<tr>
<td>No. of lymphocytes</td>
<td>70% Depletion&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(2,1)</td>
</tr>
<tr>
<td>Spleen wt.</td>
<td>Increase&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(16,1)</td>
</tr>
<tr>
<td>Peripheral nodes (wt.)</td>
<td>Increase&lt;sup&gt;c&lt;/sup&gt;</td>
<td>(16,1)</td>
</tr>
<tr>
<td>Carbon clearance</td>
<td>25–70% Decrease&lt;sup&gt;d&lt;/sup&gt;</td>
<td>(4,1)</td>
</tr>
<tr>
<td>Macrophage integrity</td>
<td>Impaired&lt;sup&gt;d&lt;/sup&gt;</td>
<td>(1,4,27–32,37)</td>
</tr>
<tr>
<td>Spleen AFC</td>
<td>10-Fold increase&lt;sup&gt;e&lt;/sup&gt;</td>
<td>(6,1)</td>
</tr>
<tr>
<td>Chronic infection</td>
<td>50% Decrease&lt;sup&gt;f&lt;/sup&gt;</td>
<td>(6,1)</td>
</tr>
<tr>
<td>Plasma corticosterone</td>
<td>100–500% Increase&lt;sup&gt;g&lt;/sup&gt;</td>
<td>(6,1)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Four days after infection.
<sup>b</sup> Although peak increases were observed on days 5–6, small differences were still observed as late as day 56 after infection.
<sup>c</sup> Days 1 through 5 after infection (K-rate determinations). K values are an index of the phagocytic activity of reticuloendothelial macrophages, and are calculated from the logarithmic slope of the carbon clearance curve depicting the rate of particulate carbon removal by macrophages from the circulating blood.
<sup>d</sup> Plaques per spleen on days 2 and 3 after infection.
<sup>e</sup> Observed 2 weeks after infection.
<sup>f</sup> Observed during the first 24 hr after LDH-virus infection.

Daily administration of the same dose of asparaginase, the level of this enzyme abruptly decreased. The most striking change occurred between day 6 and day 8. By day 14, the end of the treatment period, there were no significant amounts of asparaginase in the plasma. A reasonable interpretation is that this results from the predictable appearance, about day 7, of asparaginase antibodies. The stable level of plasma asparaginase in the virus-infected animals during this 14-day treatment is consistent with the impaired ability of the virus-infected animals to generate effective antibodies. The immunosuppressive effects of asparaginase reported by others (43) was not a significant factor at the dose levels used in the present studies.

Antibody Neutralization of Asparaginase In Vivo. In further support of the studies in vitro (Fig. 4), asparaginase neutralizing capacity in vitro of plasma obtained from mice with and without LDH-virus infection has been clearly demonstrated. Mouse plasmas containing asparaginase antibody derived from mice that were never infected by the LDH-virus show a capacity to inhibit approximately 60% of the activity of a standard asparaginase solution after incubation with such immune plasmas. When analogous plasmas obtained from mice continuously infected with the LDH-virus were tested in a similar fashion, inhibition of asparaginase activity in vitro was also observed, but at about half the level of that found in the plasma obtained from mice that had not been infected with the virus.

A direct confirmation of asparaginase antibody differences in the plasma of mice with and without an LDH-virus infection was obtained by micro double immunodiffusion with agarose gel. Precipitin lines were observed between asparaginase and plasma from noninfected mice at both 14 and 21 days after asparaginase injection, but were not seen in analogous virus-infected mice.

These and related observations that establish the changes in circulating asparaginase by direct quantitative measurements of the plasma of virus—infected and control animals will be reported separately (V. Riley, D. Spackman, and G. Kenny, in preparation).

**DISCUSSION**

Cell Types Influenced by the LDH-Virus. Experimental findings indicate that the LDH-virus can affect three cell types: macrophages and T and B cells, which are all components of the immunological apparatus.

LDH-Virus and Macrophages. Of possible relevance is the influence that this viremia may have upon the host cells that support its rapid replication (1, 4, 27–32, 37). Absolute identification of the target cells has not been established; however, the macrophage is a likely candidate since cultures support replication of the LDH-virus (27, 28). If host macrophages function in a cooperative manner in the activation of humoral antibody-forming B cells (33, 34), the enhancement or damaging effect of the virus could also be correlated with either the early stimulation of the spleen cells or their later suppression, or conceivably both. The dual virus effects on spleen AFC are illustrated in Figs. 1 and 2.

Thymus Involution and Corticosterone Elevation. The LDH-virus also induces a progressive thymus involution during the first 4 or 5 days after infection, which is correlated with a T cell lymphocytopenia. This effect is probably indirect since it appears to be mediated through the adrenal cortex, which produces an elevation in plasma corticosterone in response to
acute infection by the virus (6, 45). Corticosterone has an adverse influence upon the thymus, T cells, and macrophages, which compromises host-mediated immunity and T cell cooperative functions (6, 16, 17, 29).

**B Cell Stimulation and Inhibition.** The third cell type affected by the virus is the antibody-forming B cell, which is stimulated for a brief period followed by a long-term suppression. Although it has been assumed that the B cells are impaired, the data only establish a suppression of their antibody-forming activities.

**Influence of a Benign Virus on Malignant Processes.** Other elements relevant to a complete analysis of this complex system may remain unrecognized. However, several of the factors that alter the immunological competence and surveillance capacities of the host as a consequence of this benign virus infection have been detected and characterized (1, 6, 16–18). Clearly, the virus can alter the cancer process in various ways. Further use of this versatile model may be helpful in identifying factors that influence not only neoplastic, but also other disease processes that are responsive to the immunological competence of the host and thus important in the management of pathological problems in patients (1, 6, 26, 35, 37). These results pose the reasonable question as to whether other common, nononcogenic viruses infecting human beings may have analogous potentials.

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24. Riley, V. (1972) *Proceedings, 33rd Annual Session, American Association for Laboratory Animal Science* (St. Louis), no. 22A.