Comparison of the Immunosuppressive Effects of Asparaginases from
Escherichia coli and Vibrio succinogenes

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ABSTRACT

The immunosuppressive effects of antileukemic asparaginases isolated from Escherichia coli and Erwinia carotovora are well documented, but the basis for the immunosuppression is unclear. In addition to catalyzing the deamination of L-asparagine, the E. coli and E. carotovora enzymes also deaminate L-glutamine (glutaminase activity). Many investigators have suggested that this glutaminase activity may be the cause of immunosuppression. The role of glutaminase activity in this immunosuppression has been difficult to evaluate, largely because the lack of a glutaminase-free asparaginase obtainable in significant quantity has precluded any rigorous in vivo experimentation. This laboratory has isolated an asparaginase from Vibrio succinogenes which has potent antilymphoma activity and lacks glutaminase activity. In the present communication, we report the effects of treatment with E. coli and V. succinogenes asparaginases on specialized immune responses in BALB/c Cr ugl mice.

The immunosuppressive effects of E. coli and V. succinogenes asparaginase on the humoral response of mice during sheep red blood cell (SRBC) immunization were investigated using the plaque-forming cell assay. There was a marked reduction in the number of IgM and IgG plaque-forming cells in the E. coli asparaginase-treated group. No reduction in the plaque-forming cell response was observed in the V. succinogenes asparaginase-treated mice. Levels of antibody against SRBC as determined by hemagglutination were also depressed in E. coli asparaginase-treated mice. Splenocytes isolated from mice immunized with SRBC and simultaneously treated with asparaginase were tested for their capability to serve as effector cells in a direct-target killing assay using 51Cr-labeled SRBC’s. At all concentrations tested, the E. coli enzyme significantly suppressed the cytotoxic response of splenocytes, while splenocytes from mice treated with V. succinogenes asparaginase maintained control levels of cytotoxicity. The data provide evidence that a glutaminase-free asparaginase from V. succinogenes does not suppress the in vivo immune response of mice to SRBC as compared to the pronounced immunosuppressive effects observed in mice treated with E. coli asparaginase.

INTRODUCTION

Broome (6) first showed that an L-asparaginase isolated from guinea pig serum had antitumor activity. However, asparaginase levels in guinea pig serum are very low; hence, it proved to be an impractical source of the enzyme for clinical use. Investigators then began to consider microbial sources for the isolation of an antileukemic asparaginase. Asparaginases isolated from Escherichia coli and Erwinia carotovora are now used in the treatment of certain leukemias (2, 27). During treatment with either of these enzymes, patients frequently experience pronounced toxicity including liver and pancreatic complications and immunosuppression (9, 25). It has been suggested that the immunosuppressive effects of asparaginase therapy may be due to the capability of the enzyme to hydrolyze L-glutamine (glutaminase activity) (1, 3, 26, 28). The role of glutaminase activity in the immunosuppression associated with asparaginase treatment has remained unclear largely because the lack of a glutaminase-free asparaginase obtainable in significant quantity has precluded any rigorous experimental study. Unlike other antineoplastic enzymes, an asparaginase isolated by the author from Vibrio succinogenes has been shown to be a potent antilymphoma agent and to be highly specific for L-asparagine, lacking any appreciable glutaminase activity (12, 13). Therefore, this study was undertaken to compare the immunosuppressive effects of 2 antineoplastic asparaginases, one having the catalytic capability to hydrolyze L-glutamine and the other lacking this catalytic activity.

MATERIALS AND METHODS

Animals. BALB/c Cr ugl mice from 9 to 12 weeks of age were used in these studies. They were originally obtained from the Cancer Research Genetics Laboratory, University of California, and maintained in our laboratory by brother-sister matings.

L-Asparaginase. L-Asparaginase from V. succinogenes was purified to homogeneity as described by the author (13). The L-asparaginase, Ec-2, from E. coli was obtained from Merck, Sharp, and Dohme Laboratories, West Point, Pa. (Lot 1028A). Further purification of E. coli asparaginase by gel filtration (21) on Ultrogel AcA44 (32) (LKB Instruments Inc., Rockville, Md.) yielded a homogeneous preparation as determined by disc gel electrophoresis in the presence of sodium dodecyl sulfate (29). The purified E. coli asparaginase demonstrated the same immunosuppressive properties as did the commercial preparation.

Enzyme Activity. L-Asparaginase activity was measured by determining the amount of ammonia produced upon hydrolysis of L-asparagine as described previously (13) except that 0.01 M potassium phosphate buffer (pH 7.0) was used in the reaction mixture. Enzyme and substrate blanks were included in all assays along with a standard curve prepared with ammonium sulfate. Enzyme activity is expressed as IU (the amount of enzyme catalyzing the formation of 1 μM ammonia per min under the conditions of the assay). In all experiments, the enzyme activity of V. succinogenes and E. coli asparaginases was determined as the average IU calculated from triplicate experiments.
The percentage of lysis was calculated as follows:

\[ \text{Experimental } \% { ^{51} \text{Cr} \text{ release}} - \text{non-specific } \% { ^{51} \text{Cr} \text{ release}} = \text{Control } \% { ^{51} \text{Cr} \text{ release}} - \text{non-specific } \% { ^{51} \text{Cr} \text{ release}} \]

The experimental percentage of \( ^{51} \text{Cr} \) release was determined using splenocytes from asparaginase-treated mice immunized with SRBC, while the control percentage of \( ^{51} \text{Cr} \) release was determined using splenocytes from immunized animals not treated with asparaginase. The non-specific percentage of \( ^{51} \text{Cr} \) release was determined using splenocytes from untreated mice.

### RESULTS

#### Effects of Asparaginase on Spleen Weight

There was significant reduction in spleen weight observed in \( E. \text{coli} \) asparaginase-treated animals (Table 1). The reduction in spleen weight is directly related to enzyme dosage with as much as a 40% reduction being observed at 50 IU/injection. No significant microspleenia was observed in animals treated with \( V. \text{succinogenes} \) asparaginase even at the highest enzyme dosage.

<table>
<thead>
<tr>
<th>Enzyme dose (IU/day/mouse)</th>
<th>( E. \text{coli} ) l-asparaginase (mg)</th>
<th>( V. \text{succinogenes} ) l-asparaginase (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>136 ± 4.0*</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>150 ± 11.0</td>
<td>130 ± 3.0</td>
</tr>
<tr>
<td>10</td>
<td>104 ± 10.0</td>
<td>142 ± 2.9</td>
</tr>
<tr>
<td>25</td>
<td>99 ± 3.0</td>
<td>133 ± 7.9</td>
</tr>
<tr>
<td>50</td>
<td>80 ± 3.0</td>
<td>120 ± 6.0</td>
</tr>
</tbody>
</table>

* Mean ± S.E.

### Analysis of Data

Student's t test was utilized to evaluate observed differences between \( E. \text{coli} \) and \( V. \text{succinogenes} \) asparaginase-treated animals and controls.
E. coli asparaginase. At 25 and 50 IU, the E. coli enzyme suppressed the cytotoxic response of splenocytes to an average value of 26 and 29% lysis, respectively (Chart 1). No significant reduction of the cell-mediated cytotoxic response was observed in animals treated with V. succinogenes asparaginase. Splenocytes from mice treated with 5, 10, 25, and 50 IU per injection of V. succinogenes asparaginase maintained mean levels of cytotoxicity that were 87, 98, 76, and 92% lysis, respectively. The differences observed between E. coli and V. succinogenes asparaginase-treated mice at 10, 25, and 50 IU are significant (p < 0.001). No significant difference was observed at 5 IU (p > 0.01).

Effect of Asparaginase on the Humoral Immune Response.

The PFC response to SRBC was examined to compare the immunosuppressive effects of E. coli and V. succinogenes asparaginase on humoral immunity. The IgG PFC response per spleen is greatly depressed in E. coli asparaginase-treated mice (p < 0.001) at enzyme doses of 10, 25, and 50 IU (Chart 2A). In contrast, no significant reduction in the IgG PFC response per spleen is observed in the V. succinogenes asparaginase-treated animals (p > 0.1). Since it could be argued that the immunosuppression observed in E. coli asparaginase-treated mice could result from a nonspecific depletion of lymphoid populations, the number of PFC’s per 10⁶ splenocytes was determined (Chart 2B). The IgG PFC response per 10⁶ splenocytes is greatly reduced in the E. coli asparaginase-treated mice (p < 0.001). Animals treated with V. succinogenes asparaginase showed no reduction in the number of IgG PFC’s per 10⁶ splenocytes.

The quantitation of the IgM PFC response revealed a similar pattern of comparative immunosuppression. The IgM PFC response per spleen is greatly suppressed in E. coli asparaginase-treated mice at doses of 10, 25, and 50 IU (p < 0.001). No significant reduction in the IgM PFC response is observed in animals treated with V. succinogenes asparaginase (p > 0.05). A comparison of the IgM PFC response per 10⁶ splenocytes in E. coli and V. succinogenes asparaginase-treated mice again showed a pronounced reduction of PFC’s in the E. coli asparaginase-treated group (p < 0.001) with no significant reduction of PFC being observed in the V. succinogenes asparaginase-treated mice.

The levels of hemagglutinating anti-SRBC antibody were determined in E. coli and V. succinogenes asparaginase-treated mice (Table 2). Results of these studies parallel the PFC assay. E. coli asparaginase has a 30-fold greater glutaminase activity as compared to controls at all enzyme doses (p < 0.001). V. succinogenes asparaginase did not affect the PFC response (p > 0.1). B. effect of E. coli and V. succinogenes L-asparaginase treatment on the humoral immune response to SRBC as measured by the indirect PFC assay. Experiments were performed as described in “Materials and Methods.” Data represent the number of IgG PFC’s per spleen. Each point represents an individual animal. BALB/c(Crgl) mice were immunized on Day 0 with 1 × 10⁸ SRBC i.p. on Days 0, 1, and 2. Splenocytes were assayed for direct target cytotoxicity on Day 5. E. coli asparaginase treatment caused a significant reduction in PFC as compared with controls at all enzyme doses (p < 0.001). V. succinogenes asparaginase did not affect the PFC response (p > 0.1). C. effect of E. coli and V. succinogenes asparaginase on the humoral immune response to SRBC as measured by the indirect PFC assay. Experiments were performed as described in “Materials and Methods.” Data represent the number of IgG PFC’s per 10⁶ splenocytes. The experimental protocol is identical to that described in A. Treatment with E. coli asparaginase significantly reduced the number of PFC’s per 10⁶ splenocytes as compared to controls (p < 0.001). No significant reduction of PFC’s was associated with V. succinogenes asparaginase treatment (p > 0.1). INJ, injection.

DISCUSSION

E. coli asparaginase has a 130-fold greater glutaminase activity as compared to the almost undetectable level of glutaminase activity associated with V. succinogenes asparaginase (8, 13). Studies in vitro have suggested that glutaminase activ-
Comparing the inhibitory effects of *E. coli* and *V. succinogenes* asparaginases on the mitogen-induced blastogenic response of human lymphoid cells. At a concentration of 0.4 IU/ml, the *E. coli* enzyme inhibited the PHA-induced blastogenic response more than 95%, while the *V. succinogenes* enzyme inhibited response only 18%. Our results indicate that *E. coli* asparaginase is as much as 2- to 3-fold more inhibitory than is *V. succinogenes* asparaginase in vitro.

Other investigators have shown that an L-asparaginase lacking glutaminase activity from guinea pig serum inhibits lymphocyte blastogenesis in vitro (23). The fates of asparaginases in vivo and the lack of homeostatic control of metabolites such as L-asparagine and L-glutamine in vitro clearly limit the predictive value of such in vitro experimentation. Until now, it has not been plausible to thoroughly compare the *in vivo* immunosuppressive effects of an asparaginase having glutaminase to one lacking this catalytic activity.

Our studies indicate that *E. coli* asparaginase is highly immunosuppressive, while the *V. succinogenes* asparaginase is not. The IgM and IgG PFC responses are greatly depressed in *E. coli* asparaginase-treated mice. The PFC cell response is depressed both when considering the number of PFC's per total spleen and when considering the number of PFC's per 10^6 splenocytes. This indicates that the suppression of PFC's is not the result of a nonspecific depletion of lymphoid cells but is specific in the sense that some particular cell population within the spleen is preferentially affected. Other investigators have shown that treatment of mice with *E. coli* asparaginase preferentially affected antibody-forming precursor cells resident in the bone marrow (16). Mice treated with *V. succinogenes* asparaginase showed no reduction in their humoral immune response to SRBC. Our results also indicate that mice treated with *E. coli* asparaginase have a markedly reduced cell-mediated cytotoxic response to SRBC (Chart 1). An analysis of this cell-mediated cytotoxic reaction has revealed that anti-SRBC antibody is involved (11, 22, 31); therefore, the decrease in direct target killing could reflect a decrease in antibody synthesis. In contrast, spleen cells from animals treated with *V. succinogenes* asparaginase are not suppressed in their capability to mediate target killing.

Several studies with antitumor asparaginases have suggested that factors other than specific catalytic activity of the enzyme molecule may contribute to the relative immunosuppressive and antitumor effect of different asparaginases, e.g.,

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**Table 2**

Effects of *E. coli* and *V. succinogenes* asparaginase on the BALB/cCrj1 antibody response to SRBC

SRBC were injected on Day 0, and enzyme was injected on Days 0, 1, and 2; sera were taken on Day 5.

<table>
<thead>
<tr>
<th>Enzyme dose (IU/day/mouse)</th>
<th><em>E. coli</em> L-asparaginase</th>
<th><em>V. succinogenes</em> L-asparaginase</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>37.5 ± 5.4*</td>
<td>42.0 ± 7.6</td>
</tr>
<tr>
<td>5</td>
<td>10.0 ± 1.8</td>
<td>72.0 ± 33.0</td>
</tr>
<tr>
<td>10</td>
<td>29.6 ± 9.3</td>
<td>53.5 ± 9.3</td>
</tr>
<tr>
<td>25</td>
<td>11.5 ± 2.9</td>
<td>20.8 ± 4.4</td>
</tr>
<tr>
<td>50</td>
<td>10.5 ± 1.3</td>
<td></td>
</tr>
</tbody>
</table>

*A The hemagglutination titer of all sera treated with 0.2 M 2-mercaptoethanol was <2.0.

Mean ± S.E.
plasma half-life and antigenicity (4, 7). Based on previous work (7, 12), the differences in immunosuppression observed between \( E. \) coli and \( V. \) succinogenes asparaginase treatment cannot be explained by differences in plasma half-life of the 2 enzymes, since \( V. \) succinogenes asparaginase has been shown to have a longer plasma half-life than the \( E. \) coli enzyme does in both normal and tumor-bearing mice (7, 12).

At present, the immunosuppressive effects of asparaginase are well documented, but the basis of immunosuppression is unknown. L-Asparaginases are known to affect a number of different biological systems at different levels of cellular metabolism. The enzymes have been shown to sequentially inhibit protein, RNA, and DNA synthesis (30). L-Asparaginase has been shown to inhibit glycoprotein synthesis necessary for membrane integrity (5). Other investigators have shown that \textit{in vitro} treatment with \( E. \) coli asparaginase results in a decreased binding of a concanaavalin A to lymphoid cells followed by an inhibition of blast transformation (15), suggesting an alteration of the cell membrane. The \( E. \) carotovora asparaginase has similarly been suggested to cause alteration of lymphoid cell membranes (17). Future studies in our laboratory will focus upon using \( V. \) succinogenes asparaginase to define the role of glutaminase activity in asparaginase-induced immunosuppression. Lastly, if glutaminase activity does account for the immunosuppressive effects of asparaginase therapy, then the glutaminase-free asparaginase from \( V. \) ibrio may be a safer and more effective antileukemic agent.

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**REFERENCES**

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