Further Development of a Successful Protocol of Graft versus Leukemia without Fatal Graft-versus-Host Disease in AKR Mice*  

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SUMMARY

We previously reported a successful model for treatment of BW 5147 leukemia in AKR mice by adoptive immunotherapy using allogeneic spleen cells from C57BL/6 mice. The leukemia cells were given 3 days before initiation of therapy. Graft-versus-host reaction was prevented by treatment with spleen cells from a second allogeneic strain (CBA), followed by cyclophosphamide and syngeneic spleen cells. We now show that it is not necessary to use syngeneic spleen cells in the final transplant since H-2-compatible, allogeneic CBA cells are as effective. In addition, it is possible to initiate successful therapy 5 days after leukemia implantation providing that the initial cyclophosphamide, given in two doses of 100 mg/kg each and spaced 7 days apart, is administered prior to establishment of graft-versus-host reaction. Higher single doses of drug were followed by fatal graft-versus-host disease.

INTRODUCTION

In a previous publication (8) we presented a model system for treatment of leukemia with a combination of Cyclo® and controlled GVH reaction. In this system AKR mice were given transplantable syngeneic BW 5147 leukemic cells 3 days prior to initiation of GVH reaction with C57BL/6 spleen cells. Cyclo was used to establish the allogeneic cell transplant. In order to prevent runaway GVH reaction, rescue procedures were begun on Day 6 with a 2nd allogeneic spleen cell (CBA) transplant followed by Cyclo on Day 10 and syngeneic AKR spleen cells on Days 11 and 16. This regimen resulted in 90% survival of mice for 200 days without fatal GVH disease or leukemia.

In this report we explore some of the parameters of this model by (a) substituting allogeneic histocompatible spleen cells for the final rescue transplantation and (b) lengthening the interval of leukemia cell growth from 3 to 5 days before initiation of treatment.

MATERIALS AND METHODS

Animals. Inbred 10- to 12-week-old female AKR (H-2k) mice, CBA (H-2r) mice, and male C57BL/6 (H-2b) mice were obtained from The Jackson Laboratory, Bar Harbor, Maine, and maintained as previously described (7).

Drugs. Solutions of Cyclo, obtained from Mead Johnson & Co., Evansville, Ind., were prepared in 0.9% NaCl solution and injected within 15 min.

Antisera. Anti-AKR (D-3b) and anti-C57BL/6 (D-33) antisera were generously provided by Dr. John G. Ray, Jr., of the NIH.

Cell Suspensions. Spleen cells were prepared according to the method of Boranic and Tonkovic (2). Viable nucleated cells were counted in a hemocytometer, and the final volume was adjusted to give a cell count of 1 x 10⁶/ml. Cell viability was determined by trypan blue dye exclusion (5).

Leukemia. In 1954, BW 5147 lymphocytic leukemia originated spontaneously in an AKR mouse in The Jackson Laboratory (10) and had been maintained in our laboratory by serial s.c. transplantation in AKR mice. At autopsy microscopic involvement of the spleen, the liver, and the thymus was apparent (6). Tumor cell suspensions were prepared by mincing in Gey’s balanced salt solution, filtering through silk cloth, centrifuging at 600 x g for 10 min at 4°, removing the supernatant, and resuspending the pellet in Gey’s balanced salt solution. Viable cells were counted in a hemocytometer, and the final volume was adjusted to give a concentration of 50 x 10⁶ cells/ml. Cell viability was determined by trypan blue dye exclusion (5).

Histological Criteria. The spleen and thymus were examined histologically for features characteristic of GVH reaction (1, 7) and of leukemia (1, 3).

Cytotoxicity Assay. The method of Smith et al. (15) with minor modifications was used to determine the presence of C57BL/6 and AKR spleen cells in AKR-treated animals. Anti-D-33 and anti-D-3b antisera were used at a 1:10 dilution, which produced 95% or greater cytotoxicity with the appropriate target cell.

RESULTS

Substitution of H-2-compatible Spleen Cells for Syngeneic Spleen Cells during Rescue of Mice from GVH
Disease. In our previous report syngeneic AKR spleen cells were the final cells used to rescue the treated mice from fatal GVH disease induced by the C57BL/6 spleen cells. Since obtaining syngeneic cells is difficult in human disease, we investigated the effect of H-2-compatible, allogeneic cells for this step. AKR mice were given 10,000 BW 5147 leukemic cells i.p. 3 days before initiation of GVH reaction (Cyclo, 150 mg/kg i.p. on Day 0; 45 × 10^6 C57BL/6 spleen cells i.v. on Day 1). Rescue was initiated on Day 6 with 35 × 10^6 CBA spleen cells i.v., followed by Cyclo, 150 mg/kg i.p., on Day 10 and 35 × 10^6 CBA spleen cells i.v. on Day 11. Eleven of 15 mice (Table 1, Experiment 2) were alive 160 days after this treatment, showing that it is almost as effective as when syngeneic cells were used (Table 1, Experiment 8).

Treatment of BW 5147 Leukemia after 5 Days of Growth. In Table 1, Experiment 2, the leukemia cells had been allowed to divide for 3 days before initiation of treatment. Experiments 3 to 8 show the development of a successful treatment protocol after 5 days of growth of an initial inoculum of 10,000 BW 5147 leukemic cells. In Experiment 3 the mice were treated with Cyclo, 150 mg/kg on Day 0; 45 × 10^6 C57BL/6 spleen cells on Day 1; 35 × 10^6 CBA spleen cells on Day 6; Cyclo, 150 mg/kg on Day 10; and 35 × 10^6 CBA spleen cells on Day 11. This protocol is successful for treating a 3-day growth of leukemia cells. In this case the animals died from leukemia with a MST of 32.3 ± 0.8 days. In Experiment 4 the number of C57BL/6 spleen cells was increased to 60 × 10^6. This modification resulted in death from GVH disease (MST, 27.4 ± 6.0 days). In Experiments 5 and 6 when the initial doses of Cyclo were increased to 200 and 250 mg/kg, respectively, fatal GVH disease still resulted (MST, 27.9 ± 2.2 days, Experiment 5; MST, 24.0 ± 0.8 days, Experiment 6). Finally, in Experiment 8 the initial dose of Cyclo was split into 2 doses of 100 mg/kg, given on Days 0 and 7, with 45 × 10^6 C57BL/6 spleen cells administered on Day 8, followed by the rest of the original protocol at the usual time intervals. Six of 8 animals treated in this way were still alive at Day 100.

A control group of 7 AKR mice in which CBA spleen cells were substituted for C57BL/6 spleen cells in Experiment 8 with no further treatment, died from leukemia with a MST of 35.1 ± 5.4 days. These results established that CBA spleen cells were unable to eliminate the leukemia.

In order to determine whether this treatment eliminated the C57BL/6 spleen cells, cytotoxicity assays were performed in triplicate on Days 10 and 44 on spleen cells from 2 mice treated with the protocol in Experiment 8. The percentage of C57BL/6 spleen cells, determined with D-33 antiserum, at Day 10 was 6.6 (average of 2 animals); the remaining cells were reactive with D-3b antiserum. However, at Day 44 no C57BL/6 spleen cells were detectable, but 95% of the spleen cells reacted with D-3b antiserum.

**DISCUSSION**

In our previous report we presented a model for treatment of AKR leukemia with a GVH reaction in which we controlled the GVH reaction by a graft-versus-graft reaction.

In this report we present 2 modifications of our model. Previously, we rescued the host mice, after successful treatment of GVH reaction, with syngeneic spleen cells. Since this may not always be feasible in a clinical situation, we have substituted the syngeneic cells with cells matched at the H-2 locus. The substitution resulted in 73% survival 160 days after initiation of GVH reaction. The specific role of the H-2 and non-H-2 loci requires further experimentation. Salaman et al. (13) reported that M locus determined antigens are incapable of provoking a detectable GVH reaction as measured by spleen assay. Furthermore, Festenstein (11) has also suggested that determinants of the non-H-2 M locus have poor graft rejection-initiating properties.

Our 2nd modification was to increase the length of time between implantation of the leukemic cells and initiation of treatment. There are 2 important parameters that determine the success of our model: Parameter 1, the number of donor allogeneic cells; and Parameter 2, the ratio of the

**Table 1**

**Adoptive immunotherapy of BW 5147 in AKR mice**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cyclo (mg/kg)</th>
<th>C57BL/6 spleen cells (×10⁶)</th>
<th>CBA spleen cells (×10⁶)</th>
<th>Cyclo (mg/kg)</th>
<th>CBA spleen cells (×10⁶)</th>
<th>Leukemia</th>
<th>GVH</th>
<th>Range</th>
<th>Mean ± S.E.</th>
<th>p*</th>
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<td>1</td>
<td>150</td>
<td>45</td>
<td>35</td>
<td>150</td>
<td>35</td>
<td>8/8⁹</td>
<td></td>
<td></td>
<td>13-18</td>
<td>15.5 ± 0.6</td>
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<tr>
<td>2</td>
<td>150</td>
<td>45</td>
<td>35</td>
<td>150</td>
<td>35</td>
<td>4/15⁹</td>
<td>0/11'</td>
<td>&lt;0.001</td>
<td>33-160</td>
<td>126.3 ± 14.9</td>
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<td>3</td>
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<td>45</td>
<td>35</td>
<td>150</td>
<td>35</td>
<td>8/8'</td>
<td></td>
<td></td>
<td>30-37</td>
<td>32.3 ± 0.8</td>
</tr>
<tr>
<td>4</td>
<td>150</td>
<td>60</td>
<td>35</td>
<td>150</td>
<td>35</td>
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<td></td>
<td></td>
<td>16-37</td>
<td>27.4 ± 6.0</td>
</tr>
<tr>
<td>5</td>
<td>200</td>
<td>45</td>
<td>35</td>
<td>150</td>
<td>35</td>
<td>8/8</td>
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<td></td>
<td>22-38</td>
<td>27.9 ± 2.2</td>
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<td></td>
<td>20-26</td>
<td>24.0 ± 0.8</td>
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<tr>
<td>7</td>
<td>100</td>
<td>100</td>
<td>45</td>
<td>150</td>
<td>35</td>
<td>8/8³</td>
<td></td>
<td></td>
<td>37-48</td>
<td>42.4 ± 1.6</td>
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<tr>
<td>8</td>
<td>100</td>
<td>100</td>
<td>45</td>
<td>150</td>
<td>35</td>
<td>2/8</td>
<td>0/6'</td>
<td>&lt;0.001</td>
<td>42-100</td>
<td>88.9 ± 7.7</td>
</tr>
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</table>

* Student's t test. Experiments 3, 4, 5, 6, and 7 compared with Experiment 8.
* Control for injected leukemic cells.
* Eleven of 15 animals alive on Day 160.
* Control for Cyclo alone.
* Six of 8 animals alive on Day 100.
donor allogeneic cells to the leukemic cells. Parameter 1 determines the degree of the GVH reaction, whereas Parameter 2 determines the efficacy of the GVL reaction. We had previously found that 45 × 10^4 C57BL/6 spleen cells were adequate to cure mice given 10,000 leukemic cells 3 days prior to initiation of the GVL reaction and the administration of Cyclo, 150 mg/kg. Since this dose of Cyclo would decrease the leukemic population by 99.99% (14) and since the leukemic cells have a 16-hr doubling time (4) (confirmed in our laboratory with a long-passage AKR leukemia), we were in reality dealing with approximately 32 leukemic cells on the day the GVL reaction was initiated. In Table 1 (Experiments 3 to 8), the leukemic cells were allowed to divide for 5 days prior to treatment; thus we had approximately 256 cells with which to contend after Cyclo treatment. C57BL/6 spleen cells (45 × 10^6) were unable to overcome this number (Experiment 3). Increasing the C57BL/6 cells to 60 × 10^6 resulted in death from GVH disease (Experiment 4). Increasing the initial dose of Cyclo to 200 or 250 mg/kg did not prevent the GVH disease (Experiments 5 and 6). However, the administration of 2 doses of Cyclo, 100 mg/kg, 1 on Day 0 to establish the GVH reaction and to suppress the leukemic cells and 1 on Day 7 further to suppress the leukemic cells, resulted in survival of 6 of 8 animals for more than 100 days (Experiment 8). Cytotoxicity assays showed that AKR, but not C57BL/6 spleen cells, were present 44 days after the initiation of treatment. The presence of CBA spleen cells, however, cannot be excluded. These results can be explained on the basis of establishing a favorable ratio between the C57BL/6 cells and the leukemic cells. Although the initial dose of Cyclo reduces the population of leukemic cells to approximately 256 cells, these remaining cells would increase to 256,000 in 7 days. A 2nd dose of Cyclo would then reduce them to approximately 25 cells, the number of leukemic cells that 45 × 10^8 C57BL/6 spleen cells would overcome. Thus, in order to achieve successful treatment of AKR leukemia with a controlled GVH reaction, it is necessary to maintain a relationship between the allogeneic cells, the cells of the recipient, and the leukemic cells in which the ratio of allogeneic to leukemic cells is approximately 1 × 10^4:1 at the time GVH reaction is initiated.

Devlin et al. (9) have shown that the splenic T-cell immune system is restored 88 hr after Cyclo, 100 mg/kg. Higher doses of drug are likely to result in more profound immune suppression. If so, our observation that 200- or 250-mg/kg doses of Cyclo resulted in fatal GVH disease, whereas 2 doses of 100 mg/kg divided over a 7-day interval prevented a fatal reaction, suggests that partial restoration of host immune competence allows for significant resistance to the GVH reaction.

On the basis of the data from a 5-day growth of leukemic cells, we can speculate that treatment of advanced leukemia would be effective after reduction of the leukemic cell load to a number that could be handled by a protocol that includes the modifications described in this study.

REFERENCES

Further Development of a Successful Protocol of Graft *versus* Leukemia without Fatal Graft- *versus*-Host Disease in AKR Mice


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