Treatment of Runting Syndrome and Prevention of Primary Lymphomas in Friend Virus-tolerant Rats

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SUMMARY

A series of experiments showed that the inoculation of spleen and lymph node cells from rats immunized with Friend lymphoma (WFT-13) cells into Friend virus-tolerant rats induced the runting syndrome in nearly all cases, and immunological tolerance to WFT-13 was not broken in any survivors. The inoculation of specific immune spleen and lymph node cells admixed with normal bone marrow cells suppressed the runting death. In addition, in these animals the primary lymphomas that ordinarily occur about 200 days after neonatal inoculation of Friend virus did not appear. The mixture of immune spleen and lymph node cells and normal spleen and lymph node cells or normal thymus cells was ineffective in preventing the runting death or the incidence of primary lymphoma. Spleen and lymph node cells from normal rats or rats immunized with antigenically different AH-66 cells were also without effect. Spleen and lymph node cells from rats immunized with sheep red blood cells had a relatively high incidence of the runting syndrome; a few survivors rejected the WFT-13 transplants and also did not develop primary lymphomas. These results suggest that a supplement of hematopoietic stem cells from bone marrow will not only prevent the runting death of Friend virus-tolerant rats produced by inoculating immune lymphoid cells but will also prevent the expected occurrence of primary lymphomas.

INTRODUCTION

Rats inoculated neonatally with high doses of FV were completely tolerant to FV-specific transplantation antigens and grew well as FV-tolerant rats. FV-tolerant rats were more susceptible to a subsequent challenge with FV-induced lymphoma transplants, and they developed primary lymphomas in about 200 days (6). To inhibit development of primary lymphomas in FV-tolerant rats, attempts were made to break the tolerant state by inoculations of syngeneic spleen and lymph node cells (13, 14). The inoculation of spleen and lymph node cells from specific immune donors into FV-tolerant rats usually brought about the runting syndrome; the tolerant state was not broken in any survivors, although the titer of FV in the blood decreased markedly in all cases. In the runted rats, high-titered cytotoxic antibody to WFT-13 cells was detected, and atrophy of the thymus and enlargement of the spleen with a depletion of lymphocytes were observed. From these results, the pathological mechanism of the runting syndrome is considered to be based on an immune reaction between the inoculated lymphoid cells and FV-infected cells in the tolerant rats. Thus, the specific immune reaction of the inoculated lymphoid cells against the FV-specific transplantation antigens causes destruction of lymphoid organs and results in the runting death.

This report describes attempts to cure the runting syndrome by additional inoculations of hematopoietic stem cells from syngeneic donors and, subsequently, to prevent the development of primary lymphomas in the tolerant rats.

MATERIALS AND METHODS

Rats. An inbred strain of male and female WKA/Mk rats was used. These rats are offspring of parents maintained by consecutive brother-sister mating for more than 200 generations at the Laboratory for the Breeding of Experimental Animals, Hokkaido University, Sapporo, Japan.

FV. The FV used has been serially maintained in DHS/Mk mice by the injection of spleen homogenates. The FV was recovered from Friend-diseased spleens by the method of Chenaille et al. (4). The titer unit, expressed as MID50/ml, is the dose at which 50% of the mice are positive for the development of Friend disease as measured by the spleen focus assay method of Axelrad and Steeves (1).

Tumors. Transplantable Friend lymphoma (WFT-13) was induced in WKA/Mk rats 172 days after the neonatal injection of FV. This tumor grew well either in rats made FV tolerant by the neonatal injection of FV or in immunologically depressed rats, but it failed to grow lethally even in syngeneic normal adult rats. Detailed characteristics of Friend lymphomas have been reported elsewhere (6, 7). Transplantable ascites hepatoma (AH-66) induced in Donryu rats by p.o. administrations of dimethylnitrosobenzene was kindly supplied by Dr. Y. Tsukada, Department of Biochemistry, Hokkaido University School of Medicine, Sapporo, Japan.

1 This work was supported in part by a research grant for cancer research from the Ministry of Education of Japan.

2 Present address: Cell Biology Section, Building 37, Viral Biology Branch, National Cancer Institute, NIH, Bethesda, Md. 20014.

3 The abbreviations used are: FV, Friend virus; MID50, dose at which 50% of animals are positive for disease; SRBC, sheep red blood cells.

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Tolerant Rats. Adult rats (50 to 60 days old) given i.p. and s.c. injections of high doses of FV within 48 hr after birth were used as recipients.

Immune Sera. Sera were obtained from rats immunized 4 to 5 times s.c. with WFT-13 cells by cardiac puncture under ether anesthesia. After the blood clotted at room temperature, the sera were separated by centrifugation at 3000 rpm for 30 min. Before use, the sera were inactivated at 56° for 30 min.

Cell Preparations. Donor rats unimmunized or immunized with WFT-13 cells were killed. Spleen and lymph nodes were removed aseptically, minced, and gently blended in a loose-fitting glass homogenizer (No. 1072). The crude spleen and lymph node cell suspensions (ratio of the cell populations, approximately 2:1) were filtered through surgical gauze and washed 3 times at 1000 rpm for 5 min, after which 1 to 5 x 10^8 cells/ml were resuspended in minimum essential medium. All suspensions contained 95% or more viable cells.

Cytotoxicity Test. Cytotoxic sensitivity of lymphoid cells against the antibody was tested according to the method of Gorer and O'Gorman (5) with slight modifications. Equal volumes (0.1 ml) of serum at progressively doubled dilutions and cell suspensions (5 x 10^8 cells/ml) were incubated for 10 min at room temperature, and then 0.1 ml of guinea pig serum at a 1:2 dilution was added as a complement source. The mixture was incubated at 37° for 45 min and washed in cold minimum essential medium; the proportion of dead cells was determined microscopically with trypan blue solution. In each specimen, more than 300 cells were counted. The cytotoxic index was calculated by subtracting the percentage of unstained cells in the test serum-treated sample from the percentage of unstained cells in the control sample and dividing by the latter figure. A cytotoxicity index of more than 0.2 was regarded as a positive reaction.

RESULTS

Breakdown of FV Tolerance and Development of Runtling Syndrome. Two months after neonatal injections of high doses of FV, 63 tolerant rats were divided into 5 groups. In Group 1, 12 rats were inoculated i.p. with spleen and lymph node cells (5 x 10^7) obtained from rats preimmunized 4 to 5 times s.c. with WFT-13 cells, twice at 7-day intervals. In Group 2, 14 rats were inoculated with spleen and lymph node cells from rats immunized with AH-66 cells as in the 1st group. In Group 3, 11 rats were inoculated with spleen and lymph node cells from rats immunized with SRBC (1 x 10^8). In Group 4, 11 rats were inoculated with spleen and lymph node cells from normal rats. In Group 5 (control), 15 rats were not inoculated. To determine whether immunological tolerance to FV-specific transplantation antigens was eliminated, all rats from each group received s.c. transplants of WFT-13 cells (5 x 10^7) 2 weeks after the 2nd inoculation of lymphoid cells (Table 1). In the 1st group, 10 of the 12 rats (83%) died of the runting syndrome from 14 to 20 days after the 1st inoculation. The 2 survivors rejected the WFT-13 transplants but then died of the runting syndrome. Four of the 14 rats (29%) in the 2nd group died of the runting syndrome and 7 of the 10 survivors died from the growth of the WFT-13 transplants, while the remaining 3 survivors resisted the WFT-13 transplants but thereafter succumbed to the runting syndrome. Six of the 11 rats (55%) in the 3rd group died of the runting syndrome. However, 5 survivors rejected the WFT-13 transplants and none developed primary lymphomas until more than 300 days later, except for 1 dying of the runting syndrome. Three of the 11 rats in the 4th group died of the runting syndrome. Of the remaining 8 rats, 7 died from the growth of the WFT-13 transplants and 1 died of the runting syndrome. In the 5th group, 1 of the 15 rats developed the runting syndrome and 14 survivors died from the growth of the WFT-13 transplants.

The inoculation of lymphoid cells from rats immunized with WFT-13 cells induced a high incidence of the runting syndrome. This suggests that specific immune lymphoid cells recognize the FV-specific transplantation antigens in the tolerant rats as foreign, and the consequent immune reaction results in the development of the runting syndrome. The inoculation of lymphoid cells from rats immunized with SRBC also brought about a relatively high incidence of the runting death. Possible explanations for this are given under "Discussion."

Elimination of Cells Bearing FV-specific Cell Surface Antigens in Various Lymphoid Organs of Tolerant Rats after Receiving Immune Spleen and Lymph Node Cells. Fourteen-day-old tolerant rats were divided into 2 groups and either inoculated i.p. with immune spleen and lymph node cells (5 x 10^7) or not. Three to 4 rats from both groups were killed every 3 days after an inoculation, and the sensitivity of cells from various lymphoid organs to the cytotoxic effects of anti-WFT-13 serum was tested (Chart 1). In the tolerant rats from the control group, 30 to 40% of cells in the thymus, spleen, and bone marrow were susceptible equally to the cytotoxicity of anti-WFT-13 serum on all days tested.

### Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Spleen and lymph node cells* from rats preimmunized with WFT-13</th>
<th>No. of instances of runting syndrome/no. of rats</th>
<th>Transplantation of WFT-13 cells* (no. died/no. used)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>WFT-13 cells</td>
<td>10/12 (83%)</td>
<td>0/2 (2/2)</td>
</tr>
<tr>
<td>2</td>
<td>AH-66 cells</td>
<td>4/14 (29)</td>
<td>7/10 (3/3)</td>
</tr>
<tr>
<td>3</td>
<td>SRBC</td>
<td>6/11 (55)</td>
<td>0/5 (1/1)</td>
</tr>
<tr>
<td>4</td>
<td>None</td>
<td>3/11 (27)</td>
<td>7/8 (1/1)</td>
</tr>
<tr>
<td>5</td>
<td>Untreated</td>
<td>1/15 (7)</td>
<td>14/14</td>
</tr>
</tbody>
</table>

*5 x 10^8 spleen and lymph node cells were inoculated i.p. twice at 7-day intervals.

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In the rats from the experimental group, the cytotoxic sensitivity of these cells to antisera was still present at 3 days but negative from 6 days after the immune cell inoculation. This result indicates that the FV-infected cells, which acquired new cellular antigens, are distributed equally in the thymus, spleen, and bone marrow of the tolerant rats and are eliminated by the inoculation of the immune cells.

Inhibition of Runting Syndrome in Tolerant Rats by Inoculation of Immune Spleen and Lymph Node Cells Mixed with Normal Bone Marrow Cells or Thymus Cells. The inoculation of immune lymphoid cells into FV-tolerant rats destroyed the FV-infected cells in the lymphoid organs and the bone marrow, and the lesions could not be repaired because of loss of stem cells from bone marrow. Consequently, the progressively extensive damages to lymphoid tissues in FV-tolerant rats resulted in the development of the runting syndrome. A good supply of hematopoietic stem cells appears to be necessary to prevent the runting death in the tolerant rats inoculated with immune lymphoid cells. Therefore, experiments were performed to prevent the runting death by additional inoculations of bone marrow cells or thymus cells (Table 2). In tolerant rats inoculated i.p. with WFT-13 immune spleen and lymph node cells (5 x 10⁷) and normal bone marrow cells (5 x 10⁷) (Group 1) 4 times at 5-day intervals, 3 of the 15 rats died of the runting syndrome and the remaining 12 rats showed clinical symptoms of the runting syndrome such as loss of body weight, diarrhea, etc. (Chart 2). However, all 12 survivors that were challenged with WFT-13 at 2 weeks after the last inoculations rejected the tumor, but thereafter 3 of the rats died of the runting syndrome. The remaining 9 survivors did not develop primary lymphomas during more than 300 days of observation (see next section for a discussion). In the rats given immune spleen and lymph node cells (5 x 10⁷) and normal spleen and lymph node cells (5 x 10⁷) (Group 2), all 10 rats died of the runting syndrome in from 14 to 22 days (mean survival time, 19.5 days) after the 1st inoculation of the mixed cells. In the rats given immune spleen and lymph node cells and normal thymus cells (5 x 10⁷) (Group 3), all 9 rats died of the runting syndrome in 28 to 76 days (mean survival time, 58.9 days). Thus, mean survival time was prolonged considerably in Group 3 as compared to Group 2. All 6 rats not inoculated (Group 4) died from the growth of the tumor.

On the other hand, Table 3 shows that respective inoculations of immune bone marrow cells (Group 1), normal bone marrow cells (Group 2), immune thymus cells (Group 3), or normal thymus cells (5 x 10⁷) (Group 4) into tolerant rats did not induce the runting syndrome except for 1 case in the rats given immune thymus cells. All survivors from each group died from the growth of the WFT-13 transplants.

Prevention of Primary Lymphomas in Tolerant Rats by Inoculation of Immune Spleen and Lymph Node Cells Mixed with Normal Bone Marrow Cells. In the preceding experiments, the tolerant rats that received immune spleen and lymph node cells admixed with normal bone marrow cells had a low incidence of the runting death and resisted the WFT-13 challenge. To confirm that the development of primary lymphomas in the tolerant rats had been inhibited by inoculations of mixed lymphoid and bone marrow cells,

### Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Inoculation</th>
<th>No. of incidences of runting syndrome/no. of rats</th>
<th>Transplantation of WFT-13 cells* (no. died/no. used)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ILyC + NBMC</td>
<td>3/15 (20)*</td>
<td>0/12 (3/12)*</td>
</tr>
<tr>
<td>2</td>
<td>ILyC + NLyC</td>
<td>10/10 (100)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>ILyC + NThyC</td>
<td>9/9 (100)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Nontreated</td>
<td>0/6 (0)</td>
<td>6/6</td>
</tr>
</tbody>
</table>

* 5 x 10⁷ WFT-13 cells were transplanted s.c. 2 weeks after the 4th inoculation.

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support the growth of FV in the absence of added hematopoietic cells. When heavily irradiated mice were given injections of FV and normal spleen or bone marrow cells, FV and FV-induced tumor cells were recovered from the spleens of these mice. In our system it was shown that the cells acquiring FV-specific cell surface antigens distributed homogeneously in the spleen, bone marrow, and thymus and were eliminated by the inoculation of immune lymphoid cells. This fact suggests that the specific immune reaction of the inoculated lymphoid cells against the FV-specific transplantation antigens causes destruction of mature or immature hematopoietic cells, which are considered to be the target cells of FV, and subsequently results in the runting death. The experiments presented here demonstrated that the prevention of the runting death and the breakdown of immunological tolerance in rats were successful by the additional inoculation of syngeneic bone marrow cells. The most likely explanation for the mechanism in the prevention of the runting death and in the abrogation of the tolerant state is that new bone marrow cells replaced the FV-infected cells and repopulated lymphoid organs that had been destroyed by the administered immune lymphoid cells. The inability of admixed normal spleen and lymph node cells or thymus cells to cure the runting syndrome is due to less or lack of hematopoietic stem cells. Infection of residual FV in blood of the recipient rats to new hematopoietic stem cells cannot be overlooked. However, it has been shown in previous experiments that viremia decreased rapidly and disappeared completely within 6 days after the inoculation of immune lymphoid cells (13). Therefore, there is little chance to infect new hematopoietic stem cells with FV, and the majority of inoculated stem cells would take part in reconstitution of the damaged lymphoid organs in the runted rats.

The inoculation of the spleen and lymph node cells from rats immunized with SRBC induced a relatively high incidence of the runting syndrome compared with the inoculation of spleen and lymph node cells from normal rats or from rats immunized with AH-66 cells. However, there were 4 survivors that did develop slightly the runting syndrome and did not develop primary lymphomas during 300 days of observations. This fact might be interpreted as follows. In general, SRBC stimulated not only specific antibody-producing cells but also nonspecific antibody-producing cells and their precursor cells. The increased number of immunocompetent cells, including hematopoietic stem cells in the spleen of the donor rats, promoted the abrogation of the tolerant state and resulted in the runting death. In the 4 surviving rats the non-FV-infected hematopoietic and stem cells of the donor spleen repopulated the bone marrow and lymphoid system, consequently preventing the development of the expected primary lymphomas.

Complete immunological tolerance means that no measurable immune response can be found. In the present system this is defined as follows: (a) constant viremia with titers $\leq 10^{2.0}\text{ MIDs}_{50}/\text{ml}$ and neutralization antibody $< 1:2$; and (b) lethal growth of FV-induced lymphoma transplants and cytotoxic antibody $< 1:2$. Complete tolerance could be induced only in newborn rats given injections of high doses of FV ($10^{6.0}\text{ MIDs}_{50}/\text{rat}$) within 48 hr of birth.

### Table 3

<table>
<thead>
<tr>
<th>Inoculation</th>
<th>No. of incidence of runting syndrome/no. of rats</th>
<th>Transplantation of WFT-13 cells (no. died/no. used)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IBMC $^a$</td>
<td>0/5</td>
</tr>
<tr>
<td>2</td>
<td>NBMC</td>
<td>0/4</td>
</tr>
<tr>
<td>3</td>
<td>ITThyC</td>
<td>1/6</td>
</tr>
<tr>
<td>4</td>
<td>NThyC</td>
<td>0/4</td>
</tr>
<tr>
<td>5</td>
<td>None$^b$</td>
<td>0/6</td>
</tr>
</tbody>
</table>

$^a$5 x 10$^7$ WFT-13 cells were transplanted s.c. 2 weeks after the 4th inoculation.

$^b$IBMC, immune bone marrow cells; NBMC, normal bone marrow cells; ITThyC, immune thymus cells; NThyC, normal thymus cells.

### Table 4

Prevention of primary lymphomas in FV-tolerant rats by inoculations of immune spleen and lymph node cells and/or normal bone marrow cells

<table>
<thead>
<tr>
<th>Inoculation</th>
<th>No. of incidences of runting syndrome/no. of rats</th>
<th>No. of incidences of primary lymphomas/no. of rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IILyC $^c$ + NBMC</td>
<td>7/23 (30)</td>
</tr>
<tr>
<td>2</td>
<td>NBMC</td>
<td>4/25 (15)</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>3/24 (13)</td>
</tr>
</tbody>
</table>

$^c$The abbreviations used are the same as in Table 2.

$^c$Numbers in parentheses, percentage of rats with runting syndrome.

DISCUSSION

It is now generally accepted that FV has spleen and bone marrow cells as its target of multiplication and oncogenic transformation (2, 3, 12, 15, 18). For instance, Thompson (18) has demonstrated that heavily irradiated mice could not support the growth of FV in the absence of added hematopoietic cells. When heavily irradiated mice were given injections of FV and normal spleen or bone marrow cells, FV and FV-induced tumor cells were recovered from the spleens of these mice. In our system it was shown that the cells acquiring FV-specific cell surface antigens distributed homogeneously in the spleen, bone marrow, and thymus and were eliminated by the inoculation of immune lymphoid cells. This fact suggests that the specific immune reaction of the inoculated lymphoid cells against the FV-specific transplantation antigens causes destruction of mature or immature hematopoietic cells, which are considered to be the target cells of FV, and subsequently results in the runting death. The experiments presented here demonstrated that the prevention of the runting death and the breakdown of immunological tolerance in rats were successful by the additional inoculation of syngeneic bone marrow cells. The most likely explanation for the mechanism in the prevention of the runting death and in the abrogation of the tolerant state is that new bone marrow cells replaced the FV-infected cells and repopulated lymphoid organs that had been destroyed by the administered immune lymphoid cells. The inability of admixed normal spleen and lymph node cells or thymus cells to cure the runting syndrome is due to less or lack of hematopoietic stem cells. Infection of residual FV in blood of the recipient rats to new hematopoietic stem cells cannot be overlooked. However, it has been shown in previous experiments that viremia decreased rapidly and disappeared completely within 6 days after the inoculation of immune lymphoid cells (13). Therefore, there is little chance to infect new hematopoietic stem cells with FV, and the majority of inoculated stem cells would take part in reconstitution of the damaged lymphoid organs in the runted rats.

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Complete immunological tolerance means that no measurable immune response can be found. In the present system this is defined as follows: (a) constant viremia with titers $\leq 10^{2.0}\text{ MIDs}_{50}/\text{ml}$ and neutralization antibody $< 1:2$; and (b) lethal growth of FV-induced lymphoma transplants and cytotoxic antibody $< 1:2$. Complete tolerance could be induced only in newborn rats given injections of high doses of FV ($10^{6.0}\text{ MIDs}_{50}/\text{rat}$) within 48 hr of birth.
These rats have always satisfied the criteria described above. States of incomplete and temporary tolerance can also be produced in the present system. Rats given neonatal injections of FV, $10^{2.0}$ MID$_{50}$/rat or less, were divided into 2 groups: one is a runting group and the other is a non-tolerant group (14). Most of the rats in the former group died of the runting syndrome, and survivors produced cytotoxic antibody and resisted the FV-induced lymphoma transplants. Then, various degrees of tolerance depend on the amount of virus administered neonatally.

We cannot, however, rule out the possibilities of the continuous presence of a low-grade immune response, detectable only by refined methods or localized to the kidneys (9, 10), and the cytotoxic reaction of lymphoid cells from tolerant rats to FV-infected cells detectable in in vitro systems (1).

ACKNOWLEDGMENTS

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REFERENCES

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