Thymic Hormone Modulation of Leukemogenic Virus Replication

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SUMMARY

The effect of purified calf thymus extract, thymosin, on infection with murine leukemia virus (MuLV) (Rauscher) was studied in adult thymectomized BALB/c mice. The course of infection was determined by virus titer in the blood plasma, by enumeration of spleen cells replicating MuLV (i.e., infectious centers), and by the examination of cells for expression of virus-induced cell membrane antigen. Thymectomy (performed 1 week prior to the virus injection) decreased MuLV infection by all three parameters. However, administration of thymosin (500 μg, given in equal doses 2 days before and at the time of injection of virus) caused a marked increase of plasma titer of virus, as well as an increased number of spleen cells generating MuLV as compared to controls treated either with purified spleen fraction or with diluent (buffered salt solution). More than two doses of thymic extract (up to nine, given every 2 days after infection) had no further effect on plasma virus titer or number of splenic infectious centers. Furthermore, treatment of mice with either thymic or splenic extracts increased the incidence of virus-related membrane antigen appearing on the surface of spleen cells, as compared to diluent-treated control animals. These results indicate that thymosin may enhance infection by or replication of murine oncornaviruses.

INTRODUCTION

Cerny and Waner (7) advanced the hypothesis that activation and/or functional differentiation of immunocompetent cells play an important role in the infection and transformation of these cells by murine oncornaviruses. Antigenically sensitized cells were demonstrated to be infected, immunosuppressed, and altered, whereas virgin cells were unaffected by either Friend or Moloney leukemia virus. A more general question arises from these observations. Must the activation of lymphocytes be antigenic in nature, or is a more generalized type of stimulation, e.g., by a hormone, sufficient to bring about a virus-susceptible state?

In an attempt to investigate this problem, studies were undertaken involving the thymic hormone thymosin. Recent reviews (11, 12) give the chemical, physical, and biological properties of this substance. Briefly, thymosin is known to have the following effects: (a) it reduces the incidence of wasting disease that characteristically develops after neonatal thymectomy; (b) it accelerates the normal ontogeny of T-cells in the spleen with regard to their ability to elicit graft versus host response; (c) it accelerates the development of responsiveness of spleen cells in neonates to phytohemagglutinin in vitro; (d) it changes the response of newborn mice to sheep RBC; (e) in genetically thymusless (nu/nu) mice, thymosin restores the capacity of spleen cells to produce graft-versus-host response and to elicit a mixed lymphocyte reaction in vitro; and (f) in human and murine bone marrow cells, it brings about the appearance of cells carrying the thymocyte-marker antigen.

Since many types of "activation" (mostly in the population of T-cell precursors) appear to be engendered by thymosin, it became of interest to test the effects of this hormone on the course of a virally induced neoplasm of the reticuloendothelial system in mice.

Infection with the MuLV-R used in this study seems to require the presence of thymus (14). Rauscher (18), who discovered the virus in 1962, considered this disease to be an erythrocytopoiesis followed by a lymphoid leukemia. Others (4) report that in this disease process erythroleukemia is followed by myeloblastic leukemia. After a short latency in susceptible species, hepatosplenomegaly occurs, erythroblasts appear in the peripheral blood, and viremia is noted. Our experiments were done on adult thymectomized BALB/c mice; intact normal mice served as controls. Partially purified calf thymosin (Fraction V), a similarly partially purified fraction from calf spleen (12) or physiologically treated calf spleen fraction (17) was nonetheless used in these experiments. The course of infection was determined by the following assays: (a) enumeration of virus-releasing spleen cells in vitro (an assay for infectious centers); (b) titration of MuLV in the plasma; and (c) immunofluorescent assay for a virus-induced membrane antigen on spleen cells.

MATERIALS AND METHODS

Animals and Thymectomies. BALB/c mice (Charles River Laboratories, Wilmington, Mass.), of both sexes, were thymectomized at ages ranging from 39 to 63 days. Nonthymectomized mice served as controls. The thymectomies were performed essentially as described in Ref. 13 except that strict asepsis was not observed; a rate of success comparable to that indicated in Ref. 13 was nonetheless achieved. The mice were permitted to recover for 1 week after the operation. This time period allows endogenous thymosin levels to decline more than 90% (1).
**Thymosin.** Thymosin (Fraction V) and spleen preparation (Fraction V; see Ref. 12 for details) were generously provided by Allan L. Goldstein, Department of Human Biological Chemistry, the University of Texas Medical Branch, Galveston, Texas. Both preparations were dissolved in sterile Puck’s Saline G (Grand Island Biological Co., Grand Island, N. Y.) containing antibiotics (Microbiological Associates, Bethesda, Md.). Te mice (4/group) were injected twice (2 days prior to the infection, and on the day of infection), with 250 μg of appropriate material (in 0.2 ml) or with 0.2 ml of Puck’s Saline G, i.p. Nonthymectomized control mice, given injections of MuLV-R, were otherwise untreated.

**Virus.** MuLV-R was obtained from the American Type Tissue Culture Collection (Bethesda, Md.) as a cell-free preparation from BALB/c mice. It was passaged in BALB/c mice, once in our laboratory, using a previously described procedure (6). Mice were infected i.v. with 2 × 10^6 focus-forming units.

**Assay of MuLV Replication.** MuLV titers were determined by the in vitro assay on S+/L− cells derived from the 3T3 cell line (2, 3). S+/L− cells were cultured in McCoy’s 5A media (Microbiological Associates) with 5% fetal bovine serum (Grand Island Biological), in 35-mm tissue culture dishes (Falcon Plastic, Los Angeles, Calif.; 3 to 5 × 10^4 cells/dish). After 2 days of growth, dishes were treated with DEAE-dextran (Sigma Chemical Co., St. Louis, Mo.; 25 μg/ml for 30 min). After removal of the DEAE-dextran, either plasma or spleen cells were added, as described below. Foci of transformed S+/L− cells were scored 5 days later, following fixation of cells with a 1:10 solution of 40% formaldehyde in tap water. They were then stained for 4 min with a 0.03% solution of methylene blue in tap water. S+/L− cells (a line designated F-10) were kindly provided by Dr. David Livingston, Children’s Hospital and Harvard Medical School, Boston, Mass.

**Infectious Center Assay.** Spleen cell suspensions (pooled from 4 spleens per each group) were prepared by teasing organs on stainless steel wire screens in sterile cold Puck’s Saline G (containing penicillin and streptomycin) (Grand Island Biological), and passed several times through Pasteur pipets. After clumps were allowed to settle, single-cell suspensions were centrifuged at 225 × g for 10 min at 4° and washed once. Cells were counted and the indicated number of viable nucleated cells (in 0.5-ml volume) was plated on a dish containing DEAE-dextran-treated S+/L− cells, in duplicate. Number of foci reflect spleen cells releasing MuLV (i.e., infectious centers).

**MuLV Titer in Plasma.** Pooled plasma (from 4 mice) was collected from heparinized blood, stored at −70°, and filtered through sterile 0.45-μm Millipore filters prior to the assay. Dishes with suitably prepared S+/L− cells (see above) were treated with 0.5 ml of serially diluted plasma. Foci were calculated per total amount (μl) of undiluted plasma.

**Assay of Membrane Antigen.** The membrane antigen assay was performed on fresh, washed spleen cells as described (6). Heterologous rabbit serum against FVMA in the 1st step. This serum was raised by repeated immunization of New Zealand rabbits with FVMA-positive spleen cells from Friend virus-infected BALB/c mice. Each immunization consisted of 1 to 3 × 10^7 viable cells injected i.m. into 4 sites. An injection of 100 mg of pertussis vaccine (gift from the Massachusetts State Laboratory, Boston, Mass.) was given together with the 1st immunization. Rabbit antiserum was subsequently absorbed with blood cells, liver cells, and spleen cells pooled from uninfected BALB/c mice. The specificity of the antiserum was comparable to the isologous mouse anti-FVMA serum when tested (a) for cross-reactivity between Friend and Rauscher leukemia spleen cells (5); and (b) in mice with progressing and regressing leukemia (6), using both membrane immunofluorescence assay and cytotoxic test (J. Cerny, unpublished results). Goat anti-rabbit fluorescent conjugate (Microbiological Associates) was used in the 2nd step of the assay, after suitable dilution.

**Protein Determination.** Protein determinations on thymosin and spleen preparations were made using the method of Lowry et al. (15).

**RESULTS**

The course of MuLV-R infection in Te mice and the enhancing effect of thymosin administration are shown in Table 1 and Chart 1.

The MuLV titer in plasma of Te mice treated with Puck’s Saline G alone was 3 to 8 times lower than the titer in nonthymectomized control mice, on Day 7 after the infection (Table 1). On Day 15 (which is a separate experiment from Days 7 and 18), this difference was at least 6-fold. On Day 18, the difference in plasma titer was about 2-fold. The number of spleen cells with replicating MuLV (as reflected by assay of infectious centers in vitro at the 18th day after infection) was also about 4-fold lower in the Puck’s Saline G-treated Te mice as compared to the control. Two injections of thymosin (given 2 days prior to and simultaneously with the virus) enhanced, specifically, the MuLV replication in Te mice (Table 1). The increase of MuLV plasma titer was about 2- to 3-fold on Day 7, about 3-fold on Day 15, and about 3-fold on Day 18 after infection. Similarly, the number of spleen cells with replicating MuLV increased 2 to 3 times and almost 100 times on the 7th and 18th days after infection, respectively. Indeed, the virus replication in Te, thymosin-treated mice was even higher than that in nonthymectomized mice in the assays done on the 18th day.

The enhancing effect of thymosin was apparent regardless of the number of spleen cells or amount of plasma assayed, even though the relationship between dilution and foci was not absolutely linear. No MuLV was measurable in noninfected mice given injections of thymosin alone (Table 1).

In contrast to thymosin, 2 injections of spleen preparation (the protein content of which was determined to be comparable to that of the thymosin preparation, i.e., 85 to 95%) had very little if any effect on the infection. MuLV replication in the spleen preparation-treated, Te mice was similar to that in Te mice treated with Puck’s Saline G alone. Thus, the enhancing effect of thymosin was a specific one.

Chart 1 gives the time course for the appearance of virus-
Presence of infectious MuLV in spleen cells and plasma of Te mice receiving 2 injections of thymosin and infected with MuLV-R

<table>
<thead>
<tr>
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<th>Day 7 after infection</th>
<th>Day 15 after infection</th>
<th>Day 18 after infection</th>
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<tr>
<td></td>
<td>Spleen cells (no./dish)</td>
<td>Plasma (μl/dish)</td>
<td>Spleen cells (no./dish)</td>
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<tr>
<td>Treatment</td>
<td>MuLV-R</td>
<td></td>
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<tr>
<td></td>
<td>10</td>
<td>100</td>
<td>1000</td>
</tr>
<tr>
<td>Te, Puck’s Saline G</td>
<td>+</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Control (not thymec-tomized)</td>
<td>+</td>
<td>ND*</td>
<td>ND</td>
</tr>
<tr>
<td>Te, thymosin</td>
<td>+</td>
<td>0</td>
<td>11</td>
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<tr>
<td>Te, spleen fraction</td>
<td>+</td>
<td>0</td>
<td>3</td>
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<tr>
<td>Te, thymosin</td>
<td>-</td>
<td>0</td>
<td>ND</td>
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* ND, not done; TNC, too numerous to be counted (more than 300 foci/dish).

A ND, not done; TNC, too numerous to be counted.

DISCUSSION

Administration of partially purified calf thymosin (Fraction V) to Te mice in 2 doses, prior to and together with MuLV-R infection, markedly enhanced virus replication. Since calf thymosin represents a material foreign to the mouse and is, presumably, antigenic, control mice were given injections of comparable amounts of material (on both dry weight and protein basis) prepared from calf spleen using exactly the same procedure (Fraction V). Injection of the splenic extract produced very little, if any, effect on MuLV replication. Thus, it appears that enhancement of virus replication by thymosin is specifically related to the biological effect of the hormone on mouse cells. While 2 injections of thymosin produced a clear effect on the progress of the virus, additional doses seemed ineffective or perhaps inhibitory (Table 2).

Although the target cells of MuLV-R include cells originating in bone marrow, namely the erythroid cell precursors (16), T-cells seem to be necessary for successful infection and development of the disease. Jadin et al. (14) found that genetically thymusless (nu/nu) mice infected with MuLV-R presented no clinical symptoms of the disease. If these mice were given injections of T-cells, however, they rapidly developed the disease and succumbed to it. Furthermore, virus could be recovered from the organs of these adoptively restored mice. These observations could indicate, but...
The possible effect of calf thymosin on the course of MuLV-induced leukemia has also been investigated using the colony of carrier mice infected, congenitally, with Moloney leukemia virus (17). These mice develop thymomas at adult age; the process seems to be accelerated by injections of thymosin (M. R. Proffitt, personal communication).

The results of Jadin et al. (14) and those of our laboratory seem at variance with experiments that demonstrate the protective role of the thymus and thymosin in tumor induction by viruses. An increased oncogenic effect of polyoma virus (19) and MSV (9, 10, 20) was observed in athymic (nu/nu) mice. However, if these animals received grafts, resistance to MSV-induced tumors could be induced (20). Furthermore, repeated administration of thymosin increased the resistance of neonatal mice to tumor induction by MSV, presumably via stimulation of the immune response (21).

The 2 sets of results, those indicating an increased oncogenic potential of MuLV, on one hand, and those showing an increased immune response to virus-induced tumor, on the other, seem to reflect 2 different activities of the thymus and/or thymosin, which may involve different mechanisms. The net effect of the hormone on the outcome of oncogenic infection may vary with respect to the nature of the virus, the nature of the target cells, the mode of thymosin administration, and the physiology of the host.

In conclusion, calf thymosin has been shown here to enhance the course of MuLV-R infection in Te mice, in that it: (a) increases the replication of infectious virus in the spleen cells; (b) increases virus titer in the plasma; and (c) increases the number of cells bearing virus-related membrane antigen(s). With regard to virus replication, splenic extract had no consistent effect when compared to saline treatment. In the case of virus-induced cell membrane antigen expression, thymosin treatment caused only a marginal increase above the number of positive cells seen upon treatment with splenic extract, although both were clearly distinguishable from diluent (buffered salt solution)-treated controls.

These observations offer support for the concept that infection and alteration of lymphoreticular cells with murine oncornavirus occur more readily in cells that have reached a specific level of differentiation and/or activation, and that this stage of development can be brought about by agents such as thymic product.

ACKNOWLEDGMENTS

The authors wish to thank the following persons for their contributions to this work: Dr. Allan Goldstein, Department of Human Biological Chemistry, The University of Texas Medical Branch, Galveston, Texas, who generously provided both the preparation of thymosin and spleen extract and valuable comments; Dr. David Livingston, Children's Hospital and Harvard Medical School, Boston, Mass., for S+/L—cells; Dr. Myron E. Essex and Dr. Ronald A. Stillier, Harvard School of Public Health, for reviewing the manuscript; and Lynn M. Demler, Samuel H. Fistel, Patricia Hensgen, and Elizabeth B. Waner for skilled technical assistance.

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Cancer Res 1976;36:2048-2052.

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