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COMBINATION THERAPY OF VIRAL LEUKEMIA: STATOLON, RADIATION AND TRANSPLANTATION[1]

PAUL M. KUHNERT,[2] PENNY ERHARD,[2] BARBARA BROZOVICH, JAMES P. OKUNEWICK, and BETTY R. KUHNERT,[2] Cellular and Radiation Biology Laboratories, Clinical Radiation Therapy Research Center, Division of Radiology, Allegheny General Hospital, Pittsburgh, PA 15212

Abstract. Studies were carried out to determine the effectiveness of treating murine viral leukemia by combining statolon therapy with irradiation and transplantation therapy. The experimental design involved inoculating Rauscher leukemia-infected mice with statolon, a potent interferon inducer, prior to lethal whole-body irradiation and following hematopoietic cell transplantation. The data show that treating the leukemic animals in this manner resulted in positive responses from all indices of leukemia development investigated. These responses included a 42% decrease in spleen weight at autopsy, a 21% increase in survival at 30 days, and significantly decreased white blood cell counts and spleen weight during the treatment period. Although the effect of the combination therapy was not permanent and further refinement of the experimental protocol is necessary, it appears to be a promising method of treating viral leukemia.

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Storb *et al* (1973) have suggested that leukemia patients receiving whole body irradiation and hematopoietic cell transplantation be treated with interferon following irradiation. The basis for their suggestion was the known blocking effect of interferon on tumor virus replication and cell transformation. They hypothesized that if a leukemic agent is present in the patient and is transmitted to the susceptible donor cells, perhaps treatment with interferon may inhibit this (hypothetical) human leukemia virus and prevent it from transforming the donor cells.

One way of testing the hypothesis of Storb *et al* is to use their treatment protocol to treat a known viral leukemia in an animal model. Several animal models could be used for this evaluation, but Rauscher murine leukemia is particularly suitable for several reasons. First of all, we have shown that Rauscher leukemia recurs in mice in a manner similar to that observed in the treatment of human acute lymphoblastic leukemia, when lethal whole body irradiation is followed by hematopoietic cell transplantation (Kuhnert *et al* 1974). Further, the reinduction of the Rauscher leukemia has been shown to occur in the donor cells (Kuhnert *et al* 1974), and, studies in our laboratory (OKunewick *et al* 1972, OKunewick and Erhard 1973) and by others (Kufe *et al* 1972) have shown that Rauscher leukemia is a good model for studying the etiology of viral leukemia.

Accordingly, the purpose of this investigation was to evaluate the hypothesis of Storb and coworkers by treating Rauscher viral leukemia with statolon, a potent interferon inducer, in conjunction with irradiation and hematopoietic cell transplantation. Earlier studies showed that irradiation and transplantation therapy was capable of slowing the development of murine viral leukemia (OKunewick *et al* 1973a). Likewise, statolon therapy was extensively used to treat viral leukemia and proved to be effective under certain experimental conditions (Wheelock *et al* 1973), but no animal studies combining these 2 therapeutic techniques have been reported.

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[2]Present address: Perinatal Clinical Research Center, Department of Obstetrics and Gynecology, Cleveland Metropolitan General Hospital, Case Western Reserve Univ., 3395 Scranton Road, Cleveland, OH 44109.

MATERIALS AND METHODS

X-Irradiation. Recipient mice were exposed to 1100 R of x-rays with a G.E. Maxitron 300 x-ray unit operating at 275 kV and 20 mA with a half value layer of 1.8 mm Cu. The exposure rate was approximately 60 R/min measured within the mouse holders. A Victoreen model 570 dosimeter employing a 100 R probe was utilized to calculate R/min. The total dose was measured for three 1 minute periods, corrected for temperature and pressure, and the average R/min calculated. Mice were exposed to x-rays in groups of 10 in a plastic holder in which they were free to move.

Rauscher Leukemia Virus (RLV). Fifty SED 50 units RLV suspension were injected intraperitoneally into 12 week old mice. The preparation and assay of the Rauscher virus stock have been previously described (OKunewick *et al* 1972, 1973b).

Animals. Female SJL/J mice were utilized as recipient hosts and male SJL/J mice as donors for the hematopoietic cell transplants. They were obtained from Jackson Laboratories at the age of 8 weeks, housed 5 to a cage in filter covered cages, and maintained on autoclaved food and acid water. At 12 weeks, both the experimental and control mice were irradiated with 1100 R and within 2 hours of irradiation received an injection of spleen cells. Following transplantation, the mice were maintained 2 per cage.

Cell Transplantation. Donor spleen cell suspensions were made by cutting open the spleens of four to six 11-week-old male mice and gently forcing the cells out of the capsule into sterile Hank's solution (Mg and Ca free). The cells were then broken down further into a single suspension by repeated aspiration through a 25 gauge needle. After evaluating the number of nucleated cells per ml using a Coulter Counter, an appropriate dilution was made so that 0.5 ml of the cell suspension contained 3×10^7 nucleated cells. Recipient mice were injected with 0.5 ml of this cell suspension via the tail vein.

Vesicular Stomatitus Virus (VSV). Vesicular stomatitis virus (Indiana strain) was grown in chick embryo fibroblast cultures. The infected culture was subjected to 3 cycles of freezing and thawing in a dry ice and acetone bath. One ml aliquots were placed in sterile vials and stored at −70 °C until use. This virus preparation was found to contain 1000 tissue culture infecting doses ($TCID_{50}$) per ml when titrated on mouse L cell monolayer cultures.

Cell Cultures. Mouse L cells (L-929) employed for the assay of interferon were obtained from the American Type Culture Collection. Cells were grown in a growth medium consisting of TC Minimal Medium Eagle, Earle BSS (MEM) supplemented with L-glutamine, fetal calf serum and the antibiotic gentamicin (1, 10, 0.5 ml per 100 ml of medium respectively). Maintenance medium was the same as the growth medium except the fetal calf serum concentration was reduced to 4 ml per 100 ml of medium.

Interferon Assay. The method employed for assay of interferon was similar to that described by Wheelock (1967). The serum samples to be tested were diluted in growth medium, and 1 ml of each dilution was added to 1 day old cultures of mouse L cells grown to incomplete monolayers in screw cap culture tubes. After 20 hours of incubation at 37 °C, the cultures were washed once with 4 ml of Dulbecco's phosphate buffered solution. To each tube, 1 ml of warm protein-free growth medium was added and then 1 ml of cold growth medium containing 1000 $TCID_{50}$ of vescular stomatitis virus was inoculated. Cultures were considered to be protected when less than 10% of the cells showed cytopathic effects at a time when more than 75% of control cells exhibited cytopathic effects.

Interferon titers are expressed as reciprocals of the highest dilution of the serum employed, 1 ml of which protected cultures against challenge with standard doses of VSV. No serum was tested at less than 1:10 dilution.

Stalolon. Statolon was supplied by Eli Lilly & Co. (Lot 354-1080B-220) and was dissolved in 1% sodium bicarbonate 2 hours prior to inoculation of the mice (5 mg/mouse I.P.). It was injected 25 hours prior to irradiation (13 days after RLV inoculation) and 15 days after irradiation and transplantation (29 days after RLV inoculation).

RESULTS

Effect of Combination Therapy on Spleen Weight

Figure 1 illustrates the effect of statolon treatment on the spleen weight of 1100 R irradiated and spleen cell transplanted

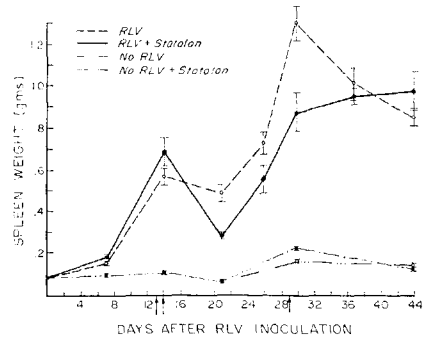

FIGURE 1. Effect of statolon on the splenomegalic response to Rauscher leukemia in 1100 R irradiated and spleen cell transplanted mice. Irradiation and transplantation were performed on day 14 (dashed arrow) and statolon was injected on days 13 and 29 (solid arrows). Each data point represents the mean ± S. E. of 10-17 mice.

mice over a period of 44 days following RLV inoculation. The gradual increase in spleen weight observed up to day 7 and the rapid increase observed from day 7 to day 14 are typical of Rauscher leukemia development and have often been used to assess the effectiveness of various types of therapy on viral leukemia (OKunewick 1972, Chirigos *et al* 1973). On day 14 all of the mice received 1100 R irradiation; thus, the rapid decrease in spleen weight from day 14 to day 21 was also expected. The leukemic mice treated with statolon prior to irradiation and transplantation, however, showed a greater decrease in spleen weight on day 21 (7 days post-irradiation) than the untreated leukemic mice. Furthermore, up to day 37 (23 days post-irradiation), mice in the statolon treated group continued to have lower spleen weights than the group treated with irradiation and spleen cell transplantation alone. The differences observed on days 21, 26, and 30 were found to be statistically significant ($P<0.05$). On days 37 and 44, the spleen weights of the Rauscher leukemic mice were similar regardless of earlier statolon treatment.

TABLE 1

Effect of statolon on white blood cell increase in 1100 R irradiated and spleen cell transplanted mice with Rauscher leukemia.*

Days after RLV Inoculation	Days after X-ray	Experimental Group			
		No RLV (n)	No RLV+ Statolon** (n)	RLV (n)	RLV+ Statolon** (n)
7	−7	6,311± 724 (16)†	7,528± 445 (10)	5,219± 766 (9)	4,475± 651 (10)
14	0	6,766± 691 (12)	1,154± 188 (9)	7,401±1386 (15)	4,019± 750 (15)
21	7	501± 128 (11)	268± 30 (2)	895± 162 (11)	321± 41 (14)
26	12	———	———	7,829±1467 (9)	2,991± 688 (9)
30	16	9,591±1634 (16)	7,868±1054 (6)	30,042±3196 (14)	11,643± 2318 (14)
37	23	———	———	40,228±7366 (8)	40,653± 7854 (19)
44	30	4,825± 627 (5)	5,144± 541 (5)	68,843±8304 (17)	50,287±12506 (14)

*1100 R irradiation and spleen cell transplant occurred on day 14.
**Statolon was injected on days 13 and 29. RLV=Rauscher leukemia virus.
†Mean±S.E.

Effect of Combination Therapy on Peripheral White Cell Counts

The effect of statolon treatment on the peripheral white blood cell increase in irradiated and spleen cell transplanted mice with Rauscher leukemia showed (on day 14) an increase in the white blood cell level of the untreated leukemic mice (table 1). This slow increase in the WBC level during the first 2 weeks of RLV infection has been reported by others (Markoe and OKunewick 1973). On day 14, the statolon treated leukemic mice did not show an increase in peripheral white cells. Apparently the injection of statolon slowed the WBC production of the experimental group. Also noticeable on day 14 was a decrease in the WBC count of control mice treated with statolon. On day 21, all groups of mice were found to have greatly decreased WBC counts. This finding is attributable to treatment with a high level of radiation and spleen cell trnasplantation on day 14. Also apparent on day 21 and on days 26 and 30 was the lower WBC counts of the statolon treated as compared to the untreated leukemic mice. These differences were statistically significant ($P<0.05$). The observed differences are apparently attributable to treatment of the leukemic mice with statolon; however, the effect of statolon was not permanent, and a further increase in the WBC count was observed on days 37 and 44.

Effect of Combination Therapy on Spleen Weight at Autopsy

One of the most readily recognizable effects of Rauscher leukemia is splenomegaly (Chirigos *et al* 1973). This parameter is often used to assess the effectiveness of various types of therapy on viral

TABLE 2

Effect of statolon on terminal spleen weight of 1100 R irradiated and spleen cell transplanted mice.

Treatment	No. of Mice	Spleen Weight at Autopsy (gm)
No RLV**	19	0.0756±0.0108*
No RLV+Statolon	18	0.0617±0.0053
RLV	19	0.7981±0.1463
RLV+Statolon	27	0.4558±0.0479

*Mean±S.E.
*RLV=Rauscher leukemia virus.

leukemia and table 2 shows the mean autopsy spleen weights of the various experimental groups of mice in this study. Since each group was treated identically with irradiation and transplantation of the same number of hematopoietic cells, the observed differences should be attributable to treatment with statolon. Comparing the statolon treated control mice with the untreated control mice, a slight decrease in mean spleen weight can be seen but this difference is not statistically significant. When comparing the average spleen weights of the treated and untreated leukemic mice, however, a significant ($P<0.05$) decrease was apparent in the statolon treated group. The mean autopsy spleen weight of statolon treated leukemic mice was 43% less than that of the untreated leukemic controls.

Interferon Production in Response to Statolon Treatment and Rauscher Virus

The levels of interferon, induced in leukemic and control mice with statolon preceding and following hematopoietic cell transplantation, (see table 3) show that 7 days after RLV inoculation there was no production of interferon in the leukemic mice in response to the Rauscher virus. On day 14, 23 hours following statolon injection and prior to irradiation and transplantation, maximal interferon production was observed in the control mice. Only 0.1 of the interferon production observed in the control mice was observed in the leukemic mice. Apparently the production of interferon was suppressed in these animals by the viral leukemia. On days 21 and 30, there was still no detectable production of interferon in the leukemic mice due to the virus. In the control mice on day 30, but not in the leukemic mice, a small amount of interferon was produced following the injection of statolon on day 29.

TABLE 3

Interferon production in statolon inoculated Rauscher leukemic (RLV) and control mice preceding and following hematopoietic cell transplantation.***

Days after RLV Inject.	Interferon Titer†	
	Control	Rauscher Leukemic
7	0	0
14	3,160	316
21	0	0
30	10	0

*Statolon was injected on days 13 and 29.
**1100 R irradiation and spleen cell transplant occurred on day 14.
†Interferon titers are expressed as reciprocols of the highest dilution of the serum employed, 1 ml of which protected cultures against challenge with standard doses of VSV. No serum was tested at less than 1:10 dilution.

Effect of Combination Therapy on Survival

Table 4 summarizes the survival data of both the statolon treated and untreated control and viral infected mice at 30 days following 1100 R X-ray exposure and spleen cell transplantation. Survival times were counted from the time of irradiation and spleen cell transplantation, *i.e* 14 days after virus infection. Among the untreated control and untreated leukemic mice, 94% and 64% survival was observed at 30 days, respectively. These values are approxi-10% higher than those observed in an

TABLE 4

Effect of statolon on 30-day survival of 1100 R irradiated and spleen cell transplanted leukemic mice.

Treatment	No. of Mice	% 30-day Survival
No RLV*	24	94
No RLV+Statolon	24	79
RLV	25	64
RLV+Statolon	27	85

*RLV=Rauscher leukemia virus.

earlier study under the same experimental conditions (Kuhnert *et al* 1974). Thirty day "rescue" was achieved in both the control and leukemic mice; however, in the control group treated with statolon, a 15% decrease in survival was observed at 30 days. Apparently, treatment with statolon decreased survival time in this experimental group. On the other hand, Rauscher leukemic mice treated with statolon showed a 21% increase in survival over the untreated leukemic mice.

DISCUSSION

From our data, it is clear that a positive response was observed on all of the indices of leukemia development investigated. These responses included a 42% decrease in spleen weight at autopsy, a 21% increase in survival at 30 days, and significantly decreased white blood cell counts and spleen weight during the treatment period. The effect of the combination therapy was not permanent, however, and the mice eventually succumbed to the viral leukemia.

The statolon dose used in the present study (5 mg/mouse) was chosen because it has been successfully used to induce interferon formation in mice (Wheelock 1967, Wheelock *et al* 1974) and because a preliminary study by us showed that it was capable of inducing large amounts of interferon in the SJL/J mouse. At this dosage level, no adverse effects have been reported but our data indicate that this dose is toxic in nonleukemic mice that have been treated with irradiation and transplantation. The toxic effects observed included a 15% decrease in 30-day survival and decreases in white blood cell counts and mean autopsy spleen weight. These side effects may, of course, be apparent in this investigation because of the unique experimental conditions. Unfortunately, little information is available concerning the toxicity of statolon to mice (Finter 1973).

Our experimental mice were injected with statolon at 2 propitious times during the course of the treatment protocal. The first injection was given 20 hours prior to irradiation and hematopoietic cell transplantation. This time was chosen because preliminary experiments showed that interferon production reached a peak in the SJL/J mouse at approximately 15 hours after statolon injection and did not begin to decline until 25 hours after injection. Therefore, the maximum titer of interferon would be present when the hematopoietic system was destroyed by irradiation and the donor hematopoietic cells were inoculated. By producing a high titer of interferon at this time, viral transformation of the donor hematopoietic cells may be theoretically blocked. The time of the second statolon injection was chosen for its therapeutic potential. De Maeyer *et al* (1967) had shown that interferon production was restored in lethally irradiated mice grafted with bone marrow cells 15 days after irradiation and transplantation. Thus, the second injection of statolon was given on the 15th day following irradiation and transplantation.

In response to the injection of statolon, Rauscher leukemic mice in the present study produced only low titers of circulating interferon. This suppression of interferon production has been reported by other investigators and is apparently an effect of the mouse leukemia viruses. According to Vandeputte *et al* (1967), mice inoculated with Rauscher virus no longer produce interferon in response to challenge with a heterologous virus. Similarly, Friend leukemic mice produce little or no interferon when induced by Sendai virus between 1 and 10 days after Friend virus inoculation (Wheelock and Larke 1968), but 12 days after Friend virus inoculation, normal levels of interferon may be produced. Wheelock *et al* (1969) showed that mice injected with statolon 3 days after Friend virus inoculation produced as much circulating interferon as normal mice. These studies and our investigation suggest that a general statement cannot be made regarding the induction of interferon in viral leukemic mice. Several factors apparently influence the response of leukemic mice to interferon inducing agents; these factors include the inducing agent, the strain of mice, and the stage of leukemia development.

It is also apparent from our data that the Rauscher leukemia virus itself did not induce interferon formation. This lack of response to Rauscher leukemia

virus has been observed by others. Pieries *et al* (1965) reported that BALB/c mice produced no interferon when infected with Rauscher virus, and Vandeputte *et al* (1967) could not detect interferon in the enlarging livers and spleens of NIMR and albino Swiss mice at various times from 16 hours to 14 days after infection with Rauscher virus.

The effectiveness of the combination therapy utilized in our investigation is apparent from the decreased WBC production and spleen weight and increase in 30-day survival time. These effects were observed in addition to those observed when treating leukemic mice with lethal whole-body irradiation and hematopoietic cell transplantation alone. They may be attributable to the use of statolon to induce interferon production in the leukemic mice but it must be noted that the production of interferon in the leukemic mice was low. In fact, interferon production in the leukemic mice was only 0.1 the amount produced in the control mice. These results suggest that other effects of statolon therapy in addition to interferon production contributed to the effectiveness of the combination therapy.

There is evidence indicating that statolon affects tumor inhibition through other mechanisms besides the well documented method of interferon induction (Metz 1975). Weislow *et al* (1973) reported that statolon abrogates the immunodepressive effects of Friend virus and permits the infected mouse to mobilize an effective humoral antibody response to leukemic cells and Toy *et al* (1973) tested several interferon inducing agents and found statolon to be the only agent capable of abrogating immunodepression and stimulating FV-cytotoxic antibody. Other possible mechanisms of tumor inhibition by statolon include the release of a lymphotoxin from lymphocytes, a mechanism reported for the interferon inducer Poly I: poly C by Dean *et al* (1972).

It is, of course, not possible to determine from our data which mechanism of statolon action resulted in the decreased rate of leukemia development. Additional studies are needed to clarify this point and to determine if the injection of interferon is more effective than the induction of interferon. Nonetheless, our studies indicate that combining statolon therapy with irradiation and transplantation therapy is a promising approach to treating viral leukemia.

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