Rauscher murine leukemia virus (R-MuLV) induces a rapidly developing erythroleukemia in BALB/c mice. Previously, we have shown that mouse interferon-α/β (Mu IFN-α/β) applied shortly after virus inoculation efficiently inhibits the leukemic process (Hekman et al., 1981). Here we describe the effect of Mu IFN-α/β on an established leukemia. Varying doses of Mu IFN-α/β were injected over 3 days, starting 8 to 12 days after virus inoculation. The effect of Mu IFN-α/β on the leukemic process was monitored by measuring the spleen weight, reverse transcriptase activity in the serum and, in selected experiments, by microscopic examination of sections of the spleen using standard histological and immunological staining techniques. Depending on the spleen weight at the start of its application (maximal about 450 mg), Mu IFN-α/β caused a dramatic reduction in the number of virus-infected erythroleukemic cells in the spleen. Also, R-MuLV disappeared from the serum within 3 days. If Mu IFN-α/β was injected into R-MuLV-infected mice with an already 10-fold enlarged spleen, it could only stop further development of leukemia. Results obtained with crude Mu IFN-α/β preparations were confirmed with absolutely pure Mu IFN-β.

Interferons are proteins with antiviral properties. Three antigenically distinct types of interferon can be distinguished: IFN-α, IFN-β and IFN-γ. IFN-α and IFN-β (Type-I interferons) are produced by incubation of cells with a virus or double-stranded RNA; IFN-γ (Type-II interferon) by antigenic or mitogenic stimulation of T lymphocytes (for review see Stewart, 1979). Most data published so far show that IFN-β (Houghton et al., 1981; Tavernier et al., 1981; Higashi et al., 1983) as well as IFN-γ (Gray and Goeddel, 1982, 1983) is the product of a single gene; IFN-α is produced by a series of more than 10 genes (Nagata et al., 1980; Brack et al., 1981; Goeddel et al., 1981; Overbach et al., 1981; Shaw et al., 1983) and can be separated into various subspecies.

In addition to their antiviral property, interferons possess a wide range of other biological activities. One of these is their ability to inhibit the growth of various types of experimental tumors in animals (for reviews see Gresser and Tovey, 1978; Stewart, 1979). Antitumor effects of interferons have also been described in humans (reviews: Strander, 1977; Billiau, 1981; DeMaeyer and Schellekens, 1983; Zoon et al., 1984). The mechanisms of the in vivo antitumor activity of interferons are still unknown. Direct effects on the tumor cell as well as anti-tumor activities mediated through the immune system may play a role in this respect.

Initial studies which showed an inhibitory effect of interferon on growth of tumors in experimental animals were carried out about fifteen years ago (for a review see Gresser and Tovey, 1978). At that time only limited amounts of relatively impure interferon preparations were available. Now, human interferons are produced in large amounts by bulk culture of lymphoblastoid cells or by bacteria, yeast or mammalian cells which are manipulated by recombinant DNA technology. These interferons are being tested for their antitumor properties in man (DeMaeyer and Schellekens, 1983; Zoon et al., 1984). Because of the complexity of the interferon system there is a need for more experiments in animal models. Results obtained in such a model — retrovirus-induced erythroleukemia — are presented here.

In earlier studies it has been reported that Mu IFN-α/β applied shortly after infection of BALB/c mice with Rauscher murine leukemia virus (R-MuLV) is able to inhibit the development of virus-induced erythroleukemia (Gresser et al., 1967, 1968). If high doses of Mu IFN-α/β are applied this inhibition is almost complete (Hekman et al., 1981). In the present study we extend these observations by analysis of the effects of Mu IFN-α/β on an already clearly established erythroleukemia. Our data show that Mu IFN-α/β, as well as pure Mu IFN-β, can not only inhibit further progress of the leukemia but also induce a rapid regression.

**MATERIAL AND METHODS**

** Animals and virus**

Animals used in this study were 4- to 5-week-old female BALB/c mice obtained from the breeding colony of Erasmus University.

Rauscher murine leukemia virus (R-MuLV) preparations were isolated from spleens of R-MuLV-infected BALB/c mice weighing at least 1 g. Spleens were homogenized in 5 volumes of phosphate-buffered saline (PBS; 0.02 M phosphate, pH 7.2, 0.15 M NaCl). Subsequently, the suspension was centrifuged (20 min, 8,000g) at 4°C. The supernatant containing the virus particles was stored at −70°C.

** Interferon**

Mu IFN-α/β was produced as described previously (Trapman, 1979; Vonk and Trapman, 1983) by incubation of mouse L-929 cells with poly (I) - poly (C) in the presence of DEAE-dextran. Mu IFN-α/β preparations were concentrated by ultrafiltration (Amicon PM10 filter), dialyzed against a 0.15 M NaCl solution, pH 2 at 4°C for at least 4 days and subsequently overnight against PBS. After centrifugation (10 min, 2,600 g), the supernatant containing essentially all interferon activity was stored at −70°C. The crude interferon preparation contained both Mu IFN-α (20 to 40% of the total activity) and Mu IFN-β (60 to 80% of the total activity). The specific activity was 0.5-1.0 × 10^6 IU/mg protein.

---

1 To whom reprint requests should be addressed.

Received: October 12, 1984 and in revised form January 7, 1985.
Purified Mu IFN-β was obtained by affinity chromatography of the crude Mu IFN-α/β preparation over a monoclonal anti-Mu IFN-α antibody agarose column (Vonk and Trapman, 1983). The specific activity of purified Mu IFN-β was 5 × 10^8 IU/mg protein. Purified Mu IFN-β was stabilized by addition of normal BALB/c mouse serum to an end concentration of 10% (v/v), dialyzed against PBS and stored at −70°C.

Interferon activity was measured in a microtiter cytopathic effect reduction assay (CPE) essentially as described (Armstrong, 1971) on L-929 cells using vesicular stomatitis virus as a challenge. Amounts of interferon are expressed in terms of international units as compared to NIH reference standard G002-904-511. Protein concentrations were determined by the method of Lowry et al., (1951) using bovine serum albumin as a standard. Before use, all interferon preparations were filter sterilized.

**Experimental procedure**

In all experiments an essentially identical procedure was followed. Groups of mice were injected intraperitoneally (i.p.) with 0.2 ml of the R-MuLV suspension. After 8 to 12 days, the animals were inoculated (i.p.) with Mu IFN-α/β or Mu IFN-β or control preparations. Interferon treatment was given daily, for 1 to 3 days. At indicated time points, mice were killed, peripheral blood was collected and the spleen weight was determined. Parts of spleens were fixed in 10% (v/v) phosphate-buffered formalin for histological examination and frozen in liquid nitrogen for immunofluorescence analysis.

**Histopathology and immunofluorescence**

For light microscopic examination, fixed parts of spleens were embedded in paraffin, then 5-μ sections were cut and stained with hematoxylin and azophloxin using standard procedures. For immunofluorescence analysis, 5-μ sections of frozen spleens were cut and incubated in appropriate dilutions of a rabbit antiserum against the R-MuLV envelope protein gp70 in PBS for 30 min at room temperature. After 3 washes with PBS, the slides were incubated with fluorescein-conjugated antibodies (horse anti-rabbit IgG, CLB, Amsterdam, The Netherlands) for 30 min at room temperature and subsequently washed 3 times in PBS.

**Reverse transcriptase assay**

As a measure of the amount of retrovirus particles in the blood, the activity of the retrovirus-specific enzyme reverse transcriptase was determined. Ten μl of serum were added to 90 μl of a 50 mm Tris-HCl buffer, pH 8.2 containing 20 mM KCl, 0.5 mM MnCl₂, 4 mM DTT, 0.1% Triton X-100, 8 μM dTTP, 5 μCi (³H)-TTP (50 μCi/mm, The Radiochemical Centre, Amersham, UK) and 0.01 Aₑ₂₆₀ units rAdT₉₀ (Boehringer, Mannheim, FRG) and incubated for 1 hr at 37°C. Subsequently, 50-μl samples were spotted on DEAE-cellulose paper and washed 3 times with 5% (w/v) Na₂HPO₄, twice with distilled water and once with ethanol. Filters were dried and counted for ³H-radioactivity in a liquid scintillation counter.

**RESULTS**

**R-MuLV-induced erythroleukemia as a model system for interferon action**

R-MuLV induces a rapidly developing erythroleukemia in BALB/c mice (Rauscher, 1962; Boiron et al., 1965). This leukemia is characterized by splenomegaly caused by extensive proliferation of virus-infected (pro)erythroblasts (Rauscher cells), the presence of large amounts of virus particles in the peripheral blood and progressive anemia. If high doses of virus are injected, animals die 3 to 6 weeks later.

Figure 1 shows as an example the increase in spleen weight and the appearance of R-MuLV particles in the peripheral blood followed in time after inoculation of a high dose of R-MuLV. During the first 3 days after injection no clear difference in the spleen weight, as compared to the normal weight of 80 to 100 mg, can be detected (Fig. 1a). Similarly, no virus particles can be detected in serum by the reverse transcriptase assay (measured in the form of ³H-TTP incorporation in polyAdT₁₀; see “Material and Methods”) (Fig. 1b). After 6 days, the spleen is clearly enlarged, to a weight of approximately 400 mg, and reverse transcriptase activity can be detected in the serum. During the following 8 days, the spleen weight rises to about 1,800 mg. During this time, reverse transcriptase activity in the serum rises to 2.4 × 10⁴ dpm ³H-incorporated in the assay.

**The effect of Mu IFN-α/β on an established erythroleukemia**

In previous experiments (Hekman et al., 1981) we reported that daily application of high doses of Mu IFN-α/β (5 × 10⁶ to 3 × 10⁵ IU) started shortly after virus inoculation was able to inhibit the development of R-MuLV-induced erythroleukemia in BALB/c mice. Here, we extend these experiments starting interferon treatment 8 to 12 days after virus infection.

In a first series of experiments we investigated the effect of Mu IFN on the leukemia by daily application of 5 × 10⁶ IU for 3 days, starting 8 days after virus infection. The interferon preparation used was a mixture of Mu IFN-α and Mu IFN-β (see “Material and Methods”). At 8 days, the mean spleen weight was 450 mg. Figure 2a shows the effect of daily injection of Mu IFN-α/β on the spleen weight of R-MuLV-infected mice followed for 3 days. Figure 2b illustrates the effect on reverse transcriptase activity in the serum. As can be seen, under the conditions used, Mu IFN-α/β was able to induce a rapid and (almost) complete regression of the erythroleukemia as indicated by the decrease in spleen weight (Fig. 2a) and reduction of the number of R-MuLV particles in the blood (Fig. 2b). The effects of Mu IFN-α/β on the leukemic pro-
cess were already perceptible after one interferon injection. After 3 days of treatment the spleen weight was almost back to normal (approximately 160 mg) and no reverse transcriptase activity could be found in the serum.

We also studied the effect of Mu IFN-α/β at a later stage of the leukemic process, starting the treatment 12 days after virus infection. At this time the average spleen weight of the infected mice was 1,000 mg. Figure 3a illustrates the effect of Mu IFN-α/β on the spleen weight of the infected mice was 1,000 mg. Figure 3a illustrates the effect of Mu IFN-α/β on the spleen weight again followed during a 3-day treatment period and Figure 3b the effect on reverse transcriptase activity in the serum. Even here, Mu IFN-α/β was constant in Mu IFN-α/β-treated mice. As observed in the previous experiment, the Mu IFN-α/β effect could already be detected after one single injection. However, in experiments starting Mu IFN-α/β treatment 12 days after inoculation of R-MuLV, we never observed a decrease in number of leukemic cells as was found in the experiment described above. In other experiments (data not shown) in which the spleen weight at the start of Mu IFN-α/β treatment was between 500 and 1,000 mg, we observed a partial regression of the erythroleukemia during a 3-day period of treatment. From these results we conclude that there is a maximal number of leukemic cells (corresponding to an approximate spleen weight of 450 mg) that can efficiently be attacked by Mu IFN-α/β in short time period.

The effect of pure Mu IFN-β on R-MuLV-induced erythroleukemia

In order to confirm that the effects caused by the interferon preparation are mediated by interferon and not by impurities present in the preparations, we investigated the effect of a completely pure interferon on the leukemic process. For this purpose we chose Mu IFN-β that can easily be purified using a monoclonal anti-Mu IFN-β agarose column (Vonk and Trapman, 1983). The results of two experiments, in which interferon treatment was started 8 and 10 days after virus application, respectively, are summarized in Figure 4. The data obtained completely confirmed our earlier observations. In a first experiment, starting with an average spleen weight of 465 mg, within 3 days this

![Figure 2](image-url)  **FIGURE 2** — The effect of daily application of Mu IFN-α/β initiated 8 days after virus inoculation on the spleen weight (a) and reverse transcriptase activity in the serum (b) of R-MuLV-infected BALB/c mice. A group of 35 BALB/c mice was injected with the R-MuLV preparation (see “Material and Methods”). After 8 days 5 mice were killed, blood was collected for the reverse transcriptase assay and the spleen weight was determined. Half of the remaining animals received Mu IFN-α/β (5 × 10^5 IU, i.p. in 0.2 ml PBS). Treatment was continued for 3 days. At each day 5 control mice and 5 interferon-treated mice were killed, reverse transcriptase activity in the serum was determined and spleen weight was measured. Each point represents the mean spleen weight (a) or reverse transcriptase activity (3H-TTP incorporation in poly A-oligodT) (b) of 5 mice ± SE (standard error).

![Figure 3](image-url)  **FIGURE 3** — The effect of daily application of Mu IFN-α/β initiated 12 days after R-MuLV inoculation on the spleen weight (a) and reverse transcriptase activity in the serum (b) of R-MuLV-infected BALB/c mice. Experimental details as described in “Material and Methods” and in the legend to Figure 2. (●—●) control group; (○—○) interferon-treated group.

![Figure 4](image-url)  **FIGURE 4** — The effect of daily application of pure Mu IFN-β initiated 8 (experiment 1) and 10 days (experiment 2) after R-MuLV inoculation on the spleen weight of BALB/c mice. A group of 20 BALB/c mice was injected with the R-MuLV preparation (see “Material and Methods”). After 8 (Exp. 1) or 10 days (Exp. 2) 5 mice were killed and the spleen weight measured. Five of the remaining mice received Mu IFN-β (2 × 10^5 IU in 0.2 ml PBS, see “Material and Methods”); the other mice were injected with a control preparation (10% mouse serum in PBS). Treatment was continued for 3 days. After this period mice were killed and the spleen weight was determined. In the Figure, mean spleen weight at the various times is indicated by a horizontal bar.
value decreased to 295 mg in the Mu IFN-β treated group, whereas in the controls it rose to approximately 665 mg. In a second experiment, starting with an average spleen weight of 570 mg, the spleen weight remained almost constant during Mu IFN-β treatment. However, in the untreated animals it nearly doubled to an average value of 1050 mg.

Histological and immunological analyses
For a more detailed investigation of the effects of Mu IFN-α/β on the leukemic (pro)erythroblasts in the spleen, we selected the experiment shown in Figure 2, in which Mu IFN-α/β treatment resulted in an almost complete regression of the spleen to normal size. Slices of the spleen were cut and stained by standard histological and immunological techniques. The morphological structure of spleens removed after 3 days of Mu IFN-α/β treatment was compared with that of spleens removed from R-MuLV-infected mice without Mu IFN-α/β treatment (Fig. 5). Moreover, spleen sections obtained after R-MuLV infection, both with and without Mu IFN-α/β treatment, were screened for the presence of virus-infected cells by immunofluorescence staining using an antiserum against the viral envelope protein gp70 (Fig. 6).

As can be seen in Figure 5a, 11 days after virus infection the normal morphological structure of the spleen has for most part disappeared: the lymph follicles are hardly detectable, and large groups of erythroleukemia cells are clearly visible (see also Fig. 5b center). Furthermore, a large number of macrophages can be seen. Spleens removed 8 days after virus infection gave a similar pattern (data not shown). In contrast to the untreated animals, the Mu IFN-α/β-treated mice showed no groups of erythroleukemia cells in their spleens. The spleen morphology seems to return to normal; lymph follicles are clearly detectable (Fig. 5c). Also, the spleen looks “empty”: large areas in which cytolysis has taken place can be seen indicating that (leukemic) cells were removed from the spleen in the Mu IFN-α/β-treated animals (Fig. 5d). Here again, large numbers of macrophages can be seen.

The results obtained by immunofluorescence analysis (Fig. 6) confirm the data described above. In spleens from untreated mice, virus-infected (erythroleukemic) cells can easily be detected, both 8 and 11 days after infection (Fig. 6a,b). After 3 days of Mu IFN-α/β treatment, fluorescent cells seem to be absent from the spleen indicating the absence of virus-infected cells (Fig. 6c). If the disappearance of virus-infected cells from the spleen is followed in time, a pattern is obtained which is in good agreement with the gradual reduction of the spleen weight (Fig. 2a). Already after one day of Mu IFN-α/β treatment, the number of virus-infected cells decreases. This process continues in the Mu IFN-α/β-treated group of mice during the
following days, whereas in the untreated group the number of virus-infected cells remains high. The results obtained with Mu IFN-α/β could be confirmed with completely pure Mu IFN-α/β (data not shown).

DISCUSSION

In this study we describe the effect of high doses of Mu IFN-α/β on the course of an already established R-MuLV-induced erythroleukemia in BALB/c mice. The results obtained show that Mu IFN-α/β is able to stop the leukemic process (Fig. 2, 3). Moreover, we found that, if the weight of the spleen does not exceed a maximal value of about 450 mg, daily injection of $5 \times 10^5$ IU of Mu IFN-α/β leads to a rapid regression as demonstrated by a substantial decrease in spleen weight and the absence of viral particles in the serum after 3 days (Fig. 2, 5 and 6). This could be achieved with crude interferon and also with pure Mu IFN-β (Fig. 4).

R-MuLV-induced erythroleukemia (like Friend MuLV-induced erythroleukemia) is a very complex process (Smadja-Joffe et al., 1975; de Both et al., 1978). The accumulation of proerythroblasts in the spleen is the result of an imbalance in cell proliferation, differentiation and degradation ("spontaneous" or regulated by the immune system) of erythroid precursor cells which are modified by the virus. Since interferons are able to modify most if not all of these processes, it is not possible to deduce from our data a clear-cut mechanism of interferon action.

In a previous study, the effect of high doses of Mu IFN-α/β applied shortly after R-MuLV infection has been described (Hekman et al., 1981). Under these conditions Mu IFN-α/β almost completely inhibited the development of R-MuLV-induced erythroleukemia. However, it could not be distinguished whether this was due to the antiviral properties of Mu IFN-α/β or to one of the other activities of Mu IFN-α/β like its cell growth-inhibitory, antigen-modulating or immunoregulatory effects. The experiments presented here concerning the effect of Mu IFN-α/β on an already established erythroleukemia almost certainly exclude antiviral action of Mu IFN-α/β.

Results obtained so far on the anti-tumor properties of IFN-α/β, both in experimental animals and in man, vary considerably. The reasons for this are at present unknown. In most studies, a partial inhibition of tumor cell growth has been observed. In two studies a rapid regression, as found in this study, has been reported. Kassel et al. (1972), using a crude Mu IFN-α/β preparation, reported a rapid interferon-induced cytolysis of an established spontaneous lymphoma in AKR mice. Cytolysis started as early as 1 hr after administration of interferon. However, their results could not be confirmed by others (Gresser et al., 1976). Belardelli et al. (1982, 1983) described the effects of Mu IFN-α/β on the growth of a Friend erythroleukemia cell line in DBA/2 mice. A decrease in the number of leukemic cells following interferon treatment was observed, but regression only started several days after inoculation of Mu IFN-α/β (Belardelli et al., 1983). Although there seem to be several discrepancies between the
results described in the above-mentioned studies and those reported here, all data indicate that virus-induced leukemias or lymphomas, and especially virus-transformed erythroleukemic cells, are highly interferon-sensitive in vivo. So far, these models are unique in the Mu IFN-αβ-mediated rapid lysis of the tumor cells. Whether the cell type or the virus-infected state plays a major role remains to be established.

The rapid analysis which is possible in the animal system described here makes it very suitable as a model for evaluation of the effects of various different Mu IFN species and subspecies. Here we report the effect of pure Mu IFN-β on leukemia. At present we are preparing various Mu IFN-α subspecies. The effects of these components in this model will be monitored as well. If Mu IFN species can be constructed which miss some of the various biological properties of interferons, these can be used for elucidation of the mechanism of interferon action in vivo. Maybe even naturally occurring Mu IFN-α subspecies which miss one of the activities, as recently found to be the case for a human IFN-α subcomponent (Ortaldo et al., 1984), can be used for this purpose.

ACKNOWLEDGEMENTS

The authors are indebted to Dr. M.E.F. Prins for help with the examination of the microscopic preparations, to Dr. J. Brouwer for the kind gift of anti-gp70 antibody and to Dr. E.C. Zwarthoff for critical reading of the manuscript. This study was financially supported by the Koningin Wilhelmina Fonds.

REFERENCES


Nagata, S., Mantei, N., and Weissmann, C., The structure of one of the eight or more distinct chromosomal genes for a human interferon-α. Nature (Lond.), 287, 401–408 (1980).


