ride density gradient. The density at the peak of infectivity for each mutant was compared. A small density difference in the range of 0.001-0.004 g/cm^3 was measured, with *m* the heavier mutant.

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Effect of Statolon on Friend Virus Leukemia in Mice.* (31870)

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In a previous communication the inhibition of Friend virus-induced splenomegaly in mice by the prior intraperitoneal inoculation of Sendai virus was reported(1). It was proposed that interferon might be involved in this virus interference since 1) Sendai virus induces large amounts of this virus inhibitor in the peritoneal cavity of normal mice, and 2) Sendai virus inoculated 1-9 days following Friend virus inoculation fails to induce normal amounts of interferon and has no Friend virus-inhibiting effect.

To delineate further the role of interferon in the Sendai virus-induced inhibition of Friend virus leukemia, a nonviral agent, Statolon,† was selected for study. Statolon, a polysaccharide produced by the mold *Penicillium stoloniferum*(2) has been reported to induce large amounts of circulating interferon in mice(3) and can protect them against lethal viral infections(4).

Materials and methods. Viruses. Friend leukemia virus (FV) was obtained from Dr. Charlotte Friend(5) in the form of infected DBA/2 mice. The enlarged spleens were removed from the mice 3 weeks after infection and a 20% suspension of them made in phosphate buffered saline (PBS). The suspension was then centrifuged at 6,000 rpm for 1 hour and the supernates were passed through a Millipore® filter with a pore size of 450 μm, quick-frozen and stored at —70°C.

Vesicular stomatitis virus (VSV) was grown in mouse L cell monolayer cultures. The infected cultures were sonicated and clarified by centrifugation at 1,000 rpm for 30 minutes; the supernates were then passed through a Millipore® filter with a pore size of 450 μm, quick-frozen, and stored at —70°C.

Cell cultures. The L strain of mouse fibroblasts derived from normal mouse skin was employed in the assay for interferon. Cells were grown in growth medium consisting of Eagle's minimum essential medium supplemented with tryptose phosphate broth (4%) and fetal calf serum (10%). The concentration of sodium bicarbonate was 1.75 g/liter, and all cell-culture vessels were gassed with 5% CO_2 in air before incubation at 37°C.

Interferon assay. The specimens to be tested for interferon were diluted in growth medium, and 1 ml of each dilution was added to 1-day-old cultures of mouse L cells grown in incomplete monolayers in screw-cap tubes. After 20 hours of incubation at 37°C the cultures were washed once with 4 ml of PBS. To each tube, 1 ml of warm protein-free

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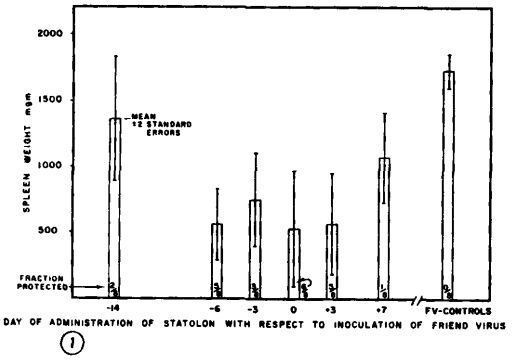

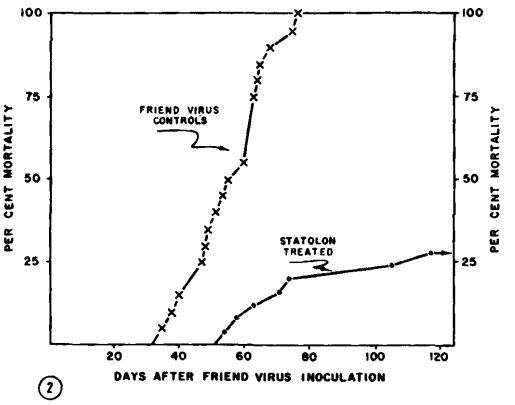

Fig. 1. Effect of Statolon on splenomegalic response to Friend leukemia virus. Fraction protected is calculated as number of mice with spleens <500 mg over total number of mice in set.
Fig. 2. Effect of Statolon on survival of Friend virus-infected mice. Statolon was injected intraperitoneally 3 days prior to Friend virus.

Eagle's medium was added, and then 1 ml of cold growth medium containing 2,000 tissue-culture infective doses ($TCID_{50}$) of vesicular stomatitis virus was inoculated. This amount of virus produced gross cytopathic effects in cultures in 24-30 hours. 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 in culture protecting units (CPU) as reciprocals of the highest dilution of the specimen, 1 ml of which protected cultures against challenge with VSV. No specimen was tested at less than 1:10 dilution.

Mice. Six-to-8-week-old DBA/2 female mice were obtained from the Jackson Memorial Laboratories.

Peritoneal washings. Mice were anesthetized with ether and exsanguinated, and the abdominal wall was shaved. The peritoneal cavity was opened and washed with 5 ml of PBS. The recovered washing was then clarified by centrifugation at 2,000 rpm for 5 minutes and assayed for interferon as described above.

Results. Production of interferon in mice in response to Statolon. The ability of Statolon to induce interferon production in the DBA/2 strain of mice was tested. Normal mice were inoculated intraperitoneally (IP) with Statolon, 5 mg per mouse, and at intervals thereafter groups of mice were bled and peritoneal cavities washed with saline. All serum and peritoneal washings were assayed for interferon activity as described in *Materials and Methods.*

As reported by others(3) interferon was detected in the serum with a maximum titer of 1,280 CPU/ml being reached at 20 hours after Statolon administration. However, little or no interferon could be detected in the peritoneal washings even at a time when high titers of circulating interferon were present.

Effect of Statolon on Friend virus leukemia. Groups of 10 mice each received one injection of Statolon, 5 mg/mouse IP, at an interval either before or after Friend virus inoculation. Friend virus inoculated intraperitoneally, 200 mouse infective dose (MID_{50}) per mouse. Twenty-one days after Friend virus inoculation all mice were sacrificed and spleens weighed.

In all experiments performed, Statolon inhibited Friend virus-induced splenomegaly, although there was some variation from experiment to experiment in the time relationship between the inoculations of Statolon and Friend virus which resulted in inhibition of splenomegaly.

Fig. 1 illustrates the results of one experiment. A marked inhibition of the splenomegalic response to Friend virus occurred in mice which received Statolon between the sixth day before and third day after Friend virus inoculation, and significant inhibition occurred when Statolon was injected 7 days

after Friend virus.

Interferon production in Friend virus-infected mice. It was of special interest to determine if Friend virus-infected mice could produce interferon in response to Statolon for two reasons: a) as shown in the preceding experiment Statolon inhibited Friend virus-induced splenomegaly when administered 0-7 days after Friend virus inoculation; b) as shown in a previous report(1), Sendai virus did not inhibit Friend virus-induced splenomegaly when administered 1-9 days after Friend virus inoculation, and markedly reduced titers of interferon were induced by Sendai virus inoculated during this interval.

It was found that mice injected with Statolon IP 3 days after Friend virus inoculation produced as much circulating interferon as did normal mice.

Effect of Statolon on survival of Friend virus-infected mice. The marked inhibition of the splenomegalic response to Friend virus by the administration of Statolon raised the question as to whether mice so protected by Statolon would eventually develop leukemia.

Twenty-five mice were given Statolon intraperitoneally, 5 mg per mouse, and 3 days later were inoculated with Friend virus 200 MID_{50} per mouse IP. Twenty non-Statolon-treated mice also were inoculated with Friend virus to serve as controls. Deaths were recorded and are illustrated in Fig. 2.

The first Friend virus control mouse died 35 days after inoculation and all control mice were dead by the seventy-sixth day. In contrast, only 7 of the 25 mice which received Statolon prior to Friend virus died of leukemia. The remaining 18 were alive 120 days after Friend virus inoculation and exhibited no signs of leukemia. Evidence for the absence of leukemia at this time was determined as follows: 1) the abdomens were shaved and spleens were visualized through the abdominal wall. All 18 mice had normal sized spleens in contrast to leukemic mice which consistently display very large spleens. 2) Peripheral white blood counts were performed on the 18 mice, all of which were within normal limits, *viz.,* 5000-15,000 cells per mm^3. In contrast, leukemic mice all have a high leucocytosis with white counts reaching over 100,000 cells per mm^3. 3) The spleens of 6 of the 18 mice were removed and extracts made. Inoculation of these extracts into normal mice failed to produce leukemia in them.

Susceptibility of protected mice to reinoculation with Friend virus. Six of the 18 Statolon-protected mice from the preceding experiment were reinoculated with Friend virus, 200 mouse infecting $doses_{50}$ per mouse, on the 120th day after their first inoculation with Friend virus. These 6 mice failed to develop Friend virus leukemia following this second inoculation of Friend virus as evidenced by absence of splenomegaly and leucocytosis 10 weeks later. The infectivity of this challenge dose of Friend virus was evidenced by the development of leukemia in 100 normal mice used in another experiment but inoculated with the same Friend virus preparation. The remaining 6 mice protected by Statolon from Friend virus leukemia in the preceding experiment received no additional treatment or inoculation and also exhibited no evidence of leukemia at the end of the 10-week interval.

Discussion. The ability of Statolon to protect completely the majority of mice from Friend virus leukemia raises important questions as to the mechanism of virus-inhibition. The induction of circulating interferon by Statolon has focused attention on this virus inhibitor as the mediator of inhibition of Friend virus. Attempts to inhibit Friend virus in mice by prior intraperitoneal administration of high titers of interferon (supplied by Dr. N. B. Finter) have been unsuccessful although Gresser has reported a inhibition of Friend virus-induced splenomegaly by repeated intraperitoneal administration of an interferon preparation (Information Exchange Group #6, Memo #220). Glasgow (personal communication) has found that repeated injections of interferon intraperitoneally have significantly inhibited Rauscher virus leukemia in mice and have prolonged their survival time.

A further argument in favor of interferon as the mediator of the inhibition of Friend virus by Statolon comes from a comparison between Statolon and Sendai virus. Statolon administered 3 days after Friend virus in-

duces normal amounts of circulating interferon and inhibits Friend virus disease, whereas Sendai virus inoculated 3 days after Friend virus neither induces normal amounts of interferon nor inhibits Friend virus disease(1). As previously reported(1), when Sendai virus is inoculated before Friend virus, it induces interferon production and inhibits Friend virus disease. Finally, the response curves to increasing doses of Sendai virus of both interferon production and Friend virus leukemia-inhibition have been found to be similar (Wheelock, E. F., Nat. Canc. Inst., in press.); optimal interferon production and Friend virus inhibition occur in response to the same dose of Sendai virus. The sum total of these pieces of evidence indicates that interferon can inhibit Friend virus and may therefore be the mediator of Friend virus-inhibition by both Statolon and Sendai virus.

There is, however, a strong argument against the role of interferon in the Statolon inhibition of Friend virus. A comparison of interferon production by the two agents (Sendai virus and Statolon) which can both inhibit Friend virus disease, reveals that Sendai virus induces large amounts of peritoneal interferon and small amounts of circulating interferon, whereas Statolon induces circulating interferon only. That circulating interferon alone is not sufficient to inhibit Friend virus leukemia is demonstrated by the failure of intravenously inoculated Newcastle disease virus (NDV) (which induces high titers of circulating interferon) to inhibit the splenomegalic response to subsequently inoculated Friend virus. These results suggest that intraperitoneal administration of Statolon may initiate events in the peritoneal cavity which lead to inhibition of Friend virus leukemia by mechanisms other than interferon. The possibility remains, however, that higher cumulative titers of circulating interferon may be reached and persist longer in Statolon-injected as compared with NDV-inoculated mice. Finally, Statolon and NDV have been shown to stimulate interferons in mice which are indistinguishable except for molecular weights(6,7,8).

The ability of mice which have been completely protected against active Friend virus by Statolon to resist rechallenge with Friend virus is of special interest. The resistance of these mice to reinoculation with Friend virus may reflect the presence of anti-Friend virus antibodies or may involve a mechanism yet to be discovered. Mice have been successfully immunized against Friend virus by prior inoculation of inactive virus. The study of agents such as Statolon which permit active Friend virus to immunize but not produce disease may provide a new approach to the prevention of virus-induced leukemia.

Summary. Statolon, an anionic polysaccharide known to induce interferon in mice, inhibits Friend virus leukemia when administered as early as 6 days before and as late as 7 days after Friend virus inoculation. The majority of Statolon-treated mice are completely protected against Friend virus leukemia and are resistant to rechallenge with Friend virus.

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