Suppression of Established Friend Virus Leukemia by Statolon

I. Demonstration of a Latent Infection in Clinically Normal Mice

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A single inoculation of statolon into mice with established Friend virus (FV) leukemia can suppress the viral infection and produce a clinical remission lasting many months. Eventually, however, most of the mice develop characteristic FV leukemia. Persistence of FV activity in the spleens of mice during clinical remission can be demonstrated by cell transfer and histopathologic studies. Transfer to normal mice of a large number of spleen cells (10^7) from mice in remission produces FV leukemia, and transfer of a small number of cells (10^2) produces immunity to FV challenge. Histopathologic examination reveals clusters of abnormal FV leukemia-like cells directly beneath the capsules of the spleens of mice in clinical remission.

Previous communications have reported the ability of certain viral and nonviral inducers of interferon, as well as interferon itself, to inhibit the course of Friend virus (FV) leukemia in mice (15, 19-21). Agents applied prior to the establishment of FV infection have varying capacities to reduce or abolish the leukemic disease. Sendai virus inoculated into DBA/2 mice prior to FV induces interferon and slows the course of FV leukemia, with complete prevention of overt disease in 10% of mice (19, 20). Statolon, an extract of *Penicillium stoloniferum* selected for its interferon-inducing and antiviral effects (10) can, when given intraperitoneally (ip) in one injection shortly before or after FV, produce a marked suppression of the development of leukemia (21). Similarly, treatment of mice prior to FV inoculation with a synthetic carboxylate copolymer results in interferon production and reduction in the splenomegalic response to FV (15). Finally, repeated inoculations of high-titered interferon itself, begun either before or up to 7 days after FV, have also been shown to reduce the splenomegalic response to FV (6-9).

Because human leukemia presents as an established disease, and therefore at the present time must be approached therapeutically rather than prophylactically, we chose, in studying the murine model, to focus our attention on interference with *established* FV infection. Treatment with interferon inducers or interferon itself has been only partially effective in modifying estab-

lished FV disease. Sendai virus or statolon, or repeated daily inoculations of interferon, administered at a time when mice were beginning to die of FV leukemia, have been shown to prolong survival by an average of 10 days (22). Treatment thus initiated at an advanced stage of the disease could not effect the complete suppression of leukemia. In this study, therefore, we directed our attention to the application of interferon inducers at the early stages of established FV infection.

This communication reports that a single inoculation of statolon, administered after FV has produced an established infection in mice, can completely suppress the clinical manifestation of FV leukemia. The persistence of FV activity in spleen cells of these clinically normal mice, leading to eventual emergence of leukemia, will be described.

MATERIALS AND METHODS

Viruses. FV leukemia was obtained from Charlotte Friend 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 was made in phosphate-buffered saline. The suspension was then centrifuged at $5,000 \times g$ for 1 hr and the supernatant fluids were passed through a membrane filter (Millipore Corp., Bedford, Mass.) with a pore size of 450 nm, frozen, and stored at -70°C . The FV inoculum used in all experiments was 2×10^8 leukemia-producing units per mouse.

Mice. Female DBA/2 mice (6 to 8 weeks old) were obtained from the Jackson Memorial Laboratories, Bar Harbor, Me.

Statolon. Supplied by Eli Lilly & Co. Indianapolis, Ind. (lot 354-869B-139), the statolon was prepared for inoculation into mice by dissolving it in 1% sodium bicarbonate.

Interferon assay. Specimens of serum were assayed for interferon according to methods described previously (21) in which interferon activity was determined on the basis of protection of mouse L-cell monolayer cultures against the cytopathic effects produced by vesicular stomatitis virus.

RESULTS

Establishment of FV infection. Detectable viremia in mice inoculated ip with FV was selected as a reliable and quantifiable criterion for established FV infection. In a study of the progression of viremia, normal DBA/2 mice were inoculated ip with FV. On selected days after inoculation, four mice were removed from the group and were exsanguinated. Their bloods were pooled, and leukemogenic virus was assayed in normal DBA/2 mice. As presented in Fig. 1, viremia was detectable at low levels by the third day after FV inoculation, and titers of FV in the blood increased rapidly thereafter. Furthermore, in experiments assessing viremia in individual mice rather than pooled groups, it was found that 95 to 100% of mice were viremic by 3 days after FV inoculation. In addition, the spleens were removed from mice 3 days after FV inoculation, and the cells were dispersed in a loose-fitting glass grinder and washed four times by centrifugation

FIG. 1. Viremia in DBA/2 mice inoculated ip with FV.

with PBS. Inoculation of these cells into mice at dilutions in which the supernatant fluids were nonleukemogenic revealed that as few as 1,000 cells could produce FV leukemia. Accordingly, day 3 was regarded in subsequent experiments as the day when FV infection was established.

Prolongation of survival of mice with established FV infection by statolon and the eventual emergence of leukemia. The survival time of 100 DBA/2 mice inoculated intravenously with statolon (in a dose of 4 mg/mouse) 3 days after FV was compared with that of 100 FV controls. At 74 days after FV inoculation, when all FV controls had died, 60% of statolon-treated animals remained alive (Fig. 2). At 130 days after FV inoculation, 30% of statolon-treated mice were alive and had spleens of normal size, as observed through the abdominal wall. A detailed autopsy of these protected mice revealed no gross evidence of FV leukemia in any organs; spleens, livers, and lymph nodes were of normal size and appearance and the mice had a normal peripheral white blood cell count and differential.

Long-term surveillance of 25 of these protected mice was maintained in an effort to assess the nature and extent of suppression of FV infection. Over an 8-month period of surveillance, leukemia emerged at random in mice which had been judged to be protected at a time when all FV controls were dead or dying (Fig. 3); of those mice which had appeared normal 78 days after FV, more than half developed leukemia and died over the course of the next 240 days. Furthermore, filtrates of both the plasma and splenic extracts from these mice in which leukemia emerged after a long latency contained leukemogenic FV.

Leukemogenic and immunogenic activity of spleen cells in statolon-protected mice. The eventual development of FV leukemia in clinically

FIG. 2. Survival of mice injected with statolon 3 days after FV as compared with FV controls.

FIG. 3. Development of FV leukemia in mice which displayed normal-sized spleens and livers 78 days after FV inoculation.

normal mice which were treated with statolon 3 days after FV inoculation suggested that the virus was constantly present in the mice from the time of inoculation. However, infective FV could not be isolated from these mice while they were clinically normal. Attempts were made, therefore, to demonstrate FV activity in their spleens by cell-transfer experiments.

Fifty DBA/2 mice were inoculated ip with FV, and 3 days later 25 were inoculated with statolon (4 mg/mouse). After 97 days, at a time when all FV control mice had died, four statolon-treated mice with normal-sized spleens were selected. The mice were exsanguinated and their spleens were removed. The spleen cells were dispersed in a loose-fitting grinder and 10-fold dilutions of cells and the respective supernatant fluids were inoculated into separate sets of mice, five mice per dilution. Seven weeks later, mice which had developed leukemia were identified by observation and palpation of large spleens through the abdominal wall, and the remaining mice were challenged with FV. In addition, five normal mice were inoculated with FV as challenge controls.

An inoculum of 10^7 spleen cells produced leukemia in normal mice (Fig. 4). Extracts of the spleens of mice developing leukemia were passed through filters of pore size 450 nm, and the filtrates produced characteristic FV leukemia in normal mice. The supernatant fluid from this high cell-number concentration was nonleukemogenic, but it was immunogenic. As few as 50 to 100 spleen cells were immunogenic; the supernatant fluid from this low cell-number suspension had no immunogenic activity.

Resistance of statolon-protected mice to rechallenge with FV. Three clinically normal

FIG. 4. FV leukemogenic and immunogenic activity of spleen cells and corresponding supernatant fluids from clinically normal statolon-treated mice.

statolon-treated mice were selected, as described in the previous section, and inoculated with FV; five normal mice served as challenge controls. After 21 days, all FV challenge controls had developed characteristic FV disease with hepato-splenomegaly, whereas the statolon-treated mice still contained normal-size spleens and livers.

Histopathology of statolon-protected mice. Histologic sections of grossly normal spleens which contained leukemogenic or immunogenic activity were prepared. As compared with normal spleens (Fig. 5, 6), the spleens from statolon-protected mice (Fig. 7, 8) contained aggregates of abnormal FV leukemia-like cells directly beneath the capsules. The spleens of mice which were inoculated with FV alone and sacrificed 48 days later (Fig. 9, 10) contained large numbers of leukemic cells which almost completely replaced the normal spleen-cell population and obliterated the normal architectural pattern.

DISCUSSION

The mechanisms by which statolon interrupts an established FV infection and suppresses the virus into a latent state are under investigation and will be the subject of subsequent reports. It is pertinent, however, to point out here that statolon induces as much interferon in mice which develop leukemia within 1 month as in mice which enter a long-lasting clinical remission. Further evidence suggests that this long-term protection against FV leukemia is probably maintained by an immunologic mechanism which is mobilized in some mice under the cover of interferon. Therefore, statolon, being a crude extract of a penicillium mold, may contain in addition to the interferon-inducing double-stranded RNA (11), a substance which stimulates an immunologic response to FV. Studies on the FV inhibitory properties of purified fractions of statolon are currently in progress.

FIG. 5-10. Low (80 $\times$; H & E) magnification illustrations are presented of the spleens from normal (Fig. 5), statolon-protected (Fig. 7), and leukemic (Fig. 9) mice. The statolon-protected and the leukemic spleens were removed 48 days after FV inoculation. In comparison with the normal spleen, the spleen from the leukemic mouse has a monotonous architecture with almost complete replacement of normal cells by leukemic cells. In contrast to the loss of architectural pattern in the spleens from the leukemic mice, the distinction between red and white pulp is preserved in the spleens from the statolon-treated mice. The small, deeply staining nuclei of lymphocytes and immature red cells immediately beneath the capsule of the normal spleen have been partially replaced in the spleen from the statolon-treated mouse by abnormal cells with lighter staining nuclei and more abundant cytoplasm. The smaller ramifications of the white pulp in the spleen from the statolon-treated mouse are more prominent than in the normal spleen, but do not contain any abnormal cells. The high magnification (500 $\times$, H & E) photomicrographs of a normal spleen (Fig. 6), statolon-protected spleen (Fig. 8), and leukemic spleen (Fig. 10) illustrate the changes in cellular population adjacent to the capsule. In the normal spleen, lymphocytes, red-cell precursors, and mature red cells are common. In the statolon-protected spleen, there is partial replacement of the normal population of cells with larger abnormal cells which have large nuclei and abundant cytoplasm. These abnormal cells resemble the FV-transformed cells present in the leukemic spleen where there has been almost complete replacement of normal cells by leukemic cells.

The prolonged survival of mice treated with statolon 3 days after FV does not represent merely a delay in the development of leukemia, for these mice, although clinically normal, contain no filterable leukemogenic FV and are resistant to FV challenge. The immune mechanism involved in this resistance to FV is probably also responsible for suppression of the FV-transformed cells which are present in the spleens of these mice.

FV-transformed cells could be demonstrated in the spleens of statolon-protected mice both histologically and by transfer of viable cells to normal mice. Large numbers of such spleen cells (10^7) produced leukemia, whereas smaller numbers of cells (10^2) produced immunity to FV. The marked differences between the number of spleen cells required to produce leukemia and to produce immunity to FV may be explained by one of two hypotheses. All FV-infected cells in a protected spleen may be of a single type, but a critical number (over 10^6) may be needed to produce leukemia when transferred to normal mice. Spleen cells inoculated in numbers below 10^7 and above 10^2 cannot produce leukemia, but they can immunize normal mice. The immunogenic activity of as few as 10^2 spleen cells from protected mice is most notable, for it implies that at least 1 in every 100 cells in the spleens of these clinically normal mice contains information relevant to FV.

The mechanism which triggers development of overt leukemia on inoculation of large numbers of leukemic cells may involve immunosuppression. Murine leukemia viruses have been shown to produce immunosuppression in mice (1-4, 14, 17, 18), and this state of immunologic paralysis or tolerance may be essential for the establishment of the virus infection and development of overt leukemia. It is quite probable that immunosuppression requires the introduction into the mouse of a critical number of virus particles or virus-transformed cells. A number of FV particles or leukemic cells below this threshold may, however, contain enough virus antigen to immunize the mouse.

Alternatively, there may be two types of FV-transformed cells in protected spleens: leukemogenic cells present in small numbers, and FV immunogenic cells present in much larger numbers. The immunogenic cells could be one of three types: they may produce FV-specific antibody itself; they may bear information for the production of FV-specific immunity (be it cellular or antibody-mediated), e.g., in the form of processed antigen; or they may produce an attenuated virus capable of infecting other cells and stimulating specific antibody production, but incapable of producing leukemia. [Several investigators have

reported the production of attenuated FV under in vitro conditions (5, 12, 13, 16, 23)]. In addition to producing immunity to FV in normal mice, such immunogenic cells would be of importance in the continued suppression of FV in protected mice.

One can account for the protective effect of the supernatant fluids from spleen-cell suspensions of protected mice by related mechanisms. The number of virus particles present may be insufficient to produce immunosuppression but great enough, at least in the supernatant fluids from the more concentrated cell suspensions, to immunize mice. Alternatively, supernatant fluids might contain an attenuated FV capable of stimulating a specific immune response.

In summary, statolon administered to mice with established FV leukemia can suppress the virus and produce a latent infection which persists for many months. The mechanism responsible for the long-term remission after statolon treatment probably has an immunological basis. Immunological mechanisms are subject to influence by a variety of factors; impairment of the integrity of these mechanisms at some stage in the life of protected mice could result in the observed emergence of overt leukemia. The sequence of events described for leukemic mice treated with statolon provides an attractive model for human leukemia, the manifestations of which may represent emergence of disease after years of latency. The factors involved in long-term protection against FV leukemia in mice and the eventual emergence of disease are under study.

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