Increased Oncogenicity of the Murine Sarcoma Virus (Moloney) by Co-infection with Murine Leukemia Viruses

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SUMMARY

The high incidence of spontaneous regressions of primary tumors induced by the Moloney sarcoma virus in adult mice was significantly altered by co-infection with Rauscher, Moloney, and Friend leukemia viruses. Intramuscular, intraperitoneal, or subcutaneous inoculation of sarcoma extracts, prepared from progressively growing tumors obtained from dually infected animals, resulted in extensive metastases. Tumor nodules were observed in several tissues and organs. Regression of primary virus-induced tumors could be altered by low concentrations of Rauscher leukemia virus. The role of the leukemia virus, acting as “helper” or as an immunosuppressor, is discussed.

INTRODUCTION

Moloney described the isolation of a virus which induces rhabdomyosarcomas in newborn mice (14). The pathologic changes produced by this virus in newborn mice and the specific relationship of the virus to muscle tissue have been reported (16, 18). Virus-induced sarcoma and the tissues of mice carrying the tumor have been studied with the electron microscope (4). Virus particles morphologically indistinguishable from the murine leukemia viruses were observed budding from the connective tissue cells.

Inoculation of MSV into adult mice results in tumor induction; however, a high incidence of regressions occurs (M. A. Chirigos, W. Turner, and B. Burka, unpublished data). Fefer et al. (7) have reported tumor regressions occurring in three strains of adult mice inoculated with MSV.

Hartley et al. (10) reported that the in vitro focus-forming effects of MSV depended on the presence of two virus particles, a defective MSV particle and a fully infectious Moloney leukemia particle, in the same cell. These results suggest that murine leukemia viruses act as “helpers” for a defective MSV particle similar to the helper effects between certain viruses of the chicken leukemia group and strains of the Rous sarcoma agent (20). The studies on the helper effect of leukemia viruses on sarcoma virus activity were carried out in a defined in vitro system with focus formation being used as an indication of virus infection.

The objective of the present study was to determine whether the regression of tumors induced in adult mice infected with MSV could be altered by co-infection with leukemia virus. In addition, the response of mice to inoculation with extracts of tumors from dually infected mice was studied. The pathogenesis occurring in these animals is described. The relationship between leukemia-virus concentration and incidence of MSV-induced tumors is presented.

MATERIALS AND METHODS

Mice. Adult male BALB/c mice, 8–12 weeks old, obtained from Simonsen Laboratory, Gilroy, California, were used.

Viruses. Two pools of Moloney sarcoma virus (SVRP-77 and SVRP-83), obtained from Dr. J. B. Moloney, were used. Virus-induced sarcoma tissue from BALB/c mice served as source material for the preparation of a cell-free concentration of the agent (16, 18). A 1-gm equivalent per ml concentrate (10°) is equal to 1 ml of final material for each gm of tissue processed. Stable, standard lots of MLV, RLV, and FLV were prepared from the viremic plasma of BALB/c mice. Plasma containing RLV was prepared using the same procedure as described for the preparation of FLV (9). Newborn animals inoculated with MLV were sacrificed 6 weeks later, and blood was collected from the brachial artery. The blood was added to an equal volume of 0.306 potassium citrate, pooled, and centrifuged (1250 × g) for 10 minutes. The plasma was recovered and again centrifuged (1250 × g) for 5 minutes to insure complete separation of cell particulates. To concentrate the virus, the recovered supernatant was centrifuged (30,000 × g) for 1 hour to sediment leukemia virus. The pelleted material was resuspended in 0.05 m sodium citrate pH 6.8, homogenized in a modified Potter and Elvehjem homogenizer and the homogenous suspension dispersed into ampules and stored at −70°C. The inocula employed were ten-fold serial dilutions of the standard virus preparations (see text).

Routes of Inoculation. Moloney, Rauscher, or Friend leukemia viruses were inoculated either i.p. or i.v. in 0.2 ml vol-
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RESULTS

Co-infection of Adult Mice with MSV and a Leukemogenic Virus. The results of dually infecting adult mice with MSV and either RLV, FLV, or MLV are shown in Charts 1 and 2. Inoculation with MSV alone caused the early induction of measurable intramuscular leg tumors (prior to 10 days). Tumor growth progressed rapidly attaining maximum size by 15 days postinfection and was histologically similar to that reported previously. From Day 15, tumor regression ensued, with complete regression occurring by the 35th day (Chart 1). No deaths occurred during the 80-day observation period (Chart 2).

In contrast, mice dually infected with MSV and RLV, FLV, or MLV showed an initial tumor regression, as evaluated from tumor size, followed by a reversion in the regression trend to progressive growth (Chart 1). The progressive trend in tumor growth was observed at 18 days (FLV), 21 days (RLV), and 24 days (MLV) following MSV inoculation.

Regression and reversion to progressive tumor growth was most marked in mice inoculated with MSV and MLV. The induction and regression of tumor growth in the control MSV, and the dually infected MSV + MLV animals, were parallel until the 24th day. After this time tumor growth progressed in the dually infected mice with 60 percent of the animals dying with tumors by the 58th day. The average tumor size and weight was 13 mm and 2.29 gm respectively. The average spleen weight was 0.40 gm. Tumors regressed completely in the remaining (40 percent) by the 65th day (Chart 1).

As can be seen from Chart 2, dual infection with RLV + MSV and FLV + MSV resulted in a 60 and 80 percent incidence of death with tumor 40 days after MSV inoculation. In contrast, infection with RLV or FLV alone resulted in no deaths of the animals which received RLV and 30 percent deaths in the FLV-inoculated group. By the 70th day, all animals dually infected with MSV and RLV or FLV died with tumors. Within the same period all animals inoculated with FLV alone died; however, only a 20 percent incidence of death occurred in the RLV-infected group.

Autopsy of mice dually infected with MSV and RLV or FLV showed the presence of leg tumors and splenomegaly. The average tumor size and weight in the MSV + RLV inoculated animals was 14 mm and 2.23 gm respectively, with an average spleen weight of 1.12 gm. In the MSV + FLV inoculated animals, the average tumor size and weight was 13 mm and 1.72 gm, with an average spleen weight of 1.98 gm.

The histologic sections prepared from mice infected with RLV + MSV revealed tumor involvement in the leg which was similar to tumors induced by MSV alone. In addition, however, tumor nodules were found in the diaphragm and in the lungs. The intense erythroblastic involvement reported to occur in mice infected with Rauscher virus alone was only slightly apparent in the liver and spleen sections and in the blood smears prepared from the dually infected animals.
Oncogenicity of Murine Sarcoma Virus

Although typical sarcomas were found in mice infected with FLV + MSV, they were infiltrated with lymphocytes, granulocytes, and connective tissue fibers arranged in a disorderly manner. Numerous fibrocytes, mast cells, and immature fibroblast-like cells with lightly staining nuclei were also found in the tumor. Further, the spleen and liver sections and the blood smears of these animals revealed the typical erythroblastic response found in animals inoculated with Friend virus alone.

Osmotic analysis of red blood cells was conducted on mice inoculated with RLV or FLV alone or combined with MSV. The red blood cell osmotic fragility pattern of the RLV + MSV-inoculated animals was normal (initial hemolysis 1.5 minutes, complete hemolysis 4.2 minutes). In contrast, the pattern observed for the FLV + MSV-infected animals was typical of a FLV infection (initial hemolysis 1.6 minutes and complete hemolysis 7.3 minutes); a sharp increase of heterogeneity in the red blood cell population was observed.

Bioassay of Cell-free Extracts of Tumor and Spleen from Animals Dually Infected with MSV and Leukemia Virus. It was desirable to determine what response(s) would occur in animals inoculated with cell-free extracts prepared from tumor and spleen obtained from dually infected mice. Three animals from each of the 3 groups which were infected with MSV and RLV, FLV, or MLV were sacrificed 38 days after MSV inoculation (Chart 1). This time was selected since complete tumor regressions had occurred in the control mice injected with MSV alone and the dually infected animals showed extensive tumors. The tumor and spleen extracts (see Materials and Methods) were inoculated into adult (8- to 10-week-old) BALB/c mice.

Maximum animal responses, i.e., tumor incidence, splenomegaly, percent death, and shorter periods to death with tumor were observed in those recipients inoculated with cell-free extracts of tumor derived from animals dually inoculated with MSV and RLV or FLV (Table 1). Inoculation of cell-free tumor extract by any of 3 routes (i.m., i.p., and s.c.) resulted in a greater than 80 percent host mortality. At the time of death animals exhibited tumor and splenomegaly. Spleen extract resulted in significant tumor incidence and/or death only when inoculated by the i.p. route.

A different pattern of response was noted in mice inoculated with tumor extracts derived from donors inoculated with MSV and MLV. A lower incidence of tumor induction and death occurred in recipients inoculated with tumor and spleen extract by the 3 routes.

Histologic Observations of Animals Inoculated with Tumor Extracts. In contrast to the mice inoculated with MSV alone, where a low incidence of metastasis was found, infection with
Table 1

<table>
<thead>
<tr>
<th>Donor mouse virus inoculation*</th>
<th>Donor tissue extract*</th>
<th>Tumor incidence (%)</th>
<th>Splenomegaly (%)</th>
<th>Death (%)</th>
<th>MST* R. of D.</th>
<th>Tumor incidence (%)</th>
<th>Splenomegaly (%)</th>
<th>Death (%)</th>
<th>MST* R. of D.</th>
<th>Tumor incidence (%)</th>
<th>Splenomegaly (%)</th>
<th>Death (%)</th>
<th>MST* R. of D.</th>
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<td>27</td>
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<td>37</td>
<td>(29-37)</td>
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Response in recipient mice inoculated with cell-free tumor or spleen extracts from donor mice. RLV, Rauscher leukemogenic virus; MSV, Moloney sarcoma virus; FLV, Friend leukemogenic virus; MLV, Moloney leukemogenic virus.

* Three donor mice were sacrificed at 38 days after infection with MSV (see Chart 1) for preparing donor tissue extracts.
+ Tissues prepared as 10 percent extracts (see Materials and Methods).
* Cell-free extracts inoculated (0.2 ml) either i.m., i.p., or s.c.
# Observations on tumor incidence based on 10 animals inoculated with each donor tissue extract.
* MST = Median survival time (days)

R. of D. = Range of death
BALB/c mice inoculated with MSV (0.1 ml of a 10⁶ concentration of SVRP-77).
cell-free extracts prepared from the dually infected donors resulted in extreme metastasis regardless of the route of inoculation. The severity of metastasis found in animals inoculated with cell-free extracts prepared from dually infected mice was in the order RLV + MSV > MLV + MSV > FLV + MSV. Tumor nodules were found in all the recipient animals inoculated with tissue extracts prepared from the 3 groups dually infected with MSV and leukemia virus (Table 1). Tumor nodules were noted mainly in striated musculature near the site of inoculation, on the diaphragm, in skull muscles, adjacent to the esophagus (Fig. 1), in areas of smooth muscles on the intestinal tract (Fig. 2) and in lung and heart. In some animals, the tumor was adjacent to and infiltrating the liver (Fig. 3), pancreas (Fig. 4), adrenal gland (Fig. 5), kidney, and brain. Tumor nodules were observed in the thorax and abdomen and often attached to the peritoneum.

Lymph node involvement was also found in a few animals. Histologic examination of these lymph nodes showed a depletion of free cells in the meshes of the stroma. The stromal sponge-like framework was filled and replaced by pleomorphic cells, some epitheloid, some round, and some resembling embryonic mesenchyme. The staining properties of these cells (stained by hematoxylin eosin and Mallory-azan or Masson's trichrome) were uniform, showing a pale, slightly red color of the cytoplasm. The nuclei were oval and chromophobic possessing large, distinct nucleoli and irregular clumps of chromatin. In severe cases only a few islands of lymphocyte clusters remained in the entire lymph node. The neoplastic cells showed a high rate of mitosis and in advanced stages were large; some were elongated and resembled the tumor cells found in or adjacent to muscle tissue.

Spleen involvement was also seen in animals inoculated with tumor and spleen extracts prepared from donors dually infected with RLV + MSV and MLV + MSV. This organ was enlarged and firm with discrete multiple cream white areas. The normal architecture of the spleen was obliterated and the splenic follicles were surrounded by pleomorphic cells similar to those described for involved lymph nodes. There was massive, diffuse infiltration of these pleomorphic cells with fibrotic involvement. Round or oval multinucleated giant cells were also present. In contrast, the spleens of recipient animals inoculated with tumor and spleen extracts prepared from donors dually infected with FLV + MSV showed the typical Friend virus-induced erythroblastic involvement.

In general, the morphology of the induced tumors in the various tissues and organs of the recipient mice was the same. Histologic appearance of the tumors was similar to that previously reported for mice inoculated with MSV, but with less differentiation towards muscle tissue cells and more toward fibrosarcomatous cells (Fig. 6).

**Relationship of Leukemia Virus Concentration to MSV Tumor Incidence.** The increased incidence of tumors observed in animals dually infected with MSV and a leukemia virus prompted a study to determine whether a dose-response relationship exists between the concentration of leukemia virus and the incidence of MSV-induced tumors (Table 2).

All animals infected with MSV alone developed leg tumors and a detectable splenomegaly. Thirty percent of the animals died by the 50th day after infection with MSV with gross symptoms of leg muscle tumor and enlarged spleens. This incidence was not seen in previous experiments (Charts 1, 2), and was attributed to the use of a more potent preparation of MSV.

A typical virus dose response was observed in animals infected with RLV. Thus, of the 3 parameters measured, splenomegaly, death, and median survival time were all found to be dependent upon the concentration of RLV injected. Only a 30 percent incidence of splenomegaly was achieved with the $10^{-5}$ dilution after 70 days of observation.

The effect of co-infection with a leukemia virus and MSV on the enhancement of progressive tumor growth and/or the increase of the death incidence is illustrated by the results shown in Table 2.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Virus inoculation (log)</th>
<th>Responses obtained in 70 days</th>
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<tr>
<td></td>
<td>RLV^a</td>
<td>MSV^b</td>
</tr>
<tr>
<td>1</td>
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</tr>
<tr>
<td>11</td>
<td>10^0</td>
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Effect of Rauscher leukemia virus dilution on Moloney sarcoma virus tumor incidence. MST, median survival time.

^a Ten adult BALB/c mice (8-10 weeks old) per group.

^b Rauscher leukemia virus diluted in phosphate buffered saline and 0.2 ml inoculated intraperitoneally.

^c Moloney sarcoma virus inoculated intramuscularly (0.1 ml of a 10° concentration of SVRP-83) five days after RLV infection.

^d Splenomegaly considered positive if palpated at 1+ or greater.

^e Dead with tumor and splenomegaly, remaining animals (70% at 70 days) showed no tumor or palpable splenomegaly.
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in Table 2. In the dually infected animals (Groups 7-10), tumor size, tumor incidence, splenomegaly, death, and median survival time did not appear to be dependent upon the dose of RLV employed. At the \(10^{-5}\) concentration of RLV (Group 11) there was only a slight difference noted in these parameters.

The effect of co-infection with leukemia virus on tumor incidence, regression, and death was most apparent in animals inoculated with MSV and low concentrations of RLV (Groups 1, 5-6, and 10-11). Seventy percent of induced tumors in the MSV-inoculated controls (Group 1) regressed as did the splenomegaly. However, 30 percent of the animals died with tumor and splenomegaly. In contrast, no tumor regressions occurred in animals dually infected with MSV and RLV at dilutions of \(10^{-1}\) through \(10^{-4}\). As expected, the pattern in the percentage of deaths occurring in animals infected with RLV alone decreased with decreasing concentrations of RLV (Groups 2-6). However, all animals infected with MSV and RLV at dilutions of \(10^{-1}\) through \(10^{-4}\) died with macroscopic leg tumors and splenomegaly. The median survival time for Groups 7-10 was much shorter than observed in Groups 2-5. A possible interpretation of these observations is that the dual infection stressed the animals leading to an earlier death than observed in mice infected with RLV alone. The reasoning, however, cannot be extended to differences obtained in Groups 6 and 11. No deaths occurred in Group 6, although 30 percent showed infection indicated by splenomegaly. In contrast, 90 percent of the dually infected animals (Group 11) died with tumor and splenomegaly.

**DISCUSSION**

Preinfection with either MLV, RLV, or FLV was effective in producing a reversal of tumor regression which is normally observed in adult mice inoculated with MSV alone. All animals co-infected with RLV + MSV and FLV + MSV died prior to 70 days. Co-infection with MLV + MSV resulted in 60 percent death by the 60th day and a 40 percent tumor regression level. Histologically, the leg tumors in these animals were similar to those induced in newborn mice infected with MSV alone (16, 18). The high incidence of tumor and the progressive growth in co-infected animals can thus be attributed to the presence of leukemia virus.

Hartley et al. (10) demonstrated that MSV produced foci of morphologically altered cells in primary MEF cultures or in a continuous line of MEF cells (CL-1). Focus formation induced by MSV in these cultures generally followed a two-hit dose-response curve which suggested the defectiveness of the MSV virions. The observation of defectiveness of MSV was confirmed when the general two-hit dose-response curve of MSV foci production was transformed to a one-hit type dose-response curve by the addition of excess MLV (\(10^{-1}\)). These studies indicated that MSV was defective in its ability to produce foci and required helper virus in form of MLV. These studies also showed that different pools of MSV contained both MSV and MLV, and that the amount of the latter virion in the pool was inversely related to the defectiveness of the MSV pool.

Huebner et al. (11) reported the development of a non-producer line of hamster tumor cells (HT-1) derived from a MSV-induced hamster tumor. The MSV genome in the HT-1 cells was "rescued" by infection of a mixed culture of HT-1 cells and MEF cells with MLV (helper virus). Rescue of the MSV genome was demonstrated by the recovery of focus-forming MSV from the tissue-culture fluid. The Rauscher and Friend leukemia viruses were also shown to act as helper viruses in this system. In addition, it was shown that extracts of tumors obtained from newborn mice inoculated with HT-1 cells and MLV, RLV, or FLV, readily produced foci in MEF cells, and sarcomas when inoculated into newborn mice. These observations indicated in vivo rescue of the MSV genome from the HT-1 cell line with helper virus (MLV, RLV, and FLV). Thus, by employing MLV, RLV, or FLV as a helper virus for in vitro or in vivo recovery of MSV genome from the non-producer HT-1 line of hamster tumor cells, pseudotypes of MSV were formed. Immunologic studies indicated that the pseudotypes consisted of the MSV genome and the envelope antigen of the helper virus (MLV, RLV, FLV, or Gross leukemia virus).

In view of these findings, the increased incidence of MSV-induced tumors by co-infection with Moloney, Rauscher, or Friend virus could be interpreted as the result of a "helper" effect. However, several factors indicate that the present results may be more complex.

The high percentage of tumor deaths and the short survival times observed in adult mice inoculated with tumor and spleen extracts that were prepared from dually infected animals indicate that the extracts contained a more virulent virus. Although few metastases were seen in animals inoculated with MSV alone (16, 18), all animals inoculated with tumor extracts, prepared from the dually infected animals, showed a high incidence of metastases. Whether the tumor nodules observed in the different organs of these animals were the result of metastases from a primary tumor or whether they were induced by virus de novo remains to be elucidated. Enhancement of pathogenicity of a nononcogenic virus (8) and increased tumor incidence (9) by dual infection with oncogenic and nononcogenic viruses in vivo have been reported.

Results reported by other investigators suggest an alternate explanation for the observed progressive tumor growths achieved in the dually infected animals. Fefer et al. (7) reported that X-irradiation of adult mice with 350 R one day prior to MSV inoculation did not affect the incidence of virus-induced tumors, but affected the fate of the tumor. Whereas all tumors induced in unirradiated adult mice completely regressed, many tumors induced in X-irradiated mice grew progressively and killed the host. No evidence for a relationship between antibody production and tumor regression was found. The authors postulated that due to the short latency period to oncogenesis by MSV there was not sufficient time for the development of immunologic competence or for the eliciting of an adequate specific-immune response. With time, however, both are attained and regression of the tumor occurs. Shachat et al. (D. A. Shachat, A. Fefer, and J. B. Moloney, Effect of Cortisone on Oncogenesis by Murine Sarcoma Virus (Moloney), unpublished data) reported that pretreatment of adult mice (BALB/c and C57BL/6) with cortisone resulted in a smaller percentage of tumor regressions occurring in cortisone-treated
animals. These observations indicate that immunologic host factors may play an important role in determining whether a primary tumor will grow or regress.

In the present study, it is possible that preinfection with leukemia virus produced an immunosuppressive effect resulting in an increased incidence of tumor growths. Several investigators have described the depressed antibody responses achieved by the injection of viruses (5, 13, 15, 19), malignant cells (6, 12), chemical carcinogens (1), and drugs (2). More recently Salaman (21) and Siegel (22) reported a depressed antibody response in mice infected with Friend or Rauscher leukemia viruses. Preliminary results of testing serum from animals infected with MSV (with regressing or regressed tumors) and sera from mice infected with MSV + RLV (bearing large tumors), show that a markedly lowered neutralizing antibody to MSV was present in the dually infected animals (W. Hooks, M. A. Chirigos, and S. Chan, unpublished data).

Thus the increased incidence of tumor growth and deaths resulting in dually infected animals reported in this study may be attributed to: (a) the leukemia virus acting as a helper for defective MSV resulting in the formation of a more virulent virus capable of inducing an increased malignancy; (b) the immunologic response of the host to MSV or MSV-specific antigens in emerging tumor cells may be altered by the infection with the leukemia virus; or (c) a combination of both conditions.

The high percentage of tumor deaths and consistent death pattern achieved in animals dually infected with MSV and serial dilutions of RLV indicate that these responses were not dependent upon the dose of leukemia virus. A high concentration of MLV helper was required in vitro to correct the defectiveness of MSV as indicated by increased foci formation by MSV in the presence of a helper (10). The titer of focus-forming MSV recovered from mixed cultures of the non-producer HT-1 line of tumor cells and MEF cells was found to be dependent upon the dose of RLV used to infect the mixed culture of cells (11). In contrast, the results obtained with the dilutions of RLV tested in the present study indicate that the enhancement of MSV was not dependent upon dose of RLV. A dilution of $10^{-5}$ of RLV was capable of exerting a helper effect.

Whether a $10^{-5}$ dilution of RLV was sufficient to alter the immunologic response of the host remains to be elucidated. Salaman (21) and Siegel (22), however, employed high concentrations of leukemia virus in their studies to effect an immunosuppressive effect.

Studies are currently in progress to further investigate interactions between MSV and the leukemogenic and nonleukemogenic viruses in attempts to elucidate the mechanism(s) responsible for the results reported herein.

REFERENCES


JUNE 1968
Fig. 1. Tumor node adjacent to the esophagus, obtained from an animal inoculated intraperitoneally with a cell-free extract of tumor prepared from a donor animal inoculated with Moloney sarcoma virus and Friend leukemogenic virus. H & E, × 85.

Fig. 2. Tumor involvement of the duodenum, obtained from an animal inoculated subcutaneously with a cell-free extract of tumor prepared from a donor animal inoculated with Moloney sarcoma virus and Rauscher leukemogenic virus. H & E, × 85.

Fig. 3. Tumor involvement of the liver, obtained from an animal inoculated intraperitoneally with a cell-free extract of tumor prepared from a donor animal inoculated with Moloney sarcoma virus and Moloney leukemogenic virus. H & E, × 140.

Fig. 4. Tumor involvement of the pancreas obtained from an animal inoculated subcutaneously with a cell-free extract of tumor prepared from a donor animal inoculated with Moloney sarcoma virus and Rauscher leukemogenic virus. H & E, × 140.

Fig. 5. Tumor adjacent to the adrenal gland, obtained from an animal inoculated subcutaneously with a cell-free extract of tumor prepared from a donor animal inoculated with Moloney sarcoma virus and Rauscher leukemogenic virus. H & E, × 140.

Fig. 6. Typical tumor, showing undifferentiated round, fusiform, and spindle cells. H & E, × 650.
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