*ERYTHROCYTIC MATURATION OF (FRIEND) VIRUS-INDUCED LEUKEMIC CELLS IN SPLEEN CLONES**

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Explants of leukemic livers or spleens[1] induced by Friend virus (FV) have produced several lines of transplantable tumors which have been in serial passage in DBA/2 mice for more than seven years. Although of low titer, the virus is still associated with these tumors, which resemble reticulum cell sarcoma.

In attempts to clarify the virus-host cell relationship, tissue culture lines have been established from these tumors.[2] The cells retain their neoplastic properties and continue to produce virus for more than two years after the initiation of the cultures. A striking characteristic of these cells is their ability to mature along the erythroid line up to the stage of the orthochromatic erythroblast.

The cells, which have been cloned *in vitro*, possess properties identical to those described for the mass cultures.[3]

In an extension of this work, the spleen colony assay[4, 5] was utilized to clone the cell lines *in vivo* in lethally irradiated mice. Under these conditions, each colony represents the population developing from a single hematopoietic cell.[6]

In the present study, the cloning efficiency and Fe^{59} incorporation of the cultured leukemic cells in syngeneic and allogeneic lethally irradiated mice was determined.

Materials and Methods.—Six- to twelve-week-old DBA/2 (Jackson Memorial Lab.), Swiss ICR/Ha (Millerton Research Farm), and F_1 (BALB/c × DBA/2) mice (bred in this laboratory) were used in the experiments. Each group consisted of 15–20 female mice. They were housed five to a cage after irradiation.

The mice were irradiated, in groups of ten each, in two 18 × 16-cm Lucite boxes, surrounded by bolus material, at 50 cm from the target of two 200-kvp X-ray generators (G.E. Maxitron units) operated at 20-ma tube current, with filtration giving a HVL of 0.9 mm Cu. The exposure at 50 cm, including scattered radiation, ranged between 124 r/min, and 146 r/min. The dose at different points within the boxes was found to vary no more than 0.6%. Nine hundred to 1000 r were given in a split dose (500–600 r followed by 400 r). This corresponds to an absorbed tissue dose of 846–940 rads. This dose has been shown to give an LD_{100}/30 days in untreated mice.[7] The probability of a hemopoietic cell's surviving in such animals is extremely small. Untreated irradiated controls, however, were always included in each experiment to evaluate the incidence of endogenous colonies. The mice were radiated in the afternoon, injected the following morning, and received the second X-ray exposure within 2 hr.

Cells from the tissue cultures were centrifuged at 700 rpm/10 min, washed once, and resuspended in Ringer's solution. A sample to which trypan blue had been added was counted in an hemocytometer and the number of the viable cells calculated. The original suspension was then diluted in Ringer's solution to the desired cell concentrations. The cell preparations were kept at 4°C throughout the experiment.

Cells directly from the tumors were obtained by transferring a 20% Ringer suspension of minced tissue to a conical tube and allowing it to stand at 4°C until the larger fragments settled to the bottom. The supernatant was then removed, the cells were counted in a 1% solution of acetic acid, and the cell dilutions in Ringer were prepared.

For spleen colony counts, the mice were sacrificed 10–12 days after inoculation. The spleens were placed in Telleyesniczky's fixatives (4.5 ml glacial acetic acid, 4.5 ml formaldehyde, 100 ml 70% ethanol) for 5 min to allow a precise demarcation of the colonies which were then counted. Duplicate counts of colonies in an individual spleen were not accurate when more than 15 colonies were present per spleen, and these were scored as confluent. Imprints of the colonies were stained

with May-Gruenwald-Giemsa. Specimens of spleen, liver, and sternum were fixed in Vandergrifft's, imbedded in paraffin, and stained with hematoxylin-eosin for histological studies. In some instances, samples of the spleen colonies were examined under the electron microscope. Finally, spleen colonies were bioassayed for transplantability, virus content, and ability to propagate in tissue cultures.

For transplantability assay, the colonies were either excised, cut into small fragments, and implanted subcutaneously (sc) in 6–8-week-old DBA/2 mice, or a 20% cell suspension of a pool of colony-containing spleens was prepared and 0.2 ml of this was inoculated intraperitoneally (i.p.) or sc.

For virus assay, a 20% homogenate was prepared from a pool of colony-containing spleens and 0.2 ml of the supernatant fluid inoculated i.p. into 6–8-week-old Swiss mice. On occasion single colonies were minced and suspended in 1 ml of Ringer's solution and 0.1 ml of the cell suspension inoculated i.p. Specimens which induced leukemia were considered positive for virus.

Fragments of individual spleen colonies were assayed *in vitro* for growth by the method previously described.[2] Spleen-cloned tissue culture cell sublines were thus obtained which could be used again to initiate spleen colonies.

To determine iron uptake, 0.6 μc of Fe^{59} (ferrous citrate, Squibb), diluted in Ringer's solution, was injected intravenously 24 hr before the animals were sacrificed. The spleens were removed and counted in a Nuclear Chicago well-type scintillation counter. With each experiment, a sample of the injected material was saved for a standard count.

Results.—Morphology of the spleen colonies: The leukemic cells produce in the spleens of the irradiated recipients colonies which are macroscopically identical to those observed by other investigators who used hematopoietic cells.[4, 5] Examination of sections and imprints of the colonies reveals a cell population consisting of cells of the erythroid series. However, many of the cells are not differentiated enough to allow a precise classification with regard to the stage of maturation. These primitive cells resemble what Metcalf *et al.*[9] call the "Friend" cell. The remainder of the population consists of proerythroblasts and erythroblasts in various stages of maturation.

Although a precise method to quantitate cell populations on imprints or smears has not as yet been devised, it appears that the proportion of differentiated to undifferentiated cells is somewhat higher in the spleen colony population than it is in the tissue culture population.

Granulocytic or megakaryocytic colonies have never been found in the spleens of the experimentally inoculated animals, whereas they have been observed in the few endogenous colonies that have arisen in the uninoculated irradiated controls.

The spleen colonies produced by all of the long-term leukemic cell tissue culture lines under study, as well as by cell suspensions derived from minced fragments of the subcutaneous transplantable tumors, from which *in vitro* lines originated, appear identical. Eleven days after cell inoculation, a typical colony contains approximately 2.6×10^6 cells. Assuming that each clone doubles exponentially, starting from the earliest precursor, the doubling time of the cells would be about 12 hours. On the other hand, the generation time is probably shorter than 12 hours, since some erythropoietic cells, such as the polychromatophilic erythroblasts, probably stop dividing after having reached a certain level of differentiation.

Bioassays: The colonies have been tested for tumor-forming capacity, infectivity of virus, and ability to re-initiate an *in vitro* culture. All colonies tested for tumor formation, in DBA/2 mice, gave rise to tumor at the site of the inoculation within 39 days. About 50 per cent of the tumor-bearing animals also had generalized leukemia. Electron microscope examination, performed by Dr. Etienne de Harven,

revealed virus particles budding from both the primitive and erythroblastic cells in the spleen colonies. Out of 19 individual colonies tested for virus content, five were positive, inducing the typical leukemia in adult Swiss mice within two months.

Every spleen colony which was assayed in tissue culture gave rise to an established cell line identical to that used to initiate the spleen colony assay. The *in vitro* assay was also useful in screening the few endogenous colonies observed in control-irradiated animals. These colonies survived *in vitro* for a limited time; within three weeks no living cells were present.

The colony-forming unit (CFU) in syngeneic mice: The relationship between the number of cells inoculated in DBA/2 mice and the mean number of spleen colonies appearing 11 days later did not deviate significantly from linearity and the intercept was not significantly different from zero. Throughout this study, the CFU was calculated from the actual number of cells inoculated and the number of colonies formed and equaled 2.20 ± 0.36 (the slope ± the standard error of the slope) per 10^4 cells inoculated.

However, since the variation of spleen colony counts increased in proportion to the number of cells injected, a transformation was considered desirable to render the variance more equal over the range studied, as required in a regression analysis. The logarithmic conversion of both the number of spleen colonies observed in each animal and of the number of cells inoculated was made. These values are plotted in Figure 1. The best fitted least-squares line with its 95 per cent confidence belts[10] for the mean number (log) of spleen colonies corresponding to a given size of inoculum is also included. The P for $b = 0$ is less than 0.001. This indicates that there is a constant proportion between the ratio of increase of the number of spleen colonies to the ratio of increase of the number of cells injected.

These data are, therefore, compatible with the assumption made in regard to normal mouse bone marrow cells,[4, 6] namely, that a single cell is able to give rise to a colony in the spleen.

Colony formation in allogeneic mice: Colony formation in Swiss mice was found to be of lower efficiency than that observed in DBA/2 mice. This was to be expected in that it had been shown that the CFU of C57Bl bone marrow cells was greatly "repressed" in allogeneic irradiated recipients, as compared with the syngeneic irradiated hosts.[11] A series of experiments was therefore carried out to compare the CFU in allogeneic Swiss and F_1 (BALB/c × DBA/2) hybrid mice as well as to confirm the linear relationship between spleen colonies formed and cells inoculated. Of interest is the fact that F_1 hybrid mice proved to be markedly more tolerant of the radiation than were the DBA/2 and Swiss mice.

The CFU in Swiss mice was 1.47 ± 0.25 per 10^4 cells inoculated, as compared to 3.5 ± 0.52 per 10^5 cells inoculated in the F_1 hybrid mice. The linear relationship with the 95 per cent confidence lines obtained from these data is shown in Figure 2 (Swiss mice) and 3*a* (F_1 hybrid mice). In both figures, a logarithmic conversion of the data such as had been described for Figure 1 was made. Figure 3*b* illustrates that the plot of the direct arithmetical values obtained in the experiments carried out on F_1 hybrid mice gives the same linear relationship shown in Figure 3*a*. The P for $b = 0$ is less than 0.001 both in Figures 2 and 3*a* and in Figure 3*b*.

In Figure 4 the interrelationships existing between the three lines shown in Figures 1, 2, and 3*a* are demonstrated.

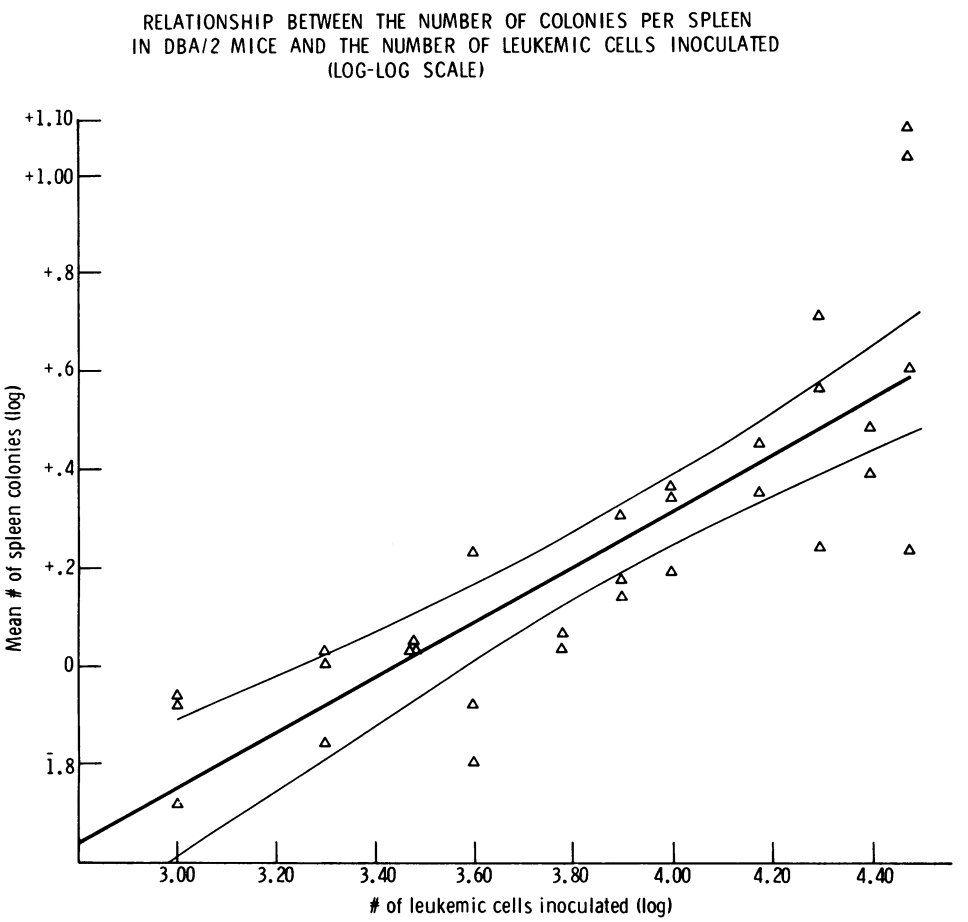

FIG. 1.—Every point represents the mean of the log of the number of colonies observed per mouse in 31 experiments. Mean number of animals per experiment, 8.8; minimum, 3.

The results indicate the presence of a repression phenomenon similar to that observed by McCulloch and Till[11] and, paradoxically, it is more marked in the F_1 hybrid than in Swiss mice. It should be noted, though, that the difference between the CFU values (Table 1) in the DBA/2 and Swiss mice is not significant at the 5 per cent level ($P > 0.1$). In contrast, the CFU value is significantly lower in the F_1 than in DBA/2 mice ($P < 0.01$), for eight times more cells are required to produce a single colony in the F_1 hybrid mice.

It is interesting to note that with increasing numbers of cells the repression may be overcome, so that as larger numbers of cells are inoculated, spleen colonies are formed with a greater efficiency than that observed in DBA/2 mice. Thus, for example, while in DBA/2 mice one gets a 2-fold increase in the number of spleen colonies for each 3.5-fold increase in the number of cells inoculated, in F_1 hybrid mice the same 2-fold increase in the number of spleen colonies was obtained with only a 2.2-fold increase in the number of cells inoculated.

The relationship of the number of spleen colonies to spleen weight and Fe^{59} incorpo-

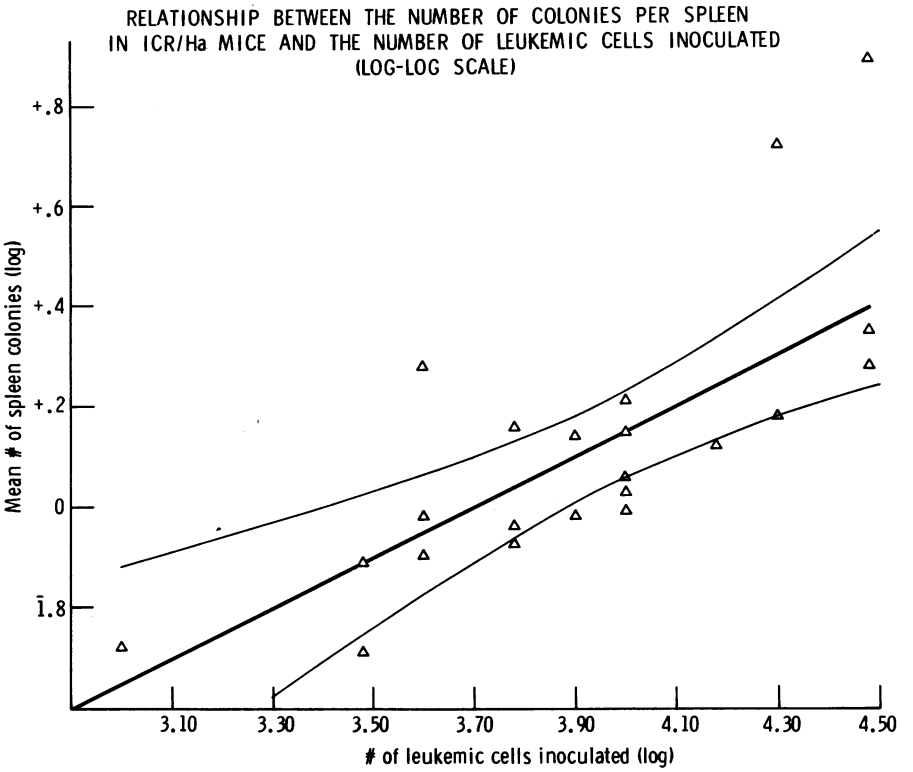

FIG. 2.—Each point represents the mean of the log of the number of colonies observed per mouse in 23 experiments. Mean number of mice per experiment, 10.9; minimum, 3.

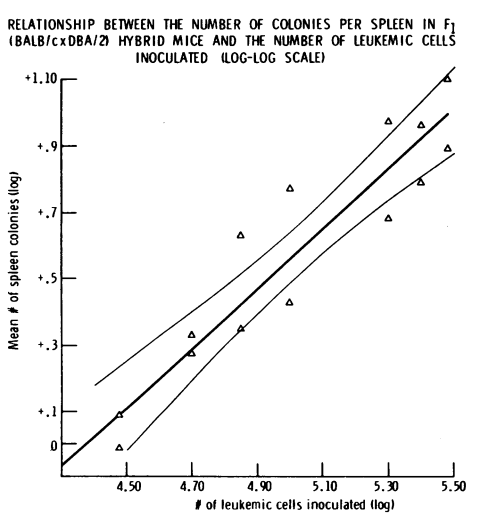

FIG. 3*a*.—Each point represents the mean of the log of the number of colonies observed per mouse in 14 experiments. Mean number of mice per experiment, 16.5; minimum, 13.

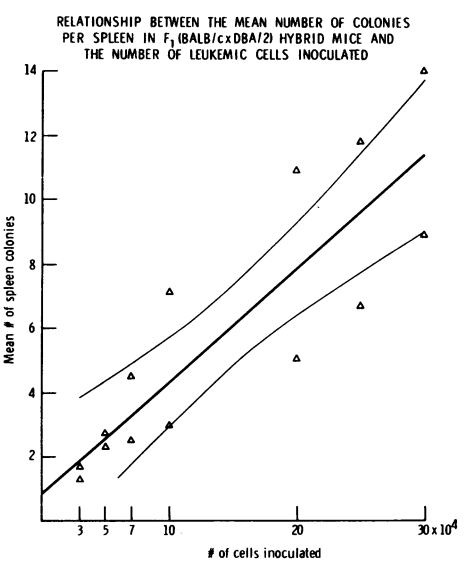

FIG. 3*b*.—Each point represents the mean number of colonies observed in the 14 experiments plotted in Fig. 3*a*.

TABLE 1

COLONY FORMATION FOLLOWING INOCULATION OF DBA/2 LEUKEMIC CELLS INTO SYNGENEIC AND ALLOGENEIC HOSTS

Number of experiments	Recipient mouse strain	CFU ± standard error of the slope/No. of cells inoculated
31	DBA/2	$2.20 \pm 0.36/10^4$ cells
23	Swiss ICR/Ha	$1.47 \pm 0.25/10^4$ cells
14	F_1(BALB/c × DBA/2)	$3.50 \pm 0.52/10^5$ cells

ration: It has been demonstrated that both spleen weight and spleen Fe^{59} uptake are directly related to the number of colonies observed in the spleens of irradiated mice inoculated with normal mouse bone marrow cells.[12] This relationship also exists in the case of the leukemic cells under study. The results obtained when irradiated F_1 hybrid mice were used as recipients are given in Figures 5 and 6. Analogous results were obtained with the other two strains of mice. Figure 5 shows a linear relationship between the number of colonies observed and spleen weight. Figure 6 also shows that a linear relationship exists between the number of colonies observed and the spleen Fe^{59} incorporation. The Fe^{59} uptake values of the spleen of irradiated uninoculated controls, whose spleens had less than 0.5 colony per spleen, were close to background levels. These data confirm similar findings obtained from tissue culture studies,[2] and suggest that erythropoiesis is taking place in the cells colonizing in the spleen.

Survival of the irradiated recipients inoculated with leukemic cells: Experiments were performed to determine whether the inoculation of leukemic cells would result in prolonged survival of the irradiated recipients. Groups of 25 mice were injected with up to 8.2×10^6 leukemic cells. No significant difference between the survival curve of these animals and that of irradiated uninoculated controls was noted. This was to be expected, for the leukemic cells have thus far demonstrated ability to mature only along the the erythrocytic line, and moreover, no reticulocytes or fully mature red blood cells have as yet been detected in the tissue culture lines.

Experiments have been undertaken to determine whether or not the ability of the leukemic cells to colonize in the spleen is affected either by the administration of erythropoietin or by previously induced plethora. Preliminary data suggest that neither factor exerts appreciable influence.

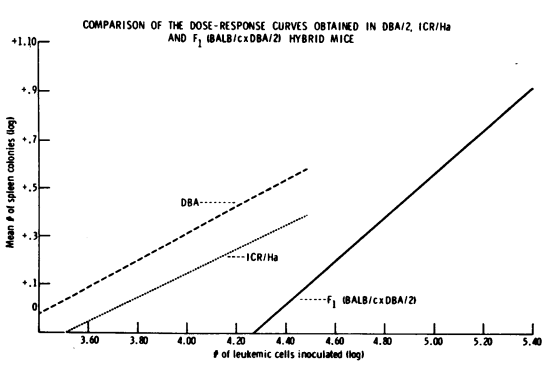

FIG. 4.—The lines in Figs. 1, 2, and 3*a* are compared to show their interrelationships. The single points have been omitted for the sake of clarity.

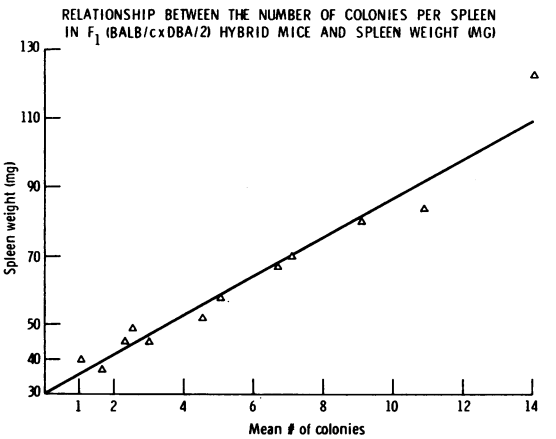

FIG. 5.—Each point represents the mean spleen weight corresponding to the mean number of spleen colonies observed in 12 experiments.

Discussion.—The findings obtained from the tissue-culture study of our mass and cloned lines of murine virus-induced leukemic cells[2, 3] are confirmed by the results of the spleen colony assay. The leukemic cells, after having been cloned *in vitro* as well as *in vivo*, retain their malignancy, replicate virus, yet differentiate along the erythrocytic line and incorporate Fe^{59}.

Despite the fact that the second dose of 400 r was given to the mice after the inoculation of the cells, it seems that these cells may have a somewhat greater colony-forming ability than normal bone marrow cells. This point is supported by recent preliminary experiments, which indeed show that when the cells are injected after the second X-ray dose, an increase in CFU's is observed.

The mechanism of the repression of the colonizing capacity of the leukemic cells in allogeneic mice, however, remains unexplained. It seems improbable that a graft-versus-host reaction occurred, since the strains used in these experiments do

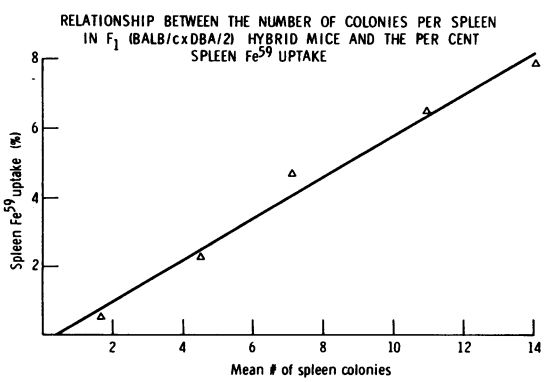

FIG. 6.—Each point represents the mean per cent spleen Fe^{59} uptake corresponding to the mean number of spleen colonies observed in 5 experiments.

not differ at the *H-2* histocompatibility locus. It seems unlikely that a mild runt disease due to other weak histocompatibility antigens might play a role. If this were to be the case, in fact, one would have expected the CFU repression to be stronger in Swiss, an unrelated strain, than in the F_1 (BALB/c × DBA/2) hybrid mice, which share all the antigens of the DBA/2 strain. Furthermore, allogeneic F_1 hybrid animals survived as well or better than the syngeneic DBA/2 mice when injected with leukemic cells. On the other hand, that a host-versus-graft reaction has taken place is unlikely in view of the supralethal dose of X-ray received by the recipients.

Worthy of further investigation is the mechanism involved in the increased rate of colonization of the leukemic cells once the repression phenomenon is broken through, for this has also been found to occur with normal mouse bone marrow cells.[13]

Finally, evidence has been obtained indicating that the virus exerts control over the proliferative capacity of the cells.[14] An almost complete inhibition of colony formation has been observed in irradiated mice previously immunized with virus from tissue culture preparations or obtained directly from filtrates of the spleens of leukemic mice.

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