*COMPLEX MITOCHONDRIAL DNA IN LEUKEMIC AND NORMAL HUMAN MYELOID CELLS**

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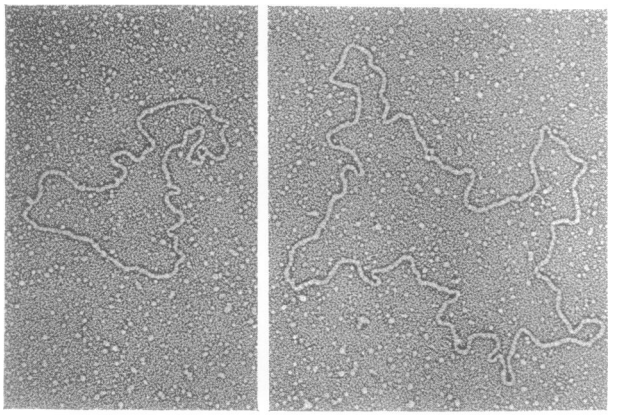
Abstract.—Mitochondrial DNA's from the peripheral blood of 14 patients with granulocytic leukemia contained a circular dimer form. No such structure could be found in M DNA's from three patients with nonmalignant proliferations of granulocytes. The frequency of the circular dimer form is reduced upon treatment with antileukemic drugs. The above results suggest that a significant relation exists between the formation and presence of the circular dimer M DNA form and granulocytic leukemia in man.

We have previously reported that animal mitochondrial DNA (M DNA) occurs in two *complex* forms[1–3] in addition to the *simple* circular form with a contour length of about 5 μ. The circular dimer form—a double-length circular molecule[1, 2]—was found in human leukemic white cells along with the catenane form,[3] in which the single-length submolecules are connected to each other like links in a chain. The presence of the circular dimer in the M DNA from the three leukemic patients studied,[2] and the absence of this form in the M DNA from normal mature human leukocytes,[2, 5] HeLa cells,[3] sea urchin eggs,[4] and a variety of normal mammalian tissues,[5] suggested to us that there might be a correlation between the occurrence of the circular dimer and human leukemia. We therefore carried out a more extensive study of the frequency of complex M DNA forms in circulating leukocytes from patients with granulocytic leukemia and, for comparison, from patients with nonleukemic leukopoietic disorders. The M DNA's from 14 leukemic patients all contained circular dimers (Fig. 1), whereas the M DNA's from three patients with normal, immature myeloid cells contained none. In the course of this study we also found that treatment with cytotoxic drugs substantially lowered the frequency of the circular dimer. The above results together suggest that a significant relation exists between the formation and presence of the abnormal M DNA form and granulocytic leukemia in man.

Materials and Methods.—Chemicals: Optical grade CsCl was obtained from the Harshaw Chemical Co. Ethidium bromide was a gift from Boots Pure Drug Co., Ltd., Nottingham, England. Sodium dodecylsulfate was obtained from the Matheson Co. All other chemicals were of reagent grade.

Preparation of leukocyte M DNA: Whole-blood samples, 10–100 ml, were allowed to settle at 4° for 2 to 20 hr. Buffy coats were drawn off and red cells were removed by hemolysis for 20 sec with distilled water. The leukocytes were spun down at 1000 $\times$ *g*, for 5 min in an International PR-1 centrifuge and resuspended in a tenfold volume of homogenizing medium: 0.21 *M* mannitol; 0.07 *M* sucrose; 0.001 *M* Tris HCl, pH 7.5; and 0.0001 *M* ethylenediaminetetraacetate (EDTA). The cells were homogenized with a tight-fitting Teflon pestle until approximately 80% of the nuclei were liberated, as observed in the light microscope. The homogenate was centrifuged at 1000 $\times$ *g* for 5



(a) (b)

FIG. 1.—Electron micrographs of M DNA isolated from leukemic leukocytes. (a) Monomeric M DNA. (b) Circular dimer M DNA.

min. The supernatant was centrifuged at 15,000 × *g* for 15 min in a Sorvall SS-34 rotor to pellet the mitochondria. The pellet was resuspended in 5 ml of homogenizing medium and placed on a step gradient containing equal volumes of 1.00 *M*, 1.50 *M*, and 1.75 *M* sucrose. The sucrose solutions contained 0.001 *M* Tris HCl, pH 7.5, and 0.0001 *M* EDTA. The tubes were centrifuged for 30 min at 20 krpm in a Spinco SW-25.1 or SW-27 rotor. The buoyant material at the 1.00 *M*–1.50 *M* interface was removed with a pipet, diluted fourfold with homogenizing medium, and pelleted. The mitochondrial pellet was resuspended in 10 ml of 0.21 *M* mannitol, 0.07 *M* sucrose, 0.05 *M* Tris HCl, pH 6.7, 0.025 *M* KCl, and 0.0025 *M* $MgCl_2$. All of the foregoing operations were at 4°.

DNase I and RNase A (Sigma Chemical Co.), 100 μg each, were added and the mixture incubated at 25° for 30 min. The reaction was quenched by the addition of EDTA, and the temperature was lowered to 0°. The mitochondria were pelleted and the DNA was isolated after the addition of sodium dodecylsulfate, CsCl, and ethidium bromide (EB), as described earlier.[1, 2, 5] In most cases, both the upper and lower fluorescent bands and the CsCl solution between them was collected from the buoyant CsCl-EB gradient. The solution was diluted with 0.01 *M* Tris HCl, pH 7.5, 0.001 *M* EDTA; and the DNA was pelleted at 45 krpm for 12 hr in a Spinco SW-50 rotor. The M DNA pellet was allowed to resuspend in 0.25–0.5 ml of supernatant and was stored at −20°.

Specimens for electron microscopy were prepared as described earlier[2] and were examined in a Philips EM-300 electron microscope. Classification of M DNA forms was performed at the microscope by direct observation on the fluorescent screen or on an auxiliary Plumbicon TV monitor system. The procedures and criteria for classifying the M DNA forms have been described.[5]

Results.—The results obtained in this study are presented graphically in a series of panels which show the M DNA and the leukocyte distributions in each blood sample. The medical diagnoses and pertinent clinical data supplied to us by our colleagues are given in Table 1, along with a summary of the M DNA distributions.

Untreated granulocytic leukemia: Figure 2*a* presents the M DNA distribution in the circulating leukocytes from M. C., a patient with chronic granulocytic leukemia. The circular dimer frequency (31%) did not change significantly in four analyses during an 18-month period. The frequency of catenated dimers (7%) is within the range found in normal mammalian tissues (6–9%).[5] The frequency of catenated higher oligomers (trimers and larger catenanes) is significantly higher (8%) than in normal tissues in which the highest frequency

TABLE 1. *Number frequency of complex mitochondrial DNA in leukemic and nonleukemic patients.*

Patient*	Diagnosis†	Chemotherapy	Circular dimer (%)	Catenated dimer (%)	Catenated higher oligomer (%)
M. C.[a]	CGL	None‡	31 ± 4	7 ± 2	8 ± 2
C. H.[b]	AGL	None	48 ± 4§	4 ± 1§	5 ± 2§
P.J.[b]	"	"	29 ± 6	6 ± 3	1.0 ± 0.9
M. W.[c]	CGL	"	21 ± 3	6 ± 2	3 ± 1
"	"	Myleran, 3 wk	7 ± 2	7 ± 2	2 ± 1
M. R.[b]	"	None	14 ± 2	7 ± 2	1 ± 0.7
"	"	Myleran, 1 wk	7 ± 2	11 ± 2	3 ± 1
"	"	" 3 wk	5 ± 1	11 ± 2	2 ± 1
M. Y.[d]	"	Myleran, 2 yr	2 ± 1	4 ± 2	2 ± 1
L. G.[b]	"	" 3 yr	2 ± 1	4 ± 1	0.1
L. S.[a]	"	" 2 yr	3 ± 1	5 ± 1	0.3
A. M.[c]	"	" 2 wk	6 ± 2	5 ± 2	1 ± 0.9
A. P.[b]	AGL	6-MP, 8 months**	1 ± 0.8	5 ± 2	0.1
L. J.[b]	"	" 5 days	2 ± 2	5 ± 2	0.6
R. B.[e]	"	" 2 wk	2 ± 1§	4 ± 2§	0.6§
E. F.[b]	At CGL	None	12 ± 2	6 ± 2	0.6
J. W.[b]	Ea CGL	"	1 ± 0.9§	3 ± 2§	0.9§
M. L.[b]	MM	"	0	6 ± 1	0.5 ± 0.3
T. D.[b]	MM	"	0	4 ± 1	0.8 ± 0.4
M. D.[c]	LR	"	0	5 ± 1	0.6 ± 0.4

* The diagnoses for the patients were supplied by: (*a*) H. R. Bierman; (*b*) R. L. Teplitz; (*c*) W. C. Moloney; (*d*) R. D. Lewis; and (*e*) R. Boardman.

† *CGL, AGL, At CGL,* and *Ea CGL* signify chronic, acute, atypical chronic, and early chronic granulocytic leukemia, respectively. *MM* and *LR* signify myeloid metaplasia and leukomoid reaction, respectively.

‡ This patient was treated periodically by leukophoresis, a procedure in which the patient's red cells and plasma are infused after removal of the buffy coat.

§ Data obtained from a lower-band M DNA in a CsCl-EB density gradient.

** This patient also received Vincristine and Methotrexate.

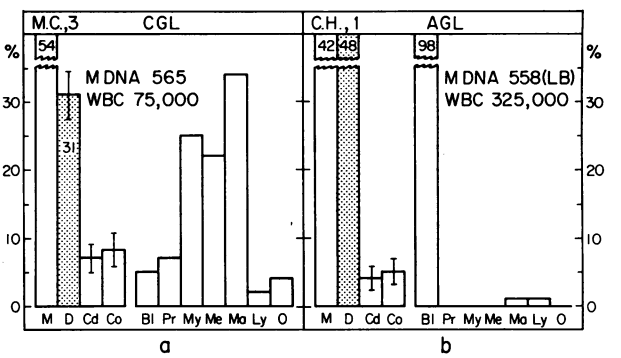


FIG. 2.—The distribution of complex forms in the M DNA obtained from two granulocytic leukemic patients who had not received cytotoxic drugs. The M DNA distribution is given in per cent at the left of each panel: Monomers, *M;* circular dimers, *D;* catenated dimers, *Cd;* and catenated higher oligomers, *Co.* The leukocyte distribution in per cent is given at the right of each panel: Myeloblasts, *Bl;* promyelocytes, *Pr;* myelocytes, *My;* metamyelocytes, *Me;* mature forms, *Ma;* lymphocytes, *Ly;* and other types, *O.* The numbers after M DNA and WBC represent the number of DNA molecules classified and the leukocyte count in cells/mm.[3] CGL and AGL stand for chronic and acute granulocytic leukemia, respectively. The number after the patient's initials is our code number for the blood sample analyzed. The error bar indicates the interval which contains the true mean at a level of confidence of 95%. The calculation takes into account only the uncertainty associated with the finite sample. The symbol *LB* stands for lower band in the CsCl-EB gradient. An analysis of a lower band rather than the combination of the upper and lower bands and the intermediate solution provides a *minimum* frequency for higher molecular weight M DNA forms.[2]

was 2 per cent.[5] We calculate that 40 per cent of the M DNA mass is in the form of circular dimers and that an additional 25 per cent is in the form of catenated DNA molecules. The catenated higher oligomers were assumed to be trimers in the foregoing calculation.

The M DNA from C. H., a patient with severe acute granulocytic leukemia, contained the highest frequency of circular dimers (48%) that we have so far encountered (Fig. 2*b*). The catenated higher oligomer frequency (5%) is again high. The circular dimer accounts for 58 per cent of the mass of the M DNA, and the catenated form for 17 per cent. The symbol *LB* in Figure 2*b* refers to a lower band in a CsCl-EB density gradient. An analysis of a lower band yields a minimum estimate of the frequency of higher molecular weight M DNA, as discussed previously.[2] The leukocytes in this blood sample consisted almost entirely of myeloblasts; we may conclude, therefore, that these blast cells contained the circular dimer and the simple M DNA forms.

The circular dimer frequency in P. J., another untreated patient with the acute form of the disease, was 29 per cent (Table 1). The per cent leukocyte distribution was as follows: myeloblasts, 81; promyelocytes, 10; mature forms, 4; lymphocytes, 4; and other types, 1.

Two additional cases of untreated granulocytic leukemia are presented below in the section on the effects of chemotherapy.

Nonleukemic leukopoietic conditions: Leukocyte distributions similar to those observed in patients with granulocytic leukemia occur in patients with myeloid metaplasia and in patients with leukomoid reactions to a variety of stimuli. Such circulating nonleukemic myeloid cells provide the appropriate controls for the proposed correlation of the circular dimer M DNA and granulocytic leukemia. The M DNA's from three such patients are presented in Figure 3. No circular dimers were detected, even though 1300–2300 molecules were examined in each sample. The circular dimer level, if not zero, is certainly less than 0.2 per cent. The catenated dimer and higher oligomer frequencies are also in the normal range at 4–6 and <1 per cent, respectively. The leukocyte distributions (Fig. 3*a* and *b*) of the two patients with myeloid metaplasia

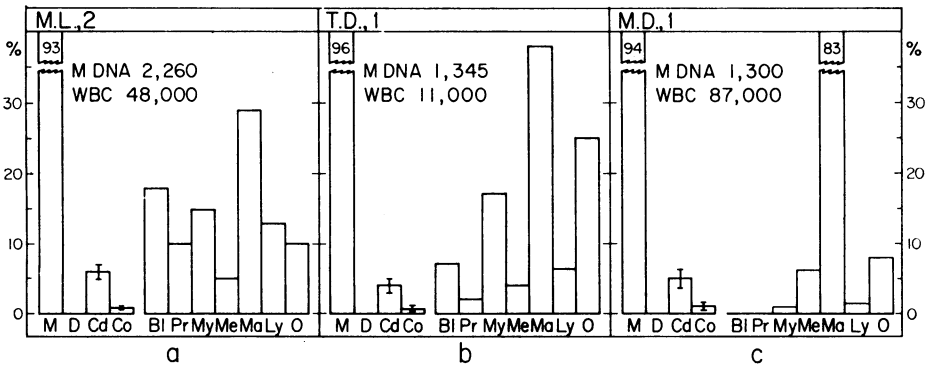


FIG. 3.—The distribution of complex forms in the M DNA obtained from two patients with myeloid metaplasia (*a* and *b*) and one patient with a leukomoid reaction (*c*). The symbols are defined in the legend for Fig. 2.

are similar to those observed in the blood of patients with chronic granulocytic leukemia (Figs. 2*a*, 4, and 5*a*, and *c*). We conclude from the above results that the circular dimer M DNA is not a constituent of normal immature and mature myeloid cells in man.

The effect of chemotherapy on the M DNA distribution in granulocytic leukemia: The M DNA from the peripheral blood of a patient with the chronic form of the disease was examined before and after therapy was initiated with the alkylating agent, Myleran—1,4 bis[methane sulfonoxyl]butane. The circular dimer frequency, which was 21 per cent before treatment began, dropped to 7 per cent after three weeks of chemotherapy (Fig. 4). A similar effect of chemotherapy was observed in a serial study of the M DNA from patient M. R. (Table 1).

The effect of treatment is illustrated further by the low levels of the circular dimer in blood samples taken from six granulocytic leukemic patients who had received cytotoxic drugs for various periods of time (Fig. 5 and Table 1). The circular dimer frequency in these samples[6] ranged from 1 to 3 per cent, in contrast to the five untreated leukemics (Table 1) in which the circular dimer frequency ranged from 14 to 48 per cent. The above effects of chemotherapy were observed consistently, although the patients had widely different leukocyte distributions.

L. J., a patient with acute granulocytic leukemia, had received the base analogue 6-mercaptopurine (6-MP) daily for only five days when the blood sample was taken for analysis (Fig. 5*e*). The white-cell distribution, which consisted almost entirely of myeloblasts, had not yet changed in response to therapy. The circular dimer level (2%) was, however, much lower than that in C. H., an untreated patient with the same form of the disease (Fig. 2*b*) whose leukocyte distribution was similar. These results raise the interesting possibility that the drug had caused selective removal of the circular dimer from the peripheral leukocytes; alternatively, a new population of blast cells with a low level of circular dimers had replaced the original population.

Atypical and early granulocytic leukemia: Patient E. F. (Fig. 6*a*) was diagnosed as an atypical granulocytic leukemic because the patient did not respond to Myleran in 1963, and bone marrow metaphase spreads did not contain the partially deleted chromosome 21 (the Philadelphia chromosome) usually present in chronic granulocytic leukemia.[7] On the other hand, the low level of

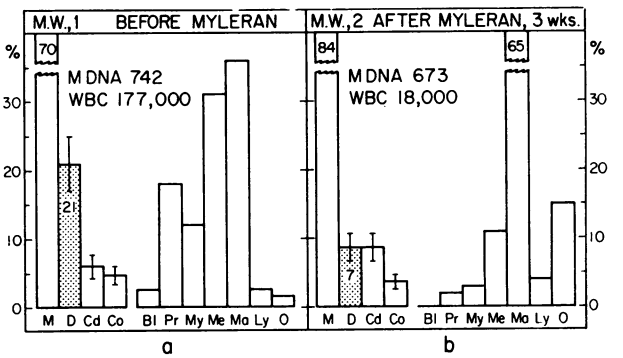


FIG. 4.—The distribution of complex forms in the M DNA obtained from a patient with chronic granulocytic leukemia before and after treatment with Myleran for three weeks. The symbols are defined in the legend for Fig. 2.

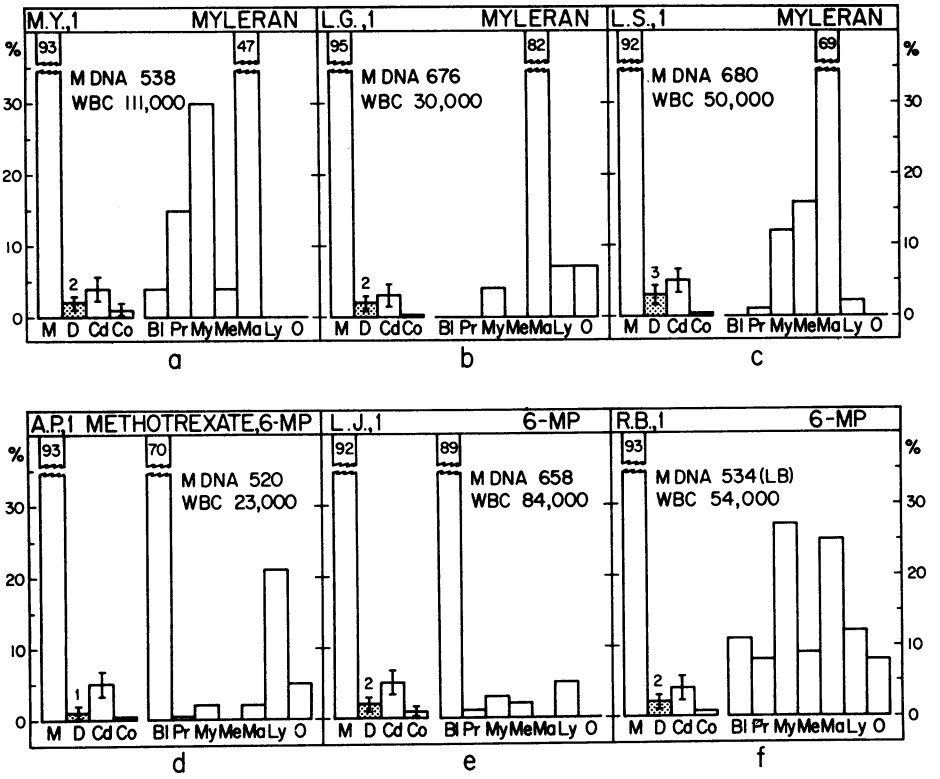


FIG. 5.—The distribution of complex forms in the M DNA obtained from three patients with chronic granulocytic leukemia (*a*, *b*, and *c*) and three patients with the acute form of the disease (*d*, *e*, and *f*) after chemotherapy. The symbols are defined in the legend for Fig. 2.

leukocyte alkaline phosphatase that is normally seen in this form of the disease was reported. The circular dimer frequency was 12 per cent. The Philadelphia (Ph′) chromosome was found in about half the bone marrow metaphase spreads of another chronic leukemic patient (J. W.) diagnosed as an early granulocytic leukemic. The M DNA in the peripheral leukocytes contained 1 per cent circular dimers.

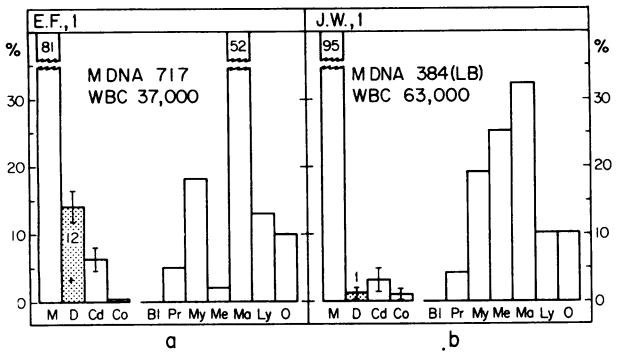


FIG. 6.—The distribution of complex forms in the M DNA obtained from untreated patients with (*a*) atypical and (*b*) early chronic granulocytic leukemia. The symbols are defined in the legend for Fig. 2.

Discussion.—Circular dimer M DNA is present in all the granulocytic leukemics that we have studied to date (Table 1). The highest frequency of circular dimers (48%) was found in a case of untreated acute granulocytic leukemia with leukocytes that contained 98 per cent myeloblasts. High frequencies of circular dimers (31 and 21%) were also observed in two cases of untreated chronic granulocytic leukemia with moderately immature leukocyte distributions. In the chronic leukemic patients with moderately mature leukocyte distributions, the frequency of circular dimers was lower: 14 per cent in an untreated case, 12 per cent in an atypical case, and 1 per cent in an early case. The above preliminary correlation of the circular dimer frequency with the immaturity of the leukocyte distribution suggests that the circular dimer frequency is related to the severity of the disease.

The effect of chemotherapy upon the circular dimer level in acute granulocytic leukemia may be dramatic: The level in two patients who had been under treatment for two weeks or less was 2 per cent, and in another, under chemotherapy for eight months, the level was 1 per cent. A drastic reduction in the circular dimer level must have occurred if it is assumed that the circular dimer levels in these patients were initially as high as in patient C. H. Serial studies are required to substantiate this reasoning.

The effect of chemotherapy upon the level of circular dimers in cases of chronic granulocytic leukemia was less pronounced. The circular dimer level was 2–3 per cent in three patients under long-term treatment with Myleran. It was 5–7 per cent in the M DNA from three patients who had received Myleran for periods of two to three weeks.

While the gene products of the M DNA in animal cells have not as yet been established, the DNA itself is known to be metabolically active. In HeLa cells it serves as a template for a rapidly labeled heterogeneous RNA found in the mitochondria and associated with the rough endoplasmic reticulum.[8, 9] The above observations, together with the report by Woodward and Munkres that a membrane structural protein is altered in a cytoplasmic mutant of *Neurospora*,[11] raise the possibility that membrane structural proteins are among the gene products of M DNA in animal cells. A defect in the regulation or the structure of such proteins in cells containing the circular dimer could contribute to the physiological properties characteristic of the neoplastic cells. A review of the evidence for a membrane theory of cancer has appeared recently.[11] At the present time we do not know whether the M DNA abnormality is an early event in the disease or a consequence of other events which change the physiology of the cell so as to permit the proliferation of the dimer M DNA form.

We noted earlier[12] that the formation of catenanes or circular dimers from circular monomers necessarily involves the cell's machinery for opening and closing the backbone chains of DNA. The various enzyme systems—enzymes, regulators, and enzyme sites—involved in DNA synthesis, recombination, and repair are thus candidates for the specific aberrations which result in the formation of the abnormal circular dimer M DNA.

Closed circular dimers and higher circular oligomers also occur as intracellular viral forms in infected bacteria[13] and animal cells,[14] and as plasmids and episomes

in bacteria.[15, 16] These simpler experimental systems may be expected to be useful in elucidating the detailed mechanism of the formation of dimers from monomers.[17, 18]

Abnormal M DNA represents the second kind of aberration to have been detected in the genetic apparatus of leukemic myeloid cells. A partially deleted chromosome, Ph′, is usually present in metaphase spreads of bone marrow cells from patients with chronic granulocytic leukemia. Aneuploidy often occurs in the cells of chronic granulocytic leukemic patients after the acute transformation of the disease has taken place,[19] and in the cells of patients with acute granulocytic leukemia. The relationship between the above chromosomal anomalies and the mitochondrial DNA anomaly is one of many problems raised by the results described in this communication.

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