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The Mechanism of Retinoic Acid Mediated Effects on Cell Growth and Differentiation

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Introduction

Retinoids (vitamin A) elicit many biological responses from cells both *in vivo* and *in vitro*. They have been shown to display distinct roles in differentiation of embryonal carcinoma (27,28), melanoma (20) and choriocarcinoma (5) cells *in vitro*. Additionally, they cause chemoprevention of cancer (2,10,14,26). It is generally believed that the biological action of retinoids is mediated by cellular retinoid binding proteins. Furthermore, a retinoic acid-binding protein in plasma membrane has also been described (7,25).

Relatively little is known about the mechanism of retinoid-induced cell differentiation. Induction of differentiation of human promyelocytic HL60 cells by retinoids, accompanied by an increase in the intracellular pH, has been described (19). Subsequently, the induction of NAD+-glycohydrolase (NADase) activity of a leukemia cell line and two human monoblast cell lines has been demonstrated (15). Recently, changes in intermediary purine metabolism which appeared to contribute to the regulation of terminal maturation in cells have also been reported (21). The concept that production of guanosine nucleotides and the activity of inosine monophosphate dehydrogenase (IMD) have an important role in regulating cell differentiation has been proposed (22). Finally, an association with growth arrest has been observed during retinoic acid-induced differentiation of F9 cells (9).

A temperature sensitive simian virus 40 (SV40) transformed fetal rat hepatocyte line (*RLA209-15*) exhibits a normal, differentiated phenotype when grown at 40°C. However, the maintenance of the transformed status is observed when grown at 33°C (4). Previous reports indicated that SV40 transformed mouse fibroblast cells were less sensitive to retinoic acid (17). Therefore, the *RLA209-15* cell line provides a more suitable model system to study the action mechanism of retinoids.

In this study we examined the effects of retinoic acid on cell growth, cell morphology and transmembrane electron transport activities of cells. The inhibition of growth and transmembrane electron transport activities is associated with a decrease in NAD+ pool which can lead to a fall of GTP formation (1,21). It has been proposed that a hypothetical cytoplasmic inducer for differentiation might exist in an uninduced state by binding to GTP (E. S. Golub, personal communication). According to this hypothesis, the fall of GTP concentration causes the dissociation of GTP from the inducer and allows the derepressed inducer to activate specific genes and therefore induce cell differentiation.

Materials and Methods

RLA209-15 fetal liver cells were cultured in α-modified minimal essential medium (α MEM, from Irvine Scientific Company, with arginine, supplemented with 0.4 mM orinthine), 100 μg/ml streptomycin, and 100 u./ml penicillin plus 4% fetal bovine serum and gassed with 5% CO_2 and 95% air as described previously (Chou and Schlegel-Haueter, 1981). After 3 days of culture at 33°C, some cells were cultured for 2 more days at 40°C to suppress transformation and cause a return to normal phenotype. The rest of cells remained at 33°C to maintain the transformed phenotype.

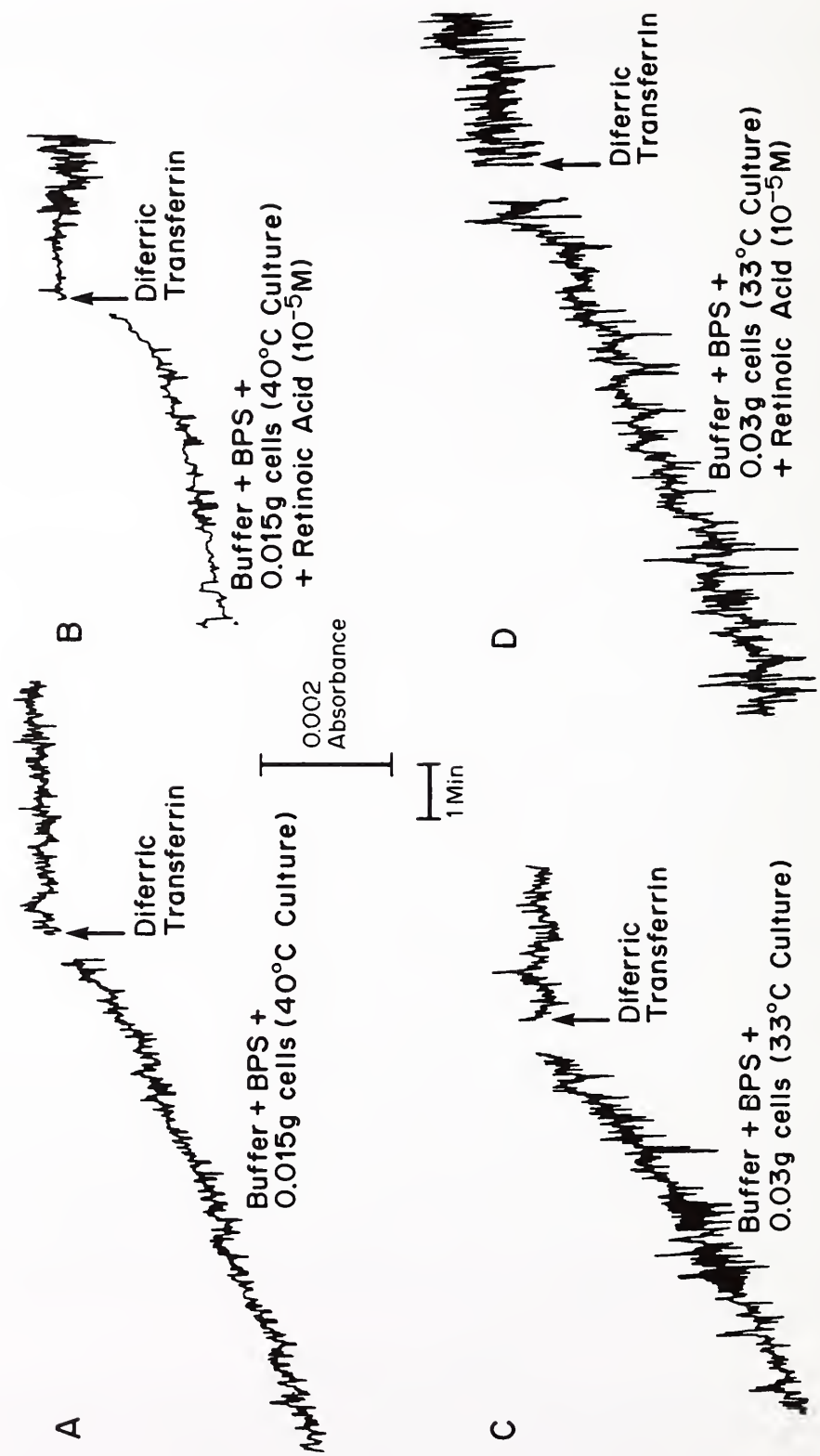

FIGURE 1. Spectrophotometer tracings of diferric transferrin reduction by transformed (33 °C) and non-transformed (40 °C) *RLA209-15* rat fetal liver cells. (A) 40 °C cells without retinoic acid; (B) 40 °C cells with retinoic acid (10^{-5}M); (C) 33 °C cells without retinoic acid and (D) 33 °C cells with retinoic acid (10^{-5}M). The reaction was run from right to left on the chart.

Cells were then harvested in the same media and washed in salts-Tris-EDTA buffer (140 mM NaCl, 2.5 mM KCl, 0.6 mM Na_2HPO_4, 25 mM Trizma base, and 0.05 mM EDTA, pH 7.4) (TD buffer). The pellet was resuspended in the same buffer to a final concentration of 0.1 gram wet weight per ml (gww/ml).

The effect of retinoids on cell growth was examined by growing cells in various concentrations of serum with and without retinoids. After 2 days of incubation at 37 °C, cells were harvested and a cell survival count was taken immediately. Survival was determined by an eosin Y exclusion method as described by Mitchell and Shrigi (24).

Assay of transmembrane electron transport activities was performed by measuring the reduction of iron in diferric transferrin (Fe_2Tf). The formation of ferrous bathophenanthroline sulfonate was determined according to Avron and Shavit (3). Absorbance change at 535 nm was subtracted from absorbance at 600 nm with the dual beam on the DW2a Aminco spectrophotometer. Extinction coefficient difference is 17.1 mM^{-1} cm^{-1}. The assay mixture in 2.8 ml TD buffer contained 3.4 μM diferric transferrin, 3.3 μM bathophenanthroline sulfonate and 0.015 gww of cells. Diferric transferrin with an absorbance ratio over 0.04 at 465:280 nm was prepared according to Karen and Mintz (18) or obtained from Miles Laboratories. Apotransferrin, used to prepare diferric transferrin, was from Sigma. Assay temperature was 37°.

The pyridine nucleotide pool was determined by extraction of NAD or NADH from cells with perchloric acid and alkali, respectively, as described previously (16). The extracted pyridine nucleotide was then quantitated using a cycling assay involving alcohol dehydrogenase (23).

Plasma membrane (PM) was isolated from rat liver as described by Goldenberg *et al.* (13). NADH diferric transferrin reductase activity with isolated PM (0.02-0.1 mg protein) was measured as described (33) or by following the oxidation of NADH (50 μM) in the presence of Fe_2Tf (3.4 μM) and 1 μM of KCN in 2.8 ml of 50 mM phosphate buffer (pH 7.2).

Results

The reduction of Fe_2Tf by cells is measured by the formation of a chelate, ferrous bathophenanthroline sulfonate in the external media since bathophenanthroline sulfonate is impermeable to cells. The spectrophotometer tracings of Fe_2Tf reduction by transformed (33 °C) and on-transformed (40 °C) *RLA209-15* rat fetal liver cells with and without all trans retinoic acid is shown in Figure 1. Retinoic acid does not inhibit the reduction rate by 33 °C transformed cells (Figure 2 and Figure 1D), however, a 67% inhibition of the reduction rate is observed with cells cultured at 40 °C (Figure 2 and Figure 1B). The inhibition is specific for all trans retinoic acid and is not observed with other retinoids such as retinol and retinyl acetate (Table 1).

RLA209-15 (40 °C) rat fetal liver cells are known to exhibit a differentiated phenotype (4). The alteration in the expression of liver functional proteins in the presence of retinoid has also been described (6). Most recently, the induction of F9 cell differentiation by retinoids accompanied by a growth arrest was reported (9). We therefore examined the growth effect of retinoids on these cells. Growth inhibition caused by retinoic acid is shown in 40° cultures only but not in 33 °C cultures as indicated by the effect of retinoic acid on cell growth (Table 2). It should be noted in this context that growth stimulation as a result of the activation of transmembrane electron transport activities (11,29,31) and growth inhibition due to the inhibition of this activity by drugs (8,30) has been demonstrated. Since retinoic acid selectivity interferes with Fe_2Tf reduction of the normal phenotype and not of the transformed cells, it is not surprising that retinoic acid causes growth arrest of the normal cells but has no effect on growth of the transformed cells. A correlated pattern of inhibition

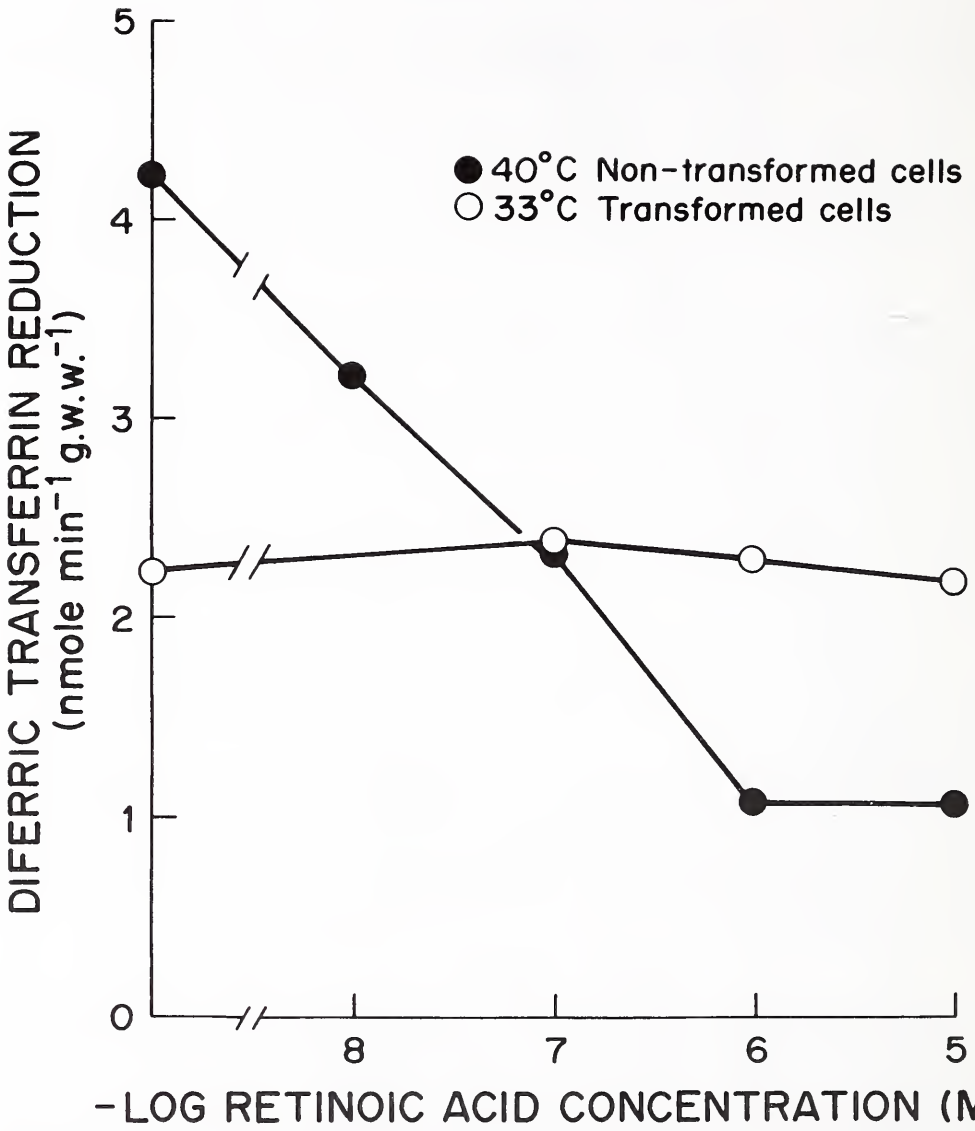

FIGURE 2. Effect of retinoic acid on diferric transferrin reduction by *RLA209-15*. Cells were grown in 33 °C and 40 °C incubator separately for 48 hr. Cells were fed fresh media containing retinoic acid (concentration as indicated on Fig. 1) daily.

TABLE 1. The Effect of Some Retinoids on the Reduction of Diferric Transferrin by Fetal Rat Liver Cells Infected with a Temperature Sensitve SV40 Virus (*RLA209-15* Cells).

Compounds	Fe_2Tf Reduction (nmoles $min^{-1}gww^{-1}$)	
	40 °C cells	33 °C cells
None	5.7	2.2
Retinoic Acid (10^{-6}M)	2.8	2.3
Retinoic Acid (10^{-5}M)	0.2	2.3
Retinol (10^{-6}M)	4.4	2.2
Retinol (10^{-5}M)	4.1	1.9
Retinyl Acetate (10^{-6}M)	4.8	--
Retinyl Acetate (10^{-5}M)	3.0	1.4

Representative of three different experiments with different batches of cells for retinoic acid inhibition.

TABLE 2. Effect of Retinoic Acid on the Growth of Fetal Rat Liver Cells Infected with a Temperature Sensitive SV40 Virus (*RLA209-15* Cells).

Calf Serum Volume Percent	Number of cells ($4\ 10^{-5}$) / 25 cm² flask			
	40°C Cells		33°C Cells	
	Without RA	With RA (10^{-5})	Without RA	With RA (10^{-5}M)
0	1.13	0.71	1.37	1.41
2.5	4.04	2.93	3.64	2.87
5	4.15	3.04	5.15	5.58
7.5	4.17	3.27	5.41	5.56
10	4.43	3.25	5.68	6.25

RA = retinoic acid

of Fe_2Tf reduction and growth inhibition in response to retinoic acid is shown between 40°C and 33°C cultures. The morphological study also shows that retinoic acid induces giant cell formation in 40°C cultures but no significant morphological changes in 33°C cultures (Figure 3). There is no morphological differences between the untreated cultures of 33°C cells and 40°C cells.

The consequence of the inhibition of electron transport should be a decrease in NAD^+. Therefore, the NAD^+ concentratiuon in 40°C and 33°C cells was determined at various times after treatment with retinoic acid. The results are seen in Table 3. It can be seen that the NAD^+ levels in 40°C cultures decrease nearly 50% whereas 33°C cultures show an insignificant change over the 36 hour period of treatment. The reduction rate of Fe_2Tf was also compared with the same batch of cells. A correlated inhibitory pattern is seen between NAD^+ level and Fe_2Tf reduction in

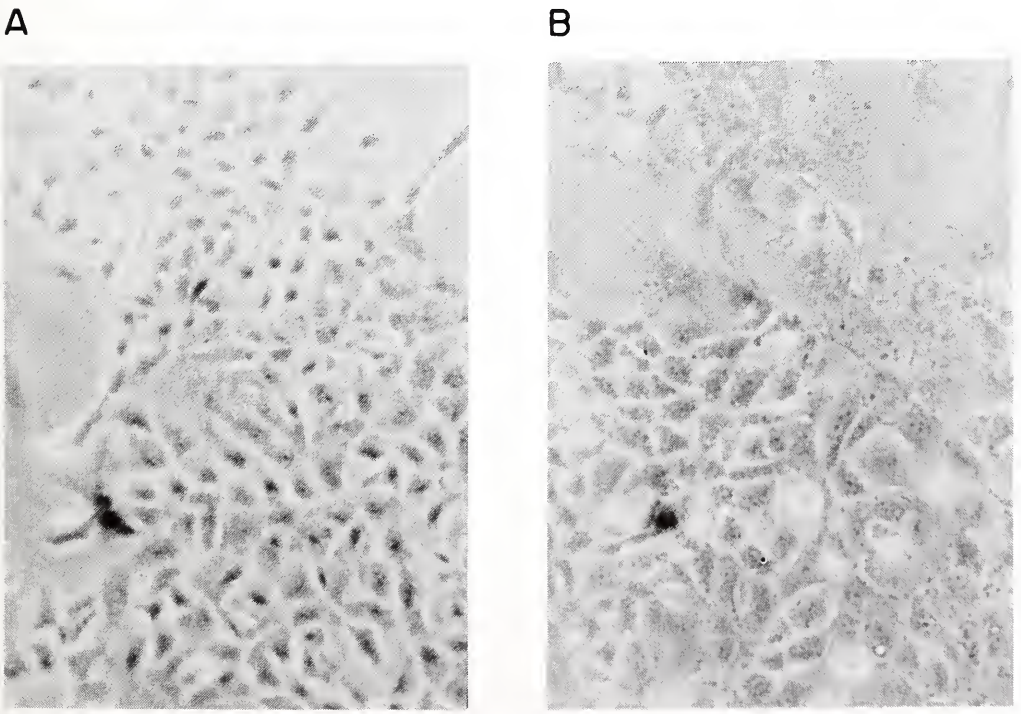

FIGURE 3. Effect of retinoic acid on the morphology of 33°C cultures and 40°C cultures. (A) 33°C cultures; (B) 40°C cultures. Cells were grown in media containing retinoic acid (10^{-5}M) for four consecutive days.

TABLE 3. Effect of Retinoic Acid on NAD+ Level and Diferric Transferrin Reduction with Fetal Rat Liver Cells Infected with Temperature Sensitive SV40 Virus (*RLA209-15* Cells).

Hour of Treatment	NAD+ (pmole/mg protein)		Fe_2Tf Reduction (nmole $min^{-1}gww^{-1}$)	
	40°C Cells	33°C Cells	40°C Cells	33°C cells
0	110.5	38.2	20.7	9.0
6	96.6	39.6	16.2	9.1
12	69.1	35.5	10.9	9.0
24	55.1	36.3	5.2	9.1
36	52.4	33.9	4.1	5.7

Concentration of retinoic acid used in the experiment is 1×10^{-5}M. The SV40 virus is expressed in the cells cultured at 33°C but not in the cells at 40°C.

40°C cells. Essentially, the reduction rate of Fe_2Tf is not changed in 33°C cells over 24 hours.

The effect of retinoic acid was also tested on NADH-diferric transferrin reductase activity of the purified rat liver plasma membrane. The assay can be performed either by measuring NADH oxidation or by measuring the formation of ferrous bathophenanthroline chelate after the addition of Fe_2Tf. The results are shown in Table 4. Both assay methods show a strong inhibition of enzyme activity by retinotic acid. We therefore believe that the mechanism for retinoic acid action begins at the plasma membrane.

Discussion

The evidence that transplasma-membrane redox systems are closely related to cell growth and development has been demonstrated previously (8, 11, 29-31). It is reasonable to consider that retinoic acid may inhibit cell growth due to its effect on redox systems. The variation in sensitivity to retinoic acid between 40°C cell cultures and 33°C cultures in this study is similar to differences in sensitivity to retinoic acid observed with mouse fibroblast cells (3T3 cells versus virus-transformed 3T3 SV cells) (17) in which an inhibition of exponential cell growth and a reduction in saturation density were compared.

Since we have shown that retinoic acid inhibits the redox system in purified plasma membrane from rat liver the site of action of retinoic acid can be at the

TABLE 4. Effect of Retinoic Acid on NADH-Diferric Transferrin Reductase of Rat Liver Plasma Membrane.

Assay Procedure for Measuring Reductase Activity	Addition	% of Control Activity
Ferrous-bethophenanthroline chelate formation	None (control)	100
	RA (10^{-7}M)	50
	RA (10^{-6}M)	29
	RA (10^{-5}M)	13
NADH oxidation stimulated by diferric transferrin	None	100
	RA (10^{-7}M)	62
	RA (10^{-6}M)	45
	RA (10^{-5}M)	42

RA = retinoic acid.

membrane (32). This is consistent with the fact that retinoic acid can induce cell differentiation even when immobilized outside the cell (34).

According to Chou's study (4), the temperature-sensitive rat fetal liver cell line, *RLA209-15* exhibited a normal differentiated phenotype characterized by increased levels of both α-fetoprotein (AFP) and albumin when grown at the nonpermissive temperature 40°C (4). The ratio of these functional liver proteins was altered, and the biosynthesis of the polypeptide chain of AFP was modified during treatment with retinoids (6). However, the synthesis of both functional proteins was inhibited following transformation by growing cells at the permissive temperature (33°C). In addition to these differences, our results indicate an alteration of the NAD^+ pool of non-transformed cells, but not of transformed cells, following the treatment with retinoic acid. The decrease in the NAD^+ pool of 40°C cultures can be a consequence of the inhibition of electron transport activity. The fall in NAD^+ level could act to down regulate inosine monophosphate dehydrogenase (IMPD), a key enzyme in purine biosynthesis. IMPD is NAD^+-dependent and very sensitive to changes of concentration of NAD^+ (1) and therefore a fall in GTP level can be a result of a decrease in NAD^+ (E. S. Golub, personal communication).

Studies in microbial systems showed inhibition of IMPD induced differentiation (sporulation) in both *B. subtilis* and yeast (12). It has been postulated that the fall in GTP derepresses an inducer molecule which then binds to methylated DNA and results in new gene transcription (E. S. Golub, personal communication). Additionally, Lucas and his coworkers showed with mammalian systems that a fall in GTP level is a first consequence of retinoic acid induction of differentiation (21). Based on these reports plus our studies, we postulate that retinoic acid reacts with a receptor molecule or a binding protein in the membrane which then reacts with a cytoplasmic inducer molecule which is in the uninduced state due to the repression by being bound to GTP. The dissociation of GTP from the inducer takes place when the fall of GTP occurs and therefore allows the now derepressed inducer to activate the specific genes and induce cell differentiation.

Since both our transformed and non-transformed cells are derived from the same clone of *RLA209-15* cells, the different response should not be due to differences in the uptake, metabolism and intracellular fate of retinoids in the cells. This system provides a useful tool for examining the mechanism of retinoic acid action in differentiation.

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