Human Lupus Inclusions and Interferon

Abstract. *Raji cells, a human B lymphoblastoid cell line of Burkitt lymphoma origin, formed lupus inclusions when grown in a medium conditioned by the growth of Raji cells whose DNA thymidine residues had been unifilarly (single-strandedly) substituted with bromodeoxyuridine. Ultracentrifugation of this medium in excess of that required to remove Epstein-Barr virus and all other known mammalian viruses did not prevent the formation of the inclusions, and treatment of the conditioned medium with pronase destroyed the activity. These results demonstrate the presence of a protein that is secreted from bromodeoxyuridine-substituted Raji cells and is capable of inducing nonbromodeoxyuridine-substituted cells to form lupus inclusions. Interferon (100 units per milliliter) was found in the conditioned medium. Inclusions also formed in Raji cells grown in fresh medium supplemented with human leukocyte or fibroblast interferon (100 units per milliliter).*

Lupus inclusions (Fig. 1) are abnormal microtubular structures that are found in reticuloendothelial cells of humans with certain diseases. Ultramorphologically the inclusions appear virus-like; they have remained a curiosity to clinicians and clinical pathologists since they were first observed by electron microscopy in the 1960's [(*1*, *2*); see (*3*) for a review]. They are found in cells associated primarily with the reticuloendothelial system in several disease conditions, including autoimmune diseases (*2*, *4*), neoplasias (*5*), and immunodeficiency disorders (*6*). In systemic lupus erythematosus they are an important diagnostic aid because they are detected in the glomerular endothelium of almost all patients and in the peripheral blood lymphocytes of more than two thirds (*4*) (Fig. 1A). It is possible experimentally to induce in certain human lymphoid cell lines structures that are ultramorphologically and ultracytochemically identical to lupus inclusions. These structures are induced by substituting 5-bromo-2′-deoxyuridine (BrdU) or 5-iodo-2′-deoxyuridine for thymidine residues in the cells' DNA (*3*, *7*).

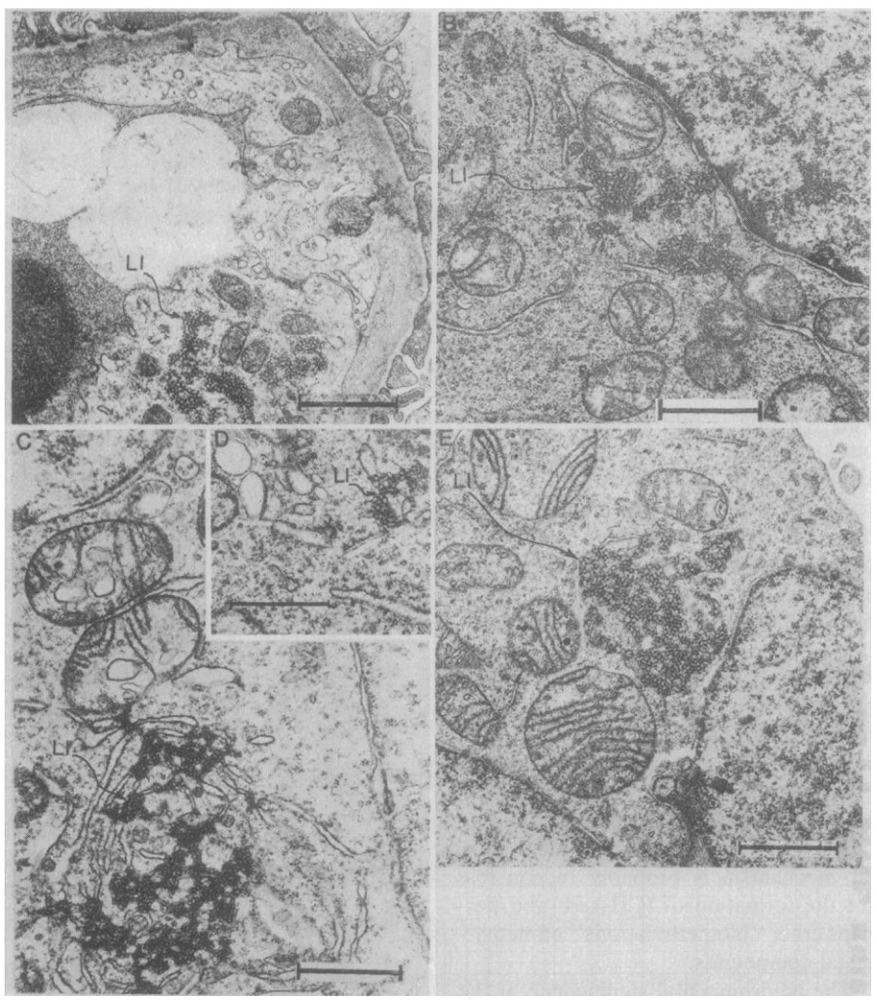

Fig. 1. Electron micrographs of lupus inclusions (*LI*) in thin sections (0.1 μm) of (A) human kidney biopsy from a patient with systemic lupus erythematosus and (B) Raji cells 70 hours after initiation of incorporation of BrdU into the cell's DNA, and in Raji cells grown under the following conditions: (C) 70 hours in conditioned medium, (D) 21 hours in conditioned medium, and (E) 70 hours in fresh medium containing 100 units of human leukocyte interferon per milliliter.

Lupus inclusions have never been purified and are not classically viral in nature (*8*). Most reports about them consist of electron microscopic and ultracytochemical observations, from which one can only infer biological and biochemical information. In the few studies of induction of lupus inclusions in human lymphoblastoid cell lines, the cells have died during and after the appearance of the inclusions (*7*). From the information available several workers have proposed that lupus inclusions are of viral origin (*2*, *4*), are insignificant markers for cellular injury (*8*), or are a key cell antigen that is intimately linked to the expression of autoimmune disease (*2*).

I have recently (*9*) obtained evidence pertinent to the biological nature of these inclusions. Raji cells, a human β-lymphoblastoid cell line originally derived from an African Burkitt lymphoma, were treated in the exponential growth phase (doubling time, 19 to 22 hours) with 65 μ*M* BrdU for one population doubling (initial density, 2×10^5 cells per milliliter); 70 hours later these cells formed lupus inclusions (Fig. 1B) (*10*). Each cell formed an average of one such inclusion, 2 μm in diameter (Table 1). At 70 hours the cell population was typically 95 percent viable but entered a cytostatic-cytotoxic phase, which resulted in an accumulation of approximately 50 percent nonviable cells within 120 hours (Fig. 2). By 330 hours the culture had returned to logarithmic growth, and more than 95 percent of the cells were viable. The lupus inclusions were no longer present. Recovered cells treated a second and third time with BrdU followed a similar growth pattern each time.

These observations showed that the cells were genetically stable with respect to their response to BrdU incorporation and the consequent transient formation of lupus inclusions. I then sought to explain the relation between the inclusions and the cytostatic-cytotoxic phase and the mode of BrdU action that causes cells to form lupus inclusions.

Raji cells not treated with BrdU readily formed lupus inclusions when grown in a medium conditioned by BrdU-treat-

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ed cells. Raji cells (2.0×10^5 per milliliter) whose DNA had been unifilarly (single-strandedly) substituted with BrdU were grown to the cytostatic-cytotoxic phase (22 to 70 hours) in fresh medium (*10*) (Fig. 2). The cells were removed by centrifugation (800*g*, 10 minutes). Residual cells and any bacteria that might have been present were removed by sterilization filtration (0.2 μm). Non-BrdU-treated cells that were added to this conditioned medium (0.25×10^5 cells per milliliter) and grown for 70 hours formed lupus inclusions with the same frequency and size as BrdU-treated cells (Fig. 1C; Table 1). At 21 hours only small inclusions of less than 1.0 μm were observed in these cells (Fig. 1D). In this BrdU induction system lupus inclusions were detected no earlier than 36 hours.

Measurement of [^{3}H]BrdU showed that only 0.22 μ*M* BrdU had been secreted into the conditioned medium (*11*). Since continuous treatment of Raji cells with up to 8 μ*M* BrdU does not induce production of lupus inclusions (*7*), the secreted BrdU, if in the form of a nucleotide, could not explain the induction of the inclusions. That the BrdU was related to their formation would be shown, however, if it was incorporated into virus particles that had been secreted into the conditioned medium and if these virus particles triggered inclusion formation. Raji cells contain approximately 50 Epstein-Barr virus (EBV) genome equivalents (*12*), and BrdU substitution activates the expression of EBV cell antigens (*13*). When non-BrdU-substituted cells were grown in conditioned medium which had been centrifuged at 100,000*g* for 60 minutes and sterilized by filtration as above, they grew normally and formed lupus inclusions by 70 hours (Table 1). Thus the etiologic agent was in the soluble fraction and EBV and all other known mammalian viruses were ruled out.

Conditioned medium was then treated with charcoal in excess of that required to remove nucleotides, including the residual BrdU (*14*). To enable cells to grow normally, this medium was supplemented with 2 percent (by volume) of a 50-fold concentrate of vitamins and amino acids (RPMI 1640) (*10*) and 2.5 percent (by volume) fetal calf serum (FCS). When non-BrdU-substituted cells were grown in this medium, inclusions appeared and grew as in the untreated conditioned medium (Table 1). Heating the conditioned medium at 56°C for 30 minutes, which would destroy weak nucleoprotein complexes, did not prevent formation of the inclusions; however, after 30 minutes at 100°C the conditioned

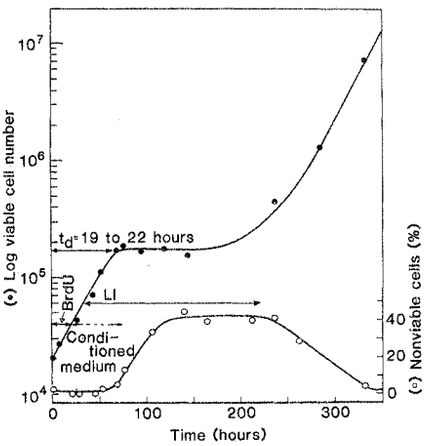

Fig. 2. Cumulative exponential growth curve of Raji cells treated with 65 μ*M* BrdU for a single mass doubling of the culture (0 to 22 hours), washed, and resuspended in fresh medium. Figure is a composite of data from three separate inductions (t_d, doubling time).

medium no longer supported their formation. This suggested that the inducer was a heat-labile protein.

The protein nature of the inducer of lupus inclusions was confirmed by showing that its activity is destroyed by digestion with pronase. Conditioned medium prepared with 1 percent FCS was digested with pronase (40 μg/ml) and then supplemented with 10 percent FCS. Raji cells did not contain inclusions after 70 hours of growth in this medium (doubling time, 18 hours, < 5 percent nonviable cells) (Table 1). However, lupus inclusions formed in Raji cells grown in a portion of the same medium treated identically except for the omission of pronase. Neither ribonuclease nor deoxyribonuclease destroyed the inducer activity. Inclusions formed when non-BrdU-substituted cells were grown in conditioned medium diluted with an equal volume of fresh medium, but not at 1/10 or 1/100 of the initial concentration. Thus a critical concentration of inducer protein is required, and the components of the fresh medium do not inhibit inclusion formation. The absence of inclusions in Raji cells grown in the conditioned medium digested with pronase indicates that the inducer was degraded to an activity less than this critical concentration.

These physiochemical characteristics of the human lymphoblastoid secretory protein are consistent with those of the lymphokine interferon (*15*). To characterize it further, portions of conditioned medium were adjusted to *p*H values between 2.0 and 11.5 (Table 1). The inducer activity, as measured by the frequency of inclusions in Raji cells, was insensitive to a *p*H of 2.0. Elution from Sephadex G-100 gave an estimated molecular weight of slightly greater than 25,000.

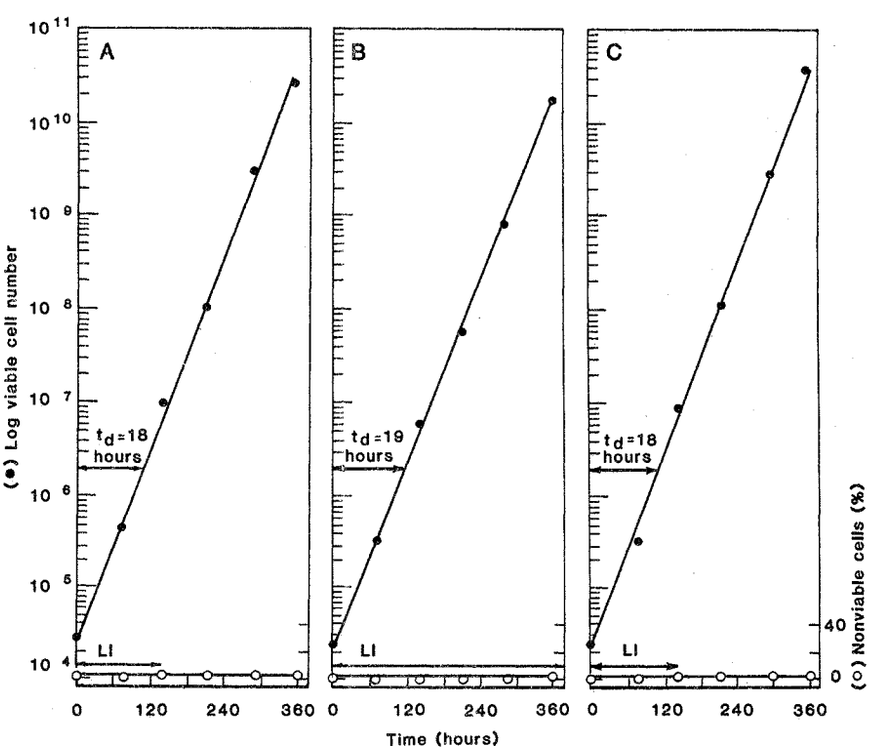

Fig. 3. Lupus inclusions were induced in Raji cells by growth for 70 hours in conditioned medium. At this time (0 hours) portions of the cells were resuspended in fresh medium (A) alone, (B) mixed equally with conditioned medium, or (C) mixed equally with spent conditioned medium.

Direct measurement of interferon in conditioned medium by a human-foreskin-fibroblast encephalomyocarditis-virus assay showed a concentration of approximately 100 U/ml. Addition of either human fibroblast or human leukocyte interferon to complete medium at this concentration (10^6 and 10^7 units per milligram of protein respectively) triggered the formation of lupus inclusions in Raji cells (Fig. 1E and Table 1), indicating that the protein secretory inducer is a type I interferon (*15*).

Cells that contained lupus inclusions after growth in the conditioned medium for 70 hours maintained exponential growth (doubling time, 18 hours, < 5 percent nonviable cells) with no cytostatic-cytotoxic phase (Fig. 3). At intervals of 70 hours these cells were resuspended to a density of 0.25×10^5 per milliliter in fresh medium or an equal mixture of fresh medium and either spent medium (removed from the flask at the time of resuspension) or conditioned medium. About 140 hours after inclusions were induced by growth in the conditioned medium, the cells grown in fresh medium and in the spent-medium mixture no longer contained inclusions, whereas the cells grown in the conditioned-medium mixture continued to form them. Doubling time and viability were not affected. The presence of these inclusions in cells that maintained normal cell growth shows that the inclusions are not an insignificant feature of dying (*7*) or injured (*8*) cells.

I conclude from these results that unifilar substitution of BrdU into the DNA of Raji cells induces the synthesis and secretion of interferon, which causes the synthesis and formation of lupus inclusions. Three other studies have reported the production of interferon by BrdU-substituted Raji cells (*16*). However, to my knowledge no one has previously shown that the formation of ultrastructures—that is, lupus inclusions—is either mediated by interferon production in these cells or stimulated by exogenous human fibroblast or leukocyte interferon.

The cell action of synthesizing lupus inclusions does not in itself lead to the continued synthesis of inclusions. Cessation of inclusion synthesis in BrdU-substituted cells appears to result from the fact that the synthesis of interferon is transient (*16*). This is consistent with the nature of unifilar substitution of BrdU into DNA, which does not cause a mutation (*17*).

The transient appearance of lupus inclusions in Raji cells after BrdU substitution and in non-BrdU-treated cells grown in conditioned medium or interferon may provide an experimental parallel to the presence of lupus inclusions in patients and to the transient appearance of these ultrastructures in newborns of mothers who have systemic lupus erythematosus or who are positive for rheumatoid factor (*18*). Recent reports of type I and type II interferons in lupus patients and in patients with related autoimmune disorders (*19*) parallel earlier reports of lupus inclusions in cells of these patients (*3*). It thus appears that interferon triggers the formation of these inclusions in vivo in a manner similar to that observed in vitro. It is unknown whether type II interferon also triggers their formation or whether the simultaneous presence of interferons I and II has a synergistic effect. The genetic range of susceptibility to induction of lupus inclusions by interferon is also unknown.

The action of interferon and the formation of lupus inclusions in patients with lupus and other disease states are not restricted to B cells. I have induced lupus inclusions in seven human lymphoblastoid cell lines, two of which are T cell lines (*20*). Neither T cell line has DNA of the EBV genome, and therefore a unique association of lupus inclusions with EBV is excluded (*21*). The possibility that induction of lupus inclusions in T and B cells by interferon in susceptible individuals has a pathologic effect on the

Table 1. Percentages of lupus inclusions in 400 cell sections of Raji cells after 70 hours of growth in the media listed. Duplicate samples of cells were fixed in 3 percent glutaraldehyde, postfixed in osmium tetroxide, embedded in Epon, and sectioned with an ultramicrotome. Thin sections (0.1 μm) were prepared from several blocks at levels of tissue separated by greater than 20 μm, the diameter of the cells to be examined. This approach approximated random sampling. Our statistical estimation of the frequency of inclusions in human lymphoma cells was based on a binomial model. The ratio of the diameter of the inclusions (2 μm) to the whole cell (20 μm) indicates a 10 percent probability (*d*) of seeing an inclusion in a random thin section of a cell that contains an inclusion. Factoring in the probability that an inclusion exists in the cell (*P*) gives a probability of observing inclusions in a cell population (P^*) of $P^* = dP$. A finding of no inclusions in *n* cell sections gives an upper 95 percent confidence limit on *P* of $\hat{P}_u^*/d$ where $\hat{P}_u^* = 1 - (.05)^{n-1}$. More than 15,000 sections of control cells have been examined without finding even one inclusion. This gives an upper limit on *P* of one inclusion in 500 cells at 95 percent confidence. Cells induced with BrdU, full-strength conditioned medium, or 100 units of interferon per millimeter have inclusions at frequencies (7 to 17 percent) consistent with one inclusion per cell.

Growth medium and conditions	Lupus inclusions (%)	Growth medium and conditions	Lupus inclusions (%)
Fresh medium		Conditioned medium (20°C, 1 hour)	
No BrdU	0.0	*p*H 2.0	17.0
BrdU (65 μ*M*)*	13.5	*p*H 4.0	12.5
Conditioned medium		*p*H 7.0	14.0
Ultrafiltered	12.5	*p*H 8.5	1.0
Supernatant (100,000*g*, 1 hour)	14.0	*p*H 10.0	3.0
Charcoal†	7.5	*p*H 11.5	5.5
Heat-treated		Conditioned medium, chromatographic fractions§	
56°C, 30 minutes	7.0	Sephadex G-25	7.5
100°C, 30 minutes	0.0	Sephadex G-100	
Diluted		1 to 33	0.0
1/2 initial concentration	6.0	34 to 66	0.0
1/10 initial concentration	0.0	67 to 99‖	4.5
1/100 initial concentration	0.0	100 to 133	7.0
Conditioned medium plus 1 percent FCS		134 to 166	0.0
Ultrafiltered	7.0	167 to 200	0.0
Heat-treated (37°C, 48 hours)	7.5	Fresh medium plus interferon (100 U/ml)	
Supplemented with enzyme (37°C, 48 hours)‡		Human fibroblast	7.0
Pronase (40 μg/ml)	0.0	Human leukocyte (two preparations)	10.5, 12.5
Ribonuclease (40 μg/ml)	6.5		
Deoxyribonuclease (40 μg/ml)	5.0		

*Cells were grown in 65 μ*M* BrdU for one population doubling (22 hours) and then in fresh medium for an additional 48 hours. † See (*14*). ‡ Plus 10 percent (by volume) FCS and 1 percent (by volume) of a 50-fold concentrate of vitamins and amino acids (RPMI 1640). §Six 100-ml portions of conditioned medium were chromatographed on a Sephadex G-25 column (90 by 2.5 cm; equilibrated with 0.1 ionic strength RPMI 1640 buffer), lyophilized, and then solubilized by adding 60 ml of glass-distilled water. Twenty milliliters were diluted to 100 ml with fresh medium, and the remaining 40 ml were chromatographed on a Sephadex G-100 column (90 by 2.5 cm) equilibrated with RPMI 1640 buffer (full ionic strength). Fractions (3 ml) were pooled as indicated and supplemented with 10 percent (by volume) FCS and 2 percent (by volume) of a 50-fold concentrate of amino acids and vitamins (RPMI 1640). Raji cells were grown in each pool for 70 hours and examined for inclusions. ‖Elution of molecular weight standards provided an estimate of 25,000 for proteins eluting at fraction 99.

equilibrium network of their immune system (*22*), leading to autoimmunity or neoplasia, is intriguing but experimentally undetermined.

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11. The BrdU concentration in the conditioned medium was calculated as (disintegrations per minute of ^3H in 100 μl of conditioned medium divided by the disintegrations per minute of ^3H in 100 μl of 65 μ*M* BrdU) × 65 μ*M*. In this study the precise calculation was (630/183,800) × 65 μ*M* = 0.22 μ*M* BrdU.
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Genetic Studies of an Acardiac Monster: Evidence of Polar Body Twinning in Man

Abstract. *Two maternally derived chromosome sets and both maternal histocompatibility antigen haplotypes were identified in the tissues of a malformed triploid acardiac twin that developed within the same chorion as its normal twin. These findings indicate that the twins arose as a result of independent fertilizations, by two different spermatozoa, of a normal haploid ovum and its diploid first-meiotic-division polar body.*

Since the middle of the 19th century, twins have been categorized as either monozygotic or dizygotic. Monozygotic or "identical" twins arise from division of a single fertilized ovum and possess identical sets of nuclear genes. Dizygotic or "fraternal" twins are the products of simultaneous fertilizations, by different spermatozoa, of two independently released ova and are genetically no more alike than full sibs (*1*). Other types of twins may result from superfecundation (fertilization of ova by separate fathers during the same menstrual cycle), superfetation (sequential conceptions during successive menstrual cycles), fertilizations of separate ova from the same corpus luteum, or simultaneous fertilization of meiotic products of the same primary oocyte, such as an ovum and its first or second polar body (*2*). Evidence for the last mechanism emerged from the following case.

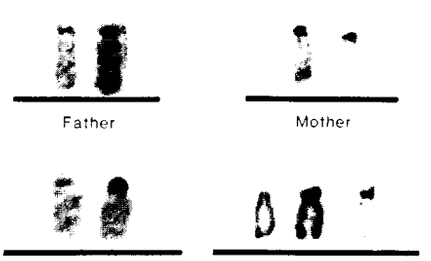

Fig. 1. Photograph of chromosomes 13 stained to show NOR heteromorphisms in the parents and twins. There is no visible silver staining at the NOR of either paternal chromosome 13, whereas there is positive NOR staining on both maternal chromosomes 13. Twin A has one chromosome 13 with positive staining and one without staining, as expected. In the cells of the triploid acardiac twin, two of the three chromosomes 13 show positive NOR staining, indicating that they are maternal in origin.

A 22-year-old primiparous black female gave birth to a normal male and his grossly malformed twin. Dissection of the malformed twin revealed holoacardius amorphus, an aberration of normal development that is found only in the twinning process (*3*). The twin placenta was monochorionic and diamniotic, with extensive vascular anastomoses between the small region supplying the acardius and the much larger region supporting the normal twin. All previously reported cases of human acardia have involved one member of a monochorionic twin pair, the single chorion being considered proof of monozygosity (*4*).

Lymphocytes from both parents and the normal twin, and skin and gut fibroblasts from the acardius, were grown by standard cell culture techniques (*5*). Chromosomes were studied after trypsin-Giemsa banding (*6*). The parents were cytogenetically normal, and the unaffected twin had a normal male (XY) karyotype. The acardiac twin had an essentially triploid XXX karyotype (*7*). Such sex discordance, remarkable in the presence of a single chorion, implied that the twins were derived from separate conceptional events involving different spermatozoa (*8*).

Additional genetic marker studies were performed to determine the parental origin of the extra haploid chromosome set in the triploid acardiac twin.

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