INDUCTION OF NUCLEAR ENVELOPES
AROUND METAPHASE CHROMOSOMES
AFTER FUSION WITH INTERPHASE CELLS

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ABSTRACT
The process of cellular fusion induced by Sendai virus in Chinese hamster cells (Don line)
afforded us the opportunity to study nuclear envelope formation around metaphase sets in
the presence of interphase nuclei, when chromosome pulverization failed to occur in such
multinucleate cells. Morphologically, the enveloped metaphase chromosomes resembled a
normal telophase nucleus, though minor differences prompted us to call it telophase-like.
Electron microscopic observations demonstrated that the membranes enveloping the
chromosomes appeared to be identical with a normal nuclear envelope. The longer the cells
were incubated with Colcemid before fusion, the higher was the number of cells with
telophase-like nuclei and the lower the percentage of cells with pulverizations. Furthermore,
the number of pulverizations bore a somewhat direct relationship to the ratio of metaphase
to interphase nuclei in multinucleate cells, and the number of telophase-like nuclei was
inversely proportional to this ratio. A hypothesis is advanced in which a balance between
the activities of a chromosome pulverization factor and a nuclear envelope formation factor,
the former in metaphase cells and the latter in interphase cells, is decisive as to the nature of
morphologic events observed in virus-induced fused cells.

INTRODUCTION
Ever since Okada et al. (29, 30) demonstrated that Sendai (HVJ) virus induced the rapid formation
of multinucleate cells in suspension, the virus has been used as a tool to study somatic cell
hybrids and heterokaryons (6, 10, 22, 38) and to study internuclear relationships in multinucleate
cells (10, 12, 14, 17, 32). In addition, extensive structural modifications of chromatin were found
to take place in multinucleate cells after treatment with a number of viruses including Sendai
virus (4, 5, 28, 34). Certain of these modifications (36) were originally termed chromosome pulverization
by Nichols et al. (27). Earlier studies from this laboratory, dealing with Chinese hamster
cells (line Don) and Sendai virus, showed that cellular fusion resulting from the viral treatment
is a prerequisite for induction of the chromosome pulverization (14, 20, 21, 37). It was further
observed that nuclei in any interphase stage of the cell cycle could be pulverized if they coexisted, as
a result of virus-induced cellular fusion, with at least one mitotic nucleus (18, 36, 37).

During the course of study on the virus-induced chromosome pulverization, we have found that
prolonged exposure of Don cells to Colcemid before fusion produced a significant number of fused cells in which the interphase nuclei were not
pulverized, despite the presence of a “metaphase
nucleus in the same cell. In these cells, observed in the light microscope, the chromosomes were found to be enclosed within an unusual baglike structure. This structure, which resembled a telophase nucleus, was found almost exclusively in fused cells that included at least one interphase nucleus. To our knowledge it represents a previously undescribed event in virus-fused cells distinct from chromosome pulverization. The detailed studies of this new phenomenon are presented in this paper.

MATERIALS AND METHODS

The pseudodiploid cell line of Chinese hamster Don cells grown in a medium consisting of Roswell Park Memorial Institute No. 1640 (25) supplemented with 20% calf serum was used throughout the experiments.

Intact or UV-inactivated Sendai virus, suitably diluted in glucose-free Hank's solution, was used to induce cellular fusion. Methods for inactivation of the virus and the preparation of virus stock were described in a previous paper (20).

Procedures for cell fusion and for slide preparation were essentially the same as those described previously (14). Cell suspensions from either log-phase monolayer or spinner cultures were exposed to Colcemid (0.08 µg/ml) for varying periods of time at 37°C. They were then treated with the virus at a dose of 1000 hemagglutinating units (HAU)/ml for 10 min before the incubation at 37°C and subsequent dilution. For light microscope studies, the cells were recovered by brief low-speed centrifugation at room temperature and suspended in 0.5% sodium citrate (16) for 10 min at room temperature, and fixed with acetic acid–methanol (1:3) solution. The cells were then spread on slides so as to leave their cytoplasm intact and stained with Giemsa's. When it was necessary to examine cells without the hypotonic citrate treatment, conventional orcein squash preparations were made. In some cases, the virus-cell suspension was directly fixed with the above acetic acid–methanol solution, air-dried on slides, and stained with Giemsa's.

RESULTS

Telophase-Like Nuclei in Fused Cells

When Colcemid was added to log-phase cultures and the cells were examined several hours later, typical "ball" metaphases (8, 9, 23) were observed, as illustrated in Fig. 1. When the culture was supplemented with virus, the ball metaphases persisted in the fused cells (Fig. 2). These were the observations when the cells were not subjected to the hypotonic citrate treatment (Materials and Methods). After treatment with hypotonic citrate, it was found that not all of the fused cells, which included both interphase and mitotic nuclei, showed chromosome pulverization; the interphase nucleus apparently remained intact. In most of such cells, the chromosomes were contained in a baglike structure (Figs. 3–5). The chromosomes in this structure were characterized by a thinner and more slender appearance than normal metaphase chromosomes. This structure resembled the nucleus of a normal telophase cell; however, the number of chromatids it contained was larger than that expected for a telophase nucleus, in which the chromatid number should be in the diploid range since the unfused cell is pseudodiploid. An additional difference from normal telophase was that in most cases the chromatids remained clearly paired (Figs. 3 and 4). For convenience, we call this nucleus TLN (telophase-like).

For electron microscope examinations, the sedimented cells obtained by low-speed centrifugation of the cell-virus preparation were fixed for 20 min in 5% glutaraldehyde buffered with 0.1 M phosphate buffer, washed several times with the same buffer, and post-fixed for 1 hr in 1% osmium tetroxide buffered with 0.1 M phosphate buffer. After dehydration with graded concentrations of ethanol, the cells were placed in propylene oxide, followed by a mixture of equal proportions of propylene oxide and Epon-Araldite mixtures. Embedding was accomplished in Epon-Araldite mixture according to the method of Mollenhauer (24). Thin sections were cut by a LKB Ultratome (LKB Instruments, Inc., Rockville, Md.), stained with uranyl acetate and lead nitrate, and examined in a JEM-7 electron microscope (Jeolco U.S.A. Inc., Medford, Mass.).

Abbreviations: EM, electron microscopy; HAU, hemagglutinating units; M/I ratio, mitotic to interphase nuclei; NE, nuclear envelope; PF, pulverization factor; TLN, telophase-like nucleus.
were occasionally found at an extremely low frequency, that is, in less than 2% of the whole mitotic cell population. As will be shown, the number of TLN in a population of fused cells was very much greater. These observations indicated that the chromosomes of the structure were derived from metaphases that existed before fusion, and that they were produced after cellular fusion with interphase cells.

**Electron Microscope Study**

For a fuller understanding of the nature of the TLN in fused cells, electron microscopy (EM) was carried out in samples which included a higher percentage of fused cells by use of 2000 HAU/ml after 5 hr of exposure to Colcemid. Part of the culture received Hanks' solution instead of virus as a control. After standing at 4°C for 10 min, both samples were incubated at 37°C, and fixed 15 and 40 min after the incubation.

Light microscope observations on the virus-treated samples harvested 40 min after incubation indicated that, out of 141 fused cells containing both interphase and mitotic nuclei, 29 (21%) showed pulverization associated with intact metaphase chromosomes, and 108 (77%) had chromosomes enclosed in the TLN.

The EM examination of the virus-treated samples was confined to fused cells which included both interphase and mitotic nuclei. In the control samples, interphase and metaphase cells were investigated. After incubation of the control samples for 40 min, membranes were not observed around the large mass of chromosomal materials, which probably resulted from aggregation of individual chromosomes after prolonged exposure to Colcemid (8, 9, 23) (Fig. 6). This material resembled HeLa metaphase cells treated with another mitotic inhibitor, vincristine (9). After incubation with virus for 40 min, most of the fused cells were found to have TLN in which similar chromosomal masses were now surrounded by double membranes (Figs. 7 and 8). These membranes closely resembled those surrounding interphase nuclei in the same cells (Fig. 7) and could not be readily distinguished from the membranes surrounding the interphase nuclei in the control sample. The only feature which might possibly distinguish the membranes in the TLN from the membranes in interphase nuclei was the paucity of nuclear pores in the virus-treated sample. Tentatively, we considered this newly-observed membrane as a normal nuclear envelope (NE). In support of this tentative conclusion was the finding of connection of this structure with the endoplasmic reticulum of the cytoplasm with its attendant ribosomes (Fig. 8).

Of interest was the finding that the new NE was incomplete after 15 min of exposure to virus (Figs. 9 and 10) and the occasional presence of a four-layered membrane structure (Fig. 9). The four-layered membranes were also seen in the cytoplasm of the control mitotic cells, together with an accumulation of layered concentric membranous elements similar to those of endoplasmic reticulum and NE (Fig. 6). A nucleolus-like structure was observed in condensed chromatin in both the control and virus-treated samples (Fig. 10). Nucleoli associated with chromatin have been observed by other workers in EM studies of metaphase chromosomes (2, 3, 7, 9). A similar observation in the virus-treated samples in the present work supports our confidence that the chromatin surrounded by the new NE is representative of the chromatin present before fusion. (The EM work did not involve hypotonic treatment before fixing as described in Materials and Methods. Therefore, pulverization of interphase nuclei in fused cells could not be examined [16, 35]).

**KINETICS OF CELL FUSION, CHROMOSOME PULVERIZATION, AND NUCLEAR ENVELOPE**

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**Figure 1**  Cells from culture treated with Colcemid for 5 hr, including cells showing a mass of clumped chromosomes and ball metaphase. Orcein squash preparation without hypotonicity. × 1300.

**Figure 2**  Cells from sample infected with virus after 5 hr Colcemid treatment: two fused cells with a mass of clumped chromosomes are shown, one containing one normal interphase and the other containing two normal interphase nuclei. × 1300.

**Figure 3**  A tetranucleate cell containing three intact interphase nuclei and one diploid chromosome set involved in a bag-like structure. Stained with Giemsa's after hypotonic treatment. × 1900.
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**FIGURE 4** A binucleate cell containing one intact interphase nucleus and one diploid set of chromosomes in a bag-like structure. $\times$ 1300.

**FIGURE 5** A trinucleate cell containing two intact interphase nuclei and several groups of chromosomes in bag-like structures. $\times$ 1300.

**FIGURE 6** Electron micrograph of an arrested cell from culture treated with Colcemid for 5 hr: a prominent nucleolus-like structure (n1) is attached to the large mass of chromosome materials (chr). The other nucleolus-like structures (n1 and n2) are seen around the chromosome mass. The n1 type is composed of predominantly fine filaments, and the n2 type of predominantly dense granules. Although several kinetochores (k) are present, no spindle tubules are seen. Membranous elements, including four-layered membranes (arrows), are present in the cytoplasm. $\times$ 18,300.
FORMATION: Cells initially in log phase were exposed to Colcemid for 5 hr and then mixed with virus. The cells were then harvested at various intervals up to 60 min. The frequency of multinucleate cells among 1500 randomly selected cells was measured by direct counts. Additionally, 200 fused cells containing at least one interphase nucleus and at least one “mitotic nucleus,” principally metaphase or prometaphase, were examined. Three kinds of fused cells were identifiable: the first consisted of normal interphase nuclei and TLN (Figs. 1–3); the second kind contained normal metaphases and pulverized nuclei (Fig. 11); and the third had both normal metaphase and interphase nuclei (Fig. 12). The numerical results of these observations are given in Fig. 13. After addition of virus, the frequency of all multinucleate cells increased rapidly, and by 10 min reached a constant value of 15–16%. Among the multinucleate cells the frequency of cells with pulverization and of those with NE increased slowly with time. Concomitantly, there was a decrease in the frequency of multinucleate cells that exhibited neither pulverization nor NE.

The frequency of pulverized cells among multinucleate cells having at least one interphase nucleus and at least one mitotic nucleus reached a plateau value of about 32% at 20 min after viral treatment. At this time, 13% of such multinucleate cells were found to show neither pulverization nor NE. It is quite probable that the chromosomes in most of these cells became surrounded by the NE during the next 10 min, since the frequency of cells with NE continued to increase during this period from 55% to 66% to reach the plateau value.

These values and their time course clearly indicate that, 5 hr after exposure of log-phase cells to Colcemid and subsequent fusion of metaphase cells with interphase cells, the nuclei of the resulting multinucleate cells can follow alternative courses. Either an interphase nucleus will be pulverized or...
the metaphase chromosomes will become enclosed in NE.

**Effect of Time of Exposure to Colcemid:** To ascertain the effect of Colcemid treatment on the induction of the NE formation around the metaphase chromosomes, the cultures were exposed to Colcemid for varying periods of time before addition of the virus. The cells were harvested 40 min later. Fig. 14 represents the proportions of the three different types of fused cells. Since the cells were still exposed to Colcemid in the presence of virus, the actual treatment with Colcemid was 40 min longer in each sample. In binucleate cells, the percentage of cells with new NE increased with time of exposure to Colcemid before fusion, from 7% initially to 38% after 5 hr, the percentage showing that pulverization decreased (Fig. 14, left). In multinucleate cells (Fig. 14, right) the frequency of NE formation was greater than in binucleate cells, and that of pulverization was less.

This finding suggests that the probability of formation of NE around the chromosomes may be dependent on the number of interphase nuclei present, because of its predominance in the multinucleate cells.

**Dose Effect of Interphase Cells on NE Formation:** The data presented in Fig. 14 were rearranged on the basis of the ratios of the number of mitotic to interphase nuclei (M/I ratio), as shown in Fig. 15. In each sample it was clear that the more interphase nuclei in the fused cells, the higher was the percentage of cells that showed the NE; conversely, the greater the number of mitotic nuclei involved, the higher was the percentage of cells that showed pulverization. In the case of an M/I ratio of 0.33, all the cells had the NE around the chromosomes; in the case of an M/I ratio of 2.0, all the cells showed pulverization.

**Discussion**

It has been fairly well established that chromosome pulverization in interphase nuclei of virus-fused multi- or binucleate cells requires the presence of at least one mitotic nucleus in the same
cytoplasm (18, 28, 36, 37). The present study describes an additional phenomenon which takes place in fused Chinese hamster Don cells containing both interphase and mitotic nuclei. This event is characterized by the appearance of a membranous structure around the metaphase chromosomes after fusion of a preformed metaphase cell with a preformed interphase cell. Fused cells of this kind never showed pulverization in the interphase nuclei. On the basis of EM observations, the membrane enclosing the chromosomes is tentatively identified as a NE.

The precise mechanism by which this structure is formed is not known, but some of the data presented permit the formulation of a hypothesis. The longer a log-phase population of cells is exposed to Colcemid, the greater is the chance that a metaphase nucleus in a subsequently fused cell containing one interphase nucleus will be surrounded by the NE. However, it is probable that the length of the period of Colcemid treatment is not the only factor to determine whether the metaphase chromosomes are surrounded by the NE. Of primary importance is the finding that the proportions of mitotic and interphase nuclei in the same cytoplasm were found to influence the probability that the induction of NE formation or pulverization would occur. Thus, it was found that the more interphase nuclei in the fused cells, the higher was the percentage of cells that showed NE formation and the lower was the percentage of cells with pulverization. The converse dose response was indicated initially from the results of Kato and Sandberg (19) and confirmed by Johnson and Rao (18), who reported that the ability of mitotic cells to induce chromosome pulverization in interphase nuclei depends on the ratio of mitotic to interphasic nuclei. The same relation was found in the present work.

To explain these results, we propose a scheme that invokes the activities of two kinds of agents, one formative and one disruptive for the NE. One
of these was recently postulated in this laboratory (36) and is concerned with the phenomenon of chromosome pulverization with its attendant loss of the NE. Evidence that such a pulverization factor, PF, is synthesized in the late G2 period of the normal Don cell cycle was recently obtained by Matsui et al. 2. The other factor(s) is concerned with NE formation and is thought to be a normal cellular component also. The PF persists in the metaphase cells after the G2-M transition, but if the cell is arrested in metaphase for a sufficient length of time, PF either disappears or reaches an ineffectual concentration so that, after fusion with an interphase cell, pulverization and loss of the NE cannot occur. At this time, the NE factor which is present in the interphase cell is able to induce formation of NE around the metaphase chromosomes. This hypothesis holds that PF and NE factor are antagonistic to each other, as strongly suggested by the dose effect data. In agreement is the finding that in some binucleate cells containing metaphase chromosomes and an interphase nucleus neither pulverization nor NE formation is observed. It will be noticed that NE is rarely seen in the unfused metaphase cells.

It is of interest to compare the NE formation studied here with that occurring in the telophase stage of normal cell division. The behavior of the NE in the mitotic cells of higher organisms has been studied by several authors (1, 3, 26, 31, 33). These workers have suggested that the fragmented NE resulting from its partial disruption in prophase migrates out into cytoplasm to become indistinguishable from granular endoplasmic reticulum. They have speculated that the NE of the daughter cells at telophase may be derived from the cytoplasmic vesicles similar to the granular

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endoplasmic reticulum. The present results are not against the concept that the newly observed NE around the chromosomes in the fused cells was supplied by the preformed membrane structures, possibly including endoplasmic reticulum present in the cytoplasm. This is supported by the fact that the four-layered membranes seen in the control metaphase cells were also seen adjacent to the NE of the TLN in fused cells. Murray et al. (26) also observed the four-layered membranes arising at the time of opening up of the NE in

prophase and involvement of similar membranes in NE reformation in daughter nuclei. Although the general nucleolar organization is lost during prophase, Hsu et al. (13) demonstrated the persistence of nucleolar substances during normal mitosis in a number of mammalian cell strains. This phenomenon appeared to be particularly pronounced in cultured Chinese hamster cells (2, 7, 11, 13), and at least a portion of the nucleolar materials was included in the daughter nuclei (2, 7, 13). The present study showed that the nucleolus-like structures present in the control metaphase cells appeared to be involved within the NE formed around the chromosomes in the fused cells (Fig. 10).

On the basis of the above findings, we have the impression at the present time that the fusion of metaphase with interphase cells can result in a cytoplasmic environment for the metaphase cell which is similar to that surrounding a telophase in normal mitosis and which leads to the membrane structures enclosing the metaphase chromosomes. An intriguing question is whether the chromosomes surrounded by the NE proceed normally into interphase stage. This possibility is under investigation.

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