CHROMOSOME PULVERIZATION IN
HUMAN BINUCLEATE CELLS
FOLLOWING COLCEMID TREATMENT

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ABSTRACT
Under the influence of Colcemid, a substantial number of binucleate human cells from a line infected with herpes-like virus was found to possess pulverized chromosomes. Although this abnormality was also detected in untreated binucleate cells, the increase in the number of pulverized cells after the addition of Colcemid was too striking to be explained by accumulation of spontaneously occurring cells in response to the mitotic inhibition by Colcemid. Furthermore, the induction of pulverization may be dependent upon Colcemid concentration. These findings imply an involvement of Colcemid in the mechanism of pulverization induction in the system studied. When tritiated thymidine was added to the culture medium simultaneously with Colcemid, the majority of binucleate cells with an intact and a pulverized chromosome set incorporated this isotope into the pulverized set only. This obviously suggests that the nuclei in the binucleate cell are asynchronous in DNA synthesis, and that this asynchrony is intimately related to the induction of the pulverization phenomenon. It seems very probable that the late S phase in the late synthesizing nuclei represents a critical stage at which damage to the chromosomes most readily occurs.

In recent years the fact that various viruses are capable of causing severe kinds of chromosome destruction has been established beyond reasonable doubt (1, 2, 5-8, 10, 11). This phenomenon, which in extreme cases is designated as chromosome pulverization, has also been noticed in plant materials after treatments with various deoxyriboside analogues (3, 13). However, the mechanisms involved in the induction of this phenomenon have remained obscure.

The present paper describes the chromosome pulverization phenomenon in a human cell line containing a substantial number of binucleate cells and the possible role of Colcemid and/or viruses in the induction of pulverization.

MATERIALS AND METHODS
All the studies reported here were made with a human diploid cell line (RPMI No. 8205) derived from the blood of a 54 yr-old woman with acute myeloblastic leukemia. This cell line was cultured originally in McCoy's medium supplemented with 20% fetal calf serum and 0.2% human serum albumin. Subsequently, the cells used in the present study were grown as suspension culture in RPMI medium No. 1640 supplemented with 10-20% fetal calf serum in 16 oz bottles made by Brockway Glass Co., Brockway, Pa. Details regarding the cell line have been reported elsewhere (4, 9). Recently, the existence of a herpes-type virus ("leukovirus") was found in this cell line.1

Experiments were conducted with cultures consisting of cells at a concentration of approximately 5 × 10⁶ per milliliter of growth medium. Demecolcin

1 The cell line was kindly supplied by Dr. G. E. Moore of our Institute. The term "leukovirus" has been used by him for the herpes-like virus found in Burkitt's lymphoma and in some of the leukocyte cell cultures derived from patients with leukemia or lymphoma.
was introduced into the cultures to a final concentration of 0.05, 0.5, and 1 μg/ml, respectively. The cells were examined at various intervals (0.5–6 hr) after addition of Colcemid. In another set of experiments, the cultures were treated with Colcemid at a concentration of 0.5 μg/ml for 0.5, 2, and 4 hr, respectively. The cells were then centrifuged and resuspended in fresh medium free from Colcemid. Cells were harvested before and after removal of the drug. The cells harvested were suspended in 0.44% sodium citrate for 30 min at room temperature, fixed for at least 1 hr in acetic-alcohol (1:3), air dried on slides, and stained with Giemsa’s. In experiments involving tritiated thymidine, cultures were treated simultaneously with Colcemid (0.5 μg/ml) and tritiated thymidine (0.2–1.0 μc/ml, specific activity 6.7 c/mmole) for periods of time ranging from 0–8 hr before fixation. When necessary, slides were stained with Giemsa’s before coating. The stain could be easily removed by rinsing the slide in 50% acetic acid. The radioautographic procedure consisted of the following steps: slides containing tritium-labeled cells were coated with Kodak NTB2 nuclear track emulsion (Eastman Kodak Co., Rochester, N. Y.), exposed for 5–7 days at 4°C, developed, and stained with Giemsa’s (9).

**RESULTS**

**Binucleate Cells**

A careful examination of over 4000 interphase cells revealed that the culture consisted of 94.4% mononucleate cells, 4.3% binucleate cells, 0.33% trinucleate cells, 0.07% tetra- or higher-nucleate cells, and 0.92% cells with an abnormally large nucleus. The proportion of each type of cell, however, varied slightly from culture to culture.

The nuclei of the binucleate cells appeared asynchronous in their DNA synthesis (9). In order to detect the length of the time-lag between the nuclei of the same cells with respect to finishing their DNA synthesis, we pulse-labeled cultures for 15 min with tritiated thymidine (1 μc/ml), and the cells were sampled at various intervals up to 8 hr after treatment. Prior to fixation, each cell sample was exposed to Colcemid (0.05 μg/ml) for 10 min. Fig. 1 depicts curves of the percentage of labeled mono- and binucleate metaphases at successive time intervals following a pulse label with tritiated thymidine. These curves provide information necessary for the determination of the duration of the
post-DNA synthetic period (G₂) in these cells. As shown in this figure, metaphases of binucleate cells in which only one-half set of the chromosomes was labeled, i.e., those of only one nucleus, appeared as early as 1 hour after tritiated thymidine treatment, whereas those having grains on both chromosome sets were detected 2.5 hr after treatment. The percentage of the former reached a peak at 3.5 hr, then decreased gradually with the lapse of time. On the other hand, the incidence of metaphasic binucleate cells with label on both chromosome sets increased rapidly and showed a maximum value at the 7th hr after treatment. The sum of the percentages of these two components is indicated by a broken line, which fits well with the labeled mitotic curve of mononucleate cells. Since the broken line could be referred to as the labeled mitotic curve of binucleate cells as a whole, it may be concluded that the duration of G₂ of binucleate cells is almost equal to that of G₂ of mononucleate ones.

On the basis of these results, the duration of the G₂ phase of the early replicating nucleus in binucleate cells can be estimated as 3.5 hr. On the other hand, the G₂ phase of the late synthesizing nucleus in binucleate cells appears to last approximately 4.5 hr. Accordingly, the time discrepancy between the two nuclei in binucleate cells with respect to completing DNA synthesis can be estimated as about 1 hr in duration. The incidence of binucleate cells in which only one of the chromosome sets was labeled showed a gradual decrease after reaching a peak at 3.5 hr (Fig. 1); therefore, a fairly large variation in the degree of asynchrony between two nuclei with respect to completing their DNA synthesis may be inferred.

**Chromosome Pulverization**

When Colcemid was added to cultures and preparations were made a few hours later, a large number of binucleate and multinucleate cells was characterized by metaphasic figures containing both extremely fragmented and normal-looking chromosomes (Fig. 2). These metaphases demonstrated a phenomenon which has been termed chromosome pulverization (5), so that we have tentatively adopted this term to describe the chromosome abnormality observed in our studies.

Pulverized chromosomes were observed exclusively in polyploid cells. Furthermore, it is of interest that in the case of tetraploid cells with chromosome pulverization the number of intact chromosomes was usually 46, the modal value of this cell line (9). This indicates clearly that these metaphases originated from binucleate cells. Therefore, it is very likely that in binucleate cells chromosome pulverization affects only one nucleus. We also observed a few polyploid cells in which all chromosomes were pulverized. However, such cells were very rare and, in addition, very difficult to distinguish from poorly fixed interphase cells. Chromosome pulverization was readily detected in trinucleate or tetranucleate cells and, in such cases, at least one of the chromosome sets was found to be intact. All metaphases showing endoreduplication were found to be devoid of pulverized chromosomes. The intensity of the chromosome pulverization varied considerably but seemed to be associated with the length of incubation with Colcemid. In the extreme cases, cells were seen in which the pulverized chromosomes were fragmented into a granular mass.

**Effects of Colcemid on Chromosome Pulverization**

To determine the role of Colcemid in the induction of chromosome pulverization, we exposed cultures to various concentrations of Colcemid: 0.05, 0.5, and 1 µg/ml. Because of the large proportion of binucleate cells among the multinucleate ones, quantitative examination of chromosome pulverization was carried out exclusively on the binucleate cell population. In Fig. 3 the percentages of cells with chromosome pulverization and the percentages of mitotic cells in the whole cell population are shown. It is noticeable that cells with pulverized chromosomes were observable in untreated control cultures, even though the incidence was very low. During the 1st hr after Colcemid treatment, the percentage of cells with pulverization remained at control level. The control level was obtained in cultures without any Colcemid. Then, this percentage showed a striking increase during the 2–4 hr period after addition of Colcemid. In the next 2 hr, the percentage of affected cells showed only a slight increase. The curves for three different doses of Colcemid were similar in their pattern, but apparently concentration dependent. Had the increase in the incidence of pulverization been a result of the accumulation of preexisting cells with pulverization in response to the mitotic arrest induced by Colcemid, the percentage should have remained at a constant level throughout the cultivation period. Furthermore, there was no con-
FIGURE 2 A binucleate cell with chromosome pulverization harvested at the 2nd hr after addition of Colcemid. One essentially diploid set is intact and the chromosomes of the other nucleus are pulverized. × 2,200

centration dependency in the mitotic inhibitory effect of Colcemid, with the doses employed in our experiments. It is of interest to note that the pattern of the appearance and increase in the incidence of pulverization coincides with that pattern of the labeled mitotic curve of binucleate cells with half-labeled metaphase plates (Fig. 1). This feature suggests a relationship of the asynchronous binucleate cells to chromosome pulverization.

In order to obtain further information concerning the effect of Colcemid on the production of pulverized chromosomes, we first treated cultures with Colcemid (0.5 μg/ml) for definite periods of time, then we removed the chemical by washing and re-suspended the cells in fresh medium. The changes in the percentages of cells with pulverized chromosomes were established at successive intervals before and following removal of Colcemid. The results are shown in Fig. 4. When Colcemid was removed 30 min after treatment, no change in the percentage of cells with chromosome pulverization occurred during the remaining culture period. When the chemical was removed at the 2nd hr, the subsequent percentage of cells with chromosome pulverization remained almost at the same level as at the time of removal of Colcemid, but at a higher level than when the drug was withdrawn at 1 hr. When the Colcemid was removed from the cultures
Figure 3 Incidences of binucleate cells with chromosome pulverization and mitotic cells at different hours after treatment with Colcemid at various concentrations.

Figure 4 The incidence of binucleate cells with chromosome pulverization before and after removal of colcemid. Arrows indicate the time of removal of Colcemid.
TABLE I

DNA Synthesis in Binucleate Cells with or without Chromosome Pulverization after Addition of Colcemid and Thymidine-\(^{3}H\)

<table>
<thead>
<tr>
<th>Time after</th>
<th>No. of metaphase binucleate cells observed</th>
<th>No. of metaphasic binucleate cells with pulverization</th>
<th>Labeled</th>
<th>Unlabeled</th>
<th>One nucleus</th>
<th>Both nuclei</th>
<th>Total</th>
<th>Normal</th>
<th>Pulverized</th>
<th>Both nuclei</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td>hr</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>465</td>
<td>458</td>
<td>0</td>
<td>0</td>
<td>458</td>
<td>1</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(100)</td>
<td>(98.5)</td>
<td>(0)</td>
<td>(0)</td>
<td>(98.5)</td>
<td>(0.2)</td>
<td>(0)</td>
<td>(1.3)</td>
<td>(0)</td>
<td>(1.5)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>494</td>
<td>451</td>
<td>7</td>
<td>0</td>
<td>458</td>
<td>8</td>
<td>0</td>
<td>27</td>
<td>1</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(100)</td>
<td>(91.3)</td>
<td>(1.4)</td>
<td>(0)</td>
<td>(92.7)</td>
<td>(1.6)</td>
<td>(0)</td>
<td>(5.5)</td>
<td>(0.2)</td>
<td>(7.3)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>546</td>
<td>326</td>
<td>85</td>
<td>39</td>
<td>470</td>
<td>4</td>
<td>0</td>
<td>65</td>
<td>7</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(100)</td>
<td>(59.7)</td>
<td>(15.6)</td>
<td>(10.8)</td>
<td>(86.1)</td>
<td>(0.7)</td>
<td>(0)</td>
<td>(11.9)</td>
<td>(1.3)</td>
<td>(13.9)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>575</td>
<td>289</td>
<td>69</td>
<td>113</td>
<td>470</td>
<td>4</td>
<td>1</td>
<td>78</td>
<td>22</td>
<td>105</td>
<td></td>
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<tr>
<td></td>
<td>(100)</td>
<td>(50.1)</td>
<td>(12.0)</td>
<td>(19.6)</td>
<td>(81.7)</td>
<td>(0.7)</td>
<td>(0.2)</td>
<td>(13.6)</td>
<td>(3.8)</td>
<td>(18.3)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>483</td>
<td>197</td>
<td>57</td>
<td>153</td>
<td>407</td>
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<td>0</td>
<td>53</td>
<td>21</td>
<td>76</td>
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<tr>
<td></td>
<td>(100)</td>
<td>(40.8)</td>
<td>(11.8)</td>
<td>(31.7)</td>
<td>(84.3)</td>
<td>(0.4)</td>
<td>(0)</td>
<td>(11.0)</td>
<td>(4.3)</td>
<td>(15.7)</td>
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Figures in parentheses indicate percentage.

after 4 hr of exposure, the percentage of affected cells, which already exceeded 10%, showed a rise in the next hour and then a sharp decline. This striking decrease in the percentage of affected cells may have resulted from the elimination of pulverized cells from the cell population after complete destruction of affected chromosomes. In view of these results, it seems probable that when Colcemid is removed from the culture cells are released from its chromosome pulverization effect. These data provide evidence for the assumption that the striking increase in the number of cells with chromosome pulverization is due to an action of Colcemid other than that related to the accumulation of cells with spontaneous pulverization in culture.

**DNA Synthesis in Pulverized Chromosomes**

The results obtained in the described experiments led us to theorize that there is a critical phase in the cell cycle in which the chromosomes are susceptible to the pulverizing effect of Colcemid. In order to ascertain this possibility, we exposed cultures to Colcemid and tritiated thymidine simultaneously, and examined labeling patterns of binucleate cells with or without chromosome pulverization. The results are presented in Tables I and II and Figs. 5–8.

Metaphasic cells with chromosome pulverization appeared at the 1st hr, and six out of seven binucleate cells with pulverization showed labeling over the pulverized chromosome set only (Table I and Fig. 5). The incidence of cells with chromosome pulverization reached a peak at the 6th hr after addition of Colcemid. The percentage at the 8th hr was lower than at the peak value. This may be due to either the death or dissolution of pulverized cells, as a result of an extreme destruction of chromosomes. Indeed, in preparations made at the 8th hr, metaphasic figures having chromosome pulverization were slightly different in appearance from those observed in the early part of the culture period, e.g., 2 hr after Colcemid addition. In the former case, one of the nuclei containing intact chromosomes did not spread well but tended to take a round shape, whereas in the other nucleus the extreme pulverization of the chromosomes led to the appearance of a homogeneous structure with interphase-like features. The differences in the morphology of the two nuclei sometimes gave a spurious impression that there were two independent cells rather than one binucleate cell. Cells showing such figures may readily cause a scoring error, resulting in an underestimation of the percentage of pulverization.

It can be seen in Fig. 5 that the majority of binucleate cells with chromosome pulverization was labeled with tritiated thymidine in the pulverized nucleus, whereas the intact nucleus remained unlabeled.
Figure 5 The incidence and the labeling pattern of binucleate cells with chromosome pulverization at different hours after addition of Colcemid and thymidine-3H.

The findings in the figures may be best explained as follows: one of the nuclei in a binucleate cell had already completed DNA synthesis at the time of addition of Colcemid and tritiated thymidine, whereas the late DNA-synthesizing nucleus was still in the S period and, hence, suffered from damage resulting in chromosome pulverization.

In order to gain further critical information on this matter, we estimated the proportion of metaphasic cells with pulverization among both unlabeled and labeled metaphasic binucleate cells, respectively. Among the labeled cell population, there were mainly two types of cells in respect to the labeling pattern of the nuclei. One type consisted of binucleate cells with a set of labeled pulverized chromosomes and a set of unlabeled intact chromosomes. The second type consisted of binucleate cells with a set of labeled pulverized chromosomes and a set of labeled intact chromosomes. Of the 300 metaphasic binucleate cells with chromosome pulverization observed, only one cell was detected to have silver grains over the set of normal chromosomes and none over the set of pulverized chromosomes. The results are illustrated in Table II and Fig. 8.

The proportion of unlabeled binucleate cells with chromosome pulverization among the total number of unlabeled binucleate cells (PU/TU) was very low and did not vary throughout the culture period. The ratio of binucleate cells with labeled pulverized chromosomes and an unlabeled set of intact chromosomes among the total population of binucleate cells in which one of the nuclei was labeled (PL1/TL1) was constantly higher than the ratio of pulverized binucleate cells with...
label on both the pulverized and intact sets among the total number of metaphasic binucleate cells in which both chromosome sets were labeled (PL2/TL2) (Tables I and II).

The data obtained seem to suggest several possibilities. Cells in the G₂ period appear not to be affected, as is indicated by a very low ratio of PU/TU. Cells in which one of the nuclei is still in the S period have the greatest chance to be affected; about one-half such cells are apparently affected, and this probability is four times as high as that of cells in which both nuclei are in the process of DNA synthesis when Colcemid is introduced. In other words, the late S phase of the late synthesizing nucleus could be the critical period in which damage can be induced.

**DISCUSSION**

The present study revealed that chromosome pulverization occurred exclusively in binucleate or multinucleate cells. This finding appears to be similar to that of Nichols et al. (6), who studied the chromosome pulverization phenomenon induced by measles virus in an in vitro system of human cells and who reached the conclusion that this phenomenon appeared in syncytia of cells. These observations were corroborated with a different virus (Sendai) by Saksela et al. (8). Furthermore, Norby et al. (7) reported that the chromosome pulverization phenomenon was dependent upon the hemolytic activity of the measles virus, which, apparently, was also responsible for the syncytium-
forming activity. The role played by Colcemid (or colchicine) in the pulverization results reported by the above authors is difficult to evaluate, since the drugs were utilized for their mitosis-arresting activity and the pulverization effect of Colcemid was probably unknown.

As previously reported by Sandberg et al. (9), it was further demonstrated in the present study that the nuclei in binucleate (or multinucleate) cells were asynchronous in their DNA synthesis, and that, when tritiated thymidine was introduced, the majority of cells with chromosome pulverization was labeled only in the pulverized half whereas the intact half remained unlabeled (Figs. 5–8). It was found that the pulverization curve did not rise immediately after the addition of Colcemid (Fig. 3) but was delayed about 1–2 hr; this suggests the amount of time required for the affected cells to reach the stage of mitosis. In the mitotic state the pulverization can be most easily recognized and quantitated. It is of interest to note that the length of this period corresponds approximately with the duration of the G2 phase of the binucleate cells (Fig. 1). The findings mentioned above strongly suggest that one of the important factors which appears to be correlated with the induction of the pulverization phenomenon is the asynchrony of DNA replication in the nuclei of binucleate cells. Moreover, it is very plausible that the period corresponding to the degree of asynchrony, i.e. the late S phase of the late synthesizing nucleus (approximately 1 hr, on the average), could be a critical stage at which cell damage leading to chromosome pulverization can be induced. In previous studies with the binucleate cell line (9) it was shown that even though the two nuclei replicated their DNA asynchronously they entered metaphase at the same time, as is evidenced by the identical ap-
TABLE II

Proportion of Binucleate Cells with Chromosome Pulverization after Addition of Colcemid and thymidine-$^3$H

<table>
<thead>
<tr>
<th>Time after Colcemid addition</th>
<th>No. of cells examined</th>
<th>No. of cells pulverized</th>
<th>PU/TU</th>
<th>No. of cells examined</th>
<th>No. of cells pulverized</th>
<th>PL1/TL1</th>
<th>No. of cells examined</th>
<th>No. of cells pulverized</th>
<th>PL2/TL2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hr</td>
<td>459</td>
<td>1</td>
<td>0.002</td>
<td>6</td>
<td>6</td>
<td>1.000</td>
<td>0</td>
<td>0</td>
<td>−</td>
</tr>
<tr>
<td>2</td>
<td>459</td>
<td>8</td>
<td>0.017</td>
<td>34</td>
<td>27</td>
<td>0.794</td>
<td>1</td>
<td>1</td>
<td>1.000</td>
</tr>
<tr>
<td>4</td>
<td>330</td>
<td>4</td>
<td>0.012</td>
<td>150</td>
<td>65</td>
<td>0.434</td>
<td>66</td>
<td>7</td>
<td>0.106</td>
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<td>78</td>
<td>0.530</td>
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<td>0.163</td>
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<td>8</td>
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<td>0.010</td>
<td>110</td>
<td>53</td>
<td>0.482</td>
<td>174</td>
<td>21</td>
<td>0.124</td>
</tr>
</tbody>
</table>

Figure 8 Proportion of binucleate cells with chromosome pulverization in the total number of binucleate cells. For explanation of PL1/TL1, PL2/TL2, and PU/TU, see text.

Since the percentage curves of pulverization showed a unique pattern in response to the addition of Colcemid, and since the incidence of chromosome pulverization may possibly depend upon the concentration of Colcemid, it may be reasonably concluded that Colcemid plays some role in inducing chromosome pulverization. However, the nature of this role remains essentially unknown. Recently, the cell line used in the present study was found to carry the "leukovirus." Evidence exists that this type of virus is capable of causing chromosome damage in mammalian cultured cells (1, 10). Therefore, a possible explanation is that Colcemid exerts its effect synergistically with the virus. However, in the light of the fact that but few binucleate cells with pulverized chromosomes occur in untreated control cultures, it seems possible that Colcemid accelerates the primary effect of the virus in the induction of chromosome pulverization. Nevertheless, almost nothing is known conclusively with regard to the exact mechanism underlying the pulverization phenomenon caused by any virus. In addition, no data are available on the occurrence of cells with chromosome pulverization in virus-carrying cell lines other than the experimentally induced ones by infecting cells with a large amount of virus. Accordingly, an alternative explanation for the results observed in the present study may be advanced: the role that the virus plays is restricted...
to the formation of syncytium cells, i.e. virus has no direct effect on pulverization induction and this phenomenon can be induced in the absence of the virus by certain cellular events taking place under an abnormal environment brought about by the fusion of cells. Colcemid may have an accelerating effect on such events. In this connection, Stubblefield (12) reported that a long exposure of culture cells to Colcemid resulted in the production of multinucleate cells, in which the nuclei were sometimes completely asynchronous in DNA synthesis. In such cells, extremely extended chromosomes were detected in the late replicating nucleus. These observations led him to propose the hypothesis that the early synthesizing nucleus sends a signal into the cytoplasm at the conclusion of its DNA synthesis period, and that the cytoplasmic environment responds with some alteration that interrupts the normal sequence of events in the late synthesizing nucleus, resulting in uncoiling of chromosomes.

It is, however, uncertain whether aberrant chromosomal figures obtained in the present study are evoked by the same mechanism as postulated by Stubblefield, even though there is some morphological resemblance between Stubblefield’s figures and ours, especially those observed in early samples after the addition of Colcemid. At the present, the data on hand are insufficient for deducing any conclusions on the feasibility of either of the two alternative explanations discussed above.

In this connection, it should be of fundamental importance to determine whether or not the pulverization phenomenon could be detected in multinucleate cells occurring in cell lines of different origin and in cell lines which are either infected with a different type of virus or completely free of virus, as well as to obtain further critical information on the action of Colcemid in the induction of chromosome pulverization. Experiments along these lines are now in progress in our laboratory.

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REFERENCES


