CHROMOSOME PULVERIZATION IN
MICRONUCLEI INDUCED BY TRITIATED THYMIDINE

TATSURO IKEUCHI, HERBERT WEINFELD,
and AVERY A. SANDBERG

From the Roswell Park Memorial Institute, Buffalo, New York 14203. Dr. Ikeuchi's present address is the Chromosome Research Unit, Faculty of Science, Hokkaido University, Sapporo, Japan 060.

ABSTRACT
Cultures of a pseudodiploid cell line (Don) of Chinese hamster origin were exposed to varying doses of tritiated thymidine (TdR-3H) for relatively long periods of time. In addition to previously observed chromosomal aberrations, such as breaks and reunions, a substantial number of interphasic cells with micronuclei and of metaphases associated with pulverized chromosomes was found; both phenomena were dependent on exposure time to and concentration of TdR-3H. The former phenomenon appeared to result from the effects of the β-emissions originating in the TdR-3H. A possible interpretation for chromosome pulverization induction is presented, emphasizing the derivation of the pulverized material from micronuclei in a common cytoplasm with a metaphase nucleus. These observations further substantiate our previously advanced hypothesis regarding the essential role played by substances present in a mitotic cell in the induction of chromosome pulverization and nuclear membrane dissolution.

INTRODUCTION
Virus-induced cell fusion has afforded us a unique system for observing two divergent phenomena: chromosome pulverization in interphase nuclei, probably related to nuclear membrane dissolution, and the formation of nuclear envelopes around metaphase chromosomes. Whether the former or the latter is encountered in a fused cell appears to be dependent on the time of examination (8). The necessary presence of a metaphase nucleus in a fused cell in order for pulverization to occur has been amply documented in our previous work (9, 23, 25) and substantiated by others (10, 21). A hypothesis advanced by us (23) relegated the virus and its products to the sole role of causing the formation of multinucleate cells. Substances present in the metaphase cell, that become part of the multinucleate cell, are then capable of causing disintegration of the nuclear membrane of the interphase nuclei resident within such a cell and, thus, lead to chromosome pulverization. The pulverization factor present in metaphase cells is dependent on protein synthesis in the G2 period, with the virus apparently playing no role in the formation or effects of the factor (16). Nevertheless, throughout these studies we have been eager to obtain a multinucleate system of the cell line used by us that is devoid and independent of virus, in order to afford us an opportunity to ascertain whether or not chromosome pulverization can indeed be induced in the absence of virus.

During our studies on Sendai virus-induced heterokaryon formation (9), we observed the presence of micronuclei in Chinese hamster cells when these were exposed to tritiated thymidine (TdR-3H)1 for 12 hr, before their exposure to the

1 Abbreviations: TdR-3H, tritiated thymidine.
virus. A significant number of these micronuclei was characterized by chromosome pulverization. The formation of micronuclei, to our knowledge, represents a hitherto unreported phenomenon induced by TdR-3H. The chromosome pulverization was easily distinguished from previously described damage of chromosomes caused by TdR-3H. These observations led us to undertake a more extensive study of the induction of micronuclei by TdR-3H and its possible utilization as a system adequate for the study of chromosome pulverization in multinucleate cells never exposed to viral products. Our hypothesis regarding chromosome pulverization was further confirmed by the present findings, which showed that induction of pulverization may occur in a nonvirus system.

MATERIALS AND METHODS

Spinner cultures of a Chinese hamster cell line (Don), grown in Roswell Park Memorial Institute No. 1640 medium supplemented with 20% calf serum (6), were used in the present study. The cell line had a sharp mode of 22 chromosomes, the diploid number of this animal, but detailed analysis of the modal cells showed a considerable variety of their karyotypes.

For all experiments cell cultures were arranged so as to contain $3 \times 10^5$ log phase cells/ml of growth medium. 6 hr later, the cells were exposed to TdR-3H (SA 6.7 Ci/m mole; New England Nuclear Corp., Boston, Mass.) at different concentrations, i.e. 0.5, 1.0, and 2.0 $\mu$Ci/ml, and harvested at subsequent periods of time up to 108 hr, which included Colcemid (Ciba Pharmaceutical Co., Summit, N.J.) treatment (0.1 $\mu$g/ml) during the last hour of culture. At 12, 36, 60, and 84 hr after the initiation of the exposure, the cultures were diluted to lower the cell concentrations to $5 \times 10^5$/ml, and the media were replaced by fresh ones, which included the same concentration of TdR-3H. This procedure was carried out not only for subcultures, but also to minimize a possible decrease in the rate of TdR incorporation by the cells due to degradation of TdR in the culture medium (14).

In order to ascertain the significance of the effect of $^3$H, other cultures were treated with nonradioactive TdR at doses corresponding to those used with the radioactive TdR, and examined in the same manner.

The methods for slide preparations for chromosomal and cytological examinations were described previously (9). For radioautographic studies, the unstained slides, coated with NTB 2 Kodak Nuclear track emulsion, were exposed for 7 days at 4°C, processed in Kodak D-19 developer, and stained with Giemsa's.

RESULTS

Cell Characteristics

One of the crucial points to establish in the present study was that the cell line used was virus-free. Repeated analyses of the Don cell line with electron microscopy over a period of years, utilizing techniques previously described by us (8), have failed to reveal the presence of virus. Before the present studies were undertaken and during their tenure, no viruses were ever visualized in the cells used, upon repeated examinations in the electron microscope. The absence of fused cells in control cultures is evidence against the presence of Sendai virus, an agent frequently used in our laboratory for other studies on chromosome pulverization. Thus, we felt fairly certain that the cells were virus-free, but even then proper controls were utilized throughout the study.

Radioautographic studies indicated that 6 hr after the initiation of TdR-3H exposure 92-94% of the cells were labeled, at 12 hr 96.5-99.5%, and later than 24 hr 100%. The findings are expected, since the generation time of the Don cells is about 12 hr and the S period lasts about 6 hr (24).

Micronuclei Formation

Exposure of the cells to TdR-3H caused an increased frequency of interphase cells having one or more micronuclei in the same cytoplasm (Figs. 1-3). In Fig. 1, it is noticeable that micro-
nucleated cells, usually having one micronucleus, were observed in untreated cultures, even though the incidence was very low (about 2.0%). During the first 6 hr after the TdR-3H treatment, the percentage of micronucleated cells remained unchanged. Then, this percentage showed a gradual increase with time, and at 108 hr it reached 45.5, 65.5, and 72.0% for each concentration of TdR-3H, thus showing, again, dose dependence. At the earlier stage of exposure, e.g. before 60 hr, the micronucleated cells contained only one or a few micronuclei besides a normal-sized main nucleus. Later, however, cells with many nuclei of different sizes were more frequently observed, making it impossible to distinguish the main nucleus from the micronuclei in a large number of cells.

**Chromosome Pulverization**

As previously reported by others (2, 4, 15, 17, 19, 26–28), the exposure of the cells to TdR-3H produced a large number of chromosomal breakages, reunions, disintegration, and so on. In addition to these anomalies, metaphase plates were accompanied by small areas containing pulverized chromatin, which appeared to be equivalent to that of one or more chromosomes characterizing a micronucleus. In their morphology and size, these chromatin masses varied considerably from finely minced chromatin particles (Figs. 3–6) to chromosomes of unusually extended appearance (Fig. 7). In this respect the characteristics of the pulverized micronuclei did not differ from those of pulverizations described by us for other micronuclei or for total nuclei in virus-fused cells (12, 25).

The percentages of cells with pulverizations of micronuclei in metaphase cells are shown in Fig. 8, under the conditions of the three different doses of TdR-3H, 0.5, 1.0, and 2.0 μCi/ml. A
Figures 4-7 Metaphase cells containing pulverized chromosomes (large arrows) showing finely minced chromatin particles (Figs. 4-6) and unusually extended chromosomes (Fig. 7). Note also the other abnormal features (small arrows) such as breaks, a fragment, and a marker chromosome. × 1900.

A comparison of the data in Fig. 6 with those of Fig. 1 indicates that as the number of cells with micronuclei increased, there appeared to take place a parallel increase in the number of pulverized micronuclei residing in the same cytoplasm with a metaphasic main nucleus. In each sample, 100–200 well-delineated metaphases were examined. Metaphases with pulverized micronuclei appeared first at 24 hr after the initiation of exposure, then strikingly increased in percentage, especially between 36 and 60 hr. The curves for the three different doses of TdR-3H were similar in their pattern, but clearly showed a concentration-dependent effect. After 108 hr of exposure, the percentages reached as high as 24.5, 37.5, and 40.0% for 0.5, 1.0, and 2.0 μCi/ml of TdR-3H, respectively. There was not a definite tendency for certain types of pulverized material described above to appear at certain periods of time of exposure.

In order to clarify the origin of the pulverized material the number of nonpulverized chromosomes in metaphases with pulverization was examined and compared with that of untreated control cells (Fig. 9). This was done in order to make sure that the development of substantial aneuploidy, particularly hyperploidy, in the cells with pulverized micronuclei did not lead to the...
phenomena observed. For this purpose, cells which had no other chromosomal abnormality besides pulverization were exclusively selected in the samples exposed to TdR-$^3$H for 36 hr. It was found that the modal chromosome numbers in both pulverized and control cells were the same, i.e., 22. This means that the micronuclei and the pulverized materials were not derived from excessive chromosomes in a metaphase set.

The incidence of cells with pulverizations was extremely high in polyploid cells (mostly in the tetraploid range). In samples harvested 36 hr after exposure, 2.7% of diploid-ranged metaphases (15 out of 552) contained pulverizations. On the other hand, polyploid cells showed this abnormality in 21.6% of the metaphases examined (13 out of 60).

**Incubation with Nonradioactive TdR-$^3$H**

In cultures treated with nonradioactive TdR-$^3$H, at concentrations equivalent to those used with radioactive TdR, neither pulverization nor micronuclei were induced, the latter remaining in frequency at control levels (Figs. 1 and 8).

**DISCUSSION**

The results of the present study bear upon two important parameters of cultured cells: the effects of TdR-$^3$H upon chromosomal morphology and behavior, and, more important to us, the induction of chromosome pulverization in micronuclei by metaphasic nuclei in a virus-free system. The discussion will concern itself with these two facets of the results.

The present study showed that prolonged exposure of Chinese hamster cells to TdR-$^3$H produced a large number of cells with micronuclei and metaphase cells with pulverizations. To our knowledge, this is a hitherto unreported effect of TdR-$^3$H. Yang et al. (29) reported that nonradioactive TdR induced chromosomal aberrations, such as breaks, gaps, and exchanges, at concentrations of 1-20 mm in several Chinese hamster cell lines. However, since the TdR doses used in the present study were extremely low (0.07-0.3 μM), it is probable that the abnormalities observed, particularly after 12 hr of culture, were not attributable to the effect of TdR per se, but to the β-radiation emitted by the $^3$H as a component of the TdR incorporated by the nuclei. In fact, in the control cultures treated with the equivalent doses of nonradioactive TdR, no significant increase in the incidence of the above abnormalities was observed. The endogenous β-radiation emitted by $^3$H may have resulted in lagging chromosomes through nondisjunction or fragments derived from chromosomal breaks in ana- or telophase stages. Both processes may lead to the production of micronuclei after cell division (1, 5).

In the previous studies on chromosome pulverization in virus-induced fused cells, the results strongly suggested that pulverization involved
interphase nuclei coexisting with at least one mitotic nucleus in the same syncytium, resulting from virus-mediated cellular fusion (9, 10, 23, 25). The necessity for the presence of a metaphase nucleus for pulverization to occur in a fused cell has been amply demonstrated. On the basis of electron microscope observations (22), we further promulgated that factors present in the metaphase may cause disintegration of the nuclear membrane of interphase nuclei, thus resulting in the appearance of chromosome pulverization (23). A direct effect of the factors on the nuclear chromatin cannot be ruled out at this time, however.

The ideal conditions for chromosome pulverization to occur in a fused cell appear to require the presence of a metaphase cell and interphase nuclei. The demonstration of asynchrony between the mitotic nucleus and the pulverized one supports this concept. Asynchrony in the cellular cycle, particularly in DNA synthesis, between the pulverized material and the intact mitotic chromosomes has been found in cultured multinucleate cells untreated with fusion virus but containing micronuclei (11, 12, 18, 30). Similar asynchrony in DNA synthesis between micronuclei and the larger nuclei has been observed by Stubblefield (24) in cultured Don cells in which multinucleation was induced by Colcemid. Furthermore, the results obtained by Kato and Sandberg (12) clearly indicated that the pulverizations were derived from micronuclei coexisting with main nuclei in the same cytoplasm; and these micronuclei replicate their chromosomal DNA asynchronously with the main nuclei (12). This assumption is in part supported by the finding that the sequence chromosome breaks, cells with micronuclei, and finally metaphase cells with pulverizations, made their appearances in the order indicated following the addition of TdR-3H to the cultures. The morphologic variety of pulverized chromosomes, ranging from minced chromatin particles to chromosomes of extended form, may be attributed to differences in the cellular cycles of the micronuclei, i.e. to the degree of asynchrony between the main nuclei and micronuclei, as shown by Johnson and Rao (10) and Sandberg et al. (23) in Sendai virus-induced multinucleate cells.

Although chromosome pulverization has been detected to date primarily in multinucleate cells in virus-treated cell cultures (10, 13, 20, 21) or cultures known to be persistently infected with virus (6, 7, 11, 12), it is apparent that, whether induced spontaneously or experimentally, chromosome pulverization occurs in interphase nuclei (micronuclei) by coexisting with a metaphase nucleus in the same cytoplasm. The role of the virus may be indirect, i.e. restricted to the formation of multinucleated cells by cell fusion, which results in the existence in the same cell of nuclei with asynchronous DNA synthesis. This assumption was further confirmed by the results of the present study, which showed in a nonvirus system the induction and increase in frequency of chromosome pulverization.

Chromosome pulverization has been recently defined by us, and a hypothesis regarding its mechanism has been advanced (23). It is probable that chromosome pulverization represents a premature occurrence of a normal event leading to premature chromosome condensation (10). Furthermore, chromosome pulverization has features which easily distinguish it from individual chromosomal breaks and rearrangements as caused by TdR-3H and a host of other agents. To date, almost all the studies on chromosome pulverization have been performed in different cell-virus systems (3, 9, 10, 13, 21, 23, 25). The virus is required for cell fusion to occur and, thus, join metaphases into a common cytoplasm with interphase nuclei, the latter nuclei being then pulverized by substances present in the metaphasic cell. Chromosome pulverization has been observed in occasional nonvirus-treated mammalian cell cultures (6, 7, 11, 12, 19, 30); but most of the cell lines utilized in the studies cited have been shown to have contained virus, probably at the time of the observations. In some of the studies referred to above (12, 19, 30), pulverized materials were found which were similar in morphology to those observed by us in the present work, and they probably represented micronuclei.

In a previous publication (8) we postulated the existence of two factors in normal cells: a pulverization factor present in metaphase cells, which leads to disintegration of the nuclear membrane before mitosis (and to chromosome pulverization under certain abnormal conditions), and another

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factor, resident within interphase cells, which plays an important role in reconstruction of the nuclear membrane following cellular division. The data presented further strengthen the concept of the pulverization factor as presented by us and rule out a role for a virus or its products in the cellular events discussed.

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REFERENCES


