PERSISTENCE OF NUCLEOLI IN SHORT TERM AND LONG TERM CELL CULTURES AND IN DIRECT BONE MARROW PREPARATIONS IN MAMMALIAN MATERIALS

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ABSTRACT

Persistent nucleoli were studied in Chinese hamster and human long term cultures, human peripheral blood short term cultures, as well as direct bone marrow preparations. No colchicine or hypotonic treatments were applied and the cells were differentially stained with the Feulgen method and light green. Nucleoli were found to persist in the three systems studied, although to a much greater extent in the long term culture. The persistent nucleolar materials were usually in the form of individualized nucleoli mainly at chromosome ends. They also sometimes existed in a fluidlike or dropletlike condition around the chromosomes. Association of acrocentrics in humans and end-to-end associations in hamsters are likely to result from persistence of nucleoli and the possible effects of colchicine and hypotonic treatments that are usually applied. Other phenomena, such as stickiness at metaphase and separation difficulties and fragmentation at anaphase, may result from persistence of nucleoli. Nucleoli were often associated with large chromosomes and sometimes at sites exhibiting faint or clear constrictions. The possibilities of a partial correspondence between sites of persistence and sites of organization, as well as of the organization of nucleolar materials at sites other than the main organizers, are discussed. The persistent nucleoli were not included in daughter nuclei. They either degenerated in the cytoplasm or were eliminated from the cell. The three systems used may represent different intensities of metabolism reflected in the amounts of nucleolar materials built up and the amount that persists.

INTRODUCTION

The nucleolar cycle is of interest in mitosis. It is thought that nucleoli usually break down at the early stages of mitosis so that by metaphase they are not detectable as organized structures. In many plant species, however, nucleoli have been found to persist during mitosis (e.g., references 2, 12, 29, 45, 53). Recently, Hsu et al. (21) have reported persistence of nucleoli in mammalian cells grown in vitro.

Nucleoli also have been found to persist in cancer cells (33, 40) and after treatments with cobalt (13) or thioacetamide (25). Arabinosylcytosine and fluorodeoxyuridine, known to inhibit or interfere with DNA synthesis, may cause or increase the frequency of persistence of nucleoli (15, 20, 27). By using cytochemical staining procedures, it has been demonstrated that certain kinds of ribonucleoproteins are distributed from the nucleus or the chromosomes to the cytoplasm during mitosis (32–34).

The present study deals with the appearance and behavior of nucleoli during mitosis in different systems, viz., long term and short term cultures as well as direct bone marrow preparations using differential staining with the Feulgen method and light green.

MATERIALS AND METHODS

Cells from the following sources were used:

Long term Monolayer Cultures

A male Chinese hamster cell line "Don," passages 41 to 46.

A human diploid cell strain "WI-38," derived from a female embryo lung, passages 23 to 30.

A diploid cell culture originated from a human testis, here called "HD," passages 2 to 11.

The Chinese hamster cell line was grown in Puck's medium supplemented with 20% calf serum and maintained in a 5% CO_2 atmosphere. For the two human cell cultures, Hank-Eagle's medium with 30% newborn calf serum was used. The cultures were grown in bottles and also on cover slips in Petri dishes.

Short term Cultures

Human peripheral blood leukocyte cultures were made as described by Moorhead et al. (36), with modifications of fixation and staining as described below.

Direct Bone Marrow Preparations

Bone marrow samples were obtained from the rat (*Rattus norvegicus*) by irrigation of the marrow cavity of the long bones of embryo rats.

Human bone marrow specimens were obtained by sternal aspiration from several patients suspected of having haematological disturbances, such as megaloblastic anemia or granulocytic leukemia.

The following technique was found to be a suitable one for the preservation, fixation, and differential staining of nucleoli and chromatin. The cells collected after short term or long term cultures were fixed directly, without any colchicine or hypotonic treatment. For an appropriate fixation of nucleoli, Kahle's modified fixative (46) composed of 95% ethyl alcohol–formaldehyde–glacial acetic acid (15:6:1) was used for 10 min. Bone marrow cells were maintained briefly in Hank's salt solution and were then treated similarly.

Kahle's modified fixative causes shrinkage and clumping together of cells. In order to have the cells in an analyzable condition, they should be made to swell and separate. For this purpose, the cells were treated with 1% Tween 80 for 1 to 2 hr, and 50% acetic acid for 1 to several hours until the white pellet of cells developed a transparent gel appearance. Although the acetic acid treatment alone may be sufficient, better spreading of cells and of chromosomes was achieved after a pretreatment with Tween 80.

After centrifugation, the acetic acid supernatant was carefully decanted and a drop or a few drops, depending on the size of the button, of 50% acetic acid were added and the cells were then resuspended. Squash preparations were made using the plastic cover slip method (38) and analyzed in a phase-contrast microscope in order to select good preparations to be stained and made permanent. After dissolving the cover slips in acetone, the slides were passed in two changes of acetone, acetone–absolute alcohol (1:1), and through an alcohol series before hydrolysis in 1 N HCl at 60° C for 8 to 10 min. The preparations were then differentially stained with the Feulgen method for the chromatin and light green as a nucleolar stain according to the method of Östergren et al. (see Reitalu, reference 40). The color pictures presented in Figs. 1 to 3 illustrate the differential staining.

Preparations were also made after colchicine and/or hypotonic pretreatments. In the case of pretreatment with colchicine, this was applied in a final concentration of 1×10^{-6} M for 2 hr. For a hypotonic treatment, medium was diluted with water (1:3 to 1:0.25) or the cells were suspended in sodium citrate (0.8%) for a period of 5 min.

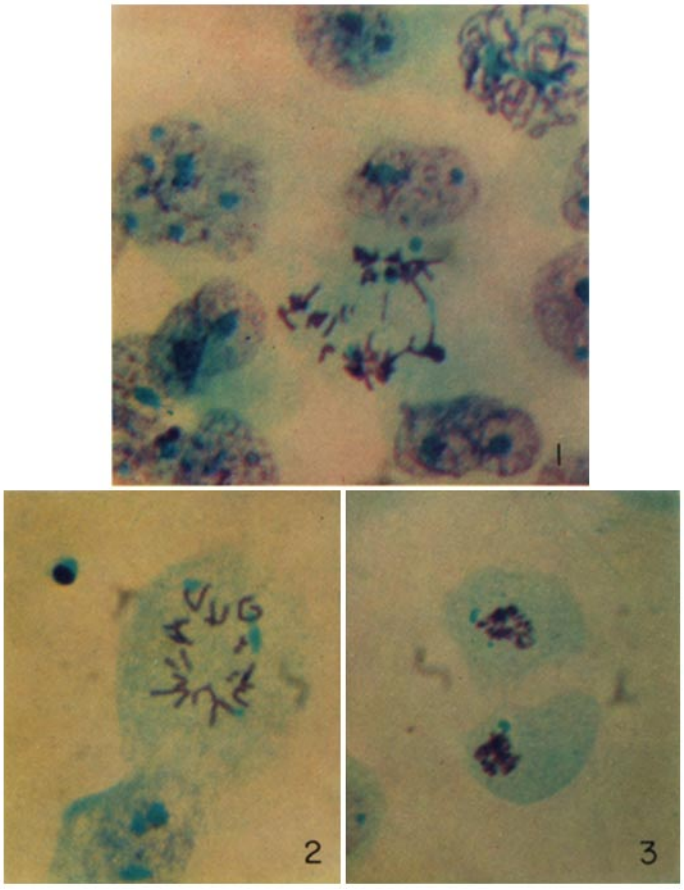
Cells grown on cover slips were washed quickly in a balanced salt solution before fixation in Kahle's modified fixative. The cover slips were then passed through an alcohol series before hydrolysis and staining, as mentioned above.

RESULTS

Effect of Colchicine and Hypotonic Treatments

During the initial phase of this study, trials with different modifications of the technique described above were made using the Chinese hamster cell line Don, the human cell culture HD, and human peripheral blood cultures in order to obtain preparations in which nucleoli were demonstrable in cells undergoing mitosis with the chromosomes in as analyzable a condition as possible.

When colchicine treatment was followed by a hypotonic treatment, as usually practiced in standard methods for preparation of metaphase chromosomes, good chromosome morphology was observed, but nucleoli seemed to disappear completely. Even a weak hypotonic treatment (1 medium to 0.25 water) for 5 min caused dissolution of nucleoli. A hypotonic treatment without



FIGURES 1 to 3 Persistence of nucleoli in the Chinese hamster cell line Don. Color photomicrographs illustrate the differential staining with the Feulgen reaction and light green.

FIGURE 1 Anaphase, with the nucleolar bridge associated with separation difficulties; other persistent nucleolar materials are close to the anaphase chromosomes. × 1210.

FIGURE 2 Metaphase, with persistent nucleoli mainly at chromosome ends. × 850.

FIGURE 3 Late telophase or early interphase, with persistent nucleoli close to the daughter nuclei but outside the nuclear membrane which starts to be visible. × 810.

preapplication of colchicine also caused dissolution and in most cases complete disappearance of nucleoli.

A colchicine treatment that was not followed by a hypotonic treatment caused stickiness and clustering of the chromosomes, but nucleoli were observed. Infrequently, metaphase plates with well spread and morphologically clear chromosomes were found, as shown in Fig. 4. In such plates persistent nucleoli could be seen, although sometimes faintly.

In Fig. 4, a colchicine treated endoreduplicated cell is clearly seen to have a large persistent nucleolus. The ends of 4 chromosomes are attached to this nucleolus. One of these chromosomes appears to be stretched at the end which is attached to the nucleolus by a faint nucleolar connection. This may imply an adhesive nature of nucleolar materials which may subject the associated chromosomes to stresses when mitotic or other forces are operative. The configuration shown in Fig. 4 (double-shafted arrow) is an example of the pos-

sible role of the persistent nucleoli in bringing about end-to-end associations between mitotic chromosomes. Smaller, individualized, or amorphous nucleolar materials are seen close to several chromosomes in this cell (arrows). It is possible that persistence of nucleolar materials could lead to lateral or any other possible kind of association as well as the end-to-end association, depending on the amounts and distribution of the materials along the chromosomes.

Long Term Cultures

The Chinese Hamster Cell Line Don

PERSISTENT NUCLEOLI AT METAPHASE: The Chinese hamster cell line Don has a modal number of 22 chromosomes. In the passages 41 to 46 studied here, aneuploidy and structural changes were frequently encountered. About 60% of the cells, however, appeared to have normal chromosomal constitutions.

Persistent nucleolar materials were of frequent occurrence at metaphase. In the majority of cases, nucleoli appeared as clear entities, usually rounded or oval in shape. They varied in size from small, hardly detectable structures to large conspicuous ones (Figs. 2, and 5 to 7). In some chromosome complements, persistent nucleolar materials were seen as droplets along the sides of many chromosomes (Fig. 9). These droplets were usually faint in color. They occurred in addition to (Fig. 5) or instead of the more darkly stained large nucleoli.

Sometimes, the persistent nucleolar material was in a fluidlike or amorphous form around the chromosomes and appeared as light green–positive substances on the surfaces of the chromosomes. This was usually encountered in metaphase plates that showed stickiness. The nucleolar materials enveloped chromosomes and were stretched between arms within or between chromosomes (Figs. 5 and 6).

The number of persistent nucleoli was determined in a total of 189 cells at metaphase and is presented in Table I. About 49% of the metaphase plates had three or four nucleoli, and only 3% had no nucleoli. Metaphase plates with many dropletlike or fluidlike nucleolar materials were not included in this table.

Persistent nucleoli were usually close to the chromosomes but occasionally separated from them. When associated with chromosomes, nucleoli were commonly adjacent to the ends (Figs. 2, 5, 6, 9, and 10). In Fig. 7 many nucleoli lie in the cytoplasm of a tetraploid cell. Some instances of nucleoli-chromosome associations are shown in Fig. 10. As can be seen in these pictures, nucleoli may be associated with any of the chromosomes in a complement. When in large numbers, they occurred adjacent to chromosomes of different sizes. Also, when the nucleolar material was persistent in a fluidlike form or had a dropletlike appearance, it was commonly found along the sides of most or all the chromosomes in the complement.

It seemed, however, that persistent nucleoli were more often associated with the large chromosomes, that is, chromosomes 1 and 2. The numbers used in this paper to designate Chinese hamster chromosomes are according to the system of Hsu and Zenzes (19). More than one chromosome can be associated with the same nucleolus (Figs.

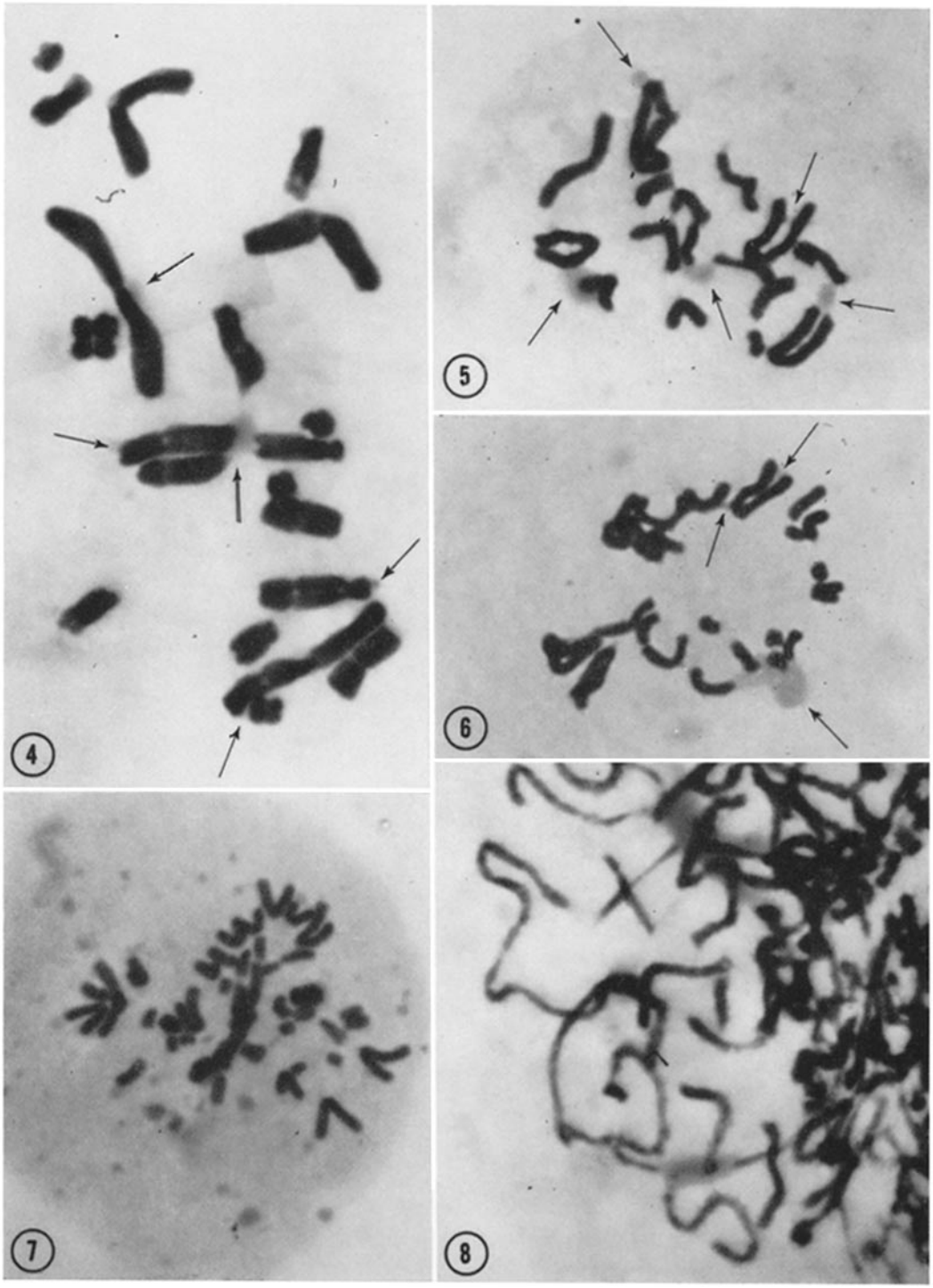
FIGURES 4 to 8 Metaphase and prophase chromosomes from the Chinese hamster cell line Don. Arrows indicate some of the persistent nucleoli.

FIGURE 4 An endoreduplicated cell at metaphase after a pretreatment with colchicine. The double-shafted arrow indicates the association of ends of different chromosomes with one large persistent nucleolus. × 2060.

FIGURES 5 and 6 Instances of stickiness between arms within and between chromosomes and of the presence of fluidlike or dropletlike nucleolar materials around and between chromosomes at metaphase. In Fig. 6, a large fusion nucleolus is present. × 1680.

FIGURE 7 Metaphase in a tetraploid cell with numerous persistent nucleoli in the cytoplasm. × 1680.

FIGURE 8 Late prophase, with association of telomeric ends of chromosomes with nucleoli. × 2060.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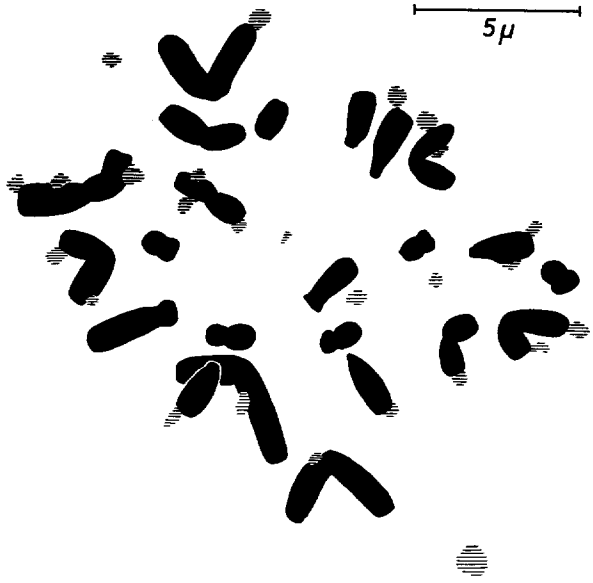

FIGURE 9 A cell from the Chinese hamster cell line Don, showing small dropletlike nucleoli associated with most of the chromosomes in the complement.

TABLE I

Number of Persistent Nucleoli at Metaphase in the Chinese Hamster Cell Line Don

No. of nucleoli	No. of cells	%
0	5	2.6
1	16	8.5
2	37	19.6
3	47	24.9
4	45	23.8
5	21	11.1
6	10	5.3
7	2	1.1
8	4	2.1
9	2	1.1
Total	189	100.1

5, 6, and 10 *p* to *r*). In such cases, nucleoli were usually large in size and may have resulted from fusion of more than one nucleolus. The association of telomeric ends of chromosomes with nucleoli was clearly demonstrated at late prophase stages, as seen in Fig. 8.

In some instances, a nucleolus was seen (Fig. 10 *a* to *c*) at the site of a secondary constriction present in the proximal region of the long arm of chromosome 1. The location of this constriction corresponds to the site in this chromosome which is highly susceptible to breakage after treatment with 5-bromodeoxyuridine (18). Nucleoli were also frequently seen close to the distal end of the long arm of chromosome 1, as seen in Fig. 10 *d* to *g*. No constriction was detectable at this specific site.

PERSISTENT NUCLEOLI AT ANAPHASE, TELOPHASE, AND INTERPHASE: Persistent nucleoli and chromosome bridges were of common occurrence at anaphase (Figs. 1, 11, and 12). Bridges and persistent nucleoli occurred in a frequency of about 54 and 78% (195 analyses), respectively. Both were seen in 44% of the cells (Fig. 11).

Persistent nucleoli at anaphase were usually close to the two daughter groups of chromosomes. In 40 anaphases that had nucleoli, 35 cells (about 88%) had most or all of the nucleoli adjacent to the chromosomes forming the two polar groups (Figs. 1 and 11). One might have expected that at a later stage of development most of these persistent nucleoli would be adherent to or included in the daughter nuclei. This did not seem to be the case, however. In 59 cells that were in late anaphase or telophase and which contained persistent nucleoli, only 5 cells (about 8%) had nucleoli close to daughter nuclei (Fig. 3). In the majority of cells (about 92%) persistent nucleoli occurred in the cytoplasm away from the two daughter nuclei (Fig. 12). Thus, it is most likely that the persistent nucleoli, although very close to the two polar groups of chromosomes at anaphase and sometimes even early telophase, become separated from them by late telophase.

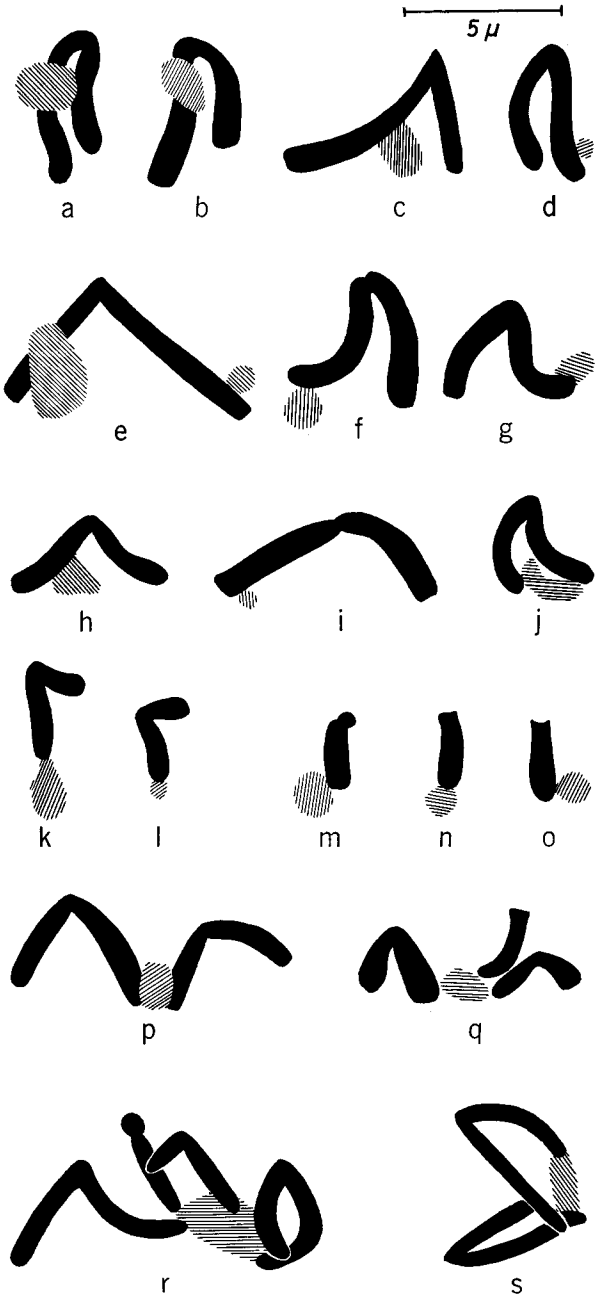

FIGURE 10 Instances of associations between nucleoli and chromosomes of the Chinese hamster cell line Don. Associations are mainly at or near the ends of the large chromosomes; *a* to *c*, associations of nucleoli with chromosome 1 at or close to a site of a faint constriction in the proximal region of the long arm; *d* to *g*, associations of nucleoli with a site close to the distal end of the long arm; *h* to *j*, associations with chromosome 2; *k* and *l*, chromosomes in the group X-Y-4-5; *m* to *o*, chromosomes in group 6 to 8; *p* to *r*, association of more than one chromosome end with persistent nucleoli; *s*, daughter chromosomes of chromosome 1 at early anaphase, both ends associated with the nucleolus.

Even when nucleoli were close to daughter nuclei, they appeared to be outside the nuclear membrane (Figs. 3 and 13).

At late telophase and early interphase stages, the "relic" nucleoli present in the cytoplasm were more darkly stained than the newly organized nucleoli inside the daughter nuclei. In later stages, the relic nucleoli became fainter, whereas the "new" nucleoli inside the nuclei became larger in size and more stainable (Fig. 14).

By the time the cells were in full interphase, the nucleoli organized *de novo* in the nucleus were large in size and darkly stained, while the persistent nucleoli were not detectable. They either

have degenerated or have been eliminated. The probable elimination of bodies which could be relic nucleoli can be seen in cells grown on cover slips, as seen in Fig. 15.

The Human Cell Cultures

PERSISTENT NUCLEOLI AT METAPHASE: The human cell cultures HD and WI-38 have normal diploid chromosome complements. Persistent nucleoli were of common occurrence in these cultures, although to a lesser extent than in the Chinese hamster cell line Don. Most of the metaphase plates (35%, 68 cell analyses) had one or two nucleoli. A maximum of seven to nine nucleoli were seen in a few cells. About 26% of the metaphase plates had no detectable persistent nucleoli.

Nucleoli in the human material apparently fused together, giving rise to a few large nucleoli, sometimes closely associated with chromosomes (Figs. 16 and 17), but more commonly separated from them (Fig. 17).

As in the Chinese hamster, nucleoli in the human cells were more often associated with large chromosomes. Nucleoli occurred frequently at the ends of chromosomes 1 to 3, also at the middle or distal third of the long arm of a chromosome from the 4 to 5 group (Fig. 22 *c* to *f*). The site of persistence in chromosomes 4 to 5 corresponds approximately to the position of the secondary constriction seen in these chromosomes (11). Instances of nucleolar associations with satellited ends of acrocentrics (chromosomes 13 to 15 and 21 to 22) were also found. This is demonstrable in earlier stages such as late prophase, as seen in Figs. 18 and 22 *g* to *i*. In these figures, associations of more than one acrocentric chromosome with the same nucleolus can be seen.

PERSISTENT NUCLEOLI AT ANAPHASE, TELOPHASE, AND INTERPHASE: In contrast to the Chinese hamster cell line Don in which nucleoli were usually close to the polar chromosomal groups in anaphase but separated from them at telophase, the human cell cultures HD and WI-38 revealed nucleoli that were usually separated from the polar groups in both anaphase (Fig. 19) and telophase.

As in the Chinese hamster cell line Don, "relic" nucleoli did not seem to be included in the daughter nuclei. In Figs. 20 and 21, two instances are presented in which degenerating nucleoli were adjacent to but outside the nuclear membranes.

Persistence of Nucleoli and Associated Aberrations

Persistent nucleoli were found to be associated with instances of separation difficulties and lagging of chromosomes (Figs. 1 and 23). Nucleoli at chromosome ends can cause stickiness of the two separating chromatids at anaphase. In most cases stickiness occurred at one chromosome end (Fig. 1), and the arms of the separating daughter chromatids sometimes became attenuated as a result of the pulling forces of the centromeres and the adhesion of ends to a nucleolus. Sometimes, this led to the formation of a nucleolar bridge between the two separating daughter chromosomes (Fig. 1). A nucleolar bridge between two daughter cells is seen in Fig. 24. The appearance of the lagging nucleolar material in the cell presented in Fig. 25 indicates partition or division of a lagging

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FIGURES 11 to 15 Cells from the Chinese hamster cell line Don, demonstrating persistence of nucleoli at anaphase and interphase.

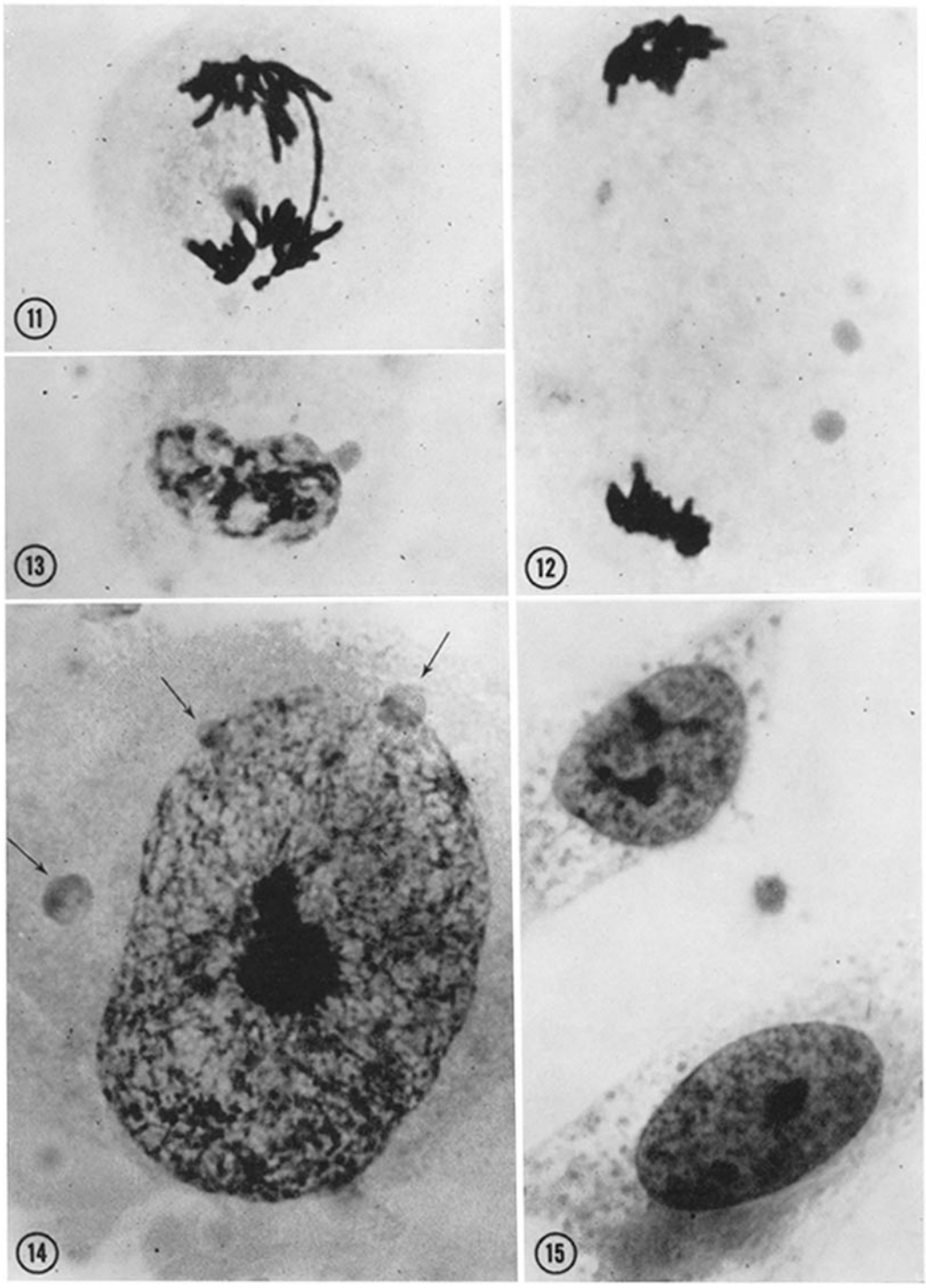
FIGURE 11 Anaphase, with a large nucleolus close to one polar group. × 1970.

FIGURE 12 Persistent nucleoli away from polar groups at anaphase. ×1970.

FIGURE 13 Early interphase, with relic nucleolus outside the nuclear membrane. × 1610.

FIGURE 14 A polyploid cell in interphase, with three relic nucleoli (arrows) in the cytoplasm. × 1970.

FIGURE 15 Elimination of a body that resembles a relic nucleolus from cells grown on a cover slip. × 1970.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persistent nucleolus and the movement of daughter nucleoli towards the poles, as a result of their chromosomal attachments.

Single or paired chromatid fragments lagging at anaphase were sometimes associated with or imbedded into nucleoli (Figs. 26 and 27), possibly owing to breakage accompanying separation difficulties in segments associated with persistent nucleoli. Persistent nucleoli were also frequently found close to chromatid bridges.

In the human cell culture HD, dumbbell-shaped nuclei commonly occurred (Figs. 28 and 29). The chromatin material at the furrow sometimes appeared stretched, connecting the two lobes of the nucleus (Fig. 28). These bilobed nuclei were probably restitution nuclei resulting from fusion of the two daughter nuclei. Chromatin bridges, separation difficulties, spindle abnormalities and persistence of nucleoli might lead to the formation of a bilobed nucleus.

The position of nucleoli in these bilobed nuclei was of interest. Nucleoli were usually in the proximity of the furrowed region, on either side of it (Fig. 28), or formed a dumbbell-shaped nucleolus which occupied the furrow and its proximal regions (Fig. 29). This may indicate that the nucleolar organizing sites were in close proximity to the furrow region. These sites might be in the chromosomes which have caused the separation difficulties and restitution in the previous division. Apparently, these dumbbell-shaped nuclei were at early interphase or mid-interphase. Either they become rounded at a later stage, since no bilobed nuclei were found entering mitosis, or they were sufficiently abnormal to prevent additional division.

The Maximum Number of Nucleoli at Late Telophase or Early Interphase

During the mitotic cycle, late anaphase and telophase are considered to be the most suitable stages for determining the maximum number of nucleoli. However, in the present material these nucleoli could more easily be determined in late telophase or early interphase. After their formation, nucleoli usually fuse together, thus becoming fewer in number than when originally organized. When cells at late telophase or early interphase are being analyzed, the levels of polyploidy should also be considered. The size of nuclei may give a relative measure for differentiating diploid from polyploid cells. This is not, however, an absolutely accurate way, and should be checked with microspectrophotometric methods.

The maximum number of nucleoli was determined in nuclei inferred, from their size relationships, to be probably diploid, and was eight (in one instance ten) in the Chinese hamster cell line Don. This indicates the possibility of the presence of eight or ten centers or sites of nucleolar organization in these cells. In the human cell culture HD, a maximum number of nine nucleoli was found.

Short Term Cultures and Direct Bone Marrow Preparations

In short term cultures of human peripheral blood leukocytes, persistent nucleoli were observed at metaphase and anaphase, but to a much lesser degree than has just been described for long term cultured cells. The nucleolar material appeared mainly in a diffuse form and was sometimes associated with chromosomes showing stickiness. In Figs. 30 and 31, two metaphase plates are exhibited. In one of these figures (Fig. 31), a nucleolus is associated with an acrocentric chromosome. Persistent nucleolar material at anaphase is shown in Fig. 32.

Infrequently, persistent nucleoli were also found in direct preparations made from rat and human bone marrow. Nucleoli were hardly de-

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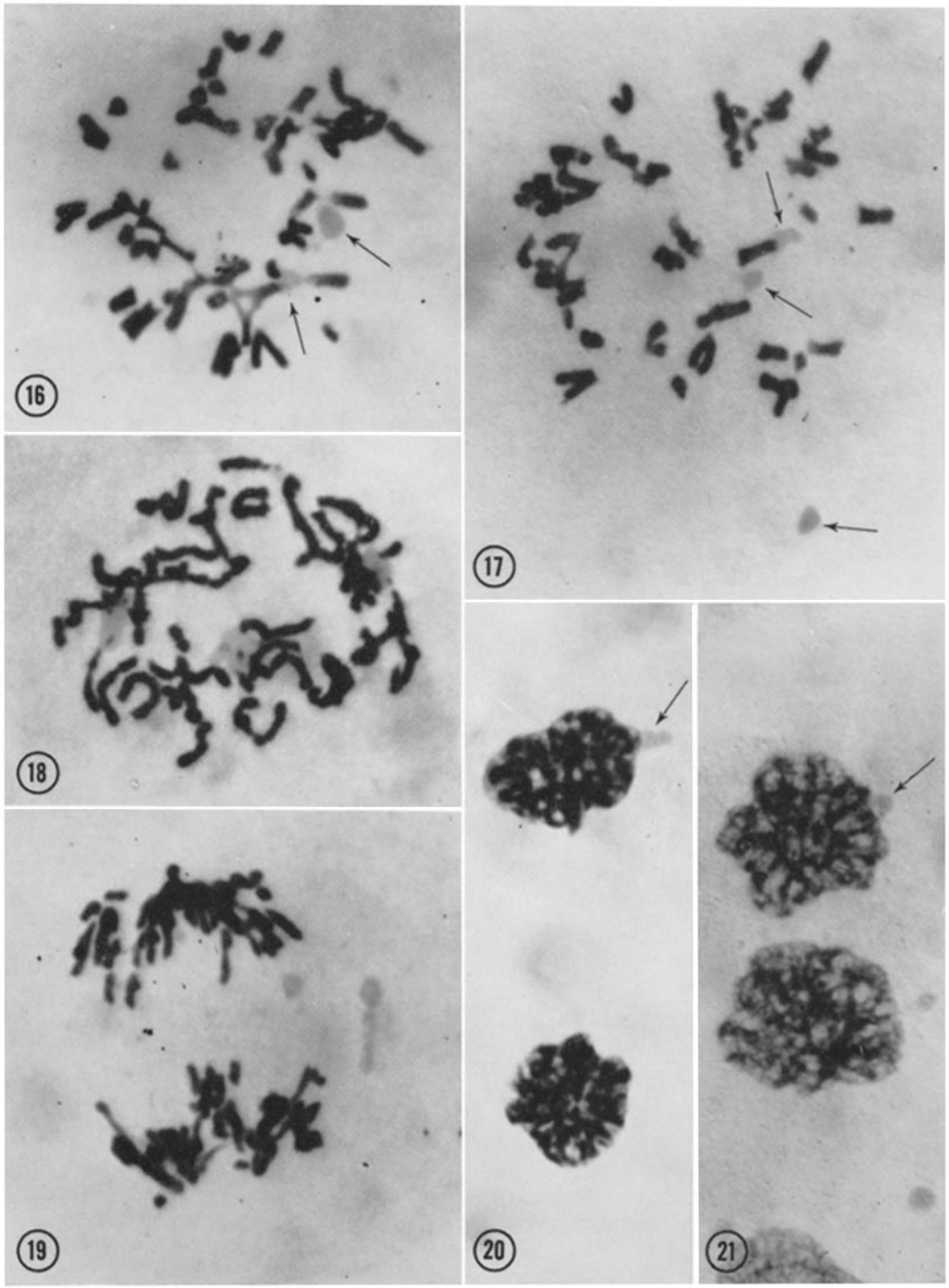
FIGURES 16 to 21 Mitotic stages from the human culture HD. Arrows indicate some of the persistent nucleoli. × 1970.

FIGURES 16 and 17 Metaphases, persistent nucleoli are both associated with chromosome ends and separated from them.

FIGURE 18 Late prophase, association of acrocentrics with nucleoli.

FIGURE 19 Anaphase, with persistent nucleolar materials not closely associated with the anaphase chromosomes.

FIGURES 20 and 21 Telophases with relic nucleoli outside the nuclear membrane.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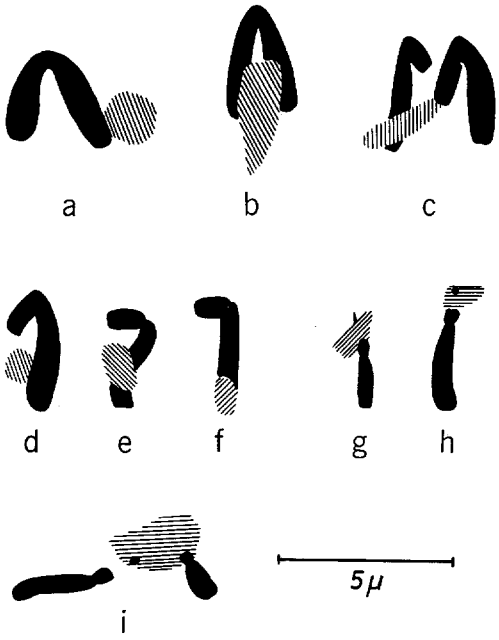


FIGURE 22 Some instances of chromosome-nucleolus associations at metaphase and late prophase in the human culture HD; *a* to *c*, nucleoli associated with large chromosomes from group 1 to 3; *c* to *f*, chromosomes from group 4 to 5; *g* to *i*, association of nucleoli with satellited ends of acrocentrics.

tectable in bone marrow cells because of the small cell size and the fact that chromosomes were usually condensed together in these cells, which did not respond well to the swelling agents applied. The amount of the persistent nucleolar material was markedly less than in the other systems studied. Fewer nucleolar materials were also present in interphase nuclei of marrow cells compared to short term cultured cells and these in turn if compared with cells grown in long term cultures. An example of persistent nucleoli at metaphase of human bone marrow cells is shown in Fig. 33.

DISCUSSION

Persistent Nucleoli

That the persistent light green–positive materials seen at metaphase were nucleolar materials is inferred from the observation that their staining reaction was similar to that of nucleoli present at prophase or interphase. Many of the sites of this persistent material correspond to the sites at which nucleoli occur at prophase. The nucleolar nature of the persisting materials is also supported by the cytochemical, radioautographic, and electron microscope observations made in similar studies recently reported by Hsu et al. (21).

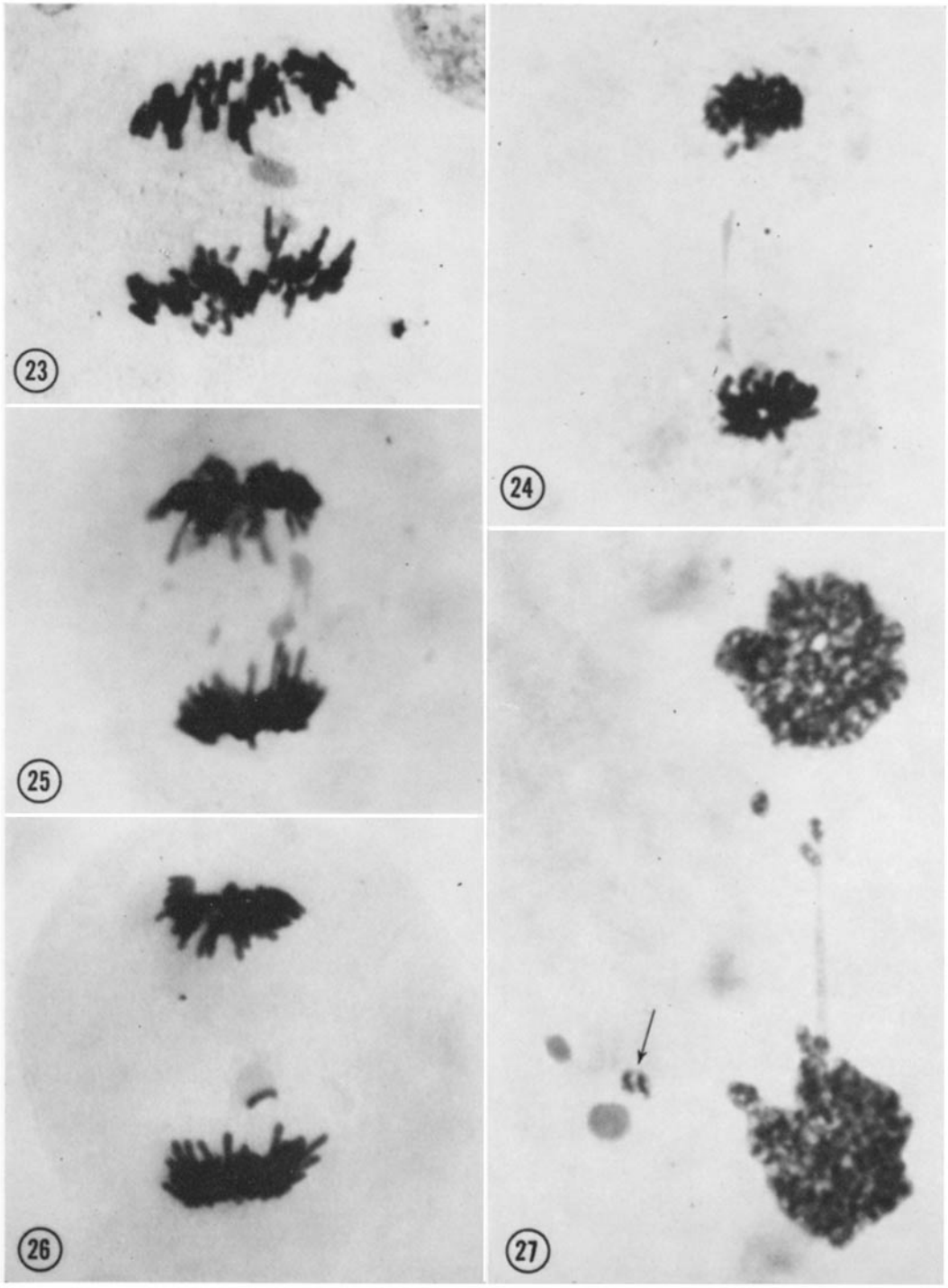
The observation that nucleoli persist in the different systems studied, viz. long term and short term cultures, and to a much lesser degree in direct bone marrow preparations, confirms and extends the observations of Hsu et al. (21) and implies that the phenomenon may occur naturally in mammalian cells. When the usual cytological methods using colchicine and hypotonic treatments are applied, nucleoli escape detection. Previously, persistence of nucleoli had been observed in various plant materials (e.g. 2, 12, 29, 45, 53). The behavior and the structural and functional characteristics of nucleoli are discussed in the reviews by Sirlin (45) and Busch *et al.* (3).

Nucleoli have been found to persist in cancer cells (33, 40) and after treatments with certain agents such as cobalt (13) and thioacetamide (25). Also, it has been reported that in the case of HeLa cells treated with puromycin the cytoplasm contained nucleoluslike inclusions (49, 50). Jones (22, 23) observed "ribosomal bodies" that were constituted from relic nucleolar materials in the cytoplasm of dividing erythroblasts.

It is likely that agents, such as arabinosylcytosine (araC) and 5-fluorodeoxyuridine (FUdR), that are known to inhibit or interfere with DNA synthesis but not RNA synthesis cause an increased frequency of persistent nucleolar materials. This has been found in grasshopper neuroblasts (27) and in the human cell line WI-38 (15). It has been suggested also for the Chinese hamster cells (20, 21). With different staining procedures using toluidine blue–molybdate, it has been shown that certain kinds of ribonucleoproteins, some of nucleolar origin, are extruded from the nucleus into the cytoplasm at the onset of metaphase (32–34).

Role of Persistent Nucleoli in Chromosome Associations

Late disappearance or persistence of nucleoli could very likely cause the association of acrocentrics in human, reported by many workers (e.g. references 10, 37) and referred to as "rosette" formations (16), and also the end-to-end associations observed here in the Chinese hamster and



FIGURES 23 to 27 Persistence of nucleoli and associated aberrations. All cells are from the human culture HD except that of Fig. 24 which is from the Chinese hamster cell line Don. × 2060.

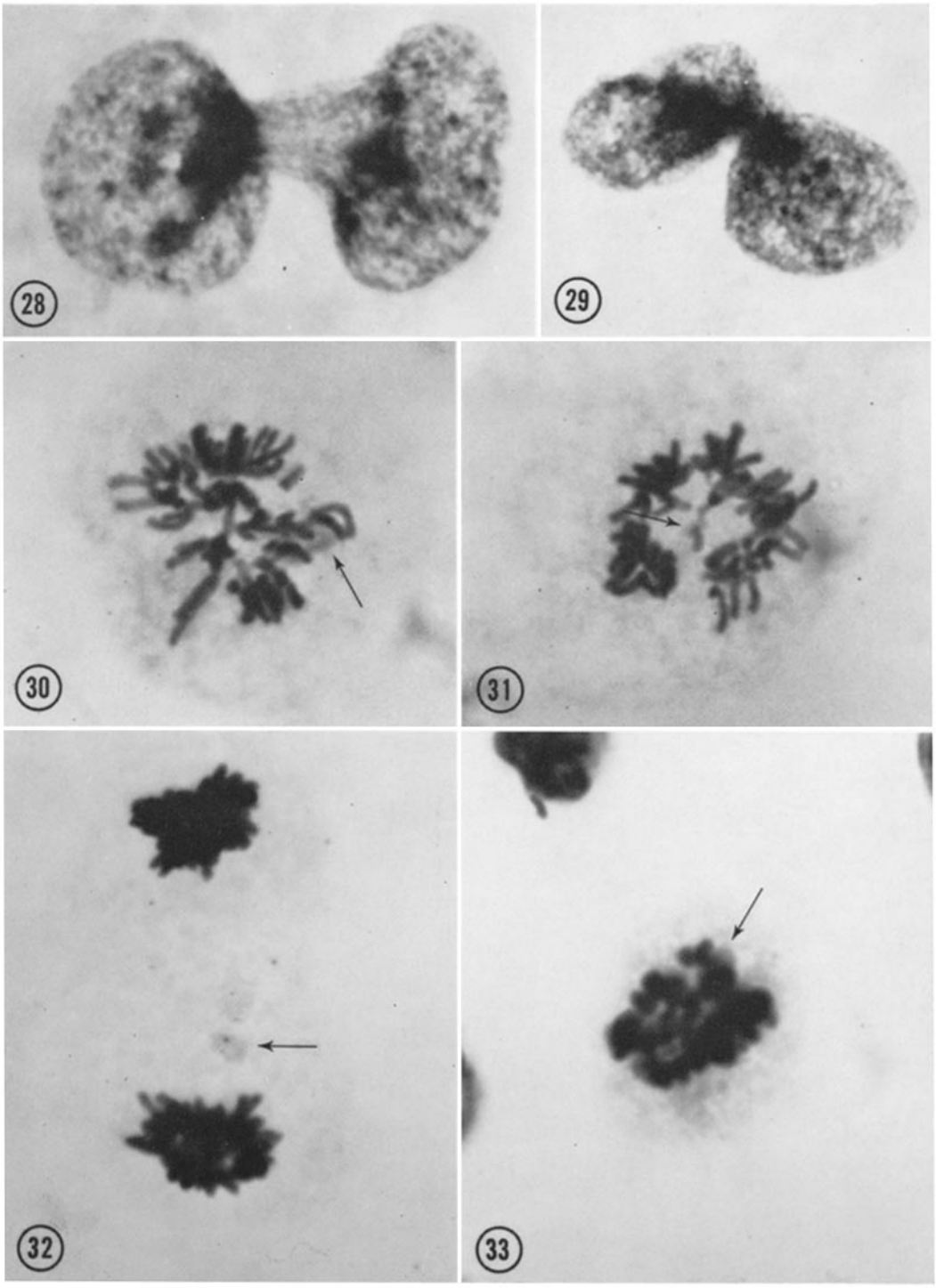
FIGURE 23 Nucleolar material associated with chromosomes slightly lagging behind the rest of the anaphase chromosomes.

FIGURE 24 Nucleolar bridge between two daughter cells.

FIGURE 25 Nucleolus apparently partitioned by movement of the daughter chromosomes associated with it.

FIGURE 26 A lagging fragment or daughter chromosome associated with a nucleolus.

FIGURE 27 Telophase, with two chromatid fragments embedded in nucleolar material (arrow); two persistent nucleoli are nearby.



FIGURES 28 and 29 Dumbbell-shaped restitution nuclei from the human culture HD. Nucleoli are organized on either side of the furrow (Fig. 28) or appear as dumbbell-shaped nucleoli occupying the furrowed region of the nucleus (Fig. 29). × 2220.

FIGURES 30 and 31 Metaphase plates from short term cultures of peripheral human blood. Persistent nucleoli are indicated by arrows. In Fig. 31, an association between a nucleolus and one acrocentric chromosome is shown (arrow). × 2220.

FIGURE 32 Anaphase in a human peripheral blood culture exhibiting lagging nucleolar material (arrow). × 2220.

FIGURE 33 Metaphase in a human bone marrow cell from a direct preparation with persistent nucleolar material (arrow). × 2680.

reported by Yerganian and Papoyan (55) in the gray hamster *Cricetulus migratorius*. Among the known colchicine effects is the clumping of chromosomes in star or ball metaphases (7). Clustering of chromosomes produced by colchicine treatment brings together nucleoli associated with different chromosomes. The subsequent hypotonic treatment causes swelling of the cells accompanied by spreading of the chromosomes, and also gradual, probably incomplete, dissolution of the nucleolar material which may maintain these associations. Nucleolar remnants on the short arms of a small acrocentric in human have been reported by Levan and Hsu (30). The intermingling of satellite strands or the possible attractions between heterochromatic nucleolar segments might cause the associations of satellited ends to be more resistant to the effects of the hypotonic treatment than end-to-end associations in general. That the acrocentrics are nucleolar organizers and exhibit associations between themselves and nucleoli has been illustrated in pachytene by Ferguson-Smith (9). Yerganian and Papoyan (55) interpreted the end-to-end associations as being due to common participation in nucleolar formation or to molecular similarities between chromosome ends leading to mutual attraction.

Sites of Nucleolar Persistence Related to Sites of Nucleolar Organization

The persistent nucleolar material when present in a fluid or dropletlike condition is usually attached to or associated with most of the chromosomes in the complement. This has been nicely elucidated with methyl green–pyronin and azure B staining procedures (21). When the nucleolar material is in the form of a number of discrete bodies, these may be associated with any chromosome, although most frequently with certain chromosomes in the complement. The possibilities of a partial, total, or no correspondence between sites of persistence and sites of organization are to be considered.

The nucleolar conditions at prophase, metaphase, or anaphase could represent an altered situation from the preceding telophase when the nucleoli were organized. Nucleoli might have fused, as they often do in interphase. There is also the possibility that they are partitioned at the onset of mitosis when the chromosomes start to spiralize and condense.

However, there are some indications which point to the possibility that some of the chromosomes and sites along which nucleoli persist might be engaged in organization or collection of nucleolar material. Thus, in the Chinese hamster cell line Don and the human culture HD, it was repeatedly observed that persistent nucleoli are associated with certain chromosomes and more often at certain sites along these chromosomes. The frequent association of persistent nucleoli with the largest chromosome in the complement (chromosome 1 in the Chinese hamster and in human) may indicate the occurrence in this chromosome of an important organizing center. Alternatively, if all the chromosomes take part in the process of nucleoli organization, more material will be synthesized along this chromosome which is the largest in the complement. Besides chromosome 1 and except for the small chromosomes 9 to 11 in the Chinese hamster, persistent nucleoli were associated mainly with long arms of the other chromosomes in the complement. In human, in addition to chromosome 1, long arms of chromosomes 2 to 5 and the satellited ends of chromosomes 13 to 15 and 21 to 22 showed frequent associations with nucleoli. Besides the observation that acrocentrics are nucleolar organizers (9), large chromosomes (possibly No. 1 and/or No. 9) have been questioned as to their role in nucleoli organization (16, 44). At late telophase stages in the Chinese hamster and human cells, at least eight to ten sites for nucleoli organization are inferred from the maximum number of nucleoli seen at this stage.

The association of nucleoli with chromosome ends is represented more clearly in the Chinese hamster cell line Don than in the human culture HD. The main sites for organizing nucleoli in the Chinese hamster may be located close to chromosome ends. Organization of nucleolar materials along the whole chromosome with accumulation at the ends could be an alternative or complementary process.

Recent works with electron microscopy (e.g. references 26, 47) have elucidated the organization of nucleoli both at the main nucleolar organizer (14) and at other sites along the chromosome. The nucleolar organizers, besides being the main producers of nucleoli, are also considered centers for collection of nucleolar substances organized at other sites along the chromosome (e.g. reference 52).

Nucleoli are known to be metabolic centers for RNA turnover. The use of labeled precursors has provided information on RNA synthetic patterns in the nucleolus as well as in the rest of the nucleus or in the chromosomes. Information gained from incorporation of labeled RNA precursors indicates that synthesis of the different kinds of RNA takes place both in individualized nucleoli and in other organizing centers in the chromatin (e.g., references 24, 39, 54). Feinendegen and Bond (8), using tritiated cytidine in HeLa cells, found that the labeled RNA in the prophase nucleus is partly contributed to the cytoplasm and partly carried with the chromosomes at metaphase. They found no evidence that the carried fraction of RNA is utilized for nucleoli organization at telophase. In neuroblasts of *Chortophaga viridifasciata* (DeGeer), nucleoli appear at mid-telophase before the start of RNA synthesis, and it has been suggested that RNA synthesized at mid-prophase and distributed to the cytoplasm at mid-mitosis is reincorporated for the organization of nucleoli at mid-telophase (42). Possible functional considerations of the nucleolar materials extruded from nuclei in fertilized eggs have been discussed by Szollosi (51). Extrusion of nucleoli or nucleolar material from nuclei has been reported also in tissue culture cells (e.g. references 28, 31).

Some of the sites at which nucleoli persist at late prophase or metaphase correspond both to secondary constrictions, such as those seen in the proximal region of the long arm of chromosome 1 in the Chinese hamster and the long arm of chromosomes 4 to 5 in man, and to constrictions such as those seen in the satellited ends of the acrocentrics in man. This indicates that these constrictions are likely to represent nucleolar organizers and/or centers at which nucleolar material collects. It might also be of interest to point out that chromosomes with which persistent nucleoli were frequently associated, such as the two largest chromosomes in the Chinese hamster and chromosomes 1 and 4 to 5 in man, are among the chromosomes which have late replicating segments in these two species (17, 43). In human, Schmid (43) pointed out the possible correlation between secondary constrictions and late replication. Late replicating regions in the Chinese hamster chromosomes (17, 48) correspond to extended or stretched segments induced by a colchicine treatment (48). The interrelationships between secondary constrictions, late replication, and persistence of nucleoli need further study.

Role of Persistent Nucleoli in the Production of Aberrations

The presence of persistent nucleoli in connection with the different aberrations at anaphase, such as separation difficulties and fragmentation, is of interest. From the present study it appears that difficulties of separation, fragmentation, lagging of daughter chromosomes, and mitotic nondisjunction might be caused by persistence of nucleoli. This does not eliminate the possibility that the persistence of nucleoli, under some of these conditions, is just an association with other factors such as stickiness or breakage and reunion. Persistence of nucleolar material could also be partially responsible for the restitution and formation of bilobed nuclei. The possibility that persistence of nucleolar materials might lead to stickiness has been pointed out also by Hsu et al. (21). Persistence of nucleoli can be added to the mechanisms that could lead to separation difficulties and pseudo chiasma formation. These mechanisms and the possible significance of such mitotic aberrations in cell differentiation have been discussed by Melander (35).

Fate of Persistent Nucleoli

Of interest is the fate of the persistent nucleolar materials in the cells undergoing mitosis. From the observations made here, it is apparent that these substances are usually not included, at least as such, in the daughter nuclei. That persistent nucleoli are not included in daughter nuclei has also been found in other materials (e.g. references 13, 33) and in time-lapse studies of living material (1). However, Hsu et al. (21), from a preliminary study using electron microscopy, pointed out that persistent nucleoli appear to be included in daughter nuclei. The degeneration or dissolution of nucleoli in the cytoplasm and possible reincorporation into the nucleus needs elucidation. The possibility that persistent nucleoli could be eliminated from cells should also be considered.

Amounts of Nucleolar Materials in the Different Systems Studied

The amount of persistent nucleolar material was more pronounced in long term cultured cells compared to short term cultured cells, and these

in turn had more than the bone marrow cells analyzed directly without preculturing. More nucleolar materials were also found at interphase in the long term cultured cells compared to the other systems. The occurrence of persistent nucleoli might result from the presence of increased amounts of nucleolar material. It remains to be determined what the nature is of the persistent nucleolar materials compared with those consumed, used up, or incorporated into the chromosomes, and also compared with the fraction that is distributed to the cytoplasm.

The transformation of the small lymphocytes of human peripheral blood into blast cells after a short term culture with phytohaemagglutinin has been shown to be accompanied by an increased metabolism of these cells (41). A pronounced increase in RNA synthesis has been found in the blast cells (6). The three systems presented here, viz. long term culture, short term culture, and direct preparation, might represent different magnitudes of metabolism reflected in the building up of nucleolar materials and also the persistence of nucleoli. It has been pointed out by Kleinfeld and Von Haam (25) that thioacetamide induces an increased rate of nuclear RNA synthesis and ribonucleoprotein build up. These authors questioned the possibility that persistence of nucleoli under these conditions may be an expression of the increased amounts of nucleolar materials. It has been emphasized by Caspersson (4, 5) that a high RNA content in nucleoli characterizes cells active in protein synthesis.

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