Histol Histopathol (1996) 11: 943-963

Histology and Histopathology

Meiosis in hematological malignancies. In situ cytogenetic morphology

H. Logothetou-Rella
Department of Experimental Physiology, Medical School, University of Athens, Athens, Greece

Summary. This is the first study on the in situ cytogenetic morphology and analysis of malignant bone marrow cells, growing attached on a culture vessel surface. It was documented that bone marrow cells, in different types of hematological malignancies, divide by meiosis giving rise to a non-repetitive aneuploidy. Male and female gametes are formed by meiosis and fertilization occurs in a life cycle of:

Gametes —Fertilization→ Embryo —Meiosis→ Gametes

Immature and mature somatic oocytes were evidenced by prophase stages of the first and diploid or hypodiploid or haploid metaphases identical to those of the second human, ovarian oocytic meiotic division, showing «XX» or «XY» sex chromosomes in female or male patients respectively. Nuclear vlimata were the male gametes showing a condensed head with tail morphology. Metaphases of nuclear vlimata were aneuploid, keeping the head with tail shape, carrying chromosomes identical to those of human spermatogonia. Somatic metaphases identical to those of human spermatocytes in meiosis II and spermatogonia were demonstrated. The process of fertilization was documented by nuclear vlima invasion into host cell metaphases, by metaphases of fertilized oocyte showing both the female chromosome «0» and the male chromatids «s» and hybrid metaphases of oocytic with somatic chromosomes. Meiosis was characterized by nuclear extrusion of chromosomes, meiotic, condensed chromosomes, nuclear vlimata, metaphase and nuclear fusion, hybrid metaphases, nuclear budding, nuclear conglomerates, nuclear bridges, chromosomal fusion substance, transfer of chromosomes and non-repetitive aneuploidy. Meiotic, Double Minute, ring and minute chromosomes extruded by nuclei were loaded with glycoproteins, glycosaminoglycans and calcium-activated neutral proteinase, distinguishing them from mitotic chromosomes. The main characteristic events of meiosis, observed in malignant bone marrow cells, were also demonstrated in fungal and rat testicular cells, known to divide by meiosis.

Key words: Nuclear vlimata, Oocytes, Fertilization, Leukemia, Cytogenetic morphology, Fungi, Testis, Calcium-activated neutral proteinase

Offprint requests to: Dr. Helen Logothetou-Rella, Department of Experimental Physiology, Medical School, University of Athens, P.O. Box 60114, GR-153 10 Agia Paraskevi, Athens, Greece

Introduction

It has recently been reported that malignant (Logothetou-Rella, 1994a), embryonic (Logothetou-Rella, 1995a), virally infected cells (Logothetou-Rella, 1995b) and phytohaemaglutinin (PHA) activated lymphocytes (Logothetou-Rella, 1994b) divide by meiosis. Meiosis in somatic cells exhibits the following characteristics: Nuclear vlimata (NVs) formation, cell to cell invasion, cell fusion into syncytia, nuclear fusion into nuclear conglomerates (NCs), metaphase fusion into hybrid cells, meiotic chromosomes, nuclear segments, fragmentation, budding and aneuploidy. Nucleus to nucleus communication is accomplished via nuclear bridges (NBs) through which chromosomes are transferred among different types of cells. Daughter cells of meiosis are parasitic and invade host cells by a process identical to sperm-oocyte fertilization or cell viral infection (Logothetou-Rella, 1994a, 1995a).

Calcium-activated neutral proteinase (CANP) is a meiotic protease found in NVs, NCs, meiotic chromosomes and extracellular matrix (ECM). Chromosome transfer and attachment on recipient host nuclei involves the chromosomal fusion substance (CFS), composed of glycoproteins, and glycosaminoglycan (GAG) bound CANP. Hence the inhibitor of CANP (CANP-I) is an anti-meiotic agent with spermicidal, anti-viral, anti-cancer and anti-inflammatory properties (Logothetou-Rella, 1994b,c, 1995a,b).

Meiosis in hematological malignancies has been documented cytologically (Logothetou-Rella, 1993). It is the purpose of this study to provide further evidence on meiosis of malignant bone marrow cells (BMC) using in situ cytogenetic morphology and analysis.

Materials and methods

Isolation and growth of bone marrow cells

A total of thirteen cases of hematological malignancies were examined. One case of erythroleukemia (EL), four cases of acute myeloid leukemia (AML-1, AML-2), two cases of acute myelomonocytic leukemia (AMMoL-M4), two cases of undifferentiated leukemia (UL-1, UL-2), one case of chronic myelogenous leukemia (CML) and three cases of acute lymphoblastic leukemia (ALL). Patients had received no treatment prior to aspirate collection.

Nucleated BMC were isolated from heparinized marrow aspirates by density gradient centrifugation (Boyum, 1968). Isolated BMC were seeded in glass petri dishes and 25 cm^2 Costar culture flasks, in RPMI-1640 (Gibco) supplemented with 10% foetal bovine serum (Gibco), penicillin (100 U/ml, Gibco), streptomycin (100 μg/ml, Gibco) and PHA. BMC were cultivated at 37 °C in a CO_2-humidified incubator for 24 to 72 hours.

BMC samples streaked in sabouraud and blood agar were incubated for 10 days for detection of possible microbial infection, or contamination.

Cytogenetic analysis, morphology, cytology and immunocytochemistry

Cytogenetic analysis of 24-48 hours BMC cultures was performed by the chromosomal spreading technique, standard Giemsa, RhG banding of Dutrillaux and Lejeune (1971) and in situ, by standard Giemsa. One to two hundred consecutive metaphases were studied in each case, including meiotic and somatic regular metaphases, as previously described (Logothetou-Rella, 1994a). For the in situ technique, petri dish BMC cultures were treated with hypotonic solution (KCl 0.075M) for 10 min, fixed in 3:1 ethanol:acetic acid, dried and stained with Giemsa.

Chromosomal preparations, besides karyotyping, were also evaluated for cytogenetic morphology, such as chromosomal morphology (condensed vs regular chromosomes) within the same metaphase and cell population, intensity of Giemsa chromosomal staining (hyperchromatic, hypochromatic), NVs, nuclear morphology, nuclear fusion, chromosomal extrusion, nuclear fragmentation and meiotic metaphases. BMC petri dish cultures treated with KCl (0.075 m) for 10 min, were fixed in 4% formaldehyde in PBS and stained with Papanicolaou (HT-Papa) for detection of nuclear bridges.

Bone marrow tissue pieces in the aspirates, from all cases, were treated with KCl (0.075 M) for 10 min, fixed in 3:1 ethanol:acetic acid for 24 hours, followed by 6:4 acetic acid:distilled water treatment for 5 min, smeared on glass slides, dried and stained with Giemsa for direct cytogenetic evaluation.

Cultured BMC and bone marrow tissues (BMT), treated with KCl (0.075M) for 10 min, smeared on slides, were fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for PAS, PAS-D, Feulgen (without counterstain) Gram staining and immunocytochemistry.

For immunocytochemical studies the avidin-biotin peroxidase complex method was applied (Hsu et al., 1981) using the antiserum against α_1-chymotrypsin (1:100, A022, Dako Corp.). Positive controls were tested for α_1-antichymotrypsin reactivity. Negative controls were used by omitting the primary antiserum.

BMC aspirates suspended in RPMI-1640 were spun at 400g for 10 min. The cell pellet was fixed in formalin, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin-eosin.

Cytogenetic morphology of meiotic fungal and rat testicular cells

Fungal cells growing in sabouraud were collected in PBS, spun at 500 g for 10 min, treated with KCl (0.075M) for 10 min, spun, spread on slides, fixed in 4% formaldehyde in PBS and used for immunocytochemistry (Hsu et al., 1981) using the antiserum against α_1-chymotrypsin (1:100, A022 Dako Corp.). KCl-treated fungal cells fixed in 3:1 alcohol:acetate, spread on slides were stained with Giemsa for cytogenetic morphology. Three types of fungus were used: *Dermatophyte microsporum* sp., *Aspergillus fumigatus* and *Candida albicans*.

Testis were excised from three-, four- and five-week-old Wistar rats. Small pieces of testicular tissues were treated with KCl (0.075 M) for 10 min, fixed in 3:1 alcohol:acetate overnight, followed by 6:4 acetic acid:distilled water treatment for 5 min, were smeared on glass slides, dried and stained with Giemsa for cytogenetic morphology.

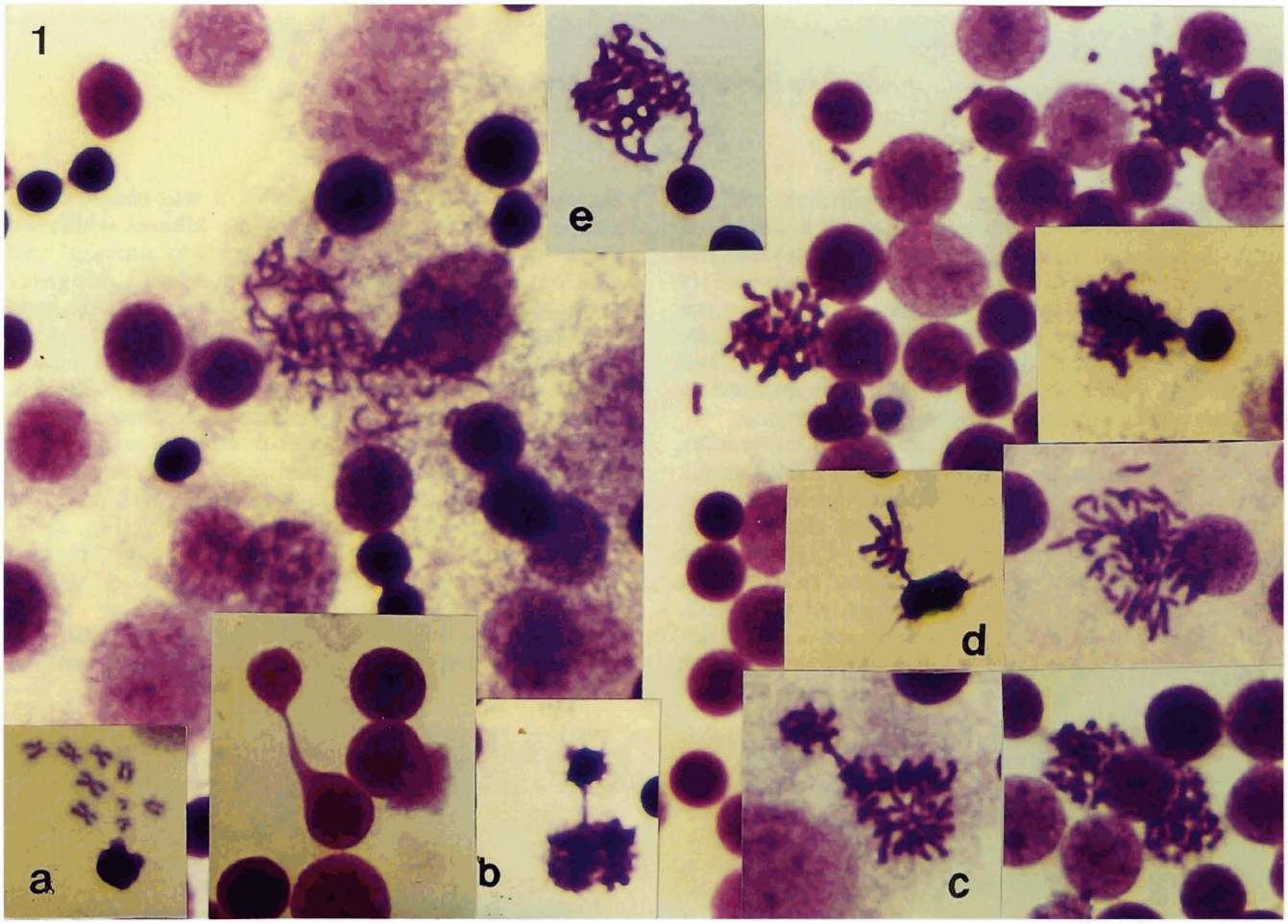
Results

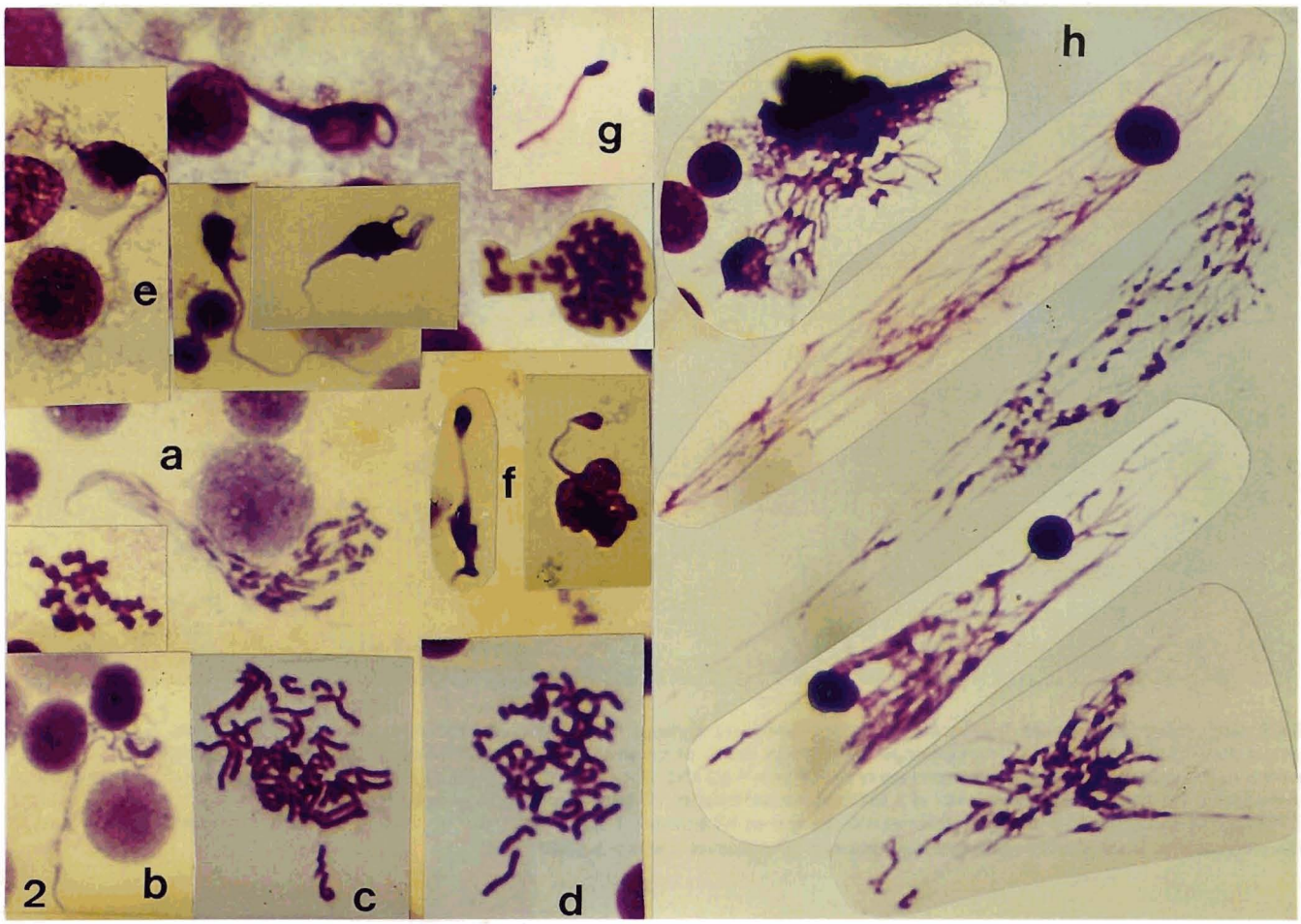
In situ cytogenetic analysis and morphology of cultured malignant BMC and tissues

All BMC in the presence of PHA remained attached on the culture vessel surface for 24 hours allowing the in

Fig. 1. Single scattered or massive extrusion of condensed, meiotic chromosomes by hyperchromatic or hypochromatic BMC nuclei. **Inset a:** extrusion of nine regular chromosomes. **Inset b:** NV of pyknotic condensed head formed by meiosis via a NB. **Insets c-e:** aneuploid NVs, formed by meiosis, attached to parent nucleus. In situ, Giemsa. x 1,000

Fig. 2. Free NVs of pyknotic, condensed head with tail or in metaphase. NVs protruding NBs or extruding chromosomes are obvious. **Inset a:** A hypodiploid NV metaphase consisting of chromosomes and fibrous chromatin. **Inset b:** A NV carrying one chromosome. **Inset c and d:** Hypodiploid NV metaphases with identical chromosomes to those of human spermatogonia. **Inset e:** a pyknotic head NV showing acrosome-like formation. **Inset f:** NV of small head. **Inset g:** NV of *Candida albicans*. **Inset h:** Leptotene and diplotene stages of BMC. In situ, Giemsa. x 1,000


1
a
b
c
d
e


2
a
b
c
d
e
f
g
h

situ cytogenetic morphology. Cell detachment and cell nodule formation started between 24 and 48 hours of cell cultivation. Only one BMC culture showed stromal cell contamination. Stromal cells were cytologically identified by their spindly elongated shape. On the second and third day of cultivation, all hematopoietic cells were detached in the medium, in the form of cell nodules while stromal cells remained firmly attached on the culture vessel surface, allowing their separation.

All cases, examined in situ, after 24 h cultivation, showed hyperchromatic or hypochromatic BMC nuclei extruding single or massive condensed hyperchromatic, hypochromatic (Fig. 1) as well as regular chromosomes in an amorphous fashion (Fig. 1a). Cell division occurred via NBs, giving rise to daughter cells, smaller than the parent, of NV morphology, of pyknotic, condensed head (Fig. 1b) or in metaphase (Fig. 1c). Since this cell division involved diminution of the parent nucleus via a NB, producing NVs, it was characterized as meiotic (GK meiosis=diminution). Meiosis within the same malignant BMC population was unequal and random, documented by the aneuploid daughter metaphases, attached to parent nucleus, retaining head with tail morphology (Fig. 1c-e). Single chromosomes away from a metaphase, shown by the in situ technique (Fig. 1), were extruded chromosomes and not artifactual, since this technique does not involve chromosomal scattering and artifacts assumed to occur by the chromosomal spreading technique.

Upon separation of daughter from parent cell, the NB constituted the tail of the daughter NV (Fig. 2a-f). Free NV metaphases consisted of chromosomes or fibrous chromatin with chromosomes (Fig. 2a). Most of the chromosomes were located in the head and single ones along the tail of the NVs. NVs carried single (Fig. 2b) or aneuploid sets of chromosomes. Chromosomes of

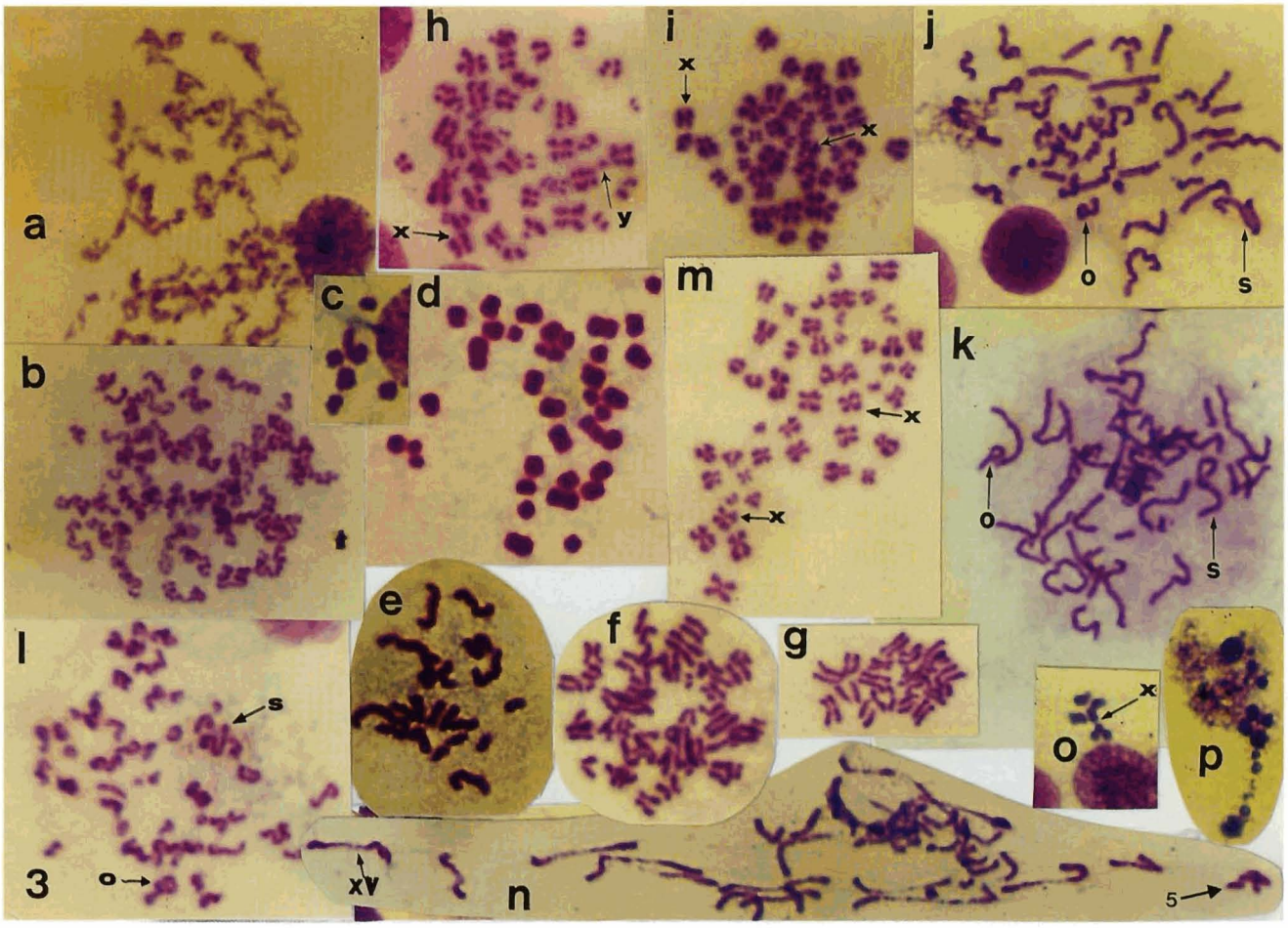


Fig. 3. BMC meiotic metaphases. **Inset a and b:** second meiotic metaphases identical to those from a human spermatocyte. **Inset c-e:** hypohaploid, hypodiploid and almost haploid metaphases identical to those of spermatogonia. **Inset f and g:** oocytes at diakinesis with bivalents. **Inset h and i:** diploid mature oocytic metaphases of 46,XY from male and 46,XX from female patients. **Inset j-l:** metaphases showing both the female chromosome «0» and male chromatids «s» of a fertilized oocyte. **Inset m:** hybrid metaphase showing oocytic and somatic reglar chromosomes. **Inset n:** metaphase showing both the oocytic chromosome «5» and an XY bivalent of human spermatocyte. **Inset o:** a hypohaploid metaphase with oocytic «X» chromosome. **Inset p:** a NV with ring and meiotic prochromosomes. Giemsa. x 1,000

NV metaphases were identical to those of human spermatogonia (Fig. 2c,d). Free NVs of various-sized condensed, pyknotic head were protruding NBs, occasionally showing acrosome-like formation (Fig. 2e). The large pyknotic head NVs morphologically resembled spermatids or spermatozoa and the small ones (Fig. 2f) the male gametes (NV) of *Candida albicans* (Fig. 2g). Hence NVs resemble the male gametes of cell systems dividing by meiosis.

Prophase stages of the first meiotic division of oocytes such as chromatin fibrils with condensed leptotene or diplotene (Fig. 2h) chromosomes were often observed in situ. BMC showed metaphases identical to those of human spermatocytes in meiosis II (Fig. 3a,b) to those of human spermatogonia (Fig. 3c-e) and to those of oocytes at diakinesis (Fig. 3f,g), irrespectively of the patient's sex. Male patients showed mature, aneuploid and diploid oocytic metaphases of 46,XY (Fig. 3h) and female patients of 46,XX (Fig. 3i). Metaphases of human fertilized oocyte showing both the female chromosome «0» and the male chromatids «S» (Fig. 3j-l) were identified (Plachot et al., 1987). Metaphases consisting of oocytic and regular, somatic chromosomes were present (Fig. 3m). Chromosome «5» of the mature human oocyte and the «X» attached to «Y» (spermatocytic bivalent) co-existed in an elongated meiotic metaphase (Fig. 3n). Chromosome «X» of the mature human oocyte was present in a hypohaploid metaphase (Fig. 3o). Many meiotic metaphases were located in diffuse nuclear material (prophasing). Best quality meiotic metaphases were obtained at 72h cell cultivation. Some NVs consisted of double minutes (DMs), ring chromosomes and meiotic prochromosomes (Fig. 3p).

The diploid karyotype of a mature, somatic BMC oocyte from a female patient (46,XX) and the haploid

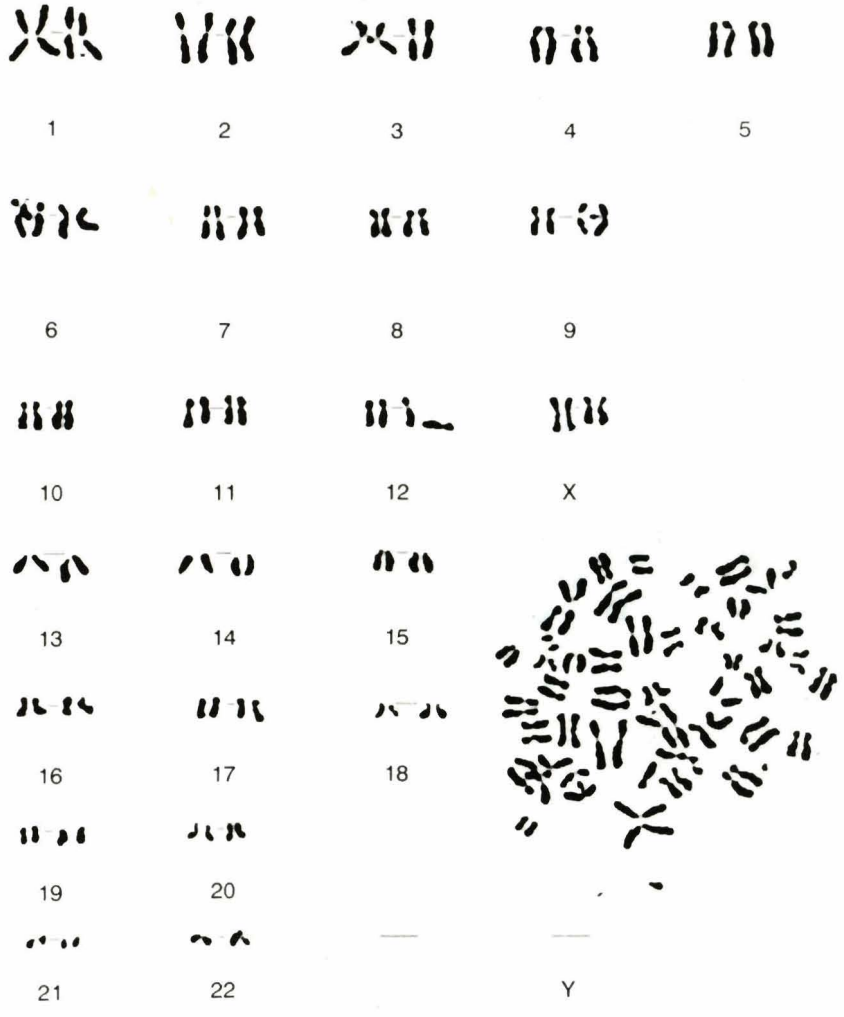


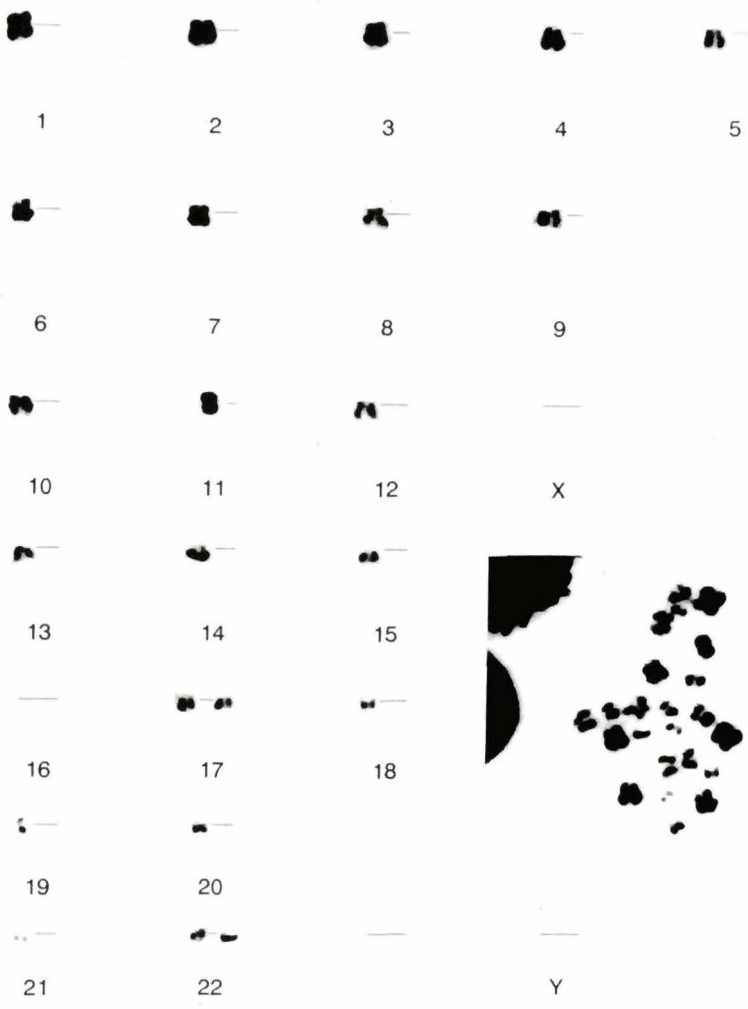
Fig. 4. A diploid BMC oocytic karyotype of 46,XX from a female patient.

oocytic karyotype of 23,-X,-Y,-16,+17,+22, from a male patient are shown in Figs. 4 and 5 respectively.

NVs, of condensed head or in metaphase, invaded the cytoplasm, the nucleus or the metaphases of recipient host cells (Fig. 6). Chromosomes of NV metaphases were first attached and then implanted into recipient host nuclei and metaphases. Decondensation of the NV head, release and implantation of its chromosomes into a host cell meiotic metaphase (Fig. 6a) was identical to sperm-oocyte fertilization. Chromatin fibrils carrying condensed chromosomes (Fig. 6b) and NV of minute chromosomes (Fig. 6c) also invaded other nuclei.

Two or more metaphases fused resulting in hybrid aneuploid metaphases (Fig. 7). Hybrid metaphases were also formed during the extrusion of chromosomes by two or more adjacent nuclei. Occasionally, extrusion and fusion of chromosomes by two oppositely-located nuclei formed hybrid metaphases of ring morphology (Fig. 7a,b).

Within the same BMC population, separate metaphases of condensed or regular hyperchromatic or hypochromatic chromosomes were observed. Hybrid metaphases could be identified by the presence of both meiotic hyperchromatic condensed and hypochromatic

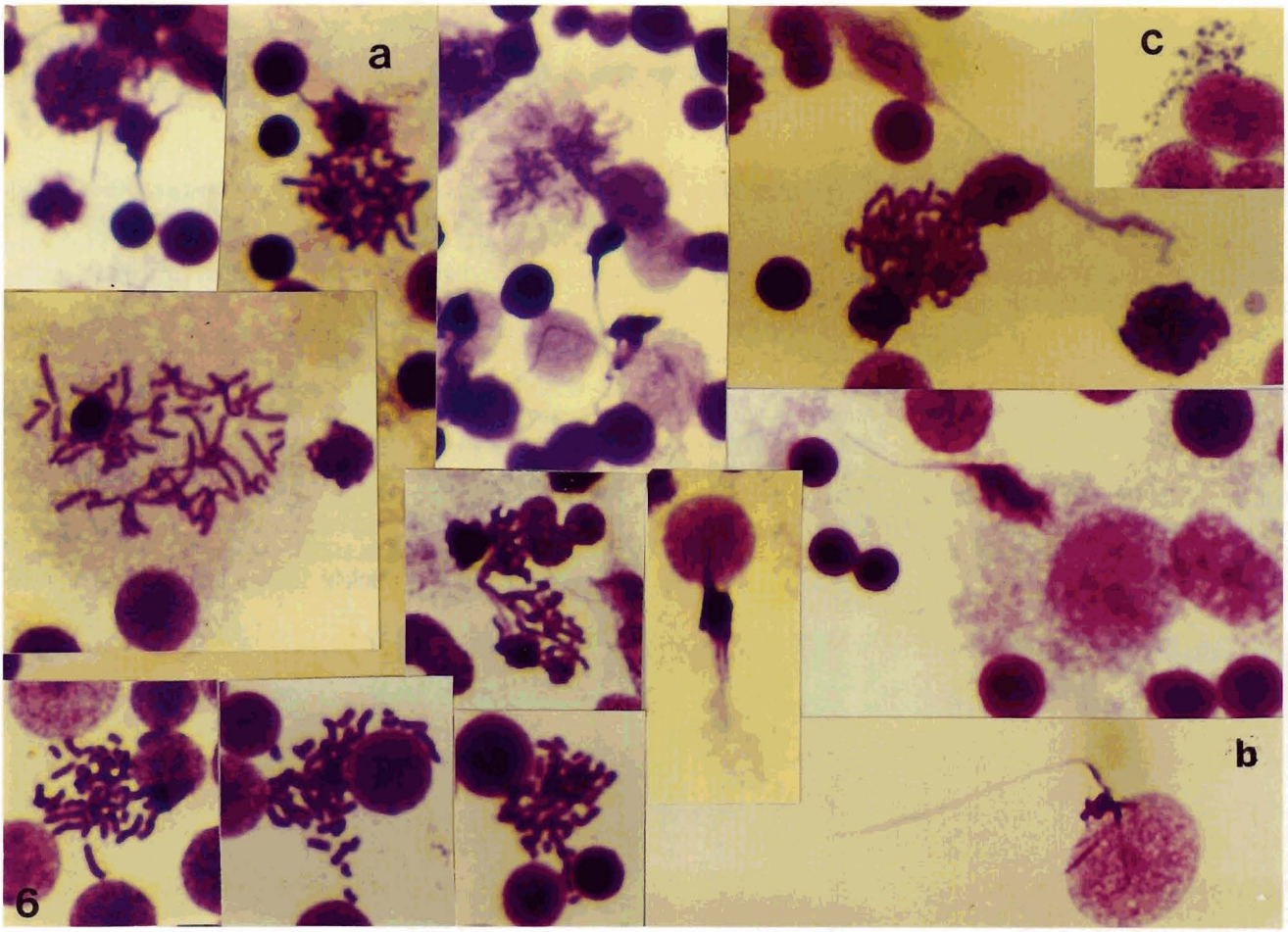


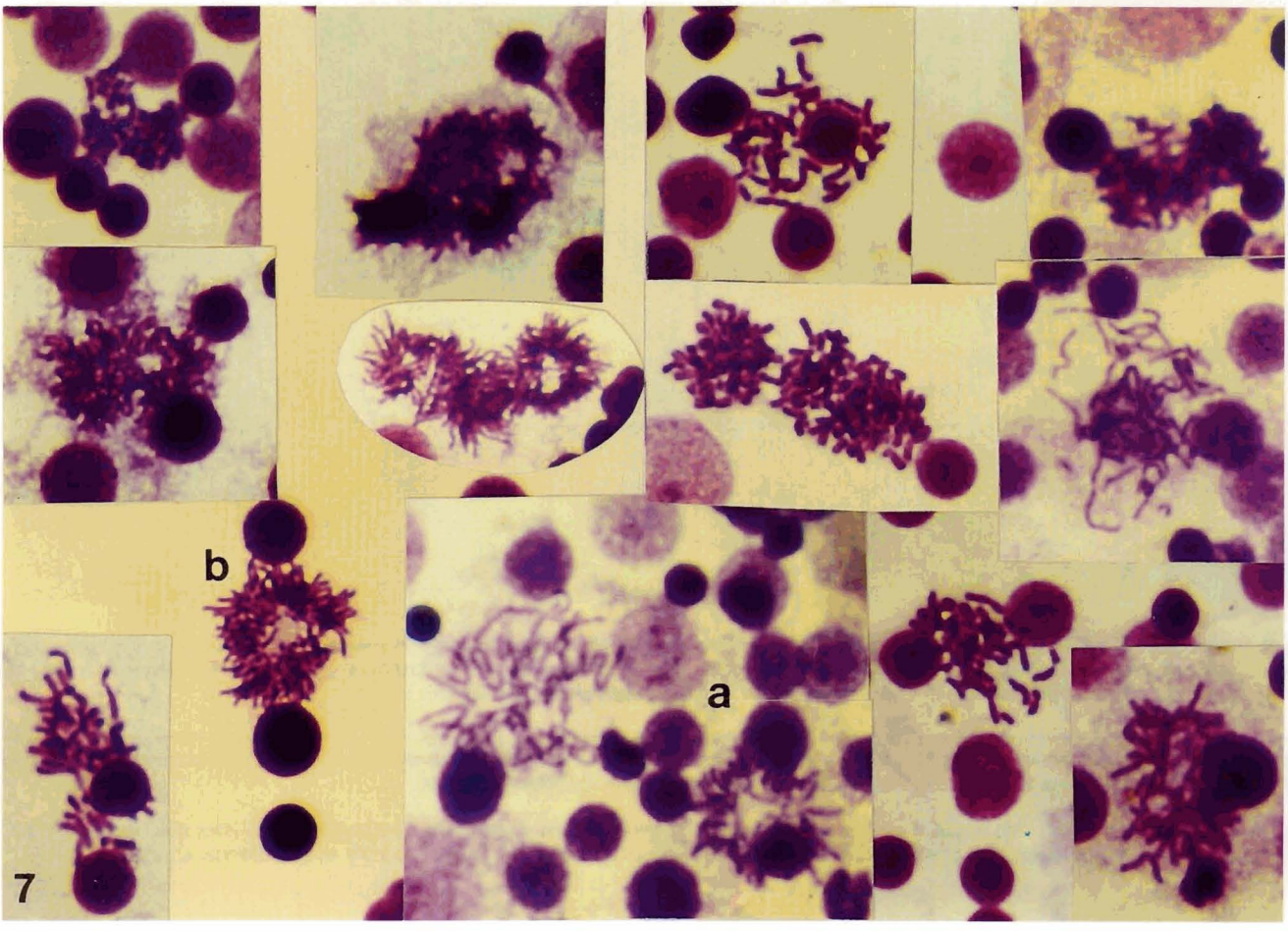
5

Fig. 5. A haploid oocytic karyotype of 23,-X,-Y,-16, +17,+22 from a male patient.

Fig. 6. NVs of condensed head or in metaphase fertilize other cells in interphase or in metaphase. **Inset a:** A NV, while attached on parent cell, fertilizes and de-condenses its chromosomes into another metaphase. **Inset b:** BMC hyphae containing chromosomes invade another nucleus. **Inset c:** DMs and minute chromosomes of a NV being implanted into another nucleus. In situ, Giemsa. x 1,000

Fig. 7. Metaphase fusion into hybrid aneuploid meiotic metaphases. **Insets a and b:** extrusion of chromosomes by two adjacent nuclei followed by metaphase fusion results in ring meiotic metaphases. In situ, Giemsa. x 1,000


a
c
b
6


b
a
7

regular chromosomes within the same metaphase (Fig. 8).

Hypochromatic or hyperchromatic nuclei and NVs fused, giving rise to various-sized NCs containing a central vacuole. Nuclear fusion was highly random as different types of nuclei (hyperchromatic with hypochromatic) fused with each other (Fig. 9). Nuclear fusion and NCs were also observed in *Candida albicans* (Fig. 9a). NCs were not inert but produced nuclei of various size and type by budding (Fig. 10). Moreover, hyperchromatic nuclei budded micronuclei which invaded other nuclei (Fig. 10). NVs carrying micronuclei (Fig. 10a) resembled fungal ascus with ascospores (Fig. 10b) and BMC nuclear budding was identical to yeast budding.

Metaphases and nuclei communicated with each other via NBs (Figs. 11, 12). NBs were identified between a NV and a resting nucleus (Fig. 11a) or a metaphase and resting nuclei or between resting nuclei (Figs. 11, 12). Each nucleus exhibited more than one NB. Chromosomes (Fig 11a,b, 12 a) and chromatin fibrils were transferred from nucleus to nucleus through the lumen of NBs. NBs, originated in the nucleus, were extensions of the nuclear membrane giving rise to multiple chromatin fibrils (hyphae) extracellularly. Some NBs contained glassy, semitransparent, solid CFS (Fig. 12b). NBs carrying ring and minute chromosomes (Fig. 12) resembled fungal hyphae containing chromosomes.

Chromosomal transfer and attachment between adjacent nuclei and nuclear fusion was associated with

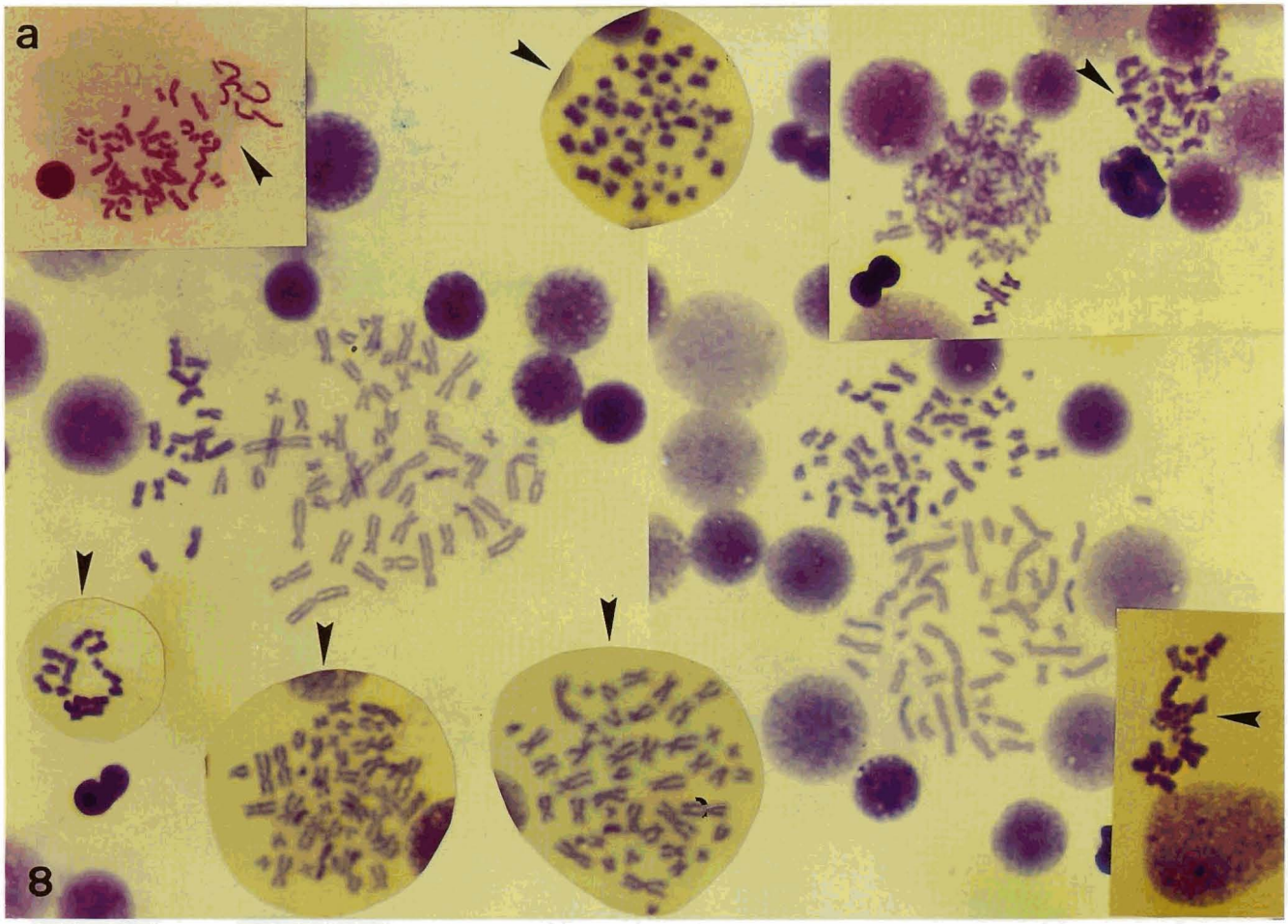
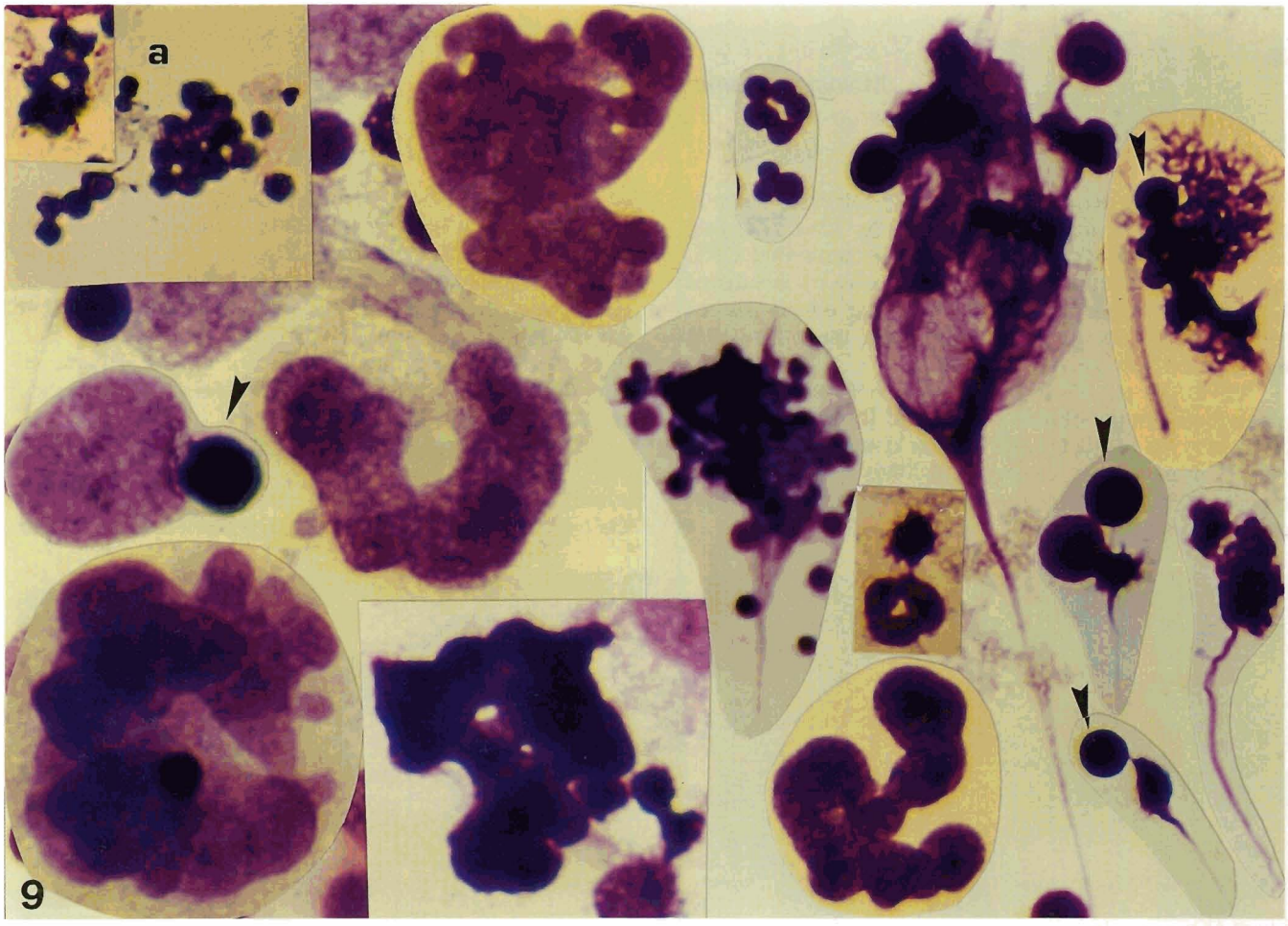
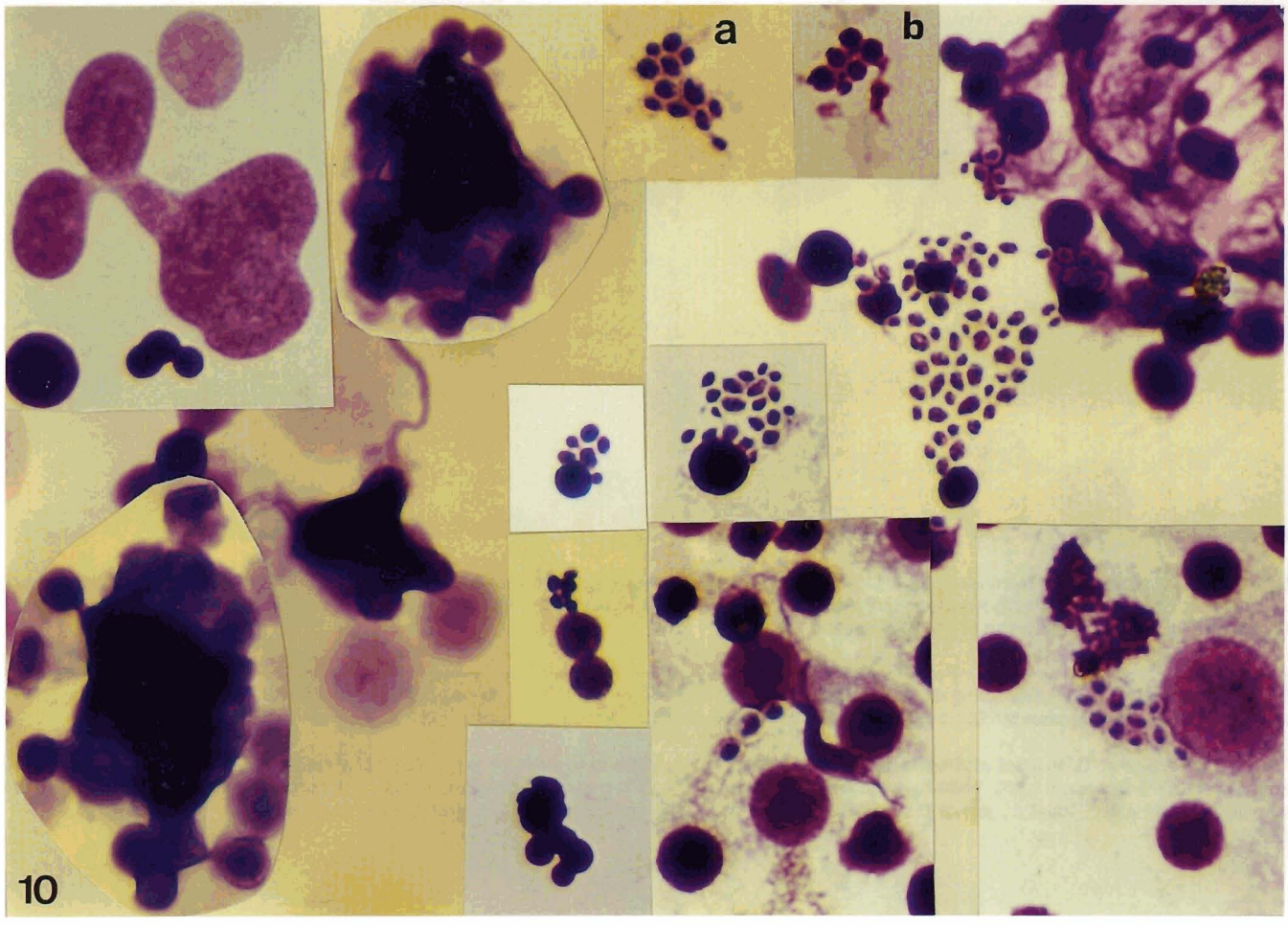


Fig. 8. Separate, side by side, diploid and aneuploid metaphases of hypochromatic or hyperchromatic chromosomes within the same BMC population. Hybrid metaphases (arrows) of mixed hyperchromatic with hypochromatic chromosomes can be observed. Giemsa. x 1,000. **Inset a:** a NV of 6 condensed meiotic chromosomes fusing with a 46,XY, metaphase in diffused nuclear material. Giemsa. x 400

Fig. 9. Hypochromatic and hyperchromatic NCs, some with central vacuole, resulted from nuclear fusion. Arrows point at hyperchromatic nuclei fusing with other types of nuclei. **Inset a:** nuclear fusion and NCs in *Candida albicans*. In situ, Giemsa. x 1,000

Fig. 10. Functional NCs are budding nuclei of various size and type. Micronuclei budded by dark nuclei are implanted into other cells. **Inset a:** A BMC NV of micronuclei resembles fungal ascus with ascospores. **Inset b:** *Aspergillus ascospores* in the form of a NV. In situ, Giemsa. x 1,000


a
9


a
b
10

CFS, which was demonstrated by both the chromosomal spreading and the in situ technique (Fig. 13a). NBs and nuclear segments were embedded in accumulated CFS, in networks, resembling fungal mycelia or dendrites (Fig. 13b). Nuclear segments containing chromosomes were heavily stained with Giemsa, while the empty ones were stained very weakly (Fig. 13c).

All cases of BMC studied in situ, showed the presence of minute chromosomes and DMs extruded by different types of nuclei and nuclear conglomerates (Fig. 14a). Minute chromosomes and DMs were scattered free, attached or implanted in host nuclei (Fig. 14b,c) resembling bacteria.

In the case of EL, hyperchromatic nuclei extruded mixed leptotene with fine pachytene short chromosomes (Fig. 15) in a colony fashion (Fig. 15a) as well as spiral and comma-shaped chromosomes (Fig. 15). Some of these nuclei were surrounded by a distinct nuclear envelope identical to that of *Candida albicans* (Fig. 15b). All BMC samples were free of fungal or bacterial contamination as there was no microbial growth detected in sabourand and blood agar.

Direct cytogenetic morphology of malignant BMT was performed in order to exclude artifactual observations assumed to occur with the BMC cultivation. BMT showed smooth diffuse nuclear

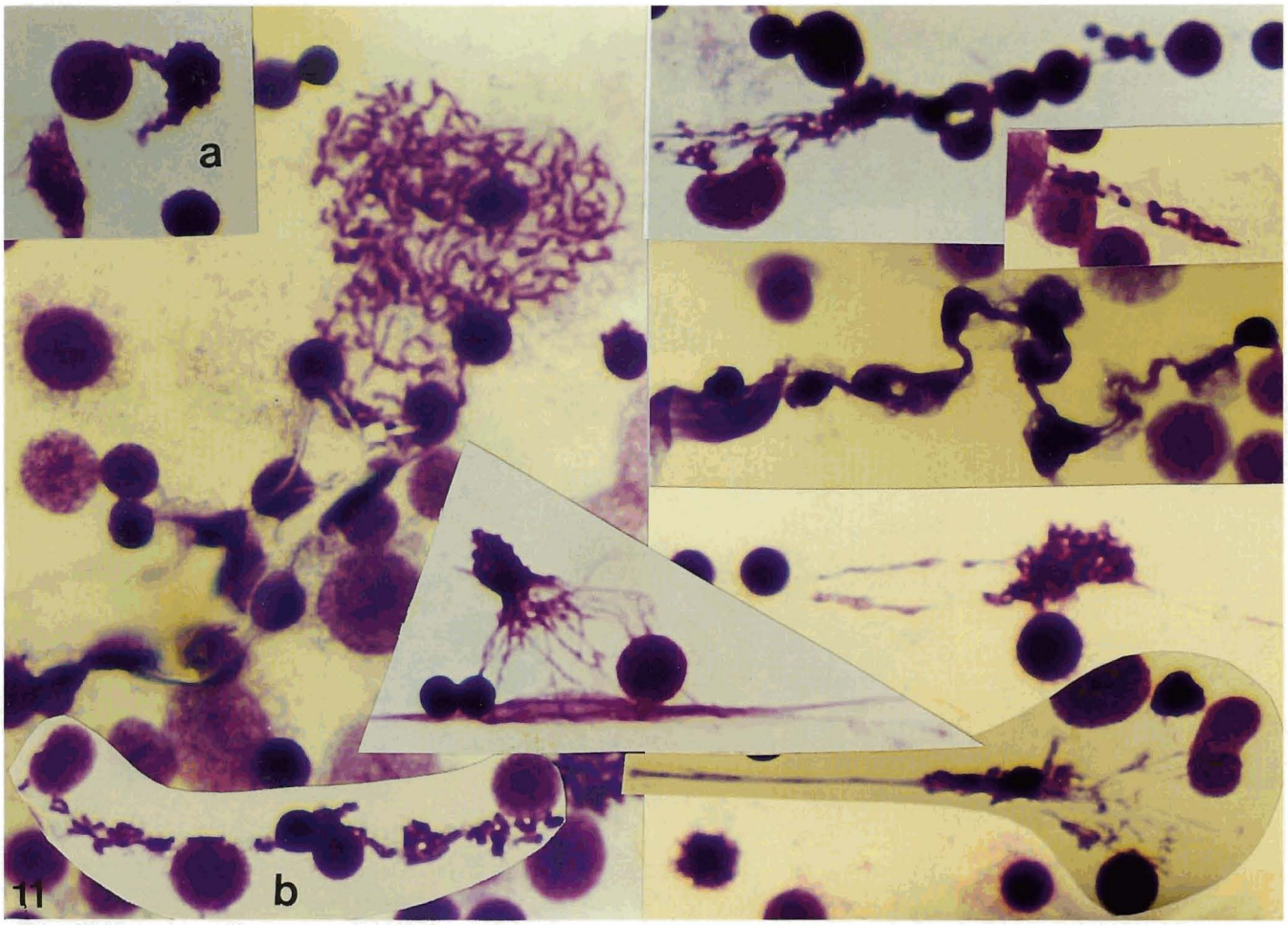
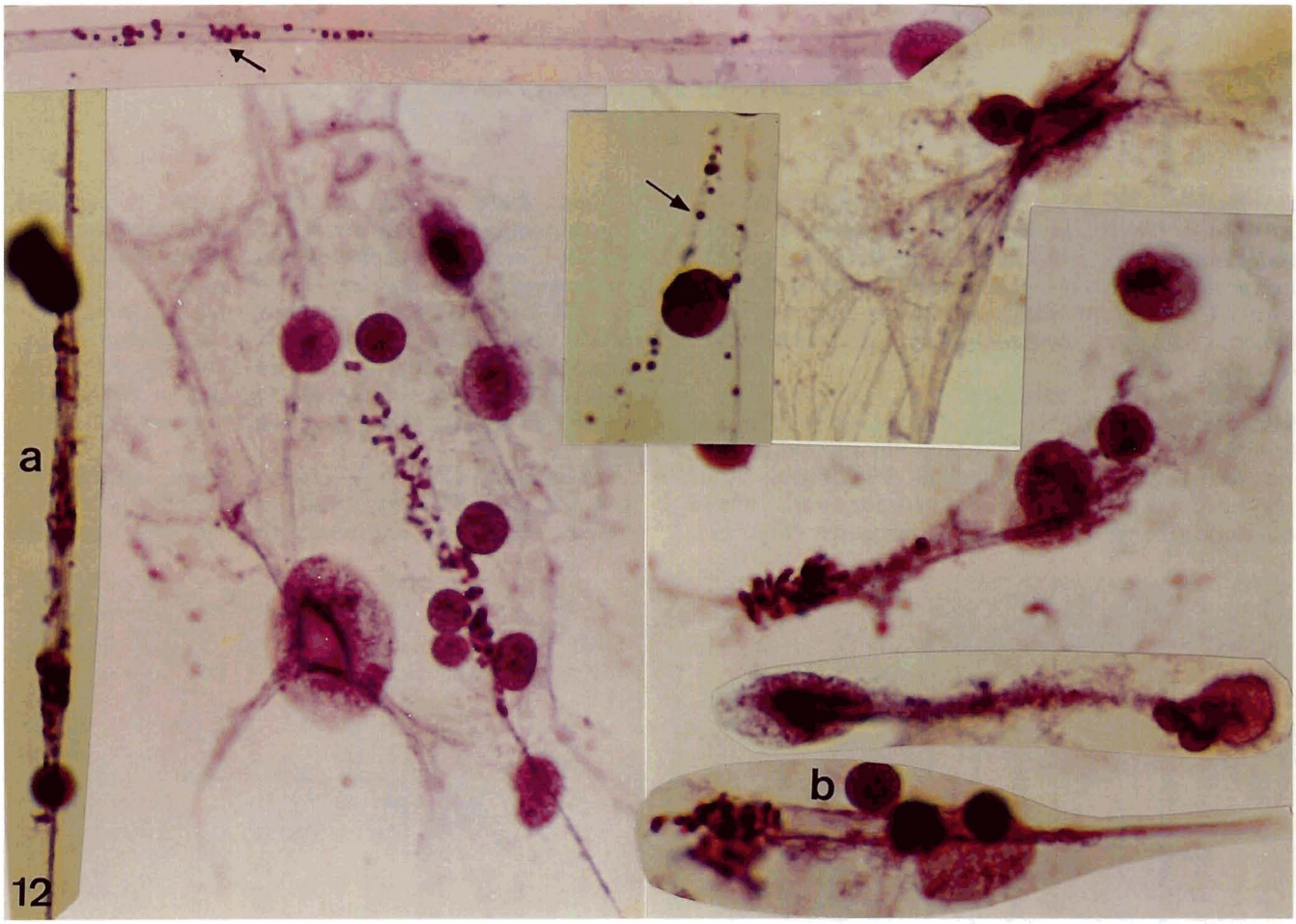
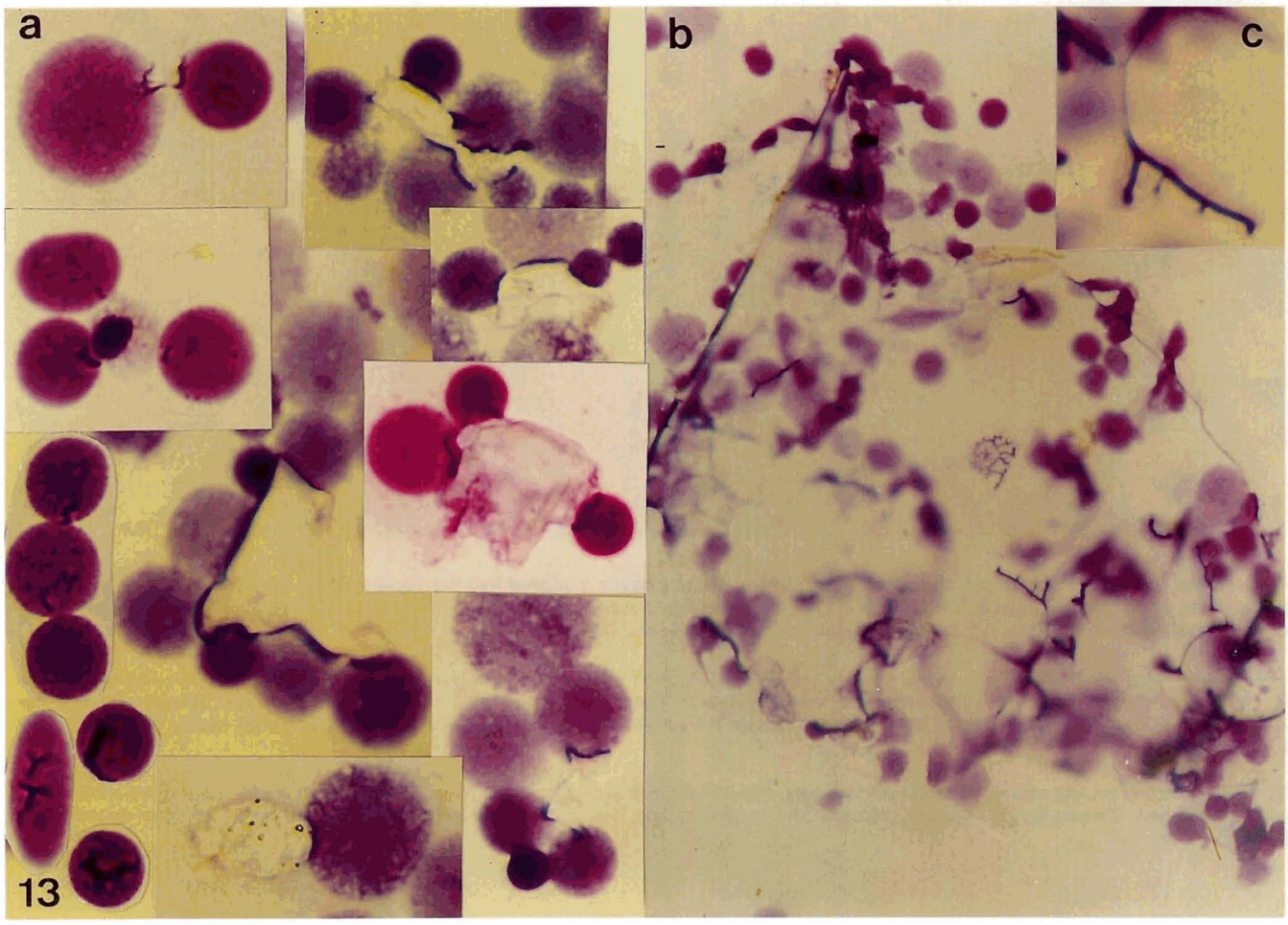


Fig. 11. NBs between cells in interphase or interphase and metaphase. Transfer of chromosomes and chromatin fibrils via NBs in obvious. **Inset a:** A NV extends a NB containing chromosomes to a nucleus in interphase. **Inset b:** extrusion and transfer of chromosomes from nucleus to nucleus via a NB disrupted by hypotonic treatment. In situ, Giemsa. x 1,000

Fig. 12. Intranuclear origin of NBs giving rise to extracellular hyphae. Arrows point at NBs containing DMs, ring or minute chromosomes. **Inset a:** a NB full of chromosomes transverses through two nuclei. **Inset b:** the glassy, translucent CFS content of NB is apparent. HT-papa. x 1,000

Fig. 13. BMC exhibiting CFS. **Inset a:** direct chromosome transfer, from nucleus to nucleus, or associated with glassy, translucent CFS. Several nuclei are fusing via CFS Giemsa x 1,000. **Inset b:** extracellular accumulation of CFS containing NBs, and nuclear segments resembling dendrites or fungal mycelia. Giemsa. x 400. **Inset c:** part of the nuclear segment containing chromosomes is hyperchromatic while the empty part is hypochromatic. Giemsa. x 1,000


a
b
12


a
b
c
13

material (Fig. 16a), NVs, NCs with central vacuole, thin chromatin fibrils in bundles, segmented linear chromosomes (Fig. 16b), DMs, thin and thick tangled nuclear hyphae (Fig. 16c-f) and NBs identical to those of cultured malignant BMC. All these nuclear forms coexisted with intact, well preserved nuclei and therefore were not artefactual.

NCs, in BMT, released scattered pulverized chromosomes (PVC) (Fig. 17a). Nuclei extruded minute chromosomes, DMs or PVC which were scattered free or passed from nucleus to nucleus. PVC consisted of chromomeres enclosed in chromosomal thread resembling bacilli containing endospores (Fig. 17). Chromomeres devoid of chromosomal thread and chromosomal threads devoid of chromomeres were visible. Meiotic metaphases were identified in BMT confirming meiotic division of BMC in vivo.

All forms of nuclear material such as nuclei, NBs wall, chromatin fibrils, nuclear segments, NCs, dissoluted nuclear material and chromosomes were positive to Feulgen stain and consisted of DNA. NCs showed Feulgen-positive and negative glassy areas (Fig. 18).

Malignant BMT cells, cultured BMC, ECM, CFS and NCs were PAS and PAS-D positive (Fig. 19). Also, meiotic, DMs, ring and minute chromosomes were PAS

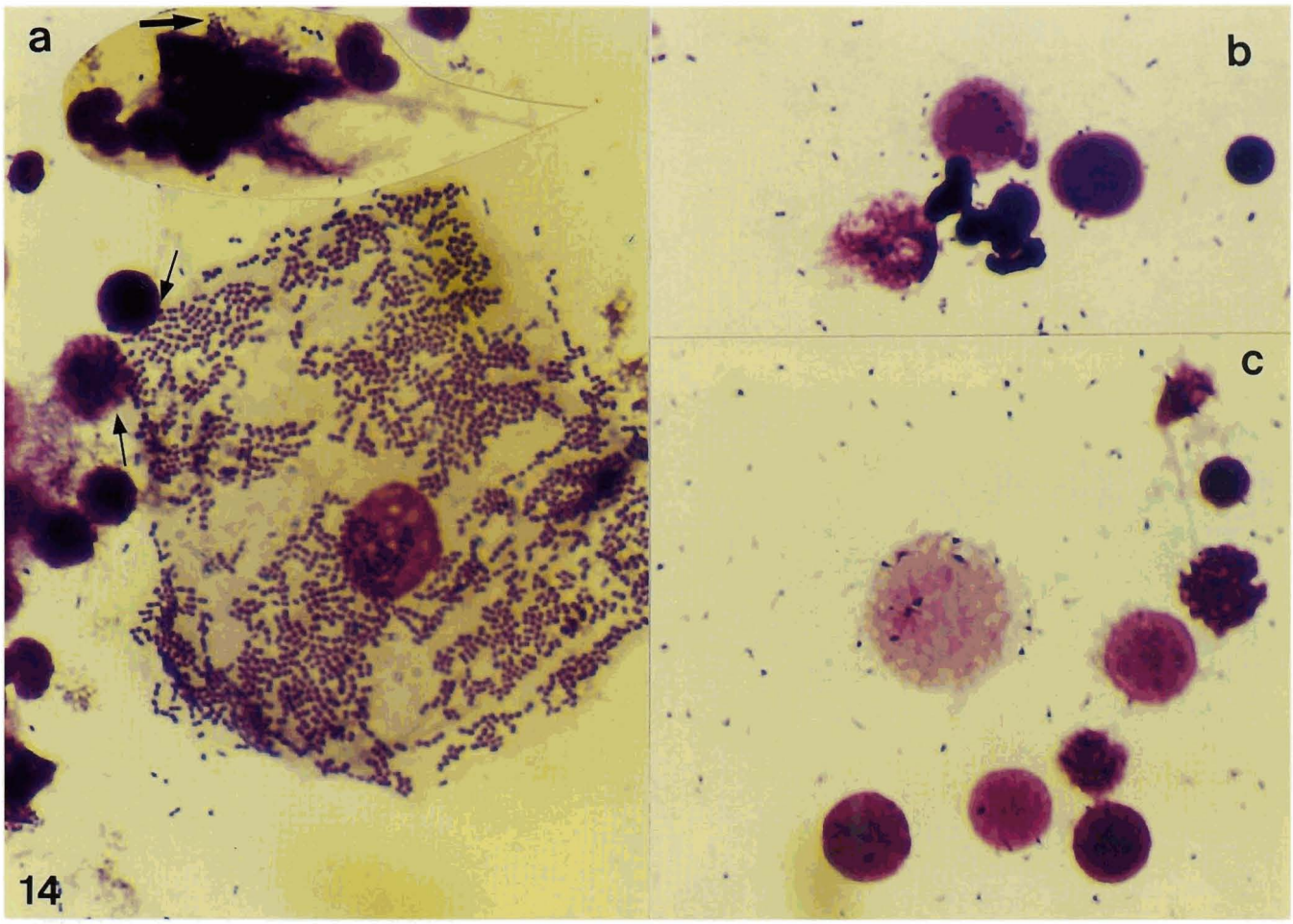
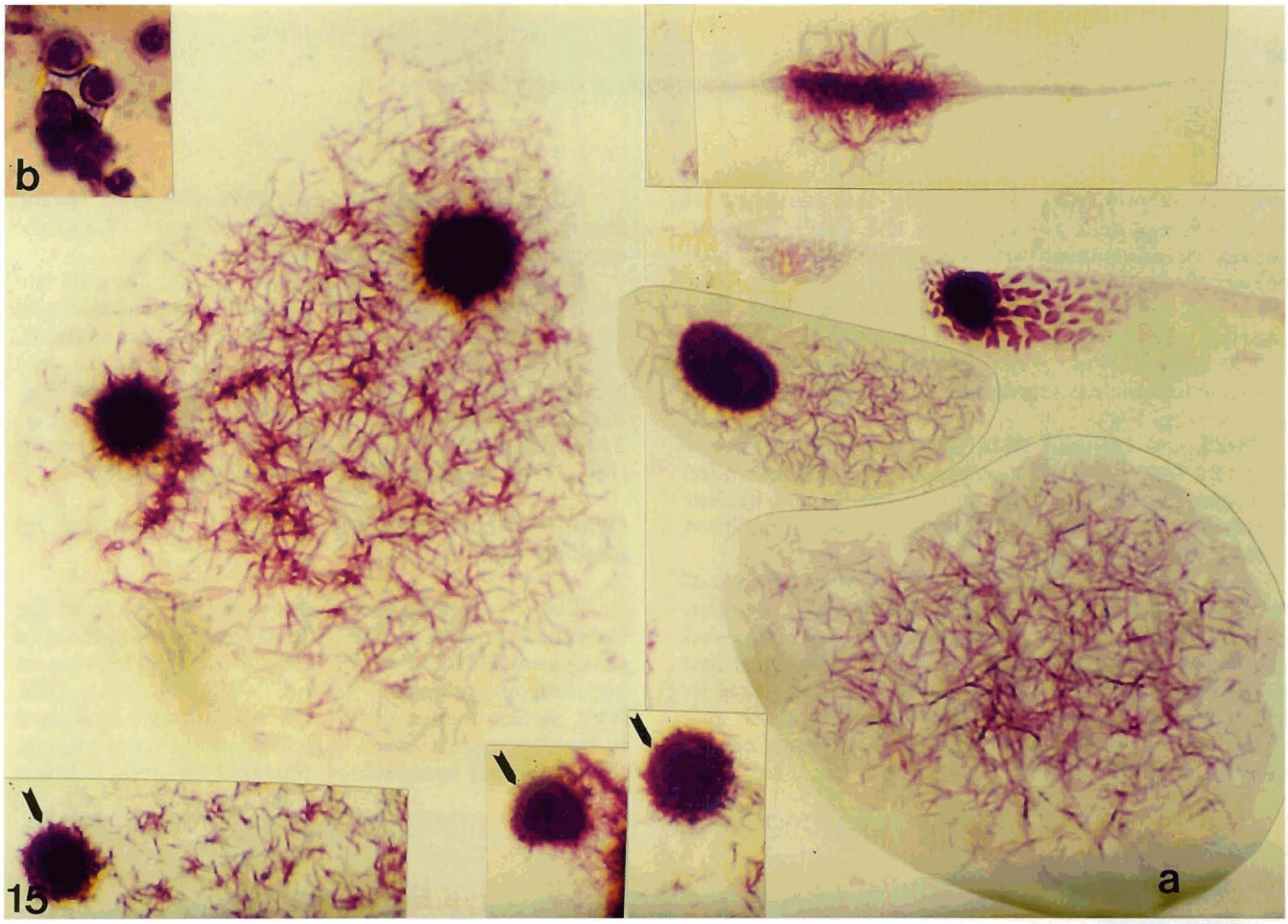
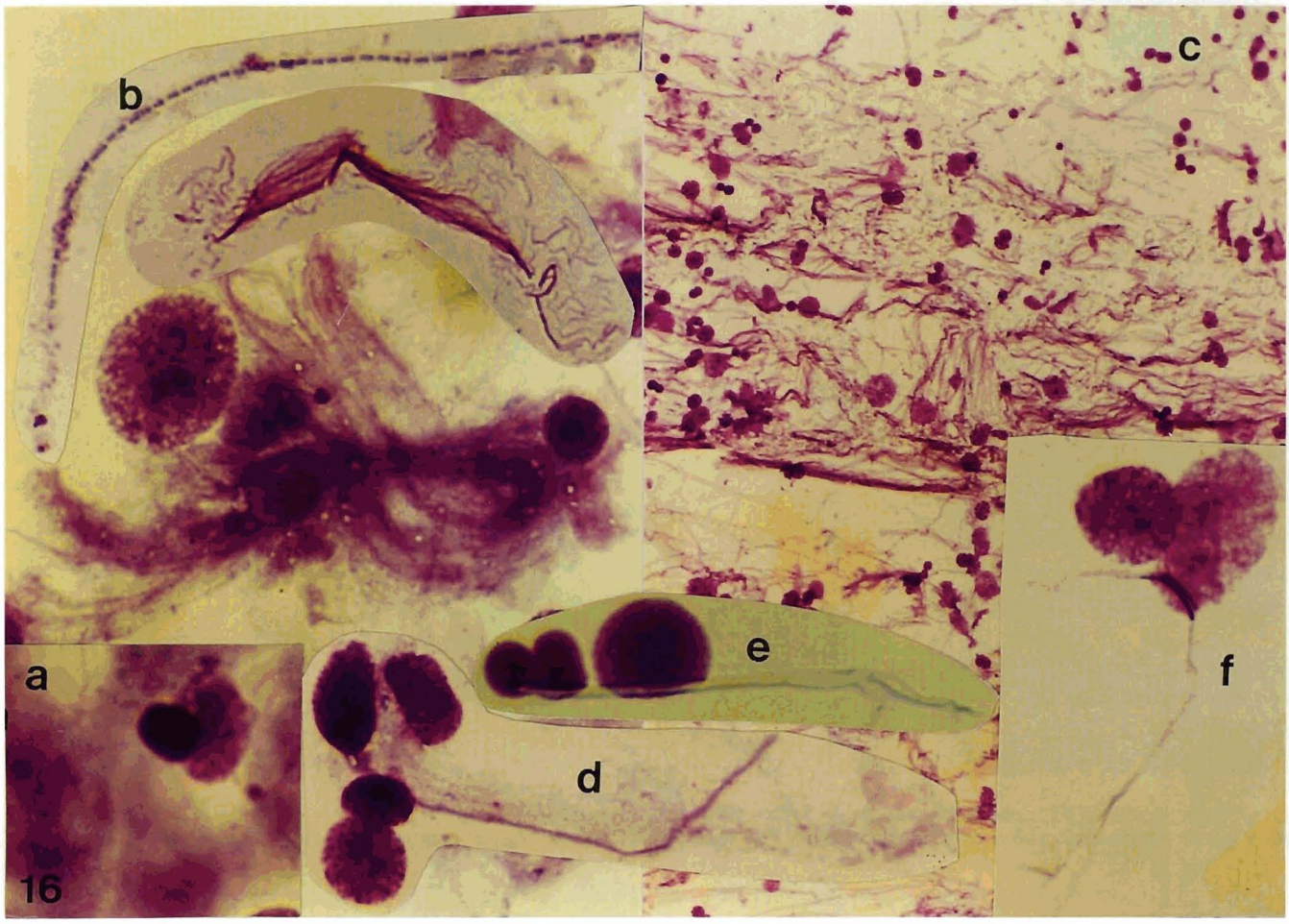


Fig. 14. Extrusion of DMs by hyperchromatic and hypochromatic nuclei (arrows), scattered free like bacteria. **Inset a:** extrusion of DMs and minute chromosomes by NC (arrow). **Inset b:** implantation of DMs into nuclei within the same BMC population. **Inset c:** minute chromosomes implanted into nuclei, behave like bacteria. Figure focused on implanted chromosomes. In situ, Giemsa. x 1,000

Fig. 15. Hyperchromatic BMC nuclei extruding fine leptotene and pachytene, spiral and comma-shaped chromosomes. Arrows point at nuclei surrounded by nuclear envelope. **Inset a:** extruded chromosomes in a colony fashion. **Inset b:** *Candida albicans* cells with nuclear envelope. In situ, Giemsa. x 1,000

Fig. 16. All forms of nuclear material in direct BMT smears. **Inset a:** diffused nuclear material coexisting with intact nuclei. **Inset b:** binary fission of linear chromosome. Giemsa. x 1,000. **Inset c:** thin and thick tangled hyphae. Giemsa. x 200. **Inset d-f:** hyphae from inset c at higher magnification. Giemsa. x 1,000


b
a
15


b
c
a
16
d
e
f

and PAS-D positive, confirming the presence of chromosome-bound glycoproteins and GAG. Regular, somatic chromosomes were PAS negative. Implanted meiotic chromosomes were surrounded by a clear halo which was obvious by PAS-D staining (Fig. 19a). NCs consisted of PAS and PAS-D positive areas blended with nuclear material (Fig. 19b). NB content showed negative to strong and nuclear segments a strong PAS and PAS-D reaction (Fig. 19c).

BMT and cultured BMC showed strong immunoreactivity for α_1-antichymotrypsin (Fig. 20). Extruded chromosomes, DMs, minute, ring chromosomes, nuclear segments, NBs, hyphae, NCs, NVs and nuclei exhibited strong immunoreactivity.

Meiotic chromosomes in the cytoplasm or nucleus of BMC were strongly immuno-stained (Fig. 20a). Regular, somatic chromosomes were negative. CFS showed negative to strong immunoreactivity. Distribution and intensity of immunoreactivity in BMC was identical to that of *Aspergillus* cells, conidiophores (Fig. 20b). and zygospores (Fig. 20c), documenting the presence of protease in meiotic cells.

Minute chromosomes and DMs of Figure 14, the chromosomes of Figure 15, PVC of Figure 17 and the nuclear segments were intensely Gram positive.

Several stages of the meiotic malignant cell life cycle can be identified in 5 μm-thick tissue paraffin sections from hematological malignancies. For example, free NVs, nuclear budding (Fig. 21a), NV fusion into NC (Fig. 21b), NV production by meiosis via NBs (Fig. 21c), large and small NCs with central vacuole (Fig. 21d), cell production by NCs (Fig. 21e) and oocytes (Fig. 21f) were shown in AML histological picture.

Cytogenetic morphology of meiotic fungal and rat testicular cells

The cytogenetic morphology and life cycle of the examined fungi exhibited identical processes to those of malignant BMC. Fungal cells showed extrusion of chromosomes, nuclear fusion, NBs (Fig. 22a), CFS, NVs, nuclear budding and NCs. Single fungal hyphae or in bundles and diffused nuclear material were present. Chromosomal morphology revealed condensed linear

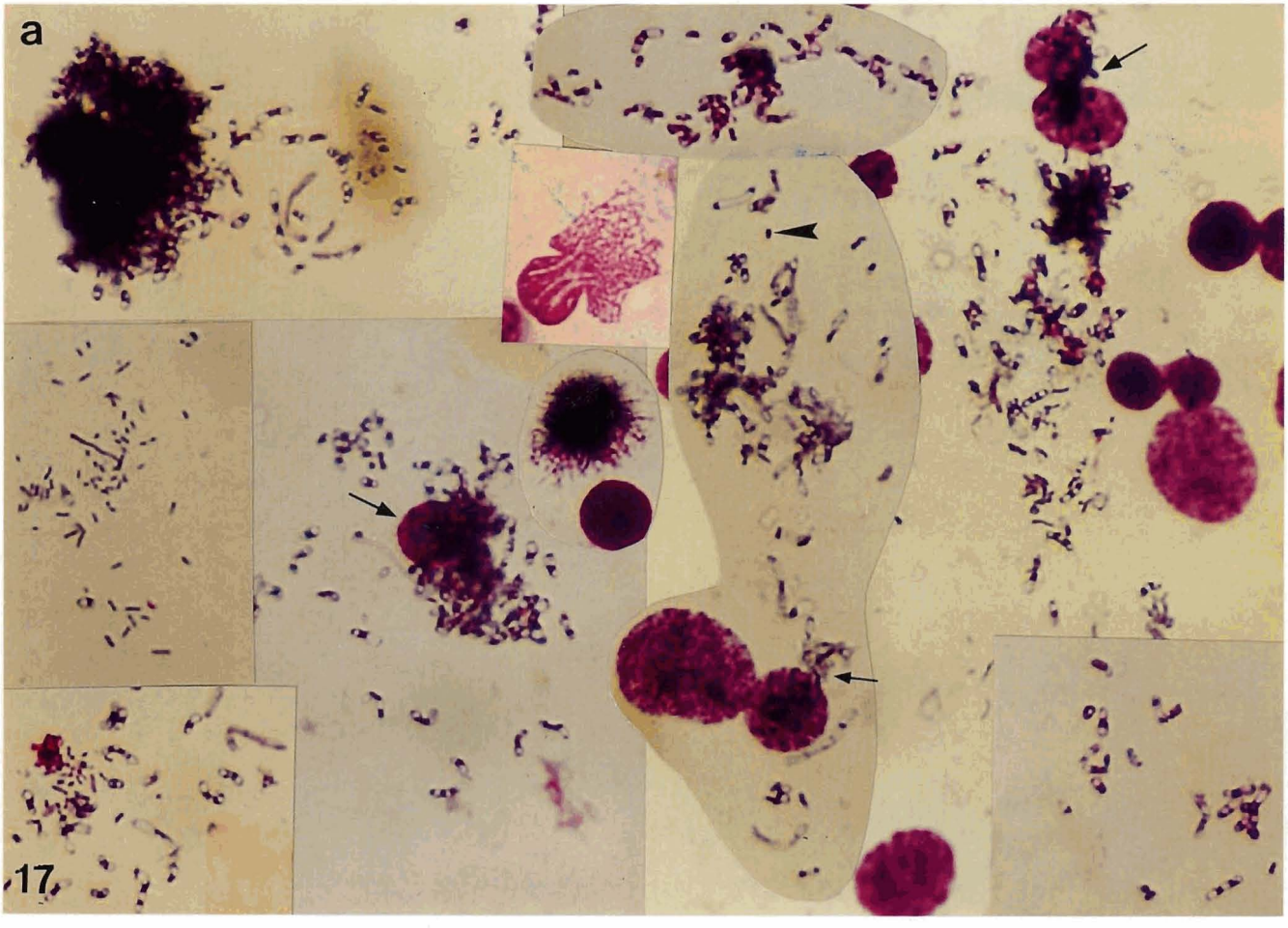


Fig. 17. DMs, minute and PVC extruded by BMT nuclei. Arrows point at PVC extruded or transfered from nucleus to nucleus. Chromosomal threads, with or without chromomeres, can be seen. Chromomeres of PVC resemble endospores of *Bacilli*. **Inset a:** NC extruding PVC. Giemsa. x 1,000

chromosomes, DMs, ring, minute and meiotic chromosomes scattered free or enclosed in hyphae (fig. 22).

Testicular cells of 3, 4 and 5 week-old rats showed amorphous nuclei connected via NBs (Fig. 23a). Meiotic division of spermatocytes was identical to that of malignant BMC, giving rise to condensed head NV or in metaphase via a NB (Fig. 23b-d). Free spermatid morphology was identical to free malignant NVs (Fig. 23e). Nuclear fusion occurred giving rise to abundant NCs of various size and shape (Fig. 23e,f), which produced complete, intact nuclei (Fig. 23f). Extrusion of chromosomes, metaphase fusion (Fig. 23g,h) and chromosomal transfer associated with CFS (Fig. 23i) were often characteristics of spermatocytes, which are well known to divide by meiosis.

Table 1. Numerical analysis of malignant BMC metaphases.

CASES OF HEMATOLOGICAL MALIGNANCY	DIPLOIDY	ANEUPLOIDY (NON-REPETITIVE)	HYPODIPLOID AND HYPOHAPLOID CHROMOSOMAL SETS OF			
			1-10	11-20	21-30	31-45
AML-1	29	71	52	6	5	8
AML-2	24	76	60	6	2	8
AMMoL-M4	23	77	50	10	1	16
UL-1	13	87	54	7	5	21
UL-2	32	68	13	12	12	31
EL	32	68	56	5	3	4
CML	44	56	23	4	5	24
$\bar{x}$	28	72	44	7	5	16

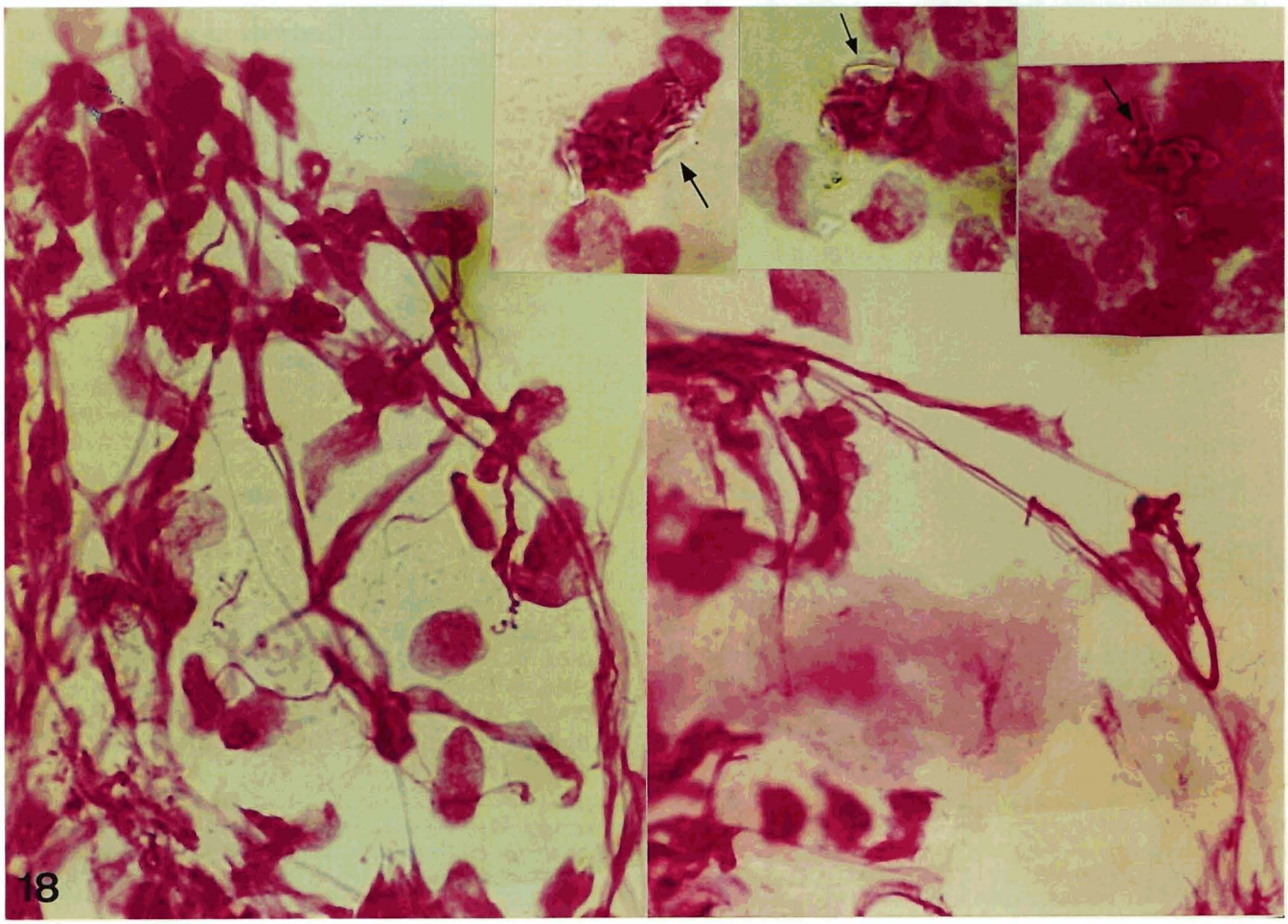


Fig. 18. Nuclear forms in BMT consisting of DNA. Hyphae, NBs and dissoluted nuclear material are Feulgen positive. Arrows point at NCs consisting of stained and unstained areas. HT-Feulgen. x 1,000

Cytogenetic analysis of cultured BMC

Cytogenetic analysis of cultured BMC showed non-repetitive aneuploidy in all cases examined (Table 1). The mean percent of aneuploidy was 72 and diploidy 28. A high percentage of the hypohaploid metaphases were in sets of 1-10 chromosomes in all cases. There was no repetitive karyotype of a particular aneuploid metaphase within the same cell culture. Repetitiveness was only numerical. Each case showed metaphases with broken or minute chromosomes and DMs. There were no structural chromosomal aberrations detected.

The non-repetitive aneuploidy caused by abnormal meiosis, metaphase fusion and abnormal fertilization shown by the in situ technique, excluded the possibility of mitotic aneuploidy or artifactual aneuploidy which is assumed to occur by the chromosomal spreading technique.

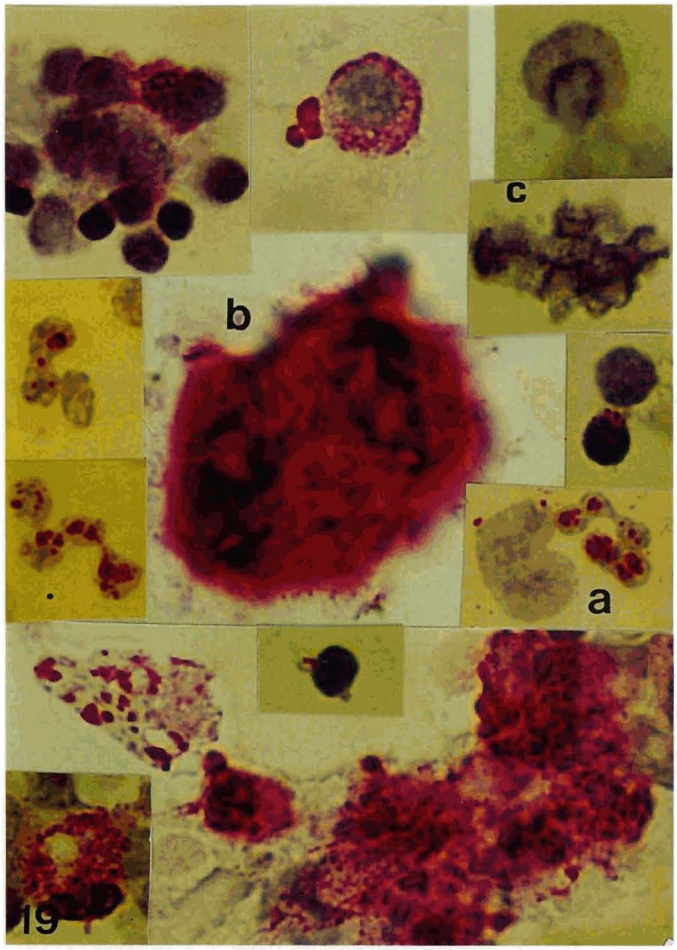


Fig. 19. NCs, meiotic, ring, extruded DMs and minute chromosomes are PAS-D positive. **Inset a:** PAS-D positive meiotic chromosomes in resting nuclei show a clear halo. **Inset b:** NC consists of glycoproteins and GAG. **Inset c:** The GAG content of NBs and nuclear segments. HT-PAS-D. x 1,000

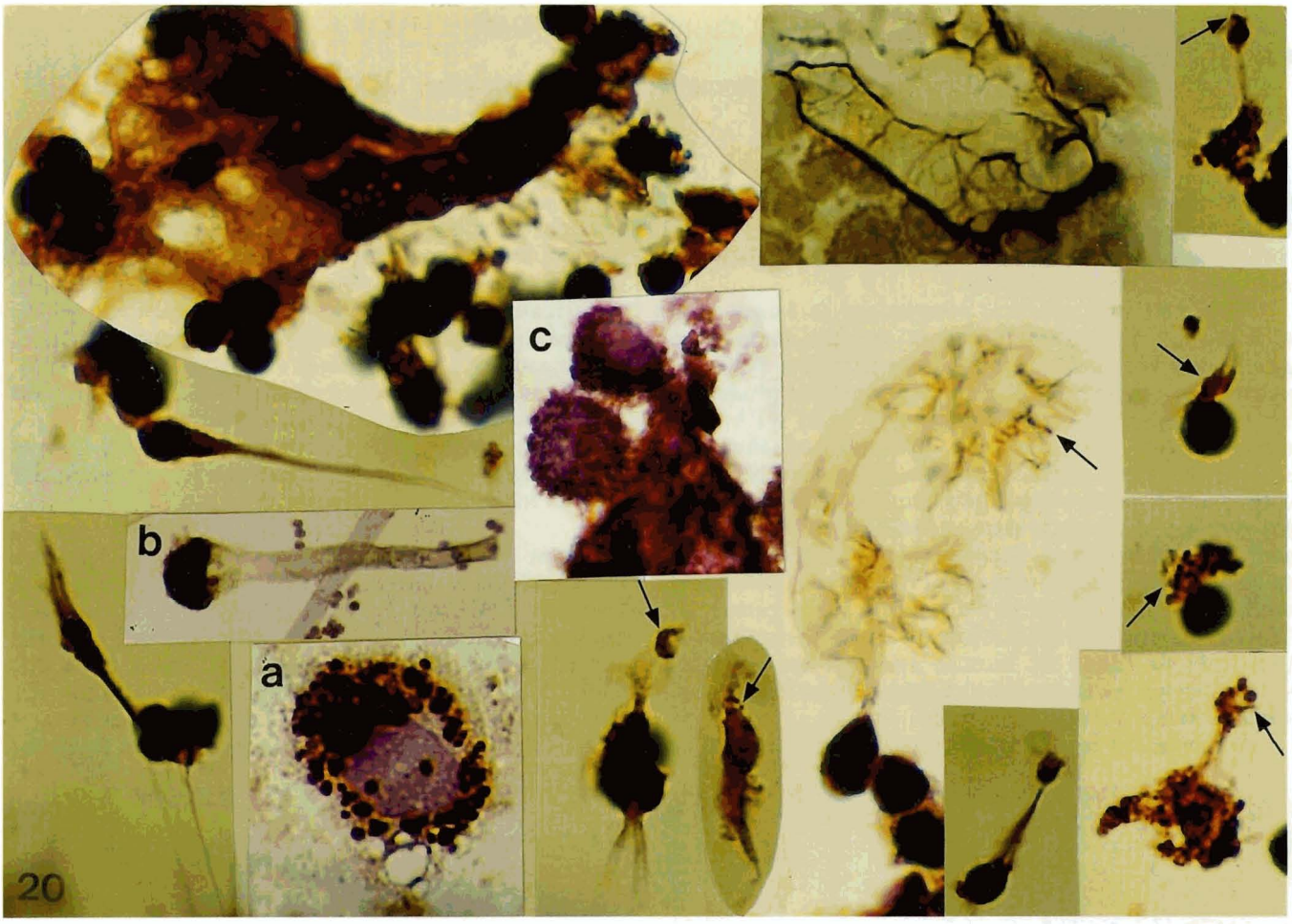
Discussion

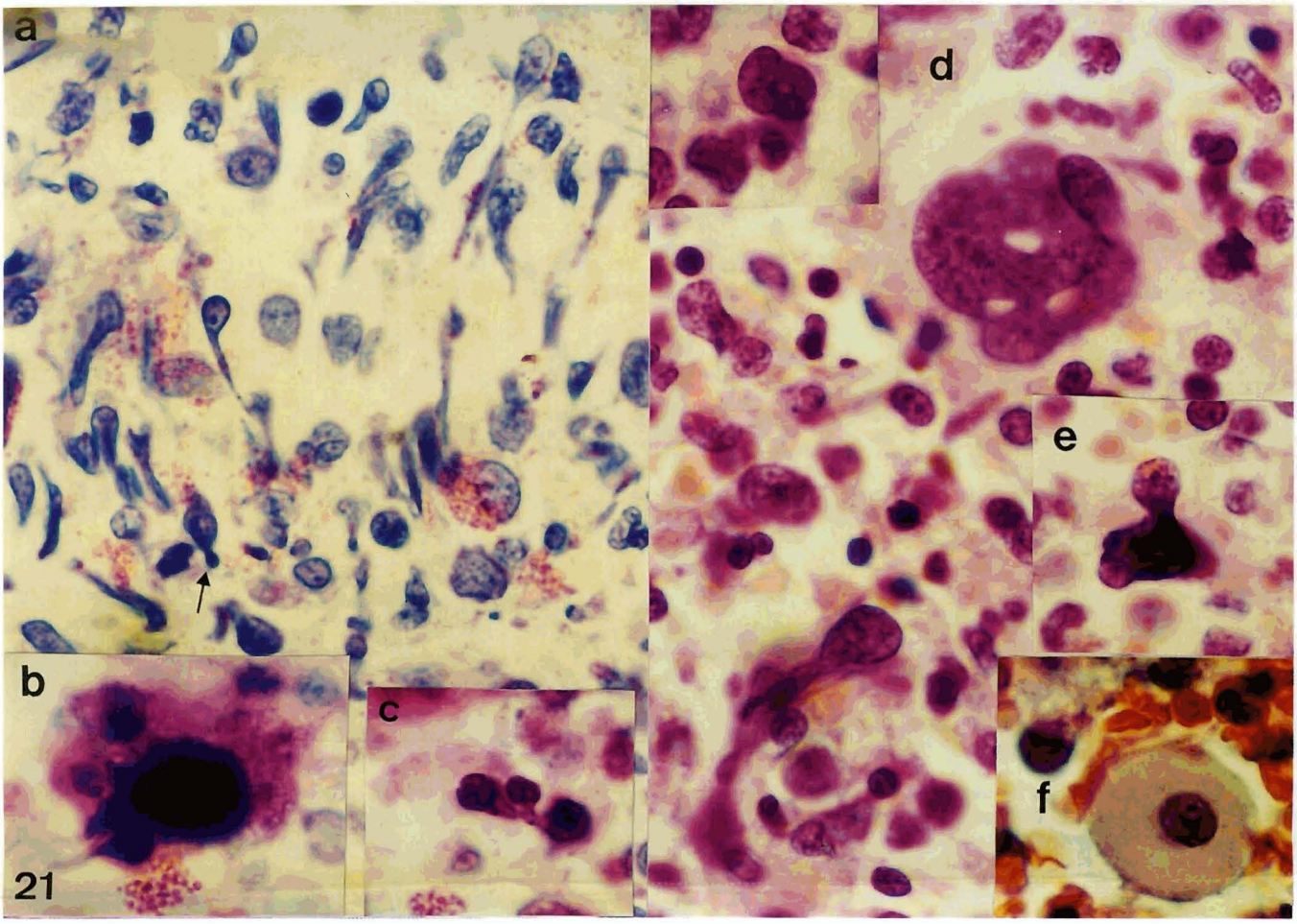
In this study in situ cytogenetic morphology was used to investigate the nuclear events of somatic cell meiosis. Cytogenetic morphology defines the morphology of cells after treatment with hypotonic solutions. Chromosomal preparation slides provide useful genetic morphological information, as hypotonic cell swelling breaks the ECM and cell membrane, exposing the nuclear events, while keeping the morphology of metaphases. The in situ technique cannot cause artifacts because it does not involve procedures such as cell trypsinization, cell transfers, centrifugation, pipetting or chromosomal suspension spreading on slides. It involves direct treatment of growing attached cells (on the culture vessel surface) with hypotonic solution, fixation and staining. It is therefore not influenced by room temperature, humidity or handling errors and can be used as a guide for cellular and genetic events. In addition, the observations of this study were demonstrated in cultivated cells and confirmed in tissue smears and paraffin tissue sections in order to exclude possible artifactual observations. This is the first study reported on the in situ cytogenetic morphology and analysis in hematological malignancies.

The present observations document that cells in different types of hematological malignancies divide by meiosis in vitro and in vivo. Meiosis was evidenced by the presence of nuclear extrusion of chromosomes, NV formation, meiotic chromosomes, nuclear and metaphase fusion, hybrid metaphases, NCs, NBs, CFS and non-repetitive aneuploidy. A new interpretation and evaluation is given on these observations, based on nuclear events, identified by the in situ cytogenetic morphology. These events are: extrusion of chromosomes, NV metaphases, nuclear, metaphase fusion and functional NCs giving rise to cells. Extrusion of chromosomes, in the form of polar bodies, is a characteristic process in human ovarian oocyte maturation by normal meiosis, giving rise to the haploid female gamete. Prophase stages of the first and metaphases of the

Fig. 20. Nuclei, NVs, NBs, NCs, nuclear segments, extruded condensed chromosomes, DMs, ring and minute chromosomes (arrows) show strong immunoreactivity for α_1-antichymotrypsin. HT-α_1-antichymotrypsin. x 1,000. **Inset a:** strongly immunostained meiotic chromosomes in the cytoplasm of a host cell. HT-α_1-antichymotrypsin. x 1,000. **Insets b and c:** distribution of α_1-antichymotrypsin in the swollen apex of *Aspergillus conidiophore and* zygosproes. HT-α_1-antichymotrypsin. x 400

Fig. 21. Meiotic events in histological picture from human AML. **Inset a:** various-sized NVs and budding of BMC can be seen. **Inset b:** fusion of a condensed head NV to a NC. Giemsa. x 1,000. **Inset c:** meiotic division of a BMC into two NVs via NBs. **Inset d:** NCs with central vacuole. **Inset e:** NC giving rise to a nucleus. HE. x 1,000. **Inset f:** oocyte in section of AML cell pellet. H-E. x 1,000


a
b
c
20


a
b
c
d
e
f
21

second oocytic meiotic division, shown in malignant BMC, strongly evidence the presence of immature and mature oocytes constituting the female, somatic gametes in a malignant cell population.

NVs exhibiting identical morphology to fungal and mammalian male gametes constitute the male-like gametes in the malignant BMC population. NVs of pyknotic, condensed head or in metaphase are produced by meiosis via a NB, keeping the head with tail morphology, just like the division of the rat spermatocyte to spermatid, shown in this study. NVs carried non-repetitive hypodiploid or hypohaploid, rarely haploid sets of chromosomes, occasionally DMs, minute and ring chromosomes, documenting abnormal meiosis as compared to normal meiosis of human spermatocytes which give rise to repetitive haploid male gametes. Moreover, the identification of BMC metaphases identical to those of human spermatogonia and spermatocytes documents the presence of male gametes within a malignant cell population.

NV invasion into host cell metaphases is indeed a process of fertilization. This is strongly evidenced by metaphases showing both the female chromosome «0» and the male chromatids «s» and hybrid metaphases of oocytic and regular chromosomes. In most mammals, fertilization occurs at the oocyte metaphase of the second meiotic division followed by decondensation of spermatozoa chromosomes (Tsafriri et al., 1983). Malignant NVs also decondense, release and implant their chromosomes into host cells in metaphase.

These observations strengthen the well known embryonic state and lead to the life cycle of malignant cells as follows:

$$\text{Gametes} \xrightarrow{\text{Fertilization}} \text{Embryos} \xrightarrow{\text{Meiosis}} \text{Gametes}$$

Fertilization and metaphase fusion occurring with malignant BMC result into hybrid aneuploid cells which continue meiotic division and growth, exhibiting the properties of meiotic embryonic cells (Logothetou-Rella, 1995a). Differences of this mechanism among the different types of hematological malignancies and between sperm-oocyte fertilization is the scope of

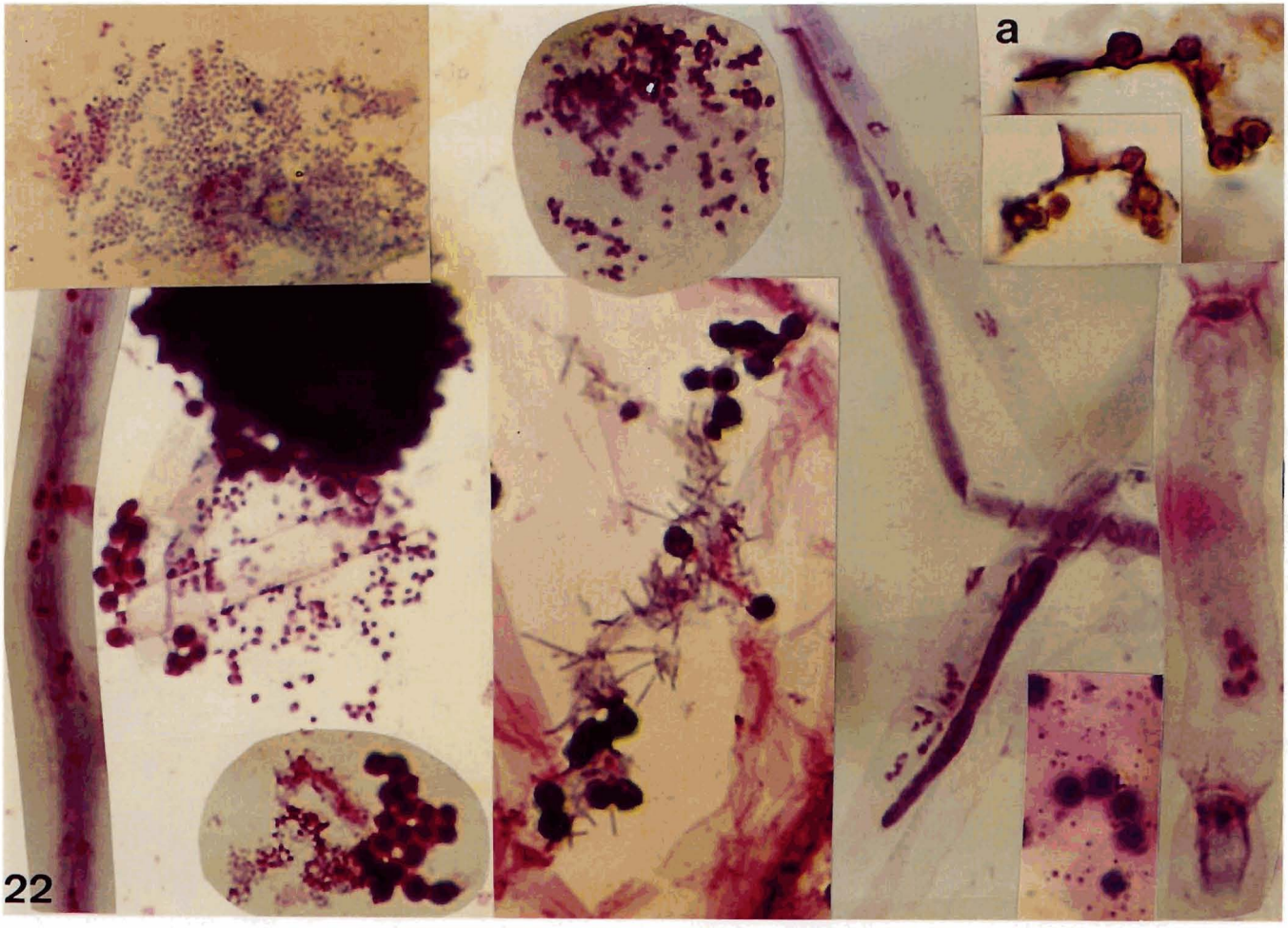


Fig. 22. Cytogenetic analysis of meiotic fungal cells showing NCs, condensed meiotic, DMs, ring and minute chromosomes scattered free or enclosed in hyphae. Giemsa. x 1,000. **Inset a:** *Candida albicans* cells communicating via NBs show high immunoreactivity for α_1-antichymotrypsin. HT-α_1-antichymotrypsin. x 1,000

another project. In this study, it is documented that germ cells and fertilization are common within the different types of these malignancies. Differences may lie within various functional genes in germ cells.

This study evidences that nuclear material of malignant meiotic cells is not wasted. All forms of nuclear material such as NVs, buds, nuclear segments, hyphae, DMs, minute and meiotic chromosomes show high attachment affinity and implantation into host cell nuclei. This property leads to saving, re-use and recycling of malignant nuclear material, resulting in cell immortality. This interpretation is strongly documented by the nuclear fusion into NCs which produce cells and are not inert or degenerating nuclear masses. NCs also shown by fungal, testicular and human embryonic cells (Logothetou-Rella, 1995a) characterize rapidly growing cells dividing by meiosis.

Invasion and implantation of all forms of malignant BMC nuclear material shown in this study, is accomplished via protease(s). NVs, DMs, minute and meiotic chromosomes were loaded with protease(s), glycoproteins and GAG. The information that meiotic chromosomes were PAS-D positive and loaded with protease(s) provides a means to distinguish meiotic from mitotic chromosomes and may be used as an index of malignant meiotic cells in tissue cell smears. The antiserum against α_1-chymotrypsin was used in this work to demonstrate CANP activity due to unavailability of antiserum against CANP (Logothetou-Rella, 1995a). Since the antiserum against α_1-chymotrypsin cross reacted with CANP and malignant BMC have been sensitive only to the CANP-I (Logothetou-Rella, 1994c), the protease(s) associated with NVs and meiotic chromosomes is CANP (Logothetou-Rella, 1995a). It has recently been reported that CANP is a meiotic protease (Logothetou-Rella, 1995b) known to hydrolyze

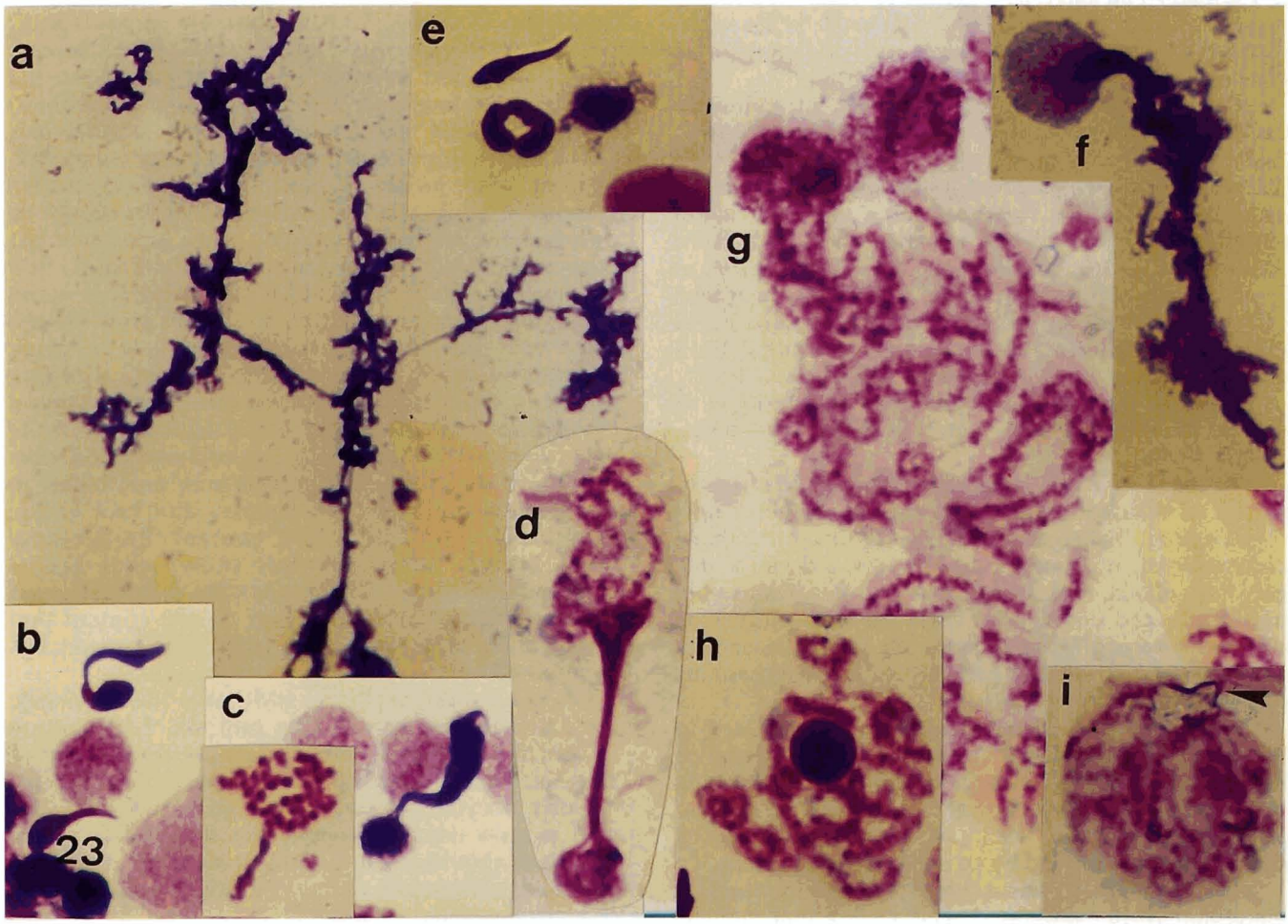


Fig. 23. Cytogenetic morphology of rat testicular tissue. **Inset a:** amorphous nuclei connected by NBs of three-week-old rat testis. Giemsa. x 400. **Insets b and c:** secondary spermatocytes dividing by meiosis, each producing one spermatid of various-size condensed head or in metaphase, via a NB, of five-week-old rat testis. **Inset d:** primary spermatocyte dividing by meiosis gives rise to a pachytene NV, of five-week-old rat testis. **Inset e:** nuclear fusion and free spermatids of NV morphology. **Inset f:** NC producing an intact nucleus of three-week-old rat testis. **Inset g and h:** extrusion of pachytene chromosomes and metaphase fusion in primary spermatocytes of five-week-old rat testis. **Inset i:** early pachytene spermatocyte shows CFS with condensed chromosome (arrow) of five-week-old rat testis. Giemsa. x 1,000

cytoskeletal proteins (Saido et al., 1994).

Chromosomal transfer from nucleus to nucleus is accomplished directly, or via NBs. Two types of NBs were identified. One type is formed between mother and daughter cells during meiotic NV formation. This NB eventually breaks upon separation of cells and comprises the tail of NVs. The other type of NB was identified between cells in interphase. Detection of chromosomes along and inside the NBs, strongly documents that genetic material is transferred and exchanged among cells via the NBs. Considering that each chromosome carries about 10,000 genes, genetic transfer among cells involves thousands of genes. Protoplasmic bridges have previously been reported as sizable communications between daughter cells during meiosis of male germ cells (Bloom and Fawcett, 1982). Moreover the presence of NBs in meiosis of rat male germ and fungal cells confirms that NBs constitute a characteristic feature of meiotic cells. NBs hidden behind the tissue ECM and cytoplasm, can only be visualized after hypotonic treatment of cells for ten min followed by fixation. Longer hypotonic cell treatment causes distortion of NBs and only remnants can be seen.

CFS was found in the lumen of NBs, intracellularly and in massive accumulations extracellularly, closely associated with chromosomal and nuclear fusion. CFS has previously been observed in malignant BMC, as the necessary substance for prophasing and cell fusion (Sandberg, 1980). This study provides further information on the composition of CFS consisting, at least, of glycoproteins and GAG-bound protease (CANP), also detected in meiotic rat male germ (Logothetou-Rella, 1995b) and fungal cells, both showing nuclear fusion. The ECM of malignant BMC, composed of GAG-bound CANP, is identical to that of solid tumor cells and embryonic cells (Logothetou-Rella, 1994d, 1995a).

Presence of PVC, DMs, ring and minute chromosomes in hematological malignancies, is not a new observation. Extrusion and implantation of these chromosomes, revealed by the in situ technique, is the new information. Ring metaphases are often observed in malignant and embryonic cells. In this study it is documented that ring metaphases are meiotic since they are formed by nuclear extrusion of chromosomes followed by metaphase fusion.

Meiotic fungal and rat testicular cells were used in this study as control meiotic cell systems in order to identify and confirm the main events such as extrusion of chromosomes, DMs, ring and minute chromosomes, metaphase fusion, NVs, NCs and NBs, which characterize meiosis and by no means define mitosis.

The presence of aneuploidy confirms meiosis of malignant BMC, since it is well known that meiosis causes aneuploidy (Goodenough and Levine, 1974). Repetitive aneuploidy caused by aberrant mitosis has been extensively studied in hematological malignancies. The non-repetitive aneuploidy, though caused by meiosis in these malignancies, is the main new observation of this study. This work reveals that aneuploidy is caused by nuclear extrusion of chromosomes, NV formation, fertilization and metaphase fusion. Thus, the cytogenetic analysis in this study showed that 72% of non-repetitive aneuploid BMC divide by meiosis and 28% of diploid ones by mitosis. The ratio of aneuploidy:diploidy may be of future prognostic and diagnostic value in malignancies, especially knowing that this ratio is reversed (very low) in late human embryonic cells (Logothetou-Rella, 1995a) with establishment of organogenesis and mitosis.

Meiosis, fertilization, embryos, cell to cell collision and interaction shown in hematological malignancies justifies the clonal evolution, subclones, karyotype instability, heterogeneity, structural aberrations, propagation and invasion of malignant cells.

The present observations are also interesting from the evolution point of view. Within a malignant BMC population all types of living cell division were observed. Malignant BMC showing binary fission of linear chromosomes, DMs and minute chromosomes, full of protease, glycoproteins and GAG, invading other cells, behave like bacteria, when considering that the bacterial nucleus and chromosome are equivalent. Budding of BMC and implantation of buds resembles yeasts and meiosis resembles that of fungi (small NV, hyphae). Also, meiosis identical to that of mammalian germ cells, fertilization and mitosis of higher eucaryotic cells is shown by malignant BMC. There is no doubt that Ogs and NVs are the link between fungal and mammalian cells (Logothetou-Rella, 1996). It is worth noting that although malignant BMT cells grow rapidly, show very few if any cells in metaphase in tissue sections identical to the rapidly growing *Candida albicans* cells which show no metaphases upon cytogenetic analysis. These observations support the hypothesis that within a malignant cell population, cells may move the evolution back and forth from higher to lower eucaryotes, to procaryotes and vice versa, depending upon cell CANP content. Preliminary work in this laboratory has shown that fungal cells contain higher CANP amounts than malignant cells and bacterial cells higher CANP content that fungal cells. More work is in progress to support this hypothesis.

In conclusion, malignant BMC give rise to somatic male and female gametes by meiosis. Fertilization occurs giving rise to embryonic cells dividing by meiosis. Recently, detection of primordial germ cells, oogonia, oocytes and embryo-like growth in malignant bone marrow tissues strengthen the observations of this study and provide more information on the embryonic state of malignant cells and stem cell renewal (Logothetou-Rella, 1996).

Acknowledgements. The author is indebted to Mrs. E. Katsarou for karyotyping the cell cultures, Mrs. M. Skoura for her expert technical assistance. Dr. C. Stamoulis and Dr. N. Tsavaris for providing bone marrow aspirates, Professors C. Kittas and S. Patsouris for providing

BMT paraffin blocks, and Mr. C.E. Harissis for his kind provision of a Nikon phase contrast microscope for the cytogenetic study of this work. The author would like to thank the Laboratory of Infectious Diseases, 1st Department Propedeutic Medicine, Athens Medical School and the Department of Microbiology, Laikon General Hosptial for providing fungi. The author is also grateful to the General Secretary of Research and Technology for supporting this project by the grant No 288.

References

Bloom W. and Fawcett S.W. (1982). A textbook of histology. Tenth Edition. W.B. Saunders Company. Igaku-Shoin/Saaunders. Tokyo pp 822-825.

Boyum A. (1968). Isolation of leucocytes from human blood. Further observations. Scand. J. Clin. Lab. Invest. 21 (Suppl. 97) 31-50.

Dutrillaux B. and Lejeune J. (1971). Sur une novelle technique d'analyse du caryotype humain. C.R. Acad. Sci. (D) Paris 272, 2638-2640.

Goodenough U. and Levine R.P. (1974). Genetics. Holt. Rinehart and Winton. Inc. New York. pp 217-220.

Hsu S.M., Raine L. and Fanger H. (1981). A comparative study of the peroxidase-antiperoxidase method and an avidin-biotin complex method for studying polypeptide hormones with radioimmunoassay antibodies. Am. J. Clin. Pathol. 75, 734-738.

Logothetou-Rella H. (1993). Spermatozoa-like cell invaders (nuclear vlimata) in human neoplasia. Histol. Histopathol. 8, 407-423.

Logothetou-Rella H. (1994a). Cytogenetic analysis and morphology of malignant nuclear vlimata. The life cycle of malignant cells. Histol. Histopathol. 9, 747-763.

Logothetou-Rella H. (1994b). Differentiation of human lymphocytes into nuclear vlimata by meiosis. The cytotoxic effect of calcium-activated neutral proteinase inhibitor. Histol. Histopathol. 9, 469-484.

Logothetou-Rella H. (1994c). The selective anticancer activity of the endogenous inhibitor of calcium-activated neutral proteinase. A histological, cytological and chemosensitivity study. Histol. Histopathol. 9, 485-493.

Logothetou-Rella H. (1994d). Glycosaminoglycan-sac formation in vitro. interactions between normal and malignant cells. Histol. Histopathol. 9, 243-249.

Logothetou-Rella H. (1995a). Nuclear vlimata and aneuploidy in embryonic cells is caused by meiosis. Behavior and properties of meiotic cells. Histol. Histopathol. 10, 541-566.

Logothetou-Rella H. (1995b). The inhibitor of calcium-activated neutral proteinase is an anti-meiotic agent. The spermicidal and anti-viral action. Histol. Histopathol. 10, 271-282.

Logothetou-Rella H. (1996). Description of primordial germ cells, oogonia, oocytes and embryo like growth in squash preparations of tissues from hematologic malignancies. Histol. Histopathol. 11, 965-984.

Plachot M., de Grouchy J., Junca A.M., Mandelbaum J., Turleau C., Coullin P., Cohen J. and Salat-Baroux J. (1987). From oocyte to embryo: A model, deduced from in vitro fertilization, for natural selection against chromosome abnormalities. Ann. Genet. 30, 22-32.

Saido T.C., Sorimachy H. and Suzuki K. (1994). Calpain: new perspectives in molecular diversity and physiological-pathological involvement. FASEB J. 8, 814-822.

Sandberg A.A. (1980). The chromosomes in human cancer and leukemia. Elsevier. New York-Amsterdam. pp 331-332.

Tsafriri A., Bar-Ami S. and Lindner H.R. (1983). Control of the development of meiotic competence and oocyte maturation in mammals. In: Fertilization of the human egg in vitro. Beier H.M. and Lindner H.R. (eds). Springer-Verlag, Berlin. Heidelberg. New York, Tokyo. pp 1-17.

Accepted June 10, 1996