

Immunoelectron microscopic study of nucleolar DNA during mitosis in Ehrlich tumor cells

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In order to investigate the DNA localization within Ehrlich tumor cell nucleoli during mitosis, two recent immunocytochemical methods using either an anti-DNA or an anti-bromodeoxyuridine (BrdU) monoclonal antibody have been applied. In both cases, the immunogold labeling has been performed on ultrathin sections of cells embedded either in Lowicryl K4M or in Epon, respectively.

Identical results are observed with both immunocytochemical approaches.

In the interphase nucleolus, besides the labeling of the perinucleolar chromatin shell and of its intranucleolar invaginations which penetrate into the nucleolar body and often terminate at the fibrillar centers, a few gold particles are also preferentially found towards the peripheral region of the fibrillar centers. In contrast, the dense fibrillar component and the granular component are never labeled. During mitosis, the fibrillar centers persist at the chromosomal nucleolus organizing regions (NOR's) and can be selectively stained by the silver method. However, these metaphase fibrillar centers are no longer decorated by the DNA- or BrdU antibodies.

These results indicate that until the end of prophase, rRNA genes are present inside the fibrillar center material, disappear during metaphase and reappear in reconstituting nucleoli during telophase. Thus, fibrillar centers appear to represent structures *sui generis*, which are populated by rRNA genes only when the nucleolus is functionally active.

In segregated nucleoli after actinomycin D treatment, the DNA labeling is exclusively restricted to the perinucleolar chromatin blocks. These findings also suggest that the DNA content of the fibrillar center material varies according to the rRNA transcription level of the cells. The results are discussed in the light of the present knowledge of the functional organization of the nucleolus.

Introduction

In modern terms, the nucleolus organizing region (NOR) is defined as a chromosomal region where rRNA genes are clustered. Numerous investigations, including observations of deletion and translocation NOR mutants in plants and

animals, *in situ* rRNA/DNA and rDNA/DNA hybridizations, have conclusively proven that the NOR's usually coincide with the secondary constrictions although the correlation is not absolute (see [9, 13, 36] for reviews).

When the chromosomes are stained with Feulgen reaction, the secondary constrictions appear, in many species, as particularly clear regions. This fact has been attributed to the low degree of DNA condensation or to a appreciable protein content in the secondary constriction [3]. Electron microscopic studies of secondary constrictions have shown that these areas predominantly consist of fine fibrillar material which has the same morphology and positivity for silver staining as the fibrillar centers (FC) in interphase (see [9] for review). However, the FC-like material is only partly integrated within chromosomes [22, 24, 27] in indentations diametrically opposed on their edge [11, 27, 37]. Although it is now well established that the FC contain a small amount of DNA (see [9] for review; [32, 38, 40]) which corresponds at least in human spermatocytes [1] to rDNA, the presence of DNA during mitosis is still a matter of controversy. Indeed, using Feulgen-like osmium-amine reaction, Hernandez-Verdun and Derenzini [15] have shown that, in metaphase chromosomes, the silver-stained material contained numerous DNA filaments, 2 or 3 nm thick, which never gave rise to nucleosomal-like structures. In contrast, on whole-mount preparations of silver-stained human metaphase chromosomes, electron microscopy revealed that the silver-stainable substance is located at the outer side of the nucleolar organizers or around them but never in the chromosomes themselves [34].

In the present work, we have attempted to study the DNA content of the fibrillar centers during the various stages of mitosis in Ehrlich tumor cells by means of immunoelectron microscopy using either a monoclonal antibody to DNA or a monoclonal antibody to bromodeoxyuridine detecting bromodeoxyuridine previously incorporated into DNA of cells.

In addition, these two immunoelectron microscope approaches have been also applied for identifying DNA within nucleoli after exposure of cells to actinomycin D, an agent known to block the rRNA synthesis and to cause segregation of the nucleolar components.

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Materials and methods

Cell culture

Ehrlich ascites tumor cells from the peritoneal cavity of C57B1 mice were cultured as monolayers in Petri dishes as previously described by Lepoint and Bassleer [18]. The medium was composed either of 45% NCTC 109 (Difco Laboratories, Detroit, MI/USA), 45% Hanks' solution or of RPMI 1640 medium (Gibco-Europe S.A., Gent/Belgium) supplemented with 10% fetal calf serum and 100 U/ml penicillin. The cells were maintained in a 5% CO₂ atmosphere. The nutrient medium was changed every day. After three days of cultivation, mitotic cells were selectively harvested [19]. A part of these cell cultures was incubated in medium containing bromodeoxyuridine (BrdU, 5×10^{-6} – 10^{-5} M) and 5-fluorodeoxyuridine (FdU, 5×10^{-7} – 10^{-6} M) for 20 h corresponding approximately to the cell cycle duration for the majority of in vitro cultivated ELT cells [17]. In addition, the medium also sometimes contained actinomycin D (0.05–100 µg/ml) during variable time.

The different cell suspensions were centrifuged at low speed to form a pellet.

Cytochemical method

Small fragments of the pellet were fixed in 1.6% glutaraldehyde for 60 min in Sorensen's buffer (pH 7.4) at 4 °C. After fixation, the cells were washed in Sorensen's buffer, and the pellet was divided into two parts.

The first one was acetylated as previously described by Wassef et al. [41]. The second one was additionally fixed for 5 min in Car-

noy's solution (3:1 ethanol/acetic acid) and then silver-stained as previously described [25] and acetylated [26]. After dehydration in a graded series of acetone, the cells were embedded in Epon 812. Ultrathin sections of the various blocks were cut with a diamond knife and mounted on copper grids.

Electron microscopic immunolocalization

Using a monoclonal antibody to DNA, small fragments of the pellet were fixed in 4% formaldehyde or in a mixture of 4% formaldehyde and 0.1% glutaraldehyde in Sorensen's buffer (pH 7.4) at 4 °C for 30 to 120 min. After fixation, the cells were washed in Sorensen's buffer and incubated in 0.5 M NH₄Cl at 4 °C for 60 min, dehydrated through graded ethanol solutions and then processed for embedding in Lowicryl K4M according to the technique of Roth et al. [30]. Some other fragments were fixed in 4% formaldehyde in Sorensen's buffer for 30 min, washed in Sorensen's buffer, incubated in pepsin in 0.1 N HCl (1 mg/ml, Merck, Darmstadt/FRG) for 15 min. After this enzymatic digestion, the cells were incubated in 5% trichloroacetic acid for 15 min at 4 °C and fixed again in 0.5% glutaraldehyde in Sorensen's buffer for 120 min. After washing in the same buffer, the cells were processed as described above.

Ultrathin sections were cut with a diamond knife and processed for the immunolabeling as previously described by Thyry et al. [40]. Identical control labelings were also performed. Monoclonal antibody (IgM) to DNA was used. The antibody bound to double and single stranded DNA but not to RNA. A detailed account of the characteristics of this antibody has been presented by Scheer et al. [32].

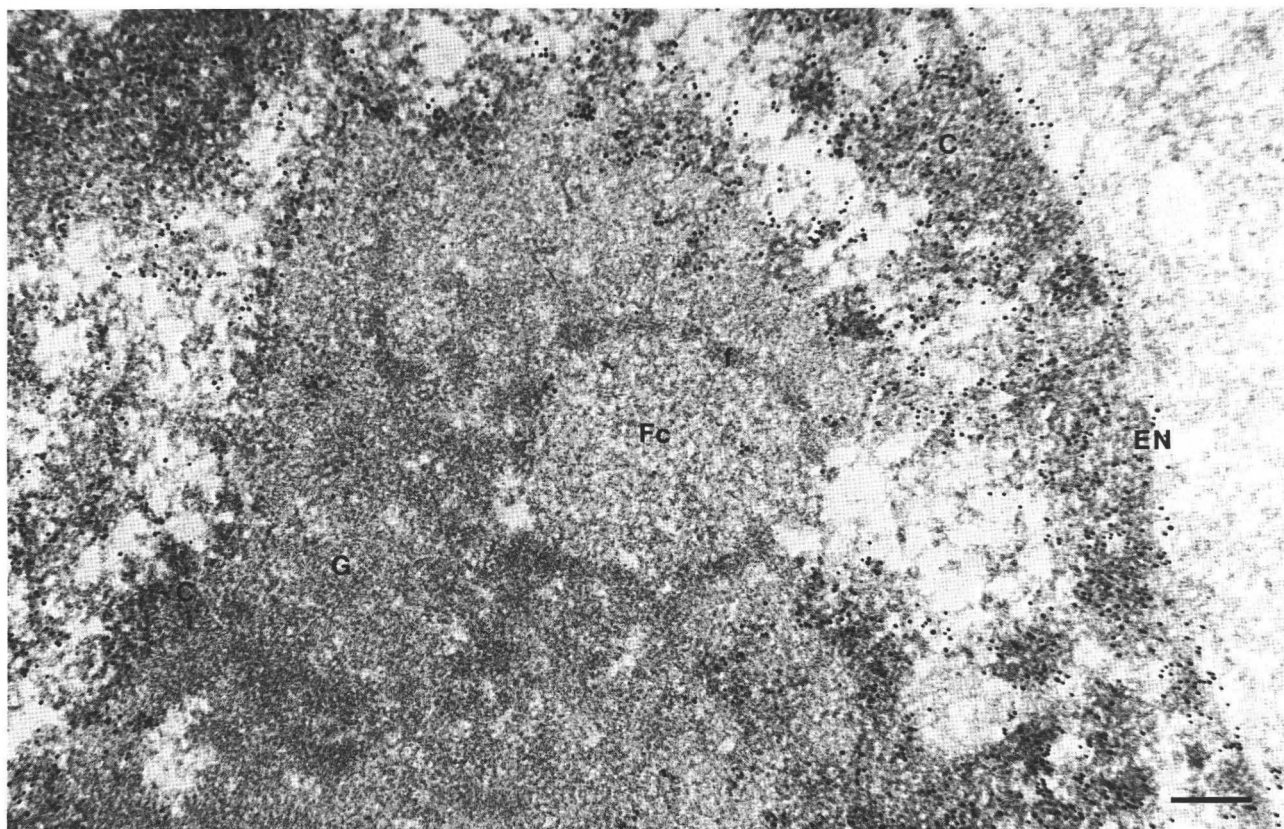


Fig. 1. Immunoelectron microscopy of nucleolar-associated DNA on a section of Lowicryl-embedded Ehrlich tumor cells using a monoclonal anti-DNA antibody. — C Dense chromatin. — EN Nu-

clear envelope. — f Dense fibrillar component. — Fc Fibrillar center. — G Granular component. 4% formaldehyde/pepsin/5% trichloroacetic acid/0.5% glutaraldehyde/Lowicryl. — Bar 0.3 µm.

Using a monoclonal antibody to BrdU, small fragments of the pellet were fixed in 4% paraformaldehyde or in a mixture of 4% formaldehyde and 0.1% glutaraldehyde in Sorensen's buffer (pH 7.4) at 4 °C for 60 min. After fixation, the cells were washed in Sorensen's buffer, dehydrated in ascending concentrations of ethanol solutions and then processed for embedding in Epon 812. Polymerization was performed at 37 °C for 3 to 5 days. Ultrathin sections of the various blocks were cut with a diamond knife and processed for the immunolabeling and its controls as previously described by Thiry and Dombrowicz [39]. A prior 5 N HCl treatment was performed for 30 min on Epon sections at room temperature before the immunolabeling. A monoclonal antibody to BrdU (Becton Dickinson, Mt. View, CA) was used.

The various sections were then stained with uranyl acetate and lead citrate before examination in a Jeol CX 100 II electron microscope at 60 kV.

Results

Interphase

Although the different fixations and embedding procedures used in the present study provide an adequate general morphology of the cell, the distinction between the various nucleolar components is sometimes complicated.

However, this discrimination can be improved by the use of enzymatic digestions. This situation is especially well displayed when the cells are treated with pepsin before the Lowicryl embedding processes (Fig. 1). The main effects of this treatment are to remove the proteinaceous matrix of nucleoli and to clear up the FC [8, 16, 29], increasing therefore the contrast between the various nucleolar components.

After the application of the postembedding immunogold labeling method involving either the monoclonal anti-DNA which specifically reacts with DNA both in double and single stranded forms or the monoclonal anti-BrdU antibody which binds to BrdU previously incorporated into DNA of cells, the same gold particles repartition in the cell is observed. Moreover, identical labeling pattern is found whatever the fixations employed in each of immunocytochemical methods. Gold particles are particularly enriched over the condensed chromatin associated with the nuclear envelope and with the nucleolus (Fig. 1). A slight labeling can be also found in the spaces between these heterochromatic blocks. Over the nucleoli, gold particles are essentially located over the shell of condensed chromatin surrounding them and over their intranucleolar invaginations. Some clumps of labeled intranucleolar chromatin even come in contact with the FC and interrupt the layer of dense fibrils surrounding them. Such chromatin regions are generally included in small nucleolar interstices. Gold particles are also present in the FC. More precisely, the labeling is preferentially distributed towards the periphery of the FC, often in close proximity to nucleolar interstices.

The dense fibrillar component and the granular component are completely devoid of gold markers. However, gold particles are present in nucleolar interstices at the periphery of the dense fibrillar component [38, 40].

Prophase

During prophase (Fig. 2), several condensing chromosomes come into contact with the nucleolus. In addition, the clumps of intranucleolar chromatin are rarely seen.

After immunolabeling of prophase cell sections, gold particles are essentially distributed over the condensing chromosomes, among others those attached to the nucleolus.

Further, a few gold particles are preferentially found at the periphery of the FC in close proximity to the contact which occurs between the condensing chromosomes and the nucleolus. The dense fibrillar component and the granular component are totally devoid of labeling.

Metaphase

When the silver-staining reaction is performed on metaphase cells, silver-stained structures appear always localized at the periphery and in contact with some chromosomes. However, as already pointed out [11, 37], various relationships between the chromosomes and the silver-stained structures can be visualized. Particularly, spherical silver-stained structures are found in indentations situated along some chromosomes (Fig. 3). Others of more elongated shape (Fig. 4) are intercalated between two portions of chromosomes. Further, in favorable sections, the spherical silver-stained structures are associated in pairs and are localized in diametrically opposed indentations of the edge of chromosomes (Fig. 5). This silver-stained material is never completely integrated within the chromosomes and consequently protude on their surface.

In Lowicryl-embedded metaphase cells, the silver-stained structures have the same morphological aspect as the FC in interphase. However, they present a variable appearance according to the fixation time in formaldehyde. So, after fixation in formaldehyde for 60 min, these structures consist of fibrillar material of low opacity (Fig. 6). In contrast, when the cells are only fixed in formaldehyde for 30 min before to be processed for embedding in Lowicryl, they appear as a network of high electron density contrasting with the silver-negative chromosomal material (Figs. 7-9).

Nevertheless, in both cases, the same gold particle repartition is observed when the immunolabeling involving the monoclonal anti-DNA antibody is performed on metaphase cell sections. Particularly, gold particles are exclusively localized over the silver-negative chromosomal material, and the silver-stained structures are always totally devoid of gold particles (Figs. 6, 7), even when these are associated in pairs (Figs. 8, 9).

Similarly, the distribution of gold particles on metaphase cells obtained with the immunocytochemical approach using the anti-BrdU antibody is exclusively seen over the silver-negative chromosomal material and not over the silver-stainable areas (Fig. 10).

Early telophase

When the silver-staining method is applied on early telophase cells, a few large roundish structures homoge-

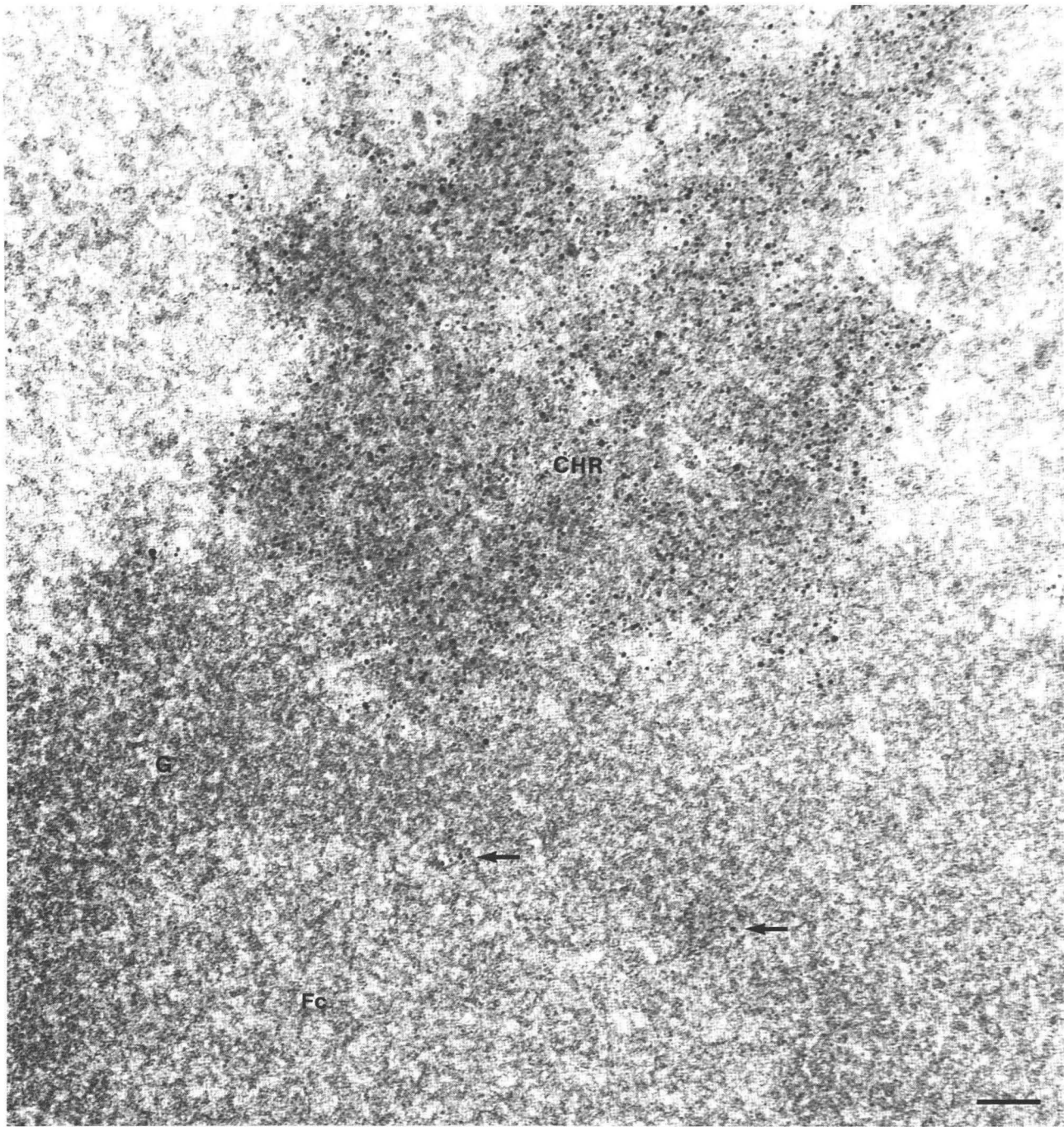


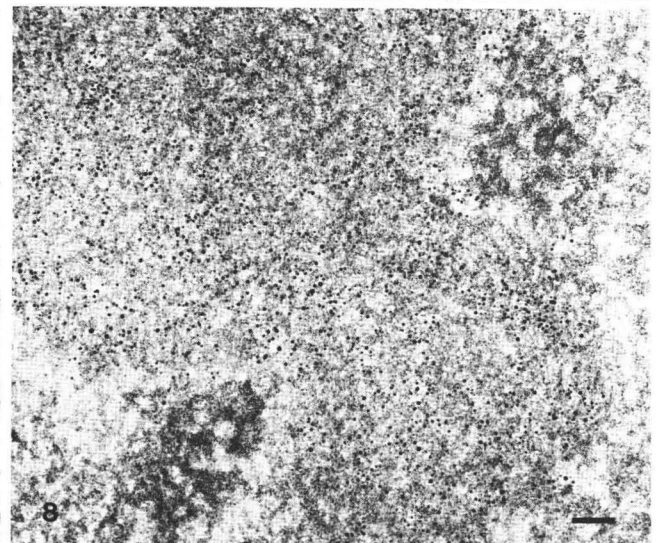
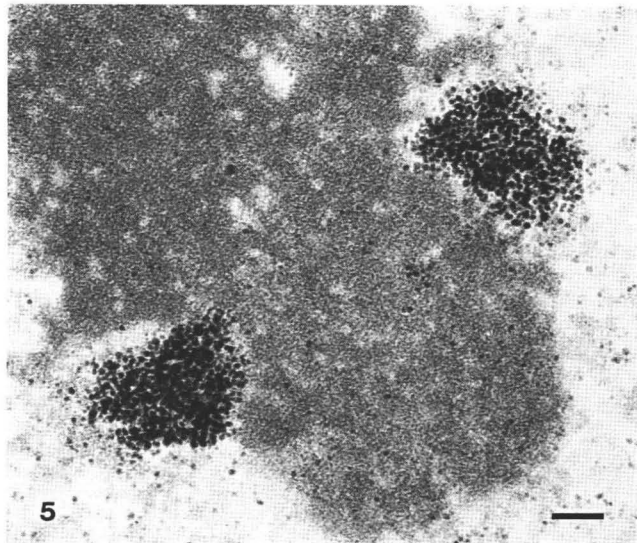
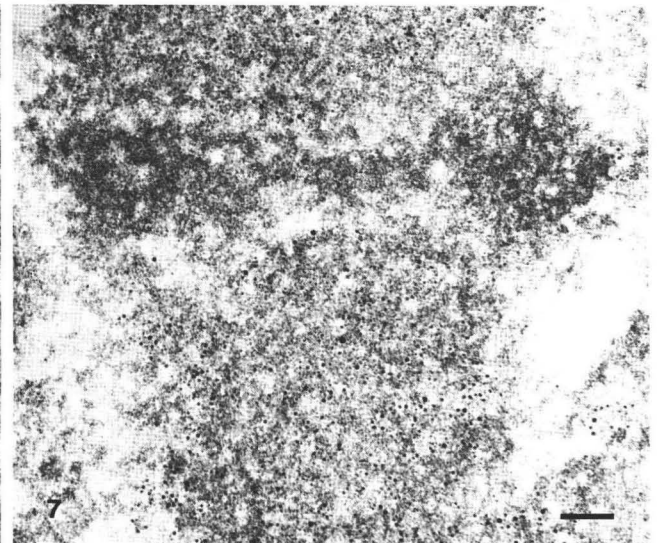
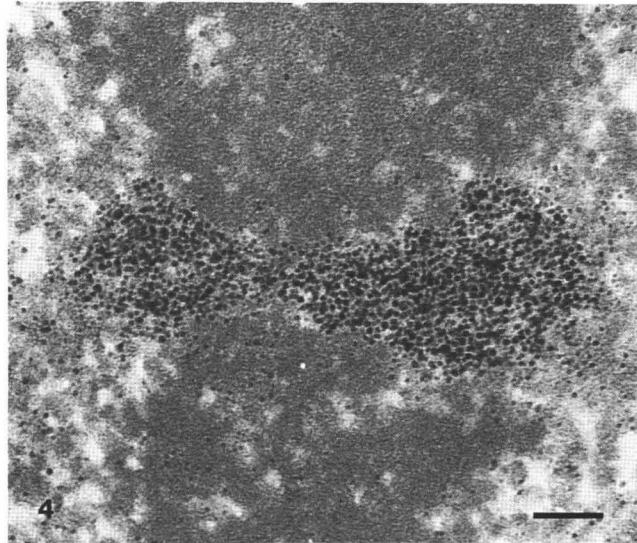
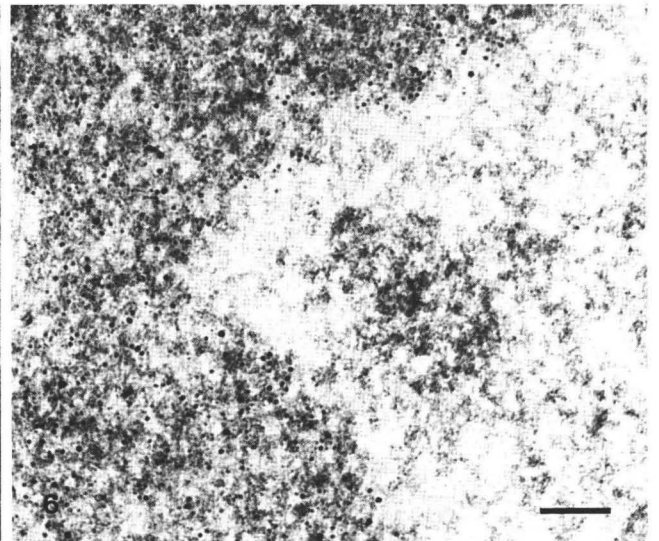
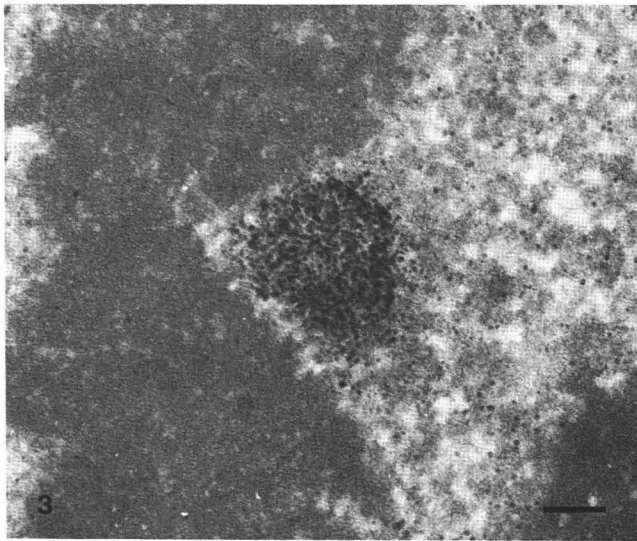
Fig. 2. Immunoelectron microscopy of DNA on a section of Lowicryl-embedded prophase Ehrlich tumor cell using a monoclonal anti-DNA antibody. A cluster of gold particles (*arrows*) is found at

the border of the fibrillar center (Fc), in proximity to a condensing chromosome (CHR) which comes into close contact with the nucleolus. — G Granular component. — Bar 0.1 μm .

neously silver-stained can be seen (Fig. 11). They are usually located at the periphery of some chromosomes. After the application of both immunocytochemical methods, these silver-positive areas are always devoid of gold particles (Figs. 12, 13) contrary to the chromosomal material which is heavily labeled.

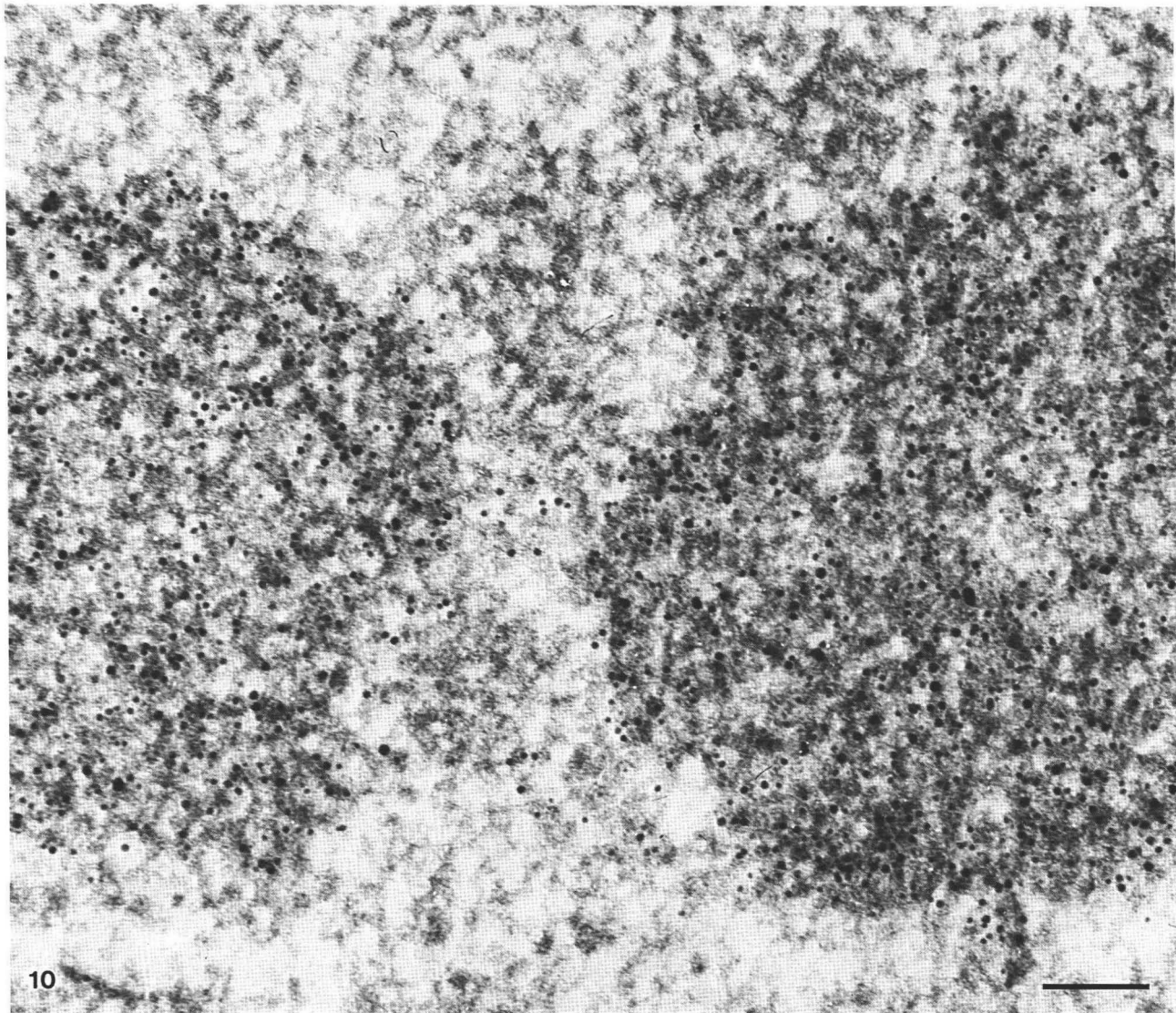
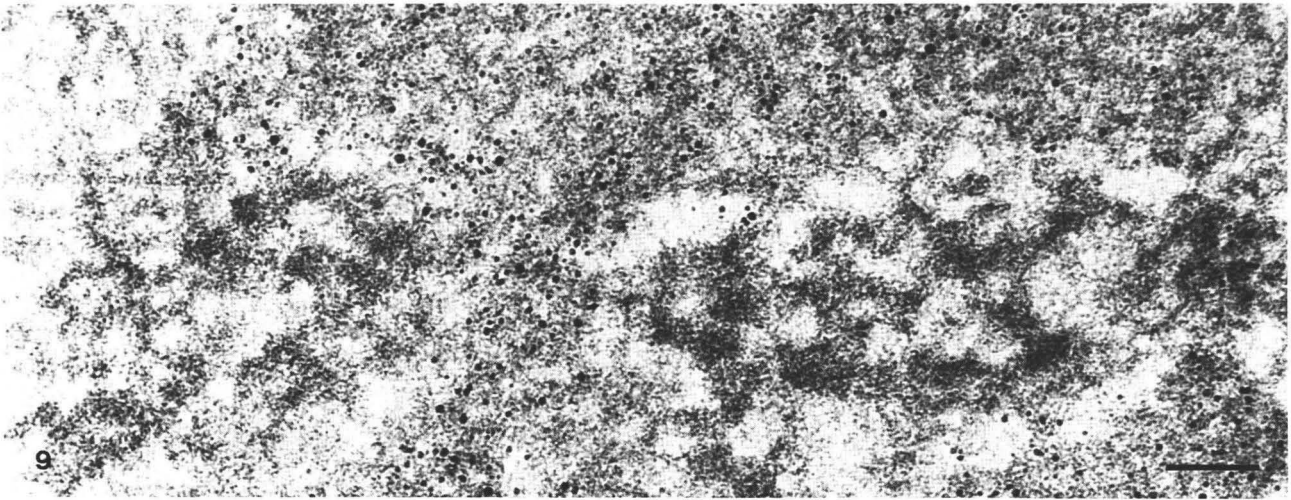
Late telophase

In the newly formed telophase nucleus, several small nucleoli can be visualized embedded in the still condensed chromatin. At this stage, the various nucleolar components are already well identifiable (Fig. 14) and discriminable by

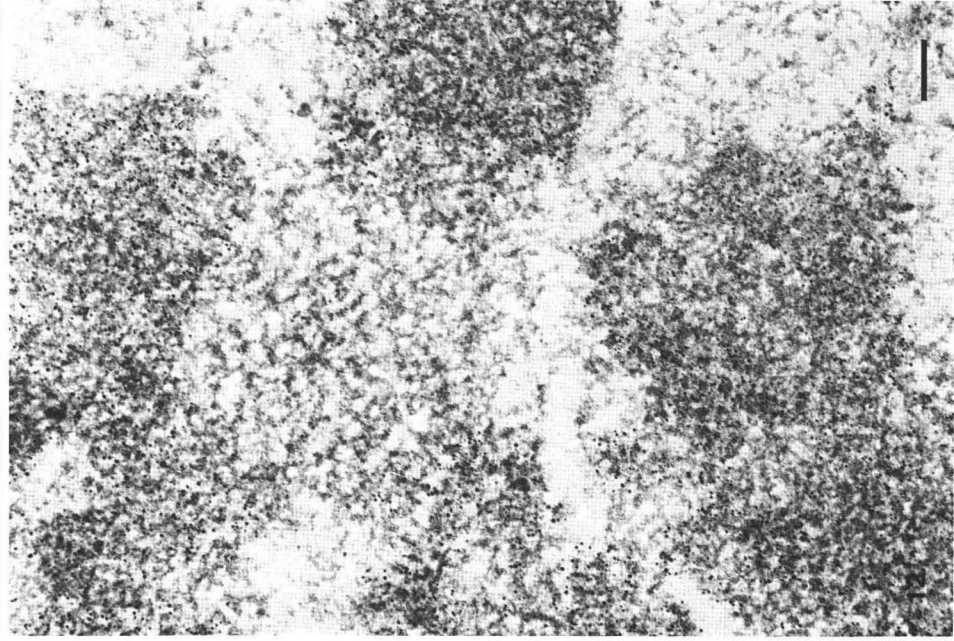
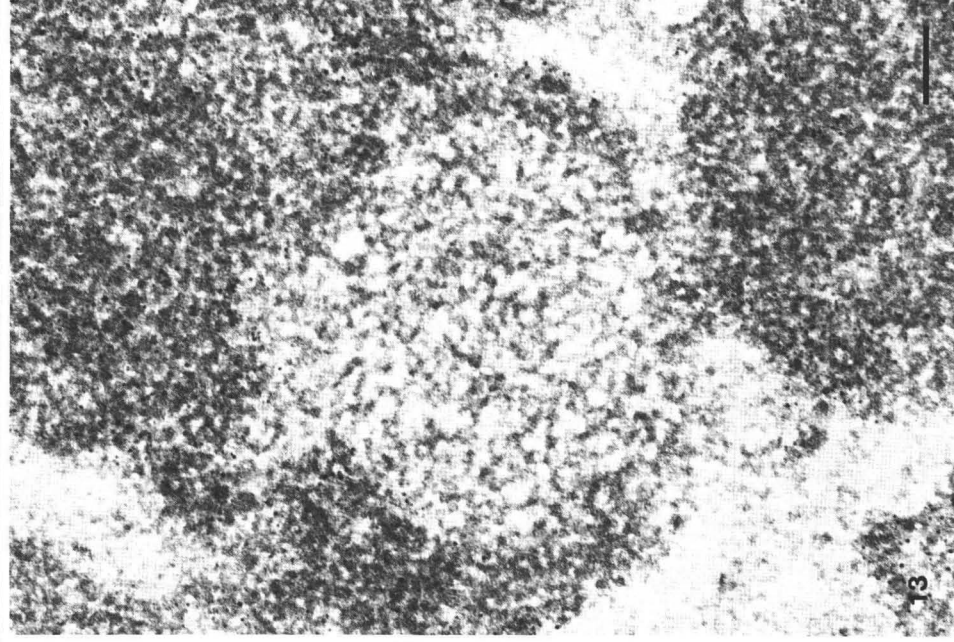
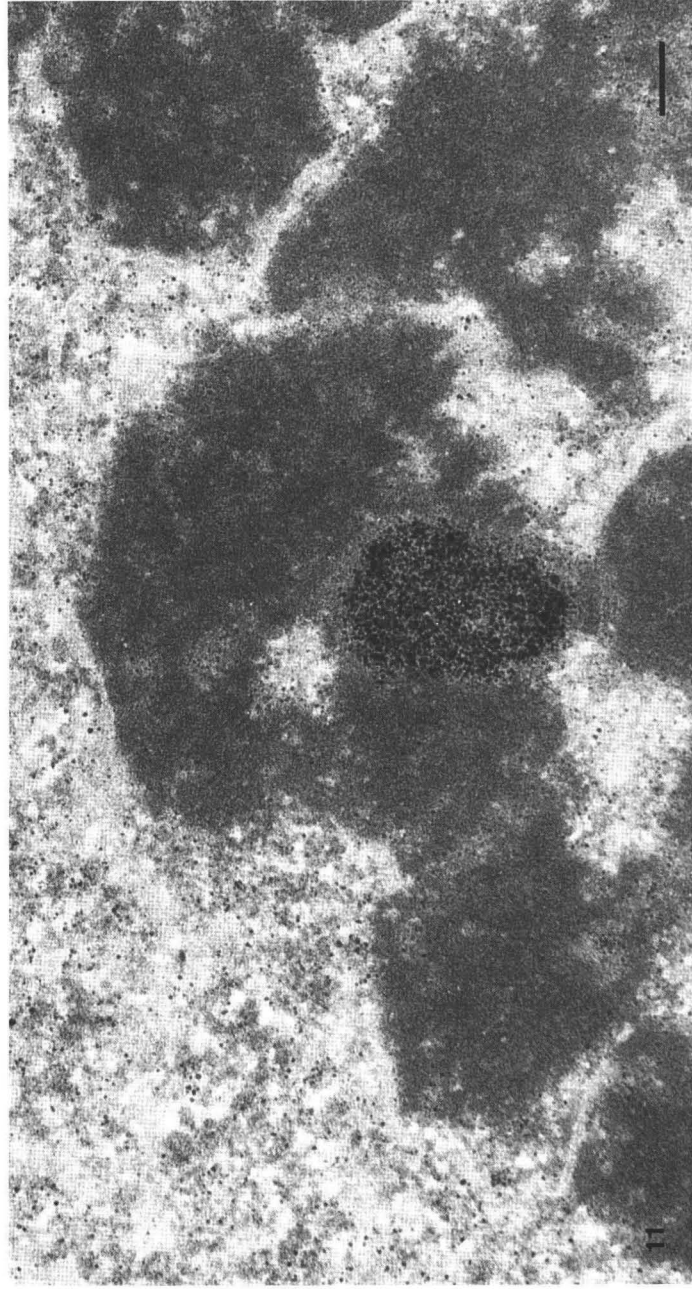


Figs. 3 to 10. Portions of metaphases in Ehrlich tumor cells after Ag-NOR staining (Figs. 3-5) or immunoelectron microscope la-

belonging either using a monoclonal anti-DNA antibody (Figs. 6-9) or using a monoclonal anti-BrdU antibody (Fig. 10).



The silver-stained material is totally devoid of gold particles. — Bars 0.1 μm .



the silver-staining method (Fig. 15). When both immunogold labeling techniques are applied on late telophase cell sections, gold particles are essentially localized over the condensed chromatin. Further, in some of these small nucleoli, gold markers are found at the periphery of the FC (Fig. 16, 17). In contrast, the dense fibrillar component and the granular component are not labeled.

Actinomycin D treatment

After actinomycin D treatment (Fig. 18), we observe a well-known segregation of the nucleolus characterized by the separation of nucleolar components into three main distinct zones: the granules, the fibrils and the FC. In addition, the intranucleolar chromatin clumps completely disappear, and only perinucleolar chromatin blocks are apparent. After immunolabeling on cells treated with actinomycin D doses ranging from 0.05 to 100 µg/ml for at least 1 h, the same repartition of gold particles is obtained in all the segregated nucleoli.

When the cells are treated for 1 to 2 h with high doses of actinomycin D (20–100 µg/ml) and the postembedding immunogold labeling technique using the anti-DNA antibody is applied, the three segregated nucleolar components are always free of gold particles. In contrast, the heterochromatinic blocks, in particular those localized at the periphery of the segregated nucleoli, are strongly labeled.

Using the immunocytochemical approach involving the anti-BrdU antibody, identical labeling pattern is seen when the cells are treated with a very low dose of actinomycin D (0.05 µg/ml) for 1 h (Fig. 18). However, it is noteworthy that at this dose of antibiotic for shorter times, a few gold particles can always be found in the segregating FC, preferentially towards their peripheral regions. The specificity of the DNA labeling after both immunocytochemical methods has been tested in several ways. First, there is virtually no labeling if the primary antibody is omitted. Next, if the antigen is specifically removed from the sections by treatment with DNase I, the labeling is almost completely abolished. Finally, when gold lacking antibody tag is used, gold particles do not bind to the sections. In addition, using the monoclonal anti-BrdU antibody, no gold particle is bound to the sections if the cells are not exposed to BrdU and FdU.

Discussion

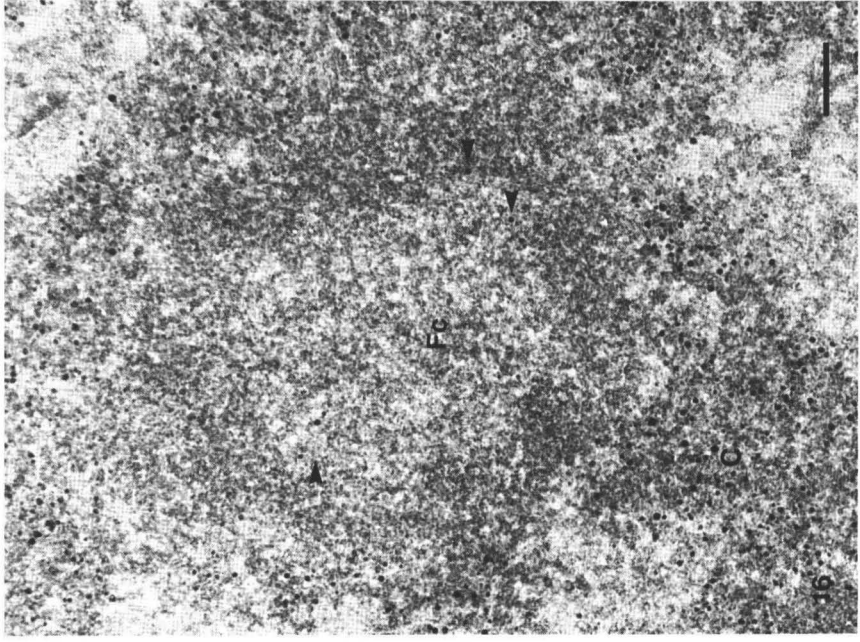
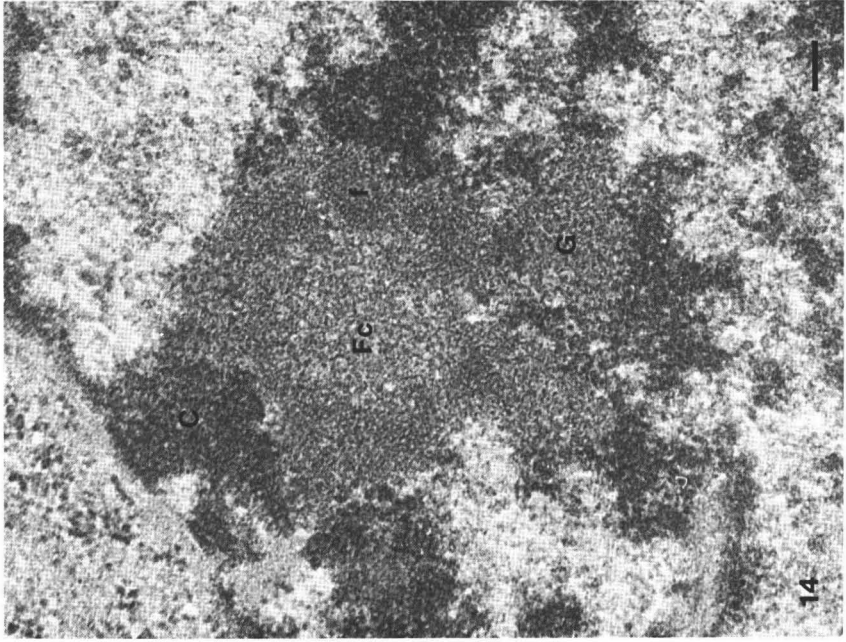
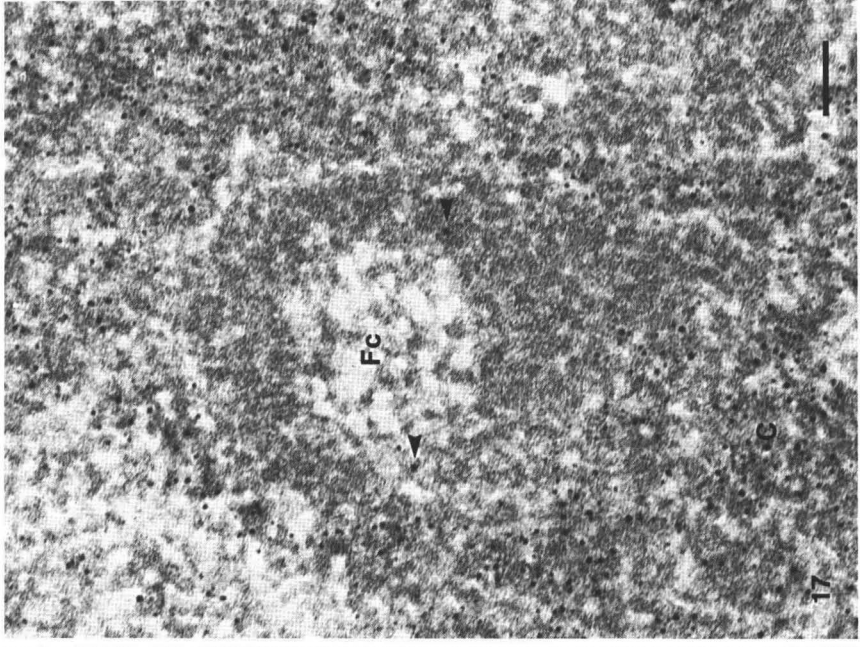
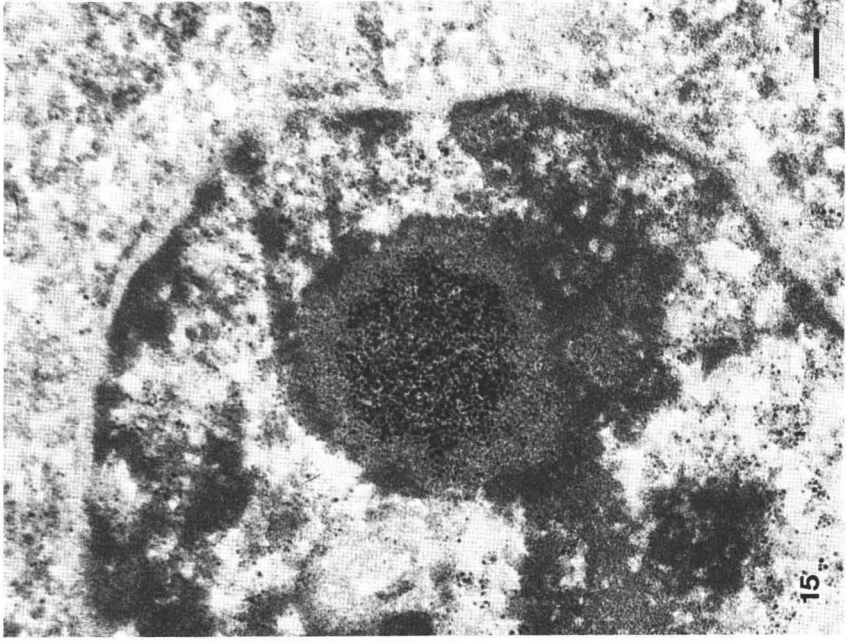
Recently, we have demonstrated the presence of a small amount of DNA in FC, of interphase nucleoli [32, 38, 40]; in the present study, which also involves the use of immunocytochemistry on Epon sections, we confirm these results. Likely, the labeling is preferentially localized at the periphery of the fibrillar centers, often in close proximity

to nucleolar interstices interrupting the layer of dense fibrils which surrounds them. In addition, the dense fibrillar component has also been found completely devoid of labeling. Although these results do not corroborate the concept that the dense fibrillar component results from the superposition of transcriptionally active rRNA genes and their primary transcription products (for review, see [9, 10, 36]), the spatial distribution of DNA obtained is consistent with the fact that rRNA transcription occurs essentially close to the boundary region between FC and the surrounding dense fibrillar component as previously discussed [38, 40].

The essential result of the present work is that the gold decoration disappears from the FC-like material during metaphase. Indeed, until the end of prophase, gold particles are found inside this material, later on the labeling completely disappears during metaphase and only reappears in reconstituting nucleoli during telophase. These results are in total disagreement with the observations performed by means of the Feulgen-like ammine-osmium reaction. Particularly, Hernandez-Verdun and Derenzini [15] have shown that silver-stained regions of metaphase chromosomes contain chromatin fibrils in a completely extended configuration which never give rise to nucleosomal-like structures. The peculiar DNA configuration was regarded as a permanent feature of intranucleolar loose chromatin agglomerates and not a consequence of transcriptional activity [4]. In view of these observations, one has to ask whether the absence of gold particles inside the silver-stained material associated with metaphase chromosomes reflects a true absence of DNA in this region or corresponds to a very low local content of DNA which does not allow detection by our immunocytochemical approach. In addition, it may also be asked whether the rDNA during metaphase does not acquire a peculiar configuration inaccessible to the antibodies.

However, identical labeling pattern is achieved by these two different high-resolution immunocytochemical approaches. Moreover, immunocytochemistry using the anti-DNA monoclonal antibody has allowed to detect a very small amount of DNA including the DNA present in amplified extrachromosomal nucleoli, chromomeres of lampbrush chromosomes, mitochondria, chloroplasts, and mycoplasma particles [32]. Further, when this antibody is used for a Lowicryl postembedding immunogold labeling approach, chromatin organized in various configurations has been revealed. For instance, it detects chromatin condensed into nucleosomes in different cell types such as the condensed chromatin associated with the nuclear envelope [14, 32, 40]. It also reveals the DNA of chromosomes from dinoflagellates [32] which are exceptionally organized since these chromosomes do not contain histones. Furthermore, by incubating mouse DNA with antibodies, Scheer et al. [32] have visualized DNA-antibody complexes in electron microscopic spread preparations. Likewise, the postembedding immunogold labeling technique using the monoclonal anti-BrdU antibody has allowed to identify various cellular sites known to contain DNA, in particular mitochondria, chromosomes and interphase chromatin [38, 39].

Figs. 11 to 13. Early telophase in Ehrlich tumor cells after Ag-NOR staining (Fig. 11) or immunoelectron microscope labeling either using a monoclonal anti-DNA antibody (Fig. 12) or using a monoclonal anti-BrdU antibody (Fig. 13). The silver staining material is totally devoid of gold particles. — Bars 0.15 µm.



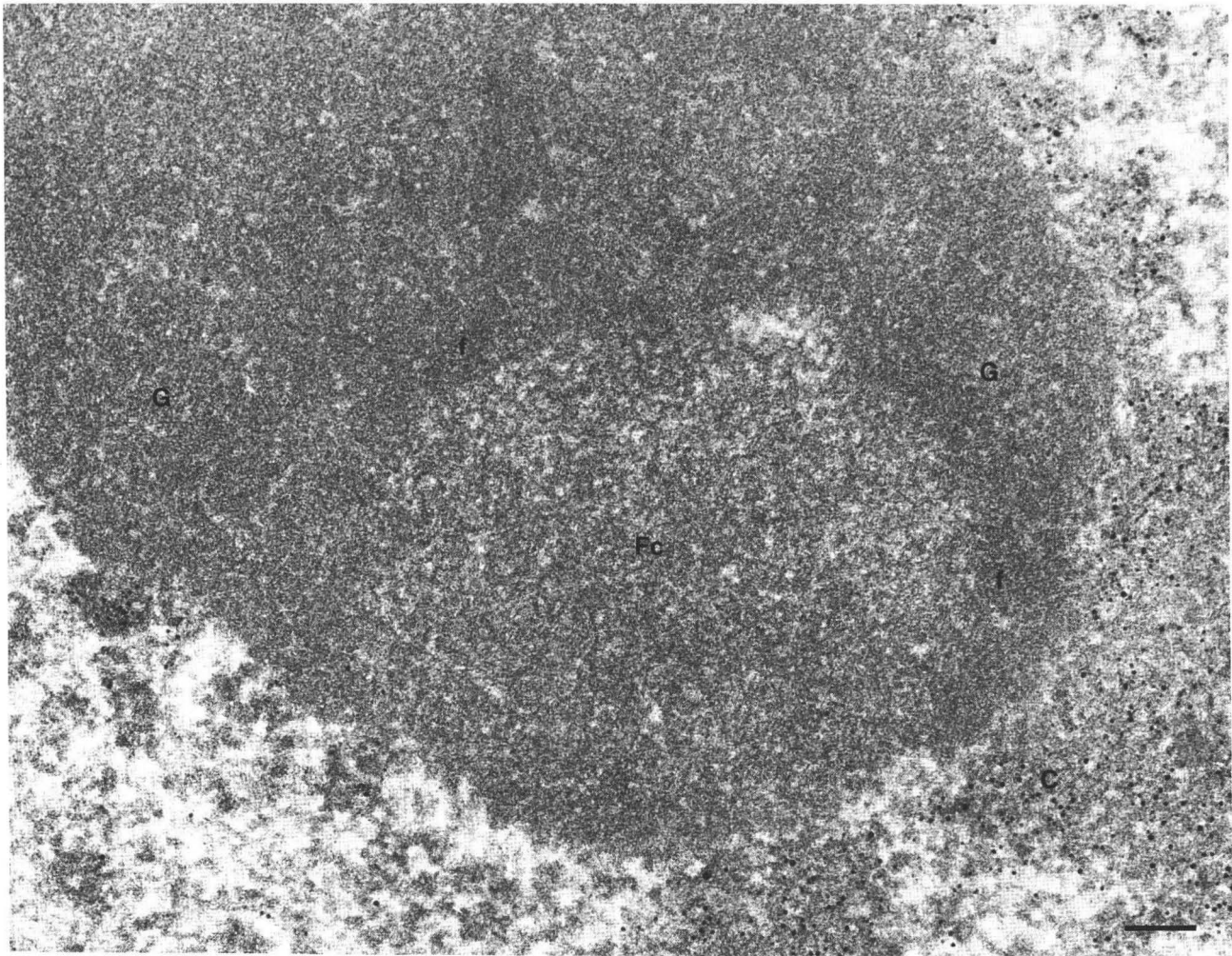


Fig. 18. Nucleolus of Ehrlich tumor cell treated with 0.05 $\mu\text{g/ml}$ of actinomycin D for 120 min and immunolabeled by a monoclonal anti-BrdU antibody. The fibrillar center (Fc), the dense fibrillar

component (f) and the granular component (G) are completely free of gold particles, only the perinucleolar blocks of dense chromatin (C) are labeled. — Bar 0.1 μm .

It is interesting to note that the absence of DNA from the FC material correlates with the transcriptional inactivity of the rRNA genes during mitosis [28]. This correlation is supported by the results obtained after blockage of rRNA synthesis induced by actinomycin D treatment. Under these conditions, the labeling obtained with both immunocytochemical approaches disappears completely from segregated nucleoli, and only condensed chromatin bulks situated at their periphery are visible. This absence

of labeling inside the FC material seems thus to reflect an inactivation of rRNA gene transcriptional activity. Now, numerous electron microscopic studies based on the chromosome-spreading technique have demonstrated that the long-term cessation of transcriptional activity of nucleolar chromatin, whether natural or induced by drugs such as actinomycin D, led to chromatin structurally indistinguishable from other inactive and condensed chromatin ([5, 31]; see [6] for review).

Accordingly, our results strongly suggest that the DNA present in the interphase FC abandons this structure during the first stages of mitosis and becomes incorporated in the adjacent chromosomal material. In metaphase cells, this DNA should be localized in the chromosomal material situated in and/or in close proximity to the secondary constriction. During telophase, the inverse phenomenon occurs, the DNA unfolds and invades the FC-like material. It is known that at this stage RNA synthesis is reactivated [19].

Figs. 14 to 17. Late telophase in Ehrlich tumor cells after acetylation (Fig. 14) or Ag-NOR staining (Fig. 15) or immunoelectron microscope labeling either using a monoclonal anti-DNA antibody (Fig. 16) or using a monoclonal anti-BrdU antibody (Fig. 17). A few gold particles (*arrowheads*) are found inside the fibrillar centers (Fc) essentially towards their peripheral regions. — C Dense chromatin. — f Dense fibrillar component. — G Granular component. — Bars 0.1 μm .

This interpretation allows to explain that in plant species characterized by a content of rRNA genes markedly higher than in animal species, most of the rRNA cistrons can be located in the heterochromatic portion of chromosomes [7, 20, 23]. It also allows to understand the results obtained by irradiation of regions outside and inside the secondary constrictions of salamander lung epithelium chromosomes using an argon laser microbeam [2]. These experiments have shown that irradiation immediately adjacent to the constriction consistently results in the loss of nucleolar organizing capacity, whereas irradiation directly inside the constriction reduces, but does not destroy, the ability to organize a nucleolus.

Further, our results support the conclusion of Schwarzscher et al. [34] that the Ag-stainable substance in metaphase cells is located outside the NOR's or around them but never in the chromosomes themselves. Why this material, which appears to be constituted of proteins originating from the interphase FC, remains in close proximity to the sites of the rRNA genes, cannot be answered at the moment. Furthermore, it would be interesting to study the distribution of RNA polymerase I which is known to be bound to the NOR's of metaphase chromosomes [33] at the electron microscopic resolution level in order to see whether this enzyme colocalizes with the FC structures. The distribution of other NOR-associated proteins such as C23 [21, 35] and topoisomerase I [12] should also be analyzed in comparison to the FC material.

Thus, although the morphological aspect of the FC persists throughout the whole cell cycle, its DNA content seems to change. Therefore, we consider the FC as a structural entity which exists independently on the presence or absence of rRNA genes. Probably the FC material which occurs close to the metaphase chromosomal region containing rRNA genes represents a proteinaceous structural framework in which the rRNA genes invade during their transcriptional reactivation.

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References

- [1] Arroua, M., M. Hartung, M. Devictor, J. Bergelefranc, A. Stahl: Localization of ribosomal genes by in situ hybridization in the fibrillar centre of the nucleolus in the human spermatocyte. *Biol. Cell* **44**, 337–340 (1982).
- [2] Berns, M., W. Cheng: Are chromosome secondary constrictions nucleolar organizers? *Exp. Cell Res.* **69**, 185–192 (1971).
- [3] Busch, H., K. Smetana: *The Nucleolus*. Academic Press, New York 1970.
- [4] Derenzini, M., A. Pession, C. Betts-Eusebi, F. Novello: Relationship between the extended, non-nucleosomal intranucleolar chromatin in situ and ribosomal RNA synthesis. *Exp. Cell Res.* **145**, 127–143 (1983).
- [5] Foe, V.: Modulation of ribosomal RNA synthesis in *Oncopeltus fasciatus*: an electron microscopic study of the relationship be-

- tween changes in chromatin structure and transcriptional activity. *Cold Spring Harbor Symp. Quant. Biol.* **42**, 723–740 (1978).
- [6] Franke, W., U. Scheer, H. Spring, M. Trendelenburg, H. Zentgraf: Organization of nucleolar chromatin. In: H. Busch (ed.): *The Cell Nucleus*. Vol. 7. pp. 45–95. Academic Press, New York 1979.
- [7] Givens, J., R. Phillips: The nucleolus organizer region of maize (*Zea mays* L.). Ribosomal RNA gene distribution and nucleolar interactions. *Chromosoma* **57**, 103–117 (1976).
- [8] Goessens, G.: Les centres fibrillaires des nucléoles de cellules tumorales d'Ehrlich. *C. R. Acad. Sci.* **277**, 325–327 (1973).
- [9] Goessens, G.: Nucleolar structure. *Int. Rev. Cytol.* **87**, 107–158 (1984).
- [10] Goessens, G., A. Lepoint: The nucleolus. Organizing Regions (NOR's): Recent data and hypotheses. *Biol. Cell.* **35**, 211–220 (1979).
- [11] Goessens, G., M. Thiry, A. Lepoint: Relations between nucleoli and nucleolus-organizing regions during the cell cycle. *Chromosomes Today* **9**, 261–271 (1987).
- [12] Guldner, H. H., C. Szosteki, H. P. Vosberg, H. J. Lakomek, E. Penner, F. Bautz: Scl 70 autoantibodies from scleroderma patients recognize a 95 KDa protein identified as DNA topoisomerase I. *Chromosoma* **94**, 132–138 (1986).
- [13] Hadjiolov, A.: *The nucleolus and ribosome biogenesis*. Cell Biology Monographs. Vol. 12. pp. 1–263. Springer-Verlag, Wien, New York 1985.
- [14] Hansmann, P., H. Falk, U. Scheer, P. Sitte: Ultrastructural localization of DNA in two *Cryptomonas* species by use of a monoclonal DNA antibody. *Eur. J. Cell Biol.* **42**, 152–160 (1986).
- [15] Hernandez-Verdun D., M. Derenzini: Non-nucleosomal configuration of chromatin in nucleolar organizer regions of metaphase chromosomes in situ. *Eur. J. Cell Biol.* **31**, 360–365 (1983).
- [16] Hubert, J.: Données préliminaires sur les «centres fibrillaires» du nucléole de certaines cellules du follicule ovarien d'un lézard: *Laecerta muralis* Laur. *C. R. Acad. Sci.* **281**, 271–273 (1975).
- [17] Lepoint, A.: Selection of Ehrlich tumor cells in different phases of the cell cycle by Ficoll gradient centrifugation. *Virchows Arch. B Cell Path.* **25**, 53–60 (1977).
- [18] Lepoint, A., R. Bassleer: Etude cytologique et cytochimique de cellules tumorales d'Ehrlich de la souris cultivées in vitro. *C. R. Acad. Sci.* **276**, 441–443 (1973).
- [19] Lepoint A., G. Goessens: Nucleologenes in Ehrlich tumor cells. *Exp. Cell Res.* **117**, 89–94 (1978).
- [20] McClintock, B.: The relation of a particular chromosomal element to the development of the nucleoli in *Zea mays*. *Z. Zellforsch.* **21**, 294–328 (1934).
- [21] Ochs, R., P. Lischwe, P. O'Leary, H. Busch: Localization of nucleolar phosphoproteins B23 and C23 during mitosis. *Exp. Cell Res.* **146**, 139–149 (1983).
- [22] Paweletz, N., M. C. Risueño: Transmission electron microscopic studies on the mitotic cycle of nucleolar proteins impregnated with silver. *Chromosoma* **85**, 261–273 (1982).
- [23] Phillips, R., D. Weber, R. Kleese, S. Wang: The nucleolus organizer region of maize (*Zea mays* L.): tests for ribosomal gene compensation or magnification. *Genetics* **77**, 285–297 (1974).
- [24] Ploton, D., A. Beorchia, M. Menager, P. Jeannesson, J. J. Adnet: The three-dimensional ultrastructure of interphasic and metaphasic nucleolar argyrophilic components studied with high-voltage electron microscopy in thick sections. *Biol. Cell* **59**, 113–120 (1987).
- [25] Ploton, D., H. Bobichon, J. J. Adnet: Ultrastructural localization of NOR in nucleoli of human breast cancer tissues using a one-step Ag-NOR staining method. *Biol. Cell.* **43**, 229–232 (1982).
- [26] Ploton, D., M. Menager, J. J. Adnet: Simultaneous high resolution localization of Ag-NOR proteins and nucleoproteins in interphasic and mitotic nuclei. *Histochem. J.* **16**, 897–906 (1984).

- [27] Ploton, D., M. Thiry, M. Menager, A. Lepoint, J. J. Adnet, G. Goessens: Behaviour of nucleolus during mitosis. A comparative ultrastructural study of various cancerous cell lines using the Ag-NOR staining procedure. *Chromosoma* **95**, 95-107 (1987).
- [28] Prescott, D.: Cellular sites of RNA synthesis. *Prog. Nucleic Acid Res. Mol. Biol.* **3**, 33-57 (1964).
- [29] Recher, L., J. Whitescarver, L. Briggs: The fine structure of a nucleolar constituent. *J. Ultrastruct. Res.* **29**, 1-14 (1969).
- [30] Roth, J., M. Bendayan, E. Carlemalm, W. Villiger, M. Garavito: Enhancement of structural preservation and immunocytochemical staining in low temperature embedded pancreatic tissue. *J. Histochem. Cytochem.* **29**, 663-671 (1981).
- [31] Scheer, U.: Changes of nucleosome frequency in nucleolar and non-nucleolar chromatin as a function of transcription: an electron microscopic study. *Cell* **13**, 535-549 (1978).
- [32] Scheer, U., K. Messner, R. Hazan, I. Raska, P. Hansmann, H. Falk, E. Spiess, W. W. Franke: High sensitivity immunolocalization of double and single-stranded DNA by a monoclonal antibody. *Eur. J. Cell Biol.* **43**, 358-371 (1987).
- [33] Scheer, U., K. Rose: Localization of RNA polymerase I in interphase cells and mitotic chromosomes by light and electron microscopic immunocytochemistry. *Proc. Natl. Acad. Sci. USA* **81**, 1431-1435 (1984).
- [34] Schwarzacher, H., A. Mikelsaar, W. Schnedl: The nature of the Ag-staining of nucleolus organizer regions. Electron- and light-microscopic studies on human cells in interphase, mitosis and meiosis. *Cytogenet. Cell Genet.* **20**, 24-39 (1978).
- [35] Spector, D., R. Ochs, H. Busch: Silver staining immunofluorescence and immunoelectron microscopic localization of nucleolar phosphoproteins B23 and C23. *Chromosoma* **90**, 139-148 (1984).
- [36] Stahl, A.: The nucleolus and nucleolar chromosomes. In: E. Jordan, C. Cullis (ed.): *The Nucleolus*. Vol. 15. pp. 1-24. Cambridge University Press. London, New York 1982.
- [37] Thiry M.: Ultrastructural study of nucleolar organizing regions by means of serial sections in Ehrlich tumour cells. *Bulletin de la Société Royale des Sciences de Liège* **4**, 527-538 (1986).
- [38] Thiry, M.: Immunoelectron microscope localization of bromodeoxyuridine incorporated into DNA of Ehrlich tumor cell nucleoli. *Exp. Cell Res.* In press 1988.
- [39] Thiry, M., D. Dombrowicz: Anti-bromodeoxyuridine monoclonal antibody: an alternative tool for the identification of replicated DNA at the electron microscope level. *Biol. Cell* **62**, 99-102 (1988).
- [40] Thiry, M., U. Scheer, G. Goessens: Localization of DNA within Ehrlich tumor cell nucleoli by immunoelectron microscopy. *Biol. Cell* **63**, 27-34 (1988).
- [41] Wassef, M., J. Burglen, W. Bernhard: A new method for visualization of preribosomal granules in the nucleolus after acetylation. *Biol. Cell.* **34**, 153-158 (1979).

Fatty acids binding to albumin increases its uptake and transcytosis by the lung capillary endothelium

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To determine whether uptake and transcytosis of albumin (A) in continuous capillary endothelia are modified when this protein carries fatty acids, the transport of albumin-oleic acid and albumin-palmitic acid complexes was compared with that of defatted albumin. The probes, either radioiodinated or tagged with 5-nm gold particles (Au), or both, were perfused in situ or injected in vivo; after 3 or 30 min lung fragments were radioassayed or examined by electron microscopy. Both in situ and in vivo, the uptake of fatty acid-carrying albumin (A-FA) was consistently 2 to 3 times higher than that of defatted A. Electron microscopy revealed that A-FA complexes tagged with gold were taken up and transported across the endothelium by plasmalemmal vesicles. Morphometric analysis showed that as compared with A-Au, at 3 min the density of (A-FA)Au bound to plasmalemmal vesicles was 2 to 3 times higher, and the extent of transcytosis was increased. Uptake of the iodinated albumin was more effectively competed by A-FA complexes than by defatted A, suggesting a higher affinity of the former for the albumin binding sites of the endothelium. The results indicate that when carrying fatty acids, albumin is taken up specifically and with high affinity by the capillary endothelium, a process that may play a role in the transport of fatty acids from the plasma to the cells where they are metabolized.

Introduction

Recent experimental data have shown that in certain vascular beds serum albumin (tagged with colloidal gold) is recognized by specific binding sites on the endothelial surface, restrictively located on the plasmalemmal vesicle membrane. It was suggested that these vesicles perform receptor-mediated transcytosis of albumin across the vascular endothelium. This specific interaction appears to be especially present in the continuous endothelium of capillaries and postcapillary venules of the lung, heart, muscles, and adipose tissue [13, 14, 23]. Specific albumin binding to microvascular endothelium in culture was also demonstrated [22].

The physiological significance of this phenomenon is not yet clearly understood. Albumin is known to be the major plasma carrier of fatty acids (FA), hormones, dyes, and a variety of drugs [19]. In the circulation, ~98% of unesterified FA are bound to albumin. We designed experiments to determine quantitatively and qualitatively the uptake of albumin-fatty acid complexes comparatively with that of defatted albumin. The lung was the organ of choice since it is known that it uses large amounts of fatty acids and that type II cells take up palmitate as an albumin-bound fatty acid [7].

Materials and methods

Reagents

Pentex bovine serum albumin crystallized was purchased from Miles Diagnostics, Kankakee, IL, oleic and palmitic acid from Merck, Darmstadt/FRG, tetrachloroauric acid from Sigma Chem. Co., St. Louis, MO, Na¹²⁵I, from Polatom/Poland, [1-¹⁴C]oleic acid and [1-¹⁴C]palmitic acid from New England Nuclear, Boston, MA.

Animals

Eighty-two male RAP mice, 25 to 30 g body weight, fasted for 16 h and given water ad libitum, were used for these experiments.

Preparation of probes

Defatted albumin (A) and albumin-carrying fatty acids (A-FA) (oleic and palmitic) were either radioiodinated for quantification of the total lung uptake or tagged with 5-nm gold particles to visualize the endothelial structures involved in the transport of these probes. In some experiments, the tracers were both radiolabeled and tagged with gold in order to substantiate by radioassay the morphometric data obtained with particulate tracers.

Albumin-oleic acid (A-OA) and albumin-palmitic acid (A-PA) complexes. A-FA conjugates were prepared according to the method of Abumrad [1], modified as follows: 1 mg/ml oleic acid (OA) in ethanol or palmitic acid (PA) in chloroform were dried under a nitrogen stream. NaOH (0.1 N) was added at FA:NaOH molar ratio of 1:1 and sonicated for 5 min. Albumin solution (1 mg/ml) in double distilled water was passed through a 0.22 µm Millipore filter, mixed under stirring with the FA-salt solution at a molar ratio A:FA of 1:5.5, and dialyzed for 48 h against phosphate-buffered saline (PBS), pH 7.4.

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