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Freeze-Dried Human Leukocytes Stabilized with Uranyl Acetate During Low Temperature Embedding or with OsO4 Vapor After Embedding

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FREEZE-DRIED HUMAN LEUKOCYTES STABILIZED WITH URANYL ACETATE DURING LOW TEMPERATURE EMBEDDING OR WITH OSO_4 VAPOR AFTER EMBEDDING

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Abstract

Two new simple stabilization procedures for freeze-dried biological material are introduced which are compatible with low temperature embedding (LTE) in Lowicryl. The first method uses a Lowicryl K11M/HM20 mixture supplemented with 0.3% uranyl acetate for LTE. For the second method polymerized Lowicryl blocks containing the freeze-dried material are exposed to OsO_4 vapor which penetrates into the Lowicryl block and stabilizes the embedded specimen. The quality of structural preservation is demonstrated with human leukocytes.

Key Words: Freeze-drying, low temperature embedding, Lowicryl HM20, K11M, uranyl acetate, OsO_4 vapor, lymphocytes, polymorphonuclear leukocytes.

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Introduction

First results obtained after freeze-drying (FD) and low temperature embedding (LTE) in the new cryosorption freeze-drying unit (CFD) of Leica (Wien, Austria) have been published in earlier papers (Edelmann, 1994a,b; Sitte *et al.*, 1994). It has been shown that good structural preservation of biological material can be obtained without the use of any chemical fixation during the preparation procedures (Edelmann, 1994a). However, depending on the type of cells and resins used labile subcellular components may be extracted from thin sections of the freeze-dried embedded material during wet cutting (Edelmann, 1994a). In order to stabilize freeze-dried specimens one may expose them to OsO_4 vapor before infiltration in a resin (see e.g., Linner *et al.*, 1986). But freeze-dried and osmicated (black) material is not well suited for low temperature embedding (LTE) in Lowicryl which requires UV polymerization.

In this paper two new simple stabilization procedures for freeze-dried biological material are introduced which are compatible with LTE in Lowicryl: (1) Uranyl acetate may be used during infiltration of the freeze-dried material with a Lowicryl K11M/HM20 mixture and (2) polymerized Lowicryl blocks containing freeze-dried specimens may be exposed to OsO_4 vapor leading to optimally stabilized preparations as seen after ultrathin sectioning and staining of the specimens. The quality of structural preservation provided by the new procedures is demonstrated with ultrathin sections of human leukocytes.

Materials and Methods

Preparation of mononuclear leukocytes (mainly lymphocytes)

Mononuclear cells were isolated from heparinized blood as described by Böyum (1968). Briefly, the anticoagulated blood was diluted 1:1 with phosphate buffered saline, layered onto Ficoll-Paque (Pharmacia, Uppsala, Sweden) and centrifuged at 400 g for 30 min. Then the mononuclear cell fraction was collected

and diluted 1:4 with modified Hank's solution (mHBSS) containing (in mM) NaCl 137, KCl 5.4, Na_2HPO_4 0.34, KH_2PO_4 0.44, glucose 5.5, $NaHCO_3$ 4.2, and 20 mg/ml albumin at pH 7.4. The cell suspension was centrifuged at 130 g for 10 min. The supernatant was removed and the cell pellet was resuspended in mHBSS. In order to wash the cells free of residual Ficoll-Paque the latter steps were repeated two times. Afterwards, the cells were resuspended in platelet poor plasma and centrifuged at 400 g for 15 min. The supernatant was removed and the cell suspension was cryofixed (see below).

Preparation of polymorphonuclear (PMN) leukocytes (mainly neutrophils, few eosinophils)

The cells were isolated from heparinized blood as recently described (Ruf *et al.*, 1992). Briefly, the anticoagulated blood was diluted 2:1 with hydroxyethylstarch (4% w/v; molecular weight 450,000; Fresenius Immuno GmbH, Heidelberg, Germany), and kept at 4°C for 15 min. The buffy coats were collected and centrifuged at 190 g for 5 min. The pellets were resuspended in mHBSS. Then residual red blood cells were lysed by addition of ice-cold distilled water. After 20 sec, isotonicity was reconstituted with a 3.5% w/v NaCl solution. The cell suspensions were again centrifuged at 190 g for 5 min and the pellets were resuspended with mHBSS. The suspensions were layered onto Percoll (Sigma, Deisenhofen, Germany) that was diluted with isotonic saline so that a final density of 1.06 g/ml was achieved, and centrifuged at 190 g for 7 min. The leukocyte pellet was resuspended and washed two times with mHBSS. The viability of the cells examined by trypan blue exclusion was 98% or higher. The cells were counted with a K1000 cell counter (Sysmex, Hamburg, Germany) and stored at a concentration of $6x10^8$ leukocytes/ml until use. The entire procedure and the storage took place at 4°C. To reconstitute the functional integrity of the cells, the suspension was warmed to 37° for 10 min. Immediately before cryofixation the warmed cell suspension was diluted 3:2 with platelet-poor plasma.

Cryofixation

Cell suspensions were cryofixed in plastic spacers in the Reichert Leica MM 80 cryofixation unit as described by Sitte *et al.* (1987). The spacers were rings of anti-adhesive plastic with an inner diameter of 2.4 mm and a thickness of 0.3 mm. 3 to 4 spacers were placed on a foam rubber support of a specimen carrier and each filled with about 1.5 μl of cell suspension. The loaded carrier was transferred and fixed upside down to the MM 80. Cryofixation of lymphocytes took place immediately afterwards. Since the polymorphonuclear cells were not concentrated to the same degree as the lymphocytes it was necessary to leave the carrier for 1 min fixed upside down at the MM 80 before cryofixation. This procedure causes a concentration of cells in the lower part of the suspension which is frozen first.

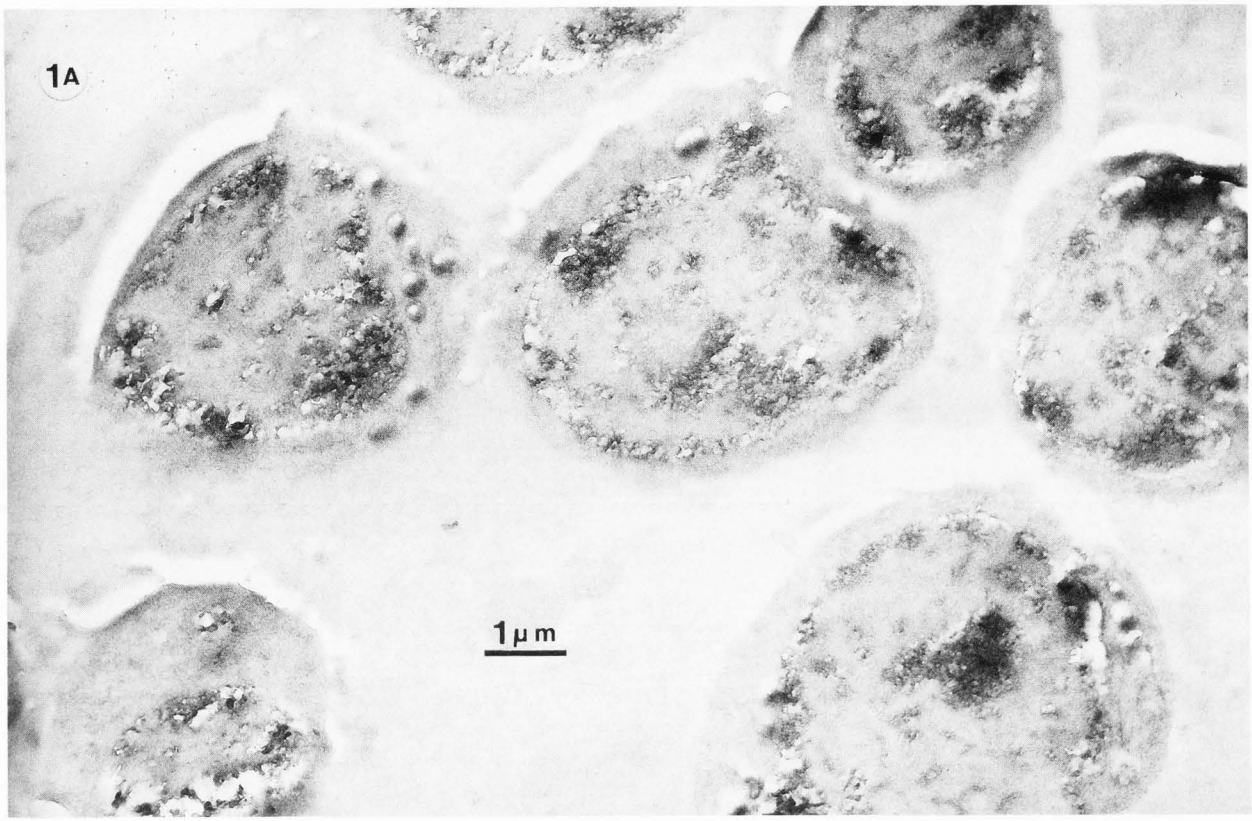
Figure 1A-F: (*next three pages*) Ultrathin sections of human lymphocytes after FD and LTE. FD of **1A**, **1B**, **1E**, **1F**: 1day at -100°C, temperature increase 0.9°C/h up to -10°C (100h), 10h -10°C. FD of **1C**, **1D**: temperature increase 0.2°C/h from -90°C to -30°C (300h), temperature increase 1°C/h from -30°C to -10°C (20h), 10h -10°C. **1A:** HM20, without poststaining. **1B:** HM20, uranyl acetate and lead citrate staining. The white areas on the right side of the cells are due to differential compression of cells and extracellular matrix during sectioning (from right to left). Note the extraction artifacts in the nuclei (heterochromatin, arrows). Freezing plane: lower right corner. **1C:** K11M/HM20, procedure P1, without poststaining. Freezing plane: lower right corner. **1D:** K11M/HM20, procedure P1, poststaining with uranyl acetate and lead citrate. The freezing plane is at a distance of about 4 μm from the right side. **1E:** HM20, procedure P2, without poststaining. **1F:** HM20, procedure P2, poststaining with uranyl acetate and lead citrate. Freezing plane: lower right corner. Figures **1B** and **1F** were obtained from the same Lowicryl block.

Freeze-drying

Transfer of the cryofixed samples into the CFD and freeze-drying was carried out as described earlier (Edelmann, 1994a). Periods of drying temperatures between -100°C and -10°C are given in the figure legends under Results.

Stabilization procedures P1 and P2 after freeze-drying

P1: After FD the specimens were infiltrated inside the CFD as described in the instruction manual of the CFD with a Lowicryl K11M/HM20 mixture at a specimen stage temperature of -25°C. This mixture was prepared by dissolving 0.4% uranyl acetate in Lowicryl K11M (without shaking this amount of uranyl acetate is dissolved over night in K11M) and by mixing 3 parts of this K11M solution with 1 part of pure HM20 (in a new syringe) yielding a Lowicryl solution with 0.3% uranyl acetate. (Note that uranyl acetate cannot be dissolved in pure HM20). 1 day after start of infiltration the UV lamp was switched on for 1 day. Afterwards the specimen stage was warmed to room temperature and removed from the CFD. The polymerized blocks were then ready for sectioning.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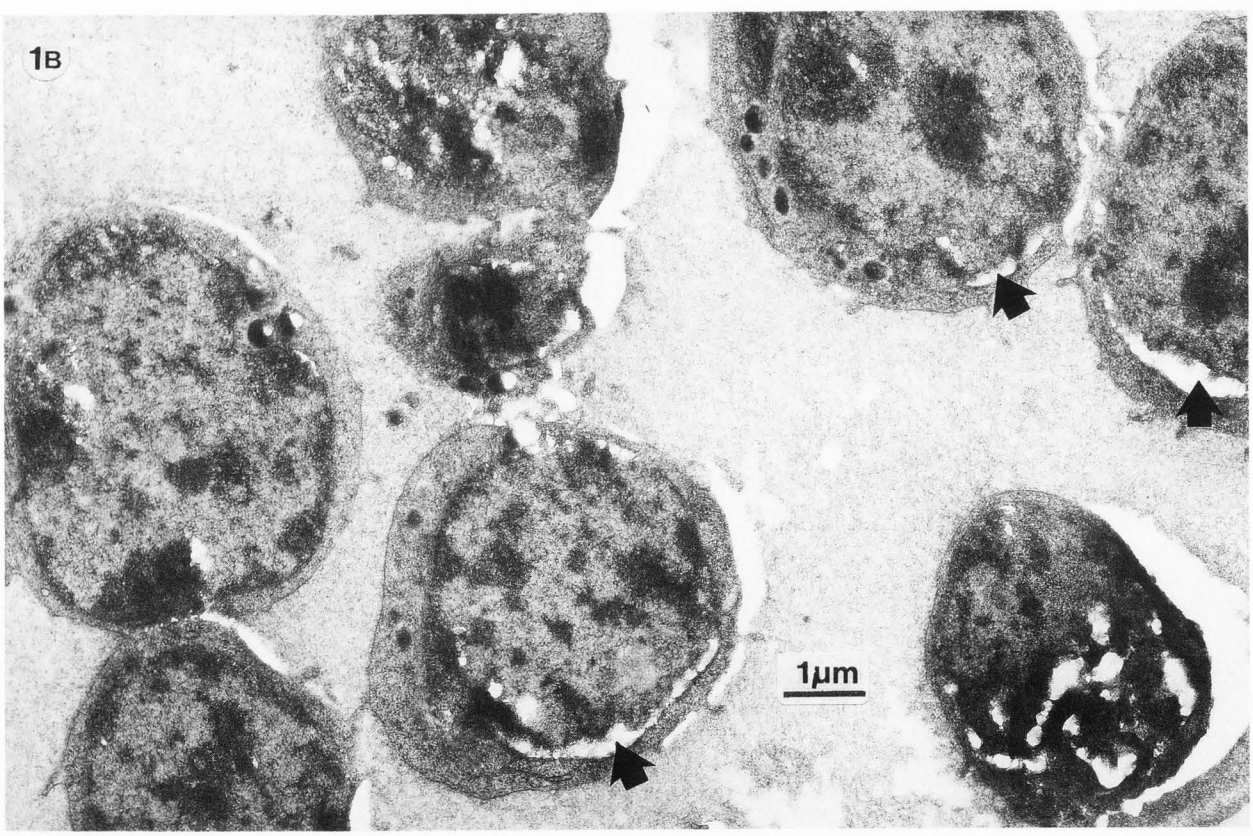
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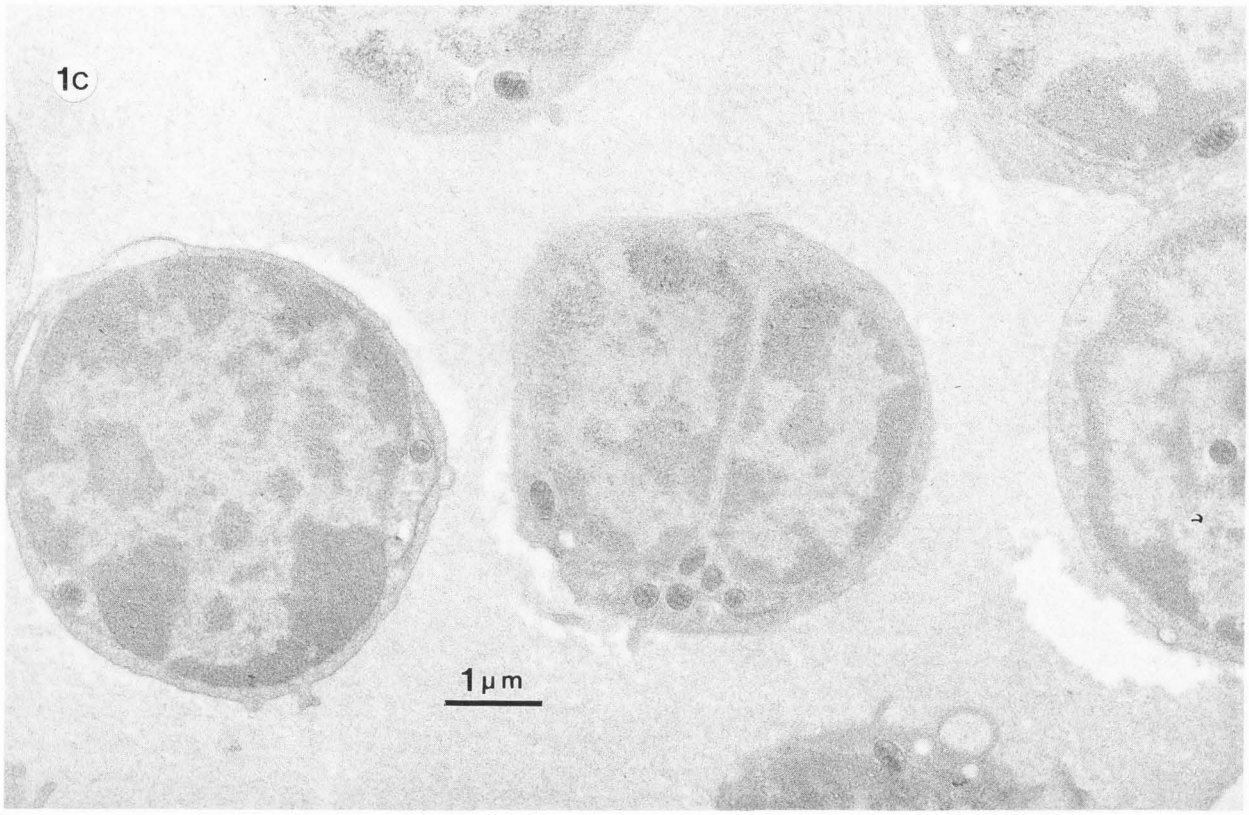
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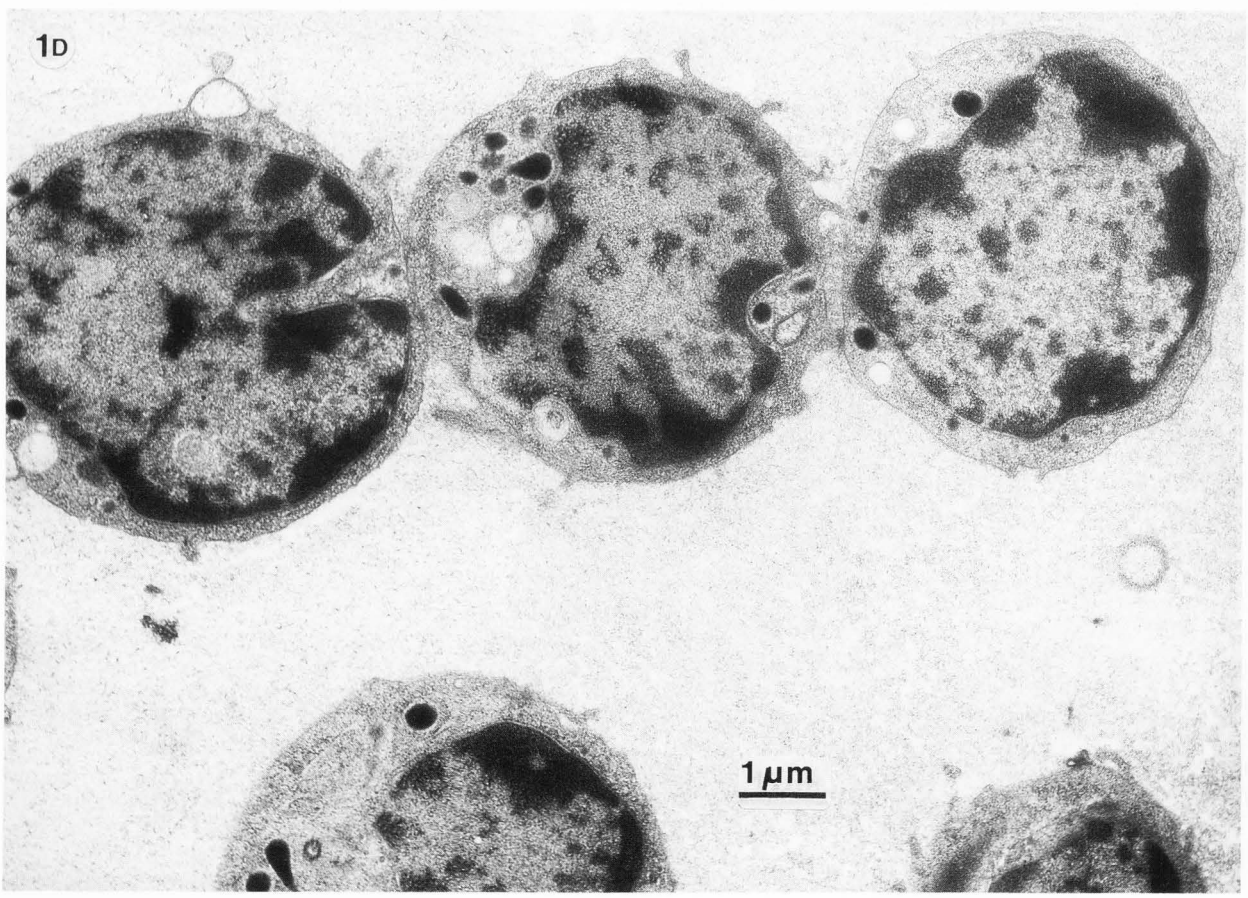
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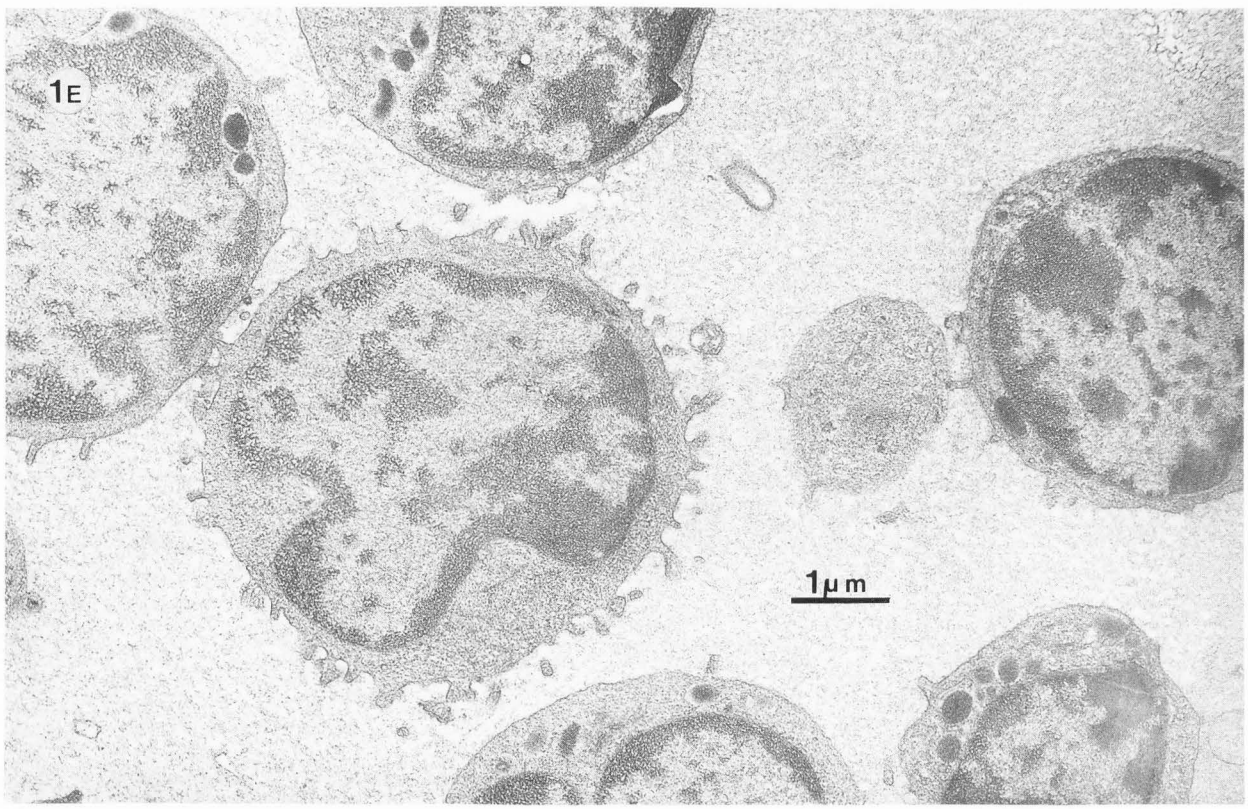
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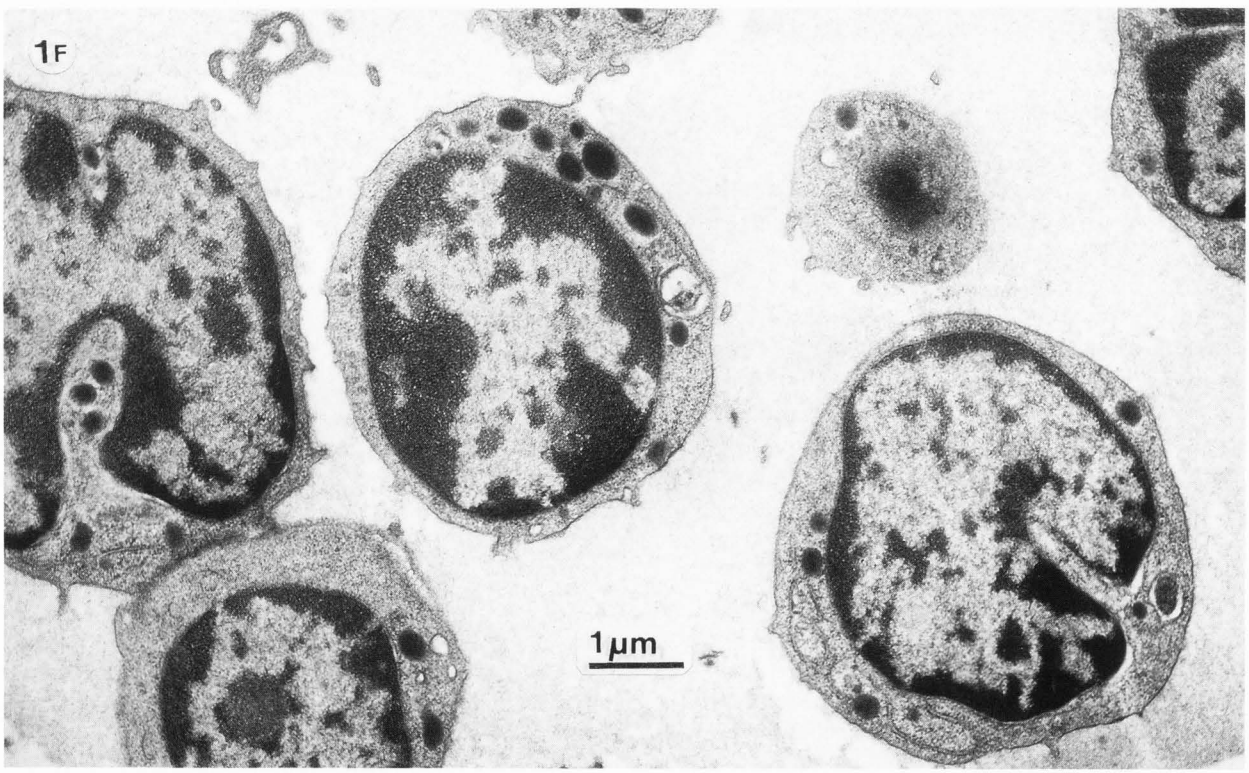

1F
1μm

P2: After FD and LTE at -25°C (infiltration of HM20 : 10 h, UV polymerization: 24 h) the polymerized Lowicryl blocks were placed for 30 min into 0.5 ml Eppendorf capsules together with a small amount (< 0.1 g) of OsO_4 crystals (as a rule the Lowicryl blocks were first sectioned until the embedded biological material appeared at the surface of the block). After exposure of the polymerized blocks to OsO_4 vapor the blocks appear dark brown and turn black after a few hours without further exposure.

Transmission electron microscopy

Ultrathin sections (≤ 80 nm) were obtained by using a Diatome (Biel, Switzerland) diamond knife (35°). The sections were either examined without poststaining or after staining with uranyl acetate and lead citrate for short periods (≤ 10 sec). The sections were photographed in a Zeiss (Oberkochen, Germany) EM 902 with energy filter at 80 kV. All figures were obtained on identical photographic paper [Agfa (Agfa-Gevaert, Leverkusen, Germany), Brovira, grade soft].

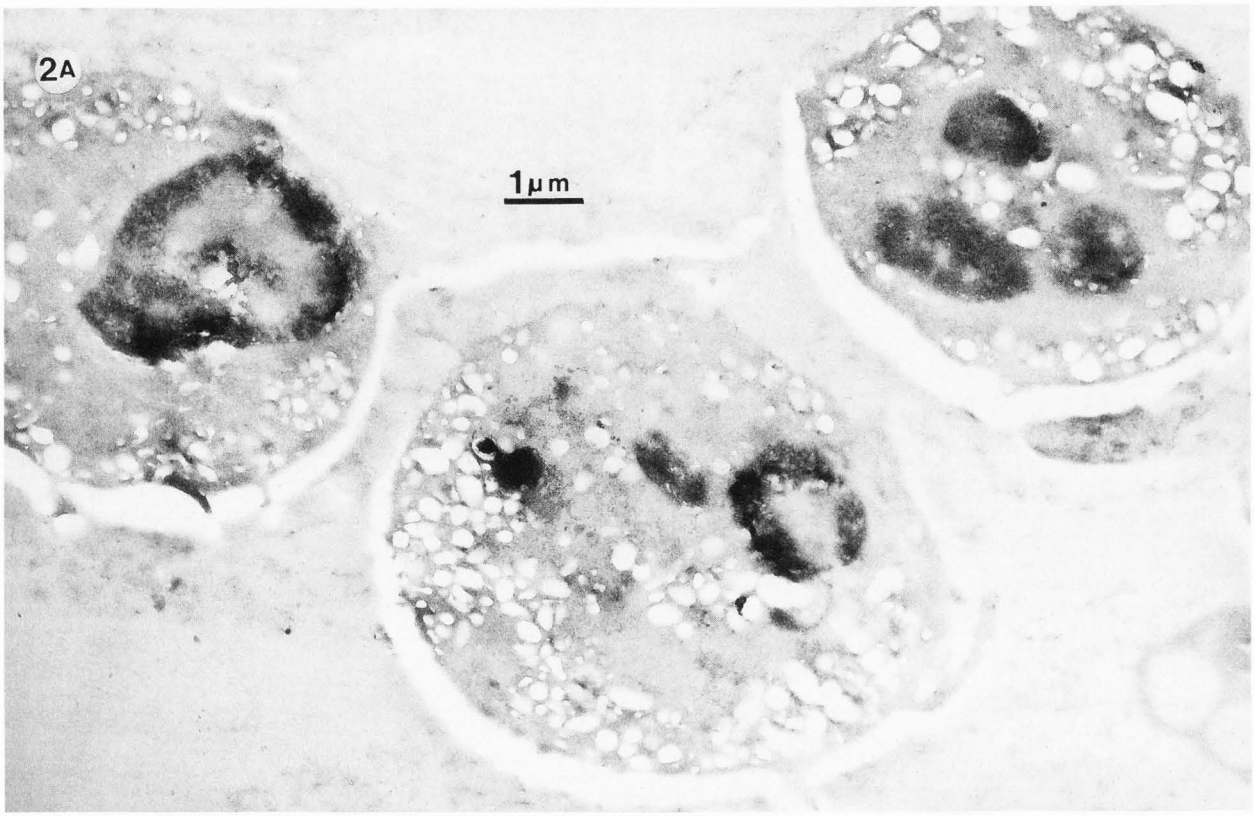
Results

Freeze-dried lymphocytes are shown in Figs. 1A-F, freeze-dried PMN leukocytes in Figs. 2A-F. Unstained and stained ultrathin sections of HM20 embedded preparations not stabilized with P1 or P2 show severe artifacts (Figs. 1A, 1B, 2A, 2B). These artifacts are distortions of nuclear material, partial separation of the cells from the surrounding Lowicryl, and extraction of cellular granules of PMN leukocytes. Similar or even more pronounced artifacts are obtained after embedding in pure K11M or in a K11M/HM20 mixture (not shown). The effect of P1 is shown in Figs. 1C, 1D, 2C, 2D. Lymphocytes appear well preserved and faintly stained without poststaining (1C) or intensely stained after poststaining (1D). PMN leukocytes are not preserved in a satisfying manner (2C, 2D). Both sections (without and after poststaining) reveal extraction of cellular granules. To be noted is however the better preservation of nuclear material in uranyl acetate treated cells and the degree of separation of the cells from the surrounding Lowicryl is reduced (compare with Figs. 2A, 2B). The effect of OsO_4 treatment of the Lowicryl blocks is demonstrated in Figs. 1E, 1F (HM20, no former uranyl acetate treatment) and Figs. 2E, 2F (K11M/HM20, after uranyl acetate treatment). All sections either without or after poststaining show a well preserved ultrastructure. Without poststaining the contrast of subcellular structures and in particular of membranes of OsO_4 treated cells is more intense than that of uranyl acetate treated cells (compare Figs. 1E with 1C and 2E with 2C). After poststaining the contrast is very much increased (Figs. 1F, 2F). PMN leukocytes embedded in pure Lowicryl (HM20, K11M or mixture) and treated with OsO_4 vapor according to P2 (not shown) are similarly preserved as those shown in Figs. 2E and 2F.

Figure 2A-F: (*next three pages*) Ultrathin sections of human PMN leukocytes. FD of **2A**, **2B** same as for **1A**, **1B**, **1E**, **1F**. FD of **2C-F** same as for **1C**, **1D**. **2A**: HM20, without poststaining. **2B**: HM20, poststaining with uranyl acetate and lead citrate. **2C**: K11M/HM20, procedure P1, without poststaining. **2D**: K11M/HM20, procedure P1, poststaining with uranyl acetate and lead citrate, freezing plane: upper left corner. Figures **2A-D** show the loss of cellular granules. **2E**: K11M/HM20, procedures P1 and P2, without poststaining. Freezing plane: lower edge. **2F**: K11M/HM20, procedures P1 and P2, poststaining with uranyl acetate and lead citrate. Freezing plane: lower left corner. **2E** and **2F** show sections from the same Lowicryl block.

Discussion

The purpose of this contribution is the introduction of two simple new methods for preparing freeze-dried embedded material for transmission electron microscopy in such a way that ultrastructural details are preserved and visualized similarly to those obtained e.g., by the freeze-substitution (FS) technique. In the first method uranyl acetate is used as a stabilization agent. From FS experiments it is well known that many cellular components and in particular lipids can be stabilized during dehydration of a cryofixed specimen in an organic solvent supplemented with uranyl acetate (see e.g., Humbel, 1984; Voorhout *et al.*, 1991). In the FD technique the dehydration is carried out without the use of an organic solvent and the stabilization - if necessary - must occur afterwards. Since many experiments with human leukocytes showed that these cells are not optimally preserved after FD, LTE in Lowicryl, and ultrathin sectioning (Figs. 1A, 1B, 2A, 2B) it was expected that one may improve the preservation of freeze-dried cells by using uranyl acetate in the embedding medium. Two reasons led to the finally adopted and described method P1: (1) A small amount (< 0.5 %) of uranyl acetate can be dissolved in Lowicryl K11M but not in HM20, (2) Ultrathin sections (≤ 100 nm) of freeze-dried preparations embedded in K11M take up a considerable amount of water and disintegrate on the water of the knife through. This phenomenon could be largely


2A
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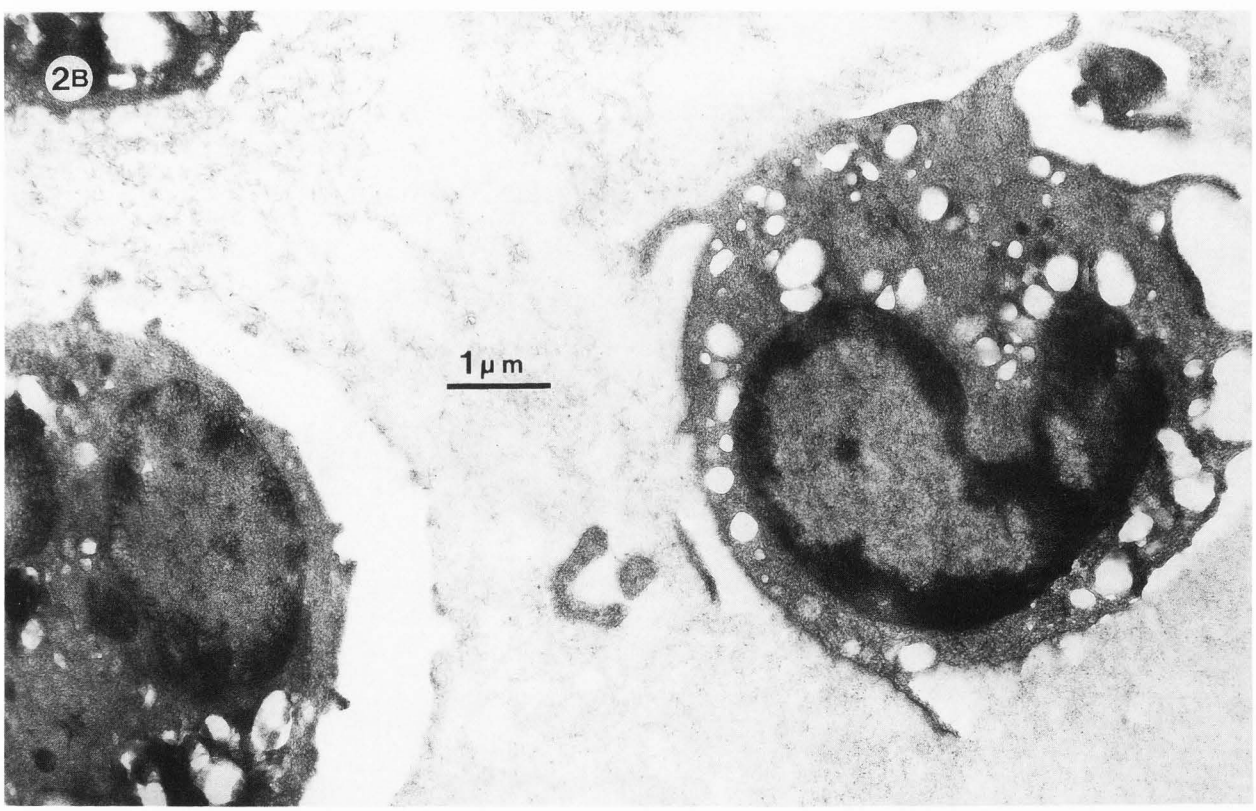
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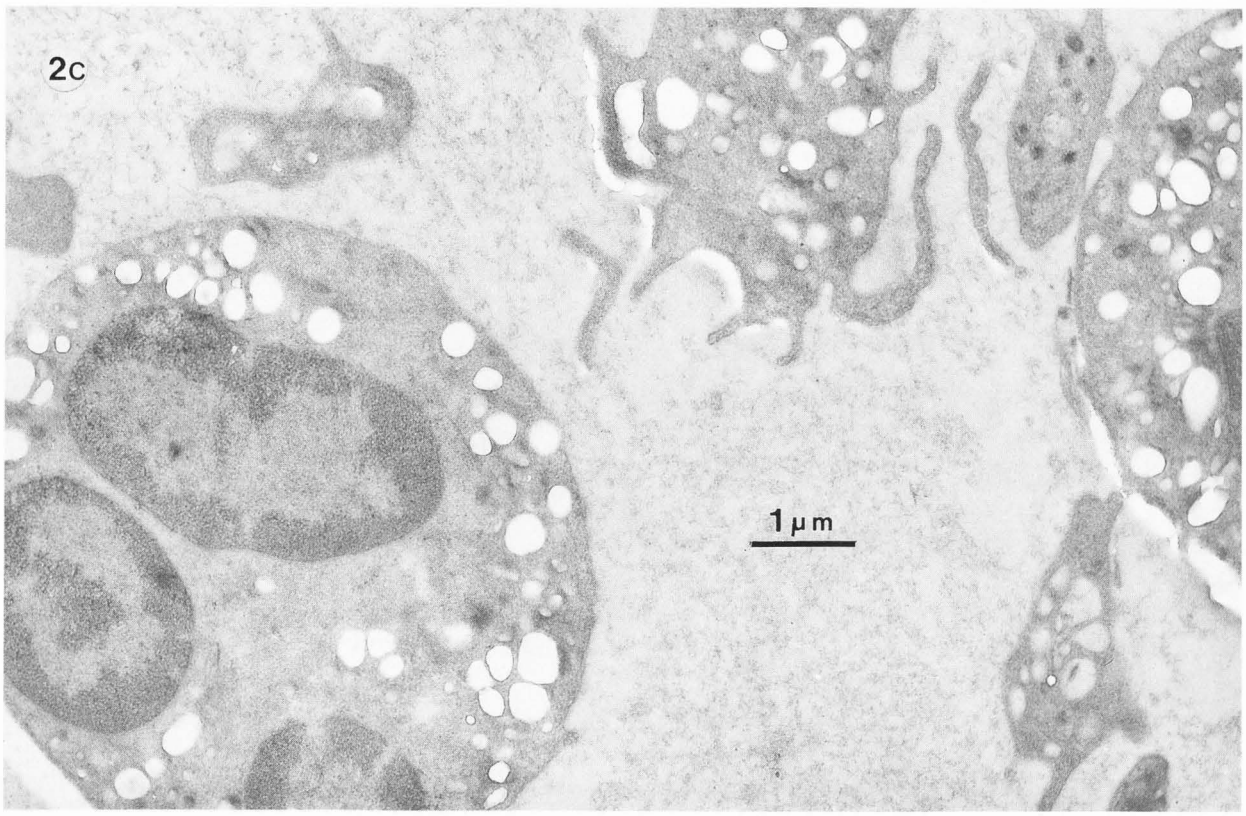
2C
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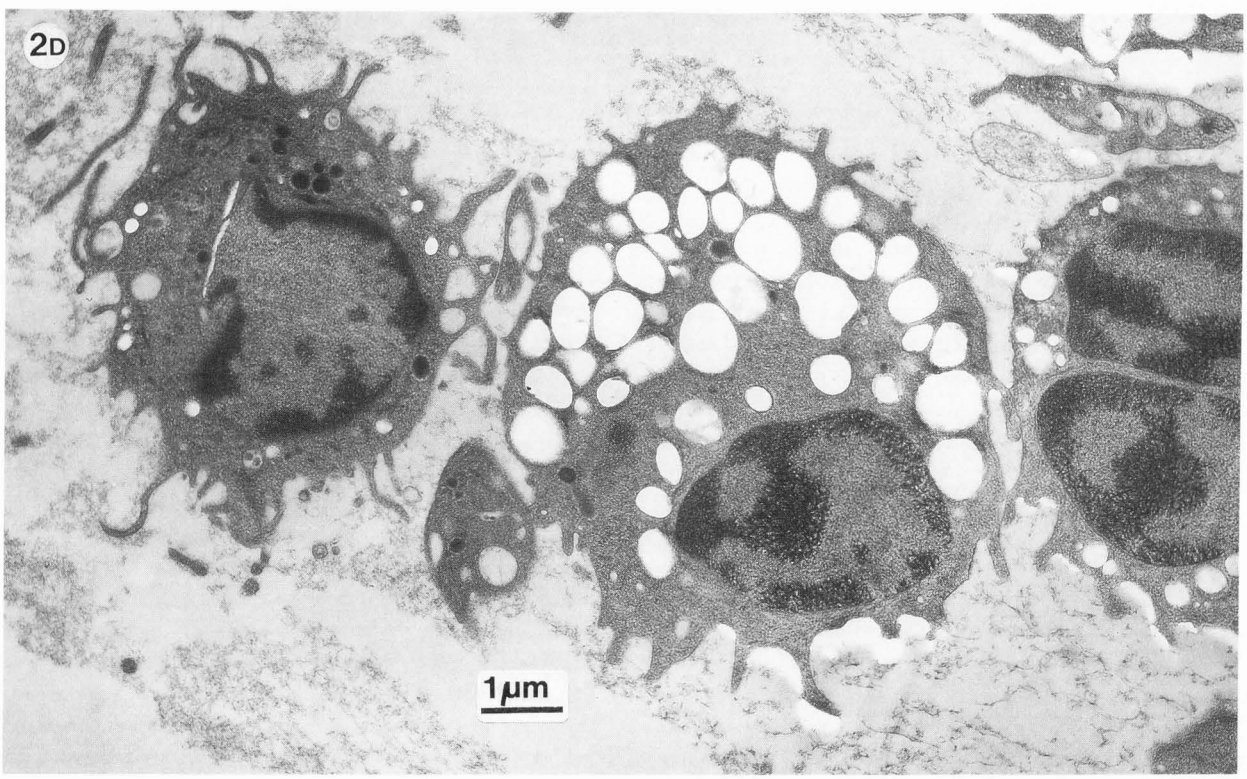
2D
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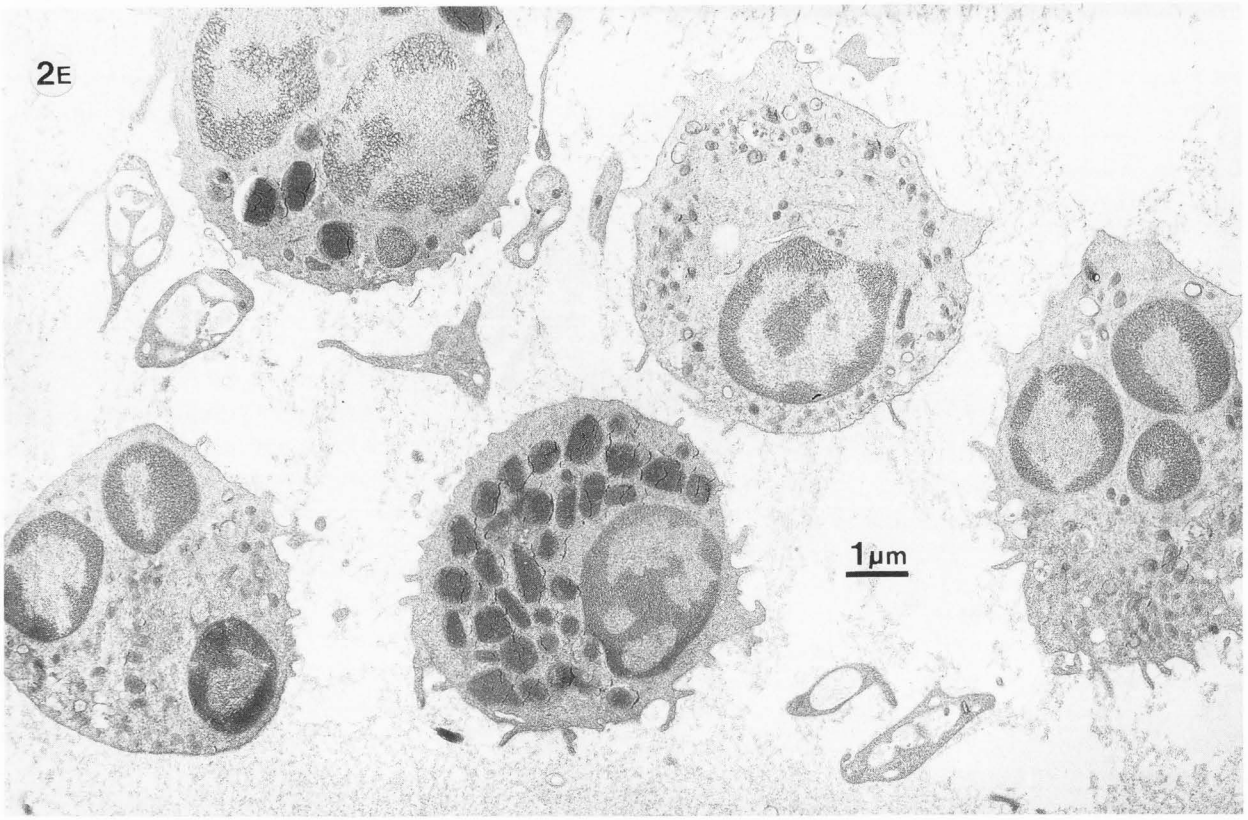
2E
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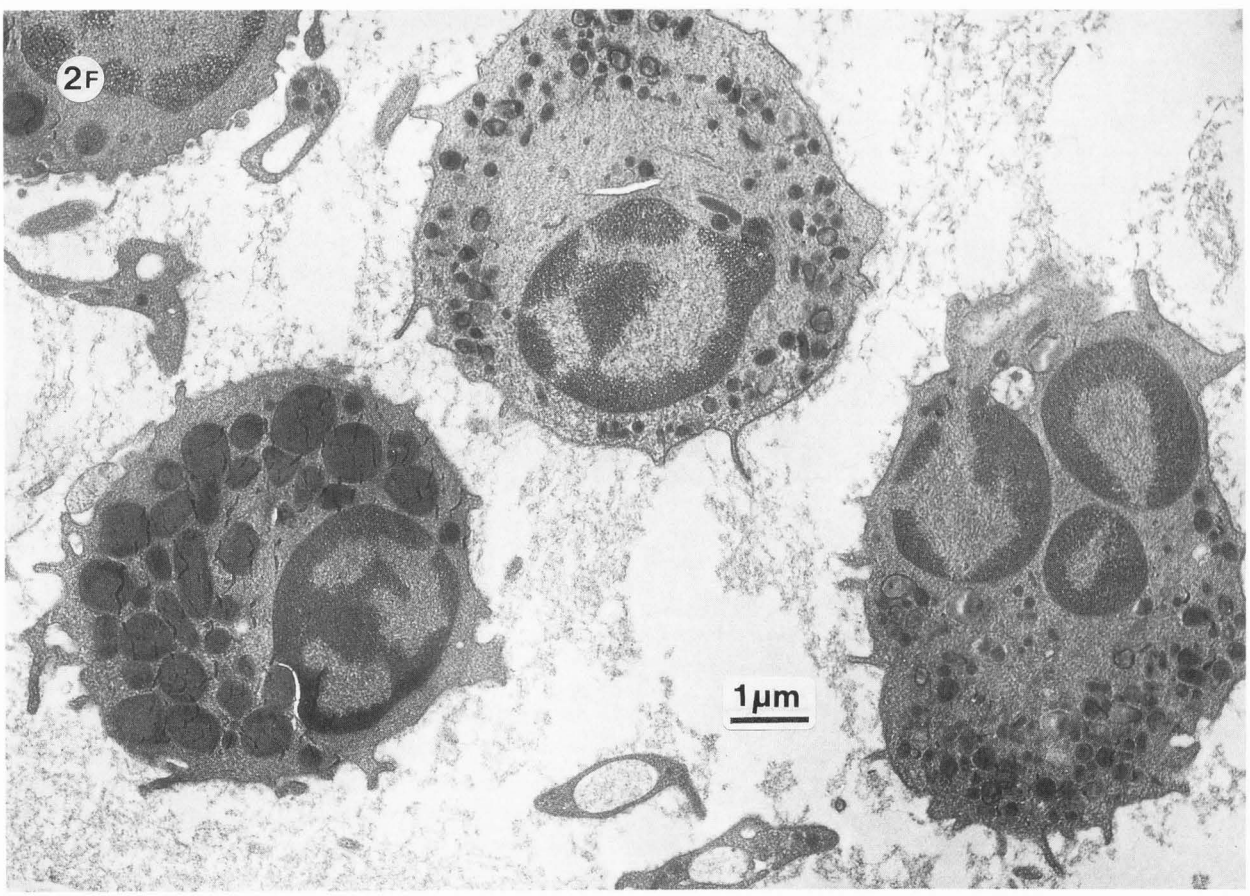

2F
1μm

reduced by using a K11M/HM20 mixture (supplemented with uranyl acetate according to P1). With this embedding medium we obtained stable ultrathin sections of well preserved human lymphocytes (Figs. 1C, 1D). However, other leukocytes were not stabilized in a satisfying manner (Figs. 2C, 2D). Fortunately subsequent experiments with OsO_4 vapor were successful: Lowicryl blocks prepared according to P1 were exposed to OsO_4 vapor (P2) and we obtained well preserved freeze-dried and low temperature embedded PMN leukocytes (Figs. 2E, 2F). Later it was found that similar results were also obtained with method P2 alone (not shown, for lymphocytes in pure HM20 see Figs. 1E, 1F).

From these positive results we conclude: during FD and also during subsequent LTE in Lowicryl a well organized but labile ultrastructure of the biological material is maintained. The freeze-dried ultrastructure can be stabilized at least partly during infiltration in a K11M/HM20 medium supplemented with uranyl acetate (P1). In addition, the freeze-dried and embedded material can be stabilized (more effectively) by exposing the polymerized Lowicryl block to OsO_4 vapor (P2). Apparently the whole labile Lowicryl-biological material-complex with its critical internal phase boundaries is crosslinked by OsO_4. Despite the fact that P2 is a more powerful and more general applicable method than P1 both methods may be used for future studies. E.g., since lymphocytes are well preserved by both methods it would be worthwhile to compare immunocytochemical results obtained from the different preparations (treated with different chemical fixatives).

One may ask whether the biological material prepared according to the described procedures is maintained in a state closer to the living state than e.g., freeze-substituted and embedded material or more specifically: is the antigenicity of macromolecules better retained in the freeze-dried preparation? Ultimately these questions have to be answered by comparative studies. At least two facts, however, speak in favor of the freeze-dried material:

(1) During FD the biological material is not exposed to dehydrating liquids (e.g., acetone, alcohol) which may alter cellular macromolecules and extract soluble substances. Although the used resin may have an influence on the biological material the already dehydrated and hence partly stabilized specimen is certainly less influenced and modified by the liquid resin than a specimen which is first exposed to a dehydrating organic solvent and then to a resin.

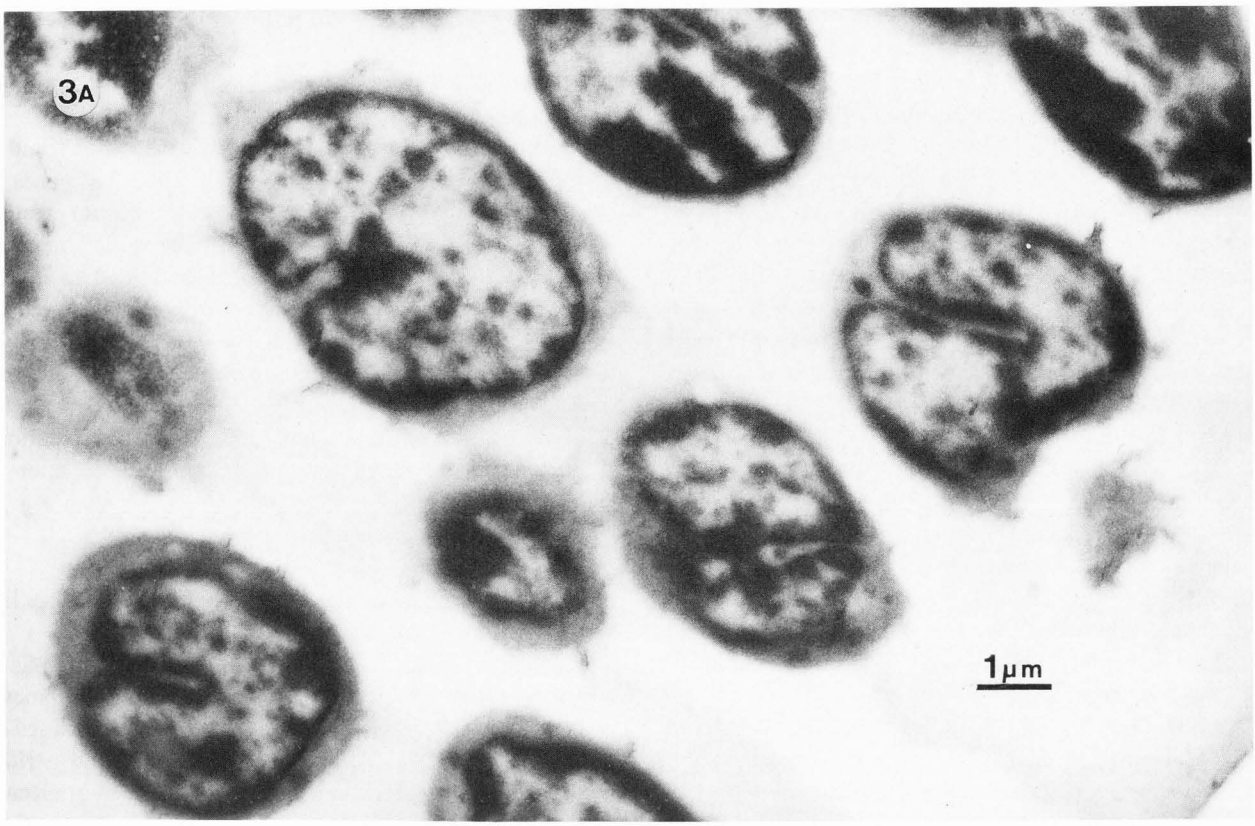
(2) Freeze-dried biological material - even if it is stabilized according to the described procedures - exhibits a very different behavior towards the electron microscopic stains uranyl acetate and lead citrate than freeze-substituted material: the freeze-dried specimen is heavily stained after very short exposure of sections to the stain (< 10 sec) whereas such an intense staining is not observed in sections of freeze-substituted material. Apparently more reactive sites are preserved in the freeze-dried specimen.

Figure 3: (*facing page*) 0.2 μm thick dry-cut unstained sections of human lymphocytes (**3A**) and PMN leukocytes (**3B**) after FD and LTE in pure HM20 (same Lowicryl blocks as for Fig. **1A** and Fig. **2A**).

Finally, Fig. 1B should be discussed: it is interesting to note that the structural preservation of the unstabilized preparation is worst in the area of best cryofixation. As discussed in earlier papers (Edelmann, 1994b; Sitte *et al.*, 1994) it is assumed that in the region of best cryofixation (vitrification) a relative strong protein-water interaction is preserved leading to a reduced rate of water removal during FD and hence to less dry areas of the biological material. If more water molecules are retained in certain areas polymerization of the Lowicryl may then be incomplete. A similar water related phenomenon may be the reason for the distortions observed in Figs. 2A-D (extraction of granules, separation of cells from the surrounding Lowicryl). One may speculate that prolonged FD at rather high temperature may reduce the water content in the critical areas and that then the cells are better stabilized even in pure Lowicryls. In the present study we have used two different FD procedures (see legend of Figs. 1 and 2) which differ mainly in the speed of water removal in the temperature range between -90°C and -30°C. Both procedures yield similar results and there is probably no reason to choose the slow procedure when freeze-drying leukocytes. The final degree of dehydration obtained by both procedures is similar because it is determined by the FD time at the highest temperature (10h at -10°C). If the more tightly bound remaining water should be reduced it would be necessary to increase the FD time at -10°C or even higher temperatures before embedding at low temperature. Whether such a strategy causes morphological changes of the freeze-dried material remains to be determined.

Acknowledgements

We would like to thank Frans A. Prins, Department of Pathology, University of Leiden, The Netherlands, for the photographs obtained by Reflection Contrast Microscopy (see Discussion with Reviewers) and Mrs. B. Reiland for typing the manuscript.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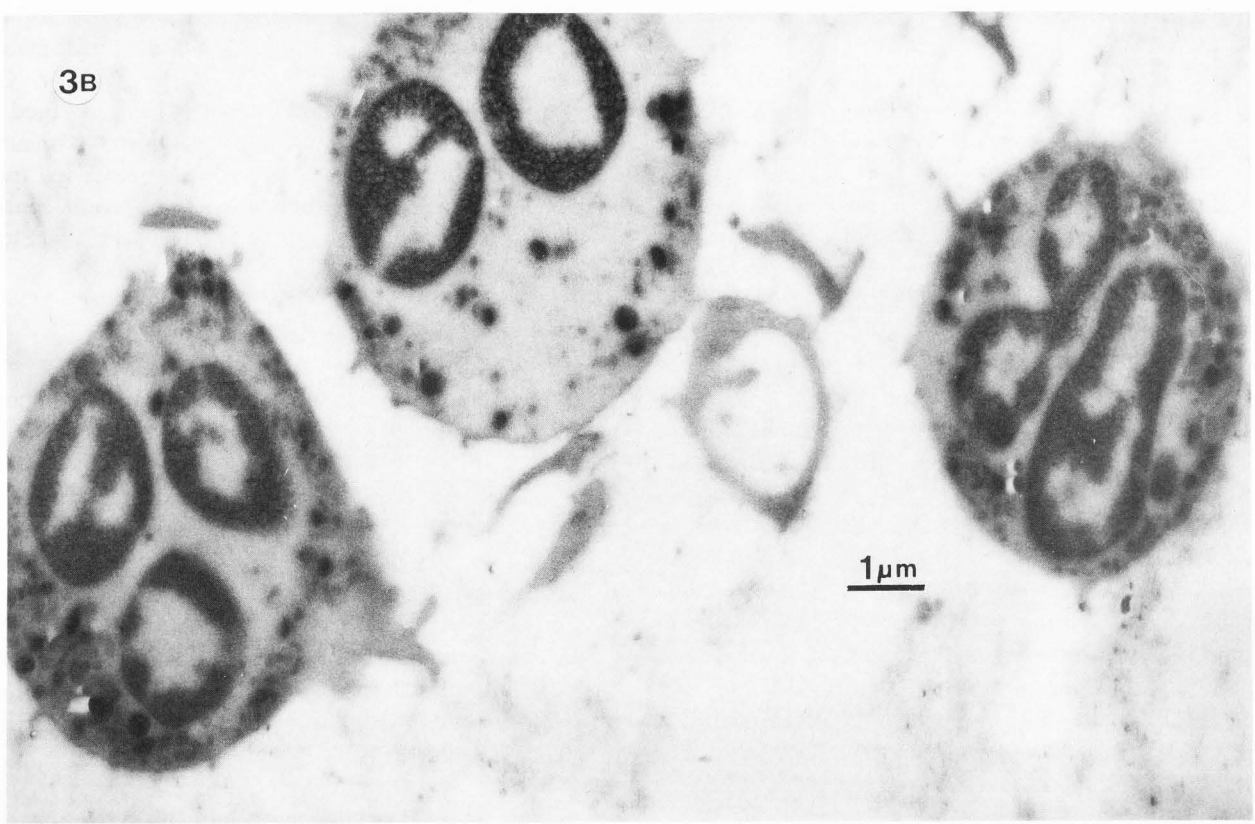
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Discussion with Reviewers

Reviewer I: What is your experience with cutting of dry sections from this material?

Authors: When using a diamond knife (35°) and an ionization device (Michel *et al.*, 1992) it poses no difficulties to produce 0.2 μm thick (blue) sections from Lowicryl (HM20 or K11M) embedded material, to place them on Formvar coated grids and to investigate them in the electron microscope. Unstained sections of freeze-dried lymphocytes and PMN leukocytes embedded in *pure* HM20 are shown in Figs. 3A and 3B. These figures show a well preserved ultrastructure of both types of cells, confirming the above mentioned statement that the ultrastructure is preserved after FD and LTE. The distortions and extractions seen e.g., in Figs. 1A, 2A are absent in dry cut sections.

Reviewer I: Have you done any studies at the light microscopy level?

Authors: Figs. 4A and 4B show photographs of freeze-dried and Lowicryl embedded lymphocytes (method P1). These figures have been obtained by Frans Prins, Leiden, after toluidine blue and acridine orange (DNA) staining of two consecutive ultrathin sections in combination with Reflection Contrast Microscopy (Leica DMR Microscope equipped for epi-illumination and adapted for reflection contrast microscopy; Leica Microscopy and Systems, Wetzlar, Germany). Such histological staining can be used as counterstaining for immunohistochemistry. Immunostaining studies with the freeze-dried, embedded material are planned. For literature on reflection contrast microscopy see e.g., Prins *et al.*, 1993, 1996; Ploem *et al.*, 1995).

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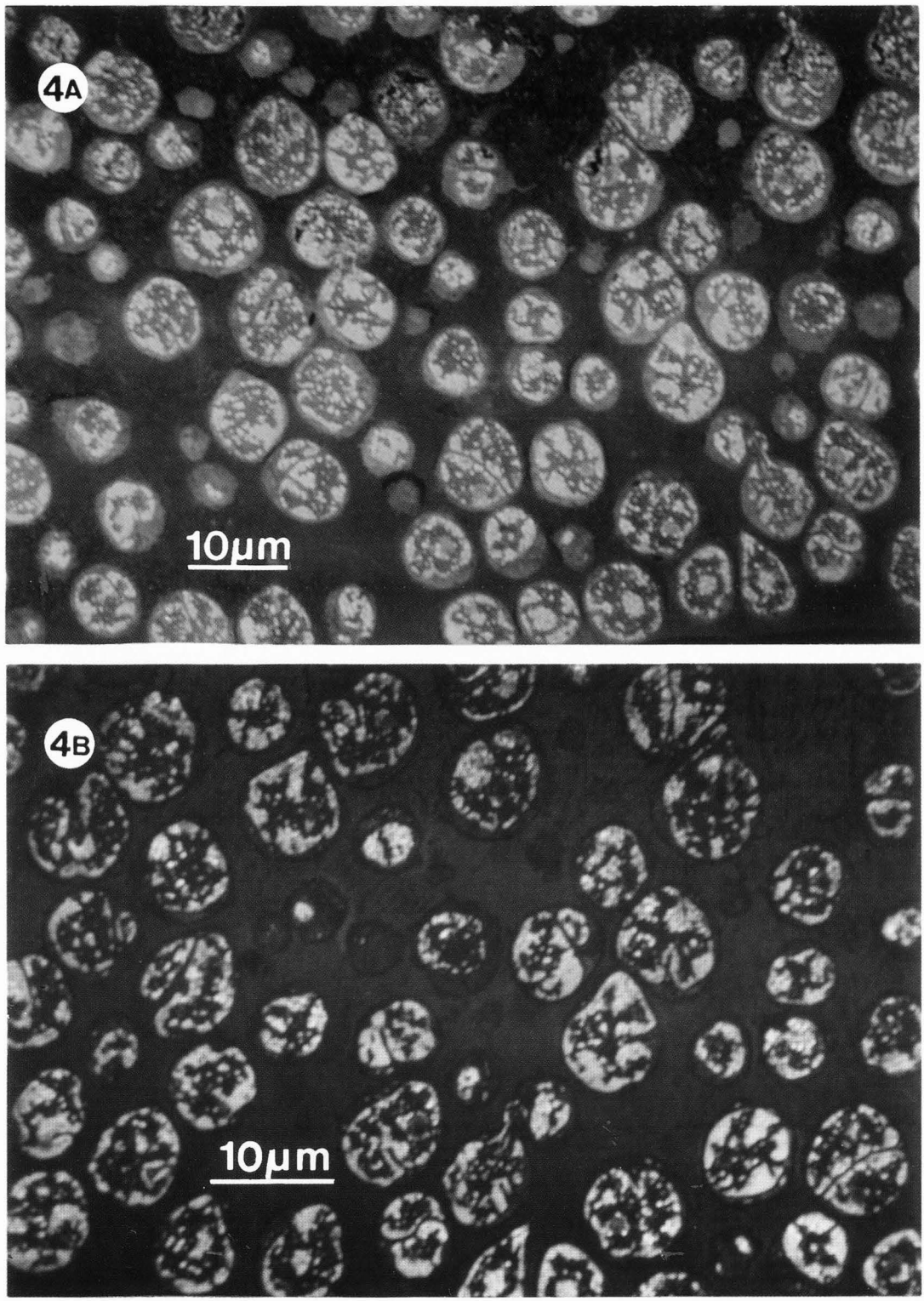

Figure 4: Sequential ultrathin ($\leq$ 80 nm) sections of human lymphocytes visualized after staining with toluidine blue (**4A**) and acridine orange (**4B**) by reflection contrast microscopy (Leica DMR Microscope equipped for epi-illumination and adapted for reflection contrast microscopy; Leica Microscopy and Systems, Wetzlar, Germany). Note that in reflection microscopy stained specimens exhibit the complementary color of the conventional transmitted light image. Photographs courtesy of F.A. Prins, Leiden.