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Cell surface characteristics and DNA content of macrophages in murine bone marrow cultures

A study using simultaneous scanning electron microscopy and fluorescence microscopy

C.H. Wouters^{1*}, J.S. van de Gevel², J.W.M. van der Meer², W.Th. Daems³, R. van Furth², and J.S. Ploem¹

¹ Department of Histochemistry and Cytochemistry, University of Leiden, Wassenaarseweg 72, NL-2333 AL Leiden, The Netherlands

² Department of Infectious Diseases, University Hospital, Rijnsburgerweg 10, NL-2333 AA Leiden, The Netherlands

³ Laboratory for Electron Microscopy, University of Leiden, Rijnsburgerweg 10, NL-2333 AA Leiden, The Netherlands

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Summary. An instrument combining scanning electron microscopy (SEM) and light microscopy (LM) was used to study the cell surface characteristics and DNA content of macrophages in murine bone marrow cultures. After a quantitative Feulgen DNA staining, the DNA content of the individual macrophages was measured and their cell surface morphology was studied immediately thereafter with the SEM part of the instrument. The cells were divided into six groups according to the number of microvilli and/or microridges present on their surface. A proportion of macrophages showed a DNA content more than occurs in diploid cells, which could indicate a future division. No special surface morphology could be detected in this cell type.

technique which enables to measure DNA and perform scanning electron microscopy on the same single cells (Wouters and Koerten 1982; Wouters et al. 1986).

Materials and methods

Culture of bone marrow cells. The techniques used for culturing bone marrow mononuclear phagocytes colonies from the mouse femur in the presence of conditioned medium on a glass surface have been described (Goud et al. 1975). In short, bone marrow cells from specific pathogen-free Swiss mice (Central Institute for the Breeding of Laboratory Animals, TNO, Bilthoven, The Netherlands) were cultured in plastic petri dishes (diameter 32 mm; Bio-Quest, BBL & Falcon Products, Becton, Dickinson & Co., Cockeysville, Md.) with a cover-glass (diameter 18 mm). Each culture contained 5×10^4 nucleated bone marrow cells per milliliter culture medium; the culture medium was composed of 60% (vol/vol) Dulbecco's modified Eagle's medium (Grand Island Biological Company, Grand Island, NY), 20% (vol/vol) horse serum (Flow Laboratories, Irvine, Scotland) and 20% (vol/vol) conditioned embryonic mouse fibroblasts medium. This conditioned medium was used as the source of colony-stimulated factor CSF-1, as described elsewhere (Goud et al. 1975; van der Meer et al. 1983). The cultures were incubated in a water-saturated atmosphere with a constant flow of 10% CO₂ in air.

Introduction

Mononuclear phagocytes originate in the bone marrow (van Furth and Cohn 1968). The most immature precursor of the monocyte, the monoblast, has been identified and characterized in liquid cultures of mouse bone marrow (Goud et al. 1975). Also in cultures of human bone marrow, this progenitor cell has been recognized.

In culture, the monoblast has the morphologic, cytochemical, and functional characteristics of a mononuclear phagocyte, albeit a primitive one (Goud et al. 1975; van der Meer et al. 1979; van der Meer et al. 1983). By division of monoblasts, promonocytes (van Furth et al. 1970) are formed; the latter cells divide again and form monocytes. Since in cultures, monocytes immediately stretch on glass surfaces and transform into macrophages they are not recognized as monocytes in bone marrow cultures under the light microscope.

A proportion of the cells with the light-microscopical morphology of macrophages divide in liquid cultures from bone marrow (van Furth et al. 1983; van der Meer et al. 1983). In a previous paper we have called these cells dividing macrophages (van Furth et al. 1983).

For the present study, we questioned whether these dividing macrophages represent a population of immature macrophages and whether we could characterize these cells. We approached this problem using a recently developed

Fixation, staining and scanning electron microscopy (SEM) processing. After culturing the cells on the cover-glass for 7 d the medium was gently removed, leaving 1 ml to cover the cells; thereafter 2 ml fixative consisting of 1% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) was added. The fixation was performed for 30 min at room-temperature. Next, the cells were washed twice in phosphate-buffered saline (PBS) and once in demineralized water. During fixation, wash-steps, staining and further processing, the cells were not allowed to air-dry. Each preparation was stained in a small petri dish by hand as described elsewhere (Wouters et al. 1986). The cells were stained for DNA with a Feulgen acriflavine-SO₂ staining in such a way that a quantitative assessment of DNA is possible (Tanke and van Ingen 1980). The Feulgen hydrolysis was performed 15 min in 5 N hydrochloric acid. After rinsing in demineralized water, the DNA of the cells was stained for 15 min with 0.01% acriflavine-SO₂. After another rinsing the preparations were placed in acid ethanol (1% hydrochloric acid w/v in 70% ethanol) for 10 min to remove non-covalently bound dye. Subsequently, the preparations were rinsed with demineralized water, phosphate buffer (pH 6.8) and demineralized water. The cells were dehydrated with an ascending ethanol series. These steps were followed by critical point drying from CO₂ after which the preparations were glued with conductive carbon cement

* To whom offprint requests should be sent

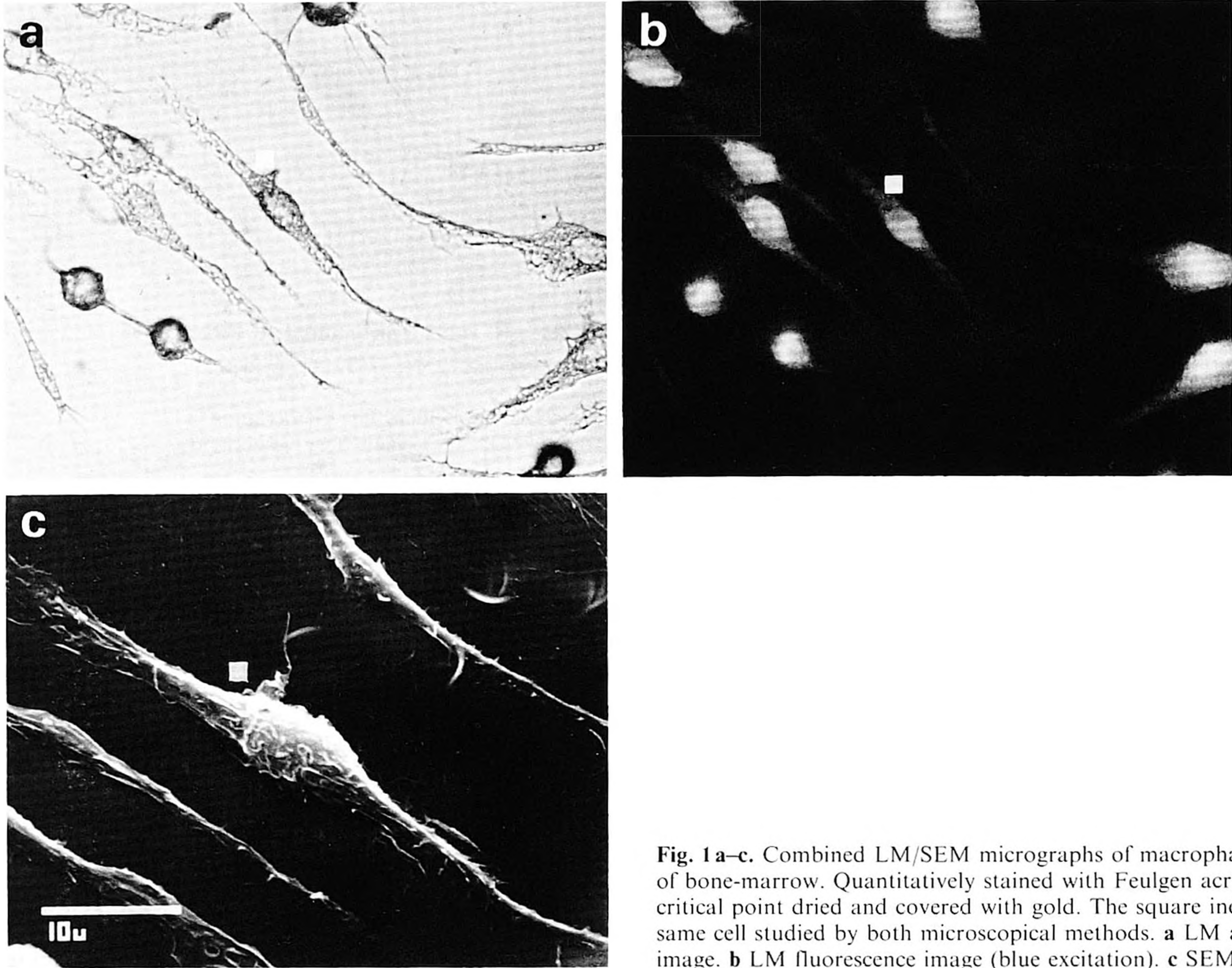


Fig. 1 a-c. Combined LM/SEM micrographs of macrophages in cultures of bone-marrow. Quantitatively stained with Feulgen acriflavine-SO₂, critical point dried and covered with gold. The square indicates the same cell studied by both microscopical methods. **a** LM absorption image. **b** LM fluorescence image (blue excitation). **c** SEM image

(Neubauer, Munster, W. Germany) to special aluminum holders with a round opening of 2 cm in the middle, and coated with a thin layer of gold. The preparations were investigated in a microscope combining light microscopy (LM) and SEM (Hartmann et al. 1978; Wouters and Koerten 1982; Wouters et al. 1986).

DNA measurements. A microfluorometer (MPV-II, Leitz, W. Germany) attached to the combined microscope made it possible to measure the DNA content of each cell individually while in the vacuum chamber. The absorption image of the acriflavine-SO₂ was used to center and focus a cell (Fig. 1a). Then a measurement was performed by exciting the nucleus with blue light for a short period and the emitted fluorescence signal was measured (Fig. 1b). The filter combination used consisted of a chromatic beam splitter (Leitz TK 500), two short pass interference filters (Leitz KP 490), a BG 38 (Schott, W. Germany) to absorb the far red, a long pass filter (Leitz LP 530), and a short pass interference filter (Leitz KP 560) (as secondary filters). The KP 560 was added to reflect the otherwise disturbing red light scattered by the gold coating. Of each preparation 100 cells were randomly measured.

Cell morphology. After each DNA measurement the cell surface morphology was examined with the SEM part of the microscope (Fig. 1c). The elongated macrophages were arbitrarily divided in six groups according to the number of microvilli and/or microridges present on their surface. Cells categorized in the first group had no or hardly any microvilli and/or microridges on their surface. Cells that were completely covered with surface structures were categorized in group six. The other cells with more microvilli and/or microridges than cells in the first group and less surface structures than completely covered were categorized in the groups 2, 3, 4 and 5.

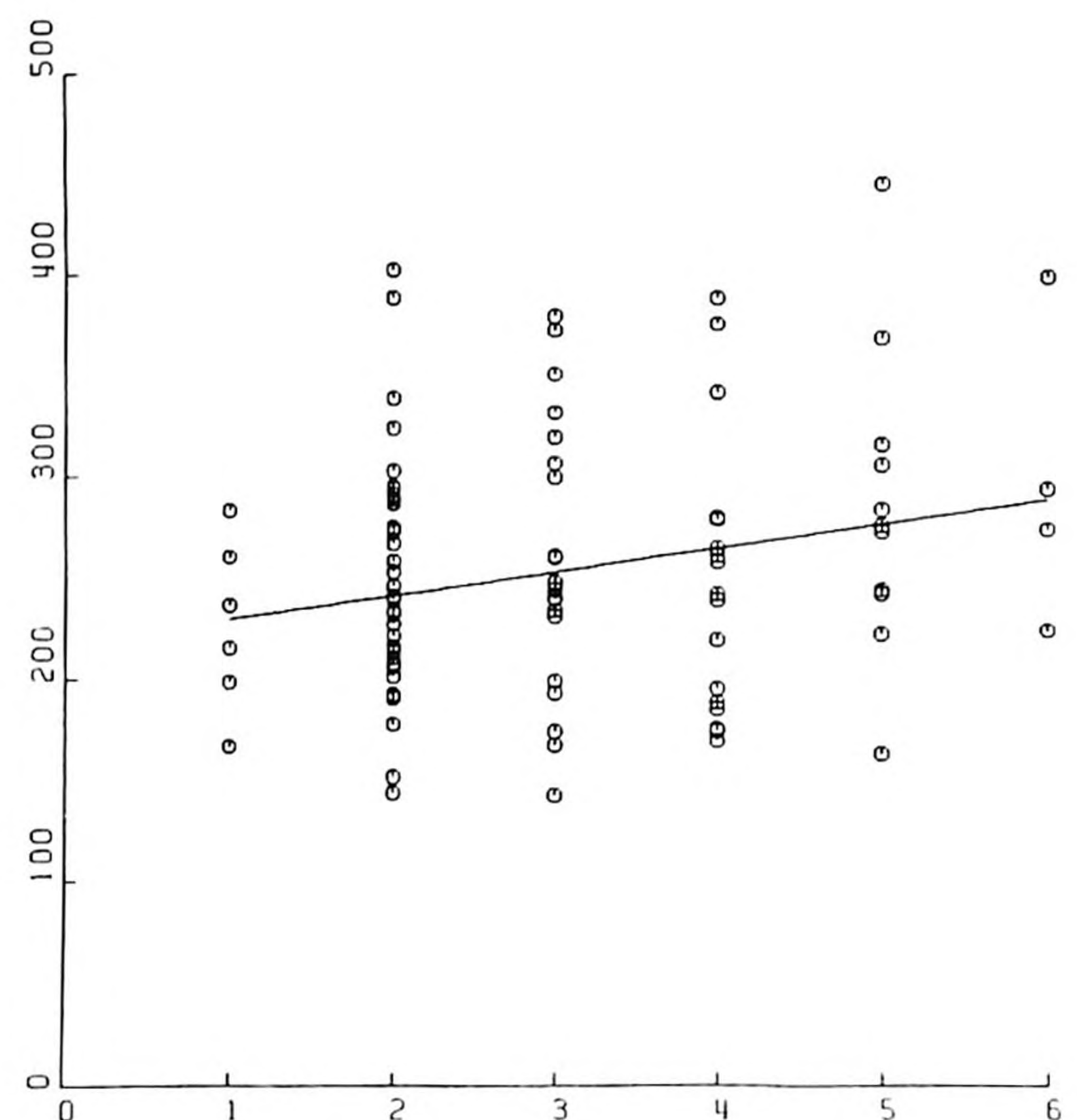


Fig. 2. Results of combined fluorescence DNA measurements and scanning electron microscopy morphology of 100 elongated macrophages cultured on glass for seven days. Abscissa: surface morphology expressed in groups 1-6 according to the number of microvilli and/or microridges. Group 1 cells with no or hardly any surface structures, group 6 cells completely covered with surface structures. Ordinate: Feulgen-DNA values expressed in arbitrary units. Linear regression line $Y = 218.2 + 11.89 X$ ($r = 0.2344$ and $P = 0.02$)

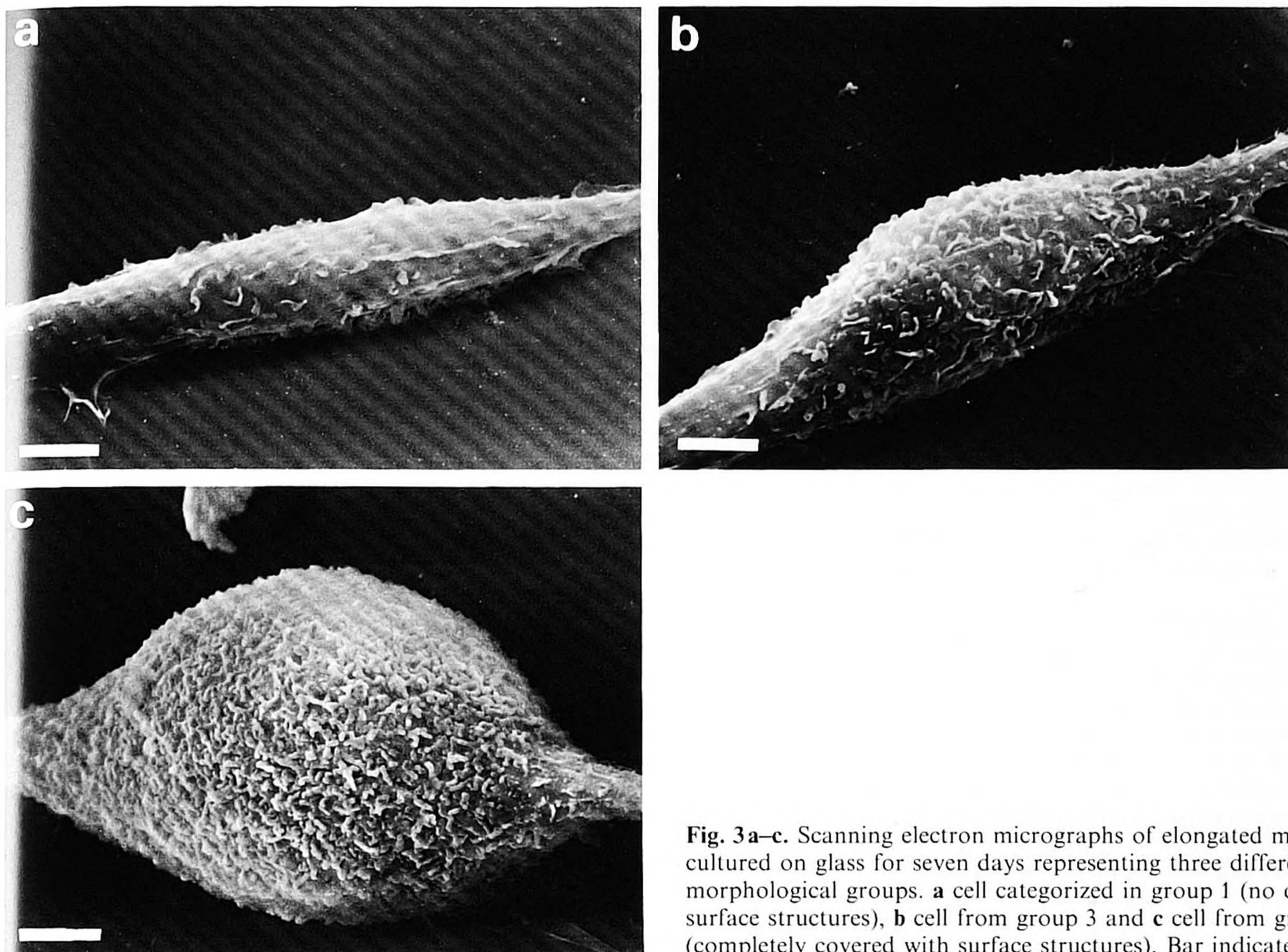


Fig. 3a–c. Scanning electron micrographs of elongated macrophages cultured on glass for seven days representing three different morphological groups. **a** cell categorized in group 1 (no or hardly any surface structures), **b** cell from group 3 and **c** cell from group 6 (completely covered with surface structures). Bar indicates 3 µm

Statistical analysis. The statistical analysis was performed by calculating the linear regression line according to standard methods (Armitage 1980).

Results

It was previously found that cells with the LM morphology of elongated macrophages had a tritiated thymidine labeling index ranging between 16% at day 7 of culture and 4% at day 21 of culture (van Furth et al. 1983). In the present experiments the measured DNA values of 100 elongated macrophages cultured on glass for seven days were compared to their type of surface morphology (Fig. 2). The expected diploid DNA content was set at about 200 arbitrary units. Six cells, which had few microvilli and/or micro-ridges and were categorized in the first surface morphology group (Fig. 3a), had a fluorescence signal (expressed in arbitrary units) that varied from 167 to 284 units.

The largest number of cells, 42 in total were categorized in the second group. Their fluorescence signal varied from 144 to 404.

In the third group, 20 cells were measured; the fluorescence signal of these cells varied from 143 to 381. An example of a representative cell for group 3 is shown in Fig. 3b.

In the fourth group, 17 cells with a fluorescence signal varying from 167 to 390 were detected.

The fifth group consisted of 11 cells with a fluorescence signal varying from 164 to 447.

Only four cells were categorized in the sixth group. Each of these cells was completely covered with cell surface struc-

tures (Fig. 3c). The fluorescence signal in this group varied from 225 to 400.

The correlation coefficient of DNA with group number was 0.23. The linear regression line of DNA (Y) on group number (X) (Fig. 2) was $Y = 218.2 + 11.89 X$. The standard error of the intercept was 16 and that of the slope was 5.0. The slope was significantly different from zero ($P = 0.02$).

Discussion

In the present paper an application of a newly described technique, simultaneous fluorescent light microscopy and scanning electron microscopy on the same single cells, is described. The question whether cells with the light-microscopical appearance of macrophages, which develop in liquid cultures from murine bone marrow, are heterogeneous with respect to DNA content can be answered in a negative sense. Macrophages with diploid and more than diploid DNA content are equally distributed among the cell types with different cell surface appearance. The heterogeneity of the surface structure of the macrophages detected by scanning electron microscopy probably represents differentiation in culture.

The linear regression line, calculated from the 100 randomly selected measured cells belonging to different morphological groups, showed that there exists some correlation between the DNA content and the cell surface morphology. Thus, cells with more surface structures have some tendency to contain more DNA.

In view of the data on DNA content and type of surface

morphology it is tempting to conclude that the different macrophages in bone marrow cultures in the presence of CSF-1 are able to divide. This conclusion is supported by the results of studies, using transmission electron microscopy and peroxidase cytochemistry in which mitotic figures of monocytes (syn. exudate macrophages), peroxidase-negative macrophages and resident macrophages were detected in these in vitro cultures with CSF-1 (van der Meer et al. 1985).

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