Department of Anatomy of the University Medical School (Director: Prof. F. Kiss)
and National Institute of Sterility Research, Budapest

NEW DATA REGARDING THE FLUORESCENCE MICROSCOPIC PICTURE OF VAGINAL EPITHELIUM

T. Donáth, Gy. Orbán and R. Gimes

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Fluorescence microscopy has been regularly used by us since 1954 for the cytodiagnostic examination of vaginal smears. Our results, obtained with a combination of the fluorochrome dyes thioflavine S and thiazine-red, were published in 1957 [9]. Since 1957 we have been working with acridine orange. According to the modification of one of us (Donáth), we treat our samples in a wet chamber. Acridine orange has been used for the purposes of exfoliative cytology by Bertalanffy et al. in the Mount Sinai Hospital of Los Angeles: applying it exclusively to fixed smears, they obtained satisfactory results [1, 2, 3].

Our present experiments had the following objects: *a)* to obtain — without the use of fixatives — quick and reliable information regarding the cellular elements of the vaginal smear; *b)* to establish the cytological pattern of the different kinds of hormonal insufficiency; *c)* to study the cytochemistry of the vaginal epithelium with a view to collecting data regarding the cytochemical properties of pathological cellular elements encountered in the vaginal secretion.

Cytotopochemistry, one of the recent branches of cytology, while not disregarding morphological criteria, endeavours to go beyond the limits of cytomorphology by determining and differentiating the chemical substances of the cells.

Papanicolaou's technique — so far the best colpocytological procedure — demands much time, needs great professional skill and is based on morphology. Of recent, cytochemical methods have gained ground, especially as regards differentiation between benign and malignant cells [5, 7, 8].

Acridine orange NO (Gurr) was first used by Strugger and — independently of him — by Bukasch and Haitinger in 1940 [14]. According to recent data the dye has a specific affinity to nucleic acids and reacts with them with intensive fluorescence [11, 12, 15]. Ribonucleic acid (RNA) produces orange-red, desoxyribonucleic acid (DNA) green fluorescence. In the course of vital and supravital examinations it was found that, while living cells take up but little acridine orange and show a green fluorescence, dead cells bind large

amounts of the dye and give, consequently, flame-red fluorescence (STRUGGER's concentration effect, 13). This classic observation made on plant cells has become a literary battleground of controversy in its application to animal cells. Acridine orange is very sensitive to pH changes: the higher the pH the more of the dye is absorbed by the cells.

Following STRUGGER's instructions, we took great care adequately to adjust the pH of the dye and to use it in adequate concentrations.

Material and method

The vaginal discharge of healthy young women in different phases of the menstrual cycle was studied in addition to the vaginal discharge of sexually mature women suffering from hormonal insufficiency and of women at the beginning or in a later phase of the menopause.

The studies were made in the wet chamber. Vaginal secretion was taken from the posterior fornix by means of a spatula, transferred to a slide, delicately mixed with a 1 : 10000 dilution of acridine orange and embedded in paraffin. The dye dilution was made of a stock solution of 0.1 per cent acridine orange in distilled water and a buffer composed of 80 per cent 1/15 M primary potassium phosphate and 20 per cent 1/15 M secondary sodium phosphate (pH 6.36). Fluorescence microscopy was done between the 3rd and the 60th minutes so as to avoid overstaining. Wet-chamber preparations may deteriorate after an hour.

We adjusted the pH of the dye solution in some cases to 3.51, 5.81, 7.63 and 8.22. Glycogen was demonstrated by the comparative use of Lugol's reaction and Cattor's method.

Simultaneously with the wet-chamber preparations smears were fixed in ethylalcohol-ether and stained with acridine orange 1 : 10000 according to Papanicolaou and methylene blue.

A larger Zeiss fluorescence unit was used. Four percent copper sulphate served for the absorption of heat rays, a Schott UGl filtre of 1.5 mm for induction and GG9 or GGl ocular suppression filtre for the elimination of UV rays. An Exacta Varex camera and Agfacolor daylight negative (17/10 Din) film were employed for colour photography; exposure time varied between 2 and 10 minutes according to the desired degree of magnification.

Results

On treatment with acridine orange the fluorescence of the epithelial cells varies according to the layer from which they originate.

The cytoplasm of superficial cells with pyknotic nuclei usually gives a very faint or no fluorescence. Such cells show sometimes brilliant red fluorescence: high magnification reveals it to be due to a great number of Döderlein's bacilli attached to the cell surface. Orange-coloured cytoplasmic granulation is observable in cells free from bacteria. The small pyknotic nucleus shows a brilliant green fluorescence which in other types is much weaker although they do not differ morphologically from the cells under review. There occurred also cells in the superficial layer which had a swollen nucleus showing a vivid red fluorescence.

The cytoplasmic fluorescence of superficial cells (with non-pyknotic nuclei) is green with a smaller number of coarse orange-stained granules. The nucleus is surrounded by a zone which contains no granules and

Fluorescence of the elements of vaginal discharge stained with acridine orange (pH 6,3)
(Number of crosses indicate intensity of fluorescence)

	Nucleus	Cytoplasm	Granules, colour	Granules, number and size	Observations
Superficial cells with pyknotic nuclei I.	green ++	0	0	0	Advanced pyknosis
Superficial cells with pyknotic nuclei II.	light green +++	greyish-green +	orange-yellow +	few-small	Recent pyknosis
Superficial cells with non-pyknotic nuclei	green ++	dark-green +	orange-yellow ++	few-coarse	
Intermediary cells	green ++	greenish-yellow ++	orange-yellow +	few-small	
Parabasal cells	yellowish ++	orange or green ++	orange-yellow +	many-small	
Basal cells	orange-yellow ++	orange or green ++	orange-yellow ++	many-coarse	
Endocervical cells	orange-yellow +++	orange-red ++	orange-yellow +	few-small	
Leukocytes I.	green ++	dark green +	orange-yellow +	few-small	Fresh
Leukocytes II.	red +++	orange-yellow +	red +++	many-coarse	Necrosed
Histiocytes	greenish-yellow ++	brownish grey +	orange-yellow ++	few—coarse	
Trichomonas	orange-yellow ++	yellowish orange +	red ++	many-small	
Döderlein's bacilli		orange-red +++			
Mucus		light-green ++			

shows no fluorescence. Neither does the cell membrane show fluorescence. Nuclear fluorescence is green.

Cells of the middle layer reveal a more vivid fluorescence than those of the upper layer: their colour is greenish yellow while nuclear fluorescence is green. The parabasal and basal cells have a granulated and brilliantly red cytoplasm and an orange-coloured nucleus. A few parabasal cells had a green cytoplasm and an orange or greenish-yellow nucleus.

Leukocytes in the wet-chamber preparations were clearly distinguished by their bright green nuclearchromatin, the faint green fluorescence of their nuclear membrane, and the dark green cytoplasm with red granules. In case of inflammation there appeared in addition leukocytes displaying a red nuclear fluorescence and leukocytes in which the entire protoplasm was filled with red granules.

The fluorescence of Döderlein's bacilli is a characteristic orange red or, at a low pH (3.1), green. Most of these bacilli are attached to the superficial cells showing nuclear pyknosis. Subsequent treatment with Lugol's solution blotted out the fluorescence of the superficial cells without affecting that of other cells.

Trichomonas show orange-yellow nuclei and a red cytoplasmic granulation: they are well distinguishable from other elements, from leukocytes with green nuclear fluorescence in particular.

Discussion

Our wet-chamber technique and the employed method of staining have the advantage of being quick, simple and of preventing cellular damage and morphological changes (change of size, shrinkage) associated with fixation. It allows a subvital evaluation of the cells in their original size and structure. Fixation — even with ether-alcohol, the best fixative for smears — is a crude operation which causes gross structural changes in the cytoplasm (the cells lose half their size) so that fixed smears are usually suitable for nuclear observations only [16], while our procedure makes it possible to follow cytoplasmic changes and so to note cytoplasmic basophilia, to observe morphological differences between the cells originating from different epithelial layers and thus to obtain a true picture of the distribution of nucleic acids.

It has been histochemically proved that acridine orange, as also other diaminoacridine derivatives, have an affinity to nucleic acids and are consequently well suited for the demonstration of these acids and for the differentiation of RNA and DNA. Acridine orange has moreover been found useful for the determination of the degree of polymerization of intracellular DNA.

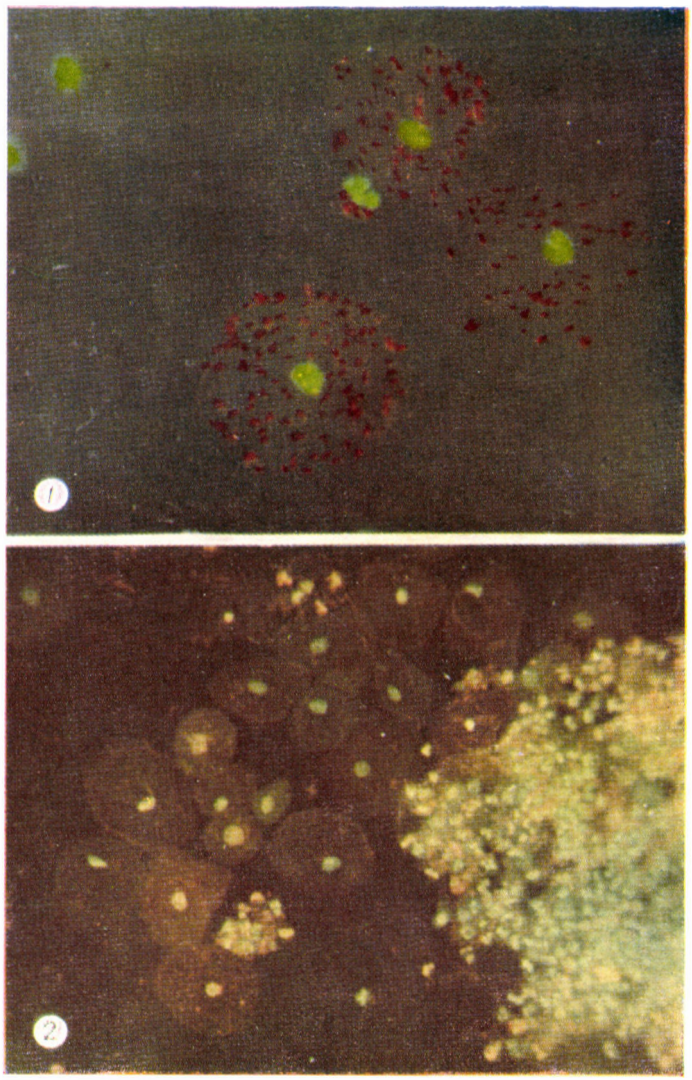
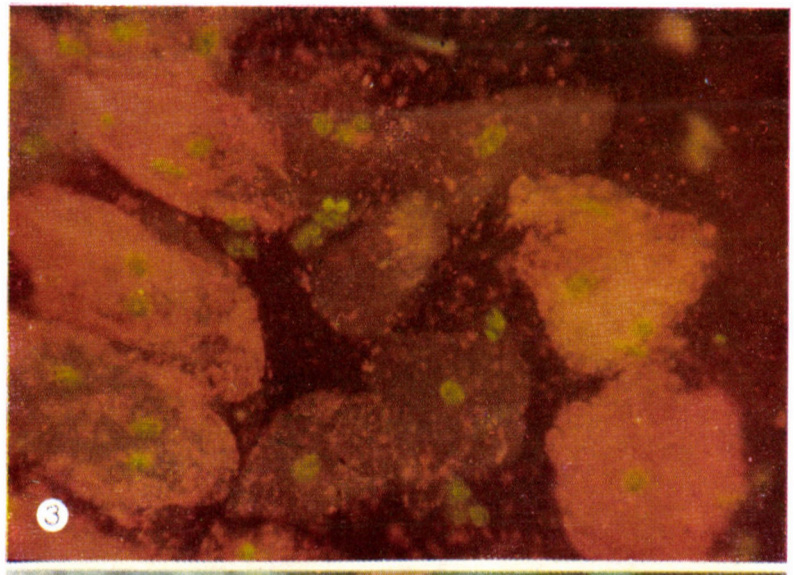


Fig. 1. The picture shows three superficial epithelial cells. Bright green nuclear fluorescence. Only orange-coloured granules produce fluorescence in the cytoplasm. Between the three cells a segmented leukocyte with green nucleus and brilliant red cytoplasmic granulation. Lacking cytoplasmic granules, only the nucleus of the other superficial epithelial cells shows fluorescence. ×400

Fig. 2. Menopause. Mostly superficial cells with non-pyknotic nuclei. Shape and arrangement of the cells illustrate the superiority of the wet-chamber method over the smear technique which injures the cells and makes them shrink. Affinity of Döderlein's bacilli to cells of the uppermost epithelial layer. Nucleus of parabasal cell shows yellowish fluorescence. ×200



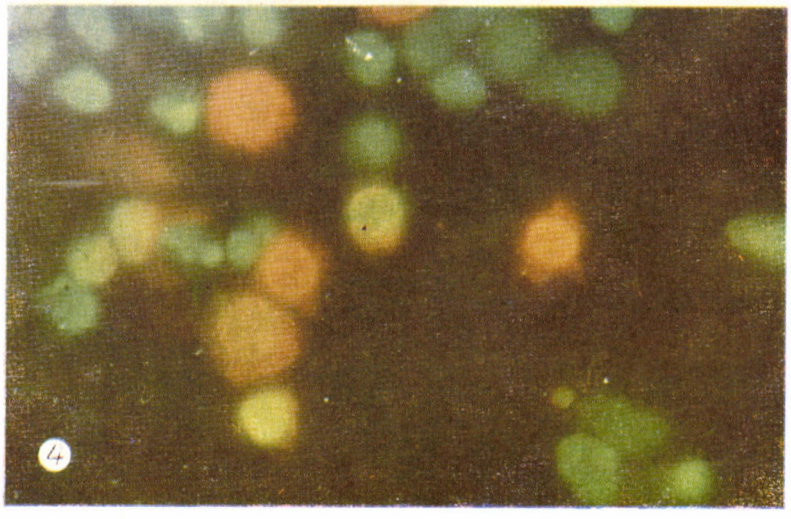


Fig. 3. The picture shows principally cells from the intermediary layer. Fluorescence of cytoplasm is dark green; of nucleus, intensive yellowish green. Orange-yellow fluorescence of inter- and intracellular bacterial flora covering the cytoplasm of some cells with a pyknotic nucleus. In the course of control experiments made with Lugol's solution, the iodine reaction blotted out the fluorescence of these superficial cells without affecting that of the others. Leukocytes show a bright green nuclear fluorescence ×400

Fig. 4. Trichomonas vaginalis and leukocytes above the two epithelial cells. The large yellow-coloured nucleus and the red cytoplasmic granules of the flagellates distinguish them sharply from the surroundings, especially from the leukocytes with their green nuclear fluorescence, ×900

Nuclear fluorescence in the cells from the uppermost layer of the epithelium was of three kinds. The major part of the nuclei gave a homogeneous and sharp fluorescence indicative of condensing DNA and incipient pyknosis. Some of the nuclei showed a considerably weaker fluorescence: these were pyknotic with completely depolymerized DNA. The cytoplasm of such cells was acidophilic which explains why only very little alkaline dye was absorbed by them. We observed occasional swollen nuclei with a brilliant red fluorescence: we regarded this as a sign not of pyknosis but karyolysis; the red fluorescence must be due to Strugger's effect, *i. e.* to the accumulation and consequent high concentration of the dye.

Progressive cytoplasmic basophilia was observed as we reached cells of the deeper layers. It manifested itself with gradual change of green into orange and orange-red cytoplasmic fluorescence. This change in colour indicates a considerable increase in the nucleic acid content of the cytoplasm (BERTALANFFY et al.). The cytoplasmic fluorescence of cells from the intermediary layers was invariably stronger than that of the superficial cells, only a few parabasal cells showed a green cytoplasmic fluorescence. In summary, we think that the intensity of fluorescence produced by acridine orange is in direct ratio to cytoplasmic basophilia.

That Döderlein's bacilli show a marked affinity to superficial cells, is in our opinion due to the high glycogen content of these cells, since only their fluorescence and not that of the other cells was distinguished on treatment with Lugol's solution. Our present observations in this respect were but an additional confirmation of similar conclusions drawn from the results of earlier experiments in which Cattor's method had been used [6].

The fact, that leukocytes give two markedly different kinds of fluorescence must in our opinion be due to differences in their age and condition: old necrosed leukocytes give a brilliant red nuclear fluorescence, while the nucleus of fresh leukocytes that have passed into the vaginal discharge quite recently, show a green colour.

While the usual dyes do not bring out the normal bacterial flora, with acridine orange this displays an intensive fluorescence. *Trichomonas vaginalis* is easily recognizable. The presence of many leukocytes does not impair the accuracy of cytodiagnosis. Cells of the vaginal epithelium can be studied undisturbed in smears containing blood because fluorescence of the erythrocytes is extinguished by their haemoglobin. One of the present authors [4] has elaborated a technique which makes it possible to induce a change in the chemical structure of haemoglobin so that erythrocytes can — if so desired — be made visible by means of fluorochromes.

Summary

Wet-chamber preparations of the cellular elements of the vaginal discharge were examined subvitally under the fluorescence microscope after staining with acridine orange. The procedure is simple, quick and gives a reliable picture in which it is easy to distinguish between different cell types. The different fluorescence of the different types of cells is attributed to differences in nucleic acid contents. The intensity of fluorescence was dependent on cytoplasmic basophilia. It is recommended that in colpocytological examination not only morphological changes occurring in nucleus and cytoplasm but also changes in cytochemistry should be studied.

Döderlein's bacilli were found to have an affinity to cells of certain types.

The method described makes it possible to conclude the degree of pyknosis from the degree of DNA polymerization and to distinguish live from dead leukocytes on the evidence of fluorescence. Bacterial flora and *Trichomonas vaginalis* are clearly distinguished.

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НОВЫЕ ДАННЫЕ К КАРТИНЕ ЭПИТЕЛИАЛЬНЫХ КЛЕТОК ВЛАГАЛИЩА ПРИ ФЛУОРЕСЦЕНТНОЙ МИКРОСКОПИИ

Т. ДОНАТ, ДЬ. ОРБАН и Р. ГИМЕШ

Под флуоресцентным микроскопом были исследованы субвитально окрашенные (в влажной камере) при помощи АО клеточные элементы выделений из влагалища, соответственно отдельным фазам. Данный метод быстрый, простой и предоставляет верную картину, позволяющую легко обособлять отдельные клетки. Встречающиеся у отдельных типов клеток разницы в цвете флуоресценции объясняются отклонением содержания нуклеиновой кислоты. Полученная окрашиванием АО интенсивность флуоресценции параллельна базофилии клеточной структуры. На основании данного метода, предоставляющего одновременно дифференцированное окрашивание ядер, предлагается, при

колпоцитологической оценке, наряду с морфологическими изменениями ядер и плазмы обратить внимание также на цитохимические изменения. В исследованиях приписывается большая роль сродству влагалищных палочек Дедерлейна к определенным типам клеток. При помощи данного метода демонстрируется на основании степени полимеризации ДНК ранняя и поздняя фазы ядерного пикноза, далее разница в флуоресценции живых и омертвелых лейкоцитов. Под флуоресцентным микроскопом прекрасно обослоьяются. бактериальная флора и трихомонасы.

NEUERE BEITRÄGE ZUM FLUORESZENZMIKROSKOPISCHEN BILD DER EPITHELZELLEN DER SCHEIDE

T. DONÁTH, GY. ORBÁN und R. GIMES

Die mit Akridinorange gefärbten Zellenelemente des Scheidenausflusses wurden den einzelnen Phasen entsprechend in Feuchtkammerpräparaten mittels (subvital) Fluoreszenzmikroskopes untersucht. Das Verfahren ist schnell, einfach und gibt ein getreues Bild, die Zellen können leicht differenziert werden. Die bei den verschiedenen Zelltypen erscheinenden Farbendifferenzen in der Fluoreszenz werden mit dem unterschiedlichen Nukleinsäuregehalt erklärt. Die mittels Akridinorange-Färbung erhaltene Fluoreszenzintensität ist der Basophilie der Zellstruktur proportional. Auf Grund der angewandten Methode, die gleichzeitig auch eine differenzierte Kernfärbung ermöglicht, wird bei der kolpozytologischen Auswertung, neben den morphologischen Veränderungen der Zellkerne und des Plasma, auch die Beobachtung der zytochemischen Veränderungen empfohlen. Der Affinität der Döderleinschen Bazillen zu gewissen Zelltypen wird eine Rolle zugeschrieben. Mittels der angewandten Methode werden auf Grund des Polymerisationsgrades des DNS die frühe und späte Phase der Kernpyknose, sowie die Fluoreszenzdifferenz zwischen den abgestorbenen und lebenden Leukozyten demonstriert. Im fluoreszenzmikroskopischen Bild können Bakterien und Trichomonas hervorragend differenziert werden.

Dr. Tibor DONÁTH
Dr. György ORBÁN
Dr. Rezső GIMES
Budapest, IX. Tűzoltó u. 58., Hungary