Reports

Use of Acridine-Orange Fluorescence Technique in Exfoliative Cytology

This is a preliminary report of a fluorescence-microscopic technique that permits rapid scanning in exfoliative cytology and promises increased insight into cytochemical changes leading to malignancy.

It is generally agreed that the Papanicolaou technique (*1*), notwithstanding its high diagnostic value, is demanding in terms of time (*2*) and does not reveal certain cytochemical properties presumably important in malignancy (*3*). Among a variety of supplementary techniques, several attempts to introduce fluorescence microscopy have been made (*2, 4–6*). The proposed methods have not been widely used because the techniques are not simpler than the routine Papanicolaou (*4*), because they do not differentiate between DNA and RNA and ask for special devices (*2, 5*) or because apparently they were not developed for diagnostic purposes (*6*).

Bertalanffy and Bickis (*7*) have described a fluorescence technique using AO (*8*), which allows for differential staining of the two nucleic acids: RNA in the cytoplasm and nucleolus gives a bright red fluorescence; DNA of the chromatin fluoresces green. The advantages of this method are (i) the simplicity and rapidity of the procedure; (ii) its applicability to living, supravital, and fixed tissues and smears; (iii) the distinction of the nucleic acids whose importance in carcinogenesis is undisputed; (iv) the brilliance of the picture obtained, allowing for evaluation of the morphological pattern as well as quick scanning under low magnification.

The method has now been applied to vaginal and cervical smears and to various types of malignant growth. Smears taken by the routine procedure, avoiding all contact with lubricants, are fixed immediately in equal parts of 95 percent ethyl alcohol-diethyl ether mixture for at least 1 hour. They are passed through alcohol 80, 70, 50 percent and distilled water, rinsed five times in 1 percent acetic acid, washed in distilled water, and stained for 3 minutes in AO (Gurr) (0.01 percent in buffer solution of 1/15*M* phosphate buffer at *p*H 6.0) at room temperature. The AO stock solution 0.1 percent is kept in the dark, and fresh dilutions are prepared once a week. The buffer is kept under refrigeration. After the preparation has been destained for 1 minute in the phosphate buffer, it is differentiated with 0.1*M* $CaCl_2$ for 30 seconds or more, until a clear outline of the nuclear structure is apparent. After a quick check under the fluorescence microscope, the slides are washed with phosphate buffer and mounted in a drop of the same buffer, with careful blotting. If it is desired, the smears may afterward be destained by transfer into 1 percent acetic acid for a short period and be processed with the standard Papanicolaou technique for comparison purposes.

The Zeiss-Winkel blue light assembly (*7*) was used. Microphotographs on Kodachrome A film give an excellent reproduction of colors.

The AO picture was checked by application of RNase (*7*) and was compared with the corresponding pictures obtained with the standard Papanicolaou technique, the fluorescence methods (*2, 4*), and toluidine-blue staining (*9*).

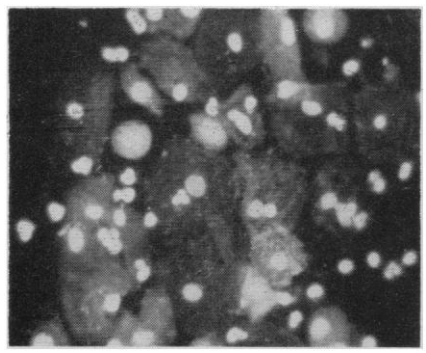

Fig. 1. Group of cells from a normal vaginal smear. Precornified squamous cells predominant, two cornified squamous cells (smaller nuclei) on the extreme right and left of the picture; inner basal cells are distinctive through their round shape, intensely fluorescing cytoplasm and large nuclei; in the lower part of the picture four outer basal cells (cytoplasm more abundant and less dense than in inner basals). Throughout the picture brilliantly fluorescing nuclei of leucocytes and lymphocytes are seen, also superimposed bacteria. 325 ×.

Elements of normal vaginal and cervical smears give the following fluorescence picture (Fig. 1) (*10*). *Cornified squamous cells*: cytoplasm greenish-gray with faint orange tinge; nuclei brilliant whitish green. *Precornified cells*: cytoplasm same as cornified, nuclei white-green to white-yellow with distinct white chromatin particles. Around the nuclei of some precornified cells orange granules (probably keratohyaline) of different size were seen, the largest approximating one-third of the size of the nucleus. *Intermediate or navicular cells*: cytoplasm with thickened cytoplasmic membrane of a faint orange color, nuclei white-green. *Outer basal cells*: cytoplasm reddish-brown (brick) color, dull, nuclei white-yellow with white chromatin particles. *Inner basal cells*: cytoplasm reddish-brown (brick) color with fine red particles (sometimes orange-reddish of much more intense hue than in outer basals), nuclei white-yellow with white chromatin particles and sometimes orange nucleoli. *Endocervical cells*: cytoplasm reddish-orange with meshlike appearance, nuclei white-yellowish with white chromatin particles and bright orange-red nucleoli. *Leucocytes*: brilliantly fluorescent white-green nuclei, lymphocytes and monocytes sometimes showing a faint greyish rim of cytoplasm. *Red blood cells*: unstained. *Histiocytes*: cytoplasm greyish, sometimes with orange particles (ingested bacteria), nuclei whitish-green of characteristic bean shape. *Mucous threads*: stained whitish-green. *Bacteria*: bright orange. *Trichomonas vaginalis*: stained bright orange with the nucleus outlined at the anterior end.

Most of the exfoliated *malignant cells* obtained in vaginal and cervical smears from cases of squamous-cell carcinoma of the cervix and the vagina yielded a fluorescence picture which was strikingly different from that of normal epithelial cells. These cells showed brilliant orange-red cytoplasm of a very intense hue and intensely yellow-fluorescent nuclei with brilliant orange-red nucleoli.

Preliminary studies of *malignant tumors* were made with carcinomas of the uterus (exfoliated smears), stomach, rectum, breast, lung, lymph nodes (metastatic) (touch preparations). The majority of malignant cells also showed intense fluorescence, orange-red in the cytoplasm and yellow in the nuclei, making them easily distinguishable from the cells of origin.

In general, the AO technique showed under *low power* the malignant cells in brilliant colors, thus calling the immediate attention of the examiner; under *high power*, since the fluorescent colors strongly enhance the contrasts, an excellent evaluation of morphological criteria was possible.

The technique suggests itself for rapid

scanning, to be followed, if necessary, by examination under high power and other procedures for differential diagnosis of the suspected cells.

An extensive investigation of vaginal and endocervical smears, with particular attention directed to nonmalignant proliferative and malignant processes, is at present being carried through. Also the applicability of the AO technique to cell suspensions and wet preparations is under investigation. Other papers giving results, as well as a more detailed description, are in preparation (*11*).

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References and Notes

1. G. N. Papanicolaou, *Atlas of Exfoliative Cytology* (Harvard Univ. Press, Cambridge, 1954).
2. R. C. Mellors, G. N. Papanicolaou, A. Glassman, *Cancer* 5, 458 (1952); *Cancer Research* 11, 267 (1951a); *Am. J. Pathol.* 27, 734 (1951b).
3. B. C. Hopman, *Am. J. Obstet. Gynecol.* 65, 1228 (1953).
4. H. P. Friedman, Jr., *ibid.* 59, 852 (1950).
5. H. A. von Schweinitz, *Acta Histochem.* 2, 25 (1955).
6. N. Schuemmelfeder, *Virchow's Arch.* 318, 119 (1950).
7. L. von Bertalanffy and I. Bickis, *J. Histochem. Cytochem.* 4, 481 (1956).
8. Abbreviations used are AO for acridine orange; DNA for desoxyribonucleic acid; RNA for ribonucleic acid.
9. W. L. Pirozynski and L. von Bertalanffy, *Arch. Pathol.* 54, 450 (1952).
10. Black-and-white photographs show neither the brilliance nor many details easily visible in the fluorescence picture. Some color photos are reproduced in 7.
11. We take pleasure in thanking Leo Kaplan, head of the pathology department of this hospital, for his valuable contribution of material and pathological diagnosis.

12 September 1956

Consistent Running Records

The two articles by M. H. Lietzke (*1, 2*) on track and other athletic records follow closely the pattern developed by previous workers in the study of the time-distance relationship. A particularly close parallel is to the pioneer work of A. E. Kennelly (*3, 4*), who plotted a straight-line log-log curve for all the activities Lietzke studied (men and women running, walking, swimming, skating, rowing; horses running, trotting, and pacing; men bicycle-riding and automobile-driving). Like other students besides Kennelly (*5–9*), Lietzke worked out a series of consistent performances (limited to men's running records) (*2*) by choosing a set of "best efforts" (220-yard, 1-mile, and 1-hour runs) that fit his curve. He then calculated the change in existing records required to reduce the times so that they would fall on the log-log straight line. Many of the calculated times are not consistent with history and experience in track athletics (Table 1), the best example being the present 1-mile record of 3 minutes, 58 seconds (3:58) and Lietzke's calculated 2-mile record of 8:22 (fractions of seconds are purposely omitted in this discussion).

The 1-mile record has been reduced in the past 50 years by more than 17 seconds, and during the same period the 2-mile record has come down about 36 seconds, with the interim marks keeping step with each other. To argue that the 2-mile distance will never be run in 8:22 would be extremely rash, but it can be predicted with reasonable certainty that the man who establishes the 8:22 mark will be able to run a mile in 3:54 or under.

A. W. Francis (*7*), using the records of 1943 when the 1-mile record was 4:02.6 and the 2-mile was 8:47.8, computed from his formula for a hyperbola that the time for the mile should be 3:58.7 and for the 2-mile 8:37.2, both slightly higher than present figures, but consistent with track history.

I was convinced many years ago (*6*) that the straight-line log-log function did not accurately fit the facts. Kennelly (*3, 4*), whose early work has never been fully appreciated by track specialists, was careful to call his logarithmic formula "An approximate law of fatigue in running animals." A modern student will be startled to find that exactly 50 years ago, Kennelly (*3*) calculated from his straight-line log-log relationship that the mile could be run in 3:58.1, but the prediction loses its force because his formula also equated this 3:58 mile with a 50.1 quarter mile run and a 23-second 220-yard run, both about 7 percent slower than the then-existing records. Lietzke, and others before him, have avoided these obvious inconsistencies by plotting their curves through the best marks only, but the inconsistencies still show in the calculated records if the attempt is made to squeeze the relationship into a straight-line log-log formula.

Table 1. Relationship between 1-mile and 2-mile running records for various dates.

Date	Record (min : sec)		Remarks
	1 mi	2 mi	
1905	4:15	9:09	
1931	4:10	8:59	Both records held by Paavo Nurmi.
1937	4:06	8:53	No great 2-mi runner in this period.
1944	4:01	8:42	Both held by Gundar Haegg (2-mi later reduced to 8:40).
1955	3:58	8:33	Iharos, the 2-mi record holder, has run 1500 m in 3:40.8.

A definitive time-distance relationship for athletic records must be based on a full knowledge of the history, experience, and practice in the various events and probably on physiological considerations as well, rather than on statistical data only. The research of Franklin M. Henry (*10, 11*) may lead to a formula that satisfies all these requirements.

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References

1. M. H. Lietzke, *Science* 119, 333 (1954).
2. ———, *ibid.* 124, 178 (1956).
3. A. E. Kennelly, *Proc. Am. Acad. Arts Sci.* 42, No. 15, 275 (1906).
4. ———, *ibid.* 61, No. 11, 487 (1926).
5. G. P. Meade, *Sci. Monthly* 2, 596 (1916).
6. ———, *New York Herald Tribune* (11 July 1926); *New York Univ. Alumnus* (Feb. 1934).
7. A. W. Francis, *Science* 98, 315 (1943).
8. B. Hamilton, *Amateur Athlete* 6, No. 2, 4 (1935).
9. ———, *ibid.* 23, No. 7, 28 (1952).
10. F. M. Henry, *Science* 120, 1073 (1954).
11. ———, *Research Quart. Am. Assoc. for Health, Phys. Educ., and Recreation* (several articles).

21 August 1956

George P. Meade is right when he mentions that a definitive time-distance relationship for track records should probably be based on physiological considerations as well as statistical data. The work of Franklin M. Henry in which an attempt is made to analyze the various biochemical factors that enter into a rate equation for running seems to me to be the correct approach to this problem. When the rate-determining processes that occur in the body during running have been sufficiently elucidated, it should be possible to derive a rate equation that will fit the pattern of observed records very closely at all distances.

In the absence of a rate equation that generated closely the pattern of the rate-versus-distance plot for all running events, I used the log-log relationship in my own calculations of the consistency of present racing records. The log-log plot seems to fit the observed records over a wider range than any other single relationship. However, the use of the log-log plot is most certainly an oversimplification. A plot of the rate equation based on physiological considerations may remove the inconsistencies that Meade has pointed out. It would be very nice if Franklin Henry could reach the point in the derivation of his rate equation where he could suitably isolate the various combinations of rate-determining biochemical reactions that occur in the body as a function of the distance run so that the shape of the rate curve could be determined prior to any consideration of the actual records. Whether this can be done remains to be seen.

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26 September 1956