RADIOISOTOPES IN PRELIMINARY SCREENING OF CERVICAL CANCER

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In an attempt to automate screening for cervical cancer, the nuclear changes observed cytologically were compared with radioactive nuclear stains in order to determine the different cellular uptakes of radioactive material by normal cells and cancer cells. Among 205 normal women, approximately 15% of false positives occurred by this technique. With invasive carcinoma, the uptake was elevated in all 25 studied. In addition, a higher percentage of patients showed elevated uptakes with cervical dysplasia. The number of cases with carcinoma in situ (four) studied is insufficient for definitive conclusions. The method is adaptable to complete automation and appears economically sound as a prescreening technique. It is recommended that the technique be applied in a much larger series during routine screening for cervical cancer.

For the last three decades the Papanicolaou technique for the detection of cervical cancer has received increasing acceptance and thus wider application, particularly for the discovery of very minimal lesions. Investigation by the American Cancer Society indicated that in 1970 40% of the population remained unscreened by this cytopathologic approach. Thus, needless morbidity and mortality continues to occur despite the potential availability of a technique that in theory should permit virtual eradication of cervical cancer.

The failure to achieve 100% application of cytologic screening appears to be related to a number of factors. This would include the limited number of available highly trained cytotechnologists and cytopathologists, difficulties in obtaining adequate clinical specimens, and certainly the cost relating both to the preparation of the smear as well as to its interpretation. In view of these factors, which may be involved in the "denial" of screening to every woman, several approaches have been made to find a replacement. Davis (*1*) and others have worked with a liquid specimen technique for preparing cellular material. This approach particularly focuses upon reaching the population that may not normally seek a medical service leading to examination and cytologic evaluation. Another attempt to expand the usefulness of the cytologic test has been concerned with finding a more rapid and less tedious approach that would simplify some phases or all aspects of laboratory evaluation. Many investigations have applied various parameters in prescreening of slides. Basically, the objectives of techniques that would serve to simplify cancer detection are dependent upon the fact that cytologic changes in nuclear structure are the most characteristic clues to cytopathologic diagnosis. Nuclear pleomorphism, pyknotic nuclei, and characteristic changes in the nucleocytoplasmic ratio are in marked contrast to the normal small nuclei of desquamated cervical epithelial cells. Moreover, multiple nuclei and significant increases in cell volume occur as alterations from the usual flat structure of the squamous epithelial cell. Whereas the highly specific differences may be readily detected, there are more subtle chemical differences with alteration in nucleoprotein structure and alteration of dye-binding properties. Historically, both changes have contributed to our current acumen. Many nuclear stains have been used in helping to visually differentiate the malignant nucleus from a nonpathologic nucleus. These chemical differences may also be important in detecting the binding and uptake of materials in the nuclei.

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Fluorescent dyes have been used which are purported to identify abnormal nuclei effectively. Individual measurement of cell properties such as nuclear size, cell volume (*2,3*), nuclear density, and even chromosome configuration have been investigated. Computerized image analysis has been attempted but becomes difficult because of the large number of fields which must be screened and analyzed as well as manual methods in selecting fields. By looking at an entire cell population with a single radioactive uptake procedure, it was postulated that a simpler method might be available and worth investigating. This report describes and gives the results of a project undertaken to test this postulate.

It seemed important to look for a new method that was automatic, inexpensive, and thus readily available. Recognizing that absolute perfection of diagnosis has not been achieved by existing methods, the investigators for this project tried to standardize a method that would be of value for prescreening and at least comparable to current methods. Because currently accepted methods have "errors" (*4,5*), we felt that a similar error rate would be acceptable if the processes were less expensive and more easily applied to fill gaps in current screening.

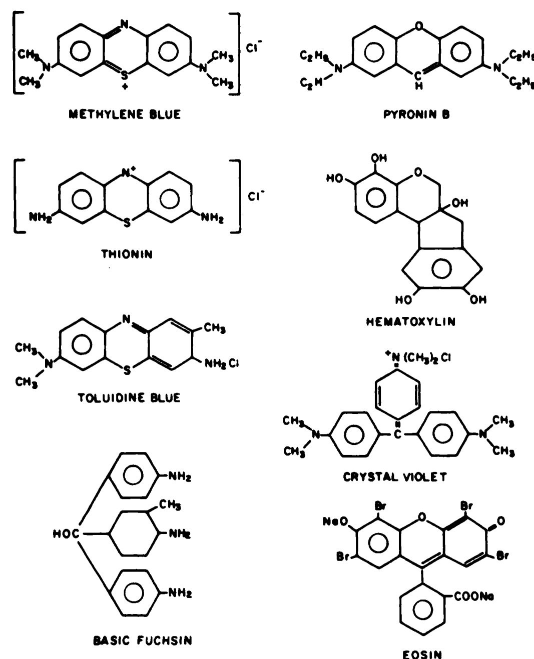

FIG. 1. Nuclear stains that were made radioactive and tested in the laboratory.

MATERIALS AND METHODS

Laboratory. A review of the various nuclear stains used in the past indicated that a large number might be available for appropriate radioactive tagging. Pilot studies with ^{14}C-methylene blue* synthesized chemically (Fig. 1) were carried out using standard Papanicolaou slide preparations followed by liquid scintillation counting. Excess variability was encountered because of the inconsistent thickness of the smear and the variable number of malignant and nonmalignant cells present. In order to control the cellular content, specimens were obtained by swabbing or scraping the cervix and suspending the cellular material in a preservative (50% ethyl alcohol), which allowed stability of the specimen at room temperature during shipment and in the laboratory from week to week as necessary for research and clinical processing. While preserving cell structure, this strength of alcohol did not significantly alter uptake of stain by the cells. The cell population of each specimen was then quantitatively evaluated using a Coulter Counter Model B with a Model H automatic size spectrum recorder. In agreement with Ladinsky (*2,3*) we found the size spectrum alone contributed information to the diagnosis of nuclear and cellular abnormality. However, in contrast to her findings, we were unable to make sufficient differentiation by this means alone. However, in the initial phases of the investigation, using a predetermined number of cells (approximately 5000–10,000), we were able to prepare standardized slides for radioactive staining and for uptake counting. The uptake per cell could be quickly related to the specific radioactivity of the staining medium and was found to be proportional to the number of cells present in any given cell population (Fig. 2).

Additional radioactive stains (Fig. 1) were obtained: ^{3}H-basic fuchsin, ^{3}H-pyronin B, ^{3}H-thionin, ^{3}H-toluidine blue, ^{3}H-crystal violet, and ^{3}H-eosin. Methylene blue was also tritiated. These were produced by catalytic exchange and had a high specific activity (1–3 mCi/mg, Schwarz Biochemicals, Inc., N.Y.). Each compound was dissolved as a stain solution using standard histologic techniques including appropriate solvent and optimum pH. Preliminary testing showed that differential uptake between cancerous and noncancerous specimens was found to some degree with all stains but seemed to be most marked with methylene blue, pyronin B, and thionin. A preliminary analysis of a small series of cancer patients and normal individuals showed that highly significant differentiation was possible using a com-

* Synthesized through the courtesy of Henry Grotta and Robert Porier, Battelle Memorial Institute, Columbus, Ohio.

bination of the cell sizing, the uptake per cell of pyronin B, and the uptake per cell of thionin. However, the high specific activity found with these stains posed problems with dye adhesion even to blank slides so that differentiation was not optimum. Accordingly, the following standardized approach was used with elimination of the slides entirely and with washing of the cells in a liquid medium by multiple centrifugation and resuspension.

Cervical cell samples, received in a 50% aqueous alcoholic solution, were gently dispersed in a loosely fitting glass homogenizer. A 0.2-ml aliquot of the well-dispersed cell suspension was transferred to a clean glass vial containing 10 ml of Isotone®. Cells were enumerated and sized with a Coulter Counter, Model B with the following settings: amplification, 2.0; aperture current, ½ ma; lower threshold, 15; upper threshold, 70. The cell suspension being counted should yield at least 2000 instrument counts. Based on the Coulter counts, 5000–10,000 cells were subsequently pipetted into a clean, 15-ml calibrated centrifuge tube. The cell suspension was centrifuged for 15 min at 3000 rpm. The supernatant solution was carefully removed by aspiration and 1 ml of the nuclear dye (^{3}H-Pyronin B, specific activity 6.5 μCi/ml, adjusted to pH 7.00 $\pm$ 0.02) was added to the packed cells. The contents were shaken on a Vortex mixer for 10 sec. The suspension was then incubated at 37°C for 10 min. The suspension was again centrifuged for 10 min at 3000 rpm to pack the cells. Excess stain was carefully aspirated and the cells were subsequently washed by centrifugation with 50% and 95% ethyl alcohol. The final cell pellet resulting from the 95% aqueous alcohol wash was dissolved with 0.3-ml of NCS® tissue solubilizer. The solubilization was complete after 2 min at room temperature. The dissolved cells were then quantitatively transferred to a 20-ml glass liquid scintillation counting vial containing 10 ml of counting solution (42 ml Liquifluor® per liter of toluene). Samples were then counted in a commercial liquid scintillation spectrometer and corrected for quenching by channels-ratio before expressing as microcuries per cell per microcurie per milliliter of dye.

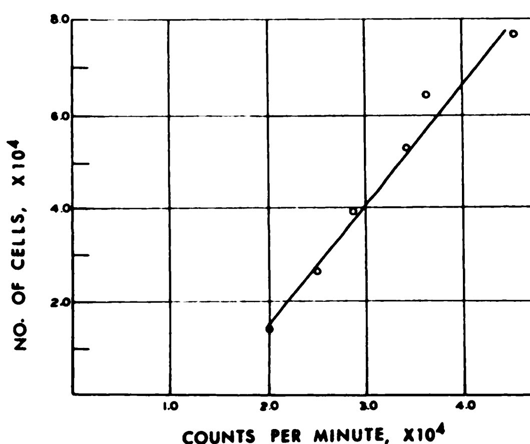

FIG. 2. Relationship between cell concentration and dye (^{3}H) uptake.

Since the day-to-day variations were significant, a blank was run each day and a pooled sample of normal suspended cells was stained with each group of unknowns. After subtracting the blank and dividing by the number of cells present, each unknown was expressed as a ratio (unitless) of itself to the daily normal. These ratios form the basis of our results and analysis.

Clinical. Although the development of this staining technique was intended to result in a prescreening procedure, it was felt that the initial clinical material used to evaluate the method should not be obtained from a general population survey but from a limited and purposely selected group of patients. In most mass screening studies the anticipated incidence of cervical malignancy might be four to six patients per 1000 of population encountered. It would therefore take at least a population of 10,000 to begin to get enough patients diagnosed by standard methods to determine the incidence of positives. Then, using known positives, the validity of a new method would usually be checked with regard to sensitivity by looking for false negatives produced by the procedure under investigation.

The initial processing presented inevitable time–expense problems that made it impractical to use in large-scale screening. If the process appeared effective, the next phase would normally be to streamline the procedure in terms of time and cost through automation so that large-scale application would be realistic.

With these thoughts in mind, it was decided to enlist the aid of a number of obstetricians, gynecologists, and pathologists geographically adjacent to the investigators' laboratories. Through the cooperation of these physicians the investigators sought to obtain 40–60 patients whose Papanicolaou smear had been interpreted as suspicious or positive. It was considered that this would be equivalent to the discovery of the same number of individuals by screening from 20,000 to 80,000 women, a population needed to help establish the false-negative rate and to evaluate dysplasia and carcinoma in situ.

Because the prescreening technique was intended to be followed by Papanicolaou smears with all high uptakes, it was felt that the backup provision re-

duced the relatively critical implications of frequent alerting of the pathologist because each such case would be subsequently evaluated without risk of mortality or morbidity, such as is associated with biopsy procedures.

The false-negative rate must be acceptable and in the range of that found by the standard Papanicolaou method. The value for false negatives varies from 3–30% depending upon the investigator (*4,5*). Evaluation of the incidence of false negatives in mass screening programs is a problem that can only be determined by prolonged and repeated followup of each patient.

In order to gather together the required positive or suspicious specimens, the physicians who agreed to assist in the project were asked to provide cell samples sent to the laboratory in suspension when patients with Classes III, IV, or V Papanicolaou smears were called back for repeat smears and/or biopsy. The results of the findings by this new uptake method could then be compared with the repeat Pap smears and the subsequent biopsy diagnoses. The physician was instructed to take his usual smear, roll the swab over the slide, and then to dip it in the preservative and stir it vigorously. Because it is impossible to ensure that the cell suspension in the preservative is identical to the slide, all suspensions were replated on slides and restained by routine methods for individual diagnosis whenever there appeared to be a discrepancy. Finally, a review of the original Pap smears and a followup of histologic slides was undertaken on all patients from other hospitals.

RESULTS

From various sources 270 vials of suspended cells were finally processed. Of these 174 were classified as "normal" and 96 specimens alerted the system. The rate of alertness is high, as it should be, because these positive or suspicious specimens were made available at our request, and the specimen, at our request, should be classified by the hospital pathologist as Classes I, IIR—suspicious, dysplasia, Classes III, IV, or V.

The basic breakdown of the 96 specimens is summarized in Table 1. Upon followup examination, these cases (96) were divided into five groups based upon histology and review of cytology including preparation of specimens from the vials actually being analyzed when the questions arose. The number of normal specimens classified by pathology was 205. The system was alerted by 31, which would result in an excess alerting rate of 15%. It is therefore anticipated that in a screening program consisting mainly of cases of these types, 85% of the "normal" ones could be eliminated without jeopardy to the patients.

TABLE 1. SUMMARY OF RESULTS OF UPTAKE WITH PYRONIN B

Final diagnosis	Cases No.	Ratio No.	≥1.50 %	Mean and s.d.
Invasive carcinoma	25	25	100	2.69 ± 1.27
Carcinoma in situ	4	2	50	2.14 ± 1.11
Severe dysplasia	17	7	41	1.67 ± 0.85
Mild-to-moderate dysplasia	19	6	31	1.51 ± 0.83
Normal or with atypical cells only	205	31	15	1.14 ± 0.42

The remaining 65 specimens were divided basically into four subgroups: invasive cancer, carcinoma in situ, severe cervical dysplasia, and mild-to-moderate cervical dysplasia. All of this mainly because we felt through followup, we would like it possible to confirm why this system had been alerted by these specimens, and the results are given in Table 1. The mean uptake ratio of each of these groups as well as the 205 "normal" cases is tabulated. It may be seen that of 25 invasive carcinomas, all 25 were detected with uptake ratios ranging from 1.54 to 7.56. The number of carcinomas in situ is not sufficient to make a broad prediction of the ultimate diagnostic potential in this condition. Two of the four cases had uptake ratios above 1.5 but one of the others was borderline at 1.45. As can be seen from the mean uptake values and their standard deviations, the groups are all significantly different from each other, with the exception of the carcinoma in situ group which is too small.

COMMENTS

In a recent review, Daly (*5*) emphasized that even with a cervical lesion that resulted in a positive biopsy, as much as 33% could turn out to be false negative with only atypical cells or no tumor cells primarily dependent upon how well the Pap smear was taken. It is obvious that any suspicious lesion deserves a biopsy whether the uptake ratio is high or low or even if the Pap smear is reported as negative. In a mass screening, such lesions should receive immediate attention. Problem cases are found in cervices that appear normal but that may be shedding cells from an endocervical lesion that is not visible. Such lesions may be picked up by the Pap smear after alerting the pathologist or by a high uptake ratio as used for this technique. Cases of dysplasia are showing increased uptake in a significant percentage of cases. If progression of the lesion

occurs, an elevated uptake should be found after two or three samplings within the critical period of time during which the dysplasia can be discovered and kept under surveillance until proper treatment can be prescribed. It is a common procedure in these dysplasia cases to manage the problem with repeated Pap smears every 2, 3, or 6 months. The economics of this method make such frequent retesting attractive. In fact, our whole philosophy may change toward more frequent testing, especially because self-administered testing is practical and simpler to carry out than a blood sugar test.

The separation of carcinoma in situ from severe dysplasia is sometimes controversial and the duration that may be involved in the change from carcinoma in situ to invasive carcinoma is an unknown and unpredictable factor. In this series there was some disagreement among pathologists concerning whether a particular patient had carcinoma in situ or severe dysplasia. For the sake of uniformity in tabulating the results, all of the diagnoses listed above were made by a single pathologist (R. Holmes). These diagnoses will remain in the province of pathologists.

Because of the economy and simplicity with which this uptake technique can be carried out, it may well be wise to reconsider the optimum frequency for doing Pap smears. Because of the relatively high alerting rate found by this technique, it is obvious that the skilled cytologist and pathologist using this technique as a prescreening approach would still have a significant number, approximately 15% of cases, requiring more detailed visual analysis. Eighty-five percent might be eliminated automatically. It might even be that a particular pathologist would prefer to set the uptake ratio somewhat lower than the 1.5 arbitrary level used in this study, thereby increasing the probability of detecting cases with dysplasia. Undoubtedly number of alerted cases would also be increased. A particular level could be adjusted to what the pathologist desired after having gained experience with the method. Set at the current level, any cytology laboratory should be able to turn out about six times as many reports with no increase in laboratory personnel. This would result in considerable savings.

In projecting how this method will be applied more generally, the three successive steps may be fully automated. The first step consists of a counting of the suspended cells and measuring cell size. Addition of the size information gives additional discrimination and increases the accuracy of the method in finding dysplasias. This step will be accomplished as a continuous flowing process like any blood-counting technique with a specimen processed in less than a minute. Second, with automatic staining and washing, using an automatic centrifuge currently under investigation, 20 specimens can be put through in less than 20 min, thereby achieving a flow rate of at least one specimen per minute. Third, with the high radioactive uptakes being encountered, it has been necessary to count only 1 min to obtain significant counts with statistically acceptable deviations.

Because each of the three steps allows an output of one specimen per minute, it is feasible to combine the three steps into a single machine so that a given specimen will move mechanically and automatically from beginning to end without human intervention, providing a final digital printout with a small calculator. At this rate over 500,000 specimens could be run a year by a single machine assuming that it had no downtime, but even with 20% downtime, 400,000 does not seem an unreasonable number of specimens for a single laboratory to process. Considering the estimated price of the apparatus involved, it appears that any laboratory doing more than 50,000 Pap smears annually would be justified economically in pursuing this prescreening approach and would benefit financially from using it.

When mass screening is stretched to try to increase the percentage of women reached, simplified techniques such as liquid samplings may provide accurate screening without expensive professional visualization and palpation. Therefore, a liquid prescreening test should strive for a low false-negative value. In this preliminary study the accuracy relative to invasive carcinoma is complete, therefore indicating further application of the method.

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