Diagnostic Reliability of the Acridine Orange Fluorescence Microscope Method for Cytodiagnosis of Cancer*

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SUMMARY

The cytodiagnostic acridine orange (AO) fluorescence microscope method was applied to exfoliated material from 4348 cases. The method utilizes increased amounts of cytoplasmic ribonucleic acid (RNA) in malignant cells to demonstrate them in brilliant orange and red fluorescence colors. Scanning smears under low magnification readily revealed the presence of cells with increased fluorescence. Usually with higher magnifications it was determined by their morphology whether such cells were cytologically negative, suspicious, or malignant. To test the diagnostic reliability of the fluorescence method, the diagnoses thus reached were compared with those obtained independently by conventional cytodiagnosis on different smears from the same exfoliated specimens. The diagnoses were in agreement with material from the female genital tract, gastric washings, and urine. Some differences in the diagnoses were encountered with exfoliated specimens from the respiratory system. Presumably these were induced by the nature of this material rather than by the method, considering the initially lower reliability of cytodiagnosis with respiratory material in general, and the excellent agreement between fluorescence and conventional diagnoses on most other material. Thus, it is felt that the AO fluorescence method in proper procedure yields as reliable results as do other cytodiagnostic techniques; this is in agreement with the findings of other workers who have tested the reliability of the fluorescence method for cytodiagnosis of cancer.

The acridine orange (AO) fluorescence microscope method for the cytodiagnosis of cancer, developed by L. von Bertalanffy (9–11), presents several advantages over conventional diagnostic methods of exfoliative cytology. Among the most important advantages is the rapid technical procedure of the AO method, enabling the cytopathologist to obtain diagnoses within less than 10 minutes after collection of the exfoliative specimen (3). Suspicious and malignant cells appear in striking, fluorescent colors in contrast to most normal cells with rather plain fluorescence. This permits the use of the fluorescence method as a prescreening procedure to eliminate the majority of normal specimens by physicians and technicians with relatively little knowledge in cytology (4). In the hands of the cytologist it greatly expedites screening, and a doubling in output of screened smears has been reported in some laboratories (14). Morphological details are shown with great clarity, allowing prompt evaluation of fluorescence-suspicious cells on a morphological basis. Moreover, the AO method demonstrates by conspicuous fluorescence bacteria, *Trichomonas vaginalis*, and fungi (e.g., *Candida albicans*). Because hemoglobin inhibits the fluorescence of erythrocytes, hemorrhagic smears are more readily evaluated by fluorescence microscopy than by other cytodiagnostic methods.

To be applicable generally, the diagnostic reliability of the fluorescence method has to closely approach or equal that of conventional cytodiagnostic procedures—for example, that of the well-established Papanicolaou technic. It must be considered, however, that the fluorescence method, which has been applied more generally only for a little over 2 years, is still greatly hampered by lack of standardization, being applied differently by various workers.

Several investigations have been published in...
which the diagnostic reliability of the fluorescence method was tested mainly by comparing fluorescence diagnoses with those obtained on the same specimens by conventional (chiefly Papanicolaou) diagnoses.

The fluorescence method has been applied both as a prescreening procedure, whereby morphological criteria are not considered, and as a method for final diagnosis of cancer. Umiker et al. (29) carried out an investigation using the fluorescence method for very rapid prescreening. Most investigations concerned with testing the reliability of the fluorescence method for final diagnosis considered not only increased cytoplasmic fluorescence but also morphological criteria of "fluorescence-suspicious" cells. Many investigations dealt primarily with exfoliated material from the female genital tract. The earliest were by L. von Bertalanffy and co-workers. One study comprised 598 female patients known to have a high incidence of malignancies of the female genital tract (9); another concerned an average population consisting of 1750 gynecological cases (6). The fluorescence method was first applied to gynecological practice by Sussman on 1050 cases (20). A large series of routine hospital material was investigated by Dart and Turner, comprising 4995 cases (14).

The fluorescence method was applied for final diagnosis to fewer cases which were not gynecological. Dart and Turner report their studies of 496 cases of "various fluids," of which only sputum was specifically mentioned (14). An informative investigation was published by Hunter et al., reporting a preliminary account of a great variety of cytological material, comprising more than 200 cases (16).

All the investigations in which the fluorescence method was used for final diagnosis based both on cytochemical and morphological criteria supplied evidence of a reliability of the method equal to that of conventional cytodiagnostic technics. Moreover, Dart and Turner reported several malignancies diagnosed by the fluorescence method which had been undetected by conventional procedures (14).

In the present investigation the reliability of the AO fluorescence method was further tested on all types of routine exfoliative material. It was thereby used to verify its applicability for the final diagnosis of the presence or absence of malignant cells. This investigation, so far comprising over 4000 cases, is still in progress. This is thus a preliminary progress report necessitated especially by the increasing application of the fluorescence method in hospitals, clinics, and in private practice.

**Cytochemical basis of the AO fluorescence method.**—The importance of ribonucleic acid (RNA) in protein synthesis of the cell is well known (12, 18). Cells engaged in active protein synthesis are usually rich in cytoplasmic RNA. For example, cells elaborating protein secretions (mucoproteins) contain abundant RNA, such as secretory endocervical cells and mucus-secreting cells of intestinal and respiratory epithelia. Similarly, active protein synthesis is requisite for the formation of mitosis of daughter cells. Consequently, cells which by division supply new cells for cell renewal (1, 17) are characterized by ample cytoplasmic RNA—e.g., the basal cells of many epithelia which give rise to new cells to replace those desquamated from the superficial epithelial cell layers.

Malignant cells proliferate at rates usually exceeding those for cell renewal of the normal cells of origin. Consequently, cancer cells usually contain RNA in amounts surpassing those of the normal cells.

However, apparently normal cells at times may also show enhanced rates of proliferation. This may occur occasionally in chronic irritations, in the presence of parasites, and in endocervical erosion. Then the cells are often present in greatly increased numbers and, as their more intense cytoplasmic fluorescence indicates, they also contain elevated RNA content. Such "active" cells originate especially from the respiratory (7) and endocervical epithelia (5) and macrophages in body fluids. Morphologically, such cells usually appear normal.

The metachromatic dye acridine orange, if applied in suitable procedure, demonstrates various amounts of RNA in cells (8). Cells devoid of or with only traces of RNA have unstained or faintly greenish fluorescent cytoplasm. Cells with low RNA content are brown, with moderate content reddish brown. If large amounts of RNA are present, the cells show bright orange or red cytoplasmic fluorescence.

When these principles are applied to exfoliated cells, superficial squamous cells, for example, reveal light green, and intermediate cells brown, cytoplasm. Basal cells and secretory endocervical cells, both dividing at moderate rates for cell renewal, normally present reddish brown cytoplasmic fluorescence. Malignant cells, because of their large RNA content, usually display bright orange to flaming red cytoplasm. Also "active" normal cells often fluoresce red, presumably because of increased RNA content.

Nuclear DNA is shown by acridine orange in green or greenish yellow fluorescence. Hyperchromatic nuclei, brought about by increased DNA
content, are readily recognized by the fluorescence method. The RNA-containing nucleoli, if sufficiently large, appear red or reddish brown.

MATERIALS AND METHODS

The exfoliative material investigated to test the diagnostic reliability of the fluorescence method was divided into two categories:

a) 2303 cases of exfoliated material from the female genital tract (cervical and vaginal), collected in gynecological office practice, with rather typical incidence of malignancies (Table 1).

b) Routine exfoliative material of all kinds (excluding female genital tract material) from a general hospital, with a relatively high incidence of malignancies (Table 2). This comprised a total of 2045 cases.

Both types of sample were processed and screened in a similar manner. With the use of phosphate buffer at pH 6 and Gurr's acridine orange, the smears were prepared and screened with fluorescence microscopes equipped with high-pressure 200-watt mercury burners. The procedure of the AO fluorescence technic has been published elsewhere (2-4).

Orange and red cytoplasmic fluorescence of cells, readily recognized under scanning power (×100), served as "warning signal." These "fluorescence-suspicious" cells were subsequently scrutinized with higher magnifications (from ×200 to ×600) for atypical morphological features of nucleus, cytoplasm, and the cell as a whole. In this way, "fluorescence-suspicious" cells proved to be either cytologically negative ("active") cells, cytologically suspicious with atypical morphology, or outright malignant. By utilizing increased cytoplasmic fluorescence as a warning signal, most normal smears could be eliminated fairly rapidly by scanning with low magnification.

The diagnoses obtained by the fluorescence method were unbiased, because the Papanicolaou and H. & E. diagnoses, to which the former was compared, were performed by hospital and clinic cytologists and cytopathologists on different smears but on smears from the same exfoliated samples. By this procedure occasional smears diagnosed by the fluorescence method were devoid of tumor cells, though malignant cells were present in some of the smears from the same sample diagnosed by conventional methods. The occasional occurrence of unequal smears from similar samples is well known. However, it was felt that uninfluenced diagnoses were more important than obtaining entirely comparable diagnoses, which could have been achieved by processing with conventional methods the same smears which had first been diagnosed by fluorescence microscopy.

TABLE 2

<table>
<thead>
<tr>
<th>Material</th>
<th>Method</th>
<th>Negative</th>
<th>Suspicious</th>
<th>Positive</th>
<th>Total no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum</td>
<td>AO</td>
<td>673</td>
<td>27</td>
<td>64</td>
<td>764</td>
</tr>
<tr>
<td></td>
<td>Pap.</td>
<td>680</td>
<td>15</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Bronchial</td>
<td>AO</td>
<td>360</td>
<td>21</td>
<td>44</td>
<td>425</td>
</tr>
<tr>
<td>secretions</td>
<td>Pap.</td>
<td>359</td>
<td>19</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>Pleural fluid</td>
<td>AO</td>
<td>271</td>
<td>13</td>
<td>69</td>
<td>353</td>
</tr>
<tr>
<td></td>
<td>Pap.</td>
<td>275</td>
<td>10</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>Ascitic fluid</td>
<td>AO</td>
<td>75</td>
<td>12</td>
<td>44</td>
<td>129</td>
</tr>
<tr>
<td></td>
<td>Pap.</td>
<td>72</td>
<td>10</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>Gastric washings</td>
<td>AO</td>
<td>116</td>
<td>2</td>
<td>17</td>
<td>135</td>
</tr>
<tr>
<td></td>
<td>Pap.</td>
<td>117</td>
<td>1</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td>AO</td>
<td>51</td>
<td>2</td>
<td>23</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>Pap.</td>
<td>52</td>
<td>1</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Miscell.</td>
<td>AO</td>
<td>139</td>
<td>6</td>
<td>18</td>
<td>163</td>
</tr>
<tr>
<td></td>
<td>Pap.</td>
<td>140</td>
<td>1</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Total:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2045</td>
</tr>
</tbody>
</table>

RESULTS

Cytodiagnoses on 2303 similar gynecological cases by the AO fluorescence and Papanicolaou's methods are shown in Table 1. These were classified into Types I to V, whereby Types I and II were cytologically negative, Type III suspicious, and Types IV and V malignant. All cervical and vaginal specimens were diagnosed independently by the AO fluorescence and Papanicolaou's methods on different smears from the same exfoliated samples.

In the material from the female genital tract, the diagnoses with the fluorescence and Papanicolaou's methods agreed in the Types IV and V category. By the fluorescence method, eight more Type III cases were diagnosed. In this series of
gynecological cases no false AO negative or positive diagnoses occurred. Positive diagnoses were confirmed by biopsies.

The results obtained on 2045 routine hospital cases, excluding gynecological, are tabulated in Table 2. The diagnoses were classified into three categories, cytologically negative, suspicious, and positive (malignant). Conventional diagnoses were achieved partly by Papanicolaou's method, partly by hematoxylin and eosin staining. For simplicity, the latter is not mentioned in the table.

The first column indicates the number of cases diagnosed as cytologically negative by the fluorescence and conventional methods. The second column shows the number of suspicious, the third of positive, cases diagnosed by both methods. In the fourth column are the total numbers of cases of each type of material.

It is a general finding that somewhat more suspicious diagnoses are obtained with the fluorescence than with conventional methods. Thus, cases diagnosed by conventional methods as negative or Type II are sometimes classified as suspicious or Type III according to the fluorescence method; this was especially the case with material from the female genital tract and sputum. This may have several reasons. It seems that increased cytoplasmic fluorescence accentuates the atypia of cells. Because changes in RNA content seem to precede morphological nuclear changes (18, 19), the fluorescence method may occasionally reveal earlier malignant or premalignant changes. Systematic follow-up studies could support the latter possibility.

Two factors account for discrepancies in the numbers of positive diagnoses obtained by the two cytodiagnostic methods. The first was that some cases diagnosed as malignant (positive) by conventional methods were considered as suspicious according to the fluorescence method, and vice versa. This, of course, is not a reflection on the diagnostic method but rather an expression of somewhat different diagnostic standards employed by different investigators. Such discrepancies also occur if similar material is diagnosed by different cytologists even when using the same cytodiagnostic method (15). More important is the occurrence of false AO negative and positive diagnoses. False fluorescence negatives were those which were AO negative-Pap. positive. Among the 4348 cases investigated, a total of nine supposedly false AO negatives were encountered. However, re-examination of the smears revealed that three of these were brought about by unequal smears from the same specimens—i.e., that the smears screened by fluorescence microscopy did not contain tumor cells. Thus actually missed by the fluorescence method were six cases, consisting of one false AO negative sputum, two bronchial secretions, one pleural fluid, and, in the miscellaneous category, one esophageal washing and one pancreatic fluid.

A total of seventeen supposedly false AO positives occurred. Sputum accounted for seven; four of these were from patients whose previous or subsequent samples were diagnosed as positive also with conventional methods, and thus three actual false AO positive sputum diagnoses remained. Of the three false AO positive bronchial secretions one was from a patient with a Papanicolaou-positive diagnosis on other specimens, two seemed actual false AO positives. There were four false AO positive pleural fluids, two from patients with pulmonary tuberculosis showing large atypical, brightly fluorescent cells, one from a case with positive sputum and bronchial secretions, and one with presumably only “active” cells. Apparently also one false AO positive ascitic fluid was because of atypical “active” cells. In the miscellaneous category, one false AO positive esophageal washing and one hydrocele aspirate, containing very atypical, bright red fluorescent cells, were AO positive—Pap. negative. Thus, of the seventeen supposedly false AO positives, only ten were from patients who did not show previous or subsequent positive diagnoses by conventional methods.

With the exception of two cases of sputum containing greenish yellow fluorescent, differentiated squamous malignant cells (21), all fluorescence-suspicous and -positive smears contained cells with increased cytoplasmic fluorescence. This was very pronounced in most cases with malignant cells, ranging from bright orange to carmine to flaming red. Malignant cells were thus readily recognized, mostly with low scanning powers. Occasionally, samples were encountered with “active” normal cells, sometimes with rather intense cytoplasmic fluorescence. The latter were distinguished from malignant cells by their otherwise normal morphological appearance.

DISCUSSION

In total, 4348 cases were diagnosed cytologically by the fluorescence method for the presence or absence of malignant cells and the diagnoses compared with those obtained independently on smears from identical specimens by conventional cytodiagnostic techniques.

Acridine orange fluorescence and conventional diagnoses agreed especially well with material from the female genital tract, gastric washings, and urine. There were smaller differences in the
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