

## THE DIAGNOSIS OF NEOPLASTIC CELLS IN SPUTUM BY TWO NEW METHODS

BY

JOHN PERRIN AND GEORGE T. LITTLEJOHN

*Clinical Laboratories, London Hospital*

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The following stains have been used in other laboratories for the demonstration of neoplastic cells in sputum: (1) haematoxylin and eosin (numerous workers including Dudgeon and Wrigley, 1935); (2) haematoxylin, orange G, light green, eosin, bismarck brown (Papanicolaou, 1942); (3) methylene blue (Schuster, 1947).

The use of the first two methods has the disadvantage that the procedure is time-consuming, since the preparation has to be fixed before it is stained, and the amount of sputum which can be examined on one slide is extremely small. The last method is rapid, gives results that compare favourably with Papanicolaou's method in proved cases of carcinoma, enables a larger quantity of sputum to be examined on one slide, but requires considerable experience to differentiate neoplastic from normal cells. It was thought that a counter-stain for cytoplasm might make diagnosis simpler, especially in the squamous cell types of carcinoma, which form at least 50% of the various morphological types encountered in sputum. Many counter-stains were tried, but most of them precipitated when mixed with the methylene blue, or failed to stain cytoplasm in vital preparations. Finally basic carbol fuchsin was found satisfactory in the proportions given below. Carbol fuchsin stains normal pharyngeal squamous epithelium brilliant red in bacteriological preparations, and it seemed likely that it would stain thickly keratinized carcinoma cells even more intensely.

### Method

The following solutions are required:

A. Methylene blue	0.5 g.	B. Basic fuchsin	1.0 g.
Glycerin	20 ml.	Absolute alcohol	10 ml.
Distilled water	80 ml.	5% carbolic acid	90 ml.

One part of B is mixed with four parts of A. These proportions may be varied slightly for different batches of stain.

The method of preparation of films of sputum is similar to that described by Schuster (1947). An early morning specimen of sputum is emptied into a petri dish. Purulent or blood-stained fragments are picked out with forceps against a black background, and placed on a slide with a drop of the stain. The two are gently warmed

on a bunsen flame, and mixed with the blade of the forceps on the slide for at least half a minute. The uniformly stained, sticky specimen is covered with a large cover slip and spread evenly by gentle pressure. Staining improves with time, but the specimen can be examined almost immediately. (In all the vital staining methods we have tried, the staining of the preparation if left for up to six hours may become so intense that earlier nuclear detail is obscured, although diagnosis need not necessarily be more difficult.) The field is searched with a 2/3 objective, and suspected areas are examined more closely with a good sixth or seventh objective.

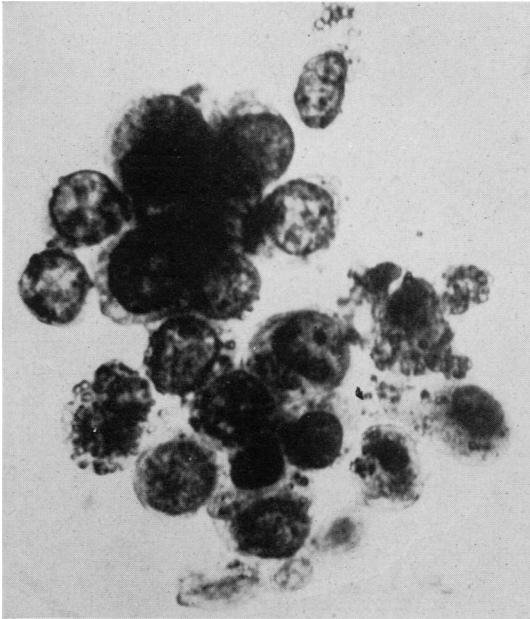


FIG. 1.

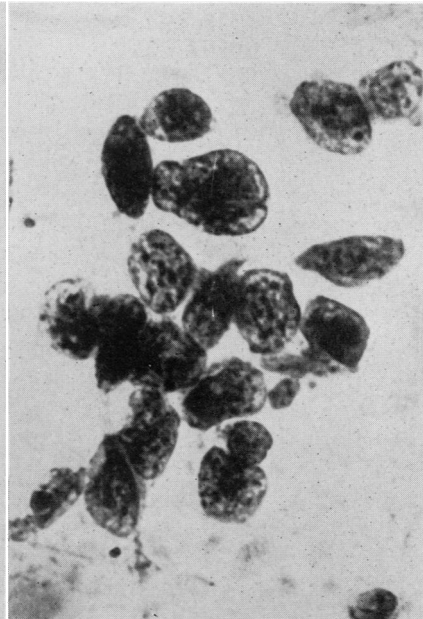


FIG. 2.

FIG. 1.—Spheroidal cell carcinoma. ( $\times 800$ . Iodine green and neutral red.)

FIG. 2.—Partly oat, partly spheroidal cell carcinoma. ( $\times 800$ . Iodine green and neutral red.)

The carbol fuchsin methylene blue stain has proved the stain of choice in the diagnosis of the group of squamous cell carcinomata, but, because the nuclear detail in oat and spheroidal cell types of carcinoma was not as good as in fixed films stained by more usual methods, a search was made for a suitable alternative stain to supplement the methylene blue fuchsin. A 2% solution of watery iodine green\* was found eminently satisfactory, and proved a great advance on either methylene blue alone or combined with carbol fuchsin. The technique of preparation of the specimen is similar to that already described. A drop of iodine green is mixed with the sputum on the warmed slide. If one part of 2% iodine green is added to 20 parts of 1% watery neutral red, the nuclear detail is further enhanced, the chromatin appearing as a crisp, black lace-work pattern, the cytoplasm remaining green (Figs. 1 and 2). This mixed stain has one disadvantage in that it will precipitate in the preparation in two to three hours. This is of little practical importance if large numbers of specimens are being rapidly examined.

\* Manufactured by G. T. Gurr.

It is worth noting that anaplastic spheroidal and oat-cell carcinoma may often be found in portions of sputum which are neither purulent nor blood-stained. An apparently unsatisfactory specimen of sputum, consisting largely of mucus and debris, may in fact contain a few cells of these types of carcinoma. In contrast squamous carcinoma is more often detected in the purulent flecks.

Using methylene blue fuchsin stain the main characteristics of the non-neoplastic cells most commonly seen in sputum are as follows.

Squamous epithelium from the superficial layers of the pharynx appears as flat, rectangular, pentagonal or hexagonal forms, with small central nuclei. Cells from the deeper layers are smaller, hexagonal or circular, frequently attached to one another in small sheets or plaques, the nucleus occupying a relatively greater proportion of the cell area.

Normal respiratory epithelium is columnar, tailed on the basement membrane surface, and in well preserved specimens has a distinct brush border of cilia. The nucleus is basal (Fig. 3).

The cytoplasm of both the squamous and the normal respiratory epithelium stains a uniform bright pink or red; the nucleus is violet or purple.

Polymorphonuclear leucocytes, as in the simple methylene blue method, take up the stains capriciously unless the preparation is well mixed and warmed. Cytoplasm is a faint pink or green, the lobed nuclei a dark purple. Macrophages can be a potent source of error in the diagnosis of carcinoma. They are invariably globular, usually contain dark pigment granules which obscure the nucleus, and tend to occur in groups; the more doubtful cells can often be identified by reference to more obvious cells in the same group. Occasionally red cells or fat particles are phagocytosed. Variation in size may be extreme. The cytoplasm stains a purplish-red and the nuclei a dark purple. Suspected neoplastic cells in the neighbourhood of macrophages should be interpreted with reserve.

Lymphocytes have a translucent pink or greenish cytoplasm and purple nuclei. Plasma cells have a more opaque purplish or violet cytoplasm, the oval-shaped and eccentric nucleus, typical of this cell, staining purple. Bacteria are purple or blue, and are frequently seen as a tracery in the superficial squamous epithelium. Curschmann's spirals stain an intense blue.

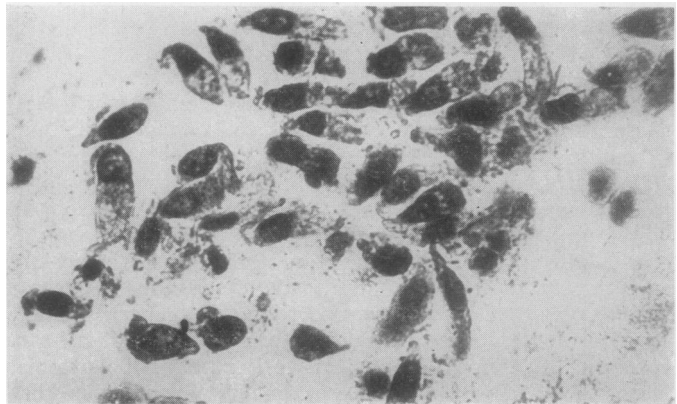


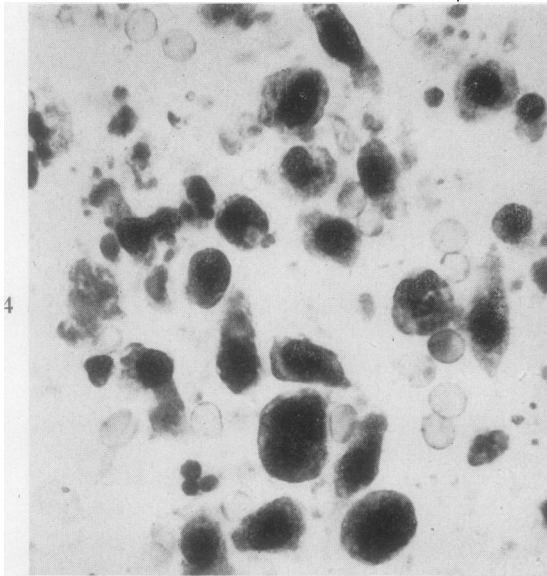
FIG. 3.—Normal respiratory epithelium. All cells similar with basal nuclei and tails, free brush border and vacuolated, foamy cytoplasm. ( $\times 500$ . Methylene blue and carbol fuchsin.)

### Classification of Neoplastic Cells in Sputum

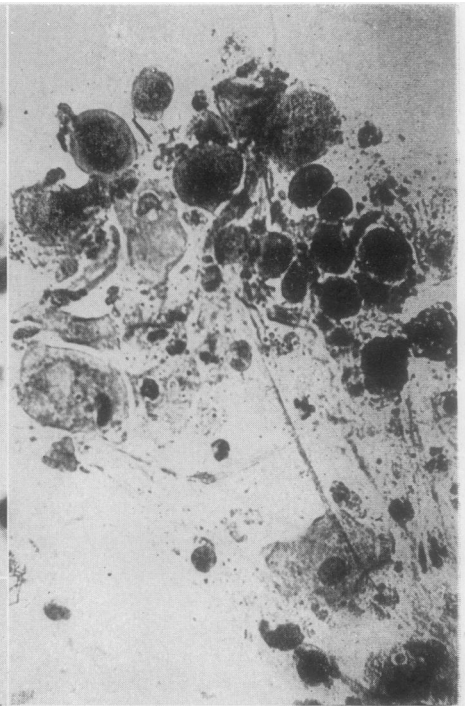
Neoplastic cells in sputum can usually be classified into two main groups according to their morphology and staining reactions. The two groups are squamous-cell carcinoma and anaplastic spheroidal-cell or oat-cell carcinoma.

**Squamous Cell Carcinoma.**—The cells of squamous carcinoma vary enormously in size and shape (Figs. 4, 5, 6, and 7). The outline is usually irregularly oval, circular, angular or jagged with irregular processes. Sometimes signet-ring forms are seen with a large central vacuole (Fig. 7). The edge may have a doubly refractile contour. Most squamous cells contain characteristically refractile granules, sometimes forming a circle round the nucleus or dispersed throughout the cell. The keratin may be whorled, similar superficially to the Hassall's corpuscles of the thymus. The cells do not "group" so well as the anaplastic spheroidal and oat-cell types, but are scattered singly, in pairs and triads, throughout the inflammatory exudate. Occasionally larger clusters are found. The cytoplasm stains intensely, and usually irregularly, in brilliant shades of deep red in the well keratinized cells, less often violet or blue-green in the more anaplastic cells derived from the basal proliferating layers. Nuclei are purple, hyperchromatic, bizarre in shape, and frequently pyknotic. Some cells have a central space, once occupied by a nucleus. Even when embedded in leucocytes, the dense red cytoplasm and the dark purple nuclei show prominently and are far easier to identify than in the simple methylene blue preparation where the cytoplasm is a pale glistening green.

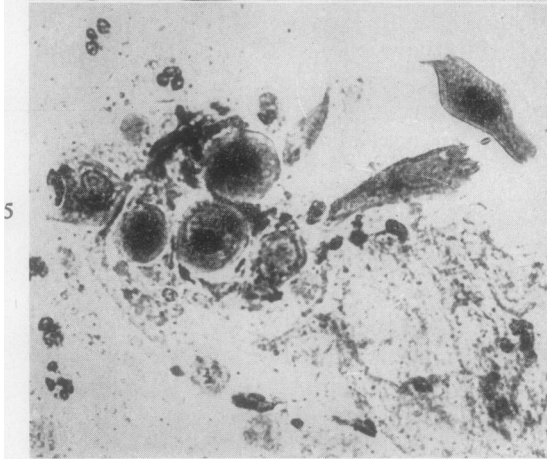
**Anaplastic Spheroidal and Oat-cell Carcinoma.**—In these types of carcinoma the cells are almost invariably in clumps and streaks (Figs. 8, 9, 10, and 11). In clumps they resemble clusters of grapes or frog's spawn. Individual cells may be spheroidal, oval, oat-shaped, or roughly polygonal. Individual size and shape may vary enormously in cells of a single group. This pleomorphism in one tumour is extremely common. With increasing experience it is becoming possible to grade the type of tumour more exactly in terms of predominant cell shape, rather than in the earlier broad distinction into squamous and oat-cell carcinoma that was originally employed. Where such small parts of large tumour masses are examined it is impossible to give such histologically exact descriptions as the morbid anatomist examining larger and more representative portions of tissue. Clusters of cells may be flattened by gentle pressure on the coverslip, a method which is helpful in ascertaining individual cell structure. The cells of these more anaplastic carcinomas take up the stain relatively slowly compared with normal tissues. Staining may not be complete in the groups of cells until after a period of 20 minutes to half an hour. Examined shortly after a preparation has been made, some cells will appear as refractile, glistening green disks, interspersed with others which are already stained a deep purple. All cells eventually are stained an equally dense purple, an intensity of staining unequalled by any other elements in the field. These dark groups of cells are easily identified under a 2/3 objective (Fig. 8). The purple staining is taken up by the nucleus which occupies most of the cell, the cytoplasm remaining unstained as a thin glistening rim of a pale green tint. It is of diagnostic importance that the cytoplasm practically never stains pink and never red as in the squamous cell



4



6



5



7

FIG. 4.—Squamous carcinoma cells. Mostly circular forms. Large hyperchromatic nuclei and densely staining cytoplasm (dark red in actual stain). A few red cells interspersed with the carcinoma cells. (×690. Methylene blue and carbol fuchsin.)

FIG. 5.—Squamous carcinoma cells. Circular forms to the left; two angular, elongated forms to the right, one with a "spinous" process, the other without a nucleus. (×490. Methylene blue and carbol fuchsin.)

FIG. 6.—Squamous carcinoma cells. Bottom right is a normal lightly stained epithelial cell from the pharynx. Nucleus is pale. Carcinoma cells on the top right. Most have densely stained cytoplasm and all have hyperchromatic nuclei. Above and to the left of the main group are more lightly stained and better differentiated carcinoma cells, the cytoplasm irregularly vacuolated and the nuclei pyknotic. (×370. Methylene blue and carbol fuchsin.)

FIG. 7.—Squamous carcinoma cells. Giant, bizarre forms, two of them resembling signet rings. (×550. Methylene blue and carbol fuchsin.)

types. This has proved a most useful extra aid in the differentiation of types of growth in doubtful cases. Nuclear detail is not good, although nucleoli and chromatin networks can be distinguished in individual cells, especially before the full intensity of the stain is acquired.

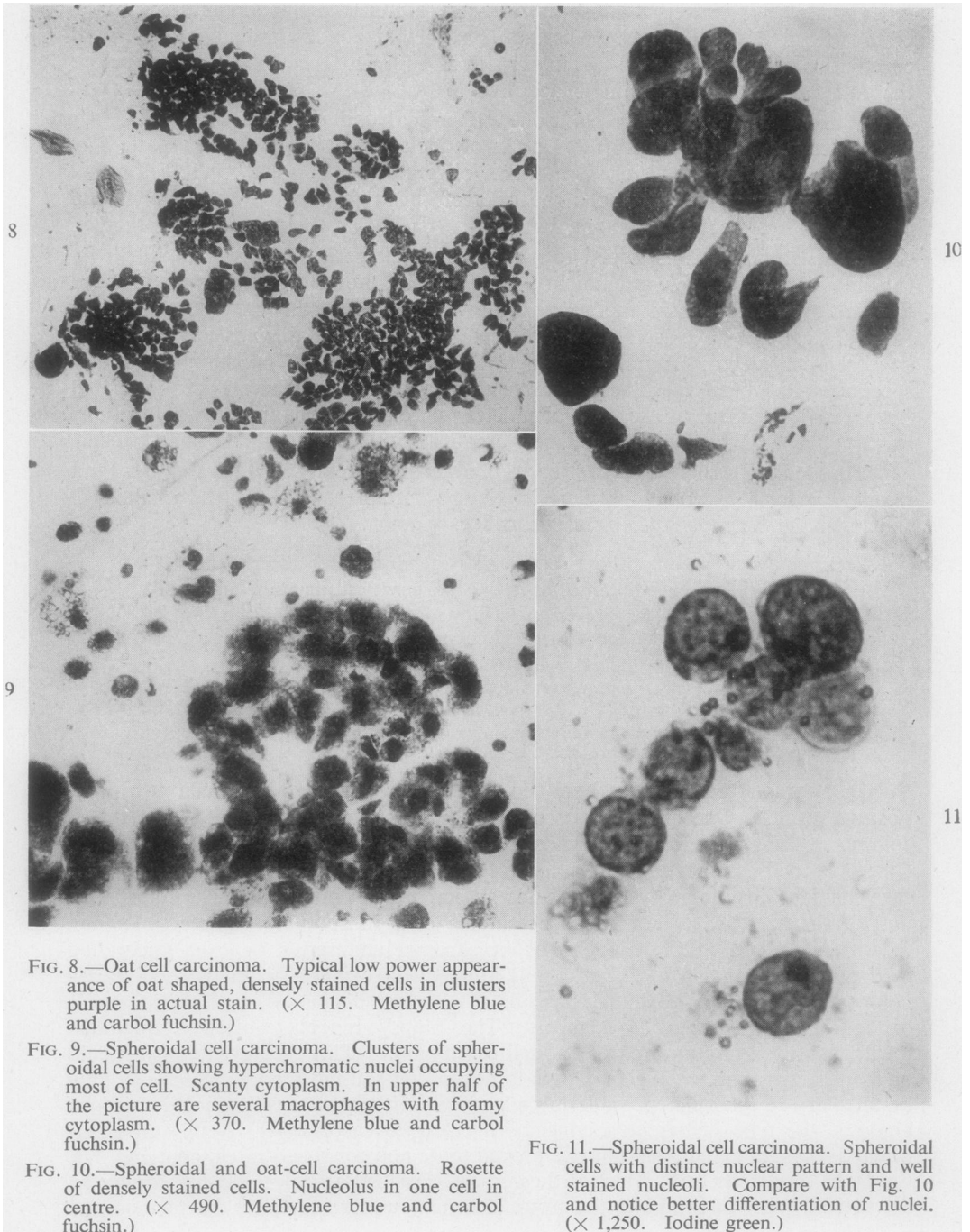
Using the iodine green stain alone, spheroidal and oat cells stain green, the nuclei being particularly distinct. Details of nucleolar structure and the pattern of chromatin filaments are visible as a darker olive green in contrast to the cytoplasm (Fig. 11). As in other methods the cells of this type are more darkly stained than normal elements of sputum, but not so darkly as to obscure the structure.

At a later date it is hoped to publish a larger series of cases examined by the above methods. A preliminary analysis has, however, already established the value of the fuchsin methylene blue stain. Using this method specimens from 174 patients have been examined where the differential diagnosis of carcinoma has been considered. A minimum of three specimens and a maximum of twelve specimens has been examined in each case, and at least three slides prepared from each specimen. Cases are completely unselected and are drawn from the wards of a large general hospital. A broad analysis of the results is appended.

The iodine green stain has been used more recently on fewer cases and requires a further probationary period, but we feel that this is the method of choice in the diagnosis of anaplastic cell carcinoma.

Total number of patients whose sputum was examined for malignant cells	174
Total number of patients diagnosed as having malignant cells in their sputum	101
Number of patients so far proved to have malignant disease of lungs by	
(a) post-mortem examination ; (b) bronchoscopic biopsy ; (c) drill biopsy ;	
(d) histological examination of lung removed at operation .....	55 (100%)
Number of patients where sputum examination was positive and confirmed by the above methods .....	33 (60%)
Number of patients where sputum examination was negative, but other laboratory examinations were positive .....	22 (40%)

In addition to the cases analysed above there have so far been two false positive results. One case diagnosed as oat-cell carcinoma proved to be a tuberculoma of lung ; the other, diagnosed in only one specimen as "suspicious of spheroidal cell carcinoma," was a case of disseminated lupus erythematosus. One case included in our analysis illustrates an important limitation in the interpretation of the presence of squamous carcinoma cells in sputum. Where squamous metaplasia occurs in the bronchi, as in chronic lung sepsis or over underlying malignant change, the metaplastic cells are indistinguishable from those of carcinoma. In this case squamous carcinoma was first diagnosed, and then oat-cell carcinoma on successive specimens. The biopsy report revealed "squamous metaplasia overlying an area of oat-cell carcinoma." This rare eventuality should be emphasized to any clinician who is to depend on sputum reports, which can only be ancillary to the other possible investigations. In two cases also included in our analysis, the type of carcinoma has been classified incorrectly. Both were diagnosed as oat-cell carcinoma, but one proved to be polygonal-cell carcinoma (Fig. 12), the other squamous cell. Most of those cases classified as polygonal-cell growth by the morbid anatomist fall into the group of squamous carcinoma by our method, the





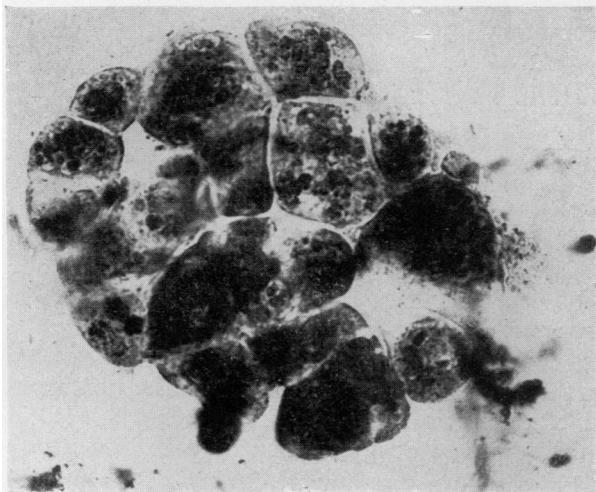


FIG. 12.—Polygonal cell carcinoma. ( $\times 385$ . Methylene blue and carbol fuchsin.)

cell exhibiting the characteristics already described for this type. Obviously the more anaplastic the squamous carcinoma, the more difficult is the differentiation of the type of growth from an examination of single cells or small groups of cells. Other workers using the methylene blue technique have pointed out the risk of error in specimens examined shortly after bronchoscopy. For the first week after bronchoscopy damaged cells sloughed from the respiratory tract could cause confusion in diagnosis. In

occasional cases examined within this period we have experienced no particular difficulty.

We believe the figure of 60% for cases diagnosed as having malignant disease of the lung by these methods will be difficult to improve upon. Mechanical factors, such as collapse of a lobe, obstruction of a bronchus proximal to the primary growth by infiltration, or, in early stages, the failure of a growth to ulcerate, all prevent the appearance of neoplastic cells in the sputum. Similarly in the copious purulent sputum of a lung abscess secondary to a carcinoma the malignant cells are rarely detected, presumably due to dilution with the purulent exudate. For these reasons we view with some suspicion reports of a higher percentage of positive findings, unless the material were selected.

No method has so far been evolved for making permanent preparations of the already stained films, an obvious drawback in reassessing a particular case where histological technique reveals a carcinoma which has been missed in the sputum.

#### Summary

A method of examination of sputum for malignant cells is described, but it is emphasized that it is complementary to the other methods of investigation, bronchoscopic biopsy and drill biopsy, and in no way replaces them. The combination of all three methods is the ideal for a clinical diagnosis in the majority of cases.

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