THE TRIALS IN THE CYTOLOGICAL DIAGNOSIS OF GASTRIC CANCER

PART Ⅲ HISTOCHEMICAL REACTION ON THE CYTODIAGNOSIS OF GASTRIC CANCER

by

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INTRODUCTION

In spite of great development of the cytology which depends not only upon the advancement of collecting-techniques of cytologic specimens (PANICO[1], ROSENTHAL[2], KLAYMAN[3] and SCHADE[4,5]) but also greatly upon the detailed study on cytological monographs (PAPANICOLAOU[6,7], GRAHAM[8] and TAKEDA[9]), definite cytological diagnosis is still difficult or even impossible.

However, considering the fact that cytological diagnosis has been contributing greatly to the establishment of early diagnosis of cancer, a more reliable and practical scale for the identification of the malignancy of the suspicious tumor cell is to be highly expected.

Although a large number of reports have been published as to histochemical stain studies of neoplastic human tissues, the application of these on exfoliative cytology has been limited only to several reactions to the cervix uteri (GROSS[10,11]) and to PAS reaction to the stomach.

The present study was undertaken to investigate a practical histochemical technique that contributes to the cytodiagnosis of gastric cancer.

MATERIALS AND METHODS

One hundred ninety-one patients with clinically evident gastric disease were examined using cytologic techniques over one year period. The following histochemical reactions were performed upon gastric cytological specimens and also upon gastrectomy materials as control.

Materials:

1) *Gastric cytological specimens*:

Smears were prepared with the specimens obtained by abrasive balloon techniques (YOSHIDA[12]).

2) *Gastrectomy materials*:

The resected stomach was collected immediately after gastrectomy and was opened routinely to examine the mucosa. Then smears were obtained by scrapping gastric lesion surfaces previously swabbed up lightly with clean cotton to remove mucous and blood, and tissue sections were prepared.

These smears and tissue sections were immediately handled by the following procedures.

Histochemical reactions :

1) *Methyl green-pyronin method* :

Using the materials fixed in CARNOY's solution, RNA and DNA were demonstrated by means of BRACHET's[13,14] methyl green-pyronin stain.

2) *PAS reaction* :

The periodic acid-Schiff reactions according to McMANUS[15,16] were applied to the materials fixed in a neutral 10 per cent formalin solution.

3) *Alkaline phosphatase* :

Alkaline phosphatase reaction was demonstrated by means of the improved GOMORI's [17] formula with an incubation time of 2 hours at about pH 9.3 and 37°C on the materials fixed in cold acetone. The incubation mixture consisted of 10 cc of 3 per cent glycerophosphate as substrate, 25 cc of 2 per cent calcium chloride, 10 gtt of 10 per cent magnesium chloride, 1 gm of sodium barbiturate and 15 cc of distilled water. Prior to incubation, the fixed tissue sections were washed with xylol, embedded in paraffin at 52°C, and cut in 6 μ thick. Light counterstain was done with MAYER's hematoxylin.

4) *Acid phosphatase* :

Reactions for acid phosphatase were carried out according to the improved GOMORI's [17,18] formula with an incubation time of 14 hours at pH 4.6 and 37°C on the materials fixed in cold acetone. The incubation mixture consisted of 40 cc of 3 per cent glycerophosphate as substrate, 20 cc of 2 per cent lead nitrate and 120 cc of distilled water. PH of incubation solution was adjusted to 4.6 with 20 cc of 0.2 M acetate buffer solution. The precipitated lead phosphate was treated with yellow ammonium sulfide to form brown lead sulfide. Prior to incubation, the fixed tissue sections were washed with xylol, embedded in paraffin at 52°C, and cut in 6 μ thick. Light counterstain was done with MAYER's hematoxylin.

5) *DPN-diaphorase* :

DPN-diaphorase was demonstrated according to FARBER's[19] histochemical method with an incubation time of 4 hours at 37°C on the materials fixed in cold acetone (−5°～0°C) for 10 minutes. The incubating mixture consisted of 1 cc of 0.1 per cent neotetrazolium chloride, 1 cc of 0.1 M sodium lactate as substrate, 1 cc of 0.4 per cent DPN and 0.5 cc of 0.1 M KCN. Incubation solution was adjusted to pH 8.5 with 1 cc of 0.1 M AMPD-HCl buffer solution. Prior to the fixation in cold acetone, tissue sections were cut in a cryostat in 10～15 μ thick at −10°C to −12°C and attached to a slide by melting slightly.

RESULTS

The following results were concerned with those obtained by gastric cytological specimens except for item 5), where the results obtained by gastrectomy materials were mentioned as well.

1) *Methyl green-pyronin method* :

Normal surface epithelium of the stomach showed moderate cytoplasmic reaction with pyronin pigment and light nuclear reaction with methyl green pigment. Goblet cells and cells of intestinal metaplasia showed moderate to marked cytoplasmic reaction and light

nuclear reaction. As to inflammatory cells including polymorphonuclear leucocytes and lymphocytes, nuclear reaction was marked and cytoplasmic reaction was very light. Nuclear reaction of plasma cells and histiocytes was as strong as that of leucocytes and lymphocytes and their cytoplasmic reaction was also remarkable.

In cancer cells, nuclear reaction was, on the whole, marked regardless of the pathohistological type. Among various types of cancer cells, the most marked cytoplasmic reaction was observed in the cellular rand of colloid carcinoma cells followed by moderate reaction in pleomorphic adenocarcinoma cells. The others showed only light cytoplasmic reaction.

Using this stain, neither fine structure of the nucleus could be observed nor the grade of reaction was not always parallel to the malignancy of cell. In addition, considerable shrinkage of the smear membrane was very often noted Because of this shrinkage which seemed to result from the effect of the fixative, Carnoy's solution, the accurate identification of exfoliated cells was difficult.

Even if it is granted that this stain demonstrates RNA and DNA content of the cell accurately, it would not serve to provide a good cytological diagnosis because of the poor presentation of cellular architecture in contrast to that of hematoxylin and eosin stain. By this method the staining of gastric cytological specimen was almost as good as that of surgical materials.

2) *PAS reaction*:

PAS positive substances in normal surface epithelium of the stomach were coarse granules arranged in supranuclear areas of the cell. In goblet cells, PAS positive material was of fine granular and diffusely distributed throughout the cytoplasm PAS reaction was most marked in intestinal metaplasia cells. Inflammatory cells showed slight PAS positive substance diffusely in the cytoplasm.

PAS reaction in cancer cells was, on the whole, very slight showing faint staining in the cytoplasm, except for signet-ring cells from colloid carcinoma with a marked positive reaction similar to those from the areas of intestinal metaplasia. Therefore, the differentiation among these cells was very difficult.

Normal squamous epithelial cells from the oroesophageal tract showed faint PAS positive coarse granules in the cytoplasm.

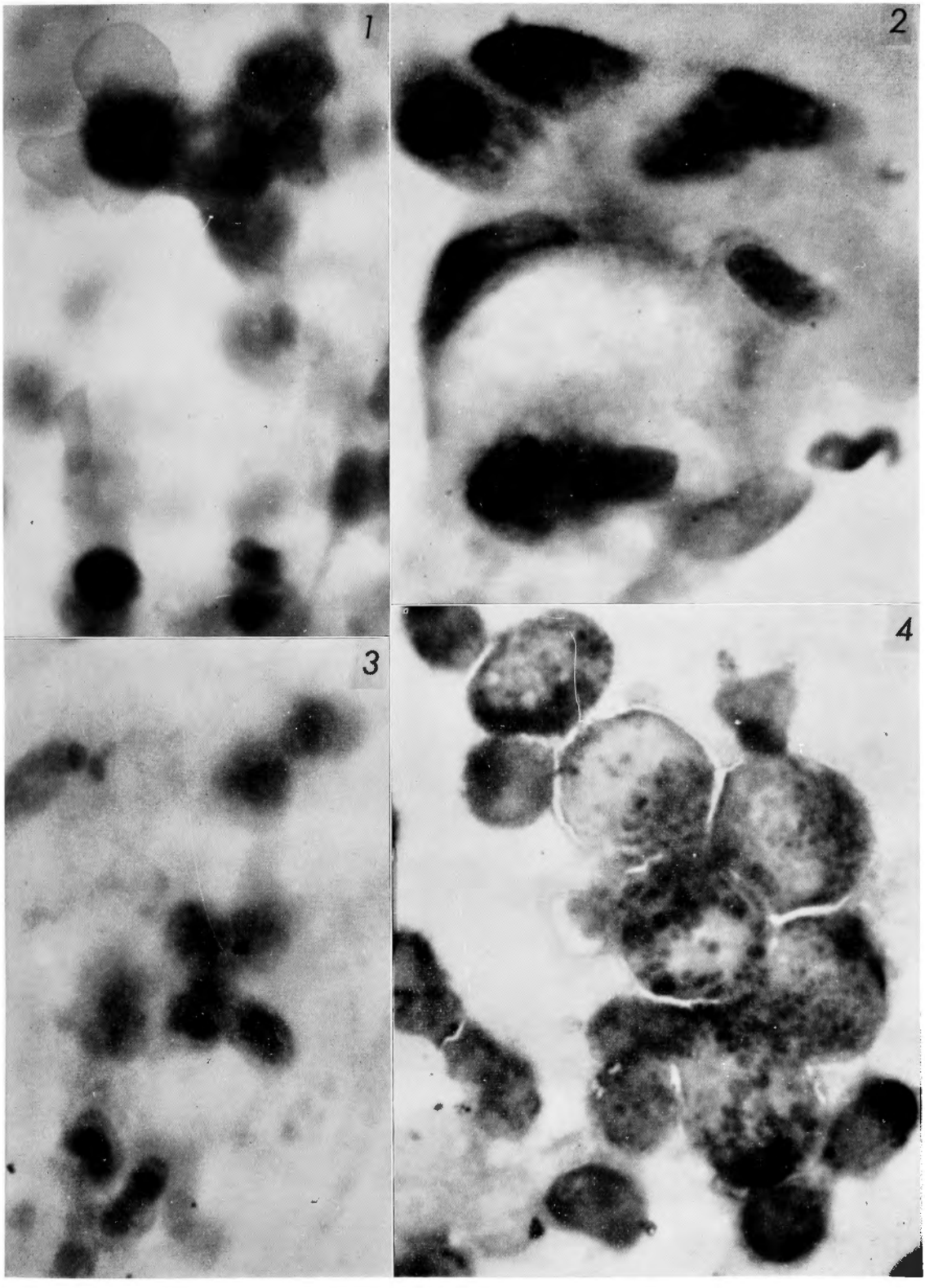
The PAS reaction activities in all cells of the gastric cytological specimens were sufficiently preserved as well as in operative materials. Though the screening of signet-ring cells found in the gastric cytological specimens was easy because of their heavy PAS reaction, the accurate identification of malignancy of signet-ring cells was impossible as

Fig. 1 : Cancer cells and inflammatory cells in the cytologic specimen showing the marked nuclear reaction with methyl green, without the distinct presentation of nuclear structure. Methyl green-pyronin stain. ×900.

Fig. 2 . A signet-ring cell in the cytologic specimen showing the marked reaction with pyronin in the cellular rand, together with normal surface epithelium of the stomach. Methyl green-pyronin stain. ×900.

Fig. 3 : Inflammatory cells in the cytologic specimen reacted markedly with methyl green-pyronin stain. ×900.

Fig. 4 : Exfoliated colloid carcinoma cells containing a lot of PAS-positive coarse granules in the cytoplasm. ×900.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mentioned above.

3) *Alkaline phosphatase*:

Normal surface epithelium of the stomach showed faint diffuse staining in the cytoplasm and pale brown fine granules in the nuclei. Inflammatory cells showed marked reaction in the nuclei and moderate reaction in the cytoplasm.

The reaction in cancer cells was light or moderate and almost without any regard to the patho-histological type. Marked reactions were observed only in some carcinoma cells which were in process of degeneration and retrogression. In cancer cells as well as in normal surface epithelium of the stomach, activity was marked in the nucleus than in the cytoplasm.

Normal squamous epithelial cells from the oroesophageal tract and micro-organism showed very light reactions.

This reaction, judging from the fact that it provides only the poor cellular structures because of its staining of vague contrast and fails to show the strong activity with cancer cells except for some of those in the stage of degeneration or retrogression, would not offer the good criteria for the exfoliative cytology, even if the activity in the cytologic specimen was as well preserved as that of the surgical material.

4) *Acid phosphatase*:

There were moderate to marked acid phosphatase activities in normal surface epithelium of the stomach. Light diffuse cytoplasmic localization was noted, whereas the nucleus showed moderate to marked reaction. Inflammatory cells showed moderate to marked reactions with coarse granular brown-black pigmentation in the nuclei without presenting fine nuclear structure.

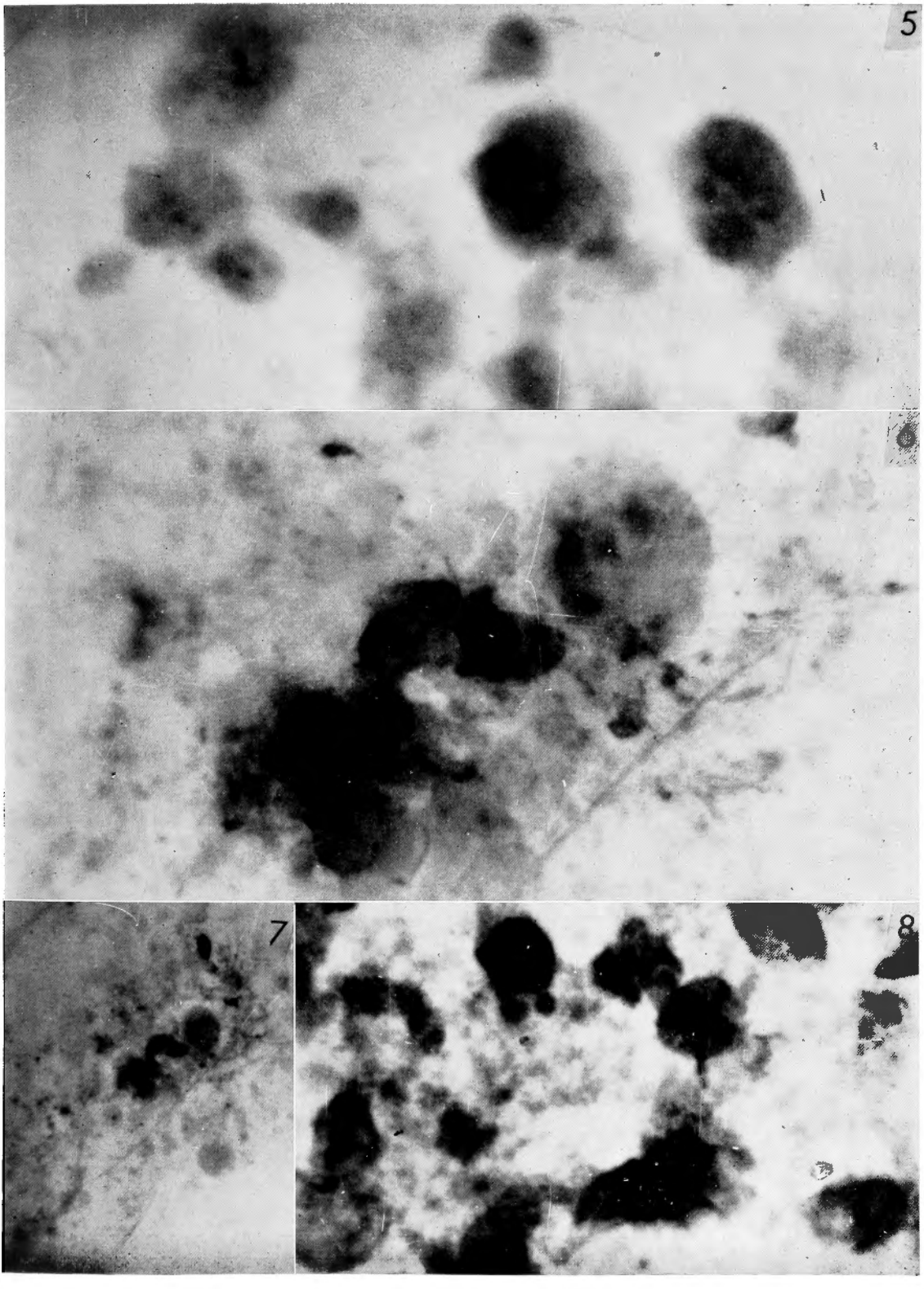
Marked reaction was observed both in inflammatory and cancer cells Among cancer cells reaction was more marked in undifferentiated cancer and primary adenocarcinoma cells than in pleomorphic adenocarcinoma and colloid carcinoma cells And fairly degenerated cancer cells showed very light or no reaction. Thus, the grade of reaction in cancer cells were very slightly related to the patho-histological type In cancer cells cytoplasmic reaction was light and diffuse, and nuclear reaction was marked revealing brown-black pigmentation as a lump in some nuclei and nuclear structure in others

Bacilli reacted moderately to markedly and normal squamous epithelial cells showed light nuclear reaction.

Similarly good preservation of activities were observed in the gastric cytological specimens as well as in the gastrectomy materials. The staining by this reaction was of high contrast. Thus, this reaction was considered to be good in identification of cancer cells because of the better preservation of activity in the gastric cytological specimens and of the strong reaction in cancer cells. This observation formed the basis of the study in

Fig. 5 : Histiocytes in the cytologic specimen with the marked alkaline phosphatase reaction both in the cytoplasm and nucleus, presenting relatively fine cellular structures. The improved GOMORI's method with light counterstain by MAYER's hematoxylin. ×1000.

Fig. 6, 7 and 8 : The cytologic specimen showing the marked alkaline phosphatase activity in degenerative carcinoma cells and moderate in other carcinoma cells. All without presenting fine nuclear structures The improved GOMORI's method with light counterstain by MAYER's hematoxylin. ×1000, ×200 and ×1000.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5) *DPN-diaphorase* :

A few of gastric cytological specimens showed very faint DPN-diaphorase activity. Namely, 21 cases turned out to be negative except for 2 cases which revealed unsatisfactory very faint diffuse red staining under microscopic examination.

In contrast to the above mentioned results obtained from the gastric cytological specimens, the smear of the lesion or the tissue section in fresh gastrectomy specimens showed following findings.

The reaction was moderate in normal surface epithelium of the stomach. Inflammatory cells showed light to moderate reaction.

As to cancer cells which, on the whole, showed the marked reaction, undifferentiated carcinoma was the most marked in its grade followed by primary adenocarcinoma and by pleomorphic adenocarcinoma. Most of degenerative cancer cells in the colloid carcinoma showed exceptionally very light or no DPN-diaphorase activity.

In all cells granular response was observed in the cytoplasm with no nuclear reaction.

DISCUSSION

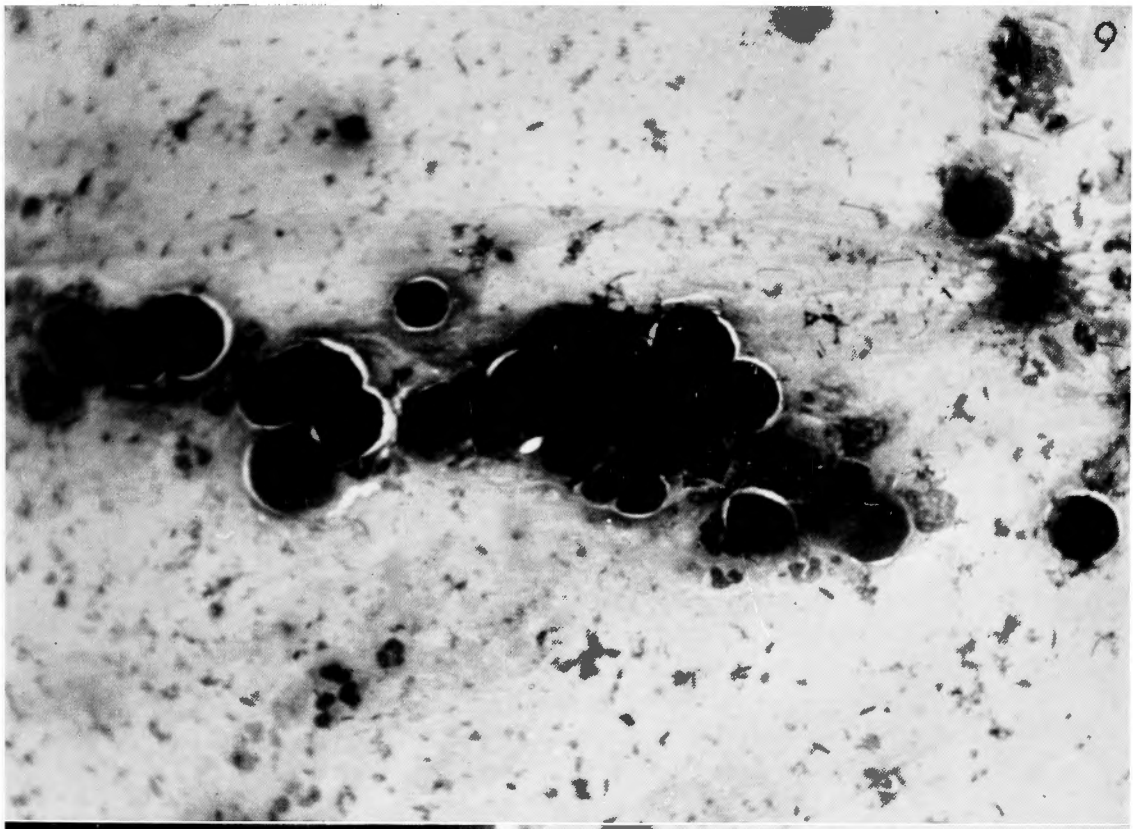
Recent great development in clinical cytologic diagnosis depends not only on the improvement of collecting technique of the cytologic specimens, but also on the careful morphological study on exfoliated cells. Among others the stain method devised by PAPANICOLAOU has been highly appreciated and widely utilized in the mordern cytology because of its detailed nuclear manifestation of exfoliated cells. And the morphological criteria for the identification of malignant cells was formed by PAPANICOLAOU, GRAHAM and TAKEDA. Though the cytodiagnosis has recently become very popular thanks to the morphological criteria, there are still not a few undistinguishable exfoliated cells even by this.

If distinct morphological demonstration of a substance either specific to malignant cells or contained in larger amount in malignant cells than in non-malignant cells is possible, the cytodiagnosis will be a definite cancer diagnostic. Up to this time, such substances have never been found out biochemically (GREENSTEIN[20], DANNENBERG[21] and NAKAHARA[22]), immunologically (SYVERTON[23]) or electro-microscopically (BRACHET & MIRSKY[24]). It may be impossible or very difficult to detect those substances by the stain technique, but in order to study the entity of cancer, the effort to detect these substances should be made not only with biochemical, immunological or electro-microscopical analysis but also with stain studies.

GROSS et al. did a histochemical study on the exfoliated cells of the cervix uteri but failed to observe the characteristic findings to cancer cells. As to the histochemical study on the gastric cytological specimens, PAS reactions alone have been investigated by many workers without obtaining any favourable findings to the exfoliative cytology.

The present study was undertaken to find out a practical histochemical reaction in

Fig. 9 and 10 : The cytologic specimen showing the marked acid phosphatase reaction in carcinoma cells revealing either brown-black pigmentation as a lump in the nucleus or nuclear structure. The improved GOMORI's PbS method with light counterstain by MAYER's hematoxylin. ×200 and ×900.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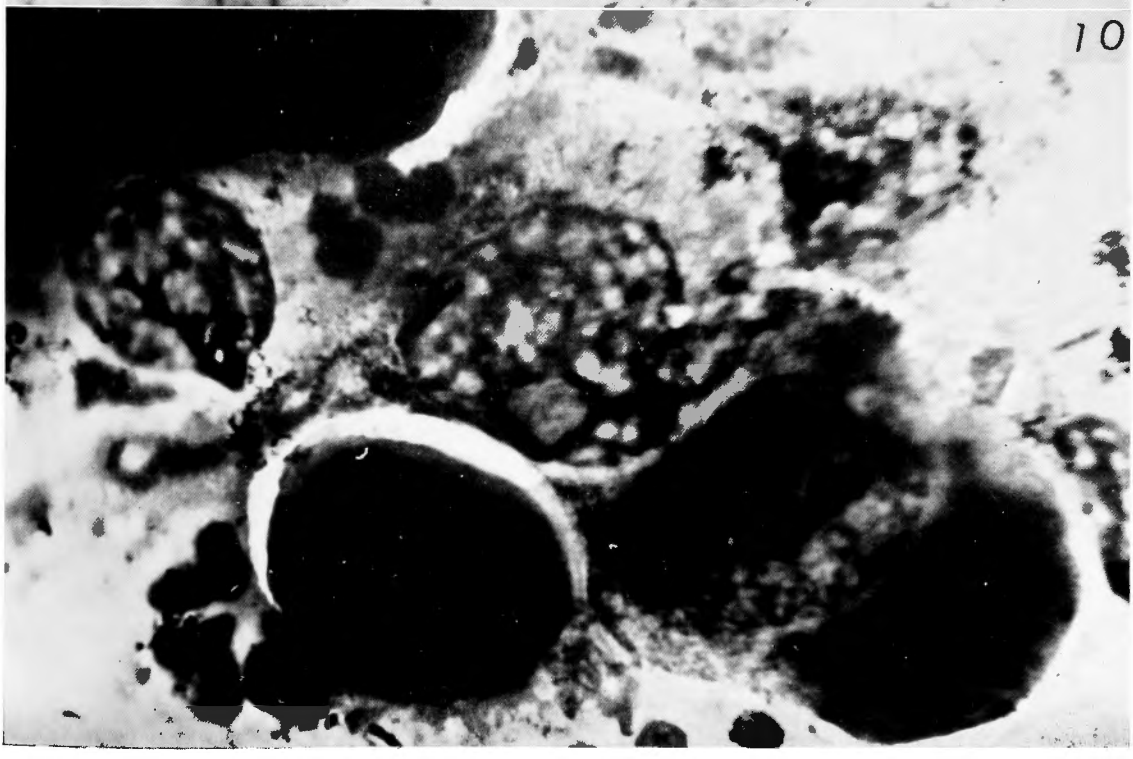
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the gastric exfoliative cytology, the activity of which could reveal both the prominent increase in the cancer cell and the good preservation in the cytologic specimen. And it was found that both acid phosphatase and DPN-diaphorase demonstrated prominent reactions to the gastric cancer cells.

In the present study, the marked acid phosphatase activity was demonstrated in the most of gastric malignant cells, though GROSS et al. reported that the grade of its activity was proportional to morphological differentiation and not to malignancy in the cervix uteri cytology.

An increase in acid phosphatase activity in the gastric cancer tissues was demonstrated by TAKAMATSU[25]. CHANGUS & DUNLAP[26] and BENDT & HOFFMANN[27] reported the increase in acid phosphatase of gastric juice in patients with gastric carcinoma. Also in the other organs, the increase in acid phosphatase was reported to occur by their malignant changes. For example, GUTMAN[28] stated long time ago that the biochemical acid phosphatase test was diagnostic for metastatic carcinoma of the prostate, and CASTLEMAN[29] emphasized recently that the acid phosphatase stain provided a good criteria for the identification of the malignant cells of the parathyroid gland. These reports as well as author's results seem to indicate that the increase in acid phosphatase activity is related not to organ specificity but to malignant changes.

As to DPN-diaphorase there was MIZUTANI's[30] report revealing its increase in human gastric cancer tissue with the exception of degenerative cancer cells in colloid carcinoma. Similar result was obtained in this study concerning both the smears and the tissue sections of surgical materials.

Among other reactions, marked PAS reaction was noted only in colloid carcinoma cells. However, this reaction was considered to be useless in identifying malignant cells since intestinal metaplasia cells showed the reaction as marked as that in colloid carcinoma cells.

Alkaline phosphatase reaction is considered to be unsatisfactory for the exfoliative cytology, as it showed only weak reaction for the gastric cancer cells except for some of those in degeneration. BENDT et al. reported marked increase in alkaline phosphatase as well as in acid phosphatase in gastric juice of the patients with gastric cancer. Difference between author's histochemical findings and their biochemical results remains to be an unsolved problem.

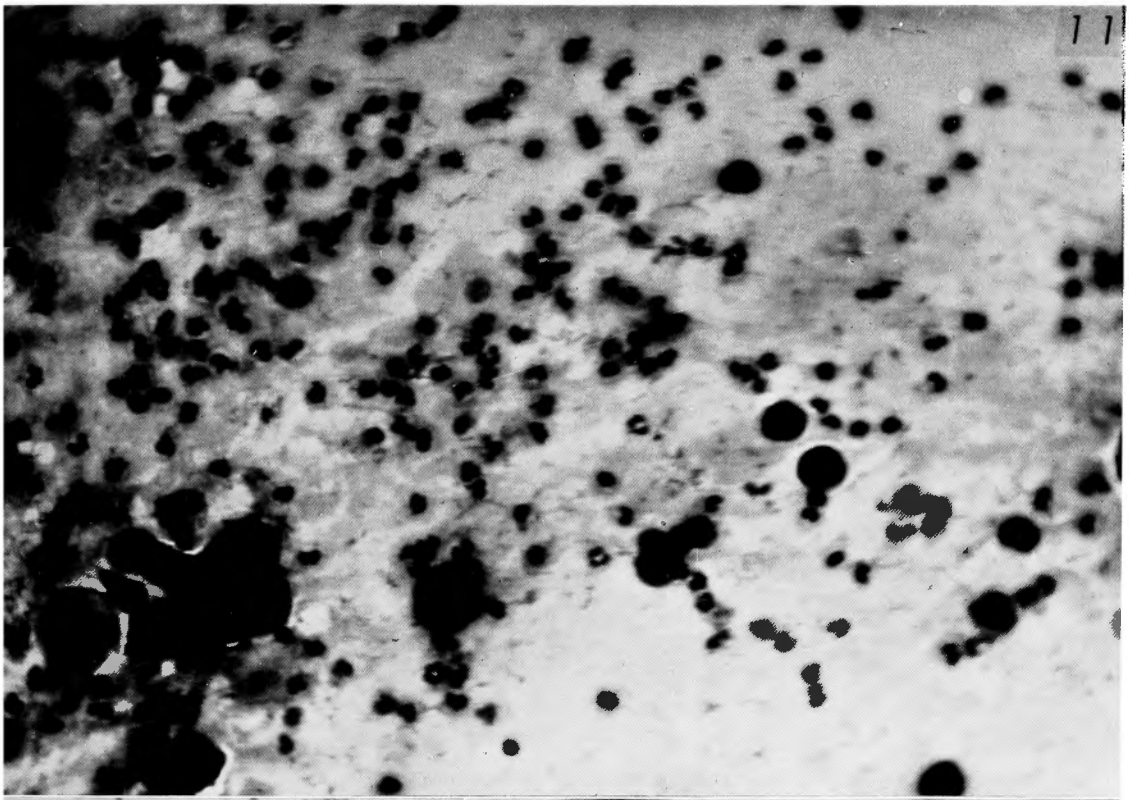
The fine nuclear structure was not obtained by methyl green-pyronin stain. Pyronin positive pigment in the cytoplasm and methyl green positive pigment in the nucleus were not helpful in identifying malignant cells, even if they did permit proper evaluation of RNA and DNA content of the cells respectively.

The preservation of the activity concerning gastric cytological specimens was good in methyl green-pyronin stain, PAS reaction, alkaline phosphatase and acid phosphatase

Fig.11 : The cytologic specimen showing the marked nuclear reaction in carcinoma cells and inflammatory cells, light in normal epithelial squamous cells and moderate to marked in bacilli. The improved GOMORI's method with light counterstain by MAYER's hematoxylin. ×200.

Fig.12 : The smear from surgical material presenting the moderate DPN-diaphorase activity in the cytoplasm of normal surface epithelium of the stomach. The FARBER's method. ×900.

Fig.13 : The smear from surgical material presenting the marked DPN-diaphorase activity in the cytoplasm of cancer cells. The FARBER's method. ×900.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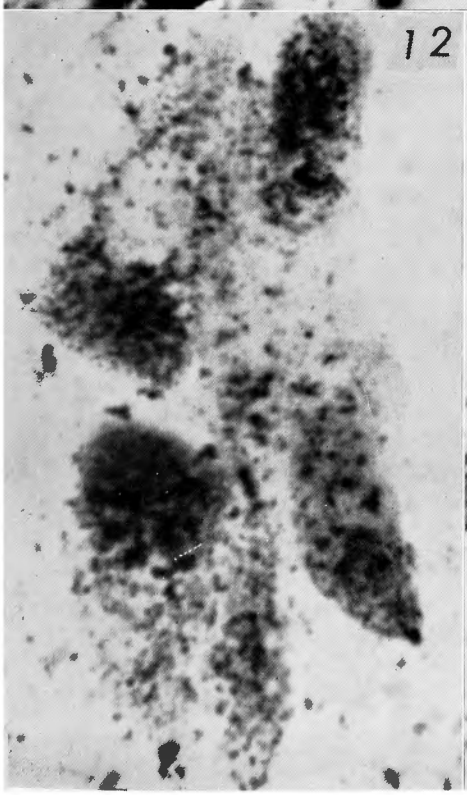
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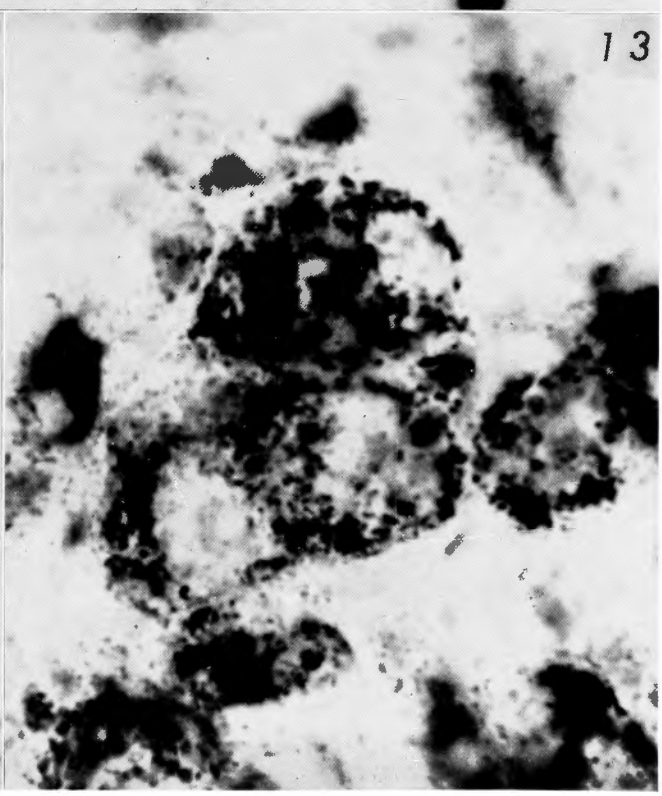
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and was not good in DPN-diaphorase, the activity of which was located in the cytoplasm and was so unstable that the clear demonstration of this was impossible, unless the material, even from the gastrectomy specimens, was treated rapidly and correctly. It should be pointed out consequently that demonstration of DPN-diaphorase activity on the degenerative cytologic specimens would be very difficult.

Therefore, it is concluded that acid phosphatase alone, among the histochemical reactions in this study, had both the prominent increase of activity in malignant cells and the good preservation of activity in the gastric cytological specimens. Another advantage of this reaction for the cytological application is that the acid phosphatase activity demonstrated by the improved GOMORI's PbS method is marked in the nucleus which is resistant to degeneration and closely related to the entity of cancer cells.

However, the acid phosphatase reaction by the improved GOMORI's PbS method has many unsolved problems yet. For example, intracellular localization of this activity is various according to the different methods such as biochemical analysis (PALADE[31]) and histochemical post-coupling (SELIGMAN[32]) or PbS method.

Study on the better stain condition of acid phosphatase for the identification of malignant cells will be undertaken in next report.

SUMMARY

In order to investigate a practical histochemical technique in the cytodiagnosis of gastric cancer, the gastric cytological specimens and the surgical materials obtained from 191 cases in total with clinical evidence of gastric disease were subjected to the reactions with methyl green-pyronin, PAS, alkaline phosphatase, acid phosphatase and DPN-diaphorase.

Acid phosphatase and DPN-diaphorase reaction showed prominency in cancer cells. For all of histochemical reactions presented in this study their activities were well preserved in the gastric cytological specimens except for DPN-diaphorase reaction.

Consequently, acid phosphatase reaction alone showed both the prominent increase of activity in gastric cancer cells and the good preservation of activity in the cytologic specimens; therefore, this reaction was considered to be useful in the gastric exfoliative cytology.

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The gist of the present study was reported at the 19th General Meeting of the Japanese Cancer Association at Tokyo (December 1960).

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(* Written in Japanese)

和文抄録

胃癌細胞診の診断適中率向上を目的とする二，三の試み
第三編 胃癌細胞診への組織化学染色法の適用

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胃癌組織に対してその意義が論じられてきた組織化学染色法の幾つかをとり挙げて胃癌細胞診に適用を試みた.

すなわち，胃疾患患者191名からabrasive balloon 法で採取した細胞診試料，並びに対照としてこれらの患者から得た手術材料に対して，methyl green-pyronin 染色，PAS 反応，alkaline phosphatase, acid phosphataseおよびDPN-diaphorase の組織化学染色を行なつた.

これらの染色の中で，胃癌細胞全般に強い活性を示したものはacid phosphatase反応であり，また，DPN-diaphorase も殆んど全ての胃癌細胞に強い活性を示した.

他の反応については，PAS反応が膠様癌細胞に強い反応を示したり，methyl green-pyronin 染色によつて胃癌細胞の核がmethyl greenで比較的強く染まつたりalkaline phosphatase の強い活性が一部の変性過程にある胃癌細胞に認められる等の所見が得られたが，いずれも，他の細胞と比較して胃癌細胞が特に強い反応を示すものではなかつた.

一方，胃細胞診試料における活性の保存性についてみると，methyl green-pyronin 染色，PAS 反応，alkaline phosphatase, および，acid phosphatase が手術材料と同程度の良い保存性を示した.

結局，この研究でとり挙げた組織化学染色法の中でacid phosphatase 反応だけが胃細胞診試料で良い活性の保存性を示し，更にまた胃癌細胞全般に強い活性を示すことが判明した.