THE EPITHELIAL CELLS IN MEGALOBLASTIC ANAEMIAS

BY

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The earliest cytological abnormalities to be recognized in pernicious anaemia were, of course, those of the blood. Apart from the various changes seen in the mature red cells, circulating nucleated precursors showed sufficiently striking alterations for Ehrlich to give them the separate name of megaloblast (Ehrlich, 1880). More recently, the granular leukocytes were found to be hypersegmented, and after the introduction of marrow puncture the gigantism of the immature granular cells became an equally familiar feature of this group of diseases.

In view of the association of anaemia, achlorhydria, glossitis, prematurely white hair, and degeneration of the spinal cord, it was not surprising to learn that cytological abnormalities can be found outside the haemopoietic system. Rubin and Massey in 1953 briefly reported the finding of enlarged nuclei in gastric mucosal cells from gastric washings of patients with pernicious anaemia, and since they were all in therapeutic remission this was presumably a feature of the permanent gastric atrophy. Graham and Rheault (1954) then described enlargement of both nucleus and cytoplasm of the squamous epithelial cells in gastric washings (cells presumably derived from swallowed saliva). This observation was supported by photomicrographs and measurements in 15 cases of pernicious anaemia in relapse, and has been confirmed by other observers; it is rapidly reversed by vitamin B₁₂ treatment. At the same time, Graham and Rheault claimed to have found that gastric columnar cells and macrophages from the same specimens also showed enlargement. The data on these were much less convincing, depending on a comparison of a single case with a single control; and in the case of the macrophages there has been no subsequent confirmation. It was also mentioned that a similar abnormality had been seen in a vaginal smear, but no data were given. Graham (1957) has also stated that the same

changes can be found in vaginal smears of young mice born of vitamin B₁₂-deficient mothers.

The enlargement of the nuclei of the buccal cells can best be studied in smears of saliva, and one of us (M. M. B.) began to measure these cells in order to determine whether the changes are specific for megaloblastic anaemias or simply related to glossitis and atrophy of the tongue. The results (Boddington, 1959) showed that similar changes are indeed found in the saliva of patients with smooth tongues due to iron-deficiency and other causes, although not to the same degree as in certain cases of megaloblastic anaemia; the same changes were also sometimes found in patients with megaloblastic anaemia whose tongues were not smooth. Nuclear enlargement was not constantly found in megaloblastic anaemias, even in the presence of some lingual atrophy.

Similar studies have been reported by Górz-Kardaszewicz (1956), Boen (1957), and Farrant (1958), all confirming that the buccal cells in pernicious anaemia exhibit nuclear enlargement but without relating the changes to the state of the tongue, so that the specificity of the abnormality remains unproved.

Meanwhile, Rubin and the Chicago school (Massey and Rubin, 1954; Massey and Klayman, 1955; Rubin, 1955, 1956) and Gardner (1956), besides confirming Graham and Rheault's findings with respect to the squamous cells of gastric washings and extending them to other types of megaloblastic anaemia, made extensive observations on the gastric mucosal cells. It appears that only a small proportion of these cells are affected, and, while the most striking abnormalities are reversed by treatment, the presence of "bland" or "active" enlarged columnar cell nuclei may persist, and is presumably related to the irreversible gastric atrophy. Neither of these types is specific for megaloblastic anaemia (Rubin, 1955; Henning and

Witte, 1957). Grable, Zamcheck, Jankelson, and Shipp (1957) measured the nuclear diameters of gastric mucosal cells and found much higher mean values in pernicious anaemia than in other conditions apart from carcinoma.

It still remained to discover whether any other cells of the body are really affected apart from those of the mouth and stomach, and, if so, whether the changes are any more specific in other areas. We have therefore studied a series of 10 cases of megaloblastic anaemia, obtaining cytological material from a variety of different sites, and comparing the morphology of the cells before and after response to treatment.

Material

Of the 10 patients studied, eight had Addisonian pernicious anaemia (with low serum vitamin B₁₂ and histamine-fast achlorhydria), one had megaloblastic anaemia of the puerperium, and one had megaloblastic anaemia with a serum vitamin B₁₂ level of 28 μ g./ml. but normal gastric acidity, probably attributable to treatment of epilepsy with phenobarbitone. Six of the patients were women and four men. All had megaloblastic marrows.

In each case at or before the start of treatment smears were made of saliva, tongue scrapings, nasal and conjunctival swabs, urinary sediment, and, in women, vaginal aspirates. The same series was repeated nine to 17 days after beginning treatment (Table I); in all cases a reticulocyte response had occurred.

Gastric washings were also examined in eight of the cases (six before and two after treatment), but paired samples were not obtained. The findings from these will form part of another series to be published.

TABLE I
CLINICAL DETAILS OF SERIES

Case No.	Sex	Age	Diagnosis	Time of Taking Second Specimen (Days after Starting Treatment)	Sources of Abnormal Cells Found in First Specimens
1	F	75	Pernicious anaemia	14	Mouth, nose, urine, vagina
2	M	46	" "	15	Mouth
3	M	51	" "	9	None
4	F	80	" "	16	Mouth, nose
5	M	70	" "	16	None
6	M	55	" "	10	Mouth
7	F	68	" "	11	" "
8	F	36	Megaloblastic anaemia of puerperium	14	Mouth, nose, vagina
9	F	49	Pernicious anaemia	13	Mouth, vagina
10	F	74	Megaloblastic anaemia in epileptic on phenobarbitone	17	Mouth, urine, vagina

Methods

The methods used in preparing the smears were as follows:

Saliva.—The patient spat into a container until about 5 ml. saliva was obtained. This was centrifuged until the cells were deposited, and the deposit was distributed on a glass slide with a platinum loop. It was immediately fixed while still wet in alcohol containing 3% acetic acid for at least 15 minutes, then stained by the method of Papanicolaou.

Air-dried films were also made and stained with May-Grünwald and Giemsa exactly as used for blood films.

Tongue Scrapings.—The tongue was scraped to one side of the midline with the narrow edge of a glass slide, and the material spread on to another slide in the same manner used in making blood films. Fixation and staining were the same as described for saliva.

Nasal Swabs.—These were taken with a dry cotton-wool "throat swab." The swab was then gently stroked on to two slides. One was wet-fixed and stained by Papanicolaou's method, the other was rapidly dried in air and stained by May-Grünwald and Giemsa. Material was obtained from 20 normal persons as well as from the anaemic subjects.

Conjunctival Swabs.—These were taken with a cotton-wool swab near to the inner canthus, and immediately smeared on to a slide. As the material dried instantaneously, only the May-Grünwald stain was used. Slides from 20 control subjects were examined as well as those from the series.

Urine.—In three of the women catheter specimens were examined; in the rest, urine was used as passed. The specimen was centrifuged immediately after voiding, and the deposit was transferred on to slides with a platinum loop. Wet-fixed films were made and stained by Papanicolaou's method, and air-dried films were also made and stained with May-Grünwald and Giemsa.

Vaginal Smears.—These were made from aspirate obtained with a vaginal pipette, and were wet-fixed in alcohol containing 3% acetic acid and stained by Papanicolaou's method. In three post-menopausal cases nuclear measurements were made before and after treatment in order to demonstrate any significant change. For this purpose, 100 squamous epithelial cell nuclei in each slide were measured in two diameters, using an eyepiece micrometer and the 1/12 oil-immersion objective. The mean diameter was calculated for each nucleus, and hence the mean nuclear diameter for the set of 100.

Apart from the vaginal cell measurements referred to, and those of buccal cells being published on page 222, measurements were only made on isolated cells. The cells of conjunctival, nasal, and urinary smears are fewer, more variable and irregularly distributed, and we felt that the statistical method applied to these would give only a false sense of accuracy.

FIG. 1.—Nasal smear in untreated pernicious anaemia (Case 1). A nasal epithelial cell is shown with an enormously enlarged abnormal nucleus. Such cells are very uncommon and most of the nasal columnar cells show no abnormality. May-Grünwald Giemsa, $\times 700$.

FIG. 2.—Nasal smear—same case as Fig. 1 after treatment. A group of nasal columnar cells showing normal morphology. May-Grünwald Giemsa. $\times 700$.

Results

Saliva and Tongue Scrapings.—In all except Case 5 the tongues showed some degree of soreness or depapillation. In all but two patients (Cases 3 and 5) the buccal squamous cells showed nuclear enlargement. The changes in these cells will not be fully described here, as one of us has given an account of a larger series which includes some of the present cases (Boddington, 1959).

In the specimens obtained after response to treatment the nucleus was reduced in size in all cases where this was initially abnormal, but completely normal levels were not always reached. For instance, in Case 2, the mean nuclear diameter before treatment was 10.3μ , and 15 days after beginning treatment it was 9.6μ , which is still above the normal level; nuclei of diameter greater than 14μ amounted to 4.8% before and 1.5% after treatment. (These have never been found in normal persons.)

Nasal Mucosal Smears.—In the controls, the nuclear diameter was usually about 14μ in air-dried films and 12μ in Papanicolaou films. There was, however, some variation between individuals, and a few large nuclei sometimes occurred in an otherwise uniform film. Nuclei with diameters up

to 16.5μ were occasionally encountered in air-dried films, where there is more variation owing to differences in spreading.

Of the series with megaloblastic anaemia, seven were considered to have nasal smears indistinguishable from normal. In three patients (Cases 1, 4, and 8) certain nasal columnar cells in the pre-treatment samples were judged to be abnormal (Figs. 1 and 2). These were rounded-up cells showing nuclear enlargement (up to 20μ diameter in air-dried films and 15μ in wet-fixed ones), and sometimes showing prominent nucleoli. In view of the variation in normals, and the small number of abnormal cells found in cases of pernicious anaemia, the evidence for any real recognizable change is meagre.

Conjunctival Smears.—Cells were scanty in films, but were fairly uniform in size and appearance. In no case was any difference noted between the cases of anaemia and the controls, or between the pre- and post-treatment samples.

Urine.—In non-catheter specimens from Cases 1, 4, and 7, most of the cells in the urinary deposit were similar to those of the vaginal smears. In Case 1, the first specimen contained some unusual cells probably from the urinary tract with a very

FIG. 3.—Urine deposit in untreated pernicious anaemia (Case 1). An epithelial cell showing nuclear enlargement and irregular hyperchromasia. Papanicolaou, $\times 2,300$.

FIG. 4.—Urine deposit—same case as Fig. 3 after treatment. Two normal urinary epithelial cells at the same magnification for comparison. Papanicolaou, $\times 2,300$.

high nucleocytoplasmic ratio and a "dotted" chromatin pattern (Fig. 3). These may be compared with normal cells in the second specimen (Fig. 4).

In the remaining three female cases, only catheter specimens were examined. In one of them, Case 10, the pre-treatment sample showed

some cells with large nuclei (up to 19μ diameter in a Papanicolaou film), a high nucleocytoplasmic ratio, hyperchromasia, and irregularities in nuclear outline, changes resembling those found in carcinoma of the bladder. The specimen obtained after treatment showed no abnormal cells.

The urine specimens from the four male cases showed no abnormalities.

Vaginal Smears.—Only three of the female cases provided comparable atrophic post-menopausal smears before and after treatment (Cases 1, 9, and 10). Owing to the hormonal changes which occur even in the elderly, it is difficult to be sure that any difference is due to the effect of treatment; these three, however, had no sign of cornification in the smears, and in Case 1 a hysterectomy and bilateral salpingo-oophorectomy had been done 18 years before.

Table II shows the figures obtained from nuclear measurements in these three cases. In Cases 1 and 10 there was a highly significant difference between the slides made before and after treatment. In Case 4 there was also a reduction in size, but the difference was not significant at the 5% level.

TABLE II
MEAN NUCLEAR DIAMETERS OF VAGINAL CELLS

	Mean Diameter and Standard Error (μ)	Standard Deviation	P
Case 1 Before B ₁₂ ..	10.11 $\pm$ 0.22	2.17	<0.001
After B ₁₂ ..	8.49 $\pm$ 0.15	1.45	
„ 4 Before B ₁₂ ..	8.63 $\pm$ 0.16	1.62	Not significant
After B ₁₂ ..	8.20 $\pm$ 0.22	2.17	
„ 10 Before B ₁₂ ..	9.48 $\pm$ 0.13	1.34	<0.001
After B ₁₂ ..	8.23 $\pm$ 0.13	1.26	

Morphologically, the vaginal cells in these three cases were of the "basal" type typical of post-menopausal atrophy. The nuclear enlargement in the pre-treatment samples was not associated with hyperchromasia; in Case 1 the nuclei had a pallid vesicular appearance (Figs. 5 and 6), while in Cases 4 and 10 they did not differ appreciably from normal. The nucleoli were not prominent. It is doubtful whether a diagnosis of megaloblastic anaemia could even be suspected from material so variable as vaginal smears, but the difference in measurements shown in these three cases, and particularly in Case 1, seems most likely to be a reflection of a morphological change due to the effect of vitamin B₁₂ deficiency and its correction.

Gastric Washings.—These were examined in eight of the present series, six in relapse and two in remission. As they form part of a larger series,

FIG. 5.—Vaginal smear in untreated pernicious anaemia (Case 1). A post-menopausal smear showing nuclear enlargement. Papanicolaou, $\times 700$.

FIG. 6.—Vaginal smear—same case as Fig. 5 after treatment. Marked reduction of nuclear diameter compared with pre-treatment sample. Papanicolaou, $\times 700$.

FIG. 7.—Sputum in untreated pernicious anaemia. A single collection of bronchial columnar cells was found showing striking nuclear enlargement. Papanicolaou, $\times 700$.

FIG. 8.—Normal sputum. A collection of bronchial columnar cells in normal sputum for comparison with Fig. 7. Papanicolaou, $\times 700$.

the details will not be given here. Only one (Case 1) showed any appreciable abnormality of gastric mucosal cells apart from the intestinal metaplasia which is recognizable in some of the cases. The abnormal cells occurred in a single fragment, and showed anisocytosis and hyperchromasia; they corresponded apparently to Rubin's "active cells." The swallowed squamous epithelial cells did not differ appreciably from those of the saliva. No abnormality was noticed in the macrophages in any of the cases.

Bronchial Epithelium.—In a case of pernicious anaemia in relapse (not included in this series) specimens of sputum containing bronchial ciliated cells were examined. Some of these showed striking nuclear enlargement in Papanicolaou films, as well as prominent nuclei (Figs. 7 and 8). Only a small group of cells was affected, the remainder being morphologically normal.

Pleural Fluid.—Pleural fluid from one case of untreated pernicious anaemia not included in the present series was examined. The patient had tabes dorsalis, and although there was histamine-fast achlorhydria and a serum vitamin B₁₂ level of 85 $\mu\text{g.}/\text{ml.}$ the bone marrow was only equivocally megaloblastic. The pleural effusion was due to congestive cardiac failure. There were abundant mesothelial cells, macrophages, and lymphocytes, and none of these showed any morphological abnormality.

Discussion and Conclusions

We hoped by this investigation to establish whether or not there is a general visible abnormality of epithelial cells in megaloblastic anaemia, such as is implicit in the paper of Graham and Rheault (1954). It appears that, in vitamin-B₁₂ deficiency and in the other anaemias which respond to folic acid, obvious abnormalities of the buccal squamous cells are the rule rather than the exception (Boddington, 1959).

Corresponding changes in the vaginal smears are difficult to establish owing to the wide variations and cyclical changes which are found normally. Our findings suggest that the nucleus can become enlarged in pernicious anaemia, but is probably only recognizable with certainty by comparing specimens before and after treatment.

The cells of the conjunctival epithelium showed no abnormalities, and those of nasal columnar epithelium only inconstant and equivocal changes. Some markedly abnormal bronchial columnar cells were seen in one sputum specimen.

The cells of the urinary tract were usually normal, but in two cases there was a reversible

abnormality for which no other cause than vitamin B₁₂ deficiency was found; this consisted of nuclear enlargement, a high nucleo-cytoplasmic ratio, and hyperchromasia only affecting a small proportion of the cells.

The "active" gastric epithelial cells described by Rubin were only found in one of six untreated cases, and "bland" nuclear enlargement of gastric mucosal cells was not seen in any of the gastric washings studied from this group of cases. (The features which may perhaps be specific for pernicious anaemia are the occurrence of fragments of epithelium showing intestinal metaplasia, with or without other pieces of normal mucus-secreting gastric epithelium, in the absence of pus.)

From the standpoint of diagnostic cytology it can be concluded that morphological changes of the epithelial cells in megaloblastic anaemias are less constant and less characteristic than the abnormalities found in bone marrow, and are only likely to be of diagnostic use in a very occasional case. This situation arose in Case 1, where, in spite of a macrocytic blood picture, an initial bone marrow smear was equivocal, and the abnormal cells of the saliva, urine, nasal and vaginal smears led to a further marrow puncture and serum vitamin B₁₂ estimation. Boen, Molhuysen, and Steenbergen (1958) have recently described a case of subacute combined degeneration of the cord in which the examination of buccal cells gave the only morphological evidence of the disease. Cases such as these are very exceptional. The most constant changes, those of the buccal epithelium, are often indistinguishable from those found in the other types of lingual atrophy, and in the average case of pernicious anaemia the other epithelia so far examined have shown no alterations at all.

From a more fundamental standpoint, it remains to be discovered whether the nuclear enlargement represents an actual increase of nuclear solids, and whether the DNA is normal in amount. The appearances in smears do not suggest that there is an increase in DNA in the majority of cases, and the comparative ranges in nuclear size are very unlike those expected if polyploidy was the explanation. In the case of megaloblasts, Reisner (1958) has recently reviewed what evidence there is on this still unsolved problem, but it would be unjustifiable to suggest that the changes seen in the epithelial cells are of the same nature.

Summary

The cytological appearances of epithelial cells were studied in 10 cases of megaloblastic anaemia,

eight of which had typical Addisonian pernicious anaemia. Cells were examined from the mouth, nose, conjunctiva, urinary tract, and vagina both before and after treatment, and observations were also made on the gastric contents in six cases before treatment and two after. Besides the enlargement of the buccal cell nuclei which was found in eight of the cases, small numbers of abnormally enlarged cells were observed in three nasal and two urinary specimens taken before treatment. In two of three post-menopausal women there was a highly significant reduction in mean nuclear diameter of the vaginal cells after vitamin B₁₂ treatment. One of the gastric washings taken before treatment showed a fragment of mucosa with irregular hyperchromatic nuclei.

All these changes were reduced or abolished by vitamin B₁₂ treatment (or folic acid in the puerperal case). No abnormality was found in conjunctival cells or in macrophages.

Bronchial columnar cells with abnormally enlarged nuclei have been found in one specimen of sputum from an untreated case of pernicious anaemia.

It is concluded that widespread epithelial cell changes can occur in pernicious anaemia, but that the appearances are inconstant and non-specific.

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