J Clin Pathol 1980;**33**:684-687

Lysosomal naphthylamidase activity as a possible aid in cytological screening

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SUMMARY Lysosomal naphthylamidase activity has been measured microdensitometrically in cells in samples obtained by cervical and vaginal irrigation from 22 cases cytologically graded I and II, 28 graded III (moderate to severe dysplasia), 22 graded IV (carcinoma *in situ*), and 15 cases (grade V) of invasive carcinoma. There was a statistically significant difference in this activity in the cells from cases of grade V as against those in the relatively normal samples (grades I and II; $P < 0.001$) and as against those of grade IV ($P < 0.005$). The method is sufficiently robust for routine use provided that it is recognised that elevated activities can be found as a consequence of other factors such as previous surgical intervention and infection with herpes simplex.

It is widely conceded that cytological screening for cervical and endometrial cancers has a significant failure rate[1] and, even when a lesion is detected, presents the problem whether or not it is invasive or potentially invasive. It is also apparent that possibly not more than about one in three cases of carcinoma *in situ* may represent a condition that will progress to invasive cancer. For these reasons, interest is focussing on the possibility of developing functional tests to complement the conventional screening procedure that is based on simple cytomorphology.

The suggestion of Sylvén and Malmgren[2] that invasive cells extrude hydrolytic enzymes, generally of lysosomal origin, indicated that the level of such enzymes inside the exfoliated cells might act as a useful functional test of malignancy. Although biochemical estimations of β-glucuronidase activity in vaginal fluid[3] failed to substantiate their earlier promise,[4,5] it was considered that cytochemical measurements of a more definitively lysosomal enzyme in the exfoliated cells might be more successful. This is related to the nature of the cytochemical lysosomal reaction: provided that a partially hydrophilic substrate such as leucine naphthylamide (for lysosomal arylamidase activity) is used as the chromogenic substrate, the full activity of the intralysosomal enzyme in relatively normal cells will not be expressed because the lysosomal membrane will retard entry of the hydrophilic substrate to the enzyme. The activity will be fully expressed if the lysosomal membranes are labilised, to render them virtually functionless as permeability-barriers, as they would be in malignant cells if indeed these extrude lysosomal enzymes into the surrounding matrix.

Received for publication 17 December 1979

Material

Cervical and vaginal irrigation samples were obtained with a plastic bellows cytopipette containing about 8 ml normal physiological saline. After centrifugation (300 *g* for 5 min) a drop of the cell deposit was placed on a glass slide which had been coated with bovine albumin; the cells were smeared over the surface and left to dry in air.

Samples were collected from nine patients with no gynaecological abnormality; 13 cases of inflammatory hyperplasia; 28 cases of moderate to severe dysplasia; 22 cases of carcinoma *in situ* (5 with evidence of microinvasion); and 15 cases of invasive carcinoma including both squamous carcinomas and endometrial adenocarcinomas.

In addition, samples were collected from patients with a variety of other gynaecological conditions such as herpes simplex infection, children of mothers exposed to diethyl stilboestrol, or patients with a previous history of cervical abnormality.

A series of samples was also obtained from patients before and after treatment by cone biopsy, punch biopsy, or cryosurgery.

Routine cervical smears collected at the same time

as the research samples were stained by the Papanicolaou method[6] and used to establish the cytological grades as listed in Table 1.

Table 1 *Coefficient of variation of naphthylamidase activity measured in duplicate smears*

Grade	*Significance*	*No. of cases*	*(%) Coefficient of variation*
I	Normal	4	14
II	Inflammatory hyperplasia	12	11
III	Dysplasia	22	13
IV	Carcinoma *in situ*	17	12
V	Invasive carcinoma	15	15
	Overall	70	13

The neoplastic grades used in this investigation are as follows:

Grade I Normal and inflammatory cell patterns.
Grade II Marked inflammatory changes.
Grade III Cell patterns reflecting dysplasia of varying degrees (CIN I, II) and possibly including some cases of carcinoma *in situ* where insufficient evidence exists to grade as IV (CIN III).
Grade IV Cell patterns characteristic of severe dysplasia/carcinoma *in situ* (CIN III).
Grade V Cell patterns characteristic of invasive carcinoma, both squamous and glandular.

All patients in grades III, IV, and V were subjected to an operative procedure, and the lesion was confirmed histologically. In the case of some grade III patients, where the tissue was obtained by punch biopsy, only the minimum condition present was established.

Methods

Unfixed smears, in duplicate where possible, were incubated in a Coplin jar containing the reaction medium at 37°C for 15 minutes. The medium[7] contained 0·1 M acetate buffer, pH 6·1 (10 ml); 0·85% sodium chloride (8 ml); 0·02 M KCN (1 ml); 1 ml distilled water containing 8 mg leucine 2-naphthylamide and 32 mg leucine amide (to 'inhibit' aminopeptidase activity). Just before use, 10 mg of Fast blue B were added, and the pH was adjusted to 6·5. At 15-minute intervals the reaction medium was replaced afresh (Fast blue B decomposes in the medium at 37°C and must be replenished), the total incubation time being 45 minutes. The slides were then rinsed in a 0·85% solution of sodium chloride at room temperature and transferred to a Coplin jar containing a 0·1 M solution of copper sulphate, again at room temperature. The cells were then mounted in aqueous Farrants' medium. Twenty of the most intensely reacted cells were measured on each of the two duplicate slides. In the preparations from malignant or premalignant lesions, these cells with maximum reactivity were identified as malignant cells but, particularly in the case of the preparations taken after treatment, decisive morphological distinction was not always possible. The measurements were made with a Vickers M85 scanning and integrating microdensitometer at 550 nm with a × 40 objective and a scanning-spot of 0·5 μm diameter. A mask, which had a diameter of 30 μm adequate to encompass the largest cells measured, was used for all measurements. The results were expressed as relative absorption as recorded by the microdensitometer.

Results

REPRODUCIBILITY

Duplicate slides from 70 samples, of all grades, were measured. The mean enzyme activity (relative absorption) per cell and the coefficients of variation between the mean values of each duplicate were calculated (Tables 1 and 2). The overall coefficient of variation was approximately 13% with no significant increase at either high or low enzyme activity.

Table 2 *Comparison of naphthylamidase activity (relative absorption) in normal and malignant samples*

Grades	*No. of cases*	*Mean*	*Standard error*
I, II	22	6·74	1·66
IV	22	22·44	5·29
V	13	74·88	16·58

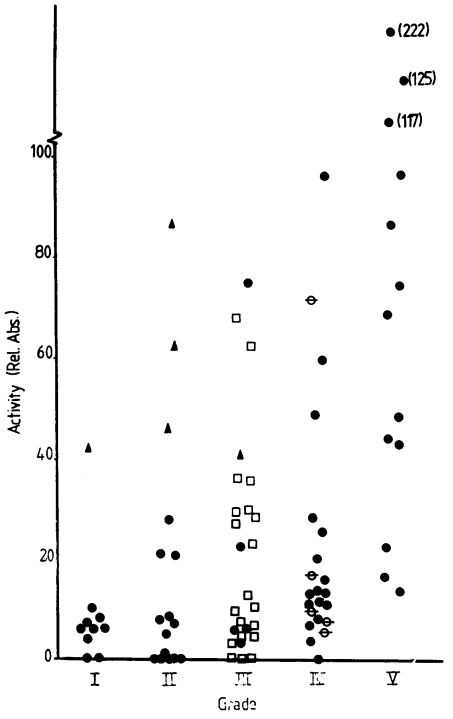
Comparison of grades I and II with grade V: $P < 0.001$.
Comparison of grade IV with grade V: $P < 0.005$.

STABILITY OF ENZYME AFTER STORAGE

Dried unfixed smears from two cases were kept at + 4°C and stained at various times up to 21 days. After four days the level of activity in the first case had dropped by approximately 5% and in the second by 15%. After 21 days the activity had dropped by 35% and 54% respectively.

LEVELS OF ENZYME ACTIVITY IN DIFFERENT CYTOLOGICAL GRADES

The mean enzyme activity was calculated for each sample and plotted in the form of scattergrams against the cytological grade from patients in whom there was also complete histological confirmation (cone biopsy or hysterectomy) or those in whom the histology was based on punch biopsy solely (Figure). It is apparent that there was a trend towards high



Scattergram showing mean value of naphthylamidase activity for each sample: ● cases in grades III, IV, and V where the grade has been confirmed histologically in specimens obtained by cone biopsy or hysterectomy; in grades I and II in smears from patients with no known gynaecological abnormality; ⊖ microinvasion; □ samples from grade III cases with histological confirmation in specimens from punch biopsy only; ▲ particular samples, discussed in the text, obtained from patients with known gynaecological abnormality.

activity in the malignant samples. Both grades III and IV had a number of samples with levels of enzyme activity within the normal range, but other samples from these two grades were clearly outside this range, having values similar to those of the malignant cases. However, as indicated in the Figure, other conditions such as herpes simplex infection, surgical intervention, or previous history of cervical malignancy also produced high levels of activity. The overall mean level of activity for normal samples (grades I and II but excluding these conditions) was 6·7 ± 1·7 (SEM). The corresponding value for the malignant, grade V, samples was 74·9 ± 16·6. When compared by the Student's *t* test the difference between these values was highly significant ($P < 0.001$: Table 3).

The overall mean activity per cell for the grade IV samples was 22·4 ± 5·3, which was significantly different ($P < 0.005$) from the values of the malignant samples (grade V). The fact that the standard deviation of the grade IV samples exceeded the mean (22·4 ± 24·2) confirmed the visual impression (Figure) that these samples were from a skewed or double population.

Table 3 *Naphthylamidase activity (relative absorption) measured pre and post punch biopsy, cone biopsy, or cryosurgery*

	Mean naphthylamidase activity	
	Pre	*Post*
	3·27	22·57
	6·36	24·73
	6·65	61·40
	13·61	32·41
	70·30	0
	19·82	29·56
	5·66	34·02
	9·17	56·70
	6·0	28·33
	6·20	28·95
	0	25·82
Mean	13·37	31·30
SD	19·50	16·45
SE	6·20	5·20

$t = 2.216$ $P < 0.025$ $df = 21$

LEVELS OF ENZYME ACTIVITY BEFORE AND AFTER SURGICAL INTERVENTION

Samples were collected from 11 patients before and after treatment by punch biopsy, cone biopsy, or cryosurgery. In all cases studied, except one cone biopsy, there was a marked increase in naphthylamidase activity after treatment (Table 3).

Discussion

In the nine grade I and in 10 of the grade II samples, the lysosomal naphthylamidase activity did not exceed 11 units of integrated relative absorption per cell; in three of the grade II specimens it was up to 28 units. In marked contrast, 10 of the grade V specimens contained activities of between 42 and 222 units per cell; three had low activities of 22, 17, and 13 units (Figure). Consequently, 10 out of 13 grade V specimens could be distinguished from those of grades I and II by this activity, three others falling within the range found in specimens from inflammatory hyperplasia.

If an activity of 30 units per cell, under these conditions, is taken as the upper limit of non-malignant activity (Figure), whether from inflammatory conditions or not, the specimens of grade IV, namely, of carcinoma *in situ*, fall into two types. Most (18/22) fell within this non-malignant range, four having markedly elevated levels. Although it would be tempting to consider that only the latter four

specimens represented carcinoma *in situ* that had invasive potentiality, it must be noted that four of the 18 specimens, with activities of less than 30 units/cell, were from cases in which histological evidence of microinvasion was detected. Thus although there is a highly significant increase in the mean available naphthylamidase activity in cells from malignant cases (grade V) when compared with the mean activity in cells of each of the other grades, as would be expected from the suggestion of Sylvén and Malmgren,[2] of lysosomal involvement in invasive propensity, it is clear that other factors can influence this activity. Thus one grade I specimen has been obtained with values of 41 units/cell; this patient had a positive lesion removed two years previously. Three specimens that cytologically were grade II also had very elevated levels of this enzyme. One, with 85 units/cell, was from a patient infected with herpes simplex; another (61 units/cell) was from a patient whose cytological test three months previously had been grade IV; one grade II (45 units/cell) and one grade III specimen (38 units/cell) were from children of mothers exposed to diethyl stilboestrol (DES) during pregnancy. Two of the three grade III specimens with the highest values had been confirmed histologically only on the basis of a punch biopsy; it is possible that a more advanced lesion existed but was not included in the specimen examined histologically; thus it is possible that they fit to the higher values of the carcinoma *in situ* specimens.

Thus although there is an overall trend (Table 2) towards considerably elevated levels in the frankly malignant cervical specimens (also seen in the few endometrial malignancies, though this field needs much greater investigation), as would fit to the concept of lysosomal involvement in the invasive process, it must be recognised that other factors can blur this distinction. This is particularly true of surgical intervention (Table 3). However, this quantitative cytochemical reaction can be helpful for a number of reasons: it does not depend on the liberal presence of malignant cells in smears from malignant cases; depending on the long-term follow-up of grade IV patients with high or low values, tests of this type have promise for future development in assessing tumour aggressiveness. On present evidence it appears to be a robust procedure, so that it is possible that smears could be made and stored (or sent through the post) to be processed and measured in a specialised centre, provided this processing is completed within a working week.

References

[1] Husain OAN, Butler EB, Evans DMD, Macgregor JK. Quality control in cervical cytology. *J Clin Pathol* 1974;**27**:935-44.

[2] Sylvén B, Malmgren H. Topical distribution of proteolytic activities in some transplanted mice tumors. *Exp Cell Res* 1955;**8**:575-7.

[3] Odell LD, Burt JC. Beta-glucuronidase activity in human female genital cancer. *Cancer Res* 1949;**9**:362-5.

[4] Fishman WH. The relationship of the enzyme beta-glucuronidase to cancer of the cervix uteri. *Bull N Engl Med Center* 1951;**XIII**:12-19.

[5] Lawson JG, Watkins DK. Vaginal fluid enzymes in relation to cervical cancer. *J Obstet Gynaec Brit Emp* 1965;**72**:1-8.

[6] Papanicolaou GN, Traut HF. *Diagnosis of Uterine Cancer by the Vaginal Smear*. Cambridge, Mass: Harvard University Press: New York: Commonwealth Fund, 1943.

[7] Bitensky L, Chayen J. In: Dingle JT, ed. *Lysosomes, A Laboratory Handbook*, 2nd ed. Amsterdam: Elsevier/North Holland Biomedical Press, 1977;209-43.

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