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Cytophotometric and flow cytometric DNA content of isolated glands in gastric neoplasia

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Abstract

The gland isolation method was applied to various gastric lesions to measure DNA content by cytophotometry and flow cytometry for the first time. By incubating and agitating fresh specimens from surgically resected stomachs in calcium-magnesium free Hanks's balanced salt solution (CMFH) containing EDTA, many neoplastic glandular epithelial cells were successfully isolated from the stroma, and their characteristic three dimensional features were seen morphologically. The DNA content of pure nuclear suspensions of isolated glands was obtained by cytophotometry and flow cytometry staining with 4′,6-diamino-2-phenylindole dihydrochloride (DAPI) and propidium iodide, respectively. Compared with histological grading, the frequency of the DNA aneuploidy of cancer with moderate or poor differentiation by cytophotometry (75%) was significantly higher than that of well differentiated cancer (25%), but the histological typing of gastric cancer DNA frequency were not correlated. This method allowed us to detect small aneuploid peaks by flow cytometry, which were previously masked by contaminating interstitial cells. The frequency of DNA aneuploidy detected by flow cytometry (87·5%) was higher than detected by cytophotometry (58·3%). The results of these studies shows the feasibility of this technique for analysing the DNA content of various lesions of the stomach.

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Keywords: gastric neoplasia, gland isolation method, cytophotometry, flow cytometry, DNA.

Many studies on the DNA content in gastric carcinomas have been performed by flow cytometry.[1–6] Most have been carried out retrospectively, using single nuclei suspensions retrieved from formalin fixed, paraffin wax embedded tissues.[1–4] With this method, however, DNA fragmentation and partial nuclei have caused large amounts of background debris and high coefficient of variation, interfering with accurate recognition of DNA ploidy of gastric neoplasms.

Conventional methods using interstitial cells as the internal diploid standard in either fixed or fresh materials could cause contamination of aneuploid tumour cell nuclei, because the ratio of tumour nuclei to interstitial nuclei is unknown and indeterminable. Moreover, a mixture of the two nuclei may prevent recognition of small aneuploid peaks or near diploid aneuploid peaks of tumour cell nuclei.[1 2]

Given these circumstances, we isolated single glands from gastric mucosal lesions without interstitial cells and were able to obtain accurate ploidies of these lesions. We report the gland isolation method,[7–12] three dimensional structures of isolated glands, and their DNA ploidies obtained by cytophotometry[11] and flow cytometry.

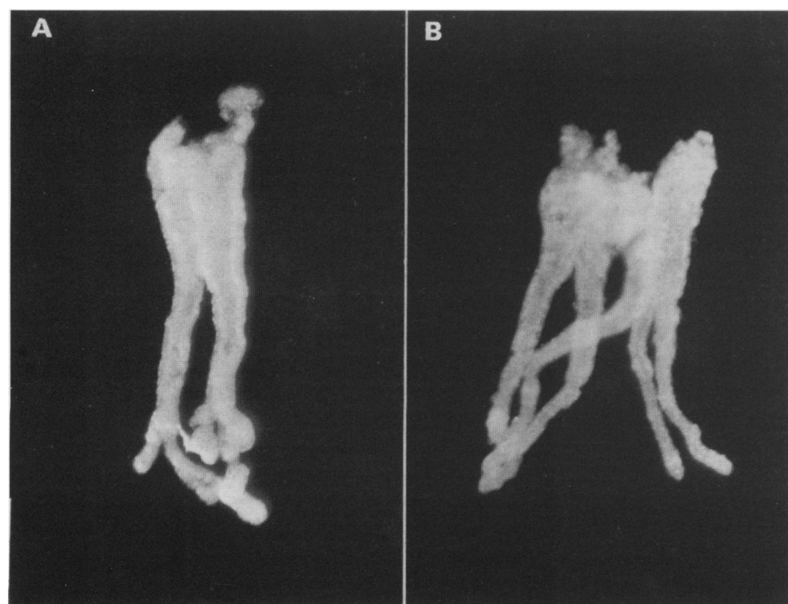

Figure 1: Single crypt epithelium of the pyloric (A) and fundic (B) mucosa as shown by stereomicroscope. The crypts are free from basement membrane of the fibrous stroma.

Methods

Samples

Seventy eight gastrectomy or biopsy specimens were obtained from 62 patients with carcinoma (48 cases), adenoma (eight cases), hyperplastic polyp (10 cases), and intestinal metaplasia (six cases). Forty two of 48 carcinoma patients (mean age: 66·3 years, 30 males and 18 females) were in the advanced stage of the disease.

All the specimens were placed in cold (4°C) calcium and magnesium free Hanks's balanced salt solution (CMFH). Small control pieces of normal pyloric and fundic mucosa were removed from regions as distant from the lesions as possible.

Gland isolation procedure

A modification of the method of Cheng was used for isolation of the gastric epithelium.[8 9]

Each specimen was cut into squares of 2–3 mm and incubated for 30 minutes at 37°C in CMFH containing 30 mM EDTA. The glands were removed by stirring in cold CMFH, and were sedimented by gentle centrifugation. The supernatant was aspirated and the glands were fixed with 70% ethanol alcohol. The mucosa remaining after isolation was fixed with 70% ethanol,[11 12] stained with haematoxylin and eosin, and subjected to pathological diagnosis. Histopathological classification was carried out based on the General Rules for Gastric Cancer Study in Japan.[1 3] In terms of histological grading for differentiation, tubular type of carcinomas was divided into well (tub 1) and moderately (tub 2) differentiated according to the degree of glandular formation of the cancer epithelium. Diffuse carcinomas were divided into the solid type (por 1) and non-solid type (por 2).

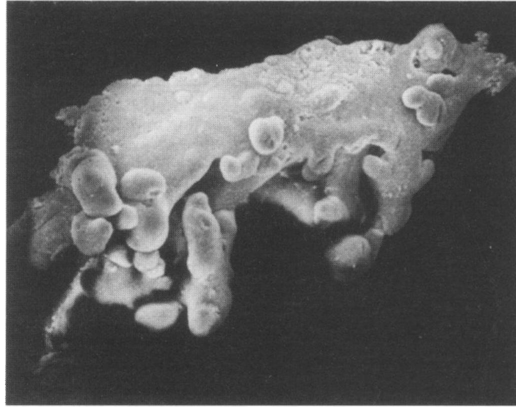

Figure 2: Histological features of papillotubular carcinoma shown by scanning electron microscope. The gland consists of small cystic and irregular branches.

Morphological findings

After fixation, epithelia consisting of normal pyloric and fundic glands, benign lesions, and mainly tubular carcinomas were observed under stereomicroscope. Some epithelia were fixed with osmium acid and observed by scanning electron microscope (ASID, 3D2 type Nihondenshi Japan). To detect intestinalised epithelium, isolated glands from the gastric mucosa were stained with alcian blue to identify goblet cells under stereomicroscope.

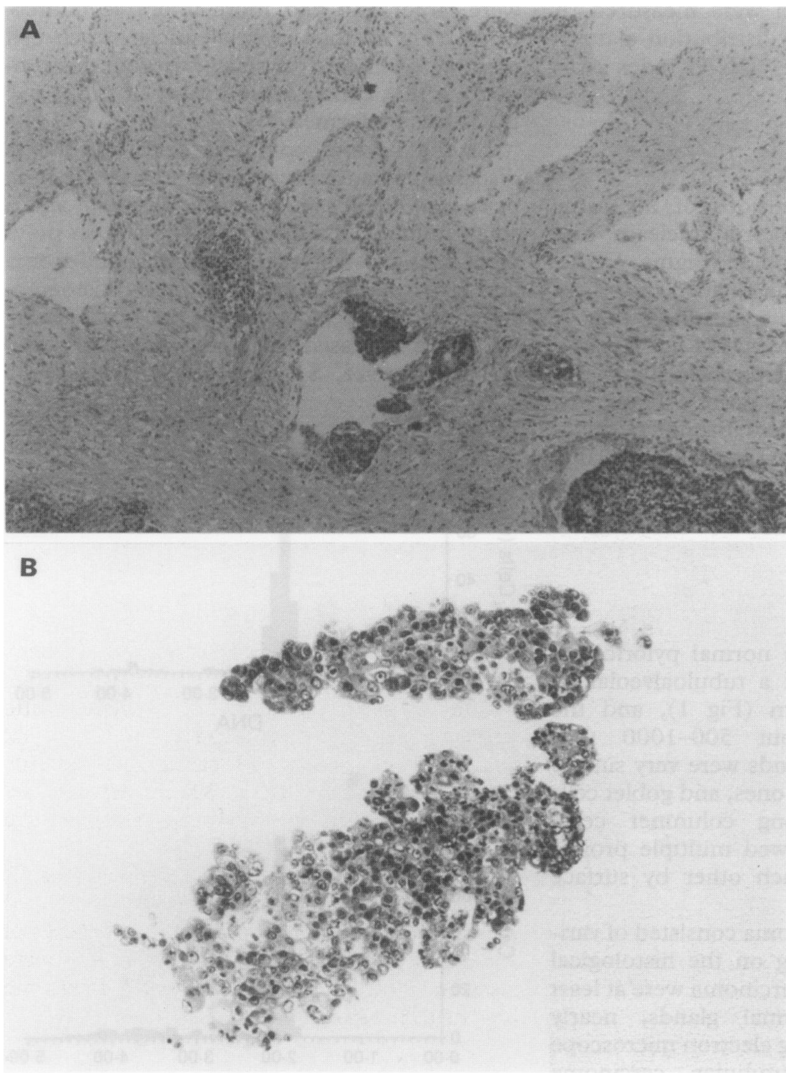

Figure 3: (A) Histological features of medullary carcinoma. Some cavities can be seen in the stroma remaining after cancer tissue isolation (haematoxylin and eosin stain original magnification ×40); (B) photomicrograph showing continuous section of isolated cancer tissue (haematoxylin and eosin stain original magnification ×100).

Cytophotometric measurement of nuclear DNA content

An aliquot of isolated epithelia was dissociated by treatment with 0·025–0·0125% pepsin (pH 1·5) for five minutes at 37°C in a water bath. The single nuclei suspensions in phosphate buffer were filtered through a nylon mesh (37 µm) and smeared on non-fluorescent glass slides with an automatic smear maker (Cytospin 3, Shandon, England), and stained with 4′,6-diamino-2-phenylindole dihydrochloride (DAPI) (Sigma, USA) at a concentration of 50 µg/ml in a buffer (pH 7·4) for 60 minutes at room temperature.[11 14–17] The nuclear DNA content was measured with an epi-illumination cytophotometer (BH2RFL, Olympus, Japan). The nuclear fluorescence intensities of 200 normal epithelial cells were measured to obtain an internal standard of diploid DNA content (2C) and 200 neoplastic

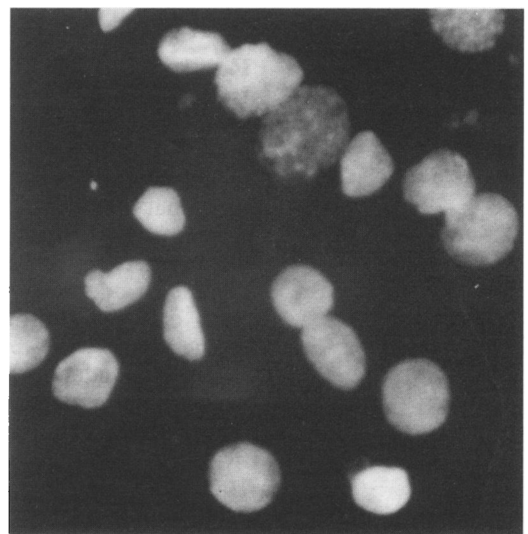

Figure 4: Cell nuclear fluorescence of carcinoma. Fluorescence intensity varied with the position in the cell cycle (original magnification ×400).

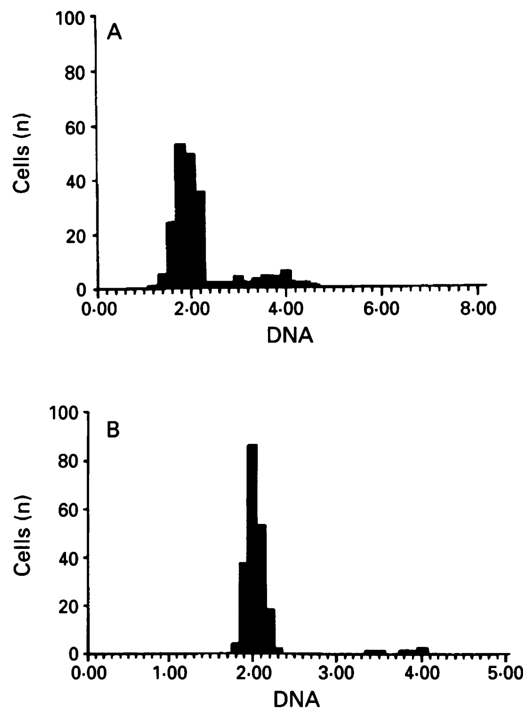

Figure 5: (A) Adenoma with some increased proliferative cells; (B) intestinal metaplasia with few S phase cells.

cells from each specimen were measured. A histogram of the DNA distribution pattern was obtained, and the DNA index was calculated.[11 17 18]

Processing for flow cytometry
The single nuclei suspensions of the same cases were treated with ribonuclease and stained with propidium iodide (Sigma, USA). In each case, samples were divided into three species of single nuclei suspensions – that is, normal cell nuclei, neoplastic cells, and mixtures of normal and neoplastic cell nuclei at a ratio of 1:1 (or at various ratios). These suspensions were on an Epics Profile (Coulter Corporation, Miami, USA) and about 20 000 cell nuclei from each sample were counted. The DNA index and coefficient of variation were also calculated.[19–21]

Results

Isolated glands from the normal pyloric and fundic mucosa disclosed a tubuloalveolar or branched tubular pattern (Fig 1), and the mean length was about 500–1000 μm. Intestinal metaplastic glands were very similar to normal large intestinal ones, and goblet cells were interspersed among columner cells. Glands of adenoma showed multiple projections connected with each other by surface epithelium.

Glands of adenocarcinoma consisted of various structures depending on the histological type. Glands of tubular carcinoma were at least twice as long as normal glands, nearly 1000–2000 μm. Scanning electron microscope examination of papillotubular carcinoma showed glands with irregular branching and length (Fig 2). Occasionally medullary carcinoma was isolated, and isolated cancer tissue

TABLE I *DNA ploidy pattern of gastric mucosal lesions classified by cytophotometry and flow cytometry.*

		Cases classified as aneuploid by			
		Cytophotometry		*Flow cytometry*	
Diagnosis	*Total*	*No*	*%*	*No*	*%*
Cancer					
tub 1	16	4	20·0	14	87·5
tub 2	22	18	81·8	20	90·1
por 1	4	2	50·0	2	50·0
por 2	6	4	66·7	6	100
Total	48	28	58·3	42	87·5
Adenoma	8	0	0	0	0
Hyperplastic polyp	10	0	0	0	0
Intestinal metaplasia	6	0	0	1	16·7

Histological classification of the gastric carcinoma was according to the Japanese Research Society for Gastric Cancer (1985). tub 1, well differentiated tubular carcinoma; tub 2, moderately differentiated tubular carcinoma; por 1 and por 2, solid type and non-solid type of poorly differentiated adenocarcinoma.

and remaining mucosa were seen as well (Fig 3).

DNA histogram by cytophotometry
Fluorescent nuclei of normal, adenoma, and carcinoma cells stained with DAPI showed different features. Nuclei of adenoma were small and spindle shaped, while nuclei of carcinoma were larger and had more intensive fluorescence (Fig 4) than normal nuclei. Each cell maintained its nuclear shape, without destruction, so that exact measurement of single cell DNA was performed.

In the normal epithelial cells, the DNA distribution pattern consisted of a single high peak in (G0/G1) phase in the 2c range and a small number of cells up to 4c with no polyploid cells exceeding the 4c range. In adenoma and hyperplastic polyp, the DNA histogram resembled the normal pattern but proliferating cells were increased (Fig 5). In intestinal metaplasia, however, S phase cells tended to be

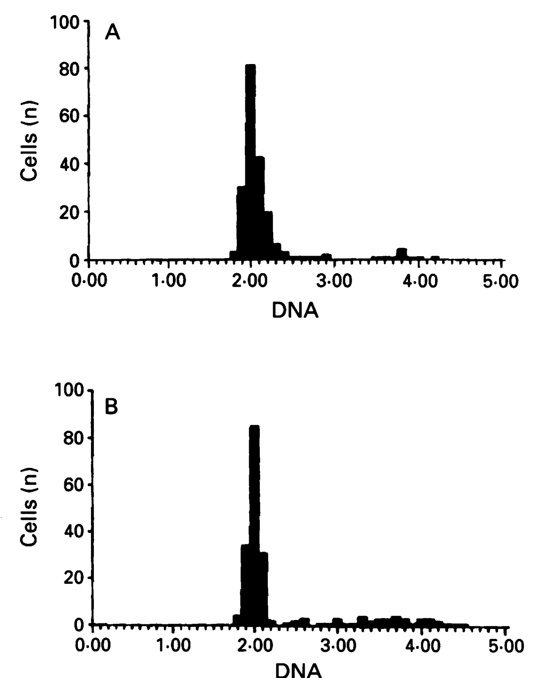

Figure 6: Representative DNA distribution pattern shown by cytophotometry. (A) Normal internal standard; (B) diploidy of the first pattern with a few higher polyploid cells.

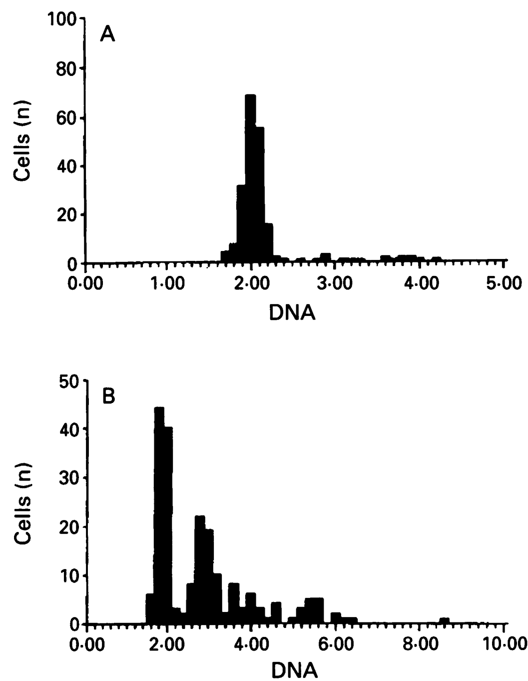

Figure 7: (A) Normal internal standard; (B) aneuploidy showing mosaicism with diploidy and tetraploidy.

diminished (Fig 5). Cancers whose predominant peak had a DNA index <1·10 were considered diploid, while those with a DNA index ≥1·10 were considered aneuploid, and three patterns were recognised.

Diploidy (20 cases) – this pattern consisted of a large population of diploid neoplastic cells with a few higher polyploid cells (Fig 6) (Tables I, IV).

Mosaic ploidy (16 cases) – this pattern showed mosaicism comprising diploid (2c), triploid (3c), tetraploid (4c) populations of proliferative cells (Fig 7). Cancer cells were classified as this pattern if a predominant peak separated from the diploid population exhibited a DNA index between 1·1 and 1·9.

Higher polyploidies (12 cases) – this pattern showed a predominant stemline at the 4c range (Fig 8). In short, the aneuploid peak separated from the G2/M peak exhibited DNA index >1·9.

Twenty eight cases (58·5%) showed aneuploidy (mosaic ploidy: 16 cases, higher polyploidies: 12 cases), and in the tubular type of carcinoma aneuploid cases of moderately differentiated carcinoma (81·8%) were more frequent than those of well differentiated (20·0%). Therefore, according to histological grading, the frequency of aneuploidy of cancer with moderate or poor differentiation was significantly higher than that of well differentiated cancer (χ^2 test: 0·0192, Table II). However, the histological typing and frequency of aneuploidy were not significantly correlated (Table III). Benign lesions were all diploid (Table I).

TABLE II *DNA ploidy and histological grading of gastric carcinomas by cytophotometry*

	Cases classified by cytophotometry	
Grading	*Diploid*	*Aneuploid (%)*
Well differentiated cancer	12	4 (25)
Moderate or poor differentiation	8	24 (75)*

Well differentiated cancer including tub 1; cancer with moderate or poor differentiation including tub 2, por 1, and por 2 (Table I) (*χ^2 test: 0·0192).

TABLE III *DNA ploidy and histological typing of gastric carcinomas by cytophotometry*

	Cases classified by cytophotometry	
Typing	*Diploid*	*Aneuploid (%)*
Cancer		
Papillary	4	0 (0)
Tubular	12	22 (64·7)
Medullary	2	2 (50·0)
Signet	2	4 (66·7)

DNA analysis by flow cytometry

Using flow cytometry, we could distinguish near diploid aneuploidy in eight cases and mosaic ploidies in six of 20 cases of diploidy diagnosed by cytophotometry. On the histogram, near diploid cases had another peak near the G1/G0 (2C) peak and exhibited a DNA index between 1·01 and 1·1 (Fig 9). Besides the three main stemlines of DNA ploidy in carcinoma shown by cytophotometry, a fourth pattern (near diploid aneuploid) was shown by flow cytometry (Table IV). The coefficient of variation of the 78 cases was between 2·03–3·68 (mean=3·12).

The frequency of aneuploidy measured by flow cytometry (87·5%) was higher than that shown by cytophotometry, especially in well differentiated carcinoma (Tables I, V). Furthermore, one of six intestinal metaplasias showed hypo-near diploid aneuploidy (Table I, DNA index=0·97). With progress of cancer stage and lymph node metastasis, the percentage of aneuploid cases decreased. There were no clear correlations, however, between other clinicopathological variables and DNA patterns (Table V).

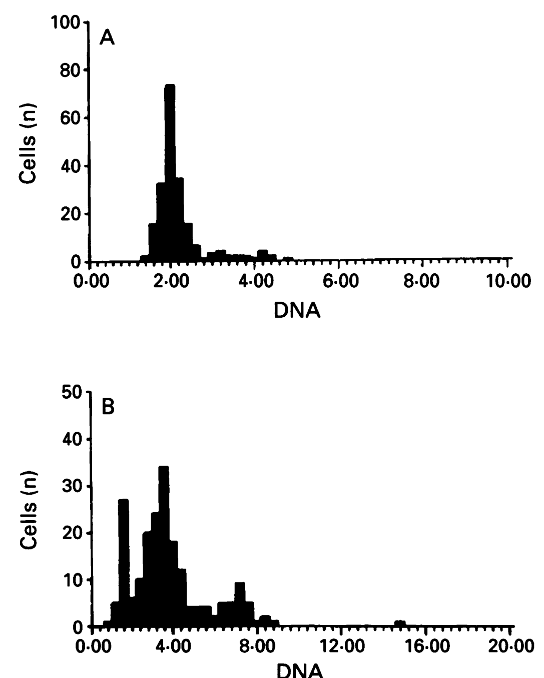

Figure 8: (A) Normal internal standard; (B) aneuploidy showing higher polyploidy by cytophotometry.

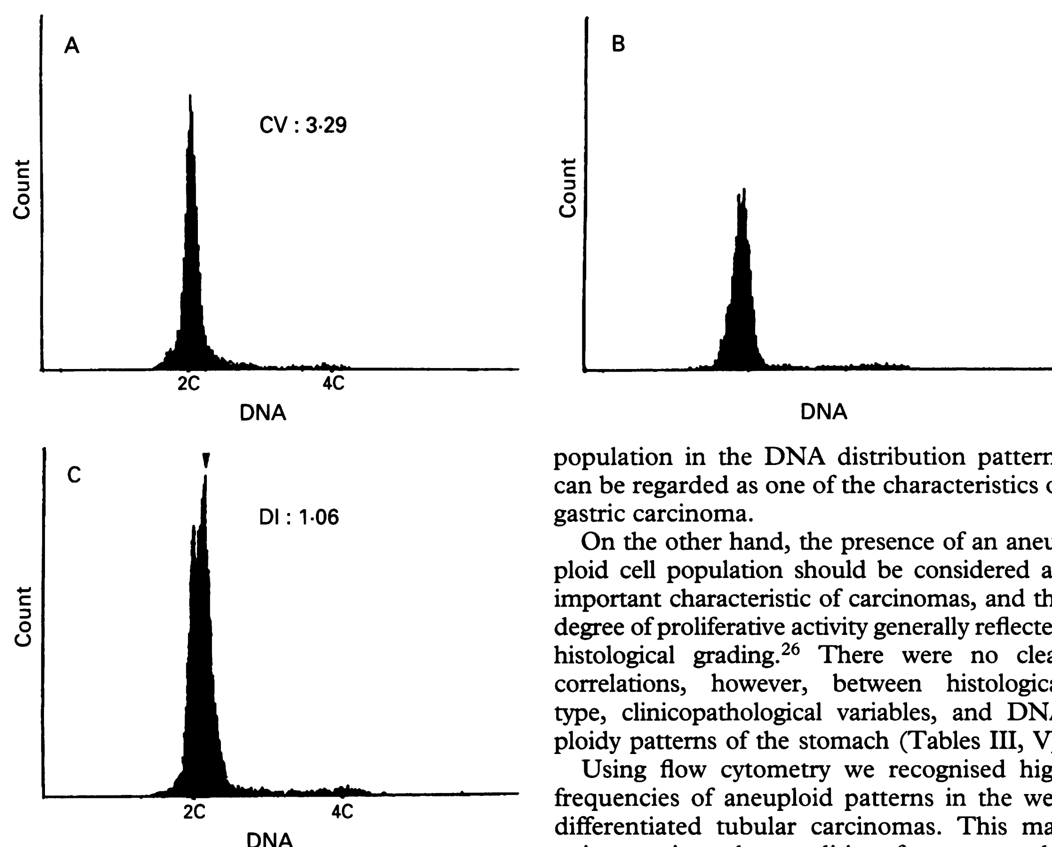

Figure 9: Flow cytometric DNA distribution pattern of near diploidy. (A) Normal internal standard; (B) cancer cell nuclei; (C) mixture of normal and cancer cell nuclei at a ratio of 1:3, as determined by flow cytometry. Near diploidy had another peak (arrowhead) near the 2C.

Discussion

Both enzymatic and mechanical methods of obtaining epithelial cells yield mixtures that include interstitial cells. Therefore, there is always doubt as to whether or not the entire epithelium is present in the isolated materials.[22–25] In case of gene analysis, including DNA measurement, of cancer tissue, contamination by interstitial cells might give rise to serious error. On the other hand, the non-enzymatic gland isolation method used here, which was based on the method by Cheng *et al*,[7–9] and modified by Arai,[11 12] yielded viable glands composed entirely of epithelial cells without contamination by interstitial cells. Most tubular glands of carcinoma could be isolated from the stroma.

The frequency of aneuploid determined by cytophotometry generally reflected the histological grade of the gastric carcinoma. As a rule, aneuploidy was more frequent in the moderately differentiated tubular carcinomas than in well differentiated ones.[17] Aneuploid cases of gastric carcinomas, were significantly more frequent (87·5%) in this study than in others[1 6] that used paraffin wax embedded tissue or frozen tissue. And all the carcinomas showed some polyploid cell nuclei on cytophotometry. Thus, existence of a polyploid cell population in the DNA distribution patterns can be regarded as one of the characteristics of gastric carcinoma.

On the other hand, the presence of an aneuploid cell population should be considered an important characteristic of carcinomas, and the degree of proliferative activity generally reflected histological grading.[26] There were no clear correlations, however, between histological type, clinicopathological variables, and DNA ploidy patterns of the stomach (Tables III, V).

Using flow cytometry we recognised high frequencies of aneuploid patterns in the well differentiated tubular carcinomas. This may point to minor abnormalities of genome at the onset of neoplasia or in low grade adenocarcinoma, which have been overlooked by ordinary flow cytometry.

Adenomas and hyperplastic polyps had no aneuploid stemline, but had more proliferative activity than normal tissue as estimated from the DNA pattern and increased number of S phase cells on cytophotometry (Fig 5). The degree of proliferative activity generally reflected the grade of histological atypia. Only one case of intestinal metaplasia in our study showed hypo-near diploid aneuploidy and decreased S phase cells, while Weiss *et al* pointed out that in some cases of intestinal metaplasia S phase cells tend to be diminished. They pointed out the same gene abnormality in other benign lesions, such as chronic gastritis and adenomas.[20]

Further investigation using this isolation method might help to analyse precancerous conditions or minimally deviating neoplasms to detect possible near diploid tumours.

TABLE IV *Ploidy distribution in 48 gastric carcinomas measured by cytophotometry and flow cytometry*

	Diploid	*MP*	*HP*	*ND*	*Total*
Cytophotometry	20	16	12	0	48
Flow cytometry	6	22	12	8	48

MP: mosaic ploidy, HP: higher polyploidy, ND: near diploidy.

TABLE V *DNA aneuploidy determined by flow cytometry and clinicopathological variables*

Cases	*No examined*	*No positive (%)*
Sex		
Male	30	26 (86·7)
Female	18	16 (88·9)
Age		
<60	8	8 (100)
⩾60	40	34 (85·0)
Grade		
Well differentiated	16	14 (87·5)
Moderate or poor differentiation	32	28 (87·5)
Stage		
I	6	6 (100)
II	18	18 (100)
III	16	12 (75·0)
IV	8	6 (75·0)
Lymph node metastasis		
Positive	40	34 (85·0)
Negative	8	8 (100)

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