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# Characterization of a Continuous Cell Line in Culture Established from a Krukenberg Tumour of the Ovary Arising from a Primary Gastric Adenocarcinoma

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**Abstract**—*A unique cell line has been established in culture from a biopsy taken from a Krukenberg tumour of the ovary arising from a primary gastric adenocarcinoma. This continuous cell line, designated KS-1, displayed epithelial-type morphological characteristics and approx. 1% of the cells had eccentric nuclei featuring the signet ring-like cells observed in the original tumour: these proved positive for neutral and acidic mucopoly saccharides. KS-1 cells have been maintained in suspension culture and had a population doubling time in logarithmic growth of approx. 28 h. They readily formed colonies in soft agar or agarose with efficiencies of 10–15%, but failed to produce tumours on inoculation into nude mice. KS-1 cells had a modal chromosome number of 60–65, with a range of 48–77%. Ten to 30 double minutes were present in most cells and several clonal marker chromosomes were identified. The effects of a 24 h in vitro exposure of KS-1 cells to a range of concentrations of 5-fluorouracil, adriamycin, mitomycin C or cisplatin have been quantitated by clonogenic assay and these values have been compared with those reported using a range of other human tumour cell lines, under comparable experimental conditions.*

## INTRODUCTION

THE ESTABLISHMENT of human tumours in continuous culture has provided a valuable source of material for studies of the biology of specific tumour types, cytogenetic analyses and *in vitro* drug sensitivity evaluations. In view of the cellular heterogeneity of tumours now widely reported [for example, 1–3], it is obviously important that a large panel of different cell lines are used in such studies. We have described previously the establishment of four new continuous cell lines derived from squamous cell carcinomas of the head and neck [4] and three from ovarian carcinomas [5]. During the course of this latter study we received a specimen for culturing from a patient with bilateral ovarian carcinoma, but suggestive of a Krukenberg tumour. Subsequently, a primary gastric carcinoma was diagnosed in this patient. Krukenberg tumours have engendered considerable controversy since their histologic appearance was originally described in 1896 [6], when they were erroneously classified as primary ovarian sarcomas. Schlagenhaufer, however, pointed out in 1902 their metastatic nature and identified the most frequent primary source as being gastrointestinal carcinoma [7]. The term 'Krukenberg tumour' was applied loosely to any adenocarcinoma metastasizing to the ovaries before definite criteria, which are currently used by the WHO classification, were proposed for the diagnosis of Krukenberg tumour: (i) cancer in the ovary; (ii) intracellular mucin production by neoplastic signet-ring cells; and (iii) diffuse sarcomatoid proliferation of the ovarian stroma [cf. 8]. In spite of its notoriety, however, the typical Krukenberg tumour remains relatively uncommon, accounting for only 3–5% of ovarian tumours in the Western world [9].

In this report we describe the establishment of a continuous cell line from this Krukenberg tumour specimen and define certain biological features with reference to *in vitro* growth characteristics and karyotype. In addition, we have demonstrated that these cells readily form colonies in semi-solid medium and therefore provide a useful system for *in vitro* drug sensitivity evaluations. To our knowledge this

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study is the first describing the establishment of a continuous human tumour cell line from this tumour type.

## MATERIALS AND METHODS

*Origin of the tumour material*

The 55-year-old patient presented in April 1984 with dyspepsia and a large solid pelvic swelling extending to her umbilicus. She had no previous history of malignancy and these symptoms had arisen within the last 2 months. At laparatomy a bilaterial ovarian tumour involving pelvic nodes, with pelvic adhesions and bowel deposits, was found. The right kidney appeared hydronephrotic. There was no liver abnormality and the omentum and mesentery was clear. Tumours were removed from both ovaries, histological examination of which showed a poorly differentiated solid adenocarcinoma with an extensive signet-ring cell component (see Fig. 1A). This was highly suggestive of secondary deposits from a gastric carcinoma ('Krukenberg tumours'), as primary signet-ring cell ovarian carcinomas are exceedingly rare. Subsequently, gastric biopsy confirmed a diffusely infiltrating primary gastric carcinoma of similar appearance (Fig. 1B). At this time residual disease was widespread in the abdomen as small sized nodules. The patient was referred for monoclonal antibody guided therapy, but before this was initiated she developed a partial and then complete obstruction. A transverse defunctioning colostomy was performed in July 1984. No further therapy was instigated before the patient's death at the end of 1984.

*Initiation and maintenance of the KS-1 cell line*

The tumour was finely minced with cross scalpels in Ham's F12 medium containing 10% foetal calf serum (FCS). The resulting cell suspension, including small tissue fragments, was transferred into tissue culture flasks (Falcon 3013E) and incubated at 37°C in 5% $CO_2$ in air. An epithelial cell line was established initially as a monolayer and subsequently in suspension culture and designated KS-1. KS-1 has been maintained in suspension culture for 2 years, passaging by dilution (1:5) in Ham's F12 medium plus 10% FCS on a weekly basis with frozen stocks established at various passages. Cultures were judged by Hoechst 33258 fluorescence, orcein staining and agar subculture to be mycoplasma free.

In vitro *growth characteristics*

The rate of cellular proliferation was measured for suspension cultures in logarithmic growth phase at a starting concentration of $2 \times 10^4$ cells per ml in Falcon 2051 tubes. Population doubling times were determined from cell counts of duplicate tubes at daily intervals over 7 days without refeeding. Cell volumes of logarithmically growing cells derived from suspension cultures were determined using the Coulter Counter Model ZBI (Coulter Electronics Inc., Luton, Beds., U.K.) and their cellular protein and DNA contents were estimated using the methods of Lowry *et al.* [10] and Burton [11] respectively. Cell cycle distributions were analysed by measurement of relative DNA contents of individual cells derived from suspension cultures using a fluorescence-activated cell sorter (FACS-1, Becton Dickinson, Mountain View, CA, U.S.A.) as described elsewhere [12]. Documented techniques were used for isozyme analyses [13] and electron microscopy [cf. 4]. Histochemical sections were prepared from cell pellets fixed in 10% formalin solution and stained with periodic acid–Schiff (PAS), alcian blue and toluidine blue stains according to standard procedures.

Anchorage-independent growth was defined as the ability to generate colonies containing 50 cells or more in semi-solid medium. Two methods were used either the 'conventional' assay employing 0.18% soft agarose [14] or the Courtenay assay using 0.3% agar [15].

*Growth in nude mice*

Female homozygous nude mice were injected subcutaneously with $10^7$ cells suspended in 0.1 ml of phosphate buffered saline.

*Chromosome analyses*

Monolayer cultures were used and grown to confluency before being split 1:2 in fresh medium. Following overnight incubation metaphase spreads were prepared according to standard procedures and analysed using trypsin G-banding.

*Drug sensitivity testing*

Logarithmically growing cells in suspension cultures were exposed to a range of concentrations of either 5-fluorouracil (Roche Products Limited, Welwyn Garden City, Herts., U.K.), adriamycin (kindly donated by Farmitalia Carbo Erba, St. Albans, Herts., U.K.), mitomycin C (Sigma Chemical Company Limited, Poole, Dorset, U.K.), or cisplatin (Sigma) for 24 h at 37°C. At the end of the exposure period, the cells were washed twice with serum-containing medium and colony-forming ability of tumour cells surviving drug treatment was measured using the Courtenay soft-agar assay [15]. The number of clonogenic cells surviving drug treatment was expressed as a percentage of untreated controls. All experimental assays illustrated were performed in triplicate on a minimum of two independent occasions.

## RESULTS

*Characteristics of the KS-1 cell line*

KS-1 cultures were propagated as rounded cells floating free in suspension and formed numerous

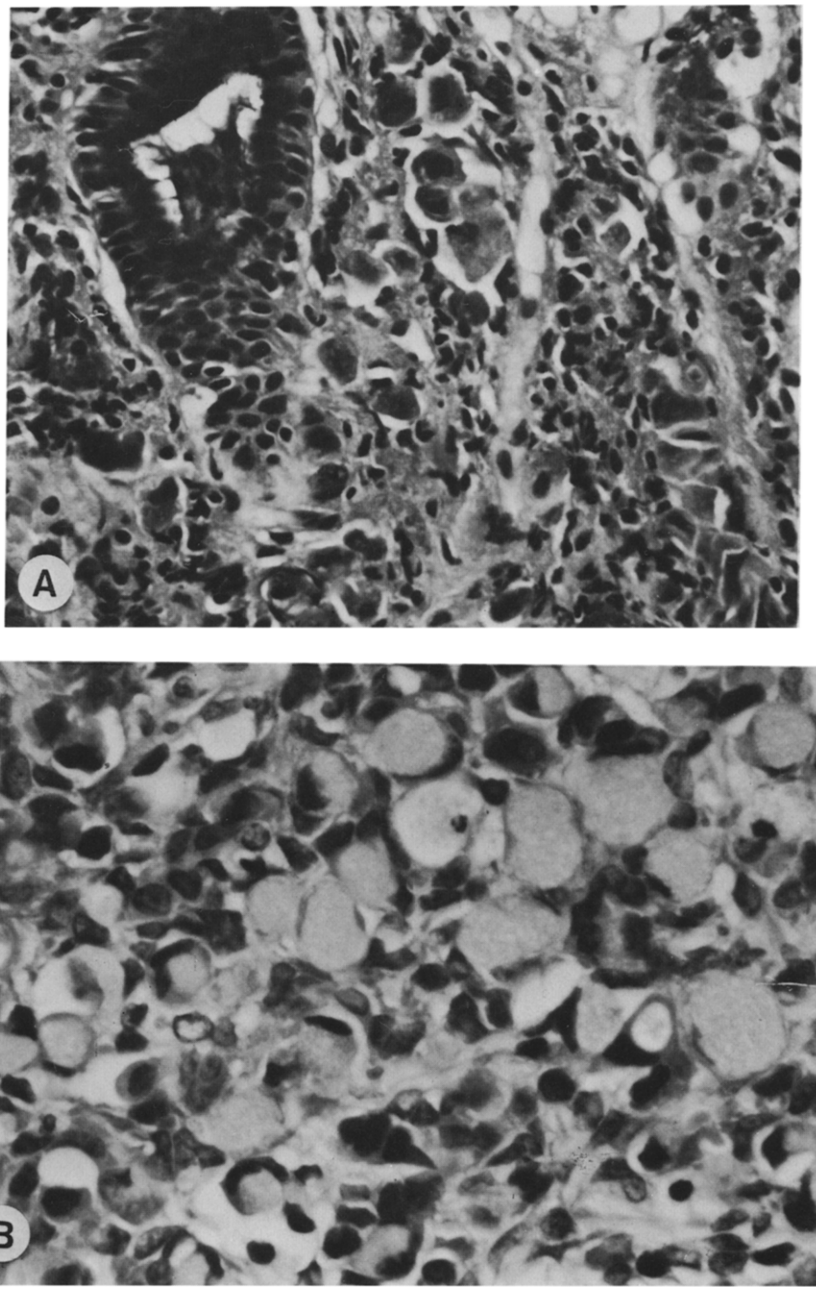
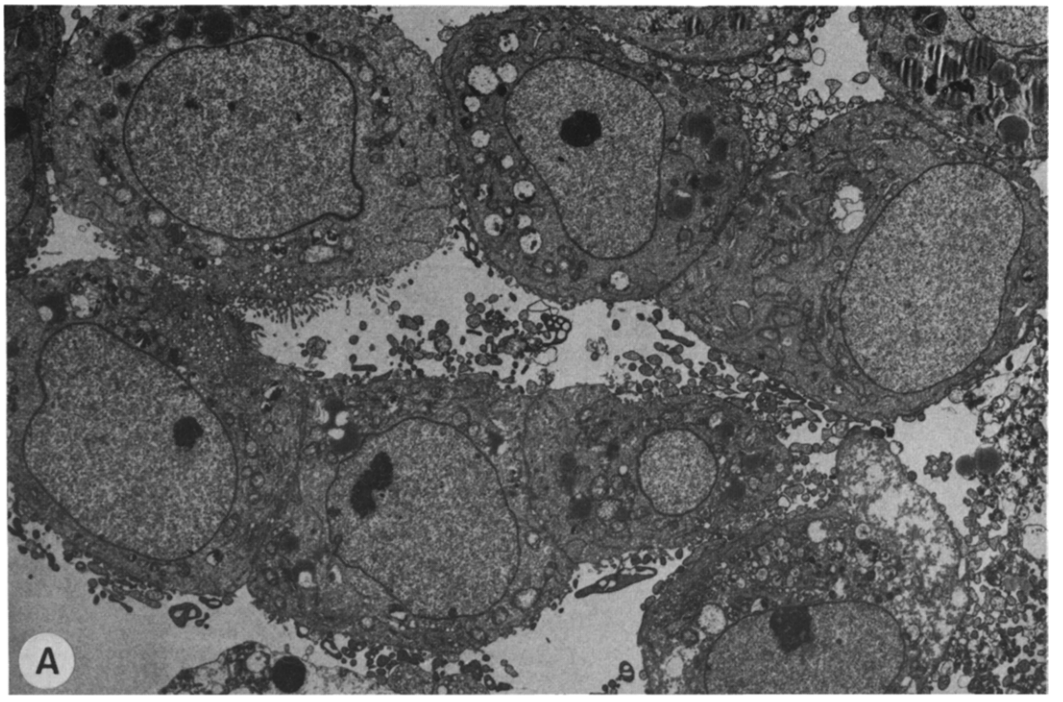



*Fig.* 1. *Histologic sections of* (*A*) *the ovarian tumour composed of pleomorphic malignant cells, some distended with mucin and displacing the nucleus* (*signet-ring cells*), *admixed with small dark ovoid cells from the ovarian stroma, H & E* × 760, *and* (*B*) *a biopsy of the primary gastric tumour showing similar poorly differentiated malignant cells infiltrating the lamina propria around benign glands, alcian blue* × 600.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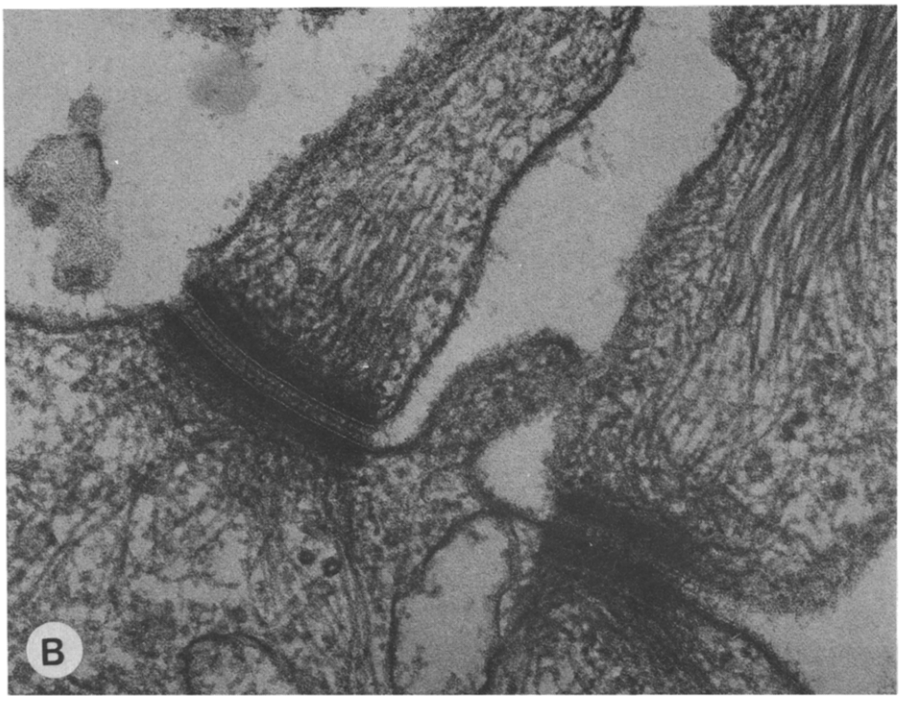
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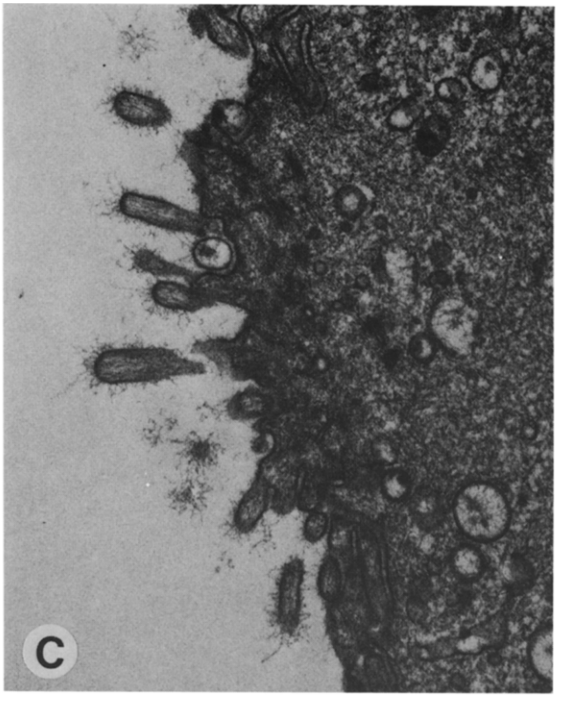


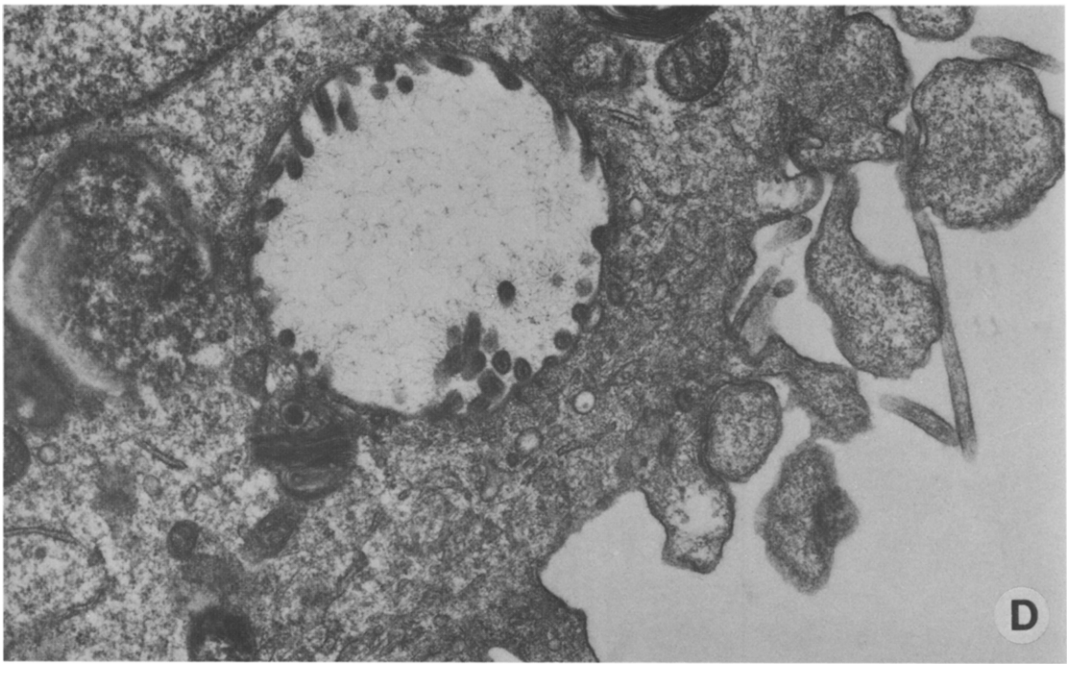


*Fig.* 2. *Electron micrographs of KS-*1 *cell, derived from cell aggregates from suspension cultures, illustrating their epithelial nature* (× 2940) (*A*), *a desmosomal contact* (× 108,000) (*B*), *surface type epithelial microvili* (× 29,400) (*C*), *some of which have intracellular villi* (× 29,400) (*D*).

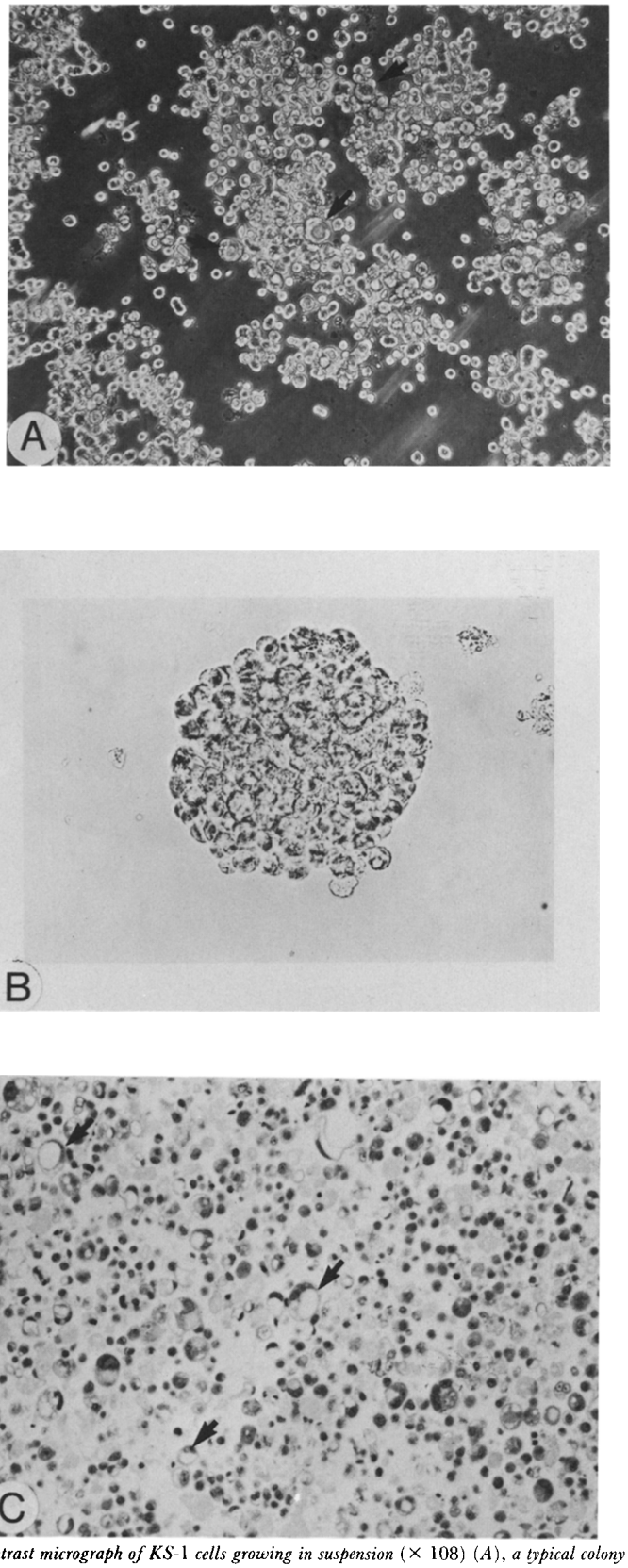


*Fig. 3. Phase-contrast micrograph of KS-1 cells growing in suspension* (× 108) (*A*), *a typical colony of KS-1 cells grown in soft agar* (× 270) (*B*) *and a haemotoxylin and eosin-stained section of a cell pellet* (*C*), *with 'signet ring' cells marked by arrows* (× 135).

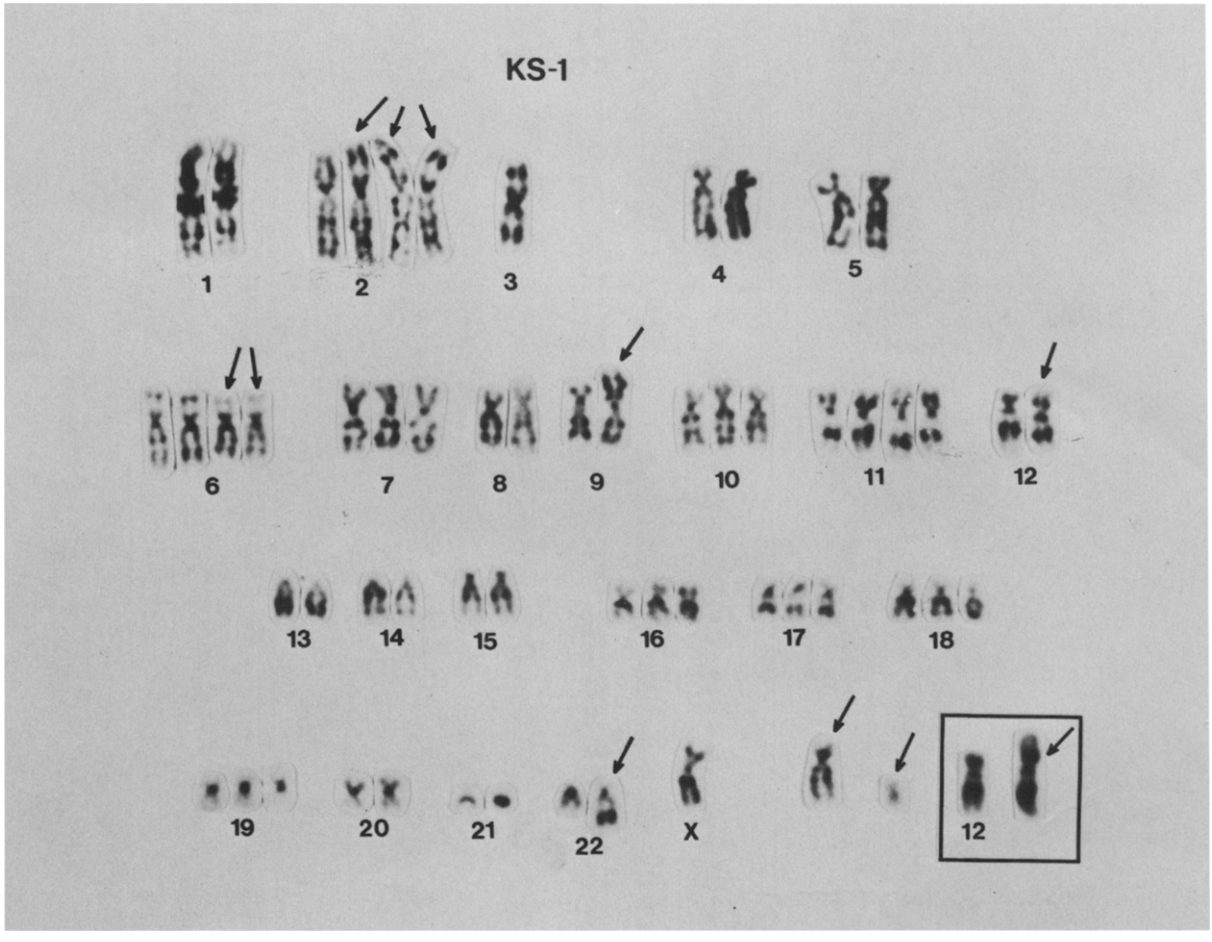


*Fig. 4. G-Banded karyotype of KS-1. Rearranged chromosomes are arrowed, placed alongside their chromosomes of origin if known. Boxed inset shows normal chromosome 12 with a rearranged chromosome from a different cell to that karyotyped.*

loosely packed aggregates of variable size, which could be easily dissociated into a single cell suspension by gentle pipetting. KS-1 cells also grew as monolayers and when examined under phase contrast microscopy displayed epithelial-type morphological characteristics. Electron microscopy of cell aggregates confirmed (see Fig. 2A) the epithelial characteristics of these tumour cells. Although many of the cells are undifferentiated, some (see Fig. 2B) retained desomosomal contact. This was of varying complexity linking adjacent cells. Surface type epithelial microvilli were present (see Fig. 2C) with attached glycoprotein strands and some had intracellular villi (see Fig. 2D). At one cell interface a tight junction was apparent. Intracytoplasmic vacuoles, some containing fat, were noted and others had apparently secretory inclusions. In general, these features were consistent with the classification of the original tumour (see Figs. 1A and 1B) from the patient as a poorly differentiated adenocarcinoma. In culture, only a small proportion of KS-1 cells (approx. 1%) (see Fig. 3A) have eccentric nuclei featuring these signet ring-like cells, which, however, proved positive for neutral and acidic mucopolysaccharides when stained with PAS, alcian blue at pH 2.5 and toluidine blue.

KS-1 cells maintained in suspension culture had a population doubling time of approx. 28 h when in logarithmic growth. Selection of cells which readily attached to plastic as monolayer cultures, resulted in a population of cells with a slightly longer doubling time in logarithmic growth phase of 36 h. In all subsequent experiments, suspension cultures of cells were used and their DNA and protein contents, cell volumes and cell cycle distributions are listed in Table 1. KS-1 cells readily formed colonies in soft agar or agarose (see Table 1 and Fig. 3B), but failed to produce tumours in nude mice when inoculated subcutaneously into 20 animals. Attempts, however, to clone out the signet-ring cell component and establish a separate line were not successful.

The isozyme profile of the KS-1 line (see Table 2) was identical (with the exception of GOTm which is not expressed in red blood cells) to that of red blood cells taken from the patient from whom the original biopsy was obtained, and was clearly distinguishable from that of HeLa cells.

*Cytogenetic analyses*

A modal chromosome number of 60–65, with a range of 48–77 chromosomes, was found in 20 metaphase spreads counted. Ten to 30 double minutes were present in most cells. Several clonal marker chromosomes were present, including a translocation chromosome consisting of the long arms of chromosomes 2 and 3, a chromosome 6 which was apparently deleted distal to band 6q23, and translocation chromosomes derived from chromosomes 9, 12 and 22 (see Fig. 4). There were often several copies of these markers, but the exact number varied from cell to cell.

In vitro *drug sensitivity evaluations*

The effects of a 24 h *in vitro* exposure of KS-1 cells to a range of concentrations of four antitumour drugs, including the three constituting the 'FAM' protocol which has demonstrated clinical activity against gastric cancer [16], are shown in Fig. 5. The $IC_{50}$ values are listed in Table 3 and compared with those reported using a range of other human tumour cell lines, under comparable experimental conditions.

## DISCUSSION

We have established a continuous cell line from a surgical biopsy taken from an ovarian tumour which was subsequently diagnosed as a Krukenberg tumour arising from a primary diffuse gastric adenocarcinoma. The *in vitro* properties and karyology of the cells have been characterized. This line is readily propagated in suspension culture as loose aggregates of cells from which a cell suspension was obtainable by simple pipetting, features previously reported for a number of gastric cell lines [18, 19]. The characteristic signet-ring component of the original tumour was also present in the cell line which proved positive histochemically for mucin secreting cells, although the masses of retained mucin distending the nucleus and cytoplasmic contents reported by Wong *et al.* [20] to be present in sections made directly from Krukenberg tumour biopsy samples were not evident in the cell line. On serial propagation in culture the proportion of these signet-ring cells declined to less than 0.1% and none could be identified in the colonies which formed readily in soft agar. We have found in the literature one other report of cultured cells having eccentric nuclei featuring signet-ring cells containing mucin [19]. These were derived from a pleural effusion from a patient with recurrent gastric cancer. This KATO II line had been maintained in culture for more than 132 passages, but no reference was made as to whether the proportion of signet-ring cells remained constant.

The *in vitro* population doubling time, DNA and protein contents, cell volumes and cell cycle distribution of this KS-1 cell line were unremarkable. Values were comparable with those reported by ourselves and others in a range of human tumour cell lines including those derived from primary or metastatic ovarian cancers and gastric tumours [for example, 4, 5, 18, 21–23]. Although these KS-1 cells readily formed colonies in soft agar, a characteristic consistent with tumourigenicity, tumours did not form following inoculation of these cells into nude mice. These two features are not invariably

*Table* 1. In vitro *characteristics of the KS-1 cell line*

| Characteristic | Determined value |
|---|---|
| Population doubling time (h): | suspension cultures 36 ± 2<br>monolayer cultures 28 ± 2 |
| μg DNA per $10^6$ cells | 20.5 ± 1.0 |
| μg protein per $10^6$ cells | 288 ± 6 |
| Cell volume ($\mu^3$): | 2668 ± 41 |
| Cell cycle distribution by FMF (%): | $G_1$ 53 ± 5; S 20 ± 2; $G_2$ + M 27 ± 2 |
| Colony-forming ability (%): | in soft agarose 9.6 ± 1.0<br>in soft agar 15.4 ± 0.5 |
| Xenograft formation in nude mice: | nil |
| Modal chromosome numbers (range): | 62–65 (48–77) |

*Table* 2. *Isozyme phenotypes*

| | $PGM_1$* | $GOT_m$ | ESD | ADA | G6P | ACP | GLO | PGD |
|---|---|---|---|---|---|---|---|---|
| KS-1 cell line | 2 | 1 | 2-1 | 1 | B | BA | 2 | 1 |
| rbc from patients | 2 | — | 2-1 | 1 | B | BA | 2 | 1 |
| HeLa cells | 1 | — | 1 | 1 | A | BA | 2 | 1 |

*$PGM_1$: first locus of phosphoglucomutase; $GOT_m$: 'mitochondrial' glutamase–oxaloacetate transaminase: ESD: esterase D; ADA: adenosine deaminase; G6P: glucose-6-phosphate dehydrogenase; ACP: acid phosphatase 1; GLO: glyoxalase; PGD: phosphogluconate dehydrogenase.

*Table* 3. In vitro *drug sensitivity evaluations*

| Drug tested (24 h exposure) | $IC_{50}$ values (μg/ml) KS-1 cells | Range of values obtained with other human tumour cell lines (No. tested)* |
|---|---|---|
| 5-Fluorouracil | 2.80 | 0.13–3.03 (15) |
| Adriamycin | 0.04 | 0.006–0.03 (18) |
| Mitomycin C | 0.014 | 0.01–0.03 (4) |
| Cisplatin | 0.25 | 0.01–0.79 (17) |

*Reproduced in part from Ref. [17].

linked, however, for example, the HOC-7 ovarian cell line [23] has a reported 10% colony-forming efficiency *in vitro* and two bladder cell lines, namely T24 and TCCSUP, with cloning efficiencies on plastic of 61% and 27% respectively [24], all failed to form tumours in nude mice. It may also be significant that three human gastric cancer cell lines which formed tumours in the cheek pouches of hamsters pretreated with rabbit antimouse thymocyte serum failed to produce even nodules following injection into athymic nude mice [18, 19].

The karyotype of KS-1 cells shows the typical pattern of a cell line derived from a solid tumour, i.e. a hypo-triploid chromosome number with several chromosome rearrangements. These rearrangements involved chromosomes 2, 3, 6, 9, 12 and 22, as well as other unidentified chromosomes. Complex karyotypes have been observed in other gastric tumours, but highly consistent chromosome aberrations have not been noted [25–28]. However this analysis is limited by the small number of gastric tumours which have been karyotyped and reported on. In one study of five primary gastric tumours, chromosome bands 1p22, 3p21 and 19p13 were frequently involved in rearrangements [27]. None of the marker chromosomes in KS-1 cells appeared to have these break points, although the selected chromosome 6 in KS-1 cells might be important since three cases reported by Ochi *et al.* [27] had deletions of the same region of chromosome 6.

This KS-1 cell line is likely to prove a useful *in vitro* model system to evaluate effects of antitumour drugs on cell survival. As illustrated in Table 3, the $IC_{50}$ values for the four drugs tested appear to fall within the ranges obtained in this laboratory using

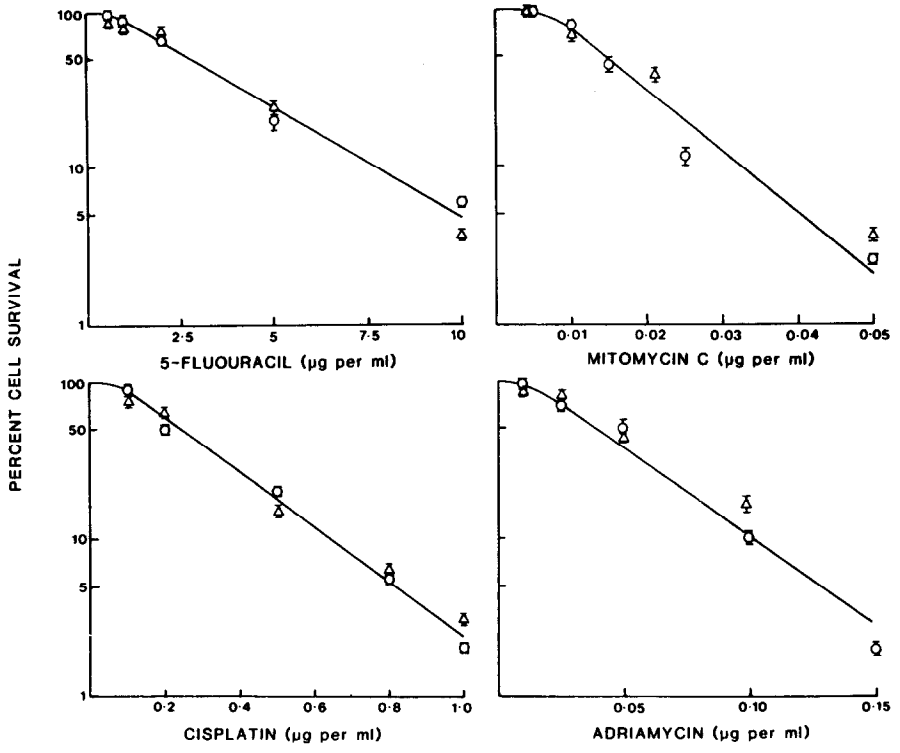


*Fig. 5. Dose–response curves following* 24 h *exposures of logarithmically growing suspension cultures of KS-*1 *cells to* 5-*fluorouracil, mitomycin C, cisplatin or adriamycin. Survival was assessed by colony formation in soft agar. Each point represents the mean of at least three estimations* ± S.E.M. *Data from two separate experiments are plotted individually as* △ *or* ○.

other cell lines derived from various types of human tumours. Interestingly, these drug concentrations are very comparable with those used by Weiss and Von Hoff [29] for their human tumour cloning assay on ovarian, gastric, pancreatic and colorectal cancers, although these authors used only a 1 h drug exposure. These values, which were considered equivalent to one-tenth of a peak plasma concentrations achievable clinically, were as follows: 5-fluorouracil 6–10 μg/ml; cisplatin 0.2–1.0 μg/ml; doxorubicin 0.04–0.40 μg/ml; mitomycin C 0.1μg/ml. Barranco *et al.* [30] reported that doxorubicin (adriamycin) was significantly more effective than 5-fluorouracil against a human stomach cancer cell line and four subclones, when compared on a dose for dose basis. Again our data are consistent with this observation.

This KS-1 cell line derived from a Krukenberg tumour therefore provides a new, and as far as we are aware, unique addition to the bank of human tumour cell lines available for use in further biological and experimental chemotherapy studies.

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