POTENTIAL PROGNOSTIC INDICATORS FOR THE RADIOTHERAPY OF CERVICAL CARCINOMA

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by

SUSAN ELIZABETH DAVIDSON MB.BS

graduate of Middlesex Hospital Medical School

University of London

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Department of Radiobiology
Paterson Institute for Cancer Research
Christie Hospital and Holt Radium Institute
Wilmslow Road
Withington
Manchester. M20 9BX

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ABSTRACT

Biopsies from 117 patients with proven cervical carcinoma were taken under anaesthesia immediately prior to radiotherapy. The Courtenay Mills soft agar clonogenic assay was used to determine the colony forming efficiency (CFE) of tumour cells from disaggregated tumours and the survival of these cells following radiation. Validation of the assay was carried out by demonstrating linearity of colony formation and production of radiation survival curves. The mean CFE was 0.18±0.49% (±1sd) with a range of 0.003 - 4.28% (based on total viable nucleated cell counts) for 84 (72%) specimens. No significant association was demonstrated between in vitro growth and either clinical stage (r=0.02) or tumour differentiation (r=0.08).

A wide range of values (0.13-0.97) for surviving fraction at 2Gy (SF2) was obtained with a mean value 0.44 (sd=0.19) for 77 tumours. There were statistically significant differences between the individual tumours (p=<.001). Heterogeneity in intrinsic radiosensitivity was not demonstrated (p=0.3) when multiple biopsies were processed independently from 18 tumours. From analysis of variance of the SF2 results it appears that the surviving fraction below 0.4 and those above 0.7 which show significant differences in radiosensitivity between pairs of tumours (p=005).

Differential cell counts were made on cytospin preparations of tumour cell suspensions. There was no correlation between either CFE or SF2 (r = -0.05, r = 0.15, respectively) and the degree of lymphocyte (r = 0.12) or macrophage (r = 0.001) infiltration.

Ki67 staining of 29 specimens gave a mean proportion of positively stained nuclei of 20.5% (sd=23). The Ki67 index was not correlated with tumour stage (r=0.58), differentiation (r=0.02), CFE (r=0.04) or SF2 (r=-0.07).

Vascularity was assessed on paraffin sections of 87 tumours. The mean intercapillary distance (ICD) was 240μm (sd=37μm) and mean proportion of vessels was 2.68% (sd=1.64%). Clinical data from 35 patients with 2 year minimum follow-up revealed no significant difference between the mean ICD or mean proportion of vessels for the group of patients which had died or recurred and the group which remained disease free using t-tests (t=0.74, p=0.47, DF=32; t= 0.6, p=0.55, DF=29 respectively).

Follow up clinical data on predictive value of SF2 - see enclosure III

LIST OF ABBREVIATIONS

APAAP	-	alkaline phosphatase - anti alkaline phosphatase
CAM	-	cell adhesion matrix
CFE	-	colony forming efficiency
DAB	-	diaminobenzidine
DMSO	-	dimethylsulphoxide
FCS	-	foetal calf serum
HPF	-	high power field
HCTA	-	human tumour clonogenic assay
ICD	-	intercapillary distance
INT	-	iododinitrotetrazolium
LI	-	labelling index
MTT	-	3-4,5 dimethyltriazol-2-yl) 25 diphenyl tetrazolium bromide
PAP	-	peroxide alkaline phosphatase
PBS	-	phosphate buffered saline
PLDR	-	potentially lethal damage repair
SF2	-	surviving fraction at 2 Gy
SLDR	-	sub lethal damage repair
TAM	-	tumour associated macrophage
TBS	-	tris buffered saline
TIL	-	tumour infiltrating lymphocyte
TLI	-	thymidine labelling index

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PAPERS ENCL.

(I) Davidson SE et al. Radiosensitivity testing of primary cervical carcinoma. Evaluation of intra- and inter-tumour heterogeneity. Radiother. Oncol. (in press).

(II) West CML et al. Evaluation of surviving fraction at 2 Gy as a potential prognostic factor for the radiotherapy of carcinoma of the cervix. Int. J. Radiat. Biol. (1989), 56: 761-765.

III Proceedings 9th International Congress of Radiation Research Eds WC Dewey, M Edington, RJM Fry, EJ. Hall, G.F. Whitmore. West CML et al: Surviving fraction at 2 Gy versus control of human cervical carcinoma - update of the Manchester Study

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1. INTRODUCTION

1.1 CLINICAL BACKGROUND

1.1.1 Incidence

Survival rates of patients treated for invasive carcinoma of the cervix have shown little improvement over the last two decades. The overall 5 year survival is 54% and is dependent on initial tumour stage, ranging from 76% for stage I to 7% for stage IV (FIGO, 1988). In the UK, cervical carcinoma accounts for 3% of cancer deaths in women (2,170 deaths per annum, CRC Figs, 1989). Between 1950 and 1988 there has been considerable variation in the age specific mortality trends. There has been a decrease in mortality in women >45 years of age and an increase in younger women 25-34 years, with the percentage of all deaths occurring in this group increasing from 2.5 to 5.5% between 1971 and 1988 (CRC Figs, 1989).

1.1.2 Prognostic Factors

Within the group of patients who have stage I disease there is variation in the 5 year survival, depending on whether the pelvic lymph nodes are involved or not, with 5 year survival being 83% in the group with negative nodes and 54% with positive nodes (FIGO, 1988). At present, stage is the most important prognostic factor in cervical carcinoma. Size of the primary tumour is important also, with local recurrence increased with bulky tumours (Jampolis et al, 1975; van Nagell, 1979; Perez et al, 1983), although the present staging system does not adequately take tumour volume into account (Hunter et al, 1986). The volume of disease has been shown to be predictive of nodal involvement as well as of outcome (Dembo et al, 1987).

Pelvic recurrence is significant in this disease and varies according to the stage of disease at presentation; 3-6% for stage I disease rising to 45% for stage III disease (Hunter et al, 1986; Davidson et al, 1989). Approximately 35% of all patients with cervical cancer will have recurrent or persistent tumour and the cause of death in 60% of these patients is related to local failure, with 80% of these recurrences occurring within 2 years of treatment (Perez, 1988) and 94% within 3 years (Jampolis et al, 1975). Of these recurrences, 75% have been shown to be situated in the parametrium and 25% are central recurrences. The majority of patients with pelvic failure also have distant metastases (Perez, 1988).

Tumour histology has been reported to be an important prognostic factor with some series demonstrating a less favourable outcome for adenocarcinoma of the cervix (Grundsell et al, 1979; Kapp et al, 1983; Milsom and Fribert, 1983), whilst others have shown no significant differences in outcome (Davidson et al, 1989; Grigsby et al, 1988). The histopathological grading of tumours gives useful prognostic information and in general histologically undifferentiated tumours have a poor prognosis. However, the morphological features of the cells do not identify all their biological properties (Tubiana, 1987). Bloom (1965) and Kapp with his associates (1983) have indicated the importance and possible difficulties encountered in identifying histological grading of cervical carcinoma. The histological cell type, as classified by Wentz and Reagan (1959), has been shown to be important in prognosis (Ng and Atkin, 1973; Van Nagell et al, 1977; Randall et al, 1988), though tumour differentation was not shown to be important in some series (Gunderson et al, 1974; Goellner, 1976; Crissman et al, 1985; Benstead et al, 1986). Vascular/lymphatic permeation has also been shown to have a significant effect on outcome (Crissman et al, 1985; Perez, 1988).

The prognostic importance of age has not been clearly defined (Dattoli et al, 1989). According to some investigators the prognosis is more favourable in younger patients (Russell et al, 1987; Meanwell et al, 1988) but has been shown to be an unfavourable

factor in other series (Lybeert, 1987; Ashby et al, 1987; Elliot et al, 1989; Dattoli et al, 1989). Of these factors, stage of disease, tumour volume and the presence of lymph node metastases are the most important factors which predict outcome.

1.1.3 Prediction of Response

Several predictors of tumour radiocurability are already used in clinical practice eg. tumour stage, size and histology, though these are non-specific and relatively imprecise (Brock et al, 1985; Peters et al, 1986). The aim of predictive assays in radiotherapy is to refine the discrimination of existing predictive factors. They can be classified as 1) direct or indirect measurement of tumour cell survival following irradiation and 2) measurement of cellular and extracellular parameters which may affect radiosensitivity (Peters et al, 1986). There have been significant efforts recently to develop predictive assays based on tumour properties. A list of potential predictive assays in clinical oncology is given below (reproduced in part from Mitchell, 1988).

It is unlikely that a single assay will predict patient/treatment outcome alone but a combination of several may add to the value of existing prognostic indicators and enable the selection of patients at high risk of recurrence or who might benefit from treatment with another modality.

1.2 INTRINSIC RADIOSENSITIVITY OF TUMOUR CELLS

1.2.1 Surviving fraction at 2Gy

"That certain tissues, particularly certain tumours, are sensitive, is the basic fact underlying all radiation therapeusis in tumours" (Paterson, 1933). Different tumour types were classified by Paterson (1933; 1936) into three groups according to their radioresponsiveness; radioresponsive (embryonic tumours, reticuloses), tumours of

intermediate radioresponsiveness (squamous cell tumours and adenocarcinomas) and radioresistant (melanomas, soft tissue and bone sarcomas). The early work attempting to relate cellular radiosensitivity with treatment outcome of Glucksman (1941) involved histological examination of biopsies of carcinoma of the cervix before and after irradiation. Assessment of these was then used to try and predict local tumour control. Agreement between cytopathological prediction and clinical outcome was shown in 87% of 150 cases. This approach however had its difficulties with many of the post-irradiation biopsies being unsatisfactory (Dobbie et al, 1962).

TABLE 1 (reproduced in part from Mitchell, 1988)

POTENTIAL PREDICTIVE ASSAYS IN CLINICAL ONCOLOGY

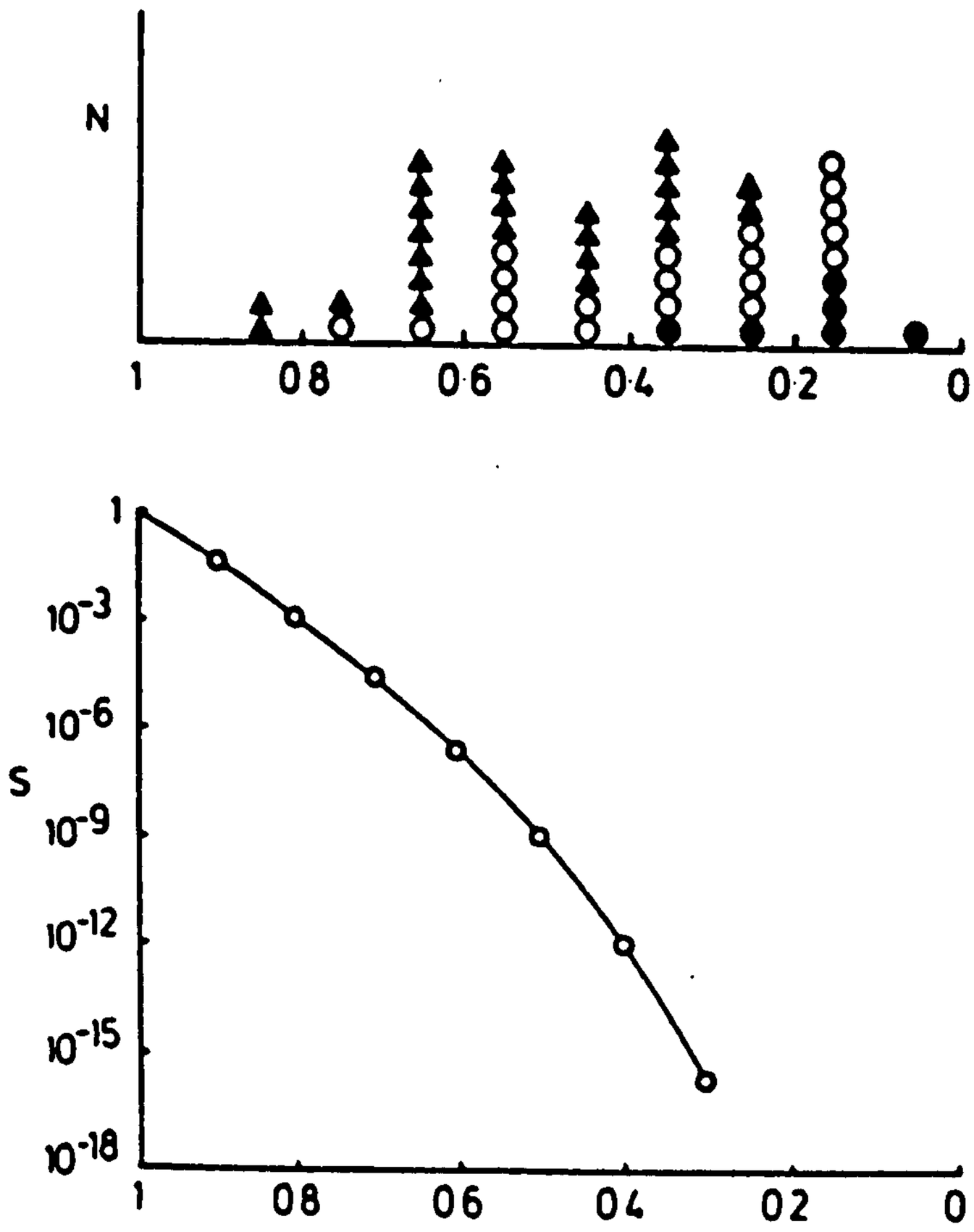
I. Direct effects of radiation on cells
   - (a) tumour cell survival
     - clonogenic assays
   - (b) growth rate
     - dye exclusion
     - tritiated thymidine incorporation
     - cell adhesive matrix
     - MTT
     - growth chamber
   - (c) cellular damage
     - DNA strand breaks
     - micronuclei formation

II. Parameters which may affect radiosensitivity
   - i) tumour cell kinetics
   - ii) DNA content/ploidy
   - iii) tumour oxygenation
     - pO_2 measurements
   - iv) biochemical assays
     - glutathione
     - gene amplification
     - membrane proteins

In the past it has been generally accepted that mammalian cells do not differ significantly in their inherent radiosensitivity. Lack of differences in the D_o values of human tumour cell lines led to the conclusion that survival parameters obtained in vitro could not explain the differences in radiocurability between tumour classes (Weichselbaum, 1976; 1980). However, this is now known not to be the case. A report from the Gustave-Roussy Institute by Fertil and Malaise (1981), confirmed by the group from the Institute of Cancer Research (Deacon et al, 1984), showed that there is a correlation between the ability to control various classes of tumour and the clonogenic survival of cell lines (derived from these classes) following exposure to 2Gy.

One hundred and one published cell survival curves for 92 human cell lines (including 64 tumour lines) were analysed in terms of parameters used to characterise cell radiosensitivity (Fertil and Malaise, 1985). It was found that 1) the initial part of the survival curve is specific to the corresponding cell line, 2) this initial part of the curve is well described by the parameter and 3) human tumour cell line radiosensitivity in terms of the surviving fraction at 2 Gy (SF2) reflects the clinical radioresponsiveness of the tumours from which the cell lines are derived. These conclusions were made on the basis of published estimates of the Prescribed Tumour Lethal Dose for various tumour types (95% control dose for tumours of corresponding histology). The degree of the correlation raised the possibility that the initial slope of the survival curve may be a major determinant of the outcome of fractionated radiotherapy. The dose of 2Gy was chosen as this was a dose found by Fertil and Malaise (1981 and Malaise et al, 1989) to give good discrimination between the cell survival curves. It is also the dose per fraction commonly used in clinical radiotherapy.

This work was further developed by Deacon et al who placed the tumour types into five categories of decreasing radiocurability with category A being radioresponsive and category E refractory (Table 2).



Key Closed circles - group A

Open circles - groups B, C and D

Closed triangles - group E

FIG.1 The implications of the value of SF2 for response to a course of 30 fractions. The lower panel shows a plot of S = $(SF2)^{30}$. Deacon et al,1984.

TABLE 2 From Deacon et al (1984)

Tumour types in decreasing order of controllability	Surviving fraction at 2Gy
A. Neuroblastoma, lymphoma, myeloma	0.19 (0.08 - 0.37)
B. Medulloblastoma, small-cell lung carcinoma	0.22 (0.1 - 0.31)
C. Breast, bladder, cervix carcinoma	0.46 (0.3 - 0.58)
D. Pancreas, colo-rectal, squamous lung carcinoma	0.43 (0.18 - 0.75)
E. Melanoma, osteosarcoma, glioblastoma, renal carcinoma	0.52 (0.48 - 0.56)

Experimental data from published survival curves were used to give further support for the importance of the clinical slope in a course of fractionated radiotherapy. Although the overall effect of fractionated radiotherapy will be modified by repair, reoxygenation and repopulation, the potential steep dependence of overall cell kill with the initial slope was indicated and differences in the SF2 values between category A and less sensitive groups were adequate to explain success or failure in achieving local tumour control (Fig. 1).

1.2.2 Differential repair

Large differences in repair capacity exist between cells. This has been demonstrated with studies looking at potentially lethal damage repair (PLDR or delayed plating repair) and split dose experiments to obtain a measure of sublethal damage repair (SLDR). PLDR has been found to vary among cell lines in vitro (Weichselbaum and Beckett, 1987) and repair capacity shown to vary amongst cell lines of the same histological cell type (Kelland and Steel, 1988). It has been suggested that differences in ability to repair damage may reflect differences in clinical radiocurability among corresponding tumours, with less responsive tumours exhibiting most PLDR (Weichselbaum and Beckett, 1987). More recent work has failed to find a correlation between the extent of PLDR and overall radio responsiveness of tumour type (Hall et al, 1988).

It has also been shown in recent work that radiosensitive cell lines may be efficient at repairing damage (Peacock et al, 1988). This has led to the suggestion that human cell lines may be "fully proficient in recovering from recoverable damage at doses below 2Gy, and at dose rates below 2Gy/minute, they do so almost completely" and that SF2 reflects the level of non-repairable radiation damage (Steel and Peacock, 1989).

1.2.3 Primary Tumour Culture

There are recognised limitations to studying the radiosensitivity of cell lines;-

1) In general there is no information on the clinical radioresponsiveness of the original tumour from which each cell line is derived. 2) The morphological characteristics of the original tumour cells have generally changed (they are frequently less differentiated in culture than in patients). 3) Only a small fraction of the original tumour population may have given rise to an established cell line (Malaise et al, 1986). Correlations based on cell lines with values described by different laboratories where methodologies may vary are open to criticism regarding interpretation. It has been demonstrated that the choice of assay may influence radiation survival curve parameters (West and Sutherland, 1986).

In vivo - in vitro comparisons in radiosensitivity have been made. Seven different cell lines were grown in vitro and as xenografts and SF2 was found to be considerably higher in vivo (Malaise et al, 1986). However, the contribution of one or more additional causes of radioresistance (eg. presence of hypoxic cells, proliferation of tumour cells during the course of treatment) does not weaken the correlation between the initial slope of the in vitro survival curve (inherent radiosensitivity) and the clinical radioresponsiveness. This was confirmed by the work of Rofstad et al (1987) on melanoma xenografts where the in vivo radioresponsiveness was positively correlated with the initial slope of the in vitro survival curve.

Rofstad et al (1987) studied the radiation sensitivity of human tumour primary cultures

using the Courtenay-Mills soft agar assay. SF2 was measured for melanoma, ovarian, breast, bladder, seminoma and head and neck tumours. The survival at 2Gy differed considerably among tumours of the same histological type. The range of SF2 values for 8 cervix tumours was 0.17 - 0.48 with a mean of 0.3. No significant difference was demonstrated between the mean SF2 values for the 7 tumour categories, but two thirds of the seminomas had a low SF2 value (< 2) and a third of the melanomas had a high SF2 value. These data are consistent with the suggestion of Fertil and Malaise (1981; 1985) and Deacon et al (1984) that the clinical radiocurability of tumours may be correlated to the cell surviving fraction at 2Gy.

1.2.4 Rapid Assay of Radiosensitivity

Recently Baker et al (1986; 1988) developed a human tumour primary culture technique which makes use of a cell adhesion matrix (CAM) for improving attachment of cells to culture plates and a highly enriched medium that support tumour cell growth. This technique measures growth of surviving cells rather than clonogenic survival. Briefly, the technique involves dissociation of a tumour biopsy into a single cell suspension. Twenty-four well plates, coated with CAM (a combination of fibronectin and fibrinopeptides), are inoculated with cells and incubated for 24 hours. Dead and non-adherent cells are removed and wells irradiated with graded doses of X-rays. The cultures are grown for 2 weeks, then stained with crystal violet and the total amount of stain uptake, expressed as optical density, measured by a video image analysis system. This measurement of staining density has been shown to be directly proportional to the total number of cells that have grown in each culture well and to be correlated with surviving fraction (Brock et al, 1985). With this method successful growth has been achieved in 80% of primary tumour specimens (Baker et al, 1986). One concern with this assay is the possible growth of normal cells that may be part of a tumour specimen (Mitchell, 1988). It has been reported that morphological, immunohistochemical and cytogenetic analyses of cultures grown from biopsies in this way, have shown that

normal cells were not present to a significant extent (Baker, 1986; 1988). However, an investigation into the ability of CAM to support the growth of normal tissues, demonstrated a predominantly fibroblastic morphology (Head et al, 1989). Tumour cultures grown using this system by the same group found that lung and breast tumour cultures were highly contaminated with fibroblasts, although a greater degree of success was seen with ovarian tumours (89% of cultures had more than 50% epithelial cell morphology).

Using this technique radiation sensitivity curves have been derived for a wide variety of tumour types including a series of head and neck tumours (Brock, 1985; 1989). These showed a wide range of SF2 values (0.10 - 0.91) with a mean of 0.33 (Brock, 1989). The radiosensitivity of tumour cells in primary culture was shown to vary from one tumour class to another in a way that is comparable to that observed for established cell lines (Malaise et al, 1989). Differences in SF2 for primary cultures and cell lines have been reported also (Malaise et al, 1987; Suit et al, 1989). Patient follow-up time is not yet long enough, though in time it will be possible to correlate these measurements of radiosensitivity with clinical outcome in the series of patients with head and neck tumours treated with surgery and radiotherapy (Peters et al, 1987).

1.3 CLONOGENICITY

The capability of a single cell to grow into a colony, which contains more than 50 cells, is a convenient proof that it has retained its reproductive integrity (Hall, 1978). The ability of single mammalian cells to proliferate into colonies was used by Puck and Marcus (1956) in a plating technique analogous to that used for the assay of bacterial cell viability. The loss of this ability as a function of radiation dose is described by the dose-survival curve.

Since its introduction in 1956, the tissue culture techniques for measuring colony-forming ability of mammalian cells have been used widely to define details of the shape and characteristics of cell survival curves, in the hope that this may provide insight to produce a better strategy for treating human tumours (Tepper, 1981). Tumours may contain proliferative cells which form a spectrum, from those with a limited number of potential divisions to those with the capacity to renew the entire cell population, including themselves (MacKillop, 1983; Selby, 1983). These self-renewing and population-renewing cells, which may constitute only a small proportion of the total population, are known as stem cells (Tepper, 1981). It may be that it is these stem cells which are responsible both for tumour repopulation after treatment and also for metastatic growth, and are therefore the primary target for any cytotoxic therapy of cancer (Selby et al, 1983).

Although it has been postulated that clonogenic assays can be used for the study of stem cells (Bruce et al, 1969), studies have shown that relatively few clonogenic cells have renewal capacity in culture (Buick and MacKillop, 1981). It may be that within the cell hierarchy there exists cells with high proliferative potential but that have no (or limited) self-renewal capacity. Therefore it is not yet known whether clonogenic assays can quantify tumour stem cells (MacKillop et al, 1983).

1.3.1 Choice of Assay

Different culture techniques are available for growing colonies directly from a variety of human tumour specimens (Hamburger and Salmon, 1977; Courtenay and Mills, 1978). A semi-solid medium suppresses the growth of most normal cells, though benign epithelial or stromal cells (Pavelic, 1980) and haemopoietic cells may occasionally proliferate (Hamburger et al, 1978), and allows the growth of cells capable of anchorage independent growth.

There is plenty of evidence of the malignant nature of the colonies from cytology (Salmon and Buick, 1979), cytogenetic studies (Trent, 1980), studies on secondary plating efficiency (Singletary, 1985) and the ability of these colony cells to produce tumours in nude mice (Pavelic, 1980).

A number of tumour classes have been studied with regard to clonogenicity; notably breast carcinoma, malignant melanoma and ovarian carcinoma (Hamburger, 1978; Tveit, 1981; Bertoncello, 1982; Ottestad, 1989). Relatively few studies have concentrated on cervical carcinomas and widely different success rates in growing colonies have been reported. Eighty eight per cent of 76 specimens grew in a study by Parker et al (1984) and only 1% (1/29) in a study by Williams et al (1983). The Hamburger-Salmon assay has been widely used since 1978 and a number of clinical trials have been carried out using this method in order to test for the validity of using clonogenic assays to predict clinical response to chemotherapy (Bertelson et al, 1984; Von Hoff, 1988; 1990; Salmon, 1990). However, it has been shown that the Courtenay-Mills assay yields higher colony-forming efficiencies than both the Hamburger-Salmon (Tveit et al, 1981; Endresen et al, 1985; Ottestad, 1988) and the Carney method, which utilised agarose instead of agar (Walls and Twentyman, 1985).

The improved clonogenicity in the Courtenay method is due primarily to the presence of rat red blood cells and low oxygen tension although the replenishable nature of the assay allows for long incubation periods often necessary for specimens cloned directly from the patient (Walls and Twentyman, 1985). Low oxygen tension of 5% produces a higher CFE than 20% oxygen shown by Tveit et al (1981), Courtenay and Mills (1978; 1984) and Gupta and Eberle (1984) showed that although there was great variation between the CFE of cells from various human xenograft lines tested, the optimal concentration could be as low as 0.1%. Bradley et al (1971) first demonstrated that washed rat and murine red blood cells improved the clonogenicity of murine marrow cells in agar and produced larger colonies. Using human tumour xenografts, Courtenay and Mills (1978) found that

red blood cells lysed before use were less effective than whole cells and that the time of lysis of the red blood cells in culture is important. This is in contrast to the findings of Besch et al (1986) who found that intact or lysed red blood cells improved the colony forming ability of breast tumour cultures. Pavelic et al (1980) did not demonstrate any increase in CFE using murine or rat red blood cells. The same report found no difference in the species of rat used to provide the red blood cells, though August rat red blood cells were found to consistently enhance colony growth of human and animal tumour cells in primary culture by Courtenay et al (1978) and Tveit et al (1981). Culture conditions can be improved and clonogenicity increased by supplementing the culture medium with growth factors and hormones. Studies involving primary human breast carcinoma cells have shown that improvements in CFE of 5 and 35 fold are possible (Hug et al, 1984; Besch et al, 1986; Ottestad et al, 1988). Epidermal growth factor (Hamburger et al, 1981; Pathak et al, 1982), irradiated lymphoblast feeder layers (Hamburger, 1983), a combination of cell line-conditioned medium and growth factors (Hug, 1984), conditioned medium from murine splenic cells (Hamburger and Salmon, 1977) and boiled rather than autoclaved agar (Thomson et al, 1983), have all been reported as promoting the growth of human tumour cells in semi-solid medium. The addition of hydrocortisone and insulin have been shown to improve colony growth by several groups (Hug et al, 1984; Kern et al, 1984; Hamburger, 1987) as has the addition of transferrin (Calvo et al, 1983).

1.3.2 Validity and Limitation of Clonogenic Assays

Primary human tumour clonogenic assays have been criticised because of: 1) the low proportion of tumours suitable for testing, 2) difficulties in preparing single tumour cell suspensions, 3) low CFEs and 4) the scoring of small colonies. The proportion of human tumours which grow with a sufficient colony forming efficiency for assessment of radiosensitivity or chemosensitivity is often less than 50% (Bertoncello et al, 1982; Selby et al, 1983) and may range from 35 - 81% depending on the research group, the

tumour type and definition of colony size (Singletary et al, 1985). In a large series of 1,014 human solid tumours (Kern et al, 1982), 690 (68%) of these tumours of various histologies showed evidence of colony formation within 2 - 4 weeks. The cloning efficiency of fresh human tumour samples is low compared to that of established cell lines with values, in general, ranging from 0.001 to 0.1%.

The presence of clumps and clusters in tumour cell suspensions has been observed by Agrez et al (1982) who used a human tumour clonogenic assay for 455 tumours and were not able to obtain preparations with exclusively single cells. Similar problems were recorded by Singletary et al (1985). In another series reported by Bertoncello et al (1982) difficulties in preparing single cell suspensions were found in 12% of ovarian tumour specimens, with only 36% of these tumours being adequate to culture. Alley and Lieber (1984) reported the presence of clumps and clusters in cell suspensions derived from human solid tumours and suggested filtration of cell suspensions, counts at Day 1, vital staining with a tetrazolium dye to facilitate colony counting and the use of 20Gy irradiated samples as negative controls to check for the presence of cells clumps in order to minimise errors.

The lack of a true radiation survival curve generated in early reports of soft agar clonogenic assays have been cited as a major criticism of their validity (Selby et al, 1983). A plateau in radiation survival curves would indicate the presence of clumps and clusters in the tumour cell suspensions (Rockwell, 1985). Meyskens et al (1983) suggested that initial survival curves obtained with the Human tumour clonogenic assay (HTCA) indicating radioresistance may have been artefactual due to the presence of clumps plated initially. It was suggested that these difficulties could be circumvented by counting "colonies" immediately after plating and subtracting the initial number of clumps from the final colony counts (Alley and Lieber, 1984; Hamburger, 1987). However, this practice is frought with theoretical and practical difficulties demonstrated by Rockwell (1985).

Technical artefacts can lead to a lack of linearity between cells plated and colonies formed (Eliason, 1985). The relationship between colony numbers and concentration of cells plated is a important parameter of clonogenic assay systems. The cloning efficiency for an ideal sample should be independent of cell concentration, thus giving a straight line through the origin when colony numbers are plotted against cell concentration. Linear relationships have been demonstrated between the numbers of colonies formed and the numbers of cells plated (Hamburger , 1978; Pavelic, 1980). Tumour samples have been reported to show increasing cloning efficiences with increasing cell concentration and also decreasing cloning efficiencies at high cell concentrations (Eliason, 1985; Page, 1988; Meyskens, 1983).

It has been shown that lethally irradiated tumour cells are capable of completing up to six divisions before dying thus producing abortive colonies (Nias, 1965). The 50 cell colony is therefore a minimum size for accurate assessment of cell survival in radiation studies. It is uncertain how often abortive colonies form after drug therapy (Courtenay, 1984). Counting colonies larger than 50 cells helps to minimise errors from cell clusters introduced into the cell suspension. Besch et al (1986), using an agar method, found identification of proper colony size to be a major obstacle in quantitating cell survival after radiation. Apparent radiosensitivity of tumour suspensions varied directly with the size used to define a colony. The use of stringent criteria for colony size resulted in conventional survival curves (Hamburger, 1987). A colony may be defined as one with a minimum horizontal diameter of 60 to 100 microns for the cell cluster. The number of cells in a three dimensional system is difficult to quantify and therefore not as precise a criterion as diameter (Singletary et al, 1985). The number of cells in a tumour colony has been demonstrated to depend on the size of the individual cells and an empirically derived formula has been used to quantitate the number of cells within a tumour colony (Meyskens, 1984).

Setting the colony size at a higher number of cell divisions reduces the overall cloning efficiency but yields more accurate assessment of true sensitivity (Nias and Fox, 1968).

1.3.3 Clonogenicity and Tumour Differentiation

The relationship between the colony forming ability of tumour cells in soft agar and the prognosis of the patient has been studied by various groups and is of considerable interest. Results from these studies (Table 3) have been inconsistent and often small numbers have been evaluated. Thus no clear conclusions can be drawn (Ottestad, 1989). It has been reported that cancer cell clones that grow in semi-solid medium may represent the most malignant and rapidly growing fractions of the heterogeneous population of primary tumours (Hug et al, 1985; Moezzi et al, 1986; Flentje et al, 1987). CFE has been shown to be both correlated (Moezzi et al, 1986; Gioanni et al, 1988) and not correlated (Smallwood et al, 1984; Dittrich et al, 1985; Trotter et al, 1985; Aapro, 1987; Cobleigh et al, 1987) with tumour grade. Similarly correlation (Mattox et al, 1980; Von Hoff et al, 1980; Bertoncello et al, 1982; Miller et al, 1983; Sutherland et al, 1983; Johns and Mills, 1983; Hug et al, 1985; Aapro, 1985; Nomura et al, 1989) and lack of correlation (Mattox, 1984; Dittrich et al, 1985; Stevenson et al, 1989; Ottestad et al, 1989) with prognosis have been reported. Out of nine breast cancer studies, two have shown a correlation, whilst three showed no correlation with patient prognosis.

Tumour growth in the clonogenic assays reflects a biologically important potential, related in at least some tumours to an abnormal DNA stemline (Verheuen et al, 1985). Smallwood et al (1984) demonstrated that the oestrogen receptor negative breast tumours had a higher Labelling Index (LI), but were not different from tumours which grew in the clonogenic assay and no correlation was demonstrated between CFE and tumour grade. Trotter et al (1985) also failed to demonstrate any relationship between thymidine labelling index and CFE in a series of twenty-eight colorectal tumours, whereas Gioanni

TABLE 3

STUDIES INVESTIGATING CLONOGENECITY AND CLINICAL OUTCOME

Tumour type	No. patients	Conclusion	Reference
Head and neck	33	Mortality increased when CFE >0.02%	Mattox, 1980
Neuroblastoma	18	Survival = 4 weeks if CFE >0.1%	Von Hoff, 1980
Ovary	29	Survival inversely related to CFE	Bertoncello, 1982
Lung	33	CFE >0.007% = 14 mth Survival <0.007% = 27 mth	Miller, 1983
Breast	66	Mortality greater with higher CFE	Sutherland, 1983
Head and neck	29	Mortality greater with higher CFE	Johns, 1983
Breast	54	CFE correlates with receptors not grade	Smallwood, 1984
Head and neck	157	CFE not related to survival	Mattox, 1984
Breast	31	Survival inversely related to CFE	Hug, 1985
Breast	87	No correlation with grade or survival	Dittrich, 1985
Colorectal	28	No correlation with grade	Trotter, 1985
Mixed	44	High CFE associated with poor prognosis	Aapro, 1985
Breast	268	CFE correlated with grade	Moezzi, 1986
Breast	61	CFE not correlated with tumour grade	Aapro, 1987
Head and neck	21	CFE not correlated with tumour grade	Cobleigh, 1987
Breast	59	CFE correlated with grade	Gioanni, 1988
Lung	68	CFE not related to prognosis	Stevenson, 1989
Breast *	138	CFE not related to prognosis	Ottestad, 1989
Breast	254	CFE related to metastatic potential of breast carcinoma	Nomura, 1989
Ovary *	121	CFE not correlated with grade: high CFE associated with poor prognosis	Tveit, 1989

* Courtenay-Mills assay used

et al (1988) showed that the tumours which grew in vitro had a higher LI than those which failed to grow.

1.4 INFLAMMATORY CELL INFILTRATES

1.4.1 Macrophages

Cell suspensions prepared from tumours may contain a large number of inflammatory cells with reported values for the proportion of macrophages ranging from 0-67% (Gauci and Alexander, 1975; Steele et al, 1984; Kelly, 1988). Host cell infiltrates have been reported to be unevenly distributed throughout a tumour being concentrated at the tumour periphery (Normann, 1985). As most studies which have quantified the numbers of macrophages in tumours have relied on methods involving tumour disaggregation, samples for disaggregation taken at the centre of a tumour may have higher tumour/macrophage ratios than samples along the invading margin of the tumour. Using these methods, different tumour-host cell ratios may be obtained when compared with earlier studies using conventional tissue sections. Early studies also experienced problems in the identification of macrophage cells as it is difficult to identify these cells with accuracy by light microscopy of conventionally stained tissue sections (Underwood, 1974). The tumour associated macrophages (TAM) recovered may be a selected population with losses occurring with disaggregation of the tumour (Takeo et al, 1986).

The work of Eccles and Alexander (1974) on experimental murine tumours demonstrated that tumours containing high numbers of macrophages showed less tendency to metastasize than those with lower numbers. A number of susbsequent studies showed that tumour associated macrophages react with tumour cells and can stimulate tumour growth in some experimental tumours (Mantovani, 1979; Evans, 1977). However, more recent studies (Talmage, 1981) on the role of TAM showed no correlation between the macrophage content and the metastatic propensity of several murine and rat tumours,

including tumour variants obtained from metastases of these tumours. The role of TAM in 77 patients with lung cancer was examined by Takeo et al (1986) and no correlation was found between the numbers of TAM recovered and the recurrence rate. This was also found to be the case when TAM density and prognosis was studied in nasopharyngeal cancer (Nomori, 1984). However, Steele et al (1984) examined the macrophage content of forty breast tumours and found that increased numbers of TAMs were in fact associated with factors that indicate a poor prognosis. As many factors influence the metastatic behaviour of tumours perhaps it is unreasonable to expect that macrophage content alone would show a good correlation with metastatic spread. The anti-tumour activity of TAM, not just the proportion of TAM recovered, may be of use in predicting patient outcome (Takeo, 1986).

Macrophages have been shown to promote tumour cell growth in vitro (Buick et al, 1980; Mantovani et al, 1979; Gabizon et al, 1980; Hamburger et al, 1983) and in vivo (Evans, 1977). There is evidence that this growth promotion is mediated by soluble factors (Gabizon, 1980). These factors may be the same or similar to those released by peripheral blood monocytes which enhance the growth of human tumours in soft agar and appear to be heat stable, non-dialyzable, acid labile proteins of 10,000 - 30,000 daltons (Hamburger, 1986; 1987).

The relationship between the degree of host cell infiltration of a tumour and its response to therapy has been less well studied. An investigation using murine tumours demonstrated that the TAM content was related to the radioresponsiveness of these tumours, with higher macrophage content conferring radioresistance (Milas et al, 1987). No correlation between TAM content and the growth rate of established tumours or metastatic spread was seen. The suggestion from this work was that the tumour macrophages may promote the survival of the tumour cells not killed by radiation.

Despite extensive investigation, the role of macrophage infiltration in the metastatic behaviour of malignant tumours remains uncertain. Little information is available on the association between the extent of macrophage infiltration and tumour progression parameters other then metastatic spread and also little on the association between TAM content and tumour response to therapy (Milas, 1987). It is clear that this role is not a simple one and that TAM are functionally heterogeneous (McBride, 1986).

1.4.2 Lymphocytes

The role of tumour infiltrating lymphocytes (TILS) has also been the subject of much investigation. The degree of infiltration has been associated with prognosis, with higher numbers of TILS being associated with longer survival (Underwood, 1974; Black et al, 1971; Hiratsuka, 1984; Svennevig, 1985; Yasumoto, 1988). This observation is often cited as evidence for an immunological response to tumours, although this is not universally accepted (Krieder, 1984). The true position may also be obscured by a publication bias for papers demonstrating a positive association with prognosis and a reluctance to publish negative or inconclusive results (Underwood, 1974). Another important factor is the absence of quantitative, morphometric measurements and of reliable sampling methods, applied to either conventional tissue sections, or to living cell suspensions obtained from dissociated tumours (Kreider, 1984).

More recent studies have looked at types of TILS in human solid tumours using monoclonal antibodies and have shown the vast majority to be T-lymphocytes (Whiteside, 1986; Miescher, 1988). Studies to examine their function have been made. Some studies have shown that freshly isolated populations of TILS from different solid tumours had depressed natural killer cell activity (Eremin, 1981; Moy, 1985), although this is not true in all cases (Meischer, 1987). It has been suggested that the poor cytotoxic actions of TIL populations may be related to cell numbers inadequate for analysis, low proportions of effector cells among TILS, possible inhibitory influences of

tumour environment (Meischer, 1986) or possible suppressive factors produced by cells in TIL populations (Vose and Moore, 1979). Lymphocyte-derived soluble mediators may play an important role in regulating the anti-tumour activity of TAMs in lung cancer because of the co-existence of a large number of lymphocytes in lung cancer tissues (Yasumoto et al, 1988). Cytolytic T lymphocyte precursors are present in human solid tumours but vary considerably amongst individual tumours (Meischer, 1987). A study on 100 patients with colorectal tumours supported the view that human tumours attract mononuclear cells and that this local reaction may influence prognosis, but the value of this parameter may be limited because of the great variance in the inflammatory reaction within the same group of survivors (Svennevig, 1984). No firm conclusions regarding a causal relationship between host cell infiltration in human tumours and prognosis can be made.

1.4.3 Eosinophils

The association of a malignant tumour with eosinophilia and tumour eosinophilic infiltrates has been long recognised (Pretlow, 1983). Cervical carcinoma is usually associated with a mixed chronic inflammatory infiltrate in the surrounding stroma (around the tumour cells), but in a small number of cases there is massive stromal infiltration by eosinophils (Lowe, 1988). A retrospective series of 1027 cases of cervical carcinoma revealed massive eosinophilia (>100 eosinophils per high power field) in 3% of cases (Lowe, 1988). From this study it was found that patients with massive eosinophila, who had no circulating eosinophilia, had a better prognosis than tumours without. Five patients who had a circulating eosinophilia, as well as eosinophilia within the tumour, had extensive disease and short survival. Other workers have also found tumour eosinophilia in cervical carcinoma to be associated with a favourable prognosis (Pastrnak, 1984). Marked eosinophilia was found to occur within 4% of these tumours which is similar to the incidence reported by Lowe (1988). In a smaller series tumour eosinophilia was reported not to be associated with prognosis, whle eosinophilia of the peripheral

blood was reported to be an adverse prognostic sign (Bostrom, 1981). There are two possible explanations for tumour eosinophilia:- 1) the tumour cells may directly manufacture eosinophil chemotactic factors and 2) the presence of eosinophilia might be part of a specific reaction of the host against the tumour (Bostrom, 1981; Pastrnak, 1984).

1.5 VASCULARITY

In view of the radiobiological effect of oxygen, knowledge of the extent to which tumours are oxygenated may be important in predicting local tumour control by radiotherapy (Peters et al, 1986). Information on the vascular supply of tumours can be of great value in reflecting the degree of oxygenation of the tissue but relatively little attention has been paid to it on routine histo-pathological classification of human tumours (Siracka, 1988). This is possibly due to the lack of methods useful in clinical practice for reliable quantitation of vascularity in biopsies.

1.5.1 Intercapillary distance

Vascularisation in carcinoma of the cervix has been studied by Kolstad (1968) using colpophotography. Assessment of the oxygenation of the tumour surface was made, using intercapillary distance as a parameter, and revealed a decrease in oxygenation with advancing stage of disease. This observation was confirmed by direct measurement of the tissue oxygen tension. The critical intercapillary distance was found to be approximately 350μm, since above this distance the incidence of necrosis rose rapidly. Kolstad (1968) was able to demonstrate a 51% local recurrence rate among 51 patients whose tumours had largest intercapillary distances greater than 400μm, compared to a 17% recurrence rate in 54 cases with greatest intercapillary distances less than 400μm. There also appeared to be different vascularisation patterns for adenocarcinomas and squamous cell tumours and the suggestion was made that these should be considered

separately when assessing the vasculature. These significant findings were confirmed by Awwad et al (1986) who measured intercapillary distance by a more precise though laborious method of analysing histological samples from 44 patients with Stages IIB and III disease of the cervix. This method involved a histochemical procedure to stain capillary endothelial cells and a projection/tracing technique was used to make the measurements. The mean intercapillary distance (304±30μm) was shown to be greater (322μm) in those patients who developed local recurrence within two years than in those whose tumours were controlled - 291μm (Awaad, 1986). This finding was shown to be independent of tumour stage and grade. However, in a series of 26 oral squamous cell carcinomas reported by Lauk and associates in 1989, the group of patients which had local control had an average median distance betwen tumour cells and blood vessels of 105μm, compared with a median distance of 76μm in the group of patients which failed treatment. The finding of a higher local control rate in oral squamous cell tumours which were less well vascularised is contradictory to the studies on cervical carcinoma. This may be because of biological differences between oral tumours and cervical carcinoma and/or incomparable treatment schedules (Lauk et al, 1989).

1.5.2 Morphometric analysis

Another method of analysing the vasculature of cervical carcinoma has been employed by Siracka and colleagues (1982; 1984) who made morphometric measurements of relative volumes of stroma, parenchyma and blood vessels. The tumours of 23 patients surviving more than 5 years following radiotherapy were found to have a stroma that was rich in blood vessels, compared to those of 22 patients who died within 5 years. The results of this retrospective study confirm Kolstad's observations that vascular density in cervix cancers is related to the radiocurability of the disease. A further retrospective study of the capillary density in nasopharyngeal tumours showed a statistically significant correlation between capillary density and survival time of patients treated with radiotherapy (Delides et al, 1988).

Intra-tumour variation of vascularity was investigated in addition to the variation between tumours (Siracka et al, 1988). It was found that there was a more or less individual pattern of vascularisation of the tumours despite a considerable intra-tumour variation of the vasculature. In the case of stage IB cervical cancers, the variation of vascular density between tumours was greater than that within them (Siracka et al, 1988). This finding was confirmed for more advanced tumours (IIA and III included) when surgically removed carcinomas were studied by taking biopsies from different sites (Revesz et al, 1989). In a further study of vascularity in cervical carcinoma, vascular density was shown to be superior to tumour grading with respect to prognostic values (Siracky et al, 1988).

For experimental squamous cell carcinomas, it has been shown that quantitative measurements of vascular sections may be of prognostic significance in that there was a positive association between relative vascular volume and radiation response in situ (Moore et al, 1985).

1.6 TUMOUR CELL PROLIFERATION

In previous decades considerable interest was given to the influence of tumour growth rate on the course of the disease. The incidence of mitotic figures in human tumours is the most direct indicator of cell production and has long been used by pathologists as a guide to proliferative activity. The discovery of the cell cycle by Howard and Pelc and the description by Taylor and colleagues of the thymidine labelling index (TLI) in the 1950's provided a new approach to tumour growth kinetics (Tubiana and Courdi, 1989). In each histological group of human tumours the individual values of the TLI vary widely although there are significant differences between means of each group. The highest mean TLIs were found in lymphomas and embryonal tumours and lowest in adenocarcinomas (Steel, 1977). The mean TLI was highest in the groups of tumours which respond to radiotherapy and chemotherapy (Tubiana and Courdi, 1989). There is

a corrrelation between the mean doubling time and TLIs within each histological group and the highest TLIs were seen in undifferentiated tumours compared to differentiated tumours of the same type (Tubiana and Courdi, 1989).

The proliferative activity of tumour cells measured by TLI has been reported to have prognostic value in breast carcinomas (Meyer et al, 1983; Tubiana et al, 1984; Silvestrini et al, 1985; 1989). TLI has also been shown to be of prognostic value in head and neck tumours with survival being lower in those patients with a high TLI (Chauvel, 1989). In a small series by Dixon and associates (1977) they were unable to demonstrate significant differences in TLI in those patients who relapsed compared to those who were cured. The measurement of TLI however is labour intensive and requires at least 10 days for processing and has never been used routinely (Tubiana and Courdi, 1989).

Flow cytometry has been used more widely to estimate the proportion of proliferating cells. The measurement of DNA content distribution provides evidence of ploidy abnormalities and approximately the proportions of cells in various phases of the cell cycle. The proportion of cells in S phase can be determined and has been shown to correlated with TLI (Tubiana and Courdi, 1989). The DNA content and S phase fraction have been found to be predictive of relapse-free and overall survival in breast cancer (Dressler et al, 1987; Kallioniemi et al, 1988), in carcinoma of the rectum (Streffer et al, 1986), ovarian tumours (Kallioniemi et al, 1988) and as a predictor of early recurrence in cervical carcinoma (Strang et al, 1987).

1.6.1 Ki67 staining

The Kiel group (Gerdes et al, 1983; 1986) has reported that a monoclonal antibody Ki67 specifically stains cycling cells. The antibody has been developed which identifies a nuclear antigen in human cells at all stages of the cell cycle except resting cells in Go (Gerdes et al, 1984). The exact nature of the antigen recognised by Ki67 has not yet

been determined and its functional role is unclear. It has been used to measure the tumour growth fraction. The growth fraction = LI/(LI)p where LI is the labelling index of the whole population and (LI)p is the labelling index of proliferating cells. A correlation has been demonstrated between the immunohistochemical labelling of cell nuclei with Ki67 and other methods of assessing cell proliferation - namely mitotic index in breast cancers (Barnard et al, 1987) and soft tissue sarcomas (Ueda et al, 1989) and in cell lines with tritiated thymidine uptake (Gerdes, 1984) and uptake of bromo-deoxyuridine in Non Hodgkin's lymphoma (Schrape et al, 1987) and S-phase fraction in breast cancers (Walker and Camplejohn, 1988).

Ki67 index was shown to be closely correlated with tumour grade in Non Hodgkin's lymphoma and breast carcinomas with the less differentiated tumours containing a higher proportion of Ki67 positive cells (Schrape et al, 1987; Barnard et al, 1987; Lellé et al, 1987; Bouzubar et al, 1989). The extent of nuclear staining did not correlate with the degree of differentiation in Walker and Camplejohn's breast study (1988) in contrast to the findings reported by Gerdes et al (1986) on breast tumours where the extent of nuclear staining was correlated to the tumour grade. However, no significant relationship between the percentage of cells labelled with Ki67 and tumour grade, cell type or mitotic index was demonstrated in a study on cervical carcinoma (Brown et al, 1988).

There was a wide range of cells that were labelled by Ki67 in the study on cervical tumours (10-50%), although a smaller variation of 10% amongst different areas of the same tumour biopsy was demonstrated (Brown et al, 1988). A similar range of labelled nuclei was seen (5-60%) in breast tumours but in 26% of tumours cytoplasmic staining was the only staining seen and 18% of specimens exhibited no staining (Walker and Camplejohn, 1988). The significance of this cytoplasmic staining is not known but it has been shown to be unrelated to proliferative activity (Gerdes et al, 1983). Aneuploid tumours have been shown to have a higher frequency of nuclear staining than diploid tumours.

Heterogeneity of Ki67 staining was marked in many carcinomas so greater numbers of cells were counted in the study of Walker and Camplejohn (1988) compared to the studies of Gerdes et al (1986) and Lellé et al (1987) and this may account for the lower percentage of nuclear staining seen in this study. The distribution of labelled nuclei varied within the individual tumours with areas of cell maturation and keratinisation showing little or no staining compared to peripheral portions which are more actively proliferating (Brown, 1988). The staining of nuclei with Ki67 was demonstrated to be confined to epithelial cells only, using a double staining procedure on fine needle aspirates of breast carcinomas (Charpin, 1989). This contrasts with the findings of Bouzubar et al (1989) who demonstrated antibody binding in the nuclei of benign components, but this was not found to be above 5%. In the study on Ki67 staining in breast carcinomas of Lellé et al (1987) the average numbers of Ki67 - positive cells was 15% (range 1-48%) for breast tumours and 4% (range 1-10%) for benign breast lesions.

The most important issue raised with the studies on Ki67 antibody staining is whether or not the variation in nuclear labelling with Ki67 (an objective measurement of cell proliferation) correlates with clinical behaviour. Retrospective studies are not possible with Ki67 because fresh (or frozen) tumour material is required as the epitope recognised by the antibody does not survive conventional fixation and processing (Hall et al, 1988). A high level of Ki67 staining was shown to be associated with early recurrence of breast carcinoma after mastectomy (Bouzabar, 1989). In a smaller series of patients with soft tissue sarcomas, the Ki67 index (number of positive tumour cells/10 High power fields) was shown to be correlated with patient outcome and was correlated with other prognostic factors (Ueda et al, 1989). The Ki67 score (positive cells/total tumour cells) was shown to be independent of lymph node status which is an important prognostic factor in breast carcinoma (Barnard et al, 1987; Bouzubar et al, 1989), though this was not supported by Lellé's data which suggested that the average number of Ki67 positive cells was slightly higher in node positive patients.

1.7 AIMS

The aims of this study were to evaluate potential prognostic factors for individuals with cervical carcinoma being treated with radiotherapy.

1. *Radiosensitivity.* Surviving fraction at 2Gy will be determined for primary cultures of tumour biopsy specimens cultured using the Courtenay-Mills soft agar clonogenic assay. The adequacy of the assay will be established and sampling errors, resolution, reproducibility, intra-tumour heterogeneity and inter-patient variation will be investigated.

2. *Clonogenicity.* The relationship between clongenic ability and both established clinical prognostic indicators and other potential prognostic factors will be studied.

3. *Vascularity.* A prospective analysis of histological specimens taken from patients whose tumours were assayed for radiosensitivity is to be carried out. Intercapillary distance will be measured for each tumour along with morphometric analyses of stroma, parenchyma, necrosis and vascular components. A comparison of the two methods will be made.

4. *Inflammatory infiltrates.* The proportions of normal host cell infiltrates in tumour cell suspensions will be examined. The relationships between these and other parameters studied are to be investigated.

5. *Ki67.* The Ki67 index of the specimens will be determined and correlated with the other parameters under study.

2. MATERIALS AND METHODS

2.1 TISSUE CULTURE MEDIA

Two media formulations were used:-

(i) High Antibiotic Medium

Basal Medium Eagle's (Gibco, Paisley) supplemented with 20µg/ml of amphotericin (Sigma) and 200µg/ml of gentamicin (Sigma) and 15mM Hepes (Gibco, Paisley).

(ii) Growth Medium for tumour culture

Ham's F12 medium (Gibco, Paisley) supplemented with:-

2µg/ml amphotericin

25 µg/ml gentamicin

The following additions were made:-

1) 10ng/ml epidermal growth factor (Sigma)

2) 10µg/ml insulin (Collaborative Research)

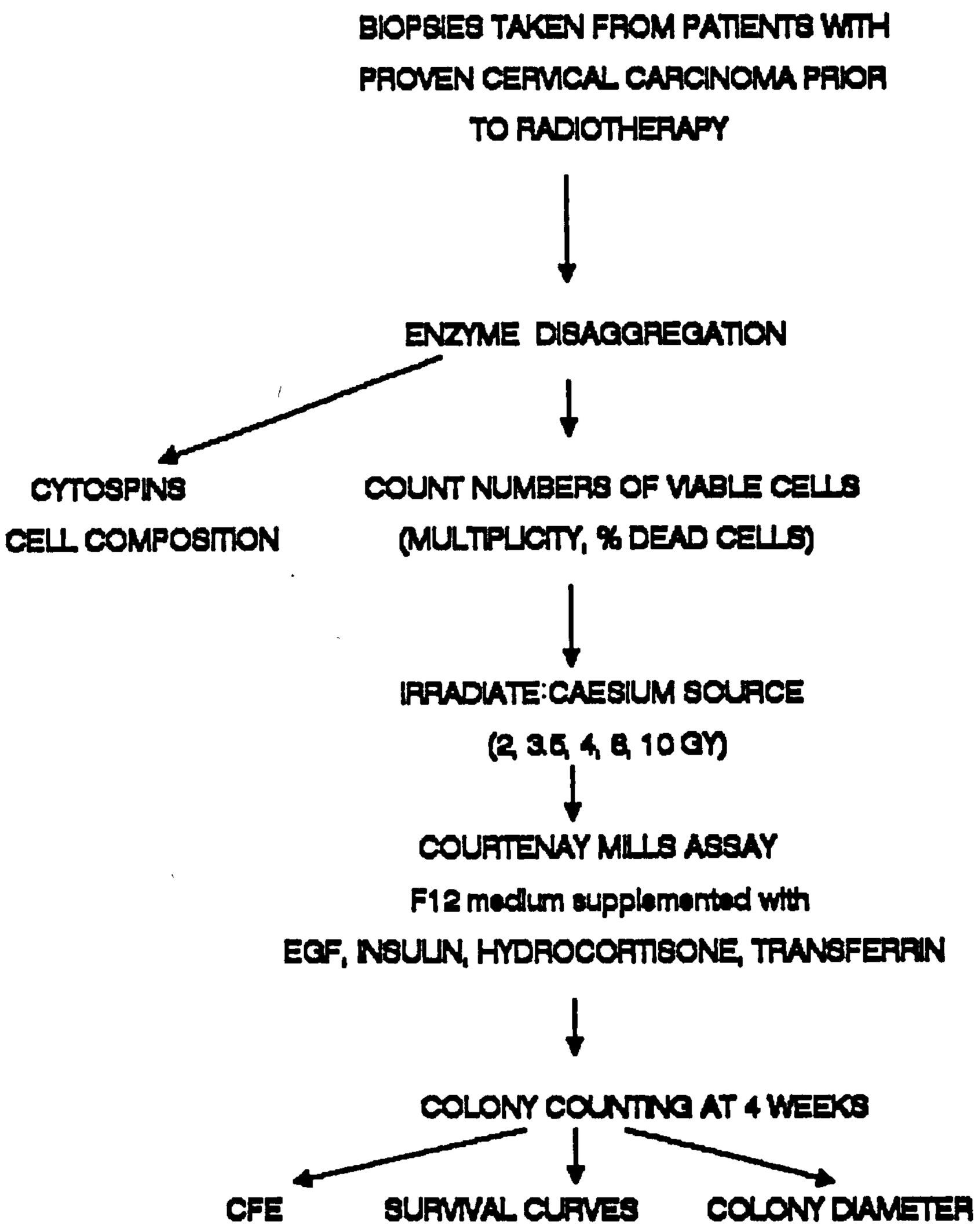
3) 0.5µg/ml hydrocortisone (Sigma)

4) 2.5µg/ml transferrin

5) 2mM glutamine (Gibco)

1ml of each (1-5) added to each 100mls of Ham's F12 and 15% foetal calf serum (FCS - Biological Industries).

FIG.2 METHOD OUTLINE



2.2 PREPARATION OF HUMAN TUMOUR SPECIMENS

Biopsies from 117 patients with proven cervical carcinoma (1987-1989) were taken under anaesthesia immediately prior to radiotherapy. Parallel specimens were sent to the histopathology department at the Christie Hospital for grading. The grading of the tumours was assessed by two histopathologists for 75% of the specimens (n=88) and were graded into well, moderately and poorly differentiated carcinomas. The specimens were transported in a sterile container to the laboratory where the specimens were weighed after removing necrotic tissue and washed in high antibiotic medium. Each sample was placed in high antibiotic medium containing 0.5mg/ml of pronase (Boehringer Mannheim), collagenase type I (Sigma) and deoxyribonuclease (Sigma) for 1.5 hours.

The supernatant was then collected and placed on ice and further disaggregation was carried out with 0.05% trypsin (Worthington) for 0.5 hour. The tumour material was filtered using a 100μm nylon screen (Nybolt) and centrifuged for 10 minutes. If red blood cells were visible in the cell pellet these were lysed using 9mls of sterile double distilled water and the material was pipetted up and down twice to mix rapidly. One ml of x10 phosphate buffered saline (PBS) was then added followed by 10mls of culture medium. The material was centrifuged for 10 minutes and the pellet resuspended in culture medium. The cell suspension was filtered through a sterile 37μm nylon filter (Nybolt) to remove any remaining cell clumps [Fig. 2].

2.2.1 Multiplicity determinations

A sample (100μl) was removed and 400μl of 0.4% Trypan Blue (Gibco, Paisley) was added. After 10 minutes viable, nucleated, trypan-blue excluding single cells, doublets and triplets as well as dead cells were counted on a haemocytometer. The number of viable cells, the viable cell yield, the multiplicity of the cell suspension and the percentage

of dead cells were calculated.

$$M = \frac{\text{no. single cells} + (\text{no. doublets x 2}) + (\text{no. triplets x 3})}{\text{no. single cells} + \text{no. doublets} + \text{no. triplets}}$$

The multiplicity of the specimens was less than 1.05 for all the specimens except 7 specimens. Multiplicity corrected counts were used in all experiments.

2.3 SAMPLE IRRADIATION

Irradiation of the cell suspensions was carried out at room temperature using a ^{137}Cs gamma source with a dose rate of 4.2Gy per minute. After determining the viable cell density of the tumour cell suspension, 1ml was placed in test tubes (Falcon 2057) at a cell density of $1x10^6$ cells/ml and $5x10^5$ cells/ml for control tubes and irradiated at doses of 2 and 3.5Gy. Where possible, doses up to 10Gy were given, and the cell suspension irradiated at 10Gy was used as a negative control. Again, the irradiated tubes were plated at more than one density if the tumour cell yield was large enough. The cell suspensions were plated without delay following irradiation.

2.4 TUMOUR CULTURE

2.4.1 Courtenay-Mills Soft Agar Clonogenic Assay

The Courtenay-Mills soft agar clonogenic assay was used to determine the CFE of tumour cells from disaggregated tumours and the survival of those cells following radiation (Courtenay and Mills, 1978).

Cell suspension (1ml, and if specimen small, 0.5ml) at 10x the density required was placed into Falcon 2057 tubes. Rat red blood cells, 1ml of a 1 in 8 dilution, was added to

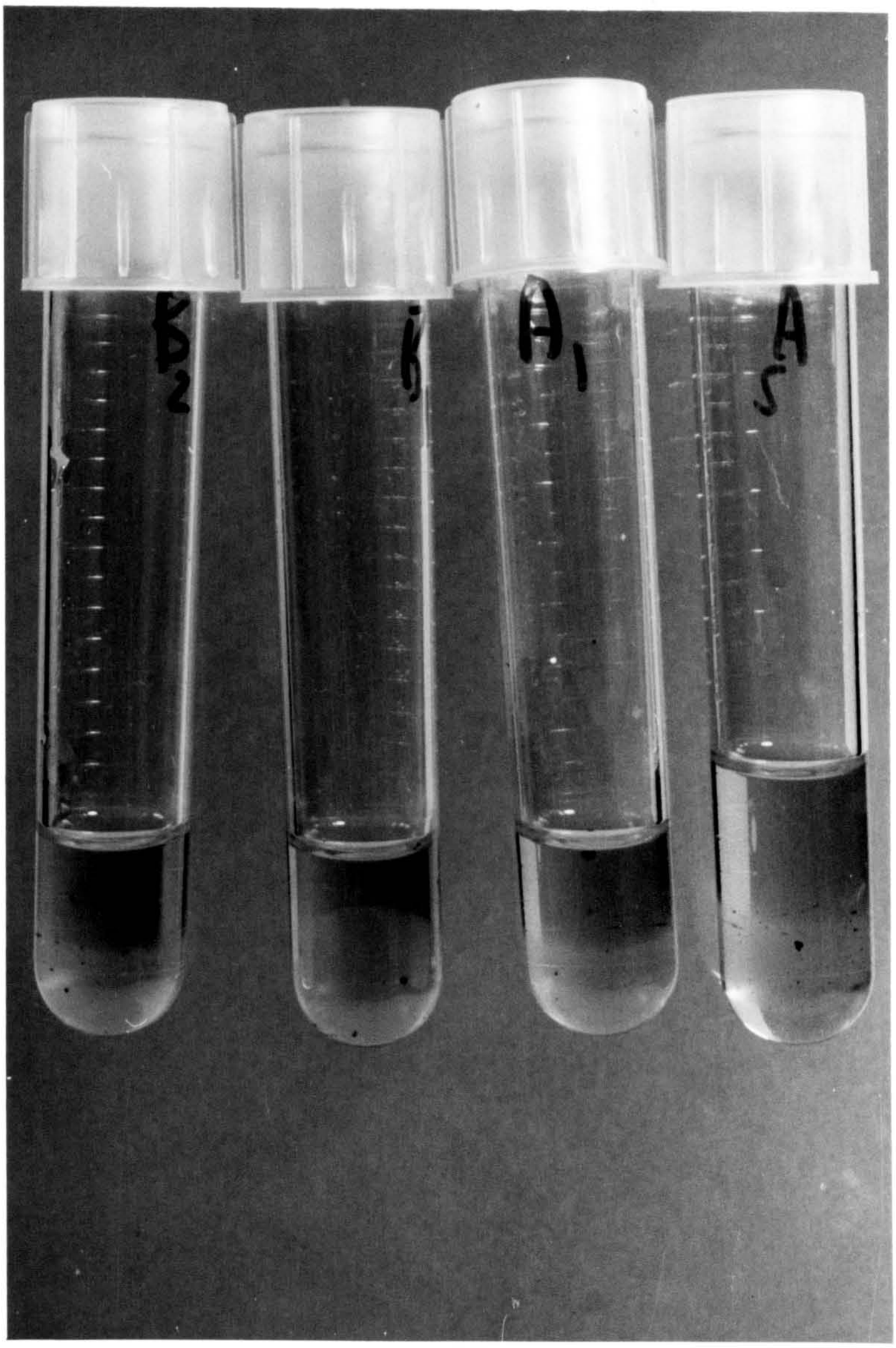


FIG.3 2057 TUBES CONTAINING COLONIES IN AGAR

the tumour cell suspension. Eight ml of 0.4% agar (stock agar was 5% Noble agar made up with double distilled water and autoclaved) was added to each 2057 tube. The medium was warmed to 37°C and the stock agar was held at 55°C and allowed to cool before adding to the medium. The 0.4% agar was pipetted up and down twice to mix the cells and 1ml aliquoted into each of 8 Falcon tubes ensuring that the tip of the pipette was at the bottom of the tube. No agar was placed on the sides of the tubes and no air bubbles were produced. The tubes were immediately placed on ice to set the agar. The tubes were placed into a 5% carbon dioxide, 5% oxygen, 90% nitrogen incubator with the caps of the tubes in the "loose" position. Occasionally, if the tumour specimen had a low cell yield, then 0.5ml of the cell suspension was placed in each tube and 4 replicates were set up for control and dose points. One ml of freshly warmed culture medium was added to each tube weekly and in the third week 1ml of medium was removed and 1ml added to each tube. After 4 weeks the tubes were removed from the incubator and 0.2ml of pre-warmed iodonitrotetrazolium violet (INT, Sigma) at 0.5mg/ml in double distilled water was added to each of the tubes which were then placed in a 20% oxygen incubator for 24 hours [Fig. 3].

2.4.2 Rat Red Blood Cells

Blood was taken from August rats via cardiac puncture. Each rat was bled every 6-8 weeks and approximately 2mls of blood was obtained per animal. The blood was processed as follows:-

1. Centrifuge blood for 10 mins at 1500 rpm and remove supernatant.

2. Add 5ml PBS, centrifuge for 10 mins at 1500 rpm and remove supernatant.

3. Add 5mls Ham's F12 + FCS, centrifuge at 2000 rpm and remove supernatant.

FIG.4 TUMOUR COLONY. Diameter = 150 um

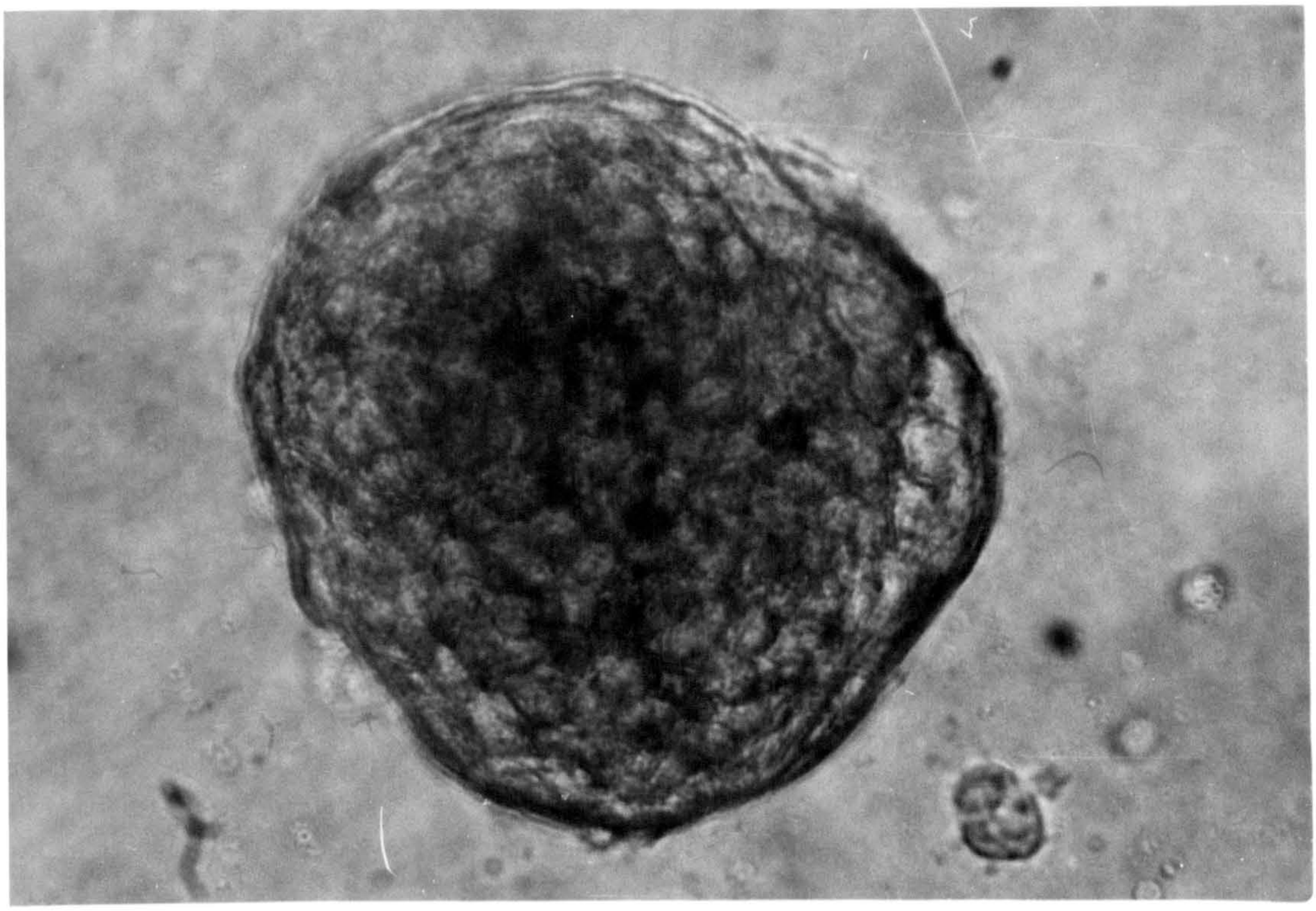
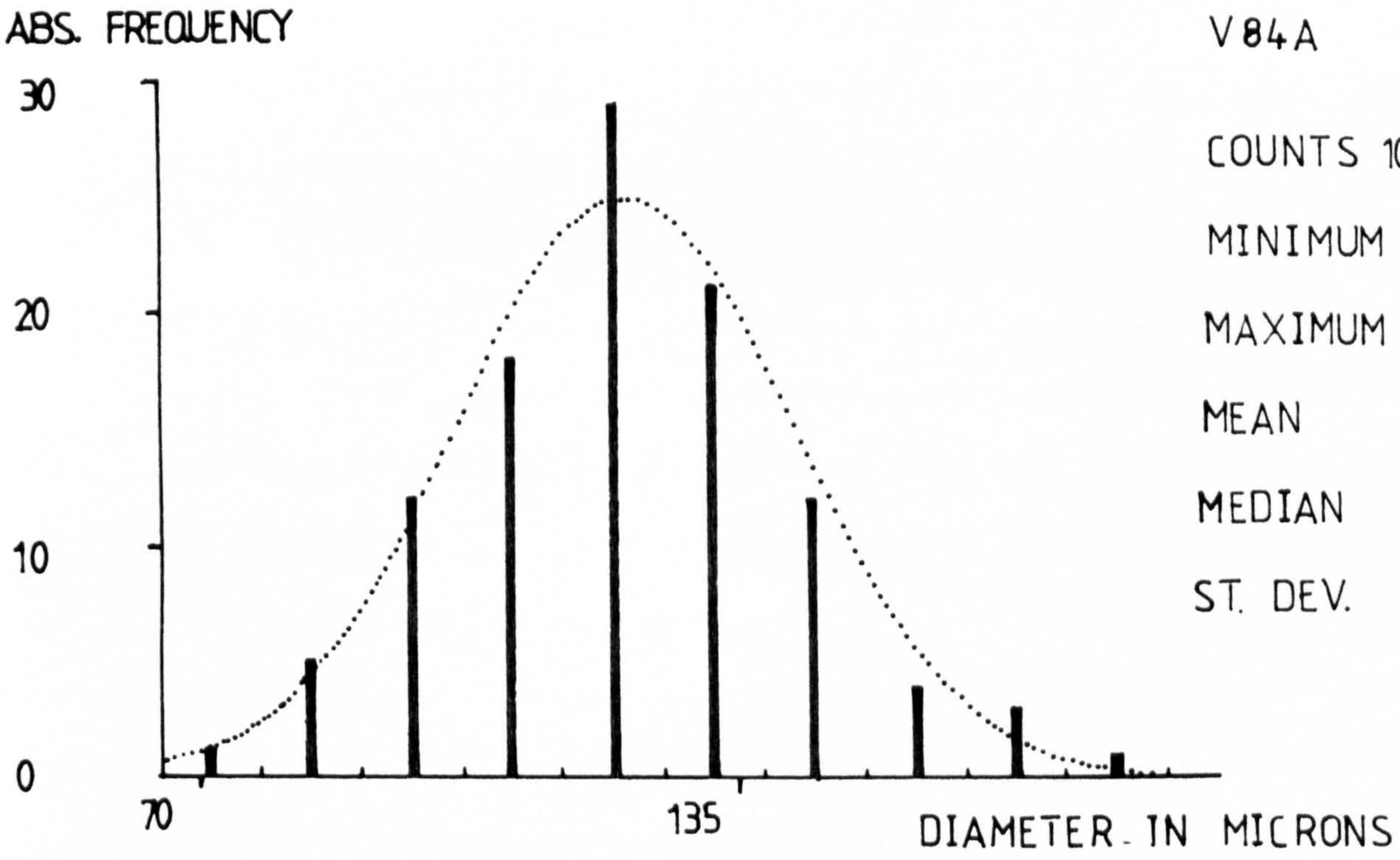


FIG.5 Histogram of colony size from tumour specimen V84A.



4. Place in a water bath at 44°C for 1 hour.

5. Add Ham's F12 + 15% FCS to the original volume and then dilute this volume of blood 1 in 8 with F12 + 15% FCS.

Diluted red blood cells were stored at 4°C for up to 2 weeks.

2.4.3 Colony Counting

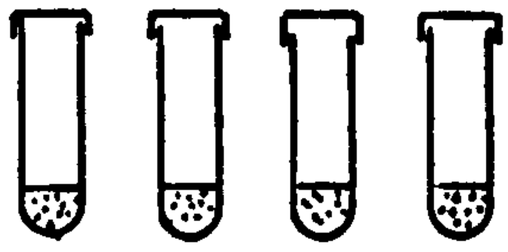
Tubes were removed from incubation 24 hours after INT addition and the medium was suctioned off. 0.2ml of 10% formol saline was added to each tube and the tubes were left overnight in a fume cupboard with the caps removed. The tubes [Fig. 3] were sometimes stored for a short time at 4°C but when colony counts were made on an experiment they were all counted on the same day.

The agar from each tube was then placed on a slide, squashed with a coverslip and colonies were counted on a light microscope [see Fig. 4]. It was noted that some tumour colonies were adherent to the plastic test tube, made more noticeable with INT staining, and care was taken to ensure that these colonies were not omitted from the colony counting. Colonies greater than 50 cells (diameter > 60 μm) were counted and the focus was continually moved up and down to ensure that all the colonies were counted. Colony counting was facilitated by using the low power and then checking the size of the colony using the high power (x400). A Kontron Mopp-Videoplan image analysis system was used to measure the diameters of the first 100 colonies for each tumour (using x100 magnification) [Fig. 5]. Colony diameters were measured by using a Zeiss light microscope equipped with a drawing projection device which allowed superimposition of drawings to the microscope field of vision. By drawing around the tumour colonies on a measuring tablet, the diameters were obtained. This provided a check on the size of the

FIG.6

THE COURTENAY - MILLS SOFT AGAR CLONOGENIC ASSAY

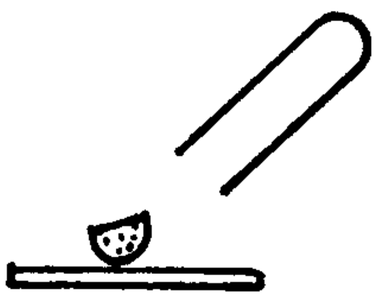
tumour cell suspension



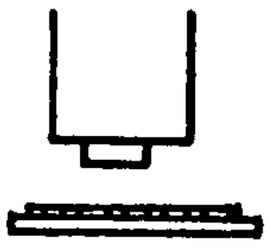
cells + rat red cells in 0.33% agar

Incubate in 5% O_2+5%CO_2

Feed weekly



Count colonies at 4 weeks



Light microscope x 400

colonies counted and enabled statistics of colony size to be obtained [see Fig. 6].

2.4.4 Frozen Tumour Storage

Where specimen size allowed, aliquots of tumour cell suspension were stored in liquid nitrogen. Cells were frozen at a cell density of 10^6 cells/ml in culture medium in 10% dimethylsulphoxide (DMSO, Analar). The cells were stored in Nunc 2ml tubes and frozen slowly above liquid nitrogen for a minimum of 6 hours and then placed in liquid nitrogen. Twelve tumour biopsy specimens were stored in liquid nitrogen before processing and extra tumour biopsies received were also frozen in liquid nitrogen, if not processed immediately. The biopsy was cut into 3-4mm pieces and washed with high antibiotic medium. The tumour pieces were placed in high antibiotic medium with 10% DMSO and frozen in nitrogen.

2.5 DIFFERENTIAL CELL COUNTS OF CYTOSPIN PREPARATIONS

Cytospin preparations of each tumour cell suspension were made using 100µl of the cell suspension at a concentration of approximately 10^5 cells/ml for each slide. Six to ten slides were prepared for each specimen. Two to four slides were stained immediately with the May Grunwald - Giemsa stain. The rest of the slides were wrapped in aluminium foil and stored in a freezer for immunohistochemical staining with monoclonal antibodies for human low molecular weight cytokeratins and human macrophages and the antibody for proliferating cells - Ki67.

2.5.1 May Grunwald - Giemsa Staining

Using air dried slides, the slides were flooded with neat May Grunwald staining solution (in methanol) and left for 2 minutes. The slides were then rinsed with tap water. The

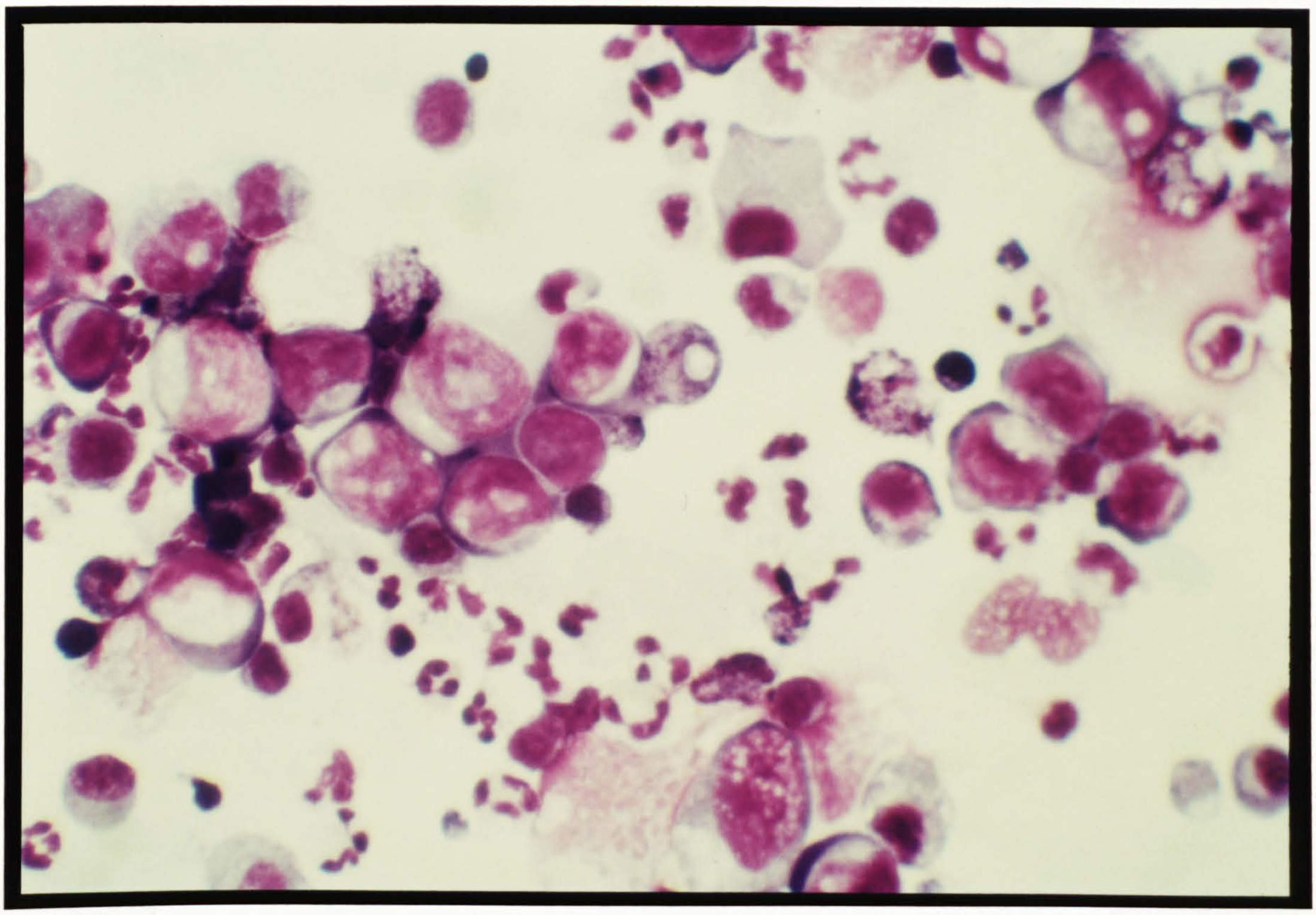


FIG.7 May Grunwald Giemsa stained cytospin of tumour cell suspension.

Giemsa stain was diluted 1.5 in 20 with Sorensen's phosphate buffer (1/15M Na_2HPO_4 + 1/15M KH_2PO_4 50:50, pH 6.8). The slides were flooded for 18 minutes, rinsed in tap water, air dried and mounted.

2.5.2 Counting

The proportion of tumour cells, macrophages, lymphocytes and granulocytes for each tumour cell suspension was determined [Fig. 7]. After staining, 500 cells on 2-4 cytospin slides were counted and scored as tumour cells, monocyte-macrophages, lymphocytes or granulocytes. Tumour cells were identified by their large size, their large euchromatic and irregular nuclei (which generally contained several large nucleoli) and their abundant, usually basophilic, cytoplasm. Macrophages were identified by their large size, eccentric, often lobulated nuclei, and abundant, usually highly vacuolated, cytoplasm. Lymphocytes were small, had a dense heterochromatic nuclear chromatic pattern and a thin rim of pale staining cytoplasm. Granulocytes were distinguished easily by their highly lobulated nucleus and cytoplasmic granules. The proportion of tumour cells in the cell suspension was also quantified by staining cytospins with the monoclonal antibody CK1 (Dako) which stains the low molecular weight human cytokeratins 6 and 18 (using the classification of Moll et al, 1982). If the tumour cells did not stain with CK1 then further immunostaining was carried out with the monoclonal CAM5.2 (Becton Dickinson) which stains the human cytokeratins 8, 18 and 19 using the Moll classification (1982). The proportion of macrophages was also determined using a monoclonal antibody for human macrophage EBM11 (Dako) due to the difficulty in distinguishing between tumour cells and macrophages in some specimens using morphological criteria alone. The antibody stains were carried out using the APAAP (alkaline phosphatase anti-alkaline phosphatase) technique. Between 300 and 500 cells were counted on 1 slide and the proportion of positive cells was expressed as a percentage of the total cells counted.

2.5.3 APAAP Staining

All staining was carried out in a humidified box at room temperature. The cytospins were fixed in acetone for 2 minutes and then transferred to Tris buffered saline (TBS). The slides were not allowed to dry out during the rest of the staining procedure as this has been shown to affect the quality of staining (Polak and Van Noorden, 1986). The primary mouse monoclonal was applied to the slide approximately 50 microlitres per slide. A 1 in 100 dilution of CK1 and a 1 in 10 dilution of EBM11 were used. The CAM5.2 antibody was used at the dilution supplied. The monoclonal antibody which stains proliferating cells Ki67 (Dako) was used at a dilution of 1 in 50. The slides were incubated for 30 minutes and then washed in TBS for 5 minutes. The secondary antibody was applied - a rabbit anti-mouse immunoglobulin (Dako) diluted 1/20 in TBS with 20% normal human serum (heat inactivated by heating at 56°C for 30 mins). The slides were incubated for a further 30 minutes and washed in TBS before applying the APAAP complex (Dako) diluted 1 in 80 in TBS. Again a 30 minute incubation was carried out and the slides were washed in TBS. The applications of the second antibody and the APAAP complex were repeated for 15 minutes. This has been shown to enhance the staining when using this technique (Cordell et al, 1984). The substrate was applied for 15 minutes. The slides were then washed in tap water to terminate the reaction and counter-stained with Mayers Haematoxylin for 3-5 minues. The slides were mounted in the water soluble Apathy's mountant.

Aliquots of the substrate used in the APAAP method were frozen in 10ml amounts.

100mls of substrate were made up as follows:-

20mg of naphthol AS MX phosphate (Sigma) dissolved in 2mls of dimethyl formamide and 98mls of 0.1M Tris (BDH) buffer (pH 8.2) were added. 100 microlitres of 1M Levamisole (Sigma) were added (blocks the endogenous alkaline phosphatase). Immediately before use of the substrate 10mg of Fast Red salt (Sigma) was added and the

substrate was filtered through a 0.45 μm filter onto the slides.

2.5.4 Double Staining using PAP and APAAP Techniques

To detect 2 antigens with mouse monoclonal antibodies, staining for one antigen was first revealed by an immunoperoxidase staining method followed by staining for the second antigen by immunoalkaline phosphatase. The cytospin preparations were fixed in acetone for 2 minutes and placed in TBS, pH 7.6.

Immunoperoxidase

The primary mouse monoclonal antibody was applied to each slide (CK1) for 30 minutes. The slides were then washed in TBS for 5 minutes and incubated with rabbit anti-mouse immunoglobulin peroxidase conjugate at a dilution of 1 in 40 (Dako) for 30 minutes. Normal human serum (1:5 diluted with TBS) was added to block cross reactivity against human immunoglobulin. The slides were washed in TBS and incubated with swine anti-rabbit immunoglobulin conjugate (Dako) at a dilution of 1 in 50 for 30 minutes and washed in TBS. The peroxidase substrate was then added (see below) to the slides for approximately 8 minutes. The slides were washed in TBS.

The immunoalkaline phosphatase staining was then carried out as described above using Ki67 as the primary monoclonal antibody. The slides were mounted in an aqueous mounting medium.

Immunoperoxidase Substrate

10mg of diaminobenzidine 4HCl (DAB, Sigma) was dissolved in 16.5mls of TBS. 17μl of hydrogen peroxide (100 volume) was added and the substrate filtered onto the slides.

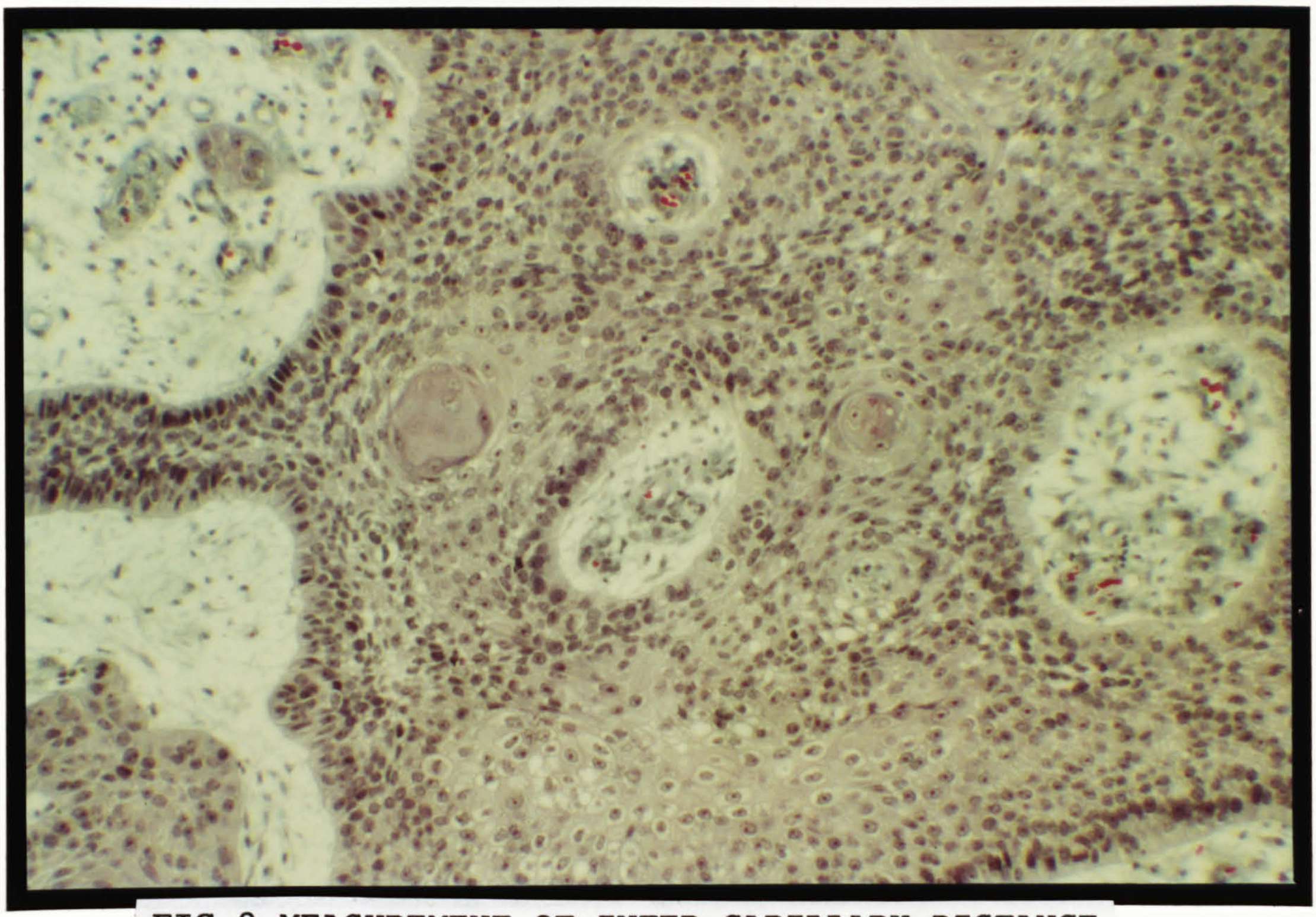
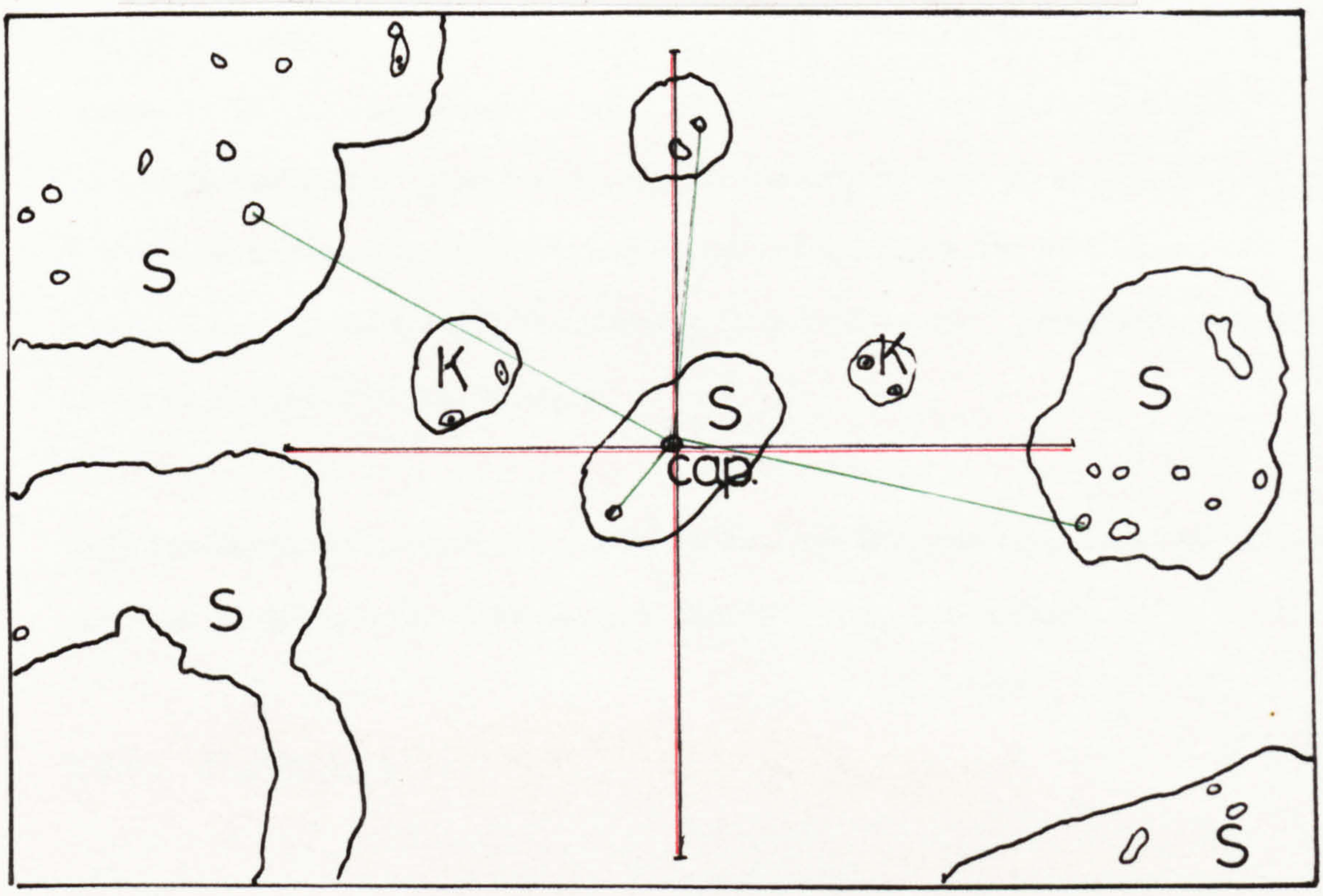


FIG.8 MEASUREMENT OF INTER-CAPILLARY DISTANCE



key: S=stroma K=keratin cap=capillary

2.6 TUMOUR VASCULARITY

2.6.1 Intercapillary Distance

Five micron thick histological sections were cut from formalin-fixed and paraffin-embedded tumour biopsies from 107 consecutive patients with cervical carcinoma. The sections were stained using the Masson-Trichrome technique. Using this technique, the green-blue colour of the connective tissue and the elastic fibres in the vascular wall, red stained erythrocytes and haematoxylin stained tumour cell areas permitted a relatively easy distinction of the parenchymal, stromal and vascular elements. Adjacent microscopic visual fields (25-40) were scored on the entire section using x100 magnification. Areas of gross necrosis and haemorrhage were avoided. An image analysis system Imagan II was used to measure the intercapillary distances. A grid in the eyepiece of the microscope was centred on a capillary vessel and a line was drawn to another capillary in each of 4 quadrants of the eyepeice, using a Leitz microscope equipped with a drawing projection device which allowed superimposition of drawings to the microscope field of vision [see Fig. 8]. By drawing the lines between the capillaries on a magnetic tablet, the intercapillary distances were obtained. For each biopsy 100-160 measurements for the intercapillary distance (ICD) were made. The computer enabled basic statistics for ICD to be obtained.

In eleven tumour biopsies the distances between capillaries in the stromal bands were measured in addition to the ICD measured over the section in its entirety.

2.6.2 Morphometry

Morphometric analyses were made on each tumour biopsy. The tissue sections were scored by using a Chalkley grid in the eyepiece of a Leitz microscope. At least 40 high power fields were scored at a magnification of x400. The cells immediately to the right of

the 25 dots in the Chalkley grid were scored. Thus 1000 counts were made and the percentage volume components of parenchyma, stroma, vessels and necrosis were determined. For 10 tumours more than one biopsy was received on which a morphometric analysis could be carried out on each biopsy.

2.7 STATISTICAL ANALYSES

The statistical analyses were carried out on a MicroVAX 3600 computer system. The Minitab statistical software was used to test the correlation between quantitative variables by the establishment of linear regression coefficients and the Chi-Square test was used for the analyses of the frequency of qualitative variables. Analysis-of-variance was carried out with the SPSS statistical package.

The radiation survival data from the tumour specimens were fitted to the linear quadratic equation using the program DRFIT on the MicroVAX. The program DRFIT is a comprehensive program for fitting survival curve data to a number of radiation survival models (Roberts, 1990). The data points are weighted by the inverse of their expected variances using the strategy of Gilbert (1969).

2.8 CLINICAL DATA

Patients with small volume stage IB or IIA disease were treated with intracavitary caesium alone with 2 insertions giving an A point dose of 67.5-75 Gy. For bulky stage IB disease or stage II disease and some stage III (no lymph node involvement demonstrated) patients received external beam irradiation to small-field, wedged inhomogeneous 3 week treatment (32.5 Gy to point B) followed by 2 intracavitary caesium insertions (A point dose of 50-60 Gy). If lymph node involvement was demonstrated patients received treatment by external beam to large hexagonal fields over 4 weeks (40 Gy point A) supplemented by a single intracavitary insertion giving

32.5-37.5 Gy to A point (Hunter et al, 1986).

The clinical data which were used to look at correlations with vascular parameters were on patients with minimum follow up time of 2 years.

FIG.9 MAY GRUNWALD GIEMSA STAINED COLONY CELLS

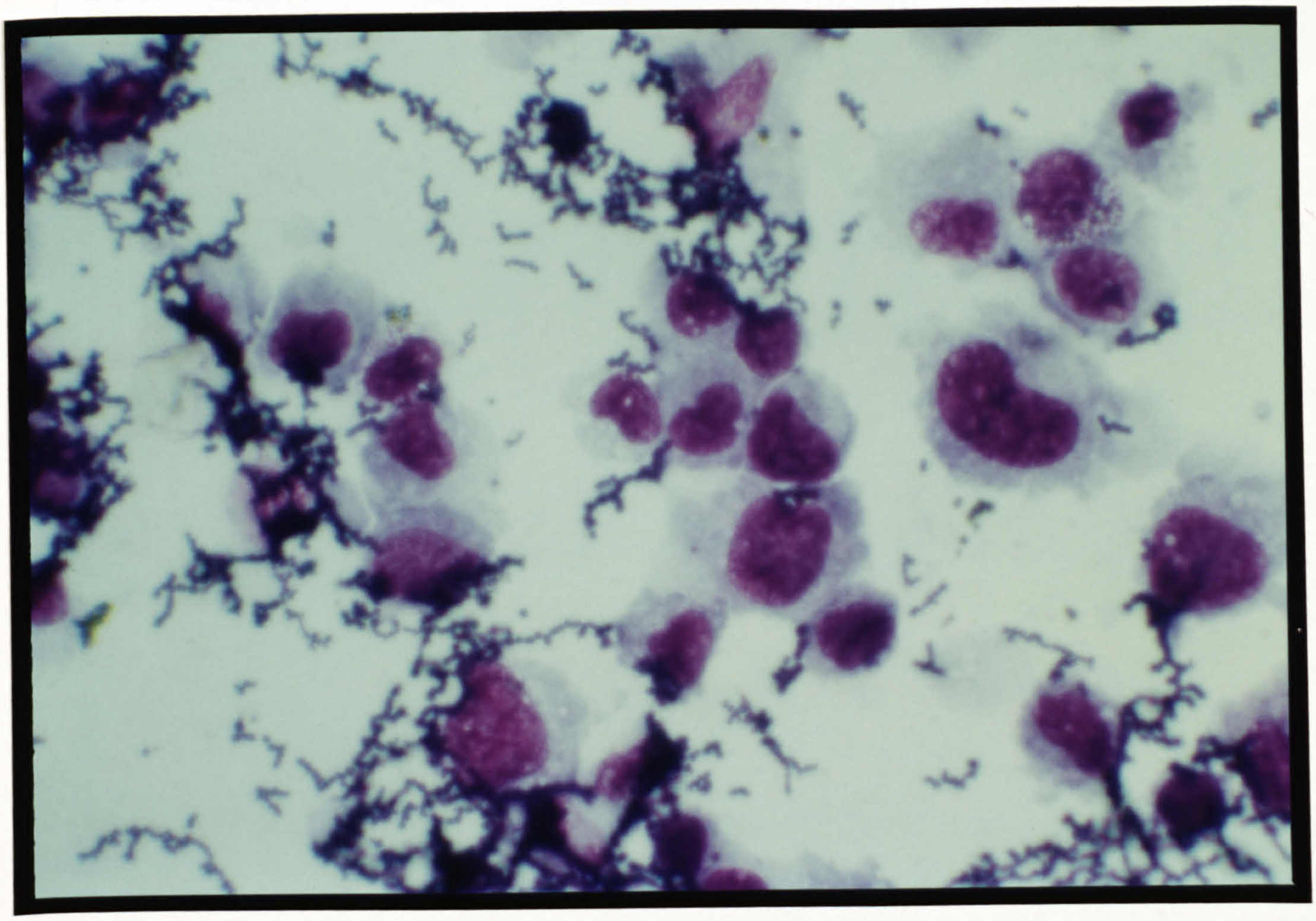
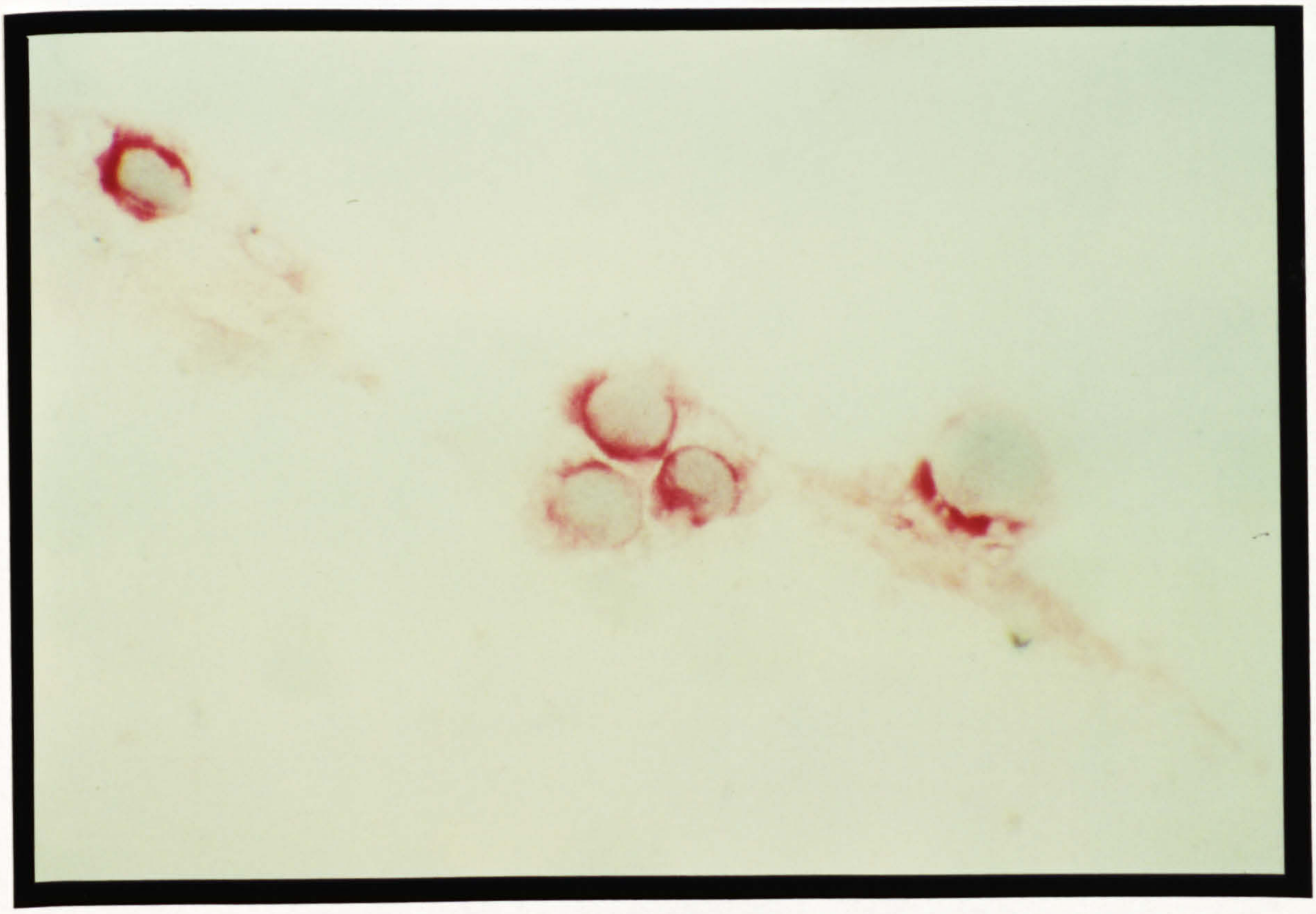


FIG.10 CK1 STAINED COLONY CELLS



3. VALIDATION OF THE ASSAY

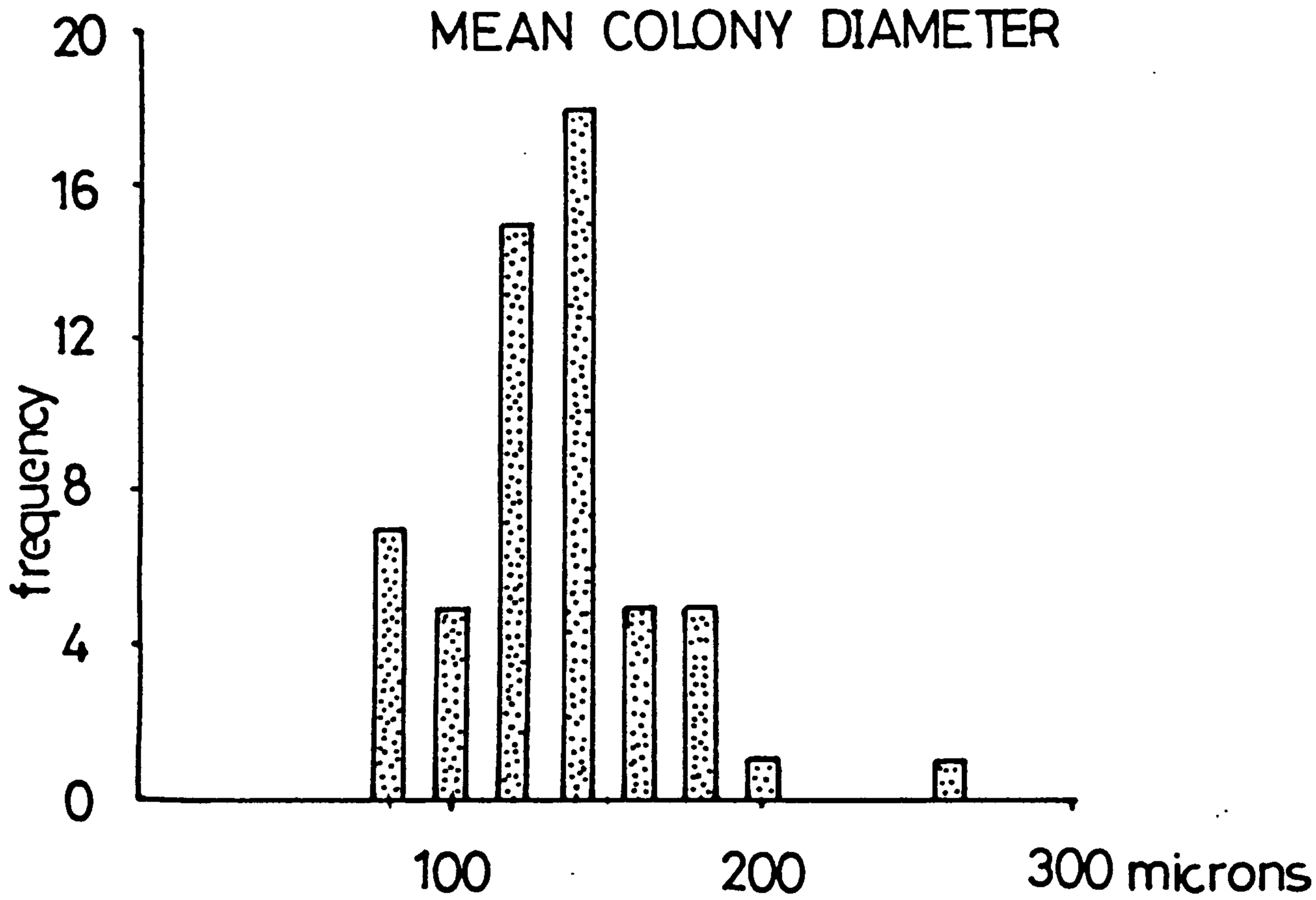
3.1 CHARACTERISATION OF COLONY CELLS

Cytospin preparations were made from colonies grown from a number of the tumour specimens. The colonies were plucked from the agar at 4 weeks using a micropipette and disaggregated in 0.5% trypsin for 30 minutes with intermittent vortexing. The cytospin preparations were stored in a freezer until staining was carried out. Some of the colonies were also embedded in either 10% gelatin or tissue embedding medium (OCT, Tissue Tek) and frozen in liquid nitrogen until antibody staining was carried out. Frozen sections of the colonies were stained in addition to the cytospins. The cytospins were stained with the May Grunwald-Giemsa technique. The colony cells had large euchromatic and irregular nuclei and many had abundant cytoplasm, typical of tumour cells (Fig. 9) and had the same morphological characteristics as the cells in the original tumour cell suspensions.

The colony cells were also stained using the APAAP immunohistochemical technique with CK1 (DAKO) and CAM5.2 (Becton Dickinson), antibodies to low molecular weight cytokeratins. The colony cells stained positively with CK1 and/or CAM5.2 (Fig. 10) which confirmed that the colony cells were of malignant epithelial origin (Bobrow et al, 1986).

Tumour colonies from specimens V76 and V79 were cultured in repeat experiments for electron microscopy. The colonies were plucked from the agar (taking as little agar as possible) and washed in tris buffered saline. The pellets of colonies were taken to the electron microscopy department within 30 minutes and were prepared for transmission electron microscopy. Lower magnification pictures of the tumour colony cells (both from moderately differentiated tumours) showed cells which had the appearances of undifferentiated tumour cells. There were areas of close approximation between the cells,

FIG.11 COLONY SIZE HISTOGRAM



but no desmosomes were seen, a common finding for poorly differentiated cells in culture. There were areas of extracellular matrix secretion, consistent with these colony cells arising from cells from the basal layer of the epidermis. Tonofilaments were not seen and would not be expected in undifferentiated cells. The cells had no features of macrophages. The appearances under EM were, therefore, those of undifferentiated tumour cells and when taken in conjunction with the results of the immunostaining provided proof that the cells which formed colonies were of malignant epithelial origin.

3.2 COLONY SIZE

The mean colony diameter measured for 57 tumours was 131±32 µm (± 1sd) with a range of 77 to 255µm (Fig. 11). The minimum colony size counted was 60µm which ensured that colonies greater than 50 cells were counted. The size of 60µm was taken as the cut off for colonies of 50 or more cells and holds true for cells less than 11µm in diameter if the empirically-derived formula proposed by Meyskens et al (1984) is used. Cell diameters were measured on a number of the tumour specimens using a Coulter Multisizer System (Coulter Electronics, Luton) counter and cells greater than 11µm were not found (n=7, mean = 10.1, range 8.5 - 11µm).

Using the formula of Meyskens et al (1984)

$$\text{no. of cells/colony} = \frac{2.4\,(\text{colony diameter})^{2.378}}{(\text{cell diameter})^{2.8}}$$

a 60µm colony = 11µm cell diameter

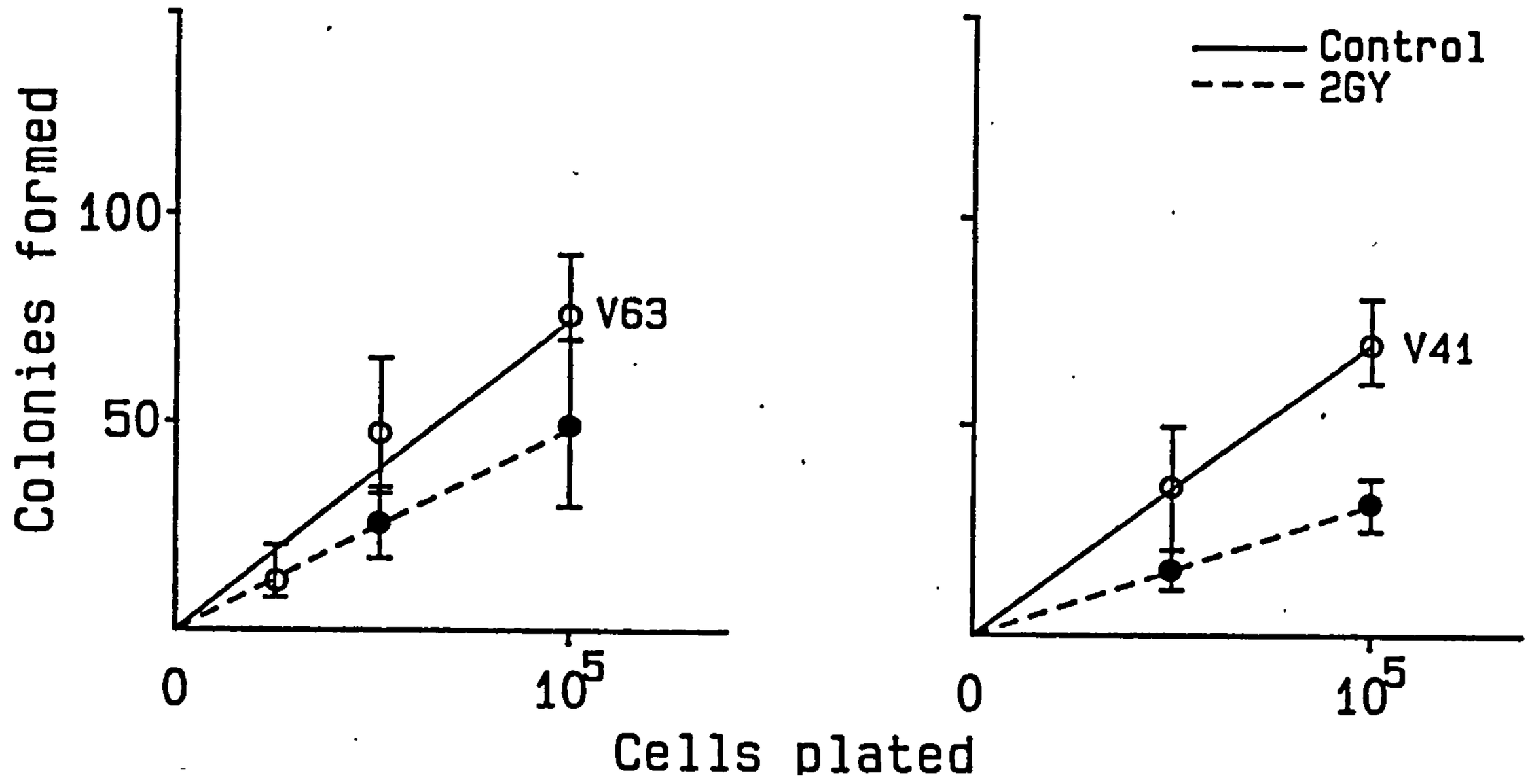
90µm colony = 16µm diameter

TABLE 4 COLONY SIZE REVIEW

Reference	Colony diameter	No. cells	Assay
Salmon et al, 1978		>40	H-S
Pavelic et al, 1980		>30	H-S
Kern et al, 1982		>20	H-S
Agrez et al, 1982	>60µm	25-50	H-S
Bertoncello et al, 1982		>40	B-M
Pathak et al, 1982	≥60µm		H-S
Sandbach et al, 1982		>50	H-S
Sutherland et al, 1983		≥30	H-S
Williams et al, 1983	60-400µm		H-S
Kirkels et al, 1983	>60-149µm		H-S
Bertelson et al, 1984	>60µm	20-40	H-S
Alley and Lieber, 1984	≥60µm		H-S
Courtenay, 1984		>50	C-M
Salmon, 1984	>60µm		H-S
Fan et al, 1984	>30µm		mod H-S
Parker et al, 1984	≥60µm		H-S
Hug et al, 1984	≥75µm	≥40	Double layer agar
Schiff and Shugar, 1984		>20	
Hofman et al, 1984	≥80µm	>40	H-S
Gupta and Eberle, 1984	≥100µm	30-50	C-M
Shoemaker et al, 1985	>60µm		H-S
Runge et al, 1985	80-100µm	30-40	mod H-S
Endresen et al, 1985	>100µm	>30	H-S and C-M
Eliason et al, 1985	≥60µm		E
Walls and Twentyman, 1985		>50	Carney and C-M
Besch et al, 1986	≥60µm		H-S
Von Hoff et al, 1986	≥200µm	>50	H-S
West and Sutherland, 1986		>50	C-M
Rofstad et al, 1987		>50	C-M
Page et al, 1988		>50	H-S
Ottestad et al, 1988	≥60µm		C-M
Tveit et al, 1988	>100µm	>30	C-M
Tveit et al, 1989	>75µm	>30	C-M

Key: H-S = Hamburger-Salmon, C-M = Courtenay-Mills, E = Eliason methyl cellulose system, B-M = Bradley-Metcalf

FIG.12 LINEARITY OF COLONY FORMATION



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The relationship between the number of cells plated and number of colonies formed for control and irradiated tubes for 2 tumour specimens. Each point represents the average for 4 or 8 replicate tubes and error bars ± 1 sem.

The number of cells in a 3-D system is difficult to quantify and therefore colony diameter provides a more precise measurement (Singletary et al, 1985). It is important to identify the proper colony size criterion to be used as the end point for quantitating cell survival. The shape of the radiation survival curves has been shown to be dependent on the size chosen to define a colony using the V79 cell line (Besch et al, 1986). From Table 4 it can be seen that a range of colony size and cell number has been used by many groups. Some tumour histologies have on average larger cells and require a greater minimum colony size eg. 100μm taken for melanoma by Tveit et al (1988). Very few studies comment on cell size though Agrez et al (1982) observed cell diameters of 14-18 microns in their series of solid tumours. Taking at least 50 cells per colony minimises errors from cell aggregates which may be introduced into the cell suspensions and is the minimum size for accurately assessing cell radiation survival.

3.3 LINEARITY OF COLONY FORMATION

It is important to establish a linear relationship between the number of colonies formed and the concentration of cells plated in clonogenic assays. This has been demonstrated in some studies (Hamburger, 1987; Pavelic et al, 1980) but not in others (Eliason et al, 1985; Page et al, 1988; Meyskens et al, 1983). In this work linearity was demonstrated with a good correlation between the number of cells plated and colonies formed , in 55 specimens. Enough cells were obtained in 19 tumour specimens to plate out more than one density for the irradiated specimens (at 2Gy) to demonstrate linearity of colony formation in irradiated specimens also (Fig. 12).

3.4 RADIATION SURVIVAL CURVES

Clonogenic assays have also been criticised in the past due to the lack of evidence of survival curves after exposure to ionising radiation (Selby et al, 1983; Meyskens, 1983). The presence of clumps has been shown to produce a plateau on the radiation survival

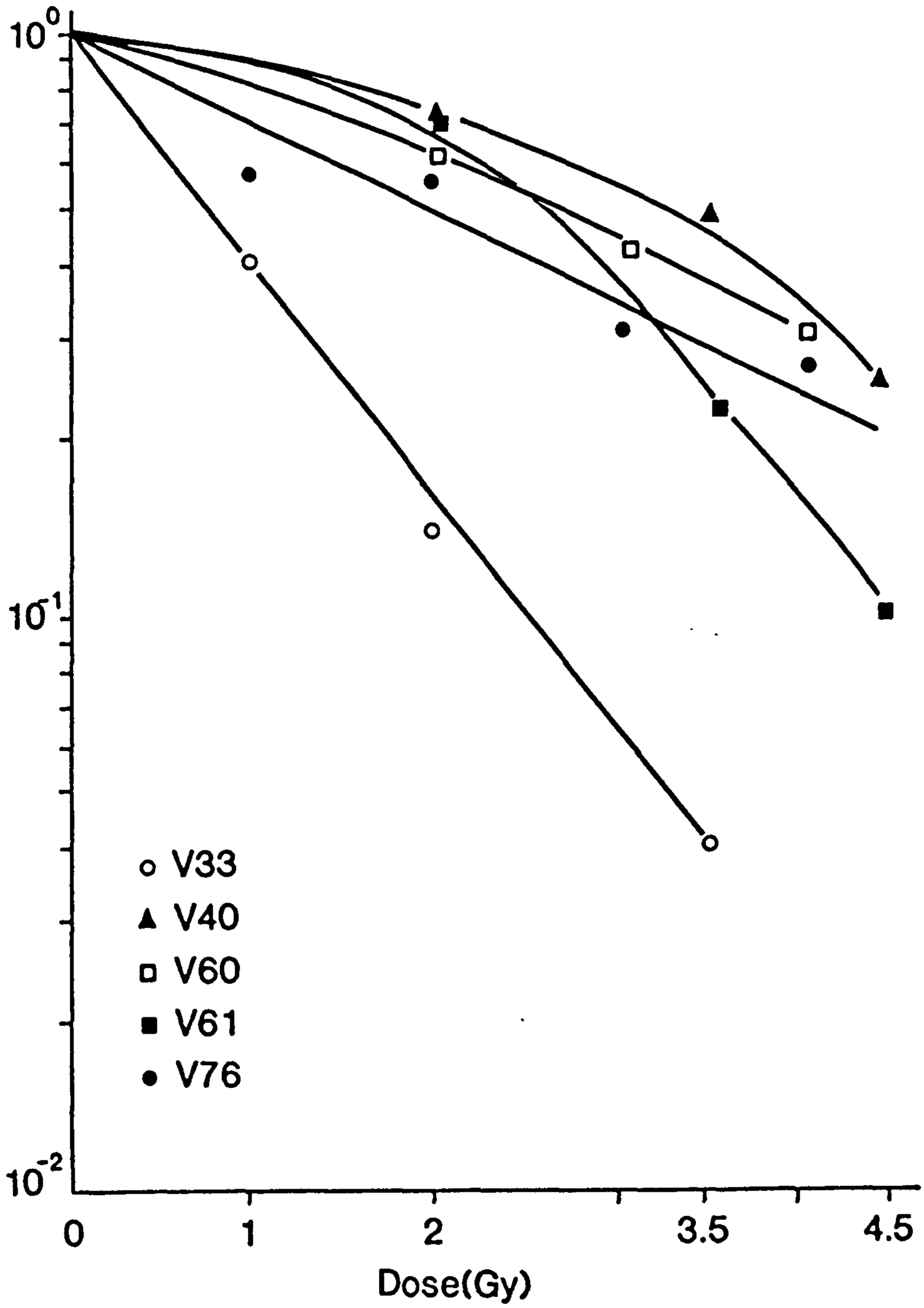


FIG.13 RADIATION SURVIVAL CURVES. Curves based on 2 - 4 experiments.(Error bars omitted for clarity, see Appendix 4).

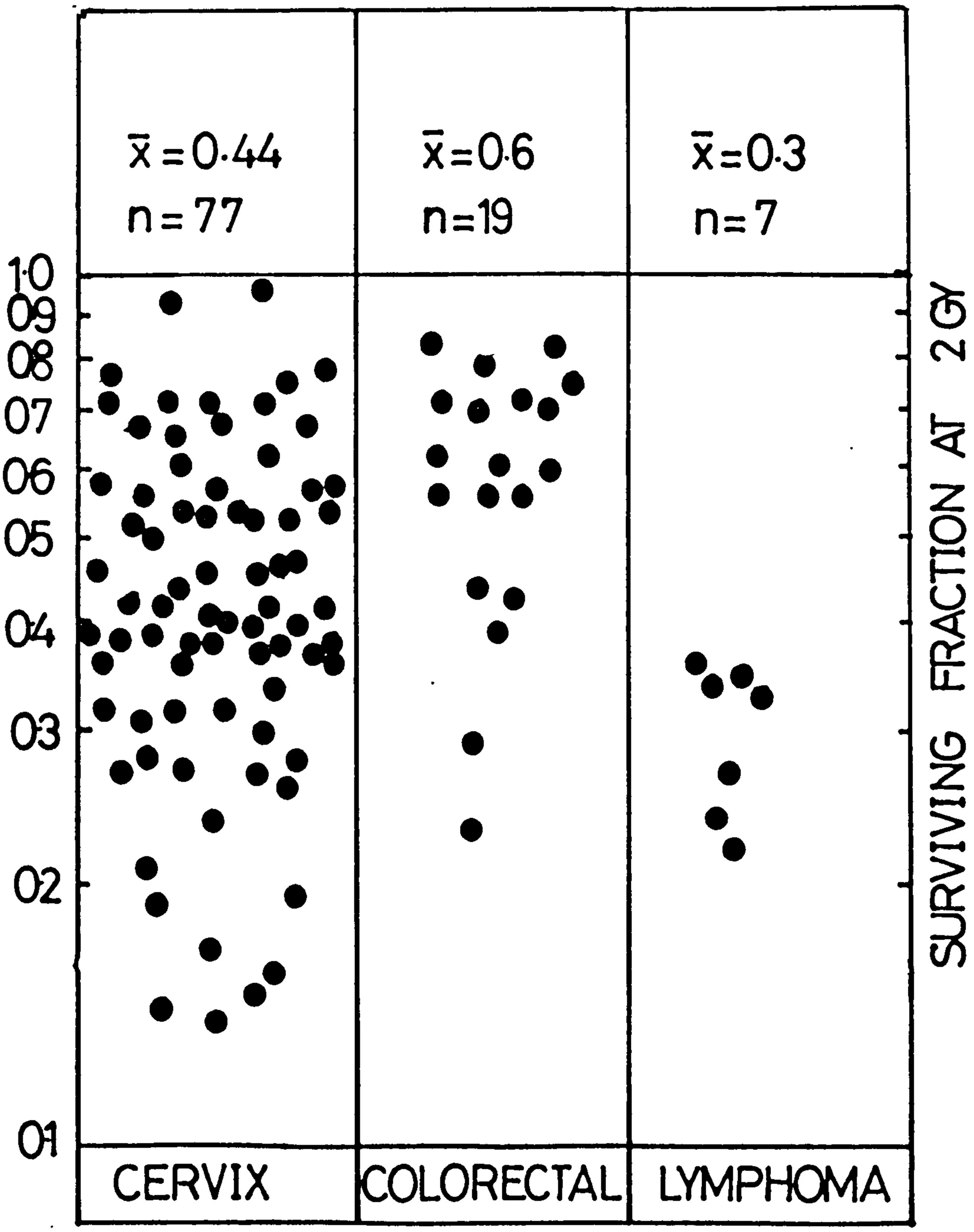


FIG.14 SF2 FOR DIFFERENT TUMOUR HISTOLOGIES
x = mean SF2value.

curves (Meyskens, 1983; Rockwell, 1985). Survival curves were produced where enough cells were obtained from specimens, with irradiation carried out at doses from 1 to 7 Gy, and irradiation at 10 Gy used as a negative control to check that cell clumps were not present in the original cell suspensions. As the colony forming efficiencies are low very few or no colonies would be seen in the 10 Gy irradiated tubes. The shapes of the survival curves established in this work are similar to those of survival curves obtained from tumour cell lines established in monolayer culture. The linear quadratic model was fitted to the curves using the DRFIT program (Chapter 2) and a wide variation in the alpha values was obtained 0.21-0.91 Gy^{-1} (Fig. 13). The survival curves plotted in figure 13 were based on 2-4 experiments and error bars have been omitted for clarity, though the errors have been listed in Appendix 4.

Surviving fraction at 3.5 Gy was determined for 39 specimens giving a mean value of 0.23 ± 0.14 (±1 sd). In all cases the SF3.5 value was lower than the SF2 value.

3.5 SURVIVING FRACTION AT 2GY FOR DIFFERENT TUMOUR HISTOLOGIES

As part of the validation of the assay colorectal tumours and lymphomas were processed using the Courtenay-Mills soft agar assay to determine SF2 values for these tumour types and to look for differences in the values obtained. SF2 was obtained for 19 colorectal tumours and 7 lymphomas (Table 5). The mean SF2 for the colorectal tumours was 0.60±0.17±0.04 (±1 sd ±1 sem) with a range of 0.23 - 0.84. The mean SF2 for the lymphomas was 0.30±0.05±0.02 (±1 sd ±1 sem) with a range of 0.22 - 0.36 (Fig. 14). A two sample t-test was carried out between the means of the cervix tumours and the colorectal tumours, t=3.27, p=0.003, DF=29, showing that the difference between the means of the two tumour groups was highly significant. Similarly, a t-test showed a significant difference between the mean SF2 of the lymphomas and the cervix tumours (t=4.82, p=0.0001, DF=22). This contrasts with the findings of Rofstad et al

TABLE 5 SF2 FOR DIFFERENT TUMOUR HISTOLOGIES

Colorectal specimens (n=19) SF2 values		Lymphomas (n=7) SF2 values	
C13	0.72	L2	0.36
C14	0.56	L3	0.22
C16	0.7	L4	0.27
C17	0.56	L7	0.33
C18	0.72	L9	0.35
C19	0.61	L10	0.24
C24	0.79	L14	0.34
C26	0.62		
C27	0.71	Ave SF2 =	0.3±0.05±0.02
C29	0.56		(±1 sd ±1 SEM)
C34	0.29		median 0.33
C35	0.6		(0.22 - 0.36)
C36	0.75		
C38	0.83		t-test - between means of
C40	0.23		cervix tumours and
C48	0.44		lymphomas
C53	0.39		t=4.82, p=0.0001,
C54	0.43		Df = 22
C60	0.84		

Ave SF2 = 0.597 median 0.61

= 0.6±0.17±0.04

(±1 SD ±1 SEM)

(0.23 - 0.84)

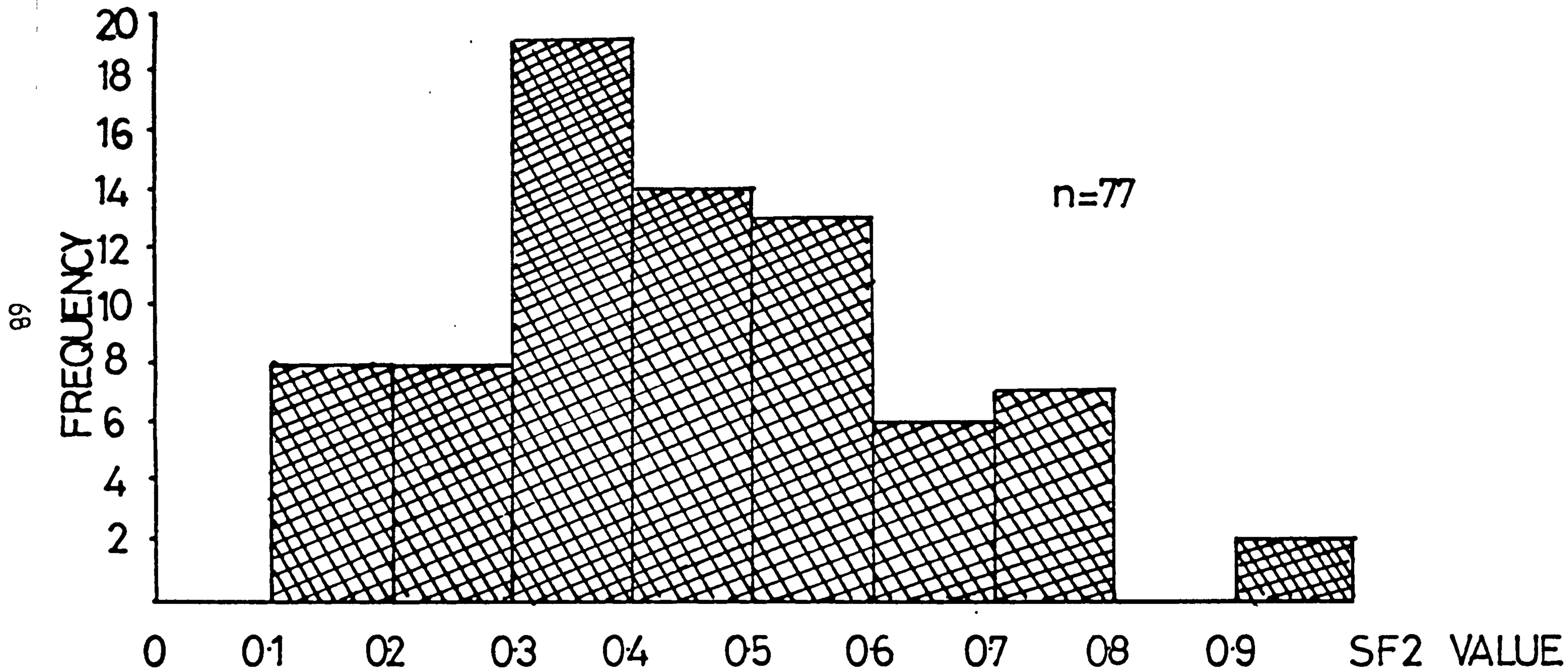
t-test - between means of cervix and colorectal tumours

t=-3.27, p=0.0028

Df = 29

(1987) who carried out SF2 determinations on 7 different tumour types and did not demonstrate a significant difference between the mean SF2 values of the 7 tumour groups using the same assay. Within each tumour group there was a wide range of SF2 values obtained, though the mean for 8 cervix tumours was lower (0.30) compared to 0.44 for 77 cervical carcinomas in this work. In the analysis of the published data by Malaise et al (1989) cells were consistently more sensitive when tested by clonogenic assay and when compared with colony formation for established cell lines (Suit et al, 1989). However, the mean SF2 for the groups of primary tumours determined by the Courtenay-Mills assay in this study are similar to the mean values obtained for cell lines derived from tumours from these classes (West et al, 1989; Suit et al, 1989). Average values were 0.49 ± 0.07 and 0.43 ± 0.06 (±1 SEM) for colorectal and cervix for published values (West et al, 1989) and 0.34 for 7 lymphoma cell lines (Fertil and Malaise, 1985). Brock et al (1989) found that the SF2 values for head and neck tumours, obtained using the CAM assay, were more sensitive than melanomas and lung adenocarcinomas. The average value for 72 squamous cell carcinoma cultures was 0.33. The reasons for these differences in the SF2 values obtained by the different groups, in particular between the values obtained with this assay and the work of Rofstad and colleagues using the same assay, are not clear. The numbers of cervix tumours in the Oslo study was only 8 and it is likely that some tumour selection occurred. In addition the plating efficiencies of the Oslo group were higher, 0.4 - 2.1% for the cervix tumours. Culture conditions have been shown to affect the radiosensitivity of cells (West and Sutherland, 1986). The SF2 values obtained from in vitro assays should not be expected to reflect simply and directly radiation sensitivity in vivo (Suit et al, 1989). It may be that the relative ranking of radiosensitivity, as measured by the SF2, will prove to be of importance within one laboratory, rather than the absolute value.

FIG.15 HISTOGRAM OF SF2 VALUES FOR CERVIX TUMOURS



4. REPRODUCIBILITY OF THE ASSAY AND INVESTIGATION OF TUMOUR HETEROGENEITY

4.1 RESULTS

Values for SF2 were obtained for 77 cervix tumours, the mean value being 0.44 (SD = 0.19) with a range of 0.14 - 0.97 (Fig. 15). The sampling errors of the cell counts, made on the tumour cell suspensions prior to plating, were of the order of 7% or less as more than 200 cells were counted for the majority of specimens. In order to eliminate the errors due to small numbers, the experimental errors were calculated for those experiments where the CFE was high ie. >100 colonies per tube. The mean intra-experimental coefficient of variation was 19% (7 - 35%, n = 12). The mean coefficient of variation for repeat experiments was 11% based on 6 tumour specimens with 2-4 experiments.

Analysis-of-variance techniques were used to determine the significance of the inter- and intra-tumour variation, using both one-way and nested designs. Duncan's Multiple Range Test was used to determine which pairs of tumours were significantly different from each other. The maximum number of specimens which could be analysed using the Multiple Range Test with the statistical package used was 50. A logarithmic transformation was applied to the CFE data in order to achieve a more normal distribution. A significance level of $p < 0.05$ was used throughout. Analysis of variance showed there were no significant differences in the SF2 values obtained between repeat experiments. Using frozen tumour cell suspensions from 23 specimens 2-4 repeat experiments were carried out with 34 repeat experiments in total. Six repeat experiments were performed using tumour biopsies which had been stored in liquid nitrogen (Fig. 16). Interestingly, there were significant differences in the CFEs of the repeat experiments ($p < 0.001$). Repeat experiments on the 3 specimens with low SF2 values (V26, V33 and V38 shown in Fig. 16) were carried out with a 6 month interval and were

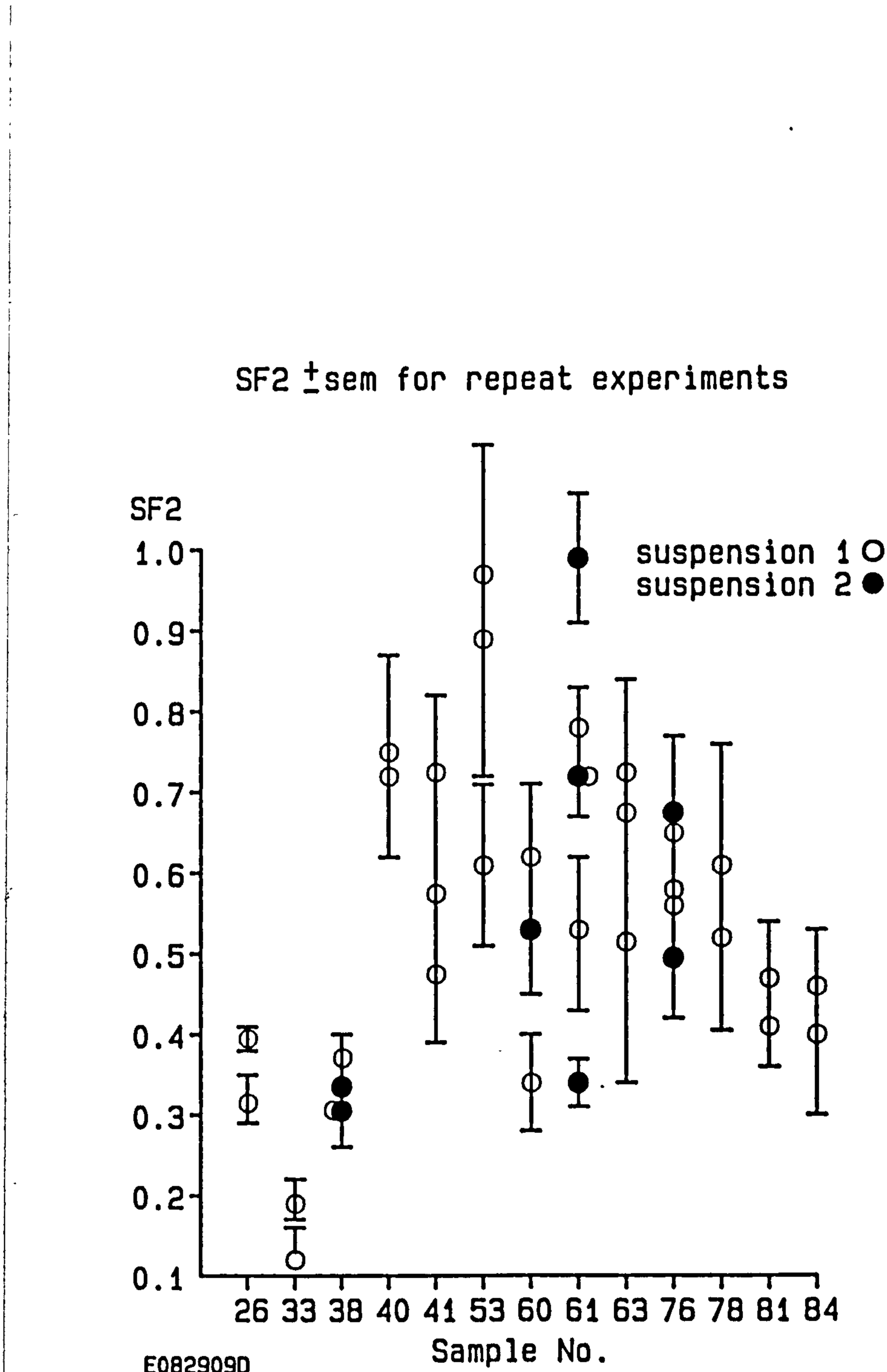


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FIG.16

Values of SF2 (± 1 SEM) obtained for repeat experiments and 4 'split' tumours. V38, V60, V61, V76.

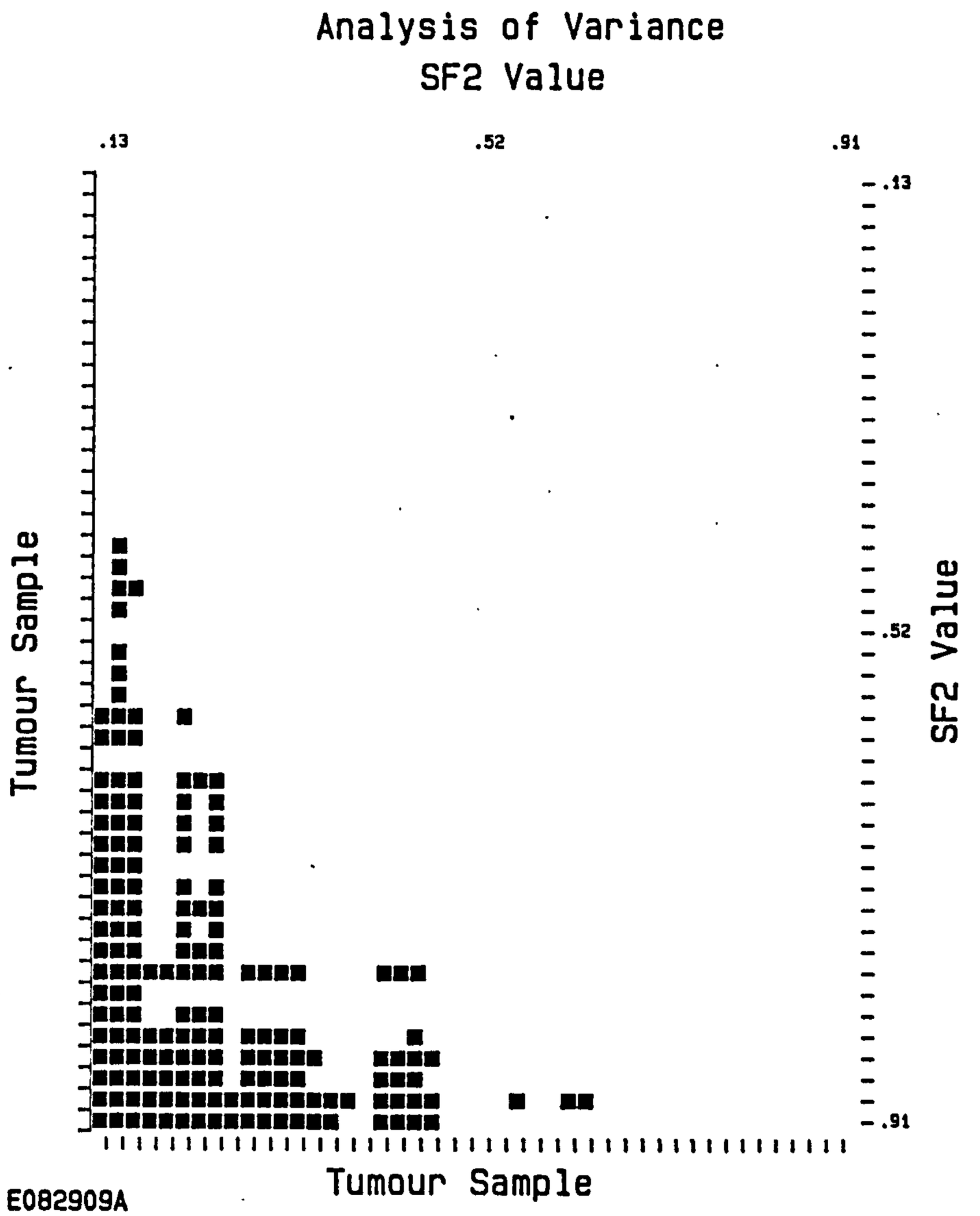

FIG.17

Pairs of tumours which are significantly different ($p<0.05$). One way analysis-of-variance followed by Duncan's Multiple Range Test. (Note SF_2 values are not on a linear scale.)

processed along with other specimens giving higher SF2 values ie. these low SF2 values were reproducible. In addition, biopsies from four tumours processed by two different experimentors gave SF2 values which were not significantly different ($p = 0.1$; V26 and V33 Fig. 16, V17 and V18 data not shown).

The overall CV for the SF2 values obtained from the different tumours was 42%. There were highly significant differences in SF2 values obtained for the different tumours ($p < 0.001$). In order to evaluate intra-tumour heterogeneity, eighteen "split" tumour experiments were carried out. Five tumours showed no growth in either culture but two SF2 values were obtained in thirteen tumours (Table 7). The differences in the CFEs of the two tumour halves processed were highly significant ($p < 0.001$). Nonetheless, the analysis of variance showed that there were no significant differences between the SF2 values obtained from each tumour part. However, the number of tumours evaluated in this way is small and larger numbers will be needed before firm conclusions regarding SF2 can be made.

In order to illustrate the discriminatory power of the assay, two further statistical analyses were performed. A Duncan's Multiple Range Test (Fig. 17), shows which pairs of tumours had significantly different SF2 values. Most values below about 0.4 are significantly different from those above about 0.6. Evaluating the 95% confidence limits for each mean (Fig. 18), shows the proportions with each SF2 value that are significantly different from the mean, given the variability in the errors of the SF2 values.

4.2 DISCUSSION

Potential limitations of any proposed predictive assay need to be explored prior to evaluation of associations with clinical outcome. It is important to determine 1) the adequacy of the assay, 2) sampling errors, resolution and reproducibility, 3) intra-tumour heterogeneity and 4) inter-tumour (patient) variation, which is the aim of

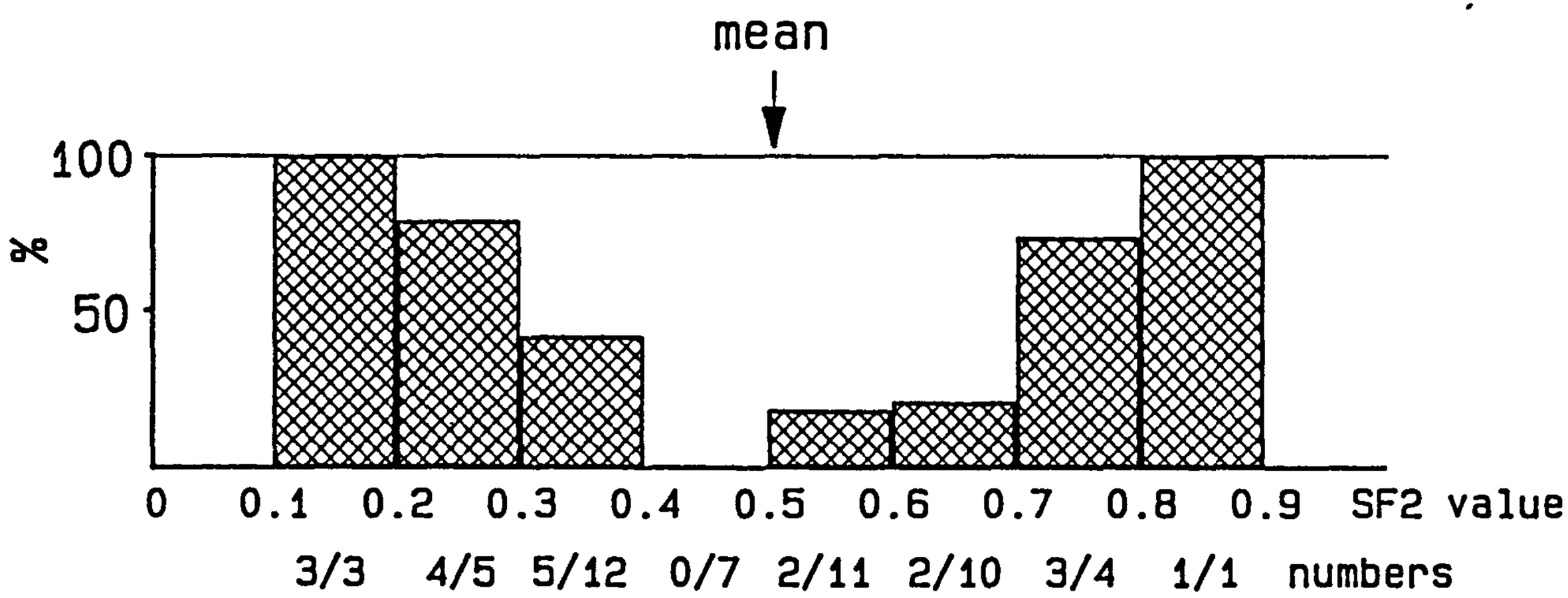

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FIG.18

The relationship between the number of cells plated and number of colonies formed for control and irradiated tubes for 2 tumour specimens. Each point represents the average for 4 or 8 replicate tubes and error bars ± 1 sem. Correlation coefficient = 0.95-1.

the assessment. A large range of SF2 values will increase the predictive accuracy while large assay errors reduce it.

Validation of the assay used in this work (see Chapter 3) was established by demonstrating good linearity between the cells plated and colonies formed. An image analysis system was used to aid colony counting and ensure that only colonies greater than 60 microns in diameter were counted.

The freezing of cells in liquid nitrogen has been demonstrated previously not to alter the radiosensitivity of cells (Rofstad et al, 1987; Selby and Steel, 1981) and these results support this. Storage in nitrogen does however affect the colony forming efficiency and for many specimens the CFE increased with repeat experiments. This may be due to the plating of increased numbers of cells with the non-viable cells exerting a feeder cell effect. No statistical difference between the SF2 values obtained from repeat experiments was seen. The mean intra-experimental coefficient of variation for the assay was 19% and the CV for repeat experiments was 11%. This compares with 9% for the repeat assays on the same tumour cell suspension with the adhesive tumour cell assay used by the group in Houston (Brock et al, 1989). The experimental errors varied amongst tumour specimens and the errors tended to be higher in the experiments where the CFE was low. Repeatability, as measured by the coefficient of variation, was investigated in reported series using clonogenic assays (Table 6).

Few series report their intra-tumour and inter-assay errors Coefficients of variation were calculated from radiation survival curves giving individual dose points and inter experimental CVs were calculated when repeat experiment data was presented. The assay errors in this work compare favourably.

TABLE 6

INTER- AND INTRA-EXPERIMENTAL COEFFICIENT OF VARIATIONS OBTAINED FOR SF2 AND CFE

(Given in text or read from radiation survival curves)

Assay	inter-expt	intra-expt	Reference
on plastic		SD of dose points 10-25% of the mean	Puck and Marcus, 1957
H - S		Mean CV=22% for PE	Hamburger et al, 1978
H - S		5-50% CV for PE on 100 expts.	Shoemaker et al, 1985
H - S		3-50% mean CV=14%	Bizzari and Mackillop, 1985
C - M	19-25% for 6 expts with cervix line for SF2		West (unpub) 1986
on plastic	12% for SF2		
H - S	25% 5 expts cervix line for SF2		
C - M		9-55% for SF2 on 5 expts	Duchesne et al, 1986
on plastic	CV=21-25% 2 expts with ovarian cell line SF2		Rofstad and Sutherland, 1988
*CAM	CV=9% for SF2		Brock et al, 1989
soft agar colony assay	CV=12%		Malaise et al, 1989

* population growth assay.

The wide range of SF2 values is similar to that obtained by others investigating head and neck tumours (Brock et al, 1989) and reflects the considerable inter-tumour heterogeneity to be found within a single tumour class. We have shown that this variation in the values is not due to intra-tumour heterogeneity. From these results there is evidence for one continuous distribution of SF2 values (Fig. 15). In the final multivariate analysis which includes patients follow up data it may be possible to identify the radioresistant and radiosensitive tumours, although it is unlikely that radiosensitivity alone will be used in predicting outcome of treatment. The range of values for a potential preditive test has to be large enough to allow discrimination between the different tumours and be large in relation to errors associated with the assay. The range of values for SF2 lends support to the concept that SF2 may be a useful prognostic test. The distribution of SF2 and the assay errors suggest that it will only be possible to distinguish between radiosensitive and more resistant tumours if the SF2 values for the former are below 0.4 and the latter are above about 0.6 or 0.7. A theoretical study investigating the effect of inter-patient variation on the accuracy of predictive assays showed that those based on intrinsic tumour cell radiosensitivity are more likely to correlate with clinical outcome than assays of tumour doubling time, hypoxic fraction or clonogen content (Tucker and Thames, 1989).

Heterogeneity within tumours is another potential limitation to radiosensitvity testing. The existence of cell subpopulations within solid tumours is well described (Heppner and Miller, 1989; Mackillop and Dotsikas, 1988). Heterogeneity in the structure and organisation of cancer tissues is also important, and one small biopsy may not represent the tumour tissue as a whole (Heppner, 1984). Heterogeneity of human tumours has been demonstrated by their DNA content (Tveit et al, 1985). In a large series of cervical tumours, flow cytometry has been used to demonstrate considerable intra-tumour heterogeneity of DNA content, as part of an investigation into the differential radioresponsiveness of this tumour class (Dyson et al, 1987). Intra-tumour heterogeneity to drug treatment has also been shown, both in vitro and in vivo (Leith and Dexter, 1986;

Tofilon et al, 1986), and had been demonstrated in studies comparing responses of metastases and primary tumour (Schlag and Schremi, 1982). In a study on intra-tumour heterogeneity of chemosensitivity of 8 primary tumour cultures, 50% discordant results of sensitivity were obtained, using a clonogenic assay, from the two halves of the biopsies processed independently (Kern et al, 1984), though the numbers were small and different tumour types were used. Relatively few investigations have been made on intra tumour heterogeneity of radiation sensitivity. Leith and colleagues (1982) showed significant differences in the intrinsic radiosensitivities of subpopulations in a human colon carcinoma cell line (DLD-1) and a lung carcinoma cell line (LX1).

Heterogeneity of intra-tumour radiosensitivity of primary human cultures have been investigated indirectly by Tofilon et al (1989) using the adhesive tumour cell assay and assays of cisplatinum induced SCE. Subpopulations, identified by cisplatinum induced SCE, were found to have differing radiosensitivities. Differences in radiation survival were detected in 50% of cultures that were predicted to have heterogenous radiosensitivity in the SCE assay.

Clearly there is evidence for the existence of sub-populations within tumours and heterogeneity of intra-tumour sensitivity to therapy. However, the results presented here suggest that intra-tumour heterogeneity is not a limitation to the use of the Courtenay-Mills assay for radiosensitivity testing for cervical tumours, ie. a single biopsy is representative of the tumour. Our results investigating the possible variation in the SF2 measurements made from different sites in one tumour using the clonogenic assay did not demonstrate any significant differences. The SF2 value obtained from a single biopsy is likely to be an average value and to reflect the response of the more sensitive cells within a tumour. If the sensitivity of these cells dominates in the radioresponsiveness of the tumour then radiosensitivity testing will be predictive.

TABLE 7

TUMOUR HETEROGENEITY

18 tumour samples processed as 2 separate cell suspensions.

No growth from 2 cell suspensions in 5 tumours.

Specimen No.	Sample A		Sample B	
	CFE ± 1SEM	SF2 ± 1SEM	CFE ± 1SEM	SF2 ± 1SEM
33	0.41 ± .03	0.13 ± .04	0.09 ± .007	0.19 ± .03
38	0.023±.002	0.31 ± .04	0.016 ± .007	0.31 ± .06
60	0.21 ± .05	0.34 ± .06	0.11 ± .01	0.53 ± .08
61	0.06 ± .02	0.35 ± .03	0.22 ± .01	0.72 ± .05
65	0.21 ± .03	0.56 ± .18	0.17 ± .05	0.35 ± .22
71	0.13 ± .08	0.31 ± .15	0.32 ± .07	0.36 ± .01
74	0.02 ± .005	0.37 ± .06	0.06 ± .007	0.42 ± .06
76	1.09 ± .07	0.68 ± .1	1.17 ± .08	0.58 ± .04
81	0.22 ± .03	0.46 ± .09	0.06 ± .006	0.47 ± .06
84	0.11 ± .01	0.46 ± .08	0.23 ± .02	0.40 ± .1
87	0.26 ± .07	0.53 ± .28	0.16 ± .03	0.70 ± .25
88	0.22 ± .04	0.44 ± .16	0.10 ± .02	0.79 ± .18
89	0.23 ± .04	0.68 ± .3	0.16 ± .03	0.70 ± .15

Reproduced in part from West et al, 1989.

5. EVALUATION OF IN VITRO CLONOGENIC GROWTH

5.1 RESULTS

One hundred and seventeen tumours (110 squamous cell carcinomas and 7 adenocarcinomas) were processed with successful growth in 84 (72%). The mean colony forming efficiency was 0.18% with a range from 0.003 to 4.28%, a normal distribution and a standard deviation of 0.49% for 80 squamous cell tumours (Figs 19 and 20). The mean CFE for 4 adenocarcinomas was 0.18 ± 0.12%. These CFEs were based on total viable nucleated cell counts. The mean CFE, corrected for the proportion of tumour cells in the suspensions, was 0.38 ± 0.64% (± 1 SD). The mean proportion of tumour cells in the suspensions was 44.5 ± 14.5% (±1 SD) with a range of 10 - 85%. There were 3 infections out of the 117 specimens (2%). A count of more than 10 colonies per tube was chosen as an arbitrary cut off point for in vitro growth. Repeat experiments were carried out on tumour cell suspensions (n = 34) and biopsies (n = 6) which had been stored in liquid nitrogen. The CFEs of the repeat experiments were higher than with the first experiments and these differences have been shown to be highly significant (Chapter 4). The CFE's of fresh specimens only have been used in the analysis of the results.

Tumour differentiation was assessed in 75% (n = 88) of the specimens by the two histopathologists at the Christie Hospital. A further 19 tumour biopsies had been assigned tumour grade by the pathologists at the referring hospital. There were 20 well differentiated tumours, 48 moderately differentiated and 39 poorly differentiated tumours. The biopsies were from patients with all tumour stages, with the majority being either Stage I or II (Table 2). The ages of the patients in this series ranged from 26 to 90 with a median age of 50 years.

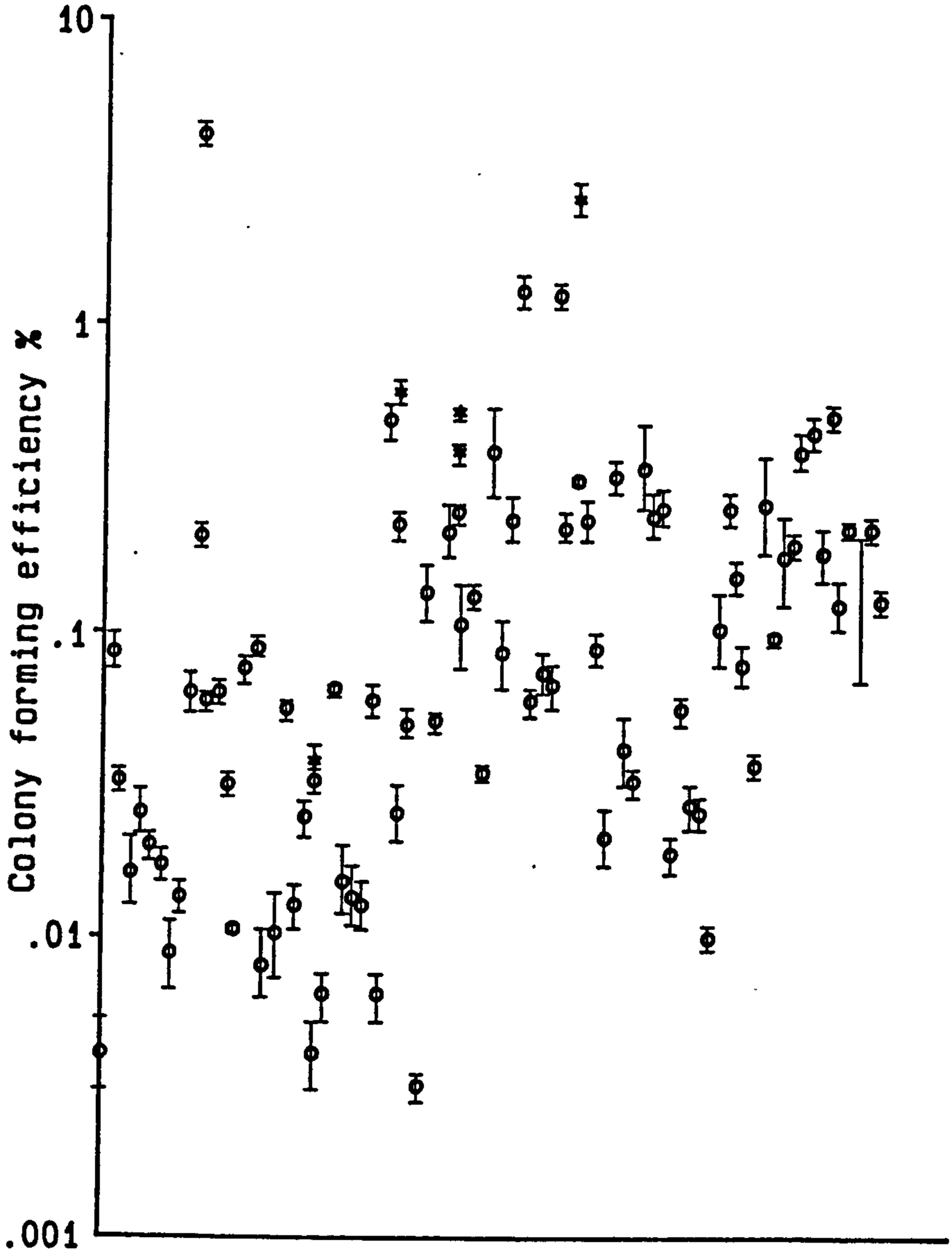

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FIG.19

Colony forming efficiencies of primary cervical carcinoma cultures [repeat experiments have been plotted with*]

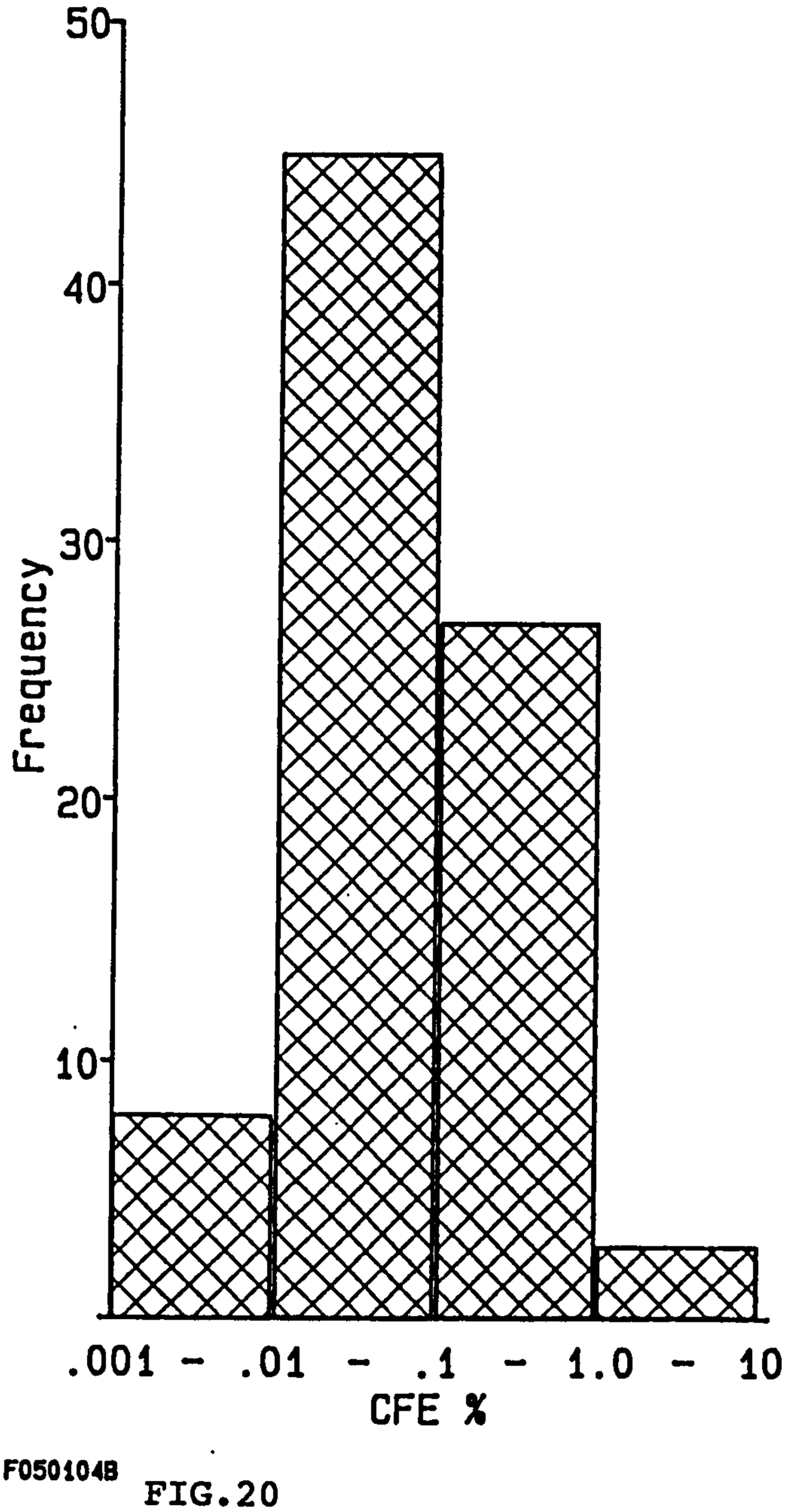

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FIG.20

Histogram of colony forming efficiency.

TABLE 8 TUMOUR STAGE

STAGE	I	II	III	IV
NO	37	39	12	5

A chi-square test was performed and showed no relationship between tumour differentiation and CFE. The CFE was divided into two groups, less than or greater than the median corrected CFE of 0.14% (Table 3). These analyses were carried out on CFEs

TABLE 9 TUMOUR GRADE AND CFE

		CFE	
		<0.14%	>0.14%
DIFF	WELL	13	7
	MOD	24	24
	POOR	25	14
		62	45

chi-square = 2.25, 2 d.f, p = 0.32

corrected for the proportion of tumour cells in the cell suspensions, although when the CFEs, based on total viable cell counts, were analysed there was no relationship demonstrated (chi-square = 0.75, 2 d.f., p = 0.75). In view of the subjective nature of the grading of tumours, the specimens which were graded by one pathologist (n = 52) were analysed separately and again no relationship between CFE and tumour grade could be demonstrated (Table 4).

TABLE 10 TUMOUR GRADE AND UNCORRECTED CFE

median CFE (uncorrected) = 0.06%, one histopathologist

		CFE	
		<0.06%	>0.06%
DIFF	WELL	5	4
	MOD	13	11
	POOR	8	11
		26	26

chi-square = 0.75, 2 d.f, p = 0.75

The tumours which grew in culture were investigated to see if this group was different from the tumours which did not grow in culture using a 2x2 contingency table and a chi-square test (Table 5). Interestingly, when a chi-square test was performed with fewer

TABLE 11 TUMOUR GRADE AND IN VITRO GROWTH

	No growth	good growth (CFE >0.14%)
WELL	5	7
MOD	11	24
POOR	9	14
	25	45

chi-square = 0.58, 2 d.f, p = 0.7

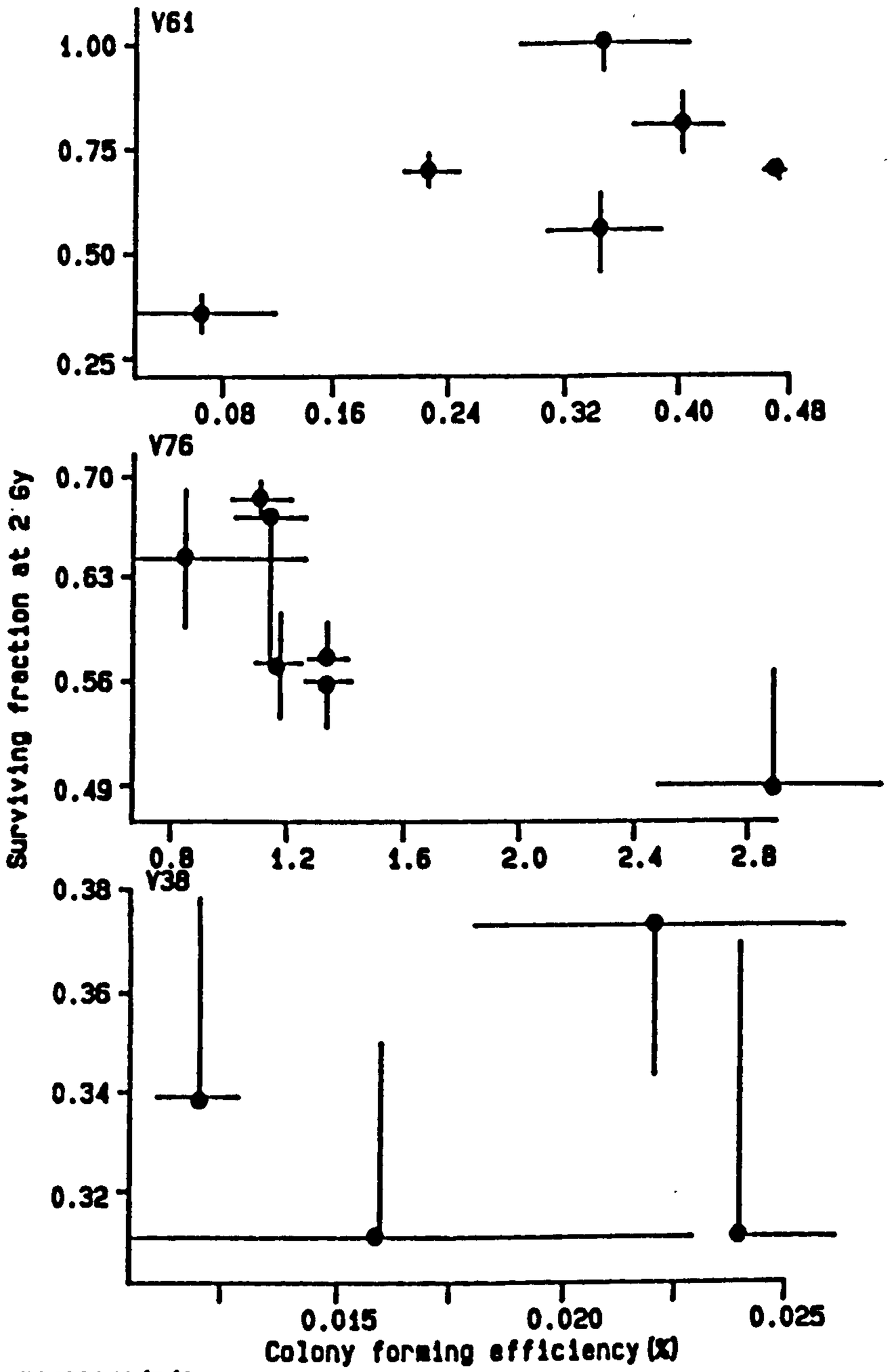

FIG.21 Surviving fraction at 2 Gy as a function of colony forming efficiency for 3 cervical carcinoma specimens. Points represent the mean and standard error of 4 - 8 replicates.

tumours (Table 6) the chi-square test showed that the tumour grade did appear to be significantly related to CFE with p = 0.02. The CFE was neither related to tumour stage (r =0.02) nor patient age (r = 0.12).

The CFE was not correlated with SF2, r = -0.06 (n = 77). In figure 21 results for 3 tumours are shown for which more than 4 repeat SF2 determinations were obtained. Correlation coefficients were 0.62, -0.81 and 0.03 for samples V61, V76 and V32 respectively. Regression analyses, weighting by the inverse variance of CFE, gave r values for V61 and V76 of 0.4 (p = 0.44) and -0.64 (p = 0.12) respectively.

TABLE 12 EARLY ANALYSIS OF TUMOUR GRADE AND CFE

	CFE	
	<0.145%	>0.145%
WELL	13	1
MOD	17	17
POOR	17	9
	47	27

chi-square = 7.92, 2 d.f, p = 0.02, taking the median CFE of 0.145% as the cut off between poor and good growth.

5.2 DISCUSSION

Relatively few series are reported to have solely concentrated on the primary clonogenic culture of human cervical carcinoma. Williams et al (1983) grew 1 out of 29 specimens, Parker and co-workers (1984) reported an 88% success rate with 76 tumours and Von Hoff (1990) reported 20% success with primary cervix tumours. No CFEs were given

in the study by Parker et al (1984) nor Williams et al (1984). The 73% success rate reported here compares favourably with these. In addition by using the Courtenay-Mills soft agar assay high CFEs have been obtained which is in agreement with the finding that higher CFEs can be obtained with the Courtenay-Mills assay when compared with the Hamburger-Salmon assay (Endresen et al, 1985; Ottestad et al, 1988).

Twenty eight per cent of the biopsy specimens received did not grow in vitro (< 10 colonies per tube). This figure agrees well with values published in the literature for other large series which have looked at different tumour classes (Ottestad et al, 1988; Tveit et al, 1989). Those tumours that do not grow well in culture may be less aggressive in their clinical behaviour (Hug, 1985; Moezzi, 1986; Flentje, 1987). However there are several reasons for not assuming that the growth of cancer cells in vitro should reflect the biological aggressiveness of a tumour and the clinical course of the disease (Ottestad et al, 1989). Good in vitro growth may express capacity for growth under specific culture conditions and does not necessarily reflect the intrinsic growth capacity of cells. There is inevitably selection of populations in colony forming assays. The results obtained to date by independent research groups have been contradictory (Table 3 in section 1.3.4) possibly because of varying tumour histologies and relatively small numbers of patients (Nomura et al, 1989).

This study failed to demonstrate any correlation between in vitro colony growth and tumour differentiation. Although it is noteworthy that analysis of the first 74 specimens using the chi-square showed a positive correlation ($p = <0.05$). This illustrates the importance of carrying out studies on large numbers of samples. Adding these results to those listed in Table 3 further contributes to the contradictory nature of the results obtained. There is no obvious trend. Five studies have shown a lack of correlation whilst two have demonstrated a correlation. Small sample size is a criticism of some studies yet of the two largest studies, one showed a correlation and the other did not. Obviously there may be differences between tumour types. However the largest number

of studies have been carried out on breast cancer and two showed the existence of a correlation, whilst three did not.

It should be noted that tumour grading is not precise. A major criticism is the issue of inter-observer reproducibility, reflected in inter-series comparisons (Contesso et al, 1989). In addition there are difficulties in defining grade using classifications of Broder or Reagan and Wentz (Goellner, 1976) and these may be added to sampling errors from the biopsies (Gundersen et al, 1974). Inter-observer reproducibility, use of different grading systems and small sample size may obscure the relationship between differentiation and in vitro growth in many series. Although more than one pathologist graded the specimens in the series reported here, when the 52 tumours graded by one pathologist were analysed, no relationship between tumour grade and CFE was demonstrated. It may be that in vitro clonogenicity, when carefully carried out under controlled conditions, it is a more objective measurement than grade.

This work shows no correlation between CFE and radiosensitivity as measured by SF2. Brenner et al (1987) reported a strong correlation between radiation sensitivity and plating efficiency for 2 cell lines. Bristow et al (1990) showed that for a panel of murine tumour cell lines, higher values of SF2 may be associated with those cell lines that have higher plating efficiencies in vitro. However the results from this study are in agreement with the findings of Fertil and Malaise (1981) who found no relationship between in vitro radiosensitivity and CFE for human tumour cell lines (n = 365). Technical artefacts such as minimum colony size counted (Nias and Fox, 1968) and culture conditions (West and Sutherland, 1986) may alter radiosensitivity and may be responsible for these differences.

Although these results have not shown a correlation between clonogenic ability and grade, grade itself is not generally accepted as predictive of outcome in carcinoma of the cervix (Ng and Atkin, 1973; Goellner, 1976; Van Nagell, 1977; Prempree, 1983). The

lack of correlation seen between CFE and other prognostic factors (eg. stage) may reflect the independence of CFE as a potential predictive factor. This is suggested also by the lack of correlation with other biological endpoints evaluated. Therefore these results do not rule out the possibility that in time CFE may prove to be a prognostic indicator for patient outcome.

6. ASSOCIATED STUDIES

6.1 TUMOUR VASCULARITY

6.1.1. Vascularity results

Of the 99 biopsies received for paraffin embedding, vascularity was assessed in 87 (88%). The remaining tumour biopsies were either too small, of too poor quality to be able to score (n = 10) or contained insufficient tumour tissue (n=2). The mean inter capillary distance (ICD) was 240 microns with a standard deviation of 37 microns (median = 239 microns), a range of 131-333 microns [Fig. 22] and a mean intra-tumour CV of 38% (n = 72). A normal distribution was obtained and the overall coefficient of variation between the tumours was 15%. The mean ICD measured in the stromal bands of 11 tumours was 139 ± 26 microns (± 1SD), with an intra-tumour CV of 18%.

There was no correlation between ICD and tumour grade, r = 0.05 (tumour grade: well diff. = 18, moderately diff. = 39, poorly diff. = 28); stage, r = 0.12 (Stage I, n = 36; Stage II, n = 30; Stage III, n = 13; Stage IV, n = 7) or radiosensitivity, as measured by SF2, r = 0.05 (n = 57). When the mean ICD for Stage I tumours (231 ± 36μm) was compared with the mean ICD for pooled Stages II, III and IV tumours (247 ± 38μm) using a t-test, the difference was approaching significance, t = 1.88, p = 0.06, DF = 71.

The morphometric analyses gave a mean proportion of vessels for the tumours of 2.68% ± 1.64% (± 1 SD) with a range of 0.6 - 11% and a CV of 61% between individual tumours [Fig. 23]. The mean proportion of parenchymal cells was 61.3 ± 17.9% (±1 SD) and the mean stromal proportion was 27.5 ± 14%. There was no correlation between the proportion of vessels and tumour grade, r = -0.1, the stage of disease r = 0.04 or with SF2, r = -0.03. Morphometry was performed on 10 tumours where two biopsies were received. A two sample t-test was carried out on the mean percentage of

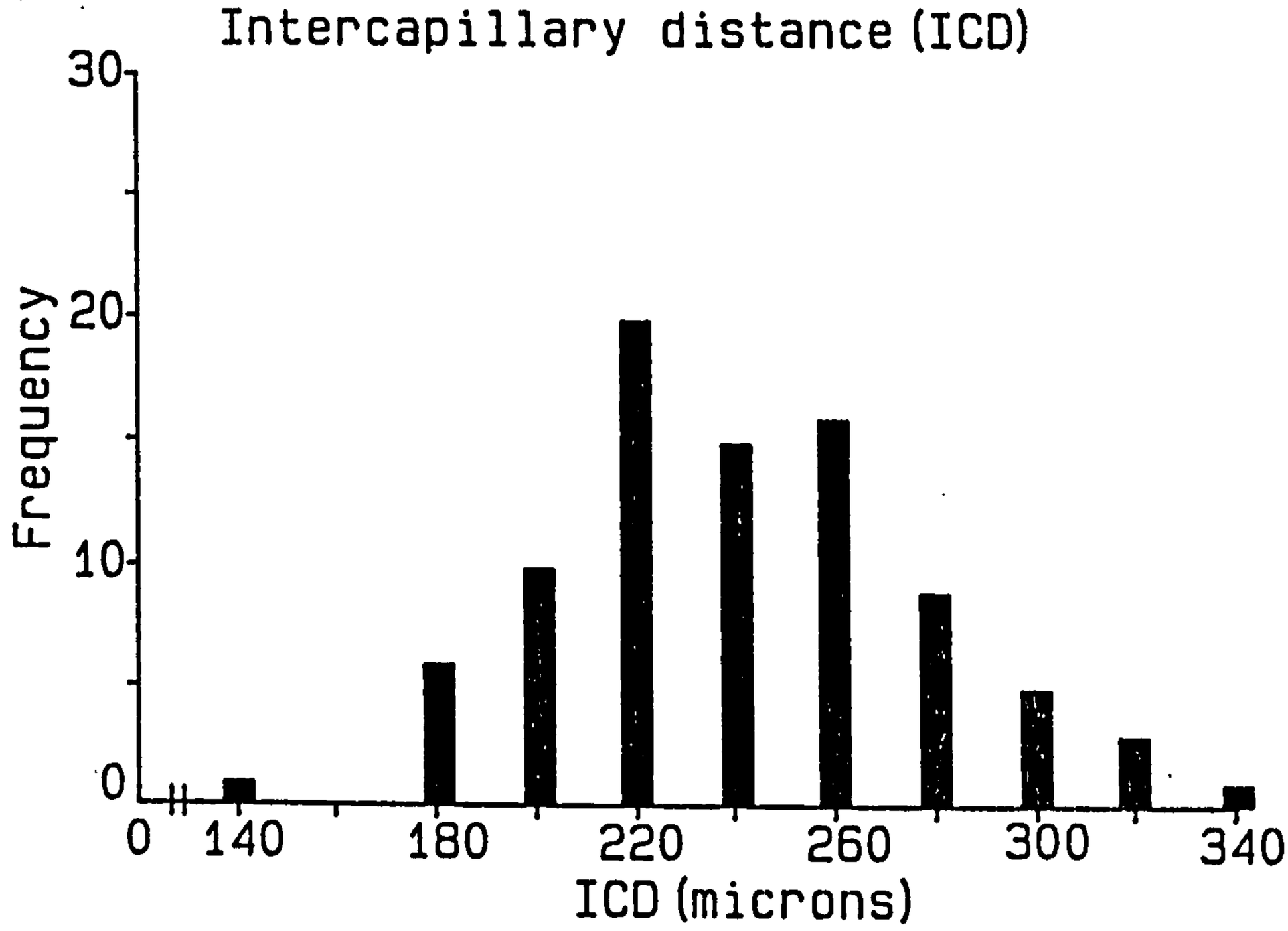

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FIG.22

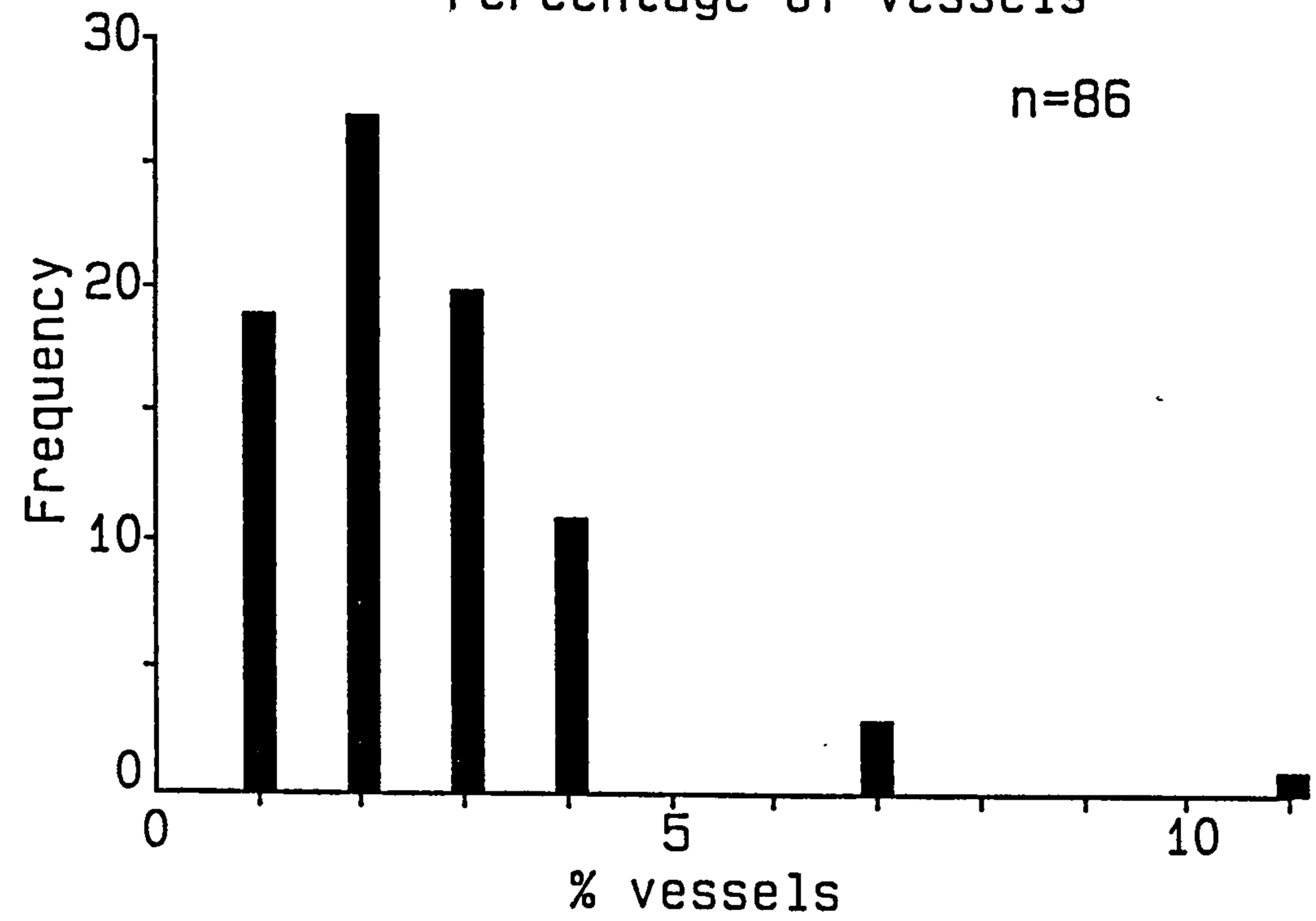

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FIG.23

vessels obtained on each biopsy (T = 0.48, p = 0.64, DF = 12) which suggested that the values were not significantly different from each biopsy. The mean ICD for 7 adenocarcinomas was 204 ± 42 microns (± 1 SD) and a range of 130.5 - 257 microns. The mean percentage of vessels in these adenocarcinomas was 3.4 ± 1.87% (± 1 SD). This mean value of ICD of adenocarcinomas was not shown to be significantly different from the value for squamous cell tumours (t = -2.33, p = 0.06, DF = 6).

Clinical data was obtained from 35 patients with at least 2 years follow up after radiotherapy (Table 14). The mean ICD of the 17 patients who were disease free at 2 years was 235 ± 32 microns (± 1 SD), with a median of 232 microns and a range of 185 - 299 microns. The mean ICD of 18 patients who had died from recurrent disease or who had recurrent disease at two years (pelvic recurrence or pelvic disease with metastases) was 244 ± 37 microns, with a median of 240 microns and a range of 193 - 333 microns. A two sample t-test was performed which failed to demonstrate a significant difference between the means of the two groups (t = 0.74, p = 0.47, DF = 32). Similarly no significant difference was seen between the mean percentage of vessels in the group which was disease free (mean = 2.42 ± 1.5%, median 2%) and the group of patients who had died or had recurrent disease (mean = 2.16 ± 1.1%, median 1.8%) with a t value of -0.6, p = 0.55 and DF = 29.

6.1.2 Vascularity discussion

TABLE 13 STUDIES ON VASCULARITY IN HUMAN TUMOURS

TUMOUR TYPE	STAGE	NO.	METHOD	CONCLUSION	REFERENCE
Cervix	I-IV	105	Colpophotography	>400µm ICD 51% recur <400µm ICD 16.7% local recurrence	Kolstad 1968
Cervix	IB & IIA	45	Morphometry	Survival > 5 years 28.9% vessels in stroma, <5 yrs 10.5% vessels in stroma	Siracka et al 1982
Cervix	IIB & III	36	Alkaline phos. technique	Mean ICD = 322µm recur at 2 yrs, mean ICD = 291µm disease free	Awwad et al 1986
Nasopharynx	III & IV	25	Morphometry	Capillary density correl. with survival time	Delides et al 1988
Oral SCC	T_2 & T_3	26	Anti-factor VIII stain	Median vessel tumour cell dist = 105µm for local control and 75µm in local recurrence	Lauk et al 1989
Cervix	IB,IIA & III	95	Morphometry	High vessel density = survival >5 yrs, low vessel density = survival <5 yrs	Siracky et al 1988
Cervix	IB,II & III	95	Morphometry	Vessel density = 25.6% >5 yr survival, vessel density = 10.6% <5 yr survival	Revesz et al 1989

The survival time in months was calculated for the patients with at least two years follow up from their radiotherapy (excluding those with metastases with no evidence of local recurrence in the pelvis, n = 29). The mean survival time was 20 months, median 23 months and a range of 5 - 30 months. There was no correlation between the survival time and ICD (r = -0.008) nor with the percentage of vessels (r = 0.08).

The measurements obtained in this study are similar to those reported by others. Awwad and colleagues (1986) obtained a mean ICD of 304µm with an intra-tumour CV ranging from 12 - 32% and an inter-tumour CV of 10%. Revesz and Siracka (1984) reported proportions of parenchymal and stromal components of around 70 and 30% respectively and a wide variation (7-22%) in the mean vascular density within a tumour. Although Kolstad (1968) suggested that adenocarcinomas should be investigated separately from squamous tumours because of different vasculature patterns in these two different

histologies, no difference was shown between adenocarcinomas and squamous tumours in this study.

It is important to assess the intra tumour variation of vascularity in addition to asessing the variation between tumours. Surgically removed specimens (Stage IB, n = 4) were looked at by Siracka et al (1988). They found that the variance in vascularity within tumours was less than that between tumours, although considerable variation in vasculature within different regions of the same specimen was demonstrated. The variation in vascular density was assessed morphometrically by Revesz et al (1989) in 14 sugically removed specimens of stage II tumours. A wide variation in vasculature of each tumour was demonstrated but the inter tumour variation was greater than the intra-tumour variation, although the tumours from one of the centres were all moderately to highly differentiated tumours and no poorly differentiated tumours were studied. The results obtained in this work are in agreement with these studies; where 10 tumours with two biopsies showed a wide variation in percentage of vessels between biopsies 0.6 - 5.8%, but these differences were not significantly different within each specimen.

No correlation was demonstrated between ICD or percentage of vessels and tumour stage ($r = 0.12$ and $r = 0.04$). This is in agreement with the findings of Awwad et al (1986) who did not show a relationship between ICD and either tumour grade or stage of disease. However, Kolstad (1968) showed an association between a large ICD and an advanced stage of disease and by pooling Stages II, III and IV found a significant difference compared to the Stage I cases. When the same exercise was carried out with this series the difference between the mean ICD of Stage I tumours and the mean ICD of pooled Stage II, III and IV cases almost reached the 0.05 level of significance ($p = 0.06$). When more tumours in Stage III and IV are analysed, there may be a correlation of ICD with stage, suggesting that the more bulky tumurs have larger ICDs.

A number of studies have shown a correlation between the degree of vascularisation of a tumour and outcome following radiotherapy for the patients from whom the tumour biopsies were obtained (see Table 13). Awwad and colleagues (1986) showed a greater ICD in 15 patients who recurred within two years compared to 21 patients who remained well at two years. Siracka and colleagues (1982; 1984; 1988), in several retrospective studies of cervical carcinoma patients, showed a relationship between the vascular density and survival and Delides et al (1988) demonstrated this in a retrospective study of 25 cases of nasopharyngeal tumour. In contrast, Lauk et al (1989), found a higher local control rate of oral squamous cell carcinoma among tumours which were less well vascularised. Whereas in the work reported here no correlation between local recurrence or survival time was found with the vascularity of a tumour (measured as ICD or morphometry). Although the follow up of the patients in this series is only two years, 80% of the recurrences that will occur, should do so within the first two years (Perez et al, 1988). It is of note that an unpublished series by Delides (1988) on bladder cancer showed no correlation between vascular density and effectiveness of radiotherapy (Revesz et al, 1989).

There are several possible explanations for the contradictory nature of these results. Firstly different techniques have been used by the various groups investigating tumour vascularity. Lauk and colleagues used an anti-factor VIII stain and Awwad used an alkaline phosphatase technique to stain endothelial cells. Lauk and colleagues (1989) also raise the issue that the technique of Masson's trichrome staining with morphometric analysis may underestimate the vasculature of poorly differentiated tumours as open and plasma perfused vessels may be hard to recognise without specific endothelial staining. The morphometry technique of Siracka et al (1982; 1984; 1988) express vascular density as a percentage of the stromal and not of the tumour component except in their earliest report where the percentage of vessels was reported (means of 2.3% and 6.3% given for the two groups of tumours). This explains the higher values for the vascular density obtained compared with the percentage of vessels found in this series using

morphometry. Delides et al (1988) also used a different method in that the number of capillaries per high power field was used as a measure of vascular density. Secondly the biopsies assessed in this study are small punch biopsies whereas the studies of Siracka et al (1988) and Revesz et al (1989) used surgically removed specimens to look at intra tumour variation in vascularity. In view of known tumour inhomogeneities sampling errors may confuse the situation. Thirdly, the majority of the studies are retrospective and have the disadvantage of patient selection (except the studies of Awwad and Kolstad). Also the patients in most of the studies are in selected stages of their disease (see Table 13); different radiotherapy schedules have been employed and investigations have been carried out on squamous cell carcinoma from different sites. These may all contribute to the varying results obtained.

It is possible that a correlation exists between ICD and clinical outcome from radiotherapy, but this study has included all stages and grades of tumours which would be better analysed by a multivariate analysis. The majority of the specimens are from patients with Stage I or II disease (Stage I = 36; Stage II = 30) and the importance of stage and size of tumour along with the presence or absence of lymph node metastases will have a considerable bearing on treatment outcome. A multivariate analysis in this prospective study may yet show the importance of tumour vasculature in predicting outcome of treatment.

TABLE 14 RECURRENCE VERSUS DISEASE FREE VASCULARITY RESULTS

RECURRENCES		DISEASE FREE	
ICD	% VESSELS	ICD	% VESSELS
193	1.6	243	2.4
257	1.6	223	1.4
213	1.1	232	0.9
216	1.7	185	3.5
234	1.2	299	2
255	1	298	3.3
197	1	202	2.3
225	2.4	187	3.7
219	3.8	223	2.5
302	1.3	248	2
256	1.9	238	0.7
260	3.9	208	1.8
274	1.9	223	3.3
277	1.0	255	7.0
208	3.4	226	1.6
247	3.6	256	0.9
333	3.2	257	1.9
228	3.4		
n = 18		n = 17	
mean = 244 ± 37 range = 193 - 333		mean = 235 ± 32 range = 185 - 299	
median = 240µm		median = 232µm	
	% vessels mean = 2.16±1.07±.25 *median = 1.8% *(±1 SD ±1 SEM)		% vessels mean = 2.42±1.49±.36 *median = 2% *(±1 SD ±1 SEM)

minimum follow up = 2 yrs

6.2 INFLAMMATORY CELL INFILTRATES

The mean percentage of tumour cells in cell suspensions prepared from the tumour specimens, determined by May Grunwald-Giemsa staining and confirmed by monoclonal antibody staining with CK1 and/or CAM5.2 (Fig. 24), was 45 ± 15% (±1 sd) with a median of 43% and a range of 10-85% (n=116). The mean percentage of macrophages was 15% ± 7% (±1 sd) with a median of 14% and a range of 4-58%. The staining was confirmed by the monoclonal antibody EBM11 (Fig. 25). In 106 specimens the percentage of tumour cells scored using both methods was in good agreement (less than 10% difference between the two methods) except in 11 specimens where the percentage of antibody staining tumour cells was less than that of the May Grunwald-Giemsa staining method. However in these specimens there was good agreement between the EBM11 staining and the May Grunwald-Giemsa staining. Three specimens showed differences in the two methods when there was a higher percentage of EBM11 staining cells than the percentage of macrophages on conventional staining. This may have been due to non-specific staining, as the percentage of tumour cells staining in these specimens was similar with the two methods. The percentage of lymphocytes ranged from 0.8 - 48% with a mean of 16 ± 9% (±1 sd) and the percentage of granulocytes ranged from 1 - 81% with a mean value of 23 ± 16% (±1 sd). In 5% of the specimens (n=6) abundant eosinophils were seen on the cytospin preparations.

The possible association between the proportions of host cells in the tumour specimens and clinical data were studied. The percentage of macrophages was not correlated with stage ($r = 0.05$), tumour differentiation ($r = -0.17$) or patient age ($r = 0.09$). There was no relationship between the percentage of macrophages and CFE ($r = -0.001$) or with radiosensitivity, as measured by SF2, $r = 0.15$. Similarly, no correlation was seen between the percentage of lymphocytes or granulocytes and tumour stage ($r = 0.03$, $r = 0.14$), tumour differentiation ($r = 0.07$, $r = 0.16$) and patient age ($r = -0.07$, $r = -0.13$). The proportion of lymphocytes was not related to CFE ($r = 0.12$) or SF2 ($r = -0.05$). No

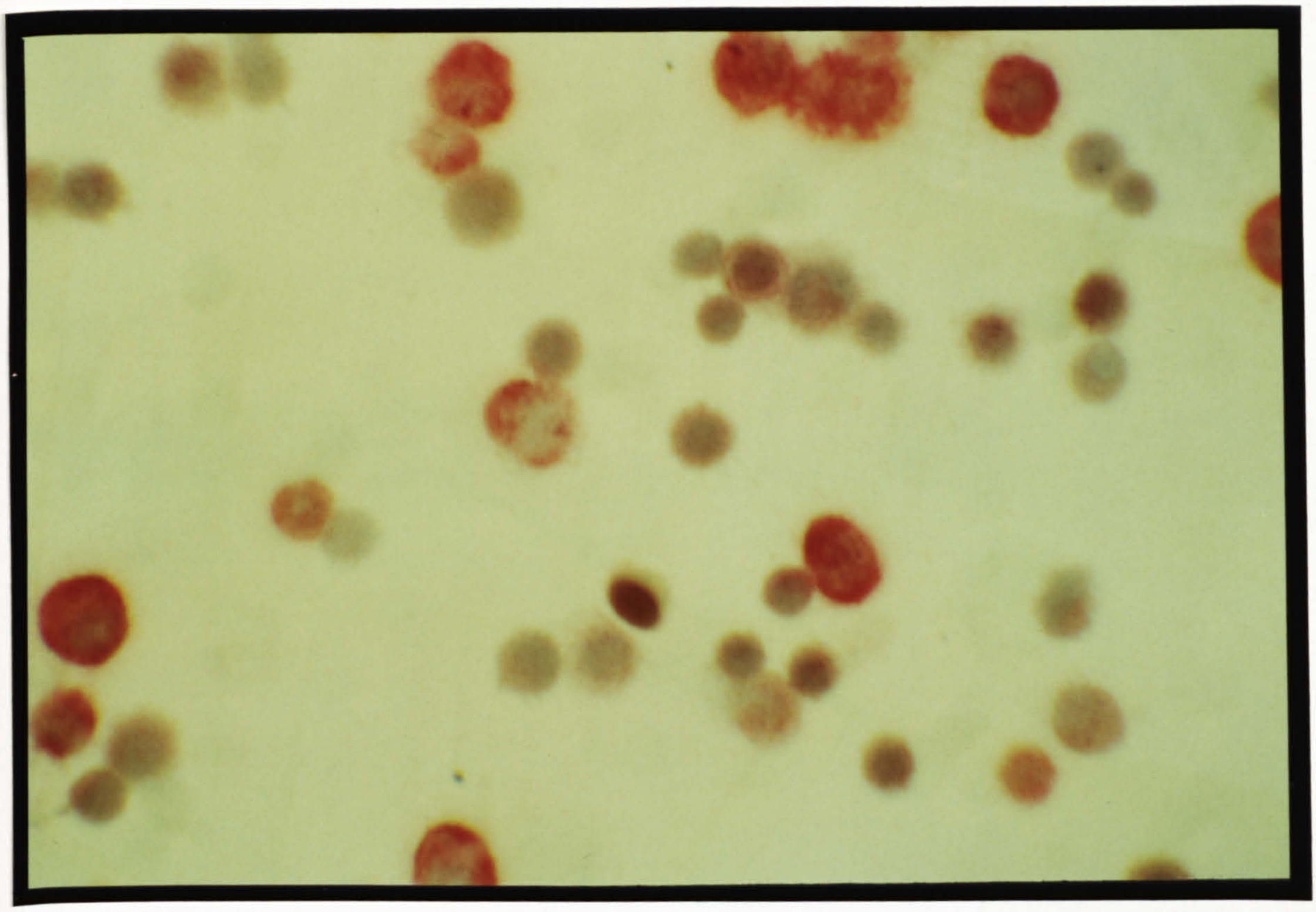

FIG.24 CK1 STAINED TUMOUR CELLS ON CYTOSPIN

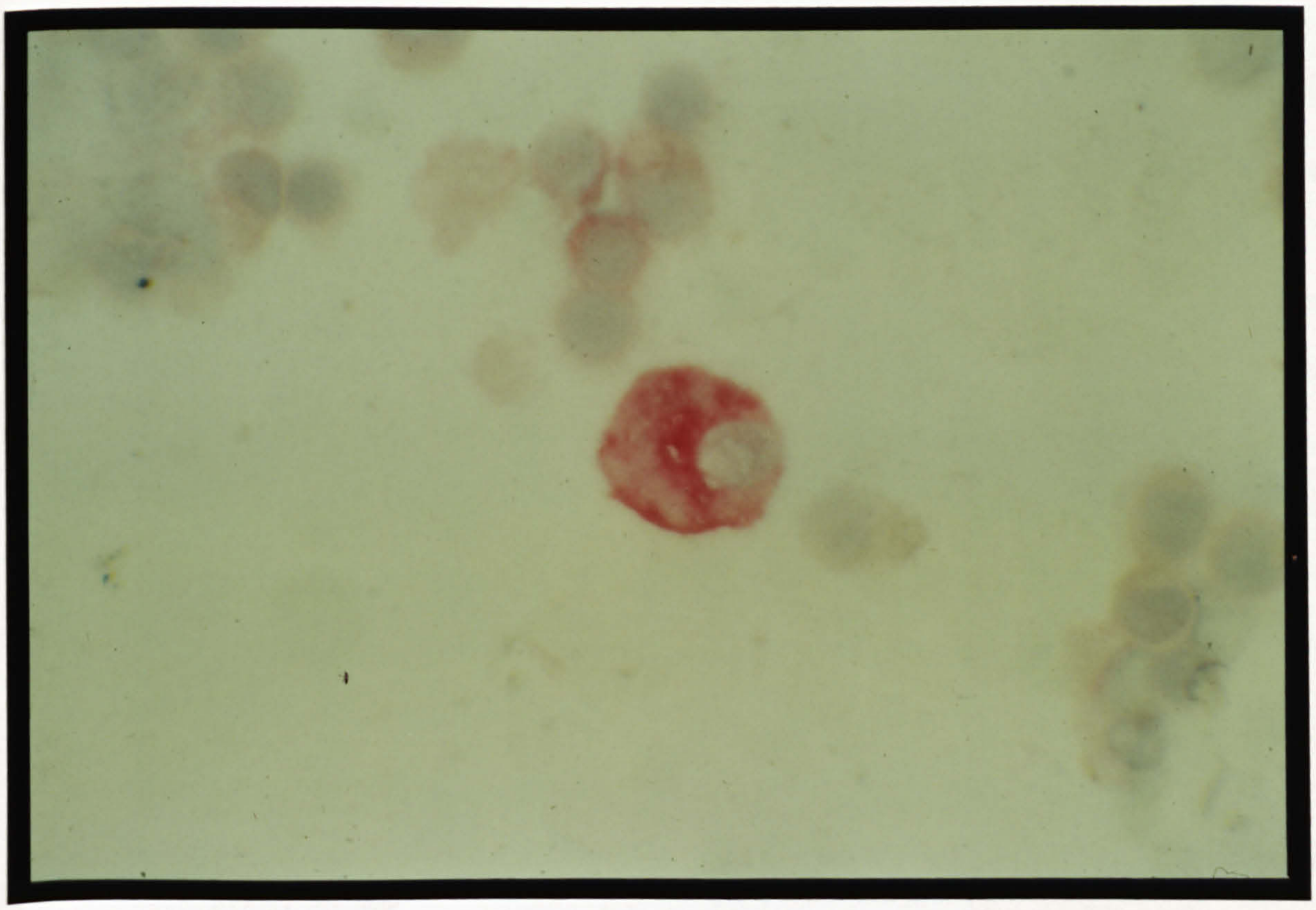

FIG.25 EBM11 STAINED MACROPHAGE ON CYTOSPIN

relationship between the granulocyte percentage and CFE (r = 0.08) or SF2 (r = 0.11) was demonstrated.

There was no significant variation in the percentage of tumour cells from two biopsies from the same specimens, processed independently, from 25 tumours (t = -0.5, p = 0.6, DF = 45). The mean percentage of tumour cells in the suspensions from 12 tumours which had been stored in liquid nitrogen prior to processing was 51 ± 8% (±1 sd). This was not significantly different from the mean tumour cell percentage of the specimens processed immediately (t = -1.25, p = 0.2, DF = 30).

6.2.1 Discussion

Infiltration of various stromal or host cells within and/or surrounding tumours has been reported to be associated with a good prognosis (Underwood, 1974; Svennevig, 1984; Steele, 1984; Luebbers, 1985). Therefore an attempt was made to quantify the host cell proportions in cell suspensions prepared from tumours and to look for correlations between the levels of inflammatory cells and other clinical and biological data. The proportions of host cells in the suspensions obtained from this series of cervical carcinomata were similar to those obtained in other series using solid tumour (lung, breast, colon and stomach tumours; Wood and Gollahan, 1977; Staren et al, 1979; Haas et al, 1990). While Svennenig et al (1979) reported lower proportions of lymphocytes and macrophages, possibly due to the mechanical method of dissaggregation used leading to selective losses. Although it has been shown that the percentage of tumour cells and macrophages obtained may be altered by enzyme disaggregation compared to values obtained using cryostat sections (Takeo et al, 1986; Costa et al, 1987). It has been demonstrated that considerable variation exists in the distribution of the inflammatory cells within tumours. Macrophages were reported in association with malignant tumours in a definable pattern which will not be seen with tumour cell suspensions (Whitworth et al, 1990) and also variation in the distribution of tumour infiltrating lymphocytes has

been described (Hiraysuka et al, 1984). Macrophages were shown to be aggregated around areas of necrosis (Svennenig et al, 1979). There are therefore likely to be sampling errors with the biopsies received which were taken from tumour areas remote from grossly necrotic areas. However, there were no significant differences shown in the percentage of host cells in the cell suspensions obtained with the 25 tumours where more than one biopsy was obtained and processed independently ($p = 0.6$), although the sampling was biased in that necrotic regions of the tumours were excluded. Eosinophils were seen to feature prominently in 6 specimens (5%) which is similar to the proportion (3%) reported by Lowe (1988) in a large series of cervical carcinomata (1027 cases). In Lowe's series these patients had a better prognosis, though Bostrom (1981) found no correlation between tumour eosinophilia and prognosis.

The in vitro growth of cervical carcinoma in this series was not influenced by the percentage of macrophages ($r = -0.001$) or by the proportions of lymphocytes ($r = 0.12$) in the tumour suspensions which is is agreement with a published series looking at ascites and pleural effusions (Hofmann et al, 1984). It has been reported that macrophages can promote the growth of tumour cells in vitro (Buick et al, 1980; Gabizon et al, 1980) with evidence that this growth promotion is mediated by soluble factors (Gabizon et al, 1980). These are possibly the same as those factors secreted by peripheral blood monocytes which enhance the growth of human tumours in soft agar and appear to be non-dialiazable, heat stable, acid labile proteins of 10,000 - 30,000 daltons (Hamburger et al, 1986; 1987). However, in this study no correlation was demonstrated between the percentage of macrophages or lymphocytes and CFE.

The macrophage content of the tumours was not demonstrated to be associated with radiosensitivity ($r = 0.15$) in this series. In contrast, the work of Milas and associates (1987) demonstrated that murine tumours with higher macrophage content were more radioresistant than those containing fewer macrophages with the suggestion that macrophages promoted the growth of surviving tumour cells. However this was a small

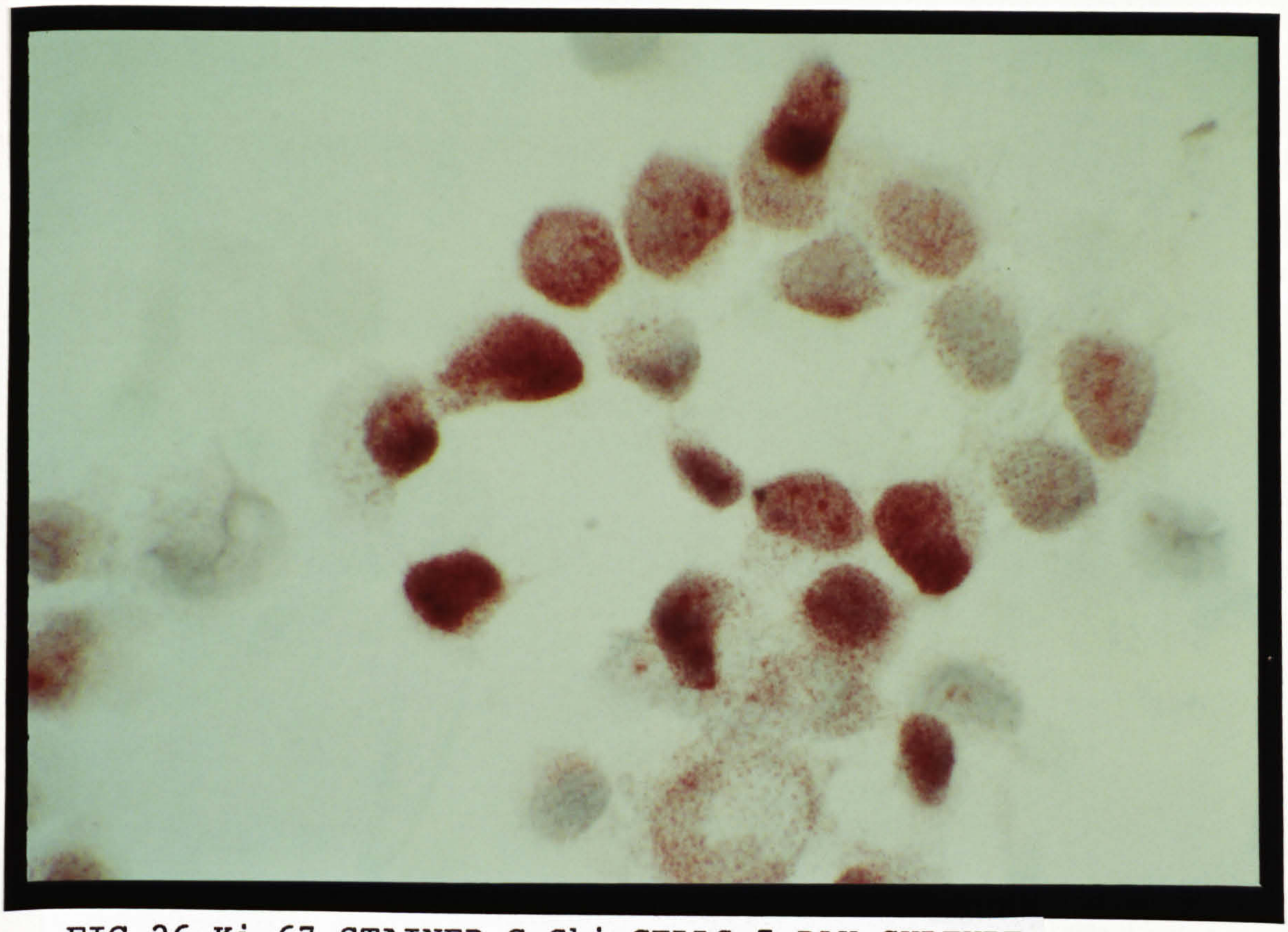

FIG.26 Ki 67 STAINED CaSki CELLS 7 DAY CULTURE

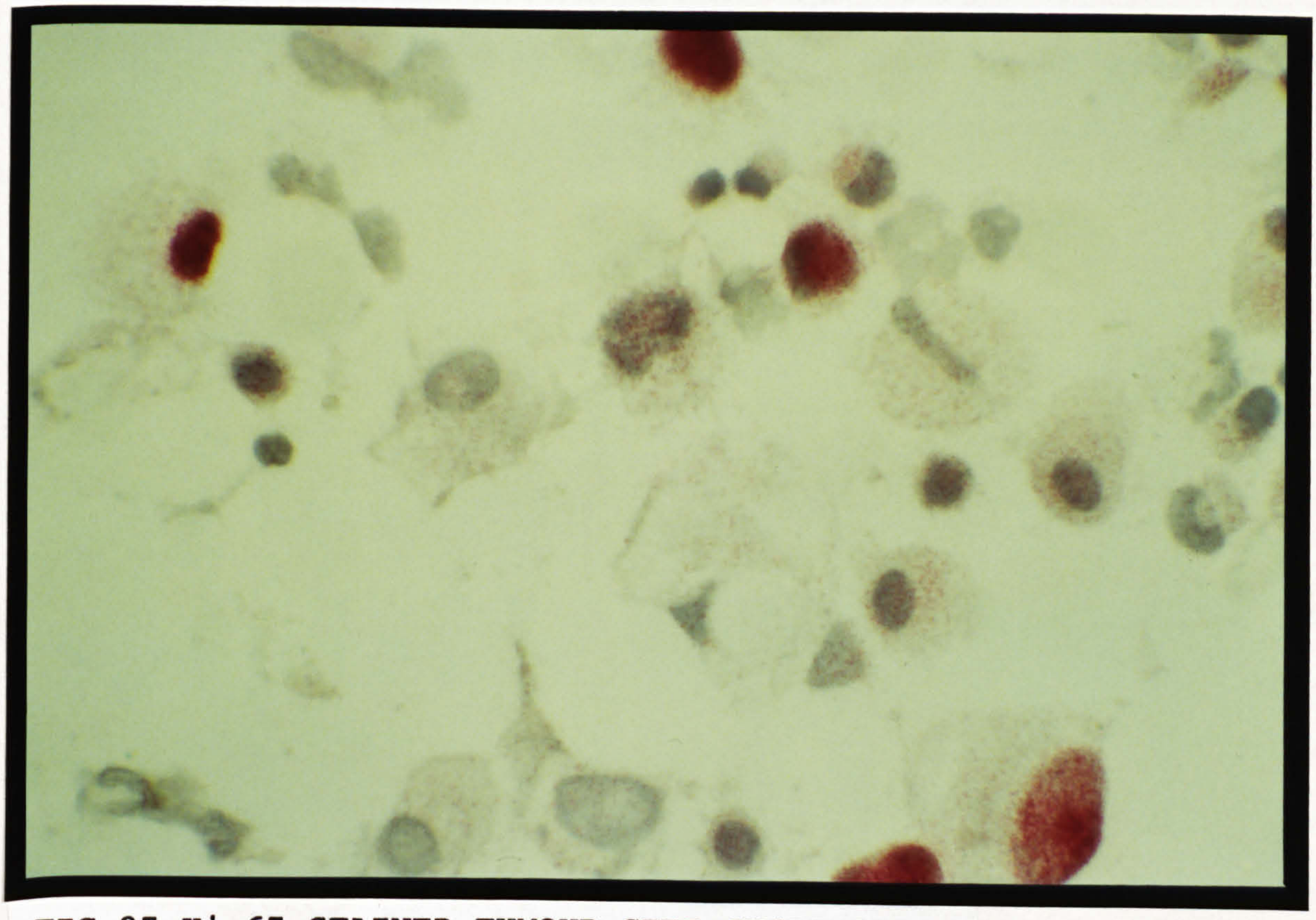

FIG.27 Ki 67 STAINED TUMOUR CELL SUSPENSION

series of murine tumours which may well differ from the behaviour of human tumours.

This work reports the findings, thus far, on a large prospective series of one tumour type and the results are similar to the findings of Steele et al (1984) on a series of 40 breast tumours in that the macrophage content was not significantly associated with any one prognostic factor. However, in the high risk group of patients the number of macrophages was shown to be higher (Steele et al, 1984). Kelly et al (1988) failed to show any correlation between macrophage content and tumour grade or stage in a small series of breast carcinomas (n = 17). This contrasts with the findings of Whitworth et al (1990) who describe fewer macrophages in larger more advanced tumours (Normann, 1985). The geometry of large solid tumours may be a factor reducing macrophage invasion into these tumours (Bugelski et al, 1987). As no correlation with stage was demonstrated in this study, sampling errors may be operating. Macrophages from tumours as with other sites of the body, display heterogeneity (McBride, 1986), though TAM subpopulations were not studied here which may have differences in function and influence on the tumour.

6.3 Ki67 INDEX

The Ki67 index

$$= \frac{\text{the number of positive cells}}{\text{the total number of cells}} \text{ expressed as a percentage x proportion of tumour cells}$$

The Ki67 staining was carried out on cytospins from 90 of the 117 tumour specimens. However 49 specimens (54%) were negative for this antibody and cytoplasmic staining, without nuclear staining, occurred in 12 specimens (29%). Nuclear staining resulted in 29 specimens giving a mean percentage of positive cells of 20.5% ± 23% and a range of 0.9 - 94% (Fig. 27). These values have been corrected for the proportion of tumour cells in the cell suspensions. Cytospins of the cervix line CaSki from 7 day and 14 day

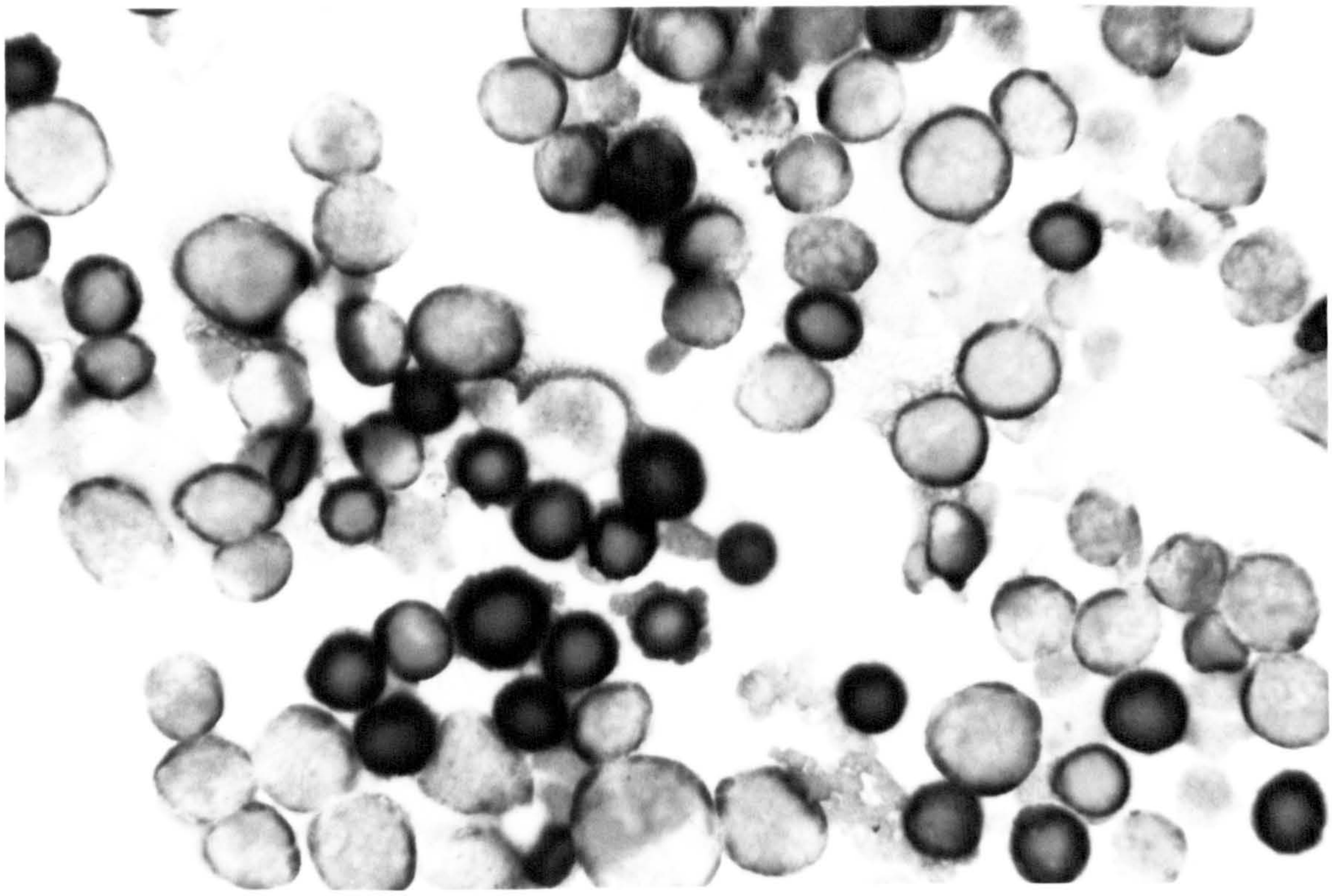

FIG.28 DOUBLE STAINED CYTOSPIN OF TUMOUR CELL SUSPENSION

Ki67 positive cells have dark nuclei and the CK1 positive cells have dark staining cytoplasm. Some tumour cells have stained positively with both antibodies and appear to be darkly stained.

cultures were used as positive controls for the Ki67 staining (Fig. 26) and 3T3 cells were used as negative controls. The 7 day cultures of CaSki gave 70 - 85% positive nuclear staining and the 14 day cultures gave 16 - 25% positivity with Ki67. Double staining was carried out with Ki67 and CK1, CAM5.2 or EBM11 on 17 specimens which confirmed that the Ki67 positive cells were among the cells that stained for low molecular weight cytokeratin antibody (Fig. 28). Possible associations between the Ki67 index and the other parameters under investigation were studied. The Ki67 index was not related to tumour stage (r = 0.58), tumour differentiation (r = 0.02) or tumour age (r = -0.03). There was also no correlation between Ki67 staining and CFE (r = 0.04) or radiosensitivity as measured by SF2 (r = -0.07).

6.3.1 Discussion

The staining of the frozen cytospin preparation from cervical carcinoma resulted in only 25% of the specimens which were labelled. Half the specimens (n = 49) were negative for Ki67 staining. However 21/50 (58%) of specimens which had been stored less than 1 year were positive for Ki67 in this series. Walker and Camplejohn (1988) reported 56% staining in their series of breast carcinomata using cryostat sections and Bouzubar et al (1989) reported 54% staining in their series of breast carcinomas. Only 10% of cytospins which had been stored in a freezer longer than a year were positive (n = 60). Some of these specimens (n = 8) were acetone fixed before storage, but this did not result in positive staining in these specimens. Walker and Camplejohn (1988) looked at storage times of 7 days (in liquid nitrogen) compared to immediate staining and found no difference in the proportion staining. However when studying the specimens in this series which were stained within 1 month of processing, a few specimens 3/9 were negative for Ki 67 with 2 of these showing cytoplasmic staining only. It is clearly not feasible to carry out Ki67 staining on specimens stored for longer than 1 year at -20°C.

The range of proportions of cells staining with Ki67 in this series was 0.9 - 94%. This compares with the range of <1 - 60% in the study of Walker and Camplejohn (1988) and 10-50% in the study of Brown et al (1988) on cervical carcinoma and 10-80% of non Hodgkin's lymphoma cells (Schrape et al, 1987). Cytoplasmic staining was seen in 25% of specimens and 18% were negative in the series of Bouzubar et al (1989). Cryostat sections were used in the study of Brown et al and it was found that 4/31 specimens (13%) showed cytoplasmic staining and had to be excluded from the study. Gerdes et al (1983) reported nuclear and cytoplasmic staining in normal human epidermal cells but do not state the percentage in which this was demonstrated. In this study on cervix tumours 29% of specimens showed cytoplasmic staining alone but only 12% of the specimens stored less than 1 year showed cytoplasmic staining. The low proportion of cells staining in this work may be due to the disaggregation procedures used on the cells which may alter the binding of the antibody. However, the porportions of Ki67 staining cells were not dissimilar from other reported series (Walker and Camplejohn, 1988; Brown et al, 1988). It is more probable that the periods and temperature of storage are important factors.

The positive controls (CaSki cells at 7 and 14 days) were positive with each batch of staining and the negative control remained unstained demonstrating that the antibody was active. The proportions of the exponential cells and plateau phase cells staining were similar to MCF-7 staining carried out by Bouzubar et al (1989). Given the low proportion of the specimens which stained for Ki67 (25%) it is perhaps not surprising that no association was seen with tumour stage though the majority of the specimens were Stage I (Stage I = 13, Stage II = 8, Stage III = 6 and Stage IV = 1) and the numbers of advanced tumours were small. There was no association with grade or patient age. No correlation was seen when these parameters were investigated in the large series reported by Bouzubar et al (1989), though an association with grade was demonstated by the work of Gerdes et al (1986).

The double staining demonstrated that Ki67 positivity in tumour cells though several series have reported staining in normal cells but in low proportions (Gerdes et al, 1983; Bouzubar et al, 1989) although Charpin et al (1989) demonstrated Ki67 staining in epithelial cells only in their series of breast carcinoma aspirates.

7. LIST OF CONCLUSIONS

1) **Radiosensitivity.** This work has demonstrated a wide range of inter-patient SF2 values. The assay results were shown to be reproducible and intra-tumour heterogeneity was not a limitation of this assay. Significant differences between the mean SF2 for different tumour histologies were obtained.

2) **Clonogenicity.** The CFE was shown not to be associated with tumour stage, tumour differentiation or patient age. It was not associated with radiosensitivity as measured by SF2.

3) **Vascularity.** Two methods of assessing vascularity namely measuring ICD and proportion of vessels were compared. Neither of these parameters were related to tumour stage, tumour grade, patient age or tumour histology. Data from patients with 2 year minimum follow up revealed no differences between the mean proportion of vessels or ICD in the tumours in the group of patients that recurred and the group which remained disease free.

4) **Inflammatory infiltrates.** The proportions of macrophages and lymphocytes were not shown to be associated with CFE, SF2, Ki67 staining, tumour stage or differentation.

5) **Ki67 index.** No relationship with tumour stage or differentiation was shown with the proportion of positively staining nuclei with Ki67. It was shown that the cytospins which were stored for more than 1 year were either negative for Ki67 or showed cytoplasmic staining.

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APPENDIX 1

No.	Age.	Stage	corrected CFE%	CFE %	SF2	SF3.5	viable yield in cells g^{-1}	Hist.	Diff.	Col.diam. um	%T	%NO	%L	%G	multi-plicity	%dead cells
V2	74	IIB		no.cols			3.56×10^{6}	SCC	poor		49	26	8	17		16
V3	67	IIB	.006	.004	(0.67)	0.59	7.16×10^{6}	SCC	poor		63	20	8	9		12
V4	35	IB bulky		no.cols			9.7×10^{6}	SCC	poor		26.5	5	5	63 +eosin.	1	17
V5	26	IVA	.44	0.084	0.42	0.27	3.4×10^{6}	adeno	poor		19	16.8	0.8	63.6	1.08	30
V6	45	IIB		no.cols			3.2×10^{6}	SCC	poor		26.5	8.5	44.5	20.5		26
V7	72	IB		no.cols			7.3×10^{5}	SCC	mod		30	8	1	52		36
V8	32	IIA		no.cols				adeno	mod		25	10.4	6	58.6		
V9	34	IB bulky		no.cols				SCC	well		53	28	2	15		
V10	51	IIB		no.cols			1.89×10^{7}	SCC	poor		31.4	14.6	13	41	1.01	16
V11	43	IIB	.054	0.031	0.68	0.46	1.55×10^{6}	SCC	poor		57	18	6	19	1.16	11
V12	32	IB	.062	0.016			3.89×10^{6}	SCC			26	14	3	57	1.004	11
V13	50	IIB		no.cols			2.55×10^{6}	SCC	poor		12.2	8.8	8.6	70.4 +eosin	1.006	12
V14	55	IVB		no.cols			6.4×10^{6}	SCC	P (small cell)		59.2	16.4	12.5	11.9	1.02	13
V15	36	IB bulky		no.cols			3.14×10^{6}	SCC	poor		45.5	16.2	21.2	17.2	1	28
V16	33	IB		no.cols			2.67×10^{7}	SCC	mod		54	14	14	18 +eosin	1.02	4
V17	46	III	.036	.025	0.52		5.37×10^{6}	SCC	poor		69	16	6	10	1.09	6
V18	65	IB	.023	.019	0.38		13.4×10^{6}	SCC			83	12	4	1	1.04	5
V19	36	IB bulky		no.cols			12.8×10^{6}	SCC	mod		64.2	13.6	18.6	3.6	1.02	24
V20	35	IIB		no.cols			7.7×10^{7}	SCC	mod		49.2	13.8	11.8	25.2	1	6

No.	Age.	Stage	corrected CFE%	CFE %	SF2	SF3.5	viable yield in cells g^{-1}	Hist.	Diff.	Col.diam. um	%T	%NO	%L	%G	multi-plicity	%dead cells
V21	66	IB	.03	0.017	(0.27)		2.37×10^{6}	SCC	poor		55.6	22.3	15	7	1	26
V22	52	IB	.024	0.02	0.5	0.23	1.25×10^{7}	SCC	mod		85	9	3	3	1.02	2.5
V23	35	IIB	.057	0.013	0.62		1.31×10^{7}	SCC	poor		22.9	6.5	18.8	51.6	1.0	16
V24	55	IIB	.14	0.06	0.57		1.97×10^{7}	SCC	mod		42.6	27.2	11.8	18.4	1.03	13
V25	43	IB		no.cols			5.54×10^{7}	adeno	well		68	16.5	11	5	1.08	5
V26	31	IIB	.63	0.295	0.32	0.195	2.64×10^{7}	SCC	mod	113	47	19.6	11.8	21.6	1.006	14
V27	64	IIA	.13	.056	0.41		3.28×10^{7}	SCC	mod		42.6	14.4	24.6	18.4	1.005	7
V28	65	IB	.18	0.064	0.27		3.73×10^{6}	SCC	poor		34.8	19.8	13	32.4 +eosin.	1.01	15
V29	62	IIB	.06	0.03	(0.53)		1.81×10^{7}	SCC	well		50	27	11	12	1.005	6
V30	44	IVB	.026	0.01			9.45×10^{6}	SCC	poor		38.8	17	15.8	29.4	1.0	7
V31	66	IIB	.31	0.072	0.54		1.46×10^{7}	SCC	mod		23.6	18	27	32.3	1.01	24
V32	54	IB		no.cols			1.72×10^{7}	SCC	well		52.8	12	32.4	2.8	1.004	32
V33	89	vaginal primary	.14	0.088	0.19	.091	3.16×10^{7}	SCC	poor	80	64.2	5.8	4	25.6	1	11
V34	29	IIA	.02	.008	(0.145)	0.77	7.29×10^{7}	SCC	poor	86	37.4	28.6	12.6	22	1.04	7.5
V35	56	IVA	.02	.012	0.37	0.12	3.10×10^{7}	SCC	mod	124	58	13	22.3	6.7	1.04	6.8
V36	74	IB	.2	.062	0.93		1.18×10^{7}	adeno	well		31	58	6.9	3.9	1.09	5
V37	37	IB bulky	.018	0.013	0.4	0.29	9.19×10^{6}	SCC	well	83	72.6	11.8	12.6	3.2	1.01	37.5
V38	54	IIB	.037	0.024	0.3	0.22	1.43×10^{7}	SCC	mod	92	65.4	16	11.6	6.6	1.002	34
V39	43	IB		no.cols			8.3×10^{6}	SCC	mod		27.2	21.4	23.8	27.6	1.013	32
V40	77	IIB	.006	.004	0.72	.45	7.27×10^{6}	SCC	poor	78	64.2	11.6	17.2	7	1.04	13
V41	43	IIIB	.017	.007	0.195	.076	3.86×10^{7}	SCC	mod	118	41.6	26.2	8.2	24.2	1	25
V42	60	IB	.12	.061	0.36	.068	7.2×10^{6}	SCC	poor	82	51.8	15.4	17.4	15.4	1.005	35
V43	77	IIA	.038	0.016	0.54		1.04×10^{7}	SCC	mod	114	42.6	23	14.2	19.8	1.036	11.5

No.	Age.	Stage	corrected CFE%	CFE %	SF2	SF3.5	viable yield in cells g^{-1}	Hist.	Diff.	Col.diam. um	%T	%MO	%L	%G	multi-plicity	%dead cells
V44	71	IIB	.032	0.018	0.21	.064	2.27×10^7	SCC	mod	101	56.6	13	20.4	9	1.007	7
V45	34	IIB	.048	.01	0.79	0.38	4.08×10^6	SCC	well	113	20.8	14.2	9.2	55.8 +eosin.	1.007	15
V46	59	IB	.23	.056	0.57		1.025×10^7	SCC	mod	106	24.6	10.2	9.6	55.6	1.014	9
V47	45	IIB	.017	.007	.4		1.39×10^7	SCC	poor	89	40.6	31.2	10.4	17.8	1.012	12.5
V48	44	IBV	.016	.005	too small		1.47×10^7	SCC	mod	159	31.8	25.8	19	23.6	1.012	17.6
V49	67	IB		INFECTED			1.97×10^6	SCC	mod		30.6	20.4	8.8	20.2	1	5.2
V50	61	IIA		too small			3.35×10^6	SCC	well						1	2.8
V51	69	IIB	.85	0.45	0.17	0.077	5.29×10^6	SCC	mod	132	52.8	28	15.4	4.2	1.015	38.5
V52	36	IIIB		no. cols			4.29×10^7	SCC	well		48.2	18.6	14.2	19	1.017	11.6
V53	43	IIA	.04	.024	.97		1.12×10^8	SCC	well	129	59.4	16.2	8	16.4	1.008	10
V54	37	IB	.14	.048	0.42		1.27×10^7	SCC	well	77	33.2	14.6	26	26.4	1	17
V55	83	IIA		no.cols			2.46×10^7	SCC	mod		41	21.7	12.2	22.2	1.005	42.6
V56	57	IIA		no.cols			1.51×10^7	SCC	mod		47.8	24.4	5.8	22.2	1.006	3.4
V57	33	IB bulky	.0069	.003			8.16×10^7	SCC	poor		43.4	25.4	7.8	23.6	1.007	7
V58	56	IB	.28	0.12			5.83×10^6	SCC	poor	114	43.2	22.8	4.8	19.4	1.047	12
V59	59	IB	.097	.046	0.31		3.96×10^7	SCC	poor	156	47.2	15	9.2	28.6	1.017	4.7
V60	50	IIB	.44	0.21	0.34		3.03×10^7	SCC	mod	156	47.8	16.8	15.8	19.8	1.016	28
V61	46	IB bulky	.53	0.22	0.72	.36	2.72×10^7	SCC	poor	148	41.2	9	37.5	12.3	1.006	16
V62	50	IB		too small			2.13×10^7	SCC			32.6	5.2	3	59.8	1.009	2.4
V63	59	IIIB	.19	.07	0.72	0.22	1.54×10^7	SCC	mod	131	37.2	8	7.2	41.2	1	18.5
V64	48	IB		no.cols			7.4×10^6	SCC	mod		37.2	15.4	41.4	6	1	11.6
V65	38	IB	.33	0.21	0.56	.125	7.69×10^7	SCC	mod	155	64.6	15.4	14.2	5.8	1.011	47
V66	68	IIA		no.cols			6.14×10^7	SCC	mod		40.6	19.2	6.6	33.6	1.005	19

No.	Age.	Stage	corrected CFE%	CFE %	SF2	SF3.5	viable yield in cells g^{-1}	Hist.	Diff.	Col.diam. um	%T	%MO	%L	%G	multi-plicity	%dead cells
V67	31	IIIB	.059	0.029	0.76		$3.88x10^6$	SCC	well	255	49	20.2	12.5	18	1.013	17.7
V68	49	IB	.9	0.37			$5.4x10^6$	SCC	mod		41	14	8	37	1	56
V69	47	IIB	.16	.08	0.24		$2.72x10^6$	SCC	mod		50	21.2	14.7	14	1.03	12.5
V70	63	IB		no.cols			$3.89x10^5$	adeno	mod		50.5	21	16.5	12	1	17.5
V71	32	primary vagina	.63	.32	.36		$1.01x10^6$	adeno squam	poor	112	51	20	11.5	18	1	36
V72	50	IIB	2.8	1.21	0.14	0.137	$1.34x10^7$	SCC	mod	131	43	12.5	20	24	1.02	9.2
V73	51	IIB	.08	.054	(0.53)		$2.36x10^6$	SCC	mod		67.5	8.5	22	1.3	1	18.2
V74	33	IB bulky	.17	.06	0.42	.12	$2.7x10^7$	SCC	poor	173	34.7	14.5	11.2	32.7	1	7
V75	56	IIIB	.14	.06	.32	.32	$6.18x10^7$	SCC	poor	194	42.4	20.8	7	29.8	1.018	11
V76	50	IIB	2.9	1.17	0.58	0.28	$7.17x10^7$	SCC	mod	122	40.5	14	21	24.7	1.01	34
V77	32	III		INFECTED			$4.5x10^6$	SCC	poor		31.8	10.2	39	19	1.02	13.5
V78	74	IB	.63	0.2	0.61	0.37	$2.97x10^7$	SCC	mod	140	32	13.4	17.2	37.4	1	47
V79	36	IIIB	.82	0.28	0.78	0.49	$1.8x10^7$	SCC	mod	138	34	10	30	26.4	1.02	23
V80	51	IIA	.57	0.23	0.36	0.24	$5.18x10^7$	SCC	mod	137	40.6	15.6	25.4	20.4	1	45
V81	34	REC	.45	0.22	0.46	0.16	$9.94x10^6$	SCC	mod	121	49.2	9	30	11.6	1	2
V82	90	IIB	.33	.08	0.27		$1.7x10^7$	SCC	well		24	12	39	24	1.007	20.8
V83	54	IB	.037	.02			$2.37x10^7$	SCC	well	133	54.6	15.2	15.6	14.8	1.003	12.5
V84	38	IIIB	.44	.23	0.4	0.18	$3.5x10^7$	SCC	mod		51.8	15.8	18.8	13.8	1.01	53
V85	40	IB	.09	.038	0.28		$5.5x10^7$	SCC	mod	138	42	13.6	25.8	18.4	1.016	19.6
V86	40	IIIB	.06	.029	0.37	0.29	$2.9x10^7$	SCC	mod	142	46	16.6	12.2	20.6	1.017	5.6
V87	67	IIIB	.82	0.25	0.53		$7.9x10^6$	adeno	poor	143	30.6	11.2	16	42.2	1.009	4.6
V88	74	IIB	.38	0.22	0.44	0.079	$4.25x10^7$	SCC	mod	147	57.6	10.2	13.2	19	1.04	23
V89	38	IB	.71	.23	.68		$1.95x10^7$	SCC	mod	170	32.6	14.4	41.8	11.4	1.003	28

No.	Age.	Stage	corrected CFE%	CFE %	SF2	SF3.5	viable yield in cells g^{-1}	Hist.	Diff.	Col.diam. um	%T	%MO	%L	%G	multi-plicity	%dead cells
V90	44	IIB	.069	0.017	0.28		$3.76x10^7$	SCC	poor		24.6	9.2	14.8	51.4	1.008	4.8
V91	51	recurr	.079	.05	0.39		$3.6x10^6$	SCC	mod		62.7	9.5	15	9	1	62
V92	48	IB	.045	.026	(0.46)		$3.9x10^6$	SCC	well		57.6	9	15.4	18	1.003	15.8
V93	47	IB bulky	.034	.024	0.38		$6.9x10^7$	SCC	well	96	70	11.8	9.6	8.6	1	34
V94	66	IIIB	.024	.009	(0.38)		$1.23x10^7$	SCC	poor		37	7.8	26.4	28.8	1.01	45
V95	62	IIB	.18	.096	0.38		$1.79x10^7$	SCC	well	180	54.8	12.8	21.6	10.8	1.008	38
V96	48	III		no.cols			$1.71x10^7$	SCC	mod		10.5	3.7	4.3	81.5 +eosin.	1.02	10.4
V97	38	IVB		no.cols			$3.1x10^7$	SCC	poor		4.7	14.6	13.2	25	1.017	7
V98	62	IB		nos.too small			$9.5x10^6$	SCC	mod		64.3	11.5	13.7	10.5	1	41
V99	58	IB bulky	.41	.22	.26	.11	$2.8x10^7$	SCC	mod	177	53.6	10.4	15.8	20.4	1.002	59
V100	50	IB		no growth			$2.15x10^7$	adeno	mod		26.2	7.8	21	45	1.01	10
V101	60	IB bulky		no growth			$1.36x10^6$	adeno squam	poor		53.8	7	25.7	13.5	1	42
V102	50	IB	.12	.068	0.38	.27	$6.86x10^7$	SCC	poor	143	56.2	7	10.2	27	1.002	12.8
V103	78	IB	.065	.028	0.32		$5.06x10^6$	SCC	poor		43.2	10	31.4	15.4	1.01	5
V104	76	III	.41	.14	0.72		$1.99x10^7$	SCC	mod	123	34.4	12.2	16	37.2	1	53
V105	32	IB	.34	0.19	0.15		$2.6x10^6$	SCC	poor	180	56.4	12	15	16.6	1	37
V106	63	IIIB	.17	.084	.35		$1.36x10^7$	SCC	mod	133	49	9.6	13.6	27.8	1.002	7
V107	31	IB	.29	0.16			$1.03x10^7$	SCC		153	56	10.6	13.6	19.8	1	4.7
V108	47	IIB	1.54	0.71	0.15	.067	$1.4x10^7$	SCC	mod		46	13.2	17	23.8	1	18
V109	65	IB	.3	0.17	0.46	0.18	$3.5x10^7$	SCC		138	57	12	13.2	17.8	1.004	10
V110	64	IIA	.44	0.21	0.54	0.26	$3.39x10^7$	SCC	poor	132	47.2	12.8	21.6	18.4	1.015	5.4
V111	52	IIA	.14	0.02	0.39	0.17	$8.4x20^7$	SCC	poor	106	13.8	5	41.4	39.8	1.003	9

No.	Age.	Stage	corrected CFE%	CFE %	SF2	SF3.5	viable yield in cells g^{-1}	Hist.	Diff.	Col.diam. um	%T	%MO	%L	%G	multi-plicity	%dead cells
V112	59	IB	1.0	0.43	0.57		2.57×10^7	SCC	well	145	42.6	20.6	19.8	17.2	1.04	3.8
V113	49	IIIB	.24	.088	.47	.2	1.39×10^7	SCC	poor	126	37.2	13.2	12	35.6	1	7.5
V114	50	IIB		INFECTED			1.59×10^6	SCC	well		29.8	10	48	12.2	1	26.3
V115	40	IIB	.55	0.195	0.48		4.56×10^6	SCC	well	142	35.6	10	13.4	41	1	24
V116	69	IIA	.36	0.12	0.16	.056	1.12×10^7	SCC	well	118	33.2	12.4	26.6	27.8	1	16
V117	1	IIIB	.38	0.2	0.42		1.26×10^6	SCC	large cell P	159	52.7	10.7	29.7	6.7	1.03	29
V118	51	IIIB	.29	0.11	0.66		5.3×10^6	SCC	mod	111	37.4	13.6	28.8	20.2	1	23.5

APPENDIX I

117 tumours processed
3 infections
Parentheses indicate small numbers

T = tumour cells
MO = macrophages
L = lymphocytes
G = granulocytes

NO.	ICD x μm	RANGE	ISD x μm	HISTOLOGY	MORPHOMETRY % relative volumes Parenchyma	Stroma	Vessels	Necrosis
V3	290	79.8-568	145	P.SCC	58.9	37.2	2.3	1.7
V4	193	86-435	-	P.SCC	56.4	41	1.6	1.1
V5	257	96-397	105	P.adeno.	69.3	5.3	1.6	23.4
V7		Too small to score						
V8	243.5	3-444	-	M.adeno.	75	19.9	2.4	2.7
V9	257	122.8-428	-	W.SCC	86.1	7.9	1.9	4.1
V10	213	109-359	-	P.SCC	77.1	19.9	1.1	1.8
V11	223	110-402	-	P.SCC	65.1	25.6	1.4	8.0
V12		No tumour on block						
V13	215.5	8.6-483	-	P.SCC	50.3	33.6	1.7	14.4
V14	234	35-416	-	P.small cell	81.2	13.8	1.2/0.8	3.9*
V15	255	93-494	149	P.SCC	92.9	4.6	1.0	1.5
V16	197	90-332	-	M.SCC	72.3	18.1	1.0	8.6
V17	225	5.6-513	-	P.SCC	28.8	26.7	2.4	42
V18		CIN on biopsy						
V19	218.5	54.7-399	-	M.SCC	63.6	32.8	3.8	0
V20	302	96.6-495	-	M.SCC	63.4	27	1.3	8.4
V21		Too small to score						
V22	232	123-509	-	M.SCC	58.1	31.4	0.9	9.6
V23	256	78-495	190	P.SCC	68.4	9.3	1.9	20.4
V24	260	107-410	-	M.SCC	55.9	35.9	3.9	5.2
V25	185	79-363	-	W.adeno	34.7	57.2	3.5	4.9
V26	274	79.6-538	170.8	M.SCC	74	8.3	1.9	15.8
V27	299	137-663	-	M.SCC	52	32	2.0	14
V28	298	4-504	-	P.SCC	20.4	54.7	3.3	21.6
V29	202	4.3-362	-	W.SCC	26.4	61.1	2.3	10.2
V30	277	119-468	-	P.SCC	51.1	37.7	1.0	10.2
V31	187	94-357	-	M.SCC	88.2	6.1	3.7	1.5
V32	208	53-445	-	W.SCC	60.9	20.5	3.4	15.2
V33	202	93-346	-	P.SCC	43	28.6	3.3/3.0	25.1*
V34	244	80-452	-	P.SCC	65.8	16.8	5.3	12.1
V35	246.8	8.6-514	-	M.SCC	67	25.1	3.6	4.3
V36	223	87-364	-	W.adeno	62.3	31.7	2.5	3.5
V37	248	107.8-433.5	-	W.SCC	87.2	9.0	2.0	1.8
V38	237.5	56.8-448	-	M.SCC	93.3	3.8	0.8/0.6	2.2*
V39	256	4.8-544	-	M.SCC	82.7	14.2	0.9	2.2

NO.	ICD x µm	RANGE	ISD x µm	HISTOLOGY	MORPHOMETRY % Parenchyma	Stroma	Vessels	Necrosis
V40	259	68-494	-	P.SCC	49.8	37.4	5.8/3.4	8.2*
V41	226.4	12-570	-	M.SCC	78.1	18.0	1.6	2.3
V42		CIN only on block						
V43	333	92-609	-	M.SCC	37.5	53.4	3.2	6.0
V45	208	85-456	133	W.SCC	59.2	37.7	1.2/2.7	1.4*
V46	223	73-394	-	M.SCC	43.2	43.7	3.3	9.8
V48	228	81-489	-	M.SCC	42.9	47.2	3.4	6.5
V49	254.8	128-613	-	M.SCC	70.6	8.2	7.0	14
V50		Too small		W.SCC				
V51	287	74.5-656	-	M.SCC	66.8	30.3	1.4	1.5
V52	229	84-396	-	W.SCC	53.7	32.1	2.1	12.1
V53	214	108-412	-	W.SCC	63.1	32.1	2.2	2.6
V54	285	11.8-610	114	W.SCC	75.9	15.9	1.0/1.2	7.2*
V55		Too small		M.SCC				
V56		Too small						
V57	257	86-496	-	P.SCC	51.9	31.6	1.1	15.4
V58	214	100-387	-	P.SCC	85.3	10.1	1.8	2.8
V59	280	48.7-522	-	P.SCC	67.3	18.4	2.3	12
V60	250	119-442	-	M.SCC	72.4	21.9	1.3	4.4
V61	272	96-488	-	P.SCC	74.5	18.7	1.1	5.7
V63	274	5-478	144	M.SCC	63.9	19.9	3.7	12.6
V64	170	7-295	-	M.SCC	31.2	56.9	2.4/2.6	9.4*
V65	257.5	12-587	-	M.SCC	57.4	32.1	2.1	8.5
V66	215	58-440	115.4	M.SCC	61.8	25.3	2.7	10.3
V67	275.6	3-474.6	-	W.SCC	38.3	53.1	2.5	6.2
V69	239	4.8-335	-	M.SCC	67	27.3	2.7	3.0
V70	130.5	55.6-368.5	-	M.adeno	37.7	52.3	4.3	5.8
V71	226.6	5.6-403	138.8	P.adeno.sq.	84.2	11.1	2.5	2.0
V72		Too little tumour on block						
V73	322	162-668	-	M.SCC	40.9	45.3	11	3.3
V74	241	69-484	-	P.SCC	79.3	14.5	3.1	3.2
V75	199	6-539	-	P.SCC	58.2	26.8	6.5	8.5
V76	231	56.8-514	-	M.SCC	63.1	29.6	3.1	4.3
V77	328	112-593	-	P.SCC	86.9	5.6	0.6	6.9
V78	221	42.8-554	-	M.SCC	64.3	27.3	1.5	6.9
V79	263	135-476	-	M.SCC	49.3	41.7	2.2/2.4	6.7*
V80	207	58-470	-	M.SCC	71.6	24.4	2.5	1.6
V81	239	94-361	-	W.SCC	62.8	29.6	2.1	5.6

NO.	ICD x µm	RANGE	ISD x µm	HISTOLOGY	MORPHOMETRY % Parenchyma	Stroma	Vessels	Necrosis
V82	252	68.8-585	-	W.SCC	64.6	20.9	4.2	10.6
V83	260	79.6-518	-	W.SCC	81.5	11.5	3.7	3.3
V84	232	66-509	-	M.SCC	68.4	21.5	23	7.8
V85	248	144-537	-	M.SCC	75.5	15.0	2.1	7.2
V86	183.5	77-384	-	M.SCC	62.3	13.8	5.1	18.8
V87	191	74-309	-	P.adeno	60.1	14	2.5	23.4
V88	246	110-441	-	M.SCC	67.1	26.4	1.9	4.6
V89	219	7.2-557	119	M.SCC	62.9	30.1	2.6/1.6	5.0*
V90	220	77-459	-	P.SCC	68	19.4	1.3	11.4
V91	277.6	88-558	-	W.SCC	60.9	34.0	0.8	4.3
V92	181	65-379	-	W.SCC	52	42.1	3.3	2.8
V93	228	98-528	-	W.SCC	56.5	39.8	3.6	0.4
V94	210	86-357	-	P.SCC	68.5	25.7	4.8	1.0
V95	250	93-477	-	W.SCC	49.2	43.8	4.1	2.9
V96	289.9	120-535	-	M.SCC	65.5	27.9	3.8/2.6	3.8*
V97	183	103-321	-	P.SCC	60	28.8	2.5	8.8
V98		Too small		M.SCC				
V99	228	80-252	-	M.SCC	76	19	4.5	0.7
V100	197	105-381	-	M.adeno	64.9	26.6	7.2	1.3
V101	235	74-465.8	-	P.SCC	57.6	25.6	2.2	14.8
V102		Too small		P.SCC				
V103		Too small						
V104		Too small						
V106	314	119-648	-	M.SCC	63.7	31.5	2.7	2.3
V107	263	97-446	-		71.4	25	1.2	1.7

APPENDIX 2 VASCULARITY RESULTS

ICD = intercapillary distance; ISD = intercapillary distance in stroma;
W.SCC = well differentiated squamous cell carcinoma
M.SCC = moderately differentiated squamous cell carcinoma
P.SCC = poorly differentiated squamous cell carcinoma

* 2 biopsies assessed separately for morphometry

no of specimens studied = 90

APPENDIX 3 Ki67 RESULTS

CaSki at 7 days 70-85% +ve

CaSki at 14 days 16-25% +ve

[UNCORRECTED FOR % TUMOUR CELLS]
[[in parentheses] corrected for % TUMOUR CELLS]

V4 cytoplasm only		
V5 neg		
	A 2 [4.8]	
V6 neg	V61 B 1.5 [3.6]	V96 neg
V7 cyto only	V62 cyto only	V97 6.3 [13.4]
		A 5 [9.3]
V8 6.8 [27.2]	V63 neg	V99 B 8.3 [14.9]
		A 29
V10 cyto only	V64 neg	V100 B 1.3 [58]
		A
V14 neg	V66 neg	V101 B neg
V15 neg	V67 neg	V102 neg
		A 0.6 [1.5]
V16 neg	V69 neg	V103 B 0.8 [1.9]
	A 0.5 [0.9]	
V17 neg	V70 B 1.6 [2.6]	V104 5.5 [16]
V20	V71A and B neg	V105 neg cyto staining
V23 cyto only	V72 neg	V106 1.5 [3]
V24 neg	V74 B neg	V107 neg
V28 neg	V75 A and B neg	V108 A cyto only
V29 neg	V76 A and B neg	V108 B 2.2 [4.8]
V30 neg	V77 A and B neg	V109 A 2 [3.8]
V32 neg	V78 neg	V109 B 2.4 [4.2]
V34 neg	V79 neg	V110 neg
V37 B 5 [6.8]	V80 neg	V111 A12.7 [92]
V38 A and B neg	V81 A neg	V111 B 14 [54]

[UNCORRECTED FOR % TUMOUR CELLS]
[[in parentheses] corrected for % TUMOUR CELLS]

V40 A 0.6 [0.9]
V41 neg
V42 neg
V43 neg
V44 neg
V46 neg
V47 neg
V51 neg
V52 neg
V53 neg
V54 B 5.3 [16]
V55 cyto only
V56 neg
V57 2.4 [5.5]
V59 5.6 [11.8]
V60 cyto only

V82 A neg
V83 A neg
V84 A and B neg
V85 neg
V86 neg
A16.6 [54]
V87 B12.8 [38]
A cyto only
V88 B 4.3 [7.4]
A 6.6 [20]
V89 B 8.3 [22]
V90 8 [32.5]
V91 A and B cyto only
V92 2 [3.5]
A neg
V93 B 2.6 [3.7]
A 7.3 [20]
V94 B 5.3 [16]
V95 neg

V112 9.4 [24.6]
V113 A 7.3 [26]
V113 B11.4 [30.8]
V114 cytoplasm only
V115 A 4.3 [21]
V115 B 6.3 [17.6]
V116 27 [81]
V117 cyto only
V118 15.2 [40.6]

$$\text{Ki67 score} = \frac{\text{positive cells}}{\text{total no. of tumour cells}}$$ expressed as a percentage

Sample No.	CFEas%(±1SEM)	SF2(±1SEM)	SF3.5(±1SEM)	SF4(±1SEM)	SF6±1SEM	SF8±1SEM	α value Gy^{-1} ±standard error
	0.29±.03	0.32±.03	0.19±.02				
V26	0.19±.016	0.64±.06					0.36±.11
	4.28±.06	0.4 ±.01	0.24±.009				
V33	0.088±.03	0.195±.03	0.091±.01				0.83±.51
	0.41 ±.03	0.13 ±.04					
				(SF4.5)			
V38	0.024±.002	0.31 ±.04	0.23±.01	0.1±.01			0.39±0.26
	0.016±.007	0.31 ±.06					
	0.012±.001	0.34 ±.04					
	0.022±.004	0.37 ±.03					
V40	0.0036±.001	0.72±.07	0.43±.09	(SF4.5)			
	0.012 ±.002	0.75±.12		0.25±.06			0.29±.21
V41	0.006±.002	0.19±.037	0.096±.01				
	0.058±.017	0.73±.094	0.17±.06				0.73±.18
	0.26 ±.07	0.58±.13					
	0.076±.009	0.48±.09					
V53	0.024±.005	0.97±.24					0.22±.16
	.2±.02	0.88±.08	0.69±.068				
	0.55 ±.04	0.61±.1	0.32±.06				
V60A	0.21±.05	0.36±.08	(SF3)				0.35±.15
	0.24±.03	0.6 ±.06	0.42±.15	0.3±.07			

Sample No.	CFEas%(±1SEM)	SF2(±1SEM)	SF3.5(±1SEM)	SF4(±1SEM)	SF6±1SEM	SF8±1SEM	α value Gy^{-1} ±standard error
V61A	0.06±.008	0.35±.03	0.24±.03	0.1±.003			
	0.34±.06	0.99±.08	0.2±.03 (SF3)	0.14±.02 (SF4)			
	0.47±.009	0.72±.01	0.27±.03				
V61B	0.22±.015	0.72±.05		(SF4)			
	0.34±.04	0.53±.1	(SF3) 0.48±.04	0.15±.05			0.21±.16
	0.4±.03	0.78±.05	0.25±.04				
V63	0.1±.03	0.72±.3	0.22±.09				
	.065±.008	.073±.13					0.55±.87
	.1±.03	0.68±.14					
V76A	2.93±.37	0.49±.08	(SF3)0.34±.04	(SF4) 0.18±.05	(SF5) 0.05±.003	(SF6) 0.09±.04	
	1.08±.07	0.67±.1	0.29±.03				
V76B	1.17±.08	0.57±.04	0.28±.06				
	1.22±.12	0.56±.03	(SF3) 0.3±.02	(SF4) 0.29±.08			0.3±.17
	0.84±.05	0.65±.05	(SF3)0.37±.04	(SF4) 0.33±.08			
V78	0.2±.02	0.61±.16	0.37±.06				
	0.34±.07	0.52±.12					

Sample No.	CFEas%(±1SEM)	SF2(±1SEM)	SF3.5(±1SEM)	SF4(±1SEM)	SF6±1SEM	SF8±1SEM	α value Gy^{-1} ±standard error
V81	0.21±.03	0.46±.09	0.085±.02				
	0.06±.006	0.47±.06	0.16±.02				
	0.072±.007	0.41±.02	0.17±.03				
V84	0.23±.02	0.4±.1	0.17±.06				
	0.11±.01	0.46±.08	0.19±.03				
	0.31±.05	0.51±.19					
	0.29±.04	0.64±.09					
V85	0.02±.005	0.4±.09					
	0.04±.012	0.28±.1					
				(SF4)	(SF6)		
V109	0.17±0.14	0.46±0.44		0.13±.03	0.017±.005		
	0.11±.014	0.56±.06	0.19±.02				

APPENDIX 4 REPEAT EXPERIMENTS AND SURVIVAL CURVE DATA

APPENDIX 5 PATIENTS WITH RECURRENCE/DIED

(* metastatic disease outside pelvis only)

	V2	Dead 19 months
	V3	Died from metastases from carcinoma of breast
	V4	Recurrence at 14 months. Died at 23 months with pelvic recurrence and mets
	V5	Died at 6 months. Recurrence after Wertheims and metastases
*	V6	Died with metastatic disease at 20 months
	V10	Died at 17 months
*	V13	Died at 29 months metastatic disease
	V14	Died at 1 months. Stage IVB disease
	V15	Recurrence at 10 months
	V16	Died at 11 months with pelvic disease
	V17	Died at 7 months. Palliative XRT. Also had carcinoma of the breast
*	V19	Died at 7 months metastases in brain and lung
	V20	Died at 23 months with pelvic disease and mets
*	V23	Died at 19 months with metastases
	V24	Died at 19 months. Had pelvic disease
	V26	Died at 14 months. Disease in pelvis
*	V30	Died at 15 months. Stage IVB at presentation, so palliative RT
*	V32	Recurrence at 17 months and mets
	V33	Palliative RT
	V35	Recurrence IVB disease
	V40	Palliative RT
*	V43	Died with metastases at 13 months
	V48	Stage IVB disease, died at 8 months
	V50	Recurrence at 21 months
*	V51	Died at 10 months
	V52	Died at 5 months with pelvic disease
*	V53	Died at 15 months with metastases
	V55	Recurrence at 18 months
	V58	Recurrence at 21 months
*	V63	Died with pulmonary metastases 11 months
	V67	Died with pelvic disease and mets 11 months
*[	V75	Died at 11 months. No cancer found at PM]
*	V77	Died at 3 months. Palliative RT because of metastases
	V80	Recurrence
	V81	Died 13 months with recurrence. Recurrence following surgery, palliative RT
	V82	Died at 7 months, residual disease. Palliative RT
	V87	Vaginal recurrence at 9 months
	V90	Recurrence
	V91	Died at 11 months with pelvic recurrence
	V96	Recurrence and paraaortic metastases
*	V97	Died of metastases at 11 months

[up to V50 - minimum 2 yr follow up; up to V97 1 yr minimum follow up]