CHEMOTHERAPY IN THE MINIMALLY-INVASIVE MANAGEMENT OF SUPERFICIAL

BLADDER CANCER

Studies of the maximum potential of intravesical thioTEPA.

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ERRATA

ABSTRACT

Fibreoptic out-patient cystoscopy has become established in the follow-up of bladder cancer, and increased the need for out-patient treatment and prophylaxis. Despite a variety of indications and a constellation of schedules, intravesical chemotherapy has been found to prevent progression and recurrence. Optimum methods remain undefined. Because of the anatomical separation of the therapeutic and toxic effects of thioTEPA, not found with more recent agents, it was thought that there might be a greater clinical opportunity to exploit positive laboratory findings.

A model course of ten instillations of intravesical chemotherapy was developed in vitro. Cells were less sensitive after treatment. Potential markers or predictors of drug resistance were studied, but no differences were identified. The addition of a solvent, dimethyl sulphoxide, did not increase the cytotoxic effect of four drugs that have commonly been used to treat bladder cancer. Adjusting pH during drug exposure to acid levels increased the effect of thioTEPA on a range of human urothelial cell lines a hundredfold. Pilot studies of amiloride gave no evidence of further effect, when used with thioTEPA.

A first prospective comparison of UK methods found flexible cystoscopy no more likely to miss tumours or induce bacteriuria than rod-lens cystoscopy.

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ACKNOWLEDGEMENTS

AUTHOR'S DECLARATION

The present studies were carried out with the benefit of the training, advice and support of patient and generous collea- gues. The Author carried out the tissue culture, the mouse xenografts, the EGFR staining, the isoenzyme electrophoresis and DNA fingerprinting, the flexible cystoscopies, and instigated and co-ordinated the other examinations.

Some techniques peripheral to my main inquiry were too skilled to learn quickly, so, while the Author read the theory, and decided on their application, the expertise of others was used as follows. Dr R. Camplejohn analysed the computer data from the cytofluorimetric specimens prepared by the Author with the advice of Dr Camplejohn's staff. The breeding and maintenance of the nude mice was the work of the Department of Biological Services, the United Medical and Dental Schools of Guy's and St Thomas' Hospitals. The Author culled the mice, harvested, fixed and embedded the tumours, and the blocks were cut, mounted, and stained for Grading by the staff of the Histopathology Department of St Thomas' Hospital. Dr Heyderman of that Department supervi- sed the Author's early immunoperoxidase tests of EGFR1 antibody on frozen cell pellets. Dr M.C. Parkinson reviewed the xenograft tumour grading, which can often be debatable among expert Histopathologists. Interpretation of metaphase spreads requires dedicated training and experience, which were exercised by Dr D. Sherr, Mrs P. Gorman, and Mrs S. Williams on cell samples

provided by the Author. In view of their findings, the Author did not learn to produce, stain, or interpret metaphase spreads from the sublines developed. The supervision and interpretation, by Dr S. Povey and Mr S. Jeremiah of the Galton Laboratory, of the isoenzyme electrophoreses and DNA fingerprints was invaluable.

Flexible cystoscopy services at Frimley Park Hospital and at St Thomas' Hospital were begun by the Author, who carried out all the examinations in Appendices 1&2, and half those in Appendix 3. The remainder in Appendix 3 were the work of Mr T.G. Liston, of the Urology Department, as the design of the study required. The microbiological samples in Appendix 2 were taken by Dr D. Muckle-Jones and the nurses of the Clinical Treatment Suite and the Surgical Unit of Frimley Park Hospital. Microbiological testing was carried out by Mrs M. Clements of the Microbiology Department, with the advice of Dr M. Williams, Consultant Microbiologist.

The preliminary findings on induced resistance were initially presented at the Guy's Hospital Urological Research Meeting, and at the British Association for Cancer Research in 1988. The DMSO study was published in Urological Research (1988) **16**, 329-331, and the study of tumour miss by fibreoptic cystoscopy was published initially as an abstract at the Vth World Congress on Endourology and ESWL, in 1987. Combined with the bacteriological data, it was also presented at the Royal Society of Medicine, in 1992.

CHAPTER 1

INTRODUCTION

EPIDEMIOLOGY

In 1986, bladder cancer affected 9,591 new patients in England and Wales (Office of Population Censuses and Surveys, 1991). The peak incidence in absolute numbers is between 60 and 79 years, but increases progressively in age-standardized data (Ibid.). In 1984, an estimated 1227 patients with the diagnosis of bladder cancer occupied hospital beds daily, and 86,330 cystoscopies with resection of bladder lesion were carried out (Hospital Inpatient Statistics, OPCS, 1984). In Scotland, the latest figures are for 1990, when 1208 new patients were registered, and in-patient discharges or deaths surveyed included 3,364 men with bladder tumours, who had stayed a mean of 7.4 days, and 1398 women, who had stayed 7.6 days on average, with a similar trend in the age-standardized data (Community Services Agency for the Scottish Health Service, 1991). Overall, the incidence is either steady or increasing (Dorn & Cutler, 1959; Cutler & Young, 1975; Ohno & Aoki, 1977). The per capita incidence, adjusted for variations in the age-structure in different countries, is greater in the industrialised nations, (Muir et al., 1989), but as the emergent nations become more influenced by tobacco use and industry, incidence there might be expected to catch up. Although smoking (Lilienfeld, Levi & Moore, 1956) and industrial carcinogens (Rehn, 1895) have long been recognised as predisposing to the

development of bladder cancer, screening programmes in the dye and rubber industries produce only a small minority of referrals, the bulk coming from the investigation of clinically apparent haematuria.

SYMPTOMATOLOGY

Over 90% of patients with superficial bladder cancer complain of symptoms at presentation (Wallace, 1985), and in 60-96% of patients, haematuria is the main complaint (Thompson, 1960; Mills, 1962; Massey, 1965; Wallace & Harris, 1965; Greene, Hanash & Farrow, 1973; Varkarakis, Gaeta, Moore, et al., 1974; Hendry, Manning, Perry et al., 1981). Cystoscopic diagnosis and resection both diagnoses and treats this symptom, but, as an indicator of the need for review, haematuria occurs too late. One author reported that 13 of 24 patients with recurrences at recurrences at review cystoscopy did not have haematuria (Miller, Mitchell & Brown 1969), and it is a clinical commonplace that recurrent haematuria rarely causes patients to present early for review.

Irritative symptoms, which are present in 20-25% of patients presenting with papillary tumours, are more commonly associated with flat carcinoma in situ (Farrow, Utz, Rife et al., 1977). Although there is little formal information on the relief of symptoms from endoscopic management, patients with papillary recurrences seldom report discomfort, and the procedure itself produces symptoms (Monsour et al., 1985; Denholm, Conn, Newsam,
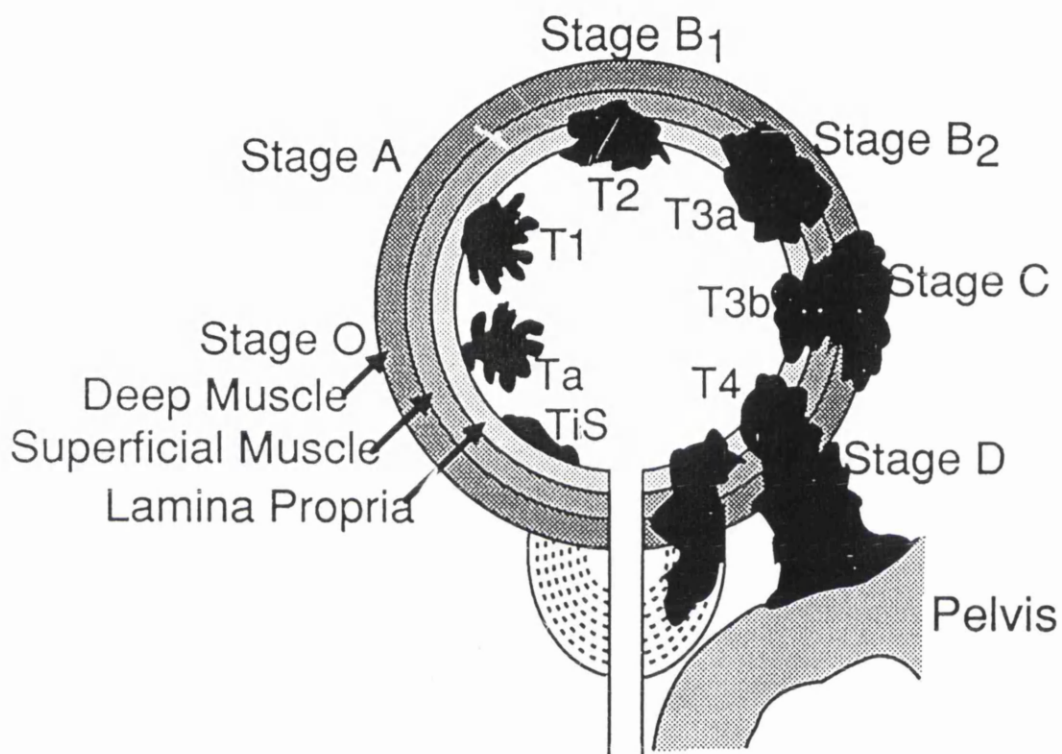
Key: Stage O-D by Jewett-Strong-Marshall classification (1946-
1956), Stages T_a-T_4 by UICC classification (1987)

FIGURE 1

COMPARISON OF AMERICAN AND TNM STAGING

et al., 1990).

PATHOLOGY

In the United Kingdom, at least 90% of tumours of the urinary
bladder derive from urothelium. The macroscopic appearance may be
of a solid, sessile lesion, or even of flat erythematous patches,
but by far the commonest appearance is of a fronded, papillary
lesion or lesions. The tumour is thought to originate in the
urothelium, and to spread within the urinary tract by implanta-
tion of shed cells (Albarran & Imbert, 1903; Boyd & Burnand,
1974), or as a result of the unmasking, at various sites, of a
widespread malignant tendency or field change (Melicow, 1952).
Spread outwith the urothelium is initially by direct invasion,
through the lamina propria, to the detrusor muscle, then the
perivesical fat and pelvic structures, with invasion of lympha-
tics, and occasionally blood vessels leading to wider metastasis.
Progression through these stages has long been associated with a
poorer chance of cure (Jewett & Strong, 1946). There have been
two main codifications of the histological extent of disease, the
Jewett-Strong-Marshall and the Tumour-Nodes-Metastasis Systems
(figure 1, fp 18). Since the latter is a specific application of
a general approach to solid tumours, it is coming to be the more
usual, even in American studies. At presentation, more than 60%
of patients have tumours limited to the surface layers (T_a or T_1
or T_{is} in the TNM Classification), with no evidence of tumour in

Author	Recurrence +	Recurrence -	Total
Loening (1980)	46	38	84
Greene (1973)*	36	64	100
Smith (1983)	51	43	94
Cutler (1982)	64	124	188
NBCOGA(1977)291	44	89	133
Total	241	358	599
%	40	60	100

* Grade I tumours only.

+ = recurrence present

- = recurrence absent

TABLE 1

NUMBERS OF PATIENTS WITH T_{a-1} BLADDER TUMOURS WHICH

RECURRED WITHIN 1 YEAR

bladder muscle on endoscopic biopsies (Cutler et al., 1982).

Because of the nature of practice in the United States of America discourages hospital referral of patients with low-risk tumours, this figure is probably an underestimate of the incidence and particularly of the position in the United Kingdom.

Flat carcinoma in situ may encompass a number of disease entities with differing prognosis, as invasion rates as low as 0% of 10 cases (Barlebo, Sorensen & Soeborg-Olsen, 1972), and 11% (Farrow et al., 1977) and as high as 61% (Daly, 1976), 73% (Utz, Hanash & Farrow, 1970) and 50 to 80% (Whitmore, 1979) have been reported. Flat carcinoma in situ is different in incidence, appearance, symptomatology and implications from papillary tran- sitional cell carcinoma, and will not be further considered here.

The present studies chiefly concern the commoner, exophytic superficial lesions. These are usually electrocoagulated or endoscopically removed at the time of diagnosis, although bi- opsies of mucosa adjacent to the tumour, or at selected distant sites may still show atypia or, occasionally, frank carcinoma in situ (Melicow, 1952; Althausen, Prout & Daly, 1976; Cutler et al., 1982; Smith, Elton, Beynon, et al., 1983). Within two years of treatment, 34-55% of patients will have a further tumour identified (Chapman, 1950; Greene, et al, 1973; Loening, Narayana, Loder, et al., 1980; Cutler et al., 1982; Smith et al.,

Author	Recurrence		
	+	−	Total
Greene (1973)*	45	55	100
Cutler (1982)	89	99	188
Chapman (1950)	23	20	43
Total	157	174	331
%	47	53	100

* Grade I tumours only

TABLE 2

NUMBERS OF PATIENTS WITH T_{a-1} BLADDER TUMOURS WITH RECURRENCE

IN TWO YEARS

1983, tables 1-2, fp 19-20). (Further tumours will not here be separated into "true recurrences" at the original site, and "new occurrences" elsewhere, as the management is essentially identical.) Whether the evidence of distant atypia is accepted as indicating that papillary tumours are only the local manifestation of urothelial field change, or whether implantation at the time of resection is the cause of heterotopic tumours later, local surgery alone does not prevent recurrence. The five-year studies found 71% recurred, range 58-84% (table 3, fp 21) At 15 years, a figure of 73% has been reported (Greene et al.,1973).

The five-year survival of patients with superficial bladder cancer (Stage A or O of the Jewett and Strong classification) has been reported to be 61-83%, with an overall figure of around 67% (Nicholls & Marshall, 1956 a&b; Barnes, Bergman, Hadley et al., 1967; Cox, Cass & Boyce, 1969; Utz, Schmitz, Fugelso et al., 1973; O'Flynn, Smith & Hanson, 1975). Separate figures for each stage at five years are unavailable in patients treated by TUR alone. Survival is a difficult end-point for evaluating treatment, both because the time taken to reach it, even in the age-group affected by bladder cancer, would prolong studies, and because other illnesses could intervene cause death. This probably contributes to the shortage of separate survival data for T_a and T_1 tumours.

Histological features of individual cells (Grading) have long

Author	Patients with Recurrence		
	+	−	Total
Chapman (1950)	12	20	32
Greene (1973)*	58	42	100
Althausen (1976)	109	20	129
England (1981)	232	100	332
Total	411	169	580
%	71	29	100

* Grade I tumours only.

+ = recurrence present

− = recurrence absent

TABLE 3

NUMBERS   OF PATIENTS WITH T_{a-1} BLADDER TUMOURS   WITH   RECURRENCE

AT FIVE YEARS

been associated with prognosis (Broders, 1922). Grade changes
may be interpreted as a progressive deterioration in the progno-
sis, but the inter- and intra-observer variations in categorizing
given slides are considerable (Ooms, Anderson, Alons et al.,
1983), making a single observation an unreliable marker of
prognosis in the individual. Where progression is defined as
increase in Grade or Stage, (e.g. England, Paris & Blandy, 1981;
Lutzeyer, Rubben & Dahm, 1982) several outcome variables are
combined and presumed to be of equal weight. Equally, studies
showing Grade as a prognostic influence usually fail to consider
the possible influence of stage (e.g. Gilbert, Logan, Kagan et
al., 1978).

Further tumours are litle more than an inconvenience, however,
unless they lead to disease progression, to worse symptoms or
poorer survival. Disease progression to involve bladder muscle or
beyond is believed to have similar implications as newly-
diagnosed disease involving the same layers, in the Jewett and
Strong (1946) and the TNM classifications (UICC, 1987), and is
therefore a significant yardstick of treatment effectiveness,
particularly from the patient's point of view. This assumption
may be an oversimplification, but this does not appear to have
invalidated Stage as a prognostic indicator.

Clinical experience suggests that superficial tumours, if they
recur, mostly remain superficial (Jewett, 1977; Marshall, 1956;

Authors	Number Progressed/Number Studied		
	T_A	T_1	Total
Cutler, 1982 $	4/120	19/78	23/198
Lutzeyer, 1982*	19/194	27/80	46/274
Heney, 1983	6/144	19/63	25/201
Total	29/458	65/221	94/673
% (95%CI)	6(4,9)	29(23,35)	14(11,17)

$ two-years' follow-up

* Progression in Grade and Stage

TABLE 4

STAGE AND NUMBERS OF PATIENTS WITH PROGRESSION AT 2-3 YEARS

Greene et al., 1973; Varkarakis, et al., 1974; Cutler et al., 1982). Fourteen per cent of a total of 673 patients with superficial bladder cancer managed by local surgery and followed for three years progressed (table 4, fp22). The five-year estimate was 28% of 345 patients (table 5, fp23). One group has reported that, at each episode of recurrence, the incidence of progresion is constant, between 5 and 10% (Mackenzie et al., 1981).

On the other hand, eighty-five percent of patients with muscle-invasive disease present de novo, without previous evidence of superficial disease (Kaye and Lange, 1982; Prout, 1982; Cutler et al., 1982). Ninety-eight percent present within one year of diagnosis, suggesting that some apparently superficial tumours were understaged at the initial assessment (Cutler, et al. 1982). Thus, the presumption that current management prevents muscle invasion is open to question.

In recent studies of patients managed with bladder conservation, T_a tumours (those contained within the epithelium, and not invading the lamina propria) show progression to muscle invasion at three years in 6% of cases, range 2-10% (table 4, fp22). At 5 years, 26%, range 17-32%, have been reported to have progressed (table 5, fp23).

T_1 tumours (those invading the lamina propria, but not bladder

	Number Progressed/Number Studied		
Authors	T_A	T_1	Total
Varkarakis, 1974	6/36	34/130	40/166
Althausen, 1976	31/110	8/19	39/129
Stephenson, 1985	7/22	9/28	16/50
Total	44/168	51/177	95/345
%(95%CI)	26(13,39)	29(17,41)	28(19,37)

TABLE 5

STAGE AND NUMBERS OF PATIENTS WITH PROGRESSION AT 5 YEARS

muscle) show 29% progression at 2-3 years, range 24-34% (table 4, fp22) and 29% at 5 years, range 26-42% (table 5, fp23). The three-year figure for all superficial tumours in these studies was 17%, range 12-23% (table 3, fp21); and the 5-year, 28%, range 24-32% (table 4, fp22). The five-year figures are somewhat surprising, at first glance, suggesting that the effect of stage on progression of superficial bladder tumours is transient, but may be influenced by the predominance of American hospital series, from which low-risk patients, particularly those with T_a disease, may have been excluded by referral bias. The series reported from the London Hospital (England et al., 1981) and of comparable size to the totals in table 5, but treated with a combination of local surgery, intravesical thioTEPA, and radical radiotherapy, distinguished the 6% rate in T_a from 28% in T_1.

Although the presence of lamina propria invasion did not appear to affect progression within five years of diagnosis in the studies reviewed above treated with local resection alone, other characteristics of the tumour may indicate low need of surveillance against progression. One group found tumour size related to progression within one year (Cutler et al., 1982), but not at two, as the incidence of progression was low. Other studies have failed to identify an association between progression and size greater than 5 cm, although there was a link between recurrence and size.

Tumour multiplicity was found to be related to progression in several studies of apparently unselected patients managed by local surgery. Percentages without denominators, ranges, or confidence intervals were reported at three years after diagnosis (Lutzeyer et al., 1982). In T_a tumours, 18% of single lesions progressed, compared with 43% of patients with multiple tumours; in T_1, 33% compared with 46%. Lerman and colleagues reported a variable length of follow-up of 103 patients. Of patients with single tumours, 5% of single "papillomata", and 14% of patients with multiple papillomata, became "carcinomata" (Lerman, Hutter & Whitmore, 1970). The false-negative rate was 5%, 95% confidence interval (0,26%), quite aceptable for selection of a low-risk group. Heney's 58 patients reflected an association between multiplicity and progression in an average follow-up of three years, with a false-negative rate of 14 (0,45)% (Heney, Ahmed, Flanagan et al., 1983). Patients with low-grade low-stage tumours followed for five years (Althausen et al., 1976) showed progression in 4% of 23 patients with single tumours, and 43% of patients with multiple tumours. The population studied was small, but it is tempting to surmise that this may be representative of a genuinely more significant role of multiplicity in low-stage low-grade disease, which is swamped by the effect of grade. The apparent significance of multiplicity has been obscured by poor presentaton of data, selected study populations, and by a failure to use a precise end-point for follow-up. Two groups have reported that tumours that are multiple at presentation are more

likely to show invasion of the lamina propria (Varkarakis et al., 1974; Williams et al., 1977). The poorer prognosis in patients with multiple tumours than those with solitary tumours (England et al., 1981b; Heney et al., 1983) might in part represent the difficulty of examining all areas of the tumour for invasion. England's group (1981b) found that patients with five or more tumours at each recurrence all progressed within 15 months, and 11/28 (39%) pT_1 G3 tumours and 29/59 (49%) of pT_1 G2 tumours progressed within 5 years, but only 8/140 (6%) of pT_a. This strongly suggests that it should be possible to select patients whose superficial tumours are unlikely to progress, for less intensive follow-up, or poor-prognosis patients who might benefit from more radical treatment.

Superficial bladder tumours are likely to remain superficial, but with recurrences, and are unlikely to decrease survival appreciably. The effect of size is unclear, and multiplicity at diagnosis, or on successive recurrences, grade and stage progressively more significant in prognosis.

CURRENT MANAGEMENT

Conventional management of superficial lesions (those that, on biopsy, do not invade detrusor muscle) involves cystoscopic diagnosis and resection, followed by surveillance, usually under general anaesthesia, one to four times per year. Usually clini-

cians in the United Kingdom advise follow-up for at least five years after the latest recurrence, as asymptomatic recurrences are common. The survival of patients with single, superficial, well-differentiated tumours approaches normal life-expectancy for age (Nicholls & Marshall, 1956b). There is little proportionate benefit in survival possible as a result of treatment. In the absence of symptoms, the primary aim of surveillance is to prevent progression.

Management by review cystoscopy has a physical and psychological morbidity (Monsour et al., 1985, Denholm et al., 1990). The number of cystoscopies required to detect one episode of recurrence was seventeen in one series (Meuleman & Delaere, 1988), although the exact number will depend on patient selection, and on the frequency of examination chosen. As patients in the age-group most affected by bladder cancer are often subject to other chronic complaints which make out-patient anaesthesia too hazardous, the surveillance of bladder cancer makes major demands on urological in-patient resources, for a relatively low tumour pick-up rate. In the main age-range for this disease, life-expectancy is already limited, and patients' priorities are likely to be different from those of younger age-groups. Demands on quality of life may be as important to the individual as potential changes in survival, particularly to those already suffering from chronic illnesses. In the United Kingdom, cystoscopic review is carried out in most cases under general

anaesthesia. Patients are predominantly male, and the S-shaped male urethra has to be distorted to accommodate the straight rod-lens cystoscope, or the bigger resectoscope. Under the same general anaesthetic, it is possible to diagnose and treat recurrent tumours by resection. In Europe and the United States of America, where the financial resources for such management are less readily available, office cystoscopy, and treatment of small tumours by cystodiathermy, are still common, as indeed they used to be in the UK. This tends to reduce the recruitment of good-prognosis tumours to hospital series, and should be remembered in interpreting series from these sources. This author finds that UK patients who have experienced rigid instrumentation under topical and under general anaesthesia find general anaesthesia highly desirable .

In the peak age-group for bladder cancer, the sixth to eighth decades, chronic conditions that complicate general anaesthesia are prevalent, such as diabetes, chronic bronchitis, and cardiovascular disease. Patients without the necessary family, transport, and telephone facilities for safe out-patient pro-cedures are more likely to depend on admission to public hospitals, where their admission competes unfavourably with those of emergency patients, for reducing numbers of beds. In this situation, it would be helpful if out-patient methods of manage-ment could be found.

Authors	No (%) Missed	Total	(95% CI)
Abu-Yousef 1984	1 (6)	17	(-5,17%)
Boccon-Gibod 1985	6 (20)	30	(6,34%)
Brun 1984	14 (29)	49	(1,46%)
Dershaw 1987	0 (0)	25	--------
Juul 1986	20 (25)	79	(16,35%)
Malone 1986	22 (50)	50	(35,65%)
Rosenkilde-Olsen 1985	6 (9)	67	(2,16%)
Vallancien 1986	15 (26)	58	(15,37%)
Total	84 (22)	375	(18,26%)

TABLE 6

NUMBER (%) OF PATIENTS MISDIAGNOSED AS TUMOUR-FREE BY

TRANSABDOMINAL ULTRASOUND

The present studies of intravesical chemotherapy developed from the introduction of flexible fibreoptic cystoscopy, which the present author had studied in both a district and a teaching hospital, and found to be acceptable to patients, less time-consuming than rod-lens examination, accurate and safe (Appendices 1-3). With the probability that diagnosis would no longer need general anaesthesia or hospital admission in the considerable majority of patients, an out-patient approach to treatment and, more immediately, to prophylaxis against recurrence and progression was needed.

LESS INVASIVE DIAGNOSIS OF RECURRENCE : ULTRASOUND

Minimally invasive follow-up by transabdominal ultrasound has a significant false-negative rate. Comparison with the findings at cystoscopy gives 0/25 (Dershaw & Scher, 1987) to 22/44 (Malone, Weston-Underwood, Aron et al, 1986) false negative ultrasounds. A summary of the data available is given in table 6 (fp28). In most of these studies, it appears that the cystoscopist was aware of the result of the ultrasound, so no assessment of the false-negative rate of cystoscopy was derived. Overall, 22(0-50)% of patients with a tumour had negative ultrasounds in these studies. Ultrasonographers providing a general ultrasonograpy service might be expected to miss more. As Boccon-Gibod and co-authors point out (Boccon-Gibod, Le Portz, Godefroy et al., 1985), there may be a greater number of flat mucosal abnormalities missed by

ultrasound, that could be detected by the addition of cytological results. Juul's group (Juul, Torp-Pedersen, Larsen et al., 1986) with combined cytology and abdominal ultrasound, still had a falsely negative test in 19% of 79 patients with tumours, 14% of all 105 negative tests. In theory, transurethral ultrasound may overcome some of the problems of the transabdominal route, but requires rigid or semi-rigid instrumentation, similar to rod-lens cystoscopy (Nakamura & Nijima, 1980; Abu-Yousef, Narayana, Franken et al., 1984; Devonec, Chapelon, Codas, et al., 1987). Ultrasound has never been able to detect carcinoma in situ, as cytology can, and cystoscopy may. The contribution of ultrasound to follow-up needs further development.

LESS INVASIVE DIAGNOSIS OF RECURRENCE: URINE CYTOLOGY

It has been argued that cytology of bladder washings can identify 88% of patients who have cystoscopically visible tumour (Flanagan & Miller, 1978), and is a valid substitute for cystoscopic follow-up. Some authors report 4% of all patients tested as having false negative cytology in their hands (Legramandi Giannoni, Ricci Barbini et al., 1989) but do not report it as a proportion of those with cystoscopic tumour, so the rate could result from studying a population with few recurrences. Cytological examination is particularly sensitive in high-grade disease (Kern, Bales & Webster, 1968), where it is probable that more radical treatment is called for (Birch & Harland, 1989; Jenkins,

et al., 1989) and in CIS that is recurrent after immunotherapy (Harland et al., 1991), where a similar radicalism could be justified. Thus, it could be argued, cytology is most effective as the horse is bolting, if not after it has bolted; but not at a time when treatment could close the stable door. In the United Kingdom, the present cytological establishment has difficulty coping with the workload generated by cervical cytology on a three-yearly screening of women over 35, so it would be hard to staff a three-monthly cytological follow-up for all patients with superficial bladder cancer. Attempts at automation have yet to gain widespread acceptance.

FIBREOPTIC CYSTOSCOPY

The application of modern fibreoptic technology to urology offers the possibility of bladder tumour diagnosis under topical analgesia in out-patients (Fowler, 1984; Powell, et al., 1984; Fawcett, et al., 1991). It now seems likely that flexible cystos-copy will greatly reduce the need for more invasive methods of diagnosis, and improve the quality of life for patients under-going follow-up.

Fibreoptic cystoscopes can negotiate the sigmoid male urethra without distorting it. As a result, the risk of injuring or abrading the urethra, and the need for anaesthesia are reduced. The proportion of cystoscopies under general anaesthesia at which

there was no recurrence was reduced in one series from 61% to 23% (Fowler et al., 1984). Patients in a London Teaching Hospital found the procedure under topical analgesia comfortable compared with previous rod-lens examinations under general anaesthesia (Flannigan et al.,1988), and in the USA, with rod-lens examinations carried out under topical analgesia on the same day (Figueroa, et al., 1984). A more recent report from Edinburgh, comparing synchronous, but non-randomised groups, found that patients having flexible examinations experienced fewer symptoms than those undergoing rigid instrumentation (Denholm et al., 1990). The present author, introducing flexible cystoscopy in a district general hospital, and then in a London teaching hospital, tested the bacteriological and diagnostic safety of the procedure, and its acceptability to patients (Appendices 1-3).

Early investigators studied induced bacteriuria indirectly. Fowler (1984) reported that bacteriuria hadbeen identified following flexible cystoscopy, but the patients had undergone other procedures also, including Marshall-Marchetti-Krantz operations. When the less elaborate aseptic precautions taken for flexible cystoscopy were used used for rod-lens cystoscopy in outpatients, the incidence of bacteriuria was 6% (Fozard, Green, Harrison, et al., 1983). The questionnaire study of Denholm and colleagues (1990) reported a higher incidence of symptoms in tthe first week after rod-lens cystoscopy than fibreoptic cystoscopy, but did not report bacteriological data. The present author, with

a group at Frimley Park Hospital, Surrey, found a lower incidence of bacteriuria in patients undergoing flexible cystoscopy than in a simultaneous, non-randomised group undergoing rod-lens cystoscopy, although the difference was not statistically significant. There was no evidence of a greater tendency to induce bacteriuria by the flexible method (Appendices 1-3).

If flexible cystoscopy is to replace rod-lens examinations in follow-up, the sensitivity of diagnosis of recurrence is crucial. As Pope and Wickham (1991) have pointed out, the view via a stationary fibreoptic instrument remains fragmented because of the fibre bundle diameter, and inferior to rod-lens optics, but as the instrument is moved in use, it is possible for the observer to integrate and intrapolate data to compensate. Because anaesthesia for rod-lens cystoscopy in the United Kingdom differs from that in Europe and in the United States of America, the present author carried out the first prospective, double-blind controlled comparison of the likelihood of missing the diagnosis of a recurrence (the false-negative rate) (Walker & Liston, 1987; Appendix 3). Practice in the USA leads to rod-lens cystoscopy being carried out under topical analgesia, so American studies do not compare the options appropriate to the United Kingdom (Clayman, Reddy & Lange, 1984; Figueroa et al.,1987). The two comparisons carried out in the United Kingdom were pilot studies of the adequacy of the procedure before its substitution for rod-lens examination, and both examinations were carried out

under general anaesthesia (Fowler 1984; Powell et al., 1984). The
study of Meyhoff's group (Meyhoff, Andersen, Klarskov et
al.,1988) used the same general or spinal anaesthetic for both
procedures. The only authors to study two relevant anaesthetic
techniques were Webb and colleagues (Webb, Butler & Fitzgerald,
1984) who reported an open comparison in 23 patients, who had
five episodes of recurrence, two of which (40%) were not
diagnosed with the flexible choledochoscope used. The tiny number
of tumour episodes available to be diagnosed defies statistical
analysis, and as the result of the flexible cystoscopy was
apparently known to the rod-lens cystoscopist, there was no
mechanism to assess the accuracy of the conventional examination.
Clayman reported missing tumour episodes in one patient with each
instrument, and one of two synchronous tumours in another
patient, but does not report the total number of tumours or
tumour episodes (Clayman et al., 1984). Meyhoff reported missing
3/15 (20%) tumour episodes on flexible cystoscopy, but none on
rod-lens examination (Meyhoff et al., 1988), when both were
carried out under spinal or general anaesthetic. Figueroa's group
missed one tumour with each instrument, used under topical
analgesia but give no denominator (Figueroa et al., 1987). Fowler
(1984) reported that there were no false-positive examinations,
when flexible cystoscopy was compared with rod-lens examination
under general anaesthesia, but gave no data on missed tumours.
The proportion of tumours missed is the relevant measurement if
flexible cystoscopy is safe to be used to avoid anaesthetic risks

in patients who do not have tumour recurrences.

Out-patient diagnosis by fibreoptic cystoscopy has developed and spread since it became a possiblity in the mid 1980s, and the need for methods of anticipating and preventing progression has been underlined.

PREDICTIVE TESTING

The test used to choose patients suitable for low-intensity surveillance requires to be a sensitive predictor of the possibility of progression (or recurrence if symptoms rather than progression are to be anticipated and prevented), whereas the emphasis of a test used to decide which patients need more radical treatment needs to be specific for high risk, to avoid over-treatment. Most prognostic tests, such as loss of blood group antigens, and even Grade and Stage have been developed retrospectively, by testing patients with known progression, using archival material, and tend to be more specific than sensitive. On the whole, the problems outweigh the potential benefit (Abel, 1988).

Abnormalities of the mucosa intervening between exophytic tumours have been generally recognised to relate to prognosis since Melicow's (1952) study of cystectomy specimens. Definitions of the abnormalities vary considerably, in addition to the incon-

Author	Antigen Normal Invasion			Antigen Deleted Invasion		
	+	−	Total	+	−	Total
Bergman (1978)	0	3	3	3	8	11
Jakse (1978)	0	18	18	9	4	13
Johnson (1980)	0	15	15	9	6	15
Lange (1978)	2	14	16	16	5	21
Limas (1979)	3	23	26	21	13	34
Newman (1980)	9	214	223	71	28	99
Total	14	287	301	129	64	193

+ = invasion ocurred

− = invasion did not occur

TABLE 7

SPECIFIC   RED   CELL ADHERENCE TEST AND   NUMBERS OF   PATIENTS

DEVELOPING PROGRESSION WITHIN 5 YEARS

sistencies of categorization mentioned above (Ooms et al., 1983), and the variable decisions whether to include carcinoma in situ associated with a papillary tumour. Further, the abnormality may be assayed on the margins of the original resection or at arbitrary points in the quadrants of the bladder. As a result, studies are rarely comparable.

Two recent studies using the National Bladder Cancer Co-operative Group criteria, over much the same duration, have tested biopsies at distant sites. Cutler's (1982) group found invasive tumours developed in 15% of patients overall, with 40% in the more dysplastic subgroup (95% confidence interval 10,70%) and a false-negative rate of 9%, which might be thought low enough to use to select patients for minimal surveillance. Heney's (1983) group, over a longer period, found a 10% (0,25%) incidence of invasion overall, 33% in those with moderate to severe dysplasia, and a false-negative rate of 8 (3,12)%.

The Specific Red Cell Adherence test was the first method of detecting changes in cellular surface antigens, which were found to be deleted in more invasive tumours studied in retrospect. A number of studies of archival biopsies (summarised in table 7, fp35) found that 5% of 301 patients with normal blood group antigens, and 67% of 193 patients with antigen deletion developed invasive lesions. The false-negative rate was 5 (3,7)%. The resulting slides cannot be kept, however, and, until the develop-

| Author | Antigen Normal | | | Antigen Deleted | | |
| | Invasion | | | Invasion | | |
	+	−	Total	+	−	Total
Finan (1982)	4	27	31	4	4	8
Stephenson (1985)	4	24	28	12	10	22
Total	8	51	59	16	14	30

+ = invasion ocurred

− = invasion did not occur

TABLE 8

BLOOD GROUP ANTIGEN (IMMUNOPEROXIDASE) AND NUMBERS OF PATIENTS

DEVELOPING PROGRESSION WITHIN FIVE YEARS

ment of the relatively weak anti-H antibodies, patients with blood-group O could not be tested. More modern immunoperoxidase studies (table 8, fp36) had a higher false-negative rate, 14 (5,24)% of 59 patients, than the initial reports, and would require further prospective study before they were used to minimise the intensity of follow-up. The Thomsen-Friedenreich or T- antigen is slightly more complex, in that it is normally masked, and is abnormal if it is unmasked, or deleted, but small studies with a three-year follow-up reported progression in 69 patients with of 7 (1,13)% of 69 patients (Coon, Weinstein & Summers, 1982; Summers, Coon, Ward et al., 1983). A similar potential use been suggested for immunohistochemical staining of cytokeratins (Abel, Cornell, Buamah et al., 1987; Feitz Beck, Smeets et al., 1985), and of the receptor for epidermal growth factor, EGFR1 (Neal, Marsh, Bennett et al., 1985).

The presence of marker chromosomes on karyotyping has been reported to have a very low false-negative rate (Sandberg, 1977; Summers et al., 1983), and in time this may be a useful investigation, but the figures so far relate to patients in whom the study could be carried out, and the test is both difficult and time-consuming to perform. Ploidy at the time of diagnosis is technically easier to measure, but has not yet been convincingly shown to predict progression (Summers et al., 1983). Various authors have examined in vitro tests on tumour biopsies, in a manner akin to antibiotic chemosensitivity testing, with the

intention of predicting, at an early stage in treatment, the drug most effective for the individual (Hamburger & Salmon 1977; Lathan, Von Hoff & Clark, 1984; Hanuske et al. 1989). The time required for cellular growth, the number of patients for whom tumour culture is unsuccessful (Salmon 1984; Sarsody et al., 1982), the proportion of false-positive and false-negative tests (Tannock, 1978), and the possibly heterogeneous response of primary versus metastatic tumours (Von Hoff & Clark 1984), have limited the benefits hoped for to the preclinical assessment of new drugs. The test bears some relation to tumour behaviour in vivo (Lathan et al., 1984). A test carried out at the start of treatment would address only inherent resistance, not resistance induced by suboptimal treatment, but if the mechanics of the test were simple enough, this would not be an overwhelming difficulty. At present, however, this test is not a reliable or efficient way of assessing the feasibility of treatment by intravesical chemo-therapy.

Of the methods of patient selection and surveillance proposed as an alternative or adjunct to rod-lens cystoscopy with electroresection, fibreoptic cystoscopy has had the greatest impact on the quality of life in patients with bladder cancer. The transformation to a largely out-patient surveillance increases the importance of any possibility of postponing or abolishing recurrence or progression by treatments that can be applied in out-patients.

IMMUNOTHERAPY

Intravesical, intradermal, and oral treatment with agents that can provoke or mimic an immune response to tumour have been used as prophylaxis against recurrence after resection, and in the therapy of polychronotopic recurrences. Intravesical interferon alpha is expensive, and early clinical studies are not encouraging (Kwok et al., 1989).

Bacillus Calmette-Guerin was first applied by both intradermal and intravesical routes in bladder cancer (Morales, 1981). It has since been reported that there is no difference in effect on the treatment of diffuse carcinoma in situ if the intradermal treatment is witheld (Lamm, De Haven, Shriver et al., 1991). There is some evidence that, in addition to reducing recurrence more than Adriamycin (Martinez-Pineiro, Jimenez-Leon, Martinez-Pineiro et al., 1990; Lamm, Blumenstein, Crawford et al., 1987) or ThioTEPA (Rodriguez-Netto, Caserta & Lemos, 1981; Camacho, Pinsky, Kerr et al., 1980; Martinez-Pineiro et al., 1990), BCG prophylaxis can delay progression (Martinez-Pineiro et al., 1990; Herr, 1991). The main drawback seems to be that BCG prophylaxis subjects a mainly asymptomatic patients to a 90% risk of cystitis, 43% of haematuria, 28% of fever, 24% of malaise (Lamm et al., 1986), and a small but recorded risk of death (Rawls et al., 1988; Deresiewicz, Stone & Aster, 1990) or of life-threatening infection (Steg, Belos, Leleu et al, 1989).

INTRAVESICAL CHEMOTHERAPY

The frequency with which cystoscopy induces its unwanted effects may be as important as their severity. Intravesical chemotherapy has been widely used to treat tumours that prove difficult to manage endoscopically, and this has been said by some to be its main role (England et al., 1981), rather than prophylaxis against the development of recurrence or progression. Generally, drugs produce complete response in one third of/f patients, partial response in one third, and no response in one third (Lum, 1983; Torti & Lum, 1984). Duration, frequency, intensity, and conditions of treatment remain to be standardised and optimal conditions to be defined. Out-patient drug treatment for bladder cancer might contribute in two ways to reducing the need for cystoscopy and for tumour resection, even if it does not cure the tumour outright.

First, it might space out recurrences. Intravesical chemotherapy has been widely used to treat superficial tumours with roughly 30% complete response, and 30% partial response (Lum, 1983; Torti & Lum, 1984). At first (Jones & Swinney, 1961) it was mainly appled to those lesions not responding to endoscopic treatment ("therapeutic" instillations), but it has also been used prophylactically, after resection of all visible tumours, both with and without maintenance treatment (Lum, 1983; Torti & Lum, 1984).

Prophylactic instillations have been reported to delay recurrence, both in terms of the interval per lesion identified (Burnand, Boyd, Mayo et al., 1976) and of recurrence rate (Schulman, Robinson, Denis et al., 1982) and time to first recurrence (Prout, Koontz, Coombs et al., 1983), although this has not been found by all investigators (NBCOGA, 1983; MRC Working Party, 1985). Patients who had responded to therapeutic thioTEPA previously, and were then randomized, in a study of thioTEPA prophylaxis, to surveillance and TUR only, had a similar disease-free interval to new patients who received prophylactic thioTEPA (Prout et al., 1983), so it may be that thioTEPA treatment selects patients with low diease activity.

Intravesical chemotherapy might offer the patient a delay of the progression of superficial tumour to one that spreads through the bladder wall to metastasise. While progression implies that the tumour has persisted or recurred, it is an additional phenomenon, and it is possible that only T_a tumours should be considered to have a low potential for progression, as T_1 lesions have, by definition, the necessary mechanisms to breach tissue layers. The low rate of progression to muscle invasion at two to three years, 6% for T_a, and 29% for T_1 (table 4, fp24), is likely to be a major obstacle to identifying differences in progression as a result of treatments. Progression to muscle invasion has not been delayed in controlled comparisons of the effect of intravesical agents (Schulman, Sylvester, Robinson et al., 1981; Heney,

Koontz, Barton et al., 1988; Rubben, Lutzeyer, Fischer et al., 1988), despite expectations (Green, Robinson, Glashan et al., 1984).

The commonest drugs in use in the United Kingdom until the recent advent of epirubicin were doxorubicin (Adriamycin), ethoglucid (Epodyl), Mitomycin-C, and triethylene thiophosphoramide (thioTEPA). The conditions and duration of administration are highly variable between studies, and optimal use of any one agent remains to be defined. ThioTEPA has been in clinical use for the longest time, and is relatively inexpensive. It has a molecular weight of 189, and is absorbed systemically from the bladder, producing myelosuppression, its main adverse effect, in about 10% of patients (Jones & Swinney, 1961; Koontz, Prout, Smith et al., 1981; Hollister and Coleman, 1980; Horn, Eidelman, Walach et al., 1981). The others, of greater molecular weight, are absorbed much less, and have their greatest toxicity in the bladder, causing cystitis and bladder contracture. The anatomical separation of thioTEPA effect and toxicity makes it theoretically possible that one effect might be enhanced without increasing the other, and was a factor in choosing thioTEPA as the main subject in the chemotherapy studies. Nevertheless, factors investigated in respect of thioTEPA may also be of relevance in the use of other agents.

INTRAVESICAL CHEMOTHERAPY: Resistance

While intensive instillations over one month can clear the bladder of multiple superficial tumours (Jones & Swinney, 1961), and single instillations after resection have been found to increase the delay per recurrent lesion (Burnand et al., 1976), prophylactic instillations at three-month cystoscopy/resection have not been found to improve the recurrence rate over resection alone in a multicentre study (MRC Working Party, 1985). When thioTEPA is used as a single dose at the time of resection (Burnand, et al., 1976; Boyd and Burnand, 1974; Pan, Slocum, Rustum et al., 1989) the mechanism of action may be different from use in a course of instillations (Jones & Swinney, 1961; Koontz et al., 1981). There is wide variation in schedules, from single doses immediately after resection, to weekly or monthly instillations (Burnand et al., 1976; England, Blandy & Paris, 1980; Schulman et al., 1982; Flanigan, Ellison & Butler et al., 1986; Zincke, Benson, Hilton et al., 1985; Koontz et al., 1985). Even among the studies of prophylaxis with thioTEPA, there is wide variation in the schedules of administration, from alternate days for a week, then three-monthly (England, et al., 1980), weekly for one month, then monthly (Schulman et al., 1982), weekly for two months, then monthly (Flanigan, et al., 1986), biweekly for five cycles (Zincke et al., 1985), monthly (Koontz et al., 1981), or three-monthly (MRC Working Party, 1985). The lack of effect in the last study may reflect the different dose-

rates, or a tendency for resistance to develop. A clear under-
standing of the process is essential if medical treatment is to
promote the well-being of patients with bladder tumours.

Cellular resistance to drug effect has been recognised since
Pasteur's era (Kossiakoff, 1887), and is a major obstacle to
curative cancer chemotherapy (De Vita, 1989). Resistance has been
found to develop in vitro to escalating concentrations of various
anticancer drugs in continuous exposure experiments (Bedford,
Shellard, Walker et al., 1987; Mc Govern, Kachel, Vijan et al.,
1988), and may be yet more of a problem with intermittent
exposures to steady concentrations used in intravesical chemo-
therapy.

Drug resistance is thought to be of two main types, inherent and
acquired, the former giving a clone a survival advantage in the
presence of the drug, and an all-or-nothing, substantial
resistance, whereas the latter is progressive, may be small in
degree, and cannot be identified in cells that have not been
exposed to the drug. This distinction is somewhat simplistic,
however, and the two characteristics are not mutually exclusive.

The hypothesis of Goldie and Coldman (1979), that spontaneous
mutations induce resistance, relies on an inherent property of
the cell, and not on the presence of the drug. Selective growth
of these resistant subpopulations would be more likely in the

presence of the agent to which they, but not their parent clone, had become resistant. This had been earlier postulated by Luria and Delbruck (1943) in the behaviour of the bacterial species E. Coli. The rate of spontaneous mutation to drug resistance has been said to be of the order of 10^{-3} to 10^{-6} mutations per cell division(Goldie & Coldman, 1979) with the presence of drug giving a further selective advantage to otherwise less vigorous mutant clones. It is argued that some slow-growing visceral tumours which shed cells copiously (because of the mechanical function of the organ of origin) may appear to be intrinsically drug resistant because, although of small volume at diagnosis, they have already undergone many doublings to reach that small tumour mass. A large, stepwise change in sensitivity could easily be understood to result from a change in the conformation of the cellular mechanism targetted by the drug, but equally, modification of rate-controlling genes might modulate, but not abolish drug sensitivity. In the more extensive studies of resistance in bacteria, there is only one example of resistance induced by means of structural alterations of a bacterial target produced by a plasmid conferring resistance. The methylation of two adenine residues on 23S RNA molecules of bacterial ribosomes, prevents binding of lincomycin or erythromycin (Davies & Smith, 1978).

There are many mechanisms which limit the effect of anticancer drugs on tumours (De Vita, 1989), including changes in permeability, repair of DNA damage and drug inactivation. The most

widely-researched phenomenon in tumour drug resistance is that of pleiotropic drug resistance, or multidrug resistance, which appears to result from modulation of an existing process, rather than the creation of a new one. Cell lines grown in escalating concentrations of a drug may be found in time to be resistant not only to that drug, but to a range of others, mainly natural products (Biedler & Riehm, 1970). This resistance has been shown to be associated with reductions in intracellular drug concentrations, and the presence of P-glycoprotein in the cell membrane (Juliano & Ling, 1976; Bech-Hanson, Till & Ling, 1976; Ling & Thompson, 1973; Kartner, Riordan & Ling, 1983). The amino acid sequence of P-glycoprotein shows structural homology with a bacterial membrane transport protein (Gros, Croop & Housman, 1986; Chen, Chin & Ueda, 1986; Gerlach, Endicott, Juranka et al., 1986; Pastan & Gottesman, 1988), and, as P-glycoprotein RNA expression has been found in high levels in normal kidney tissue, and in moderate levels in liver and colon, it is tempting to surmise that P-glycoprotein is a normal defence against environmental poisons, pumping them out of the cells in organs likely to be exposed to toxins. Its role in the development of drug resistance would not then be new or chance, but dependent on alteration of a relevant, normal process, and likely to be modulated rather than all-or-nothing. Verapamil, which is used in the treatment of cardiovascular diseases, binds to P-glycoprotein in competition with such agents as vinblastine (Cornwell, Pastan & Gottesman, 1987), and has been found to enhance the effect of

thioTEPA (Simpson, Tseng, Anderson et al., 1984). If, as this suggests, P-glycoprotein contributes to thioTEPA resistance, one mechanism of thioTEPA resistance may be subject to gradual, and perhaps inducible, variation.

On the other hand, as spontaneous mutation is a common event (Goldie & Coldman, 1979; Richter, Park & Ames, 1988) there is normally an array of DNA repair mechanisms which might be used to repair alkylation damage produced by thioTEPA. Patients with the inherited DNA-repair deficiency syndromes xeroderma pigmentosum (Arrand, Bone & Johnson, 1989), ataxia-telangiectasia (Henner & Blazka, 1986), and Bloom's syndrome (Nicotera, Notaro, Notaro et al., 1989) have a cancer-prone tendency, and cells are highly sensitive to mutational agents, including drugs and radiation. Indeed, as Harris (1985) has pointed out, drug resistance is present in normal cells, and it is sensitivity that is unusual. Ames has likewise been emphasising the natural ability of cells to resist mutagenic stimuli (Forman, 1991)

Graduated resistance can also be produced experimentally, as in bacteria (Bainbridge, 1980). Cancer cells can tolerate progress-ively increasing concentrations of drugs (Bedford et al., 1987; Mc Govern et al, 1988) by adaptation of a number of pre-existing cellular processes (De Vita, 1989). Continuous exposure to cis-platinum, for example, has been used to induce resistance (Bedford et al., 1987), and differences in cis-platinum sensiti-

vity are asssociated with differences in repair of platinum-DNA adducts (Bedford et al., 1988). As both thioTEPA and cis-platinum are thought to act mainly as alkylating agents, similar resistance to thioTEPA might be possible also.

It also remains to be explained how prophylactic intravesical chemotherapy acts, and whether exposing the urothelium to drugs in the absence of visible tumour contributes to drug resistance. This might be analogous to the development of antibiotic resistance in enteritis organisms as a result of uncritical use of antibiotics in the feed of pigs and chickens (Swann Report, 1969), leading to a resistance factor that could be transmitted between cells (Watanabe, 1963).

Agents given at the time of resection have been interpreted as preventing reimplantation of free-floating but viable tumour cells (Boyd & Burnand, 1974), but courses of instillations may act by other mechanisms. As the effect of single instillations is not to alter episodes of recurrence, but on time per recurrent lesion, it may be that the dose-rates used in this way are too low, and have a potential for the induction of resistance.

It can be difficult to tell whether resistance is inherent or acquired. Cells present after a drug exposure may be original tumour cells that were already resistant before treatment, originally sensitive cells that have been modified to become

resistant (predicating a sublethal dose-rate in the exposure), or parvenus, not present at the time of the exposure (particularly contaminants in laboratory studies). For example, Koontz' group (1981) commented on the favourable outcome of patients with multiple tumours which responded completely to a therapeutic course of thioTEPA, without further prophylaxis, confirmed later (Prout et al., 1983). This might be seen as indicating selection between clones or tumours with inherently different drug sensitivities as England's group suggested (1981). Such post hoc evidence could still be interpreted as induction of a resistance by mutation in one group (cf Goldie & Coldman, 1979), or as variations in effective dose-rate resulting from therapy, tumour mass, or from the degree of modulation of detoxification of the drug. As with antibiotic resistance in bacteria, inherent and acquired resistance are not mutually exclusive.

The characteristics of cells established as continuous lines in culture have usually been described in terms of the morphology of the cells and colonies growing on plastic; of the ability to grow as xenotransplants in immune-deficient animals; of the histology of the xenotransplants; of the pattern of isoenzymes produced; of the karyotype; or of cellular growth rates. A relationship between drug sensitivity and changes in these characteristics has not been demonstrated, with the exception of small abnormalities of karyotype, but, if altered in resistant sublines in vitro, might be useful as a clinical marker of resistance.

In response to the suggestion that antibiotic prophylaxis might promote the development of resistant bacteria, it was said that "Dead bugs don't get resistant". The same may be true of cancer cells, unless the dose-rate is inadequate to achieve cure. In the Ridgeway osteosarcoma model, Skipper and his colleagues (1986 & 1987) noted that even when treatment produced a complete response, there was a possibility of improvement in the overall cure rate by increasing the dose rate above the least that produced 100% complete remission. The implication was that, in complete response, undetectable tumour could persist, to multiply and recur later. More effective treatment is needed. Complete response is not enough.

INTRAVESICAL CHEMOTHERAPY: Optimum pH

The most effective pH for intravesical chemotherapy is not known. The pH of human urine varies between 4.5 and 9.0 and is generally slightly acidic in carnivores. At pH less than 6.0, thioTEPA decomposes, while still retaining its alkylating activity (Cohen et al., 1984), but commercial thioTEPA is supplied buffered with bicarbonate, so that when it is dissolved in water at a concentration of 1mg/ml, it has a pH of 8.2 (Groos, Walker & Masters, 1986) to keep it chemically stable. Other agents, such as adriamycin, are more effective at alkaline pH (Groos et al., 1986).

There is evidence in rodent tumours in vivo and in vitro, that thioTEPA is more effective at low pH (Phillips, Bibby & Double, 1988), and pilot studies indicate that the same is true in one human urothelial cancer cell line (Groos, et al, 1986). If this effect is general, not an idiosyncrasy of the human cell line tested, pH may be an important tool with which to maximise the desirable effects of intravesical treatments.

INTRAVESICAL CHEMOTHERAPY: Solvent Enhancement

There is a range of possible mechanisms by which the tumour may be protected from the effect of cytotoxic drugs (Sikic, 1986). Reduction in drug efflux is thought to enhance the effect of thioTEPA in the presence of verapamil (Simpson et al., 1984). The effect of thioTEPA has also been enhanced in vitro by the addition of a non-polar solvent, Tween 80, which was thought to act by increasing drug uptake into the tumour (Parris, Masters, Walker et al., 1987). Clinical studies of combined use have found variable thioTEPA absorption and toxicity (Masters, Mc Dermott, Jenkins et al., 1990). Little information is available on other potential adjuvants to thioTEPA. Dimethyl sulphoxide is a non-polar solvent which enhances the effect of anticancer agents in vitro and in vivo (Warren, Sacksteader, Jarosz et al., 1975; Tofilon, Vines & Milas, 1985), and has been used as an intravesical treatment for interstitial cystitis in humans (Stewart, Branson, Hewitt et al., 1972). Its effect on intravesical

chemotherapy is not known, but positive findings in vitro could readily be assessed in vivo, without further toxicity studies.

AIMS OF THE PRESENT STUDY

A tissue culture model of a course of ten exposures to thioTEPA, based on weekly instillations between three-monthly surveillance, was developed in three human urothelial cell lines. Sublines exposed to thioTEPA, control sublines similarly grown, but in the absence of thioTEPA, and stored parental sublines were tested in a paired fashion for sensitivity, using a single concentration of thioTEPA, to see whether drug resistance had developed. Characteristics of resistant cells were compared with parental sublines, to exclude the possibility that their resistance could result from contamination withother lines kept in the laboratory, and for potential markers of thioTEPA resistance.

The influence of pH on the effect of thioTEPA was tested in three human urothelial cell lines, to see whether the results previously obtained in one of the lines were an idiosyncrasy of that line, or pH might have a general influence on cytotoxic drug exposures in human urothelial cell lines. As a possible adjunct to treatment at optimal pH with thioTEPA, the sodium/hydrogen ion exchange blocker amiloride was tested in vitro.

The solvent, dimethyl sulphoxide, was added to solutions of four

commonly-used intravesical chemotherapeutic agents in vitro, to see whether it might enhance cytotoxicity.

CHAPTER 2

A MODEL COURSE OF INTRAVESICAL CHEMOTHERAPY:

INDUCTION OF ThioTEPA RESISTANCE

INTRODUCTION

Resistance may develop by selective survival of a pre-existing resistant subclone; by new mutation; or by induction of activity in existing enzymes. As the dose rate in clinical chemotherapy is usually limited by side-effects, the development of small degrees of resistance is likely, and might make all the difference between useful treatment and inefffective toxicity.

Chemotherapeutic agents may induce mutations, either simple point mutations, or more complex translocations or compound changes, the consequences of which will depend on the gene or genes affected. The mutations cannot be predicted, and, as there are many loci that may be afected, such a change is likely to be absent in one flask and present in another, similarly treated. Survival advantage from such mutations is uncommon, but may occur. If the drug resistance developed as a result of induction of existing cellular mechanisms, the effect in each treated flask would be comparable, and should be assessed as replicates of the same experiment.

Comparison of the effect of exposure to 40mcg/ml ThioTEPA for 1 hour.
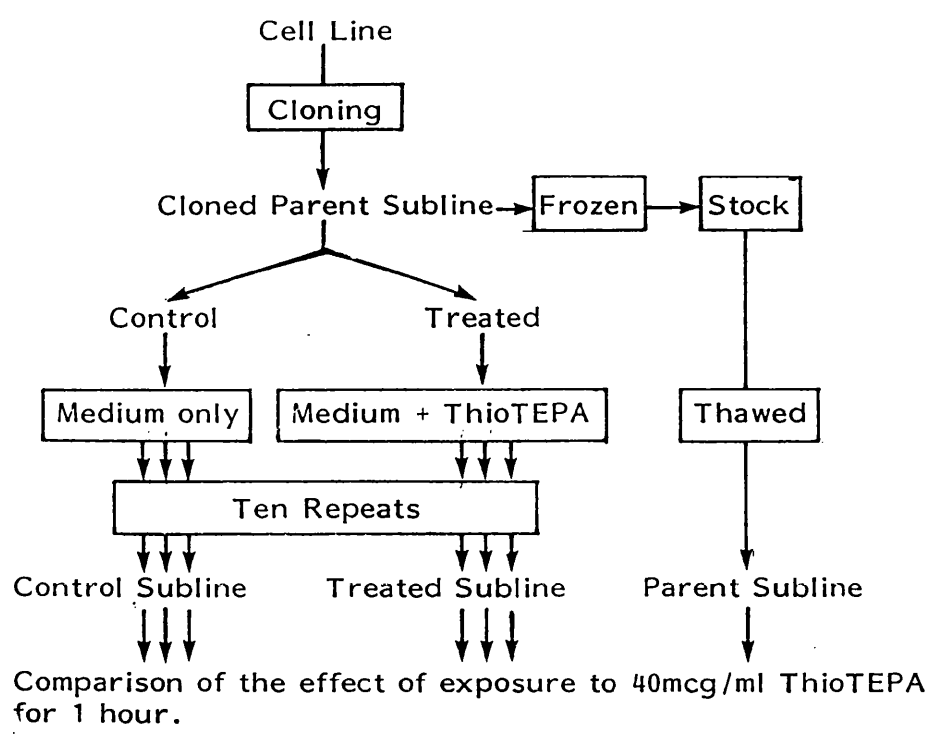
FIGURE 2

FLOW CHART OF MODEL COURSE OF INTRAVESICAL CHEMOTHERAPY

MATERIALS AND METHODS

Cloned sublines of three urothelial cell lines were exposed ten times in strict parallel, to a fixed concentration of thioTEPA. If, at any point, all cells from one flask, eg C_A, on that exposure became contaminated with fungus, or died out for any other reason, all other flasks at that exposure were also discarded, and the stocks from the previous exposure used, if all flasks (C_{A-c} and T_{A-c}) were available. If not, stocks from earlier exposures still were used, to keep all flasks of each cell subline at a parallel stage in the model course of chemotherapy.

Following these exposures, the sensitivity of the thioTEPA-treated cells was compared with that of parallel thioTEPA-free controls, and with that of stored parent cells. A flow chart of the experiment is given in figure 2 (fp54), and the details of each section are illustrated in figures 3-5 (fpp55-57).

MATERIALS AND METHODS: ThioTEPA

The thioTEPA was supplied packed in dry ice as pure powder by Cyanamid, Pearl River, USA, and diluted in sterile water for injections, BP, at a concentration of 100 mg/ml for storage in glass universal containers at -20^0C. Stock solutions of 2 mg/ml were then aliquotted into 6 ml glass bijou bottles for experimental use. The concentration chosen had resulted in less than 20%

survival in a one-hour exposure in previous dose-response experi-
ments with commercial thioTEPA. This figure was arbitrary, but
attempted to balance the rapid induction of resistance with the
most rapid recovery of stocks between exposures.

MATERIALS AND METHODS : Maintenance of Cell Lines

Cells were incubated at 36.5°C in 25cm² vented polystyrene flasks
(Nunc, Gibco, Paisley, UK) in the laboratory's standard medium,
Roswell Park Memorial Institution Medium No. 1640 (RPMI 1640, or
medium, supplied by Sigma, Poole, UK) supplemented with 1% L-
glutamine (Gibco, Paisley, UK) and 5% Fetal Calf Serum (FCS,
supplied by Flow, Irvine, UK), in an atmosphere of humidified air
containing 5% CO_2. Cells were subcultured after detatchment with
0.05% trypsin (Difco, London, UK) and 0.016% versene (disodium
Edetate, BDH Chemicals, Poole, UK) at a density between 1:10 and
1:50. For freezer storage, cells were separated by centrifugation
at 1000 rpm for 5 minutes, then resuspended in medium containing
20% FCS and 10% dimethyl sulphoxide (DMSO, BDH Chemicals) and
frozen at 1°C/min before storage in liquid nitrogen. Thawed cells
were seeded in Medium containing 20% FCS for 24 hours, to reduce
the osmotic trauma.

MATERIALS AND METHODS: MGH-U1

MGH-U1, also known as EJ, and T24 (Masters, Bedford, Kearney et
al., 1988), was derived from a poorly-differentiated human

bladder cancer (Bubenik, Baresova, Vickliky et al., 1973). It shows a number of karyotypic markers of abnormality (Vilien, Christensen, Wolf et al., 1983). Its isoenzyme profile is consistent with T24 and EJ (Masters, Hepburn, Walker et al., 1986). It readily forms tumours in nude mice, the histology of which is comparable to that of the original tumour (Vilien et al., 1983; Masters et al., 1986). On plastic, it forms irregular but compact colonies.

MATERIALS AND METHODS: RT112

RT112 is a continuous cell line, established in the Institute of Urology, in 1973, from a moderately-differentated human urothelial carcinoma. On xenotransplantation, the cells produced Grade 2 tumours histologically similar to the original biopsy. The isoenzyme profile is known (Masters et al., 1986). On plastic, it forms rounded, compact colonies.

MATERIALS AND METHODS: HU609

HU609 is a continuous cell line derived from histologically normal urothelium. It has a modal chromosome number of 45-46, and has not been found tumourigenic in nude mice (Vilien et al., 1983). Its isoenzyme profile is known (Masters et al., 1986) On plastic, it forms diffuse colonies until confluence, when there can be mutilayered growth, forming nodules.

Uncloned Cell Line

↓

Plated 20CFU/dish

↓

Single cells identified &
ringed with polystyrene tube

↓

Returned to incubator to form a colony

↓

Ring only trypsinised

↓

? ring leaking ——yes—→ discard

↓ No

↓

Subcultured into flask

↓

Freezer

↓

Thawed to form parent cell line

Control A   Control B   Control C for   Treated A   Treated B   Treated C
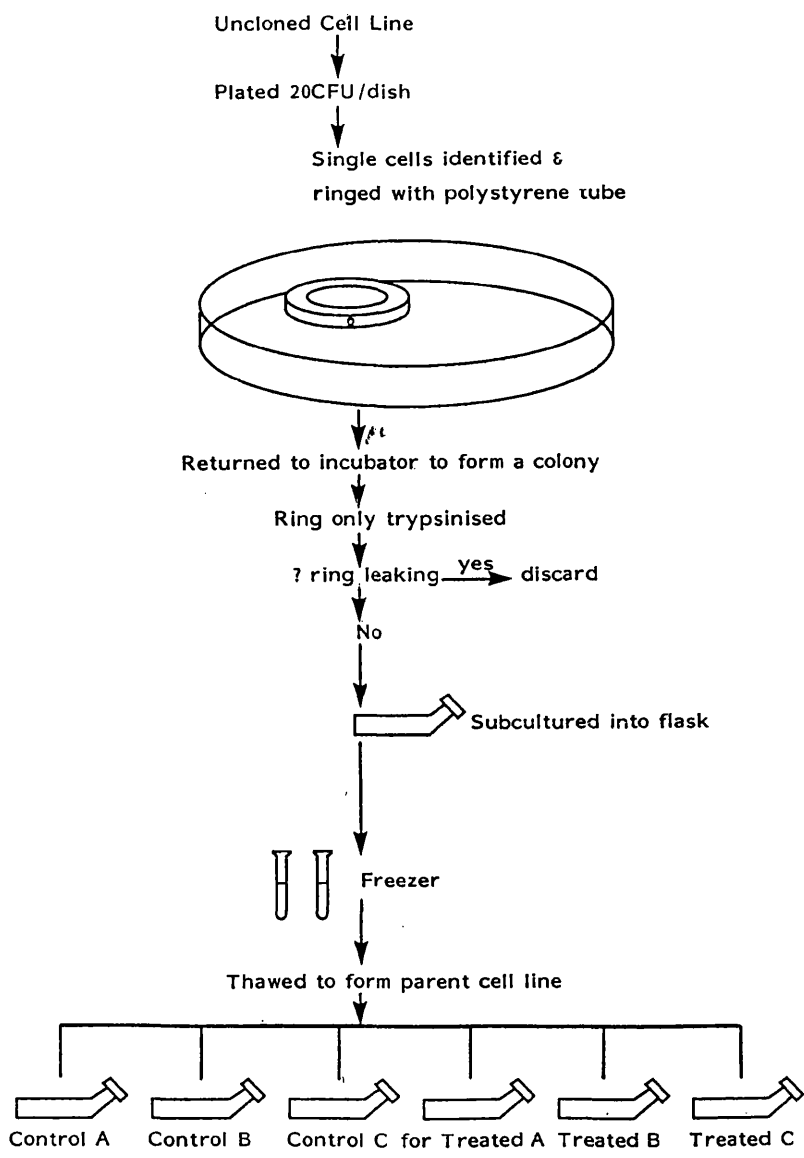
FIGURE 3

DETAILED FLOW CHART OF CLONING

MATERIALS AND METHODS : Cloning and Storage

At the beginning of the experiment, cells were cloned, by development of sublines from single cells, to reduce the possibility that the experiment would select out pre-existing drug-resistant strains. In view of the length of the model course of treatment, this was likely to be a gesture. The method was based on one described in Freshney (1987), and illustrated in figure 3, fp57. Cell suspensions, trypsinised as before, and passed through a 23G needle three times, were diluted and plated in 6 cm plastic petri dishes at a concentration equivalent to 20 viable cells per dish, and allowed to adhere overnight. Single cells were identified microscopically, and their position marked under the dish with ink. The medium was then aspirated, and the cell surrounded by a sterile ring made of a 4mm section of a 1cm diameter polystyrene tube, sealed with silicone microscope grease (Edwards, USA). Rings were placed so as to include only one cell, and only one ring was placed per dish. Medium was then instilled into the ring, and the seal checked for leakage before returning medium to the rest of the dish. Dishes were then returned to the incubator in humidified air containing 5% CO_2 at 36°C and allowed to form colonies for about one week.

The Medium was then aspirated separately from the ring and the surrounding area, and the contents of the ring trypsinised as before, checking for leakage of the trypsin/EDTA mixture. Dishes were discarded if there was any sign of leakage. The cell

Parent Cell Subline

Thawing
Growth
Subculture

Reserve

CONTROL   TREATMENT

A   B   C   A   B   C

First Exposure(E$_1$)   Medium only   Medium containing ThioTEPA
Growth/Recovery
Subculture

Second Exposure(E$_2$)   Medium only   Medium containing ThioTEPA
Growth/Recovery

Reserve Against
Infection

Subculture

Third Exposure(E$_3$)   Medium only   Medium containing ThioTEPA
Growth/Recovery

Reserve Against
Infection
Subculture

Legend:
    At each stage 2 flasks out of 3 were kept as reserve
    & 1 used for parallel subculture.

FIGURE 4

DETAILED FLOW CHART OF DRUG EXPOSURES

suspension formed was inoculated in a 25cm² plastic flasks with a vented cap, and returned to the incubator for growth. Subcultures of the cloned cells that grew were stored in liquid nitrogen in 1 ml sealed plastic cryotubes (Nunc, Gibco, Paisley, UK), as above. One cloned subline was successfully derived from each cell line.

MATERIALS AND METHODS: Induction of Resistance

Cloned sublines of the three cell lines used, HU609, MGH-U1, and RT112, were each seeded in six 25cm² plastic culture flasks (Nunc, Gibco, Paisley, UK), and divided at random into three control flasks (C_A-c), and three for thioTEPA exposure (T_A-c) for each cell line.

Treatment flasks were exposed to Medium containing 40 mcg/ml thioTEPA, and the control flasks, to Medium alone, for one hour. The Medium and Trypsin/EDTA used was always from the same bottle for each cell line at each exposure, so Control and Treated flasks were as comparable as possible. At 1 hour, the solutions were aspirated and the cells washed with 5ml Medium three times before returning them to the incubator to grow until they approached confluence.

To compensate for the different cell losses between the treated and control sublines at each exposure, treatment flasks were passaged at a ratio of 1:5, and control flasks, of 1:50. There were three C_A, three C_B, and three C_C flasks at each exposure,
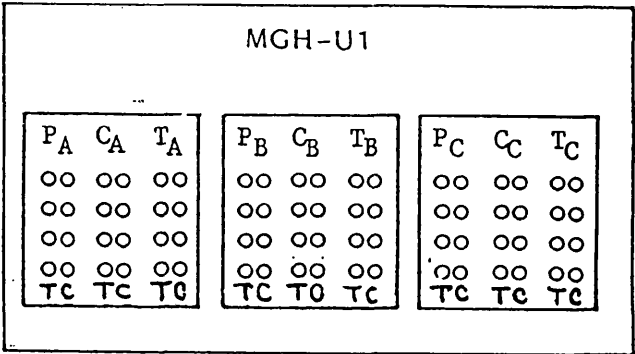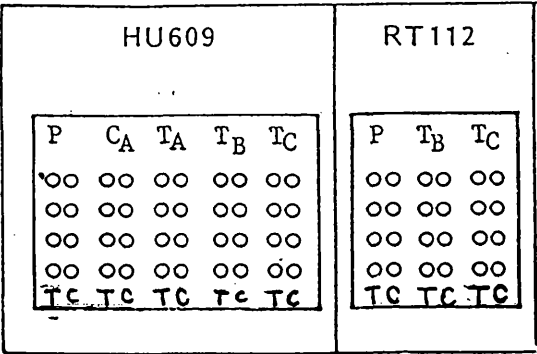
Key: P = Parent

C_A etc = Subline cultured in parallel without ThioTEPA

T_A etc = Subline cultured in parallel with ThioTEPA

T = Plated cells tested for drug sensitivity by 1hr ThioTEPA exposure

C = Plated cells used as controls without 1hr ThioTEPA exposure

Legend:

Each tray used the same bottle of control medium & the same bottle of Medium with ThioTEPA on a given day.
(i.e. 3 Control bottles & 3 ThioTEPA bottles for MGHU1.)

FIGURE 5

PAIRING OF FLASKS IN SENSITIVITY TESTING

and likewise for T_{A-C}, to enable two flasks to be kept in reserve against contamination of later exposures. At subculture, one C_A flask was seeded into three fresh C_A flasks, and so on for the other lettered Control or Treated flasks, using the same materials for all at a given stage. Ten exposures were carried out, as illustrated in figure 4 (fp58).

After the parallel induction of resistance, the cells were tested for resistance by clonogenic assay. Survival testing was carried out in parallel on parent, control and treated cells, allowing the use of a paired t-Test for comparison of most of the data. The different methods used to keep the subline survivals paired on each replicate are described in figure 5 (fp59).

MATERIALS AND METHODS : Clonogenic Assay

Clonogenic assay measures the effectiveness of a drug as the reduction in the proportion of cells retaining the ability to divide and form colonies, the mean percentage colony-forming albility or mean percentage clonogenic cell survival. As this figure can exceed 100% in some cases, and the denominator is the number of colonies formed in separate control dishes, it is not strictly a percentage but rather a proportion. Nevertheless, conventional nomenclature has been retained.

The stock cells were harvested from their flasks using Trypsin/Versine mixture (Appendix 4). A sample was then stained

with 0.1% Trypan Blue, counted, using a haemocytometer, and diluted to provide 100-200 surviving colony-forming units per dish when 200 microlitres of suspension were added, taking into account the plating efficiency of each cell line (Freshney, 1987). Cells were plated in Medium in 60mm vented plastic Petri dishes. Dishes were then incubated at 36.5°C in a humidified atmosphere of air containing 5% CO_2, to attach and to recover from trypsinization (Hepburn & Masters, 1983).

For dose-response experiments, stock thioTEPA was added to Medium to give a range of concentrations from 1 mg/ml to 80 mg/ml. The Medium used in incubation was then aspirated, and replaced with Medium containing thioTEPA or plain Medium. Dishes were incubated for 1 hour at 36.5°C in humidified air containing 5% CO_2, then aspirated of the solution used in the exposure. The cells were washed three times with Medium, and returned, in fresh Medium, to incubate in the same conditions until countable colonies had grown. The mean number of colonies (i.e. of cells surviving exposure with clonogenic powers intact) at each thioTEPA concentration was expressed as a percentage of the mean number of colonies growing in control dishes without thioTEPA. No result was recorded for a given concentration unless two or more dishes were available for that replicate of the experiment, and at least two, and usually three replicates were carried out for each value.

There were slight differences between cell lines in the

comparisons made to test for drug resistance, as it became obvious that the initial method used for MGH-U1 was very time-consuming, and that the numbers of flasks of RT112 that survived the final exposure were small. The same solutions were used to test all sublines on a single tray (Figure 5, fp59).

Initially, for MGH-U1, three separate trays were used, one for each lettered flask,A-C. As a result, three batches of Parent cells from the same flask were tested on separate trays on each day, which was avoided in other cell lines. Each tray had eight dishes of Parent cells, eight of Control cells, and eight of Treated cells. Arbitrarily, T_A was compared with C_A, and the Parental cells tested on that tray were designated P_A, and the C_B and C_C trays similarly. After aspiration of the plating Medium, half of the dishes for each subline were refilled with Medium containing 40 mcg/ml thioTEPA, and half, with Medium alone, for a one-hour exposure. After exposure, the Medium was aspirated, and the dishes washed with 3 ml Medium three times, before refilling with 5ml Medium and return to the incubator for 10-12 days to allow countable colonies to form.

The colonies were fixed in 70% methanol (BDH Chemicals, Poole) for 5 minutes, then stained with 10% Giemsa (BDH), and counted using a digital pen-marker/counter (Scientifica & Cook Electronics, UK) on a Nachet dissecting microscope at x2 magnification. The mean number of colonies in dishes of one cell type after exposure to thioTEPA was expressed as a percentage of the mean

number of colonies in dishes of that cell type not exposed to thioTEPA. The results were compared between cell types tested on the same tray, with the same chemicals.

For the comparison of sensitivity in HU609, each tray held cells of the Parental, one Control, and three Treated sublines, which were dealt with in the same way as a tray in the MGH-U1 comparison. For RT112, there were no Control sublines available, and only two Treated, but they were all tested in a paired way.

MATERIALS AND METHODS: Statistical Analysis

Mean % clonogenic cell survival otherwise known as mean % colony-forming ability was the outcome measure. The estimates were made on pre-treatment cells (P_{A-c}), control cells (C_{A-c}) and treated cells (T_{A-c}) from each cell line. Paired statistical analysis was applied only to data derived from cells treated on the same tray

Assuming that any one of the lettered flasks might have undergone a mutation that was not present in the others, each lettered flask could be compared with the Parent or with the Control subline that was exposed to the same Medium and to the same dilution of thioTEPA in Medium. If the change in sensitivity occurred as a result of a graduated phenomenon such as a rate change in an existing process, all the lettered flasks would be expected to show similar changes in sensitivity.

If the individual lettered flasks of Treated or Control cells, kept consistently apart throughout the model course of chemo-therapy, are thought sufficiently distinct to be considered different sample, but not to be a separate attempt at the induction of resistance, because they derived from the same original source, and were exposed to the same solutions, then the value of n would be tripled for a paired t-Test. As this was likely to be a contentious assumption, the following, less debatable interpretation was adopted. If all the lettered flasks represented was more of the same cells, then the survivals on each day should be averaged, to give a single Control and a single Treated value, with their respective confidence intervals. This assumption leads to the paired comparison of each of the two types of control with T_{mean}.

Cells exposed to Medium from another bottle, or on a different tray, or to thioTEPA from another dilution would not give paired data. An unpaired t-Test would be less powerful, and would need more replicates with little day-to-day variation to detect a given resistance, than would a paired experiment. Although an unpaired assay was not proposed as a first step, for reasons of economy, later assessment suggested that the day-to-day variation precluded this potentially more convincing approach.

MATERIALS AND METHODS: Dose-Response

The response of uncloned RT112, HU609, and MGH-U1, and of cloned

Replicate No.	Mean Percentage Survival					
	P	C	T_A	T_B	T_C	T_{mean}
8/9	---	7.02	25.3	24.7	26.3	25.4
10/9	19.0	---	37.2	46.8	38.9	41.0
2/11	56.8	25.5	64.0	71.9	98.2	78.0
3/11	66.1	46.9	40.6	80.6	75.8	65.7
14/11	81.8	55.8	57.0	79.3	60.9	65.7
p-value vs P	----	0.018	0.616	0.114	0.405	0.520
p-value vs C	0.018	----	0.285	0.017	0.119	0.078

p-value vs P = probability that difference from Parent cells occurred by chance, using t-Test

p-value vs C = probability that difference from Control cells occurred by chance, using t-Test

P = Parent subline

C = Control subline

T_A = Treated subline, flask "A"

T_B = Treated subline, flask "B"

T_C = Treated subline, flask "C"

T_{mean} = Treated sublines, mean of all flasks on that replication

TABLE 9

THE MEAN % CLONOGENIC CELL SURVIVAL OF PARENT (P), CONTROL (C), AND TREATED (T_{A-C}) CLONED SUBLINES OF HU609, AFTER A 1-HOUR EXPOSURE TO 40 mcg/ml OF ThioTEPA

HU609 to a range of concentrations of thioTEPA was tested in a 1-hour clonogenic assay, using commercial thioTEPA (Lederle,UK), freshly diluted each day in sterile water for injections, BP (Beechams, UK). At least two, and usually three replicates were carried out.

RESULTS

RESULTS: HU609

Table 9 gives the mean % clonogenic cell survivals of parent (P), Control (C) and Treated (T) sublines of HU609 after a one-hour exposure to 40 mcg/ml thioTEPA. One lettered flask of Control cells was tested, but data were obtained for Treated flasks A-C. T_{mean} is the average of flasks T_{A-C} on the day given. The mean % survival of uncloned HU609 cells exposed to 40 mcg/ml thioTEPA for 1-hour in a dose-response experiment was 45%, with 95% confidence interval (13,77%) (figure 6, page 66). The mean % survival of cloned HU609 cells at 40 mcg/ml thioTEPA was 11(0,43)% (figure 6, page 67).

RESULTS: HU609 Parent vs. Control Flasks

These might be predicted to have comparable results. In fact, in the three paired experiments (table 9, fp 64), the parent (P) cells had a mean clonogenic survival of 68(37,100)%, whereas the C cells had 43(34,52)%. The control flask was more sensitive to

thioTEPA, with p=0.018, n=3.

RESULTS: HU609 Parent vs. Treated Flasks

There were four experiments comparing Parent and Treated cells. There is no significant difference in survival between P, 56(13,98)%, and T_A, 50(29,70)%, T_B, 70(54,85)%, and T_C, 68(29,108)% individually, or T_{mean}, 63(38,87)%.

RESULTS: HU609 Control vs. Treated Flasks

There were four experiments which produced data for both Control and Treated cells. Replicates in which Control and T_B cells were compared gave a mean for Control cells of 34(0.79)%, and for T_B, 64(23,106)%, with p=0.017. Comparison of C with T_{mean} gave T_{mean} survival of 59(22,94)%, with p=0.08. Comparison with T_A gave p=0.29, and with T_C, p=0.12.

ThioTEPA Concentration (mcg/ml)
(1-Hour Exposure, Error Bars Represent 95% Confidence Interval).
Arrows indicate where only two replicates done.
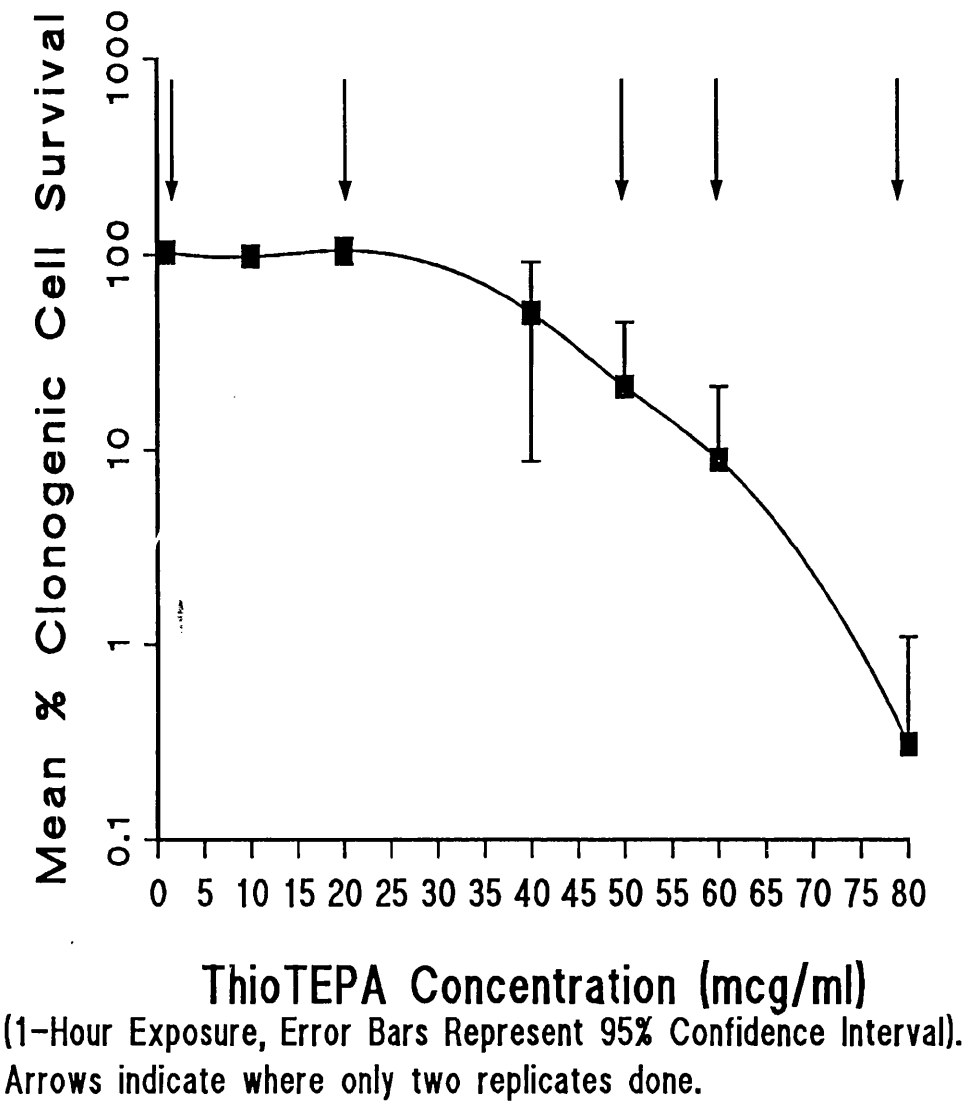
FIGURE 6

DOSE-RESPONSE OF UNCLONED HU609 TO ThioTEPA
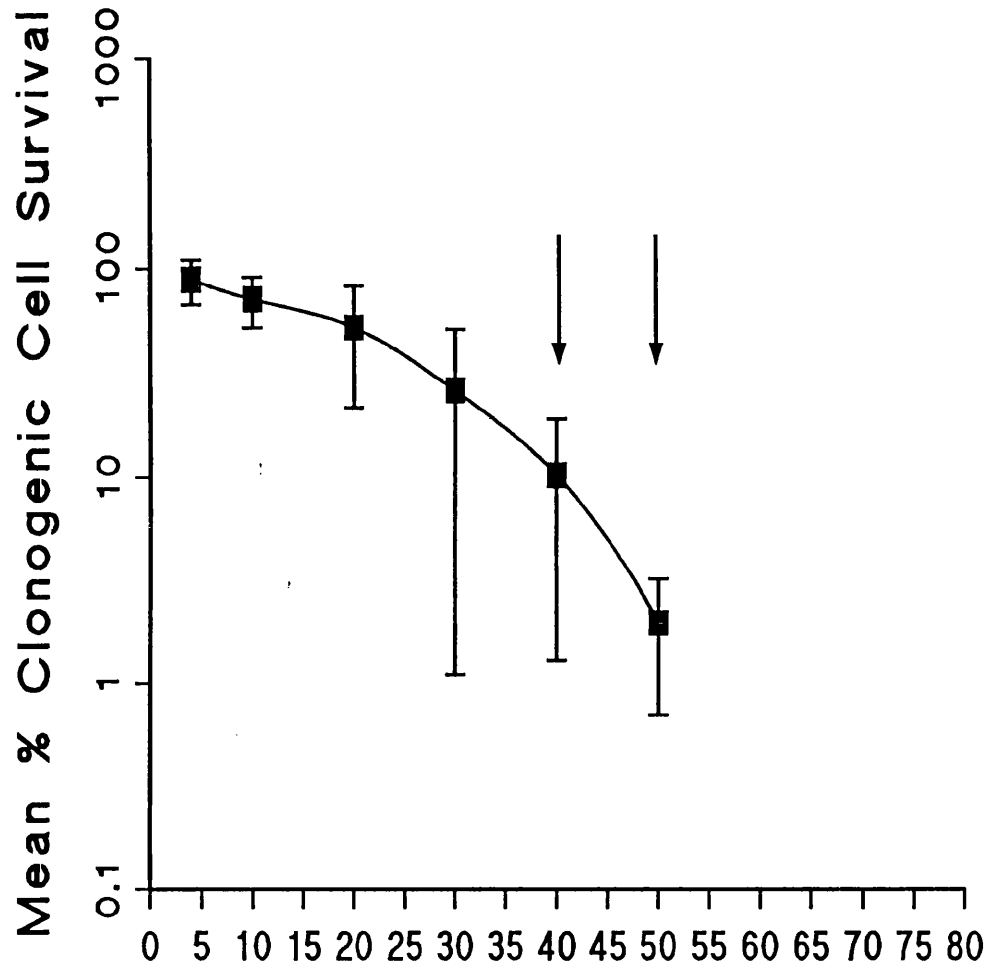
ThioTEPA Concentration (mcg/ml)
(1-Hour Exposure, Error Bars Represent 95% Confidence Interval).
Arrows indicate where only two replicates done.

FIGURE 7

DOSE-RESPONSE OF CLONED HU609 TO ThioTEPA

RESULTS: RT112

The mean % clonogenic cell survival of RT112 parent and treated cells is shown in table 10, fp 69. Because of fungal infection during the growth of the last exposure, no control cells survived to be assessed, as well as only two flasks of treated cells. The survivals of parent (P), T_B and T_C sublines were assayed using the same solution of ThioTEPA for each, on each day. The dose-response curve of uncloned RT112 exposed for 1 hour to commercial thioTEPA is shown in figure 8 (page 69). At 40 mcg/ml, the mean % survival of uncloned RT112 cells was 4.3%, with a 95% confidence interval, (0.5,8.0)%.

Application of a paired t-Test with n=3 to the mean % survival of P, 28(1,55)%, and of T_B, 65(51,77)%, T_C, 70(24,116)%, and T_{mean}, 67(44,91)%, all reach significance. The p values are 0.04, 0.02, and <0.001 respectively.

RN	P	T_B	T_C	T_{mean}
17/11	40.5	61.7	95.5	78.6
15/11	13.3	60.2	49.4	54.8
16/11	29.6	71.7	65.6	68.7

RN = replicate number

P = Parent subline

T_B = Treated subline, flask "B"

T_C = Treated subline, flask "C"

T_{mean} = Treated sublines, mean of both flasks on that replication

TABLE 9 10

MEAN % CLONOGENIC CELL SURVIVAL OF PARENT (P) AND TREATED (T_{B-C})

SUBLINES OF RT112 AFTER A 1-HOUR EXPOSURE TO 40 mcg/ml ThioTEPA
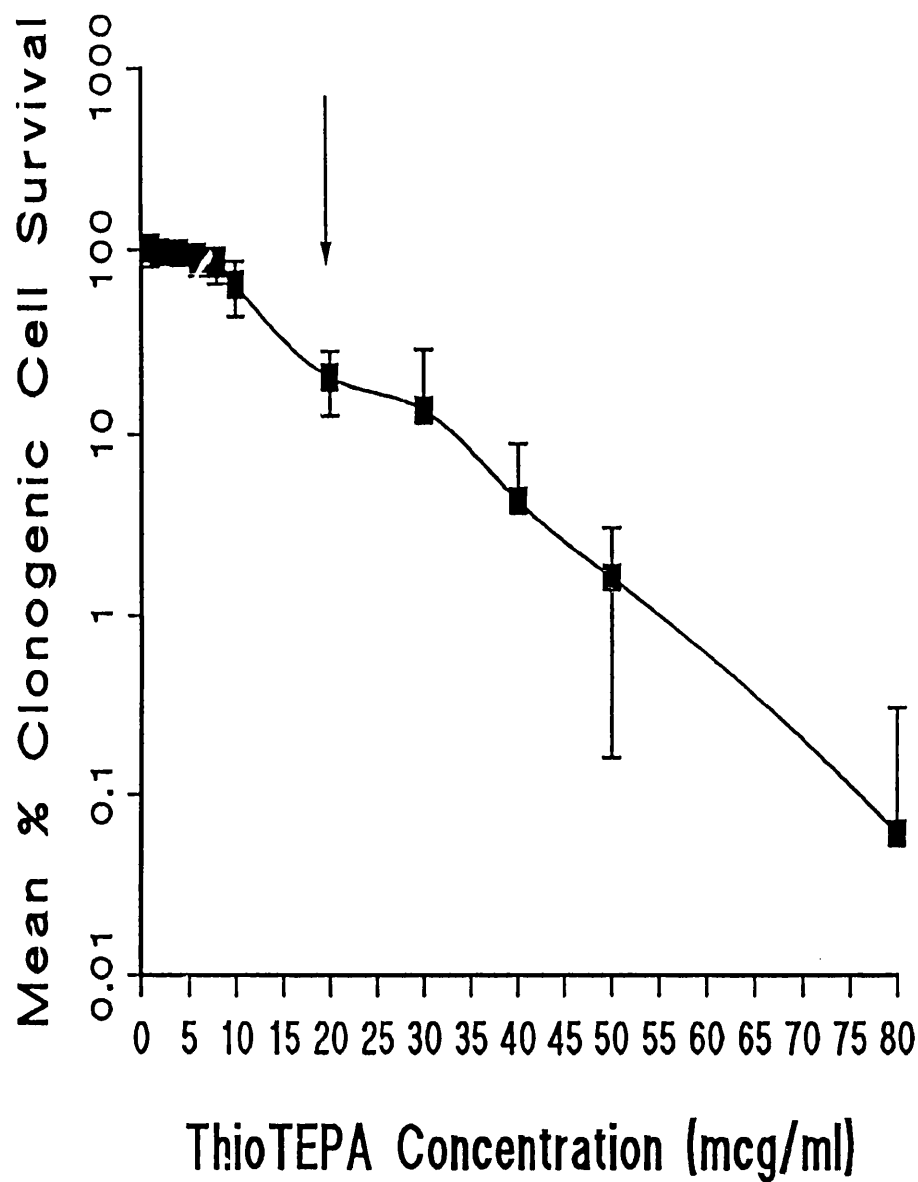
ThioTEPA Concentration (mcg/ml)

(1-Hour Exposure, Error Bars Represent 95% Confidence Interval)
Arrow indicates where only two replicates done.

FIGURE 8

DOSE-RESPONSE OF UNCLONED RT112 TO ThioTEPA

RN	P_A	C_A	T_A	P_B	C_B	T_B	P_C	C_C	T_C
15/9	19.8	20.6	31.9	22.1	16.7	35.0	22.7	15.1	30.5
6/10	32.0	24.0	55.4	83.0	----	----	44.9	49.0	64.8
12/10	16.9	16.9	40.2	23.4	40.7	65.5	19.1	30.0	45.4
13/10	0.42	0.65	7.2	2.4	3.3	8.7	6.5	6.2	18.8
14/10	10.4	6.7	36.5	8.2	10.4	25.4	2.4	2.2	11.0

RN = replicate number

P_A = Parent subline, tested with "A" flasks of Control and Treated sublines

$P_{B,C}$ = As P_A, but for "B" and "C" flasks, respectively

NB As C_B and T_B data were not obtained for Replication Number 6/10, no paired comparison can be made, and the Parent data were not included in the mean of the Parent data for the "B" flasks.

TABLE 11

THE MEAN % CLONOGENIC CELL SURVIVAL OF SUBLINES OF MGH-U1 CELLS

EXPOSED TO 40 mcg/ml ThioTEPA FOR 1 HOUR

RESULTS: MGH-U1

The pairing of data in the assays of MGH-U1 parent (P_{A-C}) control (C_{A-C}) and treated (T_{A-C}) was made closer by the testing of separate aliquots of P cells on each tray of C_A and T_A, C_B and T_B, C_C and T_C, and by the use of one bottle of each solution for each tray. Thus different sublines were treated similarly, in equal numbers. The decision to compare C_A with T_A rather than other Treated flasks was arbitrary, but there was more day-to-day variation than between T_A, T_B, and T_C. The mean % clonogenic cell survivals of each group are given in table 11, fp70. A dose-response curve for uncloned MGH-U1 cells exposed for 1 hour to commercial thioTEPA is given in figure 9, page 71. At a concentration of 40 mcg/ml, the survival was 33.8(0,76)%.

The paired data were compared by a t-Test, and for the "A", "B", and "C" sublines, the means, confidence intervals, and p-statistics are given in tables 11 & 12, fp 70-71. All comparisons indicate significant differences between Treated flasks and the two types of control, except P_B vs T_B, where there were four replicates and p=0.087. The data indicate a consistent change in ThioTEPA sensitivity at this concentration of the order of 1:2.

Flask	Mean Survival (95% CI)			p-value		
	P	C	T	P vs C	P/T	C/T
"A"	15.9	13.8	34.2	0.27	0.008	0.015
	(2,40)	(2,26)	(12,56)			
"B"	14.0	17.8	33.7	0.49	0.087	0.027
*	(0,30)	(0,44)	(0,71)			
"C"	19.1	20.5	34.1	0.67	0.01	$<10^{-3}$
	(0,40)	(0,44)	(7,61)			

* indicates 4 paired replicates, otherwise n=5

CI = 95% confidence interval

P = Parent subline

C = Control subline

T = ThioTEPA-Treated subline.

p-value P vs C = comparing Parent and Control sublines

P/T = comparing Parent and Treated sublines

C/T = comparing Control and Treated sublines.

TABLE 12

SURVIVAL (95% CONFIDENCE INTERVAL) AND RESPECTIVE p-VALUES OF PARENT, CONTROL, AND TREATED PAIRED SUBLINES OF MGH-U1

ThioTEPA Concentration (mcg/ml)
(1-Hour Exposure, Error Bars Represent 95% Confidence Interval)
Arrow indicates where only two replicates done.
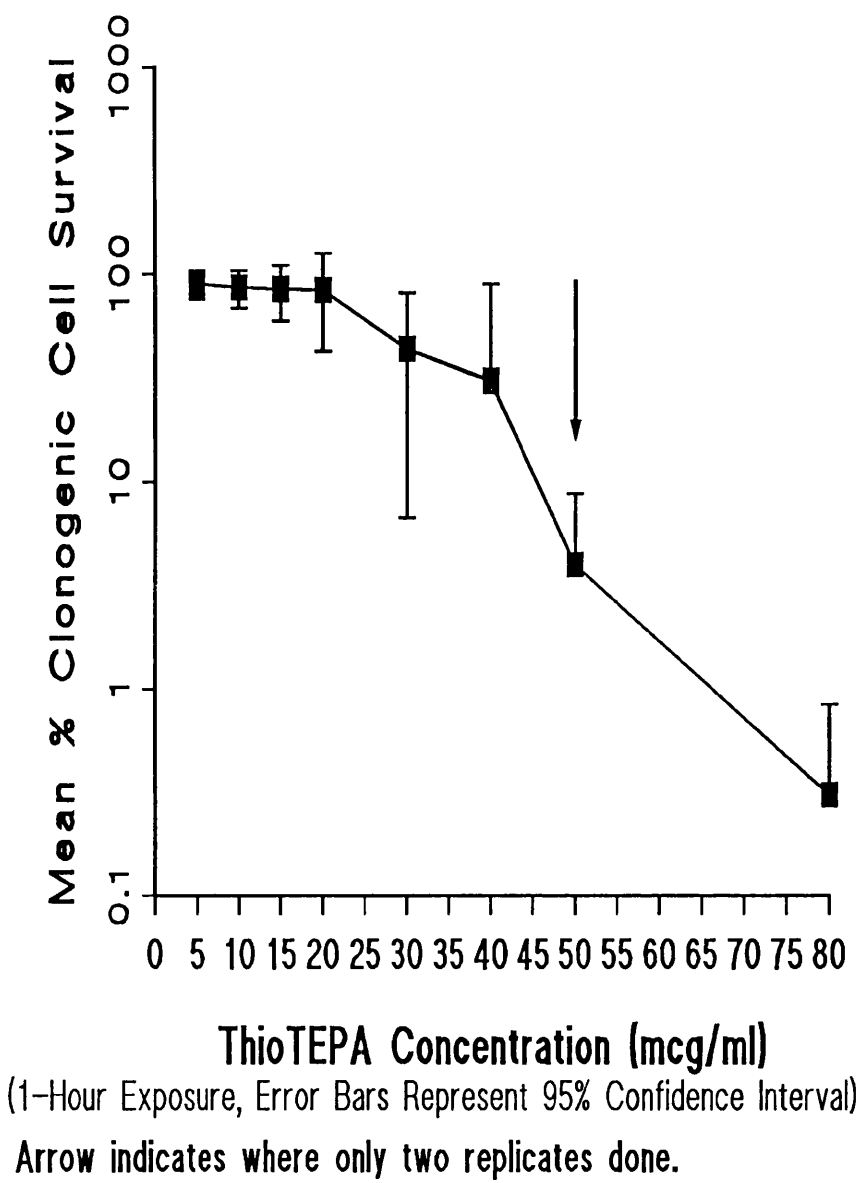
FIGURE 9

DOSE-RESPONSE OF UNCLONED MGH-U1 TO ThioTEPA

DISCUSSION

DISCUSSION: HU609

The most apparent explanation of the data is that no resistance was induced in HU609 cells by the model course of chemotherapy, but this would mean that both the T_{mean} and T_B data exhibited Type I error. The probability of the apparent resistance in T_B and T_{Mean} occurring by chance is less than 0.08, but is not nil. However, the findings in the other cell lines would then be inconsistent with those in HU609. As HU609 is derived from histologically normal urothelium, this is not inconceivable, but less likely.

The model course of chemotherapy may have induced a real but small resistance, compared with cells of the same passage, but too small for statistical significance to be demonstrated in two of the three sublines. In that case, the lack of sensitivity of the Parent subline also has to be explained. The proximity of the survivals of the T_A and T_C sublines to that of the resistant T_B does not suggest a mutational event causing resistance, as this would be more likely to induce a large change. Development of resistance would be consistent with the difference in MGH-U1, and the probable resistance in RT112. Although there appears to be a difference in survivals at 40 mcg/ml between cloned and uncloned sublines, the cloned subline was tested with pure, unbuffered thioTEPA powder, while the

uncloned cells were exposed to commercial thioTEPA, buffered with bicarbonate. The dose-responses on the uncloned cells were intended as pilots, to choose a rough concentration with which to induce resistance, rather than as touchstones of the development of resistance, for which a closely paired assay of Parent, Control and Treated cells was more accurate, and required fewer replicates.

The resistance of Parent cells compared with Control cells might result from the difference in passage. Other investigators have not found that successive subculture produced a change in sensitivity of T24 sublines (Masters & Hepburn, 1986), early and permanent cell lines derived from melanoma xenografts (Tveit, Fodstad & Pihl, 1981), and HeLa cell lines (Shoemaker, Abbott, Macdonald et al., 1983).

The Parent cells may be resistant as a result of the Dimethyl sulphoxide (DMSO) used in freezing, as the Parent subline was only two or three passages from thawing when tested. The Control cells' survival, tested without freezing after the model course of placebo treatment, is numerically closer to that of uncloned HU609 cells. DMSO was found to induce differentiation in tumour cell lines (Borenfreund, Steinglass, Korngold et al., 1975; Kim, Tsao, Siddiqui et al., 1980; Mickey, Meadows, Vassiliades et al., 1983), and to increase the effect of anticancer drugs in vivo (Warren, Sacksteader, Jarosz et al., 1975), so the decrease

observed is contrary to the previous findings. The Control results are closer to the findings in the uncloned dose-response, although not directly comparable because of the use of commercial thioTEPA in the dose-response studies.

The data may be interpreted as showing that there is no real difference as a result of the model course of chemotherapy. If so, the result for the Control C_B subline is anomalous, and should be explained. Type II error has a low estimated probability. A technical error, such as omitting the thioTEPA, was made improbable, both by the replication of the results, and by the use of a single container of thioTEPA in Medium for all cell sublines on each day.

A difference based on the condition of the Control subline when tested, such as non-logarithmic growth, was made less likely by standard and parallel maintenance of Control and Treated sublines. In all complete replicates of the experiment, the ranking of the Control subline was consistent.

The single Control flask used, chosen arbitrarily, might have become infected with mycoplasma or some other contaminant. In view of the parallel management of Control and Treated sublines, contamination from ingredients such as medium or trypsin would be likely to affect other sublines. The rank of the T_A subline is next to the Control subline, and consistently lower than the Parent or T_B or T_C sublines, and might be so affected. The

Control subline survival is comparable to that of uncloned HU609. Both comparison with the sensitivity of other Control sublines and mycoplasma staining would be useful.

The meticulous use of clean pipettes and equipment for each flask at each stage made cross-contamination of the sublines improbable, and this is further supported by the differing sensitivity results in the three cell lines. HeLa cells have not been kept or isolated in the laboratory. Microscopic morphology, isoenzyme profiles, and nude mouse xenograft tumourigenicity of all sublines of HU609, RT112, and MGH-U1 were examined and no changes were found (see Chapter 3). Growth rate estimations, which would also have been of interest, were not carried out for lack of time, but there was no marked difference, after the 1-hour exposure, between Control and Treated cells in the size of colonies (with or without thioTEPA exposure), and it seemed unlikely that this alone would explain a halving of the proportion of plated cells that go on to form colonies.

In any limited exposure assay, requiring removal of medium at the end of the exposure, it is not easy to distinguish between the aspiration of detached, but viable cells at the end of the exposure, and clonogenic death. If this applied to the present data, the model course of thioTEPA treatment would need to inhibit the detatchment of washed cells. To produce the difference seen in the assay of survival after 40 mcg/ml thioTEPA, such a hypothetical mechanical effect would have to apply more to the

Control cells than to the Treated cells, and to affect treatment
dishes in the assay more than control dishes of the same group
(Parent, Control, or Treatment), which is improbable.

A similar potential for cell loss occurs when fixing colonies,
but a differential loss, reduced by thioTEPA Treatment must again
be postulated. As dishes were inspected unfixed before fixing,
and no obvious discrepancy was noted, and as cell loss would
probably not affect whole colonies, this potential source of
error seems unlikely to be significant.

Errors due to colony counting would likewise require a difference
between Treated and Control groups. High colony densities might
lead to underestimations, particularly of the denominator of %
survival, and low densities, to overestimations, greater in the
numerator. The plating densities used were the same for P, C, and
T cells, so this is unlikely to explain the difference in the
data. There was a noticeable day-to-day variation in the mean
survivals for each subline, suggestive of a technical problem in
the experiment. This might have been a matter of the the pH or
osmolality of the RPMI 1640, the glutamine or the FCS. These were
taken from the same batch and supplier. The thioTEPA stock was
diluted in a batch, and stored at $-70^{0}C$, until use, but variation
in pipetting during dilution in Medium is a possible explanation
for the variation. The ranking of the mean % survival data does
not appear to vary, and because of the design of the experiment,
with pairing of the data, the data are not invalidated, but are

similar to a deliberate comparison of dose-response curves. In retrospect, variation as a result of thioTEPA concentration would probably have been reduced if the concentration used to test for the development in resistance had been higher, as the error in the survival is smaller , both absolutely, and as a proportion of the mean. This applies to RT112 and MGH-U1 also.

DISCUSSION: RT112

The absence of Control data limit the interpretation of the clear difference between Parent and Treated sublines. There is a difference of a factor of 2, apparent in each flask. This may represent the effect of passage, or a real consequence of treatment. The argument against variation in drug sensitivity with passage is given under HU609 Discussion. If the sensitivity of RT112 altered with passage , it would possibly resemble that seen in HU609, increasing the difference between Control and Treated sublines. The survival of the uncloned RT112 cells at a thioTEPA concentration of 40 mcg/ml was much less than either the Parent or Control RT112 sublines, and does not assist in the assessment of the data. The consistency of the difference in both available Treatment flasks does not provide evidence for a low-frequency mutation as cause of any real resistance present.

The day-to-day variation in survival was again notable, and again the design of the study compensated. These results are strongly suggestive that a graduated resistance had developed to thioTEPA

after a model course of intravesical chemotherapy in RT112 cells.

DISCUSSION: MGH-U1

The difference in sensitivity following the model course of chemotherapy was clear in this cell line. It was not present in cells of high passage that were not treated with thioTEPA. The survival of uncloned cells exposed to commercial thioTEPA is not consistent with either the Parent or the Control sublines, but is again subject to wide variation. Use of a higher concentration of thioTEPA to test sensitivity might again have reduced the potential variations in survival relating to pipetting errrors.

SUMMARY

Taking the three cell lines together, the trend is consistent towards the doubling of the survival at one thioTEPA concentration, after a series of exposures to thioTEPA modelled on a course of intravesical chemotherapy. The lack of a differential between the lines derived from histologically normal urothelium and the cancer cell lines suggests that the basic mechanisms for the development of this resistance are inherent in urothelial cells. Development of resistance was not shown to have any relation to the type of cell line.

CHAPTER 3

CHARACTERIZATION OF RESISTANT SUBLINES

The sublines surviving a model course of thioTEPA were character-
ised with two aims. Firstly, it was necessary to see whether the
resistance identified in Chapter 2 was a result of contamination
with another cell line. Secondly, markers of thioTEPA resistance
might be useful in clinical practice, to avoid fruitless treat-
ment, and might indicate a mechanism of thioTEPA resistance.

The cell lines were examined for a range of features used in the
definition and description of new cell lines. Xenograft tumouri-
genicity, isoenzyme phenotype, DNA fingerprinting, karyotype, and
colony morphology were compared. The histological appearance and
grade of the tumours was reported for comparison with the
reported appearances of the original tumour biopsy.

XENOGRAFT TUMOURIGENICITY

When MGH-U1 or RT112 cells are injected into immunocompromised
mice, almost all form tumours . With HU609, tumours do not form
(Masters et al., 1986), as with most non-malignant cells.

TUMOURIGENICITY: Cell Lines

The cell lines, previously described, were grown in 175 cm^2
flasks (Nunc, Gibco, Paisley), and trypsinised, counted in a

Neubauer counting chamber, washed three times in RPMI Medium, and resuspended in RPMI containing no FCS or glutamine, for injection.

XENOGRAFT TUMOURIGENICITY: Mice.

Male Nu/Nu BalbC mice, aged 3-12 weeks, were bred and maintained by the staff of the Biological Services Unit, The United Medical and Dental Schools, St Thomas' Campus, UK. Mice were injected subcutaneously with 1-2 x 10^6 cells suspended in 0.1 ml medium. In each subline injected was kept in separate boxes, in a Vickers Medical positive pressure isolator, in a room devoted to the keeping of nude mice. Sterilised food, autoclaved bedding and water and materials for injection were introduced into the isolator through an air-lock after disinfection with pentacetic acid. Fifteen mice were not injected, and were kept for three months as a negative control.

XENOGRAFT TUMOURIGENICITY: Tumour Harvesting

Mice were kept until three months from injection, or until the Unit Veterinarian was concerned about potential suffering, or when a tumour reached a diameter of 0.5 cm in diameter. Mice were sacrificed by etherization in air, their abdominal and peritoneal cavities examined, and all tumours excised, fixed in 10% formalin, or methacarn, paraffin embedded, sectioned, dewaxed in sequential Xylene and ethanol, and stained with Haematoxylin and

Eosin.

The slides were examined by a consultant in Urological Histo-pathology, who reported the histological appearances and Grade, for comparison with the untreated tumours, and with the published histology of the original biopsy.

XENOGRAFT TUMOURIGENICITY: Results.

All but three flank inoculations of Parent MGH-U1 cells grew tumours, and the three mice with only one flank tumour also had peritoneal tumour, as did two others (table 13, fp 82), pre-sumably from misdirected inoculations. Histology showed tran-sitional cell carcinoma Grade III in all cases.

All but four RT112 inoculations, both Parent and Treated, grew flank tumours. Two mice grew intraperitoneal tumours, with no flank tumours. Histology showed transitional cell carcinoma Grade II, except for one solid/cystic tumour with squamoid features, Grade I, in a mouse inoculated with Treated cells.

HU609 did not grow as tumour xenografts, either before or after the model course of chemotherapy.

XENOGRAFT TUMOURIGENICITY: Discussion
The tumours identified were presumed to be of human transitional cell origin, rather than mouse sarcomas, and intralesional

Cell Line	Number of Sites Injected	Number of Flank Tumours	Mice with I-P Tumours
HU609			
Parent	14	0	0
Control "C"	8	0	0
Treated "A"	8	0	0
"B"	10	0	0
"C"	8	0	0
RT112			
Parent	11	7	2
Treated "B"	8	16	0
"C"	8	16	0
MGH-U1			
Parent	16	13	5
Control "B"	6	6	0
Treated "A"	8	8	0
"B"	8	8	0
Treated "C"	6	6	0

TABLE 13

XENOGRAFT TUMOUR GROWTH OF PARENT, CONTROL, AND TREATED SUBLINES

heterogeneity was not considered here, as it had not been in previous studies (Masters et al., 1986). Control mice injected with heat-inactivated cells, or with RPMI containing no cells would have helped implicate the cells in the genesis of the tumours, and a veterinary pathologist might be asked to distinguish them from murine sarcomas, either by appearance, or by differential staining with anti-human or anti-mouse antibodies. Failing this detail, it is still possible to note that the response to the Parent, Control, and Treated sublines is the same. Resistance did not appear to affect xenograft tumourigenicity.

MORPHOLOGY

The morphology of sublines was examined by the author using a Natchet inverted microscope, without staining, and with the cells still in their flasks.

The Parent MGH-U1 cell line was composed of polygonal, stellate, and fusiform cells, in irregular colonies in which cells are mostly contiguous. The Treated subline had similar appearances.

Colonies of RT112 are defined and rounded, and composed of rounded or polygonal cells, in both Parent and Treated sublines.

HU609 cells were stellate or fusiform, and colonies were diffuse. No differences were seen between Parent and Treated sublines.

Subline	ESD	G6PD	GLO	PGM₁	PGM₃	PGP
1	1	b	2	1	ba	1
3	1	b	2	1	ba	1
5	1	b	2	1	ba	1
8	1	b	2-1	0	ba	1
9	1	b	2-1	0	ba	1
10	1	b	2-1	0	ba	1
11	1	b	1	1	a	1
12	1	b	1	1	a	1
13	1	b	1	1	a	1

TABLE 14

ISOENZYME PHENOTYPES OF PARENT, CONTROL, AND TREATED SUBLINES.

ISOENZYME PHENOTYPE

Cell lines have been distinguished by the pattern of isoenzymes they contain, as distinguished by electrophoretic mobility (Povey et al., 1976). In a case of suspected cross-contamination of 4 urothelial cell lines, the isoenzyme evidence has been confirmed by DNA fingerprinting (Masters et al., 1988). He La cells, which can be a vigorous cross-contaminant if kept in the same labora- tory, have a distinctive Type A isoenzyme of Glucose-6-phosphate dehydrogenase from that reported for MGH-U1, or RT112 (Povey et al., 1976). The sublines were examined for isoenzyme phenotypes and DNA fingerprints in the Galton Laboratory of University College London.

ISOENZYME PHENOTYPE: Materials and Methods

Cell sublines were grown in 175 cm² flasks, trypsinised, and suspended in Medium in plastic universal containers, as for xenografting. The suspensions were centrifuged at 1000 rpm for 5 minutes, the Medium aspirated, and the cells resuspended in 20 ml PBSA (Appendix 4). The washing in PBSA was repeated, with centrifugation as before. After the last wash, the cells were resuspended in 1-3 ml PBSA, depending on the size of the pellet, and aliquotted into 1ml cryotubes with a conical base (Nunc, Gibco, Paisley), and centrifuged at 1500 rpm for 10 minutes. The supernatant was then aspirated, and the cryotubes inverted on tissue to dry. The dried pellets were then stored in liquid

fp84

Lane   1   2   3   4   5   6   7   8   9.

Key: Lane 1   RT112   Parent

      Lane 2   MGH-U1   Treated C

      Lane 3   RT112   Treated C

      Lane 4   HU609   Parent

      Lane 5   MGH-U1   Control B

      Lane 6   HU609   Control C

      Lane 7   HU609   Treated C

      Lane 8   RT112   Treated B

      Lane 9   MGH-U1   Parent
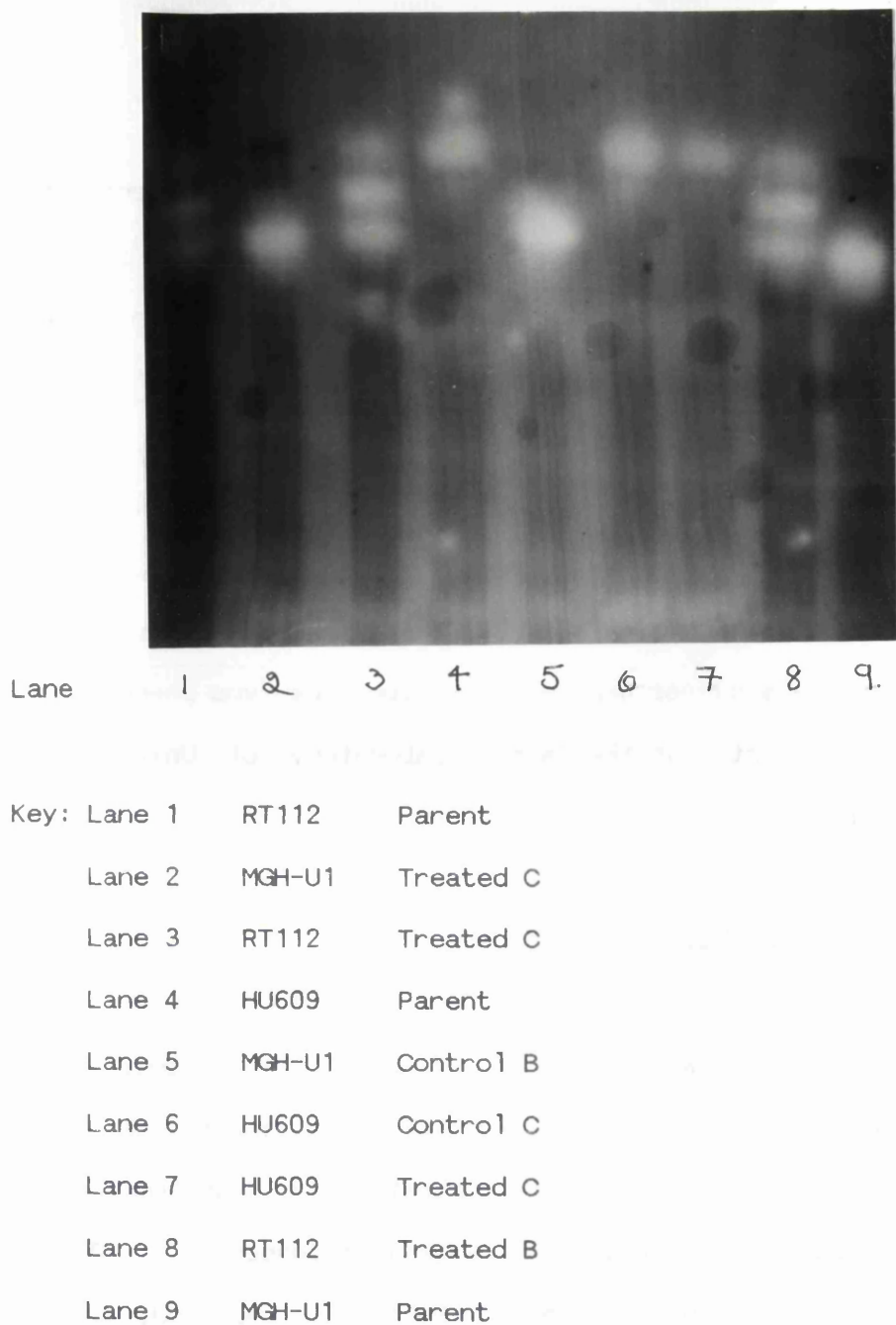
FIGURE 10

PHOTOGRAPH OF GLYOXYLASE ISOENZYME ELECTROPHORESIS GEL

FLOW CHART OF MODEL COURSE OF INTRAVESICAL CHEMOTHERAPY

nitrogen until testing for isoenzymes and DNA. The methods of isoenzyme analysis were the international standard methods, as described in Harris and Hopkins, 1976. The pellets were tested for phosphoglycolate phosphatase, glyoxylase, esterase D, and phosphoglucomutase, and glucose-6-phosphate dehydrogenase.

ISOENZYME PHENOTYPE: Results

The enzyme electrophoresis results are given in table 14, fp 83, and samples of Polaroid photographs of two of the gels, for glyoxylase, and for phosphoglucomutase, are given in figures 10 & 11, fpp84 & 85 There was no difference in isoenzyme pheotype between Parent, Control or Treated sublines examined.

ISOENZYME PHENOTYPE: Discussion

There is no evidence of cross-contamination of cell lines, or of mutations in the enzymes studied. This does not exclude pleomorphism in other characteristics of the cell lines, which might be polyclonal, but derived from the same individual, or, at a much lower probability, that contamination with a cell line with an identical phenotype in these respects, but different in others. The latter possibility was examined more closely by DNA fingerprinting.
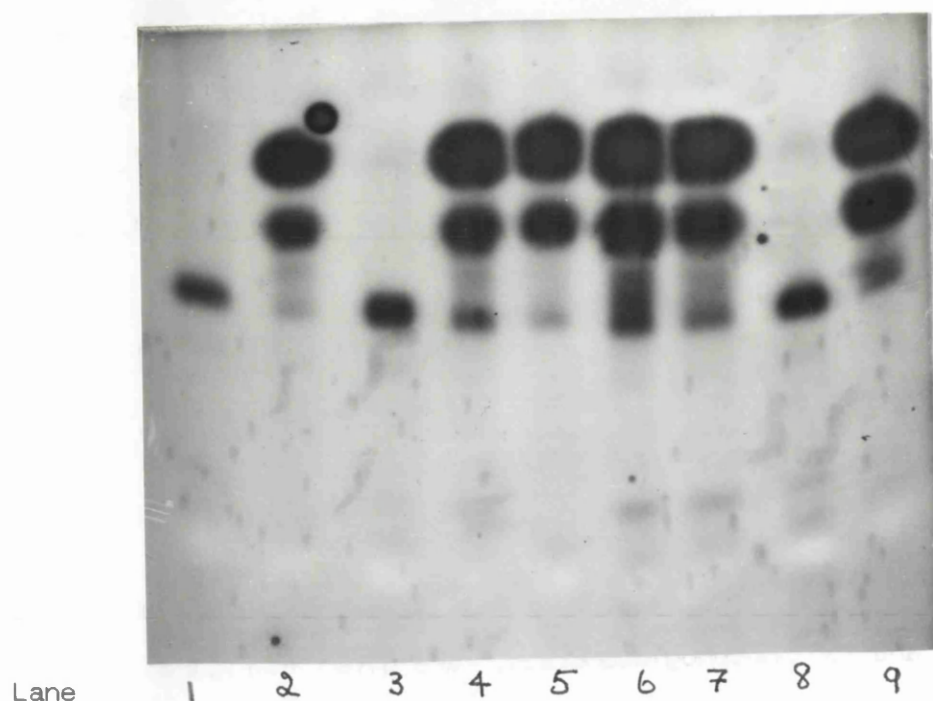
fp85

Lane   1   2   3   4   5   6   7   8   9

Key: Lane 1   RT112   Parent

     Lane 2   MGH-U1   Treated C

     Lane 3   RT112   Treated C

     Lane 4   HU609   Parent

     Lane 5   MGH-U1   Control B

     Lane 6   HU609   Control C

     Lane 7   HU609   Treated C

     Lane 8   RT112   Treated B

     Lane 9   MGH-U1   Parent

FIGURE 11

PHOTOGRAPH OF PHOSPHOGLUCOMUTASE ISOENZYME ELECTROPHORESIS GEL

DNA "FINGERPRINTING"

The principle of this method is that a certain base-pair sequence occurs at different points in the genome of different people. Cutting the chromosomes wherever that sequence appeared, using a restriction enzyme, would result in fragments of DNA with a given pattern of length and ionization for each individual. The commoner the sequence, the greater the number of fragments, and, in general, the higher the number of points of differentiation between individuals. The pattern of the fragments is distinguished by their electrophoretic mobility. The author caried out these studies in the Galton Laboratory of University College, London.

DNA "FINGERPRINTING": Materials and Methods

Abbreviations of chemicals and their suppliers are detailed in Appendix 4.

Cell pellets, prepared and stored in liquid nitrogen as described under Isoenzyme Phenotype, were suspended in 6ml STE (Appendix 4), and the total nucleic acid was extracted in phenol chloroform in an ABI Nucleic Acid Extractor (Model 340A, Applied Biosystems Inc., USA).

The DNA precipitate was dissolved in TE, and the concentration of DNA estimated by absorption spectrophotometry at 260 nm, assuming

that half the absorption was due to DNA. Five microgrammes of DNA were digested by the restriction enzyme HinF I, in the presence of spermidine, and the restriction fragments separated by electrophoresis on a 0.8% agarose gel in a buffer containing 40mM Tris/20mM sodium acetate/1mM EDTA/0.5 mcg/ml ethidium bromide (Appendix 4), using a current of 40-50 mA, at 20 V overnight.

The DNA fragments were blotted on a nylon filter (GeneScreen Plus, Du Pont, USA), by the method of Southern. The filter was prehybridised in a solution containing 4 x SSC, 0.5% SDS, and 1M Sodium Chloride at 65°C for 1 hour. The probe, p-lambda-G3, was radiolabelled with ^{32}P dCTP using the random prime reaction. The filter was hybridised in a similar solution to that used in the pre-hybridisation, but containing the labelled probe, and 5 mcg/ml salmon sperm DNA at 65°C overnight. The filter was then washed down at high stringency (0.1 x SSC and 0.1% SDS), to remove DNA probe fragments of low affinity to the blotted DNA. The filter was then exposed to Kodak X-OMAT film at -70°C overnight, and the resultant autoradiograph developed the next day using the appropriate Kodak process.

DNA "FINGERPRINTING": Results

There was no distinction between Parent, Control, and Treated sublines. The three types of subline showed no evidence of intercontamination, or of contamination from other lines kept in the laboratory, with a high degree of certainty. The autoradio-
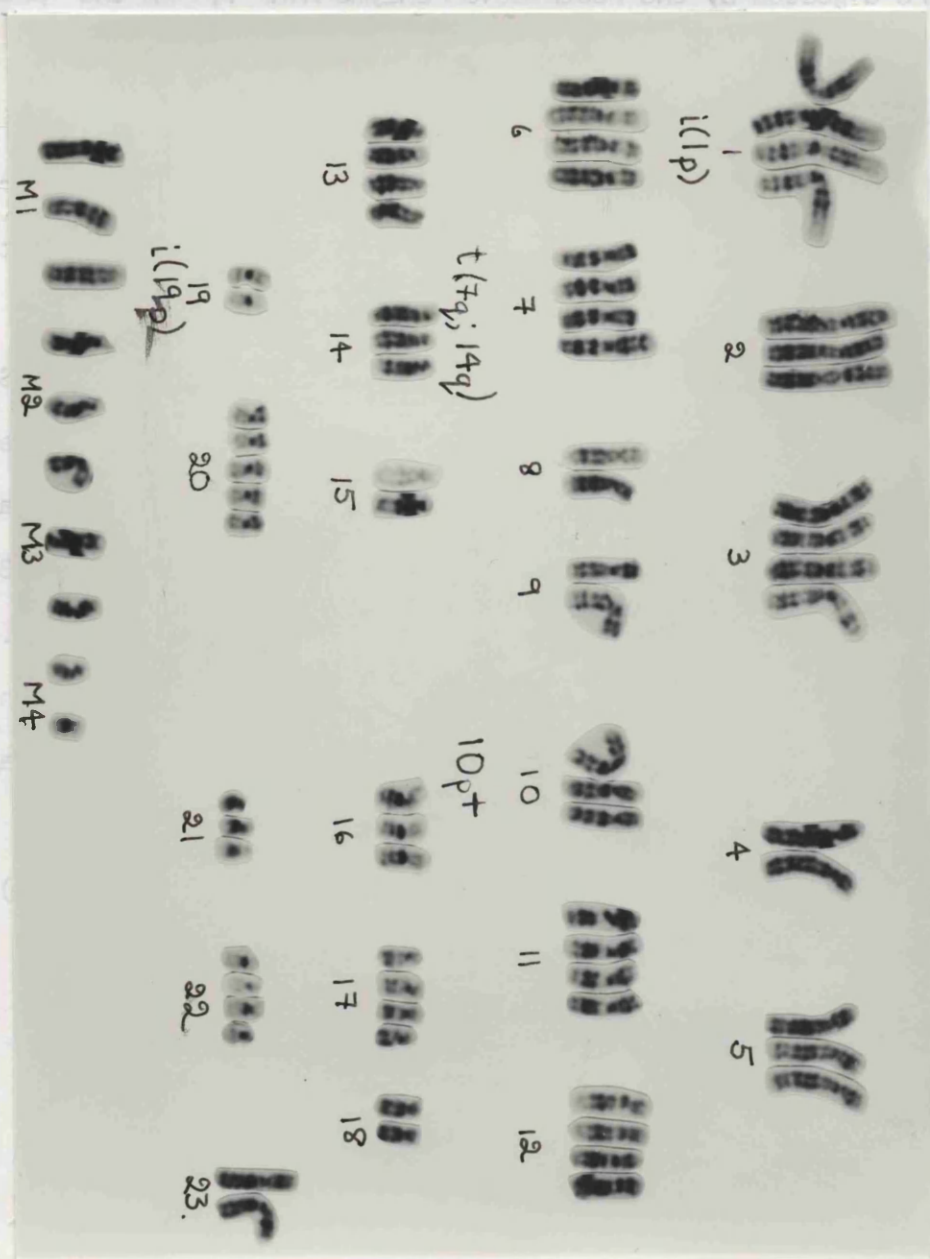
FIGURE 12

TYPICAL KARYOTYPE OF PARENT SUBLINE OF MGH-U1

graph was not preserved.

DNA "FINGERPRINTING": Discussion

The similarity between the Parent, Control, and Treated sublines is high, and for practical purposes, they are derived from the same individual. The three lines have not been intercontaminated. Some other explanation must be found for the differences betwen Parent/Control sublines, and the Treated sublines.

KARYOTYPE

Chromosomal analysis of bladder tumours by banding has been reported to be a highly sensitive predictor of the behaviour of bladder tumours (Falor & Ward, 1978; Summers et al., 1981). No single common abnormality has been confirmed as related to progression or recurrence, but the presence of marker chromosomes is generally seen as significant, and there has been interest in the role of chromosome 1 (Sandberg, 1984). There is little information on gross chromosome defects and clinical drug sensitivity.

The karyotypes of Parent and Resistant (Treated) sublines of MGH-U1, the most clearly resistant, were compared to see if any difference could be detected. This was carried out in the Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, by P Gorman and S Williams.
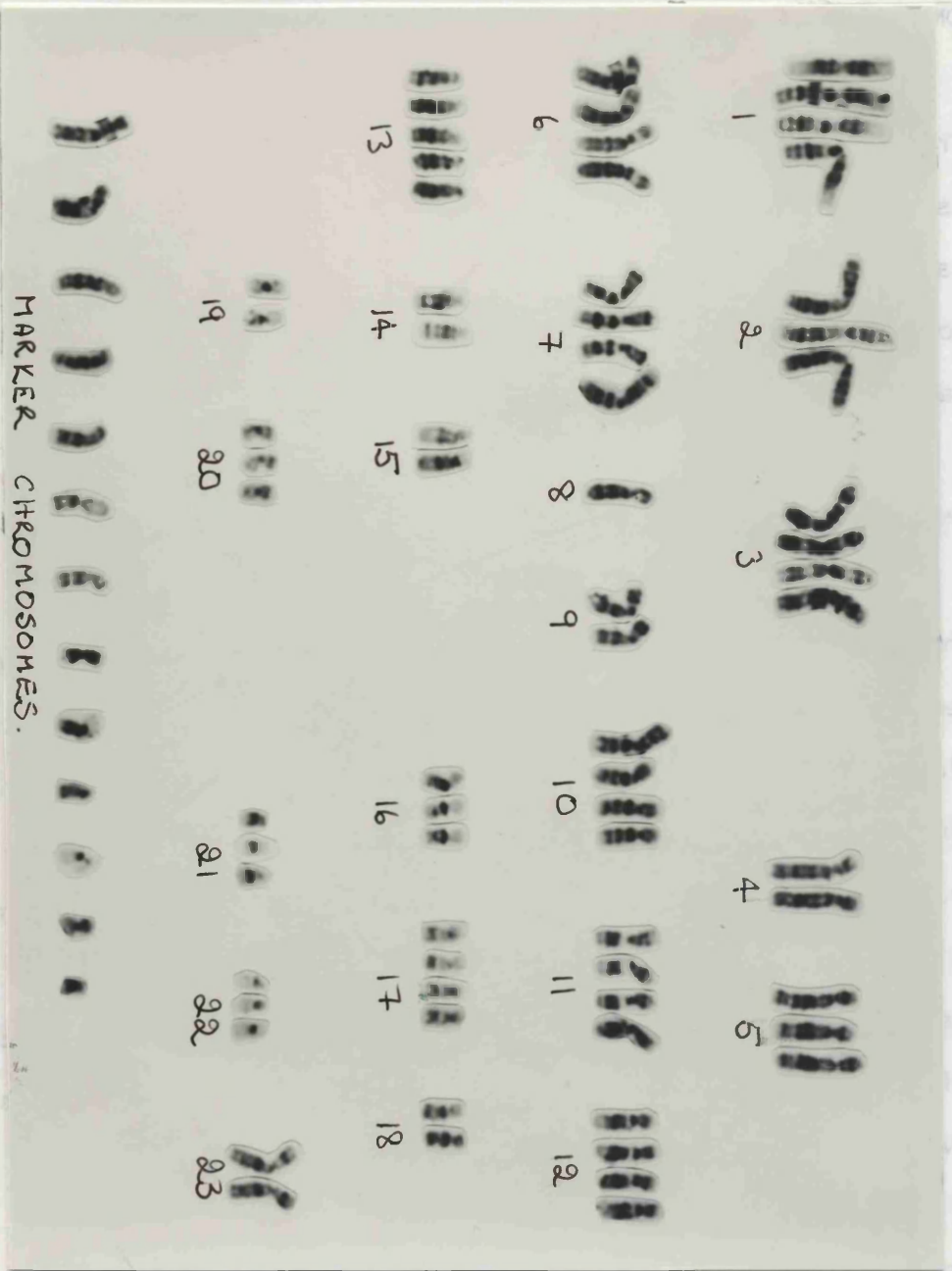
FIGURE 13

TYPICAL KARYOTYPE OF TREATED SUBLINE OF MGH-U1

KARYOTYPE: Materials and Methods

Parental and Treated sublines of MGH-U1 were exposed to 0.05 mcg/ml colcemid in Medium for 0.5 to 3 hours to induce metaphase arrest, before detachment from the flask with trypsin/versene (Appendix 4). Cells were harvested in 0.075M KCl and fixation in 3:1 methanol:glacial acetic acid. Metaphases were spread by dropping on a clean, wet slide, and air dried. Slides were banded by immersion in 2 x SSC at 60°C for 10 to 30 minutes, and stained with Wright's stain, diluted in 50% Sorensen's buffer. The method is based on that described by Benn and Perle (1986).

KARYOTYPE: Results

Nine metaphase spreads of MGH-U1 Parent cells were examined. The chromosome numbers were 71, 77, 83, 83, 83, 84, 85, and 91. The mode was therefore 83, and the karyotype hypotetraploid. The majority of chromosomes appeared normal, but there were several consistent markers, including i(1p), t(7q;14q), 10p+, and i(19p). Figure 12, fp87, shows a typical karyotype.

Eight metaphase spreads of MGH-U1 Treated cells were examined. Chromosome numbers were 75, 78, 81, 81, 82, 83, 127, 132, and the mode was 81. The karyotype was as the Parent subline, although the chromosome numbers appeared to be more variable. Figure 13, fp88, gives an example of the karyotype of a Treated cell.

KARYOTYPE: Discussion

Although the numbers of spreads examined were small, and there was no attempt to examine for double minutes, the karyotype was not so grossly changed as to be of potential value in the routine examination of bladder tumours for drug sensitivity.

EPIDERMAL GROWTH FACTOR RECEPTOR

Epidermal growth factor receptor is a transmembrane glycoprotein of low molecular weight, positive staining for which has been found to be more common in frozen section specimens of poorly differentiated bladder tumours than of well-differentiated (Neal et al., 1985). Its intracellular part is a protein kinase that has similar activity to some oncogene products (Soderquist & Carpenter, 1983), and has considerable sequence homology with the transforming protein v-erb B (Ullrich, Coussens, Hayflick et al., 1984). The antibody used by Neal's group, EGFR1, binds to the external domain of Epidermal Growth Factor (EGF) Receptor (Gullick, Marsden, Whittle et al., 1986), which is directly accessible in cell suspensions. This approach was hoped might lead to tests of urothelial cells exfoliated into bladder washings, as an outpatient diagnostic or prognostic examination. The EGFR1 staining used A431 cells as a positive control, as they are known to express high levels of EGF surface receptor (Stoscheck and Carpenter, 1983; Lin et al., 1984).

EPIDERMAL GROWTH FACTOR RECEPTOR: Method

Subconfluent 175 cm² flasks of cells were incubated for the shortest necessary time (3-5 minutes) with Trypsin/Versene mixture (Appendix 4). The cells were suspended in RPMI 1640 medium, containing 5% Foetal Calf Serum and 1% L-glutamine (Medium, Appendix 4). Cells were stained by Trypan Blue (Appendix 4) exclusion, and counted using a haemocytometer. The stock cell suspension was then spun at 1000 rpm for 5 minutes, the supernatant aspirated, and the cells resuspended in Medium, to give 10^6 cells per 200 microlitres. To three conical-bottomed cryotubes (Nunc, Gibco, Paisley, Scotland), 200 microlitres of cell suspension were added, then the tubes were kept at 4°C for 30 minutes, before addition of 5 microlitres of the first, mouse-derived antibody, EGFR1 (Dako, Amersham, UK), and 30 minutes' incubation at room temperature. After 5 minutes' centrifugation at 1000 rpm, the supernatant was aspirated, the cells resuspended in 2ml Medium, recentrifuged, and the supernatant aspirated. A mixture of the second, rabbit-anti-mouse antibody ($F(ab)_2$ frag-ments), labelled with the fluorescent agent FITC was made by adding 60 microlitres of antibody (F 313, Dako Ltd, High Wycombe, UK) to 3 ml of medium. To prevent loss of fluorescence, the mixture was protected against light. To each cell pellet, 0.5 ml of second-antibody mixture was added and mixed before incubation in the dark at room temperature for 30 minutes. When the pellets had been centrifuged again for 5 minutes at 1000 rpm, and the supernatant aspirated, the cells were washed in 2 ml medium,

centrifuged, and the supernatant replaced with 1 ml 70% ethanol for overnight fixation in the dark.

The fluorescence intensity of a minimum of 10,000 cells was measured on a Becton-Dickinson FACS cytofluorimeter, and the number of cells at each (arbitrary) fluorescence intensity was charted. Initial studies of A431 cells were compared with A431 cells from which the primary antibody had been omitted. Pilot tests of Parent and Treated cells from Chapter 2 were carried out two years later, with antibody that had passed its expiry date.

EPIDERMAL GROWTH FACTOR RECEPTOR: Results

The initial fluorescence-separation of stained and unstained A431 cells was approximately two logs of intensity. Fluorescence of A431 cells stained later, in the absence of EGFR1-free A431 controls, was apparently less marked, but the absence of con-trols, and developments in the software used made comparisons difficult to interpret reliably. No difference in fluorescence was detected between Parent, Control, and Treated sublines of the other cell lines, but the data were preliminary. Time and money were unavailable to explore this fascinating line of enquiry in more detail.

EPIDERMAL GROWTH FACTOR RECEPTOR: Discussion

The initial findings were hopeful. The separation of the

fluorescence peaks in the initial A431 cells suggested that the antibody was likely, even with a relatively simple machine, to distinguish the surface domain of Epidermal Growth Factor with some sensitivity. Regrettably, the time taken to complete the induction and testing of resistance was considerable, and the reliability of the stain was in doubt when the urothelial cell lines were tested, so it is not clear whether the cell lines did not express the receptor, whether the antibody did not adhere, or the fluorescent marker had dimmed or become deconjugated from the antibody.

CHAPTER 4

INFLUENCE OF THE SOLVENT DIMETHYL SULPHOXIDE ON THE EFFECT OF INTRAVESICAL CHEMOTHERAPEUTIC AGENTS

Intravesical chemtherapeutic agents can delay tumour recurrence (Lum, 1983) and progression (Green et al., 1984) of superficial bladder cancer. Approximately one third of patients do not respond to intravesical chemotherapy (Torti and Lum, 1984), and within six months of stopping curative treatment, recurrences can occur (Esquivel, Mackenzie & Whitmore, 1965; Oravisto, 1965). Greater effectiveness might improve these outcomes, and achieve them with less toxicity. More effective treatment is needed.

Dimethyl sulfoxide (DMSO) is a dipolar solvent used clinically as a bladder instillation for interstitial cystitis (Stewart et al., 1972). In combination with chemotherapeutic drugs, it increases antitumour activity in vivo in rats (Warren et al., 1975) and in vitro against mouse hepatocarcinoma cells (Tofilon et al., 1985). DMSO enhances the uptake of acyclovir into the skin (Spruance, et al., 1983) and of salicylates, and cisplatin through the bladder (Borzelleca, Harris & Bernstein, 1968; Schoenfeld, Bellville, Jacob et al., 1983). DMSO was therefore combined with the four most commonly used intravesical chemotherapeutic agents, and the effect on clonogenic cell survival measured in vitro, using a continuous cell line derived from a human transitional cell cancer.

MATERIALS AND METHODS

MATERIALS AND METHODS: Drugs

Stock solutions of commercial preparations of four commonly-used
intravesical chemotherapeutic agents were prepared and stored at
-20⁰C. Adriamycin (doxorubicin hydrochloride, Farmitalia Carlo
Erba, St Albans, England), Epodyl (ethoglucid, Imperial Chemical
Industries, Macclesfield, England), Mitomycin-C (Kyowa Hakko
Kogyo Co Ltd, Tokyo, Japan), and ThioTEPA (triethylene thio-
phosphoramide, Lederle Laboratories, Gosport, England) were dis-
solved in sterile water for injection, BP,(Beecham, Brentford,
England) as stock solutions, and stored at -20⁰C in glass
containers, until use. The drugs were added to tissue culture
medium in concentrations that reduced colony-forming ability by
approximately 50% after an hour's exposure: 400 ng/ml adriamycin,
50 mcg/ml epodyl, 400 ng/ml mitomycin-C, and 15 mcg/ml thioTEPA.
Dimethyl sulfoxide was supplied by Sigma Chemical Co, Poole,
England).

MATERIALS AND METHODS: Cell Line

RT112, a continuous cell line, established in the Institute of
Urology, in 1970, from a moderately-differentated human uro-
thelial carcinoma (Hastings & Franks, 1981), is described in
chapter 2.

DMSO Concentration	% Survival	95% Confidence Interval
1%	92	76, 107
2%	89	34, 143
3%	96	71, 121
4%	96	83, 108
5%	91	83, 98
6%	81	77, 85
8%	72	55, 89
10%	60	53, 67
20%	41	18, 60

Legend: The mean % colony-forming ability is over 90% for RT112

cells exposed for 1 hour to DMSO concentrations less than 5%.

TABLE 15

MEAN   PERCENTAGE CLONOGENIC CELL SURVIVAL AS A FUNCTION   OF   DMSO

CONCENTRATION (% BY VOLUME)

MATERIALS AND METHODS: Cell Culture

RT112 cells were maintained as monolayer cultures in 25 cm^2 flasks, as described in chapter 2. To minimise any changes resulting from long-term culture, assays were carried out on a restricted range of ten passages.

MATERIALS AND METHODS : Clonogenic Assay

The essentials of the clonogenic assay were as in chapter 2. A suspension of 500 colony-forming units of RT112, with a plating efficiency of approximately 40% were plated in 60 mm vented plastic petri dishes (Nunc, Gibco, Paisley, Scotland) and incubated for 48 hours at 36.5^0C in a humidified atmosphere of 5% CO_2 in air, to allow attachment and recovery from trypsinisation. To assess the dose-response to DMSO, the Medium in the dishes was then aspirated and replaced with Medium containing a range of concentrations of DMSO, or with Medium alone for controls, and returned to the incubator for an hour. After exposure, the fluid in the dishes was aspirated, the cells washed three times with 5 ml medium, and replaced with 5ml fresh Medium, before return to the incubator for 10-14 days to allow colony formation.

To assay the effect of DMSO on the four agents, the culture fluid in dishes of cells that had been plated and incubated was replaced with fresh Medium alone, or containing either 4% DMSO alone, drug alone, or drug in combination with 4% DMSO, in

% DMSO concentration (vol/vol)

(Error Bars Represent 95% Confidence Intervals.)
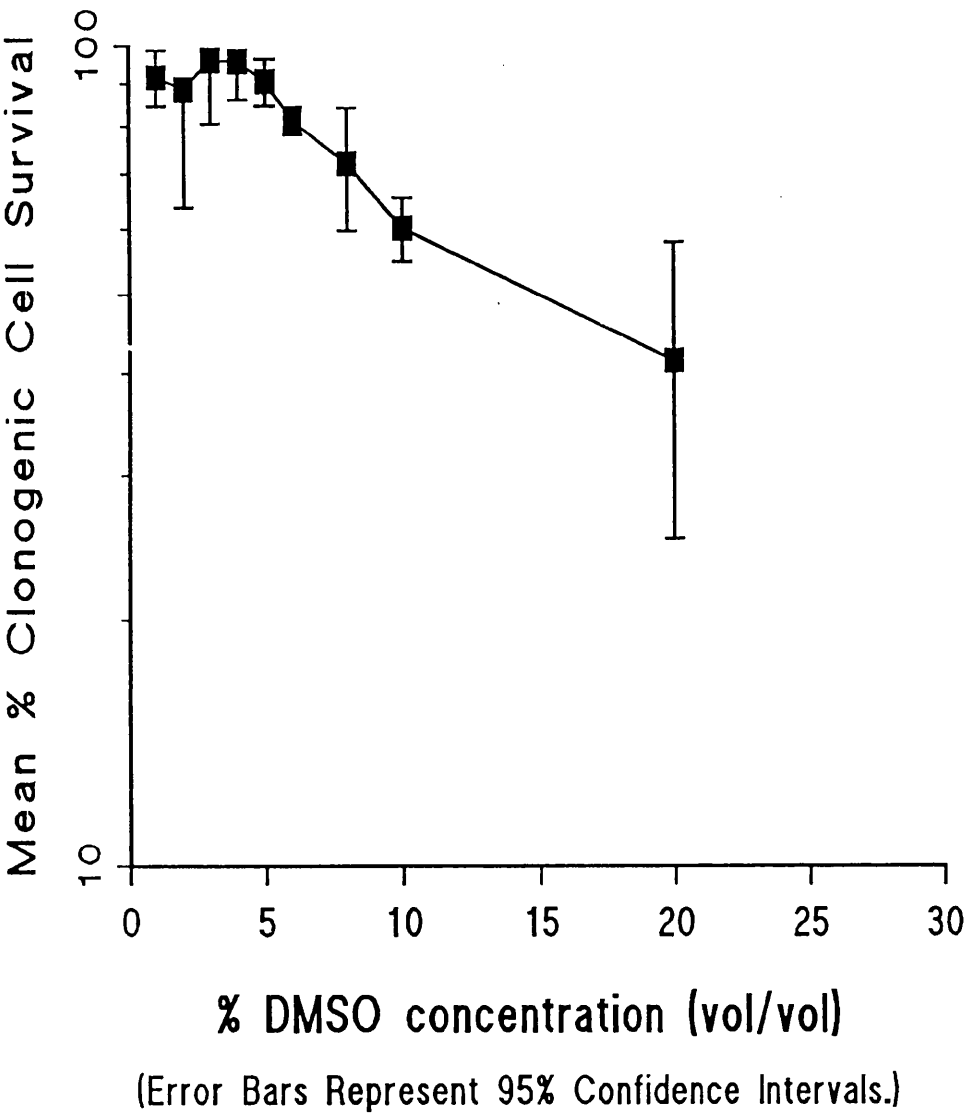
FIGURE 14

DOSE-RESPONSE OF UNCLONED RT112 CELLS TO A 1-HOUR EXPOSURE TO

DIMETHYL SULPHOXIDE (DMSO)

triplicate. After incubation for an hour, the cells were washed, allowed to form colonies for 10-14 days, fixed, stained, and counted as in chapter 2. The mean colony-forming ability was expressed as a percentage of the Medium-only controls. The data are derived from a minimum of three separate experiments for each drug.

To control for an influence of pH on clonogenic cell survival, the pH of Medium alone, or with 4% DMSO were compared after incubation for 24 hours in humidified air containing 5% CO_2 at 36.5°C. Measurements were made at 36.5°C with a Dow Corning pH meter and a KCl/ AgCl electrode (Dow Corning, Halstead, England).

MATERIALS AND METHODS: Statistical Methods

The mean percentage clonogenic cell survival of each replicate experiment was compared using an unpaired, two-tailed t-Test (Swinscow, 1976). Because replicates for drug-only and drug+DMSO assays were not always carried out on the same day, or with the same container of drug in Medium, a paired comparison was not appropriate.

RESULTS

In table 15, fp 95, and figure 14, fp 96, the dose-response of RT112 cells exposed for 1 hour to a range of concentrations of DMSO is shown. At concentrations of less than 5%, DMSO had a minimal effect on colony-forming activity. The addition of 4%

Drug	Mean % Survival	95% Confidence Interval
DMSO	92	83, 101
Adriamycin	40	20, 60
Adriamycin+DMSO	42	18, 61
Mitomycin-C	44	38, 49
Mitomycin-C+DMSO	58	41, 76
Epodyl	75	60, 90
Epodyl+DMSO	68	56, 80
ThioTEPA	50	14, 86
ThioTEPA+DMSO	51	17, 84

Legend: 4% DMSO without other drugs produced a mean % colony-forming activity (Mean % Survival) of 92%. No reduction in Mean % Survival was found when DMSO was added to thioTEPA, mitomycin-C, or adriamycin.

TABLE 16

ADDITION OF DIMETHYL SULPHOXIDE TO ADRIAMYCIN, MITOMYCIN-C, EPODYL AND THIOTEPA : MEAN % CLONOGENIC CELL SURVIVALS

DMSO to Medium increased the mean pH value by 0.16 units at a temperature of 36.5⁰C.

The mean percentage clonogenic cell survival of RT112 cells exposed for 1 hour to DMSO alone, drugs alone, and in combination is given in table 16, fp 97 and figure 15, fp 98. The large confidence intervals were produced by day-to day variation, possibly as a result of pipetting errors in drug concentration (Wilson, 1985). No enhancement of tumour cell kill was observed following the addition of DMSO.

DISCUSSION

The basic method used to test this hypothesis has been widely used (Freshney, 1987). The clonogenic assay of human bladder and testicular cancer cells in monolayer culture plated as single colony-forming units provided results consistent with the clinical response of these tumours to radiation and drug treatment in patients (Parris, Arlett, Lehman et al., 1988; Walker, Parris & Masters, 1987), Testing the sensitivity in situ after plating avoids the variable of trypsinisation after treatment inherent in transfer assays (Freshney, 1987; Hepburn & Masters, 1986), and of growth rates, and drug absorption in assays that measure cell numbers by vital staining, such as the MTT assay (Mosmann, 1983; Alley, Scudiero, Monks et al., 1988). Particularly with alkylating agents, the cytotoxic effects may not be obvious for two cell cycles after exposure (Tidd & Paterson, 1974; Fairchild,

Drug Alone/Drug + DMSO

(1-Hour Exposure, Error Bars Represent 95% Confidence Interval).
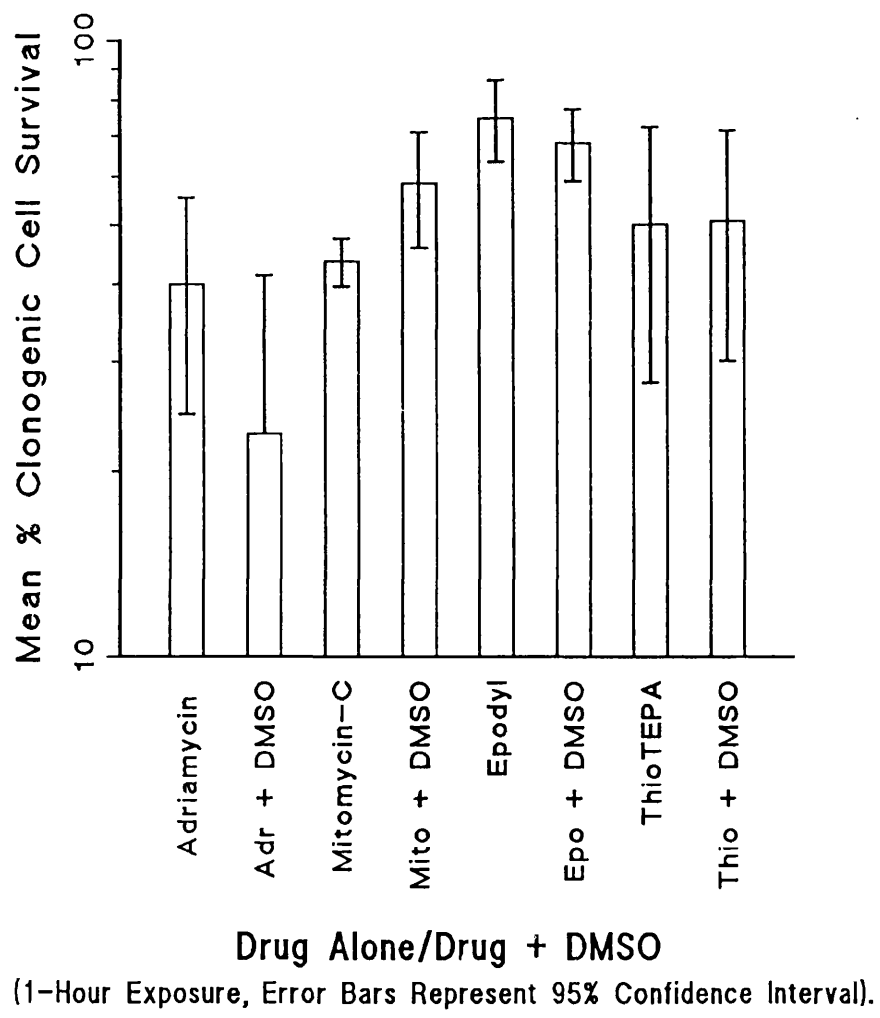
FIGURE 15

SURVIVAL OF RT112 CELLS EXPOSED TO FOUR DRUGS WITH AND WITHOUT

DIMETHYL SULPHOXIDE

Maybaum & Kennedy, 1986; Larramandy & Lopez-Larraza, 1989), so clonogenic assays are more sensitive than indirect but quick measurements of metabolic activity, such as ATP-bioluminescence (Jauhiainen, 1985). The pH alteration induced by DMSO is small, and would be expected to affect different drugs differently (Groos et al., 1986). The rise in pH found would tend to reduce the effect of Mitomycin-C and thioTEPA, but to increase the effect of adriamycin, and leave ethoglucid unchanged. With DMSO, the effect of mitomycin-C was less, but the rise in pH measured was small to account for the significant effect on clonogenic cell survival. ThioTEPA, which, like mitoycin-C is less active in alkaline conditions, was not similarly affected by the addition of DMSO. A small effect could have been masked by the experimental error in the four replicates tested. Adriamycin, which should be more effective at higher pH, did not show a consistent change. This also could be a Type II error, but there were six replicates in this case.

This study found no evidence that the addition of DMSO to instillations of the four drugs commonly used to treat superficial bladder cancer will increase their cytotoxic effect. It may reduce the effect of Mitomycin-C, but if so, this may result from altered pH.

CHAPTER 5

DOES pH INFLUENCE THE EFFECT OF ThioTEPA ON BLADDER CELLS?

Intravesical chemotherapy is an out-patient method of prophylaxis which can be applied directly and controllably to bladder tumours, with a view to delaying recurrence or progression. The optimal conditions for instillatons remain to be defined.

In 1985, a group including the author reported that the clonogenic cell survival of a human urothelial cell line, RT112, after thioTEPA exposure was lower if the instillate was acid (Groos et al., 1986). A similar phenomenon has been reported in murine colonic cancers (Phillips et al., 1988) and in vivo as a result of acidosis induced by hyperglycaemia (Osinsky Bubnovskaja, Segienko et al., 1987). In Walker carcinosarcoma cells, however, ATP bioluminescence was not reduced after ex-posures to thioTEPA at lower pH. Commercial thioTEPA powder, dissolved in water at a concentration of 1 mg/ml as recommended by the manufacturer, has a pH of 8.2 (Groos et al., 1986). Since this is likely to be the pH of the instillates used in most clinical studies, acidifying intravesical instillations may increase the clinical benefit. In vitro, a thioTEPA concentration that produced 91% survival at pH 8.2 produced 1.4% survival at pH 5.2.

To confirm that this effect was not an idiosyncrasy of RT112, three human urothelial cell lines were tested for their

clonogenic cell survival after thioTEPA exposure in medium at a range of pH.

MATERIALS AND METHODS

MATERIALS AND METHODS: Cell Lines

The cell lines used were HU609, RT112, and MGH-U1 as described in chapter 2.

MATERIALS AND METHODS: Drugs and Chemicals

A list of chemical names and their abbreviations and suppliers is given in Appendix 4.

For preliminary dose-response curves, commercial ThioTEPA (Lederle, Gosport, UK) was diluted fresh daily with sterile water for injections, to give a stock solution of 1 mg/ml before dilution in Medium to give a range of thioTEPA concentrations. ThioTEPA (triethylene thiophosphoramide) was supplied in pure powder form, at-4⁰C by Lederle Inc., Pearl River, USA, without the bicarbonate buffer added to commercial preparations, and dis-solved in sterile water for injections, BP (Beechams, UK) to give a concentration of 100 mg/ml for storage at -20⁰C in glass universals. Stock solutions of 2mg/ml were aliquotted into 6ml glass bijou bottles for experimental use, and stored at -20⁰C. The stock solutions were then added to Medium, Buffered or plain,

to give a thioTEPA concentration of 20 mcg/ml, which had produced survivals about 50% in the dose-response experiments.

Biological buffers with a low osmotic effect for their buffering power were chosen to maintain pH at 9 values between 5.0 and 9.5. Biological buffers were obtained in powder form, and diluted in Sterile Water for Injections, to give a concentration of 0.5M, and, in the case of MES, of 1 M also (MES2). In this way, inclusion of 0.4 ml stock buffer in 20 ml aliquots of buffered medium gave a concentration of 10 m mol/litre, except for MES2 at 20 m mol/litre. Solutions were filter-sterilised, with disposable (Flowfilter-D, Flow, Irvine) 20 micron filters. Stock solutions were then stored at -20°C between uses in 20 ml glass universals. To obtain a pH of 5.0, or 5.5, a concentration of 1M MES was made; of 6.0, or 6.5, 0.5M MES; 7.0, 0.5M MOPPS; 7.5, or 8.0, HEPES; 8.5, EPPS; and 9.0, CHES. Adjustments to pH were made with Molar hydrochloric acid or Molar sodium hydroxide.

The laboratory's standard medium, Roswell Park Memorial Institution No 1640 (RPMI 1640) was made up with buffers and acid or alkali, equilibrated overnight in air at 36°C, and the pH adjusted with 1N hydrochloric acid or 1N sodium hydroxide, requiring less than 5 ml in 80 ml preparations of buffered medium. This gave medium at a range of pH values from 5 to 9, that were stable over several days in air. In 175cm^2 vented plastic flasks (Nunc, Gibco, Paisley, UK) 4 ml stock buffer was added to Medium to give a total volume of 200 ml. The pH was

Nominal pH	N	Mean Diff.(CI)	p-Value
5.0	5	0.14 (0.05,0.23)	0.01
5.5	5	0.10 (-0.01,0.21)	0.07
6.0	7	0.04 (0.02,0.06)	0.01
6.5	4	0.03 (-0.1, 0.07)	0.06
7.0	7	0.07 (-0.08, 0.23)	0.30
7.5	6	0.03 (-0.004, 0.07)	0.08
8.0	6	-0.02 (-0.10,0.06)	0.49
8.5	6	0.02 (-0.02,0.06)	0.63
9.0	8	0.02 (-0.03,0.07)	0.61
"Control"	9	0.07 (-0.30, 0.17)	0.53

Mean Diff.= Mean Difference in pH (After-Before).

N= number of replicates with comparable data at the Nominal pH.

(CI)= 95% Confidence Interval

Legend: During each drug exposure, there is a small difference (maximum 0.23 pH units) in the pH measured before and after the drug exposure, but only at pH 5.0 and 6.0 does the 95% confidence interval not include zero change.

TABLE 17

THE DIFFERENCES IN pH (AFTER-BEFORE) DURING EXPERIMENTS ON

HU609 CELLS

adjusted roughly by the addition of 1N HCl to all of the flasks except the pH 9 flasks, to which 1N NaOH was added. "Control" Medium, for cells to be exposed in the laboratory standard incubator, with 5% CO_2, without buffers, was also measured into a flask. Flasks were then equilibrated overnight at 36.5^0C in a humidified atmosphere of air. Flasks for Control Medium contained Medium from the same batch as the Buffered Medium, but no buffers, acid or alkali were added. The overnight equilibration of, and later cell exposures in, Control Medium were carried out in a humidified incubator at 36.5^0C in an atmosphere containing 5% CO_2. Equilibrated Medium (Buffered and Control) was then sampled and samples kept in double-stoppered sterile plastic test tubes (Flow, Irvine, UK) in a water-bath at 36^0C to measure the pH. Fine adjustments of pH in the flasks was made with acid or alkali, and checked immediately and on the day of the experiment (the Before pH). The figures given were all measured on the day of the experiment.

MATERIALS AND METHODS: Adjusting pH

The pH of samples kept at 36^0C in a water-bath was measured with a KCl/AgCl electrode and a Dow-Corning 120 pH meter (Corning Medical and Scientific, Halstead,UK) and pH-reference buffers at pH of 4, 7, and 10 (Convol, BDH Chemicals, Poole, UK). Reference buffer solutions were made up to 500ml with deionised water, and kept in plastic universals (Alpha Laboratories, Eastleigh, UK) at -70^0C until use. A new reference buffer sample was used daily.

Nominal pH	N	Mean Diff. (CI)	p-Value
5.0	6	0.11 (-0.02, 0.25)	0.09
5.5	6	0.02 (-0.01, 0.06)	0.16
6.0	10	0.002(-0.10, 0.10)	0.96
6.5	8	0.07 (-0.03, 0.17)	0.12
7.0	11	0.08 (0, 0.17)	0.05
7.5	11	0.03 (0, 0.05)	0.03
8.0	7	0.003(-0.10, 0.11)	0.95
8.5	9	0.02 (-0.07, 0.11)	0.59
9.0	7	0.01 (-0.10, 0.11)	0.82
"Control"	11	0.08 (-0.27, 0.12)	0.39

Mean Diff.= Mean Difference in pH (After-Before).

N= number of replicates with comparable data at the Nominal pH.

(CI)= 95% Confidence Interval.

Legend: The mean differences in pH include zero in the 95% confidence interval in all cases, but at pH values of 5.0, 7.0 and 7.5, the paired t-Test shows a high level of significance, with a maximum difference of 0.25 pH units in the 95% confidence intervals. This, and the comparable points for MGH-U1 are the only evidence that a systematic change in pH occurred during the course of these experiments.

TABLE 18

THE DIFFERENCES IN pH (AFTER-BEFORE) DURING EXPERIMENTS ON RT112 CELLS

Calibration settings of +/- 0.02 units were accepted, and the electrode rinsed with deionised water and blotted with tissue between each measurement.

Although the intended or "Nominal" pH should have been stable for a given recipe, the actual value was checked at three stages in the experiment to assess the possibility of variation due to cell injury and metabolism. Samples were taken from the flasks of Buffered and Control Media that were about to be used to make up the thioTEPA and control medium at each pH, and the "Before pH" measured. After exposure, Buffered Medium was aspirated for pH measurement from two control dishes at each pH (the "After pH") and from dishes containing thioTEPA (the "Thio pH"). As the variation between the "After pH" and the "Thio pH" was less than 0.05 on all but one measurement, and as the handling of samples containing cytotoxic drugs was more complex, the measurement of the Thio pH was carried out once for each cell line.

MATERIALS AND METHODS: Experimental Method

The details of clonogenic assay, and of the dose-response curves of uncloned HU609, MGH-U1, and RT112 are given in chapter 2. A similar method was employed for the evaluation of the influence of pH. Four control dishes and four dishes for thioTEPA treatment at each pH were tested, and the mean number of colonies surviving in the thiotepa dishes expressed as a percentage of the mean number of colonies surviving in the dishes at the same pH that

Nominal pH	N	Mean Diff. (CI)	p-Statistic
5.0	4	0.09(-0.05, 0.22)	0.13
5.5	5	0.03(-0.01, 0.07)	0.13
6.0	5	-0.02(-0.12, 0.08)	0.61
6.5	5	0.07(-0.11, 0.24)	0.35
7.0	6	0.04(0.01, 0.08)	0.02
7.5	6	0.02(0, 0.03)	0.03
8.0	5	0.02(-0.12, 0.16)	0.74
8.5	5	0.04(-0.11, 0.19)	0.49
9.0	2	0.07(-1.33, 1.47)	0.64
"Control"	3	-0.13(-2.91, 2.88)	0.99

Mean Diff.= Mean Difference in pH (After-Before).

N= number of replicates with comparable data at the Nominal pH.

Legend: Significant increases in the pH at 7.0, or 7.5 are small, 0.05, and 0.03, and unlikely to introduce a bias into the survival data. At pH 7.5, the 95% confidence interval includes zero. The difficultes of measuring the pH of the "Control" medium in air are discussed in the text.

TABLE 19

THE DIFFERENCES IN pH (AFTER-BEFORE) DURING EXPERIMENTS ON

MGH-U1 CELLS

did not contain thioTEPA.

After plating in the dishes, cells were allowed 2 days to adhere, and to resume exponential growth. On the day of the experiment, the Medium was aspirated from the dishes, and each dish washed with 2 ml of Buffered Medium at the appropriate pH, to flush out any remaining Medium, and minimise variations in the pH of exposure. The wash was then aspirated, and replaced either with buffered plain Medium, or buffered thioTEPA Medium. "Control" dishes used unbuffered Medium, with and without thioTEPA, and were incubated for the duration of the exposure in the laboratory's standard conditions, humidified air, containing 5% CO_2. Dishes buffered to adjust the pH were incubated without the addition of CO_2, and the exposure time for all was one hour.

After exposure, Buffered and Control Medium was aspirated from all dishes, some being saved for "After" and "Thio" pH testing. The dishes were washed twice with 2 ml Medium, then refilled with 5 ml Medium and incubated in the presence of 5% CO_2 to allow the formation of countable colonies. This incubation took 7-10 days for MGH-U1, 10-12 days for HU609, and 12-14 days for RT112. Dishes were then rinsed, fixed, stained and counted as before.

MATERIALS AND METHODS: Statistical Methods

The mean percentage clonogenic cell survivals were treated as continuous variables, although they are derived from integer

colony counts. To assess the significance of pH variations at different times during the experiment, comparisons were made using a paired t-Test, only using replicates where data were available for both time-points.

RESULTS

The dose-response studies, with a minimum of two replicates at each point, show that at a concentration of 20 mcg/ml, the mean % clonogenic cell survival is on the sloping part of the curve for RT112, and on the plateau, or probably at the shoulder of the slopes for HU609 and MGH-U1 (figures 6-9, fpp 67,68,70,72). For simplicity, one concentration, 20 mcg/ml, was used for pH studies in all three cell lines.

RESULTS: Nominal and Measured pH

The "Before" and "After" pH values at each nominal pH were compared using a paired t-Test, for replicate experiments where both values were available on the same day. Where the After value is lower, the difference is given a negative sign (tables 17-19, fpp 102-104). The differences are significant at the 0.05 level for nominal pH values 7.0 and 7.5 for MGH-U1, 7.0 and 7.5 for RT 112, and 5.0 and 6.0 for HU 609. All of the differences were less than or, in one case, equal to, one Standard Deviation (S.D.) of the relevant mean pH. The variation in pH during one experiment does not exceed the variation between experiments on different

days,and this is concistent with the analysis of the Survival data below. The average difference between After and the Thio values for each pH, averaged among the three cell lines, is less than one S.D. of the After pH of each cell line. The difference for each cell line is less than the appropriate S.D. of the Before pH.

It might be thought that experimental replicates should be excluded from consideration if the measured pH deviated markedly from the mean measurement at that nominal pH, as that would tend to indicate a technical error. Limiting the % Survivals graphed to those relating to a pH within 0.25 pH units of the mean for each nominal pH point produced no change in the shape of the graphs for any of the cell lines, and in the case of HU609, only one point of one replicate would have been excluded. The outcome was little affected by these variations in pH.

The Mean % Clonogenic Cell Survival or Mean % Colony-Forming Ability was related to pH. It is expressed as a function of the Before pH in figures 17,18,21,22,25, & 26, and as a function of the After pH in figures 19,20,23,24,27, & 28 (see pp 107-118). Irrespective of the pH measure used, and despite the wide confidence intervals, the trend indicates reduction in survival , significant at the 5% level, as the pH approaches 5. The error bars represent the 95% confidence intervals.

Nom. pH	N	Mean pH(95% CI)	Mean % C.C.Surv. (95% CI)
5.0	4	5.26 (5.03,5,48)	0 (0)
5.5	4	5.53 (4.99,6.06)	1.9 (0,4.3)
6.0	6	6.06 (5.99,6.14)	8.3 (0,19)
6.5	3	6.51 (6.51,6.51)	46 (10,82)
7.0	5	7.07 (6.98,7.16)	63 (42,85)
7.5	4	7.65 (7.59,7.71)	77 (61,94)
8.0	4	8.27 (8.07,8.47)	94 (86,102)
8.5	4	8.58 (8.32,8.83)	87 (62,112)
9.0	6	9.11 (8.90,9.32)	80 (57,103)
Control	5	8.05 (7.42,8.67)	79 (65,93)

Mean % C.C.Surv. = Mean % Clonogenic Cell Survival

= Mean % colony-forming activity

(95%CI) = (95% Confidence Interval)

N = the number of replicates with data at each nominal pH

Nom. pH = nominal pH

Legend: These data are presented graphically in figures 16-17. There is a clear trend towards lower mean % colony-forming activity at lower pH. "Control" cells refer to assays of thioTEPA effect in the laboratory's usual CO_2 incubator.

TABLE 20

SURVIVAL OF HU609 AS A FUNCTION OF "BEFORE pH"
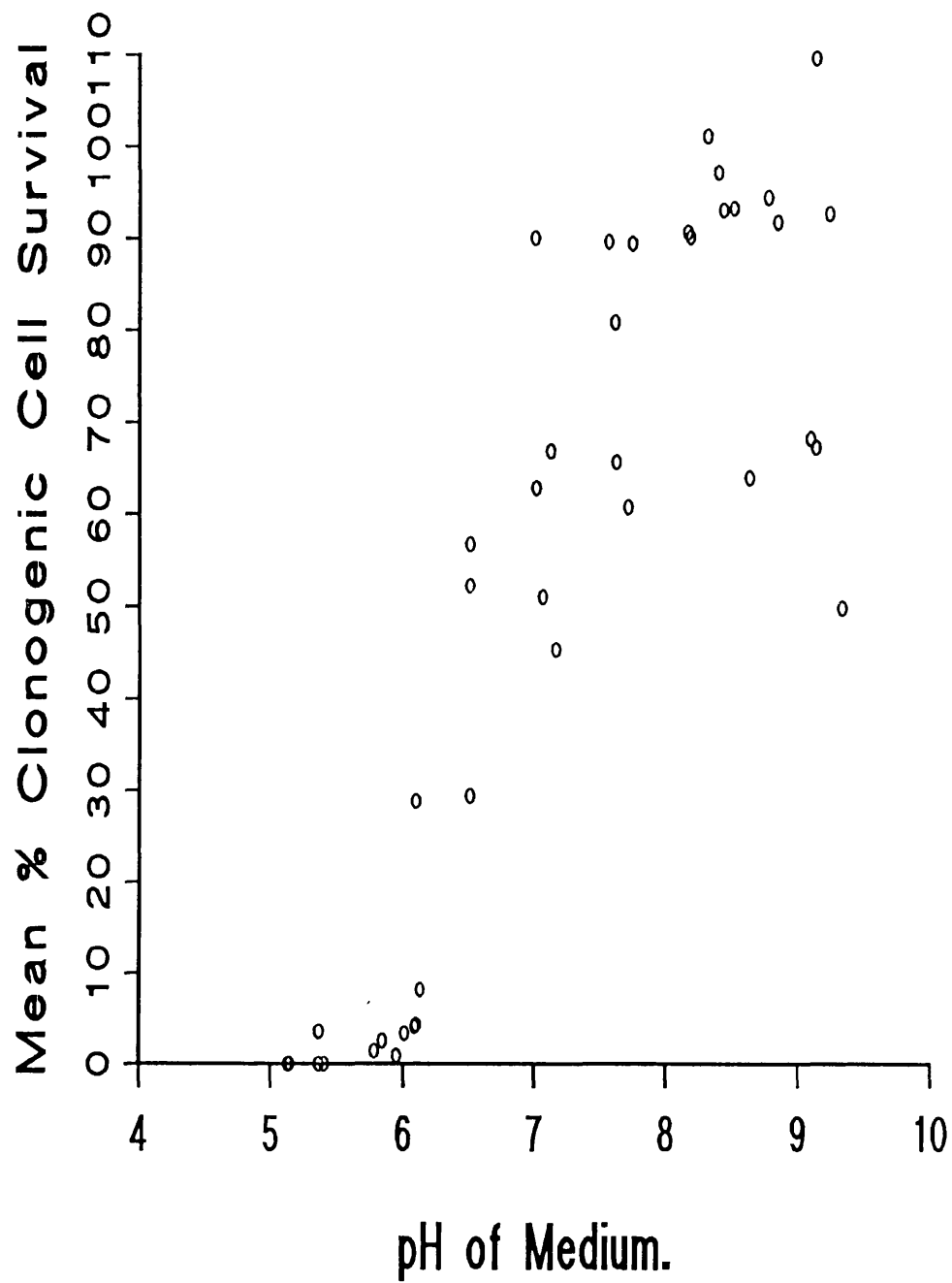
FIGURE 16

SCATTERGRAM OF SURVIVAL OF HU609 AS A FUNCTION OF "BEFORE pH"
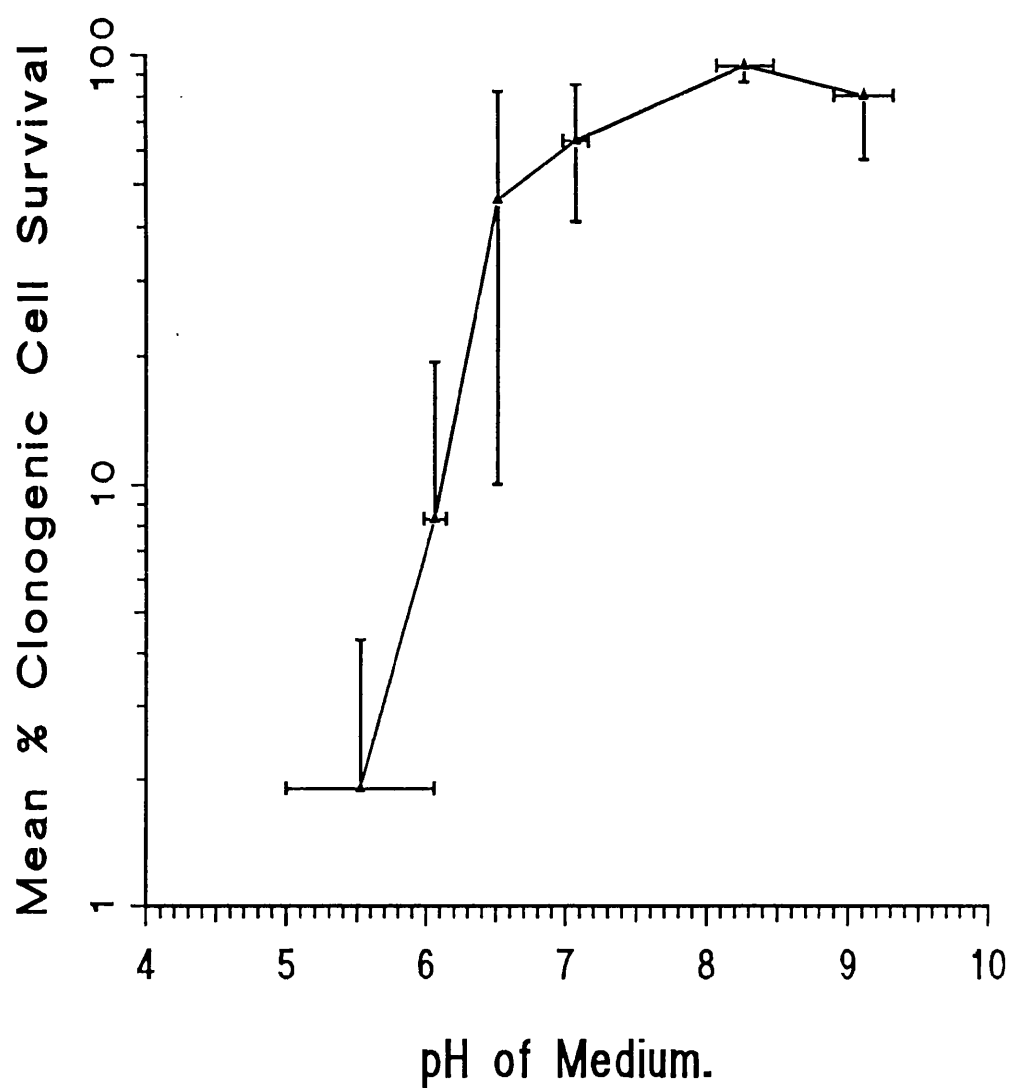
NB: at pH=5.26, the Survival was 0, and could not be plotted on log axes.

FIGURE 17

MEANS (95% CONFIDENCE BARS) OF SURVIVAL OF HU609 AS A FUNCTION OF "BEFORE pH"
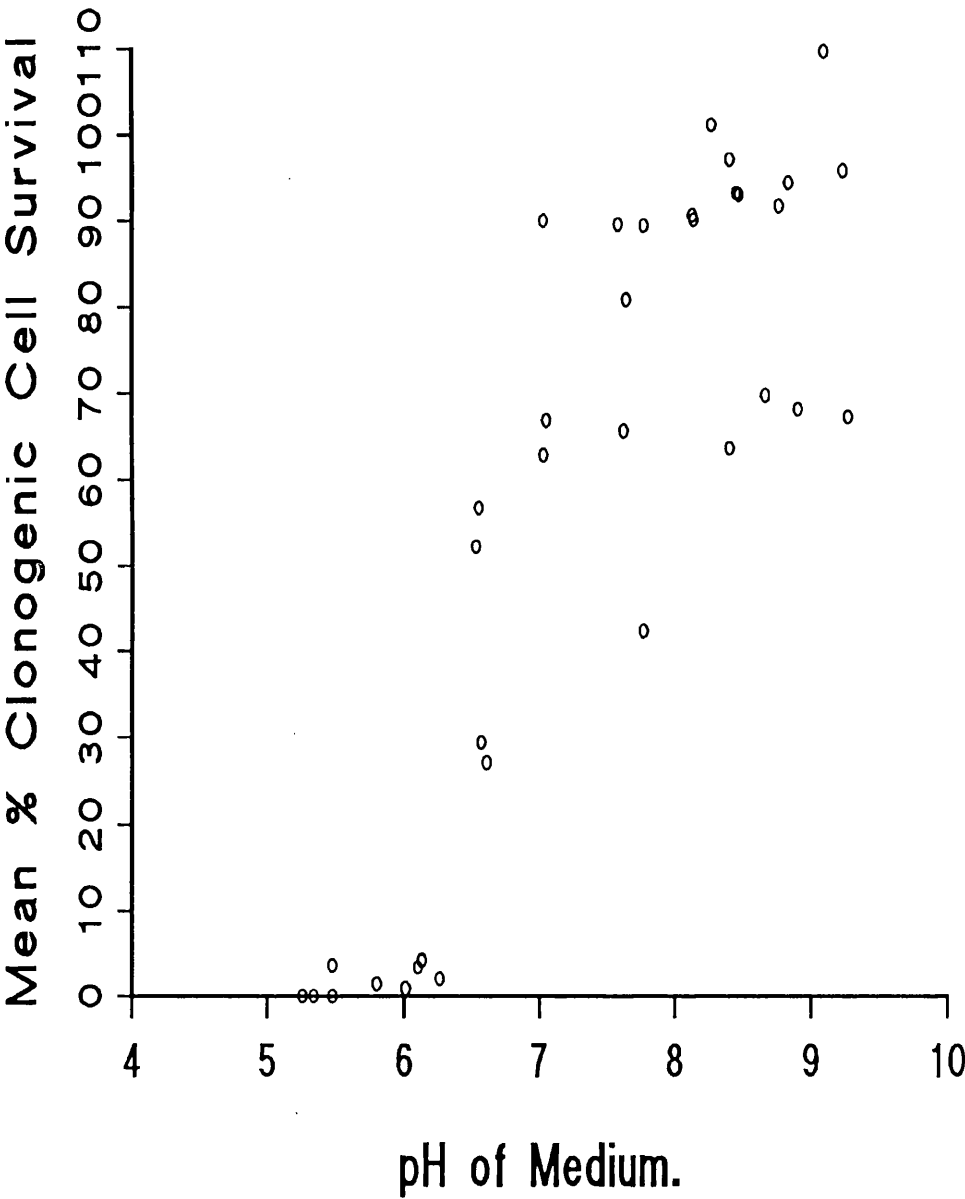
FIGURE 18

SCATTERGRAM OF SURVIVAL OF HU609 AS A FUNCTION OF "AFTER pH"
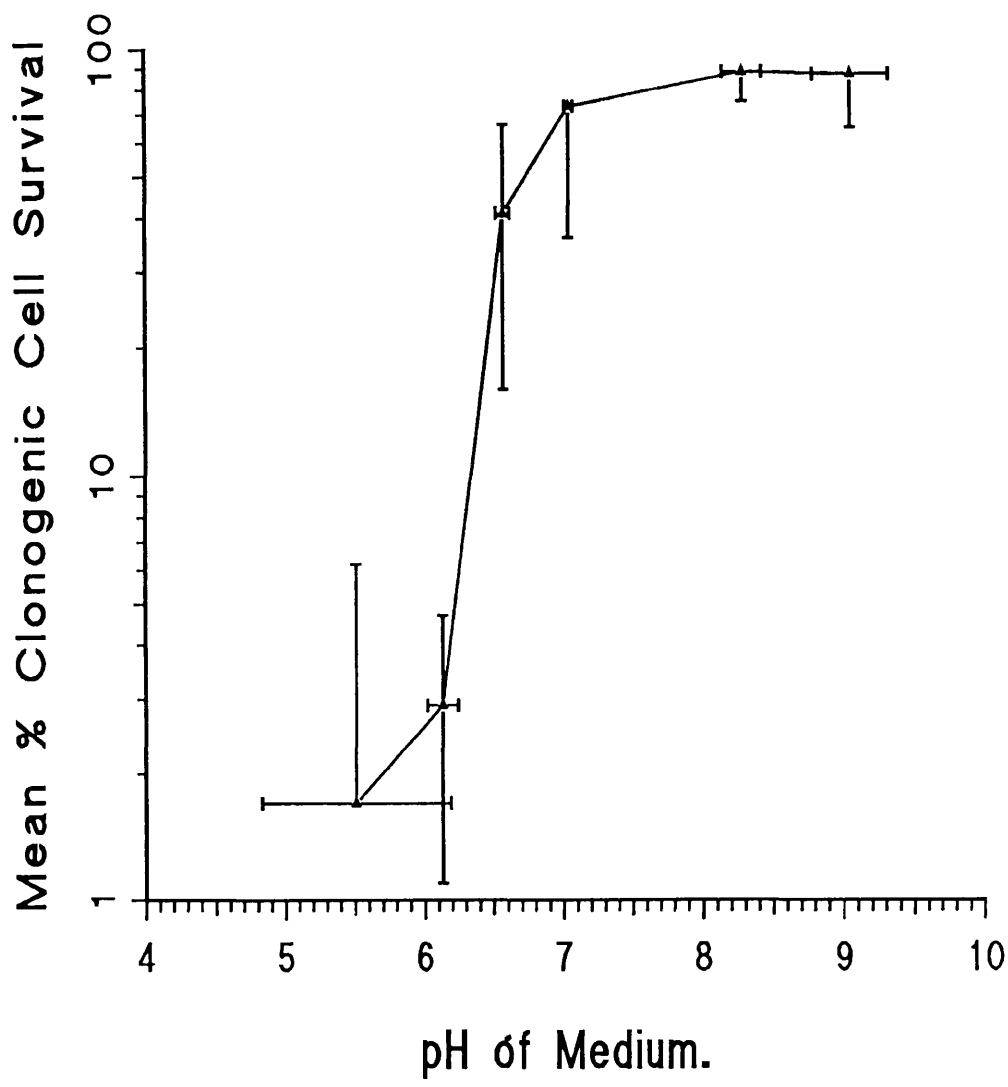
NB: The survival at pH=5.38 was 0 and could not be plotted on log axes.

FIGURE 19

MEANS (95% CONFIDENCE BARS) OF SURVIVAL OF HU609 AS A FUNCTION OF "AFTER pH"
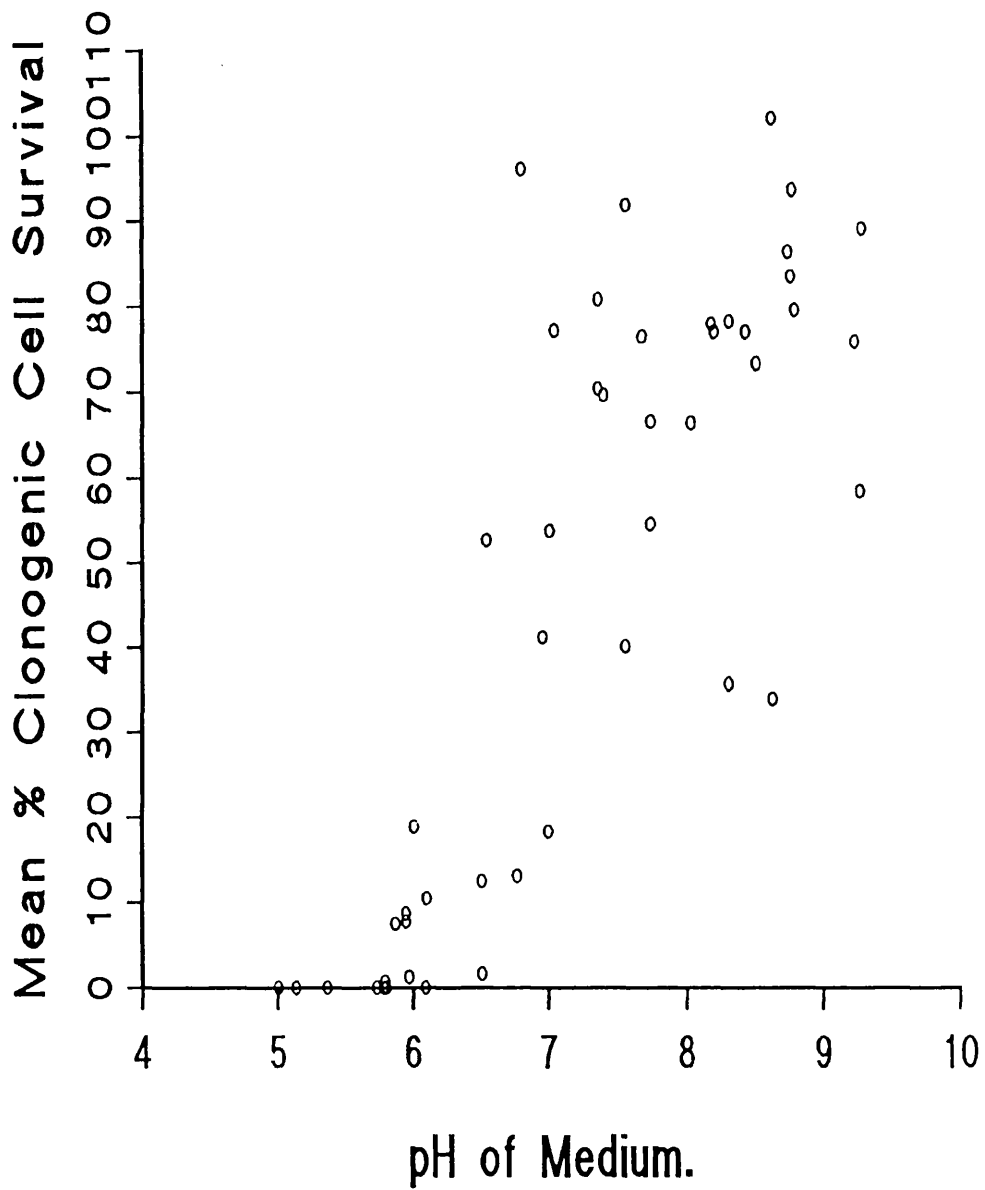
FIGURE 20

SCATTERGRAM OF SURVIVAL OF RT112 AS A FUNCTION OF "BEFORE pH"
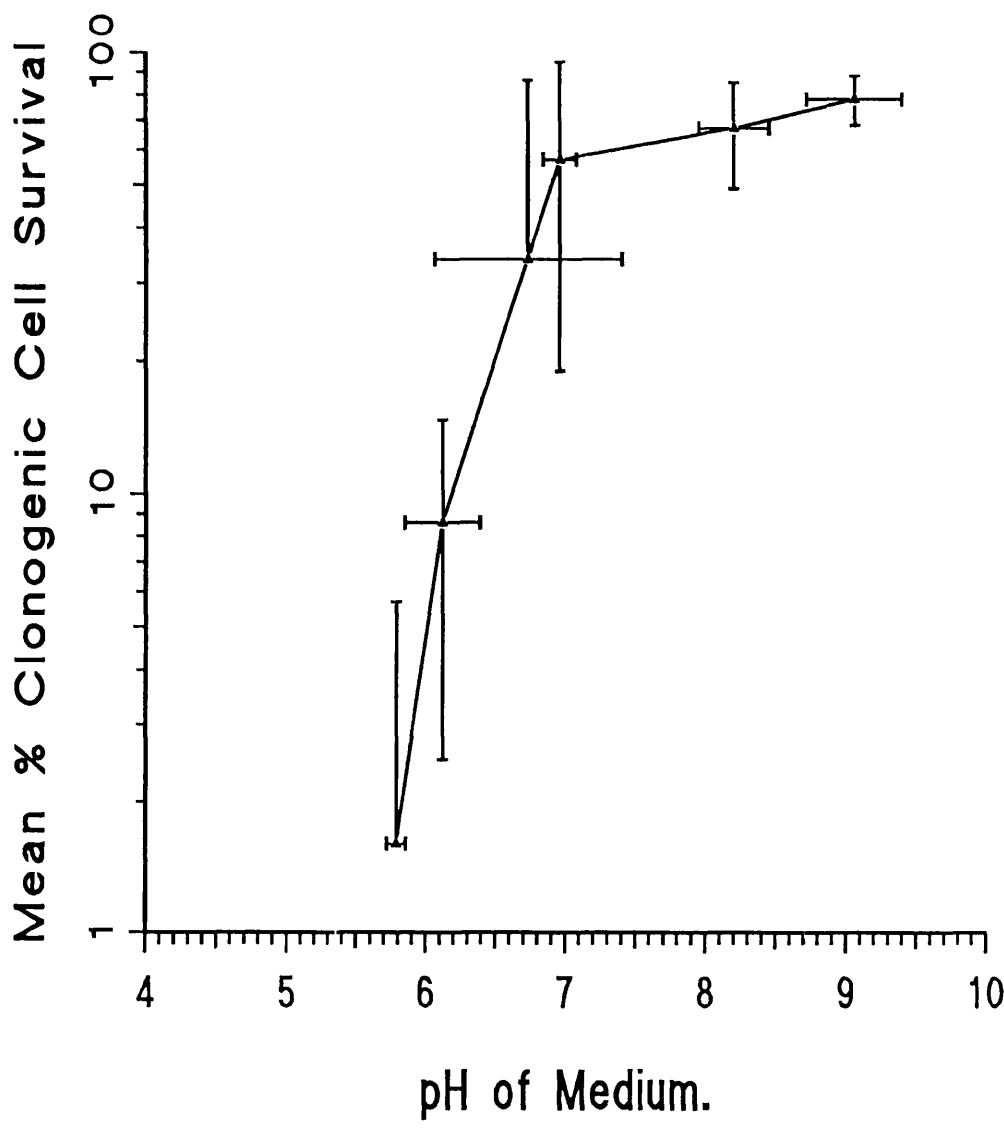
pH of Medium.

NB: at pH=5.16, the Survival was 0, and could not be plotted on log axes.

FIGURE 21

MEANS (95% CONFIDENCE BARS) OF SURVIVAL OF RT112 AS A FUNCTION OF "BEFORE pH"
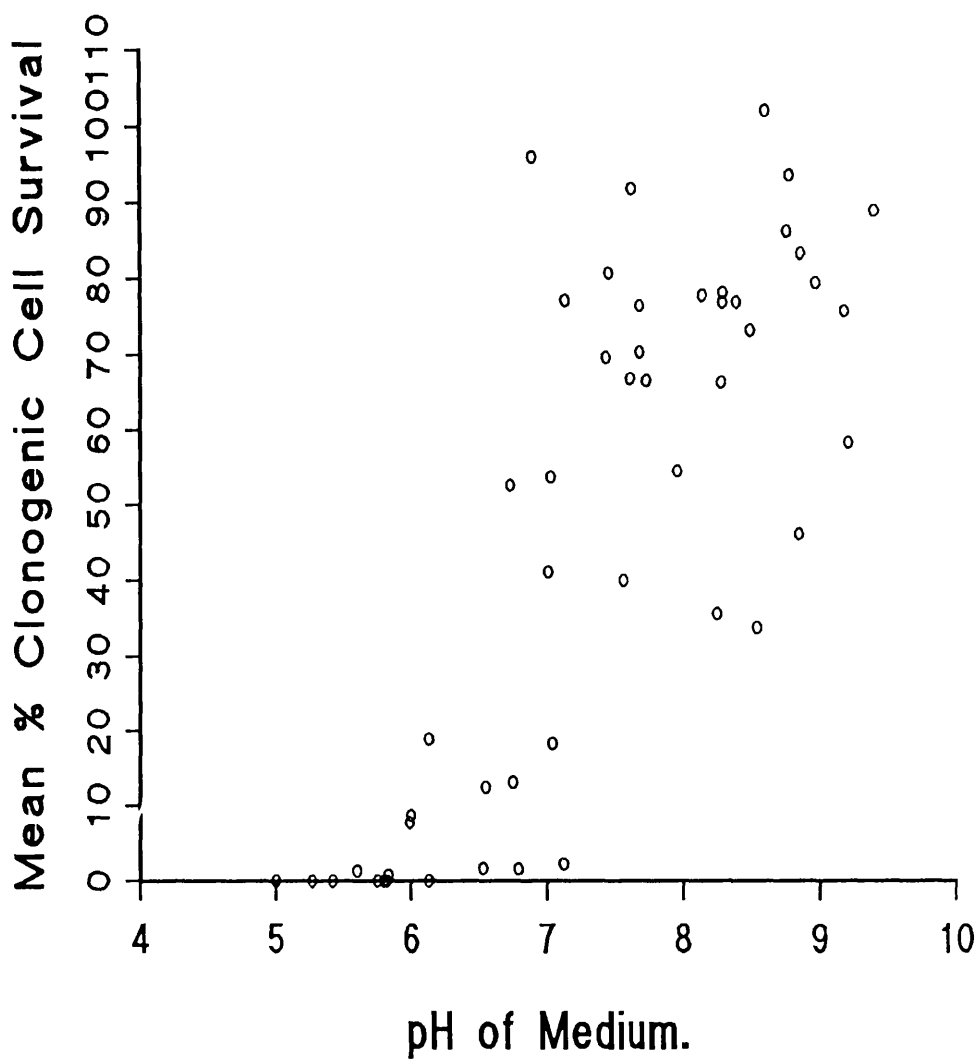
FIGURE 22

SCATTERGRAM OF SURVIVAL OF RT112 AS A FUNCTION OF "AFTER pH"
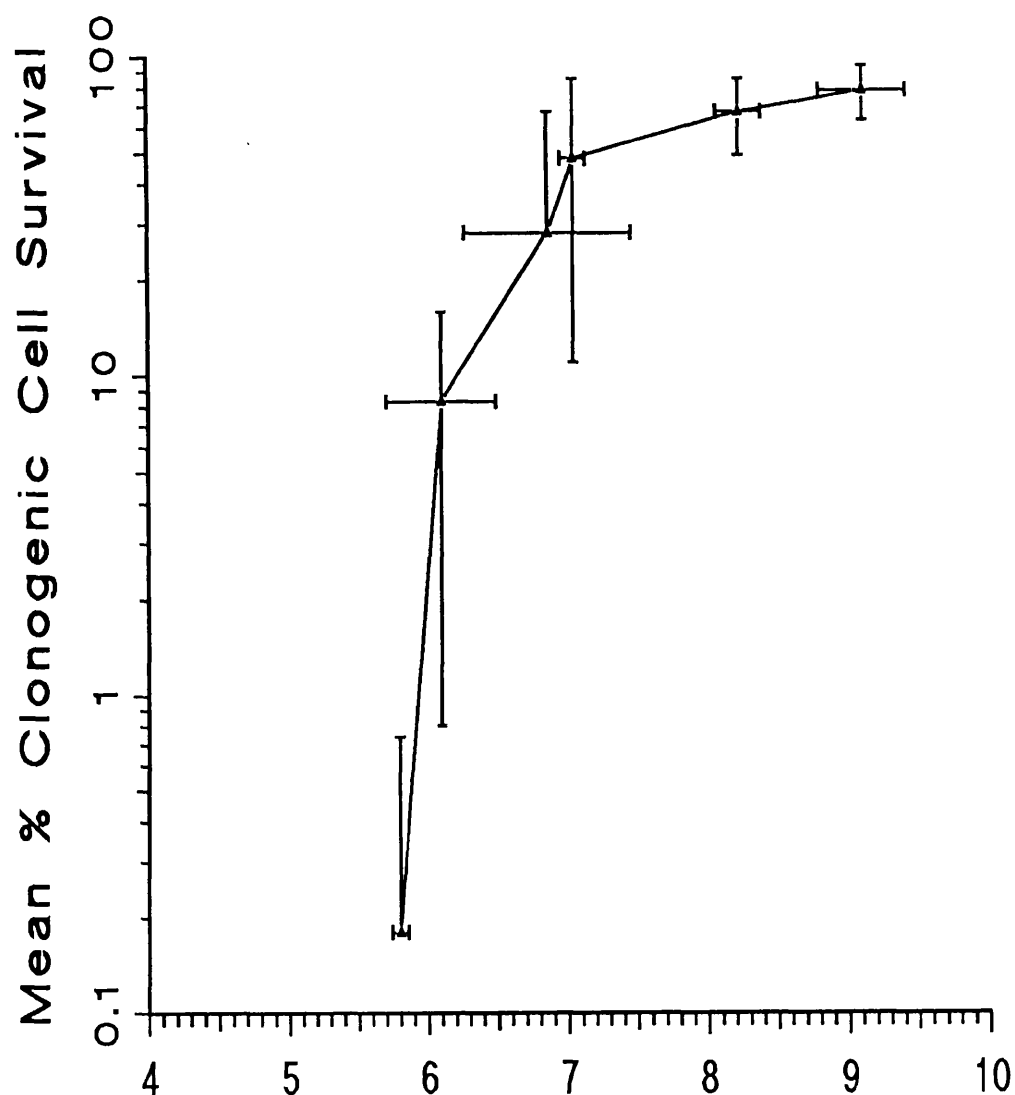
pH of Medium.

NB: at pH=5.23, the Survival was 0, which could not be plotted on log axes.

FIGURE 23

MEANS (95% CONFIDENCE BARS) OF SURVIVAL OF RT112 AS A FUNCTION OF "AFTER pH"
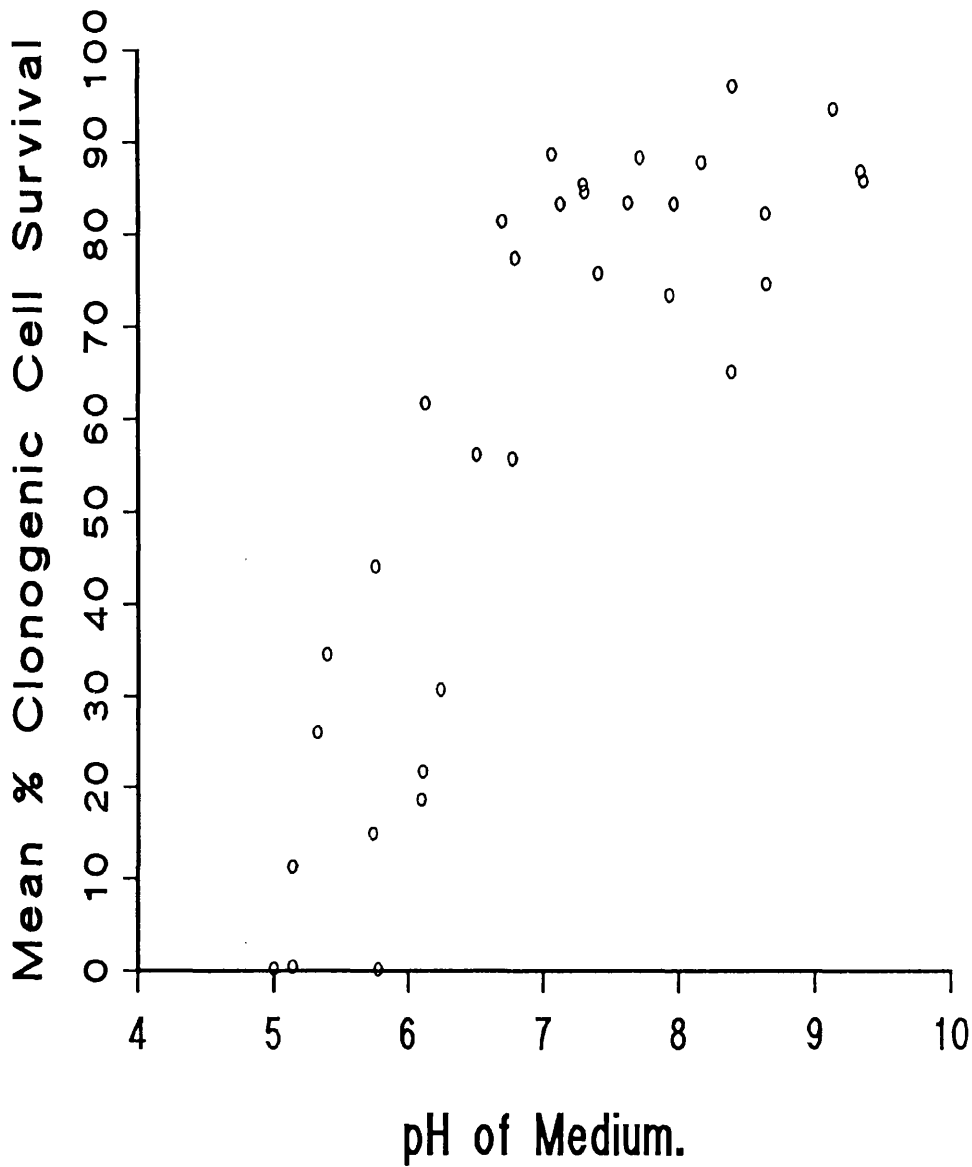
FIGURE 24

SCATTERGRAM OF SURVIVAL OF MGH-U1 AS A FUNCTION OF "BEFORE pH"
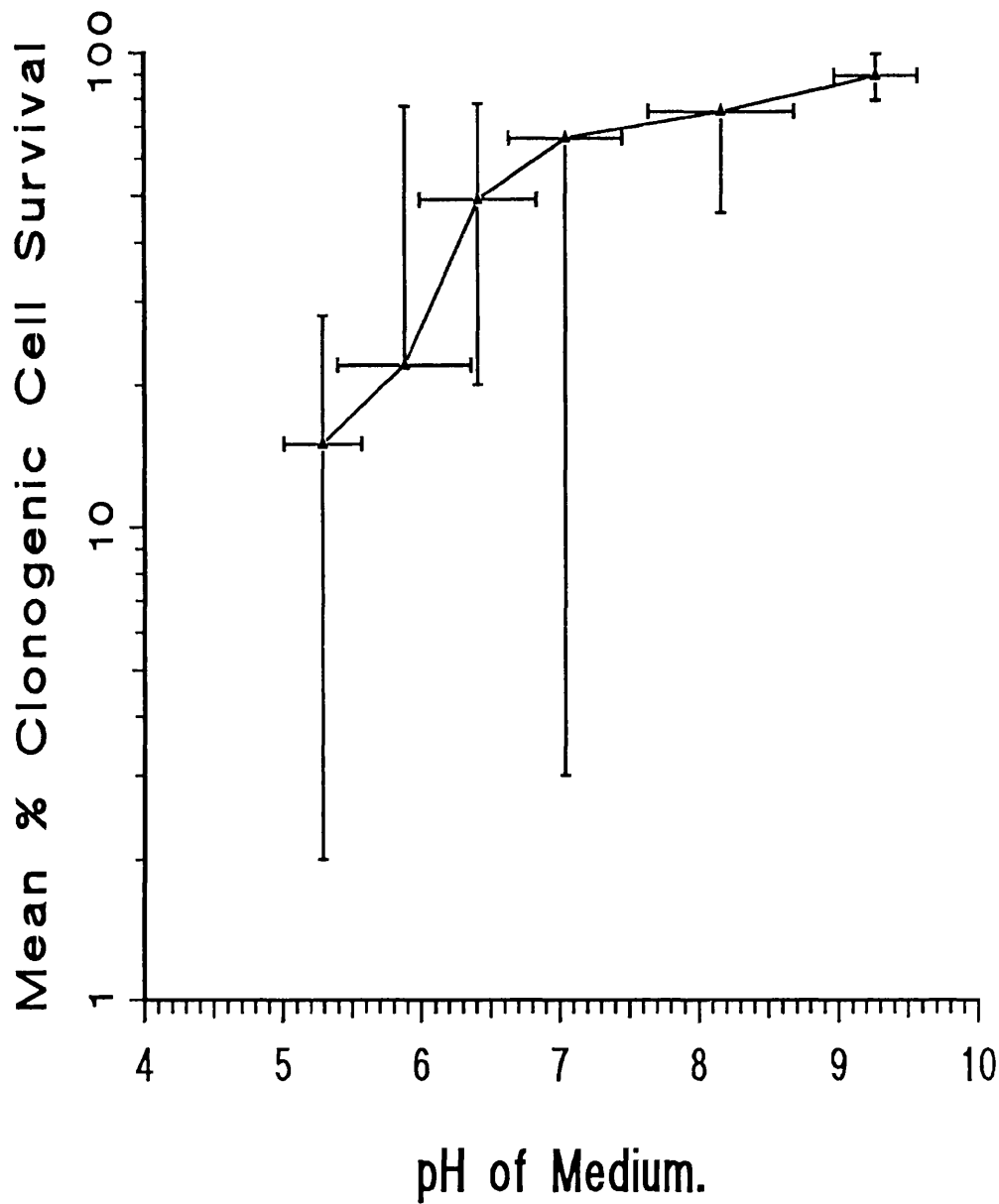
FIGURE 25

MEANS (95% CONFIDENCE BARS) OF SURVIVAL OF MGH-U1 AS A FUNCTION

OF "BEFORE pH"
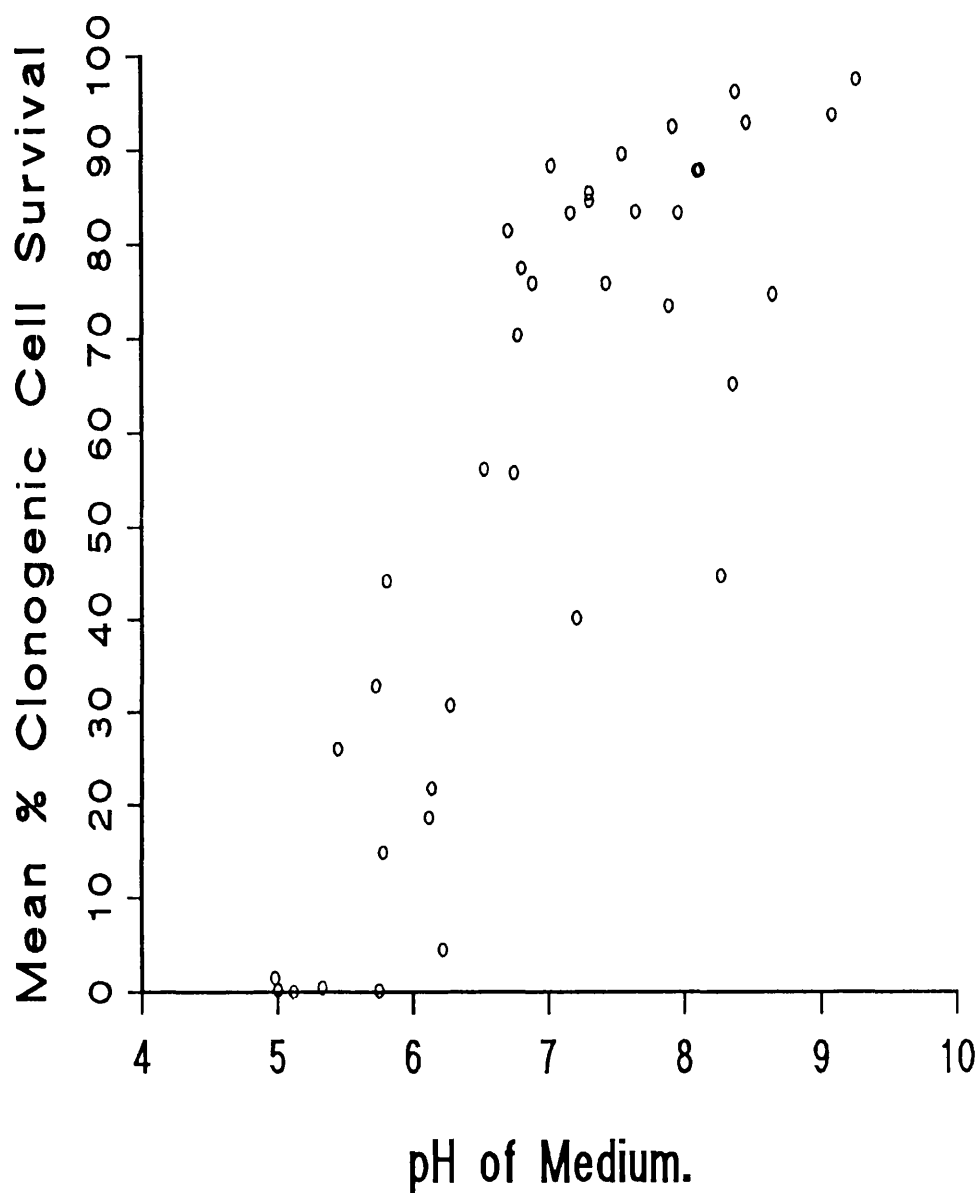
FIGURE 26

SCATTERGRAM OF SURVIVAL OF MGH–U1 AS A FUNCTION OF "AFTER pH"
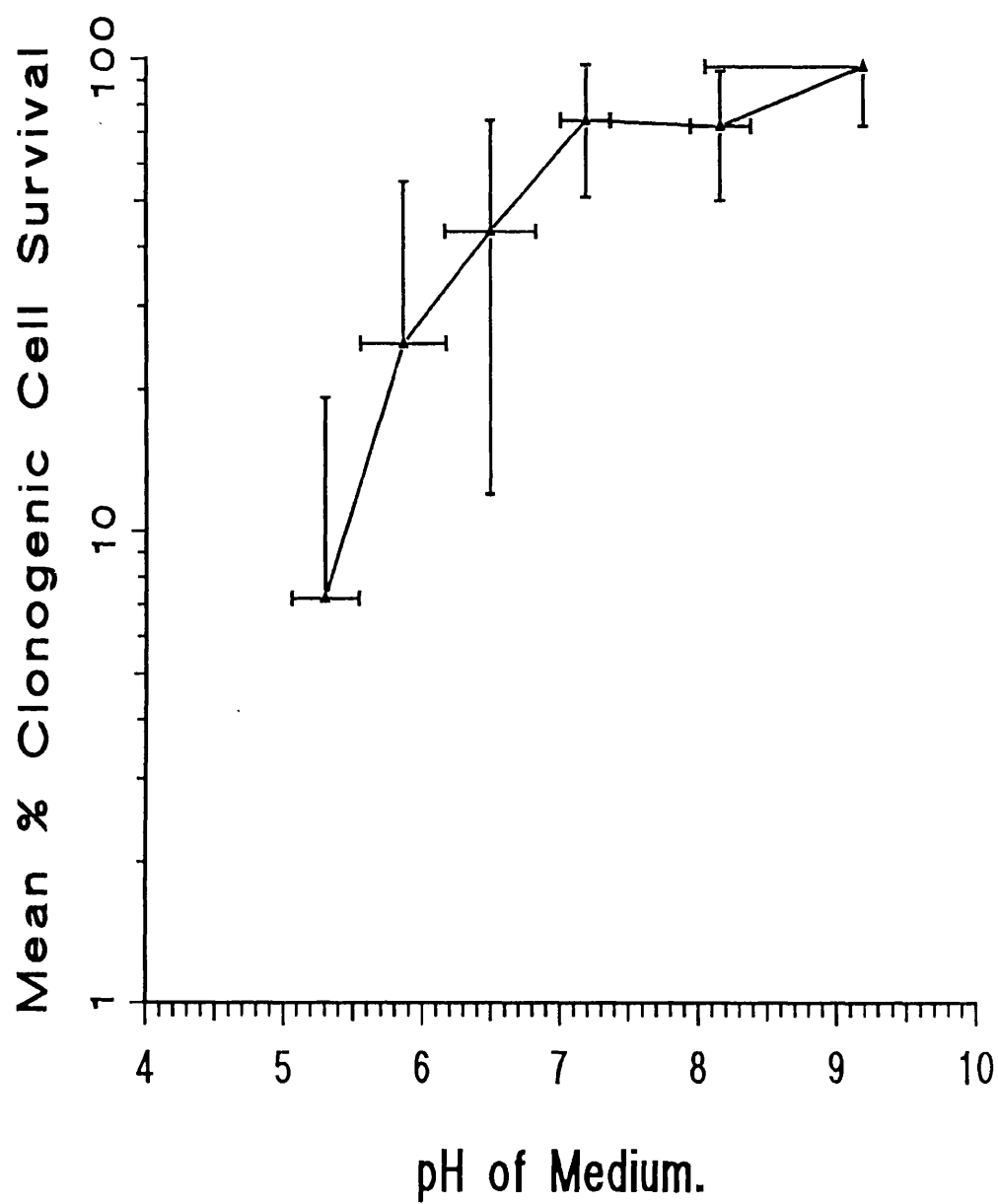
FIGURE 27

MEANS (95% CONFIDENCE BARS) OF SURVIVAL OF MGH-U1 AS A FUNCTION OF "AFTER pH"

DISCUSSION

In vitro, acid instillates decreased the Mean % Clonogenic Cell Survival of human urothelial cells exposed to 20 mcg/ml ThioTEPA for one hour, below the Survival of cells exposed in alkaline conditions.

The thioTEPA concentration used allowed some scope for the demonstration of an unexpectedly enhanced survival, ie a reduced drug effect, but left a wider range of values for increased cell kill at lower pH. Repeating the experiments with a lower concentration of thioTEPA might reduce the effect of errors in pipetting thioTEPA. On the other hand, this might extend the plateaux of the Survivals to the left, masking the effect of pH. It is particularly compelling evidence of the importance of pH in this model that, despite a choice of thioTEPA concentration that might have had no effect in HU609 and MGH-U1, the influence of pH on Survival was marked. Although the osmolarity of the buffered Medium was not measured after preparation, the amounts of acid or alkali added to adjust the pH were relatively small, and the main opportunities for variations in the osmolarity would have arisen in the preparation of the Medium. The osmolarity of batches of RPMI 1640 prepared and sterilized by the preparation unit at the Imperial Cancer Research Fund's facilities at Lincoln's Inn Fields, London, was checked routinely before use. Higher osmolarity has been found to decrease cytotoxicity in RT112, using the assay used here (Groos et al., 1986(b)).

The log scale used for the survival (ie colony-forming ability) beter reflects the logical difficulties of assessing zero or negative survival, than would a linear scale, but excludes some zero survival data given in tables 20-25, fpp 107-117, and in the scattergrams, especially at a pH of 5.0. If, as discussed above, a lower concentration of thioTEPA had been used, this difficulty also would have been avoided. In the clinical situation, it is hoped that zero tumour suvival would be a common event.

The variation in the pH values during the course of an exposure was small, and unlikely to bias the results (tables 17-19, fp 102-104). The day-to-day variation in actual pH at each Nominal value was larger, perhaps because of variation in the preparation of the medium. As adjustments to the pH of buffered Medium were avoided wherever possible, the errors in measured pH were perhaps larger than would have been ideal. Measurements in pH were particularly difficult in the Control Medium, which was unbuffered, and which, while incubated would contain dissolved CO_2 as carbonic acid. When samples, particularly small samples, were taken for pH measurement, and kept for even half an hour before measurements were taken, CO_2 was lost, even in a stoppered test tube, into the air space, causing the measured pH to be higher than the reality during incubation. The influence of the pH of the instillate on the effect of thioTEPA instillations has been observed in two bladder cancer cell lines, and in a cell line derived from histologically normal urothelium from a patient with a renal clear cell carcinoma (HU609). This confirms the

initial study in RT112 (Groos et al., 1986), and demonstrates that the effect is not a property of one cell line only. In this and in the previous experiment, the colony-forming ability retained at each pH is not expressed as a percentage of that of cells at standard pH, but of cells growing in control dishes at the appropriate pH, eliminating the known effect of pH on cell growth and survival (Eagle, 1973; Ceccarini & Eagle, 1971; Taylor, 1962). Recent studies demonstrate that pH has an effect on the activity of anticancer drugs on murine colonic tumours (Phillips et al., 1988) and on Walker 256 carcinosarcoma cell lines (Jauhiainen, Kangas, Kapyla et al., 1985) in vitro, although the latter method showed no increased effect from thioTEPA exposures at pH below 7.0. Control of pH in the bladder instillation seems to have immediate clinical application. Past studies of clinical effectiveness and toxicity may need to be reassessed, taking this variable into account.

CHAPTER 6

PILOT STUDY OF THE EFFECT OF AMILORIDE ON ThioTEPA CYTOTOXICITY AT LOW pH

Amiloride has been in clinical use as a diuretic for a number of years. It affects the control of intracellular pH by blocking Na^+/H^+ exchange across the cell membrane (Rotin, Wan, Grinstein et al., 1987). As thioTEPA had been found to be more effective in acid Medium, studies were undertaken to see whether blocking of intracellular pH-regulation by the addition of this drug might enhance the effect of thiotepa at low pH on the cell line model of superficial bladder cancer.

MATERIALS AND METHODS

MATERIALS AND METHODS: Cell Lines

The lines used were uncloned HU609, MGH-U1, and RT112, in exponential growth, as in the previous pH experiments.

MATERIALS AND METHODS: Drugs

Pure amiloride powder (MSD, UK) was diluted in water for injections (Beecham, UK) to a concentration of 100 mM, and kept at -70^0C in glass bijou containers until use. Pure ThioTEPA powder was supplied by Lederle, and diluted and stored as for the pH experiments. Drugs were diluted with Medium or buffered Medium immediately before each experiment. Actual pH was not measured, and the figures given are the nominal values for the formulations

used in the pH experiment, as the differences between pH 5 and 7 were likely to be obvious if present. The buffers used were those in the pH study (chapter 5).

MATERIALS AND METHODS: Assay Method

From the previously available dose-response curves of the cell lines to thioTEPA, a concentration of 8 mcg/ml thioTEPA was chosen to give the smallest appreciable effect, in order to give a large range of cell survival within which synergy might be detected. Cell survival was measured once for amiloride concentrations ranged above and below those found to affect regulation of intracellular pH (Roos & Boron, 1981). A concentration of 0.1 mM amiloride was selected for the pilot studies.

Cells were plated to give approximately 200 colonies in control dishes not exposed to amiloride ot to thioTEPA. Initially, dose-response below and above the 0.1 mM Amiloride concentration used by Rotin,D et al. (1987) was measured at standard pH (= approximately 7.4) in a 1-hour exposure.

To assess the combination of amiloride, thioTEPA, and low pH, Medium was buffered and equilibrated and used to wash the unbuffered Medium from the dishes as in chapter 4. Dishes containing buffered Medium alone, and with amiloride, with thioTEPA, and with both were incubated for one hour at 36.5°C in air without added CO_2, aspirated, washed with Medium, and allowed to grow as in Chapter 2. Fixation, staining and counting of

Cell Line/ Passage	RN	Mean % Clonogenic Survival at Amiloride Concentration (mM)			
		0.06	0.1	0.4	1.0
HU 609/41	25/3	100	95	99	94
MGH-U1/116	26/3	114	113	139	154
RT 112/44	26/3	109	110	109	97
RT 112/45	6/4	90	77	---	59
RT 112 mean		100	94	109	78

TABLE 26

MEAN % CLONOGENIC CELL SURVIVAL AND AMILORIDE CONCENTRATION

colonies were as described previously.

RESULTS

The minimal reduction in mean % colony-forming ability (mean % clonogenic cell survival) following exposure to a range of concentrations of amiloride are given in table 26, fp 124. Only one replicate of the experiment was carried out for HU609, and MGH-U1, and two for RT112.

The mean % colony-forming ability after exposure to amiloride and thioTEPA at pH 5 and pH 7 is detailed in table 27, fp 125. Addition of 0.1mM amiloride produced no large reduction in survival.

DISCUSSION

The absence of an effect might have been related to an inappropriate choice of amiloride concentration. Experiments using higher concentrations would be needed to clarify this.

As the stock thioTEPA had already been used with effect, loss of drug potency in storage or preparation was unlikely although it could have affected the amiloride. The low concentration of thioTEPA used might have masked a real, but small reduction of survival, perhaps up to 30%, but such a synergy would be of limited practical application.

Cell Line	pH	Number of Replicates	Amiloride	ThioTEPA	Amil + Thio
RT112	5	1	81	---	86.6
	7	0	---	---	---
MGH-U1	5	2	102	89.7	106.6
	7	1	89.0	67.2	66.2
HU 609	5	2	98.1	82.8	72.6
	7	3	102.7	106.3	94.7

TABLE 27

MEAN % CLONOGENIC CELL SURVIVAL WITH AMILORIDE, THIOTEPA, AND THE
COMBINATION OF BOTH (AMIL+THIO)

There could potentially have been a lack of susceptibility of the cell lines used to the effects of amiloride. The Na$^+$/H$^+$ exchange of MGH-U1 is known to be affected by amiloride (Rotin, et al., unpublished observations quoted in Rotin et al., 1987), so this seems improbable. Although pH was not measured, the differences in thioTEPA effect previously observed were large between pH 5 and 7 (chapter 5). The colour indicators in the Medium showed no change in pH. It may be that the acidity of the extracellular environment, combined with amiloride was insufficient to affect the intracellular environment in the time of the exposure. The previous studies (Rotin et al., 1987) used a six-hour exposure, which would not be a clinical option. They found a greater effect when amiloride was combined with the bicarbonate exchange blocking agent DIDS (4,4'-diisothiocyanostilbene 2,2-disulphonic acid) and the ionophore nigericin, which allows the exchange of intracellular K$^+$ for extracellular H$^+$. When amiloride is used alone, in a 1-hour exposure, less effect might be expected. Reduced intracellular pH, and hence altered response to thioTEPA might have been found if the amiloride had been added at low pH some time before the thioTEPA. This would be more difficult to achieve in vivo than addition of amiloride to intravesical instillations. Further, there did not appear to be any differential between tumour cell lines and HU609, which would be particularly beneficial in intravesical chemotherapy. Measurements of intracellular pH would have been interesting.

As amiloride is already in clinical use, it was considered

appropriate to assess it, rather than the other agents, in a pilot study. No data were obtained that encouraged further investigation.

CHAPTER 7

DISCUSSION OF METHODOLOGY

TISSUE CULTURE METHODS

The clonogenic assay employed is a variant on a widely-used survival assay, which measures cytotoxicity as the loss of reproductive ability, which has a clinical relevance beyond mere metabolic survival. It is conceivable that local destruction of vital structures or functions might occur in the absence of a reproductive capacity, but in practice this is rare. Other tests used to measure drug effect, such as the MTT assay (Mossman, 1983), or ATP-bioluminescence (Jauhiainen et al., 1985), with the advantage of rapidity of testing, depend on the absorption and reactions of other agents. This indirect measure might theoretically indicate positive after a recoverable metabolic insult, or negative in the presence of a DNA lesion which would prove lethal after a number of cell cycles (Tidd & Paterson, 1974; Fairchild, Maybaum & Kennedy, 1986; Larramandy & Lopez-Larraza, 1989).

Clonogenic assay methods remain to be standardized, both as to the use of feeder cell layers, and to the point at which drug exposure and trypsinizing for cell plating occur. In RT112 exposures to adriamycin and methotrexate, feeder cells appear to make no difference (Hepburn & Masters, 1986), but immediate trypsinization reduced the effect of methotrexate at higher concentrations, by comparison with plating after 24 hours. Data

on thioTEPA were not available, but the method used was the standard used in the Institute of Urology's laboratory. Using short drug exposures at 48 hours' interval after plating of exponentially-growing cells avoids the drug deterioration possible in continuous exposures, and the variable growth rate and growth fraction of cells in monolayers approaching confluence, more likely in transfer assays. The potential bias from interaction between trypsinization and drug treatment (Hepburn & Masters, 1986) is also avoided. On the other hand, cellular co-operation, which may be important in vivo, is minimised in the in-situ assay.

The test of drug sensitivity after the model course of intravesical thioTEPA can be criticised for its large day-to-day variations in survival, and for its use of a single drug concentration. The variation could have resulted from inaccurate pipetting of thioTEPA, either as a result of poor technique, of arithmetical errors in dilution, or of misidentification of stock concentratons; from degeneraton of drug in storage; or from variation in the growth status of the cells tested. As far as possible, cells were used before reaching confluence, while still in logarithmic growth. There was no evidence of a systematic decline in drug effect, and it was more likely that random pipetting errors led to variatons in the actual concentrations used.

The choice of a single value in the assessment of resistance was

based on expediency. Occasionally, there appear to be crossovers in drug dose-response curves of different cell lines, but they are sometimes less real than aparent, due to the use of standard error bars instead of 95% confidence intervals. Nonetheless, it would have been more convincing if it had been possible to carry out full dose-response curves for treated cells and controls of both types, preferably in a paired fashion, for comparison of the areas under the curves.

Because of the paired technique used in the assay of sensitivity, the power ofthe replicates carried out was considerably incresed, and the effects of day-to-day variations were reduced. For each comparison of Parent, Control, and Treated cell lines, a single preparation of Medium was used both for the drug-free control dishes, and to dilute the single container of test thioTEPA used for dishes of all sublines compared on one tray. Separate preparations were used for HU609, RT112 and MGH-U1 on each day, to avoid cross-contamination. Separate trays, eg of MGH-U1, were not paired, as different Medium and thioTEPA solutions were used, and were not similarly compared, even although an unpaired test would have been applicable, as it was obvious that the daily variation would swamp any drug effect. Although comparisons of different lettered sublines of Control and Treated cells would have been equally valid, the method used compared like letters for simplicity's sake, thus avoiding confusion of flasks.

In the model of chemotherapy, drug exposures required higher cell

densities than control exposures, to allow for cell losses from treatment. It was expected that the lower plating densities of the control flasks produced a similar number of surviving cells at the next subculture.

The results of tests of resistance in HU609 are inconsistent, but the pattern in the three cell lines, if they can be taken together, indicates a trend to development of resistance after a model course of intravesical chemotherapy. If resistance was not induced in HU609, then the difference found in T_B and T_{mean} compared with Control cells might be random, with a probability less than 0.08. Alternatively, the lack of a difference between Control cells and T_A and T_C cells has a probability less than 0.3. The T_B flask, compared with the Parent cells, had a p-value of 0.11, whereas the other Treated flasks showed much higher figures. This may indicate a peculiarity developed in T_B, such as a mutation or other low-frequency event inducing resistance. Studies with less variability would be of considerable interest.

In either event, the statistically significant difference between Parent and Control flasks remains to be explained. It might reflect an increase in sensitivity with passage, that was reversed by the model course of thioTEPA, measurably in T_B, but below threshold in other flasks. Such a change in sensitivity was not observed in MGH-U1, but this does not preclude it in HU609. The survival of uncloned HU609 cells after a 1-hour exposure to 40 mcg/ml commercial thioTEPA was 45%, 95% confidence

interval (13,77%), and cloned, 10.7(0,42.8)%, after exposure to unbuffered thioTEPA. The tests of uncloned HU609 were not intended for comparison with the resistant sublines, but to indicate a suitable concentration for the induction of re-sistance. A comparison of uncloned HU609 with C_A, C_B and C_C of HU609 would be very interesting.

The DMSO used in freezing is a known differentiating agent (Borenfreund et al., 1975; Kim et al., 1980; Mickey et al., 1983), but its influence on cell lines (chapter 6) when combined with thioTEPA at the time of exposure is likely to be neutral or to reduce survival. It might, however, be made greater by virtue of the fact that the DMSO exposure preceded the thioTEPA exposure (chapter 6).

As the Control and Treated sublines were maintained in parallel, differences in confluence or growth phase of Control subline cells at the time of testing seem unlikely. The survival of the Control subline, 43(34,52)%, was comparable with that of the uncloned cells to commercial thioTEPA, 41(28,54)%, although the effects of the commercial buffer and the cloning cannot be distinguished from the available data. The expected effect of the alkalinisation in commercial preparations would be increased survival over pure drug, so the data suggest that cloning may have reduced the survival of Control and Parent cloned cells exposed to thioTEPA. If so, the apparent increase in the survival of the Treated Cells is likely to be real. Paired comparisons of

the dose-response of uncloned, Parent, and Control cells with one drug formulation would be useful.

In any limited exposure assay, whether plating of colony-forming units occurs 2 days before exposure to the drug, as here, or after exposure, as elsewhere (Freshney, 1987), viable cells may be lost in the process of washing away the test drug. Unless the drug is postulated to have a cell-suspending effect in one subline preferentially, eg by selecting, during the model course of chemotherapy, cells that were likely to remain attached as well as viable, such a process cannot be significant in these data. A similar potential for cell loss might be postulated at the time dishes are washed and fixed for counting. In this case, there would need to be a difference between control dishes and drug-exposed dishes of the same subline, a difference that caused the loss of whole colonies rather than of individual cells within colonies. This might be a factor if the numbers of colonies per dish were small, or if the colonies contained few cells at the time of fixation, but again this seems improbable, as no major discrepancies were noted between the inspection of unfixed dishes and the colony counts.

The meticulous use of clean equipment, especially pipettes, for each flask in the model course of chemotherapy made cross-contamination within and between cell lines unlikely. This is further supported by the different senitivities of the three cell lines. Most significantly, isoenzyme testing and DNA

fingerprinting show no evidence of extraneous or contaminated sublines. He La cells have not been kept or isolated in the laboratory. Growth rate estimations, which would have been of particular interest in the HU609 comparisons, were not carried out for lack of time, but there was no marked difference in colony size as assessed by naked eye, between Control and Treated sublines, after the 1-hour exposure either to Medium alone or with thioTEPA. Behavioural changes seemed unlikely as an explanation for the data.

The absence of Control data limit the interpretation of the difference between Parent and Treated RT112 sublines. There is a difference of a factor of two, apparent in both Treated flasks. While this might represent the effect of passage, the evidence from HU609, if interpreted as due to passage, showed an increase in sensitivity in Control cells, which would be expected to amplify the factor of resistance in Treated cells compared with Control cells of similar passage. The survival of uncloned RT112 at a thioTEPA concentration of 40 mcg/ml was 4.3 (0.5, 8)%, much less than the cloned sublines, and does not illuminate the comparison of sensitivity. Indeed, it emphasises the need for caution when comparing cloned and uncloned sublines in general. The consistency of the sensitivity in both treated flasks provides no evidence of a low-frequency event, such as mutation is thought to be.

The day-to-day variation in survival was again notable, and again

the design of the study compensated. These results are strongly suggestive that a graduated resistance had developed to thioTEPA after a model course of intravesical chemotherapy in RT112 cells.

The difference in sensitivity following the model course of chemotherapy was clear in MGH-U1. It was not present in cells of high passage that were not treated with thioTEPA. This is consistent with the lack of effect of passage on drug sensitivity of MGH-U1 previously reported (Masters and Hepburn, 1986) despite evidence that MGH-U1 is polyclonal (Hastings and Franks, 1983). The survival of uncloned cells exposed to commercial thioTEPA in preliminary dose-response experiments is higher than either Parent or Control sublines, but has a wide confidence interval, which would probably mean that the apparent differences could have occured by chance. Use of a higher concentration of thioTEPA to compare Parent, Control and Treated sublines might have reduced day-to-day variations due to minor pipetting inaccuracies.

RESISTANCE: Characterisation

Although it has been reported that one clone at least of MGH-U1 does not form xenograft tumours (Hastings and Franks, 1983), tumourigenicity was not reduced in the sublines that were clearly resistant, or in the Treated RT112 or induced in HU609. The tumours induced were assumed to be of human origin, on the basis of previous findings (Masters et al., 1986). Uninjected control

mice did not grow tumours, but further controls injected with medium containing heat-inactivated or no cells would have helped implicate the cells in the genesis of the tumours. A veterinary pathologist might also have examined the lesions to characterize the species of origin. Differential staining with anti-human and anti-mouse antibodies would have been interesting. With these qualifications, it appears that there is no evidence of a change in xenograft tumourigenicity.

Morphological examination of the cell lines was rather superficial, and carried out by the present author, who has limited experience of histopathology. It was carried out rather to identify cross-contamination among the three cell lines, which have obvious differences in morphology, than to make a realistic comparison with the reported morphology of the cell lines (Masters et al., 1986). There were no changes during the couse of the experiment.

The isoenzyme analysis has been used to characterize cell lines and to distinguish them from vigorous contaminants such as HeLa cells (Povey et al., 1976). Isozyme identification of the cross-contamination of a number of apparently different cell lines has been confirmed by DNA fingerprinting (Masters et al., 1988). The distinctive type A glucose-6-phosphate dehydrogenase of HeLa cells was absent from all sublines, as has previously been reported for RT112 and MGH-U1 (Povey et al., 1976). Comparability between untreated and Treated sublines in respect of all of the

isoenzymes tested precludes neither the possibility that con-tamination had taken place with cells from an individual with a similar isoenzyme combination, nor that there might be changes in other enzymes or surface antigens during the model course of thioTEPA. Knowledge of the isoenzyme pattern of all lines used in the laboratory would have been of some help in excluding cross-contamination, but the DNA fingerprinting data identify the sublines with a high degree of certainty. For practical purposes, the Parent, Treated and Control sublines of each cell line were derived from the same person.

The karyology was not carried out to great depth, for lack of time, but it was a useful pilot study. Numbers of metaphase spreads examined were small, and no examination was made for double-minute abnormalities, or SERs, which have been associated with drug resistance. The lack of a distinction in karyotype was therefore hardly surprising.

The initial findings in A431 cells validated the technique as a potential method of quantification of cellular surface antigen expression, even with a relatively simple instrument and software. The lack of staining may have resulted from the age of the primary antibody, or from a lack of antigen in the cell lines studied. Unfortunately, the preliminary histological staining of A431 cell pellets was not carried out in any of the urothelial cell lines.

DISCUSSION: pH

In this study, the colony-forming ability of cells exposed to thioTEPA for an hour at each pH was expressed as a proportion of the colony-forming ability of cells exposed to the same pH for an hour in the absence of thioTEPA. The "Control" exposure, made in laboratory standard conditions, and without buffers, expressed survival of dishes containing thioTEPA as a proportion of dishes containing Medium alone, incubated in an atmosphere containing 5% CO_2. "Control" data were obtained as a measure of the accuracy of thioTEPA pipetting, the influence of the osmotic effect of the buffers and of the consistency of the condition of cells tested, not as a denominator for the survival of other pH values.

The titration of the Medium with acid or alkali after the addition of buffer was a potential source of osmotic variability, and might better have been avoided. On the other hand, despite a tolerance of pH 0.15 pH units before titration was used, the errors in measured pH were minimal for each Nominal pH, and the variation during the course of the experiment was small, and unlikely to bias the results (tables 17-19).

The concentration of thioTEPA chosen was rather low for HU609 and MGH-U1, if one looks at the initial dose-response curves to buffered thioTEPA, but in the pH studies themselves, the mean survivals at pH 7.0 are between 60 and 80%, and offer a small range in which it was possible to observe a loss of drug

sensitivity, with more scope for observing the reduced survival that was expected at low pH (Groos et al., 1986). In fact, there might have been some advantage in repeating the experiment with higher concentrations of thioTEPA and higher colony numbers, to reduce the effects of pipetting errors on the confidence interval of the survivals. Higher colony numbers plated in larger dishes would also have given more accurate measurement of survivals between 10^0 and 10^{-2}. The use of lower thioTEPA concentrations, while adding to the pipetting errors, might have increased the contrast between pH 5 and pH 7, and avoided the zero survivals at pH 5. Although the effect of acidity on the effect of thioTEPA might have been better elucidated by testing more points in the acid range, the effect seems clear.

In two bladder cancer cell lines, and in a cell line derived from histologically normal urothelium from a patient with a renal clear cell carcinoma (HU609), the pH of the medium during a 1-hour exposure to thioTEPA influenced the mean colony-forming ability. This confirmed the original study in RT112 (Groos et al., 1986), and demonstrated that the phenomenon was not an idiosyncrasy of that line.

AMILORIDE

The pilot assays of amiloride were not conclusive in any way, because of shortage of data, particularly control data.

It would have been more helpful to have concentrated on a single line. In view of the increased effect of thioTEPA at low pH, and of the day-to-day variations above, a low concentration of thioTEPA was used, possibly too low to produce any efffect even at acid pH. Although the concentration of amiloride was comparable to one found to be effective in MGH-U1 by others (Rotin et al., 1987), it might not have been adequate in the other lines used, or even in MGH-U1 in the assay system employed here. The six-hour exposure used by the earlier authors would not have been practical in patients, and might have led to deterioration in the effect of the thioTEPA.

DIMETHYL SULPHOXIDE

Solvents may increase the effect of anticancer drugs. As a potential adjuvant to intravesical treatment, DMSO had the advantage that a 50% solution was already in clinical intravesical use for the treatment of interstitial cystitis, so any positive interaction could directly be tested in vivo. Previous findings in animals (Thuning et al., 1983; Warren et al., 1975) and in some mouse tumour cell lines (Tofilon et al., 1985) prompted the hypothesis that the addition of DMSO to bladder instillations might increase the effect of anticancer drugs on human cell lines. In vitro observations on the enhancement of drug effect by combination with another solvent, Tween 80 (Parris et al., 1987) are supported by clinical data showing enhancement of the effect of adriamycin combined with Tween 80 in

intravesical treatment of bladder tumours (Eksborg, Edsmyr & Nasland, 1982).

The enhanced cytotoxicity observed with DMSO in animal models and the mouse hepatocarcinoma cells in vitro may represent a different phenomenon from the potentiation of antitumour effect seen with the addition of Tween 80, which is thought to act by increasing membrane permeability to drugs (Eksborg et al.,1982; Parris et al., 1987). Tween 80 was most effective with larger, relatively impermeable, ionised drugs, such as adriamycin. Although it was tenable that a dipolar solvent such as DMSO might have a similar effect, the present data do not support this hypothesis.

An alternative hypothesis concerns non-solvent properties of DMSO. In tumour cell lines, DMSO has been found to induce differentiation (Borenfreund Steinglass, Korngold et al., 1975; Kim, Tsao, Siddiqui et al., 1980; Mickey, Meadows, Vassiliades et al., 1983). Enhancement of cytotoxic drug effect was seen in murine hepatocarcinoma cells (Tofilon et al., 1985) when cells had been exposed to DMSO for 48 hours before treatment with anticancer agents, and was not seen after exposures beginning simultaneously. The animals in which DMSO enhanced the effect of chemotherapeutic drugs had been given DMSO for 6 days previously (Thuning et al., 1983). It is possible that pre-treatment of patients with DMSO might increase the effect of chemotherapeutic agents. The characteristic smell of even small amounts of DMSO

would probably make adequate length of pre-treatment unacceptable to patients. This hypothesis was not, therefore, pursued.

The present author found no evidence that the addition of DMSO to instillations of four drugs commonly used to treat superficial bladder cancer increased their cytotoxic effect. It may reduce the effect of mitomycin-C. Drug enhancement in other models may result from DMSO-induced differentiation before drug exposure.

CHAPTER 8

CLINICAL IMPLICATIONS AND CONCLUSIONS

Transurethral resection of tumours (TUR) is the first line of treatment for superficial bladder cancer. Some authors have suggested that it may cause dissemination of the tumour within the bladder (Boyd & Burnand, 1974; Soloway & Masters,1980). Summarising a number of studies, TUR is followed by recurrence in 40% of 599 patients within 1 year (table 1, fp 21); 45% of 349 patients within two years (table 2, fp 22); and 69% of 261 patients within five years (table 3, fp 23). An estimate of 4% has been made for progression of tumour stage in the first year in T_a (Heney, et al., 1982), 6% in 3 years (table 4, fp 24), and 26% of 168 patients in 5 years (table 5, fp 25). The definition of progression and the duration of follow-up have been less consistent than for studies of recurrence. In patients with T_1 tumours, one year data are few, but progression occurs in 29% of 221 patients in 3 years (table 4); and in 31% of 203 patients followed for 5 Years (table 5). Mackenzie et al.,(1981) have reported that the rate of new progression was constant at around 10% per year. The survival of patients with superficial lesions has been reported to be similar to that of a similar age-group without bladder cancer (Nicholls & Marshall, 1956), although improvements in the general standard of operative management since the data were collected are likely to have affected this comaparison still more favourably. There is some doubt as to whether conventional treatment alters the course of the disease

favourably and clear room for improvement in the results of therapy.

INTRAVESICAL CHEMOTHERAPY: Rationale.

Intravesical chemotherapy with thioTEPA can clear the bladder of multiple superficial tumours (Jones & Swinney 1961; Veenema et al.,1962; Koontz et al., 1981) with complete response in 23-56% of cases, and partial response in a further 32-41% (Prout, 1984). There are also indications that intravesical chemotherapy may prevent invasive bladder cancer (Green et al., 1984).

The intrusiveness of follow-up for superficial bladder cancer might be reduced by drug prophylaxis or treatment, and the present studies were intended to develop that contribution. By delaying recurrences, and preventing or delaying invasion, the intervals between cystoscopies might be lengthened, and the need for resection under anaesthesia reduced. Eventually, intravesical instillations might be tested in the treatment of small or single recurrences being referred for resection, either as a therapeutic option, or with a view to prophylaxis against recurrence or later progression. There may also be symptomatic advantages. Episodes of severe haematuria following thioTEPA (Treible et al., 1987) are less common than after resection of bladder tumours. As Skipper has pointed out (Skipper, Schabel & Lloyd, 1978), even within the group of complete responders, there is scope for improvement in the cure rate. Combined with a viable out-patient

cystoscopy technique suitable for use in most patients, intra-vesical treatment might reduce the demands of follow-up.

Other less invasive methods of management exist, with different advantages and limitations. Laser coagulation, which coagulates 1-2mm diameter on each application, and seals the surrounding lymphatics, which may prevent metastasis, requires expensive equipment, and special eye protection for staff. Use of haemato-porphyrins results in some generalised photosensitivity, further reducing the quality of patients' lives. Out-patient intravesical therapy offers a less invasive, and possibly less expensive method of treatment.

Intravesical Bacillus Calmette-Guerin can postpone recurrence (Rodriguez-Netto et al, 1981) and clear carcinoma in situ (Harland, Charig, Parkinson et al., 1991), but produces profound symptoms of local irritation and systemic illness, which would be hard to justify in prophylaxis against recurrence unless there was also a lasting benefit, or a reduction in progression.

INTRAVESICAL CHEMOTHERAPY: pH

In rats which have been rendered acidotic by induced hypergly-caemia (Osinsky et al.,1988), Guerin tumours show an increased sensitivity to thioTEPA, as do murine colon tumours in vitro (Phillips et al., 1988). There is a report of conflicting findings in vitro (Jauhiainen et al., 1985) probably because of

the use of ATP bioluminescence, a marker of metabolic activity, as an indicator of cyotoxicity. Drugs that act on DNA may not have their maximum effect until the second cell cycle after exposure (Tidd and Paterson, 1974; Fairchild, et al., 1986; Larramandy et al., 1989). The present study of the pH of the instillate confirms the initial study of one human urothelial cancer cell line (Groos et al., 1986), which observed that acid instillates reduce clonogenic cell survival after exposure to thioTEPA, amongst other agents. The phenomenon was observed in cells derived from histologically normal urothelium and in urothelial cancer cell lines derived from moderately differentiated tumours.

The urine of patients receiving intravesical chemotherapy may have a pH greater than 6.0 in a third of instillations and tends to become more alkaline during the period of the instillation (Groos et al., 1986). As the pH of commercial thioTEPA dissolved in sterile water at 1mg/ml is 8.2 (Groos et al., 1986), it is likely that previous clinical results reflect the use of thioTEPA at other than its most effective pH. The reported comparative studies of thioTEPA with other intravesical agents, including BCG (Schulman et al., 1982; Rodriguez-Netto et al., 1981) may be invalid because the pH of thioTEPA instillations was not optimal. The 26% complete response rate for therapeutic use in multiple and recurrent superficial tumours (Heney et al., 1988) might be improved.

The degree of difference at pH 5.5 in the effect of 20 mcg/ml thioTEPA was around two logs of cell survival, with no evidence of a plateau, so the clinical benefit might be of practical significance. Although there are considerable differences in behaviour between human solid tumour growth/regression and that of the murine leukaemia L1210 (Yankee, De Vita, Perry et al., 1968; Young and De Vita, 1970; Clarkson,et al., 1967; Whang-Peng et al., 1971; Tannock, 1978; Perry, Moxley, Weiss et al., 1966; De Vita, Denham, Perry et al., 1969; Simpson-Herren, Sanford, Holmquist et al., 1974; Mendelsohn, 1960), the log-growth model Skipper proposed (Skipper, Schabel & Wilcox, 1964; Skipper et al., 1978; Skipper, Schabel, Mellet et al., 1950) is widely accepted as descriptive of tumour chemotherapy. Increases in log-kill are important in moving from partial to complete response, and from complete response to cure, and reducing the pH of thioTEPA instillations may contribute significantly to this in vivo, as well as in vitro.

Enhancing the anti-tumour effect of thioTEPA may also reduce its unwanted effects. The dose-rate for intravesical thioTEPA is limited by myelosuppression by absorbed drug (Soloway & Ford, 1983). Myelosuppression in systemic chemotherapy has been circum-vented by autologous marrow harvesting and cryopreservation before treatment (Antman, Eder, Elias et al.; Wolff , Herzig, Fay et al.,1990), but this is highly invasive compared with conven-tional management of superficial bladder cancer. An alternative that has been used with effect in the combination chemotherapy of

advanced bladder cancer is marrow support using granulocyte colony-stimulating factors (Yagoda, A, 1988), but this is untried in thioTEPA monotherapy, and is likely to be expensive for routine use. When administered at pH 5, the weight of drug given to achieve the same cytotoxic effect locally ought to be less, in a given volume of instillate. If the bladder acts as a semipermeable membrane to thioTEPA, lower intravesical concentrations would lead to less absorption, and so to less myelotoxicity. Absorbed thioTEPA would presumably be exposed to the marrow at a higher pH than in the bladder, although the pH of the marrow, even under resting conditions, does not appear to have been measured. Thus, less toxicity might be expected in the marrow exploiting the currently-used or perhaps even higher concentrations of thioTEPA, at acid pH, with one form of marrow protection. Regrettably, planned studies of absorption were not possible.

ThioTEPA decomposes at acid pH, but without loss of its alkylating activity. The effect on bladder cancer cells is increased. The quantity of drug absorbed across the bladder at acid pH, and its consequent myelotoxic effect remain unknown. The smaller products of decomposition could be expected to diffuse, if anything, more easily than thioTEPA, increasing marrow damage, but this might be offset by a lowering of intravesical dose-rates needed to achieve the same effect. Perhaps the use of granulocyte colony-stimulating factors to support the marrow would be a possible line of approach. This would be of no benefit in the use

of mitomycin-C or adriamycin, where the limiting toxicity is bladder irritation or contact dermatitis, although pH does influence the effect of these (Groos et al., 1986).

To apply pH buffering in vivo faces further hurdles. To the dilutional effect of urine production, might be added the variability of absorption and breakdown of thioTEPA at different pH or temperature (Cohen et al.,1984), and absorption or metabolism of the buffer used. Interference from substances excreted in the urine, such as methylxanthines (Fingert, Pu. Chen et al.,1988) remains to be tested. There exist buffer systems that have been used in vivo to attain the pH required, but would have a higher osmolality which might offset (Groos et al., 1986b) the potential benefits of pH optimisation. There is the great advantage that both buffers and thioTEPA have been used in vivo, and can be tested in humans without further preliminary tests.

Other approaches to enhancing intravesical thioTEPA therapy were less promising. The amiloride pilot investigation, while quite possibly subject to Type II error, did not show a large enhancement, say of one log. No useful conclusions were derived. Probably a combination of amiloride and the stilbene, DIDS, would be more effective (Rotin et al., 1987), but DIDS is not in clinical use. DMSO may be a useful adjuvant anticancer therapy in some studies, possibly by means of inducing differentiation prior to giving therapy, but no evidence was found to suggest that it could simply be added to the intravesical instillate in the way

that Tween 80 has been (Parris et al., 1987; Eksborg et al., 1982).

INTRAVESICAL CHEMOTHERAPY: Resistance

It remains to be explained how prophylactic intravesical chemotherapy acts, and whether it contributes to drug resistance. Prophylactic use of thioTEPA following resection has reduced the number of recurrent lesions per month of follow-up (Burnand, Boyd, Mayo et al., 1976), recurrence rate (Schulman et al., 1982) and proportion of patients with recurrence (Rodriguez-Netto and Caserta, 1981) and increased the disease-free interval (Koontz et al., 1981), although not consistently (Schulman et al., 1982). Some have found it beneficial mainly in Grade I lesions (Prout, Koontz, Coombs et al., 1983). Agents given at the time of resection have been interpreted as preventing implantation of free-floating but viable tumour cells (Boyd & Burnand). As the effect of this form of chemotherapy on recurrence is not on episodes of recurrence (MRC Working Party, 1985), but on time per recurrent lesion, it might be that these low dose-rates have a potential for the induction of resistance, but limited benefit to patients.

Single post-resection instillations may have a partly mechanical effect. Implantation of disturbed tumour cells, first suggested by Albarran & Imbert (1903) in renal transitional cell carcinoma, has been reported to occur in the vault of the bladder,

possibly carried up on bubbles of gas produced by resection (Boyd & Burnand 1974). Delay in instillation of anticancer agents has been observed to permit more implantation of tumour cell suspensions after diathermy in a murine model (Pan et al., 1989). This may indicate either that the chemical effect of thioTEPA or Mitomycin-C prevents implantation, or causes detatchment or death of newly-implanted tumour cells, or that the mechanical/volumetric effects of the instillation cause an early evacuation of unattached tumour cells, or a combination. These factors may not apply in treatment of unresected tumours. It may be postulated that, as viable tumour cells can be recovered from the urine more easily in higher-grade (less differentiated) tumours than in low-grade tumours, tumour cell adhesion is stronger in more normal cells. This might then be surmised to be a possible differential on which anticancer drugs might be acting. This hypothesis might be tested by filtering or spinning the supernatant after a thioTEPA exposure in vitro, washing the cells, and then re-plating them to see how many are viable. The results of a study of irrigation alone are awaited with interest.

The consequences of treatment with suboptimal dose-rates of intravesical chemotherapy were also explored in vitro. The simplest and most credible interpretation of the data is that less than a twofold reduction in sensitivity to thioTEPA followed a model course of intravesical chemotherapy. Marked resistance to other agents, such as adriamycin (Mc Govern et al., 1988) and cis-platinum (Bedford et al.,1987) has been found to follow

continuous exposure to fixed or escalating concentrations of drugs. With the intermitent regimen used for this study, which more closely approximates to clinical treatment schedules, a lower level of induced resistance is not surprising. In the bladder, there would be more social support for tumour cells than in vitro, and a greater degree of resistance is likely than in vitro. On the other hand, because the dose-rate in vivo is usually limited by toxic effects on normal tissues, the risks and benefits are usually finely balanced, and the development of a minor degree of resistance might so mitigate the benefits of treatment as to make it worthless.

Predicting drug resistance from cellular characteristics is difficult, as is predicting any other outcome (Abel, 1988). Potential markers of drug resistance, such as p-glycoprotein, glutathione transferase activity and metallothionein expression can only indicate at best a part of the possible mechanisms (Sikic, 1986; Gerlach et al., 1986; Goldie & Coldman, 1985; Weber et al, 1989), and the cellular defences against thioTEPA effect are not yet clear. The studies carried out here to characterise the drug-resistant sublines and contrast them with the parent sublines found no distinguishing feature at this low level of drug resistance. Karyotype, isoenzymes, tumourigenicity, EGFR1 staining and DNA fingerprinting were not appreciably different in the resistant sublines. This did have the advantage that it was clear that the drug resistance was related to the drug exposures, in that it had not been produced by cross-contamination. It may

be that the tests used, particularly the chromosomal analysis and EGFR1 staining, were not sufficiently sensitive to recognise the real markers of thioTEPA resistance. It seems more likely that any changes induced by the model course of chemotherapy would be identifiable in the regulation of cellular processes, rather than in the appearance or disappearance of functions or structures such as drug inactivation or P-glycoprotein, and that the potential markers examined were unlikely to be fruitful however great the resistance induced, or however sensitive the testing. The examination of other markers, such as double minutes, in resistance, or of, e.g., EGFR1 in recurrence or progression, is not invalidated by the present findings.

The opportunities for and consequences of resistance in vivo are significant. Malignant cells not killed by treatment have an opportunity to develop resistance, and if this were not recognised, and treatment changed, patients would be exposed to the hazards of treatment without prospect of benefit. Even where complete clinical response occurs, cure may not have been achieved (Skipper, 1987; De Vita, 1986). This fits with the clinical experience that recurrence can occur after complete clearance of all visible tumour after resection or chemotherapy. Following the principles of log-kill kinetics (Skipper et al., 1964), and the mutational theory of the development of resistance (Goldie & Coldman, 1979), it would be logical to expect intra-vesical chemotherapy to be most effective where the tumour burden is small, ie after complete resection of a single tumour, whereas

its role had traditionally been seen to be in unresectable, or repeatedly recurrent disease (England et al., 1981).

Cystoscopically normal urothelium is also altered by intravesical chemotherapy (Droller & Erzoan, 1985). The field change and multihit theories of urothelial carcinogenesis imply that cystoscopically normal urothelium contains cells ripe for carcinogenesis, which might be triggered by the mitogenic and mutagenic stimulus of therapy. Green et al. (1984) found that the net effect of chemotherapy on progression in vivo was beneficial, but this may mask preventable deterioration in individual patients, as may occur in in T_{is} (Stanisic Donovan, Lebouton et al. 1987). The use of the optimal modality of treatment and the optimal method of delivery is important, as "Dead bugs don't get resistant". Where discernible improvement does not accrue to the individual, the clinician should not hesitate to institute a new approach.

CONCLUSIONS

Granted that the diagnosis of tumours by fibrescopes is adequate, there remains a deficiency in therapy compared with the rod-lens system. Opportunities do exist, however, for better instruments and techniques to be developed to perform local surgery, while this remains the simplest and least invasive form of treatment.

The application of intravesical treatment in optimal conditions

might reduce the need for surgery, even where surgery is effective. Several possible options for this approach have were studied, and one with potential clinical application has been found. Lowering the pH of the instillate increases the anticancer effect of thioTEPA in vitro. No evidence was found that this could be enhanced by the addition of amiloride. Phosphate buffer, BP, could be used as a vehicle to reduce the pH of intravesical thioTEPA instillates, in order to increase the effect of a given concentration of thioTEPA. The experiment needs to be tried.

Another way of enhancing intravesical chemotherapy might be by drug synergy, but the addition of DMSO to the instillate did not add to thioTEPA effect at pH= 7.4. Previous studies that have showed enhanced tumour shrinkage may have been dependent on a period of pre-treatment with DMSO, which might have altered the degree of differentiation of the tumour. A simple solvent effect was not thought important. Short instillations of 50% DMSO have been used in the treatment of interstitial cystitis, and patients undergoing marrow transplants tolerate small volumes of 50% DMSO systemically, in the frozen marrow suspension, but the unpleasantness of the DMSO taste would require a large improvement in prognosis to balance this disadvantage in the management of superficial bladder tumours.

Resistance to thioTEPA developed in cells surviving a model course of intravesical thioTEPA. Sublines derived from histologically normal urothelium and from urothelial tumours showed

evidence of this effect, although there was a paradoxically high survival in parent cells. Resistant cells were not distinguishable from parent or control cells in isoenzyme pattern of four enzymes, DNA fingerprint, karyotype, or nude mouse tumourigenicity. Chemotherapy therefore requires to be intensive, and there may be considerable bonuses to be obtained from the use of methods of limiting unwanted effects, such as granulocyte colony-stimulating factor for bone marrow support, to allow still higher dose-rates to be used with reduced toxicity.

The present studies, intended to promote an out-patient approach to the management of superficial bladder cancer, have taken modest steps in that direction. Intravesical thioTEPA in acid solutions is worthy of further clinical studies, particularly in those with low tumour burdens, rather than those with multiple superficial recurrent disease. Used conventionally, thioTEPA prophylaxis may induce resistance. While the need for a cure for superficial bladder cancer remains, the management must be based on the principle "Primum non nocere".

APPENDIX 1

ORGANIZATION AND ACCEPTABILITY OF A FLEXIBLE CYSTOSCOPY SERVICE

INTRODUCTION

The flexible fibreoptic cystoscope has been used under topical analgesia in English Teaching Hospitals (Fowler, Badenoch and Thakar, 1984; Powell et al., 1984) and in the USA (Soloway, 1985; Clayman et al., 1984). Although, historically, large instruments have been passed without anaesthesia (Wallace, 1978), current UK practice uses general anaesthesia in most cases. In the USA, rod-lens cystoscopies are often performed under topical analgesia. Because of the high numbers of check cystoscopies carried out in frail patients, a large number of bed-days are required for these patients, many of whom have no active cancer. Even those selected patients having out-patient examinations need a period of recovery from general anaesthesia before they can leave. If flexible cystoscopy under topical analgesia is feasible in district hospitals in the UK, it will make beds available to patients on waiting-lists for more major procedures.

METHOD: the Facilities Used

In a mixed suburban/rural area, a flexible cystoscopy service was set up, using an Olympus CHF-4B choledochofibrescope which was already available. Patients who required cystoscopy for the diagnosis of haematuria, frequency, recurrent urinary tract

infection, or pain, or voiding difficulties were offered flexible cystoscopy as an alternative to later general anaesthetic appointments. Patients who had been undergoing check cystoscopy and who had been clear for two years, and patients on follow-up for urethral stricture were also offered this option.

New patients were given a cystoscopy appiontment on the next list after their clinic appointment. Former patients attended the out-patient clinic one week before cystoscopy, to review their progress. Patients were asked to return one week after cystoscopy to pass a mid-stream specimen of urine. Patients who failed to attend were sent a further appointment. The reasons for failure to attend were obtained by enquiry where they were not volunteered.

Cystoscopies were carried out by the author in two rooms in the Central Treatment Suite, used at other times for dressings and minor procedures. As only one cystoscope was available, it was carried, sterile, between rooms, when the patient was ready to be examined. In preparation for each session, and between patients, the instrument channel was brushed through and rinsed with aqueous chlorhexidine (Hibiscrub, ICI, Macclesfield, UK), then sterilised in 2% activated glutaraldehyde (Cidex, Johnson and Johnson, High Wycombe, UK) for five minutes between patients and ten minutes before and after each session. Before use, the gluteraldehyde was washed from the instrument, and the channel flushed with sterile water for irrigation.

Whenever possible, two nurses were used, one to assist with the examination in hand, and another to help dress the previous patient and to prepare the next. Patients were shown into the room by a nurse and assisted to undress from the waist down and lie on a standard examination couch. The skin was cleansed with cetavlon/ cetrimide (Savlodil, ICI, Macclesfield, UK), and fifteen grams of 2% lignocaine gel (Biorex Laboratories, London, UK) were instilled into the urethra, and given five minutes to take effect, while the previous patient was examined, and the instrument cleaned and sterilised. While waiting, the patient was covered with a sterile towel.

The cystoscopist, the author, carried out a surgical hand scrub, and wore sterile gloves (Regent, London, UK), but surgical gowns and patient towels were not used. The bladder and urethra were irrigated through the instrument channel using Normal saline for intravenous use (Baxter,UK) given through a standard intravenous set (Travenol, UK), which was changed between patients. Each examination used between two and four hundred millilitres of saline. After cystoscopy, the patient was given a chance to dry any gel or saline, and assisted to dress. In anticipation of retention of urine, patients were asked to wait until they had voided. Where the appearances at cystoscopy were doubtful, a rod-lens examination was carried out, to avoid missing important pathology.

METHODS: Patients

Three patients opted to be examined under general anaes-
thesia. Sixty-three patients were given appointments for flexible
cystoscopy, and sixty attended, fourteen women and forty-six men,
one man on two occasions. Thirty-seven patients came for review
cystoscopy for bladder cancer, six of whom required dilatation of
a known urethral stricture in addition; ten for investigation of
haematuria; three for assessment and dilatation of urethral
strictures; four, for investigation of frequency; one, recurrent
urinary infections; one for poor stream; and in four cases, the
reason for cystoscopy was not recorded. The average age was 62
years 11 months.

RESULTS

Fifteen out of sixty patients (25%) required further management
under general anaesthesia. Four had an inadequate view. Two
patients undergoing check cystoscopy were thought by the examiner
to be unable to tolerate further fibreoptic examinations. One had
experienced difficulties with dysuria after previous rod-lens
cystoscopies, and another was anxious, cancer phobic, and had a
recurrent bladder tumour. Two others expressed a slight prefer-
ence for general anaesthesia, not precluding further fibreoscopy.
In all, six patients had bladder tumours; two, benign prostatic
hyperplasia; one, carcinoma of the prostate; one, a bladder neck
stricture; and one, a phimosis. In two patients with urethral

strictures, the review for bladder tumour was inadequate because of turbid urine. The strictures were dilated under local analgesia, and at three months, allowed unopposed passage of the fibrescope. Of the thirty-seven patients attending for check cystoscopy, seven (19%) required further assessment or management under general anaesthesia.

Failure to attend might indicate that the patient was reluctant to undergo the new procedure, but was hesitant to say so. Eighteen patients had multiple appointments, because of failure to attend. Patients who did not attend were sent another appointment in two weeks. Eight patients were given incorrect appointments, and four did not attend because of holidays or other social reasons. Two more men probably did not attend because of work problems, one being abroad, and another was due to start a night shift immediately after the examination. One of those giving a social obligation as a reason for non-attendance, and at least two others were thought likely to have been reluctant to undergo the new procedure, even although they had been given the option of conventional examination. Those with appointments problems had an average age of 58 years 3 months, as compared with the general figure of 62 years 11 months, but these are not different at the 5% level of significance.

Preparation of the patient and instillation of lignocaine took about two minutes of the appointment, the examination of the patient, one or two minutes, and the cleaning and rinsing of the

instrument two minutes. The sterilization period was used to prepare another patient, and to discuss their coming examination. As the procedure was new, appointments were scheduled every fifteen minutes.

After examination, patients were asked informally by the nurses whether they had found the procedure acceptable enough to have it again if necessary. Of 51 who did not have urethral dilatation, one reply was not recorded, and three (6%) would have preferred a general anaesthetic. One patient was cancer phobic and found all procedures difficult, and two expressed a mild preference for a general anaesthetic. Two others spontaneously expressed a preference for fibreoptic examination. Three of the ten patients who had urethral strictures dilated would not have found further local anaesthetic procedures acceptable. Of those attending for check cystoscopy, seven had lesions requiring biopsy or assessment under general anaesthesia, and 81% (95% Confidence Interval=68,94%) avoided a general anaesthetic.

DISCUSSION

The benefits offered by flexible cystoscopy relate to avoidance of admission, and to the avoidance of general anaesthesia. Initially, bladder tumour review patients with a low risk of recurrence were examined, but this service has since been extended, to reduce the number of negative in-patient examinations. This is expected to increase the proportion of patients

that require referral for general anaesthetic cystoscopy from the present 11/51=22(11,33)%, for all patients not known to have had a urethral stricture, or from 13/47=28(15,41)% of patients for tumour review or investigation of haematuria. A figure for the number of general anaesthetic cystoscopies avoided per tumour diagnosis (c.f. Meuleman and Delaere, 1989) was not valid, as patients with a recent recurrence were excluded from this study.

The avoidance of admission also has financial benefits. Over the ten-year suggested replacement life of the instrument, comparative costings favour the flexible instrument (Fowler et al., 1984; Powell et al., 1984), although more recent reports have found replacement or repair necessary after three to five years (Fawcett et al., 1991), and there may be increased costs to comply with higher standards of safety in instrument sterilization (Gibbs, 1990). The proposal in North-East Thames Region to remove glutaraldehyde from operating theatres, and to convert to centralised sterilization of supplies would have much greater implications for fibreoptic instruments than for the cheaper rod-lens cystoscopes.

Passage of a rod-lens instrument of similar size requires further sedation with benzodiazepines or opiates, and reversal with flumazenil before discharge, but does allow a limited range of surgery to be performed at the same sitting (Miller, Parry, Creighton et al., 1989). In contrast, the acceptability of the district hospital local anaesthetic fibreoptic cystoscopy service

appears to be adequate for general application, without the need for injections of sedatives or reversing agents.

The symptomatic and social convenience to the patient of a less stressful procedure, with little need for post-anaesthetic super-vision in or out of hospital are more obvious to the patient than the financial considerations. Anaesthetic hazards are particu-larly important in the elderly majority of these patients. Patients, who have previously tolerated the passage of a range of flexible and rigid instruments without analgesia or anaesthesia (Abdel-Halim, 1990, Wallace, 1978), were nevertheless likely to welcome the advent of a 15Ch, flexible instrument that could be passed with relative comfort under topical analgesia in out-patients. The present author's unquantified experience and the data of others (Flannigan et al., 1988; Figueroa, Thomas & Moon, 1987; Denholm et al., 1990) confirms patient acceptability. In view of the long-term relationship a patient expects to have with the follow-up team in a hospital, the responses to the personal enquiries about acceptability in the present study are likely to produce an over-estimate, as the results of the second question-naire in the Edinburgh study suggest (Denholm et al., 1990). At the time of the cystoscopy, 92% said that they would prefer to have this examination for future cystoscopies, but a week later, a postal questionnaire elicited such approval in only 85%. The specific questions about postoperative symptoms revealed dysuria (21%) frequency (14%) haematuria (6%) and malaise (8%) to be significantly less than in the rod-lens group at one week.

The assessment of acceptability in the present report was subject to bias. The initial selection of patients who were offered the option was not monitored, but it is not thought that clinicians excluded many, if any, patients as unsuitable for either examination. Consideration of the patient's psychological suitability for the examination will avoid some broken appointments. Patients with a high level of anxiety appeared less likely to find the service satisfactory, took longer to examine, and are probably best not cajoled or persuaded to undertake the examination. The patients having urethral dilatation might also have been more comfortable and better managed by inpatient urethrotomy. The assessment of missed appointments as an index of patient reluctance is subject to a number of complicating factors. The service was in its infancy, and administrative errors occurred in 8/58 appointments. Three patients at least were thought to have been reluctant to undergo the procedure, but the numbers were too small for analysis of the patients' ages or the sex-distribution to indicate any difference in those who did not attend. It appeared that patients were willing to try this quicker alternative.

Investigation and surveillance of bladder cancer was negative in a high proportion of patients, who had been selected for a low probability of recurrence. The "once-negative" criterion of Fawcett's study (1991) for changeover from rod-lens surveillance might be better suited to the pressures of clinical practice, and

reduce the need for admission.

The role of nurses and of reception/clerical staff in the success of such a service should not be underestimated. Cutting the ratio of nurses to cystoscopists from 2:1 to 1:1 would approximately double the appointment time, and less of the cystoscopist's time would be spent actively. If a third nurse was available, adding another instrument to the service would make certain that there was no delay while awaiting instrument sterilization. In principle, it is not essential that the examiner perform genital antisepsis and the instillation of lignocaine, but it is an opportunity to establish a rapport with the patient, and consider the clinical problem. Hypothetically, the examiner need only scrub, carry out the examination, and note the findings, but in practice, six patients per cystoscopist per hour is likely to be the maximum achievable without losing sterility, and the patient's confidence. To achieve such smooth service, co-operative staff familiar with the technique are vital.

In the six months' service reported here, 40 patients were diagnosed as clear, and not admitted for examination under general anaesthetic. Follow-up has given no reason to doubt this assessment of their condition. Against this, fourteen patients underwent flexible cystoscopy and went on to have an in-patient procedure. In the forty to eighty patient bed-days saved, approximately ten more patients from the waiting list for transurethral prostatectomy could have been treated. The gain may

be limited by theatre or operator availability, but could probably have been achieved. With a small staff, a choledocho-scope that was infrequently used in the biliary tree, and some common hospital sterile supplies, a prompt, acceptable out-patient cystoscopy service was set up, instead of an in-patient waiting-list.

APPENDIX 2

DOES FIBREOPTIC CYSTOSCOPY INDUCE BACTERIURIA?

Fever has been associated with urethral instrumentation for over one hundred years (Clark, 1883), and conventional theatre sterile procedures have been successfully devised to prevent infection. Simpler aseptic precautions as used for urethral catheterisation may be adequate in out-patients undergoing rod-lens cystoscopy (Fozard et al., 1983) and have been adopted for flexible cystoscopy (Fowler, 1984). It is essential that they be effective. The development of bacteriuria was monitored in the first patients undergoing fibreoptic cystoscopy with simpler asepsis in the Central Treatment Suite of a district general hospital. To compare the hospital prevalence of postoperative bacteriuria with reports from other centres, and with the study group, uncatheterised patients having rigid transurethral procedures in the operating theatre over the same period.

PATIENTS AND METHODS

The patients having flexible cystoscopy were as described in Appendix 1. Nine patients who had out-patient urethral diltation were excluded from the study of postoperative bacteriuria, and another twelve who had bacteriuria at presentation, required antibiotic prophylaxis, or had had antibiotics or instrumentation within the last four weeks. Four had incomplete specimens.

Thirty-five eligible patients completed the fibreoptic protocol, ten female, twenty-six male, average age 63 years 7 months, range 25 years to 82 years. None was catheterised before or after surgery. Twenty-three patients attended for check cystoscopy, eight for investigation of haematuria, three for symptoms of frequency, one for poor stream, and in one case, the reason for referral was not recorded.

METHODS: Asepsis and Fibreoptic Technique

The Olympus CHF-4B choledochofibrescope used was sterilised by immersion of the introduction tube, and irrigation of the biopsy channel with 2% activated glutaraldehyde (Cidex, Johnson and Johnson, High Wycombe, UK) for five minutes in a sterile bakelite trough or theatre washbowl. The cystoscopist (the author) carried out a surgical hand scrub, and wore conventional sterile gloves. The Cidex was rinsed from the fibrescope and channel with water for irrigation, before the cystoscopy. The patient lay supine on an examination couch with a waterproof cover, women with knees flexed and abducted during the examination. The urethral opening was washed with aqueous Cetavlon (Savlodil, ICI, Macclesfield, UK), and draped with a single sterile hand towel. Fifteen grams of 1% lignocaine gel (Xylocaine, Biorex Laboratories, London) were instilled into the urethra for five minutes to provide analgesia. The cystoscope was passed up the urethra under vision, irrigating with intravenous Normal saline through a standard intravenous set.

PATIENTS AND METHODS: Rigid Cystoscopy

Of one hundred patients undergoing rigid instrumentation, sixty-two were excluded, 43 because of failure to return to the hospial to provide a postoperative urine sample, nineteen because of preoperative bacteriuria, catheterisation, or antibiotic administration. Twenty-three required postoperative catheterisation, leaving a control group of fifteen patients undergoing rigid instrumentation of the urethra.

Rigid instrumentation was carried out under general anaesthesia, in an air-conditioned theatre suite, used by a number of surgical specialties. Patients were placed, supine, into lithotomy stirrups, and the genital area washed with aqueous cetavlon (Savlodil, ICI, UK), before draping with sterile cloth leggings and split towel. Surgeons wore theatre gowns and gloves, but masks were usually removed to prevent fogging lenses. Instruments were between 19 and 23 Charriere gauge.

METHODS: Microbiology

Mid-stream or clean-catch urine specimens were taken from all patients in the 24 hours before examination, and again on the sixth postoperative day, on the hospital premises. Specimens were taken directly to the laboratory for culture. The laboratory methods used were the standard for the hospital, and were carried out by a full-time technician, Marian Clements.

Standardised urine aliquots were plated by the filter-paper method of Leigh and Williams (1964), and the concentration of organisms calculated accordingly, from a calibration curve. This was the hospital's practice, in preference to counting bacteria. Mast Bacteriuritest Strips 12mm long were dipped in the mixed urine, and touched on to culture plates, for identification by colony morphology. Cystine-lactose-electrolyte-deficient agar (CLED, CM301, Oxoid, Basingstoke) blood agar (CM331, Oxoid, Basingstoke, with 7% horse blood, Tissue Culture Services, Slough) and MacConkey agar (CM76, Oxoid) were used for overnight culture. Gram stains were performed, and positive organisms were tested for catalase and coagulase, and negative, for oxidase and urea production. Antibiotic sensitivities were assessed on iso-sensotest agar (CM 471, Oxoid) using antibiotic-impregnated disks (Amoxycillin, 25 mcg, trimethoprim, 1.25 mcg, nitrofurantoin, 100 mcg, cephradine, 30 mcg, nalidixic acid, 30 mcg, and sulpha-methoxazole, 100 mcg, Oxoid), and comparing with standard organisms, plated daily. Microscopy was carried out on 60 microlitres mixed urine, placed in a flat-bottomed microtitre well and allowed to settle for 10 minutes before examination with an inverted microscope. The number of cell seen per high-power field was reported as the number seen per microlitre. In practice, no specimen with a growth equivalent to 10^8 organisms /litre had a count less than 10 white blood cells per high-power field.

Significant bacteriuria was defined as a growth equivalent to 10^8

organisms/litre of urine, with > 10 white cells/ml of urine.

METHODS: Statistics

Numbers of patients with significant bacteriuria were compared using the chi-squared test with Yates' correction for small numbers (Swinscow, 1976). The upper and lower extent of the 95% confidence interval for proportions were calculated as described by Gardner and Altman (1989) using the Standard Error of prportions, multiplied by 1.96, and are given in the form (lower,upper).

RESULTS

Of thirty-five eligible patients undergoing flexible cystoscopy, excluding those who had cystoscopy for urethral stricture, none had significant bacteriuria. Three more patients who were other-wise eligible, and had no rigid instrumentation, were being followed for urethral strictures. One had significant bacteriuria with coagulase-negative Staphylococcus on the sixth postoperative day (2.6(0,7)%). The patient had recurrent urethral strictures, one of which had been dilated, in the presence of a similar Staphylococcus,seven weeks before the eligible examination. Fif-teen eligible in-patients had rigid instrumentation without postoperative catheterisation. Two (13(0,31)%) developed signifi-cant bacteriuria. One had a mixed growth of predominantly Staphylococcus, and another, a pure culture of coagulase-negative

Staphylococcus. The p-value comparing the two groups was between 0.5 and 0.1.

DISCUSSION

This study addressed the possibility of ascending infection introduced by the instrumentation, similar to catheter fever (Clark, 1883). It did not measure the viability of endoscopy in the patient with pre-existing infection, as both untreated or recently treated (1 month or less) infection were a criterion for exclusion. Circumstances so pressing that an endoscopy must be performed in the presence of infection, even with antibiotic cover are less likely to arise if, as a result of better directed resources, an early alternative appointment can be offered. Equally, the transmission of viral infections was not tested, but there is only one documented example of transmission of hepatitis B by an endoscope, the channel of which had not been cleaned between patients.

The outcome measure compared, a growth equivalent to 10^5 bacteria/ml in the presence of 10 or more leucocytes/ml is a common laboratory criterion of bacteriuria that is not due to contamination. It is arguable that not all patients who meet this criterion have an infection of the urinary tract. In the absence of residual urine, bacteriuria often clears spontaneously (Thomlinson, Williams & Cope, 1968; Wettergren, Hellstrom, Stokland et al., 1990), and the presence of bacteria on this body

surface does not necessarily imply disease. Symptomatology was not recorded, and would have had some value in assessing the pathological implications of the laboratory examinations. The criteria used were, however, those used routinely by the author and his laboratory collaborators in clinical practice, are likely to be commonly accepted by urologists, and were compared with like tests in patients undergoing conventional management.

Patients having flexible cystoscopy in this study did not develop bacteriuria more frequently than patients having conventional examinations. The low incidence of bacteriuria , and its tendency to clear spontaneously indicate that it is not likely to be a large clinical problem after flexible cystoscopy.

Although patients with recent bladder tumours were more likely to be included in the rigid group, and bladder tumours have been thought to predispose to the development of bacteriuria (Appell et al, 1980), resection of bladder tumours was usually followed by catheterisation, reducing the bias towards bacteriuria in rigid instrumentation. The non-attenders for postoperative urine testing qualify the validity of the control group.

The present author has previously reported a 29% incidence (95% confidence interval 9, 48%) of bacteriuria in in-patients fol-lowing a range of transurethral procedures (Walker et al., 1984), and a figure of 2-8% has been reported for out-patients (Lytton, 1961; Hares, 1981; Richards and Bastable, 1977; Fozard et al.,

1983; Butler, 1985). To ensure that there was no major difference in bacteriuria the locality of the hospital where the study was carried out, a control group of in-patients undergoing rod-lens examination was included. A larger control group would have been desirable. The 43% of entrants to this arm who failed to provide a post-operative specimen are a potential source of bias to this measurement. The author was not always present when these patients were discharged, and relied on a number of nurses and one medical colleague to make appointments for patients to return to provide specimens on the sixth postoperative day. Given the different priorities and duties of the nurses, it is likely that this contributed largely to the difference in the numbers of patients completing the protocol. Patients who had no symptoms would be less motivated than those with urinary difficulties to return for a test. Problems severe enough to require hospitalisation would mostly have resulted in readmission to the hospital in which the study was being carried out, so the bias introduced by non-returners is likely to increase the apparent rate of post-operative bacteriuria in the control group. The post-entry exclusion from the control group of patients requiring catheterisation during their admission might have tended to exclude patients needing treatment for tumours or other problems that might predispose to the development of postoperative bacteriuria (Appell et al., 1980).

The findings of other authors (Fozard et al., 1983) suggest that the patient selection in the rigid cystoscopy group might have

increased the rate of bacteriuria in the rod-lens group. The infection rate in Fozard's patients managed with conventional draping for out-patient cystoscopy was 4(0,8.5)% , and was 6(0.9,11)% in the group in which an aseptic approach was used. These figures would compare equally well with the present study, but too few patients were included in the conventional management group for it to be possible to identify any reduction in induced bacteriuria (the examination would have to cure 0.4% of patients of pre-existing bacteriuria).

With these qualifications in mind, the difference between the new and the conventional approach in this comparison may have been a random event, as it does not reach the 95% level of statistical significance; but the incidence of bacteriuria in patients undergoing flexible cystoscopy was not greater than that in the control group, or than in other groups of patients undergoing transurethral procedures studied by the present author.

This study indicates no reason to doubt the efficacy of antibacterial precautions used in this out-patient fibreoptic cystoscopy service.

APPENDIX 3

DOES FLEXIBLE CYSTOSCOPY MISS MORE TUMOURS THAN ROD-LENS EXAMINATION?

INTRODUCTION

Data are scarce and incomplete on the likelihood of failing to identify a tumour by fibreoptic cystoscopy under topical analgesia. An examiner accustomed to cystoscopy under general anaesthesia might restrict the extent of the examination in the presence of an aware patient, causing small solitary tumours to be missed. The particulate nature of the fibreoptic picture has also been cited as giving a less adequate view (Pope and Wickham, 1991). A prospective, double-blind study was undertaken to compare the tumour episodes missed by flexible cystoscopy under topical analgesia, with that of rod-lens cystoscopy under general anaesthesia.

Rod-lens cystoscopy has often been used as a yardstick for the accuracy of alternatives such as ultrasound and of cytology in the diagnosis and follow-up of superficial bladder cancer. As a result, there are scant data on the accuracy of rod-lens cystoscopy. Positive findings on the ultrasound, that are not confirmed at conventional cystoscopy are interpreted as false-postive findings (Abu-Youssef et al.,1984; Boccon-Gibod et al., 1985; Brun, Gammelgaard & Cristoffersen, 1984; Dershaw & Scher, 1987; Malone et al., 1986; Rosenkilde-Olsen, Jorgensen, Roed-

Petersen et al., 1985; Vallencien et al., 1986). In most studies, the cystoscopist knew the ultrasound result, so it was not possible to asssess the accuracy of the cystoscopy. In the one clearly "blind" study (Rosenkilde-Olsen et al., 1985), rod-lens cystoscopies were carried out without general anaesthesia. Of 64 patients with positive ultrasound findings, six had no recurrence identified at cystoscopy, and were accounted for by "severe trabeculation, diverticula of the bladder, or impression of a small atrophic uterus into the urinary bladder". It is not recorded that the cystoscopy was repeated, so it is possible that some tumours were genuinely missed by cystoscopy. Suture material (Boccon-Gibod et al., 1985) trabeculation and prostatic hyper- trophy (Brun et al., 1984) have been reported to mimic tumours on abdominal ultrasound, but their identification on cystoscopy should not be presumed to exclude the presence of tumour.

Clayman, Reddy, and Lange (1984) report missing one of two tumours in a patient undergoing fibreoptic examination, and one patient with a tumour was not diagnosed on rod-lens cystoscopy. The total number of tumours was not given. The London Hospital group (Fowler et al. 1984), made both examinations under general anaesthesia, and did not report whether the examiners were "blind" to the alternate result. False-negative rates for fibreoptic examination were not given. Powell and colleagues, from Newcastle-upon-Tyne (1984), likewise compared both pro- cedures under general anaesthesia. Webb, Butler and Fitzpatrick (1984) reported missing two lesions from an undisclosed total

number of tumours in 25 patients, examined with the flexible instrument under topical analgesia, then with rod-lens cystoscopy under general anaesthesia, apparently by the same examiners.

A prospective, randomised,"blind" comparison of tumour-miss was therefore carried out, comparing the two options in practice in the United Kingdom, flexible cystoscopy under topical anaesthesia with rigid cystoscopy under general anaesthesia.

PATIENTS AND METHODS

For six months, consecutive patients admitted to St Thomas' Hospital for review cystoscopy for bladder tumour or for diagnosis of haematuria were asked to enter this study. Fifty-six patients gave informed consent. Three patients did not complete the protocol. One tense patient fainted after a negative flexible cystoscopy, one opted to withdraw before examination, and a third did not have flexible cystoscopy because there was not enough theatre time to perform both examinations. Thirty-four patients with bladder tumours completed the study.

After premedication for general anaesthesia, patients were examined with an Olympus CYF fibreoptic cystoscope, using lignocaine gel, and the technique decribed previously (appendix 1), in the anaesthetic room before induction of general anaesthesia for rod-lens cystoscopy. Two surgeons were allocated to either examination in paired days, on the basis of a tossed coin.

Tumours were treated under general anaesthesia, so it was not possible to randomise the order of the examinations. For ethical reasons, after recording his findings, the rod-lens cystoscopist read the results of the fibreoptic examination, and re-checked the rod-lens examination if it appeared that he had missed a tumour identified by the flexible cystoscopist. As the criterion was detection of a lesion needing management under general anaesthesia, no distinction was made between single and multiple tumours. Biopsies were not taken unless clinically indicated, as management of small lesion was normally undertaken without biopsies.

The endoscopists, the Author and a colleague (TL), were post-Fellowship registrars of more than two years' standing, and had performed several hundreds of rod-lens cystoscopies, and at least sixty fibreoptic examinations by the beginning of the study. Both were trained upper gastro-intestinal fibreoptic endoscopists.

RESULTS

Nineteen patients were diagnosed as tumour-free by both examiners. Three (9%) of the thirty-four tumours were missed by the fibreoptic endoscopist. Three others were missed by the rod-lens examination. The author missed two diagnoses on flexible cystoscopy, and one on rod-lens. All missed diagnoses were confirmed by both endoscopists after further inspection under the

same general anaesthetic, and treated at that sitting.

Tumour was missed in two of the first twelve flexible cystosco-
pies. One was on the edge of a diverticulum. Another tumour was
on the right wall of the bladder. As the cystoscopist always
stood on the patient's right, the right wall was more difficult
to examine thoroughly. The third was missed in a bladder filled
with turbid urine. Tumours missed on rod-lens cystoscopy were on
the lateral walls of the bladder. All tumours missed were less
than 5mm in diameter.

DISCUSSION

As the protocol chosen proposed that all patients in whom tumour
was diagnosed would be admitted for resectional treatment under
general anaesthesia, the number of lesions present in any one
episode of recurrence was of less significance than the fact of
recurrence. This would apply equally if treatment with chemo-
therapy was intended, but not if local laser surgery or diathermy
was planned. For that reason, the episodes of recurrence were
more appropriate measures of outcome than number of lesions. The
total number of examinations carried out in comparative studies
largely reflects the population examined, and is less important
in determining the diagnostic accuracy of bladder cancer surveil-
lance than the number of examinations at which there was
recurence to be identified. If treatment with fibreoptic diagno-
sis and intravesical therapy became routine, then the number of

lesions seen would acquire importance, as a measure of therapeutic effect on followup, and a further assessment of the ability of flexible cystoscopy to detect every lesion would become relevant. The accuracy of diagnosis of any abnormality present, the false positive rate (Fowler, 1984) is of subsidiary importance if the cystoscopic assessment can be supported by histological assessment of biopsies.

The accuracy achieved, 9% of 34 tumour diagnoses missed, 95% confidence interval 0,18%, was indistinguishable from that of conventional examination, and compares well with that of cytology and of transabdominal ultrasound (see chapter 1). In view of the unfamiliarity of the researchers with the fibreoptic cystoscope relative to the rod-lens cystoscope, higher accuracy might be expected with more experience. The element of reassessment of the bladder during the general anaesthetic, but after the test results had been noted, gives a first report of the accuracy of rod-lens cystoscopy in tumour diagnosis, as previous studies have apparently assumed that positive findings on a new test that were not confirmed by the rod-lens cystoscopist were the incorrect findings. The present study found that conventional examinations were not perfectly accurate.

In follow-up cystoscopy, where small lesions that are undetected at one sitting may be picked up three or six months later, 9% missed tumours could be acceptable, provided that the rate of progression was slow, or that the treatment that could be offered

did not prevent progression, but was palliative. For diagnostic examination, however, such a rate would give rise to concern, as there would not necessarily be a second chance to make the diagnosis. Unfortunately, there is no evidence that a single rod-lens examination would be any better. In the present study, the ability to diagnose tumours fibreoptically was as good as the best currently available.

The differing characteristics of the flexible and rigid instruments suit them to particular circumstances. The presence of turbid urine might be an indication to abandon flexible cystoscopy, and to use rod-lens cystoscopy for that patient, as the bladder can conveniently and quickly be evacuated through the cystoscope sheath. Alternatively, the bladder could be washed out through a catheter, and reviewed with the flexible cystoscope. Diverticula should be easier to examine with the flexible cystoscope, particularly larger diverticula which can be entered with the instrument. Varied levels of filling may be important to ensure true negative examinations of patients with diverticula. In view of the prognostic importance of missing a tumour in a diverticulum, urologists who have had no training in fibreoptic endoscopy may prefer to exclude patients with diverticula from fibreoptic follow-up until they have a broad experience of the technique. ""See one, do one, teach one" will no longer do" (Walker, 1991). For gastroscopy, a figure of two hundred examinations is often suggested as adequate training.

This prospective double-blind study appears to be the first substantial assessment of the accuracy of either of the practical options for cystoscopy in British practice. Flexible cystoscopy was no more likely than rod-lens examination to miss tumours. With hindsight, a lower miss rate could have been achieved in flexible examinations by a stricter definition of an acceptable view, and using catheter lavage or rod-lens examination in those below standard. Voiding immediately before the examination (Fowler, 1984) would also assist the examiner by giving a clearer view. The tumours missed on rod-lens cystoscopy were unaffected by these influences.

A first comparison of the tumour-miss rate of fibreoptic and rod-lens examinations as practised in the United Kingdom was made. Flexible cystoscopy was no more likely than rod-lens examination to miss tumours. Although a 9% miss rate might be tolerable for follow-up of patients with a low likelihood of progression before the next follow-up, it may not be acceptable in diagnostic cystoscopy. On the other hand, the current standard examination was as accurate in the present study.

APPENDIX 4

ABBREVIATIONS AND SUPPLIERS OF CHEMICALS

ABBREVIATION	CHEMICAL NAME	SUPPLIERS
Agarose	Type T low E.E.O.	Sigma, Poole, UK
CHES	(2-(N-Cyclohexylamino-) ethane sulphonic acid	Sigma, Poole, UK
commercial thioTEPA	triethylene thiophos- phoramide	Lederle, Gosport, Hants UK
EDTA	0.016% Disodium Edetate	BDH, Poole, UK
EPPS	(N-(2-Hydroxyethyl) Pi- perazine)-N'-3 Propane Sulphonic acid	Sigma, Poole, UK
FCS	Foetal Calf Serum	Flow, Gibco,Paisley, UK
Giemsa	10% aqueous Giemsa's stain	Gurr, BDH, Poole, UK
Glutamine	L-glutamine	Gibco, Paisley, UK
Hin F I	Restriction Enzyme Hin F I	BRL, Gibco, Paisley, UK
HCl	1N Hydrochloric acid (AVS Standard)	BDH, Poole, UK
HEPES	(N-(2-Hydroxyethyl) Pi- perazine)-N'-2-Ethane Sulphonic acid	Sigma,Poole, UK
IMS	70% Industrial Methyla- ted spirit	BDH, Poole, UK
MES	(2-(N-Morpholino) Ethane Sulphonic Acid	Sigma, Poole, UK

APPENDIX 4 (continued)

ABBREVIATION	CHEMICAL NAME	SUPPLIERS
MOPS	(3-(N-Morpholino) Propane Sulphonic Acid	Sigma, Poole, UK.
Na OH	1N Sodium Hydroxide (AVS Standard)	BDH, Poole, UK.
PBSA	Phosphate buffered saline "A"	Supplied by Imperial Cancer Research Fund, London, UK.
^{32}P dTCP	Radioactive deoxy Cytidine Triphosphate	Amersham International, UK
pH 4.0 buffer	Citric acid/ Sodium Citrate Reference Buffer	Convol, BDH, Poole, UK.
pH 7.0 buffer	Potassium /Hydrogen Dihydrate Orthophosphate Reference Buffer	Convol, BDH, Poole, UK
pH 10.0 buffer	Boric acid, K Cl, and Na OH Reference Buffer	Convol, BDH, Poole, UK
p lambda G3	minisatellite probe	Dr A. Jeffries, ICI, UK
RPMI 1640	Roswell Park Memorial Institute Medium 1640	Flow, Irvine, UK
Salmon sperm DNA	deoxyribonucleic acid from salmon sperm	Sigma, Poole, UK
SDS	sodium duodecyl sulphate	BDH, Poole, UK
Sorensen's buffer	$0.06M$ Na_2HPO_4 + $0.06M$ KH_2PO_4	BDH, Poole, UK

APPENDIX 4 (continued)

ABBREVIATION	CHEMICAL NAME	SUPPLIERS
SSC	0.15M sodium chloride+ 0.015M trisodium citrate pH=7	BDH, Poole, UK
STE	150mM sodium chloride+ 10mM TRIS + 10mM sodium edetate, pH=8.1	BDH, Poole, UK
sterile water	Sterile Water for Injections, BP	Beechams, Welwyn Garden City, UK
thioTEPA	triethylene thiophosphoramide	Cyanamid, Pearl River, New York, USA
Trypan Blue	0.1% Trypan Blue Stain	BDH, Poole, UK

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