LASER TREATMENT OF BLADDER CANCER

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by

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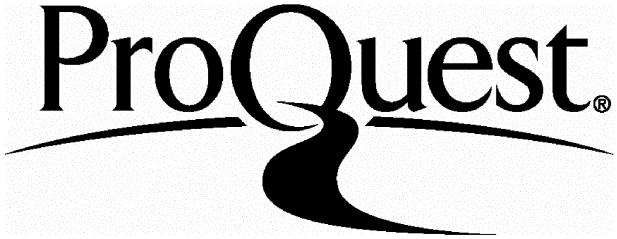

ABSTRACT

Lasers techniques are able to produce precise and sometimes unique tissue effects. These promise an improvement over the conventional techniques for treating both superficial and early muscle invasive bladder cancer.

Photodynamic Therapy (PDT) is an experimental treatment that shows great promise for treating superficial bladder cancer, especially resistant carcinoma *in situ* (Cis). Some clinical studies though have reported serious side effects, mainly in producing an irreversible functional impairment in many bladders due to fibrosis. This thesis presents a study of the effect of changes in dosimetry variables on the normal rat bladder using a new photosensitiser, aluminium chlorosulphonated phthalocyanine. The uptake of this drug into the different layers of the bladder wall has also been investigated using sensitive fluorescence microscopy techniques. The maximum concentration gradient of photosensitiser between the superficial and the deep layers of the bladder wall was reached after 24 h following administration and was increased by the photobleaching observed at low sensitiser concentrations. Morphological and functional changes (bladder capacity and compliance) were also studied and it was found that if PDT damage was restricted to the superficial layers of the bladder, the resulting functional disturbance was less severe and recovered more fully than when the muscle layers were also involved. At low concentrations of photosensitiser a selective, superficial necrosis was achieved across a wide range of light doses. If these experimental results can be achieved in clinical practice then PDT should provide an effective and bladder preserving treatment for Cis without the complications that have been seen previously.

The possible role of the flashlamp pulsed-dye laser for PDT was studied using cultured human bladder carcinoma cells (MGH-U1) sensitised with dihaematoporphyrin ether. It was found that this clinical laser was of a comparable efficacy to the more complex systems currently used for PDT.

The morphology of the coagulation produced by the neodymium:YAG laser on the pig bladder has been compared with conventional electrocautery. A marked qualitative difference was seen between these two modalities in that the laser produced a more even coagulation with little disruption of the tissue architecture. A major attraction for urologists is that, in conjunction with flexible cystoscopy, superficial bladder tumours may be laser coagulated on an outpatient basis using only topical urethral anaesthesia. A study of 33 patients with recurrent tumours treated in this way shows the convenience and economy of this technique though no reduction in the incidence of recurrences was seen after laser therapy.

PREFACE

The title of this thesis implies that it will aim to cover the whole field of laser therapy as applied to bladder cancer. This will comprise both experimental and some clinical work with a review of relevant literature.

Section 1 outlines the pathology and current treatment of bladder cancer and how laser techniques might complement or improve on conventional therapy. The principles of laser action and the several different types of lasers available are described. At present there are only 2 types of laser applications; either the thermal destruction of bladder tumours or a photo-chemical technique not requiring heat but using a photosensitising drug to produce tissue damage which is known as photodynamic therapy (PDT).

Section 2 deals with PDT in detail and describes our experimental work in this field. This comprises both *in vitro* studies on a human bladder tumour cell line and *in vivo* studies on the effect of PDT on bladder function which is the area in which most clinical problems have been encountered. It had been hoped to carry out a clinical PDT study, but due to delay in acquiring the necessary regulatory approval for the photo-sensitiser drug this has not proved possible.

Section 3 covers the use of thermal lasers to ablate bladder tumours. This is by far the commonest urological application of the neodymium:YAG laser and where the bulk of clinical experience has been gained. The experimental work presented in this section looks at the morphology of laser lesions in normal pig bladder, and compares them to conventional diathermy, as an accurate assessment of the expected tissue effect is necessary to match treatment dosimetry variables to tumour pathology. A small study in which tumour recurrences in patients have been treated without anaesthetic using a Nd:YAG laser in conjunction with flexible cystoscopy is reported together with early follow-up data.

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STATEMENT OF ORIGINALITY

The work presented in this thesis has been carried out by myself and has not been entered for a higher degree or award of this or any other University. The major element of this work involves original concepts and observations as to the effect of photodynamic therapy on the normal bladder and the means by which this might be assessed with the help of an animal model. No similar work has been published or carried out by any other group to my knowledge and I hope that it will make a contribution to the scientific understanding and clinical practice of photodynamic therapy for bladder cancer.

Other laser treatments for bladder cancer utilising the Nd:YAG laser are becoming increasingly common yet the basic tissue effects of this instrument are poorly documented in the literature and the place of laser therapy in clinical practice is not yet established. The additional studies presented here aim to clarify the morphology of the laser/tissue interaction and the likely role for this new technology in general urological practice.

LIST OF FIGURES AND TABLES

ACKNOWLEDGEMENTS

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I am indebted to the Sir Jules Thorn Charitable Trust who generously awarded me a 2 year fellowship (1988/9) for research into photodynamic therapy during which time the bulk of this experimental work was completed. Finally my dear wife Rachel has survived my long-running preoccupation with writing this thesis with her considerable good humour tested, but intact.

SECTION 1

BACKGROUND

Chapter 1

BLADDER CANCER

1.1 INTRODUCTION

Bladder carcinoma is the commonest urological malignancy requiring treatment (prostate cancer is more common in males but frequently occult) and the commonest one to cause death. In 1985, 9500 new cases were registered in the U.K. (Cancer Statistics, 1990), and in 1988 there were 4800 deaths (Mortality Statistics, 1990). It is 2.6 times more common in males and maximal around the 7th decade. Smoking increases the risk of developing the disease from 2 - 6 times. The aetiological role of industrial carcinogens, particularly certain aniline dyes and chemicals such as ß-naphthalenes which were used in the rubber industry has been recognised for years and their use is now generally banned. Nearly all bladder tumours are transitional cell carcinomas arising from the urothelium and all the trials reviewed here relate to these. Other histological types are rare and often advanced at presentation, they will be discussed briefly later.

As with other carcinomas, bladder tumours are classified by grade (G1 - 3) and stage (Ta - T4). Grade 1 (G1) indicates a well-differentiated tumour, G2 moderate and G3 a poorly differentiated tumour. Grade is the single most important prognostic feature though high grade tumours also have a positive correlation with more advanced stage.

Staging of the local tumour indicates the depth to which it has extended into the bladder wall (fig. 1.1). Superficial tumours comprise carcinoma *in situ* (Cis) and Ta which are confined to the epithelium, and T1 - into the cores of the papillae or lamina propria. Muscle invasive cancers may be T2 (into the muscle but not palpable bimanually) or T3 (subdivided rather artificially into T3a - invading more than half-way through the muscle layer, and T3b which is through the bladder wall but not invading adjacent structures). T4 tumours invade other organs such as the uterus or rectum. These are generally fixed and inoperable except for T4a (into prostate or

gynaecological organs). When a tumour has been pathologically staged either a lowercase "p" (biopsy), or an uppercase "P" (cystectomy specimen) is applied though many authors seem to interchange these or drop the "T" - e.g. T1, pT1, PT1, P1 can all refer to the same superficial tumour (UICC, 1987)

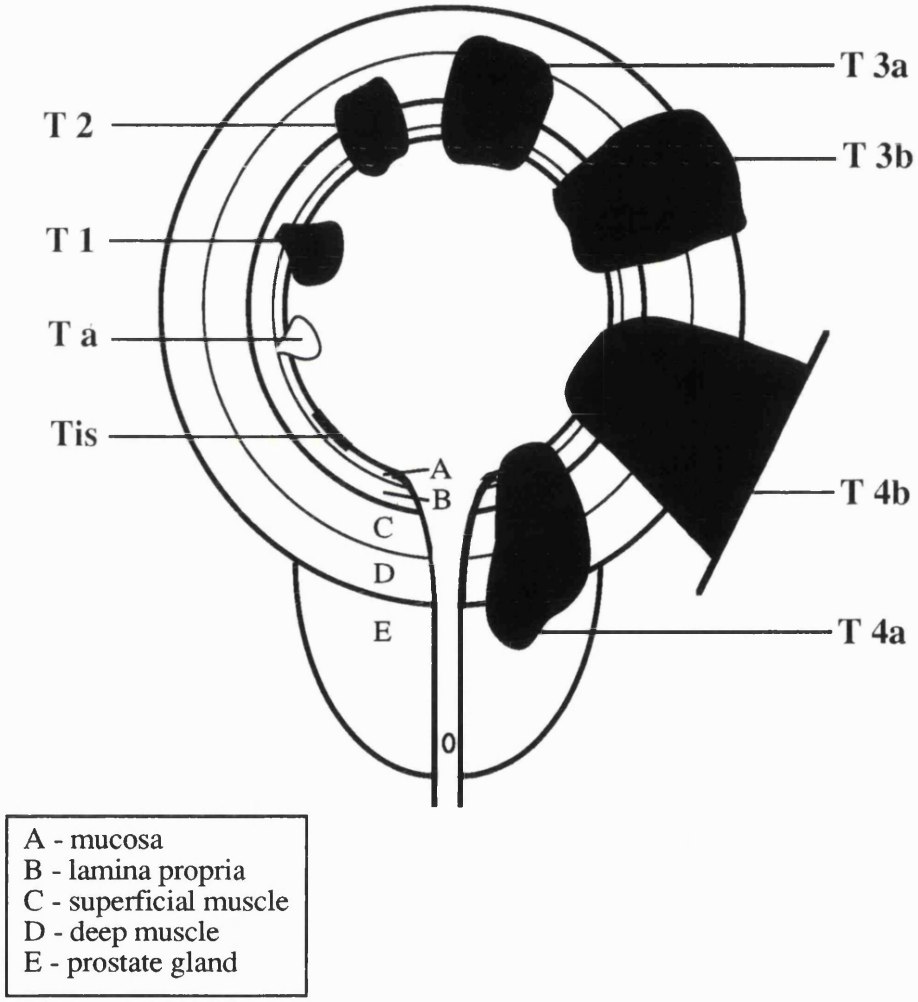


Fig. 1.1 Staging of bladder cancer

This chapter summarises the conventional treatment for bladder cancer and how much laser techniques can offer at present, or promise for the future, in the management of this varied condition.

1.2 SUPERFICIAL PAPILLARY BLADDER CANCER

The diagnosis and treatment of patients with superficial (Ta,T1) papillary bladder cancer comprise a significant proportion of a general urologist's workload. Around 70% of all superficial tumours are stage Ta, almost all of which are G1 or G2 and are most unlikely either to progress locally and become invasive or to metastasise (Abel *et al.*, 1988). Once diagnosed, however, patients are usually advised to undergo long term surveillance by regular cystoscopy as many will require repeated treatment of recurrences.

1.2.1 Treatment

Standard treatment is by transurethral resection (TUR) or diathermy fulguration and, in general, only when recurrences become particularly frequent or numerous do other adjuvant therapies such as intravesical chemotherapy become applicable. New treatment modalities that may reduce tumour recurrences in these patients would therefore have little survival benefit except in the small number of patients who present with superficial cancer which is destined to become invasive.

1.2.1.1 Prognostic indicators

The most useful of these for indicating a potentially more aggressive nature are high tumour grade, stage, size, multiplicity and rate of recurrence of new tumours, and the presence of Cis (Heney *et al.*, 1983). A patient with G2 or G3 superficial recurrences has a high chance of developing an invasive tumour whereas the only categories of patients with G1 disease who have a real risk of developing subsequent invasion are those with either multiple recurrences (greater than 5 at each cystoscopy), or those who develop disease in the upper tracts or prostatic ducts. Other prognostic factors include DNA analysis by flow cytometry as tumours with a diploid pattern have a better prognosis than aneuploid ones.

However although these techniques may have a role in routine clinical practice in the future it is doubtful whether at present they provide any more useful information over histological grade and stage alone.

1.2.1.2 Intravesical chemotherapy

The first intention in managing a patient with superficial bladder cancer must be to surgically resect or fulgurate all visible tumour. Patients who show the risk factors for developing invasive disease listed above, and those who persist in developing frequent recurrences are candidates for some form of intravesical therapy in the hope of normalising the bladder urothelium, or at least reducing subsequent recurrences and hopefully preventing progression. Treatment may be either adjuvant (prophylactic) with the aim of preventing recurrence, or therapeutic to treat established disease. Agents commonly used include epodyl (no longer available), thiotepa, mitomycin C, adriamycin or Bacillus Calmette-Guérin (BCG). There have been many trials over the years and although results vary considerably, it would appear that the chief benefit seems to be in slightly reduced numbers of recurrences and prolonged interval to first recurrence rather than in altering the natural history of superficial bladder cancer (i.e. reducing the progression rate) or giving any long term survival advantage (Herr *et al.*, 1987).

1.2.2 The pT1 G3 bladder tumour

Around 60% of invasive transitional cell tumours arise in patients with pT1 G3 disease yet these constitute less than 20% of all bladder tumours (Smith *et al.*, 1986). Furthermore these tumours are often associated with coexistent carcinoma *in situ* (Cis) which is a further high risk factor for progression (see below). Five year local recurrence rates after TUR alone are in excess of 60%, and 80% will get recurrences elsewhere in the

bladder (Jakse *et al.*, 1987). The risk of an invasive tumour developing in such a patient within 2-5 years has been estimated at around 40% overall from series comprising some 180 patients, and 60% in those who get recurrent superficial tumours (Birch and Harland, 1989).

Intravesical chemotherapy - Because of this risk most clinicians would give adjuvant intravesical therapy to patients with high grade superficial tumours though, as mentioned above, no controlled study has yet been able to demonstrate that this will reduce the progression to invasive disease in the long term (Rübben *et al.*, 1988). Time to first recurrence is delayed and the number of recurrences are reduced in the first 1-2 years and although it would see reasonable to expect that if recurrence rates are reduced so might progression rates, unfortunately this does not seem to be the case. A study of 60 patients with superficial G3 tumours treated with adjuvant intravesical chemotherapy had a similar rate of progression (40%) to that in a series treated by TUR alone (Smith *et al.*, 1986).

Radiotherapy - Radiotherapy, most commonly used for muscle-invasive bladder cancer, has been shown of benefit in the treatment of high grade superficial tumours. Most series quote freedom from progression in around 50% after 2-5 years follow-up though those who do not achieve an initial complete response do very badly (Quilty and Duncan, 1986). There also seems little benefit in irradiation after failed intravesical chemotherapy both in terms of eradicating disease and in producing severe side-effects (Sawczuk *et al.*, 1988).

Surgery - There are several studies supporting the view that the best survival rates are obtained when cystectomy is carried out early. Stöckle *et al.* (1987) reported a 90% 5-year survival in 55 patients whose bladders were removed upon diagnosis compared with only a 62% 5-year survival, albeit in a smaller number of patients, in whom cystectomy was delayed

until further recurrences had occurred. However early surgery inevitably overtreats the 15-20% of patients who will be cured by a single TUR alone and, as most urologists are loath to perform possibly unnecessary cystectomies, this is an unusual course of action, at least in the U.K. Perhaps with the increasing refinement and availability of reconstructive surgery this will begin to change.

1.3 CARCINOMA *IN SITU*

The term carcinoma *in situ* (Cis) covers a wide spectrum of disease from a flat localised area in an otherwise normal bladder which may remain relatively static and asymptomatic for years, to a generalised involvement of the epithelium producing severe symptoms (malignant cystitis) and having a high risk (60-80%) of rapid progression to muscle invasive disease by which stage the patient is invariably doomed (Utz, 1970). The histological appearances in Cis are of high grade (G3) malignant cells confined to the epithelial layer (UICC, 1981). Their propensity for exfoliation can lead to confusion in small biopsies which may be completely denuded of epithelium, although these patients will always have positive urine cytology. Dysplasia which is a benign condition suggesting urothelial instability may lead on to Cis, though severe dysplasia is morphologically indistinguishable from Cis and therefore synonymous with it.

1.3.1 Incidence

Primary Cis (unassociated with any exophytic bladder tumour) is a rare condition with a strong male predominance. Reported incidences vary considerably from as low as 0.3% of all bladder tumours in one large series (Farrow *et al.*, 1977), to 7% in a series containing a bias of tumours

induced by industrial carcinogens (Glashan, 1989). Secondary Cis in association with other transitional cell carcinomas is more common, increasing with the stage of the primary tumour, so that it is found in association with about 20% of T1 tumours and 33% of T2 tumours (Glashan, 1989). However secondary Cis is probably under-reported and will often be missed unless random biopsies of normal looking as well as the suspicious areas of bladder mucosa are taken.

1.3.2 Treatment

As there is so much variability in the natural course of a patient found to have Cis, a single treatment protocol is inappropriate. Although there is a high chance of recurrence of Cis and an overall risk of some 60% of progression to invasive cancer without adjuvant therapy (Utz *et al.*, 1980), it is reasonable just to treat conservatively by fulguration, or even by observation alone, those patients with small areas of flat asymptomatic Cis. Patients with the more aggressive widespread forms of the disease which appear raised and angry-looking at cystoscopy, invariably have symptoms of bladder irritability, reduced capacity and pain which may be most disabling and in themselves an indication for cystectomy.

Intravesical agents - The common chemotherapeutic agents such as Thiotepa (Farrow *et al.*, 1977), Mitomycin C (Heney, 1985), and Adriamycin (Jakse *et al.*, 1984) will achieve a complete response rate in at least 50% of patients with Cis. Bacillus Calmette-Guérin (BCG) seems slightly more effective with initial normalisation of the bladder epithelium in 60-70% of patients (Haff *et al.*, 1985). Herr *et al.* (1986) still had a complete remission in 50% of 47 patients at 5 years (68% initial response).

Systemic chemotherapy - Encouraging responses of extensive Cis have been achieved with intravenous cyclophosphamide which, as well as

proving more effective than intravesical mitomycin C, also led to the rapid resolution of symptoms of malignant cystitis and reversed prostatic duct involvement (Jenkins *et al.*, 1988a). Although this experience was in only 17 patients none of the 75% who responded developed disease progression during follow-up of more than 3 years. However major irritative symptoms were common, for which 2 patients required cystectomy.

Radiotherapy - Most evidence suggests that there is no place for radiotherapy in the management of primary Cis (Riddle *et al.*, 1976; Whitmore and Prout, 1982).

Radical surgery - There is little doubt that early cystectomy produces the best chance of survival from carcinoma *in situ* (Riddle *et al.*, 1976; Farrow *et al.*, 1977). The need is to identify those patients who need surgery before progression occurs.

The most satisfactory current approach is to locally fulgurate all visible tumour at the initial cystoscopy and then to repeat this with random biopsies a few weeks later. Residual disease or positive cytology should be treated with no more than 2 courses of BCG, and cystectomy performed without undue delay on those whose disease persists or later relapses.

1.4 INVASIVE BLADDER CANCER

Invasion of the muscle layers of the bladder wall by tumour is an ominous prognostic sign with an overall 5 year survival rate less than 50%. Most invasive tumours present as such (about 25% of all new bladder cancers) with only a small minority developing in patients with a preceding history of superficial carcinoma. The great majority are transitional cell carcinomas.

1.4.1 Radical Treatment

The standard treatment in the UK of non-metastatic transitional cell carcinoma which is invading muscle consists of radical cystectomy with urinary diversion or radiotherapy, either alone or in combination. There still exists some diversity of opinion as to the best management of primary, potentially curable, invasive bladder cancer largely because there have been few large, randomised prospective trials of the various treatment options available. The Institute of Urology and Royal Marsden study of T3 tumours for instance, which commenced in the 1960's, took 10 years to recruit 200 patients (Bloom *et al.*, 1982).

Radiotherapy prior to radical cystectomy offers the potential of reducing pelvic recurrence and several series have shown some benefit from this combination over surgery alone. Others though have not found any added benefit from preoperative radiotherapy (Skinner and Lieskovsky, 1984).

The lower morbidity of modern radiotherapy compared with cystectomy has led many centres to adopt radiotherapy as their primary treatment of muscle invasive bladder cancer. This will result in a complete regression in about 50% of cases though in only 60% of these will the response be durable (Quilty *et al.*, 1986). Cystectomy can then be reserved for those who fail local control and have no evidence of metastases. A recent series of 182 patients with T2 or T3 bladder cancer showed an overall corrected 5 year survival rate of 40% following radical radiotherapy as the primary treatment, with salvage cystectomy in suitable cases (18%) (Jenkins *et al.*, 1988b). Adjuvant chemotherapy has given encouraging initial response rates of 55 - 80% but no clear survival advantage over radiotherapy alone has so far emerged in randomised trials (Raghavan 1988). A current MRC trial is underway to assess the effect of combination chemotherapy before conventional treatment (radiotherapy or cystectomy).

1.4.2 Local excision

Partial cystectomy has been commonly employed for the rarer non transitional-cell carcinomas, particularly urachal tumours. There is also sometimes a role for partial cystectomy for favourably placed solitary invasive tumours in frail patients, or for those tumours arising in a diverticulum. In general, though, results are disappointing with a high incidence of local recurrence and metastases (Gill *et al.*, 1989).

Radical TUR whereby the full thickness of the bladder wall is resected has been shown to give good results in the hands of enthusiasts. Clearly though this is only suitable for relatively small tumours in extraperitoneal sites and there is a worry that the inevitable extravasation may lead to extravesical recurrence. For this reason combination with chemotherapy seems advisable (Hall *et al.*, 1984).

1.5 LASER TREATMENT OF BLADDER CANCER

1.5.1 Introduction

There has been enormous interest in the possibility that lasers could add to the current management of bladder cancer. Lasers are not magic, healing rays as they have often been portrayed but they can produce unique, precise and predictable effects on tissue which may complement existing techniques and treatments. The ability to transmit powerful light energy along fine quartz or silica fibres has opened up the whole field of therapeutic endoscopy to laser techniques. Most interest has focussed on the treatment of malignant disease of which bladder cancer is but an example. Clearly to be worthwhile a new technique must offer real advantages over existing methods, if not in overall improved prognosis then at least in terms of economy, convenience or reduced morbidity.

Most work has centred around the thermal effects of the neodymium: yttrium aluminium garnet (Nd:YAG) laser or the photosensitising abilities of certain drugs activated by specific wavelengths of laser light. The various types of urological lasers will be discussed in detail in chapter 2, but for now it is useful to reflect more generally on the introduction of laser techniques to urology.

1.5.2 Historical background

The first workers to apply laser energy to the bladder were Parsons and co-workers (1966), who exposed an opened canine bladder to 694 nm light from a pulsed ruby laser, using energy levels of 10 - 15 J. The histological appearances after 3 - 10 days showed minor damage confined to the mucosa with a sharp transition from neighbouring normal tissue. They suggested that this new energy modality might destroy bladder cancer endoscopically, provided a suitable "light pipe" could be found. This breakthrough did not come until the development of the flexible quartz fibre (Nath *et al.*, 1973) and the argon ion laser.

Several urologists have studied the argon ion laser (Staehler *et al.*, 1976). It is not ideal for urological use as it is low powered, poorly haemostatic and although readily transmitted by quartz glass fibres, only penetrates tissue for 1 - 2 mm. The blue/green argon laser light is well absorbed by haemoglobin and whilst effective for small bladder tumours (Smith and Dixon, 1984), it has in clinical practice been supplanted in this role by the more powerful, compact and reliable neodymium:YAG laser (section 2.2.1). The argon laser can be used to pump (excite) a dye laser and this configuration is still the standard light source for photodynamic therapy (Benson, 1988). Even in this field though the argon dye laser is likely to be superseded by the newer, more powerful pulsed metal vapour lasers.

Müssiggang and Katsaros (1971) were the first to describe the use of the Nd:YAG laser, for urological purposes. They realised its advantage over the carbon dioxide laser in being transmissible via flexible glass fibres, and designed a prototype laser cystoscope using bundles of thin glass fibres to transmit the energy. Unfortunately glass caused significant transmission losses, and the resultant heat generated quickly melted the adhesive used to hold the bundles together unless the whole apparatus was water cooled. They studied the effect of the pulsed Nd:YAG laser on bladder, prostate and renal tissue as well as calculi. In the bladder they were able to produce coagulation to a depth of about 1 mm using a power of 25 W. This damage was sharply demarcated from the surrounding normal tissue. Staehler and Hofstetter (1979) produced full thickness lesions (2 mm) in rabbit bladder with a power of 40 W for 2 s, in comparison to the more shallow lesions made by an argon laser.

1.5.3 Photodynamic Therapy

Photodynamic therapy (PDT) is an attractive approach to the treatment of superficial bladder cancer, especially resistant Cis. The mechanism of PDT will be described in detail in chapter 4 but in brief involves the administration of a photosensitising drug which is retained with some selectivity in malignant tissue (Benson *et al.*, 1982). Light of a specific wavelength corresponding to an absorption peak of the photosensitiser is then used to illuminate the bladder mucosa. This light is best produced by a laser and transmitted via a diffusing fibre placed centrally within the bladder at cystoscopy. Illumination activates the photosensitising drug to produce local necrosis of tumour, probably mediated via singlet oxygen, with ideally little adverse effect on adjacent normal tissue. Because PDT is not a thermal process the treatment is painless and could possibly be

carried out on an outpatient basis though all workers so far have used general or epidural anaesthesia because of the relatively lengthy illumination times required (Stamp *et al.*, 1990). Treatment of superficial bladder cancer with PDT should still be regarded as an experimental therapy whilst applications to tumours in solid "urological" organs such as the prostate are still in the pre-clinical phase.

It is in the patient with widespread Cis that PDT is most likely to be of value. It has the advantage of being a single treatment and although there are insufficient clinical data available to assess durability of response it should be possible to retreat patients who relapse without additional toxicity. Although the limitations of extravesical field changes apply to bladder PDT as well as intravesical chemotherapy it would be technically possible with currently available delivery systems to also treat the prostatic urethra and lower ureter directly.

The bladder seems ideally suited to PDT as it is readily accessible and as the entire mucosa can be treated simultaneously, areas of occult dysplasia and Cis do not have to be precisely defined. The published clinical data are discussed in detail in chapter 3, but they generally show that PDT is most effective at treating Cis, less so for superficial papillary disease, and ineffective for invasive tumour. Although good results have been achieved in terms of the eradication of superficial bladder cancer resistant to all conventional modes of treatment short of cystectomy, in around 65% of patients treated (Benson, 1986; Shumaker and Hetzel, 1987); this has been at the cost of considerable morbidity in most patients. Those serious side effects reported include severe irreversible bladder shrinkage, sometimes sufficient to require cystectomy for intolerable symptoms (Nseyo *et al.*, 1985a), and perhaps more worrying, a high incidence of upper tract obstruction and vesico-ureteric reflux (Harty *et al.*, 1989).

1.5.4 Neodymium:YAG laser coagulation

The Nd:YAG laser energy is transmitted efficiently along modern optical fibres and through water and has been used by urologists for more than 10 years. Its deep tissue penetration and excellent haemostatic properties make it the laser of choice for thermal coagulation of bladder cancer. It is with the treatment of small, superficial tumours that most expertise has been gained, notably from European centres such as Oslo (Beisland and Seland, 1986), and Hofstetter's groups in Munich and Lübeck (Hofstetter, 1987). As a result of these studies several advantages have been advanced for the Nd:YAG laser compared with standard electrocoagulation, noticeably that of improved efficacy in terms of controlling the disease with some authors reporting a much lower recurrence rate after laser coagulation compared to that seen following electrocautery (Hofstetter *et al.*, 1981; Malloy *et al.*, 1984). However there have been few prospective trials between laser and diathermy and these all have major shortcomings, as will be discussed in chapter 9. One of the most beneficial aspects of laser coagulation is that it is fairly painless and should enable, in conjunction with flexible cystoscopy, the treatment of the great majority of bladder cancer patients as out-patients without anaesthetic (Fowler, 1987).

There have also been some 7 studies, comprising around 150 patients, investigating Nd:YAG coagulation as a means of local control of invasive bladder tumours and generally showing promising results for T2 tumours Some authors quote recurrence rates of less than 10% (Staehler *et al.*, 1985; McPhee *et al.*, 1988). On the other hand laser coagulation seems to give much poorer local control of T3 tumours with recurrence rates generally in excess of 50% (Smith, 1986b; McPhee *et al.*, 1988).

With the current rapid advances in instrument technology it also seems likely that superficial low grade urothelial tumours of the kidney may soon

be treatable endoscopically, either via a percutaneous tract or more attractively via retrograde ureterorenoscopy. Ureteric tumours are already being successfully coagulated by laser with less risk of traumatic perforation or subsequent stricture than after diathermy (Schmeller and Pensel, 1989).

1.6 DISCUSSION

The role for laser treatment in bladder cancer is still evolving. Often the adoption of a new technique has as much to do with the personality of the enthusiast as with any clear evidence for its superiority over existing methods. Such has been the case with Nd:YAG laser coagulation of bladder tumours. The great vigour that accompanies the emergence of a new treatment such as PDT may lead to its wide application in a somewhat haphazard way resulting inevitably in some poor results which then bring the whole concept into disrepute. The right balance will be impossible to achieve without careful experimentation and logical clinical trials.

Widespread bladder Cis or high grade multifocal papillary carcinoma frequently herald the onset of a lethal invasive bladder cancer. Standard intravesical chemotherapy is unlikely to produce a durable response in more than 50% of patients. Early cystectomy often seems an excessively radical step for such superficial disease with the result that surgery may be delayed too long in non-responders, until the disease is incurable. An effective new treatment which allowed the patient to retain his natural bladder would therefore be very valuable and it is for this reason that PDT has aroused our interest.

There seems little doubt that PDT can destroy bladder tumours, but before it can become an accepted treatment for some resistant forms of superficial

bladder cancer there needs to be much more pre-clinical research and careful clinical trials. This is because the rapid clinical introduction of PDT in the bladder whilst it was still an under-researched treatment, led to major side-effects and there is a current need to revert to the sort of preclinical studies described in this thesis. These should investigate the effects on normal bladder as well as on tumour areas, to identify ways of reducing the inevitable side effects to acceptable levels. As these seem to be the result of necrosis followed by fibrosis in the muscle layer of the bladder, i.e. the result of damage to normal tissue rather than to tumour, these experiments can be most expeditiously performed on normal bladder. This is fortunate as an animal model of superficial bladder cancer and in particular of Cis, that closely approaches the human condition is not available.

The major part of the experimental work in this thesis (chapters 6, 7 and 8) seeks to explore the morphological and functional effects of PDT on normal rat bladder and the subsequent recovery of bladder function during healing. Although it is recognised that such work may not be directly transferable to the clinical situation nevertheless it is hoped that general principles may be established that will aid our further understanding of this exciting technique.

The place of thermal, i.e. Nd:YAG laser, coagulation in the treatment of superficial bladder cancer is by no means clear. The early laser pioneers treated large numbers of patients and claimed a significant reduction in the overall recurrence rate after laser coagulation (Hofstetter *et al.*, 1981). However these studies were not properly randomised against standard therapies and most subsequent authors have not been able to substantiate their claims, though there may be some advantage over diathermy in terms of a reduction in local recurrence rates.

An effective local treatment for early muscle-invasive bladder cancer would be a worthwhile advance but the extent of laser necrosis produced must be matched to the dimensions of the tumour being treated under conditions where one can be sure of safe healing. Therefore not only must the laser clinician know the extent of clinical disease (and here the more widespread use of improved imaging techniques should be useful), but he must also know the biological effect of the dosimetry parameters to be applied in treatment. Some studies of the morphology of Nd:YAG lesions in the pig bladder and a comparison of laser with diathermy coagulation are presented in chapter 9.

We seem therefore in the not uncommon situation with new technologies in that enough work has been done to show that a treatment is feasible but, after the initial novelty has worn off, little careful work has been done to define its proper place in the therapeutic armamentarium. In these days of restricted in-patient beds and the need for improved efficiency we should perhaps be looking at laser treatment to aid our service to patients by enabling a reduction in the need for repeated in-patient admissions and general anaesthetics. The prognosis of superficial low grade papillary bladder cancer is unlikely to be improved by Nd:YAG laser coagulation compared with diathermy coagulation as the incidence of new tumours seems unaltered. The scope for improvement is likely to be in providing more efficient treatment for the great majority of cases as outpatients without anaesthetic. If this is feasible, and there is only 1 study suggesting it may be (Fowler, 1987), then the economics of this approach, balancing the high cost of a laser against savings in bed occupancy and theatre time, need to be addressed. This was the justification for embarking on the study discussed in chapter 10.

Chapter 2

LASERS: PRINCIPLES AND EFFECTS

2.1 INTRODUCTION

Light is an essential part of photodynamic therapy which requires the production of a specific wavelength depending on the absorption spectrum of the photosensitiser used. For external illumination of small areas the filtered beam from an incandescent light source is usually adequate but it has become customary to use a laser light source as this offers several advantages. Laser light can be produced at precisely the wavelength and power required and being so pure should contribute towards a consistent biological effect, essential for the evaluation of this technique. Lasers become essential for PDT when internal organs are to be treated as it is not possible to effectively focus other forms of light down the fine optical fibres necessary for endoscopic or interstitial use.

High energy lasers producing thermal effects (e.g. coagulation or cutting), or photomechanical effects (e.g. lithotripsy) also have a place in urological practice. Of these the thermal action of the Nd:YAG laser is the most widely used and is the instrument employed for some of the work in this thesis. It is necessary, therefore, to discuss briefly how a laser works and to outline the characteristics of the relevant laser systems. This is not intended to be a comprehensive account of urological laser practice for which the reader is referred elsewhere (McNicholas, 1990).

The production of laser light requires the *stimulated emission* of photons in a controlled manner from a medium excited by an external energy source. When an atom absorbs energy it becomes excited but will soon spontaneously return to its stable ground state by releasing this absorbed energy in the form of a photon. Normally more atoms are in the ground state than in an excited state but if sufficient energy is applied to ensure that the rate of production of excited atoms is faster than that of spontaneous decay a "population inversion" results. Under these conditions a photon

released from spontaneous decay can strike an already excited atom in its path to release another photon identical to the first in terms of wavelength, direction of travel, amplitude and phase. This is the process of *stimulated emission* of radiation, first predicted in theory by Einstein over 70 years ago, and which results in an amplified cascade of these identical photons hence the acronym Light Amplification by the Stimulated Emission of Radiation - LASER.

The light is produced within an optical cavity configured in such a way that two parallel mirrors on opposite faces cause the internal reflection of those photons travelling exactly perpendicular to the mirror face whilst any others are lost. This means that the stimulated photons will also be travelling in the same direction, resulting in a narrow, precisely coherent beam. One of these mirrors is made partially reflective so that some of the light is emitted as the laser output which can be focussed into a small spot or transmitted via a flexible fibre. If light is prevented from escaping (e.g. by rotating one mirror) whilst the laser medium is being energised, then a very large population inversion builds up and upon restoring the exit path all this energy is released as a very high energy, short duration pulse; this is known as Q(quality)-switching. Other types of pulsed lasers producing their output somewhat differently by energising the laser medium using repeated electrical or optical pulses which results in a pulsed laser output.

Specific lasers derive their names from the laser medium used which may be solid (e.g. ruby, neodymium:yttrium aluminium garnet - Nd:YAG, holmium:YAG, or alexandrite), liquid (various coloured organic dyes depending on the output wavelength required) or gaseous (e.g. argon ion, carbon dioxide or metal vapour). New variants are continually coming onto the market, though these rarely represent a real advance and will not be considered here in any great detail.

2.2 LASERS FOR PHOTODYNAMIC THERAPY

All photosensitisers have an absorption spectrum with a peak in the red part of the visible spectrum. The exact wavelength depends on the sensitiser though for the most commonly used (haematoporphyrin derivative - HpD, and dihaematoporphyrin ether/ester - DHE) it is at 630 nm. Dye lasers use liquid organic dyes as the lasing medium which have a fluorescence spectrum spread over about 50 nm. The particular dye used depends on the wavelength band required and final fine tuning is achieved with a filter. The dye laser has to be excited by another laser which is termed "pumping". In theory any laser can be used for this job as long as it produces an output wavelength shorter than that desired from the dye, though in practice the efficiency is greatly reduced if they get too similar. At present, the laser sources most often used to pump a dye laser are the argon ion or copper vapour lasers. The gold vapour laser emits at 628 nm which is exactly right to excite HpD and DHE, but would not be suitable for the newer photosensitisers that generally absorb at a higher wavelength.

Other possibilities include pumping dye lasers with excimer or frequency-doubled Nd:YAG lasers (e.g. the KTP laser - potassium titranyl phosphate).

The laser output is capable of being focussed onto a very small spot (100 - 200 μ) which enables it to be coupled into the end of an optical fibre and transmitted to the target tissue. Fibres are made from a core of quartz glass or fused silica enclosed in a cladding layer of lower refractive index material to minimise transmission losses. This is then covered by a protective plastic sheath (usually PTFE - polytetrafluoroethylene). The laser light emerges from the distal end of the fibre as a slightly divergent cone so, in the absence of any focussing devices, the power density on the target tissue depends largely on the distance of the fibre from the surface.

In the work described in this thesis a copper vapour pumped dye laser system was used for the *in vivo* experiments, with aluminium sulphonated phthalocyanine (AlSPc) as the photosensitiser. A flashlamp-pumped dye laser was used for the *in vitro* work with DHE as the photosensitiser. Both of these are pulsed lasers but it should be recognised that the most commonly used laser system for PDT in most centres is still the continuous wave argon ion pumped dye laser.

2.2.1 Copper vapour pumped dye laser

The lasing medium is gaseous copper vaporised in a vacuum within a ceramic tube by repeated high voltage pulses from a thyratron (10-20 kV). This produces a population inversion within the copper atoms and a lasing action is produced yielding two wavelengths, at 511 nm (green) and 578 nm (yellow). The copper vapour laser only operates in a high frequency pulsed mode at a pulse repetition rate of 10 kHz and pulse duration of 30-50 nanoseconds (fig. 2.1).

The copper vapour laser is coupled to a dye laser so that its output passes into the dye chamber. The dye which is continuously circulating is pumped as a fine jet set at an angle (Brewster's angle) across the optical path of the laser beam resulting in a population inversion within the dye molecules. The output beam from the dye laser is passed through a birefringent filter, which can be rotated around an axis perpendicular to the laser beam. For each position of the filter only a single wavelength of light can oscillate within the dye cavity and hence fine tuning of the output wavelength is achieved. The output band of the dye laser depends on the dye used. To get an output at 630 nm suitable for HpD or DHE, Rhodamine B is the dye used, whereas an output at 675 nm necessary for AlSPc was achieved by using a mixture of 0.7 mM Ozozine 72 and 0.3 mM Rhodamine 6G.

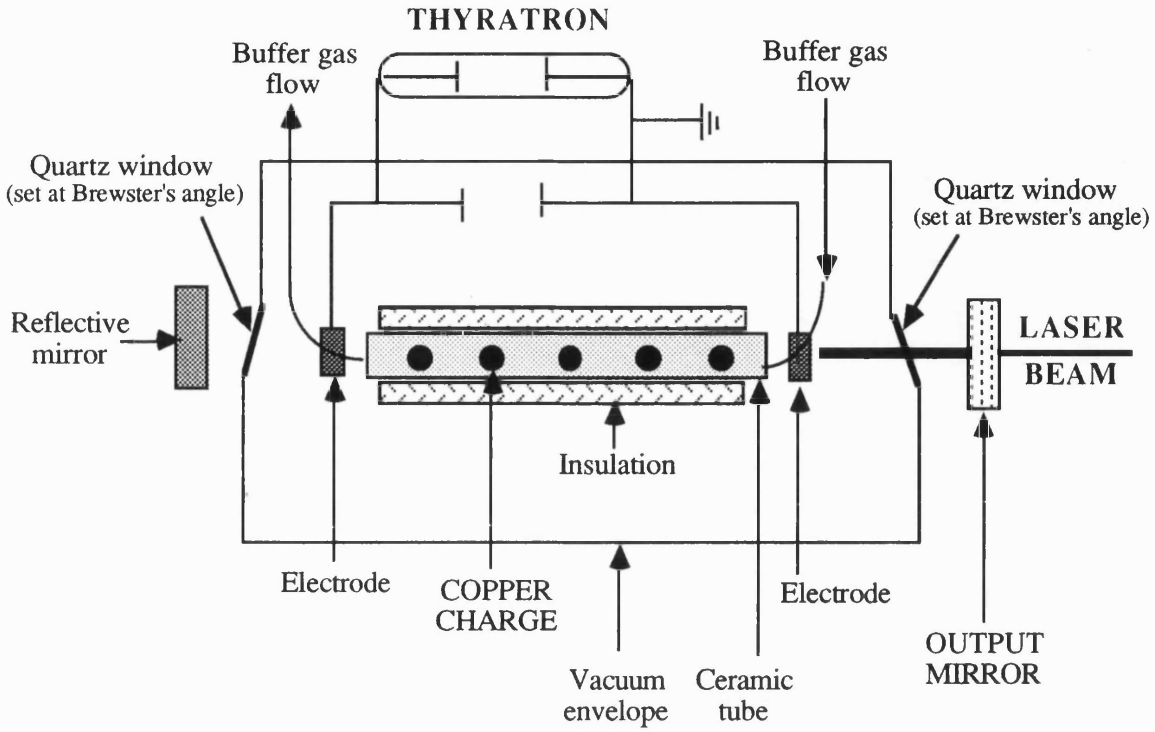


Fig. 2.1 Diagram of a copper vapour laser

The output is coupled to a dye laser (not shown)

The output powers achievable with the copper laser system can be greater than 5 W, rather more than from the argon dye laser as it is more efficient. This is not so important for most small animal work but would become vital when for instance performing whole bladder illumination clinically. This laser has also been more reliable in our experience than the argon dye and is simpler and cheaper to maintain. One disadvantage though is that the copper tube takes around 1.5 hours to reach operating temperature and peak lasing effect so this effectively rules out morning activities! Initially there was some concern as to whether the pulsed output would produce a different photodynamic effect to continuous wave light but it now seems that for all practical purposes this can be considered a continuous wave output (Cowled *et al.*, 1984; Barr *et al.*, 1989).

2.2.2 Flashlamp pumped dye laser

This laser uses a high intensity light source to excite the laser medium. A gas discharge flashlamp produces intense pulses of light, rather than continuous light, which are focussed onto a narrow dye channel by an elliptical reflector (fig. 2.2). These microsecond light pulses produce a population inversion within the dye channel. The pulse rate is many orders slower than that of the copper vapour laser (5 - 20 Hz) and large volumes of dye are pumped though the circulating system, which results in large pulse energies and peak pulse powers.

The wavelength emitted can be varied depending on the particular dye used: cresol violet will produce a wavelength of 675 nm (red) whereas coumarin dye produces a green light output (504 nm).

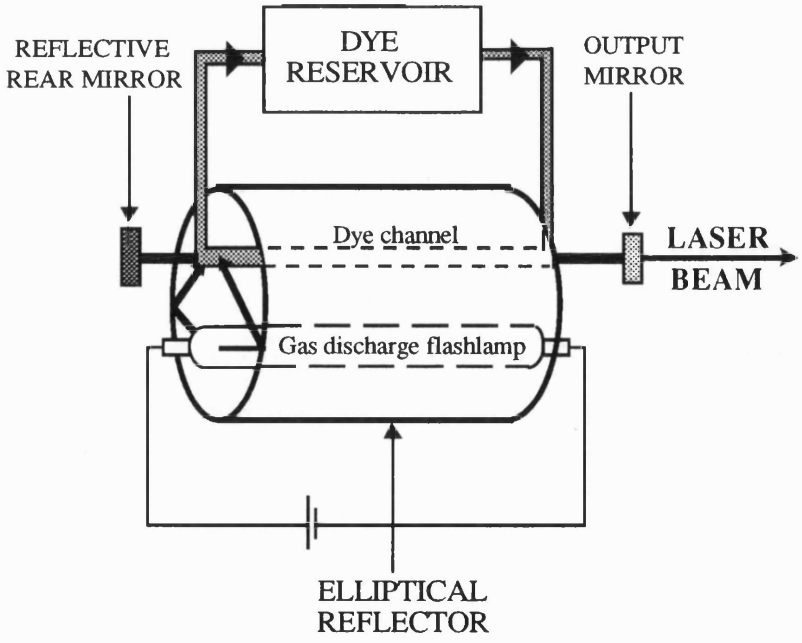


Fig. 2.2 Diagram of a flashlamp pumped pulsed-dye laser
(the water cooling jacket is omitted)

In very close proximity to tissue the intense light from a flashlamp dye laser will disrupt electron bonds, forming a tissue "plasma" and clinically its most important application is to fragment urinary and biliary calculi. If the laser fibre tip is some distance from tissue this shockwave effect is lost but it was thought interesting to see if the intense pulses of light would produce a photodynamic action and this is the basis of work described in chapter 5. The laser used was a prototype (Candela MDL-1P, Candela Corp., Natick, M.A., USA), which needed a lot more maintenance than the current clinical machines though generally it was reliable with a stable output and needed little warm-up time. It also required 3-phase electrical supply and external water supply for cooling the flashlamp.

2.3 THE NEODYMIUM:YAG LASER

The Nd:YAG laser has proved most effective as a thermal coagulator and has been used successfully to treat lesions from the external genitalia to the renal pelvis. Good transmission through water, deep tissue penetration and excellent haemostatic properties make it the laser of choice for coagulating bladder tumours. It is therefore with the Nd:YAG that most expertise has been gained, notably from European groups such as Hofstetter's who have treated over 1000 patients since 1976 (Hofstetter, 1987), and whom should be credited with the introduction of laser surgery to urology.

The laser medium is a synthetic crystal of yttrium aluminium garnet (YAG) doped with a small concentration of neodymium atoms (Nd). This is pumped by light from a high intensity krypton lamp which is focussed onto the crystal rod (fig. 2.3). Lasing is achieved between long-lived high energy states and short-lived, low energy states of the excited neodymium atoms. The output is usually in the near-infrared at 1064 nm though the

slightly longer wavelength of 1318 nm can also be obtained as well as several harmonics such as 659, 532 and 266 nm. The 1318 nm wavelength is more strongly absorbed by water than the usual 1064 nm and because of this should produce a somewhat more intense coagulation effect on tissue, but may not be as effective endoscopically in the bladder though good comparative data are lacking.

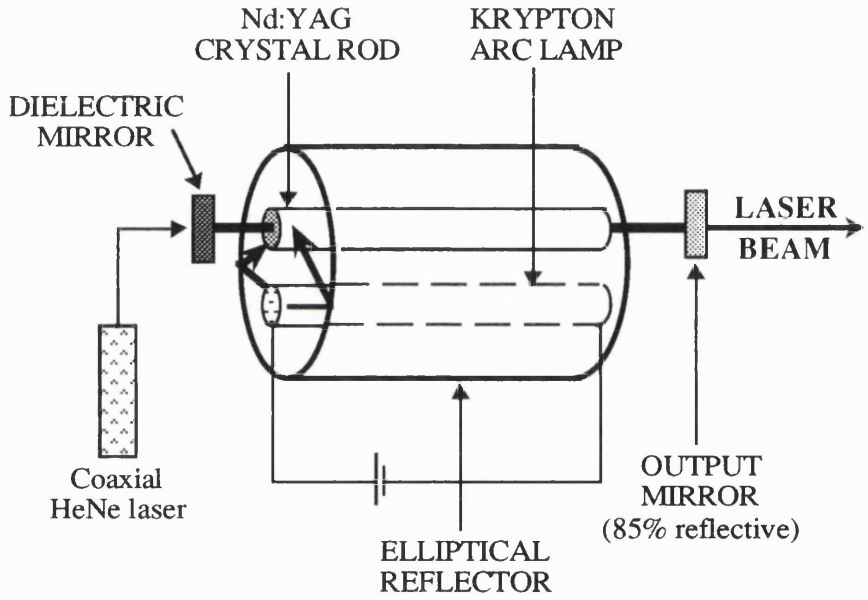


Fig. 2.3 Diagram of a neodymium:YAG laser
(The water cooling jacket is omitted)

Because the infrared Nd:YAG output is invisible, a low powered helium-neon laser (632 nm) is accurately aligned with it to give a red aiming spot. The efficiency (conversion of electrical power to light) of the Nd:YAG laser is relatively high at around 2% though the high power models with an output of 100 mW will require a 3-phase power supply and a high flow of water for cooling. For nearly all urological uses an output of 50 - 60 W is more than ample and several manufacturers have recently introduced compact, mobile machines of this power which use a standard single phase 13 A supply and do not need external cooling.

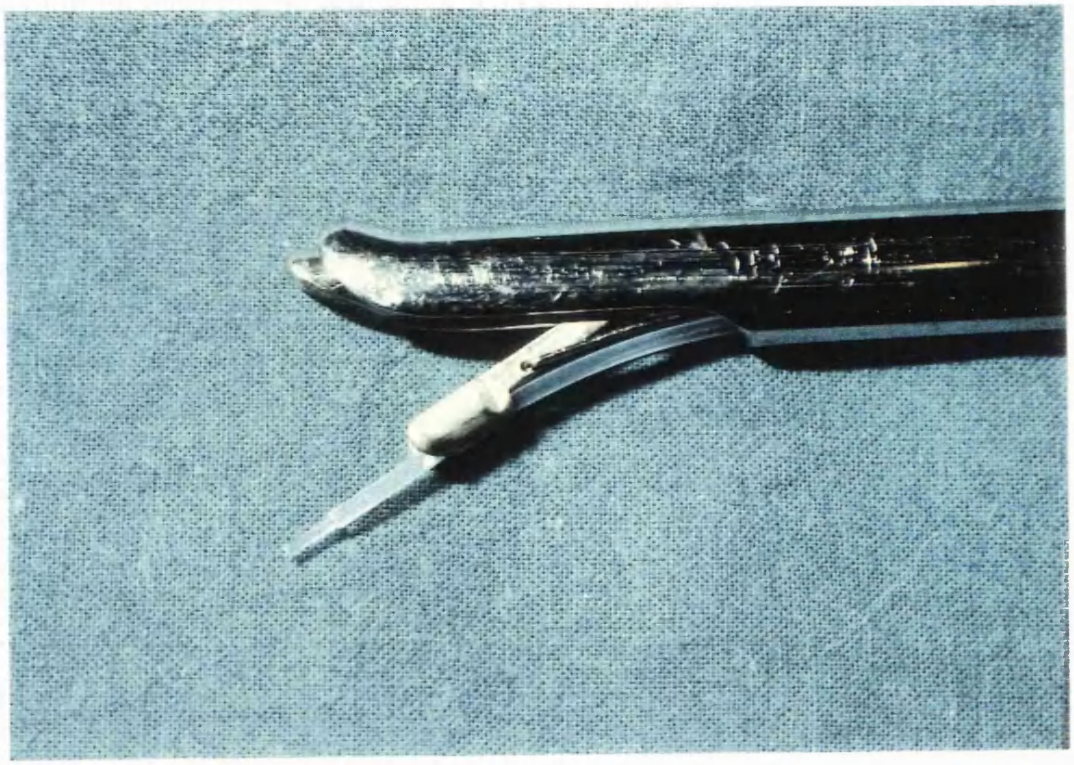


Fig. 2.4 Laser cystoscope
(the laser fibre runs through a modified Albarran bridge)

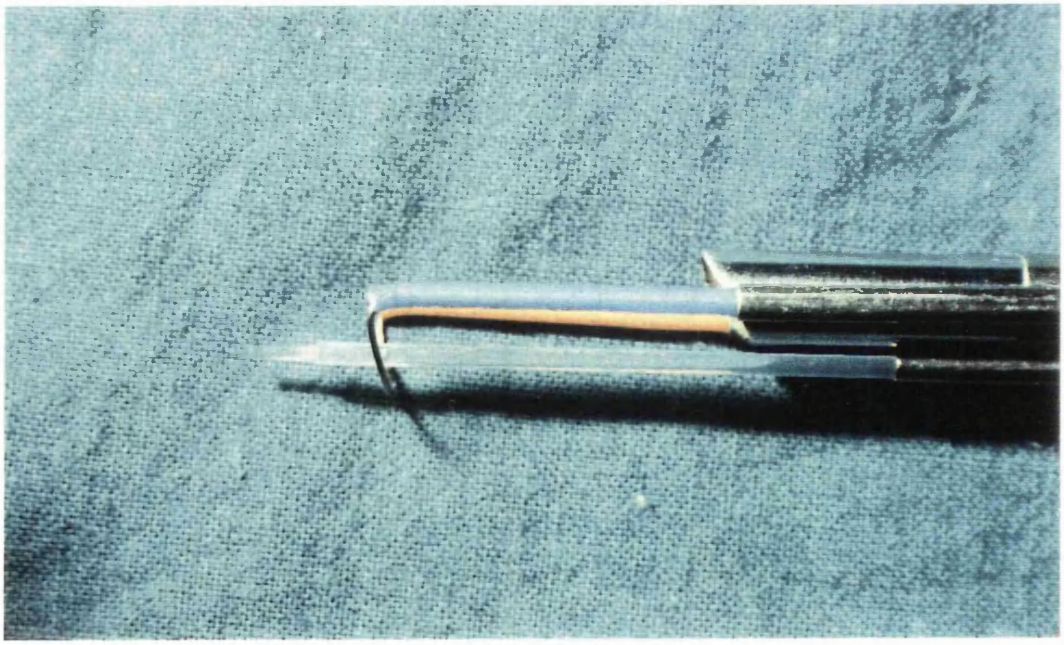


Fig. 2.5 Laser resectoscope
(the laser fibre can be used at the same time as the diathermy loop)

2.3.1 Delivery systems

Some means is required to deliver the light produced by the Nd:YAG laser to the target tissue. The output from the Nd:YAG laser can be focussed onto a spot of about 200 microns. The standard optical fibre used clinically is 600 microns which allows efficient coupling of the laser energy into the fibre. For endoscopy with flexible instruments a thinner fibre (usually 400 microns) is necessary as the rigidity of the 600 micron fibre significantly restricts the deflection range of these instruments. When working under water in the urinary tract sufficient cooling of the fibre is provided by the irrigant so a "bare" fibre is used. For external use, such as when treating anogenital condylomata, the fibre is cooled by a a flow of air in a coaxial outer sheath around it.

The Nd:YAG laser may be used with either flexible or rigid endoscopes. When using flexible endoscopes and bare fibres, care is necessary to protect the instrument working channel against accidental damage from the sharp end of the fibre (see chapter 10). For rigid endoscopy a modified Albarran bridge, which encloses the fibre for precise manipulation, is desirable (fig. 2.4), whilst a modified "laser resectoscope" (fig. 2.5) allows loop resection of a bulky exophytic tumour followed by laser coagulation of its base, without changing instruments.

2.4 LASER LIGHT INTERACTION WITH TISSUE

It is essential to understand the biological effects of laser light interaction with tissue to use lasers appropriately in medicine. On contact with tissue, photons may be reflected, scattered, absorbed or transmitted unchanged. In practice a combination of these events occurs but the only process that

produces any biological effect is absorption. Scattering (internal reflection or refraction) changes the direction of photons, though not their energy, and is caused by the heterogeneous nature of tissue resulting in variations between cells and their surrounding stroma and between differing biomolecules. Eventually the photon must reach a molecule that will absorb it or be lost from the tissue.

An illustrative example is that of external irradiation of skin (fig. 2.6). Upon striking the skin surface some of the photons are reflected immediately, all in the same direction (specular reflection); whilst others are "backscattered" in an irregular fashion (diffuse reflection).

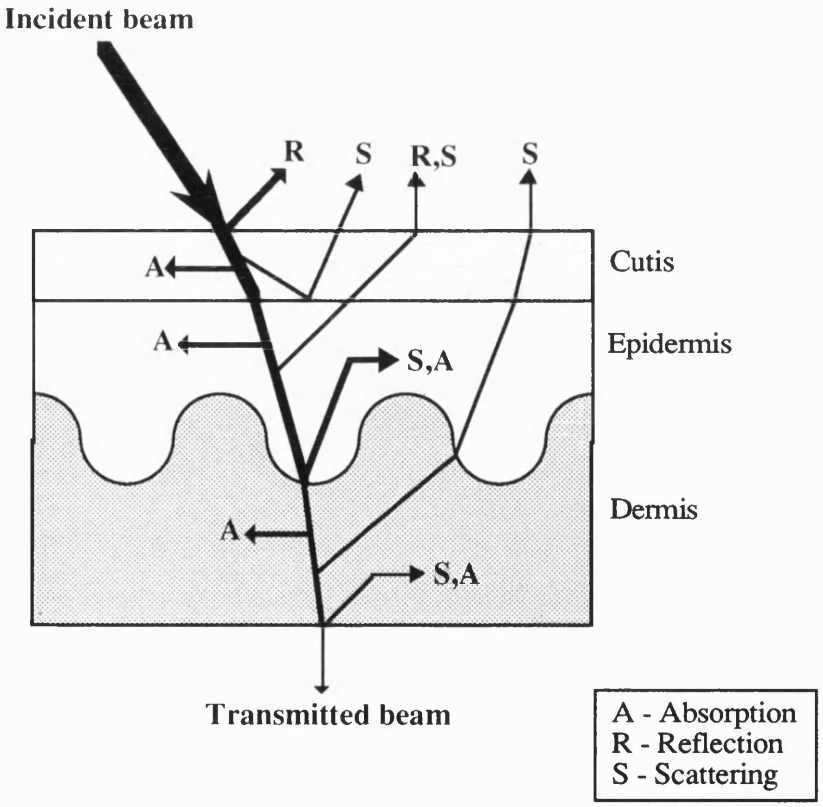


Fig. 2.6 Different types of laser light interaction with skin

Further scattering and absorption takes place within the tissue and any unabsorbed photons pass through to adjacent structures (transmission).

Transmission may be a problem with thin-walled organs such as the bladder when penetrating wavelengths of light such as the Nd:YAG laser in the bladder may cause unwanted damage to overlying small bowel.

Most of the light reflected from tissue is as a result of diffuse reflection which presents dosimetry difficulties in calculating the dose absorbed by the tissue as it is the least easy variable to measure. This will be discussed further in the context of whole bladder irradiation for photodynamic therapy as "internal reflection" in a hollow organ results in reflected light being repeatedly re-incident on another area of the tissue surface.

2.4.1 Biological effects

This is the end result of physical processes encompassing the transfer and absorption of energy from the radiation field to the biological target. To put PDT into context with other laser treatments it is useful to briefly discuss the biological effects that medical lasers can produce which are summarised below.

i) Thermal	Optical radiation is transformed into heat leading to coagulation, evaporation or carbonisation.
ii) Photomechanical	High energy pulses produce shockwaves causing photodisruption (laser angioplasty or lithotripsy).
iii) Photochemical	A non-thermal reaction of light with tissue such as in biostimulation and photodynamic therapy.

2.4.1.1 Thermal effects

The non-specific absorption of a photon by a tissue molecule will produce thermal changes if the energy is delivered more quickly than the rate at which the heat can be dissipated into the surroundings by direct conduction or via blood vessels. The most common laser actions used in medicine are

thermal producing precise coagulation or cutting actions. When using high power thermal energy the tip of the fibre should not touch the target tissue, to avoid charring, which restricts its application to external lesions or the mucosal surfaces of endoscopically accessible organs: e.g. the coagulation of small bladder tumours and bleeding peptic ulcers or malignant tumours of the oesophagus, rectum and bronchus.

The thermal damage produced reduces with the distance from the incident point, both laterally and in depth so it is usual to get a continuum of changes depending on the applied energy (fig. 2.7). The extent to which all these changes are seen in the target tissue depend both on the type of laser used and, to a lesser degree on the tissue itself.

Figure 2.8 shows the emission wavelength of the types of laser in common clinical usage. Light in the 780 - 800 nm range will penetrate tissue best as it is absorbed least by natural tissue molecules, and hence can produce deeper thermal damage. The power that can be produced conveniently at this wavelength though is less than at longer ones so, for clinical use, the Nd:YAG laser emitting 1064 nm light has proved the most effective tissue coagulator producing lesions up to 1 cm deep.

In both the ultraviolet and far infrared part of the spectrum, light is absorbed by tissue much more intensely leading to rapid vaporisation over a very short distance but little coagulation which produces a cutting effect. A cutting effect can only be produced from the Nd:YAG laser by attaching focussing tips to the optical fibre or by producing very short pulses (nsec - μsec pulse lengths). In the far infrared, at a wavelength more than 10 times that of the Nd:YAG laser, the carbon-dioxide laser light (10600 nm) is strongly absorbed by tissue water within 0.1 mm or so producing a cutting action (by vaporising rather than mechanical cleaving), with minimal thermal changes to the immediately adjacent tissue.

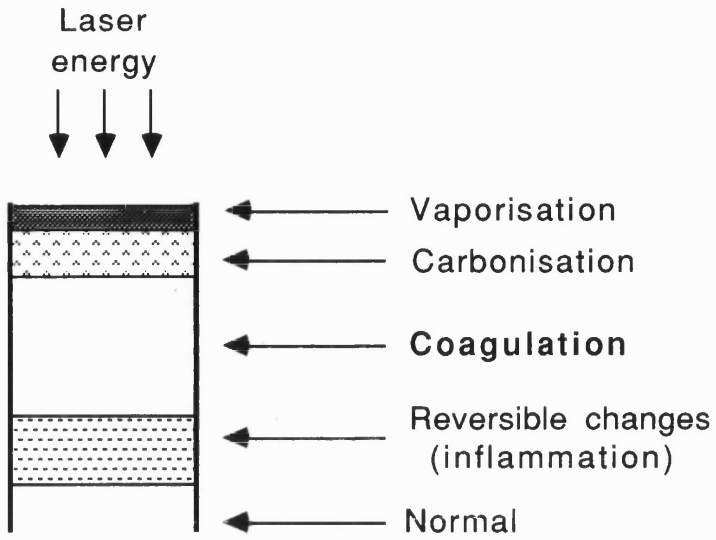


Fig. 2.7 The variation of thermal damage with tissue depth.

(A similar pattern is seen laterally from the incident point but is not shown here for clarity)

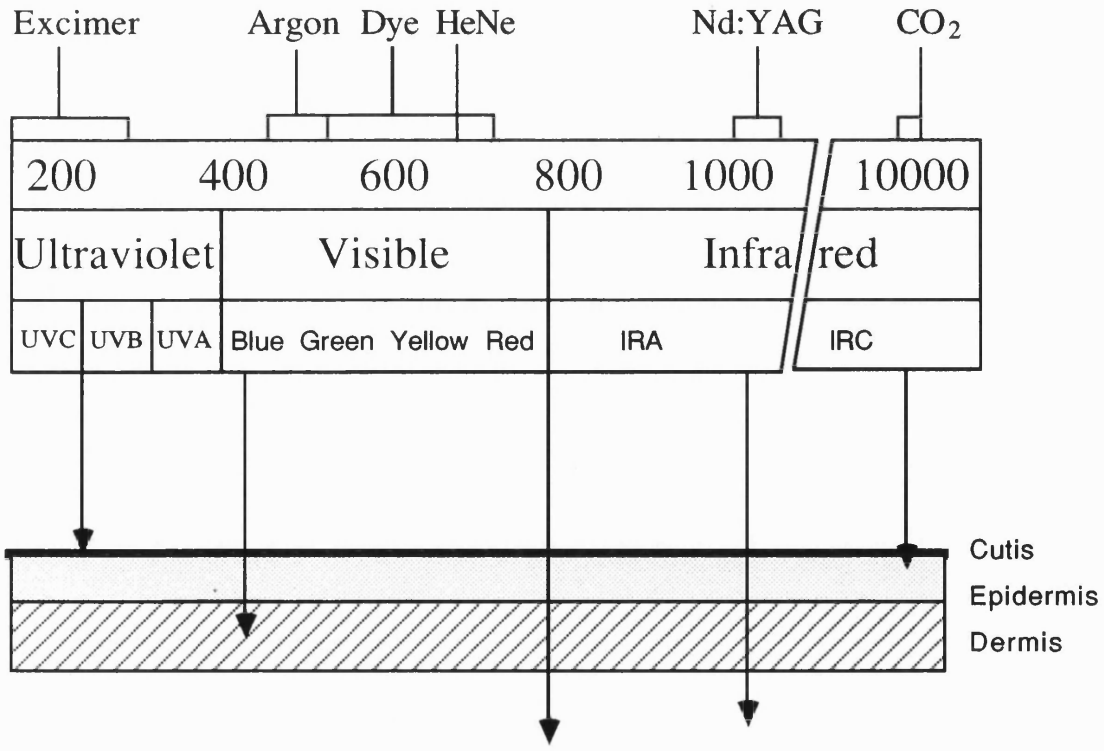


Fig. 2.8 The common clinical lasers and the effect of wavelength on tissue penetration.

2.4.1.2 Photomechanical effects

If the laser output is released in a series of very short pulses then even if the energy delivered is quite low, insufficient for a thermal effect, the peak powers can be enormous. For example an average energy of 100 mJ released as 1 μsec pulses at 5 Hz represents a pulse power of 20 kW. If this is distributed over a very small target area then quite enormous power densities are generated, in the order of megawatts/cm^2. This power can disrupt electron bonds and produce a cascade reaction leading to formation of a tissue "plasma". Brittle substances such as calculi and atheroma are fragmented by this whereas the pure thermal energy required to melt them would be unacceptably high in a clinical setting.

Both a pulsed Nd:YAG (Q-switched YAG) laser and a pulsed dye laser can produce a plasma and fragment calculi. The flashlamp pumped dye laser (section 2.2.2) was pioneered by Watson *et al.* (1987) and can be used with very small calibre ureteroscopes for easier and less traumatic endoscopy. The output wavelength which can be matched to the absorption spectrum of the target material: for example most urinary stones absorb green light well (504 nm is used clinically) whereas the adjacent wall of the ureter does not, allowing safe fragmentation of stones in this confined space. The possible use of this laser for PDT in the bladder is discussed in chapter 5.

2.4.1.3 Photochemical effects

These occur when the absorption of light by a compound results in a chemical reaction in the illuminated tissue. Examples of naturally occurring photochemical processes are photosynthesis in plants, or sunburn in Man. When this principle is used in medicine it is termed photochemotherapy (an example being the combination of a psoralen drug and ultraviolet-A light used for severe psoriasis - PUVA), or photodynamic therapy. Where PDT differs from the other laser-tissue interactions is that

relatively low powers of visible light are used, below that needed to produce a thermal effect. The cytotoxic effect is produced purely by the chemical activation of a light sensitive drug.

In summary, it is clear that the use of lasers in clinical practice is steadily increasing and urological surgery is one of the most fertile fields for their use. Laser techniques fall into 2 main categories, those that achieve results not possible in any other way and those that may improve on existing therapy. Photodynamic therapy falls into the first category whereas laser lithotripsy and coagulation of superficial bladder cancer join the latter.

The challenge is to use the unique and precise effects that lasers may produce to achieve the desired biological result, no more and no less. This is often however no easy matter and usually a lesion is overtreated to be sure of destroying it, which invariably causes unwanted damage to normal tissue. Sometimes this may not be deleterious but in other instances, such as in whole bladder photodynamic therapy, it may have serious consequences for the patient. Most of the laboratory research that comprises this thesis is aimed at exploring the biological effect produced by both PDT and thermal laser techniques on normal bladder in the hope that by matching the treatment parameters to the characteristics of the target organ an increased specificity of effect may be obtained.

Chapter 3

HISTORICAL REVIEW OF PHOTODYNAMIC THERAPY

3.1 DISCOVERY OF PHOTODYNAMIC ACTION

The ability of some substances to produce a chemical reaction in the presence of light was recognised nearly a century ago. In 1897 Oscar Raab (a student of Professor von Tappeiner in Munich) started to investigate the toxicity of an aniline dye, acridine orange, on the unicellular organism *paramecium.* He found that the time to kill *paramecia* varied widely for the same concentration of dye and then realised that this depended on the ambient light level during the experiment, so concluding that the dye rendered the *paramecia* sensitive to sunlight (Raab, 1900). This discovery led Raab's professor and other co-workers to investigate these effects more closely and soon several dyes and pigments were found with photo-sensitising properties. The term "photodynamische erscheinung" was coined by von Tappeiner and shortly after he used a topically applied solution of eosin and fluorescein to treat skin tumours which were then exposed to white light (von Tappeiner and Jesionek, 1903). Some response was apparently seen in most patients though the overall results are not clear. The same group also demonstrated that oxygen was required for this process but the realisation that photodynamic action was an oxidative reaction rather than the more complicated photobiological process proposed by von Tappeiner did not come until Smetana (1938) showed the actual uptake of oxygen during this process.

Around the same time that von Tappeiner had described photodynamic action in the laboratory the first cases of conditions now recognised as cutaneous porphyrias were being reported. Anderson (1898) studied 2 Orkney fisherman who suffered from intermittent eruptions of bullae on exposed skin associated with port-coloured urine. These attacks could be avoided by remaining indoors whilst their urine appeared dark. Analysis

of this urine revealed a pigment with a similar absorption spectrum to haematoporphyrin, which is derived from haemoglobin.

Soon haematoporphyrin was shown to have the same destructive effect on *paramecia* as acridine and on injection into white mice rendered them sensitive to light (Hausmann, 1908). In 1913 Meyer-Betz rather foolhardily injected himself with 200 mg haematoporphyrin hydrochloride and on exposure to sunlight for only a short time the following day developed a marked sunburn. His skin photosensitivity remained for 2 months.

3.2 DEVELOPMENT OF PORPHYRIN PHOTOSENSITISERS

Over the next few decades there was continued interest and research into various photosensitising compounds and their actions largely because of their apparent ability to localise in tumours (see section 3.3.1). They were a diverse group of compounds though Fowlks (1959) noted that many had a cyclic ring structure, and haematoporphyrin hydrochloride (Hp) seemed to be the most promising. However the photosensitising properties of Hp were very variable and Schwartz, working at the University of Minnesota in search of an improved photosensitiser, found that it was a crude mixture of various porphyrins with different properties. He went on to produce an improved compound by acetylating Hp with acetic and sulphuric acid which became known as haematoporphyrin derivative (HpD) (Schwartz *et al.*, 1955). Lipson refined his technique for producing HpD and confirmed its enhanced tumour localising properties (Lipson *et al.*, 1961). The essential processes in the preparation of HpD have remained unchanged since then.

HpD was still a complex mixture of porphyrins and several workers have attempted to purify it further. Dougherty and colleagues identified an new fraction from HpD by chromatography which accounted for nearly half of the mixture and was formed by hydrolysis of the acetate compounds in HpD which largely contain the tumour localising fraction. This purified material was thought to contain a higher percentage of the active ingredients of HpD and has been variously termed dihaematoporphyrin ether or ester (DHE) (Dougherty *et al.*, 1984). Others have disagreed as to the exact structure of DHE but most agree that the active components of HpD are aggregates of some kind in aqueous solution (Kessel *et al.*, 1986a). These aggregates themselves are not phototoxic due to their low quantum yield but become disaggregated within the body allowing singlet oxygen production upon exposure to light.

HpD or DHE are the photosensitisers which have been used for almost all clinical PDT to date. HpD has been available commercially for many years as Photofrin, though most studies performed after 1986 used DHE (initially called Photofrin II). DHE has now been renamed plain "Photofrin" (Lederle Laboratories) and is the preparation used in the PDT Phase III trials being conducted in several centres worldwide; although it would seem likely that many of the protocols in these clinical trials are probably inappropriate.

3.3 TUMOUR LOCALISATION OF PORPHYRINS

This putative property of the porphyrins has, more than any other, driven the interest in and the development of PDT. Some animal sarcomas were found to exhibit a red fluorescence when exposed to ultraviolet light which was attributed to endogenous porphyrins (Policard, 1924). Auler and

Banzer (1942) noted similar fluorescence from implanted tumours in rats following systemic injection of haematoporphyrin, and produced tumour damage with light exposure. These observations were confirmed by other workers in several types of human sarcomas and carcinomas (Figge *et al.*, 1948). Fluorescence was also apparent in traumatised and embryonic tissues suggesting that haematoporphyrin was taken up in rapidly growing tissues, both normal and neoplastic.

These properties were studied by many workers and there are several reports describing, with varying degrees of success, the observation of fluorescence from haematoporphyrin as an aid to locating areas of occult tumour. Some workers were able to demonstrate selective fluorescence from malignant lymph nodes in patients with head and neck cancer (Rasmussen-Taxdal *et al.*, 1955). Surprisingly the concentration of porphyrin (measured by chemical means) in organs which did not fluoresce such as normal liver was found to be as high, if not higher, than that in tumours, though this was thought to be due to light absorption by those organs. Hughes (1960) found that there was equal fluorescence from both normal and malignant tissues but the fluorescence faded more quickly from normal tissues. Lipson and co-workers conducted several studies into the use of HpD to localise tumours of the bronchus, oesophagus and cervix (Lipson *et al.*, 1964a, 1964b). This was pre-laser so they used filtered light from a carbon arc lamp at around 400 nm with the operator viewing the fluorescence through a glass filter that excluded the illuminating wavelengths. They noticed fluorescence from areas of early tumours of the larynx, oesophagus and stomach, some of which had been missed with conventional methods. Other workers found though that some benign lesions, especially ulcers, polyps and other inflammatory lesions also exhibited fluorescence, and some tumours such as sarcomas were poorly visualised. Gregorie *et al.* (1968) although they saw selective fluorescence

in 76% of patients who had biopsy proven malignancy out of more than 200 patients studied, concluded overall that this technique was not reliable enough as a test for malignancy particularly of adenocarcinomas or sarcomas. The advent of lasers and electronic signal enhancement systems has sustained the interest in HpD as a possible means of both detecting small carcinomas and delineating the extent of larger tumours, particularly in the bronchial tree, with more precision than at present (Monnier *et al.*, 1990).

3.3.1 Bladder tumour localisation with HpD

Studies of porphyrin localisation in both tumour biopsies and in the intact bladder have been reported. Kelly *et al.* (1975) working at St. Mary's in London, first demonstrated that HpD localised in human bladder cancer using an experimental model. They subsequently gave HpD to 11 patients and looked at tissue fluorescence under ultraviolet light, noting a good correlation between areas of tumour and the observed fluorescence, particularly for Cis (Kelly and Snell, 1976). Hisazumi *et al.* (1983) observed fluorescence from HpD in biopsies of bladder tumours but not in biopsies from normal urothelium in the same patients. There was great interest in this as a potential means of identifying areas of macroscopically occult tumour, especially Cis, within the bladder at the time of cystoscopy. Before this could be realised it had to be shown that HpD fluorescence was confined to and indicated all malignant or dysplastic areas and could also be detected endoscopically. Benson and colleagues at the Mayo Clinic studied bladders that were to be removed at cystectomy. They administered HpD prior to surgery and then observed and mapped out the salmon-pink fluorescence seen when the excised bladders were illuminated by ultraviolet light. The bladders were then subjected to systematic histological mapping and it was found that in every case the areas of fluorescence corresponded to areas of tumour (Benson *et al.*, 1982).

Attempts have been made to dispense with the operator needing to visually determine the areas of fluorescence, which would have the potential advantage of being less observer dependent and also enabling the examination to be carried out with normal white light rather than ultraviolet illumination. Such a system designed for bronchoscopy used "chopped" light to enable both ultraviolet excitation light and normal white light for viewing to be used simultaneously. The detection system converted the fluorescence signal to an audio tone which varied with fluorescence intensity (Cortese *et al.*, 1979; Kinsey and Cortese, 1980). However discrimination of the significance of small tonal changes was still rather subjective, so as the system was improved it evolved back to a visual assessment whereby both endoscopic and fluorescent images could be displayed simultaneously on video monitors (Benson, 1989). Other authors have developed similar systems (Lin *et al.*, 1984) but the apparatus is cumbersome, complicated and costly, and there may be some diagnostic errors as HpD will also localise in areas of bladder inflammation or injury (Selman *et al.*, 1985a). For most patients, even those with occult areas of bladder Cis, a combination of high quality urine cytology and biopsies will adequately determine tumour areas. In some situations, though probably more so in other organs such as the bronchus, the use of photosensitisers as tumour localising aids may be valuable (Monnier *et al.*, 1990).

3.4 CLINICAL STUDIES OF PHOTODYNAMIC THERAPY

3.4.1 Early studies

The first English language reports of clinical PDT came from Lipson's group when, after demonstrating that HpD would localise in tumours, they treated a patient with an ulcerating recurrent breast carcinoma using

filtered red light from a xenon arc lamp (Lipson *et al.*, 1967). Although the tumour did not disappear there was some objective evidence of response. One of Kelly and Snell's (1976) patients had an area of bladder illuminated by a mercury vapour lamp transmitted through a rigid quartz rod. Several papillary tumours were necrosed but the authors commented that improved techniques for illumination were needed. Early work with PDT therefore was largely confined to cutaneous lesions and the first British study was with superficial skin tumours of the head and neck (Carruth and McKenzie, 1985). Although no definite conclusions were drawn from the 10 patients treated, it was clear that PDT had caused tumour regression and could potentially eradicate tumours completely with few side effects and good cosmetic healing. The development of lasers and fibreoptics soon enabled powerful monochromatic light to be conveniently delivered to many sites in the body and the potential application of PDT to several cancers accessible to the endoscope has since been studied.

3.4.2 PDT in Urology

3.4.2.1 Introduction

Clinical PDT in urology has been so far confined to bladder carcinoma though advances in ureteric instrumentation and light delivery systems make upper tract urothelial tumours theoretically treatable. For solid organs such as the prostate or kidney, light can be delivered to the target area by interstitial fibres though the volume treated around each fibre site is small. In general therefore multiple fibres would be required and such applications are still in the pre-clinical phase (Pantelides *et al.*, 1990).

There are several reasons why superficial bladder cancer would seem to be the "ideal" pathology for PDT. Although the great majority of patients fall into the low risk category as discussed in chapter 1, there is a well defined

group with recurrent high grade disease whose likelihood of an early progression to muscle invasion is worryingly high. If they fail to respond to intravesical chemotherapy then cystectomy and urinary diversion is the only option. Those patients with extensive primary Cis are particularly at risk, yet it seems a shame to excise such a useful organ as the bladder when the disease only involves the urothelium. Because patients with extensive Cis have a field change dysplasia of their urothelium it is not possible to visually identify all abnormal areas. As the entire bladder mucosa can be illuminated simultaneously and these superficial lesions lie well within the depth of tissue easily treated by PDT, it offers the potential to eradicate these occult areas as well as visible tumour, at a single treatment session. How effective though is PDT?

Since Kelly and Snell's initial work there have been reports of patients with bladder cancer treated by PDT from some 10 centres published in English (2 or 3 series from China have not been reported elsewhere). These are summarised in table 3.1. The individual series are small comprising from 7 to 27 patients, the bladder pathology heterogenous (Cis to T2 tumours with some having intravesical chemotherapy or TUR prior to PDT), and the treatment parameters employed very variable. The earlier series all used HpD (dosage between 2 - 5 mg/kg) and those after 1986 generally used DHE (2 mg/kg).

3.4.2.2 Bladder illumination

Sixty two of the 144 (43%) evaluable patients received focal illumination to localised areas of tumour and 67 (47%) had illumination of their entire bladder. The latter group generally had widespread primary Cis and 10% of patients with a combination of Cis and discrete papillary tumours had both focal and whole bladder illumination. The light doses used for whole bladder illumination (5 - 70 J/cm^2) were lower than for focal illumination

(100 - 360 J/cm^2). This was both to avoid excessively long treatment times, as the powers were necessarily low because of technical limitations and the need to avoid a thermal effect, and to reduce the complications which soon became apparent after high light doses to the whole bladder with the sensitiser doses used in these studies.

Most authors used a square-cut or microlens tipped laser fibre to illuminate focal areas of bladder tumour. For whole bladder illumination the problem of even light distribution is much more difficult and has been approached in various ways by different groups. Some have filled the bladder with a diffusing medium such as dilute Intralipid, and used a special positioning catheter to centre a square-cut optical fibre (Jocham *et al.*, 1989). Although this produces an even scattering of light in a regularly shaped bladder model the positioning of the fibre tip seems to be critical (Plail *et al.*, 1990). As the smoothing effect of internal reflectance of light from the bladder surface is largely lost with Intralipid then only a small variation in position of the fibre tip or indeed a normally irregular bladder will result in loss of uniform light distribution at the bladder surface. Others have developed ingenious mechanical rotating devices for stepwise illumination of the entire bladder mucosa (Hisazumi *et al.*, 1984). Most authors though, have used some type of bulb tip diffusing fibre (Benson, 1986) centred as accurately as possible within the bladder and accepted that the light distribution will not be perfectly even, particularly around the bladder neck.

Illumination was provided by red light (630 nm) from an argon-ion pumped dye laser in all but one series, which utilised a metal vapour laser system (Williams and Stamp, 1988). All but one author illuminated between 40 and 72 h (most at 72 h) after administration of HpD/DHE though Benson (1986), treated at either 3 or 48 h with some patients

receiving bladder illumination at both 3 and 48 h. It is important that the bladder is gently distended during illumination but few give much detail about this. Water or normal saline (150-350 ml) was generally used though Tsuchiya *et al.* (1983) used gas. Nseyo's group initially used water but changed to saline at a lower filling pressure in an attempt to reduce problems with bladder irritation and contracture seen in their early patients (Nseyo *et al.*, 1985a, 1987).

3.4.2.3 Clinical results

The 10 series of PDT in bladder cancer that we are discussing comprise some 144 patients and showed an overall complete response rate at 3 months of about 65% (table 3.1). Although the definition of a "complete response" varies between the studies, in most this implies the disappearance of all visible tumour and, where available, negative mucosal biopsies and urine cytology. However several workers did not specifically define their response criteria even though they classified patients implying that this had been done (Nseyo *et al.*, 1987; Shumaker and Hetzel, 1987; Tsuchiya *et al.*, 1983; Prout *et al.*, 1987). Some workers considered a partial response to mean a greater than 50% reduction in tumour size or number though this is therapeutically fairly meaningless for bladder tumours as the patient still has active cancer! Nevertheless 22% of patients fall into this category.

Differing selection criteria, tumour pathology and treatment parameters make direct comparison difficult but there is a consensus that PDT is most effective at treating Cis, less effective for superficial papillary disease, and ineffective for invasive tumour (5 patients with T2 tumours). The best results are those of Benson (1986, 1988) who reported 27 patients with resistant Cis who refused cystectomy. Twenty three (85%) of these showed a complete initial response to PDT, the other 4 had coexistent papillary tumour which persisted though their Cis responded. Prout *et al.* (1987) on

Authors	No. Pts.	Sensitiser (mg/kg)	Interval to treatment (h)	Light dose* (J/cm^2)	Response at 3 months Complete	Persistent Tumour
Benson	15	HpD	3,48 or both	150 (F)	15	0
1986	12	(2.5-5)	3 or 48	25-45 (WB)	8	4
Harty *et al.*,	5	DHE	72	25(WB) + 100 (F)	4	1
1989	2	(2)		25 (WB)	1	1
Hisazumi *et. al.*,	9	HpD	48-72	120-250 (F)	5	4
1983,1984	2	(2-3.2)	48	10 (WB)	2	0
Jocham *et. al.*, 1989	15	DHE/HpD (2-3)	40-72	35 (15-70) (WB)	11	4
Nseyo *et. al.*,	2	DHE	72	100-200 (F)	0	2
1985a,1987	11	(2)		10-60 (WB)	3	8
	10			100-200 (F) + 5-60 (WB)	4	6
Ohi *et. al.*, 1984	11	HpD (2.5)	72	120 (F)	8	3
Prout *et. al.*,	17	DHE	48	100-200 (F)	8	9
1987	2	(2)		5.5-10 (WB)	1	1
Shumaker and Hetzel, 1987	13	DHE (2)	72	25 (WB)	12	1
Tsuchiya *et. al.*, 1983	8	HpD (2.5)	48-72	120-360 (F)	8	0
Williams and Stamp, 1988	10	DHE (2)	72	10-15 (WB)	4	6
	144				94 (65%)	50 (35%)

* F = focal, WB = whole bladder treatment

Table 3.1 Results of photodynamic therapy in bladder carcinoma

the other hand, reported on 19 patients the great majority of whom had papillary bladder cancer alone, and found that only 47% had a complete response.

The only British series so far reported (Williams and Stamp, 1988; Stamp *et al.*, 1990), comprised 10 patients with multiple superficial bladder tumours (Cis, Ta and T1) that had not responded to intravesical chemotherapy. A complete initial response was seen in 4 patients and a partial response in another 4. Only 2 patients though remained tumour free (in excess of 2 years) and 7 ultimately developed an invasive cancer of whom 2 died.

There are few other published data on the durability of the response of bladder tumours to PDT to suggest whether or not PDT offers any worthwhile improvement in either recurrence rate, time to progression, or survival. Although these series do include patients followed up for up to 3 years rarely is the median follow-up time given and it would seem that the great majority of patients were followed for less than 1 year, some for only a few months (3 weeks to 4 months - Hisazumi *et al.*, 1983, 1984). The high rate of response seen, of Cis in particular, may be a reflection of this. Benson (1988) reported a 50% recurrence of focally treated Cis at 1 year though most recurrence was in previously uninvolved areas and was successfully retreated with PDT. Jocham *et al,* (1989) reported that 9 out of 15 patients with Cis remained clear with follow up in excess of 2 years after whole bladder PDT. Two became clear with a second treatment and 4 had recurrences after a mean of 20 months.

Resistant Cis - It is clear that this group of patients have the most to gain from PDT as their only alternative is cystectomy. The above mentioned studies contained 47 patients who had failed intravesical therapy (though BCG was not available in most cases and no comparative trial of PDT vs.

BCG has been performed). They had all either refused or been considered unfit for cystectomy. All but 1 of these 47 patients had an initial complete response to PDT (negative cytology and biopsies) though again there are inadequate follow-up data to assess durability of the response. Patients who had focal treatment of Cis tended, not surprisingly, to relapse in other areas so whole bladder treatment is to be preferred

3.4.2.4 Discussion

At first sight, these results seem impressive, especially as they have been achieved largely in patients who have failed all other modalities of treatment short of cystectomy. However the complication rate was alarming, as will now be discussed.

Functional impairment - Most authors state that their patients develop irritative symptoms following PDT but these are often described as "transient" and of no particular worry. There has almost certainly been a gross under reporting of side effects by the enthusiasts of PDT, often no doubt because they have not been specifically looked for, and many patients would have had some pre-existing irritative symptoms anyway. All authors who did look specifically at bladder function after PDT found serious impairment in a high percentage of patients. Nseyo *et al.* (1985a) reported severe side effects in all of an initial series of 6 patients undergoing whole bladder PDT. Two patients had such a marked reduction in bladder capacity with incontinence that they required a cystectomy for these PDT induced symptoms alone, although their bladders were found to be clear of tumour. Following animal experiments they recommended treatment parameters which produced better results in a subsequent 15 patients, with on average only a 20 percent reduction in bladder capacity which recovered within 3 months (Nseyo *et al.*, 1987). However, the Sheffield group (Williams and Stamp, 1988; Stamp *et al.*, 1990) using these

supposedly improved parameters, still found severe dysuria and reduced bladder capacity in all their patients. Some were hospitalised for several weeks and one patient was left with a bladder capacity of only 50 ml after voiding a sloughed "cast" of bladder mucosa. Jocham (1989) reported a reduction in bladder capacity in many of his patients of more than half, and 3 out of 15 still had a 70% reduction 1 year after PDT. Some authors have suggested not giving PDT to patients who have a bladder capacity less than 150 ml (quite common) or whom have received radiotherapy (rare for superficial tumours) as they seem to have the worst complications.

Upper tract complications - Both ureteric reflux and ureteric obstruction were seen in the initial series of Nseyo *et al.* (1985a) but in general no problems with the upper tracts have been mentioned by other authors until a recent report by Harty *et al.* (1989), in which he studied the side effects after PDT in a series of 7 patients who were given whole bladder treatment. Five patients had a significant reduction in bladder capacity after PDT which persisted at 1 year in 4 of them. Deep bladder biopsies showed marked fibrosis in the muscle layer. These 4 patients also developed persistent bilateral hydronephrosis and grade 4 vesicoureteric reflux. Interestingly the 1 patient in this series who escaped these problems still had a complete response; he had received only two-thirds the intended dose of DHE.

Cutaneous photosensitivity - Any drug given systemically will be distributed throughout the body and so inevitably, photosensitisers will be taken up to some extent in the skin. Unfortunately, HpD and DHE absorb light well throughout the range of wavelengths in sunlight which means that patients may develop cutaneous photosensitivity to ambient light. This may last several weeks and require the patient to remain in subdued lighting and to wear protective clothing. This is a major factor against

patient tolerance of PDT with these sensitisers and despite warnings, most series report a small number of patients who develop significant sunburn. Other ways of administering photosensitisers have been tried with the aim of avoiding skin sensitivity. Direct intra-tumour injection is only suitable for solitary bladder tumours (Amano *et al.*, 1988) and would clearly not be feasible for Cis. Intravesical administration of HpD has not been successful (Benson, 1988), and our studies on the intravesical uptake of phthalocyanine (chapter 6) also show that although significant amounts are absorbed by normal bladder, the uptake can be patchy and unpredictable.

The most likely solution to this particular complication lies in newer photosensitisers which, although still given intravenously, are either cleared more rapidly from the skin or, like the phthalocyanines, have a localised absorption peak (fig. 3.1), at a longer wavelength than HpD, away from the main concentration of solar emission, which produces much less of a skin reaction (Tralau *et al.*, 1989). When using such a sensitiser at low dose levels, the problem of cutaneous sensitivity to sunlight is likely to be virtually eliminated. An alternative approach may be to use singlet oxygen quenchers which can be given after PDT to reduce skin sensitivity in animals, though their clinical efficacy is not yet proven (Dillon *et al.*, 1988).

3.4.2.5 Dosimetry

It is perhaps not surprising that there have been problems encountered in PDT of the bladder as there are very little data available on which to base the light and sensitiser doses used clinically. This is illustrated by the wide range of values for the most important treatment parameters which have been chosen by the authors of the published clinical series of PDT for superficial bladder cancer listed in table 3.1.

- Sensitiser dose: HpD 2 - 5 mg/kg
  DHE 2 mg/kg (all authors)

- Time between sensitisation and light exposure: 3 - 72 h
- Light dose: whole bladder 5 - 70 J/cm^2
  focal treatment 100 - 360 J/cm^2

At the photosensitiser concentrations commonly used clinically, the light dose seems critical. However it is not easy to calculate accurately the dose absorbed by the mucosa and the problem of achieving an even light distribution has been approached in several ways as already described. The light dose given is by custom merely the energy of the primary beam divided by the estimated surface area of the bladder (assuming it to be a sphere of known volume). This is certainly the easiest way of expressing it and may be adequate to compare results between clinical series but is no way close to the important value which is the light dose absorbed by the malignant epithelium. Because the highly reflective internal surface of the bladder acts somewhat like an integrating sphere the incident light dose in certain areas can be considerably greater (direct plus reflected light) than the output of the fibre. Multiple isotropic detectors placed in close proximity to the bladder wall can be used to monitor this incident light which it may be rather more important to measure than just the fluence of the primary beam emitted from the fibre tip (Marynissen *et al.*, 1989).

All of these methods require considerable enthusiasm and patience to use so simplification of light delivery would make PDT easier to perform. One way of doing this, as mentioned already, is by utilising the photobleaching that occurs at low sensitiser concentrations. The investigation of this important concept is one of the major aspects of the work presented in this thesis and will be discussed in detail later. A possible disadvantage though is likely to be that when using these low sensitiser concentrations the rather high total light doses inevitably required will require long treatment times with currently available laser systems.

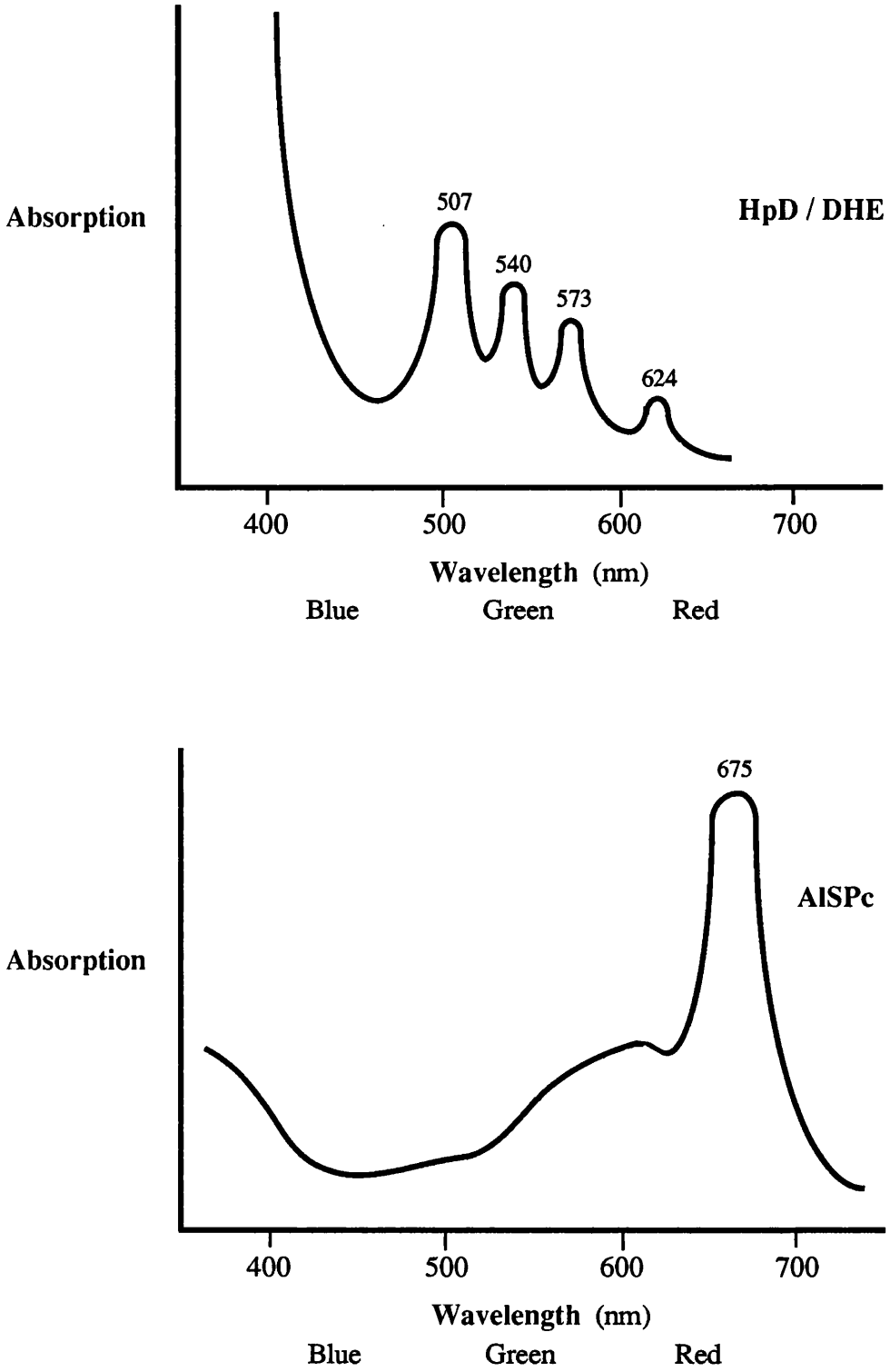


Fig. 3.1 Absorption spectra for HpD and AlSPc
(after Bown *et al.*, 1986)

An alternative approach to the problem of avoiding unwanted PDT damage in the muscle layers of the bladder is to activate the photosensitiser with a wavelength of light less penetrating than the red light (630 nm) used in all clinical studies with HpD and DHE. Green light would be expected to penetrate the bladder wall only 1 - 2 mm and HpD will absorb in the green spectrum (a minor peak at 507 nm) rather better than it does at 630 nm. Haemoglobin however also absorbs strongly here, so any surface bleeding may shield underlying tumour and the reduced internal reflection of green light within the bladder will make dosimetry more difficult. There are no published clinical data as yet using green light to address these points. Activation with green light though is not possible for the phthalocyanines and most of the newer photosensitisers under study which only absorb in the red part of the visible spectrum. Green light would also not be suitable for any other clinical application of PDT apart from bladder Cis.

3.4.3 Overview of PDT in other specialties

Although PDT may well become most useful for the treatment of resistant superficial bladder cancer, the majority of the clinical data presently available come from other specialities. The largest studies are with tumours of the respiratory tract though there is also experience with gastrointestinal and brain tumours as well as cutaneous cancers, especially of the head and neck.

3.4.3.1 Bronchial cancer

The largest studies come from Japan where Professor Hayata's group has treated over 160 patients in the last decade. The results from early tumours in 40 patients, for whom surgery was not possible in over half, seem particularly encouraging with a complete remission rate approaching 80% and reports, albeit rather anecdotal, of some long term cures (Kato *et*

al., 1989). The major side effects were seen in treating bulky obstructing lesions in that repeated bronchial toilet was necessary to keep the airways patent, a problem not seen with Nd:YAG laser therapy where the necrotic tissue can be cleared immediately. Delayed haemorrhage from the raw tumour bed though can be fatal. This should have been predicted from experimental studies as the maximum depth of PDT effect can only be in the order of 4-5 mm, which would leave residual viable and highly vascular tissue exposed when the superficial portion destroyed by PDT sloughed (Cortese and Kinsey, 1982).

Apart from the treatment of tumours, the utilisation of photosensitiser fluorescence as an aid to the detection and delineation of early tumours, particularly synchronous lesions seems promising and has been discussed earlier in this chapter. Further study will be necessary before to establish whether the increased complexity required for these techniques compared with standard white light bronchoscopy is justified

3.4.3.2 Gastrointestinal tumours

Like the situation in the bladder, PDT seems to have a fairly non-selective effect against tumours of the upper gastrointestinal tract, requiring quite extensive damage of adjacent normal tissue to obtain an adequate response in the cancer. More selective damage to experimental colonic tumours has been achieved but this requires careful manipulation of the treatment parameters used so that photobleaching of sensitiser in the normal colon occurs but not in the tumour; this situation has not yet been achieved clinically (Barr *et al.*, 1990a). Worthwhile palliation of the dysphagia caused by advanced oesophageal tumours has been obtained with PDT and the risk of serious secondary haemorrhage seems less than in the bronchus though has been reported in colonic tumours (Barr *et al.*, 1990b). The major potential advantage for PDT over thermal methods of tumour

ablation in the GI tract such as the Nd:YAG laser, is that there is little risk of causing a perforation after PDT even from full thickness damage to the bowel wall as there is relative sparing of submucosal collagen so that the mechanical strength is maintained (Barr *et al*, 1987a). However it is with early tumours that the best results have been achieved, some groups from China and Japan reporting disease free survival in excess of 2 years (Jin *et al.*, 1987).

PDT may also prove to be a useful modality for treating small tumours where the patient is unsuitable for surgery or later relapses locally with, for instance, an anastomotic recurrence (Barr *et al.*, 1990b). Another interesting finding is that the normal pancreas is very resistant to PDT damage whereas pancreatic tumours are considerably more sensitive. Thus selective destruction of pancreatic cancer may be possible though the clinical applications of this have not yet been explored.

3.4.3.3 Brain tumours

The brain is a particularly exciting area for PDT as very high levels of photosensitiser drugs can be achieved in glial tumours compared to normal brain due to the disruption of the normal blood:brain barrier by the tumour (in the order of 30:1). Unfortunately damage is still not entirely selective after surface illumination which it needs to be as normal brain cannot regenerate. Improved methods of light delivery however, either interstitially or peroperatively after tumour resection to treat residual infiltrating tumour cells, promise a useful therapeutic advance on the rather depressing results of conventional treatment for gliomas (Muller and Wilson, 1990).

3.4.3.4 Tumours of the head and neck

It has already been mentioned that the first clinical work of PDT in this country was with head and neck tumours. Most of these whilst locally

invasive metastasise late, and the anatomical clearance preferred for wide surgical excision is rarely available. The Southampton group has been treating head and neck tumours for some years and although there is no controlled trial from this centre, they have produced long standing control of some unresectable tumours (Carruth and McKenzie, 1985). In addition PDT seems to be a suitable means of treating multiple basal cell carcinomas (Buchanan *et al.*, 1989).

3.5 THE WAY FORWARD

So what is the current status of PDT? From the literature it has become apparent that PDT is only likely to be of value as a primary treatment for small tumours because of the limited depth of penetration of the activating light. The volume of necrosis that can be obtained from a single fibre is much less than that from thermal lasers such as the Nd:YAG. The likely place for PDT is not therefore to destroy large amounts of tissue but to kill localised areas of tumour cells, most usefully in sites where they may not necessarily be apparent visually. For large tumours though it could still be valuable as a adjuvant treatment to "sterilise" the tumour bed once the main bulk of disease had been removed by surgery.

Bladder Cis is therefore such an attractive pathology for PDT as it is very superficial, tends to be multifocal and difficult to delineate precisely, and is a serious disease difficult to eradicate with existing therapies. However with so many serious problems encountered, it is perhaps surprising that anyone is still trying to use PDT in the bladder. Several of the clinical pioneers did decide to stop clinical work and go back to the laboratory to try and solve the problems (Nseyo *et al.*, 1985b, 1988; Reed *et al.*, 1989). However, even now, a survey of the literature in this field shows that there

is a remarkable absence of basic studies on exactly what PDT does to normal and neoplastic tissue in the bladder. There are very few background data on which to base the light and sensitiser doses used clinically, and the values that have been used must be regarded as little more than inspired guesses.

As has happened on many occasions in the history of medicine, the clinicians have tried to run before their scientific colleagues have taught them to walk! Often this empirical approach has worked, but for PDT in the bladder clearly it has not as the role for PDT in this disease is still evolving. The great vigour that accompanies the emergence of a new treatment such as PDT may lead to its wide application in a somewhat haphazard way resulting inevitably in some poor results which then bring the whole concept into disrepute. Phase III clinical trials of PDT for bladder cancer are under way in several countries both for resistant Cis and as prophylaxis against recurrent papillary disease. There must however be some doubt about whether these trials are appropriate in our current state of knowledge as the protocols being used are essentially the same as those that have produced irreversible damage to bladder muscle in many of the patients whom have been treated over the last few years. The right balance may be difficult to achieve and indeed will be impossible without careful experimentation and logical clinical trials.

The work described in the section 2 of this thesis is designed to explore the actions of PDT on the normal bladder, and in particular how dosimetry variations will affect the biological result. The aim of this work is to suggest ways in which the clinical application of PDT may be directed so as to minimise the problems seen in these pioneering clinical series, which have detracted from the wider development of this promising technique, and still achieve the desired clinical effect.

Chapter 4

THE NATURE OF PHOTODYNAMIC THERAPY

4.1 PRINCIPLES OF PHOTODYNAMIC ACTION

4.1.1 Introduction

Photodynamic action requires a photosensitiser, light, oxygen and a tissue substrate. When the photosensitiser absorbs light it can either release it, usually in the form of fluorescence or heat, or interact with tissue to form toxic free radicals, which can then damage adjacent cells. These processes will now be considered in more detail.

Normally the photosensitiser molecules are in a singlet ground state with paired electron spins. The absorption of light leads to an excited singlet state where the electrons are "pushed" into an outer valence ring though the spins are still paired. This high energy state is very unstable and shortlived (in the order of 10^{-9} secs) before it returns to the ground state by 1 of 2 main mechanisms or is degraded; these processes are competitive (fig. 4.1).

i) Firstly the excited state can decay directly to the ground state by releasing light at a longer wavelength than the exciting light in the form of fluorescence or heat. Detection of such fluorescence is the main way of assessing photosensitiser distribution and hence identifying probable areas of the malignant or dysplastic tissue which retain these drugs with some selectivity. This will be discussed in more detail in section 4.3.

ii) Around 50% (the actual percentage is known as the quantum yield and is specific to each sensitiser) of the excited singlet molecules are converted to a more stable triplet state by a process called intersystem crossover which involves inversion of the electron spin such that the electrons are no longer paired. As this excited triplet state is much longer lived than the excited singlet state (0.5 msec) it can react with tissue elements in two ways, either directly (Type I phototoxicity) producing hydroxyl and superoxide radicals or, more importantly, by transferring its energy to molecular oxygen

thereby producing singlet oxygen (Type II phototoxicity). Singlet oxygen is very unstable (lifetime around 0.1 msec) and reacts with cells only in its immediate vicinity causing oxidation of cellular components leading to cell death and is thought to be the cytotoxic agent responsible for photodynamic action (Dougherty *et al.*, 1983). This is a threshold effect, and a certain quantity of singlet oxygen must be produced before a cell is killed. The photosensitiser molecules then return to the ground state and, as they have not been chemically transformed, can be activated again by absorbing another photon and so the cycle is repeated. Clearly this is a very efficient process as a single photosensitiser molecule may generate many times its own concentration of singlet oxygen provided of course that there is an adequate supply of molecular oxygen.

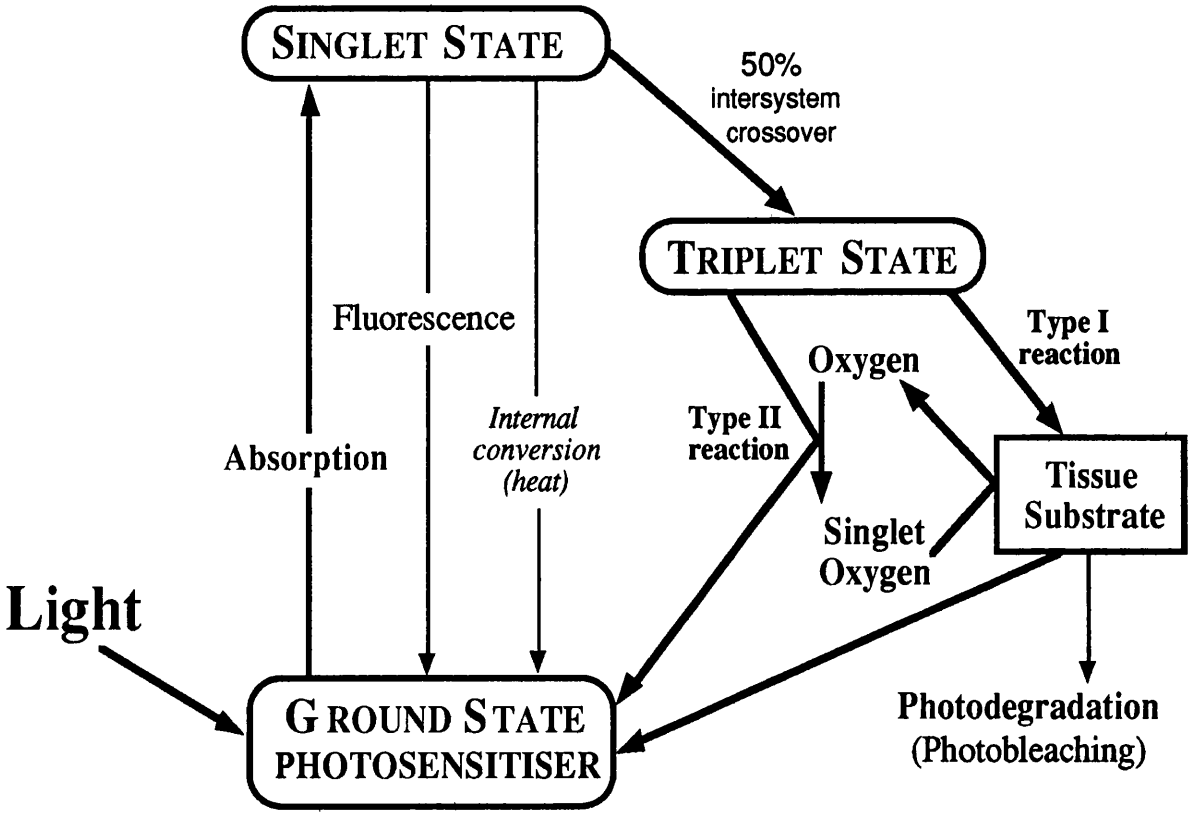


Fig. 4.1 Mechanism of photodynamic action

iii) Finally, the activated molecules can go through a different process in which they are destroyed by the same activating light (photobleaching). This means that the total quantity of photosensitiser in tissue is steadily reduced as the tissue is exposed to light. This has important implications for protecting tissues with low concentrations of photosensitiser as will be discussed later in this chapter.

4.1.2 Singlet oxygen

Weishaupt *et al.*, (1976) were the first to suggest that singlet oxygen was the cytotoxic agent generated during PDT as they demonstrated that cell cultures were protected from photodynamic damage when a singlet oxygen "scavenger" was present. Others have since confirmed this so the belief that this type II reaction is the predominant mechanism in PDT is largely based on this indirect evidence (Valenzo, 1987).

Comparatively little is known about singlet oxygen considering it is generally assumed to play such a pivotal role in PDT. This is largely because it has so far proved impossible to detect *in vivo* as it is present in minute amounts and has a very short lifetime. Its mode of action is thought to be by producing irreversible oxidation of certain amino acids (cysteine, histidine, methionine, tryptophan and tyrosine), the unsaturated bonds in lipids, and some nucleic acids which will interfere with the normal function of many cellular components and reactions. Singlet oxygen has been shown *in vitro* to damage the cell membrane (Kessel, 1976), mitochondria and DNA (Moan *et al.*, 1982). Of these it would appear that the more important effect is the peroxidisation of fatty acids in the cell membrane (Spikes, 1984). Because of its very short lifetime, singlet oxygen can only react within about 0.1 microns of its origin (Moan, 1985) which should limit photodynamic damage to tissue actually containing the photosensitiser.

Slight variations in the chemical structure of photosensitisers can greatly alter their ability to produce singlet oxygen. For instance the incorporation of certain metals such as aluminium and zinc in the phthalocyanine photosensitiser molecule will increase the triplet and hence the singlet oxygen yield, though other metals (e.g. copper) will reduce it (Rosenthal *et al.*, 1987).

4.1.3 Cellular effects

Many authors over the years have confirmed that illuminating sensitised cells in tissue culture will kill them (Moan, 1986a). Therefore there is clearly a direct cellular toxicity of PDT which as mentioned above may be via damage to the cell membrane, intracellular organelles especially the mitochondria or to chromosomes, though no mutagenic effect of PDT has been demonstrated. The distribution of photosensitisers within the cell also depends on time factors as early on after administration most is found in association with the cell membrane though later on, fluorescence studies have found HpD intracellularly, associated with the mitochondria and nuclear envelope (Moan, 1985). Direct binding of the drug to the target is not required being a less specific, though still localised, "shrapnel effect".

What is disappointing is that there is no convincing evidence for an increased susceptibility to photodynamic damage in malignant as opposed to benign cells, notwithstanding the possible limitations of how "benign" a cell line can be that will grow in culture. Henderson *et al.* (1983) looked at PDT on a variety of malignant and benign cell lines and found no difference in susceptibility as did Shulok *et al.* (1986) in normal and neoplastic rat bladder cells. Other workers though have found a increase in both uptake of photosensitiser in malignant cells (Christensen *et al.*, 1983), and their destruction following PDT (Andreoni *et al.*, 1983).

4.1.4 Vascular effects

It was evident quite early on in the investigation of photodynamic action that there was a marked effect on blood vessels. Some of the early work on this aspect did not monitor temperature and as tungsten light sources were generally used there may have been some thermal contribution to the observed vascular disruption reported (Smetana, 1928; Blum, 1941). Castellani *et al.* (1963) illuminated the mesentery of sensitised mice. They kept the tissues moist and cooled below 37°C during irradiation and still observed agglutination of erythrocytes and circulatory stasis which progressed to ischaemic necrosis of the surrounding tissue.

There is convincing evidence that the major part of the PDT effect *in vivo* results from damage to tumour blood vessels rather than a direct action on the malignant cells themselves, although recently it has been suggested that the bladder may be an exception (see below). Studies on the distribution of photosensitisers in animal tumour models demonstrate that the relatively small degree of selectivity of uptake of photosensitisers that is seen in tumours is due to their retention in the vascular stroma (Bugelski *et al.*, 1981). The most obvious early effect of PDT is to shut down small blood vessels leading to decreased tumour blood flow shortly after phototherapy (as measured by a radioactive microsphere technique), which has been shown to correlate with tumour regression (Selman *et al.*, 1984). Endothelial cells are probably damaged first (Berenbaum *et al.*, 1986), although it is not yet clear how this leads to the vasoconstriction and closing of vessels that has been observed experimentally in both normal and tumour tissue (Star *et al.*, 1986). The release of vasoactive substances from mast cells may have a role here. Vascular shutdown is observed prior to major cellular destruction (Henderson *et al.*, 1985a).

Henderson and colleagues also performed an elegant series of experiments showing that cells from animal tumours treated at levels certain to kill them *in vivo*, would survive if transplanted or cultured immediately after PDT but showed a progressively reduced survival if this was delayed. This reduced clonogenicity decreased along the same timescale as the survival of cells taken from untreated tumours rendered anoxic by killing the animal (Henderson *et al.*, 1984).

Other workers have recently suggested that there may be a difference in the form of vascular damage between different photosensitisers. Using the rat ear chamber model and illuminating 24 h after administration of the photosensitiser, Stern *et al.*, noticed that following PDT with DHE there was early haemorrhage and disruption of small venules whilst after aluminium sulphonated phthalocyanine (AlSPc) the main features were a pronounced spasm and vessel shutdown. After both drugs the typical neovascularisation usually seen in this model due to the implanted chamber was abolished (S Stern *et al.*, unpublished work, presented at 3rd Congr. Int. Photodynamic Assoc., Buffalo, New York, 20 July 1990).

It is also possible that there may be a difference in the relative importances of direct versus vascular-mediated damage between different tissues, and furthermore that even in the same tissue this relationship may alter with the time delay between sensitisation and illumination. Clearly there is a lot more photosensitiser in the blood vessels early on (see chapter 6), whereas after 24 h it is predominantly in the tissues. Only 1 group of workers has looked at the microcirculation in the normal bladder after PDT (Reed *et al.*, 1989). They used DHE and a rat model in which the bladder with all its neurovascular connections intact was mobilised and, with the animal on its side, suspended in a tissue bath to be illuminated and viewed under a microscope. They found much more marked changes in the bladder

microcirculation (as assessed by red blood cell column diameter), when illumination was given 30 min after sensitisation compared to when given 48 h afterwards. In the 30 min group, blood flow ceased in all small blood vessels, and slowed in 75% of arterioles and 44% of venules due to mural thrombosis though only rarely was true vasoconstriction noted. In the late treated group there was some thrombus formed but no cessation of blood flow, and a perivascular reaction was observed. The authors contrast these results with similar experiments on rat cremaster where thrombosis and vasoconstriction was clearly evident at 48 h and question whether the bladder smooth muscle behaves in the same way as the striated muscle preparations (e.g. hamster cheek pouch, rabbit or rat ear chamber etc.) that most other investigators have used. They suggest that direct cytotoxic effects, rather than vascular mediated damage were responsible for the fibrosis that developed in the bladder smooth muscle of 80% of their small clinical series (Harty *et al.*, 1989).

4.1.5 Role of oxygen

Molecular oxygen, as has already been mentioned, is essential for photodynamic action in order that singlet oxygen may be produced. Smetana (1938) was the first to demonstrate that molecular oxygen was taken up during photodynamic action though it had long been realised to have an important role. Tissue oxygenation is dependent on an intact blood supply and several workers have shown that if the organ or tumour undergoing PDT is rendered temporarily anoxic then the effect produced is much reduced or abolished. Gomer and Razum (1984) looked at the PDT effect on the skin of the leg of albino mice. They found that if the blood supply to the limb was temporarily interrupted during PDT no damage occured, though the levels to which tissue oxygen tension had to fall were not investigated. Certainly in tissue culture where oxygen is present at higher

saturation than *in vivo*, a considerable reduction in oxygen level is needed to inhibit cell death after PDT. Moan and Sommer (1985) found that if the oxygen tension in the cell culture medium was reduced below 10 mmHg, then there was a 50% inhibition of photodynamic cell kill. This is of some concern in the treatment of larger tumours which are likely to contain a hypoxic cell fraction, unlikely to be sensitive to the direct effects of PDT.

It would appear at first sight that we have a somewhat contradictory situation here in that oxygen is necessary for photodynamic damage yet the main mode of action *in vivo* is to damage tumour vasculature which will reduce oxygen supply to the target tissue. The resultant tissue hypoxia may limit direct tumour cell kill (Henderson and Fingar, 1989). Fingar *et al.* (1988) artificially raised tissue oxygen tension in a mouse tumour model and found that the effectiveness of PDT was enhanced. However the time scale of these actions is difficult. Any direct cell kill there might be from PDT must be initiated during phototherapy as the action of singlet oxygen is localised and short-lived, though may not be detectable until later. The interruption of vascular supply is a delayed event adding the "coup de grace" to already compromised cells. Clearly though in human tumours some parts will be relatively hypoxic and it might be expected that these cells will be resistant to PDT damage and therefore impossible to eradicate. This however may not necessarily be the case as such cells might eventually be killed by the secondary failure of their vascular supply. It should be remembered though, that because of the physical limitations of light penetration, PDT using surface illumination is only applicable to relatively thin tumours anyway (no more than 4-5 mm) which are not likely to contain significantly hypoxic elements. Hypoxic cell fractions may become more of a problem though if the interstitial light delivery techniques presently being developed do allow the effective illumination, and hence possible treatment of much larger tumours.

4.2 PHOTOSENSITISERS

Any compound that will absorb light and produce cytotoxic radical species can be used as a photosensitiser. The production of singlet oxygen from the excited triplet state of a photosensitiser is energy and tissue dependent. Therefore to produce singlet oxygen a photosensitiser must first absorb light with greater photon energy than its excited triplet state which theoretically limits it to the visible spectrum. Doiron (1984) calculated that infrared light has insufficient energy to produce the excited triplet state of known photosensitisers and the maximum wavelength of light that will do this is about 750 nm.

The wavelengths of light that a photosensitiser can absorb depend on its structure, in particular the length of the conjugated pathway (chain of single and double bonds) around a molecule. Red light is the most deeply penetrating in living tissue as few natural biomolecules have a long enough conjugated pathway to absorb it whereas shorter wavelength light (blue and green) is absorbed more rapidly. Therefore ideal photosensitisers tend to be large molecules. In the past a number of cationic dyes have been studied including acridine, fluorescein, rhodamine, and various "blues" such as nile blue, victoria blue and methylene blue; but as already mentioned it is only the porphyrins that have been used to any great extent so far.

4.2.1 Newer photosensitisers

The photosensitisers that have been used for essentially all clinical PDT to date are HpD or DHE though these are far from ideal as photosensitisers as they are incompletely defined mixtures whose composition and stability between preparations varies, and which are difficult to assay. There are

several promising new groups of drugs being developed, especially the metallophthalocyanines (tetra-azoporphyrins) which have similar biological properties to the porphyrins, but are easier to handle chemically and assay in tissue and have much more suitable light absorption spectra (Ben-Hur and Rosenthal, 1985a; Bown *et al.*, 1986). However despite extensive experimentation with these compounds, especially the aluminium sulphonated phthalocyanine used in this work, they are not yet ready for clinical trials.

Other photosensitising drugs that show promise include the chlorins which appear effective both in tumour localisation and absorption of red light (Beems *et al.*, 1987). Synthetic porphyrins called purpurins have been synthesized by several groups (Morgan *et al.*, 1987). These can be made with greater purity than HpD/DHE and also absorb further into the red, e.g. benzoporphyrin derivative absorbs at 690 nm. Other experimental drugs such as naphthalocyanines and bacteriochlorophylls absorb at even longer wavelengths, above 750 nm, which should make them suitable to use with the new generation of cheap semi-conductor lasers. Although there are some dyes that absorb light in the near infrared region (around 1 μm) where tissue is most transparent, their triplet state energies are too low to generate sufficient singlet oxygen for effective photodynamic action.

4.2.2 Ideal properties of a photosensitiser

For most clinical situations where PDT will be used a deep as possible penetration of tissue is required, though bladder Cis may be an exception as was discussed earlier. Therefore an ideal photosensitiser should have a single absorption band in the far red of the visible spectrum, with little absorption across the main concentration of solar emission (below 600 nm) to minimise skin photosensitivity. It should be relatively easy to synthesise in a pure and consistent form, be non toxic and photochemically stable

during storage. It is clearly desirable that a photosensitiser should have a strong affinity for malignant tissue, though complete selectivity of uptake is unlikely to be achieved and processes such as photobleaching may be important in providing an alternative mechanism of selectivity. It should have a high quantum yield with a long-lived triplet excited state to produce the most efficient yield of singlet oxygen and other reactive radicals. The ability to detect and assay the drug *in vivo* by fluorescence or chemical methods is important and has been widely used. However it is also preferable that these estimations correspond to the biological effect achieved, as there may be a considerable difference in photochemical activity between drug aggregates (e.g. dimers) and the monomeric species.

Table 4.1 compares haematoporphyrin derivative (HpD) and aluminium sulphonated phthalocyanine (AlSPc) in these respects.

Desirable properties	**HpD**	**AlSPc**
Easy to synthesise	no	yes
Photochemically stable	no	yes
Non toxic	yes	probably
A single major absorption peak	no	yes
Absorption peak in red spectrum	630 nm	675 nm
High quantum yield	0.5 - 0.9	0.6
Long lived triplet state	0.5 msec	> 0.5 msec
High yield of reactive radicals	yes	yes
Easily detected *in vitro* and *in vivo*	no	yes
Fluorescence corresponds to photoactivity	no	yes
Selectivity for malignant tissue	poor	poor

Table 4.1 Comparison of the main photochemical properties of HpD and AlSPc

The following sections will discuss the properties of the haematoporphyrin and phthalocyanine photosensitisers in more detail prior to presenting the experimental work carried out with these drugs. AlSPc has been chosen by our Unit as the most promising of the second generation of photosensitisers to replace HpD, and has been undergoing extensive preclinical investigation across several specialties since 1984. Other groups worldwide are studying the alternative compounds outlined above, several of which show promise but will not be considered further here.

4.2.3 Porphyrin photosensitisers

The development of the porphyrin photosensitisers has been outlined in Chapter 3. It has been mentioned that both HpD and DHE are complex mixtures of compounds with varying tumour localising and photosensitising properties whose exact chemical nature has still not been clarified. Around 50% of HpD comprises several porphyrins including haematoporphyrin, protoporphyrin and hydroxyvinyldeuteroporphyrin, which are photosensitisers but poor tumour localisers. The remainder comprises an undefined hydrophobic component which is the tumour localising agent and forms the bulk of the purified product DHE, extracted by Dougherty (Dougherty *et al.*, 1984). There has been spirited debate as to whether DHE is an ether or an ester, and in reality it is probably contains both ether and ester-linked porphyrin units ranging from dimers to oligomers, the amount of ether-linked porphyrin increasing with storage at room temperature (Kessel *et al.*, 1987).

The proposed structure of Hp is shown in fig. 4.2. It has a strong tendency to form large aggregates (molecular weights from 20 000 - 30 000) which tend to bind to cells where they may remain for some time. These aggregates can be detected by fluorescence but are poor photosensitisers as

their yield of singlet oxygen is low (Moan and Sommer, 1984). HpD is also unstable in solution, being degraded by light and temperature. The thawing and refreezing of porphyrin solutions may give unreliable results.

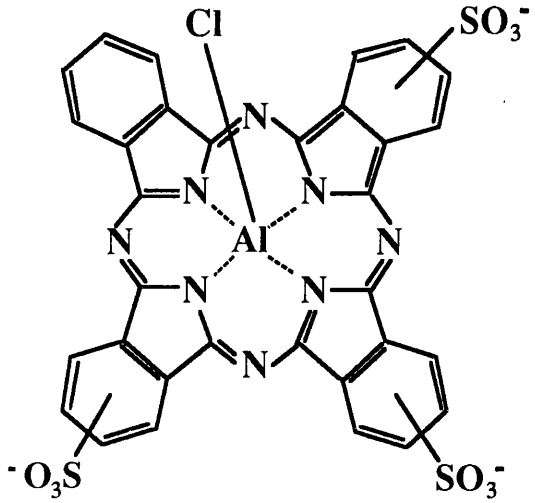
4.2.3.1 Mechanism of tumour uptake of porphyrins

It would appear that the retention of porphyrins in malignant tumours is the result of general differences in the physiological environment of the tumour rather than any specific property of the malignant cells themselves. Often after injection of HpD into an animal bearing a transplanted tumour, higher levels are seen in organs such as the liver and spleen than in the tumour, particularly in associations with mast cells and macrophages. These cells are also plentiful in tumour stroma and may well be responsible for localising porphyrins. The rather leaky vasculature and incompetent lymphatics of tumours should further encourage the trapping of HpD bound to serum proteins, in the extracellular fluid. HpD may also bind to low density lipoproteins (LDL), numerous receptors for which are found in tumour cells associated with membrane synthesis. There appears to be a correlation between LDL receptor levels and sensitiser distribution (Kessel, 1986b).

4.2.4 Phthalocyanine photosensitisers

The phthalocyanines have similar biological properties to the porphyrins but are easier to handle chemically and assay in tissue. Aluminium sulphonated phthalocyanine (AlSPc) has a single strong absorption band at 675 nm, where tissue penetration is good as there is little unwanted absorption by natural biomolecules, with a high yield of singlet oxygen. It appears non toxic in animals and though not yet licensed for clinical use, is under detailed experimental study in several centres (Bown *et al.*, 1986; Chan *et al.*, 1986; Spikes, 1986)

AlSPc



Hp

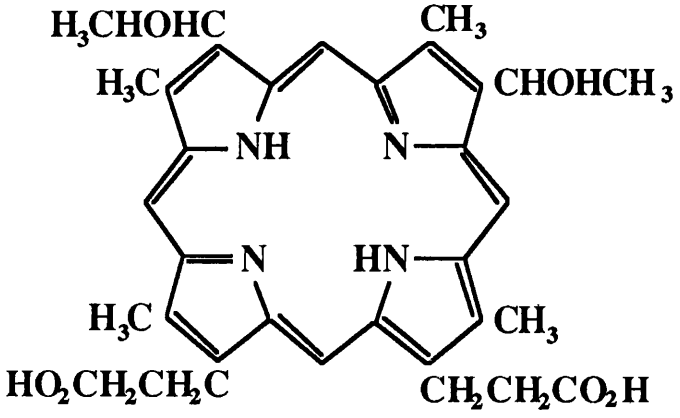


Fig. 4.2 The planar structures of aluminium sulphonated phthalocyanine (AlSPc) and haematoporphyrin (Hp)

The chloride ligand attached to AlSPc minimises aggregation and may be substituted by others during sulphonation. The longer the conjugated pathway around the molecule the longer the wavelength of light that can be absorbed.

Phthalocyanines are structurally related to porphyrins in that they are tetraazoporphyrins and their molecules have a similar planar ring structure (fig. 4.2). Biochemically they comprise 4 isoindole units linked by nitrogen atoms, whereas the porphyrin ring consists of 4 pyrrole units linked by methine carbon atoms. Because the conjugated pathway around the hydrocarbon ring is longer they will absorb light of a rather higher wavelength than the porphyrins, the exact wavelength being dependent on the metallic ion incorporated. Although they have some absorption in the ultraviolet range (345 nm), there is only a single strong peak in the visible spectrum at 675 nm for AlSPc (fig. 3.1). This peak represents almost a 50 times stronger absorption than HpD has at 630 nm (Spikes, 1986), and

should result in a greater photodynamic effect though the differences are not as vastly great as this might suggest. AlSPc's low absorption at UV and visible wavelengths is likely to minimise cutaneous photosensitivity which will be of major clinical benefit (Tralau *et el.*, 1989). Naphthalocyanines have the longest conjugated pathway and will absorb light out to 780 nm.

Pure phthalocyanines are not water soluble but they can easily be made so by sulphonation of the metallic compound by treatment with fuming sulphuric acid (McCubbin, 1985). This process results in a mixture of molecules containing from 1 - 4 sulphonate groups per molecule with an average of around 3 in the compound supplied to us for this work from the Royal Institution. The exact composition is determined by partitioning the phthalocyanine with an insoluble cation which forms an organic complex soluble in chloroform but not in water. The optical density of the fraction partitioned in chloroform compared to that in aqueous solution will indicate the degree of sulphonation. Because the mono-, di-, tri- and tetra-sulphonated derivatives have different charges they have differing solubilities *in vivo,* the greater the degree of sulphonation the greater the water solubility and vice versa for lipid solubility, and hence will have differing photochemical properties. Recent studies in our Unit have shown that the relatively hydrophilic tetra-sulphonated preparation (AlPcS4) tends to remain in the extracellular space whereas the more lipid soluble di-sulphonated derivative (AlPcS2) localises more intracellularly (Chatlani *et al.*, 1991). Berg *et al.* (1989) found that the cellular uptake of AlPcS1 (the most lipid soluble) was 10 times higher than that of AlPcS4 (the least lipid soluble). Other groups have incorporated insoluble phthalocyanine into liposomes as a means of transport and targeting tumour cells (Jori, 1989).

Like HpD and DHE, AlSPc forms aggregates in tissue, though rather less readily, but the important feature is that phthalocyanine aggregates do not

appear to be active as photosensitisers nor do they exhibit fluorescence. This means that fluorescence detection is specific for the photoactive compound, in contrast to the porphyrins where all forms with varying activities are fluorescent. Aggregation is dependent upon the degree of sulphonation so AlPcS1 will form aggregates much more readily than AlPcS4. The efficiency of cell inactivation, higher for increasing lipid solubility, also increases with decreasing number of sulphonate groups with the exception of AlPcS1 which is less efficient than AlPcS2 but more photoactive than either AlPcS3 or AlPcS4. This implies that the aggregated species are photoinactive (Berg *et al.*, 1989).

4.2.4.1 Development of phthalocyanines for PDT

Preparations of copper phthalocyanines have been used for some years as selective histological stains for certain polysaccharides and connective tissue elements. Phthalocyanines have also been used in industry for photo-disinfecting textiles, and as dyestuffs on account of their intense blue/green colouring. Their first potential use in cancer therapy was suggested nearly 30 years ago when several groups noticed that they were taken up well by experimental brain tumours in mice. Frigerio (1962) proposed that linking phthalocyanines to radionuclides might be an effective method of targeting radiotherapy for brain tumours. Increasing interest in PDT lead to their potential as tumour photosensitisers being studied in a variety of tissue culture and experimental tumour systems. Various metal atoms were chelated with phthalocyanines and were found to have a marked effect on photosensitising ability, aluminium and zinc being the most effective as producers of singlet oxygen whereas copper is not (Rosenthal *et al.*, 1987). This group was also the first to produce water-soluble sulphonated phthalo-cyanines and to show that molecular oxygen was necessary for their activity, a small reduction in pO_2 reducing the photoinactivation of Chinese hamster cells by half (Ben-Hur and Rosenthal, 1985b).

The mechanism of action of the phthalocyanines is similar to that of the porphyrins except that the longer triplet lifetime should result in a higher yield of singlet oxygen (Spikes and Bommer, 1986). They have been shown to be efficient photosensitisers both *in vitro* (Chan *et al.*, 1986) and *in vivo* (Barr *et al.*, 1987b) though are not any more selective for malignant tissue than HpD (Tralau *et al.*, 1987). Like HpD, AlSPc seems to exert a major effect via the tumour vasculature. Selman *et al.* (1986) measured blood flow in experimental tumours, using a radioactive microsphere technique, both before and after PDT with AlSPc. Blood flow in the tumours was found to decrease by 85% compared with controls, which were sensitised but shielded during light exposure, following PDT and haemorrhage and coagulation necrosis were observed later. This group had previously reported similar results using HpD (Selman *et al.*, 1984).

Most of the work presented in this thesis has used the AlSPc mixture but more recently the use of high pressure liquid chromatography has enabled the purification of enough of the di-sulphonated derivative to use experimentally. This compound (AlPcS2) is also proceeding slowly through the extensive pre-clinical assessments required before the phthalocyanines can be used in patients.

4.3 SELECTIVITY OF PHOTODYNAMIC EFFECT

The driving interest in PDT has centred around the concept that it should be possible to kill tumours without damaging normal tissue. Unfortunately this is rather an optimistic oversimplification. It is very difficult to produce truly selective tumour necrosis with PDT based on selective uptake of the photosensitiser, as this can only be done under special circumstances

with careful manipulation of all the treatment variables involved (Barr *et al.*, 1990a). Under the conditions described in virtually every published paper on PDT, if tumour and normal areas are exposed to the same light dose, there is damage to both. However, it is now becoming apparent that the *nature* of the biological effects of PDT on normal and malignant tissues is different from those produced by other forms of local injury such as thermal coagulation or ionising radiation. For example, tissue architecture and tensile strength are much better preserved after PDT than after thermal injury. Experimental studies of rat colon show that despite a full thickness necrosis after PDT there was no reduction in wall strength as measured by gaseous bursting pressures, in contrast to comparable thermal lesions produced by a Nd:YAG laser which proved much weaker (Barr *et al.*, 1987a). The reason for this seems to be that PDT has little effect on submucosal collagen in the colon whereas heat destroys it. Interestingly the collagen in blood vessels on the other hand is easily damaged by PDT (Nelson *et al.*, 1988). In the treatment of human disease the understanding and exploitation of these differences is likely to be more important than trying to limit effects to tumour areas. Even though normal tissue may be damaged along with the tumour, if this heals by regeneration of normal tissue as seems to be the case after PDT, then the net effect is selective eradication of the tumour.

Any form of local treatment has some degree of selectivity. Therapy with ionising radiation or with a thermal laser such as the Nd:YAG will produce the greatest effect in the area that receives the highest dose. If the light used for PDT is directed solely at tumour tissue, then naturally necrosis is confined to the tumour area. Much higher degrees of selectivity are possible by controlling where the light is directed than can be achieved solely by differences in the uptake of the sensitiser between normal and neoplastic areas. However in many instances where PDT is likely to be

useful, particularly for early multifocal tumours, it will not be possible to localise all the neoplastic areas precisely so both normal and tumour areas will be exposed to similar light doses. The understanding of the nature and healing of photodynamic damage to normal tissue is therefore fundamental to the establishment of those treatment parameters that will eradicate tumour but not cause irreversible damage to normal tissue.

One method that has been suggested to enhance PDT selectivity and effectiveness is to also heat the tumour. It may seem strange to want to combine PDT with hyperthermia as one of the distinguishing features of PDT is that it is a non-thermal process. Nevertheless several workers have found a synergistic effect when PDT is followed by hyperthermia (in the order of 42.5 - 45 °C), both *in vitro* and *in vivo* (Christensen *et al.*, 1984; Henderson *et al.*, 1985b). This potentiating effect is lost when the time delay between PDT and hyperthermia is increased much beyond 2 - 3 h, and it is thought that hyperthermia inhibits the repair of sublethal PDT damage.

Other workers have tried to improve the targeting of photosensitiser by combination with monoclonal antibodies (Mew *et al.*, 1985). This sounds a promising technique but can be no better than the specificity of the linked monoclonal for the tumour cells, which is often disappointing.

4.3.1 Selectivity of PDT in the bladder

The side effects of PDT on the bladder that have been encountered in clinical studies, and which were discussed in chapter 3 (section 3.4.2.4), appear to be the result of necrosis followed by fibrosis in the muscle layer of the bladder, i.e. the result of damage to normal tissue rather than to tumour. These results beg the question of how much selectivity there really is under the conditions being used at present. Also, bearing in mind the superficial nature of the pathology we are trying to treat (bladder Cis),

should we be perhaps looking for selectivity between superficial and deep layers of the normal bladder wall rather than between normal and neoplastic mucosa?

Much of the experimental work on PDT has stressed the importance of selective uptake of photosensitisers using tumours transplanted subcutaneously in mice and rats compared with the adjacent skin and muscle. However, what is important clinically is the difference in uptake or retention between a tumour and the *adjacent normal tissue in which that tumour arose* (or into which the tumour has spread); it is surprising how few data are available in the world literature on this crucial point. There have been many reports, both clinical and experimental, on selective fluorescence of malignant tumours after porphyrin sensitisation, but this is normally excited by ultraviolet light, which penetrates less than 0.1 mm into tissue so one is only looking at surface differences between tumour and normal areas. It is difficult to correlate these measurements with concentrations of photosensitiser in the bulk of the tumour though these techniques may have a useful role in diagnosing areas of endoscopically occult malignancy (Lin *et al.*, 1984; Benson, 1989). Work on animal tumours of the colon and pancreas has shown that the best selectivity of uptake achieved with either HpD or AlSPc is only in the order of 2-3:1 between tumour and adjacent normal tissue. The major exception to this is for intracranial gliomas where the ratio is considerably greater at 28:1, most likely due to the breakdown of the blood brain barrier in tumour areas (Tralau *et al.*, 1987). Little such data are available for bladder tumours.

4.3.1.1 Experimental studies on normal bladder

The first objective in planning PDT experiments on the bladder must be to decide what biological effect is desired and then to manipulate all the

variables involved to see if this effect is achievable. It is surprising that so little has been done along these lines.

In whole bladder treatments, no-one has shown necrosis limited to the tumour areas, and instead large areas of mucosa have sloughed which must be presumed to include both normal and neoplastic regions. These have healed with regeneration of normal mucosa which for patients with Cis means that their tumour has been eradicated, although the price paid was scarring and a permanent impairment of detrusor muscle function. Thus it seems less important to attempt any form of selectivity between normal and abnormal mucosa, but highly appropriate to try and limit the damage to the mucosa and submucosa and leave the muscle intact. Experiments designed to answer this question can be carried out on normal animals, which is simpler than finding an adequate animal model of *in situ* bladder cancer.

Two sets of variables can be manipulated. Those related to the photo-sensitising drug (e.g. dose, route of administration and time between sensitisation and light exposure), and those related to the light (wavelength, power, exposure time and geometry of light distribution). It is only by performing careful and logical experiments that the problems that exist with clinical dosimetry may be properly addressed. The aim of the experimental work presented in this thesis is to investigate the effect of varying these basic dosimetry values on the biological result.

4.3.2 Fluorescence

All photosensitisers will fluoresce when exposed to light of a wavelength corresponding to an absorption peak (fig. 3.1). The emitted light is of a longer wavelength than the exciting light energy and may be detected visually, photographically, or by a variety of sensitive electronic imaging systems. Normally ultraviolet light is used to excite fluorescence from

porphyrins, the largest absorption band is around 405 nm, and the resulting reddish fluorescence detected at 630 nm. Fluorescence has been widely used to localise photosensitisers, both to identify putative areas of malignant tissue and to investigate the mechanism of photodynamic action. The bladder is one organ in which such localisation seems useful (Benson *et al.*, 1982) though the equipment required is costly and the technique complicated (section 3.3).

Fluorescence measurements though have several potential drawbacks notably that it is questionable whether or not the observed fluorescence closely mirrors photoactivity, given that the non-active porphyrin aggregates also fluoresce (Moan and Sommer, 1981; Berns *et al.*, 1984). With the phthalocyanines however the main aggregates formed are dimers whose fluorescence appears to be negligible and whose photoactivity is also very low compared to that of monomers (McCubbin, 1985; Spikes and Bommer, 1986). The question of the correlation between fluorescence and photoactivity has been examined previously for AlSPc (Barr *et al.*, 1988; Berg *et al.*, 1989), from which it was concluded that fluorescence detection is selective in that only photoactive monomers are detected. Therefore fluorescence estimations of phthalocyanines are probably more relevant in most instances than chemical assays of sensitiser concentration as the inactive aggregates are ignored. Another potential difficulty in relating fluorescence intensity to sensitiser concentration in tissue is that some tissues are efficient quenchers of fluorescence. For instance, very little fluorescence is seen from normal liver after the administration of HpD even though sensitiser levels are high as measured by radiolabelling techniques (Gomer and Dougherty, 1979). Also the very light used to excite fluorescence may itself degrade the image by causing photo-bleaching. This can be minimised by using a very sensitive imaging system that only requires an extremely low-powered excitation source (chapter 6).

4.3.3 Photodynamic thresholds and photobleaching

Photobleaching, the irreversible degradation of some of the excited triplet state molecules, was initially thought to be a disadvantageous property for a photosensitiser as it indicated that it was photochemically unstable. This can occur through reaction of the activated photosensitiser either with singlet oxygen or with tissue components such as amino acids. AlSPc is known to be extremely stable against attack by singlet oxygen (McCubbin, (1985; McCubbin and Phillips, 1986) unlike many porphyrins, but it has been shown that phthalocyanines can react with amino acids such as cysteine (Darwent *et al.*, 1982), leading to the formation of less stable phthalocyanine ionic and radical species. These may undergo further reactions resulting in decomposition of the phthalocyanine ring and irreversible degradation (fig. 4.1). This phenomenon was first described for porphyrins *in vitro* by Moan (1986b), and later *in vivo* by Mang *et al.* (1987) who showed a loss of fluorescence and reduction in extractable porphyrin from a mouse mammary tumour. Similar responses have been since demonstrated in other tumour models and it has become clear that photobleaching could be a valuable mechanism by which tumour selectivity in PDT may be enhanced. How might this work?

The amount of singlet oxygen produced depends on the total "photodynamic dose" which is a product of the total light energy absorbed at each point and the tissue concentration of photosensitiser at the time of light exposure. There is reasonable reciprocity between the sensitiser concentration and the light dose in the ranges most workers have studied; i.e. for a given tissue effect if the sensitiser concentration is doubled then the light dose is halved (Bown *et al.*, 1986; Barr *et al.*, 1987b; Profio and Doiron, 1987). However photodynamic damage is a threshold effect, and a minimum amount of singlet oxygen must be produced before a cell is killed. This reciprocity

therefore fails for low concentrations of photosensitiser which cannot produce sufficient singlet oxygen, however high the light dose given.

In effect then, there are two threshold conditions that must be satisfied before a PDT effect can be produced. The first is a minimum tissue concentration of photosensitiser below which it may be inactivated by photodegradation before sufficient singlet oxygen is produced to cause any biological damage. Even if there is enough photosensitiser there must of course also be enough light, which results in a second threshold for the total photodynamic dose. We have already seen that the difference in photosensitiser concentration between tumour and normal tissue is rarely more than about 2:1. However if treatment variables can be manipulated such that the concentration of photosensitiser in the tumour is above the first threshold level, whereas it is below it in the adjacent normal tissue then a truly selective tumour destruction should occur. The only two requirements are that the absorbed light dose in the tumour is sufficient to satisfy the second threshold condition for total photodynamic dose, and the fluence rate is not high enough to cause thermal damage.

These threshold values are likely to vary considerably, not only between experimental animals and Man but also between different organs and tissues. The depth of tissue damage produced using these low photosensitiser concentrations is less than would be the case at a higher photodynamic dose when larger amounts of singlet oxygen are being generated, only about 2 mm; though this is quite adequate for bladder Cis. Careful experimentation is therefore going to be necessary to achieve these theoretical results in clinical work, but the principle has been shown to work in animal colon cancers sensitised with AlSPc (Barr *et al.*, 1990a) and in some other tumours using HpD (Potter *et al.*, 1987). Barr and colleagues concluded that low concentrations of AlSPc in normal colon

were photodegraded too rapidly for the threshold light energy absorption to be reached, whereas in tumour, containing about twice as much AlSPc as normal tissue, the threshold dose was reached making selective tumour destruction possible. MacRobert *et al.* (1989) have calculated that significant photodegradation may occur in a comparatively short time, in the order of 10^3 absorption cycles, using typical PDT energy doses. This principle of photobleaching enhancing selectivity of necrosis is explored for the normal bladder in the work presented in chapters 6-8.

Photobleaching as a means of reducing the period of cutaneous photosensitivity following PDT with porphyrins has been suggested (Boyle and Potter, 1987). They were able to show in mice that repeated low doses of light degraded the sensitiser in skin such that there was no reaction to a subsequent large dose. If this worked in Man, the typical period of sensitivity to daylight after porphyrin photosensitisation might be reduced from six weeks to one week.

SECTION 2

EXPERIMENTAL STUDIES ON PHOTODYNAMIC THERAPY

Chapter 5

THE PHOTODYNAMIC ACTION OF A PULSED DYE LASER ON HUMAN BLADDER CANCER CELLS SENSITISED WITH DHE

5.1 INTRODUCTION

The complications encountered in clinical bladder PDT with porphyrin photosensitisers have already been discussed in chapter 3. The damaging event appears to be the activation of photosensitiser in the muscle layers of the bladder wall causing tissue damage that heals with sufficient fibrosis to produce a significant reduction in compliance and functional capacity. Until photosensitiser drugs that are more selective for neoplastic tissue are developed the only possible ways of limiting damage to the muscle layer are by adjusting dosimetry parameters such that either one or other of the following conditions occurs:

i) The concentration of photosensitiser in the muscle never reaches the threshold level required to yield enough singlet oxygen to result in photodynamic necrosis, independent of the light dose.

ii) The light dose reaching the deeper tissue layers is insufficient to produce sufficient singlet oxygen from the photosensitiser molecules to cause necrosis even in the presence of adequate amounts of the sensitiser (the second threshold condition).

The first statement implies that there should be a gradient of photosensitiser distribution between the superficial (epithelium and lamina propria) and deep (muscularis) layers of the bladder wall; a concept which will be studied more fully in the following chapters. The only way though of satisfying the second condition and reducing the light dose reaching muscle whilst still giving enough surface illumination for a PDT effect in the epithelium, is to use a less penetrating wavelength of light.

All published clinical PDT studies have used HpD or DHE as the photosensitiser activated by red light with a wavelength of 630 nm as red light penetrates tissue well because it is poorly absorbed by naturally occurring

biomolecules. The maximum penetration of light is at around 780 - 800 nm in most tissues but current photosensitisers cannot absorb such long wavelengths and the photon energy of this light is insufficient for an adequate yield of singlet oxygen (Doiron, 1984). For most applications of PDT it would be advantageous to treat the maximum bulk of tissue possible though this will never be more than about 5 - 6 mm (around 4 penetration depths in most tissues). For superficial bladder carcinoma this is not necessary and as we have seen is probably responsible for the major complications encountered. Furthermore, as has already been discussed, 630 nm corresponds to a relatively minor peak in the absorption spectrum of HpD / DHE, whereas there is rather stronger absorption through most of the rest of the visible spectrum, e.g. a peak for green light around 507 nm. Therefore not only might a stronger photodynamic effect be expected for the same dose of green as compared to red light but also the depth of tissue penetration by green light would be much less at only 1-2 mm (van-Gemert *et al.*, 1985), so affording some protection to the deeper layers of the bladder wall. Of course it is not possible to activate the phthalocyanine photosensitisers with green light as they do not absorb at this wavelength so it is necessary to use a porphyrin photosensitiser (HpD/DHE) for studies involving green light.

There are several types of laser that will produce green wavelength of light. The most obvious option is to use the direct output of an argon ion laser rather than coupling it to the dye laser necessary to produce red light. This would also result in much higher output powers (typically 7 W at 514 nm from a laser system that would give only 1 W in the red). The copper vapour laser also produces a high output of green light (511 nm). Neither of these lasers though have any other urological application and the argon ion laser in particular has several disadvantages in terms of its bulk, poor reliability and expense that make it less than satisfactory for clinical use.

The flashlamp pumped dye laser which produces pulsed green light (504 nm) has already been described (section 2.2.2) and is routinely used for fragmenting ureteric calculi (Coptcoat *et al.*, 1987). Although it is a more expensive laser than the copper vapour laser there would be an advantage for Units already possessing one to have another possible use for it.

There are very few studies investigating the suitability of the pulsed dye laser for PDT. There was no report of its efficacy *in vitro* prior to undertaking this work and only 2 *in vivo* studies neither of which showed an effect. These studies will be discussed later, but briefly, Bellnier *et al.* (1984) used a murine tumour model sensitised with HpD and although observing some damage after illumination with a pulsed dye laser, attributed this to thermal effects. Barr *et al.* (1989) did not produce any PDT effect on normal rat colon sensitised with AlSPc. These latter authors expressed doubt as to whether the slow pulse repetition rate of this type of laser was adequate to produce a high enough singlet oxygen yield for efficient photodynamic action.

It was considered, nevertheless, that these experiments were not conclusive and that the rather more tightly controlled environment of *in vitro* experiments would be most likely to answer these points. Would green light PDT effectively kill bladder tumour cells in culture and would the particular qualities of this type of pulsed laser prove advantageous over the more commonly used light sources?

5.2 MATERIALS AND METHODS

The author had no prior experience of *in vitro* techniques, so began this work with a period of instruction in the cell culture laboratories at St. Paul's hospital under the guidance of Dr. J.R.W. Masters. Adequate proficiency was acquired in performing standard procedures involved in:

- cell freezing and thawing and culture medium preparation.
- routine maintenance and subculture of continuous cell lines.
- cell counting and viability assessment with a haemocytometer.
- techniques of *in situ* clonogenic assay.
- fixing, staining and counting cell colonies.

5.2.1 Cell culture techniques

A human continuous bladder carcinoma cell line (MGH-U1) was maintained in monolayer culture in RPMI 1640 medium, (Gibco Europe Ltd., Paisley, Scotland) supplemented with 5% heat-inactivated fetal bovine serum (Sera-Lab Ltd., Crawley Down, England) and 2mM L-glutamine (Flow Laboratories Inc., Irvine, Scotland), at 36.5°C in a humidified atmosphere of 5% CO_2 in air. The flasks were examined daily and when the cells were becoming confluent they were routinely subcultured to allow them to continue growing logarithmically. Subculture was also performed to obtain a single cell suspension for the clonogenic assay described below.

5.2.1.1 Cell subculture

Subculture was performed in the sterile environment of a laminar flow cabinet after first checking the culture flask under an inverted microscope to exclude any bacterial or fungal contamination. Fresh medium was prepared by adding 25 ml fetal bovine serum and 5 ml L-glutamine (kept frozen at -20°C and defrosted in a 37°C water bath immediately prior to

use) to 500 ml RMPI 1640 medium (stored at 4°C). The necks of all containers were flamed in a bunsen burner both before and after removing their lids to ensure sterility. The enzyme mixture was prepared by adding 4 ml of an aqueous solution of 0.05% trypsin (1:250; Difco Laboratories, London) to a 16 ml aliquot of 0.016% versene (EDTA disodium salt; BDH Chemicals, Poole, England).

The flask containing the cells to be subcultured was tilted onto its side and the medium aspirated from one corner taking care that the tip of the Pasteur pipette did not scrape cells off the bottom of the flask. The cells were then gently rinsed with 5 ml phosphate-buffered saline (PBS) to remove any remaining serum which would inactivate the trypsin. Next 4 ml of the trypsin/versene mixture were added, the flask was gently rocked so that the monolayer was covered and after 30 s most of the trypsin/versene was aspirated, leaving only a thin film to cover the cells. The flask was then returned to the incubator until all the cells had rounded up indicating that they had detached from the flask base (usually 5-10 min) and could be dislodged by tapping the side of the flask. A single cell suspension was produced by forcefully syringing 10 ml medium repeatedly up and down through a 25 gauge needle. For routine maintenance of the flasks 1 ml of this suspension was placed into 2 new 25 cm^2 Falcon flasks and made up to 5 ml with fresh medium. Flasks were then labelled and one was added to the old passage number. The medium was changed the following day to remove any dead cells and the cells were used over a restricted range of 10 passages to minimise any changes occurring as a result of long term culture. No antibiotics were used.

5.2.1.2 Cell counting and preparation of culture dishes

Exponentially growing cells were enzymatically detached from the flask base, and a single cell suspension was produced as described above. The

degree of dilution of this cell suspension needed to produce the required number of cells in the experimental dishes was calculated as follows.

The counting chamber of a haemocytometer (Neubauer) was cleaned with alcohol and a coverslip applied. Equal amounts (0.2 ml) of the cell suspension and of a 0.1% solution of trypan blue were drawn up with a micro-pipette, mixed together and a small volume of the resulting solution aspirated into the tip of a Pasteur pipette. Applying a drop to each side of the coverslip drew in, by capillary action, the exact amount needed to fill the counting chambers of the haemocytometer which was then examined under a microscope. Dead cells would take up the stain, appear dark blue and could be excluded from the count. If the cells appeared clumped this indicated that a single cell suspension had not been achieved and then the flask suspension was syringed back and forth a few more times before repeating this step.

The 4 counting areas in the haemocytometer chamber each represented an area of 1 mm^2 and were subdivided into 16 smaller squares. The depth of the counting chamber beneath the coverslip was 0.1 mm and therefore the volume of each area was 10^{-4} ml. The number of viable cells within the grid confines of each of these areas was counted and this process repeated 4 times with a new solution of the cell suspension and trypan blue. Cells lying across the boundary lines at the top and left-hand side of the counting square were counted in whilst those lying across the bottom and right-hand lines were excluded. A typical set of values is illustrated below.

Haemocytometer readings -	i)	73, 66, 50, 68	(mean 64.25)
	ii)	71, 66, 55, 59	(mean 62.75)
	iii)	65, 65, 68, 69	(mean 66.75)
	iv)	74, 56, 59, 52	(mean 60.25)

Overall mean value = 63.5

Concentration of viable cells = $63.5 \times 2^{*} \times 10^{4} = 127 \times 10^{4}$ cells/ml

* (the factor of 2 allows for the dilution in trypan blue)

Now that the cell concentration of the single cell suspension was known then the dilution required in order to seed the required number of cells into each of the experimental dishes could be calculated.

Each dish would contain 0.2 ml cell suspension and 2.8 ml medium
150 cells were required per dish
concentration of cell suspension required was 750 cells/ml

Serial dilutions of cell suspension with fresh medium required were

	1 : 9	giving a cell concentration of	12.7×10^4 cells/ml
then	1 : 16	giving a cell concentration of	7.5×10^3 cells/ml
then	1 : 9	giving a cell concentration of	7.5×10^2 cells/ml

Sufficient 3.5 cm petri dishes for each experiment were prepared (5 replicates for each parameter to be studied plus controls) containing 2.8 ml prewarmed and gassed medium, seeded with 0.2 ml diluted cell suspension. Out of the 150 cells seeded, the MGH-U1 cell line would be expected to yield about 100 viable colonies per dish which are about the maximum that can be conveniently counted in a 3.5 cm dish. The dishes were returned to the incubator for 48 h to allow the cells to attach and attain exponential growth prior to any experimental procedure.

The medium was then replaced with either fresh medium alone for some of the controls or with medium containing the photosensitiser dihaematoporphyrin ether (DHE-Photofrin II, Photomedica Inc., Ravitan, N.Y.). DHE was received from the manufacturers as a frozen concentrate containing 2.5 mg DHE/ml. This was prepared for each experiment by thawing the required amount and diluting in fresh medium 1:11.5 to produce a concentration of 200 μg DHE/ml. This solution was sterilised by passing through a millipore filter and further diluted as required. The concentration most often used was 20 μg/ml (section 5.3.1), 0.3 ml of the initial solution being added to each culture dish containing 2.7 ml medium.

After a 1 h incubation in DHE solution the dishes were washed with fresh medium to remove any unbound DHE and then placed in 1 ml PBS to prevent the cells from drying out during the subsequent light exposure.

All dish procedures on sensitised cells were performed under very low level indirect lighting (by necessity at night!) and sensitised cells remained protected by aluminium foil from scattered light except during the actual exposure. At all other times, including during incubation, the dishes were also covered with foil. Two sets of control dishes were used, the ones used to calculate survival rates being unsensitised cells exposed to light. A second set of sensitised controls manipulated in the same way as the test replicates but without the actual light exposure would reflect any toxicity from ambient or inadvertent illumination which if apparent led to the whole experiment being repeated.

5.2.1.3 *In situ* clonogenic assay

Following light exposure, given as described below, the cells were washed 3 times in fresh medium and incubated for a further 7-8 days during which times the viable cells would form colonies. Any dishes with macroscopic contamination were discarded. After this time a couple of dishes would be checked under an inverted microscope to ensure that there were adequate sized colonies present (minimum 50 cells). The medium was poured off and the dishes washed under gently running water then fixed in methanol for 10 min. The dishes were washed again and colonies stained by adding 5 ml of 10% Giemsa (Giemsa's stain, BDH chemicals) for 5-10 min. Stained dishes were washed in tap water and left to dry in air before the colonies were counted using a binocular dissecting microscope (fig. 5.1).

Five replicates were exposed initially at each parameter tested. Despite meticulous attention to dish handling techniques the large number of procedures on each dish, and the transport to and from the laser room (in

another building), did result in some becoming infected. The minimum acceptable number of surviving dishes for any parameter tested was 3, so if loss from infection resulted in less than this, then the whole experiment was repeated. This occured in over half of all experiments (8 out of a total of 15 major experiments). In most cases this was due to dish contamination but on 2 occasions was as a consequence of laser malfunction and once there were too few colonies, most likely due to inadvertent illumination.

Cell survival (colony forming ability) was calculated as the mean number of colonies of a minimum of 3 dishes at each treatment point expressed as a percentage of the mean of the controls. All experiments were repeated 3 times to plot cell survival curves. The data shown by these curves, therefore, represent the means and variation *between* experiments rather than those results within individual experiments. Discrete experiments (defined as having only a single variable) might be combined on a single occasion to comprise dishes treated with different variables; e.g. DHE and AlSPc and the same light exposure, or cells treated at different laser powers; generally up to the maximum of about 60 plates that could be conveniently managed at one time. Each experiment though would be repeated on 3 separate occasions with a new cell passage and new controls.

The mean values for each series of observations may be compared by calculating overall mean percentage cell survival values and the standard error of these means. These calculations together with the data from each experiment are shown in appendix 1. Statistical significance was assessed by the Student's t test for unpaired values. It is a reasonable assumption that cell numbers plated out into each dish would be normally distributed for this analysis to be valid. In view of the relatively small numbers of observations being compared it was considered that non-parametric tests would be unlikely to provide sufficient sensitivity to make even large

differences between groups statistically valid. Nevertheless it is not intended that undue emphasis should be placed on such statements of significance as the differences between, say, the individual laser powers employed are of secondary importance to the overall effectiveness of PDT.

5.2.2 Experiment design

Experiments were designed to address the following questions.

i) To assess any direct toxicity of DHE on MGH-U1 cells.

ii) To assess the effect of strong white light on sensitised cells and any difference in sensitivity to white light between cells sensitised with DHE and those sensitised with AlSPc.

iii) To assess the effect of pulsed green laser light at varying powers and pulse repetition rates on both unsensitised cells and cells sensitised with DHE.

5.2.2.1 Direct toxicity of DHE

Initial experiments were designed to assess whether DHE caused any direct toxicity to this cell line, and to determine a suitable concentration for use in subsequent laser experiments. MGH-U1 cells, prepared as described above, were incubated in the dark for 1 h in medium containing DHE at concentrations of 0 (controls), 5, 10, 15, 20, 25, 30 and 50 μg DHE/ml. Five replicate dishes were used at each concentration. After this period the cells were washed 3 times with fresh medium and returned to the incubator for 7 days before the *in situ* clonogenic assay was performed. This experiment, as with all others described below, was repeated on 3 seperate occasions to calculate cell survival rates.

5.2.2.2 The effect of white light

These experiments were now repeated using the same concentrations of DHE (0 - 50 μg/ml), but this time both sensitised cells and controls were

exposed to strong white light for 30 min. The light source used was the standard fluorescent lighting of the laminar flow cabinet.

The effect of exposure to white light was compared between cells sensitised with either DHE or AlSPc, as an indication of the magnitude of cutaneous photosensitivity that might be expected *in vivo*. This is the only instance that AlSPc was used as it would not be suitable for the laser experiments using green light and should not confuse the overall aim of these experiments. Less photodynamic effect would be expected on those cells sensitised with AlSPc due to its more localised absorption of visible light than DHE (fig. 3.1). Cells were incubated for 1 h with either DHE or AlSPc (both at 20 μg/ml) prior to exposure to bright white light for 2, 5, 10, 15 and 20 min. Controls comprised sensitised cells kept in the dark.

5.2.2.3 The effect of pulsed green laser light

The laser light source used was a flashlamp pumped dye laser (Candela MDL-1P, Candela Corp., Natick, M.A., USA - fig. 5.2), emitting green light (504 nm) transmitted along a 200 micron quartz fibre which was led into the laminar flow cabinet so that the cells could be illuminated under sterile conditions. This is the same clinical laser used by urologists for lithotripsy of ureteric calculi and its properties have already been described in section 2.2.2. The laser output was controlled by a foot switch and electronic timer allowing accurate short exposures.

The tip of the freshly cleaved laser fibre was positioned in a clamp approximately 12 cm above the culture dish, with its lid removed. At this distance the divergence of the laser beam was sufficient to illuminate the whole dish evenly. Incident light energy was measured with a power meter at the level of the dish. This meter had a 1 cm^2 aperture placed over its sensor so that the laser output could be adjusted to give an average power density of 50, 75 or 100 mW/cm^2 (close to maximum available power).

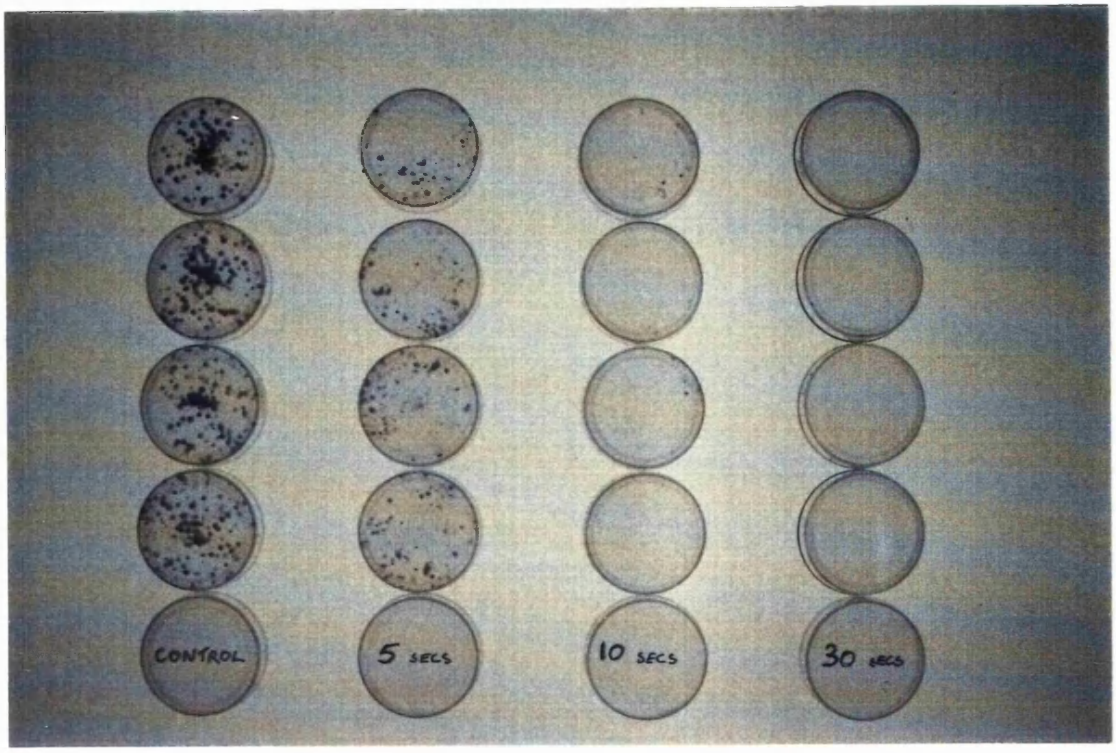


Fig. 5.1 Typical results of sensitised cells showing viable colonies after light exposure (stained with 10% Giemsa).

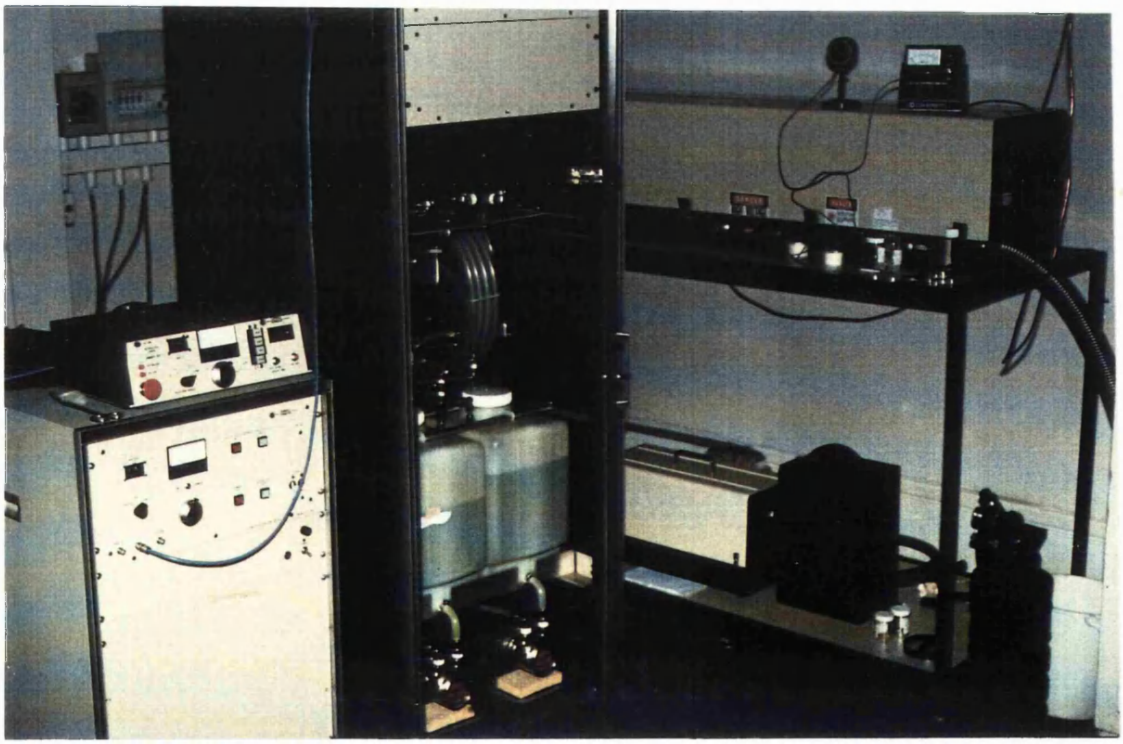


Fig. 5.2 The Candela flashlamp pumped dye laser used in this study. (The dye reservoirs can be seen at the bottom of the cabinet)

As Barr *et al.* (1989) had suggested that the low repetition rate (5 Hz) that they had used from a similar laser to our own was the main reason for a lack of effect, most of these experiments were performed at 20 Hz which is towards the higher end of the available frequency range from this laser. The frequency usually chosen for clinical lithotripsy is 10 Hz and the average power drops off quite rapidly much above 20 Hz due to insufficient recycling time for the flashlamp capacitor. The pulse length was 1 μs representing a pulse energy density (at 20 Hz) of 2.5 - 5 mJ/cm^2 and peak power density of 2.5 - 5 kW/cm^2. The output power was measured after each set of exposures to confirm that it had remained stable, which was almost always the case. Variations up to 5% were accepted but on the rare occasions that output had drifted further than this the laser was recalibrated before the next set of exposures.

Three different experiments using laser light were performed. Initially any possible direct cytotoxic effect of the laser light alone was investigated on unsensitised cells exposed to the maximum laser power used in these studies (average power density of 100 mW/cm^2). Five replicate dishes were exposed to laser light at this fluence rate and 20 Hz pulse frequency for 5 - 30 s. Control dishes comprised unexposed cells.

Then sensitised replicates were exposed to a range of 3 laser powers (average power densities of 50, 75 and 100 mW/cm^2) at a pulse repetition rate of 20 Hz, and exposure durations from 2-15 s. Initially a 30 s exposure was also used but this resulted in total cell kill with 50 mW/cm^2. Control dishes comprised both unsensitised cells exposed in the same way (to pick up any direct toxicity of either DHE or the laser), and sensitised cells kept in the dark (to pick up any toxicity due to inadvertent light exposure during the multiple manipulations of the culture dishes).

Finally the effect of reducing the laser pulse frequency was investigated. Sensitised cells were exposed to an average power density of 50 mW/cm^2 delivered at either 5 Hz or 20 Hz for exposures of 2-15 s. As the average power was the same for both the peak pulse power density was therefore 4 times higher at the slower frequency (a massive 10 kW/cm^2) than that delivered at 20 Hz. Control dishes comprised both unsensitised cells exposed to laser light and sensitised cells kept in the dark.

5.3 RESULTS

Detailed cell survival data are given in appendix 1. The values presented in this section are the means and standard errors for each set of experiments. Each data point therefore represents the mean ± SEM of 3 experiments, with each experiment consisting of 3-5 dishes at each point, depending upon the level of dish loss from contamination and other causes.

5.3.1 Direct toxicity of DHE and the effect of white light

For cells kept in the dark there was no significant toxicity observed throughout the range of DHE concentrations from 0-50 μg/ml (fig. 5.3). Marked toxicity however was evident on exposure of sensitised cells to the strong white overhead illumination of the laminar flow cabinet. Colony forming ability was reduced, compared to unsensitised cells similarly exposed to light, to 55% at a DHE concentration of only 5 μg/ml. There were only 2 colonies seen in 12 plates treated at 15 μg/ml DHE and no surviving colony seen at the higher concentrations. A concentration of 20 μg/ml DHE with a 1 h incubation was therefore chosen as standard sensitisation for subsequent laser exposures for two reasons. Firstly there

would be no direct toxicity of the DHE on the cells. Secondly as it was clearly possible to achieve a total kill of sensitised cells with white light using this concentration of DHE, then that indicated that there was enough sensitiser present, so if the pulsed laser was to prove effective for PDT it would be reasonable to expect a significant effect from it at this drug level.

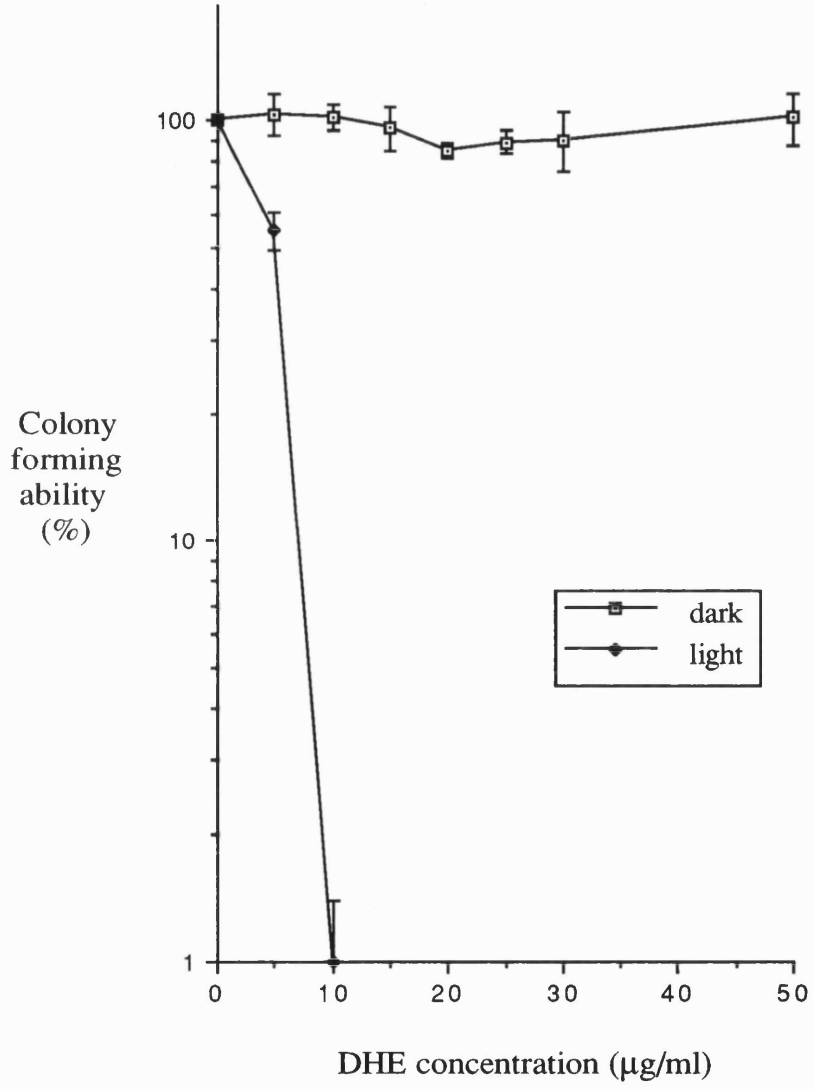


Fig. 5.3 The effect of exposure to white light for 30 min on MGH-U1 cells sensitised with DHE (1 h incubation).

The sensitivity to white light of cells sensitised with DHE was compared to that of cells sensitised with AlSPc (both at 20 μg/ml for 1 h). Again there

was marked toxicity in the DHE group, with a reduction in mean colony forming ability to 57% ± 10% at 10 min exposure and to 10% ± 5% at 20 min (fig. 5.4). The cells sensitised with AlSPc showed no toxicity at 20 min (mean = 102% ± 10%). The difference between the curves from the 2 sensitisers was statistically significant ($p < 0.05$) after 10 min. It was not considered necessary to have a control group of unsensitised cells exposed to the cabinet lighting as it is a basic assumption that standard conditions are not detrimental to cell survival and an effect here would negate any conclusion being drawn from cell culture work in general.

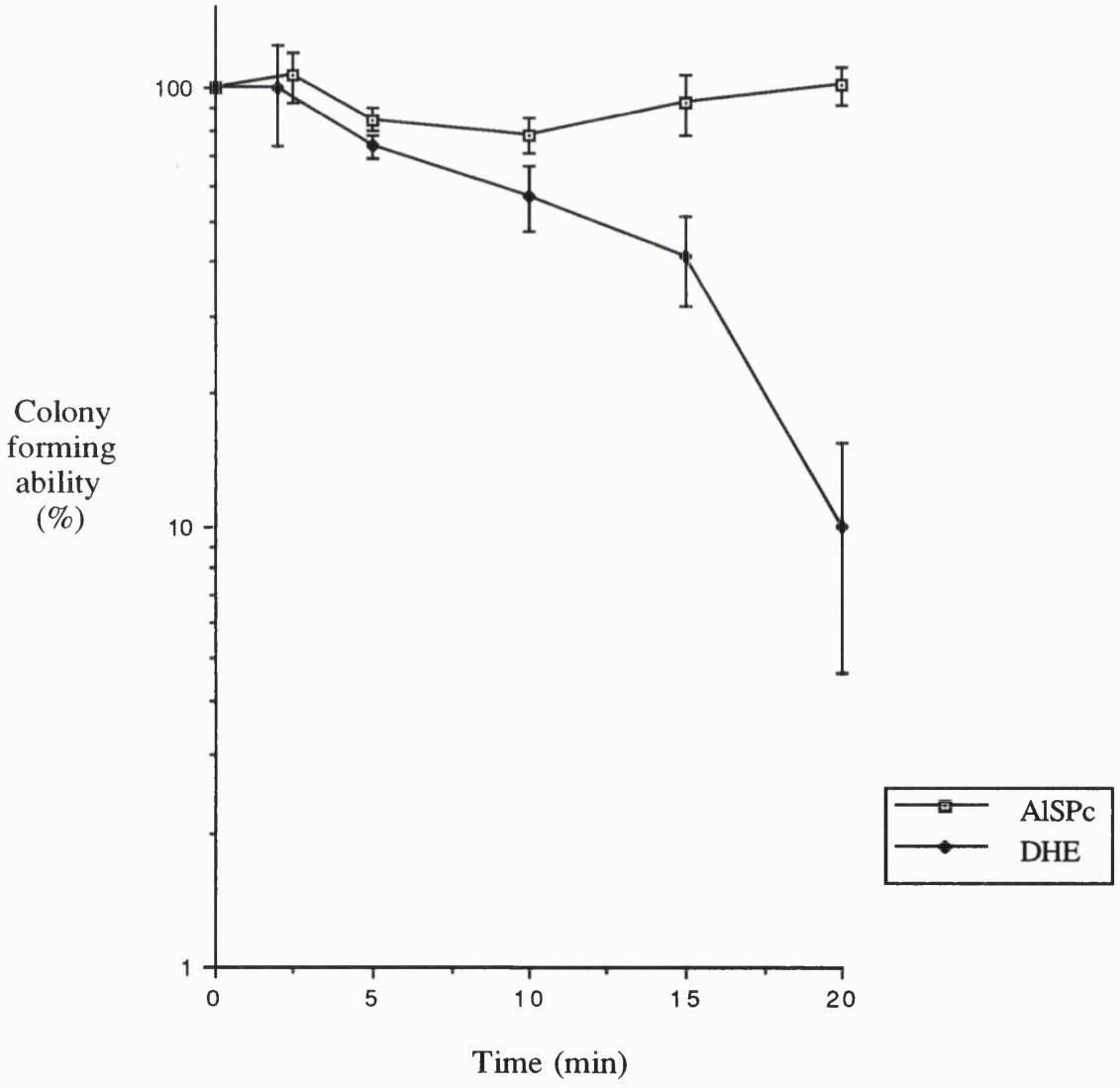


Fig. 5.4 The effect of exposure to white light on MGH-U1 cells sensitised with either DHE or AlSPc (20 μg/ml for 1 h)

5.3.2 The effect of pulsed green laser light

5.3.2.1 Laser light on unsensitised cells

Laser light alone, delivered as described in section 5.2.2.3, was not cytotoxic to unsensitised cells (fig. 5.5). No reduction in survival was seen when using the highest fluence rates employed in any of these experiments (100 mW/cm^2) and exposure times up to 30 s (colony forming ability at 30 s compared with unexposed controls = 94 ± 6%). No subsequent laser exposure on sensitised cells was for longer than 15 s.

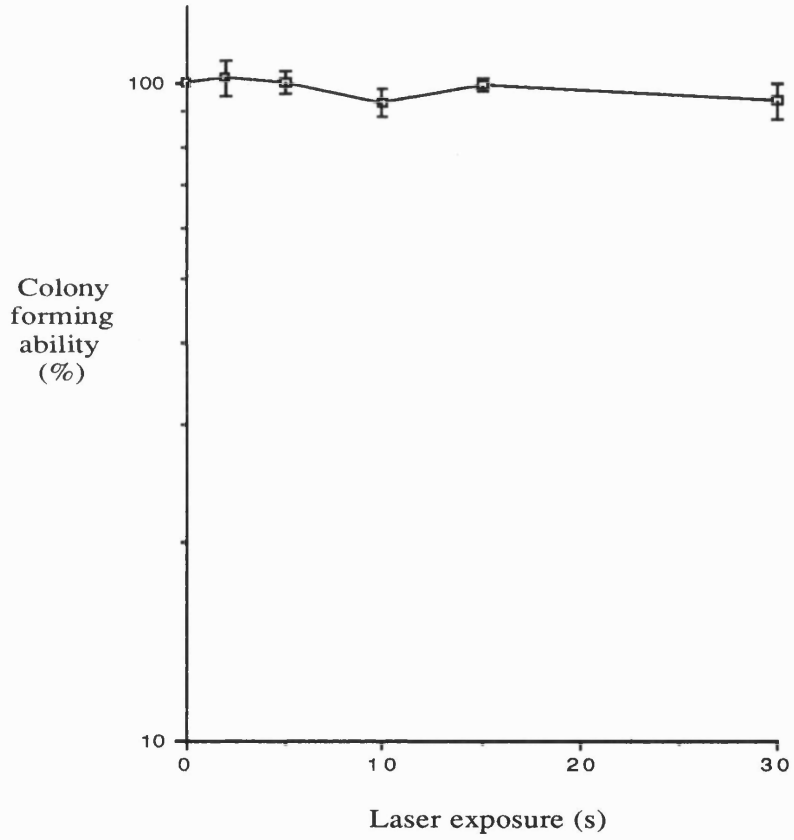


Fig. 5.5 The effect of exposure to pulsed green laser light (504 nm at 100 mW/cm^2) on unsensitised MGH-U1 cells

5.3.2.2 Laser light on sensitised cells

The effect of increasing exposures to 3 laser power densities (50, 75 and 100 mW/cm^2) delivered at the same pulse frequency (20 Hz) was studied.

Figure 5.6 shows the survival curves for sensitised cells exposed to these fluences for 2, 5, 7 and 10 s. Similar shouldered curves were obtained at each power level studied. A 90% reduction in colonies (LD_{90}) was achieved with projected exposures of 6 s at 100 mW/cm^2, 7.3 s at 75 mW/cm^2 or 9.6 s at 50 mW/cm^2. No colony was seen after either a 10 s exposure at 100 mW/cm^2 or a 15 s exposure at 75 mW/cm^2 and very few colonies after 15 s at 50 mW/cm^2. There was a significant difference ($p < 0.05$) between all curves and controls and between 50 and 100 mW/cm^2.

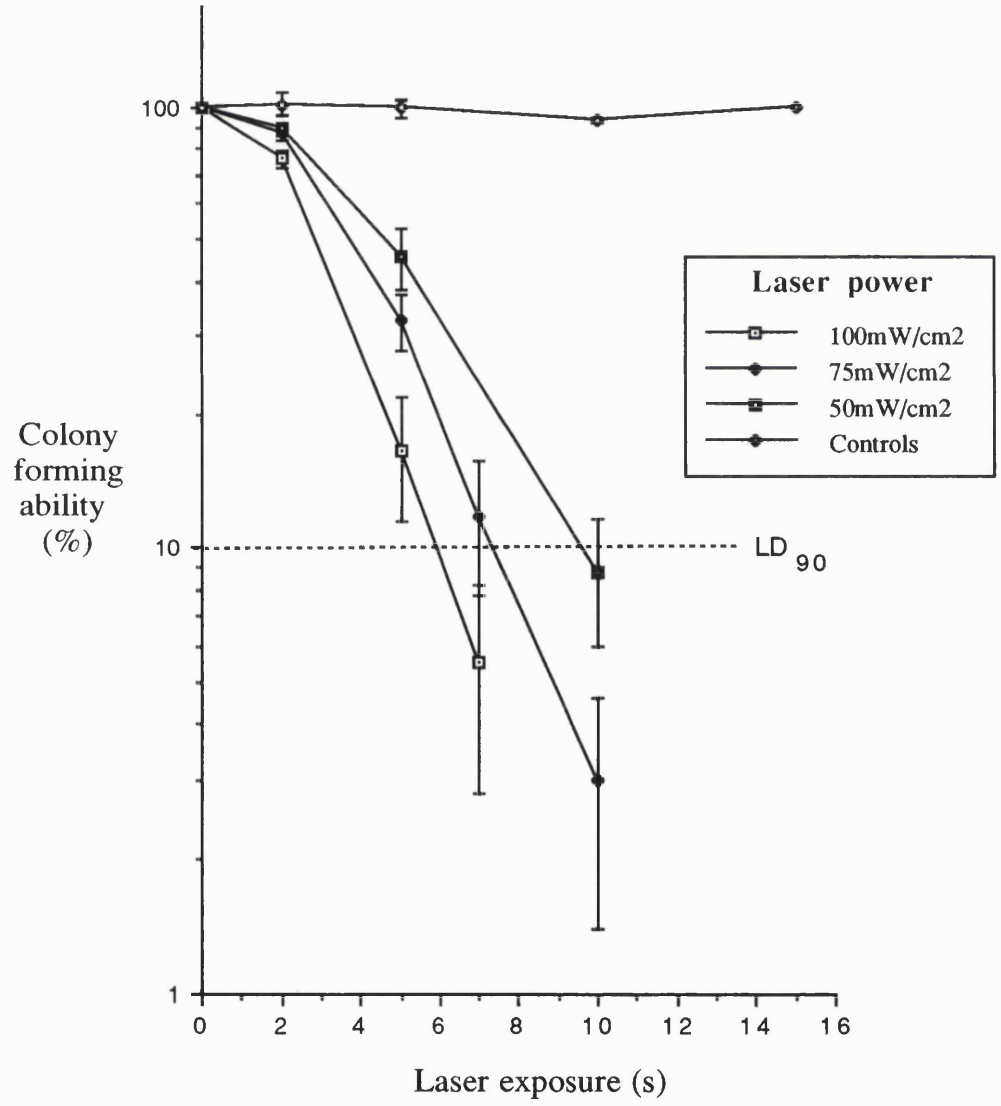


Fig. 5.6 Survival of cells sensitised with DHE (20 μg/ml for 1 h) exposed to laser light (504 nm) at varying power densities (controls - 100 mW/cm^2 laser light alone)

These values expressed in terms of the total light dose necessary to produce a LD_{90}, represent energy densities of 0.48, 0.55 and 0.6 J/cm^2 at 50, 75 and 100 mW/cm^2 respectively. When energy density is plotted against cell survival for each of the 3 laser powers studied the resultant survival curves are very similar (fig. 5.7). Allowing for the variation inherent in any biological system they can be taken as the same (all p values < 0.05). This indicates that cell survival is dependent on the total light dose (energy) and independent of the power at which this is delivered.

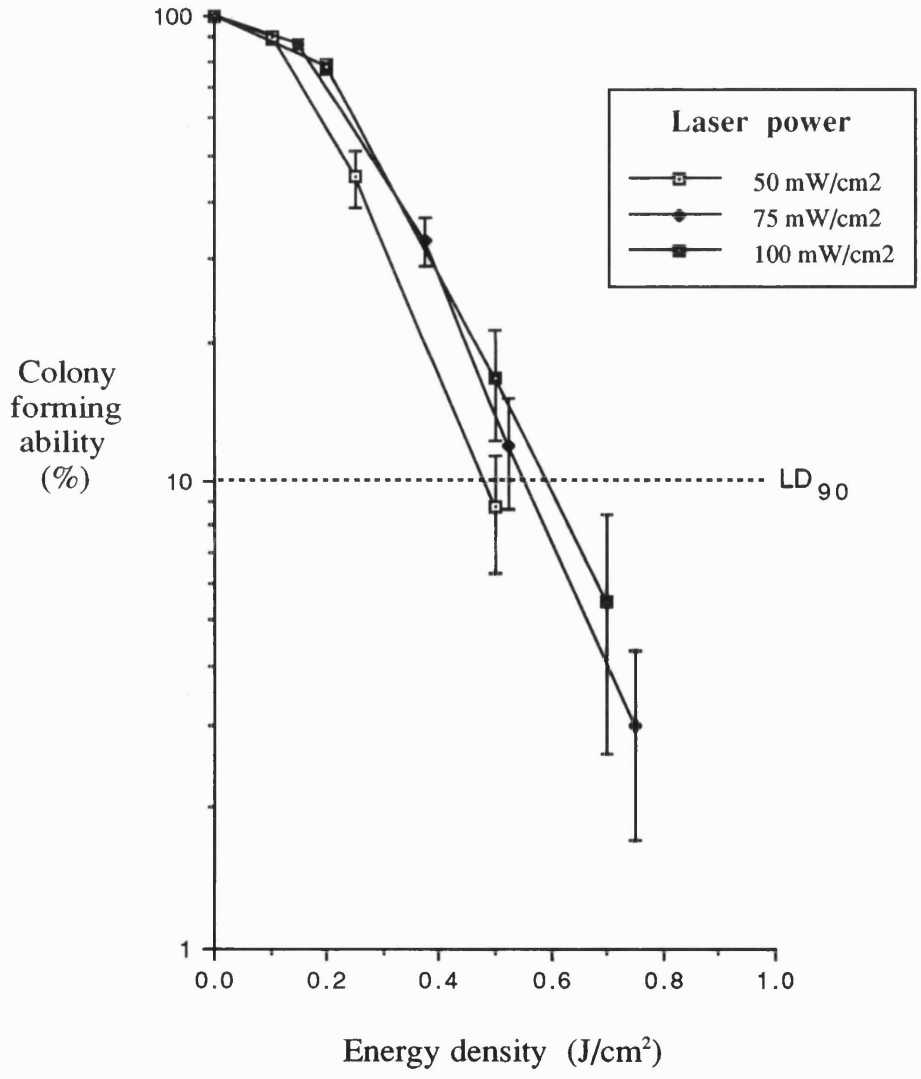


Fig. 5.7 Cell survival related to energy fluence
(same data as in fig. 5.6)

The initial shoulder to the above survival curves suggests that the sensitised cells are better able to withstand short exposures. Figure 5.8 illustrates this by plotting survival against laser power for exposures of 2, 5 and 10 s. The cell kill achieved with short exposures therefore, even at high powers, is less efficient than that seen after longer exposures. This graph does not compare points at the same delivered energy but rather indicates the likely extrapolation if a given energy was delivered in a short exposure time rather than in a long one.

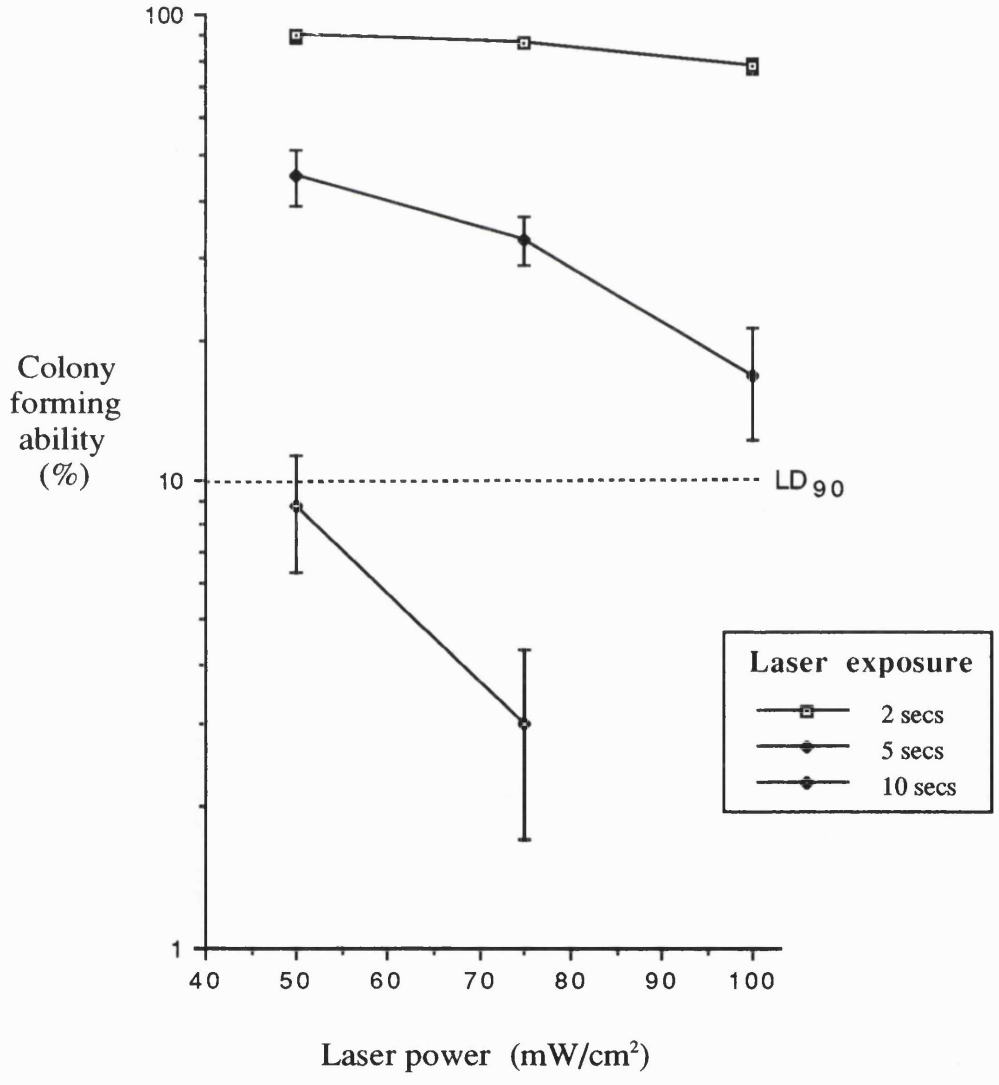


Fig. 5.8 Effect of the duration of light exposure time on cell survival
(MGH-U1 cells sensitised with DHE 20 µg/ml, 1 h)

5.3.2.3 The effect of a change in pulse frequency

All previous laser experiments have used a pulse frequency of 20 Hz. Comparative experiments were done reducing this to 5 Hz but maintaining the average power density at 50 mW/cm^2. The fourfold increase in pulse fluence this required was close to the maximum possible and unless the laser was in optimum alignment was difficult to achieve. Therefore only 2 instead of the normal 3 experiments could be satisfactorily completed so error bars are not given in fig. 5.9. The survival curve obtained is very similar though, to that achieved with the same average power delivered at 20 Hz (data from fig. 5.6) which is shown for comparison.

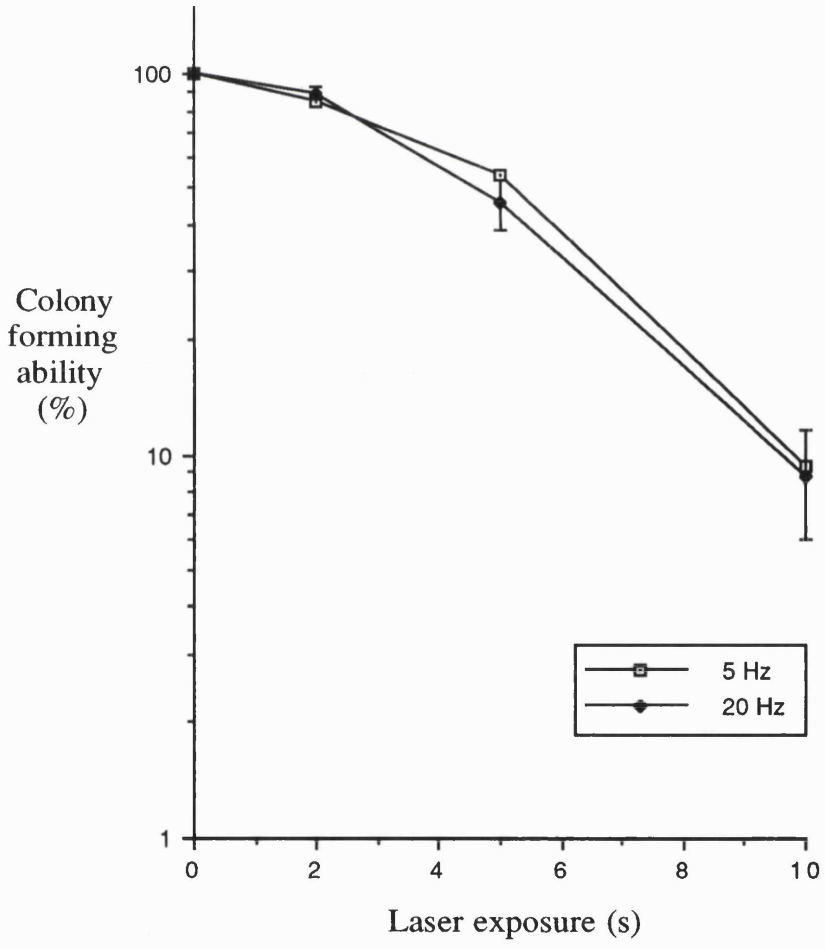


Fig. 5.9 The effect of varying the pulse repetition rate at an average power density of 50 mW/cm^2 (MGH-U1 cells, DHE 20 μg/ml)

5.4 DISCUSSION

It was reassuring to find that DHE alone, at concentrations up to 50 μg/ml, did not cause any direct toxicity to the MGH-U1 bladder tumour cell line used in these experiments (fig. 5.3), as some other workers have reported an effect. Chan *et al.* (1986) found a toxicity of 20% at 25 μg/ml, and 60% at 100 μg/ml using HpD on fibroblast cultures. Interestingly they found much less direct toxicity with AlSPc (< 10% at 100 μg/ml). The MGH-U1 cell line was used here as it had become the standard stock for other work in our Unit on testing chemotherapeutic agents for bladder cancer. It is a robust and quickly growing line and there is no reason to suppose that these results would have been different using any other bladder cancer cell line.

The high loss from contamination was a cause of concern and several factors are likely to contributed to this apart from this researcher's relative inexperience in cell culture techniques. There were a large number of procedures on each dish and as the sensitive cells had to be protected from light during these, the very low level indirect lighting used made it difficult to maintain the highest sterile standards. Controls showed that there was no unintentional phototoxicity but it may have been possible to manage with slightly brighter conditions. The extra experimentation that would have been involved to establish maximum safe levels of ambient illumination, however, was not thought worthwhile. Dishes also had to be transported outside to another building where the laser was housed, again increasing the risk of contamination and prolonging the time spent outside the incubator. In retrospect it might have been a good idea to routinely add antibiotics to the culture medium though this has not been the general practice in our Unit.

The decision to use a concentration of 20 μg/ml DHE and a 1 h incubation for all the subsequent work was based on the results of exposure to bright white light for 30 min which showed no surviving colony at concentrations above 15 μg DHE/ml (fig. 5.3). The 1 hour incubation time was both a convenient period and is also the conventional duration for which intravesical chemotherapeutic drugs are instilled in clinical practice. It was concluded that if the laser were to prove effective then 20 μg/ml DHE would be sufficient to achieve a complete cell kill whereas a higher concentration whilst not detrimental to cells kept in the dark would increase the risk of inadvertent damage during dish handling. This latter possibility was also protected against by the 2 sets of controls already described. Other workers have used varying concentrations of photosensitiser in their *in vitro* experiments; e.g. Camps *et al.* (1985) used 10 μg/ml HpD incubated for 2 h, Cowled *et al.* (1984) and Gomer *et al.* (1985) used 25 μg/ml HpD for 1 h, whilst Bellnier and Lin (1985) used 50 μg/ml DHE for 12 h.

This study demonstrates that the pulsed output at 504 nm from a flashlamp pumped dye laser whilst having no effect on unsensitised human bladder carcinoma cells *in vitro* (fig. 5.5) will rapidly kill sensitised cells (fig. 5.6). This is interesting as it has not been previously shown, but the important question is whether or not pulsed light might be a more effective activator of sensitised tissue than the continuous wave (CW) laser light already widely used for PDT which has been shown to be effective both *in vitro* and *in vivo*.

Pulsed lasers differ from CW lasers in that they emit light of very short pulses with extremely high peak power. There are two main types of pulsed lasers; metal vapour lasers (usually copper) emitting nanosecond pulses at high frequency (above 10 kHz), and flashlamp pumped dye lasers

used here, which emit longer pulses (1 - 2 μs) at low frequency (5 - 20 Hz) with peak powers in the kilowatt range. These laser types have been discussed in more detail in chapter 2.

Several workers have shown metal vapour lasers to have an equivalent effect *in vitro* to CW lasers (Cowled *et al.*, 1984). Evidence for the efficacy of flashlamp pumped dye lasers *in vitro* was not available prior to this study and their action *in vivo* is less certain. Bellnier *et al.* (1984) compared the effect of a CW argon-ion pumped dye laser on a mouse transplantable bladder tumour sensitised with HpD, to that of a similar flashlamp pumped dye laser to our own. Their dosimetry variables were rather different to ours in that they used very slow pulse rates (2 - 4 Hz) and high power densities (average 0.25 - 1 W/cm^2) to give pulse energies of 100 - 250 mJ/cm^2. Our pulse energies were much lower (2.5 - 5 mJ/cm^2) and even with a much quicker repetition rate we still used a rather lower overall power density (50 - 100 mW/cm^2). In some tumours they produced temperatures in excess of 50°C and although significant tumour regression occurred this was also seen in control animals and so they concluded that it was the result of thermal damage alone.

The prospect of producing a thermal (i.e. non PDT) effect from this laser was of concern as although the average power density was low there was the possibility of a transient thermal effect during each pulse and it should be remembered that this will be produced at *some* power level. Dish temperatures were not measured in our experiments both as sterility would have been further compromised and as short lived effects would have been very difficult to record anyway. The lack of an effect, though, in unsensitised cells when exposed to laser light at the highest power levels used in any experiment effectively excludes this (fig. 5.5).

Barr *et al.* (1989) did not demonstrate a PDT effect on rat colon with a similar laser to ours though they used a slower repetition rate (5 Hz) and a phthalocyanine photosensitiser (AlSPc). Although they used a similar average power to this work the tip of the laser fibre was in contact with the colon and photomechanical damage was seen in some animals. When the fibre was directed from a distance, no effect was seen. They used pulse energy densities of 25 mJ/cm^2 and suggested that this high energy level would excite almost all the sensitiser molecules to the photoactive triplet state very early in the pulse, with the result that most of the photons in the pulse are "wasted" as the sensitiser has become saturated due to depletion of the absorbing ground state molecules.

The cytotoxic singlet oxygen is generated from the interaction of oxygen with the triplet state of the sensitiser which, for both phthalocyanines and porphyrins, has a lifetime somewhat longer than the pulse length generated by the flashlamp pumped dye laser (triplet state lifetime in fibroblast cells *in vitro* approximately 8 μs; Truscott *et al.*, 1988). In theory therefore, only 1 excitation cycle can be initiated each pulse, i.e. in these experiments 20 cycles per second but only 5 cycles per second in the experiments described by Barr *et al.* (1989). They explain the failure to produce any PDT damage in their model by suggesting that this seemingly rather inefficient activation process may limit the overall yield of singlet oxygen to below the threshold required for a PDT effect. The decision to use a pulse frequency of 20 Hz was based on our analysis of saturation fluence under these experimental conditions and the inability of our pulsed dye laser to maintaining a reliable output at frequencies in excess of 20 Hz.

5.4.1 Saturation fluence

These restrictions however do not apply in the current experiments since it can be shown that the pulse fluences employed here (2.5 - 5 mJ/cm^2) are

below the saturation threshold of 10 mJ/cm^2 for DHE at 504 nm which is derived as follows (Lechnish *et al.*, 1976). At this excitation wavelength the absorption cross section of DHE is 4 x 10^{-17}cm^2 per molecule and from the saturation condition (absorption cross section x $n_s = 1$), the saturation photon fluence (n_s) = 2.5 x 10^{16} photons/cm^2 or equivalently 10 mJ/cm^2. This figure is an underestimate since the analysis assumes a triplet quantum yield of unity.

Therefore the pulse energies used in these experiments could be at least doubled before the photosensitiser became saturated. Although 20 Hz was the only frequency studied in detail some comparative experiments were done at 5 Hz. Here an average power density of 50 mW/cm^2 was employed resulting in a pulse fluence of 10 mJ/cm^2, the upper limit of the saturation threshold derived above. The survival curve obtained at 5 Hz was very similar to that seen at 20 Hz as would be expected if the above argument, that photosensitiser saturation would not occur at pulse fluences below about 10 mJ/cm^2, was true (fig. 5.9).

The calculations for the saturation threshold of AlSPc are different in that the absorption cross section of AlSPc at 675 nm is 6 x 10^{-16} cm^2 per molecule and the saturation photon fluence = 2.5 x 10^{16} photons/cm^2, corresponding to a pulse energy fluence of only 1 mJ/cm^2. Clearly the AlSPc would be saturated by the pulse energies of 25 mJ/cm^2 employed by Barr *et al.* (1989). Likewise the pulse energies of 100-250 mJ/cm^2 employed by Bellnier *et al.* (1984) are more than 10 fold in excess of that needed to saturate the HpD molecules. Clearly the repetition rate chosen is of prime importance if sensitiser saturation is to be avoided. In contrast, the nanosecond pulsed copper vapour laser producing an average power of 100 mW/cm^2 delivers a pulse fluence of only 10 μJ/cm^2 so there in no danger of saturating either porphyrin or phthalocyanine molecules.

5.4.2 Comparison with CW lasers

How then do the results obtained here compare to those that have been demonstrated by other workers on various cell lines with HpD or DHE? Camps *et al.* (1985) looking at the PDT effect of CW red light (630 nm) on prostate cancer cells sensitised with HpD, found an energy density in excess of 20 J/cm^2 at 100 mW/cm^2 reduced cell viability to 10% (LD_{90}). Cowled *et al.* (1984) using a higher power density, needed a similar energy density to achieve 50% cell kill with Raji cells, though a different assay method was used. Gomer *et al,* (1985; 1986) studying *in vitro* PDT with Chinese hamster ovary cells found, using red light, an LD_{90} of 0.25 J/cm^2 but when using relatively low level fluorescent illumination (0.35 mW/cm^2), lower energy densities were needed (LD_{90} = 0.05 J/cm^2). Bellnier and Lin (1985), using a similar cell line to ours and CW red light found an LD_{90} of 0.15 J/cm^2. Even these 5 studies show a 100 fold difference in energy densities for the same PDT effect, a variation attributable in part to different assay methods and light sources. We are not aware of any other study looking at the PDT effect of either CW or pulsed green light on a comparable cell line to ours so all one is able to conclude is that the low frequency pulsed green light used here produces an effect of the same order as that generally reported by these other authors using CW red light (LD_{90} = 0.54 J/cm^2). It might be expected that green light, which is absorbed more strongly by porphyrins than red light, may be more effective but this question could only be addressed if the same experimental set-up was used for each wavelength.

Camps *et al.* (1985) concluded that it was the length of light exposure needed to achieve cell kill which was important rather than the total light dose (energy density), so that cellular repair mechanisms for sublethal damage would be overcome. In contrast, Gomer *et al.* (1985), found no

significant dose-rate variation and felt that the cell killing was dependent on total light dose. The shouldered cell survival curves from our experiments (fig. 5.6) indicate the ability to repair sublethal damage though, like Gomer *et al.* (1985), we found that cell kill depended on the total light dose rather than the power density. Very short exposures which fall on the shoulder of the survival curve (e.g. 2 s), are unable to produce a high cell kill even at very high power densities as they presumably do not saturate cellular repair mechanisms. If the curves in fig. 5.8 are extrapolated it is clear that merely increasing the laser power for a 2 s exposure is not going to produce a worthwhile cytotoxic effect, whereas longer exposures which have moved away from the shoulder of the curve are more effective.

Care is needed when interpreting the results of *in vitro* PDT studies as they may not relate directly to clinical use. Oxygen, which is essential for photodynamic action, is present at higher saturation in cell culture than *in vivo*, possibly enhancing the effect. As has been discussed previously in chapter 4, several *in vivo* studies have shown that a major part of the PDT effect results from damage to tumour blood vessels (Selman *et al.*, 1985b; Star *et al.*, 1986; Fingar *et al.*, 1988), whereas *in vitro* damage clearly can only result from direct toxicity (Shulok *et al.*, 1986). Nevertheless the mechanism of action is probably the same.

5.4.3 Conclusions

These experiments demonstrate that the flashlamp dye laser emitting pulsed green light has a powerful PDT effect on human bladder carcinoma cells *in vitro*, sensitised with DHE. Survival curves indicate the ability to repair sublethal damage after exposures up to about 4 s, but with longer ones a greater cell kill can be achieved. The degree of cell killing achieved is related to the total light energy delivered rather than the power employed. For the variables tested here an LD_{90} was achieved at a mean energy

density of 0.54 J/cm^2 (range 0.48 - 0.6 J/cm^2). Comparison with other studies using CW red light is difficult owing to differences in cell lines and methodology, but it would seem that this laser is at least as effective for PDT as the CW argon laser. Other workers who have found a flashlamp pumped dye laser ineffective for PDT have used pulse fluences in excess of the saturation threshold of their photosensitiser which is estimated for DHE to be 10 mJ/cm^2 per pulse. The saturation threshold for AlSPc is only 1 mJ/cm^2 so it is unlikely that this laser would be effective with this drug. For those urology units which already possess a flashlamp pumped dye laser, PDT may provide a second use for this instrument though further studies will be required to confirm these results *in vivo*. We have been unable to carry out such studies, as our flashlamp dye laser is a clinical instrument in an area where animal work is not permitted.

The initial question posed as to whether or not pulsed light might be *more* effective than CW light for PDT cannot be answered by these results. It is unlikely that 20 Hz is the optimum repetition rate to use, merely one dictated by the characteristics of the available laser. A somewhat faster rate (say around 100 Hz) would avoid any chance of photosensitiser saturation even at high average powers, yet still retain the very high peak power that distinguishes pulsed from CW light and is the quality most likely to produce a superior PDT effect in terms, for instance, of swamping cellular repair mechanisms. It is unlikely that the flashlamp pumped laser could achieve these rates but some other types of lasers (excimer and KTP pumped dye lasers) are able to, though our Unit does not have access to one. The use of green light to reduce the depth of penetration in the bladder for PDT with porphyrins has also received little attention but this is an avenue that should be further explored if the aim of efficient PDT of Cis without the troublesome side effects which have been encountered with the use of CW red light is to be achieved.

Chapter 6

UPTAKE AND MICROSCOPIC DISTRIBUTION OF ALUMINIUM SULPHONATED PHTHALOCYANINE IN NORMAL RAT BLADDER

6.1 INTRODUCTION

The search for better photosensitising drugs than HpD and DHE has led to the development of several new classes of compounds as outlined in section 4.2.1. The phthalocyanines, and in particular the aluminium sulphonated derivatives, have aroused considerable interest and data on their use as effective experimental photosensitisers for PDT are rapidly accumulating (Tralau *et al.*, 1987). As was discussed in chapter 4 the phthalocyanines have several advantages over HpD or DHE in terms of chemical purity and stability, and a major absorption peak at 675 nm at which wavelength there is deeper tissue penetration than at the 630 nm used for HpD and DHE.

Although in almost every other likely application of PDT a deep tissue effect is desirable this is not the case when treating superficial bladder cancer. The significant complications seen clinically with HpD, including bladder irritability and a greatly reduced bladder capacity, seem to be the result of unwanted photosensitiser activation and subsequent fibrosis in the deep muscle layers of the bladder wall (Nseyo *et al.*, 1987; Harty *et al.*, 1989). It was suggested in chapter 5 that the only way of limiting damage to the muscle layers, apart from using a less penetrating wavelength of light which is of course not possible with the phthalocyanines, would be to choose a drug dose such that concentration in the muscle was below the threshold level needed for tissue necrosis at the time of illumination whilst that in areas of tumour was above it. All photosensitising agents, AlSPc included, are relatively poorly selective for neoplastic tissue when compared with the adjacent normal tissue in which the neoplasm arose. As the problems with PDT usually relate to normal tissue damage it is appropriate and quite adequate to address this problem first by measuring photosensitiser distribution in normal tissue.

The normal way to measure the tissue concentration of a photosensitiser is by means of a chemical extraction assay. This technique cannot provide information on the microscopic distribution of the drug within the bladder wall, which in this case is the most important factor. Standard fluorescence microscopy does not have the sensitivity required to accurately record very low levels of photosensitiser fluorescence as the ultraviolet excitation commonly used will lead to image distortion due to natural background tissue auto-fluorescence and sensitiser photodegradation.

Our Unit has developed a CCD (charge coupled device) imaging system (Barr *et al.*, 1988) which is connected to a standard fluorescence microscope. This system produces high quality colour images which can be digitally analysed to determine relative photosensitiser fluorescence intensities in various parts of the tissue section. Fluorescence of AlSPc is only seen from photoactive components, the non-active aggregates do not fluoresce. This means that the technique is a sensitive indicator of the biologically important fraction of the drug unlike extraction assays which would measure all forms of the photosensitiser.

The work presented in this chapter studies the microscopic distribution of AlSPc in sections of normal rat bladder at different drug dosages and time intervals after sensitisation. The standard method of assessing photo-sensitiser concentration in tissue is by a gross tissue extraction assay. Whilst this type of assay cannot give information about the microscopic distribution of photosensitiser it does allow assessment of general uptake and elimination, and the relationship between plasma and tissue drug levels. Such an assay was performed, therefore, as it was expected that some useful comparisons may be drawn between this method of assessing sensitiser distribution and the fluorescence studies.

All published studies of PDT in the bladder have used intravenous photosensitisation so although this route was predominantly employed here, the absorption of AlSPc after its intravesical instillation has also been assessed. Photobleaching, which is the irreversible degradation of photosensitiser molecules by the activating light itself, is a potentially useful therapeutic phenomenon (section 4.3.3) but has not been demonstrated before at a microscopic level in tissue. Therefore we have investigated if this CCD imaging system could image photobleaching *in vivo*. The information from such studies is required to predict the optimum treatment parameters for clinical PDT in the bladder.

6.2 MATERIALS AND METHODS

6.2.1 Photosensitiser

Aluminium sulphonated phthalocyanine (AlSPc) was obtained from Ciba-Geigy as a sterile powder which was reconstituted in 0.9 % saline to make a stock solution of 2 mg/ml and sterilised by passing through a millipore filter. This solution was kept out of direct light but no other special precautions were needed and it would remain chemically stable for many months though its chemical composition was periodically checked by chromatography (departmental photochemist) to ensure consistency. The stock solution was diluted as required in saline just before use.

This preparation of AlSPc is a consistent water-soluble mixture containing a mean of 3 sulphonate groups per molecule (range 2 - 4), and has been the drug used for most of the experiments described in this thesis (see section 4.2.4). With increasing study of AlSPc, though, it has been decided that the more lipid soluble disulphonated derivative (AlPcS2) is to be the

preparation that will be developed and tested for regulatory approval prior to clinical use. This compound (purified in Professor D. Phillip's laboratory, Department of Chemistry, Imperial College, London) was used in some of the experiments on intravesical uptake of the photosensitiser and also for the chemical extraction assays.

6.2.2 Rat bladder model

Normal bladder specimens were obtained from adult female Wistar rats (weight approximately 200 g). The rat was chosen as having the largest bladder available on an economic scale and was readily available and easy to work with. Ideally a large mammal with a bladder more similar to Man would have been used but this was not practicable considering that large numbers would be required. The rat bladder although much thinner (0.6 mm) than the human organ has the same basic structure of a transitional-cell epithelium, lamina propria and muscle layer.

The rats were housed in standard conditions and in particular were not kept in a darkened room which would have been necessary to prevent any side-effect due to cutaneous photosensitivity had a porphyrin photosensitiser been studied. There was no restriction or modification to their diet (a commercial belittled feed) and they had free access to water. All procedures except the weighing of animals were done under a general anaesthetic, administered as described below. No antibiotic was used.

6.2.2.1 Anaesthesia

The requirements for a general anaesthetic for this work was that it should be easy to administer and maintain, and be sufficiently profound and long lasting to enable a range of procedures, including laparotomy, of up to 60-90 min duration to be performed. Good muscle relaxation and adequate post-operative analgesia were also required. It was decided to use a

parenterally administered anaesthetic rather than inhalational agents on the advice and experience of other workers in our Unit and therefore two standardised regimes were adopted.

For a short-acting restraint alone to enable intravenous injection of photosensitiser, Hypnorm (fentanyl and fluanisone; Jansen Pharmaceuticals) was given intramuscularly at a dose of 0.3 ml/kg. For all the other procedures, such as the intravesical sensitisation described later in this section and the work in chapters 7 and 8, which required a somewhat longer period of anaesthesia, a larger dose of Hypnorm (0.5 ml/kg) was combined with diazepam (0.5 mg/kg). This combination gave a good degree of muscle relaxation and adequate postoperative analgesia.

The onset of anaesthesia would occur within 5 - 8 min of injection. The unconscious animal was placed supine on a cork board and its paws gently restrained by securing them with adhesive tape. The airway was kept open by the natural neck extension that occurred in the supine posture and no problem with airway obstruction was encountered. Evaluation of the depth and duration of surgical anaesthesia obtained by this technique, which is necessary when measuring bladder function, is reported in chapter 8. A few of those animals receiving a 60 min installation began to lighten, as judged by any response to a sharp noise, towards the end of this period and received a further half of the original anaesthetic dose.

6.2.2.2 Intravenous photosensitisation

The AlSPc stock solution was diluted as required in saline to maintain a convenient volume of 0.25 - 0.75 ml and administered by tail vein injection using a 1 ml syringe and 25 gauge needle in animals anaesthetised as described above. The tail was cleaned under warm running water prior to injection which also made the veins more prominent. The injection site chosen was usually quite distal where the tail was thinner and the veins

more visible on its lateral aspects. The blue colour of the AlSPc made it easy to detect any extravasation immediately and if this did occur it was usually right at the beginning of the injection. A further attempt would be made but, rarely, if this also failed the animal was discarded from that experiment.

Animals were divided into 2 treatment groups (12 rats per group) and 1 control group (3 animals). Each treatment group received a different dose of AlSPc (either 0.5 or 5 mg/kg). These doses were chosen as being either extreme of the dose range used for the studies of bladder morphology and function after PDT which will be described in chapters 7 and 8. Controls received 0.5 ml saline. Four animals were sacrificed, by carbon dioxide asphyxia, from each group and 1 from the control group at times following sensitisation of 1, 24 and 72 h and their bladders processed as described in section 6.2.3.

6.2.2.3 Intravesical photosensitisation

The direct absorption into the bladder wall of a solution of AlSPc administered intravesically for either 30 min or 60 min (the usual duration for clinical intravesical chemotherapy) was studied. The anaesthetised animals were gently secured supine on a cork board and the bladder was catheterised and first emptied of urine before 0.3 ml of the sensitiser solution was instilled.

Catheterisation was performed with a 18 gauge Teflon IV cannula passed over a nylon "guidewire" and the bladder was gently emptied of any urine present. It was found necessary to use a guidewire (a length of no.1 nylon suture) as the urethral meatus opens into a short (3 mm) vestibule and is not easily visible externally. Gentle probing with the guidewire whilst maintaining counter traction on the adjacent skin with fine forceps was the surest way of catheterising the urethra without trauma (see fig. 8.3). With

the guide in the bladder the lubricated cannula was easily introduced over it using a screwing motion and the 18 G size proved a snug fit in the urethra which prevented leakage around it during the experiment.

A short lower midline abdominal incision was made to visually check both that the bladder had been completely emptied of urine before instilling the photosensitiser and that no air bubble had been introduced (this was easy to ascertain through the translucent bladder wall). Direct vision was also useful to avoid any trauma to the bladder wall from the catheter as sensitiser might preferentially adhere to damaged mucosa. The catheter was withdrawn into the urethra and left in place with a bung inserted and the incision was then taped shut. After the chosen instillation period the catheter was advanced a little and the sensitiser solution drained out. The bladder was then gently washed out 3 times with saline to remove any surface dye, before the animal was killed and the bladder processed.

Animals were divided into 2 groups (either AlSPc mixture or AlPcS2), each of 12 rats plus 2 controls. Three concentrations of photosensitiser solution were used in each group (2 animals each) for both 30 and 60 min; in fact an equimolar dose of AlPcS2 was given as the S2 is a slightly smaller molecule - molecular weights of S2 = 770, mixture = 881. These corresponded to 1%, 5% and 20% dilutions of the 2 mg/ml stock AlSPc solution, i.e. 0.02, 0.1 and 0.4 mg/ml AlSPc. No allowance was made for the very small dilution of the photosensitiser solution that would have occurred due to urine production during the test period (about 0.08 ml/h).

6.2.3 Preparation of specimens for fluorescence scanning

After sacrifice the bladder was exposed via a lower abdominal incision (or the existing incision was just un-taped in the intravesical groups) and dissected free from its apex to the bladder neck removing any perivesical

connective tissue. The bladder was then catheterised so that any urine which might contain photosensitiser could be gently washed out with 3 changes of saline and then inspected to ensure that it was empty. It was then minimally distended, just enough to smooth out mucosal folds, with the 0.3 ml of OCT medium (Tissue-Tek, Miles Laboratories Inc.). This was recommended to provide support to the delicate bladder wall during tissue sectioning. Identification of the urethra was facilitated by insertion of a short length of size 1 nylon suture through the cannula prior to its removal. The urethra was then mobilised until sufficient length was obtained to clip across it and the bladder removed and placed in isopentane (2-methyl butane) which had been cooled in liquid nitrogen until viscous.

Specimens froze immediately in this solution and were then stored in a liquid nitrogen tank until required. Duplicate 10 μm transverse frozen sections were cut at 4 levels from each intact bladder using a Cryocat E microtome (Reichert Ltd) and mounted on 2 glass slides. One slide was kept at -70°C until used for the fluorescence studies and the other fixed and stained with haematoxylin and eosin (H & E) to enable subsequent orientation of the fluorescence image.

6.2.4 Fluorescence detection

An inverted microscope (Olympus IMT-2) with epifluorescence and phase contrast attachments was used, with excitation light, provided by an 8 mW helium-neon laser emitting at 632.8 nm (fig. 6.1). A liquid light guide was used to direct the laser output via a 10 nm band pass filter (Omega Optical Inc., Vermont, USA), centred at 633 nm to remove any extraneous light, into the dichroic mirror housing for standard epifluorescence excitation. The principal advantage of using a helium-neon laser, apart from its spectral purity, is that tissue auto-fluorescence is significantly reduced for

this relatively long excitation wavelength compared to that seen with conventional UV lamp illumination.

Microscope slides on which the frozen tissue sections were mounted in wells were allowed to defrost at room temperature immediately prior to imaging and were placed face down on the specimen stage. Specimens were centred and focussed under phase contrast illumination, with the room otherwise darkened, before the 633 nm laser excitation was switched in. The resulting phthalocyanine fluorescence was detected using a long pass filter (Schott RG665) and a band pass filter (Omega Optical, Inc.) which transmitted in the range 665 - 700 nm and covered the main fluorescence band of these sensitisers (Spikes, 1986). The fluorescence image was captured by a cooled charge-coupled device (CCD) camera (Wright Instruments, model 1, resolution 600 x 400 pixels, fig. 6.2) connected to the microscope. An IBM PC controlled the camera exposures and was used for digital image processing, storage and display.

The advantages of using a cooled slow-scan CCD camera over video imaging systems include much higher sensitivity, direct digital image integration and a linear response over a signal range of 10^4 in magnitude. The cooling (by liquid nitrogen) largely eliminates "noise" and the high sensitivity allows low power excitation and short integration times (in the order of 10 s) which prevent the occurrence of sensitiser bleaching that may distort the image with longer times. Tissue auto-fluorescence from control sections amounted to only 1 - 2 counts on an image scale of 10^3.

The program software enabled fluorescence intensity to be digitally quantified either by box superimposition over several representative areas of the tissue section (usually 3 were chosen), or by line integration across the full thickness of the appropriate tissue layer (figs. 6.3).

Data and fluorescence images could be stored for further analysis though as this required up to 1 megabyte of disk space for each image they were only retained temporarily. Permanent copies of the fluorescence images were made, including all the illustrations in this chapter, by photographing them directly from the screen of the high resolution colour monitor using a single lens reflex camera (Olympus OM-2).

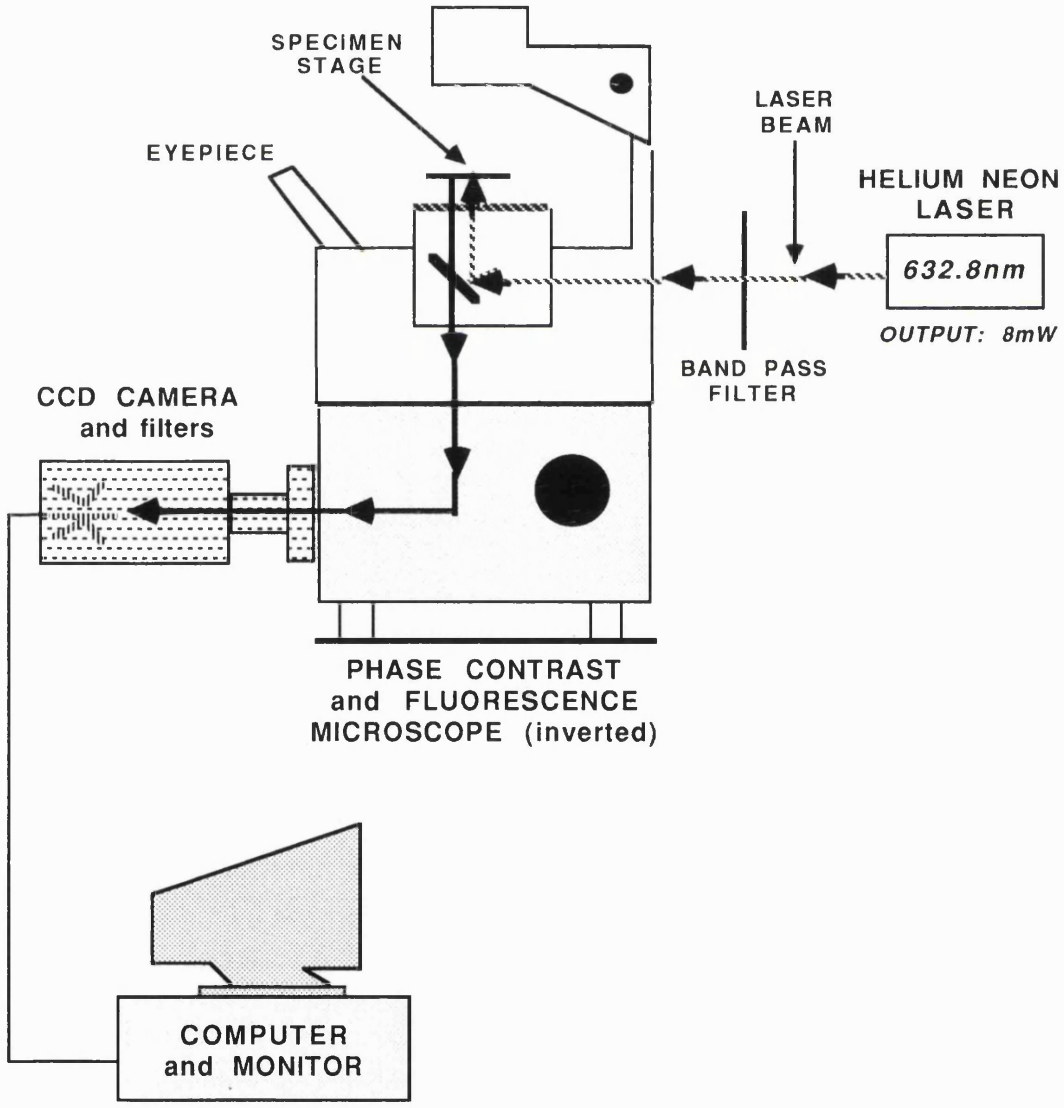


Fig. 6.1 Fluorescence imaging system

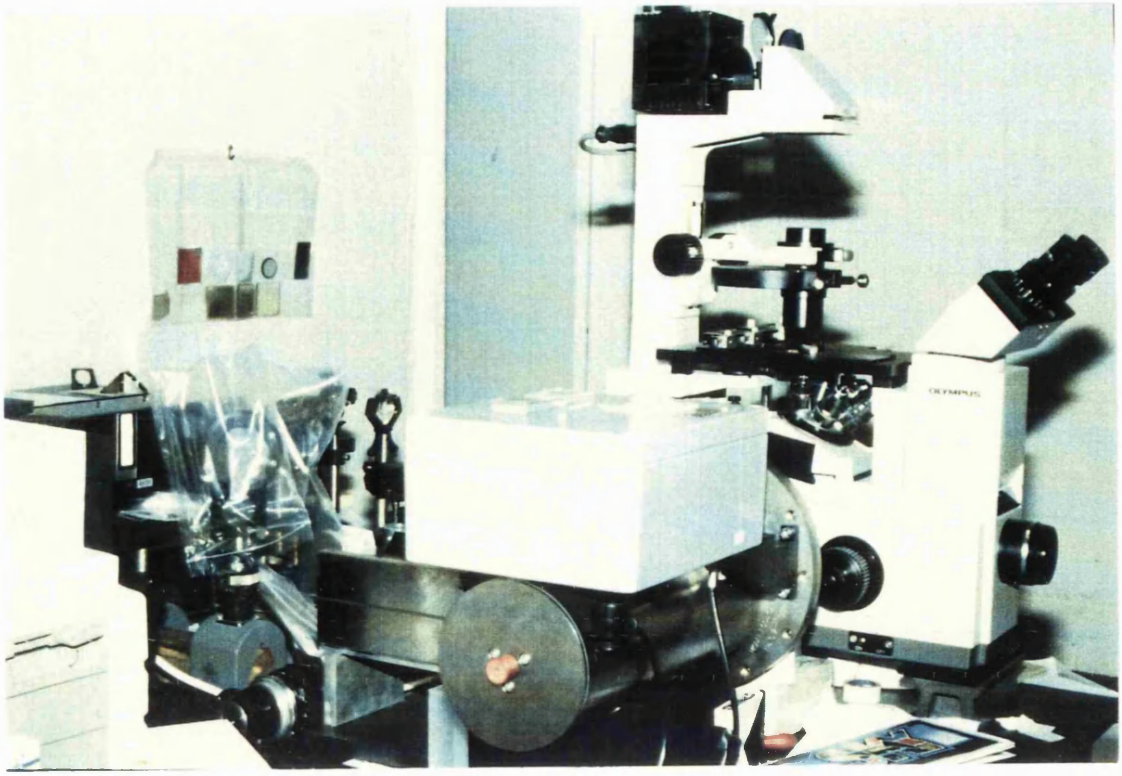
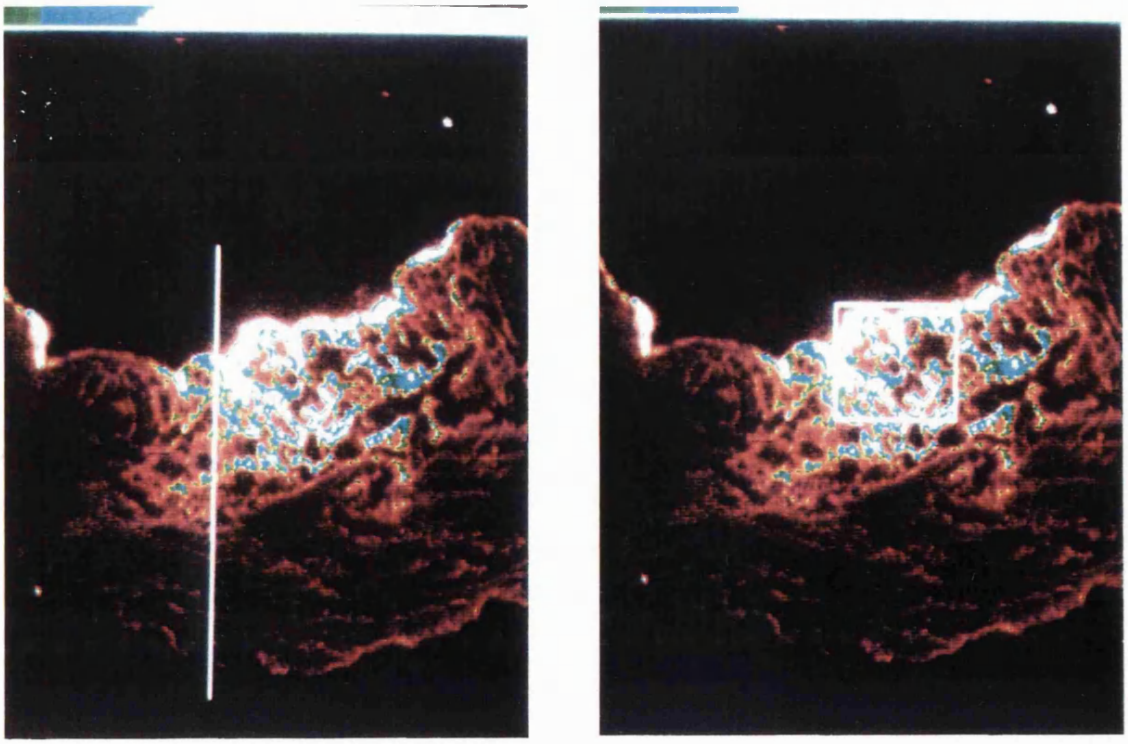


Fig. 6.2 CCD camera attached to the fluorescence microscope



Figs. 6.3 Line or box integration for fluorescence measurement

6.2.5 Light exposure for photobleaching studies

Photobleaching refers to the destruction of the photosensitiser molecules by the illuminating light itself and may have a therapeutic benefit in some situations (section 4.3.3). It would be expected that this phenomenon may be demonstrated by a reduction in the fluorescence observed from tissue following PDT treatment.

Animals were divided into 2 treatment groups (6 rats in each group) plus 2 control groups (2 rats). Both the treatment and control groups received either 0.5 or 5 mg/kg AlSPc by tail vein injection. After 24 h their bladders (not controls) were illuminated *in vivo* using the same technique employed for the main part of the work in this thesis, which is described in detail in chapter 7. Briefly, red light (675 nm) from a copper vapour pumped dye laser was transmitted via a 200 μm laser fibre introduced per-urethrally, within a diffusing catheter, and positioned centrally in the bladder of anaesthetised animals. The bladder was filled with 0.3 ml saline. The interval of 24 h between sensitisation and light treatment was chosen purely as a convenient time when the initial fluorescence studies had shown a favourable distribution of photosensitiser (and for consistency with the main experiments described in chapters 7 and 8). It seems unlikely, though, that this time is critical within the period 24 - 72 h.

An average laser power output of 100 mW was employed which was below the level at which any thermal effect might be expected (see section 7.2.1). The treatment time was adjusted to give 3 animals in each treatment group a calculated incident light dose to the whole bladder of either 20 or 80 J/cm^2, the latter corresponding to a treatment time of 2000 s. The bladder was exposed via a lower abdominal incision during light exposure to facilitate optimum positioning of the cannula. The output from the fibre

was also checked after each exposure, and at intervals during the longer exposures, though it rarely required adjustment.

Animals were sacrificed immediately following light treatment and their bladder removed and processed for fluorescence scanning as described above. The effect of a cessation of blood flow (to reduce the oxygen available to the tissue) on the photobleaching of sensitiser fluorescence was also studied in a separate group of 4 animals who had received the higher dose of AlSPc (5 mg/kg) and were killed (by intracardiac injection of barbiturates) just before the start of light exposure (either 40 or 80 J/cm^2).

6.2.6 Pharmacokinetic studies by chemical extraction

This last part of the study was set up at the time when the S2 preparation was just becoming available so therefore this compound was used.

The rats were divided into 8 groups of 5 each plus 5 controls. Treatment animals received 1 mg/kg AlPcS2 by tail vein injection, control animals saline. This dose was chosen as the minimum (as AlPcS2 was in short supply) likely to produce adequate tissue levels for assay based on the experience of colleagues studying distribution in the rat colon. One group of rats was killed at each interval of 1, 3, 6, 12, 24, 48, 72 h and 1 week after sensitisation. Blood (2-3 ml) was collected by cardiac aspiration, placed in heparinised tubes, spun down and the plasma separated before freezing. The whole bladder was removed intact immediately after death and any extravesical tissue trimmed off. It was then opened and gently washed in saline to remove any urine prior to freezing. A kidney was also removed as example of a solid organ with a major vascular supply and also frozen until required. The concentration of AlPcS2 in plasma, the whole bladder and in renal parenchyma was later measured by an alkali extraction assay, details of which may be found in appendix 2.2.

6.3 RESULTS

Observations on the microscopic distribution of AlSPc fluorescence within the wall of the normal rat bladder after both intravenous and intravesical sensitisation will be presented first, followed by the demonstration of the phenomenon of photobleaching *in vivo*. Finally the results of the chemical extraction assay of photosensitiser from whole bladders will be given.

Fluorescence images are shown from representative sections of normal rat bladder together with the corresponding H & E stained sections, where appropriate, to aid orientation. The false colour bar shown along the top of these images indicates the level of fluorescence intensity with dark red being the weakest and white the strongest. The numerical values attached are linear units but not absolute values as they depend largely on the exposure time chosen for the CCD camera. This was selected, as with any photograph, to give a good density negative for reproduction but in general it proved possible to keep the same exposure time (10 s) for sections between which intensity measurements are compared in this section. The intensity value does allow a direct comparison between fluorescence in various areas of a tissue section and between different sections. The mean values given below are derived from computer averaging within boxes drawn over representative full thickness areas of the section (fig. 6.3). Three values were obtained from each section, 4 sections were examined from each bladder specimen and 4 specimens were obtained at each time point studied. The fluorescence intensity so derived is still open to some subjective variability between specimens and this should be remembered when drawing conclusions from these results.

Control sections from unsensitised animals showed negligible auto-fluorescence, less than that from the weakest areas of any of the sensitised

sections, so are not illustrated here. Any visible fluorescence thus seen is entirely due to the administered phthalocyanine and indicates the concentration of photoactive drug within various areas of the tissue section.

6.3.1 Microscopic tissue distribution of AlSPc

6.3.1.1 Intravenous administration

At 1 h fluorescence was seen in all layers of the bladder wall particularly in the well vascularised submucosa (fig. 6.4). As might be expected shortly after intravenous injection a high level of fluorescence was seen in vascular endothelium (figs. 6.5). By 1 h there was approximately twice the level of fluorescence activity in the superficial layers than that in the muscle layers and by 24 h after sensitisation a marked gradient of between 3:1 and 4:1 had developed (figs. 6.6, 6.8). This differential was maintained to at least 72 h (the last observation interval) though the absolute fluorescence values reduced by approximately 10 - 20% over this time period (figs. 6.7, 6.8).

These data are presented in appendix 2.1 and summarised in fig. 6.8. Fluorescence readings from bladder wall muscle at 24 h following sensitisation with 5 mg/kg AlSPc were only 38±12% (mean ± S.D.) of their value at 1 h compared with a much higher relative level of 75±6% from the superficial layers. At 72 h after sensitisation these values were reduced to 30±10% from the muscle layer and 68±8% from the superficial layers. This demonstrates there to be a ratio of fluorescence intensity (i.e. a concentration gradient of AlSPc) between superficial and deep layers of the bladder, of 2:1 at 1 h, 4:1 at 24 h and 4.6:1 at 72 h. The values obtained after a much lower dose of sensitiser (0.5 mg/kg AlSPc) showed a similar pattern of uptake with overall fluorescence intensities ranging approximately between 8 - 12% of the corresponding values seen following 5 mg/kg AlSPc (fig. 6.8).

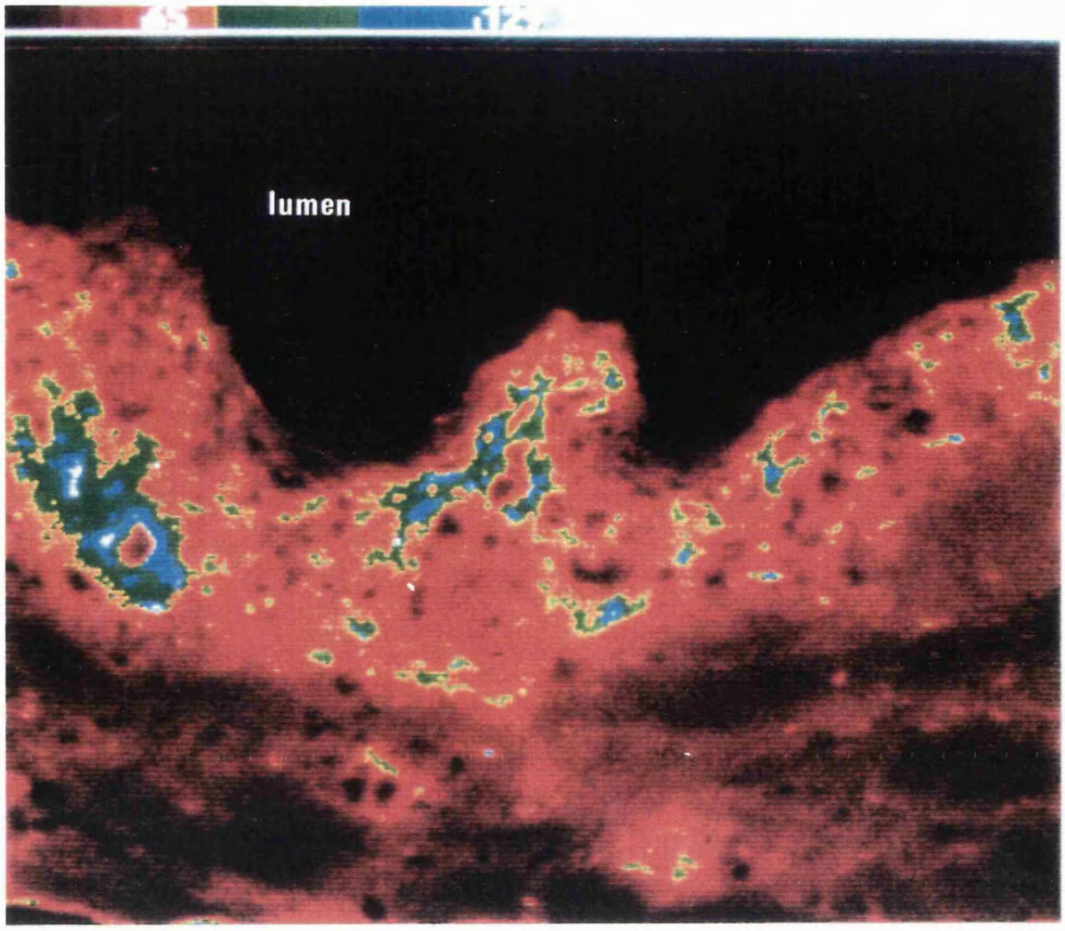
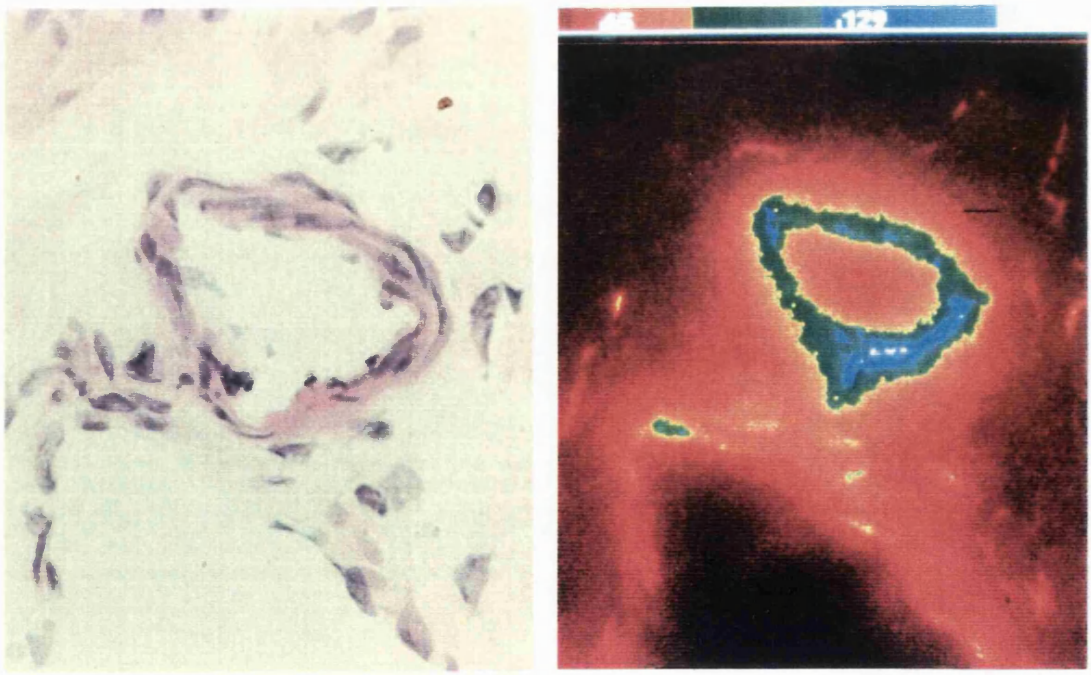
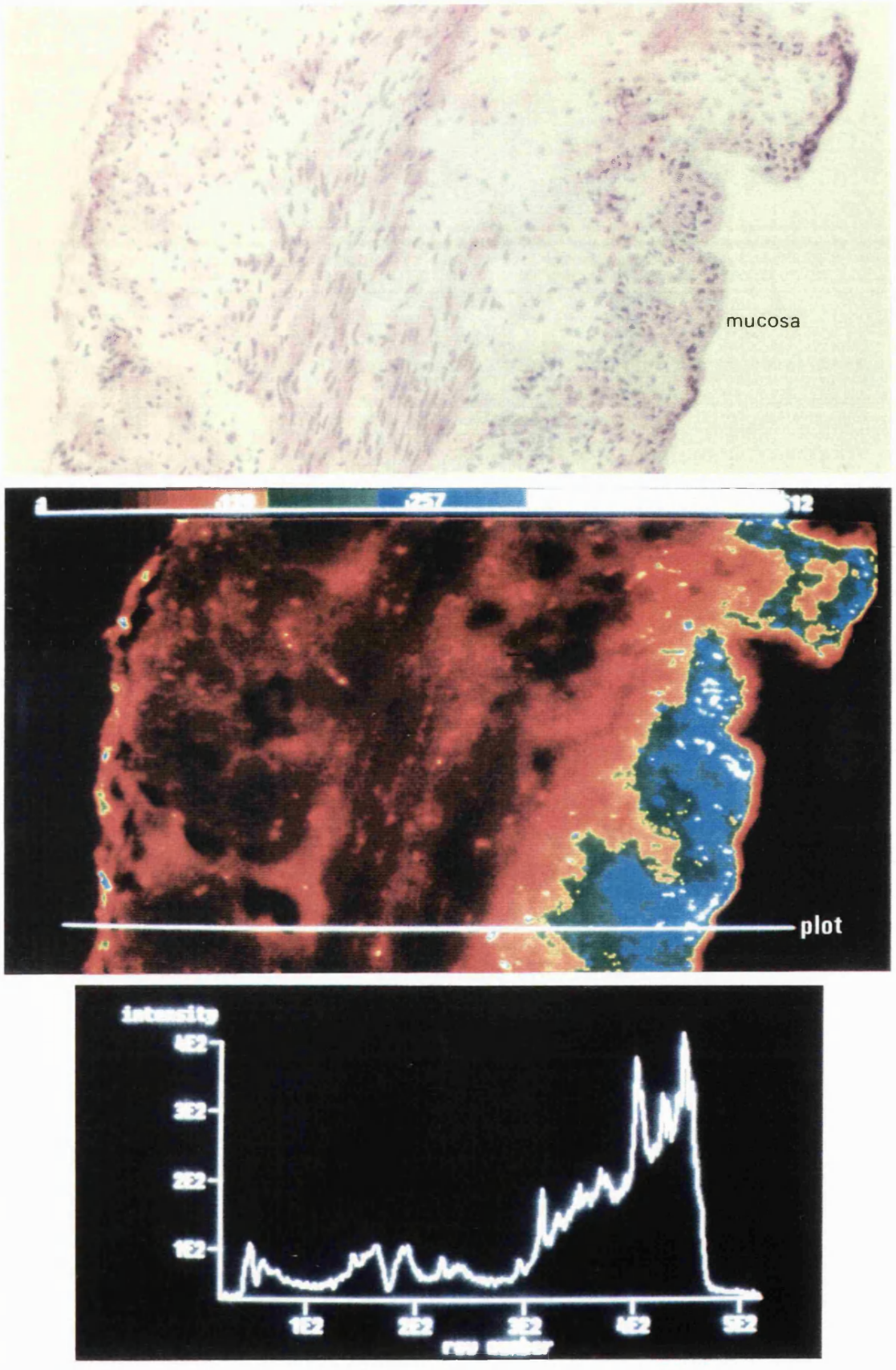


Fig. 6.4 Fluorescence image 1 h after sensitisation with AlSPc (0.5 mg/kg)

The false colour scale indicates fluorescence intensity which is high in well vascularised areas such as the submucosa.

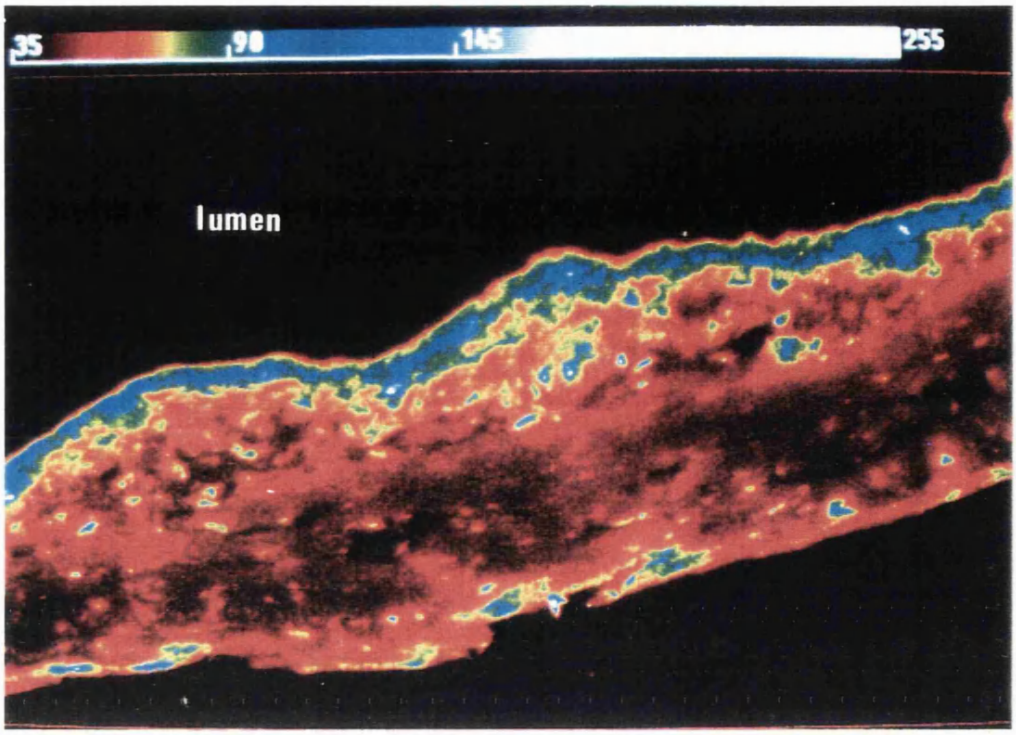


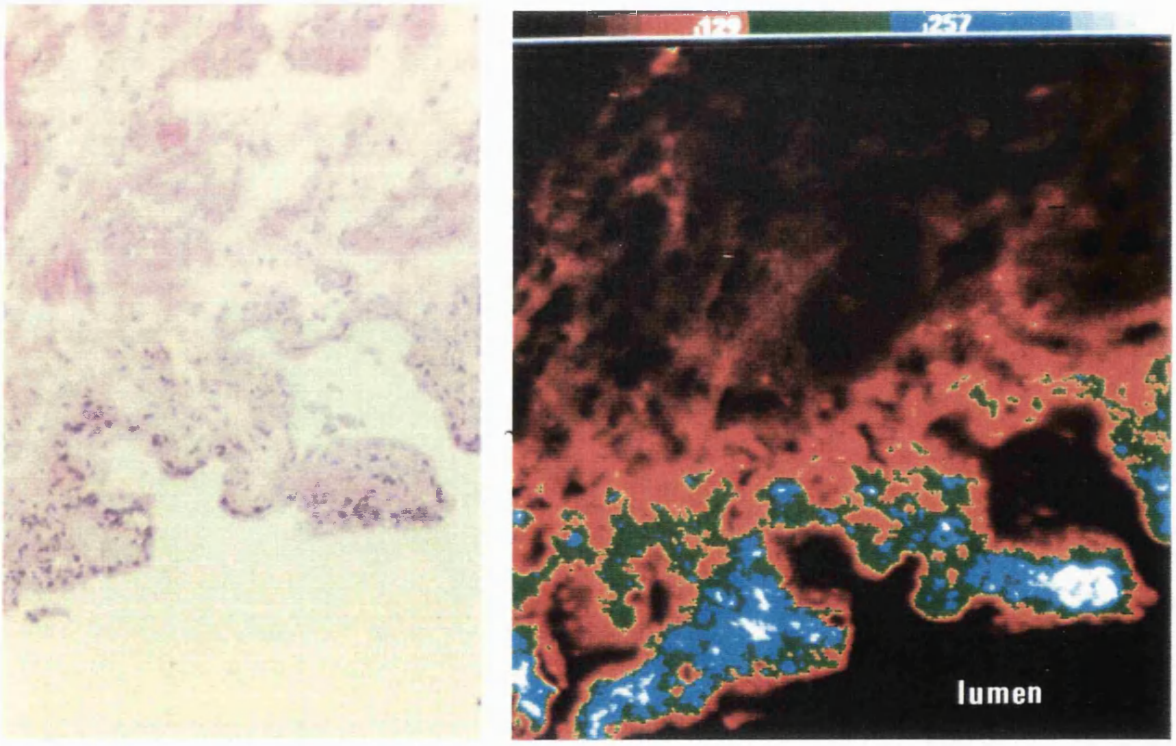
Figs. 6.5 Strong fluorescence at 1 h in vascular endothelium



Figs. 6.6 24 h after sensitisation with 5 mg/kg AlSPc

H & E stained section is shown for orientation. A ratio of 3.5 -4:1 in fluorescence intensity now exists between the superficial layers (mucosa and lamina propria) and the deeper (muscle) layers of the bladder wall as illustrated in the plot taken along the white line.





Figs. 6.7 Fluorescence at 72 h after sensitisation with 5 mg/kg AlSPc

Results are shown with both AlPcS2 (above) and AlSPc mixture (below). The gradient between the superficial and deeper layers is similar to that seen at 24 h though the level of fluorescence intensity is lower

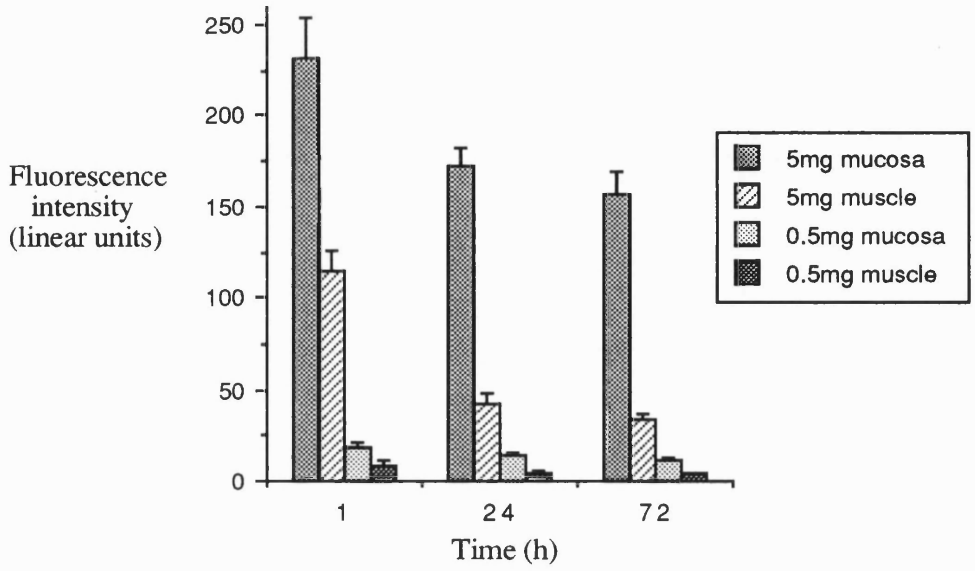


Fig. 6.8 Fluorescence of AlSPc in the bladder after IV sensitisation with either 5 or 0.5 mg/kg AlSPc (mean values ± 1 SD)

It is probably not appropriate to subject this data to statistical analysis of significance using student's t test as the assumption of normality cannot be made from subjective observations such as fluorescence. Despite this limitation the means and SD's are shown in appendix 2.1 which indicate that the reduction in fluorescence between 1 and 24 h seen in all 4 groups, but not between 24 and 72 h, and between readings from the superficial layers compared to the muscle coat at all points was significant ($p<0.05$). Non parametric tests (Mann-Whitney U) failed to detect any significance.

6.3.1.2 Intravesical administration

In contrast to the generally even distribution of AlSPc in the bladder after I.V. injection, the uptake of intravesically administered AlSPc mixture was found to be patchy and rather less predictable. High levels of fluorescence, which extended across the full thickness of the bladder wall, were seen in some areas but in other areas adjacent to these there was often either virtually no uptake or just a very superficial coating (fig. 6.9). Because of this variability it was not felt appropriate to attempt a quantitative comparison with the levels of fluorescence recorded after IV sensitisation.

The highest levels of fluorescence seen were several times greater than those achieved after IV administration but in all of the 12 bladders examined there were large areas of minimal or zero uptake. Interestingly there did not appear to be any clear correlation between fluorescence distribution or intensity and either the concentration of sensitiser instilled or whether it was left in contact with the urothelium for 30 min or 1 h.

The AlSPc mixture used is a relatively hydrophilic molecule due to the large predominance of the trisulphonated fraction, but it would be expected that the uptake of the purified disulphonated derivative (AlPcS2), which is more lipid soluble, might be more effective. AlPcS2, administered in equimolar concentrations to the AlSPc mixture, did appear to be taken up much more evenly than the mixture in the most superficial layers of the bladder wall. Although this uptake was considerably more uniform than that seen with the mixture there were still a few areas where only minimal uptake was apparent so no figures for fluorescence intensity will be given.

Of more interest, however, was the observation that in none of the 12 bladders examined was there any major fluorescence from the muscle layer In most cases the signal from the muscle hardly exceeded the background intensity (fig. 6.10). This produced an extremely high ratio of fluorescence intensities between the superficial and deep layers, much greater than that seen after intravenous administration and often in the order of 20:1. The graph superimposed on fig. 6.10 demonstrates this very strong, localised uptake in a typical specimen.

The histological damage seen with PDT after intravesical administration of either AlSPc mixture or S2 compound shows some similarities with the distribution of photosensitiser observed by this method. Those results will be presented in chapter 7.

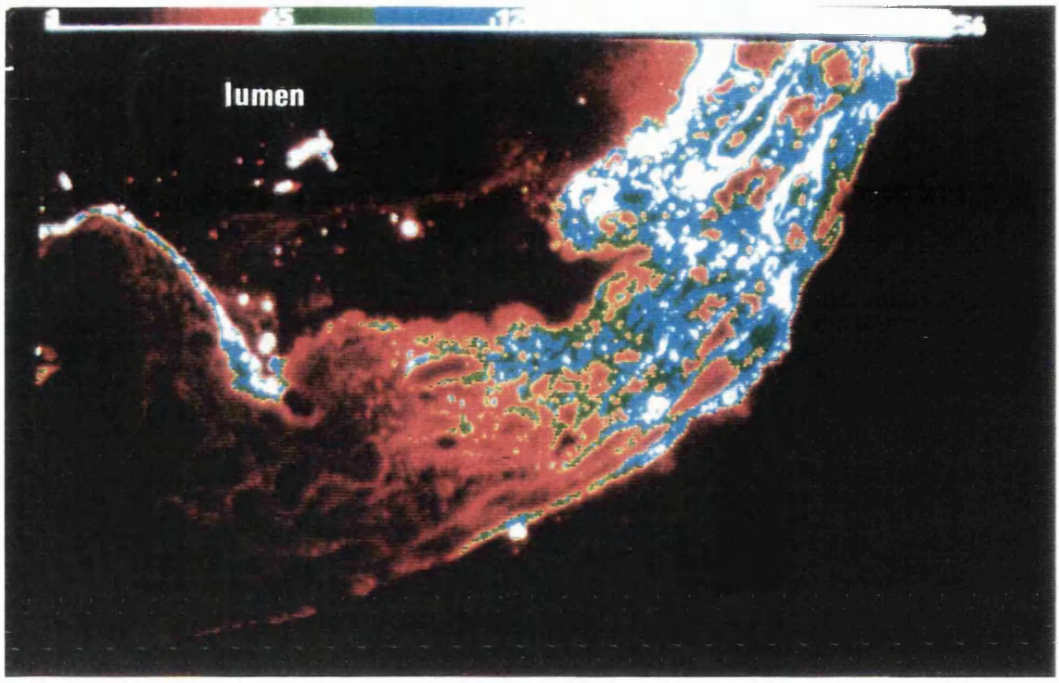


Fig. 6.9 Intravesical administration of AlSPc mixture (100 µg / ml, 1 h)

Note high though variable fluorescence throughout the bladder wall

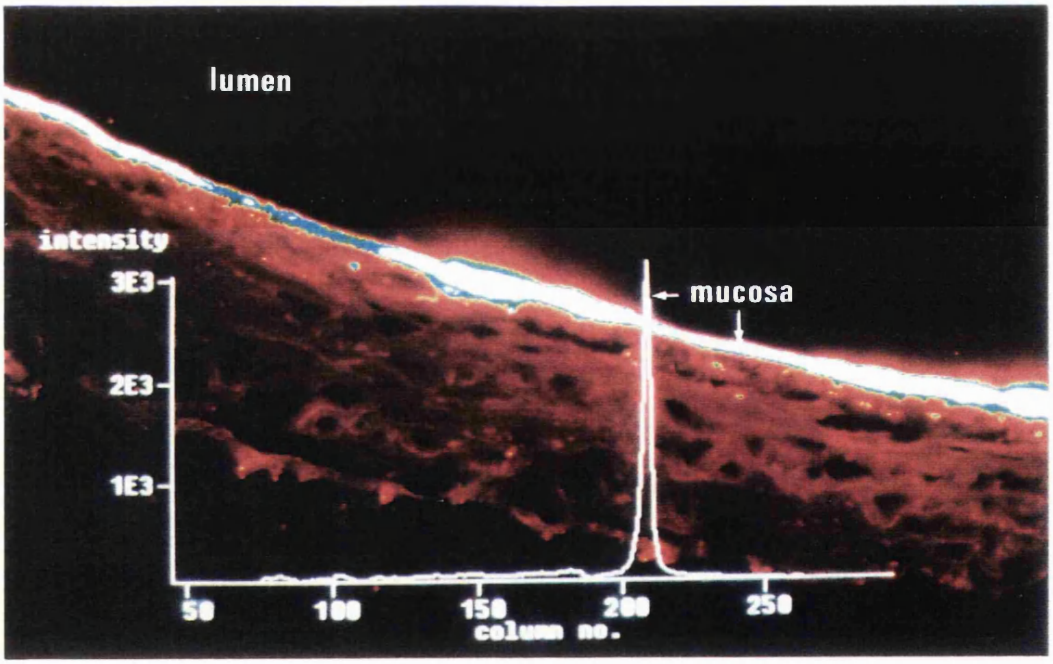


Fig. 6.10 Intravesical administration of AlPcS2 (100 µg / ml, 1 h) showing a much more superficial and even uptake compared to the AlSPc mixture

Note that the fluorescence from the muscle layer is essentially nil (background) producing a very large ratio between superficial and deep layers

6.3.2 Photobleaching

The highest light fluence given (80 J/cm^2), resulted in an almost complete degradation of the sensitiser at both doses studied (0.5, and 5 mg/kg AlSPc, 24 h prior to light exposure). A fluorescence scan is shown from an animal who had received 5 mg/kg AlSPc and even here there is only a thin rim of superficial fluorescence remaining (figs. 6.12). The lower light dose of 20 J/cm^2 (6 animals) caused less photobleaching (fig. 6.13), only reducing the fluorescence in muscle by 20 - 30 % compared with unexposed controls, and by 10 - 15 % in the superficial layers. By causing a proportionally greater reduction in the fluorescence from the muscle layer, photobleaching had the important effect of increasing the ratio of the fluorescence intensities between the superficial layers (mucosa / lamina propria) and the deep layers (muscle) to 4.5 - 6:1 after 20 J/cm^2 and to greater than 10:1 after 80 J/cm^2 (compared with 3.5 - 4:1 in unexposed controls). After the high light doses (figs. 6.12) the absolute levels of fluorescence, however, were very greatly reduced, to levels likely to be below the threshold for a PDT effect.

When an animal was killed immediately before irradiation it would be expected that tissue oxygenation would be much lower. A light dose of 80 J/cm^2 given subsequently to 2 animals produced minimal (< 10%) photo-bleaching (fig. 6.14). This is in marked contrast that seen earlier *in vivo* (fig. 6.12). The lower light dose of 40 J/cm^2 had no measurable effect.

6.3.3 Tissue distribution of AlPcS2 by chemical extraction

The data from the tissue extraction assays are given in appendix 2.2.2 and summarised here in fig. 6.11. The concentrations of AlPcS2 seen in the bladder after IV administration of 1 mg/kg were very similar to those in the kidney from 6 h onward. Initial uptake, however, was higher in the kidney than in the bladder, as might be expected from the former's greater

blood supply, with peak concentrations in the kidney measured after 1 h at around twice those in the bladder (0.73 vs. 0.37 μg AlPcS2/g tissue) at this time. The sensitiser concentration in the kidney fell gradually thereafter whereas that in the bladder increased so that at 6 h they were very similar (0.47 μg/g in kidney, 0.49 μg/g in bladder) and remained so until 24h. From then the concentration in the bladder reduced more quickly though levels at 72 h were still greater than two-thirds of the maximum reached at 6 h. The increase in AlPcS2 in the bladder between 1 h and 6 h was statistically significant (1 h = 0.37 ± 0.027 μg/g, 6 h = 0.49 ± 0.042 μg/g; P = 0.001) as was the drop by around 30% between 3 and 7 days ($p<0.05$).

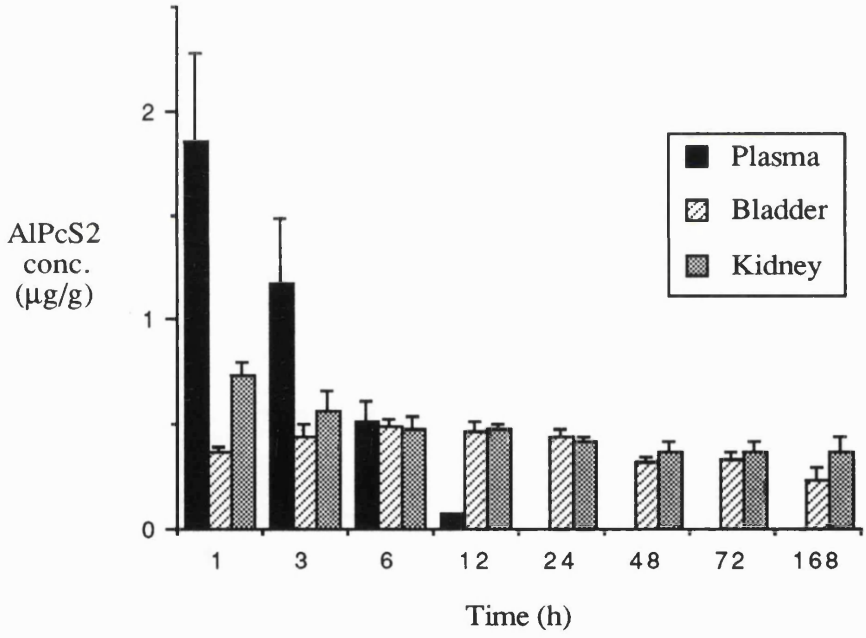
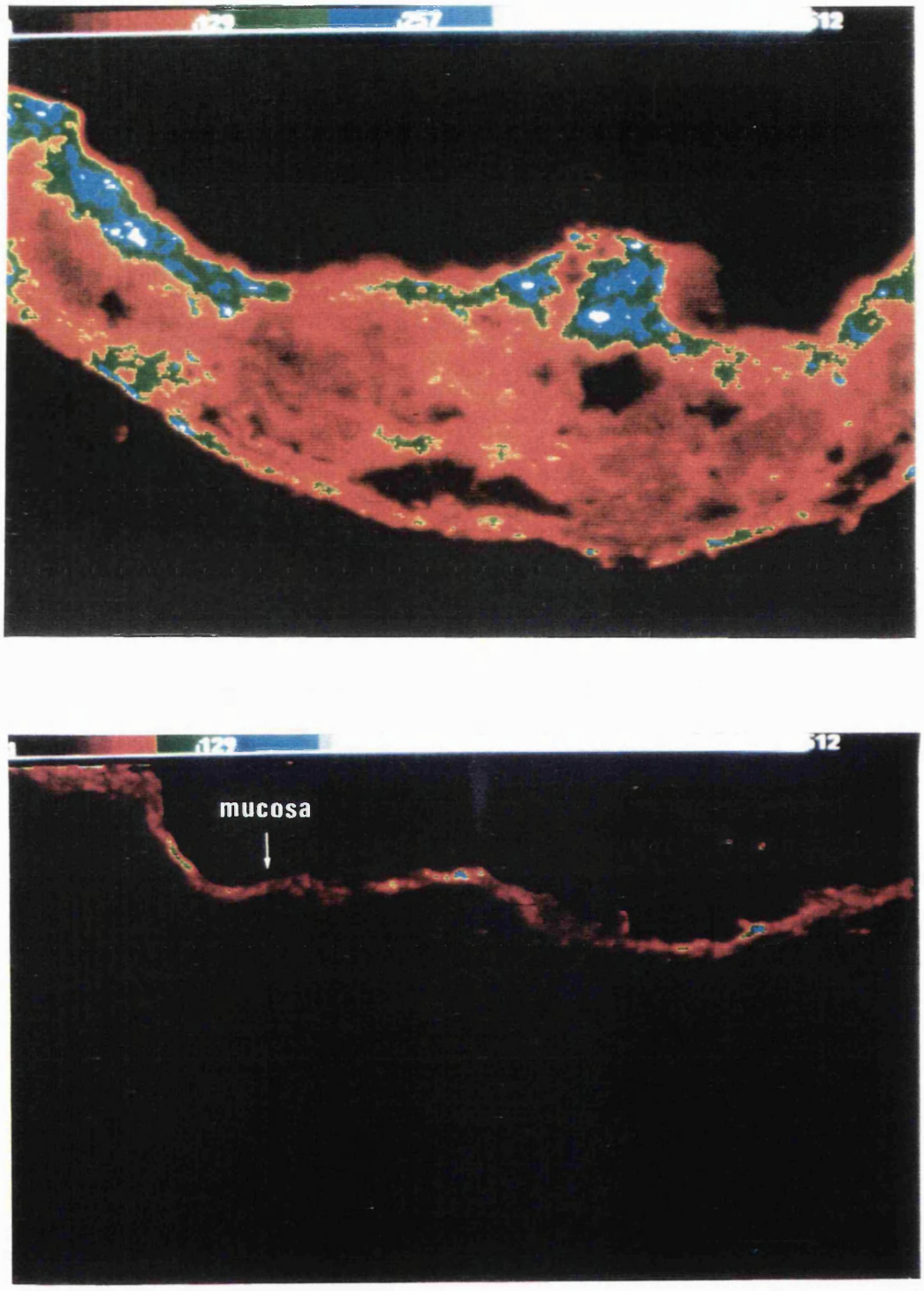


Fig. 6.11 Concentration of AlPcS2 in whole bladder and kidney compared with plasma levels after IV sensitisation (1 mg/kg)
(values given represent means ± 1 SD)

Plasma levels of S2 fell rapidly from the start, becoming almost undetectable at 24 h in contrast to the fairly constant tissue levels at this time. It was noted above that the fluorescence values for AlSPc mixture in the bladder reduced by the order of 15% between 24 h and 72 h. The fall in gross S2 concentration during this time was similar at nearly 25%.



Figs. 6.12 Photobleaching effect of 80 J/cm^2 red light (675nm) *in vivo* on bladder 24 h after sensitisation with 5 mg/kg AlSPc

The upper scan shows a control section (24 h after 5 mg/kg AlSPc but no light). Following 80 J/cm^2 intravesical illumination (lower picture) most of the sensitiser has been bleached out leaving only a thin rim of fluorescence visible in the mucosa.

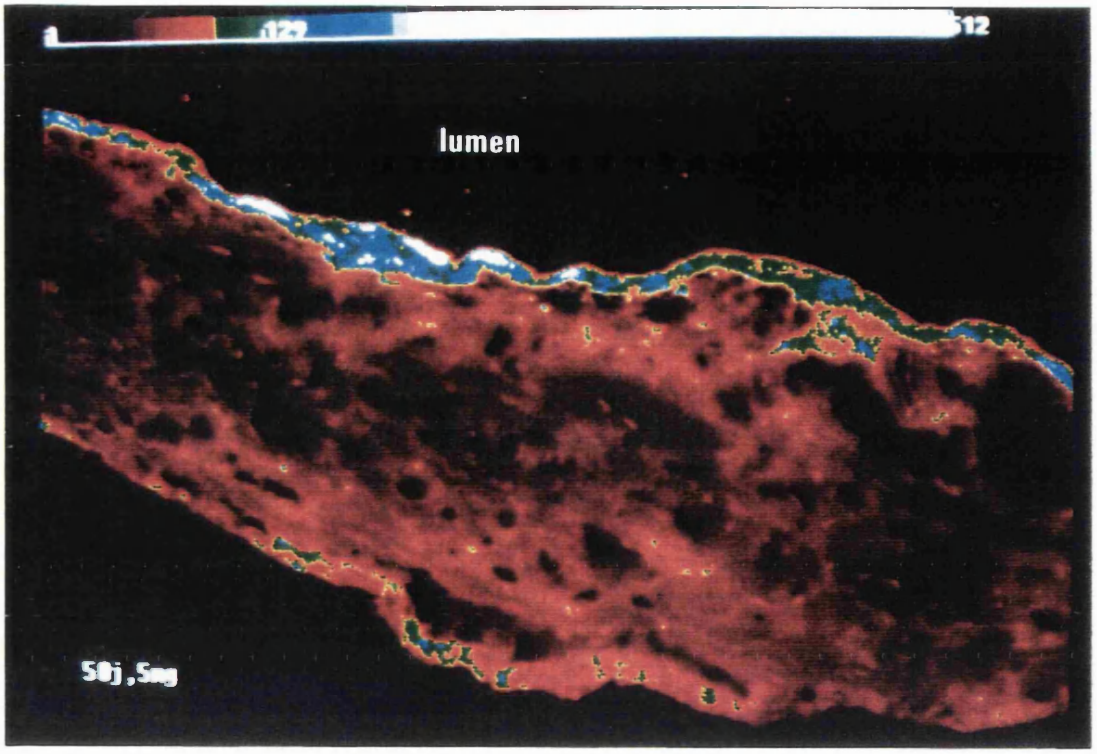


Fig. 6.13 Photobleaching after 20 J/cm^2 red light (5 mg/kg, 24 h)

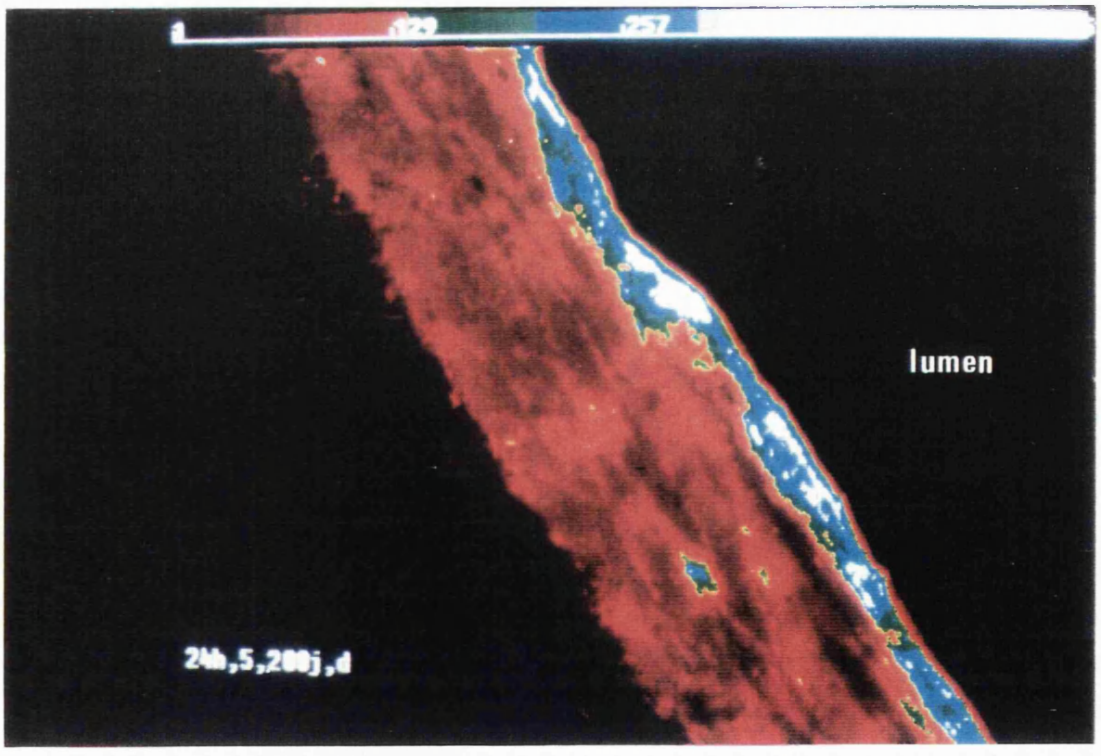


Fig. 6.14 Effect of cessation of blood flow on photobleaching

(Same parameters as fig 6.12, *ex-vivo*. Note there is very little photobleaching)

6.4 DISCUSSION

The work presented in this chapter has described the quantitative imaging of phthalocyanine fluorescence in rat bladder by a highly sensitive detection system. This has not been previously demonstrated in this way by any other group and as far as we are aware is the first time that photobleaching of any sensitiser in tissue has been demonstrated at the microscopic level.

Fluorescence imaging of porphyrins has been used by many workers both as a diagnostic aid to identify areas of increased uptake which may indicate dysplastic or neoplastic tissue (section 3.3) and to quantitatively assess the amount of photosensitiser in tissue. However as we have seen, one needs to ask which forms of the photosensitiser molecules exhibit fluorescence as they may be photochemically inactive aggregates (section 4.3.2). This is much more of a problem with HpD than with AlSPc. A chemical assay of the total amount of sensitiser dye present in the tissue is adequate when looking at general pharmacokinetic trends (fig. 6.11) but results from such techniques must be interpreted with care as for instance the amount of drug aggregates formed will depend on several variables, particularly drug concentration. Also a chemical assay will say nothing about the photo-sensitiser distribution within the different tissue layers.

The ultimate aim of studying the distribution of photosensitiser in animal bladder must be to derive guidelines of help in defining optimum treatment parameters for human disease. This is not simply a question of just getting as much of the drug as possible into the bladder but of maximising the ratio of the amount in tumour compared to that in areas of normal tissue that may be damaged by PDT, i.e. normal bladder muscle. Although there are several factors that will influence this, the duration of the interval between photosensitisation and treatment is probably the most important and the easiest to adjust. The role of photobleaching and the variability of uptake

dependent on the method of delivery of the drug also seem relevant but the possible importance of these factors has received little attention.

6.4.1 Timing of light exposure

Tissue sensitiser concentration will depend on the time lapse before light treatment as well as the administered dose. The ideal is not known and it was the aim of these experiments to help clarify this optimum time. Most reported series have treated at 48 - 72 h after sensitisation. Benson's group (1986) treated at 3 h as the *in vivo* fluorescence of bladder tumours was greatest at this time. A short interval may not allow the most advantageous ratio of concentration between tumour and normal tissue to develop.

Initially after injection there was a high level in normal tissue especially in well vascularised submucosa and serosa, and of course in the endothelial cells themselves. There was no actual increase in fluorescence from the levels observed after 1 h so this gradient after 24 h compared with 1 h is most likely to be due to a more rapid elimination of AlSPc from the muscle layer rather than from the superficial layers.

Others have noticed that a slower clearance of sensitiser from malignant tissue results in a concentration difference. This ratio in rat colonic tumours has been found by some of my colleagues to be greatest at 48 h, though only about 2:1 (Tralau *et al.*, 1987), and it would not seem unreasonable to expect a similar situation in bladder carcinoma. This work shows that after the first 24 h the ratio between normal mucosa and muscle remains fairly constant at between 3 - 4:1, and absolute values decline by less than 20% between 24 h and 72 h. It would therefore seem most appropriate to treat bladder tumours within this time period, say around 48 h after sensitisation. Later than this confers no advantage in the distribution of photosensitiser in normal tissue and it is at 48 h that the most

advantageous ratio between tumour and normal tissue may be expected, though this should be clarified when studies of AlSPc uptake in man are possible. Ideally photosensitiser distribution should also be checked in an animal bladder tumour model though these are not very representative of the human condition, in particular a pure Cis cannot be reliably produced.

6.4.2 Intravenous or intravesical administration?

All reported studies, both clinical and experimental, have administered the photosensitiser systemically until very recently (Bachor *et al.*, 1992). There are, however, clear advantages to topical (i.e. intravesical) application as widely practised for other cytotoxic agents. These include the probable avoidance of systemic toxicity, especially cutaneous photosensitivity, and the possible advantage of getting high levels of drug into superficial malignant tissue with less penetration of, and subsequent unwanted damage to, the deeper bladder wall muscle layers. In this work the absorption of AlSPc mixture after intravesical instillation proved unreliable with patchy uptake into the bladder wall that could be full thickness in some places and minimal in others. There is no clear explanation for this, in particular the uptake did not directly relate to the main dosimetry variables of concentration or instillation time. It will be seen in chapter 7 that this unpredictable intravesical absorption appears entirely consistent with the morphological results seen following PDT and intravesical sensitisation.

Bachor *et al.* (1992) using AlSPc mixture and a rat bladder tumour model found a more superficial, though no more selective, uptake of the dye into tumour following intravesical compared with intravenous administration. Tumour destruction after PDT was not enhanced but there was less damage to normal bladder wall following intravesical administration. Their model used a bladder tumour cell line transplanted submucosally so that the

urothelium remained intact over it. Drug uptake might be expected to be modified, hopefully increased, by the abnormal epithelium of neoplastic or dysplastic areas which may absorb more easily than normal "waterproof" urothelium.

Work with the S2 component seemed more promising with a very superficial, though quite even, uptake leading to a much higher gradient (x 20 or more) of sensitiser concentration between superficial and deep layers than was seen after intravenous administration. These preliminary results should be interpreted with some caution as until the study cited above, no other group had demonstrated effective photosensitisation after the intravesical administration of any photosensitiser. Further studies are clearly needed in clinically relevant material, particularly with the promising S2 preparation, but it is a reasonable hope that when these are possible and with improved formulation it is likely that intravesical administration of the photosensitiser will be the route of choice. Until then the intravenous route will remain the standard.

6.4.3 Photobleaching

During light exposure some photosensitiser molecules become photo-degraded and will neither produce singlet oxygen nor fluoresce. In this work photobleaching has been demonstrated at the microscopic level and been shown to require an intact blood supply. These experiments suggest that the rate of bleaching depends rather more on the light dose than on the photosensitiser concentration. This has the effect of reducing the amount of sensitiser in areas of lower concentration by a greater proportion than in areas of higher concentration. This would explain the increases in the ratio of fluorescence intensities between the superficial and deep layers of the bladder wall (from around 3:1 to in excess of 5:1).

Specific estimations of photobleaching rates are outside the scope of this present work but it is clear that the larger light doses almost entirely degraded the photosensitiser. It is likely that the latter part of these longer exposures would in effect be wasted, as the drug levels had been reduced below the threshold level for a PDT effect (see below). The light dose at which this would occur is likely to be between 20 and 40 J/cm^2 at the drug doses used here. The factor of reassuring clinical relevance is that as long as the photobleaching range is entered the effect of prolonged illumination becomes self limiting. It must be accepted that light distribution over the bladder surface will never be perfectly isotropic so it would prove advantageous to merely stipulate the minimum light dose and ensure that an adequate treatment is given to all areas without dangerously overtreating those areas inevitably receiving a higher irradiance.

There are two conditions to be satisfied before a PDT effect can occur. Firstly enough singlet oxygen must be produced to cause tissue necrosis. This requires a minimum tissue concentration of photosensitiser as below this threshold level it is destroyed by photobleaching before sufficient singlet oxygen has been produced, whatever the light dose. Secondly a given tissue effect depends on the total photodynamic dose (the product of sensitiser concentration and light dose). If the sensitiser concentration is increased, the light dose required is reduced and vice versa. This relationship is generally reciprocal except for low concentrations of photosensitiser near the threshold level where photobleaching becomes more important (Potter *et al.*, 1987). The therapeutic ideal is to manipulate the difference in concentration of photosensitiser between the urothelium and underlying muscle to achieve photoactive conditions in the former but sub-threshold concentrations in the muscle. In this situation photobleaching of the

sensitiser in the muscle layers would spare it from unwanted damage during light exposure though still produce tissue necrosis in the mucosa.

In theory it should be possible to use photobleaching to increase the selectivity of clinical PDT, though, in practice this will not be at all straightforward as it will be very difficult with the variability of clinical material to ensure that the dye concentration in the muscularis does not exceed the threshold level. This implies the use of a relatively low concentration of photosensitiser and a necessarily larger light dose than has been generally employed before. Some additional selectivity in clinical practice may result from the small differential in photosensitiser concentration to be expected between bladder tumour and adjacent normal mucosa. The application of these parameters to an animal model of bladder function with the aim of producing a reliable necrosis of the bladder mucosa without damaging the underlying muscle is described in the next chapter.

6.4.4 Chemical assay or fluorescence?

The most common method of assessing photosensitiser concentration is to perform an extraction analysis of the type described here. This has the advantage of being quick and relatively simple and of giving a quantitative value for each sample. Drug levels can be assessed in plasma as well as tissue but there are a couple of main limitations in that both active and inactive forms (usually aggregates) of the drug are measured and there is no information on the drug distribution within that tissue. Quite apart from the latter factor it is clear that the results from the extraction assay do not closely mirror the fluorescence data. Drug levels in the bladder measured by chemical analysis increased by one third between 1 and 24 h and had only just fallen back a little by 72 h. Fluorescence levels in the

superficial layers on the other hand fell by 25 - 30% between 1 and 24 h, though by only a little more at 72 h. In the deeper layers the fall was between 50 and 60% by 24 h before levelling out, giving rise to the concentration gradient previously emphasised.

It is recognised that an important factor in this discrepancy may have been the use of AlSPc mixture for the fluorescence studies and AlPcS2 for the extraction analysis. This was unavoidable as the latter study was carried out later when the mixture was no longer available. The chemical differences between the compounds are slight although the slightly greater lipid solubility of the S2 is likely to prolong its retention in tissue. With increasing time after administration there will also be metabolites formed which in general are not biologically active as they will not generate singlet oxygen not fluoresce. They will, though, still be assayed by chemical means which would explain the sustained or increased drug levels measured by the extraction assay during the first 24 h compared to the reduction in fluorescence during the same period. It is concluded, therefore, that the data provided by this fluorescence technique are more sensitive in terms of assessing biological activity as well as providing important information on the microscopic distribution of photosensitiser than the standard chemical extraction assay.

Chapter 7

MORPHOLOGICAL CHANGES IN THE NORMAL RAT BLADDER FOLLOWING PHOTODYNAMIC THERAPY WITH PHTHALOCYANINE PHOTOSENSITISATION

7.1 INTRODUCTION

Since the potential of PDT in bladder carcinoma was first demonstrated by Kelly and Snell in 1976, well over 100 clinical cases have been reported (table 3.1). In general these have shown promising control of resistant disease though, as was discussed in chapter 3, this was at the cost of significant morbidity in most patients. All patients receiving whole bladder PDT experienced irritative symptoms which were often severe. Some authors have reported muscle fibrosis and permanently reduced bladder capacity, incontinence and upper tract deterioration (Harty *et al.*, 1989), with symptoms that may themselves necessitate cystectomy (Nseyo *et al.*, 1985a; Jocham *et al.*, 1986). Prolonged skin photosensitivity may also be troublesome with porphyrin photosensitisers.

Clinical PDT, as with many new medical treatments, has evolved on a largely empirical basis whilst there are still fundamental questions regarding its mechanism of action and optimum treatment parameters unanswered. These include the choice of photosensitiser and the optimum drug dose; the time delay between sensitisation and treatment to allow for the most advantageous distribution between tumour and normal tissue, and the wavelength and energy of the activating light. It is clearly unrealistic to hope that photosensitising drugs will only be retained by malignant or dysplastic cells so it becomes essential therefore when treating a whole organ such as the bladder to understand the action that PDT will have on the normal tissue so that dosimetry parameters may be developed which will produce the desired effect in areas of tumour but will minimise unwanted damage and the complications which have been seen in clinical practice so far.

The first objective in planning PDT experiments on the bladder must be to decide what biological effect is desired and then to manipulate all the

parameters involved to see if this effect is achievable. It is surprising that so little has been done along these lines.

The clinical reports described in chapter 3 have suggested that the main problem is scarring in the muscle causing irreversible changes in bladder function. In whole bladder treatments, no-one has shown necrosis limited to the tumour areas, and instead large areas of mucosa have sloughed which must be presumed to include both normal and neoplastic regions. These have healed with regeneration of normal mucosa which for patients with Cis means that their tumour has been eradicated. Thus it seems less important to attempt any form of selectivity between normal and abnormal mucosa, but highly appropriate to try and limit the damage to the mucosa and submucosa and leave the muscle intact. Experiments designed to answer this question can be carried out on normal animals, which is much simpler than finding an adequate animal model of *in situ* bladder cancer.

Two sets of treatment parameters can be manipulated. Those related to the photosensitising drug (e.g. dose, route of administration and time between sensitisation and light exposure), and those related to the light (wavelength, power, exposure time and geometry of light distribution).

The main consideration for the sensitiser is how the variables mentioned above affect its distribution. For localisation within different layers of the bladder wall, the best technique is fluorescence microscopy and these studies were described in the chapter 6. These indicated that a more even distribution was achieved after intravenous than after intravesical administration of AlSPc, and that the optimum (largest) gradient between superficial and deep layers occurred at around 24 - 48 h after sensitisation. Estimations of the biological effects of variations in drug dose cannot be made from fluorescence measurements so an *in vivo* treatment model is therefore necessary to assess these.

In vivo experiments are also required to assess most of the dosimetry variables of the activating light. The wavelength used to activate AlSPc has to be 675 nm, with porphyrins it is usually 630 nm though almost any visible wavelength up to 635 nm may be used. Few workers have explored the different effects likely when using green light and HpD and there are no published data to indicate whether green light may be more appropriate for PDT of superficial bladder cancer. Likewise the sensitiser and light doses might be expected to be inversely related for a given effect but this view fails to take account of any effect of sensitiser photobleaching at low drug doses, or of saturation or possible thermal effects at high light levels.

As has already been discussed, there seems little justification for those parameters that have been used in clinical PDT and the aim of the work presented in this chapter is to investigate the morphological changes seen after PDT for a range of sensitiser and light doses. The functional changes that occur consequent to these will be investigated in the following chapter. The normal female rat bladder was used as this is large enough to catheterise both for intravesical light delivery and for the measurement of intravesical pressure. A rather larger bladder would have been easier to work with but, as previously explained, this was not practicable.

In this animal model the aim is to produce a dependable necrosis of the superficial layers of the bladder whilst leaving the muscle layers intact. The dosimetry tolerances that might allow this degree of discrimination in the very thin bladder wall of the rat are likely to be quite narrow. If this can be achieved, however, it seems reasonable to propose that in the clinical setting not only will it be possible to destroy all abnormal bladder urothelium, but also that the inevitable damage to adjacent normal tissue will not result in permanent disability.

7.2 MATERIALS AND METHODS

7.2.1 Phototherapy

A copper vapour laser (Oxford Lasers) was used, pumping a dye laser to emit red light at 675 nm (fig. 7.1). This type of laser has been described in chapter 2. It produces very short (40 ns) pulses of light at a very fast repetition rate (12 kHz) and has been shown to have the same biological effect as a CW laser (Barr *et al.*, 1989). The output was via a 200 μm quartz fibre and was set to an average power of 100 mW as measured by an external power meter. This power level was chosen as a convenient value well below the expected maximum output of the dye laser where the output would be stable and, more importantly, sufficiently low not to have any danger of producing thermal damage which would cloud the observed PDT effect. This was confirmed both by the controls in these experiment and also previously by myself and colleagues in several organ systems.

The laser fibre tip was freshly cleaved prior to each session with a tungsten carbide blade after stripping off the terminal 1 cm of protective cladding. The spot pattern from the tip was checked to be circular with well defined edges. The stability of the power output from the laser was monitored during each exposure by the laser's own in-line power meter and very rarely required even minor adjustment during illumination. It was also re-checked after each exposure, and halfway through the longer exposures (as energy densities of 80 and 200 J/cm^2 represented long exposure times of around 30 and 75 min respectively), using the external power meter. The design of the catheter assembly (see below) made it easy to replace the fibre in the same position after these checks and re-inflate the bladder to the same treatment volume.

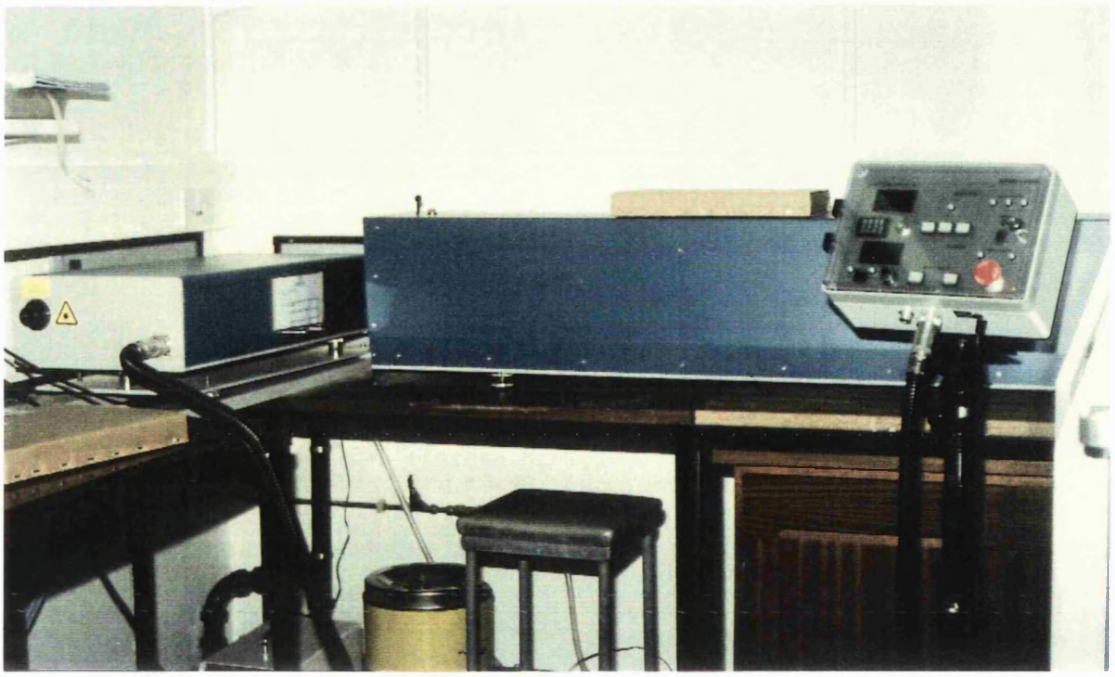


Fig. 7.1 Copper vapour pumped dye laser (Oxford Lasers)

(the dye laser is the smaller blue box with the laser aperture on the extreme left)

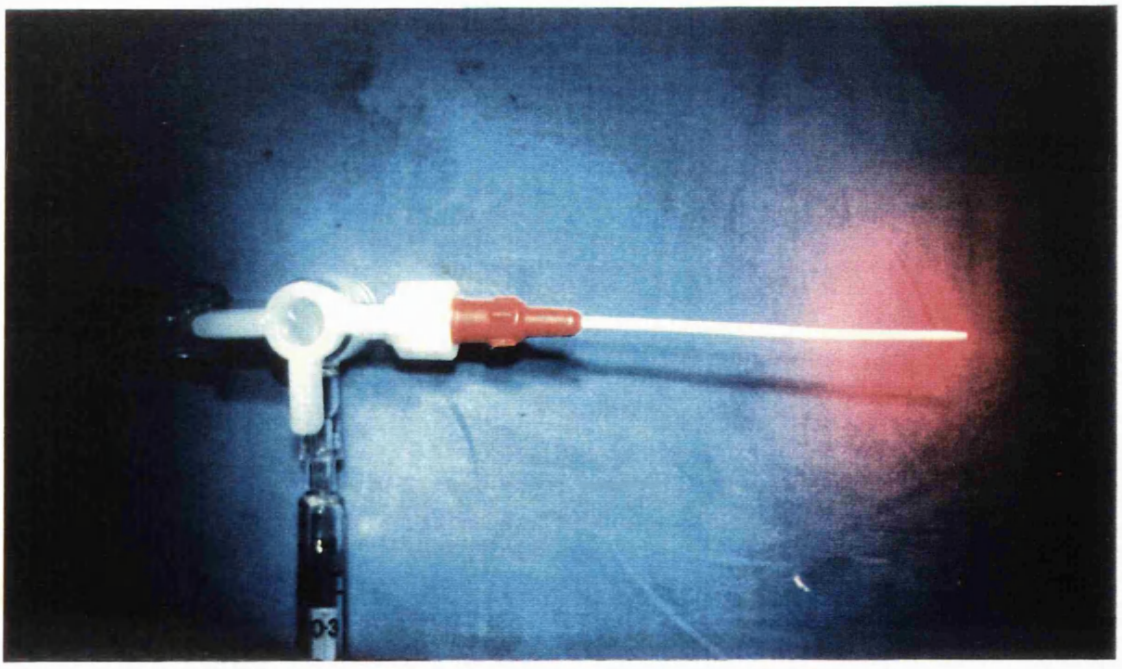


Fig. 7.2 Laser fibre within diffuser cannula

(The laser light is diffused by a teflon cannula through which a known amount of saline can be passed to distend the bladder)

7.2.1.1 Light delivery

The method of bladder illumination is shown in fig. 7.2. The anaesthetised rats were catheterised as described in section 6.2.2.3. The bladder was exposed via a lower midline abdominal incision and the residual urine, and any air introduced during catheterisation, was gently aspirated through the 18G cannula without damaging the bladder mucosa. A 1 ml syringe was attached to the side arm of a 3-way tap connected to this cannula and the bladder was inflated with 0.3 ml saline for all treatments.

The distance from bladder neck to apex was measured with callipers after filling to the treatment volume as just described. The laser fibre was introduced through a rubber injection port, by reverse passage through a 19G needle, for the required distance such that when this was connected onto the straight arm of the 3-way tap the tip of the fibre lying within the cannula was just behind the centre point of the bladder and the end of the cannula was just touching the apex, as shown in fig. 7.3.

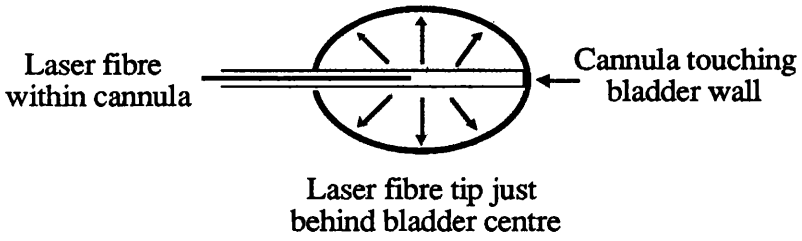


Fig. 7.3 Diagram of bladder illumination

The opaque teflon cannula acted as a diffuser for the laser light (fig. 7.4) as well as enabling the laser fibre to be positioned along the longitudinal axis of the bladder. Central placement of this cannula was done visually as it was easy to see through the translucent bladder wall, although this was more difficult in the saggital (anteroposterior) plane than in the coronal (left to right) plane. This was because the bladder angled forward a little

so that the cannula tended to lie closer to the back of the bladder than to the front. This problem was overcome by vertically positioning a pair of forceps in a retort stand such that its tips pushed down either side of the bladder on a piece of surgical drape. This both allowed the bladder to fall back a little into the pelvis such that the cannula would lie more centrally, and also shielded adjacent bowel from possible PDT damage itself due to the transmitted light (fig. 7.5).

When the laser output needed to be checked during the exposure the fibre was withdrawn from the bladder by disconnecting at the 3-way tap thereby leaving the cannula in place. The rubber port through which it passed ensured that the fibre was held to the correct length and replaced in exactly the same position.

The volume chosen to fill the bladder (0.3 ml) just unfolded the mucosa rather than distending it and consequentially thinning its wall (maximum capacity was usually greater than 1 ml). Overdistension has been implicated in an excessive depth of PDT effect (Nseyo *et al.* 1988). This standard inflation aimed to ensure a constant bladder surface area for illumination in all animals. The bladder was assumed to be spherical to calculate its surface area so that the necessary treatment times required to give incident light doses ranging from 20 - 200 J/cm^2 could be derived as shown below.

Bladder volume $v = \frac{4}{3} \pi r^3$ Surface area $s = 4 \pi r^2$

for $v = 0.3$ then $r = 0.42$ and $s = 2.22$ cm^2

$$\text{power density (mW/cm}^2\text{)} = \frac{\text{treatment power (mW)}}{\text{surface area (cm}^2\text{)}} = \frac{100}{2.22} = 45 \text{ mW/cm}^2$$

$$\text{treatment time (secs)} = \frac{\text{light dose required (J/cm}^2\text{) x 1000}}{45}$$

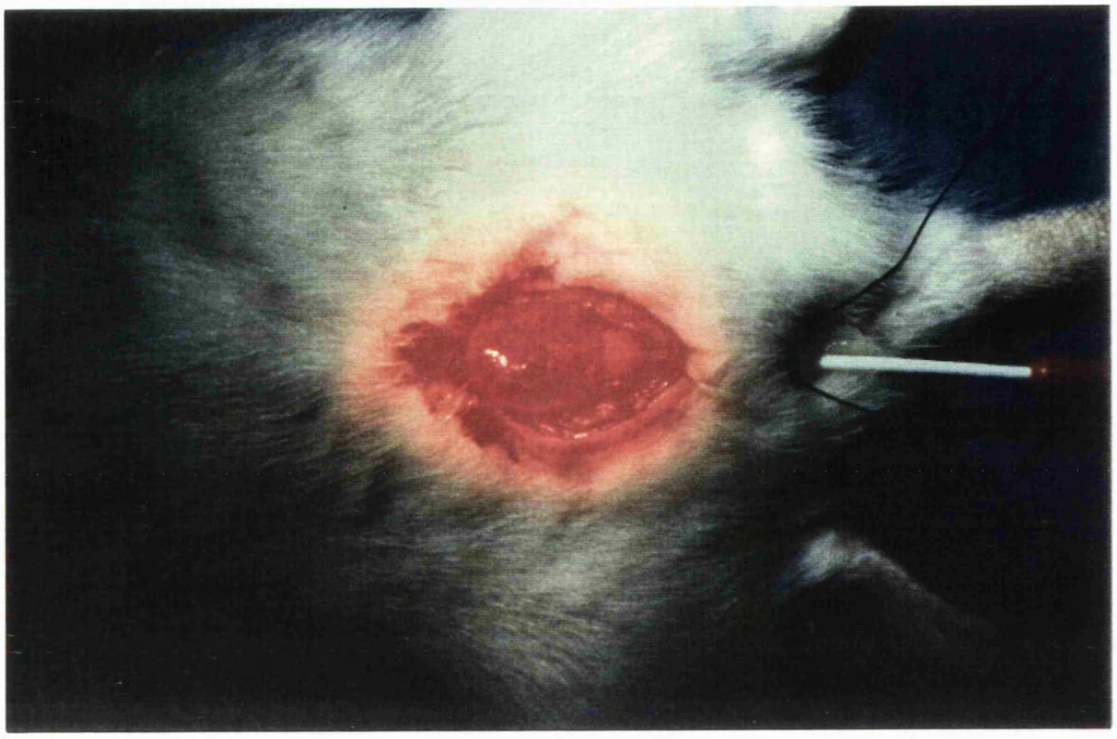


Fig. 7.4 Laparotomy to expose bladder and illustrate illumination from diffused laser fibre

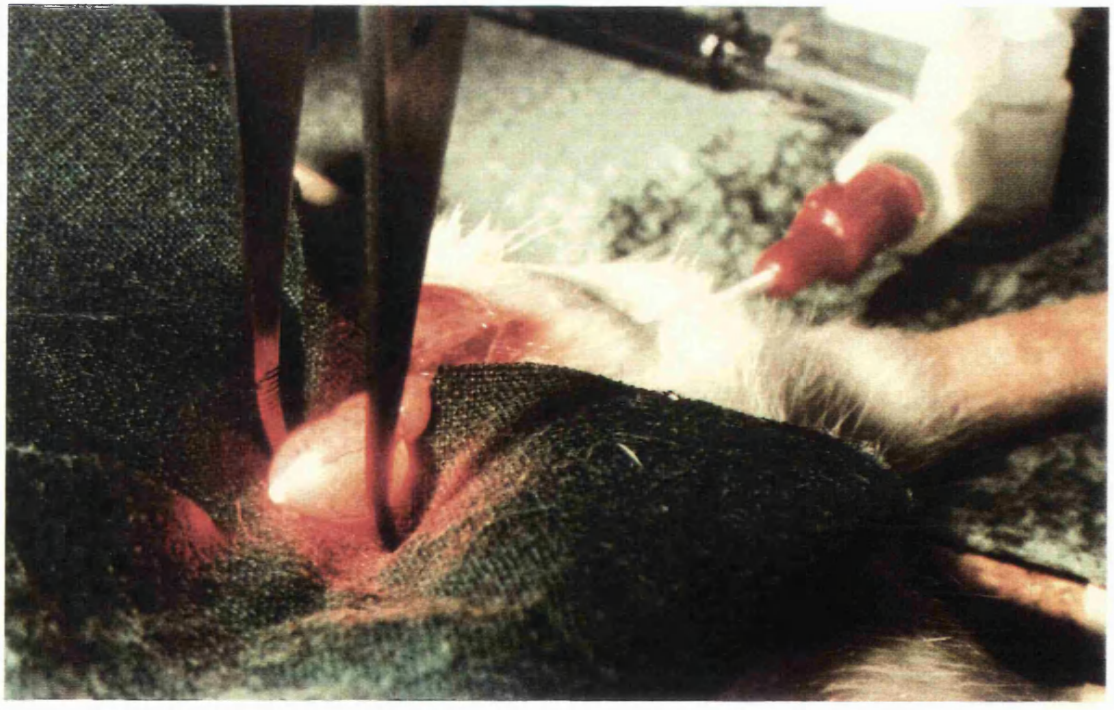


Fig. 7.5 The adjacent bowel is shielded from transmitted light

(gentle downwards pressure with forceps allows the bladder to fall back into the abdomen and the cannula to lie centrally within it. Note the very small area of high intensity forward illumination from the cannula)

7.2.1.2 Light distribution

This method of illumination was essentially an uncapped cylindrical diffuser as it was necessary to introduce the cannula into the bladder over a guidewire and also to regulate bladder volume. However the tip of the cannula was in contact with the dome of the bladder so that the high intensity forward beam was restricted to this tiny area which it was considered would not unduly influence the results. This will be elaborated on later but in brief this small area never perforated though usually looked a little different (more inflamed) than the rest of the bladder. As the rat bladder is a slightly elongated rather than truly spherical a cylindrical output was useful, though volume calculations on this basis would not be feasible. Some of the difficulties associated with accurate light dosimetry that were encountered will be discussed later but it is appropriate here to describe the method used to validate light distribution from this delivery system in terms of producing an acceptably even bladder illumination.

The evenness of the light distribution from the diffusing cannula was studied in 2 respects using a photodiode detector fibre (acceptance angle 42°). First the irradiance output from the diffusing cannula, with the laser output set to 100 mW, was measured in air by moving the detector fibre in 1 mm steps parallel to and 2 mm away from the axis of the cannula using a micrometer stage (fig. 7.6). The tip of the laser fibre was positioned 1 cm short of the end of the cannula as would have been the case within the typical rat bladder. The levels of irradiance recorded along the cannula were found to be very similar on each of the 3 occasions this experiment was repeated so a single typical plot only is shown in fig. 7.8. The maximum lateral irradiance was found to be at a level 1 - 2 mm in front of the fibre tip with a smooth decrease on either side. A much higher, (usually 2.5 - 3 times the maximum lateral irradiance) but very localised

illumination came from the end of the cannula and is not illustrated in this plot as it was not recordable with the detector fibre parallel to the cannula.

These measurements were next repeated to record the light transmitted through the wall of an *in vivo* bladder arranged as in fig. 7.3. These measurements are of light transmitted through the thin wall of the rat bladder whereas the important amount is of course the dose absorbed, though this should be directly proportional assuming an uniform thickness and optical quality of the tissue. The detector fibre was moved along, and 2 mm away from, the saggital axis of the bladder and measurements taken at 2 mm intervals (fig. 7.7).

There was a somewhat greater variation in these measurements than in those from the diffuser cannula alone, largely due to the slightly different shapes of the individual bladders. The results of measurements on 3 bladders, though, indicated a similar situation in the general pattern of the transmitted light. One representative plot is shown in fig. 7.9. Light transmission through the bladder wall whilst not isotropic, was slightly more even than that seen with the diffuser cannula alone. It is likely that this smoothing effect was the result of internal reflection within the bladder cavity. The level of transmitted illumination shown in fig. 7.9 is approximately two-thirds of that seen in fig. 7.8, suggesting that in all these PDT experiments on the rat bladder only one-third of the light dose is being absorbed within the bladder wall and contributing to the observed biological effect. Illumination was rather lower over the bladder neck and apex but fairly even over the central third of the bladder from which all the histological sections would be taken. Again there was a very small area of high intensity forward projection where the open end of the cannula abutted the bladder wall. This was measured at between 2.5 - 3.5 the level of the maximum lateral irradiance and is demonstrated in fig. 7.5.

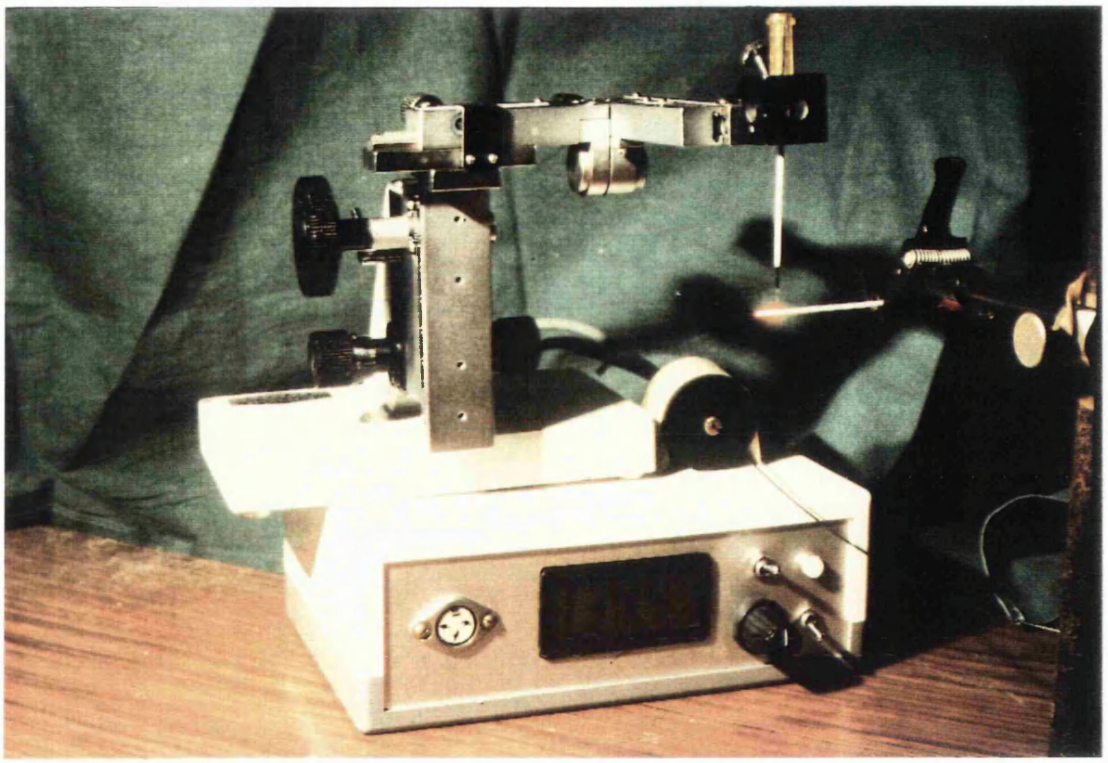


Fig. 7.6 Irradiance measurement from a diffused fibre

(The detector fibre (vertical) is moved along the source fibre (horizontal) on a micrometer stage. The intensity (linear units) is displayed on a meter)

Fig. 7.7 Irradiance measurement from an intact bladder

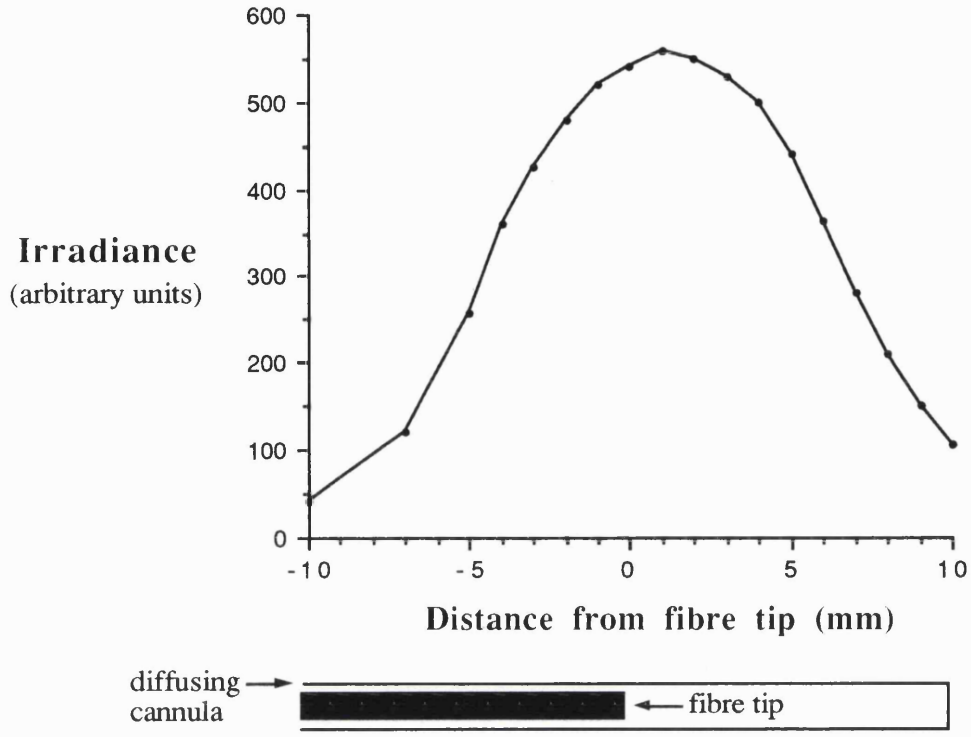


Fig. 7.8 Plot of fibre irradiance (in air)

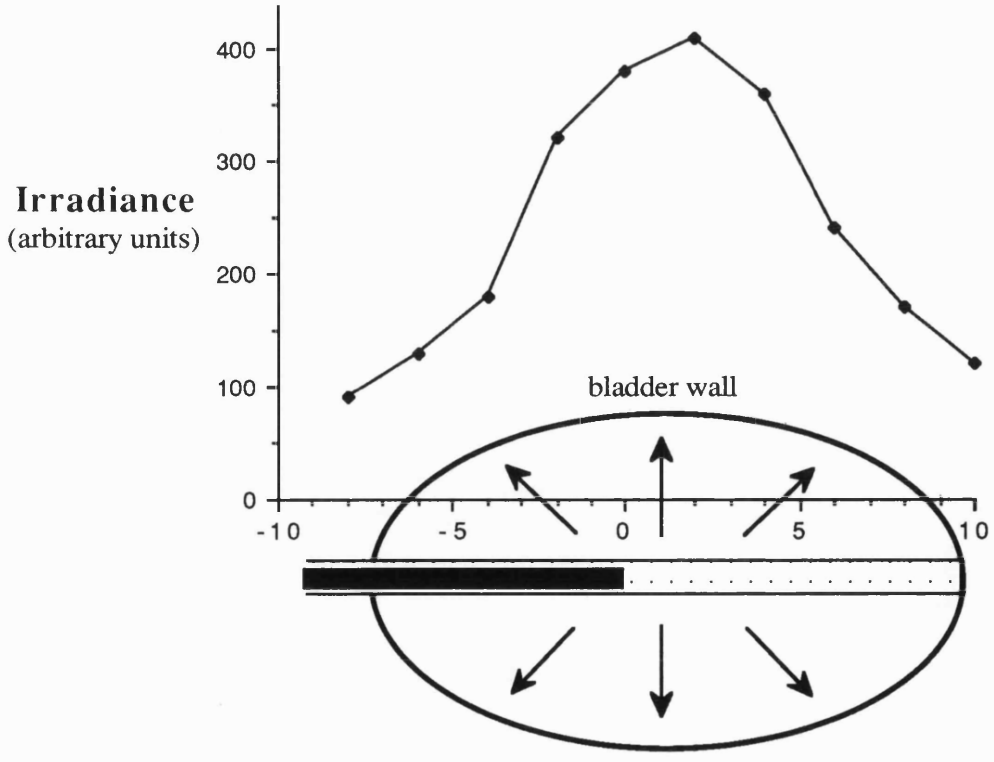


Fig. 7.9 Plot of fibre irradiance (*in vivo*)

7.2.2 Experimental groups

Adult female Wistar rats (weight approximately 200 g) were used for all these experiments. This model has been described in chapter 6 and will not be elaborated further here. The morphological effect of PDT after both intravenously and intravesically administered AlSPc mixture was studied.

7.2.2.1 Intravenous photosensitisation

Three dose levels of AlSPc (0.5, 1.5 and 5 mg/kg), administered by tail vein injection, were studied first in 12 animals (3 in each dose group plus 3 controls). Light treatment comprised a single dose of 20 J/cm^2 intravesical red light (675 nm) given 24 h after sensitisation as the fluorescence studies described in chapter 6 had indicated that this would be the most appropriate time interval at which to expect the maximal gradient in tissue sensitiser concentration between the superficial and deep layers of the bladder wall.

These first animals were killed at 1 week and the intention was to treat another 36 animals (plus controls) such that a total of 3 bladders from each dose group would be examined at intervals following PDT of 1 day, 4 days, 1 week, 3 weeks, and 3 months, to assess both the acute tissue response and the subsequent healing process. It was soon apparent that the higher doses were very toxic, with no animal treated with 5 mg AlSPc/kg surviving for more than 1 week, so this dose group was discontinued after the first 6 animals had been treated. As there was a large difference in effect between 0.5 and 1.5 mg/kg, an intermediate 1 mg/kg dose was added.

The effect of increased light dose (40, 80 and 200 J/cm^2) was looked at in a second series of 54 animals receiving either 0.25, 0.5 or 1 mg AlSPc/kg, as the combination of 1.5 mg AlSPc/kg and 40 J/cm^2 light was also found to be too toxic. Three animals from each group were treated with the 3 higher light doses and sacrificed after 4 days, 1 week, and 3 weeks. An

additional control group comprised 6 animals (3 at 80 and 200 J/cm^2 examined at 1 week) and was particularly important here as it would be expected that any unwanted thermal effect would be most likely after more prolonged illumination. In fact as no thermal effect was apparent at these fluences the controls for the lower light doses were unnecessary but they guarded against any other effect that might have occured as a result of unforeseen experimental variability. Treatment parameters which proved fatal, or caused sufficient morbidity for the animal to be sacrificed, were only repeated if their validity was thought insufficient.

7.2.2.2 Intravesical photosensitisation.

Three concentrations of intravesically administered AlSPc solution (0.02, 0.1 and 0.2 mg/ml) were studied. Control animals received saline. Each drug concentrations was instilled for either 30 or 60 min as described in chapter 6, after which time the sensitiser solution was drained and the bladder gently washed out twice with saline to remove any non-absorbed sensitiser immediately prior to light treatment (20 J/cm^2). Bladders were examined after 4 days, 1 week and 3 weeks and a total of 60 animals were used (3 in each of the 18 groups plus 6 controls). Three additional control animals had a 60 min instillation of the 0.2 mg AlSPc/ml solution but no illumination. Their bladders were examined at 1 day, 4 days and 1 week for any possible direct toxic effect of the sensitiser.

The purified S2 preparation (section 6.2.1) only became available after the main body of this work had been completed, but as the fluorescence studies (section 6.3.1.2) had suggested that the intravesical uptake of S2 was rather more superficial and uniform than that of the mixture, a limited assessment of this drug was made. Nine animals were treated with an intravesical solution of 0.2 mg AlPcS2/ml for 60 min followed by 20 J/cm^2 light. Three bladders were each examined after 4 days, 1 week and 3 weeks.

7.2.3 Histological studies

The morphological effects of PDT on the normal rat bladder were studied in all animals that survived the combinations of sensitiser and light dose described above. The rats were sacrificed (by CO_2 narcosis) at the above specified intervals from 24 h to 3 months. The bladders were catheterised, emptied of urine and distended with 0.3 ml of 10% formaldehyde (i.e. the treatment volume). A lower abdominal incision was made and the bladder mobilised fully. A silk tie was placed round the urethra before this was divided to prevent the bladder collapsing. Bladders were fixed in 10% formaldehyde solution prior to routine histological processing which was done at the ICRF histopathology laboratory in Lincoln's Inn Fields.

The fixed bladders were embedded on end so that when the blocks were cut complete transverse sections were obtained. It has already been mentioned that light distribution was most even, and the intensity greatest, over the central third of the bladder so sections were routinely cut from this area. Usually 4 levels were examined. Some comparative sections cut more towards the bladder neck or the apex in 10 specimens did not show any consistent variation from those taken within the central area. In the bladders treated at the higher doses a very small apical area of discolouration would often be seen in the first 2 weeks which corresponded to the position of the open end of the diffuser cannula. Microscopically this indicated a transmural inflammation with associated haemorrhage.

Standard haematoxylin and eosin staining (H & E) was used throughout though some duplicate sections were stained with van Gieson and haematoxylin (HVG) to assess collagen deposition during healing. All slides were assessed by the author, but not in a blinded fashion, and the percentage area of bladder wall where necrosis of either the superficial or deep layers was seen assessed as 0, <10, 10-25, 25-50, 50-75 or 75-100%.

7.3 RESULTS

There were a wide range of effects noticed following the PDT described in the previous section. The magnitude of these ranged from little discernible change to rapid death, and in general were more dependent upon the dose of photosensitiser rather than the light dose. It is convenient to consider these changes in terms firstly of effects on the whole animal, then macroscopic changes to the bladder and associated tissue and finally the spectrum of microscopic damage and repair. The results of intravesical sensitisation will be presented last of all.

7.3.1 Whole animal effects

No ill effect was seen in any control animal (light but no photosensitiser). The higher doses of PDT proved very toxic, with no animal treated with 5 mg AlSPc/kg and 20 J/cm^2 of light surviving for more than 1 week, so this dose group was discontinued after the first 6 animals. In this group, from the initial recovery from anaesthetic, the animals would appear less active than expected. By the next day they would remain lethargic and unwilling to feed or drink. All would be incontinent as judged by urine soaking of lower abdominal fur though only in one or two was slight haematuria noticeable. The rats would progressively lose weight, up to 30% of initial values by 4 days, and appear more unwell, often shivering and completely immobile. No antibiotics nor other supportive measures were used that might have enabled some animals to survive this period which was almost certainly due to septicaemia though no blood culture was performed. The great majority of deaths (or the need to kill an ill animal) occured within 3 days and if a rat survived 7 days it usually recovered. A significant morbidity was noticed in 80% (12 of 15) of the animals treated with 1.5 mg/kg AlSPc and 20 J/cm^2 of light and mortality was 40%; this rose again to 100% after 40 J/cm^2 (3 animals). Table 7.1 summarises these findings.

AlSPc Dose (mg/kg)		Light Dose (J/cm^2) 20	40	80	200
0.5	Morbidity	0	0	22	33
	Mortality	0	0	0	0
1	Morbidity	40	78	100	100
	Mortality	0	22	67	100
1.5	Morbidity	80	100	-	
	Mortality	40	100	-	
5	Morbidity	100	-		
	Mortality	100	-		

Table 7.1 **Percentage incidence of morbidity and mortality in the treatment groups.**

Lower doses of AlSPc were associated with less morbidity and no death occurred in animals treated with 20 J/cm^2 light after either 0.5 or 1 mg/kg AlSPc. Transient lethargy or incontinence was seen in 40% of the 1 mg/kg group but no adverse effect in any of those treated after 0.5 mg AlSPc/kg.

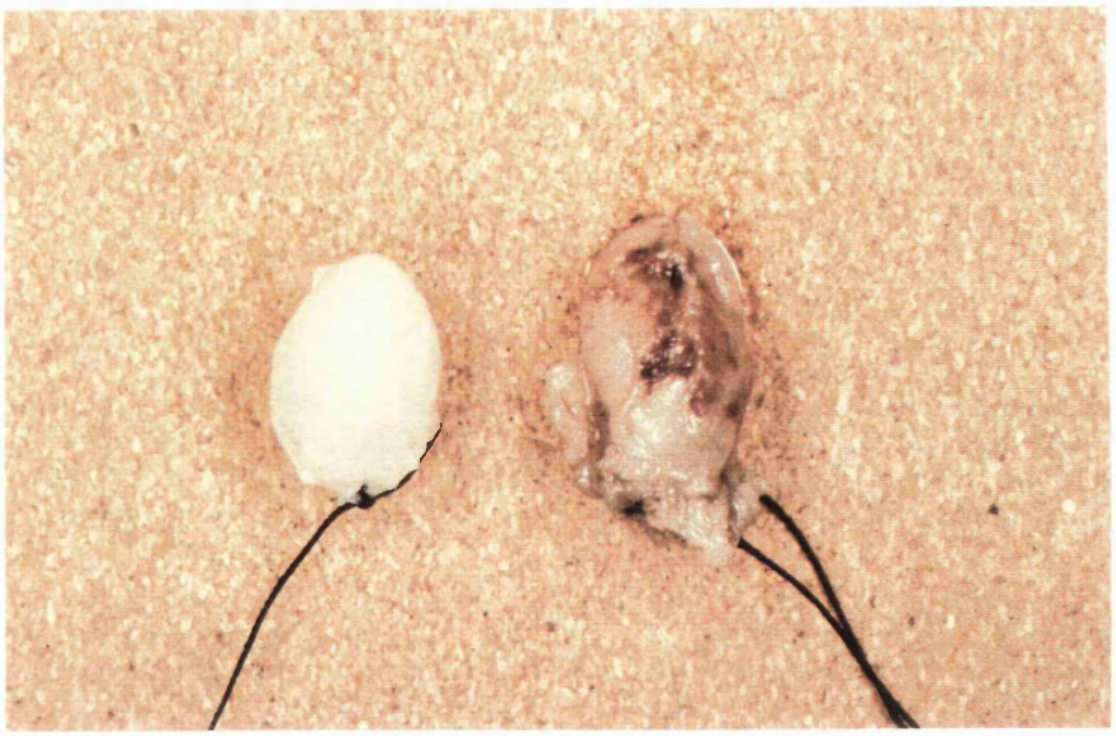
As the photosensitiser dose was reduced a higher light dose could be given before encountering significant morbidity and mortality. It is important to note that these adverse effects did not seem merely proportional to the PDT dose (the product of sensitiser dose and light dose) as increasing the drug dose above 1 mg/kg was more harmful than corresponding increases in light dose at lower sensitiser levels. Indeed with 0.5 mg/kg AlSPc the highest light dose studied, 200 J/cm^2, did not cause any mortality and little extra morbidity over the 20 J/cm^2 group. The implication of this finding will be discussed later. However longer treatment times required top-up increments of anaesthetic to be given approximately every 30 - 40 min (200 J/cm^2 represented an illumination time of nearly 75 min) and 3 animals in these groups failed to recover from the anaesthetic.

7.3.2 Macroscopic changes

The substantial mortality that occured following the higher doses of AlSPc studied and only 20 J/cm^2 of whole bladder illumination has already been mentioned. At autopsy large areas of the bladder would appear necrotic and the released urine would be purulent. Often multiple small renal abscesses were seen (figs. 7.10), usually associated with dilated ureters which would seem to indicate that reflux of infected urine had occurred. These may, though, have been a manifestation of systemic sepsis but no abscess formation was seen in any other organ and these renal changes were only seen in the more severely affected animals.

After lower doses (1.5 mg AlSPc/kg and below, and 20 J/cm^2 light) the bladder and the immediately adjacent perivesical fat usually appeared uniformly oedematous and often reddened. The small apical area where the open tip of the cannula had been in contact with the bladder mucosa would be a little darker indicating greater damage with some haemorrhage but this area never perforated. The adjacent small bowel had been shielded from transmitted illumination during light exposure and in no instance was any damage apparent to this. Damage to small bowel has been seen by other workers if this precaution is not taken (H. Barr, personal communication 1988). It would not have been possible to completely shield the pelvic organs lying directly behind the lower bladder without fully mobilising it but a careful inspection of these at the time of cystectomy did not indicate any injury even in the higher treatment groups.

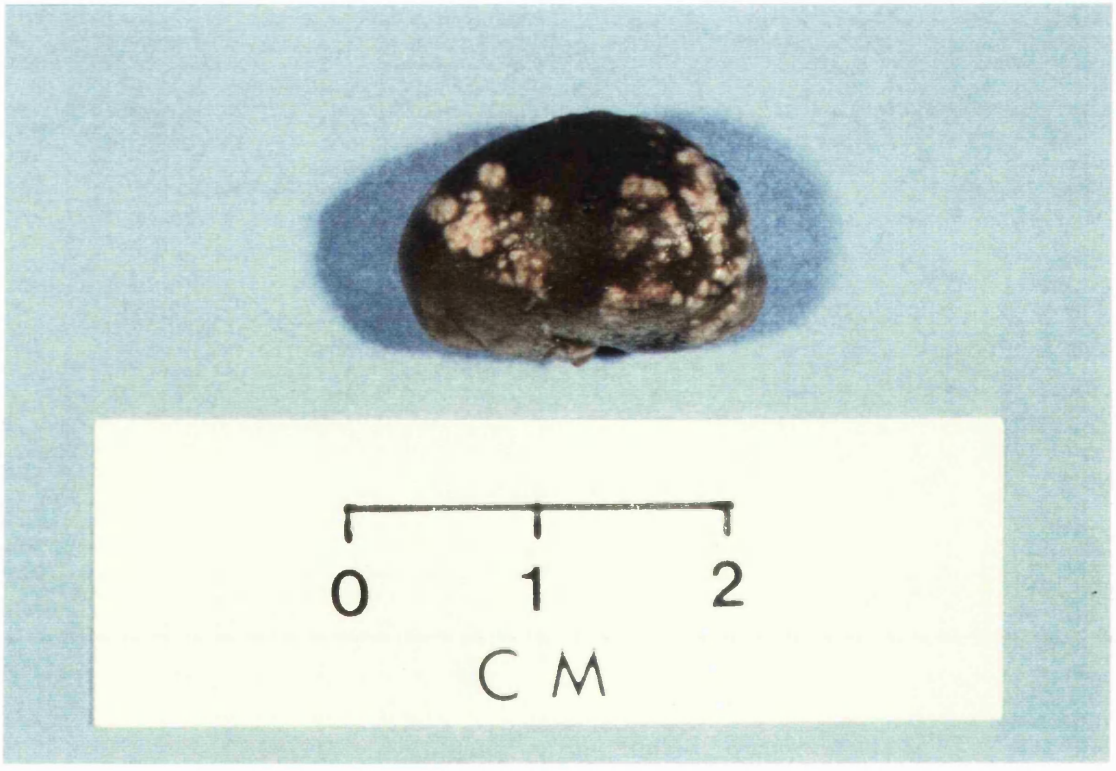
In the case of the majority of animals treated at lower doses, that appeared well at the time of sacrifice, very little external damage to the bladder was apparent. The mucosa was not inspected, though, as this would have have necessitated opening the bladder and disrupting the tissue architecture for subsequent histological assessment.



Figs. 7.10 Necrosis produced by high doses of PDT

Bladder on right is 3 days after PDT (5 mg/kg AlSPc 24 h prior to 20 J/cm^2 light @ 675 nm) and shows multiple areas of necrosis. Control bladder is shown on left (20 J/cm^2 light only).

Typical appearance of a kidney 3 days after these treatment parameters showing multiple small abscesses is seen below.

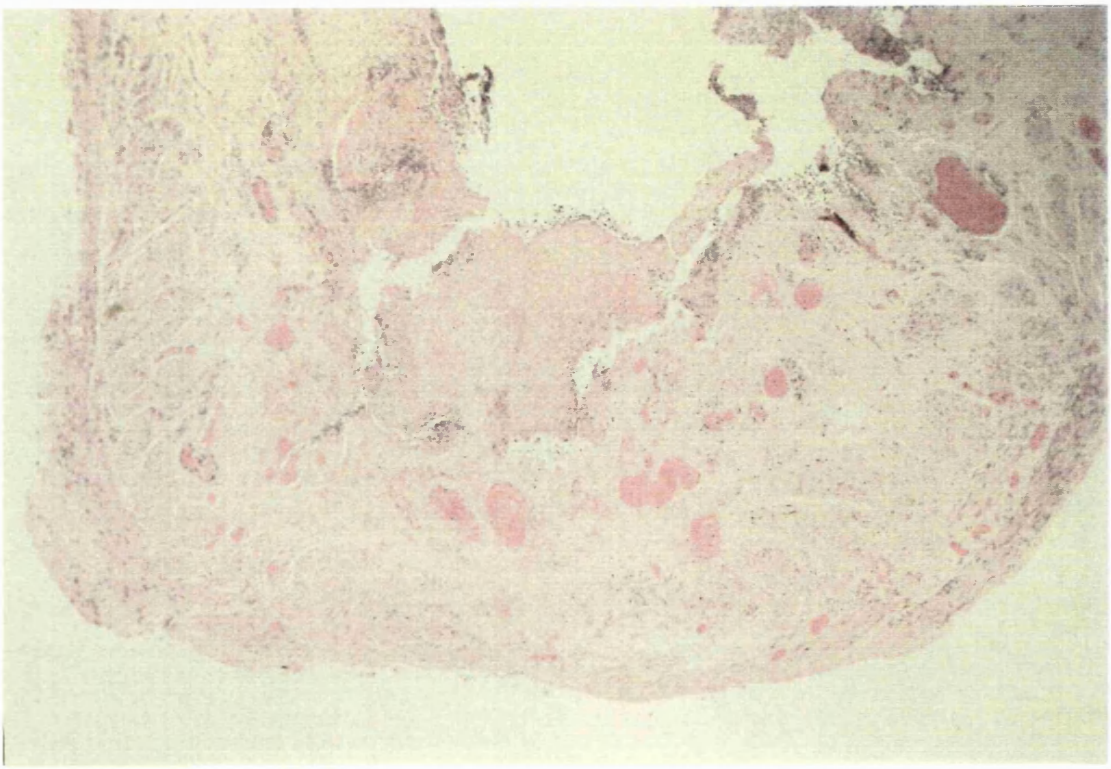


No control bladder showed any sign of thermal injury and there was no problem with infection or breakdown of the abdominal incision, the skin sutures were usually chewed off by the animals within 4 - 6 days.

7.3.3 Microscopic changes

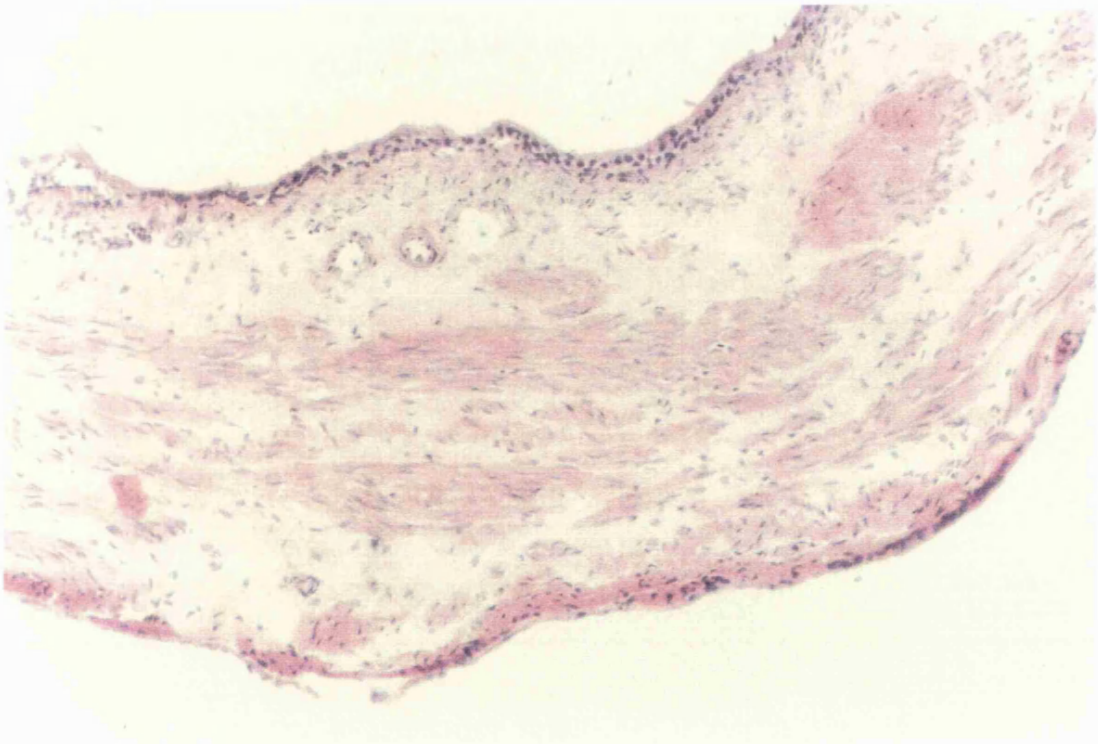
The extent of the tissue damage produced depended on the treatment parameters chosen, i.e. sensitiser concentration and light dose. Early acute inflammatory changes and oedema were seen predominantly in the submucosa, with urothelial destruction by 2 - 3 days. The maximum effect was seen after 4 - 6 days and at the higher sensitiser doses which resulted in a high mortality (2.5 - 5 mg/kg), there was florid acute inflammation throughout the bladder wall with large areas of muscle necrosis. At the intermediate doses there was no surviving urothelium seen, and a largely transmural inflammation (figs. 7.11). Vascular changes were amongst the earliest seen (by 24 h) and were particularly prominent in the submucosal blood vessels. Extensive coagulation was the most common finding though extravasation and haemorrhage were also seen.

A lower dose of AlSPc (in the order of 1 mg/kg) with 20 J/cm^2 light only produced superficial damage, the muscle layer being unaffected. With 0.5 mg/kg AlSPc a light dose in the order of 40 J/cm^2 was necessary to produce a consistent epithelial destruction (fig. 7.12), and with 0.25 mg/kg no significant PDT effect was produced even with 200 J/cm^2 of light. The relationship between sensitiser and light dose in producing either superficial damage (typified by loss of the mucosa and inflammation of lamina propria), or deep necrosis (with significant involvement of the muscle layers also) is summarised in table 7.2 below. On the whole there was a reassuring degree of consistency in the effect seen between individual bladders within each treatment group.



Figs. 7.11 Acute inflammation of the full thickness of the bladder wall. Note coagulation in submucosal blood vessels

4 days after PDT with 1.5 mg/kg AlSPc, 24 h prior to 20 J/cm^2 light @ 675 nm. Sections were taken from the middle region of both the treated (above) and the control bladders (shown below for comparison - no AlSPc, 4 days after 20 J/cm^2)



This assessment was by its nature rather empirical relying on the author examining 4 sections from the central third of each bladder. In practice, though, PDT damage was usually either superficial, or it was transmural in most areas. There were only very few occasions when the acute inflammatory changes after PDT were into muscle but did not penetrate to the deeper levels of this layer. As the assessment was only intended to be a qualitative one it is not possible to tabulate the findings in any more meaningful a way. There was less difference between the various sections from each individual bladder than between different bladders given the same treatment and in general this latter variation was acceptable. The percentage areas of bladder damage given indicate the authors assessment of the average surface area affected across the range of specimens studied (up to 9) at each treatment dose. The time of maximum damage referred to in table 7.2 was usually at either 4 days or 1 week after the light exposure.

AlSPc Dose (mg/kg)	Extent of necrosis	Light Dose (J/cm^2) 20	40	80	200
0.25	Superficial	0	0	0	0
	Into muscle	0	0	0	0
0.5	Superficial	< 10	10-25	50-75	75-100
	Into muscle	0	0	0	< 10
1	Superficial	25-50	75-100	100	Died
	Into muscle	0	10-25	50-75	-
1.5	Superficial	75-100	Died	-	-
	Into muscle	25-50			
5	Superficial	Died	-	-	-
	Into muscle	-			

Table 7.2 Effect of AlSPc and light dose on PDT necrosis in the superficial and deep layers of the rat bladder wall.

The figures in the "light dose" columns represent the average area (%) of the bladder necrosed at the time of maximum damage.

The effect of increasing the light dose up to 200 J/cm^2 (longer exposures at the same fluence rate), which was studied on animals receiving the lower doses of AlSPc (0.5 - 1 mg/kg) deserves special mention. Unsensitised controls showed no changes at any of these levels thereby excluding a thermal component to the observed effect.

At the lowest of these sensitiser dosages (0.5 mg/kg), the light dose needed to produce a given depth of tissue damage became less critical. It can be seen from these results that using 0.5 mg/kg AlSPc the desired effect of urothelial loss without damaging muscle could be achieved with light fluences between 40 and 200 J/cm^2 (table 7.2). This increased latitude is thought to be the result of sensitiser photobleaching and is likely to be of major clinical importance, as will be discussed later.

Healing of PDT damage occurred rapidly and wholly. Superficial damage would heal by complete regeneration of normal tissue within 2 weeks to give an appearance indistinguishable from the control sections. Even where the initial inflammatory changes were transmural, complete repair had occurred by 3 weeks in surviving animals (fig. 7.13).

Clinical studies have cited muscle scarring as the cause of the impaired bladder function (Harty *et al.*, 1989). However in this work no long term histological abnormality in any surviving animal has been demonstrated after the acute inflammatory process had settled. This involved a careful study not only of the standard H & E stained sections but also of adjacent sections stained for collagen with HVG. In no case was there any sign of muscle fibrosis nor any long-term change in the amount or pattern of collagen deposition seen in the sections (fig. 7.14). There appeared to be some apparent increase in collagen in the acute phase but these sections may have been misleading as the considerable amount of tissue oedema present

would have the effect of separating collagen fibres and giving a false impression of expansion. At least there was no destruction of collagen by PDT, as would be expected in a thermal injury for instance, which is consistent with other workers' findings (Barr *et al.*, 1987a).

7.3.4 Intravesical administration of AlSPc

PDT following the intravesical administration of AlSPc mixture produced patchy and unpredictable results often with areas of full thickness damage adjacent to those of minimal change (fig. 7.15). There was a much greater variability between specimens than was seen after parenteral administration and the observed damage did not correlate well with either the sensitiser concentration used, the time it had been in contact with the bladder mucosa or the treatment light dose. This is consistent with the results of the fluorescence studies described in chapter 6 which had shown an irregular uptake of AlSPc into the bladder wall.

The rather more limited assessment of intravesical AlPcS2 seemed more promising (this compound was only available late on in this work after all the bladder function studies had been completed using the AlSPc mixture). Fluorescence localisation had shown a very superficial uptake of the S2 drug with minimal penetration to the deeper layers (section 6.3.1.2) and after light exposure, damage seemed much less extensive than that possible following either intravenous sensitisation or intravesical administration of the mixture. The low-power section in fig. 7.16 demonstrates the much more uniform mucosal loss. The medium-power plate shows some basal mucosal cells which may be either the result of early regeneration or incomplete loss. Typical coagulation in submucosal blood vessels is well demonstrated and the muscle layer is seen to be undamaged. It is of some interest that no full thickness necrosis was observed in any section.

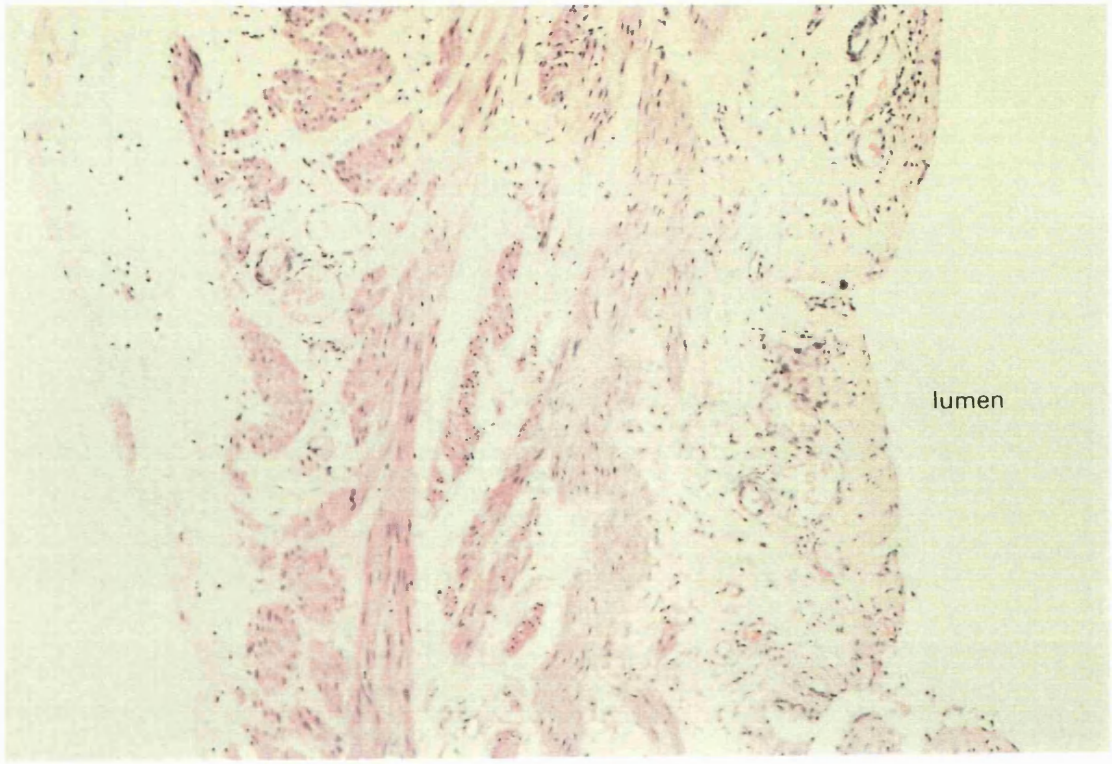


Fig. 7.12 Photodynamic damage limited to the superficial layers of the bladder after reducing the photosensitiser dose and increasing the light dose.

Note epithelial loss and some acute inflammation and oedema of the lamina propria but no damage to muscle. (4 days after PDT with 0.5 mg/kg AlSPc, 24 h prior to 80 J/cm^2 light @ 675 nm)

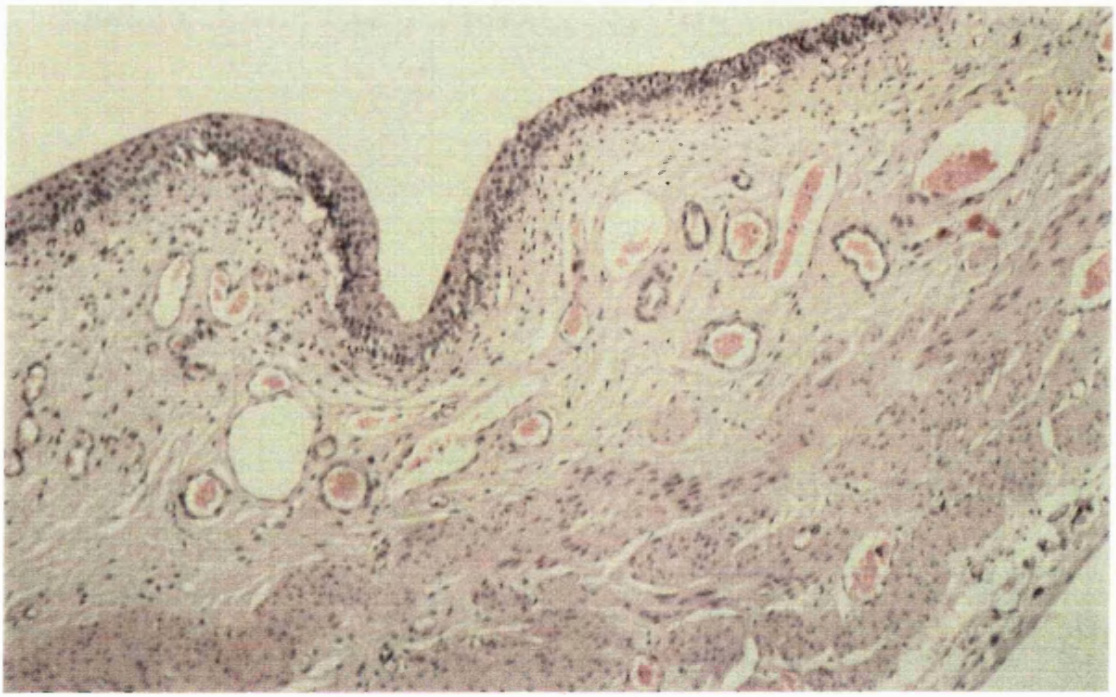


Fig. 7.13 Healing with complete regeneration of bladder wall architecture after 21 days

(Same treatment parameters as fig. 7.11)

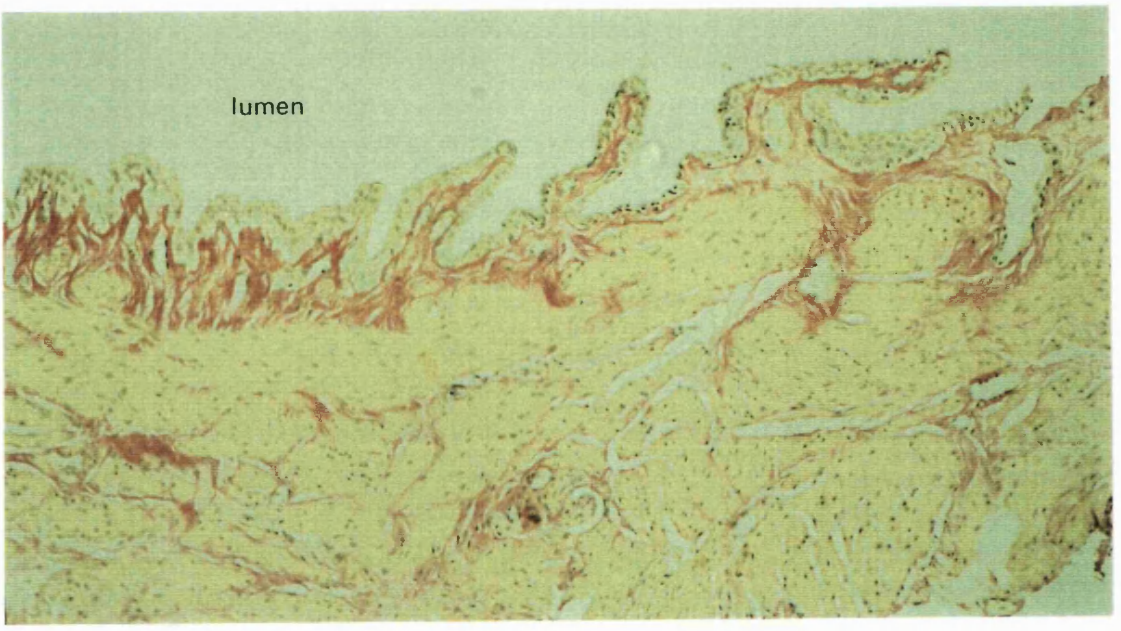


Fig. 7.14 HVG stained section of bladder wall 3 months after PDT when there was still some impairment of bladder function.

(1.5 mg/kg AlSPc and 20 J/cm^2 light) No increase in collagen deposition (pink / red staining) is seen

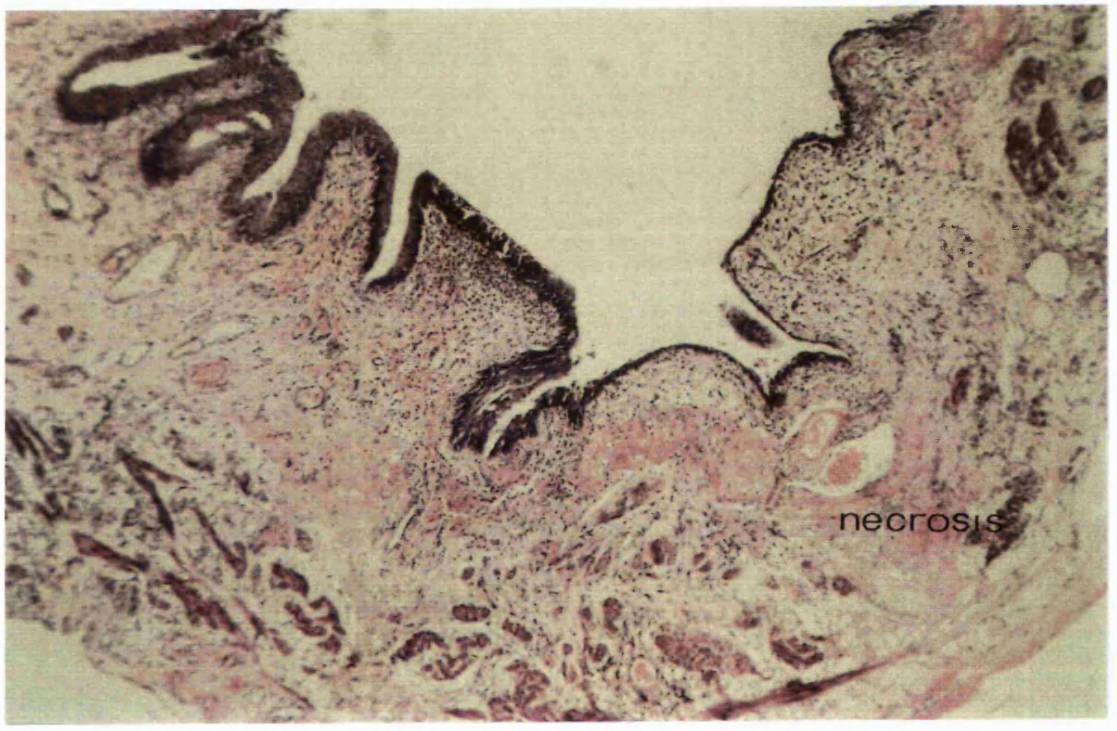
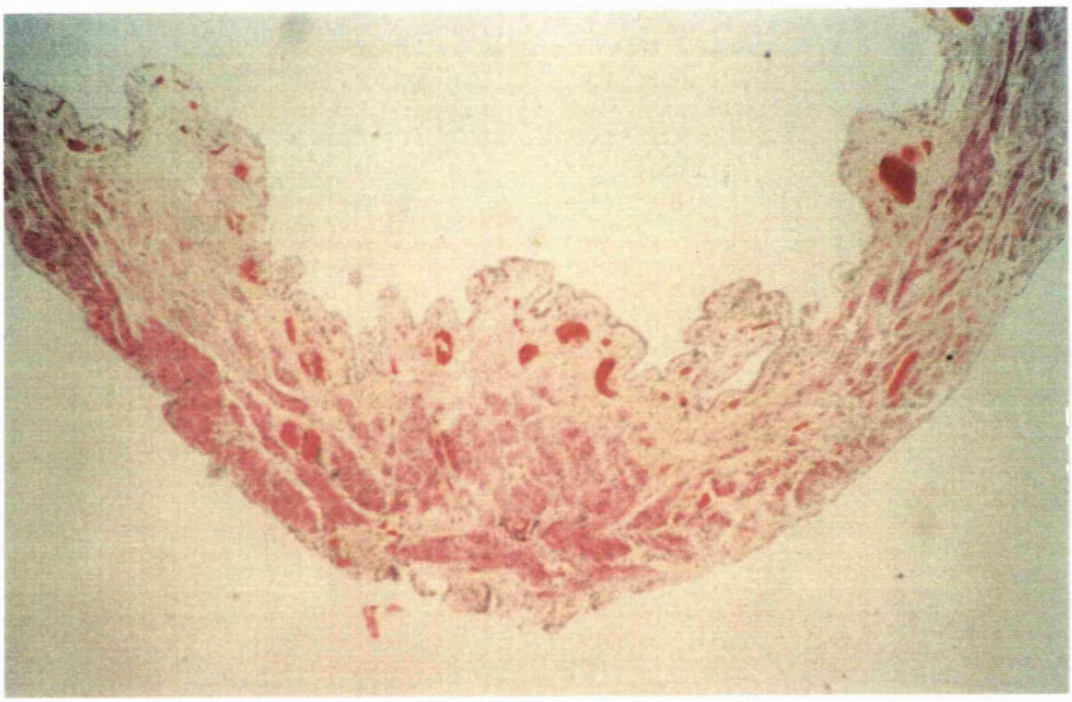


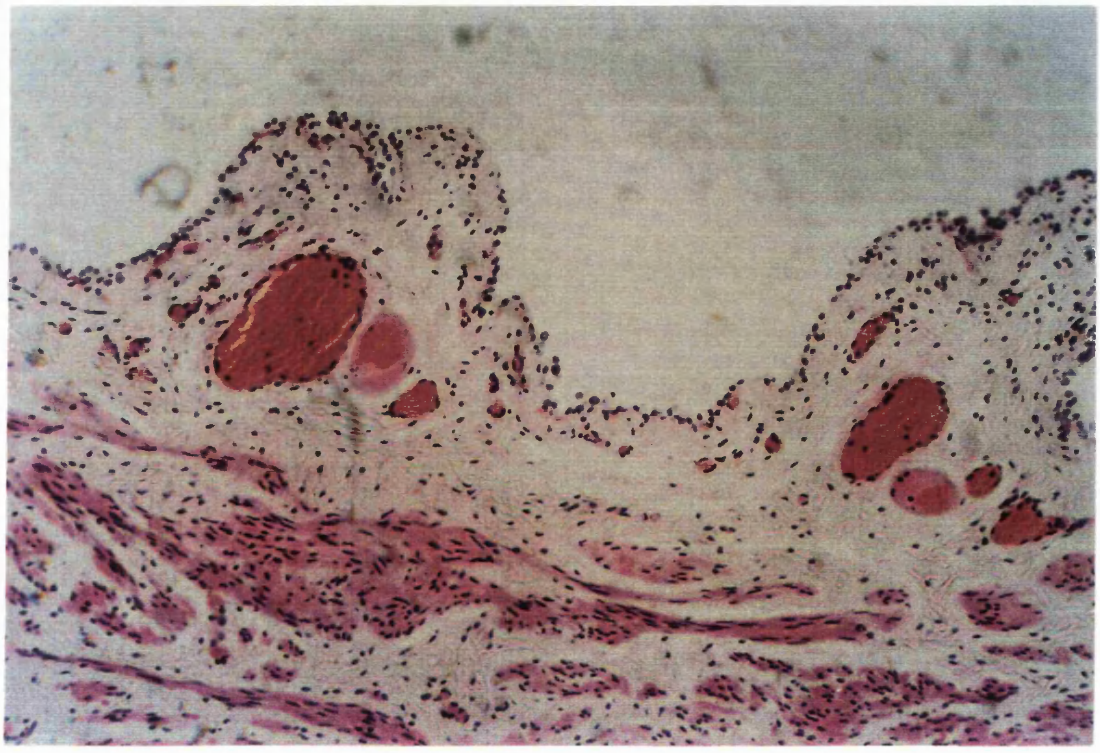
Fig. 7.15 PDT after intravesical administration of AlSPc mixture

Patchy damage is seen with an area of full thickness necrosis adjacent to an area showing the urothelium intact and superficial oedema only. (4 days after PDT with 20 J/cm^2 light @ 675 nm delivered after sensitisation by the intravesical administration of 0.3 ml of a 0.2 mg/ml solution of AlSPc, mixture, for 1 h)



Figs. 7.16 PDT after intravesical AlPcS2 showing a more even effect. (same treatment parameters as in fig. 7.15)

The lower section is a higher power view from the right end of the section above and shows more clearly the epithelial loss with no muscle damage. The coagulation seen in the submucosal blood vessels is well demonstrated.



7.4 DISCUSSION

The experiments described in this chapter have demonstrated a range of tissue effects produced by PDT on rat bladder. The aim has to been to cause a comprehensive necrosis of the superficial layers of the bladder wall which does not result in significant damage to the deeper muscle layer. One is unlikely to be able to rely in the clinical setting on selectivity of the PDT effect to spare normal epithelium and yet destroy all areas of rather thicker malignant epithelium and small exophytic tumours. As the entire bladder surface will be treated simultaneously this requires a uniform effect which in turn necessitates an even illumination, in the absence of significant photobleaching, assuming that there will be consistent uptake of photosensitiser. This is a critically important factor as areas receiving less than the intended light dose (i.e. furthest away) will be undertreated whereas receiving most those (i.e. the closest) may be damaged excessively. It is in the control of this variable that most dosimetry inaccuracies arise as there are at least 5 factors that need to be considered in this model.

In this work a cylindrical diffuser positioned along the longitudinal axis of the bladder was used to diffuse the light from the laser fibre tip. This arrangement is recognised to have several shortcomings particularly in that although there was a fairly even illumination over the mid-part of the bladder this tailed off significantly at both ends (fig. 7.9). All sections studied were taken from the middle third of the bladder and should at least be consistent between groups. Indeed when macroscopically assessing the bladder there was no impression that damage was greatest in this area even at the highest doses (figs. 7.10). The inexact positioning of the fibre along the central axis of the bladder was another source of error tending to lie closer to the posterior wall despite efforts to correct this (fig. 7.5). Bladder wall asymmetry is another factor that could not be assessed or

compensated for, though may be minimised by maximally inflating the bladder which was not thought advisable for reasons mentioned earlier. The high forward beam through the open ended cannula was thought to be less important than these other factors as it was so localised. It was not possible to cap the cannula as it needed to be introduced over a guidewire. Finally there may have been unrecognised variations in the laser output or degradation of the fibre tip during treatment. This was guarded against by not only monitoring the laser's in-line power meter during the exposure but also by checking that the output from the fibre tip had not reduced at the end of the exposure, and at intervals during the longer exposures as already described. It was very unusual to find even a minor error here.

All these sources of inaccuracy will be relatively much greater in the small rat bladder compared to Man but even in a 300 cc bladder a variation of only 6 mm in central positioning will lead to an error at the bladder surface of ± 18 %, and that does not allow for any bladder wall asymmetry (Nseyo *et al.*, 1991). Clinical workers have used diffusing solutions such as dilute Intralipid to scatter light from a square-cut fibre tip in the bladder (section 3.4.2.2). However although this more evenly distributes the primary beam, light loss within the medium is increased and surface reflectance from the bladder wall is much reduced. Internal reflectance makes a significant contribution to the evenness of the overall light distribution such that if it is reduced by a diffusing medium, the uniformity of light at the mucosa is impaired even more by slight errors in precise central positioning of the fibre or by a non spherical bladders. As both these conditions were likely in this model Intralipid diffusion was not pursued further but an intensive assessment of light dosimetry was considered outside the scope of this present work and the interested reader is referred elsewhere (Marynissen *et al.*, 1989; Plail *et al.*, 1990).

The best solution may have been to use a miniature bulb-tip fibre but a small enough device was not available when this work was started. Even bulb-tip fibres tend to scatter more light forward than backwards and are more likely to deteriorate than plane-cut fibres. In addition one ideally needs a method for assessing fluence at the bladder wall.

It has been shown that the true light fluence incident on the mucosa is much greater than the calculated fibre tip fluence due to significant internal reflectance from the bladder mucosa (Marynissen *et al.*, 1989). The important light dose in determining PDT effect, however, is not the amount that falls on the bladder mucosa but the actual amount of light absorbed by the photosensitiser molecules in the bladder wall. Therefore individual photons may be internally reflected within the bladder cavity several times (and each time contribute to a high incident fluence) but it is only when they strike a molecule of the photosensitiser and are absorbed by it (which can only occur once) that any photodynamic action may result. This absorption cannot be accurately estimated particularly as in this rat model the bladder wall is thin enough to transmit unchanged a large amount of the incident light dose at this wavelength (probably at least two-thirds). It is accepted convention, despite these shortcomings, to describe the treatment light dose in terms of the incident non-scattered fluence at the fibre tip divided by the surface area illuminated, which is what has been done in these experiments. For all these shortcomings the method used here was consistent in that light distribution and absorption would have been broadly the same in all the bladders treated thus allowing a valid comparison to be made between the treatments groups studied.

These comments illustrate how difficult it has proved to quantify light dosimetry in all studies of PDT in the bladder. Some work has begun to address these problems and more effective delivery systems are being

developed which will hopefully enable bladder PDT to become less cumbersome and unpredictable. One laser catheter assembly in particular looks promising, comprising a translucent balloon inflated to distend the bladder into an sphere of accurately known dimensions and fixing a bulb-tipped catheter at its centre. A detector fibre incorporated into the balloon monitors light fluence at the bladder wall (Nseyo *et al.*, 1991).

The clinical results so far reported for PDT in bladder cancer using HpD suggest that it will be most useful when applied to the whole bladder mucosa to treat multifocal Cis and diffuse abnormal areas which may not be apparent visually. In these situations the effect on normal bladder tissue becomes important as the ideal photosensitiser with a total selectivity for malignant and dysplastic tissue has yet to be developed. An initial period of bladder irritability is of course to be expected after whole bladder PDT as there is a florid acute inflammatory response which however much one can manipulate conditions will inevitably damage normal mucosa if it is going to treat neoplastic areas effectively. It would seem from these experiments, though, that even totally necrosed urothelium will, at least in rats, regenerate normally after PDT with AlSPc within 2 - 3 weeks.

This process of repair is most likely to be by regeneration of residual epithelial cells in surviving areas. If the entire bladder mucosa has been necrosed re-epithelialisation may occur from the proximal urethra and terminal ureters. Wishnow *et al.* (1989) performed an elegant series of experiments in which the entire canine bladder mucosa was denuded with a Nd:YAG laser using a sapphire tip. Regenerating mucosa was seen spreading away from the urethra and ureteric orifices by 1 week and a complete re-covering with a hyperplastic epithelium had occured by 3 weeks which had returned to normal appearances within 3 months. They produced isolated pouches from denuded bladder and found that those

containing an ureteric orifice, and hence a source of transitional cells, regenerated normal mucosa whereas those that did not include an ureteric orifice remained denuded. In patients with Cis of the bladder it is well recognised that there may be involvement of the terminal ureters or prostatic fossa. It would therefore seem prudent to exclude this before PDT was performed as these areas are inadequately treated by whole bladder illumination alone and residual tumour at these sites would seem to doom PDT to failure. However there is a strong likelihood that one could treat both these sites with PDT after the bladder had healed. There would be no technical difficulty in illuminating either the lower ureter or prostatic fossa with an appropriate cylindrical diffusing fibre and they should re-epithelialise without scarring, though a period of stenting or catheterisation would be advisable.

The problems reported in clinical series using porphyrins result from PDT damage to the deeper muscle which appears to heal less completely than urothelium with at least some fibrosis (Harty *et al.*, 1989), resulting in a permanent and often severe reduction in bladder capacity and compliance. In this study, no long term histological abnormality was demonstrated in those surviving animals whose bladder muscle was damaged following PDT with AlSPc, but this is more likely to be an limitation of the model rather than a true difference between the porphyrins and the phthalocyanines.

The assessment of histological damage in this work is recognised to be somewhat qualitative and open to bias. An assessment of the slides by an observer unaware of the treatment protocols might have avoided the latter but would have involved other colleagues significantly in this work.

The rat bladder is clearly much thinner than that in Man and is easily penetrated by the 675 nm light used here so that, if anything, rather more permanent damage might be expected. However less was seen in this study,

but this is probably because any distressed animals were killed and no supportive treatment such as antibiotics was used. Therefore the more severely affected cases, which may have shown bladder fibrosis upon healing, did not survive. This is not going to be the case in Man as although some patients have become quite toxic after PDT even to the extent of voiding a sloughed "cast" of their bladder mucosa (Williams and Stamp, 1988), no fatalities have been reported. However in order to avoid these unwanted effects in patients it does seem advisable to try and limit all damage caused by PDT to the more superficial layers of the bladder wall.

7.4.1 Improving selectivity of PDT

The factors determining PDT effect and how these may be modified to improve selectivity of action have been discussed in chapter 4. It was concluded that it was less important to attempt to achieve any form of selectivity between normal and abnormal mucosa than to try and limit the damage to the mucosa and submucosa and leave the muscle intact. Perhaps the most attractive way of achieving this is the hope that if the sensitiser was administered directly into the bladder, as is of course the case with all other drugs used to treat superficial bladder cancer, then uptake and the resultant PDT effect would be superficial. It was disappointing to get such unreliable results therefore after the intravesical instillation of the AlSPc mixture but further study on this is still required, particularly using the more lipid soluble AlPcS2 preparation the preliminary experience with which reported here is rather encouraging. It should be pointed out that the intravesical administration of HpD has not been successful either (Lin *et al.*, 1984), but means of enhancing uptake into urothelial cells using penetrating agents seem more promising (Rosenberg and Williams, 1986). It would be expected that the direct uptake of the photosensitiser into malignant urothelium and areas of dysplasia may be better than in the

normal bladder as the protective, waterproof umbrella cell layer will have been disrupted by neoplastic change. Evidence for the clinical efficacy or otherwise of intravesically administered AlPcS2 will have to wait, though, until approval for human administration is forthcoming.

The PDT effect depends on the tissue concentration of photosensitiser at the time of treatment and the light dose applied. Experiments on other tissues (particularly the colon) have established that there is a threshold level for the tissue concentration of photosensitiser at the time of light exposure below which insufficient singlet oxygen is generated to cause tissue damage, even in the presence of excess light (Barr *et al.*, 1990a). This is because the sensitiser is destroyed by photobleaching as previously discussed (section 4.3.3). Above this threshold reasonable reciprocity is seen between the sensitiser concentration and light dose required to produce a given biological effect. Photobleaching was initially thought to be a disadvantage but if it were possible to achieve a concentration of photosensitiser in muscle below the threshold level, whilst that in the superficial layer remained adequate for photodynamic damage, then there might be a mechanism for limiting PDT effects to the superficial layers. The threshold dose of photosensitiser is of course likely to vary for different time intervals between sensitisation and illumination so this should be standardised. In clinical practice the best ratio between tumour and the adjacent normal tissue, which is reached some 48 h after sensitisation, would be further expected to enhance selectivity, though this is unlikely to be by more than 2 or 3:1.

The experiments described in this chapter confirm that with a low dose of AlSPc (0.5 mg/kg in the rat, 24 h prior to light exposure), damage can be limited to the superficial layers in a way that is independent of the light dose, a therapeutic ratio not seen when using higher concentrations of

sensitiser (table 7.1). The only requirements are that sufficient light is delivered to every point on the bladder surface and that the fluence rate is not high enough to cause thermal effects. It would be a great advantage in clinical PDT to have to ensure only that each part of the bladder received a *minimum* light dose without concern over adverse effects from too much illumination in some areas. This would reduce many of the difficult dosimetry problems associated with the accurate calculation and even distribution of light over the entire bladder mucosa, to which end much effort has been directed as discussed above. That is not to say that light dosimetry in the bladder will not still be important as an estimation of absorbed dose will be necessary, but merely that some unevenness of illumination between different areas of the bladder may be accepted and not lead to inconsistent clinical results.

7.4.2 Conclusions

The aim of the work described in this chapter has been to investigate the morphological effects of PDT with AlSPc on a normal bladder model. Using whole bladder illumination with red light (675 nm) 24 h after intravenous sensitisation, a wide spectrum of tissue damage has been produced, the maximum effect developing 4 - 6 days after treatment. High photosensitiser and light doses in the rat produced a full thickness bladder wall necrosis, vesicoureteric reflux and renal damage with a high resultant mortality. These findings are a more extreme example of the major complications that have been seen clinically with HpD. It was only by using low doses of photosensitiser (0.5 mg/kg) that it was found possible to reliably destroy the urothelium, which rapidly regenerated, but not cause necrosis of the muscle layers. This is most likely due to the protective effect of sensitiser photobleaching in the muscle layers of the bladder at such low concentrations. The light dose required is higher than when using

larger amounts of photosensitiser but less critical, which should offset many of the dosimetry difficulties encountered in clinical use. As long as damage was limited to the superficial layers of the bladder, PDT caused no apparent long term morbidity, though a pronounced initial inflammatory reaction is inevitable.

The administration of AlSPc mixture by intravesical instillation proved rather unreliable with patchy, though often extensive damage being produced after illumination. There was no clear correlation between the dosimetry parameters employed (i.e. concentration and instillation time) and the degree of necrosis produced. Preliminary studies using the more lipid soluble S2 preparation of AlSPc have indicated that this may prove more satisfactory than the mixture but further clinical experimentation will be necessary to clarify the situation. Certainly there is a considerable attractiveness to the concept of intravesical administration of the photo-sensitiser which will provide a stimulus for further investigation of this aspect of sensitiser uptake.

This work has not looked at the effect of PDT on bladder tumours but in logically investigating the effects of a new therapy it is important to study the effect on normal tissues first, not withstanding the difficulties of producing an adequate animal model of superficial bladder cancer. This is because in whole bladder treatment, the situation in which PDT is most likely to be used clinically, the great majority of the bladder surface treated will comprise normal tissue. Treatment of this normal tissue must not result in unacceptable side-effects.

The next chapter presents what we believe to be the most important aspect of this work, the effect of PDT on bladder function, and discusses the correlation of these changes with the degree of tissue damage produced as seen in this chapter.

Chapter 8

FUNCTIONAL CHANGES IN THE NORMAL RAT BLADDER FOLLOWING PHOTODYNAMIC THERAPY WITH PHTHALOCYANINE PHOTOSENSITISATION

8.1 INTRODUCTION

Disturbances in bladder function are universal following PDT and most authors mention this. However the low prominence that these side-effects are given in most reports would suggest that there is little to worry about. However those authors who have carefully documented bladder function describe widespread and serious complications (see section 3.4.2.4). In particular Harty *et al.* (1989) noted a significant and permanent reduction in bladder capacity with ureteric reflux and hydronephrosis in 4 out of the 7 patients they treated. Often trabeculation of the bladder wall and widely open ureteric orifices were found at cystoscopy and deep biopsies showed extensive fibrosis and loss of smooth muscle cells. This was not an isolated report and similar problems have been reported by other groups (Nseyo *et al.*, 1985a; Williams and Stamp, 1988; Jocham, 1989). Both Nseyo and Jocham have commented that cystectomy was sometimes necessary for intractable frequency, though this may be masked by gross ureteric reflux and dilatation allowing a functionally significant urine capacity to be carried in the upper tracts! The net result is that most centres have stopped using PDT in the bladder and those still doing so are taking a lot more care with dosimetry (W Star, 1990, personal communication).

It is not going to be possible to avoid some irritative symptoms in the initial phase because the whole object of the treatment is to necrose areas of malignant urothelium which implies, as the degree of selectivity of PDT for malignant tissue is fairly poor, that a lot of normal mucosa will be destroyed as well. Even if good selectivity were possible, many of the patients with resistant Cis for whom PDT would be appropriate have a field change involvement of the whole bladder. It is apparent that those patients with persisting impairment of bladder function have suffered PDT damage to the bladder musculature which has healed with scarring rather

than by regeneration of normal tissue. The bladder urothelium seems to regenerate more normally after extensive loss presumably by growing in from surviving peripheral cells. The work presented in chapter 7 showed that in the rat this occurs within 3 weeks and therefore the aim should be to restrict PDT damage to the superficial layers of the bladder in the hope that the ensuing inflammatory changes will settle more quickly than if the muscle layers are also involved.

It has been seen in the previous chapter that it is possible to limit necrosis following PDT to the superficial layers which appear to heal satisfactorily by regeneration so that after 3 weeks or so the bladder looks histologically normal. This was more reliably the case when low doses of photosensitiser were employed and it was suggested from these results and from the fluorescence studies presented in chapter 6 (section 6.4.2) that although the amount of illumination required to get a adequate response was higher, the consequent photobleaching of sensitiser in the deeper tissue protected the smooth muscle from significant damage.

It is important to see if these concepts can be shown to reduce the degree and duration of functional disturbance after PDT and these experiments can be carried out quite adequately on the same model that was used for the morphological studies.

8.2 MATERIALS AND METHODS

8.2.1 Animals, photosensitiser and phototherapy

Similar Wistar rats were used for this study and most of the conditions described in section 7.2.1 apply here also so will not be repeated. Only AlSPc mixture was studied, given intravenously at either 0.5, 1 or 1.5

mg/kg to 4 animals in each group. A standard light dose of 20 J/cm^2 of 675 nm red laser light to the whole bladder was given 24 h after sensitisation. The high mortality and morbidity encountered in the group treated with 5 mg/kg and 20 J/cm^2 light precluded sufficient long term survivors for accurate assessment and so they are excluded from analysis.

8.2.2 Evaluation of anaesthetic technique

The requirements for a general anaesthetic for this work was that it should be easy to administer and maintain, and be sufficiently profound and long lasting to enable a range of procedures of up to about 60 min duration to be performed. Adequate post-operative analgesia was also required together with a good degree of abdominal muscle relaxation in order to neutralise any possible influence of intra-abdominal pressure on the pressure recorded within the bladder. It has been standard practice in our Unit to use intramuscularly administered anaesthetic agents to rats (and other rodents) in preference to inhalational anaesthesia, so there is little local experience with the latter method.

Evaluation of the depth and duration of surgical anaesthesia obtained by my standard technique was obtained by a separate experiment involving 6 rats. Anaesthesia was induced with Hypnorm (0.5 ml/kg) and diazepam (0.5 mg/kg), given by a single injection into the thigh muscle. Stimuli were then applied after a sufficient depth of anaesthesia had occurred to enable the animal to be restrained which took between 5 - 8 min from the time of injection. Three stimuli were thought to be potentially useful in assessing depth of anaesthesia:

i) movement in response to urethral catheterisation.
ii) movement in response to a sudden, sharp noise (finger snapping close to the animal's head.
iii) a drop of water onto the mouth and observation of a lick response.

Two other stimuli were investigated; the application of a drop of water to either the eye, eliciting a blink response, or to the nostril producing a twitching of the nose. Both these stimuli produced a response in all animals throughout the period of anaesthesia so were not discriminatory.

Responses were graded as nil, slight or moderate. A slight response implies a weak, non-purposeful reaction to the stimulus and which did not indicate any awareness. A moderate response needed to be more sustained and purposeful such that the animal would have moved if it were not restrained, and would have required further sedation to proceed with an experiment. These observations were recorded at 5 min intervals after induction (defined as the minimum time until the animal did not resist gentle restraint) until there was no response after which time they were repeated every 10 min. As the depth of anaesthesia lightened the stimuli were again repeated at 5 min intervals until a moderate response was recorded.

The results, which are shown in table 8.1, demonstrate that the sharp sound was just the most powerful of these stimuli and the duration of the complete loss of response to this provocation following a single injection ranged from 35 - 65 min. The recovery of a reaction to urethral catheterisation started on average some 5 - 10 min after this. The lick response disappeared quickly and only returned when the animal was practically awake again, so was not thought to be an adequate determinant. In practice it was found that when a rat would not respond to catheterisation then there was never any reaction to laparotomy.

The duration of some of the longer experiments was found to exceed the length of effective anaesthesia from a single dose, so when the animal was found to be lightening (by a response to sound), a second administration of

half the initial drug dose was given. Initially the same dose had been repeated but this lead to 3 instances early on in this work where animals failed to recover consciousness without any obvious explanation except probable anaesthetic overdose. The top-up with just a half dose proved safe in all subsequent instances.

Time after induction (min)	STIMULUS		
	catheter	sound	water
0	2,2,2,2,2,1	2,2,2,2,2,2	1,0,0,0,0,0
5	1,1,0,0,0,0	1,1,1,1,0,0	0,0,0,0,0,0
10	0,0,0,0,0,0	1,1,0,0,0,0	0,0,0,0,0,0
15	0,0,0,0,0,0	0,0,0,0,0,0	0,0,0,0,0,0
20	0,0,0,0,0,0	0,0,0,0,0,0	0,0,0,0,0,0
30	0,0,0,0,0,0	0,0,0,0,0,0	0,0,0,0,0,0
40	0,0,0,0,0,0	0,0,0,0,0,0	0,0,0,0,0,0
50	0,0,0,0,0,0	1,0,0,0,0,0	0,0,0,0,0,0
55	1,1,0,0,0,0	1,1,1,0,0,0	0,0,0,0,0,0
60	1,1,1,1,0,0	2,1,1,1,1,0	0,0,0,0,0,0
65	1,1,1,1,1,0	2,2,1,1,1,0	0,0,0,0,0,0
70	2,1,1,1,1,1	2,2,2,2,1,1	0,0,0,0,0,0
75	2,2,2,1,1,1	2,2,2,2,2,1	0,0,0,0,0,0
80	2,2,2,2,2,1	2,2,2,2,2,2	0,0,0,0,0,0
85	2,2,2,2,2,2	2,2,2,2,2,2	1,0,0,0,0,0
90	2,2,2,2,2,2	2,2,2,2,2,2	1,1,0,0,0,0
95	2,2,2,2,2,2	2,2,2,2,2,2	1,1,1,0,0,0

Table 8.1 Response to stimuli during anaesthesia in 6 rats

Responses are graded as nil (0), slight (1) or moderate (2)

Each column of data represents results from a single animal and is presented in the same order for each stimulus

8.2.3 Assessment of bladder function

All animals had their bladder function assessed before PDT and at intervals following as specified below. Control groups comprised unsensitised animals also treated with 20 J/cm^2. These sequential measurements were

made on the same animals so clearly they could not be used for histological assessment until the end of the 3 month study period. Therefore the later comparisons between function and morphology are for the most part made on different groups of animals albeit treated under the same conditions.

A filling cystometrogram (CMG) was performed using modified clinical urodynamic apparatus. This comprised a shortened saline-filled 3.5 French gauge epidural catheter connected to a standard urodynamic pressure transducer. A 3-way tap enabled this line to be used for both filling and pressure measurements (fig. 8.1). The transducer was connected to a digital meter which was calibrated against a saline manometer and maintained a linear recording of pressures up to 80 cm H_20 (fig. 8.2).

The rat urethra was sufficiently dilated with graduated cannulae passed over a nylon "guidewire" (fig. 8.3), to enable the epidural line to be introduced into the bladder through the cannula. The end of the catheter was positioned in the middle of the bladder as judged by previous measurements of urethral length and the markings on the catheter. The first compliance measurement would require a laparotomy for light treatment so this enabled the correct positioning of the catheter to be determined. There were several side holes near the tip of the catheter to ensure that it would not be occluded by resting against the bladder mucosa. The catheter was held in place by a silk tie (just visible in fig. 8.2) placed just tightly enough around the margin of the urethral meatus to prevent leakage (unless at a very high pressure) but not to occlude the cannula. The bladder was allowed to empty and particular care taken to exclude air bubbles in the line before connecting to the transducer. This was zeroed and then the bladder was slowly filled (0.25 ml/min) from a 1 ml syringe connected via the 3-way tap. Because of the common filling/recording line it was found that artefacts were produced if the pressure was measured whilst filling.

The most accurate method was to stop filling after each increment of 0.25 ml had been instilled (up to 1 ml), and allow the pressure to stabilize before recording it. More frequent estimations did not improve the accuracy of the subsequent pressure/volume curve.

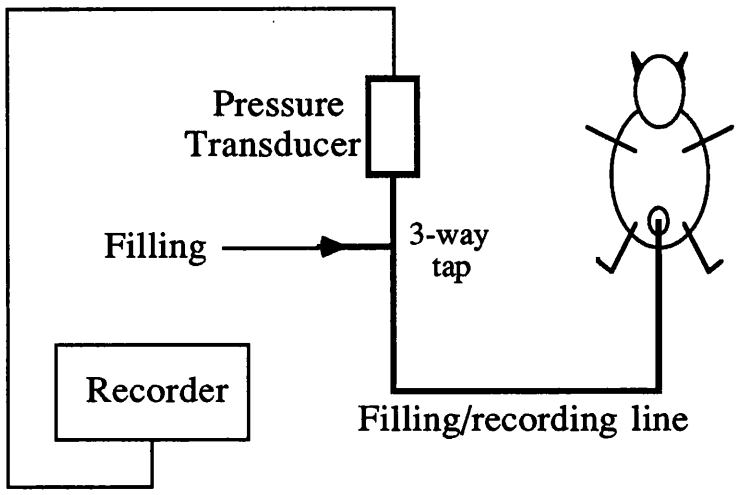


Fig. 8.1 Apparatus for cystometry

Initially a pump-driven syringe had been used to fill the bladder and a pen-chart recorder to provide a continuous filling trace. Unfortunately the errors and artefacts produced by this system, particularly in accurately delivering the very small volumes required, led to a return to manual filling and the very accurate and responsive direct transducer readings.

These measurements were performed sequentially on the same groups of animals before PDT and at 4 days, 1 week, 2 weeks, 1 month and 3 months afterwards (fig. 8.3). These pressures were total bladder pressures (P_{ves}) rather than the true subtracted detrusor pressure calculated in clinical urodynamics, but were measured under general anaesthesia so should equate with detrusor pressure. This view was confirmed by preliminary studies which had shown no difference between P_{ves} measured with the abdomen either intact or opened to neutralize any component due to intra-abdominal pressure.

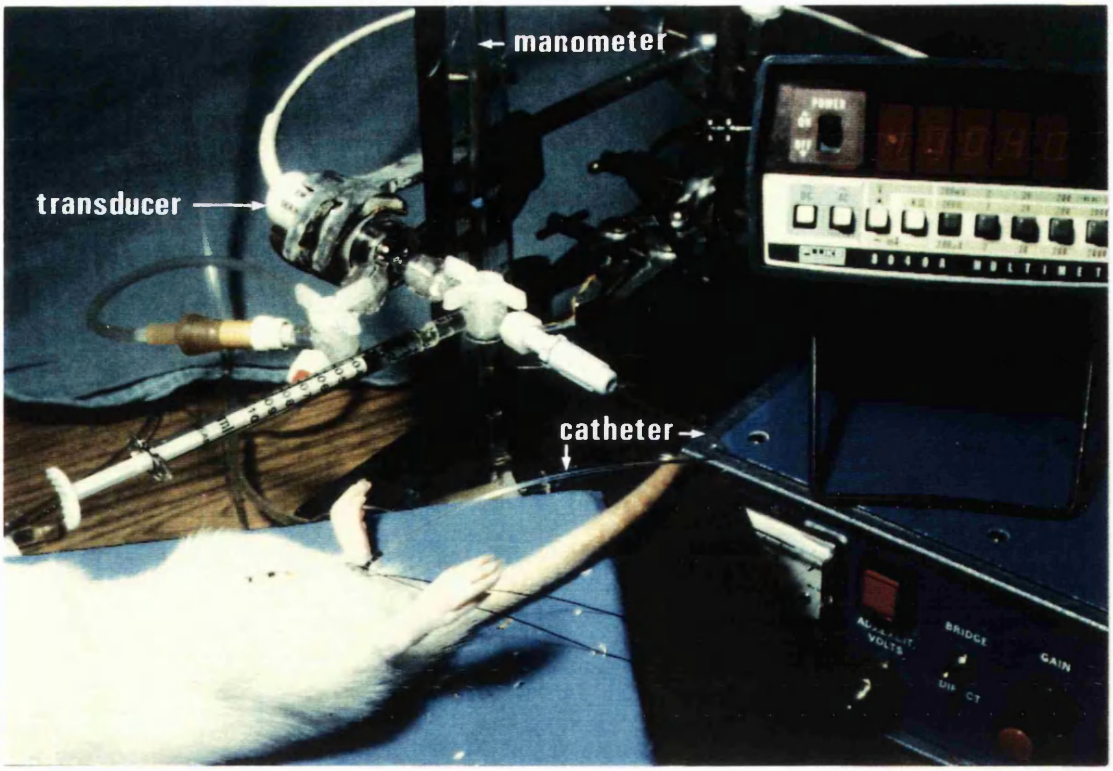


Fig. 8.2 Bladder pressure measuring system comprising a fine fluid-filled catheter connected to a pressure transducer producing a digital recording. The manometer used to check the transducer calibration can also be seen.

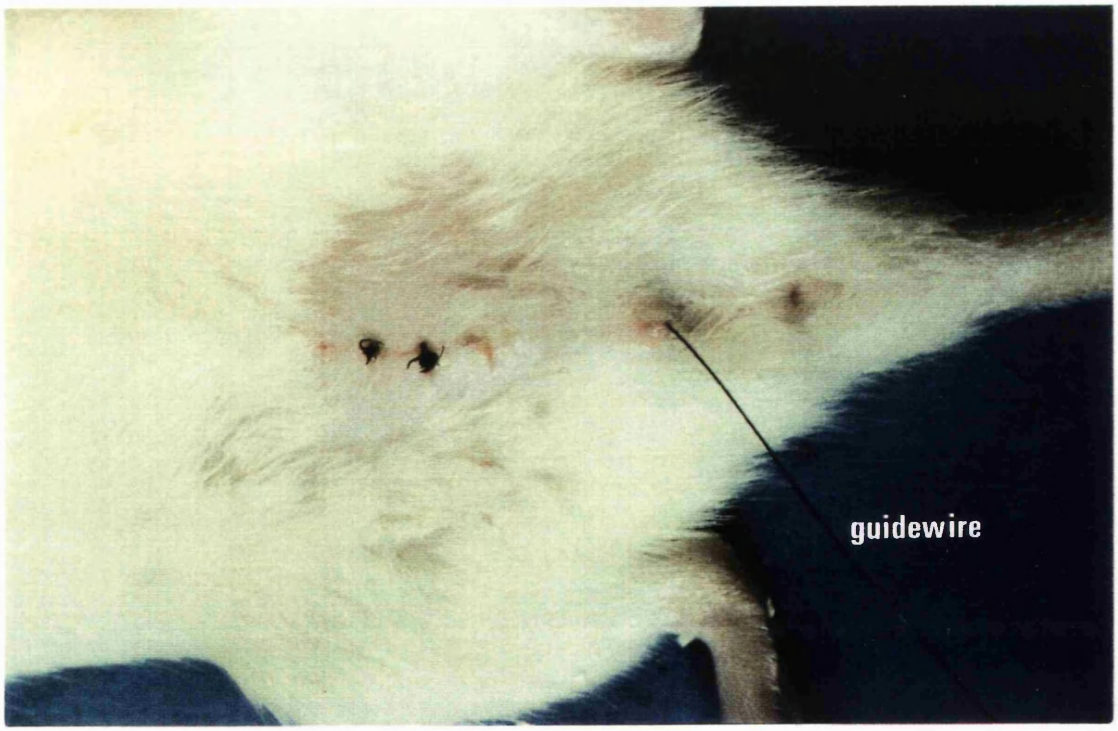


Fig. 8.3 Repeat CMG's were performed without laparotomy

8.2.3.1 Bladder compliance

Changes in bladder compliance after PDT were calculated as follows:

i) Sequential filling CMG's were performed on each rat in the control group and in all treatment groups at each of the time intervals specified above (i.e. before light treatment and at 4 days, 1 week, 2 weeks, 1 month and 3 months afterwards).

ii) The P_{ves} at each of the 4 volume increments measured (0.25, 0.5, 0.75 and 1 ml), was expressed as a multiple of the corresponding pre-treatment value for that animal. This resulted in 4 values of pressure ratio for each study in each animal. The mean of these 4 values was taken to represent the change in compliance at that point in time, compared to the pre-treatment situation, and termed the **compliance ratio (CR)**. The actual pressures measured are given in appendix 3.3, but by expressing the results in this way it was possible to derive a single value (mean compliance ratio) to describe compliance for each treatment group at each time point and thereby readily compare changes both with time (pre-treatment CR would be 1), and between the treatment groups studied (0.5, 1 and 1.5 mg/kg AlSPc, 20 J/cm^2 light) and the control animals (light but no AlSPc).

A compliance ratio of 2 therefore represented an average 2 fold increase in bladder filling pressure (P_{ves}) over the volume range tested, indicating a halving of bladder compliance. In those animals with very high pressures it was often not possible to fill a full 1 ml into the bladder before leakage around the catheter occurred or there was vesico-ureteric reflux. In these cases the compliance ratio has been calculated from fewer volume points, though in at least 3 animals for each point. It is recognised that the mean CR is only a convenient approximation to the gradient of the filling curve and may be skewed by 1 extreme value, though this was not a problem.

8.2.3.2 Bladder capacity

This was measured separately after each compliance assessment had been completed, though under the same anaesthetic, by slowly filling the bladder with saline from a manometer set at a constant pressure head of 30 cm H_20. Apart from the pre-treatment assessment when a laparotomy was performed for the bladder illumination, all of the subsequent repeat studies were done with the abdomen closed. It was therefore important to make sure that the bladder had been emptied fully before this part of the study and here a gentle suprapubic pressure was sometimes helpful. The capacity was taken as the amount emptied from the bladder after filling ceased rather than the amount instilled. This was to correct for any vesico-ureteric reflux or urethral leakage that may have occurred, the former of which was seen particularly in those animals given the higher doses of PDT. If a spurious result was suspected the assessment was repeated 2 or 3 times but despite this there may still have been some over-estimation of true bladder capacity in those more severely affected animals.

Data and statistical methods - The complete data for each animal are shown in appendix 3. Results are given in the next section generally as individual plots for each animal followed by a summary graph showing the changes in mean value for each group. Standard errors are shown generally to make the summary graphs more legible, though ranges of values and confidence limits are quoted were appropriate, and standard deviations are given in appendix 3. Animals were randomly allocated from stock to each test group so may be considered representative of a larger population of female rats. The intention of these results is to indicate general trends rather than specific differences but some differences are emphasised with levels of significance calculated by the Student's t test.

8.3 RESULTS

8.3.1 Bladder compliance

Figure 8.4 shows the pressure/volume curve in the normal rat bladder. This is constructed from the pre-treatment cystometrograms obtained from 40 consecutive animals (full data in appendix 3.1).

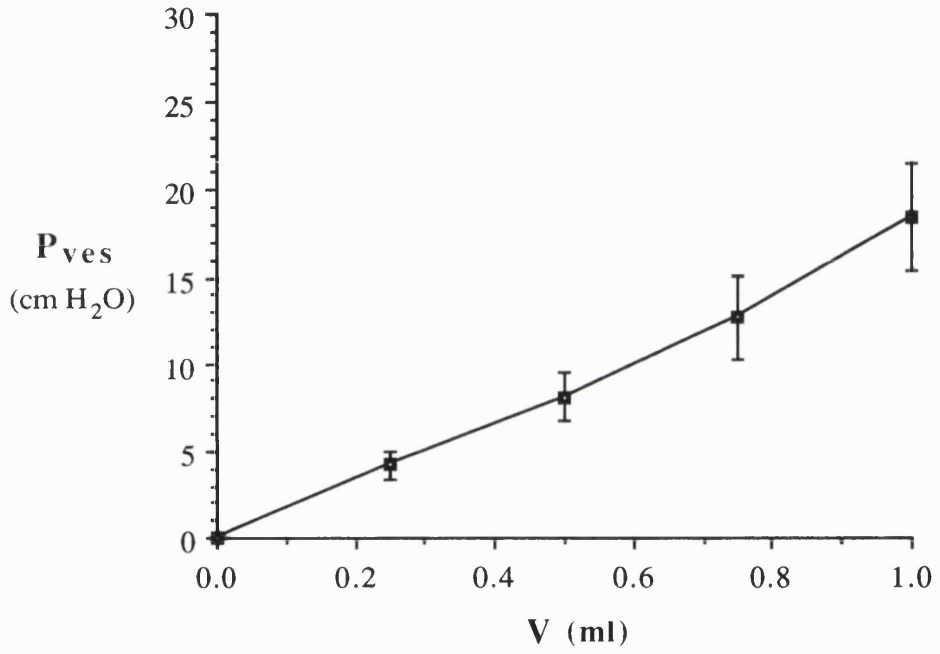


Fig. 8.4 Pressure/volume curve in 40 normal rat bladders
(values are means ± 95% confidence limits)

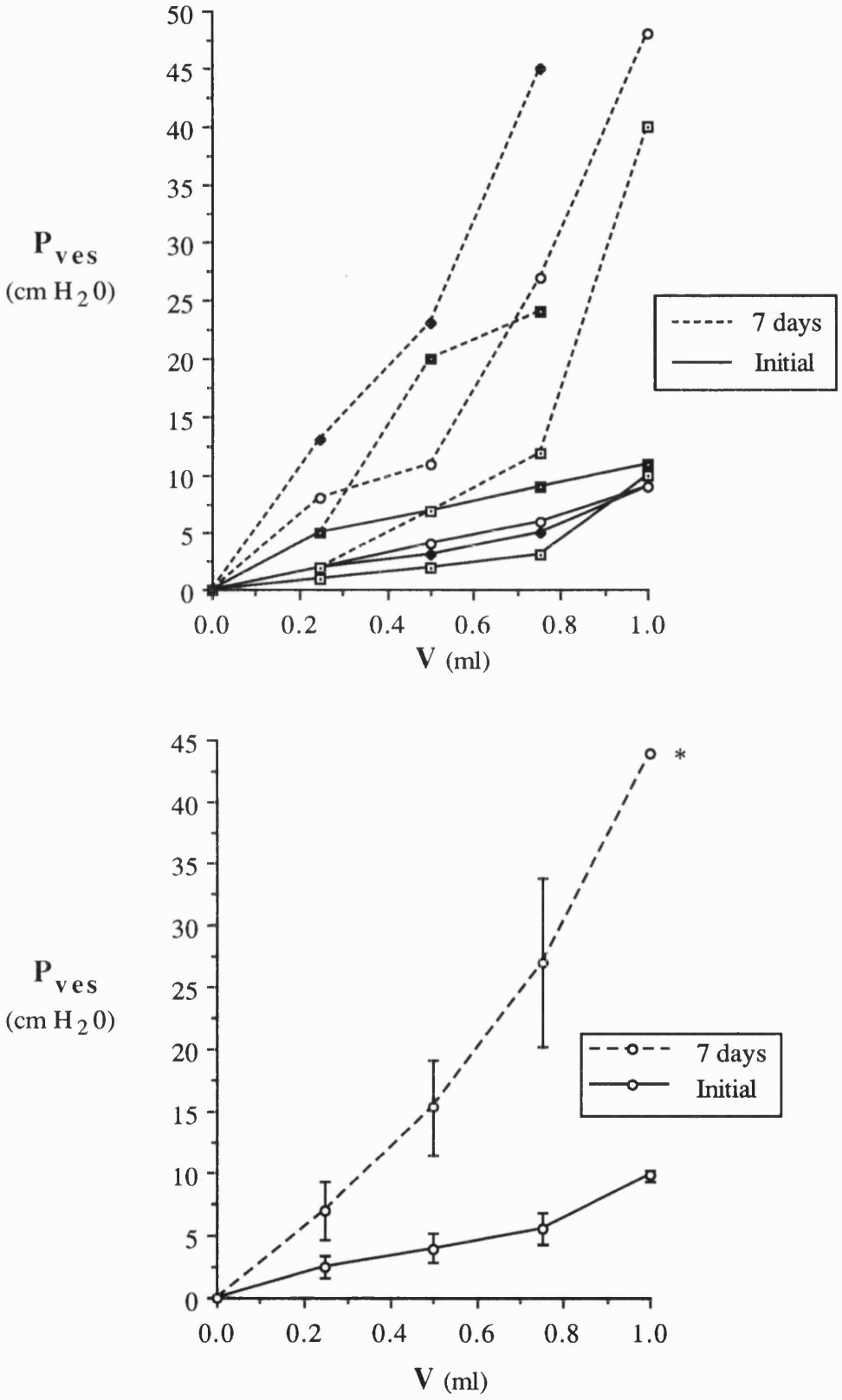
It can be seen from this graph that the intravesical pressure rises steadily during filling to about 18 cm H_20 at a volume of 1 ml instilled. This is somewhat different to the situation in Man where there is very little pressure rise until capacity is reached. It will be apparent from the appendix that the range of values is quite large and the error bars shown in fig. 8.4 represent 95% confidence limits of the mean (SEM x "t" value taken from probability tables).

Figures 8.5 illustrate how the compliance ratio is derived by demonstrating the reduction in compliance that occurred after PDT in a single test group (1 mg/kg AlSPc), at a single point (7 days) after PDT. The CMG curves obtained from the same 4 animals both before and 7 days after PDT are shown (each curve separately in fig. 8.6a and the mean values for greater clarity in fig 8.6b). The actual measurements are given in appendix 3.2.

It can be seen that the filling pressures are much higher after treatment and indeed it was only possible to instil a full 1 ml into 2 of the 4 animals without leakage and/or reflux. The ratio of the post-treatment to the initial pressures gives a mean compliance ratio in this case of 4.4. A single value is thus obtained at each measurement time after PDT to show both the magnitude of the initial effect and the trend and time scale for recovery of bladder compliance.

All animals received 20 J/cm^2 of whole bladder illumination with 675 nm red light 24 h after sensitisation. It is stressed that the results presented in figs. 8.5 comprise the data from only 1 group of animals (1 mg/kg AlSPc) at only 1 assessment point 7 days after PDT. Full data for all 4 groups at the 6 assessment times are given in appendix 3.3 and therefore only the summary graphs will be shown here.

As has been mentioned earlier none of the 4 animals treated with sensitiser doses greater than 1.5 mg/kg survived more than 1 week so they are excluded from this analysis. If any animal from the other groups did not survive a further one was treated so that at least 3 animals in each dose group successfully completed the 3 month study period. Their results will be presented in turn (complete series only).



Figs. 8.5 Filling pressures in rat bladder after PDT

CMG curves in the same 4 animals before and 7 days after PDT (1 mg/kg AlSPc, 24h prior to 20 J/cm^2 light). Individual curves shown in top graph, means ± SEM shown in lower graph (* 2 values only)

8.3.1.1 Control group

There was no mortality nor any morbidity in this group and 4 animals successfully completed the compliance measurements at 4,7,14,30 and 90 days after light treatment with 20 J/cm^2 but no photosensitiser. The first cystometrogram was performed at laparotomy for the bladder illumination but, as with the other groups, the abdomen was not opened subsequently.

If there was no change in the bladder compliance following PDT then the mean compliance ratio would remain at 1. The controls did not show any significant change in CR after light exposure as illustrated in fig. 8.6 but the bladders did show a tendency to become more compliant with time (mean CR were 1.0, 1.1, 0.88, 0.79 and 0.7 at 4, 7, 24, 30 and 90 days respectively). The numerical designation of each animal relates to the order of presentation of data in the tables shown in appendix 3.3.

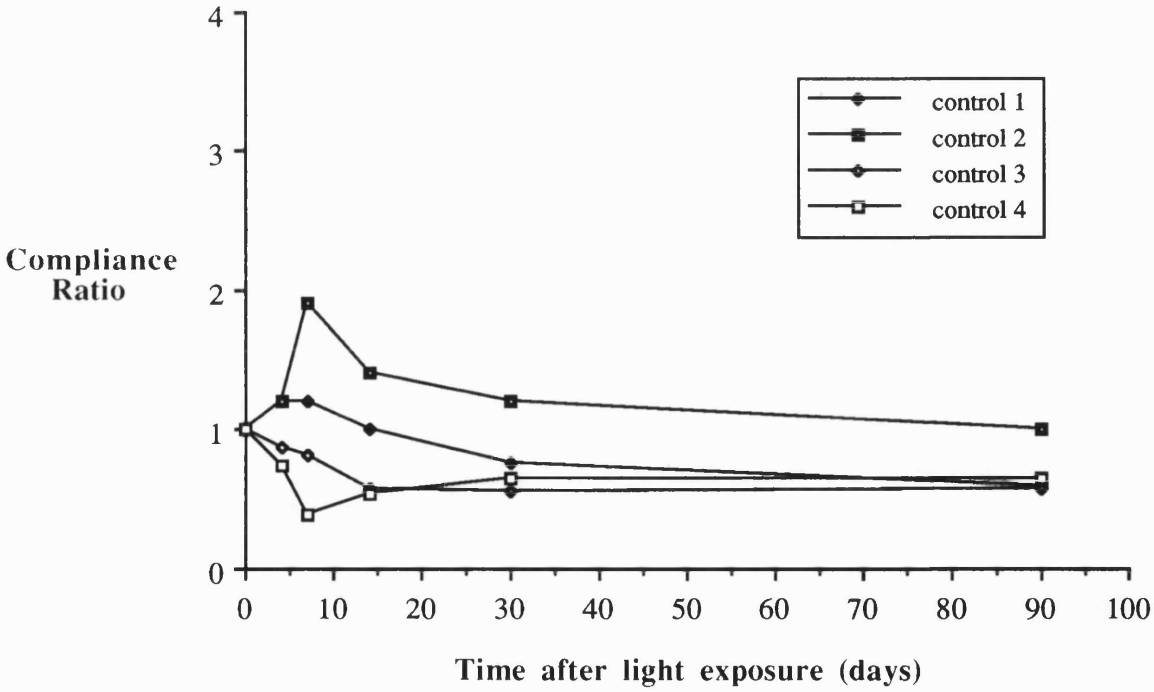


Fig. 8.6 Changes in compliance ratio in the 4 control animals studied (20 J/cm^2 light but no AlSPc)

8.3.1.2 0.5 mg/kg AlSPc group

There was no mortality in this group and no significant morbidity, as assessed by incontinence, anorexia or weight loss. Figure 8.7 shows the changes in CR for each individual animal in this group. There was an initial rise in mean compliance ratio to 2.4 at 4 days (range 1.1-4.3; difference not significant at the 5% level vs. the controls), though 1 of the 4 animals (no. 3) was rather more affected than the others.

This disturbance in bladder function was short-lived and, except for animal no. 3, had returned to normal levels by 1 month (mean CR = 1.3, range 1 - 1.9). The final assessment at 3 months indicated a generally greater compliance than before PDT (mean CR = 0.59, range 0.43 - 0.89). It seems likely that this is largely due to an increase in bladder capacity as a result of the general growth of the animals during this time.

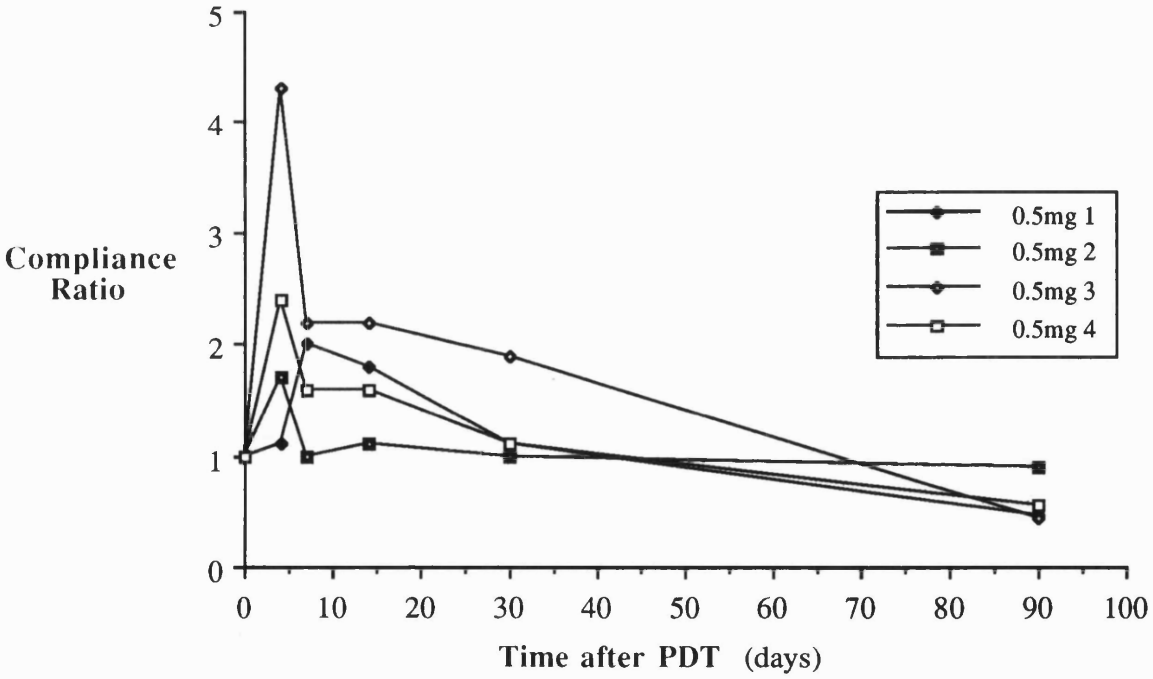


Fig. 8.7 Changes in compliance ratio after PDT in 4 animals (0.5 mg/kg AlSPc 24 h prior to 20 J/cm^2 light)

8.3.1.3 1 mg/kg AlSPc group

There was no mortality nor morbidity in the 4 animals comprising this treatment group either, though a much greater disturbance in compliance was seen initially than in those rats treated with 0.5 mg/kg AlSPc (fig. 8.8).

The mean CR reached a maximum at the 4 day assessment of 7.1 (range 3.6 - 11). This had again largely recovered by 2 weeks (mean CR = 1.5, range 0.9 - 1.9), and was back to pre-PDT levels by 1 month (mean CR = 1.1, range 0.5 - 1.6). As with the lower sensitiser dose all animals had a greater bladder compliance at 3 months than they had before PDT (mean CR = 0.71, range 0.53 - 0.85).

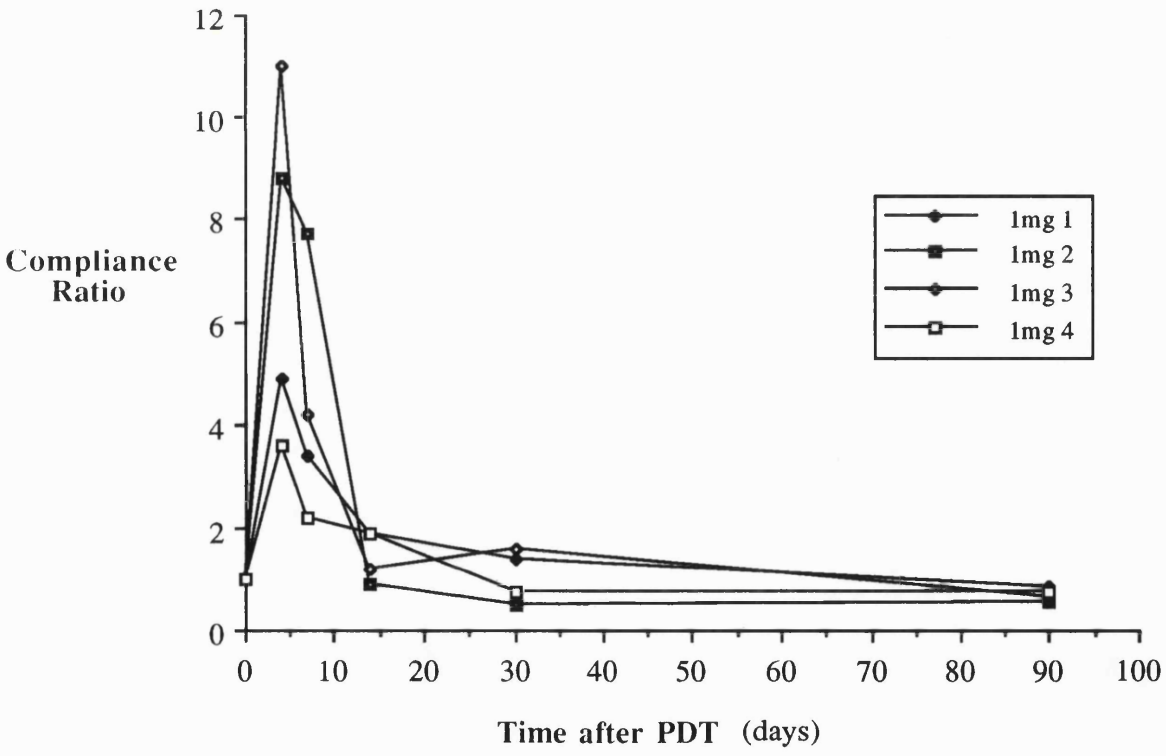


Fig. 8.8 Changes in compliance ratio after PDT in 4 animals (1 mg/kg AlSPc 24 h prior to 20 J/cm^2 light)

8.3.1.4 1.5 mg/kg AlSPc group

There was a much more severe effect noted in this group. Nine animals were treated in order to achieve survival in 3. Two animals died at 6 and 18 days after PDT and 2 others were killed as they were judged to be suffering at 3 and 4 days. At autopsy all had grossly inflamed bladders, peri-vesical oedema and bilateral renal abscesses. Two others failed to recover from the anaesthetic after their cystometrogram at 7 days no doubt largely due to their generally weak condition giving a direct mortality of 57% (4 ex 7) and overall mortality of 67% (6 ex 9).

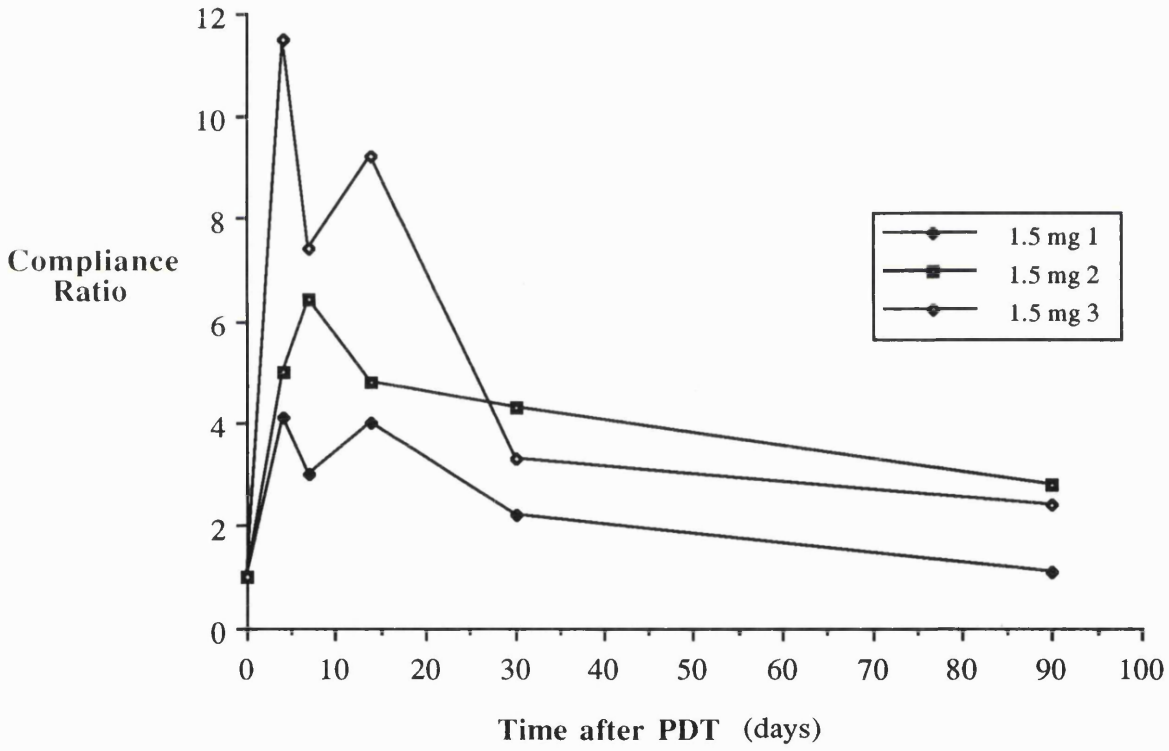


Fig. 8.9 Changes in compliance ratio after PDT in 3 animals (1.5 mg/kg AlSPc 24 h prior to 20 J/cm^2 light)

Surviving animals were clearly unwell, showing all the signs mentioned previously and with a weight loss of 10 - 15%. The initial disturbance in compliance was no different from those animals treated with 1 mg/kg (mean CR 6.9, range 4.1 - 11.5) but this probably represents close to the

maximum disturbance recordable as it can be seen from the data in appendix 3.3 that in none of the animals was it possible to instil 1 ml at either 4 or 7 days after PDT and only in 1 of the 3 at 14 days. Additionally, of course, the more severely affected animals did not survive.

Unlike the previous groups where CR had returned to pre-treatment levels by 1 month this group showed a significant elevation at both 1 month (mean CR = 3.3, range 2.2 - 4.3) and at 3 months when no animal had recovered to the level of its pre-PDT values (mean CR = 2.1, range 1.1 - 2.8; $p < 0.05$ vs. controls).

Figure 8.10 summarises the previous 4 graphs showing the changes in mean CR (standard errors shown to maintain legibility). This clearly shows the transient disturbance of compliance after treatment with either 0.5 or 1 mg/kg AlSPc, but a prolonged impairment in the 1.5 mg/kg group.

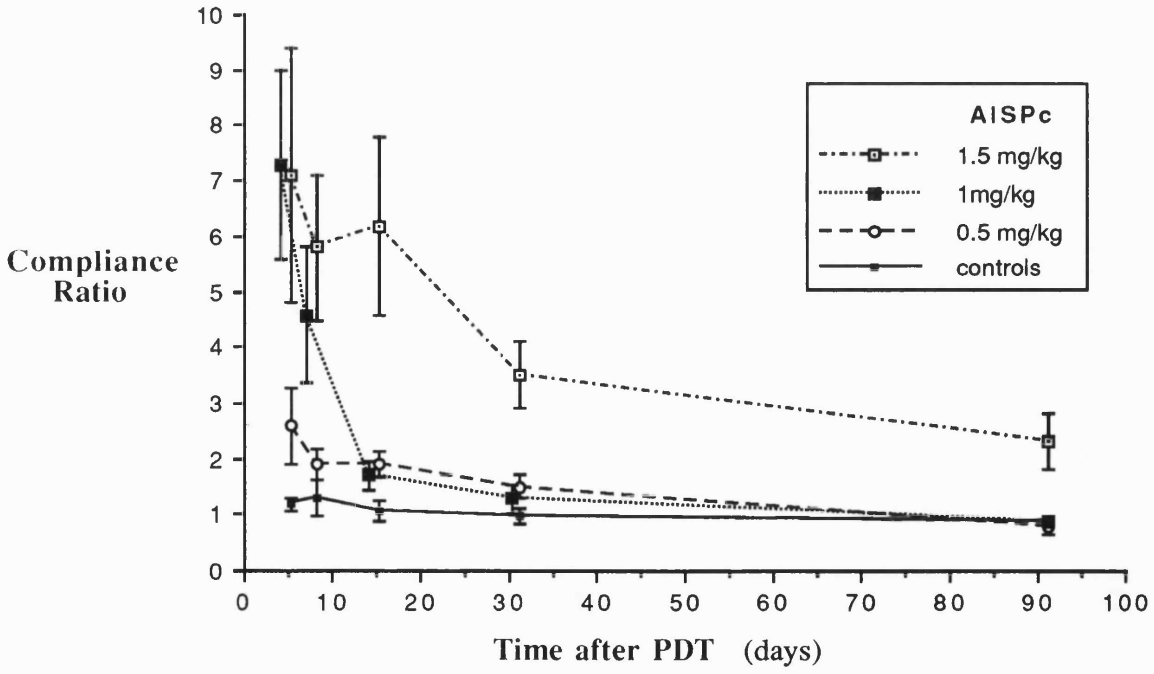


Fig. 8.10 Changes in mean compliance ratio after PDT

Mean ± SEM of at least 3 animals per point
(0.5-1.5 mg/kg AlSPc 24 h prior to 20 J/cm^2 light)

8.3.2 Bladder Capacity

The initial mean bladder capacity (at 30 cm H_20) in the control group was 1.04 ± 0.02 ml (range 1.0 - 1.1 ml). This was very similar to that seen in each of the 3 treatment groups before PDT (0.5 mg/kg - 1.03 ± 0.13 ml, 1 mg/kg - 1.25 ± 0.06 ml, 1.5 mg/kg - 1.12 ± 0.04 ml).

It will be seen from fig. 8.11 that bladder capacity in the control animals during the first 2 weeks of the study actually increased significantly to 1.99 ± 0.27 ml ($p < 0.05$ - complete data given in appendix 3.3), probably because the 4 cystometrograms within that time period had a cystodistension effect. Thereafter bladder capacity fell a little, being 39% greater at 3 months than it was at the start of the study (mean 1.45 ml, range 1 - 1.85). This final increase was broadly in line with the animals' growth during this period.

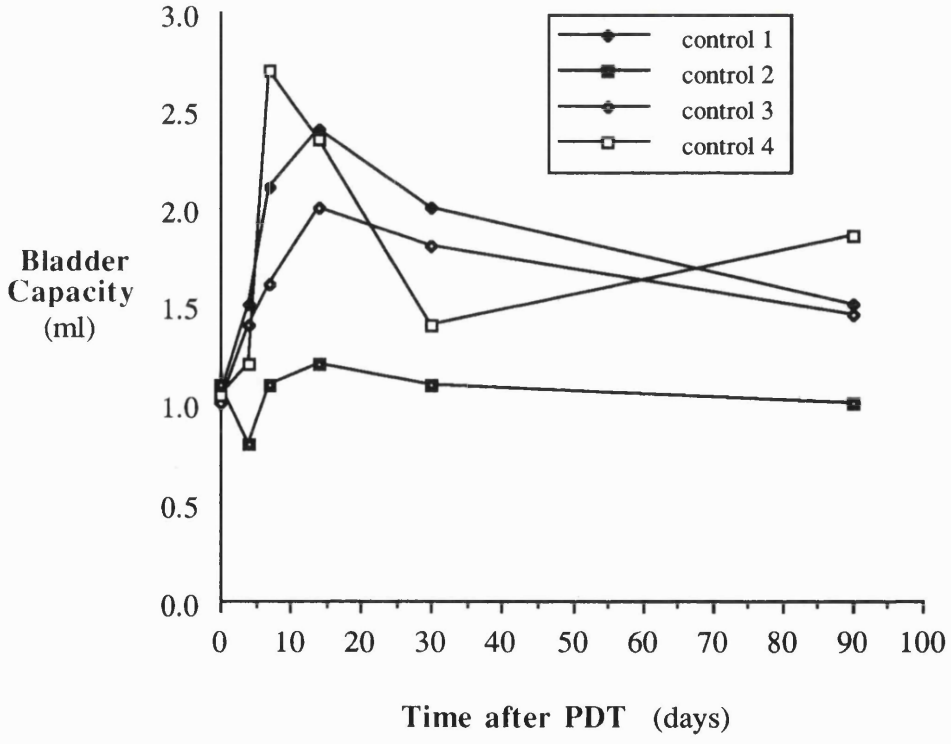


Fig. 8.11 Changes in bladder capacity in the 4 control rats studied (20 J/cm^2 light but no AlSPc)

Rat no. 4 showed the most marked increase in capacity in the control group from 1.2 ml at 4 days after illumination to 2.7 ml at 7 days. It should be no surprise to see that this animal also showed the largest increase in compliance (CR = 0.39 - fig. 8.6) during this time. In only 1 animal (no. 2) was there a small initial fall in bladder capacity.

In view of this marked change in the bladder capacity of the controls it was felt essential to correct the results from the treated animals for this change to allow a valid assessment of the effect due purely to PDT. The following 3 graphs (figs. 8.12 - 8.14) show the actual bladder capacity recorded in each animal within the 3 treatment groups at each of the assessment times (data from appendix 3.3). Figure 8.15 presents the mean values for each treatment group which have been corrected for the corresponding controls (data from appendix 3.4). The numbers given to the individual animal's plots corresponds with the order of the data in the appendices.

The 0.5 mg/kg AlSPc group (fig. 8.12) all showed an initial slight reduction in their bladder capacity (at 4 days mean capacity = 0.75 ml, range 0.6 - 0.95 ml). This drop had been reversed by 7 days after PDT and remained constant up until the 1 month assessment, though allowing for the expected increase with the repeated measurements this still represented a reduction, as will be seen later.

The rats given 1 mg/kg AlSPc prior to light treatment had a more dramatic initial reduction in their bladder capacity (fig. 8.13) mirroring the compliance findings. All but 1 were severely reduced at 4 days but had returned to pre-PDT, though not control levels, by 2 weeks.

The changes in bladder capacity measured in the 3 surviving animals treated with 1.5 mg/kg AlSPc and 20 J/cm^2 whole bladder illumination are shown in fig. 8.14.

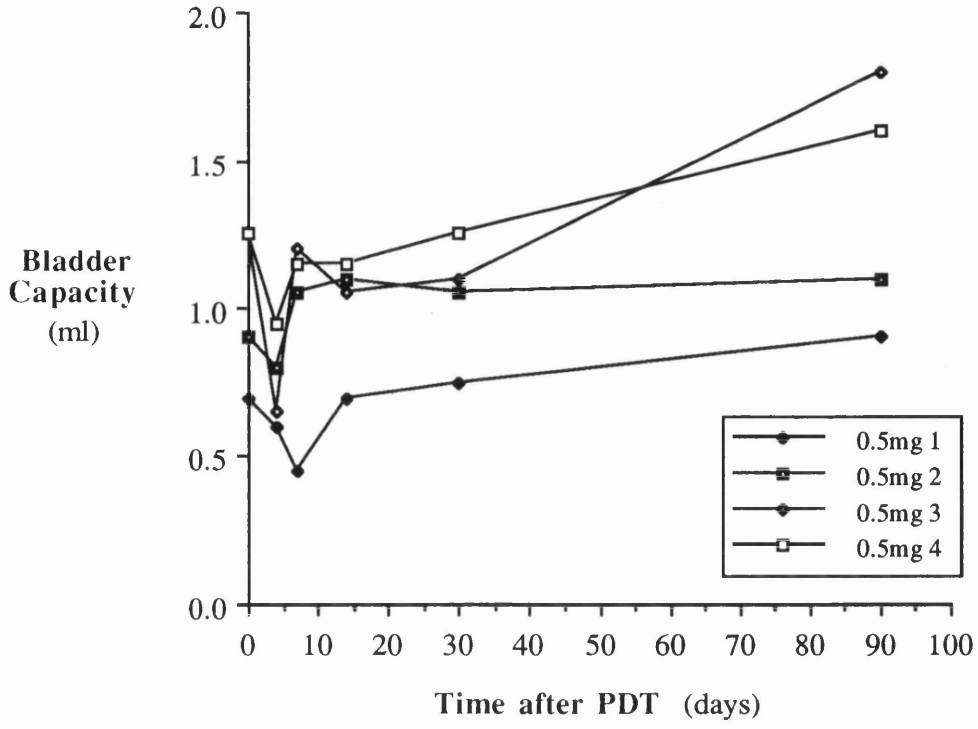


Fig. 8.12 Changes in bladder capacity after PDT in 4 rats (0.5 mg/kg AlSPc 24 h prior to 20 J/cm^2 light)

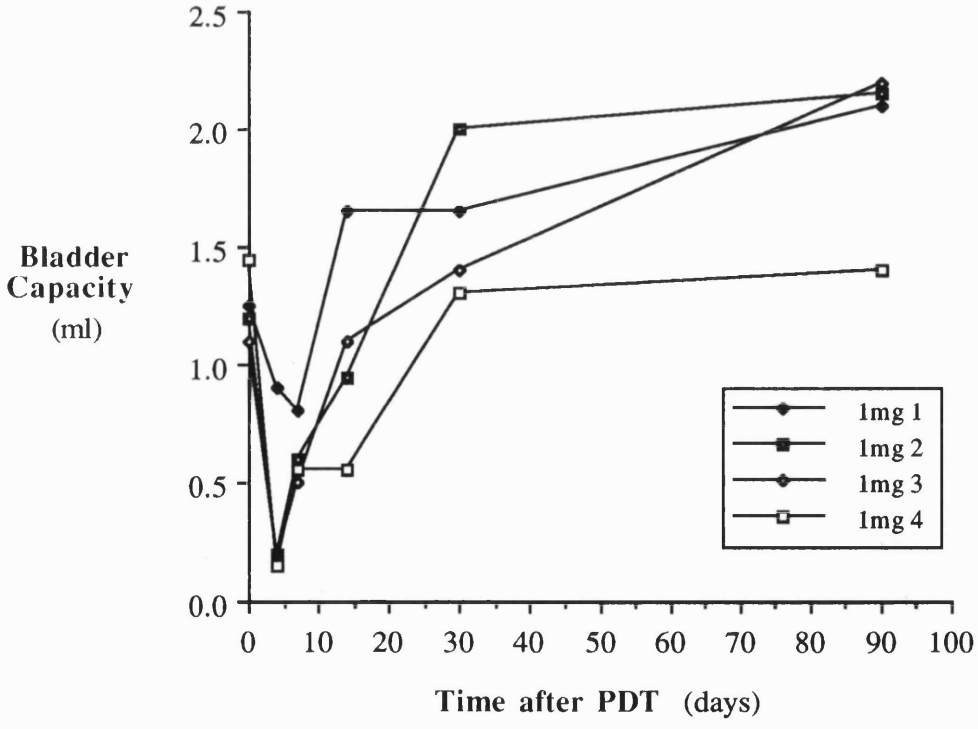


Fig. 8.13 Changes in bladder capacity after PDT in 4 rats (1 mg/kg AlSPc 24 h prior to 20 J/cm^2 light)

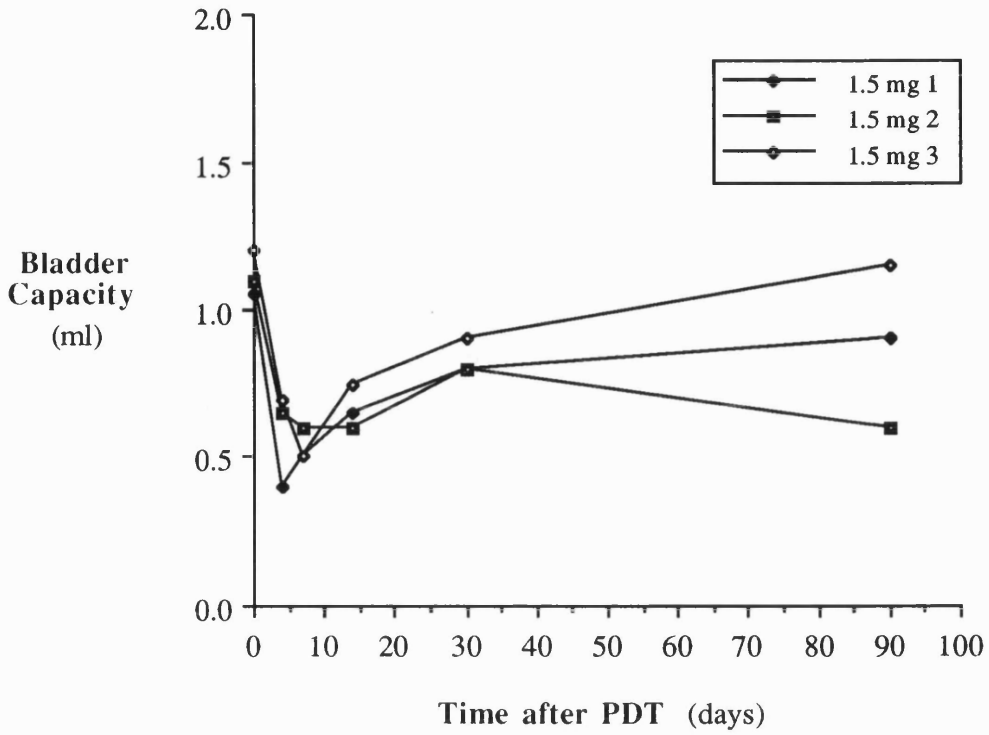


Fig. 8.14 Changes in bladder capacity after PDT in 3 rats (1.5 mg/kg AlSPc 24 h prior to 20 J/cm^2 light)

There was clearly some variation between individual animals in the same group but the trends are similar. For instance all PDT treated animals had an initial reduction in bladder capacity. All those given either the 0.5 or 1 mg/kg AlSPc doses had regained or exceeded their initial capacity by 3 months (figs. 8.12 and 8.13), none of those in the 1.5 mg/kg group had done so (fig. 8.14). The initial reduction in bladder capacity was severe in many cases and in 23 animals given 1 mg/kg AlSPc went down to only 0.2 ml. Those given 1.5 mg/kg AlSPc may seem to be less affected initially than the 1 mg/kg AlSPc group but the differences were not significant and the high mortality already mentioned in the former group should be borne in mind, i.e. those shown here were the least severely affected. Three of the 4 animals who had been treated with 1.5 mg/kg AlSPc and who died after the 4 day CMG (and are therefore not included in this analysis) had bladder capacities below 0.2 ml at this time.

Figure 8.15 compares the changes in bladder capacity after PDT in each of the 3 treatment groups. All figures are the mean percentage change in capacity for each group (i.e. taking the pre-treatment capacity as 100), having corrected these values for the corresponding control group at each time point (the "corrected" values given in appendix 3.4).

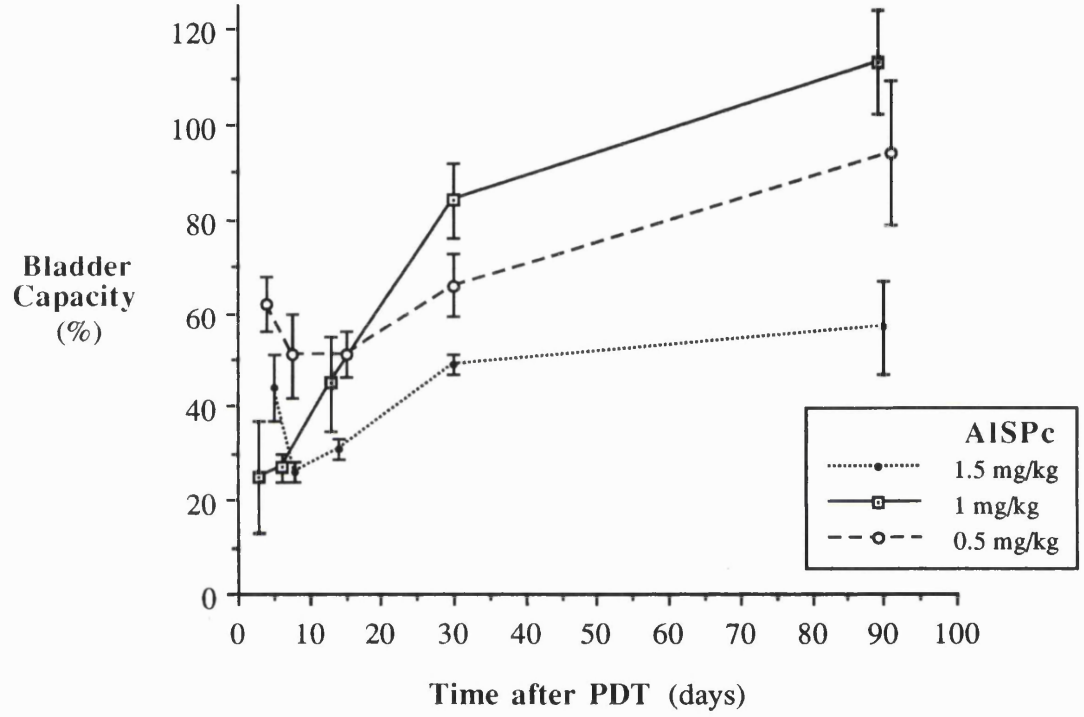


Fig. 8.15 Changes in rat bladder capacity after PDT
(means ± SEM corrected for controls, initial value for all groups = 100)

Bladder capacity was reduced by 50% in the 0.5 mg/kg group and by nearly 75% in the 1 and 1.5 mg/kg groups within the first week after PDT. Recovery started after 2 weeks, a complete recovery being seen with lower doses of sensitiser but, as has been seen with the compliance results, those animals treated with 1.5 mg/kg AlSPc did not regain more than half their expected capacity at 3 months. At this point the slope of the 1.5 mg/kg graph suggests that this impairment is likely to be permanent.

8.4 DISCUSSION

Although the rat bladder clearly differs from the human in terms of wall thickness and capacity this model can provide useful information about functional changes following PDT. Using modified clinical urodynamic equipment a consistent pressure/volume curve was constructed (fig. 8.4) for bladder filling. The requirement to express bladder function in a simple, ideally numerical, way that could be used to directly compare the changes occurring after PDT and in the subsequent healing phase led to the creation of the term "compliance ratio" (CR). This as has already been mentioned is the ratio of the pressures measured at each volume increment at the assessment points after treatment, to those obtained initially.

There are several points to be considered concerning the applicability of this experimental model. Bladder behaviour (in Man at least) has a significant sensory component which may exert a large influence on voiding parameters and cannot be assessed comprehensively by a study of storage function. It is recognised that many drugs, including anaesthetic agents, atropine and opiates, have a inhibitory effect on bladder contractility so the performance of such a study under general anaesthetic is likely to lower bladder pressures and abolish micturition reflexes. However it does have the advantage of a controlled experimental environment and in general we are not concerned with very subtle changes but variations of at least 50%. As the main long term side-effects of PDT are largely those of reduced bladder capacity due to fibrosis the anaesthetic objections are somewhat overcome. Alternative ways of looking at bladder function such as micturition cycles are variable and time consuming when frequently repeated and would not enable compliance to be assessed.

Another concern is the effect that repeated studies themselves may have on bladder function. The control animals showed both a decrease in CR of

30% and a rise in bladder capacity of 40% (nearly all of which occured in the first 2 weeks), during the 3 month test period. This early increase in bladder capacity in particular is likely to have been the result of what are in effect repeated cystodistensions together with an element due to the growth of the animals during this time. For this reason the results given in fig. 8.15 were corrected for the bladder volume of the relevant controls.

Bladder compliance is the relationship between volume and pressure and is defined as a given increase in bladder volume divided by the increase in intravesical pressure that occurs during that change in volume; i.e. the slope of the pressure/volume curve. It is a term widely used to describe bladder function but usually only in terms of a "normal" or "reduced" compliance.

$$\text{compliance} = \frac{\Delta V}{\Delta P} \quad (\Delta V = \text{change in volume; } \Delta P = \text{change in pressure})$$

A normal compliance therefore indicates little rise in pressure for a given volume and low compliance the reverse (a high compliance implies a large capacity as well as little rise in pressure). In the rat there is a steady and gradual increase in pressure during filling (fig. 8.4), a less compliant system than in normal Man where little rise in pressure is seen throughout the functional bladder capacity. The CR value for each cystometrogram, which is an reflection of the average gradient of the pressure/volume curve, was calculated for each animal such that the mean for each treatment group expressed bladder compliance as a single figure for each observation point with a value of unity implying no change (fig 8.10).

The same light dose (20 J/cm^2) was used for all groups to enable the effect of varying sensitiser doses to be assessed. In the rat even low doses of AlSPc (0.5 mg/kg) still produced a marked initial reduction in both bladder compliance and capacity due to the acute inflammation but these improved to within the pre-treatment levels after about 3 weeks. An intermediate

dose of 1 mg/kg resulted in a early increase in CR to more than 7 which was probably the upper limit for this value as after that the pressure needed to fill even a small volume into these intensely inflamed bladders lead to leakage around the filling catheter and ureteric reflux, as already discussed, which complicated the measurements. However despite such a large initial disturbance in bladder function, recovery to pre-treatment values occured within 1 month. After PDT with a higher dose of 1.5 mg/kg AlSPc not only was the initial functional impairment severe but it is important to note that recovery was incomplete with a CR over 2 and a bladder capacity barely more than half the pre-treatment values at 3 months.

We are aware of only 1 other study of the effect of PDT on bladder function in an experimental model. Nseyo and colleagues (1988) looked at the effect of varying light doses (0 - 64 J/cm^2) and bladder filling pressures (30 or 60 cm H_20) during PDT on the normal canine bladder. They had concluded from a previous study that the canine bladder was fairly resistant to PDT in that a light dose less than 30 J/cm^2 to the whole bladder caused damage to the superficial layers only, above 35 J/cm^2 some muscle damage occurred, and more than 70 J/cm^2 was required to produce any full thickness damage (Nseyo *et al.*, 1985b). They performed weekly cystoscopies and measured bladder capacity, at the treatment filling pressure, until such time as bladder capacity returned to its pre-treatment value whereupon cystectomy was performed. Although they did not see any permanent reduction of bladder capacity in surviving animals, those dogs in which they used a high bladder filling pressure (60 cm H_20) had a more delayed recovery (mean 10.5 weeks) and all had a degree of muscle damage ranging from neovascularity to necrosis. Therefore thinning of the bladder wall by overdistension may well extend the expected depth of damage. None of the animals treated at the lower filling pressure showed any significant muscle abnormality. They used a sensitiser dose well above

threshold (DHE 2 mg/kg), so higher light doses not surprisingly produced more histological damage than lower ones though the small numbers of animals (total of 15 in all groups) studied showed quite a degree of variability.

We did not investigate the effect of different bladder filling pressures believing that it is important to only minimally distend any bladder for PDT, especially one so thin as in the rat. The treatment volume of 0.3 ml corresponded to a mean intravesical pressure of about 5 cm H_20 (fig. 8.4). Nseyo *et al.* (1988) did not include any assessment of the acute histological effects of PDT so it is not known which dosimetry parameters produced a clinically appropriate degree of damage that might have been expected to eradicate superficial bladder cancer. Indeed the bladder capacity of some of the treated dogs recovered more quickly than the average control animal that had not received any photosensitiser. This would suggest that either they were producing a thermal effect though the maximum power density of 22 mW/cm^2 was less than employed here (43 mW/cm^2) and our controls were unaffected, or that there was a detrimental effect from prolonged bladder distension with water. This is more likely as one of their controls died from water intoxication.

8.4.1 Correlation of functional and histological changes

The magnitude of the changes in bladder function described above, both in the acute phase after PDT and during healing seemed to be related, as might be expected, to the degree of tissue damage produced. An initial acute inflammatory reaction involving the entire bladder mucosa and lamina propria is bound to cause marked changes in bladder function but the important factor seems to be damage to the bladder wall muscle. By comparing the histological changes described in chapter 7 with the

functional changes it is clear that when treatment parameters are chosen that do not damage muscle, then the resulting functional impairment is less severe, and recovers more quickly and completely. Table 8.2 has been constructed from the results in all animals who underwent cystometric assessment, categorised as to the presence or absence of initial muscle damage as evident from other animals treated with the same parameters that were subjected to histological examination.

	4 days		2 weeks		3 months	
	Cap.*	Comp.*	Cap.*	Comp.*	Cap.*	Comp.*
Muscle intact	41%	4.1	48%	1.8	104%	0.98
Muscle damaged	25%	7.2	32%	6.5	53%	3.4

Table 8.2 **Effect of initial muscle damage from PDT on the recovery of bladder function in rats treated with 20 J/cm^2 light and 0.5 - 5 mg/kg AlSPc**

* Cap. = Bladder Capacity (at 30 cm H_20) as a percentage of that in control animals treated with light alone.

* Comp. = Bladder Compliance Ratio.

It has been stressed earlier that despite persisting functional disturbances no significant histological abnormality was detected after the acute inflammatory process had settled e.g. in those treated with 1.5 mg/kg AlSPc and examined at 1 or 3 months. This perhaps is not surprising given that many patients with bladder dysfunction have normal looking bladders on light microscopy. We looked only at gross structure and the deposition of collagen. Ultrastructure studies or immunocytochemical techniques may however show abnormalities and should be the subject of further study.

8.4.2 Conclusions

The aim of the work described in this chapter has been to investigate the functional effects of PDT with AlSPc on a normal bladder model. It is the severe and often permanent reduction in bladder function reported by several clinical workers following PDT that has been the major hindrance to the more widespread clinical development of this promising technique. There has been little effort directed at basic biological research to explore the tissue effects of PDT on an animal bladder model to anticipate the problems that may be expected in Man, and methods whereby these might be overcome. The effect on normal bladder has been studied in this work mainly because the potentially most useful urological application of PDT, bladder Cis, involves treating the entire bladder and it is the consequent damage to normal rather than tumour tissue that results in the serious side-effects previously discussed.

We found that provided treatment parameters were chosen that would not be expected to damage the bladder wall smooth muscle then there was only a transient disturbance of bladder compliance and capacity though this could still be profound due to a marked inflammatory response. This was easiest to ensure by using a low enough dose of AlSPc (1 mg/kg or less at the light dose of 20 J/cm^2 studied here) at which levels the photobleaching of sensitiser in the deeper layers of the bladder wall should reduce the amount of singlet oxygen produced to below the threshold needed for a PDT effect. Under these conditions it may not be necessary to deliver a precise light dose to every part of the bladder, but merely an minimum effective dose to all points, with no limit to the maximum at any point provided of course that no thermal damage resulted. Therefore some of the major problems associated with precise, even illumination of the entire bladder mucosa should be somewhat lessened, although the high total light

doses needed may require long treatment times with currently available laser systems.

Taking these findings together with the histological effects of PDT presented in chapter 7, it was possible in our animal model to achieve a uniform destruction of the mucosa with PDT that spared the underlying tissue from significant damage. This rapidly healed by the regeneration of normal mucosa and bladder function as assessed by measurements of capacity and compliance returned to normal within 1 month. It seems likely that in Man the careful adjustment of treatment variables and the use of minimum concentrations of photosensitiser in particular, should minimise unwanted damage to the detrusor muscle and avoid permanent functional impairment.

This work has been performed with AlSPc which is not yet approved for clinical use. However it seems likely that similar results should be obtained using HpD though this has not been investigated to our knowledge. It is important that such basic experiments are carried out before embarking on clinical studies, as the data from such work should indicate the way to refine the several dosimetry variables and to avoid the major clinical complications that have been seen with whole bladder PDT to date. This technique may then offer a valuable alternative to existing management in cases of resistant Cis and superficial bladder carcinoma.

For future work it would be advisable to do some similar experiments on a larger animal bladder such as the minipig or dog. This would allow the use of a light delivery system similar to that used clinically, and a sufficiently thick-walled bladder to more accurately discriminate between the depth of damage produced at different doses. In addition an implanted bladder tumour model may prove feasible, and relevant to the question of producing selective necrosis.

SECTION 3

NEODYMIUM:YAG LASER THERAPY

Chapter 9

THE USE OF THE Nd:YAG LASER FOR THE TREATMENT OF BLADDER CANCER

9.1 INTRODUCTION

In this chapter the published clinical and experimental work dealing with the application of the Nd:YAG laser to the treatment of bladder cancer will be reviewed. Studies of the thermal coagulation of bladder cancer with the Nd:YAG laser have been much larger and more numerous than those investigating PDT but although these techniques have been developing for more than 10 years the place of the Nd:YAG laser in the therapeutic process has not yet been defined.

Although there are some data on the laser treatment of invasive bladder tumours it is with superficial bladder cancer that most experience with the Nd:YAG laser has been gained. The surveillance and treatment of patients with superficial bladder cancer constitute a significant proportion of a general urologist's workload and the conventional techniques of diathermy resection and fulguration are undoubtedly effective. Laser enthusiasts have proposed several advantages over electrocoagulation particularly in terms of a reduction in recurrences and increased convenience and economy by enabling outpatient treatment in many cases. Many clinicians however will need convincing of real advantages to justify the use of an expensive laser over conventional diathermy so what evidence is there for these claims ?

9.2 SUPERFICIAL BLADDER CANCER

There are around a dozen published clinical series of laser treated superficial bladder tumours in the English literature comprising over 800 patients (table 9.1). Most of the early work was pioneered by the Germans in the late seventies, particularly Hofstetter's groups, and they deserve the credit for the birth of urological laser surgery. However there are often

few published experimental data to back up the dosimetry parameters they recommended and few randomised or comparative studies comparing laser with standard electrical coagulation. There are only 2 small studies from UK centres (Fowler, 1987; Randall and Arkell, 1987). Several series have also included some patients with muscle invasive tumours and these have therefore been extracted as far as possible from the data shown in table 9.1.

9.2.1 Clinical results

Some authors have reported a much lower recurrence rate after laser coagulation compared to that seen with electrocautery (Hofstetter *et al.*, 1981; Malloy *et al.*, 1984). Hofstetter's group in particular claimed an impressively low local recurrence rate of only 1.5% after laser therapy though these patients were not randomised as part of a prospective study including a conventional treatment arm. This centre later reported a small prospective, randomised study which showed that recurrence rates after laser treatment (7/44) were less than after TUR (15/22) (Hofstetter, 1987). However numbers were small, follow-up time between the various treatment groups varied widely, and the effect due purely to laser therapy was clouded as most patients also received intravesical chemotherapy with mitomycin C.

The reported rates for the local recurrence of bladder tumours following conventional treatment range from around 25-50% (e.g. 26% - National Bladder Cancer Collaborative Group A, 1977a; 32% - Loening *et al.*, 1980; 50% - Boyd and Burnand, 1974). The reasons proposed for the apparent superiority of laser treatment over traditional electroresection include the efficient sealing of submucosal lymphatics by laser, and the non-contact destruction of the laser reducing the liberation and subsequent implantation of viable tumour cells.

Authors	No. of patients	Tumour details	Treatment details	Follow-up (months)	Recurrences
Beisland *et al.*, 1985	85*	T1, G1-3	TUR first > 1.5 cm	3-24	8% local, 41% new
Beisland and Seland, 1986	97* (randomised laser or TUR)	T1, G1-3	Laser - 47 pts TUR - 50 pts	> 48 > 48	nil local, 20% new 26% local, 22% new
Fowler, 1987	39	Ta-T1, G1 (< 0.7 cm)	Laser alone < 40 W	not stated	3% local, 9% new
Hofstetter, 1981	203	Ta-T3a, G1-3	TUR first > 2 cm	2	20% local
Hofstetter, 1987	66 (laser or TUR ± mitomycin C)	Ta-T2	Laser - 44 pts ** TUR - 22 pts **	27-41 9-25	28% overall 42% overall
Malloy, 1986	56*	Ta-T1	35-40 W TUR first > 2.5 cm	> 24	32% overall
Okada *et al.*, 1982	45	36 pts - Ta-T1 9 pts - T2	50 W TUR after > 1 cm	2-22	24% overall
Randall and Arkell, 1987	12	Ta-T1, G 1-2	40 W	3-8	50%
Rothenberger *et al.*, 1983	44	8 pts < 1.5 cm, G 1-2 36 pts > 1.5 cm, G 1-3 (staging not specified)	Laser alone < 45 W TUR first > 1.5 cm	48 48	nil 28% overall
Shanberg *et al.*, 1987	32*†	Ta-T1	35-40 W TUR also > 2.5cm	24	nil local, 16% new
Smith, 1986a,	93	Ta-T1	35-40 W	3-58	6% local, 32% new
Staehler *et al.*, 1985	38*	Ta-T1, G1-3 (solitary tumours only)	TUR first if 'large'	24 (mean)	3% local, 57% new

Table 9.1 - Recurrences after laser treatment of superficial bladder tumours

* included invasive tumours shown separately where possible in Table 9.2

** adjuvant mitomycin C given to 30(73%) of laser group and 15(68%) of TUR group

† 3 pts with Cis who also received intravesical BCG excluded

Zimmerman *et al.* (1984) injected Indian ink into the dome of rat bladders and found that by coagulating a rim of bladder around the injection site with the laser they could prevent the uptake of ink by the regional lymph nodes. Similar experiments in our Unit though have shown that electro-coagulation can also prevent drainage of ink to these draining lymph nodes (T McNicholas, unpublished work).

See and Chapman (1987) used a transplantable transitional cell tumour model to study the liberation of cells produced during either electro-resection followed by diathermy of the tumour base, or laser coagulation. They found that more than 6 times as many viable cells were released by diathermy as by laser treatment though it is not clear whether the release of such cells if similarly produced in man would increase the chance of successful implantation and tumour recurrences. It seems reasonable though that this factor may be expected to contribute to the differences in recurrence rates observed by some groups after laser treatment.

The results of most clinical trials however, whilst suggesting that there is less chance of local tumour recurrence after laser treatment, do not convincingly demonstrate a reduction in the rate of new tumour recurrences elsewhere in the bladder (table 9.1).

Perhaps the best study available comes from the Oslo group comparing local recurrence after Nd:YAG treatment to that following traditional electroresection and coagulation (Beisland and Seland 1986). In this prospective randomised study 97 patients received either transurethral resection or laser treatment if small, superficial Ta/T1 tumours were found. These had to be small enough to be removed by one pass of the resectoscope loop (less than 6 mm). Those found to have larger though still superficial (Ta/T1) tumours underwent conventional tumour resection

first before randomisation to either have laser coagulation of the resected area 2 weeks later or to no further treatment. They observed no local recurrence, with follow-up in excess of 2 years, in patients treated with the laser compared to 26% in those who had received no follow-up treatment. This though was seen only with the larger tumours as small tumours tended not to recur whatever their mode of treatment. Recurrences elsewhere in the bladder were around 20% in both groups. This study's shortcoming is that for the larger tumours it compared the laser with *no* additional treatment; they really should have had a group that was retreated after 2 weeks with electrocoagulation. A previous study from the same group with shorter follow-up had shown a higher incidence of both local recurrences and new tumours after laser coagulation (Beisland *et al.*, 1985).

9.2.2 Convenience and economy

If the laser is sited in the main operating theatre it can only add to the complexity of a routine urological list. Even the more modern laser systems are more complicated to use than a diathermy generator and the nursing staff need to be trained in the operation of the laser and in the care and sterilisation of the fibre. Also the necessary safety precautions tend to slow down procedures and laser coagulation is generally rather more time consuming to perform than diathermy largely due to the relatively small laser spot size that requires multiple "shots" to all tumours. Therefore it is hardly surprising that often the laser lies idle in the corner of the operating theatre during routine lists.

Many urologists have introduced outpatient flexible cystoscopy lists as an efficient way of managing a large proportion of the "routine check" patients. When tumour recurrences are noted it is quite feasible to coagulate them with the Nd:YAG laser causing very minor discomfort to

the patient and little disruption to the list. There are also clear medical advantages for the many patients who are relatively elderly and unfit and whom would otherwise require repeated anaesthetics over many years. The resultant savings in terms of bed occupancy, theatre and anaesthetic costs can be considerable particularly if the laser is sited in a multi-specialty endoscopy unit where its use can be shared with other users primarily gastroenterologists and chest physicians. The sharing of a laser which could not possibly be fully utilised by even the largest urology department is essential to achieve cost-effective use of such an expensive item of equipment. Our experience of such outpatient laser treatment of patients with recurrent bladder cancer is presented in chapter 10.

9.2.3 Complications

The only significant complication of laser irradiation of the bladder, as already mentioned, is the possibility of damaging an adjacent loop of bowel from forward scattering of energy through the bladder wall. This is most unlikely using the energy parameters recommended. Hofstetter (1987) had only 3 patients out of more than 1000 in whom secondary intestinal perforation occurred, when too high a power (in excess of 80 W) was inadvertently delivered. Cos and Di Sant'Agnese (1988) found in dog bladder that bowel injury occurred with a 7 s irradiation (7 x 1 s pulses) at 60 W, or more than 10 s at 40 W. Also in contrast to electrical coagulation obturator nerve stimulation is not seen with the laser. It is prudent though, as well as considerate to a conscious patient, to avoid thinning the bladder wall by any more distension than is required merely to unfold the mucosa. The risk of perforation of the bladder itself, not unknown when resecting a tumour with diathermy especially in a thin-walled bladder, is much reduced with laser energy.

9.2.4 Patient selection and tumour staging

One of the criticisms often directed at laser therapy is that inadequate material is available for histological examination, particularly in respect of determining tumour stage which is the single most important prognostic feature. Biopsies of the lesion can be taken prior to laser treatment which may confirm the presence and grade of tumour but the base cannot be adequately sampled. Pre-treatment biopsy also negates any advantage there might be in the non-contact aspect of the laser technique and will potentially release viable tumour cells. It is quite adequate though to use post-laser biopsies for histology as the tissue architecture is minimally altered (Pavone-Macaluso *et al.*, 1990), in contrast to the situation after diathermy. It still is not possible to biopsy the tumour base unless the coagulated tumour mass is first detached, though the advent of automatic biopsy needles for use with rigid endoscopes (Cook Urological Inc.) enable a full thickness core of tissue to be taken through the tumour base.

We feel though that the index tumour should be resected conventionally so that an adequate deep biopsy of the tumour base may be obtained and complete histological information is available at the start. Those patients with papillary low grade superficial disease are then followed up on the outpatient laser list initially at 3 months and then at increasing intervals as long as they remain clear. The rare patients who develop sessile tumours, large numbers of recurrences or high grade malignant cells on cytology should revert to conventional treatment.

In this way the risk of significant understaging seems to be very low and large numbers of patients fit into this category in whom routine histology is unnecessary. This view is supported by a series of 121 patients treated by laser for superficial bladder cancer. Only 3 patients developed an

invasive tumour and none of these lesions occurred at the site of a previously treated tumour (Smith and Middleton, 1988). Most local "recurrences" are most likely the result of inadequate initial treatment and this is related to tumour size.

9.3 INVASIVE BLADDER CANCER

9.3.1 Introduction

The conventional "curative" management of a localised, invasive bladder tumour would be resection of as much tumour as possible into the muscle layer of the bladder wall followed later, after further assessment of both the patient and the stage of the tumour, by radical cystectomy with urinary diversion or by radiotherapy, either alone or in combination. It has been suggested that in selected patients the deep thermal effects possible with the Nd:YAG laser would be as effective as radiotherapy or cystectomy. The only other local approach would be to perform a full thickness resection of the bladder wall which would lead to considerable extravasation and would be unsuitable for tumours on the intraperitoneal area of the bladder. There is the worry that this would spread viable tumour cells to the extravesical tissues, so advocates of this approach have combined it with adjuvant systemic chemotherapy (Hall *et al.*, 1984). Although good results have been achieved this seems unlikely to become a common form of management.

When one is considering endoscopic treatment of a potentially lethal muscle invasive tumour it is important to be confident that a safe and consistent full thickness necrosis can be produced in the clinical setting. Despite these difficulties several authors have reported promising results with Nd:YAG treatment of invasive bladder cancer (Smith, 1989).

9.3.2 Clinical results

Most authors treated invasive tumours in a broadly similar manner. The patient has a conventional resection of their initial tumour deep enough for adequate staging. The subsequent laser irradiation is generally carried out under general anaesthetic after an interval of 1 - 2 weeks when the adherent blood clot has separated. A higher power than is generally used for superficial tumours (45 - 50 W) is applied in overlapping pulses to ensure 4 - 5 s treatment to each point of the tumour and surrounding mucosa. The characteristic blanching is less apparent in a previously resected field, so particular care has to be taken to ensure an even irradiation. A catheter is not needed post-operatively and patients can usually be treated as day cases.

The reported series of Nd:YAG treated patients with invasive cancer, for which there is adequate information about stage and treatment parameters, involve quite small numbers of patients (table 9.2). Beisland *et al.* (1985) reported a group treated by electroresection followed by laser irradiation of the tumour base which included 15 patients with stage T2 - 3 tumours. During a fairly short follow-up (less than 2 years) they noticed local recurrence of tumour in 13% whereas the occurrence of new tumours elsewhere in the bladder was 40%. This same group conducted a prospective randomised trial that included 25 patients with T2 tumours, 15 of whom had laser treatment after TUR, and 10 of whom had TUR only (Beisland and Seland, 1986). After 2 years all were still alive, with 11 (73%) of the laser group free from recurrence in the treated area compared with only 4 (40%) in the TUR alone group. The recurrence of superficial disease elsewhere in the bladder was similar in both groups. Longer follow-up (54 - 78 months) of the laser treated group showed no further local recurrences after 1 year and a 75% rescue by either radiotherapy or cystectomy of those whom relapsed (Beisland and Sander, 1990).

Authors	No. of patients	Tumour details	Treatment details	Follow-up (months)	Recurrences
Beisland *et al.*, 1985	15	T2-3, G 2-3	45-50 W TUR first > 1.5 cm	3-24	13% local, 40% new
Beisland and Seland, 1986	25 (randomised laser or TUR)	T2, G1-3	15 pts - TUR first then laser to base 10 pts - TUR alone	> 48 > 48	20% local 60% local
Malloy, 1986	20	T2	35-40 W TUR first > 2.5 cm	> 24	50% overall
McPhee *et al.*, 1988	32	12 pts - T2 7 pts - T3a 13 pts - T3b,T4	50-80 W (diffuser tip fibre)	6-72	nil local, 40% new T1 T3a - 57% local T3b-T4 - 100% local
Shanberg *et al.*, 1987	18	T2-T3	35-40 W TUR also > 2.5cm	early cystectomy in 78%	T2-38%, T3-80% (residual at cystectomy)
Smith, 1986b	22	T2-T3	45-50 W after TUR	> 6	T2-17%, T3-58%
Staehler *et al.*, 1985	22	T2-T3a, G1-3 (solitary tumours only)	TUR first	24 (mean)	9% local

Table 9.2 - Recurrences after laser treatment of invasive bladder tumours

Smith (1986b) reported 21 patients with muscle invasive tumour. Fifteen patients had T2 or T3 tumours and 8(53%) were disease free at a mean follow up of 1 year. Results not surprisingly were better in less advanced disease. Four out of 5 patients with T2 tumours were clear at 1 year as were 3 out of 6 with clinical stage T3a though 1 developed metastases, but only 1 out of 4 with T3b remained clear. The 2 clinically pT3a patients without metastases who failed were later staged PT3b after cystectomy. Six patients with bulky T4 disease all seemed to be symptomatically improved after palliative laser treatment. One delayed perforation of the sigmoid colon was seen but no incidence of small bowel damage.

McPhee *et al.* (1988) treated 32 patients with a higher laser power (50 - 80 W) but used a diffusing tip to increase spot size and therefore power density was probably less than that used by other groups. There was one postoperative death (cardiac) but no instance of bowel perforation. Crude survival for T2 and T3 tumours was 58% (50% disease free) at follow up between 4 months and 6 years. They again got good results in T2 tumours, with no local recurrence in 12 patients after follow up from 6 - 72 months, though 40% developed T1 recurrences elsewhere in the bladder. Four out of 7 patients with T3a cancer recurred and 3 died (only 1 with cancer). All 13 patients with stage T3b or T4 tumours died from tumour progression.

Impressively low recurrence rates after laser treatment of T2 tumours were reported by Staehler *et al.* (1985) though not by all workers. Shanberg *et al.* (1987) performed elective cystectomy with 2000 rad adjuvant radiotherapy within a few weeks of laser treatment of invasive tumours in 14 of their 18 patients. There was a high incidence of residual tumour in these specimens, especially those with T3 disease, though this may have been related to the low laser powers used (35 - 40 W).

9.4 MORPHOLOGY OF EXPERIMENTAL LASER LESIONS

When setting out to treat bladder tumours with the Nd:YAG laser it is important to consider the sort of lesion that is required to achieve this aim in a safe and consistent manner. Clearly for effective treatment of invasive tumours one should be aiming for a full-thickness coagulation of the bladder wall but this may be difficult to produce reliably in the clinical setting. The dosimetry recommendations of the early laser enthusiasts, most commonly 40 W for 2-4 s (Hofstetter and Frank, 1980; Staehler *et al.*, 1981a), are widely quoted with little supporting laboratory evidence. There are few published data from experiments comparable to clinical practice to guide the clinician in his choice of those power and energy parameters that might be expected to produce a given depth of necrosis and therefore match the tissue effect to the extent of the tumour being treated.

When monopolar electrical energy is applied to the bladder surface it passes along lines of least resistance to earth, and in a layered structure such as the bladder will spread in a somewhat irregular way along tissue planes and blood vessels for instance, in contrast to producing a rather more even lesion in a more homogeneous tissue such as liver. Laser energy though is not affected by tissue electrical conductivity (though it is by optical properties) and will tend to produce an even effect in all tissues. Therefore lasers are said to produce a deep and well-demarcated coagulation in the bladder whereas diathermy produces a more superficial, diffuse lesion (fig. 9.1). One might expect then to find careful studies comparing the morphological effect of these two energy modalities on bladder tissue. This is sadly not the case as is illustrated by work presented by Keiditsch *et al.* (1981) on the comparative tissue effects of diathermy and the Nd:YAG laser on rat and rabbit bladder, where no indication of the power or energy doses employed or the experimental method was given.

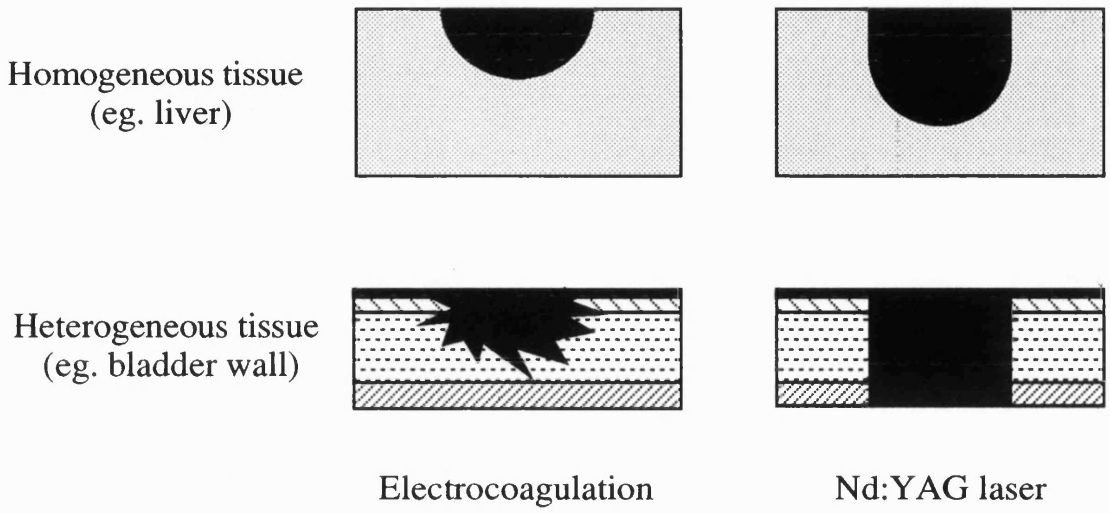


Fig. 9.1 Comparative schematic representation of lesions produced by electrocoagulation and Nd:YAG laser irradiation (after Keiditsch, 1986)

Furthermore even recent published experimental work on the effect of the Nd:YAG laser on the bladder is contradictory, and most of the key studies have significant shortcomings, particularly in that often they looked only at the immediate tissue effect which will underestimate the true extent of damage. For example Stein (1986), looking at immediate damage, found the laser effect more limited than previously thought, whereas Cos and Di Sant'Agnese (1988), examining lesions after 1 week, produced a deeper coagulation and a high incidence of bladder and small bowel perforation.

The aim of the work presented in this section was to study lesions produced in the pig bladder by Nd:YAG irradiation in a controlled setting, and to compare these laser effects to those produced by conventional diathermy under similar conditions. Many authors tend to relate the power employed to the tissue effect and whilst this is true for either very low or very high powers, it is the total energy absorbed by the tissue that is usually the most important factor though in a clinical setting this can be very difficult to regulate consistently when moving the laser beam over the target area.

9.4.1 Materials and methods

The foremost requirement for an model to assess the morphology of laser and diathermy lesions, and to derive clinically relevant conclusions, was that the bladder wall architecture and thickness should resemble the human as closely as possible. This precluded the use of rats as their bladders are so small and so we considered either the dog or minipig. The decision to use the minipig was largely one of economics and feasibility as the animal facility could only house a single large animal species at any one time and other workers were already engaged on pig work. Licence approval was also designed to encourage the use of minipigs in preference to dogs wherever possible. It will be seen later that if these considerations had not applied, the slightly thicker dog bladder may have been more satisfactory.

Five female minipigs (wt. 25 - 35 kg) were used in this study. They were premedicated with intramuscular ketamine (10 mg/kg), diazepam (0.5 mg/kg) and atropine (0.6 mg) which produced excellent sedation and allowed anaesthesia to be induced with gaseous agents only. General anaesthetic was maintained with a mixture of halothane, nitrous oxide and oxygen using a close fitting snout mask and a spontaneous breathing circuit. It was not found necessary to intubate the animals as there was no airway problem and muscle relaxation was quite adequate for these procedures. The depth of anaesthesia was monitored by observing the respiratory pattern and the pupil response. The animals were placed supine on a warming blanket and their core temperature was monitored with a rectal thermometer. Postoperative analgesia was provided by pethidine (50 mg) given IM during recovery from anaesthesia and further doses were seldom required. Animals were recovered in a warm cubicle with television monitoring and were not replaced in their cage until able to stand, usually within 1 hr. There was no anaesthetic or post-operative complication.

The bladder was exposed via a lower midline incision and was filled to 100 ml with either standard irrigation solutions of saline or glycine (for the diathermy experiments) at room temperature, through a 16 gauge cannula inserted into dome of the bladder. This volume was enough to unfold the bladder mucosa but without over-distending and thinning the bladder wall.

The original intention was to cystoscope the minipigs to avoid the need for a laparotomy. That was the reason for using females but although it was possible to pass a cystoscope per-urethrally it was found that the posteriorly angled and rather long urethra resulted in poor access to a large part of the dome and anterior bladder wall, the most accessible area at laparotomy. Furthermore unless the number of lesions made in each bladder were to be restricted to say 6-8 it might be difficult to identify the appropriate areas later and in the case of lower energy parameters, that would produce very little damage, this might not be possible. It was considered essential, therefore, to mark the position of lesions as they were made and as this required exposure of the bladder it was found more satisfactory to pass the laser fibre or electrode either via a cystoscope through a cystotomy in the dome of the bladder (fig. 9.2), or along a cannula inserted through the bladder wall (fig. 9.3). The use of a cystoscope enabled the energy to be delivered under direct vision and with the laser fibre was the best method of ensuring a consistent stand-off distance and spot size. It was only feasible to introduce the cystoscope sheath through a single point in each bladder (in the dome), so it was less easy to get an acceptably perpendicular approach to the mucosa in this way when treating both sides of the bladder. Passing the laser fibre or diathermy electode via a fine cannula opposite the treatment area was better in this respect and became the favoured method except for the "area" lesions (see later). The laser fibre would be advanced to just touch the mucosa and then be withdrawn 2 mm before firing.

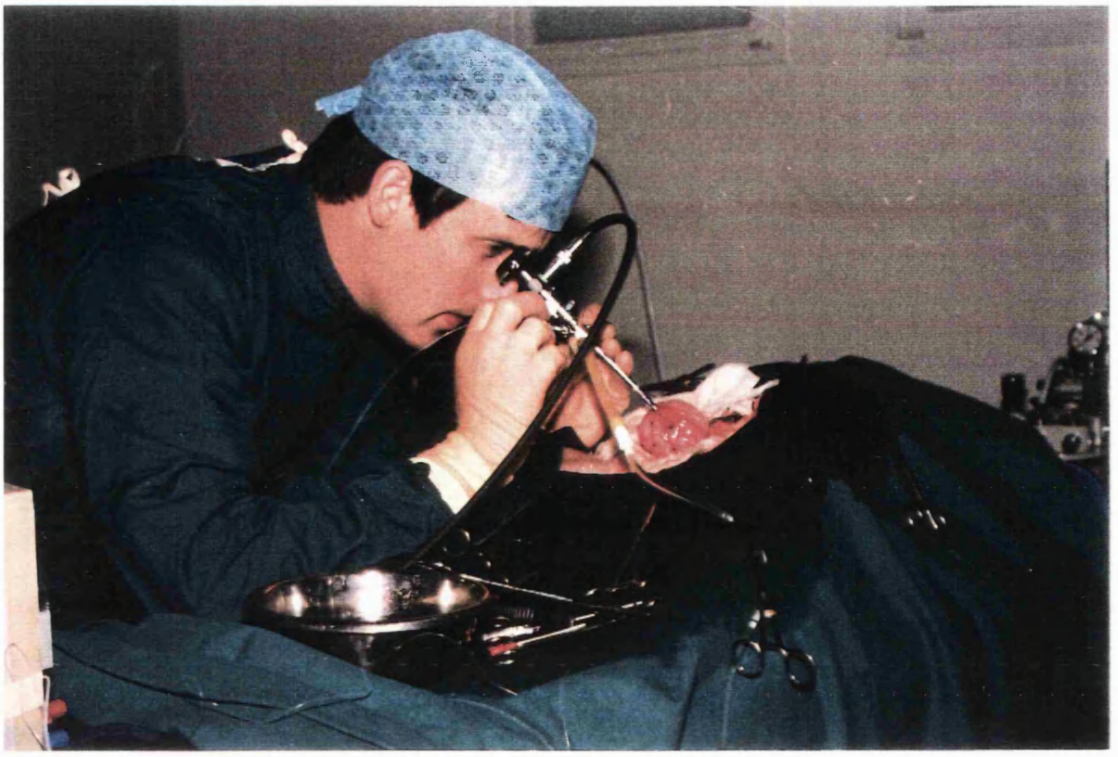


Fig. 9.2 Laser cystoscope inserted via a cystotomy in the dome of the bladder

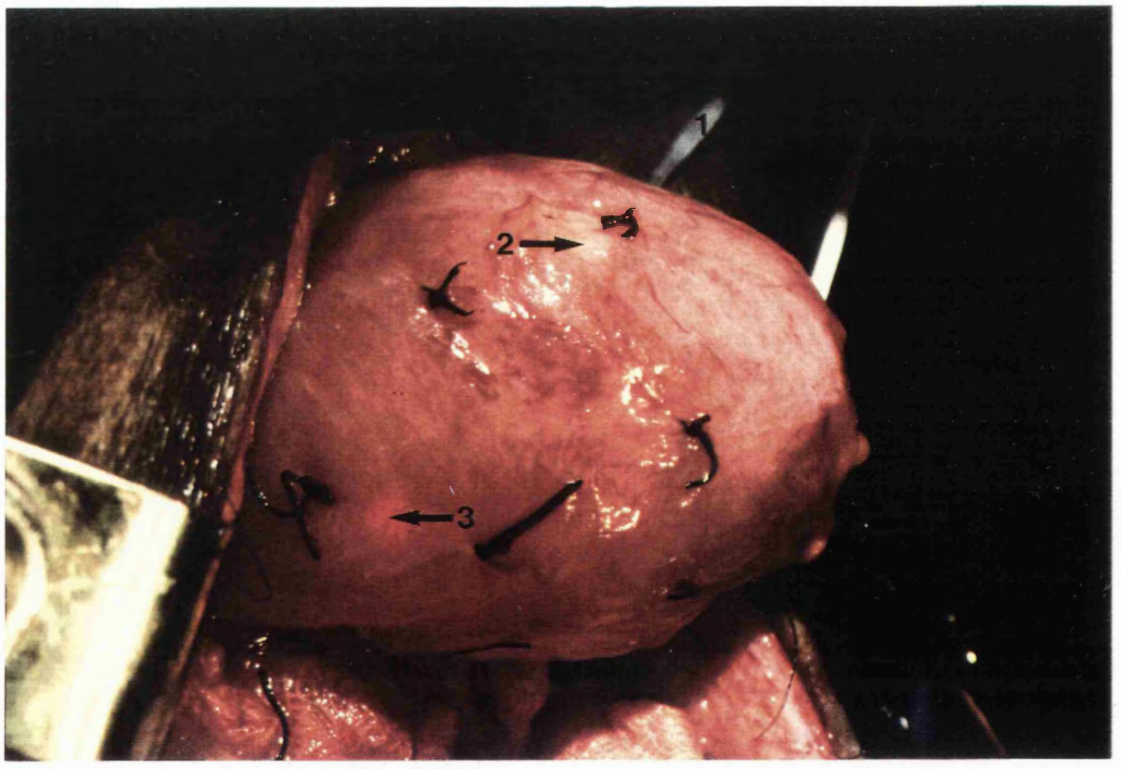


Fig. 9.3 HeNe aiming beam seen through the bladder wall
(laser fibre - '1', lesion - '2', aiming beam - '3')

Lesions were made with both the laser and diathermy as described below. These were arranged in 3 horizontal rows, 2-3 cm apart on both sides of the bladder giving about 24 lesions per bladder. Each treatment variable studied was repeated 5 times in several bladders to maximise the range of biological variation that might be expected. An urethral catheter was left sutured in after operation in the first 2 animals. However this did not last long before being chewed off so was subsequently abandoned, and no instance of delayed perforation at a cystotomy or lesion site was encountered. Animals were killed 5 days later and their bladders removed, distended to 100 ml with 10% formaldehyde and fixed. The individual lesions were identified (see below) and processed for histological examination. Standard H & E stained sections were cut and the maximum depth of lesion was measured to the nearest 0.1 mm using a microscope. Micrometer measurements were taken of bladder strips before and after processing to correct for the specimen shrinkage that occured during the dehydration and embedding procedures.

9.4.1.1 Energy sources and lesions

The Nd:YAG laser used (Fiberlase - Living Technology Ltd, Glasgow) had a maximum output of 100 W at 1064 nm which was transmitted via a 600 micron "bare" laser fibre. The incident power from the end of a freshly cleaved fibre was checked with the internal power meter prior to the creation of lesions at each power level studied. The fibre tip was positioned approximately 2 mm from the mucosa for each exposure and the helium-neon aiming beam could be clearly seen through the bladder to enable accurate marking of lesions by a coded serosal suture (fig. 9.3).

Single diathermy lesions were made with a 5 Fr button electrode placed in light contact with the mucosa. Most area lesions (see below) were produced under vision with this electrode but a few (9) were made with a

resectoscope loop for comparison. A standard clinical diathermy unit (Eschmann TD 411-S) was used which has an output calibrated in watts as well as the more usual numerical scale (in these experiments 20 W, 40 W and 60 W output corresponded to coagulation settings of 2, 3.5 and 4.5 respectively but these probably bear an inexact relationship to the actual power delivered).

Two sorts of lesions were produced by both laser and diathermy energy:

i) **spot lesions** - a single pulse of either 2 or 4 s exposure at powers of 20, 30, 40, 50 and 60 W (the diathermy effect was studied at either 20, 40 or 60 W only). This produced a reproducible lesion that could be used to compare dosimetry parameters.

ii) **area lesions** - these simulated the effect achieved clinically when coagulating a small tumour. The laser fibre, diathermy electrode or loop was manipulated under endoscopic control and enough energy was delivered, again at powers of 20-60 W, to produce an area of "white coagulation" approximately 0.5 cm^2. This blanching is generally taken as indicating that enough energy has been delivered for adequate coagulation of a superficial tumour.

9.4.2 Results

The data on the depth of the lesions produced in these experiments are presented in tables 9.3 and 9.4. The fresh specimens were found to have shrunk by an average 20% during histological processing so the mean values given in these tables have been increased to correct for this in the summary of these data shown in table 9.5. Where a lesion had penetrated the full thickness of the bladder wall its depth is given as the thickness of the bladder wall at that point and such instances are indicated in bold type.

		Depth of necrosis with Nd:YAG laser in pig bladder (mm)	
Power (watts)	**Duration (s)**	**Individual lesions**	**Mean depth**
20	2	1.0, 1.2, 1.3	1.2
	4	1.0, 1.5, 1.6	1.4
	Area	1.7, 2.5, **3.0**	2.4
30	2	1.6, 1.5, 1.8	1.6
	4	2.0, 2.4, **2.5**	2.3
	Area	2.0, 2.2, **2.1**	2.1
40	2	1.2, 1.7, **1.5**	1.5
	4	1.6, 2.1, **2.8**	2.2
	Area	1.0, 2.3, **2.0**	1.8
50	2	1.8, 2.0, 2.2	2.0
	4	2.5, **2.6, 3.0**	2.7
	Area	**2.3, 2.4, 2.6**	2.4
60	2	1.8, **2.2, 2.3**	2.1
	4	**2.0, 2.5, 2.5**	2.3
	Area	**2.0, 2.3, 2.5**	2.3

Table 9.3 Depth of necrosis produced by laser (to nearest mm)

Values in bold indicate that the lesion was full thickness

Some lesions were excluded as they could not be accurately identified either in the gross specimen or when the blocks were cut (this proved more of a problem at the lower energies). Where there were more than 3 lesions for evaluation, only the 3 thickest have been included.

		Depth of necrosis from diathermy coagulation in pig bladder (mm)	
Power (watts)	Duration (s)	Individual lesions	Mean depth
20	2	0.7, 0.8, 1.0	0.8
	4	0.8, 1.1, 1.5	1.1
	Area	1.1, 1.2, 1.4	1.2
40	2	1.4, 1.1, **2.3**	1.6
	4	2.0, **2.3**, **2.6**	2.3
	Area	1.5, 1.6, 1.8	1.6
60	2	1.4, 1.1, **1.6**	1.4
	4	1.6, **1.5**, **2.3**	1.8
	Area	1.8, **1.8**, **2.0**	1.9

Table 9.4 Depth of necrosis produced by diathermy

Values in bold indicate that the lesion was full thickness.

The first measurement in each row relates to a lesion made with the diathermy loop, the last 2 relate to lesions with the button electrode

Power (watts)	Duration (s)	Mean depth of necrosis in pig bladder (mm)	
		Nd:YAG laser	Diathermy
20	2	1.5	1.0
	4	1.8	1.4
	Area	**3.0**	1.5
30	2	2.0	-
	4	**2.9**	-
	Area	**2.6**	-
40	2	**1.9**	**2.0**
	4	**2.8**	**2.9**
	Area	**2.3**	2.0
50	2	2.5	-
	4	**3.4**	-
	Area	**3.0**	-
60	2	**2.6**	**1.8**
	4	**2.9**	**2.2**
	Area	**2.9**	**2.4**

Table 9.5 **Mean depth of both laser and diathermy coagulation in pig bladder**

values in bold type indicates at least 1 lesion was transmural

The mean values given in tables 9.3 and 9.4 have been corrected here by the average shrinkage (20%) that occurred during processing in 10 carefully measured samples to arrive at the "true" depths given above.

It was clear that both energy modalities were able to produce effective full thickness lesions in the relatively thin-walled (mean 2.6 mm) pig bladder. This will have clearly underestimated the maximum potential penetration and not surprisingly it will be noted that many of the lesions made at the higher powers fall into this category. Indeed it was unusual **not** to produce a transmural coagulation with either laser or diathermy electrode at settings greater than 30 W for 4 s. At least 1 "area" lesion made with the laser at all powers studied was full-thickness though this was less common with the button electrode. None of the lesions made with the diathermy loop was transmural whereas half of those produced by the button electrode at 40 W and all but one at 60 W were full thickness. The laser or diathermy electrode also produced a generally deeper coagulation than the same energy parameters applied to the diathermy loop.

The appearance of white coagulation used as the visual endpoint in the "area" lesions resulted in a depth of necrosis generally between that obtained at 2 and 4 s (more reliably so with the laser). The laser energy level most often recommended for treating superficial bladder tumours (40 W for 2 s) produced a coagulation depth averaging 1.9 mm, though one of these lesions was transmural.

What was particularly striking, though, was the marked qualitative difference between the two modalities when used at equivalent energies. Lesions produced with the diathermy button electrode at the lower energies showed a distinct tissue graduation between the various thermal effects (see chapter 2, fig. 2.12) ranging from surface vaporisation, carbonisation, through true coagulation to a zone of recoverable thermal injury (fig. 9.5). Figure 9.6 shows a lesion produced by the same energy of laser irradiation (20 W for 4 s) where the transition between these zones is less noticeable though the depth of effect is similar. At 40 W (4 s) this difference is more

marked (figs. 9.7 and 9.8), with the laser lesions showing coagulative necrosis, some superficial haemorrhage and loss of nuclear staining in muscle fibres, but little alteration of the tissue architecture. Increasing the power to 60 W for 4 s (figs. 9.9 and 9.10) produced the most striking differences with considerable structural disruption following diathermy, particularly close to the point of contact, but a homogeneous laser lesion.

The lateral margins of coagulation were sharply demarcated from normal tissue following both energy modalities and the base in partial thickness lesions was also fairly smoothly defined. Laser lesions were generally a little more sharply defined than those from diathermy but the differences were not marked and in particular a significant spread of diathermy effect in an irregular fashion along tissue planes was not apparent. The inevitable slight variations in the pressure with which the diathermy electrode was applied to the mucosal surface seemed to be the main factor in the slight variability seen in the geometry of diathermy lesions.

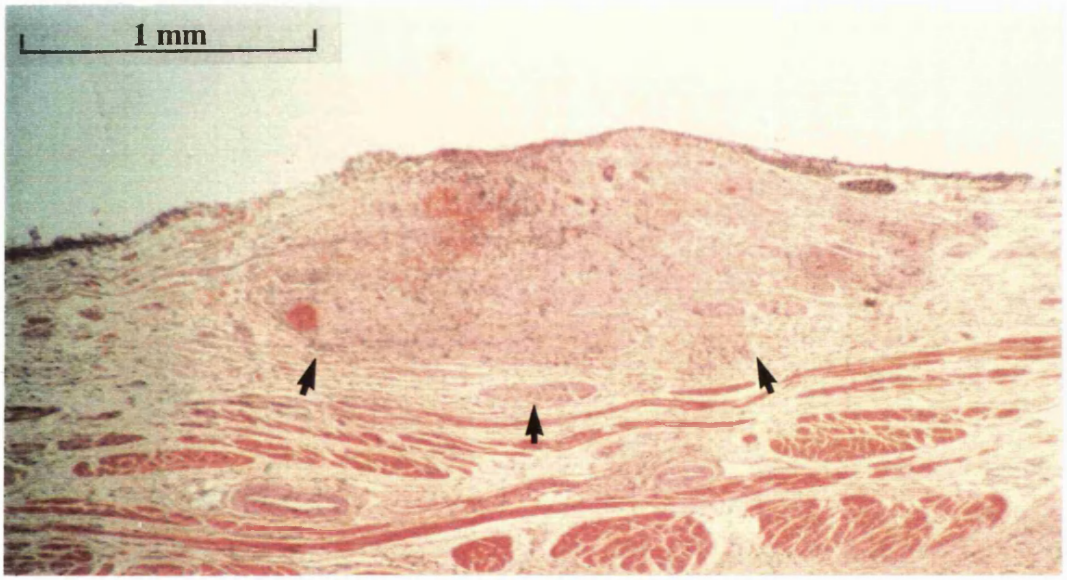


Fig. 9.4 Superficial coagulation from the diathermy resectoscope loop (20 W for 4 s)
(compare with effect of diathermy electrode - fig. 9.5)

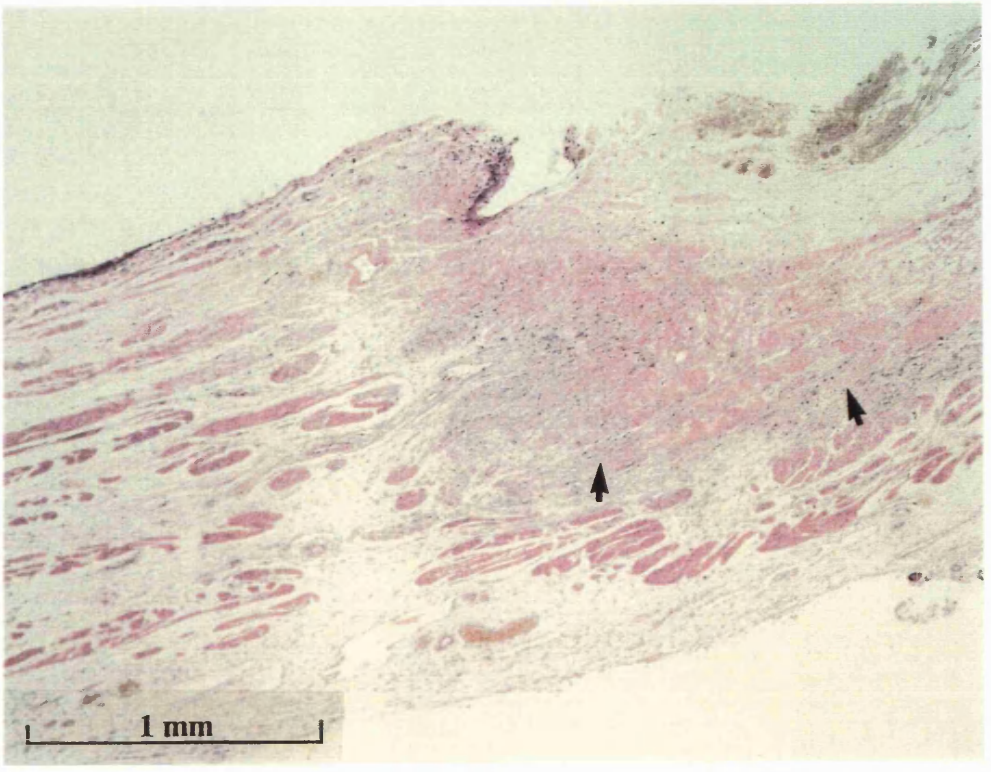


Fig. 9.5 **Diathermy electrode 20 W for 4 s**
(note graduated tissue effect as in fig. 2.7)

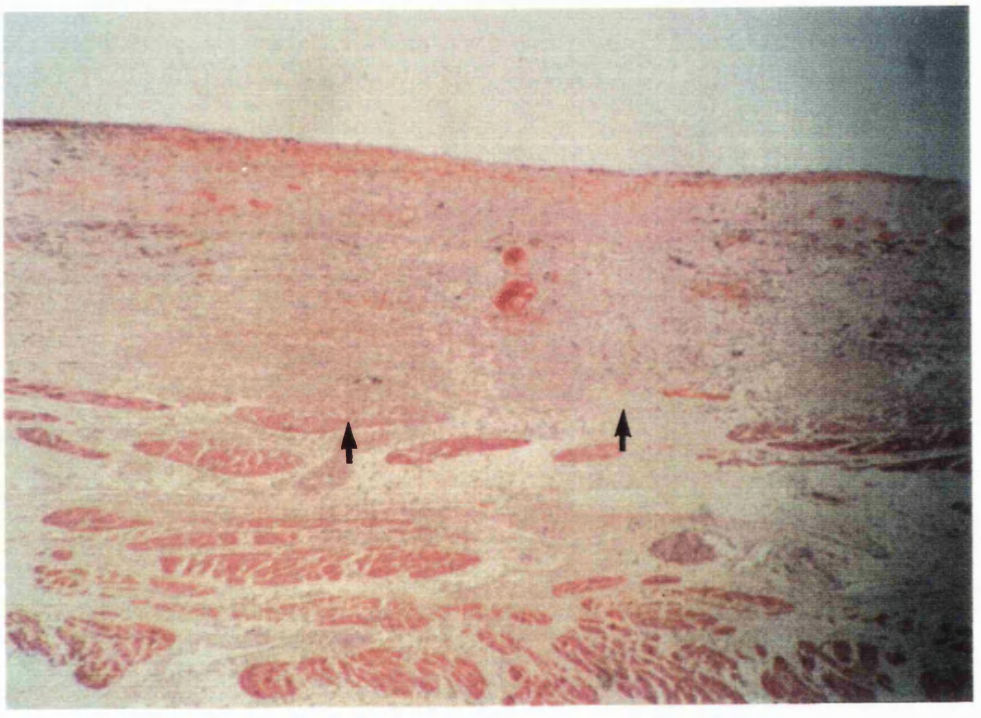


Fig. 9.6 **Nd:YAG laser coagulation 20 W for 4 s**
(note the somewhat more uniform effect)

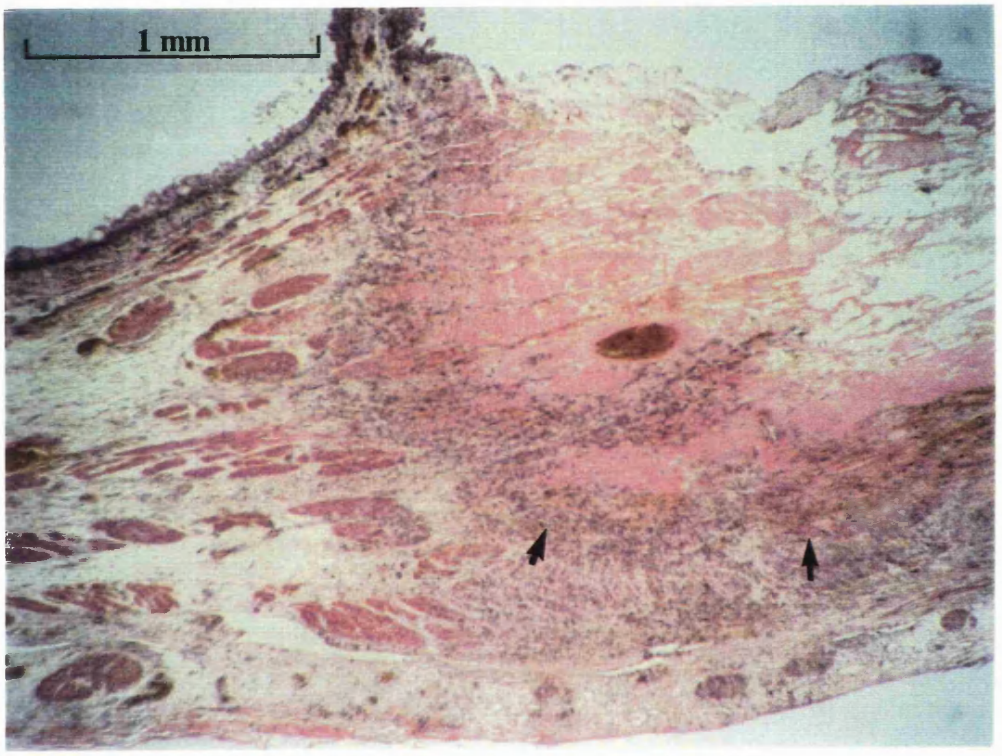


Fig. 9.7 Diathermy electrode 40 W for 4 s

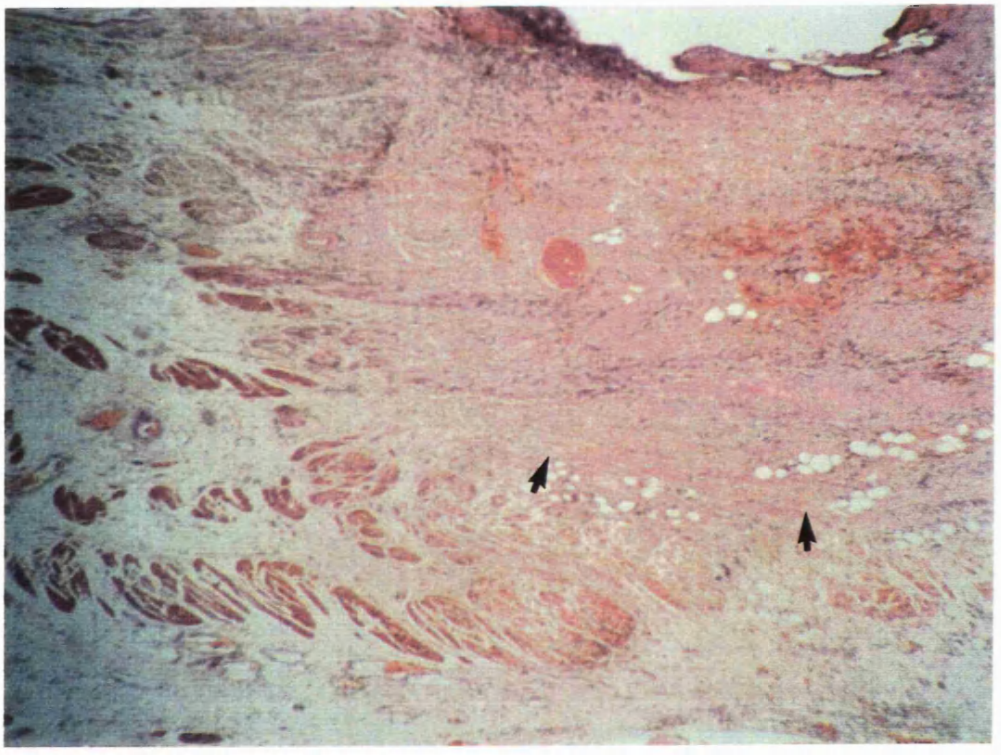


Fig. 9.8 Nd:YAG laser coagulation 40 W for 4 s
(similar depth but more even coagulation)

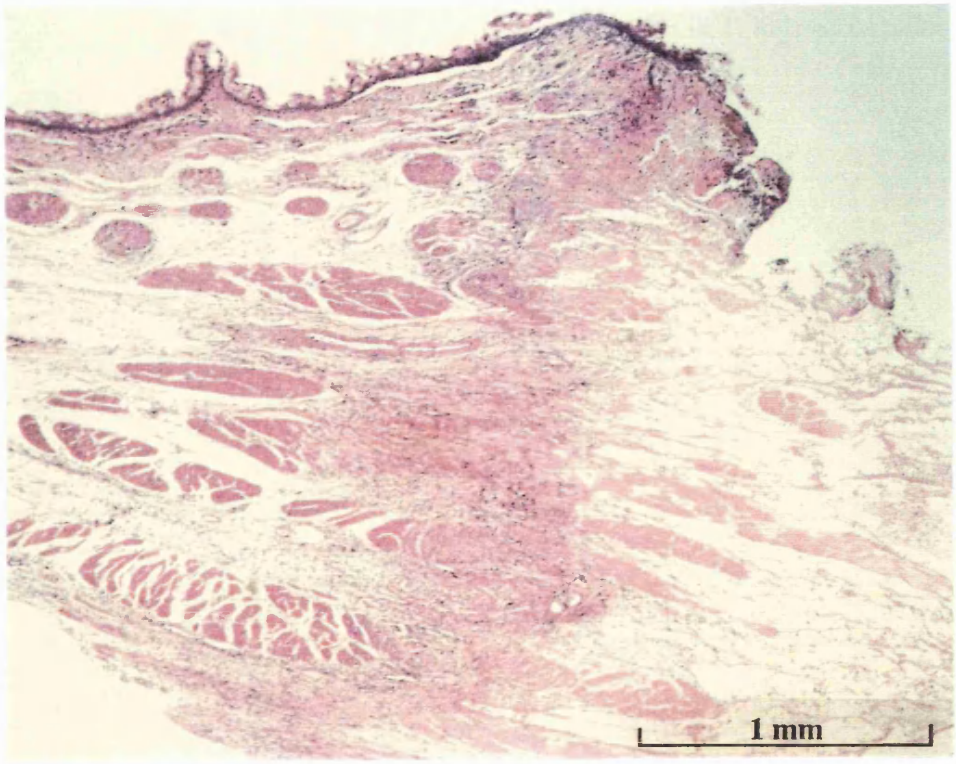


Fig. 9.9 Diathermy electrode 60 W for 4 s
(note considerable disruption of tissue architecture)

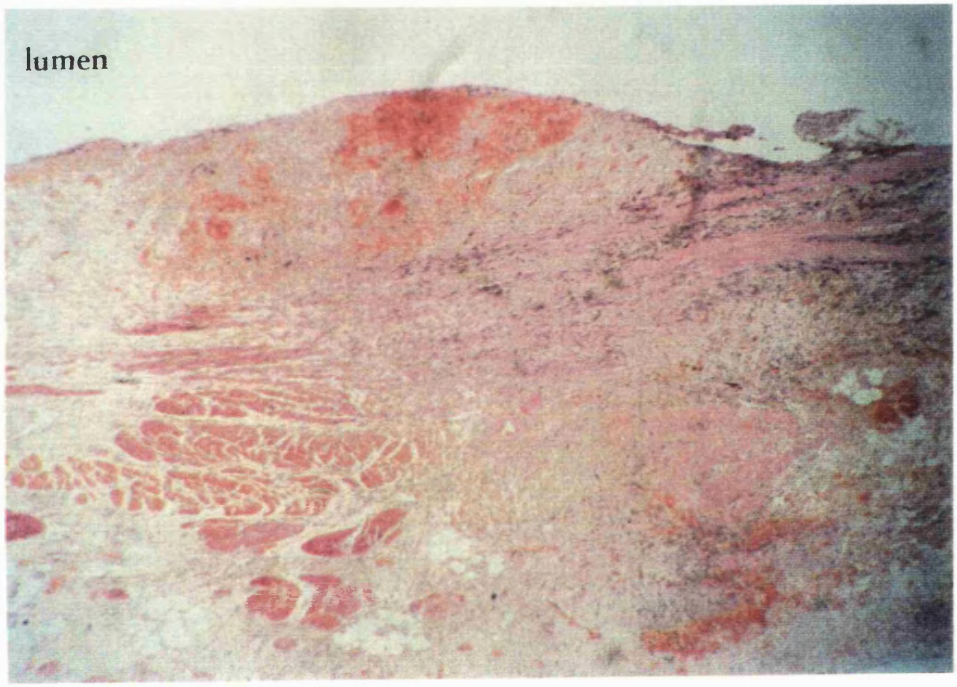


Fig. 9.10 Nd:YAG laser coagulation 60 W for 4 s
(note the much smoother transmural coagulation)

9.5 DISCUSSION

As Nd:YAG lasers are becoming more widely available to urologists, usually in the context of a multispecialty laser unit, clinicians want to know whether or not they have any advantages over conventional techniques, i.e. electrocautery. The early laser enthusiasts from Hofstetter's Munich group did much to promote the Nd:YAG laser in Urology, treating large numbers of patients with superficial bladder carcinoma and claiming a significant reduction in total recurrences after laser treatment (Hofstetter *et al.*, 1981). Other authors though have only seen a reduction in *local* recurrences after laser treatment (table 9.1), still implying however that laser coagulation is more effective at destroying malignant cells than diathermy, presumably by producing a more even and deeper coagulation. It is for the quantification of such laser effects that the available studies need to be reviewed.

9.5.1 Experimental studies

Several authors have explored the laser dosimetry parameters that might produce a safe, full thickness coagulation of the bladder wall. In some of the earliest reported work, Hofstetter and Frank (1980) measured the serosal temperatures reached in specimens of fresh human bladder. A power of 50 W applied for 4 s raised the temperature of the outer bladder wall to 65°C, enough to produce a full thickness thermal necrosis, but adjacent loops of small bowel were not heated beyond 45°C. The depth of necrosis obtained with electrocautery was much less though unfortunately the dosimetry data given is scanty. Further work from this group, largely extrapolated from serosal temperature profiles in thinner rabbit bladders, suggested that 45 W for 2-4 s would produce an even tissue necrosis to 4 mm but a greater penetration was not possible (Pensel *et al.*, 1981).

Staehler *et al.* (1981a) calculated that 200 - 300 J of laser energy would be necessary to achieve a transmural necrosis in human bladder and this could be obtained by 3 pulses of 40 W for 2 s each. This statement was based on experiments using fresh tissue producing a sustained serosal temperature of 60°C which they considered adequate for a full thickness necrosis though no histological confirmation of this was shown. They concluded however that 40 W for 4 s was most appropriate as it would allow for some safety margin. This dose produced small bowel perforation in around 20% of rabbit bladders (whereas 40 W for 2 s did not), but only 1 case of perforation occurred in their clinical series and that was when a power of 70 W was inadvertently delivered (Staehler *et al.*, 1981b).

Stein (1986) investigated both Nd:YAG and CO_2 laser penetration in the bladder and kidney. He found that a greater effect was produced when using room temperature irrigant than with either very cold (2°C) or very hot (85°C) irrigant. This is easily explicable as in the case of the very cold bladder the laser energy needs to raise the temperature excessively whereas at the other extreme only a small additional rise is necessary to produce carbonisation and increased backscattering which then reduces laser penetration, though in any case the entire bladder will have been "cooked" at such a high temperature. Their conclusion that room temperature irrigant provides the ideal compromise of a heat-sink to reduce carbonisation but not excessive cooling may be true but is not supported by their data as other temperatures, e.g. 37°C, were not studied. Room temperature irrigant (22 - 25°C) was used for our work as this is universally the case in clinical practice for convenience rather than optimum effect. Stein could produce a maximum depth of lesion in the dog bladder of only 2.6 mm, using 50 W for 4 s, whilst the dose that is generally recommended to treat

superficial tumours, 40 W for 2 s, penetrated less than 1 mm. In our work 40 W for 2 s produced a deeper effect (average 2.9 mm).

All these studies though suffer by looking only at the immediate effect of laser energy, often on dead tissue. This may give an inappropriate under-estimation of the laser effect as the maximum damage is not apparent for at least 4 days (Bown *et al.*, 1980). Only 1 other group have studied the morphological effect of laser coagulation after such an interval.

Cos and Di Sant'Agnese (1988) studied the histological effects in the pig bladder 1 week after endoscopic Nd:YAG irradiation. They irradiated areas approximately 1.5 cm in diameter using laser powers from 10 - 60 W for 7 - 30 s either continuously or in 1 s pulses. Although it may seem desirable to try and reproduce a "clinical" lesion in this way it is inevitably far more subjective than a spot lesion and even coverage, especially with the shorter exposures, is likely to be near impossible. For example they state that a focussed spot diameter of 2 mm was used; 56 of these will fit into a 1.5 cm diameter circle so this implies an exposure time of only 0.13 - 0.54 s on each fresh target area, very small energy densities compared with other workers' stated parameters. In practice of course the laser effect spreads out and adjacent pulses overlap but this is difficult to quantify scientifically and compare with others' results. This is why we used both spot and area lesions in this work, but instead of giving a set amount of energy to the area lesions chose the clinically more useful, but less quantifiable, visual endpoint of even "white coagulation".

Despite these apparently low energy densities Cos and Di Sant'Agnese (1988) produced rather deep lesions in their pig bladders. They found that pulsed irradiation produced a deeper effect than the same energy given in a continuous form. Limiting the laser power to 30 W for 15 s produced lesions up to 4 mm deep, as did 10 W for 30 s. A transmural necrosis

(stated thickness 4-5 mm) resulted from 40 W either for 10 s continuously or for 7 pulses of 1 s. Perforation either of the bladder or, when the bladder dome or posterior wall was irradiated, of the adjacent small bowel was seen with higher powers and/or longer exposures. The authors state that depth of effect was directly proportional to the applied energy but their data would seem to show that power is the more important variable as 40 W for 10 s (400 J) produced a deeper lesion than 30 W for 15 s (450 J). They then concluded that really the clinician should be guided by the visual appearances but should be wary of using more than 30 W and 10 pulses for a 1.5 cm diameter sessile tumour. So much for precise science!

9.5.1.1 Dosimetry recommendations

If anything is clear from the studies discussed above it is that no hard and fast dosimetry recommendations can be made! One of the difficulties is with a suitable animal model. The thickness of the minipig bladder used here was about half than of Man, with the result that most lesions made at powers above 30 W were transmural (i.e. in excess of our average bladder wall thickness of 2.6 mm). This makes any further discrimination of maximum penetration impossible, and is the major shortcoming of this study. Although the visual appearances of white coagulation seemed to be achieved by much less than 4 s exposure on each spot area, the lesion was often deeper than a 4 s pulse at that power. This suggests that in practice there was inevitably considerable overlapping of the adjacent fields.

A thicker animal bladder, such as the dog, was not possible due to facilities, expense and licence conditions. The ideal experiment would be to laser human bladders several days prior to cystectomy, but the ethics of this are questionable and no comprehensive such study has been reported. It seems unlikely that experiments on dead tissue have much to offer in predicting the extent of a fully developed lesion as it will be considerably under-

estimated. A pilot study we carried out on post-mortem dog bladder specimens prior to this work failed to penetrate deeper than the superficial muscle (less than 0.5 mm) even using a power of 60 W for 5 s.

It has been often stated that the depth of diathermy coagulation is less and more irregular than that produced by the Nd:YAG laser. This statement need qualifying because our results seem rather inconsistent with the usual view of diathermy producing irregular lesions as indicated in fig. 9.1. It is clearly possible to produce a very effective transmural coagulation in the pig bladder with modern diathermy, and by extrapolation well into the muscle layer of a human bladder (thickness approx 5-7 mm). Also a similar depth of penetration was achieved from roughly equivalent energies with either the laser or a diathermy electrode (figs. 9.5-9.10). The coagulation resulting from the resectoscope loop diathermy though was relatively superficial, extending only 1-2 mm into the bladder wall despite the endoscopic appearance of "white coagulation". This appearance using the Nd:YAG laser reliably signified a coagulation depth greater than 2 mm and usually 3 mm.

The most striking feature was the great qualitative difference between electrocautery and laser. Even in the transmural laser injury there was little difficulty in identifying the several layers of the bladder wall, often some haemorrhage was seen superficially but muscle fibres for example were readily recognisable. Although no mechanical tests were done, and no instances were encountered in this study, the likelihood of a perforation through such an area seems much less than at the site of extensive diathermy damage, where vaporisation, cavitation and disruption of the tissue architecture could be most dramatic (fig. 9.9). Care is necessary to avoid bladder perforation with diathermy particularly in thin walled bladders with extensive tumours, whereas this is most unlikely to occur

with laser coagulation although the laser carries a much greater risk of damaging adjacent bowel from forward scattered energy. It is not surprising that biopsy specimens taken after laser coagulation are usually perfectly adequate for grading and staging tumours (Pavone-Macaluso *et al.*, 1990) whereas the report of "severe diathermy artefact" is more usual following electrocoagulation.

Not withstanding the previous comments about the difficulty of making recommendations for clinical dosimetry based on animal work it is possible to suggest the following:

i) Using the Nd:YAG laser for superficial bladder tumours (e.g. at out-patient flexible cystoscopy), a coagulation deeper than 1 mm is not necessary so a power of 20 - 30 W for 2 s is adequate, and safe.

ii) When used in conjunction with rigid cystoscopy or following the preliminary resection of larger tumours a deeper coagulation with 2 second pulses of 30 - 40 W is appropriate.

iii) Coagulation resulting from loop diathermy is relatively superficial so after initial resection of a tumour the loop should be exchanged for a button or ball electrode for adequate treatment of the tumour base, if the laser is not being used.

iv) The aim of producing an effective transmural coagulation in human bladders to treat early invasive disease is possible with 50 - 60 W for 2 - 4 s but should be considered experimental; a synchronous open / endoscopic procedure may be less likely to damage adjacent bowel.

9.5.2 Conclusions

9.5.2.1 Superficial bladder cancer

The initial hope that Nd:YAG laser coagulation would provide a more effective treatment for superficial bladder cancer than electrocautery has

not been realised. Overall recurrence rates are not significantly reduced though most authors have reported fewer local recurrences. Furthermore no evidence has yet been presented to show a superiority over conventional treatment in either the incidence of disease progression to muscle invasion or in survival. Therefore laser coagulation needs to offer other advantages, most probably in terms of economy or convenience of treatment, if these techniques are to justify a wider adoption in general urological practice.

The Nd:YAG laser in conjunction with flexible cystoscopy can make a useful contribution to the management of a large proportion of bladder cancer patients by enabling anaesthetic free, outpatient treatment of their recurrences. The laser technique is easy to learn and as long as certain selection criteria are applied there is no real risk of tumour understaging. Considerable benefits may be had in terms of convenience for the patient and a more economical use of health-care facilities, especially as part of a multispecialty laser unit. Our experiences with this method of treatment are described in the following chapter.

9.5.2.2 Invasive bladder cancer

The development of a muscle invasive bladder tumour carries a very poor prognosis in that only around 50% of treated patients will survive 5 years. Radical cystectomy is often inappropriate in the elderly patient whereas radiotherapy can also result in considerable morbidity. Even after radical treatment for apparently localised disease, many patients will die of metastases. Therefore it would seem logical to pursue methods of effective local control that carry less morbidity than either radical surgery or radiotherapy perhaps combined with adjuvant chemotherapy to "mop up" unrecognised micro-metastases. As the Nd:YAG laser has been shown to produce a deep coagulation without loss of bladder wall integrity it merits further study as to whether an effective treatment for localised muscle

invasive tumours is possible. Surprisingly few authors have addressed this point directly and their studies, as with many trials of "new" treatments, tend to consist largely of patients who are either unfit for, or whom refuse conventional treatment (table 9.2).

Despite these problems it would seem that Nd:YAG irradiation after initial endoscopic resection will reliably control T2 tumours in over 80% of patients. The difficulty is with the more advanced lesions and particularly the risk of understaging T3b tumours which are not adequately treated by laser. The main problem is in being unable to guarantee a consistent full thickness necrosis in the clinical setting despite what seems to be possible experimentally. One possible solution to this might be to irradiate the tumour, if appropriately situated, from the serosal aspect also; ideally via a laparoscope. This would also prevent possible damage to overlying bowel from high energies of Nd:YAG irradiation fired "blind" from within the bladder. Another exciting prospect is the use of multiple low-power fibres placed directly into the tumour with the development of the lesion being monitored by endoscopic ultrasound.

Until more accurate dosimetry is possible it seems prudent to select patients carefully for curative laser treatment with either T2 tumours or those with T3a cancer who are unsuitable for standard treatments. Palliative laser coagulation may also have a useful role as a haemostatic treatment for troublesome and resistant haemorrhage from incurable tumours. However it should be stressed that laser therapy for invasive bladder tumours is still in its infancy and at the present time should only be realistically applied to superficial bladder tumours. It seems likely though that when more patients have been treated with the Nd:YAG laser, the outcome will be comparable with the best results after radical surgery or radiotherapy but with only a fraction of the morbidity seen after these conventional treatments.

Chapter 10

OUTPATIENT LOCAL ANAESTHETIC TREATMENT OF SUPERFICIAL BLADDER CANCER USING FLEXIBLE CYSTOSCOPY AND Nd:YAG LASER

10.1 INTRODUCTION

There is an increasing tendency in surgical practice, encouraged by the Government and the Royal Colleges, to treat more patients on a day-case or outpatient basis. This can produce significant economies particularly in terms of saving on inpatient beds and nursing costs though often requires more skilled medical attention (the Royal Colleges recommend that day surgery is performed by senior staff to minimise complications), and is more demanding of community medical services. The use of improved local anaesthetic techniques, often combined with reversible sedation, has enabled many common operations to be done without a general anaesthetic and has been shown by their proponents to result in dramatic waiting list reductions (Birch *et al.*, 1990). In addition the introduction and steady proliferation of new technologies, including lasers, is resulting in more surgical procedures becoming minimally invasive or even non-invasive. Percutaneous and laparoscopic surgery are rapidly replacing several standard open operations for the majority of cases, and urologists have been in the forefront of the development of both these and non-invasive techniques such as extracorporeal shockwave lithotripsy.

10.1.1 Flexible cystoscopy

Flexible cystoscopes though several times more expensive than their rigid counterparts, are becoming increasingly popular for outpatient diagnostic and minor therapeutic manoeuvres (e.g. biopsy and stent removal) as they need only topical urethral anaesthesia for male patients, and none at all in women. Fibreoptic endoscopes have been used in gastroenterology for over 25 years and although the first reported use of a flexible instrument to view the bladder came from Japan in 1973 (Tsuchida and Sugawara), it was

not until much later that any real experience with these instrument was reported (Burchardt, 1982). Not surprisingly the early flexible cystoscopes were derived from gastroenterological endoscopes, usually choledocho-scopes, and were less manoeuvrable and of larger calibre than the present purpose designed instruments, the first of which was introduced by Olympus in 1986. Flexible cystoscopy is now well established and has been shown to be at least as accurate in diagnosis (Clayman *et al.*, 1984), more efficient in practice (Fowler *et al.*, 1984), and more acceptable to patients than conventional rigid endoscopy (Flannigan *et al.*, 1988). The many applications of this technique have been reviewed by Kennedy and Preminger (1988) and we have compared elsewhere the technical attributes of the currently available instruments (Pope and Wickham, 1991).

The main use of an outpatient flexible cystoscopy list is to provide an efficient means of surveillance for a large proportion of the "routine check" bladder tumour patients who have a relatively low risk of recurrence. When a recurrence is noticed however it is rather less satisfactory to then have to re-book the patient for a general anaesthetic list. It is of course possible to use fine diathermy electrodes with flexible cystoscopes but these are distinctly uncomfortable, particularly on or close to the trigone. This usually necessitates parenteral analgesia and sedation which seems to negate the major advantage of flexible cystoscopy, although injecting local anaesthetic under the tumour is feasible. The discomfort from Nd:YAG laser energy seems much less than that from diathermy, though no controlled trial of the two modalities has been published.

We have studied the feasibility and effectiveness of providing laser treatment for the routine outpatient follow-up of bladder cancer patients over a 2 year period at University College Hospital, London.

10.2 PATIENTS AND METHODS

10.2.1 Patients and instruments

A routine flexible cystoscopy list has been established at University College Hospital since 1986. It largely comprises just over 100 of the lower risk "routine check" patients in addition to diagnostic cystoscopies where malignancy is not suspected. The period of this study ran for 2 years (April 1988 - April 1990) with follow-up data to the end of 1990. During this time 33 patients (19 male, 14 female; mean age 67 years, range 46-85) who developed superficial bladder tumour recurrences were entered into the study. Four other patients had recurrences either considered too large or numerous for laser therapy (2), or in an inaccessible place for adequate access with the laser fibre (just inside the bladder neck, and within a diverticulum). Original histology, available for 28 patients, showed only 1 invasive tumour (pT2 G2). Most patients had G1 disease though 10 had presented with pT1 G2 tumours. The average precedent diagnosis was 6 years (range 3 months to 21 years) though in those for whom this was the first recurrence (12 patients) this time was rather shorter (mean 2.8 years).

All examinations and treatments were performed on an outpatient basis using 2% lignocaine gel as topical anaesthesia in men. An Olympus CYF-1 flexible cystoscope was used (Keymed, U.K.) for both diagnosis and treatment, in conjunction with a Nd:YAG laser (Flexilase - Living Technology, Glasgow - fig. 10.1). This had a power output of 1 - 100 W and required 3-phase power and water cooling. A voided urine sample for cytology was collected at every visit. Tumours were not routinely biopsied when the visual appearances were indicative of a superficial recurrence. Biopsies were more likely to be taken of suspicious mucosa or from the site of laser treatment at follow-up. Examinations were repeated at increasing intervals from 3 months to 1 year as long as the bladder remained clear.

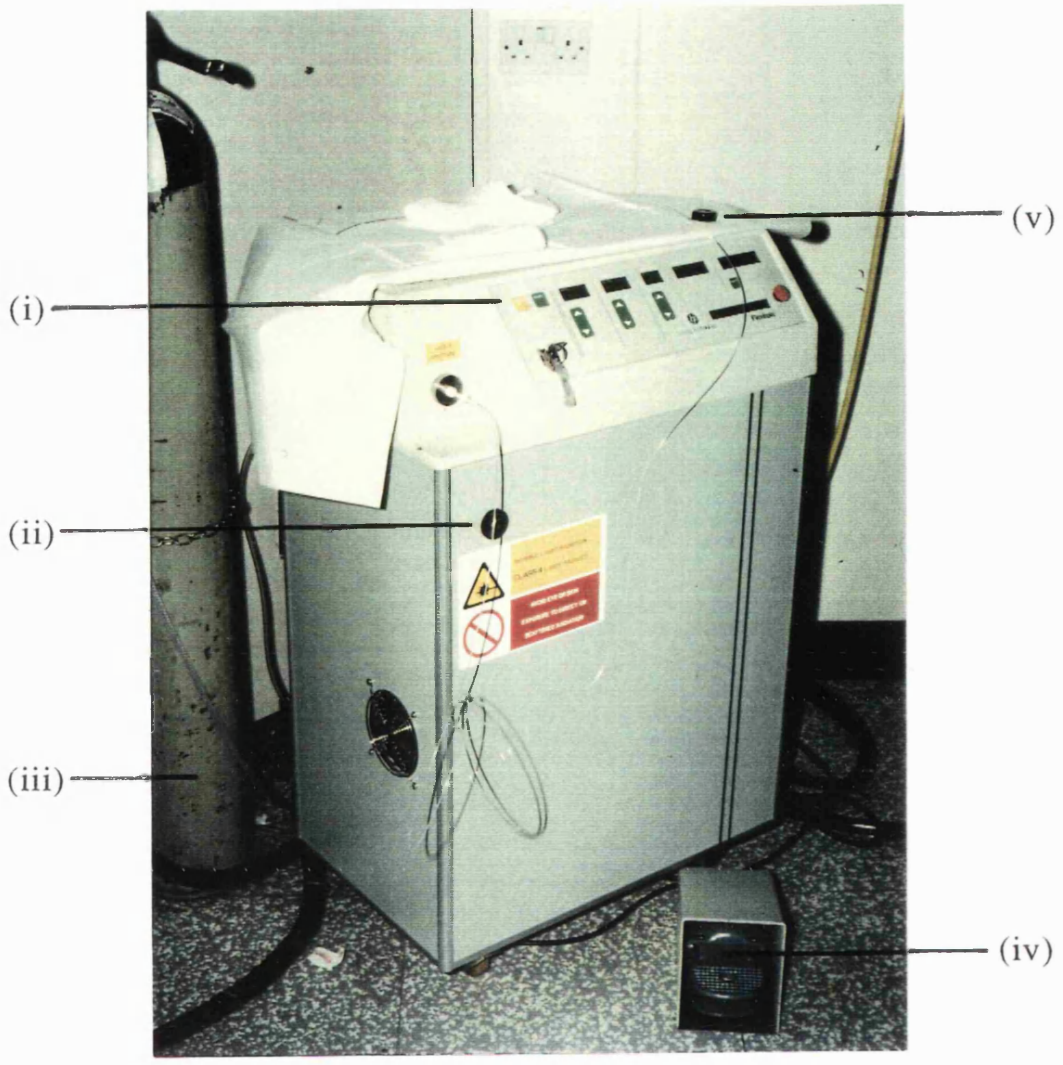


Fig. 10.1 The Nd:YAG laser used in this study (Flexilase - Living Technology, Glasgow) with fibre connected

Key

(i) disable/enable buttons

(ii) calibration port - the fibre is fired into this prior to use and the integral power meter calibrates the output of the laser head to the output at the fibre tip.

(iii) compressed air supply - used to cool laser fibres used either externally or in air filled organs.

(iv) foot pedal - covered to reduce risk of accidental firing.

(v) protective filter for cystoscope eyepiece.

10.2.2 Organisation of the Laser Unit

A Nd:YAG laser is the most powerful of the medical laser systems and if fired inappropriately or accidentally may damage the endoscope and injure the patient, the operator or other theatre personnel. The most important risk is of eye damage as the invisible infrared beam will be focussed onto the retina. Therefore there are strict safety rules laid down by Department of Health regulations that govern the construction and operation of a laser unit, under the guidance of an appointed laser safety officer. A list of medical staff authorised to use the laser is maintained, all of whom have been suitably instructed in operating and safety procedures.

The laser is sited in a controlled area from which any stray laser energy cannot escape. The doors have external warning notices and are controlled by interlocks which prevent the laser operating with them open. The laser itself has several safety features, including shutters to prevent firing unless a suitable delivery system is connected and a prominent safety cut-out button. A self-test check of the fibre output is run through before it can be used which will detect any unsuspected damage. The disable/enable control should not be activated until the laser is ready to fire which is accompanied by an audible alarm when the cowled foot pedal is depressed. When the laser is being used outside the body all personnel including the patient wear approved eye protection, and for endoscopic use a suitable infrared filter is fitted over the eyepiece to absorb backscattered energy.

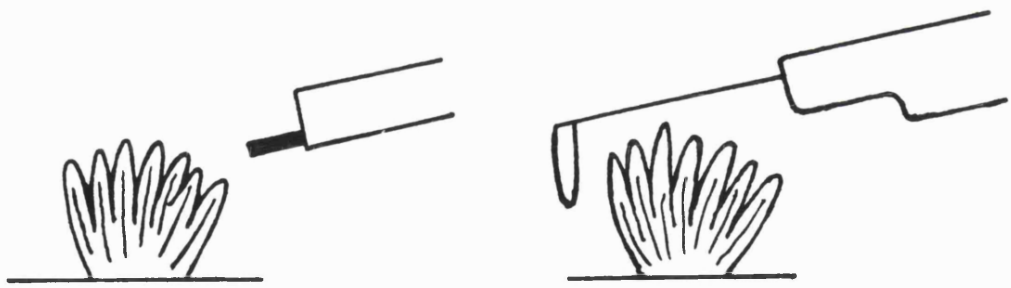
10.2.3 Laser technique

A "bare" urological fibre was used as no additional cooling other than the irrigant fluid is required. This can be water or saline rather than the more expensive glycine solution necessary when using electrocoagulation. A protective eyepiece filter is required, as mentioned above.

The fibre is "stroked" linearly across the tumour keeping 2-3 mm away. Initially a power of 40 W for 2 s on each portion of the tumour was employed but this was later reduced to 25-30 W when the results from the dosimetry experiments described in chapter 9 became apparent. The surface blanching effect taken as the visual endpoint of treatment will indicate that a 2-3 mm depth of coagulation has been achieved. There was no suitable still camera available to me for endoscopic photography but the typical appearance of "white coagulation" is shown in fig. 10.3. Some authorities (e.g. Hofstetter, 1987) recommend first treating an area of normal mucosa approximately 0.5 cm around the tumour to seal off lymphatics and small blood vessels. The theoretical benefits of this in reducing recurrence rates seem greater than the practical ones and as the laser effect will extend for several millimetres beyond the irradiated area this is probably unnecessary. Therefore just a small rim of normal looking mucosa around the base of the tumour was treated unless carcinoma *in situ* was present. Most awake patients were aware of the laser energy and usually described it as a suprapubic burning or stinging sensation which was only rarely uncomfortable as long as short pulses of about 1 s were given with a short interval to permit heat dispersal. Coagulation on or close to the trigone seemed more uncomfortable but no additional analgesia or sedation was needed in any patient. A catheter is not necessary as there is no bleeding with laser coagulation. Neither of course is there any of the obturator nerve stimulation sometimes caused by diathermy current.

Precise positioning of the tip of the laser fibre is less easy with flexible compared to rigid endoscopes particularly when a significant angulation is necessary to reach the tumour. This is largely a matter of practice as one does not have the same instinctive feel for the tip of the instrument that one gets from a rigid cystoscope, and the narrow laser beam requires accurate

aiming to cover the target area evenly. In addition the flexible endoscope naturally creeps along the bladder wall so that one tends to approach tumours more obliquely. This is no problem for the resectoscope loop that reaches past the tumour but with a laser fibre the energy penetrating the furthermost part of a sizeable tumour may be insufficient to coagulate it thoroughly (fig. 10.2). In this situation it is quite safe to exceed the normal power as the tangential incidence of the beam reduces the power density considerably. In such cases the bladder was re-examined about a month later, when adequately coagulated tumour had sloughed, in case there was any residual tumour which needed a further treatment. Another solution is to detach the coagulated tissue immediately with biopsy forceps to reveal any untreated tumour. This procedure tended to impair the view somewhat but was found useful on occasions.



(a) (b)

Fig. 10.2 Tangential incidence on a sizeable tumour is less desirable for a laser fibre (a) than for a diathermy loop (b)

These techniques are easily mastered though there are several safety points particularly pertinent to the use of lasers with flexible cystoscopes that need emphasising as these instruments are several times more expensive than their rigid counterparts and quite easily damaged. Even relatively minor

damage may require the costly replacement of most of the instrument as selective repairs to the small calibre components are rarely feasible, unlike in other thicker fibrescopes.

The most serious problem is that of damage to the endoscope by the laser fibre itself. The tip of the standard 600 micron bare fibre is sharp and stiff and, in attempting to pass it down a working channel thrown into a sharp bend by a serpentine prostatic urethra, it is easy to impinge on and pierce the channel lining if forced, the resulting hole allowing fluid inside the instrument. This is of course only a problem when treating male patients. Other potential sites of damage are at the angle where the side entry port joins the endoscope body and at the distal flexible tip if this has not been straightened out in the bladder. The more flexible 400 micron fibre available from some manufacturers is only slightly better in this respect than the standard 600 micron fibre, but there are several solutions to these difficulties.

If use of the laser is expected the fibre may be threaded into the cystoscope prior to the examination, but this severely restricts irrigant flow. A second method is to withdraw into the straight anterior urethra before inserting the fibre though this means repeating the most uncomfortable part of the procedure, passing the distal sphincter mechanism. The best solution we have found is to insert the fibre coaxially inside and just short of the end of a piece of fine bore tubing (made from the outer sheath of a gas-cooled fibre). The leading soft end of this introducer will guide the fibre safely round bends in the working channel and can be withdrawn once inside the bladder. This problem has not been addressed adequately by the fibre manufacturers though we managed to get hold of one prototype with a retractable outer sheath that worked quite well although the retraction mechanism was too awkward to use one-handed as designed (fig. 10.4).

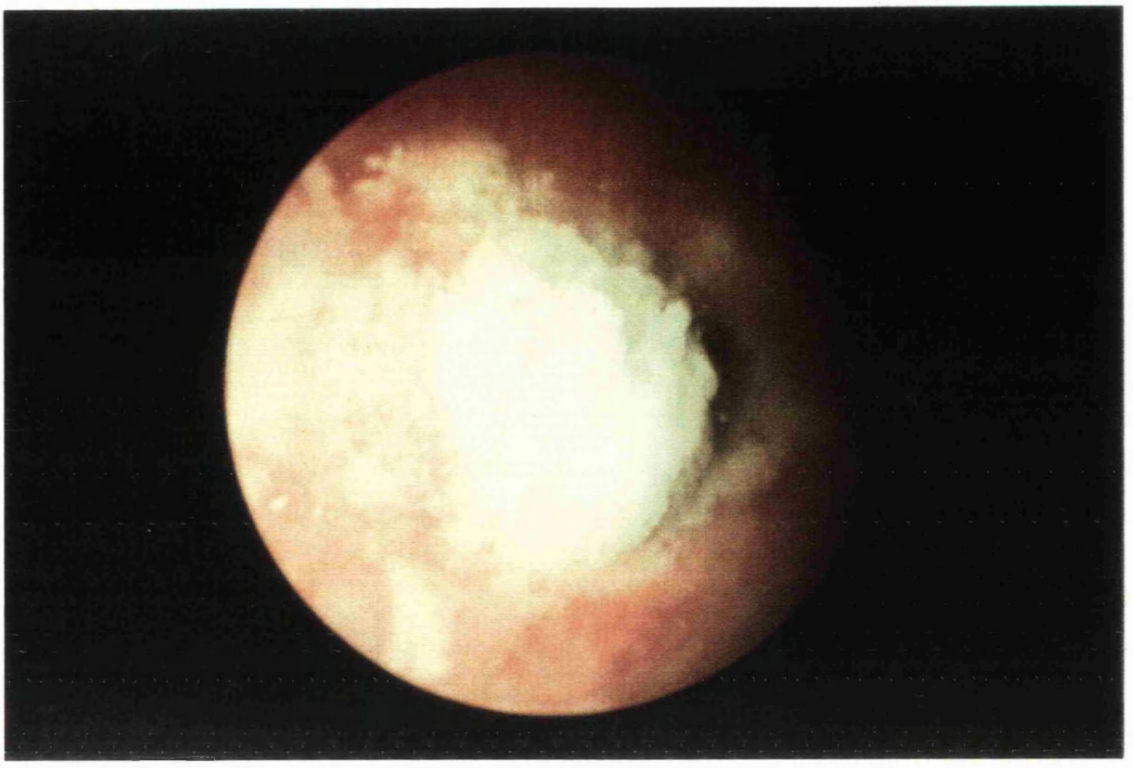


Fig. 10.3 Endoscopic appearances following laser coagulation of a small bladder tumour (By courtesy of Mr TA McNicholas)

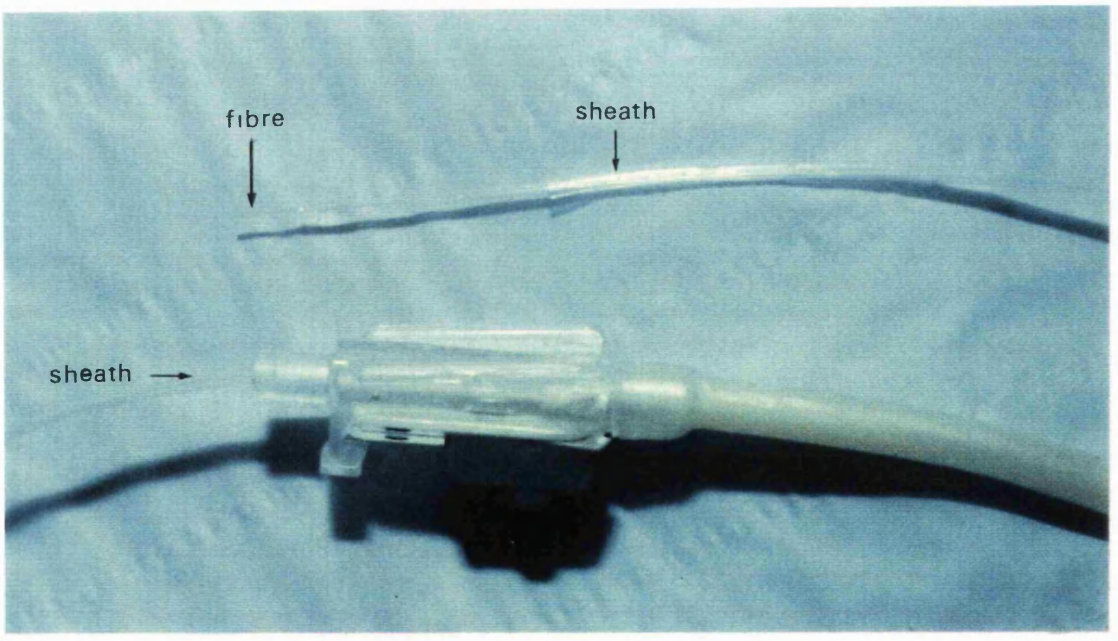
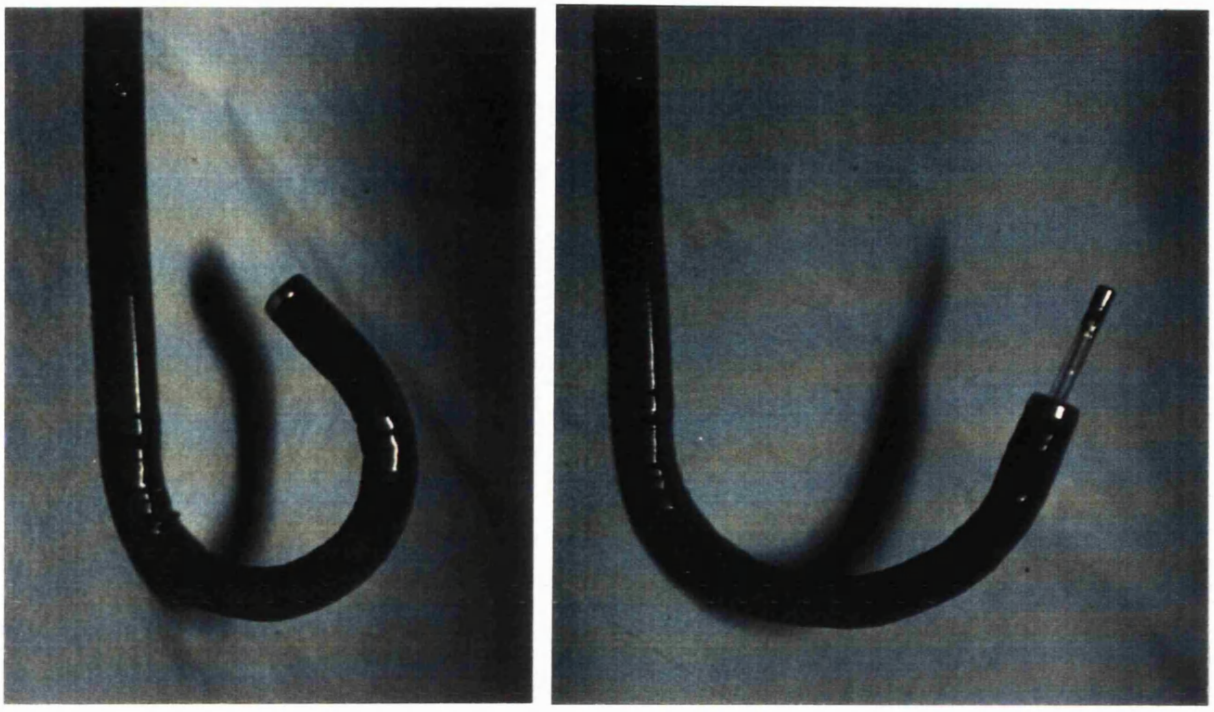


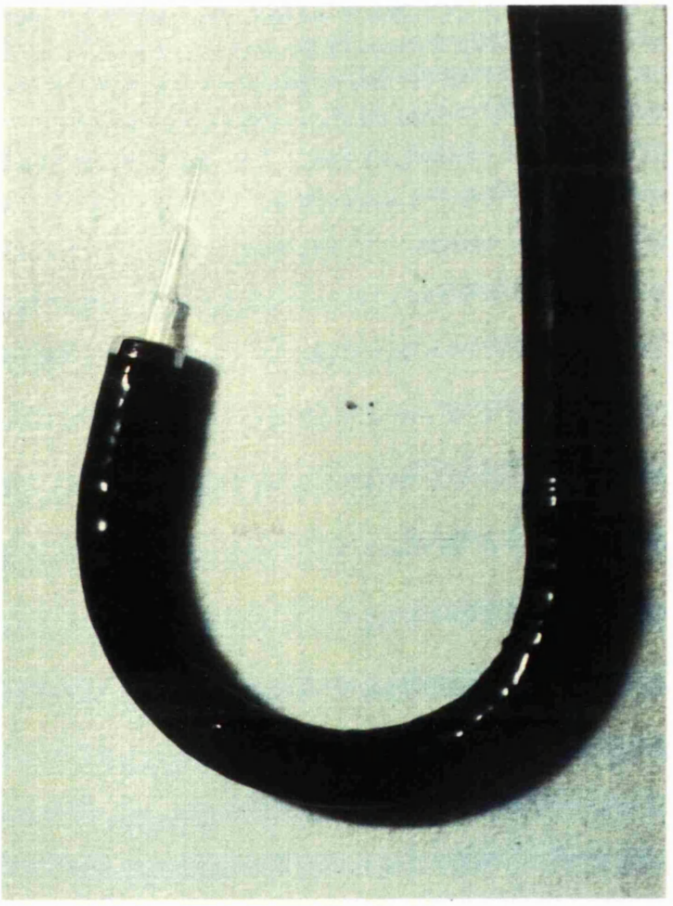
Fig. 10.4 400 micron fibre with a retractable outer sheath to aid insertion through flexible endoscopes

(the locking mechanism seen at the bottom of the picture enables the thin protective sleeve to be withdrawn into the thicker proximal fibre)

The 600 micron fibre is also stiff enough to greatly restrict the maximum angulation of the flexible cystoscope and can make for difficult access to tumours near the bladder neck when the full deflection is needed. Often a recurve "J" manoeuvre, approaching the bladder neck from above, will be successful but care must be taken not to damage the emerging cystoscope with the forward scattered beam (fig. 10.5). The increased flexibility of the newer 400 micron fibres greatly reduced this problem but they are not yet readily available from most manufacturers (fig. 10.6). It should be self evident that the laser must not be fired unless both the tip of the fibre and the helium-neon aiming spot are visible. It is not unknown for a 600 micron fibre to crack under maximum deflection and yet retain integrity due to the teflon sheath, until the laser is fired into the optical bundle! In this case the fibre tip will appear to manipulate normally but the aiming spot will be lost. After laser use all endoscopes should be leak tested so that if any damage has been done this is recognised before it may be made irreparable by immersion in sterilising solutions.

Fig. 10.5 Recurve "J" manoeuvre useful for getting at lesions close to the bladder neck





Figs. 10.6

Reduction in the maximum deflection (above) of the Olympus cystoscope caused by the stiff 600 micron fibre (top right). The 400 micron fibre is much less restrictive (opposite)

10.3 RESULTS

Of the 33 patients enroled into the study 1 was lost to follow-up after the initial treatment. Three patients are known to have died from other diseases; at their last cystoscopies (13, 14 and 20 months after entry), 1 was free of bladder tumour and the other two had small recurrences.

There was no major complication, in particular no instance of small bowel perforation. Two patients reported moderate haematuria, in 1 who was on warfarin this lasted for 4 days following his first treatment; in the other haematuria was observed between 3 and 7 days after the largest amount of energy delivered on any occasion in this series (5600 J to 2 large tumours). In both cases this settled spontaneously. One patient had an episode of retention 2 months after laser treatment, treated by temporary catheterisation at a local hospital, which may have been associated with urinary infection. One patient complained of frequency and dysuria, with sterile urine, for some days after extensive lasering. No patient found the laser unduly uncomfortable and additional analgesics were not required though mucosa on or near the trigone could be quite sensitive. Three patients have subsequently required conventional resection under general anaesthetic. One had a residual abnormal area after lasering which did not settle fully, this was resected and histology showed chronic inflammatory changes only. Another had tumour around the bladder neck (and in other sites which were successfully treated), which was not possible to treat adequately due to its position, and the third patient developed multiple recurrences after 9 months which were considered too extensive for laser coagulation.

At initial presentation there were an average of 2.1 tumours per patient (range 1-15). Energy levels delivered averaged 1130 J (range 170-5020 J) at a typical power of 30 W (range 20-40 W). Since then 84 recurrences

have been treated in 20 patients (62%) on a further 40 occasions, during a mean follow-up period of 18 months (median 17.5 months, range 6-32 months). There was only a loose correlation between the number of tumours treated and the laser energy delivered (fig. 10.7). All follow-up times given here are from entry to the patient's most recent cystoscopy. Twelve patients (38%) have been free from recurrence after 1 laser treatment for an mean of 17 months (median 16 months, range 6-28 months).

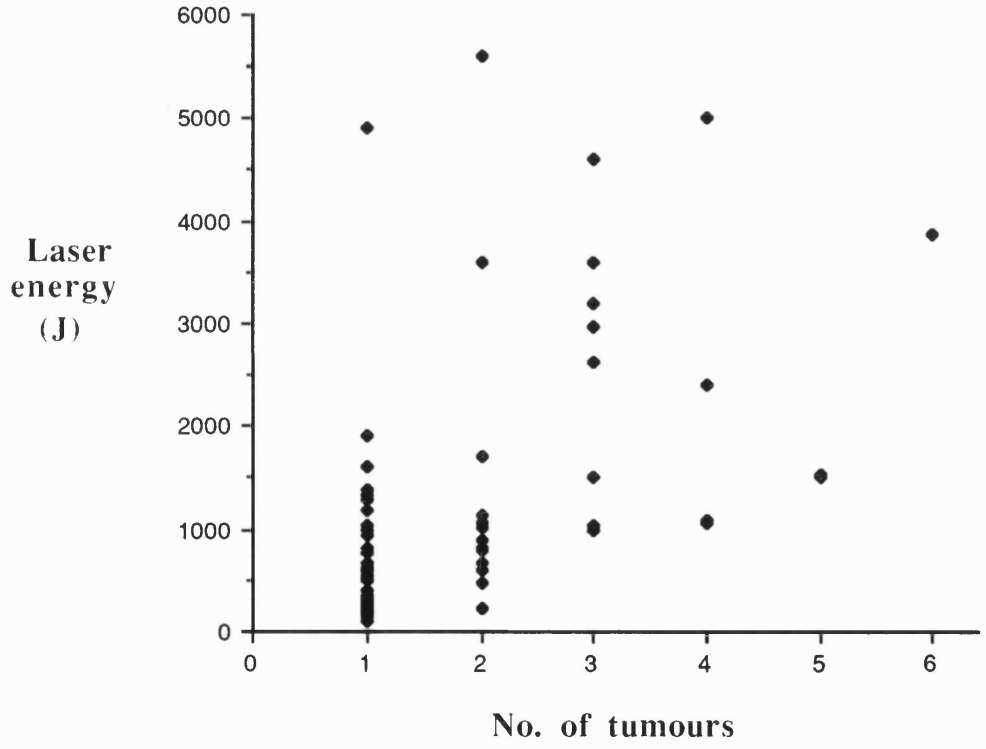


Fig. 10.7 Relationship between the number of tumours treated on each occasion and the laser energy delivered

(excludes one treatment of 15 tumours with 4130 J)

The average recurrence free interval before the start of laser treatment was just over 2 years (range 3 months to 4 years). Twenty six patients have been followed for more than 12 months (median 20 months) and of these 17 (65%) have recurred at some stage, most of them (65%) within 1 year.

Of the 9 patients with more than 2 years follow-up (median 28 months), 6 (67%) have recurred. Some tumours were undertreated initially as 8 patients were found to have residual or recurrent tumour in the previously treated area at the first follow-up, and 11 by 6 months. Usually these were the larger tumours and further laser treatment led to resolution in all but 1 case, mentioned above, who had a recurrence at the bladder neck. All patients have had at least one clear cystoscopy at some stage though 2 patients needed 3 laser treatments before this was achieved (fig. 10.8).

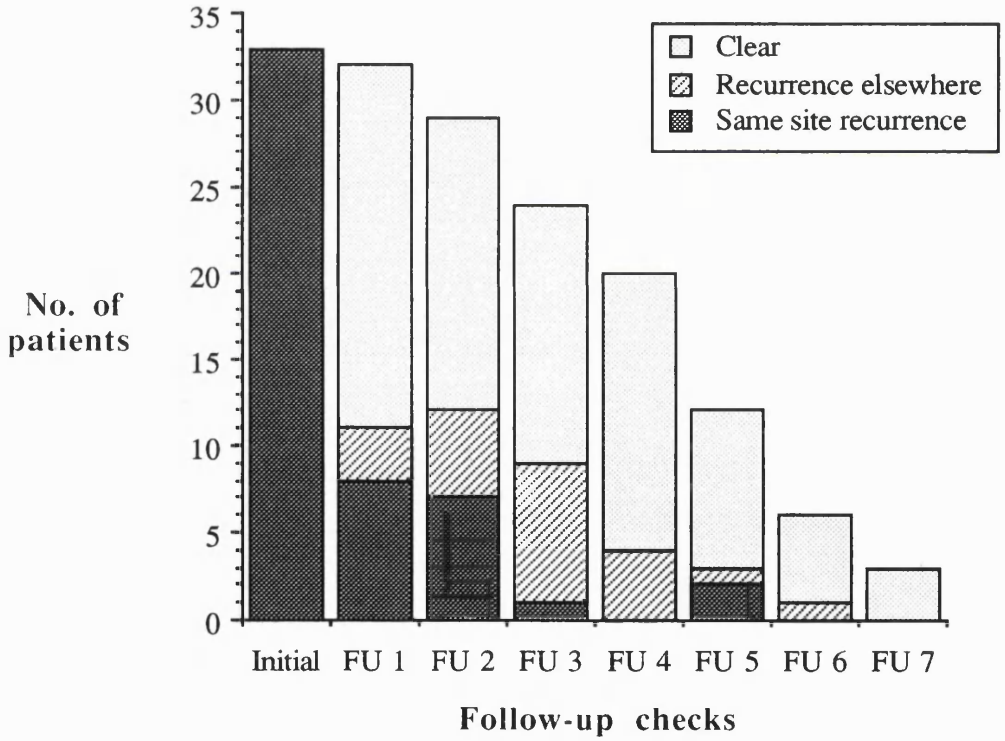


Fig. 10.8 Recurrence status at follow-up examinations (if both same and distant sites shown as distant)

As the length of follow-up has increased proportionally more recurrences have occured at distant sites to the presenting tumour (fig. 10.8). Of the 20 patients (out of 32 evaluable) who had recurrences, 16 (50%) have relapsed in a previously treated area at some stage and 15 in other areas. Twenty-

six patients have been followed up for more than 1 year (median 20 months, range 12 - 32). In the first 12 months follow-up of these patients, 54 recurrences were treated on 28 occasions in 17 patients. These were evenly split between the same site (28) and distant recurrences (26). Nine patients have been followed up for more than 2 years (median 28 months). Six of these patients developed recurrences during the first 12 months follow-up, 4 at a previously treated site and at other sites in all 6. In their second year of follow up only 3 recurred, none in their original area. Of the 29 patients still under active follow-up, 25 (86%) were clear at their most recent cystoscopy a mean of 18 months from entry to this study.

No patient had a previous history of upper tract transitional cell carcinoma and routine urography was not performed. 121 urine cytology results were available which reflect 80% of the cystoscopies performed, the shortfall being accounted for by failed or unsatisfactory samples or missing reports. The results are summarised below. If equivocal results were counted as positive, then positive cytology was reported in only 18% of cases where tumour was noted at cystoscopy. Negative cytology was obtained on 93% of occasions when the bladder appeared clear. False positive cytology, as judged by 2 consecutive normal cystoscopies with biopsies and a normal IVU, occured twice in the same patient. Two patients had an equivocal cytology and a clear cystoscopy, in 1 of these there was macroscopic tumour at the next visit. Seven (46%) of the positive or suspicious cytology results were seen in patients with previous G2 disease (30%).

		Cystoscopy findings	
		positive	negative
Cytology	positive	6	2
	suspicious	5	2
	negative	51	55

10.4 DISCUSSION

The aim of introducing Nd:YAG laser coagulation to an existing flexible cystoscopy list was to maximum the potential for outpatient treatment of superficial bladder tumour recurrences. The laser has the advantages over electrocoagulation of requiring no additional anaesthetic and being a non-contact method of destruction. This latter feature helps to preserve the clarity of view down the flexible cystoscope as minimal simultaneous irrigation is available with the working channel occupied by a fibre or electrode. To recommend this method of treatment however it should be shown to be both safe, effective and acceptable to the patient.

Fowler (1987) has reported the only other study, to our knowledge, of outpatient laser coagulation of bladder tumours using flexible cystoscopy. This comprised 39 patients with 5 or fewer low grade (G1) superficial recurrences smaller than 7 mm. Six treatment failures were accounted for by technical difficulties in 4 (mainly awkwardly situated tumours) and pain in 2. Fowler gave systemic diazepam to most patients but this delayed their recovery and did not seem to reduce any discomfort they might experience. The length of follow-up was not specified, so was presumably quite short, but the quoted recurrence rates were low at only 1 patient with residual tumour and 3 patients with new tumours. This is a much lower figure than we found but is unfortunately fairly meaningless without good follow-up data. Our patients had more tumours initially (average 2.1 compared with 1.2) and were not selected to exclude patients with G2 disease or larger or more numerous recurrences.

As long as the standard safety procedures are followed then laser treatment is safe for the operator. It is less safe for the endoscope and there can be few laser units, ours included, where an instrument has not been damaged

at some stage either by breaching the lining of the working channel with the sharp end of a fibre or by firing the laser within, or too close to, the tip of the endoscope. This normally happens in situations of poor access or visibility where it is clearly better to give up and come back another day. Keymed are the only equipment distributor not to exclude accidental laser damage from the insurance cover available for their instruments, but this may change and subsequent repairs to the image bundle can easily cost several thousand pounds. Again it should be emphasised that the laser should not be fired unless both the end of the fibre and the aiming beam can be clearly seen. Improvements in the design of delivery systems are urgently needed to reduce the risk of damaging the lining of the fibrescope working channel during introduction of the fibre.

The treatment complication that worries urologists most is the risk of secondary small bowel perforation due to forward scattered energy from intraperitoneal areas of the bladder. This possibility has probably been over-emphasized (section 9.2.3), especially using the energy levels recommended here, and we would suggest that the conscious patient is unlikely to tolerate the energy levels at which this would be a real risk. Overdistension with consequent thinning of the bladder wall should though be avoided and it is our practice to turn off the irrigation completely once the bladder mucosa has been adequately unfolded as unlike electroresection the laser does not cause bleeding or release debris to cloud the view.

This study provides yet more evidence to suggest that the initial optimism of laser coagulation reducing recurrence rates elsewhere in the bladder is unfounded. The experimental evidence from Zimmerman *et al.* (1984) and See and Chapman (1987), discussed in chapter 9, that the laser both seals submucosal lymphatics and reduces the number of viable tumour cells shed into the bladder cavity has not been shown to be relevant in Man. It is

more likely that the recurrence rate at other sites in the bladder is a reflection of the degree of mucosal instability in a "field-change" disease and will be independent of the method of treatment. All the patients in this study are selected in the sense that they had already shown the ability to recur after treatment of their primary tumour so it is perhaps not surprising that almost half (47%) did relapse at other sites within a mean follow-up time of 18 months (range 6 to 32 months). Only a quarter of those patients who relapsed did so exclusively in a previously treated area.

Critics of laser therapy often cite as a major disadvantage the fact that biopsy material is rarely taken. If the flexible cystoscopy list includes patients for diagnostic endoscopy then it will not be infrequent to find a new tumour and there is a strong temptation in such cases to go ahead and treat it on that occasion. It is essential that a full histological assessment can be made on the index tumour so a biopsy must be deep enough to include the base of the lesion and preferably bladder wall muscle also. This in itself is quite difficult to achieve with the small biopsy forceps that are used with a flexible cystoscope but also will inevitably cause bleeding which, by the time the specimens have been obtained and the laser fibre threaded down, usually results in an unacceptably impaired view as there is minimal irrigant flow with the working channel occupied. Some authors have found it difficult to control the bleeding from a biopsy site with the laser alone (Arkell and Randall, 1988) and although we have not, it is advisable to turn the power up to 50 W for a couple of short bursts. It has already been mentioned that post-laser biopsies should yield adequate histological information (Pavone-Macaluso *et al.*, 1990) but we find the process unsatisfactory and still feel that the first tumour should be resected conventionally, though the base may then be laser coagulated.

Two main aspects concerning the effectiveness of laser coagulation with a flexible cystoscope must also be considered. Firstly are the results using a flexible cystoscope inferior to those obtained with a rigid cystoscope and the laser, and secondly would diathermy be just as good as the laser? It might be expected that operative procedures with a flexible instrument are going to be inherently more difficult than with a rigid cystoscope. Lesions at the bladder neck for instance are easily seen with a flexible cystoscope but getting onto them is more difficult whereas the converse is often the case for tumours on the anterior bladder wall. The tendency to approach tumours tangentially can be a problem particularly on the bladder base and lateral walls (fig. 10.4), and if there is a significant exophytic growth the base and furthermost portion are likely to be inadequately treated. After initial coagulation the denatured tumour may be detached with biopsy forceps to reveal untreated tissue to which a second treatment is given, but if this is not done the patient should be brought back in 3-4 weeks to check for residual tumour. This can be quite time consuming and we would suggest that conventional resection is more satisfactory for tumours larger than 2-3 cm. The incidence of residual tumour at the 3 month check in this study (24%) is higher than other authors using the laser with rigid cystoscopes have reported, and also than in Fowler's study (1987). Part of this is no doubt due to the "learning curve" of this technique but nevertheless if it is a persisting finding one might conclude that the optimum treatment parameters are not yet clear. However it is far less inconvenient to bring the patient back for an outpatient check than for an inpatient general anaesthetic procedure and all but 2 patients, who needed 3 sessions, were clear after a second treatment.

Could the same effect be achieved with diathermy as with the Nd:YAG laser? It is slower and technically more difficult to coagulate a tumour

with the laser because of the need to precisely manipulate the small aiming beam to cover the target area evenly. Add to that the extra care needed to prepare the fibre and operate the laser and it is not difficult to see why there are few laser enthusiasts amongst urologists. In the absence of an improvement in disease control the main benefit is that the relatively painless laser technique can be used on an awake patient. In none of these patients did treatment have to be terminated due to discomfort and indeed most patients said it was better than going to the dentist! Although there has been no study presented to my knowledge of bladder tumour diathermy in conjunction with flexible cystoscopy, the rather small electrodes that are available for these instruments are likely to be more difficult to use than a laser fibre and the operative view much less satisfactory.

One is therefore left with standard techniques under local anaesthetic, perhaps supplemented with systemic sedation. We are all aware that it is usually possible to pass a rigid cystoscope in the female under local anaesthetic with little discomfort and to gently diathermy small tumours but very few urologists do this routinely and it is certainly not appreciated by most male patients. Others have demonstrated the wide range of procedures that can be performed by local infiltration of local anaesthetic supplemented with benzodiazepine sedation, so-called "sedoanalgesia". The sedative element can be rapidly and effectively reversed after operation easily permitting day case, if not exactly outpatient treatment. The advocates of such techniques point to greater efficiency by freeing in-patient general anaesthetic lists from the sort of case that we are discussing here (Birch *et al.*, 1990). However this alternative still requires the same facilities as a standard operating list apart from the anaesthetist.

No patient refused to undergo further laser treatment if required and most expressed a strong preference for this form of management compared with

their earlier experiences of in-patient general anaesthetic treatment. There are also clear medical advantages for the many patients who are relatively elderly and unfit and whom would otherwise require repeated anaesthetics over many years. As long as the procedure is adequately explained, few patients given the choice will opt for conventional in-patient treatment (Flannigan *et al.*, 1989). Those that do are usually elderly women who can find flexible cystoscopy somewhat embarrassing, though nothing like as undignified as rigid cystoscopy under local, or those who believe that the more thorough history taking and examination necessary prior to a general anaesthetic is beneficial!

The capital cost of a Nd:YAG laser is high (approx. £50 000) and with annual running costs amounting to some £5000 it is a very expensive facility to be allowed to sit idle. Therefore multidisciplinary use is essential if it is to be cost effective. Ideally the laser should be sited in an endoscopy or day surgery unit, not in the main theatres which would unnecessarily complicate the aim of outpatient treatment for urology patients. In our Unit around 800 Nd:YAG laser treatments are performed annually, the gastroenterologists accounting for some 65% of these and the chest physicians 20%.

Urological use is around 80 treatments per year of which half are bladder tumours and the remainder condylomata. One laser session per week is allocated to the urology list though in hospitals where there is a greater load of bladder cancer patients this might need to be increased. The use of the laser adds £20 to the cost of a flexible cystoscopy calculated as £16 for the capital and running costs of the laser and £4 each time for a reusable fibre. There seems to be a tendency towards regarding laser fibres as disposable and manufacturers, not surprisingly perhaps, seem to be leading this by marketing their fibres as "single use only" products. It would of

course be very nice to have a new fibre for each patient as this would obviate the need to clean and sterilise it in gluteraldehyde between cases and to periodically recleave the fibre tip and calibrate its output to ensure maximum performance. However the cost (around £40 - 50 per fibre) is not justified for this type of application and we have re-used the same fibre for many months until it became too short, after numerous cleaves, to reach from the laser to the patient!

If each laser cystoscopy saves a day in hospital and a slot on a general anaesthetic operating list the cost benefit per procedure is around £200. The use of water or saline rather than glycine for cystoscopy and no need for a catheter post-operatively can further reduce costs. As a rough estimate there will be an average of 1 positive cystoscopy per year for each patient on routine bladder cancer surveillance so for a typical urology department with 250 such patients there is a potential annual cost benefit in outpatient laser cystoscopy of some £50 000.

Of course the main cost benefits are to be found in gastroenterology where laser techniques bring even greater savings largely by reducing the hospital stay for patients with bleeding peptic ulcers and obstructing gastrointestinal tumours compared with conventional surgical treatment. It has been calculated that a laser centre in a large hospital might yield savings of around £1 million per year (Bown, 1990).

This study also enabled us to assess the merit of the cytological examination of a voided urine specimen which is a standard investigation in diagnosis and follow-up of bladder cancer patients. However the value of this in assisting management is not clear though it is recognised that the sensitivity of urine cytology is strongly dependent upon the tumour grade. The National Bladder Cancer Collaborative Group A (1977b) reported a 43% sensitivity for grade 1 and 2 tumours and a 93% sensitivity for G3 disease.

In carcinoma *in situ* the accuracy approaches 100% (Highman, 1988). Hastie *et al.* (1990) found that although the overall sensitivity in a population of check cystoscopy patients was 57% this was skewed by a 100% prediction of G3 tumours. When looking only at his patients with previous G1 disease, positive cytology was obtained in just 17% of those with recurrences at cystoscopy. In this study, also of predominantly low grade tumours, the finding of a positive urine cytology had a sensitivity of 18% and a specificity of 73%. A negative result had a sensitivity of 52% and a specificity of 93%. As the results were not available until after the cystoscopy a false positive was the only report to influence management. Although on only 4 occasions (in 3 patients) these did represent 27% of the entire positive results and in 2 patients significant extra investigations were initiated for no benefit. The high false negative rate (82%) rules out cytology as an acceptable substitute for cystoscopy as follow-up in these patients if it is hoped to pick up small, low grade recurrences and therefore its routine use cannot be clearly justified.

10.4.1 Conclusions

The Nd:YAG laser can maximise the potential of flexible cystoscopy to treat superficial bladder cancer recurrences on an outpatient basis without anaesthetic. The techniques are safe and easy to learn and in a well run Unit cause little delay to what would otherwise be only a diagnostic procedure. As one's ability and confidence with the laser increases, larger and more numerous tumours can be tackled and therefore almost all patients become suitable for outpatient treatment though an index tumour, particularly numerous recurrences or those larger than 2-3 cm should be dealt with by conventional resection. Laser coagulation is unlikely to prove a more effective means of treatment than electrocoagulation in terms of reducing either the recurrence rate or the risk of developing muscle

invasion, the latter being very rare anyway for well differentiated superficial tumours. The potential worry therefore that one might miss such progression through not routinely taking deep biopsies or performing bimanual examinations under anaesthetic should not be exaggerated.

Patients find laser coagulation a very acceptable treatment and no session had to be abandoned due to discomfort. Most patients expressed a strong preference for this form of therapy over standard inpatient treatment and as well as the considerable social convenience to most patients of not having to be frequently admitted to hospital there are medical advantages in avoiding regular anaesthetics in a generally elderly population.

The Nd:YAG laser is an expensive piece of equipment and will be most effectively employed if it is conveniently located, say in the endoscopy suite, where its use can be shared between several specialties. Although the major benefits in economy and efficiency of health care delivery will be seen by other specialties, a typical urology department would benefit from 1 - 2 laser sessions per week and might be expected to achieve savings in the order of £50000 annually. This would hopefully lead to a reduction in waiting lists as the more expensive inpatient surgical facilities could be concentrated on more major cases which would be a better use of the increasingly restricted numbers of beds available in most hospitals.

SECTION 4

SUMMARY

Chapter 11

GENERAL CONCLUSIONS

11.1 INTRODUCTION

Bladder cancer is the most common malignant disease treated by urologists. It is a heterogeneous condition whose management may require the services and tax the ingenuity not only of the urologist, but also the oncologist, the radiotherapist and perhaps in the future the "lasertherapist". Notwithstanding some of the newer chemotherapy regimes that have been developed, the treatment of most patients has hardly changed for many years. Lasers are playing an increasing role in many areas of medicine as diverse as ophthalmology and gynaecology. Their value lies in the ability to focus a high intensity beam of light into a fine flexible fibre which can deliver precise tissue effects to most parts of the body. Some of these effects are of particular relevance to the widening applications of "minimally invasive therapy" whereas others such as PDT may open up completely new ways of treating localised tumours.

One of the dangers with high technology is of letting enthusiasm get in the way of sound common sense and logical clinical practice. All too often we see new developments applied rapidly and often inappropriately to patients leading to poor results and disillusionment which takes much more careful study and development to rectify. Bown (1990) has suggested the steps along which the introduction of a new laser treatment should proceed.

i) Identification of a clinical need.

ii) Experiments to understand the biology of the laser tissue interaction required to establish the most appropriate laser and the best way to use it.

iii) Pilot clinical trials to show that the laser technique is safe and produces the desired effect.

iv) Controlled clinical trials to compare the laser treatment with conventional alternatives.

v) Once the role of laser treatment on its own is established, to assess whether it can usefully be combined with other treatment.

vi) If the safety, efficacy and superiority of the laser approach are confirmed, to carefully define the patient group who will benefit most and make the treatment generally available.

All too often the laser pioneers have concentrated on i) and iii) to the exclusion of the others, especially stage ii). All these steps though cannot be covered by one worker and at the root of many problems is the classical divide between the clinician and scientist. To bridge this gap requires expertise in biology, physics, photochemistry and experimental pathology integrated with the clinical sciences, a breadth of knowledge available to very few centres anywhere in the world. Therefore closer collaboration between different research groups will be needed in the future.

This thesis has investigated 2 laser techniques which promise to enhance the conventional management of superficial bladder cancer; PDT an experimental treatment for resistant Cis and multifocal superficial tumours, and Nd:YAG laser coagulation for small, low grade recurrences.

11.2 PHOTODYNAMIC THERAPY

PDT has been studied by many research groups world wide with numerous claims made for its efficacy over a period of more than 10 years. It would seem to hold particular promise for treating various types of superficial bladder cancer, especially resistant Cis. Why then is it not yet an established treatment? The main reason is that clinical PDT, as with many

new medical treatments, has evolved on a largely empirical basis whilst there are still fundamental questions regarding its mechanism of action and optimum treatment parameters unanswered. It is not surprising therefore that many reports of its use are anecdotal and few publications give much convincing evidence of clinical efficacy for the management of Cis, or indeed for most other conditions for which it has been tried. Some serious complications have been reported and the development of an irritable contracted bladder or upper tract damage secondary to ureteric obstruction or reflux, are tragic sequelae for a patient in whom PDT has successfully eradicated resistant and potentially lethal carcinoma. It seems likely that had more experimental studies been carried out *prior* to the clinical application of PDT in the bladder then the incidence of these complications would have been drastically reduced.

We have investigated the effects produced by PDT on the normal bladder to try and identify the range of dosimetry variables that will damage the superficial layers of the bladder leading to loss of the mucosa but not irreversibly damage muscle. Only by achieving necrosis of normal mucosa is one likely to be sure in the patient with Cis that all areas of the thicker, malignant mucosa will also have been destroyed, because although there will be some selectivity due to increased uptake of photosensitiser in tumour this is unlikely to be sufficient to ensure the reliable eradication of all abnormal areas whilst leaving benign mucosa intact. Fortunately the mucosa seems to heal rapidly by the regeneration of normal tissue from the margins of the treated areas, i.e. the lower ureters and bladder neck in a successful whole bladder treatment, but deeper tissue is less forgiving with strong evidence that significant side effects result from muscle damage which heals with fibrosis and scarring (Harty *et al.*, 1989). The need to comprehensively damage the mucosa but leave underlying muscle unimpaired might seem rather a fine distinction to be made in practice and

there is some concern that even if this can be achieved in carefully controlled animal experiments it will be much more difficult to produce in patients where dosimetry and biological variations are likely to be greater than in an animal model. A treatment with a low safety margin is unlikely to become widely adopted, particularly if it also technically complex (temperamental and expensive lasers, elaborate delivery and monitoring systems for bladder illumination, stringent post-operative precautions to avoid sunlight etc.). Therefore any factor that might simplify PDT deserves full investigation and, as already introduced in sections 4.3.3 and 6.4.3, photobleaching is one such potentially useful quality seen with photosensitising drugs.

Photobleaching is an attractive mechanism for which may be important in limiting PDT effects to the superficial layers of the bladder. The degradation of the photosensitiser without producing enough singlet oxygen to cause tissue damage would be expected if the tissue concentration of sensitiser drug was below the photodynamic threshold in muscle but above it in mucosa and submucosa. Photobleaching of AlSPc was demonstrated *in vivo* by our CCD fluorescence technique though rather more investigation will be needed to accurately delineate photobleaching rates in tissue. This approach has the added attraction that as the sensitiser is bleached out of the muscle layer, no PDT damage should be produced however high the light dose. The experiments in chapter 7 confirm that with a low dose of AlSPc, damage can be limited to the superficial layers in a way that is largely independent of the light dose, the only requirements being that sufficient light is delivered to all points on the bladder surface and that of course the fluence rate is not high enough to cause thermal effects. With higher sensitiser doses there is much less latitude in light fluence before wide-spread damage is caused to the normal bladder, including transmural inflammation and extensive necrosis. Of course this model cannot indicate

to what degree the damage inflicted on areas of tumour will exceed that on normal tissue but as has been stressed already, the selectivity based on a differential uptake of photosensitiser alone is likely to be quite small and to provide an insufficient margin of safety for clinical needs.

In order to determine the optimum dosimetry variables for PDT it is surely essential to know how the sensitiser is distributed in tissue with time as one of the cornerstones of PDT is the belief that a selective distribution will develop between benign and malignant tissue. Most reported work has looked at variations in surface fluorescence from sensitised tissue which is inappropriate when the important factor is the distribution in depth within the tissue (i.e. between mucosa and muscle). The novel fluorescence laser microscopy technique described in chapter 6 provides the most accurate and sensitive way of quantifying photosensitiser distribution within normal or tumour tissue. This technique is also more reliable than chemical extraction assays as it does not measure the biologically inactive photo-sensitiser aggregates. We found that the preferential loss of photosensitiser from the muscle of the bladder wall allows a gradient of 3-4:1 between superficial and deep layers of the normal bladder wall to become established within 24-48 h after sensitisation. It was reassuring to find that the timescale for optimum distribution was not too critical which will be an advantage clinically, though this would not be known without this type of experiment. Of course fine-tuning will be necessary when it is possible to biopsy and study phthalocyanine distribution in real tumours in patients but it seems likely that a worthwhile gradient can be achieved.

Merely producing an adequate morphological effect in the bladder after PDT is by itself not sufficient. Unlike nearly all other organs in which PDT has been studied the bladder is unique in that disturbances of function, both in storage and voiding parameters, are readily apparent to the patient.

No noticeable effect will result from even severe damage to the amount of tissue likely to be treated by PDT in the gastrointestinal or respiratory tracts as long of course that perforation does not occur (obstruction resulting from circumferential treatment is also a small risk). In most instances of bladder treatment, though, the entire lining will be affected so therefore any assessment of PDT should address the inevitable functional disturbances that will follow, rather than the morphological changes alone.

Our urodynamic studies on the rat bladder model at a range of times from a few days to 3 months after PDT (chapter 8) indicate that when treatment parameters are employed that would be expected to restrict necrosis to the mucosa and submucosa then the subsequent reduction in compliance and capacity during the healing period is less, lasts a shorter time (less than 1 month) and is more completely reversible than when the muscle layers are also damaged. It is not possible to avoid initial side effects completely as there is a marked inflammatory response where the mucosa sloughs, but if these results can be extrapolated to Man, they should reduce complications to an acceptable level, with little worry of the risk of causing a permanent reduction in bladder compliance or capacity.

There is general agreement that dosimetry is a major problem in treating bladder cancer but most workers see uniform illumination of the entire bladder mucosa as the challenge. We would suggest that the only requirement for the *light* is that the amount at every point should be above a certain threshold. The real challenge comes in getting the dose of *sensitiser* right so that PDT effects are limited to the superficial layers of the bladder. This optimum is likely to be much lower than the values currently used clinically. Of course, one must ask what depth of necrosis is required as when using low sensitiser doses this will be limited, but quite adequate to treat Cis.

The most obvious solution to try for the problem of how to get a higher dose of photosensitiser into the superficial layers of the bladder is to administer it topically as is done for all the chemotherapeutic drugs used for superficial bladder cancer, though it is fair to say that the selectivity of uptake of these into tumour cells in Man is poorly documented. Our investigations of both fluorescence and tissue effect after the intravesical administration of AlSPc mixture have shown uptake to be patchy and unpredictable which is in line with the unsatisfactory results obtained by other workers using porphyrins, though comprehensive data on this are lacking in the literature. Preliminary results with the recently available S2 compound which is more lipid soluble then the AlSPc mixture have been encouraging in that fluorescence in particular seems rather more even and largely confined to the superficial layers giving a larger concentration gradient (determined by fluorescence intensity) between superficial and deeper layers. However the all important question of how this uptake is modified by areas of tumour or dysplasia cannot be adequately answered in this, or indeed any, animal model and ideally needs to be studied in patients, which in the first instance could be carried out by instilling AlPcS2 into the bladders of patients undergoing cystectomy (see 11.2.1).

The most suitable laser system for PDT is also unclear. It seems likely that the continuous wave argon pumped dye laser systems which have been used in nearly all clinical PDT series will be superseded by the pulsed copper vapour pumped dye lasers which are generally more powerful (necessary for illuminating a large area such as the entire bladder) and seem more reliable. However these are still very bulky, expensive and rather too complicated to be ideal as clinical instruments. Over the next few years much cheaper, more compact and reliable semi-conductor lasers are likely to become available for use with sensitisers that absorb at the longer

wavelengths, but it is only very recently that their specifications have started to come close to those required for PDT, in particular their power output is currently insufficient for clinical use in the bladder. We looked at the PDT effect available from the flashlamp pumped dye laser (chapter 5) and concluded that *in vitro* it had a comparable efficacy on a human bladder cancer cell line to the other types of laser usually employed for PDT. The flashlamp laser has the advantage of already being in clinical use for lithotripsy of urinary and biliary tract stones and may also prove to be the laser of choice for angioplasty and the treatment of port wine stains. The optimum pulse repetition rate is likely to be somewhat faster than that achieved by the current instruments in order to prevent photosensitiser saturation, especially with porphyrins, and clinical efficacy still needs to be proven. The likelihood of the green light output of this laser preventing too deep a PDT effect also seems logical but despite several groups studying porphyrin photosensitisation with green light in the bladder (J L Williams, 1991, personal communication), no clear benefit over red light has yet been demonstrated.

11.2.1 Further work

Most of the work presented in this thesis has used AlSPc as the photosensitiser which we believe has major advantages over HpD but does not yet have approval for clinical use. Clearly when this is obtained it will be necessary to verify to what extent these results can be achieved in Man.

The first stage will be to sensitize patients undergoing cystectomy and look at the fluorescence distribution in tumour and normal bladder wall before actually treating anyone. The expected absence of cutaneous photosensitivity should make this possible whereas it would be unethical to do so with porphyrins. Only then could one suggest the most appropriate dose to use

and the optimum time interval between sensitiser injection and bladder illumination. The aim would be to achieve a concentration of photo-sensitiser in abnormal tissue just enough to allow a PDT effect (even though this might require quite a high light fluence) with a sub-threshold concentration in normal tissue. In practice this sub-threshold concentration is rather more likely to be achieved in the deeper muscle layers rather than also in normal urothelium. The assumption that human bladder tissue is similarly sensitive to PDT damage as animal tissue is of course implied but at least it is a logical place to start. The likely effect of intravesical administration of photosensitiser, particularly any differential uptake into malignant over normal tissue, would also be answered by relatively simple clinical studies.

Once this basic information is available the question of the optimum light dose for whole bladder illumination would be addressed by a limited clinical trial. For PDT to have any chance of becoming widely adopted in urology the actual procedure of light delivery needs to be relatively straightforward. It is envisaged that a simple bulb-tip diffuser type of laser fibre would be positioned centrally within the minimally distended bladder as judged by trans-abdominal ultrasound (accepting that such placement will only be moderately accurate). Two, or perhaps more, detector fibres would be attached to the bladder mucosa to monitor the light fluence incident on the bladder mucosa at clinically relevant areas. An integral delivery/monitoring catheter of the type developed by Nseyo *et al.* (1991) would simplify bladder illumination which would continue until the desired minimum energy density had been delivered. This value would doubtless need some experimentation to determine but, using low concentrations of photosensitiser, is likely to be between 40 and 80 J/cm^2. All patients would be closely followed with flexible cystoscopy for assessment of efficacy and

by pre- and post-treatment urodynamic studies for evidence of any disturbance of bladder function.

Only then would one proceed to treat larger numbers of patients, ideally in the context of a clinical trial e.g. comparing PDT with BCG for Cis or resistant multifocal papillary tumour. The development of other urological applications of PDT such as for upper tract urothelial tumours, or for benign or neoplastic conditions of the prostate gland (using interstitial fibres), should ideally wait until the basic principles of PDT in the bladder are established. PDT is a very attractive concept for treating upper tract urothelial tumours as it could be delivered from below without the need for endoscopic control and would be rather safer than thermal energy in terms of avoiding a perforation of the rather thin wall of the renal pelvis.

It is likely that our scientist colleagues will develop new sensitisers and more precise ways of delivering them to tumour tissue, e.g. incorporated into liposomes or bound to monoclonal antibodies. However these advances must be balanced by work designed to establish how the biological principles studied in animals can be applied to treating real cancers growing in real patients. Few if any workers carried out these sort of basic studies in either animals or humans before embarking on clinical trials but surely it should be incumbent on them to do so. The clinical use of PDT requires a new way of thinking about treating human disease and it will take a lot of time, effort and high quality data to establish its efficacy. It is to be hoped that this technique will offer a valuable alternative to our current management in cases of resistant Cis and superficial bladder carcinoma but only careful and logical scientific experiments and rational clinical trials will tell us if photodynamic therapy is really here to stay.

11.3 NEODYMIUM:YAG LASER COAGULATION

In the 10 years that the Nd:YAG laser has been in clinical use there have been great technical improvements such that modern instruments are easy to use and generally reliable. Some of the new medium power lasers, suitable for all urological uses, are surprisingly compact and mobile and whilst not cheap they cost no more than many other items of complex medical equipment. Why is it then that so few urologists utilise them in their routine clinical practice? The answer is probably that most urologists see no good reason to change their current methods but hopefully this view is changing as it is becoming clear that lasers do indeed have a useful role to play particularly in the treatment of bladder cancer.

The experimental work presented in chapter 9 showed that the Nd:YAG laser will produce a sharply demarcated coagulation in the pig bladder. The tissue architecture remains intact compared with the considerable disruption seen after diathermy. Even using a low laser energy (2 s pulse at 20 - 30 W) the depth of penetration was more than adequate to coagulate small superficial recurrences with minimal risk of damaging adjacent structures from forward scattering of the beam. Our animal model was limited by a rather thin bladder wall which prevented an assessment of the likely depth of lesion produced with higher laser energies that would be appropriate for achieving a reliable transmural coagulation in Man. The probable efficacy of interval laser coagulation in preventing local recurrence of T2 invasive bladder cancer as demonstrated by several authors (Staehler *et al.*, 1985; Beisland and Sander, 1990) is an exciting concept that merits further development, particularly of the role of interstitial coagulation (see below).

Chapter 10 dealt with our experiences of using the Nd:YAG laser and flexible cystoscopy to treat bladder tumour recurrences in outpatients without anaesthetic. We have shown that this technique is applicable to almost all patients on the routine "check cystoscopy" list for bladder cancer surveillance who will periodically develop superficial recurrences (around one third did so during a 2 year period). No other series of similarly treated patients has been presented with follow-up data to compare with this study but it would seem that the incidence of new tumours is not reduced by laser compared with historical series of patients receiving diathermy coagulation (47% with follow-up from 6 - 32 months). The fine laser fibre is rather more difficult to accurately direct with a flexible cystoscope than it would be for instance through a rigid laser cystoscope with Albarran attachment which increases the chance of under-treating some tumours especially if sizeable or awkwardly situated. Therefore it is advisable to get all patients back for further examination within 3 months as 22% of ours were found to have a small amount of residual tumour.

The value of this technique lies in its convenience and economy rather than in a superiority of disease control. These patients constitute a significant percentage of routine admissions to general urology wards and we believe that these beds which are becoming increasingly under pressure in the current climate of inpatient bed cuts would be better allocated to more major cases. The emphasis on audit of clinical practice is likely to further show the benefits achieved by lasers applied to many other conditions in medicine and surgery. Hopefully this will lead to the establishment of properly funded, multi-specialty laser centres where clinicians with different interests can work together with specialists in the basic sciences in the optimum environment to develop and enhance these techniques for the overall benefit of patient care.

11.3.1 Further work

Longer follow up and increased numbers of patients with superficial bladder cancer will be necessary to confirm the benefits put forward for this work so the study presented in chapter 10 will continue. One aim in particular is to improve the ablation rate of tumours at the first treatment, but whether this reflects a learning curve effect or indicates a need to apply more energy is not yet clear.

We already use the laser to treat resistant condylomata of the urethra and external genitalia with better results than standard treatments and this service is being developed. The Nd:YAG laser has also been shown in our Unit to be effective and less invasive than other treatments for the rather more rare cases of urethral and penile haemangiomas and carcinoma of the penis (T McNicholas, 1990, personal communication).

There are other applications of thermal laser energy that offer promise. Recently we have commenced basic experimental work to assess the lesions produced in the bladder and kidney by low power interstitial fibres to extend our Unit's experience of interstitial hyperthermia in the liver (Steger *et al.*, 1989). This technique has exciting potential to treat parenchymal tumours of the kidney and prostate percutaneously, though the small volume of tissue coagulated by a single fibre will require a multiple fibre array or a special diffusing device to treat most of the lesions found in practice. It should be possible to more accurately match the depth of laser necrosis to the extent of the tumour using interstitial techniques so localised invasive bladder tumours may be more safely and effectively treated this way than by high laser powers applied to their resected surface. If a localised invasive cancer were situated on the intraperitoneal aspect of the bladder a combined approach of laser

coagulation from both the mucosal and serosal (via a laparoscope) surfaces should allow effective control of tumour in the deeper layers of the bladder wall (and also allow pelvic node sampling for more complete staging information).

Low power Nd:YAG irradiation applied via a diffusing cylinder (or balloon) is showing promise in enlarging an obstructed lumen due to such diverse pathology as malignant stricturing of the gut or airways or in bladder outflow obstruction due to benign prostatic hyperplasia. The potential role of interstitial PDT, particularly in the prostate, has already been mentioned.

Minimally invasive treatment of ureteric and renal urothelial tumours by laser is quite feasible and should be investigated further. The Institute of Urology has reported the percutaneous treatment of urothelial tumours of the renal pelvis with diathermy (Nurse *et al.*, 1990) and it seems likely that the Nd:YAG laser will have advantages here particularly in lessening the risk of perforating the relatively thin-walled renal pelvis, though PDT may be even safer in this respect. The use of a flexible endoscope should enable less accessible areas of the kidney to be reached ideally in a retrograde fashion. The number of potential patients is likely to be small however as they are usually those who have either a single kidney or other medical reasons for renal preservation.

11.4 LASER TREATMENT OF BLADDER CANCER - AN INTEGRATED APPROACH

Where does laser therapy now fit into the overall management of bladder cancer? As Nd:YAG laser coagulation is the only modality currently available for general clinical use this will be considered first.

The patient who presents for the first time with bladder cancer is likely to have had the diagnosis made at outpatient flexible cystoscopy. He should not be treated at this stage but booked for an inpatient cystoscopy where the tumour is resected, deep biopsies taken from its base to include muscle, biopsies of any other abnormal mucosa and 4 quadrant "random" biopsies together with bimanual examination under anaesthetic. Voided urine cytology will be repeated after treatment to check that, if positive initially, it is now negative. The upper urinary tracts will have been assessed by urography. Provided the tumour is superficial and not high grade (i.e. up to pT1 G2) the patient reverts to the outpatient flexible cystoscopy list with laser coagulation of recurrences as they occur. Initial follow-up at 3 months would be rapidly lengthened to 1 year for pTa G1 patients who would be discharged after being free from recurrences for 5 years. All patients with pT1 and/or G2 disease would be followed more frequently with biopsies of recurrences to look for any progression and random mucosal biopsies and urine cytology assessment if there had been any suggestion of associated Cis. If recurrences became too large (> 1.5 cm), too numerous (> 10) or awkwardly situated (e.g. bladder neck), patients would revert to inpatient treatment until the situation stabilised or more aggressive therapy was required.

Patients with initially muscle invasive bladder tumours or those that become so are in a different category and their treatment cannot be generalised. If bladder conservation seems possible then that should be considered, but not at the risk of missing the opportunity for a cure. A localised, early invasive tumour (T2 and perhaps some T3a) without associated Cis should be considered for resection followed by laser coagulation of its base. The difficulty is that despite the most careful pre-operative staging a significant number of tumours thought confined to the

bladder wall (T3a) actually turn out to be through it (T3b) and would not be suitable for laser coagulation. Nevertheless it seems reasonable to offer such conservative treatment to selected patients as the results can be excellent and salvage cystectomy may be offered if there is failure of local control. The question of adjuvant radiotherapy or chemotherapy after laser coagulation has not yet been addressed.

PDT is still a considerable way off becoming a generally available treatment. If the problems already mentioned can be overcome, and we believe that they can, then PDT will be of prime use in those patients with widespread Cis who have failed therapy with the standard intravesical agents such as BCG and are facing a cystectomy. PDT may well be shown to be superior to BCG and might therefore become a first-line treatment for Cis but this is pure supposition. Furthermore the application of PDT to patients with multiply recurrent superficial papillary tumours after resection of visible disease seems a promising approach in an attempt to normalise the unstable urothelium.

11.4.1 The role for a Laser Unit

Laser techniques are going to play an increasing part in the medicine and surgery of the future and should, for the present at least, be seen as an adjunct rather than as a replacement for current treatments, which if applied logically and carefully may enhance therapy in several respects. Clinicians from many specialties will require training and experience in their use within a framework that provides the necessary technical support and opportunity for research and development. These aims are most economically achieved by establishing a laser centre headed by a clinician who would coordinate laser-based activities within that hospital. A physicist (often doubling as the laser safety officer) would be necessary to

ensure mechanical efficiency as well as compliance with the relevant regulations on installation, operation and training.

Ideally such a unit would be centrally located, probably based on a multi-disciplinary endoscopy unit with a high-power Nd:YAG laser as the core service but with additional laser facilities (e.g. CO_2, pulsed dye, argon, excimer) for other specialties such as gynaecology, dermatology, and ophthalmology. There would be the facilities for general anaesthesia, radiological and ultrasound screening and day case surgery. This should lead to less duplication of expensive equipment required by more than one specialty. In this way the great majority of all laser surgery could be carried out in a single unit and the occasional need for say a Nd:YAG laser in the main operating theatres could be met by a portable machine. There would be other advantages in that the nursing staff would all become familiar with handling laser equipment and the organisation of "hands-on" training courses, and demonstrations for scientific meetings would be more easily accomplished for little extra outlay (e.g. simple audio-visual links).

There is a case for a laser unit along the above lines being established in every large district hospital which rather than being an extravagance should lead to significant savings as a result of more efficient practices. The teaching centres will continue to take a lead in the pre-clinical development of new techniques for which extensive laboratory and other research facilities are necessary. However these centres require adequate funding so that a continuing programme of research and development is established which is vital if the NHS is to fully benefit from the exciting new treatments promised through laser technology.

APPENDICES

Appendix 1

Cell Culture Data - from chapter 5

1.1 Direct toxicity of DHE on MGH-U1 cells (fig. 5.3)

1.1.1 Unexposed cells

Cells incubated in the dark for 1 h in DHE solution at concentrations from zero (controls) to 50 μg DHE/ml. Numbers given are of colony counts from *in situ* clonogenic assays (all to 1 DP).

DHE concentration (μg/ml)							
0	**5**	**10**	**15**	**20**	**25**	**30**	**50**

Experiment no.1 (mean and percentage survival in bold type)

97	102	97	92	98	99	96	119
102	88	105	84	65	109	99	91
90	108	103	82	72	85	92	108
106	91		90			105	115
98.8	**97.3**	**101.7**	**87**	**78.3**	**97.7**	**98**	**108.3**
100	**98.5**	**102.9**	**88.1**	**79.3**	**98.9**	**99.2**	**109.6**

Experiment no.2

115	124	143	113	98	89	92	116
124	162	141	90	89	83	65	93
111	143	105	85	107	105	61	66
	151	145			97		70
116.7	**145**	**133.5**	**96**	**98**	**93.5**	**72.7**	**86.3**
100	**124.3**	**114.4**	**82.3**	**84**	**80.1**	**62.3**	**74**

Experiment no.3

96	68	86	94	76	67	90	95
78	82	72	105	65	82	76	104
88	74	71	110	105	76	105	112
82			99	67		102	101
86	**74.7**	**76.3**	**102**	**78.3**	**75**	**93.3**	**103**
100	**86.9**	**88.7**	**118.6**	**91**	**87.2**	**108.5**	**119.8**

Overall mean percentage cell survivals and *standard errors (SEM)*

100	**103.2**	**102**	**96.3**	**84.8**	**88.7**	**90**	**101.1**
	11.1	***7.4***	***11.3***	***3.4***	***5.5***	***14.1***	***13.9***

1.1.2 Cells exposed to white light

Cells incubated in the dark for 1 h in DHE solution at concentrations from zero(controls) to 50 μg DHE/ml and then exposed to strong white light for 30 min.

DHE concentration (μg/ml)							
0	5	10	15	20	25	30	50
Experiment no.1 (mean and percentage survival in bold type)							
104	63	1	1	0	0	0	0
92	35	1	0	0	0	0	0
108	28	0	0	0	0	0	0
89	49	0	0	0	0	0	0
98.3	**43.8**	**0.5**	**0.3**	**0**	**0**	**0**	**0**
100	**44.6**	**0.5**	**0.3**	**0**	**0**	**0**	**0**
Experiment no.2							
123	64	2	1	0	0	0	0
130	59	0	0	0	0	0	0
105	66	3	0	0	0	0	0
120	80	1	0	0		0	0
120.3	**67.3**	**1.8**	**0.3**	**0**	**0**	**0**	**0**
100	**55.9**	**1.5**	**0.2**	**0**	**0**	**0**	**0**
Experiment no.3							
89	45	1	0	0	0	0	0
114	81	0	0	0	0	0	0
109	73	0	0	0	0	0	0
98	62	0	0	0	0	0	0
102.5	**65.3**	**0.3**	**0**	**0**	**0**	**0**	**0**
100	**63.7**	**0.3**	**0**	**0**	**0**	**0**	**0**
Overall mean percentage cell survivals and *standard errors (SEM)*							
100	**54.7**	**0.8**	**0.2**	**0**	**0**	**0**	**0**
	5.5	***0.4***	***< 0.1***				

1.2 Effect of white light on cells sensitised with DHE or AlSPc

1.2.1 Cells sensitised with DHE (fig. 5.4)

Cells incubated in the dark for 1 h in DHE solution (concentration 20 μg DHE/ml) and then exposed to white light for exposures varying from 0-20 min. Numbers given are of colony counts from *in situ* clonogenic assays (all to 1 DP only).

Light exposure (min)					
0	2	5	10	15	20
Experiment no.1 (mean and percentage survival in bold type)					
135	151	93	75	66	12
116	140	88	76	48	4
102	126	77	62	41	5
127	136			50	
120	**138.3**	**86**	**71**	**51.3**	**7**
100	**115.3**	**71.7**	**59.2**	**42.8**	**5.8**
Experiment no.2					
134	88	92	56	28	8
127	41	99	49	33	1
140	59	74	45	31	4
111		82			
128	**62.7**	**86.8**	**50**	**30.7**	**4.3**
100	**49**	**67.8**	**39.1**	**24**	**3.4**
Experiment no.3					
90	127	65	69	49	22
78	109	83	62	38	21
75	96	61	55	51	9
		57	49		15
81	**110.7**	**66.5**	**58.8**	**46**	**16.8**
100	**136.7**	**82.1**	**72.6**	**56.8**	**20.7**
Overall mean percentage cell survivals and *standard errors (SEM)*					
100	**100.3**	**73.9**	**57**	**41.2**	**10**
	26.4	***4.3***	***9.7***	***9.5***	***5.4***

1.2.2 Cells sensitised with AlSPc (fig. 5.4)

Cells incubated in the dark for 1 h in AlSPc solution (concentration 20 μg AlSPc/ml) and then exposed to white light for exposures varying from 0-20 min. Numbers given are of colony counts from *in situ* clonogenic assays (all to 1 DP).

Light exposure (min)					
0	**2**	**5**	**10**	**15**	**20**
Experiment no.1 (mean and percentage survival in bold type)					
125	110	102	88	76	105
112	95	104	81	84	116
134	91	91	74	73	109
127		86	91	94	
124.5	**98.7**	**95.8**	**83.5**	**81.8**	**110**
100	**79.3**	**76.9**	**67.1**	**65.7**	**88.4**
Experiment no.2					
98	145	108	95	96	104
107	107	81	62	132	111
97	116	82	59	120	138
82				98	114
96	**122.7**	**90.3**	**72**	**111.5**	**116.8**
100	**127.8**	**94.1**	**75**	**116.1**	**121.7**
Experiment no.3					
96	102	59	85	81	69
58	81	55	74	63	80
65	66	70	51	77	62
			58	65	
73	**83**	**61.3**	**67**	**71.5**	**70.3**
100	**113.7**	**84**	**91.8**	**97.9**	**96.3**
Overall mean percentage cell survivals and *standard errors (SEM)*					
100	**106.9**	**85**	**78**	**93.2**	**102.1**
	14.4	***5***	***7.3***	***14.7***	***10***

1.3 Effect of laser light on unsensitised MGH-U1 cells (fig. 5.3)

Unsensitised cells exposed to pulsed green laser light (at 20 Hz, 504 nm, 100 mW/cm^2) for exposures from zero (controls) to 30 s. Numbers given are of colony counts from *in situ* clonogenic assays (all to 1 DP only).

Laser light exposure (s)					
0	**2**	**5**	**10**	**15**	**30**
Experiment no.1 (mean and percentage survival in bold type)					
112	118	105	125	99	116
107	124	112	114	100	129
124	123	122	102	119	104
99	114	120	106	125	121
110.5	**119.8**	**114.8**	**111.8**	**110.8**	**117.5**
100	**108.4**	**103.9**	**101.2**	**100.3**	**106.3**
Experiment no.2					
75	89	81	57	90	64
69	81	70	66	62	65
72	61	76	70	67	66
74		67	76	58	58
72	**77**	**73.5**	**67.3**	**69.3**	**63.3**
100	**107**	**102.1**	**93.5**	**96.3**	**87.9**
Experiment no.3					
92	86	92	80	84	72
85	84	80	67	86	88
81	61	64	75	93	62
88					86
86.5	**77**	**78.7**	**74**	**87.7**	**77**
100	**89**	**91**	**85.5**	**101.4**	**89**
Overall mean percentage cell survivals and *standard errors (SEM)*					
100	**101.5**	**99**	**93.4**	**99.3**	**94.4**
	6.2	***4***	***4.5***	***1.5***	***6***

1.4 Effect of laser light on MGH-U1 cells sensitised with DHE

(Data relating to figs. 5.6 and 5.7)

Cells incubated in the dark for 1 h in DHE solution (concentration 20 μg DHE/ml) and then exposed to pulsed green laser light (at 20 Hz, 504 nm) exposures varying from 0-15 s at power densities from 50-100 mW/cm^2. Numbers given are of colony counts from *in situ* clonogenic assays (all to 1 DP only). Controls as for fig. 5.5.

1.4.1 Sensitised cells exposed to 50 mW/cm^2 laser light

Laser light exposure (s)				
0	**2**	**5**	**10**	**15**
Experiment no.1 (mean and percentage survival in bold type)				
93	112	50	9	1
96	77	72	8	1
115	117	49	6	0
112	98	57	11	0
104	**101**	**57**	**8.5**	**0.5**
100	**97.1**	**54.8**	**8.2**	**0.5**
Experiment no.2				
97	83	23	3	0
100	80	31	2	0
113	91	24	1	0
		23	1	
103.3	**84.7**	**25.3**	**1.75**	**0**
100	**82**	**24.5**	**1.7**	**0**
Experiment no.3				
125	105	60	10	0
112	100	80	10	0
134	124	73	16	0
127		50	13	
124.5	**109.7**	**65.8**	**12.3**	**0**
100	**88.1**	**52.9**	**9.9**	**0**

Experiment no.4

97	86	42	18	0
98	74	44	12	2
89	96	46	14	1
93		57	14	0
94.3	**85.3**	**47.3**	**14.5**	**0.8**
100	**90.5**	**50.2**	**15.4**	**0.8**

Overall mean percentage cell survivals and *standard errors (SEM)*

100	**89.4**	**45.6**	**8.8**	**<1**
	3.1	***7.1***	***2.8***	

1.4.2 Sensitised cells exposed to 75 mW/cm^2 laser light

Note: some of the control replicates are the same, in those instances when a single experiment covered exposures at more than 1 power.

Laser light exposure (s)					
0	**2**	**5**	**7**	**10**	**15**

Experiment no.1 (mean and percentage survival in bold type)

93	85	37	21	3	0
96	78	52	11	3	0
115	87	44	19	5	0
112	91	35			
104	**85.3**	**42**	**17**	**3.7**	**0**
100	**82**	**40.4**	**16.3**	**3.6**	**0**

Experiment no.2

126	119	33	6	0	0
116	97	31	5	0	0
119	105	21	3	0	0
116					
119.3	**107**	**28.3**	**4.7**	**0**	**0**
100	**89.7**	**23.7**	**3.9**	**0**	**0**

Experiment no.3

105	94	34	17	5	0
95	76	29	17	7	0
98	101	34	15	6	0
102	83	36	12	4	
100	**88.5**	**33.3**	**15.3**	**5.5**	**0**
100	**88.5**	**33.3**	**15.3**	**5.5**	**0**

Overall mean percentage cell survivals and *standard errors (SEM)*

100	**86.7**	**32.5**	**11.8**	**3**	**0**
	2.4	*4.8*	*4*	*1.6*	

1.4.3 Sensitised cells exposed to 100 mW/cm^2 laser light

Note: some of the control replicates are the same, in those instances when a single experiment covered exposures at more than 1 power.

Laser light exposure (s)				
0	**2**	**5**	**7**	**10**

Experiment no.1 (mean and percentage survival in bold type)

93	88	27	10	0
96	84	30	8	0
115	71	18	4	0
112		33		
104	**81**	**27**	**7.3**	**0**
100	**77.8**	**26**	**7**	**0**

Experiment no.2

126	88	12	0	0
116	91	13	1	0
119	82	9	2	0
116	82		1	
119.3	**85.8**	**11.3**	**1**	**0**
100	**71.9**	**9.5**	**0.8**	**0**

Experiment no.3

106	72	13	3	0
122	84	3	3	0
104	81	7	0	0
120	80	3	2	
113	**79.3**	**6.5**	**2**	**0**
100	**70.2**	**5.8**	**1.8**	**0**

Experiment no.4

97	79	21	14	2
98	85	25	12	1
89	76	20	12	0
93		30	9	0
94.3	**80**	**24**	**11.8**	**0.8**
100	**84.8**	**25.5**	**12.5**	**0.8**

Overall mean percentage cell survivals and *standard errors (SEM)*

100	**76.2**	**16.7**	**5.5**	**<1**
	3.3	*5.3*	*2.7*	

1.4.4 Cell survival related to energy fluence (fig. 5.7)

The data presented in fig. 5.6 are redrawn in fig 5.7 to relate cell survival to the total laser energy delivered. The table below shows the energy density (in J/cm^2) delivered at each parameter examined.

Power density (mW/cm^2)

Exposure time (s)	**25**	**50**	**75**	**100**
2	0.05	0.1	0.15	0.2
5	0.125	0.25	0.375	0.5
7	0.175	0.35	0.525	0.7
10	0.25	0.5	0.75	1
15	0.375	0.75	1.125	1.5

1.5 Cell survival related to light pulse frequency (fig. 5.9)

Cells incubated in the dark for 1 h in DHE solution (concentration 20 μg DHE/ml) and then exposed to pulsed green laser light (504 nm, 50 mW/cm^2) for exposures varying from 0-10 s at a pulse repetition rate of 5 Hz. Only 2 experiments were carried out at this pulse rate, the results are compared in fig. 5.9 to those at 20 Hz.

Laser light exposure (s)			
0	**2**	**5**	**10**

Experiment no.1 (mean and percentage survival in bold type)

59	49	33	6
63	55	40	8
67	59	38	9
55	51	36	5
61	**53.5**	**36.8**	**7**
100	**87.7**	**60.3**	**11.5**

Experiment no.2

104	78	48	5
106	95	57	9
118	89	54	11
113	97		6
110.3	**89.8**	**53**	**7.8**
100	**81.4**	**48.1**	**7.1**

Overall mean percentage cell survivals

100	**84.6**	**54.2**	**9.3**

Comparison with results for 50 mW/cm^2 at a pulse rate of 20 Hz

Overall mean percentage cell survivals

100	**89.4**	**45.6**	**8.8**
	3.1	*7.1*	*2.8*

Appendix 2

Fluorescence Data - from chapter 6

2.1 Fluorescence from rat bladder after intravenous sensitisation with either 0.5 or 5 mg/kg AlSPc

The nature of fluorescence microscopy is essentially qualitative. This technique, though, does allows fluorescence in areas of interest to be quantified by expressing it in arbitrary (though linear) units of fluorescence intensity. As this is subject to observer error in sampling several (3-5) readings were taken from boxes drawn over representative areas of each section (fig. 6.3), and at least 3 sections examined from each bladder. The values given below are the average readings from each bladder (to the nearest 5 units for larger values) and the overall means. The readings from control bladders were so low that they are not given.

Time after sensitisation	5 mg/kg AlSPc		0.5 mg/kg AlSPc	
	mucosa	muscle	mucosa	muscle
1 h	200	125	15	10
	240	105	18	7
	260	100	22	12
	220	125	21	8
MEAN	**230**	**114**	**19**	**9.3**
S.D.	**22**	**11**	**2.7**	**1.9**
24 h	185	45	15	5
	180	36	11	6
	165	50	12	4
	160	41	16	3
MEAN	**173**	**43**	**13.5**	**4.5**
S.D.	**10**	**5.1**	**2.1**	**1.1**
72 h	160	33	11	4
	175	29	11	3
	150	35	12	5
	140	38	14	3
MEAN	**156**	**33.8**	**12**	**3.8**
S.D.	**13**	**3.3**	**1.2**	**0.8**

2.2 Extraction Analysis of AlPcS2 Concentration

2.2.1 Assay method

1. Specimens of sensitised and control tissues frozen until required.
2. Place 0.1 g of tissue in test tube and add 10 ml of 0.1 M sodium hydroxide (NaOH).
3. Place test sample and controls (unsensitised tissue) into a water bath for 4 h at 50°C shaking vigorously at intervals.
4. After 4 h a clear solution should be present without any need for centrifugation.
5. Sample fluorescence is measured on a spectrofluorimeter set as follows and the concentration of AlSPc is determined by reference to the standard curves (see below).

excitation wavelength	-	605 nm
emission wavelength	-	675 nm
slit width	-	10 nm
emission cut off filter	-	645 nm

Preparation of standard curves

A stock solution of 0.2 μg/ml AlSPc is diluted in 0.1 M NaOH to produce concentrations ranging from 0 - 0.1 μg/ml in steps of 0.01 μg/ml. 1.5 ml of this solution is added to an equal amount of either NaOH solution or the control tissue solution prepared as per steps 1 - 4 above, to get 2 series of samples and produce 2 standard curves. The first curve, using NaOH solution, is used to determine the concentrations of AlSPc that will lie on the straight part of the "curve" for accurate reading so that if the test tissue sample is too concentrated it may be diluted appropriately. The curve using control tissue extract is used to correct for the effects of absorption, autofluorescence and quenching by normal tissue and allow for a more accurate quantification of the AlSPc concentration.

Calculation of sample AlPcS2 concentration

The fluorescence value for the sample is plotted on the standard curve with control tissue extract to give a value in μg/ml of sample extract. This is multiplied by 100 plus any dilution required to get a reading on the straight part of the standard curve, to give a final concentration in μg/g tissue.

2.2.2 Assay data (fig. 6.11)

The data tabulated below were obtained from an extraction assay of AlPcS2 performed as described above. The author sensitised the animals, collected and prepared the specimens but did not perform the assay (see acknowledgements). The concentration of sensitiser in plasma, whole bladder and kidney was determined to the nearest 0.05 micrograms AlPcS2 per gram of specimen. Each value relates to an individual animal but the 3 specimens from each rat do not necessarily appear in the same row of the table.

ND = AlPcS2 not detected in the sample (i.e. less than 0.05 μg/g AlPcS2)

	Tissue concentration of AlPcS2 (μg/g)		
	Plasma	**Bladder**	**Kidney**
1 h	1.3	0.35	0.65
	2.05	0.4	0.75
	2.55	0.35	0.85
	1.6	0.4	0.7
	1.7	0.35	0.7
mean	**1.84**	**0.37**	**0.73**
S.D.	**0.43**	**0.02**	**0.07**
3 h	0.9	0.55	0.55
	0.85	0.35	0.5
	1.6	0.4	0.5
	1.35	0.45	0.75
		0.45	0.5
mean	**1.18**	**0.44**	**0.56**
S.D.	**0.31**	**0.07**	**0.1**
6 h	0.45	0.45	0.6
	0.4	0.5	0.45
	0.6	0.5	0.45
	0.45	0.45	0.4
	0.65	0.55	0.45
mean	**0.51**	**0.49**	**0.47**
S.D.	**0.1**	**0.04**	**0.07**

	Plasma	Bladder	Kidney
12 h	0.08	0.4	0.5
	0.07	0.45	0.5
	ND	0.55	0.45
	ND	0.45	0.5
	ND	0.45	0.45
mean	**0.075**	**0.46**	**0.48**
S.D.		**0.05**	**0.02**

24 h	ND	0.4	0.45
	ND	0.45	0.4
	ND	0.4	0.4
	ND	0.5	0.4
	ND	0.45	0.45
mean		**0.44**	**0.42**
S.D.		**0.04**	**0.02**

2 days	ND	0.3	0.35
	ND	0.35	0.35
	ND	0.35	0.35
	ND	0.3	0.35
	ND	0.3	0.45
mean		**0.32**	**0.37**
S.D.		**0.02**	**0.04**

	Plasma	Bladder	Kidney
3 days	ND	0.3	0.35
	ND	0.3	0.35
	ND	0.35	0.3
	ND	0.4	0.45
	ND	0.3	0.35
mean		**0.33**	**0.36**
S.D.		**0.04**	**0.05**

	Plasma	Bladder	Kidney
7 days	ND	0.3	0.5
	ND	0.15	0.35
	ND	0.3	0.3
	ND	0.2	0.3
	ND	0.2	0.4
mean		**0.23**	**0.37**
S.D.		**0.06**	**0.07**

Appendix 3

Bladder compliance and capacity data - from chapter 8

3.1. Filling pressures in normal rat bladder (fig. 8.4)

These data comprise the pressure/volume recordings from 40 rats before they underwent PDT. Each row comprises the pressure measurements from an individual animal at the 4 volume points shown and are given to the nearest cm H_20. (-) indicates incomplete data.

	Intravesical pressure (cm H_20) at 4 bladder volume points			
	0.25 ml	0.5 ml	0.75 ml	1 ml
1	3	5	7	27
2	12	13	18	21
3	8	10	12	13
4	7	12	13	20
5	7	15	18	33
6	5	13	25	30
7	5	10	15	22
8	3	5	8	20
9	7	17	23	37
10	3	5	5	17
11	3	8	13	25
12	4	7	12	19
13	5	7	9	13
14	3	10	13	22
15	6	7	8	13
16	2	5	7	10
17	2	3	8	20
18	3	8	2	13
19	2	2	3	10

	0.25 ml	0.5 ml	0.75 ml	1 ml
20	3	7	13	30
21	2	3	5	8
22	3	8	17	25
23	5	8	17	22
24	4	9	12	27
25	4	10	23	38
26	2	3	5	6
27	2	4	7	8
28	2	3	6	8
29	2	3	13	20
30	3	7	9	11
31	1	2	3	10
32	2	3	5	9
33	2	4	6	9
34	5	7	9	11
35	4	20	33	-
36	5	10	25	-
37	3	17	28	-
38	7	7	12	-
39	8	17	20	-
40	7	8	20	-

Summary (all values to 1 DP)

volume	0.25 ml	0.5 ml	0.75 ml	1 ml
no. values	40	40	40	34
Mean	4.2	8.1	12.7	18.4
St. Dev.	2.3	4.6	7.5	8.9
SEM	0.4	0.7	1.2	1.5

3.2 Filling pressures in rat bladder before and after PDT (fig. 8.5)

Each row in the table below comprises pressure measurements (to the nearest cm H_20) from an individual animal at the 4 volume points shown, both before and 7 days after PDT (1 mg/kg AlSPc, 24h prior to 20 J/cm^2 light). This particular treatment group is shown as illustrative of the change following PDT in moderately affected animals.

The figures in brackets are the ratio of that pressure to the pre-treatment value. The mean of these values for each animal derives the compliance ratio (CR) (see also figs. 8.8 and 8.10).

(-) indicates that it was not possible to get a valid measurement as the very high pressure resulted either in leakage around the catheter or ureteric reflux.

	Intravesical pressure (cm H_20) at 4 bladder volume points								
	0.25ml		0.5ml		0.75ml		1ml		Compliance Ratio
	pre	post	pre	post	pre	post	pre	post	
1	1	2(2)	2	7(3.5)	3	12(4)	10	40(4)	3.4
2	2	13(6.5)	3	23(7.7)	5	45(9)	9	-	7.7
3	2	8(4)	4	11(2.8)	6	27(4.5)	9	48(5.3)	4.2
4	5	5(1)	7	20(2.9)	9	24(2.7)	11	-	2.2
Mean	**2.5**	**7.0**	**4.0**	**15.3**	**5.8**	**27**	**9.8**	**44**	**4.4**
St. Dev	**1.7**	**4.5**	**2.2**	**7.5**	**2.5**	**13.6**	**1.0**	**-**	**2.4**
SEM	**0.9**	**2.3**	**1.1**	**3.8**	**1.3**	**6.8**	**0.5**	**-**	**1.2**

3.3 Bladder compliance and capacity data

These data relate to figs. 8.6 - 8.10 (compliance) and figs. 8.11 - 8.15 (capacity). The Compliance Ratio (CR) is derived from the pressure ratios given in brackets after the actual recorded pressures. Each section between the bold lines comprises data from 1 animal in the dose group given. The order of these is that used for the corresponding graphs (animal 1 - 4 etc.). The 2 tables at the end of this section summarise the data for each group.

Controls	Intravesical pressure (cm H_2O) at 4 bladder volume points				Bladder capacity	Compliance Ratio
Time	0.25 ml	0.5 ml	0.75 ml	1 ml		
pre - PDT	3 (1)	5 (1)	8 (1)	17 (1)	1.05	1
4 days	5 (1.7)	7 (1.4)	8 (1)	12 (.71)	1.5	1.2
7 days	5 (1.7)	7 (1.4)	9 (1.1)	10 (.59)	2.1	1.2
14 days	5 (1.7)	5 (1)	7 (.88)	8 (.47)	2.4	1.0
30 days	3 (1)	4 (.8)	6 (.75)	8 (.47)	2.0	0.76
90 days	2 (.67)	3 (.6)	5 (.63)	8 (.47)	1.5	0.59
pre - PDT	3 (1)	8 (1)	13 (1)	25 (1)	1.1	1
4 days	3 (1)	10 (1.3)	21 (1.6)	20 (.8)	0.8	1.2
7 days	7 (2.3)	19 (2.4)	20 (1.5)	35 (1.4)	1.1	1.9
14 days	5 (1.7)	12 (1.5)	16 (1.2)	32 (1.3)	1.2	1.4
30 days	4 (1.3)	9 (1.1)	14 (1.1)	28 (1.1)	1.1	1.2
90 days	3 (1)	7 (.88)	13 (1)	30 (1.2)	1.0	1.0
pre - PDT	3 (1)	8 (1)	17 (1)	25 (1)	1.0	1
4 days	3 (1)	7 (.89)	12 (.71)	22 (.88)	1.4	0.87
7 days	3 (1)	7 (.89)	10 (.56)	20 (.8)	1.6	0.81
14 days	3 (1)	5 (.63)	7 (.41)	7 (.28)	2.0	0.58
30 days	2 (.67)	6 (.75)	8 (.47)	9 (.36)	1.8	0.56
90 days	2 (.67)	8 (1)	8 (.47)	4 (.16)	1.45	0.58
pre - PDT	2 (1)	3 (1)	13 (1)	20 (1)	1.05	1
4 days	1 (.5)	4 (1.3)	8 (.62)	11 (.55)	1.2	0.74
7 days	1 (.5)	2 (.67)	3 (.23)	3 (.15)	2.7	0.39
14 days	1 (.5)	3 (1)	4 (.31)	6 (.3)	2.35	0.53
30 days	2 (1)	3 (1)	4 (.31)	5 (.25)	1.4	0.64
90 days	2 (1)	3 (1)	4 (.31)	5 (.25)	1.85	0.64

0.5mg/kg AlSPc Time	Intravesical pressure (cm H_20) at 4 bladder volume points 0.25ml	0.5ml	0.75ml	1ml	Bladder capacity	Compliance Ratio
pre - PDT	7 (1)	17 (1)	23 (1)	37 (1)	0.7	1
4 days	8 (1.1)	20 (1.2)	27 (1.2)	37 (1)	0.6	1.1
7 days	17 (2.4)	25 (1.5)	28 (1.2)	103 (2.8)	0.45	2.0
14 days	5 (.71)	16 (.94)	70 (3)	100 (2.7)	0.7	1.8
30 days	5 (.71)	12 (.71)	35 (1.5)	53 (1.4)	0.75	1.1
90 days	3 (.43)	5 (.29)	14 (.61)	20 (.54)	0.9	0.47
pre - PDT	3 (1)	5 (1)	12 (1)	17 (1)	0.9	1
4 days	5 (1.7)	8 (1.6)	17 (1.4)	33 (1.9)	0.8	1.7
7 days	3 (1)	5 (1)	10 (.83)	20 (1.2)	1.05	1.0
14 days	3 (1)	7 (1.4)	10 (.83)	17 (1)	1.1	1.1
30 days	3 (1)	6 (1.2)	13 (1.1)	12 (.71)	1.05	1.0
90 days	3 (1)	5 (1)	11 (.92)	10 (.59)	1.1	0.89
pre - PDT	2 (1)	3 (1)	5 (1)	6 (1)	1.25	1
4 days	7 (3.5)	15 (5)	20 (4)	28 (4.7)	0.65	4.3
7 days	3 (1.5)	6 (2)	12 (2.4)	16 (2.7)	1.2	2.2
14 days	4 (2)	8 (2.7)	10 (2)	12 (2)	1.05	2.2
30 days	3 (1.5)	7 (2.3)	10 (2)	11 (1.8)	1.1	1.9
90 days	1 (.5)	1 (.33)	2 (.4)	3 (.5)	1.8	0.43
pre - PDT	2 (1)	4 (1)	7 (1)	8 (1)	1.25	1
4 days	5 (2.5)	8 (2)	14 (2)	25 (3.1)	0.95	2.4
7 days	4 (2)	6 (1.5)	10 (1.4)	12 (1.5)	1.15	1.6
14 days	3 (1.5)	6 (1.5)	10 (1.4)	17 (2.1)	1.15	1.6
30 days	2 (1)	5 (1.3)	7 (1)	8 (1)	1.25	1.1
90 days	1 (.5)	3 (.75)	3 (.43)	4 (.5)	1.6	0.55

Bladder capacity is defined as the volume released (ml) after slowly filling the bladder at a pressure head of 30 cm H_20.

1 mg/kg AlSPc Time	Intravesical pressure (cm H_20) at 4 bladder volume points 0.25ml	0.5ml	0.75ml	1ml	Bladder capacity	Compliance Ratio
pre - PDT	1 (1)	2 (1)	3 (1)	10 (1)	1.25	1
4 days	8 (8)	10 (5)	12 (4)	27 (2.7)	0.9	4.9
7 days	2 (2)	7 (3.5)	12 (4)	40 (4)	0.8	3.4
14 days	3 (3)	4 (2)	5 (1.7)	8 (.8)	1.65	1.9
30 days	2 (2)	2 (1)	5 (1.7)	8 (.8)	1.65	1.4
90 days	1 (1)	2 (1)	3 (1)	4 (.4)	2.1	0.85
pre - PDT	2 (1)	3 (1)	5 (1)	9 (1)	1.2	1
4 days	11 (5.5)	37 (12)	-	-	0.2	8.8
7 days	13 (6.5)	23 (7.7)	45 (9)	-	0.6	7.7
14 days	2 (1)	3 (1)	4 (.8)	7 (.8)	0.95	0.9
30 days	1 (.5)	2 (.67)	2 (.4)	4 (.44)	2.0	0.5
90 days	1 (.5)	2 (.67)	3 (.6)	3 (.33)	2.15	0.53
pre - PDT	2 (1)	4 (1)	6 (1)	9 (1)	1.1	1
4 days	17 (8.5)	53 (13)	-	-	0.2	11
7 days	8 (4)	11 (2.8)	27 (4.5)	48 (5.3)	0.5	4.2
14 days	1 (.5)	3 (.75)	8 (1.3)	19 (2.1)	1.1	1.2
30 days	3 (1.5)	6 (1.5)	9 (1.5)	16 (1.8)	1.4	1.6
90 days	2 (1)	3 (.75)	3 (.5)	4 (.44)	2.2	0.67
pre - PDT	5 (1)	7 (1)	9 (1)	11 (1)	1.45	1
4 days	9 (1.8)	22 (3.1)	53 (5.9)	-	0.15	3.6
7 days	5 (1)	20 (2.9)	24 (2.7)	-	0.55	2.2
14 days	5 (1)	15 (2.1)	23 (2.6)	-	0.55	1.9
30 days	3 (.6)	4 (.57)	7 (.78)	12 (1.1)	1.3	0.76
90 days	2 (.4)	5 (.71)	7 (.78)	13 (1.2)	1.4	0.77

(-) indicates that it was not possible to get a valid measurement as the very high pressure resulted either in leakage around the catheter or ureteric reflux.

1.5 mg/kg AlSPc Time	Intravesical pressure (cm H_2O) at 4 bladder volume points				Bladder capacity	Compliance Ratio
	0.25 ml	0.5 ml	0.75 ml	1 ml		
pre - PDT	5 (1)	7 (1)	9 (1)	13 (1)	1.05	1
4 days	18 (3.6)	32 (4.6)	-	-	0.4	4.1
7 days	10 (2)	23 (3.3)	33 (3.7)	-	0.5	3.0
14 days	9 (1.8)	27 (3.9)	57 (6.3)	-	0.65	4.0
30 days	5 (1)	15 (2.1)	20 (2.2)	43 (3.3)	0.8	2.2
90 days	3 (.6)	6 (.86)	13 (1.4)	20 (1.5)	0.9	1.1
pre - PDT	2 (1)	3 (1)	8 (1)	20 (1)	1.1	1
4 days	10 (5)	20 (6.7)	27 (3.4)	-	0.65	5.0
7 days	16 (8)	22 (7.3)	32 (4)	-	0.6	6.4
14 days	6 (3)	15 (5)	50 (6.3)	-	0.6	4.8
30 days	9 (4.5)	19 (6.3)	24 (3)	63 (3.2)	0.8	4.3
90 days	7 (3.5)	12 (4)	17 (2.1)	30 (1.5)	0.6	2.8
pre - PDT	2 (1)	2 (1)	3 (1)	10 (1)	1.2	1
4 days	15 (7.5)	27 (14)	38 (13)	-	0.7	11.5
7 days	13 (6.5)	16 (8)	23 (7.7)	-	0.5	7.4
14 days	10 (5)	18 (9)	42 (14)	87 (8.7)	0.75	9.2
30 days	8 (2)	10 (5)	12 (4)	21 (2.1)	0.9	3.3
90 days	3 (1.5)	7 (3.5)	10 (3.3)	12 (1.2)	1.15	2.4

(-) indicates that it was not possible to get a valid measurement as the very high pressure resulted either in leakage around the catheter or ureteric reflux.

3.4 Summary of bladder compliance and capacity data (figs. 8.10 & 8.15)

	Compliance Ratio				
	4 days	7 days	14 days	30 days	90 days
controls	1.2	1.2	1.0	0.76	0.59
	1.2	1.9	1.4	1.2	1.0
	0.87	0.81	0.58	0.56	0.58
	0.74	0.39	0.53	0.64	0.64
Mean	**1.0**	**1.1**	**0.88**	**0.79**	**0.7**
St. Dev	0.23	0.64	0.41	0.29	0.2
SEM	0.12	0.32	0.2	0.14	0.1
0.5 mg/kg	1.1	2.0	1.8	1.1	0.47
	1.7	1.0	1.1	1.0	0.89
	4.3	2.2	2.2	1.9	0.43
	2.4	1.6	1.6	1.1	0.55
Mean	**2.4**	**1.7**	**1.7**	**1.3**	**0.59**
St. Dev	1.4	0.53	0.46	0.42	0.21
SEM	0.69	0.27	0.23	0.21	0.11
1 mg/kg	4.9	3.4	1.9	1.4	0.85
	8.8	7.7	0.9	0.5	0.53
	11	4.2	1.2	1.6	0.67
	3.6	2.2	1.9	0.76	0.77
Mean	**7.1**	**4.4**	**1.5**	**1.1**	**0.71**
St. Dev	3.4	2.4	0.51	0.52	0.14
SEM	1.7	1.2	0.25	0.26	0.07
1.5 mg/kg	4.1	3.0	4.0	2.2	1.1
	5.0	6.4	4.8	4.3	2.8
	11.5	7.4	9.2	3.3	2.4
Mean	**6.9**	**5.6**	**6.0**	**3.3**	**2.1**
St. Dev	4.0	2.3	2.8	1.1	0.89
SEM	2.3	1.3	1.6	0.6	0.51

	Bladder Capacity (at 30 cm H_20)					
	pre PDT	4 days	7 days	14 days	30 days	90 days
controls	1.05	1.5	2.1	2.4	2.0	1.5
	1.1	0.8	1.1	1.2	1.1	1.0
	1.0	1.4	1.6	2.0	1.8	1.45
	1.05	1.2	2.7	2.35	1.4	1.85
Mean	1.04	1.23	1.88	1.99	1.58	1.45
% change	**1**	**1.18**	**1.81**	**1.91**	**1.52**	**1.39**
SEM	0.02	0.15	0.33	0.27	0.19	0.16
0.5 mg/kg	0.7	0.6	0.45	0.7	0.75	0.9
	0.9	0.8	1.05	1.1	1.05	1.1
	1.25	0.65	1.2	1.05	1.1	1.8
	1.25	0.95	1.15	1.15	1.25	1.6
Mean	1.03	0.75	0.96	1.0	1.04	1.35
% change	1	0.73	0.93	0.97	1.0	1.31
corrected	**1**	**0.62**	**0.51**	**0.51**	**0.66**	**0.94**
SEM	0.13	0.06	0.09	0.05	0.07	0.15
1 mg/kg	1.25	0.9	0.8	1.65	1.65	2.1
	1.2	0.2	0.6	0.95	2.0	2.15
	1.1	0.2	0.5	1.1	1.4	2.2
	1.45	0.15	0.55	0.55	1.3	1.4
Mean	1.25	0.36	0.61	1.06	1.59	1.96
% change	1	0.29	0.49	0.85	1.27	1.57
corrected	**1**	**0.25**	**0.27**	**0.45**	**0.84**	**1.13**
SEM	0.06	0.12	0.03	0.1	0.08	0.11
1.5 mg/kg	1.05	0.4	0.5	0.65	0.8	0.9
	1.1	0.65	0.6	0.6	0.8	0.6
	1.2	0.7	0.5	0.75	0.9	1.15
Mean	1.12	0.58	0.53	0.67	0.83	0.88
% change	1	0.52	0.47	0.60	0.74	0.79
corrected	**1**	**0.44**	**0.26**	**0.31**	**0.49**	**0.57**
SEM	0.04	0.07	0.02	0.02	0.02	0.1

Appendix 4

Publications and Presentations arising from this work

4.1 Publications

Pope A J, Masters J R W, MacRobert A J (1990) The photodynamic effect of a pulsed dye laser on human bladder carcinoma cells *in vitro*. Urol Res 18:267-270.

McNicholas T A, Pope A J (1990) Laser treatment of urological tumours 1: transitional cell tumours. In: McNicholas T A (ed) Lasers in urology: principles and practice, Springer-Verlag, London, p.41-63.

Pope A J, Bown S G (1991) The morphological and functional changes in rat bladder following photodynamic therapy with phthalocyanine photosensitization. J Urol 145:1064-1070.

Pope A J, Bown S G (1991) Photodynamic therapy. Br J Urol 68:1-9.

Pope A J, MacRobert A J, Phillips D, Bown S G (1991) The detection of phthalocyanine fluorescence in normal rat bladder wall using sensitive digital imaging microscopy. Br J Cancer 64:875-881.

4.2 Personal presentations to scientific meetings and learned institutions

The effect of photodynamic therapy on bladder compliance. RSM (Urology section) Annual Meeting, London, May 1988.

Photodynamic therapy in the bladder. Middlesex and University College Medical Research Club, June 1989.

The effect of photodynamic therapy using phthalocyanine sensitisation on bladder compliance - an animal study. Annual Meeting of the British Association of Urological Surgeons (BAUS), Jersey, June 1989.

The morphological effect of the neodymium:YAG laser compared with electrocautery on the bladder. BAUS Annual Meeting, Jersey, June 1989.

Photodynamic therapy of bladder tumours - experimental studies. British Medical Laser Association (BMLA) Annual Meeting, London, Oct 1989.

Laser tissue effects and delivery systems. BAUS Instrument Study Day, Royal College of Surgeons, Nov 1989 (invited lecture).

Experimental aspects of photodynamic therapy in the bladder. British Photobiology Society Annual Meeting, Dec 1989.

Lasers and endoscopes - the urinary tract. Laser applications in medicine, University of Essex, Feb 1990 (invited lecture).

The correlation of structural changes with bladder function after photodynamic therapy using phthalocyanine sensitisation. Laser 90 - BMLA International Congress, Manchester, Feb 1990.

The detection of phthalocyanine fluorescence in normal rat bladder wall using sensitive digital imaging microscopy. Laser 90 - BMLA International Congress, Manchester, Feb 1990.

Neodymium:Yag laser versus electrocautery: comparative effects on the bladder. Laser 90 - BMLA International Congress, Manchester, Feb 1990.

The morphological and functional effects of photodynamic therapy using a new phthalocyanine photosensitiser on rat bladder. American Urological Association Annual Meeting, New Orleans, May 1990.

Comparative effects of the neodymium:YAG laser and electrocautery on the bladder. European Association of Urology (EAU), 1Xth Congress, Amsterdam, June 1990.

The correlation of structural changes with bladder function after photo-dynamic therapy using phthalocyanine sensitisation. EAU, 1Xth Congress, Amsterdam, June 1990.

Experimental aspects of phthalocyanine photodynamic therapy in the bladder. Netherlands Cancer Institute, June 1990 (invited lecture).

Phthalocyanine photodynamic therapy in normal bladder: A need to limit muscle damage to preserve bladder function? (poster) International Photodynamic Association, 3rd biennial meeting, Buffalo USA, July 1990.

Outpatient laser treatment for urologists. BAUS Annual Meeting, Glasgow, June 1991.

Appendix 5

Abbreviations

(note: SI units and other common abbreviations are not listed)

AlSPc	aluminium sulphonated phthalocyanine (mixture)
AlPc(S1-4)	aluminium (mono-tetra) sulphonated phthalocyanine
BCG	Bacillus Calmette-Guérin
CCD	charge coupled device
Cis	carcinoma *in situ* (of the bladder)
CMG	cystometrogram
CR	compliance ratio
CW	continuous wave (laser output)
DHE	dihaematoporphyrin ether/ester
H&E	haematoxylin and eosin
HeNe	helium neon (laser)
HpD	haematoporphyrin derivative
HVG	haematoxylin van Gieson
IV	intravenous
IVU	intravenous urogram
KTP	potassium titranyl phosphate
LDL	low density lipoprotein
Nd:YAG	neodymium:yttrium aluminium garnet (laser)
PBS	phosphate-buffered saline
PC	personal computer (as in IBM-PC)
PDT	photodynamic therapy
PTFE	polytetrafluoroethylene
SE(M)	standard error (of the mean)
TUR	trans-urethral resection
UV	ultraviolet

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Barr H, Traulau C J, Boulos P B, Krasner N, Clark C G, Bown S G (1990a) Selective destruction of dimethyl-hydrazine rat colon cancer using phthalocyanine photodynamic therapy. Gastroenterology 98:1532-1537.

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REVIEW

Photodynamic Therapy

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Photodynamic therapy (PDT) is an innovative approach to the local ablation of malignant tumours. It has attracted a great deal of interest as a possible means of selectively destroying small cancers. The concept is simple:

> "A photosensitising drug is given intravenously and over the subsequent few days this is retained selectively in the tumour area while it is lost from adjacent normal tissue. Light at a specific wavelength corresponding to an absorption peak of the photosensitiser is then used to activate this drug to produce local necrosis in the tumour and leave the adjacent normal tissue undamaged."

Unfortunately this is rather an optimistic oversimplification. It is extremely difficult to produce truly selective tumour necrosis with PDT based on selective uptake of the sensitiser, and this can only be done under special circumstances with careful manipulation of all of the treatment parameters involved. Under the conditions described in virtually every published paper on PDT, if tumour and normal areas are exposed to the same light dose, there is damage to both (Barr *et al.*, 1990). However, it is now becoming apparent that the *nature* of the biological effects of PDT on normal and malignant tissues is different from that produced by other forms of local injury such as thermal coagulation or ionising radiation. For example, tissue architecture and tensile strength are much better preserved after PDT than after thermal injury as PDT has little effect on collagen whereas heat destroys it (Barr *et al.*, 1987). In the treatment of human disease, understanding and exploitation of these differences are likely to be more important than trying to limit effects to tumour areas. Even though normal tissue may be damaged along with the tumour, if this heals by regeneration of normal tissue, as seems to be the case after PDT, then the net effect is selective eradication of the tumour.

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Historical Perspective

The first clinical use of PDT was in 1903, when von Tappeiner and Jesionek treated skin tumours with topical eosin followed by exposure to white light, and the technique has slowly evolved since then. The photosensitisers that have been used for essentially all clinical PDT to date are haematoporphyrin derivative (HpD) or dihaematoporphyrin ether/ester (DHE), which is a more purified preparation of HpD (Dougherty *et al.*, 1983). However, both HpD and DHE are far from ideal photosensitisers as they are incompletely defined mixtures of porphyrins whose composition and stability between preparations varies, and which are difficult to assay. Several promising new groups of drugs are being developed, especially the phthalocyanines, which have similar biological properties to the porphyrins but are easier to handle chemically and assay in tissue and have much more suitable light absorption spectra (Bown *et al.*, 1986). However, these are not yet ready for clinical trials.

Lipson *et al.* (1961) showed that HpD seemed to have an affinity for malignant tumours in man. Later they treated a patient with recurrent breast cancer using filtered red light from a xenon arc lamp, with apparent resolution of visible disease. It was not until 1975 that the potential of PDT in bladder carcinoma was demonstrated by Kelly and Snell. They subsequently gave HpD to 11 patients and looked at tissue fluorescence under ultraviolet

light, noting a good correlation between areas of tumour and the observed fluorescence, particularly for carcinoma *in situ* (Kelly and Snell, 1976), a finding confirmed by Benson (1986). One patient had an area of bladder illuminated by a mercury vapour lamp transmitted through a rigid quartz rod. Several papillary tumours were necrosed but the authors commented that improved techniques for illumination were needed. Early work with PDT therefore was largely confined to skin lesions until the development of suitable lasers and fibreoptics.

Mechanism of Action

In tissue, the photosensitiser is activated by absorbing light. One of 3 competing processes can then occur (Fig.).

(i) The activated photosensitiser molecules can react with tissue oxygen to produce singlet oxygen, which is thought to be the cytotoxic agent (Dougherty *et al.*, 1983). This is a threshold effect and a certain quantity of singlet oxygen must be produced before a cell is killed. The photosensitiser molecules then return to the ground state and can be activated again.

(ii) The activated molecules can fluoresce (emit light) and then return directly to the ground state, whereupon they can be activated again.

(iii) The activated molecules can go through a different process in which they are destroyed by the same activating light (photodegradation). This means that the total quantity of photosensitiser in tissue is steadily reduced as the tissue is exposed to light. This has important implications for protecting tissues with low concentrations of photosensitiser, as will be discussed below.

Many studies have confirmed this direct cellular toxicity on a wide range of normal and neoplastic cells *in vitro* (with no real difference in susceptibility between normal and neoplastic cell lines), but *in vivo* the most obvious early effect of PDT is to shut down small blood vessels. Endothelial cells are probably affected first, although it is not yet clear how this leads to the vasoconstriction and closing of vessels that have been observed experimentally in both normal and tumour tissue (Star *et al.*, 1986). Studies on the distribution of photosensitisers in animal tumour models demonstrate that the relatively small degree of selectivity of uptake of photosensitisers that is seen in tumours is due to their retention in the vascular stroma (Bugelski *et al.*, 1981). At a cellular level *in vivo*, as with the *in vitro* studies, there is no difference in uptake between malignant cells and the normal cells from which the malignancy arose.

Light and Lasers

The main requirement for the therapeutic light is that the wavelength used should match an absorption peak of the photosensitiser. In addition, the depth of tumour that can be treated by PDT below the illuminated tissue surface is largely limited by the depth to which light will adequately penetrate that tissue. The most penetrating wavelengths to use are in the red and near infrared part of the spectrum, but even using these, the maximum depth of necrosis possible in most tissues is typically no more than about 5 mm. For this reason, PDT is best for small tumours, although larger lesions can be treated by inserting the laser fibre directly into the target tissue. HpD and DHE both have only a

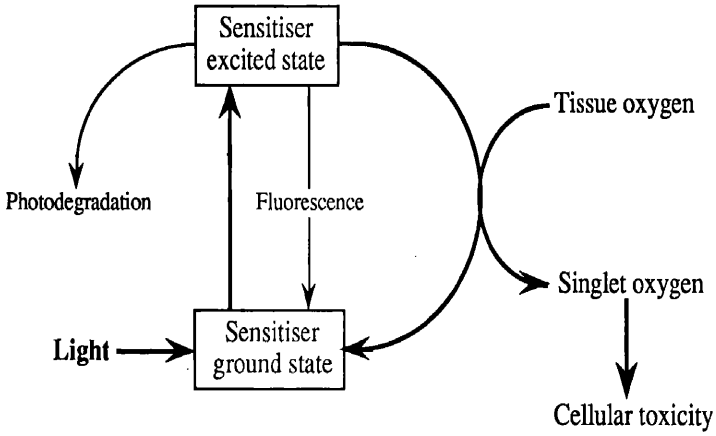

Fig. Outline mechanism of photodynamic action. Light energy absorbed by the photosensitiser molecules elevates them to an excited state. This is short-lived and can react to produce singlet oxygen which is highly toxic and causes local cell death. Some of the excited photosensitiser molecules may decay back to the ground state by emitting light (fluorescence) or may become irreversibly degraded.

minor absorption peak for red light (at 630 nm), although newer sensitisers generally have their major absorption bands at longer wavelengths for greater tissue penetration (*e.g.* aluminium sulphonated phthalocyanine at 675 nm, benzoporphyrin at 700 nm and silicon naphthalcyanine at 776 nm).

Laser light is not an absolute requirement for PDT but is just a convenient way of producing high intensity light at exactly the right wavelength in a small enough beam to be focused into a thin optical fibre for endoscopic use. At present, the most suitable laser sources are argon pumped dye lasers or copper vapour pumped dye lasers, as the wavelength of the output beam can be tuned to match the absorption peak of the photosensitiser being used. The gold vapour laser emits at 628 nm, which is exactly right to excite HpD and DHE but would not be suitable for the newer sensitisers. Other possibilities include pumping dye lasers with excimer and frequency-doubled neodymium: YAG lasers. Over the next few years much cheaper, more compact and reliable semi-conductor lasers are likely to become available for use with sensitisers that absorb at the longer wavelengths, but it is only very recently that their specifications have started to come close to those required for PDT.

Clinical Applications

The bladder seems ideally suited to PDT as it is readily accessible and the entire mucosa can be treated simultaneously. Resistant superficial bladder cancer is usually multifocal, and areas of occult dysplasia and carcinoma *in situ* (Cis) do not have to be precisely defined for PDT to be effective. Advances in ureteric instrumentation and light delivery systems make upper tract urothelial tumours theoretically treatable. There is also the important consideration that these superficial lesions lie well within the depth of tissue easily treated by PDT.

The other 2 important hollow organs in which PDT may have a useful role to play in tumour therapy are the gastrointestinal tract and the airways, although tumours in these organs are usually much larger than those in the bladder at the time of presentation. It is seldom appropriate to use PDT alone for these tumours unless they are small but it may be of value as adjuvant therapy if the main bulk is removed by other techniques. For solid organs such as the prostate, light can be delivered to the target area by interstitial fibres but the volume treated around each fibre site is small. In general, therefore, multiple fibres would be required and such applications are still in the preclinical phase (Pantelides *et al.*, 1990). There are numerous clinical studies showing anecdotal success with PDT for tumours of the skin, head and neck and brain, although as yet no controlled trials.

Clinical Results in Bladder Cancer

There are several reported series of PDT in bladder cancer that give an average "complete response" rate at 3 months of about 65% (Table). This implies the disappearance of all visible tumour and, where available, negative biopsies and urine cytology. Differing selection criteria, tumour pathology and treatment parameters make direct comparison difficult but there is a consensus that PDT is most effective at treating Cis, less effective for superficial papillary disease, and ineffective for invasive tumour. The best results are those of Benson (1986, 1988), who reported 27 patients with resistant Cis who refused cystectomy; 23 of these (85%) showed a complete response to PDT but the other 4 had coexistent papillary tumour which persisted, although their Cis responded. Conversely, Prout *et al.* (1987) reported on 19 patients, the majority of whom had papillary bladder cancer alone, and found that only 47% had a complete response. The only British series so far reported (Williams and Stamp, 1988; Stamp *et al.*, 1990) comprised 10 patients with multiple superficial bladder tumours (Cis, Ta and T1) that had not responded to intravesical chemotherapy. A complete initial response was seen in 4 patients and a partial response in another 4. Only 2 patients remained tumour-free (in excess of 2 years) and 7 ultimately developed an invasive cancer, of whom 2 died.

There are few other published data on the durability of the response of bladder tumours to PDT to suggest whether or not PDT offers any worthwhile improvement in either recurrence rate, time to progression or survival. Benson (1988) reported a 50% recurrence of focally treated Cis at 1 year, though most recurrence was in previously uninvolved areas and was successfully re-treated with PDT. Jocham *et al.* (1989) reported that 9 of 15 patients with Cis remained clear with follow-up in excess of 2 years after whole bladder PDT; 2 became clear with a second treatment and 4 had recurrences after a mean of 20 months.

Complications Following PDT to the Bladder

At first sight these results seem impressive, especially as they have been achieved largely in patients

Table Results of Photodynamic Therapy in Bladder Carcinoma

Authors	*No. of patients*	*Sensitiser (mg/kg)*	*Interval to treatment (h)*	*Light dose (J/cm²)*	*Response at 3 months*	
					Complete	*Persistent tumour*
Benson	15	HpD	3, 48 or both	150 (F)*	15	0
(1986)	12	(2.5–5)	3 or 48	25–45 (WB)*	8	4
Harty *et al.*	2	DHE	72	25 (WB)	1	1
(1989)	5	(2)		25 (WB) + 100 (F)	4	1
Hisazumi *et al.*	9	HpD	48–72	120–250 (F)	5	4
(1983, 1984)	2	(2–3.2)	48	10 (WB)	2	0
Jocham *et al.*	15	DHE/HpD	40–72	35 (15–70) (WB)	11	4
(1989)		(2–3)				
Nseyo *et al.*	11	DHE	72	10–60 (WB)	3	8
(1987)	10	(2)		100–200 (F) + 5–60 (WB)	4	6
	2			100–200 (F)	0	2
Prout *et al.*	17	DHE	48	100–200 (F)	8	9
(1987)	2	(2)		5.5–10 (WB)	1	1
Shumaker and	13	DHE	72	25 (WB)	12	1
Hetzel		(2)				
(1987)						
Tsuchiya *et al.*	8	HpD	48–72	120–360 (F)	8	0
(1983)		(2.5)				
Williams and Stamp	10	DHE	72	10–15 (WB)	4	6
(1988)		(2)				
Total	133 (100%)				86 (65%)	47 (35%)

*Denotes focal or whole bladder treatment.

who have failed all other modalities of treatment short of cystectomy. However, the complication rate was alarming, as will now be discussed.

Functional impairment. Most authors state that their patients develop irritative symptoms following PDT but these are often described as "transient" and of no particular worry. There has almost certainly been a gross under-reporting of side effects by the enthusiasts of PDT, often no doubt because they have not been specifically sought, and many patients would have had some pre-existing symptoms anyway. All authors who did look specifically at bladder function after PDT found serious impairment in a high percentage of patients. Nseyo *et al.* (1985) reported severe side effects in all of an initial series of 6 patients undergoing whole bladder PDT. Two patients had such a marked reduction in bladder capacity with incontinence that they required a cystectomy for these PDT-induced symptoms alone, although their bladders were found to be clear of tumour. Following animal experiments they recommended treatment parameters which produced better results in a subsequent 15 patients, with on average only a 20% reduction in bladder capacity which recovered within 3 months (Nseyo *et al.*, 1987). However, the Sheffield group (Williams and Stamp, 1988; Stamp *et al.*, 1990), using these supposedly improved parameters, still found severe dysuria and reduced bladder capacity in all of their patients. Some were hospitalised for several weeks and 1 patient was left with a bladder capacity of only 50 ml after voiding a sloughed "cast" of bladder mucosa. Jocham *et al.* (1989) reported a reduction in bladder capacity in some of their patients in excess of 50% and 3 still had a 70% reduction 1 year after PDT. Some authors have suggested not giving PDT to patients who have a pre-treatment bladder capacity less than 150 ml (quite common) or who have received radiotherapy (rare for superficial tumours) as they seem to have the worst complications.

Upper tract complications. Both ureteric reflux and ureteric obstruction were seen in the initial series of Nseyo *et al.* (1985) but in general no problems with the upper tracts have been mentioned by other authors until a recent report by Harty *et al.* (1989) in which they studied the side effects after PDT in a series of 7 patients who were given whole bladder treatment. Five patients had a significant reduction in bladder capacity after PDT which persisted at 1 year in 4 of them. Deep bladder biopsies showed

marked fibrosis in the muscle layer. These 4 patients also developed persistent bilateral hydronephrosis and grade 4 vesicoureteric reflux. It is interesting that the 1 patient in this series who escaped these problems still had a complete response; he had received only two-thirds of the intended dose of DHE.

Cutaneous photosensitivity. Any drug given systemically will be distributed throughout the body and so it is inevitable that photosensitisers will be taken up to some extent in the skin. Unfortunately, HpD and DHE absorb light well throughout the range of wavelengths in sunlight, which means that patients may develop cutaneous photosensitivity to ambient light. This may last several weeks and require the patient to remain in subdued lighting and to wear protective clothing. This is a major factor against patient tolerance of PDT with these sensitisers and despite warnings, most series report a small number of patients who develop significant sunburn. Other ways of administering photosensitisers have been tried with the aim of avoiding skin sensitivity. Direct intra-tumour injection is suitable only for solitary bladder tumours (Amano *et al.*, 1988) and would clearly not be feasible for Cis. Intravesical administration of HpD has not been successful (Benson, 1988) and our studies on the intravesical uptake of phthalocyanine show that although significant amounts are absorbed by normal bladder, the uptake is patchy and unpredictable (Pope *et al.*, 1990).

The most likely solution lies in newer photosensitisers which, although still given intravenously, are either cleared more rapidly from the skin or, like the phthalocyanines, have a localised absorption peak at a longer wavelength than HpD, away from the main concentration of solar emission, which produces much less of a skin reaction (Tralau *et al.*, 1989). Using a phthalocyanine sensitiser at low dose levels, the problem of cutaneous sensitivity to sunlight is likely to be virtually eliminated. Another approach may be to use singlet oxygen quenchers which can be given after PDT to reduce skin sensitivity in animals, though their clinical efficacy is not yet proven (Dillon *et al.*, 1988).

With so many serious problems it is perhaps surprising that anyone is still trying to use PDT in the bladder. Several of the clinical pioneers did decide to stop clinical work and go back to the laboratory to try and solve the problems (Nseyo *et al.*, 1985, 1988; Reed *et al.*, 1989). However, even now, a survey of the literature in this field shows that there is a remarkable absence of basic studies on exactly what PDT does to normal and neoplastic tissue in the bladder. There are very little background data on which to base the light and sensitiser doses used clinically, and the values that have been used must be regarded as little more than inspired guesses. This is illustrated by the wide range of values used for the most important treatment parameters given in published clinical series of PDT for superficial bladder cancer (Table):

Sensitiser dose:	HpD 2–5 mg/kg DHA 2 mg/kg (all authors)	
Time between sensitisation and light exposure: 3–72 h		
Light dose:	whole bladder	5–70 J/cm^2
	focal treatment	100–360 J/cm^2

As has happened on many occasions in the history of medicine, the clinicians have tried to run before their scientific collagues had taught them to walk! Often this empirical approach has worked, but for PDT in the bladder, clearly it has not. Phase III clinical trials of PDT for bladder cancer are under way in several countries both for resistant Cis and as prophylaxis against recurrent papillary disease. There must, however, be some doubt about whether these trials are appropriate in our current state of knowledge, since the parameters being used are essentially the same as those that have produced irreversible damage to bladder muscle in many of the patients treated over the last few years.

Selectivity of PDT

The side effects of PDT on the bladder outlined above appear to be the result of necrosis followed by fibrosis in the muscle layer of the bladder, *i.e.* the result of damage to normal tissue rather than to tumour. Much of the attention focused on PDT stems from the possibility of selective destruction of malignant tumours, but these results must make us ask how much selectivity there really is under the conditions being used at present. Also, bearing in mind the pathology we are trying to treat, should we be looking for selectivity between the superficial and deep layers of the normal bladder wall rather than between normal and neoplastic areas? Much of the experimental work on PDT has stressed the importance of selective uptake of photosensitisers using tumours transplanted subcutaneously in mice and rats compared with the adjacent skin and muscle. However, what is important clinically is the difference in uptake or retention between a

tumour and the *adjacent normal tissue in which that tumour arose* (or into which the tumour has spread); it is suprising how little data are available in the world literature on this crucial point. There have been many reports (both clinical and experimental) on selective fluorescence of malignant tumours after porphyrin sensitisation, but this is normally excited by ultraviolet light, which penetrates less than 0.1 mm into tissue, so that one is only looking at surface differences between tumour and normal areas. It is difficult to correlate these measurements with concentrations of photosensitiser in the bulk of the tumour, though these techniques may have a useful role in diagnosing areas of endoscopically occult malignancy (Lin *et al.*, 1984). Work on animal tumours of the colon and pancreas has shown that the best selectivity of uptake achieved with either HpD or aluminium sulphonated phthalocyanine (AlSPc) is only in the order of 2-3:1 between tumour and adjacent normal tissue. The major exception to this is for intracranial gliomas, where the ratio is considerably greater at 28:1, most likely due to the breakdown of the blood brain barrier in tumour areas (Tralau *et al.*, 1987). Little such data are available for bladder tumours.

Any form of local treatment has some degree of selectivity. Therapy with ionising radiation or with a thermal laser such as the neodymium: YAG will produce the greatest effect in the area that receives the highest dose. If the light used for PDT is directed solely at tumour tissue, then naturally necrosis is confined to the tumour area. Much higher degrees of selectivity are possible by controlling where the light is directed than can be achieved solely by differences in the uptake of the sensitiser between normal and neoplastic areas. For bladder Cis, however, we are dealing with a situation when one cannot localise all neoplastic areas precisely and both normal and tumour areas will be exposed to similar light doses. The understanding of the nature and healing of PDT damage to normal tissue is therefore fundamental to the establishment of those treatment parameters which will eradicate tumour but not cause irreversible damage to normal tissue.

Experimental Studies on Normal Bladder

The first objective in planning PDT experiments on the bladder must be to decide what biological effect is desired and then to manipulate all of the parameters involved to see if this effect is achievable. It is surprising that so little has been done along these lines.

The clinical reports described above have shown that the main problem is scarring in the muscle causing irreversible changes to bladder function. In whole bladder treatments, no-one has shown necrosis limited to the tumour areas, and instead large areas of mucosa have sloughed which must be presumed to include both normal and neoplastic regions. These have healed with regeneration of normal mucosa which for patients with Cis means that their tumour has been eradicated. Thus it seems less important to attempt any form of selectivity between normal and abnormal mucosa, but highly appropriate to try to limit the damage to the mucosa and submucosa and leave the muscle intact. Experiments designed to answer this question can be carried out on normal animals, which is much simpler than finding an adequate animal model of *in situ* bladder cancer.

Two sets of parameters can be manipulated: those related to the photosensitising drug (*e.g.* dose, route of administration and time between sensitisation and light exposure) and those related to the light (wavelength, power, exposure time and geometry of light distribution).

The first consideration for the sensitiser is how it is distributed. For localisation within different layers of the bladder wall the best technique is fluorescence microscopy. In experiments using AlSPc as the sensitiser, immediately after intravenous administration, similar concentrations are found in all layers. However, after 24 to 48 h there is 3 to 4 times as much photosensitiser in normal mucosa and submucosa as in the underlying muscle; this is due to more rapid clearance from the muscle layers and seems the best time interval to use (Pope *et al.*, 1990). Experiments on other tissues (particularly the colon) have established that there is a threshold level for the tissue concentration of photosensitiser at the time of light exposure below which insufficient singlet oxygen is generated to cause tissue damage, even in the presence of excess light (Barr *et al.*, 1990). This is because the sensitiser is destroyed by photodegradation (photobleaching).

Putting both these results together, it would be expected that if the tissue concentration of photosensitiser was below the threshold in muscle but above it in the mucosa and submucosa, then there might be a mechanism for limiting PDT effects to the superficial layers. This approach has the added attraction that as the sensitiser is photobleached out of the muscle layer, no PDT damage should be produced however high the light dose. Experiments confirm that with a low dose of AlSPc (0.5 mg/kg in the rat, 24 h prior to light exposure), damage can

be limited to the superficial layers in a way that is independent of the light dose, the only requirements being that sufficient light is delivered to every point on the bladder surface and that the fluence rate is not high enough to cause thermal effects. Urodynamic studies on rat bladders at a range of times from a few days to 3 months after PDT indicate that if necrosis can be restricted to the mucosa and submucosa, then the subsequent reduction in compliance and capacity during the healing period is less, lasts a shorter time and is more completely reversible than when the muscle layers are also damaged (Pope and Bown, 1991). It is not possible to avoid initial side effects completely because there is a marked inflammatory response where the mucosa sloughs, but if these results can be extrapolated to man they should reduce the complications to an acceptable level and there should be little permanent reduction in compliance or capacity.

Few other workers have studied the effect of PDT on bladder function in an experimental model. Nseyo *et al.* (1988) looked at the effect of varying light dose and filling pressure during PDT on the canine bladder, which they had previously found to be relatively resistant to PDT. Although they did not see any permanent reduction of bladder capacity in surviving animals, higher filling pressures produced a more delayed recovery and were associated with greater muscle damage (thinning of the bladder wall during overdistension may well extend the expected depth of damage). They did not alter the sensitiser dose (well above threshold), so not surprisingly higher light doses produced more long-term damage than lower ones, though the small numbers of animals studied precluded any assessment of the acute histological effects, particularly as to whether a clinically appropriate degree of photodynamic damage had been achieved.

Light Delivery and Dosimetry

At the photosensitiser concentrations commonly used clinically, the light dose seems critical. However, it is not easy to calculate accurately the dose absorbed by the mucosa. The problem of achieving an even light distribution has been approached in several ways. Some workers have filled the bladder with a diffusing medium such as dilute intralipid and used a special positioning catheter to centre the laser fibre (Jocham *et al.*, 1989). Others have developed ingenious mechanical rotating devices for stepwise illumination of the entire bladder mucosa (Hisazumi *et al.*, 1984). Most authors have used some type of bulb tip diffusing fibre (Benson, 1986) centred as accurately as possible within the bladder and accepted that the light distribution will not be entirely even, particularly around the bladder neck. Multiple isotropic detectors placed in close proximity to the bladder wall can be used to monitor the incident light and, since there is a high internal reflectance from the bladder wall, it may be important to measure this light rather than just the fluence of the primary beam emitted from the fibre tip (Marynissen *et al.*, 1989).

All of these methods require considerable enthusiasm and patience to use, so that simplification of light delivery would make PDT easier to perform. One way of doing this, as mentioned already, is by using the photobleaching which occurs at low sensitiser concentrations. Under these conditions it may not be necessary to deliver a precise light dose to every part of the bladder, but merely a minimum effective dose to all points, with no limit to the maximum at any point. Therefore some of the difficult problems associated with the accurate calculation and even distribution of light over the entire bladder mucosa would become less important, although the high total light doses needed may require long treatment times with currently available laser systems.

An alternative approach to the problem of avoiding unwanted PDT damage in the muscle layers of the bladder is to activate the photosensitiser with a wavelength of light less penetrating than the red light (630 nm) used in all clinical studies with HpD and DHE. Green light would be expected to penetrate the bladder wall only 1 to 2 mm and HpD has a strong absorption peak in the green spectrum (at 507 nm). Haemoglobin also absorbs strongly here, however, so that any surface bleeding may shield underlying tumour and the reduced internal reflection of green light within the bladder will make dosimetry more difficult. There are no published clinical data as yet using green light to address these points. Activation with green light is not possible for the phthalocyanines and most of the newer photosensitisers under study, which absorb only in the red part of the visible spectrum. Green light would also not be suitable for any other clinical application of PDT apart from bladder Cis.

Photodynamic therapy has been studied by many research groups world-wide with numerous claims made for its efficacy over a period of more than 10 years. It would seem to hold particular promise for treating various types of superficial bladder cancer, especially resistant Cis. Why then is it not yet an

established treatment? The main reason is that clinical PDT, as with many new medical treatments, has evolved on a largely empirical basis whilst fundamental questions regarding its mechanism of action and optimum treatment parameters remain unanswered. It is not surprising, therefore, that few publications give convincing evidence of clinical efficacy for the management of Cis, or indeed for most other conditions for which it has been tried, and some serious complications have been reported. The development of an irritable contracted bladder, or upper tract damage secondary to ureteric obstruction or reflux, are tragic sequelae for a patient in whom PDT has successfully eradicated resistant and potentially lethal carcinoma. One does wonder if more experimental studies *prior* to the clinical application of PDT in the bladder could have reduced the incidence of these complications.

We believe that PDT has great potential in urological oncology, although the clinical results achieved so far have not been satisfactory. PDT is a topic that requires expertise in a range of both clinical and basic science fields and few people have a wide enough spectrum of knowledge to realise which are the most critical gaps in our knowledge and hence where the research effort should be directed. There is general agreement that light dosimetry is the biggest problem in treating bladder cancer but most workers see uniform illumination of the entire bladder mucosa as the challenge. We suggest that the only requirement for the *light* is that the amount at every point should be above a certain threshold. The real challenge comes in getting the dose of *sensitiser* right, so that PDT effects are limited to the superficial layers of the bladder. This optimum is likely to be much lower than the values currently used clinically. Of course, one must ask what depth of necrosis is required, as when using low sensitiser doses this will be limited but quite adequate to treat Cis.

New sensitisers and more precise ways of delivering them to tumour tissue (*e.g.* incorporated into liposomes or bound to monoclonal antibodies) may be developed but this must be balanced with work designed to establish how the biological principles studied in animals can be applied to treating real cancers in real patients. The clinical use of PDT requires a new way of thinking about treating human disease and it will take much time, effort and high quality data to establish its efficacy. Only careful and logical scientific experiments and rational clinical trials will tell us if photodynamic therapy is really here to stay.

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A User's Guide to Flexible Cystoscopes

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Summary—This survey, carried out on behalf of the BAUS Instruments Committee, aims to assist the urologist in choosing a flexible cystoscope. The 5 most commonly available flexible cystoscopes (from ACMI, Olympus, Pentax, Storz and Wolf) were assessed in a clinical setting for handling, image quality and user satisfaction. Several technical characteristics, including deflection range, irrigant flow rate and optics, were tested in the laboratory. Whilst all instruments were satisfactory, the favourite of our testing panel was the Olympus CYF-2, which also came out best in the technical assessment and has the most comprehensive service support. The cheaper Storz instrument also performed well.

Flexible cystoscopes are becoming increasingly popular for out-patient diagnostic and minor therapeutic manoeuvres (*e.g.* biopsy, stent removal) needing only topical urethral anaesthesia. Although fibreoptic endoscopes have been used in gastroenterology for over 25 years and the first reported use of a flexible instrument to view the bladder came from Japan in 1973 (Tsuchida and Sugawara), it was not until 1982 that any real experience with these instruments was reported by Burchardt.

Not surprisingly, the early flexible cystoscopes were derived from gastroenterological endoscopes, usually choledochoscopes, and were less manoeuvrable and of larger calibre than the present purpose-designed instruments, the first of which was introduced by Olympus in 1986. Flexible cystoscopy is now well established and has been shown to be at least as accurate in diagnosis (Clayman *et al.*, 1984), more efficient in practice (Fowler *et al.*, 1984) and more acceptable to patients than conventional rigid endoscopy (Flannigan *et al.*, 1988). The applications of this technique have been reviewed by Kennedy and Preminger (1988).

Many urologists will consider purchasing a flexible cystoscope and an increasing number of endoscope manufacturers are entering the field. This report, whilst no substitute for personal trial, aims to offer some guidance in selection.

Materials and Methods

The 5 most commonly available flexible cystoscopes in the UK were chosen for this assessment. They were the ACMI AFC-1 (distributed by Cory Bros), the Olympus CYF-2 (Keymed), the Pentax FCY 15P (Pentax, UK), the Storz 11272 (Rimmer Bros) and the Wolf 7305 (Richard Wolf, UK). The more expensive Pentax FCY 15H differs from the FCY 15P in having a connecting lead for automatic flash photography and some minor differences in control layout. The new Storz flexible cystoscope (11275), which is claimed to be improved in several respects, was not available at the time of testing. The instruments tested were demonstration models provided by the distributors and the assessment was in 2 parts.

User test

Each instrument except for Pentax was assessed by the same 5 urologists, with varying prior experience of flexible cystoscopy, on routine check cystoscopy lists. This was a subjective opinion of handling (ease of insertion, controls, manoeuvrability, *etc*) and of image quality. Instruments were then graded by each urologist for overall satisfaction in use. The Pentax cystoscope was tested subsequently in

Based on a Poster Demonstration at the 46th Annual Meeting of the British Association of Urological Surgeons in Scarborough, July 1990

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Urological Research

The photodynamic effect of a pulsed dye laser on human bladder carcinoma cells in vitro

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Summary. The photodynamic effect of a pulsed flashlamp pumped dye laser on cultured human bladder carcinoma cells was studied. MGH-U1 cells were incubated for 1 h in dihaematoporphyrin ether (DHE) and then exposed to green laser light (504 nm, 20 Hz) for varying laser power densities (50–100 mW/cm^2 and exposure times (2–15 s), representing incident pulse energy fluences of 2.5–5 mJ/cm^2 and energy densities of 0.1–1.5 J/cm^2. The cell survival was measured by clonogenic assay and controls exposed to either laser light alone or DHE in the dark showed no cytotoxicity. Sensitised cells were killed by energy densities of less than 1 J/cm^2 ($LD_{90} = 0.54$ J/cm^2). This demonstrates the probable effectiveness of a pulsed dye laser for photodynamic therapy provided that pulse fluence are below the saturation threshold of the photosensitiser (10 mJ/cm^2).

Key words: Photodynamic Therapy – Bladder cancer – Pulsed dye laser

Photodynamic Therapy (PDT) is an experimental technique which has the potential to treat malignant disease in many parts of the body, particularly superficial multifocal tumours. A photosensitising drug is administered which is taken up throughout the body. There appears to be some selective retention in neoplastic tissue over the next 24–72 h and then the tumour area is illuminated, usually by laser, with light of a specific wavelength corresponding to one of the absorption peaks of the photosensitiser. This leads to activation of the photosensitiser with production of a highly reactive form of tissue oxygen called singlet oxygen. Cell death results from oxidation of essential cellular components both in the tumour cells themselves and in vascular endothelial cells leading to disruption of its blood supply. One major application of PDT may be in the treatment of superficial bladder carcinoma, especially resistant carcinoma in situ (Tis), in which encouraging results have been reported by several authors [4, 5, 15, 16, 18]. PDT however can have serious side effects apart from the inevitable cutaneous photosensitivity which may last several weeks. There is usually severe bladder irritability with a significant incidence of a permanent reduction in bladder capacity, and even ureteric reflux leading to upper tract dilation [13]. Sometimes symptoms may be severe enough to require cystectomy [14]. This damage is most likely due to activation of photosensitiser in the muscle layers of the bladder which leads to fibrosis and subsequent contraction with reduction in compliance and functional capacity.

Most research and all published clinical work on bladder PDT have used porphyrin photosensitisers, either haematoporphyrin derivative (HpD) or the purified preparation dihaematoporphyrin ether (DHE). These are activated by red light (630 nm) from an argon ion laser pumping a rhodamine B dye laser.

Red light is generally used for PDT as it penetrates tissue well (up to 1 cm), but this is unnecessary for the treatment of Tis and probably causes the troublesome side effects mentioned above. One possible way of avoiding this might be by using green light which only penetrates 1–2 mm into tissue [22]. HpD also has a much stronger absorption peak for green light (around 507 nm) than for red light, and therefore a greater photodynamic effect would be expected. Recently the metal vapour lasers, particularly copper, have been developed for PDT and although they offer several advantages over the argon laser they also have no other urological application.

The flashlamp pumped dye laser is routinely used for fragmenting ureteric calculi [8]. This laser produces low frequency (less than 30 Hz) pulses of green light (504 nm) of short pulse length (1 μs) and very high peak powers in excess of 5 MW. A further use for this laser would be desirable and enable PDT to be undertaken without the expense of another laser.

Materials and methods

A human continuous bladder carcinoma cell line (MGH-U1) was maintained in monolayer culture in RPMI 1640 medium (Gibco Europe Ltd., Paisley, Scotland), supplemented with 5% heat-

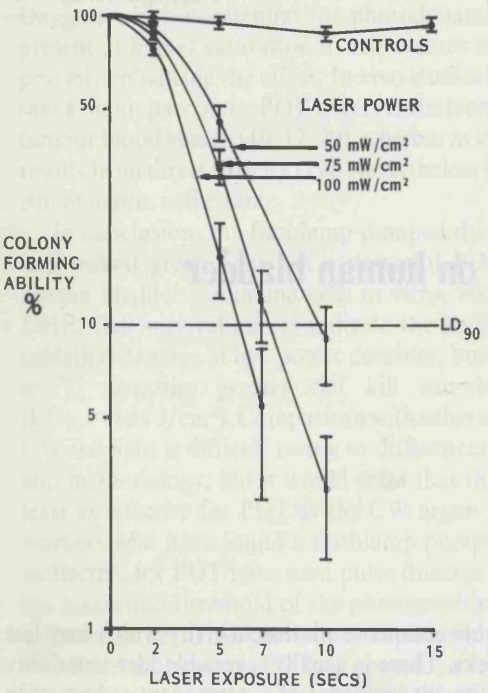

Fig. 1. Survival curves for MGH-U1 cells sensitised with DHE (20 μg/ml) and exposed to pulsed green light (514 nm, 20 Hz) from a flashlamp dye laser

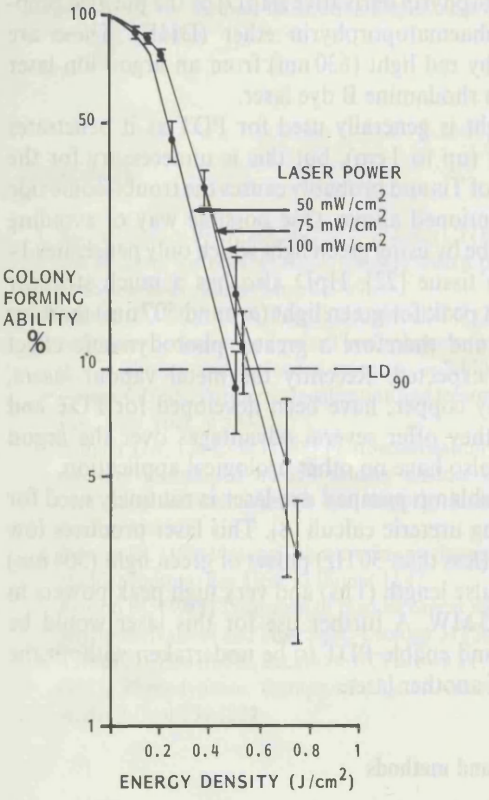

Fig. 2. Cell survival related to energy fluence at varying power densities

inactivated fetal bovine serum (Sera-Lab Ltd., Crawley Down, England) and 2 mM L-glutamine (Flow Laboratories Inc., Irvine, Scotland), at 36.5°C in a humidified atmosphere of 5% CO_2 in air. The cells were routinely subcultured using an aqueous solution of 0.05% trypsin (1:250; Difco Laboratories, London) and 0.016% versene (EDTA disodium salt; BDH Chemicals, Poole, England). The cells were used over a restricted range of 10 passages to minimise any changes occurring as a result of long term culture. No antibiotics were used.

For photodynamic treatment exponentially growing cells were enzymatically detached, and a single-cell suspension was produced by repeated syringing through a 19 gauge needle. Viable cell numbers were estimated by trypan blue dye exclusion with the use of a haemocytometer. Approximately 150 cells were plated into 3.5 cm petri dishes containing 3 ml prewarmed and gassed medium, yielding about 100 viable colonies per dish. The cells were incubated for 48 h to allow the cells to attach and attain exponential growth.

The medium was then replaced with either fresh medium alone for the controls or with medium containing DHE (Photofrin II, Photomedica Inc., Ravitan, N.Y.) sterilised by passing through a millipore filter. All work was performed under very low level indirect lighting. Dishes were additionally protected from light, except during actual exposure, by covering with aluminium foil. After a 1 h incubation in DHE solution the dishes were washed with fresh medium to remove any unbound DHE and then placed in 1 ml phosphate-buffered saline (PBS) to prevent the cells from drying out during subsequent light exposure.

Light treatment was given as described below, following which the cells were washed again in fresh medium and incubated for a further 7 days. Colonies were fixed in methanol and stained with 10% Giemsa (Giemsa's stain, BDH chemicals) prior to counting. Colonies consisting of a minimum of 50 cells were scored with the use of a binocular dissecting microscope. The mean number of colonies of a least 3 dishes at each point was expressed as a percentage of the controls to calculate cell survival (colony forming ability). All experiments were repeated at least 3 times to plot cell survival curves.

A flashlamp pumped dye laser (Candela MDL-1P, Candela Corp., Natick, M.A., USA) was used, emitting green light (504 nm) transmitted along a 200 micron quartz fibre. This was positioned 12 cm above the culture dish, with the lid removed, thereby illuminating the whole dish evenly. Incident light energy was measured with a power meter at the level of the dish and the laser output adjusted to give an average power density of 50, 75 or 100 mW/cm^2 at 20 Hz. The pulse length was 1 μs representing a pulse energy density of 2.5–5 mJ/cm^2 and peak power density of 2.5–5 kW/cm^2. Measurements were repeated after each set of exposures to confirm that the power output was stable. 5 replicates were exposed at each parameter tested.

Results

Initial experiments were designed to assess whether DHE caused any direct toxicity, and to determine a suitable concentration for use in subsequent laser experiments. MGH-U1 cells were incubated for 1 h in medium containing DHE at concentrations of 0–50 μg/ml. There was no direct toxicity throughout this range of DHE concentrations, though Chan et al. [7] found a toxicity of 20% at 25 μg/ml, and 60% at 100 μg/ml using HpD on fibroblast cultures. In clinical work DHE is generally administered at half the dose of HpD. When these experiments were repeated, and the cells exposed to strong white light for 30 mins, there was marked toxicity. Colony forming ability was reduced to 55% at a DHE concentration of 5 μg/ml, and there were no surviving colonies seen at concentrations above 15 μg/ml. A phototoxic concentration of 20 μg/ml DHE was therefore chosen for subsequent laser

exposures. Other workers have used varying concentrations of photosensitiser in their experiments; eg. Camps et al. [6] used 10 μg/ml HpD incubated for 2 h, Cowled et al. [9] and Gomer et al. [11] used 25 μg/ml HpD for 1 h, whilst Bellnier and Lin [3] used 50 μg/ml DHE for 12 h.

Figure 1 shows cell survival curves for varying laser exposures at power densities of 50, 75 and 100 mW/cm^2. 90% cell kill (LD_{90}) was achieved with exposures of 6 s at 100 mW/cm^2, 7.5 s at 75 mW/cm^2 or 10 s at 50 mW/cm^2. These LD_{90} values, expressed as the total light dose received by the cells, represent energy densities of 0.59, 0.55 and 0.48 J/cm^2 respectively. The energy density curve for each of the 3 laser powers studied is similar (Fig. 2), and the initial shoulder to the survival curves indicates that the cells are better able to withstand very short exposures which do not overwhelm cellular repair mechanisms. The cell kill achieved for very short exposures even at high powers though, is less efficient than that seen with longer exposures.

Discussion

This study demonstrates that the pulsed output at 504 nm from a flashlamp pumped dye laser kills sensitised human bladder carcinoma cells in vitro. Earlier studies showed that CW red light (630 nm) from an argon ion pumped dye laser kills tumour cells sensitised by HpD or DHE both in vitro and in vivo. The continuous wave laser therefore is already widely used for PDT.

Pulsed lasers differ from CW lasers in that they emit light of very short pulses with extremely high peak power. There are two main types of pulsed lasers; metal vapour lasers (usually copper) emitting nanosecond pulses at high frequency (above 10 kHz), and flashlamp pumped dye lasers which emit longer pulses (1–2 μs) at low frequency (5–20 Hz) with peak powers in the kilowatt range. Several workers have shown metal vapour lasers to have an equivalent effect in vitro to CW lasers [9]. Evidence for the efficacy of flashlamp pumped dye lasers in vitro was not available prior to this study and their action in vivo is less certain. Bellnier et al. [2] concluded that the toxicity they observed in transplantable transitional cell carcinoma in mice was the result of thermal damage alone. Although dish temperatures were not measured in our experiments the controls exclude a thermal effect. Barr et al. [1] did not demonstrate a PDT effect on rat colon with a similar laser to ours though they used a slower repetition rate (5 Hz) and a phthalocyanine photosensitiser.

It has been suggested that the very high pulse energy of the flashlamp pumped dye laser excites almost all the sensitiser molecules to the photoactive triplet state before completion of the pulse, with the result that most of the photons in the pulse are "wasted" as the sensitiser has become saturated due to depletion of the absorbing ground state molecules [1]. The cytotoxic singlet oxygen is generated from the interaction of oxygen with the triplet state of the sensitiser which has a lifetime of approximately 8 μs [21]; somewhat longer than the pulse length used here. In theory therefore, only 1 excitation cycle can be initiated each pulse, ie. in these experiments only 20/s. This limits the overall yield of singlet oxygen, below the threshold required for a PDT effect. These restrictions do not apply in our experiments unlike those of Bellnier et al. [2] and Barr et al. [1] since it can be shown that the pulse fluences employed here (2.5–5 mJ/cm^2) are below the saturation threshold (10 mJ/cm^2) for DHE at 504 nm. At this excitation wavelength the absorption cross section of DHE is 4×10^{-17} cm^2/molecule and from the saturation condition (absorption cross section $\times n_s = 1$), the saturation photon fluence (n_s) $= 2.5\times10^{16}$ photons/cm^2 or equivalently 10 mJ/cm^2. This figure is an underestimate since the analysis assumes a triplet quantum yield of unity. Therefore the pulse energies used in these experiments could be at least doubled before the photosensitiser became saturated. Barr et al. [1] used pulse energies of 25 mJ/cm^2 and a phthalocyanine photosensitiser (AlSPc) which is saturated at a pulse energy fluence of only 1 mJ/cm^2. Bellnier et al. [2] used very high pulse energies of 100–250 mJ/cm^2 per pulse from a similar laser to ours and a slow pulse repetition rate of 2–4 Hz causing thermal tumour damage and clearly also saturating the photosensitiser (HpD).

Other workers have demonstrated PDT responses on various cell lines with HpD or DHE. Camps et al. [6] looking at the PDT effect of CW red light (630 nm) on prostate cancer cells sensitised with HpD, found an energy density in excess of 20 J/cm^2 at 100 mW/cm^2 reduced cell viability to 10% (LD_{90}). Cowled et al. [9] using a higher power density, needed a similar energy density to achieve 50% cell kill with Raji cells, though a different assay method was used. Gomer et al. [11, 12] studying in vitro PDT with Chinese hamster ovary cells found, using red light, an LD_{90} of 0.25 J/cm^2 but when using relatively low level fluorescent illumination (0.35 mW/cm^2), lower energy densities were needed ($LD_{90} = 0.05$ J/cm^2). Bellnier and Lin [3], using a similar cell line to ours and CW red light found an LD_{90} of 0.15 J/cm^2. Even these 5 studies show a 100 fold difference in energy densities for the same PDT effect, a variation attributable in part to different assay methods and light sources. Our low frequency pulsed green light produces a PDT effect comparable to CW red light ($LD_{90} = 0.54$ J/cm^2.

Camps et al. [6] concluded that it was the length of light exposure needed to achieve cell kill which was important rather than the total light dose (energy density), so that cellular repair mechanisms for sublethal damage would be overcome. In contrast, Gomer et al. [11] found no significant dose-rate variation and felt that the cell killing was dependent on total light dose. The shouldered cell survival curves from our experiments indicate the ability to repair sublethal damage though, like them, we found that cell kill depended on the total light dose rather than the power density. Very short exposures which fall on the shoulder of the survival curve (e.g.2 s), are unable to produce high cell kill even at very high power densities, whereas longer exposures of similar total energy are more effective (e.g. from Fig. 1 there is a 78% survival at 100 mW/cm^2 for 2 s (0.2 J/cm^2), compared with only a 60% survival at 50 mW/cm^2 for 4 s.

Care is needed when interpreting the results of in vitro PDT studies as they may not relate directly to clinical use.

Oxygen, which is essential for photodynamic action, is present at higher saturation in cell culture than in vivo, possibly enhancing the effect. In vivo studies have shown that a major part of the PDT effect results from damage to tumour blood vessels [10, 17, 20], whereas in vitro damage results from direct toxicity [19]. Nevertheless the mechanism of action is the same.

In conclusion, the flashlamp pumped dye laser emitting pulsed green light has a powerful PDT effect on human bladder carcinoma cells in vitro, sensitised with DHE. Cell survival curves indicate the ability to repair sublethal damage at low power densities, but with higher energy densities greater cell kill can be achieved ($LD_{90} = 0.54\,J/cm^2$). Comparison with other studies using CW red light is difficult owing to differences in cell lines and methodology, but it would seem that this laser is at least as effective for PDT as the CW argon laser. Other workers who have found a flashlamp pumped dye laser ineffective for PDT have used pulse fluences in excess of the saturation threshold of the photosensitiser which we estimate to be $10\,mJ/cm^2$ in our experiments. This is of interest to urologists as PDT may provide a second use for this type of laser. Further studies will be required to confirm these results in vivo but it is likely that the use of pulsed green light and porphyrin sensitisation will enable efficient PDT of carcinoma in situ of the bladder without the troublesome side effects which have been encountered with the use of CW red light.

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THE MORPHOLOGICAL AND FUNCTIONAL CHANGES IN RAT BLADDER FOLLOWING PHOTODYNAMIC THERAPY WITH PHTHALOCYANINE PHOTOSENSITIZATION

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ABSTRACT

We have studied photodynamic therapy (PDT) in the rat bladder with a new photosensitizer, aluminium sulfonated phthalocyanine (AlSPc) given intravenously and intravesically. The microscopic distribution of photosensitizer fluorescence in the bladder wall was studied by laser fluorescence microscopy. Prior to PDT the bladder capacity and compliance were assessed by filling cystometry. Intravesical red light (675 nm.) from a copper vapour pumped dye laser was used to activate the photosensitizer using light doses of 20 to 200 J/cm.^{2} Urodynamic and histologic changes were studied at intervals for up to three months.

The fluorescence studies showed that AlSPc was eliminated from the deeper muscle layers more quickly than from the superficial layers of the bladder wall so that by 24 hours there was four times as much fluorescence from the mucosa and lamina propria compared to the deeper muscle. Control bladders illuminated with laser light alone showed no effects at these light doses. Animals treated 24 hours after sensitization showed a reduction in bladder capacity of up to 78% (20 J/cm.^{2} light and 1.5 mg./kg. AlSPc). An initial reduction in compliance recovered in two weeks after low doses (0.5 mg./kg.) of AlSPc but was still abnormal at three months after higher doses (1.5 mg./kg.); though there was no long term histologic abnormality seen.

Aluminium sulfonated phthalocyanine is a promising photosensitizer for bladder photodynamic therapy and using low doses of the drug it is possible to produce a superficial necrosis without muscle damage across a range of light doses. This heals by epithelial regeneration with no long term functional impairment. Direct absorption of this photosensitizer following intravesical administration seems unreliable.

KEY WORDS: Photodynamic therapy, bladder function, phthalocyanines

Photodynamic therapy (PDT) is an experimental technique which offers the potential for selectively destroying malignant tumors, especially small multifocal lesions. It involves the systemic administration of a photosensitizing drug which is retained in neoplastic tissue with a small degree of selectivity. This drug is then activated by light of a specific wavelength corresponding to one of its absorption peaks and produces a local cytotoxic effect mediated by singlet oxygen. The main component of PDT damage is produced by its effect on vascular endothelial cells leading to a reduced tumor blood flow.[1] The bladder seems ideally suited to PDT as it is readily accessible endoscopically and the entire mucosa can be treated simultaneously with high powers of monochromatic light from a dye laser. Resistant superficial bladder cancer is usually multifocal, and areas of occult dysplasia and carcinoma in situ (Cis) do not have to be precisely defined for photodynamic treatment to be effective. Since the potential of PDT in bladder carcinoma was first demonstrated by Kelly and Snell,[2] over 100 clinical cases have been reported which have shown promising control of resistant disease though at the cost of significant morbidity in most patients. All patients receiving whole bladder PDT experienced irritative symptoms which were often severe. Some authors have reported muscle fibrosis and permanently reduced bladder capacity, incontinence and upper tract deterioration,[3] with symptoms which may themselves necessitate cystectomy.[4,5] Prolonged skin photosensitivity may also be troublesome with porphyrin photosensitizers.

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Clinical PDT, as with many new medical treatments, has evolved on a largely empirical basis whilst there are still fundamental questions regarding its mechanism of action and optimum treatment parameters unanswered. These include the choice of photosensitizer and the optimum drug dose; the time delay between sensitization and treatment to allow for the most advantageous distribution between tumor and normal tissue, and the wavelength and energy of the activating light. It is clearly unrealistic to hope that photosensitizing drugs will only be retained by malignant or dysplastic cells so it becomes essential therefore when treating a whole organ such as the bladder to understand the action that PDT will have on the normal tissue so that dosimetry parameters may be developed which will produce the desired effect in areas of tumor but will minimise unwanted damage and the complications which have been seen in clinical practice so far.

All clinical studies have used hematoporphyrin derivative (HpD), or its more purified derivative dihematoporphyrin ether/ester (DHE), activated by 630 nm. red light. These compounds are far from ideal as photosensitizers being incompletely defined mixtures of porphyrins whose composition and stability between preparations varies and which are difficult to assay. There are several promising new groups of photosensitizing drugs being developed, especially the phthalocyanines which have similar biological properties to the porphyrins but are easier to handle chemically and assay in tissue. Aluminium sulfonated phthalocyanine (AlSPc) has a single strong absorption band at 675 nm., where tissue penetration is good as there is little unwanted absorption by natural biomolecules, with a high yield of singlet oxygen. Its low absorption at UV and

visible wavelengths is likely to minimise cutaneous photosensitivity which will be of major clinical benefit.[6] It appears nontoxic in animals and though not yet licensed for clinical use, is under detailed experimental study in several centres.[7,8,9]

This paper looks at the microscopic distribution of AlSPc in the normal rat bladder, and the morphological and functional changes seen after PDT for a range of sensitizer and light doses. In this model the aim of PDT is to produce a dependable necrosis of the superficial layers whilst leaving the muscle layers intact and not causing any irreversible functional impairment.

MATERIALS AND METHODS

Animals and photosensitizer. Adult female Wistar rats (source - Imperial Cancer Research Fund; weight approx 200 gm.) were used for these experiments. All procedures were done under general anaesthetic using intramuscular Hypnorm 0.5 ml./kg. (fentanyl and fluanisone; Jansen pharmaceuticals) and diazepam 0.5 mg./kg. Aluminium sulfonated phthalocyanine (AlSPc) was obtained from Ciba-Geigy and made up as a sterile solution of two mg./ml. in saline. This preparation is a consistent water-soluble mixture of molecules containing predominantly three sulfonate groups per molecule (range one to four) though it is possible to extract pure preparations of the specific sulfonated derivatives whose photoactivity and solubility vary considerably. It was administered by tail vein injection and diluted as required to maintain an injection volume of 0.25 to 0.75 ml.

Photosensitizer distribution. The fluorescence distribution of AlSPc in the normal rat bladder was studied using laser fluorescence microscopy coupled to a cooled CCD (charge coupled device) imaging system with computerised image processing. This analysis enables a quantitative assessment of photosensitizer fluorescence in the various layers of the bladder wall which is not possible with chemical extraction assays of whole bladder. This technique will be reported more fully elsewhere,[10] but briefly its advantages over conventional fluorescence microscopy or video imaging systems include much greater sensitivity and direct color digital image analysis over a linear signal range of 10.[4] There is minimal tissue autofluorescence nor unwanted sensitizer quenching as the high sensitivity allows low-power excitation (at 632.8 nm. from a helium-neon laser), and short integration times.

Fluorescence was studied at intervals from one to 72 hours after intravenous injection of varying doses of AlSPc (0.5 to 5 mg./kg.). Ten micron frozen sections were cut transversely from whole bladder specimens after any urine present, which might contain photosensitizer, had been gently washed out and the bladder distended to 0.3 ml. with OCT medium (Tissue-Tek, Miles Laboratories, Inc.) prior to placement in liquid nitrogen. The direct absorption of AlSPc mixture was assessed either 30 or 60 minutes after the intravesical administration of 0.3 ml. of the two mg./ml. solution diluted by a factor of five to 100 times, as these would be the most useful instillation periods for clinical intravesical therapy.

Photodynamic therapy. Rats were sensitized with AlSPc (0.5 to 5 mg./kg.) 24 hours prior to light exposure as our fluorescence studies indicated that this was the most appropriate time interval to achieve the optimum concentration ratio between the superficial (mucosa and lamina propria) and deep (muscle) layers of the bladder wall. The rat bladder was catheterized with a 18 gauge Teflon cannula. This diffused the light from a 200 μm. laser fibre which was positioned inside the cannula just short of its tip and centrally within the bladder. The resultant light distribution whilst not perfectly isotropic (highest straight ahead, though over a very small area, and lowest at the bladder neck) did provide acceptably even illumination throughout the rat bladder. A copper vapour laser (Oxford Lasers) was used, pumping a dye laser to emit red light at 675 nm. This laser produces 40 ns pulses of light at 12 kHz and was set to an average power output of 100 mW.

The bladder was filled to a volume of 0.3 ml. with saline and assumed to be spherical to calculate the treatment times required to give incident light doses of 20-200 J/cm.^{2} The bladder was exposed through a lower abdominal incision and during light exposure the adjacent bowel was shielded to prevent PDT damage from forward scattering, as the rat bladder is both thin enough to transmit a significant amount of the light, and is also largely intraperitoneal. The power output from the laser was monitored during each exposure by an in-line power meter and adjusted if necessary, though in general it remained stable. The output from the fibre was also checked after each exposure, and at intervals during the higher energy treatments, using an integrating sphere power meter.

Preparation of tissue specimens for histology. The morphological effects of PDT on the normal rat bladder were studied for all combinations of sensitizer and light dose described above. The rats were sacrificed at intervals from 24 hours to three months, and their bladders distended with 0.3 ml. of 10% formaldehyde prior to excision and routine histologic processing. The blocks were cut so that complete transverse sections were obtained from the central part of the bladder where illumination was most even. Standard hematoxylin and eosin (H and E) staining was used though some slides were stained with van Gieson and hematoxylin to assess collagen deposition during healing. Control animals were treated for similar times at 100 mW but without photosensitization.

Assessment of bladder function after PDT. A filling cystometrogram (CMG) was performed using modified urodynamic apparatus. This comprised a shortened saline-filled epidural catheter connected to a standard urodynamic pressure transducer. A three-way tap enabled this line to be used for both filling and pressure measurements. The transducer was connected to a digital meter which was calibrated against a saline manometer and maintained a linear recording of pressures up to 80 cm. H_2O.

The rat urethra was dilated up to 16 gauge using graduated cannulae passed over a guidewire, sufficient to permit the epidural line to be introduced into the bladder through the cannula. The bladder was allowed to empty and particular care was taken to exclude air bubbles in the line before connecting to the transducer. This was zeroed and then the bladder was slowly filled (0.2 ml./min.) from a one ml. syringe connected via the three-way tap. Artefacts were produced if the pressure was measured whilst filling and the most accurate method was to stop filling after each increment of 0.25 ml. (up to one ml.) had been instilled and allow the pressure to stabilize. These measurements were performed sequentially on the same animals before PDT and at four days, one week, two weeks, one month and three months afterwards. From three to five animals were used for each combination of variables studied. These pressures are total bladder pressure (P_{ves}) rather than the true subtracted detrusor pressure but were measured under general anesthesia. Preliminary studies had shown no difference between P_{ves} measured with the abdomen either intact or opened to neutralize any intra-abdominal pressure component.

Bladder compliance. Changes in bladder compliance after PDT were calculated as follows:

i) A filling CMG was done at the time intervals specified above.

ii) The P_{ves} at each volume increment measured (0.25, 0.5, 0.75 and one ml.), was expressed as a ratio of the pretreatment value for that animal. As these pressure ratios were reasonably constant over this volume range their average was taken to represent the change in compliance at that point in time and termed the Compliance Ratio. This gave a single value to compare compliance changes both with time, and between the treatment groups (0.5,

one and 1.5 mg./kg. AlSPc, 20 J/cm.^{2} light) and the control group which comprised animals treated with light but no AlSPc.

A compliance ratio of two therefore represented an average two fold increase in P_{ves} over the volume range tested and indicated a halving of bladder compliance. In those animals with very high pressures it was often not possible to fill more than about 0.5 ml. before leakage occurred around the catheter or there was vesico-ureteric reflux. In these instances the compliance ratio has been calculated from fewer volume points, though in at least three animals for each point.

Bladder capacity. This was measured separately from the compliance assessment, though at the same time intervals after PDT, by slowly filling the bladder with saline at a constant pressure head of 30 cm. H_2O. The capacity was taken as the amount emptied from the bladder when filling ceased rather than the amount instilled. This was to correct for any vesico-ureteric reflux which may have occurred and which was seen particularly in those animals which received the higher doses of PDT. Despite this there may still have been some overestimation of true bladder capacity in those more severely affected animals.

Statistical methods. Results given are the mean ± standard errors for each group of animals tested. Levels of significance quoted were calculated by the Student's *t* test (unpaired observations).

RESULTS

Photosensitizer distribution. At one hour after intravenous sensitization strong fluorescence was seen in all layers of the bladder wall particularly around blood vessels. By 24 hours a gradient of 3-4:1 in fluorescence intensity had developed between the superficial and deep layers of the bladder wall. This ratio reflects the more rapid clearance of photosensitizer from the muscle layer and remained similar, though absolute values reduced by some 10%, between 24 hours and 72 hours. As only the phthalocyanine monomers fluoresce, and dimers and other aggregates have a very low photoactivity, this technique only identifies the biologically active sensitizer.[11] We therefore chose 24 hours as the time interval between sensitization and bladder illumination for all our studies.

Morphological changes: macroscopic. The highest dose of AlSPc studied (five mg./kg.) resulted in a substantial mortality from septicaemia (in excess of 80%) after only 20 J/cm.^{2} illumination. At autopsy large areas of the bladder would be necrotic (fig. 1), with purulent urine, dilated ureters and multiple small renal abscesses. Lower doses of AlSPc were associated with less morbidity (characterized by lassitude, anorexia and incontinence) and no deaths occurred in animals given less than 1.5 mg./kg. AlSPc (20 J/cm.^{2} light). The bladder and immediately adjacent perivesical fat often appeared uniformly edematous after these lower doses when examined subsequently, though no damage occurred to adjacent organs.

Microscopic. The extent of the tissue damage produced depended on the treatment parameters chosen, ie. sensitizer concentration and light dose. Early acute inflammatory changes and edema were seen predominantly in the submucosa, with urothelial destruction by two to three days. The maximum effect was seen after four to six days and at the higher treatment doses, which resulted in a high mortality, there was florid acute inflammation throughout the bladder wall with areas of muscle necrosis. At intermediate doses (fig. 2), there was no surviving urothelium seen (sections were taken from the middle region of the bladder), and a largely transmural inflammation and coagulation in small blood vessels in the submucosa. In areas even where these inflammatory changes were transmural, complete repair had occurred by three weeks in surviving animals (fig. 3). A lower dose of AlSPc (in the order of one mg./kg.) with 20 J/cm.^{2} light only produced superficial damage, the muscle layer being unaffected (table 1). With 0.5 mg./kg. AlSPc a light dose in excess of 40 J/cm.^{2} was necessary to produce a consistent epithelial destruction (fig. 4), and below 0.5 mg./kg. no significant PDT effect was apparent in any layer of the bladder wall, even after high light doses. No change was seen in the controls.

The effect of increasing the light dose up to 200 J/cm.^{2} was studied on animals receiving the lower doses of AlSPc (table 1). Unsensitized controls showed no changes at these levels thereby excluding a thermal effect. As the sensitizer dose was reduced, the light dose needed to produce a given depth of tissue damage became less critical, and at 0.5 mg./kg. AlSPc the desired effect of urothelial loss, without damaging muscle and with complete healing by regeneration of normal tissue, could be achieved with light fluences between 40 and 200 J/cm.^{2}

We found that PDT following the intravesical administration of AlSPc produced patchy and unpredictable results often with areas of full thickness damage adjacent to those of minimal change (fig. 5). Fluorescence studies confirmed an irregular uptake of AlSPc which seemed largely independent of either sensitizer concentration or time in contact with the bladder

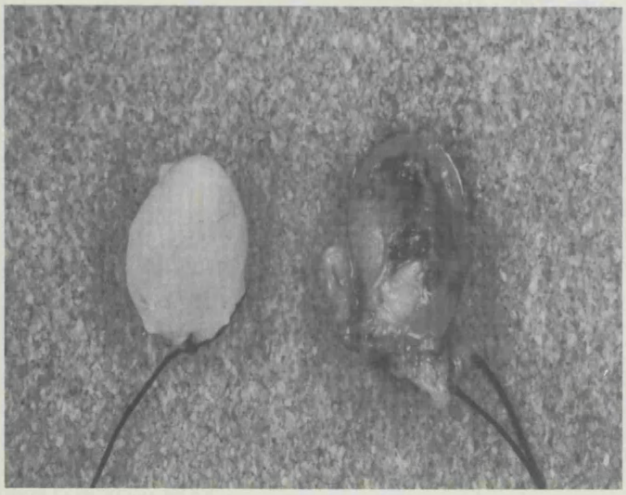

FIG. 1. Necrosis produced by high doses of PDT. Bladder on right is 3 days after PDT (5 mg./kg. AlSPc 24 hours prior to 20 J/cm.^{2} light @ 675 nm.) and shows multiple areas of necrosis. Control bladder is shown on left (20 J/cm.^{2} light only).

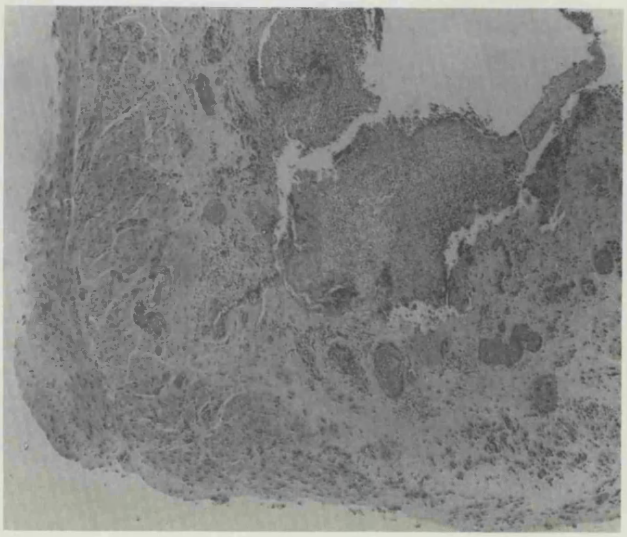

FIG. 2. Acute inflammation of full thickness of bladder wall. Note coagulation in submucosal blood vessels. (4 days after PDT with 1.5 mg./kg. AlSPc, 24 hours prior to 20 J/cm.^{2} light @ 675 nm.).

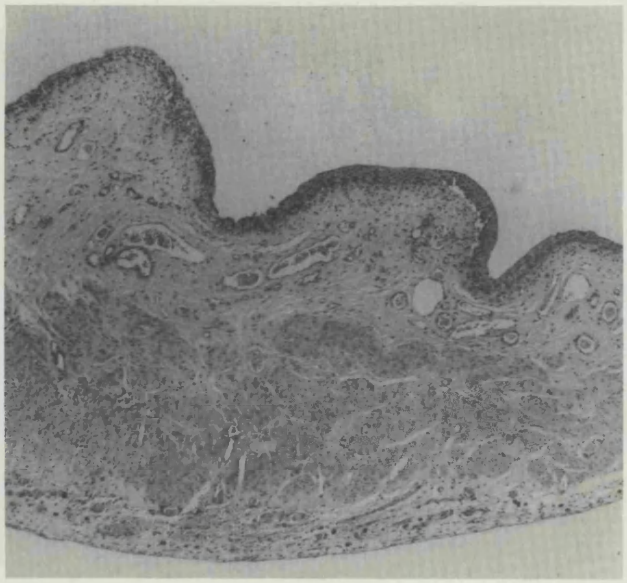

FIG. 3. Same treatment parameters as fig. 2 after 21 days to demonstrate healing with complete regeneration of bladder wall architecture.

TABLE 1. *Effect of AlSPc and light dose of PDT necrosis in the superficial and deep layers of the rat bladder wall*

AlSPc Dose (mg./kg.)	Depth of Necrosis	Light Dose (J/cm.^{2}) 20	40	80	200
0.5	Superficial	<25	50–75	100	100
	Into muscle	0	0	0	0
1	Superficial	50–75	100	100	Died
	Into muscle	0	25–50	100	
1.5	Superficial	100	Died	—	—
	Into muscle	25–50			
5	Superficial	Died	—	—	—
	Into muscle				

The figures in the 'light dose' columns represent the approximate area (%) of the bladder necrosed at the time of maximum damage.

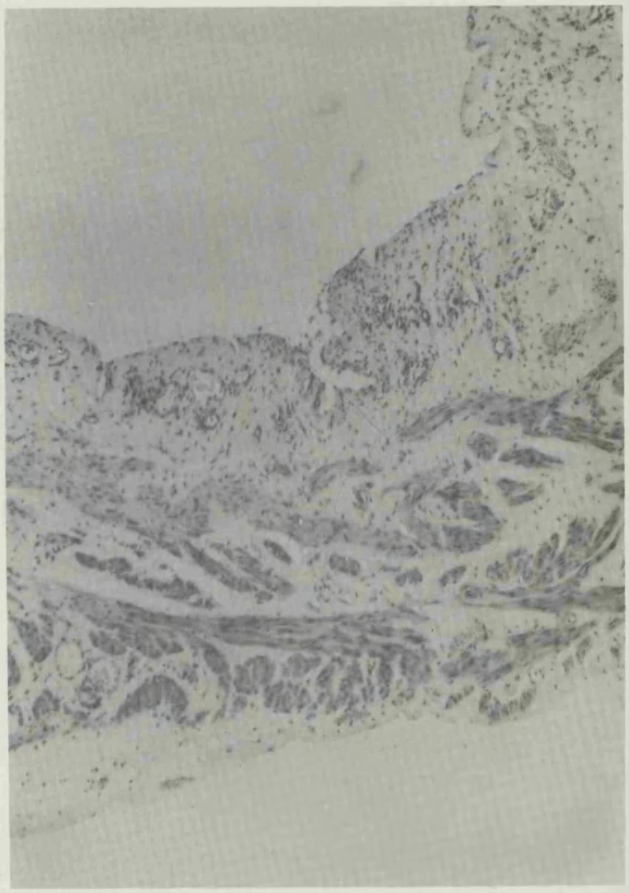

FIG. 4. Photodynamic damage limited to superficial layers of bladder after reducing the photosensitizer dose and increasing light dose. Note epithelial loss and some acute inflammation and edema of lamina propria but no damage to muscle. (4 days after PDT with 0.5 mg./kg. AlSPc, 24 hours prior to 40 J/cm.^{2} light @ 675 nm.).

FIG. 5. PDT after intravesical administration of AlSPc. Patchy damage is seen with area of full thickness necrosis adjacent to area showing urothelium intact and superficial edema only (4 days after PDT with 20 J/cm.^{2} light @ 675 nm. delivered after sensitization by the intravesical administration of 0.3 ml. of a 0.2 mg./ml. solution of AlSPc, for one hour).

mucosa. Our preliminary studies with the purified di-sulfonated AlSPc fraction (AlPcS2), which is more lipid soluble than the predominantly tri-sulfonated AlSPc mixture used in this work, show some improvement in these respects.

Functional changes: bladder compliance. Intravesical pressure in the normal rat bladder rises gradually during filling to about 10 cm. H_2O at a volume of one ml. Fig. 6 shows an example of the filling curve after treatment compared with that in the same animals prior to PDT. The compliance ratio in this case was 4.7 ± 1.4. Controls (no AlSPc, 20 J/cm.^{2} light dose) showed no significant change in compliance after PDT (fig. 7). There was a marked initial change in those rats given 1.5 mg./kg. AlSPc with a compliance ratio of 7.4 ± 2.7 at four days and 3.3 ± 1.1 at four weeks. Even at three months it was still significantly raised (CR = 2.2, $p < 0.05$ vs. controls). Animals given 0.5 mg./kg. AlSPc had an early rise in compliance ratio to 2.4 ± 0.7 which returned to the level of the control animals by one month. An intermediate dose of one mg./kg. AlSPc produced a marked increase in compliance ratio (7.3 ± 1.7 at four days) which quickly returned to normal. The considerable mortality in animals treated with sensitizer doses greater than 1.5 mg./kg. precluded any accurate assessment of urodynamic changes in this group.

Bladder capacity. The initial mean bladder capacity was 1.06 ± 0.28 ml. This was reduced to between 22 ± 3% (1.5 mg./kg. AlSPc) and 40 ± 5% (0.5 mg./kg. AlSPc) of control values within the first week after PDT (fig. 8). Recovery started after two weeks but animals treated with 1.5 mg./kg. AlSPc did not regain more than half their capacity at three months ($p < 0.05$ vs. controls). A more complete recovery was seen with lower doses of sensitizer. The magnitude of these changes in bladder function both in the acute phase after PDT and during healing

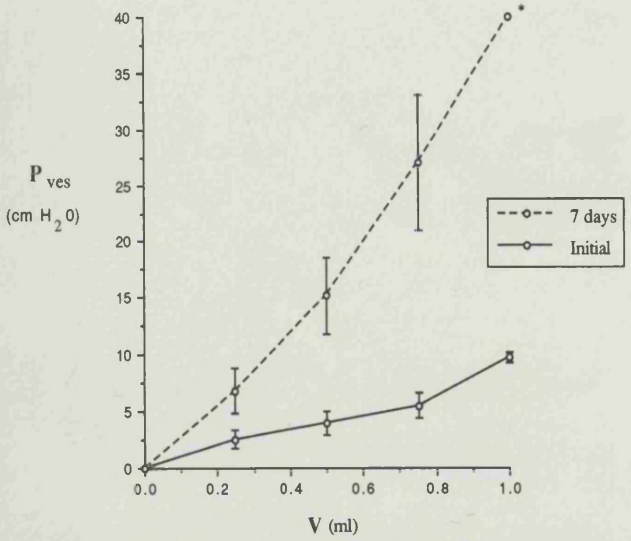

FIG. 6. Filling pressures in rat bladder after PDT. Mean ± SEM in same 4 animals (except * single value) prior to PDT (one mg./kg. AlSPc given 24 hours before exposure to 20 J/cm.^{2} light @ 675 nm.) and 7 days after PDT. P_{ves} = bladder pressure. V = volume of saline instilled.

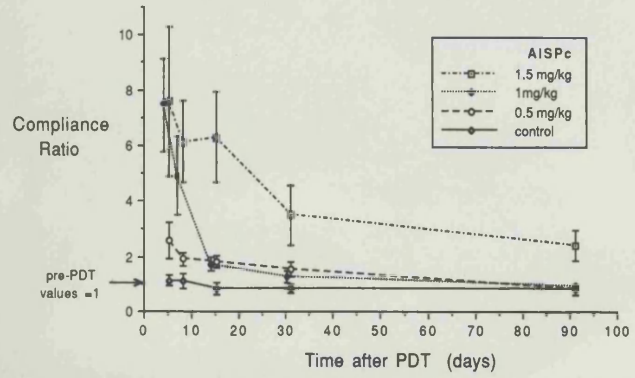

FIG. 7. Change in bladder compliance ratio after PDT; 0.5-1.5 mg./kg. AlSPc 24 hours prior to 20 J/cm.^{2} light @ 675 nm., mean ± SEM in at least 3 animals per point. Controls received 20 J/cm.^{2} light alone.

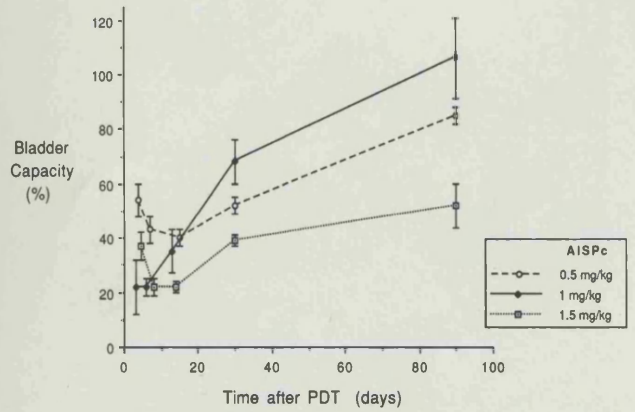

FIG. 8. Change in rat bladder capacity after PDT; 0.5-1.5 mg./kg. AlSPc 24 hours prior to 20 J/cm.^{2} light @ 675 nm., mean ± SEM in at least 3 animals per point. Bladder capacity is expressed as percentage of that in control animals who received 20 J/cm.^{2} light alone.

seemed, as might be expected, related to the degree of tissue damage produced (table 2). However there was no long term histologic abnormality apparent in any surviving animals after the acute inflammatory process had settled even when there was still some functional impairment. In particular no muscle fibrosis nor any change in the amount or pattern of collagen deposition was evident from sections stained with van Gieson's and hematoxylin.

TABLE 2. *Effect of muscle damage from PDT on bladder function in rats treated with 20 J/cm.^{2} light and 0.5–1.5 mg./kg. AlSPc*

	4 Days		4 Weeks		3 Months	
	Cap.*	Comp.*	Cap.	Comp.	Cap.	Comp.
Muscle intact	38%	2.6	57%	2.4	85%	1.1
Muscle damaged	22%	8.4	38%	6.1	53%	3.8

* Cap. = Bladder capacity (at 30 cm. H_2O) as a percentage of that in control animals treated with light alone.
* Comp. = Bladder Compliance Ratio.

DISCUSSION

The clinical results so far reported for PDT in bladder cancer using HpD suggest that this treatment is likely to be most effective in cases of carcinoma in situ (Cis), less effective in superficial papillary disease and ineffective for invasive tumor. It is therefore likely that PDT will become most useful when applied to the whole bladder mucosa to treat multifocal Cis and diffuse abnormal areas which may not be apparent visually. In these situations the effect on normal bladder tissue becomes important as the ideal photosensitizer with a total selectivity for malignant and dysplastic tissue has yet to be developed. An initial period of irritability is of course to be expected after whole bladder PDT as there is a florid acute inflammatory response which, however much one can manipulate conditions, will inevitably damage normal mucosa, though it would seem from the present experiments that injured urothelium at least will regenerate normally after PDT with AlSPc. The problems reported in clinical series using porphyrins result from PDT damage to the deeper muscle which appears to heal less completely than urothelium and with at least some fibrosis,[3] resulting in a permanent and often severe reduction in bladder capacity and compliance. We could not demonstrate any long term histologic abnormality in our animals whose bladder muscle was damaged following PDT with AlSPc, but feel that this is more likely to be a limitation of our model rather than a true difference between the porphyrins and phthalocyanines. Therefore in order to avoid these unwanted effects in patients it seems advisable to try and limit the initial damage caused by PDT, to the superficial layers of the bladder wall.

Although the rat bladder clearly differs from the human our model does provide useful information about functional changes. High photosensitizer and light doses in the rat can produce full thickness bladder wall necrosis, vesico-ureteric reflux and renal damage. These findings are a more extreme example of the major complications which have been seen clinically with HpD. It may be that those authors who have not commented on reduced bladder capacity after whole bladder PDT may not have looked specifically for it, or that it was partly concealed by significant reflux and hydronephrosis as was discovered on cystography in four out of five of Harty's patients with severe irritative symptoms.[3] In our animals even quite low doses of PDT still produced a marked initial reduction in bladder compliance and capacity due to the acute inflammation, but these improved to within normal limits after about one month. It is clear though that when treatment parameters are chosen which do not damage the bladder wall muscle, then the resulting functional impairment is less severe, and recovers more quickly and completely than after those which do (table 2).

Several methods have been suggested to improve PDT selectivity including combination with hyperthermia[12] and with monoclonal antibodies.[13] Porphyrin sensitizers are more efficiently activated by green light than by red, and the more limited depth of tissue penetration at this wavelength may be an advantage rather than a drawback when treating Cis, though absorption by hemoglobin is theoretically undesirable and there

is as yet no published clinical experience with green light. Direct intratumor injection of the photosensitizer may be suitable for discrete bladder tumors[14] but would clearly not be feasible for Cis. The intravesical administration of HpD has not been successful,[15] but means of enhancing uptake into urothelial cells using penetrating agents seem more promising.[16] Our results with the intravesical instillation of AlSPc were disappointing as this would be an attractive method of administration. Further study on this is still required, particularly using AlPcS2, and it is possible that the direct uptake into malignant urothelium and areas of dysplasia may be better than in the normal bladder.

The effect of PDT depends on the tissue concentration of photosensitizer at the time of treatment and the light dose applied. Tissue concentration in turn will depend not only on the administered dose of photosensitizer and its distribution in the target tissue but also on the time interval before irradiation. There is a threshold level below which insufficient singlet oxygen is generated to cause tissue damage even in the presence of excess light.[17] This is due to photobleaching of the sensitizer as discussed below. Above this threshold there is reasonable reciprocity between the sensitizer concentration and light dose required to produce a given biological effect. The therapeutic ideal is to manipulate the difference in concentration of photosensitizer between tumor and normal tissue to achieve a photoactive concentration in tumor but a subthreshold concentration in adjacent normal tissue. Unfortunately this ratio between tumor and adjacent normal tissue is unlikely to be more than 2-3:1,[18] which is reached some 48 hours after sensitization. These values come from gross tissue assays but our results show that the microscopic distribution of photosensitizer also varies with time and, as it is lost more rapidly from the muscle, a gradient of 3-4:1 develops between superficial and deep layers of the bladder wall, which remains fairly constant between 24 and 72 hours. When added to the expected higher levels in neoplastic tissue there should be a biologically useful difference in sensitizer concentration between abnormal urothelium and muscle.

Photobleaching, whereby the photosensitizer itself is degraded by exposure to light,[19] was initially thought to be a disadvantage. However this property could be exploited to enhance the desired biological effect if it were possible to achieve a concentration of photosensitizer in muscle below the threshold level, whilst that in the superficial layer remained adequate for photodynamic damage. Our results indicate that with a low dose of AlSPc it is indeed possible to produce just superficial damage across a wide range of light fluences, a therapeutic ratio not seen when using higher concentrations of sensitizer. It would be a great advantage in clinical PDT to have to ensure only that each part of the bladder received a minimum light dose without concern over adverse effects from too much illumination in some areas. This would reduce many of the difficult dosimetry problems associated with the accurate calculation and even distribution of light over the entire bladder mucosa to which end much effort has been directed.[20]

In conclusion, we have found aluminium sulfonated phthalocyanine to be a promising photosensitizer for bladder PDT. It is retained preferentially in the superficial layers of the normal bladder 24 to 48 hours following injection and after irradiation with red light (675 nm.), a reproducible mucosal destruction can be achieved which heals by regeneration of normal tissue. There is only a transient disturbance of bladder compliance and capacity as long as a low enough dose of AlSPc is used. This is most likely due to the protective effect of sensitizer photobleaching in the muscle layers of the bladder. The light dose required is higher than when using larger amounts of photosensitizer but less critical, which should offset many of the dosimetry difficulties in clinical use. It is likely that similar results may be obtained with low doses of HpD, though this has not been investigated to our knowledge and we feel that it is incumbent on workers in the PDT field to carry out these basic experiments before embarking on clinical studies. It should be possible to refine dosimetry parameters to avoid the major complications previously seen with whole bladder PDT so that this technique may offer a valuable alternative to existing management in cases of resistant Cis and superficial bladder carcinoma.

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The detection of phthalocyanine fluorescence in normal rat bladder wall using sensitive digital imaging microscopy

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Summary The ability to detect photosensitisers in tissue at a microscopical level is important when studying photodynamic therapy (PDT) in both normal and malignant tissue. We have studied the fluorescence distribution of aluminium sulphonated phthalocyanine (AlSPc) in the normal rat bladder using a cooled CCD (charge coupled device) imaging system with computerised image processing. This system makes it possible to carry out a quantitative assessment of photosensitiser fluorescence in the various layers of the bladder wall. The highest fluorescence intensities were obtained within 1 h of intravenous administration but there was little selectivity of uptake between layers. AlSPc was eliminated from the deeper muscle layers more quickly than from the superficial layers of the bladder wall so that by 24 h a 4:1 ratio of fluorescence intensity was apparent which persisted at least until 72 h, although the absolute amount of photosensitiser declined. Following irradiation by red light (675 nm), photobleaching of the sensitiser in the deeper layers further increased this ratio. Direct absorption of AlSPc by the bladder wall following intravesical administration proved unreliable.

The use of phthalocyanines, and in particular the aluminium sulphonated derivatives, as experimental photosensitisers for photodynamic therapy (PDT) is developing rapidly. These substances have several advantages over haematoporphyrin derivative (HpD) in terms of chemical purity and stability, and a major absorption peak in the red part of the spectrum (675 nm) where there is better tissue penetration. AlSPc has been shown to be an effective photosensitiser in animal tumour models for which there appears to be a similar degree of tumour retention as is found for HpD (Tralau *et al.*, 1987).

Clinical series of bladder PDT using HpD have shown some significant complications including bladder irritability and a greatly reduced bladder capacity, which are probably the result of unwanted photosensitiser activation and subsequent fibrosis in the deep muscle layers of the bladder wall (Nseyo *et al.*, 1987; Harty *et al.*, 1989). Many photosensitising agents are relatively poor localisers, achieving only a 2 or 3:1 ratio in tumour *vs* normal tissue. It is therefore important to measure the photosensitiser distribution in normal tissue to minimise adverse effects. In particular it is desirable to limit the effects to the urothelial and submucosal layers as the most suitable clinical indication for PDT in urology appears to be superficial bladder cancer, especially resistant carcinoma *in situ*. Gross tissue extraction measurements cannot give this information whilst standard fluorescent microscopy does not have the required sensitivity to record low level images free from distortion due to natural background tissue auto-fluorescence and sensitiser photodegradation. Initial experiences with a CCD imaging system have been reported by us previously (Barr *et al.*, 1988), but the system is now greatly enhanced producing high quality colour images which can be digitally analysed to determine relative photosensitiser fluorescence intensities in various parts of the tissue section (Figure 1). This apparatus could also be used to study the distribution of porphyrins with suitable adjustments to the excitation and detection wavelengths. In this paper we report studies of AlSPc distribution in normal rat bladder and how this is influenced by photodegradation. This information is required to optimise the treatment parameters for clinical PDT in the bladder.

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Materials and methods

Photosensitiser

Aluminium sulphonated phthalocyanine (AlSPc) was obtained from Ciba-Geigy and used after dissolving in 0.9% saline. This preparation is a mixture of molecules containing on average three sulphonate groups. A preparation of the purified disulphonated fraction (S2 – Porphyrin Products Inc., Logan, Utah) was used for some of the experiments of intravesical uptake of the photosensitiser.

Preparation of specimens for fluorescence scanning

Normal bladder tissue was obtained from female Wistar rats (approximately 200 g) which received varying concentrations (0.5–5 mg kg^{-1}) of AlSPc (mixture) by tail vein injection. At intervals after injection the animals were sacrificed and the bladder catheterised with a fine cannula so that any urine which might contain photosensitiser could be gently washed out. Their bladders were then distended to 0.3 ml with OCT

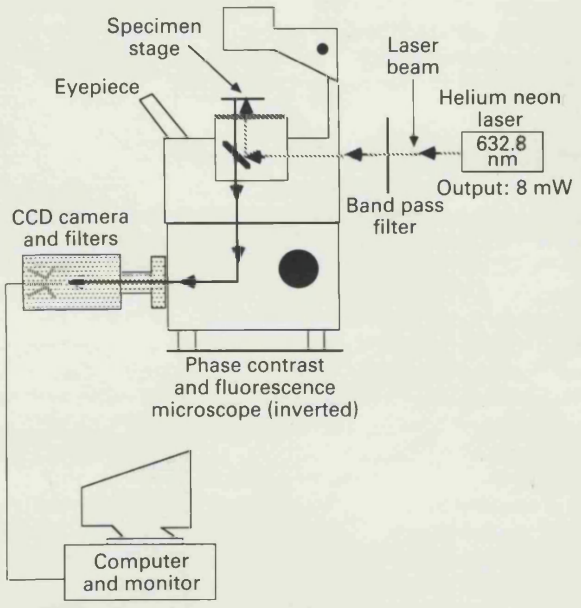

Figure 1 Fluorescence imaging system.

medium (Tissue-Tek, Miles Laboratories Inc.) prior to removal and placing in isopentane (2-methyl butane) which had been cooled in liquid nitrogen for a few minutes. Specimens froze immediately in this solution and were then stored in liquid nitrogen. Paired 10 μm transverse sections of intact bladder were cut using a Cryocat E microtome (Reichert Ltd), several from each block. Half were used for the fluorescence studies and the others stained with haematoxylin and eosin (H&E) to enable orientation of the image.

The direct absorption of intravesically administered photosensitiser was studied after first catheterising and emptying the bladder in anaesthetised rats. 0.3 ml of A1SPc solution was instilled for either 30 min or 1 h after which the bladder was gently washed out several times with saline to remove any surface dye, before processing as described above. Both A1SPc mixture and the more lipid soluble S2 fraction were used at concentrations ranging from 0.02–0.4 mg ml^{-1}.

Fluorescence detection

An inverted microscope (Olympus IMT-2) with epifluorescence and phase contrast attachments was used, and excitation light was provided by an 8 mW helium-neon laser emitting at 632.8 nm (Figure 1). A liquid guide was used to direct the laser output via a 10 nm band pass filter (Omega Optical Inc., Vermont, USA), centred at 633 nm to remove extraneous light, into the dichroic mirror housing for standard epifluorescence excitation. The phthalocyanine fluorescence was detected using a long pass filter (Schott RG665) and a band pass filter (Omega Optical, Inc.) which transmitted in the range 665–700 nm and covers the main fluorescence band of this sensitiser. The principal advantage of using the helium-neon laser, apart from its spectral purity, is that tissue auto-fluorescence is significantly reduced for this relatively long excitation wavelength compared to that seen with conventional UV lamp illumination.

Fluorescence was imaged using a cooled charge-coupled device (CCD) camera (Wright Instruments, model 1, resolution 600 × 400 pixels). An IBM PC with a high resolution colour monitor controlled the camera operation and was used for digital image processing, display and storage. The advantages of using a cooled slow-scan CCD camera over video imaging systems have been discussed previously (Barr *et al.*, 1988) but briefly they include much higher sensitivity, direct digital image integration and a linear response over a signal range of 10^4 in magnitude. The high sensitivity allows low power excitation and short integration times which prevents the occurrence of sensitiser bleaching that may distort the fluorescence image. Tissue auto-fluorescence from control 10 μm frozen section amounts to only 1–2 counts on an image scale of 10^3 employed in this work. Fluorescence was digitally quantified by box superimposition over several representative areas of the tissue section covering the full thickness of the appropriate tissue layer.

Light exposure for photobleaching studies

The rat bladder was catheterised with an 18 gauge Teflon cannula under general anaesthesia. This evenly diffused the light from the 200 μm laser fibre which was positioned inside the cannula just short of its tip and centrally within the bladder. A copper vapour laser (Oxford Lasers) was used, pumping a dye laser to emit red light at 675 nm. This laser produces very short (40 ns) pulses of light at 12 kHz and was set to an average power output of 100 mW. This is well below the power level at which a thermal effect was seen (unpublished results). The fluid filled bladder also acts as a heat sink.

The bladder was filled to a volume of 0.3 ml with saline and assumed to be a perfect sphere to calculate the treatment time needed to give a light dose of 20–80 J cm^{-2}. This does not take account of internal reflectance within the bladder so the true incident energy density may be greater than this. The bladder was exposed through a lower abdominal incision and during light exposure the adjacent bowel was shielded to prevent PDT damage from forward scattering, as the rat bladder is both thin enough to transmit a significant amount of the light, and is also largely intraperitoneal. The power output from the laser was monitored during each exposure by an in-line power meter and adjusted if necessary, though in general it remained stable. The output from the fibre was also checked after each exposure and at intervals during the higher energy treatments. Animals were sacrificed immediately following treatment and the bladder removed and processed for fluorescence scanning as described above. The effect of a cessation of vesical blood flow on the photobleaching of sensitiser fluorescence was also studied in animals killed (by intracardiac injection of barbiturates) just before the start of light exposure.

Results

Intravenous administration

Fluorescence images are shown from representative sections of normal rat bladder together with the corresponding H&E stained slides. Control sections showed negligible auto-fluorescence. At 1 h fluorescence was seen in all layers of the bladder wall with fluorescence intensities in the well vascularised layers of submucosa and serosa about twice that in the muscle layers (Figure 2). Vascular endothelium fluoresced brightly at this time. By 24 h after sensitisation a more marked gradient of between 3.5 and 4 had developed between the A1SPc fluorescence intensity in the superficial *vs* muscle layers (Figure 3), and this differential was maintained at 72 h. This gradient was due to more rapid elimination from the muscle layers; fluorescence readings from muscle were 45 ± 5% at 24 h and 40 ± 5% at 72 h of their value at 1 h compared with levels in the superficial layers of 70 ± 10% and 62 ± 8% respectively.

Intravesical administration

We found the uptake of intravesical A1SPc mixture to be patchy and unpredictable which mirrored the results in our animal PDT studies (Pope & Bown, 1991). In some areas high levels of fluorescence extending through the full thickness of the bladder wall were seen adjacent to areas in which there was no uptake (Figure 4). As the A1SPc mixture used is relatively hydrophilic due to the predominance of the trisulphonated fraction, we also investigated uptake of the purified disulphonated derivative which is more lipid soluble and may penetrate the bladder wall more effectively. This S2 fraction did appear to be taken up more evenly than the mixture and did not penetrate the deeper layers (Figure 5). However the distribution of the photosensitiser within the bladder wall was still not as even as that seen after intravenous administration.

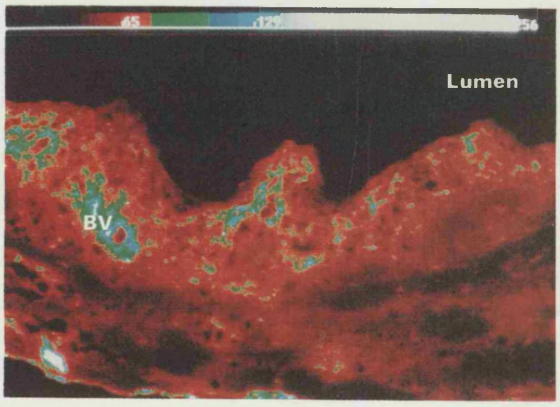

Figure 2 Fluorescence image 1 h after sensitisation with 0.5 mg kg^{-1} A1SPc showing high fluorescence in the endothelium of submicosal blood vessels. The upper colour bar scale indicates fluorescence intensity. Final magnification × 120.

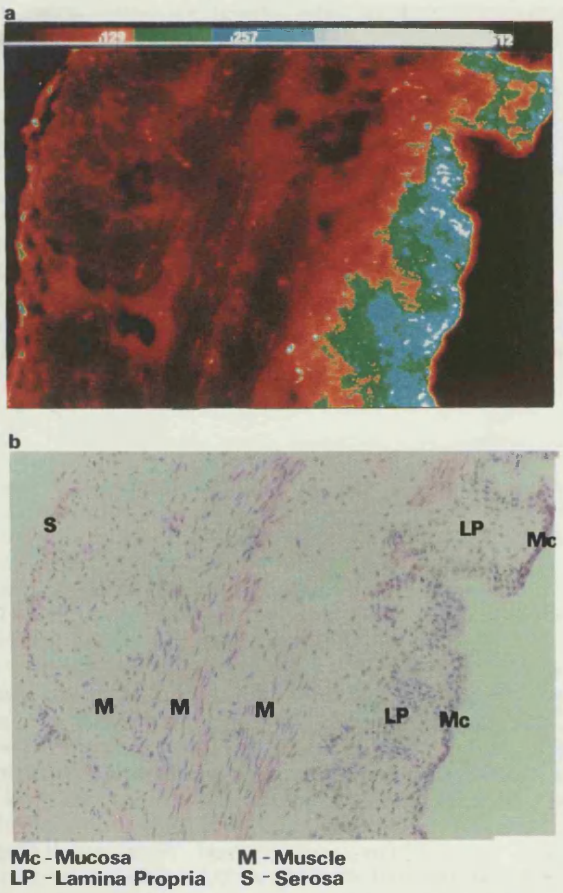

Figure 3 **a**, 24 h after sensitisation with 5 mg kg^{-1} A1SPc. A ratio of 3.5–4:1 in fluorescence intensity now exists between the superficial layers (mucosa and lamina propria) and the deeper (muscle) layers of the bladder wall. **b**, H&E stained section corresponding to **a**. Final magnification × 450.

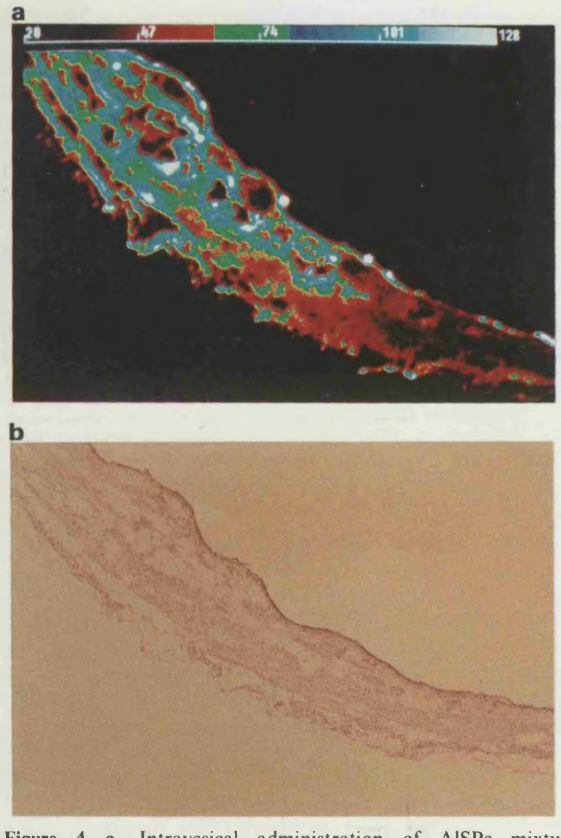

Figure 4 **a**, Intravesical administration of AlSPc mixture (100 μg ml^{-1}, 1 h), showing high though variable fluorescence throughout the bladder wall. **b**, H&E stained section corresponding to figure 4a. Final magnification × 60.

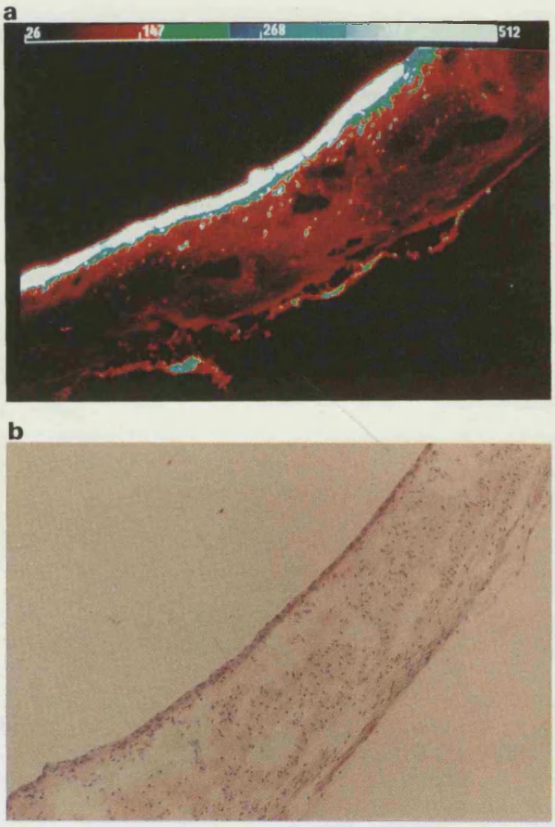

Figure 5 **a**, Intravesical administration of A1SPc S2 (100 μg ml^{-1}, 1 h) showing more superficial uptake compared with A1SPc mixture. **b**, H&E stained section corresponding to **a**. Final magnification × 60.

Photobleaching studies

A high light fluence (80 J cm^{-2}) *in vivo* resulted in almost complete degradation of the sensitiser throughout the dose range studied (0.5–5 mg kg^{-1} A1SPc, 24 h prior to light exposure), with only a thin rim of superficial fluorescence remaining (Figure 6b). Smaller light doses caused a lesser degree of photobleaching (Figure 6a) and a dose of 20 J cm^{-2} only reduced fluorescence in muscle by about 25% compared with unexposed controls, though by proportionally less than this in the superficial layers. This had the result of increasing the ratio of the fluorescence intensities between mucosa/lamina propria and muscle to about 5:1 after 20 J cm^{-2} and greater than 10:1 after 80 J cm^{-2} (compared with 3.5–4:1 in unexposed controls). If the circulation was arrested immediately before irradiation then minimal photobleaching of the fluorescence image occurred even using high light doses (Figure 6c).

Discussion

In this work we present the quantitative imaging of phthalocyanine fluorescence in the bladder using a highly sensitive detection system. This is the first time that the distribution of A1SPc fluorescence in the bladder has been demonstrated in this way and to our knowledge the first report of photobleaching of any photosensitiser in tissue resolved at the microscopic level.

The porphyrins are the most commonly used photosensitisers but in the case of HpD or DHE, which are complex and poorly defined mixtures of products with varying fluorescence properties, it is questionable whether the observed fluorescence closely mirrors photoactivity (Moan & Sommer, 1981; Berns *et al.*, 1984). The main aggregates formed with phthalocyanines are dimers whose fluorescence appears to be negligible and whose photoactivity is also very low compared

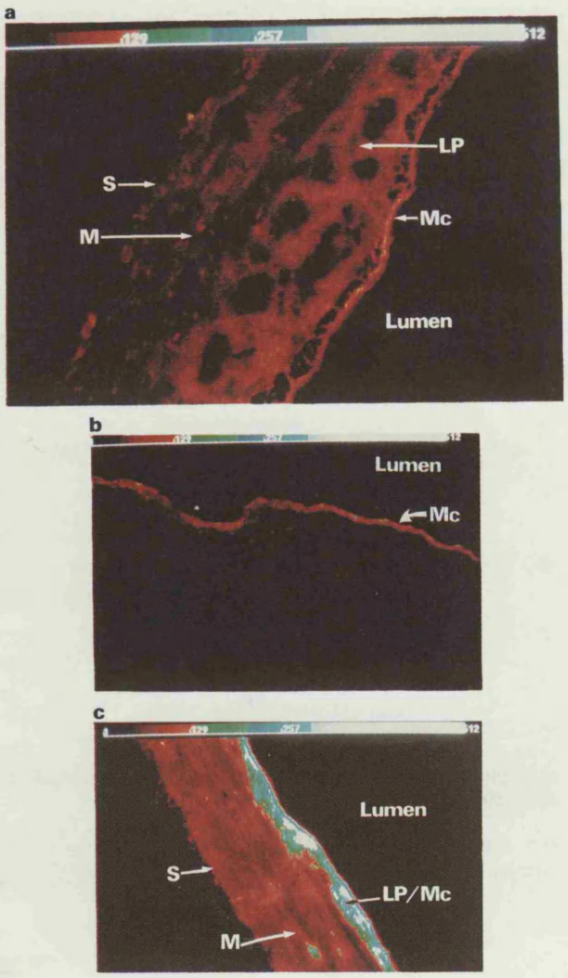

Figure 6 Photobleaching effect of red light (675 nm) *in vivo* on bladder 24 h after sensitisation with 5 mg kg^{-1} AlSPc (same as Figure 3a). The sensitiser is progressively degraded with increasing fluence (**a** – after 40 J cm^{-2}) and only a thin rim of fluorescence is seen in the mucosa after 80 J cm^{-2} (**b**). If the circulation is arrested before irradiation little loss of fluorescence is seen (**c** – 80 J cm^{-2} *ex vivo*).

to that of monomers (McCubbin, 1985; Spikes & Bommer, 1986). The interpretation of the correlation between fluorescence and photoactivity has been examined previously for AlSPc (Barr *et al.*, 1988; Berg *et al.*, 1989), from which it was concluded that fluorescence detection is selective in that only the photoactive monomers are detected. Therefore these fluorescence estimations reflect the concentration of photoactive sensitiser in tissue which is more relevant than the total amount which will include inactive aggregates.

Timing of light exposure

Tissue sensitiser concentration will depend on the time lapse before treatment as well as the administered dose. Again the ideal is not known though most reported series have treated at 48–72 h after sensitisation. Benson's group (1986) treated at 3 h as *in vivo* fluorescence of bladder tumours under UV light was greatest at this time. Not withstanding the difficulties in relating HpD fluorescence to photoactivity, the timing of light delivery should be when the most advantageous ratio of concentration between tumour and normal tissue occurs, which is not necessarily when the highest levels are seen in tumour. Initially after injection there is also a high level in normal tissue especially in well vascularised areas such as the submucosa and serosa. It is only later that the slower clearance of sensitiser from malignant tissue results in a concentration difference. This ratio in rat colonic tumours has been found in our laboratory to be greatest at 48 h, though only about 2:1 (Tralau *et al.*, 1987), and it would not be unreasonable to expect a similar situation in bladder carcinoma. We have shown in this paper that the ratio between normal mucosa and muscle remains fairly constant, at between 3–4:1, though absolute values decline, between 24 h and 72 h so it would therefore seem most appropriate to treat bladder tumours around 48 h after sensitisation. Later than this confers no advantage in the distribution of photosensitiser in normal tissue and it is at 48 h that the most advantageous ratio between tumour and normal tissue may be expected, though this should be clarified when studies of AlSPc uptake in man are possible.

Photobleaching

During light exposure some photosensitiser molecules become photodegraded and will neither produce singlet oxygen nor fluoresce. This phenomenon was first described *in vitro* by Moan (1986), and later *in vivo* by Mang *et al.* (1987) who showed a loss of fluorescence and reduction in extractable porphyrin from a mouse mammary tumour. A similar response has been demonstrated in other tumour models. In this work we have demonstrated photobleaching at the microscopic level and shown that this process requires an intact blood supply and has the effect of reducing the amount of photoactive sensitiser in the deeper muscle relative to the superficial layers of the bladder wall. It may be possible to use this phenomenon to increase the selectivity of clinical PDT.

There are two conditions to be satisfied before a PDT effect can occur. Firstly enough singlet oxygen must be produced to cause tissue necrosis. This requires a minimum tissue concentration of photosensitiser as below this threshold level it is destroyed by photobleaching before sufficient singlet oxygen has been produced, whatever the light dose. Secondly a given tissue effect depends on the product of sensitiser concentration and light dose for which there is reciprocity in the ranges most workers have studied (Bown *et al.*, 1986; Barr *et al.*, 1987; Profio & Doiron, 1987). If the sensitiser concentration is increased, the threshold light dose is reduced and vice versa. This reciprocity is lost for low concentrations of photosensitiser near the threshold level (Potter *et al.*, 1987) where photobleaching becomes more important. The therapeutic ideal is to manipulate the difference in concentration of photosensitiser between the urothelium and underlying muscle to achieve photoactive conditions in the former but sub-threshold concentrations in the muscle. In this situation photobleaching of the sensitiser in the muscle layers will spare it from unwanted damage during light exposure though still produce tissue necrosis in the mucosa. Additional selectivity in clinical practice may result from the small differential in photosensitiser concentration to be expected between bladder tumour and adjacent normal mucosa.

In conclusion this fluorescence technique defines and quantifies photosensitiser distribution in tissue on the microscopic level. We have studied AlSPc, but with appropriate wavelength adjustment, it would be equally applicable to the porphyrins. High magnifications may be used to localise fluorescence to cellular structures as sensitivity is much greater than with conventional fluorescence microscopy. Unwanted photobleaching during acquisition of the image is not a problem because of the low excitation powers used, therefore rates of photobleaching of low sensitiser concentrations caused by *in vivo* irradiation can be accurately assessed (unpublished data). We found that a fluorescence intensity gradient of 3–4:1 between superficial and deep layers of the normal bladder wall was established from 24 h sensitisation due to slower elimination from the superficial layers, and it is suggested that the optimum time to treat bladder tumours is around 48 h. If low photosensitiser doses, and necessarily higher light doses, are employed then photobleaching may further improve the selectivity of PDT damage between the

superficial and deep layers of the bladder wall. We have since applied these parameters to an animal model of bladder function, and produced a reliable necrosis of the bladder mucosa without damaging the underlying musculature (Pope & Bown, 1991). This has healed by the regeneration of normal tissue without the permanent reduction in bladder capacity or compliance which has been seen in some clinical series subsequent to muscle layer fibrosis.

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