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Technical report

Determination of X-ray-induced damage to the murine colon using tissue compliance measurements

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A proctometroscope has been developed to measure the mechanical functioning of the colon in a murine model. A balloon-tipped probe is inserted into the colorectal region of anaesthetized mice and inflated hydraulically, at a constant rate, by a motor-driven syringe. Balloon pressure and volume are monitored, processed and plotted on a $X-Y/t$ recorder. The balloon is inserted within the irradiated area, such that the centre of the balloon is 1 cm proximal to the anus, and inflated up to either a maximum volume of 100 μl or a maximum pressure of 100 cmH_2O, depending on which occurs first. Compliance ($\Delta V/\Delta P$) of the colon was measured at various intervals following X-irradiation, a dose-dependent decrease being observed at 24 weeks. This decrease was progressive with time out to 72 weeks postirradiation. The compliance measured, in both control and irradiated groups, was not dependent on the inflation rate used to make the determination, and no iatrogenic effects have been detected after repeated probing during a long-term study. The use of different anaesthetic agents was also investigated, no significant difference between the compliance of mice anaesthetized with the different agents being detected. Use of this technique provides fully quantitative data on the function of the colon following radiation injury, and provides an alternative to other physiological assays. The technique is non-destructive, rapid, easy to use and non-invasive to the tissue, thus the onset and progression of damage can be followed in each mouse over long postirradiation periods.

1. Introduction

The doses of radiation delivered in curative radiotherapy inevitably damage the normal healthy tissue adjacent to the cancerous site. Cancers of the prostate, bladder, ovary, cervix and uterus rank amongst the ten most common in occurrence in the UK. In the treatment of cancers of the lower abdomen the descending (or rectosigmoid) colon is considered a dose-limiting normal tissue (Jackson 1976). Incidence rates of large bowel complications occur after a variable latent period, ranging from a few weeks or months to over 5 years, and vary from 0·25% to 4% (Kuipers 1982, Schofield *et al.* 1983) but may reach 18% (Fajardo 1982, Montana and Fowler 1989). Chronic damage, i.e. episodic diarrhoea, proctitis, haemorrhage, ulceration and intestinal obstruction due to a fibrous stricture (Jackson 1976, Porquier *et al.* 1982) can be managed conservatively, but some may be progressive

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and become so debilitating to the patient that segmental resection and anastomosis is required, in spite of the aggravated risk of surgical complications (Palmer and Bush 1976, Jackson 1976; Browning *et al.* 1987). A physiological assay which enables the onset and pathogenesis of this damage to be monitored would be valuable both to the clinic and to the laboratory.

There are few techniques available for the study of colorectal damage in small laboratory animals, those currently available being semiquantitative, invasive or involving sacrifice of the animal (Black *et al.* 1980, Hubmann 1981, Dewit *et al.* 1987, Breiter *et al.* 1989). Balloon probes have been used extensively in the clinic for the measurement of pressure responses in a variety of clinical abnormalities, e.g. oesophageal varices (Gertsch and Meister 1987), ulcerative colitis (Farthing and Lennard-Jones 1978), chronic constipation (Preston *et al.* 1983) as well as for studying the normal physiology of the colorectum (Lipkin *et al.* 1972, Davenport 1971). Manometric measurements have also been made clinically to monitor colorectal function after X-irradiation (Varma *et al.* 1985) but no such study has taken place in small laboratory animals, presumably due to the difficulties in making a probe of suitable dimensions.

The present study reports the development of a proctometroscope for use in small laboratory animals, and describes various modifications to the original machine which enable parameters other than compliance to be measured.

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2. Materials and methods

2.1. *Animals and irradiation procedure*

Unanaesthetized barrier-maintained isogenic female CBA/HtGy F TO mice, aged 16 weeks, were irradiated (total pelvic irradiation) with single doses, 5–20 Gy, of X-rays, control mice being sham-irradiated. Animals were kept in groups of six in 20 cm × 26 cm cages, and were supplied food and water *ad libitum*. Temperature and humidity in each of the animal rooms were controlled at 22 ± 2°C and 50% H_2O respectively, with light being supplied on a 14 h on–10 h off schedule, and 12 changes of air taking place per hour.

Radiation was delivered using a 250 kV X-ray unit (Pantak Ltd, Windsor, UK), located in the animal unit, operating at 240 kV and with a tube current of 15 mA. The beam was filtered with 0·25 mm Cu and 1 mm Al (HVL 1·3 mm Cu) and was delivered at a dose rate of 3·97 Gy/min. Individual mice were restrained by means of a close-fitting Perspex box (Terry 1984) with an aperture in the front to allow free air flow. Lateral movement was prohibited by the box dimensions and forward movement by taping the animal's tail after pulling it through a hole in the drop-in rear door. Animals were acclimatized to the boxes for 20 min prior to irradiation. The boxes were placed on the irradiation jig assembly such that the mice were held in a vertical position, heads down, to exclude any small bowel from the field, irradiation taking place in an anterior–posterior siting. Positioning and dose distribution were checked by radiography and thermoluminescent dosimetry (TLD microrods were placed in various sites in cadavers and irradiated to 3 Gy). The focal skin distance was 20 cm, 19 ± 0·5 mm of colon proximal to the anus was irradiated, the kidney received 14 ± 1% of delivered dose, liver 6 ± 0·8% and heart 3 ± 0·3%. In all procedures involving mice, Home Office guidelines were strictly observed.

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2.2. *Equipment and electronics*

It was decided at the outset that the instrument used to determine compliance (from the maximum point of the pressure–volume curves) should be simple to operate and flexible enough to make it suitable for other measurements such as recording intraluminal pressures and contractions. Sensitive differential pressure transducers are readily available and one of these (Sensym Controls LX1601D) was initially used. This is a temperature-compensated, semiconductor strain-gauge bridge with integral amplifier, having a sensitivity of $\sim 1{\cdot}4\,mV/mmH_2O$ pressure. The pressure transducer was found to be vulnerable to corrosion when water was used as the transmission fluid. This was later replaced by a transducer (type LX06005) together with a high-stability, low-drift, dc preamplifier with a gain of 100. As an additional precaution the fluid used in the system was changed from water to a low-viscosity silicone oil to prevent further deterioration of the sensors.

A very simple prototype instrument was constructed first. This consisted of a fixed-speed, reversible syringe drive mechanism (inflation rate 100 μl/min) coupled to a resistive linear position transducer to indicate volume. A 'T'-piece at the syringe output was connected to the pressure transducer input port and to a balloon-tipped probe consisting of a hypodermic needle surrounded by a high compliance latex membrane (condom type) at the tip (Figure 1). The probe was designed such that only lateral expansion would occur during balloon inflation, and that the point of maximal inflation of the balloon would occur 1 cm proximal to the anus, directly within the irradiated area. The system was first evacuated and then filled with fluid and sealed. Simple analogue signal conditioning amplifiers were used to scale the two transducer output voltages so that a standard X-Y analogue plotter could be used to trace out the characteristic pressure–volume curve. The main limitations of the instrument were that only a constant rate of inflation was possible, that intraluminal pressures and contractions could only be investigated using an X–Y/t plotter and that relatively frequent zeroing of the pressure transducer trace was required, primarily due to a small, but non-negligible, degree of hysteresis exhibited by the balloon.

A slightly different approach was taken in the design of the final instrument. In this case a linear stepper motor (0·025 mm/step, 350 steps/s, four phase) was used to drive the syringe. A variable rate of inflation (from 10 to 1000 μl/min) is thus readily achieved by varying the repetition rate of the pulses energizing the motor; in addition no position transducer is required since an estimate of volume can be obtained by counting the number of motor pulses. A simplified diagram of the electronic control unit is shown in Figure 2. A four-digit up–down counter is used to count the motor pulses and incorporates a presettable register which can be used to set a limit for the maximum change in volume, disabling the motor pulses. A second counter, coupled to a digital-to-analogue counter, can be used either to provide an output voltage proportional to volume to count clock pulses (providing a linear ramp voltage output with the motor pulses disabled), thus converting the 'volume' axis of the plotter to a 'time' axis for the recording of intraluminal pressures and contractions. Push-buttons can be used at any time to inflate, deflate or hold the balloon inflation. A 'new' zero is achieved by resetting the motor pulse counter. The limit and zero outputs of the motor pulse counter can be used either to stop inflation (or deflation) when the limit (or zero) is reached, or to reverse the current cycle following the limit (or zero).

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The pressure transducer output is amplified to provide full-scale ranges of 199·9, 19·99 or 1·999 mmH_2O. This output is then fed to a digital autozero loop which maintains the signal level and generates an error signal proportional to the difference between the 'current' and true zero. This difference can be subtracted at any time from the signal and subsequently held in a digital follow/hold circuit. The 'pressure' scale can thus be zeroed at any time by triggering the follow/hold circuit; such a push-button-operated system is considerably less prone to operator error than a conventional rotary control. A solenoid-operated value—coupled to the pressure transducer, syringe and balloon probe—is used to open the hydraulic system to atmosphere when the zero pressure button is activated, thus establishing a true zero pressure reference. However, the pressure signal can be zeroed without opening this valve when the instrument is set to record changes of pressure, i.e. contraction/intraluminal pressure. The processed signal is applied to a digital voltmeter and to the axis of the plotter. In addition, it is internally connected to a

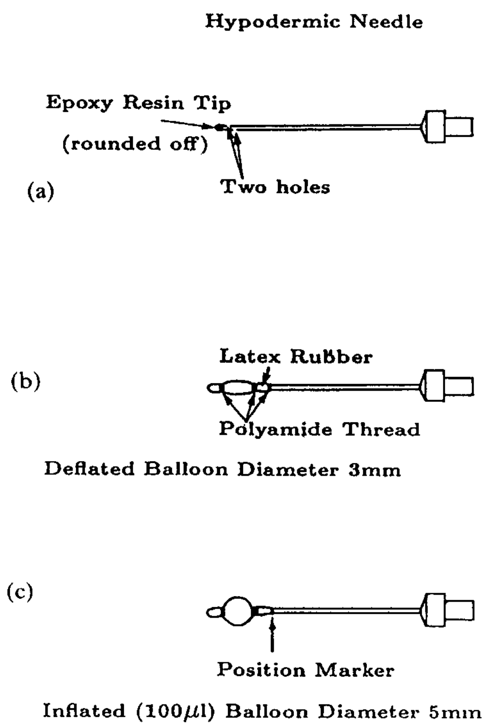

Figure 1. Probe design. (a) Hamilton CTEE hubbed hypodermic needle, point style 3, shaft length 51 mm, o.d. 1·24 mm, i.d. 0·84 mm. Tip is sealed with epoxy resin and rounded off for ease of insertion. Two holes, diameter approximately 1 mm, are ground on opposite sides of the shaft 2 mm and 4 mm from the tip respectively. (b) High-compliance latex rubber (condom type, London Rubber Company) is placed over the tip and tied with monofilament polyamide thread (Ethilon) to allow only lateral expansion of the balloon during inflation. (c) The rubber is tied off further along the shaft and sealed with epoxy resin. Insertion of this position ensures that the centre of the balloon is 1 cm proximal to the anus.

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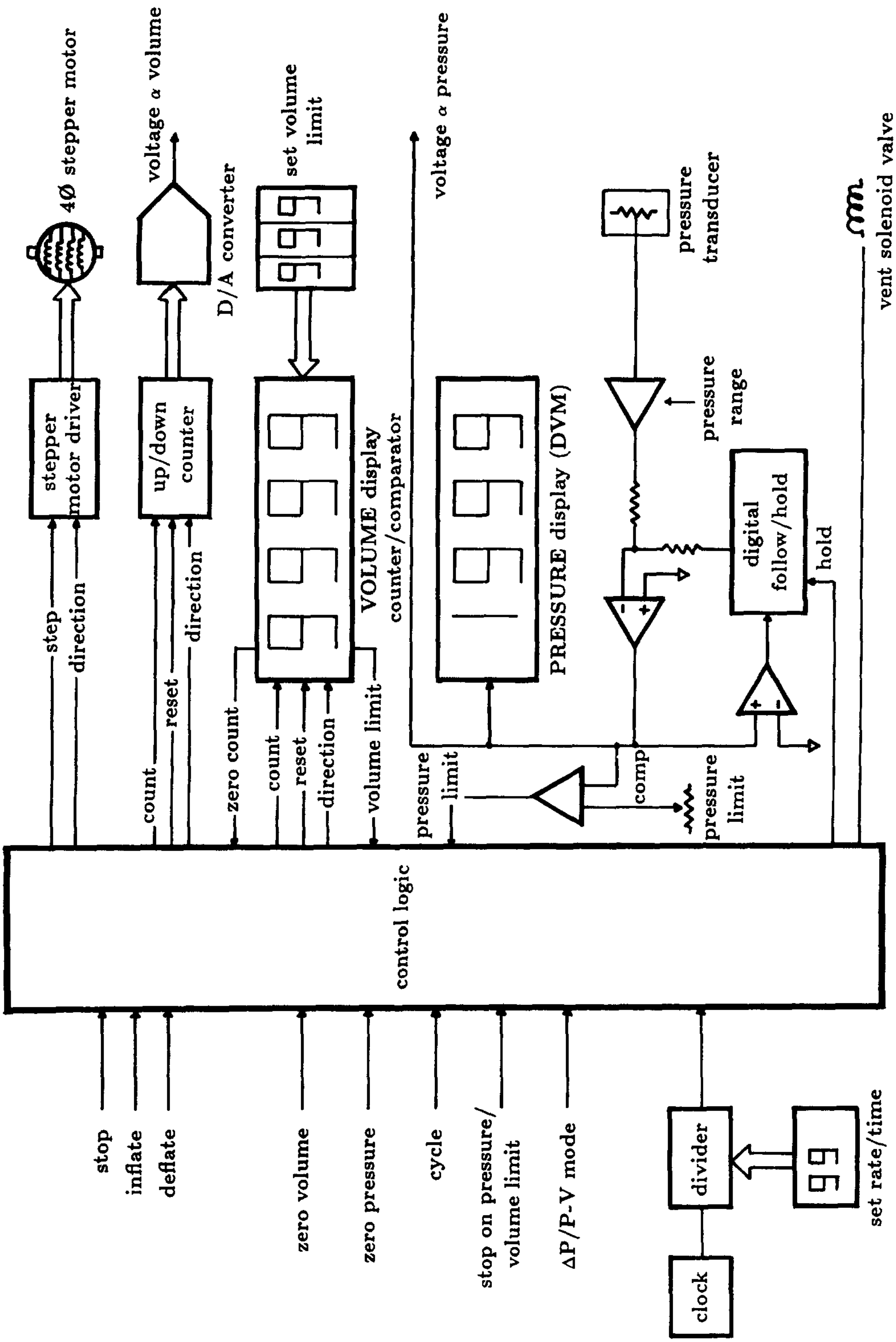

Figure 2. Block diagram of the electronic processing unit.

comparator which works in much the same way as the volume limit to stop or reverse the cycle once a preset pressure limit is reached.

Although the system described here provides outputs in analogue form, digital data are available from the motor pulse counter (volume) and digital voltmeter (pressure) and the device could be readily interfaced to a microcomputer for data logging and experiment control. The system can be seen in use with the syringe drive mechanism in Figure 3. The drive mechanism is constructed from thick aluminium sheet and incorporates 'luer' fittings throughout. A 250 μl 'luer' tip syringe is used (Hamilton 1000 series High Performance Gas Tight Syringes), coupled to a junction block; this connects to a male 'luer' output port, for connection of the balloon probe, to the pressure transducer and the solenoid-operated venting valve. This in turn is connected to a two-way manual valve, one input port of which is normally connected to a fluid reservoir at atmospheric pressure. The other input port can be connected to a small vacuum pump which is activated only when the system needs to be filled. After evacuation, the manual valve is coupled to the reservoir, which then fills the system. It is essential to exclude all air bubbles from the system, otherwise erratic results obtained due to the essentially adiabatic compression of any gas in the system.

2.3. *Testing procedure*

Following irrigation of the intestine with water, and palpation of the abdomen to facilitate removal of any faeces from the colon, colonic compliance was measured in methoxyflurane-anaesthetized mice at 24 weeks postirradiation and at various time intervals thereafter. With the centre of the lubricated balloon positioned 1 cm proximal to the anus the system was vented to atmospheric pressure and balloon inflation performed at a rate of 100 μl/min up to either a preset maximum volume change of 100 μl or a preset maximum pressure change of 100 cmH_2O. Inflation was immediately followed by a deflation cycle. The time taken for the overall measurement (i.e. insertion, inflation and deflation) was 3 min per mouse.

Compliance is a parameter used in a number of investigations to monitor tissue function (lung: Goldstein *et al.* 1979, Hesterberg *et al.* 1981; colon: Varma *et al.*

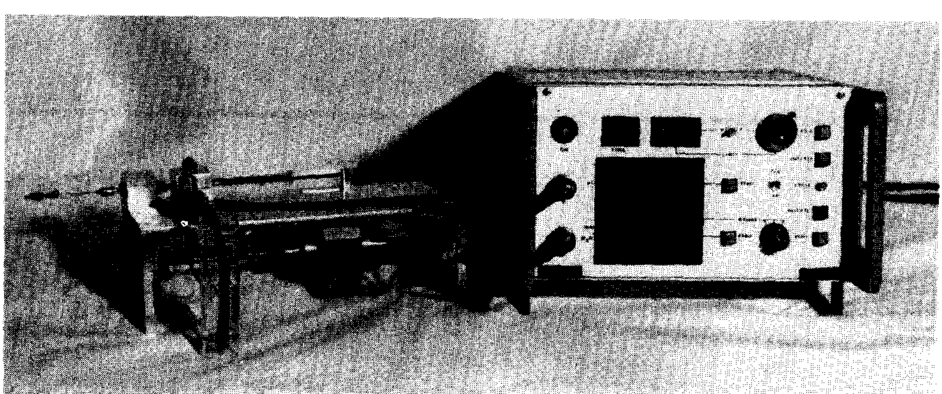

Figure 3. The proctometroscope in use, checking balloon characteristics by inflation in air. Left: The syringe drive mechanism with attached probe and fluid reservoir to allow balloon filling. Right: Electronic processing unit.

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1985, Varma and Smith 1986). Colonic compliance, a measure of the expandibility of the wall of the colon, was examined in the present investigation by monitoring the changes in volume with changes in pressure ($\Delta V/\Delta P$). The compliance of the balloon was taken into account by using the following equation:

$$\text{Tissue compliance} = \frac{V_m}{(P_m - P_{air})}$$

Where V_m and P_m are the volume and pressure corresponding to the point of maximal inflation, and P_{air} is the pressure of the balloon in air at volume V_m. The trace of the balloon inflation in air is important not only to take into account balloon compliance but also to allow inter-experimental comparisons. One balloon normally lasts between 2 and 3 weeks before its inflation/deflation characteristics change. For comparisons to be valid the new balloon, made immediately prior to testing, must have characteristics which match those of the balloon used for the previous time-point. Variation of balloon compliance was of the order of 3%, which was considered acceptable (Martin *et al.* 1988).

Since measurements routinely took place at an inflation/deflation rate of 100 μl/min, while the animals were anaesthetized with inhalation anaesthesia using methoxyflurane (Metofane), experiments were carried out to examine the effect on colonic compliance of varying either the anaesthetic or inflation rate. The effect of altering the anaesthetics was investigated in control animals aged 38 weeks. The other inhalation anaesthetics used were: diethyl ether or Ethrane (2-chloro-1,1,2-trifluoroethyldifluromethyl ether), or an i.p. injection of pentobarbitone sodium (Sagatal 0·6 mg/ml). All are non-selective CNS depressants. At 76 weeks postirradiation the effect of altering the inflation rate was investigated in a control group ($n=3$) and groups of irradiated animals; 11 Gy ($n=4$), 14 Gy ($n=4$) and 17 Gy ($n=5$). Immediately following a normal measurement, carried out at 100 μl/min, a second cycle commenced at 300 μl/min, which was followed by a third cycle, again at 100 μl/min. Possible iatrogenic effects of the technique were investigated by carrying out measurements on animals at 52 weeks postirradiation, having never been probed previously.

One set of animals, containing groups irradiated with either 14 Gy ($n=4$) or 17 Gy ($n=5$), was probed on four occasions prior to this measurement (i.e. at 24, 32, 40 and 48 weeks postirradiation) and the results at 52 weeks compared to a parallel set of animals, irradiated at the same time with the same doses, but never previously probed.

2.4. *Statistical analysis*

Significance of differences between groups of mice was assessed by paired or unpaired Student's t-test.

3. Results

Figure 4 shows typical traces of balloon inflation in air, in a control animal 52 weeks after a single-dose irradiation of 17 Gy and in an age-matched control. Irradiation causes a marked change in the type of trace obtained, with compliance decreasing from 1·96 μl/cmH_2O in the control animal to 0·38 μl/cmH_2O in the irradiated animal. In all cases, elastic hysteresis is observed, i.e. forces are not conservative; the work done in returning the balloon to its original shape is less than

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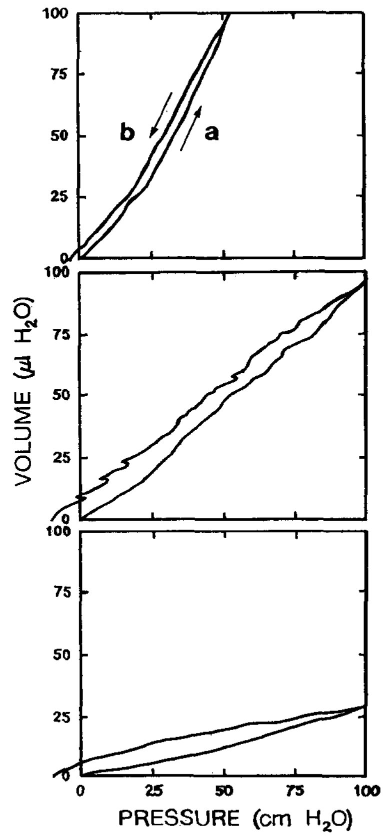

Figure 4. Raw data from the chart recorder. Top panel: balloon characteristics by inflation in air. Direction of cycle is shown by arrows (a = inflation, b = deflation). Middle panel: trace from inflation in a control mouse aged 68 weeks (compliance 1·96 µl/cmH_2O). Botton panel: trace from inflation in an age matched mouse 52 weeks after irradiation with 17 Gy.

the work done to deform it (i.e. inflation). In most biological materials the slope of the hysteresis curve is steeper on removal of the stress, implying that energy has been lost in its deformation (Watters *et al.* 1985a). In view of the complexity of interpretation of hysteresis effects in such a physiological system, only compliance measurements were extracted from pressure–volume curves. Energy loss, calculated from the area within the pressure–volume hysteresis loop, showed no correlation with radiation dose.

With age there was a slight, but non-significant, increase in colonic compliance of unirradiated control animals (1·06 ± 0·08 µl/cmH_2O aged 40 weeks, 1·13 ± 0·08 aged 88 weeks, $n = 24$, no significant difference, $p = 0{\cdot}55$). All data from irradiated animals were expressed as percentage of control compliance at each time. Figure 5 shows the dose–response curves obtained from those animals surviving at 24, 40, 52 and 72 weeks postirradiation. Although compliance decreases with increasing dose there appears to be a dose-independent plateau over the range 4–12 Gy. For each dose group the decrease in compliance is progressive with time to 72 weeks

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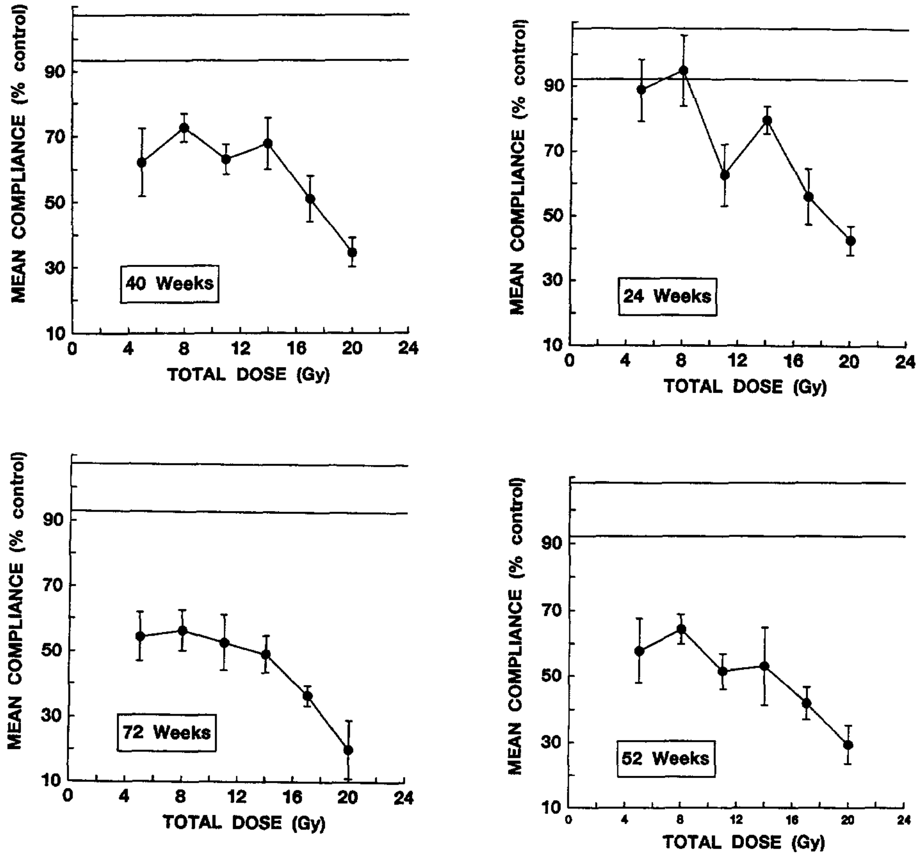

Figure 5. Dose–response curves 24, 40, 52 and 72 weeks postirradiation. Compliance decreases with increasing dose of radiation and is progressive with time. Shaded area represents control value ± 1 SEM. Each point represents the mean of four to six animals ± 1 SEM.

postirradiation. Although compliance was measured at earlier times, it was only by 24 weeks that a dose response was seen. Animals died at a constant rate throughout this time period, primarily due to stenosis. The LD_{50} values at 48 and 60 weeks postirradiation, based upon logit analysis of lethality plots, were 20·8 ± 1·1 Gy and 19·4 ± 0·7 Gy, respectively.

Nutritional status of the mice was examined by monitoring changes in weight. The absolute weights of irradiated groups did not at any time reach control values. This altered nutritional status of irradiated animals was not due to cage effects (Martin 1990).

The use of either Sagatal, enflurane or diethyl ether did not significantly alter colonic compliance of control animals as opposed to Metofane-anaesthetized animals (p = 0·08, 0·12 and 0·77 respectively). Metofane was used for all experiments. Altering the inflation/deflation cycle rate did not significantly alter the characteristics of the traces or the compliance values in either control or irradiated animals. Values of 0·94 ± 0·17 $\mu l/cmH_2O$ were obtained for the control group (n = 3, ± 1 SEM) and 0·58 ± 0·06 $\mu l/cmH_2O$, 0·43 ± 0·05 $\mu l/cmH_2O$ and

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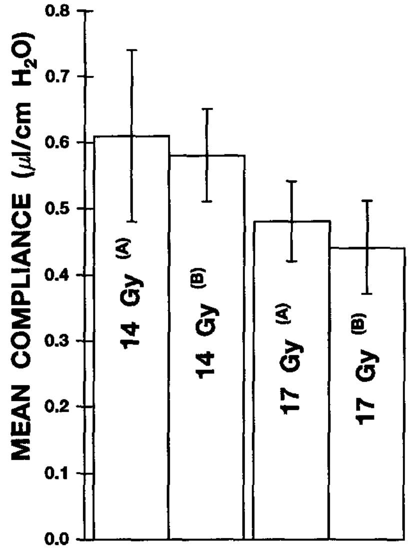

Figure 6. Bar graph of mean compliance 52 weeks postirradiation with either 14 Gy or 17 Gy. (A) Animals tested on four previous occasions, each separated by eight weeks. (B) Age matched animals irradiated at the same time but never measured prior to this occasion. Error bars (± 1 SEM) indicate that there is no significant difference in the compliance values obtained.

$0{\cdot}28 \pm 0{\cdot}01$ $\mu l/cmH_2O$ for the 11 Gy, and 17 Gy groups, respectively. Data in Figure 6 indicate that prior use of the probe on four separate occasions, all separated by 8 weeks, did not significantly alter the compliance when measured at 52 weeks postirradiation.

4. Discussion

Balloon probes have been used extensively in the study of colonic physiology and disease states for many years (Lipkin *et al.* 1962, Davenport 1971, Suzuki *et al.* 1980, Varma and Smith 1984). The colonic compliance of unirradiated control animals in the present study appeared to increase slightly with the age of the animal. In rats (Watters *et al.* 1985a) and in humans (Watters *et al.* 1985b) it has been observed that the strength and elasticity of the colon may first rise during growth and then decline as the post-mature animal ages. The general picture is one of the colon becoming mechanically less efficient with ageing. Watters *et al.* (1985a,b) attributed this either to increased flexibility of the collagen fibres or to a decrease in the integrity of the connective tissue.

The decreased compliance after X-irradiation in the present study has also been reported clinically during the investigation of chronic radiation proctitis; the altered compliance, which was not obvious radiologically, was suggested on the basis of supporting histological evidence to be due to smooth muscle hypertrophy and myenteric plexus damage (Varma *et al.* 1985). Histological evidence of smooth muscle cell degeneration and mechanical hypertrophy of the muscularis externa was obtained 24–32 weeks postirradiation from studies being run in parallel with the present investigation. There was no evidence of submucosal fibrosis. The

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relaxation ability of this smooth muscle layer was tested by the administration of papaverine (5 mg/kg). However, since methoxyflurane produces CNS depression, the relative importance of muscle relaxation and peripheral nervous system (PNS) involvement cannot be dissociated (Martin 1990). Further work is required on unanaesthetized animals before this question can be properly addressed. Lundbeck *et al.* (1989), whilst examining radiation damage to the bladder, also observed an altered muscle appearance which correlated with a decreased bladder capacity. They, as with the present investigation, could not dissociate the muscularis involvement from pharmacological interactions involving cholinergic, adrenergic or non-cholinergic, non-adrenergic innervation.

It appears from the present study that there may be two competing processes involved in the observed decrease in colonic compliance after X-irradiation; age tending to increase compliance (due to a decrease in the integrity of the connective tissue or the collagen fibres becoming more flexible (Watters *et al.* 1985,b), with X-irradiation damage tending to decrease it. This decrease is not due to a radiation-induced fibrosis since a parallel biochemical investigation showed no net change in the metabolism or net deposition of collagen (Martin 1990). Preliminary results from a collaborative project under way with Dr G. G. Miller (Cross Cancer Institute, Alberta, Canada) indicate, however, that there may be changes in the relative proportion of different collagen isotypes after irradiation.

Use of the proctometroscope to measure compliance offers a relatively simple, rapid and practical method of evaluating the physiology of colonic function in both healthy and diseased small laboratory animals, and is a technique which can be applied clinically. Work is continuing on further characterization of the radiation response, investigating fractionation response, compliance during the acute phase of damage and the time required between measurements before iatogenic effects become important.

Since the proctometroscope is also capable of measuring changes in pressure with time, it may be useful to examine whether colonic pressure or gastrointestinal movement is altered after irradiation. Otterson *et al.* (1988) implicated altered motility of the small intestine after X-irradiation in absorptive abnormalities during the prodromal and acute phases of radiation sickness in dogs. Their findings suggested that the generation of abnormal motor patterns may be one of the major factors in early radiation-induced diarrhoea. Caution should be observed when using the proctometroscope to study these parameters, since a balloon probe responds to mechanical deformation produced by contraction as well as recording pressure changes, making no distinction between the two. Since contraction can occur without significant changes in intraluminal pressure, and changes in pressure can be produced by distant contraction, a record obtained from a balloon probe may be misleading (Davenport 1971). By adapting the system so that an open-ended catheter is used instead of the balloon probe this problem can be circumvented. The system may also be adapted for use in different anatomical sites of different species by altering the design of the probe.

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