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RELATIONSHIP BETWEEN VILLOUS SHAPE AND MURAL STRUCTURE IN NEUTRON IRRADIATED SMALL INTESTINE

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Abstract

Previous work on irradiation of mouse small intestine has assessed the changes produced by counting crypts/microcolonies, scoring villous shape or examining morphological changes in specific parts of the wall.

This paper used scanning and transmission electron microscopy to study the effects of whole body irradiation with 5 Gy neutrons on the surface and internal features of the intestinal wall of CFLP mice, 1 day, 3 days and 7 days after treatment.

Empirical scores from the ultrastructural findings were inserted into a Morphological Index display calculated from analytical data based on cell counts and area measurements obtained from resin histology sections. The final data display showed that the neutron irradiation produced marked structural changes in different cells and tissues by 1 day. These changes were maximal at 3 days with substantial improvement by 7 days.

When this data display was compared with scores taken from scanning electron microscopy of the mucosal surface, the change in villous shape from erect fingerlike projections to lower profiles less suited to absorption was seen to correlate more with changes in the smooth muscle than with the epithelial cryptal compartment.

Key Words:- Scanning electron microscopy, transmission electron microscopy, neutron irradiation, Morphological Index, villous scores.

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Introduction

One principal aim of radiobiology research is to understand better the side-effects of anti-tumour therapy, in order that treatment schedules may be designed to minimise discomfort and late effects. This requires that basic research be undertaken to investigate the changes in neighbouring normal tissues when tumours are irradiated. Small intestine is at risk during abdominal irradiation and the importance of the radiation response in this organ is reflected in early papers (Quastler, 1956) and substantial reviews (Potten et al, 1983; Potten, 1990).

Although crypt/microcolony counting was for many years the standard method used to assess radiation induced changes to the small intestine (Withers and Elkind, 1970), other approaches have been used. For example, since the absorptive function of the small intestine is dependent on the villous projections of the mucosal surface, radiation induced changes in villous shape have been extensively examined using scanning electron microscopy (Carr and Toner, 1972; Anderson and Withers, 1973: Carr et al, 1979, 1981; Friberg, 1980) and scored to give a semi-quantitative assessment (Carr et al, 1983). One of the important findings of this approach has been the fact that villous and cryptal compartments do not respond identically to radiation (Carr et al, 1979), thereby inferring that knowledge of changes in microcolony numbers, an epithelial compartment, may not in itself be sufficient to produce a complete understanding of the behaviour of irradiated small intestine, but that other tissue types may also be affected and in turn have an influence on villous shape.

The hypothesis that the villous shape was affected by the neuromuscular component of the intestine stemmed from the quick villous collapse and recovery seen after hyperthermia (Carr et al, 1983, Kamel et al, 1985). This hypothesis was tested by the use of reserpine, a pharmacological agent chosen because of its effects on adrenergic neurones and the likelihood therefore that it might mimic radiation induced changes in the intestinal wall (Indran et al, 1985, 1991). Application of the system of villous scoring already established

for the study of radiation induced changes in villous shape showed that the overall effects of radiation and reserpine were indeed approximately equivalent, indicating that at least some of the radiation induced shape changes may be due to neuromuscular effects, rather than being necessarily secondary to cryptal, epithelial damage.

The fact that villous shape alters synchronously with other structural effects of radiation was also explored using resin histology and transmission electron microscopy (Carr et al, 1984) and ultrastructural changes in several cell types were demonstrated in neutron and gamma irradiated intestine (Carr et al, 1985). There has, however, been little discussion of the way in which the response of different cell types to insult might vary or of the possibility that cells might produce secondary effects on each other, although one study has measured area related villous measurements and cell counts in a comparison of control and cytosine arabinoside treated intestinal epithelial and stromal compartments (Wright et al, 1989). There have, however, been general comments on how morphological radiation damage contained patterns common to several tissues (Fajardo, 1989) and the need for an understanding of the pathophysiology of multiple cell populations (Rubin, 1989).

In response to this need for further data, recent work (Carr et al, 1991a), based on cell counts and tissue areas, has compared the response of several cell types, showing that different small intestinal cells and tissues respond differently. The approach also uses control-referenced ratios in a Morphological Index data display summarising the quantitative changes for constituent cells, tissues and total damage (Carr et al, 1991a).

To date, the Morphological Index approach has been used to highlight differences between the effects of a) neutron and X-ray treatments (Carr et al, 1991a) and b) abdominal and whole-body X-irradiation (Carr et al, 1990, 1991b) and, in addition has been used to investigate the effects of radioprotectants misoprostol and WR-2721 (Carr et al, 1991c). The Morphological Index display has so far only included data based on histological measurements on resin sections, but there is a need to modulate the resulting figures with subcellular information obtained using transmission electron microscopy, such as that previously reported by Quastler and Hampton (1962) or Lieb et al (1977).

The present paper aims to integrate the information now available on radiation induced changes in villous shape (Scanning electron microscopy - SEM) with histological (light microscopy - LM) and ultrastrucural data (transmission electron microscopy - TEM) collected on the varying responses in different cells and tissues within the intestinal wall in order to test further the hypothesis that the villous changes are related more to neuromuscular effects than to cryptal epithelial damage.

Because of the large number of parameters scored and the complexity of different cellular responses and interactions, the study has been designed, in the first instance, to be limited to a single radiation dose (5 Gy Neutrons) and to the period of acute injury (1-7 days). It is feasible that the work could be extended, using the final integrated SEM, LM, TEM data approach, to compare differential effects of radiation quality and dose at late, in addition to early, times after irradiation.

Materials and Methods

Experimental design

The data are drawn from two experiments, one carried out to investigate the effects of radiation on villous shape and cellular ultrastructure and the other to study the changes induced in different cells and tissues of the small intestine. The animals used in each case were 10 - 12 week old female CFLP mice, with 3 animals per point. The scanning and transmission electron microscopy experiments were carried out on specimens 15 cms from the pylorus, to allow comparison with previous work on villous shape (Carr et al, 1983).

The full circumference resin histology was carried out on duodenum, chosen to allow comparison with work done on endocrine cell changes after irradiation; a description of these results has already been given, without integrating them with the villous scores and ultrastructural findings (Carr et al, 1991a).

Each experiment had its own control specimens, with irradiated material compared with appropriate untreated material. For the comparison of villous shape, ultrastructural changes and cell and tissue effects, the two experiments have been pooled.

Radiation

Unanaesthetized, unrestrained mice were placed in a Perspex box and given a whole-body dose of 5 Gy cyclotron-produced neutrons (mean energy 7.5 MeV, with a gamma-ray contamination of 4%) at a dose rate of 0.45 Gy min^{-1} and a beam current of 100 μ A. Sham-treated animals were treated as above but were not irradiated: these tissues were collected 8 days after sham treatment.

Villous scoring and transmission electron microscopy

Animals were killed by cervical vertebral dislocation. Six animals per time point were examined.

For each animal, pieces of small intestine (2-3cms long), were fixed with 5% glutaraldehyde (buffered with Millonig's phosphate buffer) at 4.0°C (pH 7.4) for 1 to 3 weeks, using the method described by Carr and Toner (1972). Following fixation, the gut was opened longitudinally opposite the line of the mesentery and washed out with buffer solution. Suitable portions of tissue were slightly stretched and fixed by pins on pieces of cork. The specimens were dehydrated and then critical-point dried and mounted on aluminium stubs with a carbon conductive cement or silver paint. They were then coated with either gold or gold/palladium for 4 minutes using a Polaron E5000S sputter-coater.

The specimens were examined in a JEOL T300 scanning electron microscope. The whole area of each specimen (1.5 cm x 1 cm) was examined.

Specimens were scored according to the score system described by Carr et al (1983) to quantify villous collapse through clearly defined stages linked to changes in villous area (Altmann, 1974). The stages and scores are as follows:-

Stage	Score
Normal, erect (VE) or laterally collapsed villi (VL)	0
Vertically collapsed villi (VV)	1
Horizontal villi (VH)	1
Conical villi (VC)	3.5
Rudimentary villi (VR)	6
Absent villi (VA)	8.5

For each animal, the final score was calculated as the mean value of the different scores for the different villous patterns as observed in 10 scanning micrographs taken at 200 times magnification.

For TEM, 1-2 mm fragments from an area opposite the mesentery were taken. Processing for TEM, microtomy and examination in the transmission electron microscope were done routinely. A 1 μm thick section stained with toluidine blue was examined before the block was trimmed for cutting thin sections. Electron microscopy was carried out using Philips EM200 and Philips EM301 instruments.

Full circumference resin histology and compilation of Morphological Index data collection

The methods and results for this part of the work have already been published (Carr et al, 1991a). However, the technique can be summarised briefly as follows -

Specimens of mouse duodenum were fixed as above. Tissue was embedded in Epon 812 (Emscope Laboratories, Ashford, Kent, U.K.) and sections stained with methylene blue and basic fuchsin (Aparicio & Marsden, 1969).

Counts were made per circumference for major cell types and structural elements such as nerve plexuses, some villous, some cryptal and some deep to the crypts. Area measurements were made of all four tissues, epithelium, connective tissue, muscle and nerve. Data were also collected for the numbers of crypts/microcolonies and mitotic figures.

Counting was done blind on coded material. A standard approach was maintained by one experienced observer instructing and spot checking other observers.

The data were subjected to one-way analysis of variance and multiple comparisons between means were tested by using the Scheffe-F method (Sokal & Rohlf, 1981).

The data were also entered into an equation, to produce Morphological Indices for tissues and a total Index, giving an indication of total structural damage. The equation for tissues and cells is as follows in equation (1)

$$(1)\quad M^{o}I = {}^{x}\Sigma_{n=1}\{TWF_{n}.TD_{n}.[{}^{y}\prod_{m=1}{}^{m}CD]\}$$

where Morphological Index ($M^{o}I$) is the total figure of structural change for any specimen or group, calculated by entering into a mathematical model data for tissue weighting factors, tissue deviations and cell deviations.
Tissue Weighting Factor (TWF) is the proportion of the total section area taken up by any one tissue.
Tissue Deviation (TD) is the amount by which the experimental tissue deviates from control i.e. the ratio of experimental to control specimens with regard to tissue areas.
Cell Deviation (CD) is the amount by which the number of cells or structural features of a particular type in the experimental situation deviates from that of control i.e. the ratio of experimental to control counts.
x is the number of tissue compartments, in this case four i.e. epithelium, stroma or connective tissue, muscle and nerve; and y is the number of cell types or structural units counted.

A Cell Weighting Factor could also be applied, reflecting the relative proportion of each cell type or structural feature. In the testing of the model, this factor is taken as 1, indicating equal weighting for each cell type and simplifying the calculation. All deviation ratios are less than 1, as the direction of change from control is not considered.

In order to include an extra level of information, a new factor is included as follows in equation (2) -

$$(2)\quad M^{o}I = {}^{x}\Sigma_{n=1}\{TWF_{n}.TD_{n}.[{}^{y}\prod_{m=1}{}^{m}CD].[{}^{m}UD]\}$$

where UD = ratio of treated to control empirical scores for ultrastructural features.

Results

Although large numbers of slides and micrographs were examined, the aim of the work was the collection of quantitatve data. The bulk of the illustrations in this paper are therefore made up of tabular presentation of data, although a few representative micrographs have been included.

Villous shape

Untreated and sham irradiated duodenal villi were in general erect (Figure 1 inset), slightly flattened in cross section and with slightly tapering tips. One day after neutron irradiation, the villi were still erect, but with signs of fusion or swelling and superficial sloughing of cells. Three days after irradiation, the villi were lower, often with a conical shape, although sometimes the villi collapsed laterally to one side. Maximum damage was seen 7 days after treatment, with the changes similar to those seen earlier, but to a greater extent (Figure 1).

The villous scores are shown in Table 1, with mean values, ranges and standard deviations all given. Statistical comparison of the raw data using the Student's t test showed that the

7 day point was the only one to show a statistically significant change after treatment by comparison with the control group. However, the micrograph analysis was done using a subjective assessment, with erect villi seen at all time points and over reliance on statistical testing may be unwise. Note should therefore also be taken of the fact that the mean value of the villous score shows increasing damage from 1 day onwards.

Table 1

Mean villous score values at different times after 5 Gy whole body neutron irradiation

Time point	Score ± standard deviation (range in brackets)
Controls, sham irradiated	0.25 ± 0.29 (0.0-0.5)
1 day	0.8 ± 0.26 (0.5-1.0)
3 days	1.0 ± 0.49 (0.5-1.5)
7 days**	1.6 ± 0.56 (1.0-2.25)

Note that when this score system was developed (Carr et al, 1983), the baseline for normal villous shape was defined as 0, with increasing damage producing a higher score. This is different from the Morphological Index approach where all irradiated data are compared to a control value of 100%.

** Statistically significantly different from control after Student's t test

Ultrastructural changes

The preliminary resin histology study of small sections of duodenal tissue showed that the histological features of the control specimens were all normal and there were no abnormal features of note when thin sections were examined with a transmission electron microscope. In particular, the villous stroma was close-packed and the epithelial cells showed little separation (Figure 2). After irradiation, changes were seen at all time points and in several types of cell, with both epithelial and stromal cells often showing marked signs of separation (Figure 3). It is evident that many epithelial cells exhibit a multinucleate condition or have a multilobular nucleus following irradiation (Figure 4). The presence of dense bodies within the cytoplasm is also a common feature (Figure 4). The data are displayed in Table 2, where the parameters used are grouped according to the type of tissue studied, with epithelial, stromal, muscle and nerve in different categories.

Subsequent to the initial assessment, a score of 1 was alloted to all normal structures, a value of less than 1 indicating either positive or negative deviation from this and representing an increase in the number of abnormal features present or a decrease in the normality of the structures seen. A total figure for ultrastructural damage for each tissue was calculated as the mean of the individual tissue values in Table 2 and is given in Table 3.

Table 2

Scores for transmission electron microscope results.

Epithelium (villous)		1d	3d	7d
Nucleus/	shape	+	++	+++
	bodies	-	+	++
	nucleoli	+	+	++
Cytoplasm/	Golgi	+	+	ND
	mitochondria	+	++	+++
	inclusion bodies	-	+	++
	microvesicles	++	+++	+
Membrane/microvilli		-	+	++
Epithelium (cryptal)				
Cell separation, shape		+	++	+++
Paneth cell structure		-	+	-
Nuclei		+	++	+++
Nucleoli		+	+	ND
Inclusion/general		-	++	+
	crystalline	-	++	+
Membrane/microvilli		-	ND	+
Connective Tissue				
Cell separation		+	++	+++
Oedema		+	+	+
Cytoplasmic vacuoles		+	++	+++
Inclusion bodies		+	+	++
Capillary endothelium, blebbing, inclusions		+	+	+
Muscle				
Rarefaction, vacuolation, myofilamentous degeneration		+	++	+++
Nerve				
Dense bodies		ND	ND	+

* including sham irradiated
KEY - Normal; +, ++, +++ represent minimal, moderate and substantial changes respectively
ND - No data

Table 3

Mean figures for ultrastructural changes*

Tissue	Con	1d	3d	7d
Epithelium	1	0.88	0.70	0.57
Connective tissue	1	0.80	0.72	0.60
Muscle	1	0.80	0.60	0.40
Nerve	1	ND	ND	0.80

* Calculated from values arbitrarily assigned to deviations from normality listed in Table 2. A Score of 1 = normality; 0.8 = (+); 0.6 = (++); 0.4 = (+++); 0.2 = (++++)

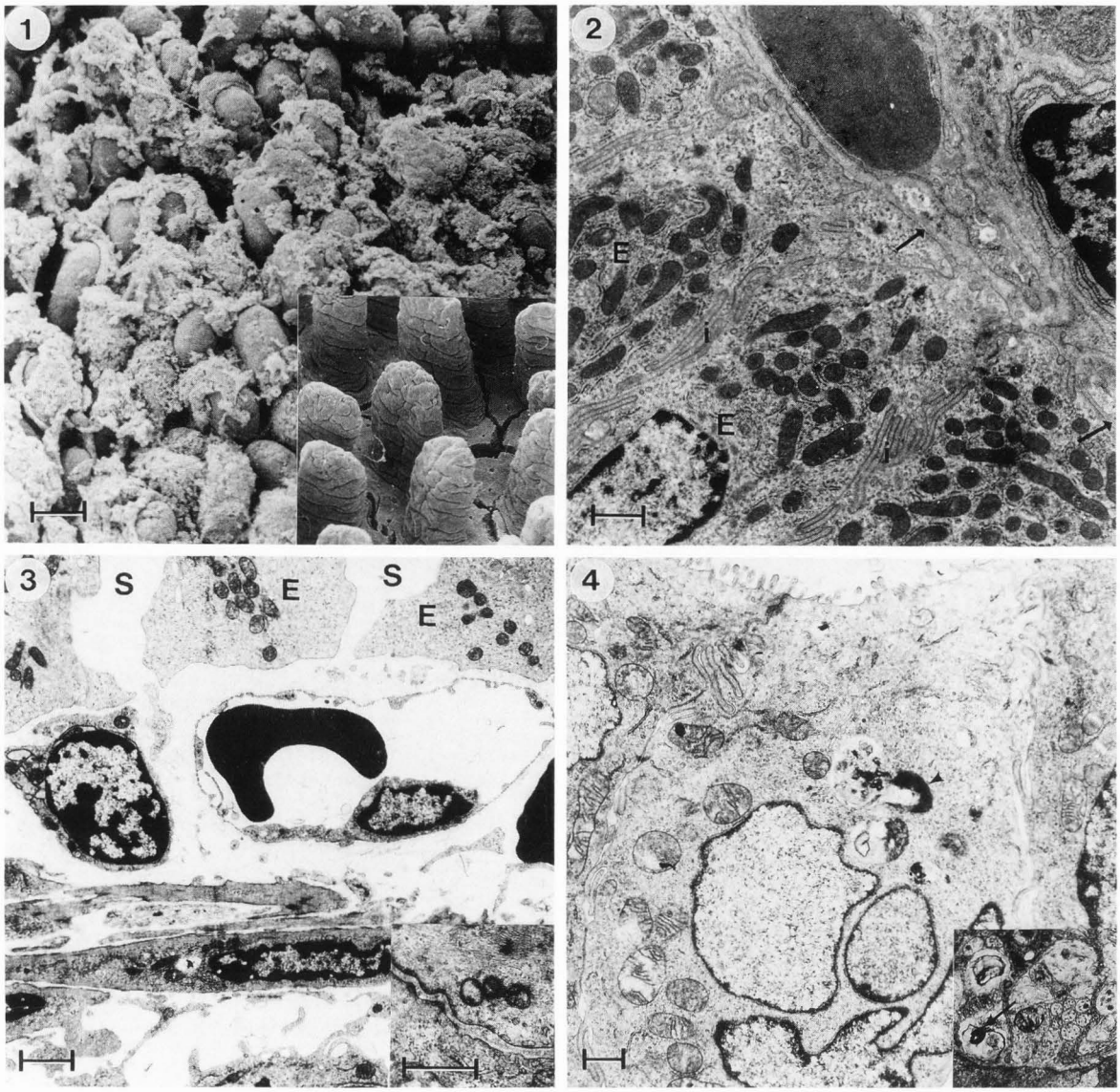

Fig. 1: Scanning electron micrographs of small intestinal villi from 7 day neutron irradiated and control (inset) mice. Note that iradiated villi are covered by prominent mucus and debris whereas control villi are clean, finger-shaped and erect.
Bar = 100 μm

Fig. 3: Transmission electron micrograph of the villous epithelial/stromal boundary 7 days after 5 Gy neutron irradiation showing large spaces (S) with no interdigitations between adjacent epithelial cells (E). The stromal cells show irregularity and are also well separated from each other with large intracellular spaces. Note also a dilated blood capillary.
Bar = 1 μm
Inset: Myofilaments in a nearby smooth muscle cell are slightly separated, 3 days after irradiation.
Bar = 1 μm

Fig. 2: Transmission electron micrograph of the villous epithelial/stromal boundary in an untreated control mouse. Cell membranes and the lateral surface of the epithelial cells (E) show prominent interdigitations (i). The closely packed underlying villous stromal structures can also be seen adjoining the epithelium at the basal lamina (arrow).
Bar = 1 μm

Fig. 4: Transmission electron micrograph of a cryptal epithelial cell 3 days after 5 Gy neutron irradiation. The cell is multinucleate or contains a multilobed nucleus. Note the presence of dense inclusion bodies (arrow) in the cytoplasm.
Bar = 1 μm
Inset: Nerve profile in villous stroma 3 days after irradiation which shows dense bodies (arrow).

Integration of ultrastructural deviations into Morphological Index data display

In order to put the Morphological Index data in context with other methods of assessing intestinal damage, the results for the numbers of jejunal crypts and mitotic figures are reported first, with standard errors in brackets and asterisks marking values statistically significantly different from the corresponding control figure -

Crypts/ circumference: untreated control/ 229.8 (±54.5), sham irradiated/ 154.0 (±8.9), 1 day/ 169.2 (±17.0), 3 days/ 118.9* (±20.9), 7 days/ 100.3* (±6.2).

Mitotic figures/ circumference: untreated control/ 118.7 (±33.7), sham irradiated/ 61.4* (±2.9), 1 day/29.5* (±1.8), 3 days/35.1* (±2.7), 7 days/ 63.6* (±6.0).

The three level Morphological Index display for the 3 time points studied is given in Figure 5. Apart from the ultrastructural information, already described in this paper, the data is taken from previously published work on jejunal specimens from the same strain of mice, irradiated with the same dose of neutrons (Carr et al, 1991a).

Figure 5 contains a large amount of information, summarising the response to radiation of the four main tissues, based on area changes, counts per circumference for ten cell types or structures such as vessels and nerve plexuses and changes at the ultrastructural level. The data display can be broken down into several parts. There are Tissue Weighting Factors, based on areas of the tissues, which allows for the relative abundance of each in an estimate of overall change in the organ. In addition to this, the display has three sections, each showing deviations from the control: the sections show change in tissues (Tissue Deviation), cells (Cell Deviation) and subcellular structures (Ultrastructure Deviation).

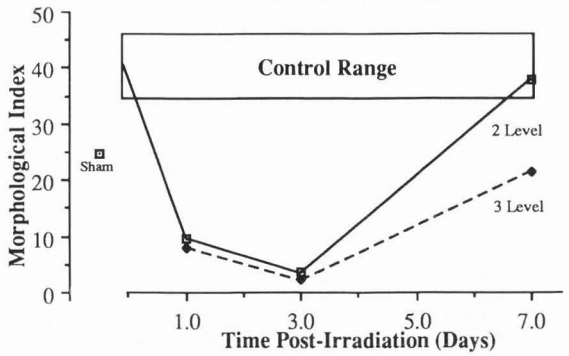

Fig. 5: Multiple Morphological Index display summarising tissue, cell and ultrastructural information. The graph at the top plots the overall structural changes, while the three tables give information on proportion of areas taken up by each tissue and ratios of treated to untreated data for cells and ultrastructural scores. The Tissue Indices are obtained by multiplying the figures within each horizontal line, the total Index figure by adding the relevant Tissue Indices. Asterisks represent individual parameters which are significantly different from control.

	TWF	TD	CD				2 Level Tissue Index		UD	3 Level Tissue Index
E	70.5	0.95	Enterocyte 0.64	Goblet 0.95	Endocrine *↓ 0.11	Paneth 0.87	3.90		0.88	3.43
CT	21.7	1.00	Villous Stromal Cells (0.66)²		Submucosal Arterioles (0.60)²		3.40		0.80	2.72
M	7.5	0.83	Outer Muscle Nuclei (0.80)²		Inner Muscle Nuclei (0.75)²		2.24		0.80	1.79
N	0.3	0.50	Auerbach *↑ (0.63)²		Meissner (0.70)²		0.03		ND	0.03
5 Gy Neutron 1 Day					2 Level MoI (analytical)		9.57		3 Level MoI (empirical)	7.97

	T W F	T D	C D				2 Level Tissue Index	U D	3 Level Tissue Index
			Enterocyte	Goblet	Endocrine	Paneth			
E	63.4	0.85	*↓ 0.30	*↓ 0.45	*↓ 0.06	0.64	0.23	0.70	0.20
			Villous Stromal Cells		Submucosal Arterioles				
CT	27.9	0.87	*↓ $(0.31)^2$		$(0.67)^2$		1.05	0.72	0.76
			Outer Muscle Nuclei		Inner Muscle Nuclei				
M	8.4	0.83	$(0.83)^2$		$(0.67)^2$		2.16	0.60	1.30
			Auerbach		Meissner				
N	0.3	0.50	*↑ $(0.45)^2$		$(0.88)^2$		0.02	ND	0.02
5 Gy Neutron 3 Days					2 Level MoI (analytical)		3.51	3 Level MoI (empirical)	2.28

	T W F	T D	C D				2 Level Tissue Index	U D	3 Level Tissue Index
			Enterocyte	Goblet	Endocrine	Paneth			
E	73.2	0.84	0.80	0.93	0.86	0.85	33.44	0.57	19.06
			Villous Stromal Cells		Submucosal Arterioles				
CT	19.3	0.97	*↓ $(0.47)^2$		$(0.76)^2$		2.39	0.60	1.43
			Outer Muscle Nuclei		Inner Muscle Nuclei				
M	7.2	0.87	$(0.97)^2$		$(0.58)^2$		1.99	0.40	0.79
			Auerbach		Meissner				
N	0.3	0.50	$(0.77)^2$		$(0.99)^2$		0.09	0.80	0.07
5 Gy Neutron 7 Days					2 Level MoI (analytical)		37.90	3 Level MoI (empirical)	21.35

The tables above and at left are part of Fig. 5 (see figure 5 legend).

The display shows that several individual cell parameters are statistically significantly different from the corresponding controls (marked with an *), as follows - apparent decrease in endocrine cell numbers and increase in number of Auerbach's plexuses 1 day after irradiation, decrease in enterocytes, goblet cells, endocrine cells and stromal cells and continuing increase in number of Auerbach's plexuses at 3 days and a decrease in the number of villous stromal cells by 7 days.

Two subtotal Indices for each tissue are shown, one for a two level Index (Tissues and cells) and the other a three level Index (Tissues, cells and ultrastructure): each is obtained by multiplying the Tissue Weighting factor by the appropriate Tissue, Cell or Ultrastructural Deviation. The total three level Index for the whole organ is obtained by adding the Tissue Indices and can be either a two level Index (containing tissue and cell information) or a three level Index (containing in addition ultrastructural data). The two level figure is an analytical Index, based on the data measured using objective criteria. The ultrastructural deviation is, however, based on a subjective score, giving an empirical Index and inclusion of this material in the final display ensures that the final Index is also empirical.

The display shows that, while Tissue Indices for epithelium, connective tissue and nerve all show a drop at 1 day and 3 days, with recovery by 7 days, muscle has a different pattern, with damage still dropping at the last time point. The final figure for each time point shows that the structural change is at a maximum 3 days after treatment, with recovery clearly seen by 7 days.

Relationship between surface and internal changes

Statistical analysis of the villous scores indicates that only at 7 days is there a significant difference between the control and the irradiated material. However, at 1 day, there are villous shapes which are not seen in the control specimens, indicating that statistical analysis of score values, although useful for highlighting marked changes, hides important trends in the changes in the villous shape. The data for this parameter is therefore best analysed by looking at the micrographs qualitatively and at trends in the score values (Table 4). The trend in villous shape change is that the damage is identified at 1 day and increases through 3 and 7 days.

To compare the villous shape with other data, statistical testing can be used to assess the cell deviations and analytical Indices. None of these parameters show the same pattern of damage through these time points. The empirical results can only be assessed by looking for trends. Table 4 lists the empirical parameters and indices which show this trend. These include the three level muscle Index and several ultrastructural features.

Table 4

Comparison of trends with time in villous scores, indices and parameters

Parameter	1d	3d	7d
Villous Scores	↓	↓↓	↓↓↓
Epithelial 3 level index	↓	↓↓	↑↑↑
CT 3 level index	↓	↓↓	↑
Muscle 3 level index	↓	↓↓	↓↓↓
Nerve 3 level index	↓*	↓↓*	↑↑
Villous nuclear shape	↓	↓↓	↓↓↓
Mitochondria	↓	↓↓	↓↓↓
Cryptal cell separation	↓	↓↓	↓↓↓
Cryptal cell nucleoli	↓	↓↓	↓↓↓
CT cell separation	↓	↓↓	↓↓↓
CT vacuoles	↓	↓↓	↓↓↓
Muscle ultrastructure	↓	↓↓	↓↓↓

KEY

↓	deviation at 1 day by comparison with control
↓↓	further deviation at 3 days
↓↓↓	further deviation at 7 days
↑	return towards control level
↑↑	marked return towards control level
CT	connective tissue
*	2 level $M^{O}I$ only

Discussion

The discussion of the material reported in this paper falls into two separate parts, consideration of experimental design and biomedical implications of the data.

Experimental design

The data stem from two different sets of experiments, in which the common features are that the studies were carried out by the same collaborating research groups, with mouse strain, sex, age, radiation conditions and collection procedures being standard. The variations are precise site or size of specimen taken and the type of statistical analyses used to assess the data, since these varied with the specific needs of the project or changed as knowledge or expertise improved.

The bringing together of the two sets of data allows a better understanding of radiation induced structural changes in two adjacent regions of proximal small intestine, an organ with one principal function, that of absorption. The integration of the results assumes that duodenal villous scores can be used alongside jejunal information from the same mouse strain. Comparison of previously published material shows that the mean value for jejunal villous scores at 3 days for two different strains of neutron irradiated mice is 0.85 (Carr et al, 1983, 1984), while that quoted in this paper for CFLP mice is 1.0, well within the same range. Likewise, although the absolute figures for the previous data on time related neutron induced changes (Carr et al, 1984) give lower villous scores throughout the experiment for jejunum, nonetheless the trend upwards from 1 day through to 5 days is similar to that reported here. The only previous data on duodenum is for gamma irradiated tissue (Indran et al, 1985, 1991), where the figures compare well with the corresponding data for gamma irradiated jejunum (Carr et al, 1983). It therefore seems

appropriate to compare the duodenal villous scores with the jejunal resin histology data.

With respect to ultrastructural information, there have been several reports of such damage to epithelial cells (Quastler and Hampton, 1962), and also to connective tissue, nerve and muscle (Lieb et al, 1977, Abbas et al, 1990a,b, Carr et al, 1985). The ultrastructural information included in this paper confirms the range of changes seen and since duodenal and jejunal morphology are similar both in control specimens and after irradiation (Abbas, 1990), the jejunal information described in this paper can be used to extend the description of the changes from the cellular to the subcellular.

The general approach to the Morphological Index display has already been described (Carr et al, 1991a). However, since the approach is a new one, some brief comments on the methods can be given here. The whole approach of bringing together data of different types is necessary because of the non-standard response of the different cells and tissues to irradiation. The production of a summary figure by integrating data from different tissues and cells allows comparison of different points within an experiment, and gives information on the likely overall effect.

The form of the equation is based on the hypothesis that an organ's response may involve all its major cell and tissue types. The first principal assumption made in the construction of equation (1) is that tissues are considered independently of each other and therefore adjacent tissues and the corresponding Tissue Deviations should appear under the summation in equations (1) and (2). On the other hand, constituent cells within a tissue are regarded as mutually interdependent and are related via multiplicative procedures. One advantage of the approach is that all deviation ratios are directly control referenced, allowing all Index figures to be directly compared across radiation schedules. This contrasts with the technique used for the calculation of relative biological effectiveness (RBE), where data calculated for any one schedule comparison are based on one end point and cannot easily be used for comparison with other schedules.

When the ultrastructural data are entered into the Morphological Index display in order to obtain a three level Index figure, only one extra ratio per tissue has been used, representing the mean of the changes seen in various aspects of the organelle or membrane changes. Entry of all individual scores separately would imply that each ultrastructural reading was related to other subcellular changes, an assumption which is not necessarily valid. It would also be more difficult to standardise the entries for the four tissues, since tissues with a greater collection of micrographs would appear to have greater subcellular damage. For these two reasons, it has been decided to use the approach described, which also allows the Index display to show clear comparison between the two level and three level Indices.

The fact that the two level Index is analytical, while the three level Index is empirical has already been described in the Results section. The previous paper (Carr et al, 1991a) states that the total Morphological Indices for the 1, 3 and 7 day points for the 2 level analytical Index are different from each other. However, no such comment can be made for the 3 level Indices, because the ultrastructural results were scored from a pool of micrographs for all the animals, therefore not allowing separate Indices to be prepared and compared within and among groups.

Biomedical implications

The experimental design section has described that the villous scores are in the region of those collected previously (Carr et al, 1984, Indran et al, 1985, 1991). The untreated and sham control levels show that there is little villous collapse in the control population, suggesting good gastrointestinal health. The scores shown in Table 1 show that a villous shape change occurs 1 day after treatment. The score continues to rise till 7 days and extension of the experiment beyond this (Kamel, 1985), shows that at 9 days the villi are beginning to return to normal, indicating that 7 days is the time of peak damage.

The integration of the ultrastructural information into the Morphological Index display shows that the ultrastructural deviation figures reduce the total Index further at all time points, particularly so at 7 days. This changes the shape of the graph little by comparison with the two level curve, where damage was seen at 1 day, maximum at 3 days and compensated for by recovery by 7 days. The conclusion here is that the use of techniques other than light microscopy allows the recording of unsuspected changes, such as those visible in Paneth cells or muscle layers. Further information could be added from other techniques, such as histochemistry or immunocytochemistry. The paper also reports on data for crypt counts and mitotic figures, since these provide information on changes to these parameters, which have been more widely studied by other groups. It should be noted that these measurements, like the Morphological Index, represent a final figure for changes happening within a composite cell population. The mean number of crypts in control animals was observed as 229.8 ($\pm$54.5). This value may seem high, especially when compared to another study on the same strain of mouse (by Hornsey et al., 1988) who calculated the number of crypts to be 180-190 per circumference. In the present study, the larger figure may be explained by an unusually high reading from an individual animal but given the equally high standard error the results still fall within the expected range.

The main aim of the work was to explain the change in villous shape, since this is important for the absorptive process. The comparison of trends in Table 4 shows that several data sets have a similar pattern to that of the villous scores, with damage increasing with time to 7 days. The muscle Index also drops steadily to the 7 day value. At tissue level then, there is a suggestion that the muscle layer may be linked to the villous shape.

When the ultrastructural information is examined, there are three groups of data with the appropriate pattern of response. The first group includes epithelial nuclear shape and mitochondria, epithelial cryptal cell separation and cryptal nucleoli. It is possible that these are secondary to the change in villous shape rather than being implicated in its cause. The reduction in number of epithelial cells following radiation induced disruption of proliferative capacity could lead to some of the changes observed. The second group of parameters with the appropriate pattern of response includes connective tissue separation and vacuoles, the former of which could be caused by oedema, which could certainly cause villous shape changes. However, the most interesting ultrastructural change with the response similar to that of the villous shape is the presence of rarefaction, vacuolation and myofilamentous degeneration in the smooth muscle cells, since this is in line with the tissue trend already discussed. This finding supports the report of smooth muscle damage underlying villous shape change after reserpine treatment or gamma irradiation (Indran et al, 1985, 1991). However, it does not support the report that 5 days after treatment with 5 Gy neutrons no gaps were observed between irradiated smooth muscle cells (Carr et al, 1984): the discrepancy may be due to the fact that the gaps between the muscle were so obvious in that study that they may have distracted attention from more subtle changes in the cytoplasm. The 1984 study was also the first time that smooth muscle changes had been seen and since then has warranted closer attention.

The current paper therefore adds further support to the hypothesis that radiation induced effects in the wall of mouse small intestine are caused by changes in cells and tissues of several types and that these changes are not necessarily similar to the decrease in crypt/microcolony numbers. The change in villous shape is accompanied by changes in epithelial cell shape and in the stroma, but is most closely linked to alterations in the smooth muscle component of the wall.

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Discussion with Reviewers

L.G. Friberg: In radiotherapy, neutron irradiation is a rare and special technique and 5 Gy a neither low nor high dose. Why did you choose neutron technique instead of gamma-irradiation and why just and only a single dose of 5 Gy?

Authors: The group has studied the effects of several different radiation schedules, including gamma rays, X-rays, neutrons and heavy ions. Where possible, a low, medium and high dose is included, the high dose to allow identification of all possible morphological signs of damage. When a time course is being studied, however, a lower dose is used to minimise the loss of animals. Of the experiments carried out, the neutron time course for a dose of 5 Gy was the first one to be completed, with data available from light and electron microscopy.

L.G. Friberg: Pelvic irradiation is common in the clinic and upper abdomen irradiation is a more seldom treatment. The villi of jejunum, especially in the rat, are high, gracile and fragile in SEM-preparation. Why not study the ileum and simulate clinical work by giving partial body irradiation to the pelvic region instead of whole body irradiation where other parameters may influence the results?

Authors: Partial body irradiation experiments have been done for X-ray treatments. Most of the group's previous work has used proximal rather than distal small intestine. To allow comparison with this previous work, the use of distal small intestine is avoided. The villi of the mice used here have not proved excessively fragile in SEM-preparation.

L.G. Friberg: A single dose of 5 Gy by gamma-irradiation gives only a minor tissue effect as well as functional disturbance and a repair after 7 days. To confirm our ideas of a total expression of effects by means of a formula, would it not be valuable to study effects of a single dose of 7, 9 or 10 Gy?

Authors: The effects of different doses have been studied following treatment with partial body X-rays.

M. Albertson: Do the early effects of neutron radiation observed in the present animal study provide any basis for hypothesis as to the reported effects (in particular manifest late effects) observed after neutron radiation of human tumours, despite toxicity having been tolerable?

Authors: The effects seen here certainly point to the possibility of the involvement of neuromuscular and stromal components in the development of late effects.

M. Albertson: Are further investigations with longer observation periods planned?
Authors: Data are being collated for whole body neutron and X-ray effects 11 days after treatment. Investigations on fractionated doses and late effects are included in the group's long term plans.

R. Poley: While it does make sense that villous changes are almost closely linked to alterations in the smooth muscle component of the wall, as the experiments have demonstrated, what about changes in the microvascular compartment? Would one not expect tropic changes which may also be captured by a morphologic index display?
Authors: Changes in the vascular compartment are already included in the Index display. Villous microvascular changes have been reported following X-irradiation (Abbas B, Boyle FC, Wilson, DJ, Nelson AC, Carr, KE, 1990, Radiation induced changes in the blood capillaries of rat duodenal villi: a corrosion cast, light and transmission electron microscopical study, J. Submicrosc. Cytol. Pathol., 22, 63-70; Abbas B, Hume SP, McCullough JS, Wilson DJ, Stewart PC, Carr KE, 1990, Early morphological changes in blood capillaries of mouse duodenal villi induced by X-irradiation, J. Submicrosc. Cytol. Pathol., 22, 609-614). Data are currently being collected for the 5 Gy neutron experiment. The reason that such data are not routinely inserted into the Index display is that they are collected on transverse sections of villi, while the rest of the Index display refers to transverse sections of the intestine itself.

R. Poley: Is the cellular separation typical for neutron-irradiated intestine? Is it a nonspecific finding or simply an epiphenomenon of injury to the stromal apparatus of the cells?
Authors: The cellular separation is also seen following X-irradiation. It could be caused by vascular changes, alterations to the basal lamina or to other parts of the non-epithelial compartments.

R. Poley: What is the explanation for the increasing number of Auerbach's plexus with increasing irradiation? Is this increase relative, i.e., as compared with the decrease of other cell parameters or is the increase to be understood in absolute terms?
Authors: The increase is probably an apparent one, perhaps caused by post-treatment swelling leading to previously small plexus profiles being sufficiently visible to be counted.

Reviewer IV: Why were these times selected?
Authors: The times were selected to straddle the likely maximum changes visible during the acute phase of damage. Data are available for a 6 hour time point for the Morphological Index (Carr KE, McCullough JS, Nunn S, Hume SP, Nelson AC, 1991a, Neutron and X-ray effects on small intestine summarised using a mathematical model/paradigm, Proc. Roy. Soc. Series B, 243, 187-194), but cell death is not a particularly dramatic feature.