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THE MECHANISM OF THE ANTI-TUMOUR ACTION

OF FLAVONE ACETIC ACID

AND ITS XANTHENONE ACETIC ACID ANALOGUES

by

LEON JONATHAN ZWI

A thesis submitted in accordance with the requirements

for the degree of

Doctor of Philosophy

in the field of Pathology

University of Auckland, May 1992
ABSTRACT

The search for new compounds with anti-tumour activity is driven by the high mortality from disseminated visceral carcinomas, for which few effective treatments exist. The experimental agent flavone acetic acid (FAA) has stimulated interest because of its broad spectrum of activity against solid tumours in mice. This remarkable activity suggested an unusual mechanism of action. The aim of the thesis was to elucidate the mechanism of action of FAA and its analogues in mouse tumours, particularly the rapid induction of haemorrhagic necrosis.

Light and electron microscopic studies of Colon 38 tumours treated with 1.2 mmol/Kg FAA revealed a sequence of tumour cell separation and rounding, focal apoptosis, and finally confluent necrosis with blood vessel engorgement and rupture within 4 - 8 h. This was accompanied by a loss of energy metabolites. The combination of apoptotic and necrotic modes of cell death suggested that both ischaemic and non-ischaemic mechanisms were involved.

The role of blood flow failure was further investigated in Colon 38 and EMT6 tumours using a double label fluorescent vessel-labelling technique, which demonstrated a progressive and sustained fall in blood flow, beginning within 15 min of treatment. A mathematical model of ischaemic tumour cell killing using data from measurements of tumour perfusion, the rate of FAA-induced cell killing and the rate of cell death following global ischaemia indicated that mechanisms in addition ischaemia were involved. However, comparison of the resistance to FAA of avascular intraperitoneal tumour multicellular spheroids and vascularised tumours suggested that the major component of the anti-tumour effect was indeed blood vessel-dependent.

The vessel-independent effects of FAA were then studied in vitro, using EMT6 tumour multicellular spheroids which had been placed for six days in the peritoneal cavities of mice to become infiltrated by macrophages and lymphocytes. These ex vivo spheroids were more that twice as sensitive as spheroids grown entirely in vitro, when exposed to 2.5 mM FAA for 24 h. This provides evidence for immune cell mediation of the FAA anti-tumour effect.

To determine the relative importance of the vessel dependent and the immune mediated effects of FAA, a novel vascularised spheroid tumour system, which contained both avascular and vascular components, was developed and studied. FAA treatment caused necrosis only in the vascularised component, preceded by a loss in perfusion. The avascular tumour tissue also showed evidence of some cell loss by apoptosis. Thus, in tumour tissue not relying directly on blood vessels for metabolite exchange, only limited cell killing occurs, suggesting that treatment with FAA-like agents alone would have limited ability to cure.
The anti-tumour activities of a series of xanthenone acetic acid (XAA) analogues of FAA, were then compared with FAA by examination and measurement of morphological changes in subcutaneous tumours and spheroids, and by measurement of blood perfusion in tumours. Those XAA analogues which were active in inducing haemorrhagic necrosis also caused FAA-like morphological changes in tumours and spheroids, and inhibited blood flow, indicating a similar mode of action to FAA. The parent XAA and FAA were approximately equipotent, and 5-methyl XAA and 5,6-dimethyl XAA were about ten times as potent as FAA. 3-O-methyl XAA, 8-methyl XAA and xanthenone-4,5-diacetic acid showed no FAA-like activities.

Examination of non-tumour tissues after therapeutic doses of FAA and 5,6-dimethyl XAA showed cytolytic lesions, including necrosis, in the peripheral lymphoid tissues, the thymus and the uterus. Thus, although these agents lack the myelosuppressive effects of conventional chemotherapeutic agents, cell killing is not entirely specific for tumours. The sensitive tissues, including tumours, have in common a high content of macrophages or neutrophils, and regions of low vascular density.

The thesis studies have shown that FAA and its analogues differ from conventional cytotoxic (anti-proliferative) agents in their mechanism of action. Direct toxicity against tumour cells is of minor importance compared to macrophage mediated cytotoxicity and perfusion failure. If biological activity can be demonstrated in humans, FAA-like agents may show clinical utility in combination with radiotherapy, conventional or hypoxia selective cytotoxic agents.
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<tr>
<td>3OMX</td>
<td>3-O-methyl xanthenone-4-acetic acid</td>
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<td>5MX</td>
<td>5-methyl xanthenone-4-acetic acid</td>
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<tr>
<td>8MX</td>
<td>8-methyl xanthenone-4-acetic acid</td>
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<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AVS</td>
<td>Avascular spheroid</td>
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<tr>
<td>BCG</td>
<td>Bacille Calmette Guerin</td>
</tr>
<tr>
<td>BDF&lt;sub&gt;1&lt;/sub&gt;</td>
<td>(C52BL/6JxDBA/2J)&lt;sub&gt;F&lt;sub&gt;1&lt;/sub&gt;&lt;/sub&gt; hybrid</td>
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<tr>
<td>CP</td>
<td>Creatine Phosphate</td>
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<tr>
<td>DMBA</td>
<td>7,12-dimethylbenz[a]anthracene</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DMX</td>
<td>5,6-dimethyl xanthenone-4-acetic acid</td>
</tr>
<tr>
<td>Eq.</td>
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<td>FAA</td>
<td>Flavone-8-acetic acid</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GM</td>
<td>Growth medium</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
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<td>Hoechst 33342</td>
<td>Hoechst 33342</td>
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<td>HX</td>
<td>Hypoxanthine</td>
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<td>i.d.</td>
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<tr>
<td>i.m.</td>
<td>Intramuscular(ly)</td>
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<tr>
<td>i.v.</td>
<td>Intravenous(ly)</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>MTD</td>
<td>Maximum tolerated dose</td>
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<tr>
<td>NAO</td>
<td>10-nonyl acridine orange</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>P</td>
<td>Probability</td>
</tr>
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<td>TNF</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<tr>
<td>s.c.</td>
<td>Subcutaneous(ly)</td>
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<tr>
<td>s.e.m.</td>
<td>Standard error of the mean</td>
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<tr>
<td>vol/vol</td>
<td>Volume/volume</td>
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<td>Vascularised spheroid</td>
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<td>Weight/volume</td>
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<tr>
<td>X</td>
<td>Xanthene</td>
</tr>
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<td>XAA</td>
<td>Xanthenone-4-acetic acid</td>
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<tr>
<td>XDA</td>
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CHAPTER 1

CANCER THERAPY AND FLAVONE ACETIC ACID

1.1. Cancer chemotherapy perspective

Cancer is a major cause of death world-wide, but the incidence of the different types varies greatly from region to region (Newell, 1985). For example, Japanese migrants to the United States have a lower incidence of carcinoma of the stomach than those in Japan, while other tumours common in the United States increase in incidence in the migrant group (Haenszel & Kurihara, 1968). This suggests that environmental factors are important and that, once identified, can be avoided. Thus prevention may be an effective (and generally desirable) method to reduce cancer mortality. However, the potential of this approach is limited by the small number of environmental factors which have been identified, and by the difficulty involved in educating and convincing whole populations to avoid exposure to carcinogens where possible.

In nearly all deaths from neoplastic disease, the tumour has metastasised from its site of origin, or has shown extensive local infiltration, limiting curative surgical excision. Thus early detection of cancer and identifiable pre-cancerous conditions offers another effective strategy, but one that is limited to those tumours which are clinically accessible. Success would be unlikely with tumours characterised by long periods of asymptomatic growth (ovarian cancers), tumours which are diffuse by nature (leukaemias and some lymphomas) or tumours which tend to metastasize at an early phase in development (carcinoma of the breast).

During the next few decades, the major hope for preventing cancer deaths overall, and the only hope for those individuals who will present with, or progress to advanced malignant disease, lies in the development of more effective methods of killing cancer cells which have spread beyond the reach of current surgical procedures: radiotherapy, chemotherapy and immunotherapy. Although these types of treatment have resulted in cures and palliation in several tumour types, many common malignancies do not respond to available therapies, and researchers continue to seek new agents and methods of administration.
1.1.1. Thesis overview

New chemotherapeutic agents originate from two major sources: screening of large numbers of biological and synthetic compounds for anti-tumour activity, and deliberate design of chemicals for this purpose, sometimes by modification of existing compounds. Flavone acetic acid (FAA) (Figure 1.1) belongs in the former category, as it was originally synthesized (as the diethylethanolamine ester) as a possible anti-inflammatory agent, but was found to be active in tumours in vivo in a National Cancer Institute screen (Plowman et al., 1986). Xanthenone acetic acid (XAA) (Figure 1.1) and its derivatives are of the second type, as they were synthesised as FAA analogues for the purpose of improving anti-tumour activity (Atwell et al., 1989).

FAA has generated considerable interest both in the laboratory and the clinic because of its interesting activity profile in mice: activity is only seen in vivo and against solid tumours, and nearly all solid tumours appear to be sensitive (summarised in Section 1.6.; Table 1.1, p. 44). These findings have interested tumour biologists because they suggest a novel mechanism of action for this agent. Clinical interest has stemmed largely from the broad spectrum of activity of FAA, particularly against resistant mouse colonic carcinomas and against resistant human colonic adenocarcinomas grown as xenografts in mice (Table 1.1). Unfortunately, clinical trials have not shown anti-tumour activity. The reason for this difference in activity in mice and humans may emerge when the mechanism of action of this compound is better understood. Elucidation of the anti-tumour mechanisms of FAA and its analogues is the major objective of this thesis.

Chapter 1 reviews historical aspects of chemotherapy and radiotherapy, the reasons for resistance to these treatments, and approaches to overcome resistance including the search for new tumour targets. "Target" is defined as the characteristic of a particular tumour that determines its sensitivity to damage, relative to normal tissues, for a particular agent. A target may exist at various levels of organisation within the tumour. Whereas most agents depend on delivery to, and interaction with, individual tumour cells, other types of treatment (most still experimental) depend on some feature of the tumour at the tissue level, and so may be inactive in vitro. FAA appears to belong to the latter group. This thesis aimed to determin at which level of tumour organisation the FAA target exists, and its exact nature.

For this reason, aspects of the structure and physiology of solid tumours are reviewed, particularly their blood supply (Section 1.3), microenvironment (Section 1.4), and interactions with the immune system (Section 1.5). All of these are of therapeutic interest, and the possibility that FAA acts through them is considered. The literature review then focusses on the macrophage, and a major macrophage product, tumour necrosis factor-α (TNF; Sections 1.5.3. - 7.). The similarities between
FIGURE 1.1

FLAVONE ACETIC ACID

XANTHENONE ACETIC ACID
TNF and FAA are then discussed (Section 1.5.8.) and the small literature on FAA at the time of commencement of the thesis is reviewed (Section 1.6). The first chapter concludes with a statement of the aims of the thesis (Section 1.6.3., p. 47).

Chapters 2 to 7 give an account of the experimental work. That described in Chapter 2 investigated the mechanisms of injury of tumour tissue in vivo, and suggested that both ischaemic and non-ischaemic effects occur. Chapter 3 reports studies that directly examined the role of failure of the tumour blood supply, and which concluded that perfusion failure (ischaemia) is a major component of the anti-tumour action of FAA. The investigations comprising Chapter 4 examined the nature of the non-ischaemic component in vitro in tumour multicellular spheroids, and provided evidence that macrophages also mediate cytotoxicity. A direct comparison of the vascular and the immune mediated effects is described in Chapter 5. Research described in Chapter 6 compared the activities of XAA and its derivatives with those of FAA, and the studies of Chapter 7 examined the toxic effects of therapeutic doses of FAA and 5,6-dimethyl XAA in non-tumour tissues of the mouse. The thesis concludes with Chapter 8 which integrates the findings, discusses the implications, and suggests directions for further investigation.

1.1.2. The development of chemotherapy

The history of cancer chemotherapy has many parallels with that of antimicrobial chemotherapy in its search for substances with selective toxicity. The term "chemotherapy" was applied by Paul Ehrlich early this century for chemicals used to treat systemic parasitic infections. His systematic evaluation of synthetic compounds for activity against bacterial and protozoal infections in laboratory animals led him to the discovery of salvarsan (arsphenamine), which is active against spirochaetes and trypanosomes (Marshall, 1964).

Following Ehrlich's successful use of animal models in infectious disease research, George Clowes developed inbred rodent strains that could carry transplanted tumours, against which potential anti-cancer agents could be screened. The application of alkylating agents to neoplastic diseases was the next major advance (reviewed by DeVita, 1985). These were originally developed as chemical weapons, but aroused interest as anti-lymphoma agents. This occurred because bone marrow and lymphoid hypoplasia were observed in seamen accidentally exposed to alkylating agents following an explosion in Naples harbour during World War II. Clinical trials were conducted and remissions were seen, but all the tumours recurred (Marshall, 1964).
Techniques developed in the 1960's for studying the cell cycle clarified certain critical differences between cells from normal adult tissues and cancer cells (Denekamp, 1982a). Skipper's studies on the L1210 mouse leukaemia demonstrated the relationship between number of cells inoculated, doubling time, and survival time, and showed that anti-cancer agents killed cells by first order kinetics (number killed is proportional to initial number) rather than zero order kinetics (Skipper et al., 1964), as had previously been assumed. The differences in growth fraction (proportion of cells actively proliferating) between cancers and normal tissues was soon implicated as the basis of the differential toxicity observed when effectively treating rapidly-growing tumours in experimental systems (DeVita, 1985).

The use of multi-agent chemotherapy has resulted in the successful treatment of many cancers in the last two decades. Cures are now seen with certain haematological malignancies, embryonic tumours, germ cell tumours and other solid tumours of childhood (Devita, 1985). Chemotherapy now has an established (but limited) role as an adjuvant to surgery and radiotherapy in the treatment of carcinoma of the breast (Bonadonna et al., 1976; Bonadonna, 1989), and the use of chemotherapy combined with radiotherapy and surgery in small cell carcinoma of the lung has had a small but definite effect on the long-term survival rate (Blechman, 1990). However, for most adult carcinomas, the role of chemotherapy has been limited to inducing small extensions in survival or palliation, and for many tumours chemotherapy has yet to make any contribution to treatment.

1.1.3. Resistance to treatment

Resistance of tumours to chemotherapy results from a combination of environmental and genetic factors. Some types of tumour cells are intrinsically resistant to current chemotherapeutic agents (e.g. carcinoma of the large bowel - Moscow & Cowan, 1988), or develop resistance after exposure. Resistance in the latter situation is determined by the selection of genetically resistant cells (DeVita, 1983; Moscow & Cowan, 1988). Many mechanisms are thought to mediate resistance including decreased net drug uptake, increased drug catabolism, and increased capacity to repair sub-lethal damage (Moscow & Cowan, 1989).

The major environmental causes of resistance in solid tumours are the consequences of an inadequate blood supply (Sutherland, 1988; Vaupel et al., 1989). Tannock (1968) noted that regions furthest from the blood vessels in tumours had the smallest growth fractions, explained by poor delivery of nutrients and accumulation of waste products in these regions. Such cells are resistant to cycle-specific agents (DeVita, 1985). In addition, the accumulation of lactic acid leads to a fall in the
pH of the extracellular fluid, impairing the diffusion of weakly basic drugs into the cells (Denny & Wilson, 1986). Cytotoxic drugs with a high affinity for substances within the cell (particularly DNA), are limited in their diffusion to those cells remote from blood vessels (Ozols et al., 1979; Durand, 1986, 1989; Los et al., 1989).

As with chemotherapy, resistance to radiotherapy is determined both by intrinsic cellular characteristics (Deacon et al., 1984) and by the environment, in this case primarily the oxygen tension. In the decades following the first application of radiotherapy for the treatment of tumours, it became evident that the tissue oxygen tension influenced the response of the irradiated tissues.

The history of the relationship between tumour vascularity, hypoxia and response to radiation has been reviewed by Kolstad (1964). Schwarz in 1909 noted a reduced reaction to radium irradiation if the skin was blanched by pressure or suction. Twenty years later Mottram correlated tumour radio-responsiveness with vascularity. In the 1950s bacteria, and later tumour cells, were shown to be three times more sensitive to irradiation in the presence of oxygen. Most experimental tumours are now known to contain hypoxic cells (Denekamp et al., 1982a; Moulder & Rockwell, 1984; Vaupel et al., 1989) and the degree of hypoxia in human tumours has been correlated with the outcome after radiotherapy (Kolstad, 1964; Dische et al., 1983; Gatenby et al., 1988; Dische, 1989).

Thus, in both chemotherapy and radiotherapy, environmental resistance is a consequence of ineffective blood flow. However, the direct causes of resistance differ in importance. Hypoxia per se appears to be critical in radiotherapy, whereas drug delivery and a low proliferation rate are probably the major problems in chemotherapy. The exact reason for resistance may be important in considering the strategies to improve therapy. These include manoeuvres to increase blood oxygen content, and to increase tumour blood flow (reviewed by Hirst, 1986; Jirtle, 1988). The first applies mainly to radiotherapy, while the second has the potential to improve the response to chemotherapy as well. Decreasing blood flow could also be used to advantage in cancer treatment, either to sensitise tumours to hyperthermia (Jirtle, 1988), to trap cytotoxic agents within tumours (Stratford et al., 1988), to activate hypoxia-selective cytotoxic agents (Chaplin, 1989), or to kill tumour cells directly by ischaemia (Section 1.2.3.).

Genetic resistance may also be modified physiologically. An example is the use of calcium channel blockers to inhibit P-glycoprotein, which mediates multiple drug resistance in carcinoma of the large bowel and other tumours (Moscow & Cowan, 1988). However, if the effectiveness of existing treatments cannot be improved by such physiological interventions, an alternative approach is to seek a completely new basis for selective anti-tumour toxicity.
1.2. Finding a new basis for selective toxicity

Lack of progress in the systemic treatment of solid malignancies raises doubts about the benefits of developing further cytotoxic agents that damage DNA or otherwise interfere with cell division (Workman 1989). The common tumours in humans are slow-growing due to both low growth fractions and high cell loss factors (Wright, 1984). The low growth fraction makes the tumour cells less sensitive to cycle-selective agents than the bone marrow and intestinal epithelium, and these limitations have encouraged the search for agents directed at different targets.

1.2.1. New tumour cell targets

1.2.1a. Tissue specificity

One approach is to target the tissue type from which the tumour has arisen. Such tissue specificity was the basis of the preparation of tissue extracts (chalones) in the 1960s and 1970s, which showed tissue-specific mitotic inhibition (Houck and Attallah, 1975). In fact targeting of a specific tissue type is already used in cancer therapy: corticosteroids cause intermitotic death in normal and malignant lymphoid cells, and are part of modern treatment protocols for lymphoid malignancies (Lester & Ulmann, 1990).

Tumour cell targeting with antibodies, often raised against tissue-specific antigens, has become an active area in experimental therapeutics (Embleton, 1987a). Monoclonal antibody technology has increased our capacity to discover new tumour-related antigens, and radio-labelled antibodies have been used successfully in imaging tumours in humans. Despite tumour regressions in experimental animals, radio-isotopes have not yet been concentrated sufficiently in human tumours to be therapeutically useful (Embleton, 1987a; Sivolapenko et al., 1989). The distribution of anti-tumour antibodies in human tumours after systemic administration is heterogeneous (del Vecchio et al., 1989), probably due to the low vascular density found in solid tumours (Vaupel et al., 1973), the limited diffusion of large molecules such as antibodies (Jain & Baxter, 1988), and the likelihood that not all tumour cells will express the target antigen (del Vecchio et al., 1989). The most promising application of antibody targeting is probably linkage to an enzyme that converts an inactive pro-drug into a cytotoxic substance within the tumour (Bagshawe et al., 1988). This technique would overcome the problem of antibody delivery, because the high concentrations of small cytotoxic molecules generated in parts of the tumour could diffuse to antibody-deficient regions.

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Research on growth factors in the abnormal proliferative activity of tumour cells has suggested another potential avenue of attacking tumours. An example is the self-stimulating (autocrine) growth factor bombesin, which is important in the growth of human small cell carcinoma of lung (Cuttita et al., 1985). Clinical trials with antibodies to bombesin have been undertaken, but results to date have been disappointing (Carney, 1989).

1. 2. 1c. Immunotherapy

Immunotherapy aims to kill tumour cells either by the administration of immune cytotoxic cells or cytokines, or by stimulating the immune system within the host to exhibit anti-tumour activity. Early attempts to stimulate host defences include those of William Coley who gave cancer patients extracts of bacteria and succeeded in achieving some tumour remissions (Coley-Nauts et al., 1953). Recently interferons have been successfully used in the treatment of hairy cell leukaemia and some solid tumours, and interleukin-2 (IL-2) and TNF have also shown limited clinical activity (Sikora, 1989).

1. 2. 1d. Other cell-directed agents

Other new cell-targeted approaches include the development of agents with selective affinity for particular DNA nucleotide sequences, drugs which interfere with critical signalling systems that are overactive in malignant cells, and agents which have tumour-selective effects on the cell membrane (Workman, 1989).

1. 2. 2. Therapies directed at hypoxic cells

Radiobiologically significant hypoxia is rare in normal tissues, but is usual in solid tumours, particularly when these are large (Denekamp, 1982a; Vaupel et al., 1989). Although hypoxia is the cause of resistance to treatment by radiation and some cytotoxic agents, its restriction to tumour tissue suggests a possible basis for selective toxicity. Hypoxia selective cytotoxic agents would have to exploit some difference between hypoxic and well oxygenated cells, including a low oxygen tension (Denny & Wilson, 1986), a high concentration of lactic acid and a low extracellular pH (Tannock & Rotin, 1989), and a high glucose requirement (Song et al., 1978). Most interest has centered on
activation of cytotoxins by low oxygen concentration, and some compounds with in vitro and in vivo hypoxia selective cytotoxicity have been found. These include the nitroimidazole aziridine derivative RSU 1069 (Chaplin et al., 1986b), the benzotriazine di-N-oxide, SR 4233 (Zeman et al., 1986), and the quinone, porfiromycin (Keyes et al., 1985). The development of an agent with clinically useful hypoxia selectivity seems feasible on physico-chemical grounds, and is stimulating much research. The restriction of FAA activity to established solid tumours in vivo suggests a role for hypoxia in its mechanism of action, and this possibility is investigated in this thesis (Chapters 4 and 7).

1. 2. 3. Therapies aimed at the tumour vasculature

Blood vessels in tumours are characterised by active growth and peculiar structural features, neither of which is found in normal tissues (Section 1. 3.). For this reason the tumour vasculature has been considered a potential target. The proliferation rate of tumour endothelial cells exceeds that of those in normal tissues by a factor of 20 (Denekamp, 1982b), and the possibility of identifying an antigen specific for tumour endothelial cells, or for proliferating endothelial cells, has been suggested (Denekamp, 1982b).

Following their discovery of tumour angiogenic factors, Folkman and his group have been searching for inhibitors of angiogenesis. A variety of experimental tumours have regressed after treatment with a combination of heparin and hydrocortisone, or their synthetic analogues (Folkman, 1986). This group have recently synthesised potent non-toxic anti-angiogenenic analogues of the fungus-derived antibiotic fumagillin, which inhibits the growth of a wide variety of mouse tumours (Ingber et al., 1990). The tumour vasculature as a target for cancer therapy has been reviewed recently by Denekamp (1991).

Hyperthermia and photodynamic therapy both probably involve vessel damage as a major component of their anti-tumour effects (Star et al., 1986; Nishimura et al., 1988; Yasumasa et al., 1988; Dodd et al., 1989; Evans et al., 1990). No drugs in current clinical use have been shown to exert an anti-tumour effect by stopping blood flow. The development or discovery of such agents could be useful in the treatment of resistant solid tumours, and the possibility that FAA acts by causing perfusion failure is an interesting possibility which is investigated in this thesis.

The next two sections discuss the biology of the tumour vasculature, and its effects on the tumour microenvironment.

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1.3. Tumour vessels and blood flow

Neoplastic cells exist in two forms in vivo: dispersed (as single cells or small aggregates in body fluids or tissues), and as solid tumours. The mode of growth is determined largely by cell type. As a rule, leukaemias are dispersed and carcinomas form solid tumours, but often a neoplasm will show both solid and dispersed forms of growth in the same patient (Figure 1.2). Malignant effusions are common in many carcinomas, such as those of the ovary. Many solid tumours have an infiltrative margin, and this component of the tumour shows some similarities to dispersed neoplasms, as do micrometastases. The form of the neoplasm may affect its response to treatment. Anti-proliferative agents and radiotherapy would be most effective against dispersed cells, which usually exist in environments where adequate exchange occurs with the blood. Hypoxia selective and vessel directed agents would be effective only against solid tumour masses.

Thus defined, solid tumours of all types share structural and physiological characteristics, the most important being the development of new tumour blood vessels, which must accompany any accumulation of neoplastic cells above a critical tumour size (Folkman, 1985, 1990). The following account of the tumour vasculature serves as a background for the discussion of the low exchange environment (Section 1.5.), and as a basis for the experiments on solid tumours described later (Chapters 2, 3, 5 & 6).

1.3.1. Tumour neovascularisation

Tumour neovascularisation begins when a group of malignant cells expands by cell division. At first metabolite exchange occurs by diffusion from the surrounding tissue, but when the mass reaches 2 - 3 mm in diameter, further growth depends on the production by the tumour cells of tumour angiogenic factors, which stimulate the growth of new vessels into the tumour (Folkman, 1985; Presta & Rifkin, 1988). The pre-vascular and vascular phases of tumour growth have been described as being sequential (Folkman, 1985).

Angiogenesis is a continuous process in tumours, as in the embryo, while in adults new vessels grow only in response to specific stimuli (wounds, inflammation) and in the renewing tissues of the female genital tract. The sequence of events in all types of angiogenesis is thought to be similar and includes the outgrowth of endothelial cell buds from venules or capillaries. These begin to canalise and elongate by endothelial cell mitosis in their mid-portions. They finally join one another to form
FIGURE 1.2 - GROWTH PATTERNS OF SOLID AND DISPERSED TUMOURS

a. A human kidney which has been diffusely infiltrated by a large cell lymphoma (arrows), without expanding the tissue. This tumour formed *expansile* masses in other parts of the body at postmortem. (Formalin-fixed postmortem specimen, actual size).

b. Histology of the lesion in a. showing a glomerulus (G), a tubule (T) and an artery (A), native structures remaining after diffuse infiltration by large-cell malignant lymphoma cells (arrows). (Light photomicrograph, H&E-stained section, bar = 50 μm).

c. Human cardiac atrium showing a pericardial nodule (arrowheads) composed of acute myeloid leukaemia cells. (Light photomicrograph, H&E-stained section. Bar = 500 μm).

d. Higher magnification of the edge of the tumour deposit shown in c. It comprises a solid (expansile) component (right) and the diffuse infiltration (left) of the myocardial fibres by leukaemic cells (arrows). This was the only solid tumour found postmortem, but the leukaemia was present in diffuse form in the peripheral blood, the bone marrow and several other organs. (Light photomicrograph, H&E-stained section. Bar = 50 μm).
loops, allowing blood flow to commence (Folkman, 1985). Unlike blood vessels, lymphatic vessels do not grow into tumours (Wiig et al., 1982; Folkman, 1985) and this probably contributes to high intra-tumour pressure (Section 1.4.2.; Jain & Baxter, 1988).

1.3.2. Structure of the tumour vasculature

The pattern of the tumour vasculature depends on the tumour type and the site of growth (Falk, 1978, 1980). Thus various tumours grown in the rat kidney replace the normal cells but retain much of the original vascular structure of that organ (Gullino & Grantham, 1962), while human renal cell tumours are usually expansile and contain a dense plexus of new vessels (Robbins et al., 1984). Variation in the vascular pattern also occurs between tumours of the same histological type (Solesvic, 1982), and even within individual tumours (Warnke et al., 1987).

Blood flow can be observed directly in early tumours growing in transparent chambers. Reinhold (1971), using implanted fragments of C3H mouse mammary carcinoma noted venous and capillary vessels growing into the graft by three days. Arterial flow began one or two days later. New vessels continually emerged, while others were stretched or occluded. Warren and Shubick (1966) observed both neovascularisation and the incorporation of normal venous vessels into melanomas growing in hamsters (Figure 1.3). Peters et al. (1980) described fluctuations in blood flow, and even changes in flow direction. Arterio-venous anastomoses were observed in all the above studies. Similar findings have been reported in a variety of other experimental tumours (Gullino, 1975; Warren, 1979; Endrich et al., 1982).

Histological studies of spontaneous mouse mammary C3H tumours (Falk, 1980) have confirmed the findings of the chamber studies and demonstrated the vascular changes at a more advanced stage of tumour growth. The native vessels were engulfed and greatly modified. The artery increased its diameter four-fold, and the branches abruptly lost their muscle coats on entering the tumour. With increasing size, the arterial channels became kinked and obstructed. The capacious venous system was particularly susceptible to pressure effects of the growing tumour (Falk 1980).

1.3.3. Physiological control of tumour vessels

The physiology of tumour blood flow is of interest in experimental tumour therapy because of the therapeutic potential of manipulating tumour blood flow (Section 1.1.3.). In the context of this thesis, it is important to consider how physiological mechanisms might mediate any blood flow changes.
FIGURE 1.3 - TUMOUR VASCULARISATION AND GROWTH

Tracings from photographs of a melanoma transplant growing in a hamster cheek pouch (from Warren and Shubik, 1966). 1 - 4. A tumour fragment (t) is placed between two veins (v) and an artery (a). Vessels grow towards and into the graft. 5 - 8. The tumour grows rapidly incorporating and modifying veins (v'), but pushing aside the artery.
caused by FAA administration. The influence of vasoactive agents on tumour blood flow has been reviewed by Mattsson and Peterson (1981) and Jirtle (1988). Accounts of the responsiveness of tumour vessels vary. Krylova (1977) found no vessels capable of regulating flow in any of the mouse tumours she studied. Mattsson et al. (1977) failed to find catecholamines in the vessels of either of two rat tumours by formalin-induced fluorescence. Wickenham et al. (1984) showed a progressive loss of responsiveness of mammary vessels to vasoactive agents as spontaneous C3H mouse mammary tumours increased in size. This finding corresponds with Falk's (1980) study on this tumour (preceding paragraph), in which arteriolar muscle was replaced by collagen. However, Mattsson et al. (1980) showed decreased clearance of ^133^Xe from tumours injected with noradrenaline, and similar results were found by Tveit et al. (1984) in DMBA-induced rat mammary carcinomas after systemic noradrenaline.

This variation in response is probably determined by the agent used, the number of normal vessels incorporated into the tumour, the degree to which these vessels retain physiological responsiveness, and the extent to which the tumour vessels are in series or in parallel with those of surrounding tissues. Some tumours retain much of the vasculature of the organ they invade (Gullino & Grantham, 1962; Skinner et al., 1990), and this correlates with retention of physiological responsiveness (Gullino & Grantham, 1962).

Although the results discussed above vary with tumour type and site, some generalisations can be made (Mattsson & Peterson, 1981; Jirtle 1988): 1) Arterial tumour vessels are maximally dilated and will not dilate further in response to vasodilatory stimuli. 2) Incorporated vessels may constrict in response to some vasoconstrictor agents (e.g. noradrenaline). 3) Most vasoactive agents cause opposite effects in tumour and normal vessels. For example, hydralazine dilates normal vessels, increases flow, and thus diverts blood away from the less responsive tumour vessels ("steal effect").

1.3.4. The endothelial layer

The endothelial layer influences the movement of both blood and extracellular fluid, particularly in the capillary beds. Vessels in different tumours differ in their permeability to large molecules (Sands et al., 1988). The high permeability found in some tumours allows up to 10% of the plasma flow to seep out into the interstitium, causing haemoconcentration in the venules (reviewed by Jain, 1988). Interstitial fluid flow is required for the delivery of large molecules (Swabb et al., 1974), and may thus be important in treatments employing antibodies or cytokines (Section 1.4.2.).
Several types of endothelial layer have been found in normal and tumour tissues (reviewed by Jain, 1988). In normal tissues, the type depends on functional requirements: the endothelium of the glomerulus is fenestrated, while that in the brain is continuous. Most types of endothelium found in normal tissues have also been reported in tumours, including those of the fenestrated type (Warren, 1970; Underwood & Carr, 1972). Some tumour endothelial layers are incomplete and the vascular channels are partly formed by tumour cells (Warren et al., 1966), and capillaries are often fragile with a tendency to haemorrhage (Warren, 1970). Tumour vessels were found by Underwood and Carr (1972) to be more permeable to albumin than those of normal tissues. Tumour capillaries differ from those of normal tissues in their response to drugs. Histamine and serotonin open tight junctions between endothelial cells in the Rd/3 transplanted rat sarcoma, but not in normal tissues (Underwood & Carr, 1972), and similar results have been seen in rat sarcomas by Papadimitriou and Woods (1975). More recent studies have shown increased permeability of tumour capillaries after treatment with interleukin-1 (Braunschweiger et al., 1988), and that endothelial cells are damaged by interferon-α/β (Dvorak & Gresser, 1989). Endothelial cells may play a role in the regulation of blood flow, since they can contract in response to serotonin (Majno et al., 1969). Endothelial cells also have a major role in the control of coagulation and platelet function, and exhibit pro-coagulant activity in response to TNF (Clauss et al., 1990).

1.4. The low exchange environment

The peculiarities of tumour vascular structure discussed above result in regional differences in ability to exchange substances with the blood. Some regions are low in oxygen, and become acidic because the lactic acid produced by the hypoxic tumour cells accumulates. Gullino et al. (1965) showed that the pH of interstitial fluid from a variety of rat tumours was 0.2 to 0.4 units lower than that of subcutaneous tissues. Vaupel et al. (1981) using fine microelectrodes found a wide range of oxygen pressures in C3H mammary carcinomas, with a modal class of 0 - 5 mmHg, with low readings more common in larger tumours. pH readings also varied widely (5.8 - 7.2). Similar results have been obtained by others (Jahde et al. 1982; Jahde & Rajewsky, 1982; Endrich et al., 1982).

The determinants of this heterogeneous exchange are of particular interest, and require review for the following reasons. First, since this type of heterogeneous microenvironment has not been reported in normal tissues, it could include regions which are selectively sensitive to new anti-tumour agents, and could be responsible for the tumour selectivity of FAA. Second, this thesis uses
histological analysis as a major technique, and so the spatial arrangement of low exchange regions needs to be reviewed. In addition, these low exchange regions may be resistant to various anti-tumour treatments, as discussed in Section 1.1.3.

Three determinants of tumour environmental heterogeneity will now be discussed, two spatial and one temporal. The first is large intercapillary distances, which cause chronic hypoxia. The second is high intra-tumour pressures, which affect mainly the centres of some large tumours, and have several secondary effects. The temporal factor is blood flow fluctuation.

1.4.1. Distance of tumour cells from the vasculature

As tumours increase in size, the vascular density decreases (Vaupel et al., 1973), and not all tumour cells can adequately exchange nutrients and waste products with the blood (Section 1.1.3). The first study to describe the spatial arrangement of these regions was that of Thomlinson and Gray (1955). In a histological study of human lung squamous carcinomas they observed a layer of viable tumour cells about 100 μm thick, situated between the vascularised stroma and necrotic zones. The thickness of this layer corresponded to the calculated diffusion distance of oxygen, suggesting that oxygen was the nutrient critical for maintaining viability in vivo. They concluded that the viable cells closest to the necrotic zone are hypoxic. The thickness of the viable cell layer can be altered by varying the oxygen content of the inspired air (Tannock, 1970; Hirst et al., 1991). Because the growth and remodelling of the vascular structure probably takes hours to days, the hypoxic state of these cells is stable or chronic, and such cells have a decreased proliferative activity in experimental (Tannock, 1968) and human tumours (Rabes et al., 1979, 1985). Both chronic and acute hypoxia (Section 1.4.3) contribute to radiation resistance (Sutherland & Franko, 1980).

Chronic hypoxia has been modelled in vitro by multicellular spheroids grown in tissue culture medium (Sutherland, 1988), an in vitro tumour model system used extensively in this thesis. The surface cells of spheroids correspond to the para-vascular cells in tumours, and the hypoxic cells lie distant from the medium close to the necrotic zone. Substances other than oxygen (eg. other metabolites and drugs) may also be limited in diffusion in tumours and spheroids (Sutherland, 1988), to create a "low exchange" environment. The limited diffusion of the fluorescent compound Hoechst 33342 (H33342) has been used as the basis for fluorescence-activated cell sorting (FACS) of hypoxic and well-oxygenated cells from spheroids (Durand, 1982). Similar methods have been used to separate cells from tumours into populations defined by their distance from functional vessels (Chaplin et al., 1985; Minchinton et al., 1990), and to visualise vessels and para-vascular tumour cells in frozen sections (Trotter et al., 1989a).
1.4.2. High intra-tumour pressures

Low exchange microregions of the type discussed above relate to high intercapillary distances. Heterogeneity in exchange conditions also occur at a higher level of tumour organisation, and this is determined by intra-tumour pressure, as discussed below.

Gullino et al. (1964) measured interstitial pressures of up to 16 mmHg in micropore chambers embedded in various rat tumours, compared to 7 - 9 mmHg in normal subcutaneous tissue. Pressure effects were also evident in Walker 256 carcinomas growing in the rat testis (Paskins-Hurlburt et al., 1982), where blood flow per unit mass decreased after the third day post-implantation, coinciding with an increase in intra-tumour pressure.

Wiig et al. (1982) measured tumour interstitial pressures with wick-in-needle cannulae in DMBA-induced rat mammary tumours. Pressure was higher in larger tumours, and toward the centres of individual tumours, the highest pressure recorded being 23 mmHg. Suggested causes included the absence of lymphatic drainage, a high permeability of the tumour vessels to plasma proteins, hypoxic swelling of tumour cells and continued proliferative activity. The possible contribution of encapsulation in causing a "compartment syndrome" (similar to the muscle compartment syndromes seen by surgeons) was considered. In an accompanying paper, Wiig (1982) found that venous pressures (~ 13 mHg) were lower than those for interstitial pressures in many tumour centres. He thus concluded that in some parts of larger tumours, blood flow is determined not by the arterio-venous pressure differences, but rather by closure of venous-end capillaries by high external pressure. Lower blood flows at the tumour centre have been confirmed by observations in diverse tumour sytems (Straw et al., 1974; Jirtle, 1981; Endrich et al., 1982; Blasberg et al., 1985; Tozer et al., 1990), and perfusion per unit mass has also been seen to fall with increasing size (Endrich et al., 1982; Sevick & Jain, 1989; Tozer et al., 1990).

Apart from decreased blood flow, another effect of high intra-tumour pressures could be diminished exchange of large molecules from the blood, including the therapeutically interesting antibodies and cytokines mentioned in Section 1.2.1. The reason is that large molecules permeate tissues largely by the mass movement of fluid filtering out of the circulation (Swabb et al., 1974), and this would be opposed by high interstitial pressures (Jain & Baxter, 1988, Boucher et al., 1990).

The occurrence of high intra-tumour pressure and its effects depend on the tumour type, and particularly on its mode of growth. Falk (1978) described tumours which appeared to have low tissue tension ("lax tumours") growing around a central vascular supply, which were more sensitive to radiation than their "tense" counterparts, implying that the former have a better blood supply. Similarly, blood flow in human medulloblastomas growing in the brains of athymic rats differed
according to the tumour growth pattern. Blood flow exceeded that of normal white matter when
tumours grew in an infiltrative manner, but was lower when forming a solid tumour mass (Warnke
et al., 1987).

While pressure effects would hinder some types of treatment, they could also help to sensitise
tumours to vessel-directed therapies. Damage to the vessel wall resulting in exudation or haemorrhage
could further increase the intra-tumour pressure and so cause widespread capillary collapse and
ischaemic necrosis. The possibility that FAA acts by increasing intra-tumour pressure has been
proposed (Evelhoch et al., 1988), and is considered again briefly in the final chapter (Section 8. 1. 2.).

1. 4. 3. Fluctuating blood flow

Exchange conditions also vary with time, due to fluctuations in the perfusion of individual tumour
blood vessels, as discussed below. The resulting fluctuations in the tissue concentration of oxygen has
been called "transient" or "acute" hypoxia. It is worth noting that acute hypoxia is not always transient
(i.e. reversible), and if permanent will eventually lead to necrosis of dependent tumour cells.

Fluctuating blood flow with a periodicity of 2 - 3 minutes was observed in BA 1112
rhabdomyosarcomas growing in transparent chambers by Intaglietta et al. (1977). The observation that
acute hypoxia further increased the resistance of spheroids to radiation in high doses, suggested to
Sutherland and Franko (1980) that chronic hypoxia alone cannot account for all radiobiological
hypoxia in tumours. Brown (1979) found radiobiologically hypoxic cells in tumours 24 h after killing
the chronically hypoxic cells with high doses of misonidazole, interpreted as suggesting the presence
of acutely hypoxic cells. More direct evidence for acute hypoxia comes from Chaplin et al. (1986a)
who injected the diffusion-limited fluorescent dye Hoechst 33342 into mice bearing SCC VII tumours,
to selectively stain tumour cells close to functioning blood vessels (see also Section 1. 4. 1.). Sorting
the tumour cells by flow cytometry showed a correlation of sort fraction with sensitivity to irradiation
in small tumours, whether the dye was given as a bolus prior to, or as an infusion during, irradiation.
With larger tumours, this correlation was seen only when the dye was infused during the irradiation
period. This difference is best explained by the occurrence of intermittent blood flow in larger
tumours, and estimates of vessels with fluctuating blood flow of up to 20% were made in SCC VII
tumours using this technique and microscopic methods (Chaplin et al, 1987). The technique has now
been further refined and applied also to KHT sarcomas (Minchinton et al., 1990). Trotter et al. (1989a,
1991) have confirmed the presence of fluctuating blood flow in SCC VII tumours by showing that
different vessels were labelled by two fluorescent dyes injected intravenously 20 minutes apart.
The exact physical basis for these fluctuations and the determinants of their periodicity are unknown, but have been attributed to the disorganized and inefficient state of the tumour vasculature, which is more pronounced in larger tumours (Vaupel, 1977). This is supported by the correlation between acute hypoxic fraction and tumour size (Jirtle and Clifton, 1978; Brown, 1979; Chaplin et al., 1986b; Trotter et al., 1991). More fluctuating flow is also seen in the central, than in the peripheral parts of tumours (Trotter et al., 1991). These findings suggest a role for high intra-tumour pressures. However, these factors would not explain why the resulting hypoxia should be fluctuating rather than stable. It is possible that in tumour regions where transmural pressure gradients are low, very small perturbations, such as those caused by body movements, could determine whether local perfusion occurred. Vasomotion in the arterial supply could also explain the fluctuations, as suggested by Trotter et al. (1991).

Fluctuations in blood flow may have therapeutic implications because acutely and chronically hypoxic cells may differ in sensitivity to treatment. In addition, blood flow fluctuations could complicate the measurement of tumour blood flow, as is discussed in Chapter 3.

1.4.4. The necrotic zone

The necrotic zone is of interest because it may occupy the greater part of the tumour (or the multicellular spheroid) and influence tumour growth and response to treatment. The exchange of substances between the blood and the dense gel-like necrotic zone is exceptionally slow (Goldacre & Sylven, 1962). Necrotic material contains substances which are toxic to cells (Sylven & Homberg, 1965), and which may retard the growth of adjacent viable cells (Freyer, 1988). Release of enzymes and other cell constituents into the interstitial space could affect the activities of some therapeutic agents. Large amounts of nucleosides from DNA breakdown might diminish the activity of antimetabolites such as methotrexate, and intact DNA fragments could bind to DNA-affinic drugs limiting their uptake into the cells (Tattersall, 1981).

Necrosis is the ultimate consequence of deteriorating exchange with the blood, and therefore has the same causes as low exchange (Sections 1.4.1. & 2.). Thus cells become necrotic after they have become separated from their supplying vessels by about 100 mm (Thomlinson & Gray, 1955), due to proliferative activity of the intervening cells (Tannock, 1968). This type of necrosis is identified microscopically. Necrosis can also be appreciated macroscopically in the centres of tumours, and this distribution corresponds with that of high intra-tumour pressures, which could cause vessel collapse and necrosis.
A study of (non-drug-induced) necrosis in Colon 38 tumours is reported in Chapter 2, and this is followed by further discussion of the pathogenesis of spontaneous necrosis (Section 2. D. 1., p. 65).

1. 4. 5. Implications of microenvironmental heterogeneity

The position of the tumour cell relative to the nearest functional blood vessel and to the tumour surface, provides major sources of variation even without considering the temporal irregularities of tumour blood flow. The implications for cancer therapy have been discussed in detail in Section 1. 1. 3. and Sections 1. 4. 1 - 3. To summarise, the occurrence of low exchange regions could decrease the effectiveness of anti-tumour agents directed at the individual cells of the tumour, but on the other hand these regions may form the basis for new tumour targets.

1. 5. Immune cell cytotoxicity in solid tumours

1. 5. 1. Immune cells in human and experimental tumours

The role of the immune system in controlling tumour growth is controversial, but is of interest for two reasons. The immune system may be involved in suppressing tumour growth, especially after treatment has reduced tumour load to low levels (DeVita, 1985), and there is therapeutic interest in artificially stimulating the immune system to kill tumour cells. Arguments against a natural role for the immune system in tumour suppression are that the immune system has evolved primarily in response to foreign organisms, and that the tumour types which arise in immunosuppressed individuals are those of the lymphoid system (which is already abnormal), or those likely to be caused by viruses (eg. squamous carcinoma of the cervix) (Robbins et al., 1984; Purtilo, 1987). On the other hand, many human tumours have immune cells infiltrating their substance or (more often) their associated host-derived stroma, and in some tumour types, the degree of immune cell infiltration correlates with improved prognosis (eg. carcinoma of the large bowel; Jass et al., 1987).

The investigation of the role of the immune system in cancer is confounded by differences between human tumours and experimental tumours in animals. Although spontaneous murine tumours may be comparable to human tumours in their immunogenicity, most experiments are performed on
transplanted or culture-adapted tumours, which may elicit a specific immune response even though they are grown in same species and strain of origin (Denekamp, 1982a). On the other hand, low-incidence, spontaneous tumours show little immunogenicity in the strain of origin (Hewitt, 1978), but are available in few laboratories.

Many human tumours contain both lymphocytes and macrophages, which may constitute a significant fraction of the total cell population (Svennevig & Svaar, 1979), and are known to outnumber tumour cells in particular tumour types, such as the poorly-differentiated nasopharyngeal carcinoma (Robbins et al., 1984). Lymphocytes are of T-cell or B-cell type, but K-cells and NK-cells are not often detected (Vose & Moore, 1985). Both cytotoxic and helper T-cells are found, but the role of cytotoxic T-cells in anti-tumour activity is limited by the finding that MHC class I antigens are expressed in less than 50% of human malignant tumours (Vose & Moore, 1985), and there is little evidence that immune effects play any role in the response of tumours to anti-tumour therapy (Embleton, 1987b).

1.5.2. Specific and non-specific anti-tumour effects

Immunological reactions to tumours may be directed against a specific tumour antigen, or may be non-specific. The former involve the T-cell system, and possibly specific antibodies, though antibodies may actually inhibit T-cell cytotoxic responses ("enhancement"; Robbins et al., 1984). Macrophages may participate in specific anti-tumour immunity by killing antibody-coated tumour cells (Adams & Hamilton, 1988), becoming cytotoxic in response to stimulation by specific T-helper cells (Urban et al., 1988), or by stimulating cytotoxic T-cells via monokine release. Non-specific anti-tumour activity does not require specific recognition of antigens on tumour cells, and the basis for the ability of certain effector cells (NK-cells and macrophages) to lyse tumour cells rather than non-tumour cells is not understood.

1.5.3. Macrophage tumouricidal activity

Macrophages become cytotoxic to tumour cells after a series of stimuli, and different levels of cytotoxicity are exhibited depending on the degree of stimulation. Thus after exposure to BCG in vivo, macrophages will kill some tumour cells, but the addition of bacterial endotoxin (lipopolysaccharide) enhances this activity, probably by inducing the release of TNF (Adams & Hamilton, 1988). The
stimulation of macrophages to exhibit high levels of anti-tumour cell activity is termed "activation".

Macrophage cytotoxicity after BCG stimulation is specific for tumour cells and non-tumour cells are not affected. Close and prolonged contact between macrophage and target is required. The secretion of a neutral serine protease seems to be a major component of this activity (Adams 1980). A high macrophage density, and an effector:target cell ratio of at least unity, are critical for cell killing in vitro. The in vivo significance of this zero-order mechanism would be limited to very small tumour loads (Steward et al., 1988). However, this tumour-specific cytotoxic effect may play a part in the elimination of small numbers of cells remaining after chemotherapy or radiotherapy (DeVita, 1985).

In addition to the production of a cytolytic protease, other cytotoxic mechanisms may contribute to macrophage cytotoxicity (Steward et al., 1988). These include the production of hydrogen peroxide and the superoxide anion, which have a shorter time course for killing than that described above, and may act against some leukaemic cells. Another is L-arginine-dependent cytotoxicity, which involves the production of nitric oxide by macrophages (Hibbs et al., 1988; Keller et al., 1990). There are many other macrophage products (Nathan, 1987), including various neutral and acidic hydrolases, which enable the macrophage to remove dead and damaged tissues from wounds. Some of these may also be cytotoxic to tumour cells.

The role of the various cytokines in macrophage cytotoxicity is complex and probably involves direct toxicity against some tumour cells, the stimulation of cytotoxic activity in macrophages, and effects on other targets such as the tumour vasculature. Macrophages are often activated by synergistic stimuli. For example, one study investigated the interactions of the lymphokine interferon-γ (IFN-γ) and the monokines interleukin-1 (IL-1) and TNF, in activating macrophages to kill tumour cells (Chen et al., 1987). The target cell, L5178Y, was resistant to direct killing by these monokines, even in combination. Macrophage mediated cytotoxicity was only seen if low concentrations of IFN-γ were combined with low concentrations of either IL-1 or TNF. Antiserum to IL-1 blocked the effects of IFN-γ plus TNF, and antiserum to TNF blocked the combined action of IFN-γ and IL-1, indicating that the one monokine stimulated the release/production of the other. High concentrations of IFN-γ were capable of activating the macrophages to kill tumour cells in the absence of either monokine, but this effect could be blocked by antibodies to either IL-1 or TNF. Similar findings have been reported by Hori et al. (1989).

In summary, macrophages can kill tumour cells, and this involves complex interactions of several cytokines. Whether this cytotoxic activity is important in vivo, and its importance relative to specific immunity, are unknown.
1.5.4. Tumour necrosis factor-α

TNF appears to play a central role in macrophage-mediated tumour cell killing. TNF was shown to be the major mediator of haemorrhagic necrosis induced by endotoxin in tumours (Carswell et al., 1975), and of endotoxic shock (Tracey et al., 1986). The gene for TNF is situated within the major histocompatibility complex (chromosome 6 in humans), close to the gene for lymphotoxin (also called tumour necrosis factor-β; Semenzato, 1990). The amino acid sequences have now been determined for mouse, rabbit and human TNF (Beutler & Cerami, 1987). The monomer has a molecular weight of 17 300, and TNF is thought to exist in a dimeric or trimeric form, with a "jelly-roll" configuration unique among human proteins, but also found in viral coat proteins (Jones et al., 1989). The major source is the macrophage, but other cells, particularly those of the immune system, also produce TNF. Production is stimulated in primed macrophages by endotoxin, IFN-γ, interleukin-2, immune complexes and viruses (Semenzato, 1990). In addition to its anti-tumour properties, TNF has a wide variety of physiological effects, both within and beyond the immune system (Beutler & Cerami, 1987; Semenzato, 1990), including inhibition of lipoprotein lipase, changes in muscle membrane potential, activation of osteoclasts, release of IL-1 from monocytes and endothelial cells, and activation of neutrophils.

1.5.5. Direct tumour cell toxicity of TNF

Some but not all types of tumour cell are killed directly by TNF (Sugarman et al., 1985), but the mechanism and basis for sensitivity have not yet been explained. TNF binds to specific cell surface receptors and is then internalised and processed in lysosomes to smaller fragments, which may mediate the cytotoxic effect, as suggested by Liddil et al. (1989). Sensitivity does not appear to be related to the number of specific cell membrane receptors (Sugarman et al., 1985; Spriggs et al., 1988). However, comparison of TNF-resistant L929 variants with the sensitive parental line, showed a 50% decrease in lysosomal enzyme content (Liddil et al., 1989), and these authors were able to inhibit cytotoxicity with lysosomal inhibitors. TNF did not decrease the synthesis of DNA, RNA, protein or ATP, or damage the DNA until cell death was apparent morphologically. Exactly what role the tumour cell lysosomes play in the cytotoxic action of TNF is unknown, but it is of interest that numerous lysosomes appeared in the cytoplasm of tumours treated with TNF in vivo (Shimomura et al., 1988). TNF causes oxidative damage to target cells (Zimmerman et al., 1989), and the production of cytotoxic levels of the hydroxyl radical anion have been reported (Yamauchi et al., 1989). However,
depletion of glutathione did not increase cell sensitivity to TNF (Liddil et al., 1989). Other factors associated with resistance to TNF include a closely-packed cell colony morphology (Matthews & Neale, 1989) and the ability of target cells to themselves produce TNF (Spriggs et al., 1988; Niitsu et al., 1988), but the relationship of these phenomena to resistance is unknown.

1. 5. 6. Indirect anti-tumour effects of TNF

While direct TNF cytotoxicity is of mechanistic interest, it may not be important in vivo. While in vitro toxicity is seen only in a limited number of cell lines (Sugarman et al., 1985), TNF has a broad spectrum of activity in vivo (Haranaka et al., 1984) suggesting that other mechanisms are involved. Subcutaneous Friend leukaemia tumours responded to TNF, while the same cells grown as ascites tumours did not (Proietti et al., 1988). Differences between in vitro and in vivo activity have also been observed in other tumour types (reviewed by Shimomura et al., 1988). Damage to the tumour vasculature and/or thrombosis have been reported in several studies (Asher et al., 1981; Proietti et al., 1988; Watanabe et al., 1988; Shimomura et al., 1988; Havell et al., 1988), and the anti-tumour effect has been inhibited by the prothrombin depressant dicoumarol in one study, suggesting a causal role for thrombosis in the anti-tumour action (Shimomura et al., 1988). Various effects on endothelial cells have also been reported. TNF causes morphological changes and cytostatic effects in endothelial cell layers (Stolpen et al., 1986), inhibits angiogenesis in an in vitro system (Sato et al., 1987), and induces pro-coagulant activity (Clauss et al., 1990). In other experiments, TNF functions as an angiogenic factor (Leibovich et al., 1987). Clearly the activities of this substance are complex and depend on the exact experimental conditions, interactions with other cytokines, and TNF concentration. The importance of dose has been clearly demonstrated in vivo by Kallinowski et al. (1989), who showed that while high doses of TNF inhibited tumour growth, low doses increased growth, possibly by an angiogenic effect. This apparent dual role for TNF in tumours, as an anti-tumour agent and an angiogenesis factor, is discussed further in Chapter 5.

1. 5. 7. The role of specific immunity in TNF activity

T-cells could interact with TNF either by moderating its effects, or by controlling its endogenous release by other stimuli such as endotoxin. The tumour necrotising effect of TNF does not appear to depend on the presence of an intact T-cell system, since tumour necrosis occurs in nude mice and
thymectomised mice. However an intact immune system has a marked effect on tumour cure rate (Haranaka et al., 1984; Havell et al., 1988; North & Havell, 1988). Similarly, immunogenic tumours showed greater growth delays than related non-immunogenic tumours, although necrosis was indistinguishable in the different tumour types (Asher et al., 1987). North and Havell (1988) concluded from their observations that two distinct phases of anti-tumour effect were involved: an early (<24 h) T-cell-independent haemorrhagic (necrotising) effect, and a slower T-cell-dependent effect required for cure. Asher et al. (1987) noted a significant lymphocytic infiltrate around their immunogenic tumours in the days following treatment, and also suggested that cure depended on a combination of an early non-specific, and a later specific immune effect. It is not clear from the above studies whether T-cell cytotoxicity was stimulated directly by TNF, or merely by the presence of necrotic tumour tissue.

1.5.8. Relationship of TNF to FAA

The similarity between TNF and FAA was first noted by Baguley et al. (1989), who found that the haemorrhagic necrosis induced in Colon 38 tumours by the two agents were microscopically indistinguishable (compare Figure 1.4, p. 41 and Figure 2.2, p. 58). At the commencement of the studies reported in this thesis TNF had not been shown to mediate FAA actions. The possible relationships between these substances are: 1) FAA could be a TNF agonist. 2) FAA could be an endotoxin agonist, causing the release of TNF. 3) FAA could act via a separate but converging pathway.

Even if FAA acts as an endotoxin agonist, mediators other than TNF could be involved. North and Havell (1988) found that although TNF was produced within the tumour after treatment with endotoxin, antibodies to TNF did not completely inhibit the haemorrhagic activity of endotoxin. This was interpreted by the authors as suggesting the action of other mediators (though poor penetration of tumour tissue by the large immunoglobulin molecule may be the explanation). Similarly, FAA may involve the production and release of several cytokines. Alternatively, FAA may act independently of the endotoxin pathway: another tumour necrosis factor-like substance has been identified in the serum of mice treated with streptococcal lipoteichoic acids (Usami et al., 1987), and such mediators or some other undiscovered mechanisms may play a role.

Even if FAA does function purely as a stimulant for the production of TNF or as a pure TNF agonist, this small synthetic molecule could have therapeutic advantages over TNF. At less than 1% of the size of the active TNF trimer, FAA would have better tissue-penetrating properties. FAA is
Colon 38 tumour growing in a BDF1 mouse, treated 24 h before excision with 300 000 units of TNF i.p. This field shows tumour tissue which has survived treatment (above) and was indistinguishable from untreated tumours. Necrotic tumour tissue (below) shows loss of cytoplasmic staining and nuclear pyknosis (shrinkage with increased staining). Compare with necrosis following FAA treatment (Figure 2.2). (Tumour from the experiments of Baguley et al., 1989).

Light photomicrograph, H&E-stained section. Bar = 50 μm.
relatively simple to synthesise, and so is readily available for experimental use. If clinically active, commercial production of this substance would allow widespread use. Even if chemically pure, TNF preparations vary with species (Kramer et al., 1988), and method of preparation (whether prepared by endotoxin stimulation or by recombinant technology; Havell et al., 1988). Finally, the simple structure of FAA lends itself to the preparation and testing of analogues, which may show better activity than FAA itself.

1.6. Flavone acetic acid

Flavone is a naturally-occurring plant substance, a member of the large and ubiquitous flavonoid group, which has diverse biological activities in animal cells (Havsteen, 1983). Flavone acetic acid (FAA) is a synthetic compound, which failed to show cytotoxic activity against P388 leukaemia cells in vitro, but was highly active in vivo against the chemoresistant Colon 38 tumour (Plowman et al., 1986). Table 1.1 (overleaf) illustrates the diversity of the tumours which have shown sensitivity. These include colorectal tumours which are resistant to several anti-proliferative agents. The MAC 16 tumour did not respond to any of seven standard anti-cancer agents, but was sensitive to FAA (Bibby et al., 1988). Table 1.1 shows that FAA is active against tumours implanted at various sites, haematogenous metastases in the liver, tumours arising in the colon after carcinogen administration, and human xenografts growing in mice.

Resistance to FAA has been seen in leukaemias and other ascites tumours (Plowman et al., 1986; Finlay et al., 1988; Bibby et al., 1987; O'Dwyer et al., 1987; Ching & Baguley 1989a) and in vitro (Schroyens et al., 1987; Finlay et al., 1988; Bibby et al., 1989b). The resistance of small lung metastases to FAA has been ascribed to their early stage of development (Finlay et al., 1988) or their site (Bibby et al., 1989b). Generally, human tumour xenografts grown in mice seem to be responsive to FAA (Table 1.1; Baguley, personal communication). However, only one of three human xenografts was sensitive in the studies of O'Dwyer et al. (1987), though the mode of growth of these tumours (whether dispersed in ascites, or forming tumour nodules) was not described. The importance of mode of growth is discussed further in Chapter 5.

In contrast, no activity has been seen in humans in early clinical trials (Kerr et al., 1987). The reason for the marked differences in activity in humans and mice is of considerable interest, because of the hope that varying mode of administration of FAA, or developing an analogue active in humans, could result in effective treatment for resistant advanced solid tumours.
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<th>COMMENT</th>
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<tr>
<td>Colon 38 (s.c.)</td>
<td>1.0 mmol/Kg (i.p.) x2; d2; d9</td>
<td>growth delay, survival, cure</td>
<td>Ascites tumour resistant</td>
<td>Plowman et al., 1986</td>
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<td>Colon 38 (s.c.)</td>
<td>1.2 mmol/Kg (i.p.) x1</td>
<td>necrosis, growth delay</td>
<td>Similar histology after TNF</td>
<td>Baguley et al., 1989</td>
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<td>Colon 38 (s.c.)</td>
<td>0.6 mmol/Kg (i.v.) x3; 4d.a.</td>
<td>growth delay (41 days)</td>
<td>Colon 38 cells resistant</td>
<td>Chabot et al., 1989b</td>
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<td>(various)</td>
<td>life extension</td>
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<td>Bissery et al., 1988</td>
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<td>MAC 26 colon (s.c.)</td>
<td>1.1 mmol/Kg (i.p.) x2; 7d.a.</td>
<td>cures (10/10)</td>
<td>Ascites tumour resistant</td>
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<td>MAC 16 colon (s.c.)</td>
<td>0.7 mmol/Kg (i.p.) x2; 7d.a.</td>
<td>growth delay, cures (8/10), necrosis</td>
<td>Tumour highly resistant to other agents</td>
<td>Bibby et al., 1988</td>
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<td>P815 mastocytoma (s.c.)</td>
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<td>Ascites tumour resistant</td>
<td>Ching &amp; Baguley, 1989a</td>
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<td>necrosis, growth</td>
<td>No effect in ascites, early lung metastases,</td>
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<td>growth delay,</td>
<td>Increased effect with SR4233</td>
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<tr>
<td>sarcoma (i.d.)</td>
<td>(i.p.) x1</td>
<td>necrosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3LL lung tumours (i.m.</td>
<td>0.5 mmol/Kg</td>
<td>decreased</td>
<td>Prevention of metastases</td>
<td>Desoize et al., 1989</td>
</tr>
<tr>
<td>lung)</td>
<td>(i.p.) x2; 7d.a.</td>
<td>tumour burden</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RENCA (renal)</td>
<td>0.9 mmol/Kg</td>
<td>Cures</td>
<td>Marked increase in cures with IL-2</td>
<td>Wiltrout et al., 1988</td>
</tr>
<tr>
<td></td>
<td>(1/2i.v.,1/2i.p.)</td>
<td></td>
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<tr>
<td></td>
<td>x1</td>
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Abbreviations: subcutaneous (s.c.), intraperitoneal (i.p.), intravenous (i.v.), intradermal (i.d.), intramuscular (i.m.), day (d), days apart (d.a.).
The present Section reviews the literature on FAA available at the commencement of the experimental work of the thesis. All subsequent publications are referred to in the appropriate experimental chapters, or in the concluding chapter.

1.6.1. Early pharmacokinetic and mechanistic studies

Compared to normal human myeloid progenitor cells, human tumour cell lines in vitro show no specific sensitivity to FAA (Schroyens et al., 1987). Capolongo et al. (1987) showed decreased synthesis of DNA, RNA and protein after exposure to FAA, but no evidence of DNA damage. However, the concentrations and duration of exposure to FAA used in these studies would be lethal in vivo (Zaharko et al., 1986).

The in vivo studies have investigated the pharmacokinetics and distribution of FAA in various tissues, and have examined differences in experimental animals and humans. Humans metabolise FAA more quickly than mice, and different metabolites are produced (Cummings et al., 1989). Plasma protein binding of FAA is higher in humans than in mice (Bibby et al., 1987). Zaharko et al. (1986) showed that the "therapeutic window" (effective but non-toxic dose range) is small, and that no activity was seen if plasma concentrations did not reach 0.4 mM (100 µg/ml), and that toxicity (and anti-tumour activity) could be of two types: immediate, due to high peak levels, and delayed, due to plasma levels above 0.4 mM for a prolonged time. The steep dose response and small therapeutic range have been explained by the non-linear pharmacokinetics of FAA, in which its rate of removal is decreased at higher doses (Chabot et al., 1989a; Damia et al., 1988; Gouyette et al., 1988).

In summary, FAA differs from most anti-proliferative drugs in its broad spectrum of activity, and in its greater activity in vivo, against solid rather than dispersed tumours, and against established rather than early tumours. An additional finding of note was that unlike anti-proliferative agents, FAA induced haemorrhagic necrosis within 4 - 24 h of treatment (Smith et al., 1987), similar to the effect seen with TNF (Section 1.5.4.). These unusual features suggest that FAA has a novel mechanism of action. Three hypotheses have been proposed to account for these unusual characteristics (Cummings & Smyth, 1989).
1.6.2. Hypotheses to explain FAA activity

1.6.2a. Direct cytotoxicity after in vivo activation

The apparent discrepancy between in vitro and in vivo activity led Chabot et al. (1989b) to propose activation of FAA to a more cytotoxic species in vivo. These authors demonstrated cytotoxic activity in the plasma of treated mice, which was higher than equivalent concentrations of FAA added to plasma from untreated mice. Furthermore, the addition of liver microsomal preparations enhanced the cytotoxicity of FAA in vitro. In this study, no attempt was made to isolate the cytotoxic substances, and the possibility that these were cytokines or other cytotoxic macromolecules released by FAA was not excluded. The report of single strand DNA breaks after FAA treatment in vivo (Bissery et al., 1988) has been interpreted (Cummings & Smyth, 1989) as supporting a direct cytotoxic effect of FAA or a metabolite. However, this effect was not seen in vitro (Capolongo et al., 1987), and the DNA damage seen by Bissery et al. (1988) was later thought to be secondary to tumour cell death (Evelhoch et al., 1988). Further evidence against the in vivo activation hypothesis emerges in Chapters 3 and 5.

1.6.2b. Stimulation of immune-mediated cytotoxicity

The hypothesis of in vivo activation does not explain the FAA-resistance of ascites tumours (Section 1.6.1), or the interesting findings of Finlay et al. (1988). In their study, diffusion chambers containing Lewis Lung tumour cells were incubated in the peritoneal cavities of mice treated with FAA. The cells did not undergo necrosis, as did subcutaneous Lewis Lung tumours, suggesting that a critical host cellular component, possibly immune effector cells, was lacking. The similar necrotising effect of FAA and TNF (Section 1.6.1; Figure 1.4) also supports an immune mechanism.

Several studies have provided evidence of immune effector cell effects (reviewed by Wiltrout & Hornung, 1988). Natural killer (NK) activity was induced in spleen cells after FAA administration (Ching & Baguley, 1987), an effect mediated by interferons (Hornung et al., 1988a). Life extension in mice bearing RENCA murine renal cell carcinomas by FAA, was improved by the co-administration of interleukin-2 (IL-2), which increased both NK-cell activity, and number of cures (Wiltrout et al., 1988). The resistance of treated mice to re-challenge with RENCA tumours indicated a role for specific T-cell immunity (Hornung et al., 1988b). Thus it appears that tumour damage after FAA treatment includes a late T-cell-dependent action, which increases cure rate. Whether the early necrotising phase of FAA activity also requires T-cells is not known, though the necrotising activity...
of the drug in xenografts in nude mice, suggests that it is T-cell-independent (see also Section 8.1.3d., p. 185).

There are now reasons to doubt the involvement of NK-cells in the necrotising action of FAA (Ching & Baguley, 1989a): FAA causes haemorrhagic necrosis in a NK-resistant tumour, and NK-induction does not correlate with necrotising activity. More interest is now being focussed on the macrophage. Anti-tumour cytotoxic activity is enhanced in macrophages by exposure to FAA in vitro (Ching & Baguley, 1988), and this effect is inhibited by dexamethasone. TNF, a macrophage product, was suspected of being the mediator of this effect, but the cytotoxic activity could not be demonstrated in macrophage supernatants. The effector:target cell ratios used in these experiments were considerably higher than those found in vivo, and so the importance of this process in anti-tumour activity has not been established. In Chapter 4, the role of immune effector cells is investigated in vitro in spheroids, in which the effector:target ratio is similar to that of tumours.

1.6.2c. Inhibition of tumour blood flow

The indirect mechanism suggested by Finlay et al. (1988) could involve modulation of the tumour physiology rather than mediation by immune effector cells. Evelhoch et al. (1988) studied the ATP levels and blood flow in Glasgow osteogenic sarcomas using nuclear magnetic resonance spectroscopy. They showed a marked fall by 4 h in tumour perfusion as well as tumour ATP levels, but were unsure which effect was primary. Indeed, since obvious histological tissue damage was seen by 4 h in Colon 38 tumours by Smith et al. (1987), both of these physiological effects could have been secondary to tissue damage in the tumours. Therefore, the investigations described in Chapters 2 and 3 examined the effects of FAA on perfusion and morphology from 15 min onwards.

1.6.2d. Specificity for tumour microenvironment

A fourth hypothesis, which has not previously been suggested, is that the greater cytotoxicity of FAA in vivo could reflect increased potency of this drug under the microenvironmental conditions peculiar to tumours, such as hypoxia, low pH or nutrient deficiency. Agents of this type have been discussed above (Section 1.2.2.). This hypothesis is tested specifically in Chapters 4 and 7.
1.6.3. AIMS OF THE THESIS

The failure of FAA to show clinical activity (Kerr et al., 1987) despite outstanding activity in mice, is the most important reason to establish the mechanism of the anti-tumour action of this interesting compound. With this knowledge clinical administration and monitoring could be optimised. If FAA itself ultimately fails in the clinic, knowledge of the mechanism of the anti-tumour action in mice may assist in the design and evaluation of analogues which are active in humans. Alternatively, the results of such research may indicate a fundamental difference in the physiology of mice and humans which would preclude any agent of this type exhibiting clinical activity.

The major aim of the thesis is to explain the mechanism by which FAA causes early tumour cell death, and any later T-cell-dependent phase of tumour cell eradication is not investigated. In explaining its mechanism of action, it will be necessary to account for the selectivity of FAA for tumour tissue rather than normal tissues, and this requires examination of the toxic effects of FAA on normal tissues. Finally, the thesis compares FAA with some xanthenone acetic acids with structural similarities to FAA (Atwell et al., 1989), to determine whether they operate by similar mechanisms, and to assess whether these analogues may be superior to FAA in the clinic.

Summary of aims:

1. To test the four hypotheses discussed in Section 1.6.2. as explanations for the unusual anti-tumour activity of FAA in mice, and to determine, in detail, the mechanism of its necrotising action (Chapters 2 - 5).

2. To compare the actions of FAA with those of its xanthenone acetic acid analogues (Chapter 6).

3. To examine the toxicity of FAA in normal tissues, and the basis of the selective toxicity of the drug (Chapter 7).
CHAPTER 2

MECHANISMS OF FAA-INDUCED INJURY IN TUMOUR TISSUE:
A MORPHOLOGICAL AND METABOLIC STUDY

2.1. Introduction

A dramatic feature of the anti-tumour action of FAA is its induction of haemorrhagic necrosis (Baguley et al., 1989). This process has been noted in various tumours (see Table 1.1, p. 43), but all these reports describe the morphological features at only one or two intervals after treatment. The evolution of the necrotic process, and the morphological changes at early times after treatment, have not been described previously.

Sequential morphological examination of tumours after FAA treatment has the potential to demonstrate the mechanism of tumour cell death, and could provide clues to the tumour selectivity of this unusual agent. Furthermore, the tumour blood vessels, considered to be a potential target for the action of FAA (Section 1.6.2c., p. 46), could be examined directly. Morphological studies could also provide supportive evidence for the other hypotheses proposed in Section 1.6.2. If necrosis was found to begin in tumour tissue distant from the vasculature, this would suggest that the low exchange environment determines the selective toxicity of FAA for tumours. If changes were seen in the number or nature of immune or inflammatory cells within the tumour, this would provide evidence for the operation of immune mechanisms.

The investigations reported in this chapter describe the progression of morphological changes in Colon 38 tumours from 30 min to 24 h after treatment with FAA. These changes in tumour cells and blood vessels are then compared to those which occur following global ischaemia. In addition, concentrations of the high energy metabolites ATP and creatine phosphate (CP) in Colon 38 tumours were measured to determine whether energy depletion is an early event after FAA administration.
2. M. Methods

2. M. 1. Mice and tumours

C57BL/6J, DBA/2J and Balb/C breeding pairs were obtained from the Jackson Laboratory, Bar Harbour, Maine, USA, and were bred in the Auckland Cancer Research Laboratory under conditions of controlled lighting, constant temperature and humidity with sterile bedding, water and food.

The Colon 38 tumour, used in previous FAA studies (Table 1.1, p. 43), is a well-differentiated colonic adenocarcinoma, originally induced in a C57BL/6J mouse by 1,2-dimethylhydrazine (Corbett et al., 1975). Tumour stocks were obtained from the Mason Research Institute, Worcester, USA, and after in vivo passage, were stored at -196°C. Tumours were passaged by implantation of 1 mm³ tumour fragments s.c. into the flanks of BDF₁ [(C52BL/6JxDBA/2)F₁] mice anaesthetised with i.p. pentobarbital (90 mg/Kg) as described by Baguley et al. (1989). The mice used weighed 18 - 25g, and the tumours 0.3 - 1.0g.

2. M. 2. Flavone acetic acid

FAA was dissolved in 5% wt/vol sodium bicarbonate. Solutions were made up fresh or stored overnight at -20°C. FAA solutions were exposed to normal laboratory lighting, but not to direct sunlight, which causes loss of activity (Rewcastle et al., 1990a). FAA was given i.v. in a volume of 10 μl/g mouse weight, either at 30 min, 1 h, 2 h, 4 h, 8 h or 24 h before sacrifice. The maximum tolerated dose of FAA in Colon 38-bearing BDF₁ mice, 1.2 mmol/Kg (BC Baguley, personal communication), was used in all experiments.

2. M. 3. Histological assessment

Mice (4 - 5 per time point) were killed by cervical dislocation, the tumours were removed, incised to aid fixation, and immersed in 4% formaldehyde for at least 24 h. The tumours were then cut into 3 mm thick slices and processed in a Tissue-Tek VIP 2000 automated tissue processor, in which the tissue was dehydrated in increasing concentrations of ethanol, exposed to graded ethanol-xylol mixtures and embedded in paraffin wax. The blocks were then sectioned at 5 μm intervals on a
microtome, and the sections stained with Ehrlich's haematoxylin and eosin (Culling, 1974). The slides were viewed with a Nikon Optiphot microscope at a magnification of 40x. Quantitation of necrosis was performed using an 81-square eyepiece grid. Each square was counted either as viable, recent necrosis, or pre-existing necrosis according to the criteria discussed in Section 2. R. 2. The entire tumour area on each slide (between 6 and 17 fields) was counted.

To simulate total ischaemia, Colon 38 tumours (4 per time point) were excised and allowed to autolyse in a nitrogen atmosphere at 37°C, and the histological changes at 1 - 48 h were compared with those which followed FAA treatment.

2. M. 4. Electron microscopy

Tumours and samples from the caecum, pancreas, adrenal gland, kidney and lung were rapidly excised after killing the mice (2 per time point) and the tissue quickly cut into 1 mm blocks with razor blades in cold phosphate-buffered 2.5% glutaraldehyde (pH 7.4). The fragments were fixed and stored for up to 3 weeks in glutaraldehyde solution, and then further processed in bottles on an inclined rotator. After rinsing in phosphate buffer the blocks were post-fixed in cacodylate-buffered osmium tetroxide (30 min), and stored in cacodylate buffer. They were then dehydrated in 50, 70 and 100% ethanol, anhydrous ethanol, transferred to propylene oxide, propylene oxide-resin (1:1), and then into 100% epoxy resin (EMbed-812) under vacuum. After polymerisation the resin blocks were sectioned at intervals of 2 μm, and the sections stained with toluidine blue and examined with a light microscope. Ultrathin sections (90 nm) of selected areas of interest were then cut using an ultramicrotome with a diamond knife, collected onto copper grids, stained with lead citrate and uranyl acetate, and examined with a Philips 410 LS transmission electron microscope.

2. M. 5. Tumour ATP and related metabolites

Treated and untreated mice bearing Colon 38 tumours (4 - 5 per timepoint) were killed by cervical dislocation and the tumours rapidly excised and frozen in liquid nitrogen. Tumour levels of ATP, ADP, AMP, adenosine, inositol monophosphate, inositol, hypoxanthine plus xanthine, uric acid and CP were determined by high performance liquid chromatography using the method of Holliss et al. (1984). For this, the frozen tissue was pulverised, extracted into 0.6 M perchloric acid and neutralised. Samples were injected into a chromatography column supplied by a Waters 510 HPLC pump using
a mobile phase with decreasing polarity, controlled by a Waters Automated Gradient Controller at a flow rate of 1.2 ml/min, through a Absorbosphere HS column (7 μm, 250 x 4.6mm). The eluate was monitored with a 440 Waters 254nm fixed wavelength detector.

2. R. Results

2. R. 1. Histology of untreated Colon 38 tumours

The untreated Colon 38 tumour showed the features of a well-differentiated adenocarcinoma (Figure 2.1) similar in appearance to that illustrated in the original description of this tumour (Corbett et al., 1975). While the tumour histology varied from area to area in amount of necrotic tissue and in the density of blood vessels and fibrous stroma (discussed below), the cytological appearances of individual tumour cells were uniform, and cells were regularly arranged around small uniform gland spaces (acini). The acini were closely packed, with little interstitial matrix or fibre in most areas, but the tumours had fibrous capsules from which occasional septa extended into the tumour (Figure 2.1a).

The blood vessels varied in size and density; in most areas they were sparse, small, and inconspicuous by light microscopy (Figure 2.1a,g). Less common were thin walled sinusoids of large diameter which lay between nodules or papillae of tumour tissue (Figure 2.1b,d). Most vessels within the tumour were of capillary type, consisting simply of an endothelial lining surrounded by a small (but variable) amount of loose connective tissue stroma. Thrombi were rarely seen. Occasionally normal host tissues such as nerves or arteries were seen in stromal septa. Among the tumour cells, there were scattered single small round cells with indented or bean-shaped nuclei with condensed chromatin, having the appearances of macrophages (Figure 2.1c).

Necrosis was seen in all untreated tumours, and this ranged in volume fraction from 5 - 51%. Necrosis was defined as confluent zones of irreversible cell injury, which was recognised by cell membrane damage, loss of cytoplasmic staining, coarse clumping of nuclear chromatin, karyorrhexis (nuclear fragmentation), karyolysis (loss of nuclear staining) or nuclear pyknosis (shrinkage with increased staining).

There were two patterns of necrosis in untreated tumours. In the more common pattern (called Type 1), small foci of necrosis situated at a distance from tumour blood vessels alternated with viable areas (Figure 2.1b,d-f). Where extensive, Type 1 necrosis surrounded zones of viable tumour cells
adjacent to blood vessels (Figure 2.1e) but did themselves contain vessels. Type 1 necrosis was characterised by progressive disintegration of the cytoplasm and nucleus of tumour cells with increasing distance from the vessels, and affected regions contained angular, haematoxyphilic bodies (Figure 2.1f).

Type 2 necrosis showed well-demarcated, confluent, sometimes extensive, areas of morphological change, which were remarkably uniform within any individual focus. Loss of staining and fragmentation of the cytoplasm, karyolysis and nuclear pyknosis were seen, but there was little karyorrhexis (Figure 2.1g-i). Such necrotic areas usually contained blood vessels which were directly in contact with necrotic tumour tissue (Figure 2.1h), and which themselves showed features of necrosis (loss of staining of endothelial nuclei). In some cases, these tumour vessels were congested, and diffuse haemorrhage was often seen in the necrotic focus.

2. R. 2. Histological effects of FAA treatment

FAA treatment resulted in the appearance of focal morphological changes in Colon 38 tumours which became more extensive with time after treatment, until eventually most of the tumour was necrotic. However, even after 24 h, small areas of apparently unaltered tumour tissue remained in some tumours.

The earliest morphological changes in tumours were loss of cohesion, rounding of cells and coarse clumping of nuclear chromatin. Occasional foci displaying this feature were seen after 30 min in both plastic embedded tumours as pale staining areas, sometimes with interstitial haemorrhage. By 2 h confluent areas of cell rounding and separation were evident in all tumours (Figure 2.2a-c), accompanied by scattered nuclear fragmentation (Figure 2.2b,d). After 4 h, such changes were widespread and affected most of the tumour (Figure 2.2e-h). Blood vessels of all sizes showed marked congestion with focal disruption and haemorrhage by 4 h (Figure 2.2.h). Vascular occlusion by thrombi was rare, and was not noticeably more frequent than in untreated tumours. No increase was noted in the number of lymphocytes, macrophages or neutrophil leukocytes in FAA-treated tumours.

There was no recognisable pattern in the distribution of these foci of histological change. Those regions most distant from the vasculature, and at the margins of pre-existing necrotic zones (hypoxic cells), showed no increased tendency to be affected. Rather, the affected areas included vessels, as seen in Type 2 spontaneous necrosis.

Cytoplasmic and nuclear degeneration progressed so that after 8 h the apparently unaffected areas of tumour cells could easily be distinguished from necrotic tissue (Figure 2.2i-m). These areas
were often situated adjacent to either the tumour capsule or septa, or were in regions where stroma between tumour cells was increased (Figure 2.2k).

Necrosis following FAA treatment differed from both types of necrosis seen in untreated tumours, since it was never more than 24 h old, and neither karyolysis nor haematoxophilic bodies were present. This recent necrosis could thus be clearly distinguished from pre-existing necrosis, and scored separately. The following results for recent necrosis are expressed as the percentages (mean ± s.e.m.) of total tumour volume (viable tumour plus pre-existing necrosis). Untreated tumours scored 5 ± 3% for recent necrosis, and values for tumours 1 h and 2 h after treatment were not significantly different (3 ± 2% and 4 ± 1% respectively). However, by 4 h, the necrotic fraction had increased markedly to 94 ± 2%.

Sections from the caecum, pancreas, kidney, adrenal and lung examined 24 h after FAA treatment showed no histological changes.
FIGURE 2.1 - HISTOLOGY OF UNTREATED COLON 38 TUMOURS

a. Tumour surrounded by a fibrous capsule (C), from which septa (arrows) extend into the tumour. The cells are mostly arranged in round or oval acini of regular size. Vessels in this field are small and associated with little connective tissue stroma (small arrowheads), or more abundant stroma (large arrowheads).

b. Clusters of tumour acini (T) are surrounded by wide diameter sinusoidal vessels (V). A small amount of connective tissue stroma (S) is seen. Zones of necrosis (N) are present within the tumour tissue.

c. Detail of a tumour blood vessel (V) which is lined by a single layer of endothelial cells. Associated fibrous stromal tissue is seen (upper left corner). A macrophage with a characteristic bean-shaped nucleus (arrow) is present between tumour cells.

Light photomicrographs, H&E-stained paraffin sections, bars = 50 μm.
FIGURE 2.1 (CONTINUED) - HISTOLOGY OF UNTREATED COLON 38 TUMOURS

d - f. Type 1 necrosis. d. Closely packed small vessels (upper right) and widely spaced large sinusoids (lower left) are seen in this field. Necrosis (N) is seen at a distance from the sinusoids. e. Extensive necrotic material (N) surrounds viable tumour cells adjacent to blood vessels (arrowheads). f. Detail of Type 1 necrosis (N) which is characterised by nuclear fragments (arrows) and haematoxyphilic bodies (arrowheads) with progressive degradation of cellular material as the distance from the tumour vessel (V) increases.

g - i. Type 2 necrosis. g. A small, well-demarcated necrotic focus (N) showing uniform degeneration of tumour cells is seen within highly vascular tumour tissue. h. The necrotic zone (arrows) contains necrotic tumour cells and a necrotic blood vessel (arrowhead). i. Detail of necrotic/viable cell interface (arrows). The cytoplasm of necrotic cells stains faintly, and nuclei show pyknosis (solid arrowheads) or karyolysis (open arrowheads). However, the degree of cell breakdown does not increase with distance from the interface (compare with f.)

Light photomicrographs, H&E-stained paraffin sections, bars = 50 μm.
FIGURE 2.2 - HISTOLOGY OF FAA-TREATED TUMOURS

a - d. 2 h post-treatment. a. This field shows an area of decreased tissue staining (upper left). Small foci of pre-existing necrosis (N) are also present. b. In the more lightly-stained regions (upper right) tumour cells show decreased cytoplasmic staining and are separating. Vessels show congestion (arrowheads), and haemorrhage is seen among dissociated cells (arrow). c. Detail of cell dissociation and rounding (arrow). Some apoptotic cell fragments are also seen (arrowheads). d. Area of cell fragmentation without cell rounding.

Light photomicrographs, H&E-stained paraffin sections, bars = 50 μm.
FIGURE 2.2 (CONTINUED) - HISTOLOGY OF FAA-TREATED TUMOURS

e - h. 4 h post-treatment. e. Confluent affected area showing decreased tissue staining (lower half). The upper part of the field is largely unaffected, and consists of pre-existing necrosis (N), and viable tissue around vessels (arrowheads). f. Cell dissociation and fragmentation are more extensive than at 2 h (cf. Figure 2.2b). Residual viable tissue is associated with vascularised stroma (arrowheads). g. Detail of dissociating tumour cells showing rounding, margination of nuclear chromatin (arrowheads) and fragmentation of cells (arrows). h. A tumour vessel (V) is congested by red cells, which are haemorrhaging into the disintegrating tumour tissue (arrows). Endothelial cells (arrowheads) appear to be intact.

Light photomicrographs, H&E-stained paraffin sections, bars = 50 μm.
i, j. 8 h post-treatment. i. A single focus of residual viable tumour tissue (arrow) is surrounded by confluent necrosis. j. Acini are partly dissociated and cells have pyknotic nuclei. Haemorrhage is seen between tumour cells (arrows).

k - m. 24 h post-treatment. k. A focus of residual viable tumour tissue (centre) adjacent to capsule (C), surrounded by confluent necrosis (N). The vessels are associated with fibrous stroma (pale-staining tissue, arrows). l. Necrotic region, including areas in which acini can still be recognised (centre, upper right), and areas in which cells are dissociated and show nuclear fragmentation (periphery). m. Detail of necrotic cells showing nuclear pynosis (arrowheads), or fragmentation (arrows).

Light photomicrographs, H&E-stained paraffin sections, bars = 50 μm.
2. R. 3. Ultrastructure of untreated Colon 38 tumours

Electron microscopy demonstrated features characteristic of a well-differentiated adenocarcinoma (Figure 2.3). Tumour cells had well-developed junctional complexes between adjacent apical surfaces, from which a few short microvilli projected into the lumen of the acinus (Figure 2.3c,d). The cytoplasm contained many polyribosomes, some mildly dilated endoplasmic reticulum, moderate numbers of mitochondria and occasional Golgi complexes (Figure 2.3a-d). Vessels were lined by a single layer of endothelial cells (Figure 2.3a,b), sometimes with pericytes on their abluminal surfaces (Figure 2.3a). The endothelial cells formed intercellular junctions with one another in most places (Figure 2.3a,b), but occasionally discontinuities were seen and some endothelial cells were fenestrated (Figure 2.3b). In most areas collagen fibres and interstitial matrix between tumour cells or vessels were sparse.

There were some foci of spontaneous necrosis (Figure 2.3e,f) in which tumour cells showed rupture of cell membranes, with increased granularity and electron density of both cytoplasm and nuclei. Although some cell fragmentation was seen, cell rounding was not a feature, and cell junctions were still present between degenerating cells (Figure 2.3f).

2. R. 4. Ultrastructural effects of FAA treatment

In the foci of tumour cell dissociation (described in Section 2. R. 2.) there was dissolution of cell junctions and rounding of tumour cells (Figure 2.4a-e). The nuclear chromatin of some tumour cells had become marginated and coarse, and mitochondria showed a slight increase in electron density (Figure 2.4c,d). Some tumour cells also showed fragmentation into spherical globules, some containing nuclear fragments, which is characteristic of apoptosis (Figure 2.4e). Focal membrane loss and cytoplasmic degeneration were also seen, particularly after 4 h (Figure 2.4f). Endothelial cells did not show these changes even when surrounded by affected tumour cells (Figure 2.4e), or when the vessels were engorged. Neither thrombosis nor infiltration of vessels by neutrophils was seen.
FIGURE 2.3 - ULTRASTUCTURE OF UNTREATED COLON 38 TUMOURS

a, b. Vascular structure. Tumour vessels are mainly of capillary type with little connective tissue stroma. a. This field shows a tumour vessel lined by an non-fenestrated endothelial layer (arrowheads). A pericyte cell body (P) and processes (arrows) are seen in the space between the endothelial layer and the basal parts of the tumour cells (T). b. In this field the endothelial layer shows gaps (open arrows) and junctions (solid arrows) between cells, and fenestrae within cells (arrowheads). No pericytes are seen. E = endothelial cell nucleus.

c, d. The gland lumen. A few short surface microvilli (arrows) project into the lumen (L), which contains degenerate cellular material. Well-formed junctional complexes (arrowheads) are seen between the apices of the tumour cells. Mitochondria (M), mildly-dilated rough endoplasmic reticulum (E), and a centriole (C) are seen.

e, f. Spontaneous necrosis: e. An intact tumour cell (T) is attached to a degenerating tumour cell containing electron-dense bodies (arrowheads). The upper part of the field shows tumour cell fragments with loss of cell membranes, granularity of the cytoplasm and coarse condensation of the nuclear chromatin. f. Detail of two cells undergoing necrosis, showing nuclear chromatin condensation (N), and cell and nuclear membrane damage (arrowheads). The cells remain in close contact, and a cell junction (arrow) is preserved.

Transmission electron micrographs, uranyl acetate and lead citrate stain, bars = 2 μm.
FIGURE 2.4 - ULTRASTRUCTURE OF FAA-TREATED COLON 38 TUMOURS

a, b. 30 min post-treatment. A few tumour cells show early changes of apoptosis: loss of cohesion with cell rounding, and peripheral condensation of nuclear chromatin (arrowheads). The cell membranes appear intact and there is no cytoplasmic degeneration.

c - e. 60 min post-treatment. c, d. Acini show loss of cell junctional complexes at luminal margins (arrowheads), and irregular arrangement of the cells around the lumina (compare with Figure 2.3 c,d). The mitochondria (M) show increased electron density. e. Tumour cells (T) show apoptosis, with cell rounding and fragmentation. The capillary (C) shows an intact endothelial layer (arrowheads), and contains a neutrophil leukocyte (N) and platelets (arrows).

f. 4 h post-treatment. In this field the changes are those of necrosis, with maintenance of acinar structure, and marked degenerative changes in the tumour cells. These include rupture of the basal cell membrane (arrows), and marked swelling of the endoplasmic reticulum and other organelles.

Transmission electron micrographs, uranyl acetate and lead citrate stain, bars = 2 μm.
2. R. 5. Histology of globally ischaemic Colon 38 tumours

Excised tumours incubated at 37°C under nitrogen to simulate in vivo ischaemia showed only subtle changes in morphology after 4 h, but all showed increased nuclear chromatin staining. By 24 h the outlines of the acini were blurred because of rupture of the basal cell membranes (Figure 2.5), but the loss of structure was much less severe that that seen 24 h after FAA (Figure 2.31). Individual cells maintained cohesion, and cell rounding and nuclear fragmentation, features characteristic of FAA-treated tumours, were not seen at any stage.

2. R. 6. Effects of FAA on ATP and related metabolites

ATP and CP concentrations fell progressively over 4 h (Figure 2.6A). This was accompanied by a transient rise in ADP and AMP levels (Figure 2.6B), with peaks at 30 min and 1 h respectively, and a gradual rise in purine catabolites hypoxanthine plus xanthine, and uric acid (Figure 2.6C). Adenosine, inosine monophosphate and inosine also showed an increase in concentration with time (data not shown).

These changes began early, with a significant rise in AMP and ADP levels at 15 and 30 min, respectively (P < 0.05).
Colon 38 tumour 24 h after excision, incubated at 370C. (a). The acini have become separated, but the cells of each acinus maintain cohesion. Detail of fields from tumours after 4 h (b), 8 h (c) and 24 h (d) of global ischaemia. They show mild changes which progress slowly including a slight increase in nuclear staining, and disruption of the cell membrane and cytoplasm at the periphery of the acini (arrows), which themselves remain intact.

Light photomicrographs, H&E-stained paraffin sections, bars = 50 μm.
FIGURE 2.6 - EFFECT OF FAA ON ENERGY METABOLITES

Tumour levels of ATP and CP (A), ADP and AMP (B), and uric acid and hypoxanthine + xanthine (HX + X) (C), after treatment with 1.2 mmol/Kg FAA.

Zero time points represent untreated tumours. Points represent means ± sem. n = 4, 5.
2. D. Discussion

This chapter has shown that most cell death in Colon 38 tumours occurs between 2 h and 4 h after FAA treatment. Initially this is evident as apoptosis but later necrosis supervenes. The discussion below considers spontaneous necrosis in Colon 38 tumours, how this differs from the morphological changes following FAA treatment, and the interpretation of the FAA-induced morphological and metabolic changes in terms of the mechanism of action of this drug.

2. D. 1. Spontaneous necrosis in Colon 38 tumours

Necrosis was seen in untreated Colon 38 tumours, and two patterns were observed. The more common pattern (Type 1) always occurred at a distance from the tumour vessels. This is consistent with cell death occurring through the depletion of oxygen as it diffuses through, and is metabolised by, intervening viable tumour cells, as originally described by Thomlinson and Gray (1955) (Section 1.4.1., p. 31). Two features of this pattern suggest such a mechanism: 1) the spatial relationship to the blood vessels, and 2) the progression of cell fragmentation with increasing distance from the vessels. The latter suggests that necrotic material is being continuously added at the interface with viable tissue as the tumour grows.

The second pattern of necrosis (Type 2) has not been previously reported and has the appearances of infarction (cell death following acute ischaemia). Such a mechanism is suggested by the observed sharp demarcation from viable tumour tissue, and the uniformity of degenerative changes within the necrotic focus, which includes blood vessels. This is consistent with acute irreversible cessation of blood flow in a vessel or group of vessels. Recognition of this particular type of spontaneous necrosis is important in investigations of this kind because it could otherwise be confused with necrosis due to anti-tumour therapies.

It is suggested that each type of necrosis corresponds to a different type of hypoxia (discussed previously in Section 1.4.4., p. 34): chronic hypoxia would lead to Type 1 necrosis, and acute hypoxia, if irreversible, would lead to Type 2 necrosis. Both types of necrosis are frequently seen in human malignant tumours of diverse histological types (unpublished observations).

The morphological effects of FAA treatment differed from those of spontaneous necrosis and global ischaemia. In the latter two situations, the changes were those of necrosis, while FAA-treated tumours had features of both necrosis and apoptosis. The relative importance of these two processes varied with interval after FAA treatment: the earliest changes were mainly apoptotic, but at later times only necrotic changes were evident.

The earliest evidence of cell injury was margination and fragmentation of the nuclear chromatin with disintegration of tumour cells into ovoid cytoplasmic bodies containing nuclear fragments, which is characteristic of apoptosis. This process occurs in a wide variety of physiological and pathological conditions. It is the major mechanism responsible for the reduction in cell mass and tissue remodelling in embryogenesis, and for the removal of injured cells following radiotherapy, chemotherapy and immune mediated cell damage (reviewed by Wyllie, 1981; Kerr et al., 1984; Wyllie, 1987).

Unlike necrosis, apoptosis does not disrupt tissue architecture or elicit an inflammatory response in normal tissues, though these features might be difficult to appreciate in tumours. While necrosis is often the consequence of the interruption of energy metabolism due to ischaemia, apoptosis is an active process which requires energy and protein synthesis (Kerr et al., 1984). Ultrastructural studies of apoptosis have shown early margination of chromatin, protrusion of cytoplasmic blebs (containing nuclear fragments and organelles), the separation of these blebs into intact ovoid bodies, and their phagocytosis by other parenchymal cells or macrophages. On the other hand, necrosis is characterised by disintegration of the cell structure (Searle et al., 1975; Wyllie, 1981; Kerr et al., 1984). Because of the rapidity of the apoptotic process, it is difficult to detect by light microscopy unless large numbers of cells are simultaneously affected (Kerr et al., 1984), as was the case in the present study.

The changes of necrosis, such as breaches in the cell membrane and degeneration of organelles, began within 30 min of treatment and became the prevalent type of cell death between 4 and 8 h. There was thus considerable temporal overlap of the two processes. The variation in appearance of the degenerating cells 24 h after FAA treatment (Figure 2.2l,m), suggests that some tumour cells (those with intact pyknotic nuclei) became necrotic following pure ischaemia, while others (with fragmenting nuclei) were undergoing apoptosis when interrupted by ischaemia causing energy depletion.

The similarity of haemorrhagic necrosis following both FAA and tumour necrosis factor-α (TNF) has been noted previously (Baguley et al., 1989), and the possibility that TNF mediates the anti-tumour effects of FAA has been considered (reviewed in Section 1.5.8., p. 40). The morphological effects of TNF on tumour cells in vitro are similar to those seen in this in vivo study. TNF may cause either an apoptotic or a necrotic type of cell death in vitro, depending on the cell line (Laster
Electron microscopy has shown loss of junctions in human glioblastoma cells exposed to TNF (Rutka et al., 1988). However, the effects of TNF on tumours in vivo show some differences from the present findings. In Meth A fibrosarcomas TNF causes thrombosis (Watanabe et al., 1988; Shimomura et al., 1988), accompanied by endothelial damage apparently mediated by neutrophil leukocytes (van de Weil et al., 1989). Vascular changes of this type have not been described after FAA in this or other studies (Bissery et al., 1988; Shimomura et al., 1988; Baguley et al., 1989; Sun & Brown, 1989) and suggest that TNF is not the sole mediator of FAA activity.

2. D. 3. Non-ischaemic mechanisms of tumour cell killing

The occurrence of apoptosis after FAA treatment suggests the contribution of an early non-ischaemic component of tumour cell killing, since this energy-requiring process would not be expected to occur as a consequence of ischaemia. This is confirmed by the failure to detect apoptosis, cell dissociation or cell rounding either in globally ischaemic tumours or in areas of spontaneous necrosis in untreated tumours.

The nature of the stimulus causing apoptosis after FAA has not been determined in this study. While ischaemia can be excluded, direct FAA cytotoxicity and immune-mediated tumour cell damage remain potential causes of apoptosis. The former appears unlikely, because FAA is only weakly cytotoxic in vitro (Finlay et al., 1988; Chabot et al., 1989b), though in vivo activation to a more cytotoxic compound (Chabot et al., 1989b) is not excluded. The present study does not offer any support for immune mediation. Treated tumours showed no differences in content or appearance of lymphocytes or macrophages but immune effects mediated by cytokines or other immune cell secretory products cannot be detected histologically. The role of immune effector cells is investigated in Chapter 4.

2. D. 4. Ischaemic mechanisms of tumour cell killing

Confluent tumour cell necrosis was seen 8 h after FAA treatment and is likely to be due to the interruption of blood flow. Such a vascular mechanism is supported by the focal nature of the necrosis (which resembles Type 2 spontaneous necrosis), the direct morphological evidence of vascular damage and congestion, and the metabolic effects of FAA treatment. Blood flow is known to fall by 85% in Glasgow osteogenic sarcomas within 4 h of FAA treatment (Evelhoch et al., 1988), and Bibby et al. (1989a) observed a fall in blood flow in MAC 26 colonic tumours after 2 h. The role of ischaemia and the tumour vasculature in cell killing is investigated directly in Chapters 3 and 5.
2. D. 5. The distribution of necrosis

Within 8 h of FAA treatment most of the tumour tissue was necrotic, and thus the distribution of residual viable tissue could be easily discerned. This was usually associated with host stromal tissues: the tumour capsule, fibrous septa, and fibrous tissue interspersed between tumour cells. The Colon 38 tumours from the experiments of Baguley et al. (1989), and various carcinomas and sarcomas studied by Dr Sally Hill, CRC Gray Laboratory, UK, have been reviewed by the author, and show a similar pattern after FAA treatment.

The persistence of residual viable tumour cells beneath the capsule has been reported previously after treatment with FAA (Bissery et al., 1988), TNF (Asher et al., 1987; North & Havell, 1988) and hyperthermia (Nishimura et al., 1988). The last two of these treatments are thought to involve vascular mechanisms. Nishimura et al. (1988) reported that the more resistant of two tumour types had a higher content of interstitial stroma, which was thought to provide better physical support for the thin-walled tumour vessels. It is possible that a similar selective resistance to FAA is conferred by the presence of fibrous stroma, which may support the thin-walled vessels after the cells loose cohesion in the early stages after treatment.

Whatever the basis of resistance, this finding could have significance in the treatment of human tumours with FAA-like agents, since several human tumours (including many carcinomas of breast, Robbins et al., 1984) have a high content of fibrous stroma.


The progressive morphological damage seen in Colon 38 tumours treated with FAA was accompanied by loss of ATP and CP, with a transient rise in ADP and AMP, before their progressive oxidation to uric acid. The fall in ATP and CP was more marked and rapid than that seen in human neural tumour xenografts after treatment with anti-proliferative agents (Naruse et al., 1985), in agreement with the studies of Evelhoch et al. (1988) in Glasgow osteogenic sarcomas. However, in the present studies ATP and CP stores were still 1/3 of control levels 4 h after FAA treatment, while only 4% of tumour cells were histologically viable at this time. This suggests that loss of viability precedes depletion of high energy compounds in parts of the tumour.

The metabolic changes begin early, as shown by the significant rise in tumour AMP levels within 15 min of FAA treatment. This could be consistent with a direct effect of FAA on the energy metabolism of the tumour cells, but the progressive breakdown of the purine nucleotides towards uric
acid, suggests that the depletion of high energy metabolites is secondary to failure of tumour perfusion, as occurs in experimental myocardial ischaemia (Jennings et al., 1981).

2. D. 7. Conclusions

Colon 38 tumour cells develop apoptosis within 30 min of FAA treatment. At later times tumour cell death is manifest as necrosis. Tumour necrosis is associated with the loss of high energy metabolites and blood vessel disruption, suggesting that ischaemia plays a major role.

The distribution of necrosis suggests that proximity to host connective tissues may confer resistance to FAA, a finding of potential clinical significance.
CHAPTER 3

THE ROLE OF ISCHAEMIA IN THE ANTI-TUMOUR ACTION OF FAA

3.1. Introduction

Although no direct evidence for immune mechanisms or blood flow failure was found in the investigations of the previous chapter, the presence of both necrosis and apoptosis in the tumour cells suggested that both ischaemic and non-ischaemic mechanisms were involved. This chapter specifically investigates the role of ischaemia in the anti-tumour effect by measuring the early changes in tumour perfusion by single and double labelling techniques, and by determining the causal relationship between changes in perfusion and the anti-tumour effect.

To measure tumour perfusion, a double label fluorescent intravascular marker technique, based on that of Trotter et al. (1989a), was used, in which tumours are stained in vivo by the i.v. injection of two fluorescent dyes. The first label, Hoechst 33342 (H33342), was used as a diffusion marker in tumour cell spheroids by Durand (1982), and was first used in vivo by Reinhold and Visser (1983) to demonstrate endothelial cells. Its ability to stain paravascular tumour cells was later used for the assessment of spontaneous fluctuations in blood flow (Chaplin et al., 1987) and reductions in tumour blood flow caused by misonidazole either alone (Murray & Randhawa, 1988) or in combination with melphalan (Murray et al., 1987). The second label was the carbocyanin dye DiOC₆(3), first used as a diffusion marker in tumour cell spheroids by Olive and Durand (1987). Trotter et al. (1989a) then used it in a double label fluorescent intravascular marker study in which an i.v. injection of H33342 was followed after 20 min by an i.v. injection of DiOC₆(3). The mismatch of staining by the two dyes was used to demonstrate spontaneous fluctuations in tumour blood flow (reviewed in Section 1.4.3., p. 33). In the present study, both single and double label techniques were used to measure changes in tumour blood flow after FAA treatment.

Proof of causality requires that the putative cause (ischaemia) be both sufficient and necessary for the effect (tumour cell killing). Therefore, to determine whether perfusion failure is sufficient to explain the degree of cell kill seen after FAA treatment, the rate of cell death in globally ischaemic tumours and those treated with FAA were compared using a simple mathematical model. To determine whether perfusion failure is a necessary component of FAA activity, clonogenic cell yields from avascular i.p. spheroids and vascularised i.m. EMT6 tumours were compared after FAA treatment.
3. M. Methods

3. M. 1. Mice and tumours

Subcutaneous Colon 38 tumours were implanted into BDF₁ mice as described in Section 2. M. 1. (p. 49). The breeding of Balb/C mice was the same as for BDF₁ mice. The EMT6 tumour cell line was derived from the KHJJ mouse mammary carcinoma, which developed spontaneously from an implanted hyperplastic mammary alveolus (Rockwell et al., 1972). The EMT6 line can be propagated in culture, used in clonogenic assays (without agar) and grown in Balb/C mice to form solid tumours, which have a loose spindle cell histological pattern. The Auckland subline EMT6/Ak derived from the EMT6/Io subline, and kindly provided in 1984 by Dr. Ian Tannock, Ontario Cancer Institute, Canada, was maintained in culture using growth medium (GM) consisting of α Minimum Essential Medium with 5% (vol/vol) heat-inactivated (56°C, 40 min) fetal calf serum (FCS). New cultures were initiated every three months from frozen stock. EMT6 tumours were grown by i.m. injection of 10⁶ EMT6 cells into the thighs of Balb/C mice. The mice were used at about 11 days after inoculation when the tumours weighed 0.2 - 0.9 g, and the mice weighed 20 - 30 g.

3. M. 2. FAA and fluorescent perfusion markers

For single label blood flow and clonogenic experiments, FAA was dissolved in 5% (wt/vol) sodium bicarbonate. In double label blood flow experiments, the sodium salt of FAA was dissolved in 5% (wt/vol) D-glucose to 2.4 mM. H33342 was dissolved in 5% (wt/vol) D-glucose at 1.62 mM (1.0 mg/ml) for the single-label, or 4.87 mM (3.0 mg/ml) for the double-label experiments. DiOC₃(3) was dissolved in 75% (vol/vol) dimethylsulfoxide in water. These solutions were stored at -20°C. In the double-label experiments, the H33342 and FAA solutions were thawed and mixed in equal proportions immediately before use. For controls, the H33342 was mixed with an equal volume of 5% D-glucose. Volumes used for injection were 5 µl/g mouse weight for DiOC₃(3), and 10 µl/g for FAA, H33342 or mixtures of the two.
3. M. 3. Assessment of tumour perfusion: single label study

FAU was injected i.p. at a dose of 1.2 mmol/Kg, or an equivalent volume of 5% sodium bicarbonate solution was injected into control mice, followed 15 min to 24 h later by an i.v. injection of H33342 (16.2 µmol/Kg). Five minutes after the mice received the dye, they were killed by cervical dislocation, the tumours were excised, frozen rapidly in liquid nitrogen-cooled Freon 12 and stored at -80°C. Frozen sections 10 µm thick were cut from the mid-plane of the tumours on a cryostat, were air-dried, mounted in 10% (vol/vol) glycerol in 0.9% (wt/vol) saline, and viewed under a Nikon Optiphot microscope with a EF-D episcopic fluorescent attachment and a UV1A filter block (excitation maximum 365 nm, barrier 400 nm). H33342 staining was seen as a blue fluorescence of endothelial and perivascular tumour cell nuclei. Using a 64-point eyepiece grid and a magnification of 100, 16 non-overlapping contiguous fields were counted for each tumour. Points were scored as H33342 positive, necrosis, or artefact.

Points were scored positive in the fluorescent zone around the vessel and in its non-fluorescing lumen. Necrosis was identified either by its dull yellow autofluorescence or by its refractile appearance by light transmitted though a lowered condenser. These methods for identifying necrosis were validated by cutting consecutive sections from a few tumours and staining them with heamatoxylin and eosin. Artefact points included folds and clefts in the section and points falling outside the tumour or on the capsule. These were subtracted from the total count.

3. M. 4. Assessment of tumour perfusion: double label studies

Mice with s.c. Colon 38 tumours or i.m. EMT6 tumours received two i.v. injections: the first contained FAA (1.2 mmol/Kg mouse weight) plus H33342 (24.4 µmol/Kg), and the second contained DiOC7(3) (0.42 µmol/Kg), injected 15 min, 30 min, 1 h, 2 h and 4 h later. Control mice received H33342 only in the first injection. The mice were killed after a further 5 min. No external heating was applied before injection, except for immersion of the tail in warm water, to minimise the effects of temperature on tumour blood flow (Reinhold & van den Berg-Blok, 1987). The mice were unrestrained between injections, except for the "zero" time point, when the first injection was followed within one minute by the second.

Excised tumours were frozen, sectioned, and examined as described above. H33342-positive points were counted with a UV1A filter block, and then DiOC7(3)-positive points were counted with a B2A filter block (excitation maximum 450 - 480 nm, barrier 520 nm) on the same field. DiOC7(3)
staining was seen as a green fluorescence of endothelial and perivascular tumour cell cytoplasm. Because diffusion of DiOC₆(3) from the vessels occurred only after sections were mounted in glycerol (Section 3. R. 2.), each section was monitored and counted when the diffusion distances of the two dyes were the same, usually 30 - 60 min after mounting. Twenty five fields were then assessed on a 24-point counting grid for Colon 38 tumours and on a 64-point grid for (the less vascular) EMT6 tumours, both at a magnification of 100. Points were scored positive in areas of peri-vascular fluorescence, and in vessel lumina surrounded by fluorescence. In EMT6 tumours, fields with a zero score for both dyes, seen mainly in the necrotic centres, were excluded and, when necessary, further sections were cut at 200 µm steps into the block, so that sufficient fields were obtained. Tissue from the liver, spleen, kidney and small intestine of one of the 4 h post-treatment Balb/C mice was also examined to detect any changes in blood flow occurring in non-tumour tissues.

3. M. 5. Clonogenic assays on i.m. EMT6 tumours

FAA in 5% sodium bicarbonate was given i.v. to mice bearing i.m. EMT6 tumours. The dose response and time course of tumour cell killing were determined by tumour dissociation and in vitro clonogenic assay based on that of Brown et al. (1979). Tumours were removed under sterile conditions, minced with scalpels, and digested for 1 h at 37°C (with magnetic stirring) with 60 mg of tumour mince/ml of an enzyme cocktail consisting of 0.5 mg/ml Pronase, 0.2 mg/ml DNase, and 0.2 mg/ml collagenase in GM plus 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells were washed twice by centrifugation (145g, 8 min) and cell numbers determined with a Coulter ZF electronic particle counter. Up to 10⁵ cells in GM plus antibiotics were seeded in 60 mm dishes and cultured for seven days in a CO₂ incubator. Colonies were stained with 0.5% methylene blue in 50% ethanol and those with more than 50 cells were counted. To determine the rate of cell death after global ischaemia, nitrogen-asphyxiated mice were maintained at 37°C in nitrogen-purged bottles for 1 - 6 h, and the tumours assessed for clonogenicity.

The results are expressed in the following terms: Plating efficiency is the fraction of cells which form colonies on plating (clonogenic cells). Cell yield is the number of cells per unit volume of spheroid as detected by Coulter counting after dissociation. Clonogenic cell yield is the product of the above two measures.
3. M. 6. Effects of FAA on EMT6 i.p. spheroids and i.m. tumours

EMT6 spheroids were initiated by incubating 7 x 10^4 cells in 15 ml GM with 10% (vol/vol) FCS in bacteriological-type plastic dishes, and were then transferred to spinner flasks (after Sutherland & Durand, 1976) and grown to ~1 mm diameter. Between 20 and 25 spheroids were introduced into the peritoneal cavity of each Balb/C mouse, anaesthetised with 90 mg/Kg pentobarbital i.p., through a 16.5 gauge needle (modified from Lord, 1980). Six days after implantation, the mice were given i.v. or i.p. FAA in 5% sodium bicarbonate at a dose of 0.8 mmol/Kg. At least 12 spheroids were recovered from each mouse 18 h after treatment. Spheroids were washed twice in phosphate-buffered saline and dissociated in GM containing 0.5 mg/ml Pronase and 0.2 mg/ml DNase, and clonogenic assays performed as described above.

3. R. Results

3. R. 1. Single label blood flow study: Colon 38 tumours

Untreated Colon 38 tumours showed a dense network of fluorescent vessels, with occasional non-staining regions of necrosis, identified as described in Section 3. M. 3. Treated tumours showed progressively larger confluent zones of non-perfused tumour tissue, as time following FAA injection increased. These zones could be differentiated from zones of established necrosis by their lack of yellow autofluorescence and refractility. The effective perfusion volume showed a progressive fall to undetectable levels 24 h after treatment (Figure 3.1), and the earliest time at which treated tumours were significantly different from controls was 2 h (P < .05).

3. R. 2. Double label studies: fluorescent dyes in control tumours

The staining of H33342 was stable for individual perfused vessels and was clearly identifiable up to 4 h after i.v. administration, so that it was suitable as a marker of pre-treatment blood flow. Investigation of DiOC6(3) as a second (post-treatment) blood flow marker showed it to be lethal to BDF_1 mice at the dose used by Trotter _et al._ (1989a) in SCCHV-bearing C3H/He mice, and a four-fold
decrease in dose was required. The fluorescence intensity of DiOC₇(3) was much lower than that of H33342, and in contrast to the latter marker, showed little diffusion beyond the endothelium in the 5 min between its administration and removal of the tumour. However, slow diffusion of DiOC₇(3) into the para-vascular tumour tissue did occur after mounting in glycerol saline, while the H33342 staining pattern changed little, even after 6 h. The correspondence between the diffusion distances of the two fluorescent labels was closest between 30 and 60 min after mounting (Figure 3.2), at which time the point counts were made, allowing a direct comparison of the pre-treatment and post-treatment perfusion patterns.

3. R. 3. Staining patterns in control EMT6 tumours

EMT6 tumours had large central necrotic zones and lower vascular densities than those of Colon 38 tumours. The constituent vessels were small and, in some regions, the staining intensities of both dyes was low. Spontaneous loss of perfusion of 5% or more, identified as areas of single labelling, occurred in some areas in 8/18 of the control EMT6 tumours during the time between administration of the two labels, but no significant trend with time was observed (Figure 3.3).


All tumours examined 15 min or more after FAA treatment showed localised areas in which the blood vessels stained with H33342 but not with DiOC7(3) (Figure 3.2c,d). This loss of perfusion was a patchy phenomenon; it occurred in all vessels in the involved areas, and only rarely were isolated single vessels affected. These areas were not confined to either the centre or the periphery of the tumour. In Colon 38 tumours, densely vascular areas (Figure 3.2a,b) appeared more resistant to perfusion failure at early times. Figure 3.3 shows the decline in perfusion as a fraction of pre-treatment perfusion with time after FAA treatment. The FAA-treated tumours showed a significant (P < .05) decline in perfusion 15 or more minutes after FAA administration. Sections of liver, spleen, kidney and small intestine from a tumour-bearing Balb/C mouse 4 h after treatment with FAA 1.2 mmol/Kg, showed no evidence of any change in blood flow.
FIGURE 3.1 - PERFUSION OF COLON 38 TUMOURS AFTER FAA - SINGLE LABEL

The change in volume of vascular perfusion in Colon 38 tumours with time after FAA, 1.2 mmol/Kg i.v., as determined by the fraction of points positive for H33342, divided by the total points counted over the tumour (including necrotic tumour). Values are means ± s.e.m. (n = 4). The cross-hatched area represents the mean ± s.e.m. for control tumours (n = 7), one for each time point. Values for controls showed no significant trend with time, but treated groups were significantly different from controls (P < .05) at 2 h and later.
FIGURE 3.2 - FLOURESCENT STAINING OF COLON 38 TUMOURS

Fluorescent photomicrographs from two areas of the same Colon 38 tumour after i.v. treatment with 1.2 mmol/Kg FAA. H33342 was injected with the FAA, and DiOC$_7$(3) was administered 15 min later. a, b. Densely vascular regions showing the same pattern of vascular staining with H33342 (a), and DiOC$_7$(3) (b). c, d. A less vascular area of the tumour in which the lower part of the field was perfused with H33342 (c) but not with DiOC$_7$(3) (d).

Fluorescent photomicrographs, bar = 50 μm.
FIGURE 3.3 - PERFUSION OF TUMOURS AFTER FAA - DOUBLE LABEL

Time course of perfusion changes in Colon 38 and EMT6 tumours after FAA, 1.2 mmol/Kg i.v., as indicated by the percentage of H33342-positive grid points also stained with DiOC7(3). Values are means ± s.e.m. Controls (open circles) n = 3; FAA-treated (solid circles) n = 5. Treated groups are significantly different (P < .05) from controls 15 min or more after treatment.
3. R. 5. Fluctuating perfusion in tumours

When H33342 and DiOC₆(3) were administered up to 4 h apart, the ratio of point counts was close to unity, particularly for the Colon 38 tumour (Figure 3.3), which suggested little fluctuation in perfusion. Lack of concordance in the two point counts usually appeared because of 1) differences in diffusion distances of the two markers, or 2) lack of staining of the paravascular zone by DiOC₆(3) because the adjacent vessel was not transected, thereby precluding diffusion of the stain after mounting. Therefore, direct evaluation of fluctuating perfusion required direct scoring of vessels. One hundred vessels, defined by a lumen surrounded by a rim of H33342 staining, were examined in each of three EMT6 and three Colon 38 60-minute control (non-FAA-treated) tumours for DiOC₆(3) staining. In Colon 38 tumours, all vessels were double-labelled indicating the absence of vessel opening or closure. However in EMT6 tumours, ten percent of the vessels stained with H33342 were not labelled with DiOC₆(3), and none stained with DiOC₆(3) alone. This indicates vessel closure, but not opening, during this time.


The dose response to FAA for cell killing tested after 24 h in EMT6 tumours (Figure 3.4A) showed no effect below 0.4 mmol/Kg. A steep decline in clonogenicity was seen between 0.4 and 0.8 mmol/Kg, after which the limits of the assay were reached. Doses above 0.8 mmol/Kg were lethal in some Balb/C mice beyond 9 h. This lethality contrasts with the results in BDF₁ mice in Chapter 2 in which no deaths occurred within 24 h even when 1.2 mmol/Kg FAA was given. The time course of tumour cell killing (Figure 3.4B), measured over 24 h after a dose of 0.8 mmol/Kg, showed a steady decline in clonogenicity.

There was a continuous decrease in the cell yield with both increasing dose and time after administration as shown by the divergence of the curves for plating efficiency and clonogenic cells per gram in Figure 3.4. By 24 h after a dose of 0.8 mmol/Kg, the cell yield had decreased by a factor of 12 (P< .05). The morphology and size distribution of the colonies arising from treated and untreated tumours were indistinguishable.

Rates of cell killing in EMT6 tumours in the total absence of blood flow are shown in Figure 3.5. Plating efficiency declined after 2 h and reached the limits of detection at 6 h, a rate of killing much faster than that seen with FAA. This was accompanied by a decreased yield of tumour cells, as occurred following administration of FAA at 0.8 mmol/Kg.

3. R. 8. Modelling FAA-induced cell killing as an ischaemic process

Using data from the time course of perfusion failure in the EMT6 tumours (Figure 3.3) and the rate of cell death in globally ischaemic tumours (Figure 3.5), a mathematical model was developed to test the hypothesis that ischaemic injury could account for the cytotoxic activity of FAA in EMT6 tumours. The assumptions made were that 1) tumour cell death is secondary to irreversible failure of regional tumour perfusion, 2) the kinetics of cell death in a region of total perfusion failure are the same as those of a whole tumour in which blood flow ceases, and 3) the ratio of DiOQ(3) to H33342 points quantitatively reflects the changes in perfusion. The blood flow as a fraction of that in control tumours was well fitted \((r = .94)\) by a biexponential, with a least squares fit to the experimental data (Figure 3.3). The fraction of total tumour \(F\) which has normal blood flow is then given by the expression:

\[
F = 0.54e^{-0.1t} + 0.46e^{-0.2t},
\]  

[Eq. 1]

where \(t\) is the time in hours after FAA treatment. The survival of cells following total failure of blood flow was fitted to a single exponential \((r = .94)\) with a time delay; the data from Figure 3.5 were used. If the surviving fraction is \(S\) and the time in hours is \(t\), then

\[
S = 1, \quad \{t < 1.2\}; \quad S = e^{-2.3(t-1.2)}, \quad \{t > 1.2\}
\]  

[Eq. 2]

The tumour can be considered to be composed of three fractions. The first, \(S_a\) is fully viable and has an unaltered blood flow (Eq. 1). The second, \(S_b\), has been ischaemic for \(t\) less than 1.2 h and is still fully viable (calculated from Eq. 1). The remaining fraction, \(S_c\), is composed of cells that have been ischaemic for more than 1.2 h, and are dying at an exponential rate. This fraction was divided arbitrarily into 0.25 h cohorts (measured from the onset of ischaemia), the sizes and survival of which were calculated from equations 1 and 2, respectively.
Figure 3.6 shows the curve predicting survival with time after treatment with 1.2 mmol/Kg FAA. The model was tested by determining the numbers of clonogenic cells in i.m. EMT6 tumours up to 6 h after treatment with FAA at this dose. The observed survival was in reasonable agreement with the prediction up to 4 h, but measured values for most tumours were much lower than predicted by the model at later times.


Figure 3.7 shows the effects on survival of EMT6 cells from i.m. tumours and i.p. spheroids 18 h after treatment with 0.8 mmol/Kg FAA. A small but statistically significant decrease in the number of clonogenic cells occurred after i.v. FAA treatment (1.1 ± 0.03, vs. 2.1 ± 0.12 for untreated peritoneal spheroids; P < .01), but this change contrasts with the 3.7 log_{10} fall in clonogenicity seen in FAA-treated i.m. tumours under these conditions (Figure 3.4).
FIGURE 3.4 - CELL DEATH IN EMT6 TUMOURS AFTER FAA

A. Dose response at 24 h. B. Time course at the dose of 0.8 mmol/Kg. Plating efficiency (open circles) and clonogenic cells/g tumour tissue (solid circles) are shown. Values are arithmetic means ± s.e.m. for 3 to 5 tumours for treated groups or 12 tumours for controls. Symbols in parentheses indicate that, for one of three tumours from which no colonies were recovered, a plating efficiency of \(10^{-5}\) was assumed.
FIGURE 3.5 - CELL DEATH IN GLOBALLY ISCHAEMIC EMT6 TUMOURS

Tumours were maintained at 37°C post mortem. Plating efficiency (open circles) and clonogenic cells/g tumour tissue (solid circles) are shown. Values are arithmetic means ± s.e.m. for 3 to 4 tumours. Symbols in parentheses indicate that, for one of three tumours from which no colonies were recovered, a plating efficiency of $10^3$ was assumed.
FIGURE 3.6 - FAA-INDUCED CELL KILLING AS AN ISCHAEMIC PROCESS

Time course of cell killing (percentage of control clonogenic cells/g tumour tissue) in EMT6 tumours after i.v. FAA, 1.2 mmol/Kg. Each circle represents a single tumour. The solid line represents the rate of killing predicted by a simple model in which tumour cell death is caused by ischaemic injury resulting from FAA-induced inhibition of blood flow (see text).
FIGURE 3.7 - CELL DEATH IN SPHEROIDS AND TUMOURS AFTER FAA

Clonogenic cells/g from EMT6 spheroids or tumours 18 h after i.p. or i.v. administration of 0.8 mmol/Kg FAA to mice bearing i.p. spheroids or i.m. tumours. In vitro spheroids were direct from culture, and peritoneal spheroids were from untreated control animals. Values are means ± s.e.m. for three populations of spheroids each from a different mouse. Data for control and treated tumours (tr. t.) are replotted from Figure 3.4B.
3. D. Discussion

This chapter has shown that a failure of tumour blood flow is a major mechanism in the anti-tumour action of FAA. The evidence includes the demonstration of an *early and sustained* decline in tumour perfusion, the rapid fall in clonogenic cell yield in globally ischaemic tumours, and the requirement for tumour vessels for the full anti-tumour effect. The discussion below considers the sensitive double label method used to demonstrate the early perfusion effect of FAA, the potentially confounding phenomenon of transient changes in blood flow, the evidence for the hypotheses of FAA mechanisms proposed in Section 1.6.2. (pp. 45 - 46), and the possible mechanisms of the perfusion failure observed.

3. D. 1. Assessment of tumour perfusion

The demonstration of an early and irreversible fall in tumour perfusion relied on the use of intravascular fluorescent markers. In the present single label study, point counting included all vascular and perivascular cells stained intensely with H33342 as well as intraluminal areas, to provide a measure of the fraction of tumour volume that was well perfused (close to a functional vessel). This approach differed from that used in previous studies, which estimated actual intravascular volumes (Murray *et al.*, 1987; Murray & Randhawa, 1988), or counted transected vessels (Chaplin *et al.*, 1987; Trotter *et al.*, 1989a). Counts were made of grid points, rather than vessels because it was difficult to define individual vessels in some tissue sections, particularly with H33342 when vessels were closely spaced in areas of high vascular density (Colon 38) or near branching points (EMT6, in the double label study).

The point-scoring technique used here suffers from the disadvantage of requiring subjective determination of the threshold of staining intensity needed for a positive count. However, it offers advantages in being at least partially sensitive to quantitative changes in perfusion in individual vessels (resulting in an altered H33342 diffusion distance) rather than an all-or-none score of vessel patency. This technique allowed the demonstration of a continuous decline in the perfusion of Colon 38 tumours to undetectable levels 24 h after treatment with FAA.

There were large variations in the H33342 point count between different tumours, and this was not due to differences in extent of pre-existing necrosis because the inter-tumour variance was not decreased when necrotic regions were excluded (data not shown). Perhaps it was because of this
inter-tumour variation that no statistically significant change from controls was demonstrated until 2 h after treatment.

The greater precision of the double vascular marker technique allowed the demonstration of a significant diminution of perfusion within 15 min of FAA administration in both Colon 38 and EMT6 tumours. The advantages of this method stem from the direct comparison of pre- and post-treatment perfusion patterns in each tumour. Exactly the same points in each field were evaluated, thus minimising the variation due to heterogeneity of the vascular density and the statistical error involved in point-counting techniques. The method gives an estimate of the volume fraction of the tumour with a functional blood supply rather than an absolute measure of perfusion. It is relatively insensitive to decreases in perfusion within individual vessels and thus may overestimate blood flow at later times.

Although H33342 was found to be a suitable dye for assessment of vascular perfusion, DiOC₆(3) was toxic and differed in diffusion characteristics from H33342. For this reason, new fluorescent vascular markers were sought, and DiOC₆(3) was replaced by a superior dye (Section 5. D. 7., p. 149) for the studies reported in Chapters 5 - 7.

3. D. 2. Spontaneous changes in tumour perfusion

It was necessary to consider the possibility of spontaneous fluctuations in regional perfusion, since this phenomenon (discussed in Section 1. 4. 3., p. 33) could complicate the measurement of treatment-induced perfusion changes. Fluctuations in perfusion have been described in SCCVII tumours (Chaplin et al., 1987; Trotter et al., 1989a). The possibility that H33342 may itself decrease perfusion has also been raised (Smith et al., 1988), and a marked, but transient, effect has now been demonstrated by Trotter et al. (1990). In the present double label study there was no evidence of vessel closure or opening in any control Colon 38 tumours. However, in control EMT6 tumours, occasional areas of vessel shutdown were seen that could have been caused by a flow-modifying effect of H33342, but it is also possible that flow in the delicate and sparse vasculature of this tumour was affected by handling or constraining of the mice. No vessels opened within the 60 min interval between injections in either Colon 38 or EMT6 tumours. Therefore, fluctuating blood flow does not appear to be a general characteristic of all solid tumours.
3. D. 3. The evidence for an ischaemic FAA mechanism

The demonstration of a significant fall (20 - 40%) in tumour vascular perfusion within 15 min of treatment with FAA is notable because no other structural or physiological changes (apart from the rise in AMP levels; Section 2. R. 6., p. 62) have been shown to occur this early. This suggests that blood flow failure plays a causal role in tumour cell killing. All other changes reported following FAA treatment occur at later times. Evelhoch et al. (1988) showed decreased tumour ATP levels and blood flow in Glasgow osteogenic sarcomas only after 4 h. The earliest morphological changes in Colon 26 and Colon 38 tumours were also apparent at this time (Smith et al., 1987). In MAC 26 tumours, both histological and perfusion changes have been reported at 2 h (Bibby et al., 1989a), and the studies in Chapter 2 indicate that focal morphological changes occur between 30 and 60 min. Ching and Baguley (1989b) found a decrease in the number and activity of tumour-associated cytotoxic effector cells 1 h after FAA treatment, and interferon has been detected 1.5 h after treatment of mice bearing renal cell carcinomas (Hornung et al., 1988a).

However, the early onset of the blood flow effect does not prove causality, and further experiments were performed to determine whether ischaemia is necessary and sufficient for tumour cell killing. To test whether inhibition of blood flow alone is sufficient to account for the observed rate of FAA-induced cell killing, the latter was measured in globally ischaemic tumours maintained at 37°C post mortem, rather than by application of a vascular clamp in vivo. Tumour clamping might be expected to lower tumour temperature and thus underestimate ischaemic damage. Cell viability fell much more rapidly in post mortem EMT6 tumours in the present experiments than in the clamped SaF sarcomas of Denekamp et al. (1983). The rapid rate of cell death in globally ischaemic tumours relative to that seen after FAA is consistent with the hypothesis that perfusion failure is responsible for FAA-induced cell killing. A simple mathematical model that predicts the time course of cell death on the basis of the rate of perfusion failure and the rate of cell death in the ischaemic zone, shows good agreement with observed values for cell killing induced by 1.2 mmol/Kg FAA over the first 4 h (Figure 3.6). However, at later times, FAA-induced cell killing is more rapid than expected for ischaemia alone (see Section 3. D. 4., overleaf).

To determine whether ischaemia is necessary for cell killing, the response to FAA of avascular i.p. EMT6 spheroids was compared to that of vascular i.m. EMT6 tumours. The relative resistance of spheroids to FAA provides strong evidence that interruption of blood flow is a major component of the anti-tumour action of this drug. The requirement for a host component for FAA activity has been previously demonstrated by the lack of FAA activity against Lewis lung tumours grown in peritoneal diffusion chambers (Finlay et al., 1988), but this result could reflect either the lack of vascular supply,
or the absence of host immune cells. Intraperitoneal EMT6 spheroids, which lack vessels but are infiltrated by host immune cells (Lord, 1980) were therefore used to distinguish between these two possibilities. In the present studies FAA had only a minor effect against peritoneal spheroids (0.4 log₁₀ vs. 3.7 log₁₀ for i.m. tumours), even when the drug was administered by the i.p. route to provide high local concentrations.

One difficulty with the above experiment was that the spheroids differed from i.m. tumours in both size and site, and this issue is addressed and resolved in Chapter 5. Because the EMT6 tumour is immunogenic (Denekamp, 1982a), caution is required in generalising the results of the spheroid experiments, but such immunogenicity might be expected to increase sensitivity to immune mechanisms of cell killing.

The requirement for a tumour vasculature is consistent with the low activity of FAA against tumour cells in ascites tumours (Plowman et al., 1986), in diffusion chambers (Finlay et al., 1988), or in early tumours (Finlay et al., 1988; Smith et al., 1988) that may be in a pre-vascular growth phase. The relationship between blood flow failure and the anti-tumour effect is further supported by the observations of Hill et al. (1989, 1991), who showed that tumours in which FAA causes the greatest falls in perfusion also have the longest growth delays, which could reflect the amount of induced necrosis.

3. D. 4. The evidence for non-ischaemic FAA mechanisms

The mathematical model of FAA-induced cell killing shows that after 4 h this is more rapid than predicted for pure ischaemia. This may reflect an underestimation of the degree of perfusion failure at later times, or some other mechanism of injury. Non-ischaemic mechanisms are also likely to operate, as suggested by the morphological findings of apoptosis presented in the previous chapter. This is further supported by the results of the present spheroid experiments, which showed a fall of almost 50% in clonogenic cell content in the complete absence of a tumour vasculature.

In studies by others, FAA prevented the growth of Colon 38 tumour fragments as early as two days after implantation (Plowman et al., 1986), by which time vascularisation may not have been established. FAA also prevented metastasis to the lungs of 3LL lung tumour cells from i.m. tumours in mice, rather than destroying established metastases (Desoise et al., 1989). The latter study raises the possibility that non-ischaemic anti-tumour effects could have clinical significance.
The nature of the non-ischaemic mechanisms is not clear from the present studies, but the known presence of immune effector cells within the spheroids suggests that FAA may stimulate these cells to exhibit cytotoxicity. The role of such cells is studied in Chapter 4.

3. D. 5. The evidence against in vivo activation of FAA

Whatever the relative contributions of ischaemic and non-ischaemic mechanisms, FAA appears to exert its anti-tumour effects by some interaction with the host (blood vessels and/or immune effector cells), and not as a direct cytotoxic agent. The hypothesis that FAA is activated to a cytotoxin in vivo (Chabot et al., 1989b; Section 1. 6. 2a., p. 45) is not supported by the findings of the present studies.

First, the mode of cell death appears to be different from that of cytotoxic agents. In both FAA-treated and ischaemic EMT6 tumours, cell killing was reflected by both a decline in plating efficiency and in cell yield. The former is suggestive of lethal DNA damage, while the latter implies increased cell disintegration or fragility, and points to a non-nuclear target such as the cell membrane. While these findings are consistent with either an ischaemic or an immune mechanism of cell killing by FAA, a fall in cell yield is not seen after treatment with most cytotoxic agents. The decline in plating efficiency indicates lethal DNA damage, and this is consistent with apoptosis (Eastman, 1990) seen in Colon 38 tumours in the previous chapter. Furthermore, the nature of the DNA damage following both FAA and global ischaemia appears to differ from that following conventional cytotoxic agents: the colony size distributions were indistinguishable from those of control tumours, a finding in distinct contrast to that seen with most cytotoxic agents and radiation (Elkind & Whitmore, 1967). DNA damage following FAA treatment (Bissery et al., 1988), has been interpreted as secondary to cell death following non-DNA injury (Evelhoch et al., 1988).

The strongest evidence against the in vivo activation hypothesis is the resistance of i.p. spheroids to FAA, relative to i.m. tumours, both of which were exposed to the the drug in vivo.


Comparison of the effects of FAA in tumour tissue and various normal tissues by the double label technique (Section 3. R. 4.) showed loss of perfusion only in tumour tissue. However, the conclusion that FAA-induced changes in blood flow are tumour specific is provisional because, as noted by Smith et al. (1988), H33342 has limitations as a vascular marker in most normal tissues due to high vascular...
densities. Any blood flow changes in normal tissue must however be quantitively minor because the dramatic inhibition observed in tumour tissues would clearly be lethal if critical normal tissues were similarly affected. No perfusion loss was found in non-tumour tissues by Honess and Bleehan (1991) in their FAA study. Any explanation of the mechanism of the blood flow changes should explain this tumour specificity as well as the focal nature of the early perfusion changes.

3. D. 7. The mechanism of perfusion failure

The appearance of foci of perfusion failure which progressively increased in size with interval after treatment suggests a positive feedback mechanism in the action of FAA. This is supported by the sharp dose response of EMT6 tumours to FAA (Figure 3.4A). One mechanism suggested is the depletion of endothelial or tumour cell ATP, leading to cell swelling and further ischaemia following vascular collapse (Evelhoch et al., 1988). Factors that could contribute to vascular collapse include a fall in arterial pressure, noted as a dose-limiting toxic effect of FAA in humans (Kerr et al., 1987), and the high interstitial pressures measured in mouse tumours (Gullino et al., 1964; Wiig et al., 1982; Boucher et al., 1990; Section 1.4.2., p. 32). The latter pressures might contribute to the tumour specificity of the blood flow effect. The unresponsiveness of the tumour vasculature to physiological or pharmacological stimuli because of the paucity of smooth muscle (Mattsson & Peterson, 1981; Section 1.3.3., p. 29) suggests that a direct vasoconstrictive effect by FAA is unlikely. However, endothelial cells do contract in response to the platelet product serotonin (Majno et al., 1969), a substance implicated in the mechanism of action of tumour necrosis factor-α (TNF; Manda et al., 1988).

Immune mechanisms could also explain the occurrence of positive feedback. The monokine TNF is known to stimulate macrophages in an autocrine manner (Hori et al., 1987, 1989), and this monokine has procoagulant and other effects on endothelial cells (Semenzato, 1990; Clauss et al., 1990; Murray et al., 1991).

More information is required about the events following FAA administration to fully understand the complex interactions which lead to perfusion failure and haemorrhagic necrosis. This subject is discussed further in the final chapter (Section 8.1.2., pp. 181-183).

The early, rapid and sustained loss of blood flow plays an important causative role in haemorrhagic necrosis. Ischaemia produces a decline in EMT6 cell viability which is sufficiently rapid to account for the major component of the FAA anti-tumour effect. The relative resistance of i.p. spheroids to FAA also indicates that a vascular component is of key importance in cell killing, and argues that metabolic activation of FAA in vivo, or immune cell effects, cannot be solely responsible for activity. However, ischaemia does not account for all tumour cell killing. The investigation of the non-ischaemic mechanisms is the subject of the next chapter.
CHAPTER 4
THE ROLE OF MICROENVIRONMENTAL FACTORS AND IMMUNE EFFECTOR CELLS IN FAA-INDUCED TUMOUR CELL INJURY

4. I. Introduction

Chapter 3 demonstrated the importance of blood flow failure in the anti-tumour action of FAA, but suggested that non-ischaemic mechanisms also contribute to tumour cell killing. This was also indicated by the occurrence in FAA-treated tumours of apoptosis (Chapter 2), a mode of cell death which cannot occur in conditions of nutrient deprivation (Kerr et al.). These non-ischaemic mechanisms may be important, not only because they could kill a significant number of tumour cells directly, but also because these mechanisms may play a role in perfusion failure.

The aim of the study reported in the present chapter was to establish the nature of the component of tumour cell damage that is independent of vascular effects. These non-ischaemic mechanisms were examined in EMT6 spheroids, which resemble EMT6 tumours but lack blood vessels. These spheroids, some of which were infiltrated by immune effector cells, were used to test two of the hypotheses proposed earlier (Section 1. 6. 2., pp. 45 - 46): that FAA selectively kills tumour cells in the low-exchange environment of the tumour, and that FAA stimulates immune effector cells to kill tumour cells. The next sections of the introduction review the use of spheroids for investigating the effects of hypoxia on anti-tumour therapy, and the application of immune cell-infiltrated spheroids to the investigation of immune-mediated anti-tumour effects.

4. I. 1. The spheroid as a model of the tumour microenvironment

Spheroids, and cells from dissociated spheroids, were used in the present experiments to examine the influence of the tumour cell microenvironment on the activity of FAA. Spheroids have been used previously in tumour research (reviewed by Sutherland, 1988) because they model the microenvironments found in tumours, while retaining many of the advantages of simpler in vitro systems. They have been used for studying the limited diffusion of oxygen, nutrients and drugs through tumour tissue, which results in resistance to radiation and chemotherapy (Section 1. 1. 3., p. 20). The response of the individual cell to radiation depends on its location: the central hypoxic cells are relatively
resistant to radiation (Giesbrecht et al., 1981) and anti-proliferative drugs (Wilson et al., 1981; Erlichman & Vidgen 1984). On the other hand, hypoxia selective cytotoxic agents selectively kill the inner cells of spheroids (Sutherland 1974). If hypoxia selectivity were the explanation for the in vivo activity of FAA, greater activity of the drug against cells in intact spheroids might be expected.

A more sensitive method to identify hypoxia selectivity was also used: comparison of cell killing in single cell suspensions under oxic and anoxic atmospheres. The increased sensitivity of this approach has been shown with nitracrine, which exhibits marked hypoxia selectivity by this method (Wilson et al., 1984), but not in spheroids, because of activation and binding in the outer oxic cells (Wilson et al., 1986). However, this method is limited in not assessing features of the low diffusion environment other than hypoxia.

4.1.2. Infiltration of spheroids by immune cells in the peritoneum

The technique of introducing EMT6 spheroids into the peritoneal cavities of mice to allow their infiltration by immune effector cells was first described by Lord et al. (1979). This group examined the type of immune cells infiltrating spheroids, and the cytotoxicity of these cells in mice sensitised to killed EMT6 cells. More than half of the total cells in such spheroids were found to be host cells, and similar fractions were found in EMT6 intra-muscular tumours (Lord, 1980).

In the previous chapter, intraperitoneal spheroids were treated with FAA in vivo, to compare the responses of avascular tumour tissue with vascularised tumours growing in skeletal muscle. A small but a significant FAA-induced cytotoxic effect was seen in the spheroids. In the present studies immune cell-infiltrated spheroids were removed from untreated mice (ex vivo spheroids), and the infiltrating host cell population was characterised by flow cytometric assessment of cell ploidy and surface markers. Both ex vivo spheroids and spheroids grown entirely in vitro (in vitro spheroids), were exposed to FAA in vitro, in order to determine whether damage to the tumour cells is a direct effect of the toxicity of FAA, or due to stimulation of immune cell cytotoxicity by the agent. Both clonogenic assays and morphological examination were used as endpoints.

In addition, the possibility of an interaction between hypoxia and immune effector cells was investigated. Hypoxia is known to stimulate macrophages to produce an angiogenic factor (Knighton et al., 1983), shown to be tumour necrosis factor-α (TNF; Liebovich et al., 1987). FAA might synergise with the hypoxic stimulus to produce this or other cytokines in cytotoxic quantities. Therefore the effect of oxygen tension on FAA activity in ex vivo spheroids was assessed.
4. M. Methods

4. M. 1. EMT6/Ak spheroids

The growth of spheroids has been described in Section 3. M. 6., p. 74. For clonogenic assays, the volume \( V \) of each spheroid was estimated before dissociation by measuring two diameters \( d \) of each spheroid using a grid in the eyepiece of an Olympus IM inverted microscope, and applying the formula

\[
V = \frac{\pi}{6} d_1^2 d_2
\]

where \( d_1 \) was the smaller diameter.

4. M. 2. Oxic and anoxic exposure of EMT6 cells to FAA

Single cell suspensions of EMT6 cells were obtained by dissociation of spheroids in an enzyme cocktail consisting of 0.5 mg/ml pronase and 0.2 mg/ml DNase in growth medium (GM; Section 3. M. 1., p. 71) with magnetic stirring for 20 min at 37°C. The effect of total anoxia on FAA cytotoxicity in stirred suspension cultures of the EMT6 tumour cells was assessed by the method of Wilson et al. (1984). A gassing rig supplied a constant flow of either air or nitrogen (<10 ppm oxygen), each with 5% carbon dioxide, to a series of magnetically stirred bottles, each containing \( 10^6 \) EMT6 cells/ml in 4 ml GM with 10% (vol/vol) fetal calf serum (FCS) plus antibiotics. The bottles containing the incubation medium, with dissolved drug at 1.25x final concentration, and the cells at 5x final concentration, were gassed for 1 h. Drug exposure was then initiated by adding 1 ml of the cell suspension to the drug-containing bottles. Samples were withdrawn for clonogenic assay at various times with a spinal needle inserted through the gas exhaust vent.

4. M. 3. Clonogenic assays

Clonogenic assays were performed on cell suspensions as described in Section 3. M. 5. (p. 73).
4. M. 4. Eosin exclusion fraction

The fraction of dissociated cells which were impermeable to eosin (viable cells) was assessed after adding eosin Y to 0.4% wt/vol and counting the cells in a haemocytometer (between 85 and 230 cells per specimen). Small cells in specimens from ex vivo spheroids were assumed to be host cells and were not counted.

4. M. 5. Ex vivo (peritoneal) spheroids

Spheroids (0.5 - 1.0 mm diameter) were injected into the peritoneal cavities of mice (15 - 30 per mouse), as described in Section 3. M. 6. (p. 74). Six days after implantation, the mice were killed by cervical dislocation, the peritoneal cavities opened, and the spheroids recovered (~60% yield) under sterile conditions. The spheroids were washed twice in phosphate-buffered saline (PBS) before further use.


Ex vivo and in vitro spheroids were disaggregated (Section 4. M. 2.) and the cells washed in PBS by centrifugation at 365g x 5 min and resuspended. DNA content and cell surface markers were assessed by flow cytometry. To measure the DNA content, the washed cells were permeabilised by resuspension in 0.5% vol/vol Tween 20 in PBS plus 2% FCS (PBS/FCS), washed in PBS and resuspended in 20 µg/ml propidium iodide (PI), a fluorescent DNA-binding compound.

Alternatively, cell suspensions incubated for 20 min with a 1:10 dilution of a mouse monoclonal IgG antibody against either Mac-1, Ly-1, or Thy-1 antigens (markers for macrophage /monocytes, B-cells and T-cells respectively; for sources see Materials, p. 197). The cells were then washed in PBS/FCS and incubated in goat anti-mouse immunoglobulin bound to fluorescein isothiocyanate (FITC) at a final concentration of 9.1 µg/ml for 20 min. This served as the signal for the flow cytometer. The cells were finally washed and resuspended in PBS/FCS plus 20 µg/ml PI. All incubations were carried out on ice to inhibit antibody capping and internalization.

Flow cytometric analysis of the cell suspensions was performed on a FACS IV 440 flow cytometer with a 2 W argon laser tuned to an excitation wavelength of 488 nm at a power of 150 mW. A 530 nm band pass filter was used for FITC fluorescence, and a 620 nm filter was used for PI.
fluorescence. 1.5 x 10⁴ cells were counted for each sample and the results were stored in a VAX computer. Data were processed using the Consort 40 software. In the fluorescent antibody studies, dead cell signals were excluded from data collection by their PI fluorescence.


Between one and five spheroids (ex vivo or in vitro) were placed in Falcon 2057 tubes with 10ml GM with 5% FCS plus antibiotics and the drugs to be tested. The tubes were sealed to include ~1.5 ml of air and rotated (50 rpm) on a roller wheel at 37°C. Spheroids were exposed to FAA either continuously for intervals ranging from 2 to 24 h, or were exposed for 2 h or 4 h and then transferred to FAA-free GM for a total incubation of 24 h. In other experiments, spheroids were exposed to phorbol myristate acetate (PMA) for 24 h under the same conditions. The effect of dexamethasone on FAA activity was also measured.

4. M. 8. Effect of oxygen concentration on spheroid response to FAA

Ex vivo spheroids were exposed to 2.5 mM FAA for 24 h in spinner flasks in an atmosphere of oxygen, air or nitrogen (each with 5% carbon dioxide). Four spheroids, each from a different mouse, were placed in each of six spinner flasks in a 37°C waterbath, with 25 ml of GM, with or without FAA. The total gas flow was maintained at ~100 ml/min during the first seven hours of the incubation, the flasks were then sealed for twelve hours, and gas flow resumed for the final five hours. The 20% oxygen flasks were flushed with gas at the start of the experiment and then sealed.

4. M. 9. Light and electron microscopy of spheroids

After completion of each of the 20 experiments that included morphological studies, spheroids were fixed in 4% formaldehyde for light microscopy as described in Section 2. M. 3. (p. 49). Multiple 5 μm sections were cut from each spheroid and stained with haematoxylin and eosin (H&E). The slides were examined after each experiment, and the histological features noted. When several experiments were completed, the slides were randomly coded and scored blind for each of several histological features (see Section 4. R. 5.). In addition, the thickness of the viable rim of each spheroid was measured, and
the volume fraction of histologically intact cells in the viable rim was determined by point counts using a 100-point eyepiece grid at 400x magnification. Cells were scored as histologically intact (viable) when the cell outline was complete, the nucleus was intact with dispersed chromatin, and there was no cytoplasmic fragmentation.

The spheroids were prepared for electron microscopy as described in Section 2. M. 4., p. 50.

4. M. 10. Statistical analysis

Data from the clonogenic assays, measurement of spheroid volume fraction and morphological scoring were tabulated and compared using RS/1 statistical software on a Digital Professional 350 computer. If the programme showed the data to be normally distributed, the two data sets were compared with the Student t test. For data not normally distributed (all morphological feature scores), the Mann-Whitney non-parametric test was applied, whether or not dispersions were equal by the Ansari-Bradley test.

Experimental determinations were compiled as the means ± s.e.m. Separate experimental "determinations" were defined as values from 1) in vitro spheroids incubated in separate tubes, or 2) ex vivo spheroids derived from separate mice, but not necessarily incubated in separate tubes.

4. R. Results

4. R. 1. Effect of hypoxia on the direct toxicity of FAA

Figure 4.1 shows the surviving fraction of dissociated EMT6 spheroid cells exposed to FAA for up to 4 h at 2.5 or 5 mM, underoxic and anoxic conditions. No cytotoxicity was seen. Higher concentrations of FAA could not be tested as the drug precipitated slowly from the medium. FAA cytotoxicity was also not seen against EMT6 cells in intact in vitro-grown spheroids (data not shown).
EMT6 spheroids (grown *in vitro*) were dissociated in a single flask, and samples of the cell suspension were incubated with 2.5 mM (circles) or 5.0 mM (triangles) FAA under oxic or anoxic atmospheres. The surviving fraction is the ratio of the plating efficiencies of FAA-exposed cells, and time-matched oxic and anoxic untreated cells (corrected for changes in cell density). Points represent single values, or the mean ± s.e.m. of two values. Data are pooled from two experiments. No cytotoxicity is evident.
4. R. 2. Composition of host cells infiltrating \textit{ex vivo} spheroids

Flow cytometric analysis of DNA content and immune cell surface markers identified host cells in the suspensions prepared from \textit{ex vivo} spheroids. Figure 4.2 shows the histograms for the antibodies to the different cell surface markers, each of which identifies a population with high epitope density. Table 4.1 shows the numbers of cells staining with each surface marker, as well as the number of diploid (host) cells present in the dissociated cell population. The results show that diploid cells, T-cells, B-cells and macrophages were present only in \textit{ex vivo} spheroids, and accounted for between 40\% (diploid fraction) and 55\% (sum of fractions for all surface marker-positive cells) of the total population.

4. R. 3. FAA cytotoxicity in intact \textit{in vitro} and \textit{ex vivo} spheroids

\textit{Ex vivo} spheroids differed from \textit{in vitro} spheroids in viable cell content and in response to FAA. Figure 4.3. shows that spheroids grown in the peritoneal cavity for 6 days had a lower cell density (cell yield) than spheroids grown entirely \textit{in vitro}. Exposure to 2.5 mM FAA for 24 h decreased the cell yield in both types of spheroid, but the effect was significantly greater (P < .005) with the \textit{ex vivo} spheroids. The effect of FAA on plating efficiency was much more marked in \textit{ex vivo} spheroids. Clonogenic cell yield, the product of cell yield and plating efficiency, was reduced 80\% by FAA in \textit{ex vivo} spheroids, but only 34\% in \textit{in vitro} spheroids.

Microscopic examination of dissociated cells in the presence of eosin showed lethal cell damage only after \textit{ex vivo} spheroids were exposed to FAA (P < .05; Figure 4.3D). The degree of cell killing measured by this method was lower than that seen for plating efficiency (15 vs. 45\%).
Representative histograms for spheroid cell populations derived by dissociation of spheroids after incubation in the peritoneal cavities of mice for six days (ex vivo spheroids). The cells were incubated with Ly-1 (B), Thy-1 (C), Mac-1 (D), or no primary antibody (A), and then with a secondary antibody-fluorescein conjugate. Intensity of fluorescence is shown on the horizontal axis (logarithmic scale, arbitrary units). The large peaks represent autofluorescence and/or non-specific binding by cells of the fluorescent conjugate. The small peaks to the right in graphs B - D, are thought to represent specific binding by the primary antibody, since this peak is absent from A. Histograms from in vitro spheroid cells (not shown) resembled A in lacking the second peak.
### Table 4.1 - Percentage of Host Cells in Spheroids

<table>
<thead>
<tr>
<th>Spheroid Source</th>
<th>Diploid Cells (B-Cells)</th>
<th>Ly-1 (T-Cells)</th>
<th>Thy-1 (T-Cells)</th>
<th>Mac-1 (Macrophages)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>In Vitro</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Ex Vivo</em></td>
<td>40 ± 3</td>
<td>19 ± 3</td>
<td>21 ± 1</td>
<td>15 ± 1</td>
</tr>
</tbody>
</table>

Ploidy was determined from histograms of propidium iodide-stained spheroid cells made permeable with detergent. The values for surface markers were determined by subtracting the histograms for controls not exposed to primary antibody (Figure 4.2A), from each primary antibody-labelled histogram (Figure 4.2B-D). For *ex vivo* spheroids, the values are means ± s.e.m. for four spheroid cell populations, each derived from a separate mouse (4 - 11 spheroids per mouse). *In vitro* spheroid cell populations consisted of tetraploid (tumour) cells only, and showed no specific staining for any immune cell surface markers.
EMT6 cell survival after exposure of intact *in vitro* and *ex vivo* spheroids to 2.5 mM FAA for 24 h. The graphs on the left show the effects of FAA on the two spheroid types (error bars represent s.e.m.). The graphs on the right show the % decrease caused by FAA in each spheroid type (error bars represent root mean square error). Clonogenic cell yield = cell yield x plating efficiency. The data were pooled from two experiments. For *ex vivo* spheroids, each determination was performed on 3 - 9 spheroids taken from each of four different mice. Significant differences are indicated (* : $P \leq .05$; ** : $P \leq .01$; *** : $P \leq .005$).
4. R. 4. Light microscopy of spheroids

In vitro spheroids were composed of an outer viable zone, which merged gradually with the inner necrotic zone (Figure 4.4a). The outer zone consisted of loosely arranged, randomly-orientated bundles of large spindle shaped cells with a single layer of large round cells on the surface. Mitoses were numerous, particularly in the outer layers. Occasional nuclear fragments within the viable cell layer indicating spontaneous apoptosis were seen. The necrotic zone was occupied by a continuous coagulum of cell debris including pyknotic nuclei and their fragments.

Ex vivo spheroids were less uniform in size and shape than those grown in vitro, and tended to be ovoid rather than spherical. Microscopically, they were similar in structure (Figure 4.4b,f), and differed only in containing macrophages distributed evenly through the viable zone (Figure 4.4f). These were identified as smaller round cells with a moderate amount of cytoplasm and indented nuclei with margined chromatin. Lymphocytes could not be identified with certainty by light microscopy.

In vitro spheroids exposed to FAA showed an increase in cell fragments within the outer viable cell zone, but no other changes (Figure 4.4c,g).

Ex vivo spheroids underwent marked morphological changes when exposed to FAA. Macroscopically they appeared more translucent, and were fragile on handling. Microscopy revealed that this was due to partial dissolution of the central necrotic debris and decreased cellularity of the outer zone (Figure 4.4d). Any residual necrotic material showed loss of staining of the nuclear remnants. The innermost viable cells (hypoxic region) were round and swollen, and showed increased cytoplasmic eosinophilia with focal cytoplasmic disruption and nuclear lysis. The loss of central necrotic debris and hypoxic cell swelling resulted in a clear demarcation between the necrotic and the viable zones. The cellular density of the viable cell layer appeared to be decreased, and remaining cells were mainly round or oval in profile, having lost or retracted their characteristic long cell processes. This was accompanied by widespread cytoplasmic fragmentation (apoptosis), sometimes resulting in clusters of round cytoplasmic bodies (Figure 4.4i). Mitoses were rare and were seen only in 5/36 experimental determinations (two spheroid sections examined in each). Macrophages were evenly distributed through the viable cell zone, as in untreated ex vivo spheroids.
4. R. 5. Scoring histological features of spheroids

The above investigation of morphological changes induced by FAA was undertaken with the observer cognizant of the treatments used, the intention being to identify qualitative drug-dependent changes. To provide a more quantitative assessment, the slides from all in vitro exposure experiments were reviewed in random order, and evaluated "blind". In addition to measurement of viable rim thickness and volume fraction of viable cells (Section 4. M. 7.), other morphological features not amenable to direct quantitation were assigned a score of 0 - 3 according to the degree of expression of the particular feature (see Figure 4.4 for examples):

\textit{Necrotic dissolution:} Based on the amount of empty space in the necrotic centres of the spheroid, and the loss of stainable nuclear fragments.

\textit{Hypoxic zone swelling:} Swollen cells over >1/4 of the circumference in this zone were scored 1, over the whole circumference scored 2, and when thicker than one layer, scored 3.

\textit{Necrotic zone demarcation:} An impression, at low magnification, of an increased demarcation between the viable and necrotic zones. Gradual transition scored 0, clear demarcation 3.

\textit{Cell rounding:} Based on the degree of shortening or loss of cell processes from the cells in the outer zone, so that the EMT6 cells had round or oval profiles. Spindle-shaped cells prominent scored 0, all round or oval cells, 3.

\textit{Cell fragmentation:} A score of 0 was given for occasional nuclear fragments in the outer zone. A score of 3 was given for abundant fragments.

\textit{Decreased mitoses:} A score of 0 was given for five or more mitoses per section of spheroid. A score of 1 was assigned for 2 - 4 mitoses per spheroid section, a score of 2 for 1 - 2 mitoses per 2 sections, and a score of 3 for no mitoses seen in 2 sections.

The scores for morphological features are summarised in Figure 4.5A. The measurements of rim thickness showed high variability and no differences between the experimental groups, and are not shown. FAA caused falls in viable cell volume fraction in both \textit{in vitro} and \textit{ex vivo} spheroids.
(P < 0.0001; Figure 4.5C), but the effect was almost twice as great with ex vivo spheroids. The results showed the same trends as those for cell yield after dissociation (Figure 4.3B). Apart from hypoxic zone swelling, all morphological indices for FAA-treated ex vivo spheroids had mean scores of 2.4 or more, and their means were significantly different from those of untreated ex vivo, and treated in vitro, spheroids (Mann-Whitney test, P ≤ 0.01). On the other hand, FAA-treated in vitro spheroids had mean scores of < 0.4 for all features except cell fragmentation and decreased mitoses. Cell rounding was the best discriminant between the effects seen in ex vivo and in vitro spheroids. These results corresponded with and confirmed the morphological descriptions above (Section 4. R. 4.).

To facilitate comparison between experiments using the morphology scoring system, a compound score was calculated for each spheroid assessed, to reflect the overall effect. This "Activity Index" (Figure 4.5 B) is the sum all five scores which were highest in ex vivo spheroids exposed to 2.5 mM FAA for 24 h: necrotic zone dissolution, necrotic zone demarcation, decreased mitoses, cell rounding and cell fragmentation. Hypoxic zone swelling, which was higher in transiently exposed spheroids (Section 4. R. 8.; Figure 4.8) was not included. The Activity Index and measurements of viable cell volume fraction, were used in assessing activity in later experiments in this chapter (Sections 4. R. 7. - 11.), and also for the comparison of xanthenone acetic acid analogues in Chapter 6.
FIGURE 4.4 - HISTOLOGICAL EFFECTS OF FAA ON SPHEROIDS

a. Untreated in vitro spheroid composed of randomly-orientated bundles of intact large spindle-shaped cells in the outer zone (O). A layer of large round cells is present on the surface. The innermost (hypoxic) portion of the outer zone merges gradually with the inner necrotic zone (I). The latter is composed of degenerating cellular material including pyknotic nuclei and their fragments.

b. Untreated ex vivo spheroid showing similar features to a.

c. In vitro spheroid exposed to 2.5 mM FAA for 24 h. The cell density of the outer zone is slightly decreased, but the structure is similar to that shown in a and b. Scores (see Section 4. R. 5.) for all morphological features in the particular spheroids shown in a to c were zero.

d. Ex vivo spheroid exposed to 2.5 mM FAA for 24 h. The outer zone shows a decrease in density, and the tumour cells have become shorter and more round. The necrotic zone is largely cleared of cell debris and very few pyknotic nuclei are seen. This zone is well demarcated from the outer zone. Intact cells at the interface are swollen. This spheroid scored 3 for all morphological features except hypoxic zone swelling, for which it scored 1 (Activity Index = 15).

Light photomicrographs, H&E-stained paraffin sections. Bar = 50 μm.
FIGURE 4.4 (CONT.) - HISTOLOGICAL EFFECTS OF FAA ON SPHEROIDS

e - i. Detail of outer zone. e. Untreated in vitro spheroid composed of spindle-shaped cells. f. Untreated ex vivo spheroid showing similar appearance to e. A mitotic figure (arrow) is seen in a tumour cell. The small round cell (arrowhead) is interpreted as a host cell. g. FAA-treated in vitro spheroid composed of spindle-shaped cells with a few tumour cell fragments (arrows). h. FAA-treated ex vivo spheroid showing a decrease in cell density, tumour cell rounding and fragmentation, and a macrophage (arrow). i. Plastic-embedded section of FAA-treated ex vivo spheroid, showing cell rounding, a macrophage (arrow) and an apoptotic cell (arrowhead).

Light photomicrographs, e - h: H&E-stained paraffin sections; i: toluidine blue-stained plastic section. Bars = 20 µm.
EMT6 spheroids were exposed to 2.5 mM FAA for 24 h. The number of determinations, and the key to the hatch patterns used in all three graphs, are shown in graph C. A: A score of 0 - 3 was assigned to each of the six morphological features shown in the bar graph. B: The Activity Index is the sum of scores for all morphological features shown in A, except hypoxic cell swelling. C: The cell volume fraction in the viable rim was determined by point counting.

The values are means ± s.e.m. The data were pooled from 14 experiments. FAA caused significant differences (vs. no FAA), as indicated on the graphs (*: P ≤ .01; **: P ≤ .0001). Effects of FAA were significantly different (P ≤ .01) for in vitro vs. ex vivo spheroids, for all eight measures.

Untreated spheroids showed wide spaces between EMT6 tumour cells (Figure 4.6a) even at the outer surface, where the cells were joined by interdigitations of the cell membranes and a few poorly-formed desmosome-like junctions (Figure 4.6c). The tumour cells possessed long cell processes, a few microvilli, strands of endoplasmic reticulum and mitochondria and a few free ribosomes (Figure 4.6e). There was no evidence of gland formation in this poorly-differentiated mammary carcinoma. The cells in the hypoxic zone were of similar appearance, and these merged with cells undergoing autophagy (Figure 4.6l,m) and fragmentation of cell membranes, cytoplasmic contents and nuclei at the edge of the necrotic zone (Figure 4.6f). Macrophages and lymphocytes were identified in untreated ex vivo spheroids (Figure 4.6n,o), evenly distributed through the viable cell layer, though they were less obvious than might be expected from the FCM results. No neutrophils or other granulocytes were seen.

FAA treatment caused some degeneration and fragmentation of in vitro spheroid cells, as seen by light microscopy, and also the appearance of small round lysosome-like electron dense bodies in the outer two cell layers (Figure 4.6g). These were also seen in ex vivo spheroids (Figure 4.6h). The cells in the middle of the viable zones of ex vivo spheroids were much more round or oval than controls (Figure 4.6d,i), lacking long cell processes and possessing fewer microvilli (Figure 4.6i). Clusters of membrane-bound cell fragments were also seen, each apparently derived from a single tumour cell by apoptosis (Figure 4.6j,k). In contrast, the swollen cells of the hypoxic zone showed the marked degenerative changes of necrosis (Figure 4.6l,m), similar to, but more severe than, those seen in this zone in untreated spheroids (Figure 4.6f). Macrophages (Figure 4.6p,q) were evenly distributed in the viable zone. Some of these contained large vacuoles and increased amounts of phagocytic material, but were otherwise similar to those of controls.
FIGURE 4.6 - ULTRASTRUCTURE OF EMT6 SPHEROIDS

a-d. Effects of FAA treatment on cells in the middle of the outer zone.  

a. Untreated in vitro spheroid composed of spindle-shaped cells, round in cross section. The cells have long tapering processes, sometimes closely applied to other cells (arrowheads). In this field, many processes are seen in cross section (large arrows). Numerous fine microvilli protrude from the cell surfaces (small arrows).  
b. Untreated ex vivo spheroid, similar in appearance to a.  
c. FAA-exposed in vitro spheroid, similar in appearance to a and b.  
d. FAA-exposed ex vivo spheroid. All cells are round or oval in profile, and no longer show long cytoplasmic processes. Microvilli are reduced.

Transmission electron micrographs, uranyl acetate and lead citrate stain. Bar = 2 μm.
e, f. *Detail of untreated spheroids.* e. Surface cells of untreated *in vitro* spheroid. Wavy microvilli project from the spheroid surface, or interdigitate between cells. Cell junctions are few (arrowhead), and are not associated with tonofilaments. The cytoplasm contains many polyribosomes (circle), some rough endoplasmic reticulum (small arrows), and a few mitochondria (large arrows). f. The necrotic/viable interface of an untreated *in vitro* spheroid. Three necrotic cells and one intact cell (C) are seen. The necrotic cells show condensation of the nuclear chromatin, coarsely granular cytosol, dilatation of the cytoplasmic membrane systems (small arrows), and breaches in the cell membrane (large arrows).

Transmission electron micrographs, uranyl acetate and lead citrate stain. Bars = 2 μm.
FIGURE 4.6 (CONT.) - ULTRASTRUCTURE OF EMT6 SPHEROIDS

g - i. Non-lethal effects of FAA.  g. Surface of an in vitro spheroid showing the appearance of lysosome-like bodies (arrows).  h. Lysosome-like bodies at the surface of an ex vivo spheroid (arrows).  i. Detail of a cell from an FAA-treated ex vivo spheroid, showing rounding of the cell contour and reduction in microvilli. No abnormalities are seen in the nucleus or cytoplasm.

Transmission electron micrographs, uranyl acetate and lead citrate stain. Bars = 2 µm.
j, k. Apoptotic cell death in FAA-exposed ex vivo spheroids. j. Clusters of cell fragments from the middle of the viable zone. k. Detail of apoptotic cell fragments showing nuclear fragments with condensed chromatin (large arrows). The cell membranes of the fragments are intact, and although there is vesiculation and swelling of the endoplasmic reticulum (small arrows), the mitochondria (open arrows) are intact.

l, m. Necrotic cell death in FAA-exposed ex vivo spheroids. l. Swollen, degenerate cells in the hypoxic zone. The cell at the top of the field shows karyolysis and cytoplasmic disruption. The cell below appears viable, but is enlarged and contains autophagic vacuoles (V). m. Detail from l.

Transmission electron micrographs, uranyl acetate and lead citrate stain. Bars = 2 μm.
FIGURE 4.6 (CONT.) - ULTRASTRUCTURE OF EMT6 SPHEROIDS


Transmission electron micrographs, uranyl acetate and lead citrate stain. Bar = 2 μm.
4. R. 7. Effects of FAA concentration

*Ex vivo* spheroids exposed to FAA for 24 h showed a progressive decrease in the cell volume fraction of the viable outer zone and an increase in the Activity Index with increasing concentrations of FAA up to 2.5 mM (Figure 4.7). In some spheroids exposed to concentrations of FAA below 2.5 mM, the density of the cells in the viable zone showed regional variation with a lower density seen centrally (Figure 4.8a). When evaluated blind, this type of regional variation was found in 7/21 spheroids exposed to 0.25 - 2.0 mM FAA, in 1/29 spheroids exposed to 2.5 mM FAA, and in 3/21 control spheroids.

4. R. 8. Effects of FAA exposure time

A decrease in cellularity of the inner part of the viable zone, similar to that seen in Section 4. R. 7., was seen in 7/10 *ex vivo* spheroids exposed to 2.5 mM FAA for 2 - 7.5 h (Figure 4.8b), although in this study no significant changes were found on quantitation of cell volume fraction in the viable zone.

In an attempt to mimic the falling plasma levels following a single dose of FAA *in vivo*, *ex vivo* spheroids were exposed to 2.5 mM FAA for 2 h or 4 h, followed by incubation in non-FAA-containing GM for the remainder of the 24 h incubation period. These spheroids did not show significant changes in the outer zone compared to spheroids exposed to FAA for the full 24 h, but did show marked hypoxic zone cell swelling together with a small degree of necrotic dissolution. Quantitation confirmed that the highest scores for hypoxic cell swelling occurred in such spheroids (2.8 ± 0.2, n = 6; 2 h and 4 h values combined), significantly higher than those for continuous 24 h exposure (1.5 ± 0.1; P ≤ .01; Figure 4.5A). Scores for the other morphological indices and viable cell volume fraction were not significantly different from those of untreated spheroids (results not shown).
FIGURE 4.7 - EFFECTS OF FAA CONCENTRATION ON EX VIVO SPHEROIDS

Cell volume fraction in the viable rim (A) and Activity Index for morphological features (B) after exposure of ex vivo spheroids to FAA for 24 h. For ex vivo spheroids, data were pooled from four experiments, n = 4. The in vitro values are reproduced from Figure 4.5 B & C, for comparison.
a. *Ex vivo* spheroid exposed to 0.25 mM FAA for 24 h.  
*b. Ex vivo* spheroid exposed to 2.5 mM FAA for 4 h. Both spheroids show loss of nuclear remnant staining in the inner necrotic zone (compare with Figure 4.4a-c), and a decrease in cell density in the inner half of the outer zone (dotted lines).

c. *Ex vivo* spheroid exposed to 2.5 mM FAA for 4h, and then incubated in FAA-free growth medium for 20 h. There is some dissolution and loss of nuclear material in the necrotic zone. A thick layer of swollen degenerating tumour cells staining intensely with eosin is present at the viable/necrotic zone interface (dotted line). This layer of swollen cells is thicker than that seen after continuous FAA exposure for 24 h (see Figure 4.4d).  
*d. Higher magnification of the swollen degenerate cells.*

Light photomicrographs, H&E-stained paraffin sections. Bars = 50 μm.

Dexamethasone partially inhibited the morphological effects of FAA, as shown in Figure 4.9, but there was some variability between individual spheroids. In some cases, spheroids incubated in the same tube, but from different mice, showed effects varying between no inhibition and total inhibition of the FAA effect. Dexamethasone without FAA had no observable effect on the morphology of ex vivo spheroids, and had no effects on in vitro spheroids, with or without FAA.

4. R. 10. Effects of phorbol myristate acetate (PMA)

When ex vivo spheroids were exposed for 24 h to PMA (0.40 - 6.34 mM), the morphological changes observed in some ways resembled those of FAA (Figures 4.10 & 4.11). The most marked of these was cell rounding, but scores were also elevated for cell fragmentation, necrotic dissolution and necrotic demarcation. There was however no decrease in mitoses. No changes were seen in in vitro spheroids.

4. R. 11. Effects of oxygen concentration on ex vivo spheroids

Varying the oxygen tension did not influence the response of spheroids to FAA (Figure 4.12) except that mitoses were less frequent in the absence of oxygen. The results for both control and FAA-exposed spheroids, under all three atmospheres, were similar to those obtained when the incubations were carried out in sealed tubes containing a small amount of air, as described earlier (Figure 4.5B,C).
**FIGURE 4.9 - EFFECTS OF DEXAMETHASONE**

*Ex vivo* spheroids were exposed to 1 μM dexamethasone (DX) for 3 h, then FAA was added to a concentration of 2.5 mM, and the spheroids exposed for a further 24 h. **A:** Viable cell fraction. **B:** Activity Index. Values are means ± s.e.m. The numbers of determinations are shown in **A.** Dexamethasone significantly decreased the effect of FAA on cell volume fraction (P < .005) and on Activity Index (P < .01). Data were pooled from four experiments.
Ex vivo spheroids were exposed for 24 h to PMA (0.40, 1.58 or 6.34 6M, values from all concentrations pooled). A: Cell volume fraction. B: Activity Index. Values are means ± s.e.m. The numbers of determinations are shown in A. PMA caused significant effects (compared to no drugs) in cell volume fraction (P < .01) and Activity Index (P ≤ .01). Data were pooled from two experiments.
FIGURE 4.11 - HISTOLOGICAL EFFECTS OF PMA ON EX VIVO SPHEROIDS

a. The transition between the outer zone containing viable cells (O) and the inner necrotic zone (I) is indistinct, as in untreated spheroids (Figure 4.4 a,b), but there is some loss of staining of nuclear remnants in the necrotic centre. b. Detail of outer zone. Cell rounding is the most striking feature, and is more marked and generalised than in FAA-treated tumours (Figure 4.4 d,h). However, unlike FAA-treated spheroids, the cell density is not decreased, cell fragmentation is seen only in scattered foci (arrows), and mitoses are frequent (arrowhead). This spheroid was exposed to PMA 0.04 μM for 24 h, but the appearances were the same when concentrations of 1.58 and 6.34 μM were used.

Light photomicrographs, H&E-stained paraffin sections. Bars = 50 μm.
**FIGURE 4.12 - EFFECT OF OXYGEN CONCENTRATION**

*Ex vivo* spheroids were incubated in spinner flasks for 24 h under different atmospheres. Values are means ± s.e.m., n = 4.
4. Discussion

This chapter has investigated the activity of FAA against EMT6 spheroid cells in vitro, and shows that FAA exerts a small degree of direct cytotoxicity against EMT6 tumour cells. Greater cytotoxicity was observed in ex vivo spheroids, indicating the importance of an indirect anti-tumour effect. The following discussion considers the evidence for hypoxia as a factor in the cytotoxicity of FAA, the content of host immune cells in ex vivo spheroids, their role in the observed cytotoxic and morphological changes, and the part that these changes play in the anti-tumour mechanism of FAA in vivo. The possibility of synergy between FAA and low exchange environmental conditions in stimulating macrophages is considered, and further applications for the ex vivo spheroid tumour system are discussed.

4. D. 1. Direct cytotoxicity of FAA

The present studies have shown that FAA exhibits both direct and indirect toxicity against EMT6 tumour cells. Direct toxicity decreased clonogenic cell yield by 34%, and morphometrically determined cell volume fraction by 26% in in vitro spheroids. The increased numbers of cell fragments in the viable rims of in vitro spheroids exposed to FAA suggest that apoptosis was responsible for this cell loss.

There was no evidence that the direct toxic effect was influenced by the microenvironmental factors that occur in solid tumours (Section 1.4., p. 30). FAA was not toxic to EMT6 spheroid cells under anoxic or oxic conditions. Both intact and dissociated spheroids were resistant to FAA. Morphological changes in ex vivo spheroids were not affected by oxygen tension. Thus, conditions of the low exchange environment do not appear to sensitize EMT6 cells to FAA. Indeed, Bibby et al. (1989b) found that HT 29 colon carcinoma cells were four times more resistant to FAA when grown as spheroids compared to monolayers, further evidence against the hypothesis that tumour selectivity in vivo is due to some effect of the low exchange environment on direct cytotoxicity.

4. D. 2. Immune cell infiltration of EMT6 spheroids

Flow cytometry showed that ex vivo spheroids contained a population of diploid cells, and cells which stained with antibodies to specific markers of macrophages, T-lymphocytes and B-lymphocytes. These
findings confirm those of Lord et al. (1979), who first incubated EMT6 spheroids in the peritoneal cavities of mice to allow their infiltration by immune effector cells. They characterised the infiltrating cells and investigated the cytotoxicity of these cells in spheroids from non-immunised mice and those immunised with killed EMT6 cells. In their studies, the infiltrating cells were counted on cytocentrifuge preparations, and included macrophages, lymphocytes and granulocytes, the ratios depending on the time the spheroids were in the peritoneum and whether the mouse had been immunised or not.

There was a prima facie discrepancy in the present study between the high content of host cells in ex vivo spheroids (about 50%, Table 4.1), and their lack of prominence in histological and ultrastructural sections (Figures 4.4f & 4.6b). This could be due to the large differences in the volumes of immune cells and EMT6 tumour cells. Host cells are similarly inconspicuous in the light micrographs of Lord et al. (1979), who also estimated a high fraction of infiltrating immune cells on cytocentrifuge preparations.

A major difference in results between the present study and those of Lord’s group is that, in addition to lymphocytes and macrophages, Lord’s studies also found granulocytes in the spheroid infiltrate (Lord, 1980; Lord & Burkhardt, 1984; Wilson & Lord, 1987), though the fractions retrieved after similar incubation conditions differed between the different studies. In the present experiments, macrophages and lymphocytes were seen by light and electron microscopy, but no granulocytes were identified despite their distinctive morphology. The possibility of mistaking apoptotic cells for degenerating granulocytes has been noted (Kerr et al., 1984), and this distinction was also found to be difficult in cytocentrifuge preparations in the present studies. It is likely that granulocytes are not present in ex vivo EMT6 spheroids from non-immunised mice, but this requires confirmation using specific granulocyte markers.

4. D. 3. Host mediated cytotoxicity of FAA

Ex vivo spheroids exposed to FAA showed significantly greater falls in cell yield, plating efficiency, clonogenic cell yield and eosin exclusion than in vitro spheroids. This corresponded with specific morphological changes indicating cell injury, seen only in ex vivo spheroids. Quantitation of these changes confirmed significant differences between in vitro and ex vivo spheroids, and these effects varied as a function of FAA concentration. The most likely basis of these differences is the presence of the immune cell infiltrate in ex vivo spheroids, but the action of some diffusible "humoral" factor from the host, or some other modification in EMT6 cell sensitivity as a result of growth of spheroids.
in vivo, could not be excluded. Small spheroids were therefore incubated in the peritoneal cavities of mice within diffusion chambers, and these spheroids were recovered and exposed to FAA in vitro (results not shown). Because of poor recovery and coalescence of the spheroids, most of the morphological features could not be evaluated, but the characteristic cell rounding effect was completely lacking, providing evidence against sensitisation by a diffusible host factor. However, this possibility requires further investigation because a recent study has shown that conditioned medium from endotoxin-exposed macrophages stimulated EMT6 cells to produce nitric oxide (Amber et al., 1991), a substance which may mediate some of the cytotoxic effects of FAA (Thomsen et al., 1990). The theoretical possibility therefore exists that ex vivo spheroids may be sensitised to FAA as a result of cytokine exposure in vivo.

Dexamethasone, which inhibits diverse immune activities, partially inhibited the FAA morphological effects. This favours an immune cell mediated mechanism of killing in ex vivo spheroids, and agrees with the results of Ching and Baguley (1988), who also inhibited FAA-stimulated macrophage-mediated cytotoxicity with dexamethasone. Their experiments used higher effector:target cell ratios and lower concentrations of FAA.

The mechanisms involved in cell killing by FAA appeared to differ according to the location of the cells within the spheroid. In the innermost zone of the viable rim, cell swelling was accompanied by progressive cell membrane, cytoplasmic and nuclear degradation, all characteristics of necrosis. In the remainder of the outer rim, clusters of apoptotic cell fragments were present between widely separated intact cells, which had become round or oval in shape, with loss of their characteristic long cell processes. Despite these morphological changes, many of these cells retained their clonogenicity, since plating efficiency in ex vivo spheroids treated with FAA fell by only 45%.

The cell rounding and apoptosis seen were also evident in Colon 38 tumours treated with FAA in Chapter 2. Their appearance in the present studies confirms that the changes seen in Colon 38 tumours were not due entirely to loss of blood flow, and could have been due partly to immune cell injury.

In addition to cell injury, another major morphological effect was the dissolution of the central necrotic material, which usually occupies the entire central zone in EMT6 spheroids. Both amorphous cytoplasmic material and nuclear fragments were lysed, suggesting the action of hydrolytic enzymes. These could have been released from the degenerating tumour cells themselves. Treatment of tumours with TNF (a monokine which may mediate the anti-tumour effects of FAA; Section 1. 5. 8., p. 40; Mahadevan et al., 1990), has caused lysosomes to become more clearly visible in tumour cells (Shimomura et al., 1988), and the activation of lysosomes has been implicated in the mechanism of action of TNF (Section 1. 5. 5., p. 38; Liddil et al., 1989). In the present studies, lysosome-like
granules appeared in tumour cells after FAA exposure, but they were few in number, occurred only in cells at the spheroid surfaces, and were observed in both ex vivo and in vitro spheroids.

Alternatively, lytic enzymes could be released from macrophages, which are known to secrete a variety of products, including many enzymes, on activation (Nathan, 1986). One such product is "cytolytic protease", a major mediator of the anti-tumour activity of macrophages in vitro (Adams & Hamilton, 1988). This serine protease can be inhibited by the natural peptide aprotinin (Adams 1980). Spheroids were therefore incubated with FAA and aprotinin but no inhibition of the FAA effects was seen; nor did aprotinin prevent haemorrhagic necrosis when given i.p. 4 h before FAA treatment (results not shown). However, the possibility has not been excluded that other hydrolytic enzymes play a part in dissolution of the necrotic coagulum and the observed cytotoxic effects.

4. D. 4. Which infiltrating cells mediate the FAA-induced effects?

Evidence in favour of the macrophage as the major mediator of the effects seen in spheroids includes the dissolution of the necrotic material in the spheroid centre (above section). A role for macrophages is further supported by the resemblance of the FAA effect to that seen after exposure to PMA of ex vivo, but not in vitro, spheroids. PMA is known to activate macrophages to produce the superoxide radical anion (Baggiolini & Wymann, 1990), which may synergise with cytolytic protease in mediating macrophage cytotoxicity (Adams et al., 1981). Marked cell rounding as well as cell fragmentation and dissolution of the central necrotic material were seen after both FAA and PMA. A major difference was that mitoses were not decreased after PMA. These findings suggest that some, but not all, of the biological actions of these two drugs are similar. Macrophages in vitro are stimulated by FAA to exhibit anti-tumour cytotoxicity (Ching & Baguley, 1988), and to produce nitric oxide (Thomsen et al., 1990), which enhances the cytotoxic effects of TNF (Higuchi et al., 1990).

Of the other cells identified, T-cells are only cytotoxic in EMT6 spheroids after prior immunisation of the mouse to EMT6 antigen (in the absence of FAA stimulation, Lord & Burkhardt, 1984). T-cells do not contribute to the cytotoxicity of peritoneal exudate cells stimulated by FAA (Ching & Baguley, 1988). NK cells are induced in vivo by FAA (Ching & Baguley, 1987), but these cells are probably not involved in its anti-tumour action (Ching & Baguley, 1989a). NK cells have not been found in peritoneal EMT6 spheroids (Wilson & Lord, 1987). The possible cytotoxic role of B-cells requires investigation, since they are known to produce interferons and TNF in response to FAA (Futami et al., 1991).
The population of infiltrating immune cells did not exhibit significant spontaneous (FAA-independent) cytotoxicity: plating efficiency (Figure 4.3B) and viable cell volume fraction (Figure 4.5C) showed no significant differences between in vitro and ex vivo spheroids. This finding agrees with that of Wilson and Lord (1987), who found no anti-EMT6 activity in macrophages, even from immunised mice, despite the cytotoxicity of these macrophages to WEHI-164 cells. This observation was interpreted as reflecting the resistance of EMT6 cells to TNF (at the levels of secretion found in unstimulated macrophages), as this monokine mediates the WEHI-164 cytotoxicity of spheroid-infiltrating macrophages (Wilson et al., 1989). In summary, both the present experiments and literature suggest that FAA exerts its cytotoxic effect via the activation of macrophages.


Microenvironmental factors do not appear to make a major contribution to the direct toxicity of FAA (Section 4. D. 1.). However, the observation that hypoxia stimulates macrophages to secrete an angiogenic factor (Knighton et al., 1983), which appears to be TNF (Liebovich et al., 1987), raises the possibility that hypoxia or some other characteristic of the low exchange environment could synergise with FAA to enhance its effects in tumours or in ex vivo spheroids.

The morphological appearances of FAA-treated ex vivo spheroids supported this hypothesis. Exposure of ex vivo spheroids to concentrations of FAA below 2.5 mM, or for times less than 24 h, often resulted in a gradient of cell density in the viable zone, with the lower density occurring centrally (Figure 4.8a,b). This suggested that the central region was more sensitive to toxic effects of FAA. Furthermore, the mode of cell death differed with location in the spheroid: only in the innermost part of the viable cell zone did cell death have the features of necrosis.

In an attempt to enhance this effect, ex vivo spheroids were exposed to FAA in vertical unagitated tubes for up to 4 h at 37°C, incubated for the remainder of the 24 h period in FAA-free GM on a roller wheel, fixed and examined histologically (results not shown). The growth medium around the spheroids changed colour while unagitated, indicating a fall in pH, and presumably an accentuation of the other conditions of the low exchange environment. However, the morphological appearances of these spheroids were similar to those of transiently FAA-exposed spheroids which were continuously agitated (Section 4. R. 8.). Varying the oxygen concentration during FAA exposure in well-stirred cultures from 0 - 95% also had no effect on ex vivo spheroid morphology (Section 4. R. 11.).

There was thus no consistent evidence from the present in vitro studies that microenviron-
mental factors play any part in the tumour selectivity of FAA. However, a role for such factors in vivo cannot be excluded since the selective effect could occur on the vasculature. Hypoxia has been shown to alter the cytoskeletal structure and to increase the permeability and pro-coagulant activity of bovine adrenal microvascular endothelial cells (Ogawa et al., 1990). Similar procoagulant effects have been seen after FAA treatment in vitro (Murray, et al., 1991), and it is possible that the hypoxic and FAA stimuli could synergise. The role of microenvironmental factors is further discussed in Chapter 7 in relation to the similarities in vascular patterns in tumours and in FAA-sensitive non-tumour tissues.

4. D. 6. Relevance to the anti-tumour action of FAA

While the previous chapter demonstrated the importance of ischaemic mechanisms in the anti-tumour activity of FAA, the present study has shown the existence of non-ischaemic mechanisms which may also contribute to tumour cell killing. Spheroid infiltration by host immune cells substantially and significantly increased their sensitivity to the cytotoxic action of FAA in vitro. Direct cytotoxicity of the drug against EMT6 cells (discussed in Section 4. D. 1.) has a minor effect compared to the host-cell mediated anti-tumour activity (discussed in Section 4. D. 3.). The greatest cytotoxic effect observed was an 80% reduction in clonogenic cell yield in intact ex vivo spheroids. However, this is much smaller than the effect seen in vascularised i.m. EMT6 tumours in Chapter 3 (Figure 3.7, p. 85), despite exposures to high concentrations of FAA continuously for up to 24 h in the in vitro studies, which confirms the requirement for blood vessels for the maximum anti-tumour effect of FAA in vivo.

The concentration of FAA used in most of the experiments, 2.5 mM, is comparable to that found in vivo in the plasma of mice after bolus injection of effective doses (Zaharko et al., 1986; Damia et al., 1988; McKeage et al., 1991). In vivo peak plasma concentrations exceed 2.5 mM after a (non-toxic) dose of 1.1 mmol/Kg, but fall well below 1 mM within 2 h (Damia et al., 1988). A plasma concentration of 2.5 mM cannot be sustained for 24 h in vivo without lethality (Zaharko et al., 1986; Damia et al., 1988; Bibby et al., 1988; 1989a; Chabot et al., 1989a). The dose-response relationship (Figure 4.7), although not well defined, shows no evidence of a threshold, so that the lower blood concentrations found in vivo after the peak, may still kill tumour cells by this mechanism. If this killing has the characteristics of a zero order rather than a first order process, then its contribution to fractional cell kill could be much greater in combination with an ischaemic mechanism which reduces the absolute tumour cell number (see also Section 1. 5. 3., pp. 36 - 37).
4. D. 7. Utility of the *ex vivo* spheroid tumour model

*Ex vivo* EMT6 spheroids resemble EMT6 tumours in histological appearance, in microenvironmental heterogeneity, and in content of immune effector cells (Lord, 1980). They offer the advantage of uniformity, and are more versatile under experimental conditions. Their difference from actual tumours is their total lack of blood vessels, and *ex vivo* spheroids were therefore useful in investigating the non-vascular components of FAA activity. *Ex vivo* spheroids are thus intermediate in cellular complexity between *in vitro* spheroids and vascularised tumours.

The endpoints used in the present studies were clonogenic assay and morphological evaluation. The latter included a morphological scoring system, which allowed objective confirmation of the relationship between morphology and treatment. The good correlation between the clonogenic assays and the scoring of morphological features suggested that the latter could be used as a reliable endpoint. The morphological scoring system was simple and rapid, and enabled detection of regional differences in the effects of FAA, and could produce a graded score as seen in the dose response experiment (Figure 4.7).

The *ex vivo* spheroid model is a novel *in vitro* tumour system which allows the investigation of host cell-mediated anti-tumour effects under conditions which closely resemble those existing in actual tumours. This applies to the relationship between tumour cells themselves and the microenvironment in which they exist (Sutherland, 1988), and the relationships between the tumour cells and infiltrating host cells (Lord, 1980). This may be important because some immune cell effects occur only at short range, due to a requirement for cell to cell contact (Section 1. 5. 3., p. 37), or the production of labile mediators (eg. nitric oxide) or high molecular weight products with low diffusion coefficients (eg. cytokines). *Ex vivo* spheroids could thus also be used to investigate other anti-tumour agents which may stimulate macrophages, including cyclophosphamide (Stoychkov *et al.*, 1979; McBride *et al.*, 1987), doxorubicin (Stoychkov *et al.*, 1979) and phototherapy (Evans *et al.*, 1990). The effects of xanthenone acetic acids on *ex vivo* spheroids are reported in Chapter 6.
4. D. 8. Conclusions

EMT6 spheroids introduced into the peritoneal cavities of mice are extensively infiltrated by lymphocytes and macrophages. The greater sensitivity of such ex vivo spheroids to FAA provides evidence that immune effector cells mediate the non-ischaemic component of the FAA anti-tumour action. This may contribute significantly and independently to in vivo anti-tumour activity. There is no evidence from these studies that FAA and hypoxia synergise to activate macrophages for tumour cell killing.
5.1. Introduction

The preceding chapters of the thesis have suggested that both ischaemia, caused by an irreversible regional loss of blood flow, and immune mechanisms, mediated primarily by macrophages, play a part in the anti-tumour action of FAA. Modes of cell death for the two mechanisms appear to differ: a necrotic type of cell death follows ischaemia (Chapter 2), and immune mediated cell death occurs mainly by apoptosis (Chapter 4).

The spheroid studies in Chapter 3 suggested that loss of perfusion was the major anti-tumour effect, since vascularised i.m. EMT6 tumours were much more sensitive to FAA than were i.p. EMT6 spheroids. While the resistance of spheroids to FAA in these studies was attributed to their lack of blood vessels, other site- or size-related factors could not be excluded. It has been suggested that the site of the tumour is important in determining its responsiveness to FAA (Bibby et al., 1989b). With regard to size, Finlay et al. (1988) found that small lung metastases of the Lewis lung tumour failed to respond to FAA treatment, while larger lung nodules and subcutaneous implants of this tumour were sensitive.

To determine the relative roles of the vascular and avascular components in the anti-tumour action of FAA, a new experimental model was developed. The process of attachment and vascularisation of EMT6 spheroids in the peritoneum was examined, and vascularised tumours were found which were directly comparable in site and size range to avascular spheroids (AVS). Since these vascularised spheroids (VS) were themselves composed of a vascularised core surrounded by an avascular outer layer, direct comparison of the effects of FAA on vascular and avascular tissue within the same individual tumour was possible. The histological changes occurring in AVS, VS and small deposits of infiltrating peritoneal tumour were compared. A double-label fluorescent vascular dye technique, based on that developed by Trotter et al. (1989a), and similar to that used in Chapter 3, was used to assess the changes in blood flow in the VS.
5. M. Methods

5. M. 1. Histological studies

Spheroids were implanted into the peritoneal cavities of mice, as described in Section 3. M. 6. (p. 74), but were used after seven days instead of six, to allow attachment and vascularisation of a larger fraction of the spheroids. The mice in the treatment group \((n = 7)\) were injected with FAA 0.8 mmol/Kg in 5% wt/vol sodium bicarbonate by the i.v. or i.p. route, and were killed after a further 18 hrs (on day 8). Untreated spheroid-bearing mice \((n = 2)\) were killed after the same interval. The peritoneal cavities of the mice were opened, the free spheroids were collected, and those spheroids which had become attached to host structures were excised with a cuff of adjacent tissue. The spheroids were fixed in either 4% formaldehyde or 2.5% phosphate-buffered glutaraldehyde (pH 7.4). The formalin-fixed tissue was embedded in paraffin, and 5 \(\mu m\) sections were cut and stained with haematoxylin and eosin (H&E), as described in Section 2. M. 3. (p. 49). Glutaraldehyde-fixed spheroids were embedded in epoxy resin and 2 \(\mu m\) sections cut and stained with toluidine blue, as described in Section 4. M. 9. (p. 97). Multiple sections were examined from each spheroid. The thicknesses of the viable rim and the avascular zone were measured with an eyepiece micrometer, and the results expressed as the means ± s.e.m.

5. M. 2. Fluorescent vascular marker studies

Mice were used eight days after the introduction of spheroids into the peritoneal cavities for these experiments, to correspond with the eight-day total experiment time for the histological studies. Three mice each received two i.v. injections. The first contained 80 mM FAA (sodium salt), 3.25 mM Hoechst 33342 (H33342) and 5% wt/vol D-glucose. The volume injected was 10 \(\mu l/g\) body weight (FAA dose 0.8 mmol/Kg; H33342 dose 32.5 \(\mu mol/Kg\)). In control mice \((n = 3)\), the first injection contained only H33342. The second injection, given 4 hrs later, contained 2 mM 10-nonyl acridine orange bromide (NAO), 4% vol/vol dimethylsulfoxide (DMSO) and 5% wt/vol D-glucose, and was given at an NAO dose of 20 \(\mu mol/Kg\). The mice were killed 5 minutes after the second injection, and free and attached spheroids were collected or excised and rapidly frozen in liquid nitrogen-cooled Freon 12. Frozen sections 10 or 16 \(\mu m\) in thickness were cut at 160 - 200 \(\mu m\) intervals, and viewed under a Nikon Optiphot microscope, as described in Sections 3. M. 3. & 4. (p. 72). The UV1A filter
block was used to visualise H33342, which fluoresced blue (nuclei), and the B2A filter block was used to visualise NAO, which fluoresced green (cytoplasm). Sections were viewed dry or mounted in 10% vol/vol glycerol saline.

5. R. Results

5. R. 1. Histology of untreated spheroids

Eight days after the introduction of EMT6 spheroids into the peritoneal cavities of mice, there was mild abdominal distension by blood-stained ascites. More than half of the introduced spheroids were recovered from each mouse, and about 20% of these were attached to host tissues, and appeared as cream-coloured spherical or ellipsoidal protrusions varying in size from barely visible to 4 mm in diameter. These were adherent to the peritoneal surfaces of the anterior abdominal muscles, the diaphragm, the omentum and mesentaries. A frequent site of attachment was the needle track through which the spheroids had been introduced. None of the spheroids was haemorrhagic. The majority of the attached spheroids were found by microscopy to be vascularised, and between one and eleven vascularised spheroids were found in each mouse.

AVS, whether free or attached, were composed of an outer zone of viable spindle-shaped cells, 145 ± 12 µm (n = 8) thick, surrounding an inner necrotic zone, which consisted of disintegrating cytoplasmic and nuclear material (Figure 5.1a). Scattered macrophages, identified by their smaller size, indented nuclei and more marginated chromatin, were distributed evenly in the viable zone. AVS were similar in appearance to the untreated ex vivo spheroids on day 7, as described in detail in the previous chapter (Section 4. R. 4., p. 104).

Most VS were composed of a solid mass of tumour cells and showed no evidence of central necrosis (Figure 5.1b,c). Blood vessels were present in their central regions, but never in the outer zone. The mean distance from the spheroid surface to the most superficial vessel in each spheroid was 142 ± 10 µm (n = 7), similar to the thickness of the viable zone of the AVS. In a few spheroids, the central zone showed features of both VS and AVS with tumour cells and blood vessels near the pole adjacent to the attachment site, and necrotic material towards the unattached pole (Figure 5.1d). These appearances were interpreted as reflecting recently-initiated incomplete vascularisation (Figure 5.6c, p. 144). Host cells were evenly dispersed within the VS by light and electron microscopy, including
macrophages, fibroblasts, lymphocytes and granulocytes.

Small groups of tumour cells were also seen in the adjacent host tissues including skeletal muscle (Figure 5.1c) and peritoneal fat, usually near the point of attachment of a spheroid. These infiltrative tumour deposits differed from VS in having a high vascular density and vessels close to the peritoneal surface. Several such areas were present in each mouse.

5. R. 2. Fluorescent markers in untreated VS

Attached spheroids excised 5 min after simultaneous administration of both H33342 and NAO showed staining of the tumour cells located within about five cell diameters of the blood vessels, with H33342 staining the nuclei and NAO the cytoplasm (Figure 5.2a,b). The diffusion distances of the two dyes from the vessels was initially similar whether viewed wet or dry, but within 15 min wet-mounted sections showed progressive diffusion of NAO into previously unstained areas. The pattern of staining with H33342 was more stable, but when given 4 h before sacrifice, diffusion in vivo caused most cells in the VS to show some fluorescence, although the para-vascular cells remained brightest (Figure 5.2c).

Both dyes identified functional blood vessels as being restricted to the central regions of the VS. An outer rim of non-fluorescent tissue, varying in thickness, but usually less than 140 μm, confirmed the histological observations that this zone was avascular (Figure 5.2a-d). Weakly fluorescent cells at the surface of VS (Figure 5.2a,b), were also present on both the free and adherent AVS, and were assumed to be due to staining via the peritoneal fluid.

In the untreated VS, vessels identified by one fluorescent marker were invariably also stained by the other, even when the dyes were injected 4 hrs apart, indicating that no opening or closure of tumour vessels occurred during this period. Adjacent host tissues showed confluent staining by both dyes (Figures 5.2c,d; 5.5c), presumably due to their high capillary density.
a. Avascular spheroid (AVS) consisting of an outer layer of spindle-shaped tumour cells (O) and an inner zone of necrotic cells (I).

b. Vascularised spheroid (VS) consisting of spindle cells with no evidence of necrosis. Blood vessels (arrows) are present in the inner zone (I), but not the outer zone (O).

c. VS showing attachment to the peritoneal surface and underlying skeletal muscle. Some tumour cells (arrows) are infiltrating within the muscle and beneath the mesothelium.

d. Spheroid in which the process of vascularisation appears incomplete. Blood vessels and loosely-packed tumour cells occupy part of the core (V), while a small, lens-shaped necrotic zone (N) persists below the outer viable zone (O).

Light photomicrographs. a-c: H&E-stained paraffin sections; d: toluidine blue-stained plastic section. Bar = 20 \mu m.
FIGURE 5.2 - FLUORESCENT STAINING OF UNTREATED VS

a, b. Staining of para-vascular tumour cell nuclei (arrows) by H33342 (a) and cytoplasm by NAO (b), both given 5 minutes before sacrifice. The avascular zone (A) shows no fluorescence except at the surface (arrowheads).

c, d. VS with underlying peritoneal fatty tissue. H33342 injected 4 hrs, and NAO 5 minutes before sacrifice. H33342 fluorescence (c) is more diffuse after 4 hours than in a., but the position of a vessel (arrow) can still be determined by the brighter fluorescence. The avascular zone (A), is faintly fluorescent in this section. The pattern of NAO staining (d) is similar, indicating that the vessel is still functioning after 4 hrs. The fatty tissue (F) stains evenly with both dyes, intense in the case of NAO.

Fluorescent photomicrographs of air-dried frozen sections. Bar = 50 µm.
5. R. 3. Histology of spheroids after FAA treatment

Treatment of spheroid-bearing mice with FAA resulted in morphological changes in both AVS and VS. Treated AVS had thinner rims of viable cells (90 ± 2 µm, n = 49) than did untreated AVS (145 ± 12 µm, n = 8), but were otherwise indistinguishable.

Treated VS appeared red in colour. All 25 VS examined histologically showed similar changes regardless of size (0.4 - 4 mm in diameter). The central zone was haemorrhagic, and the tumour cells showed margination of chromatin, nuclear pyknosis, and fragmentation of nuclei and cytoplasm (Figure 5.3). The haemorrhagic necrosis involved the entire vascularised zone in 20/25 VS, but small residual islands of histologically viable tumour cells were found in the remaining five. The outer avascular zones of all treated VS were composed of cells identical in appearance to those of untreated VS (Figure 5.3), with fine chromatin and mitotic activity, but measured only 60 ± 4 µm (n = 16) in thickness. The changes were the same whether the FAA was administered i.v. or i.p.

The central necrotic zones of treated VS differed from those of treated and untreated AVS in that numerous red blood cells were seen dispersed among the necrotic debris and in congested vessels (Figure 5.3c,d). In addition, the necrotic process was less advanced in treated VS, with less degradation and dissolution of the nuclei and cytoplasm of the dead tumour cells. These distinctions were clearly evident in the few incompletely vascularised spheroids from treated animals, where the two types of necrotic zone were contiguous (Figure 5.3c).

The blood vessels in VS showed a variety of changes 18 hrs after FAA treatment. The most frequent were congestion and haemorrhage (Figure 5.3d), but vascular occlusion by fibrin and/or platelet thrombi was not common, and was sometimes seen in tumour regions which did not show necrosis.

5. R. 4. Histology of infiltrative tumour deposits

The deposits formed by tumour cell infiltration of sub-mesothelial fat showed similar FAA-induced changes to those seen in VS. In superficial zones, where tumour cells were growing close to the mesothelium, necrosis was rare despite the presence of numerous thrombi in associated vessels (see also Section 5. R. 5; Figure 5.4a,b). However, the deeper masses of invasive tumour were necrotic (Figure 5.4a). No histological effects were seen in the small tumour infiltrates of skeletal muscle. Neither thrombosis not necrosis was seen in fat or skeletal muscle not infiltrated by tumour.
a. VS inner core (I) shows haemorrhagic necrosis. The speckled appearance is due to both scattered red blood cells and pyknotic tumour cell nuclei, both of which stain heavily with toluidine blue. The dead tumour cells are round or oval, while the cells of the outer viable zone (O) are spindle-shaped.

b. Higher magnification from the same section as a. showing the inner zone (I) with cell fragmentation (arrowheads) and extravasation of red blood cells (arrows).

c. Incompletely-vascularised spheroid after FAA treatment. The outer viable zone (O) extends the length of the photomicrograph. The upper part of the field is not vascularised and has not been obviously affected by the treatment. This necrotic zone (N₁) lacks blood vessels and haemorrhage, and resembles that of an AVS (Figure 5.1a). The lower vascularised portion of the spheroid contains a necrotic zone (N₂) in which cell breakdown is less advanced. Vessels are packed with red blood cells (arrows).

d. A higher magnification of the right-hand vessel indicated in c., containing red blood cells, which are also seen (arrows) in the interstitium among the necrotic tumour cells.

Light photomicrographs. a, b: toluidine blue-stained plastic sections. c, d: H&E-stained paraffin sections. Bars = 20 \( \mu \text{m} \).
FIGURE 5.4 - EFFECTS OF FAA ON INFILTRATING TUMOUR DEPOSITS

a. A deep nodule of tumour (D) shows necrotic changes, while the superficial nodule (S) shows numerous vessels distended by thrombi (arrows).

b. Higher magnification of a. showing fibrillar material (arrows) in the blood vessels, but an absence of necrosis in associated tumour cells.

Light photomicrographs, H&E-stained paraffin sections. Bars = 20 μm.
5. R. 5. Effects of FAA on fluorescent marker distribution

An initial injection of FAA plus H33342 was followed 4 hrs later by an injection of NAO. The distribution of H33342 in treated VS (Figure 5.5a,c) was similar to that in untreated VS (Figure 5.2a,c), but NAO staining was totally absent in 13/18 VS (Figure 5.5b,d), and only an occasional NAO-positive vessel was seen in the other five VS. The surface layer showed fluorescence with both dyes (Figure 5.5a,b), as seen with non-FAA-treated spheroids. Normal tissues excised with VS showed both H33342 and NAO staining. However, some loss of NAO staining was seen in normal tissues close to the attachment sites of VS (Figure 5.5d).
FIGURE 5.5 - PERFUSION LOSS IN VS AFTER FAA TREATMENT

a. VS consisting of an avascular zone (A) with surface fluorescence, and a vascularised zone indicated by intense H33342 fluorescence around vessels (arrows).

b. Same field as a. showing NAO fluorescence on the surface of the avascular zone (A), but no fluorescence in the underlying vascular zone.

c. VS consisting of a vascular zone (V) showing H33342 fluorescence, and an avascular zone (A), lying on the peritoneal surface and underlying skeletal muscle (M).

d. Same field as c. showing NAO fluorescence is absent from the vascularised core (V), and from much of the adjacent skeletal muscle (M), indicating failure of perfusion to these regions.

Mice were given FAA and H33342 i.v. at the start of the experiment. NAO was given after 4 hours, 5 minutes before sacrifice. Fluorescent photomicrographs of air-dried frozen sections. Bar = 50 μm.
5. D. Discussion

Vascularised spheroids contain both avascular and vascular tumour tissue, and both types of tissue responded to FAA, though necrosis was only seen in the vascularised core (summarised in Figure 5.6, overleaf). The first four sections below discuss the implications of these findings. Sections 5. D. 5. and 6. discuss the angiogenic mechanisms in the VS system, and the possible applications of VS to other fields in experimental anti-cancer therapy. Section 5. D. 7. considers the benefits of NAO as a new fluorescent perfusion marker for double label studies.

5. D. 1. Effects of FAA on VS

The presence of both vascular and avascular tissue in the same tumours allowed the role of the vasculature in the anti-tumour action of FAA to be examined. The vascularised core of the tumours invariably showed a severe loss of perfusion at 4 h, and haemorrhagic necrosis 18 h after FAA treatment. The original outer viable cell layer of the spheroid, which consistently failed to become vascularised, showed no qualitative histological change after FAA. This is the first direct evidence that it is the vascularised core (the vascularised cores) in which perfusion fails, that later become necrotic. There was no minimum size required for these effects. This is consistent with the finding that vascularised Walker 256 carcinoma peritoneal tumour deposits in rats regress after treatment with the angiogenesis inhibitor protamine, while tumour cells growing as thin avascular layers in the same animals persist (Heuser et al., 1984). These findings illustrate both the potential and the limitations of an approach to tumour treatment based on attacking the tumour vasculature. The dependence of the FAA effect on the presence of a vasculature may explain the results of O’Dwyer et al. (1987). In that study, 2/3 human tumours growing i.p. in mice were resistant to FAA treatment, but the mode of tumour growth of the i.p. tumour tissues was not reported (see also Section 1. 6., p. 42).

The distribution of necrosis in FAA-treated VS confirms that the vasculature plays a major role in the anti-tumour action of this drug. However, the presence of blood vessels alone is not sufficient, since the highly vascular sub-mesothelial infiltrative tumour deposits did not undergo necrosis in their superficial parts despite widespread thrombosis of the associated vessels. It is possible that thrombosis occurred later in these regions than in VS allowing insufficient time for the morphological changes of cell death to develop. A more likely explanation is that although FAA caused thrombosis of the vessels, the superficial tumour cells were close enough to the mesothelial surface for effective
FIGURE 5.6 - VASCULARISATION AND FAA TREATMENT OF SPHEROIDS


d. Vascularised spheroid. e. FAA-treated vascularised spheroid.
metabolite exchange by diffusion alone. This implies that vascular failure, rather than the release of a cytotoxic factor from vessels, best explains the vascular dependence of tumour cell killing. Furthermore, the high vascular densities seen in normal sub-mesothelial fat, the diffuse mode of tumour cell infiltration into that tissue, and the presence of vessels very close to the mesothelial surface, all suggest that the thrombosed vessels were pre-existing host vessels incorporated into the tumour during its infiltrative growth. An implication would be that FAA tumour selectivity (demonstrated by a lack of thrombosis or necrosis in non-tumour tissues) resides in the association of blood vessels with tumour cells and/or their accompanying immune effector cells, rather than in some peculiarity of the new tumour vessels themselves.

5. D. 2. Modes of cell death in VS and AVS

Tumour cell death can occur by various mechanisms in this spheroid system and these can be differentiated histologically. Central necrosis occurs spontaneously in tumour cell spheroids of all types beyond a certain size, and is the result of the exhaustion of oxygen and other nutrients diffusing in from the surface, and the accumulation of waste products in the centre (Sutherland, 1988). This implies that as the spheroid grows, dead cells are continuously being added to the necrotic zone, and would explain the advanced degree of cellular degradation seen in the central regions of untreated AVS. This corresponds morphologically to Type 1 spontaneous necrosis in Colon 38 tumours (Section 2. R. 1., p. 51), and presumably reflects the same mechanism.

A second type of cell death was seen in the centres of FAA-treated VS. This appears to result primarily from ischaemia due to vascular obstruction occurring within 4 hrs of treatment, and results in a more homogeneous, less advanced degree of cell breakdown (at 18 h) than that described above, and is similar to that seen in FAA-treated Colon 38 tumours after 24 h (Section 2. R. 2., p. 52).

A third type of cell death, FAA-related but independent of vascularisation, manifests as the approximately 50% narrowing of the viable rim thickness in FAA-treated AVS. This corresponds with a similar decline in clonogenic cell yield described after FAA treatment of AVS in Chapter 3 (Figure 3.7, p. 85). This effect was also seen in vitro as a decline, of similar magnitude, in both clonogenic cell yield and viable cell volume fraction in ex vivo spheroids (Chapter 4: Figures 4.3 & 5, pp. 103 & 109). The mode of non-ischaemic cell death after FAA appears to be apoptosis (Chapters 2 & 4). This process can be difficult to detect by light microscopy (Kerr et al., 1984), which could explain the absence of any qualitative morphological effects in FAA-treated AVS. In contrast, marked morphological changes were seen in ex vivo spheroids treated in vitro (Section 4. R. 4., p. 104),
possibly because the exposure to FAA was greater in those experiments. The role of macrophages in mediating these effects has been discussed in Sections 4. D. 3. and 4. (pp. 125 - 128).

The findings of the present study show that immune-mediated apoptotic cell death occurs not only in regions which are destined to undergo ischaemic necrosis, but also in avascular tumour tissue, and so may contribute independently to the overall anti-tumour effect.

5. D. 3. The role of thrombosis in perfusion failure

In the present study, thrombi were seen in vessels within tumours, and these could have played a part in the blood flow failure observed. The presence of apparently viable tumour cells between thrombosed vessels in the superficial infiltrative tumour deposits, suggest that thrombosis is not simply a consequence of necrosis. Thrombosis could explain the extension of perfusion failure into surrounding normal tissues seen in the fluorescent marker studies. FAA has been shown to cause a coagulopathy in mice soon after administration (Murray et al., 1989), but thrombosis of tumour vessels has not been reported in the literature, and was not seen at increased frequency in FAA-treated Colon 38 tumours (Section 2. R. 2., p. 52). Few thrombi were seen in the necrotic VS cores, where the necrotising effect was greatest. Thus the role of thrombosis in causing perfusion failure remains unclear.

5. D. 4. The role of immune mechanisms in perfusion failure

The decreased thickness of the viable rims of AVS after FAA treatment indicates that vessel-independent cell killing does occur (Section 5. D. 2.), and this type of cell loss appears to be mediated by macrophages (Sections 4. D. 3. & 4., pp. 125-128). The immune effector cells may also mediate the perfusion effects. FAA has immunostimulatory activity (Ching & Baguley, 1987, 1988, 1989a) and macrophage products share with FAA the capacity to cause tumour necrosis (Baguley et al., 1989). There is evidence from the literature that this activity is mediated, at least in part, by vascular mechanisms. Algire et al. (1947) first showed that the anti-tumour action of endotoxin involved vascular damage and decreased blood flow. Tumour necrosis factor-α (TNF), now thought to be the major (Carswell et al., 1975), but not exclusive (North & Havell, 1988; Johnson et al., 1991), mediator of the endotoxin anti-tumour effect, has been shown to cause tumour vessel haemorrhage and thrombosis (Watanabe et al., 1988). However, attempts to inhibit the anti-tumour effect of TNF by blocking coagulation pathways have produced variable results (Watanabe et al., 1988; Shimomura et al., 1988).
Other monokines may also be involved. Tumour necrosis preceded by vessel leakiness has been observed after treatment with interleukin-1 in RIF-1 and Panc02 tumours (Braunschweiger et al., 1988), and in RIF-1 and MGT tumours (Johnson et al., 1991). Interferon α/β injected directly into or around Friend erythroleukaemia cell tumours also caused tumour necrosis, accompanied by damage to endothelial cells (Dvorak & Gresser, 1989). The early production of mRNAs for interferons and TNF in vivo in response to FAA treatment (Mace et al., 1989), argues for the participation of these monokines in the FAA mechanism. Recently, Mahadevan et al. (1990) have inhibited the blood flow effects of FAA with anti-TNF antiserum.

In agreement with the studies of Chapter 2 (Section 2. R. 4., p. 59), there was no morphological evidence of selective endothelial cell damage by FAA in VS, even at the ultrastructural level (results not shown). This leaves the possibility that mechanical factors such as changes in vascular and interstitial pressure could cause perfusion failure (discussed in Section 8. R. 2., pp. 181-183).

5. D. 5. Tumour neovascularisation in VS

The vascularised spheroid system provides new insights into tumour angiogenesis. This process has been extensively studied previously by observing the growth of new vessels into implanted tumour fragments (reviewed in Sections 1. 3. 1. & 2., pp. 25 - 29). Those studies have identified an early avascular phase of tumour growth, which is followed by vascularisation and accelerated growth (Folkman, 1985). However, the persistence of a solid component of the tumour which remains avascular and dependent entirely on diffusion from outside the tumour, co-existing with vascularised tumour tissue, has not been noted previously. The ability to identify an avascular zone in the VS system by conventional histology and with i.v. markers probably relates to the symmetrical, polypoid shape of the tumours, with the vascular supply passing in through only a small region of the spheroid surface. This mode of growth may be due to resistance of the peritoneal cavity to spheroid attachment. The frequent implantation of spheroids at the needle track site supports this suggestion. In contrast, one would expect that an angiogenic response from multiple directions into a tumour implant would obliterate any avascular zone by invading though it.

The failure of vascularisation of the outer zone is not a manifestation of incomplete vascularisation, because 1) the process of vascularisation occurs rapidly after attachment, as shown by the relative rarity of attached non-vascularised, and incompletely vascularised, spheroids (stages b and c in Figure 5.6); and 2) the avascular zone is rather uniform in thickness.

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The major stimulus for neovascularisation in tumours is thought to be the production of tumour angiogenic factors (reviewed by Presta & Rifkin, 1988). There appears to be no evidence in the literature suggesting that tumour angiogenesis is influenced by oxygen tension, or other environmentally determined factors. However, vascularisation in non-tumour tissues does occur as an adaptive response to hypoxia, as in chick chorio-allantoic membranes incubated at low oxygen concentrations (Dusseau & Hutchins, 1988). In addition, the release of a macrophage angiogenic factor only at low oxygen tension (Knighton et al., 1983) implies a possible role for hypoxia in wound- and tumour-related angiogenesis. In the present experiments, the restriction of neovascularisation to the central zone of the spheroids suggests that hypoxia or related environmental factors may be necessary for this process in tumours as well. Macrophages, present in both AVS and VS, may have been involved. An alternative explanation for the avascular zone is a loss of angiogenic factors from this zone by diffusion into the peritoneal fluid.

5. D. 6. The vascularised spheroid tumour system

The ex vivo peritoneal spheroids used in Chapter 4 represent an intermediate stage between spheroids grown entirely in vitro and tumours (Lord et al., 1979; Section 4. D. 7., p. 130), since they contain an immune cell infiltrate. The vascularised peritoneal spheroid system described in this chapter represents a further intermediate stage, in which a vascular component is added. The experiments were completed before necrosis reappeared due to the inadequacy of the vascular supply that develops with increasing tumour size (Vaupel et al., 1973). This novel tumour system has potential application not only in the study of the basic biology of tumour angiogenesis, and in the evaluation of anti-tumour agents thought to act by inhibition of tumour blood flow, but also in studying the effect of drug diffusion in the treatment of intra-cavity malignancies.

The persistence of an avascular zone in VS could have consequences in studies of drug diffusion, if a multicellular avascular layer is present in other intra-cavity solid tumour systems. Los et al. (1989) found different concentration gradients of cisplatinum in solid peritoneal CC531 colonic adenocarcinomas after i.v. and i.p. treatments. The presence of an avascular layer on the peritoneal surface of the tumour deposits was not examined in that study, and may have contributed to the higher concentrations found at the tumour surfaces after i.p. treatment. Such effects could have implications in the development of i.p. therapies in humans. In the present experiments, and those reported in Section 3. R. 9. (p. 81), no differences were seen in the effects of i.p. and i.v. treatments with FAA, probably because FAA appears to diffuse easily though tumour tissue (Bibby et al., 1989b).
5. D. 7. Fluorescent markers

The double fluorescent marker study used was based on that of Trotter et al. (1989a), and similar to that described in Chapter 3, but introduced a new second vascular marker with improved characteristics. H33342 is a useful first vascular marker because of its low toxicity, its limited diffusion in tumour tissue and the stability of its staining pattern with time (Trotter et al., 1989a). However the previously used second marker, DiOC₇(3), showed acute toxicity at doses required for adequate tissue fluorescence, and showed different diffusion properties to those of H33342 (Section 3. R. 2., p. 74).

After testing several alternative fluorescent compounds, NAO was found to function as a tumour vascular marker, and was superior to DiOC₇(3) in several respects. NAO diffuses out of the vessels \textit{in vivo} as does H33342, avoiding the apparent perfusion mismatch occurring with DiOC₇(3), which only diffuses out of the vessels after the sections are cut and mounted and therefore requires that the vessel be transected (Section 3. R. 2., p. 75). The choice of NAO allowed the use of dry sections, which are more stable than wet-mounted sections, and provided advantages for photography or quantification by morphometry. NAO also has superior aqueous solubility, and does not show acute toxicity at doses required to mark tumour vessels with high fluorescent intensity. Toxicity at later times, and rapid diffusion though tumour tissue \textit{in vivo} relative to H33342, did however limit its use to that of a second label administered shortly before sacrifice. NAO was also more susceptible to photobleaching than DiOC₇(3).

NAO is a highly fluorescent lipophilic acridine quaternary salt which is concentrated in mitochondria, even after osmotic shock or uncoupling of respiration (Ratinaud et al., 1988). A direct effect of FAA on mitochondrial metabolism or physical integrity can therefore be excluded as a reason for failure of tumour cells in affected regions to take up this dye, and lack of fluorescence can thus be confidently attributed to a lack of perfusion at the time of the second injection.

The fluorescent marker studies confirmed the distribution of blood vessels in the VS seen in histological sections, with the presence in all VS of a uniform outer avascular zone surrounding vascularised tumour tissue. This confirmation was necessary, since the presence of small or collapsed vessels in the outer zone could not be confidently excluded on histological sections alone. Observations in FAA-treated VS showed a marked loss of tumour perfusion by 4 h, similar to that seen in Chapter 3, even though the dose of FAA was reduced by 1/3. In that study, EMT6 tumours not treated with FAA showed a small loss in tumour perfusion over 4 h (Section 3. R. 5., p. 79), but in the VS experiments reported here, no vessels either opened or closed during this period, possibly because of the smaller size of these tumours.
5. D. 8. Conclusions

The vascularised spheroid is a novel tumour system, which provides evidence that low exchange environmental conditions are a requirement for tumour angiogenesis. This tumour system has permitted the role of the tumour vasculature to be examined from a new perspective. The major findings were:

1) FAA causes necrosis in the same regions in which perfusion failure is seen at an earlier stage, providing good evidence for a causal link between these two phenomena. 2) Avascular tumour tissue shrinks in volume, probably due to apoptosis, and thus makes an independent contribution to the anti-tumour action of FAA. Further preliminary findings in infiltrative tumour deposits were: 1) FAA may cause thrombosis in blood vessels associated with tumour cells. 2) Obstruction of vessels is not sufficient to kill associated tumour cells if the cells are not fully dependent on the affected vessels. These findings confirm that certain populations of tumour cells, which are independent of the tumour vasculature, are relatively resistant to FAA.
CHAPTER 6

THE ANTI-TUMOUR ACTIVITY OF XANTHENONE ACETIC ACIDS:

COMPARISON WITH FAA

6.1. Introduction

The lack of clinical activity of FAA (Kerr et al., 1988), and its low potency compared to other chemotherapeutic agents, has prompted the search for more potent analogues likely to show clinical activity. Most modifications of the structure of FAA have resulted in loss of activity, and those analogues that have retained activity have not shown any obvious advantages over FAA (Atassi et al., 1985; Atwell et al., 1989). An exception is xanthenone acetic acid (XAA), which can be regarded as a "fused" flavone compound (Figure 6.1; Rewcastle et al., 1989).

Some substituted derivatives of XAA cause widespread haemorrhagic necrosis like FAA, while others show no such activity. Study of the structure-activity relationships for mono-substituted XAAs has shown that both the type and position of the substituent are important. Methyl-, methoxy-, and chloro-substituents are generally more active, suggesting that lipophilicity rather than electronic properties are important for activity. Substitution at the 5 and 6 positions often enhances potency, while in other positions, potency is diminished or activity lost. 5-methyl XAA (5MX) is the most potent of the mono-substituted XAA derivatives in causing haemorrhagic necrosis (Rewcastle et al., 1989). Study of di-substituted derivatives has revealed a high potency for several compounds that include a 5-methyl substituent, with one of the highest potencies found in 5,6-dimethyl XAA (DMX) (Rewcastle et al., 1990b). These studies of XAAs assessed anti-tumour activity in vivo by measuring haemorrhagic necrosis and tumour growth delay, but did not examine the mechanisms.

The present study was therefore undertaken to determine 1) whether the compounds which cause haemorrhagic necrosis also cause a reduction in tumour blood flow, 2) whether active XAAs cause FAA-like morphological changes, 3) whether the mechanisms of action of the active XAAs are the same as those for FAA, and 4) the basis of the higher potency of 5MX and DMX.

During the course of the present experiments, attempts were made to improve the double label fluorescent marker technique for the assessment of tumour perfusion. The technique and its modifications are reviewed and evaluated in the Appendix (p. 194).
FIGURE 6.1 - STRUCTURES OF FAA AND XAA

FLAVONE-8-ACETIC ACID

XANTHENONE-4-ACETIC ACID
6. M. Methods

6. M. 1. Drugs

For the in vivo experiments, XAA, substituted XAAs, and hydralazine HCl, were dissolved in 5% (wt/vol) D-glucose for i.v. injection. Vincristine sulphate was obtained as a 1 mg/ml solution, and injected undiluted. For the spheroid experiments, FAA and XAAs were dissolved in distilled water at 100 mM, filter sterilised, and stored at -80°C. These were added to the incubation tubes containing growth medium and ex vivo spheroids, as described in Section 4. M. 7. (p. 97). Experiments were conducted under normal laboratory lighting conditions, as described for FAA (Section 2. M. 2., p. 49).

6. M. 2. Maximum tolerated dose (MTD) and tumour necrotising activity

The MTD for i.v. administration in tumour-bearing BDF₁ mice (weight 17 - 25g), and the necrotising activity of: XAA; the mono-substituted XAA analogues 5MX, 8-methyl XAA (8MX), 3-O-methyl XAA (3OMX); and the di-substituted XAA analogue, DMX, were determined as follows. The dose was escalated in 1.5-fold steps and the mice monitored for up to 50 days; deaths usually occurred by 24 h. In the case of FAA, XAA and DMX, the dose at which the first death occurred was then reduced by 10%, and confirmed to be non-lethal by further testing in at least three mice. For the other compounds, the MTD was taken to be the highest non-lethal dose tested.

Necrotising activity was assessed on histological sections of Colon 38 tumours taken 24 h after treatment. Slides were overlaid with an acetate sheet marked with a 0.4 mm interval grid, and the number of grid intersection points overlying necrotic and viable tissue counted (Baguley et al., 1989).

The progression of DMX-induced histological changes was determined by examination of tumours 1, 2, 4, 8 and 24 h after treatment (four tumours at each time), as described for FAA in Section 2. M. 3. (p. 49).

6. M. 3. Perfusion studies

Changes in tumour perfusion were assessed in BDF₁ mice (weight 17 - 25g) bearing Colon 38 tumours (0.35 - 0.80g), using a double label fluorescent marker technique, similar to that used previously
The only difference was that the dye Hoechst 33342 (H33342) was not mixed with the drug to be tested before injection because, with some XAAs, precipitation occurred. Instead, the two substances were given as separate injections, each at half the volume and twice the concentration used in Chapter 5. H33342 was administered first, followed within 2 min by the test drug. Thirty or 240 min later, 10-nonyl acridine orange (NAO) was injected i.v. and the mice were killed 5 min later. The tumours were then excised and rapidly frozen. Sections of the tumours cut at 10 μm intervals were examined under a fluorescent microscope. Three tumours were examined for each agent after each of the two intervals. Twenty five non-overlapping fields were scored on one frozen section from each tumour.

A score of 0 - 4 was assigned for each of the two dyes for each field, according to the fraction of the area (to the nearest quarter) which was fluorescent (eg. a score of 1 was given for a fluorescent fraction of 1/8 - 3/8).

FAA and the XAAs were administered at the MTD. Vincristine was used at a dose of 5.4 μmol/Kg (5 mg/Kg), which is non-lethal at 24 h, but results in fatal myelosuppression after a few days. Hydralazine was given at a dose of 50 μmol/Kg (10 mg/Kg). Slides were coded so that all determinations were performed without knowledge of the drug used or the time interval after injection of the test drug.

6. M. 4. Spheroid experiments

The details of the methods used in the spheroid experiments are given in Sections 3. M. 6. (p. 74). Briefly, EMT6 spheroids were grown in vitro (in vitro spheroids), and some were recovered from mice after growth for six days in the peritoneal cavity (ex vivo spheroids). Spheroids of both types were exposed to FAA and XAAs at a concentrations of 2.5 mM (and also 0.25 mM for 5MX and DMX) for 24 h in tubes on a roller wheel, then processed for histological examination, and scored. The volume fraction of viable cells in the outer rim and the Activity Index (the sum of scores from 0 - 3 for five histological features) were assessed for each spheroid, as described in Sections 4. M. 9. (p. 97) and 4. R. 5. (p. 105). The XAAs tested included those assessed for necrotising activity (listed in Section 6. M. 2.), as well as XAA with a second acetic acid group added at the 5 position: xanthone-4,5-diaceitic acid (XDA). Statistical significance was determined by the Student t test for cell volume fraction, and by the Mann-Whitney test for the Activity Index, as in Section 4. M. 10. (p. 98).
6. R. Results

6. R. 1. Necrotising activity in Colon 38 tumours

Table 6.1 shows the MTD for each XAA analogue, and the mean percentage of tumour area which was necrotic after 24 h. According to the criteria of Baguley et al. (1989), FAA, XAA, SMX and DMX were active (> 50% necrosis), while 8MX and 3OMX were inactive. The distribution and appearances of the necrotic tumour tissue was qualitatively similar after treatment with all active compounds.

At 1/12 of the FAA dose, treatment with DMX caused 100% necrosis in all tumours, and the development of necrosis followed a similar time course to that seen for FAA in Section 2. R. 2. (p. 52). After 2 h small areas of cell rounding, fragmentation and vascular congestion were seen, and this change had become widespread after 4 h (Figure 6.2), and was accompanied by congestion and rupture of blood vessels. After 24 h confluent necrosis was present.

The activities of vincristine and hydralazine were also assessed, each at the doses used in the blood flow studies (Section 6. M. 3.). Like FAA, vincristine caused haemorrhagic necrosis, but hydralazine did not produce any histological effects.

6. R. 2. Perfusion studies

The results of the perfusion studies are summarised in Figure 6.3. Control tumours, from mice injected with fluorescent compounds only, showed similar staining patterns for both dyes, although 6/15 tumours contained areas of up to 5% that stained with the first dye only. This effect was not dependent on time, since the means ± s.e.m. after 30 min and 4 h were similar (94 ± 7; 93 ± 4). The non-necrotising analogues 30MX and 8MX had no apparent effect on perfusion. FAA and vincristine caused significant (P < .001) reductions in perfusion after 4 h. XAA reduced perfusion in two of three tumours, but the results were not significantly different from controls. Significant (P < .01) falls in perfusion were seen after 30 min in the tumours treated with 5MX and DMX, and the extent of perfusion decreased further by 4 h. Hydralazine caused a 30% (P < .01) fall in perfusion at 30 min, but by 4 h perfusion had returned to control levels. The pattern of perfusion loss was the same for all agents, that with increasing effect there was an increase in the size, rather than the number, of non-perfused areas, as described in Section 3. R. 1. (p. 74). The dose response relationship for DMX (Figure 6.4) indicates a threshold with no effect on blood flow below half the MTD.
### TABLE 6.1 - DRUG-INDUCED NECROSIS IN COLON 38 TUMOURS

<table>
<thead>
<tr>
<th>DRUG</th>
<th>DOSE* (mmol/Kg)</th>
<th>% NECROSIS**</th>
</tr>
</thead>
<tbody>
<tr>
<td>UNTREATED</td>
<td></td>
<td>10 ± 4</td>
</tr>
<tr>
<td>FLAVONE ACETIC ACID (FAA)</td>
<td>1.2</td>
<td>94 ± 3</td>
</tr>
<tr>
<td>XANTHENONE ACETIC ACID (XAA)</td>
<td>0.80</td>
<td>91 ± 4</td>
</tr>
<tr>
<td>5-METHYL XAA (5MX)</td>
<td>0.15</td>
<td>96 ± 4</td>
</tr>
<tr>
<td>5,6-DIMETHYL XAA (DMX)</td>
<td>0.10</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>8-METHYL XAA (8MX)</td>
<td>0.50</td>
<td>20 ± 13</td>
</tr>
<tr>
<td>3-O-METHYL XAA (3OMX)</td>
<td>0.45</td>
<td>23 ± 13</td>
</tr>
<tr>
<td>XANTHENONE-4,5-DIACETIC ACID (XDA)**</td>
<td>1.9</td>
<td>40 ± 10</td>
</tr>
<tr>
<td>VINCRISTINE</td>
<td>0.0054</td>
<td>96 ± 3</td>
</tr>
<tr>
<td>HYDRALAZINE</td>
<td>0.05</td>
<td>9 ± 1</td>
</tr>
</tbody>
</table>

* The dose used was the maximum tolerated dose (MTD) for FAA and the XAAs after i.v. administration.

** Mean ± s.e.m., number of tumours = 3, 4 (18 for untreated tumours).

*** This result is included for comparison with the spheroid results on this compound, although XDA was not tested in the present experiments. Values (for i.p. administration) have been provided by Prof. BC Baguley (unpublished).
Morphological changes 4 h after treatment with 0.1 mmol/Kg DMX. The glandular pattern of the Colon 38 tumour is still discernible in most areas, but there are foci of cell rounding and dissociation (large arrows), and widespread fragmentation of tumour cells (small arrows). Congested blood vessels (arrowheads) are also present. Compare with FAA-treated tumour, Figure 2.2c-h (p. 57).

Light photomicrograph, H&E-stained paraffin section. Bar = 50 μm.
Perfusion changes at 30 min and 4 h after i.v. injection of various drugs at the doses shown in Table 6.1 (points are offset for clarity). The scores are the ratios of NAO-positive (representing final perfusion) to H33342-positive areas (representing pre-treatment perfusion), and each value is the mean of three determinations. The vertical bars show the s.e.m. The dotted lines represent the means ± s.e.m. for untreated tumours; n = 10 (30 min), n = 5 (4 h). Perfusion at 4 h was significantly different from controls at 4 h for FAA, 5MX, DMX and vincristine (P < .001) and at 30 min for 5MX, DMX and hydralazine (P < .01).
FIGURE 6.4 - EFFECT OF DMX DOSE ON TUMOUR PERFUSION

Loss of perfusion in Colon 38 tumours 4 h after an i.v. injection of DMX at doses of 0.045, 0.065 and 0.10 mmol/Kg. Values are means ± s.e.m. for three tumours. The zero dose value was derived from five mice which received injections of only the two fluorescent dyes, 4 h apart.
6. R. 3. Effects of XAAs on ex vivo EMT6 spheroids

The viable cell volume fractions, and the composite scores of morphological features (Activity Index) in EMT6 spheroids, are shown in Figure 6.5, and the histological appearances are illustrated in Figure 6.6. Exposure of spheroids for 24 h to FAA, XAA, 5MX and DMX, all active in the necrosis assay (Section 6. R. 1.), and all causing a fall in blood flow (Section 6. R. 2.), resulted in decreases in viable cell volume fraction and increases in the Activity Index. For both measures, these effects were significantly (P < .05) more marked for ex vivo spheroids. The cell volume fractions were lowest for 5MX and DMX.

8MX, which showed no necrotising or blood flow effects (Sections 6. R. 1. & 2.), caused a fall in cell volume fraction and a small rise in the Activity Index, but these changes were not significantly greater in ex vivo than in in vitro spheroids. XDA and 3-OMX, also both inactive (Sections 6. R. 1. & 2.), caused no effects in either in vitro or ex vivo spheroids, which were indistinguishable from untreated spheroids in histological appearance, cell volume fraction and Activity Index.

DMX at the concentration of 0.25 mM caused morphological changes comparable (Figure 6.6) to those following FAA and XAA at 10 times that concentration. The quantitative scores (cell volume fraction 21 ± 3; Activity Index 13.3 ± 0.9) were also similar to those of FAA and XAA at the higher concentration. 5MX at 0.25 mM showed activity in some spheroids (Figure 6.6), but the quantitative scores (53 ± 8 & 3.3 ± 0.3) were not significantly different from controls (61 ± 5 & 0.4 ± 0.3).
FIGURE 6.5 - SCORING OF MORPHOLOGICAL CHANGES IN EMT6 SPHEROIDS

Histograms showing the cell volume fraction (A) and Activity Index (B) after the incubation of *in vitro* spheroids (open bars) and *ex vivo* spheroids (hatched bars) with drugs at a concentration of 2.5 mM for 24 h. The numbers in the bars represent the numbers of determinations, and apply also to the corresponding bars in B. Error bars represent s.e.m. *Ex vivo* spheroids showed significantly (P ≤ .05) greater effects (*) than *in vitro* spheroids for both measures after exposure to FAA, XAA, S5MX and DMX.
FIGURE 6.6 - HISTOLOGICAL EFFECTS OF XAA s ON SPHEROIDS

a - d. *Ex vivo* spheroids lack histological changes 24 h after exposure to no drug (a), or 2.5 mM 30MX (b), XDA (c) or 8MX (d). The outer viable cell zone (above), contains spindle cells, poorly demarcated from the inner necrotic zone (below). The latter consists of a solid coagulum of cell debris including nuclear fragments, rather more sparse in d.

e - h. *Ex vivo* spheroids show histological changes 24 h after exposure to 2.5 mM FAA (e), 2.5 mM XAA (f), 0.25 mM 5MX (g), or 0.25 mM DMX (h). The outer viable cell zone (above) shows a decrease in cell density, and cells show rounding, with loss of long cell processes. Nuclear fragments are seen in the viable cell zone. The necrotic and viable zones are well-demarcated due to partial dissolution of the inner necrotic zone (below).

Light photomicrographs, H&E-stained paraffin sections. Bar = 50 μm.
6. D. Discussion

6. D. 1. FAA-like activities of XAA analogues

This study demonstrates a relationship between tumour necrotising activity, inhibition of tumour blood flow, and cell killing in immune cell-infiltrated spheroids. The parent XAA, 5MX and DMX showed all these activities, and the nature of the morphological changes produced were the same as with FAA.

The occurrence of apoptosis, necrosis and vascular changes in DMX-treated tumours suggests that, as for FAA, both ischaemic and non-ischaemic mechanisms are involved in the anti-tumour action of active XAAs. The fluorescent marker studies confirm a role for perfusion failure (ischaemia) in the anti-tumour action of the active XAAs; the immune infiltrated spheroid studies suggest that immune mediated effects are also important. These observations provide strong evidence that the mechanisms of action of FAA and the active XAA analogues are the same.

No FAA-like activities were seen with 3OMX and XDA. 8MX did show some cytotoxicity, but this was not significantly enhanced by infiltrating immune cells, indicating that this toxicity is direct.

The induction by FAA and its active XAA analogues of cytotoxic effects in spheroids, decreases in tumour perfusion, and induction of tumour necrosis, suggests that these events are causally connected, and are probably mediated by the same receptor, possibly situated on the macrophage and/or the endothelial cell.

6. D. 2. Potencies of the XAAs

Preliminary information has been acquired from the present studies about the relative potencies of the XAAs, although full dose-response studies have not yet been performed. The MTD for i.v. administration for each drug has been determined, and the necrotising activity was measured at this dose. While XAA showed only a slightly greater potency than FAA, 5MX and DMX showed tumour necrotising activity at least as great as FAA at doses 8-fold and 12-fold lower, respectively. Administration of doses of active XAAs below the MTD results in a decrease in, or a loss of, anti-tumour activity (BC Baguley, personal communication). This is similar to FAA (Figure 3.4, p. 82), in which there was a steep dose response to maximal activity, followed by toxicity after a small additional dose increment. Thus, the therapeutic window appears to be no wider for the XAAs than for FAA.
The greater potency of 5MX and DMX was also evident in the perfusion experiments. Declines in perfusion were at least as marked with doses 8- and 12-fold lower respectively than used for FAA. The dose response curve for DMX showed a high threshold followed by a sharp transition to maximal effect, as seen for the anti-tumour effect of FAA (Figure 3.4, p. 82).

The greater potency of 5MX and DMX \textit{in vivo} could reflect a difference in the pharmacokinetics of FAA and the XAA analogues, or alternatively, a more powerful agonist action at the receptor level. The \textit{in vitro} evaluation of these compounds using spheroids supports the latter explanation, as morphological effects were seen in spheroids at 1/10 of the FAA concentration. The pharmacokinetic study of FAA, XAA and DMX by McKeage \textit{et al.} (1991) further supports this view. They found that the time-concentration integrals ("areas under the curve") for DMX were less than 1/4 of those for FAA (at the MTDs), and that the plasma concentrations of unbound drug were up to 23 times lower. Thus the higher potency of DMX \textit{in vivo} cannot be explained by higher exposure of tumour cells to the drug.

Higher potencies for 5MX and DMX have also been demonstrated \textit{in vitro} by others. Ching \textit{et al.} (1991a) have shown enhancement of macrophage-mediated cytotoxicity by low concentrations of 5MX, and macrophage nitric oxide production was elicited by low concentrations of 5MX and DMX (Thomsen \textit{et al.}, 1990).

6. D. 3. Effects of hydralazine and vincristine on tumour perfusion

The perfusion effects of hydralazine and vincristine were evaluated in parallel with FAA and the XAAs. Hydralazine is known to reversibly decrease tumour perfusion by decreasing systemic arterial pressure (Jirtle, 1988; Chaplin, 1989). It is conceivable that a fall in arterial pressure might cause a uniform decrease in individual tumour vessels, which would not be perceptible as a perfusion loss using the double fluorescent label method, which can only detect focal perfusion failure (Section 3. D. 1., p. 86). It was therefore of interest that focal areas of complete perfusion loss were found 30 min after treatment with hydralazine, indistinguishable in appearance from those seen after FAA treatment, although with hydralazine such perfusion defects dissappeared after 4 h. Similar results have been reported by Trotter \textit{et al.} (1989b). One implication of this finding is that systemic hypotension could play a part in the perfusion failure effect which follows FAA and its analogues (discussed further in Section 8. 1. 2c., p. 182).

The interest in examining the possible perfusion effects of vincristine arose from the discovery that this agent also causes FAA-like rapid haemorrhagic necrosis (Baguley \textit{et al.}, 1991). Another agent
previously shown to decrease blood flow is misonidazole (Murray & Randhawa, 1988), a compound which may also cause necrosis (Brown et al., 1978). The finding of a marked loss of perfusion following vincristine treatment raises the possibility that blood flow effects may contribute to the anti-tumour action of other anti-tumour agents. The occurrence of both haemorrhagic necrosis and perfusion failure following administration of vincristine, which is different to FAA in its other activities, strengthens the causal connection between these two phenomena, first shown in Chapter 5 (Section 5. D. 1., p. 143). The relationship between these FAA-like activities and the well-known anti-mitotic effects of this agent, is unknown.

6. D. 4. The double label perfusion technique

The double label technique used in this chapter for assessing tumour perfusion differed from that described in previous chapters in method of quantitation. There were also some differences in the results. These differences are discussed in the Appendix (p. 194).

6. D. 5. Conclusions

FAA and its analogues XAA, 5MX and DMX have similar ischaemic and non-ischaemic mechanisms of action, and appear to act on the same receptor. The higher potency of 5MX and DMX in vivo is probably due to a more powerful stimulus at this receptor.

Systemic hypotension could play a role in the focal perfusion defect in tumours seen early after FAA treatment.

Haemorrhagic necrosis in tumours is preceded by an irreversible decline in perfusion after treatment with vincristine, suggesting that a similar sequence may occur for other causes of rapid necrosis in tumours.
CHAPTER 7

TOXIC EFFECTS OF FAA AND 5,6-DIMETHYL XANTHENONE ACETIC ACID:
CELL KILLING IN NON-TUMOUR TISSUES

7.1 Introduction

The utility of any anti-cancer agent is dependent on the differential between its effect on the tumour and on normal tissues. The previous chapters have addressed the mechanism of the anti-tumour action of FAA and its xanthenone acetic acid (XAA) analogues. The present study examines their effects on non-tumour tissues. This could provide information relevant to the management of patients in clinical trials, and may also help our understanding of the basis of the tumour selectivity of these agents.

In a clinical situation, anti-proliferative drugs kill rapidly dividing cells and cause myelosuppression and alopecia, but these are not seen after FAA treatment. Instead, the dose-limiting effect of FAA in clinical trials has been hypotension (Kerr et al., 1987, 1989; Weiss et al., 1988), with blood pressures as low as 75/55 mmHg recorded (Weiss et al., 1988). Other adverse effects have included headaches, sensations of warmth, abdominal cramps, nausea, visual blurring, metallic taste sensation and small rises or falls in body temperature. One case of autoimmune thrombocytopenia (Kerr et al., 1989), and one case of persistent postural hypotension due to autonomic neuropathy (Lewis et al., 1989), have been reported. One fatality has occurred, 1 h after FAA infusion, but post mortem examination failed to identify the mechanism of death (Weiss et al., 1988).

In experimental animals, the toxicity of FAA varies with species. In dogs, hypersalivation, emesis, diarrhoea and abnormal movements have been seen, but no gross or microscopic changes were observed to explain these toxic effects (O'Dwyer et al., 1987). In mice, lethargy, decreased physical activity, rough hair coat, hunched posture, discharge from the eyes, emaciation and convulsions have been reported (O'Dwyer et al., 1987). Deaths usually occurred within 48 h, and were preceded by a drop in body temperature (Hill et al., 1989).

FAA also affects the blood and circulatory system. Murray et al. (1989) described a coagulopathy resembling disseminated intravascular coagulation in mice early after FAA treatment. Smith et al. (1991) measured a fall in circulating platelet numbers in mice, and increased permeability of, and neutrophil adhesion to, the endothelial cells of the hepatic sinusoids. An interesting observation has been the increased toxicity of FAA in mice bearing tumours (Murray et al., 1989; Hill et al., 1989,
1991; Smith et al., 1991). Peak plasma levels of more than 2 mM, or plasma levels of more than 0.4 mM maintained by i.v. infusions for 24 h, are lethal in mice (Zaharko et al., 1988).

The mechanisms involved in the toxicity of FAA are poorly understood, and the sites at which it exerts these effects have not yet been determined. In Chapter 2, a selection of non-tumour tissues were examined histologically after FAA treatment (Section 2. R. 2., p. 52). The choice of organs examined in that study was based on the distribution of the toxic effects of tumour necrosis factor-α (TNF) (Tracey et al., 1986). In Chapter 3 a few non-tumour tissues were assessed for focal changes in perfusion (Section 3. R. 4., p. 75). However, no effects were observed in non-tumour tissues in either of these studies. In the present investigation the toxicity of FAA and its potent analogue 5,6-dimethyl XAA (DMX) were studied by the histological examination of tumours and a wide variety of non-tumour tissues from mice treated with therapeutic doses of these agents. In addition, double label fluorescent marker studies were performed to examine the vascular patterns of untreated non-tumour tissues, and to monitor any changes in perfusion after treatment with FAA.

7. M. Methods

7. M. 1. Histological studies

Male and female hybrid (C52BL/6JxDBA/2J)F₁ (BDF₁) mice with or without subcutaneous Colon 38 tumours (0.4 - 1.2g) were treated with i.v. FAA or DMX dissolved in 5% wt/vol sodium bicarbonate in a volume of 10 μl/g mouse weight. Male and female Balb/C mice bearing i.p. EMT6 spheroids implanted seven days previously were treated with i.v. or i.p. FAA, as described in Section 5. M. 1. (p. 133). The number of mice in each experiment is shown in Table 7.1. Tumour and non-tumour tissues, including small and large intestine, pancreas, testis, ovary, uterus, lung, adrenal, kidney, bone marrow, spleen, thymus and lymph nodes, were excised 1 - 24 h after drug treatment. The specimens were processed, sectioned at 5 μm, stained with H&E, and examined by light microscopy as described in Section 2. M. 3. (p. 49).
7. M. 2. Fluorescent vascular marker studies

The methods for examination of changes in non-tumour tissues were the same as those used previously for vascularised spheroids (Section 5. M. 2., p. 133). Briefly, three spheroid-bearing Balb/C mice each received two i.v. injections (10 μl/g mouse weight), the first containing 3.25 mM Hoechst 33342 (H33342) plus 0.8 mM FAA, and the second containing 2 mM 10-nonyl acridine orange (NAO) given 4 h later. The mice were killed after a further 5 min and frozen sections of the three spleens, six gut lymphoid nodules and two lymph nodes were examined by fluorescence microscopy. Three control mice received only H33342 in the first injection.

In other experiments to examine and compare the distribution of the two dyes in non-tumour tissues, two EMT6 spheroid-bearing mice were each given a single injection containing both dyes, and the tumours, and non-tumour tissue samples were removed after 5 min, rapidly frozen, and sections examined by fluorescence microscopy.

7. R. Results

7. R. 1. Histological effects of FAA and DMX

Confluent areas of haemorrhagic necrosis were seen in all tumours from mice treated with FAA, as described in Sections 2. R. 2. (p. 52) and 5. R. 3. (p. 138). No necrosis, thrombosis or haemorrhage was observed in the non-lymphoid tissues of the small and large intestine, liver, pancreas, testis, ovary, fat, skeletal muscle, lung, adrenal, kidney or bone marrow. However, focal cell lysis or necrosis was seen in lymphoid tissues and in the uteri of drug-treated mice which were examined 8 and 24 h after treatment (and less commonly at earlier times). The frequency with which these tissues were affected is shown in Table 7.1.

Gut lymphoid nodules (Figure 7.1b-f) were affected most frequently. FAA caused confluent necrosis of the B-cell lymphoid follicles but not the T-cell para-follicular zones (Figure 7.1b,c). This was accompanied by thrombosis of blood vessels in all affected gut lymphoid nodules (Figure 7.1c), and often by haemorrhage, so that they appeared red macroscopically. Treatment with 0.065 or 0.10 mmol/Kg DMX also caused histological changes in gut lymphoid nodules. In one gut lymphoid nodule, confluent haemorrhagic necrosis with vessel thrombosis was seen (Figure 7.1d). In the ten
other gut lymphoid nodules in which effects were seen, lymphocyte death was more focal, and also involved the para-follicular zone (Figure 7.1e,f). These foci showed nuclear fragmentation without associated haemorrhage or vessel thrombosis, appearances of apoptosis.

Inguinal and axillary lymph nodes (Figure 7.1g,h) from mice treated with FAA or DMX showed changes in the B-cell follicles similar to those seen in gut lymphoid nodules. In addition, the lymph node sinuses (structures not found in other lymphoid tissues) were depleted of lymphocytes.

Follicular necrosis was less frequent in the spleen (Figure 7.1i,j) than in gut lymphoid nodules or lymph nodes following FAA treatment. DMX treatment produced scattered clusters of fragmenting lymphocytes, suggesting apoptosis, in the white pulp (Figure 7.1j). The red pulp was congested in spleens from all drug-treated animals.

After FAA treatment, the thymus glands (Figure 7.1k,l) of 3/4 tumour-bearing mice showed confluent nuclear fragmentation, affecting the entire cortex. The loss of thymocytes from this normally highly cellular zone blurred the demarcation between cortex and medulla (Figure 7.1k). Some of the pyknotic or fragmented nuclei were arranged in round clusters (Figure 7.1l) suggesting that they had been phagocytosed by macrophages. No loss of cells was evident in the medulla. Neither haemorrhage, vascular congestion nor thrombosis was seen, and the residual non-thymocyte elements including vessels, reticulin fibres and epithelial cells were intact. The appearances thus indicated selective confluent apoptosis of cortical thymocytes. In the four thymus glands examined, these changes affected the entire cortex in three, but the whole organ had a normal histological appearance in the other.

The uterine changes after FAA or DMX varied from focal superficial endometrial necrosis (Figure 7.1m) to extensive necrosis extending into the myometrium (Figure 7.1n). Endometrial neutrophils, some undergoing fragmentation, were numerous in the superficial endometrium of both treated and untreated uteri. Occasional thrombi were seen.

Although haemorrhage was sometimes seen in association with necrosis in the above tissues, there was no direct evidence of selective damage to endothelial cells in tissues which showed cytolytic effects (findings similar to those reported in FAA-treated tumours, Section 2. R. 4., p. 59).

7. R. 2. Fluorescent marking of non-tumour tissues

Both H33342 and NAO marked the endothelial cells and the parenchymal cells within about five cell diameters of the vessels. In peripheral lymphoid tissues (Figure 7.2a,b), the thymus (Figure 7.2c,d), and the superficial layer of the myometrium (Figure 7.2e,f) the intercapillary distances were noted to
be wide, with a high proportion of unstained parenchymal cells at a distance from the vessels. This uneven pattern of staining was similar to that seen in tumours (Chapters 3, 5 & 6; Trotter et al., 1989a). However in other tissues including non-lymphoid gut tissue (Figure 7.2a,b), pancreas, liver, kidney, adrenal, fat and skeletal muscle, inter-vessel distances were smaller and these tissues were uniformly stained.

7. R. 3. Effects of FAA treatment on perfusion

Gut lymphoid nodules removed 4 h after FAA treatment frequently showed small haemorrhages macroscopically. However, all vessels were labelled with both dyes, and no definite foci of perfusion failure were found. Nor did the liver, non-lymphoid intestinal tissue, mesenteric fat or skeletal muscle, seen incidentally in the tissue sections, show effects on perfusion.
### TABLE 7.1 DISTRIBUTION OF CYTOLYTIC LESIONS

<table>
<thead>
<tr>
<th>MICE</th>
<th>TUMOUR</th>
<th>DRUG</th>
<th>DOSE (mmol/Kg)</th>
<th>TIME (hrs)</th>
<th>N*</th>
<th>GUT LYMPHOID NODE</th>
<th>LYMPH SLEEN</th>
<th>THYMUS</th>
<th>UTERUS</th>
<th>TUMOUR</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6lb/C</td>
<td>EMT6</td>
<td>FAA</td>
<td>0.8</td>
<td>18</td>
<td>7</td>
<td>4/6**</td>
<td>0/2</td>
<td>1/5</td>
<td></td>
<td>7/7</td>
</tr>
<tr>
<td>BDF₁</td>
<td>Co38</td>
<td>FAA</td>
<td>1.2</td>
<td>8-24</td>
<td>19</td>
<td>14/14</td>
<td>6/10</td>
<td>8/17</td>
<td>3/4</td>
<td>7/7</td>
</tr>
<tr>
<td>BDF₁</td>
<td>none</td>
<td>FAA</td>
<td>1.2</td>
<td>24</td>
<td>4</td>
<td>4/4</td>
<td>3/3</td>
<td>1/4</td>
<td>0/4</td>
<td>2/3</td>
</tr>
<tr>
<td>BDF₁</td>
<td>Co38</td>
<td>DMX</td>
<td>0.065-0.10</td>
<td>8-24</td>
<td>12</td>
<td>8/12</td>
<td>4/6</td>
<td>6/8</td>
<td>0/4</td>
<td>1/4</td>
</tr>
<tr>
<td>both strains, tumours; untreated</td>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td>0/7</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/10</td>
</tr>
</tbody>
</table>

* Number of mice. ** Fraction of mice in which the tissue showed drug-induced cell death.

Between 1 and 6 (mode = 2) gut lymphoid nodules, and up to 3 (mode = 1) lymph nodes were examined from each mouse.
a. Gut lymphoid nodule, untreated. Two small B-cell follicles (arrows) are surrounded by the T-cell para-follicular zone. The mucosa, including glands opening into the intestinal lumen (L), is seen below.

b, c. Gut lymphoid nodules, FAA-treated. b. The B-cell follicle (B) and part of the para-follicular zone is necrotic but some para-follicular cells (T) are not. This nodule shows ulceration of the mucosa (arrows). c. The para-follicular zone (T) is preserved while the B-cell follicle (B) shows karyolysis, karyorrhexis and vessel thrombosis (arrows). (Tumour-bearing Balb/C, 0.8mmol/Kg FAA, 18h).

d - f. Gut lymphoid nodules, DMX-treated. d. This B-cell follicle (B) is necrotic, and a thrombosed vessel (arrow) is present. e. This nodule shows focal fragmentation of lymphocytes (arrows) in both the follicle and the para-follicular zone. f. Detail (from e) of cell fragmentation in the para-follicular zone. (Tumour-bearing BDF1, 0.1mmol/Kg DMX, 8h [d] & 24h [e,f]).

Light photomicrographs, H&E-stained paraffin sections. Bars = 20 μm.
FIGURE 7.1 - HISTOLOGY OF NON-TUMOUR TISSUES (CONT.)

LYMPH NODES AND SPLEEN

**g, h. Lymph nodes.** g. Untreated lymph node consisting of two B-cell follicles (B) surrounded by the T-cell para-follicular zone, which merges with the lymph node sinuses (S). h. DMX-treated lymph node showing cell lysis in a follicle (arrows), and depletion of lymphocytes in the sinuses (S). (Tumour-bearing BDF, 0.1mmol/Kg DMX, 8h).

**i, j. Spleen.** i. Untreated spleen, with white pulp consisting of T-cell zone (T), and a B-cell follicle (B) showing a few prominent tingible body macrophages (arrows) containing nuclear fragments. Red pulp (R) surrounds the lymphoid tissue of the white pulp. j. DMX-treated spleen showing coalescing clusters of fragmenting lymphocytes (arrows) in the white pulp. (Tumour-bearing BDF, 0.1mmol/Kg, 8h).

Light photomicrographs, H&E-stained paraffin sections. Bars = 20 μm.
FIGURE 7.1 - HISTOLOGY OF NON-TUMOUR TISSUES (CONT.)

UTERUS AND THYMUS

k, l. Thymus. k. Untreated thymus consisting of highly cellular cortex (C), and medulla (M).
l. FAA-treated thymus showing depletion of lymphocytes in the cortex (C), which contains nuclear
fragments, either scattered (small arrows), or clumped (large arrows). No damaged cells are seen in
the medulla (M). (Tumour-bearing BDF1, 1.2 mmol/Kg FAA, 24 h).

m, n. Uterus. m. DMX-treated uterus showing superficial endometrial necrosis (arrows). (Tumour-
bearing BDF1, 0.1 mmol/Kg DMX, 8 h). n. FAA-treated uterus in which necrosis (arrows) extends
from the endometrium (E) into the myometrium (M). (Non-tumour-bearing BDF1, 1.2 mmol/Kg, 24 h).

Light photomicrographs, H&E-stained paraffin sections. Bars = 20 μm.
FIGURE 7.2 - DISTRIBUTION OF FLUORESCENT MARKERS
IN UNTREATED NON-TUMOUR TISSUES

a, b. Gut lymphoid nodule. H33342 (a) and NAO (b) staining is limited to para-vascular cells indicating a low vascular density in the T-cell (T) and the B-cell zone (B) of the lymphoid nodule. In the mucosal glands (G) all cells fluoresce.

c, d. Thymus gland. H33342 (c) and NAO (d) fluorescence is limited to para-vascular cells, in both the medulla (M) and cortex (C).

e, f. Uterus. H33342 (e) and NAO (f) both stain most cells in the endometrium (E), indicating a high vascular density in that region. Fluorescence is limited to para-vascular cells in the superficial myometrium (M).

Fluorescent photomicrographs of air-dried frozen sections. Both dyes were injected together i.v. 5 min before removing the tumour. Bar = 20 µm.
7. D. Discussion

7. D. 1. Cytolytic and vascular effects in non-tumour tissues

The mode of cell killing in non-tumour tissues following FAA or DMX treatment was similar to that in tumours, with evidence that both apoptosis and necrosis were involved. The mode of cell killing was determined partly by tissue type: pure apoptosis was seen in the thymus, and necrosis was seen in the uterus. In peripheral lymphoid tissues, the mode of cell death was influenced by the agent: DMX usually caused apoptosis, and haemorrhagic necrosis was seen after FAA. It is possible that necrosis supervenes when the toxic stimulus is sufficient to cause vascular damage, adding an ischaemic component to cell killing. This is consistent with finding thrombi in vessels in or around necrotic lymphoid follicles, but not in association with focal lymphocyte lysis or in the thymus. Thrombosis is likely to be a consequence, rather than a cause of the cytolytic changes observed, because focal defects in perfusion were not detected in lymphoid tissues examined 4 h after FAA treatment.

7. D. 2. The lymphotoxic effects of FAA and DMX

The cells killed included both B-cells (lymphoid follicles) and T-cells (thymic cortex). These are unusual changes, but do occur in other circumstances in humans. Depletion of thymic lymphocytes is seen in fetuses or neonates stressed by hypoxia, though the cell loss is more focal (Wigglesworth, 1984), and lysis of lymphoid follicles has been described in the lymph nodes of patients dying of diphtheria (Leech et al., 1990). It is not understood why an all-or-none effect was seen in the response of lymphoid tissues to FAA, although this does suggest that after a threshold level of toxic stimulus, a positive feedback effect occurs, as has been suggested for tumours (Section 3. D. 7., p. 91).

The results of the present study have recently been confirmed by Ching et al. (1991b), who have shown a decline in the number of lymphocytes in the peripheral blood, the thymus and the spleen after FAA, DMX, and XAA. They observed the same histological changes in the thymus and spleen with all three drugs. In their study the lymphotoxic effect appeared to be indirect, since it was more marked in vivo. In contrast, myelotoxic effects were not seen; indeed myeloid precursors showed increased proliferative activity. Eliot et al. (1990) have found a direct cytostatic effect, due to inhibition of ornithine decarboxylase, in human peripheral and intestinal lymphocytes exposed to FAA in vitro.
7. D. 3. The basis of the tissue selectivity of FAA and DMX

The occurrence of cytolysis in non-tumour tissues provides an additional perspective on the basis of tissue selectivity of FAA and its XAA analogues. Although lymphoid tissues share with tumours a high rate of cell proliferation, this does not appear to be the determinant of tissue selectivity (as with conventional cytotoxic agents, Section 1. 1. 2., p. 20). The rapidly-dividing small bowel crypt cells, which are sensitive to conventional cytotoxic agents (Searle et al., 1975), were not affected by FAA or DMX treatment. The results of Ching et al. (1991b), who demonstrated stimulation of growth of myeloid cells, also argue against proliferation rate as the determinant of sensitivity.

Tumours and lymphoid tissues both contain large numbers of immune effector cells including macrophages and NK-cells (Svennevig & Svaar, 1979; Vose & Moore, 1985; Ching & Baguley, 1989b), and FAA increases the cytolytic activity of such cells (Ching & Baguley, 1988, 1989a). In the present study, patterns of necrosis matched the normal distribution of macrophages in lymphoid tissues. Macrophages are sparse in the thymic medulla, which was not sensitive to FAA in mice, but are numerous in the cortex (Ruco et al., 1989). In peripheral lymphoid tissues, phagocytic (tingible body) macrophages are most numerous in the B-cell germinal centres (Figure 7.1i; Baroni et al., 1987). Neutrophils, present in large numbers in the superficial endometrium, could explain the sensitivity of the uterus to necrosis if these cells respond to FAA. In this regard it is of interest that neutrophils have been implicated in uterine necrosis following toxic doses of TNF by Shalaby et al. (1989). These authors also mentioned focal cell lysis in lymphoid follicles and the thymus. This suggests that TNF mediates some of the cytolytic effects of FAA in normal tissues, as suggested previously for tumours (Section 2. D. 2., p. 66).

Alternatively, the critical common factor could be the low vascular densities observed in all the affected tissues. This could cause relatively poor exchange between tissue and circulation, resulting in hypoxia and a low extracellular pH (Vaupel et al., 1981). The latter could lead to increased intracellular concentrations of FAA as it is a weak acid (Denny & Wilson, 1986). Hypoxia stimulates the production of a macrophage angiogenic factor (Knighton et al., 1983), which is presumably TNF (Leibovich et al., 1987). FAA also stimulates the production of TNF (Mace et al., 1990), and could thus synergise with the hypoxic stimulus to release this monokine in cytotoxic quantities. The activity of any TNF produced could be enhanced by the presence of tumour-derived tissue factors, which enhance the procoagulant activity of this cytokine (Clauss et al., 1990). Finally, the large intercapillary distances could retard the clearance of cytotoxic substances produced in the tissues in response to FAA. Low tissue blood flows could also limit the delivery of TNF inhibitors, which have been found in high concentrations in human plasma (reviewed by Loetscher et al., 1991). Although the spheroid
experiments of Chapter 4 provided no evidence that hypoxia or other microenvironmental factors influence the response of tumour cells to FAA in vitro, other elements such as endothelial cells present in vivo, in both tumours and non-tumour tissues, might respond with greater sensitivity to FAA under low exchange conditions (see also Section 4. D. 5., p. 128).

7. D. 4. Relationship of cytolytic lesions to systemic toxicity

The tissue necrosis observed is unlikely to explain directly the lethal effects in mice, since damage to lymphoid organs should not cause immediate compromise of vital functions. However, if such effects occur in humans, immune function could be affected. Endometrial necrosis may have contributed to the vaginal bleeding seen in one patient who became thrombocytopenic after FAA treatment (Kerr et al., 1989).

7. D. 5. Comparison of FAA and DMX

The distribution and appearances of the cytolytic or necrotic changes were similar after treatment with both FAA and DMX. This supports the conclusions of the previous chapter that the mechanisms of action of FAA and its active XAA analogues are the same. The results of the comparison are consistent with those of Ching et al. (1991b) that these drugs have different potencies, but have the same qualitative effects on lymphoid tissues. One difference noted in the present study was that DMX usually caused focal cell lysis in peripheral lymphoid tissues rather than the haemorrhagic necrosis which was frequently seen after FAA treatment. Because a wide range of doses was not tested for the two agents, these morphological differences could simply indicate that non-equivalent doses for the two drugs were used, rather than an intrinsic difference in toxicity or mechanism. There is no indication from the present studies that the morphological differences observed point to an improved therapeutic ratio for DMX.
7. D. 6. Conclusions

FAA and DMX have cytolytic effects not only in tumours, but also in lymphoid tissues and the uterus, though other tissues are not affected. Both apoptotic and necrotic modes of cell death occur. The cytolytic effects appear to be independent of perfusion failure in non-tumour tissues. The distribution of the cytolytic lesions suggests various possible determinants of tissue selectivity, including concentrations of immune effector cells and low vascular density.
This chapter considers the findings of the various sections of the thesis, together with research of others on FAA reported since the thesis commenced. It then discusses the implications of these findings for further research, and for the testing and use of FAA and related agents in the clinic.

8.1. The mechanisms of the anti-tumour action of FAA

The major aims of the thesis (Section 1.6.3., p. 47) were to determine the mechanism of the anti-tumour action of FAA and its XAA analogues, to elucidate the basis for their selective toxicity, and to define the toxic effects of these agents in normal tissues.

The research embodied in this thesis shows that the anti-tumour effect of FAA involves two distinct mechanisms: haemorrhagic necrosis, due to the failure of the tumour blood flow, and immune cell mediated apoptosis, which contribute separately to tumour cell killing. The next three sections discuss these mechanisms and the sections following consider other hypotheses proposed for the anti-tumour effect of FAA.

8.1.1. The role of perfusion failure in the the anti-tumour effect

The confluent distribution of necrosis in Colon 38 tumours after treatment with FAA, and the engorgement and rupture of tumour vessels (Section 2. R. 2., p. 52) provided strong but indirect evidence that ischaemia is an important factor. Using the double label perfusion technique developed in Chapter 3, focal defects in tumour vascular perfusion were demonstrated as early as 15 min after treatment, earlier than any other change. Peters et al. (1991) have recently reported similar results. The case for a causal relationship between perfusion failure and the anti-tumour effects was supported by the in vivo spheroid studies (Section 3. R. 9., p. 81), which showed that tumour tissue not dependent on blood vessels is resistant to FAA. The rate of cell death in globally ischaemic tumours undergoing autolysis at 37°C, was sufficiently rapid to account for FAA-induced cell killing in the first 4 h after treatment.

The importance of perfusion failure, and its relationship to haemorrhagic necrosis, were confirmed in vascularised spheroids in Chapter 5. This novel tumour system not only avoided the
complications of spontaneous necrosis, but also provided both vascularised and avascular components which could be directly compared. FAA-induced necrosis was seen only in the vascularised zone, and was preceded by loss of perfusion. This shows that the same regions which suffer perfusion failure later become necrotic, confirming the causal relationship between the two processes.

The examination of infiltrative EMT6 tumour deposits in Chapter 5 (Section 5. R. 4., p. 138) revealed interesting findings regarding the mechanism of the perfusion effect and cell killing. The deep infiltrative tumour deposits, apparently supplied by incorporated host vessels, became necrotic after FAA treatment. Thus the anti-tumour specificity of the perfusion and cytotoxic effects appears to reside in the association of the native vessels with tumour tissue, rather than some special characteristic of new tumour vessels.

8.1.2. The mechanism of perfusion failure

The studies of the thesis did not succeed in identifying the cause of perfusion failure. Tumour necrosis factor-α (TNF) appears to be a major mediator of the perfusion effect (Mahadevan et al., 1990), but the role of interferons, also produced in response to FAA (Mace et al., 1990) is not known. This section considers the possible roles of the coagulation system, intra-tumour haemorrhage and systemic arterial hypotension. The relationship between the immune effects and perfusion failure is discussed in Section 8.1.3c.

8.1.2a. The coagulation system

Thrombi were rarely seen in FAA-treated Colon 38 tumours (Section 2. R. 2., p. 52) or in vascularised EMT6 spheroids (Section 5. R. 3., p. 138), and there have been no reports in the literature of FAA-induced thrombosis. In contrast, TNF has been shown to cause thrombosis (discussed in Section 1. 5. 6., p. 39). Murray et al. (1989) did detect a coagulopathy following FAA administration by examination of the blood constituents, but no thrombi were seen in several FAA-treated tumours studied at the CRC Gray Laboratory (MC Murray, personal communication). The author reviewed the histology of CaNT, CaRD, CaGF, CaRH, CaHAL and SaS tumours treated with FAA at the Gray Laboratory, and thrombi were identified only in CaHAL tumours, although all six showed FAA-induced necrosis. If activation of the coagulation system is involved in the FAA mechanism, the paucity of histologically obvious thrombi must be explained.
Activation of the coagulation system may act together with other mechanisms to obstruct blood flow, particularly via effects on endothelial cells. FAA increases endothelial cell procoagulant activity (Murray et al., 1991), and increases binding of neutrophils to endothelial cells (Smith et al., 1991), which may lead to endothelial damage. FAA also increases the permeability of endothelial cell layers to large molecules (Smith et al., 1991). The latter effect could raise the interstitial pressure and lead to haemoconcentration in tumour capillaries, which would predispose to thrombosis. A possible explanation for the paucity of thrombi seen after FAA in the studies of the thesis and those of others, is that thrombosis occurs only in very small foci, and that most of the obstruction to blood flow is caused by non-thrombotic coagulation which propagates widely from the thrombotic foci, due to stagnation and hypercoagulability of the blood within the tumour. Blood coagulating under these conditions would be expected to have a similar morphological appearance to liquid blood that had coagulated after immersion in fixative for morphological study, and thus would not be obvious as a cause of vascular obstruction.

8. 1. 2b. Haemorrhage

While haemorrhage may be secondary to necrosis, and a consequence of vessel weakening due to massive tissue dissolution, haemorrhage has been identified in areas of tumour cell dissociation as early as 30 min after FAA treatment (Section 2. R. 2., p. 52). Even a small haemorrhage from a tumour vessel on the arterial side of the tumour vasculature, might transiently raise local tissue interstitial pressure, compressing neighbouring low-pressure vessels, and thus play a causal role in perfusion failure and subsequent necrosis. The local tissue damage and stagnation of blood in the ruptured vessel could also initiate coagulation, which might spread through other stagnant vessels in the region of the disturbance. In this way several factors might contribute to perfusion failure.

8. 1. 2c. Arterial hypotension

Systemic arterial hypotension induced by hydralazine is capable of causing focal defects in tumour perfusion (Section 6. R. 2., p. 155; Trotter et al., 1989b). Hypotension has also been suggested as a contributor to decreased tumour perfusion after FAA due to the systemic vasodilator effects of nitric oxide produced by macrophages (Thomsen et al., 1991). However, the fall in systemic arterial pressure seen after FAA is small, and shows a poor correlation with the decrease in tumour blood flow, compared to hydralazine (Stone et al., 1992). It therefore seems more probable that the major causes of perfusion failure are local.
8. 1. 2d. Approaches to further investigation

In order to more precisely understand the mechanism of perfusion failure, further in vivo studies will be required. Inhibition of the necrotising effect of FAA with heparin has been attempted (BC Baguley, personal communication), but preliminary results are not conclusive. Thurston et al. (1991) have failed to inhibit the anti-tumour activity of FAA with both heparin and ancrod. Platelet inhibitors such as dipyridamole and dextran have not yet been tested. In addition to their pivotal role in thrombosis, platelets contain serotonin, which causes contraction of endothelial cells (Majno et al., 1969), and serotonin receptor antagonists have been shown to prevent tumour growth delay caused by TNF (Manda et al., 1988). Similar studies with FAA are in progress (Baguley et al., 1992).

The various events postulated above to contribute to perfusion failure and haemorrhagic necrosis might occur in anatomically-distinct parts of the tumour vascular tree. While haemorrhage would be more probable close to the arterial end (Section 8. 1. 2b.), vascular engorgement suggests distal obstruction, perhaps in the venules draining the tumour. The site of obstruction could be investigated by making vascular casts at various times after FAA administration. The vascularised spheroid system would serve well in such studies because the small size and predictable shape of the tumours should allow easy identification of the small number of vessels in the tumour stalk.

8. 1. 3. Non-ischaemic anti-tumour mechanisms

The identification of both necrotic and apoptotic modes of cell death in Colon 38 tumours after FAA treatment indicated that both ischaemic and non-ischaemic tumour cell killing occurs (2. D. 3., p. 67), and this was confirmed in the studies of Chapter 3 (Section 3. D. 4., p. 89). The spheroid studies of Chapter 4 showed that the non-ischaemic component includes a small direct toxic effect (4. D. 1., p. 124), and a larger indirect effect (4. D. 3., p. 125). This section discusses the involvement of macrophages in the indirect cytotoxic effect, the relevance of this mechanism to the overall anti-tumour effect of FAA, the possible causal relationships between the immune and the perfusion effects, the role of specific immunity, and the relationship between FAA and TNF.

8. 1. 3a. The role of macrophages

Although the thesis studies did not establish exactly which cell type was responsible for the cytotoxic and morphological effects observed, the dissolution of the central necrotic debris suggested the activity
of hydrolytic enzymes released by macrophages. This is consistent with the findings of Ching and Baguley (1988), that macrophages mediate FAA cytotoxicity. However, such macrophage activation does not correlate completely with haemorrhagic necrosis: FAA does not enhance the cytotoxicity of macrophages from endotoxin-resistant C3H/HeJ mice, but does cause haemorrhagic necrosis in M16C tumours growing in this mutant strain (Ching & Baguley, 1989c). Further experiments with immune infiltrated spheroids are required to test whether macrophages, rather than lymphocytes, are responsible for the effects observed. This might be possible using spheroids infiltrated by the immune cells in vitro, (after Sutherland et al., 1977), with pure preparations of one immune cell type. The nature and role of any chemical mediators has not yet been adequately examined. Preliminary experiments with the protease inhibitor aprotinin (Section 4. D. 3., p. 127) failed to inhibit the morphological changes induced by FAA, but a variety of inhibitors should be studied in this spheroid system. Enzyme, cytokine and other biological activities could be conveniently assayed in spheroid supernatants or homogenates, or in intact or dissociated spheroids.

8. 1. 3b. The relevance of immune mediated cytotoxicity

Whether immune cell toxicity is relevant to the overall anti-tumour effect in vivo is uncertain. It is feasible, for example, that direct immune cell toxicity is limited to regions which are in any case destined for perfusion failure. However, apoptotic cell loss from the necrosis-resistant outer viable zone of spheroids (Section 5. R. 3., p. 138) suggests that non-ischaemic mechanisms have the potential to make an independent contribution to the anti-tumour effect.

These results also provide a new perspective on the question of whether ischaemic or non-ischaemic cell killing is more important: the answer depends partly on the relative amounts of vascularised and avascular tumour tissue, and this may vary greatly. For example, in vascularised spheroids this would depend on the size of the spheroid, with the avascular fraction decreasing as the tumour enlarged. In leukaemias or ascites tumours, the cells are dispersed and thus not vessel-dependent, and only the relatively inefficient immune-mediated cytotoxic component of the FAA effect could operate. The results are thus consistent with early observations (reviewed in Section 1. 6., p. 42) that FAA is most effective against established solid tumours.

8. 1. 3c. The relationship of immune effects and perfusion failure

The structure-activity relationships for both the immune cell stimulatory effects and tumour perfusion failure after treatment with a series of XAA analogues of FAA, is preliminary evidence for a close
The role of specific (T-cell mediated) immunity in the anti-tumour action of TNF has been discussed in Section 1.5.7. (p. 39). As this cytokine plays a major role in the mechanism of action of FAA (Mahadevan et al., 1990, Mace et al., 1990), it is not surprising that similar results have now been found with FAA. Bibby et al. (1991) have shown that tumour necrosis occurs in both thymectomised and intact mice, but tumour growth delay is seen only in the latter. Pratesi et al. (1990) have reported similar findings. This suggests that tumour necrosis is independent of specific immune effects, and is consistent with its occurrence in human tumours growing in nude mice (Section 1.6.; Table 1.1, p. 43). However, tumour growth delays have been seen in some tumours growing in athymic mice (Ching et al., 1992), and so the role of specific immunity is not yet clear.

T-cells do appear to play a role in the response of some tumours to FAA, and IL-2 enhances their activity to improve the tumour cure rate (Hornung et al., 1988a,b). The T-cell component was not investigated in the thesis, since only short term (≤ 24 h) assays of anti-tumour activity (necrotic fraction and clonogenic assay) were performed. The issue of the relevance of specific immunity in
transplanted mouse tumours to those growing in humans, has long been controversial, and has recently been reviewed by Scott (1991).

8. 1. 3e. The relationship of FAA and TNF

The similarity in the effects of FAA and TNF in vivo led to early speculation about the relationship between these two substances (discussed in Section 1. 5. 8., p. 40). Anti-TNF antibodies inhibit both the perfusion effect (Mahadevan et al., 1990) and the anti-tumour effect (Pratesi et al., 1990) of FAA, confirming the important role of TNF in the actions of FAA. However, not all FAA effects are mediated simply by the release of TNF into the circulation. For example, FAA and TNF synergise both in the induction of tissue factor from endothelial cells, and in anti-tumour activity (Murray et al., 1991). Furthermore TNF, but not FAA, synergises with RSU 1069 in killing tumour cells in vivo (Edwards et al., 1991b). Ching and Baguley (1989c) have induced haemorrhagic necrosis with FAA in M16C tumours growing in C57H/HeJ mice, in which macrophages are incapable of producing normal amounts of TNF in response to endotoxin. The authors argue that other cytokines induced by FAA (eg. interferons) help to bypass the deficient pathway in the macrophages to allow cytotoxic activation. Thus FAA appears to stimulate various host cells to produce a number of different products, which act together against tumours.

8. 1. 4. The role of the tumour microenvironment

Hypoxia, or some other microenvironmental factor resulting from low exchange with the circulation, was considered as a possible basis for the tumour selectivity of FAA. Sensitisation to the effects of FAA by a low exchange environment would result in positive feedback as the tumour perfusion decreased (Section 3. D. 7., p. 91), which could help explain the focal nature of the defects in perfusion, and the steep dose response of FAA. The studies of Chapter 4 failed to show that hypoxia per se activated FAA to become a direct cytotoxin, or that it sensitised immune effector cells to FAA. There have been no reports in the literature to suggest a role for microenvironmental factors in the activity of FAA. However, a more complex role for a low exchange environment cannot be excluded. The correlation of sensitivity to FAA with low vascular density in normal tissues (Section 7. D. 3., p. 177) does raise the possibility of a contribution of some microenvironmental factor to tissue selectivity, perhaps in conjunction with macrophage content. The role of nutrients other than oxygen could also be tested in spheroids by varying their concentrations in the growth medium. However, the
influence of accumulation of metabolites would be more difficult, since these could not easily be varied independently of each other, or independently of the tumour cell density.

8. 1. 5. Activation of FAA *in vivo*

The hypothesis of *in vivo* activation of FAA proposed by Chabot *et al.* (1989b) was based on the difference between *in vitro* and *in vivo* activity of the drug. These authors also observed that plasma from FAA-treated mice, and FAA to which liver microsomes had been added, were cytotoxic (Section 1. 6. 2a., p. 45), but the putative toxic metabolites have not been characterised. This hypothesis would not explain the resistance to FAA of tumour cells in peritoneal diffusion chambers found by Finlay *et al.* (1988), the low level of its activity against peritoneal EMT6 spheroids (Figure 3.7, p. 85), or the difference in effect of FAA against the inner vascular and the outer avascular zones of vascularised spheroids (Section 5. R. 3., p. 138). Nevertheless, the findings of Chabot *et al.* (1989b) are interesting and remain unexplained, and characterisation of the cytotoxic substances could be of mechanistic interest.

8. 1. 6. Mechanisms of the xanthenone acetic acid (XAA) analogues

The XAA analogues that showed necrotising activity in Colon 38 tumours, also inhibited tumour perfusion, and caused FAA-like morphological changes *in vitro* in immune cell-infiltrated spheroids, and *in vivo* in Colon 38 tumours (Section 6. D. 1., p. 163). These experiments have provided further evidence that active XAAs have a similar mechanism of action to FAA, and possibly stimulate the same receptors on the same target cells. Although the higher potency of some XAA analogues was confirmed, the question of the relative therapeutic ratios for the XAAs has not yet been properly investigated. It is important to continue to compare FAA and its XAA analogues, and to determine whether any differences identified are likely to indicate clinical activity.

8. 1. 7. Toxic effects of FAA and 5,6-dimethyl XAA (DMX)

Prior to the studies reported in this thesis, the cytolytic effects of FAA appeared to be confined to tumour tissue. Although toxic effects of FAA were recognised, specific tissue lesions had not been
described. Chapter 7 details necrotic and apoptotic changes occurring in non-tumour tissues after therapeutic (non-toxic) doses of FAA and DMX. Although these changes do not explain the lethal effects of these agents, they may provide clues to the basis of tumour selectivity. This information also may be of use in predicting adverse effects of these drugs in future clinical trials.

8.2. Clinical implications of FAA mechanisms

8.2.1. Perfusion failure in anti-cancer treatment

The apparently irreversible inhibition of tumour blood flow by FAA and its analogues is of therapeutic interest, and the development of strategies for treatment of tumours by attacking the vasculature has been an important goal in cancer research (Denekamp, 1982b, 1991). There has also been considerable interest in the modification of tumour blood flow to increase the efficacy of therapeutic agents (reviewed by Jirtle, 1988). For example, the anti-tumour activity of melphalan has been enhanced by combination with misonidazole (Murray et al., 1987) and hydralazine (Stratford et al., 1988; Chaplin 1989), both of which reversibly reduce blood flow. The potentiation of cytotoxic effect is thought to result from melphalan being trapped in the tumour before plasma concentrations fall, although with misonidazole other sensitising interactions are also possible.

FAA differs from most vasoactive agents because inhibition of tumour blood flow is sustained, thus killing tumour cells by ischaemic injury. The obstruction to flow appears to be irreversible in affected vessels because of the extensive disruption of the tumour architecture that occurs between 2 and 24 h after FAA treatment (Section 2. R. 2., p. 52). This differs from the persisting or increasing blood flow observed after single treatments with cyclophosphamide (Braunschweiger 1988) or radiation (Kummermehr & Buschmann, 1987), which indicates the maintenance of vascular structure that can act as a framework for rapid tumour regrowth from residual viable cells (Kummermehr & Buschmann, 1987).

Other agents, which like FAA and the XAAs, cause irreversible inhibition of tumour blood flow as well as haemorrhagic necrosis, include photodynamic therapy and hyperthermia (Section 1. 2. 3., p. 24), and the mitotic inhibitors vincristine, vinblastine, vindicine and colchicine (Baguley et al., 1991). Such treatments should offer a number of potential therapeutic advantages, including non-selective elimination of all tumour sub-populations dependent on the affected vessels, irrespective of whether they are cycling or non-cycling, well-oxygenated or hypoxic. The indirect mechanism of
action should also make unlikely the emergence of treatment-resistant clones and may exploit common features of tumour vasculature to provide activity against a broad range of solid tumours.

8.2.2. Combinations with other treatments

Many solid tumour are likely to have dispersed or diffusion-dependent components (Section 1.3., p. 25), and these would be relatively resistant to FAA treatment (Section 3. D. 3., p. 88). Therefore FAA would be most useful in combination with other anti-cancer agents. The evaluation of such combinations was beyond the scope of the thesis, but because the mechanistic findings of the thesis could influence the choice and timing of such combinations, they will be discussed briefly below.

8.2.2a. Hypoxia selective cytotoxic agents

Sun and Brown (1989) examined the combination of FAA and the hypoxia-activated agent SR 4233 in SCC VII and RIF tumours. The amount of hypoxic tissue in tumours increased as FAA-induced perfusion failure progressed. The combination of FAA and SR 4233 increased tumour levels of the activated metabolite of SR 4233. When both drugs were given within 1 - 2 h of one another, the anti-tumour effects were found to be more than additive compared to the effects of either drug alone. The authors caution that this combination may not result in any therapeutic gain, since SR 4233 also increased the toxicity of FAA.

An interesting mechanistic point is that SR 4233 caused no necrosis when used alone, so that the increased necrosis seen when both drugs were used appears to be an enhancement of the action of FAA by SR 4233 (as indicated in the title of the paper), rather than the activation of SR 4233 by FAA-induced hypoxia (as suggested in the discussion of the paper). Thus, the results of this experiment did not support the initial rationale for this combination: expansion of the hypoxic fraction to enhance the action of a hypoxia selective agent. However, the observation of synergism between these agents is of interest both in terms of the development of future therapies, and because it may reveal information about the mechanisms of action of FAA. The enhancement of the anti-tumour activity (and the toxicity) of FAA is consistent with the observation that diverse anti-cancer treatments enhance the cytotoxicity of macrophages (cyclophosphamide and doxorubicin; Section 4. D. 7., p. 130), or inhibit blood flow (vincristine; Section 6. R. 2., p. 155). It is of interest that Edwards et al. (1991b) also demonstrated synergism of FAA with SR 4233, but not with another hypoxia selective
agent RSU 1069. This supports the view that FAA-induced hypoxia in not the only mechanism of synergism with SR 4233.

8. 2. 2b. Conventional anti-proliferative agents

FAA-like agents could be used, like surgery, to decrease tumour bulk, and so theoretically reduce the number of cycles of anti-proliferative agents required for cure. This could decrease the risks of both the toxic effects of anti-proliferative agents on the bone marrow and other sensitive tissues, and the risks of persistence or emergence of treatment resistant clones of tumour cells. FAA-like agents could also be used like hydralazine, to trap the more diffusible anti-proliferative agents, such as melphalan, in the tumour (Stratford et al., 1988). Because the washout of cytotoxin from the FAA-induced necrotic zone would be very slow, the reservoir of cytotoxin might continue to diffuse to surrounding viable tumour cells which had escaped the action of FAA.

8. 2. 2c. Radiation

Radiation, like anti-proliferative drugs, could theoretically complement the action of FAA, by killing those well-oxygenated cells that are dispersed at the margins of the tumour, and which might thus be resistant to FAA. A study on the combination of FAA infusion and irradiation (De Neve et al., 1990) showed a greater than "log-additive" effect of the two agents when they were given at the same time, but not apart. However, the doses of both agents used in this experiment were low, as were the levels of clonogenic cell kill. Because of the complex effects of FAA, and the potential for low perfusion to reduce oxygenation and thus reduce effectivenes of radiation (Edwards et al., 1991a), this combination still requires further study.

8. 2. 2d. Immunotherapy

FAA has been combined with IL-2 in experimental animals and in a clinical trial. The increase in life extension in mice bearing RENCA renal tumours using this combination has been described (Wiltrout et al., 1988; Section 1.6. 2b., p. 45). Since no separate assessment was made of the early phase of tumour cell killing, it is not possible to say whether IL-2 enhanced the early necrotising effect, or the later specific anti-tumour immune effect, to eradicate residual tumour cells. The latter explanation appears more likely, and is consistent with the observed resistance of mice to re-challenge with the
RENCA tumour (Hornung et al., 1988b). This question is of importance in extrapolating these experimental findings to humans, in whom specific anti-tumour immunity appears to be less important (Section 1. 5. 1., p. 35).

One clinical phase II trial with metastatic melanoma (Thatcher et al., 1990) has also used these two agents. Responses were seen, but were no more frequent than with IL-2 alone.

8. 2. 2d. Other vessel-directed agents

Synergy may result from FAA and other agents involving vascular mechanisms, including TNF (Murray et al., 1991) and hyperthermia (Horsman et al., 1991).

8. 2. 3. Reasons for the failure of FAA in the clinic

Considering the remarkable activity of FAA against tumours in mice, it is disappointing that no anti-tumour activity has been seen in any of the reported clinical trials (Kerr et al., 1987; Weiss et al., 1988; Kerr et al., 1989; Havlin et al., 1991). There are four possible explanations for this apparent lack of activity in humans.

1) FAA is weakly active in humans, but this activity has not been detected because of a low sensitivity of the endpoints used in clinical trials. It is possible that examination of tumour histology, or measurement of tumour blood flow during treatment (eg. by positron emission tomography or nuclear magnetic resonance), would reveal an effect on the tumour. Such studies are needed, because a subclinical level of activity may still be of therapeutic interest in combination with other types of treatment, and could possibly be improved by optimising administration.

2) FAA is potentially active, but it has not yet been administered optimally, or at a sufficient dose. Although FAA is metabolised more quickly in humans than in mice (O'Dwyer et al., 1986), concentrations of FAA measured in human tumours are in the same range as tumours in mice receiving curative doses of the drug (Damia et al., 1990). Humans may then require a greater exposure to FAA than mice. If hypotension is the dose-limiting toxic effect, perhaps higher doses could be tolerated if used in conjunction with pressor agents, as has been demonstrated in sheep treated with IL-2 (Zeilender et al., 1989). New treatment schedules should consider the observations made in experimental systems, including the high plasma threshold level before activity is seen (Figure 3.4A,
p. 82; Zaharko et al., 1986), the possibility of low-dose tolerance (a phenomenon noted with endotoxin and TNF: Lepe-Zuniga & Klostergaard, 1990; Takahashi et al., 1991), and the adverse effects of alkalisation (Futami et al., 1990).

3) FAA is not active against tumours in humans, but FAA analogues may be active. Despite the apparent similarities between FAA and its active XAA analogues, there may be differences in receptor specificity, which could result in activity in humans. This possibility could perhaps be investigated by using human tumour spheroids infiltrated by human immune effector cells in vitro.

4) Neither FAA, nor any FAA analogue will be active in humans, because humans lack some critical biochemical pathway or receptor. Evidence against this hypothesis is that natural killer cell activity and interferon production have been observed in some patients receiving the drug (Urba et al., 1988). However, natural killer activity does not correlate with anti-tumour activity in mice (Ching & Baguley, 1989a), and it is still not known whether humans can respond to the anti-tumour effects of FAA.

8. 3. Concluding remarks

In the last few decades, multi-agent chemotherapy has been widely applied, providing a high cure rate for several uncommon types of disseminated malignant disease. Chemotherapy has also been used effectively as an adjuvant to surgery and radiation for certain solid tumours, and has resulted in life extension and palliation for many types of cancer. With the exception of the glucocorticoids, all of the more commonly used chemotherapeutic agents are thought to act by damaging proliferation-related targets (anti-proliferative agents). This may partly explain their lack of effectiveness against the visceral carcinomas of adulthood. The other major factor limiting cure is the emergence of resistance following repeated exposures of the tumour to the agents.

Prior to the advent of chemotherapy, one of the only forms of treatment available for disseminated malignant disease was the intra-tumoural and intramuscular injection of "Coley’s toxins", which were bacterial endotoxin (lipopolysaccharide) preparations (Editorial, 1934). Coley, among others, achieved as many as 30 cures using this type of therapy (Coley-Nauts et al., 1953), but there is no record of the total number of patients treated, and there is little documentation of the adverse effects of the therapy. However, these cures (seen mainly in sarcomas, but also in some carcinomas) indicate that at least some tumours in humans are capable of responding to endotoxin treatment. Endotoxin has
since been shown to cause necrosis in mouse tumours, accompanied by a failure of the tumour vasculature (Algire et al., 1947). Carswell et al. (1975) showed that TNF is the major mediator of the necrotising effect of endotoxin, and there have been several reports (reviewed in Section 1.5.6., p. 39) that this involves vascular damage. The thesis has shown that FAA involves similar vessel-dependent and immune mechanisms, and again TNF appears to be a major mediator (Mahadevan et al., 1990; Mace et al., 1990).

The continued investigation of the endotoxin type of anti-tumour effect, whether provoked by endotoxin, TNF or FAA-like small molecules, is important not only because of the well-documented cases of success with endotoxin referred to above, but also because of the theoretical possibility that therapies involving vascular and immune mechanisms could synergise well with anti-proliferative agents (Section 1.3., p. 25; Sections 8.2.1. & 2.). To establish whether tumour necrosis could be induced in humans by these agents, and so play a role in the therapy of human tumours, future studies will have to further define the mechanisms of action of the different agents, and determine whether these could operate in human patients.

Regarding the future of research on FAA and related compounds, the failure of FAA in the clinic, for whatever reason (Section 8.2.3.), is likely to decrease enthusiasm (and funding) for this type of research, because of pressure for clinically applicable results. In the author’s view, it would be a loss for the understanding of tumour biology if research on FAA were to wane before some of the questions discussed above are answered.
APPENDIX

The double label fluorescent marker technique: additional experiments and final discussion

This appendix to the thesis compares the perfusion experiments of the various chapters of the thesis, re-evaluates the original material from the double label study of Chapter 3, and proposes future improvements to the technique.

The double label scoring system used in Chapter 6 (Section 6. M. 3., p. 153) differed from that of Chapter 3 (Section 3. M. 4., p. 72), in that whole fields were assessed for fluorescent area, instead of counting grid points. The Chapter 6 technique was much simpler and more rapid than point counting, although a comparison of the two scoring methods (below) showed similar results. In the Chapter 6 study a small fall in perfusion was seen in tumours from mice not receiving vasoactive or anti-tumour agents, to about 95% of control levels (Section 6. R. 2., p. 155). A similar fall in perfusion was also seen in Chapter 3 with EMT6 tumours, but not with Colon 38 tumours (Figure 3.3, p. 78), and not with the vascularised EMT6 spheroids studied in Chapter 5 (Section 5. R. 2., p. 135). The reason for this variation is not known. The different scoring system does not appear to be the explanation, because the three control tumours from the Chapter 3 study, which were scored again by the present technique still yielded scores of close to 100% (Figure A.1, p. 196). None of the studies showed spontaneous opening of previously closed vessels (Sections 3. R. 5., p. 79; 5. R. 2., p. 135; 6. R. 2., p. 155), in contrast to the results of Trotter et al. (1989a, 1991) in SCC VII tumours.

The inhibition of blood flow seen 4 h after FAA treatment in the Chapter 6 study (Figure 6.3, p. 158) agrees with that seen in the experiments of Chapter 3 (Figure 3.3, p. 78). However, the Chapter 6 study did not detect perfusion changes at 30 min. This difference, and the failure of one of the three XAA-treated tumours to show any decrease in perfusion, may have been due to inaccuracies in injection volumes. This could have occurred since the test compounds were injected in half the volumes used in the experiments of Chapters 3 and 5 (Section 6. M. 3., p. 153). Total injection volumes were kept low to prevent excessive changes in blood volume, which might have influenced tumour blood flow. Thus in a 20 g mouse, the test drug was injected in a volume of 0.1 ml, and a loss of 0.01 ml into the subcutaneous tissues, and perhaps out of the injection wound, would reduce the dose by 10%. This could be critical for the activity of such compounds, since the responses of tumours are sensitive to small changes in dose, both for FAA (Figure 3.4, p. 82) and for DMX (Figure 6.4, p. 159).

Although the double label fluorescent technique was useful in detecting small regional changes in extent of tumour perfusion, there are some criticisms of the application of the technique. A fault
in the perfusion experiments of Chapter 3 was that the scoring of fluorescence was not performed "blind", and it is conceivable that observer bias occurred, facilitated by the continuous decrease in fluorescent staining from the para-vascular cells to the hypoxic zone. Another criticism is that different methods of scoring were used in Chapters 3 and in Chapter 6.

For these reasons, new sections were cut from a selection of stored frozen tumours from the Chapter 3 study, and the sections were scored without knowledge of the treatment history, by the scoring method used in Chapter 6. Figure A.1 shows the new scores plotted against the original scores for each tumour. The good agreement between each pair of values indicates 1) that significant bias did not occur in the original experiments reported in Chapter 3, and 2) that the two methods of scoring give similar results.

In order to retain the advantages of double labelling while eliminating the potential disadvantages of morphological methods (unrepresentative sampling, artificial distinction between fluorescent and non-fluorescent areas, observer bias), attempts were made to extract both NAO and H33342 from whole tumours, and to use the fluorescence ratio as a measure of perfusion. While NAO could be quantitatively extracted from tumours, there was interference with H33342 extraction and/or fluorescence. This effect may have been caused by blood, because fluorescence was lower when tumours were haemorrhagic. This resulted in an unacceptably high variance in the fluorescence ratios of control tumours, and the technique was abandoned. If the problem of H33342 extraction could be overcome, and extraction ratios from untreated tumours were found to vary little, then double label studies could be performed without requiring histological sections.
Colon 38 tumours from the double label perfusion study of Chapter 3, selected to cover a range of point-count scores, were re-sectioned and scored by the area assessment method described in Section 6. M. 3. The graph shows the relationship of the point score to the area score for each tumour. The horizontal axis represents the ratio of DiOC(3)- to H33342-positive area, and the vertical axis represents the ratio to DiOC(3)- to H33342-positive points. The tumours used were: untreated, injected with dyes 30 min apart (closed circles), or treated with i.v. FAA, 1.2 mmol/Kg, 15 min (triangles), 30 min (open circles), 60 min (inverted triangles), 120 min (diamonds), or 240 min (squares) before excision.
MATERIALS AND EQUIPMENT

MATERIALS

α Minimal Essential Medium

Antibodies:

Anti-MAC-1

Anti-Ly-1

Anti-Thy-1

Goat-anti-mouse
Ig - FITC

Cacodylate

Cacodylate buffer

Collagenase

DiOC₃(3)

Dimethylsulfoxide

DNase

D-glucose

Gibco-BRL, Auckland, NZ.

MCA-745, culture supernatant monoclonal IgG. Designation M1/70.15.1, Seratec, Kidlington, England.

Culture supernatant monoclonal IgG. Designated 53-7.313, Prof JD Watson, Department of Molecular Medicine, Auckland University School of Medicine.

Ascites preparation, monoclonal IgG, from T24 hybridoma grown in Balb/C mice. Prof JD Watson, Department of Molecular Medicine, Auckland University School of Medicine.

Tago, Inc., Burlingame, CA, USA.

Merck, Schuchardt (Munich), Germany.

Stock solution:
sodium cacodylate 10.7 g
double distilled water to 250 ml
pH 7.4

Working solution:
diluted x 2.

Sigma Chemical Company, St. Louis, Missouri, USA.

Molecular Probes Inc., Eugene, Oregon, USA.

Serva Fine Chemicals, Westbury, New York, NY, USA.

Sigma Chemical Company, St. Louis, Missouri, USA.

Riedel-de Haen AG, Hanover, Germany.
<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier/Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dexamethasone</td>
<td>David Bull Laboratories, Mulgrave, Victoria, Australia.</td>
</tr>
<tr>
<td>EMbed-812</td>
<td>Electron Microscopy Sciences, Fort Washington, PA, USA.</td>
</tr>
<tr>
<td>Eosin Y</td>
<td>Sigma Chemical Company, St. Louis, Missouri, USA.</td>
</tr>
<tr>
<td>FAA</td>
<td>Dr Kenneth Paull, Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD, USA.</td>
</tr>
<tr>
<td>FAA, sodium salt</td>
<td>Prepared by Mr Graeme Atwell, Auckland Cancer Research Laboratory, Auckland, NZ.</td>
</tr>
<tr>
<td>Fetal calf serum</td>
<td>Phoenix Chemicals Ltd., Auckland, New Zealand.</td>
</tr>
<tr>
<td>Freon 12</td>
<td>E. I. Du Pont de Nemours &amp; Co., Inc., Wilmington, Delaware, USA.</td>
</tr>
<tr>
<td>glycerol saline</td>
<td>glycerol (BDH, Poole, England) 10% vol/vol in PBS.</td>
</tr>
<tr>
<td>Hoechst 33342</td>
<td>Serva Fine Chemicals, Westbury, New York, NY, USA.</td>
</tr>
<tr>
<td>methylene blue</td>
<td>Serva Fine Chemicals, Westbury, New York, NY, USA.</td>
</tr>
<tr>
<td>10-nonyl acridine orange bromide (NAO)</td>
<td>Prepared as a 50 mM (stock solution) in DMSO, diluted to 2 mM (solution for injection) in 5% wt/vol D-glucose.</td>
</tr>
<tr>
<td>osmium tetroxide</td>
<td>Probing &amp; Structure, Kirwan, Queensland, Australia. 1% (wt/vol) in cacodylate buffer.</td>
</tr>
<tr>
<td>penecillin</td>
<td>Sigma Chemical Company, St. Louis, Missouri, USA.</td>
</tr>
<tr>
<td>pentobarbital</td>
<td>Auckland Hospital Pharmacy, Auckland Hospital, Auckland, New Zealand.</td>
</tr>
<tr>
<td>Phorbol myristate acetate (PMA)</td>
<td>Serva Fine Chemicals, Westbury, New York, NY, USA.</td>
</tr>
</tbody>
</table>
Phosphate buffered saline (PBS) (Sodium potassium phosphate-buffered, 0.9 N sodium chloride, pH 7.2)

- anhydrous $\text{KH}_2\text{PO}_4$ 0.20 g
- anhydrous $\text{Na}_2\text{HPO}_4$ 1.15 g
- KCl 0.20 g
- NaCl 8.00 g
- milliQ-filtered water 1 L

KH$_2$PO$_4$ Riedel-de Haen AG, Hanover, Germany.

Na$_2$HPO$_4$ Merck, Schuchardt (Munich), Germany.

NaCl Serva Fine Chemicals, Westbury, New York, NY, USA.

KCl BDH, Poole, England.

Pronase Sigma Chemical Company, St. Louis, Missouri, USA.

Propidium iodide Calbiochem Corporation Australia, Ltd, Alexandria, NSW, Australia.

Sodium bicarbonate Labsupply Pierce, Birkenhead, Auckland, NZ.

Streptomycin Sigma Chemical Company, St. Louis, Missouri, USA.

Tween 20 Aldrich Chemical Company, Milwaukee, Wisconsin, USA.

XAA and substituted XAAs Synthesised by Dr GW Rewcastle and Mr GJ Atwell, Auckland Cancer Research Laboratory, Auckland, NZ.
Absorbosphere HS chromatography column
Coulter ZF electronic particle counter
FACS IV 440 cell sorter
Argon laser
Nikon Optiphot microscope
Olympus IM inverted microscope
Philips 410 LS transmission electron microscope
Tissue Tek 2000
Waters chromatography equipment

C18, 7 μm, 250 x 4.6 mm.
Alltech Associates, Deerfield, Ill, USA.
Coulter Electronics, Inc., Hialeah, Florida, USA.
Becton Dickinson, Mountain View, CA, USA.
Coherent Inc., Palo Alto, CA, USA.
Nippon Kogaku K.K., Tokyo, Japan.
Olympus Optical Co. Ltd., Tokyo, Japan.
Philips Nederland, Eindhoven, Netherlands.
Miles Scientific, Naperville, IL, USA.
Millipore Corporation, Bedford, MA, USA.
REFERENCES


