AN INVESTIGATION OF ASPECTS OF NORMAL AND ABNORMAL WOUND RESOLUTION

NJ Brown

Experimental Pathology,

St.Bartholomew's Hospital Medical College,

Charterhouse Square,

London, EC1M 6BQ

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Bismillah irahma niraheem

This thesis is dedicated to my parents for their unwavering support and to my wonderful and loving husband for recognising falsehood when I had been blinded, alhamdulillah

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ABSTRACT

Keloids are classically regarded as scars that 'outgrow the boundary of the original injury'. Ambiguous data concerning certain characteristics of keloid fibroblasts (such as proliferation rates and collagen production), however, have served only to confuse researchers. The lack of an *in vivo* model and of detailed clinical accounts are added problems. In this study, a murine granulomatous tissue resolution model was used to investigate the profile of a number of cytokines suspected to be involved in the aetiology of keloids.

The results obtained from these experiments were then extrapolated to clarify the observations made in keloids. The results of these extrapolated comparisons revealed elevated levels of interleukin (IL)-4, IL-10, vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and transforming growth factor beta (TGF)-β in keloid samples and decreased levels of interferon (IFN)-γ and IL-2.

The murine model was also used to investigate the implications of the low levels of IFN-γ known to be present in the serum of keloid patients and shown here to be present in clinical samples. The results were found to support the hypothesis that the addition of IFN-γ reduces the fibrosis so typical of keloids by rectifying the abnormality of the absence of IFN-γ content. The same model was used to provide evidence that the inhibition of VEGF activity in resolving wounds may halt the development of keloid lesions.

The immunohistochemical profiles of cellular proliferation, apoptosis, lymphokines and cytokines for resolution (and, to a certain extent, the model) were used to compare with the abnormally resolved wounds which were available as clinical samples. Immunohistochemistry was also employed to describe the cellular nature of the clinical tissue samples in detail and to facilitate the development of the following hypothesis for keloid formation and propagation: lymphocytes migrate to the site of an (alleged) endogenous antigen present in the skin. The nature of these lymphocytes is characteristic of a type 2 immune response, they produce IL-4 (and IL-10) which in turn inhibit(s) the production of IFN-γ and IL-2. Aside from this immunological response, wound resolution is taking place: fibroblasts are producing PDGF, EGF and TGF-β to aid matrix remodelling and collagen synthesis. The provisional matrix is being vascularised by the action of VEGF, to allow the replenishment of nutrients; regression of blood vessels occurs through the action of an apoptosis-dependent mechanism, as does the 'normalisation' of fibroblastic populations. The keloid scar continues to grow after the cessation of resolution because the immune response to the 'endogenous' antigen continues and the lymphocytes continue to migrate to the site of the wound and continue to stimulate fibroblast proliferation and collagen production through the release of IL-4.

Chapter 1

INTRODUCTION

CONTENTS

1.10 WOUND HEALING

Under normal conditions, in normal individuals, the body's response to injury whether the injury is induced surgically or traumatically is immediate and effective. Cutaneous wound healing is a natural mechanism which operates optimally in most circumstances and generally requires no therapeutic intervention. Research carried out in this area aims to elucidate each step, and all components thereof, from the infliction of the injury through to the resolution of the wound. Some workers aim to develop therapies which will provide routes resulting in scarless healing; in adult tissue, with our current understanding, however, this would mean no healing at all.

Not all wounds are equal. This is a fact better understood by clinicians and surgeons than by researchers. The infliction of the injury dictates the subsequent healing process: first intention healing occurs quickly with minimal granulation tissue and scar formation (granulation tissue is characterised by the large-scale regeneration of the tissue microvasculature and the reinstatement of macrophage, lymphocyte and fibroblast populations) and is generally associated with surgical cuts or small skin wounds; second intention healing (which the work in this thesis is concerned with) occurs with granulation tissue formation, wound contraction and reepithelialisation, the

scar formed is less cosmetically acceptable than that formed as a result of first intention healing and infection and/or tissue loss may be involved; third intention healing occurs when wound closure is hampered or delayed by prolonged debridement or by the application of treatment, respectively (for reviews, see Bertone, 1989 x2).

Simplistically, wound healing can be viewed as a three step process (for review, see Clark, 1993): inflammation; tissue regeneration; and tissue reorganisation. Each step is highly organised and complex with numerous components. The three steps overlap and the terminology is used by researchers to allow visualisation and to provide order (any time periods given for healing processes are approximations only and tend to vary from individual to individual and injury to injury).

Step 1: Inflammation

Inflammation is an area of research in itself and will not be described in great detail here. It involves the prevention of extensive blood loss and the closing of the wound via clot and scar formation. Reepithelialisation, neovascularisation and decontamination by phagocytosis occur. The

inflammatory phase, if it proceeds without complication, takes place in the first four days post-injury (Hardy, 1989).

Step 2: Tissue regeneration

Four days post-injury, the neodermis begins to form. The behaviour of the granulation tissue which consists of newly formed blood vessels, macrophages, fibroblasts and loose connective tissue is directed by the interaction of a procession of cytokines with the provisional matrix (Raghow, 1994). The regeneration process takes approximately three weeks with wound contraction occurring between seven and fourteen days. Collagen type III has been detected in human skin wounds as early as 2–3 days post injury (Betz *et al*, 1993), but the majority of it is quickly replaced by the highly cross-linked collagen type I by approximately day seven or eight. Type I procollagen is absent from fibroblast granules by day nine or ten (Moulin, 1995).

Step 3: Tissue reorganisation

This stage of healing can continue for months or even years. Early phases involve scar formation and an increase in wound strength, the latter effect

being caused by the realignment and rearrangement of collagen fibres. Subsequent phases involve a decrease in the number of cellular populations present within the wound, blood vessel regression and collagen remodelling, all of which result in the injured area becoming more like normal tissue in appearance and structure (Hardy, 1989).

It is not intended to give a detailed account of every aspect of cutaneous wound healing here but, for the purpose of this thesis, it is necessary to explain the importance of certain components of the process. These components are as follows: angiogenesis; fibroblasts; collagen; and mast cells.

1.11 The role of angiogenesis in wound healing

Angiogenesis is the process of new blood vessel formation. Although adult cutaneous vasularisation is generally quiescent, adult skin has the ability to initiate neovascularisation in response to pathological stimuli (termed secondary angiogenesis; Detmar, 1996). In the context of healing wounds, the role of secondary angiogenesis is to supply the injured area with oxygen and nutrients. This is a very important role. In fact, Hunt and Van Winkle went as

far as to say, 'as neovascularisation goes, so goes the wound' (Hunt and Van Winkle, 1976).

Angiogenesis begins as early as two days post-injury, new blood vessels at the wound site arise from existing vessels bordering the damaged area. Hyperpermeability of microvessels always precedes and accompanies angiogenesis (Dvorak *et al*, 1995 x2). Endothelial cells become activated and then undergo migration, which occurs within a provisional matrix which endothelial cells are able to synthesise as they proceed (Folkman and Shing, 1992). Migration of endothelial cells is followed by proliferation, which ultimately occurs within the neovasculature because endothelial cells at the tip of the capillary do not divide (Ausprunk and Folkman, 1977). The newly formed vessels transport nutrients and oxygen throughout the tissue. As healing progresses and the injured tissue is repaired, the capillaries regress and the area begins to resemble normal tissue once more.

Research carried out to study angiogenesis by the use of tumours has indicated that the local inhibition of new blood vessel formation may be therapeutic. Detmar (1996) suggested that there are currently more than 300 ongoing clinical trials testing angiostatic agents for the treatment of human cancers. The suggestion that certain inflammatory skin pathologies, such as psoriasis, are aided by a tumour-like ability to promote enhanced angiogenesis is not new (Dvorak, 1986). Could this theory be developed to include

cutaneous healing abnormalities? Angiogenesis is essential to inflammation and wound healing, and any malfunction could have far-reaching effects. In view of this, Detmar (1996) suggested that psoriasis could be treated by the inhibition of angiogenesis, and that certain pathologies featuring delayed wound healing could benefit from the induction of angiogenesis.

1.12 The role of fibroblasts in wound healing

A great deal of work carried out over many years has established that, during wound healing, fibroblast populations progress through four different phenotypes (Welch *et al*, 1990; for review, see Clark, 1993): proliferative; migratory; synthetic (synthesising extracellular matrix components); and contractile (now termed myofibroblasts; Gabbiani *et al*, 1971; Hirschel *et al*, 1971; Gabbiani *et al*, 1972; Ryan *et al*, 1974).

During the first three days post-injury, fibroblasts in the subcutaneous tissue beneath the cutaneous wound proliferate, on day four they move quickly into the wound (Knox *et al*, 1986). It is thought that fibronectin receptors expressed in the first three days prohibit migration, and it is unclear whether or not migratory fibroblasts are able to proliferate. Once established within the wound, the fibroblasts begin to deposit collagen and fibronectin (Welch *et al*,

1990). Collagen I production is maximal at day seven; around this time, cell-matrix connections are formed and fibronectin receptors reappear. By approximately day nine, collagen synthesis has arrested and fibroblasts, which are now of the contractile phenotype (myofibroblasts), are anchored and aligned along the deposited collagen fibres. Contraction takes place between days seven and fourteen and occurs as the attached fibroblasts retract their pseudopodia (Bell *et al*, 1983). The ultimate fate of the wound myofibroblasts when their activities are completed is now recorded to be apoptosis (Desmouliere *et al*, 1995).

It is clear that the fibroblast is a very important component of wound healing. The data underlying the above preview of the general activities of fibroblasts in the wound are impressive with respect to order and efficiency. *In vitro* studies using fibroblasts have illustrated the consistency of fibroblasts in continuing their functions in a variety of conditions, as cells they are resilient and adaptable (for review, see Clark, 1993). It is now widely accepted that cytokines are a modulatory influence (perhaps even the most important influence) on the behaviour of fibroblasts, whether *in situ* or *ex situ*.

1.13 The role of collagen in wound healing

To date, numerous collagen subtypes have been identified. Six of which have been definitively identified in skin: types I, III, V and VI are interstitial collagens; types IV and VII are found in basement membranes. Immunohistochemical localisation has been used to analyse the time-dependent appearance of each of these types of collagen throughout the wound healing process (Vialle-Presles *et al*, 1989; Betz *et al*, 1992 x3; Betz *et al*, 1993; Oono *et al*, 1993). Collagen III appears as early as 48 hours post-injury (Kurkinen *et al*, 1980), it is thought to be involved in the process of wound contraction (Ehrlich, 1988). Procollagen type III is present for approximately two or two and a half days. Immunolabelling for collagen type III in wound samples often indicates a wound age of less than six days (Betz *et al*, 1993).

Type I procollagen can be detected in cytoplasmic granules once fibroblasts have migrated into the wound; when the collagen matrix has accumulated, type I procollagen disappears (usually by day nine or ten; for review, see Clark, 1993). Changes in the alignment of collagen fibres strengthen the wound, and within approximately two weeks all collagen type III is completely replaced by the stronger, more mature collagen type I. By the end of the three weeks, the amount of collagen present has long since

plateauxed but the tensile strength is still only 15% of that of normal tissue (Howes and Harvey, 1929; Levenson, 1962).

Remodelling of the newly synthesised area can continue for years; collagen synthesis and breakdown occurs at elevated rates compared with that of the site prior to injury, although there is evidence that neighbouring tissues also show enhanced collagen turnover (Merritt *et al*, 1975; for review, see Hardy, 1989).

1.14 The role of mast cells in wound healing

Mast cells have been found in elevated numbers in pulmonary fibrosis, keloids, hypertropic scars, scleroderma and wound healing. These observations have led to increased interest in the role of mast cells in fibrosis and their possible interactions with fibroblasts (for review, see Claman, 1985).

Mast cells are resident in skin, they produce interleukin 4 (Bradding *et al*, 1992) and 'degranulate' to release mediators when activated through the cross-linking of their high-affinity immunoglobulin E receptors by antigen. *In vitro*, mast cell survival and growth is enhanced by interleukins 3, 4 and 10 alone and in synergy (Thompson-Snipes *et al*, 1991; Tsuji *et al*, 1995). The presence of mast cells in co-culture with fibroblasts enhances the migration

and proliferation of fibroblasts into artificial wounds (Levi-Schaffer and Kupietzky, 1990). Activated mast cells induce fibroblast proliferation and collagen production (Levi-Schaffer and Rubinchik, 1995).

Kinetic studies have demonstrated that mast cell numbers fall early in the wound healing process but after one or two weeks there is a great increase in numbers. This initial decrease may be caused by emigration, but it is more likely to be caused by degranulation taking place in the early stages of healing. This hypothesis is consistent with the suggestion of mast cell degranulation being the early source of interleukin 4 release during the development of an immune response, and that the later source of interleukin 4 release is lymphocytes (for review, see Claman, 1985). There is also evidence that the activation of mast cells coincides with the initiation of skin fibrosis in some diseases (Claman, 1985). In this case it is possible that the mast cells are providing substances which promote fibrosis, such as histamine (Claman, 1985).

Studies of T cell, mast cell and fibroblast interactions during wound healing are still in the early stages, but it is clear that mast cells play a critical role.

1.20 CYTOKINES

Cytokines are relatively small secreted regulatory polypeptides which control the growth, differentiation and functions of tissue cells. They are protease-resistant, soluble and stable (probably because of their general structure which includes protective carbohydrates and disulphide bonds). Almost all are found extracellularly and interact with specific target cells. Their production is specific, they are transient, rarely found in the circulation, and they have high biological redundancy.

Producer cells respond to an external stimulus and initiate the appropriate response in neighbouring target cells through the action of cytokines. Or, as is the case in the nervous system, a constitutive level of cytokine production may be necessary for cell survival and selection.

Experiments with cytokine gene knockout mice (Tavernier, 1993) have demonstrated that very few cytokines are essential to life – there are many others which are suited to compensate for absences. Cytokines, therefore, are now generally accepted as being widely pleiotropic in action.

Another important characteristic of cytokines and their actions is secondary production. Cytokines can stimulate the production, by target cells, of positive and negative regulatory cytokines in an autocrine or paracrine way

(Arai *et al*, 1990). Because of the pleiotropic behaviour of cytokines, however, it is very difficult to study secondary production or, indeed, any cytokine action *in vivo* (for review, see Carter and Swain, 1997). Serum levels of cytokines are rarely detectable. Reasons for cytokine elusiveness include the close proximity of target and responder cells (Metcalf, 1991), the fact that production is directional and in small quantities (Poo *et al*, 1988), the destruction of the cytokine by the target cell (Nicola *et al*, 1988), the possible binding of the cytokine to extracellular matrix components (Gordon, 1991), and the incidence of high levels of circulating soluble cytokine receptors (Fernandez-Botran, 1991; Arend, 1993; for a general overview, see Nicola, 1994).

1.21 Cytokines and T cells

It is now well established that murine and human $CD4^+$ and $CD8^+$ T cells exist as subsets (for review, see Romagnani, 1991; Paul and Seder, 1994; Mosmann and Sad, 1996). Moreover, these subsets have defining characteristic cytokine secretion patterns and are associated with certain pathological conditions. The type 1 and type 2 subsets interact with one another through cross-regulation. It has been suggested that this attribute will provide a means to manipulate

lymphocytes and the subsequent immune responses in the context of certain pathologies and to improve the overall condition of patients.

Antigenic stimulation leads to a transient burst of cytokine production by activated T cells. The nature and concentration of the antigen dictates the pattern of cytokine production, in nonpathological situations. Levels of type 1 cytokines, such as interleukin 2 and interferon gamma, are elevated in efficient responses against intracellular pathogens, whereas type 2 cytokines, such as interleukins 4 and 10, are elevated in allergic responses and helminth infections. Type 1 cytokines activate macrophages, increase antigen presentation, phagocytosis, macrophage killing of intracellular pathogens, and are involved in delayed-type hypersensitivity responses and long-term immunity. Type 2 cytokines increase immunoglobulin E expression and mast cell and eosinophil degranulation in response to metazoan parasites, which is not always beneficial to the host (for review, see Powrie and Coffman, 1993).

It is not clear how the immune response matches the type 1 or type 2 T cell response to a particular pathogen (Mosman and Sad, 1996). Perhaps the soluble or particulate nature of the antigen or even its concentration (type 1 responses are associated with moderate doses of antigen, type 2 responses are associated with a range of doses) has some influence over the immune polarisation. Some researchers report that antigens stimulate a type 1, type 2

and type 1 pattern chronologically, whereas others report that the pattern is type 2, type 1 and type 2 (Mosman and Sad, 1996).

Skin-generated immunity is dominated by T cells, they mediate some delayed-type hypersensitivity responses (which can descibed as type 1 responses) and some atopic responses (which can be said to feature type 2 responses). Antigen presenting cells which are resident in the skin are as follows: Langerhans' cells in the epidermis and dendritic cells and macrophages in the dermis (Zierhut *et al*, 1996). γ/δ T cells are a prominent population within epithelial tissues such as skin but knowledge of their function and specificity is poor. They share features with innate immunity cells and respond much faster to antigenic stimulation than do α/β T cells, which suggests that their response influences the subsequent cytokine production by the late α/β T cell arrivals. There is also evidence that γ/δ T cells exist in type 1 and 2 subsets (Boismenu and Havran, 1997).

$CD8^+$ T cells also feature type 1 and type 2 subsets, the major difference between $CD8^+$ and $CD4^+$ T cell subsets is that once the type has been set for $CD8^+$ cells it cannot be reversed (Mosman and Sad, 1996). They are associated with the same cytokine profiles as are $CD4^+$ T cells, although it appears that $CD8^+$ T cells favour the development of the type 1 subset and that more interleukin 4 is required to develop the type 2 subset.

Both type 1 and type 2 $CD8^+$ T cells are thought to be cytolytic. Killing of infected cells by $CD8^+$ T cells does not occur through the effects of cytokine secretion, however, it usually occurs through apoptosis (Carter and Dutton, 1996).

To date, the only cell marker which allows the differentiation of type 1 and type 2 subsets is CD30 (CD, cluster of differentiation). Both subsets have been documented to express it, but expression by type 2 cells is thought to be much stronger and more sustained; indeed, a rare $CD30^+$ population has been found to contain cells specific for a recently exposed allergen (see, Mosman and Sad, 1996), a situation associated with type 2 responses. The mechanism of the differentiation of T cells into the $CD4^+$ and $CD8^+$ subsets is thought to be the same, with the cross-linkage of the CD30 marker stimulating type 2 cells preferentially (Carter and Dutton, 1996). And it appears that repeated antigenic stimulation drives the immune response towards the type 2 subset (Carter and Dutton, 1996).

Now that it has become possible to classify the T cell subsets involved in immune reactions by the detection of CD30 expression and cytokine secretion patterns, it may also be possible to suggest methods of therapeutic intervention.

Although it has not yet been documented which T cell subset(s) is/are involved in each wound healing stage, there are many opportunities for control

via cytokine neutralisation or cytokine administration (for review, see Miossec, 1993; Powrie and Coffman, 1993). For example, the beneficial effects of the administration of interferon gamma to hypertropic scars and the administration of interleukin 4 to schleroderma patients (Miossec, 1993).

1.22 Cytokines and wound healing

Because wound healing involves the growth, interaction, differentiation and activity of cells and because the release and action of cytokines is the mechanism of communication between responding and target cells, much work has been done to elucidate each finite step connecting cytokine expression and appearance with the effects of subsequent cellular responses in the healing process (for reviews, Kovacs and DiPietro, 1994; Slavin, 1996). Many reviews and primary publications have attempted to elucidate the role of cytokines in healing, but have generally focused on 'growth factors' such as transforming growth factor beta, with 'lymphokines' such as interleukin 2 getting little attention so far (for an example, see Martin *et al*, 1992).

Interferon gamma

Interferon gamma is produced by T cells (specifically $CD8^+$ cells and types 1 and 0 $CD4^+$ cells [type 0 $CD4^+$ T cells are those which are classified as being neither type 1 nor type 2. It is unclear whether this subset represents an initial subset consisting of all $CD4^+$ T cells prior to type 1/type 2 differentiation or if it represents a third distinctive and individual subtype]) and natural killer cells (Farrar and Schreiber, 1993). Interferon gamma antibodies protect mice against the generalised Schwartzman reaction (a hypersensitivity reaction induced by lipopolysaccharide administration; Billiau *et al*, 1987).

Interferon gamma is synthesised and secreted via antigen stimulation of T cells; maximal synthesis occurs 12–14 hours following stimulation. It has an antiproliferative effect on tumour cells (Farrar and Schreiber, 1993) and enhances immunoglobulin secretion by B cells (see Nicola, 1994). Interferon gamma also causes nonspecific cell-mediated responses and is necessary for the resolution of microbial infections (Farrar and Schreiber, 1993; Buchmeier and Schreiber, 1985) and has been implicated in the development of autoimmune disease (Sarvetnick *et al*, 1988).

T cells mainly produce interferon gamma in response to soluble antigens (for review, see Trinchieri and Perussia, 1985), which induces similar

effects to that of the adaptive immune system. Mast cells and fibroblasts express interferon gamma receptors, as do other cells (for review, see Hill *et al*, 1992).

Interferon gamma and wound healing

Interferon gamma downregulates angiogenesis *in vitro* in a dose-dependent manner via the inhibition of endothelial cell proliferation and growth (Sato *et al*, 1990; Tsuruoka *et al*, 1988). It inhibits collagen lattice contraction and delays wound closure *in vitro* (Granstein *et al*, 1989; Dans and Isseroff, 1994). Work carried out *in vitro* and by immunohistochemistry, immunofluorescence and Western blotting has shown that interferon gamma increases the production by fibroblasts of glycosaminoglycans and collagenase (Duncan and Berman, 1989). It has been shown to decrease alpha smooth muscle actin mRNA and protein levels and the proliferation of human fibroblasts *in vitro* (Desmouliere *et al*, 1992).

Interferon gamma can act in a proinflammatory or anti-inflammatory way, depending on the model being used (Heremans *et al*, 1989; Granstein *et al*, 1989), it decreases collagen synthesis *in vivo* (Granstein *et al*, 1989; Granstein *et al*, 1990; Guruyjeyalakshmi and Giri, 1995) possibly via the

downregulation of transforming growth factor beta mRNA expression (Guruyjeyalakshmi and Giri, 1995). This modulation of transforming growth factor beta mRNA activity could explain the ability of interferon gamma to decrease extracellular matrix component release, collagen synthesis, angiogenesis, wound contraction and its ability to increase collagenase synthesis. Kovacs and DiPietro (1994) suggest that interferon gamma may function as an endogenous mediator to terminate the collagen synthesis phase of wound healing. Its appearance early and late in the wound healing process, although not in the middle stages, reiterates this suggested influence.

Interleukin 2

The most important function of interleukin 2 is growth promotion of T cells (for review, see Smith, 1980; Nicola, 1994). It is synthesised and secreted in response to the activation of T cells. Antigen-specific effector cells are then generated via the binding of autocrine and paracrine interleukin 2 to its receptor. Interleukin 2 knockout mice demonstrate normal thymocyte and peripheral T cell development (Schorle *et al*, 1991; Kundig *et al*, 1993).

Interleukin 2 also has a role in the differentiation of T cells and is able to stimulate the production of itself and of interferon gamma (Farrar *et al*,

1982; Howard *et al*, 1983). Interleukin 2 is also able to induce an inflammatory response by the activation of natural killer cells (for review, see Oleksowicz *et al*, 1994) and to increase immunoglobulin production by B cells (for review, see Hill *et al*, 1992).

Interleukin 4 is a homeostatic regulator of interleukin-2-induced interferon gamma production; that is, the production of interferon gamma by interleukin-2-stimulated cultures is inhibited by the addition of recombinant interleukin 4 (Bello-Fernandez *et al*, 1991).

Interleukin 2 and wound healing

Very little work has been carried out in the area of interleukin 2 action in wound healing. Although, an *in vivo* study carried out in rats demonstrated increased fresh and fixed wound breaking strength and increased collagen synthesis (as measured by hydroxyproline content levels). This enhancement was attributed to the induction of secondary cytokine production in response to the interleukin 2 stimulation of T cells (Barbul *et al*, 1986).

Most of the *in vivo* work carried out using interleukin 2 has centred on its ability to induce lymphocyte-activated killer cells to destroy cancer cells

(for review, see Hill *et al*, 1992). Clinical trials have provided positive results but it is clear that the administration of interleukin 2 alone is toxic to patients.

Interleukin 4

Interleukin 4 is primarily produced by T cells, it induces immunoglobulin class switching to immunoglobulin E expression, augments immunoglobulin G_1 production (Finkelman *et al*, 1990; Kuhn *et al*, 1991) and is a vital component of T cell subset differentiation into type 2 and away from type 1 (Sadick *et al*, 1990; Swain *et al*, 1990; Abehsira-Amar *et al*, 1992; Chatelain *et al*, 1992; Kopf *et al*, 1993). Mast cells, basophils and natural killer 1.1^+ cells also produce interleukin 4 (Scott *et al*, 1990; Seder *et al*, 1991; Bradding *et al*, 1992; MacGlashan *et al*, 1994). It has been suggested that either one of these cell types could provide the initial pulse of interleukin 4 release which is required for the development of the type 2 T cell clones (Scott *et al*, 1990; Flamand *et al*, 1990; Bradding *et al*, 1992; Schroeder *et al*, 1995; Huels *et al*, 1995). Cross-linkage of the T cell receptor and the immunoglobulin E receptor is a requirement for the induction of interleukin 4 release by T lineage, mast or basophil cells (for review, see Nicola, 1994).

Interleukin 4 also has potent antitumour action *in vivo* (Tepper *et al*, 1989; Golumbek *et al*, 1991). The discovery of this activity has led to clinical trials, the results of which have been promising. The therapeutic ability of interleukin 4 seems to lay in the induction of cytotoxic eosinophil activity (Tepper *et al*, 1992) and immunity involving tumour-specific cytotoxic T cells (Golumbek *et al*, 1991).

Interleukin 4 and wound healing

In vitro data indicate that interleukin-4-stimulated collagen synthesis by dermal fibroblasts occurs in a dose-dependent manner. Under certain conditions it has been documented to be more potent in stimulating the release of collagen than is transforming growth factor beta (Fertin *et al*, 1991). Indeed, fibroblasts do express interleukin 4 receptors (Lowenthal *et al*, 1988) and have been shown to proliferate in response to its presence (Monroe *et al*, 1988), and interleukin 4 has been shown to be fibroblast chemotactic (Postlethwaite and Seyer, 1991). Postlethwaite and co-workers (1992) have also demonstrated that dermal fibroblasts are stimulated to produce collagen types I and III and fibronectin in response to interleukin 4.

The implication of this evidence, with respect to cutaneous wound healing, is that interleukin 4 can be classified as a 'fibrogenic' cytokine. That is, in addition to the clinical role that interleukin 4 has in inflammation, immune response development and immunoglobulin class switching, it could have great influence on the matrix deposition, tissue regeneration and tissue reorganisation components of wound resolution.

Interleukins 4 and 10 synergise to inhibit type 1 T-cell-mediated immunity *in vivo*, although not via interferon gamma inhibition (Powrie *et al*, 1993). Interleukins 3, 4 and 10 synergise to enhance the survival of mast cells *in vitro* (Tsuji *et al*, 1995). Interleukin 4 inhibits interferon gamma production; and immunoglobulin E production by normal human lymphocytes induced by interleukin 4 is suppressed by interferon gamma (Pene *et al*, 1988).

Interleukin 10

Interleukin 10 inhibits the proliferation of type 0, 1 and 2 T cells in humans (for review, see De Vries, 1995). It does not inhibit interleukin-2-induced T cell proliferation, although interleukin 2 produced by resting and activated T cells is inhibited by interleukin 10 at the mRNA and protein level (De Waal *et al*, 1993). Interleukin 2 is released four hours after activation and interleukin

10 is released 24 hours after (De Waal *et al*, 1991; Yssel *et al*, 1992; Mosmann, 1994).

In humans, interleukin 10 is produced by and downregulates the functions of type 1 and type 2 T cells. It inhibits antigen-induced production of interferon gamma by type 1 T cells and of interleukin 4 by type 2 T cells (Del Prete *et al*, 1993). Interleukin 10 is a natural suppresser of irritant skin responses via the inhibition of inflammatory cytokines (Berg *et al*, 1995).

Interleukin 10 and wound healing

Interleukin 10 is produced by mast cells and keratinocytes (Moore *et al*, 1990; Rivas and Ullrich, 1992; Euk and Katz, 1992). It inhibits collagen type I release by fibroblasts and fibroblast proliferation (Van Vlasselaer *et al*, 1993). At the mRNA level interleukin 10 increases collagenase production and decreases collagen type I production (Reitamo *et al*, 1994).

Epidermal growth factor and wound healing

Epidermal growth factor is a mitogen for fibroblasts and epithelial cells and is present in normal human skin and keratinocytes (Nanney *et al*, 1984; Laato *et al*, 1987; Nickoloff *et al*, 1988; Grant *et al*, 1992). It increases collagen production via the stimulation of the proliferation of granulation tissue fibroblasts (Laato *et al*, 1987). Epidermal growth factor decreases the amount of hydroxyproline in cultured cells, which is commonly used as a measure of collagen content, but actually increases the overall collagen level presumably through the increased number of fibroblasts present.

It has been shown to enhance reepithelialisation in partial thickness wounds in a dose-dependent manner and increases the thickness of the neodermis (Nanney, 1990). In clinical trials in human partial thickness wounds, however, there was no significant difference between control and treated sites (Cohen *et al*, 1995).

Platelet-derived growth factor in wound healing

Platelet-derived growth factor is produced by fibroblasts, smooth muscle cells, macrophages/monocytes and platelets (for review, see Ross *et al*, 1990).

Platelet-derived growth factor reversed radiation effects on wound healing in rats (Mustoe *et al*, 1989); wound breaking strength increased by 50% and was attributed to fibroblast migration rather than to collagen production (Tan *et al*, 1995). It increases collagenase synthesis in cultured human fibroblasts (Bauer *et al*, 1985) and granulation tissue deposition in full thickness guinea pig wounds (Hill *et al*, 1991) and in wounds featuring a Hunt–Schilling chamber (Schilling *et al*, 59; Grotendorst *et al*, 1985). It increases the influx of procollagen-containing fibroblasts between days 2 and 21 in wound healing experiments but its effects are transient, there are no known differences between control and treated wounds when healing has been completed (Deuel and Kawahara, 1991).

Platelet-derived growth factor increases neovascularisation but has no effect on endothelial cells; it increases reepithelialisation but has no demonstrable effect on keratinocytes (Deuel and Kawahara, 1991).

Basic fibroblast growth factor and wound healing

Basic fibroblast growth factor has a different nature compared with other growth factors such as epidermal growth factor, platelet-derived growth factor and transforming growth factor beta because it is not released into the extracellular space in a soluble form (for reviews, see Gospodarowicz, 1990; Nicola, 1994). It exists intracellularly and its mechanism of release is unknown. It binds heparin sulphate strongly and is mainly found bound to it in the extracellular matrix. It has been shown that the association between basic fibroblast growth factor and heparin sulphate is so intimate that the formation of their complex is a prerequisite for binding to the basic fibroblast growth factor receptor. It is from its position in the extracellular matrix that basic fibroblast growth factor exerts its mitogenic effects on fibroblasts, endothelial cells and other cell types. The mitogenic action of basic fibroblast growth factor correlates with its appearance in the nucleus where it tends to localise, rather than in the cytoplasm. This nucleic localisation follows cytoplasmic synthesis or internalisation.

Basic fibroblast growth factor has a vital role in healing, it is released from damaged cells and induces proliferation. It increases granulation tissue formation and accelerates wound healing and reepithelialisation. *In vitro* it has

been shown to increase collagenase levels and to have no effect on collagen content when added alone; when added along with transforming growth factor beta, however, collagenase levels were decreased (Edwards *et al*, 1987). Pierce *et al* (1992) found that basic fibroblast growth factor induced an angiogenic response which delayed wound maturation and suggested that its presence may cause the 'loss' of some signal which initiates the cessation of repair.

Basic fibroblast growth factor is produced by T cells, platelets, keratinocytes, fibroblasts, macrophages and endothelial cells. It is found in the sweat glands of normal human skin (Cordon-Cardo *et al*, 1990). It stimulates the release of collagenase, also in human skin (Buckley-Sturrock *et al*, 1989). It is a mitogen for endothelial cells but does not directly stimulate angiogenesis (Knighton *et al*, 1990). Work in an *in vivo* model showed that basic fibroblast growth factor decreases DNA, protein and collagen content (Broadley *et al*, 1989).

It has been suggested that the release of heparanase from platelets or neutrophils may be the stimulation for basic fibroblast growth factor release from its complex with heparin sulphate in the extracellular matrix (Ishai-Michaeli *et al*, 1990).

Vascular endothelial growth factor

Vascular endothelial growth factor is unique among growth factors in its specificity, its mechanism of release and its distribution (for reviews, see Klagsbrun and Soker, 1993; Dvorak *et al*, 1995). It initiates the hyperpermeability of blood vessels and neovascularisation and can enhance any process which involves angiogenesis, for example tumour growth (Plate *et al*, 1992; Jin Kim *et al*, 1993), rheumatoid arthritis (Koch *et al*, 1994), inflammation (Dvorak *et al*, 1995) and wound healing (Brown *et al*, 1992). It is a potent mitogen for endothelial cells and it appears that it is totally endothelial-cell-specific in its action.

Vascular endothelial growth factor is expressed by keratinocytes (Ballaun *et al*, 1995) and there is no evidence that endothelial cells actually produce it; it is thought that, once released, it is taken up by endothelial cells. It is not thought that the cells of blood vessels release it and it has been shown that it is only expressed during active angiogenesis. During tumour growth, tumour cells close to the blood vessels express it. Vascular endothelial growth factor does not actually have any direct positive or negative effects on tumour cells, its control is exerted purely through its action on the process of angiogenesis. It is a heparin-binding growth factor and may be released from

sites in the extracellular matrix during wound healing in much the same way as is basic fibroblast growth factor.

The mechanism of release is not known, but, in tumour growth, it is thought that hypoxia is the stimulus.

The expression of receptors for vascular endothelial cell growth factor are confined to the endothelial cells of proliferating blood vessels.

Transforming growth factor beta and wound healing

Briefly stated, the role of transforming growth factor beta in wound healing is to 'protect' the extracellular matrix and connective tissue: it increases extracellular matrix deposition, decreases proteolytic enzyme release and increases proteolytic enzyme inhibitor release (for review, see Nicola, 1994). It is released by platelets, activated macrophages and monocytes. It has both proinflammatory and anti-inflammatory actions and is chemotactic for monocytes and fibroblasts, rather than being an inducer of proliferation. Its release is stimulated both by autocrine and paracrine means.

In vitro, exogenous transforming growth factor beta inhibits interleukin-2-dependent T cell proliferation and is itself produced by activated T cells (Kehrl *et al*, 1986). Also *in vitro*, it has been shown to increase the

expression of procollagen I. When added alone to fibroblast cultures it is unable to influence collagenase production, when added together with epidermal growth factor or basic fibroblast growth factor, however, collagenase levels increase (Edwards *et al*, 1987).

A single topical dose of transforming growth factor beta increases the synthesis of extracellular matrix and granulation tissue components as well as wound breaking strength in normal wounds Cromack *et al*, 1990). When applied to partial thickness porcine skin wounds, it increases connective tissue volume and collagen content and maturity as well as angiogenesis (Lynch *et al*, 1989). It has been shown that the inhibitory effects of interferon gamma on the expression of procollagen and collagen types I and III is caused by the inhibition of the activities of transforming growth factor beta (Gurujeyalakshimi and Giri, 1995). Indeed, extensive *in vivo* experiments involving the application of recombinant transforming growth factor beta and antibodies to it showed that the antibodies reduced the number of blood vessels and the content of collagen and fibronectin present in wounds (Shah *et al*, 1992). Scarring was increased in those wounds which had been treated with the antigen and was reduced in those treated with antibody, although tensile strength was not affected in either case. Transforming growth factor beta induces fibroblast proliferation probably via the production of platelet-derived growth factor and other growth factors, not directly (Rodemann and Bamberg,

1995), it also inhibits the synthesis of collagenase and stimulates the production of collagen at the mRNA level. The authors of this piece of work (Rodemann and Bamberg, 1995) declare that transforming growth factor beta is the major cytokine responsible for fibrotic deposition.

Transforming growth factor beta mRNA expression is induced after wounding in adult skin, although not in foetal tissue. Foetal wounds heal without scarring, but if transforming growth factor beta is applied to the wound scar formation occurs (Lin *et al*, 1995).

Transforming growth factor beta exists in three forms: termed one, two and three. Type 1 alone increases the production of collagen types I and III in cutaneous rat wounds. Type 3 alone decreases the production of collagen types I and III and scar formation. The neutralisation of types 1 and 2 together results in reduced scarring (Shah *et al*, 1995). Other *in vivo* work carried out in pigs, however, proved inconclusive, thus leading some to resolve that the exact roles of the three forms of transforming growth factor beta are still unclear (Levine *et al*, 1993).

1.23 Measuring cytokine production

Technical advances have provided several analytical procedures which allow the investigation of cytokine presence (for review, see Carter and Swain, 1996). Each approach has advantages and disadvantages which must be considered when selecting a suitable method to investigate specific activities.

Levels of cytokines can be assessed by polymerase chain reaction analysis, this method is sensitive and quantitative. Whole organ homogenates and bulk cultures are suitable samples, but heterogeneous tissues, such as skin, can only provide very nonspecific data.

Amounts of cytokines secreted into culture medium can be inferred or quantified by enzyme-linked immunosorbance assay (ELISA) or bioassay, respectively (the ELISA bioassay has the advantage of only measuring bioactive protein). Unfortunately, these approaches measure cytokine uptake and secretion together.

A new technique developed which permits cytokine antibodies to enter cells and to be analysed by fluorescent antibody cell sorting (FACS) allows the observation of single cells producing cytokines. This allows statistical analysis and the observation of cells which may be producing more than one cytokine at a time, the expression of certain cell markers can also be localised.

Unfortunately, for this technique, the cells must be removed from their natural environment, the amount of cytokine(s) produced cannot be quantified and, most importantly, many cell types do not survive the necessary isolation and permeabilisation processes.

In situ cytokine mRNA levels can be measured by *in situ* hybridisation and *in situ* cytokine protein levels can be measured by immunohistochemistry and immunofluorescence. In immunohistochemical analysis, cell types can be 'identified' by nuclear counterstaining and it has the distinct advantage of observing the cells in their physical environment. The disadvantages include technical difficulty and the fact that comparisons can only be made within the same experiment, and it is, therefore, not the ideal technique for quantitation.

Western blotting is a variation on the theme of immunohistochemistry. Antibody staining is used once the cytokine proteins have been separated by size with a gel. Whole organ homogenates and cell fractions work well and even heterogeneous tissues, such as skin, can be analysed. Unfortunately, this technique does not demonstrate which cells have produced the protein.

1.30 CELL DEATH

1.31 Apoptosis

Apoptosis is a fast growing area of research. The term itself is still being defined and redefined (Hockenbery, 1995; Kane, 1995; Schwartz and Osborne, 1993). As a form of cell death, it has been clearly classified as distinct from necrosis. And as the process of apoptosis has been elucidated this distinction has become clearer (Majno and Joris, 1995). Indeed, light microscopy and straightforward histological techniques are still the most effective method of identification and observation of apoptotic cells (Stewart, 1994). Apoptosis is a form of homeostasis and maintenance not only for the immune system (Goldstein *et al*, 1991) but also for tissues such as the skin (Olson and Exerett, 1975; Weedon *et al*, 1979).

1.32 Necrosis versus apoptosis

DNA fragmentation is among the first manifestations of the apoptotic cascade. Endonuclease digestion of DNA at exposed sites between nucleosomes results

in the liberation of fragments of 180–200 base pairs (and in multiples thereafter). Unfortunately, the origin of this endonuclease activity is not yet known.

Grossly described, the cell condenses and fragments. The fragments are phagocytosed by macrophages (for review, see Goldstein *et al*, 1991). That apoptosis does not generate an inflammatory response is often part of the definition used to separate necrosis and apoptosis. But it is not clear how the phagocytes find the apoptotic cells. Apoptotic cells do not attract lymphocytes or neutrophils but the phagocytic macrophages do migrate (albeit a small distance) towards their targets. Evidence of an apparent inflammatory response when apoptosis occurs on a large scale has led to many workers doubting the definitive understanding that apoptosis means no inflammation (for review, see Majno and Joris, 1995).

In cells undergoing necrosis, DNA is not cleaved into discrete fragments and DNA degradation actually occurs quite late in the dying process. Inhibition of protein or RNA synthesis does not prevent necrosis, which indicates that the synthesis of new molecules is not a requirement. In contrast to the condensation observed in apoptosis, necrotic cells swell as the plasma membrane is breached (Goldstein *et al*, 1991).

1.33 Apoptosis and lymphocytes

Peripheral homeostasis of T cells is regulated by Fas (CD95) and the Fas ligand (FasL). Surface expression of FasL is high on activated T cells, and T cells can secrete soluble FasL in an autocrine and a paracrine manner, that is T cells can kill one another. But not all T cell death in the periphery is mediated by Fas and FasL: other routes involve the cytotoxic T lymphocyte associated molecule 4 (CTLA-4), the tumour necrosis factor receptor and perforin release (for review, see Kruisbeck and Amsen, 1996). There is no evidence that any of these methods of cell death favours a particular lymphocyte subset type, although there is some evidence of an association of $CD4^+$ T cells and Fas and of $CD8^+$ T cells and the tumour necrosis factor receptor (Kruisbeck and Amsen, 1996). Documenting the apoptosis of antigen-specific T cells is difficult *in situ* because many activated T cells home to the liver or gut for apoptosis. Chronic antigen exposure causes functional silencing of CD4 cells and eventual elimination, in CD8 cells chronic antigen exposure results in the downregulation of coreceptor expression and eventual elimination.

Fas is expressed by T and B cells and other hemopoietic cells and is the primary mediator of cell death in $CD4^+$ T cells, antigen–receptor binding activates the cell and increases Fas expression. FasL expression is of a lower level than that of Fas. Expression of FasL is induced on mature $CD4^+$ and

$CD8^+$ T cells following activation but is not seen on other hemopoietic cells. The eye and testes express FasL constantly. If activated T cells are stimulated by antigen, they produce interleukin 2 and then express Fas and FasL before dying. Fas only mediates cell death in T cells as a result of repeated antigen activation (for review, see Van Parijs and Abbas, 1996; Osborne, 1996).

Cell death caused by insufficient antigenic stimulation can be prevented by costimulation with CD28 and by interleukin 2. In contrast, cell death caused by Fas and FasL linkage is actually enhanced by interleukin 2 and is not blocked by costimulation (Lenardo, 1991). Interleukin 4 rescues type 2 T cells from glucocorticoid-induced apoptosis, interleukin 2 rescues type 1 T cells. The reasons for this are not known (Cory, 1995).

1.34 Apoptosis and nonimmune cells

As was described above, apoptosis is intimately involved in immune responses. Apoptosis is also vital to skin maintenance, and to skin pathologies because so many result through immunological mechanisms (Weedon *et al*, 1979). Lichen planus is the most studied skin disease, with respect to apoptosis. It has been suggested that the degeneration of the epidermal cells,

which is characteristic of lichen planus, is due to apoptosis (Weedon *et al*, 1979).

In normal healthy skin, desquamation is the route of epidermal cell maintenance and keratin bodies are now recognised as being apoptosed keratinocytes which have dropped below the basement membrane. Enhanced numbers of epidermal cells undergo apoptosis following exposure to ultraviolet radiation (Grubauer *et al*, 1986).

In cutaneous wound healing, the ultimate fate of the contractile myofibroblasts after their contribution to wound contraction had long been a mystery. It had been suspected that apoptosis was involved (Kischer, 1992). Desmouliere and co-workers (1995) have now confirmed that as the granulation tissue resolves and scar formation progresses, the decrease in the number of myofibroblasts present is caused by apoptosis.

Interest in apoptosis has increased because of the overall biological significance of it as a phenomenon and because of the myriad of implications for human disease. The role of apoptosis in cancer is a major source of new research: most anticancer drugs induce apoptosis in sensitive cells, and it was recently demonstrated that the majority of tumour promoters are potent apoptosis inhibitors (for review, see Orrenius, 1995).

1.35 Apoptosis analysis

Because DNA cleavage during apoptosis generates characteristic fragments, the classical biochemical method of analysis is to use agarose gels. Necrotic samples produce a 'smear' on the gel, apoptotic cells produce a 'ladder'. This method, however, does not allow the *in situ* observation of apoptotic cells. Trypan blue dye uptake signifies whether the cells are viable or not but is unfortunately nonspecific.

Microscopic observation of apoptosis is possible but demanding, evidence suggests that the apoptotic process is often complete within minutes and that the subsequent apoptotic body formed is visible with a light microscope for between 12–18 hours only. Light microscopy is the most straightforward method of recognising apoptotic cells in cell suspensions and tissue sections. And acridine orange staining gives excellent results. It differentially stains double stranded nucleic acids as opposed to single stranded nucleic acids. Denaturation of DNA is performed by hydrochloric acid and is then stained with acridine orange. When bound to double stranded nucleic acids and upon excitation with blue light, acridine orange fluoresces green. When bound to single stranded nucleic acids and upon excitation, it fluoresces red. During apoptosis, DNA is more sensitive to denaturation and

therefore apoptotic cells are more red than green and nonapoptotic cells are more green than red.

The most accurate method of *in situ* histochemical detection of apoptosis involves the labelling of the newly exposed free ends of DNA which are generated during the apoptotic fragmentation process. The DNA fragments are labelled with dioxygenin and then bound by an antibody peroxidase conjugate. Staining is achieved by the addition of the relevant substrate. This method allows the visualisation of intact apoptotic nuclei and apoptotic bodies in tissue sections, and is more sensitive than DNA agarose gels (Studzinski, 1995).

1.40 KELOIDS

Keloids are abnormal cutaneous scars (for reviews, see Osman *et al*, 1978; Murray, 1993; McGrouther, 1994; Berman and Bieley, 1995). They are classically regarded as more of a cosmetic problem than a medical problem. Keloid lesions are indistinguishable from normally healing wounds and the formation of normal scars until, at some stage and via some unknown mechanism, excessive fibrosis develops and the scar tissue outgrows the site of the original injury. Hypertropic scars and keloid scars are often studied along side one another, but there are marked differences between the two conditions (for reviews, see Sahl and Clever, 1994 x2). Hypertropic scars do grow larger than normal scars would but, generally, they do not outgrow the area of the original wound site. There have been many attempts to clearly define the two abnormalities as distinct and to separate them clinically; it remains, however, that the only way to identify either one in opposition to the other is through histological examination (see below and, De Limpens and Cormane, 1982; Kischer, 1984; Kischer *et al*, 1990; Rudolph, 1991; McGrouther, 1994; Ehrlich *et al*, 1994; Munro, 1995).

There appears to be a recessive genetic predisposition to the formation of keloid lesions (Omo-Dare, 1975) and their formation is more common in

black and oriental skin than in white (Doyle-Lloyd and White, 1991). It has been observed, mainly by clinicians, that anatomical areas of high skin tension and mechanical activity, such as shoulders, form keloids more frequently than other areas although it is not clear why (Murray, 1993). Types of trauma which have been documented to induce keloid formation include the following: cartilage and tissue grafting (Ofodile and Wood, 1992); circumcision (Warwick and Dickson, 1993); dental treatment (Ow, 1989); Norplant insertion (Nuoro and Sweha, 1994); lightning strike (Resnik and Capland, 1994); hypodermic abuse (Scott *et al*, 1994).

Treatments attempted have been largely unsuccessful. The general lack of understanding of the processes involved in the formation and propagation exacerbates the problem of designing effective methods of prevention and therapy. Nevertheless, many attempts have been made: pressure dressings (Kischer *et al*, 1978); carbon dioxide laser (Norris, 1991); cryosurgery (Zouboulis *et al*, 1993); lasers (Alster and Williams, 1995); surgical excision with second intention healing (Glenn *et al*, 1995); X-rays (Norris, 1995); and steroids (Boyadjier *et al*, 1995). The most successful approaches to date involve interferon administration and silicone dressings (Berman and Duncan, 1989; Larrabee *et al*, 1990; Granstein *et al*, 1990; Wong *et al*, 1995) usually accompanied by surgical excision.

A model of keloid formation has yet to be developed. Lesional implantation onto the backs of mice has been attempted (Shetlar *et al*, 1985; Kischer *et al*, 1989; Waki *et al*, 1991; Shetlar *et al*, 1991) and one report claiming that a small percentage of rabbits incidentally developed keloids following a very involved and unique protocol appeared in 1959 (Chytilova *et al*). Unfortunately, as is the case with treatment, the development of a model is hampered by the lack of knowledge of keloid aetiology.

1.41 Fibroblasts and collagen in keloids

Fibroblasts explanted from keloid lesions have been studied extensively. The results of the multitude of experiments which have been carried out are conflicting.

Knapp *et al* (1977) demonstrated that keloids contained a heterogeneous population of fibroblasts which produced twice the quantity of collagenase when compared with normal fibroblasts. In addition to this, keloid fibroblasts produced elevated levels of glycosaminoglycans. It was unclear, however, whether the population of fibroblasts producing the excessive collagenase were the same as those producing the glycosaminoglycans. Jutley *et al*, (1993) demonstrated that there was only a negligible difference in the

rate of keloid fibroblast proliferation and normal skin fibroblast proliferation. Two years later, Nakaoka *et al* (1995) presented evidence that keloid fibroblasts constituted a homogeneous population and that they actually proliferated at an elevated rate. The addition of hydrocortisone slowed the growth of keloid fibroblasts without any effect on collagen production (Russel *et al*, 1978; Trupin *et al*, 1983).

Work carried out on fibroblasts isolated from acne keloids by Jutley and co-workers (1993) was based on the assumption that keloids contain 'normal' and 'abnormal' fibroblast populations. Total collagen type I levels were higher than those for hypertropic scars and normal skin samples. Keloids and hypertropic scars also contained more collagen type III than did normal skin. Lee and co-workers (1992) reiterated the theory of a 'manic' subpopulation of keloid fibroblasts over producing collagen type I with results illustrating higher mRNA levels of procollagen type I than normal fibroblasts and that the ratio of collagen types I:III was significantly elevated in keloids. Younai and co-workers (1994) performed a detailed study which revealed that keloid fibroblasts produce four times more collagen than fibroblasts grown from hypertropic scars and twelve times more than those from normal skin.

Ala-Kokko and co-workers (1987) tentatively stated in their abstract that 'fibroblasts isolated from keloids often synthesise normal amounts of collagen' because they found that the collagen content of keloids was

approximately the same as for normal skin and that the rate of procollagen production by keloid fibroblasts was not significantly different from that of those derived from normal skin. They were tentative because, even without conclusive evidence, there is a central paradigm that keloid fibroblasts 'must' produce elevated quantities of collagen. This assumption is prevalent irrespective of the evidence.

Friedman *et al* (1993) measured the transcription rates of collagen types I and III and found that the ratio of types I:III was very high, the reason being the abnormal amount of collagen type I present. The authors used the results to propose a mechanism for keloid growth based on the lack of downregulation of collagen type I production when healing had been completed. Abergel *et al* (1985*) also found the collagen types I:III ratio to be elevated in keloids and later found that this increase could be traced back to the levels of RNA (1987). Interestingly, Knapp *et al* (1977) only found collagen type I in normal skin, normal scars and keloid scars. Despite this, however, it is generally accepted that the ratio of collagen type I to type III is high in keloids. Why this should be so is not yet clear.

Di Cesare *et al* (1990) documented that levels of collagen type III were elevated in keloids and that the collagen that was present had reduced cross-linking. The reduction in cross-linkage, however, was not caused by a lack of lysyl oxidase, the cross-linking enzyme, nor was it caused by the enzyme

being inactive. An earlier study (Knapp *et al*, 1977) had demonstrated much the same, with the authors reporting normal or elevated levels of lysyl oxidase but, again, the amount of cross-linking in keloids was reduced.

It is not only the biochemical collagen composition of keloids which differs from normal samples, the histological collagen distribution is also altered. The collagen fibres in normal skin and normal scars lies parallel to the surface of the epidermis and is closely-packed. In hypertropic scars the collagen bundles are flatter than those in normal samples and have less structure although the fibres are still roughly parallel to the surface of the skin. The collagen fibres in keloid lesions are very disorganised, there are almost no discrete bundles at all, and those that are present are laid in haphazard sheets with random orientation and the fibres are of inconsistent lengths (for information on collagen fibres, see Hunter and Finlay, 1976; Ehrlich *et al*, 1994; Stewart, 1995).

Another major ultrastructural difference between hypertropic scars and keloids is the presence and absence of myofibroblasts, respectively. Several studies have been carried out to confirm the situation one way or another but the results were contradictory more often that they were confirmatory. James *et al* (1980) stated after the histological and microscopical analysis of one keloid lesion which 'was found to be comprised entirely of myofibroblasts' that indeed the presence of these contractile cells were a component of keloid

formation. In 1982 Kischer *et al* provided evidence that both hypertropic scars and keloids contained myofibroblasts. Six years later (Matsuoka *et al*, 1988) histological and microscopical evidence was published that myofibroblasts were in fact absent from keloid lesions! Gabbiani and co-workers (1994) carried out electron microscopy, light microscopy and *in vitro* analyses on hypertropic scars and keloids and declared that although myofibroblasts were present in hypertropic scars, they were absent from keloids (Ehrlich *et al*, 1994). The evidence presented thus far, therefore, has remained inconclusive. Considering Gabbiani's experience in the study of myofibroblasts, however, there is a tentative consensus that keloids do not contain myofibroblasts.

1.42 Mast cells and histamine in keloids

Hakanson *et al* (1969) and Cohen *et al* (1972) demonstrated higher than normal levels of histamine (a mast cell product) in keloid scars. Histamine is known to increase collagen deposition and to be a competitive inhibitor of the cross-linking enzyme lysyl oxidase (for review, see Smith *et al*, 1987). This evidence has lead to hypotheses that, although the enzyme is present and active, the increased quantity of histamine present in keloid scars is preventing the enzyme from performing its function.

1.43 The immunology of keloids

The slow development and growth of keloids and the rapid regrowth of the lesion following surgical excision mimics the pattern of an immunological response to antigen; that is, sensitivity to antigen, memory development and adaptive response when reintroduced to the sensitising antigen (Kazeem, 1988). The first evidence for an immune response being involved in keloid formation was presented in 1951 by Mowlem. Mowlem carried out histological analysis of hypertropic scars and keloid lesions and found that there were large numbers of lymphocytes resident in the dermis usually associated with hair follicles. He even went as far as to suggest that the antigen was present in the follicles and that it was probably keratin.

Delayed-type hypersensitivity investigations of keloid formers has led to suggestions that the formation of the keloid scar is a component of an hypersensitivity cell-mediated immune response (for a review of the immunological aspects of keloid formation, see Placik and Lewis, 1992).

Reports documenting the levels of immunoglobulin, complement and MHC molecules have been conflicting and generally inconclusive (see Placik and Lewis, 1992; Cohen *et al*, 1979). Increased immunoglobulin E levels do

appear to correlate with keloid formation, however, and these theories have gone even further and have suggested that mast-cell-derived products, the release of which is stimulated by immunoglobulin E, may play a role in keloid growth.

The fact that human keloid lesions can be transplanted onto the backs of athymic nude mice (the execution of which was an attempt at designing a model), the result of which is a decrease in size suggests an autoimmune component. That is, without immunological stimulation the keloid does not grow. Interestingly, transplantation of keloid lesions between human subjects and even in different anatomical areas of the same patient were unsuccessful, the tissues were not tolerated (Calnan and Copenhagen, 1967).

Mowlem is not the only researcher to nominate an antigen. Suggested antigens for this immunological response have included melanin and collagen (Oluwasanmi, 1974) and sebum (Yagi *et al*, 1979; Fasika, 1992). Despite many attempts, however, neither the nature, the identity nor the origin have been elaborated. And, as yet, there has been no large-scale study to ascertain if keloid formers have any systemic dysfunction in their immune responses or not.

Keloids can take years to grow and the same patients are able to form them in different areas on their bodies, but there are no confirmed reports of full-body, systemic reactions characteristic of an autoimmune response (it is

known that systemic lupus erythematosus (SLE) is more common in black people than in white people and in women more than men; keloids are also more common in black skin than white and in women than men but this phenomenon has generally been explained by the higher number of women having their ears pierced, a situation which is less definitive in the present day. Is it possible that the positive correlation of females and keloid formation has a less obvious association?).

1.44 Cytokines and keloids

Tritiated thymidine experiments adding a variety of cytokines to the culture media sustaining keloid fibroblasts have shown that they reacted as normal fibroblasts to platelet-derived growth factor, transforming growth factor beta 1, interferon gamma and histamine. But when epidermal growth factor was added, the level of thymidine incorporation was enhanced (Kikuchi *et al*, 1995). The keloid fibroblasts in this study produced 4.4 times more collagen than normal fibroblasts. When interferon gamma was added, the production of collagen type I was decreased in both normal and keloid fibroblasts with the difference being more marked in keloids. Transforming growth factor beta 1 increased the production of collagen type I by both cell types. And histamine

increased the production of collagen type I in keloid fibroblasts only. The addition of transforming growth factor beta inhibited the epidermal-growth factor-stimulated growth of normal fibroblasts and fibroblasts from normal scars, but actually increased the growth of fibroblasts explanted from keloids under the same conditions. Tan *et al* (1993*) reported decreased collagen type I gene expression in keloid fibroblasts in response to basic fibroblast growth factor. Babu *et al* (1992) claimed that total protein synthesis by normal dermal fibroblasts increased in response to transforming growth factor beta 1 but had no effect on keloid fibroblasts.

Fibroblasts taken from the expanding edge of the keloid lesion actively express collagen type I genes at a much higher level than they do type III, and the presence of transforming growth factor beta 1 is associated with areas of active collagen type I production (Peltonen *et al*, 1991). The authors of this work suggested that endothelial cells in the area of the keloid lesion expansion were producing transforming growth factor beta 1 which stimulated the autocrine production of more transforming growth factor beta 1 which, in turn, stimulated the fibroblasts to produce excessive collagen type I. Haisa *et al* (1994) reported that keloid fibroblasts were more chemotactically and mitogenically responsive to platelet-derived growth factor than were normal fibroblasts. And that there was no difference in response between the two cell types when epidermal growth factor or fibroblast growth factor were added.

The enhanced response of keloid fibroblasts to platelet-derived growth factor was mediated by increased levels of receptors (almost four or five times more compared with normal fibroblasts).

Another report demonstrated that keloid fibroblasts produced twelve times more collagen than hypertropic scar and normal fibroblasts. And that the administration of transforming growth factor beta increased the level 2.7-fold in keloid fibroblasts without having any effect on hypertropic scar or normal fibroblasts. The addition of neutralising antibodies to transforming growth factor beta decreased the rate of collagen synthesis by keloid fibroblasts by 40% but had no effect on the two other cell types (Younai *et al*, 1994).

1.50 CONCLUSION

It seems that the phenomenon of keloids is best regarded as a problem waiting to be solved. Evidence provided by many studies carried out over many years reveals who is more likely to develop keloids and what the clinical outcome will be. There is no evidence of a systemic abnormality in the characteristics of keloid patient fibroblasts nor in their wound healing ability. But there is a local malfunction which results in excessive collagen deposition. Much more work needs to be carried out on the identification of molecules and mediators present within the keloid and the wound bed which preceded it. It is rather unethical, however, to biopsy patients throughout the healing process whether or not it were possible to guarantee that a keloid would result. And the lack of understanding of keloid aetiology has thus far prevented the development of an *in vivo* model.

1.60 AIMS

Ultimately, the work in this thesis is aimed at 'mapping' keloid scar samples. The first step in this objective is to attempt to identify the cell types present within the lesion and the accompanying dermis through the immunolocalisation of CD cell surface markers. Once it has become possible to suggest the composition of the tissues, it is feasible to locate mediators within the samples and speculate on their roles in keloid formation and propagation.

It is hoped that by the end of the work it will be possible to identify a mediator (or a group of mediators) which is a viable culprit for the orchestration of keloid formation. The candidates selected to identify cells within the tissues are as follows: CD4, CD8, CD30, CD31, CD58; and those mediators selected to study the development and propagation of keloids are interferon gamma, interleukins 2, 4 and 10, epidermal growth factor, platelet-derived growth factor, basic fibroblast growth factor, vascular endothelial growth factor and transforming growth factor beta.

Previous work has demonstrated that the administration of interferon gamma has a therapeutic influence on both keloid lesions and hypertropic scars. And that neutralising the activity of vascular endothelial growth factor

inhibits the growth of tumours. Also to be included in this thesis, are investigations of the effect of neutralising the action of interferon gamma on the production of hydroxyproline and glycosaminoglycans in a model featuring a granulation tissue component and, using the same model, the effect of neutralising the action of vascular endothelial growth factor on the development of new blood vessels. It is hoped that evidence obtained from these studies will permit the proposal of a mechanism responsible for the beneficial effect of the administration of interferon gamma to keloid lesions and whether or not the inhibition of new blood vessel formation could allow the regulation or prevention of keloid growth.

Chapter 2

MATERIALS AND METHODS

CONTENTS

2.10 SUPPLIERS

2.11 Animals

Tuck Original (TO) mice were used throughout.

2.12 Chemicals

Any inorganic chemicals used to prepare buffers and solutions were purchased from Merck Ltd (Analar grade). Industrial methylated spirit (IMS) and all stains and dyes were also supplied by Merck Ltd.

2.13 Antibodies

bFGF (polyclonal rabbit antibovine)	British Biotechnology Ltd
Biotinylated secondary antibodies	Vector Laboratories
EGF (polyclonal rabbit antimurine)	Genzyme
PDGF (polyclonal goat antihuman)	British Biotechnology Ltd
TGF-β (polyclonal chicken antiporcine)	British Biotechnology Ltd
Vectastain elite ABC kit	Vector Laboratories
IFN-γ (monoclonal hamster antimurine)	Genzyme

CD markers (monoclonal mouse antihuman)	Serotec
IL-2, 4, 10 (polyclonal goat antihuman)	R and D Systems
CD30 marker (monoclonal mouse antihuman)	Serotec
IL-2 (monoclonal rat antimurine)	Genzyme
IFN-γ (polyclonal goat antihuman)	R and D Systems
EGF, PDGF, bFGF, TGF-β (polyclonal goat antihuman)	R and D Systems
VEGF (polyclonal rabbit antihuman)	Oncogene Science

2.14 Antigens

VEGF (recombinant human)	R and D Systems
bFGF, EGF, PDGF, TGF-β (recombinant human)	Calbiochem
IL-2, 4, 10 (recombinant murine)	Genzyme
IL-2, 4, 10 (recombinant human)	PeproTech
IFN-γ (recombinant human)	PeproTech
IFN-γ (recombinant murine)	Genzyme

2.15 Miscellaneous

Other biochemical reagents were purchased from Sigma with the exception of the following:

Mycobacterium tuberculosis (stains C, DT, PN)	Central Veterinary Supplies

2.16 Human tissues

Clinical samples were collected from consenting patients being treated at St Bartholomew's and Middlesex Hospitals. The collection of keloid tissues consisted of 111 samples (n = 111); 52% of which were taken from female patients; 70% of which were black skin samples, 20% of which were oriental or asian samples and 10% of which were white samples (approximately). Patient history was unknown as was anatomical area any treatment administered was also unknown. The collection of normal tissues used in these studies consisted of 15 samples (n = 15) and was taken from a variety of anatomical areas, from both sexes and from a variety of skin colours. Normal tissues were mainly removed from patients undergoing cosmetic surgery and were, therefore, assumed to be nonpathological.

2.20 HOUSING AND HUSBANDRY

Female Tuck Original (TO) mice were used throughout (25–30g). Ten animals were housed in each cage. Cages were plastic and lined with sawdust, SDS pellets and water were provided without restriction. Environmental conditions such as humidity, light and temperature were controlled and maintained.

2.30 MURINE AIR POUCH MODEL

The discussion section included in Chapter 3 explains the benefits of the use of this model for work of the type included in this thesis.

2.31 Formation of pouch

On day 0, 3ml air was injected into the dorsal subcutaneous tissue to form a pouch. Twenty-four hours later, 0.5ml 0.1% volume/volume croton oil in Freund's complete adjuvant (5mg/ml *Mycobacterium tuberculosis* [strains C, DT and PN] in Freund's incomplete adjuvant) was injected into the inflated pouch. Animals were allowed to recover and then sacrificed at 3, 7, 14, 21 or 28 days by carbon dioxide inhalation.

2.32 Removal of experimental tissue

The fur on the back of the animal was shaved and the whole pouch carefully dissected, any inflammatory exudate was removed. The upper portion of the pouch was excised and prepared for histological study (2.80) and the lower portion for biochemical analysis (2.70), or the whole pouch was recovered and prepared for dry weight investigation (dried in oven for 2 days at 56° C).

2.40 INVESTIGATING MURINE AIR POUCH VASCULARITY (Kimura *et al*, 1985)

2.41 Tissue digestion

The whole pouch was carefully dissected from the animal and placed in digestion buffer (12 units/ml papain, 1mM ethylenediaminotetracetic acid [EDTA] in 0.05M phosphate buffer [containing KCl and KH_2PO_4] pH7.0) at 56°C for 2 days (1ml aliquots were used in assays).

2.42 Formation of vascular cast (Modified from Kimura *et al*, 1986)

Animals with a 7 day air pouch (2.30) were anaesthetised with 0.5ml Hypnorm/Hypnovel intraperitoneally (1 part Hypnorm, 1 part Hypnovel, 2 parts water). 1ml 3% carmine solution (10% carmine dye, 5% gelatine) was injected into the tail vein and the carcasses chilled to solidify the gelatine. The pouch was then recovered and digested (2.41), 1ml 5M sodium hydroxide was added to solubilise the dye and the samples then diluted 1:20 with 5M sodium hydroxide. Particulates were removed using 0.22μm Whatman filters and 200μm of each sample placed in a 96-well plate. The amount of carmine present was measured using a Biotek (model EL310) microplate reader at 490nm.

2.43 Air pouch clearing

Animals with a 7 day air pouch were injected with carmine (2.42), the dorsal fur was shaved and the top area of the pouch excised. Tissues were washed in Hanks buffered saline solution and immersed in 10% formal saline (4% formaldehyde in 0.9% aqueous sodium chloride) for 2 days. The tissues were then dehydrated using ascending concentrations of alcohol and cleared in Cedar Wood oil for 2 weeks.

2.50 INVESTIGATING MURINE AIR POUCH TISSUE GLYCOSAMINOGLYCAN (GAG) CONTENT

A standard curve was constructed using chondroitin sulphate with digestion buffer (2.41) as diluent. 10µl aliquots of digested samples (2.41) were mixed with 200µl DMB solution (16mg 1,9-dimethylmethylene blue chloride in 5ml IMS and 95ml formate buffer, 2g sodium formate, 19ml 90% formic acid made to 1 litre using distilled water) in wells of a 96-well plate. The plates were allowed to stand for 1 minute and absorbance read using a Biotek (Model EL310) microplate reader at 535nm.

2.60 INVESTIGATING MURINE AIR POUCH TISSUE HYDROXYPROLINE (HPr) CONTENT (Woessner, 1961)

A standard curve was constructed using hydroxyproline diluent (10ml 4M sodium hydroxide with 10ml 0.47M citric acid). Digested samples (2.41) were centrifuged at 700g for 10 minutes and 100µl aliquots evaporated overnight at 80–100°C. Samples were reconstituted using 100µl 4M sodium hydroxide and agitated until mixed. Samples were incubated at 120°C for 3 hours and evaporated overnight then reconstituted with 100µl 4M sodium hydroxide and 100µl 0.47M citric acid. 25µl of each sample were placed into the wells of a 96-

well plate and 125µl of chloramine T added (0.14g chloramine T, 1ml propan-2-ol, 1ml distilled water, 8ml citrate buffer: 10g citric acid monohydrate, 14.5g anhydrous sodium acetate, 6.8g sodium hydroxide in 150ml water. 2.4ml glacial acetic acid was added. pH adjusted to 6.0 and solution made to 200ml. Stored under toluene at 4°C) before a 20 minute incubation at room temperature. 125µl DMAB/perchloric acid solution was added (1.7g dimethylaminobenzaldehyde [DMAB] was dissolved in 7ml propan-2-ol. 3ml 60% perchloric acid added and the solution maintained at 65°C to prevent precipitation) followed by a 15 minute incubation at 65°C. Absorbance was read using a Biotek (Model EL310) microplate reader at 560nm..

2.70 BIOCHEMICAL ANALYSIS: WESTERN BLOTTING

The rationale for using this method of biochemical analysis are outlined in Chapter 1.

2.71 Sample preparation

Air pouch tissues (2.30) were dissected and the lower part homogenised in 1ml of 1mM phenylmethylsulphonyl fluoride, 70mg/ml pepstatin A, 0.01% leupeptin (weight/volume) in 50mM Tris (6.06g Tris HCl and 1.39g Tris Base in 1 litre of

water), pH 7.4 before 1ml gel loading buffer was added (50mM Tris HCl, 10% SDS, 10% glycerol, 10% 2-mercaptoethanol, 2 mg/ml bromophenol blue) and the samples boiled for 3 minutes. The samples were centrifuged at 10 000g for 10 minutes and the protein content calculated, see below.

2.72 Assay to measure protein content of air pouch samples (Bradford, 1976)

A standard curve was constructed using bovine serum albumin (BSA) and sample homogenising buffer (2.71). 10µl of each sample was placed in the wells of a 96-well plate and 200µl Bio-Rad Protein Assay Dye Reagent added (made up according to instructions). Absorbance was measured at 595nm using a Biotek (Model EL310) microplate reader. Results are expressed as mg/ml of protein.

2.73 Electrophoresis of air pouch samples

Electrophoresis was executed as instructed using the Bio-Rad Mini-Protean II Electrophoresis Cell on a 15% discontinuous (Laemmli) polyacrylamide gel. Combs used were 0.75mm thick and contained ten 36µl wells. Equivalent quantities of protein (20µl, 1mg/ml concentration) were loaded into each well to

allow comparison of samples. Gels were run at 90V for 5 minutes and then at 180V for 45 minutes, or until the dye front had reached the base of the gel.

2.74 Transfer of proteins from gels

Transfer was executed as instructed using the Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell. Blotting media used was 0.45μm nitrocellulose membrane. Transfers were run at 100V for 1 hour.

2.75 Immunoblot detection of transferred VEGF antigen

The nitrocellulose membrane containing the transferred antigens was incubated overnight in 5% dried nonfat milk in TBS (200mM Tris HCl, 500mM sodium chloride, pH7.5) to block free protein binding sites. Membranes were washed in TTBS (TBS with 0.05% Tween-20) with gentle agitation, for 5 minutes, 3 times before incubation with the primary antibody diluted in TBS to the optimum concentration (x500) and refrigerated overnight. The membranes were washed as before and biotinylated secondary antibody applied (diluted 1:200 in TBS). Two hours later, the wash step was repeated and the streptavidin-biotinylated alkaline phosphatase solution applied (diluted 1:200 in TBS) for a further 2 hours. Following incubation with the alkaline phosphatase complex, membranes

were washed thoroughly in TTBS (15 minutes, 3 times). The substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) was prepared according to instructions and applied for 10–30 minutes, or until colour development was complete. The development reaction was stopped using distilled water.

2.80 HISTOLOGICAL ANALYSIS

2.81 Tissue preparation

1cm^2 pieces of tissue taken from the upper most part of the air pouch (2.30), or from human samples, were frozen in n-hexane (precooled in liquid nitrogen) and stored under liquid nitrogen until use.

2.82 Wax embedding

1cm^2 pieces of tissue taken from the upper most part of the air pouch (2.30), or from human samples, were fixed in 10% formal saline for 48 hours. The tissues were rinsed in 70% aqueous alcohol and then emmersed in 70% aqueous, 90% aqueous and 100% pure alcohol for 24 hours each and two changes of toluene

for 48 hours each. Wax embedding was carried out at 56%. Tissues were sectioned at 4μm on a base-sledge microtome (Leitz).

2.83 Cryotomy

Tissues were attached to brass cryostat chucks using OCT chilled in dry ice. Chucks were then mounted in a Bright Cryostat (Model 5030), cooled before use to -25°C. 7μm sections were cut, thaw-mounted onto TESPA (3-aminopropyltriethoxy silane)-coated 4-spot PTFE slides (5 minutes in acetone, 5 minutes in 4% TESPA in acetone and air dried) and stored at -20°C until use.

2.84 Fixation (Bancroft and Stevens, 1982)

Sections were fixed in 4% paraformaldehyde (prepared by heating 8% aqueous paraformaldehyde solution to 60°C, concentrated sodium hydroxide was added until the solution cleared. The mixture was allowed to cool and an equal volume of 0.2M phosphate buffer pH7.6 added) for 1 hour and then rehydrated using distilled water.

2.85 Histological stains

Van Gieson's

Sections were stained with celestine blue for up to 15 minutes, or until nuclei were intensely stained, and washed in tap water. This was followed by 5 minutes in Mayer's haematoxylin and a tap water wash. Slides were then rinsed in distilled water before being stained for 2 minutes with Van Gieson's solution and rinsed again. Tissues were dehydrated using ascending concentrations of alcohol, cleared in xylene and mounted using DPX.

Stains mature collagen fibres deep red, muscle and fibrin yellow and nuclei black.

Haematoxylin and eosin

Sections were stained with Harris's haematoxylin for 5 minutes and washed with distilled water. Staining was differentiated using acid/alcohol (70% aqueous alcohol, 1% concentrated hydrochloric acid) and the slides washed in tap water until a blue colour developed. Sections were stained with 1% aqueous eosin Y for 1 minute and then washed for 5 minutes in tap water. Tissues were

dehydrated using ascending concentrations of alcohol, cleared in xylene and mounted using DPX.

Stains nuclei blue, extracellular matrix components and cytoplasm various shades of pink.

Toluidine blue

Unfixed sections were dipped in toluidine blue (0.25%) in veronal acetate buffer (0.1M pH4.5) for 10 seconds. Sections were then washed in distilled water, dehydrated and mounted.

Stains glycosaminoglycans purple, nuclei and other cell components blue and mast cell granules pink.

Acridine orange

Unfixed sections were immersed in one part acridine orange (0.1%) in distilled water, nine parts Krebs–Ringer solution (pH6.2) for approximately 15 minutes. The sections were blotted dry and a drop of Krebs–Ringer solution used as mountant.

Fluorescent staining of nuclei and fibrous tissue green, nucleoli and cytoplasmic nucleic acids red. Black background.

2.90 IMMUNOHISTOLOGIAL ANALYSIS

Immunocytochemistry (the use of labelled antibodies as specific reagents for the localisation of antigens *in situ*) was extensively used in the research for this thesis because it allows the visualisation of cells and mediators *in situ*. That is, one aim of the work in this thesis was to identify cells within the tissue samples and any mediators present as well as their location. It was hoped that this evidence could be used to speculate on the possible mechanisms of keloid formation and propagation.

2.91 Tissue preparation

As 2.81

2.92 Cryotomy

As 2.83

2.93 Fixation

Sections were fixed in acetone for 10 minutes and allowed to air dry before use.

2.94 Immunohistological staining

Following rehydration of the tissues in 10mM phosphate buffered saline pH 7.4 ([PBS] 80g NaCl, 2g KCl, 11.5g Na_2HPO_4, 2g KH_2PO_4 in 10 litres of water), endogenous peroxidases were quenched (0.3% hydrogen peroxide in methanol) for 30 minutes and the sections washed for 5 minutes, 3 times in PBS. The appropriate serum (diluted 1:200 in 0.1% essentially globulin free BSA in PBS) was used to block nonspecific binding of immunoglobulin G for 30 minutes. Primary antibody diluted to optimum concentration (in 0.1% sodium azide, 0.1% Triton X100, 0.01% BSA in PBS) was applied and the sections incubated at 4°C overnight. Sections were then washed for 5 minutes, twice in PBS containing 0.1% Triton X100 and once in PBS before being incubated for a further 30 minutes in a biotinylated secondary antibody diluted 1:200 in PBS containing 1.5% normal serum. Following incubation with the secondary antibody, sections were washed for 5 minutes, twice in PBS containing 0.1% Triton X100, once in PBS and incubated for 30 minutes with Vectastain ABC horseradish peroxidase (made up according to instructions in 5ml PBS

containing 0.5M sodium chloride). Unbound complex was removed by washing for 5 minutes, 3 times in PBS and then 5 minutes in 0.05M Tris HCl pH7.6. The substrate, 3,3'-diaminobenzidine tetrahydrochloride (DAB) was prepared according to instructions (10mg DAB in 20ml 0.05M Tris HCl pH7.6 with 3.4 weight/volume hydrogen peroxide) and applied for 1–5 minutes, until colour development was complete. The development reaction was stopped using water. Tissues were counterstained using Mayer's haematoxylin, washed in tap water, differentiated using acid/alcohol (70% aqueous alcohol, 1% concentrated hydrochloric acid), dehydrated in ascending concentrations of alcohol, cleared in xylene and mounted using DPX.

2.100 *IN SITU* END LABELLING

2.101 Tissue preparation

As 2.81

2.102 Cryotomy

As 2.83

2.103 Fixation

Sections were fixed in 10% buffered formalin for 10 minutes, post-fixed in ethanol:acetic acid (2:1) for 5 minutes at -20°C and used immediately.

2.104 *In situ* end labelling

In situ end labelling was carried out as instructed using the ApopTag *In Situ* End Apoptosis Detection Kit – Peroxidase. Sections were counterstained using Mayer's haematoxylin.

2.110 STATISTICS

A statistics package for social sciences was used in the analysis of *in vivo* experimental data. Statistical significance calculated using the Mann–Whitney U-test. The Mann–Whitney test is a nonparametric statistical test which identifies differences between two conditions of one independent variable (Hinton, 1995).

Quantitative data collected from immunohistochemical analysis were analysed without the aid of a computer package (Rowntree, 1991; Bland, 1996);

the statistical significance was determined using the Mann-Whitney test. Results are included in the relevant areas of the text.

Chapter 3

CHARACTERISATION OF MURINE AIR POUCH TISSUES AND HUMAN TISSUES

CONTENTS

3.10 INTRODUCTION

Because the profile of normal wound resolution in the murine air pouch model is to be used to illustrate the changes in vascularity and glycosaminoglycan and hydroxyproline content in a 'normal' response, as well as for comparison with the abnormal human keloid tissues, it is necessary to describe aspects of the model in detail. Such aspects include the appearance and behaviour of various cell types, cytokine involvement, vascularity development and glycosaminoglycan and hydroxyproline distribution. This chapter aims to describe the tissues and to establish those facts which will aid the interpretation of the results of subsequent experiments.

3.11 The normal murine air pouch model timecourse

This model was originally developed to study the role of angiogenesis in granulation tissue formation and resolution (Kimura *et al*, 1985). Since then it has been used to study many processes including granulation tissue contraction (Appleton *et al*, 1992) and the effects of transforming growth factor beta on matrix deposition (Appleton *et al*, unpublished data). It is an immune driven model and has a strong T cell component. A study by

Appleton and co-workers (1993) on the spatial and temporal distribution of cytokines throughout the timecourse furthered the understanding of the roles of these mediators in granulation tissue formation and wound resolution.

The execution of the murine air pouch model was described in Chapter 2. The tissue dry weight, carmine content and vascularity are displayed in Figures 3.1, 3.2 and 3.3 (Appleton *et al*, 1993).

Tissue dry weight (mg)

Granulation tissue dry weight was 47.7 ± 3.8mg at day 3 and rose to a peak at day 7 (120.9 ± 4.4mg), tissue was harvested at this timepoint and used for the subsequent vascularity experiments. Following day 7 the granulation tissue dry weight fell gradually to 51.4 ± 3.4mg at day 28 (Figure 3.1).

Carmine content (mg)

Carmine content at day 3 was 0.45 ± 0.21mg. The peak of carmine content occurred at day 5 (0.99 ± 0.06mg) and then fell steadily to day 28 (0.43 ± 0.02mg; Figure 3.2).

Vascularity (μg/mg)

Vascularity of air pouch tissues also peaked at day 5 (14.84 ± 0.5μg/mg) after a level at day 3 of 9.59 ± 0.33μg/mg. The vascularity fell sharply at day 7 (8.14 ± 0.2μg/mg) and wavered thereafter through to day 28 (8.34 ± 0.2μg/mg; Figure 3.3).

Figures 3.4, 3.5, 3.6 illustrate the histology of the murine air pouch at days 3, 7 and 28, respectively.

Day 3 histology

At day 3 the murine air pouch tissues displayed large concentrations of inflammatory cells which were predominantly polymorphonuclear neutrophils but also included macrophages, fibroblasts and some lymphocytes. Blood supply to the granulation tissue was provided by capillaries which were probably derived from existing vessels and were seen mainly in the area of granulation tissue just beneath the skeletal muscle layer. The extracellular matrix displayed a small quantity of collagen fibres in the area of granulation tissue close to the skeletal muscle layer. Mast cells were present in the dermis, some were degranulated, although very few were seen in the granulation tissue (Appleton *et al*, 1993; Figure 3.4).

Day 7 histology

Maximum tissue dry weight occurred day 7 (this was evident by the increase in depth of tissue). The granulomatous/granulation tissue itself was heterogeneous in terms of cell type distribution throughout the depth of the tissue. The area bordered by the skeletal muscle featured a loosely packed matrix embedded with fibroblasts and macrophages, blood vessels were sparse

compared with the neighbouring granulomatous/granulation tissue region, the cellular nature of which was approximately the same as that of day 3 (Appleton *et al*, 1993; Figure 3.5).

Day 14 histology

At day 14, individual regions in the granulation tissue were more pronounced. The region below the skeletal muscle layer featured a more densely packed matrix and increased numbers of embedded fibroblasts; some macrophages and lymphocytes were also present. The blood vessels were more prominent than at day 3 and the neighbouring granulation tissue region was similar in appearance to that of day 7. The area of granulation tissue bordering the pouch cavity appeared to be approximately the same in nature as that of day 3 (Appleton *et al*, 1993).

Day 21 histology

At day 21 different regions were evident in the granulation tissue layer, with the area below the skeletal muscle featuring a fibrotic extracellular matrix

containing numerous blood vessels, although the area was not densely packed there were many fibroblasts and increased numbers of mast cells. The amount of collagen had increased and there were many fibroblasts and some macrophages and lymphocytes. The tissue region close to the pouch interface resembled that of day 3 (Appleton *et al*, 1993).

Day 28 histology

By day 28 the granulation tissue area below the skeletal muscle layer had increased in depth and mature blood vessels were evident. The number of mast cells throughout the granulation tissue had decreased and collagen fibres appeared to be more numerous and more mature (Appleton *et al*, 1993; Figure 3.6).

Figure 3.1 A graph to show the total dry weight (mg) of murine air pouch tissues sampled throughout the timecourse of 3–28 days

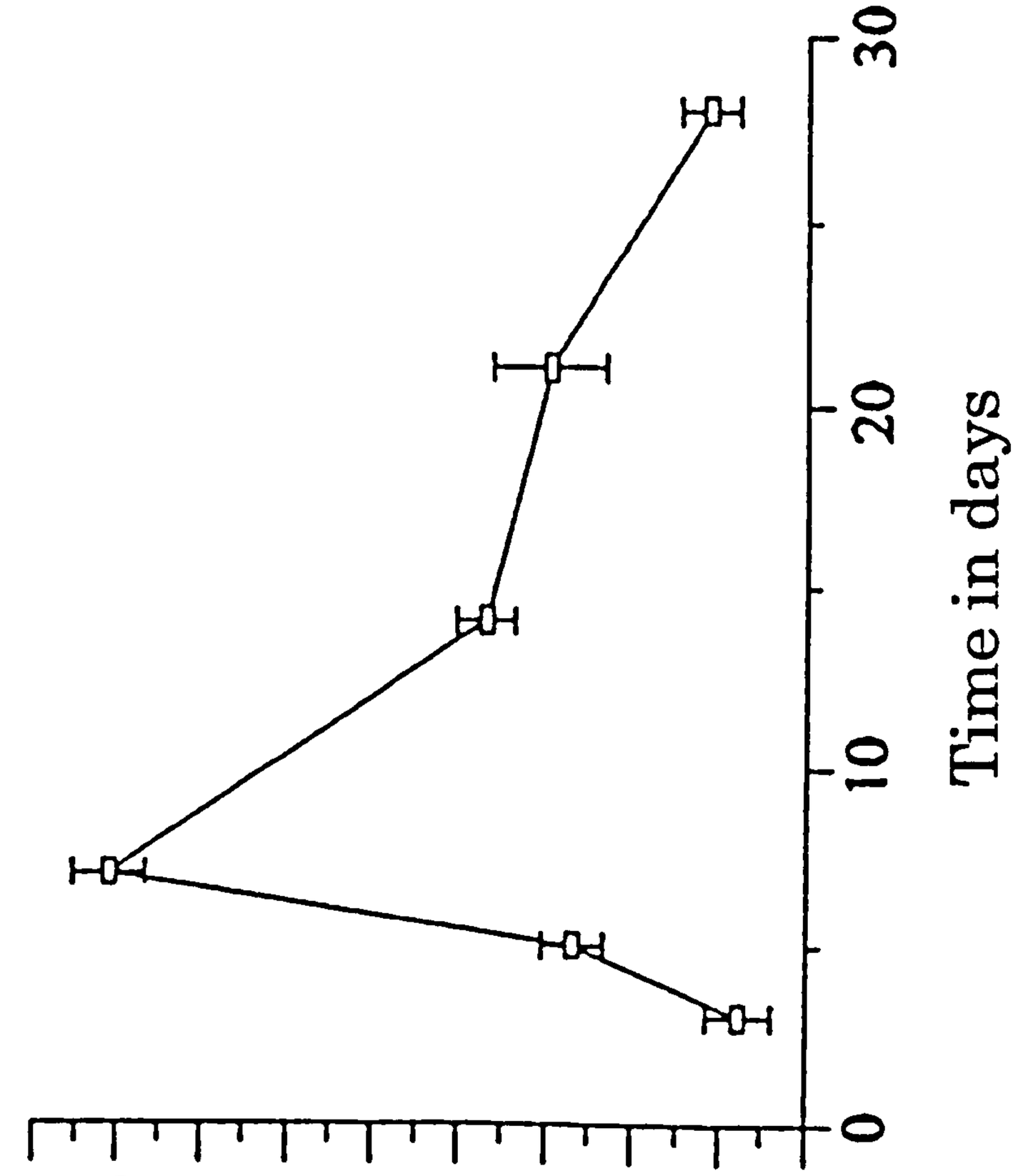


Figure 3.2 A graph to show the total carmine content (mg) in murine air pouch tissue samples taken throughout the timecourse of 3–28 days

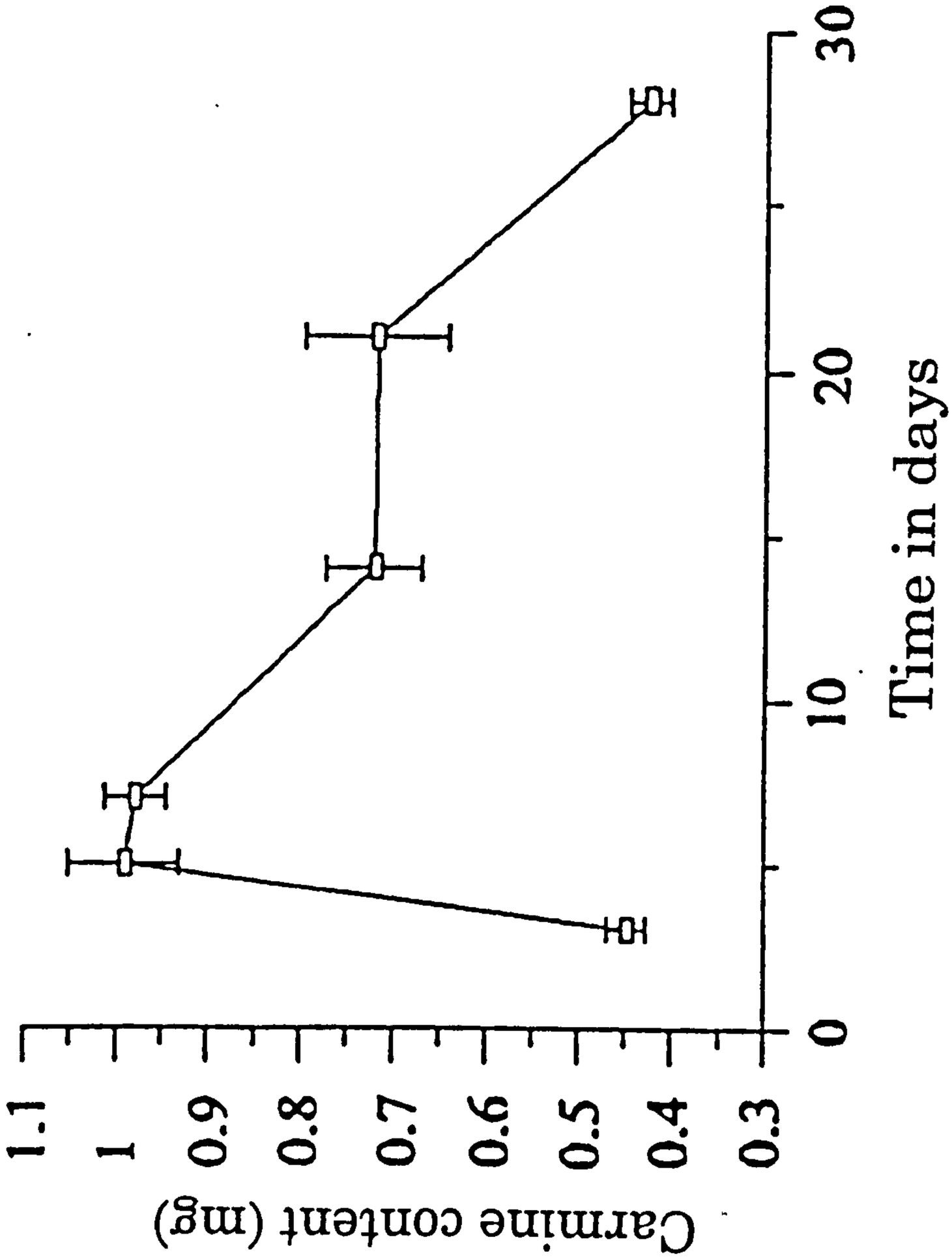


Figure 3.3 A graph to show the ratio of carmine (μg) to tissue dry weight (mg) in murine air pouch tissues taken throughout the timecourse of 3–28 days

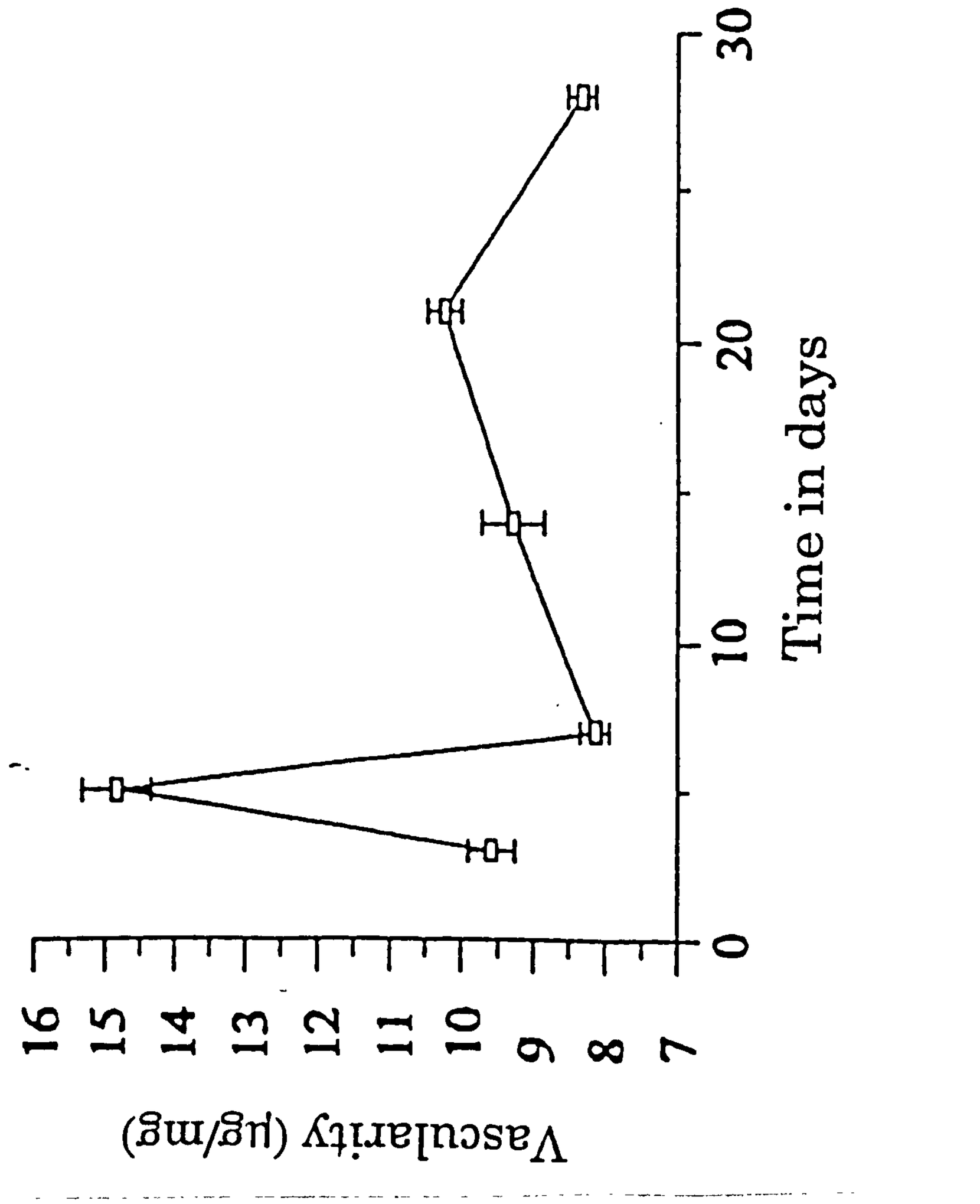


Figure 3.4 A micrograph to illustrate the histology of murine air pouch tissue sampled at day 3 of the timecourse (magnification x20, representative of n=12)

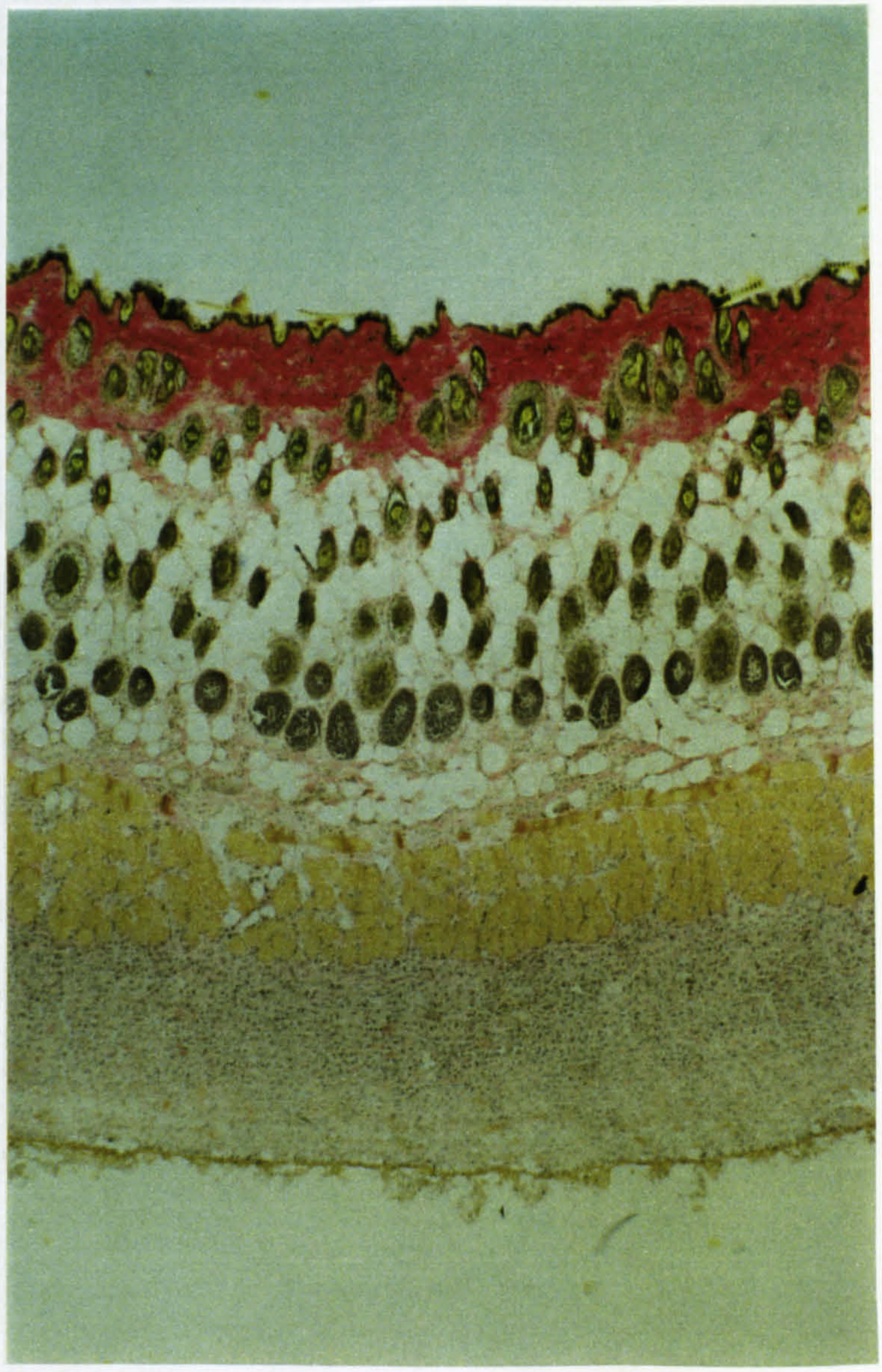


Figure 3.5 A micrograph to illustrate the histology of murine air pouch tissue sampled at day 7 of the timecourse (magnification x20, representative of n=12)

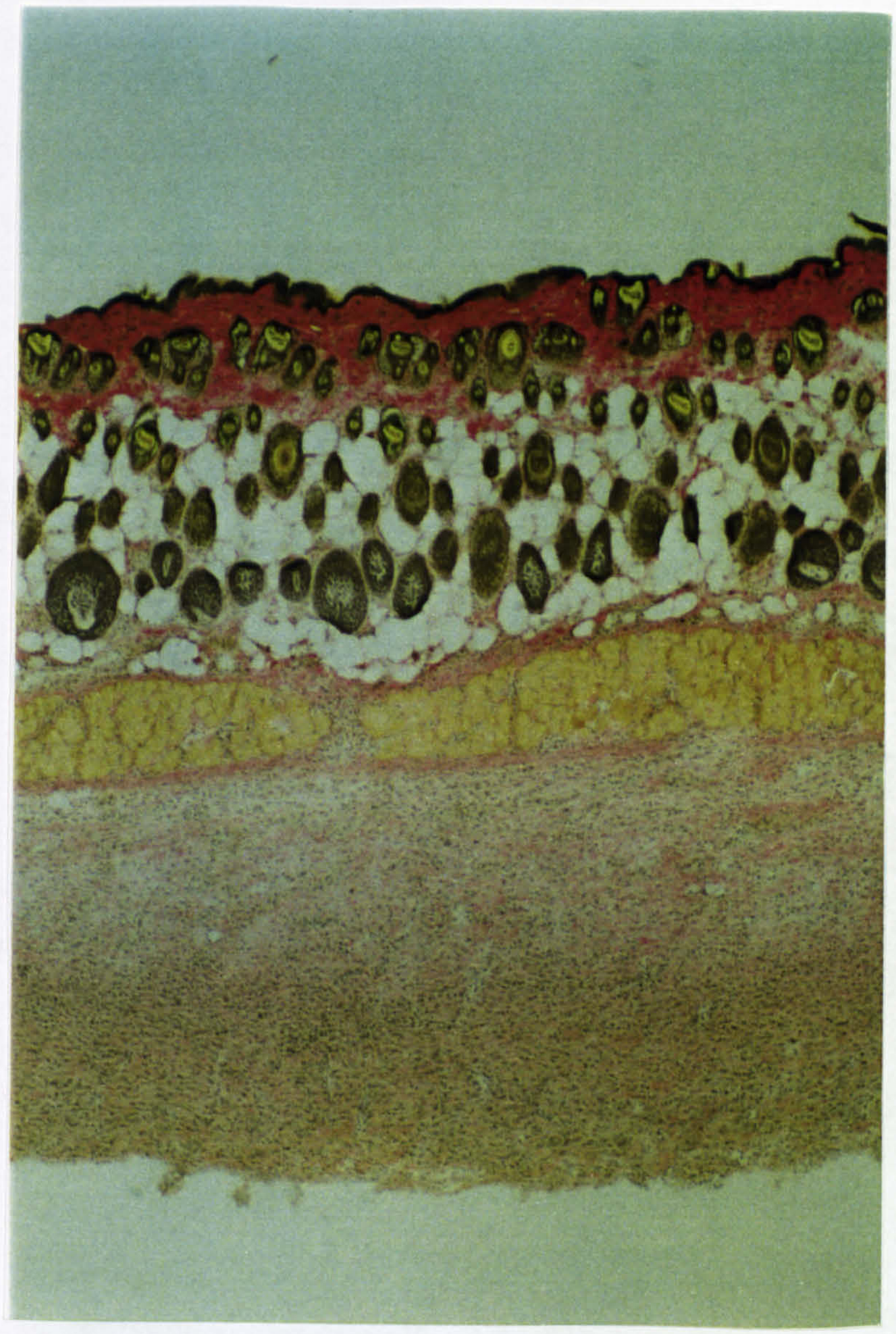
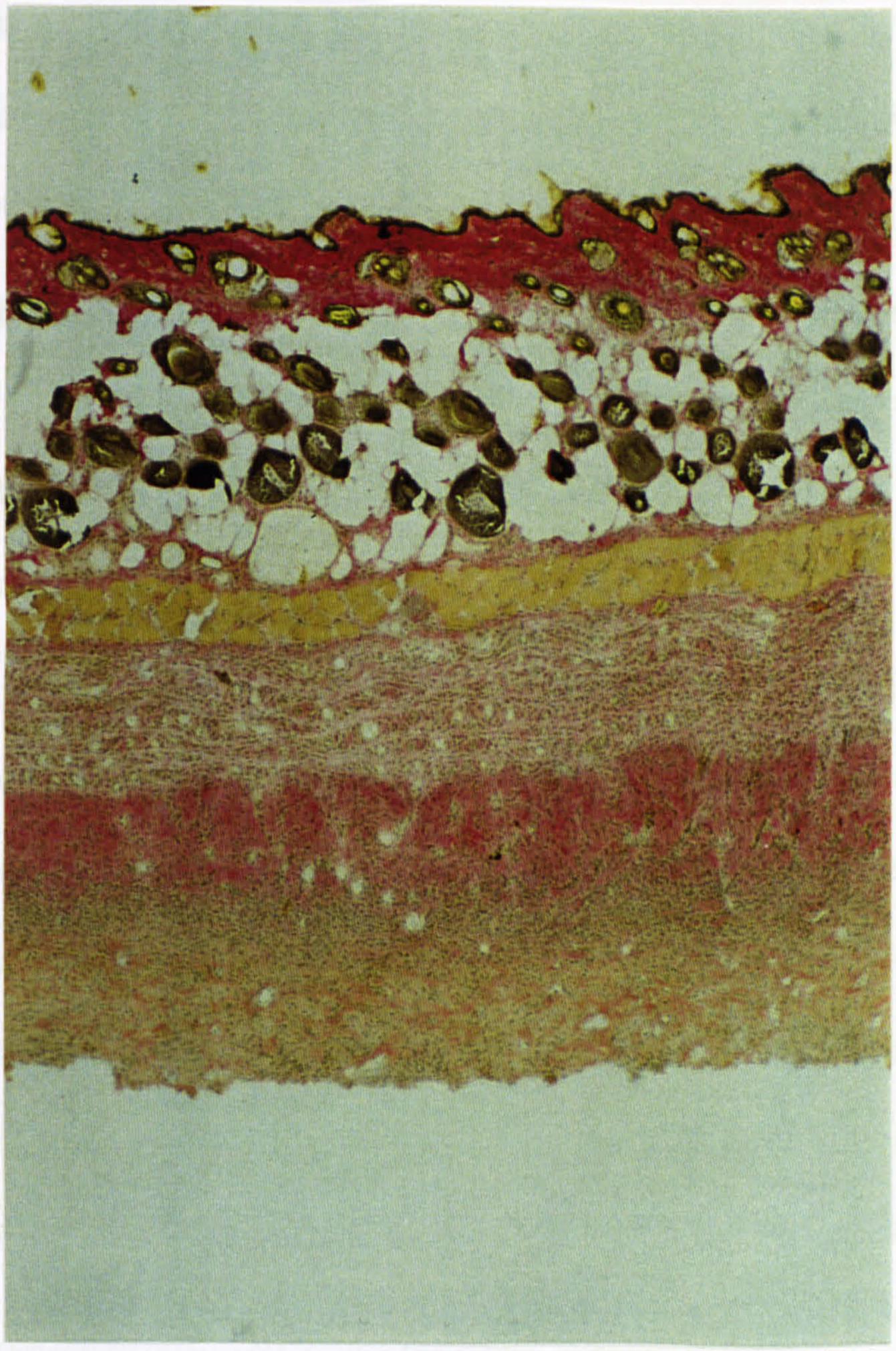


Figure 3.6 A micrograph to illustrate the histology of murine air pouch tissue sampled at day 28 of the timecourse (magnification x20, representative of n=12)



3.12 CD4 and CD8 markers in murine air pouch tissues

The cell surface marker CD4 is involved in the recognition by T cells of foreign antigens in association with MHC class II molecules. The administration of CD4 antibodies inhibits the functions of T cells *in vivo* and *in vitro* (Barclay, 1994). CD4 is expressed by those peripheral blood T cells which are CD8 negative (approximately two thirds of the total).

CD8 is involved as a co-receptor with those T cell receptors restricted to MHC class I molecules in the recognition of foreign antigens. It is expressed by those peripheral blood T cells which are CD4 negative (approximately one third of the total).

Studies of normal murine skin samples (see Table 3.1) showed that almost one half of the dermal hair follicles contained some $CD4^+$ T cells. By day 3 there were numerous cells in the dermis (more than $CD8^+$ T cells) and throughout the granulomatous/granulation tissue. A general increase in the number of $CD4^+$ T cells was evident at day 7, and remained consistent through day 14 (Figure 3.7). By day 21, $CD4^+$ T cells numbers had decreased in the dermis but had increased in the granulomatous/granulation tissue, particularly around the lower area of the tissue close to the pouch. $CD4^+$ T cells were

fewer in number in the granulomatous/granulation tissue by day 28, and were approximately equivalent to the pattern seen at day 3.

Figures 3.7 and 3.8 illustrate the distribution of CD4 and CD8 lymphocyte markers at day 14, respectively.

Figure 3.7 A micrograph to illustrate the immunolocalisation of the CD4 cell surface marker in a day 14 murine air pouch tissue (magnification x50; dilution 1:50; representative of n=12)

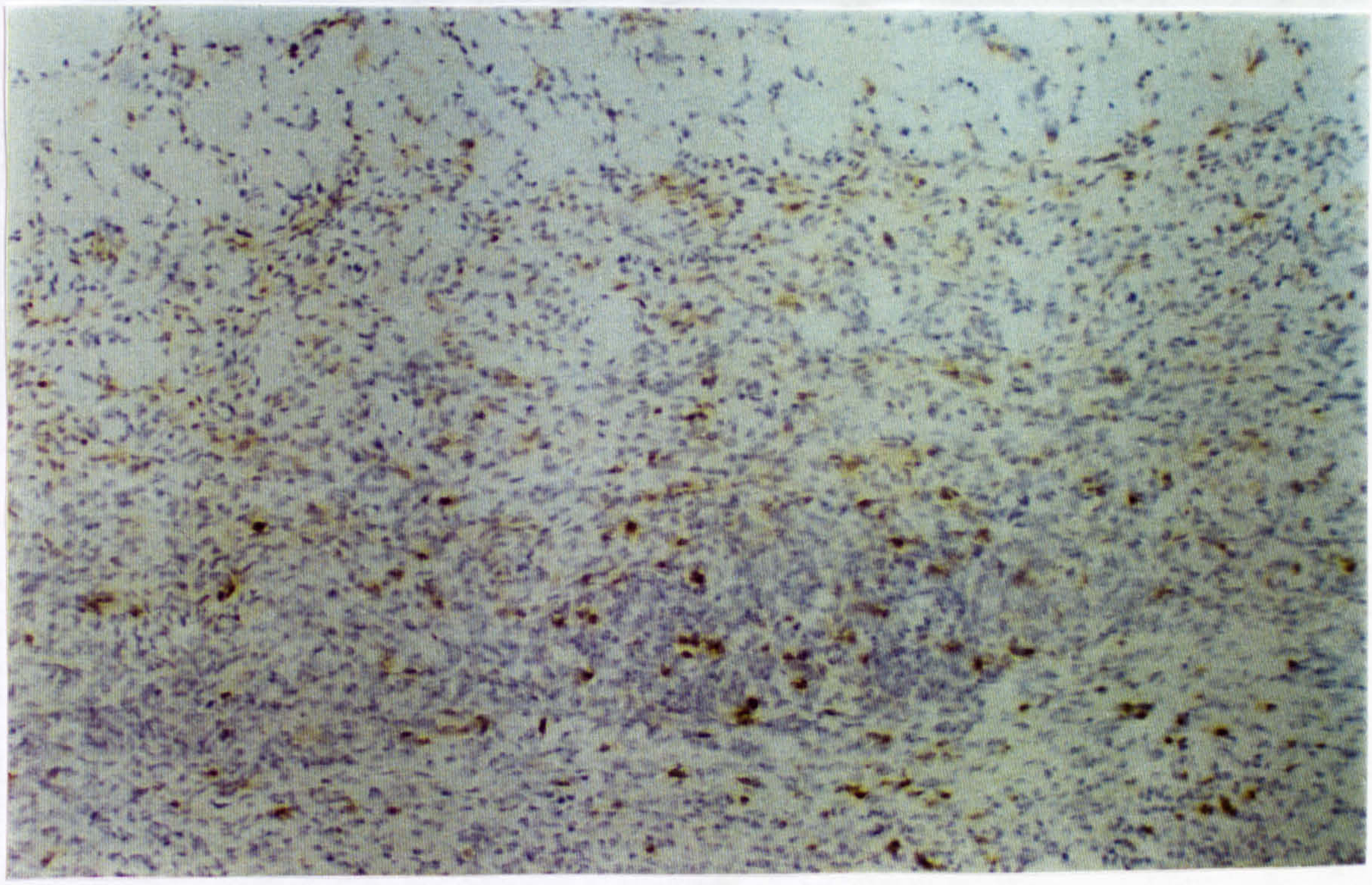
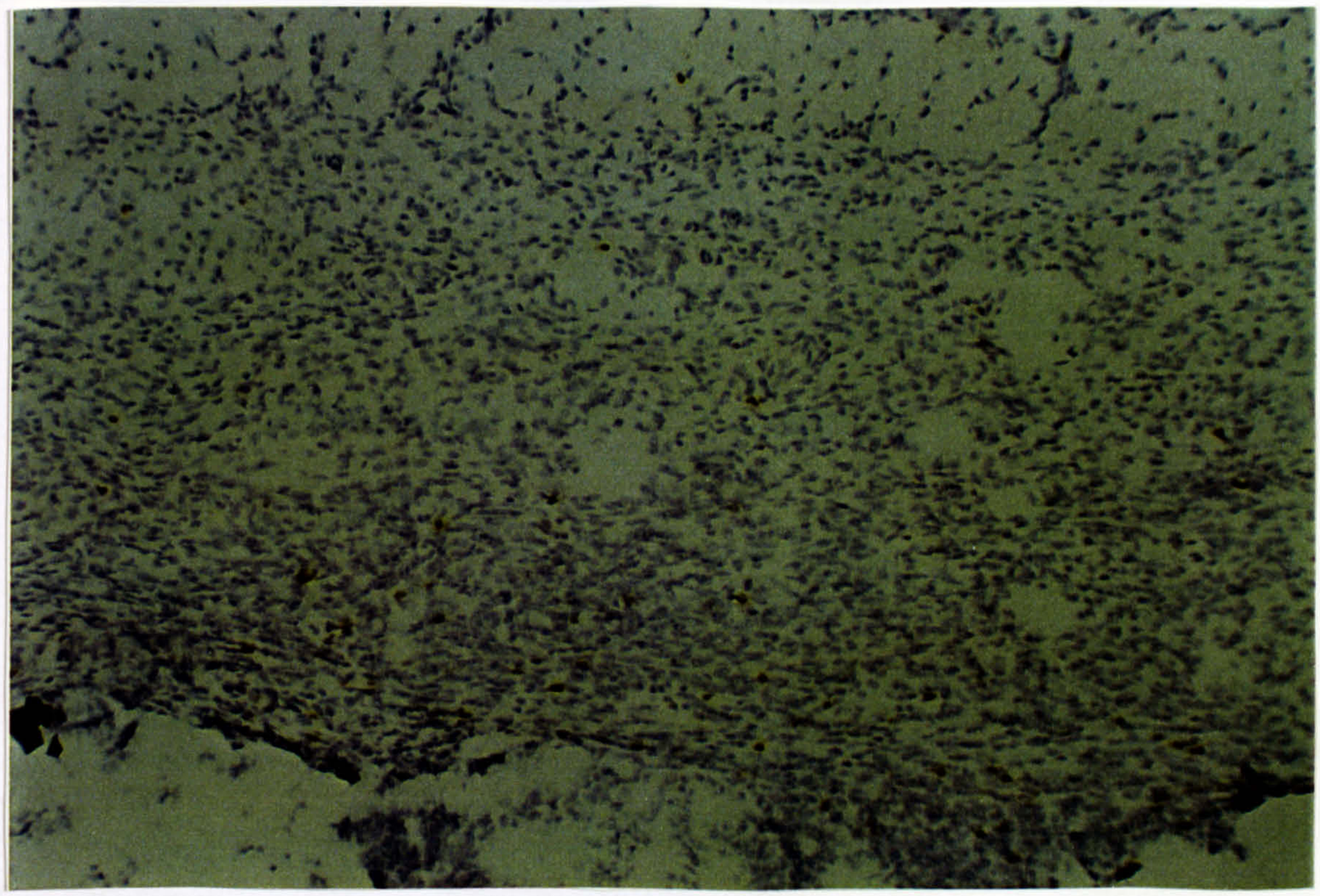


Figure 3.8 A micrograph to illustrate the immunolocalisation of the cell surface marker CD8 in a day 14 murine air pouch tissue (magnification x50; dilution 1:50; representative of n=12)



Control murine skin samples displayed isolated single $CD8^+$ T cells in dermal hair follicles, although the numbers were almost negligible. By day 3 there had been a small increase in numbers of $CD8^+$ T cells in the dermis and there were now some present in the granulomatous/granulation tissue. By day 7 there were more $CD8^+$ T cells in the granulomatous/granulation tissue, in the area close to the pouch cavity, and some in the dermis, a pattern which was consistent through to day 14 (Figure 3.8). At day 21 less immunolabelled $CD8^+$ T cells were seen in the dermis; numbers in the granulomatous/granulation tissue were approximately the same as for day 14. The number of $CD8^+$ T cells present at day 28 was more than that seen at day 3 but was less than that at day 7, and the distribution was more diffuse.

*Table 3.1: Quantitative data for the immunolocalisation of cell surface markers CD4 and CD8 in the murine air pouch timecourse. Results are expressed as the mean and standard error (±) of positively stained cells in n = 4 tissues, 10 fields of view per tissue (4mm^2, x20). The Mann-Whitney U-test was used to investigate statistical significance ($P < 0.05$ = *), groups were compared with day 3 data*

Day	Region	CD4	CD8
3	D	49.0±2.9	4.0±0.8
3	GT	29.8±3.6	6.8±1.0

7	D	28.0±1.4*	7.0±1.8
7	GT	65.8±4.1*	32.3±2.5*
14	D	29.5±2.4*	15.3±2.5*
14	GT	106.0±5.9*	39.0±5.5*
21	D	21.5±2.1*	6.3±1.5
21	GT	134.0±6.7*	38.8±3.9*
28	D	50.5±6.0	5.8±2.2
28	GT	30.0±5.7	14.8±3.1*

(D, dermis; GT, granulation/granulomatous tissue)

3.13 Human tissues

The accessory structures of the skin, such as hair follicles, sebaceous glands, sweat glands and hairs, mainly lie in the dermis (Figure 3.10).

B cells are rarely found in normal control skin samples, whereas T cells (normally T helper cells) are present in the dermis usually associated with hair follicles, sebaceous glands, sweat glands or blood vessels and the epidermis. Generally, 'free' lymphocytes are not seen. Small numbers of macrophages are present, and are spread diffusely (Martin and Muir, 1990). Dendritic epidermal T cells found in association with keratinocytes of the epidermis express the γ/δ antigen receptor (Havran *et al*, 1991).

Figures 3.9 and 3.10 represent sections through normal human tissue sampled from the chest area of a caucasian patient.

Figure 3.11 represents a cross-section though a typical keloid sample taken from the face of a black patient.

Figure 3.9 A micrograph to illustrate the histology of normal human skin (magnification x20, representative of n=15)

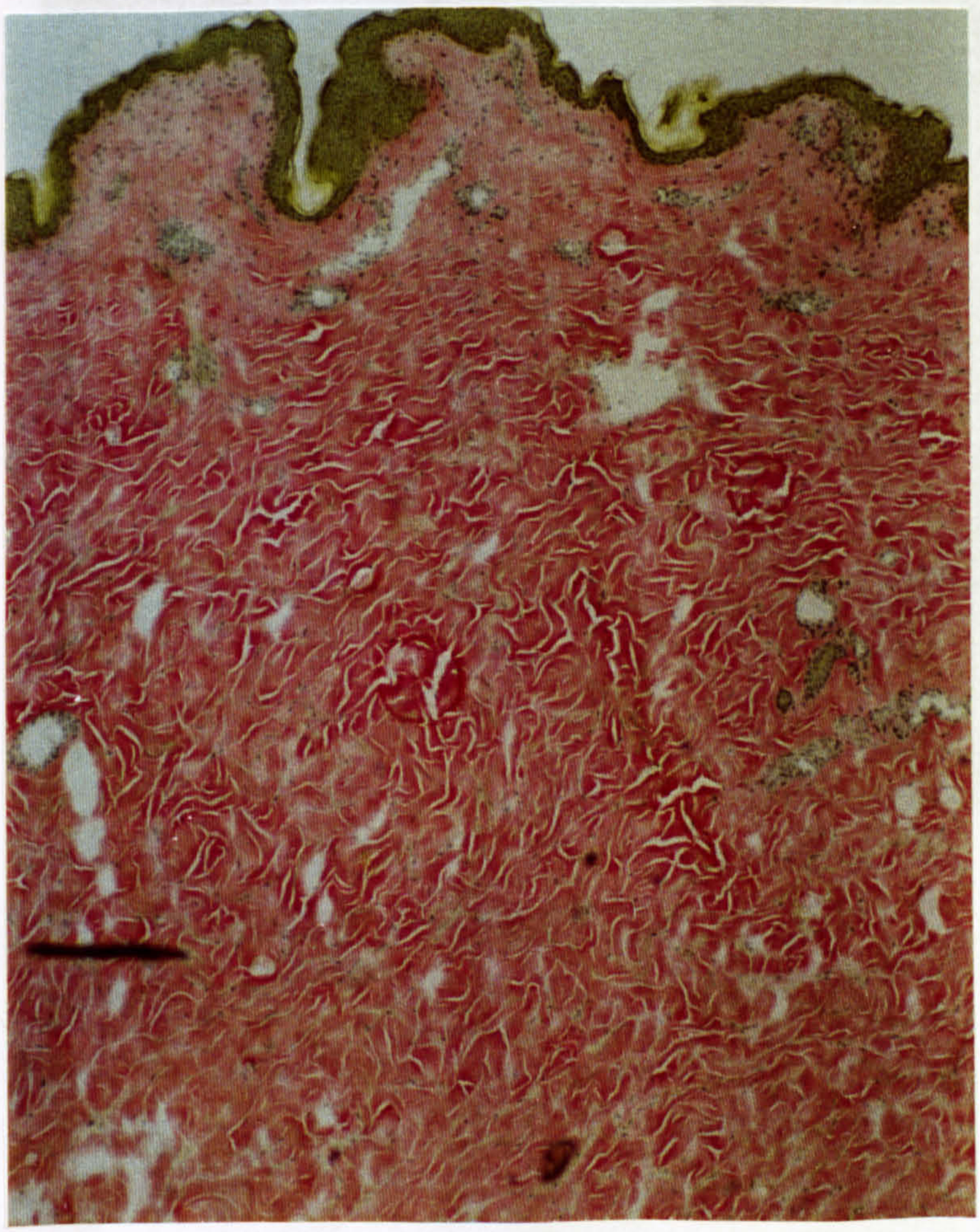


Figure 3.10 A micrograph to illustrate the histology of normal human skin (magnification x20, representative of n=15)

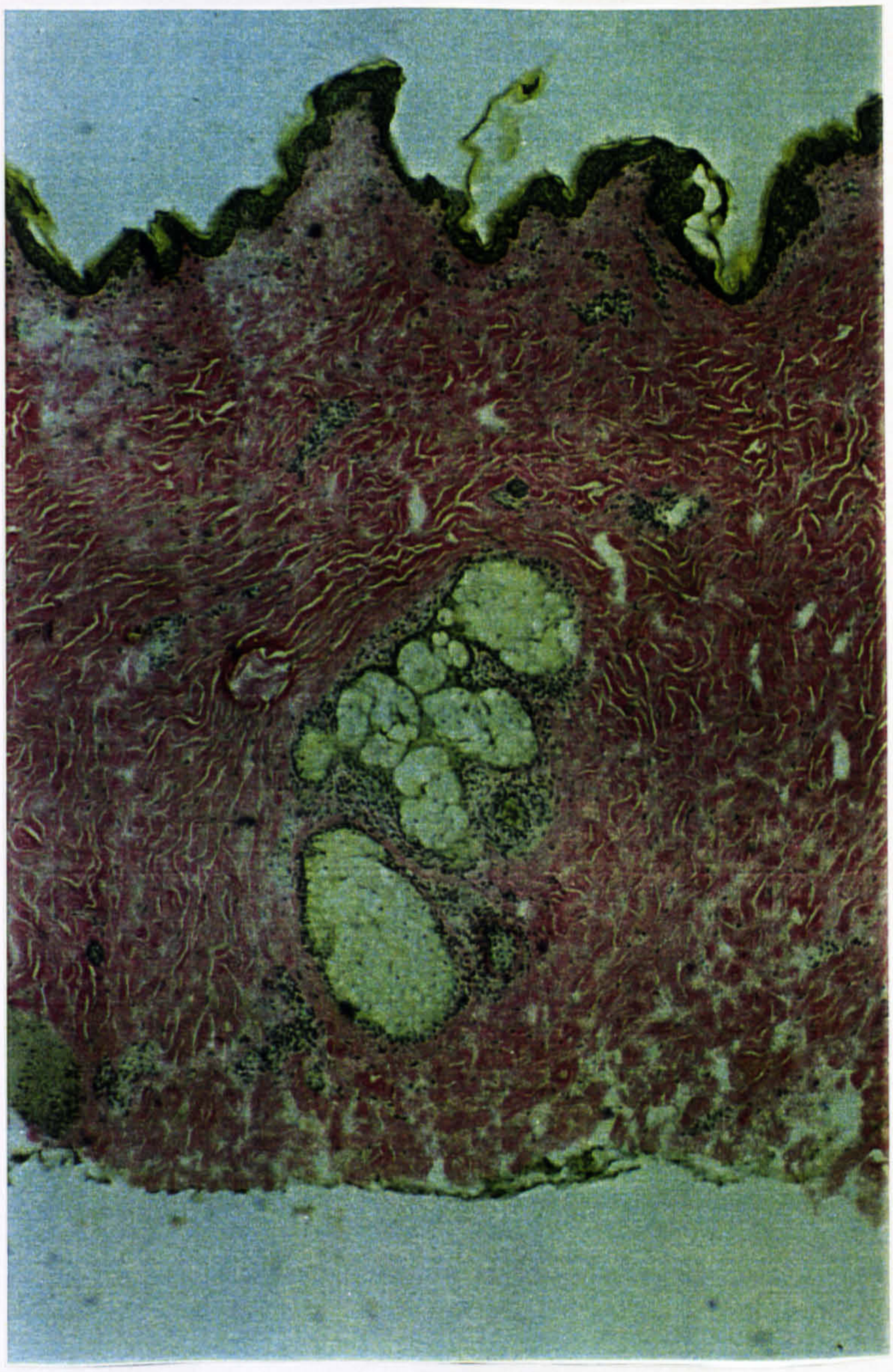
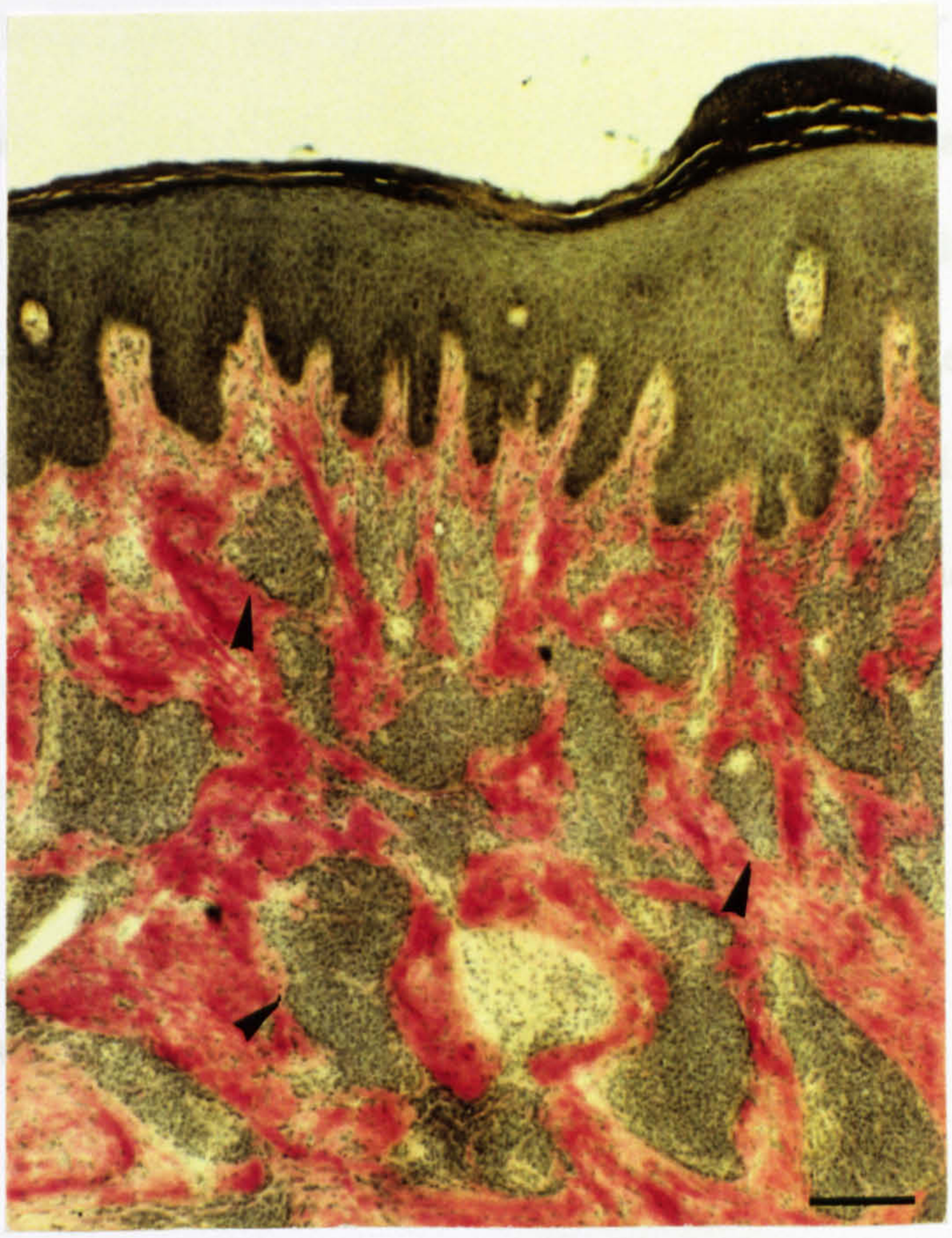


Figure 3.11 A micrograph to illustrate the histology of human keloid tissue (magnification x20, representative of n=55), the arrows point out populations of lymphocytes. Note the numerous lymphocytes in focal aggregates and the excessive collagen deposition in the dermis



Two very important differences between normal skin samples and keloid tissues are clear in Figures 3.9, 3.10 and 3.11: numerous pockets of lymphocytes in the upper dermis and areas stained intensely for collagen in the keloid.

Martin and Muir (1990) performed an extensive investigation of lymphocytes in normal skin, wounded tissues, hypertropic scars and keloid samples. They documented no B cells and increased numbers of T helper lymphocytes. Also consistent with observations made during the research for this thesis was their description that lymphocytes were concentrated around hair follicles and the invading edge of the keloid lesion. In addition, they introduced valuable evidence that the lymphocyte presence was consistently intense whatever the age of the keloid scars.

3.14 CD58 marker in human keloid tissues

The cell surface marker CD58 is strongly expressed on macrophages and associates with CD2 in order to enhance antigen-specific T cell activation. That is, CD2 and CD58 form an adhesive pair between T cells and antigen-presenting cells in order to induce a response to antigen.

Using CD58, the pattern of macrophage presence in keloid tissues was shown to be an even distribution of isolated cells throughout the dermis and numerous cells 'buried' in masses of lymphocytes in the dermis. Virtually no CD58 immunolabelling was present in the keloid lesion itself (see Figures 3.12, 3.13 and Table 3.2).

Figure 3.12 A micrograph to illustrate the immunolocalisation of the cell surface marker CD58 in human keloid tissue (magnification x100; dilution 1:50; representative of n=75)

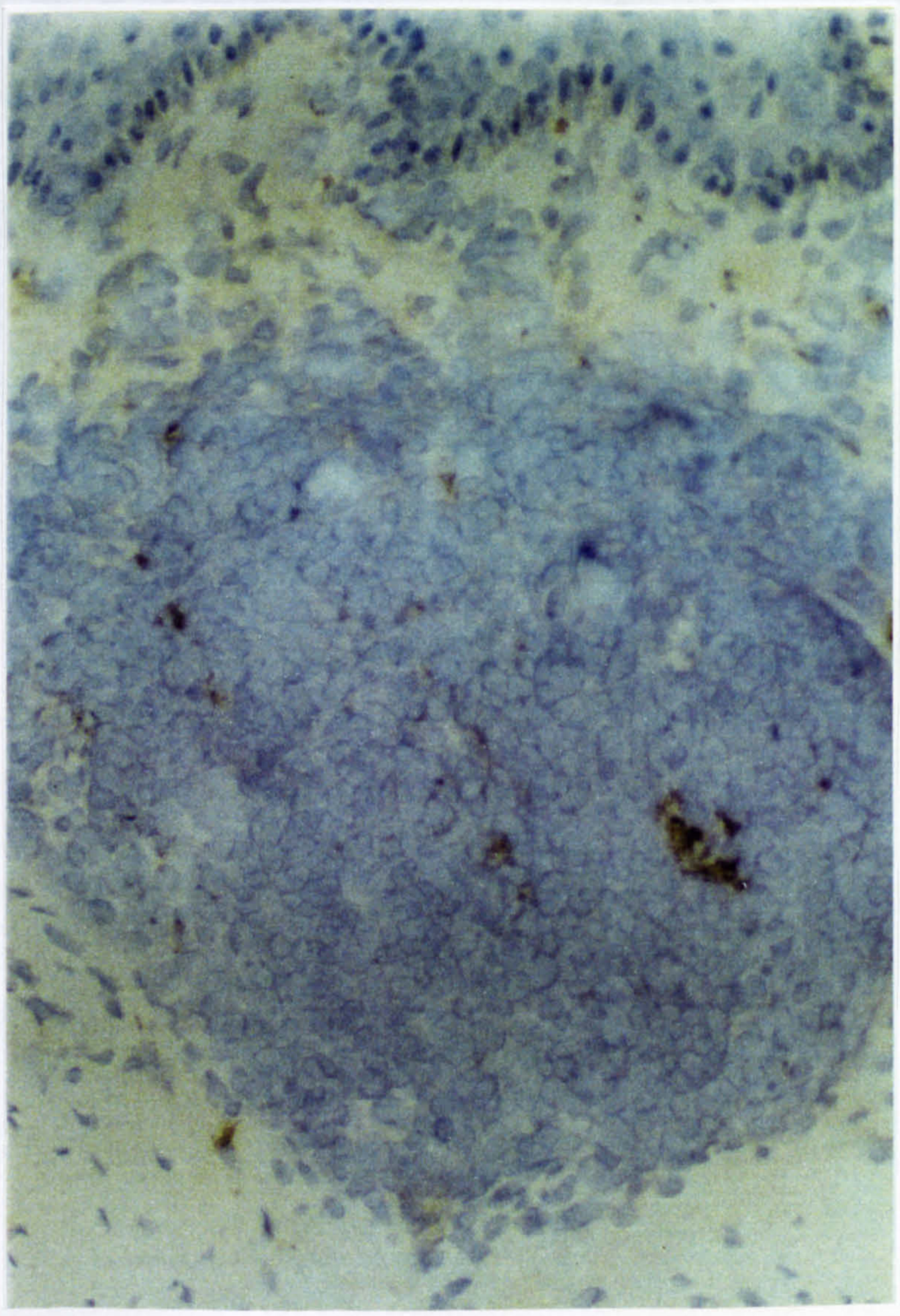
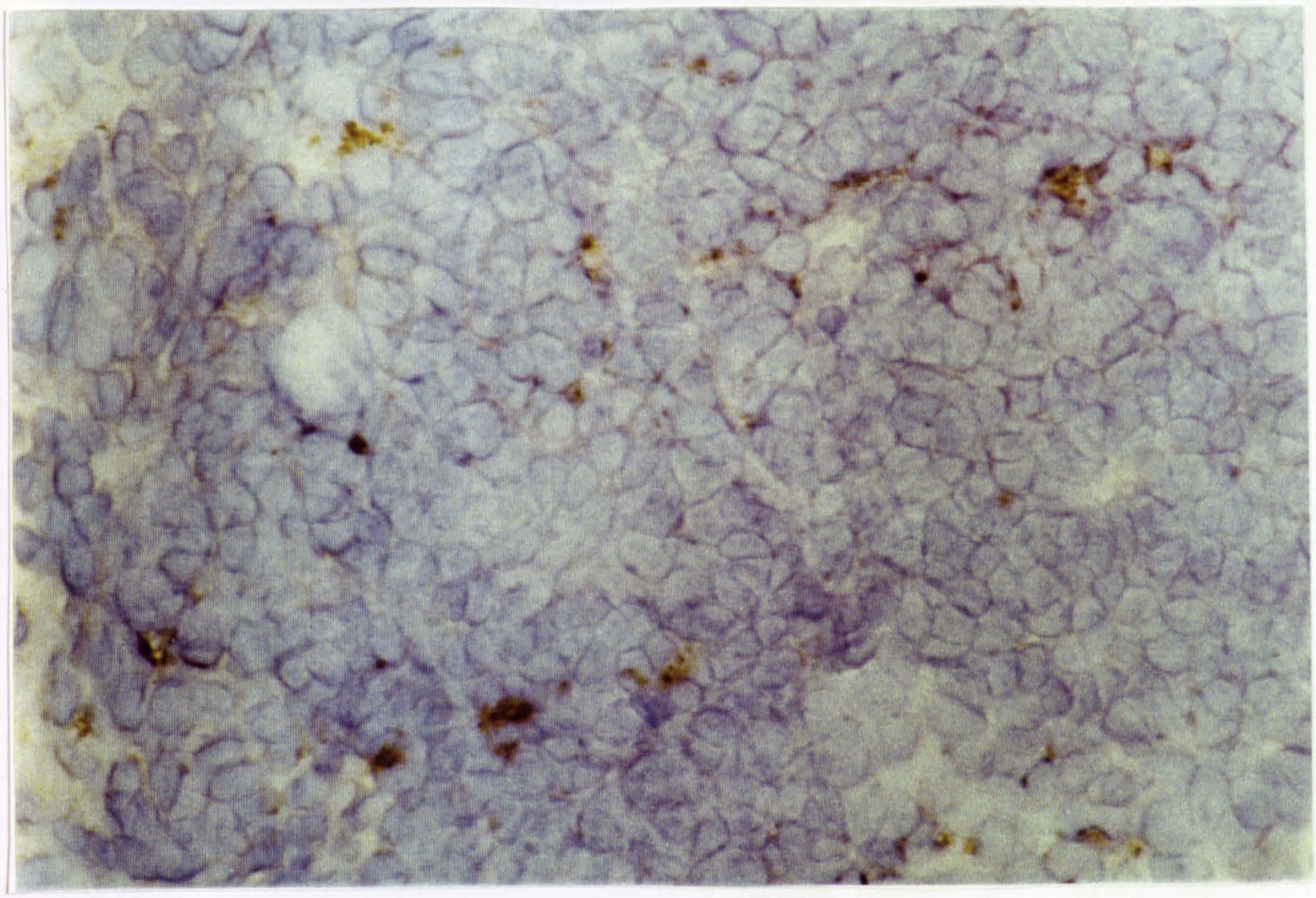


Figure 3.13 A micrograph to illustrate the immunolocalisation of the cell surface marker CD58 in human keloid tissue at higher power (magnification x200; dilution 1:50; representative of n=75)

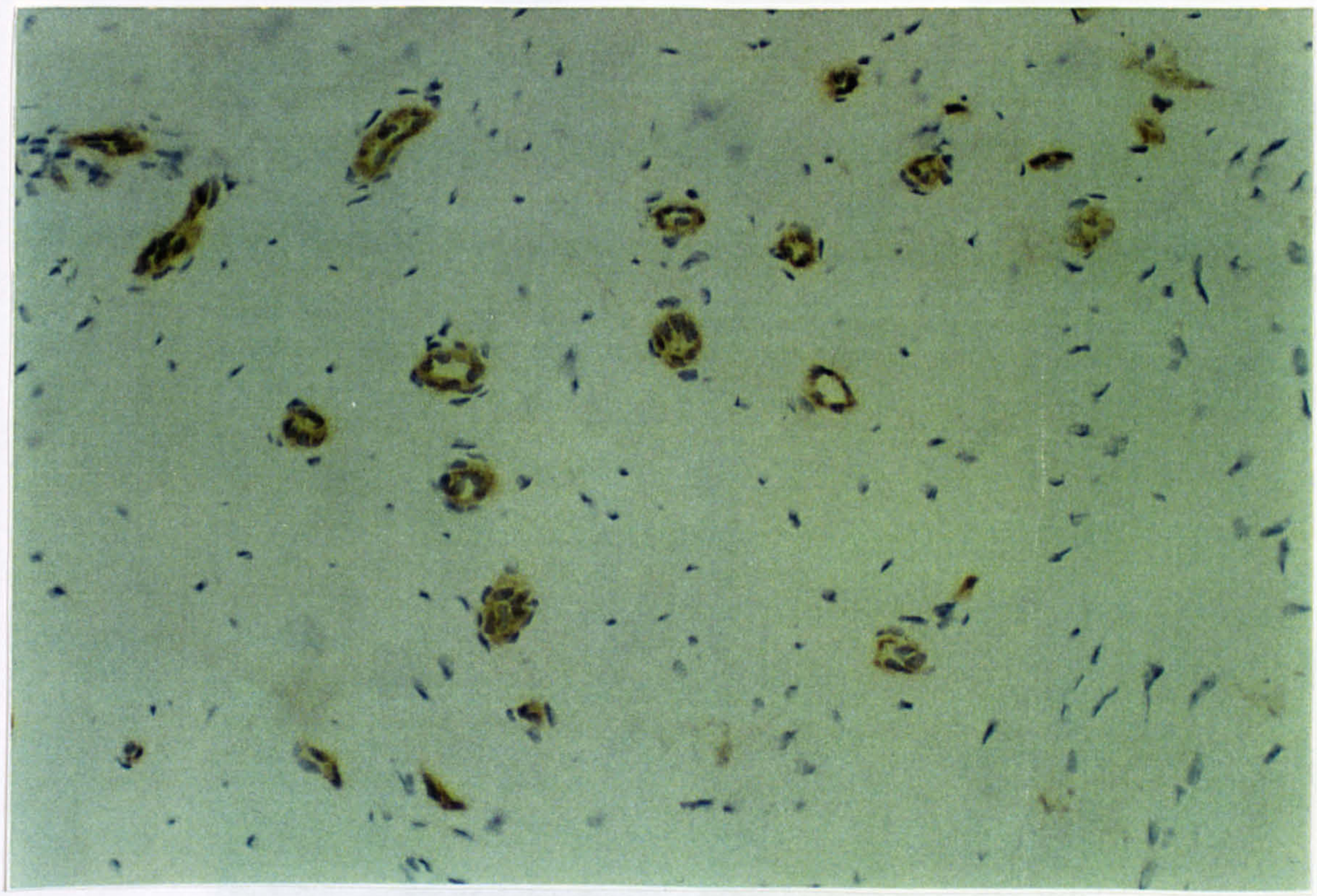


3.15 CD31 marker in human keloid tissues

The cell surface marker CD31 (also known as PECAM, platelet endothelial cell adhesion molecule 1) is highly expressed on endothelial cells and is concentrated at the junction between them. It is present on platelets and some macrophages (Barclay, 1994).

Figure 3.14 shows intense immunolabelling for CD31 in endothelial cells (or the junctions between them) in blood vessels in the keloid lesion, very few isolated cells are stained (that is, cells which are unlikely to be endothelial cells and which are possibly macrophages) which is consistent with the lack of CD58 immunolabelling in the lesion (see Figures 3.12, 3.13 and Table 3.2).

Figure 3.14 A micrograph to illustrate the immunolocalisation of the cell surface marker CD31 in human keloid tissue (magnification x100; dilution 1:50; representative of n=75)



3.16 CD4 and CD8 markers in human keloid tissue

Figures 3.15 and 3.16 represent $CD4^+$ and $CD8^+$ immunolocalisation in a keloid sample. The number of $CD4^+$ T cells in the dermis present is striking, as is the number of $CD8^+$ T cells. In addition to the $CD4^+$ T cell aggregations of lymphocytes in the keloid dermis, small groups of lymphocytes (and sometimes single lymphocytes) were present throughout the samples (see Table 3.2).

Figure 3.15 A micrograph to illustrate the immunolocalisation of the cell surface marker CD4 in human keloid tissue (magnification x50, dilution 1:50, representative of n=75)

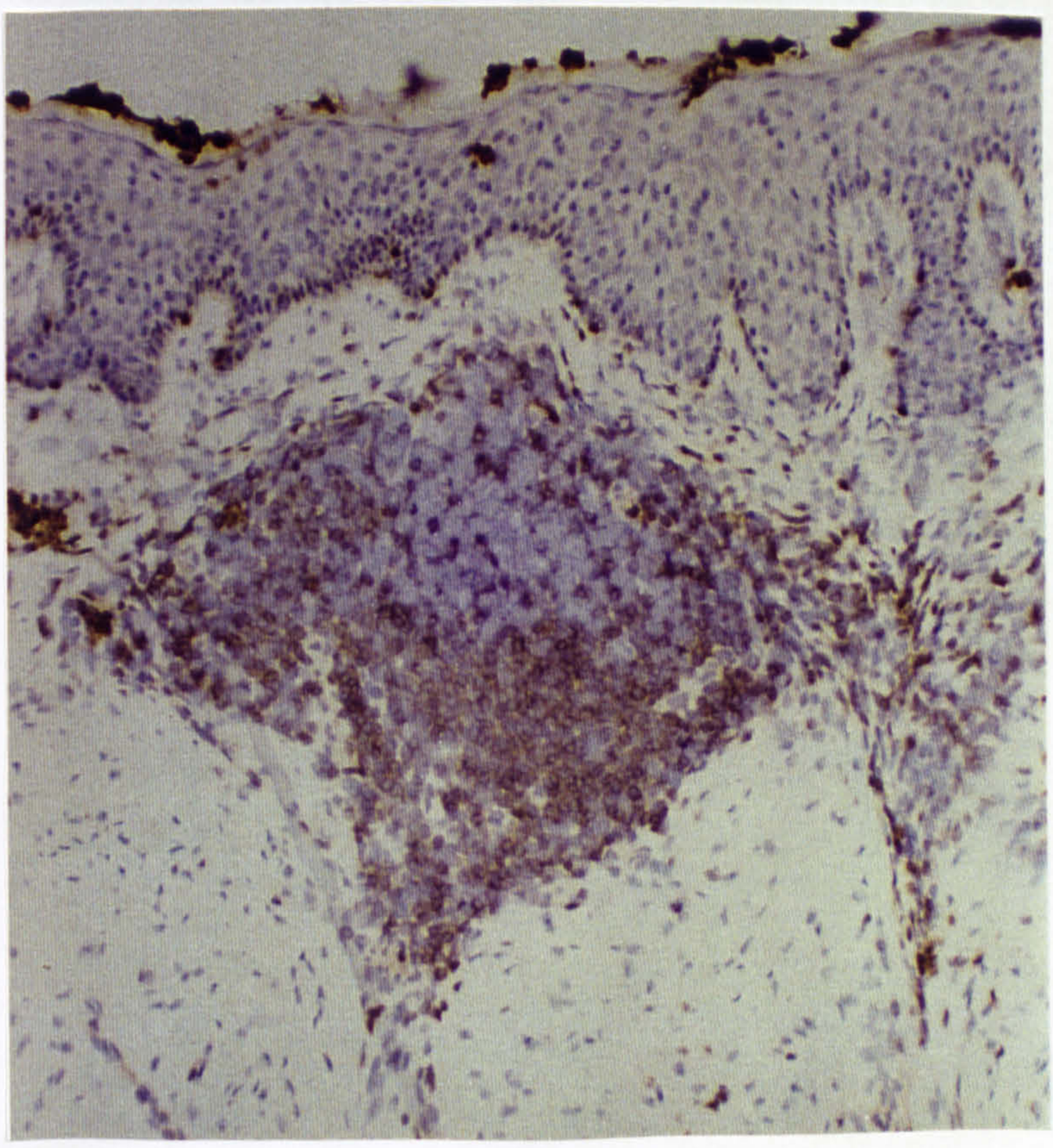
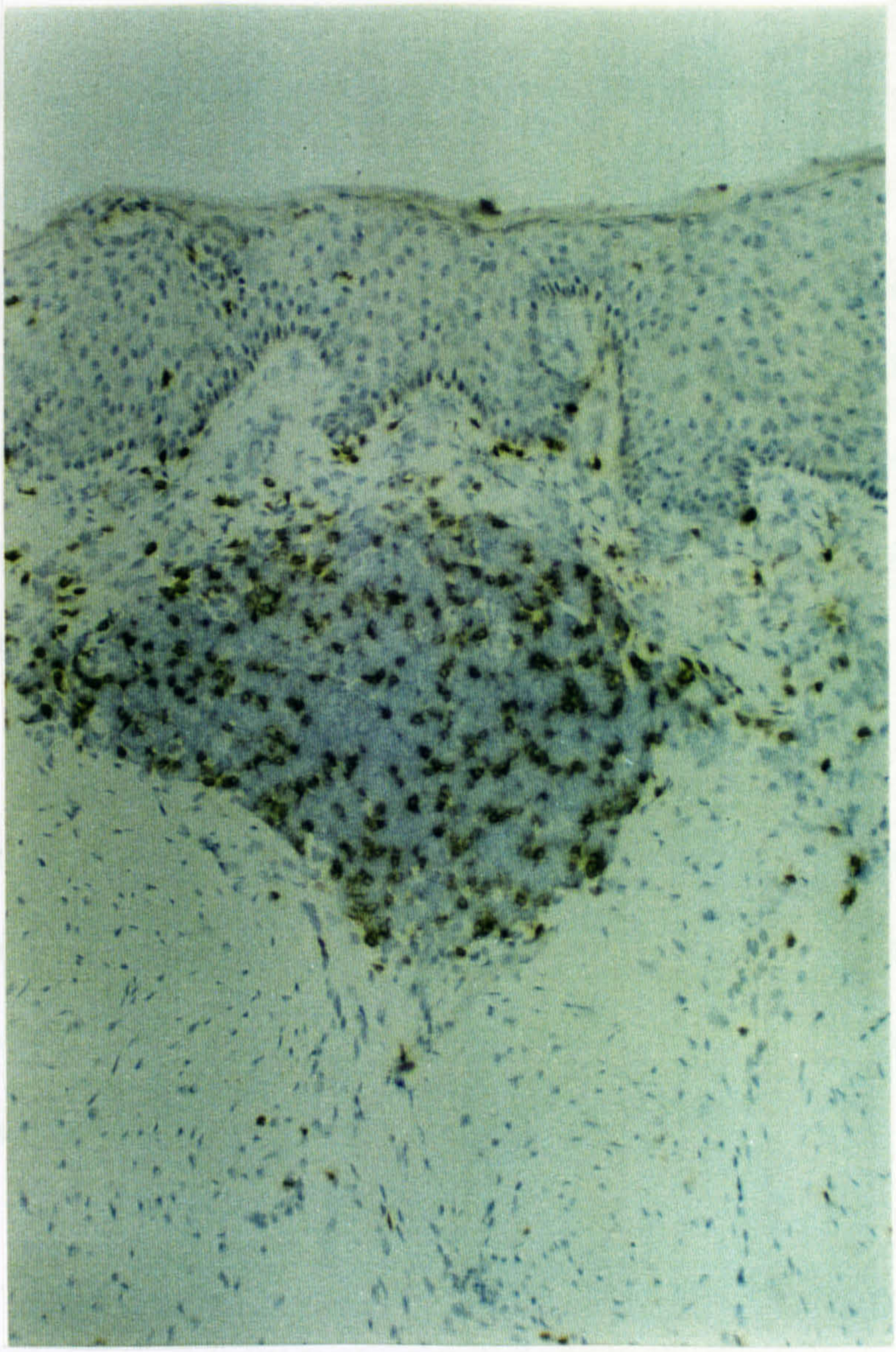


Figure 3.16 A micrograph to illustrate the immunolocalisation of the cell surface marker CD8 in human keloid tissue (magnification x50, dilution 1:50, representative of n=75)



3.17 CD30 marker in human keloid tissues

The function of the CD30 molecule is currently unknown; recently, however, Del Prete and co-workers (1995) have demonstrated its association with the development (in differentiation or activation) of murine type 2 T cells. The work was initially carried out *in vitro* and was then tested *in vivo* by measuring circulating levels of CD30 immunopositive T cells in atopic and nonatopic patients. The results showed that the presence of small numbers of circulating $CD4^+CD30^+$ cells in patients the type 2 allergic response characteristic of atopy. The $CD4^+CD30^+$ T cells were also shown to produce type 2 cytokines when stimulated with antigen.

As Figure 3.17 shows, CD30 immunolocalisation was evident for a proportion of lymphocytes in a focal aggregate present in the keloid dermis (see Table 3.2). Figure 3.18 is a $CD30^+$ cell shown at higher magnification to confirm its cell surface expression.

Figure 3.17 A micrograph to illustrate the immunolocalisation of the cell surface marker CD30 in human keloid tissue (magnification x100, dilution 1:50, representative of n=75)

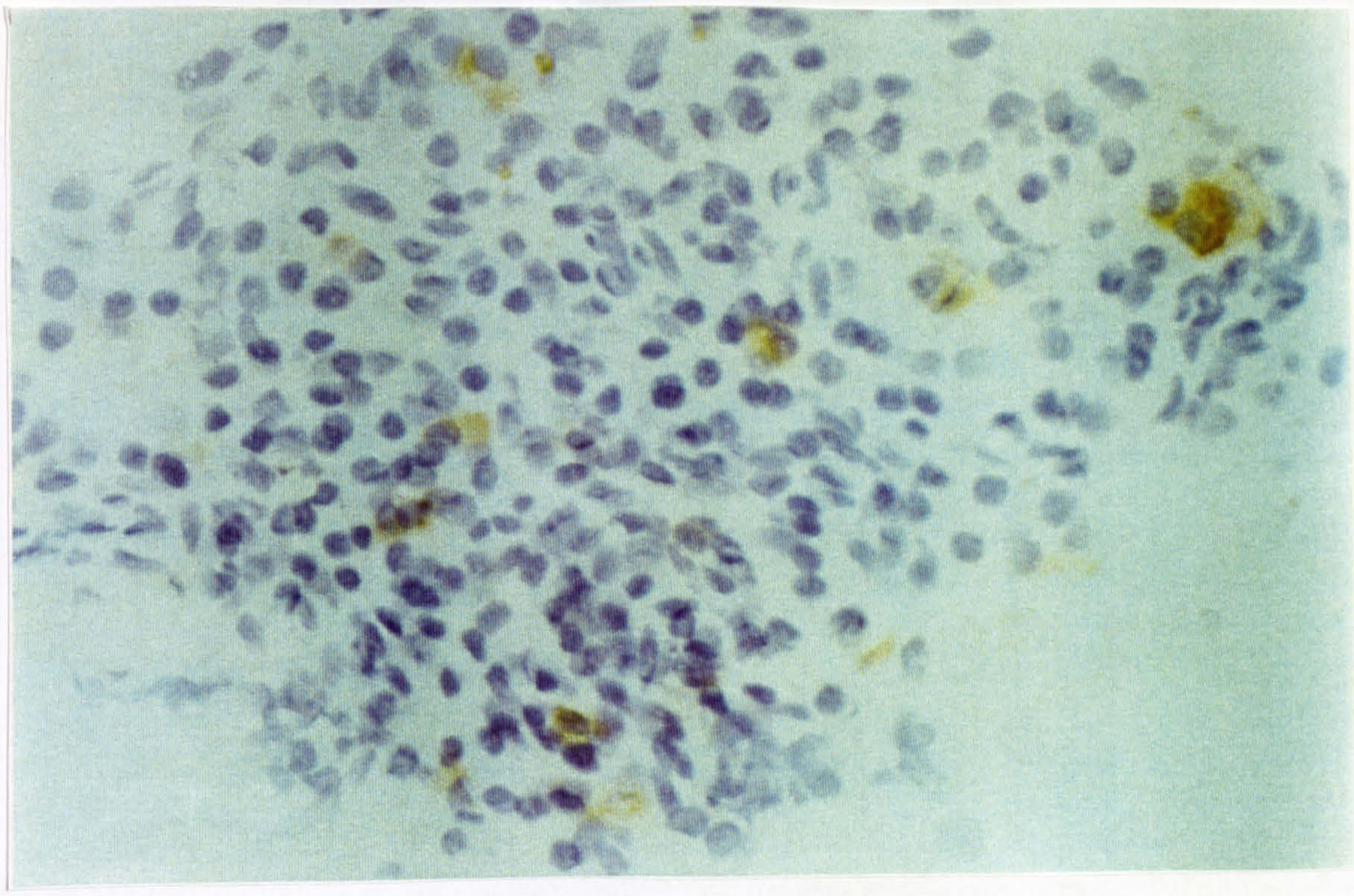


Figure 3.18 A micrograph to illustrate the immunolocalisation of the cell surface marker CD30 in human keloid tissue at higher power (magnification x500, dilution 1:50, representative of n=75)

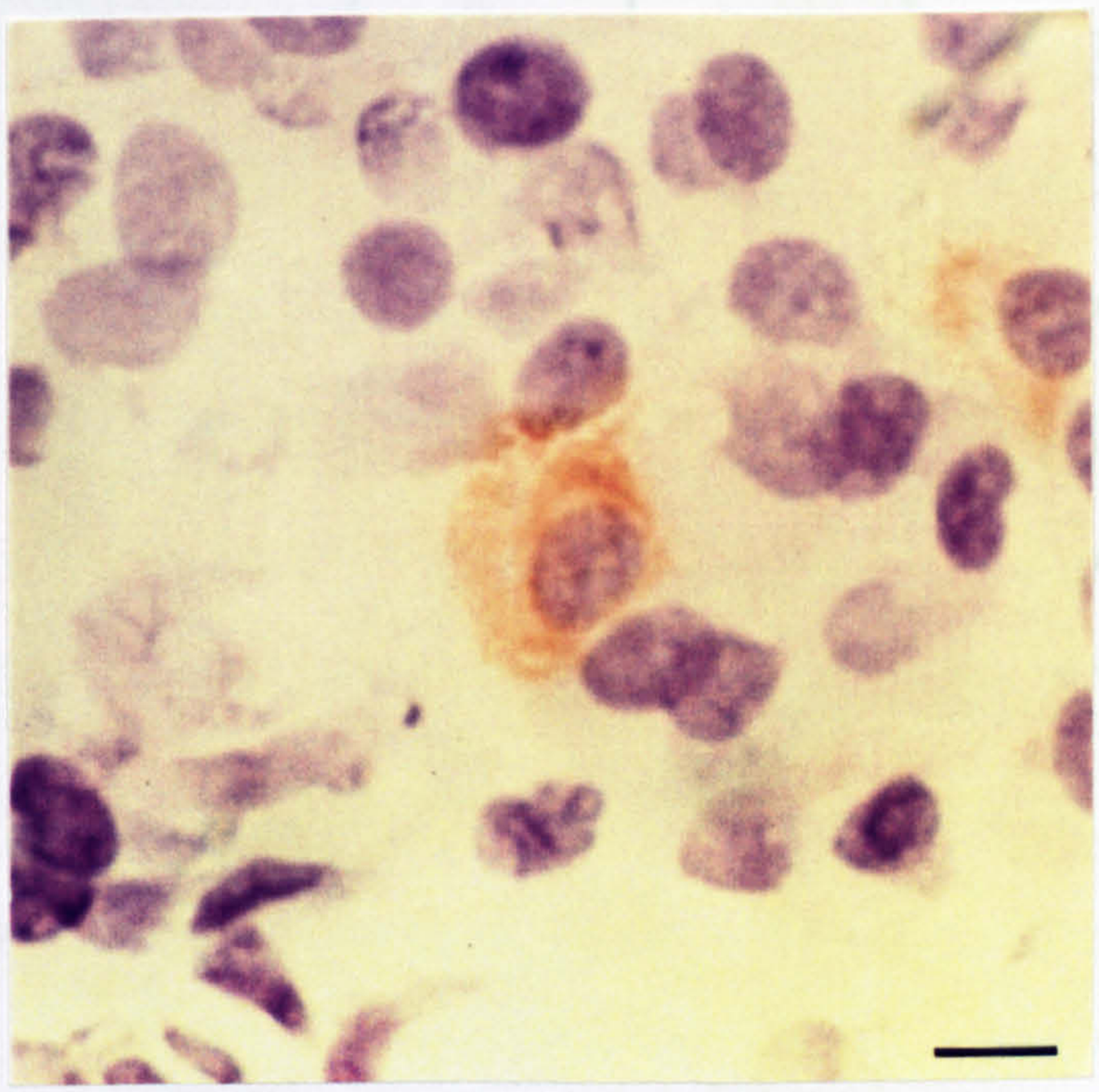


*Table 3.2: Quantitative data for the immunolocalisation of cell surface markers in normal human skin and keloid tissue. Results are expressed as the mean and standard error (±) of positively stained stained cells in n = 4 tissues, 10 fields of view per tissue (4mm^2, x20). The Mann-Whitney U-test was used to investigate statistical significance (P < 0.05 = *)*

Region	CD4	CD8	CD30	CD31	CD58
NS, D	5.0±1.8	5.3±2.1	1.3±1.3	30.5±10.1	26.8±9.1
KS, D	3946.3 ±137.3*	281.5 ±34.7*	128.3 ±11.5*	289.5 ±26.2*	111.0 ±11.7*
KS, L	305.3 ±16.4*	49.5 ±5.0*	24.5 ±2.6*	186.3 ±10.5*	32.3 ±3.6

(D, dermis; L, lesion and interface; NS, normal sample; KS, keloid sample)

3.20 DISCUSSION

The challenge of selecting the most appropriate *in vivo* model with which to compare the isolated 'snap shots' (which were available as clinical samples) of wound healing abnormalities was demanding.

Hypertropic scars and keloids are often discussed in the same breath, but so far it has been impossible to develop a model of the formation of either one. The major contributing factor preventing the development of the models is the general lack of understanding of exactly what the malfunction is which leads to the development of keloid (or hypertropic) scars. Added problems include the definition of keloid scars, which is still a source of controversy, the fact that even in known keloid-formers it is not clear that a keloid will form until it actually has (this is mainly because clinical observation is still the only absolute way to define and describe a keloid) and the mass of conflicting publications concerning the behaviour of keloid fibroblasts in culture and their heterogeneity.

Keloid samples used in the investigation in this thesis varied in size, age, growth position and patient sex, age and skin colour. Studies were carried out using both frozen and wax embedded sections with n numbers ranging from 50–75 per experiment. Normal control samples were also taken from a

variety of patients; however, the sample region was usually the chest area (by nature of the cosmetic operations undertaken).

Wound healing research has progressed from the early Nobel prize winning *in vitro* work carried out by Carrell (Carrell and Burows, 1910) to more specific, detailed *in vitro* work to establish fibroblast production of collagen and collagenase (Stearns, 1940) and from linear, open (Stein and Keiser, 1971) and burn wounds (Horton et al, 1990) *in vivo* to sponge implants (Vilijanto, 1964) and chamber implants (Schilling *et al*, 1959). Every piece of work carried out has a role in the development of our current understanding of wound healing. Parameters commonly studied *in vivo* include wound breaking strength, hydroxyproline/collagen deposition and the nature and accumulation of wound fluid. In each case, the *in vivo* model should be selected carefully in order to study specific features.

The study of cellular and matrix components and their interactions is best evaluated histologically (Cohen and Mast, 1990). The use of stains and antibodies allows the observation of processes in a 'fly on the wall' manner, although quantitation is difficult. Absolute values of negative versus positive immunolabelling are almost beyond current histological techniques (Carter and Swain, 1997) but with careful execution comparisons can be made between different samples in the same experiment.

The murine air pouch model is not traditionally regarded as a wound healing model, this is particularly evident in the opinions held by clinicians and surgeons (an excellent review of wound healing and its models by two plastic surgeons omits it completely, Cohen and Mast, 1990). Whether or not an individual researcher considers it as such depends upon the definition used: the murine air pouch model features granulation tissue formation, wound contraction (and the presence of myofibroblasts), collagen deposition and injury resolution. What it does not feature is an open injury, reepithelialisation, blood loss and clotting etc. As Drs Cohen and Mast state, 'A variety of investigative models are available for the study of wound healing, yet there is no single best model. Each study protocol must be designed with full recognition of the advantages and disadvantages of the model(s) to be used and the results must be interpreted accordingly' (Cohen and Mast, 1990).

A central theme of this thesis was to use the murine air pouch model as a comparison with keloid samples, the model is appropriate for this approach because, as mentioned above, it features injury resolution. The aspects of healing which it does not feature, such as blood loss, clotting etc, are not in consideration in this particular study. The main reason for this is that the clinical samples necessary for such investigations (that is, samples taken at timepoints from injury to keloid formation) were unavailable. This approach,

however, does not assume that these aspects of wound healing are not relevant to keloid formation or propagation.

3.30 QUANTITATION OF IMMUNOHISTOCHEMISTRY DATA

As will be mentioned in Chapter 4, a computerised method of quantitation of immunocytochemical results was attempted using SeeScan. SeeScan is a basic form of light microscopical image analysis which permits the visualisation of the microscope stage (and slide) using a computer monitor.

It is an excellent technique to aid quantitation in cell smears and cell fractions. The main problem with its use in the quantitation of cells within tissue sections is its inability to discern subtle variations in colour contrast of staining. This limitation is exacerbated by the fact that the monitor being used is usually black and white (it was in this case).

Human keloid lesional tissue is dense and collagenous and is cradled on approximately 70% of its perimeter by human dermis, which is less dense and much softer in texture. In order to section through the full depth of the samples and not cause the dermis and lesion to separate, it is necessary to cut thicker sections than would be the case for other types of tissue. Unfortunately by adding thickness ('depth') to the sample, difficulty in visualisation is also

added. It becomes even more difficult to discern cell types when there are likely to be several layers of cells within the depth of tissue. It also becomes more difficult to discern variations in shade if the three-dimensional effect is emphasised by the added depth of the section. It was the combination of these difficulties coupled with the extensive heterogeneity of keloid samples which prevented the application of SeeScan in this thesis.

In view of the emphasis placed on immunocytochemical analysis in the research for this thesis and the very individual nature of the samples to be studied, it was necessary to develop a suitable method for quantitation of positively stained cells. All murine and human samples were subjected to the quantitation method and the results for individual antibodies and samples are included in the relevant areas of text throughout the thesis. The method gives an approximation of the cells within an area which are positively stained for a given antibody.

A further issue to address when using immunocytochemistry is how to nominate which cells can be considered to be 'positively stained'. In this case, this problem was compensated for (initially) by using various concentrations of antibody and analysing the optimum concentration for effective staining and minimum nonspecific background colouration. The actual dilution used in each experiment is given in brackets in the relevant areas of text throughout the thesis. Decisions of which individual cells were or were not positively

stained were taken with care and were based on the mean of sample populations (n numbers are given in relevant areas of text throughout the thesis).

The results of the quantitative analysis are expressed as the mean and standard error of n = 4, 10 fields of view per tissue (randomly generated geography). The data were collected using a graticule, cells crossing the upper and right grid lines were excluded from the total.

Chapter 4

IMMUNOLOCALISATION OF CYTOKINES IN HUMAN TISSUES

CONTENTS

4.10 INTRODUCTION

Keloids are cutaneous lesions acquired through abnormal wound healing. They feature excessive collagen deposition (according to the general consensus), reduced collagen cross-linking (Di Cesare *et al*, 1990) and the absence of myofibroblasts (Ehrlich *et al*, 1994). The scar tissue outgrows the boundary of the original injury and can be itchy and painful (Rudolph, 1991).

Numerous cytokines are known to be involved in normal wound healing and their general effects are well established (see Chapter 1). In abnormalities of repair, such as keloids, however, the information regarding the distribution and possible effects of these factors is scarce.

Epidermal growth factor (EGF) is a mitogen for endothelial cells, T cells and epidermal cells (Knauer and Cunningham, 1983; Acres *et al*, 1985). It promotes the reepithelialisation of partial thickness wounds and increases wound strength in full thickness wounds (Nanney, 1990; Brown *et al*, 1988). EGF is present in most bodily fluids, although tissue concentration is usually low (Savage *et al*, 1986).

Platelet-derived growth factor (PDGF) is a chemoattractant for monocytes, fibroblasts and polymorphonuclear neutrophils (Deuel *et al*, 1982; Seppa *et al*, 1982). It stimulates wound healing in healing impaired genetically diabetic rodents and accelerates granulation tissue formation in full thickness

wounds (Greenhalgh *et al*, 1990; Hill *et al*, 1991). Platelets are the primary source of PDGF in wounds, although it is also released by macrophages, endothelial cells and stimulated fibroblasts (for review, see Antoniades, 1991).

Basic fibroblast growth factor (bFGF) is a mitogen for keratinocytes, fibroblasts, promotes the division of endothelial cells and is a potent angiogenic factor (Gospodarowicz *et al*, 1986). It accelerates wound healing in genetically diabetic animals and downregulates collagen gene expression in keloid fibroblasts *in vitro* (Greenhalgh *et al*, 1990; Tan *et al*, 1993*). bFGF is produced by fibroblasts, macrophages, endothelial cells and smooth muscle cells (for review, see Baird and Bohlen, 1990).

Vascular endothelial growth factor (VEGF), also termed vascular permeability factor, is the most potent angiogenic factor known. It increases vascular permeability and induces the division of endothelial cells (Leung *et al*, 1989). VEGF has been shown to be a potential therapeutic target for tumour treatment through experiments using monoclonal VEGF antibodies to block VEGF activity and tumour cell growth *in vitro* (Jin Kim *et al*, 1993) as well as work proving that tumour angiogenesis is regulated by VEGF activity *in vivo* (Plate *et al*, 1992). VEGF is produced by keratinocytes and tumour cells and is taken up by endothelial cells (for review, see Dvorak *et al*, 1995 x2) and induces the synthesis of interstitial collagenase in endothelial cells although not in dermal fibroblasts.

Transforming growth factor-β (TGF-ß) increases the deposition of extracellular matrix components, this is achieved by increasing the production of collagen and protease inhibitors and decreasing the production of proteolytic enzymes (Ignotz and Massague, 1986; Edwards *et al*, 1987). It accelerates the healing of full thickness wounds and of wounds impaired by steroids (Quaglino *et al*, 1990; Beck *et al*, 1991). TGF-ß is produced by platelets, lymphocytes, fibroblasts and macrophages (for review, see Roberts and Sporn, 1990).

A comprehensive study (Appleton *et al*, 1993) mapping the temporal and spatial immunolocalisation of EGF, bFGF, PDGF and TGF-ß in the murine air pouch model demonstrated that these cytokines, although possessing a wide range of properties, play intricate and intertwining roles in the healing process. In the murine air pouch model the initial release of cytokines is achieved through the degranulation of platelets in response to injury. It is suggested that continued release, which is required for wound resolution, is provided by macrophages and fibroblasts. Western blotting for VEGF in this model has shown a protein level peak around day 3, just prior to the timepoint of maximum vascularity (Appleton *et al*, 1996; see Chapter 5).

These two studies by Appleton and co-workers have illustrated that cytokines are important mediators in wound resolution, what has not been confirmed, however, is their roles in abnormal healing pathologies. This study was designed to investigate the distribution of cytokine immunolabelling in

keloid tissues, in comparison to normal skin, and to extrapolate their influence in the aetiology of this condition.

4.20 RESULTS

4.21 Epidermal growth factor

Immunoreactivity for EGF in normal human skin was restricted to the epidermis, hair follicles and some blood vessels. No immunoreactivity was seen in other cellular populations (see Table 4.1).

In the dermis of the keloid tissues, intense labelling was evident in the acinus cells of the sebaceous glands, the endothelial cells of the blood vessels, hair follicles, epidermis and the myoepithelial cells of the sweat glands, EGF immunoreactivity was observed in some fibroblasts and macrophages (Figures 4.1a and b, 4.2 and Table 4.1).

4.22 Platelet-derived growth factor

The immunolocalisation profiles for PDGF in normal skin (see Table 4.1) and keloids was similar to that observed with EGF.

In general, staining intensity and distribution was greater in the keloid dermis than in the normal skin. Immunoreactivity was observed in the epidermis, hair follicles, sebaceous glands and the endothelial cells of some blood vessels of both skin types; merocrine sweat glands, macrophages and fibroblasts were labelled for PDGF in keloid dermis only (Figures 4.3, 4.4 and Table 4.1).

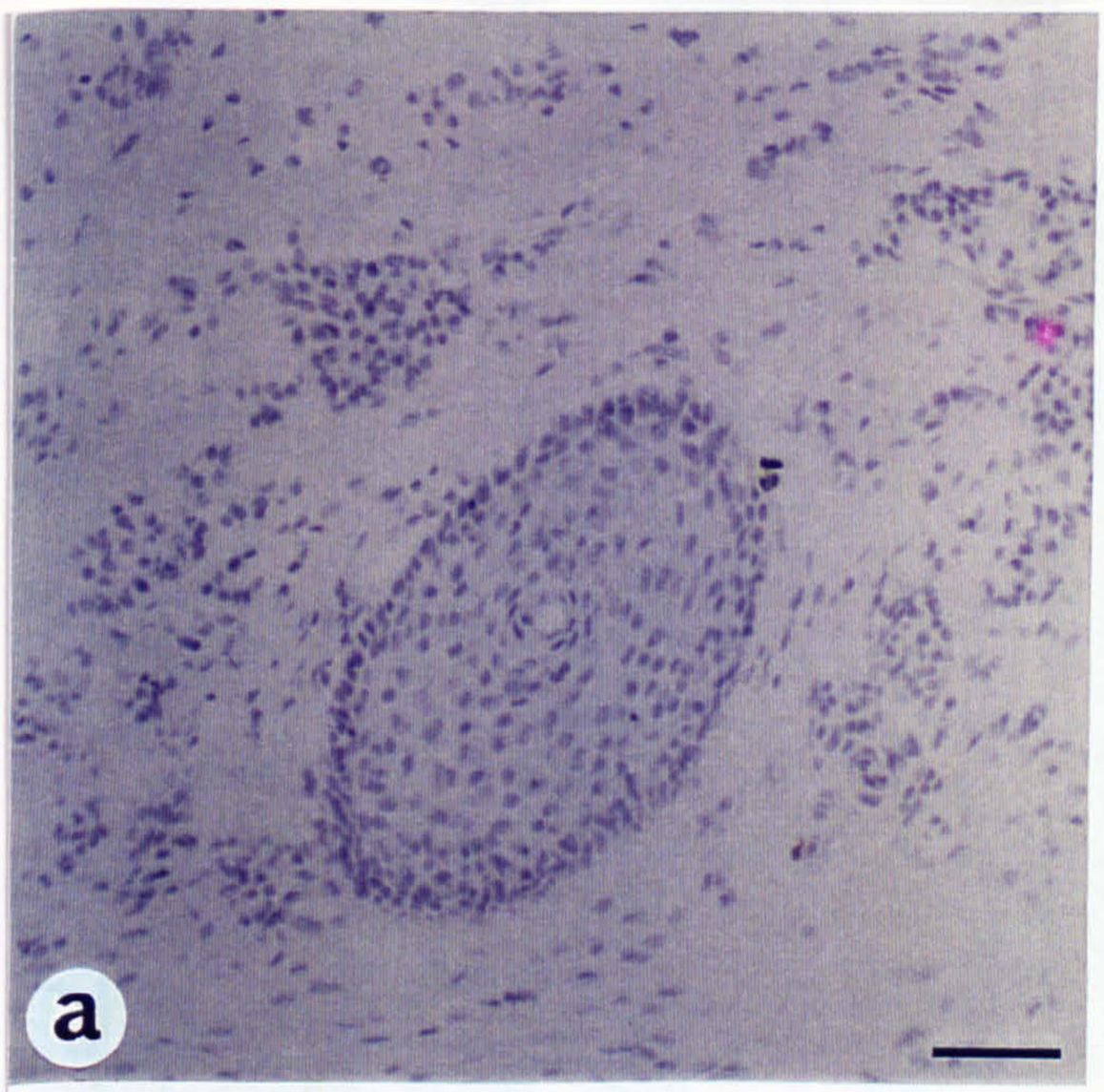
*Table 4.1: Quantitative data for the immunolocalisation of cytokines in normal human skin and keloid tissue. Results are expressed as the mean and standard error (±) of positively stained stained cells in n = 4 tissues, 10 fields of view per tissue (4mm^2, x20). The Mann-Whitney U-test was used to investigate statistical significance (P < 0.05 = *)*

Region	EGF	PDGF	bFGF	TGF-β	VEGF
NS, D	14.5±2.9	43.3±4.6	47.3±2.9	8.3±2.2	27.0±5.1
KS, D	156.0 ±10.7*	152.3 ±11.2*	166.3 ±6.0*	53.3 ±4.6*	101.0 ±8.0*
KS, L	14.8 ±1.1*	65.3 ±5.9*	164.5 ±11.6*	9.3 ±2.3*	31.5 ±9.7

(D, dermis and interface; L, lesion; NS normal sample; KS, keloid sample)

Figure 4.1 Micrographs to illustrate the immunolocalisation of EGF, bFGF and TGF-β in keloid dermis. Preabsorption of the antibody results in complete amelioration of labelling a), whereas in b) serial sections labelled with EGF demonstrates specific localisation to the hair follicles of the keloid dermis (bar represents 20μm, dilution 1:200, representative of n=75). In c) localisation of bFGF to the endothelial cells of a capillary in the keloid dermis is shown (bar represents 5μm, dilution 1:200, representative of n=75). TGF-β immunoreactivity associated with a sweat gland in the keloid dermis is illustrated in d) (bar represents 10μm, dilution 1:100, representative of n=75).

See over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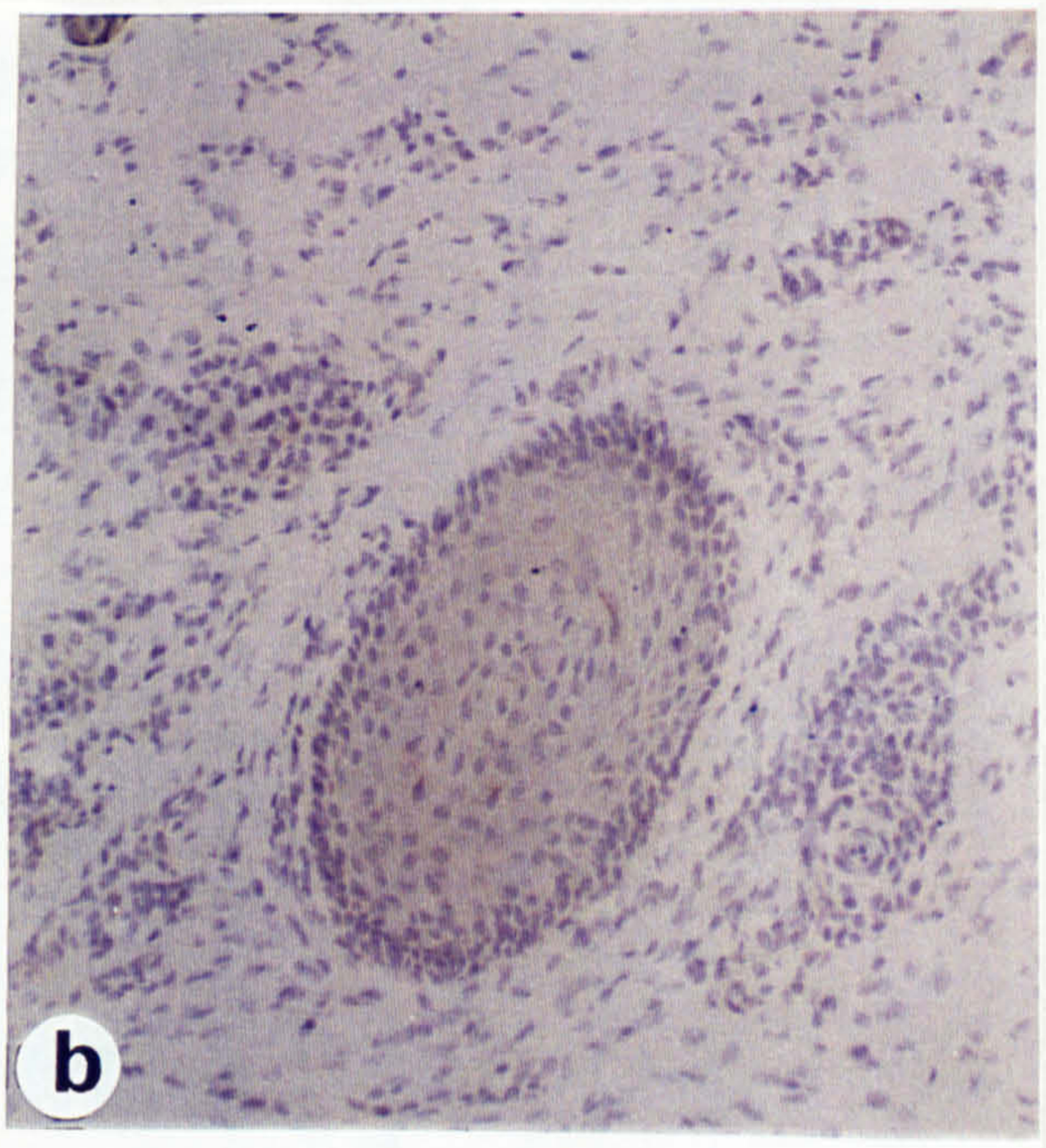
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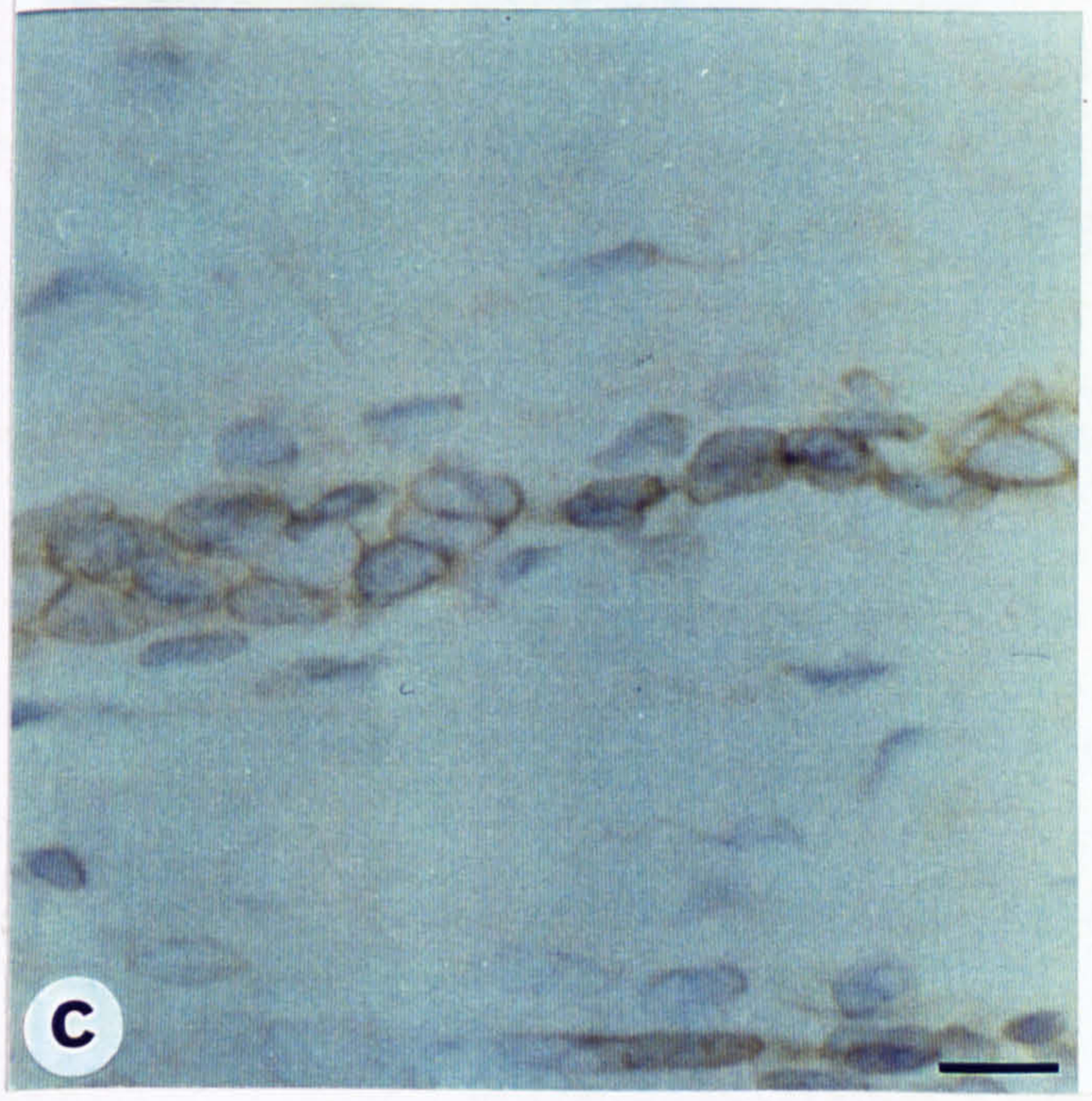
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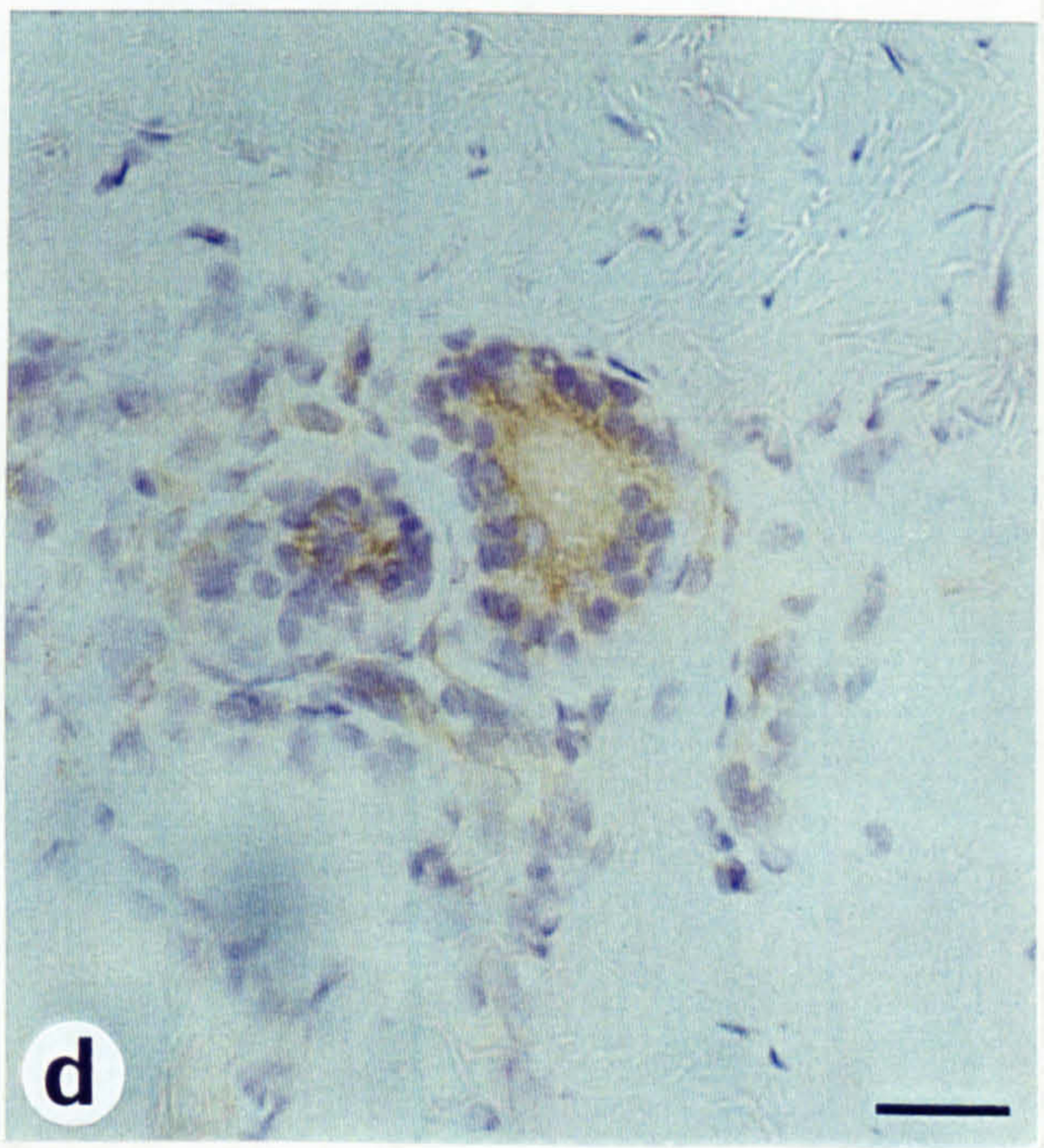

d

Figure 4.2 A micrograph to illustrate the immunolocalisation of EGF to sweat glands in keloid tissue (magnification x100, dilution 1:200, representative of n=75)

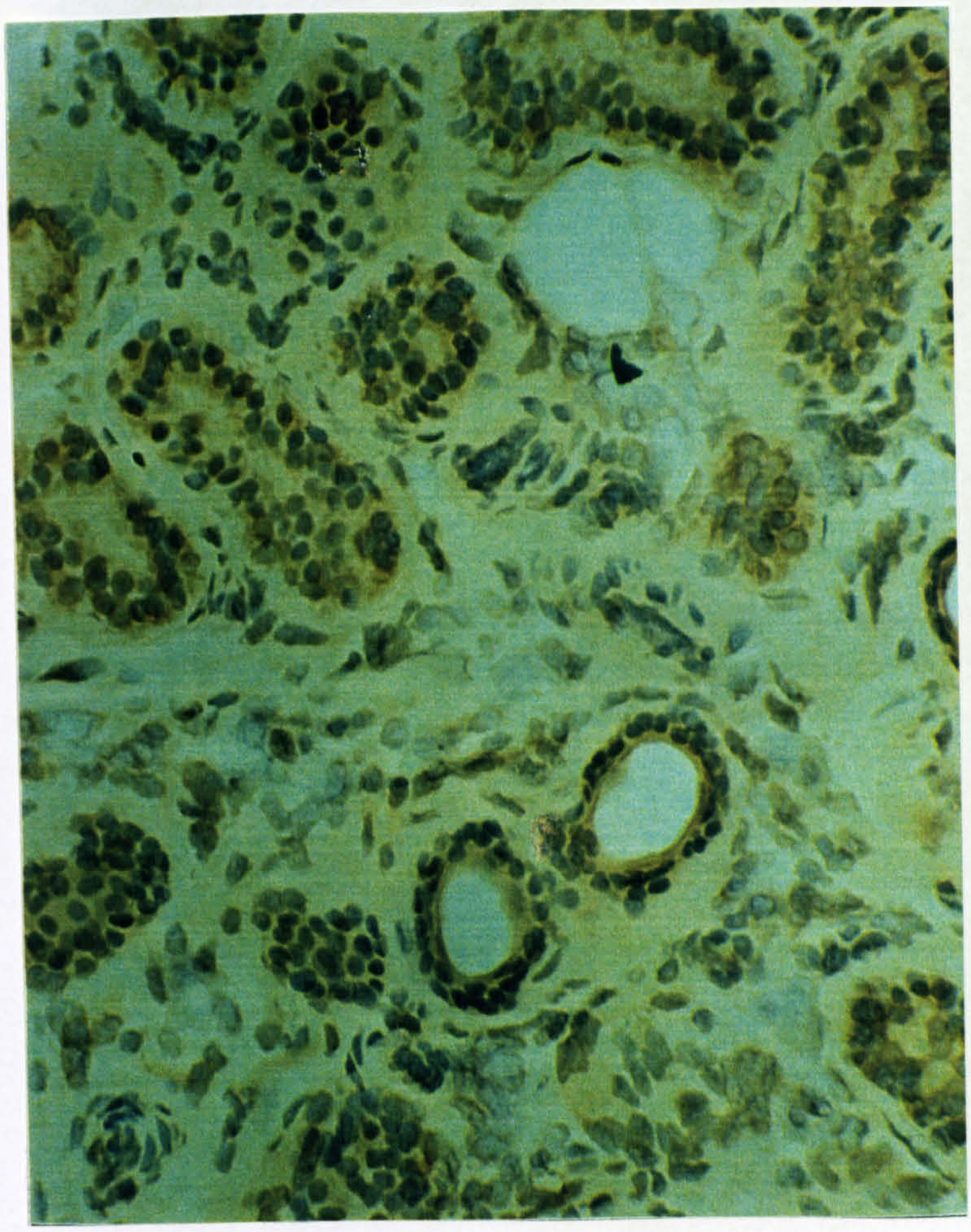


Figure 4.3 A micrograph to illustrate the immunolocalisation of PDGF in keloid dermis (magnification x100, dilution 1:200, representative of n=75)

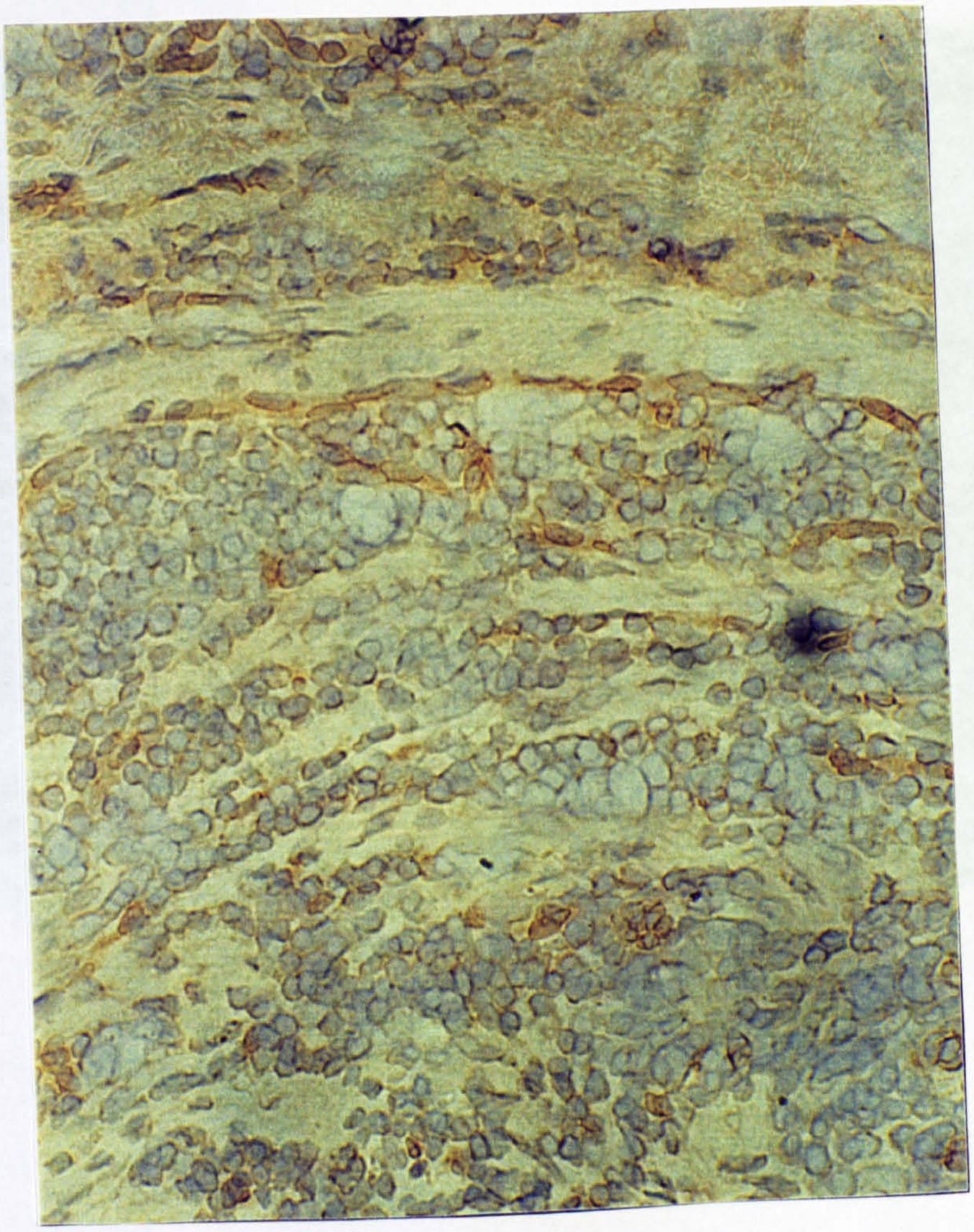
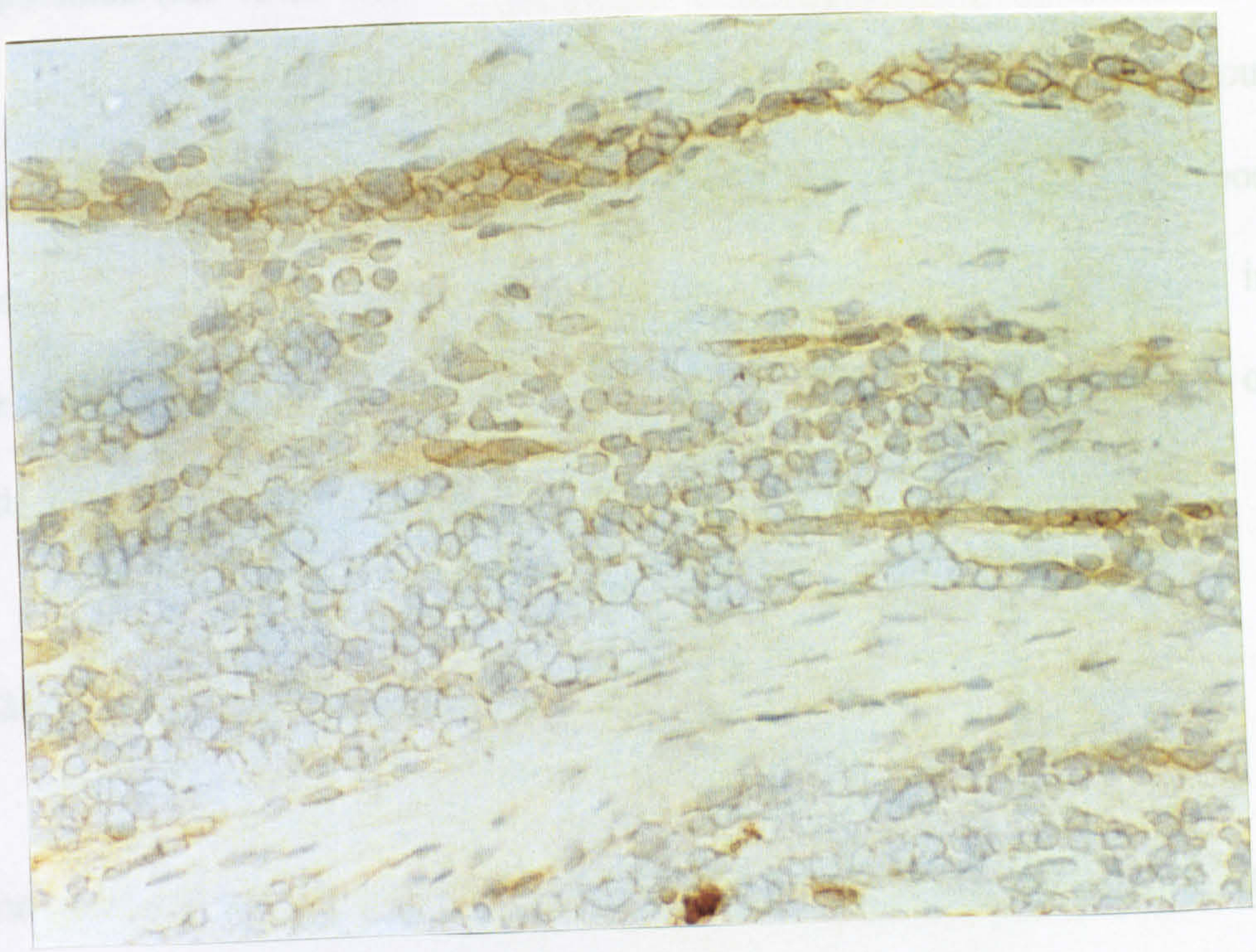


Figure 4.4 A micrograph to illustrate the immunolocalisation of PDGF to a blood vessel in keloid tissue (magnification x100, dilution 1:200, representative of n=75)



4.23 Basic fibroblast growth factor

Low levels of bFGF immunoreactivity were evident in hair follicles, sebaceous glands and blood vessels of the normal skin although not in other cellular populations (see Table 4.1).

Intense immunolabelling was observed in dermal structures throughout the keloid tissues: hair follicles, sweat glands, sebaceous glands and blood vessels. Immunoreactivity was evident in most fibroblasts and macrophages in the keloid lesion and the dermis. bFGF was not detected in the epidermis of either skin type (Figures 4.1c, 4.5, 4.6 and Table 4.1).

4.24 Vascular endothelial growth factor

Immunoreactivity of VEGF in normal skin was confined to the endothelial cells of a minority of blood vessels and to low levels in hair follicles (see Table 4.1). Immunolabelling was not observed in other cellular populations in either tissue.

The keloid tissue exhibited intense labelling for VEGF in the endothelial cells of most of the blood vessels in the dermis and lesion as well as hair follicles (see Figure 4.7 and Table 4.1).

Figure 4.5 A micrograph to illustrate the immunolocalisation of bFGF to a hair follicle and lymphocytes in keloid dermis (magnification x50, dilution 1:200, representative of n=75)

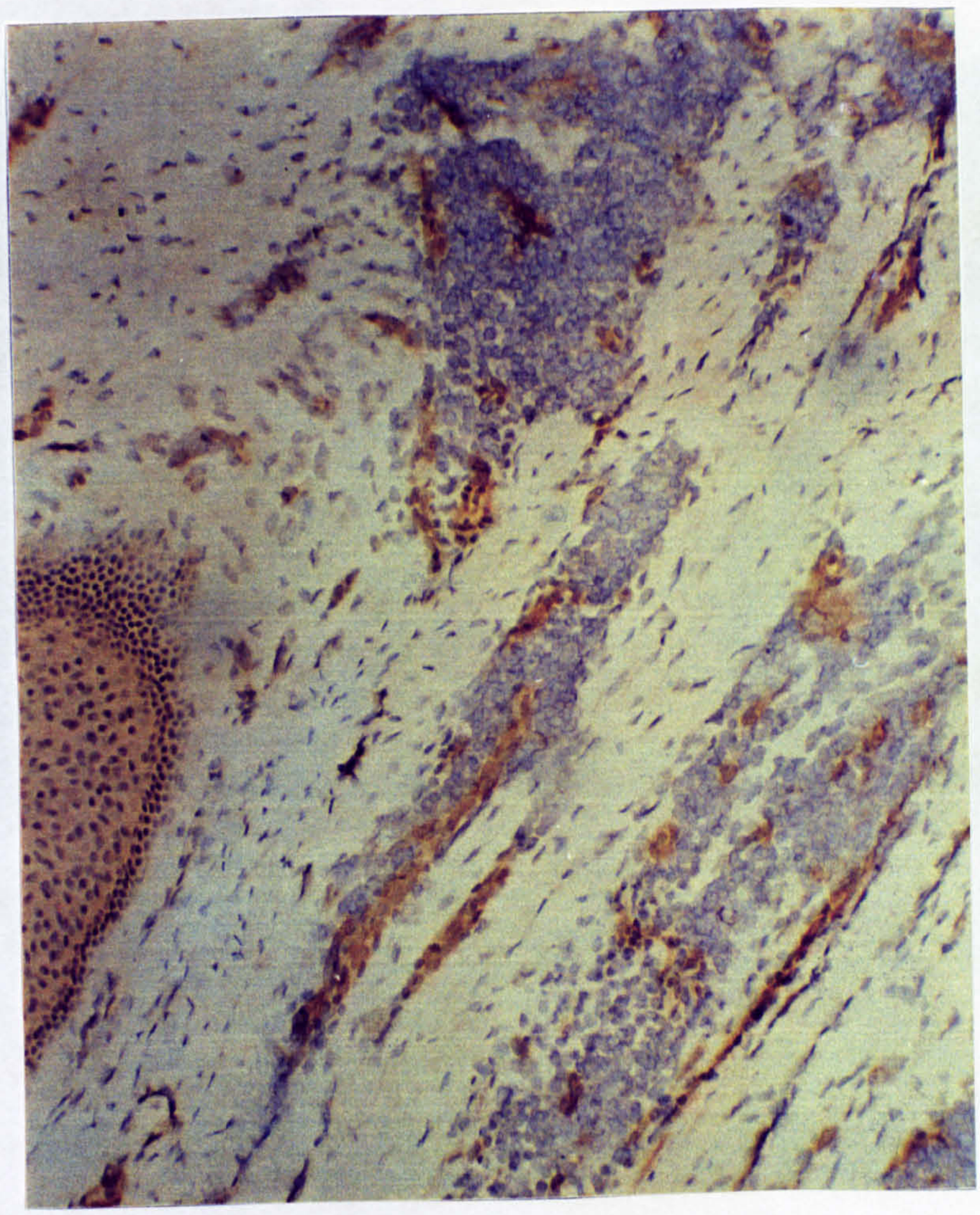


Figure 4.6 A micrograph to illustrate the immunolocalisation of bFGF to a blood vessel in keloid tissue (magnification x200, dilution 1:200, representative of n=75)

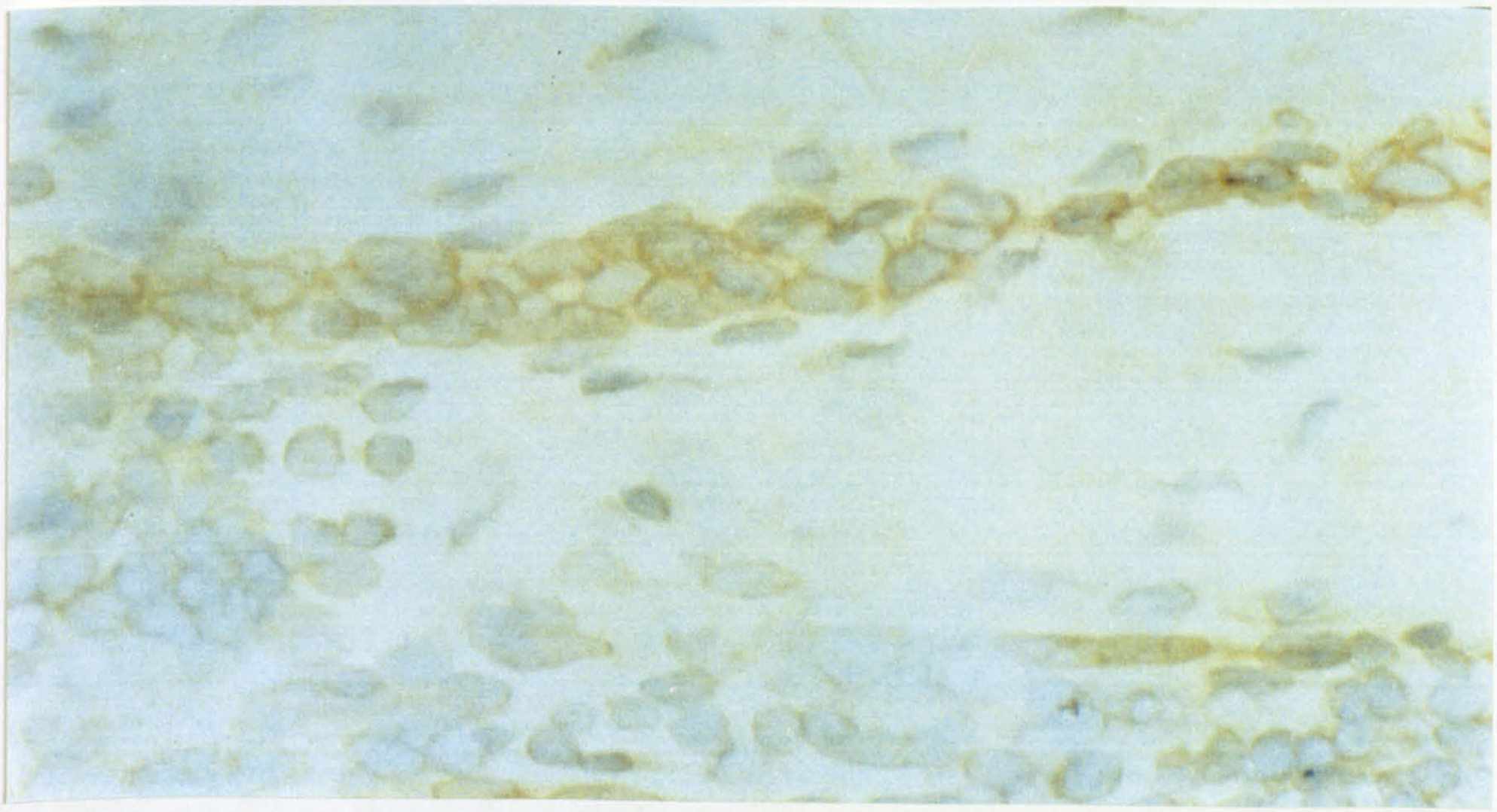
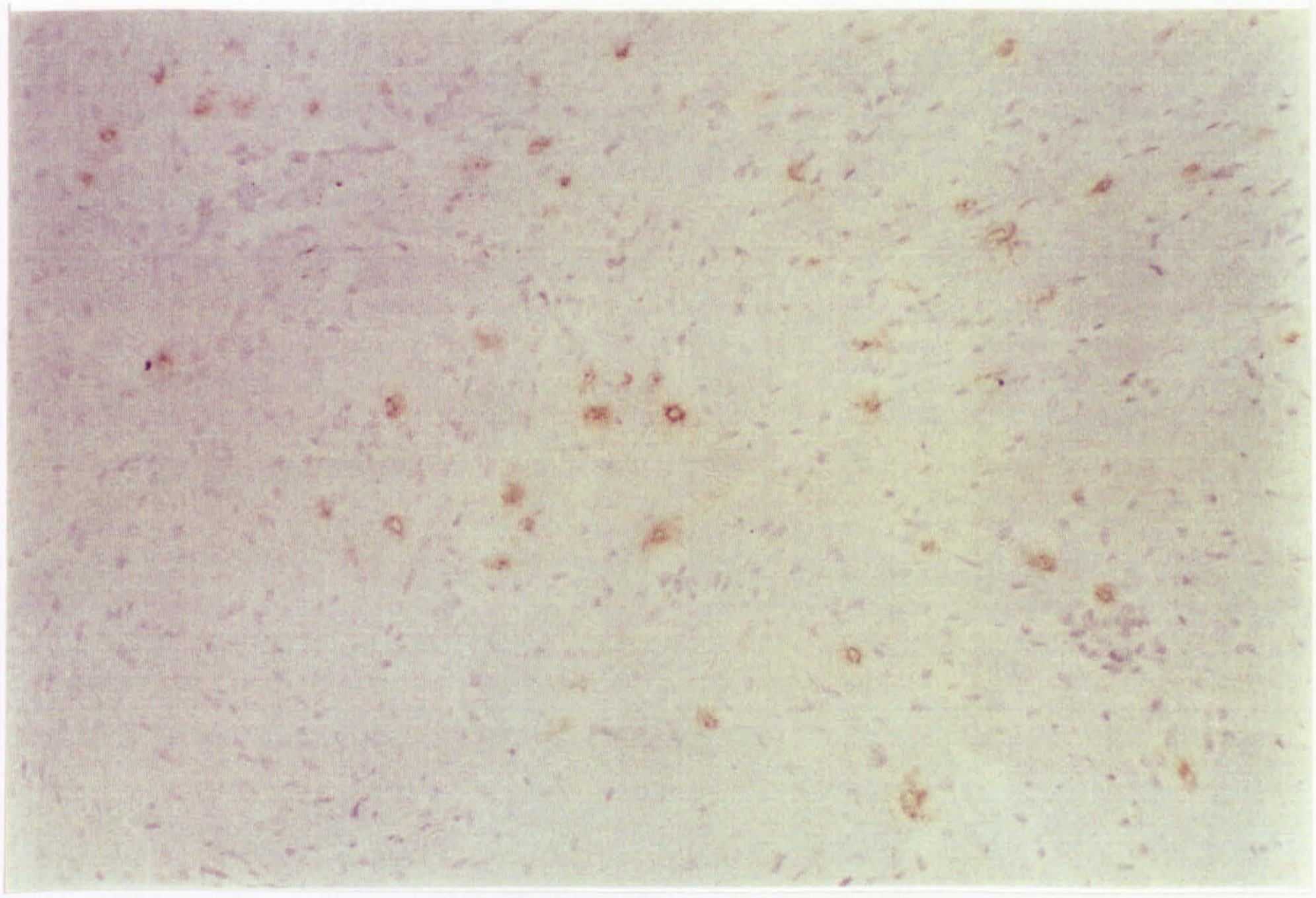


Figure 4.7 A micrograph illustrating the immunolocalisation of VEGF in keloid dermis. Specific localisation to the endothelial cells of capillaries is shown (bar represents 250 μm, dilution 1:100, representative of n=75)



4.25 Transforming growth factor beta

Low levels of immunoreactivity were observed in a minority of macrophages in the normal skin although not in other cellular populations (see Table 4.1).

Immunoreactivity to TGF-ß was found in hair follicles, sebaceous glands and merocrine sweat glands in the keloid dermis. Macrophages and fibroblasts in the dermis exhibited labelling although fibroblasts in the keloid itself did not. TGF-ß immunolabelling was not detected the epidermis of either skin type (Figure 4.1d and Table 4.1).

4.30 FURTHER ANALYSES

Quantification studies using SeeScan were attempted, it was impossible, however, to discern cell types within tissue sections (see Chapter 1). Western blotting was also attempted, sample preparation, however, proved unsuccessful. This failure was caused by the collagenous nature of the keloid lesions; homogenisation, freeze-fracturing and vibrational-fracturing were all tested for suitability but efforts were unfortunately unsuccessful.

4.40 DISCUSSION

Although much research has been carried out to elucidate the aetiology of keloids, the mechanisms involved in their formation are still unknown. Wound healing is a complex cascade of events initiated in response to injury. There is an acute inflammatory phase and the deposition of a provisional extracellular matrix, followed by collagen synthesis and wound resolution. Injuries resulting in keloid growth do not enter the final remodelling phase leading to normal scar formation. Many cytokines are important to normal wound healing, the relative quantities and distribution of these mediators reflect their roles in the resolution of injury. In this study cytokines were located immunohistologically within normal and keloid tissues with a view to suggesting their contribution to the continued expansion of the keloid lesion.

EGF has many roles in the normal healing process. In the current study, increased levels of EGF were found in association with blood vessels, macrophages and fibroblasts in the keloid tissue. EGF acts as a mitogen for a variety of cell types and stimulates the release of collagenase (Delany and Brinkerhoff, 1992). It is possible that the distribution pattern observed in this study is consistent with EGF involvement in wound remodelling, through the production of collagenase.

The capacity of PDGF to induce both the synthesis and the breakdown of extracellular components provides it with a vital role in the resolution of wounds. The majority of macrophages and fibroblasts in the dermis of the keloid were immunolabelled for PDGF. These results suggest PDGF production by macrophages and the action of PDGF on the fibroblasts leading to the production of collagenase and collagen (Narayanan and Page, 1983; Bauer *et al*, 1985). This hypothesis is corroborated by the finding that keloid fibroblasts display an enhanced response to PDGF as well as having elevated levels of PDGF α receptors (Haisa *et al*, 1994). This suggests both positive and negative roles for PDGF in the aetiology of keloids through the production of collagen and collagenase, respectively.

bFGF promotes angiogenesis and upregulates the expression of VEGF (Stavri *et al*, 1995). Neutralising antibody treatment of bFGF *in vivo* has revealed its importance at the early stages of wound repair (Broadley *et al*, 1989), it increases granulation tissue cellularity and reduces collagen content. A notable feature of the character of bFGF is that it is mainly intracellular, the mechanism of release is not known although it has been suggested that heat shock may achieve it (Jackson *et al*, 1992). The presence of substantial levels of bFGF in keloid dermis and lesion is consistent with the general stimulatory effects of bFGF on angiogenesis and VEGF expression.

Increased levels of VEGF in keloid samples indicates the development of new blood vessels, because when endothelial cell proliferation has ceased transcription levels are reduced (Breier *et al*, 1992). In tumours, VEGF release is stimulated by hypoxia and reduced oxygen tension (Jim Kim *et al*, 1993; Plate *et al*, 1992), it is not yet absolutely clear if keloids display evidence of hypoxia or necrosis, although it has been suggested (Kischer, 1992). In the case of keloids, therefore, the stimulus for VEGF expression is unclear. Given the abundant presence of cytokines in the keloid tissue, the stimulus in this case is more likely to be the presence of TGF-ß, bFGF and/or PDGF, each of which have been shown to increase the production of VEGF (Finkenzeller *et al*, 1992; Pertovaara *et al*, 1994; Stavri *et al*, 1995). There are many blood vessels throughout the keloid and the dermis, it is possible that the persistence of VEGF-stimulating growth factors could be the cause.

TGF-ß increases the deposition of extracellular matrix components by increasing the production of collagen and protease inhibitors and decreasing the production of proteolytic enzymes (Ignotz and Massague, 1986; Edwards *et al*, 1987). In keloid samples, TGF-ß was found in both fibroblasts and macrophages around the perimeter of the lesion. This observation is consistent with extracellular matrix deposition occurring in this area, suggesting the increase in growth of the lesion is propagated at its perimeter. Previous *in vivo* work has shown that dermal wounds treated with neutralising antibody to TGF-ß healed

without scarring, strongly suggesting that TGF-ß is required for scar formation. Those wounds treated with TGF-ß itself showed increased and more disorganised collagen content (Shah *et al*, 1992; Shah *et al*, 1995). Disorganised collagen arrangement caused by reduced cross-linking is present in keloid lesions although the enzyme, lysyl oxidase, which is required for the formation of mature cross-links is present and active (Di Cesare *et al*, 1990). It is possible, therefore, that the presence of increased levels of TGF-ß in the keloid lesion could account for the disordered collagen arrangement.

The results of this study have demonstrated notably increased levels of bFGF and VEGF in keloid samples. Each of these cytokines alone or both of them acting synergistically, therefore, may be responsible for the increased number of blood vessels observed in keloid tissue. Because angiogenesis (new blood vessel formation) is a prerequisite for granulation tissue growth and, hence, wound resolution, this may indicate that the manipulation of bFGF or VEGF activity may be therapeutically useful. Furthermore, the results have demonstrated increased levels of TGF-ß. It is possibel that the action of this cytokine on extracellular matrix deposition may account for the large collagenous lesion typical of keloids.

4.50 CONCLUSION

In vitro studies have shown that keloid fibroblasts display enhanced proliferative responses to EGF (Kikuchi *et al*, 1995) and TGF-ß (Russell *et al*, 1988), enhanced mitogenic responses to PDGF (Haisa *et al*, 1994) as well as displaying reduced collagen I production in response to bFGF (Tan *et al*, 1993*; Tan *et al*, 1993). The results of this study suggest that an excess of these factors is present in keloid tissues. What is not clear is why this should be so; the propagation of the lesion, however, is clearly related to the continued cytokine presence. Cells produce mediators in response to some stimulus until that stimulus is lifted or until inhibition occurs through the action of another mediator. Keloid patients do not display effects of systemic upregulation of cytokines; indeed, not all injured areas will result in keloid formation in susceptible individuals. Although cytokines provide therapeutic opportunities, therefore, it is unlikely that they are the initial cause of keloid formation. It is necessary to identify the persistent stimulus responsible for the propagation of the lesion.

Chapter 5

THE ROLE OF VEGF IN THE MURINE AIR POUCH MODEL

CONTENTS

5.10 INTRODUCTION

The process of new blood vessel formation, angiogenesis, is critical to the propagation of certain pathophysiological conditions. One such well documented condition is the growth of tumours. In this particular instance, a lack of sufficient oxygen and nutrients in the centre of the tumour mass stimulates the growth of blood vessels into its centre. A number of cytokines, for example bFGF (Lobb *et al*, 1985; Schweigerer *et al*, 1987; Gospodarowicz, 1990), have been implicated in this process and recently a potent endothelial-cell-specific mediator has provided fascinating new insight into angiogenesis. Vascular endothelial growth factor (VEGF), also termed vascular permeability factor (VPF), has been shown to be an absolute requirement for tumour growth (Jin Kim *et al*, 1993). Its mitogenic effects are restricted to endothelial cells (Leung *et al*, 1989), an attribute which makes VEGF unique among cytokines. As already mentioned, VEGF is angiogenic *in vivo* (Leung *et al*, 1989) and is a potent vascular permeability factor. These observations have prompted the suggestion that VEGF is the angiogenic factor which causes tumour growth and the vascular permeability factor which causes the persistent hyperpermeability of microvessels so characteristic of healing wounds (Klagsbrun and Soker, 1993).

As documented in Chapter 4, blood vessels in keloid tissue display intense immunoreactivity for VEGF. Although it is widely accepted that keloids

feature extensive vascularisation, evidence of a role for angiogenesis in keloid formation and propagation has not yet been presented. Colville-Nash *et al* have previously demonstrated that angiogenesis is maximal at day 7 in the murine air pouch model (1995), and there is a lot of information available on the profile and distribution of various cytokines in this model (Appleton *et al*, 1993). In this study, therefore, the effects of neutralising antibody treatment to VEGF was examined using the murine air pouch model.

5.20 DOSING REGIME

Animals were dosed twice weekly, intraperitoneally, from day 1, with 10mg polyclonal antihuman VEGF, 10mg control immunoglobulin (Ig) G or phosphate buffered saline (PBS). The pouch was allowed to develop for 7 days.

5.30 RESULTS

5.31 Air pouch tissue dry weight (mg)

Tissue dry weight was evaluated at day 7 (the timepoint at which angiogenesis is maximal in this model). Treatment with anti-VEGF resulted in a statistically

significant decrease in tissue dry weight (43.07 ± 1.32mg) compared to IgG control (62.5 ± 2.85mg [P<0.05]) and PBS (59.82 ± 4.23mg; Figure 5.1).

5.32 Air pouch carmine content (μg)

Carmine content at day 7 for the PBS group was 87 ± 8.7μg which was significantly increased (P<0.05) compared with the anti-VEGF group (24.1 ± 3.3μg). The carmine content of the anti-VEGF group was significantly decreased (P<0.05) when compared with the IgG control group (50.6 ± 6.5μg; Figure 5.2).

5.33 Air pouch vascularity (μg carmine/mg tissue dry weight)

A decrease in vascularity accompanied that of tissue dry weight. Anti-VEGF treatment resulted in a significant reduction in vascularity from 0.78 ± 0.08μg/mg (P<0.05) in the PBS control group and 0.7 ± 0.09μg/mg (P<0.05) in the IgG group to 0.42 ± 0.07μg/mg in the treatment group (Figure 5.3).

Figure 5.1 A graph to show granulomatous tissue dry weight (mg) in the PBS, IgG and anti-VEGF groups (results are expressed as mean ± standard error for n=15 animals per group [experiment was carried out three times, data presented is that of a single experiment])

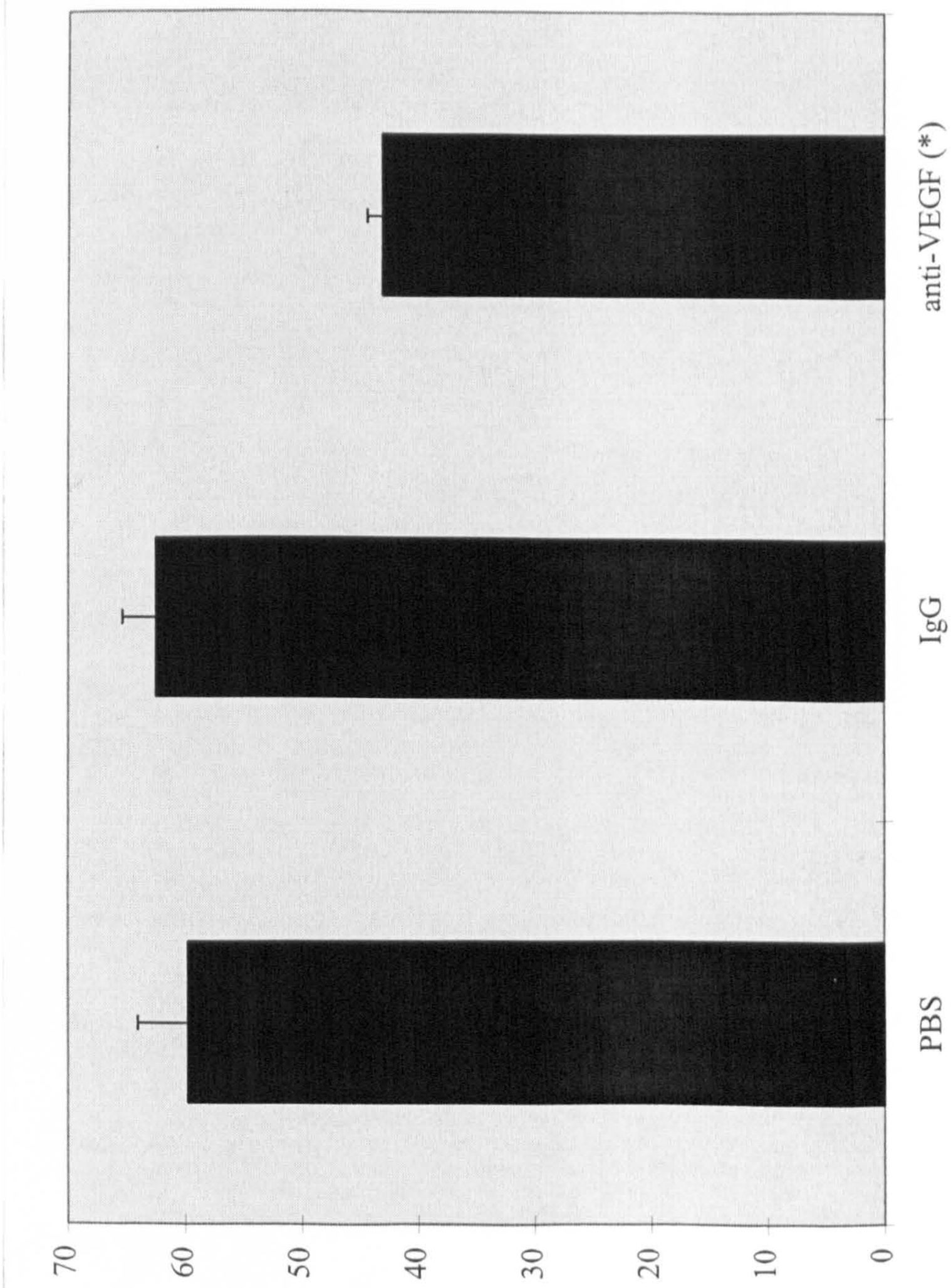


Figure 5.2 A graph to show total carmine content (μg) in the PBS, IgG and anti-VEGF groups (results are expressed as mean ± standard error for n=15 animals per group [experiment was carried out three times, data presented is that of a single experiment])

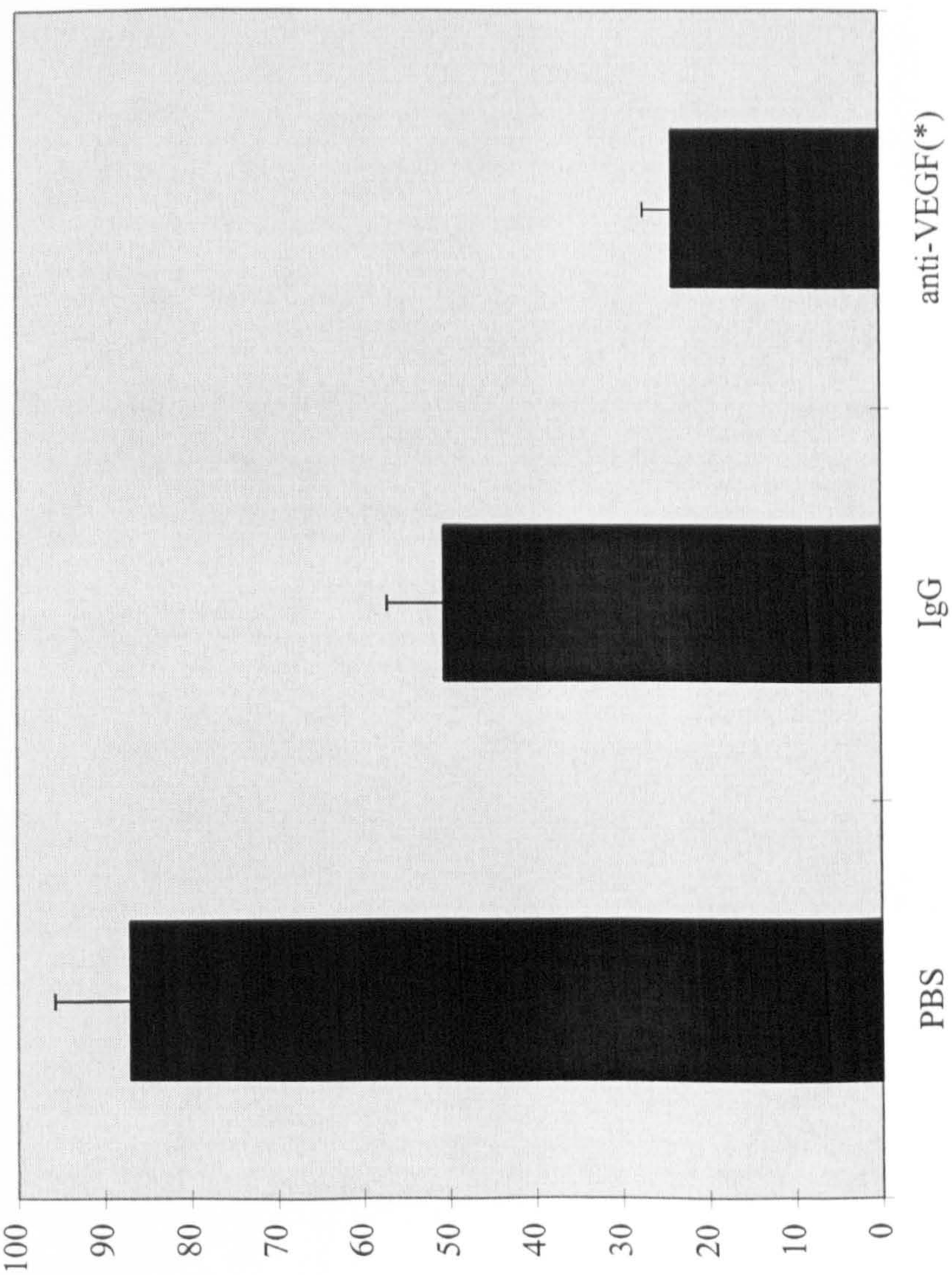
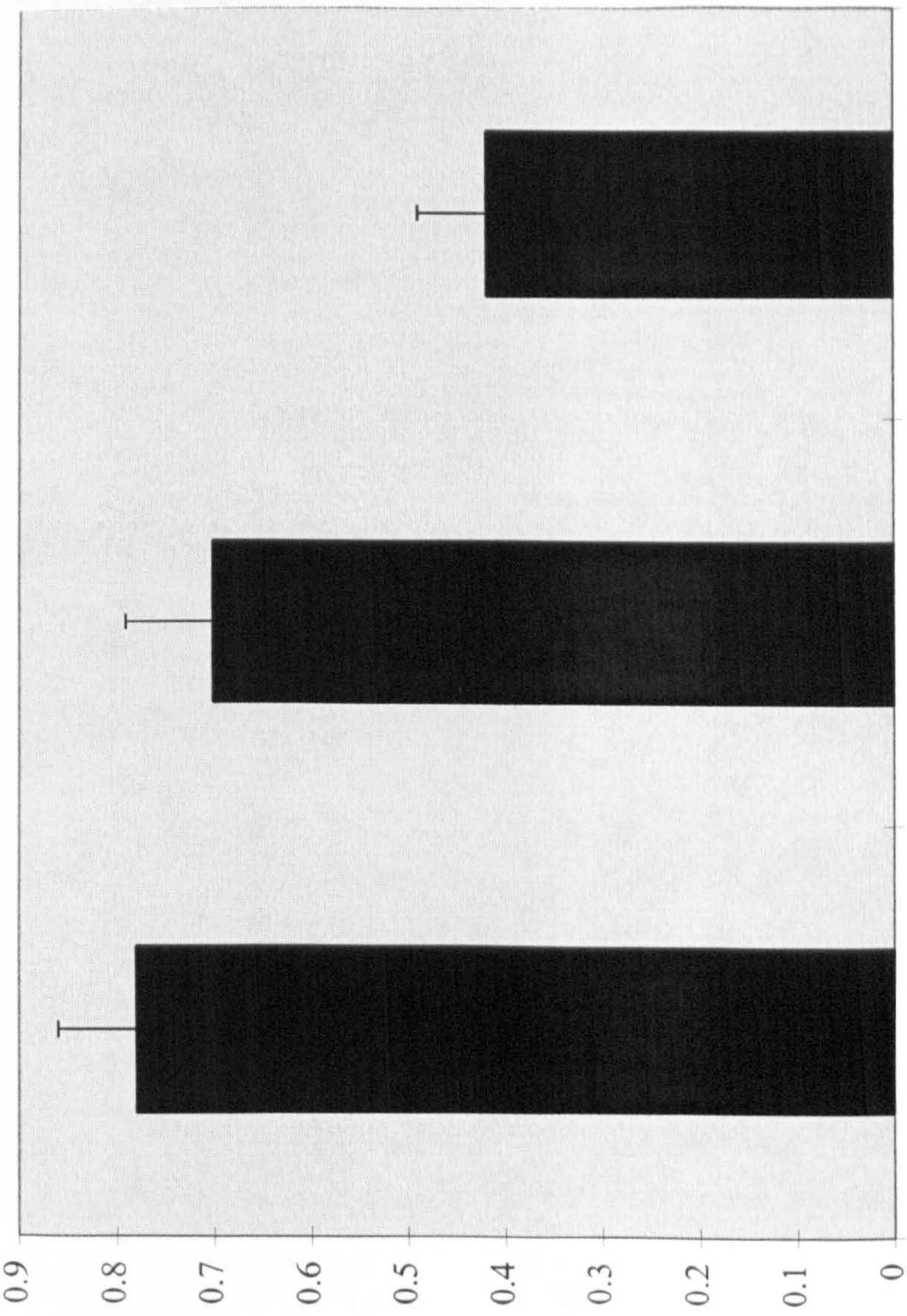


Figure 5.3 A graph to show vascularity (μg carmine/mg tissue dry weight) in the PBS, IgG and anti-VEGF groups (results are expressed as mean ± standard error for n=15 animals per group [experiment was carried out three times, data presented is that of a single experiment])



5.34 Clearing

The results of cedar wood oil treatment are shown in Figure 5.4: a) represents the PBS control sample; b) represents the IgG treated group; and c) represents the anti-VEGF treated group. It is clear from Figure 5.4 that anti-VEGF treatment has greatly reduced the number of blood vessels present in the day 7 air pouch samples. In Figure 5.4c, the carmine is retained within the pre-existing blood vessels of the dermis.

5.35 Immunolocalisation of CD31/PECAM

Figure 5.5 shows that neutralising antibody treatment to VEGF has resulted in a marked reduction in the number of blood vessels present in the granulomatous/granulation tissue.

5.36 Western blot (1mg/ml protein, 20μl/well)

VEGF protein occurred at approximately 23kDa (as expected). VEGF was high up to 24 hours, still present at day 3 with levels at subsequent timepoints being significantly reduced. (Levels of protein were highest before day 3.) VEGF protein was not detected in normal skin (Figure 5.6).

Figure 5.4 Micrographs to show the vascularity of skin samples of the murine air pouch model (a = PBS, b = IgG, c = anti-VEGF, magnification x200, representative of n=15)

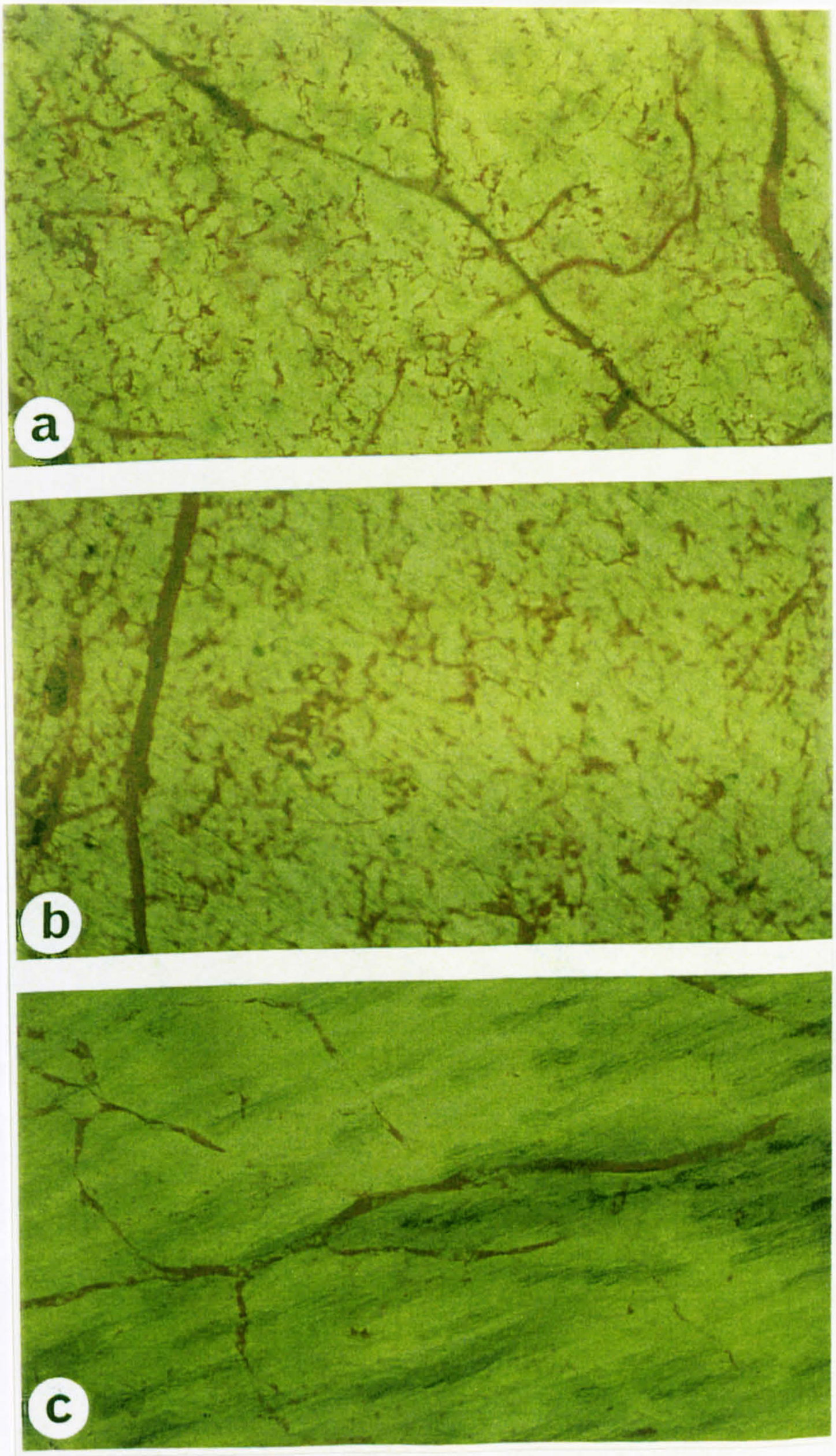


Figure 5.5 Micrographs to show the immunolocalisation of PECAM in tissue from the murine air pouch model (magnification x100, dilution 1:50, representative of n=25, a = control, b = anti-VEGF group)

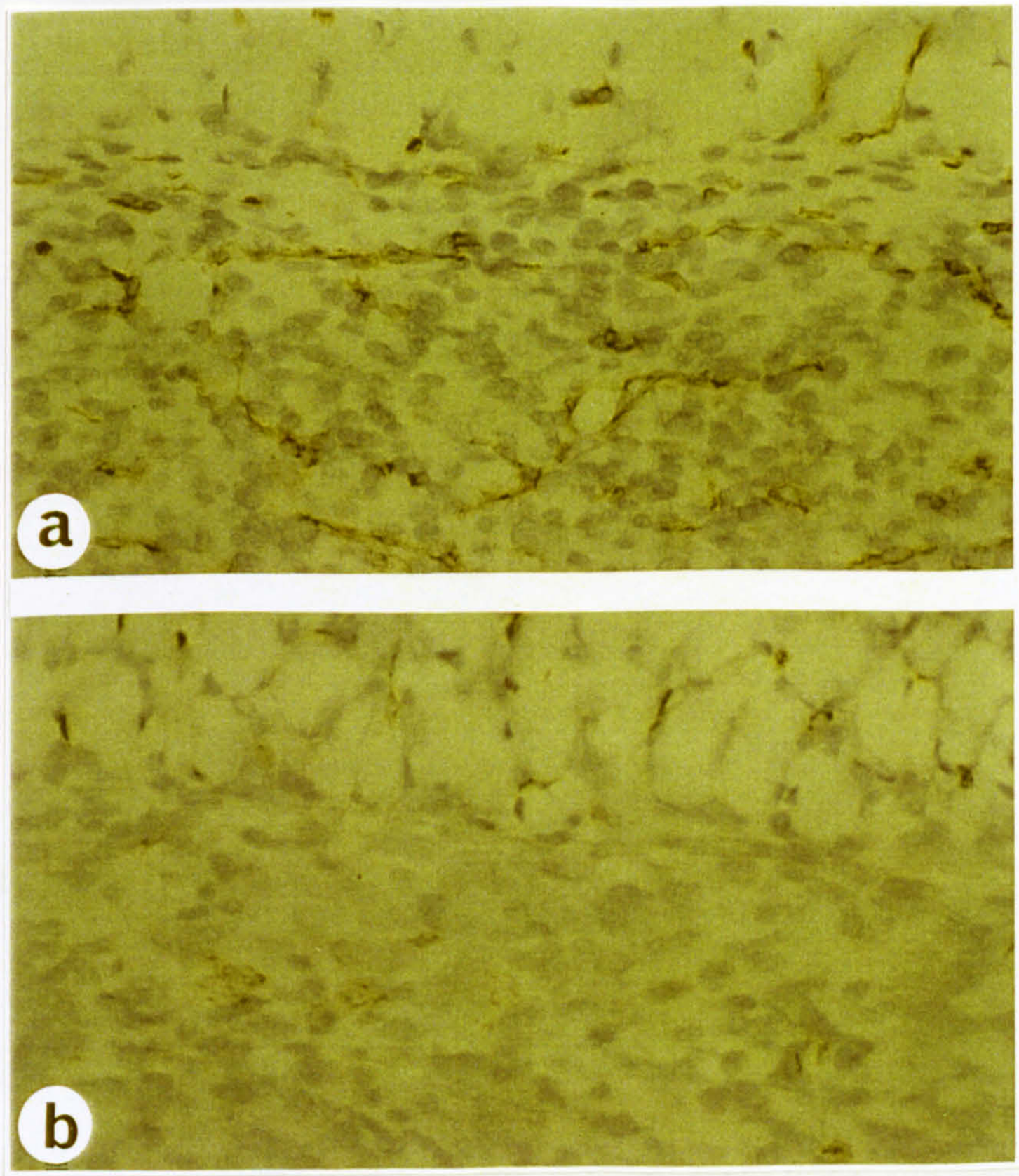
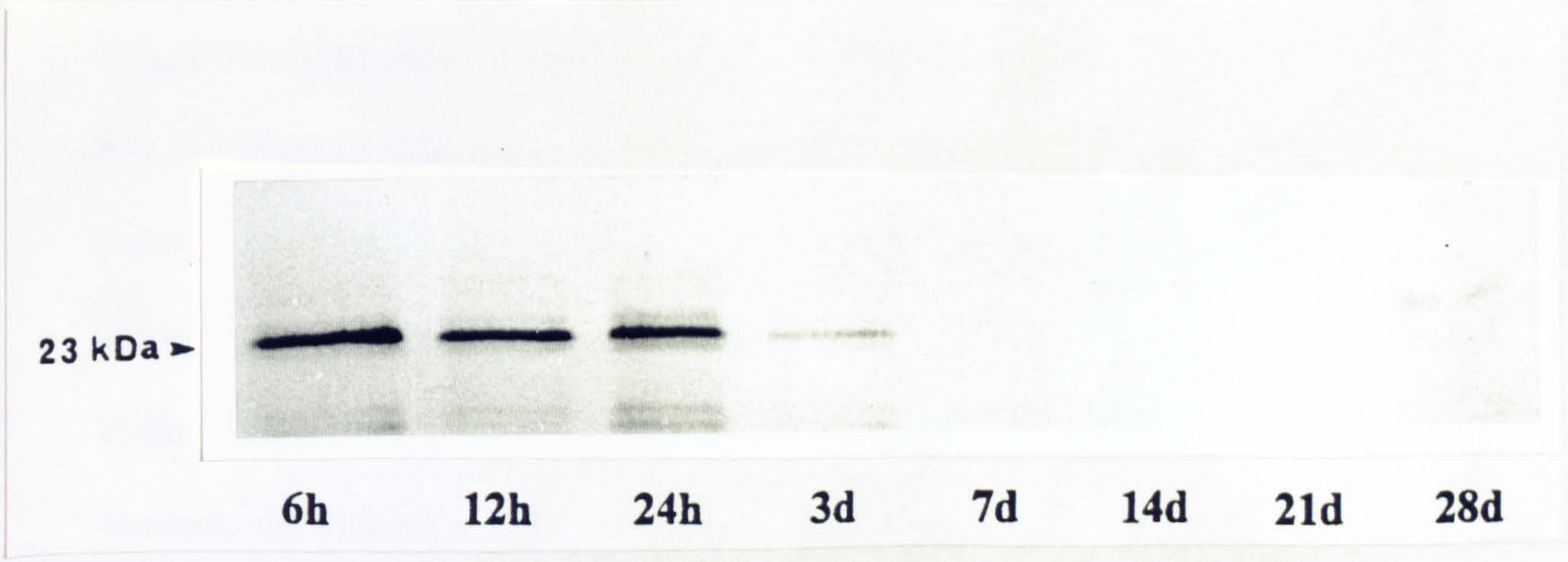


Figure 5.6 A micrograph to show the Western blotting results for VEGF in tissues taken throughout murine air pouch tissues



5.40 DISCUSSION

There is an increasing body of evidence to suggest that VEGF may be the most important factor in tumour angiogenesis. It is the only known endothelial-cell-specific mitogen (Leung *et al*, 1989), it is angiogenic *in vivo* (Leung *et al*, 1989; Klagsbrun and Soker, 1993) and treatment with anti-VEGF significantly reduces angiogenesis in a number of tumour models (Jin Kim *et al*, 1993). This study

shows that anti-VEGF treatment can also significantly reduce pouch tissue dry weight as well as suppress the vascularity in the murine air pouch model (and by inference, wound resolution).

bFGF upregulates the expression of VEGF in vascular smooth muscle cells (Stavri *et al*, 1995) and these two cytokines act synergistically to induce endothelial cell proliferation (Pepper *et al*, 1992). Pepper *et al* also demonstrated that low levels of TGF-ß potentiate the actions of bFGF and VEGF, whereas higher levels of TGF-ß attenuate them (1993). Appleton *et al* have previously shown that bFGF is present throughout the air pouch model and that TGF-ß levels are low at day 3 but increase thereafter (1993). The results of this study, therefore, are consistent with active angiogenesis until approximately day 7 followed by a reduction, possibly caused by the presence of increasing levels of TGF-ß, as the tissue resolves. Also, termination of endothelial cell proliferation results in a decrease of VEGF production (Breier *et al*, 1992); hence, the finding that VEGF protein is mainly present upto day 3 only, in this model, suggests active angiogenesis at that timepoint. Neutralising the action of VEGF from day 1, therefore, would result in significant inhibition of angiogenesis. This hypothesis is corroborated by the observation that anti-VEGF treatment resulted in drastically reduced blood vessel formation (Figures 5.4 and 5.5).

Much of the initial work on VEGF was carried out within the context of tumours and their growth. In the case of tumours, VEGF release is associated

with reduced oxygen tension and hypoxia in the centre of the growing mass. The stimulation of VEGF activity in wound healing is still not clear; Brown *et al* (1992) suggested that VEGF may be acting as a mitogen for endothelial cells in the wound bed and as a promoter of the deposition of an extravascular fibrin matrix via its vascular permeability abilities, but did not offer any suggestion as to what the mechanism of VEGF stimulation and release may be.

The results here suggest that VEGF activity is a vital component of the resolution of the murine air pouch model. The inhibition of this activity resulted in a statistically significant reduction in vascularity and granulomatous/granulation tissue dry weight. Granulation tissue has an extensive neovasculature embedded within a loosely assembled matrix of collagens, fibronectin and proteoglycans. Fibroblasts within the granulation tissue acquire the ability to contract the collagenous matrix thus contributing to wound closure and healing. The formation of granulation tissue is an essential transitory stage in the progression to efficient healing, any inhibition of its formation will delay wound resolution.

5.50 CONCLUSION

The evidence provided by many studies indicates that VEGF is very important in the angiogenic process; a process that facilitates the continued growth of tumours *in vivo* and, as demonstrated by these results, influences granulomatous/granulation tissue development and resolution. Is it possible that VEGF is involved in the formation and/or propagation of keloid lesions?

The results of immunocytochemical analyses of keloid tissues have revealed high levels of immunolabelling for bFGF and VEGF in the dermis and in the lesion; whereas TGF-ß was located only in the dermis and at the leading edge of the lesion, not actually within it where the growth of blood vessels would be occurring (Chapter 4). Blood vessels were found throughout the tissue and the majority were immunolabelled for bFGF and VEGF (characterisation of blood vessels was carried out using antibodies to CD31/PECAM; Chapter 3). VEGF activity in wounds quickly returns to normal levels within days of healing (Brown *et al*, 1992) and indeed VEGF is only expressed during active angiogenesis (Breier *et al*, 1992) as is confirmed by the results obtained in the murine air pouch model which show levels of VEGF activity building to day 7, the timepoint of maximum tissue dry weight, and decreasing at subsequent timepoints as resolution progresses. Keloid tissues, however, feature consistently high levels of VEGF protein throughout the extensive vascular network. This

evidence suggests that active angiogenesis is still ongoing in scars which may be several years old.

The abundant presence of angiogenic factors (bFGF, TGF-β and VEGF) in keloid samples suggests a role for them in the propagation of the lesion. It is possible, therefore, that therapeutic neutralisation of local VEGF activity could provide a means to halt the continued growth of the lesion.

Chapter 6

IMMUNOLOCALISATION OF LYMPHOKINES IN MURINE AIR POUCH TISSUES AND HUMAN TISSUES

CONTENTS

6.10 INTRODUCTION

The dermis of keloid scars features increased numbers of T cells in comparison with normal skin and abnormal scars (see Chapter 3; Martin and Muir, 1990). The greatest concentrations of lymphocytes are observed at the invading edge of the lesion and in association with hair follicles. The lesion is composed of disordered collagen bundles (Hunter and Finlay, 1976) interspersed with fibroblasts (Matsuoka *et al*, 1988) and mast cells (Craig *et al*, 1986).

In normal wound healing, T cells are involved in the modulation of fibroblastic activity. This has been demonstrated *in vivo* by depleting mice of T cells, which resulted in impaired collagen deposition and decreased wound breaking strength (Peterson *et al*, 1987).

It is possible to define T cell subsets by their pattern of cytokines secreted in response to antigen (for reviews, see Romagnani, 1991; Carter and Dutton, 1996). These subsets are termed type 1 and type 2 and have been identified for $CD4^+$, $CD8^+$ and γδ T cells. Type 1 $CD4^+$ cells produce interleukin (IL)-2 and interferon (IFN)-γ and inhibit the development of type 2 cells; type 2 $CD4^+$ cells produce IL-4 and IL-5 and inhibit the development of type 1 cells (Goldman *et al*, 1991). (The situation for $CD8^+$ and γδ T cells is

more complex and will not be dealt with here, although it is probably suffice to say that $CD8^+$ cells are involved in immune suppression.)

IL-10 is produced by type 1 and type 2 human $CD4^+$ cells, it inhibits type 1 $CD4^+$ cell development both alone and in synergy with IL-4 (Yssel *et al*, 1992; Powrie *et al*, 1993; Del Prete *et al*, 1993).

In vitro activated mast cells enhance fibroblast proliferation and collagen production (Abergel *et al*, 1985; Levi-Schaffer and Rubinchik, 1995) possibly via histamine release (Sandberg, 1962). Keloid samples contain elevated levels of histamine and increased blood vessels in the dermis. This suggests a role for histamine and blood vessels in the propagation of keloid lesions (Hakanson *et al*, 1969; Cohen *et al*, 1972; Kischer *et al*, 1978; Smith *et al*, 1987).

Human skin mast cells produce IL-4 in response to immunoglobulin-E-dependent stimulation (Bradding *et al*, 1992). It has been suggested that this activity triggers the development of $CD4^+$ cells of the type 2 subset (Bradding *et al*, 1992), although natural killer cells and basophils have also been implicated (Bendelac, 1995; Porcelli and Modlin, 1995; Yoshimoto *et al*, 1995). IL-4-transgenic mice develop high levels of circulating immunoglobulin E (Tepper *et al*, 1990), whereas IL-4-deficient mice cannot produce IL-4 nor do they develop IgE responses (Kuhn *et al*, 1991). In a random study carried out on 1206 individuals, a positive correlation was

shown between the level of IgE-associated responses and keloid formation (Smith *et al*, 1987).

There is overwhelming evidence in favour of an immunological component to keloid formation (for review, see Placik and Lewis, 1992); however, the initiation and perpetuating force are unknown. This study was designed to investigate the distribution of lymphokines (IL-2, IL-4, IL-10 and IFN-γ) in keloid tissue and to propose their roles in the formation and propagation of the lesion.

In order to speculate on the roles of these mediators in abnormal healing it was necessary to further investigate their roles in an animal model. Immunohistochemical analysis was carried out on tissues from the murine air pouch model at different stages of resolution. It was intended that demonstrating the presence or absence of these four lymphokines, as well as their patterns and relative distribution, would provide further evidence of their roles in normal resolution and that the insight gained could be employed to further explain the cause and exacerbation of keloids.

6.20 MURINE AIR POUCH MODEL TISSUE RESULTS

6.21 Interleukin 4

Immunolabelling for IL-4 at day 3 was restricted to hair follicles, at day 7 additional immunoreactivity was evident in lymphocytes in the area of granulomatous/granulation tissue adjacent to the skeletal muscle layer. This gradual increase in positivity continued through to day 14, with labelling of fibroblasts and lymphocytes in the granulation tissue. The epidermis and hair follicles in the dermis also displayed immunoreactivity at day 14. Levels of IL-4 reached a peak at day 21, with increasing numbers of lymphocytes and fibroblasts labelling. Immunoreactivity had reduced throughout the tissue by day 28 and levels were comparable to those at day 7 (see Table 6.1).

6.22 Interleukin 10

As was the case for IL-4, immunoreactivity for IL-10 at day 3 was restricted to hair follicles. Lymphocytes in the area of granulomatous/granulation tissue adjacent to the skeletal muscle were immunolabelled at day 7, as were dermal hair follicles. By day 14, fibroblasts, macrophages and lymphocytes in the granulation tissue were intensely labelled, numbers were higher than those for

IL-4 at the same timepoint. A further increase in positivity was evident at day 21 with fibroblasts in the lower region of the granulation tissue and with lymphocytes, macrophages and blood vessels in the upper region of the granulomatous/granulation tissue being strongly labelled. This pattern of distribution remained consistent through day 28 (see Table 6.1).

6.23 Interferon gamma

By day 3 immunoreactivity was evident in hair follicles and lymphocytes in the area of granulomatous/granulation tissue adjacent to the skeletal muscle. By day 7, IFN-γ levels had decreased throughout the tissue with only the hair follicles displaying positivity. There was an increase in immunoreactivity at day 14 to approximately equivalent levels to those at day 3 although, in contrast to the distribution at the earlier timepoint, labelled lymphocytes were located in the lower area of the granulomatous/granulation tissue. This increase in positive labelling continued throughout the remaining timepoints, with fibroblasts in the upper region and lymphocytes in the lower region of the granulation tissue displaying intense positivity (see Table 6.1).

6.24 Interleukin 2

Of the four lymphokines studied only IL-2 was present in control skin samples, and in this tissue its distribution was restricted to the epidermis. Dermal lymphocytes, hair follicles and the epidermis displayed immunoreactivity for IL-2 at day 3. Levels remained consistent at day 7 although the distribution had altered to include a few dermal blood vessels and granulomatous/granulation tissue lymphocytes. Immunoreactivity for IL-2 reached a peak at day 14 and levelled off at day 21, with lymphocytes and blood vessels in the granulation tissue being labelled as well as dermal lymphocytes, hair follicles and the epidermis. By day 28 the level and distribution of immunoreactivity had decreased to approximately that of day 7 (see Table 6.1).

*Table 6.1: Quantitative data for the immunolocalisation of lymphokines in the murine air pouch timecourse. Results are expressed as the mean and standard error (±) of positively stained stained cells in n = 4 tissues, 10 fields of view per tissue (4mm^2, x20). The Mann-Whitney U-test was used to investigate statistical significance (P < 0.05 = *)*

Day	Region	IFN-γ	IL-2	IL-4	IL-10
3	D	51.0±5.6	25.5±4.0	8.3±1.3	16.5±2.6
3	GT	20.3±1.7	11.5±1.7	2.3±1.3	2.0±0.8
7	D	30.0±4.7*	22.5±8.4	8.3±2.2	28.0±3.6*
7	GT	13.0±2.2*	14.8±2.5	14.3±3.0*	25.5±2.9*
14	D	51.0±2.9	67.5±3.7*	24.5±5.6*	40.3±3.8*
14	GT	29.5±5.9*	70.3±8.2*	50.5±3.7*	69.0±2.2*
21	D	69.3±5.6*	69.5±5.2*	31.0±1.8*	37.3±5.9*
21	GT	40.5±2.1*	72.5±5.6*	87.3±4.8*	82.0±3.7*
28	D	89.0±5.9*	28.0±3.7	14.3±2.6*	30.8±3.9*
28	GT	40.0±1.8*	21.8±3.3*	20.8±4.1*	84.0±3.7*

(D, dermis; GT, granulomatous/granulation tissue)

6.30 DISCUSSION

These results illustrate the relative distribution of IL-2, IL-4, IL-10 and IFN-γ in the context of a model of normal granulomatous/granulation tissue resolution. Generally, in this model the levels of immunoreactivity for all four lymphokines was shown to be increasing through 7–21 days, this time period is associated with matrix remodelling. Fibroblast proliferation is maximal at day 7 with persistently high levels through to day 21 (Kimura *et al*, 1985). The same pattern is observed for lymphocytes (Chapter 3; Kimura *et al*, 1985) and the quantity of collagen fibres (Chapter 7; Kimura *et al*, 1985). Matrix remodelling requires the synergism of mediators that exert positive and negative effects on collagen deposition and breakdown, the distribution pattern of the four lymphokines shown here is consistent with previous investigations: IL-2 is known to increase hydroxyproline content and wound breaking strength *in vivo* (Barbul *et al*, 1986); IL-4 stimulates fibroblast proliferation and collagen production (Fertin *et al*, 1991; Postlethwaite *et al*, 1992); IL-10 and IFN-γ have both been shown to behave as regulators of fibrosis; and, IFN-γ inhibits collagen production by fibroblasts as well as stimulating collagenase release (Seder *et al*, 1992; Reitamo *et al*, 1994).

As will be discussed in Chapter 7, IFN-γ is important early in the murine air pouch model, and indeed it is present in a greater number of cells

compared with the three other lymphokines investigated here. Granstein *et al* (1989) demonstrated that the *in vivo* administration of IFN-γ reduced the acute inflammatory response until approximately 72 hours after injury at which time the expected, although delayed, inflammatory response was evident. The group proved that this suppression was largely caused by angiostatic effects. Angiogenesis is maximal at day 7 in the murine air pouch model and these immunocytochemical results show that IFN-γ levels are lowest at that timepoint. It is possible, therefore, that IFN-γ is behaving as an anti-inflammatory agent at day 7.

By day 28 there is a clear dichotomy in lymphokine presence: immunoreactivity for IL-10 and IFN-γ is high; immunoreactivity for IL-2 and IL-4 is low. These findings are inkeeping with previous investigations of these particular lymphokines in wound resolution. It is possible that, at day 28, IL-10 and IFN-γ are acting as antifibrotic agents, with IL-10 inhibiting collagen I production and IFN-γ stimulating collagenase release, whereas IL-2 and IL-4, factors which would ordinarily increase collagen production and fibroblast proliferation, are at reduced levels.

6.40 HUMAN TISSUE RESULTS

6.41 Interleukin 4

Normal skin displayed immunoreactivity for IL-4 in the epidermis and hair follicles only (see Table 6.2).

In the keloid dermis immunoreactivity for IL-4 was observed in the epidermis, hair follicles, mast cells, lymphocyte populations, fibroblasts and some blood vessels, a minority of blood vessels were also labelled in the keloid lesion. Fibroblasts within the lesion were not labelled for IL-4 (Figures 6.1, 6.2, 6.3 and Table 6.2).

6.42 Interleukin 10

Immunoreactivity for IL-10 in normal skin was confined to hair follicles and the epidermis (see Table 6.2).

The epidermis, hair follicles, lymphocytes, blood vessels and fibroblasts in the keloid dermis were all positively labelled. Fibroblasts in the keloid lesion did not display immunoreactivity for IL-10 (Figures 6.1a and b, 6.4 and Table 6.2).

Figure 6.1 Micrographs illustrating the immunolocalisation of IL-4 and IL-10 in keloid tissue. Preabsorption of the antibody results in complete amelioration of labelling a) whereas b) serial sections labelled with IL-10 demonstrates specific localisation to hair follicles in the keloid dermis). In c) and d) localisation of IL-4 to mast cells and lymphocytes respectively is shown (bar represents 250μm, dilution 1:100, representative of n=75)

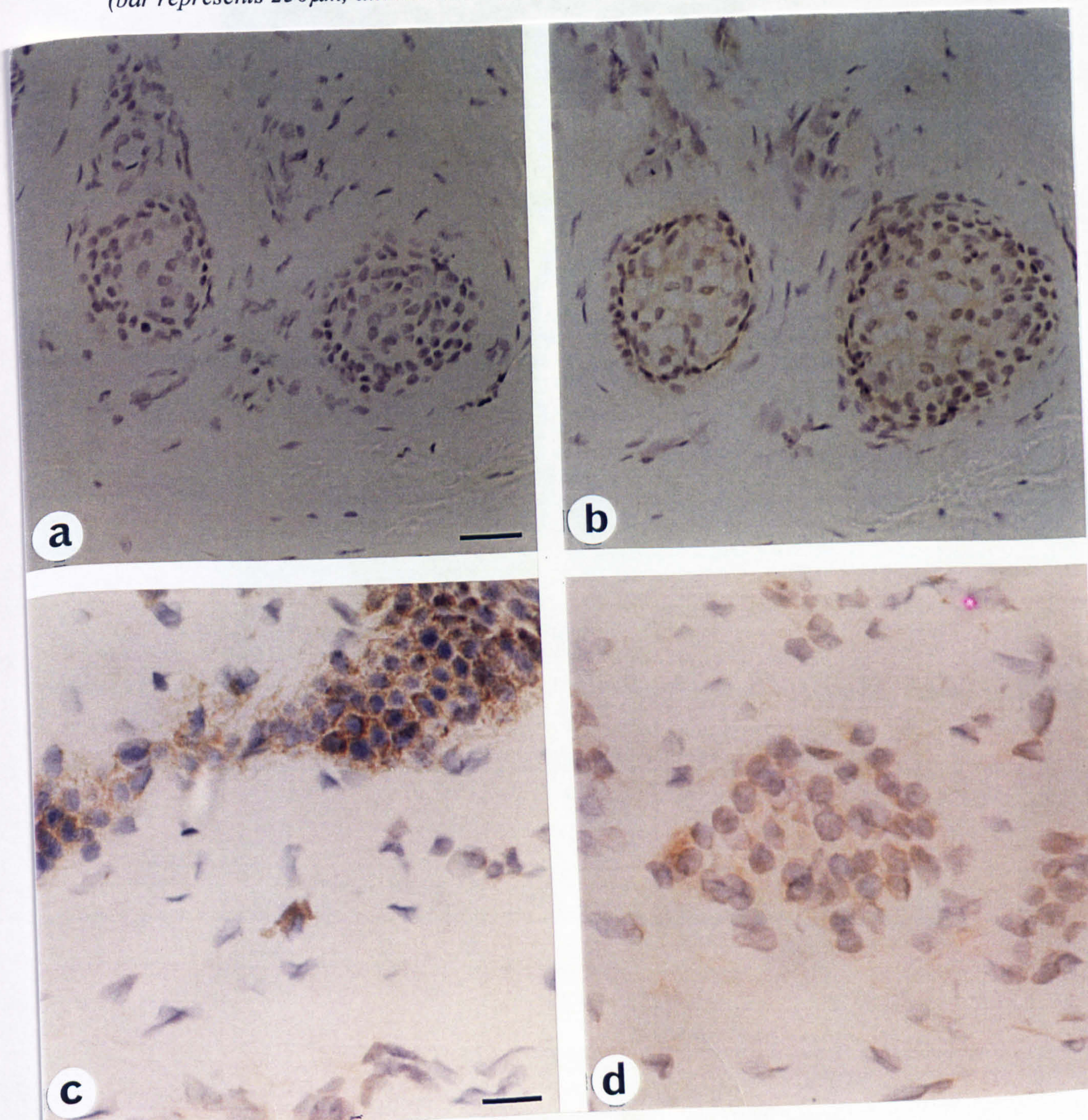


Figure 6.2 A micrograph to illustrate the immunolocalisation of IL-4 to lymphocytes in the dermis of a keloid sample (magnification x100, dilution 1:100, representative of n=75)

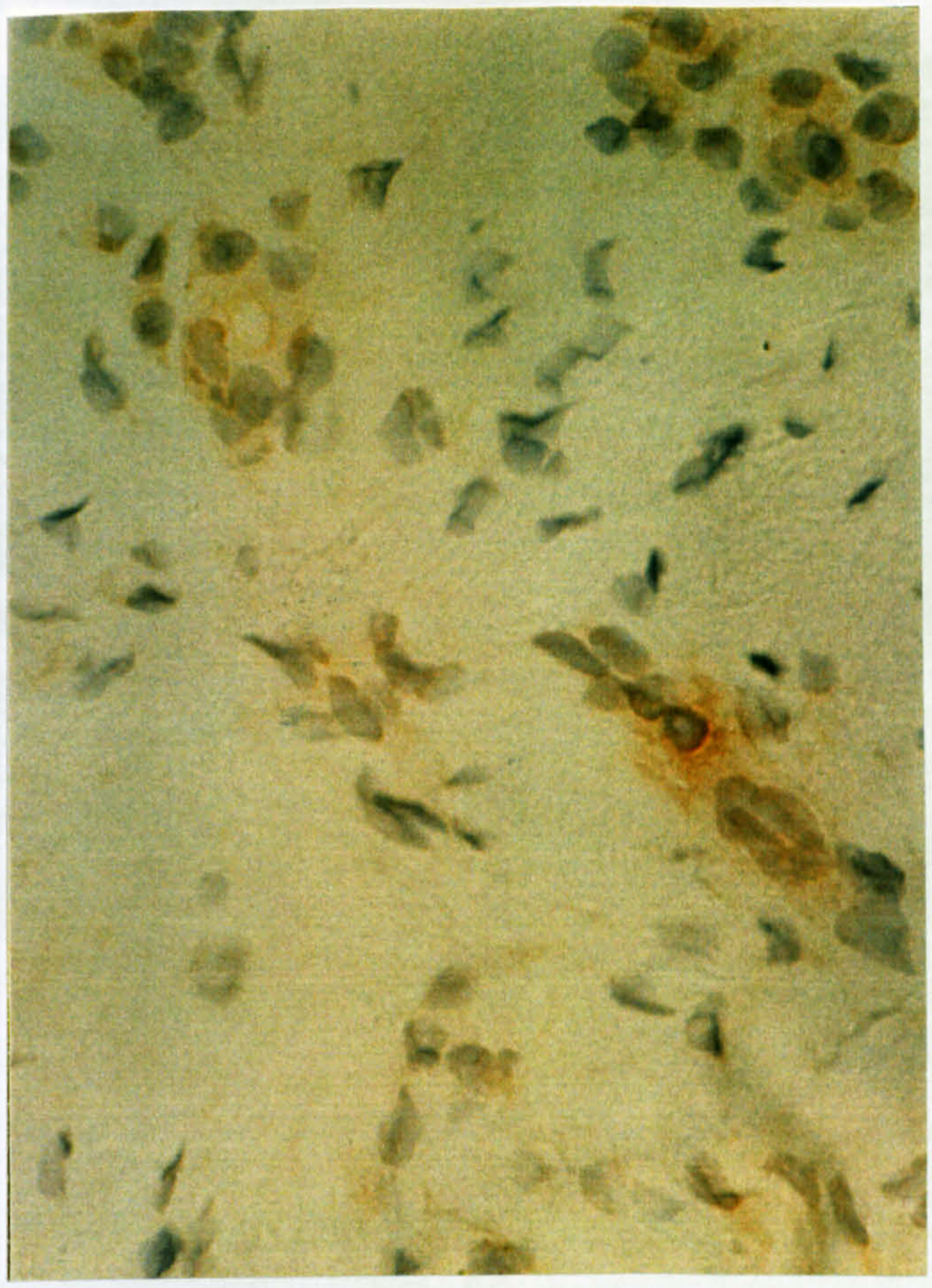


Figure 6.3 A micrograph to illustrate the immunolocalisation of IL-4 to lymphocytes in the dermis of a keloid sample at higher power (magnification x200, dilution 1:100, representative of n=75)

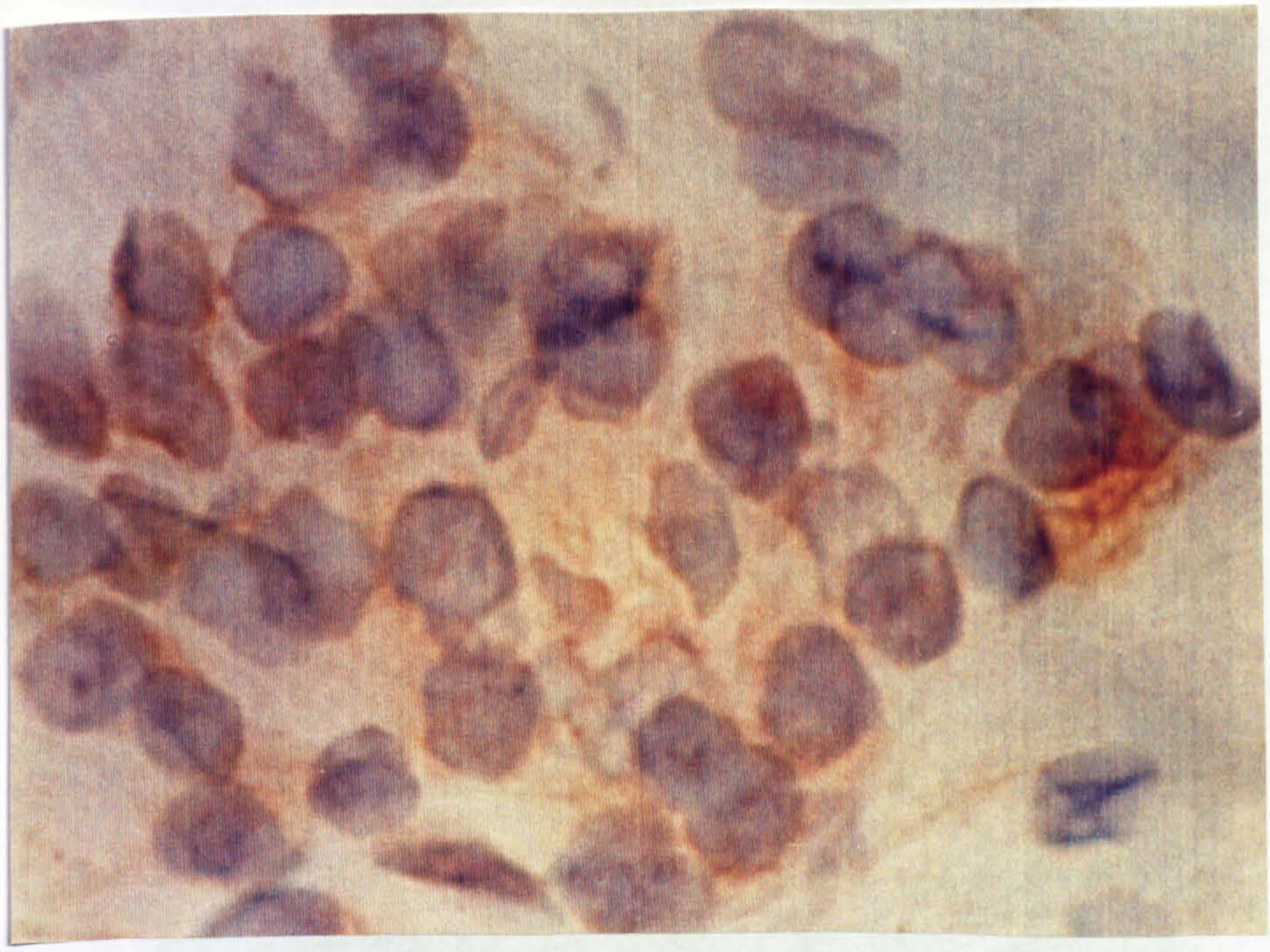
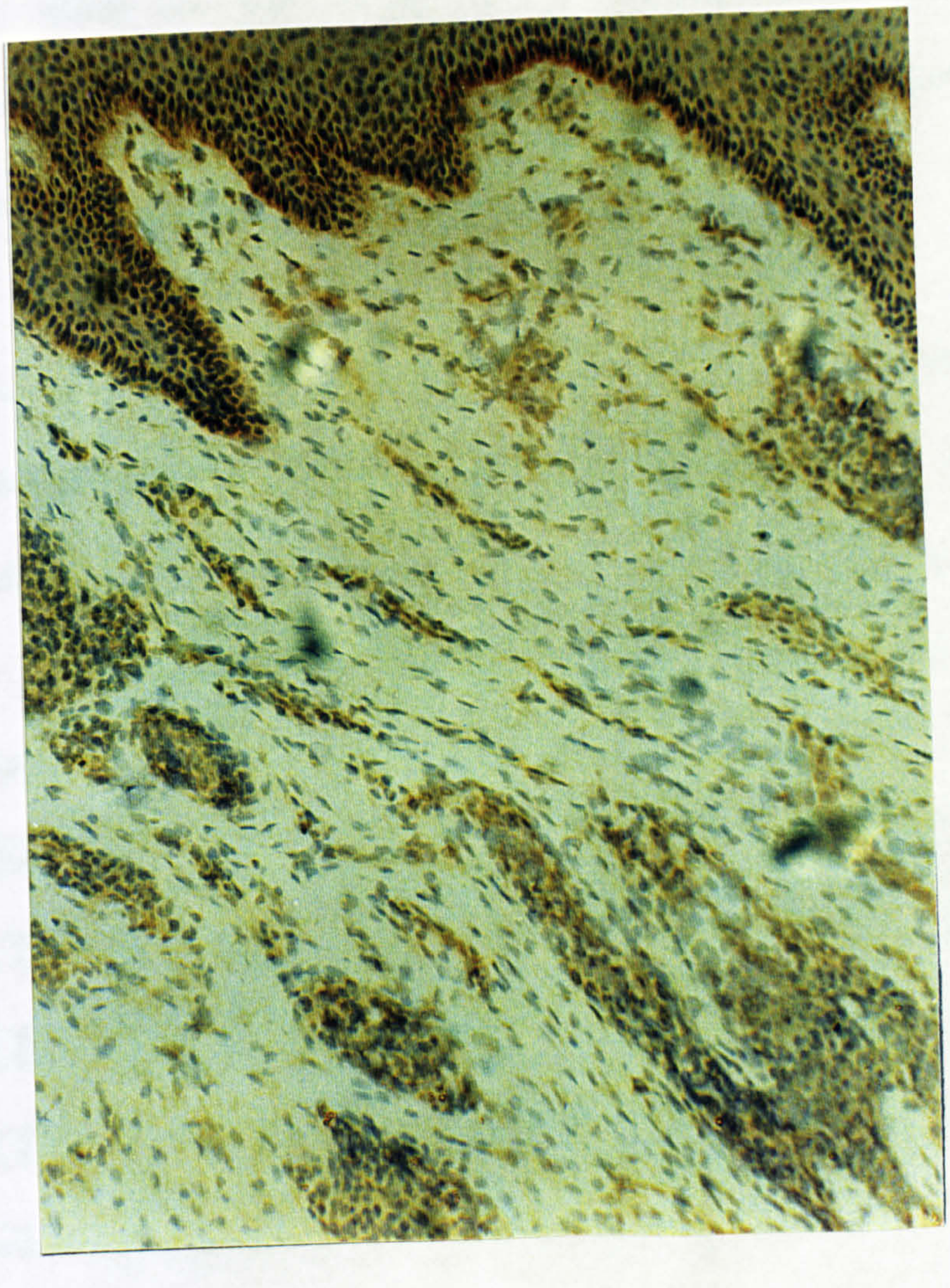


Figure 6.4 A micrograph to illustrate the immunolocalisation of IL-10 in a section taken through the epidermis and dermis of a keloid sample. Note the numerous positively labelled lymphocytes (magnification x50, dilution 1:100, representative of n=75)



6.43 Interferon gamma and interleukin 2

Immunoreactivity for IFN-γ and IL-2 was not seen in any cellular population in the keloid dermis or lesion (see Table 6.2). Positive labelling was seen in the control tissue, fibrocontractile capsule formed around silicone gel implants, which has been shown to be associated with delayed-type hypersensitivity, a type 1 response (Narini *et al*, 1995), thus proving the efficacy of the antibodies used.

*Table 6.2: Quantitative data for the immunolocalisation of lymphokines in normal human skin and keloid tissue. Results are expressed as the mean and standard error (±) of positively stained stained cells in n = 4 tissues, 10 fields of view per tissue (4mm^2, x20). The Mann-Whitney U-test was used to investigate statistical significance (P < 0.05 = *)*

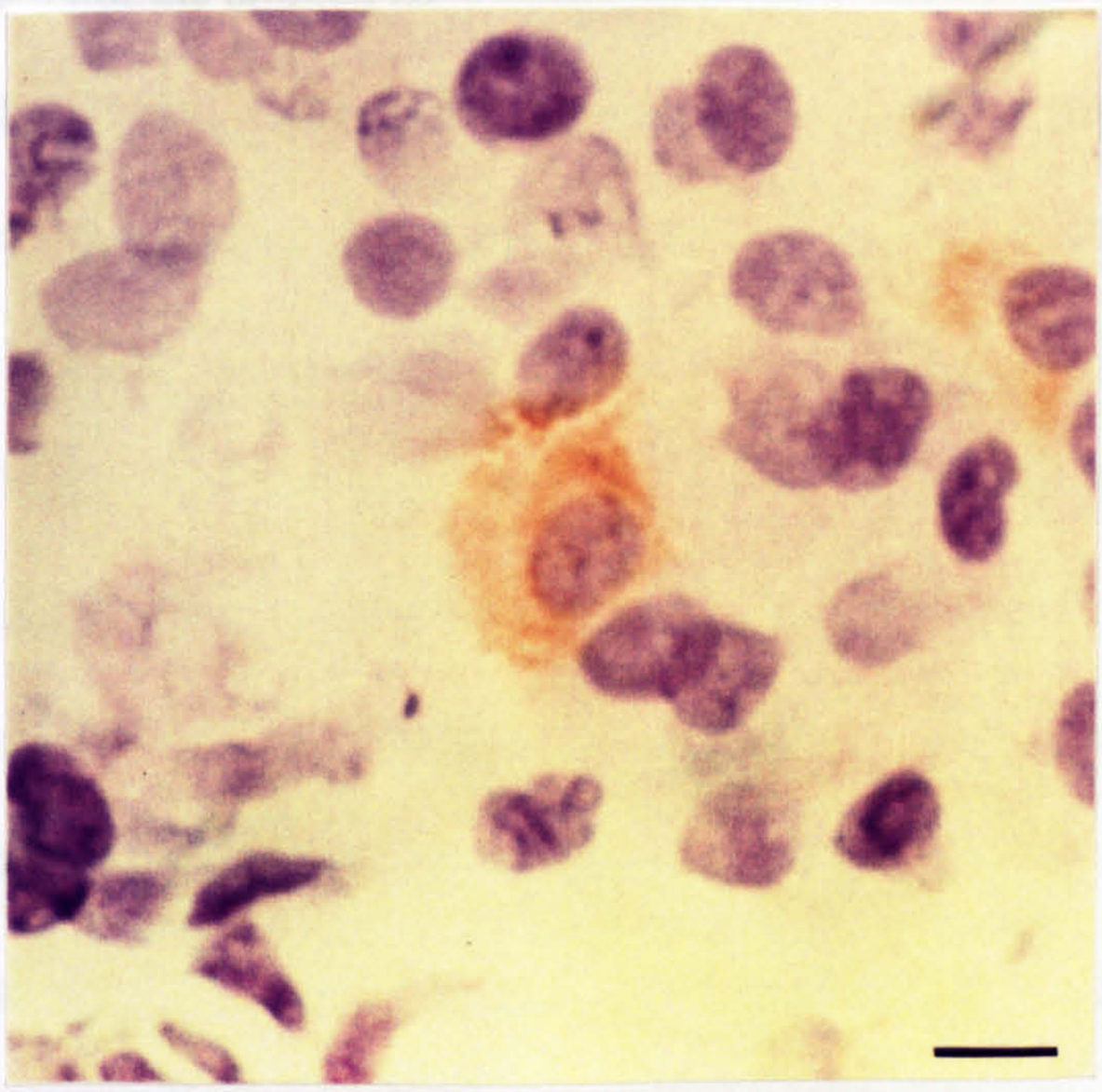
Region	IFN-γ	IL-2	IL-4	IL-10
NS, D	2.0±0.8	10.5±3.9	23.5±2.9	36.8±4.0
KS, D	3.5±1.3	4.5±1.7	116.5±4.7*	111.0±7.1*
KS, L	3.0±2.4	1.5±0.6*	55.0±2.9*	112.5±8.9*

(D, dermis and interface; L, lesion; NS normal sample; KS, keloid sample)

6.44 CD30 cell marker

Normal skin and control tissue were not labelled in any area. A proportion of lymphocytes (see Chapter 3 for $CD4^+$ distribution) in the keloid dermis displayed positive immunoreactivity for CD30 (see Figure 6.5 and Chapter 3).

Figure 6.5 A micrograph to show the immunolocalisation of CD30 to lymphocytes in keloid dermis. Specific immunoreactivity to CD30 is illustrated in a proportion of lymphocytes in keloid dermis (bar represents 65μm, dilution 1:50, representative of n=75)



6.50 DISCUSSION

Keloids feature abnormal collagen composition (DiCesare *et al*, 1990), extensive lymphocyte populations in the dermis (Martin and Muir, 1990) and increased mast cell and histamine content (Hakanson *et al*, 1969; Cohen *et al*, 1972; Kischer *et al*, 1978; Craig *et al*, 1986; Smith *et al*, 1987). The scar tissue outgrows the boundary of the original injury and as such can become disfiguring and incapacitating. Mowlem suggested in 1951 that there may be an immunological component to keloid formation (Mowlem, 1951). In spite of extensive research to test this hypothesis (Chytilova *et al*, 1959; Osman *et al*, 1978; Yagi *et al*, 1979; DeLimpens and Cormane, 1982; Fasika, 1992), neither the mechanism nor the antigen have been identified.

Cytokines have many important roles in repair processes (for reviews, see LeRoy *et al*, 1990; Lowry, 1993; Kovacs and DiPietro, 1994; Paul and Seder, 1994; Holtmann and Resch, 1995). IL-4 is produced by mast cells and activated $CD4^+$ cells of the type 2 and type 0 subsets (Del Prete *et al*, 1991; Bradding *et al*, 1992) and type 2 $CD8^+$ cells (Carter and Dutton, 1996). It is a pleiotropic cytokine with modulatory effects on diverse cell types including T cells, fibroblasts, endothelial cells, keratinocytes and mast cells (for review, see Vellenga *et al*, 1993). A profibrotic role for IL-4 is based on the observation that IL-4 stimulates fibroblasts to proliferate and secrete collagen

(Fertin *et al*, 1991; Postlethwaite *et al*, 1992). The presence of IL-4 in cultures of type 0 lymphocytes favours the development of the type 2 subset and inhibits the development of cells into the type 1 subset and the production of cytokines thereof (Abehsira-Amar *et al*, 1992).

The results of this study of keloid tissue indicate intense immunolabelling for IL-4 in mast cells and lymphocytes. In conjunction with the presence of immunoreactivity to the $CD4^+$ type 2 specific marker, CD30 (Del Prete *et al*, 1995), this suggests that the $CD4^+$ cells present in the keloid dermis are characteristic of type 2 cells. The production of IL-4 has been shown to increase immunoglobulin E levels (Pene *et al*, 1988). An increase in immunoglobulin E levels heightens the sensitivity of mast cells to degranulation when bound to antigen (Roitt *et al*, 1993), this results in the release of IL-4 and histamine (Sandberg, 1962; Bradding *et al*, 1992). Keloids feature elevated levels of both these mediators (Hakanson *et al*, 1969; Cohen *et al*, 1972; Kischer *et al*, 1978; Smith *et al*, 1987). Polarisation of the cellular immune system towards a type 2 response could ultimately maintain the fibrotic nature of the keloid lesion, via prolonged collagen production by fibroblasts under the influence of IL-4. The studies carried out in the murine air pouch model which demonstrates a decrease in IL-4 levels as resolution takes place reiterate this possibility. In normal wound healing, collagen III acts

as a scaffold for collagen I deposition (Nimni, 1983), a process which leads to remodelling and ultimately to wound resolution.

Collagen I gene expression is inhibited by IL-10 in human skin fibroblasts (Reitamo *et al*, 1994) and collagen I production is inhibited in bone marrow cells (Van Vlasselaer *et al*, 1993). In the latter instance, no effect on proliferation was seen (for review, see Mosmann, 1994). The balance of collagen I and collagen III is disrupted in keloid tissue, with the ratio of type I to type III being higher than that of normal tissue (Lee *et al*, 1992). The results of this study show increased immunoreactivity for IL-10 throughout the keloid dermis in comparison to normal skin, possibly suggesting that the presence of increased levels of IL-10 may be an attempt by the tissue to rectify the increase in the ratio of type I to type III collagen seen in keloids. This study, therefore, provides evidence that the overproduction of IL-10 may be a countermeasure to interrupt the continued collagen I deposition which may otherwise contribute and exacerbate the ineffective wound resolution leading to the subsequent growth of the keloid lesion.

IFN-γ is produced by activated $CD4^+$ cells of the type 1 subset, type 1 $CD8^+$ cells and natural killer cells (Reiter, 1993; for review, see Carter and Dutton, 1996). It inhibits collagen production by fibroblasts and can disrupt an established immune response, shifting it from type 2 to type 1 (Seder *et al*, 1992), resulting in the inhibition of IL-4 production by lymphocytes. The

findings from the murine air pouch model experiments that IL-10 and IFN-γ levels increase in the advanced stages of wound resolution (see murine air pouch model results), at a time when it is expected that fibrotic deposition is being curtailed, whereas increased levels of IL-10 and decreased levels of IFN-γ are consistently found in keloids suggest that remodelling of the matrix is dysfunctional in keloid lesions.

It is possible that the release of IL-10 is an attempt to inhibit excessive collagen I deposition, but that the collagen levels continue to increase in the absence of the IFN-γ-stimulated production of collagenase. This study has shown that no cellular elements in the keloid dermis displayed immunoreactivity for IFN-γ. Further evidence for immune system polarisation is provided by the lack of immunoreactivity for the type 2 specific product, CD30. Injections of IFN-γ into keloid lesions provided positive results with diminished scar size (Granstein *et al*, 1990). These changes have been attributed to the inhibitory effects which IFN-γ exerts on the production of collagen. The results of the investigation presented here (Chapter 6) suggest that it is possible that the lack of IFN-γ may also effect the lymphocyte subset present within the tissue and, therefore, may prevent any further progression to normal wound resolution.

The major findings of this study are the absence of immunoreactivity for cytokines characteristic of the type 1 subset, and the abundant presence of

cytokines characteristic of the type 2 subset. Previous work has demonstrated that a proportion of the lymphocytes in keloids are of the T helper subset (Martin and Muir, 1990; Chapter 3). The results presented here extend these observations and have strongly suggested, by immunolocalisation of the CD30 marker, that the $CD4^+$ lymphocytes present are characteristic of the type 2 subset.

6.60 CONCLUSION

The lymphokines investigated in this study have many different actions in a variety of situations. In addition to the roles they play in the modulation of immune responses, they are intimately involved in the healing of wounds and in fibrotic deposition. These attributes implicate roles for IL-2, IL-4, IL-10 and IFN-γ not only in the immunological component of keloid formation and propagation but also in the fibrotic component. Indeed, these results give rise to possible approaches for intervention. For example, if keloid formation is initiated by the type 2 polarisation of the immune response (perhaps caused by the persistence of antigen within the wound), a situation which would ensure a predominance of IL-4 and IL-10, suitable therapy could be to sufficiently reverse the immune response polarisation to aid successful healing. This could

be achieved by blocking IL-4 action, or by administering IFN-γ (an approach already successfully employed in clinical trials, Granstein *et al*, 1990).

It is interesting to note that Narini *et al* (1995) have shown that the presence of silicone gel in breast implants polarises the immune response towards the development of type 1 cells. Furthermore, it has recently been demonstrated that the topical application of silicone cream can dramatically improve the resolution of keloids (Wong *et al*, 1995). Taken together, these data suggest that driving the immune response away from type 2 predominance, by the application of type 1 cytokines, can improve wound healing in keloids.

It is difficult to test these hypotheses in the absence of an *in vivo* model of keloid formation and designing a suitable model is almost impossible as long as the nature of the antigen remains elusive. These results do help to further the understanding of keloid propagation, however, if not the initiation.

Chapter 7

THE ROLE OF IFN-γ IN THE MURINE AIR POUCH MODEL

CONTENTS

7.10 INTRODUCTION

Interferon-gamma (IFN-γ) has been proven to illicit many effects not only *in vitro* (Table 7.1) but also *in vivo* (Table 7.2), and the administration of anti-IFN-γ has been shown to protect mice against the generalised Schwartzman reaction, initiated by lipopolysaccharide injection (Billiau *et al*, 1987).

Table 7.1:

IN VITRO EFFECTS	REFERENCES
Inhibits wound contraction	Dans and Isseroff, 1994
Inhibits angiogenesis	Sato *et al*, 1990
Increases collagenase and glycosaminoglycan levels	Duncan and Berman, 1989

Table 7.2:

IN VIVO EFFECTS	REFERENCES
Decreases transforming growth factor ß mRNA levels	Gurujeyalakshmi and Giri, 1995
Decreases procollagen mRNA levels	Gurujeyalakshmi and Giri, 1995
Decreases collagen levels	Granstein *et al*, 1989
Inhibits wound closure	Granstein *et al*, 1989
Decreases neutrophil presence	Granstein *et al*, 1989

IFN-γ has already been clinically tested to treat a range of abnormal cutaneous conditions (Granstein *et al*, 1990; Larrabee *et al*, 1990; Pittet *et al*, 1994). A trial involving keloid patients and intralesional injections of IFN-γ (0.01 or 0.1mg recombinant IFN-γ into one lesion and diluent into another, 3 times per week for 3 weeks, measurements taken at 8, 15 and 22 days) provided evidence that this cytokine can influence the human cutaneous healing process. When administered to keloid sites (Granstein *et al*, 1990), lesional size was reduced by almost one third. Histological studies of the treated dermal area

revealed the presence of increased numbers of inflammatory cells accompanied by reduced quantities of thickened collagen bundles and reduced numbers of active fibroblasts.

A similar trial in 1990 (Larrabee *et al*) involving keloids and hypertropic scars yielded comparable results.

A further trial (Pittet *et al*, 1994), involved intralesional injections of IFN-γ (200μg twice per week for 4 weeks, with a 12 week post-dosing follow-up) into hypertropic scars and Dupuytren's disease nodules. Clinical observations included reduced lesional height and firmness, effects which persisted throughout the follow-up period. Histological studies showed a reduction in alpha smooth muscle actin expression in hypertropic scars (myofibroblasts are allegedly responsible for tissue retraction and excessive connective tissue production, the expression of alpha smooth muscle actin is an indirect indication of the presence of myofibroblasts). No change in collagen content was detected in response to treatment when collagen III and total collagen were studied histologically. *In vitro* experiments revealed antiproliferative effects on fibroblasts by IFN-γ after 5 days of culture (IFN-γ was added 24 and 96 hours after plating [passage 5] and was applied for 5 days). It was suggested in the study that IFN-γ had reduced alpha smooth muscle actin expression, indicating a reduction in myofibroblast activity/presence, which had eased wound deformation.

In vivo experiments using a murine cutaneous healing model (Granstein *et al*, 1989) demonstrated that systemic administration of IFN-γ ($8.7x10^3$ units/hour over 14 days using a pump implanted subcutaneously or intraperitoneally) dampened the inflammatory response in early healing and delayed wound closure. It was suggested in this study that the inhibition of fibroblast proliferation and collagen production were responsible for the delay in wound closure. This study demonstrated that systemic administration of IFN-γ could influence the resolution of cutaneous wounds *in vivo*.

Normal wound healing involves the production of glycosaminoglycans which bind the wound fluid into a gel during the inflammatory stage of healing (days 0–3). The deposited glycosaminoglycans provide a temporary scaffold for neovascularisation, cell migration and the laying down of collagen fibres. Wound contraction generally begins at 7–8 days and serves to reduce the size of the area to be repaired. The fibroblastic stage often extends to approximately 3 weeks and is essential to strengthen the wound via collagen deposition and maturation.

IFN-γ increases glycosaminoglycan (Duncan and Berman, 1989) production, is angiostatic (Sato *et al*, 1990; Tsuruoka *et al*, 1988) and inhibits fibroblast proliferation (Pittet *et al*, 1994), collagen production (Granstein *et al*, 1989), alpha smooth muscle actin expression (Pittet *et al*, 1994) and wound closure (Granstein *et al*, 1989; Dans and Isseroff, 1994).

The murine air pouch model exhibits an acute inflammatory stage (0–3 days), wound contraction (5–7 days) culminating in a remodelling and resolving stage (7–28 days). Following the immunocytochemical finding that IFN-γ was absent for keloid samples (Chapter 6), although present throughout the resolving and remodelling stage of the air pouch model, an experiment blocking the activity of IFN-γ and observing the subsequent effects on granulation tissue dry weight, glycosaminoglycan content and hydroxyproline (an index of wound reparative collagen deposition) content was designed. The aim being to collect data on the effects of IFN-γ on resolution and to use this data to extrapolate its role in the mechanisms leading to keloid formation and propagation.

7.20 DOSING REGIME

Animals were dosed twice weekly, from day 1, with either 10μg/animal neutralising antibody to IFN-γ (hamster anti-mouse; dose recommended by manufacturer), hamster immunoglobulin (Ig) G or phosphate buffered saline (PBS). 12–15 animals from each group were sacrificed at days 3, 7, 14, 21 or 28.

7.30 RESULTS

7.31 Air pouch tissue dry weight (mg)

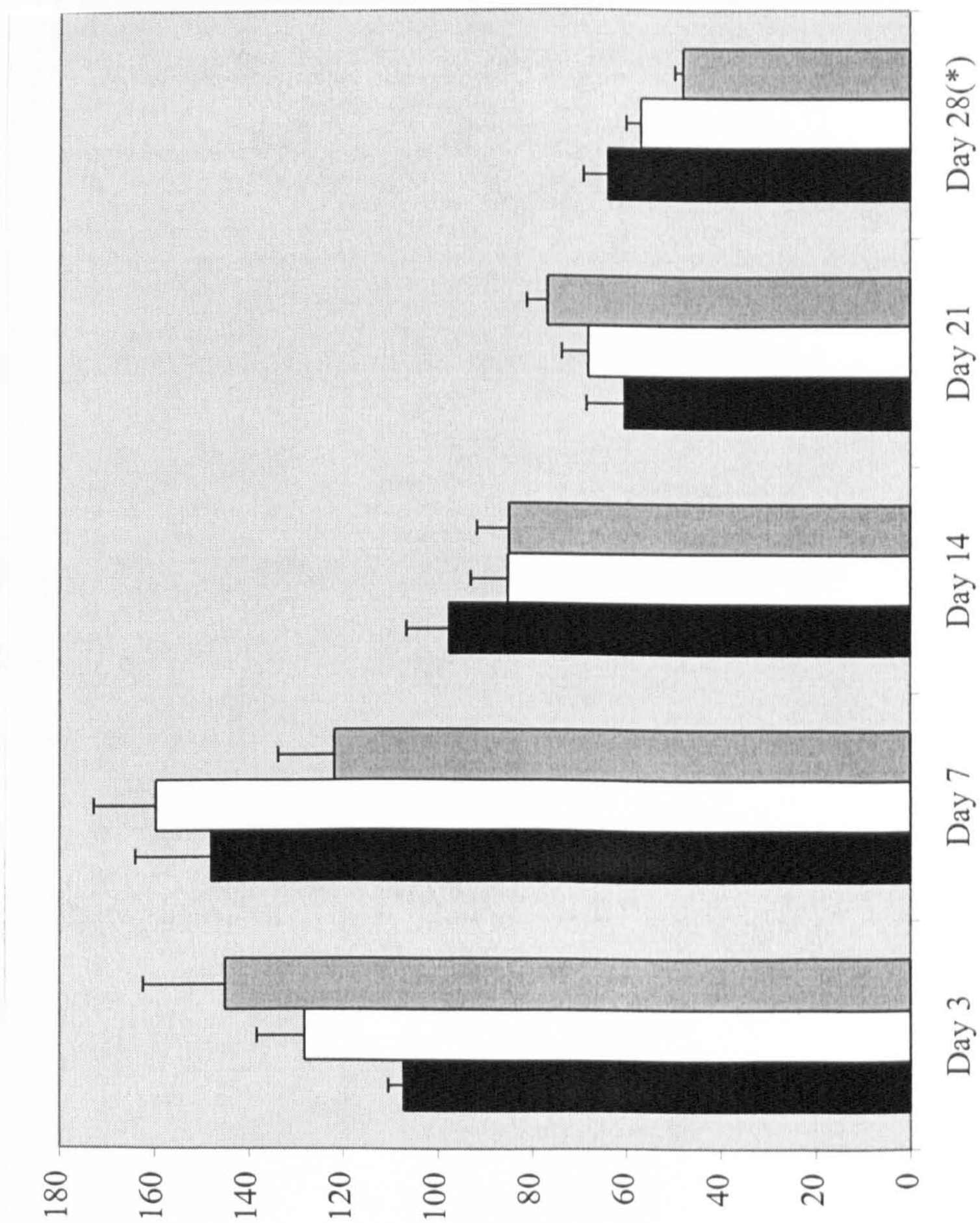
Table 7.3: Tissue dry weight (Mean ± standard error)

	PBS	IgG	anti-IFN-γ
Day 3	107.2±3.3	128.8±10	145±17.4
Day 7	148±16	159.7±13.1	121.8±11.9
Day 14	97.37±9	84.9±7.9	84.7±6.8
Day 21	60.3±8	67.9±5.6	76.5±4.5
Day 28	63.9±5	56.9±3	48±1.7*

No statistically significant increase in tissue dry weight was achieved at day 3 with anti-IFN-γ treatment compared with IgG although there was a modest difference, raising the level almost to that of the IgG control group at day 7. There was a substantial reduction in dry weight with anti-IFN-γ treatment at day 7, although statistical significance was not reached. By day 28 the reduction

achieved statistical significance when compared with the IgG control group (Figure 7.1).

*Figure 7.1 A graph to show the profile of tissue dry weight (mg) in relation to the murine air pouch timecourse. Results are expressed as mean ± standard error (3–28 days, * P<0.05, n=12–15 [experiment was carried out three times, data presented is that of a single experiment]). Black column = PBS, white = IgG, grey = anti-*IFN-γ



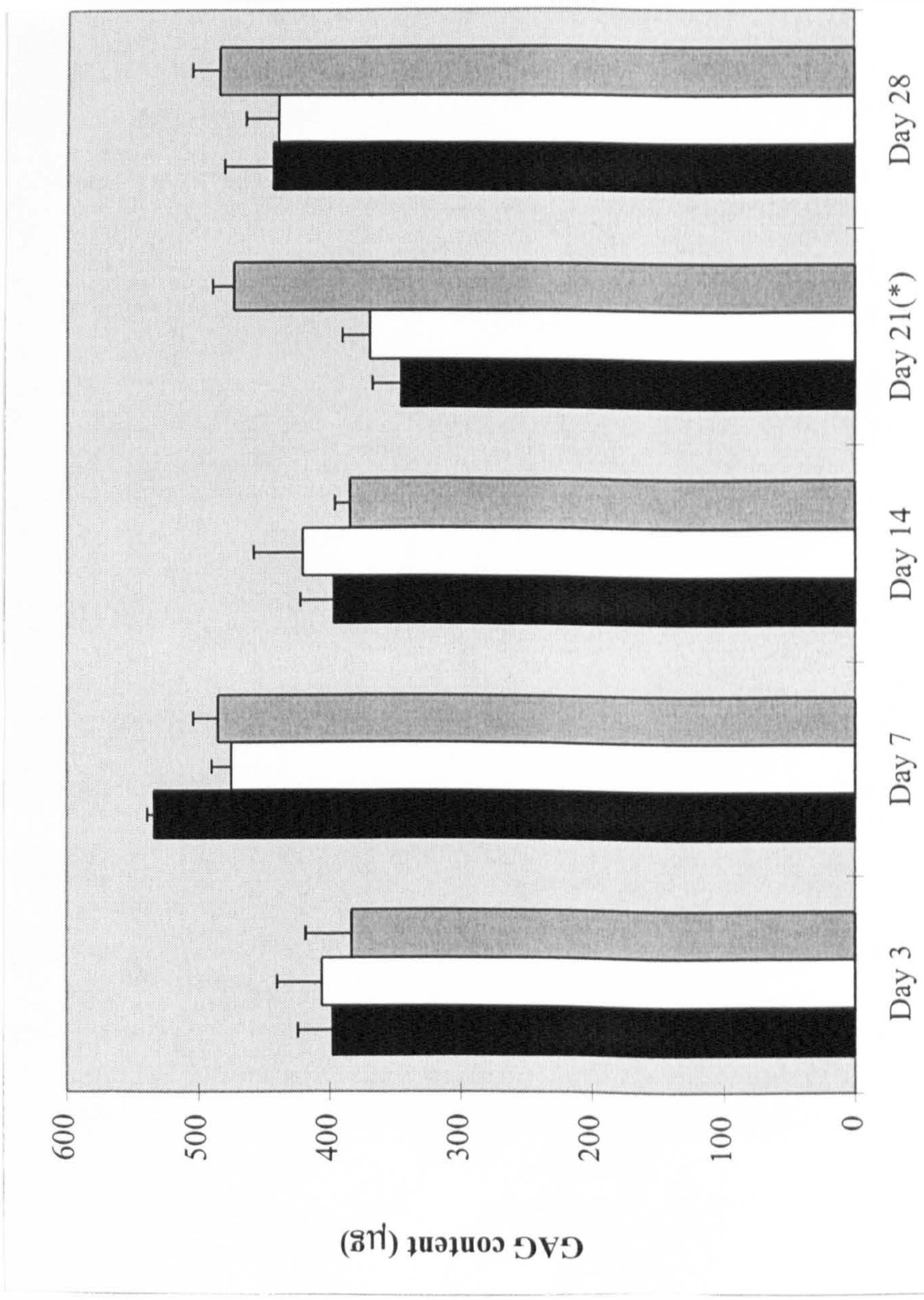
7.32 Air pouch glycosaminoglycan (GAG) content (μg)

Table 7.4: Glycosaminoglycan content (Mean ± standard error)

	PBS	IgG	anti-IFN-γ
Day 3	398±26	406±34	383±35
Day 7	534±5	475±15	485±19
Day 14	397±25	420±38	384±12
Day 21	345±22	369±21	473±16*
Day 28	443±37	439±25	484±21

Blocking antibody treatment to IFN-γ reduced the total glycosaminoglycan content compared with the IgG group at days 3 and 14 and increased it at day 7. By day 21 the day 14 decrease was reversed with a statistically significant increase in glycosaminoglycan content in the anti-IFN-γ group compared with the IgG control. Levels of glycosaminoglycan at days 7, 21 and 28 were approximately equivalent within the treated and untreated groups (Figure 7.2).

Figure 7.2 A graph to show the total glycosaminoglycan content (μg) of murine air pouch tissues in relation to the timecourse of 3–28 days. Results are expressed as mean ± standard error (P<0.05, n=12–15 [experiment was carried out three times, data presented is that of a single experiment]). Black column = PBS, white = IgG, grey = anti-IFN-γ*



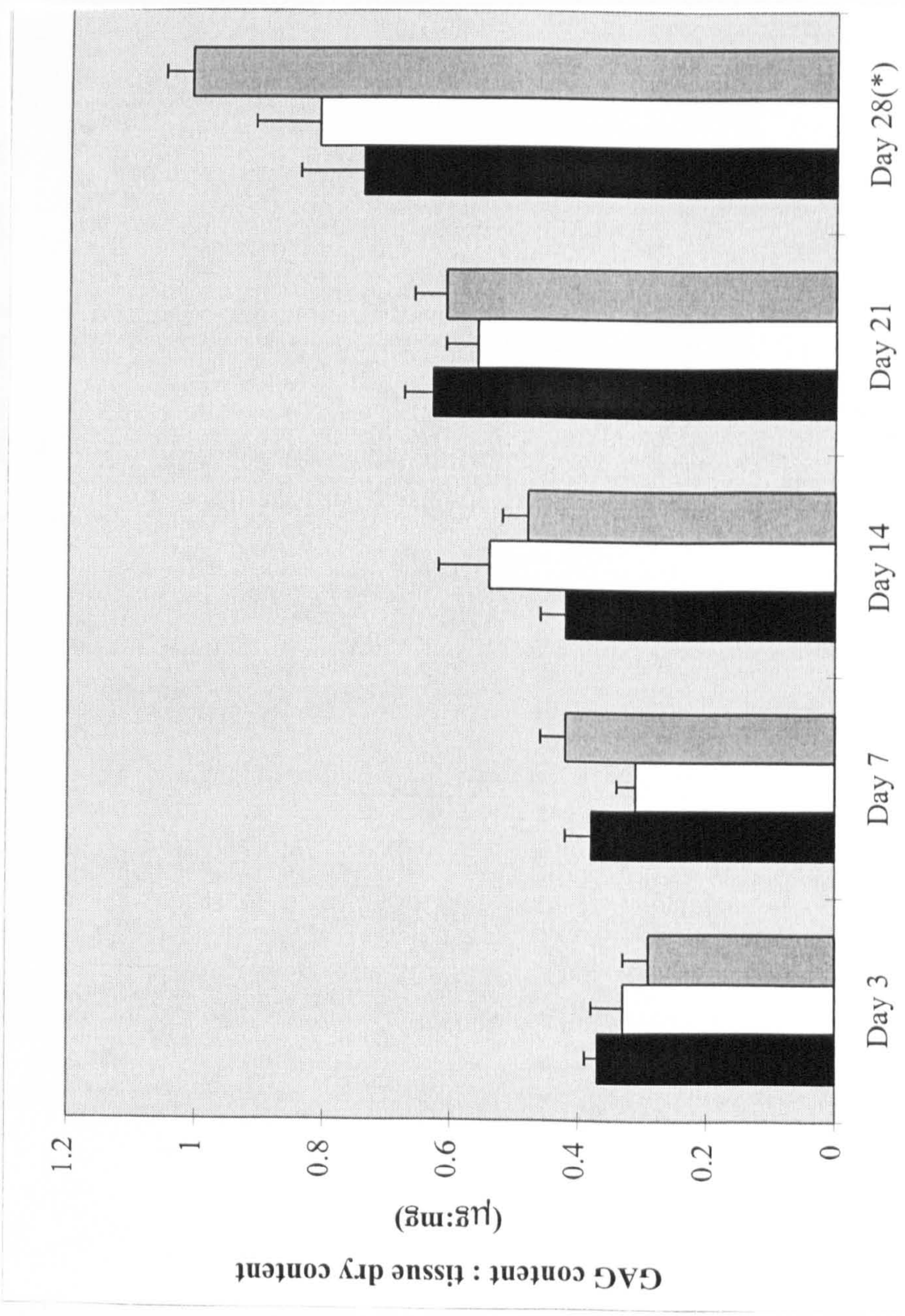
7.33 Ratio of glycosaminoglycan content : tissue dry weight (μg:mg)

Table 7.5: Glycosaminoglycan content : tissue dry weight (Mean ± standard error)

	PBS	IgG	anti-IFN-γ
Day 3	0.37±0.02	0.33±0.05	0.29±0.04
Day 7	0.38±0.04	0.31±0.03	0.42±0.4
Day 14	0.42±0.04	0.54±0.08	0.48±0.04
Day 21	0.63±0.045	0.56±0.05	0.61±0.05
Day 28	0.74±0.1	0.81±0.1	1.01±0.042*

The overall trend for the glycosaminoglycan : dry weight ratio was an increase throughout the time course in all groups, with statistical significance in the treatment group at day 28 compared with IgG. Neutralising antibody treatment to IFN-γ reduced the glycosaminoglycan : dry weight ratio in comparison to the IgG control at day 3, the reduction was close to statistical significance (Figure 7.3).

Figure 7.3 A graph to show the ratio of glycosaminoglycan content (μg) to tissue dry weight (mg) in murine air pouch tissues throughout the timecourse of 3–28 days. Results are expressed as mean ± standard error (P<0.05, n=12–15 [experiment was carried out three times, data presented is that of a single experiment]). Black column = PBS, white = IgG, grey = anti-*IFN-γ



7.34 Air pouch hydroxyproline (HPr) content (μg)

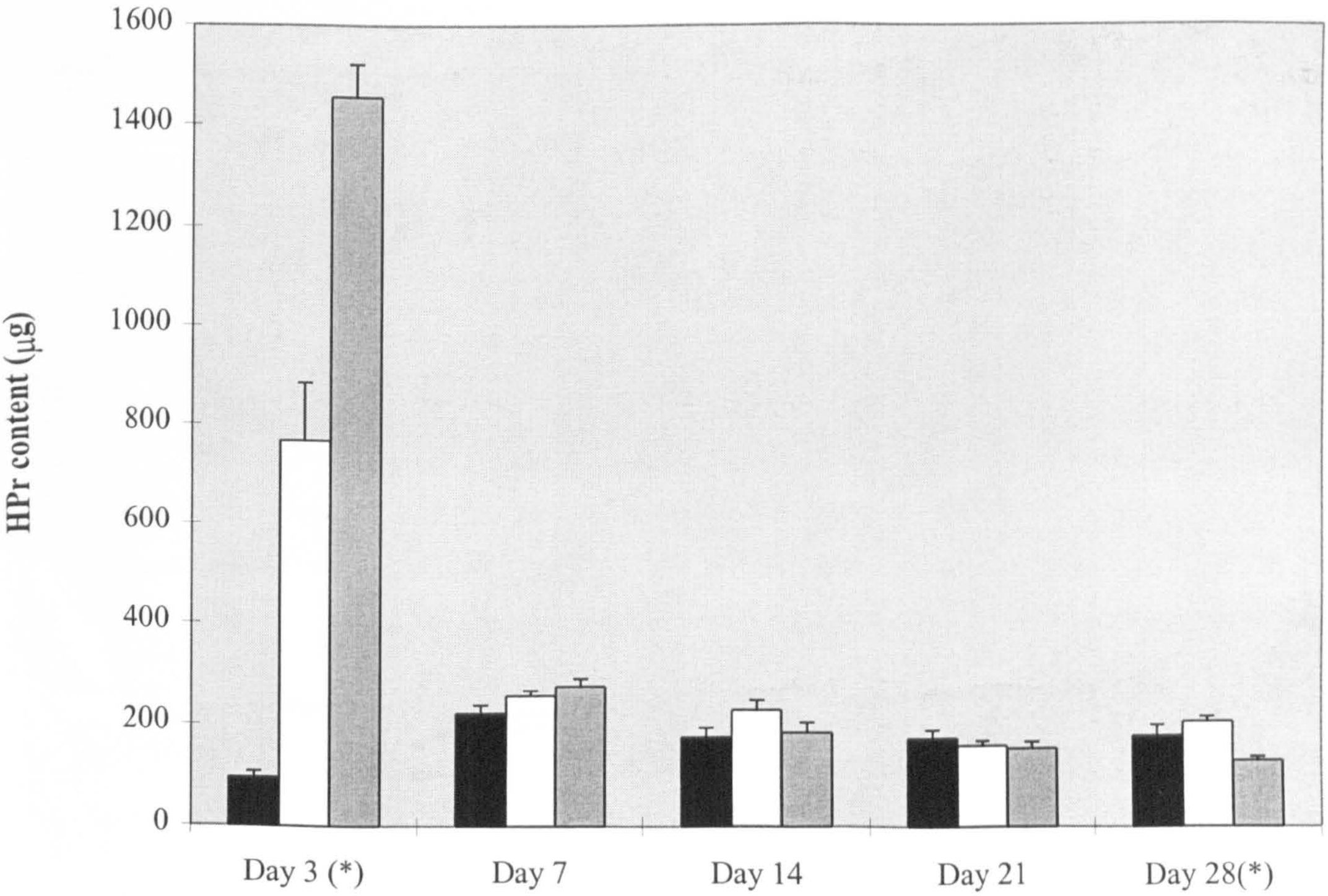
Table 7.6: Hydroxyproline content (Mean ± standard error)

	PBS	IgG	anti-IFN-γ
Day 3	194.7±13.0	767±119	1456±66.6*
Day 7	222±16	256±9.8	275±16
Day 14	172.5±19	226±20.4	182±20
Day 21	170±16	155.7±9	150±12.4
Day 28	172.5±22.5	201.3±10	125.3±7.1*

A statistically significant increase in tissue hydroxyproline content was observed at day 3 in the treatment group compared with the IgG group. Levels were also elevated at day 7 in comparison with the IgG control, although not significantly. Hydroxyproline content in the IgG group at day 14 was higher than that of the treatment group. At day 21 the anti-IFN-γ treatment had marginally decreased hydroxyproline content compared with the IgG control. There was a statistically

significant decrease in hydroxyproline content in the treatment group compared with the IgG group (Figure 7.4).

*Figure 7.4 A graph to show total hydroxyproline (mg) content in relation to time in the murine air pouch model. Results are expressed as mean ± standard error (3–28 days, * P<0.05, n=12–15 [experiment was carried out three times, data presented is that of a single experiment]). Black column = PBS, white = IgG, grey = anti-*IFN-γ



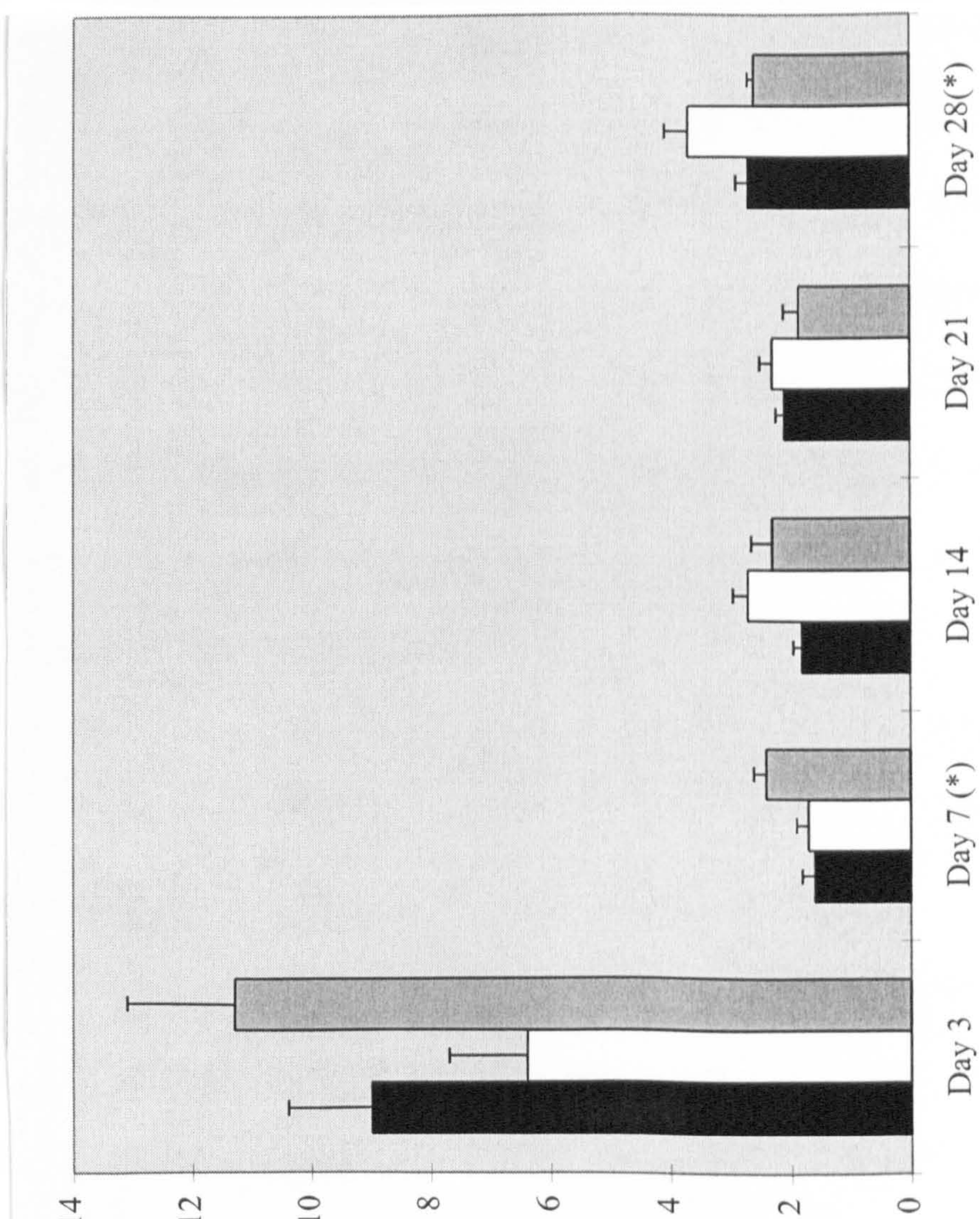
7.35 Ratio of hydroxyproline content : tissue dry weight (μg:mg)

Table 7.7: Hydroxyproline content : tissue dry weight (Mean ± standard error)

	PBS	IgG	anti-IFN-γ
Day 3	9±1.4	6.4±1.3	11.3±1.8
Day 7	1.6±0.2	1.7±0.2	2.4±0.2*
Day 14	1.8±0.15	2.7±0.25	2.3±0.34
Day 21	2.1±0.14	2.3±0.2	1.86±0.25
Day 28	2.7±0.2	3.7±0.38	2.6±0.11*

Until day 14 the ratio of hydroxyproline : dry weight in the anti-IFN-γ treated groups was elevated, when compared with the IgG control, reaching statistical significance at day 7. At days 21 and 28 the levels were lower in the treatment group than in the IgG control, although statistical significance was reached only at day 28 (Figure 7.5).

Figure 7.5 A graph to show the ratio of hydroxyproline content (μg) to tissue dry weight (mg) throughout the murine air pouch timecourse of 3–28 days. Results are expressed as mean ± standard error (P<0.05, n=12–15 [experiment was carried out three times, data presented is that of a single experiment]). Black column = PBS, white = IgG, grey = anti-*IFN-γ



7.40 DISCUSSION

IFN-γ is classically regarded as an antifibrotic cytokine (Kovacs and Di Pietro, 1994). This is mainly because research has shown that it can enhance collagenase production (Duncan and Berman, 1989), thus preventing the deposition of excessive levels of collagen and it can increase hyaluronic acid levels (Granstein *et al*, 1989), a glycosaminoglycan which is abundant in foetal wounds (in foetal wounds, which heal without scar formation, hyaluronic acid constitutes the majority of the matrix; see, Siebert *et al*, 1990). There is also evidence that IFN-γ is able to inhibit transforming growth factor ß production at mRNA level (Gurujeyalakshmi and Giri, 1995); the presence of which would otherwise contribute to scar formation (Shah *et al*, 1992), and *in vitro* and *in vivo* experiments have clearly demonstrated that both procollagen and collagen synthesis are inhibited by IFN-γ.

Collectively these attributes have elected IFN-γ as the solution to all fibrotic maladies (Vilcek *et al*, 1985), and indeed clinical trials have proved promising.

The aim of this study was to investigate the effects of anti-IFN-γ on the content of glycosaminoglycan and hydroxyproline in the developmental and resolving phases of the murine air pouch model. Blocking the action of IFN-γ can sufficiently downregulate the inflammatory response so as to protect mice

from the generalised Schwartzman reaction (Billiau *et al*, 1987). This is only achieved, however, because the inflammatory reaction is driven by IFN-γ release in this model. Studies claiming that IFN-γ treatment can normalise a disordered healing process, therefore, suggest that the absence of IFN-γ is the pathological cause. Is it possible that the absence of IFN-γ in keloid samples, as shown in Chapter 6, is the cause of the excessive fibrotic deposition so characteristic of these lesions?

Neutralising antibody treatment to IFN-γ markedly altered the tissue dry weight profile compared to controls (Figure 7.1). Maximum tissue dry weight in the treatment group was at day 3 and was approximately equivalent to the maximum tissue dry weight in the IgG control group at day 7. Tissue dry weight in the treatment group at day 21 was similar to the level in the IgG control group at day 28. Because of Home Office restrictions, the length of the experiment and, therefore, resolution in this model is taken to be day 28. Anti-IFN-γ treatment caused a statistically significant decrease in tissue dry weight at that timepoint, however, which suggests that the wound was still resolving after 28 days.

On the basis of this single parameter, it would appear that blocking the action of IFN-γ has accelerated progression through the model (wound resolution). This conclusion is consistent with the evidence that IFN-γ administration delays healing *in vivo* (Granstein *et al*, 1989). In the same study,

the acute inflammatory response at 48 hours was greatly reduced in IFN-γ animals and an increased response was seen at 72–96 hours as though the timecourse had 'shifted' or had been temporarily paused. Transmission electron microscopical analysis proved that this effect was caused by changes in local blood flow. This angiostatic, anti-inflammatory activity of IFN-γ is also implicated in the experimental results presented in this chapter. Although no measurements were made, it is possible that blocking the action of IFN-γ has hastened maximum tissue dry weight to from day 7 to day 3 via angiogenic/proinflammatory behaviour.

Glycosaminoglycan content in anti-IFN-γ treated groups was reduced at days 3 and 14, approximately equivalent at day 7 and elevated at days 21 and 28 when compared with IgG controls. IFN-γ has been shown to increase glycosaminoglycan levels *in vitro*, reduced levels in response to anti-IFN-γ, therefore, as shown at the early timepoints, is expected. The elevation of glycosaminoglycan levels at days 21 and 28 in the treatment groups is in opposition to the findings of Granstein *et al*, 1989, who state that as connective tissue content falls from day 14 glycosaminoglycan content increases in experiments administering IFN-γ to wounded mice. It is difficult to explain this anomaly; it is interesting to note, however, that keloid lesions and Dupuytren's nodules also contain higher concentrations of glycosaminolgycans than controls (Flint *et al*, 1978; Abergel *et al*, 1985*). Levels of the fibrotic cytokine

transforming growth factor ß are elevated at days 21 and 28 in the murine air pouch model (Appleton *et al*, 1993) and the presence of IFN-γ inhibits the actions of transforming growth factor ß at the mRNA level (Gurujeyalakshmi and Giri, 1995). It is possible, therefore, that the inhibition of transforming growth factor ß action is lifted when IFN-γ is blocked and so glycosaminoglycan levels increase.

Figure 7.4 shows total hydroxyproline levels in samples, a quantity which broadly represents collagen content. Blocking IFN-γ activity at day 3 caused a significant increase in hydroxyproline levels. A more modest elevation was also present at day 7. At days 14, 21 and 28 reduced levels of hydroxyproline were present in the treated tissues, the reduction was statistically significant at day 28. IFN-γ reduces collagen production at mRNA level via the inhibition of transforming growth factor ß expression (Gurujeyalakshmi and Giri, 1995). Transforming growth factor ß levels are normally low at day 3 in this model (Appleton *et al*, 1993), an increase in hydroxyproline levels when IFN-γ activity is blocked suggests that transforming growth factor ß has recovered reactivity and is stimulating the production of hydroxyproline. By days 21 and 28 the healing tissues are highly collagenous and of reduced size. Anti-IFN-γ treatment caused a reduction in granulation tissue dry weight at these timepoints (Figure 7.1), with significance at day 28. When hydroxyproline levels were normalised for reduced tissue dry weight (Figure 7.5) it was evident that

anti-IFN-γ treatment had not had significant effects. (Normalisation minimalises differences in hydroxyproline content because collagen accounts for the majority of the tissue dry weight at the later timepoints, Granstein *et al*, 1990.) Although, therefore, Figure 7.4 suggests a reduction in hydroxyproline levels at days 21 and 28, with anti-IFN-γ treatment, there appears to have been no overall effect. It is important here to note that clinical analyses of keloid and normal skin samples have shown them to contain roughly equivalent values of hydroxyproline concentration/mg tissue dry weight content (Abergel *et al*, 1985). Figure 7.5 illustrates that anti-IFN-γ treatment increased the value of the hydroxyproline : tissue dry weight ratio at earlier timepoints, with significance at day 7, but decreased after day 14. This evidence further suggests that blocking the activity of IFN-γ 'accelerates' wound resolution.

7.50 CONCLUSION

Clinical trials documenting reduced keloid lesion size with the administration of IFN-γ injections have not yet included biochemical analyses of collagen content. Histological examinations indicate reduced quantities of thickened collagen fibres (Granstein *et al*, 1990) but no more. Therefore the actual mechanism of IFN-γ action in these trials is unknown. The lack of an *in vivo* model of keloid formation makes intensive investigation of the mechanisms involved very difficult. Analysing glycosaminoglycan and hydroxyproline levels in a normal resolving model, as was done here, however, has suggested that a lack of IFN-γ does not cause excessive fibrotic deposition, in fact, on the contrary, it accelerates healing. Although, therefore, IFN-γ administration may be useful therapeutically, its effects on collagen levels do not provide evidence for prophylactic application. Indeed, Granstein *et al* reported that placebo and IFN-γ-injected excision sites of a keloid former revealed a keloidal regrowth in both areas, with the IFN-γ-treated lesion of reduced size compared to the placebo-treated area (1990).

Chapter 8

THE ROLE OF APOPTOSIS IN KELOID FORMATION AND THE MURINE AIR POUCH MODEL

CONTENTS

8.10 INTRODUCTION

The balance between cellular proliferation and cell death is a vital component of both normal and abnormal biological processes. The balance is a fine one and it is not difficult to envisage the results of a malfunction in one or the other activity. There are numerous forms of cell death (for review, see Majno and Joris, 1995), many of which have roles in growth and maintenance (eg in embryonic development) whereas others have been shown to be involved in pathology (eg diphtheria, in which necrosis in the tracheobronchial epithelium leads to the discharging of a leathery, whitish pseudomembrane formed by leukocyte and fibrin accumulation). Exact definitions of particular types of cell death are not yet clear because of the wide range of physiological areas, cell types and stimuli involved. The subject is currently enjoying plenty of interest and special attention, however.

Apoptosis is a form of cell death that occurs in a controlled manner and is characterised by the condensation of components of the cell, a process which is usually followed by phagocytosis by macrophages. It is a process that can be easily studied through the application of *in situ* end labelling (see Chapter 2); during apoptosis the cell DNA is broken down into multiple segments of 185 base pairs which exposes molecular endings which are biochemically specific and can be identified routinely (Wyllie, 1980).

During normal wound healing, new tissue is formed which contains numerous tortuous capillaries (see Chapter 5) and extensive fibroblast populations embedded in a collagenous matrix, this is a transitional state which ultimately progresses to an acellular collagenous scar. It is obvious, therefore, that cellular proliferation and cell death are at the forefront of wound resolution. Previous work carried out in the murine air pouch model (Appleton *et al*, unpublished data) has illustrated that, in this model, at day 3 there are proliferating macrophages and fibroblasts in the granulomatous/granulation tissue area with macrophages also proliferating in the skeletal muscle, presumably traversing from the dermis. By day 5 there is a significant decrease in the number of inflammatory cells in the dermis generally with proliferating macrophages still evident in the upper area of the granulation tissue. The number and distribution of proliferating fibroblasts remains consistent throughout the timecourse. The results prompted the group to conclude that the major source of inflammatory cells is migration, followed by proliferation and further migration through the smooth muscle layer to the granulomatous/granulation tissue.

After day 5 it is suggested that the newly-formed blood supply to the granulation tissue is the source of the remaining proliferating inflammatory cells. Once this migration and proliferation is over what happens to the inflammatory cells and fibroblasts? For fibroblasts, this question has been answered by Gabbiani and co-workers (Desmouliere, 1995). This group employed *in situ* end

labelling to demonstrate that, as the wound resolves and a scar develops, the number of fibroblastic and vascular cells undergoing apoptosis increases. They go on to suggest that 'this is the mechanism of granulation tissue evolution into a scar'. They go even further to suggest that pathological scarring, such as keloid formation, could result if these granulation tissue cells (fibroblasts and vascular cells) are not eliminated.

The normal histology of keloid tissue reveals that the centre of the lesion is largely avascular and acellular, which is in contrast to the surrounding area of dermis which features a rich vascular supply and numerous cells of varying types. This study was designed to investigate the proliferation and apoptosis taking place in the keloid lesion and in the surrounding area, with a view to extrapolating their effects on keloid propagation.

8.20 RESULTS

8.21 Murine air pouch tissue apoptosis data

In situ end labelling

Using *in situ* end labelling, no apoptotic cells were found in control skin samples. By day 3 there were cells undergoing apoptosis in the basal layer of the

epidermis and hair follicles. Some apoptotic cells were present in the dermis but very few and of debatable lineage. By day 7 a few apoptotic cells were visible in the upper area of the granulomatous/granulation tissue, possibly fibroblasts, a pattern which was repeated with increasing numbers throughout the remaining timepoints. Cells undergoing apoptosis were evident very close to blood vessels in the dermis from day 14 onwards, although it was difficult to conclusively discern if they were endothelial cells (Figure 8.1 and Table 8.1).

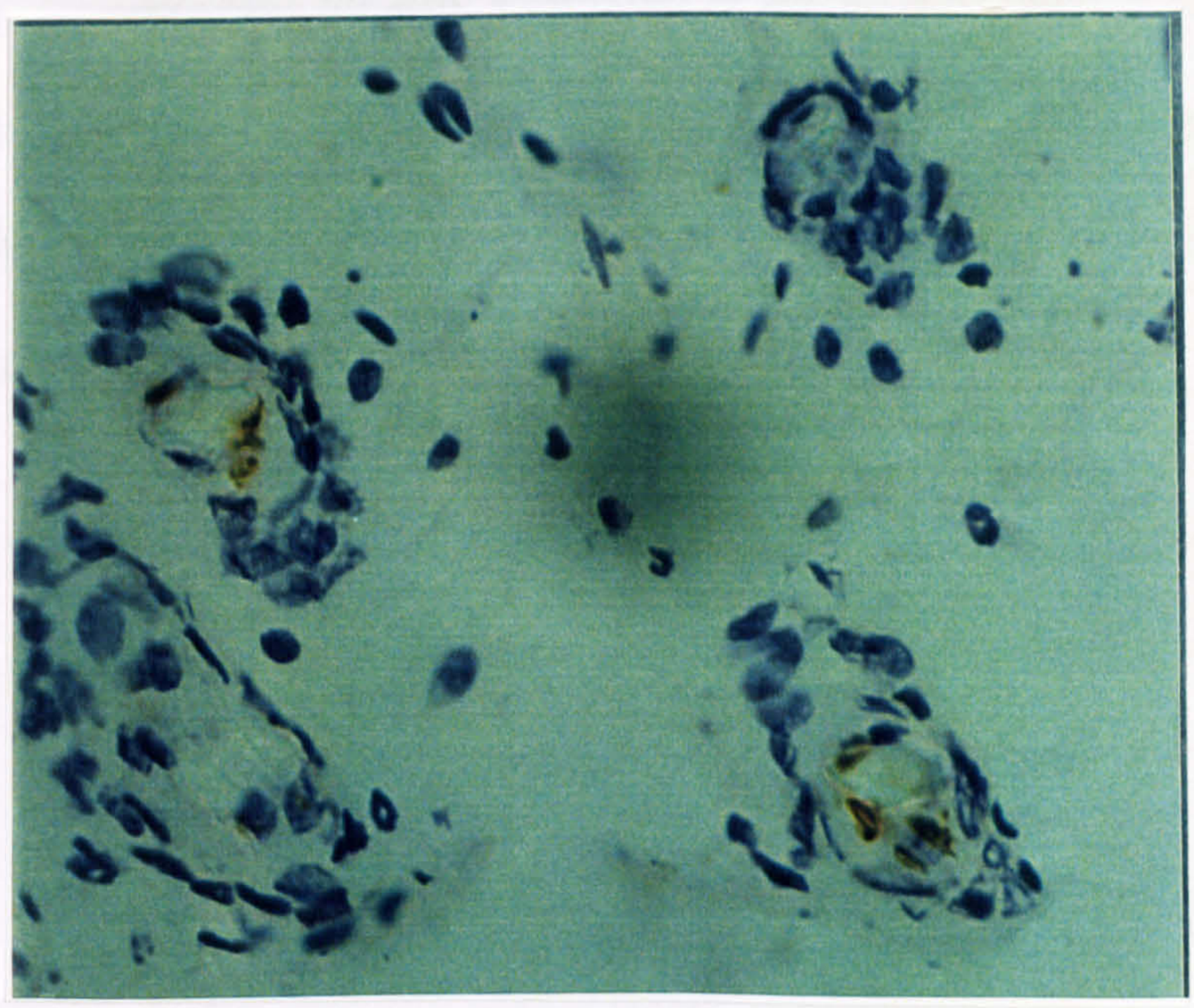
*Table 8.1: Quantitative data for the apoptosis of cells in tissue taken from the murine air pouch timecourse. Results are expressed as the mean and standard error (±) of positively stained stained cells in n = 4 tissues, 10 fields of view per tissue (4mm^2, x20). The Mann-Whitney U-test was used to investigate statistical significance (P = 0.05 = *). Groups are compared with day 3 data*

Day	Region	*in situ*
3	D	11.5±1.3
3	GT	1.0±0.8
7	D	15.5±1.3*
7	GT	19.0±1.8*
14	D	15.8±1.3*
14	GT	29.5±1.3*
21	D	23.0±2.2*

21	GT	34.5±3.1*
28	D	46.3±3.6*
28	GT	62.5±2.9*

(D, dermis; GT granulomatous/granulation tissue)

*Figure 8.1 A micrograph illustrating the presence of apoptotic cells in the hair follicles of tissue sampled at day 3 of the murine air pouch (*in situ *analysis; magnification x100; n=12–15)*

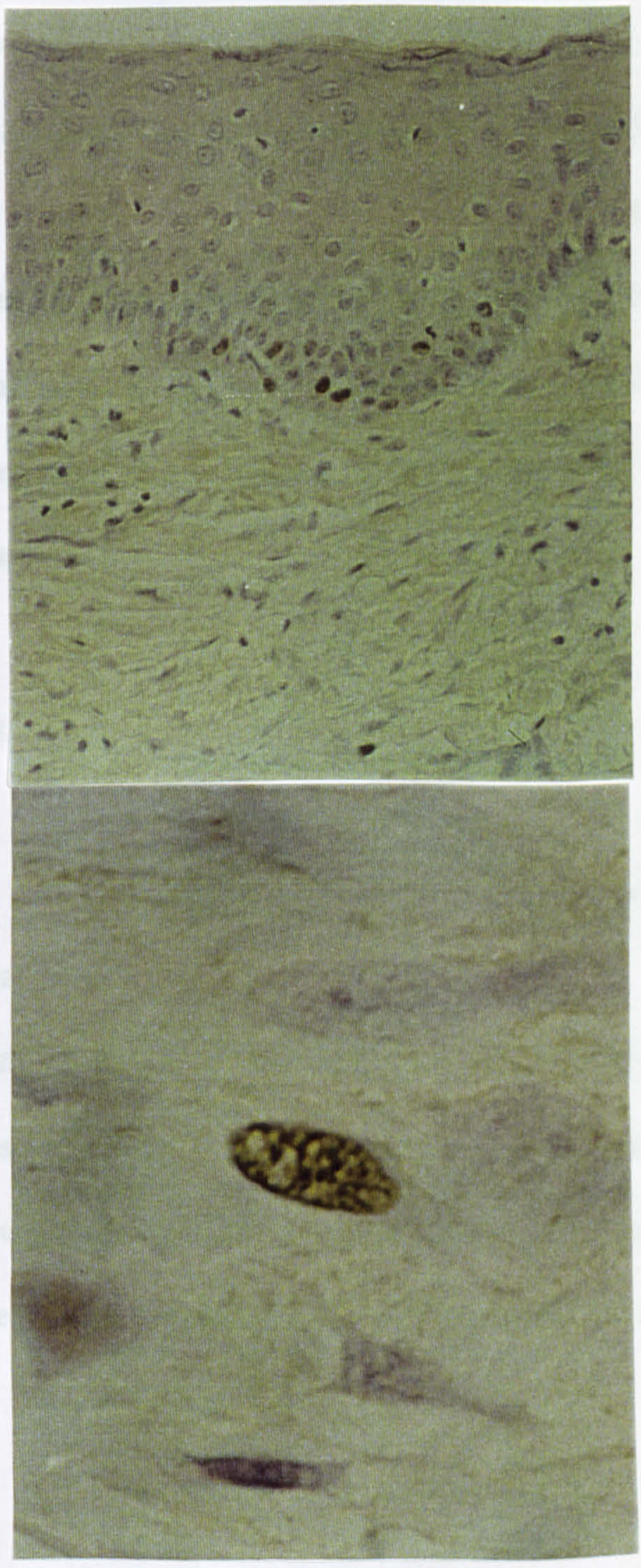


8.22 Human tissue proliferation data

Proliferating cell nuclear antigen immunolocalisation

Using immunolabelling with proliferating cell nuclear antigen (PCNA) antibodies, numerous proliferating cells were seen in the basal layer of the epidermis in both the normal and keloid samples. Almost no proliferating cells were present in the normal dermis. This is in contrast to the area of dermis adjacent to the keloid lesion featuring immunolabelled fibroblasts, although the positive cells accounted for only a minority of the total fibroblastic population. There were no proliferating cells in the lesion itself, although the morphology of the fibroblasts present was consistent with those immunolabelled in the dermis (Figure 8.2 and Table 8.2).

Figure 8.2 Micrographs to illustrate the immunolocalisation of PCNA to epidermal cells in keloid tissue (above), magnification x100 (dilution 1:50, representative of n=25). Immunolabelling of a fibroblast at high power is shown in (below), magnification x500 (dilution 1:50, representative of n=25)



8.23 Human tissue apoptosis data

In situ end labelling

Using *in situ* end labelling, apoptotic cells in the normal human samples were restricted to the basal layer of the epidermis.

In the keloid dermis and epidermis, numerous cells displaying positivity were seen. No apoptotic cells were present in the more collagenous acellular regions of the lesion, although numerous positively labelled cells were evident in the keloid dermis. A small proportion of apoptotic cells, presumably endothelial cells, were observed in blood vessels of the dermis. Areas of necrosis were also present in the keloid dermis (Figure 8.3 and Table 8.2).

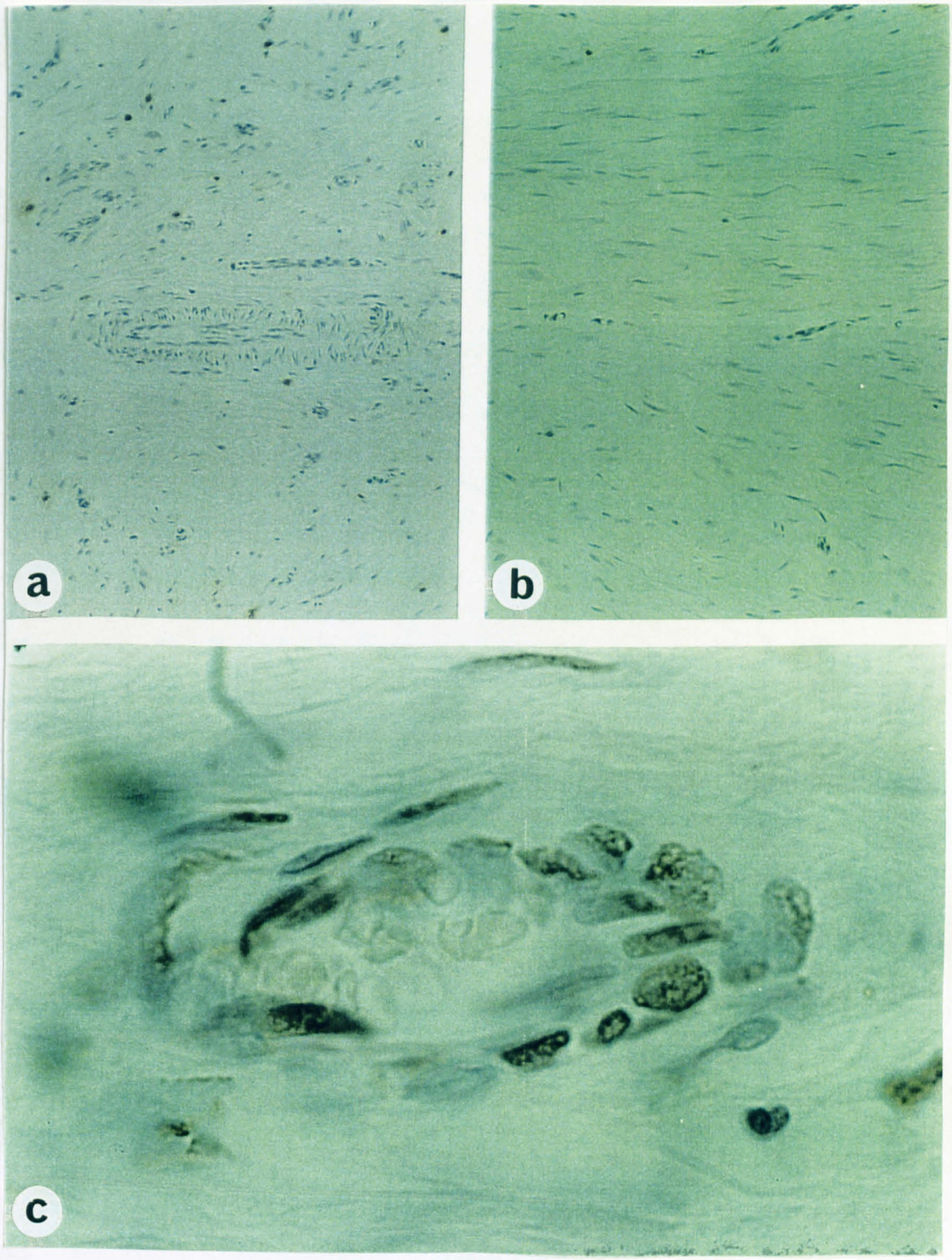
Acridine orange staining

Using acridine orange staining, a few apoptotic keratinocytes were seen the dermis of normal skin.

Apoptotic keratinocytes were also present in the area of dermis adjacent to the keloid lesion, numbers of which decreased nearer to the lesion. Although apoptotic cells were observed at the interface between the dermis and the lesion, none were present in the centre of the lesion (Figure 8.4 and Table 8.2).

Figure 8.3 Micrographs to illustrate immunoreactivity for DNA strand breaks in keloid dermis a), magnification x50 and the mid region of the lesion b), magnification x50. Blood vessel endothelial cells undergoing apoptosis are shown in c), (magnification x500, representative of n=25). See over

Figure 8.4 Micrographs to illustrate acridine orange staining in normal human dermis in a). Apoptotic cells present in keloid tissue are shown in b) and c) shows the area of keloid at the interface between the dermis and lesion. It is interesting to note the absence of apoptotic cells in the lesion and the presence of apoptotic cells in the keloid dermis. Apoptotic cells are stained red, nonapoptotic cells are stained green and background is stained black (magnification x100, representative of n=25). See over


a
b
c

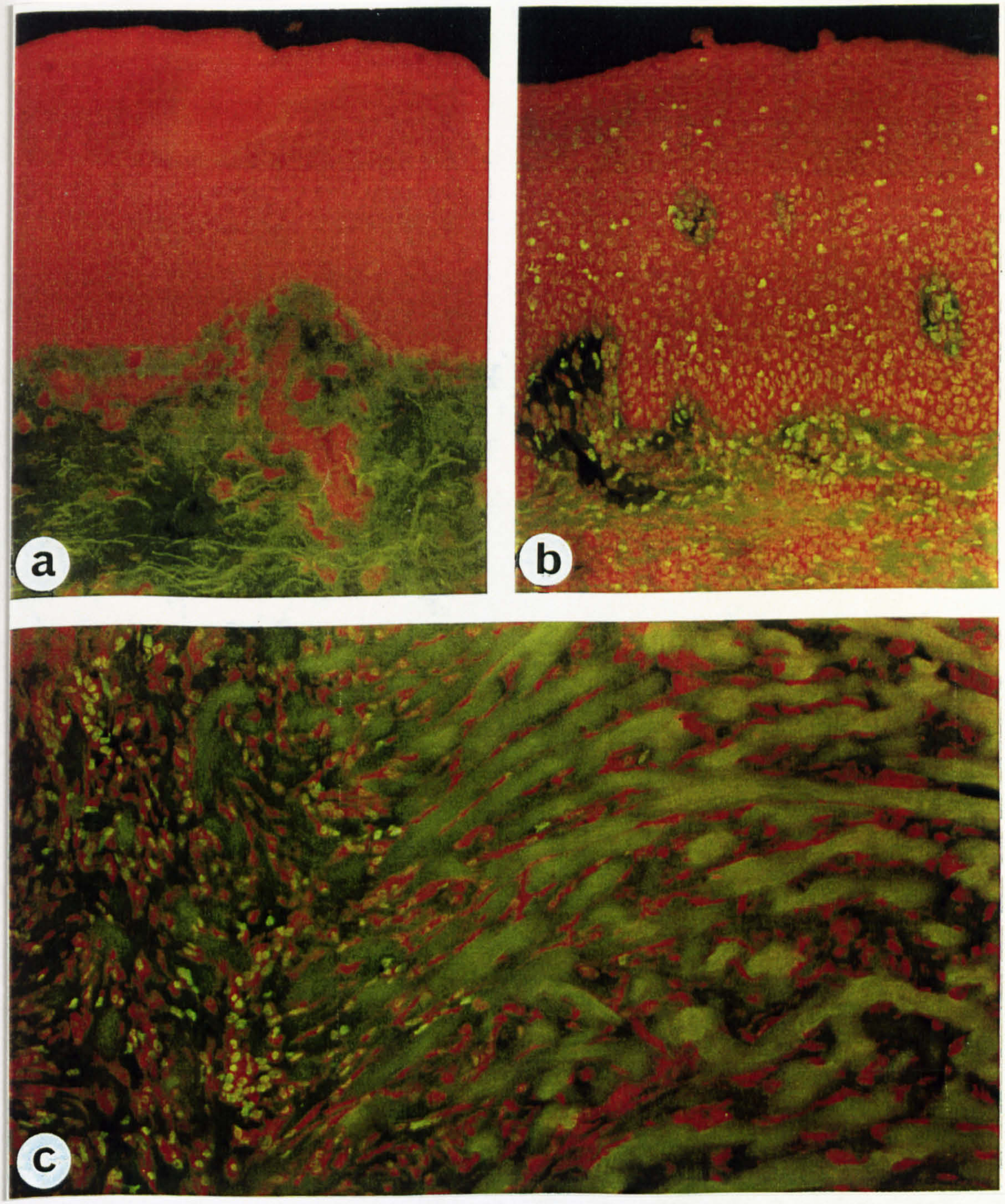

a
b
c

*Table 8.2: Quantitative data for the proliferation and apoptosis of cells in normal human skin and keloid tissue. Results are expressed as the mean and standard error (±) of positively stained stained cells in n = 4 tissues, 10 fields of view per tissue (4mm^2, x20). The Mann-Whitney U-test was used to investigate statistical significance (P = 0.05 = *)*

Region	PCNA	AO	*in situ*
NS, D	37.0±3.2	20.5±2.6	15.5±3.1
KS, D	149.3±3.1*	205.5±7.6*	136.5±41.9*
KS, L	15.0±4.1*	29.0±8.8	4.3±3.2*

(D, dermis and interface; L, lesion; NS, normal sample; KS, keloid sample; PCNA, proliferating cell nuclear antigen; AO, acridine orange; IS, *in situ*)

8.30 DISCUSSION

It seems inevitable from accrued knowledge that the fibrous keloid lesion is formed with the involvement of apoptosis. Gabbiani and co-workers (Desmouliere *et al*, 1995) documented that as a wound resolves and a scar forms the number of fibroblastic cells undergoing apoptosis increases. The results presented here of apoptotic analyses in the murine air pouch model reiterate this. The area in the centre of the keloid lesion is largely acellular and avascular whereas the dermal area contains fibroblasts and large numbers of lymphocytes and blood vessels, as does the interface between the two. This study was

designed to investigate whether visualising those cells which are proliferating or apoptosing, and their relative positions, could provide clues to the mechanisms involved in the formation and propagation of keloid lesions and to compare the data to that of the murine air pouch model.

The results demonstrated that, although numerous lymphocytes and fibroblasts were present in the dermis of the keloid samples, only a small proportion of the fibroblastic population were positive for PCNA. This suggests that migration of lymphocytes, rather than proliferation, is responsible for their abundant presence in the keloid dermis. This hypothesis is consistent with previous suggestions that an endogenous antigen is the persistent driving force of keloid development. Indeed, if an antigen were resident in the skin, which was stimulating the migration of lymphocytes to the area, its prolonged presence would explain the apparent uninterrupted cycle of cytokine production and fibrotic deposition. There have been some suggestions as to the nature of the antigen, for example keratin, melanin and sebum (for reviews, see De Limpens and Cormane, 1982; Placik and Lewis, 1992), but there has been no confirmation yet.

Although *in vitro* data has not clearly indicated increased proliferation rates for fibroblasts derived from keloid lesions compared with those derived from normal human skin samples, a study claimed that keloid fibroblasts do indeed proliferate at a higher rate than do normal fibroblasts (the study was

carried out using PCNA antibodies; Nakaoka *et al*, 1995). The latter study was flawed, however, because it was assumed that the fibroblasts at the centre and edge of the lesion were of the same phenotype. The results presented here clearly show that proliferating fibroblasts are restricted to those areas associated with dense lymphocyte populations and vascularity, and not to the central region of the lesion. This is consistent with the findings of Chapters 4 and 6 which indicate that the area associated with fibroblast proliferation is also an area rich in numerous cytokines and growth factors, whereas the centre of the lesion is not. Unfortunately, with the presence of heterogeneous cellular populations and the intricate interplay of numerous mediators *in vivo*, interpretation of *in vitro* data must be done very carefully. Again the absence of an animal model of keloid formation is a major problem.

Hypoxia in keloid lesions has been suggested by light and electron microscopy (Kischer, 1992), a situation that could lead to the production of vascular endothelial growth factor (VEGF), as shown in tumours (Schweiki *et al*, 1992), and the induction of apoptosis. The results presented in Chapter 4 establish that keloids samples display increased immunoreactivity for VEGF and the results in Chapter 5 suggest that VEGF could be important in the resolution of wounds. Apoptosis and VEGF localisation was confined to the blood vessels in the region of keloid close to the dermis, whereas neither apoptosis nor

necrosis was seen in the centre of the lesion. It is possible, therefore, that blood vessel regression in the lesion is apoptosis dependent.

The results obtained in the murine air pouch model confirm the suggestion that apoptosis occurs naturally during the resolution of injury; and the results in human samples are inkeeping with Kischer's proposal that apoptosis may occur during the transition from granulation tissue resolution to scar formation in keloids as well as in normal scars (1992). Also demonstrated here (through the study of human samples) is that it is possible that proliferation, apoptosis and necrosis can proceed simultaneously with distinct roles. It is possible that as the keloid matures, apoptosis and necrosis remove certain cellular populations which results in the characteristic avascular, fibrotic lesion; whereas, the proliferation of fibroblasts in the dermis propagates the growth and fibrotic deposition of the lesion.

8.40 CONCLUSION

By observing the origin (proliferation) and demise (apoptosis and necrosis) of particular cell types, as well as their relative locations, it is possible to hypothesise their roles in the aetiology of keloids. The results presented in this chapter are largely consistent with those from previous chapters: although keloids are an abnormal form of wound resolution, those mediators present and the characteristic pattern of cellular distribution and lesional growth suggest a normal response to an abnormal, persistent stimulus.

Chapter 9

CONCLUSIONS

9.10 SUMMARY OF FINDINGS

The results of immunolocalisation studies suggesting the presence and distribution of CD cell surface markers in keloid tissues were documented in Chapter 3. CD31, the marker strongly expressed by endothelial cells, was immunolocalised in association with blood vessels throughout the keloid lesion. CD31 is also expressed by some macrophages, it was assumed that the few isolated cells immunolabelled for CD31 in the keloid tissues were macrophages. These cells were dotted around the keloid dermis in low numbers.

CD58 is a more selective macrophage marker. In keloid tissues, $CD58^+$ cells were found by immunolocalisation to be evenly distributed around the dermis with some also present 'buried' in the masses of lymphocytes so characteristic of keloid tissues. There were no $CD58^+$ cells in the keloid lesion itself.

The characterisation of the many lymphocytes present within the keloid tissues was attempted using immunolocalisation of CD4 and CD8 cell surface markers. CD4 is associated with the recognition of antigens bound by MHC class II molecules, CD8 is associated with the recognition of antigens bound by MHC class I molecules. The majority of lymphocytes in the keloid dermis were immunolocalised to be $CD4^+$, although there were also small groups present throughout the tissue. Numbers of $CD8^+$ lymphocytes were also substantial, although reduced in comparison with $CD4^+$ cell numbers.

The CD30 cell surface marker has been documented to be preferentially expressed by type 2 CD4 cells. Immunolocalisation studies suggested that a proportion of lymphocytes present in the keloid dermis were labelled for CD30.

These results have suggested the following detailed description of the cellular nature and structure of keloid tissues, which has previously been unavailable. The keloid dermis

contains aggregates of large numbers of lymphocytes (in comparison with normal skin) some of which are $CD8^+$, the majority of which are $CD4^+$. Individual lymphocytes are also present scattered around the tissue, particularly in association with hair follicles and the invading edge of the keloid lesion. In addition to the classification of the lymphocytes as $CD4^+$ or $CD8^+$ a number are characterised as being of the type 2 subset through the immunolocalisation of the CD30 cell surface marker. The keloid dermis is extensively vascularised, the lesion does not contain a vascularised network. Macrophages are present buried in the groupings of lymphocytes in the keloid dermis, although there are a few individual cells present in isolation. The lesion itself does not contain macrophages.

The results of immunolocalisation studies using antibodies to cytokines were documented in Chapter 4. Cytokines have been shown to be the foremost mediators involved in intercellular communication. Enhanced presence of EGF, PDGF, bFGF, VEGF and TGF-β was displayed in keloid samples (compared with normal samples). Distribution of labelling was generally confined to the dermis of the samples and structures therein. Most notably, distribution of TGF-β immunolocalisation was evident around the perimeter of the lesion. It is generally accepted that the growth of the keloid lesion occurs from the perimeter. It is also generally accepted that TGF-β is a powerful force in the deposition of fibrotic and scar tissues in any healing situation. These results strongly suggest, therefore, that TGF-β is probably involved in the excessive fibrotic deposition present in keloid samples. The localisation pattern of VEGF in keloid tissues (VEGF is only expressed during active angiogenesis) suggests a greatly increased number of blood vessels in the dermis (compared with normal samples), the lesion does not display VEGF immunoreactivity (which is consistent with the localisation pattern of CD31 shown in Chapter 3).

The *in vivo* experiments documented in Chapter 5 using antibodies to neutralise the activity of VEGF showed that the presence of VEGF (and hence angiogenesis) is a strong driving force in the development of granulomatous/granulation tissue and its resolution. As mentioned above, the dermal area of keloid samples features elevated levels of VEGF (in comparison with normal samples). This suggests that, in the same way that TGF-β is probably involved in the deposition of fibrotic and scar tissues in keloids, VEGF is probably involved in the development of the extensive vascular network present in the keloid dermis. And that the abundance of this angiogenic factor plays an active role, regardless of the age of the keloid, in lesional propagation.

The results of immunolocalisation studies documented in Chapter 6 suggest that type 2 lymphokines (ie IL-4 and IL-10) are present in large quantities in keloid samples (in comparison with normal samples) and that type 1 lymphokines (ie IL-2 and IFN-γ) are absent. Added evidence suggestive of the nature of the immune response underway in keloids is the presence of labelling for the type-2-characteristic cell surface marker, CD30. The results of immunolocalisation studies of IL-2, 4, 10 and IFN- γ in murine air pouch tissues suggest that at the later timepoints when resolution is underway, levels of IL-10 and IFN-γ are high and levels of IL-2 and 4 are low. These findings are in contrast to the levels of these mediators in keloid tissues, which revealed an approximately inverse pattern.

It has previously been shown that IFN-γ is a strong influence in the reduction of the size of fibrotic lesions, the *in vivo* experiments documented in Chapter 7 using antibodies to neutralise the activity of IFN-γ showed that it does not play a central role in the resolution of

granulomatous/granulation tissue in this model. That is, it probably plays a late role in the resolution of injury. Indeed, immunolocalisation of IFN-γ showed that it was more prominent in the later, rather than earlier, (resolution) phases of the murine air pouch model.

Cellular proliferation analyses of keloid samples documented in Chapter 8 illustrated that very little proliferation is underway. Apoptosis analyses illustrated that there are enhanced levels of apoptosis taking place (in comparison with normal samples). Apoptosing fibroblasts are present only in those areas featuring lymphocytes and vascularity, fibroblasts in the keloid lesion (the few that exist) are not undergoing apoptosis. In addition to this, certain areas of vascularity around the perimeter of the lesion show evidence of endothelial cell apoptosis.

It is recognised that immunohistological studies are sufficient to suggest the location of mediators *in situ*, but that they do not permit the absolute suggestion of mediator action, activity or effects. In order to investigate these aspects of mediator characteristics it would also be necessary to visualise mediator receptor and mRNA levels within the tissues. An alternative approach would be to perform multiple immunolocalisation labelling within the tissue samples. This technique would permit the localisation of the mediators as well as the relevant cell surface markers within the same tissue section, in order to provide further evidence of which cell types were positive for which mediators and where those cells were.

Whenever suggestions are made in this thesis concerning mediator roles, therefore, it is necessary to consider the limitations of data provided by immunolocalisation experiments.

Evidence collected in this thesis goes a long way towards providing an understanding of certain documented findings relevant to the formation and propagation of keloid lesions. The

following hypothesis is consistent with documented evidence and the results presented in this thesis: an injury occurs, the wound healing process is initiated and an antigen at the site is discovered by a patrolling lymphocyte. The lymphocyte journeys back to the bone marrow/thymus/lymph node and proliferates, the lymphocyte progeny migrate to the original site of antigen and release mediators in order to quell its effects. An unspecified event triggers an unspecified source of IL-4 (possibly mast cells) to become active, which reinforces that the lymphocytes are type 2 and that IL-2 and IFN-γ, which may otherwise interfere with the immunological action against the antigen and inhibit excessive fibrotic deposition, are absent. The type 2 products stimulate the deposition of fibrotic components by fibroblasts and the production of themselves, meanwhile the antigen is still present, more lymphocytes migrate providing more type 2 mediators. Fibroblasts continue to respond to the presence of elevated levels of type 2 mediators and to the fibrotic cytokines (EGF, PDGF, TGF-β) produced during normal resolution; wound healing and fibrosis deposition progress unabated at the same time that vascularisation continues under the direction of high levels of VEGF and bFGF. A mass of disorganised collagen has been deposited through the action of TGF-β and fellow growth factors and the absence of the usually fibrosis-regulating IFN-γ, inhibited by the action of the type 2 mediators, perpetuates this cycle. The unfortunate cycle of events is repeated as more lymphocytes migrate to the antigen and less IFN-γ and IL-2 and more IL-4 and IL-10 are produced. The levels of fibrotic growth factors present continue to rise, more deposition occurs and the area of tissue begins to grow larger than the wound site.

The fact that lesional growth occurs from its perimeter suggests that the antigen is probably present in the dermis. The fact that the keloid grows beyond the boundary of the wound site suggests that the antigen is discovered anew at the invading edge of the reaction.

In vitro experiments using keloid fibroblasts do not confirm current knowledge of the character of that particular cell type. Is it possible that all fibroblasts throughout keloid formers are dysfunctional if the sufferer has normal fibroblastic processes elsewhere *in situ*?

Evidence that the serum of keloid patients contained elevated levels of IL-4 and IL-10 and reduced levels of IFN-γ (as the keloid samples do) would certainly be alarming, more so if the patients were tested in the absence of a keloid lesion and the same results were to be obtained. If an individual were to permanently suffer from full-body dysfunctions of this magnitude, keloid growth would probably be a small manifestation of the problem when compared with accompanying effects. This seems to concur with the general belief that the problem is local to the site of keloid growth.

Many questions remained unanswered, such as the following:

Why do sufferers not develop keloid scars at every injury site?

Why is the condition more common in black and oriental skins types than in white skin?

Why is it more common in females than in males?

It is suggested that the information provided in this thesis agrees with aspects of the commonly held hypothesis that keloid growth and regrowth are intricately entwined with an intense and complex immune response which has a local initiation. This thesis attempts to provide researchers with a 'map' of cell types, cytokines, proliferation and apoptosis in keloid tissues. As well as a clearer picture of the likely causes of the continued growth of keloid lesions. The *in vivo* results documented in this thesis provide some enlightenment of the *in*

situ effects of abnormal levels of VEGF and IFN-γ, which is the case in keloid samples, on the resolution of granulomatous/granulation tissue, and, by inference, injury resolution.

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PUBLICATIONS

FULL PAPERS

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