

CHARACTERISTICS OF EPIDERMOLYSIS BULLOSA
SKIN FIBROBLASTS IN VITRO

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I, Catherine Ann Oakley, declare that this thesis has been composed by myself and that the research described within it is my own.

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ABSTRACT

Epidermolysis bullosa (EB) is a heterogeneous group of rare hereditary mechanobullous diseases. Three different sub-groups exist: simplex (EBS), with blistering within the epidermis; junctional (EBJ) with blistering at the dermo-epidermal junction and dystrophic, either dominantly (EBDD) or recessively (EBDr) inherited where blisters form within the dermis. The aim of this project was to characterise collagen, collagenase and glycosaminoglycan (GAG) metabolism in fibroblasts cultured from the skin of patients with different forms of EB and to determine as far as possible how two drugs currently used to treat the disease, phenytoin and vitamin E, affect these functions.

Thirty patients donated skin samples to establish fibroblast cultures and fibroblast lines were cultivated from 18 (7 from EBS, 6 from EBDr, 3 from EBDD and 2 from EBJ). Twenty four skin samples were examined by electron microscopy for confirmation of diagnosis and to study certain ultrastructural features of the disease.

No difference was found between the proliferation rates of any of the EB groups and an age-matched healthy control group (mean \pm SEM: $88 \pm 13\%$). Similarly no statistically significant difference was found between the GAG outputs of control and EB groups.

Protein synthesis in the EB group differed significantly from the controls, with a selective increase in collagen synthesis over synthesis of non-collagenous protein. Collagenase levels were highest in the EBDr fibroblast cultures and the bulk of the enzyme appeared to be in the active form. The EBS cultures gave collagenase levels which were intermediate between those of controls and EBDr, and most of the

enzyme was in the latent form which requires activation by trypsin in vitro.

Phenytoin did not affect the proliferation rate of any of the groups but vitamin E caused a significant increase in the rate of proliferation of control fibroblasts. Phenytoin produced a decrease in protein synthesis at 300-500 μM . The effect of phenytoin on GAG output differed between groups; the drug increasing GAG accumulation in controls whilst depressing levels in the EBS group. Vitamin E did not directly affect GAG secretion.

INTRODUCTION

INTRODUCTION

In the late nineteenth century a group of congenital blistering diseases was recognised, to which Koebner in 1884 gave the name "epidermolysis bullosa hereditaria". This name has since become widely accepted and has come to encompass a spectrum of clinically differing hereditary conditions. Fortunately EB remains a relatively rare disorder, with probably less than 1,000 patients recorded in Britain. Where intra-familial marriages are more common - in the Middle East, Asia, or where communities have been extremely isolated in the past - the incidence of EB is increased, recessive genes and mutations having a greater chance of being expressed.

Unfortunately "epidermolysis bullosa" (EB) is a misnomer for a heterogeneous group of mechanobullous diseases united by the inherent tendency of the skin to blister either spontaneously, or after banal trauma. EB differs from other blistering conditions, in that it is the result of a congenital defect and unlike the pemphigus, pemphigoid and dermatitis herpetiformis disorders, does not involve any aspect of the immune system. Inheritance of the disorder is either by autosomal dominant or recessive genes; blistering can be in the epidermis, as the name would imply, at the junction between epidermis and dermis, or within the dermis. Clinically the disorder ranges from a single blister on one hand, through to the mutilating polydysplasia of the most severe form.

The simplest form of classification uses the level at which cleavage of the skin occurs, determined either by light or electron microscopy. All blisters cleaving in the epidermis are diagnostic of the EB simplex group, all forms of which, as far as is known, are

<u>GROUP</u>	<u>VARIANT</u>	<u>INHERITANCE</u>	<u>SCARRING</u>	<u>SITE OF CLEAVAGE</u>	<u>CHARACTERISTIC FEATURES</u>
SIMPLEX					
Weber-Cockayne:	localised	D	-	B, Sb	Localised to extremities. Worse in hot weather. No scarring.
Koebner:	generalised	D	-	B	Generalised blistering, as above.
Herpetiformis Dowling-Meara		?	-	B	Clumped tonofilaments (using EM).
Ogna		?D	-	Sb	Bruising skin fragility, linked with GPT locus.
Mottled pigmentation		D	-	Sb	Patches of hyper and hypopigmentation.
JUNCTIONAL					
Herlitz:	lethalis	r	-	11	Abnormal hemidesmosomes, death at or soon after birth.
Non-lethal:	generalised	r	-	11	Abnormal hemidesmosomes, survival with generalised blisters, no scarring.
Non-lethal inverse		r	-	11	As above except blistering is chiefly on the trunk.
Localised		r	-	11	As above, blistering mainly of hands and feet
Progressiva (neutrophica)		r	-	11	Normal hemidesmosomes, deposits of amorphous material in the 11.
DYSTROPHIC					
Cockayne-Touraine:	localised	D	+	d	Blistering of the hands and feet heal with scarring and milia.
Pasini:	generalised	D	+	d	Albopapuloid papules, blistering more generalised, scarring and milia.
Hallopeau-Siemens:	generalised	r	+	d	Widespread blistering, often mutilating and lethal, scarring and milia.
	localised	r	+	d	Blistering restricted to the extremities, scarring and milia.
	inverse	r	+	d	Blistering confined to specific areas of the body.
D	autosomal dominant inheritance				
r	autosomal recessive inheritance				
B	cleavage occurs through the basal cell				
Sb	cleavage occurs suprabasally				
11	cleavage occurs through the lamina lucida				
d	cleavage is in the dermis below the basal lamina				

dominantly inherited. No disruption of the dermis occurs and therefore no scarring is seen when the blisters heal. All blisters occurring between the epidermis and dermis are diagnosed as junctional EB, which is recessively inherited, whilst blisters arising within the dermis are categorised as the dystrophic variety, which can either be dominantly or recessively inherited (Table 1).

The Simplex Group (EBS) is composed of six different variants according to the classification of Gedde-Dahl (1981), or five variants according to Eady and Tidman (1983). The latter authors disregard the Mendes da Costa variant included by Gedde-Dahl, on the grounds that it is not a mechanobullous disorder. The five variants include:

- 1) Weber-Cockayne: localised. Blistering is restricted to the hands and feet. The blisters occur at or above the level of the basal cell (Haneke and Anton-Lamprecht, 1982), and high temperatures seem to exacerbate the condition.
- 2) Koebner: generalised. Blistering is widespread and more severe than the localised variety. It occurs at the level of the basal cell (Baker, 1982).
- 3) Herpetiformis Dowling-Meara. This form is similar in clinical appearance to the Koebner variant and can only be distinguished by electron microscopy. Ultrastructural examination reveals electron-dense clumps or clusters of tonofibrils in the epidermis (Dowling and Meara, 1954; Anton-Lamprecht et al, 1979; Niemi et al, 1983). Curiously this variant differs from the localised and generalised simplex forms in that high temperatures ameliorate the blistering (Gedde-Dahl, 1981).

- 4) Onga. This is characterised by a congenital bruising tendency of the skin and traumatic serous blisters of the hands, feet and occasionally elsewhere (Gedde-Dahl, 1971). In this type of EB a genetic linkage with the erythrocyte glutamic pyruvic transaminase locus has been established (Gedde-Dahl, 1981).
- 5) Mottled pigmentation. This is characterised by patches of lighter and darker skin 2 to 5 mm diameter. Hyper and hypo-pigmentation is diffusely delineated and may be localised on extremities or found over the whole body. Blistering is usually widespread (Gedde-Dahl, 1981).

The Junctional Group is composed of five recessively inherited variants, according to the classification of Gedde-Dahl (1981) or three according to Eady and Tidman (1983). These include:

- 1) Herlitz: letalis. In this variant patients die at or soon after birth (Fig. 1). The blisters form between the basal cell plasma membrane and the basal lamina. Ultrastructurally the hemidesmosomes are abnormal, lacking the sub-basal dense plaque and having a rudimentary plasma membrane attachment plaque: they are also thought to be fewer in number (Hashimoto et al, 1976a; Anton-Lamprecht and Schnyder, 1979).
- 2) Non-lethal, generalised. This variant is similar in clinical and ultrastructural appearance to the lethal variety but despite widespread blistering, the patient is able to survive into adulthood. Blisters heal without scarring (Pearson et al, 1974; Hashimoto et al, 1976a).
- 3) Non-lethal, inverse. This is characterised by junctional blisters located on the trunk, preferentially affecting the groin, axillae and neck (Gedde-Dahl, 1981; Eady and Tidman, 1983).

FIG. 1

EBL patient: showing the severity and widespread distribution of blisters.

FIG. 2

EBDD patient: showing milia (arrow) and atrophic skin at a predilected site.

FIG. 3

EBDr patient: showing the widespread serous blisters which encrust and heal leaving scars.

FIG. 4

EBDr patient: showing "mittening" (total syndactyly) of the hands.

1



2



4



3



Neonatal blisters disappear during the first 3 to 5 months of life and recur more frequently from about 4 years onwards (Gedde-Dahl, 1981).

Included in Gedde-Dahl's classification (1981) are

- 4) Non-lethal, localised. This variant has localised junctional blistering of the hands and feet.
- 5) Progressiva (neutrophica). This is characterised by the presence of normal hemidesmosomes at the dermo-epidermal junction. Deposits of amorphous material have been noted within the lamina lucida (Gedde-Dahl, 1971). The onset of blistering is usually from early childhood (4 years) to adolescence and this variant primarily affects the hands and feet (Gedde-Dahl, 1981).

The Dystrophic or dermolytic form of EB is divided into dominantly and recessively inherited groups, both characterised by blisters which heal leaving scars or atrophic skin and various degrees of nail dystrophy. The dominantly inherited group contains two variants, though whether one is a more extreme clinical expression of the other is unknown.

- 1) Cockayne-Touraine: localised. This is characterised by blisters affecting the extremities, including the elbows and knees (Hashimoto et al, 1976). Atrophic skin and milia are common at the predilected sites (Fig. 2).
- 2) Pasini: generalised. Blistering is usually more generalised and severe than the Cockayne-Touraine form. The Pasini form of dominant dystrophic EB is distinguished by the spontaneous appearance in late childhood or adolescence of albopapuloid papules on the body. An abnormality of the anchoring fibrils is thought to be the causal defect in this disease (Hashimoto et al,

1975) and an aberration of glycosaminoglycan synthesis is thought to be related to the appearance of the white papules (Sasai et al, 1973; Bauer et al, 1979).

The Recessively inherited Group of dermolytic EB includes three variants:

- 1) Hallopeau-Siemens: generalised. This is characterised by widespread serous blisters from the time of birth, which heal with scarring and milia (Fig. 3). There is commonly extensive involvement of the mouth and pharynx, with frequent occurrence of oesophageal strictures. Blistering of the extremities is often so severe that it leads to mutilating polydysplasia (mittening) of the hands and feet (Fig. 4). In its worst form this type of EB can be fatal but it should not be confused with the junctional letalis (Herlitz) variant. Ultra-structurally, anchoring fibrils are rarely seen in the severe recessive dystrophic type (EBDr) and this deficiency has been proposed as a primary causal defect (Briggaman and Wheeler, 1975a). However it is now uncertain whether this feature is an outcome of abnormal collagenase metabolism, which is associated with severe EBDr (Hashimoto et al, 1976b; Bauer, 1982).
 - 2) Localised dystrophic recessive. This is characterised by dermolytic blistering which is largely restricted to the extremities.
 - 3) Inverse dystrophic recessive. This is characterised by the distribution of blisters on the trunk, predominantly around the groin, axillae, neck and lumbar regions.
- Bart's Syndrome, the hereditary, congenital, localised absence

of skin from the lower legs, with blistering of the skin, mouth and nail abnormalities, was previously thought to be a separate mechanobullous variant. It is now understood to be a feature of any of the major variants of EB (Wojnarowska et al, 1983) and therefore does not constitute a separate category.

Also excluded from the classification is a form of blistering disease known as acquired EB (or EB aquisita). It is now accepted that this is distinct from other hereditary mechanobullous disorders, in having an immunologically based defect. Studies have shown deposits of immunoglobulins A, M and complement C_4 at the dermo-epidermal junction in both involved and non-involved skin (Nieboer et al, 1980; Yaoita et al, 1981). The onset of EB aquisita is late in life (over 50 years) and it appears to be more closely related to the pemphigoid disorder of the skin than to EB.

The genetic relationship between the variants within each group is poorly understood, despite the extensive work by Gedde-Dahl (1971). It is currently thought that where discrimination of a variant is made on a quantitative basis, for example: EBS Weber-Cockayne from Koebner; EBDr generalised from localised or EBDD Cockayne-Touraine from Pasini, there may be a similar pathogenetic defect caused by slightly different mutations at the same gene locus (Gedde-Dahl, 1981). However between groups and between the variants within a group, where clinical discrimination is based on qualitative differences, it is thought that separate mutations at different gene loci must be responsible, although phenotypic appearances may be deceptively similar. The diverse range of variants in the EB group as a whole make classification difficult and has complicated attempts to determine the pathogenetic defects underlying each particular variant.

Little is known about the causal defect in EBS: disruption of the basal cell implies some inherent defect within the cell, perhaps the over-production of an enzyme or the failure to produce a vital constituent. Equally, the significance of dermal influences on the epidermis in this disorder is unknown, although the dermis does influence epidermal metabolism elsewhere (Fleishmayer and Billingham, 1968; Woodley et al, 1980a; King and Tabiowo, 1980). Similarly, although the hemidesmosomes are known to be abnormal in some patients with junctional EB, the cause of the hemidesmosome deformity is unknown; the epidermis or dermis being capable of producing enzymes which could affect the integrity of the hemidesmosome and therefore the dermo-epidermal bond. More is known about the dermolytic, dystrophic EB types, largely from the work of Dr. E.A. Bauer's research group at St. Louis, U.S.A. Their studies have confirmed that an aberrant collagenase is produced in excess in the severe EBDr variant. They have also investigated the abnormal glycosaminoglycan metabolism in the dominant dystrophic Pasini variant. As is explained in chapter 1, the culture of fibroblasts from the skin of patients with EB represents one of the few feasible avenues of research, both in terms of defining the abnormality and in assessing the effect of drugs on fibroblast metabolism.

It was therefore the aim of the present project to study the skin fibroblasts of the major EB variants with the intention of highlighting any metabolic defects by direct comparison with fibroblasts from the skin of normal healthy subjects. In this way the findings of previous investigators could be confirmed or questioned, and new aspects of the disease might receive attention.

Very few drugs have been used to help ameliorate the particularly severe dystrophic variants, they include: corticosteroids, phenytoin

and vitamin E. None of these drugs have met with any long term success. Phenytoin is now considered to be useful for some patients with EBDr (Bauer and Cooper, 1981) but the unpredictable responses of patients to this drug make it an unsatisfactory and unreliable form of treatment. Unfortunately, it is currently one of the few therapies to offer even limited success. Vitamin E has been used to treat both Simplex (Ayres and Mihan, 1969) and dystrophic forms of EB (Wilson, 1964; Smith and Michener, 1973) apparently with some success but unfortunately few trials have been reported and without scientific investigation of its value as a potential moderator of EB, true clinical effectiveness is difficult to assess. The hazards of long term corticosteroid therapy limit their use, particularly where young children are concerned.

Although retinoids have not yet been used in the treatment of EB, they are reported to induce the synthesis of a fibroblast-derived inhibitor of collagenase activity in vitro (Bauer et al, 1982). It is possible that after further research this group of compounds will be of benefit to patients with dystrophic EB.

It should be understood that hope for an outright "cure" for this group of diseases is unrealistic and beyond the scope of this project. However, further research is essential to distinguish the basic underlying causes, in conjunction with a search for drugs which can ameliorate, or rectify the various conditions. This project represents a step in that direction.

The thesis has been divided into chapters according to the particular aspect of the fibroblast being examined: for example collagen, collagenase and glycosaminoglycan metabolism. Each chapter is self-contained with its own introduction and discussion.

The basic structure of the skin is described in chapter 2, along with conclusions drawn from ultrastructural examination of some of the patients involved in this study. The examination was not on a morphometric basis but merely useful in confirming the diagnosis whilst providing an insight into any ultrastructural defects that might be present. Chapter 1 includes an introduction to fibroblast culture and describes the basic procedures and techniques involved, as well as providing a morphological comparison of EB and control fibroblasts.

CHAPTER 1

FIBROBLAST CULTURE

FIBROBLAST CULTUREINTRODUCTION

A logical approach to improving the **treatment** of a disease requires that the underlying causal defects are understood. In a disease such as EB, where there is enormous heterogeneity in clinical appearance and severity, one of the few avenues of investigation is to examine the source of the defect directly, by fibroblast and/or keratinocyte culture, since skin is primarily a product of these two cells.

Keratinocyte culture is still a relatively recent innovation, beset by problems: chiefly the difficulty in maintaining cultures over long periods isolated from important dermal and serum borne factors which must influence keratinocyte differentiation and the difficulty in raising enough keratinocytes to perform, and repeat, experiments. Fibroblast culture, on the other hand, provides a comparatively easy method of analysing the production of dermal components, using the large numbers of cells which can be obtained from a single skin biopsy. Clearly both fibroblast and keratinocyte culture techniques are important in the investigation of skin diseases and ideally should be conducted concurrently. Unfortunately technical and physical practicalities often make this impossible and a choice has to be made between the two lines of investigation.

The more severe types of EB, such as EBDr, involve dermolytic blisters with subsequent scarring. It was therefore the aim of this study to establish cultures of EBDr fibroblasts, as well as fibroblasts from the other EB variants for comparative purposes; then to determine whether their characteristics, including proliferative capacity, morphology, glycosaminoglycan secretion, collagen and collagenase synthesis, differed from normal fibroblasts. Hopefully

this would give some indication of the pathogenetic defect(s), which could facilitate investigations into how to rectify, or at least ameliorate, the disease.

The culture of fibroblasts *in vitro* has limitations and drawbacks but studies in vivo cannot easily be made without inflicting undue discomfort on patients with EB. The limitations of the system lie in the artificial nature of the culture conditions - isolated from serum-borne factors in the body and other cell types: in a situation where the fibroblasts are forced to divide and form a confluent layer of closely associated cells. The subdivision of confluent-layers of cells (passaging) means that the fibroblasts' surface proteins, including receptors, are stripped off by the general protease trypsin: the cell is then forced to replace these components on its surface so that it can readhere to the substratum.

As a consequence of these constraints of culture, fibroblasts have only a limited life span in vitro. There has been much research into ageing in vitro (Hayflick, 1965): it is now accepted that with increasing age in tissue culture a decreasing number of cells are capable of division - approximately 99% undergoing mitosis at passage 8, whilst only approximately 50% have this capacity at passage 47 (Merz and Ross, 1969). There is still some question regarding the effect of the donor's age on the proliferative capacity of cells in culture. Vracko and McFarlane (1980) found that the cumulative replicative life span of normal fibroblasts ($n = 10$) decreased as a function of the donor's age; it was not affected by the freezing and storage process but was affected by the quality of the foetal bovine sera used in the culture media, which seemed to differ between lots. Ryan et al (1981), studying skin fibroblasts from monozygotic

twins, concluded that the donor genotype was the most important determinant of cell proliferation and replicative life span in vitro. The problem of ageing in culture is further complicated by the knowledge that fibroblasts from the papillary dermis exhibit a greater proliferative capacity than those from the reticular dermis in vitro (Harper and Grove, 1979). These authors suggest that the shortened life span of human skin fibroblasts from older donors may be due to the absence or reduced number of papillary fibroblasts resulting from age-related dermal atrophy.

In order to avoid complications related to donor age, and induced ageing of cells in culture, all experiments in this study were performed between passage 4-10 and the mean age of control donors was matched as closely as possible with those of the EB patients. Apart from determining the mean proliferation rates of all the cell lines used in this study, it was necessary to identify the concentration range of phenytoin and vitamin E with the greatest effect on proliferation, thereby showing which concentrations should be used in other experiments.

In the current study the morphology of all the fibroblasts cultured was studied and representative cell lines were fixed and stained for comparison. A further attempt to study the extracellular morphology was made by indirect "staining" of the fibronectin extracellular matrix of the fibroblasts. The method of Hsieh et al (1980) was used, which involved binding fluorescein isothiocyanate (FITC)-conjugated gelatin onto the fibronectin associated with the extracellular matrix of the cell (Chen et al, 1978; Singer, 1979). This method is based on the strong affinity between gelatin and fibronectin (Engvall and Ruoslahti, 1977) combined with the ease with which the

fluorescent tag can be conjugated to the gelatin. The extracellular matrix can then be visualised using an ultraviolet emission microscope.

A recent study of EBDr fibroblasts in vitro suggested that they differed from normal control fibroblasts, not only in their reduced capacity to contract collagen lattices but also in their morphology (Ehrlich et al, 1983). The findings of the present study were compared and discussed in view of those of Ehrlich et al (1983).

METHODS

Patients

A total of 30 patients were biopsied for fibroblast culture; 12 EBS, 10 EBD_r, 6 EBDD and 2 EBL (see Table 2 and Fig. 5 for details). The clinical diagnosis, based on the patient's previous history and condition at the time of presentation was agreed by the attending physician and later confirmed by electron microscopy in 24 patients (see Table 4). Normal skin to provide fibroblasts which would serve as controls in experiments was taken from the forearm of healthy volunteers with their informed consent. Three strains of control fibroblasts were donated by other centres (Table 3).

Initiation of Fibroblast Cultures

The biopsy site was sterilised with a Savlon wipe: local anaesthetic (1-2% xylocaine without adrenalin) was injected into the site and either a punch or incision biopsy excised. (A general anaesthetic was used in 4 patients who were undergoing plastic surgery at the time of biopsy - see Table 2). The biopsy was halved if electron microscopy of the sample was required; otherwise the skin was placed in a universal container full of "transport medium" for between 1-16 hours.

Transport Medium: Dulbecco and Eagle medium with

20% foetal calf serum

4 mM glutamine

200 units/ml Penicillin-streptomycin

100 units/ml mycostatin

(all from Gibco-Europe, Paisley, Scotland)

The biopsy was washed five times in sterile phosphate buffered

TABLE 2

PATIENTS INVOLVED IN EB STUDY

all biopsies were taken from uninvolved sites except EB32

No	Sex	Age (years)	Type	Biopsy	Site	Blister sites	Cell line established
EB4	F	37	S(W-C)	p	f	hands + feet, mild	
EB6	M	22	S(W-C)	i	u.a.	feet, mild	
EB8	M	39	S(K)	p	f	generalised severe	
EB9	M	42	S(W-C)	p	f	feet, mild	+
EB10 ^a	F	31	S(W-C)	p	f	hands + feet, mild	+
EB11 ^a	F	12	S(W-C)	p	f	hands + feet, mild	+
EB12 ^a	M	8	S(W-C)	p	f	hands + feet, moderate	+
EB23	F	3	S(W-C)	p*	f	hands + feet, mild	+
EB24	F	29	S(W-C)	p	f	hands + feet, mild	
EB28	M	8	S(W-C)	p	f	feet, mild	
EB29	M	32	S(W-C)	p	f	feet, mild	+
EB30	F	19	S(W-C)	p	f	hands, feet + friction sites, mild	+
EB32	F	1	S(D-M)	i	u.th	generalised	
EB26	F	3/12	L	i	ch.	atrophic scarring generalised, very severe	+
EB27	M	2	L	i	ch.	generalised, severe	+
EB1	F	15	Dr(H-S)	p*	u.th	generalised, poor	+
EB2	F	13	Dr(H-S)	i*	u.th	generalised, severe	+
EB3	F	23	Dr(H-S)	i	u.th	generalised	
EB5	M	47	Dr(H-S)	p	f	generalised, poor	+
EB14	F	2	Dr(H-S)	i	f	generalised. Died aged 3	+
EB15	M	15	Dr(H-S)	i	f	total syndactyly, very severe	
EB18	F	36	Dr(H-S)	i	f	generalised, moderate	+
EB19	M	33	Dr(?)	i	f	mode of inheritance uncertain, moderate	
EB22	F	32	Dr(H-S)	p	f	total syndactyly, very severe	
EB31	M	4	Dr(H-S)	i*	u.th	extremities	+

No	Sex	Age (years)	Type	Biopsy	Site	Blister sites	Cell line established
EB7	M	34	DD(C-T)	p	f	hands, feet + mouth	
EB16 ^b	M	18	DD(C-T)	p	f	hands, feet + friction sites	+
EB17 ^b	F	40	DD(C-T)	p	f	hands, feet + friction sites	+
EB20 ^c	M	15	DD(C-T)	p	f	haemorrhagic blisters, mouth	
EB21 ^c	F	38	DD(C-T)	p	f	extremities, mouth	
EB25	M	16	DD(C-T)	i	k	extremities no nail dystrophy	+

- * : biopsies taken under general anaesthetic
- a : EB10, 11, 12 - mother, daughter and son respectively
- b : EB16 and 17 - son and mother respectively
- c : EB20 and 21 - son and mother respectively
- S(W-C) : Simplex Weber-Cockayne
- S(K) : Simplex Koebner
- S(D-M) : Simplex herpetiformis Dowling-Meara
- Dr(H-S) : Dystrophic recessive, Hallopeau-Siemens
- DD(C-T) : Dystrophic dominant, Cockayne-Touraine
- L : Junctional, letalis: Herlitz
- p : punch (either 4 or 5 mm) biopsy
- i : incisional biopsy
- f : forearm
- u.a. : upper arm
- u.th : upper thigh
- k : knee
- ch : chest

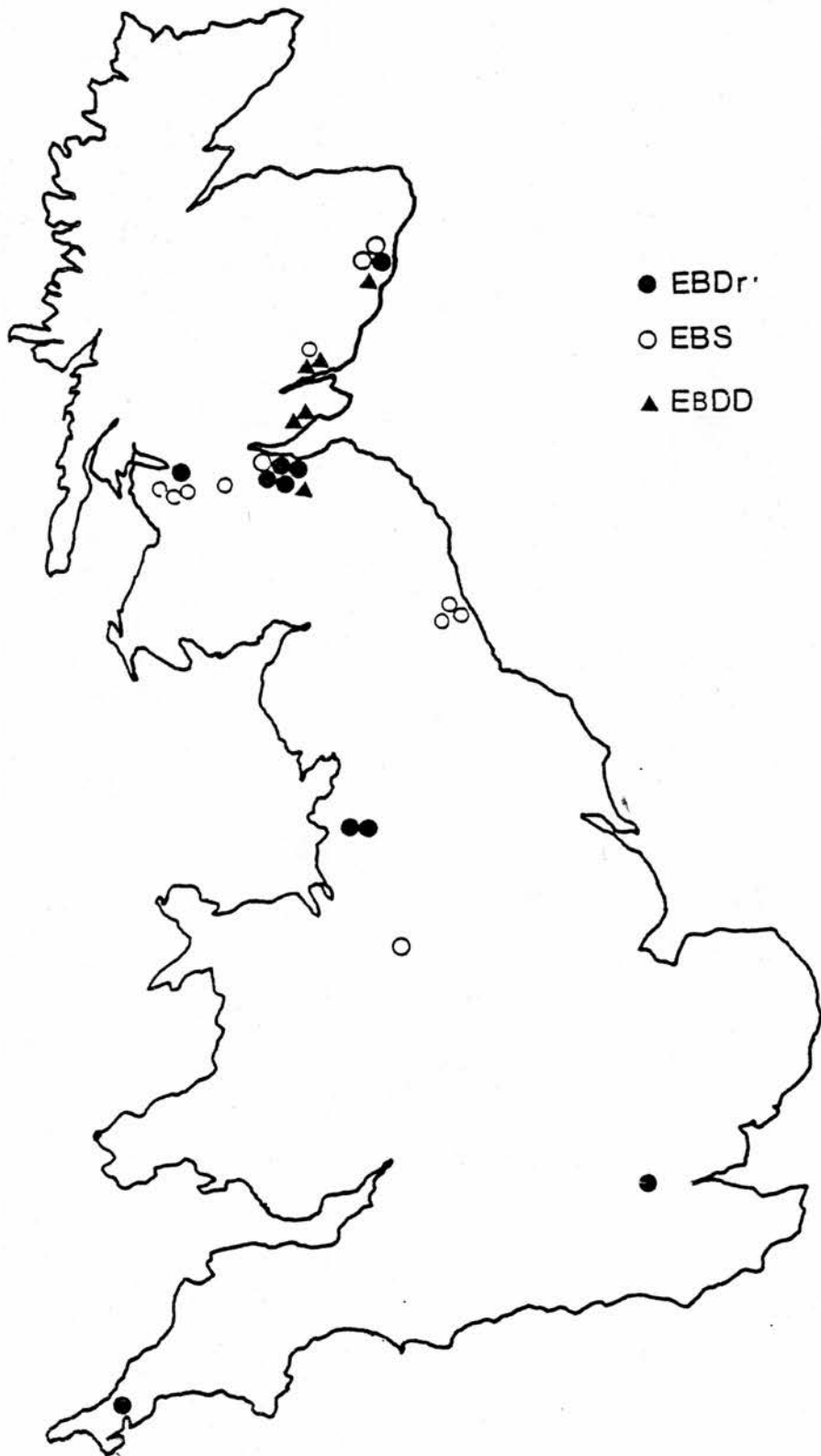
TABLE 3

CONTROL CELL STRAINS USED IN THE STUDY

No	Sex	Age	Biopsy	Site	Cell line
NF2	F	22	p	f	+
NF3	M	5	Donation, Sick Children's Hospital, Glasgow.		+
NF4	M	13	"	"	+
NF5	M	37	p	f	+
HSF8	F	25	Donation, Royal Hospital for Sick Children, Edinburgh.		+
HSF11	M	26	p	f	+
HSF13	M	41	p	f	+
HSF14	F	23	p	f	+
HSF17	M	41	p	f	+
HSF21	M	41	p	f	+
HSF22	M	19	p	f	+
HSF24	M	31	p	f	+
HSF26	M	40	p	f	+

FIG. 5

Distribution of EB patients involved in this study



(N.B: The two EBL patients were visitors to Britain from Saudi Arabia undergoing treatment at the Royal Hospital for Sick Children, Edinburgh)

saline (PBS), excess fat was removed using a sharp disposable scalpel and the skin was cut into 1mm x 1mm pieces. Groups of explants (4-8) were placed in each well of a 6 well culture dish (Linbro; from Flow Laboratories, Irvine, Scotland) and a sterile glass cover-slip placed over them: complete medium was then added.

Complete Medium: Dulbecco and Eagle medium with
20% foetal calf serum
4 mM glutamine
100 units/ml penicillin-streptomycin

Dishes were incubated at 37°C in a 5% CO₂ : air atmosphere. Medium was changed every second day until a healthy outgrowth of fibroblasts was seen emerging from beneath a layer of epithelial cells.

Trypsinisation and first passage

When fibroblast growth was established around the explants (average 31 days; range 12-55 days), the wells were washed twice with phosphate buffered saline. The glass cover slips were removed using sterile forceps and placed cells uppermost in another sterile dish with 1 ml of 0.5% trypsin-versene solution (Gibco-Europe, Paisley, Scotland). Trypsin-versene was also added to the wells containing explants. The dishes were incubated at 37°C for 10 mins or until the fibroblasts surrounding the explant had detached from the substrate and were rounded in appearance, progress being followed using an inverted phase-contrast microscope. The trypsinised cells were immediately transferred to a universal container of complete medium and centrifuged at 1,000 rev/min for 5 mins.

The supernatant was poured off and "fresh" complete medium

added, the pellet of cells was dispersed using a sterile Pasteur pipette. The cells were then transferred to a small plastic flask 25 cm² growth area (Nunc, Gibco-Europe, Paisley, Scotland) and maintained at 37°C in a 5% CO₂ : air atmosphere. This was regarded as the first cell line passage. A cell line is defined as the collection of cells arising from a primary culture at the time of the first passage. The term implies that cultures contain numerous lineages of cells originally present in the primary culture. This is different from a cell strain which is derived from either a primary culture or a cell line by the selection or cloning of cells with specific properties or markers which persist during subsequent cultivation (Paul, 1972).

Once the fibroblasts reached confluence, they were thoroughly washed with phosphate buffered saline (PBS) before being trypsinised, as described, and placed into a larger flask (85 cm² growth area). In this way many millions of fibroblasts could be obtained from a single biopsy. After the second passage cells were routinely cultured in complete medium containing only 10% foetal calf serum, which was changed thrice weekly.

Freezing and storage of Fibroblasts

By the 3rd or 4th passage, large enough numbers of fibroblasts were obtained to permit some experiments and enable the surfeit to be stored in liquid nitrogen. The pellet of trypsinised cells was resuspended in 2 mls of complete medium: a small aliquot was removed and the total number of cells/ml ascertained using a haemocytometer. Dimethylsulphoxide (DMSO) was added to the cell suspension to give a final concentration of 10%. The cells were

transferred to small plastic vials (Nunc, from Gibco-Europe, Paisley, Scotland), at approximate concentrations of 10^6 /ml/vial. The vials were immediately placed in a biological freezer for a controlled reduction in temperature nominally $1^\circ\text{C}/\text{min}$ before being placed in liquid nitrogen, at -196°C , until the fibroblasts were required for experimental use.

To retrieve cells from liquid nitrogen storage, the plastic vial was immediately placed in a water bath at 37°C ; as soon as the cell suspension defrosted it was transferred to a universal container of complete medium and centrifuged (1,000 rev/min for 5 mins), removing the DMSO in the supernatant. The fibroblasts were resuspended in fresh medium, transferred to a suitable flask, equilibrated with a 5% CO_2 : air atmosphere and incubated at 37°C .

Determination of Viability

To test the viability of cells after recovery from freezing or after treatment with a drug, the pelleted fibroblasts were resuspended in a small volume of PBS. Trypan blue (0.5% solution in 0.85% saline: Flow Laboratories, Irvine, Scotland) was added to 0.1 ml of the cell suspension to give a final concentration of 0.0625% trypan blue. After 2 minutes the viable cells which had excluded the blue dye and the non-viable stained cells were counted. A total of 200 cells from 4 different fields of the haemocytometer was used. The number of viable cells obtained was expressed as a percentage of total number of cells counted.

Determination of Proliferation Rates

The proliferation rate of each cell line was determined between passages 4 and 6. Fibroblasts were seeded in 7 mls of complete

medium containing 10% foetal calf serum at approximately 10^5 cells/25 cm² in flasks and incubated at 37°C in a 5% CO₂ : air atmosphere. Next day (day 1 after seeding) the medium was replaced with 7 mls of fresh complete medium. On day 3 four flasks were withdrawn, trypsinised and the mean cell number determined using a Coulter counter (model DN). The remaining four flasks received fresh medium on day 3 and 4: the final cell counts were determined on day 6, using the Coulter counter.

The proliferation rate was expressed as a percentage of the mean increase in cell number between days 3 and 6.

Normal control cell lines: HSF22 and HSF14 were used to examine the effect of phenytoin and vitamin E on the rate of fibroblast proliferation over large concentration ranges. The initial procedure was the same as for the determination of proliferation rates, previously described. On day 3 four flasks were removed, trypsinised and the mean cell number determined using the Coulter counter. The remaining flasks were divided into groups of four which then received phenytoin (in 50% ethanol: final conc. 0.08%) at 1, 10, 50, 100, 150, 200, 250, 300, 400 and 500 µM, leaving a group of flasks untreated to act as controls. Similarly vitamin E was added (in acetone: final conc. 0.2%) at a final concentration of 10, 50, 100, 250, 300, 400, 500 and 750 µg/ml. On day 4 all flasks received fresh medium containing the appropriate drug concentration. All the flasks were trypsinised and the final cell numbers were counted on day 6. The mean increase (or decrease) in cell number at each concentration was expressed as a percentage of the mean change in cell number between day 3-6 in untreated cultures i.e. as the mean percentage increase (or decrease) in proliferation rate at day 6.

Histochemical Staining

Fibroblasts were grown on glass cover slips in 6-well dishes until they reached the required cell density. The cells were then washed several times with PBS, and the cover slips with adhering cells were removed and left to dry at room temperature (12-15 mins). The fibroblasts were fixed in methanol (5 mins), then stained with May-Grunwald (12 mins) followed by Giemsa (12 mins). The coverslips were washed twice (5 mins each) in water and left to dry. They were mounted on glass microscope slides using DPX mountant.

Investigation of pericellular fibronectin

The procedure followed was that of Hsieh et al. (1980). Gelatin 20 mg/ml was mixed with fluorescein isothiocyanate (FITC: Sigma Poole, England) 40 µg/ml in 0.1M carbonate-bicarbonate buffer, pH 9.3, in the dark for 18 hours at room temperature. Unconjugated dye was removed by dialysis for 24 hours against PBS followed by gel filtration through a Sephadex G-25 column (Pharmacia, Uppsala, Sweden). The FITC-gelatin complex was collected in 2 ml aliquots: a 0.1 ml sample from each aliquot was diluted 10-fold with PBS and the optical density was read at 490 nm in a Unicam SP600 spectrophotometer. This enabled aliquots containing the highest concentration of FITC-gelatin to be identified by light absorption of the yellow FITC dye itself. Aliquots were stored at -20°C.

Fibroblast lines for staining were grown on glass coverslips (22 mm x 22 mm) in 6-well dishes. The cells were washed twice with PBS, before addition of 50 µl FITC-gelatin in 1 ml of serum-free complete medium, in which they were incubated at 37°C for 30 mins. The excess FITC-gelatin complex was removed by three 10 min washes

in PBS. Finally the cells were sandwiched in glycerine:FA buffer (9:1) between a glass microscope slide and a larger (22 mm x 32 mm) coverslip, sealed with varnish and viewed under an ultra-violet emitting Leitz microscope.

RESULTS

As indicated in Table 2 only 18 cell lines were established from the 30 biopsies taken for culture purposes (a 60% success rate) and success in establishing primary cultures did not seem to vary between the different EB groups: EBS 7/12, EBD_r 6/10, EBDD 3/6 and EBL 2/2. Of the unsuccessful biopsies, 9 produced enough fibroblasts to reach the first passage but failed during or just after the first trypsinisation.

A comparison of the different rates of proliferation between EB and control groups is seen below:

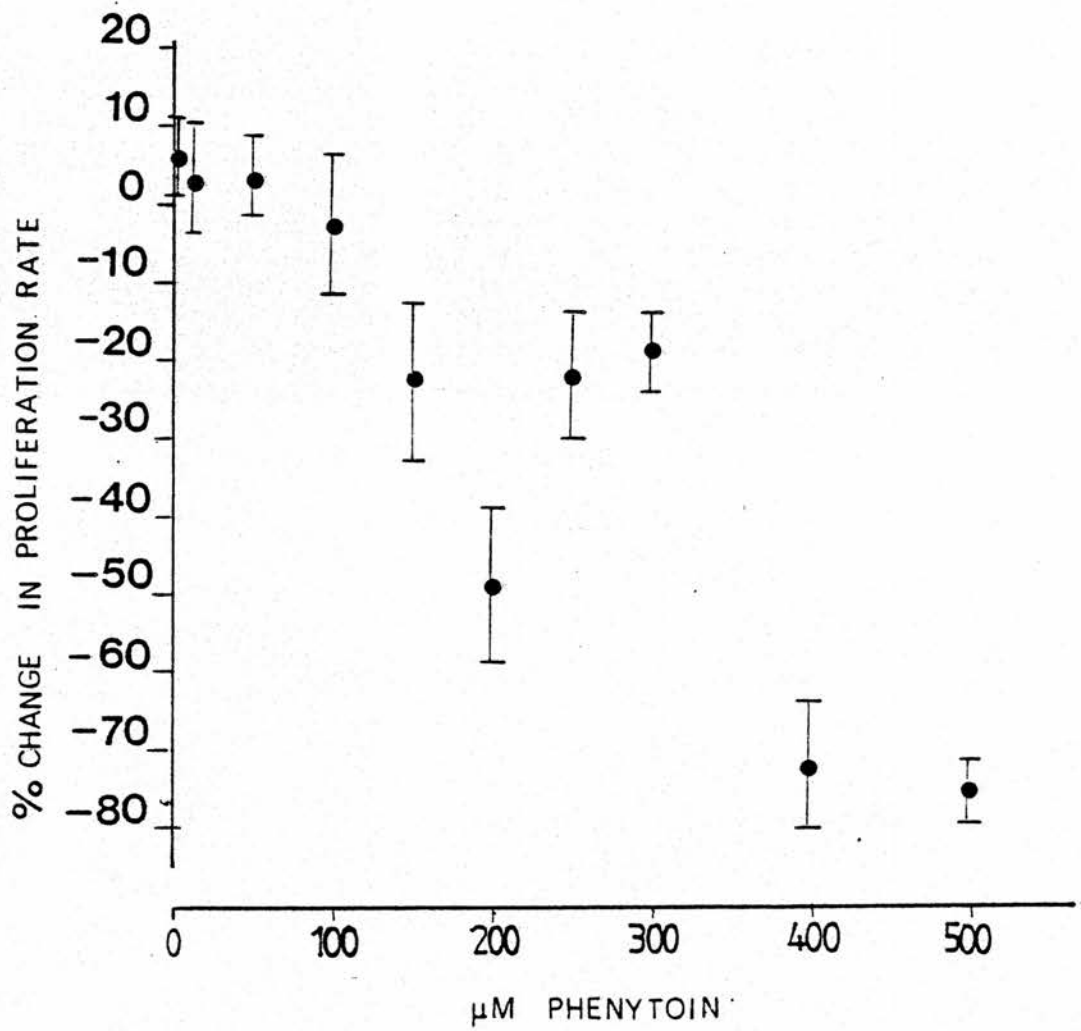
	N	EBS	EBD _r	EBDD
Number of cell lines	10	7	6	3
Mean age (years)	28.3 ± 4	21 ± 5	19.5 ± 7	24.6
Proliferation rate (%)	87.8 ± 13	123 ± 16	98 ± 29	106

Mean ± SEM

No significant differences in proliferation rate were found between any of the groups studied. There was no significant correlation ($r = -0.05$) between proliferation rate and the age of the donor in this study.

The responses of fibroblasts to phenytoin and vitamin E are seen in Figs. 6 and 7. Phenytoin appeared to depress the proliferation rate with increasing concentrations: a maximum 75% decrease in the proliferation rate was seen at 500 μ M phenytoin. The overall effect of vitamin E was to stimulate proliferation at concentrations over 150 μ g/ml: at lower concentrations proliferation was inhibited.

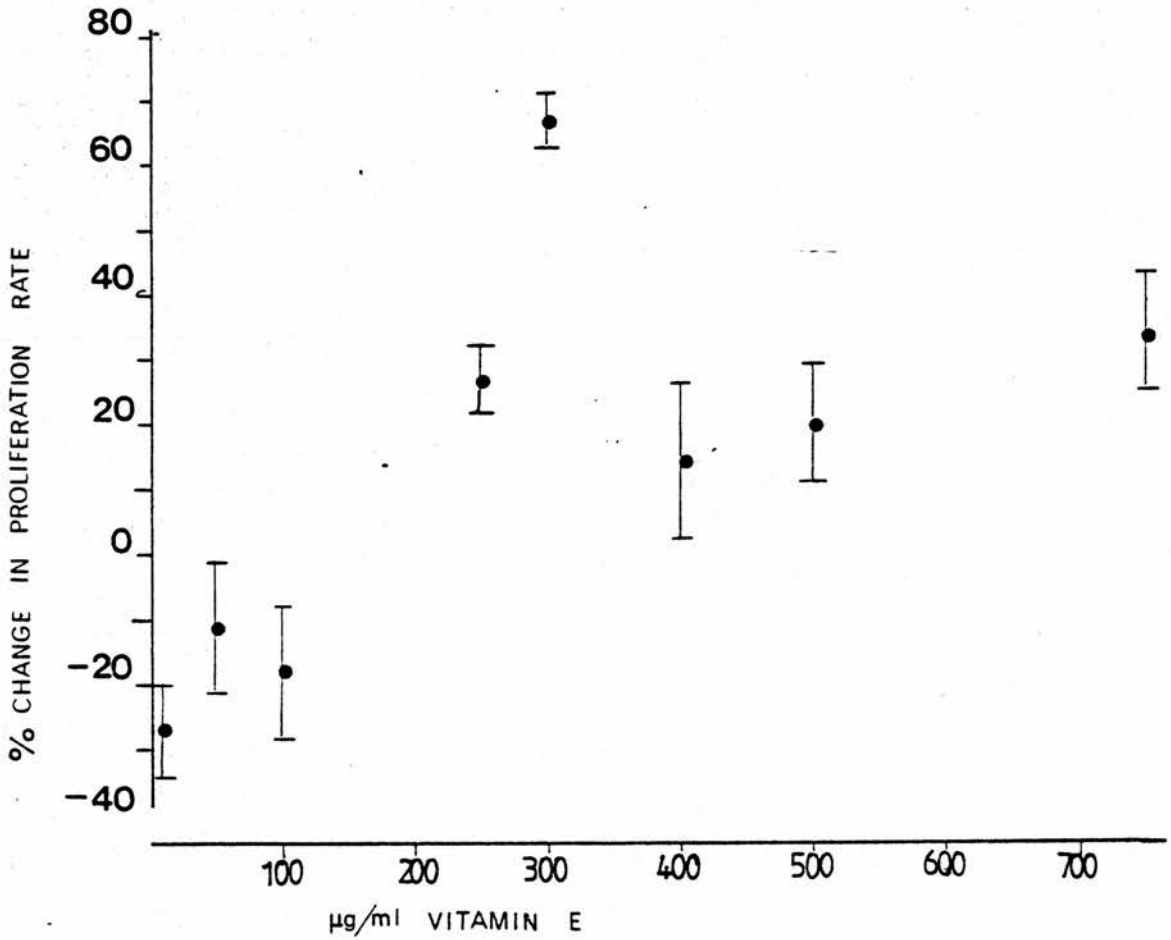
Dose-response with phenytoin



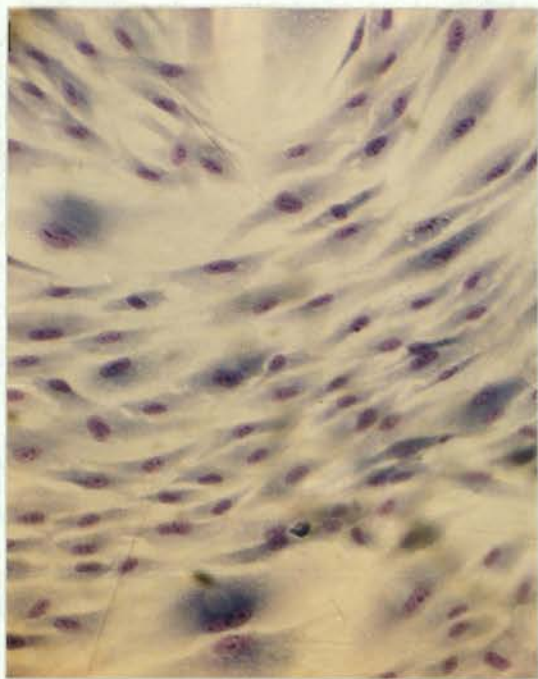
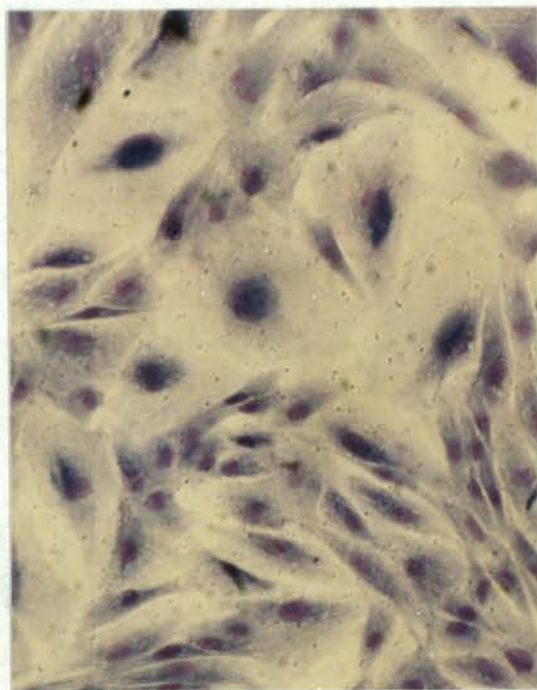
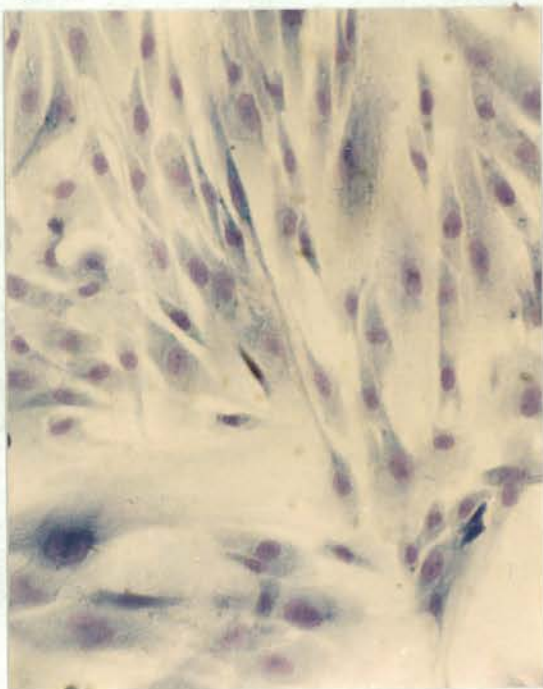
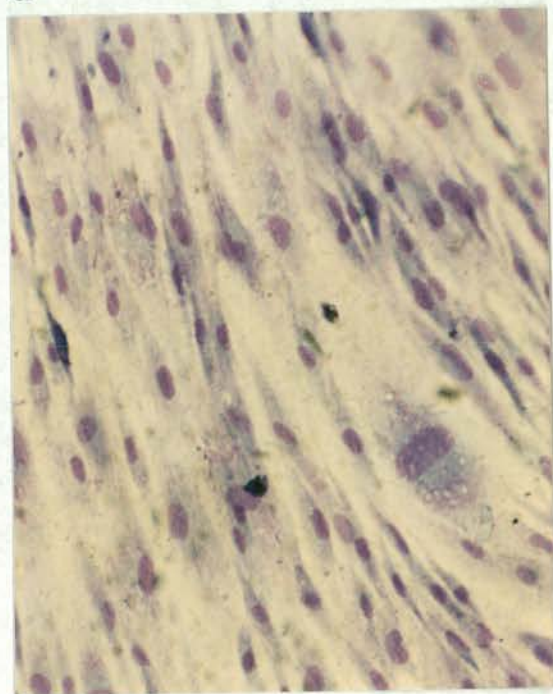
Mean (\pm SEM) percentage change in proliferation rate with phenytoin, using four individual observations at each drug concentration on one control cell line.

FIG. 7

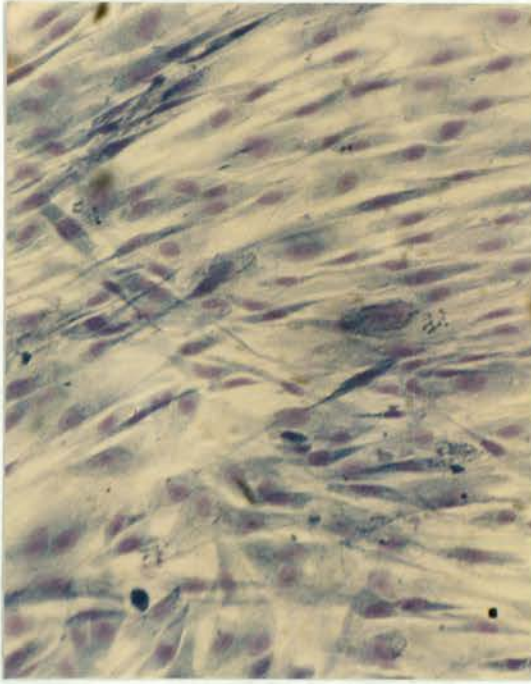
Dose-response with vitamin E



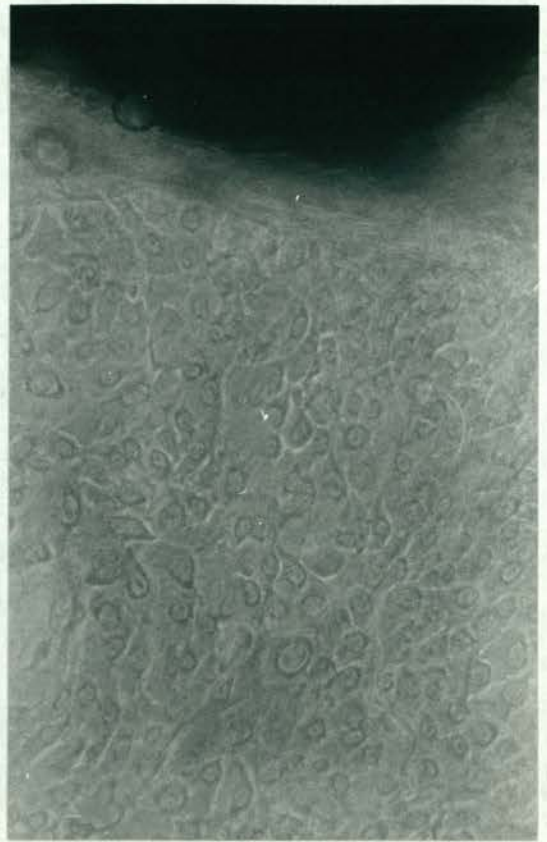
Mean (\pm SEM) percentage change in proliferation rate with Vitamin E using four individual observations at each drug concentration from one control cell line.

a**b****c****d**

9



12



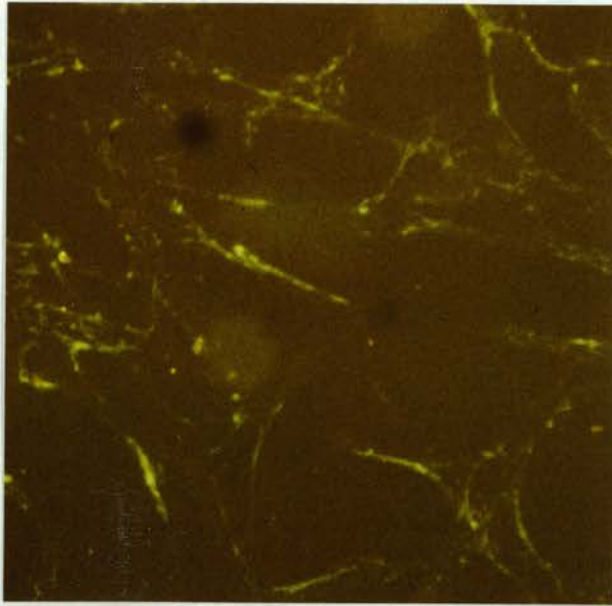
10a



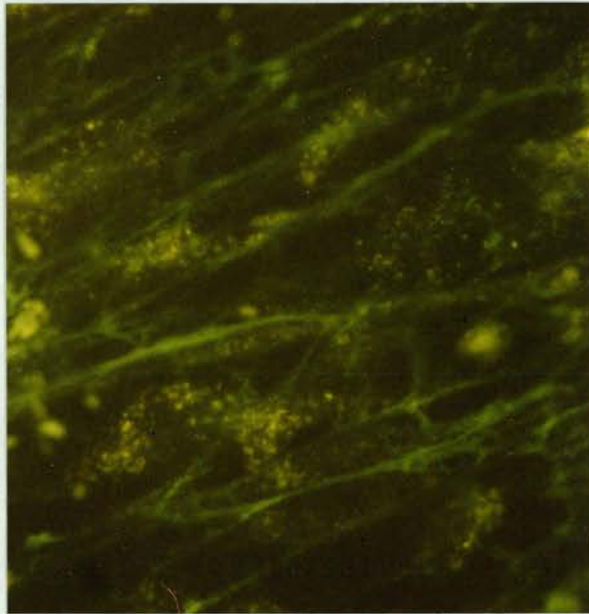
b



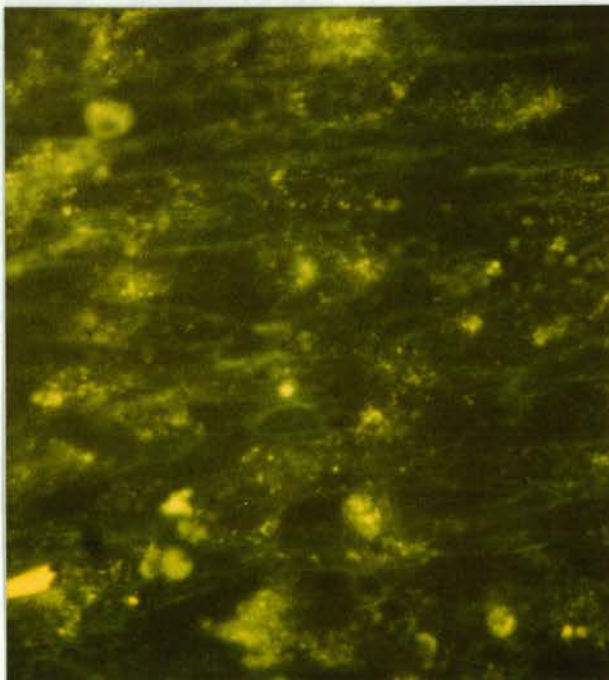
11 a



b



c



These experiments were repeated several times and although there was some variation in the response of the same cell lines, the figures given are representative.

Comparison of the morphology of representative cell lines showed variation in the size and shape of the fibroblasts and general morphology of the cultures. No particular characteristic was special to any one group whether control or EB (Fig. 8). The relative density of each culture at the time of examination inevitably affected the appearance of the cells: confluent cultures characteristically showed the "swirling" pattern associated with fibroblast cultures, whilst sparsely seeded cultures tended to lack a particular pattern and the filopodia-forming intercellular connections were much more obvious. Crossing-over of cells was observed both in control and EB groups but only when cultures became particularly dense (Fig. 9). This phenomenon could occur before the culture reached confluence if seeding had not been uniform, making some areas much more dense and therefore likely to have fibroblasts growing over the top of their neighbours, as described by Ehrlich et al (1983).

The similar patterns of FITC-fluorescence, indicated no obvious difference between groups (Fig. 10). Although this pattern developed and altered as the density of the culture increased, (Fig. 11), thin tangled fibrils becoming more dense (reflected by an increase in the intensity of fluorescence), eventually becoming ill-defined and finally the fibrillar pattern was replaced by sporadic, more punctate, patches of fluorescence. In no group was the pattern obviously different provided the cell density and the time in culture were the same. No quantitative differences between groups was detected, indicating similar extracellular fibronectin levels.

DISCUSSION

There was considerable variation in the time taken to establish the primary culture. This variability seemed to be unrelated to the donors' ages or the severity of their disease. Other factors such as the depth of the biopsy, the size of the pieces into which the biopsy was subdivided, and the number of skin bacteria present at the time of biopsy, seemed to be of importance. Most of the indigenous bacteria were successfully disposed of, firstly by skin disinfection, and secondly by the penicillin and streptomycin in the transport and complete medium. If bacterial contamination became a serious threat to the primary culture a small dose of another antibiotic (such as Gentamycin) was included in the medium of the affected well and was usually most effective.

In one unsuccessful biopsy fibroblasts failed to appear from the explant: in a further two unsuccessful biopsies there were too few fibroblasts to attempt subculture. The remaining nine biopsies produced enough fibroblasts to warrant an attempt at subculturing and it was at this stage - the first passage - that success or failure to establish a fibroblast line seemed to occur. In three of these unsuccessful biopsies, areas of "ghost-like" impressions of cells were seen following removal of the coverslip and transfer to trypsin-versene. The reason for this is unknown, but the trauma and tension applied to the surface of the fibroblast during the lifting and removal of the cover-slip may have been sufficient to disrupt the cell membrane. It is also possible that due to the location of certain areas of cells, between explants and close to the centre of the coverslip, fresh medium did not always reach the cells, causing premature exhaustion of intracellular resources and consequent death of the fibroblasts.

Although not all of the explants from any single biopsy established a sheet of epithelial cells before fibroblasts emerged (Fig. 12), nearly every biopsy did exhibit some epithelial growth. These large flat polygonal cells usually appeared within the first week in culture, extended to surround the explant and then regressed as fibroblasts emerged from beneath and between the epithelial cells. This pattern of growth has been well recorded by Van der Shueren et al (1980).

After the first passage the fibroblasts were heterogeneous in shape and size, some cells were spindle shaped - with a single axis of symmetry; some were sail shaped - with two axes of symmetry, and some were large and irregular in shape (pleomorphic) (Hennis et al, 1981). There is some evidence to suggest that the sail-shaped and pleomorphic cells are derived from the spindle-shaped cells by selection in vitro (Hennis et al, 1981). However, without being able to quantify the observation it was the impression in this study that spindle shaped cells tended to proliferate faster than the large cells and that the ratio of small to large cells tended to increase rather than decrease with time in culture.

The heterogeneity seen between the cell lines of each group in this study precludes general statements about differences between control and EB fibroblasts.

By examining the fibronectin of the fibroblasts' extracellular matrix, a further attempt was made to find differences between control and EB fibroblasts, either in the pattern of the matrix or in its rate of formation. The extracellular matrix is a dense network of fibres of different diameters which encircle the entire cell surface. Cell-cell contact is required to initiate the formation of these

fibres which are composed almost entirely of fibronectin (>90%). As cell density increases with prolonged time in culture (>14 days), a second intercellular matrix composed of collagen is produced (Chen et al, 1978). The matrix seen by FITC-gelatin staining is very similar to that visualised by scanning electron microscopy (Chen et al, 1978; Hsieh et al, 1980).

No differences could be seen between the intercellular matrices of either control, EBDr or EBS cell lines. They all developed a fine fibrous mesh-work by day 2 after seeding, providing that the seeding density was great enough to allow immediate cell contact. The fine fibrillar pattern did however alter with time in culture (Fig. 11), probably as a result of the developing collagenous extracellular matrix.

Amongst recent work on EBDr fibroblasts was a study of their behaviour within a collagen matrix (Ehrlich et al, 1983): these authors found that apart from a reduced capacity to contract the collagen lattice the EBDr fibroblasts had an altered morphology. They compared only two EBDr cell strains with three control cell strains, including strains derived from the parents of one of the EBDr patients. Donor ages were not given and must therefore be a consideration in any conclusions drawn from the results. The EBDr fibroblasts were reportedly abnormal, differing from controls by growing over neighbouring cells prior to the cultures becoming confluent (although the cells illustrating this abnormality do appear to be at a high density). No mention was made of differences in the proliferative capacity of control and EBDr cells, which could have some bearing on the findings. The EBDr fibroblasts were reported to form a greater number of "cross-overs" at greater

angles than control cells; but unfortunately no estimate of statistical error is given to indicate the certainty and reproducibility of this observation. It was also observed that the normal cells formed filopodia at opposite poles of the cell, whilst EBDr fibroblasts formed filopodia all around the periphery of the cell. This difference in morphology was thought to be ultimately responsible for the poor lattice-contracting powers of the EBDr fibroblasts.

It is possible that the morphological differences between control and EB groups found by Ehrlich et al (1983), would not have been detected by the methods adopted in this study and it is also possible that if seeded onto a collagen substratum the cells would behave differently. The present study showed no differences in the cellular or extra cellular morphology of control and EB cells grown on a glass coverslip.

No statistical difference was found between the mean proliferation rates of control and EB fibroblast lines, indicating that in this respect there is no evidence of hyperactivity in the EB fibroblasts. An increased proliferative capacity in vitro could have reflected a similar potential in vivo, thereby increasing levels of fibroblast products in the dermis. This does not appear to be the case and any other differences in fibroblast characteristics which were observed do not result in an increased proliferative capacity. In this study there was no significant correlation between the age of the donor and proliferation rate of the fibroblasts. This may be because the majority of donors were young adults and to show an age-related decline in proliferation rate, more donors from the extremes of the age range would be required.

Apart from using the concentrations of vitamin E and phenytoin

adopted by earlier investigators, it was necessary to establish at which concentrations the drugs exerted an effect. Unfortunately there was substantial variation in the results with phenytoin, even using the same control cell line. This may be attributable to the binding of phenytoin by serum albumin with variation between different batches of serum. There was also a problem of precipitation of phenytoin at concentrations higher than 200 μM . This may have added another variable to the system. Such variability, in response of fibroblasts to a drug, differs from previous investigations with other drugs (Priestley and Brown, 1980), indicating that phenytoin is the cause of the variability found in the present study. However based on these results and the data of other investigators (Shafer, 1960; Houck et al, 1972; Blumenkrantz and Asboe-Hansen, 1974 and Bergenholtz and Hännström, 1979), phenytoin was used at 50-200 μM for further studies.

The response of a control cell line to vitamin E was rather less variable than that seen with phenytoin. As the changes in proliferation were greatest between 10-300 $\mu\text{g/ml}$ vitamin E, the concentrations adopted for further experiments were 10, 50, 100 and 250 $\mu\text{g/ml}$.

SUMMARY

- 1) Biopsies were taken from 30 EB patients and 18 produced viable cell lines.
- 2) The mean proliferation rate of the various EB groups did not differ significantly from that of the control group, which was $88\% \pm 13$ (SEM; n = 10).
- 3) Great heterogeneity of cell shape and size was seen both within and between cell lines of both the EB and control groups: but no particular characteristic was limited to a particular group.
- 4) Dose-response curves were obtained for the effect of phenytoin and vitamin E on the proliferation of a control fibroblast strain: the phenytoin seemed to inhibit proliferation whilst the vitamin E tended to stimulate fibroblast proliferation.

CHAPTER 2

ULTRASTRUCTURE

ULTRASTRUCTURE

INTRODUCTION

The use of the electron microscope to visualise the architecture of the skin has greatly enhanced our knowledge and understanding of structure with relation to function. The ultrastructural characteristics of normal skin have been well documented (Zelickson, 1967) and will not be dealt with in detail here. However it is necessary to clarify a few important aspects of skin structure if abnormalities, or changes, seen in bullous conditions are to be understood.

The basic division of skin into epidermis and dermis distinguishes cells of an ectodermal origin from the inner cells of mesodermal source. The epidermis is divided into further layers, or strata, reflecting various stages in the differentiation of basal cell to corneum cell, to the point at which the cell is sloughed off. The basal cell layer therefore represents a resident population of dividing keratinocytes, a stem cell population for the rest of the epidermis. This cell layer is anchored to the basal lamina - the electron-dense dividing line between dermis and epidermis - by hemidesmosomes and the cells to each other by desmosomes. Intracellularly tonofibrils - part of the cytoskeleton - converge on these points of contact providing strength and form to the basal cell. The basal cells contain the expected organelles - mitochondria, endoplasmic reticulum, ribosomes, lysosomes and have a large electron-dense stained oval nucleus. Included in the basal cell layer are melanocytes and sometimes Langerhans cells, though they are more frequently found in the overlying stratum spinosum.

The stratum spinosum, or prickle cell layer, is so called

because of the cellular conformation adopted at this stage in differentiation. Numerous cytoplasmic projections and ridges are seen with desmosomes appearing as prickles at the end of such protrusions. In this cell layer keratohyalin first appears and becomes associated with the tonofibrils.

In the overlying stratum granulosum the keratinocytes become more flattened and elongated. The cells of this layer characteristically contain keratohyalin granules, which form on the tonofibrils. These gradually increase in quantity until eventually they coalesce within the cells, just below the stratum corneum. As the keratohyalin accumulates the nucleus of the cell disintegrates and the cytoplasmic organelles disappear. As the cell approaches the stratum corneum the desmosomal contacts change, with the electron-dense attachment plaque disappearing in the overlying cornified keratinocyte.

In the stratum corneum the final product of basal cell differentiation is seen - a flattened anuclear cell, lacking in any cytoplasmic organelles and containing only fine filaments of keratin embedded in an amorphous matrix. As the desmosomes finally degenerate the keratinocyte can finally be sloughed off.

The dermis is largely an acellular matrix of collagen, elastin and ground substance interspersed with resident fibroblasts and other motile cell types. The dermis spans the region between the basement membrane zone and the subcutaneous fat and can be divided into two regions according to the size and arrangement of collagen fibres. The papillary dermis is proximal to the basement membrane zone, usually constituting only 10% of the full dermal thickness; the underlying region is the reticular dermis.

The abundant randomly orientated collagen fibres, with their characteristic cross-banding are the most obvious ultrastructural

feature of the dermis. Type I collagen forms the majority of the reticular dermal collagen, ~~having thick fibres 60-100 nm in diameter.~~ Type III collagen ~~forms a thinner fibre 20-40 nm in thickness and~~ accounts for only 10-15% of the dermal collagen.

The other fibrous components of the dermis are the elastic fibres. These fibres have two components; a microfibrillar scaffolding embedded in a matrix of elastic cement constituting 90% of the fibre, hence ultrastructurally the fibres appear as rather amorphous electron-dense bands in the dermis. The ground substance, composed largely of glycosaminoglycans, is not usually visualised.

Fibroblasts are responsible for the production of constituents of the dermis, as well as the synthesis of various enzymes necessary for the maintenance and regulation of the dermal components. Such metabolically active cells contain extensive endoplasmic reticulum, Golgi apparatus and ribosomes.

Biochemical aspects of the dermo-epidermal junction

The region separating the dermis from epidermis is the dermo-epidermal junction or basement membrane zone. This site of attachment of epidermis to dermis has been well reviewed (Briggaman and Wheeler, 1975b; Briggaman, 1982; Prunieras et al, 1983). The junction can be divided into four components using the electron microscope: the basal cell plasma membrane, the lamina lucida, the basal lamina and the sub-basal lamina fibrous components.

The basal cell plasma membrane is usually highly convoluted and is clearly recognised as the membrane boundary between cell and lamina lucida, interrupted at intervals by electron-dense hemidesmosomes. Beneath the plasma membrane is the electron-lucent lamina lucida 20-40 nm thick. Anchoring filaments traverse the

lamina lucida. These filaments are most dense beneath the hemidesmosomes, where they emerge from a sub-basal electron-dense plaque and extend into the underlying basal lamina. The composition of these filaments is unknown although they are susceptible to digestion by bacterial collagenase and elastase (Kobayasi et al, 1977).

Other components of the lamina lucida, but not exclusive to it, (reviewed by Stanley et al, 1982; Briggaman, 1982) include laminin, bullous pemphigoid antigen, heparan sulphate proteoglycan and fibronectin, determined by indirect immunofluorescent techniques (Fyrand, 1979; Hintner et al, 1980; Foidart et al, 1980).

Laminin is a non-collagenous glycoprotein (Timpl et al, 1979) vital in the adherence of epithelial cells specifically to Type IV basement membrane collagen (Terranova et al, 1980). It is a large asymmetrical molecule consisting of three polypeptide chains cross-linked by disulphide bonds (Hogan, 1981). In wound healing laminin is laid down with Type IV collagen after re-epithelialation has commenced (Stanley et al, 1981a). At the ultrastructural level the formation of the basal lamina is accompanied by the simultaneous emergence of Type IV collagen and laminin (Hintner et al, 1980). The close association of laminin and Type IV collagen is seen in the production of a fine fibrillar matrix produced in culture by a keratocarcinoma-derived endodermal cell line (Leivo et al, 1982). Kariniemi et al. (1982) found that epidermal cells in culture do not deposit laminin. This could explain the findings of Woodley et al (1980a) indicating that the presence of living dermis is required to produce Type IV collagen and therefore perhaps other components of the basement membrane zone.

Bullous pemphigoid antigen is another glycoprotein, recognised

by immunoreactivity with bullous pemphigoid antibodies from patients with that disease. The antigen appears to consist of a heterogeneous group of closely related glycoproteins (Zhu and Bystryn, 1983).

The antigen isolated from skin consists of two components of 20,000 daltons and 9,200 daltons (Diaz et al, 1977), whilst cultured epithelial cells produce a high molecular weight ($\sim 220,000$ dalton) bullous pemphigoid antigen (Stanley et al, 1981b). The antigen is of epidermal origin (Diaz and Marcelo, 1978) forming at the junction between viable human epidermis and non-viable (irradiated) dermis (Woodley et al, 1980b). Similarly the antigen can be detected in re-epithelializing superficial wounds at the dermo-epidermal junction where laminin and Type IV collagen were not initially present (Stanley et al, 1981a).

A heparan sulphate proteoglycan of approximately 750,000 daltons has been isolated from the basement membrane and is thought to be located in the lamina lucida (Hassell et al, 1980). It contains approximately equal amounts of protein and covalently linked heparan sulphate. Its function and mode of interaction with other components of the lamina lucida and basal lamina are still unknown.

Fibronectin has been identified at the dermo-epidermal junction (Fyrand, 1979; Foidart and Yaar, 1981) as well as being widely distributed throughout most of the tissues of the body (reviewed by Yamada and Olden, 1978; Ruoslahti et al, 1981; Stasko and De Villez, 1982). It is present in the plasma membrane of the basal cells and in the lamina lucida, however no correlation between specific areas of cell-substrate adhesions and fibronectin location has been established (Couchman et al, 1979). Skin fibroblasts produce fibronectin both in vivo and in vitro (Stenman and Vaheri, 1978;

Fyrand, 1979; Couchman et al, 1982): there is also an abundance of fibronectin in serum, consequently the origin of fibronectin located within the basement membrane zone is uncertain.

Fibronectin is a glycoprotein made up of two subunits of 220,000 daltons linked by disulphide bonds. It may exist as a dimer or as a multimer. The subunits may not be identical, thus producing an asymmetrical molecule capable of localised folding. The role of fibronectin at the basement membrane is unclear. It is known that epithelial cells in vitro produce and deposit fibronectin. Indirect immunofluorescent techniques have been used to show fibronectin at the margin of attaching epithelial cells and also in the peripheral cells of spreading cell foci (Kariniemi et al, 1982). In contrast the work of Federgreen and Stenn (1980) suggested that in vitro fibronectin was not essential for epithelial cell spreading. This work, however, did not take into account the effects of endogenous fibronectin. It therefore seems likely that fibronectin plays some role in the adhesion of epithelial cells to the substratum whether in vitro or in vivo. It has also been suggested that fibronectin might be involved in the orientation of regenerating epithelial cells which proliferate at the basement membrane (Stenman and Vaheri, 1978).

The major component of the basal lamina is Type IV collagen; also present are small amounts of Type V collagen. These collagens differ from Types I - III in their lack of banding and fibrillar appearance (reviewed by Kleinmann et al, 1981; Sage, 1982). Immunoelectron microscopy was used to locate Type IV collagen at the basement membrane in vivo (Foidart and Yaar, 1981) and it can be shown that the development of the basal lamina in organ culture is accompanied by the appearance of Type IV collagen and laminin

(Hintner et al, 1980).

The production of Type IV collagen is intriguing. Laurie et al (1980) failed to show immunostaining in the cells associated with the basement membrane, whereas the basement membrane itself stained strongly. They presumed that the development of the basal lamina is a transient activity that occurs at an early stage in development and that once established the basal lamina required only minimal synthesis of Type IV collagen to maintain its structural integrity over long periods. Woodley et al (1980a) showed that keratinocytes next to a non-living substrate failed to produce Type IV collagen but once in contact with a living dermis an ultrastructurally recognisable basement membrane was laid down. This is in contrast to the earlier work of Briggaman et al (1971) who in a series of recombination experiments, showed the basal lamina to be of epidermal origin, being formed when living epidermis was recombined with dead dermis. However, because the dead dermis was supported on well vasculated living chick chorioallantoic membrane, Woodley et al (1980a) argued that the epidermis was indeed receiving material from living cells. Some controversy remains as to whether a viable dermis is necessary for the development of the basal lamina which is ultimately of epidermal origin. It is interesting that Type IV collagen could not be detected using indirect immunofluorescent techniques against epithelial cells in culture. Two explanations are possible; firstly factors that only the dermis can provide are required or secondly that production of Type IV collagen is slow or non-existent, unless wounding of the epidermis occurs, in which case other factors are released and may stimulate Type IV collagen synthesis by basal cells. These factors are not produced in a culture system and

therefore no production of this collagen occurs.

The structure and properties of Type IV collagen are further mentioned in chapter 3. One of the most intriguing properties is the preferential adhesion of epidermal cells to a substrate of Type IV collagen, compared to Types I - III (Murray et al, 1979); fibroblasts attach equally well to all four collagen substrates. The slow attachment rate of epithelial cells is thought to be due to de novo synthesis of an attachment protein (Murray et al, 1979) - probably laminin in the light of current knowledge. Laminin is known to be closely associated with Type IV collagen in producing a fine fibred matrix in cell cultures (Leivo et al, 1982).

The final component of the basement membrane zone are the sub-basal lamina anchoring fibrils. These are believed to be of dermal origin because dermal viability is essential for their formation (Briggaman et al, 1971). Although their biochemical composition is unknown, the work of Kobayasi et al (1977) suggests a collagenous component. The fibrils were found to be sensitive to clostridal collagenase but insensitive to elastase, trypsin and the reducing agent dithioerythritol.

With an ever increasing knowledge of the structure and biochemical composition of normal skin it is possible to examine abnormalities and diseases of the skin, with a view to highlighting the causal defect. Electron microscopy has proved a useful tool in the large and heterogeneous EB group of diseases. Clearly some variants such as the mild EBS Weber-Cockayne type do not require such sophisticated technology for diagnosis but even so examination of the ultra-structure of the skin from this group may provide a clue in the pathogenesis of the disease. For this reason a number of skin

biopsies from EB patients were examined using electron microscopic techniques.

MATERIALS AND METHODSPATIENTS

Twenty four patients were studied, 9 with EB simplex, 13 with dystrophic EB and 2 with junctional EB (see Table 2 and 4 for details). The clinical diagnosis, based on the patient's family and previous history and signs of the disease at presentation, was proposed by the attending physician. For comparisons with normal skin electron micrographs from the collection at the Department of Dermatology, Royal Infirmary, Edinburgh, were used.

PROCEDURE

The biopsy was halved: one half was prepared for tissue culture (page 16) and the other, for electron microscopy, was immediately cut into 1mm x 1mm pieces and immersed in 3% glutaraldehyde in 0.1M sodium cacodylate buffer, with 2 drops of 1% calcium chloride (pH 7.4). After 3 hours at room temperature the tissue was rinsed twice in 0.1M cacodylate buffer (pH 7.4) where it remained overnight at 4°C. The next day samples were post-fixed in 2% osmium oxide in distilled water for 1 hour at 4°C. This was followed by dehydration of the tissue by immersion in an ascending series of alcohols for 15 minute periods; 70%, 95% and 4 changes of absolute ethanol. The samples were then transferred into fresh Spurr resin comprising:

Vinyl cyclohexene dioxide (resin)	16.1 gms
Nonenyl succinic anhydride (hardener)	43.4 gms
Diglycidyl ether of propylene glycol (plasticiser)	9.8 gms
Dimethylaminoethanol (accelerator)	0.7 gms

The resin was changed after 1 hour, 3 hours, 16 hours and 24 hours respectively, all at room temperature. The tissue was

finally transferred to fresh resin, orientated in Taab Capsules, and polymerised at 60°C for 24 hours.

The blocks were sectioned on an LKB ultratome III. Sections of silver interference colour (50nm) were mounted on uncoated 200 mesh copper grids. Routine staining was carried out using a saturated solution of uranyl acetate in 70% methanol for 10 minutes followed by lead citrate (Reynolds, 1963) for 3 minutes. Sections were examined using a Philips 301 electron microscope operating at an accelerating voltage of 60 kV.

All processing for electron microscopy was very kindly performed by Dr. J.A. Ross of the Department of Dermatology, Royal Infirmary, Edinburgh.

TABLE 4

PATIENTS EXAMINED BY ELECTRON MICROSCOPY

<u>NO</u>	<u>SITE OF CLEAVAGE</u>	<u>NOTEABLE FEATURES</u>
EB6	None	Multiple duplication of the basal lamina
EB8	None	Some perinuclear oedema
EB9	None	Slight intracellular oedema in basal cells
EB10	None	Some duplication of the basal lamina
EB11	None	None
EB12	None	None
EB23	Intraepidermal	Cytolysis of the basal cell, numerous vacuoles in the basal cells.
EB24	None	None
EB32*	Intraepidermal	Cytolysis of basal cell, clumped tonofibrils
EB26	Junctional	Abnormal hemidesmosomes
EB27	Junctional	Abnormal hemidesmosomes, "torn off" phenomenon, basal lamina duplication, vacuoles in basal cells
EB1	Intradermal	No anchoring fibrils seen, disrupted basal lamina
EB14	Intradermal	No anchoring fibrils seen
EB15	Intradermal	No anchoring fibrils seen
EB18	Intradermal	No anchoring fibrils seen
EB19	No cleavage	Basal lamina normal, anchoring fibrils present though possibly rudimentary
EB31	Intradermal	Cleavage incomplete, anchoring fibrils present, possible collagen dissolution
EB22	Intradermal	Single anchoring fibril present
EB7	Intradermal	Anchoring fibrils present, varying amounts of material attached to the basal lamina
EB16	None	Some deposits of fibrillar material in the dermis, basal lamina duplicated in places
EB17	None	Granular body in the dermis, duplication of basal lamina, anchoring fibrils present
EB20	None	Basal lamina duplication, anchoring fibrils present
EB21	None	Granular body in the dermis anchoring fibrils present
EB25	None	Herniation of the basal lamina

* biopsy taken from an involved site



RESULTS

EB Simplex

As these biopsies were taken from clinically normal skin (except EB32, which was taken from an involved site), blisters were not evident; only in one patient EB23 did blistering arise spontaneously during the biopsy procedure.

In the majority of EBS skin samples the ultrastructural changes were minimal and comprised the following:

- 1) Perinuclear oedema in some but not all of the basal cells (Figs. 13, 14).
- 2) Occasional perinuclear and cytoplasmic vacuolation (Fig. 14). Extensive cytoplasmic vacuolation and dissolution of the cytoplasm in cells close to the cleavage site in EB32 (Fig. 15).
- 3) When cleavage was induced by the biopsy procedure (EB23), the split occurred intra-basally; similarly where the biopsy was taken from an involved site (EB32) blistering was at the same level (Figs. 15, 16). The floor of the blister is composed of fragmented basal cells (Fig. 16).
- 4) In one patient (EB32) striking granular globules were evident in the upper layers of the stratum spinosum (Fig. 17). These masses of electron-dense granular material were located in the cytoplasm adjacent to the attachment plaque of the desmosomes; possibly comprised of bundles of tonofibrils tightly clumped together. Tonofibrils can be seen merging with the globules (Fig. 17). Normal desmosomes and tonofibrils were seen in the same section. In the same patient, there was further evidence of granular clumping at the level of the blister roof (Fig. 15).

EB Junctional-lethalis

Skin taken from non-involved sites in two infants with junctional EB blistered spontaneously during the biopsy procedure. Electron microscopy showed:

- 1) The cleavage was between the basal cell plasma membrane and the basal lamina. In the blister roof (Fig. 18) the plasma membrane remained intact: hemidesmosomes, with anchoring filaments still attached to them were visible, although few anchoring filaments were to be seen other than in the region of the hemidesmosomes. The disrupted hemidesmosomes frequently lacked the sub-basal electron-dense plaque, normally located immediately beneath the hemidesmosomes; seen as a thin dark line in the lamina lucida (Fig. 19).
- 2) Cleavage was not always "clean cut"; some darkly staining fragments of the basal cell plasma membrane could be seen attached to the basal lamina in the blister floor, their hemidesmosomes intact (Fig. 20). The hemidesmosomes again lacked the sub-basal plaque but anchoring filaments were present.
- 3) Perinuclear oedema and numerous vacuoles were striking features of the basal cells in the blister roof (Fig. 21). The intercellular junctions, desmosomes, were normal in appearance: the tonofilaments, though present, were disrupted by the large vacuoles of the basal keratinocytes.
- 4) The dermis of the blister floor (Fig. 20) appeared normal: anchoring fibrils attached to the basal lamina, extended into the dermis. The basal lamina was irregular and diffuse in appearance; attached to it was some amorphous material and possibly some anchoring filaments.

Some areas of the skin did remain intact. Although the basement membrane zone looked normal at first sight some features of interest were noted:

- 1) The plasma membrane attachment plaques of the hemidesmosomes were of variable electron density and the hemidesmosomes lacked the sub-basal electron-dense plaque (Fig. 22). Tonofibrils were seen above the hemidesmosomes, within the cytoplasm. A number of microcytotic vesicles were present in the cytoplasm above the plasma membrane of the basal keratinocyte.
- 2) The basal lamina was discontinuous and of variable density: some basal lamina duplication or malformation has occurred (Fig. 23). Normal-looking anchoring fibrils were seen attached to the basal lamina, at both levels, where duplication had occurred.
- 3) There was no indication of an abnormality in the width of the lamina lucida, which contained anchoring filaments and amorphous material, neither of which appeared to be as dense beneath the hemidesmosomes as they are in normal skin. Nor was there any evidence of an abnormality within the dermis.

EB dystrophic recessive - Hallopeau Siemens

In patients with EBDr Hallopeau-Siemens it is very difficult to obtain skin from a non-involved site. In all individuals except one, the skin blistered just with the trauma of the biopsy procedure though occasionally intact areas could be found.

- 1) The place of cleavage was in the upper dermis just beneath the basal lamina (Figs. 24, 25). The epidermis looked normal. The basal cells had normal desmosomes and hemidesmosomes. Their cytoplasm was full of tonofibrils and a large number of densely

staining melanosomes. The intercellular space was wider than is normal but such oedema may be expected in the roof of any blister (Fig. 26). A contiguous basal lamina with some attached amorphous material was seen at the under surface of the roof (Fig. 26) and at higher magnification (Fig. 24), appeared more diffuse and disrupted. The lamina lucida was normal where the basal lamina is well defined. Fine anchoring filaments were seen traversing the lamina lucida and the electron-dense sub-basal plaque was visible beneath the hemidesmosomes.

- 2) There was often very little evidence of anchoring fibrils attached to the dermal aspect of the basal lamina, though it is possible that 4 or 5 degenerative anchoring fibrils could be discerned in Fig. 24. This seemed characteristic of other areas of the blister roof from this particular patient and of the other patients examined. However in one patient, EB22, a single, well defined fibril was evident (Fig. 27).
- 3) In the floor of the blister both large and small collagen fibrils were to be seen randomly orientated within the dermis. Banding could be seen on the thick collagen bundles even in the proximity of the blister cavity, although there was some disruption of the actual collagen bundles closest to the blister floor (Fig. 25).

Dermal separation was incomplete in just one patient, EB31, and this provides interesting information concerning the pre-blistered state of the dermis. At the apex of the narrower infolds or involutions of the highly convoluted basal lamina, cross-banded anchoring fibrils could be seen (Fig. 28). However elsewhere along the basal lamina very few normal fibrils were visible although some degenerative fibrils may be present. The collagen of the dermis appeared to be undergoing focal degenerative changes and areas of

few collagen fibres are filled with amorphous material; the fibres themselves varied in electron density along their length, particularly where they are located close to these regions of electron-lucent amorphous material, although cross-banding was still clearly discernible. Some variation in the diameter of the collagen fibres was obvious, noticeably the particularly large fibre ~100 nm in width.

Stages of degeneration of the collagen fibres just beneath the basal lamina was shown well in Figs. 28 and 29.

- a) The beginning of degradation an area or focal point of electron lucency in the collagen fibre; banding was still clearly visible.
- b) Almost complete disruption of the collagen fibre; banding has disappeared.
- c) The complete disruption of the fibre.

Occasionally the formation of a blister cleft could be seen in close proximity to an abnormal-looking fibroblast (Fig. 30). Again interruption and disintegration of the collagen fibres was clearly visible, especially in the remaining fibres spanning the cleft itself. The fibroblast seemed to be undergoing cytolysis and a neighbouring fibroblast, seen at higher magnification in Fig. 31, has undergone complete cytolysis. Its plasma membrane could still be seen surrounding the cavity.

This association of cleft formation with the presence of a fibroblast was again seen in Fig. 32. Disintegration of collagen could be seen above a fibroblast. The cytoplasm of this cell appears more rarified than is usual, mitochondria and a number of vacuoles are present.

EB dystrophic dominant: Cockayne-Touraine

Only one skin sample taken from a non-predilected site in a dominant dystrophic patient (EB7), blistered during the biopsy. Generally the skin remained intact so the diagnosis was based on the mode of inheritance and clinical features including whether previous blistering had produced scarring and milia, the severity of blistering and the absence of albopapuloid lesions on the body.

- 1) In the single blister examined the plane of cleavage was beneath the basal lamina within the papillary dermis (Fig. 33). The basal cells, hemidesmosomes and anchoring filaments appeared to be normal. The basal lamina itself was continuous and well defined. Beneath the basal lamina amorphous material could be seen. There was variation in the amount of tissue remaining attached to the basal lamina after cleavage (Fig. 34). At higher magnification, (Fig. 35), this material appeared to be composed of anchoring fibrils and a large amount of fine fibrillar material. The anchoring fibrils varied in appearance from apparently normal to small, rudimentary fibrils. Some collagen fibres with clear cross-banding could be seen in the blister roof.
- 2) The blister floor shows two distinct populations of normal-looking collagen fibres (Fig. 33). The thicker type I fibres, diameter 75 nm, were separated by thinner, 10 nm diameter type III fibres (Fig. 36). There was no evidence of collagen degradation.

In the intact skin from the same patient, EB7, normal anchoring fibrils were seen extending into the dermis (Fig. 37). In other areas the fibrils were missing or of abnormal appearance. The basal lamina was rather ill-defined in this section of intact skin.

The dermis appeared to be structurally normal. The intact skin of the other patients examined exhibited some interesting features:

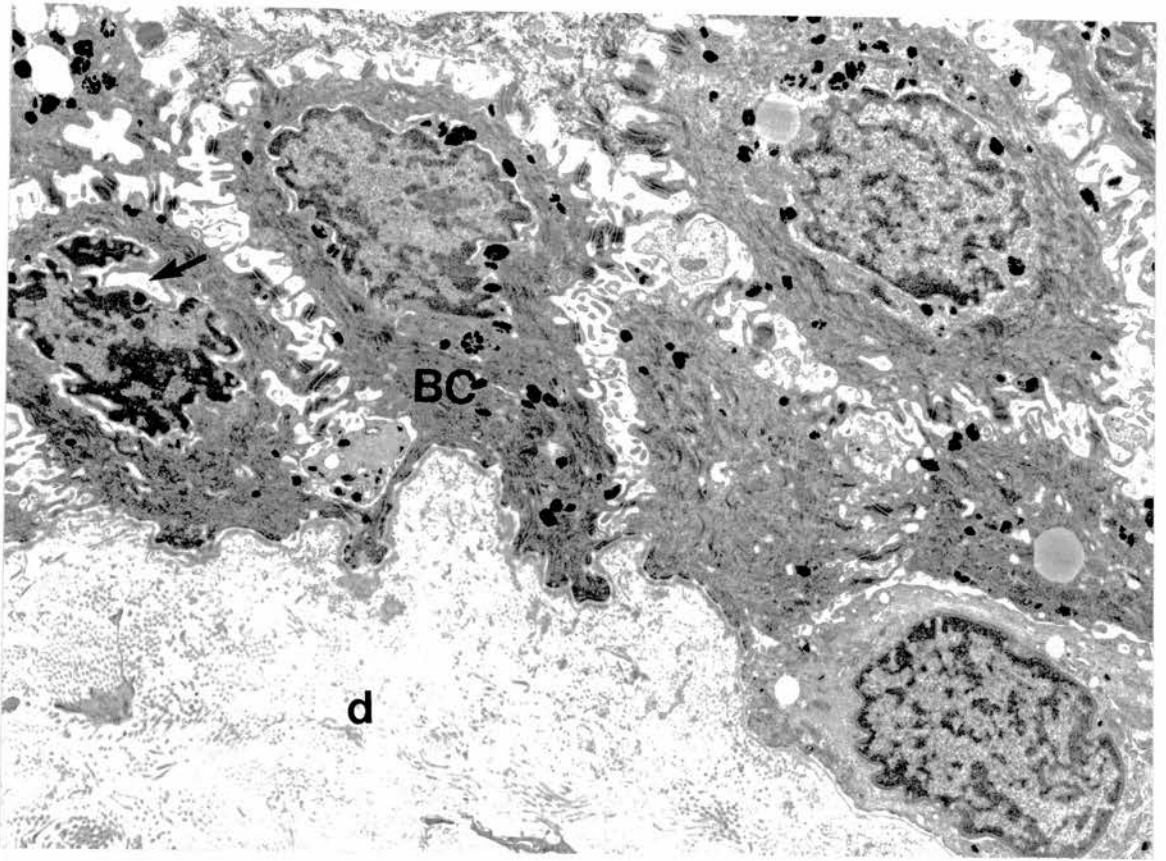
- 1) The dermis of two adult patients, EB17 and 21, contained filamentous-amorphous bodies (Figs. 38, 39) up to 5.3 μm in diameter. Both were of fibrillar appearance but in Fig. 39 the body had a densely-stained centre. The body itself was surrounded by large collagen fibres and above it there has been some disruption of the dermis; fine filamentous material was visible, as well as some darkly-stained granular material. Normal cross-banded anchoring fibrils were seen attached to the basal lamina directly above the fibrillar body.

A dermal abnormality was also noted in the biopsy from EB16. Fig. 40 illustrates this and shows condensed material within the dermis, and fine amorphous material surrounding intact collagen fibrils.

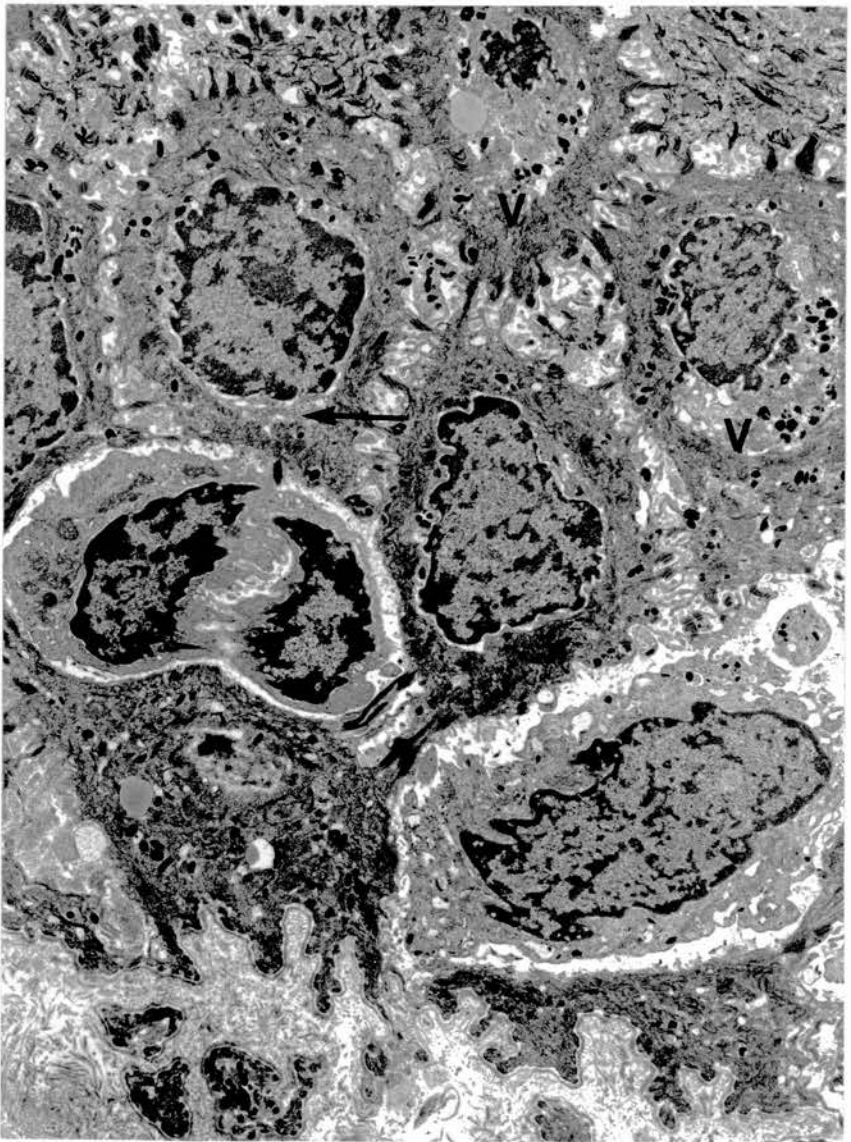
- 2) In three patients, EB16, 17 and 21, there was splitting or duplication of the basal lamina (Fig. 41). Unlike the duplication often seen beneath melanocytes or dividing basal cells, the basal lamina was of irregular thickness, becoming diffuse in areas and could be seen "trailing" down into the dermis, particularly in Fig. 42. This may be related to the observation in Figs. 33 and 36 of some basal lamina apparently deep within the dermis of a blister floor.
- 3) Yet another abnormality of the basal lamina could be seen in Fig. 43. The basal lamina, of irregular thickness and definition, was interrupted by herniations of the overlying basal cells. Otherwise normal hemidesmosomes, anchoring filaments and fibrils were present. The dermis again contained ill-defined amorphous

material surrounding apparently normal collagen. A large herniation, almost isolated in the dermis, with some disruption of the adjacent tissue is shown in Fig. 44.

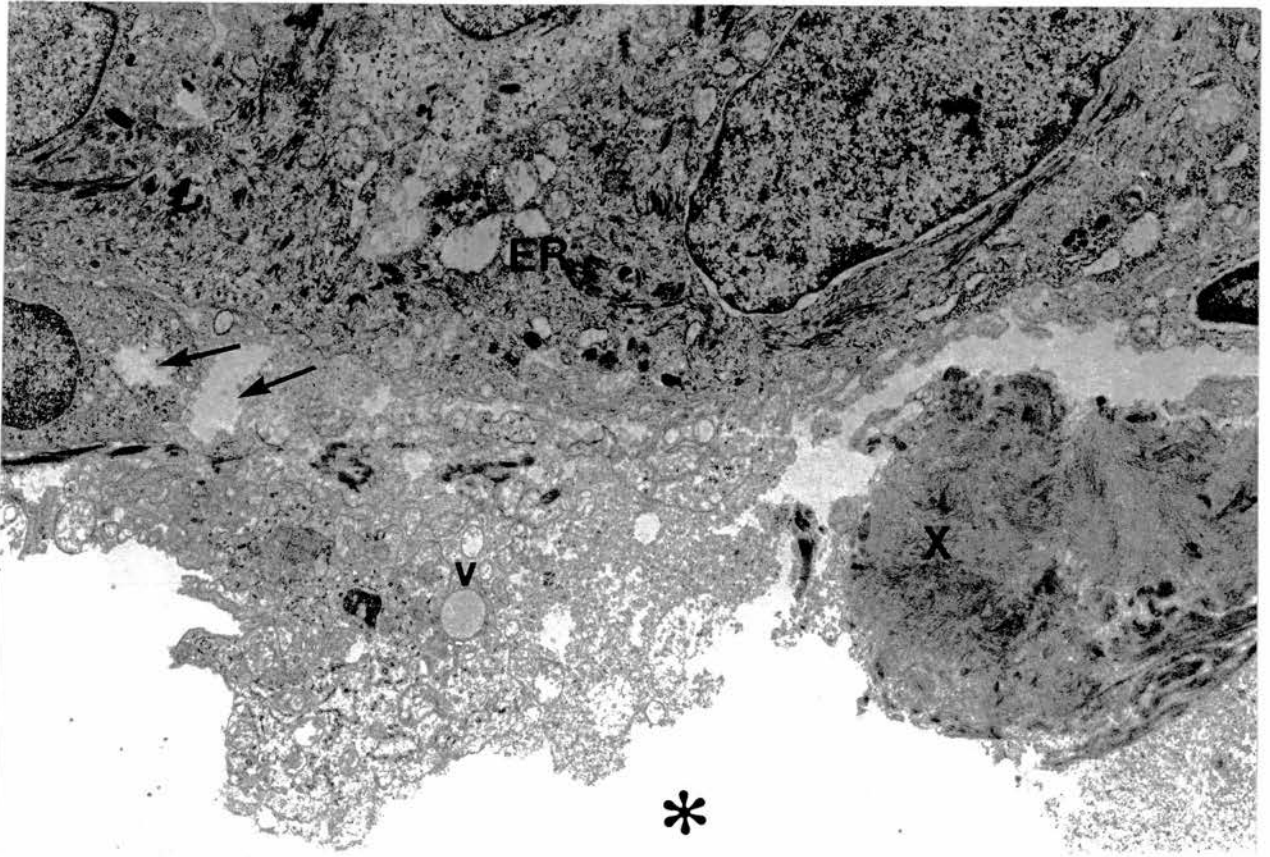
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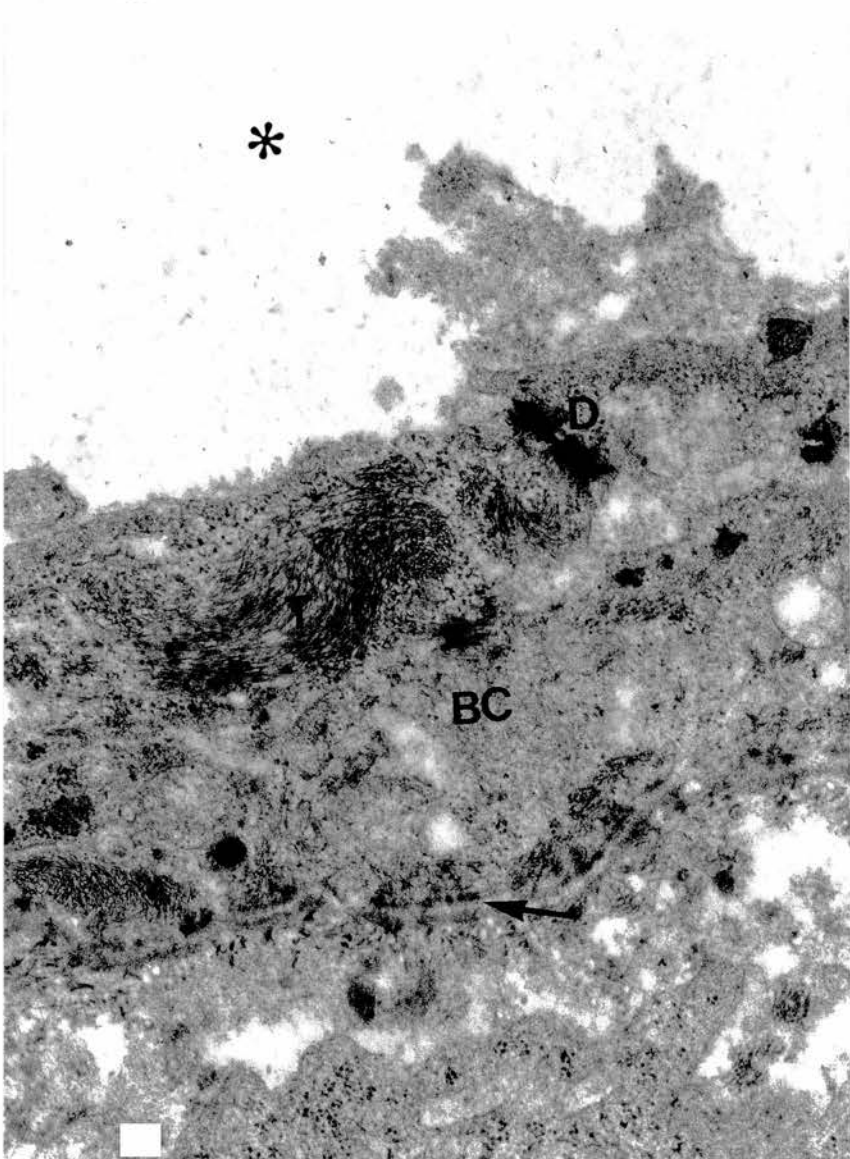
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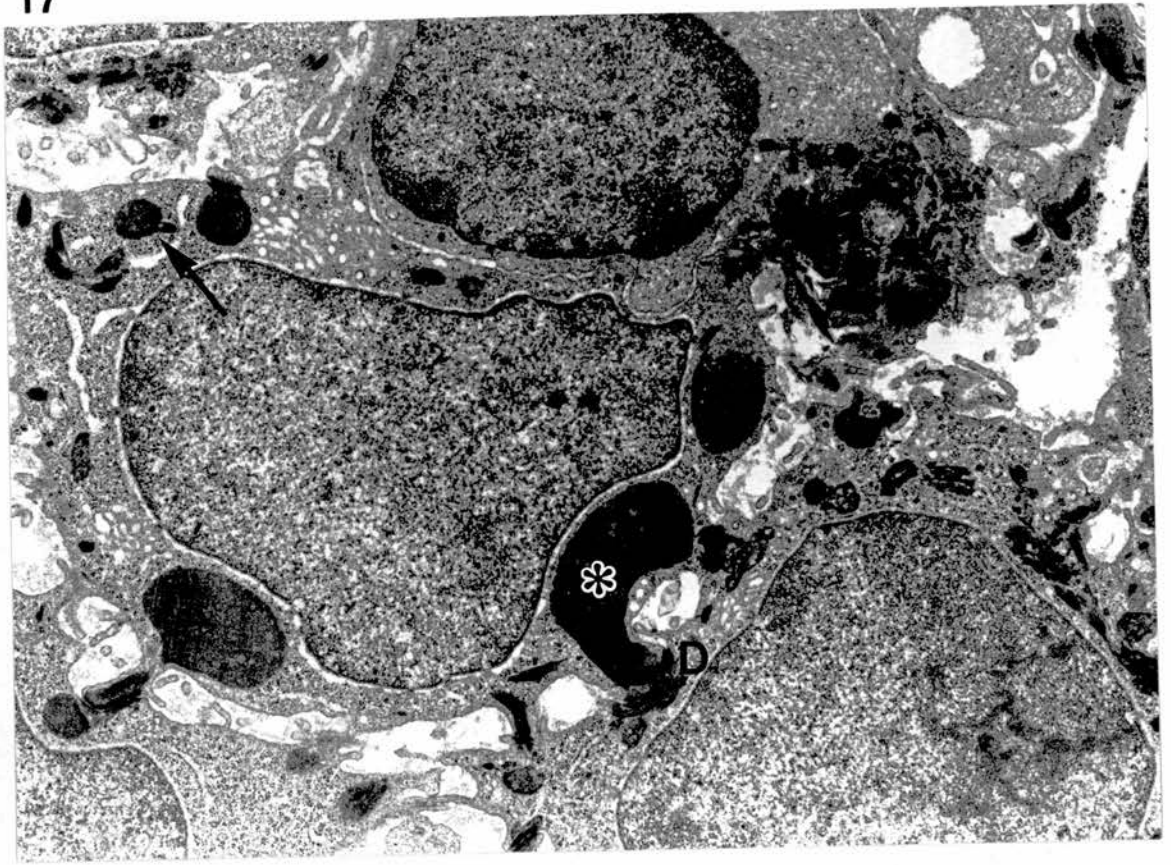
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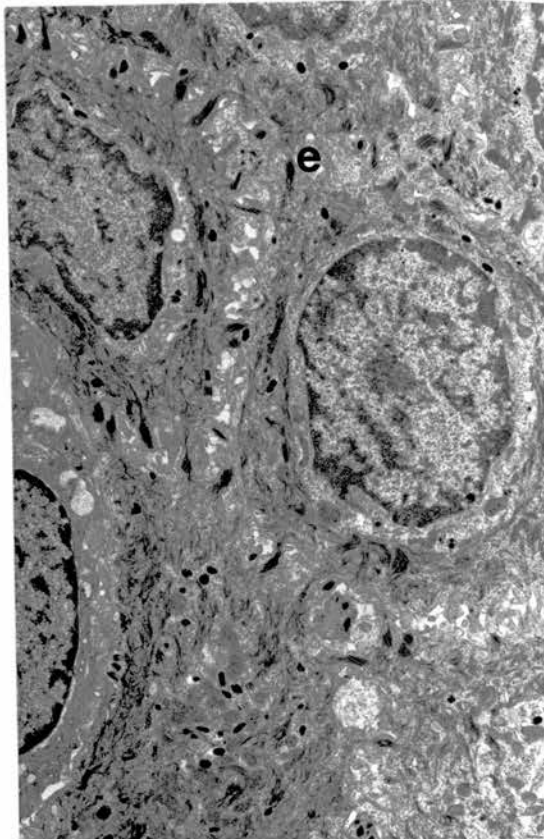
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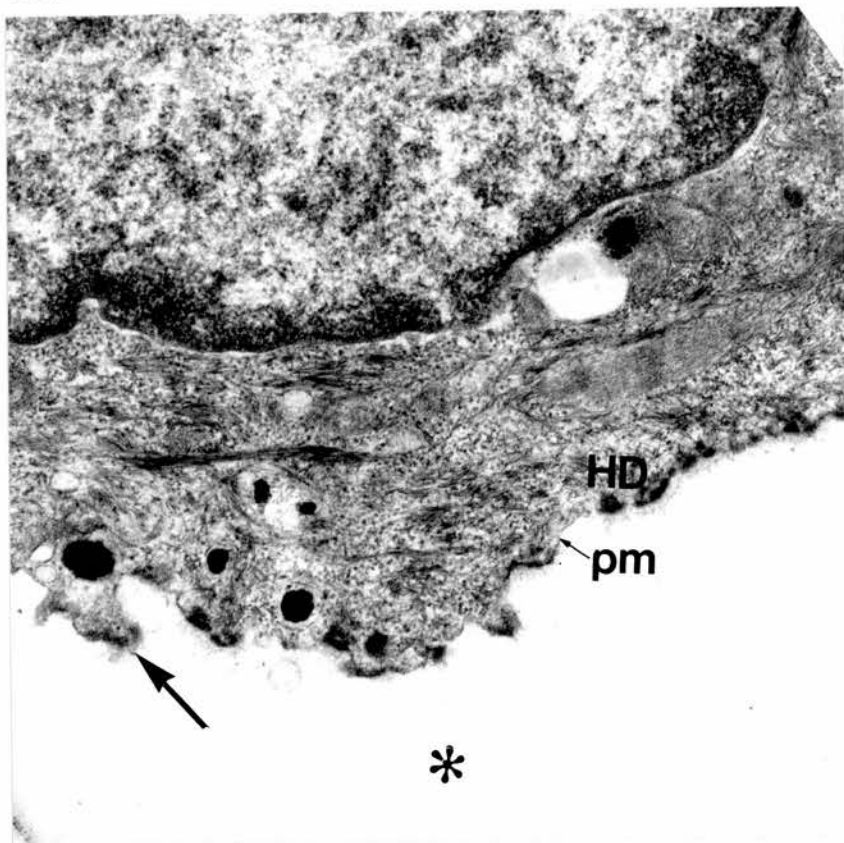
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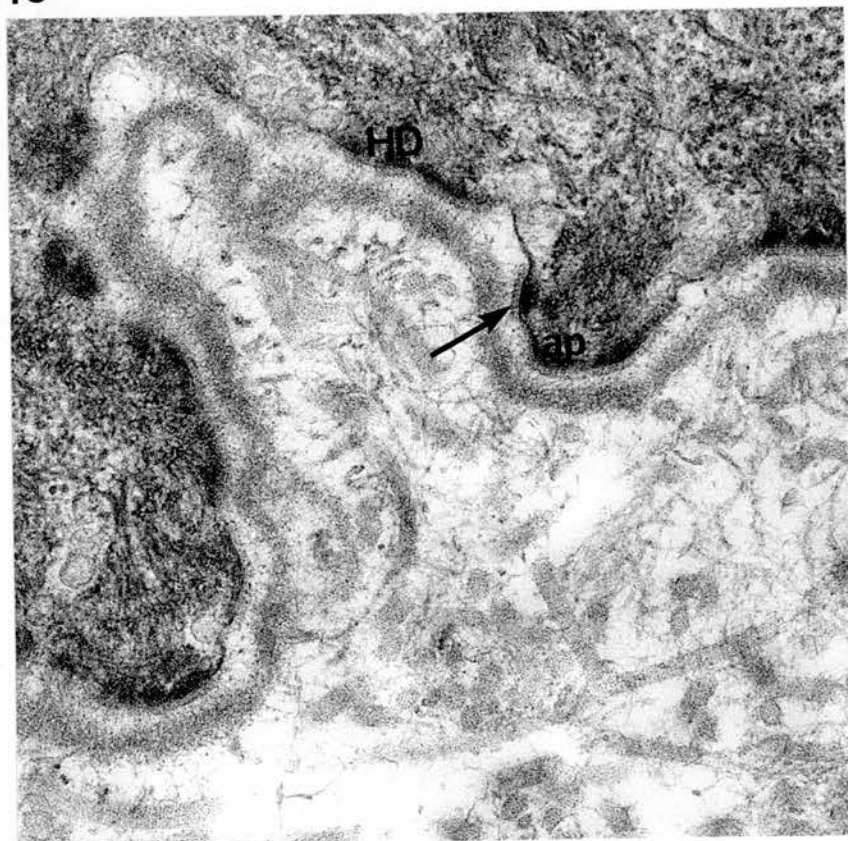
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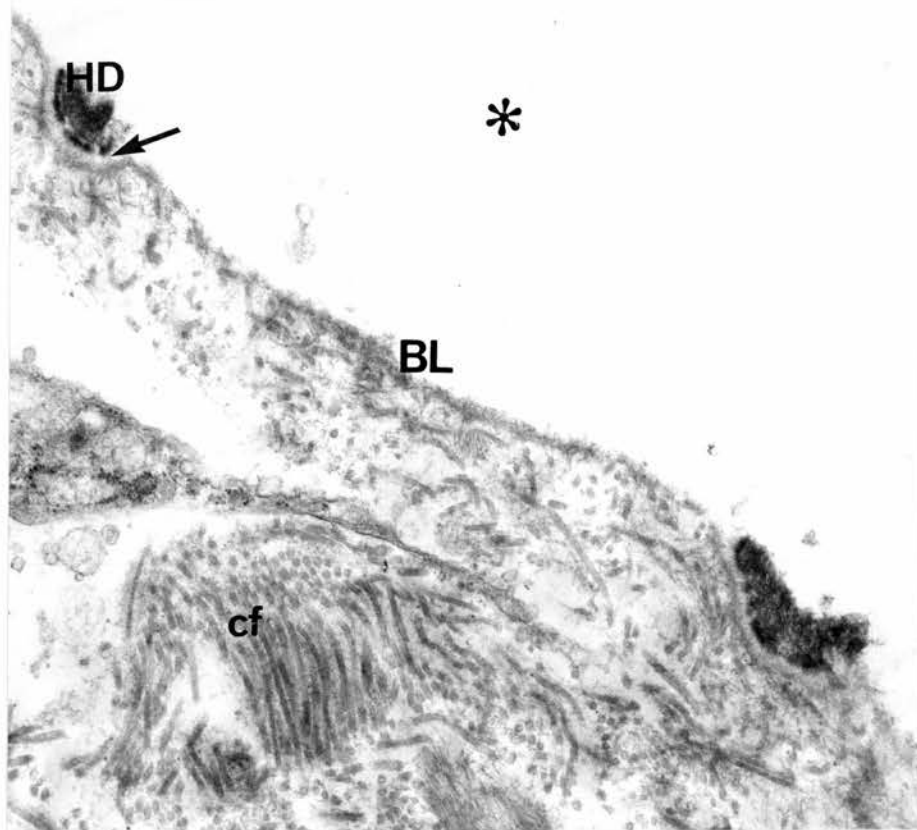
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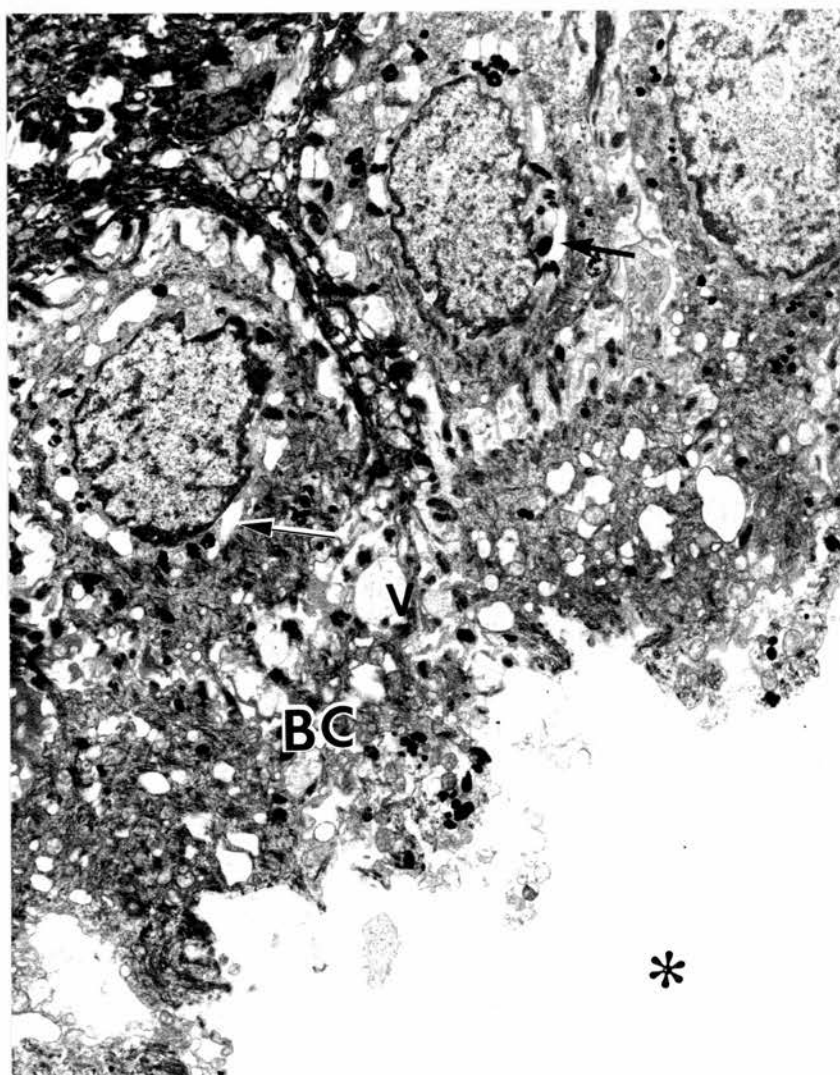
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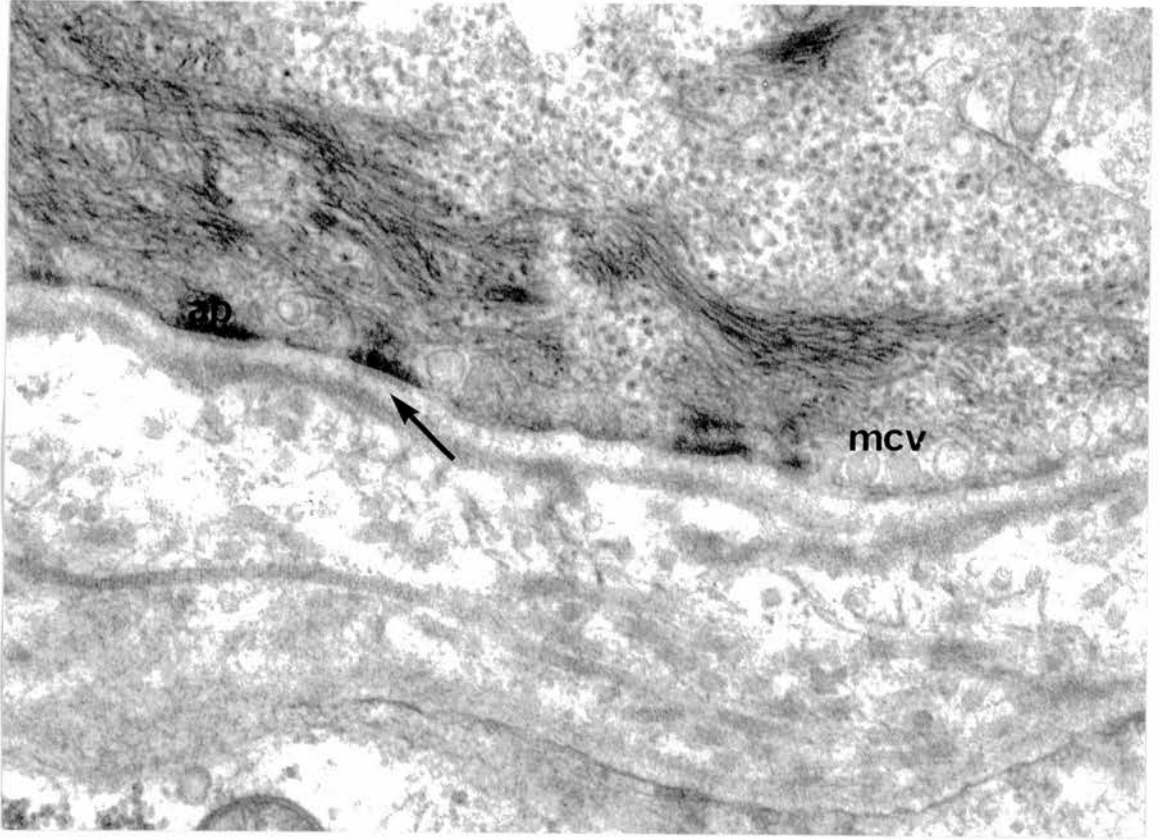
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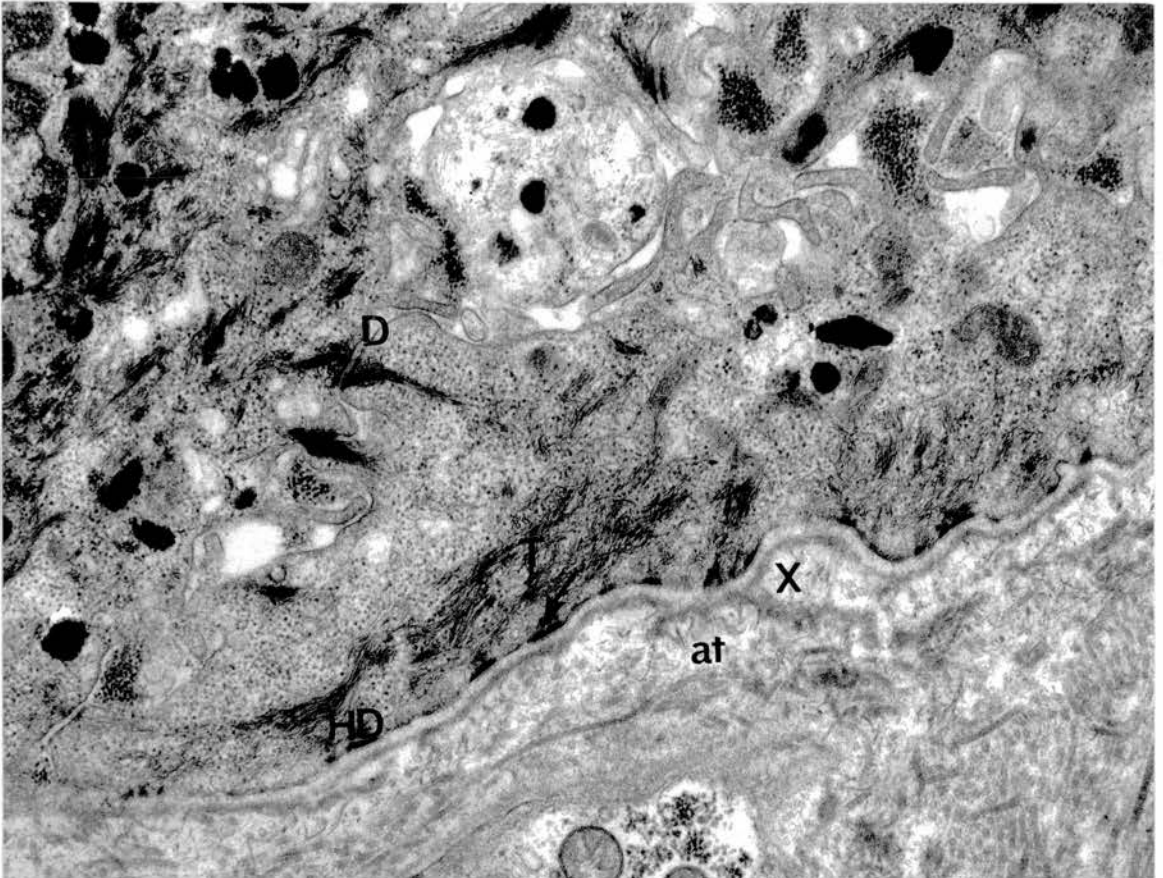
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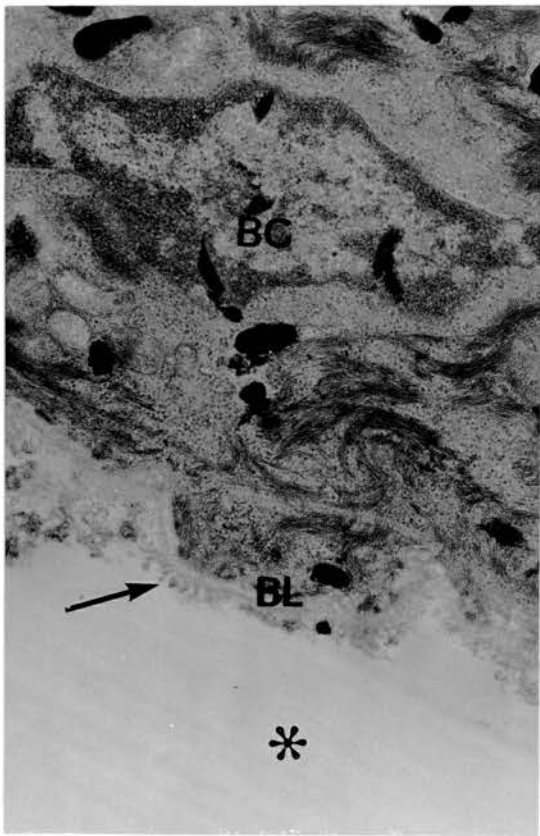
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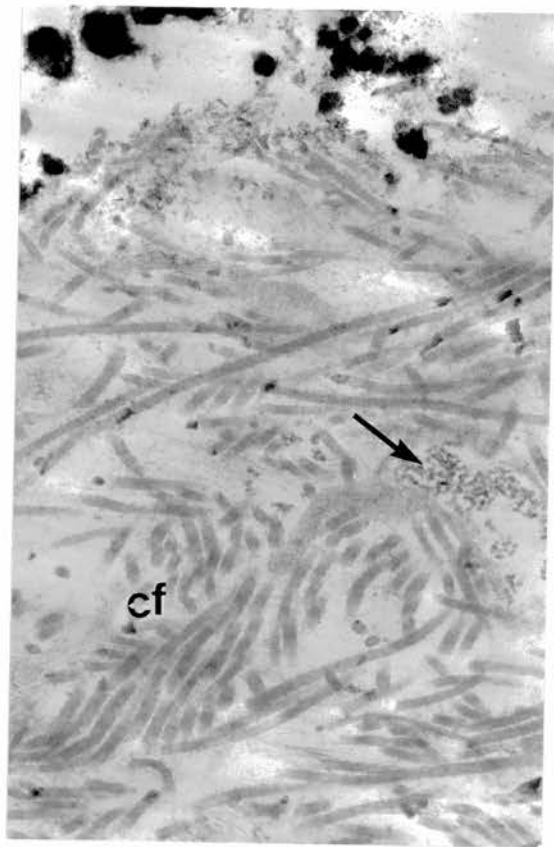
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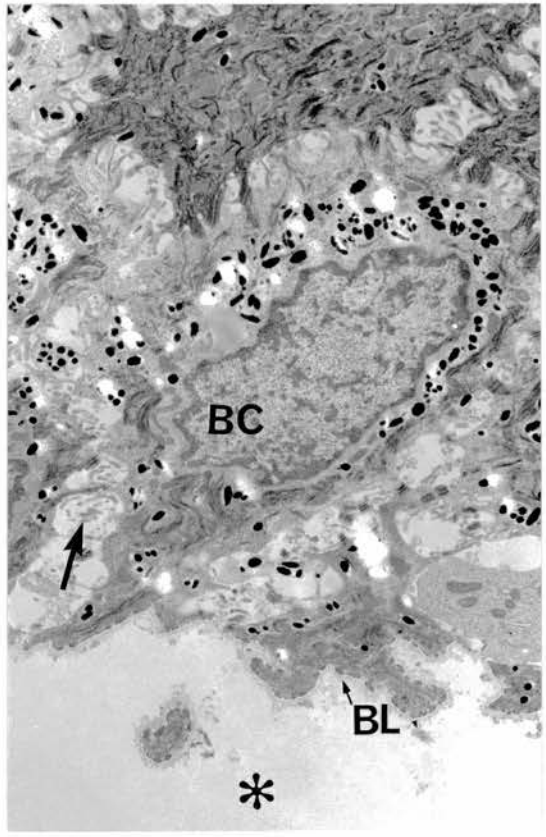
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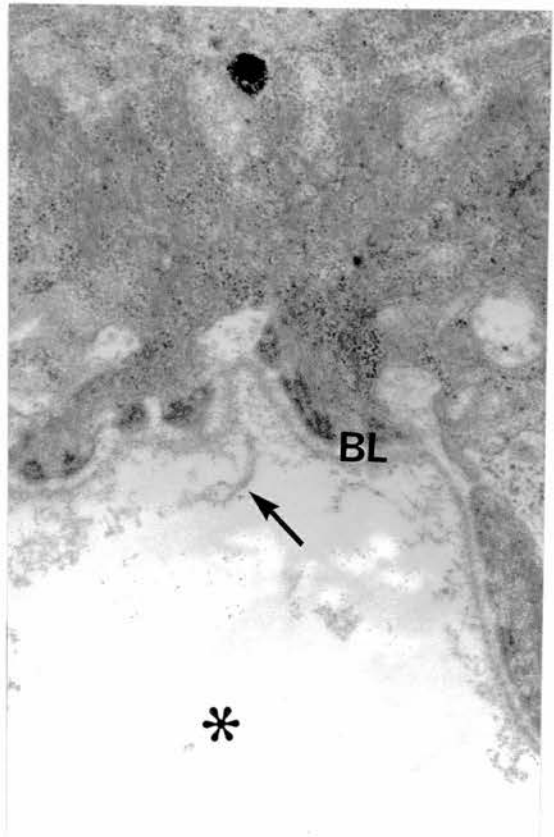
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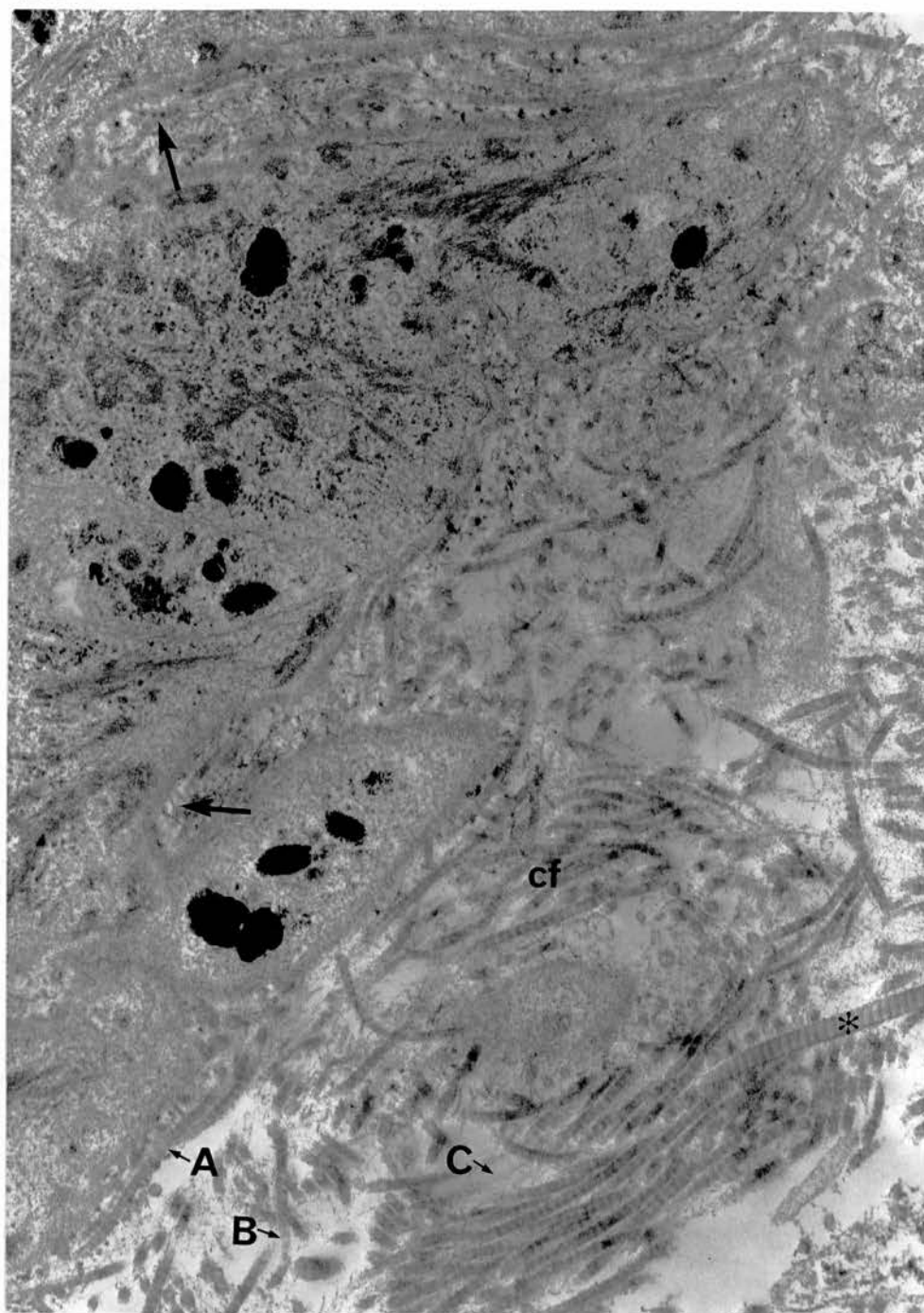


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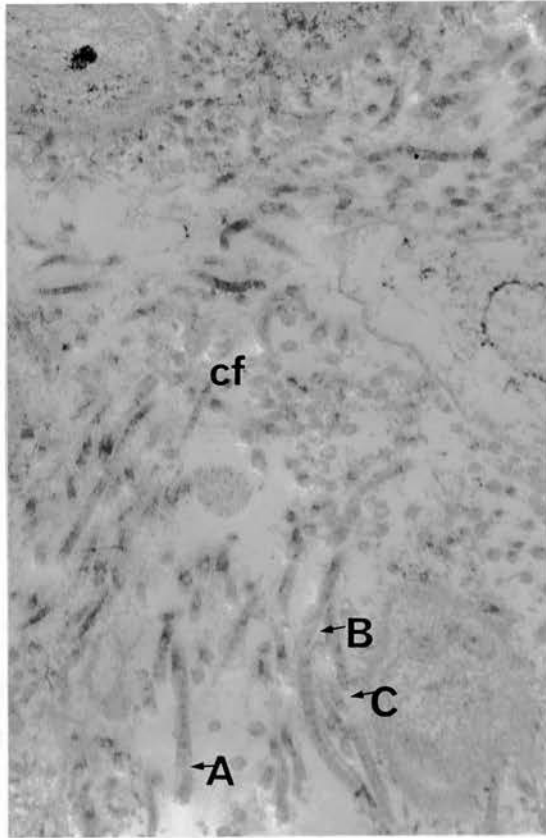


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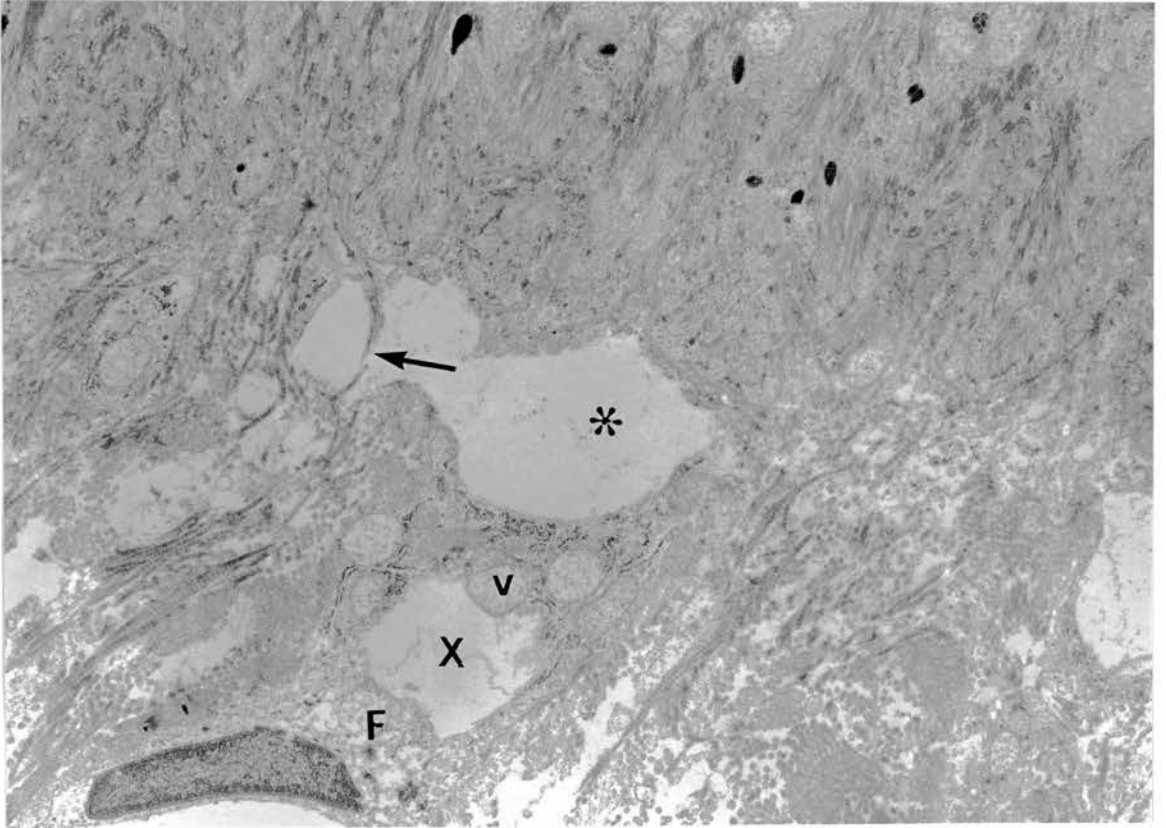




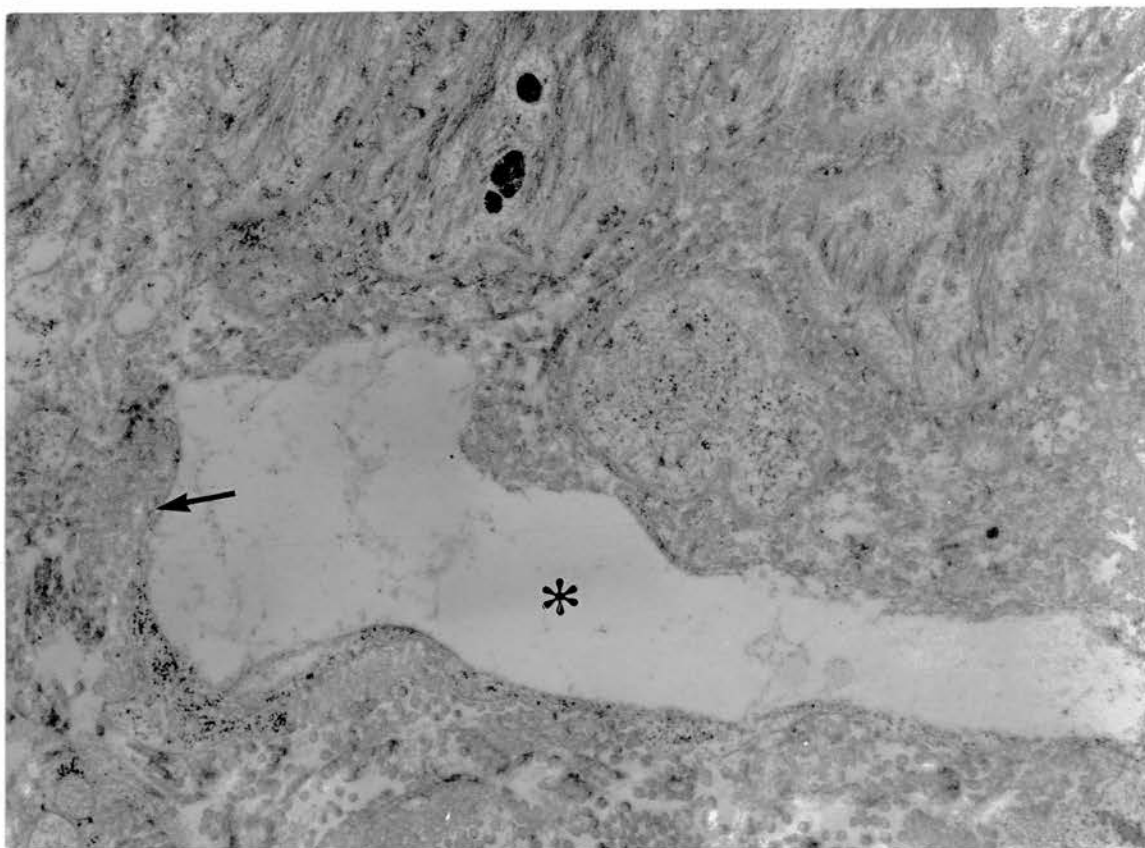
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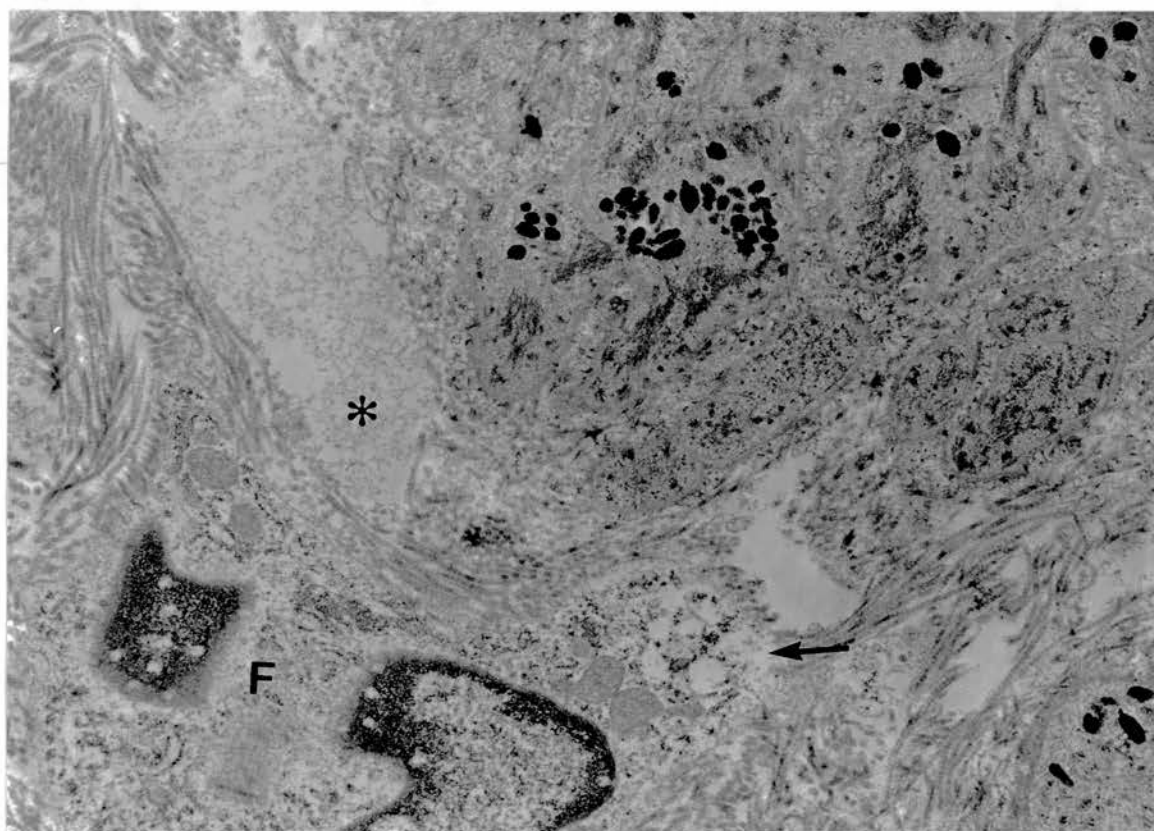
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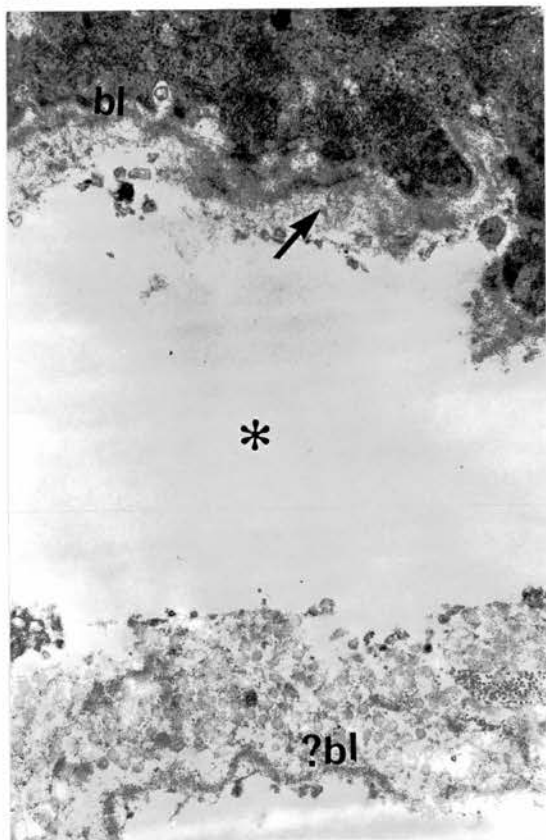
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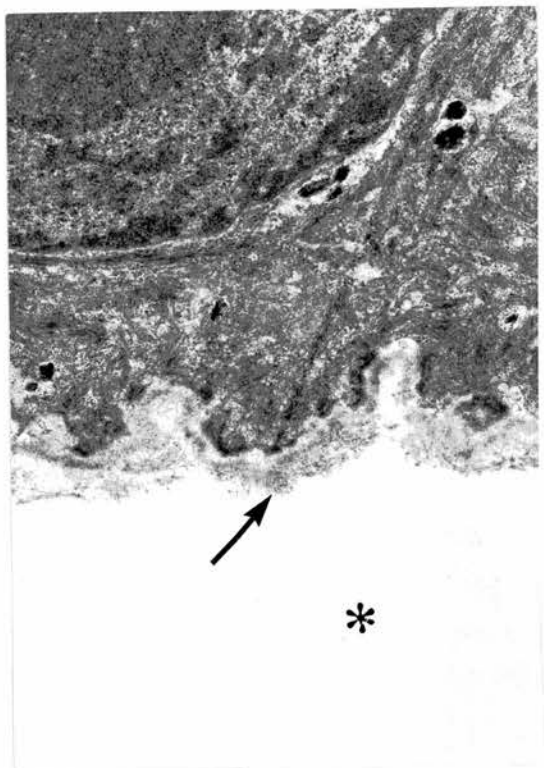
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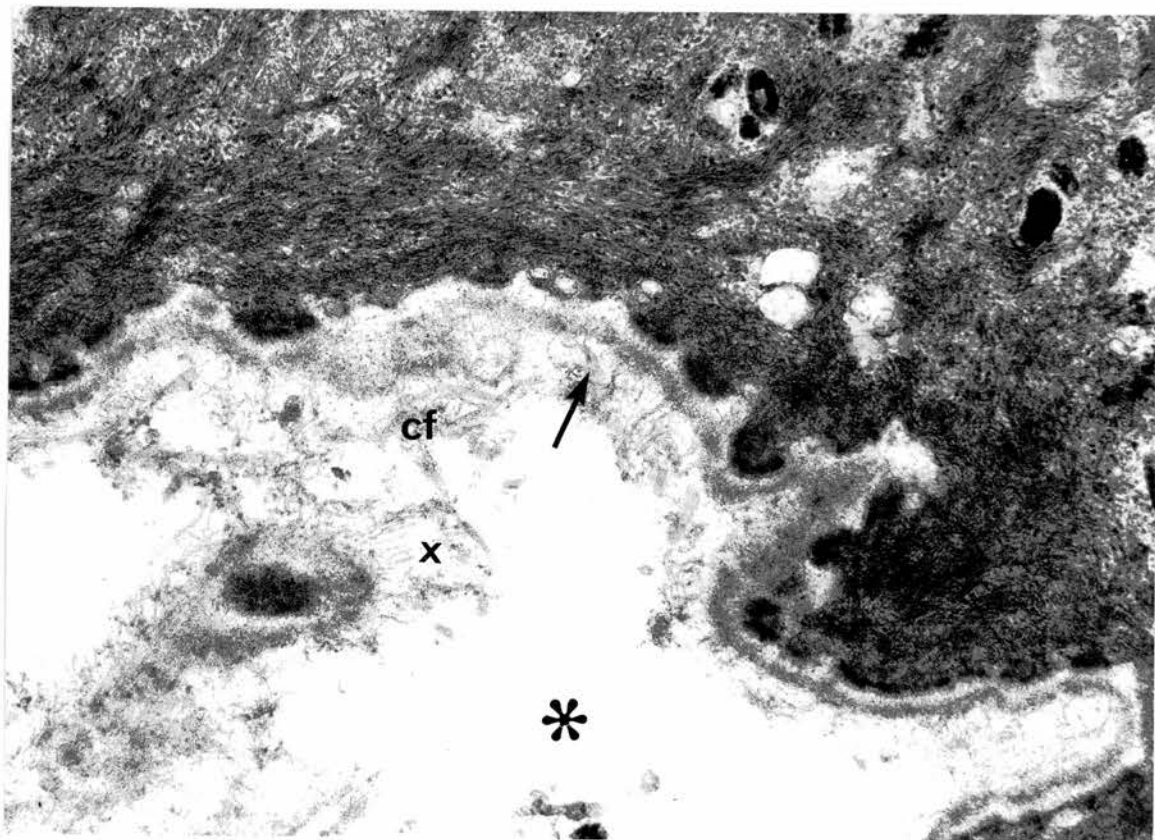
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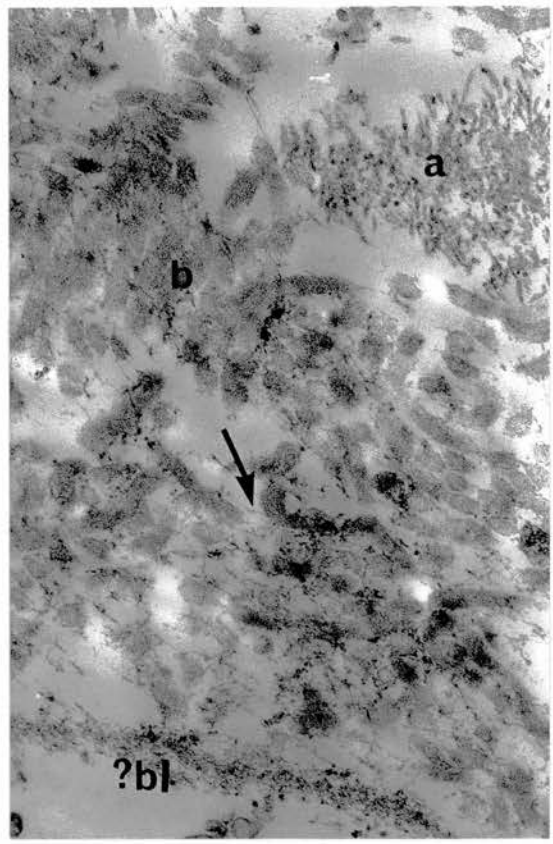
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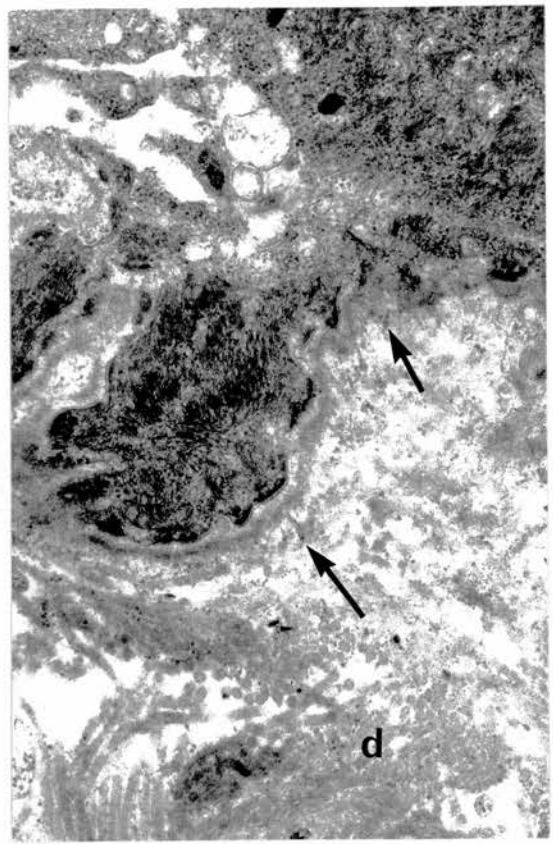
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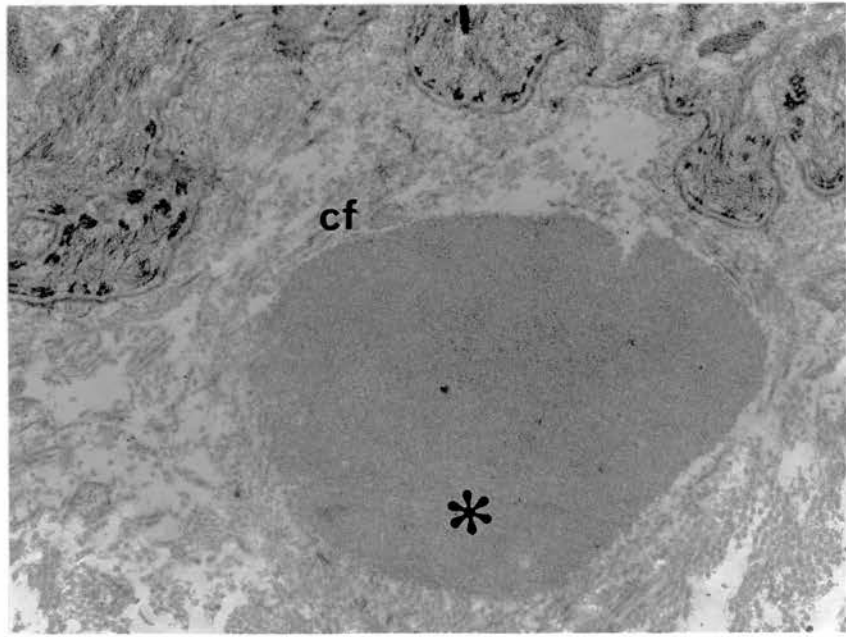
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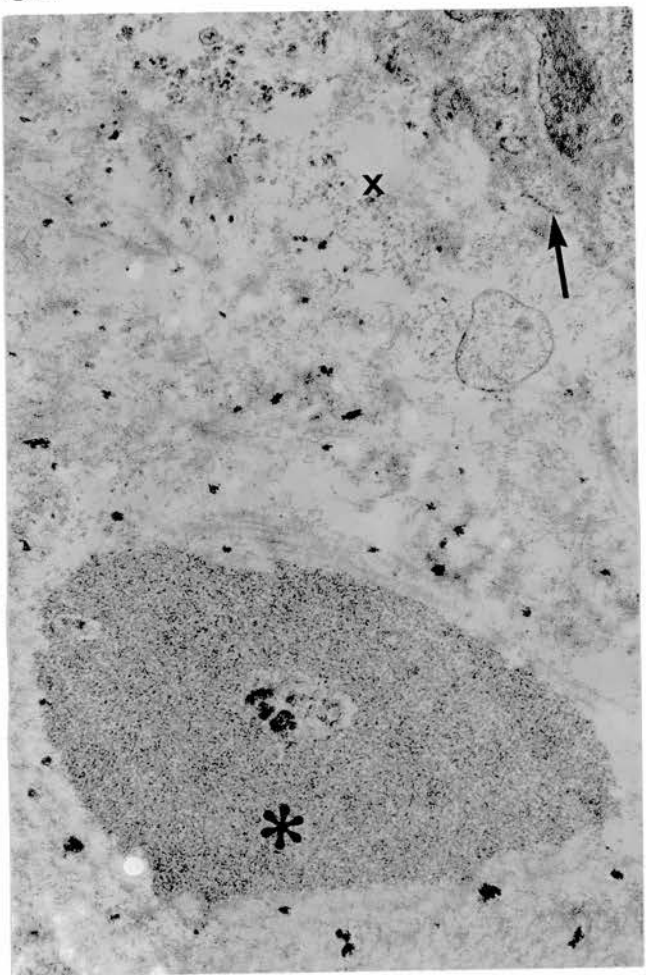
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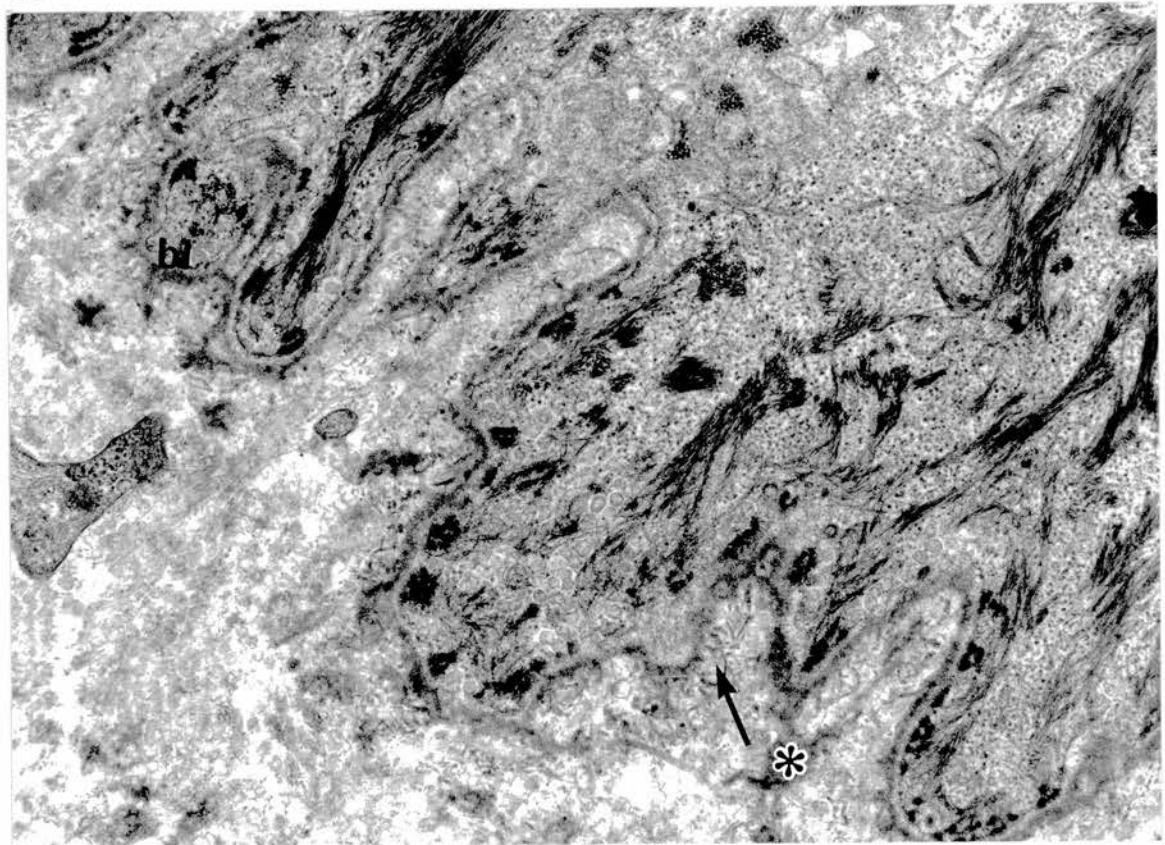
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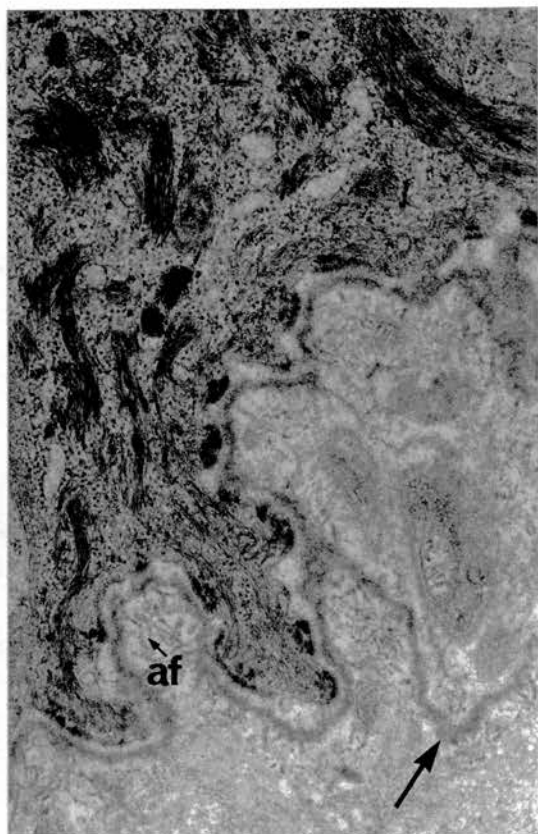
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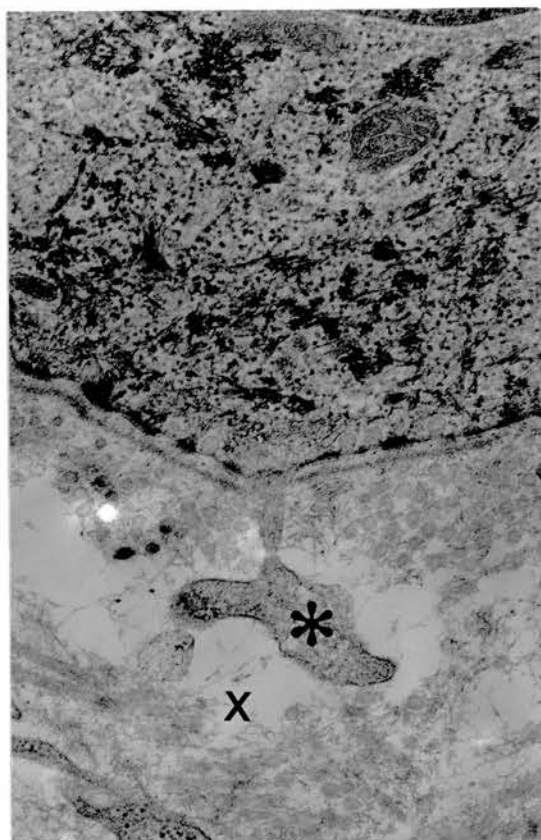
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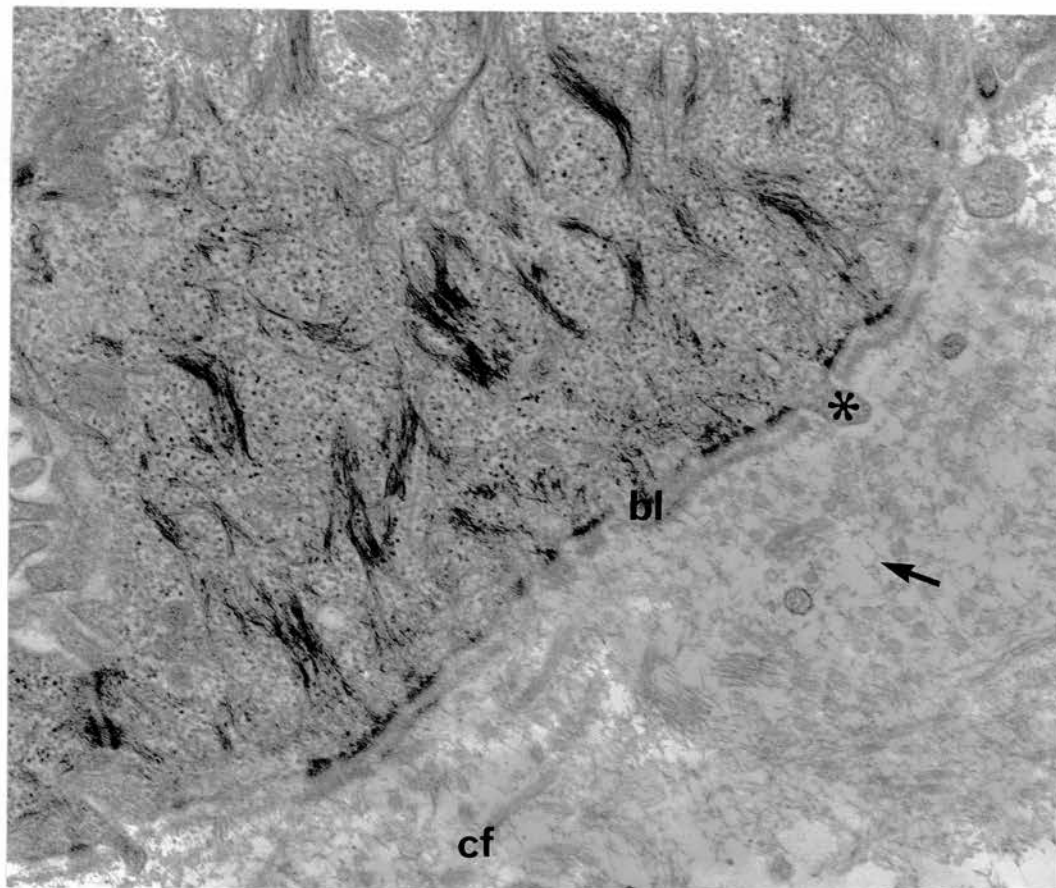
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DISCUSSION

EB Simplex

Ultrastructural examination of the skin from non-involved sites of patients with EBS has only limited diagnostic value: clinical assessment alone should be sufficient to distinguish the localised Weber-Cockayne variety. However, in the more severe forms of EBS where extensive blistering occurs, such as in EBS generalisata: Koebner (Baker, 1982); or the recently recognised EB herpetiformis Dowling-Meara (Gedde-Dahl, 1981; Niemi et al, 1983), electron microscopy is useful in confirming the diagnosis. The EB herpetiformis Dowling-Meara variant can only be distinguished from other EBS variants by examining the ultrastructure for the characteristic electron-dense globules located in the upper epidermis.

Previous studies of Weber-Cockayne mild EBS involved the production of fresh blisters by heating followed by friction (Haneke and Anton-Lamprecht, 1982) or by friction alone (Pearson, 1967). Interestingly, the plane of cleavage in the experimentally induced blisters was not the same as that of spontaneously developed blisters.

In the initial phase of blister formation (Haneke and Anton-Lamprecht, 1982) reported oedema of the subnuclear cytoplasm to be one of the characteristics; with "dilution" of the ribosome-rich cytoplasm, in which small holes appeared and gradually enlarged. Pearson (1967) described a similar change involving perinuclear swelling, distortion of the organelles in that region and partial loss of cytoplasmic constituents. Perinuclear oedema and cytoplasmic vacuolation was clearly seen in the present study (Figs. 13, 14). Arguably this could be an artefact of fixation; however this is unlikely as cells in a different area but from the same section

appeared normal (Fig. 58).

In this study, no changes in the tonofilaments were seen in the mild EBS Weber-Cockayne skin, in contrast to Haneke and Anton-Lamprecht (1982), who suggested that the tonofilaments were slightly more condensed than normal in an induced blister from the skin of mild EBS. As friction and heat can produce such changes in the tonofilaments (Pearson, 1965; Hunter et al, 1974), the importance of condensed tonofilaments in EBS is questionable.

The discrepancy between the level of cleavage in spontaneous and induced blisters is interesting. Haneke and Anton-Lamprecht (1982) found the plane of cleavage of induced blisters to be through the basal cells, whilst spontaneous blisters occurred at or between the horny and granular layer: Hintner et al (1981), using immunofluorescent staining of antigenic determinants to components of the epidermis showed the supra-basal split to be between the stratum corneum and stratum granulosum in EBS. It seems that unless experimentally induced, presumably thereby exploiting a pathogenetic defect, blistering in mild EBS occurs at a higher level than the basal cell layer. In other forms of EBS the defect in the basal cells may be more severe, consequently blister formation occurs at this level following trauma (Baker, 1982). A clue to the nature of the pathogenetic defect may lie with the observations of Niemi and Kanerva (1981): the distension of the rough endoplasmic reticulum and enlargement of the mitochondria could indicate the over-production of protein, perhaps a protease, resulting in the abnormalities associated with EBS.

Niemi et al (1983) described the abnormal tonofilament formation of irregular electron-dense globules, now recognised as the primary

diagnostic feature of EBS herpetiformis Dowling-Meara (Fig. 17). The development of these abnormal structures is unlikely to be an artefact as some relatively normal desmosomes can be seen in surrounding cells. Such clumping of tonofilaments has also been noted in epidermolytic hyperkeratosis (bullous ichthyosiform erythroderma) (Niemi et al, 1983). The relevance of this feature to the intra-basal cell cleavage seen in this variant remains a mystery.

Junctional EB - letalis

Five types of junctional EB have been recorded (Gedde-Dahl, 1981); the two cases examined here, (siblings), fit the group described by Herlitz (1955), i.e. EB hereditaria letalis.

Pearson (1962, 1967) was amongst the first to establish by electron microscopy that the blister occurred between the plasma membrane and the basal lamina, and that consequently this group was a separate entity from both the simplex and dystrophic groups. Recently Hashimoto et al (1976a) and Anton-Lamprecht and Schnyder (1979) have conducted more detailed electron microphotography studies and conclude that abnormalities of the hemidesmosomes are the primary causal defect in this variety of EB. The evidence from this current study supports an abnormality of the hemidesmosomes but indicates that this may not be the primary defect.

The presence of abnormal hemidesmosomes, having a rudimentary attachment plaque in the plasma membrane and lacking the sub-basal dense plaque, agrees with the findings of Rodeck et al (1980). This could indicate an abnormality in the mode of attachment of the anchoring filament to the hemidesmosome; representing a weak link in the attachment of epidermis to dermis. However it is uncertain whether the lack of a substantial sub-basal plaque affects attachment of

filament to hemidesmosome, as some amorphous material, which may include anchoring filaments, remains attached to the hemidesmosomes following cleavage. This was also noted by Pearson et al (1974).

Furthermore the "torn off" phenomenon described by Hashimoto et al (1976a) and illustrated by Fig. 20, suggests either that not all hemidesmosomes have the same inherent weakness; or that the abnormality in structure is not as crucial as has been supposed. The lack of a sub-basal plaque at these sites, has not apparently weakened the bonding of plasma membrane to basal lamina. Disruption of the plasma membrane itself has had to occur before dermo-epidermal separation could take place. The significance of abnormal hemidesmosomes is still further questioned by the finding that junctional cleavage can occur where hemidesmosomes are structurally and numerically normal (Tidman and Eady, 1983).

The significance of the microcytotic vesicles is uncertain. They are related to the lysosomal system: possibly having the potential to release lysosomal enzymes, capable of degrading collagen, proteoglycans and protein (Lazarus et al, 1975; Etherington, 1977).

Further evidence that the causal abnormality may be within the basal cell itself comes from the presence of numerous vacuoles and disruption of the cytoplasmic inclusions (Fig. 21) in areas of separation. This is unlikely to have been fixation artefact in material that was otherwise well fixed, not all cells had marked perinuclear oedema, or abnormal cytoplasm: Pearson (1962) mentions vacuoles present in basal cells of intact skin. Moreover he mentions that electron microscopic observations were "impaired by the lack of good cellular detail"; with the significant addendum that he did not

think poor fixation and processing to be responsible as other material processed simultaneously did not show such abnormalities. Hashimoto et al (1976a) also referred to such basal cell abnormalities but regarded them as secondary.

It is therefore conceivable that the causal defect lies within the basal cell itself, perhaps over production of a protease, which consequently could cause changes in the structural integrity of the hemidesmosomes and/or disruption of the underlying anchoring filaments. Only the slightest trauma would then be necessary to cause blistering. Obviously further work on enzyme production, or control, in the basal cells of patients suffering from junctional EB is necessary to confirm whether the causal factor is simply hemidesmosome abnormality, or whether lysosomal enzymes are involved. If an abnormality of the hemidesmosomes is the primary defect then some abnormalities in desmosome formation might be expected elsewhere in the basal cell layer: this is not apparent in this study. Bauer and Eisen (1978) found increased levels of collagenase activity in vivo but not in vitro in junctional EB; this they regarded as a secondary effect due to chronic wound healing. The significance of this finding, along with the possibility of abnormal enzyme production in the epidermis remains to be answered.

EBDr - Hallopeau-Siemens

Until recently there has been some controversy regarding the primary causative factor in the aetiology of EBDr Hallopeau-Siemens.

Pearson (1962) suggested that the marked degeneration of dermal collagen might be due to the presence of abnormal collagen or that a "collagenase-like" substance might be active. This hypothesis was substantiated by the findings of Eisen (1969b) who found collagenase

levels to be increased six fold above the normal. However Briggaman and Wheeler (1975), extending their work on the formation of anchoring fibrils and basal lamina in skin (1971), conducted a series of epidermal-dermal recombination experiments using EBDr and normal skin. By replacing EBDr epidermis on normal dermis and vice versa, they showed that EBDr dermis was not capable of producing anchoring fibrils and consequently concluded that the primary defect lay, not with excess collagenase production, but with impaired formation of anchoring fibrils.

More recently, following extensive ultrastructural studies on EBDr, Hashimoto et al (1976b) concluded that the collagen and anchoring fibril degradation was secondary to the primary defect, probably related to an increase of skin collagenase activity. This would fit in with the findings of Eisen (1969b) and Bauer (1977b) who found high collagenase levels in patients with EBDr.

The ultrastructural findings of this study are similar to those of Pearson (1962) and Hashimoto et al (1976b). Cleavage of the skin occurs in the upper papillary dermis just beneath the basal lamina. The basal lamina though often ill-defined is usually continuous. Its lack of definition in the blister roof may well be due to the trauma of blistering, oedema of the blister cavity placing the roof under stress.

Where blistering has occurred, only amorphous material can be seen attached to the dermal aspect of the basal lamina. Anchoring fibrils were rarely identified in the blister roof of severe cases. This does not however rule out their presence in non-blistered skin of EBDr patients and, indeed, on examination of intact skin from one patient with milder expression, cross-banded and apparently

normal anchoring fibrils could be seen at the apex of tight involutions of the basal lamina. Such regions may be protected by the topography of the skin and therefore less susceptible to attack by collagenolytic enzymes.

The findings of Briggaman and Wheeler (1975) may have been misinterpreted. It seems possible that normal anchoring fibrils do not develop in EBDr dermis because the collagenolytic enzyme content of the dermis does not allow their normal formation rather than because the dermis is incapable of producing the fibrils.

Further evidence for collagenase being the primary causative agent can be found by examination of the collagen fibres of the papillary dermis where they appear to be in various stages of dissolution. Although this might relate to the angle at which the block has been cut, or the orientation and conformation of the collagen fibres themselves, it does seem that in several instances interrupted collagen fibres are connected by a line of amorphous material, indicating that dissolution of the fibres has occurred. Also, in agreement with Pearson (1962), a number of unusually thick collagen fibrils were seen. Phagocytosis of these larger fibrils was not however observed, in contrast to his report. The significance of these larger fibres is uncertain.

The association of degenerative changes in a fibroblast, as seen in Fig. 30, with the apparent beginning of cleavage formation and dissolution of collagen in the surrounding dermis is of particular interest. It could be argued that the degenerative changes in the fibroblast are due to artefacts of fixation. This specimen has indeed suffered from poor fixation, as indicated by the state of the mitochondria in the overlying basal cells of the

epidermis. However it seems unlikely that the complete destruction of a fibroblast should have occurred (Fig. 31) in the same section as another unaffected fibroblast (Fig. 32). Fig. 30 shows the degenerative changes occurring both within and around a fibroblast. Perhaps the dissolution of the surrounding collagen combined with degeneration of dermal fibroblasts, together weaken the upper dermis to such an extent that even the slightest trauma causes cleavage within the skin.

It therefore seems likely that dissolution of the collagen fibrils and anchoring fibrils are the main causative factors in blister formation in EBD. The presence of degenerating fibroblasts in the upper dermis and the disintegration of collagen in the immediate proximity of these cells also indicate that the fibroblasts are the most probable source of active collagenase and other proteases.

The evidence from this study suggests that the dermis of EBD patients can produce anchoring fibrils but that they are probably destroyed on exposure to the active collagenase of the dermis. This is substantiated by the work of Kobayasi et al (1977), who showed that the anchoring fibrils were indeed sensitive to bacterial collagenase.

An abnormality in collagen synthesis cannot be discounted. Larger fibrils are present in the dermis, though this may not be significant in blister formation unless the fibrils themselves are more susceptible to enzyme attack. However there is no evidence to support this view.

EBDD - Cockayne-Touraine

There have been few ultrastructural reports on either the dominant dystrophic Pasini or Cockayne-Touraine types of EB, but

Hashimoto et al (1975, 1976c) studied 4 patients of each type. They thought that the anchoring fibrils were of the utmost importance in the Pasini type, being rudimentary in areas of non-predilection as well as at sites of blister formation. Furthermore, they concluded that the mechanism of blistering was the same in both dominant dystrophic types, having found that anchoring fibrils were markedly reduced in predilected sites, but normal or only mildly reduced in non-predilected sites of the Cockayne-Touraine type. Although the assumption that more numerous rudimentary anchoring fibrils would reflect increased severity of clinical expression seems logical, this was not found. The significance of anchoring fibril numbers for the integrity of skin is further questioned by the work of Tidman and his colleagues (1983). Their morphometric studies on anchoring fibrils show that, although there is no difference between the sexes, there are regional differences over the body - fewer anchoring fibrils being found in the arm compared with the thigh or lower leg.

The present study has involved only the Cockayne-Touraine type of dominant dystrophic EB, none of the patients having the allopapuloid papules on their trunk - which distinguish the Pasini type. The findings of this study differ from those of Hashimoto et al (1976c) with regard to the relevance of the anchoring fibrils in blistering. Normal anchoring fibrils can be clearly seen in the roof of a blister (Fig. 35), as well as longer thinner fibrils (Fig. 33). This is in contrast to Hashimoto et al (1976c) who could not demonstrate anchoring fibrils in blistered areas of skin. Furthermore the presence of large cross-banded collagen fibres in the blister roof (Fig. 35) indicates that cleavage can occur at a lower level than the dermal anchoring site of the fibrils. This is substantiated

by the varying amounts of amorphous material located in the blister roof (Fig. 34), indicating that cleavage is not simply related to the defective structure of the anchoring fibrils. However it seems that the anchoring fibril attachment site is disrupted in some instances, as indicated by the fan-like filamentous ends of the fibrils being free in the blister roof.

Other abnormalities of the patients in this study, include the presence of quantities of filamentous-amorphous material surrounding the dermal collagen in all of the patients; the presence of large fibrillar bodies in two of the adult patients, and abnormalities of the basal lamina in three of the patients.

The presence of filamentous material and amorphous bodies are noteworthy. Fibrillar bodies were noticed in the dermis of the Pasini type patients of Hashimoto et al (1975). He concluded that these bodies were related to the basal lamina; suggesting that they might be derived from the herniated portions of the basal cells, with subsequent reparation of the basal lamina resulting in duplication. However in our patients exhibiting such bodies there was no evidence of basal lamina herniation; although in one patient, EB25, herniation and protrusions of the basal cells into the dermis were evident, but no large fibrillar bodies were seen. Such findings suggest that the Cockayne-Touraine variety of EB has a similar pathogenetic origin to the Pasini variety, differing only in its milder clinical expression, and absence of papules on the trunk. The lack of papules may represent a less significant disturbance of GAG levels in the skin, the Pasini type having been shown to involve an impaired degradation of GAG in the skin (Sasai et al, 1973; Endo et al, 1974; Bauer et al, 1979). Perhaps the filamentous, amorphous

material seen amongst the collagen fibres of the dermis (Fig. 36) represents a peculiarity in GAG metabolism which may be related to the fibrillar bodies. A disturbance in GAG metabolism could affect the integrity of the dermis by altering collagen-GAG interaction, with consequent aberrant fibril formation (Mathews, 1965; Mathews and Decker, 1968). The attachment of the anchoring fibrils to such abnormal collagen fibres might then be destabilised, allowing cleavage of the dermis to occur. A similar mechanism may also be at work in the basal lamina, disrupting the stable meshwork of the Type IV collagen, thus enabling herniations to occur, as well as the splitting and duplication of the basal lamina seen in Figs. 40, 41.

The cause of blister formation in dominant dystrophic patients therefore seems to be far more complex than simply the anchoring fibril defects and reduction in their numbers, suggested by Hashimoto et al (1976c); if, indeed, they are involved in blister formation at all. The cause may be in the over production and reduced degradation of GAGs, which interact to cause aberrant collagen production, whether it be the collagenous component of the anchoring fibrils, the basal lamina itself, or the Type I and III collagens of the dermis.

SUMMARY

Electron microscopy was used to examine skin biopsies from patients with EB and was primarily helpful in distinguishing 4 main groups, but also provided evidence to suggest certain pathogenetic mechanisms.

EBS

Diagnosis of the Dowling-Meara Herpetiformis variant can only be made by EM. Intra-epidermal blistering was provoked by the biopsy procedure in the severer EBS Koebner and Dowling-Meara Herpetiformis, but in mild EBS only cytoplasmic dilution, perinuclear oedema and distortion of the organelles in keratinocytes were seen. In mild EBS a defect may arise within the basal cell that is not expressed until temperature, or friction, causes cleavage higher in the epidermis; whilst in severe EBS the defect cannot be adequately compensated for, and blistering consequently occurs in the basal cell layer.

EBL

Although structurally abnormal hemidesmosomes were seen, lacking the sub-basal electron-dense plaque, it was suggested that this is not the primary defect because a) anchoring filaments are still attached to hemidesmosomes in the roof of the blister and b) the "torn off" phenomenon illustrates that hemidesmosome attachment is sufficiently secure to withstand the trauma of cleavage. The significance of microcytotic vesicles is questioned, particularly if the primary defect lies within the basal cell itself, possibly an abnormality in metabolism of a protease(s), eventually disrupting components of the lamina lucida.

EBDr

The suggestion that absence of anchoring fibrils might be the primary defect in EBDr is questioned. Anchoring fibrils are present in mild EBDr and their frequent absence in blister roofs may be due to their destruction, perhaps because of elevated collagenase and/or other protease levels. There is some evidence of collagen dissolution and fibroblast degeneration in mild EBDr where cleavage has not yet occurred. It is proposed that increased collagenase activity, and/or other proteases, is responsible for the apparent lack of anchoring fibrils, disruption of the dermis and blister formation.

EBDD

A similar pathogenesis is considered for Cockayne-Touraine and Pasini EBDD, comparing these findings to the published reports on the Pasini variant. Whether the anchoring fibrils are the primary causal defect is questionable as they are present in intact, and in the roof of blistered skin. An abnormality of the basal lamina is suspected, being malformed and discontinuous in places enabling herniations of the basal keratinocyte into the dermis to occur. Fibrillar bodies are present in two of the patients studied. The amount of amorphous material seen, may relate to a GAG abnormality and may interfere with collagen metabolism - perhaps causing an instability within the dermis, hence blister formation.

CHAPTER 3

COLLAGEN

COLLAGEN

INTRODUCTION

Collagen is a major structural component of the body, being found in skin, bone, dentine and cartilage. It is invariably an extracellular protein, providing a scaffolding, or matrix around which tissues and organs can be organised. The form of the matrix is largely dependent on the type of constituent collagen and its interactions with the other components. There are known to be at least six different types of collagen, all having the same basic structure: three intertwined, helical chains composed of repeating triplet amino acid sequences (reviewed by Woodhead-Galloway, 1980; Weiss and Jayson, 1983).

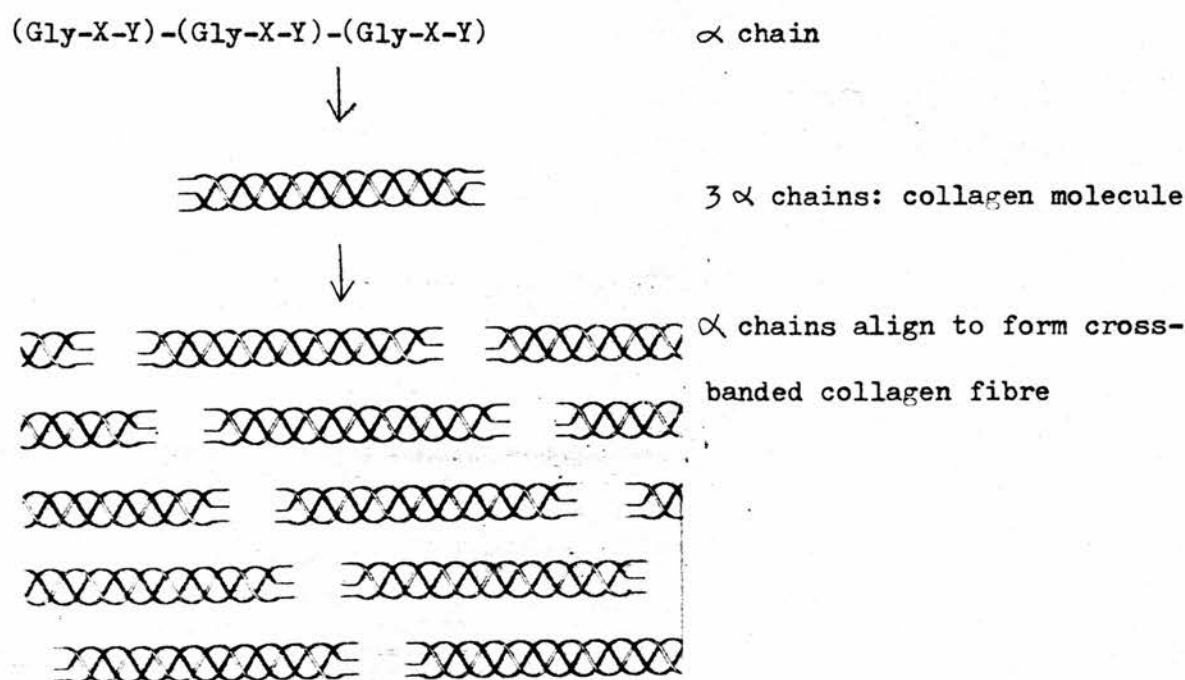
Skin fibroblasts produce fibrillar collagen, which together with glycosaminoglycans and various glycoproteins, notably fibronectin, constitute the dermal matrix. In man 72% of the dermis, by weight, is composed of collagen, which is predominantly of fibrillar collagen Types I (85-90%) and III (10-15%) and is divided into papillary and reticular regions by the orientation and thickness of the collagen fibres. Disruption of the basic scaffolding must necessarily have severe consequences for the structural integrity of the skin, as is the case with recessively inherited dermolytic EB. It is therefore vital to investigate and understand collagen metabolism in the skin of patients with EB, in order to further our knowledge of the disease.

Basic structure of collagen

The basic unit of a collagen fibre is a triple helical molecule composed of three peptide chains, known as alpha (α) chains. Each chain contains numerous repeating sequences in which every third

amino acid is glycine (Gly-X-Y). The "X" position in the triplet is frequently proline, which represents 10% of the total amino acids in collagen. The "Y" position is frequently occupied by hydroxyproline, which is produced by post-translational modification of proline. Similarly lysine and hydroxylysine are commonly found in X - Y positions (reviewed by Prockop, 1980, 1982).

These triplets determine the folding of the chains into a unique triple helical conformation. Proline and hydroxyproline, because of their ring structure give the triple helix its rigidity. Glycine, the smallest amino acid, fits into the restricted space where all three α chains come into contact at the middle of the triple helix, explaining the necessity for the recurring triple amino acid sequence. Where X and Y positions are occupied by amino acids other than proline and hydroxyproline, they provide precisely-positioned reactive groups that determine lateral and longitudinal assembly of the molecules into fibres. Differences between the various classes of collagen are due to subtle differences in amino acid sequence.



Four major classes of collagen are now recognised and 6 unique collagen types are distinguished by differences in α chain composition and conformation of the collagen molecule. Types I, III, IV and to a lesser extent V are constituents of skin. Type I, produced by the fibroblasts of the dermis, comprises 80-90% of the total collagenous protein, Type III 10-15% and Types IV and V constitute less than 5% (Epstein and Munderloh, 1978; Uitto et al, 1980). Type I collagen is located throughout the dermis. Although Type III was once thought to be restricted to the papillary dermis (Fleishmajer et al, 1980), it is now thought to be found throughout the dermis (Epstein and Munderloh, 1978; Fleishmajer et al, 1981). Type IV collagen is confined to the basement membrane of the dermo-epidermal junction, more specifically to the basal lamina (Foidart and Yaar, 1981). Type V is also associated with the basal lamina but may not be an integral component: it is thought to be associated with cell surfaces and is not restricted to the skin. The precise nature and function of this collagen is still largely unknown (reviewed by Sage, 1982).

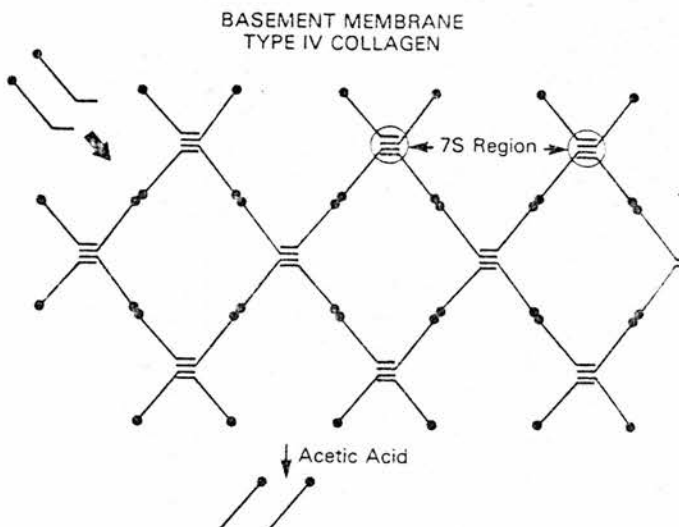
Types I and III are typically fibrillar collagens, being composed of parallel arrays of collagen molecules due to their ability to form and maintain extensive interactions along the length of the triple helix. Type I collagen is a heteropolymer consisting of two identical $\alpha 1(1)$ chains and one genetically distinct $\alpha 2$ chain (Piez et al, 1963). A Type I trimer has also been reported (Uitto, 1979) in which three $\alpha 1$ chains rich in hydroxylysine are folded together.

Type III collagen exists solely as a trimer. Although it was thought that the chains were joined by disulphide bonds, it is now suggested (Burgeson, 1982) that these bonds may be an artefact of purification.

TABLE 5CHAIN COMPOSITION OF VERTEBRATE COLLAGENS (Burgeson 1982)

<u>Class 1</u>	<u>Major structural collagens</u>	
Type I		$[\alpha 1(I)]_2 \alpha 2$
Type I trimer		$[\alpha 1(I)]_3$
II major		$[\alpha 1(IIM)]_3$
II minor		$[\alpha 1(IIm)]_3$
III		$[\alpha 1(III)]_3$
<u>Class 2</u>	<u>Basement membrane collagens</u>	
Type IV		$[\alpha 1(IV)]_2 \alpha 2(IV)$
<u>Class 3</u>	<u>Pericellular collagens</u>	
Type V		$[\alpha 1(V)]_2 \alpha 2(V) "AB_2"$
		$[\alpha 1(V)]_3 [\alpha 3(V)]_3$
		$\alpha 1(V) \alpha 2(V) \alpha 3(V)$
Type VI? (minor cartilage collagens)		E 3
		F 3
<u>Class IV</u>	<u>Collagens with discontinuous triple helix</u>	
EC		EC 3
HMW LMW (Rease and Mayne, 1981)		Disulphide-bonded high
Acidic and basic collagenous fragments		molecular weight aggregates
(Furuto and Miller, 1980)		

Type IV collagen (pages 45-47) differs from the fibrillar types I and III, being more of an amorphous felt-like matrix (Sage, 1982), the basic unit being a heteropolymer (Crouch et al, 1980). Recently, however, tetramers of Type IV collagen have been identified (Duncan et al, 1983). Because glycine is not present in every third triplet sequence of amino acids in Type IV collagen, there is distortion of the triple helical structure producing regions of non-helical form (Bornstein and Sage, 1980). It is currently thought that the Type IV molecules form an interlocking network via the association of the amino helical ends of each of four molecules. This alignment of molecules produces a stable protease-resistant "7S" region, so called because of its sedimentation coefficient (Kuhn et al, 1981). The carboxy-terminal globular domains are free to interact with like regions of adjacent molecules, thus completing the network, as illustrated in the following diagram.



A MODEL FOR THE ARRANGEMENT OF TYPE IV COLLAGEN MOLECULES IN THE
BASEMENT MEMBRANE

The regions of non-helical structural domain increase the flexibility of the molecule and may enable a greater interaction with non-collagenous components of the basement membrane, such as fibronectin and bullous pemphigoid antigen.

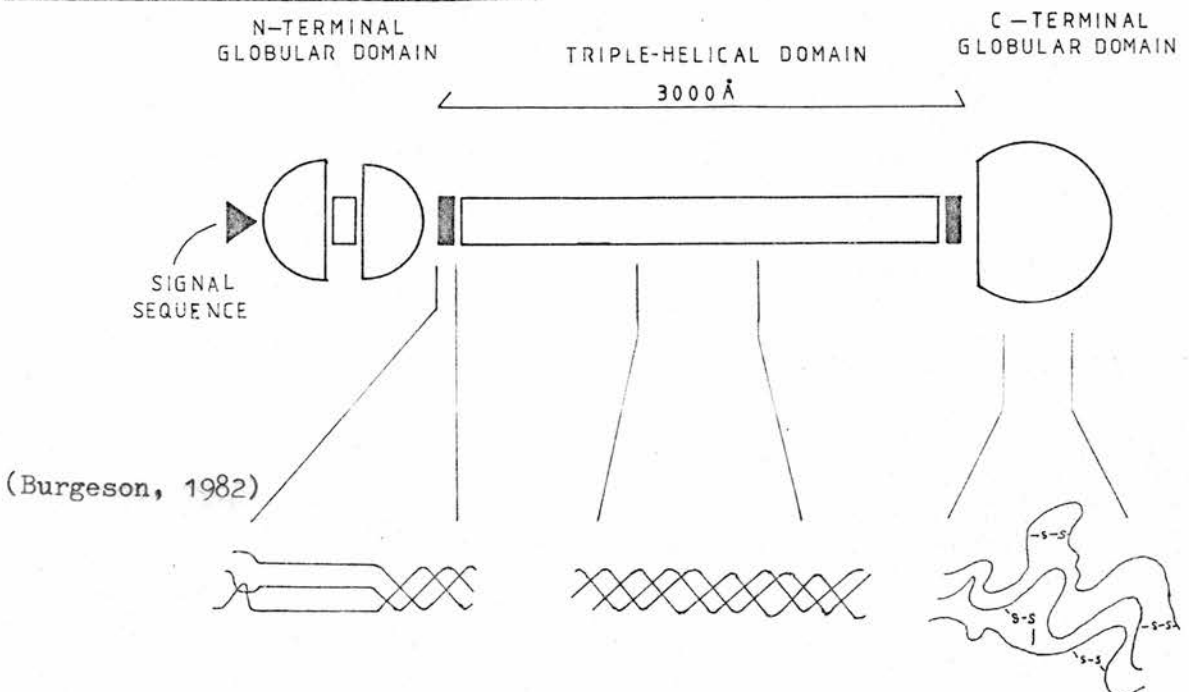
Both Type I and III collagens are susceptible to human skin collagenase, which specifically cleaves the triple helix at a site one quarter of the molecule's length, 300 nm from the NH₂ terminal. Type IV collagen is resistant to the attack of human skin collagenase (Welgus et al, 1981) but some endogenous proteases can degrade basement membrane collagen (Liotta et al, 1979).

Synthesis of Collagen

Collagen synthesis by skin fibroblasts is a complex and highly regulated orchestration of intracellular and extracellular events. It has been well reviewed by Fessler and Fessler (1978), Prockop et al (1979), Prockop (1982) and Bauer and Uitto (1982).

Each of the genetically distinct collagen types is synthesised as a procollagen precursor molecule, composed of pro α chains longer than the final product due to the presence of additional propeptides, as illustrated in the diagram below.

DIAGRAM OF PRO-COLLAGEN MOLECULE



Following transcription of the gene for a particular α chain and modification of the resulting heterogeneous nucleic RNA, messenger RNA is translated on the rough endoplasmic reticulum to produce single α chains. These chains then undergo hydroxylation regulated by prolyl and lysyl hydroxylase. Some of the proline and lysine residues are thus converted to hydroxyproline and hydroxylysine respectively. Ascorbic acid (vitamin C) is an essential cofactor for the hydroxylation of proline (Booth and Uitto, 1981). At this early stage glycosylation also occurs, galactosyl residues being attached to the chains by galactotransferase, followed by the attachment of glucose to certain galactosyl residues. The degree of glycosylation is affected by both the primary structure and the rate of triple helix formation. Type I procollagen requires 10 minutes for the chains to form a triple helix, and exhibits least glycosylation. In contrast Type IV procollagen is rich in glycosylated hydroxylysine residues, requiring 60 minutes for triple helix formation (reviewed by Bauer and Uitto, 1982).

Preliminary association of the three α chains occurs by way of disulphide bonds between the half-cystine residues on the carboxy terminal extensions of the pro α chains, before the triple helix can form. The procollagen molecule is then transported across the plasma membrane of the fibroblast and further modification occurs extracellularly. The procollagen is soluble at this stage, but once conversion to collagen occurs precipitation is spontaneous. Goldberg (1979; 1982) has recently isolated an unidentified glycoprotein on the fibroblast (3T3) plasma membrane which acts as a specific collagen binding site and is presumed to participate in some phase of the extracellular maturation of collagen. Specific procollagen

peptidases remove the amino and carboxy terminal extensions from the procollagen of Types I, II and III. In contrast Type IV apparently retains large non-helical structural domains removed from the fibrillar types of collagen at this stage (Crouch and Bornstein, 1979). The model proposed by Goldberg and Burgeson (1982) for subsequent collagen fibrillogenesis supposes that once soluble surface-bound collagen aggregates reach a limiting size, they detach from the cell and then combine to form insoluble fibrils.

Lysyl oxidase then facilitates the conversion of lysine and hydroxylysine to corresponding aldehydes, which can interact with similar residues on other fibrils to form a covalent cross-link (Siegel and Fu, 1976). As collagen matures in the skin and the number of cross-links increase, it becomes increasingly insoluble.

Regulation of Collagen Synthesis

Such a hierarchical assembly of several different types of collagen by skin fibroblasts requires an extremely complex regulatory system. Regulation of synthesis operates at any stage in the pathway, from the nuclear events of gene selection transcription and processing of the messenger RNA, to the cytoplasmic hydroxylation and glycosylation to the extracellular events of propeptide cleavage and fibril formation (reviewed by Pinnell, 1982). See Table 6 for a summary of the steps in metabolic processing.

As well as endogenous regulation of collagen synthesis, many exogenous factors play an equally important role. The importance of ascorbate in the hydroxylation of proline is fully recognised (Peterkovsky, 1972; Murad et al, 1980). Ascorbate acts primarily on the amount of hydroxyproline synthesised rather than on the amount

TABLE 6

STEPS IN METABOLIC PROCESSING (Muller et al, 1981)

<u>MODE</u>	<u>ENZYME</u>	<u>LOCATION</u>
Selection of structural genes	?	Nucleus
Transcription	Polymerase	Nucleus
Processing	Splicing enzymes	Nucleus
Translation of α chain m RNA	Multi enzyme complex	Endoplasmic reticulum polyribosomes
Hydroxylation	Peptidyl proline hydroxylase	E.R. cisternae
	Peptidyl lysine hydroxylase	
Glycosylation	Galactosyl transferase	E.R. cisternae
	Glucosyl transferase	
Chain selection	Not known	E.R. cisternae
Molecular assembly	Not known	E.R. cisternae
Helix formation	Not known	E.R. cisternae
Translocation	Not known	Golgi complex
Pro-collagen-collagen conversion	Procollagen peptidases	Extracellular space
Fibre formation		Extracellular space
Cross linking	Lysyl oxidase	Extracellular space

of prolyl hydroxylase (Booth and Uitto, 1981). Ascorbate therefore directly affects the efficiency of triple helix formation and the subsequent secretion of procollagen.

Metal cofactors are equally important, ferrous iron and manganese being necessary for hydroxylation and glycosylation while calcium and copper are vital for the extracellular metabolic events.

Using human skin fibroblasts in culture as a model (Freiberger et al, 1980; Booth et al, 1980; Uitto et al, 1980; Booth and Uitto, 1981) it can be shown that hormones and serum-borne factors influence collagen synthesis. Tan et al (1981) showed that normal human serum could stimulate synthesis of procollagens Type I and III. Conversely the amino terminal extension propeptides of procollagen Types I and III inhibit Type I procollagen synthesis (Wiestner et al, 1979), and proline analogues inhibit procollagen production (Tan et al, 1983). Parathyroid hormone specifically inhibits collagen synthesis by decreasing levels of procollagen messenger RNA (Kream et al, 1980).

However, in the in vitro model system for collagen synthesis, artificial constraints and conditions are automatically introduced, such as the composition of culture medium, cell density and pH. Most of these parameters are now well documented and understood (Booth et al, 1980; Freiberger et al, 1980). Optimal Type I and III procollagen synthesis is achieved in "complete" medium containing 20% foetal calf serum, supplemented with 25-50 µg/ml ascorbic acid, at a pH between 7.5 and 7.8 and synthesis is maximal just after visual confluency. Earlier, Steinberg (1973) showed that in relation to total new protein, collagen synthesis was less in logarithmically growing, than in stationary phase cultures.

The effects of both density and cellular ageing have been examined

in normal and keloid skin fibroblasts (McCoy et al, 1982). A gradual decrease in collagen synthesis with ageing in culture, was found in both normal and keloid cells, despite the latter having elevated levels of collagen synthesis. Therefore in considering collagen synthesis in vitro it is important to be aware of the constraints imposed by the system.

Interactions of Collagen

Collagen has important interactions with other constituents of the dermis, particularly fibronectin and glycosaminoglycans (GAG). The role of fibronectin in cell attachment to collagen has been comprehensively reviewed (Kleinman et al, 1981; Kleinman and Wilkes, 1982). Briefly fibronectin is a large glycoprotein (220,000 daltons) found on the cell surface and in extracellular matrices. It is now well established that fibronectin plays a role both in vivo and in vitro in fibroblast attachment to collagen, having specific domains for collagen binding (Ruoslahti et al, 1982). Binding to collagen occurs along the fibre's length with a regular periodicity (Furcht, 1980).

FIG. 45 COLLAGEN-FIBRONECTIN-CELL INTERACTION

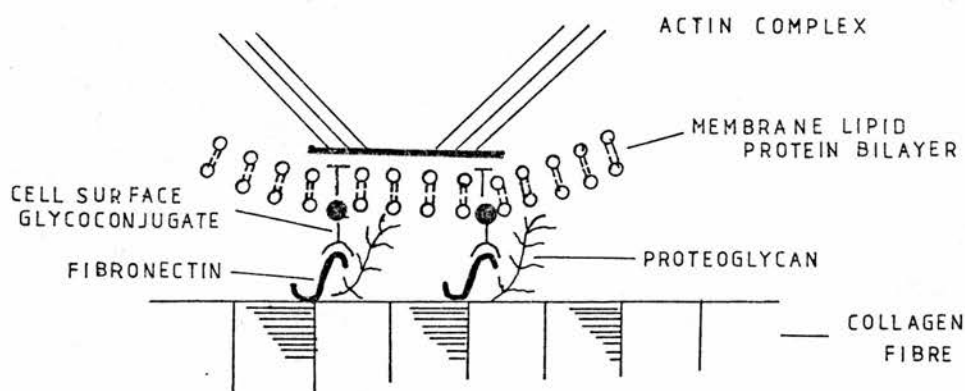


Diagram showing the interaction between a collagen fibre and the surface of a fibroblast. The collagen contains specific sites to which fibronectin binds: the fibronectin contains another region that specifically recognises the cell surface.

(Kleinman et al, 1981)

Fibronectin is an important component of adhesive interactions in vivo (Fig. 45) and is most likely to be equally significant in cell-matrix interactions in vivo.

The interaction of collagen with GAG is not yet fully understood. However it is clear that the hydrated GAG gel interacts with the collagen fibres of the dermis to provide tensile strength with flexibility. The binding of GAG to collagen by electrostatic interaction has been reviewed (Lindahl and Hook, 1978). Mathews (1965) proposed a model for such an interaction between chondroitin sulphate and collagen, in which the protein core of the GAG was aligned with the collagen molecules by way of the chondroitin sulphate side chains. Such interactions may be an important factor in the organisation

and functioning of connective tissue during growth and development (Mathews and Decker, 1968).

Effects of Phenytoin on Collagen

The relationship between phenytoin and collagenase is dealt with in Chapter 4. Phenytoin, an anticonvulsant drug (Merritt and Putnam, 1938), was found to cause hypertrophy of the gums in some epileptic patients (Aas, 1963). Amongst its other properties was improvement of wound healing (Shafer et al, 1958).

The early investigations (Houck et al, 1960; Houck, 1962, 1965; Bright, 1963) showed that phenytoin altered the chemistry of the skin, notably by increasing collagen content. Two alternative but not mutually exclusive explanations were proposed: the excess of collagen could be due to an increase in collagen synthesis (Hassell et al, 1976, 1982) or to a decrease in collagen degradation (Bergenholtz and Hånström, 1979; Hånström et al, 1979).

Fibroblasts removed from the hypertrophic gingivae of five epileptics exhibited a two-fold increase in protein synthesis, 20% of the protein synthesised by the affected cells being collagen compared to the 11% of the total protein produced by control cells (Hassell et al, 1976). This suggested that the drug selects for, or induces, fibroblasts with enhanced levels of protein synthesis and collagen production. This work did not exclude the possibility that collagen degradation was altered or that both synthesis and degradation were affected. Hassell (1982) confirmed that there was more extracellular protein in cultures of fibroblasts from hypertrophic gingivae, including elevated amounts of collagenase compared with normal controls. However the collagenase was less active than that

from untreated controls. Reduced degradation, combined with increased synthesis of collagen, are therefore important actions of phenytoin on human gingival fibroblasts in some epileptics.

Bergenholtz and Hånström (1979), using cat palatal mucosa in organ culture, showed that up to 20 µg/ml phenytoin had no influence on collagen synthesis. This agrees with some of the results of Hassell et al (1976), who showed that collagen synthesis was not affected by 5 µg/ml phenytoin in fibroblasts from non-responding patients i.e. epileptics who did not develop gingival hypertrophy, and fibroblasts from normal human gingivae. Similarly Blumenkranz and Asboe-Hansen (1974) found no change in collagen synthesis by chick embryo tibiae fibroblasts at 44 µg/ml: only at high concentrations 137 µg/ml was collagen production inhibited (500 µM). Bergenholtz and Hanstrom (1979) did find a selective increase in accumulation of mature collagen over newly synthesised collagens, indicating an effect of the drug on degradation.

Hånström et al (1979) also considered the effect of phenytoin on enzyme release in relation to collagen degradation in the feline palatal mucosa, using β -glucuronidase and lactate dehydrogenase as markers for lysosomal and cytosolic enzymes respectively. The activities of both enzymes were reduced on treatment with phenytoin, suggesting that the drug might have a non-specific effect upon membrane function. This agrees with the findings of Perry et al (1978), who showed that phenytoin blocked the resting sodium channels of the membrane, causing hyper-polarisation and reducing intracellular calcium.

Hånström et al (1979) suggested that the reduction in lysosomal enzyme activity, caused by phenytoin, might affect collagenase

activity because less protease would be available to activate latent collagenase. Although this partially explains the findings of Hassell (1982), this does not indicate why the collagenase produced is of a lower activity following in vitro activation by trypsin. It is possible that phenytoin causes the production of an aberrant collagenase, or an increase in an endogenous inhibitor of collagenase activity.

Collagen Investigations

As the collagen status of EB fibroblasts had not been studied the following experiments were designed to establish whether their collagen synthesis was comparable with that of normal control fibroblast lines. A reduction in synthesis could exaggerate the effect of increased collagenase activity by exacerbating the imbalance between enzyme and substrate.

The effect of phenytoin and vitamin E on collagen synthesis in relative and absolute terms was also examined using skin fibroblasts. This was done to determine whether the response of skin fibroblasts to phenytoin is similar to that already described for gingival fibroblasts (Hassell et al, 1976) and cat palatal mucosa (Bergenholtz and Hänström, 1979). The effect of vitamin E on collagen synthesis in skin fibroblasts is unknown and was therefore investigated.

METHODComparative Collagen Assay

Three cell lines, a control, an EBS an EBDr or another (EBDD, EBL), from donors of similar ages, were seeded in complete medium containing 10% foetal calf serum into each of seven plastic culture dishes. Each dish (Linbro; Flow Laboratories, Paisley), had 6 flat-bottomed wells with a surface area of 9.62 cm^2 per well and received 10^5 cells/well. The dishes were incubated at 37°C in a 5% CO_2 : air atmosphere. After two days the cells received medium with 10% foetal calf serum supplemented with $50 \text{ }\mu\text{g/ml}$ ascorbic acid. Forty eight hours later $10 \text{ }\mu\text{Ci/ml}$ ($5\text{-}^3\text{H}$)proline in medium with 2% foetal calf serum and $50 \text{ }\mu\text{g/ml}$ ascorbic acid but without antibiotics, was added at 1 ml/well to 5 of the 7 dishes. The remaining dishes received identical treatment but without the (^3H)proline. All the dishes were incubated as before for 24 hours. The unlabelled dishes were then used for cell counts.

The 5 radioactively-labelled dishes were cooled to 4°C and the medium was removed. The cells were rinsed with 1 ml of phosphate buffered saline which was added to the medium. The cells were swollen with 1 ml of distilled water at 4°C for 30 minutes, after which they were removed from the dishes and effectively homogenised with a jet of distilled water dispensed through a fine hypodermic needle several times. Trichloroacetic acid (TCA) was added to both the medium and cellular fractions, to give a final concentration of 5% and precipitate protein at 4°C .

The resulting protein precipitates were collected by centrifugation at 2000 rpm (4°C) and washed successively with ethanol containing 2% sodium acetate, ethanol-ether (3:1) and ether.

The dried protein pellets were then redissolved in 0.5 mls 0.2N KOH, neutralised with 0.2 mls of 0.4N HCl and diluted with 2 mls of Tris-HCl buffer containing 10 mM calcium chloride and 10 mM N-ethyl maleamide (NEM) (Sigma Chemical Co.) pH 7.6. Each fraction was subsequently divided into two equal aliquots, one of which received 100 units of collagenase (Advanced Biofacturers Corporation, Lynbrook New York), the other received buffer alone. All aliquots were incubated overnight at 37°C. Protein was then reprecipitated in cold 5% TCA containing 0.25% tannic acid, washed as before and dried. The final residues were dissolved in 1 ml hyamine hydroxide and added to 10 mls NE233 Scintillation fluid (Nuclear Enterprises, Edinburgh). Samples were counted in a Packard Tri-carb 300 liquid scintillation counter with automatic quench correction of cpm to dpm based on external standard channel ratios.

As discussed later (page 139) the ABC collagenase used is virtually free of non-specific proteases and the NEM added to the buffer should inhibit the action of any non-specific proteases already present in the culture preparation.

To examine the effect of phenytoin and vitamin E on collagen synthesis, a similar method to that just described was adopted, except that only one cell line was used in each experiment; three concentrations of drug were tested simultaneously. Consequently 32 wells were seeded at 10^5 cells/well for ^3H -labelling, whilst 16 wells were seeded for cell counts.

The drug was added with the (^3H) proline, 8 wells receiving no drug at all, while a similar number of wells each received phenytoin at 100, 300 and 500 μM dissolved in 50% ethanol. Equivalent concentrations of the drug were added to the 16 wells without

(³H) proline. Vitamin E dissolved in acetone was added to culture media at a concentration of 100, 300 and 500 µg/ml.

RESULTS

Cell lines used for comparison are given below, all were between passages 5-10.

<u>CONTROL</u>	<u>EBS</u>	<u>EBDr</u>	<u>EBDD</u>	<u>EBL</u>
NF2	EB9	EB1	EB16	EB27
NF3	EB10	EB2	EB17	
NF4	EB11	EB5	EB25	
NF5	EB12	EB14		
HSF8	EB23	EB18		
HSF9	EB30	EB31		
HSF14				
HSF22				

Relative collagen synthesis - the percentage of total protein synthesis which is collagen - was calculated by the method of Diegelmann and Peterkofsky (1972). Three EBDD lines and 1 EBL line were included in the study, though no conclusions have been based on those results.

The mean results for collagen synthesis between groups are given in Table 7, and Fig. 46 a, b and c.

TABLE 7

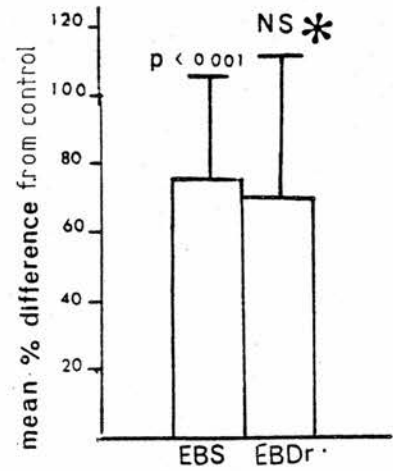
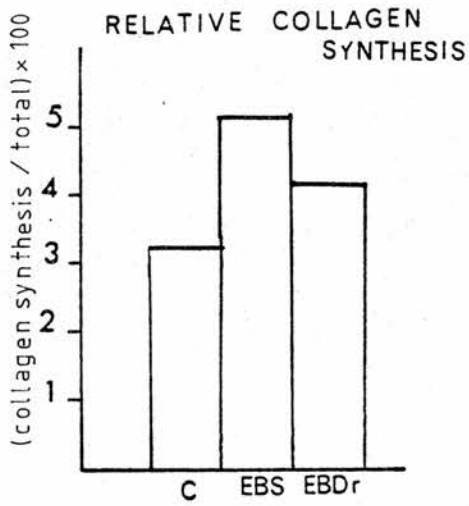
RESULTS FOR COLLAGEN AND NON-COLLAGEN PROTEIN SYNTHESIS IN EB AND CONTROL GROUPS

	<u>CONTROL n = 6</u>	<u>EBS n = 6</u>	<u>EBDr n = 6</u>	<u>EBDD n = 3</u>	<u>EBL n = 1</u>
R.C.S.	3.2 ± 0.5	5.1 ± 0.9	4.1 ± 0.8	4.9	3.2
dpm collagen/ 10 ⁵ cells	4,314 ± 664	8,672 ± 1,845	6,778 ± 1,039	9,627	8,364
dpm non-collagen/ 10 ⁵ cells	29,189 ± 5,842	33,648 ± 8,871	38,077 ± 9,240	33,709	51,164

MEAN ± SEM

FIG. 46

a)



b)

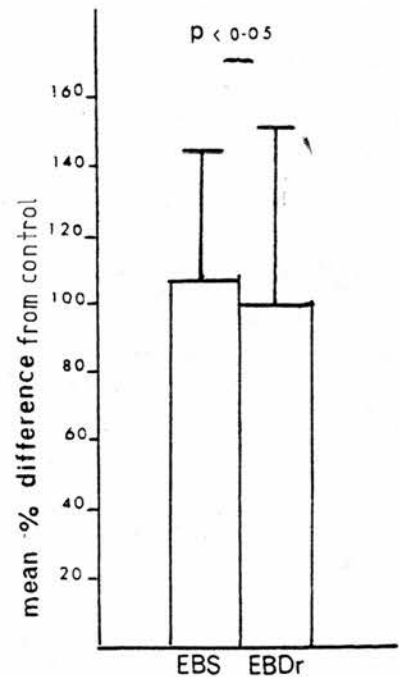
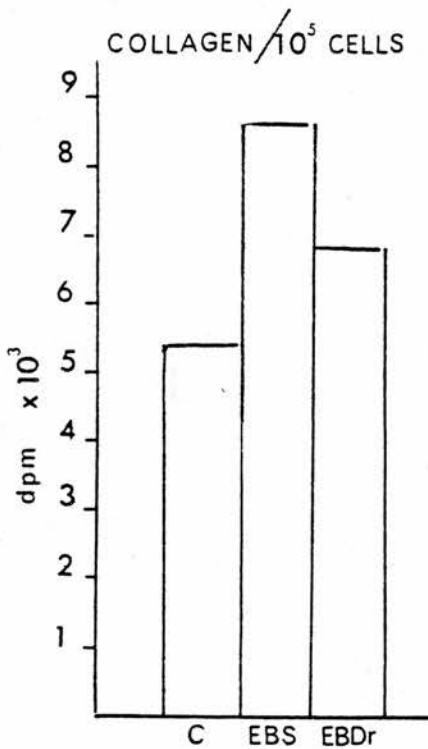


Fig. 46 (a) and (b) show the mean relative collagen synthesis and mean collagen synthesised per 10^5 cells respectively in control, EBS and EBDr groups with the corresponding mean (\pm SEM) percentage difference between the EB groups and controls adjacent. The students-paired t-test was used to test the significance of the difference between controls and EB groups.

FIG. 46

c)

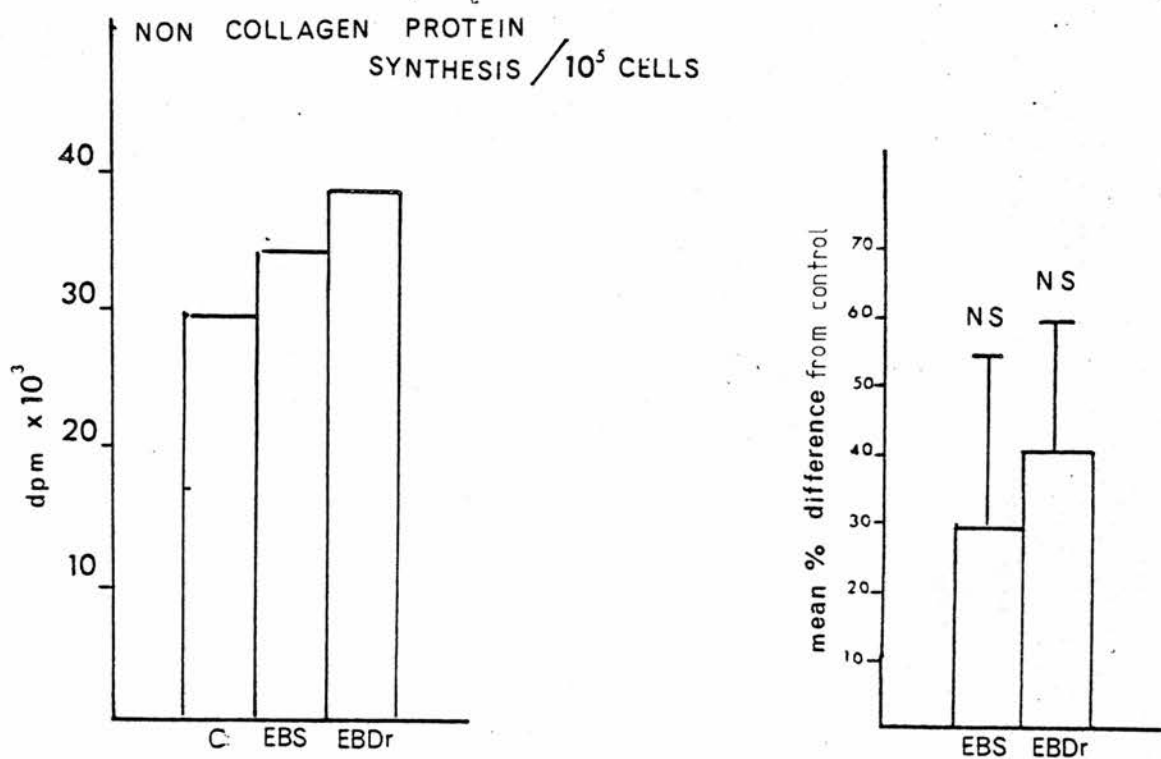


Fig. 46 (c) shows the mean non-collagen protein synthesis per 10^5 cells in control, EBS and EBDr groups with the corresponding mean (\pm SEM) percentage difference between the EB groups and controls adjacent. The students-paired t-test was used to test the significance of the difference between controls and EB groups.

Although the variation in $\text{dpm}/10^5$ cells between experiments was quite large, the experimental design enables a Student's paired t-test to be used to test the significance of the difference between the EB group and its corresponding age-matched control within each experiment. The results, expressed as the mean percentage difference between control and EB groups (Fig. 46), indicate that relative collagen synthesis was increased by 73% in EBS ($p < 0.001$) whereas the 69% increase for the more variable EBDr data was not statistically significant. However collagen synthesis per 10^5 cells was elevated by approximately the same amount in both EBS (108%) and EBDr groups (100%) over controls ($p < 0.05$). That is to say, collagen synthesis per cell was approximately double that of controls, although again there was considerable variability between experiments.

Non-collagen protein synthesis per 10^5 cells was very variable, consequently the differences were not statistically significant.

The percentage of collagen and other non-collagen protein secreted into the medium was also determined. Approximately 50% of the labelled collagen synthesised over 24 hours was found in the medium, and approximately 18% of labelled non-collagen protein, with no significant differences between groups.

As only three EBDD cell lines were used in experiments statistical analysis is invalid. However the results suggest a similarity in both collagen and non-collagenous protein synthesis between the EBDD and EBS groups, the EBDD group differing from controls in all these aspects. The relative collagen synthesis in the single EBL cell line examined did not differ from the control group; although collagen and non-collagen protein synthesis were above the mean control values, they fall within the control range (collagen/ 10^5 cells 1,613-15,899,

non-collagen protein/ 10^5 cells 11,998-60,738). The age of the EBL patient (2 years) may explain the higher levels of protein synthesis generally.

The effect of phenytoin and vitamin E at various concentrations was assessed on 6 cell lines - 2 controls, two EBS and two EBDr. The intention was to ensure that representative lines from each group did not differ from each other in their response to the drug, having established that differences do exist in collagen synthesis between groups. As no difference in response to the drug was observed, the data for the 6 strains was treated as a single group. The raw data are given in Tables 8 and 9. Statistical analyses, however, were performed on normalised results, owing to the large variation between each cell line caused by extraneous experimental conditions. To normalise the raw data in each experiment, the mean and standard deviation in the response of a cell line to a particular drug concentration, were determined. The mean was then subtracted from each item of data and the result was divided by the standard deviation. This ensured that large variations between experiments did not mask a real effect of the drug and allowed direct comparisons across experiments. Having normalised all the data, a non-parametric method of statistical analysis was adopted - the Mann-Whitney U-test - to evaluate differences between groups.

TABLE 8

EFFECT OF PHENYTOIN ON PROTEIN SYNTHESIS Mean results (\pm SEM) as raw data

PHENYTOIN	0	100	300	500 μ M
R.C.S.	3.43 \pm 0.4	3.25 \pm 0.6	3.88 \pm 0.6	3.78 \pm 0.6
dpm collagen/ 10^5 cells	10,980 \pm 1,370	9,685 \pm 611	10,895 \pm 1,831	9,612 \pm 1,955 ^a
dpm non-collagen protein/ 10^5 cells	50,543 \pm 6,392	48,537 \pm 6,441	46,699 \pm 7,837 ^b	44,146 \pm 5,736 ^c

a: -12% p<0.02; b: -11% p<0.02; c: -18% p<0.001 (Mann-Whitney U-test on normalised data)

TABLE 9

EFFECT OF VITAMIN E ON PROTEIN SYNTHESIS Mean results (\pm SEM) as raw data

VITAMIN E	0	100	300	500 $\mu\text{g/ml}$
R.C.S.	3.8 \pm 0.7	2.89 \pm 0.6 ^a	3.47 \pm 0.6	3.29 \pm 0.3
dpm collagen/ 10^5 cells	8,823 \pm 1,786	7,671 \pm 1,370	7,838 \pm 1,582	7,722 \pm 1,532
dpm non-collagen protein/ 10^5 cells	43,008 \pm 5,581	50,086 \pm 9,232 ^b	42,357 \pm 8,718	41,123 \pm 6,708

a: -13% $p < 0.02$; b: +14% $p < 0.001$ (Mann-Whitney U-test on normalised data)

The results indicate that phenytoin at 500 μM (137 $\mu\text{g/ml}$) caused a significant reduction in both collagen and other protein synthesised, relative collagen synthesis was therefore not affected. At 300 μM (82 $\mu\text{g/ml}$) only the amount of non-collagen protein synthesised was significantly affected by the drug, the normalised data indicated that synthesis was suppressed by 11% ($p < 0.02$).

Vitamin E only significantly affected protein synthesis at 100 $\mu\text{g/ml}$, increasing non-collagen protein synthesis by 14% ($p < 0.001$). This increase caused a corresponding depression of 13% in the relative collagen synthesis.

DISCUSSION

Relative and absolute collagen synthesis in control and EB groups showed some significant differences. Perhaps most surprisingly, because of its apparent lack of dermal involvement, the EBS group showed the greatest difference from the controls, both in relative and absolute terms.

Relative synthesis in EBS was increased by 73% ($p < 0.001$), reflected by an increase of 108% in absolute collagen synthesis. There was no significant difference between non-collagen synthesis in EBS and controls. The relevance of these findings is hard to assess; the finding that trypsin-activatable levels of collagenase (pages 147-163) are slightly higher than controls (14%), may be relevant. Increased collagen synthesis could represent a response by the fibroblast to increased collagenase activity, or alternatively such an increase in collagen synthesis could have affected regulation of collagen degradation, thereby promoting increases in collagenase levels. Because the increase in collagenase activity was only comparatively small and the increase in collagen synthesis comparatively large, it is possible that an equilibrium has been established, enabling the maintenance of an overtly normal dermis.

However, this equilibrium was seemingly disturbed in the EBDr group. Collagen synthesis per cell was significantly increased, being approximately double that of controls and only slightly less than the EBS group. Neither relative collagen synthesis, nor non-collagen protein synthesised per cell significantly differ from controls despite non-collagen protein being elevated 40% ($\pm 19\%$ SEM) because of the large variability between experiments. Therefore the general increase in protein synthesis combined with the 38% increase

in collagenase activity, indicated by the collagenase data (pages 151-157) could combine to cause destabilisation of the dermis. It is possible that the increase in collagen per cell is a response to the excess collagenase, the alternative - that excess collagenase is a response to increased collagen is conceivable but unlikely as it is known that higher collagenase levels in EBDr are due to the presence of more messenger RNA coding for collagenase, implying increased gene coding capacity (Kronberger et al, 1982).

Such upsets in dermal homeostasis have previously been noted. It is now thought that amongst the contributing factors in scleroderma, characterised by extensive induration and fibrosis of the skin (reviewed by Leroy, 1982), is an increased biosynthesis of both Types I and III collagen whilst collagenase levels remain unaltered (Uitto et al, 1979). It would be interesting to know whether Type I and III collagens are produced in the usual ratios in EBS and EBDr. Particularly, as Type III collagen is degraded faster than Type I by collagenase (Welgus et al, 1981).

It is interesting that a large collagen fibre (~100 nm) was noted in an electron micrograph of EBDr skin (Fig. 28 EB31) in agreement with the observations of Pearson (1962). Typically, EB31 showed no significant change in relative collagen synthesis but synthesis of collagen per cell was increased by 65%. It is possible that the production of large fibres is related to the increase in collagen synthesis observed.

There have been many studies of the effect of phenytoin on gingival fibroblasts and cat palatal mucosa, but only Houck et al (1972) have used human skin fibroblasts as a model, using only two cell strains to test phenytoin at 100, 10 and 1 μ M. It should be

noted that the optimum range of serum concentration for epileptics is 10-20 $\mu\text{g/ml}$, i.e., 40-80 μM (Reynolds et al, 1976), therefore the concentrations used in the present investigations are well in excess of therapeutic levels.

The results show that at high concentrations of the drug both protein and collagen synthesis are significantly depressed. This is in agreement with Blumenkrantz and Asboe-Hansen (1974), who found that large concentrations of phenytoin, 500 μM to 8 mM, could reduce all protein synthesis including collagen in chicken embryo tibia fibroblasts. At the highest concentrations, the drug is probably having a toxic effect on the cells. The viability of cells subjected to (100-500 μM) phenytoin in the present study did not differ from untreated cells (90-95% viability) when determined by trypan blue exclusion (see p. 23).

At 300 μM only other protein synthesis was significantly depressed. Bergenholtz and Hånström (1979) also found changes in protein synthesis, although it is uncertain whether these were a real effect of phenytoin, or as they suggest, an artefact of their organ culture system.

Although 300 μM is well above the therapeutic limit, the implications of an inhibition in non-collagenase protein are obvious to EBDr, where production of collagenase could be curtailed. The likelihood of this happening in vivo is small as such levels of the drug could not be realised. Plasma proteins particularly albumin bind to 90% of phenytoin (Richens, 1979), so that intolerably high doses of phenytoin would therefore be necessary if plasma concentrations were to approach 100 μM .

The finding that up to 500 μM phenytoin fails to alter collagen synthesis is in agreement with Houck et al (1972) and Hassell et al (1976). The latter authors found changes in collagen synthesis

only in the gingival fibroblasts of epileptic patients who had responded to phenytoin by producing gingival overgrowths. No change was observed in fibroblasts from controls and non-responding patients.

Phenytoin has been found to have a rather generalised effect on membrane function, altering the release of both cytoplasmic and lysosomal enzymes (Hänström et al, 1979). This work also verified that phenytoin was responsible for a reduction in collagen degradation rather than any change in synthesis, postulating that collagenase release might similarly be inhibited. Without knowing whether collagen degradation is reduced in human skin fibroblasts treated with phenytoin, it is difficult to say whether the drug is likely to have a beneficial effect on EBDr skin or not. Clinical evidence suggests that phenytoin is only useful in a subgroup of EBDr patients (Bauer et al, 1980). It is possible that this group equates with the group of epileptics who respond to the drug by producing gingival overgrowths. In epileptics this characteristic has been attributed to selection of a unique population of gingival fibroblasts inherently capable of higher collagen and protein synthesis and "switched on" by phenytoin (Hassell et al, 1976). Perhaps a similar mechanism is at work in the skin of EBDr patients. Obviously further work is required to establish whether collagen degradation is affected by phenytoin in human skin and whether this is a general occurrence or a trait of only certain susceptible cell lines.

The only significant effect of vitamin E was seen at 100 µg/ml. At this concentration non-collagen synthesis was increased by approximately 14% ($p < 0.001$), and relative collagen synthesis was

correspondingly decreased. Very little is understood about the affect of vitamin E on skin. It is known that collagen metabolism is affected from investigations by Brown et al (1967) into vitamin E deficient rats. Newly synthesised collagen from these rats was unstable compared to collagen from normal rats, indicating an abnormality in the process of cross-linking.

However, more is known about the effect of vitamin E at the cellular level, where it serves as both an antioxidant and a structural component of the membrane (Lucy, 1972; Betteger et al, 1980; Giasuddin and Diplock, 1981). It is thought that vitamin E stabilises membranes by way of specific physicochemical interactions between its phytyl side chain and the fatty acid chain of polyunsaturated phospholipids. It inhibits the oxidative destruction of polyunsaturated fatty acids in cells and their membranes high in polyunsaturated fatty acids and can prevent the degradation of membrane phospholipids by membrane bound phospholipases in vivo (Lucy, 1972).

Exactly how this related to protein synthesis in fibroblasts is uncertain. Certainly the stabilizing of membranes, including lysosomal membranes, and effects on permeability could be important if neutral proteases as well as collagenase are involved in the pathogenesis of EBDr (Takamori et al, 1983). It is curious that the amelioratory effects attributed to vitamin E extend from EBDr to EBS-Weber-Cockayne type (Ayres and Mihan, 1969). It is possible that membrane stabilization has an important role in both these types of EB, with the lysosomes of the basal cell in EBS, as opposed to the fibroblast in EBDr, being the most likely target for vitamin E action.

The beneficial effects of increasing non-collagenous protein

synthesis are equivocal without knowing whether enzyme release and activity is affected by vitamin E. It is possible that if the vitamin does have a role in promoting and stabilizing cross-linking of collagen, then collagen degradation might be altered without collagen synthesis being affected by the drug. In the case of EBDr a decrease in the rate of collagen degradation would probably be beneficial.

SUMMARY

Collagen synthesis was compared between control and various EB fibroblast lines. The relative collagen synthesis in EBS was significantly elevated (73% $p < 0.001$) over control levels. Collagen synthesis per cell was approximately doubled in both EBS and EBDr cell lines compared with controls ($p < 0.05$). Non-collagen protein synthesis was not significantly altered in any of the groups investigated.

The effects of phenytoin and vitamin E on various fibroblast lines were studied. Phenytoin significantly decreased non-collagen protein synthesis at 300 μM whilst depressing both collagen and other protein synthesis at 500 μM ($p < 0.02$).

Vitamin E only had a significant affect at 100 $\mu\text{g/ml}$, depressing relative collagen synthesis by stimulating the synthesis of non-collagenous protein by 14% ($p < 0.001$).

CHAPTER 4

COLLAGENASE

COLLAGENASEINTRODUCTION

The controlled remodelling of connective tissue during the growth and development of an organism is of great physiological importance. It requires not only a precise regulation of the enzymes involved but also that they have a high degree of specificity for the substrate. In vertebrates such a system was first recognised in the Anuran tadpole Rana catesbeiana, when Gross and Lapiere (1962) detected and assayed the protease which caused resorption of the tadpole's tail in vivo, and solubilised fibrillar calf skin collagen in vitro.

Collagenolytic enzymes were then detected in other connective tissues including the involuting rat uterus (Jeffrey and Gross, 1967), human rheumatoid synovial tissue (Evanson et al, 1967) and human skin (Eisen et al, 1968). At first human skin collagenase could only be detected in culture, using serum-free medium to exclude serum inhibitors. Eisen (1969a) investigated the distribution of collagenase in normal human skin by dissecting samples into epidermal, upper dermal and lower dermal layers. He concluded that human skin collagenase, unlike that from the Anuran tadpole, was a product of the dermis, and specifically, the upper dermis. Reddick et al (1974), using immuno-cytochemical techniques, later showed collagenase to be specifically located in the papillary dermis and suggested that fibroblasts were responsible for its production.

Investigations then followed to isolate, purify and ultimately characterise the collagenase protein. Eisen et al (1968) found that the enzyme degraded the collagen molecule into quarter and three quarter fragments. Bauer et al (1970) found that there appeared to

be two enzyme proteins involved; as determined by their electrophoretic mobility in a polyacrylamide gel. Furthermore, on raising antibodies to one of the enzyme bodies he found the two to be extremely closely related, with similar antigenetic specificities. This work also showed inter-species differences between the enzymes, bacterial, crustacean and vertebrate enzymes being antigenically distinct.

The complexity of the enzyme system was increased by the discovery that collagenase was released into culture medium in a latent form, which requires protease (trypsin) activation in vitro (Vaes, 1971). Vaes postulated that there must be another factor, or agent, released by the skin fibroblasts in culture, which could activate the latent collagenase and which was also elicited by trypsin activation. He did not think that the latent collagenase was an enzyme-inhibitor complex. The following year Vaes (1972) isolated an activator molecule similar in size to the collagenase molecule itself, i.e. 100,000 daltons. Unfortunately there have been no corroboratory studies concerning the activating agent.

To overcome the problem of assaying two almost identical species of collagenase which could apparently be either in an inactive, pro-collagenase form, or appear as an active enzyme, two methods of enzyme assay were developed. Firstly Bauer et al (1972) developed a radioimmunoassay that could provide a specific and accurate quantitation of human skin collagenase in vitro and in vivo. After enzyme purification on a Sephadex column, antibodies were raised against the collagenase: as cross reactivity occurred between the zymogen and active enzyme, the total immunoreactive enzyme protein could be assayed whether it was activated or not.

Secondly Bauer et al (1975), in a systematic investigation of

procollagenase activation in vitro, found that different concentrations of trypsin produced activation optimally over a period of 10 minutes at 25°C. In this study they also demonstrated the presence of a collagenase inhibitor capable of abolishing over 90% of the collagenase activity whilst displaying virtually no inhibition of trypsin. This inhibitor was thought to be a product of skin fibroblasts themselves and could explain the previous inability to detect collagenase activity in fibroblast cultures (Reddick et al, 1974). Welgus et al (1979) later isolated an inhibitor from the medium of human skin fibroblasts in serum-free conditions, with a molecular weight of approximately 31,000 daltons. The production of latent collagenase is not, however, purely a characteristic of human skin. Werb et al (1977) showed that procollagenase from rheumatoid synovial cells was activated by plasmin, itself activated from plasminogen by an agent thought to be produced by the synovial cells.

Electrophoresis of collagenase, isolated from the media of human skin fibroblasts in culture (Stricklin et al, 1977; 1978), revealed not just pro- and active enzymes but two pairs of enzymes - an upper doublet of 60,000 and 55,000 daltons, which upon trypsin activation lost a 10,000 dalton fragment producing the lower 50,000 and 45,000 dalton species respectively. This work also revealed a second mode of activation, that of autoactivation; whereby latent enzyme without protease interaction, or a detectable change in molecular weight, could become active (Valle and Bauer, 1979).

Interestingly, Bauer (1977a) showed that the specific activity of collagenase increased 6-10 fold shortly after confluence, implying that synthesis and/or release of enzyme changed with culture density. Curiously, after confluence the amount of immunoreactive collagenase had increased only 2 fold, whilst the increase in specific activity

of the enzyme was disproportionately large. This disparity must mean that although enzyme secretion and probably synthesis increases, some other factor controlling enzyme activity must decrease - perhaps an endogenous inhibitory factor of the collagenase.

Other factors that affect collagenase activity include the divalent calcium ion (Ca^{2+}). This ion is necessary for the thermostabilization of the enzyme, whether in the presence of substrate or not. Increasing collagenase activity with increasing Ca^{2+} concentrations is associated with an increase in thermostability. There is however no evidence that Ca^{2+} plays any role in the binding of collagenases to their substrate (Seltzer et al, 1976).

It is now thought that the microtubule system is intimately involved in the synthesis and/or degradation of newly synthesised collagenase (Bauer and Valle, 1982). The microtubular agent colchicine, in low concentrations (10^{-6}M), produced a 3-fold increase in trypsin-activatable collagenase in culture medium, without an increase in total protein synthesis.

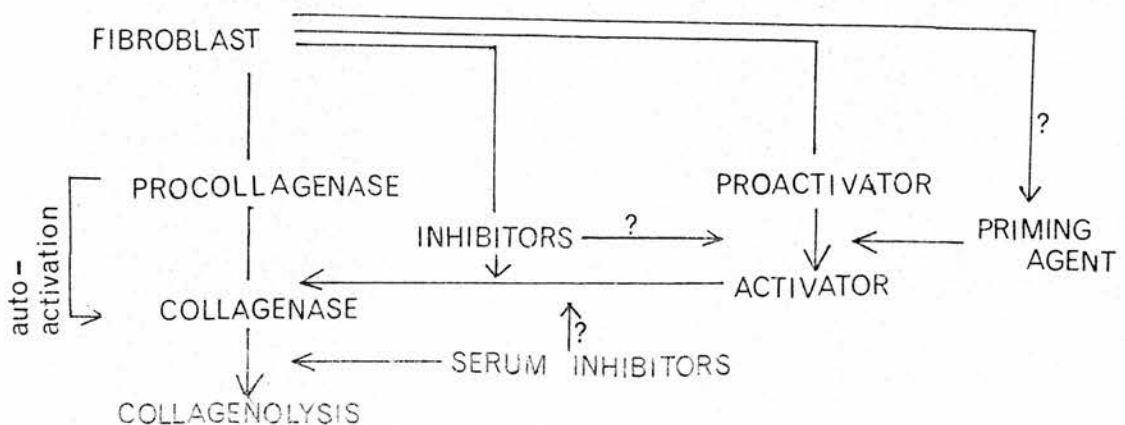
More is now known about the specificity and interaction of the enzyme with its substrate (Welgus et al, 1981). Human skin collagenase successfully degrades collagen Types I, II and III but not IV or V and although it does degrade collagen from different species, it is most active against specific homologous collagen Types I and III. The cleavage of Type III collagen occurred at 10 times the rate of homologous Type I collagen. The presence of the triple helical conformation of the constituent collagen fibrils was an absolute requirement for the differential selectivity of susceptible collagen types to fibroblast collagenase: following substrate denaturation, collagen Types I - III were degraded at approximately equal rates.

Despite the failure of normal skin fibroblast collagenase to degrade Type IV or V collagen, protease extracted from an invasive tumour has been shown to degrade Type IV collagen (Liotta et al, 1979).

The kinetics of the collagen-collagenase reaction have been examined (Welgus et al, 1980): the degradation process was extremely slow, one molecule of human skin collagenase degrading approximately 25 molecules of guinea pig Type I fibrillar collagen per hour at 37°C. The explanation appears to be that only 10% of the collagen molecule in the fibrillar form is accessible to the enzyme for binding prior to degradation of the substrate.

The control of collagenase activity is obviously extremely complex. There is substantial evidence that the enzyme is secreted in a latent form and therefore requires interaction with other factors for activation, unless autoactivation occurs. The importance and relevance of autoactivation in vivo is still unknown. There is evidence that as well as procollagenase the fibroblasts secrete inhibitors of the active enzyme, representing another possible point of control. Furthermore, large inhibitory molecules such as the α 2-macroglobulin in serum are potent inhibitors of activated collagenase prior to substrate binding.

FIG. 47



SCHEME OF SOME OF THE INTERACTIONS LIKELY TO BE INVOLVED IN THE CONTROL OF FIBROBLAST COLLAGENASE ACTIVITY

The interaction of these factors as well as the intracellular controls over collagenase synthesis, compound the complexity of the system (Woolley and Evanson, 1980). Moreover there is now evidence that epidermal cell products can influence stromal cell response. Activators and inhibitors from the epithelial cells of rabbit cornea have been isolated and shown to affect collagenase activity of the underlying stromal cells (Johnson-Wint, 1980).

COLLAGENASE AND EB

An abnormality in collagenase production is thought to be a causal factor in the pathogenesis of epidermolysis bullosa dystrophica recessive (EBDr).

Pearson, (1962) was amongst the first electron microscopists to note the degenerative changes in the dermal collagen near the dermo-epidermal junction and to suggest that collagenolysis might be occurring. Eisen (1969b) followed up this study by investigating the collagenolytic activity from the involved and uninvolved skin of five patients with EBDr. He found that involved skin had a 6-fold increase in enzyme activity, with a smaller increase in the uninvolved skin, compared with normal control skin. Curiously Eisen also concluded that the epidermis of EBDr skin had even more enzyme activity than the underlying papillary dermis. He regarded increased collagenase synthesis as the most likely explanation but thought there might also be an associated deficiency of collagenase inhibitor in the serum of these patients. Lazarus (1972) came to rather different conclusions, suggesting that increased local levels of collagenase are a secondary tissue reaction to chronic injury. He found that 2 out of 9 EBD patients had minimally decreased serum collagenase inhibitor. It now seems likely that this

early experiment failed to detect the latent enzyme either because of the interaction of active enzyme with serum inhibitors, or simply failure to activate what is now known as procollagenase.

With greater knowledge of collagenase and improved techniques Bauer et al (1977) quantitated the amounts of immunoreactive protein present in generalised and localised EBDr, EBL and EBDD. Elevated levels of immunoreactive human skin collagenase were found in the blistered skin of all types of EB examined and significantly higher collagenase levels were found in the uninvolved skin of EBDr and EBL. Unfortunately uninvolved skin is often a misnomer, particularly for EBL and severe EBDr, blistering almost invariably occurring during biopsy in these types of EB. Therefore the results may be somewhat misleading, particularly if induction of a blister elevates collagenase levels. This investigation did not examine the specific activity of the enzyme present but concluded that the significant increase in concentration of enzyme protein in clinically uninvolved skin of EBDr patients was of major importance. Bauer et al suggested that the excess collagenase may be responsible for the absence of anchoring fibrils noted by Hashimoto et al (1976b) and also Briggaman and Wheeler (1975a).

Bauer (1977b) further demonstrated that the collagenase from two EBDr patients was not only produced in excess but also in an aberrant form. The procollagenase had a decreased affinity for the Ca^{2+} co-factor required for enzyme activity and thermal stability: consequently it was more thermo-labile at low Ca^{2+} concentrations than control enzymes. The resulting collagenase therefore had a diminished specific activity when expressed as activity per unit of immunoreactive protein, with average values 39% and 16% of control

enzymes. Such a disparity between increased amounts of enzyme and decreased activity poses the intriguing problem of whether there is sufficient enzyme present to increase the rate of collagen degradation in vivo. Bauer concluded that over-production, or decreased degradation of a defective collagenase enzyme retaining considerable activity in vivo was the likely cause of blistering in EBDr.

A comparative study of EBDr, EBDD, EBL and EBS (Bauer and Eisen, 1978) using tissue culture techniques revealed that, in contrast to his earlier findings (Bauer et al, 1977), only EBDr showed increased immunoreactive collagenase levels, with a mean value 328% of that in control cultures. Within the 10 EBDr cell strains used in the study, there was great variation in the results. The specific activity in 8 out of the 10 EBDr strains was examined. In 3 out of 8, the activity was decreased, in a similar number the activity was normal and in only 2 out of 8 was there a slight increase in activity. The disproportionate increase in immunoreactive enzyme protein, which could be structurally altered, was thought to be the reason for the decrease in collagenase activity per unit immunoreactive protein; hence the greatly reduced catalytic efficiency in EBDr collagenase. No studies on the collagenase activity in the other types of EB cell strains were performed.

Studies were then concentrated on the precise characteristics of EBDr collagenase. Valle and Bauer (1980) found that the initial rate of accumulation of intracellular enzyme as well as the rate of secretion were increased in 2 EBDr cell lines examined. They concluded that excess accumulation of enzyme must be related to increased synthesis, particularly as the rate of degradation of the enzyme did not differ from controls. Comparison of the electrophoretic mobilities of the EBDr procollagenase and collagenase with control

enzyme showed no difference between them, suggesting that the aberration in EBDr collagenase must be a very small alteration in structure affecting the function of the enzyme. Stricklin et al (1982) verified that EBDr collagenase was indeed similar in size to that of normal human skin fibroblasts; with zymogens of 60,000 and 55,000 daltons, which lost a 10,000 dalton fragment to form active enzymes. The active collagenase had similar reaction rates and substrate specificity for the various human collagen types as did normal human collagenase. The amino acid composition of EBDr and normal collagenase was analysed as well as a comparison of cyanogen bromide digestion products separated by gel electrophoresis. The amino acid analysis showed that differences could be seen between proenzyme quantities of proline, glycine, half cystine, methionine and tyrosine residues, whilst the active enzymes were quite similar in composition. Digestion product patterns of procollagenase revealed small but reproducible differences: EBDr lacked one of the resolved peptide bands seen in normal enzyme digests and had two extra bands not seen in the control. This provided strong evidence of structural differences between normal and EBDr procollagenases. The study also confirmed that the marked decrease in thermal stability could largely be attributed to the decrease in affinity for the stabilizing metal co-factor Ca^{2+} in the pure enzyme. It therefore seems that in EBDr there is an increased synthesis of a structurally abnormal collagenase.

Analysis of messenger ribonucleic acids (mRNA), the intermediary between coded gene and protein synthesised, shows that although total translational activity was equal in normal and EBDr mRNA preparations, there was a 3.5 - 10 fold increase in translatable collagenase mRNA

from EBDr cell lines (Kronberger et al, 1982). This confirms that the increased collagenase synthesis is due to elevated levels or preferential translation of collagenase mRNA.

It seems possible that the final explanation may be within the collagenase gene code itself. Gene duplication can occur within the DNA of a chromosome and it is possible that a fault or slight change in the nucleic acid sequence coding for collagenase occurred when the gene duplicated - thus increasing the amount of translatable collagenase DNA and subsequent mRNA, whilst accounting for a defect in the pro-collagenase produced.

It is known that human skin collagenase is most efficient at degrading Type III collagen, cleavage occurring at 10 times the rate of homologous Type I collagen (Welgus et al, 1981). Type III collagen forms the thinner fibrils of the dermis, accounting for only 10-15% of the dermal collagen; immunotechniques suggest that most is found in the upper papillary dermis (Fleishmajor et al, 1980), though there is evidence to suggest even distribution throughout the dermis (Epstein and Mulderloh, 1978). The consequence of increased collagenase levels would be the rapid destruction of the thinner Type III fibrils and hence the site of cleavage would be in the upper papillary dermis, as indeed it is in EBDr. If the anchoring fibrils contain a helical collagen element, susceptible to human skin collagenase, and it is known that they are susceptible to bacterial collagenase digestion (Kobayasi et al, 1977), their apparent absence in EBDr could be due to early destruction by the increased levels of collagenase.

PHENYTOIN AND COLLAGENASE

So far only two drugs have emerged that might ameliorate the effect of increased collagenase in patients suffering from EBDr - phenytoin and vitamin E. Both have been used for rather tenuous reasons, with rather disappointing and debatable results.

Phenytoin (5-5 diphenylhydantoin; dilantin sodium) is an anti-convulsant drug long used in the treatment of epileptics (Merritt and Putnam, 1938). One of the side effects observed in epileptic patients was a fibrous outgrowth of connective tissue in the gums, so-called gingival hyperplasia (Aas, 1963). It had already been noted in a series of experiments by Houck (1962, 1963) that phenytoin affected the composition of the skin in rats. Houck found that while dermal water and fat decreased, a unique insoluble non-collagenous protein appeared and the drug also caused an increase in acid soluble and insoluble collagen in the dermis. Bright (1965) confirmed that dermal collagen increased, because the excretion of degraded collagen in the form of proline and hydroxyproline fell in rats receiving phenytoin. Such changes could not be explained by increased fibroblast proliferation alone, for although phenytoin and some of its analogues accelerated the proliferation of fibroblasts in tissue culture, only phenytoin and its analogue 1-allyl-5-phenyl-hydantoin produced significant changes in skin in vivo (Shafer, 1960, 1961; Houck, 1963).

Hassell et al (1976) confirmed that the gingival overgrowth was predominantly collagen. On culturing the gingival fibroblasts he found that their total protein synthesis had been stimulated by the drug, with collagen synthesis increasing from 11% of the protein synthesised to 20%. They concluded that the drug appeared to select

for fibroblasts characterised by enhanced levels of protein synthesis and collagen production. It is not, however, certain whether it is phenytoin or its major metabolite, 5-(parahydroxyphenyl)-5-phenylhydantoin (HPPH) converted by liver hydroxylases, that causes the gingival hypertrophy (Hassell and Page, 1978).

In contrast to the idea that phenytoin specifically stimulated collagen synthesis, Bergenholtz and Hånström (1979), using radioactive labelling methods, found that phenytoin did not influence collagen or non-collagen synthesis but that the effect was on the rate of collagen degradation. Hånström et al (1979), using cat palatal mucosa in organ culture, found that phenytoin caused a 36% inhibition of the cumulative release of hydroxyproline into the medium, i.e. a decrease in collagen degradative products. Simultaneously there were 23% and 30% reductions in β -glucuronidase and lactate dehydrogenase respectively, which Hånström et al regarded as markers for lysosomal and cytosolic enzyme levels. They concluded that phenytoin, or its chief derivative HPPH, may interfere with collagen degradation by blocking enzyme release from cells.

Hassell (1982) again demonstrated that fibroblasts from phenytoin-enlarged human gingivae secreted 100% more extracellular protein, including collagen, as determined by radioimmunoassay. However despite the elevated levels of collagenase, Hassell found no difference in the specific activity of control and treated cells: for some reason the procollagenase activation step was blocked by the effects of phenytoin. Hassell postulated several mechanisms by which this might occur and concluded that the drug probably selects for a subpopulation of gingival fibroblasts, which are inherently capable of increased protein synthesis.

There is therefore some evidence that phenytoin does affect collagen catabolism in gingival fibroblasts by inhibiting various factors and enzymes involved but the exact mode of action of phenytoin is uncertain. There is no recorded evidence that phenytoin can cause sclerotic changes in the otherwise normal skin of epileptics, as might be expected if human skin collagenase is affected simultaneously.

PHENYTOIN AND EB

Despite this lack of any link between human skin collagenase and phenytoin, Eisenberg et al (1978) used pharmacological doses of phenytoin to treat two EBDr patients and also conducted in vitro experiments on 5 EBDr patients, 2 EBDD patients, and 2 EBD patients of unknown genetic heritage.

The clinical trial showed phenytoin to have dramatic results with only two weeks of therapy. Blister counts dropped from 32 to 3 blisters per week and the collagenolytic activity of the skin assayed simultaneously seemed to fall sharply. However the graph in the text indicated that the effect required six weeks rather than two to appear. A second patient also showed dramatic decreases in blistering.

Eisenberg et al measured the collagenase and collagen peptidase activity in skin samples, obtained by removing the roof of a freshly-induced blister. The sample would therefore contain very little dermis, if any at all, in EBDr patients where the blister forms just beneath the basal lamina. It should also be remembered that Bauer et al (1977) found elevated levels of immunoreactive skin collagenase in extracts of blistered skin from EBDr, EBDD and EBL patients but using culture techniques only EBDr strains gave consistently elevated collagenase levels (Bauer and Eisen, 1978): the possibility being

that induction of a blister provides sufficient disruption of the skin to alter collagenase levels in any type of EB, including EBDD which Eisenberg included in his study.

Predictably, Eisenberg's group found elevated collagenase activity 3 - 24 fold in the blistered skin of their heterogeneous group of EB patients, and a 19 - 52 fold increase in collagen peptidase activity compared with two normal control samples.

To examine the direct inhibitory effect of phenytoin in vitro, Eisenberg et al used skin collagenase from two dystrophic patients, one of which may not have been a recessive type. The collagenase activity in these patients was not assessed against any normal controls. Furthermore the patients do not seem to have been included in the previous enzyme analysis, making comparisons of collagenase activity dubious especially as Eisenberg et al used (^{14}C) collagen substrates of different specific activity. Nevertheless a single, but different, drug concentration was used with samples of collagenase extracted from the two patients. The result showed that in one patient 55 μM phenytoin caused a 27.4% inhibition and in the other patient 110 μM phenytoin caused a 51.05% inhibition: no experimental errors or statistical information regarding the reliability of these results were provided.

Eisenberg et al concluded that phenytoin inhibits collagenase and collagen peptidase in pharmacological doses. Unfortunately there is, in my opinion, insufficient evidence in this paper alone to substantiate such a conclusion, although the findings warrant further experimental investigation.

Bauer et al (1980) performed a clinical trial based on Eisenberg's conclusions using phenytoin at a dose of 8 $\mu\text{g}/\text{ml}$ in blood

in 17 patients with EBDr. The response was variable: 5 of the 17 gave no response and could not be attributed to the clinical state of the patients. The 12 patients who responded with a reduction in blisters, did seem to show dose-dependence. Bauer et al also examined the direct effect of phenytoin on purified human collagenase: contrary to Eisenberg's results he found no inhibition. Bauer postulated that phenytoin may act on another protein that may secondarily inhibit collagenase: phenytoin added to human skin explants in culture caused a decrease in collagenase activity, paralleled by an inhibition of immunoreactive collagenase protein in the medium, which was dose-dependent. This suggests that phenytoin diminishes synthesis and/or secretion of the enzyme. Furthermore no change was found in total protein synthesis at these drug doses. These findings conflict with those of Hassell (1982) regarding the effect of phenytoin on collagenase production in gingival fibroblasts.

Long term trials of more than 75 weeks clearly show that the EBDr patients can be divided into those who respond to treatment and those who do not (Bauer and Cooper, 1981).

As in the studies of gingival hyperplasia it seems that phenytoin elicits changes in only certain individuals suffering from EBDr. Consequently it is difficult to assess the beneficial affects and to determine why only certain individuals are predisposed to its influence.

VITAMIN E AND COLLAGENASE

Vitamin E (D-alpha-tocopherol) has been less extensively studied in the treatment of epidermolysis bullosa. Only a few trial studies have been recorded (Wilson, 1964; Ayres and Mihan, 1969; Sehgal et al, 1972; Smith and Michener, 1973 and Michaelson et al, 1974).

Wilson (1964) used the drug to treat a severe case of EBD_r with some improvement whilst Ayres and Mihan (1969) successfully treated a patient with the Weber-Cockayne variant (simplex). In a double-blind cross-over study Smith and Michener (1973) showed that two sisters with a "mild form of recessive EBD", underwent a convincing amelioration in blistering whilst on vitamin E treatment.

Michaelson et al (1974) using three patients with unclassified dystrophic EB examined the effect of vitamin E on the disease and the skin collagenase levels in two patients before and during treatment. One patient remained free of blisters for 4 months, the second for 12 months. In the third patient bullae began to reappear after three months and an increase in vitamin E dosage was ineffective. The collagenase levels in skin explants of two patients studied showed that initially there was a 15% and 21% level increase of collagenase activity in involved skin in terms of percentage of labelled collagen solubilized after 4 days at 37°C. After 30 days of vitamin E therapy this was reduced to 3% and 2% respectively. Unfortunately the authors do not specify whether the pre-treatment skin sample was of a blister (involved predilected) and whether the sample during treatment was not (uninvolved predilected), as might be expected considering the clinical improvement recorded. Also adequate controls were lacking.

There is little other evidence to suggest that vitamin E affects collagenase levels. The work of Brown et al (1967) suggests that the metabolism of collagen in the skin of vitamin E-deficient rats was abnormal: rats raised on a vitamin E-deficient diet had more soluble collagen in their skins than their controls. This collagen did not form gels as readily in vitro, nor were the gels as stable at 4°C, as gels from normal rats. Vitamin E may therefore influence the

formation of intermolecular and intramolecular cross-linkages in collagen fibrils either directly or indirectly through changes in GAG metabolism. The mechanism is uncertain and whether this is the primary affect of vitamin E on skin is unknown.

METHOD

COLLAGENASE INVESTIGATIONS

The aim of the following experiments was firstly to determine whether phenytoin and vitamin E are capable of influencing collagenase activity directly; secondly to compare collagenase status of representative strains from each variety of EB with control cell lines.

Before testing the drugs against commercial (bacterial) collagenase, or the collagenase in fibroblast culture medium, it was necessary to prepare a radioactively-labelled collagen substrate. This was done by the method of Lefevre et al (1979) using (^3H) acetic anhydride to label the acid-soluble collagen. The collagenase activity reported is therefore a measure of the (^3H) counts left in solution after the residual undigested collagen fibrils have been precipitated. It was necessary to test the substrate against non-specific protease degradation and for background levels of radioactivity following the labelling procedure. Trypsin was used as a non-specific protease, fibrillar collagen being highly resistant to attack because of triple helical conformation.

Substrate labelling method

Two hundred mg of acid-soluble calf skin collagen, (Type III) (Sigma Chemical Co. Ltd.) were solubilised in 90 mls of 0.01% acetic acid by stirring overnight at 4°C . The pH of the collagen solution was adjusted to 8.0 with 1M NaOH and 1M K_2HPO_4 and this pH was maintained throughout the reaction procedure with adjustment when necessary.

Three mls of benzene were added to the 250 μl of acetylating

agent, (^3H) acetic anhydride (100mCi/ml; 500 mCi/mmol: Amersham International). One hundred μl aliquots of the diluted (^3H) acetic anhydride were then added to the collagen solution at 6 minute intervals over a period of 3 hours. The solution was kept at 4°C and stirred continually.

One hour after the final addition of acetylating agent the reaction was stopped by adjusting the pH to 4.0 with glacial acetic acid. The benzene was then removed by gently bubbling nitrogen through the solution for 4 hours at 4°C .

Excess radioactive label was removed by dialysis at 4°C against distilled water (15 l/day) for 30 days. The final dialysis was against 5 l of 0.01% acetic acid for 24 hours. The labelled collagen solution was then stored at 4°C .

The specific activity of the mixture was measured using a Packard Tri-carb 300 liquid scintillation counter. Twenty five μl aliquots were added to 1 ml of water and measured in 10 mls of scintillant composed of Triton X-100 (Koch-Light Laboratories Ltd.) and NE 233 (Nuclear Enterprises Edinburgh) in a 1:2 ratio.

To test the purity of the substrate the action of 50 μg trypsin (Type III bovine pancreas, Sigma Chemical Co. Ltd.), in 25 μl Tris-HCL buffer containing 5 mM CaCl_2 pH 7.5 was compared to a range of collagenase concentrations (Advanced Biofactures Corporation, U.S.A.) chromatographically purified form III collagenase and a Tris-HCL buffer blank. The collagenase from ABC has previously been found to be largely free from other non-specific proteases (Miller and Udenfriend, 1970; Verbruggen et al, 1981). It should be noted that bacterial collagenase will attack the non-helical α chains at many loci. This differs from human skin collagenase which only cleaves native, triple

helical collagen specifically at a position one quarter of the distance from the COOH terminal end of the molecule.

Assay for purity of substrate - method

Test solutions containing 0.5 Units (U) - 250 U of collagenase in Tri-HCL buffer were added to 250 μ l (\sim 400 μ g) labelled substrate and the final volume was made up to 775 μ l with Tris-HCL buffer. Similarly 50 μ g trypsin was added to 250 μ l of substrate and 500 μ l buffer added, the blank, necessary to determine background levels of activity, contained 525 μ l buffer and 250 μ l substrate only.

Samples were incubated for 2.5 and 5 hours at 37°C. The residual fibrillar substrate was then precipitated by adding 100 μ l 4% phosphotungstic acid and 100 μ l 4M HCL. After 15 minutes the samples were centrifuged at 8500 g in a Beckman J2-21 centrifuge for 10 minutes. One hundred μ l aliquots of supernatant were used for scintillation counting.

Test for inhibition of collagenase by phenytoin or vitamin E - method

Phenytoin (5.5 diphenylhydantoin sodium salt: Sigma Chemical Co. Ltd.) was dissolved in 50% ethanol (EtOH) so that 25 μ l aliquots were added to 750 μ l test solution gave a final concentration of 50, 100, 150 and 200 μ M phenytoin. Triplicate test solutions contained 250 μ l labelled substrate, 100 U collagenase (A.B.C.) in 500 μ l Tris-HCL-CaCl₂ buffer. Four controls were prepared:

- 1) 100 U collagenase and no drug, to determine the maximum number of counts which could be released during the course of the assay.
- 2) 100 U collagenase and 25 μ l of 50% EtOH alone, to test the effect on the collagenase of the vehicle in which the drug was dissolved.

- 3) 50 μg trypsin to determine the non-specific counts released.
- 4) No enzymes, buffer only to ensure that the background level of radioactivity was constant after preparation of the substrate.

Vitamin E 1D α tocopherol acetate (Sigma Chemical Co. Ltd.), was dissolved in acetone so that 25 μl aliquots added to 750 μl test solution gave a final concentration of 10, 50, 100 and 200 $\mu\text{g}/\text{ml}$ of vitamin E. As described above the solutions were in triplicate, using 250 μl of labelled substrate and 100 U collagenase in 500 μl Tris-HCl-CaCl₂ buffer. Controls were those previously described except that acetone replaced 50% EtOH as the drug vehicle.

All the tubes were then incubated at 37°C for 2.5 hours.

The substrate was precipitated by adding 100 μl 4% phosphotungstic acid and 100 μl 4M HCl. After 15 minutes the samples were centrifuged at 8500 g for 10 minutes. One hundred μl aliquots of supernatant were counted in a Packard scintillation counter as previously described.

Assessment of collagenase status in representative cell strains - method

Each human skin fibroblast line, Table 10, obtained and cultured as previously described, was seeded equally into three large plastic flasks (175cm²).

TABLE 10

CELL LINES USED IN COLLAGENASE STUDIES

NORMAL	PASSAGE NO.	EBDr	PASSAGE NO.	EBS	PASSAGE NO.	EBDD	PASSAGE NO.	EBL	PASSAGE NO.
NF5	P5	EB1	P4	EB9	P9	EB16	P8		
HSF14	P5	EB2	P4	EB12	P5	EB17	P9		
HSF22	P7	EB14	P6	EB29	P4	EB25	P5		
HSF26	P7	EB18	P4	EB30	P7	-		EB26	P5

The cells were routinely cultured in complete medium containing 10% foetal calf serum, in an atmosphere of 5% CO₂ in air at 37°C. Medium was changed thrice weekly and the cultures observed daily. At confluence, the medium was removed and the cells were washed thoroughly with 5 changes of PBS. A minimum amount (20 mls) of serum-free medium was applied to the cells and the cultures were re-incubated for 72 hours. The medium was then removed from the cells and the media from the 3 flasks of each cell line pooled. The total cell number was estimated by counting an aliquot of cells in the Coulter counter.

The pooled medium from each cell line was concentrated 10-fold to a final volume of 6 mls by vacuum dialysis at 4°C through Millipore immersible-CX ultra filters, nominal pore size 10,000 daltons. The concentrate was then dialysed against Tris-HCl-CaCl₂ buffer for 24 hours at 4°C. The resulting preparation was either assayed immediately or stored at -20°C for a few days until required.

Collagenase assay

The method was based on that of Bauer et al (1975), using several different trypsin concentrations to activate the latent

enzyme in the crude medium extract.

Duplicate 100 μ l aliquots of pre-prepared medium were activated, using final concentrations of 0, 0.2, 0.4, 0.6, 0.8, 1.0 and 2.0 μ g trypsin (Type III bovine pancreas, Sigma Chemical Co.) per sample, at 25°C for 10 minutes. The reaction was then stopped with a 5-fold excess of soya bean trypsin inhibitor (B.D.H.) and the sample was diluted with 275 μ l Tris-HCl buffer before adding 250 μ l of the labelled collagen substrate. Various controls were necessary:

- 1) A blank containing substrate and no crude medium sample to determine background levels of radioactivity.
- 2) Labelled substrate and trypsin at 20 μ g/ml, without trypsin inhibitor, to ascertain the maximum level of counts released from the substrate by any uninhibited non-specific proteases.
- 3) 100 U of collagenase (A.B.C.), to assess the effect of collagenase activity on the substrate and to provide a constant for comparisons between experiments.

The samples were then incubated at 37°C for 4 hours and processed as previously described.

RESULTSSubstrate labelling and purity - Results

The specific activity of the substrate, based on 200 mg protein, was 2.5×10^7 dpm/mg, the total activity in 125 mls being 2.25 mCi. This was comparable with the results of Lefevre et al (1979) who achieved a specific activity of 5.93×10^6 dpm/mg collagen, using an (^3H) acetic anhydride label of 5 fold lower activity (100 m Ci/mmol) than was used in this experiment. The following results are given in terms of the 100 μl aliquots and not the total radioactivity released into the test solution (975 μl total final volume).

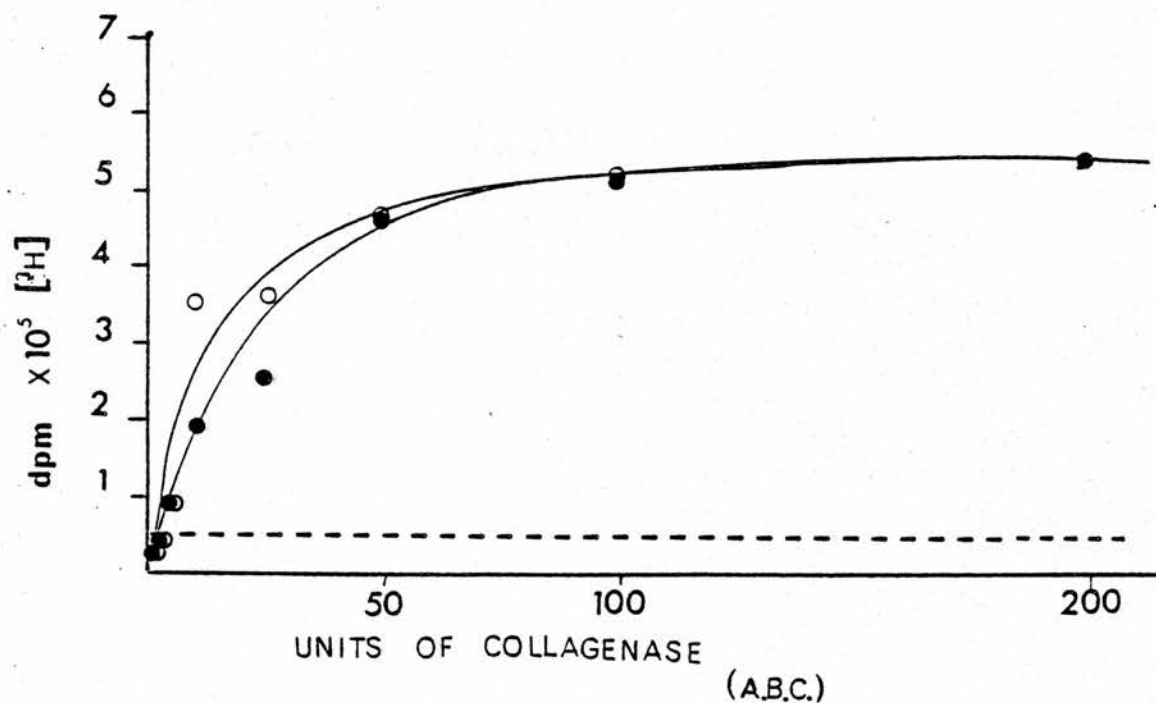
The purity of the acetylated substrate was determined by digestion with the non-specific enzyme trypsin. In 2.5 hours 48,500 dpm were released, representing 9% of the total collagenase labile radioactivity (maximum 5.5×10^5 dpm) as illustrated in Fig. 48. Similarly in 5 hours 10% of the total collagenase-labile radioactivity was released by 50 μg of trypsin. The background, as determined by the sample without enzyme, represented only 2.9% of the total radioactivity released by collagenase (Table 11).

TABLE 11 SUBSTRATE SPECIFICITY

	<u>per 100 μl aliquot</u>
Total radioactivity dpm	1.03×10^6
Maximum collagenase releasable dpm	5.5×10^5
Mean trypsin releasable dpm	4.85×10^4
Background level dpm	2.11×10^4

FIG. 48

Collagen lysed over 2.5 hrs and 5 hrs at 37°C with increasing units of commercial (bacterial) collagenase.



- - ^3H released ($\text{dpm} \times 10^5$) from 250 μl of labelled collagen substrate following incubation with collagenase for 2.5 hrs.
- - as above, following incubation with collagenase for 5 hrs.
- - level of ^3H released following incubation with 50 μg of trypsin for both 2.5 and 5 hrs.

Increasing the incubation period of enzyme and substrate from 2.5 h to 5 h did not appear to alter the total number of dpm released (Fig. 48). This agrees with Lefevre et al (1979) who found that the use of larger amounts of substrate - 570 μg (cf 400 μg) enabled a sensitive detection of enzyme to be made, which was not improved by incubation times longer than 2.5 h; smaller amounts of substrate required much longer incubation times for equivalent sensitivity. Therefore seemingly large amounts of substrate 250 μl (400 μg) were used in the collagenase studies.

Inhibition of collagenase by phenytoin and vitamin E - Results

TABLE 12

	<u>dpm released</u>	<u>\pm SEM</u>	
Blank	18,363	\pm 1,724	CONTROLS FOR SPECIFICITY OF THE SUBSTRATE
50 μg trypsin	50,891	\pm 2,397	
100 U collagenase	478,726	\pm 10,663	
100 U collagenase +			
<u>25 μl 50% EtOH</u>	<u>472,789</u>	<u>\pm 9,601</u>	THE EFFECT OF PHENYTOIN ON BACTERIAL COLLAGENASE
50 μM phenytoin	474,877	\pm 10,260	
100 μM	456,869	\pm 17,287	
150 μM	447,724	\pm 10,190	
200 μM	458,039	\pm 4,171	
100 U collagenase +			
<u>25 μl acetone</u>	<u>524,986</u>	<u>\pm 12,884</u>	THE EFFECT OF VITAMIN E ON BACTERIAL COLLAGENASE
10 $\mu\text{g}/\text{ml}$ vit. E	494,243	\pm 6,060	
50 μg	497,409	\pm 5,550	
100 μg	446,965	\pm 30,679	
200 μg	502,019	\pm 10,506	

From Fig. 49a based on the data in Table 12 it can be seen that phenytoin at 50-200 μM does not impair bacterial collagenase function. Although a small decline in collagenase activity was seen, maximum 5% at 150 μM phenytoin, a finding which was reproducible between experiments; in no experiment was the decline significantly different from control values.

The effects of vitamin E on collagenase were rather curious (Fig. 49b). The results suggest that the acetone in which the drug was dissolved (final concentration 1.6%), may have enhanced the action of the collagenase: 10% more radioactivity was released when 25 μl acetone was included in the enzyme and substrate test solution compared with the enzyme alone. None of the vitamin E concentrations significantly inhibited the activity of the collagenase. These findings were reproducible.

Collagenase status in representative cell lines - Results

Collagenase activity was assessed as dpm collagen substrate released per 10^6 cells. Fig. 50 illustrates the results of individual experiments contributing to the mean result for each concentration of trypsin used in the experiment. The results from each experiment are pooled in Fig. 51, based on Table 13. As the results have been presented as dpm/ 10^6 cells, the data for radioactivity released by the trypsin control is not directly comparable. However the mean value of trypsin dpm was $(40,569 \pm \text{SEM } 1,433)$ compared with the range of dpm released by the prepared media before cell number is taken into account $(44,933 \pm 1,008 - 110,931 \pm 1,869)$. This means that the media released 1% - 13% of collagenase labile radioactivity over the trypsin background level.

The effect of phenytoin on (bacterial) collagenase activity as determined by dpm released from 250 μ l of ^3H collagen substrate after incubation at 37°C for 2.5 hrs. (Vehicle for phenytoin : 50% ethanol.)

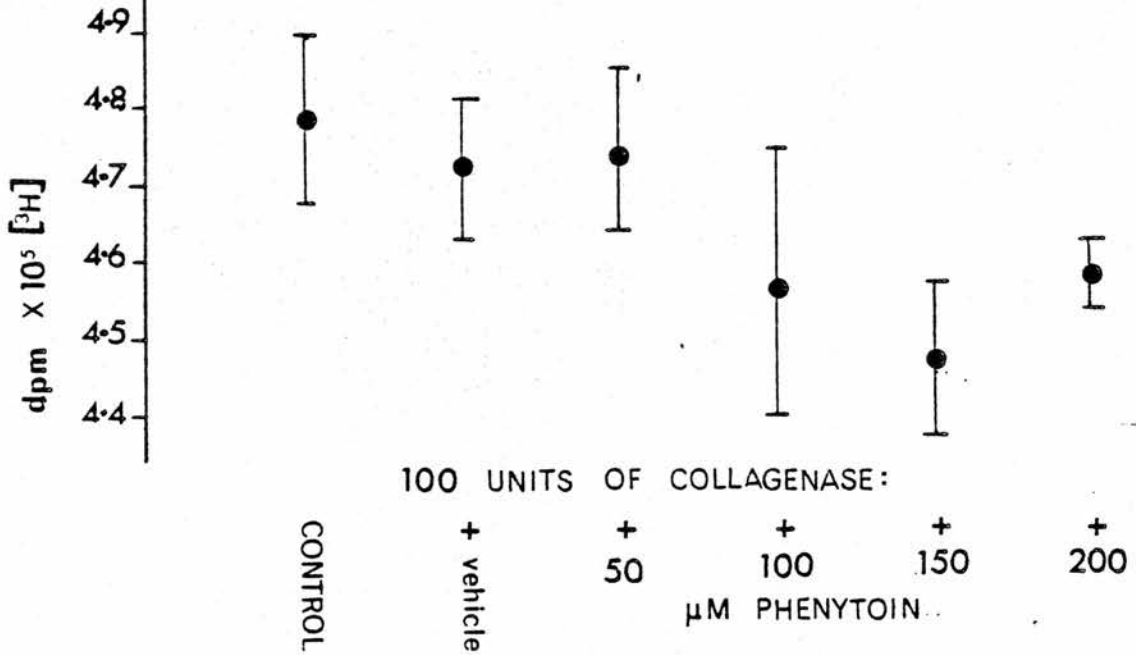


FIG. 49(b)

The effect of vitamin E on (bacterial) collagenase activity as determined by dpm released from 250 μ l of ^3H collagen substrate after incubation at 37°C for 2.5 hrs. (Vehicle for vitamin E : acetone.)

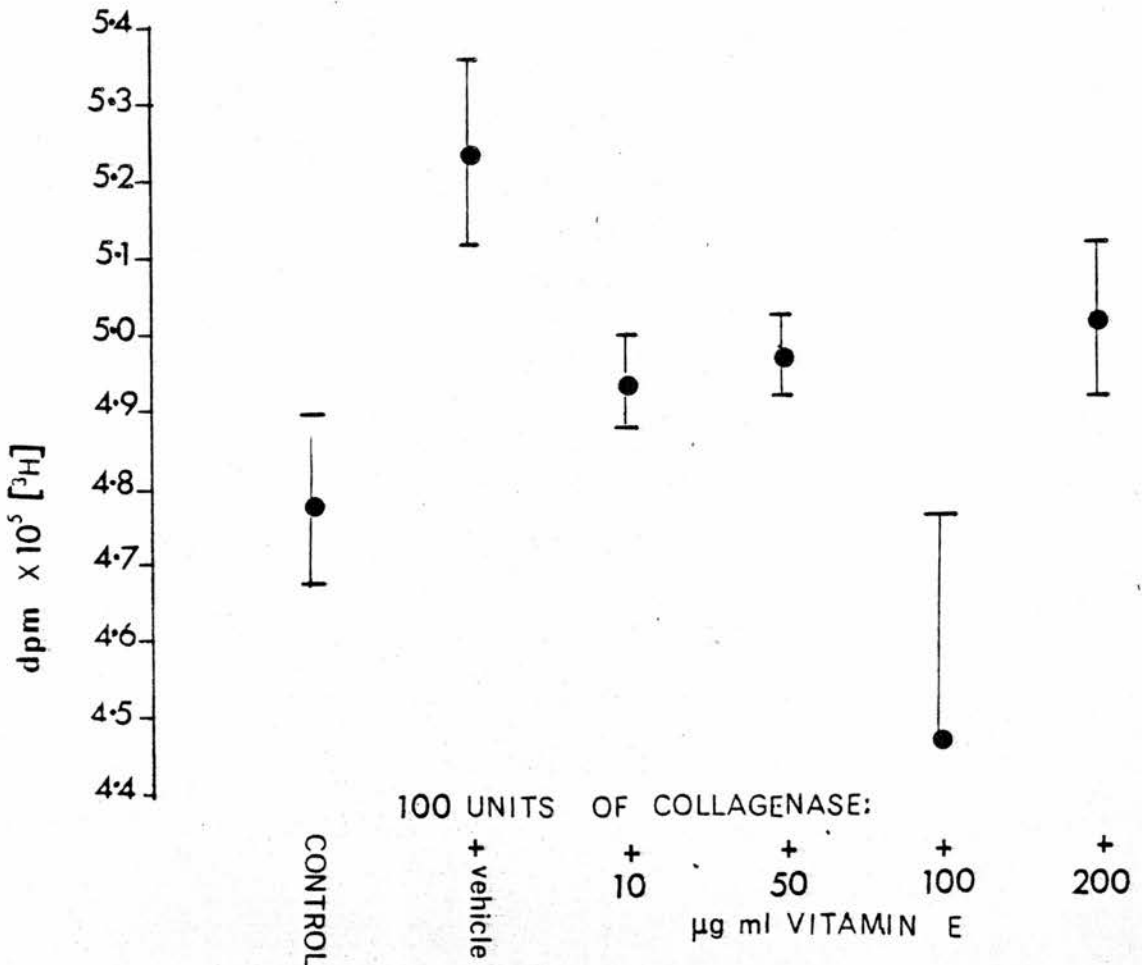


FIG. 50

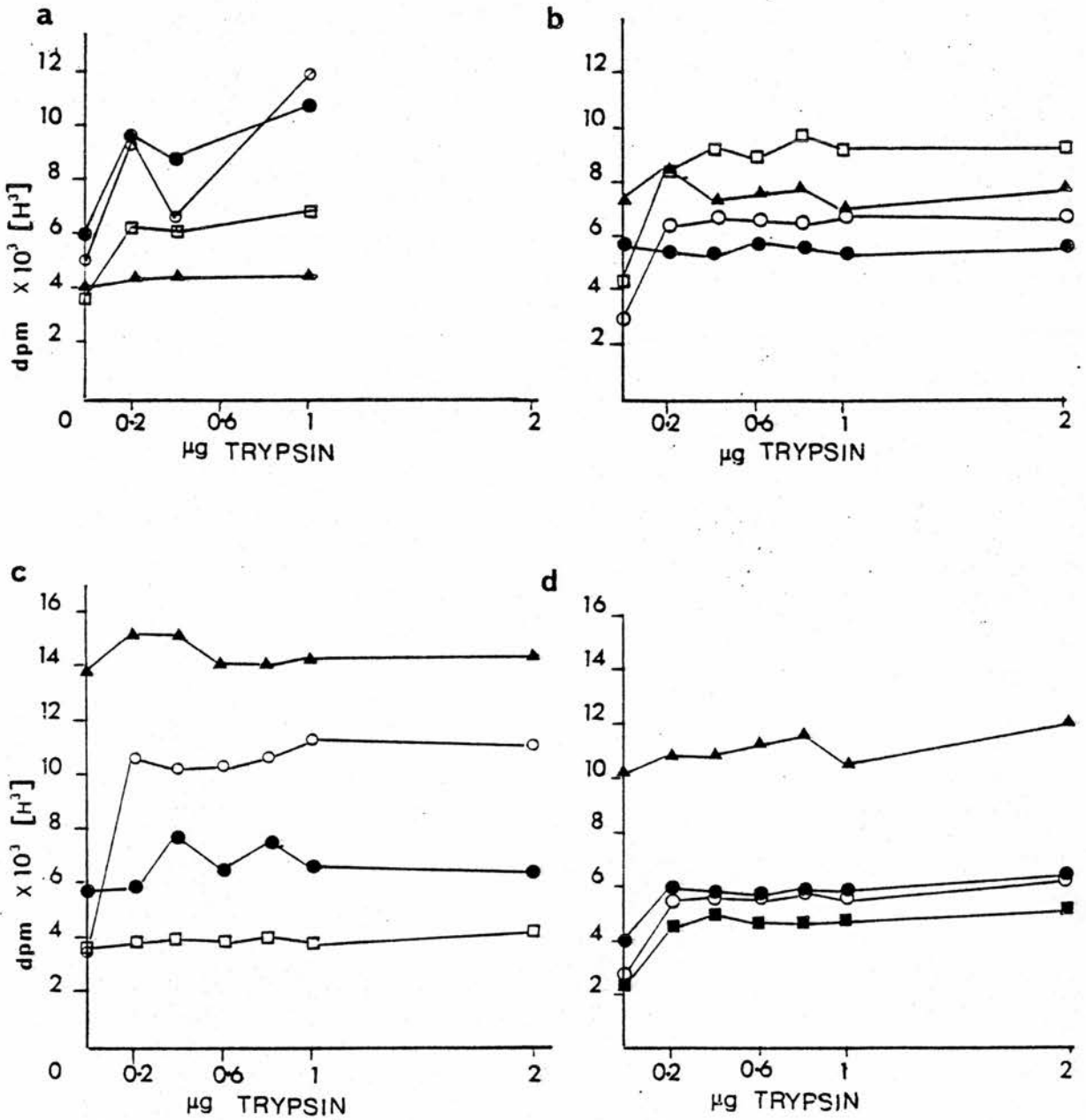


Fig. 50 (a), (b), (c) and (d) illustrates the activation patterns of representative cell lines in four different experiments: ● - controls; ○ - EBS; ▲ - EBDr; □ - EBDD and ■ -EBL. The dpm $\times 10^3$ released from ³H labelled collagen after activation of collagenase with trypsin at 25°C for 10 mins and incubation at 37°C for 4 hrs.

FIG. 51

Mean activation pattern of all the cell lines (control n = 4, EBS n = 4, EBDr n = 4; EBDD n = 3, EBL n = 1) in each group given as dpm x 10³ released from ³H labelled collagen after activation of collagenase with trypsin at 25°C for 10 mins and incubation at 37°C for 4 hrs.

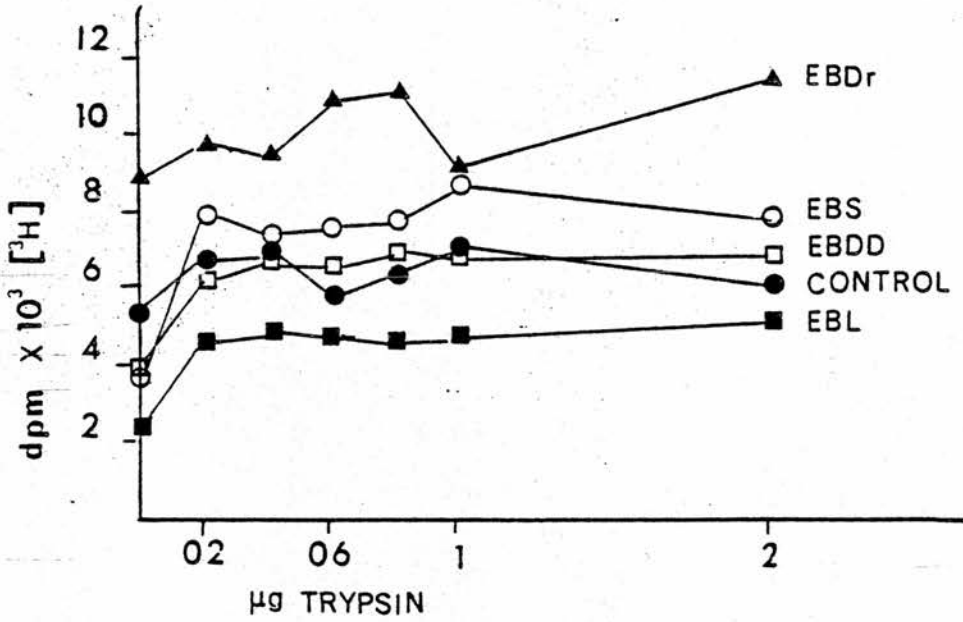


FIG. 52

A comparison of mean initial levels of collagenase activity prior to trypsin activation (blank), with the mean activated level of collagenase for each group after incubation with trypsin (cross hatched) at 25°C for 10 mins (Mean ± SEM) ● - control, ▲ - EBDr, ○ - EBS, □ - EBDD

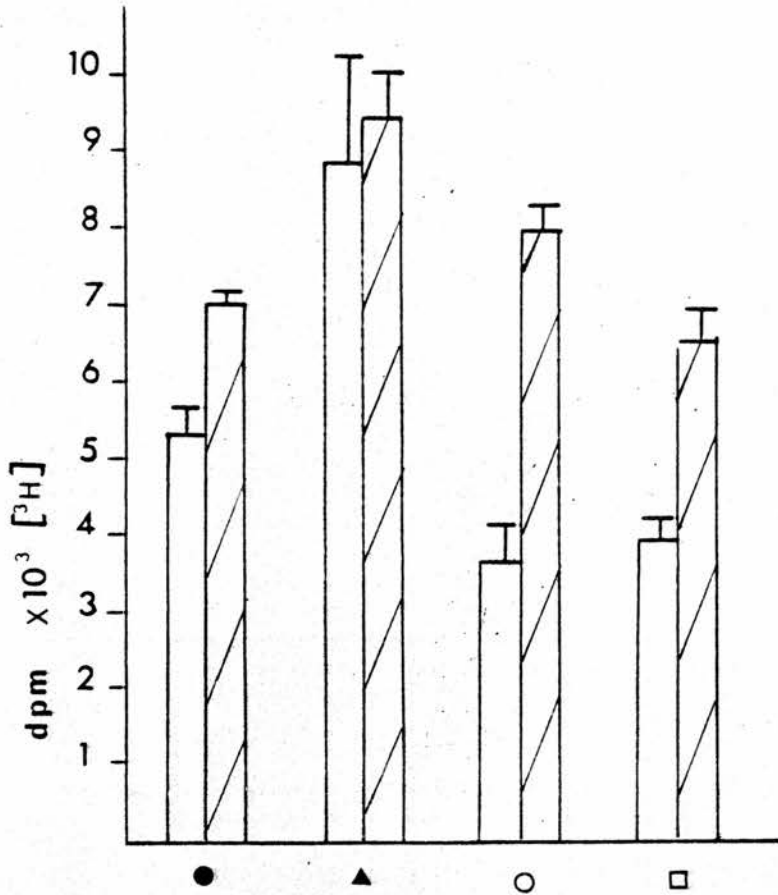


Fig. 50 illustrates the large variation in activated collagenase levels between cell lines and between experiments. This is not because of variation in experimental technique or conditions, as the result for a control of 100 U collagenase remained relatively constant across experiments (mean $393,765 \pm 11,482$). Fig. 51 shows clearly that the levels of collagenase after activation are generally highest in the EBDr cell lines, with cells from the EBS group falling between control and EBDr levels.

Fig. 50 also indicates that the collagenase level is relatively constant regardless of the trypsin concentration used in activation. Therefore to obtain overall levels of collagenase activity in the different groups before and after activation, the results for final levels of collagenase activity have been pooled (Table 14, Fig. 52).

TABLE 14 INITIAL AND MEAN ACTIVATED LEVELS OF COLLAGENASE ACTIVITY IN EB AND CONTROL GROUPS

	NORMAL	EBDr	EBS	EBDD	EBL
Initial level	5286 ± 300	8847 ± 1353	3615 ± 458	3963 ± 153	2349
Activated level of collagenase activity	6931 ± 246	9406 ± 560	7928 ± 308	6518 ± 357	4748

\pm SEM

TABLE 13 COMPARISON OF COLLAGENASE ACTIVITY, AFTER ACTIVATION WITH VARIOUS CONCENTRATIONS OF TRYPSIN,

IN EB AND CONTROL GROUP

MEAN RESULTS FOR ALL THE EXPERIMENTS POOLED

Trypsin conc. ($\mu\text{g/ml}$)	NORMAL	EBDr	EBS	EBDD
0	5286 \pm 453	8847 \pm 2062	3615 \pm 556	3964 \pm 225
0.2	6753 \pm 949	9639 \pm 2255	7989 \pm 1242	6128 \pm 1261
0.4	6844 \pm 790	9418 \pm 2318	7232 \pm 1042	6561 \pm 1525
0.6	5979 \pm 290	10947 \pm 1929	7423 \pm 1450	6450 \pm 2491
0.8	6322 \pm 624	11060 \pm 1879	7681 \pm 1500	6854 \pm 2840
1.0	7096 \pm 1224	9021 \pm 2140	8606 \pm 1450	6657 \pm 1530
2.0	6074 \pm 179	11426 \pm 1918	7988 \pm 1521	6746 \pm 2461

MEAN \pm SEM

In assessing the results it is necessary to take into account the variation between cell lines as well as between groups of cells. A hierarchical or nested analysis of variance was performed on the control and EBDr groups of cell lines, as they apparently showed the greatest mean difference (Fig. 51).

The values in Table 15 show that the probability of the variation between groups being greater than the between cell line variance, was not significant. That is to say that the variation seen between individual cell lines within a group was great enough to mask any significant variation between the normal and EBDr groups, or any other groups. Unfortunately in one of the four experiments (Fig. 50a) the EBDr levels of collagenase activity were lower than the control and both control and EBDr results in this experiment represented the opposite extremes of the group ranges.

(Expts. 1-4 normal range 5022-11205 EBDr 4079-15464

Expts. 2-4 normal range 5022-8743 EBDr 7021-15464).

If this experiment is ignored and a nested analysis of variance applied the results just fail to be significant (Table 16).

TABLE 15

NESTED ANALYSIS OF VARIANCE APPLIED TO EXPTS. 1-4

Source of variation	Sums of Squares	Degrees of Freedom	Mean Squares	F
Between groups normal EBDr	147.055	1	147.055	1.085
Between cell lines within a group	812.897	6	135.483	386.25
Between counts within cell lines	30.867	88	0.351	-

TABLE 16

NESTED ANALYSIS OF VARIANCE APPLIED TO EXPTS. 2-4

Source of variation	Sums of Squares	Degrees of Freedom	Mean Squares	F
Between groups normal EBDr	453.284	1	453.283	6.585
Between cell lines within a group	275.331	4	68.833	98.13
Between counts within cell lines	46.296	66	0.702	-

The probability of variation between groups divided by variation between cell lines within a group being significant is ($0.1 > p > 0.05$) - thus giving some indication of the validity of the difference between EBDr and control cell lines based on a minimal number of experiments.

A non-parametric method of analysis, the Mann Whitney U-test, when applied to the comparative mean results (Table 13) for activation at various concentrations of trypsin, indicated that the EBDr and EBS levels of collagenase activity were composed of independent populations of results, both groups having a p value of 0.001. EBDD did not differ from the controls, having a p value of 0.469. This method of analysis does not take into account the variation within the mean results but again indicates a difference between EB groups and controls. Obviously several more comparative collagenase assays are necessary to make a statistically significant and therefore conclusive statement about the differences between normal and diseased cell lines.

Fig. 50 also shows an interesting difference between the amount of activation of procollagenase produced by the smallest (0.2 μ g) concentration of trypsin. The results show that there is the greatest difference between initial levels and activated levels of collagenase in the simplex group (Table 17).

TABLE 17 COMPARISON OF THE DIFFERENCE BETWEEN INITIAL AND ACTIVATED LEVELS OF COLLAGENASE ACTIVITY IN EB AND CONTROL GROUPS

	Initial levels	0.2µg trypsin activated level	Difference	% increase	Mean ± SEM
Control	5618	9568	3950	70	30 ± 18
	5705	5470	-235	-4	
	5884	5999	110	2	
	3938	5974	2036	52	
Simplex	5195	9543	4348	84	124 ± 26
	3064	6448	3384	110	
	3535	10603	7068	200	
	2665	5362	2697	101	
EBDr	4143	4354	211	5	9 ± 2
	7304	8273	969	13	
	13840	15100	1260	9	
	10100	10828	728	7	
DD	3895	6133	2238	57	52 ± 23
	4382	8309	3927	90	
	3614	3943	329	9	

The Mann-Whitney U-test, which ascertains whether two independent groups have been drawn from the same population, was used to test the significance of comparative percentage increase in collagenase activity, because it can be applied to very small samples.

The results confirm that the simplex group is an independent population, $U = 0$, where $n_1 = 4$, $n_2 = 4$, $p = 0.014$. A significant difference therefore exists between these two groups, control and simplex, in their activation characteristics, but not between control and EBDr groups ($U = 8$, $p = 0.557$), or between the controls and EBDD. Within a group the change in collagenase activity can be tested for significance using a paired-sample t test. Although the number in each group is small ($n = 4$) the following results were obtained:

Controls	$t = 1.312$	Not significant
EBS	$t = 3.943$	$p < 0.05$
EBDr	$t = 3.08$	$p < 0.1$
EBDD	$t = 1.70$	Not significant ($n = 3$)

It is therefore possible to say that the difference in collagenase activity is significantly increased by trypsin activation at the 95% confidence limits in EBS. Thus in the EBS group there is a large significant change in activity and in no other group is this change statistically significant.

DISCUSSION

Neither phenytoin nor vitamin E had a direct effect on the activity of bacterial collagenase in this system. Eisenberg et al (1978) used collagenase extracted from the skin of two patients with epidermolysis bullosa to test directly the effect of phenytoin on activity. As has already been noted, adequate controls were lacking in his experiments, but Eisenberg concluded that at 55 μM there was a 27.4% inhibition and at 110 μM a 51.05% inhibition of collagenase activity. These drug concentrations compare with the concentrations used in the present investigation where there is no evidence that phenytoin has the direct effect claimed by Eisenberg. This conclusion therefore agrees with Bauer et al (1980), who found no inhibition in enzyme activity at approximately 30 μM , with maximal inhibition of purified skin collagenase being 5.5% at about 70 μM phenytoin. Bauer's data indicated that phenytoin affected the skin fibroblast itself, in culture, and by doing so decreased the synthesis or secretion of collagenase.

It would be interesting to determine whether the major metabolite of phenytoin, 5-(parahydroxyphenyl-5-phenyl-hydantoin) also has this affect on the synthesis of collagenase by fibroblasts in vitro, particularly as HPPH can cause gingival hypertrophy in cats (Hassell and Page, 1978).

There is no conclusive experimental work, to date, involving vitamin E in collagenase metabolism or activity. The results of this experiment indicate that vitamin E does not act directly on collagenase. Curiously it seems that the vehicle acetone alters the collagen substrate; perhaps by making the site of enzyme attachment and action more readily available to the collagenase, or

by affecting the collagenase molecule itself, and improving its efficacy. This finding is purely an artefact of the experimental design. Taking into account the effect of the vehicle alone on the collagenase, vitamin E failed to produce any significant diminution in collagenase activity in this experimental system.

Although the collagenase activity in too few cell lines has been determined to enable categorical statements, there is sufficient evidence to give a strong indication of qualitative differences in collagenase levels between the various groups.

Clearly there is considerable variation between cell lines within a group (Fig. 50). The nested analysis of variance showed that the variation between cell lines within a group was statistically significant and therefore sufficient in any other parametric analysis to nullify variation seen between groups. Consequently although the within-group pooled results for non-activated and activated collagenase (Table 15), and also the mean values plotted for each trypsin concentration (Fig. 51), indicate that the EBDr cell lines have a higher initial collagenase level and also remain above the control group when activated, it is not possible to perform a parametric test (t test) to substantiate the findings. Similarly the simplex group, which appears to have lower levels of collagenase prior to activation than the controls, apparently contains greater levels of procollagenase and is therefore more markedly stimulated by trypsin, the final collagenase activity exceeding that of the control group. The non-parametric method of statistical analysis, the Mann-Whitney U-test was used to determine whether the pooled results (Table 13, Fig. 51) were independent of each other. By this method the EBDr

and EBS mean activated collagenase results were significantly different from the control ($p < 0.001$) whilst EBDD did not significantly differ from the control.

Such heterogeneity in results has been recorded before by Bauer and Eisen (1978), who found great variation within groups. Their EBDr group varied not only in collagenase activity but also in levels of immunoreactive collagenase present in culture medium. In one out of four experiments in the present study, the EBDr cell line appeared to have less collagenase activity than the controls. Interestingly three out of eight in Bauer and Eisen's study (1978) also had lower activity than controls. It is also noteworthy that where the EBDr cell lines in Bauer's investigations were similar or above normal, the magnitude of increase above control levels (28%) was not dissimilar to that found in the present study (36%). The tentative conclusion is therefore that the EBDr cell lines do possess greater levels of collagenase activity prior to and following activation in vitro but that the scale of the difference is not very great and is highly variable.

Bauer and Eisen (1978) also examined EBS, EBDD and EBL for levels of immunoreactive collagenase i.e. total collagenase present whether active or not. They found that these three groups and controls showed no significant difference in levels of collagenolytic protein present as detected by a specific antibody against the enzyme. In the present study the EBDD group did not seem to differ from controls in the amount of active collagenase present, neither did the single letalis cell line. However the EBS group is intriguing in that although the initial level of collagenase, prior to activation, appears to be within the normal range, once activated elevated levels of collagenolysis can be detected compared with controls. As Bauer

and Eisen failed to detect any difference in the simplex group using immunoreactive techniques, it is interesting to speculate whether some of the procollagenase in this group is masked by an inhibitor of its activity, which upon exposure to trypsin renders the zymogen active. The presence of such an inhibitor-enzyme complex might alter the antibody recognition site (epitope) on the procollagenase molecule, preventing detection by immunoreactive techniques. The idea that there is some endogenous inhibitory or controlling factor in the media of cultured fibroblasts is not new. Bauer (1977a) found a disproportionate (6-10 fold) increase in enzyme activity compared with 2 fold levels of immunoreactive protein, just after the cells had reached confluence; the point being that although collagenase synthesis and secretion doubles, some other element in the control of activity diminishes thus enabling collagenase activity to increase 6-10 fold.

It is possible that collagenase or procollagenase from the simplex group is complexed with an inhibitor or somehow compromised so that trypsin activation had a greater effect than on any other group. The Mann-Whitney U test indicated that only the simplex group, when compared with the control, represented a significantly different population of results ($p = 0.014$). Similarly using a paired t-test, the difference between levels of activity before and after activation was only significantly different in the simplex group ($p < 0.05$). The EBDr cell lines although showing a consistent activation pattern demonstrate only a very slight increase in activity (9%) upon exposure to trypsin. The reason for this is probably the increased thermolability of the EBDr procollagenase enzyme, due to its reduced affinity for the Ca^{++} ion (Bauer, 1977b; Stricklin et al, 1982).

Alternatively it could be due to the diminished production of the inhibitor postulated to be responsible for the altered simplex activation characteristics.

The relationship of these results to the situation in vivo can only be a matter of speculation at present. Clearly an excess of thermally-unstable collagenase, with a preference for Type III collagen in the dermis, has the potential to produce blisters with cleavage in the papillary dermis. The relationship between collagenase levels and the presence or absence of anchoring fibrils remains uncertain. The electron microscopy in the present study suggests that with one exception (Fig. 27) in the severe form of EBD_r, no anchoring fibrils are present. However in a milder localised form of EBD_r (EB31) anchoring fibrils can be seen at the apex of a tightly convoluted dermo-epidermal junction (Fig. 28). As collagenase levels are higher in the involved skin of EBD_r and in severe EBD_r any skin taken for ultrastructural purposes spontaneously becomes "involved" i.e. blisters, the likelihood of finding intact anchoring fibrils, if they are susceptible to collagenase, is greatly diminished. Hence they may be present in mild EBD_r where the skin remains virtually intact on biopsying.

The results of this study and those of Bauer and Eisen (1978) indicate that EBDD cells in culture resemble controls in their collagenolytic activity. The uninvolved skin of EBDD patients in vivo also showed no significant difference in activity (Bauer et al, 1977). Blister formation was associated with significant increases in tissue levels of immunoreactive human skin collagenase however. Unfortunately Bauer et al give no data for the collagenase levels in a friction-blistered control group, the possibility being

that induction of a blister may automatically alter collagenase activity in that area. The elevated collagenase levels of EBDD in involved skin would therefore be a secondary consequence of blister formation occurring for other reasons.

Collagenase production in the EBS group remains an intriguing and unsolved problem. Blistering in this group occurs in the epidermis with no evidence of dermal involvement and yet the results of this study suggest an excess production in the dermis, not necessarily excess or increased activity. Perhaps the simultaneous presence of an inhibitor prevents the full potential of the collagenase being expressed and thus prevents formation of blisters within the dermis. The role of the dermis, if it has one, in blistering within the epidermis remains completely obscure.

SUMMARY

Collagenase exists as a pro- and active enzyme, the conversion being made in vitro by trypsin. Autoactivation can occur. EBDr cell lines are known to produce excess amounts of aberrant collagenase with a reduced catalytic efficiency. Phenytoin has been found to affect collagenase activity in some EBDr cell lines in vitro and to be beneficial to only some EBDr patients clinically. Little is known about vitamin E and it has been used to treat various types of EB with varying degrees of success.

The findings of this investigation suggest that:-

- 1) Neither phenytoin nor vitamin E directly inhibits the action of bacterial collagenase on a labelled collagen substrate.
- 2) EBDr cell lines have elevated levels (~ 36%) of collagenase activity over controls.
- 3) Little change is seen on activation of EBDr collagenase indicating that most must already be present in an active form.
- 4) There is large variation between cell lines in levels of collagenase activity.
- 5) The EBS levels of collagenase fall between EBDr and control levels but were not significantly greater than controls.
- 6) The EBS group undergoes a significant increase in levels of active enzyme after trypsin activation, with a 124% increase compared with a 30% increase in activity in controls.
- 7) The EBDD group and single EBL cell line failed to differ from the controls with respect to the characteristics examined.

CHAPTER 5

GLYCOSAMINOGLYCANS

GLYCOSAMINOGLYCANSINTRODUCTION

Apart from collagen, the other major constituent of the dermis is the "ground substance" or extracellular matrix. This matrix is composed of a variety of complex macromolecules vital to the regulation and maintenance of the integrity of the dermis and of the skin as a whole. These macromolecules are predominantly proteoglycan consisting of polysaccharide covalently linked to protein. The polysaccharide constituent chains, collectively known as glycosaminoglycans (GAG), include hyaluronic acid, chondroitin 4 and 6 sulphates, dermatan sulphate, keratan sulphate, heparan sulphate and heparin (reviewed by Lamberg and Stoolmiller, 1974; Kennedy, 1979; Silbert, 1982).

Proteoglycans were previously termed "mucopolysaccharides", before the importance of the protein backbone was fully appreciated. Mucopolysaccharides were introduced to describe hexosamine-containing polysaccharide materials of animal origin, usually associated with viscous secretions. The term "mucopolysaccharide" is less frequently used today, being imprecise. The term "glycoprotein" covers a range of covalent carbohydrate-protein macromolecules of similar composition, which have been classified according to their biological function and are a separate group from the proteoglycans, as understood today.

Glycosaminoglycans form a chemically related group, each GAG consisting of a chain of alternating monosaccharide units. These are an acidic monosaccharide, D-glucuronic acid or L-iduronic acid (except for keratan sulphate which contains galactose rather than uronic acid) and a basic monosaccharide, galactosamine or glucosamine. These monosaccharides are either N-acetylated or

N-sulphated: the resultant disaccharide therefore has acidic properties. Variation between the various GAG types occurs in differences in: polysaccharide chain length, the extent of sulphation, the position of the sulphate groups, the relative proportion of the uronic acids and differences in mode of linkage to protein.

Hyaluronic acid and chondroitin are the only non-sulphated GAGs occurring naturally; hyaluronic acid being a polymer of repeating disaccharide units of N-acetyl-D-glucosamine and D-glucuronic acid, (chondroitin is an isomer of hyaluronic acid). The chain length of HA can vary from 100-20,000 disaccharide units depending on the tissue from which it was extracted. Embryonic tissues, vitreous and synovial fluids are rich in hyaluronic acid and it represents ~50% of the GAG in skin.

The chondroitin 4 and 6 sulphates are the sulphated variants of chondroitin, consisting of N-acetyl-D-galactosamine and D-glucuronic acid. They exhibit considerable structural heterogeneity both with respect to the degree of sulphation and chain length. The chondroitin sulphate chains are much shorter than those of hyaluronic acid, being composed of 20-100 repeating disaccharide units. The chondroitin sulphates are the major GAG of cartilage but constitute only 10% of the GAG in skin.

Dermatan sulphate is an isomer of chondroitin 4-sulphate in which the D-glucuronic acid units have been replaced by L-iduronic acid. The structure is different from chondroitin 4-sulphate only in the orientation of the carboxy group on every other monosaccharide residue along the chain. However its physiological properties differ from the chondroitin sulphates. In skin dermatan is sulphated at the 4-position (and forms 40% of the total GAG) and in the umbilical

cord at the 6-position, suggesting that differences in biological function may originate in subtle structural variations.

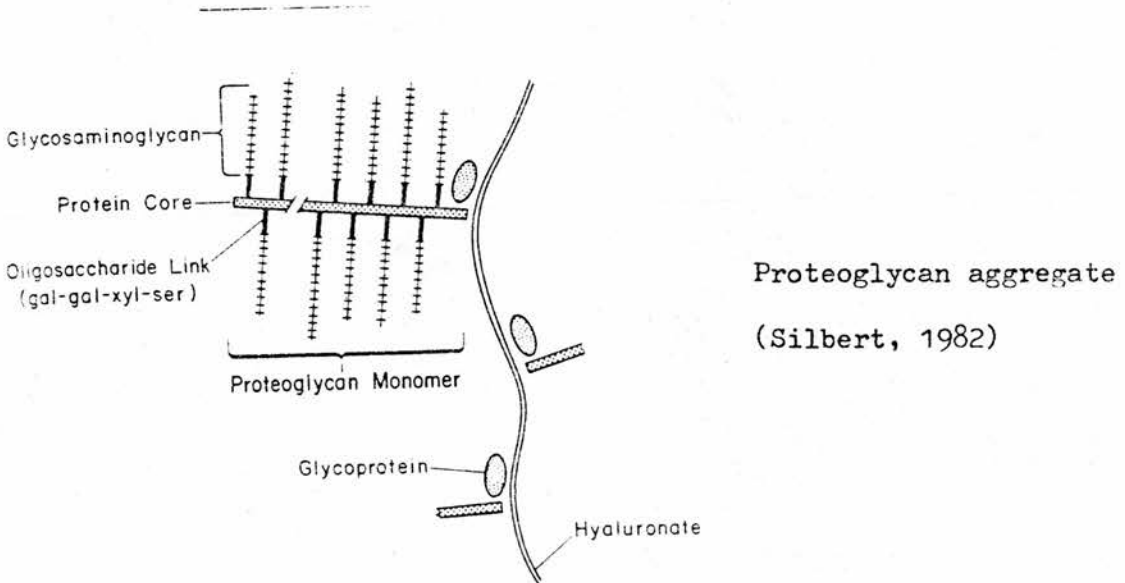
Keratan sulphate contains no hexuronic acid, having instead D-galactose linked to N-acetyl-D-glucosamine. Varying amounts of mannose, fucose, sialic acid and N-acetyl galactosamine, as well as other monosaccharides provide a large degree of structural heterogeneity. Keratan sulphate has not been isolated from the skin.

Heparan sulphate and heparin, consist of alternating D-glucosamine and D-glucuronic acid units, as does hyaluronic acid. However in contrast to the latter, they contain considerable amounts of L-iduronic acid. The glucosamine moieties in heparan sulphate may be N-acetylated or N-sulphated and in heparin nearly all glucosamine moieties are N-sulphated.

The GAG-chondroitin sulphates, dermatan sulphate, heparin and heparan sulphate are each attached to a protein core (Fig. 53) by means of an oligosaccharide link. Each GAG is linked at its reducing end to a trisaccharide of galactosyl-galactosyl-xylose. The latter is linked to the hydroxyl group of serine in the protein chain. As the first sugar of the GAG chain is always glucuronic acid, which is added to the terminal chain by a specific enzyme, differing from the enzyme involved in the incorporation of glucuronic acid into the polysaccharide chain, it is therefore considered as part of the linkage group.

FIG. 53

THE STRUCTURAL RELATIONSHIP BETWEEN THE GAG COMPONENT AND A
PROTEOGLYCAN AGGREGATE AS A WHOLE



The precursors of GAG synthesis are uridine nucleotide-sugars, derived from D-glucose by several monosaccharide interconversions involving pyrophosphorylase type reactions. The intracellular levels of the various sugar precursors are important in the regulation of synthesis due to feedback inhibition. The ordering of the sugar sequence is not specified by a template but by the specificity of the individual enzymes - glycosyl transferases - which catalyze transfer of the monosaccharides to the growing chain.

It is now understood that the core protein and glycosyl transferases are synthesised in the rough endoplasmic reticulum, where the initiation of GAG chains and synthesis of the linkage

region also occurs. The growing chain passes from rough to smooth endoplasmic reticulum and then to the Golgi apparatus where sulphation and completion of the chain occurs. The product is transferred to the exterior by vacuoles.

The half-life of GAGs in vivo has been found to be relatively short. Hyaluronic acid in the skin of young rabbits and rats has a half life of 2-5 days (Schiller et al, 1955), dermatan sulphate 7-14 days in young animals although it may be longer in older animals (Schiller et al, 1956; Davidson and Small, 1963). Chondroitin sulphate has a half life of 17 days in rats.

Since the enzymatic degradation of GAG was first noted following the injection of "spreading factor" from testicular extracts and Indian ink, into skin (Hoffman and Duran-Reynals, 1931), investigations into the enzymes involved in GAG degradation have been conducted. The best known endoglycosidase is testicular hyaluronidase, degrading hyaluronic acid into fragments of various sizes by attacking β -linked N-acetylglucosaminyl groups at random within the chain (Weissman et al, 1954; Weissman, 1955). The chondroitin sulphates are also known to be degraded by "hyaluronidase" (Meyer and Rapport, 1950) although the presence of sulphate groups seems to hamper the speed of the reaction, chondroitin being similar to hyaluronic acid in reaction kinetics. Although hyaluronidase has been isolated from rat skin (Cashman and Laryea, 1969) embryonic chick skin, and chick fibroblast cultures (Orkin and Toole, 1980), it has not been found in human skin fibroblast cultures. No skin endoenzymes capable of degrading dermatan sulphate or heparan sulphate have yet been discovered. Enzymes do however exist in the liver and spleen capable of degrading heparin and heparan sulphate.

A number of exoglycosidases are known to exist including β glucuronidase, galactosidase and N-acetyl-hexosaminidase. These enzymes, by definition, are not capable of cleaving an intact GAG, they attack a terminal non-reducing sugar only after an endoenzyme has begun degradation. Similarly the sulphatases have no action on the intact GAG. The group of mucopolysaccharide storage diseases (the mucopolysaccharidoses), in which there is an excessive accumulation of acidic polysaccharides in tissues (and cultured fibroblasts), accompanied by an altered urinary excretion profile, illustrate the importance of exoglycosidase enzymatic regulation of GAG metabolism by cells. The absence of enzymes such as α -L-iduronidase and β -glucuronidase, as well as other lysosomal glycosidases and sulphatases, have been found to be responsible for eight varieties of mucopolysaccharidoses affecting skeletal and connective tissue as well as mental development (Dorfman and Matalon, 1976; Fluharty, 1982).

Degradation of the core protein of proteoglycans may be important in the turn-over and therefore regulation of GAG synthesis, illustrated by the injection of papain into rabbits' ears. This resulted in rapid loss of rigidity of the ear with a simultaneous mobilization and excretion of GAGs (Thomas, 1964).

The function of GAGs in skin and the significance of their interaction with other components of the dermis are highly complex and not yet completely understood. Taken as a group, GAGs influence the hydration, permeability and elasticity of the dermis (Kennedy, 1979), as well as being involved in the adhesion (Schubert and La Corbiere, 1980) aggregation and motility of fibroblasts in vitro (Underhill and Dorfmann, 1978; Abatangelo et al, 1982).

Hyaluronic acid is the predominant GAG (80-90%) synthesised by skin fibroblasts in culture medium and presumably secreted into the interstitial spaces in vivo (Gallagher et al, 1980; Fukui et al, 1981; Slack et al, 1982). Hyaluronic acid is thought to be chiefly responsible for determining the water content of skin: because of its intrinsic high degree of hydration and interactions between its own molecules, the passage of interstitial water is impeded, consequently increasing tissue rigidity and resistance to compression (Kennedy, 1979).

Investigations into the mechanism of cell adhesion in vitro have shown that GAGs are of importance. It has been found that fibronectin has specific binding sites for hyaluronic acid, heparan sulphate and will bind, though to a lesser extent, heparan sulphate (Yamada et al, 1980). Heparan sulphate is understood to be important in binding extracellular fibronectin to the cell surface, the fibronectin then links the cell to the substratum. However the accumulation of hyaluronate-chondroitin complexes at these adhesion sites appears to cause a destabilisation of binding through interactions between hyaluronic acid and the cell surface fibronectin, possibly by altering or hampering heparan sulphate binding (Rollins and Culp, 1979; Laterra et al, 1980; Schubert and La Corbiere, 1980; Abatangelo et al, 1982). Hyaluronic acid is also known to be important in cell-cell adhesions and interactions (Underhill and Dorfman, 1978).

Although it has become clear that heparan sulphate is important in cell substrate adhesion in vitro (Laterra et al, 1980), the work of Gallagher et al (1980) demonstrates that culture conditions may not accurately reflect the situation in vivo. Their work showed that whilst heparan sulphate was the major sulphated GAG produced by

human skin fibroblasts when cultured on a plastic substrate, dermatan sulphate synthesis could be increased three-fold on a collagen substrate and consequently became the major sulphated GAG synthesised. This work also demonstrated that reduced levels of sulphated GAG present in the culture medium could be attributed to their interaction with the collagen gel upon which the fibroblasts were being grown.

The interaction of GAG with collagen has previously been mentioned (p 98). There is substantial evidence that the organisation of collagen fibrils in tissues, is related to the amount and type of proteoglycan present (Mathews, 1965; Mathews and Decker, 1968; Oegema et al, 1975). It has been shown that the precipitation of collagen fibrils at 37°C is markedly reduced in the presence of small amounts of proteoglycan monomer. The protein core of the monomer does not itself retard fibrillogenesis although it does bind to collagen. Therefore it is the interaction of GAG with collagen that appears to be of significance, the GAGs binding to collagen by electrostatic forces.

The sulphated GAGs have been shown to inhibit leucocytic lysosomal enzyme activity in vitro to different degrees, heparin being the most effective. The type and apparent distribution of the sulphate bond are the important factors in determining the degree of interaction (Avila, 1978). Whether this finding has any significance to fibroblast lysosomal enzyme activity is unknown.

Cultured skin fibroblasts have made a useful model for studying factors which might affect GAG production, both in diseased conditions: such as mucopolysaccharidoses (Frantoni et al, 1968) and EB (Bauer et al, 1979); as well as for examining the constraints of culture conditions: such as cellular ageing in vitro

(Schachtschabel and Wever, 1978; Matuoka and Mitsui, 1981; Vogel et al, 1981); pH of the culture milieu (Lie et al, 1972) and the effects of drugs on fibroblasts in vitro (Priestley and Brown, 1980). However the in vitro model is complicated by the finding that cell density (cells/cm²) can influence the rate of GAG synthesis and secretion in culture, GAG secretion per cell decreasing with increasing cell density (Morris, 1960; Hronowski and Anastassiades, 1980; Oakley and Priestley, 1983). The present investigation confirmed and attempted to take into account the influence of cell density on GAG secretion.

GAG and EB

An abnormality in GAG production has been linked with the pathogenesis of EBDD-Pasini, the variant in which albopapuloid nodules occur on the body. The work of Sasai et al (1973) suggested that in their single patient the nodules occurred because of an increase in the amount of degraded sulphated GAG in the skin. Endo et al (1974) examined the urinary GAG profile of the same patient and found the major GAG present to be partially degraded dermatan sulphate. Lower levels of partially degraded chondroitin sulphate, the major GAG in normal urine, were found. They concluded that the substance accumulated in the skin was most probably dermatan sulphate.

In a more extensive study using seven EBDD-Pasini patients, Bauer et al (1979) were able to show that the disturbance in GAG metabolism was a genetic characteristic of these patients. Their study of cultured skin fibroblasts revealed an increased accumulation of the sulphated GAGs both intra- and extracellularly, probably due to increased synthesis; degradation rates were comparable to control

values. This aberration in GAG metabolism was found to be unique to the Pasini variant of EB. Bauer et al (1979) suggested that the presence of excess sulphated GAGs in the dermis might impair collagen fibril formation resulting in an intrinsic instability which could result in trauma-induced blistering.

The effect of phenytoin on the degradation of sulphated GAGs was examined in organ cultures of cat palatal mucosa (Hänström and Jones, 1979) and in cultures of fibroblasts from both normal and phenytoin-enlarged gingivae (Kantor and Hassell, 1983). The results of Hänström and Jones (1979) indicated that phenytoin inhibited the extracellular degradation of sulphated GAGs without influencing degradation in the intracellular GAG pool. In accordance with the finding that phenytoin reduced collagen degradation (Bergenholtz and Hänström, 1979; Hänström et al, 1979), Hänström and Jones (1979) proposed that the same mechanism inhibiting cytosolic and lysosomal enzyme release, was responsible for the reduced extracellular sulphated GAG degradation. Kantor and Hassell (1983) also found an increase in the accumulation of sulphated GAGs, but ascribed the increase to elevated synthesis and not decreased degradation. This agrees with their previous results indicating elevated anabolic activity in fibroblasts from phenytoin-enlarged human gingivae (Hassell et al, 1976; Hassell, 1982). As phenytoin apparently increases sulphated GAG accumulation, whether by increased synthesis or decreased degradation, it is unlikely to be of any benefit to patients with the EBDD-Pasini variant.

No investigations into the effect of phenytoin on GAG production in either control or EB skin fibroblasts have been made. It was therefore the aim of this investigation to establish the level of

GAG production in various representative cell lines and then to examine the effect of phenytoin at various concentrations on GAG output. Simultaneously it was possible to assess any changes in the proliferation of the fibroblasts induced by the drug.

Phenytoin has been implicated in hyperplastic enlargement of human gingivae. Hassell et al (1976), however, pointed out that "hyperplasia" was a misnomer, as the resident connective tissue cells to not appear to be abnormal in morphology or number (Han et al, 1967). Recently Modeer et al (1982) have demonstrated that the major metabolite of phenytoin 5-(parahydroxyphenyl)-5-phenylhydantoin (p-HPPH) decreased the number of gingival fibroblasts per culture, by interference with the mitotic process, without affecting the total protein and DNA-content of the cultures. In contrast, the experiments of Shafer (1960), using "fibroblast-like" cells originally isolated from human gingivae, indicated that cell proliferation in vitro was stimulated (103%) at concentrations up to 200 µg/ml. Houck et al (1972), using human skin fibroblasts in culture, showed that phenytoin increased cell population doubling times between 2-20 µg/ml, (i.e. decreased proliferation rate), the optimal concentration being 5 µg/ml phenytoin. The recent work of Al-Ubaidy et al (1981) suggests increased mitotic activity in enlarged gingivae isolated from responsive epileptics and also in rat fibroblasts treated with phenytoin in vitro, supporting the view of gingival enlargement as hyperplasia. This work (Al Ubaidy et al, 1981) fails to define the concentration of phenytoin used, the number of cultures and biopsies and no indication of experimental error is given. Thus the question of hyperplasia versus hypertrophy of phenytoin-enlarged gingivae remains equivocal.

The effect of vitamin E on the proliferation and GAG secretion of a group of normal control cell lines was also studied in the present work; no such studies having previously been reported.

METHODThe effect of phenytoin and vitamin E on GAG accumulation and fibroblast proliferation

GAG production was measured in all the fibroblast lines used in the current study. To determine the effect of phenytoin on GAG output and its influence on growth in individual cell lines, 6 lines of control, EBS and EBDr were used:

N	EBS	EBDr
NF5	EB9	EB1
NF6	EB10	EB2
HSF8	EB11	EB5
HSF14	EB12	EB14
HSF17	EB29	EB18
HSF24	EB30	EB31

Fibroblasts were seeded at 10^5 cells/flask in complete medium containing 10% foetal calf serum, into 24 Nunc plastic flasks (25 cm² growth area) and incubated at 37°C in a 5% CO₂ : air atmosphere. The medium was changed on day 1 after seeding. On day 3, 4 flasks were removed and cell numbers determined, following trypsin-versene treatment, using a Coulter counter. Phenytoin at 50, 100, 150 and 200 µM was added to the medium, using 4 flasks per concentration, leaving an untreated group as a control both for GAG output and for fibroblast proliferation relative to day 3. (Similarly in other experiments vitamin E was added at 10, 50, 100 and 250 µg/ml on day 3 to the six control strains examined.) Twenty four hours

later the medium was removed and replaced by "fresh" medium containing the same drug concentrations.

On day 6 after seeding, the medium was removed from the flasks and 1 ml of phosphate buffered saline, used to rinse the cells, was added to each sample. The final cell number was determined after trypsinisation using the Coulter counter. The proliferation of the fibroblast lines was expressed as a percentage of the mean increase in cell numbers between days 3 and 6. Cells treated with drug were compared in terms of the percentage change in growth rate, relative to day 6.

The medium was dialysed against 0.1M sodium acetate buffer pH 4.9 for 3 days before being concentrated to about 3 mls using Lyphogel (Gelman-Hawksley Ltd). Each sample was digested with 2 mg hyaluronidase (Sigma type 1) overnight at 37°C, before precipitation of protein at 4°C in 5% trichloroacetic acid (TCA). After centrifugation at 18,000 g for 30 mins, the uronic acid content of the supernatant was assayed using metahydroxydiphenyl reagent (Blumenkrantz and Asboe-Hansen, 1973) with a glucuronolactone standard. The GAG secreted, in terms of uronic acid output from each culture group, was determined by subtracting the uronic acid content of each batch of medium (assayed along with the experimental samples). Output was calculated as $\mu\text{g}/10^7$ cells/48 hours from the final cell counts.

RESULTS

The relationship between GAG ($\mu\text{g}/10^7$ cells/48 hrs) accumulation in culture medium and cell density can be seen in Fig. 54 using nine lines of control (normal) fibroblasts. This hyperbolic curve can be transformed to a linear relationship by plotting the GAG data on a logarithmic scale (Fig. 55). The relationship between cell density and the GAG levels can then be expressed as

$$y = -0.0922x + 2.6191$$

$$(r = -0.4902, df = 87, p < 0.001)$$

where y is the log GAG/ 10^7 cells/48 hrs, x is cell density as 10^5 cells/ 25 cm^2 . The slope of the line is therefore an index of the change in GAG secretion with increasing cell density.

GAG secretion in the different groups of fibroblasts is compared in the following table (Table 18).

No significant difference can be detected between groups, both EBS and EBD_r values falling within the normal range. (EBDD is too small a group for statistical analysis.

Using the equation derived above to predict whether the final cell density has influenced the value for mean GAG secreted, it can be seen that the predicted values, except in the EBDD group, fall within the range of observed values; confirming the similarity between control, EBS and EBD_r groups.

The effect of phenytoin on the rate of fibroblast proliferation and on GAG secretion can be seen in Fig. 56 a and b. Clearly phenytoin had no significant effect on the proliferation rate of any group at the concentrations used in these experiments, although there was large intra-group variability. It can therefore be assumed that any changes in GAG accumulation must be a direct effect of the phenytoin and not

FIG. 54

The relationship between GAG output $\mu\text{g}/10^7$ cells/48 hrs and cell density using nine different control cell lines.

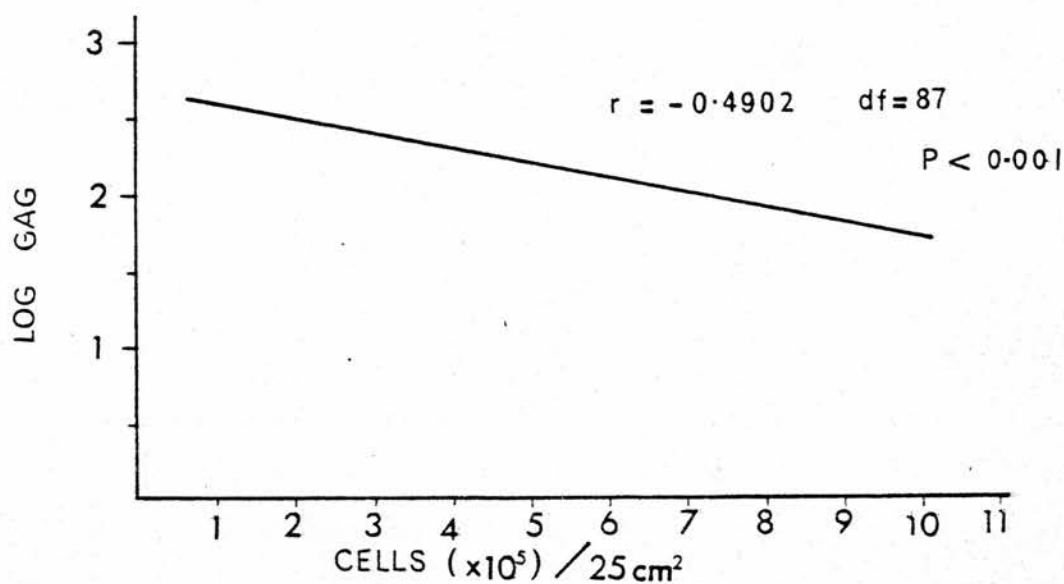
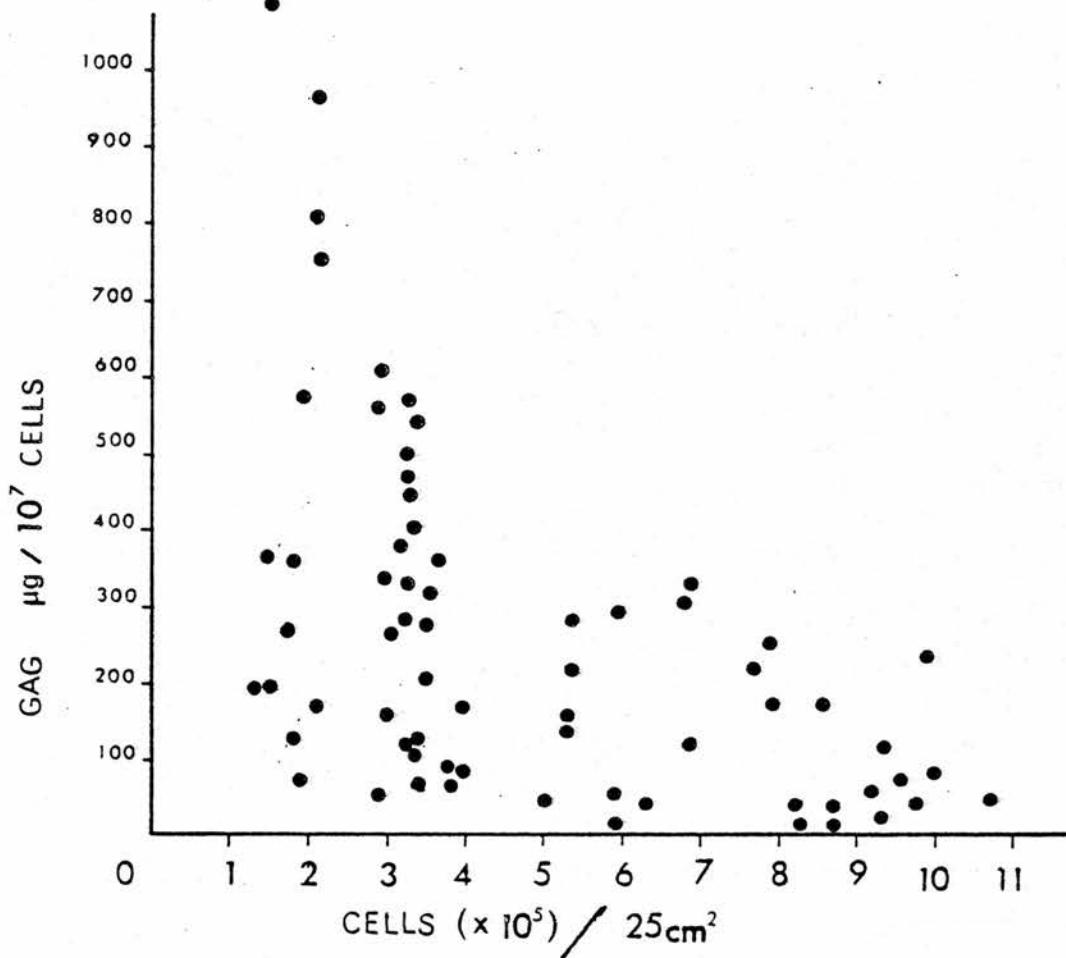


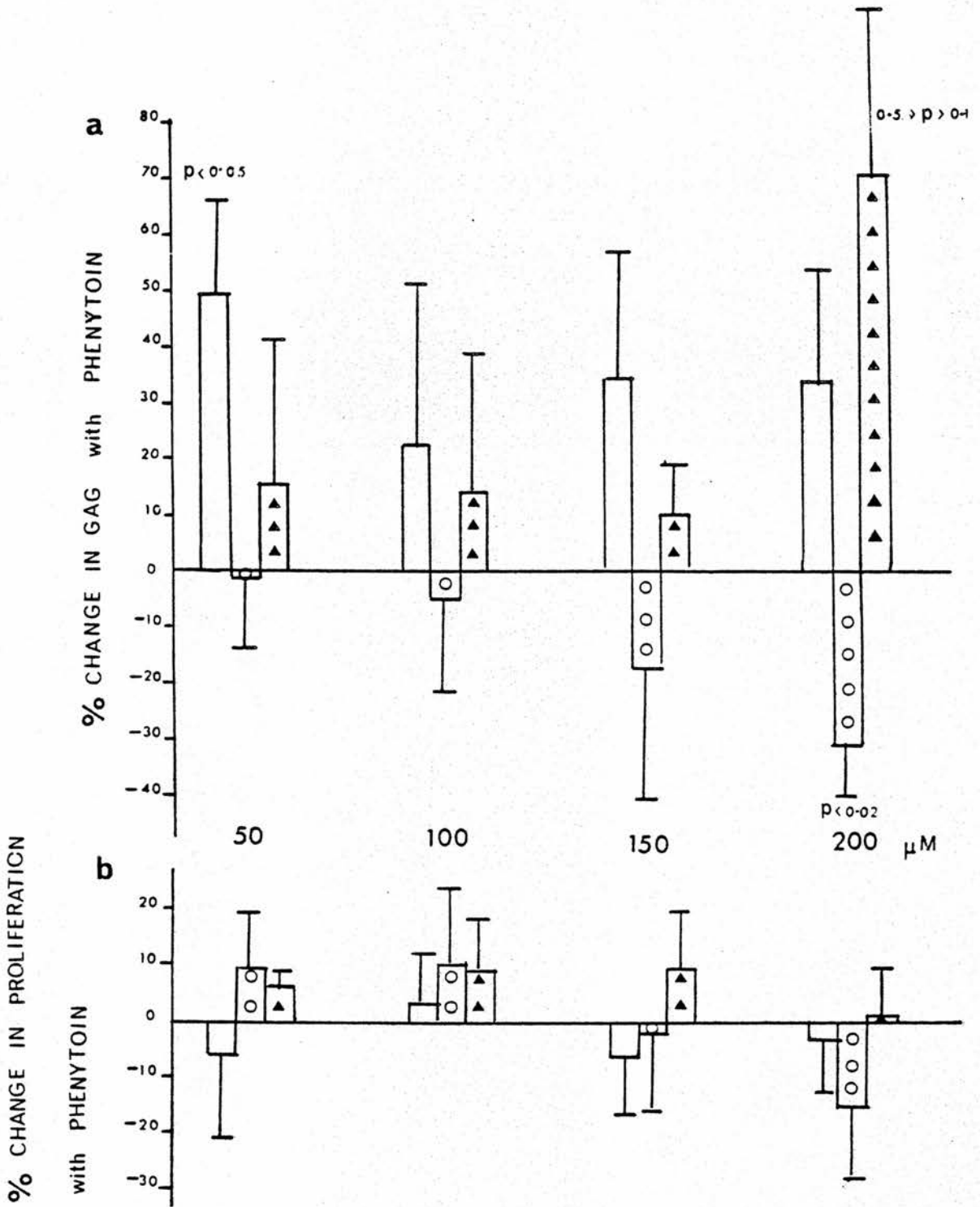
FIG. 55

The relationship between log GAG output $\mu\text{g}/10^7$ cells/48 hrs and cell density in control fibroblast cell lines. Linear regression analysis based on 89 individual observations from nine different cell strains.

TABLE 18

A COMPARISON OF GAG SECRETION IN CONTROL AND EB FIBROBLAST GROUPS

	N	EBS	EBDr	EBDD
Number of cell lines	10	7	6	3
Mean cell density 10 ⁵ cells/25 cm ²	5.48 ± 0.88	6.49 ± 0.82	6.27 ± 1.48	5.02
GAG				
ug/10 ⁷ cells/48 hrs	134 ± 28	138 ± 37	99 ± 17	234
	Mean ± SEM			
(predicted) GAG from Fig. 55 ug/10 ⁷ cells/48 hrs	(130)	(105)	(110)	(143)



(a) - Mean (\pm SEM) percentage change in GAG accumulation in (blank) control, \circ - EBS, and \blacktriangle - EBDr cell lines (n = 6) with increasing concentrations of phenytoin.

(b) - Mean (\pm SEM) percentage change in proliferation of control, EBS and EBDr cell lines (n = 6) produced by increasing concentrations of phenytoin.

an indirect result of differences in the final cell density due to an altered proliferation rate.

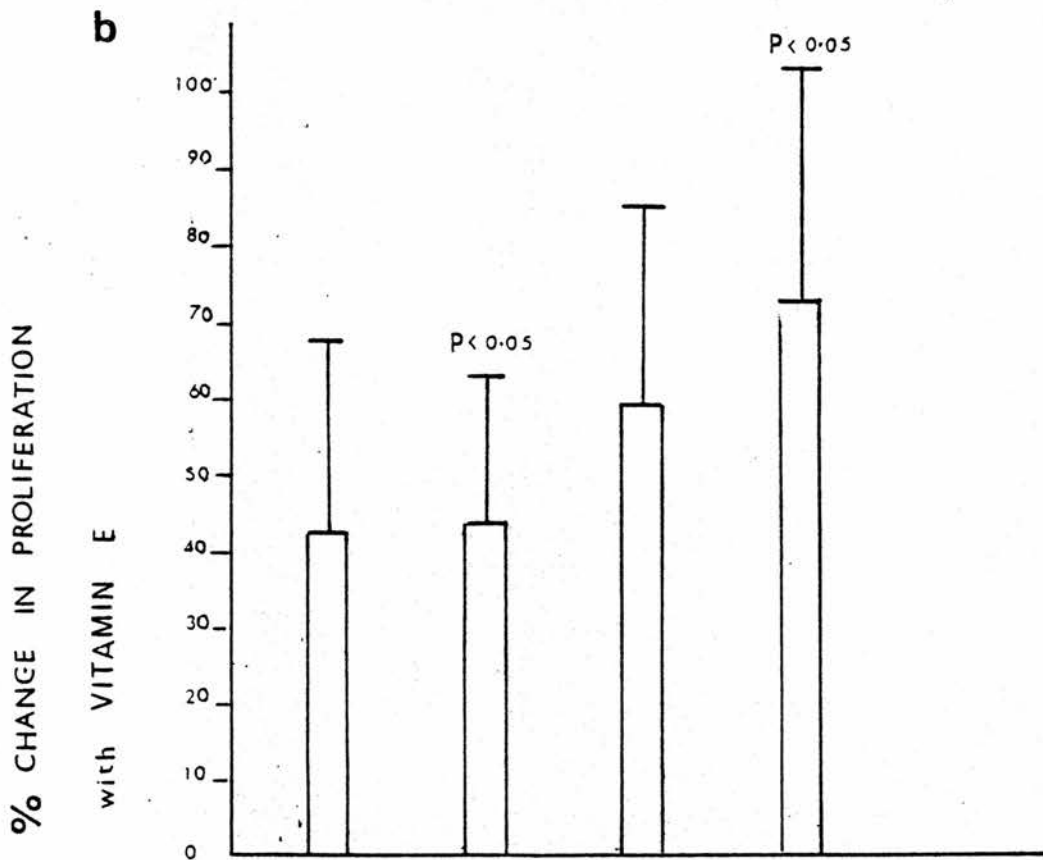
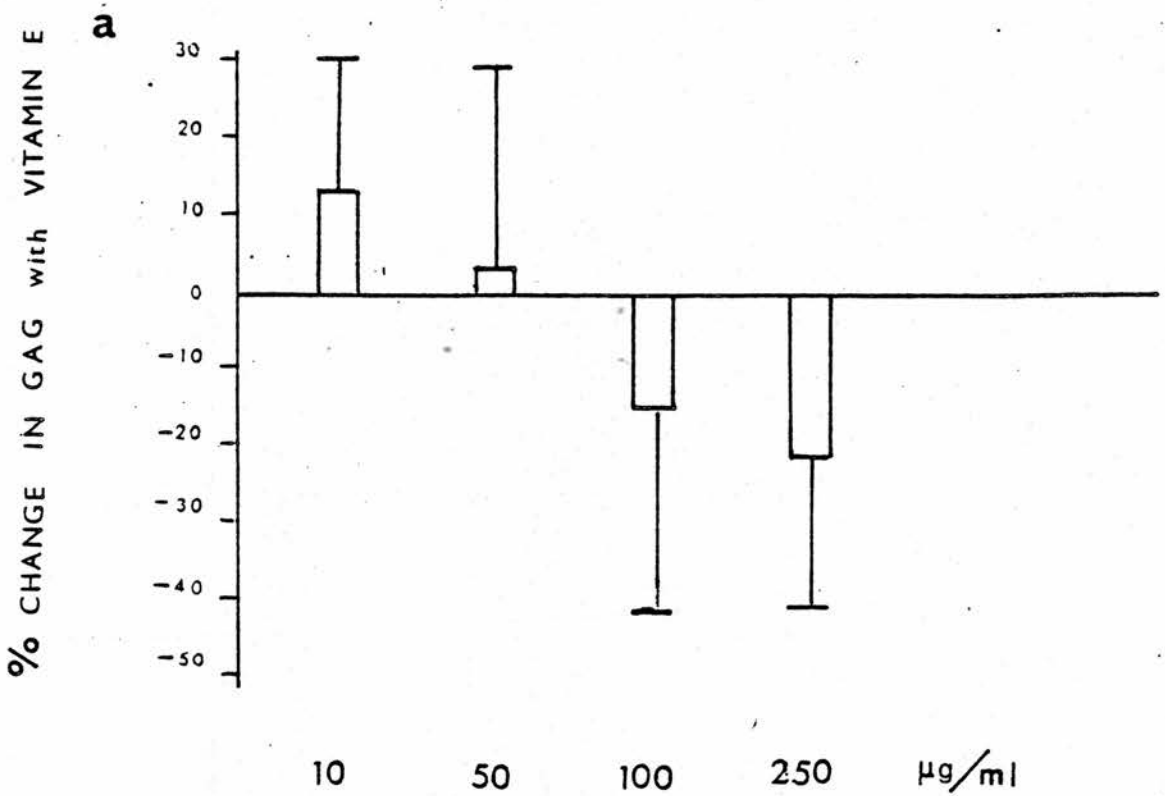
The effect of phenytoin on GAG secretion is more complex, with different effects on the four groups. The drug appears to have increased GAG concentration in the media of control cell lines (30%) at 50 μM phenytoin ($p < 0.05$; Students paired t-test). In contrast GAG levels in the simplex group were depressed with increasing phenytoin concentrations, maximal reduction in GAG (32%) occurring at 200 μM ($p < 0.02$; Students paired t-test).

Maximal elevation of GAG induced by phenytoin is seen in the EBDr group at 200 μM , but the 70% increase fails to be significant ($0.5 > p > 0.1$) due to the variability in response to phenytoin within the group.

The significance of the variation in response to phenytoin between groups was assessed using the non-parametric Mann-Whitney U-test. No statistical difference was observed between control and EBDr groups at any given concentration of the drug. In contrast the difference in response of EBS compared to the control group was significant at every concentration except 100 μM phenytoin. (50 μM : $p < 0.008$ $U = 3$; 150 μM : $p < 0.047$ $U = 7$; 200 μM : $p < 0.004$ $U = 2$).

Effect of Vitamin E on proliferation rate and GAG secretion in control cell lines

On a group of six controls, the mean effect of vitamin E was to increase the rate of fibroblast proliferation (Fig. 57b). However there was a large intra-group variation in response. Using the non-parametric Wilcoxon matched-pairs signed-ranks test, significant changes ($p < 0.05$) were seen at 50 and 250 $\mu\text{g/ml}$ vitamin E,



- (a) - Mean (\pm SEM) percentage change in GAG accumulation in control cell lines (n = 6) with increasing vitamin E concentrations.
- (b) - Mean (\pm SEM) percentage change in proliferation of control cell lines (n = 6) produced by increasing concentrations of vitamin E.

the proliferation rate being increased by 43% and 73% respectively.

The mean effect on GAG levels in control fibroblasts is shown in Fig. 57a. At no vitamin E concentration was a statistically significant change in GAG accumulation found. To determine whether the change in GAG secretion was attributable to the change in proliferation rate and therefore the final cell density, the predicted GAG output for the mean cell density at each concentration was calculated (Table 19).

The percentage changes in predicted GAG levels induced by the drug, given the associated change in mean final cell density, are of the same magnitude as those experimentally observed. The largest discrepancy between predicted and observed GAG values is seen at 10 $\mu\text{g/ml}$ vitamin E, where the percentage change in observed GAG (1%) is much less than was predicted by the graph (-22%). This suggests that there may be an increase in GAG accumulation attributable to the direct effect of the drug. From Fig. 57a, illustrating the mean percentage change in GAG secretion, it can be seen that this increase is in fact reflected in the experimental data: 10 $\mu\text{g/ml}$ vitamin E causing a mean percentage change in GAG of +13% (± 17).

TABLE 19

A COMPARISON OF OBSERVED GAG AND PREDICTED GAG VALUES GIVEN THE FINAL CELL DENSITY AT

VARIOUS VITAMIN E CONCENTRATIONS

VITAMIN E CONC (ug/ml)	0	10	50	100	250
Mean cell density	5.45	6.63	6.27	6.51	7.27
% change	-	22	15	19	33
% change in GAG predicted by Fig. 55	-	-22	-16	-20	-32
Mean GAG observed ug/10 ⁷ cells ± SEM	216 (± 24)	219 (± 36)	179 (± 20)	150 (± 45)	141 (± 32)
% change		1	-17	-31	-35

DISCUSSION

The measurement of GAG production in culture medium is complicated by the inverse relationship between cell density and GAG secretion (Hronowski and Anastassiades, 1980; Oakley and Priestley, 1983) and the age related decline in GAG synthesis (Schachtschabel and Wever, 1978; Vogel et al, 1981). All GAG analyses were therefore performed on cell lines between passages 4-10: in the experimental procedure any cultures that failed to reach a density of 2.5×10^5 cells/25 cm² were discounted from the results. Cultures which had a final cell density greater than this were located on the asymptote of the hyperbolic density versus GAG output curve and therefore were not expected to be unduly biased by cell density. Ideally all measures of GAG should be made at identical cell densities: in practice this is impossible because different rates of proliferation and different plating efficiencies create the differences seen in final cell density. However by using the linear relationship between cell density and the logarithm of GAG secretion, it was possible to predict the GAG values that might be observed at particular mean cell densities: thereby confirming that the experimental results were not unduly biased. It is interesting that the EBDD group (n = 3) does seem to have higher values of GAG secretion than are predicted. Although there is insufficient evidence to be statistically significant, this result could indicate further similarities (p. 83) between the EBDD-Cockayne-Touraine and Pasini variants. Bauer et al (1979), using three EBDD-CT fibroblast strains, concluded that there was no difference between them and controls; it is however, possible that relatively small differences in GAG secretion could be masked by differences in the final cell density.

In terms of overall GAG production, there appears to be no difference between either EBS or EBDr and control fibroblasts. However, percentage differences between the GAG levels in EBS and controls in response to phenytoin are striking; the GAG output of controls and EBDr being generally increased, whilst the simplex group show a reduction in levels of GAG in the medium.

The finding that the GAG content of the culture medium is higher after phenytoin treatment of EBDr and control cell lines agrees with the findings of Hänström and Jones (1979) and Kantor and Hassell (1983). The former postulated that phenytoin was hindering extracellular GAG degradation but not intracellular catabolism: whilst the latter authors thought the increase was due to elevated synthesis. In the present study it is impossible to say whether phenytoin is increasing GAG synthesis or decreasing the rate of degradation.

The intra-group variability in response to phenytoin was also noted by Hänström and Jones (1979). In their work only 3 out of 5 cultures of cat palatal mucosa showed an inhibition of GAG degradation by phenytoin. Similar variability was seen in the present cell culture system.

The reason for such variability and the reason for the opposite response of the simplex group is unknown. The EBS cell lines could either have synthesised less GAG or increased the rate of GAG degradation with increasing phenytoin concentration. Without further investigations it is impossible to know which is the more likely explanation, or why the simplex group should differ from controls and EBDr in their response.

The finding that the proliferation rate was not significantly altered by phenytoin in any of the groups studied is in contrast to

Shafer (1960), Houck et al (1972) and Al-Ubaidy et al (1981). Shafer (1960) did, however, note differences between the response of different cell types, rat fibrosarcoma cells showing no detectable change in growth rate. Whether the high serum concentration - 30% foetal calf serum, the age of the cell cultures - passage 17 - or their source - human gingivae, were relevant in determining the response to phenytoin is unknown. Houck et al (1972) found that the maximal response in growth rate of fibroblasts to phenytoin occurs at 5 µg/ml (19 µM) phenytoin, well below the lowest concentration used in the present study (50 µM). The data of Houck et al (1972) were derived from only two fibroblast cell lines, and in view of the variability in response to phenytoin seen in the present study, it is possible that they are not truly representative of a larger collection of fibroblast lines.

The clinical relevance of the present findings is hard to assess without knowing whether the change in GAG levels present in culture medium is due to increased synthesis or decreased degradation in the case of controls and EBDr and whether decreased synthesis or increased degradation is responsible for the 32% depression in GAG concentration seen in the simplex group. Whether, as suggested by Hänström and Jones (1979), an inhibition of degradative enzymes is the answer, or whether an increase in synthesis is responsible (Kantor and Hassell, 1983), an increase in the GAG content of the dermis might be beneficial. The work of Mathews and Decker (1968) indicated that GAGs affect collagen fibril formation and aggregation in solution. Chondroitin 4 and 6 sulphates and hyaluronate, over a certain molecular weight, retarded the rate of collagen fibril formation but increased the degree of aggregation of fibrillar collagen. It is possible that in vivo such properties are

important in stabilising the dermis of EBDr patients, although the variability in response of fibroblast strains to the drug make phenytoin's usefulness in vivo unpredictable.

Vitamin E caused no statistically significant change in GAG accumulation in culture medium at 10, 50, 100 and 250 $\mu\text{g}/\text{ml}$. It did however significantly increase the proliferation rate of the fibroblast lines up to 72% at 250 $\mu\text{g}/\text{ml}$ vitamin E ($p < 0.05$). The decline seen in GAG accumulation, though not significant, is probably a direct result of the increase in proliferation and therefore the higher final cell densities observed. The stimulation of proliferation may explain the rather generalised beneficial effects attributed to vitamin E after treatment of patients with different EB variants including simplex and dystrophic types. An increase in the number of fibroblasts with increased non-collagenous protein synthesis (see p. 106) might help stabilise the dermis. How this might apply to the intra-epidermal blistering types is unknown. Further research is required to answer such questions.

SUMMARY

- 1) No significant difference in the amount of GAG secreted into the culture medium was detected between control, EBS and EBDr fibroblast groups (n = 6): the mean GAG value for control cell lines was $134 \mu\text{g}/10^7$ cells (± 28 SEM n = 10).
- 2) Phenytoin at 50 μM increased GAG levels by 30% ($p < 0.05$) in control cell lines but at 250 μM GAG levels in the EBS group were depressed by 32% ($p < 0.02$). The mean proliferation rate was unaffected in all groups.
- 3) The main difference in response to phenytoin in terms of GAG secretion of the EBS and control group was statistically significant ($p < 0.05$) at all drug concentrations, except 100 μM phenytoin.
- 4) Vitamin E at 50 and 250 $\mu\text{g}/\text{ml}$ increased the rate of proliferation of control fibroblasts by 43% and 73% respectively ($p < 0.05$); no significant changes in GAG secretion were observed.

GENERAL DISCUSSION

GENERAL DISCUSSION

The aim of this project was to study fibroblasts from the skin of EB patients, with the intention of uncovering any metabolic differences which could separate them from the fibroblasts of normal, healthy subjects; and to examine the effects of phenytoin and vitamin E on fibroblast function. There were however several problems in fulfilling such a task.

Apart from the scarcity of patients, one important difficulty lay in defining correctly the class of EB in each case - the lack of familiarity with this rare disease making precise diagnosis difficult at first. In this study the variants which were quantitatively different: EBS Weber-Cockayne and Koebner; EBDr Hallopeau-Siemens and the localised variant, were regarded as single groups. Unless these variants are shown to be caused by different pathogenetic defects, rather than distinguished by somewhat arbitrary clinical demarcations of the body, it seems reasonable to make such generalisations for investigative purposes. Some variants are so rare that sub-groups would have to be very small, making the significance of results obtained questionable, unless a much longer term and more widespread search can be made to collect sufficient material of each particular type. However, in terms of clinical diagnosis and therefore of prognosis, it is probably of more value to recognise all the variants; a patient with localised EBDr having a much better prognosis than a counterpart with EBDr Hallopeau-Siemens.

Having found the patient, classified the type of EB and collected a skin biopsy, further difficulties arose during fibroblast culture. The time taken to obtain adequate numbers of fibroblast cultures was an important consideration. Three to four months could

elapse from the time of biopsy until enough fibroblasts were obtained for use in experiments. It was impossible to obtain all the skin biopsies at the same time, and only 6 to 8 could be cultured simultaneously, consequently a disproportionate amount of time was spent simply establishing the material for investigation.

The validity of cell culture systems in research remains a controversial subject. In culture, cells are isolated from all endogenous influences, including the many serum-borne factors, from other types of cell and from the influence of their immediate surroundings. This must affect their behaviour and function to a considerable extent. In addition, the substratum on which the fibroblast lives in vitro, i.e. glass, plastic or collagen, can influence GAG metabolism (Gallagher et al, 1980) and probably other aspects of cell behaviour too.

However, alternative means of direct investigation are impractical at present. Collagen matrices, more closely mimicking in vivo conditions, might be preferable but their use adds further complexities. The alterations in cell metabolism or behaviour should apply equally to all the cell lines grown in culture in this study, and differences detected between groups of cell lines should be valid. The underlying metabolic defects in mucopolysaccharidoses and the Ehlers-Danlos syndrome have already been successfully determined by using cultured fibroblasts.

Collagen metabolism is the most important of the fibroblast characteristics examined in culture. As the most severe form of EB is the dermolytic, dystrophic type, and because collagen synthesis had not previously been examined in EB, it was important to investigate this aspect of EB fibroblast function. Both EBS and EBDr cell lines

were found to produce more collagen, in absolute terms, than equivalent control cell lines. However relative collagen synthesis in EBDr cell lines did not significantly differ from controls. This indicates a general increase in protein synthesis in the EBDr group reflected in the results for non-collagen protein synthesis, which although were not statistically significant due to the large intra-EBDr group variability, were increased $40\% \pm 19$ (mean \pm SEM) over equivalent controls and must therefore be considered relevant. Such a general increase will, presumably, include collagenase, which represents 0.1-1% of the protein synthesised by normal skin fibroblasts (Valle and Bauer, 1979). It has been shown previously (Kronberger et al, 1982) that collagenase synthesis is disproportionately increased in EBDr cell lines, due to increased quantities of mRNA coding for collagenase. Therefore the inherent imbalance between collagen and collagenase production persists despite an increase in absolute collagen synthesis in the EBDr cell lines, (detected as a 36% increase in collagenase activity over that of controls in the present study.) As a result of this imbalance in collagen metabolism and the lack of any other metabolic abnormalities, it would seem the most likely cause of disruption and destruction of the dermis in the EBDr variant.

Curiously, fibroblasts from the EBS group were found to differ from controls in several ways. This had not been anticipated, as EBS is usually taken to be a disorder of the epidermis. Collagen synthesis was significantly increased, both in relative and absolute terms, over equivalent control and EBDr groups, whilst collagenase activity for the group as a whole was intermediate between that of the control and EBDr groups. It is possible that the increased collagen synthesis adequately compensates for any slight increase in

collagenase levels. A second finding with EBS fibroblasts was that the difference between pre- and post-activation levels of collagenase differed significantly from corresponding controls: much more of the collagenase in the EBS group apparently being in the pro-collagenase form. Possibly the EBS fibroblasts produce more collagenase inhibitor, or less endogenous collagenase activator (the equivalent to trypsin in vitro), and therefore any imbalance in collagenase metabolism is better controlled. This important difference between EBS fibroblast characteristics and those of controls warrants further research. Ideally collagen synthesis and collagen breakdown should be measured in the same terms (e.g. $\mu\text{g collagen}/10^6 \text{ cells/hour}$), so that the balance can be more easily assessed. Furthermore the type of nascent collagen and the relative proportions in which the various types are produced by fibroblasts, are important considerations and require further investigation. An abnormality in the basic collagen molecule could affect collagenous components of the dermis, including such things as the anchoring fibrils by rendering the collagen more susceptible to collagenase attack; thus providing a possible explanation for the apparent lack of anchoring fibrils in EBDr skin (Briggaman and Wheeler, 1975).

These results suggest similarities between the simplex and EBDr groups that have not been appreciated hitherto; whether such anomalies within the dermis of the simplex group affect the epidermis is unknown. It is possible that the dominant gene(s) causing blistering within the epidermis are linked with (or close to) the recessive trait altering collagen and collagenase metabolism, which affects the dermis. In EBS a mutation in the dominant gene affecting the integrity of the epidermis is inherited along with the collagen-

collagenase genes, whose products, although differing from controls, compensate each other so that the dermis appears to be normal. In EBD_r only the collagen-collagenase gene products are imbalanced, the work of Kronberger et al (1982) suggests that multiplication as well as mutation of the collagenase gene has occurred, which could result in disruption of the dermis. Such genetic speculation is, however, beyond the scope of this project.

Unfortunately too few dominant dystrophic cell lines were obtained to make the results significant. However the data support the idea that EBDD has a different aetiology from the recessive dystrophic group since collagenase activity was the same as in controls. Relative and absolute collagen synthesis seemed to be higher than controls and on a par with EBS levels, but variation between the three lines was so considerable that it would be unwise to speculate from this finding.

The close similarity of the data with those from the simplex group for collagen and non-collagen protein synthesis, is notable and deserves further study.

An abnormality in glycosaminoglycan production has previously been attributed to the Pasini variant of EBDD (Bauer et al, 1979). The GAG results obtained in this study, taking into account the final cell density of the cultures, indicate that the Cockayne-Touraine group may also involve an aberration in GAG metabolism, though probably to a lesser extent than the Pasini variant. An increased accumulation of GAG within the dermis may lead to collagen destabilization and disruption of the dermis. Obviously, many more cell lines need to be studied.

The single EBL cell line appeared to resemble most closely

those in the control group, suggesting no overt abnormality of fibroblast metabolism. In particular there is no evidence, from this study or from the data of other workers (Bauer and Eisen, 1978), to suggest that excessive collagenase activity features in the pathogenesis of EBL. Therefore there is no reason to suppose that phenytoin would be useful in the treatment of EBL; and in fact the two patients involved in this study did not benefit from a course of phenytoin treatment. Further research, using more cell lines, is needed to consolidate these findings.

It proved difficult to assess the effect of drugs on the various fibroblast characteristics examined. The difficulties lay in obtaining reproducible results and in the variability in results between cell lines of the same group. The reason for the poor reproducibility of results, particularly where phenytoin is concerned, is unlikely to be the inherent variability in the culture system. Previous work has shown that consistent results can be obtained using fibroblast culture to test the effect of various corticosteroids (Priestley and Brown, 1980; Priestley et al, 1983) and other drugs. The most likely explanation of such experimental variability, is the binding of phenytoin to serum albumin: the amount bound may differ from experiment to experiment depending on small variations in the amount of albumin present in the foetal calf serum in the culture medium. It is not known whether the variability in the response of cell lines to phenytoin reflects the variability in response of patients in vivo, but the work of Bauer et al (1980) indicates that this might be the case. They found that in a patient unresponsive to phenytoin only a small reduction in collagenase expression could be achieved by adding the drug to

skin fibroblasts in culture, in contrast to the more positive response of fibroblasts from patients who were responsive to the drug. It has been suggested that "responsiveness" to phenytoin is genetically modulated, both in the treatment of EBDr (Bauer et al, 1980) and in the development of gingival hyperplasia (Hassell et al, 1976).

Phenytoin had no statistically significant effect on the mean proliferation rate of any of the groups, although there were effects on individual cell lines. At high concentrations (500 μ M) phenytoin depressed both collagen and non-collagen protein synthesis by 10-20%, whilst at 300 μ M only non-collagen protein synthesis was depressed. Bauer et al (1980) found that phenytoin reduced synthesis and/or secretion of collagenase at 10 μ g/ml (\approx 37 μ M). The small reduction in non-collagen protein synthesis found in the present study might include a decrease in collagenase synthesis. No alteration in the percentage of nascent collagen or other protein secreted by the cell into the culture medium was detected: the results of this study therefore suggest that diminished collagenase activity with phenytoin treatment is due to reduced synthesis. Phenytoin had no significant effect on GAG production in EBDr cell lines, although opposite responses were seen in EBS and control groups. Control cell lines accumulated more GAG in the culture medium at 50 μ M, whilst for EBS cell lines GAG levels were depressed by a similar amount (32%) at 200 μ M. This also points to a further difference between control and EBS fibroblasts.

The response of fibroblasts to vitamin E differed from their response to phenytoin, and was more reproducible. Vitamin E caused a dose-dependent increase in the rate of proliferation of control

fibroblasts, without significantly altering GAG secretion. Vitamin E also stimulated synthesis of non-collagenous protein by 14%, depressing relative collagen synthesis. The clinical implications of these findings are very difficult to assess. An increase in non-collagenous protein synthesis in the EBDr group, could be detrimental to the patient, favouring the over-production of an aberrant collagenase. However it is not known whether all non-collagenous protein synthesis is stimulated to the same degree. Perhaps levels of inhibitors of collagenase are elevated to the same or a greater extent as collagenase levels. There have been very few large clinical studies on the effects of vitamin E; certainly Wilson (1964) reported a marked improvement in one patient with "severe EBDr", as did Seghal et al (1972) with their three patients with dystrophic recessive EB. Vitamin E has also been used to treat the EBS Weber-Cockayne variant, reportedly with considerable success (Ayres and Mihan, 1969). Very little is known about the effect of vitamin E on human skin: much more research and properly organised long term trials are needed before it can be recommended as a reliable treatment for EB.

This project has fulfilled its aims, in that it does represent a step towards an understanding of some of the underlying defects involved in the heterogeneous EB group. Advances in unravelling the complexities of such a rare disease are necessarily slow but progress is being made, and must continue. Future research should involve determining the true differences between variants in order to avoid further confusion in the diagnosis and treatment of what may be completely different conditions with a similar clinical appearance. Such research should include detailed biochemical

studies of both fibroblasts and keratinocytes, from all of the recognised EB variants. Having established the basic pathogenetic defect(s), the possibility of finding and testing potentially effective drugs becomes more realistic. In the short term, however, the finding that retinoids can suppress fibroblast collagenase in vitro (Bauer et al, 1982) points the way to clinical trials of these drugs in EBDr.

Parallels exist between the state of our knowledge of EB today and our past knowledge of other confusingly heterogeneous disorders, such as the Ehlers-Danlos syndrome and the mucopolysaccharidoses; now reasonably well understood conditions, bringing hope of successful treatment. It is to be hoped that progress leading to a more complete understanding of EB will continue to be made.

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