HUMAN KERATINOCYTE CULTURE AND GRAFTING TECHNIQUES USED IN THE TREATMENT OF EXTENSIVE FULL THICKNESS BURN WOUNDS AND CHRONIC LEG ULCERS

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DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

J. B. Beder

(Name of candidate)

18th day of May 1992

DEDICATION

I would like to dedicate this work to
my parents Sonia and the late Jack Beder,
my sisters Eunice and Avril Beder,
they all gave me tremendous encouragement during the course of this dissertation.

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LIST OF ABBREVIATIONS

b-FGF	-	basic Fibroblast Growth Factor
BSA	-	Burn surface urea
CEA	-	Cultured Epidermal Allograft
DMEM	-	Dulbecco's Modification of Eagle's Medium
DNA	-	Deoxyribonucleic Acid
EGF	-	Epidermal Growth Factor
E.M.	-	Electron Microscope
GM-CSF	-	Granulocyte Macrophage Colony Stimulating Factor
HIV	-	Human Immunodeficiency Virus
HLA	-	Human Leukocyte Antigen
IL	-	Interleukin
KGF	-	Keratinocyte Growth Factor
L.M.	-	Light Microscope
MHC	-	Major Histocompatability Complex
mRNA	-	messenger Ribonucleic Acid
PBS	-	Phosphate Buffered Saline
PDGF	-	Platelet Derived Growth Factor
rpm	-	revolutions per minute
SSG	-	Split thickness Skin Graft
TGF-&	-	Transforming Growth Factor alpha
TGF-b	-	Transforming Growth Factor beta
TNF	-	Tumour Necrosis Factor
3T3	-	Tritiated Thymidine

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2. Beder, J.B., Thornley, A.L. and Veale, R. Human keratinocyte culture techniques in extensive burn wounds. The Journal of Trauma and Emergency Medicine. 1990 Jan/Feb; 7 (1): 29-32.

3. Beder J.B. Cultured allografts. A letter appearing in the Continuing Medical Education Journal (CEM). 1990 Jan; 8(1): 48.

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5. Beder, J.B., Thornley, A.L., Veale R., Kadwa, A.M., Scott, E. Healing chronic leg ulcers cultured keratinocyte allografts. This paper was presented at the annual Congress of The Surgical Research Society of Southern Africa held in Bloemfontein from 29 April to 1 May 1990. This paper was the runner-up for the best paper presented at the above congress.

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7. Beder, J.B. Healing full thickness burns with various types of cultured epidermal grafts - a comparative study. This paper was presented at the 24th annual meeting of the British Burn Association held at the post-graduate centre, Stock Mandeville, Aylesbury, Bucks, England, United Kingdom, on the 10th May 1991.

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CHAPTER ONE

1.0 HUMAN KERATINOCYTE CULTURE AND GRAFTING TECHNIQUES USED IN THE TREATMENT OF EXTENSIVE FULL THICKNESS BURN WOUNDS AND CHRONIC LEG ULCERS.

1.1 Introduction

Skin loss occurs in diverse clinical situations including burns, trauma, leg ulcers, skin diseases including epidermolysis bullosa and surgical wounds. Small skin lesions usually heal spontaneously, but epidermal replacement is often required in large, persistent defects. The conventional technique of covering skin defects is by use of full- or split- thickness skin grafts. These skin grafts are composed of epidermal and dermal components.

Over the past 16 years culture techniques have been devised to grow the keratinocyte component of the epidermis to form confluent stratified sheets to be used as keratinocyte-allografts or autografts (1, 2). These keratinocyte grafts, derived from small biopsy specimens, and expanded a few hundred times, can be used to cover the skin defects caused by burns, chronic leg ulcers, trauma and cellulitis induced defects, as well as surgical fasciotomy induced defects.

1.1.1 Architecture of the epidermis

The epidermis consists of multiple layers of keratinocytes. Mitosis occurs mainly in the basal layer, which rests on the basement membrane. Mitoses are also known to occur in the suprabasal cells, since extremely thin slices of epidermis taken above the basal layer from the sole of the foot or the palm of the hand, take after grafting (3).

As the basal keratinocytes divide and differentiate, they are pushed upwards to the more superficial layers of the epidermis. They progressively enlarge, lose their nucleus and most cytoplasmic constituents and when reaching the most superficial layer, are no more than squames containing mainly keratin. The accumulated layers of terminally differentiated cells at the surface of the epidermis protect the underlying living layers from desiccation and mechanical damage (4).

One is able to identify three different zones of suprabasal keratinocytes in histological

sections of epidermis. Cells in the spinous layers are larger than cells in the basal layer and have abundant desmosomes. In the granular layer, the cytoplasm of keratinocytes contain keratinohyalin granules, thought to be involved in the aggregation of filamentous protein aggregates. In the outer cornified layers, the cells have lost their nuclei and cytoplasmic organelles. These cells are full of involucrin and keratin filaments and contain an insoluble protein envelope, about 12nm thick, which is closely opposed to the inner surface of the plasma membrane (5).

The first attempts in growing skin in the laboratory involved organ culture (6) and explants (1, 7 - 9). From the explant bathed in culture medium, keratinocytes grew down and over the surface of the culture dish. This was followed by stratification and differentiation. The main disadvantages here were that the explants did not produce confluent stratified keratinocyte sheets for grafting and the fibroblasts derived from the explants, competed with the keratinocytes for surface space on the culture dish.

1.1.2 The morphology of cultured keratinocyte grafts

Keratinocytes grown to confluence on plastic are found to consist of a sheet of up to 12 cell layers lacking the clear histological division into strata of a normal epidermis. The basal cells are flat as are those of the intermediate layers which show occasional keratohyalin granules and a few membrane coated granules. Desmosomes are present between the cells and microvillous processes on all cell surfaces except the attachment face of the basal layer. In the upper layers there are anucleated cells with thickened cell envelopes but a stratum corneum is absent (10 - 12). Langerhans cells are absent whilst melanocytes survive and proliferate in culture with keratinocytes (13). Keratinocytes play an active role in inducing melanocyte growth (14).

1.1.3 Keratinocyte immunohistochemistry

Immunohistochemical studies have shown the presence of bullous pemphigoid antigen on the basal aspect of the basal cells but the absence of other basement membrane components, such as laminin, type IV and type VII collagens and epidermolysis aquista antigen (10). Auböck (11) showed the presence of pemphigus, bullous pemphigoid and HLA class I antigens and involucrin (an envelope protein). Surprisingly, vimentin, a mesenchymal marker, was found in the basal cells of the cultured epidermis. Compton (15) showed the existence of electron dense zones on the basal plasma membrane resembling immature hemidesmosomes. After

detachment from the culture flask, large membrane bound blebs developed in their place.

1.1.4 Keratinoycyte aging

Neonatal foreskin has 50 - 60 population doublings before senescence. The older the donor, the fewer doublings can the keratinocytes undergo in culture (16). The cell cycle time of normal skin keratinocytes in vivo varies from 50 hours to more than 300 hours (17). In culture, the doubling time is more rapid in addition to a higher growth fraction (77%) being present compared to either normal or hyperproliferative skin
(18).

To what extent do, the age of the donor, the cell type, the preparation of the keratinocytes for culture, the growth factors and the growth medium used all play a role with respect to keratinocyte growth potential and ultimately their ability to form confluent stratified epidermal sheets. These factors and others were investigated and the data analysed.

1.1.5 The application of keratinocyte sheets

A major application of keratinocyte sheets is in the treatment of extensive (more than 40% burn surface area (BSA)), full thickness burns. Keratinocyte sheets can complement other areas grafted with split thickness skin graft (SSG), in cases where not enough SSG is available. To date, results using keratinocyte grafts have been variable (19 - 23).

The use of keratinocyte sheets in burns has been extended to covering partial thickness burns and SSG donor sites. The rationale behind this is that the keratinocyte grafts speed up healing at these sites. Innovative ways of using these cultured grafts in treating burn patients is proposed and the results presented in this dissertation. Also an overall methodology with respect to the application of the cultured keratinocyte sheets to skin defects irrespective of aetiology is proposed and has been tested.

Chronic indolent leg ulcers have been treated in various ways in an attempt to obtain healing. Application of various antiseptic solutions and dressings has been the mainstay of long term conservative management. The cause should be found and treated appropriately, if possible. Split thickness skin grafts have been the surgical treatment of choice. This method has not always proved successful due to poor tissue perfusion and sepsis. An alternate surgical approach is the application of cultured keratinocyte allografts. These are applied to a healthy

clean wound bed in combination with leg elevation and leg protection. Various researchers have applied these keratinocyte sheets with fairly good results
(24 - 27). Do these allograft keratinocytes covering the defect remain there indefinitely or are they replaced by the patients own keratinocytes? The solution to this question has been elucidated in this dissertation. Is it possible to predict with any degree of accuracy the length of time that an ulcer of a specific size will heal following the application of a cultured keratinocyte sheet? This possibility has been investigated here.

1.1.6 Composite grafts

Keratinocyte sheets only replace the lost epidermis, but what of the dermal component? An extension of keratinocyte culture is the recent development of dermal equivalents, forming complex cultures in order to mimic skin. This is an attempt to reduce contractures and make for a more stable graft. Various substances such as bovine collagen (28), chondroitin - 6 - sulphate (28) and human types 1 and 3 collagen (29) have been tested. The problem appears to be durability following application to the wound bed. The keratinocyte sheet, requiring nutrients, may be required to be applied after the dermal equivalent has first become vascularised. A model for a composite graft is proposed and discussed.

1.1.7 Ai

a) The culturing of confluent stratified epidermal sheets in the laboratory which are able to be used as substitutes for skin grafts in extensive burns and other conditions resulting in skin loss.

b) To bring cultured keratinocyte sheets to the patient in an academic hospital in South Africa.

c) The most appropriate method of application of a cultured keratinocyte sheet to an area of skin loss, irrespective of aetiology that will result in visible skin formation, thus speeding up healing of the skin defect.

d) The ultimate aim is to create a technology that is reproducible, simple, giving maximum benifit to the patient and thus reducing patient morbidity and mortality and being cost effective.

e) To improve on existing skin equivalents, such as the addition of a dermal component. Choosing the most appropriate substance as a dermal component, which will produce a durable, effective and cosmetically acceptable composite graft, ultimately obviating the need to harvesting large areas of the patient's own skin for the provision of skin for skin cover.

The specific conditions to which these confluent stratified keratinocyte sheets were applied include extensive and small full thickness burns and chronic leg ulcers of various aetiologies.

CHAPTER TWO

2.0 A COMPARATIVE STUDY OF VARIOUS KERATINOCYTE CELL LINES USED IN THE SYNTHESIS OF CONFLUENT STRATIFIED KERATINOCYTE SHEETS.

2.1 Introduction

In 1975, Rheinwald and Green (1) successfully cultured keratinocyte sheets from single cell suspensions. The cells, derived from trypsinized skin biopsy specimens, are grown in Dulbeco's Minimum Essential medium, Ham's F12 medium (in a ratio of 1:1), bovine calf serum and various mitogens. Boyce and Ham (2), using serum free medium and Bovine Pituitary Extract also produced uniformly confluent stratified keratinocyte sheets. Using either of the above methods, one can expand a $1cm^2$ skin biopsy specimen to $2m^2$ of cultured epithelium within about 6 weeks.

These keratinocyte grafts or cultured epidermal grafts can be used as autografts or allografts in the treatment of a number of skin conditions resulting in skin loss. They include burns, chronic leg ulcers, degloving injuries and following removal of giant congenital naevi, to name but a few.

2.1.1 The regulation of epidermal cell proliferation

The epidermis can be used as a model to investigate certain basic processes which are found in all regenerating tissues. There appears to be 2 different types of dividing keratinocytes, as seen by analysing cell kinetics of murine epidermis. One of the types, the stem cell, has the capacity for unlimited proliferation. These stem cells can either give rise to other stem cells or else to the second type of keratinocytes, called transit amplifying cells. These transit amplifying cells are programmed to undergo terminal differentiation after a finite number of rounds of division
(5).

Proliferation in the epidermis depends not only on the nature of the dividing cells, but also upon the external signals to which they respond (30). Various growth factors have been found which influence the growth and proliferation of keratinocytes (31, 32). These factors include transferrin, insulin, triiodothyronine, epidermal growth factor, hydrocortisone and

cholera toxin (32, 33). Fibronectin, derived from 3T3 murine fibroblasts, acting as feeders (1), cause the spreading of cells as well as strengthening the attachment of adjacent keratinocytes (34).

Cholera toxin increases the proportion of small cells in the colonies (33), but does not appear to speed up the time taken for confluence to occur (unpublished findings). Epidermal growth factor (EGF) and the structurally related polypeptide, transforming growth factor alpha (TFG-&), both increase the life-span of keratinocytes (by affecting cell division and maturation), and stimulate the lateral migration of the peripheral zone of dividing cells in expanding colonies (34). Growth factor stimulators, some as yet unidentified, are found in Bovine Pituitary Extract (35), (most notably type II basic fibroblast growth factor), bovine calf serum and in medium conditioned by 3T3 murine fibroblast feeder cells (1). Human keratinocyte growth factor (KGF), derived from fibroblasts, appears to exert a paracrine effect on adjacent epithelial cells, resulting in their proliferation (36, 37).

Keratinocytes not only respond to growth regulating molecules, but they also express them. Coffey et al. (31) showed that cultures of normal human keratinocytes synthesize TGF-α . The addition of EGF or TGF-α to the medium induces TGF-α gene expression (31). TGF-α is present in and secreted by all living layers of normal epidermis (31).

2.1.2 Differentiation of keratinocyte grafts in vitro and in vivo

Keratinocyte differentiation can be determined from their expression of keratin filament proteins, envelope proteins (involucrin) and membrane glycoproteins (38). Keratins are major structural proteins of all epithelial cells which are the most invariable characteristic of the epithelial cell type (39). 19 distinct keratin polypeptides are expressed in man, which are divided into type I and type II classes based on their molecular weight and iso-electric point. The keratin profile of a tissue is closely related to its differentiated state (39).

In keratinocytes cultured in plastic, K1 and 10 expression is reduced suprabasally but expression of hyperproliferation keratin 6 and 16 is induced (12, 40). Some cells also express simple epithelial keratins (15, 40); some of these cells may be appendageal cells in the culture. Mucosal keratins 4 and 13 are also found. The cornified envelope protein precursor, involucrin, a marker of cellular differentiation, expressed in the upper cell layers in skin, is found in the epibasal layer in keratinocyte cultures (12).

As one has seen above, the following major factors affect keratinocyte growth potential in vitro

a) The age of donor.
b) Cell type and shape (41, 42, 43).
c) Growth factor stimulates and growth factor depressants synthesized by other cells or by keratinocytes themselves.
d) The time at which markers of cellular differentiation (e.g. involucrin) are produced resulting in the maturation and terminal differentiation of the keratinocytes (44).
e) Cell size with respect to differentiation (45).
f) The time interval between harvesting and the initiation
g) The growth medium used.

Some of these factors were investigated and the data analysed. There does not appear to be any comparative studies comparing keratinocytes obtained from neonatal and older donors. Is there a significant difference in their growth rates? Although it has been found that certain growth factors appear to enhance multiplication and spreading of keratinocytes in culture, are they all necessary? Professor H. Green, himself, admits that EGF is not entirely necessary for culturing keratinocytes in order to form confluent stratified sheets. Cholera toxin also does not significantly reduce the time interval between monoclonal colony formation and confluence (unpublished findings).

Are antibiotics and fungicides added to contaminated cultures, effective not only in killing the contaminant but also in not diminishing the growth potential of the keratinocytes? Do keratinocytes obtained from primary cultures have a faster growth rate compared to keratinocytes obtained from skin biopsy specimens? Does monoclonal colony appearance depend upon the time of harvesting to culturing? In this study one has attempted to answer these questions and pose a few more.

2.2 Materials and Methods

The methodology of obtaining and culturing keratinocytes is similar to that described by Rheinwald and Green (1). This is summarized below and specific modifications are included.

2.1.1 Obtaining and testing keratinocytes

A site for full thickness donor skin measuring 2 - 4 cm^2 (figure 1) was chosen in the case of the burn patient (appendix II B). Allograft skin was obtained from neonatal or young adult circumcisions (appendix II A) and excess skin from certain plastic surgical procedures, such as breast reductions. Either a blood sample or a suspension of cultured keratinocytes derived from a particular donor, in the case of allografts, was tested for the presence of, or antibodies directed against the Human Immunodeficiency virus and the Hepatitis B virus (appendix VIII). Only cell lines testing seronegative for both viruses were used as allografts.

2.2.2 Processing of skin specimen

The skin specimen was placed in a bacterial petri dish and excess fascia was removed with the aid of fine toothed forceps and a scalpel blade. Within 2 hours of harvesting, the skin specimen was placed in a petri dish containing 0.17% trypsin and incubated at 37°C in a carbon dioxide incubator for 20 - 40 minutes; or at 4°C in a fridge over night. The epidermis separated from the dermis at the level of the stratum basale (figure 2). With the aid of curved glass pipettes 5 - 10 x 10^3 basal cells/ml were scrapped off and placed into a culture dish containing growth medium solution, (DMEM: Ham's F12, 1:1, and 10% bovine calf serum) (appendix I A) and mitomycin treated murine fibroblast feeder cells, (appendix I B), (figure 3). Any remaining basal keratinocytes still adherent to the epidermis were removed by gentle suction of these pieces of epidermis into and out of a graduated 10ml pipette attached to a suction device.

Additional skin specimens were cut up into 1mm^2 pieces and placed onto 6cm^2 petri dishes. Sticking to the surface of the dish being achieved by air drying. These explants were cultured in the same growth medium as the single keratinocytes. The cells derived from both types of cultures were used to initiate secondary and tertiary cultures. Harvesting was carried out when subconfluent colonies were present.

2.2.3 Culturing keratinocytes

When monoclonal colonies first form (on average within 2.7 days), (figure 4), growth factor supplements were added to the culture medium, which was changed every second day. But as the colonies became subconfluent, and the pH indicator changed more quickly from a dark pink to a yellow, (indicating consumption of nutrients within the medium) they were fed every day. Following the removal of the old medium, 4ml more medium was added to the 9cm dishes and 8ml more medium was added to the 14cm dishes.

Human fibroblasts may initially compete with the multiplying keratinocytes for surface space (figure 5a). These fibroblasts were removed together with the feeder cells with the aid of EDTA/glucose, which caused the release of fibroblasts from the surface of the petri dish a few minutes before the keratinocytes, thus having a time dependant action. After washing the keratinocyte colonies with phosphate buffered saline, new 3T3 fibroblasts were added.

If the human fibroblasts were not a problem, then the original feeders, belonging to the murine strain J_2, tended to detach from the surface of the petri dish long before confluence was reached with the specific serum used (since this is serum dependant).

After confluence and stratification have been achieved, (figure 5b), (on average within 24.6 days), the epidermal sheet was ready to be used as a graft. The epidermal sheets were lifted off the surface of the petri dish by the addition of Dispase II for 40 minutes.

2.2.4 Preparation of keratinocyte sheets for grafting

The petri dishes were placed either in an incubator or on a glass sheet, placed over a bowl of water at 37°C. In the latter case a sterile green sheet was placed over the petri dishes. At least once during the 40 minute time interval the Dispase II was swirled over the sheets in order to re-establish an even distribution. The Dispase II was removed and the epidermal sheet was washed in phosphate buffered saline. The petri dishes, thus containing epidermal sheets and PBS were placed on ice until grafting took place (figure 10).

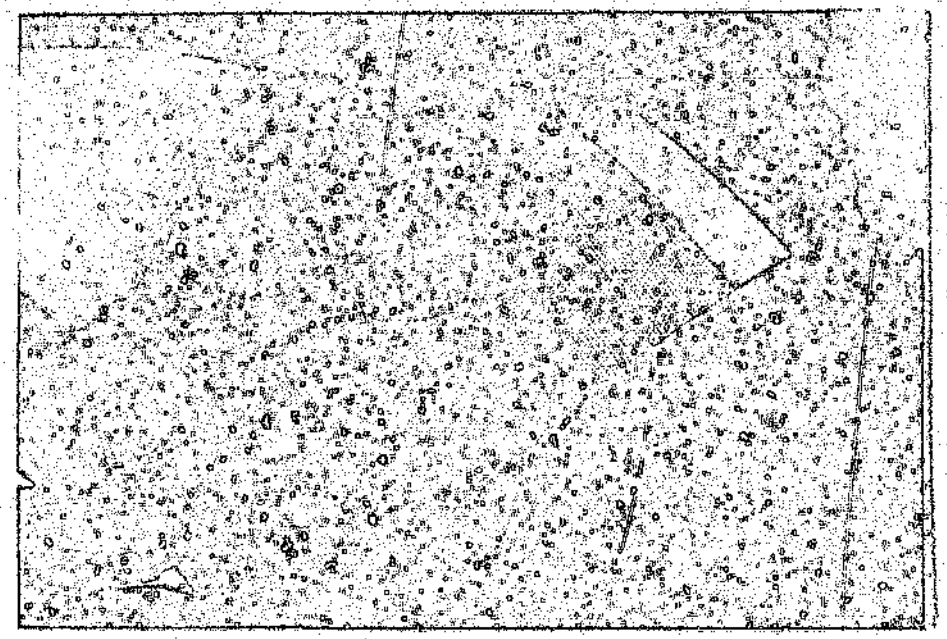


FIGURE 1.

An example of a 4cm by 4cm partial thickness skin biopsy specimen. From it was derived autologous keratinocytes for culture, which multiplied to form confluent stratified keratinocyte sheets.

FIGURE 2. TRYPSIN TREATED SKIN

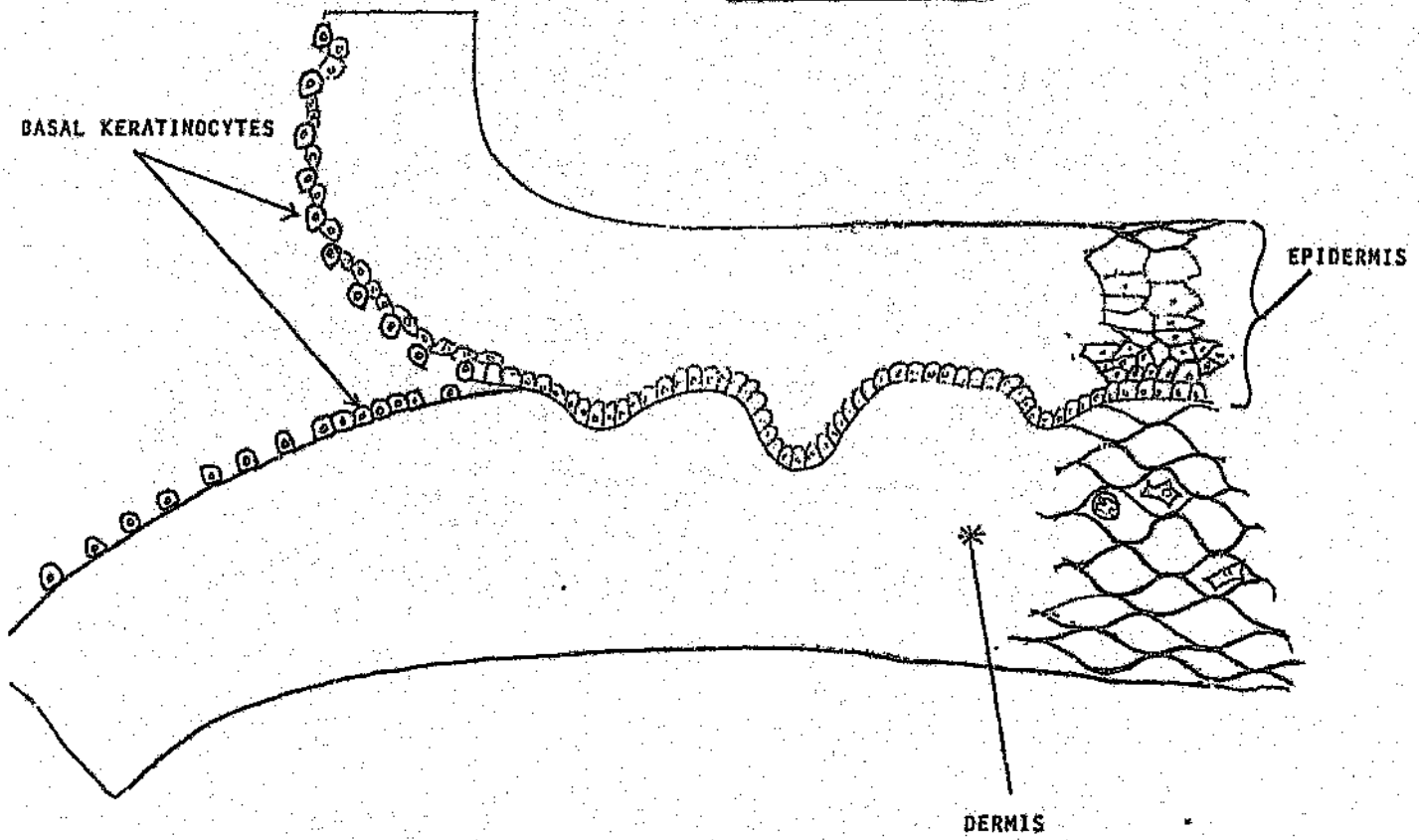


FIGURE 3. CULTURE COMPLEX

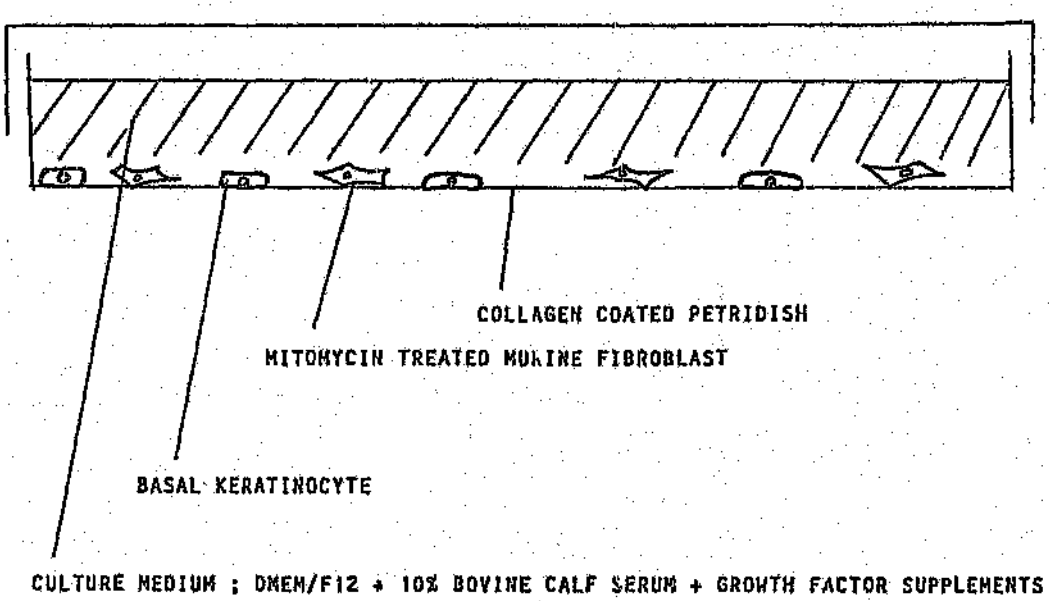


FIGURE 4 (a)

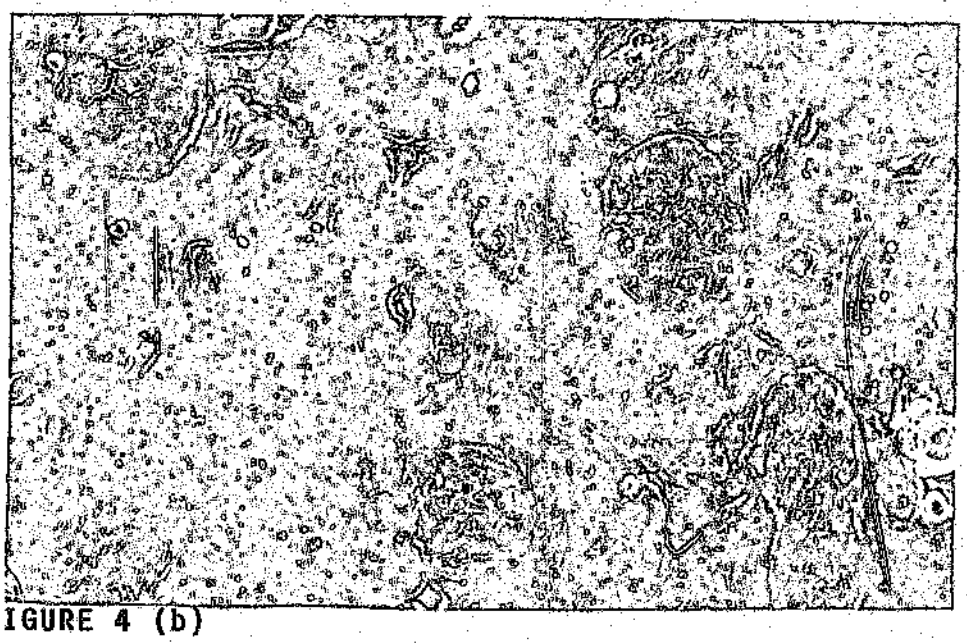


FIGURE 4 (b)

FIGURE 4.

Monoclonal keratinocyte colonies surrounded by mitomicin treated murine fibroblasts.

4(a). A monoclonal keratinocyte colony composed of 11 cells. Two fibroblasts can be seen on either side of this colony. (mag. 400x).

4(b). A number of expanding monoclonal keratinocyte colonies are seen. Surrounding these colonies are mitomicin treated 3T3 murine fibroblast feeders. (mag. 100x).

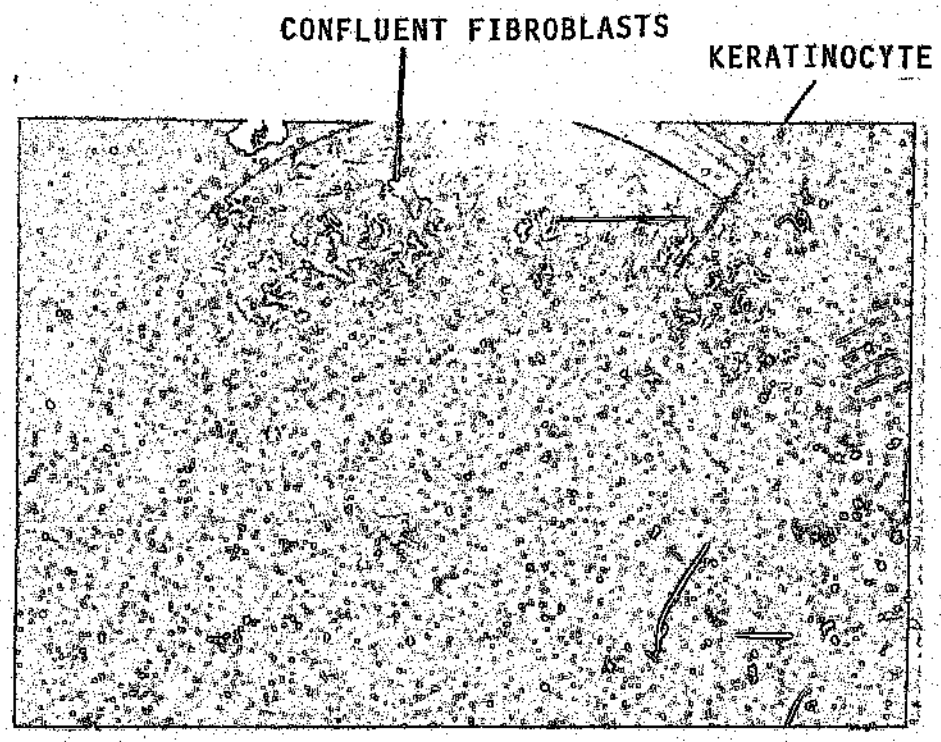


FIGURE 5(a).

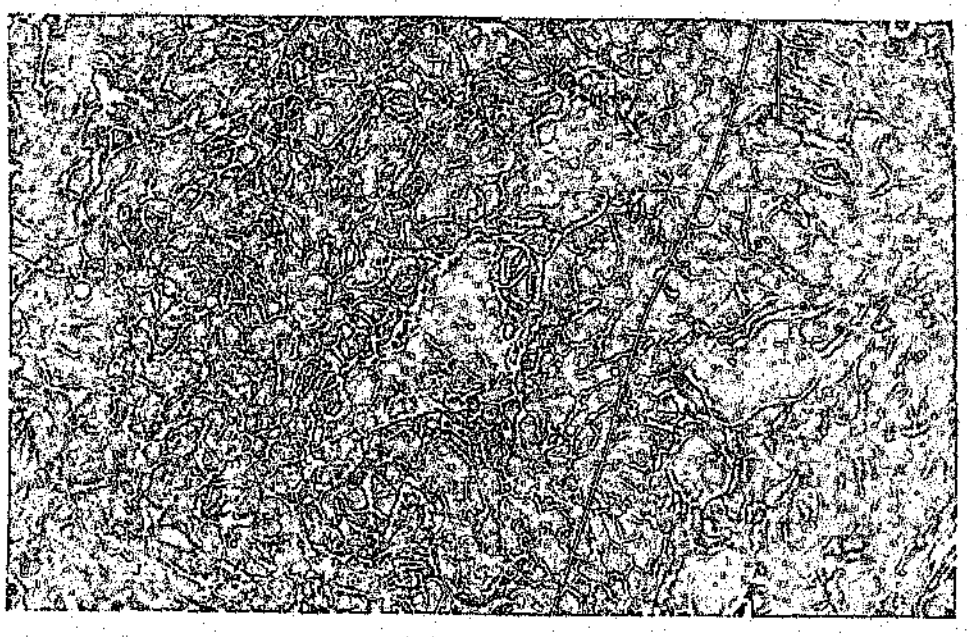


FIGURE 5(b). (mag. 10x).

FIGURE 5.

Examples of keratinocyte sheets and human fibroblast overgrowth.

5(a). An example of human fibroblast overgrowth. The whorl pattern is concentrations of human fibroblasts that have formed confluent sheets, preventing growth of human keratinocytes.

5(b). A confluent stratified keratinocyte sheet. Two large superficial cells can be seen. This keratinocyte sheet is ready for use as a graft.

2.3 Results

2.3.1 THE DEVELOPMENT OF A SKIN GRAFT MODEL: THE HISTOLOGICAL APPEARANCE OF THE CULTURED EPIDERMAL SHEETS

2.3.1.1 L.M. Appearance

After confluence and stratification have been achieved and the basal cells appeared small and uniform, the epidermal sheet was ready to be used as a graft. The epithelia ranged in thickness from 4 to 7 cell layers. They were composed of a basal layer with small polygonal or cuboidal cells and several `intermediate layers' with progressively flattened cells, and at the surface, one or two `upper layers' where nuclei were present (figures 6a and 6b).

2.3.1.2 E.M. Appearance

Electron microscopic examination of these sheets confirm that dendritic cells (Langerhans cells) were usually absent, but not always and that a multilayered Malpighian-type epithelium with keratinocytes at various stages of differentiation could be seen (figure 7). Basal cells were organised into a single layer of closely associated cells, with a distinct nucleus, cytoplasmic organelles and intermediate filaments, but hemidesmosomes were absent at the basal side of the cells. `Intermediate layer' cells were rather elongated and contained numerous organelles, tonofilaments and keratohyalin granules (figure 7). They were separated from each other and from the basal cells by intercellular spaces with numerous interdigitations of the plasma membrane, visible gap junctions and well defined desmosomes (figure 9). One or two `upper layers' with nucleated cells, a electron-dense cell envelope were seen at the surface (figure 8). The above findings are similar to those found by Merick et al. (46).

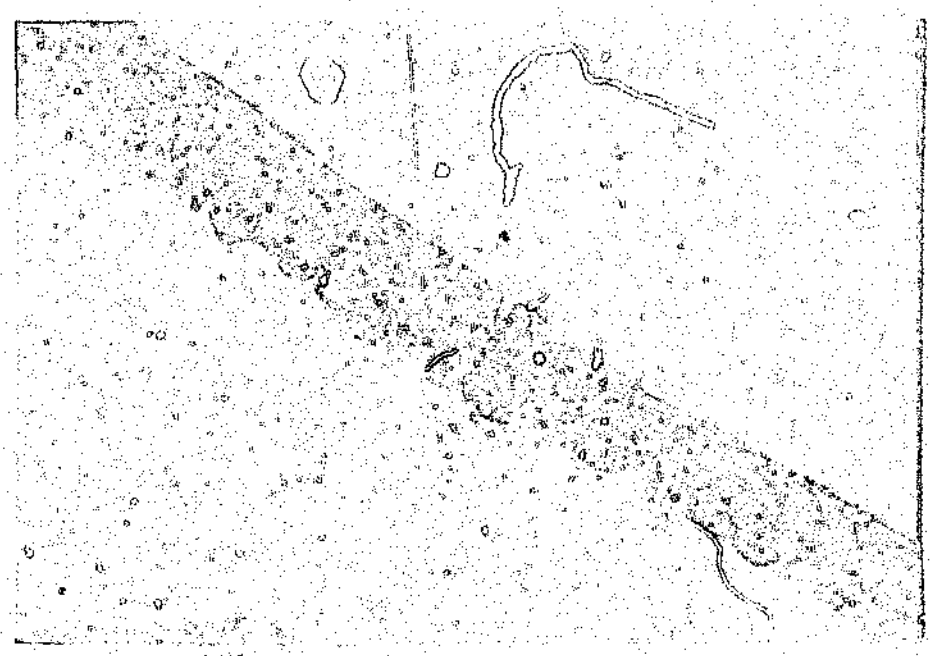


FIGURE 6 (a)

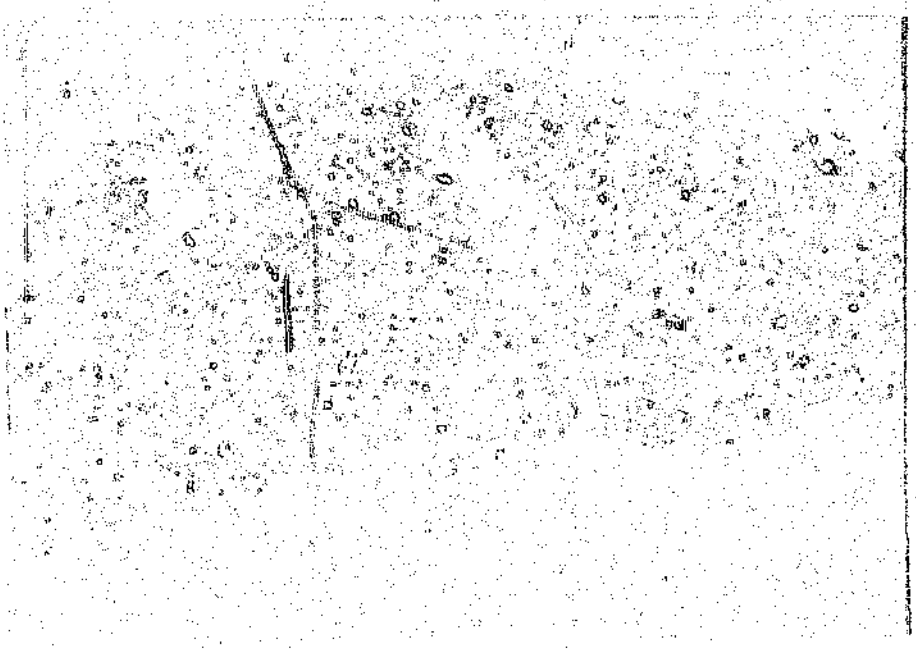


FIGURE 6 (b)

FIGURE 6.

L.M. sections through a confluent stratified keratinocyte sheet. (a. mag. 100x and b. 400x). The number of layers vary from 4 to 7 cell layers thick. The basal keratinocytes are small, round to cuboidal. Dispase II, which is used to lift the epidermal sheet from the surface of the petridish, has the propensity of causing the the basal cells to round up. In the intermediate region the cells are large and polygonal, while at the surface, the cells are flattened.

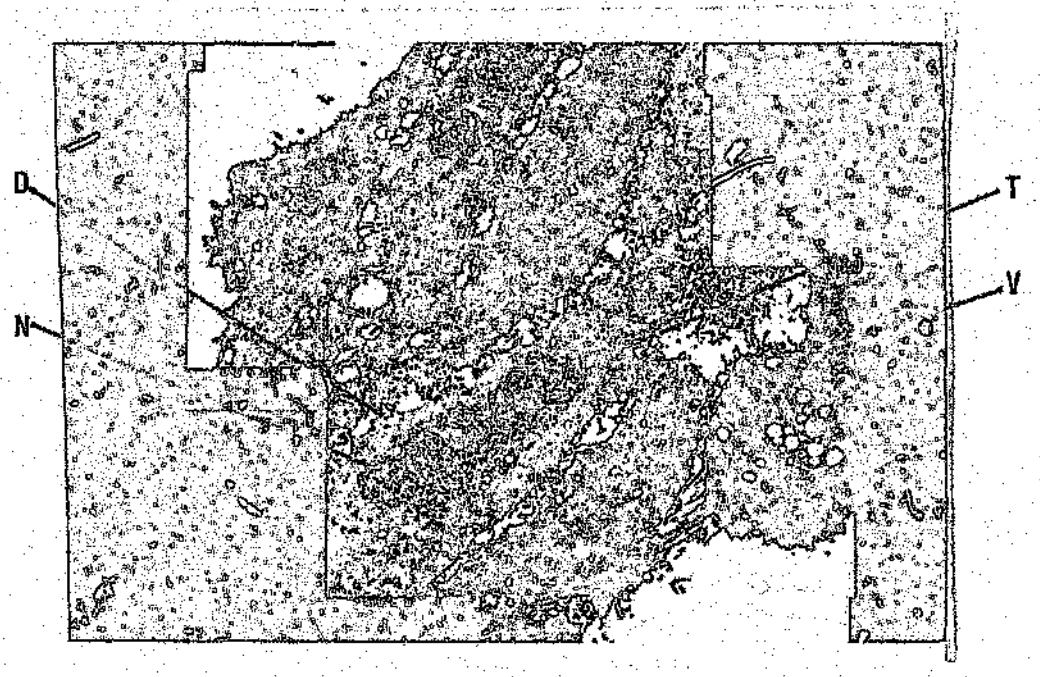


FIGURE 7.

E.M. section through a stratified epidermal sheet. In the lower right hand corner 2 basal cells containing a number of vesicles can be seen. The more superficial cells contain fewer vesicles and more keratinohyalin granules. (mag. 1000x).

N = nucleus
D = desmosomes and interdigitations
V = vessicle
T = tonofilaments

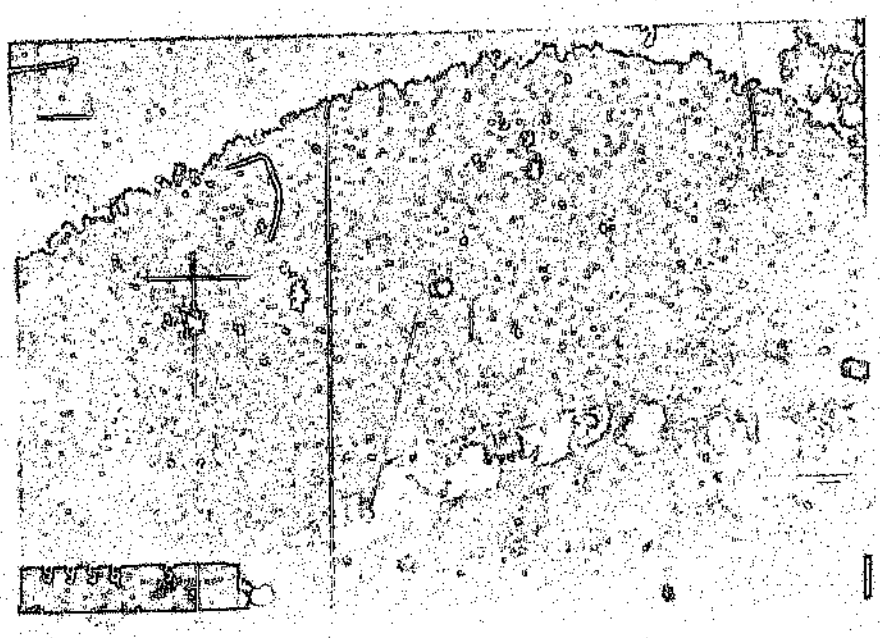


FIGURE 8.

E.M. section through a superficial flattened anuclear keratinocyte. (mag. 15000x). Keratin is deposited within the cell.

FIGURE 9.

E.M. section through a round basal keratinocyte. The basal keratinocyte contains a large nucleus and nucleolus. This cell actively divides to produce the suprabasal cells. Vacoules can be seen within the cytoplasm. The surface projections contain (D), desmosomes (maculae adherens) which connect adjacent cells to each other. Desmosomes are particularly well developed in the normal epidermis where they occur as intercellular bridges between the cells. (mag. 15000x). T = tonofilaments.

FIGURE 10.

A confluent stratified keratinocyte sheet floating in phosphate buffered saline.It has lifted off the surface of the petridish with the aid of dispase II.

2.3.2 The temporal progression of cultures to stratification

There does not appear to be a significant difference in the growth rate between neonatal and older keratinocytes, when comparing the time at which stratification is reached after first plating out single cells.

Average time to reach stratification:

24.1 days - for neonatal keratinocytes

25 days - for older keratinocytes

2.3.3 Factors affecting the progression to stratification

a) Monoclonal colony appearance depends on the time of harvesting to culturing. The monoclonal colonies which formed from day 4 and later, were first cultured at least 12 hours after harvesting. Prior to culturing, the explants were kept in the fridge at 4° C in 0.17% trypsin over night. Whereas the monoclonal colonies that formed from day 2 and 3 were derived from explants whose cells were put down for culture within 2 hours after harvesting.

For neonatal cells lines:

if this time was more than 12 hours: monoclonal colonies formed on average by 4 days;

if this time was less than 12 hours: monoclonal colonies formed on average by 2.3 days;

For older cells lines:

if this time was more than 12 hours: monoclonal colonies formed on average by 4.6 days;

if this time was less than 12 hours: monoclonal colonies formed on average by 3 days;

For all cells lines: (neonatal and older cell lines)

if this time was more than 12 hours: monoclonal colonies formed on average by 4.3 days;

if this time was less than 12 hours: monoclonal colonies formed on average by 2.7 days.

The ultimate viability of the cell line may also be affected.

b. Certain cell lines (e.g. cell lines Kit, Defratas, Lui and Reece) did not require growth factor supplements to form stratified sheets.

c. Cell cultures infected by bacteria had to be discarded, since antibiotic administration was never completely successful.

d. Cell cultures infected by fungi were more amenable to fungicides and recovered to go on to form confluent stratified sheets (e.g. cell line Br).

e. Secondary cell cultures reached confluence and stratification faster (by 20 days on average), than primary cell cultures (by 24.6 days on average).

f. Cryopreserved cells (appendix III) tended to take longer time to grow out and did not always grow to form confluent sheets.

TABLE 1.

PRIMARY COLONIES — burn patients

Cell Line	Monoclonal Colonies	Subconfluent Colonies	Confluence	Stratification
Rain	4 days	12 days	16 days	27 days
Moses	4 days	20 days	25 days	35 days
Else	4 days	7 days	11 days	21 days
Jonas	3 days	8 days	10 days	20 days
Virginia	3 days	10 days	14 days	24 days
Grobler	4 days	12 days	14 days	17 days
Lindiwe	4 days	8 days	10 days	20 days
Braam	3 days	10 days	13 days	25 days

The above date represents the fastest growth rate for each cell line, as seen in a single culture dish.

GRAPH 1.

Age of Cell Line vs Stratification Time

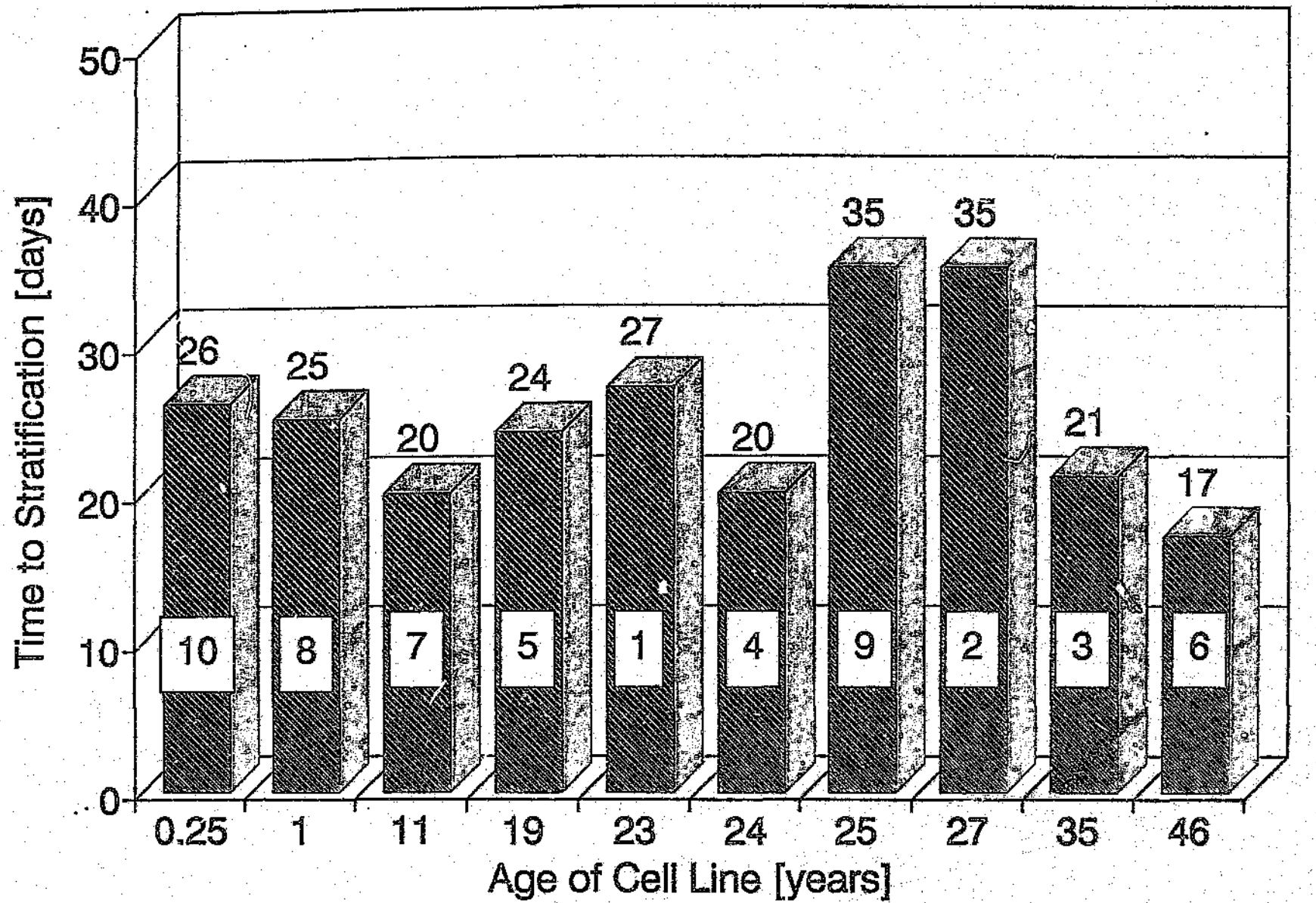


TABLE 2.

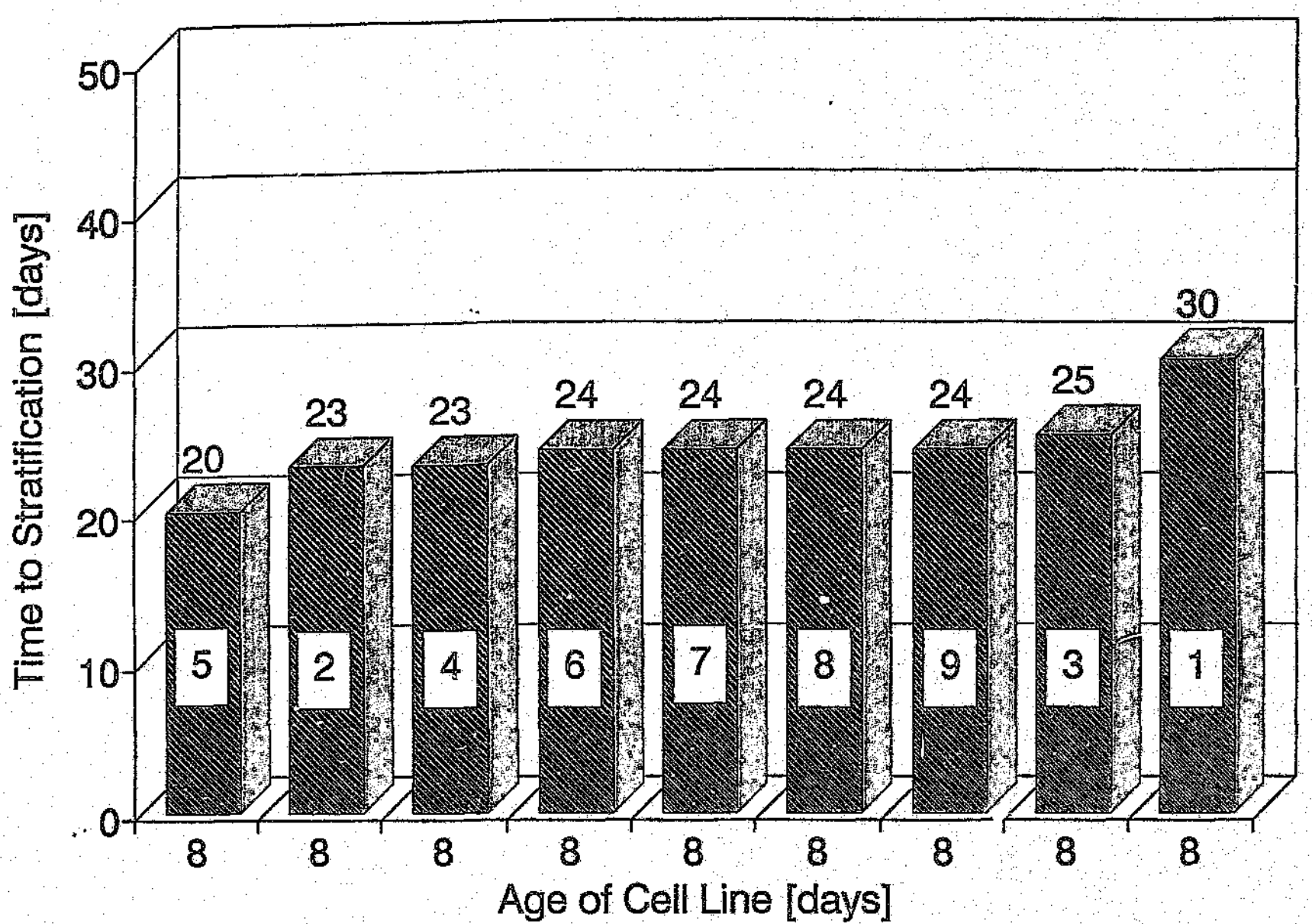
PRIMARY COLONIES — cell lines

Cell Line	Monoclonal Colonies	Subconfluent Colonies	Confluence	Stratification
K: (8 days)	day 3	15 days	20 days	30 days
B: (8 days	day 4	10 days	13 days	23 days
D: (8 days)	day 4	10 days	13 days	23 days
G: (8 days)	day 4	10 days	13 days	20 days
BR: (27 years)	day 8	18 days	28 days	35 days
Tim (3 months)	day 3	10 days	16 days	26 days
N: (8 days)	day 3	8 days	10 days	20 days
Kit: (8 days)	day 2	10 days	14 days	24 days
Defratas:(8 days)	day 2	10 days	14 days	24 days
Lui (8 days)	day 2	10 days	14 days	24 days
Reece (8 days)	day 2	10 days	14 days	24 days

The above date represents the fastest growth rate for each cell line, as seen in a single culture dish.

GRAPH 2.

Age of Cell Line vs Stratification Time



2.4 DISCUSSION

From the data presented one sees that there is a relatively wide variation with respect to the time at which confluence and subsequent stratification occurs. This being from 10 days to 28 days, for the former, and from 17 days to 35 days for the latter.

2.4.1 Multiplication time is keratinocyte specific

The culture methodology did not vary, thus it may be concluded that there is an inter-individual variation between specific cell lines. The keratinocytes derived from a specific cell line have an inbuilt 'clock', dictating the rate of cellular multiplication. This does not appear to be age dependant, since the time to reach confluence for the keratinocytes derived from the 8 day old foreskins, varied from 10 days to 20 days. This confirms a finding by various researchers, who have identified cultured keratinocytes having different cell cycle times. They have shown that the smallest cells have the greatest clone forming ability (47, 48, 49).

2.4.2 The importance of plating out concentration

Another factor to be considered is the plating out concentration of keratinocytes. A lower concentration is said to speed up the time at which confluence is reached (50). At a so called ideal plating out concentration, keratinocytes grown on plastic, both in serum containing and serum free medium, divided rapidly with a mean cell cycle time of 22 - 24 hours. The cells pass through multiple population doublings and form stratified cultures with poor cornification. Keratinocytes undergo a number of population doublings before senescence depending on the age of the donor; neonatal foreskin having probably 50 - 60 population doublings but declining with donor age (16).

2.4.2 Site of keratinocyte harvest

The skin from which the keratinocytes are derived is of importance. Retro-auricular skin and foreskin produce numerous fibroblasts, which compete with the keratinocytes for surface space. Whereas breast skin appears to produce fewer fibroblasts and the keratinocytes can form fairly large monoclonal colonies before the human fibroblasts have to be removed with

the aid of EDTA/glucose. By adding numerous fibroblast feeder cells at an early stage in keratinocyte culture, the human fibroblasts tend not to be a competitive problem.

2.4.4. The affect of trypsin exposure and time before culturing

Trypsin may adversely affect keratinocyte growth and multiplication. This is deduced from the finding that there was at least a full 24 hour to 5 day delay in the formation of monoclonal colonies in those keratinocytes which were exposed to trypsin for more than 4 hours. Besides the long exposure time to trypsin (more than 12 hours), the relatively longer time after harvesting to placement of the cells into culture medium compared to those cells which were put down within 2 - 4 hours after harvesting, may also be the cause for significantly affecting the increased time taken for monoclonal colony formation.

2.4.5 Keratinocyte multiplication and growth factors

Although the various growth factor supplements enhance keratinocyte growth and multiplication in specific ways, they did not appear to be required in all the cell lines cultured. This may be due to the fact that the bovine calf serum used may itself contain some of these growth factors. The reason why some cell lines did not respond to them, may be due to them being present in too small amounts, in a certain batch, or that certain keratinocytes, from specific cell lines, are more responsive (receptor differences) than others to the various concentrations of growth factors present within the serum. This has as yet to be proven.

Keratinocytes synthesize and respond to a wide range of growth factors and an increasing number of these have been detected in the keratinocyte supernatant (31, 51 - 59); interleukins 1, 3, 6 and 8, transforming growth factors alpha and beta (TGF-α, TGF-ß), granulocyte macrophage colony stimulating factor (GM-CSF), basic fibroblast growth factor (b-FGF), platelet derived growth factor (PDGF), and tumour necrosis factor (TNF). Roles for these growth factors have been proposed in the regulation of keratinocyte turnover and immune function in normal and diseased skin.

TGF - α is mitogenic for keratinocytes in vitro (55) and also enhances keratinocyte migration (60). The serum may contain antimitogenic substances such as TGF-ß (61) which inhibits keratinocyte multiplication in vitro, but enhances keratinocyte differentiation (62) and fibronectin production (63). TGF-ß (64) and a partially characterised factor (65) produced by keratinocytes accelerate epithelialization of split thickness wounds in pigs. IL-1 (66) and

6 (67) and b-FGF (68) are mitogenic to keratinocytes. IL-3, IL-6 and GM-CSF stimulates haemopoiesis, lymphopoiesis and inflammatory and immune cell chemotaxis and activation (69), they may gain access to the circulation and modulate systemic inflammatory reactions. IL-6 is a pyrogen, stimulates production of acute phase proteins by the liver (70) and increased serum levels are found in burns (71) and psoriasis (67).

These cultured keratinocyte sheets have clinical applications. What is their application to burn therapy and other conditions resulting in skin loss? Is there a difference between cultures grown from the patient's own skin and those grown from donor skin? Is the surgical method involved in the application of the keratinocyte grafts operator dependant?

CHAPTER THREE

3.0. THE PREPARATION AND GRAFTING OF CULTURED EPIDERMAL SHEETS

3.1. Introduction

Human keratinocytes can be cultured from single cell suspensions in order to form confluent stratified epidermal sheets (1). They have been applied to a variety of skin defects either as cultured autografts or as cultured allografts. The majority of the experience gained with respect to their use is in their application to full thickness burns and leg ulcers.

Gallico, O'Connor, et al. (72) have described a method for culturing as well as the application of these grafts to the wound bed. This report describes new methods for the preparation and grafting of keratinocyte sheets.

To ensure the best possible take, preparation is as important as ensuring that one has a healthy graft. In this chapter, steps are outlined that should be followed if one is to successfully apply cultured confluent stratified keratinocyte sheets to areas of skin loss. This is a user friendly model, but for the best results, attention must be paid to the details highlighted in this chapter.

3.2 Material and Methods

3.2.1 Preparation of keratinocyte sheets for grafting

A site for donor skin measuring 2 - 4cm^2 (figure 1) chosen in the case of a burn patient (appendix II B). Allograft skin was obtained from neonatal or young adult circumcisions (appendix II A) and excess skin from certain plastic surgical procedures, such as breast reductions. This allograft skin must test seronegative for the hepatitis B virus as well as the human immunodeficiency virus (appendix VIII). The skin specimen was placed in a bacterial petri dish and excess fascia was removed with the aid of a watch maker forceps and a scalpel blade. This was followed by placing skin specimen, covered by 0.17% trypsin, in an incubator for 20 - 40 minutes, or at 4°C in a fridge over night. The epidermis separated from the dermis at the level of the stratum basale. With the aid of curved glass pipettes 5 - 10 x 10^3 basal cells/ml was scrapped off and placed into a culture dish containing growth

medium solution (DMEM: Ham's F12 (1:1) and 10% bovine calf serum) and 3T3 murine fibroblast feeder cells (appendix I B). When monoclonal colonies first formed (on average within 2.7 days), growth factor supplements (appendix I A) were added to the culture medium, which was changed every second day.

After confluence and stratification have been achieved (on average within 24.6 days), the epidermal sheet was ready to be used as a graf.. The epidermal sheets were lifted off the surface of the petri dish by the addition of Dispase II (appendix I A) for 40 minutes. The petri dishes were placed either in an incubator or on a glass sheet, placed over a bowl of water at 37°C. In the latter case a sterile green sheet was placed over the petri dishes. At least once during the 40 minute time interval the Dispase II was swirled over the sheets in order to re-establish an even distribution. The Dispase II was removed and the epidermal sheet was washed in phosphate buffered saline. The petri dishes, thus containing epidermal sheets and PBS were placed on ice until grafting took place (figure 10).

FIGURE 11.

Here one sees a keratinocyte sheet being lifted out of the 9cm (diameter) petridish with the aid of 2 blunt forceps. Orientation of the sheet is important. The basal surface must be applied to the donor surface.

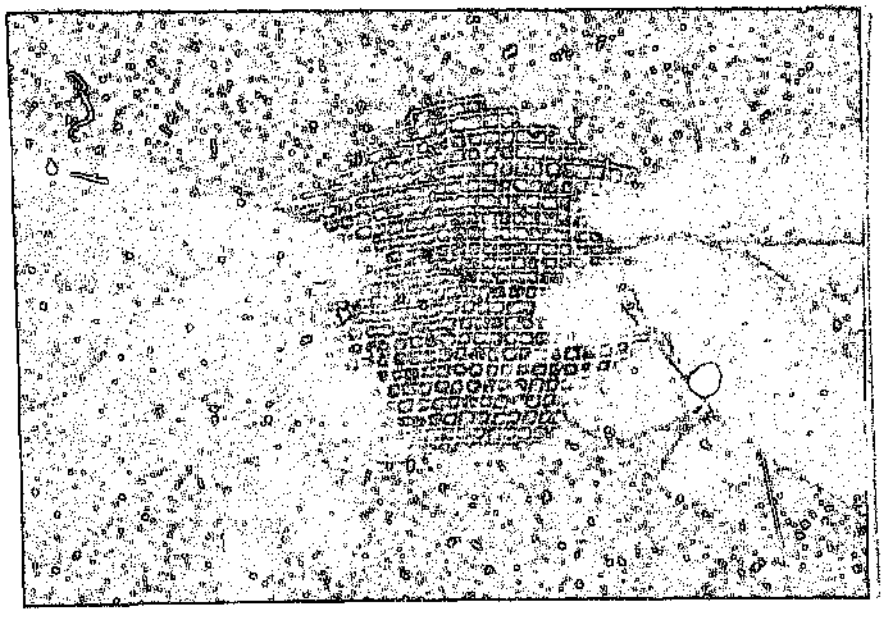


FIGURE 12.

Seen here, the keratinocyte sheet is applied first to the paraffin gause and stretched out to its maximum extent. Alternatively, it may first be applied to the donor site and the paraffin gause is placed above it.

FIGURE 13.

The modified Norwich cage. The paraffin gause is tented over the leg ulcer which has been covered with a cultured keratinocyte sheet allograft. Over this has been placed malleable aluminum bars, covering one side with foam rubber and secured with elastoplast.

FIGURE 14.

The elastomesh stocking has been applied over the malleable aluminum bars.

FIGURE 13.

The modified Norwich cage. The paraffin gause is tented over the leg ulcer which has been covered with a cultured keratinocyte sheet allograft. Over this has been placed malleable aluminum bars, covering one side with foam rubber and secured with elastoplast.

FIGURE 14.

The elastomesh stocking has been applied over the malleable aluminum bars.

3.2.2 Grafting of full thickness burn wounds

The removal of inflammatory necrotic tissue by chemical debridement exposes the underlying vascular tissue. The patient was given a general anaesthetic. Debridement of the burns was carried out, until slight bleeding occurred. On any exposed subdermal structure noevascularisation occurs, resulting in the formation of granulation tissue. Muscle fascia is highly vascular thus will bleed following debridement. A solution of 1:100 000 of adrenaline was applied to the wound bed with the aid of abdominal swabs. When haemostasis was achieved, piperacillin powder was sprinkled into the graft bed, (be it granulation tissue or vascular adipose tissue) and the excess antibiotic was removed by using dry swabs to dust away excessive powder.

The epidermal sheet was lifted out of the Petri dish with the aid of 2 blunt forceps (figure 11), in the case of the 9cm sheet. The 14cm sheet was placed on the recipient site with the aid of 4 forceps, thus requiring an assistant. The basal surface of the epidermal sheet was applied to the surface of the burn wound. The epidermal sheet was spread out to its maximum extent and stapled into place (figure 12).

In the case of the compound graft, 1:3 meshed split thickness skin autograft was placed over the cultured autologous or allogeneic epidermal sheet. This in turn was stapled into place.

The dressing applied were similar to those described by BTI* (73) and included paraffin gauze, coarse gauze, orthopaedic wool and crepe bandage. If on day 4 or 5 the dressing was wet and foul smelling, infection has occurred and they must be removed. The burn wound was then covered with saline soaked gauze. As long as the grafts were dry, the dressings could be left in place, for up to 14 days and then changed every second day until complete healing has occurred .

*= Biosurface Technology Incorporated

3.2.3 Grafting of leg ulcers and other skin defects

The state of the ulcer bed is important. It must contain healthy red granulation tissue that bleeds slightly when rubbed. The most recent pus swab must be negative.

Piperacillin powder is applied and then dusted off. This is followed by the application of a cultured epidermal allograft sheet to the ulcer bed with the aid of non-toothed forceps.

One of 2 types of dressing is applied:

a) Closed method - paraffin gauze, coarse gauze, orthopaedic wool and crepe bandage are applied in that order.

b) Open method - the modified Norwich cage. Here the epidermal sheet, after being placed on the ulcer bed, is exposed to air. A sheet of paraffin gauze is tented above the graft. The grafted ulcer is protected from mechanical damage by the application of malleable aluminum bars which are padded with foam rubber. These bars are able to be moulded into a protective cage. Over the bars an elastomesh stocking is applied (see figures 13 and 14), (74).

3.2.4 Follow-up following grafting of leg ulcers and other skin defects

In the case of the closed method, the dressings were removed at day 4 or 5 post-grafting. Thereafter, the dressings were changed at every second day until complete healing of the ulcer had occurred, the dressing being the same as the initial dressing.

If the grafting was carried out on an out-patient basis, then the patient was told to remain in bed with the leg elevated for at least 5 days post-grafting. Following complete healing, the patient was told to protect the area of previous ulceration from

any type of trauma. Graduated elastic stockings were also prescribed.

3.3 Discussion

By making use of this grafting procedure, one has achieved relatively good "take", as seen by visible skin formation both in the case of full thickness burns as well as in the case of a variety of lesions, resulting in skin loss in the leg (Beder et al. in press, (75, 76).

Besides the adherence of the cultured keratinocyte sheet to the wound bed, on histological examination one sees the epidermis becoming more stratified. This is what is meant by the word 'take'. Initially no new dermis is present. The new epithelium of the wound is thicker than normal epidermis although it appears thin and friable to the naked eye (120). However, it becomes thinner as it matures and acquires an attachment to the underlying connective tissue.

The use of a bowl of water heated to 37°C eliminates the requirement of an incubator close to the operating theatre. Biosurface Technology Inc. (73) actually make use of a portable incubator. The keratinocyte sheets already lifted off from the culture flask and attached to a backing material, are thus transported to the operating theatre. This method was assessed, but has 2 drawbacks. Firstly, the keratinocyte sheet being 9cm in diameter, shrunk to less than half that size on being placed on a paraffin gauze backing. Secondly, by the time the keratinocyte sheet reached the operating theatre, it had almost completely disintegrated.

An important component of this grafting procedure is the application of the antibiotic powder piperacillin. It appears to improve percentage take. Piperacillin is toxic to Pseudomonas spp. which are common bacterial contaminants of burn wounds especially within the hospital environment. This antibiotic is also toxic to a wide variety of gram negative and gram positive bacteria. No allergic phenomena were seen in patients to which it was administered. Many researchers (77, 78) reject the use of topical antiseptics, since they are toxic to keratinocytes at very low concentrations. But in this study the topical antibiotic piperacillin did not appear to be toxic to cultured keratinocytes.

Other researchers tried to counteract the effect of infection by grafting onto freshly excised muscle fascia or onto 2-3 day old granulation tissue (23).

The use of the grafting methodology described in this paper has enabled one to obtain results comparable to those obtained by the Boston group (79) in the treatment of burn patients with cultured keratinocyte autografts.

This paper attempts to bring to the attention of surgeons involved in skin grafting, a proven methodology used for grafting cultured keratinocyte sheets to any skin defect, irrespective of its aetiology.

In the following 2 chapters, the clinical outcome of cultured keratinocyte sheets applied to burns, chronic leg ulcers and other lesions resulting in skin loss, is assessed and the results analysed.

CHAPTER FOUR

4.0 HEALING FULL THICKNESS BURNS WITH CULTURED EPIDERMAL GRAFTS

4.1 Introduction

Patiens presenting with burns damaging more than half of the body surface area have too few donor sites to provide enough split skin grafts to resurface the area after surgical excision.

The definition of a full thicknes burn implies total destruction of the entire skin and dermal appendages, obviat ng the possibilty of epithelial regeneration, save from the margins (implying the need for skin grafting). The full-thickness burn may extend deep into the underlying muscle, viscera and even bone (121).

The use of cultured epidermal grafts (keratinocyte grafts) to treat patients with life-threatening burns has been carried out since 1981 (19) with variable degrees of success. Perusal of the literature shows that the success rate using this method to treat full thickness burns has been limited (19, 20, 21, 22, 23).

The first 2 cases which were grafted with cultured autografts resulted in approximately 50% "take", as seen by visible skin formation (19). Subsequently in 1984, two children receiving 95% full thickness burns, had 60% - 80% 'take' (72). But controversies in the literature with respect to "take" abound. A number of researchers (20, 21, 22, 23) have reported relatively low percentage of "take" either at day 5 or at day 14 in the case of cultured autografts. Two studies (22, 23), showed that "take" was better in patients younger than 18 years compared with older patients. Apparent good results were seen in deep partial thickness burns (23). But here dermal elements such as hair follicles and sweat glands contributed greatly to supplying multiplying keratinocytes.

Good 'take' occurred when Clarke (80) used cultured allografts and cultured autografts in the following manner; highly meshed (6:1) split thickness autograft was applied to the wound bed over which cultured keratinocyte sheets were applied. Whether the skin covering the interstistices was of culture origin or not, was not able to be determined.

Madden (81) grafted patients with cultured allogeneic keratinocyte sheets. Percentage `take' varied from 0 - 50%. These full thickness burns appeared to heal primarily by contracture and re-epithelialization from the wound edges. In the cases where cultured allografts were applied to deep second degree burns, healing appeared to be speeded up by their use, compared to if they were left to heal alone. The allografts may have induced healing by secreting growth factors even in the absence of a physical 'take'.

Many researchers (77, 78) reject the use of topical antiseptics since they are toxic to keratinocytes at very low concentrations. But in the study presented here, a technique using a topical antibiotic was used which was not toxic to cultured keratinocytes. I report the treatment of 10 patients using various combinations of cultured epidermal sheets and their application to the wound bed. Use of an antibiotic powder has reduced infection enabling a better and more reliable comparison of auto-, allo-, and compound grafts.

4.2 Patients and Methods

Ten patients, all with full thickness burns are presented. Please refer to appendix V for patient details. The patients received one of the following types of grafts:

4.2.1 Cultured allografts
4.2.2 Cultured autografts
4.2.3 Highly meshed split thickness skin grafts applied over cultured allografts or autografts (here called the compound graft).

The culture method used to generate epidermal sheets from suspensions of single keratinocytes is that described by Rheinwald and Green (1).

4.2.1 Cultured allografts

Five patients were grafted with cultured allografts.
They were divided up into 2 groups.
a) Those patients presenting with large areas of full thickness burn injury - 3 patients.
b) Those patients presenting with small areas of full thickness burn injury - 2 patients.
Percentage 'take' was assessed at day 14. 'Take' implies visible skin formation both clinically and histologically.

4.2.2 Cultured autografts

Five patients received cultured autografts.

4.2.3 The compound grafts

Four patients were grafted with cultured allograft or autograft, over which a highly meshed split thickness autograft was applied. In one patient, a biopsy of the area grafted with a compound graft was taken for histological analysis.

I recognise that the patient numbers are low but there was a problem with respect to patient avalability and follow-up. However, the numbers correlate well with the numbers presented in previous studies (19, 21-23).

4.3 Results

4.3.1 Cultured allografts

The 3 patients, with extensive burns, presented with no or low take at day 14 (see table 3). The 2 patients with small burns had good rates of healing by day 14 post grafting (see table 3, graph 1, figure 16).

4.3.2 Cultured autografts

The results of the 5 patients receiving cultured autografts are set out in table 5, graph 4 and figures 17 and 18.

4.3.3 The compound grafts

The percentage `take' by day 14 was good (see graph 5 & table 5). In the case of the patient, Miss R.M., the compound graft on the right calf was compared with a similar area on the left calf, which had been grafted with a 1:3 meshed split thickness autograft alone. The cosmetic appearance of the compound graft is more acceptable than that of the latter graft (see figures 20 and 21).

Sections of the biopsy specimen taken from an area grafted with the compound graft, show skin in which the overlying epidermis shows disorganisation of the dermal - epidermal junction. Within the dermis stellate fibroblasts, haemosiderin and extra-vasated red blood cells are noted. The latter are distributed around proliferating vascular channels.

4.3.4 Comparison of graft types

Graph 6 portrays a comparison between healing rates of full thickness burns to which various types of cultured grafts were applied. The best take was seen in the case of the compound graft.

Comparing the rate of healing up to 14 days post grafting of large full thickness burns after the application of cultured autografts and cultured allografts, one finds that there is a significant difference. Cultured autografts result in faster healing compared to cultured allografts. Using the Mann Whitney test $p = 0.0497$.

During the course of this study an interesting correlation relating to the percentage take was noted between patients presenting with smal full thickness burns and leg ulcers. In the case of cultured allografts being applied to relatively small full thickness burns, percentage take is comparable to lower leg ulcers of similar size (76).

TABLE 3 CULTURED ALLOGRAFTS

Patient	Age	Size of Burn	Percentage Healing by 14 days
Mr G.	46 yrs	282 cm^2	20
Mr B.H.	1 yr	1240 cm^2	0
Mr J.	24 yrs	64 cm^2	50
Mr R.N.	23 yrs	25 cm^2	100
Mr E.N..	34 yrs	125 cm^2	90

TABLE 4 CULTURED AUTOGRAFTS

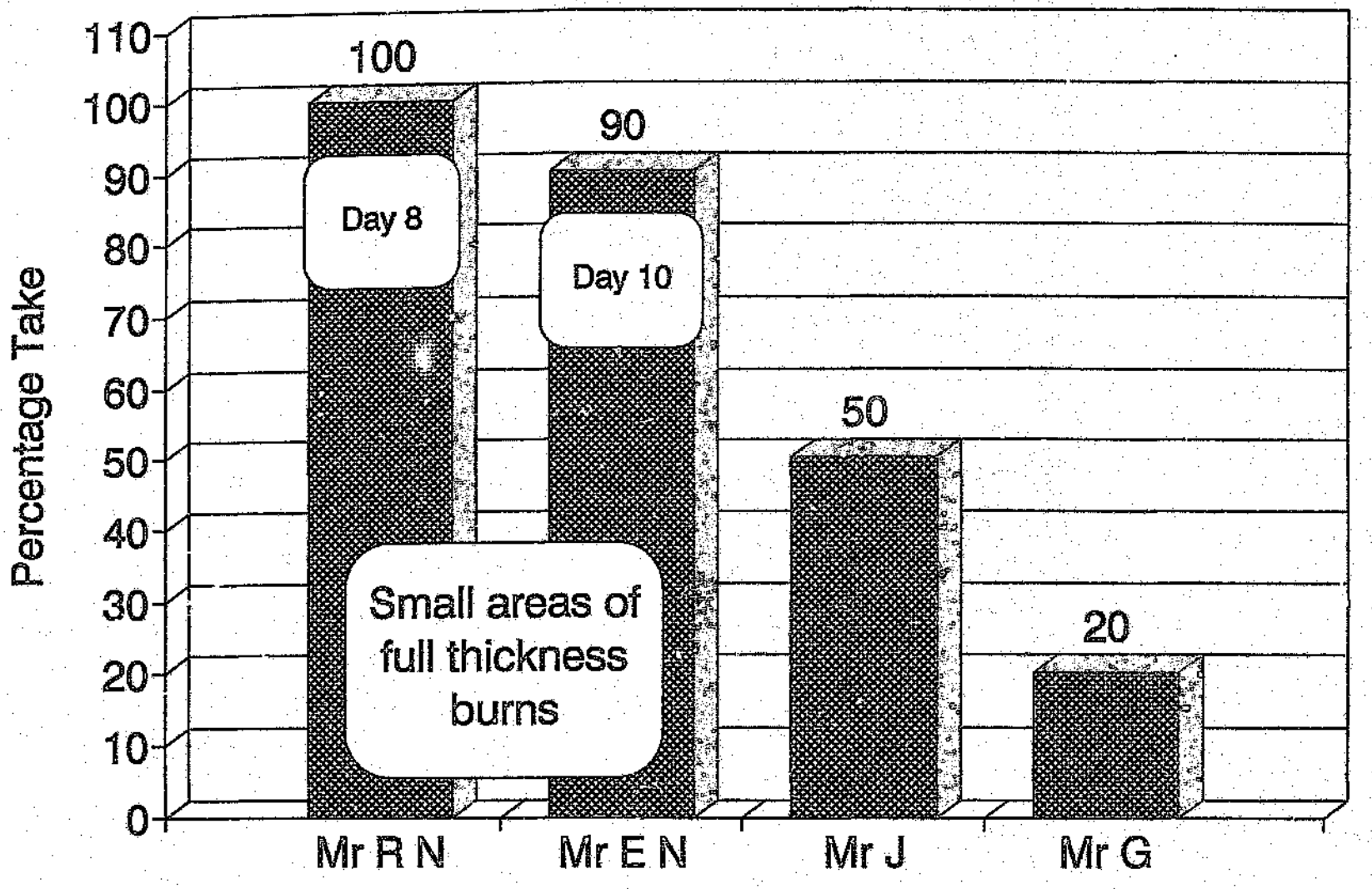
Patient	Age	Size of Burn	Percentage Healing by 14 days	
Mrs E.	35 yrs	127 cm^2	50	
Miss L.Z.	11 yrs	108 cm^2	92.6	
Mr J.	24 yrs	218 cm^2	98	
Mr M.	42 yrs	36 cm^2	11.1	(Pseudomonas spp infection present)
Miss V..	19 yrs	96 cm^2	97	

TABLE 5 COMPOUND GRAFTS

Patient	Age	Size of Burn	Percentage Healing by 14 days
Mrs E.	35 yrs	156 cm^2	100
Miss L.Z.	12 yrs	5 cm^2	100
Mr R.M.	23 yrs	144 cm^2	100
Miss V..	19 yrs	96 cm^2	100

GRAPH 3.

The Cultured Allograft



Percentage Healing by Day 14

FIGURE 15.

Patient B H used to illustrate cultured epidermal grafts acting as excellent biological dressings. A clean full thickness burn wound is shown 17 days after the application of cultured epidermal allografts.

FIGURE 16 (a)

FIGURE 16.

Patient Mr E N used to illustrate the stages of healing following the application of a cultured epidermal allograft.

a) The area of full thickness burn which is about to be grafted.
b) Four days after applying the cultured epidermal allograft. The size of the granulation tissue has markedly decreased.
c) Sixteen days after grafting, 92% of the area grafted is covered by newly formed skin.

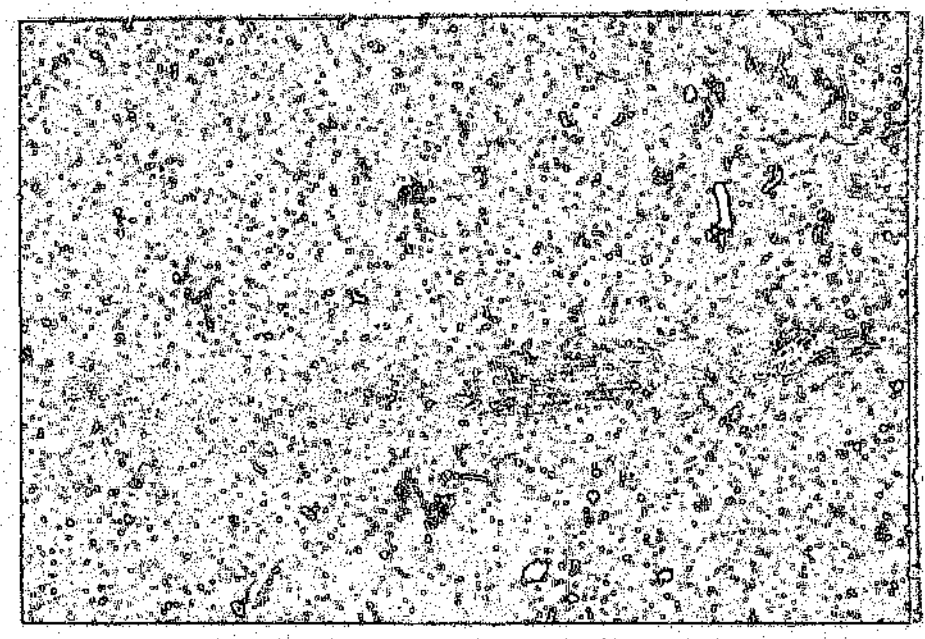
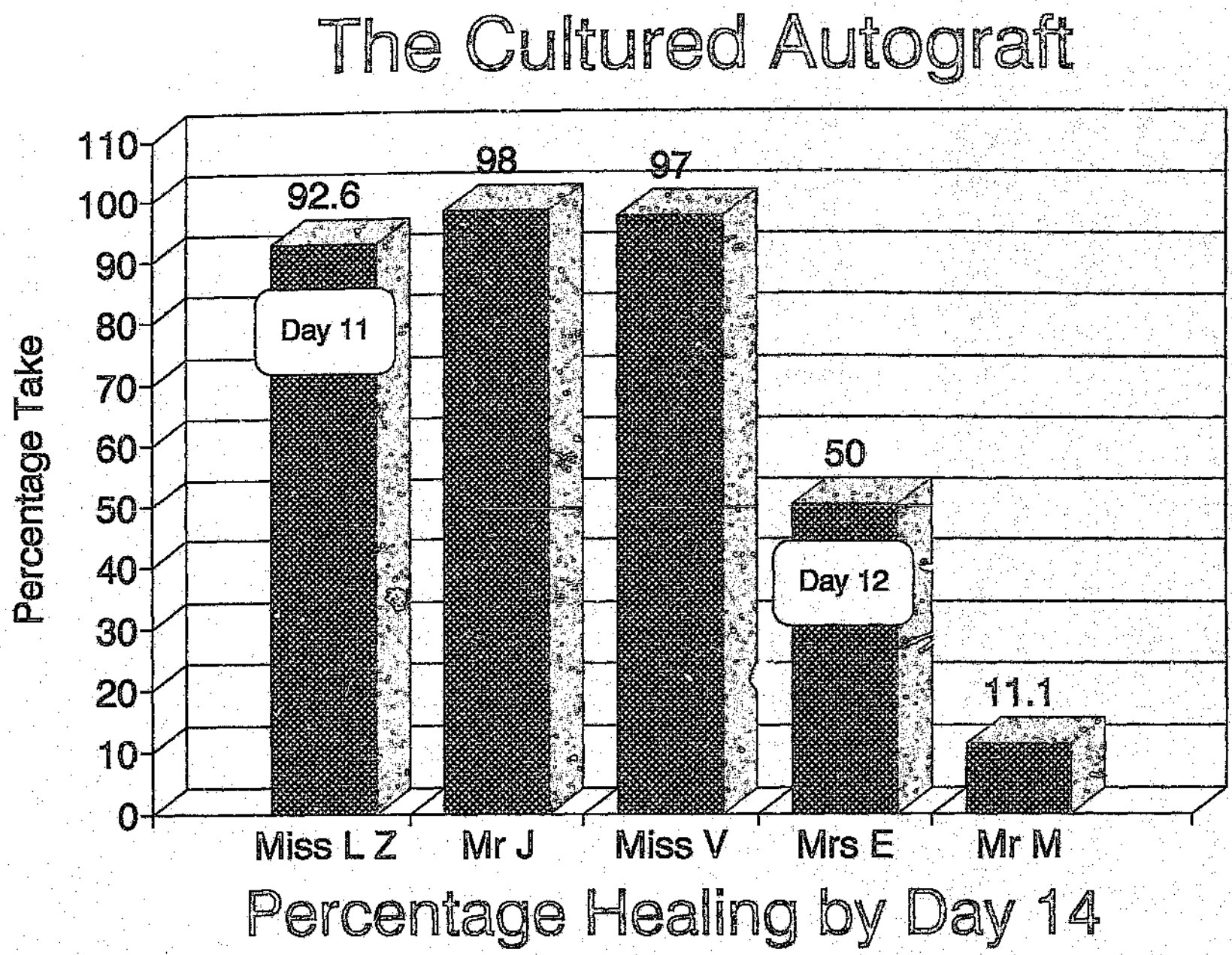


FIGURE 16 (c)

GRAPH 4.


The Cultured Autograft
Percentage Take
110
100
90
80
70
60
50
40
30
20
10
0
92.6
98
97
50
11.1
Day 11
Day 12
Miss L Z
Mr J
Miss V
Mrs E
Mr M
Percentage Healing by Day 14

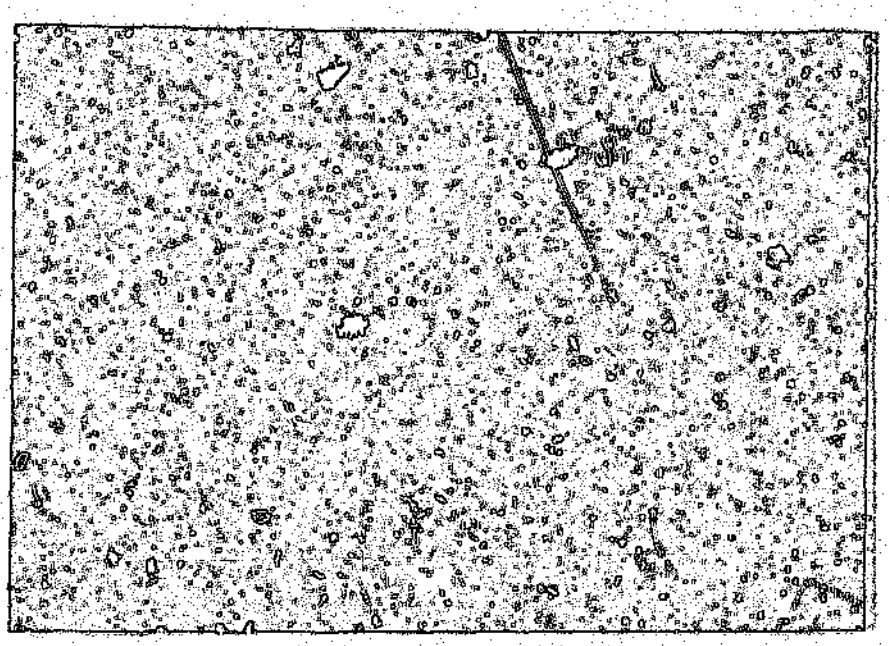


FIGURE 17 (a)

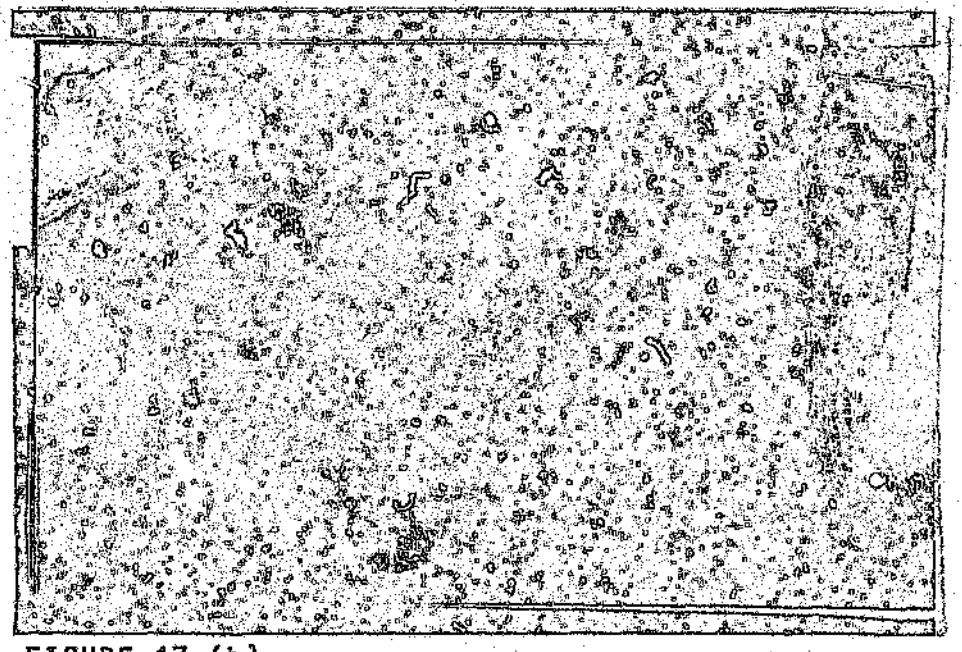


FIGURE 17 (b)

FIGURE 17.

Patient Miss L Z used to illustrate the stages of healing following the application of cultured autografts.

a) At the time of grafting the cultured autografts to the anterior chest wall.

b) One month after grafting reveals the taken cultured autografts. The brown spotted area, to the right of the area to which cultured epidermal autografts were applied alone, was were a compound graft was applied.
(See appendix V page 132 for the data sheet pertaining to this patient).

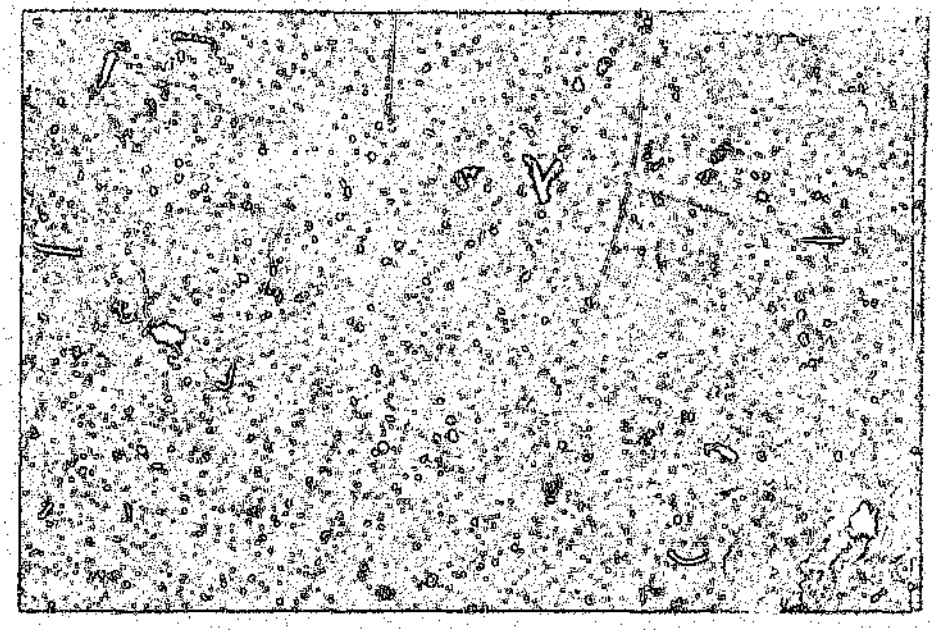
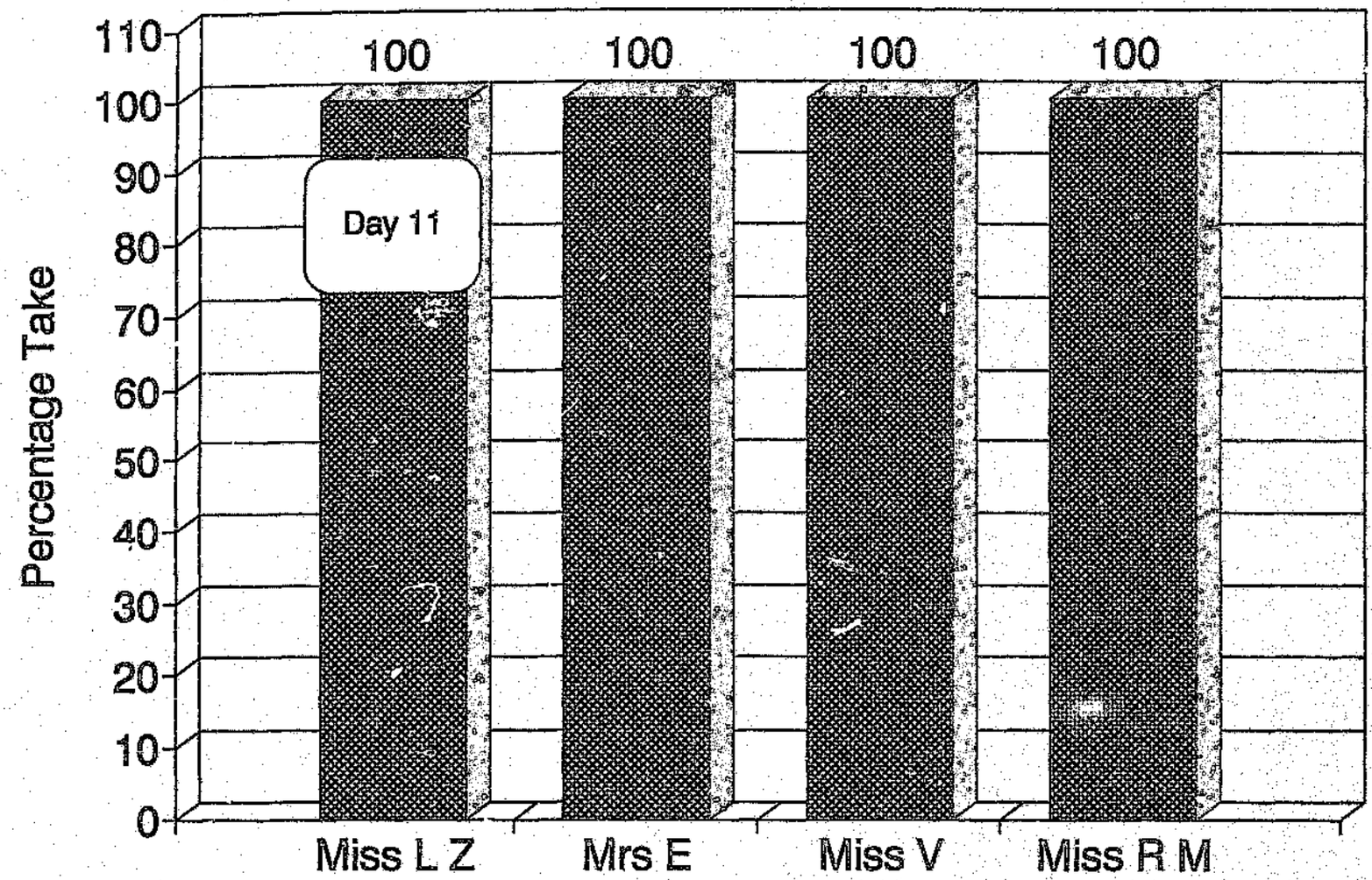


FIGURE 18.

An example of a healing full thickness burn 14 days after cultured autograft application. The anterior upper arm was grafted.
(See appendix V page 134 for the data sheet pertaining to this patient).

GRAPH 5.

The Compound Graft



Percentage Healing by Day 14

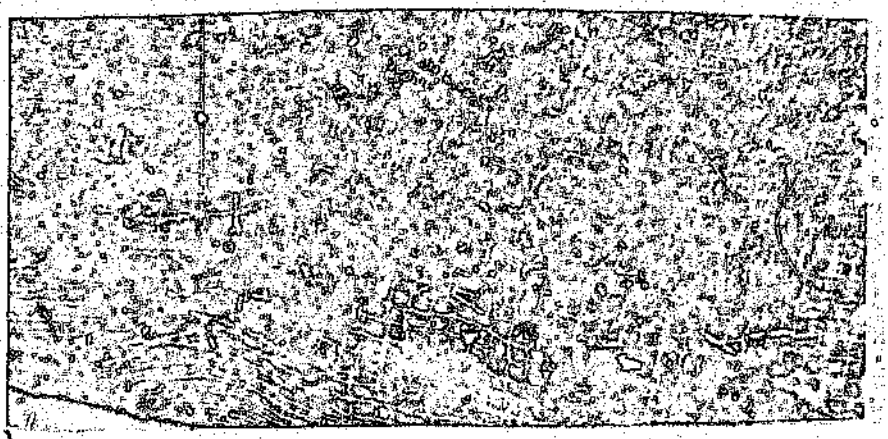


FIGURE 19 (a)

FIGURE 19 (b)

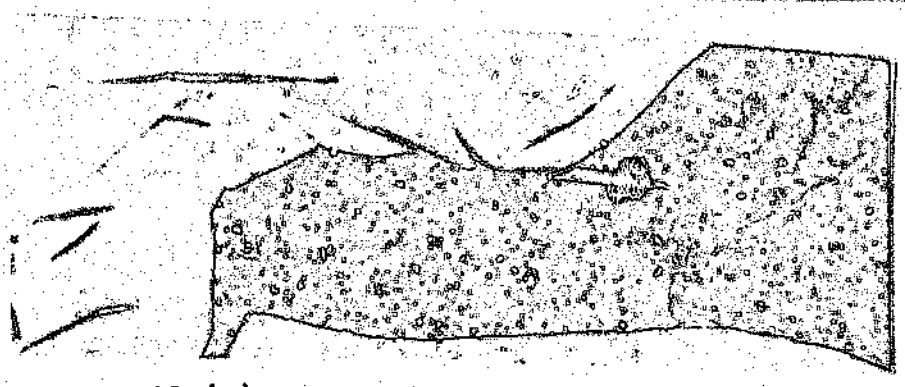


FIGURE 19 (c)

FIGURE 19.

Patient Miss R M used to illustrate the stages of healing following the application of a compound graft.

a) Three days post-grafting of the compound graft reveals a dry clean graft. This is the first sign of take.
b) 14 days after grafting some of the overlying SSG has sloughed, but in its place new skin is seen.
c) 23 days post-grafting reveals a smooth dark brown skin which appears completely different from surrounding grafted areas.

(See appendix V page 124 for the data sheet pertaining to this patient).

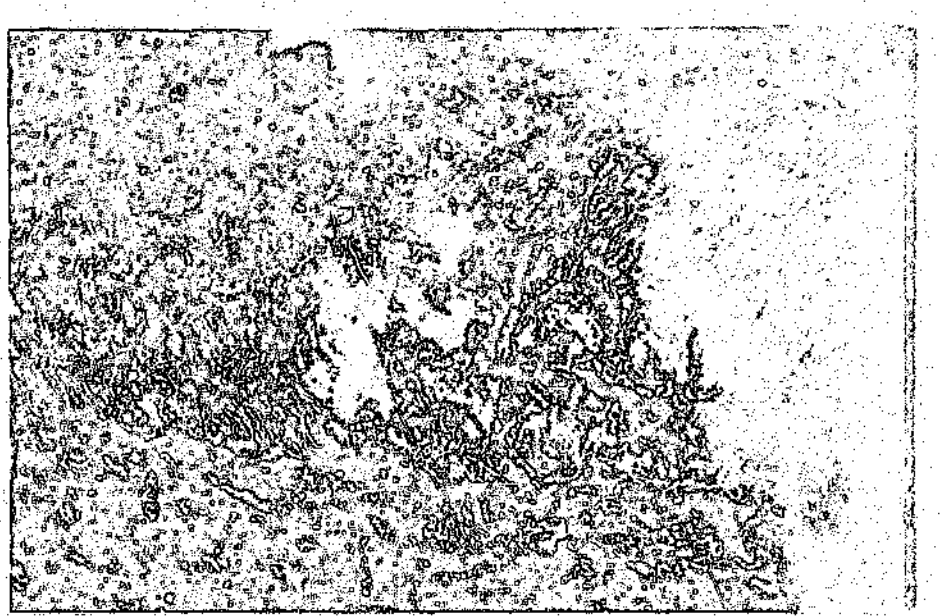


FIGURE 20.

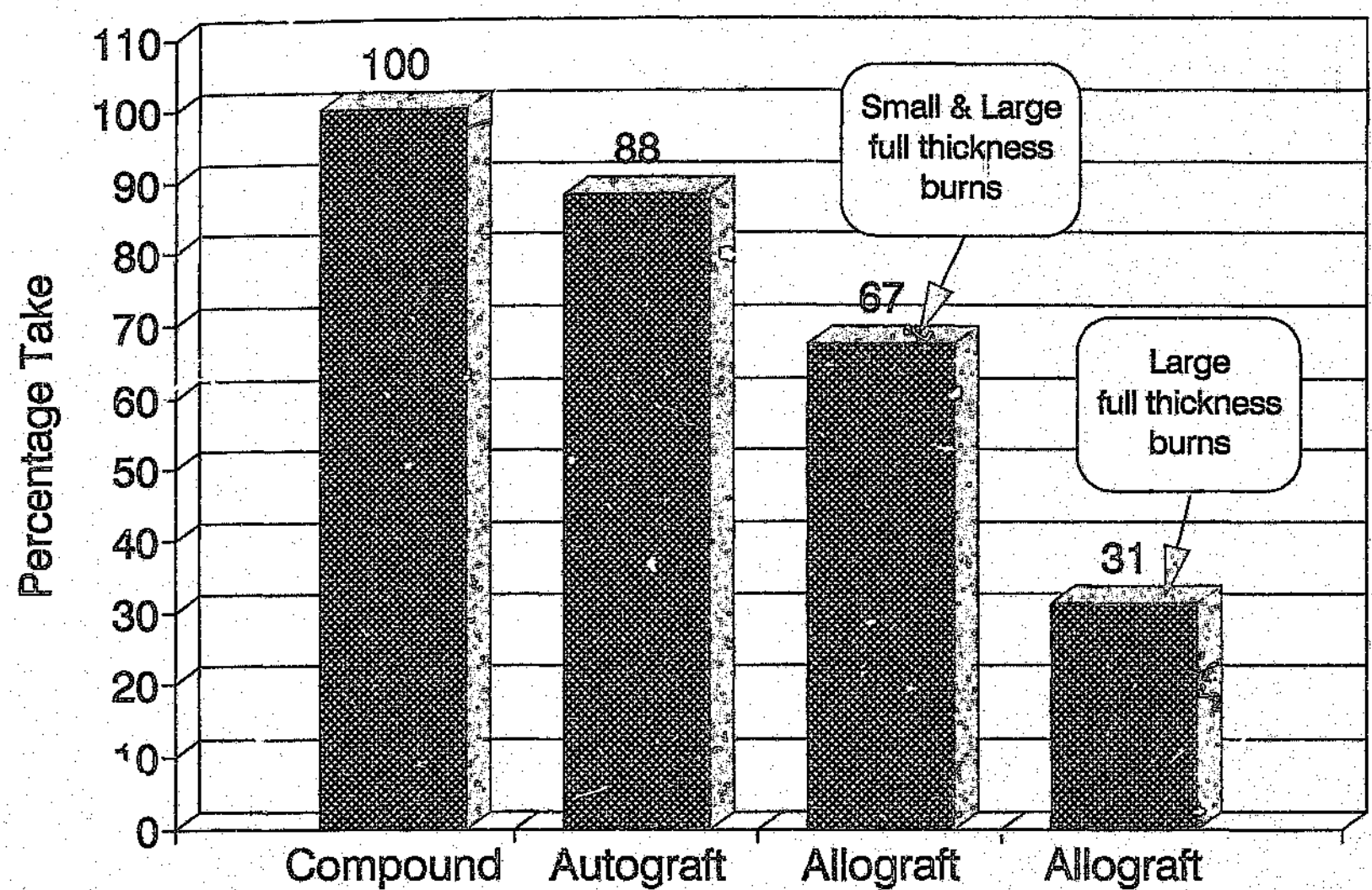
Patient Miss R M showing an area grafted with a highly meshed split thickness skin graft alone. The unacceptable cosmetic appearance is seen.

FIGURE 21.

PatientMiss R M, demonstrating the compound graft 23 days following application. This graft is relatively smooth. Compare with figure 20.

GRAPH 6 -

Comparison of Graft Types



Percentage Healing by Day 14

4.4 Discussion

This study is an initial comparison of three different types of cultured keratinocyte grafts and their application to the wound bed of burn injury patients.

This technique of keratinocyte grafting has the obvious advantage of the huge expansion of the initial skin biopsy. Green (82) has shown that starting from a small biopsy, 1cm² initially, a 100 fold amplification can be achieved in 2 weeks and up to a further 10 fold increase after sub-cultivation.

Cultured allografts have the advantage over autografts in that they are readily available for use on the newly burnt patient. Cultured autografts require approximately three weeks to form confluent stratified epidermal sheets before they are ready for use as grafts (82).

4.4.1 The fate of allograft keratinocytes

As seen in this study, the take of cultured allografts in extensive burns is significantly less compared to the take seen with cultured autografts (tables & graphs 3 & 4). Initial reports stated that cultured allografts appeared to take since Langerhans cells are lost after 7 - 10 days in culture (83, 84). Langerhans cells are antigen presenting cells in skin which are responsible for the rejection of allogeneic skin. But by the use of DNA hybridization techniques in sex mismatched donors and recipients, whereby the Y-chromosome of donor origin was labelled (figure 22) it was shown that donor keratinocytes could only be detected up to day 4 post-grafting on to full thickness burns (Burt, et al. - 85) and leg ulcers (Beder, et al. - 76). There appears to be an immunological rejection, postulated on the basis of MHC class I antigens present on the keratinocytes themselves (Gielen - 86).

In spite of low 'take' rates and eventual replacement by host keratinocytes (87, 88), one patient demonstrated that cultured allografts acted as good temporary biological dressings, possibly by preventing bacterial invasion, contamination of the underlying wound bed and protein and electrolyte losses (figure 15).

At the time when this study was carried out the use of cadaver skin as well as pig skin in burn patients was not permitted by law. Special government approval had to be obtained before these types of tempory biological cover could be used.

In addition, the cultured keratinocyte allografts and autografts appear to stimulate the patient's own non-transplanted keratinocytes, present at the wound edges and dermal appendages, to multiply faster and result in a more rapid skin coverage of the wound than is expected where no graft was applied. In this study, this was seen when cultured allografts were applied to small full thickness burns (table & graph 3). Cultured keratinocyte grafts were also found to provide pain relief within 24 hours after grafting. Even on relatively avascular subcutaneous fat the cultured keratinocyte grafts 'took' as seen in figure V.6.

In the absence of a dermal component wound contracture as part of the process of wound healing is inevitable. In humans wound contracture accounts for approximately 10-20 percent of wound closure of wounds healing by secondary intention. With specific reference to patient Mr E.N., (SEE FIG. 16), inspite of wound contracture accounting for some degree of wound closure, following wound healing he did not develope a claw-like hand posture . He had a good range of hand movements. The skin creases seen are due to the thin nature of the newly formed skin..

4.4.2 The advantages of compound grafts

For compound grafts, hypertrophic scarring is suppressed (figure 21). This finding is similar to that reported by De Luca (23). This study has gone one step further and compared 2 sites to demonstrate that the compound graft gives cosmetically superior results compared to split thickness skin grafts used alone (80, 81) (figures 20 and 21).

4.4.3 The topical use of piperacillin

The percentage take of cultured autografts was good in this study (table 4). The topical use of the antibiotic piperacillin appeared to improve percentage take. Other researchers tried to overcome bacterial contamination by grafting cultured autografts onto cleanly desloughed viable muscle fascia or 2 - 3 day old granulation tissue (20, 23). Piperacillin is toxic to Pseudomonas spp. and to a wide variety of gram negative and gram positive bacteria. No allergic phenomena were seen in the patients to which it was administered. When omitted, take was greatly reduced.

Before Piperacillin powder was applied Pseudomonas aeruginosa was cultured from burn

wound areas which were grafted with cultured keratinocyte grafts (refer to patients Mr M and Miss R in appendix V). No or little new skin formation occured (see fig. V.2). This was in spite of cleaning the wound with iodine solution and then washing this off with normal saline. Following the application of Piperacillin powder the rate of 'take' improved dramatically as seen by the results presented in the dissertation. Biosurface Technologies in Boston U.S.A, apply neomycin or a similar antibiotic to the burn wound before applying the cultured keratinocyte sheet (personal communications with Dr Kehinde). In a different area of burn in the case of Miss R, the burn wound was prepared with the application of Piperacillin powder and the take was 100 percent following the application of a compound graft (see fig. 19). The small amount of Piperacillin powder applied and the fact that new skin formation occured following the application of cultured keratinocyte sheets shows that this antibiotic was not toxic to the cultured keratinocytes.

4.4.4 Other factors determining `take'

In this study the percentage `take' was not related to the age of the patient. A similar finding was seen in over 370 patients treated by Biosurface Technology in Boston as reported by Odessey R. (79), but this is contrary to the findings obtained by De Luca et al. (23). Other factors determining `take' of cultured keratinocyte grafts include the following: the time from confluence to the time of grafting is crucial. If the keratinocyte graft is used too early, it has a tendency to disintegrate. Whereas, used too late, the viability of the basal keratinocytes are greatly reduced, resulting in a poorer `take'. The quality of the graft bed, with respect to the presence of bacterial contamination, pus, urine and blood will all hinder graft take. Hence, the use of piperacillin on the graft bed prior to grafting, in order to reduce the chances of bacterial contamination. But the application of any antiseptic such as silver sulfadiazine, Eusol or Betadine, kills the cultured graft. If blood tracts under the cultured epidermal sheet following grafting, the relatively firm application of the orthopaedic wool and crepe bandage will remove the blood from under this impermeable graft. Also, cuts (holes) are made into the graft at regular intervals allowing excess blood to escape from under the graft. The first layer of dressing applied over the cultured graft must be non-adherent thus reducing damage to the graft. For this purpose paraffin gauze was used. Also, the degree of pressure applied to the graft site must be of such a nature as not to damage the cultured graft. Finally the time interval after grafting when the paraffin gauze backing is first removed, is of importance, since if removed too soon, the cultured epidermal sheets are damaged.

The delicate nature of the cultured grafts was evidenced in one case where the newly formed skin from cultured autograft application partially stripped off (figure 18). At day 14, the newly formed skin is more stable, a finding previously reported (15). The instability of the cultured graft may be attributed to the reported absence of type IV basement membrane collagen and anchoring fibrils (10) although this is controversial (89).

Other disadvantages of cultured grafts is that the newly formed skin is tender to touch at one month post-grafting. However at 6 months, the keratinocyte grafts are similar to skin grafts in thickness, durability and texture, but have a smoother and shinier appearance (90, 15).

The results of the study demonstrates that the cultured epithelium can successfully re-epithelialize full thickness burn injury. But what of other skin conditions resulting in skin loss, such as chronic stasis ulcers, arterial ulcers, diabetic ulcers, trauma induced skin defects and so on? Would cultured keratinocyte allografts or autografts have a role to play in speeding up their rate of healing? Would these grafts be advantageous in other respects, such as pain relief, reduce hospital stay and improve quality of life?

CHAPTER FIVE

5.0 HEALING CHRONIC LEG ULCERS AND OTHER SKIN DEFECTS, USING CULTURED KERATINOCYTE ALLOGRAFTS - INCLUDING AN ASSESSMENT OF THEIR SURVIVAL

5.1 Introduction

An ulcer is a discontinuity in, or erosion of, the skin. Chronic ulceration of the leg affects approximately 1% of European populations (91). This percentage is probably similar for the South African Black population. An ulcer represents a failure of the normal processes of skin healing and is often the end result of a number of different factors or their interactions (91, 92).

When one manages ulcers it is essential to attempt to identify and treat the primary cause or causes of the failure of wound healing. The cause of leg ulcers are classified in table 1 below.

5.1.1 Pathophysiology of venous stasis ulcers

Burnand and Browse (93) found that induced venous hypertension enlarged the capillary bed, allowing the escape of fibrinogen, which deposed as a 'cuff' around the capillaries; the cuff blocked the diffusion of oxygen and caused anoxic tissue necrosis. Angel and Colleagues (94) have suggested that the final tissue destruction is caused by free radicals, such as superoxide $OH^{\cdot}$ or singlet oxygen OH, generated by abnormal enzyme reaction in ischaemic and infected tissue. Arterial ulcers on the other hand are due to occlusive vessel disease. Both these types of ulcers are characterised by their chronicity and recurrence (91).

5.1.2 Methods of treating chronic indolent leg ulcers

Ulcers can be treated conservatively, which is usually the case, or surgically. Conventional conservative management entails the use of a variety of antiseptic solutions (including Eusol, acetic acid and povidine - iodine), in combination with daily dressing, pressure stockings, bed - rest and leg elevation. The antiseptic solutions are toxic to healing skin, and they together with daily dressings are costly to the patient. One of the surgical methods involves

applying split-thickness skin autografts as the method of choice for leg ulcers, which however has met with varying degrees of success (92). The above surgical method involves subjecting the patient to general anaesthesia, which has its potential risks, especially in the age group in which these leg ulcers tend to occur. Also one requires healing of a painful donor site as well as of the leg ulcer itself. Healing will not occur until the concentration of bacteria is reduced to below 10^5 organisms / gram of tissue, since this concentration implies tissue colonization and invasion. It must also be borne in mind that many topical agents are toxic to granulation tissue. Therefore, although they counteract infection, they also retard wound healing (77, 78).

As an alternative to the above, the treatment of chronic leg ulcers with cultured keratinocyte allograft sheets is proposed after the initial infection has been treated successfully. With chronicity, the margins and base of the ulcer develope fibroblastic proliferation, scarring and the accumulation of lymphocytes, macrophages and plasma cells (120). Following the removal of this inflammatory necrotic tissue by chemical debridement the underlying tissue is vascular. Onto this bed the cultured keratinocyte grafts were applied. In the text, an area of lower leg skin loss is used as a synonomous term for chronic leg ulcer. Since all the lower leg skin defects presented in this dissertation fulfilled the above criteria and are thus representative for this study.

The most common type of venous abnormality affecting the venous drainage of the lower limb is simple saphenofermoral incompetence, which leads to varices (91). Ulceration only occurs if the perforating veins also become incompetent, allowing transmission of pressure from the deep to the superficial system, so that sustained venous hypertension develops. Specifically in this case of venous hypertensive ulcers, healing of skin is required before definitive surgery can be carried out. Definitive surgery entails locating and tying off the venous perforators. Healthy healed skin is required in this case so that the surgical incision is not in an ulcerating contaminated area. This allows for favourable healing of the surgical wound.

5.1.3 Risk factors affecting ulcer healing

Harrison has shown that various risk factors (92) act as good prediction indicators with respect to outcome following split-skin grafting of varicose leg ulcers. The risk factors include: the presence of major medical problems; especially ischaemic heart disease or cerebro-vascular disease; multiple hospital admissions; multiple medications; problems

with immobility; arthritis or multiple falls; local leg problems; either ischaemic previous deep vein thrombosis or a long (greater than 5 years) history of leg ulceration; and whether or not the patient lived alone.

The presence of 3 or less risk factors resulted in 75% of the ulcers healing. Four or more risk factors resulted in only 12% healing. These risk factors would most certainly also apply in the case of cultured epidermal allografts applied to varicose and other types of leg ulcers.

The time which it takes for a healthy skin wound to heal is proportional to the size of the wound. If adverse factors are present then this time is increased proportionally. Twelve of the 14 patients presenting with lower leg skin loss had 4 or more risk factors that predispose to poor wound healing as assessed by Harrison (92).

5.1.4 Treating leg ulcers with cultured keratinocyte allografts

The first documented use of cultured keratinocyte autografts in the treatment of leg ulceration was reported in 1983 by Hefton (24). The three ulcers treated healed within 3 - 5 weeks post-grafting and remained healed for up to 2 years. Leigh (25) had similar results after applying cultured autografts to 12 chronic venous stasis leg ulcers. But the cultured keratinocyte sheets derived from the elderly patients were found to be extremely fragile as the cells are less proliferative compared to younger cells in culture. In all cases there was noticeable pain relief within 24 hours after application of the cultured keratinocyte sheets (26, 95).

Due to the low colony forming ability of these autologous keratinocytes of the elderly patients presenting with leg ulcers, cultured keratinocyte allografts were tested in the treatment of chronic lower leg ulcers. 59 chronic venous ulcers and 11 rheumatoid ulcers were treated with keratinocyte allografts (26). After removal of the dressings at 5 days, 29% showed islands of epithelium growing within the ulcerated area, whilst in 44% - there was a visible migration inwards of the epithelial edge and a reduction in ulcer size. In 27% no effect was seen. Phillips (27) found that 73% of the 36 skin ulcers of various aetiologies healed completely in an average of 3.3 weeks when treated with keratinocyte allografts. No significant difference in the mean-healing time of venous ulcers treated with fresh allografts (23 days) or cryopreserved allografts (26 days) was noted in one comparative study (95).

None of the above researchers have correlated the size of ulcer with respect to the rate of

healing. An equation to this effect is proposed below. The age of the patient with respect to the rate of healing has also not been investigated previously. Also in this study the healing rate of leg ulcers of various aetiologies are compared, as well as the viability and efficacy of cultured keratinocytes allograft sheets against conservative management of chronic indolent lower leg ulcers.

5.1.5 Keratinocyte allograft survival

In skin grafts the main antigen presenting cells are Langerhans cells, which form 5% of the total mixed epidermal cell population. These cells present antigen to helper-T cells and may initiate rejection of a transplant by expressing class II histocompatibility molecules and secreting cytokines. Langerhans cells are lost 7 - 10 days after a single cell suspension of mixed epidermal cells is cultured in vitro, as determined by the expression of HLA-DR antigens (83). The mixed epidermal cell lymphocyte reaction is lost in parallel, which appears to confirm the loss of the Langerhans cells. Endothelial cells, which also have the ability to express class II antigens and thus play a role in rejection reactions, are also lost during culture of keratinocytes from mixed suspensions. Clinically and histologically no overt signs of cultured allograft rejection has been seen (Beder et al - in press, 75).

If chronic leg ulcers heal faster with the use of cultured epidermal allograft application, compared to conservative management, what is inducing this increased healing rate? Is it a growth factor(s) in the medium in which the cultured grafts were bathed during cellular multiplication, for example an unknown factor in the bovine serum or in bovine pituitary extract? Is it a factor(s) produced by the multiplying keratinocytes themselves in concentrations not normally present within normal skin? To determine the latter indirectly, one proceeded to determine if the actual transplanted allogeneic keratinocytes survived following transplantation and for how long.

Auböck implied that keratinocyte allografts survived indefinitely (11) but Burt (85) and Brain (87) have shown that this is not the case in their studies of burns and shavings, respectively. To see if the findings obtained by the above researchers were comparable to what occurred in the case of keratinocyte allografts applied to leg ulcers, in situ DNA hybridization was used.

In 1982 Cooke et al characterised a sequence that was repeated in tandem with 2000 copies of the Y chromosome (96). This constitutes a fifth of the DNA and is located on the tip of the long arm of the Y chromosome. Page of Boston has characterised a similar sequence (personal communications). A simple reproducible method to detect this multiple copy human gene has been established (97). Therefore the sex of cells present in skin biopsy specimens taken from healing leg ulcers of female patients were determined. The ulcers had been grafted with cultured allogeneic keratinocyte sheets derived from unrelated male donors.

TABLE 6
CAUSES OF LEG ULCERS

A) VASCULAR
- i) Venous (due to venous hypertension)
- ii) Arterial (ischaemic)
- iii) Lymphatic

B) VASCULITIS
- i) Collagen diseases

C) HAEMATOLOGICAL
- i) Anaemias

D) INFECTIVE
- i) Primary - due to a specific agent
- ii) Secondary - complication of other conditions

E) METABOLIC
- i) Diabetic
- ii) Nutritional deficiency
- iii) Uraemia

F) SKIN MALIGNANCIES
- i) Primary
- ii) Secondary

G) PHYSICAL AGENTS
- i) Trauma - including burns
- ii) Drugs

The above table has been modified from C. Khoo (91).

5.2 Patients and Methods

Fourteen patients, all with lower leg skin loss, are presented (please refer to appendix VI for patient details). All were grafted with cultured keratinocyte allograft sheets. The patients grafted presented with one of the following:

a) Venous stasis ulceration.
b) Arterial ulceration.
c) Fasciotomy induced skin defects.
d) Trauma induced skin loss accompanied by cellulitis and abscess formation.

The keratinocytes were obtained from neonatal foreskin or from young adults who had undergone various plastic surgical procedures (appendix II). These keratinocytes were grown into confluent stratified epidermal sheets using the method described by Green et al. (82). The donors had tested seronegative for the Hepatitis B virus as well as for the HIV (appendix VIII), (98).

I recognise that the patient numbers are low, but there was a problem with respect to patient availability and follow up. Also the numbers correlate quiet well with the numbers presented in previous studies (24-27).

Thorough conservative management involved applying betadine (medicated iodine) dressings three times a day, (layers consisting of betadine, cotton gause and crepe bandage), until the infected slough had been removed. This was followed by the application of saline, paraffin gause, cotton gause and crepe bandage, in that order, three times a day.

Patients Mrs M and Mr E (see figures 23 and 24 respectively), had numerous attempts at skin grafting with split thickness skin but the grafts did not take.Although arterial ulcers are usually found in a more distal position, patient Mrs M (see fig. 23), was considered to have a recurrent arterial ulcer.

5.2.1 Grafting methodology and follow-up

For the grafting methodology of leg ulcers and other skin defects and their follow-up, please refer to chapter two on page 36.

5.2.2 Biopsies used for in situ DNA hybridization

These biopsies were taken from 6 female patients. Two patients had biopsies taken on day 4 post grafting and one each on days, 5, 8 and 16 post-grafting. The biopsies, taken under local anaesthesia, were taken from areas of the ulcers that were clinically healing. Half of each biopsy specimen was mounted in paraffin and stained with the routine haematoxylin and eosin stain. The other half of each biopsy specimen was analysed for the presence of cells containing the Y chromosome, using in situ DNA hybridization with a biotinylated Y-probe (pDP 105), (appendix IV). Normal male skin and prostatic tissue, which had undergone benign hypertrophy, were used as positive controls. Normal female skin was used as a negative control. The method is based on that described by Burns J. et al. (97) (See figure 22).

Each section was scanned for the presence of male cells, which were detected by the presence of a black dot in the nucleus. The black dot representing the Y-probe.

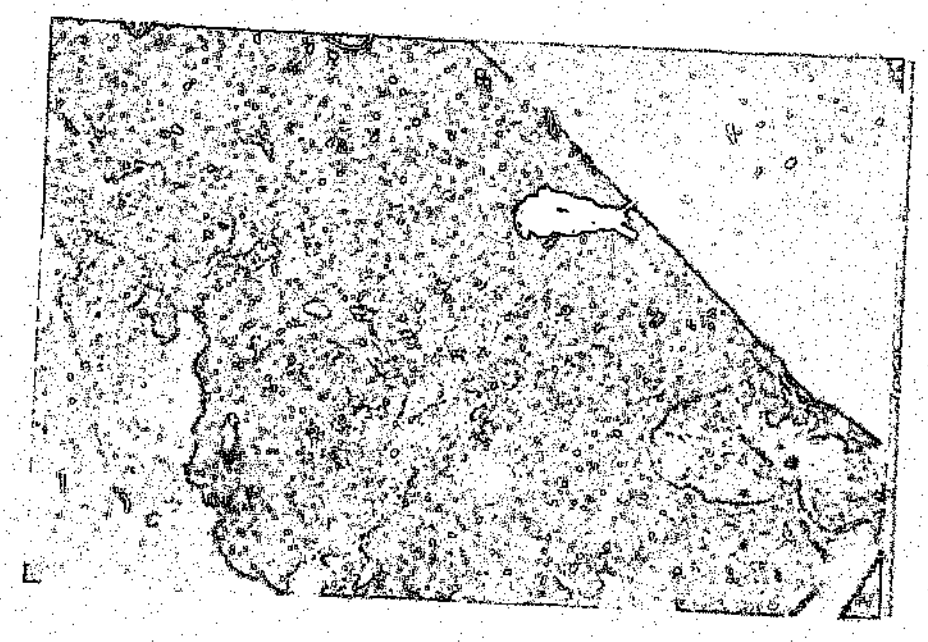


FIGURE 22 (a)

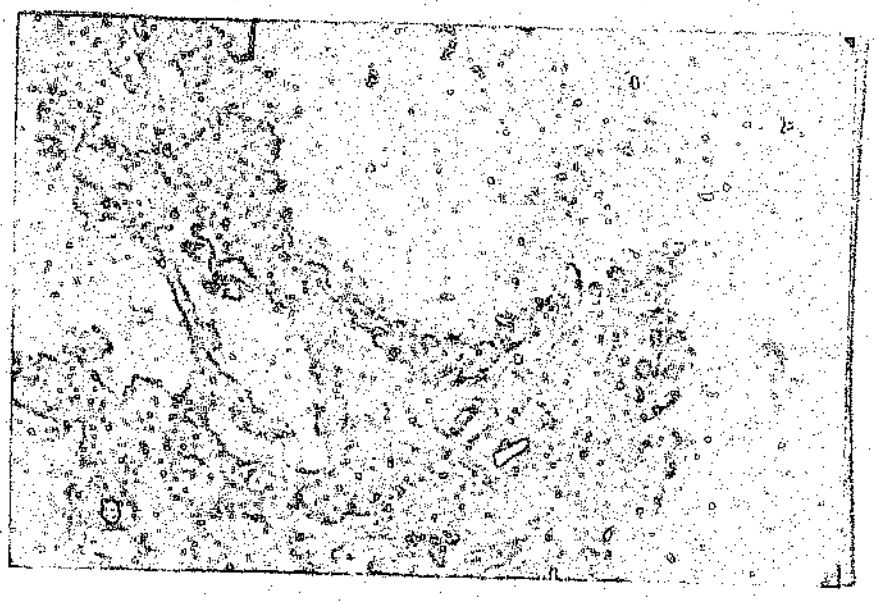


FIGURE 22 (b)

FIGURE 22.

Examples of controls used to demonstrate the presence of the male Y - chromosome.

a) A section through normal prostatic tissue. The dark dots indicate cells containing the the Y - chromosome. This was used as a control. (mag. 10x).

b) A L.M. view through normal male skin showing cells staining purple brown. This was used as a control (mag. 10x).

5.3 Results

5.3.1 Average healing time

Cultured keratinocyte allograft sheets were applied to 17 lower leg skin defects. 14 skin defects present for 4 weeks to 25 years, measuring from 3,5cm² to 64cm², healed completely within an average period of 18.1 days. The smallest ulcer took 3 days to heal and the largest took 27 days.

One patient, Mrs E.M. was lost to follow-up when her 2 ulcers, measuring 40cm² and 16cm² had healed by 90% and 87.5% respectively, by 16 days. No healing occurred in the case of Mr S., who presented with a syphilitic ulcer, from which Methicillin resistant Staphylococcus aureus was cultured.

Follow-up has ranged from 12 to 24 months. To date, all of the above 14 ulcers have remained healed except for 2, one being a venous stasis ulcer and the other being a diabetic ulcer.

5.3.2 An equation relating time to heal and ulcer size

Combining all the different types of ulcers a histogram comparing days to healing versus size of the lower leg skin defect after application of the first cultured epidermal allograft (CEA) was drawn (graph 7). The numbering of each bar is from the youngest to the oldest patient (1 through to 13). From this graph one can see that there appears to be no significant difference with respect to the patient's age and the rate of healing. Overall, graph 7b shows a linear relationship between the above 2 parameters.
From graph 7b, the following equation to predict the time it will take for an ulcer of a specific size to heal is given below:

$$Y = 2.2186 + 0.4387X$$

Where Y = days taken for complete healing to occur after application of a single cultured epidermal allograft.

X = size of skin defect (cm^2)

The above equation is valid for leg skin defects up to a size of $63cm^2$. Above this size its validity cannot be guaranteed.

5.3.3 Findings following the application of multiple CEA

The application of a second cultured epidermal allograft to the leg ulcer speeded up healing by an average of 10.2 days. This is seen in graph 8 of days to healing versus size of the lower leg skin defect after the application of a first, second and in one case, a third cultured epidermal allograft (see figures 23 and 24). Within 12 subjects grafted, the application of CEA resulted in pain relief within 24 - 48 hours after grafting.

When applied as the first or second CEA, the quality of the CEA did not appear to significantly alter the rate of healing. However, when a poor quality graft was applied to one area of a lesion and a good quality graft to another area, a difference with respect to the rate of healing was noted. In a single case studied it took 11 days for the former area to heal and 26 days for the latter area to heal (see figure 25).

From table 14, one sees that only up to day 4 post-grafting, were donor keratinocytes identified in biopsy specimens. Thereafter the donor keratinocytes were not identified and appeared to have been replaced by the patient's own keratinocytes.

Table 7. LEG ULCERS DUE TO A FASCIOTOMY INDUCED SKIN DEFECT

Patient	Duration of Conservative Rx	Time to healing after CEA applied
Mrs J	56 days	3 days - left thigh (3.5 cm^2) 90% healing - 11 days - left calf (27 cm^2)
Mrs H	4 weeks	15 days (16 cm^2)

Table 8. LEG ULCERS DUE TO ARTERIAL INSUFFICIENCY

Patient	Duration of Conservative Rx	Time to healing after CEA applied
Mrs M	2 months	27 days (63 cm^2)
Mrs LB	4 years	16 days (7.5 cm^2)

Table 9. LEG ULCERS DUE TO TRAUMA FOLLOWED BY CELLULITIS AND SKIN LOSS

Patient	Duration of Conservative Rx	Time to healing after CEA applied
Mrs GM	4 weeks	41 days and 23 days after CEA No. 2 (28 cm^2)
Mrs DM	39 days	31 days (60 cm^2)

Table 10. LEG ULCERS DUE TO DIABETES

Patient	Duration of Conservative Rx	Time to healing after CEA applied
Mrs R	2.5 months	19 days (12 cm^2)

Table 11. LEG ULCERS DUE TO SYPHILIS

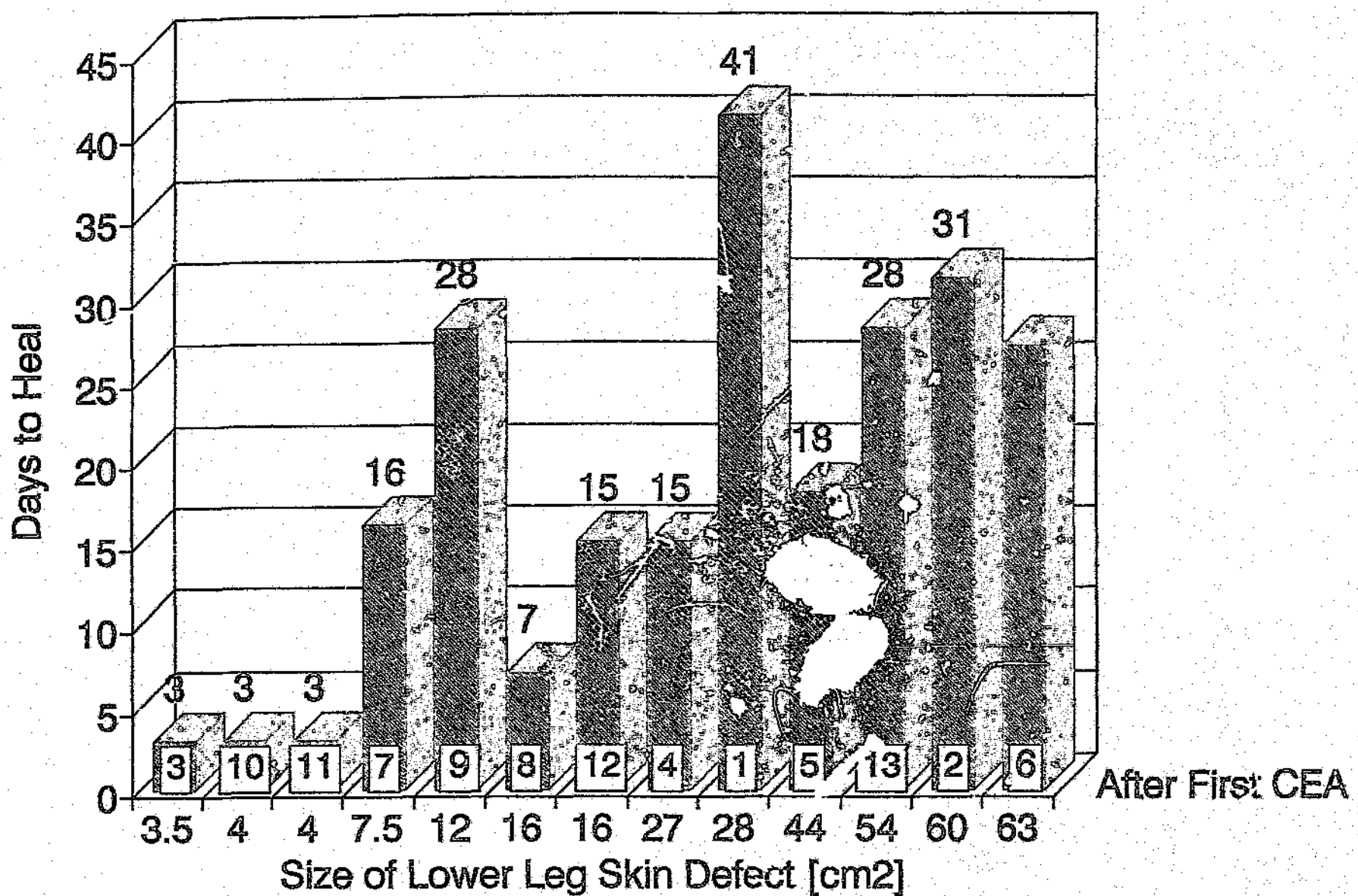
Patient	Duration of Conservative Rx	Time to healing after CEA applied
Mr S	5 years	no healing occurred (8 cm^2)

Table 12. LEG ULCERS DUE TO VENOUS HYPERTENSION

Patient	Duration of Conservative Rx	Time to healing after CEA applied
Mrs MM	3 months	28 days (12 cm^2)
Mrs S	6 months	30 days (54 cm^2)
Mr E	15 months	18 days (44 cm^2)
Mr WM	9 months	7 days (16 cm^2)
Mrs EM	25 years	16 days*
Mrs N	6 months	3 days (4 cm^2)
* = It took 16 days for the two ulcers, measuring in size 40 cm^2 and 16 cm^2, to heal by 90% and 87.5%, respectively, before the patient was lost to follow-up.		

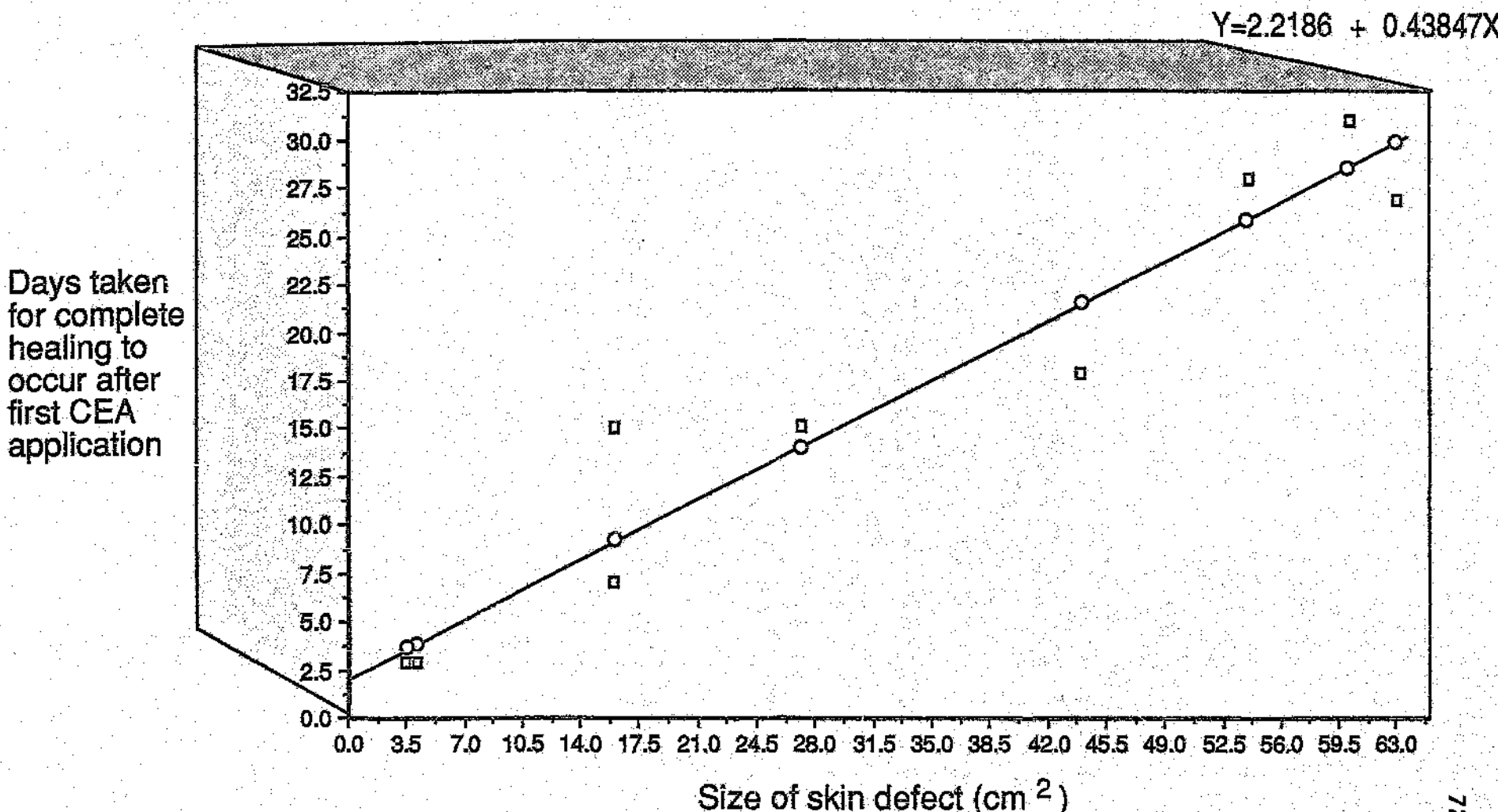
GRAPH 7a.

Days to Heal vs Size



GRAPH 7b

Days to heal vs size of leg skin defect



GRAPH 8.

Days to Heal vs Size

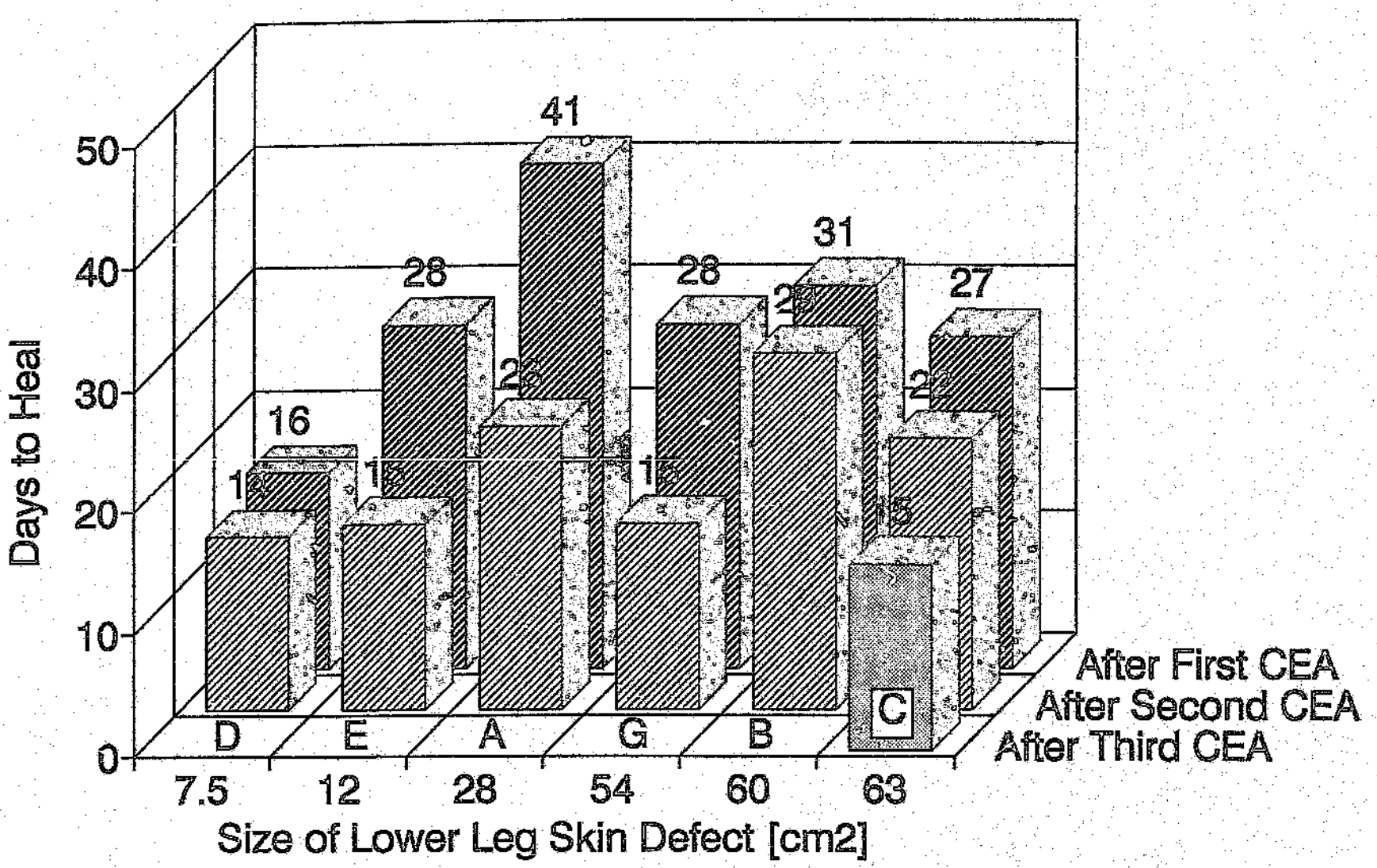


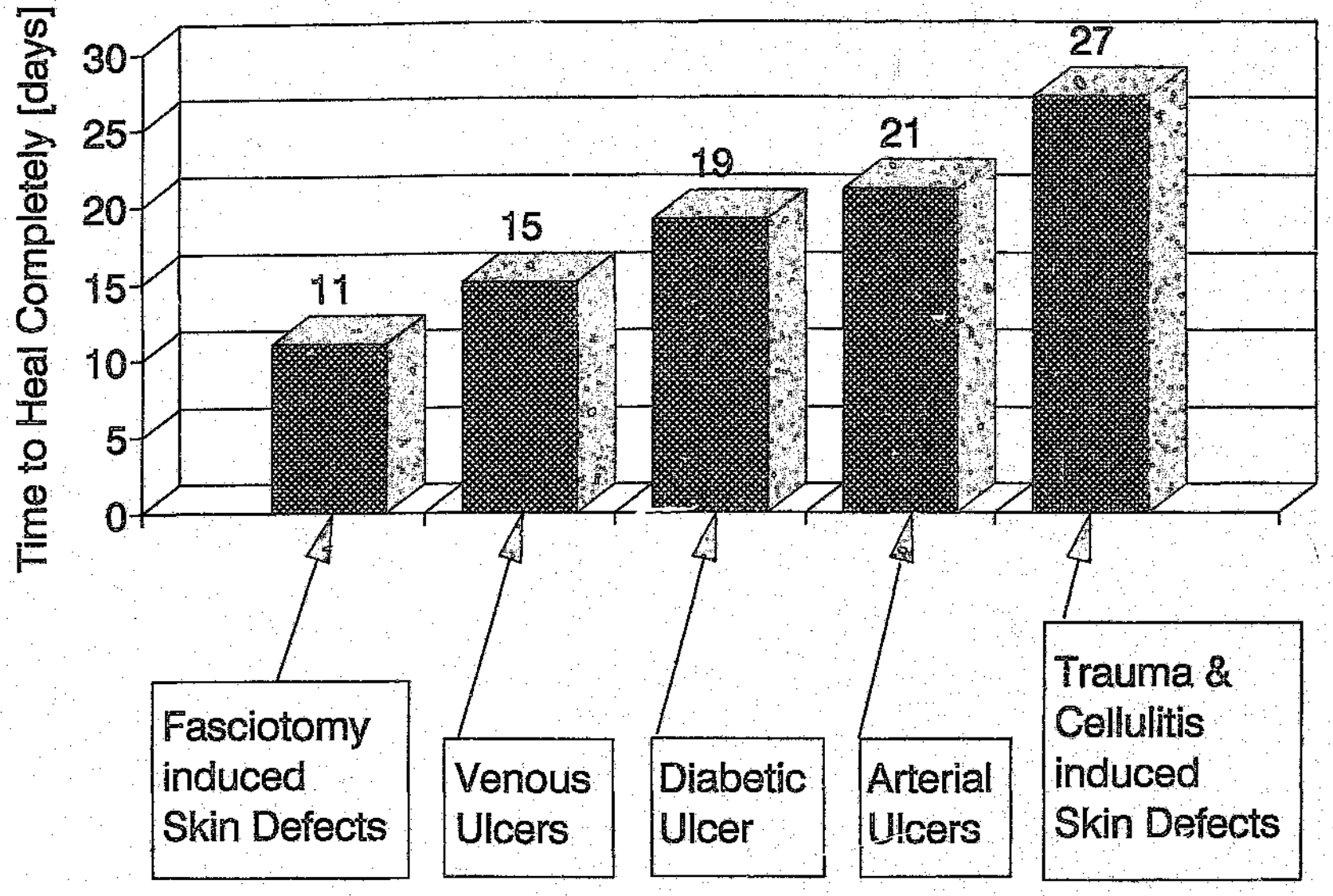
Table 13.

MEAN TIME TAKEN FOR VARIOUS TYPES OF LOWER LEG SKIN DEFECTS TO HEAL

VENOUS ULCERS	:	14.8 days
ARTERIAL ULCERS	:	21.5 days
FASCIOTOMY INDUCED SKIN DEFECTS	:	11 days
TRAUMA & CELLULITIS INDUCED SKIN DEFECTS	:	27 days
DIABETIC ULCERS	:	19 days

GRAPH 7.

Comparing Lower Leg Skin Defect Types



Patient	Day post grafting, on which biopsy taken			
	Day 4	Day 5	Day 8	Day 16
Miss DM	1 (+ ve)			
Mrs LB	1 (+ ve)			1
Miss GM		1		
Mrs M			1	
Mrs S			1	
Mrs EM			1	
Unless indicated, the biopsy samples did not contain male cells and thus were negative.				

TABLE 14.

Table 14 tabulates biopsy specimens taken at various days post CEA application. To each specimen a biotinylated DNA probe, specific for the Y chromosome, was added. If detected, then a black dot was seen in the nucleus of a male cell, being of donor origin.

FIGURE 23 (a)

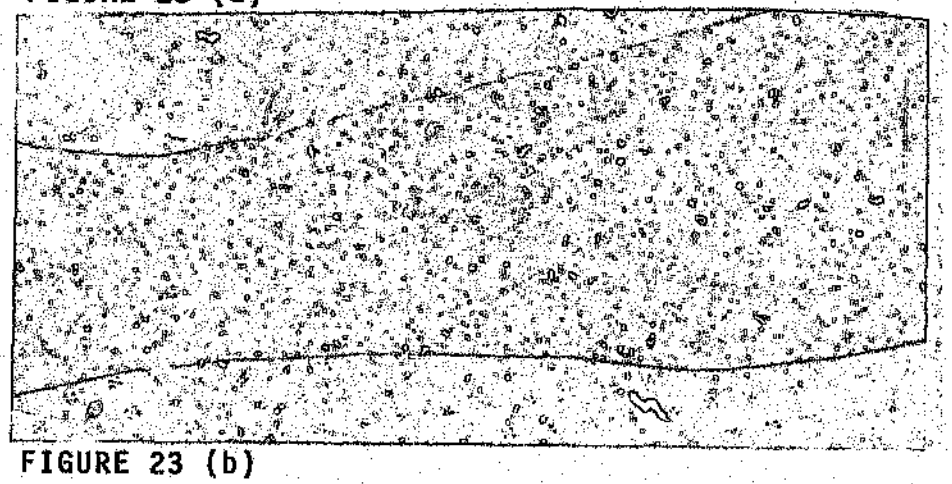


FIGURE 23 (b)

FIGURE 23.

Patient Mrs M used as an example to show the stages of healing following the application of a cultured keratinocyte allograft to a chronic arterial leg ulcer.

a) The leg ulcer before the first cultured epidermal allograft was applied.
b) 3 days after the first cultured epidermal sheet was applied. Two areas of new skin formation is seen within the ulcer bed itself. New skin is also seen along the margin of the ulcer.
c) 27 days after the first cultured epidermal allograft application. The leg ulcer is covered by new skin. In total 3 cultured keratinocyte sheets were applied.

(See appendix VI page 171 for the data sheet pertaining to this patient).

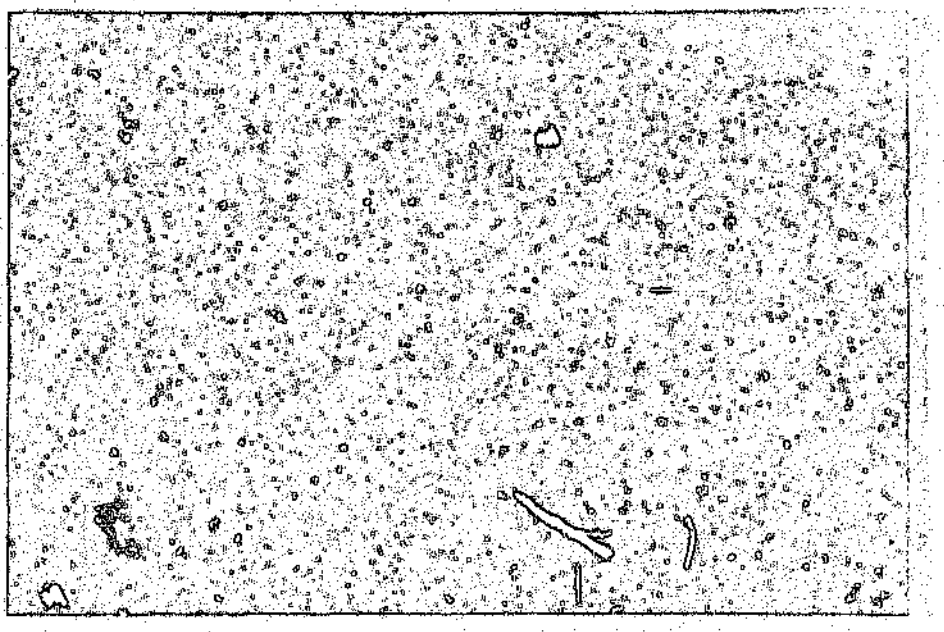


FIGURE 23 (c)

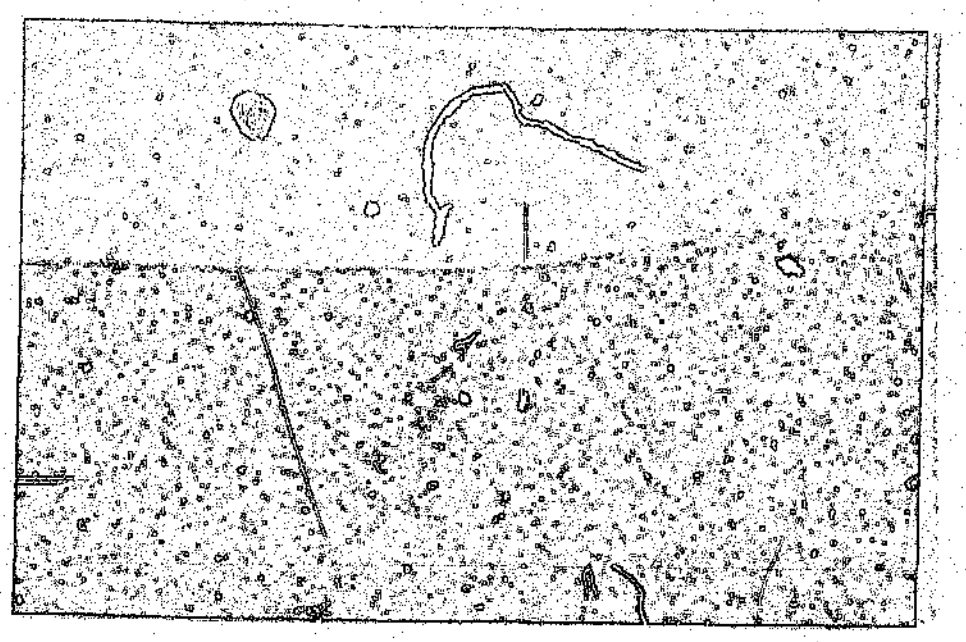


FIGURE 24 (a)

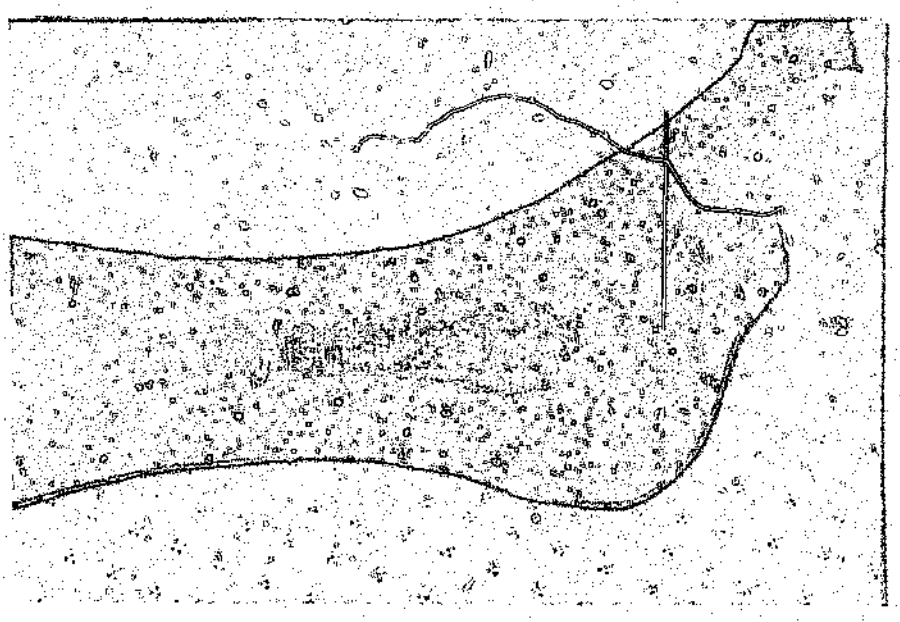


FIGURE 24 (b)

FIGURE 24.

Patient Mr E used as an example to show the stages of healing following the application of a cultured keratinocyte allograft to a chronic leg ulcer.

a) The leg ulcer showing islands of newly formed skin 8 days after grafting with an epidermal allograft culture.
b) 18 days after CEA application, a well healed ulcer is seen.
c) A close-up view of the healing ulcer (day 16)

(See appendix VI page 150 for the data sheet pertaining to the above patient).

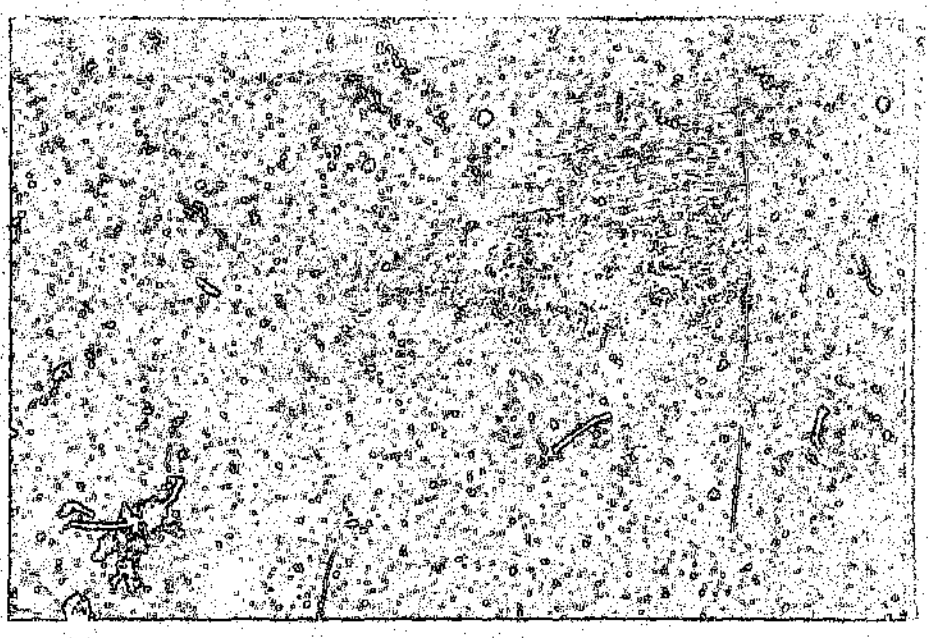


FIGURE 24 (c)

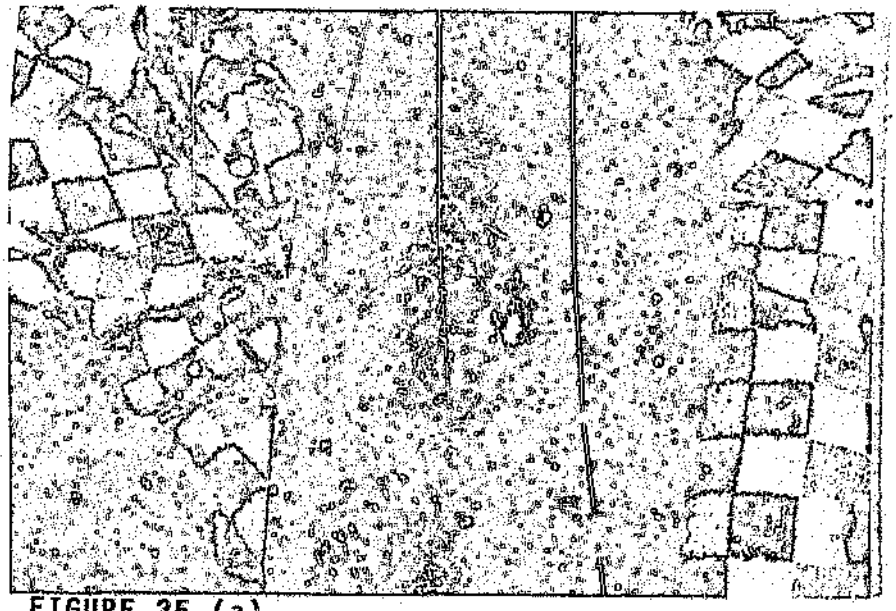
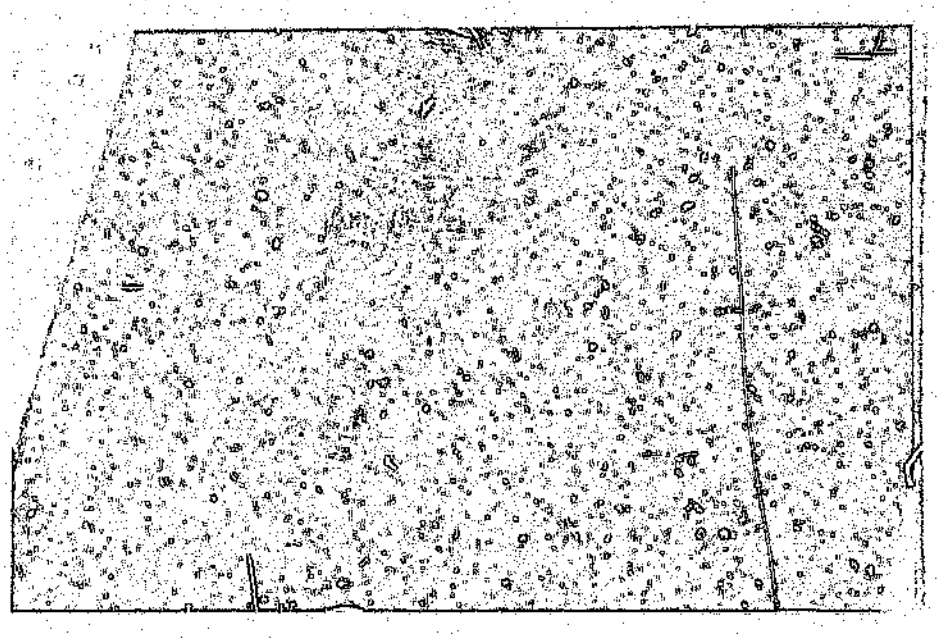


FIGURE 25 (a)

FIGURE 25.
Patient Miss D M used to compare the stages of healing following the application of 2 types of keratinocyte allografts to a trauma and cellulitis induced leg ulcer.
a) The leg ulcer seen prior to grafting.
b) The leg ulcer after the good quality graft was applied to the lateral side (day 25) and a poor quality graft was applied to the medial side (day 27). The area to which the good quality CEA was applied reveals more newly formed skin compared to the part to which a poor quality CEA was applied.
(See appendix VI page 155 for the data sheet pertaining to the above patient).



MEDIAL

FIGURE 25 (b)

5.4 Discussion

Multiple CEA applications appear to speed up ulcer healing. This is not surprising in light of the identified growth promoting factors found in the supernatant of cultured keratinocytes (31, 51 - 59). These factors probably accelerate multiplication of the patient's own marginal keratinocytes as well as of those keratinocytes found in the dermal appendage remnants.

5.4.1 Growth promoting substances secreted by keratinocytes

The cultured keratinocytes secrete basement membrane proteins, (collagen types IV - VII, laminin, fibronectin and basic protein antigen), and extracelluar matrix, which all aid cell migration, as well as growth factors, which stimulates multiplication of the hosts keratinocytes (20, 51 - 59). These growth factors include: Interleukins 1, 3, 6 and 8, transforming growth factors alpha and beta (TGFα, TGFß), granulocyte macrophage colony stimulating factor (GM-CSF), basic fibroblast growth factor (b-FGF), platelet derived growth factor (PDGF), and tumour necrosis factor (TNF-α). These factors regulate cell division, cell migration, cell differentiation and function of epidermal, dermal and even immunocompetent cells. There is growing evidence that some keratinocyte derived growth factors are involved in wound healing (99, 100), b-FGF and TGF-α are angiogenic factors stimulating endothelial cell migration and proliferation (101). TGF-ß and PDGF are both chemoattractants to fibroblasts and stimulate their division and collagen production, furthermore, they augment dermal wound healing in rats (102, 103). A recently characterised growth factor, derived from dermal fibroblasts, called keratinocyte growth factor (KGF), appears to exert a paracrine effect on adjacent epithelial cells, resulting in their proliferation (37). KGF may act by preventing the degradation of DNA in the keratinocyte stem cells, as seen in the case of erythropoietin on erythroid progenitor cells (104), thus resulting in more stem cells multiplying and not differentiating into transit amplifying cells.

5.4.2 Fate of cultured allogeneic keratinocytes

This study which showed that only up to day 4 post-grafting (table 14), could donor keratinocytes be found in biopsy specimens for healing ulcers grafted with CEA sheets, confirms the finding obtained by Burt et al. (85) and Van der Merwe et al (105) - in the case of burns, and Brain et al (87) - in the case of deep dermal wounds. Since no evidence of rejection was noted either macroscopically, in the form of erythema or blistering, or microscopically, which normally presents with leucocyte invasion and epithelial vacuolation,

a physical replacement of the allograft, rather than a rejection, appears to occur.

5.4.3 The advantages of keratinocyte allograft application

In spite of the above findings, one sees in figures 23 b) and 24 a), that following CEA grafting, islands of newly formed skin is present. Healing of the ulcer in this way was completely different in patient Mr E., where previously it had healed only from the margins, following conservative management. Many of the patients treated were of negroid origin. Healing rates following CEA application were comparable to their caucasian counterparts.

In this study, following CEA application, no hypertrophic scar formation occurred with ulcer healing. This has also been seen when these grafts were applied to split thickness skin graft donor sites (23). Also pain relief was seen to occur in patients within 24 hours after CEA application. This finding has also been noted by 2 other researchers (24, 25).

Teepe (95) found that venous ulcers healed twice as fast as rheumatoid ulcers (57 days). In this present study, 7 venous ulcers healed within an average time of 15 days, compared to the healing of, 1 diabetic ulcer (19 days), 2 arterial ulcers (21.5 days) and 2 trauma and cellulitis induced skin defects (27 days). Only the 2 fasciotomy skin defects healed faster on average (11 days) compared to the venous ulcers. Since the numbers of the various types of lower leg skin defects are small and one must also take into account the relative sizes of these defects, these results will only be verified when a larger number of patients are grafted (See table 13 and graph 9).

Two venous ulcers re-occurred within one month and the one diabetic ulcer re-occurred within 1 year after grafting. All the other 12 lower leg skin defects of various aetiologies remained healed after a follow-up period lasting from 12 - 24 months.

5.4.4 Potential risks of keratinocyte allograft application

Potentially there are risks attached to the clinical use of keratinocytes that have been cultured in the presence of mitogens, with the possibility of spontaneous keratinocyte transformation in vitro and subsequent malignancy after grafting. A spontaneously transformed keratinocyte cell line has been reported (106) but such transformation appears to be rare. Langerhans cells are a target for the human immunodeficiency virus (98) but keratinocytes do not appear to be. Also, antibodies to foetal bovine serum have been detected in patients on which

cultured keratinocyte grafts have been applied, but to date they have not produced any problems of graft rejection, anaphylaxis, or serum sickness (107).

Thus before cultured keratinocyte sheets are applied to the patient, the actual keratinocytes of donor origin must be tested for the presence of the hepatitis B surface antigen and an antigen of the HIV. The donor's blood on the other hand must be tested for the presence of antibodies to the human immunodeficiency virus. This was carried out for all cell lines used in this study.

5.4.5 Interpretation of results

A clinical trial is difficult to interpret because of multifactorial influences upon the events of graft take and wound healing. Here one has tried to eliminate some of these influences, firstly by the individual who cultures the keratinocytes, developing a grafting methodology which he carried out himself in applying the grafts to the wound bed. This gives one the insight into the fragility of the grafts and hopefully giving these grafts the best opportunity to take. The elimination of infection by the use of the antibiotic piperacillin, in a topical form, has gone a long way in eliminating wound infection, and thus producing better results with respect to take. Protection of the graft site, using the open or closed methods as described in this paper, and meticulous follow-up should enable one to achieve the best results possible.

Whether better results will be achieved using a composite graft, where one has a dermal equivalent, is still under investigation. In burns, all the dermal components were present within 6 months following cultured autograft application to viable fascia (15). Thus the requirement of a dermal equivalent, which first requires vascularization before a cultured epidermis will survive above it, is still debatable.

The major advantages of using a cultured allograft for the healing of lower leg skin defects are:

a) Grafting can be carried out on an out-patient basis, resulting in no or reduced hospital stay,
b) No general anaesthesia is required, with its potential risks, especially in the elderly patient,
c) No painful donor site, as is required in the case of application of a split thickness skin graft,

d) One does not require healing of both the donor site as well as of the leg ulcer. Good healing rates occurred especially in those cases where thorough conservative management resulted in no healing.

This method of treating skin loss of various aetiologies appears to be not only cost effective for the hospital and the patient, but also patient friendly. These features are felt to out-weigh the few short comings. Cultured keratinocyte allograft application to all relatively small skin defects must now be considered as a viable alternative to split thickness skin grafting.

Would a composite graft, composed of an artificial dermis covered by a cultured epidermal sheet, improve the results obtained with cultured keratinocyte sheets alone? How would one go about constructing such a graft? What components would be required? Would the composite graft strengthen the durability of the healed skin and also prevent contractures? These possibilities have to be investigated further.

CHAPTER SIX

6.0 THE CONSTRUCTION OF A COMPOSITE SKIN GRAFT

6.1 Introduction

The problem with cultured confluent stratified keratinocyte sheets is attachment to the wound bed. If not firmly attached, they tend to form blisters especially if blood tracts between the graft and the wound bed. Also it takes time for vascularization of the graft to occur. Since no dermal component is present contractures tend to occur.

6.1.1 The advantages of the presence of a dermis

Keratinocyte grafts take better on dermis than on fascia, muscle or fat possibly due to the dermis being more vascularized (108). Dermal allografts of low immunoreactivity have been successfully used to support the growth of keratinocyte sheets (109 - 112). Based on the success of the above, dermal equivalents are now being developed to provide the keratinocyte sheet with a stable base prior to grafting. The dermal equivalent should prevent contractures occurring following grafting. The artificial dermis provides a lattice for the dermal fibroblasts to become embedded and to lay down recipient collagen in a similar manner to that dictated by the foreign collagen already present.

6.1.2 The use of collagenous substrates

Unfortunately collagenous substrates, i.e. the dermal equivalents, which are stable in vitro cultures, are unstable and degrade rapidly on the wound bed. It is thought that the overlying keratinocyte sheets have a poor survival rate due to not getting adequate nutrients from the dermal equivalent. It takes some time before the dermal equivalent becomes vascularized. Therefore take may be improved by first placing the stable dermal equivalent on the wound bed and after it is vascularized, placing the cultured epidermal sheet above it.

In spite of the above findings, composite grafts appeared to take well when transplanted on to full thickness burns in rats (113), and athymic nude mice (29). The dermal equivalent varied in composition from simple type 1 collagen (113), human types 1 and 3 collagen (29), bovine collagen and chondroitin - 6 - sulphate all being impregnated with fibroblasts (28).

An epidermis was regenerated without adnexal structures on connective tissue that resembled undamaged dermis. However when transplanted onto full thickness defects on pigs, this substrate provoked an intense foreign body reaction (114).

Yannas (115) used a highly porous cross linked collagen - glycosaminoglycan substrate seeded with autologous dermal and epidermal cells derived from a pig. A confluent epidermis was seen in vivo at 14 days and a new basement membrane, containing the collagen IV 7-S domain, thought to be important in the development of its lattice-like structure, was not detected until day 23. At 4 months fewer anchoring filaments were seen compared to normal skin, but were found to be of equal number at 1 - 2 years.

The above confirms the clinical findings of bulla formation in grafted cultured autologous epidermis seen in patient Mr J. (see table 4, figure 18), with a cleavage plane immediately below the anchoring fibrils (116, 15, 112, 10), which remain attached to intact lamina densa in the blister roof (116, 112). At 4 months it took 17 minutes for suction blisters to form with a cleavage plane below the lamina densa, whereas it took 65 minutes to raise blisters with a cleavage plane at the lamina lucida in normal skin (10).

Burn wounds grafted with cultured autologous keratinocytes and fibroblasts attached to collagen - glycosaminoglycan substrate, had an epithelium with a normal stratified appearance overlying the remnants of the substrate which had developed a vascular supply at 9 days post-grafting (28). Early anchoring fibril formation requires components from keratinocytes and fibroblasts both of which were present in the composite culture. Thus at the dermo-epidermal junction the lamina densa was continuous with multiple hemidesmosomes and a few anchoring fibrils. The basement membrane developed an undulating appearance with dermo-epidermal interdigitations suggestive of rete ridges at 4 weeks.

Melanocytes which are generally present in vitro in keratinocyte cultures, have been observed in grafted epidermis with visible re-pigmentation from a few weeks to several months. Langerhans cells which after serial cultivation of keratinocytes, are not present in vitro, repopulate the grafted epidermis within one week (12), with normal numbers found between 2 months and 1 year (15).

Compton et al have followed up patients grafted with cultured epithelium for 5 years (15). They found at 2 weeks inflammation in the granulation tissue had resolved. At 2 months, as

the cellularity decreased, collagen content increased, the collagen bundles being just sub-epithelial and parallel to the epidermis. A bilayered dermis appeared at 2 - 3 years. Deeper elastic fibres appeared at 3 years, becoming more diffuse at 4 - 5 years, at which time the initial random vascular pattern formed a superficial plexus parallel to the epidermis, sending capillary arcades into the papillae between the rete ridges.

Would a composite graft, composed of an artificial dermis (collagen gel, chrondroitin-6-sulphate and GAGs) covered by a cultured epidermal sheet improve the results obtained with cultured keratinocyte sheets alone? Possibly, once the composite graft has taken, it may strengthen the durability of the healed skin and also prevent contractures. These possibilities have to be investigated further. Thus in this paper a composite graft is proposed. Its components are described and the final product is shown.

6.2 Methods

6.2.1 Construction of the composite graft

6.2.1.1 Components

a) VITROGEN 100 Collagen - a sterile solution of purified, pepsin - stabilised dermal bovine collagen dissolved in 0.012N HCL.

b) FIBROBLASTS
   i) Mitomycin treated murine 3T3 fibroblasts.
   ii) Human derived fibroblasts, being of autologous or allogeneic origin.

c) KERATINOCYTES - being of human allogeneic or autologous origin.

6.2.1.2 Construction

a) Preparation of the neutralized, isotonic Vitrogen 100 Collagen solution
   1. Mix 8ml of chilled vitrogen 100 Collagen with 1ml of 0.1M NaOH and 1ml of 10X phosphate buffered saline solution.
   2. Adjust the pH of the solution to 7.4 (± - 0.2) by the addition of a few drops of 0.1M HCL or 0.1M NaOH. The pH of the solution can be monitored by the use of a pH meter.

b) Mixing of the constituents

1. A suspension of human or murine fibroblasts were obtained by treating a culture dish obtaining these cells with a solution of trypsin/EDTA. To this suspension the growth medium DMEM/Ham's F12, containing Bovine calf serum, was added. This suspension was spun down at 20 000 rpm for 5 minutes. The supernatant was removed. The neutralized, isotonic Vitrogen 100 Collagen solution was added to the cell pellet and mixed, by pippeting the solution up and down. The collage solution / cell mixture was placed in a 12cm petri dish, which was placed in the carbon dioxide incubator at 37°C. It took 15 minutes for gelation to occur.

2. Keratinocytes from a 12cm culture dish containing keratinocyte colonies which were subconfluent, were obtained by treating such a dish with trypsin/EDTA. The keratinocyte suspension, to which DMEM/Ham's F12 growth medium and Bovine calf serum was added, was centrifuged at 20 000 rpm for 5 minutes. The supernatant was removed. The cells were resuspended in the growth medium and serum mentioned above. This suspension was now placed on top of the collagen gel, which has been impregnated with fibroblasts. The keratinocytes settled down on the surface of the collagen gel, grew out to form a confluent stratified epidermal sheet.

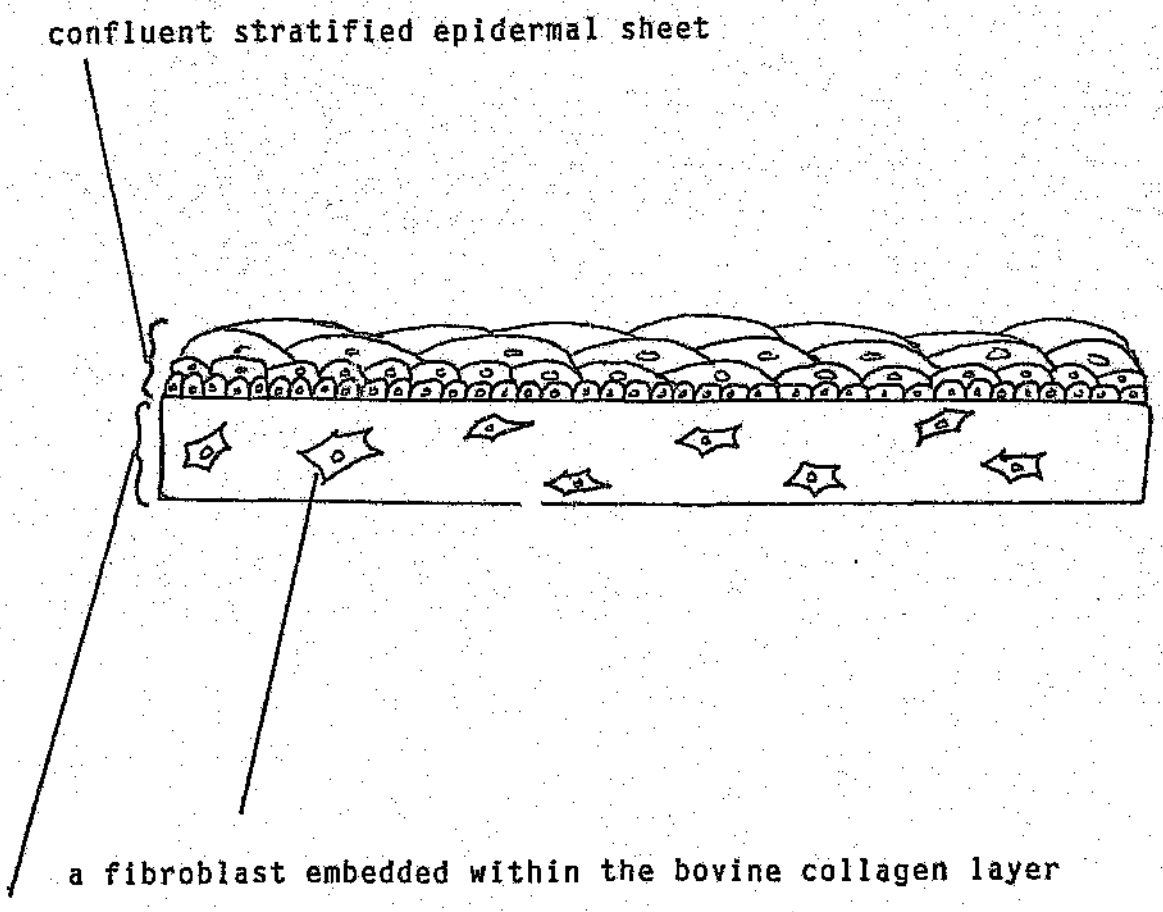


FIGURE 26.

The components of a composite graft which is ready for grafting.

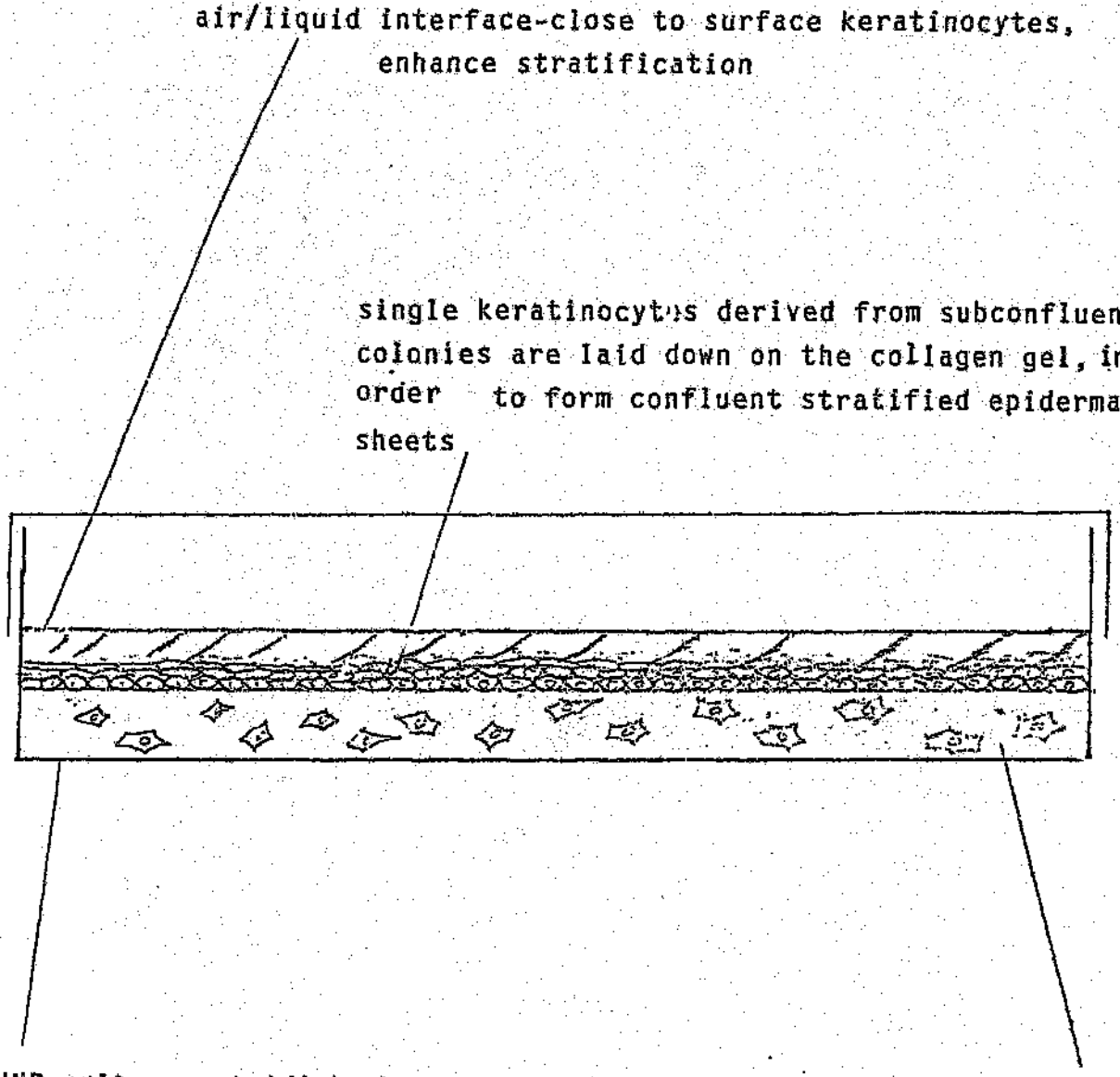


FIGURE 27.

The composite graft within the culture dish.

FIGURE 28 (a)

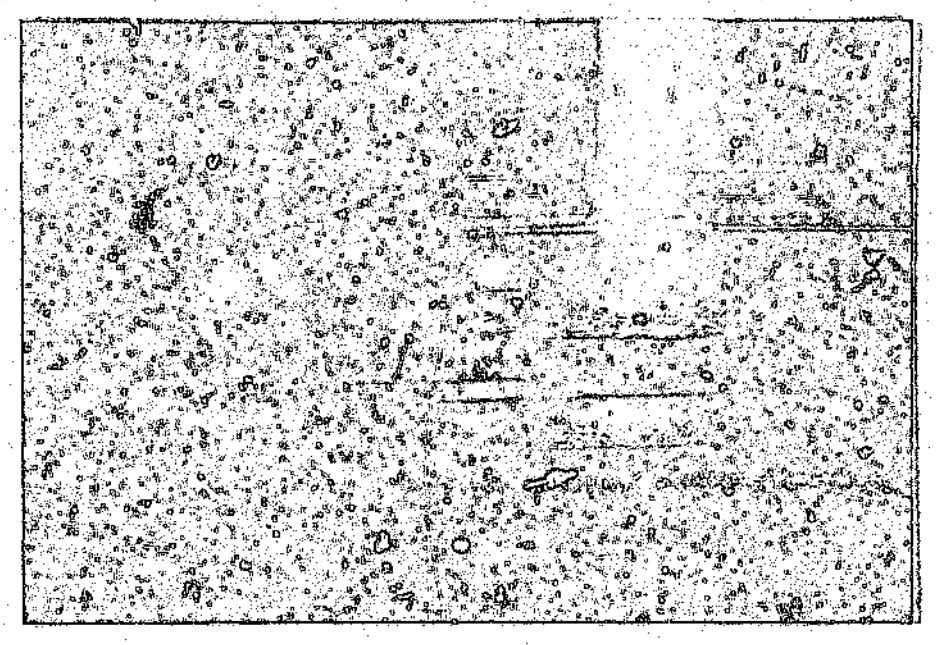


FIGURE 28 (b)

FIGURE 28.

A comparison between 2 collagen gel layers of different thicknesses.

a) The bovine collagen gel layer, being the artificial dermal component, is seen in a 6cm diameter culture dish.
b) The collagen gel layer is seen in a 9cm diameter culture dish. This thinner layer would probably be more permable for nutrients to diffuse through to the overlying cultured epidermal sheet in vivo.

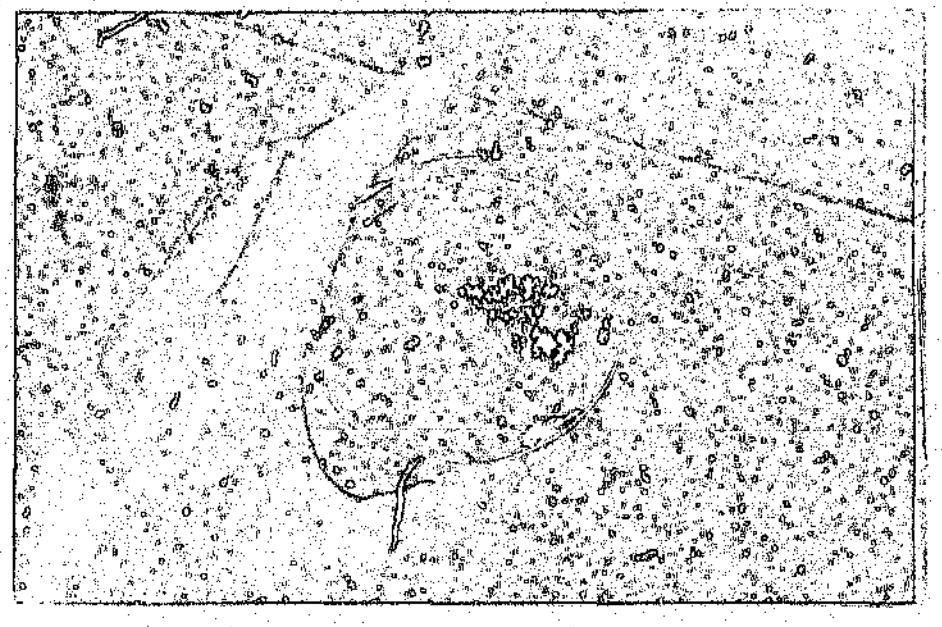


FIGURE 29 (a)

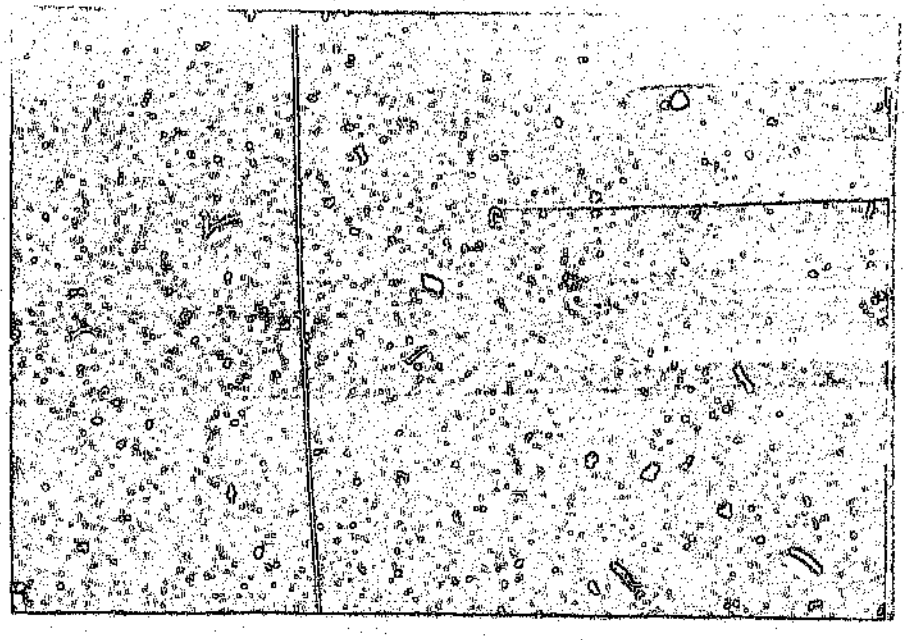


FIGURE 29 (b)

FIGURE 29.

The composite 'skin' graft.

a) Appearance from above.

b) The red dots are growing keratinocyte colonies.

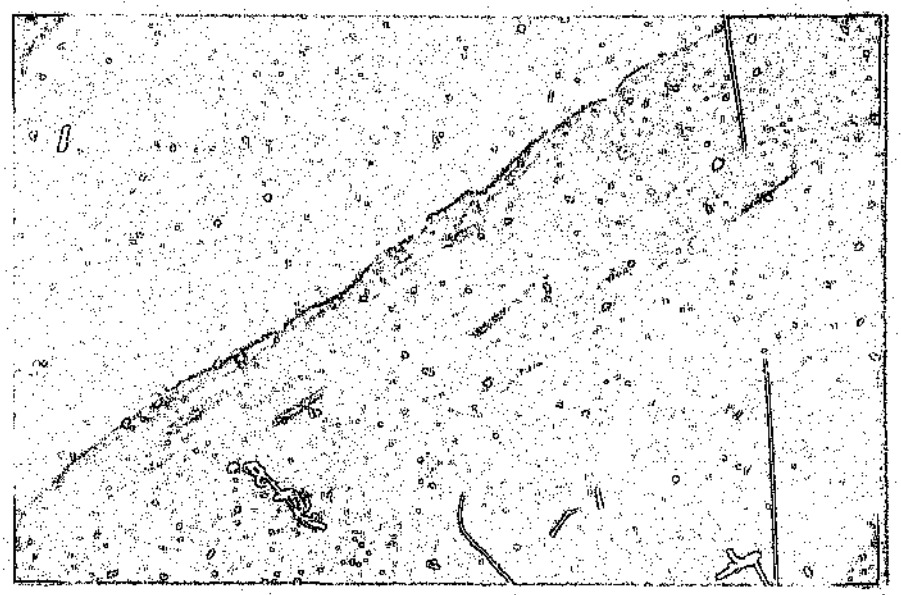


FIGURE 30 (a)

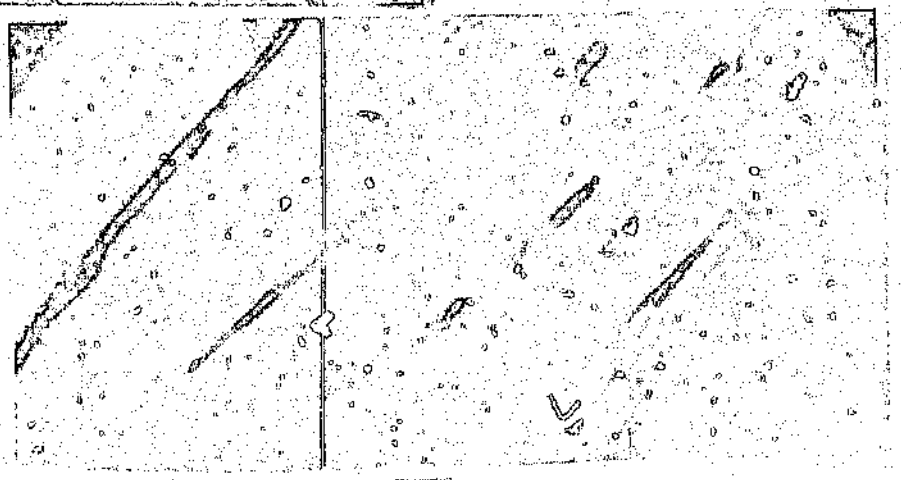


FIGURE 30 (b)

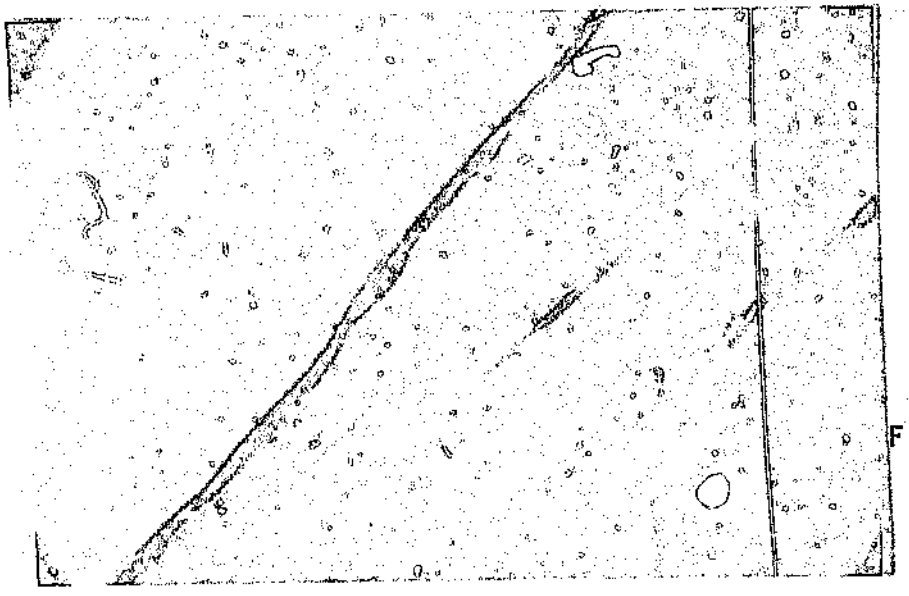


FIGURE 30 (c)

FIGURE 30.

L.M. sections through a composite graft.

a) The top surface is covered by a keratinocyte sheet. (mag. 10x).
b) The bovine collagen base is impregnated with human fibroblasts. (mag. 40x).
c) The surface can be seen to be composed of a confluent stratified keratinocyte sheet which is at least 2 cell layers thick. (mag. 100x).

6.3 Discussion

The bovine collagen gel acts as an artificial dermis. It also acts as a lattice work for the fibroblasts, enabling them to secrete their own collagen and laying it down in a uniform fashion. Thus the bovine collagen should eventually be replaced by human collagen.

6.3.1 Modifications to the collagen gel

The figures (figure 31), of the composite grafts show the overlying cultured epidermal sheet lying above the collagen gel, in which fibroblasts are embedded. The thickness of the collagen gel may be of importance when the graft is applied to the graft bed. If it is too thick then the overlying keratinocyte sheet may not get an adequate nutrient supply from the blood vessels within the donor bed.

6.3.2 The clinical application of an artificial dermis

Vascovali (117) applied a dermis of human collagen, chitosan and glycosaminoglycans to a patient after burn excision followed by autologous keratinocyte sheet 18 days later when the dermis was vascularised. 75% of the keratinocyte autograft was lost but in intact areas a reconstructed full thickness skin was observed.

Hansborough (28) used a fibrovascular ingrowth, but with a non-porous surface on which autologous keratinocytes were cultured. These composite cultures were placed on wounds of 4 burn patients after cadaver allograft removal. At 5 - 6 days epithelial foci were evident and at 9 days epithelial areas were well keratinized and beginning to desquamate. Take was observed in 9 of 13 grafts and at 4 weeks the surface was smooth with a good subjective resistance to shear and a relat. ely normal epidermal and dermal structure. In a more recent study (118) one autograft and two allograft composite cultures of human keratinocytes on fibroblast containing rat tail collagen gels were transplanted onto patients after tattoo excision down to fat. They reported rapid take of all three grafts with good cosmetic appearance and only 29% wound contraction in one case. A biopsy of one sex mismatched allograft at 4 weeks showed a fully differentiated epidermis and a mature dermis. However sound biological evidence of allograft survival was not provided. There is still controversy about the requirement for a dermal substrate at all since long term follow-up of early patients treated with keratinocyte autografts placed on muscle fascia without a substrate layer showed the eventual development of a normal dermis 5 year post grafting (15). This will only be

solved by direct comparison in controlled studies.

□ □ □ □ □ □ □ □

CHAPTER SEVEN

7.0 HUMAN KERATINOCYTE CULTURE AND GRAFTING TECHNIQUES IN PERSPECTIVE

7.1. Introduction

7.1.1 Is the in vitro model an appropriate one?

The time intervals - from monoclonal colony formation to confluence, and from confluence to stratification, are they acceptable? These time intervals most probably can be shortened by a few days, by carrying out the steps described in chapter two. This entails using smaller plating-out concentrations, reducing time exposure to trypsin and reducing the time interval between harvesting and culturing.

7.1.1.1 How does one know that this multilayered keratinocyte sheet showed signs of stratification

The answer to this seen in the L.M. and E.M. histological appearance of the keratinocyte sheet.

a) The basal layer being composed of small polygonal or cuboidal cells containing numerous vesicles and no basal hemidesmosomes.
b) The intermediate layer cells being elongated and containing numerous organelles.
c) One or two layers of more flattened nucleated cells were seen in the upper layers.

By carrying out strict aseptic techniques prevents the cultured keratinocytes from becoming contaminated by bacteria and/or fungi. What is important, is to use a cultured confluent keratinocyte sheet that has recently reached maximum stratification. One does not want to use an `old' cultured keratinocyte sheet, since the number of actively multiplying keratinocytes are reduced and the percentage take is reduced proportionately (refer to figure 25). Keratinocytes derived from older patients have fewer population doublings before senescence compared to neonatal keratinocytes (16).

Certain growth factors affect keratinocyte multiplication and migration in various ways. The

most potent factor being keratinocyte growth factor (KGF) produced by fibroblasts. This growth factor accelerates epithelialisation.

7.1.2 Is the clinical application of this method a useful one?

With specific reference to burns, the efficacy of 3 types of grafts containing the cultured keratinocyte sheet, were demonstrated. The so called compound graft giving the best results with respect to take, durability and cosmesis. The cultured keratinocyte autograft gave better results with respect to percentage take compared to cultured keratinocyte allografts. Initially, these latter 2 types of grafts had a tendency for blister formation, but with time the newly formed skin became thicker and more durable. This correlated with the findings of Compton et al. (15). To support the hypothesis proposed in this dissertation that the compound grafts may suppress hypertrophic scarring a large trial has to be undertaken.

Since infection is a major limiting factor with respect to cultured keratinocyte grafting, the results were standardized by adhering to the grafting protocol, particular reference being paid to the application of the antipseudomonal drug, piperacillin. The results obtained in the burn patients treated with keratinocyte grafts correlated with those obtained in over 370 patients treated by Biosurface Technology in Boston (79). At this centre the keratinocyte sheets were cultured according to the method described by Rheinwald and Green (1).

Leg lesions other than burns, grafted with cultured allografts, gave pleasing results. These results were able to be processed to produce a linear equation allowing one to predict the time that it would take for a leg ulcer of a given size to heal following the single application of a cultured keratinocyte allograft sheet.

The results following the application of cultured keratinocyte sheets to extensive full thickness burns, leg ulcers and other conditions resulting in skin loss, admirably demonstrate that these sheets enhance the rate of healing as seen by visible skin formation. But do these transplanted keratinocytes survive? In chapter five, it was demonstrated with the use of a Y-chromosome probe that the allograft keratinocytes could not be detected beyond day 4 post-grafting. In spite of this finding they appeared to visibly enhance wound healing. The postulated mechanism being that the keratinocytes impart some growth factor(s) which stimulate the patients own keratinocytes to multiply faster than usual.

The above management of leg ulcers and extensive burns represents a significant advance

with respect to wound care. It appears to reduce morbidity and mortality. The draw-backs concerning durability of the newly formed skin may possibly be overcome with the use of the proposed composite graft described in the previous chapter. This composite graft mimics the major skin components.

The composite graft should not only prevent contractures but should also provide a lattice work in which fibroblasts, endothelial cells and other dermal cells can proliferate and secrete structural and functional proteins.

Cell culture is rapidly opening up new previously unimaginable fields of endeavour having diverse applications. Not only regarding gross clinical applications but also with regard to furthering our understanding of basic cellular processes. This therefore will enable us to harness the cell for the benefit of mankind.

APPENDIX I

A) List of culture media and solutions:

1) Eagle's Minimal Essential Medium (Earl's salts) - Flow Laboratories.

2) Dulbecco's Modification of Eagle's Medium - Flow Laboratories.

3) Hams F12 Medium - Flow Laboratories.

4) Foetal calf serum (fortified with iron) - Hyclone Laboratories.

5) Bovine serum - Flow Laboratories.

6) Trypsin - Sigma - used at 0.17% in calcium/magnesium free phosphate buffered saline.

7) Ethylenediaminetetra acetic acid (EDTA) - BDH - used at 0.1% in calcium/magnesium free phosphate buffered saline.

8) Pencillin - Novo Industries used at 100 units/ml.

9) Streptomycin - Novo industries used at 50ug/ml.

10) Fungizone - GIBCO - used at 1ug/ml.

11) Mycostatin (Nystatin) - Squibb Laboratories - used at 25 units/ml.

12) Gentamycin - Sigma - used at 100ug/ml.

13) Mitomycin C - Sigma - used at 4ug/ml for 2 hr.

14) Epidermal Growth Factor (EGF) - Sigma used at 10ug/ml.

15) Adenine - Boeh. & Mannh. used at 2.42mg/ml.

16) Insulin - Sigma - used at 5ug/ml.

17) Hydrocortisone - Sigma - used at 0.5ug/ml.

18) Transferrin - Sigma - used at 5ug/ml.

19) Tri-iodotyronine - Sigma - used at 2X 10^{-9}M.

20) Linoleic acid - Highveld Biological used at 5ug/ml.

21) Cholera toxin - Sigma - used at 100ng/ml.

22) Calcium/magnesium - free phosphate buffered saline (CMF-PBS) -8.0g NaCl;0.2g KCI;0.2g KH_2PO_4;2.9g $Na_2HPO_4.12H_20$: PH 7.2 made up to 1 litre.

23) Phosphate buffered saline (PBS) - 8.0g NaCl;).2g KCI;
0.2g KH_2PO_4;2.9g $Na_2HOP_4.12H_20$;0.13g $CaCl_2.2H_20$;
0.1gMgCl2.6H20;pH 7.2 made up to 1 litre.

24) Petri dishes - various sizes - Nunclon.

25) Dispase II - Sigma, St Louis,MO. U.S.A.

26) Piperacillin-Ledele Laboratories, Isando Transvaal, South Africa.

The water used in both the preparation of culture media and the washing of culture glassware was produced by either ultra-filtration and polishing through a Milli-Q system (millipore) or by double glass distillation (Fissions).

Sterilization of culture glassware was achieved by autoclaving (Glaxo-Allenbury) or in some instances by 'dry-heat' sterilization at 180°C for a minimum of 3 hr.

Culture media and the various other solutions were filter sterilized (0.22um pore size) using 5% CO_2 in air, divided into relevant aliquots and stored at 4°C if to be used immediately otherwise at -20°C for up to 3 months.

B) Preparation of 3T3 feeder layers

Confluent cultures of 3T3 murine fibroblasts were incubated with 4ug/ml mitomycin C for two hours, which made them reproductively inactive. These cultures received three washes with sterile PBS followed by a further two with culture medium prior to being plated out as feeder support. Plating density was $7.5X10^5$ cells/64cm petri dish.

APPENDIX II

THE KERATINOCYTE CELL LINES

The keratinocyte cell lines were used to form confluent stratified epidermal-like sheets used as allografts or autografts.

A) Foetal and neonatal cell lines;

1. MAC-derived from a term foetus.
2. K-derived from an eight day old foreskin.
3. Mey-derived from an eight day old foreskin.
4. G-derived from an eight day old foreskin.
5. B-derived from an eight day old foreskin.
6. D-derived from an eight day old foreskin.
7. Chinn-derived from a ten day old foreskin.
8. Tim-derived from a three week old foreskin.
9. S-derived from a ten day old foreskin.
10. Lui-derived from a ten day old foreskin.
11. Reece-derived from a ten day old foreskin.
12. J-derived from a ten day old foreskin.
13. K't-derived from a ten day old foreskin.
14. C-derived from a ten day old foreskin.
15. H-derived from a ten day old foreskin.

All these cell lines tested seronegative for the Hepatitis B virus as well as for the Human Immunodeficiency Virus. These cell lines were all frozen down in 1ml cryopreservation tubes.

B) Adult cell lines:

1. Rain-23 year old black female.
2. Moses-42 year old black male.
3. Else-35 year old black female.
4. Jonas-24 year old black male.
5. Virginia-19 year old black female.
6. Grobler-46 year old white male.
7. Lindiwe-12 year old black female.
8. Braam-1 year old white male.
9. Edwin-54 year old black male.
10. Defontes-42 year old white male.
11. Dulfred-18 year old black male.
12. Zuma-21 year old black male.

APPENDIX XII

CRYOPRESERVATION OF HUMAN KERATINOCYTES

Cells marked for cryopreservation were harvested from subconfluent colonies. The fibroblast feeders were first removed with the aid of EDTA/glucose being applied for 120 seconds. This was followed by washing with PBS. The cells were removed from the surface of the culture dishes with the aid of Trypsin/EDTA, incubated in a CO_2 incubator for 20-40 minutes at 37°C.

The cells were spun down at 20 rpm. for 5 minutes. The supernatant was removed and cryopreservation medium is added. 10 000 cells per millilitre were preserved in a 1ml Nunc cryopreservation tube. The cryopreservation tubes were stored in a -70°C freezer.

CRYOPRESERVATION OF KERATINOCYTE CELL LINES

The cryopreservation medium:

Bovine calf serum-20%
Glycerine-15%
Dulbeco's Modified Eagles Medium/Ham's F 12 medium as 1:1-65%

APPENDIX IV

The hybridization and detection of biotinylated DNA probes in fixed tissues.

IV.1 Materials

IV.1.1 Materials required for nick translation of DNA probes.

Vial 1. DNase I:
0.5 mg/ml in 100mM $MgCl_2$

Vial 2. DNase I dilution buffer:
10mM Tris HCI pH 7.5, 1mg/ml BSA

Vial 3. 10X reaction buffer:
0.5M Tris HCI pH 7.5,50mM $MgCl_2$

Vial 4. Deoxynucleotide solution:
0.3mM dATP,0.3mM dGTP, 0.3mM dCTP in 50 mM Tris HCl pH 7.5.

Vial 5. Bio-11-dUTP:
0.3mM in 50mM Tris HCl pH7.5

Vial 6. TTP:
).3mM in 50mM Tris HCl pH 7.5. The TTP may be used in a control reaction in place of Bio-11-dUTP.

Vial 7. DNA polymerase I
3 units/ul in 0.1M sodium phosphate buffer pH 7.2,
50% glycerol (v/v),1.0mM DTT

Vial 8. Stop buffer:
200mM EDTA

Vial 9. Control DNA
0.25mg/ml lambda DNA in 50mM Tris HCl pH 7.4, 1mM EDTA.

Vial 10. Y chromosomal DNA:
pDP 105 - supplied by Dr M Ramsey of the Department of Human Genetics, South African Institute for Medical Research with permission from Dr D Page.

Buffer: 0.06g Tris HCl,0.19g EDTA,500ml distilled water,pH 7.5.

IV.1.2. Materials required for hybridisation mot biotinylated DNA probes in fixed tissues

a) 1X phosphate buffered saline (1X PBS):
7.6g of 130mM sodium chloride (NaCl)
1g of 7mM dibasic sodium phosphate (Na_2HPO_4)
0.47g of 3mM monobasic sodium phosphate (NaH_2PO_4)

b) Phosphate buffered saline, EDTA (PBS-EDTA):
130 mM naCl
7mM Na_2HPO_4
3mM NaH_2PO_4
1.9g of 5mM EDTA

c) 20X SSPE:
210.384g of 3.6M sodium chloride
31.2g of 0.2 M sodium phosphate pH 7.4
7.445g of 0.02M EDTA PH 7.4

e) Deionized formamide

f) 50% Dextran sulfate:2.5g of dextran sulfate in 5ml PBS.

g) 50% Formamide, 0.1XPBS:
Combine 25ml deionized formamide,5ml 1X PBS and 20ml deionized or distilled water.

IV.1.3 Materials required for the detection of hybridized biotinylated DNA probes in fixed tissues

a) 1X Phosphate buffered saline (1X PBS)
130mM NaCl
7mM Na_2HPO_4
3mM NaH_2PO_4

b) Complex Dilution Buffer:
12.114g of 0.1M Tris (pH 7.4)
5.844g of 0.1M NaCl
0.0136g of 0.1mM $ZnCl_2$

c) Detection Buffer
12.114g of 100mM Tris HCl pH 9.5
5.844g of 100mM NaCl
2.063g of 10mM $MgCl_2$

d) Substrate stock solution:
Store at 4°C in the dark
50mg/ml 5- bromo - 4 chloro -3-indolyl phosphate (BCIP) in anhydrous dimethylformamide.

e) Chromogen stock solution:
Store at 4°C in the dark
Suspend 75mg nitroblue tetrazolium (NBT) in 0.7ml anhydrous dimethylformamide. When suspended, add 0.3ml deionized water to dissolve fully.

f) Reaction mixture (make fresh for each use):
To 5ml detection buffer (solution 3) add 22ul chromogen stock solution, (NBT,solution 5) and mix. Then add 16.50ul substrate stock solution, (BCIP,solution 4).

IV.2 Methods

IV.2.1 The Method for nick translation of DNA probes with biotinylated nucleotides

IV.2.1.1 Introduction

Nick translation is based on the introduction of random scissions or 'nicks' by pancreatic DNase.E.coli DNA polymerase I then catalyzes the addition of nucleotide resides to the 3'-hydroxyl terminus of a nick, with the simultaneous elimination of nucleotides from the 5'-phosphoryl terminus. There is no net DNA synthesis. As nucleotides are removed from the 5'-phosphoryl terminus and new ones are added to the 3'-hydroxyl terminus, the nick is removed, or translated linearly along the strand. In the presence of biotinylated nucleotide(s) pre-existing unmodified nucleotides in the DNA strand are replaced by biotinylated analoge.

Enzo Diagnostics (119) provided the nick translation kit, which follows established protocols using Bio-11-dUTP, a TTP analogue, as the biotinylated nucleotide. Because nick translation is a template dependent reaction, the number of biotinylated nucleotides incorporated is determined by the A-T composition of the DNA. With this procedure 20-60% of the TMP resides are replaced with Bio-11-dUMP. Nick translation is used for biotinylating double- stranded DNA greater than 1kb. Nick translation is followed by DNA hybridization and detection of the biotinylated DNA probe.

Here is described a procedure for detection of biotinylated pHY2.1 in situ on the Y chromosome in tissues derived from healing ulcers 4,58 and 16 days after grafing with cultured keratinocyte allograft sheets. The method is based on that described by Burns J,et al (97).

IV.2.1.2 Methods

a) Nick translation reaction

The following reaction is for labelling 1ug DNA. To the eppendorph centrifuge tube add the following:

1. 20ul of 10 X reaction buffer - vial 3.
2. 20ul deoxynucleotide solution - vial 4.
3. 20ul Bio-11-dUTP - vial 5.
4. 8ul Y chromosomal DNA - vial 10. (pDP 105).

OR

5. 16ul Control DNA - vial 9.

Bring the volume to 168ul with distilled water.
To this 168ul add 16ul DNA polymerase I - vial 7.

b) Dilution of DNase I

Mix the following:
1. 1ul DNase I - vial 1.
2. 49ul DNase I dilution buffer - vial 2.

This forms a 1:500 dilution.

c) Mixing the solution constituted for the nick translation reaction with diluted DNase I

Mix the 174ul obtained in part a) with 16ul of diluted DNase I obtained in part b). This mixture is mixed carefully in an Eppendorph centrifuge for 1 minute and then incubated at 14°C for 2 hours.

This is followed by the addition of 28ul stop buffer - vial 8. Now one has a solution containing a total of 308ul, which is centrifuged for 2 minutes followed by incubating a 65°C for 10 minutes.

To the 308ul solution add 30.8ul of 3M sodium acetate - pH 5.2. This is followed by the addition of 700ul of 95% ethanol. The solution is centrifuged for 3 minutes and then stored at -70°C for 15 minutes.

The DNA is recovered by centrifugation for 5 minutes. The supernatant is discarded. The pellet is washed twice with 700ul 80% ethanol. Each time the solution is centrifuged for 5 minutes and the supernatant is removed.

The pellet is dried by placing the tube in a vacuum dessicator for a short time. The DNA is dissolved in 10mM Tris HCl pH 7 5, 1mM EDTA, getting a concentration of 30ng/ml.

In order to get the above concentration take 5ml of the above buffer and take off 20ul and add 20ul of the DNA probe.

One now has the DNA probe.

IV.2.2 The method hybridization and detection of biotinylated DNA probes in fixed tissues

IV.2.2.1 Obtaining and preparing the biopsy specimens.

Punch biopsy specimens (2mm) were taken under local anaesthesia at 4,58 and 16 day intervals from areas of the wound (leg ulcer) that were clinically healing. These biopsies were taken from female patients who were grafted with cultured keratinocyte sheets obtained from male donor. The controls were male skin and prostatic tissue obtained from an individual who presented with benign prostatic hypertophy.

Sections (3um) were cut from tissue fixed in formal saline and embedded in paraffin wax. The sections were mounted on slides that had been cleaned, washed, air, dried and coated with triethoxysilyl adhesive.

Sections of the same tissue were stained with haematoxylin and eosin after dewaxing and rehydration. These sections were used for routine histology.

The sections for in situ DNAhybridization were washed in xylene, graded concentrations of alcohol followed by distilled water. This was followed by treating the sections for 20 minutes at 37^{o}C with Proteinase K (0.5mg/ml in PBS-EDTA). The reaction is stopped by washing the slide with solution 2 (PBS.5mM EDTA) for 10 seconds. Excess liquid is wiped off and the slide is air dried.

Now the tissue section is ready for DNA hybridization.

IV.2.2.1 Preparation of the hybridisation mixture

The following components were mixed thoroughly:

200ul deionized formamide,
40ul 20X SSPE,
40ul deionized water.

The above three components were heated to 65°C. This was followed by the addition of 40ul of 50% dextran sulfate. The test tube was cooled and 80ul of the DNA probe was added.

IV.2.2.3 In situ hybridisation

20ul of hybridization mixture was applied to each specimen on a microscope slide and cover with a glass coverslip. The covered slide was placed on to the 92°C heating block for 10 minutes, in order to denature the double stranded DNA.

The slides were then removed from the heating block and hybridization was allowed to proceed at room temperature (20-30°C) for 20 minutes. The coverslip was washed off gently with PBS. This was followed by the application of 200ul of 50% formamide,0.1X PBS, to each slide, which were allowed to stand at room temperature for 10 minutes.

The slides were rinsed in solution 2 for 5 minutes at room temperature. Excess moisture was tapped off the slide. The samples were now ready for visualization using a biotin-recognizing signal detection generating system.

IV2.2.4 Detection of biotinylated DNA in fixed tissues

The slides were removed from PBS. 10ul from 10 ml of complex dilution buffer were removed and 10ul of DETEK 1-alk complex. 100ul of this well-mixed diluted complex to each slide were added. Incubated at 37°C for 30 minutes. This was followed by rinsing for 10 seconds under a steady stream of PBS. Gently blotted dry with the aid of filter paper.

The reaction mixture were added and the slides were incubated at room temperature, in the dark, for 30 minutes. Checked under the microscope for colour. Positives appeared as dark brown precipitates. The slides were rinsed in water and counter-stained with 1% fast green in 1X PBS, if desired.

APPENDIX V

Data sheets and figures of burn patients treated with cultured keratinocyte sheets. The data sheets were used to obtain tables 3 - 5 and graphs 3 - 6. The figures aim to illustrate the progress of healing for each patient referred to on the accompanying data sheet.

PATIENT: Miss R M : 23 year old female (Black female patient)

BURN SURFACE AREA: 55%, of this 50% full thickness burn

LOCALISATION: anterolateral aspect of lower leg, to which a CEA was applied. Both arms and legs as well as face and chest.

DAYS FROM INJURY TO GRAFTING: 62 days

AREA GRAFTED WITH CULTURED EPIDERMAL ALLOGRAFT (cm^2): 144 cm^2
Over the cultured epidermal allograft a highly meshed (1;3) autologous SSG was applied.

NUMBER OF TIMES CEA APPLIED: once

PERCENTAGE TAKE PER APPLICATION: 100% - seen at 14 days post-grafting.

FINAL ESTIMATE % CEA OF BSA: 8

COSMETIC APPEARANCE OF AREA GRAFTED WITH CEA:
A smooth dark brown skin seen 23 days after grafting.
A comparative area grafted with 1;3 meshed SSG (autograft) alone, has a marked diamond meshed appearance 23 days after grafting.

PATIENT: Master B H : 1 year old (European male patient)

BURN SURFACE AREA: 80%. 50% full thickness.

LOCALISATION: Face, arms trunk upper half of both anterior thighs.

DAYS FROM INJURY TO GRAFTING: 31 days

AREA GRAFTED WITH CULTURED EPIDERMAL ALLOGRAFT (cm^2): Lower chest and anterior abdominal wall as well as inner aspects of both arms and forearms.

NUMBER OF TIMES CEA APPLIED: once

PERCENTAGE TAKE PER APPLICATION: 0%

FINAL ESTIMATE % CEA OF BSA: 90%

CONCLUSIONS: Seventeen days after CEA applied the areas grafted were clean and failed to grow any bacteria after repeated swabs were taken. In the presence of a negative nitrogen balance and poor oxygenation CEAs act as excellent temporary biological dressings.

COSMETIC APPEARANCE OF AREA GRAFTED WITH CEA: red granulation tissue seen.

FOLLOW-UP: Patient died 2 months following burns, due to respiratory distress syndrome caused by smoke inhalation.

PATIENT: Mr R N : 23 years (Black male patient)

BURN SURFACE AREA: 9%. .' full thickness burn.
Total BSA = 112.5 cm^2.

LOCALISATION: Right arm and dorsum of hand

DAYS FROM INJURY TO GRAFTING: 19 days

AREA GRAFTED WITH CULTURED EPIDERMAL ALLOGRAFT (cm^2): 25

NUMBER OF TIMES CEA APPLIED: once

PERCENTAGE TAKE PER APPLICATION: 100% - occurring 8 days after application.

FINAL ESTIMATE % CEA OF BSA: 22.2% (66.7% of full thickness burn area)

CELL LINE USED: H

COSMETIC APPEARANCE OF AREA GRAFTED WITH CEA:
Pink smooth skin.

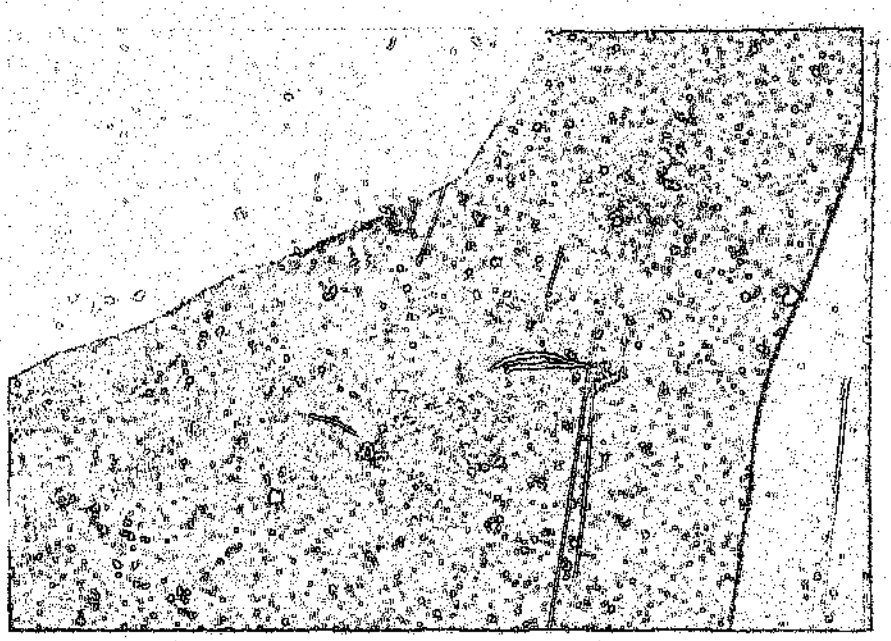


FIGURE V.1

The dorsum of the hand of patient Mr R N, a close-up view of the healed burn. The 2 scabs will fall off to reveal new underlying skin.

PATIENT: Mr M : 42 years (Black male patient)

BURN SURFACE AREA: 38% - all full thickness burn

LOCALISATION: Arms and legs. Area on posterior aspect of right calf grafted with CEA.

DAYS FROM INJURY TO GRAFTING: 72 days

AREA GRAFTED WITH CULTURED EPIDERMAL ALLOGRAFT (cm^2): 36 cm^2

NUMBER OF TIMES CEA APPLIED: once

PERCENTAGE TAKE PER APPLICATION: 11.1% seen at 14 days post-grafting despite the wound bed being infected with Pseudomonas aeruginosa.

FINAL ESTIMATE % CEA OF BSA: 4.5%

COSMETIC APPEARANCE OF AREA GRAFTED WITH CEA:
smooth pink skin seen.

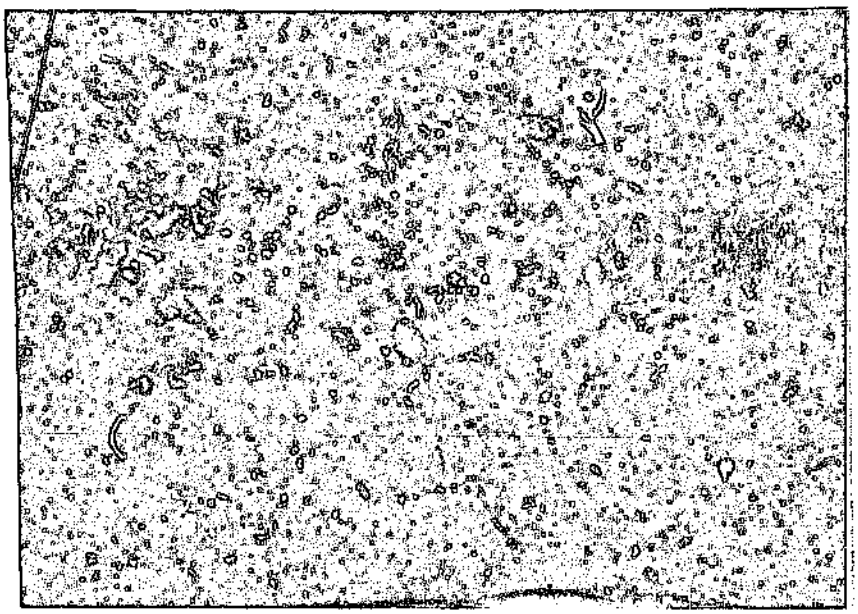


FIGURE V.2

The right calf of patient Mr M. reveals a small area of taken CEA seen 14 days folling grafting. When the dressings were removed on day 3, a Pseudomonas aeruginosa infection was noted. Following this case, all subsequent cases had the grafted area treated with piperacillin prior to grafting.

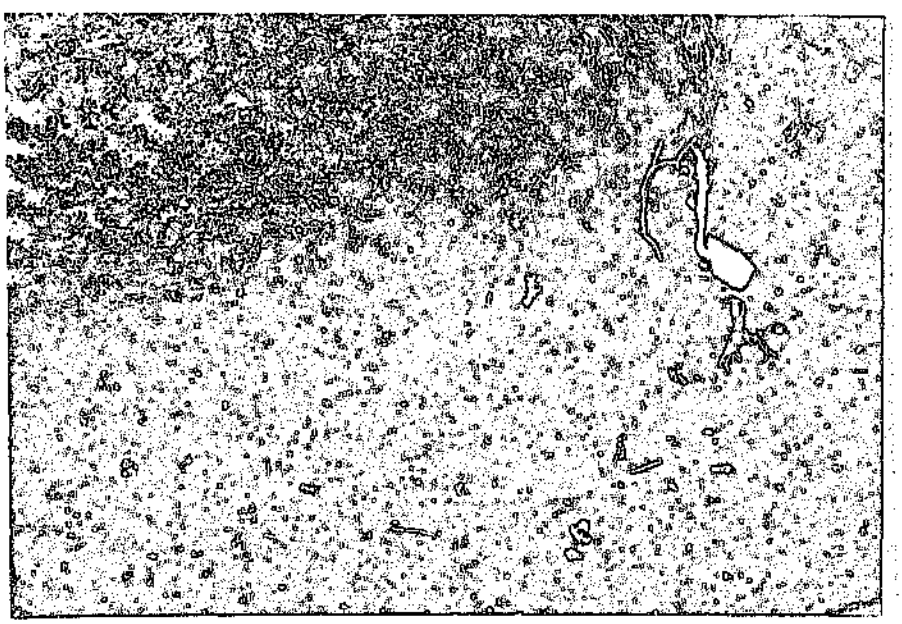


FIGURE V.3

An area on the left calf of patient Mrs E. 12 days after application of a compound graft reveals a taken graft.

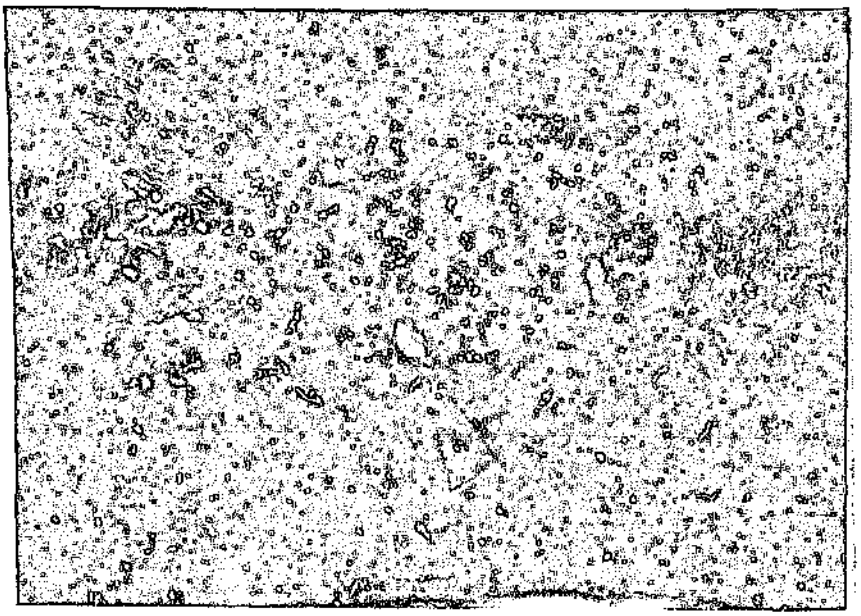


FIGURE V.2

The right calf of patient Mr M. reveals a small area of taken CEA seen 14 days folling grafting. When the dressings were removed on day 3, a Pseudomonas aeruginosa infection was noted. Following this case, all subsequent cases had the grafted area treated with piperacillin prior to grafting.

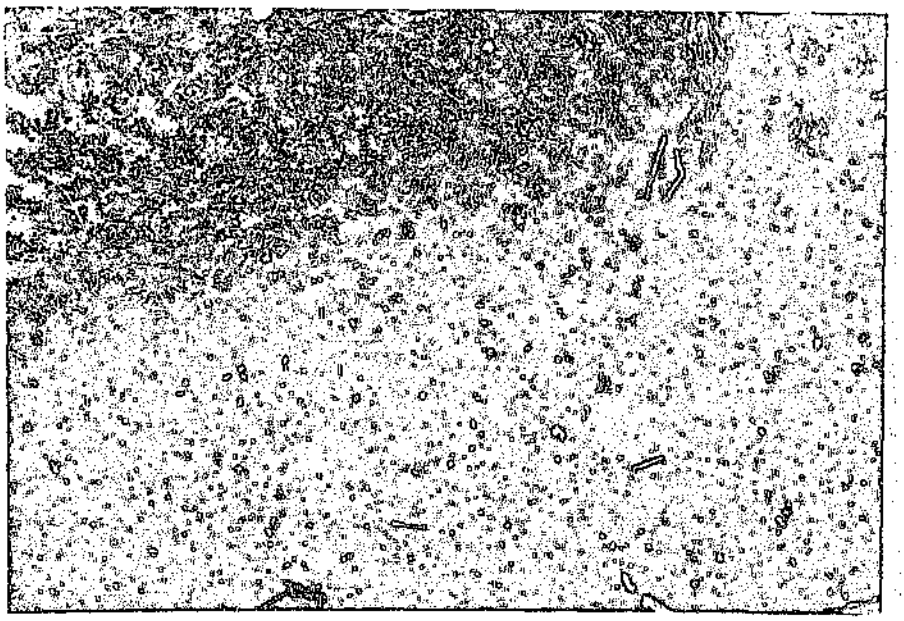


FIGURE V.3

An area on the left calf of patient Mrs E. 12 days after application of a compound graft reveals a taken graft.

PATIENT: Mrs E : 35 years (Black female patient)

BURN SURFACE AREA: 18% - all full thickness burn

LOCALISATION: Both legs

DAYS FROM INJURY TO GRAFTING: 62 days

AREA GRAFTED WITH CULTURED EPIDERMAL ALLOGRAFT (cm^2): 64 cm^2 -area on posterior aspect of right thigh and calf. A meshed (1;1.5) SSG backing was applied.

AREA GRAFTED WITH CULTURED EPIDERMAL AUTOGRAFT (cm^2): 127 cm^2 -to the above area. Part had SSG backing.

NUMBER OF TIMES CEA APPLIED: once.

PERCENTAGE TAKE PER APPLICATION: Of the area covered by the SSG backing 100% take seen 12 days after grafting-156cm^2.
Of the area covered by the cultured autograft 32cm^2 showed visible skin formation, this represents 50% of this area grafted.

FINAL ESTIMATE % CEA OF BSA: 45%

COSMETIC APPEARANCE OF AREA GRAFTED WITH CEA: Area grafted with the compound graft was brown in appearance with a diamond mesh pattern. The area grafted with a CEA alone was pink in appearance.

PATIENT: Mr J : 24 years (Black male patient)

BURN SURFACE AREA: 18% - all full thickness burn

LOCALISATION: Both arms

DAYS FROM INJURY TO GRAFTING: 27 days

AREA GRAFTED WITH CULTURED EPIDERMAL ALLOGRAFT (cm^2): 64 cm^2 on anterior aspect of right forearm just above the wrist.

GRAFTED WITH CULTURED EPIDERMAL AUTOGRAFT (cm^2): 218 cm^2 applied to anterior arm and forearm (R).

NUMBER OF TIMES CEA APPLIED: once

PERCENTAGE TAKE PER APPLICATION: Of cultured allograft, 50% at 14 days post grafting.
Of cultured autograft, 98% at 14 days post grafting.

FINAL ESTIMATE % CEA OF BSA: 38.9%

CELL LINE ORIGIN: Autograft confluent and stratified at 22 days from initiating cultures.
Allograft derived from cell line Virginia and took 24 days to culture.

APPEARANCE OF AREA GRAFTED WITH CEA: Smooth pink skin formed.

REMARKS: On day 14 part of the newly formed skin lifted off from the graft bed, but subsequently reattached. This may be due to anchoring fibrils being deficient in type IV collagen.

PATIENT: Miss L Z : 12 years (Black female patient)

BURN SURFACE AREA: 46%,of this 40% was full thickness burn

LOCALISATION: Anterior chest wall, arms and legs as well as face.

DAYS FROM INJURY TO GRAFTING: 28 days

AREA GRAFTED WITH CULTURED EPIDERMAL AUTOGRAFT (cm^2): 108 cm^2

AREA GRAFTED WITH THE COMPOUND GRAFT (CEA + 1/3 MESHED SSG): 5cm^2

NUMBER OF TIMES CEA APPLIED: once

PERCENTAGE TAKE PER APPLICATION: 11 days post grafting 92.6% 'take' seen. Area grafted with compound graft-100% 'take' seen at 11 days post grafting.

FINAL ESTIMATE % CEA OF BSA: 38.9%

COSMETIC APPEARANCE OF AREA GRAFTED WITH CEA:
Pink smooth skin, tender on palpation over site grafted with a cultured epidermal autograft alone. Light brown skin seen at the compound graft site. Through the smooth thin skin small blood vessels can be seen.
The light brown skin appears slightly thicker than the pink skin.

PATIENT: Mr E N : 34 years (Black male patient)

BURN SURFACE AREA: 40%. 20% full thickness

LOCALISATION: head, arms and back

DAYS FROM INJURY TO GRAFTING: 38 days-when cultured allograft applied

AREA GRAFTED WITH CULTURED EPIDERMAL ALLOGRAFT (cm^2): 125 cm^2

NUMBER OF TIMES CEA APPLIED: once

PERCENTAGE TAKE PER APPLICATION: 40% (≈50cm^2 seen 4 days after grafting)
90%, seen 16 days after grafting.

FINAL ESTIMATE % CEA OF BSA: 6.25% (12.5% full thickness burn)

COSMETIC APPEARANCE OF AREA GRAFTED WITH CEA:
Pink and smooth.

CELL LINE USED: SM

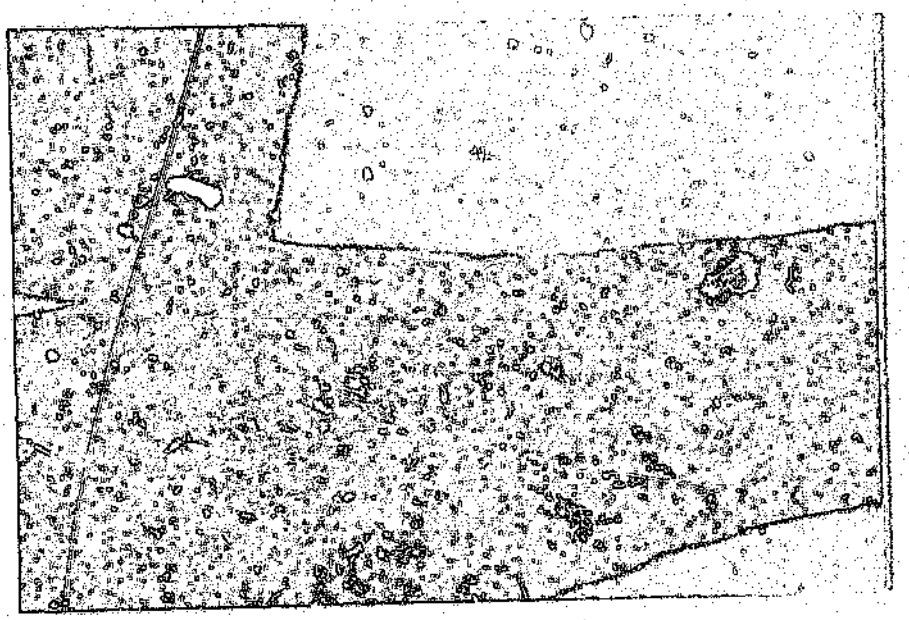


FIGURE V.4 (a)

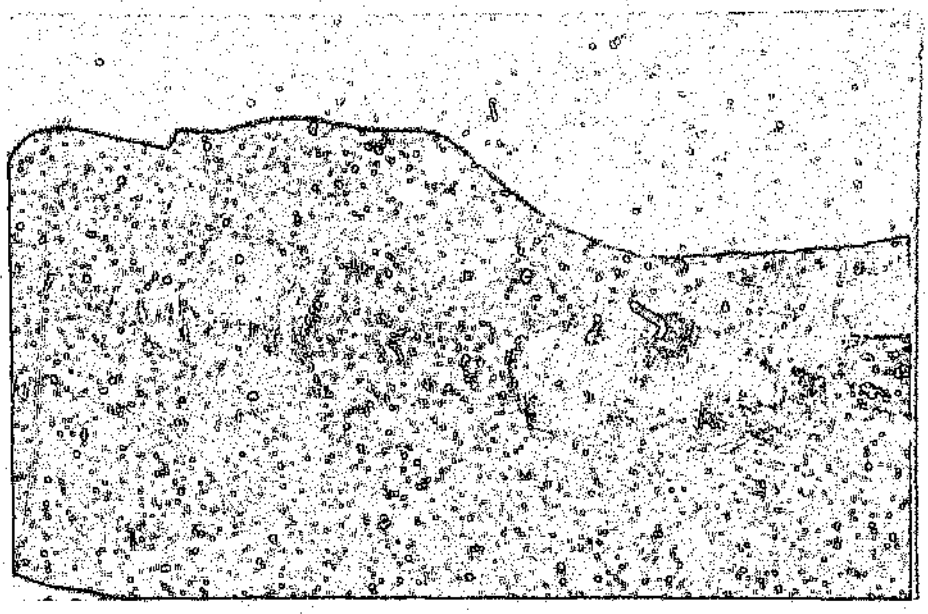


FIGURE V.4 (b)

FIGURE V.4

The dorsum of the left hand of patient Mr E.N. before (Figure V.4 (a)) and after (Figure V.4 (b)) cultured epidermal allograft application. Figure V.4 (b) shows amarked reduction in the size of the remaining burn wound 4 days after grafting.

PATIENT: Mr G : 46 years (European male patient)

BURN SURFACE AREA: 80%

LOCALISATION: Whole body except part of back and flanks.

DAYS FROM INJURY TO GRAFTING: 14 days

AREA GRAFTED WITH CULTURED EPIDERMAL ALLOGRAFT (cm^2): $282cm^2$

NUMBER OF TIMES CEA APPLIED: once

PERCENTAGE TAKE PER APPLICATION: 20% at 14 days after grafting
Only the area grafted with a CEA applied to right anterior thigh revealed some new skin.

FINAL ESTIMATE % CEA OF BSA: 6.25%

LIMITING FACTORS: Contamination of grafted areas with faeces. Also the disinfectant Eusol was applied inadvertently to some of the areas grafted.

COSMETIC APPEARANCE OF AREA GRAFTED WITH CEA:
Pink smooth skin visible.

FOLLOW-UP: Patient died 6 weeks after sustaining his burns.

PATIENT: Miss V : 19 years (Black female patient)

BURN SURFACE AREA: 30% - all full thickness burn

LOCALISATION: Buttocks, full extent of both legs

DAYS FROM INJURY TO GRAFTING: 21 days

AREA GRAFTED WITH CULTURED EPIDERMAL AUTOGRAFT (cm^2): $192cm^2$ - half of this area, $96cm^2$ - was grafted with a the compound graft.

NUMBER OF TIMES CEA APPLIED: once

PERCENTAGE TAKE PER APPLICATION: 97% at 14 days after applying CEA alone.
At 14 days 100% of area grafted with the compound graft.

FINAL ESTIMATE % CEA OF BSA: 16.7%

LIMITING FACTORS: The patient constantly urinated over her legs.

COSMETIC APPEARANCE OF AREA GRAFTED WITH CEA:
Half the area grafted was brown and smooth. This area received a the compound graft. The other half, which received a CEA only, was pink and smooth.

FOLLOW-UP: Patient died 2 months after sustaining her burns.

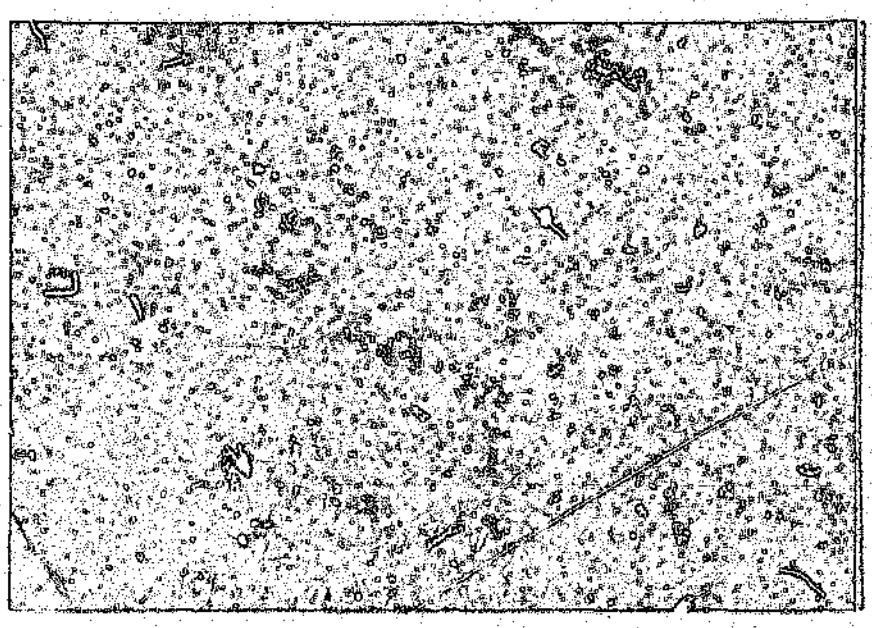


FIGURE V.5 (a)

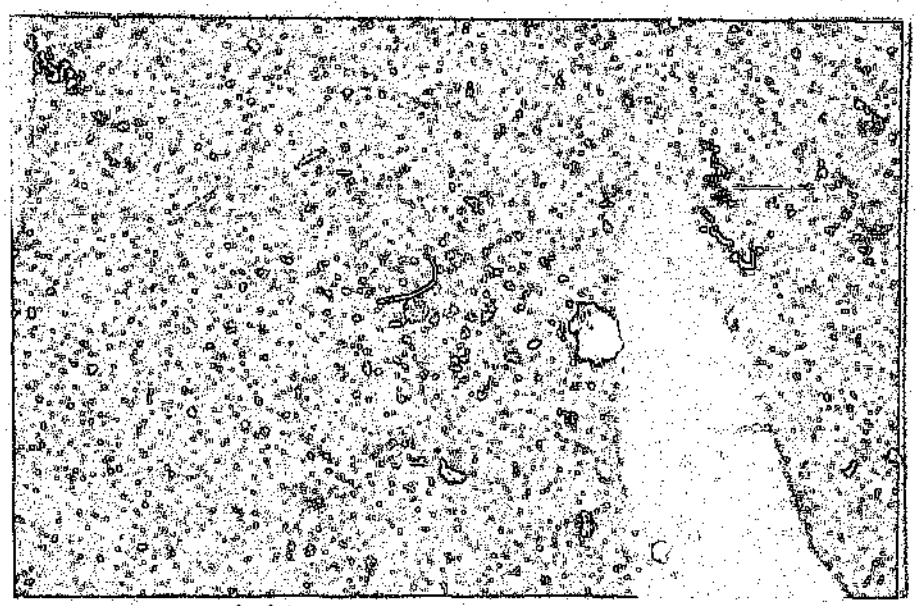


FIGURE V.5 (b)

FIGURE V.5

The anterior aspect of the left calf of patient Miss V. grafted with a cultured epidermal autograft. Figure V.5 (a) shows the area grafted following a sloughectomy. Figure V.5 (b) taken 7 days after grafting, reveals islands of newly formed skin. These islands appear to be predominantly located close to the staples. This could be due to the CEA being more securely attached to the grafted bed in these regions.

FIGURE V.6 (a)

FIGURE V.6 (b)

FIGURE V.6

The posterior aspect of the left calf of patient Miss V. grafted with a compound graft. Figure V.6 (a) was taken just after grafting. To the open areas cultured epidermal autografts were applied. Figure V.6 (b) reveals new skin formation 12 days after grafting. 100% take is seen in the area to which the compound graft was applied. 65% of the area covered by the CEA shows visible skin formation.

APPENDIX VI

Data sheets and figures of patients with leg skin defects treated with cultured epidermal allografts. The data sheets were used to obtain tables 7-14 and graphs 7a, 7b, 8 and 9. The figures aim to illustrate the progress of healing for each patient referred to on the accompanying data sheet.

PATIENT: Mrs E M : 80 years (Black female patient)

AETIOLOGY OF LEG ULCER: venous hypertension

DURATION OF LEG ULCER: 25 years

DIMENSIONS OF LEG ULCER: Two ulcers on medial aspect of right lower leg measuring 10cm by 4cm ($40cm^2$) and 4cm by 4cm ($16cm^2$)

PREVIOUS MODALITIES OF TREATMENT;
CONSERVATIVE: Betadine dressings - daily

SURGICAL-SSG: nil

TIME OF CULTURED EPIDERMAL ALLOGRAFT APPLICATION: 9/7/90

NUMBER OF APPLICATIONS OF CEA (DISTINGUISHING BETWEEN 'GOOD' AND 'POOR' QUALITY CEA): A single application of a 'good quality' graft.

PERCENTAGE TAKE AFTER EACH CEA APPLICATION: 16 days after CEA application the two ulcers decreased in size to $4cm^2$ (90% 'take') and $2cm^2$ (87.5%'take') respectively.

NUMBER OF DAYS FOR COMPLETE HEALING TO OCCUR: The patient was lost to follow-up.

CELL LINE USED: Hamilton

FOLLOW-UP: The patient was lost to follow-up.

BIOPSY TAKEN: Yes, 8 days after CEA was applied.

FIGURE VI.1 (a)

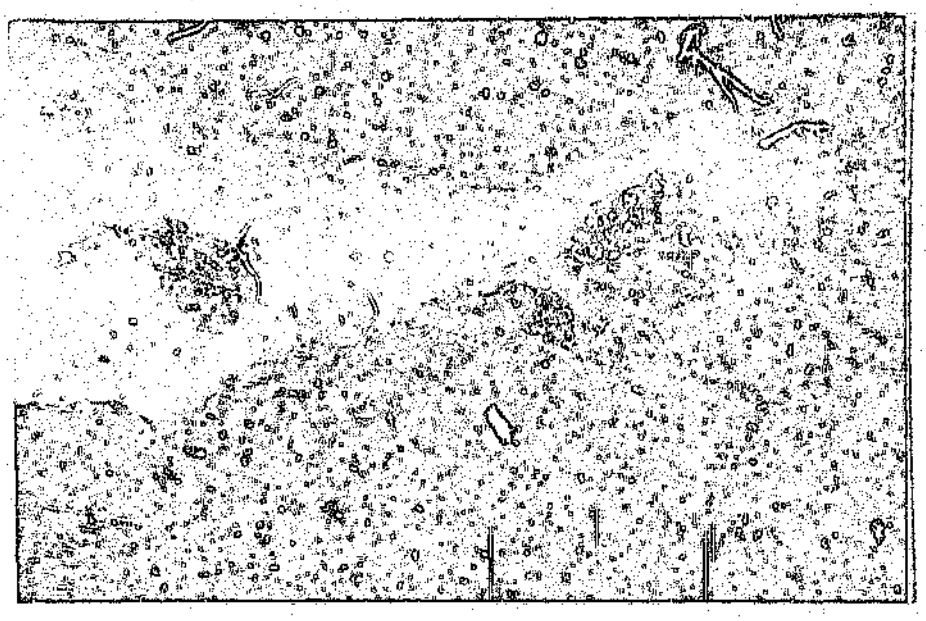


FIGURE VI.1 (b)

FIGURE VI.1

Venous hypertensive ulcers present on the medial aspect of the right leg of patient Mrs E.M.. Figure VI.1 (a) The leg ulcer before CEA application. Figure VI.1 (b) 16 days later reveals a smaller ulcer.

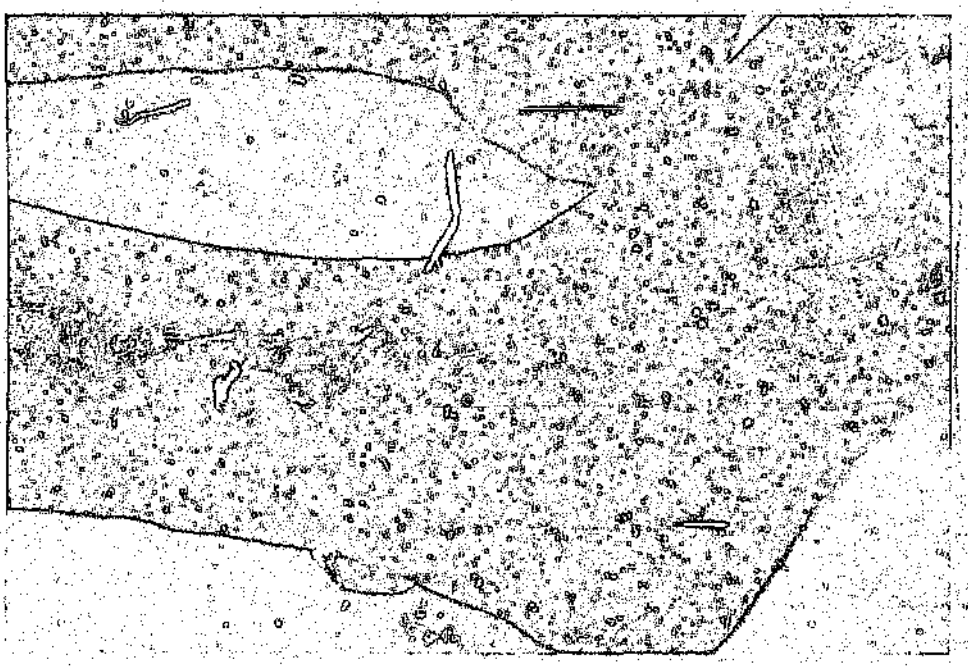


FIGURE VI.1 (a)

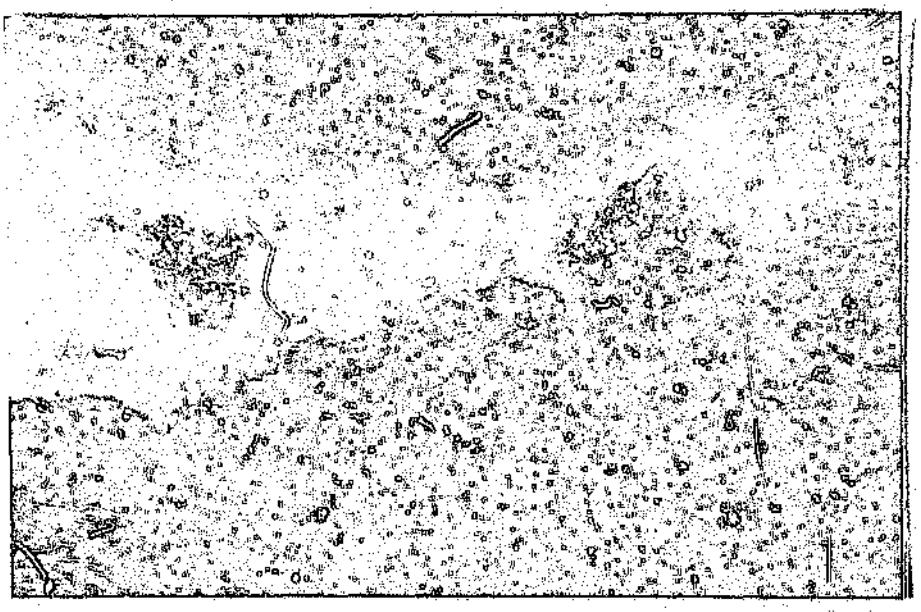


FIGURE VI.1 (b)

FIGURE VI.1

Venous hypertensive ulcers present on the medial aspect of the right leg of patient Mrs E.M.. Figure VI.1 (a) The leg ulcer before CEA application. Figure VI.1 (b) 16 days later reveals a smaller ulcer.

PATIENT: Mr W M : 56 years (Black male patient)

AETIOLOGY OF LEG ULCER: venous hypertension

DURATION OF LEG ULCER: 9 months

DIMENSIONS OF LEG ULCER: Left leg, medial aspect - 4cm by 4cm ($16cm^2$)

PREVIOUS MODALITIES OF TREATMENT;

CONSERVATIVE: Daily betadine dressings, continuously for the last 2 months.

SURGICAL-SSG: nil

TIME OF CULTURED EPIDERMAL ALLOGRAFT APPLICATION: 9 months after first appearance of ulcer

NUMBER OF APPLICATIONS OF CEA (DISTINGUISHING BETWEEN 'GOOD' AND 'POOR' QUALITY CEA): Single application of a 'poor quality' graft

PERCENTAGE TAKE AFTER EACH CEA APPLICATION: 100%

NUMBER OF DAYS FOR COMPLETE HEALING TO OCCUR: 7 days

FOLLOW-UP: To date the ulcer has remained healed

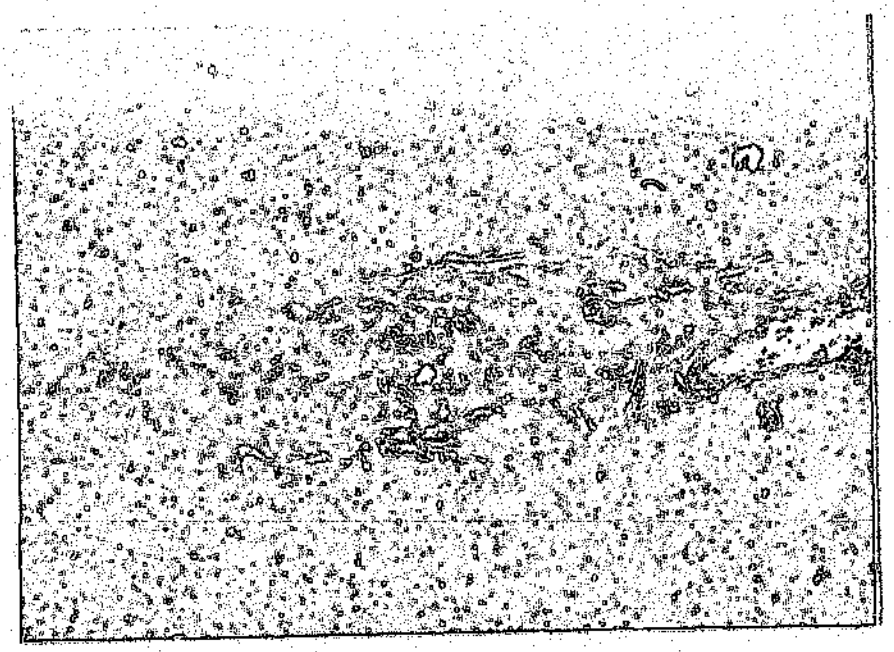


FIGURE VI.2 (a)

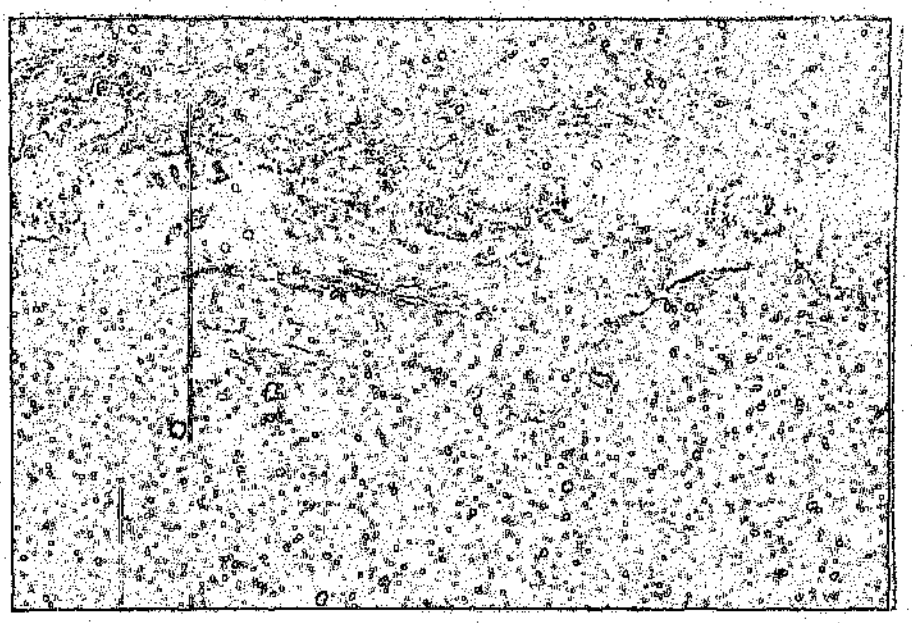


FIGURE VI.2 (b)

FIGURE VI.2

The medial aspect of the left leg of patient Mr W.N. shows a venous hypertensive ulcer.
Figure VI.2 (a) shows the ulcer prior to CEA application.
Figure VI.2 (b) shows the healed ulcer 7 days later. The scab over the central part of the healed ulcer will fall of in time.

PATIENT: Mrs N ; 65 years (European female patient)

AETIOLOGY OF LEG ULCER: venous hypertension

DURATION OF LEG ULCER: 6 months

DIMENSIONS OF LEG ULCER: 2cm by 2cm

PREVIOUS MODALITIES OF TREATMENT;
CONSERVATIVE: Intermitant betadine dressings

SURGICAL-SSG: Single application - but did not 'take'.

TIME OF CULTURED EPIDERMAL ALLOGRAFT APPLICATION: 6 months after ulcer first arose

NUMBER OF APPLICATIONS OF CEA (DISTINGUISHING BETWEEN 'GOOD' AND 'POOR' QUALITY CEA): One 'good' quality CEA.

CELL LINE ORIGIN: Dulfred - 18 year old foreskin

PERCENTAGE TAKE AFTER EACH CEA APPLICATION: 100% take within 3 days.

NUMBER OF DAYS FOR COMPLETE HEALING TO OCCUR: 3 days

FOLLOW-UP: Patient lost to follow-up

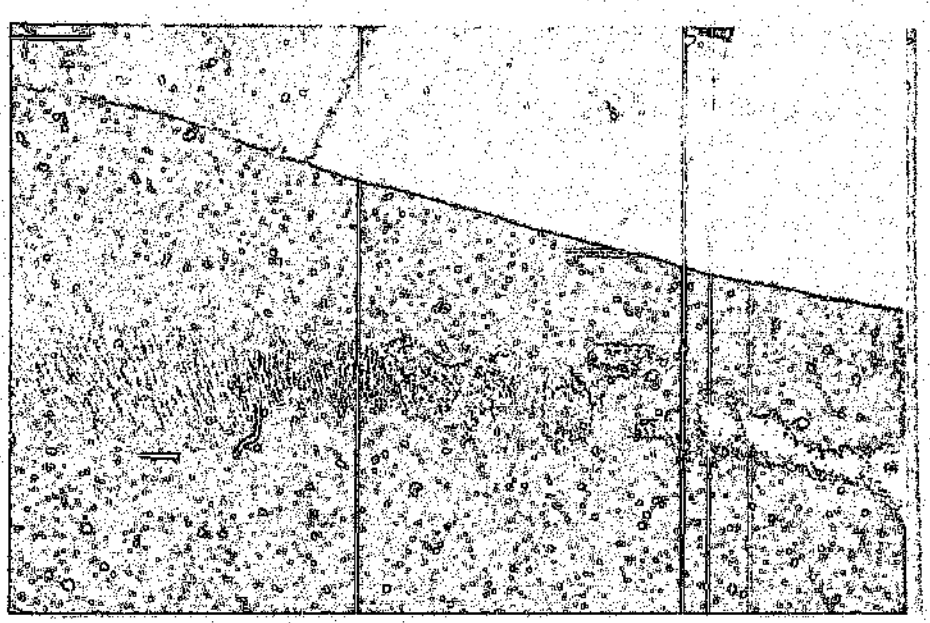


FIGURE VI.3 (a)

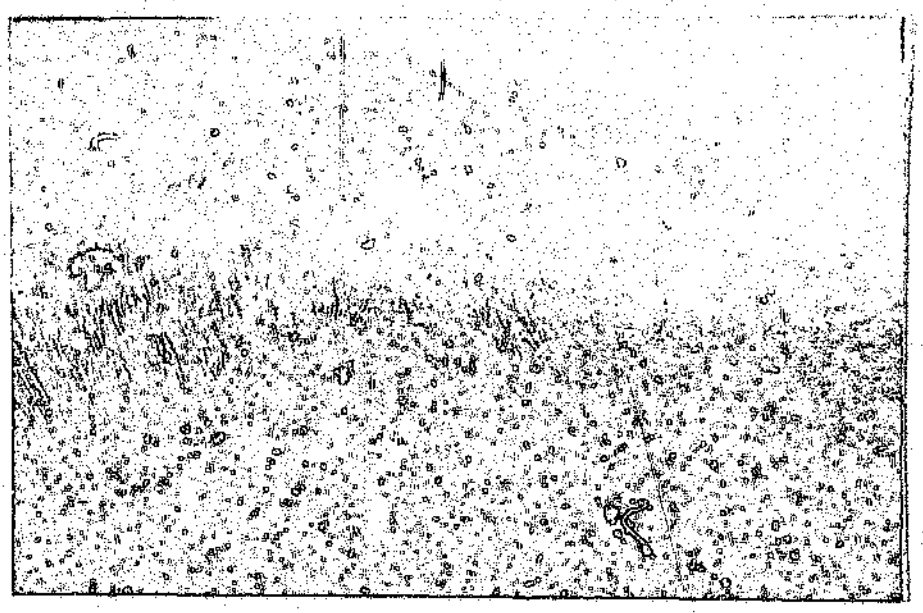


FIGURE VI.3 (b)

FIGURE VI.3

Two ulcers on the medial aspect of the left leg of patient Mrs N. treated with CEAs.
Figure VI.3 (a) shows the ulcers prior to CEA application. Cellulitis is also present.
Figure VI.3 (b) shows the healed ulcers 3 days after CEA application.

PATIENT: Mrs M : 52 years (Black female patient)

AETIOLOGY OF LEG ULCER: Arterial insufficiency Present on lateral aspect of right calf.

DURATION OF LEG ULCER: 2 months

DIMENSIONS OF LEG ULCER: Length - 9cm
Breadth - 7cm, narrowest part of breadth - 2cm

PREVIOUS MODALITIES OF TREATMENT;
CONSERVATIVE: 10 days of Betadine dressings, three times a day.
No visible healing noted.

SURGICAL-SSG: nil

TIME OF CULTURED EPIDERMAL ALLOGRAFT APPLICATION: 10 days thorough conservative management.

NUMBER OF APPLICATIONS OF CEA (DISTINGUISHING BETWEEN 'GOOD' AND 'POOR' QUALITY CEA): Three 'good' quality grafts
Second CEA applied 3 days after first CEA.
Third CEA applied 7 days after second CEA.

PERCENTAGE TAKE AFTER EACH CEA APPLICATION: First-CEA- three days after application 45% of area grafted showed new skin formation. Three patches of new skin seen as well as 0.5cm margin present.
Second CEA-10 days after initial CEA, 80% of ulcer healed.

NUMBER OF DAYS FOR COMPLETE HEALING TO OCCUR: 27 days

FOLLOW-UP: To date ulcer has remained healed.

BIOPSY TAKEN: Yes, on day 8 post first CEA application.

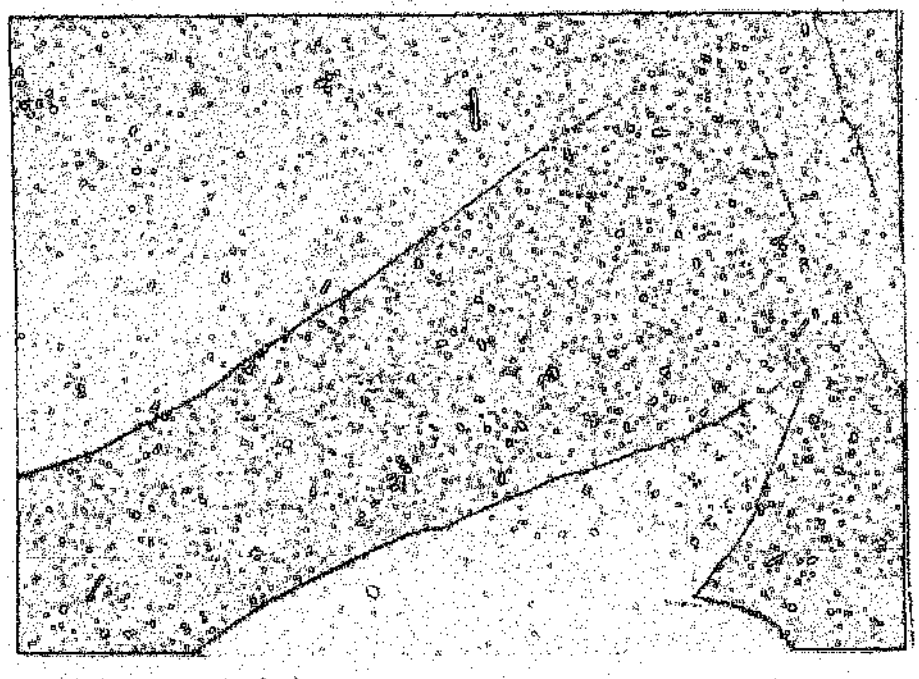


FIGURE VI.4 (a)

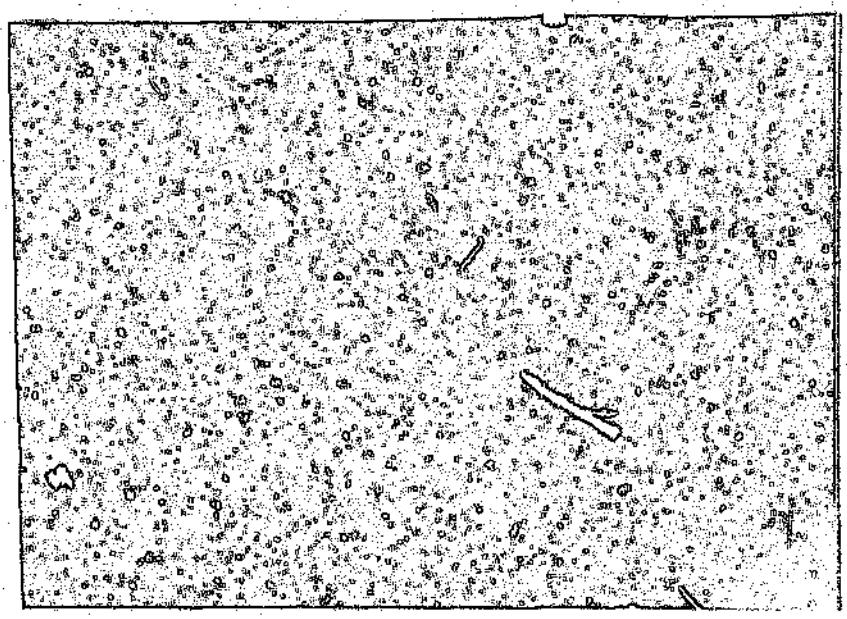


FIGURE VI.4 (b)

FIGURE VI.4

The healing arterial leg ulcer of patient Mrs M.
Figure VI.4 (a) 7 days after second CEA application and 10 days after first CEA application. The ulcer has decreased in size and the lower part has healed completely.
Figure VI.4 (b) is a close-up view of the healed lower part of the ulcer.

PATIENT: Mrs S : 75 years (European female patient)

AETIOLOGY OF LEG ULCER: venous hypertension

DURATION OF LEG ULCER: 6 months

DIMENSIONS OF LEG ULCER: Length - 9cm
Breadth - 6cm, at narrowest point - 2cm

PREVIOUS MODALITIES OF TREATMENT:
CONSERVATIVE: Betadine dressings applied three times a a day for 6 months.

SURGICAL-SSG: nil

TIME OF CULTURED EPIDERMAL ALLOGRAFT APPLICATION: 6 months after ulcer first appeared.
Second CEA applied 15 days after first CEA.

NUMBER OF APPLICATIONS OF CEA (DISTINGUISHING BETWEEN 'GOOD' AND 'POOR' QUALITY CEA): Two 'good' quality grafts applied.

CELL LINE ORIGIN: Dulfred - 18 year old foreskin - first CEA.
Tim - 8 day old foreskin - second CEA

PERCENTAGE TAKE AFTER EACH CEA APPLICATION: 50% 'take' 15 days after first CEA applied.
90% 'take' of original area grafted 13 days after second CEA application.

NUMBER OF DAYS FOR COMPLETE HEALING TO OCCUR: 30 days after first CEA application

FOLLOW-UP: The ulcer has remained healed for up to one year.

BIOPSY TAKEN: Yes, 8 days after first CEA was applied.

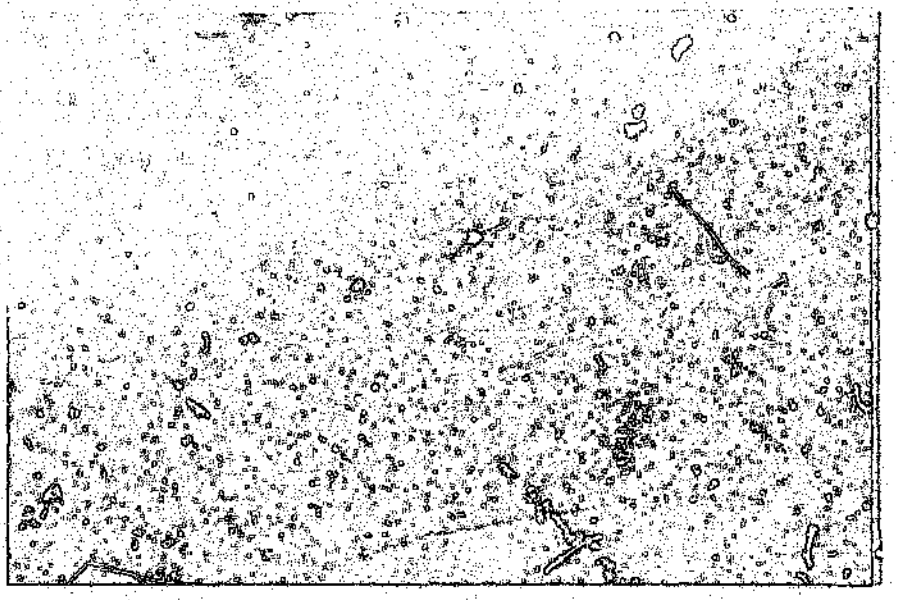


FIGURE VI.5

A venous hypertensive ulcer on the medial aspect of the left leg on the patient Mrs S.. 16 days after grafting with a CEA reveals a healing ulcer.

PATIENT: Mr E : 50 years (European male patient)

AETIOLOGY OF LEG ULCER: Venous hypertension
This venous stasis ulcer was present on the medial aspect of left leg just above the medial malleolus.

DURATION OF LEG ULCER: 15 months

DIMENSIONS OF LEG ULCER: Length - 11cm
Breadth - 4cm

PREVIOUS MODALITIES OF TREATMENT:
CONSERVATIVE: 9 weeks of Betadine dressings twice a day.

SURGICAL-SSG: Previous application of SSG 12 weeks previously resulted in partial healing but subsequent break down.

TIME OF CULTURED EPIDERMAL ALLOGRAFT APPLICATION: 9 weeks after complete break down following SSG application.

NUMBER OF APPLICATIONS OF CEA (DISTINGUISHING BETWEEN 'GOOD' AND 'POOR' QUALITY CEA): Single application of a 'good' quality CEA.

CELL LINE ORIGIN: Br-derived rom auricle of a 27 year old male.

PERCENTAGE TAKE AFTER EACH CEA APPLICATION: 100% 'take' 18 days after CEA application.

NUMBER OF DAYS FOR COMPLETE HEALING TO OCCUR: 18 days

FOLLOW-UP: To date the ulcer has remained healed.

PATIENT: Mr S : 50 years (European male patient)

AETIOLOGY OF LEG ULCER: Syphilis

DURATION OF LEG ULCER: 5 years

DIMENSIONS OF LEG ULCER: 4cm by 4cm

PREVIOUS MODALITIES OF TREATMENT;
CONSERVATIVE: Daily Betadine dressings

SURGICAL-SSG: Single application of SSG 12, which had sloughed

TIME OF CULTURED EPIDERMAL ALLOGRAFT APPLICATION: 1 week after SSG attempted.

NUMBER OF APPLICATIONS OF CEA (DISTINGUISHING BETWEEN 'GOOD' AND 'POOR' QUALITY CEA): Single application of a 'good' quality CEA.

DRESSING METHOD: Open method using the modified Norwich cage.
This enables one to visualise the progress of the area grafted with the CEA.

PERCENTAGE TAKE AFTER EACH CEA APPLICATION: 0%

NUMBER OF DAYS FOR COMPLETE HEALING TO OCCUR: 0 days

FOLLOW-UP: All attempts at healing have failed to date.
The limiting factors being the nature of the ulcer (syphilitic) and the presence of a Methicillin resistant Staphylococcus aureus infection in the ulcer bed.

PATIENT: Mrs R ; 64 years (European female patient)

AETIOLOGY OF LEG ULCER: Diabetic ulcer present on dorsum of left foot just above the lateral three toes.

DURATION OF LEG ULCER: 2.5 months

DIMENSIONS OF LEG ULCER: Length - 6cm
Breadth - 2cm

PREVIOUS MODALITIES OF TREATMENT:
CONSERVATIVE: Daily Betadine dressings for 1.5 months

SURGICAL-SSG: nil

TIME OF CULTURED EPIDERMAL ALLOGRAFT APPLICATION: 2.5 months after ulcer occurrence.

NUMBER OF APPLICATIONS OF CEA (DISTINGUISHING BETWEEN 'GOOD' AND 'POOR' QUALITY CEA): Two 'good' quality CEA applied.
The second CEA applied three days after first CEA.

PERCENTAGE TAKE AFTER EACH CEA APPLICATION: Three days after first CEA applied no new skin formation seen. Two days after second CEA applied a margin of new skin seen.

NUMBER OF DAYS FOR COMPLETE HEALING TO OCCUR: 19 days

FOLLOW-UP: The area grafted has remained healed for up to 1 year.

CELL LINE ORIGIN: Neonatal foreskin

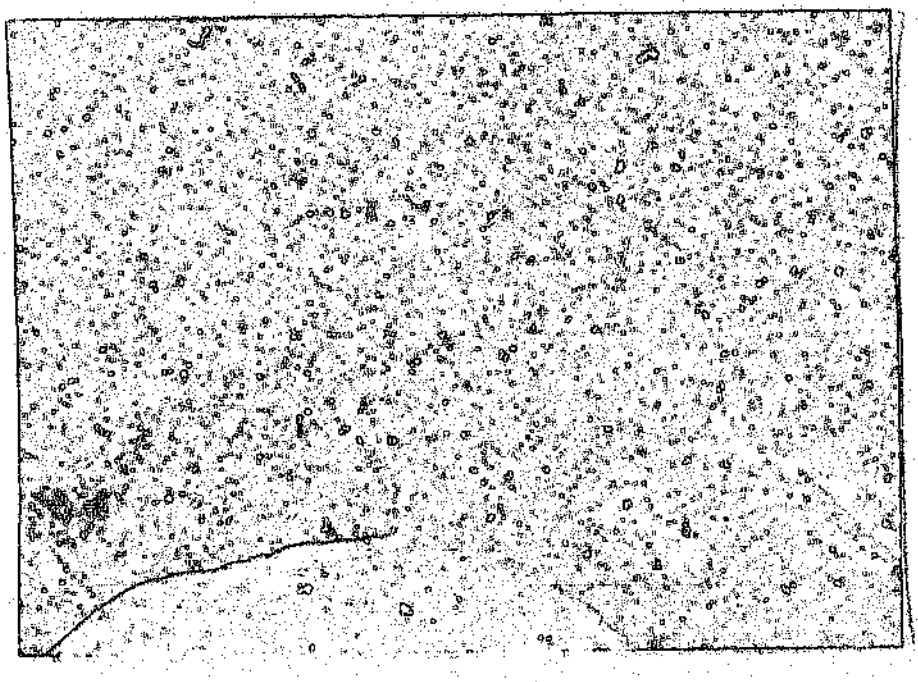


FIGURE VI.6 (a)

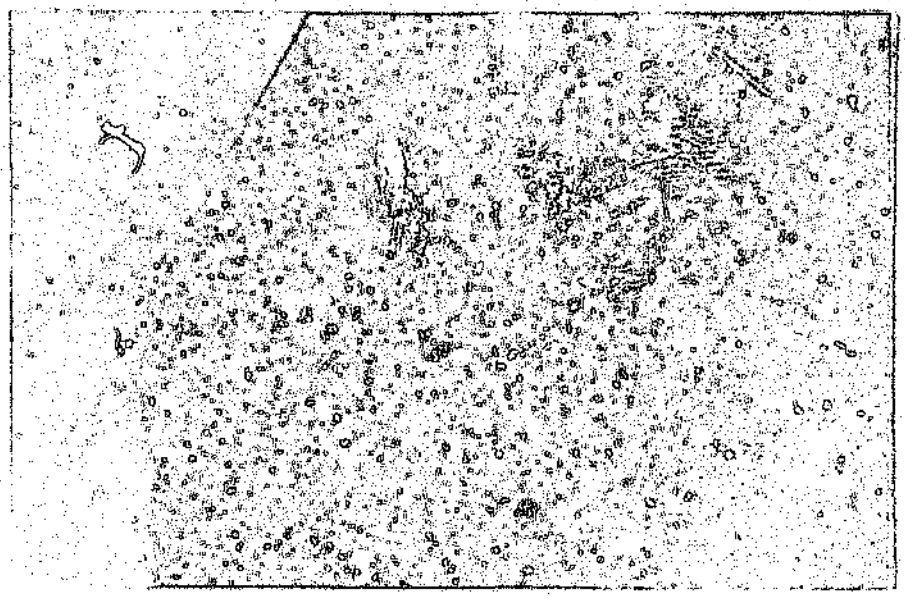


FIGURE VI.6 (b)

FIGURE VI.6

The diabetic ulcer of patient Mrs R. present on the dorsum of the left foot, just proximal the three lateral toes.

Figure VI.6 (a) The ulcer just prior to grafting.

Figure VI.6 (b) The ulcer 4 days after CEA application.

Figure VI.6 (c) 19 days after first CEA application reveals the healed non-infected part of the ulcer. The right unhealed part of the ulcer contains infected necrotic

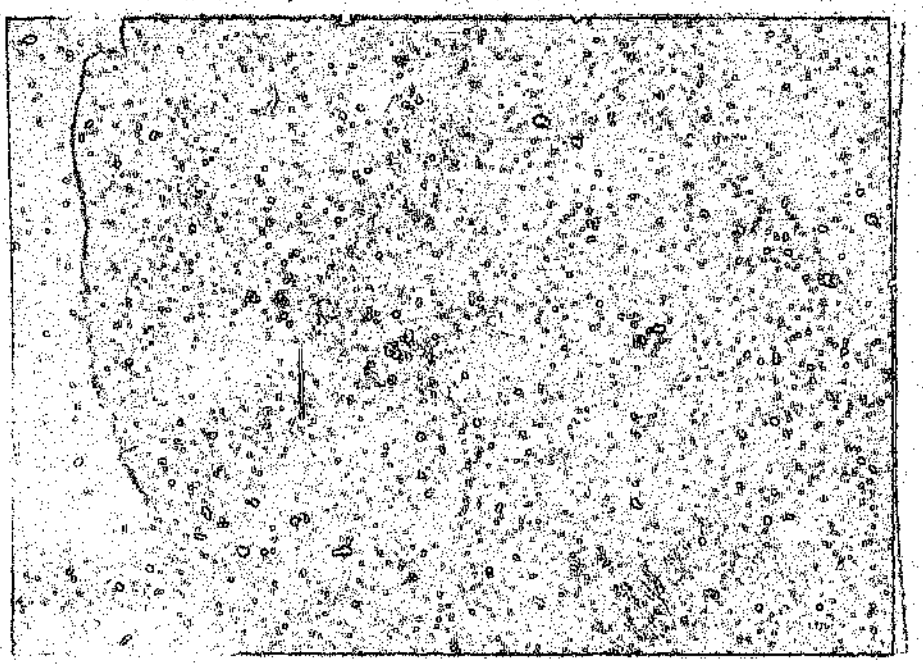


FIGURE VI.6 (c)

PATIENT: Miss D M : 32 years (Black female patient)

AETIOLOGY OF LEG ULCER: Skin loss just below the knee due to an accident which was followed by cellulitis and abscess formation.
Drainage procedure resulted in skin loss.

DURATION OF LEG ULCER: 39 days

DIMENSIONS OF LEG ULCER: 12 cm^2 by 5cm^2 (60cm^2)

PREVIOUS MODALITIES OF TREATMENT;
CONSERVATIVE: Daily Betadine dressings

SURGICAL-SSG: nil

TIME OF CULTURED EPIDERMAL ALLOGRAFT APPLICATION: First and second CEA applied at 39 and 41 days respectively.

NUMBER OF APPLICATIONS OF CEA (DISTINGUISHING BETWEEN 'GOOD' AND 'POOR' QUALITY CEA): Half the skin defect was grafted with a 'poor' quality CEA and the other half was grafted with a 'good' quality CEA.
The first type of CEA was applied two days before the second type of CEA.

PERCENTAGE TAKE AFTER EACH CEA APPLICATION: 26 days after the 'poor' quality graft was applied, 80% of this area grafted showed visible skin formation.
11 days after the 'good' quality graft was applied new skin was seen in 90% of this area grafted.

NUMBER OF DAYS FOR COMPLETE HEALING TO OCCUR: Approx. 31 days - according to the patient, who attended the clinic after this date.

FOLLOW-UP: The lesion has remained healed for over 3 years.

BIOPSY TAKEN: Yes, four days after first CEA was applied.

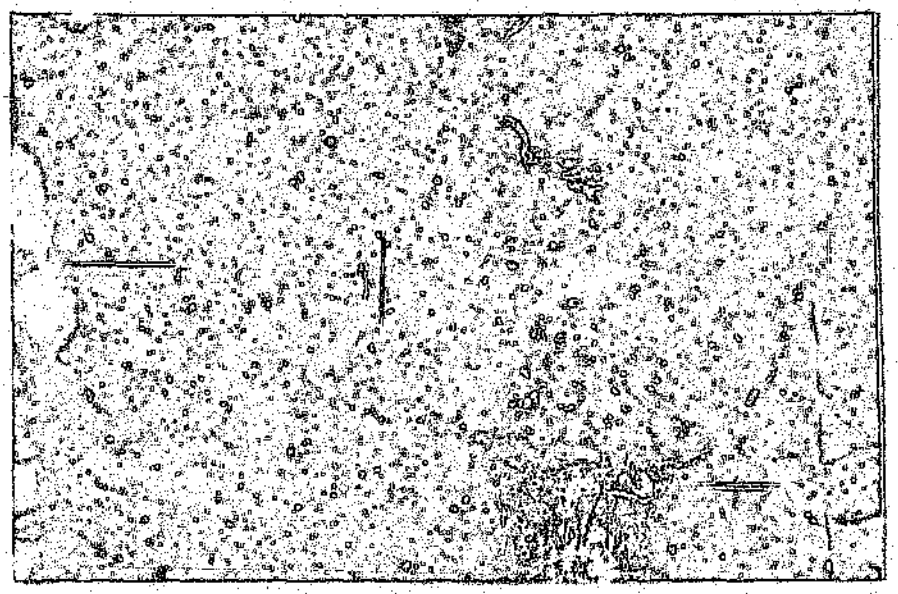


FIGURE VI.7 (a)

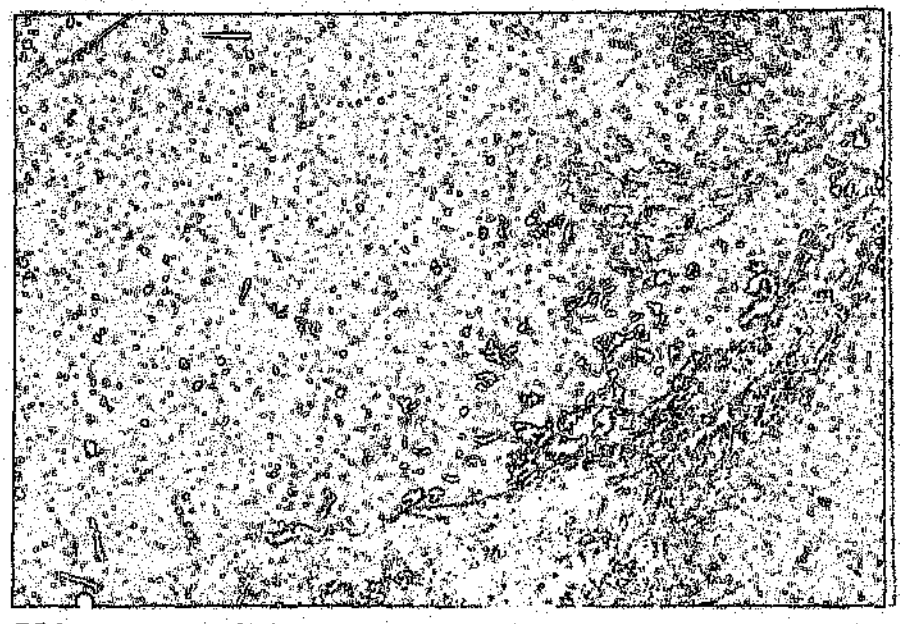


FIGURE VI.7 (b)

FIGURE VI.7

The trauma and cellulitis induced skin defect of patient Miss D.M., present below the right knee. Figure VI.7 (a) The skin defect prior to grafting. Figure VI.7 (b) 4 days after applying a poor quality CEA to the medial half of the defect. Figure VI.7 (c) 26 days after poor quality CEA application and 11 days after good quality CEA application to the respective areas. Figure VI.7 (d) A close-up of (c) showing the healing ulcer. The left side, to which the good quality CEA was applied has healed almost completely. Figure VI.7 (e) The healed leg skin defect. Figure VI.7 (f) A close-up of (e).

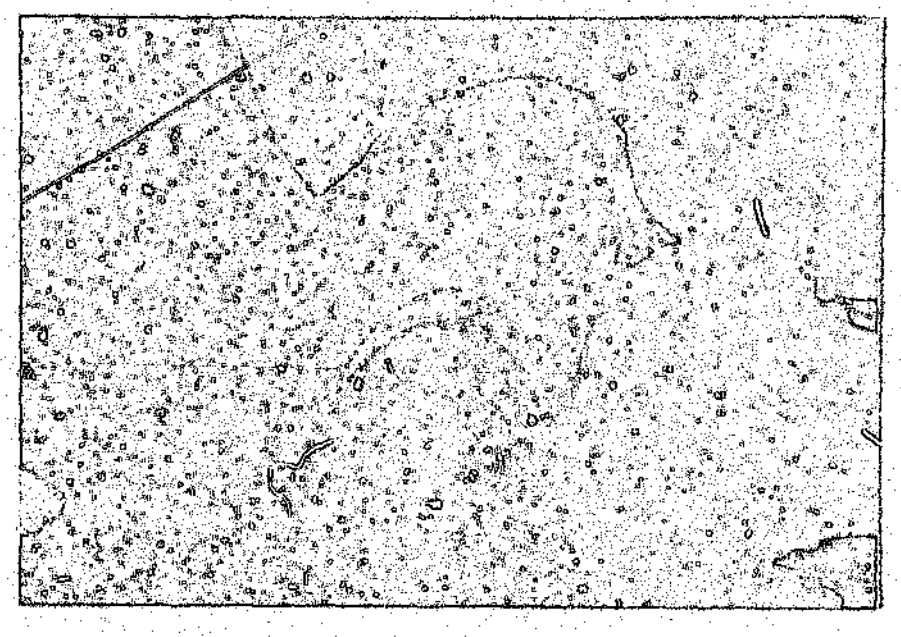


FIGURE VI.7 (c)

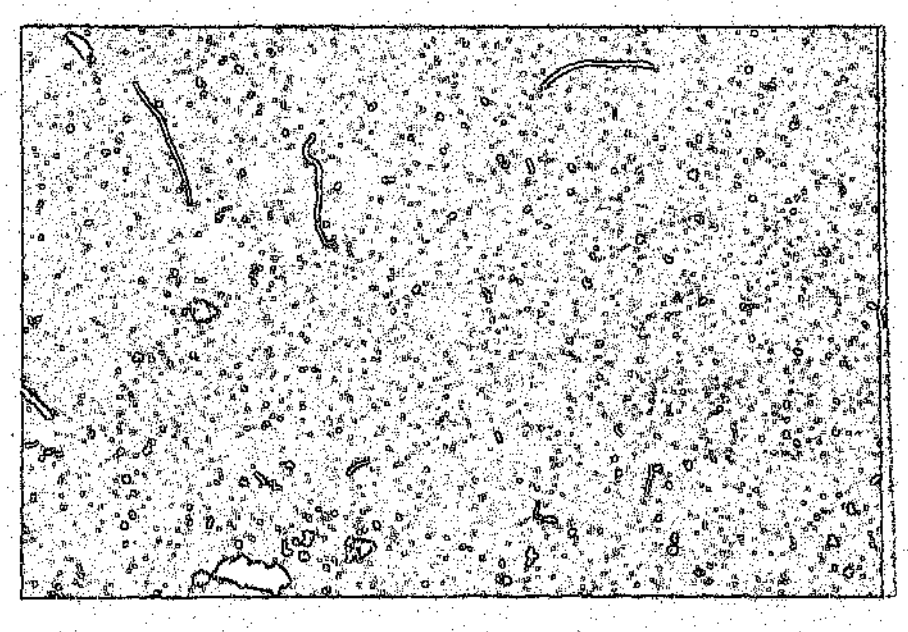


FIGURE VI.7 (d)

FIGURE VI.7 (e)

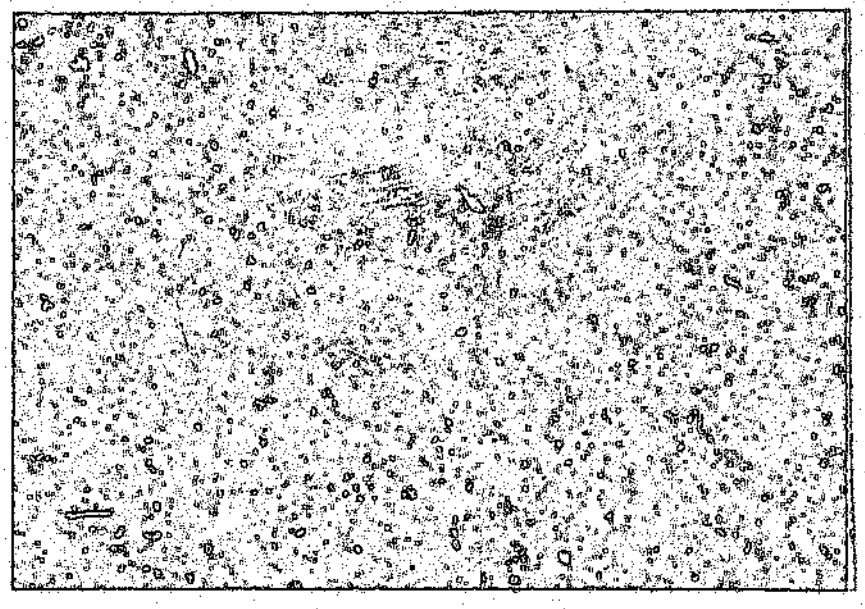


FIGURE VI.7 (f)

PATIENT: Miss G M : 14 years (Black female patient)

AETIOLOGY OF LEG ULCER:	Cellulitis and knee abscess with resulting skin loss following the drainage procedure.
DURATION OF LEG ULCER:	4 weeks
DIMENSIONS OF LEG ULCER:	7 cm by 4 cm (28 cm^2)
PREVIOUS MODALITIES OF TREATMENT:	
CONSERVATIVE:	Daily Betadine dressings
SURGICAL-SSG:	nil
TIME OF CULTURED EPIDERMAL ALLOGRAFT APPLICATION:	First graft applied on 12/6/90 Second graft applied on 4/7/90
NUMBER OF APPLICATIONS OF CEA (DISTINGUISHING BETWEEN 'GOOD' AND 'POOR' QUALITY CEA):	Two 'good' quality grafts applied
PERCENTAGE TAKE AFTER EACH CEA APPLICATION:	67.9% occurring 18 days after first CEA applied. Complete healing occurred 23 days after second CEA application.
NUMBER OF DAYS FOR COMPLETE HEALING TO OCCUR:	41 days after first CEA applied. But 23 days after applying the second CEA.
FOLLOW-UP:	The lesion remained healed to date.
CELL LINE USED:	Ma3
BIOPSY TAKEN:	Yes, five days after first CEA was applied.

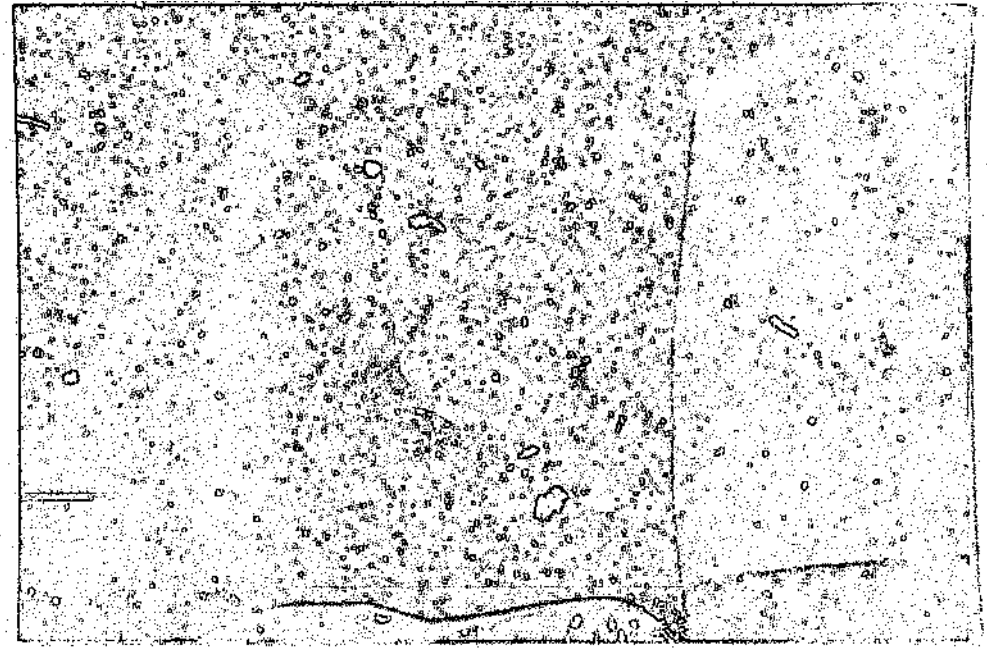


FIGURE VI.8 (a)

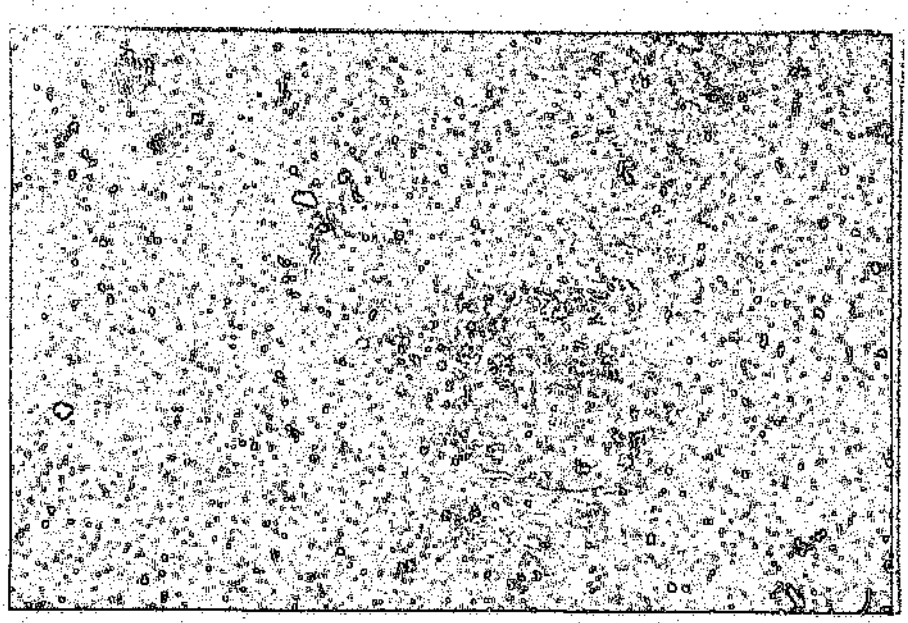


FIGURE VI.8 (b)

FIGURE VI.8

The trauma and cellulitis induced skin defect of Miss G.M. situated below the left knee. Figure VI.8 (a) The skin defect before grafting. Figure VI.8 (b) 18 days after first CEA application reveals a healing margin. Figure vi.8 (c) 25 days after first CEA application shows a healing ulcer but also breakdown in the top right hand corner. Figure VI.8 (d) 41 days after first CEA application reveals a healed ulcer. Intervening sepsis accounted for the relatively long healing time.

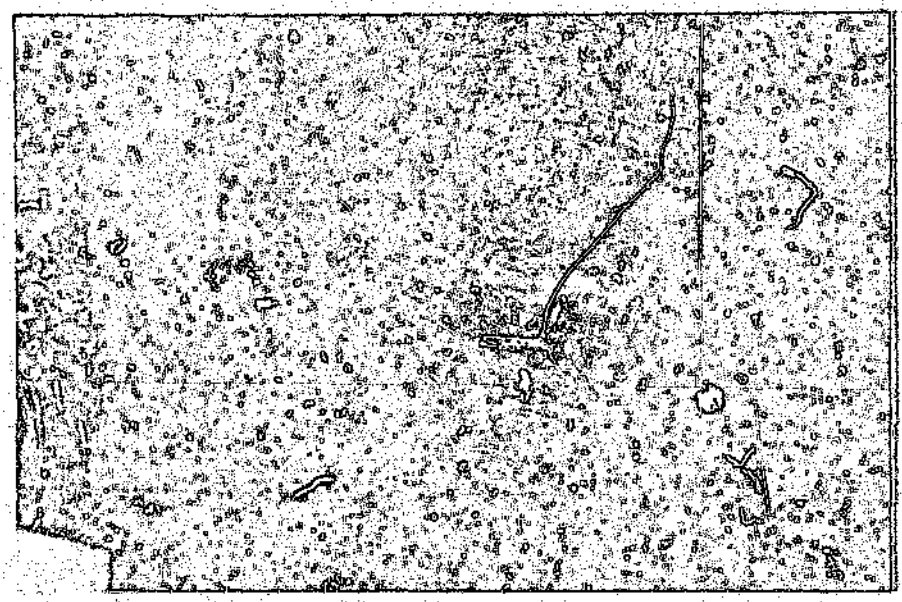


FIGURE VI.8 (c)

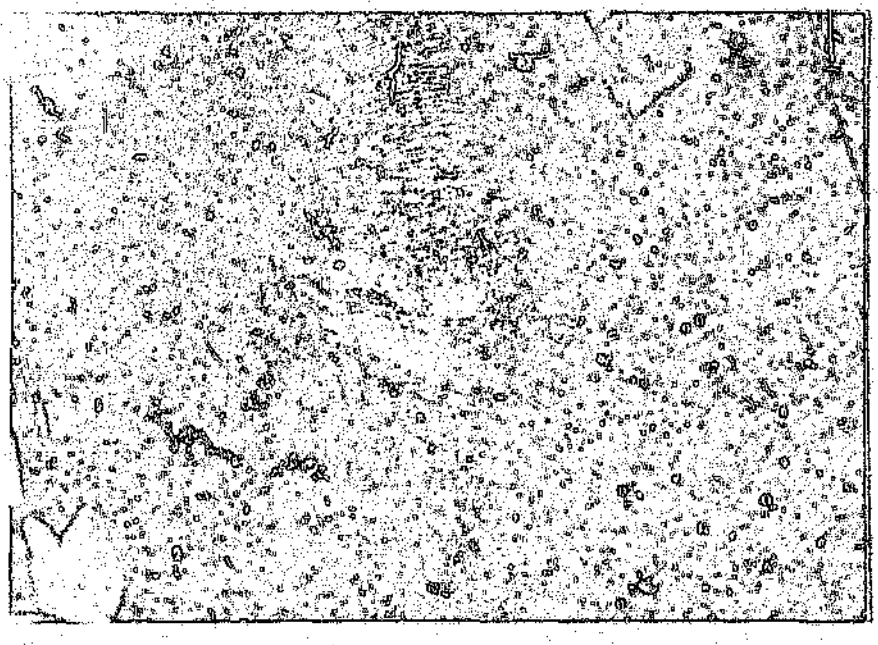


FIGURE VI.8 (d)

PATIENT: Mr H : 70 years (European male patient)

AETIOLOGY OF LEG ULCER:	Fasciotomy induced skin defect on medial aspect of right calf.
DURATION OF LEG ULCER:	4 weeks prior to CEA application.
DIMENSIONS OF LEG ULCER:	Length - 3cm Breadth - 2cm at its maximum extent.
PREVIOUS MODALITIES OF TREATMENT: CONSERVATIVE:	Twice daily Betadine dressings
SURGICAL-SSG:	nil
TIME OF CULTURED EPIDERMAL ALLOGRAFT APPLICATION:	4 weeks after embolectomy.
NUMBER OF APPLICATIONS OF CEA (DISTINGUISHING BETWEEN 'GOOD' AND 'POOR' QUALITY CEA):	Two, both being of 'good' quality.
PERCENTAGE TAKE AFTER EACH CEA APPLICATION:	First CEA-healing margin seen 1 month later, resulting in skin defect being 30% small. Second CEA applied 1 month after the first, revealed in 90% of the original skin defect being healed 12 days later.
NUMBER OF DAYS FOR COMPLETE HEALING TO OCCUR:	15 days from second graft application. 1.5 months from first CEA application.

FOLLOW-UP: To date the lesion has remained healed. With time the new skin and underlying connective tissue strengthened.

PATIENT: Mrs M M : 63 years (Black female patient)

AETIOLOGY OF LEG ULCER: venous hypertension

DURATION OF LEG ULCER: 3 months

DIMENSIONS OF LEG ULCER: 4cm by 3cm, present on the medial aspect of the right lower leg.

PREVIOUS MODALITIES OF TREATMENT;
CONSERVATIVE: Daily betadine dressings applied for 3 months.

SURGICAL-SSG: nil

TIME OF CULTURED EPIDERMAL ALLOGRAFT APPLICATION: 3 months

NUMBER OF APPLICATIONS OF CEA (DISTINGUISHING BETWEEN 'GOOD' AND 'POOR' QUALITY CEA): Twice, 10 days apart. First with a good quality' graft followed by a 'poor quality' graft.

PERCENTAGE TAKE AFTER EACH CEA APPLICATION: After first CEA application, 50% 'take' - 4 days later. After second CEA application, 70% 'take' at 8 days and 97.9% 'take' at 12 days.

NUMBER OF DAYS FOR COMPLETE HEALING TO OCCUR: 28 days (15 days after second CEA application)

FOLLOW-UP: Ulcer has remained healed for over 3 years.

CELL LINE USED: Hamilton - which took 20 days to become confluent and stratified and thus ready to be used as a graft.

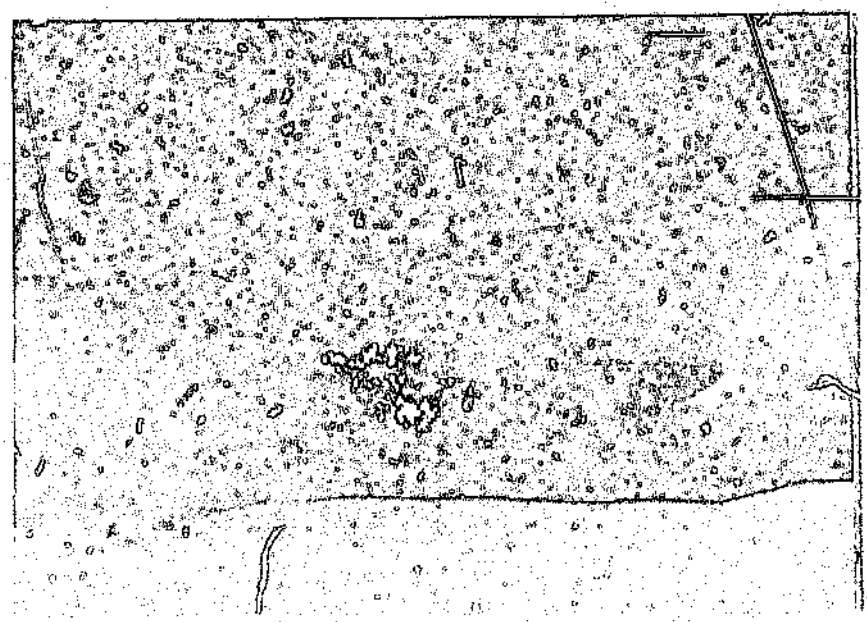


FIGURE VI.9 (a)

FIGURE VI.9 (b)

FIGURE VI.9

A fasciotomy induced skin defect on the medial aspect of the right calf of patient Mr H., which was covered with CEAs.
Figure VI.9 (a) The defect before grafting.
Figure VI.9 (b) The almost completely healed fasciotomy induced skin defect 13 days after the second CEA application.
Figure VI.9 (c) 15 days after second CEA application reveals a healed fasciotomy skin defect.

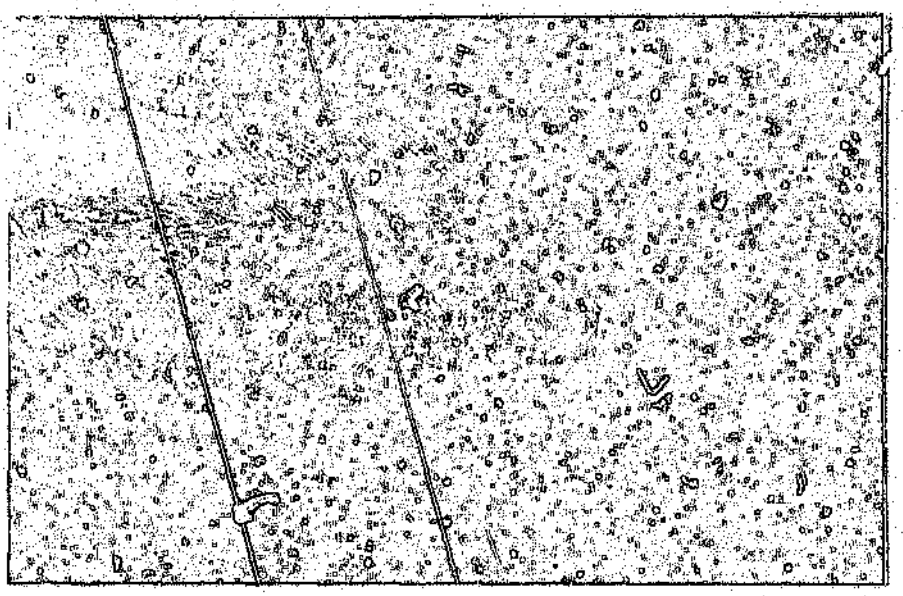


FIGURE VI.9 (c)

PATIENT: Mr J : 34 years (Black male patient)

AETIOLOGY OF LEG ULCER: Fasciotomy induced skin defect

DURATION OF LEG ULCER: 56 days

DIMENSIONS OF LEG ULCER: Two fasciotomy induced skin defects
- Left Thigh 0.5cm by 7cm = 3.5cm^2
Left calf - 3cm by 9cm = 27cm^2

PREVIOUS MODALITIES OF TREATMENT;
CONSERVATIVE: Daily Betadine dressing for 56 days

SURGICAL-SSG: nil

TIME OF CULTURED EPIDERMAL ALLOGRAFT APPLICATION: 57 days

NUMBER OF APPLICATIONS OF CEA (DISTINGUISHING BETWEEN 'GOOD' AND 'POOR' QUALITY CEA): A single application of a 'poor quality' CEA.

PERCENTAGE TAKE AFTER EACH CEA APPLICATION: Left thigh - complete 'take' 3 days following application.
Left calf - 90% 'take' 11 days following application.

NUMBER OF DAYS FOR COMPLETE HEALING TO OCCUR: Left thigh - 3 days
Left calf - the patient did not return for follow-up.

FOLLOW-UP: The patient did not return for follow-up.

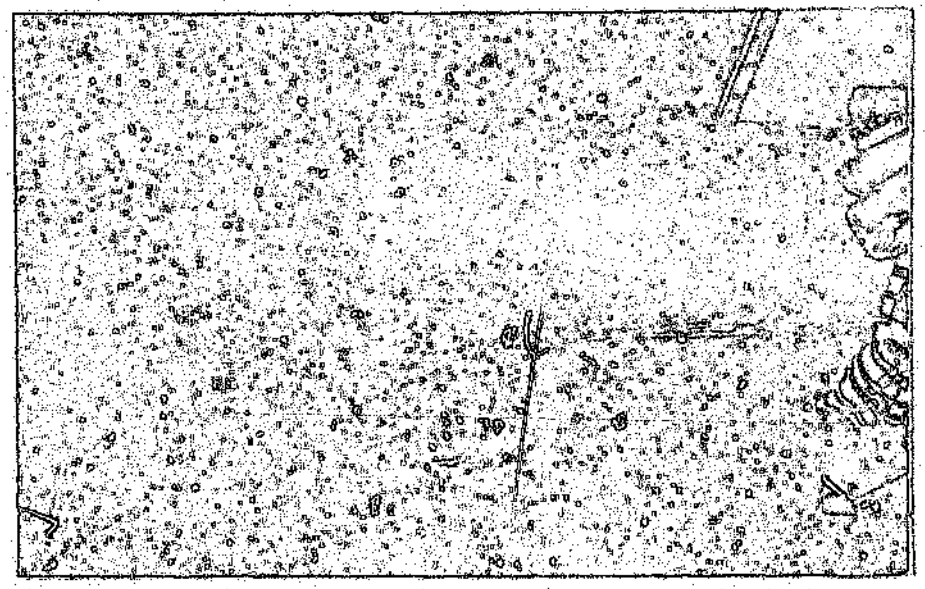


FIGURE VI.10 (a)

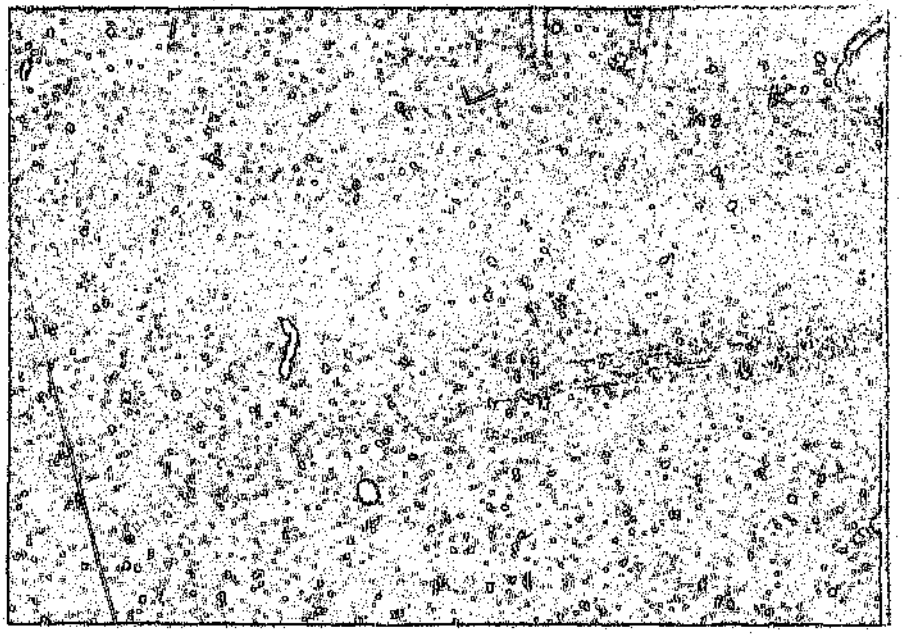


FIGURE VI.10 (b)

FIGURE VI.10

A fasciotomy induced skin defect on the medial aspect of the left leg of patient Mr J. which was grafted with a CEA.

Figure VI.10 (a) The extent of the defect.
Figure VI.10 (b) 3 days following CEA application reveals a closed fasciotomy wound on the thigh.

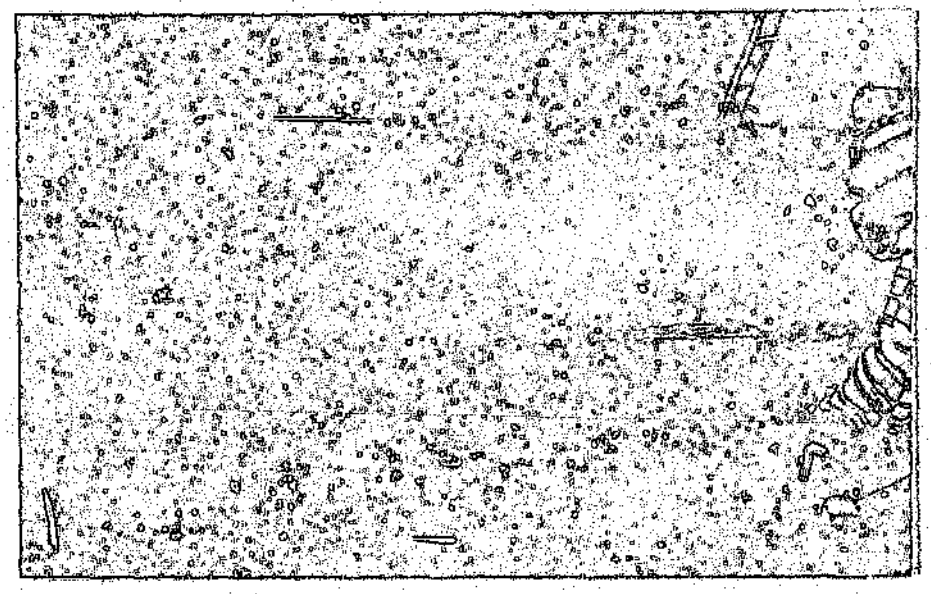


FIGURE VI.10 (a)

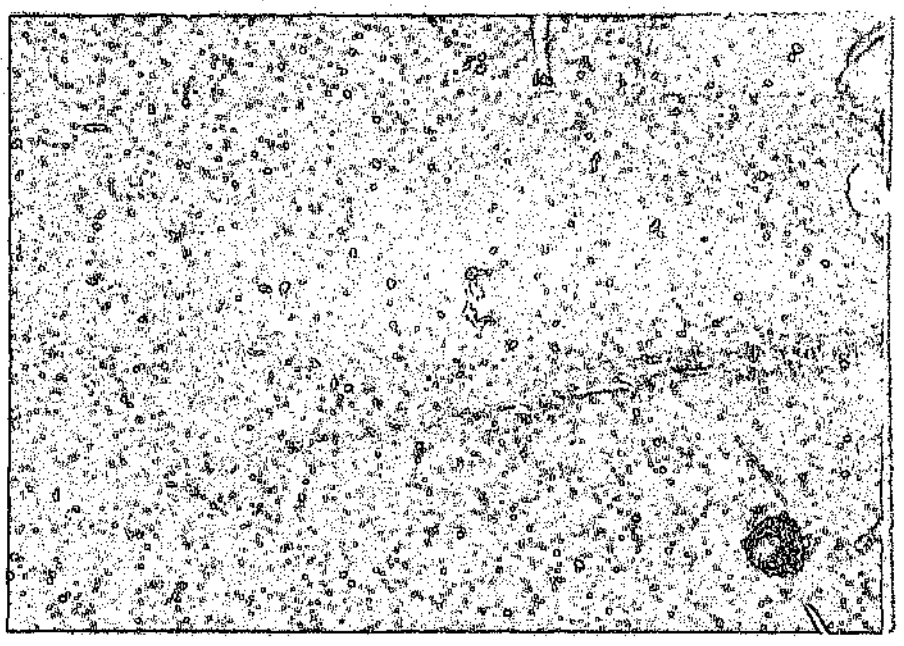


FIGURE VI.10 (b)

FIGURE VI.10

A fasciotomy induced skin defect on the medial aspect of the left leg of patient Mr J. which was grafted with a CEA.

Figure VI.10 (a) The extent of the defect.
Figure VI.10 (b) 3 days following CEA application reveals a closed fasciotomy wound on the thigh.

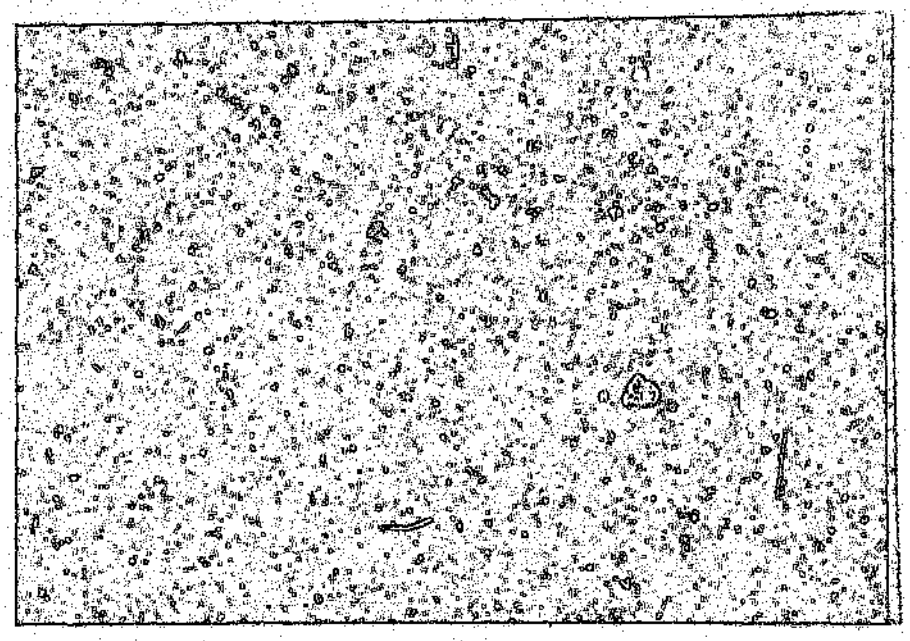


FIGURE VI.10 (c)

11 days after CEA application reveals that the skin defect has healed by 90%.

PATIENT: Mrs L B : 52 years (Black female patient)

AETIOLOGY OF LEG ULCER: Arterial insufficiency

DURATION OF LEG ULCER: 4 years

DIMENSIONS OF LEG ULCER: 2.5cm by 3cm (7.5cm^2)

PREVIOUS MODALITIES OF TREATMENT:
CONSERVATIVE: Daily Betadine dressings for 4 years

SURGICAL-SSG: Nil

TIME OF CULTURED EPIDERMAL ALLOGRAFT APPLICATION: 4 years after ulcer first appeared

NUMBER OF APPLICATIONS OF CEA (DISTINGUISHING BETWEEN 'GOOD' AND 'POOR' QUALITY CEA): Two 'good' quality CEA applied within an interval of 4 days.

PERCENTAGE TAKE AFTER EACH CEA APPLICATION: Four days after the first CEA applied, the ulcer area had decreased in size by 45%.
Five days after application of second CEA (9 days after applying first CEA) 80% of ulcer had healed.

NUMBER OF DAYS FOR COMPLETE HEALING TO OCCUR: 10 days

CELL LINE USED: Mac

BIOPSY TAKEN: Two ; one at 4 days and one at 16 days.

FOLLOW-UP: To date the lesion has remained healed. Prior to CEA application the leg ulcer was painful, but after CEA application the pain disappeared.

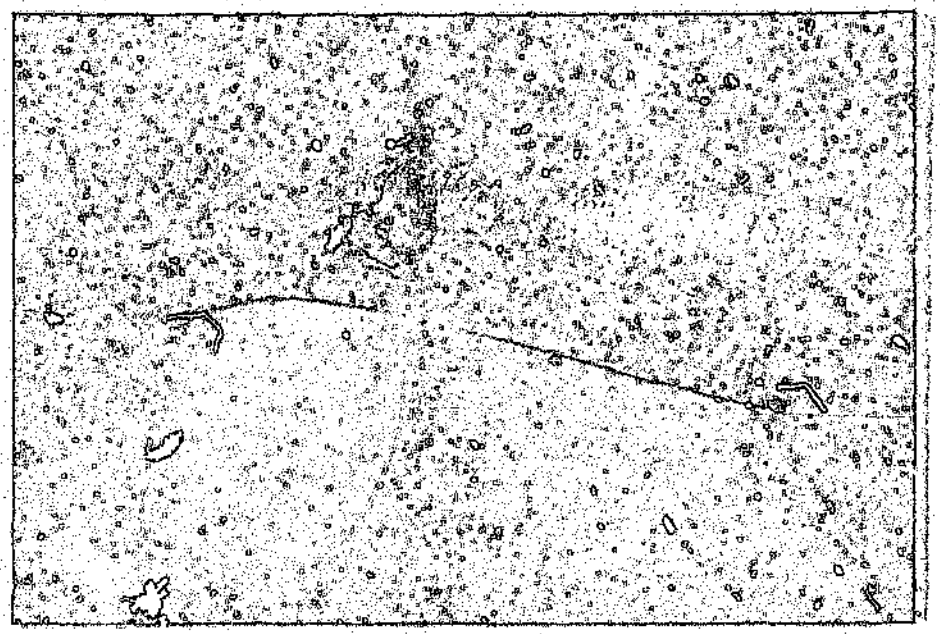


FIGURE VI.11 (a)

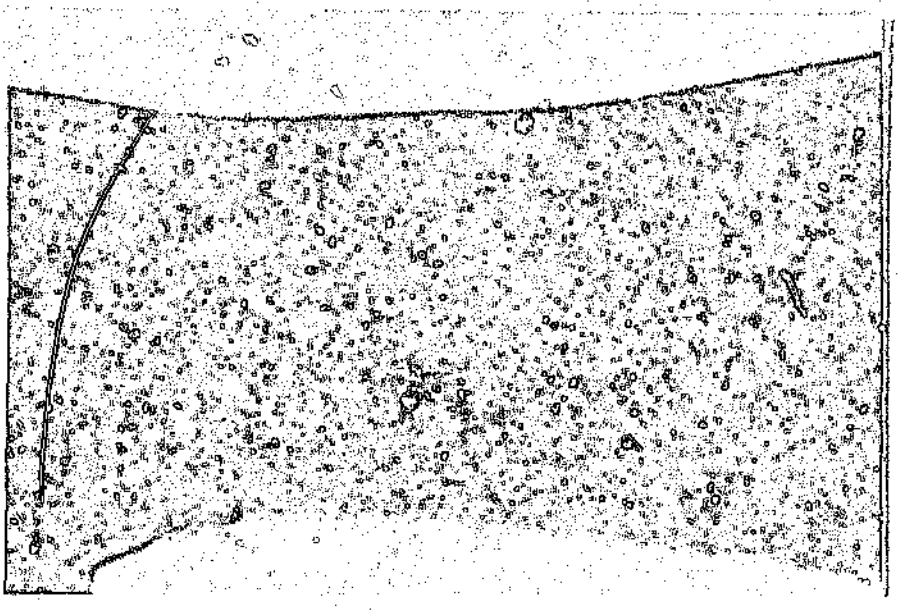


FIGURE VI.11 (b)

FIGURE VI.11

A ulcer caused by arterial insufficiency on the right leg of patient Mrs L.B..
Figure VI.11 (a) The ulcer before first CEA application.
Figure VI.11 (b) 9 days after first CEA application shows 80% healing of the ulcer.
Figure VI.11 (c) A close-up view of fig. VI.11.

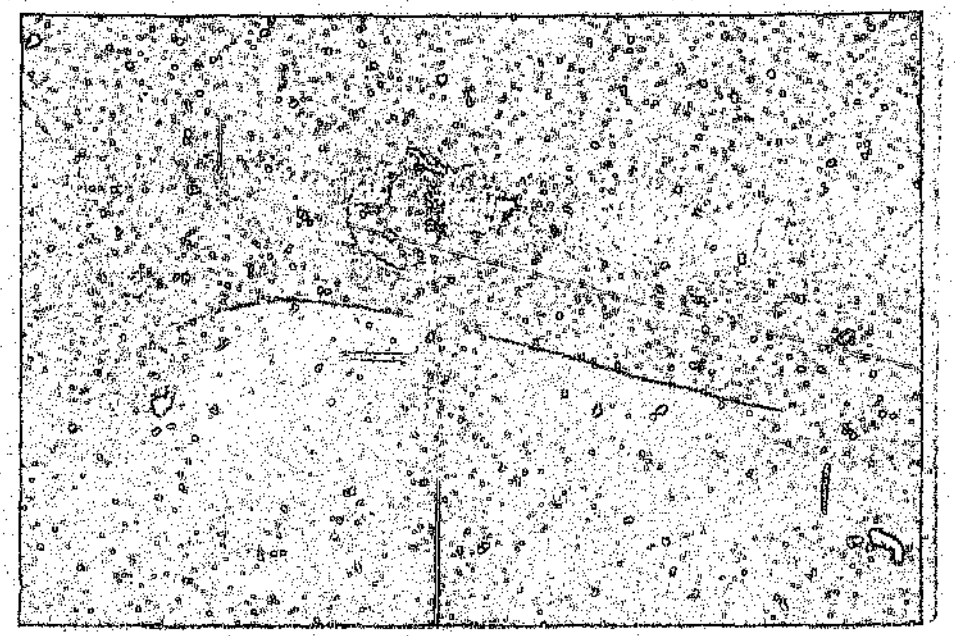


FIGURE VI.11 (a)

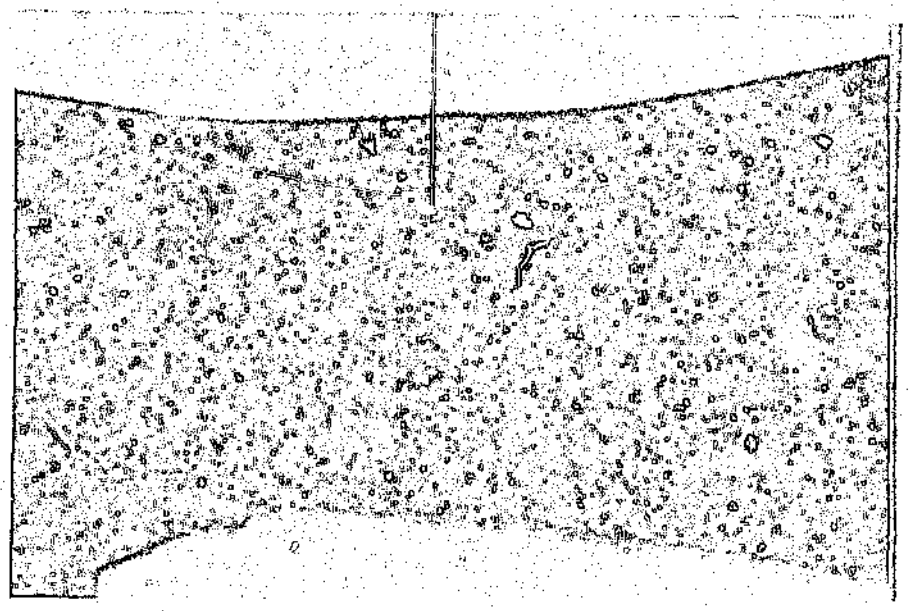


FIGURE VI.11 (b)

FIGURE VI.11

A ulcer caused by arterial insufficiency on the right leg of patient Mrs L.B..
Figure VI.11 (a) The ulcer before first CEA application.
Figure VI.11 (b) 9 days after first CEA application shows 80% healing of the ulcer.
Figure VI.11 (c) A close-up view of fig. VI.11.

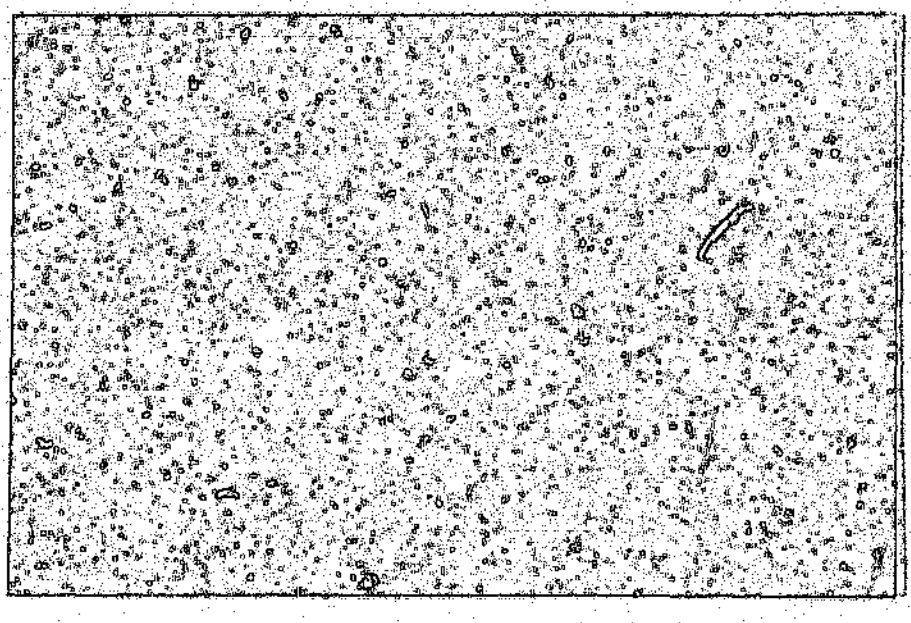


FIGURE VI.11 (c)

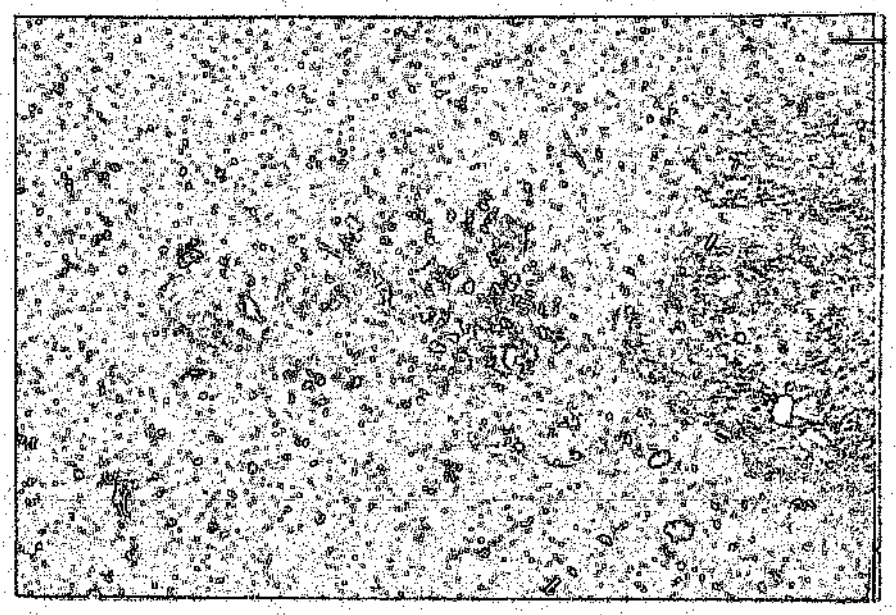


FIGURE VI.12 (a)

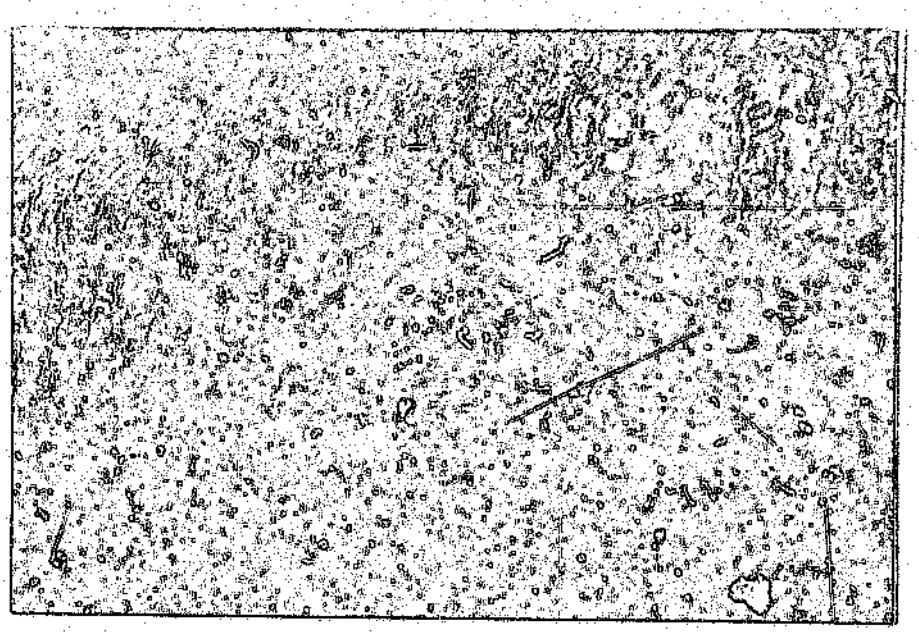


FIGURE VI.12 (b)

FIGURE VI.12

An arterial insufficiency ulcer on the lateral aspect of the right calf of patient Mrs M.M..
Figure VI.12 (a) The ulcer before first CEA application.
Figure VI.12 (b) 4 days after the first CEA was applied reveals new skin formation.
Figure VI.12 (c) 8 days after second CEA application (18 days after first CEA), shows the ulcer well on its way to being healed.

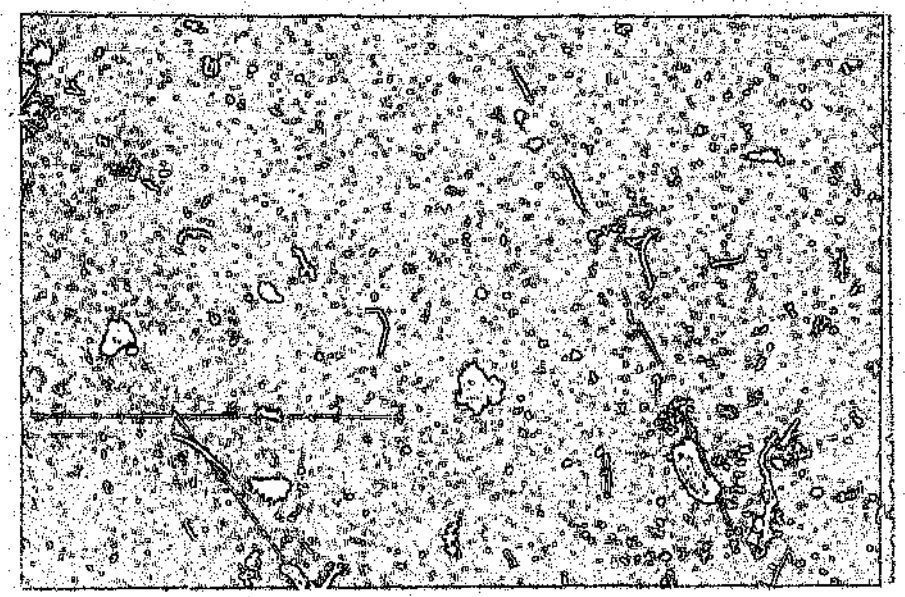


FIGURE VI.12 (c)

APPENDIX VII

Clearance certificate protocol.

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS

CLEARANCE CERTIFICATE

PROTOCOL NO: 54/2/89

PROJECT : HUMAN KERATINOCYTE CULTURE TECHNIQUES USING FOETAL AND ADULT KERATINOCYTES WITH PARTICULAR REFERENCE TO EXTENSIVE BURN WOUNDS AND CHRONIC LEG ULCERS

INVESTIGATOR/S : DR J B BEDER

DEPARTMENT : ZOOLOGY

DATE CONSIDERED : AD HOC

RECOMMENDATION OF COMMITTEE :

NOT APPROVED	
APPROVED	X

subject to the following conditions:

Date : 3/4/89 CHAIRMAN : P E Cleaton-Jones
Professor P E Cleaton-Jones

"INFORMED CONSENT" forms attached - where applicable.
FURTHER "I/C" FORMS AVAILABLE AT FACULTY OFFICE

DECLARATION BY INVESTIGATOR/S

To be completed in duplicate and ONE copy returned to the OFFICE OF THE DEPUTY REGISTRAR (Research), ROOM 10002, 10th Floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorised to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions.

Should any departure be contemplated from the research procedure as approved I/we undertake to resubmit the Protocol to the Committee.

DATE : 24/4/89 SIGNED : J.B. Beder

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
OFFICE OF THE DEPUTY REGISTRAR (Research)

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS

CLEARANCE CERTIFICATE PROTOCOL NO: 54/2/89

PROJECT : HUMAN KERATINOCYTE CULTURE TECHNIQUES USING FOETAL AND ADULT KERATINOCYTES, WITH PARTICULAR REFERENCE TO EXTENSIVE BURN WOUNDS AND CHRONIC LEG ULCERS

INVESTIGATOR/S : DR J B BEDER

DEPARTMENT : ZOOLOGY

DATE CONSIDERED 10 MARCH 1989

RECOMMENDATION OF COMMITTEE :

NOT APPROVED [ ]

APPROVED [X] subject to the following conditions

Date : 13/3/89 CHAIRMAN : .....
Professor P E Cleaton-Jones

"INFORMED CONSENT" forms attached - where applicable.
FURTHER "I/C" FORMS AVAILABLE AT FACULTY OFFICE

DECLARATION BY INVESTIGATOR/S

To be completed in duplicate and ONE copy returned to the OFFICE OF THE DEPUTY REGISTRAR (Research), ROOM 10002, 10th Floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorised to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions.

Should any departure be contemplated from the research procedure as approved I/we undertake to resubmit the Protocol to the Committee.

APPENDIX VIII

An example of results for patients tested for Hepatitis B virus and the HIV.

REPORT/VERSLAG

DEPARTMENT OF NATIONAL HEALTH AND POPULATION DEVELOPMENT
DEPARTEMENT VAN NASIONALE GESONDHEID EN BEVOLKINGSONTWIKKELING

THE NATIONAL INSTITUTE FOR VIROLOGY
DIE NASIONALE INSTITUUT VIR VIROLOGIE

PRIVATE BAG X4 SANDRINGHAM, 2131
PRIVAATSAK X4 SANDRINGHAM, 2131
☎ 640-5031 FAX/FAKS: 485-1410
Tel. Ad.: 'VIRUS', SANDRINGHAM
4-27409 SA

UR REFERENCE :
NS VERWYSING: 890.1378/R06633/B1

DATE OF REPORT/DATUM VAN VERSLAG: 20/11/89

SENDER/AFSENDER:
SUPERINTENDENT
JOHANNESBURG HOSPITAL
PRIVATE BAG X39

JOHANNESBURG
2000

PATIENT/PASIËNT:
LIU H MRS
1038207

WARD/SAAL: JOHANN. BURG
277

IAGNOSIS:
IAGNOSE :

INVESTIGATION REQUESTED/ONDERSOEK AANGEVRA:
HEPATITIS B

PECIMEN/MONSTER: BLOOD

DATE TAKEN/DATUM GENEEM:
16-11-89
DATE RECEIVED/DATUM ONTVANG:
17-11-89

RADIOIMMUNOASSAY FOR
HEPATITIS VIRUS

HEPATITIS B VIRUS
Anti HBc(HBc Antibody).....NEGATIVE

HBs Ag(HBs Antigen).....NEGATIVE

OMMENTS
EPATITIS B SURFACE ANTIBODY TEST WILL BE DONE ON THIS SPECIMEN IF EQUESTED.

DEPT. OF NATIONAL H. & P.D.
AND POPULATION DEVELOPMENT
NATIONAL INSTITUTE FOR VIROLOGY
1989 -11- 20
JOHANNESBURG
NASIONALE INSTITUUT VIR VIROLOGIE
DEPT. VAN NASIONALE GESONDHEID
EN BEVOLKINGSONTWIKKELING

n Verslag in die ander amptelike taal sal verskaf word op versoek.

FOR DIRECTOR/VIR DIREKTEUR
Dr D J Martin

Nº 740237

TH
DIE

CTOR / TER HEAD OF DEPARTMENT
DRESS / RES JOHANNESBURG HOSPITAL

PAT / PAS

ADD / ADR

POSTAL CODE / POSKODE

SPITAAL / SPITAAL

WARD / SAAL 46G

HOSP. CLASS / HOSP. KLAS 02

HOSP. NO. / HOSP. NO. 10562 89

RACE / RAS W SEX / GESLAG F DATE OF BIRTH / GEBOORTEDATUM 30 4

SPECIMEN / MONSTER V

INVESTIGATION / ONDERSOEK HTLV III

PROV. DIAGNOSIS / VOORLOPIGE DIAG. (AIDS)

COLLECTION / INSAMELINGS

DATE / DATUM 5.6.87 TIME / TYD 15 3

HUMAN IMMUNODEFICIENCY VIRUS SEROLOGY / SEROLOGIE VIR MENSLIKE IMMUNGEBREKVIRUS

0877

CISER

HIV-1 ANTIBODIES / HIV-1 TEENLIGGAME

		Negative/Negatief	Positive/Positief	
Enzyme immunoassay Ensiem - immuunessai	(EIA) (EIE)	☑	☐	
Indirect flourescent antibody test Indirekte flouressensie teenliggaamtoets	(IFA) (IFT)	☐	☐	IgM ☐

INTERPRETATION

The presence of HIV-1 antibodies is not diagnostic of AIDS, but merely indicates exposure to the human immunodeficiency virus.

INTERPRETASIE

Die teenwoordigheid van HIV-1 teenliggame is nie diagnosties van VIGS nie en dui slegs op blootstelling aan die mensmse immuungebrekvirus.

WESTERN BLOT / "WESTERN" -- KLAD

p 18	☐	p 31	☐	p 51	☐	p 66	☐
p 24	☐	gp 41	☐	p 55	☐	Negative/Negatief	☐

INTERPRETATION

The presence of antibodies to GP41 and P24 is indicative of exposure to HIV-1. The GP41 is specific for HIV-1 and the detection of antibodies to this protein is essential for the diagnosis of HIV-1 infection. As the P24 is shared by other human retroviruses, it is therefore non-specific. Antibodies against this protein alone are not diagnostic of HIV infection. Furthermore, antibodies to P24 may disappear with the onset of AIDS.

INTERPRETASIE

Die teenwoordigheid van teenliggame teen GP41 en P24 dui op blootstelling aan HIV-1. Die GP41 is spesifiek vir HIV-1 en die opsporing van teenliggame teen hierdie proteïen is noodsaaklik vir die diagnose van HIV-1 infeksie. Aangesien die P24 deur ander menslike retrovirusse gedeel word, is dit derhalwe nie-spesifiek. Teenliggame slegs teen hierdie proteïen is nie diagnosties vir HIV-infeksie nie. Daarby kan teenliggame teen P24 verdwyn wanneer VIGS ontwikkel.

SEROLOGY 9/87

APPENDIX IX

Type of form used when taking skin biopsy specimens from volunteers.

TOESTEMMING TOT OPERASIE/CONSENT TO OPERATION

T.P.H. 3

HOSPITAAL/HOSPITAL _____ KLINIESE DEPART./CLINICAL DEPT. _____ AFDELING/WARD _____

Ek, _____ die ondergetekende, verleen hiermee my toestemming tot die
I, the undersigned hereby consent to the administration of a

toediening van 'n algemene of ander narkose en vir die uitvoering van 'n _____ operasie,
general or other anaesthetic and to the performance of an operation,

waarvan die aard en moontlike gevolge aan my verduidelik is en wat ek begryp, op *myself/my kind/my eggenoot/my eggenote,
the nature and possible effects of which have been explained to me and which I understand, on *myself/my child/my husband/my

die pasiënt _____ Ek verleen ook my toestemming tot sodanige verdere of alter-
wif:/the patient (Naam van pasiënt/Name of patient) I also consent to such further or alternative operative measures

atiewe operatiewe handelinge as wat nodig gevind mag word gedurende die verloop van bogenoemde operasie,
as may be found necessary during the course of the abovementioned operation.

*Ek verstaan dat 'n versekering nie gegee is nie dat die operasie deur 'n bepaalde geneesheer gedoen sal word.
I understand that an assurance has not been given that the operation will be performed by a particular medical practitioner.

*Handtekening van pasiënt/vader/moeder/voog/eggenoot/eggenote/naasbestaande (hoedanigheid)/Superintendent
Signature of patient/father/mother/guardian/husband/wife/near relative (capacity)/Superintendent.

_____

Getuies 1. _____ Datum _____ Tyd _____
Witnesses Date Time
2. _____

*Haal deur wat nie van toepassing is nie./Delete that which is not applicable.

BESONDERHEDE VAN OPERASIE/PARTICULARS OF OPERATION

Datum _____ Operasiesaal _____
Date Theatre

Duur van operasie: Van _____ vm./nm. tot _____ vm./nm.
Duration of operation: From a.m./p.m. to a.m./p.m.

Chirurg _____
Surgeon

Assistent _____
Assistant

Aard van operasie _____
Nature of operation

Handtekening van Chirurg/Signature of Surgeon

Narkotiseur _____
Anaesthetist

Assistent _____
Assistant

Aard van Narkose _____
Nature of Anaesthetic

Handtekening van Narkotiseur/Signature of Anaesthetist

Merk met 'n kruisie in toepaslike blokkies
Mark with a cross in the appropriate blocks

	Voltallig Complete	Nie voltallig Incomplete
Deppers Swabs		
Instrumente Instruments		
Hegtingsnaalde Suture needles		
	Gebruik & afgehaal Used & removed	Nie gebruik/ Not used
Toerniket Tourniquet		
	Getal Number	Tipe Type
Dreinerings-buise Drains		
Kateters Catheters		
Tamponne Plugs		
Monsters Specimens		

e.a. _____
a.o.

Gekontroleur deur (Handtekening en Rang):
Checked by (Signature and Rank):

1.) _____ (Opskropverpl.) (Scrub Nurse)

2.) _____ (Mede-kontroleur) (Co-checker)

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