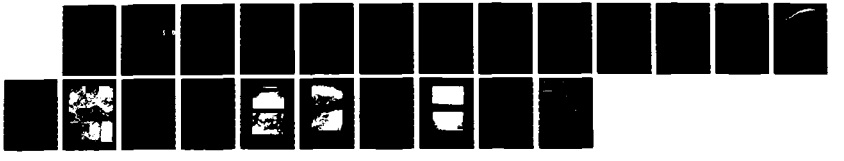
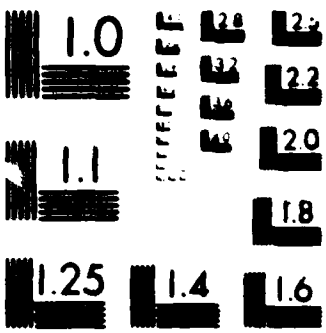
AD-A175 643 EXPERIMENTAL TREATMENT FOR BURN VICTIMS IN FIELD HOSPITALS(U) MASSACHUSETTS INST OF TECH CAMBRIDGE DEPT OF MECHANICAL ENGINEERING I V YANNAS ET AL. AUG 85 1/1

UNCLASSIFIED DAMD17-83-C-3203 F/G 6/5 NL




1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2

AD _____



# EXPERIMENTAL TREATMENT FOR BURN VICTIMS IN FIELD HOSPITALS

Annual/Final Report

I.V. Yannas and E.M. Skrabut

August 1985



Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-83-C-3203

"Original contains color plates: All DTIC reproductions will be in black and white"

Massachusetts Institute of Technology
Cambridge, Massuchusetts 02139

SECURITY CLASSIFICATION OF THIS PAGE

# REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188
Exp. Date: Jun 30, 1986

| | |
|---|---|
| 1a. REPORT SECURITY CLASSIFICATION: Unclassified | 1b. RESTRICTIVE MARKINGS |
| 2a. SECURITY CLASSIFICATION AUTHORITY | 3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited |
| 2b. DECLASSIFICATION/DOWNGRADING SCHEDULE | |
| 4. PERFORMING ORGANIZATION REPORT NUMBER(S) | 5. MONITORING ORGANIZATION REPORT NUMBER(S) |
| 6a. NAME OF PERFORMING ORGANIZATION: Massachusetts Institute of Technology; 6b. OFFICE SYMBOL (If applicable) | 7a. NAME OF MONITORING ORGANIZATION |
| 6c. ADDRESS (City, State, and ZIP Code): Department of Mechanical Engineering, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139 | 7b. ADDRESS (City, State, and ZIP Code) |
| 8a. NAME OF FUNDING/SPONSORING ORGANIZATION: U.S. Army Medical Research & Development Command; 8b. OFFICE SYMBOL (If applicable) | 9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAMD17-83-C-3203 |
| 8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, Maryland 21701-5012 | 10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. 62772A; PROJECT NO. 3S162. 772A874; TASK NO. AF; WORK UNIT ACCESSION NO 269 |

11 TITLE (Include Security Classification)
(U) Experimental Treatment for Burn Victims in Field Hospitals

12 PERSONAL AUTHOR(S)
I. V. Yannas and E. M. Skrabut

| 13a TYPE OF REPORT | 13b TIME COVERED | 14. DATE OF REPORT (Year, Month, Day) | 15. PAGE COUNT |
|---|---|---|---|
| Annual/Final* | FROM 8/1/83 TO 7/31/85 | August 1985 | |

16 SUPPLEMENTARY NOTATION
Annual for the period 9/84-7/85
Final for the period 8/1/83-7/31/85

| 17 COSATI CODES | | | 18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number) |
|---|---|---|---|
| FIELD | GROUP | SUB-GROUP | |
| 06 | 05 | | |
| 06 | 16 | | |

19 ABSTRACT (Continue on reverse if necessary and identify by block number)


We have initiated a program to adapt an artificial skin, which we have previously used to treat over 55 severely burned patients, for use in field hospitals.

During this contract we have succeeded in redesigning the original preparation procedure to produce a satisfactory "Stage 1" artificial skin with apparently unlimited shelf life. Additional animal studies are needed to confirm the preliminary positive results. Second, we have developed specialized ultrastructural procedures and completed exploratory work which aims to convert the current donor-dependent process ("Stage 2 artificial skin") into a donor-independent process by seeding our artificial


Continued on reverse

| 20 DISTRIBUTION/AVAILABILITY OF ABSTRACT | 21 ABSTRACT SECURITY CLASSIFICATION |
|---|---|
| ☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS | |

| 22a. NAME OF RESPONSIBLE INDIVIDUAL | 22b TELEPHONE (Include Area Code) | 22c. OFFICE SYMBOL |
|---|---|---|
| Mrs. Judy Pawlus | 301-663-7325 | SGRD-RMI-S |

DD FORM 1473, 84 MAR — 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE

#19 Continued

skin membranes with specially selected heterologous epidermal cells instead of autologous cells. To this end we have tested the hypothesis that a population of heterologous epidermal cells which has been depleted of Langerhans cells in our laboratory by use of a variety of procedures can yield a satisfactory donor-independent artificial skin without use of immunosuppression. Third, we have demonstrated that the viability of epidermal cells persists following 6-month storage at -196°C while preliminary results show that 7-day storage at -80°C preserves a fraction of the in vitro viability and a fraction of the skin grafting performance of epidermal cells. Fourth, we have discovered a new procedure for controlling the ability of collagen fibers to aggregate platelets and we can fabricate either the well-known hemostatic collagens which clot blood efficiently or the novel collagens prepared in our laboratory which do not induce clotting. This basic information will be used to design membranes for skin grafting in a way that would greatly reduce the time required to achieve hemostasis in the operating room.

Our long-term goal continues to be the design of a refrigerated membrane which upon thawing can be used to graft large, full-thickness skin wounds, eventually yielding by a single application, new, nearly physiologic integument within 2 weeks with prevention of scarring and contraction. The anticipated benefits of such a device are decreased mortality rate, reduction of the time spent in the operating room, reduction of hospital stay and reduction of follow-up surgery (reconstruction).

## TABLE OF CONTENTS

## A. MILITARY SIGNIFICANCE

Several methods are commonly used to cover full-thickness wounds in burn treatment clinics. From all points of view the most satisfactory method involves use of a fraction of the patient's own skin. In this procedure, the split-thickness autograft, a thin layer of skin, which includes not only the complete epidermis but a fraction of the dermis as well, is surgically removed from an intact area of the patient's body and grafted onto the burn wound. While the split thickness autograft might solve the problem of preventing water loss and infection in a burn patient, it does have serious disadvantages. It leaves a scar, it is less functional than normal skin, it is cosmetically inferior and the procedure causes additional discomfort to a patient who is already very ill. Also, when the burn damage is excessive, there may not be enough intact skin left to cover the large wounds.

When autografting is not possible, wounds may be covered with specially prepared pigskin. Commercially available, this graft is prepared by removing split thickness portions of porcine skin. These xenografts can cover the wound satisfactorily for about 3-5 days. They are usually removed or "changed" after a few days and under ordinary circumstances do not vascularize or "take". Cadaver allografts vascularize and take but begin to reject in about 14 days, although immunosuppression, which increases the risk of infection, can prolong significantly the life of such a graft. There is no satisfactory solution to the problem of wound closure when there are insufficient donor sites to secure autograft.

There is need for a membrane, available in large quantities in a variety of sizes and three-dimensional shapes, which can be used to treat large, full-thickness excised burn wounds in the various parts of the body not requiring use of immunosuppression and without inflicting additional trauma in its use. We define "treatment" as the long term control of wound contraction and scar formation in addition to prompt control of infection and exudation over more than 50% of body surface area following a single application (grafting) of the membrane on the fully excised area.

We have previously demonstrated procedures for solving part of this problem, by controlling infection and exudation with experimentally wounded guinea pigs and with patients who had sustained massive skin loss from burn injury. In addition, we have demonstrated procedures for controlling contraction and scar formation in extensively burnt humans by use of a double (rather than single) grafting operation (Stage 1 artificial skin). Finally, we have recently succeeded in controlling scar formation and skin contraction with guinea pigs, which carried full-thickness excised wounds, by a single grafting operation (Stage 2 artificial skin). Clinical trials will soon begin using this procedure which appears essentially to solve the full surgical problem as defined in the preceding paragraph.

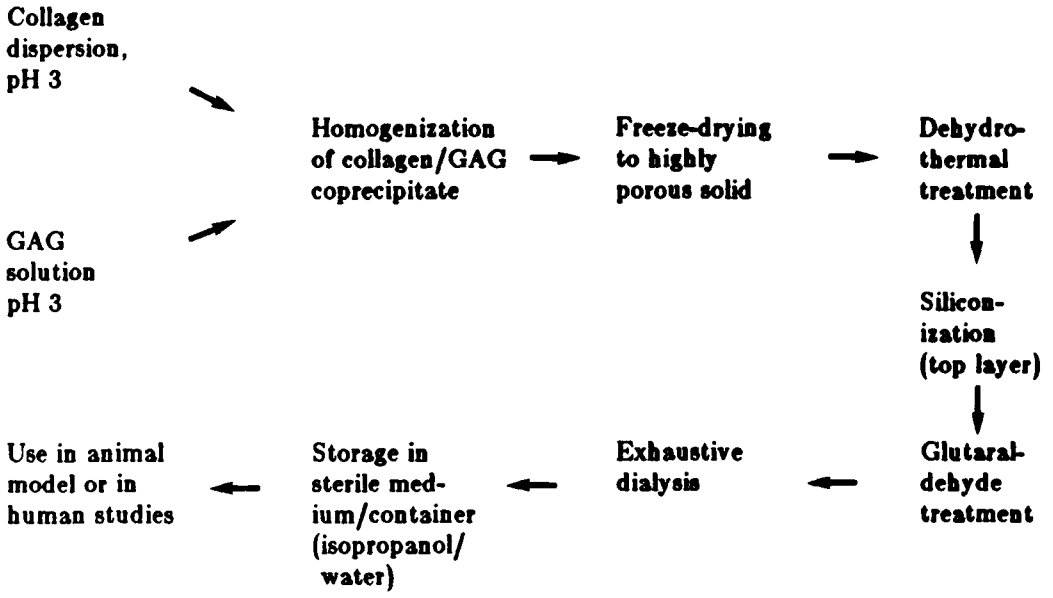
The successful use of these membranes, which are sometimes referred to as "artificial skin", to the treatment of over 55 severely burned patients at Massachusetts General Hospital suggests the modification of these membranes to devices which can be used to graft large excised wounds on shipboard and in field hospitals. We have already modified the process for preparing these membranes and have thereby extended their shelf life indefinitely. This accomplishment reduces the problem of resupply.

## B. OBJECTIVES

Our long term objective is to design a membrane which, after being thawed from storage at -80°C and grafted onto a full-thickness skin wound without extensive control of capillary bleeding, can promptly control fluid loss and infection and can lead to regeneration of a neodermis and a neoepidermis within 2 weeks.

## C. METHODOLOGY

1. Fabrication of Stage 1 membranes. A detailed discussion of the bovine hide collagen preparations and chondroitin 6-sulfate preparations used has been published elsewhere [1-3] and will not be repeated here. The flowsheet below shows schematically the method currently used to prepare Stage 1 membranes. The acronym GAG refers to the glycosaminoglycan, chondroitin 6-sulfate.




2. Control and determination of pore structure, collagen/GAG ratio and crosslink density of collagen-GAG membranes. The pore volume fraction, the mean pore width and the mean aspect ratio of pores (a measure of pore shape) of collagen-GAG membranes can be controlled by adjusting the conditions of freeze drying and the result can be determined by use of quantitative stereological procedures which have been described previously in detail [4]. The mean pore width was maintained at $120 \pm 50$ $\mu$m.

The collagen/GAG ratio was maintained constant at 92/8 and was determined using a hexosamine assay which has been published [2].

The average molecular weight between crosslinks, $M_c$, a reciprocal measure of crosslink density, was controlled at the level $12750 \pm 3300$ and will be determined using procedures which have been described in detail elsewhere [5].

3. Preparation and testing of membranes differing in their ability to aggregate platelets. Collagen which does not react with platelets [6-8] was prepared by treatment at pH 3 over 24 hours, followed by addition of chondroitin 6-sulfate, freeze drying, vacuum oven treatment, treatment with acetic acid at pH 3 over 24 hours followed by treatment with 0.25% w/w glutaraldehyde at pH 3 and ending with dialysis against pH 7 buffer. Collagen which aggregates platelets in the well-known vigorous manner was prepared by omitting in the above procedure all steps which involve treatment in acetic acid (pH 3) and in glutaraldehyde.

4. Preparation and testing of membranes which incorporate epidermal cells (Stage 2 or Stage 3). The basic process of epidermal cell separation and graft seeding currently proceeds as follows: A small area of guinea pig skin is harvested, washed with buffered saline, and dissociated with trypsin in phosphate-buffered saline (pH 7.2) for 40 minutes at 37°C [9]. A suspension of epidermal cells is

prepared by discarding the top epidermal layer, placing the bottom dermal layer in Eagle's minimum essential medium (Dulbecco's modification) containing 10% fetal calf serum, vortexing the medium, separating the suspended cells from the pieces of dermal tissue by filtration, and centrifuging the cell suspension until the desired density of viable cells is attained [10].

The cells are seeded into the porous membrane by mild centrifugation. The centrifuge bucket is lined with a membrane holder designed to maintain the plane of the membrane normal to the centrifugal force vector with the collagen-GAG layer facing the vector, pipetting the cell suspension into the membrane holder, and operating the centrifuge at 50 x g for 15 minutes or at 500 x g for 10 minutes [10].

5. Preparation and testing of membranes which incorporate epidermal cells depleted of Langerhans cells. Seeding of the CG membrane with corneal keratinocytes. The cornea is a structure which is well known to be devoid of Langerhans cells. This and the fact that the cornea is not vascularized are reasons frequently implicated in explaining the successful corneal allografts seen commonly in ophthalmology. These grafts are able to survive for years and do not require immunosuppressive agents. The cornea could therefore provide a source of epithelial cells which are Langerhans-cell free [11].

Streilein, et al. [12] examined the immunologic potential of corneas when they were grafted heterotopically on the thoracic wall of recipient mice. It was found that corneal grafts disparate at H-1K elicited an immune response while corneal grafts disparate at Ia were not observed to be rejected over a follow-up period of 45 days.

Cultured epithelial cells of human cornea, conjunctiva, and skin were studied by Sun and Green [13], They found very litle difference in a variety of differentiation markers present on keratinocytes and proposed that the external phenotypic difference seen in vivo was due to external modulation. The incorporation corneal epithelial cells into a Stage 3 artificial skin graft could potentially be important in understanding keratinocyte differentiation.

Observations of graft rejection. Preliminary past experience with collaen-GAG membranes which have been seeded with untreated or partly treated epidermal cells has shown a clear difference between grafts which are accepted by the host and those which are rejected. Grafts which are rejected do not form neodermal confluence, and have increased amounts of serous exudate beginning about 9-10 days post grafting. This gross observation may be less than optimal in grafts which have reduced immunogenicity. To avoid this difficulty, histological specimens were taken from the maturing grafts.

In Stage 2 artificial skin grafts it was shown that, epidermal cells which are seeded close to the woundbed (away from the silicone layer) divide and form epidermal clusters with a central core of keratin (keratin pearls). These keratin pearls migrate slowly up through the wound and are spontaneously ejected by day 25. The keratin pearls have been shown to be useful markers for rejection as a lymphoid infiltrate forms around keratin pearls in cases where the seeded cells are being rejected [10]. Since keratin pearls are not seen in unseeded membranes they provide an unambiguous marker for seeded cells.

6. Preparation and testing of membranes which incorporate cryopreserved epidermal cells. Our effort focused on resolving the major problem which we observed during cryopreservation of epidermal cells at -80°C. Briefly, culture of the cell preparation following cryopreservation treatment at -80°C gives a preparation which is dominated by fibroblasts and contains epidermal cell colonies as a minor constituent. This result is most probably associated with a lower sensitivity to freeze-thawing which fibroblasts exhibit compared to epidermal cells. Our previous work shows, however, that freeze-thawing at -196°C does not give rise to such a problem.

Epidermal cell preparations were enriched by centrifuging the standard cell preparation over a Percoll (Silica Particles coated with polyvinylpyrrholidone) gradient. Alternate methods to centrifugation, such as velocity sedimentation at 1 x g [14] could also be applied to separate populations of cells which give high and low epidermal colony forming efficiencies.

## D. RESULTS

We present below the major results obtained during the course of this contract. We refer for additional details to the Annual Summary Report Contract No. DAMD17-83-C-3203, dated September 1984 [15]. The discussion presented in the section below emphasizes results that were obtained between September 1984 and March 1985.

The term "Stage 1 artificial skin" refers below to a bilayer membrane, comprising a top thin layer of silicone rubber bonded onto a bottom layer of a biodegradable polymer prepared by covalently crosslinking chondroitin 6-sulfate to collagen fibers [1-3,16,17]. Stage 1 membranes are not seeded with epidermal cells prior to grafting. During the period 1979-1985 Stage 1 membranes have been produced almost entirely by Dr. I.V. Yannas and his coworkers in the Fibers and Polymers Laboratories, MIT and have been used to treat over 55 patients, all victims of severe burns, at Massachusetts General Hospital by Dr. J.F. Burke and associates [18]. Stage 1 artificial skin induces spontaneous partial regeneration of a new dermis. The "neodermis" produced thereby requires final grafting with a very thin autoepidermal layer obtained by use of the dermatone [18].

The term "Stage 2 artificial skin" refers to a Stage 1 membrane which has previously been seeded with autologous epidermal cells prior to grafting on animals [9]. Stage 2 membranes induce regeneration not only of a neodermis but of a neoepidermis as well and it leads to complete wound closure by mature epithelium within 10-14 days following grafting [5,9]. Preparations are currently under way to test clinically Stage 2 artificial skin with severely burnt patients at Massachusetts General Hospital.

For the purpose of this report we will use the term "Stage 3 artificial skin" to denote a Stage 1 membrane which has been seeded with heterologous, rather than autologous, epidermal cells. As currently envisaged, Stage 3 membranes will be seeded with heterologous cells which, having been depleted from their Langerhans cell content, would induce synthesis both of a neodermis and a neoepidermis, much like Stage 2 artificial skin, and would not require use of immunosuppressive agents.

The acronym CG denotes the crosslinked collaen-glycosaminoglycan (or collagen-GAG) polymer which constitutes the bottom layer of the artificial skin [2].

1. Method for manufacturing artificial skin in the dry state. At the present time Stage 1 artificial skin is stored in 70% aqueous isopropanol at 4°C. Storage of collagen-GAG membrane in aqueous media leads to hydrolysis and reduces the shelf-life of these membranes. Storage in the dry state would not only extend the shelf life indefinitely but would also reduce the weight of the device to a small fraction of its current value thereby simplifying transport and deployment.

Studies showed that a final freeze-drying treatment applied to the collagen-GAG (CG) membrane reduced the porosity significantly and made the membrane useless as a graft when the CG layer was freeze dried out of distilled water. Freeze drying of the CG layer out of 70% isopropanol and 10% isopropanol led to apparent retention of the porosity, as studied by scanning electron microscopy (SEM). However, animal studies using the CG membrane that had retained its pore structure almost intact following this second (and terminal) freeze drying treatment showed histologically that there was a significantly higher incidence of undigested CG fibers by day 14 and 21 than was the case when the CG membrane had not been freeze dried for a second time. In addition, the histological studies showed the presence of many multi-nucleated giant cells associated with the CG fibers [15].

This data suggested to us that, even though the apparent porosity of the CG fibers remained intact following the most useful of the various freeze drying procedures, the "degree of subdivision" of the CG fibers may not have been preserved as well. Accordingly, it was recently hypothesized that freeeze drying out of aqueous isopropanol caused deswelling of the CG fibers and, therefore, the fibers clustered together in sufficiently large bundles to slow down biodegradation of the CG graft and to elicit the presence of giant cells.

We subsequently freeze dried CG membranes out of 0.05 M acetic acid, a medium which is known to cause swelling and dispersion of collagen fibers (see section 6 below). In these experiments the CG

membranes were prepared as described previously [2,3], however, following the crosslinking treatment in glutaraldehyde [2] the membranes were dialyzed extensively in 0.05 M acetic acid (rather than in water or aqueous isopropanol) and freeze-dried from that medium (pH 3.2). Freeze drying proceeded by first placing the swollen membrane on a shelf maintained at -65°C in a Virtis LN10 freeze-dryer; after freezing for one hour, vacuum was pulled, the condenser temperature was set at -100°C and the shelf temperature was raised to -5°C. Following freeze-drying the CG membranes were bonded to a preformed silicone layer and stored until use. Prior to surgery the bilayer membranes were hydrated in 70% isopropanol and were dialyzed extensively in saline before being used to graft full-thickness skin wounds in guinea pigs. Specimens of the freeze-dried CG membranes were viewed in a scanning electron microscope (SEM). Details of the animal model which we have used over the years are presented elsewhere [4,10,15].

Recent histological studies of grafts freeze-dried out of acetic acid showed that the presence of giant cells was reduced and the number of obviously undigested CG fibers by day 21 was negligible compared to grafts which were freeze-dried out of 10% isopropanol. The wound contraction rate, another method of assaying the ability of a graft to act as an effective wound cover [15], showed a wound contraction half-life of 16 $\pm$ 2 days for the experimental membrane which had been freeze-dried out of acetic acid compared to a value of 18 $\pm$ 2 days for controls which were not freeze-dried and required storage in the wet state. Our results are illustrated in Figure 1.

Although some additional animal studies are necessary to confirm these findings it appears very likely that the correct choice of the liquid medium out of which the membrane is freeze dried dominates its performance as a graft. We tentatively conclude that the first task proposed in this contract has been successfully completed and, therefore, Stage 1 artificial skin can be stored in the dry state. In addition, clinical studies need to be done in the future to test the performance of these new grafts.

2. Method for hydrating the CG membrane within several minutes using standard hospital solutions. The successful preparation of acceptable Stage 1 artificial skin which can be stored in the dry state led us to initiate studies for rapid hydration of CG membranes. Previously we found that use of a surfactant, sodium dodecyl sulfate, could reduce the hydration time of freeze dried CG membranes from about 10 hours (no surfactant present) to less than one hour (surfactant present). Nevertheless, no determination has yet been made of the minimum concentration of surfactant necessary or of the possible effects of residual (not removed by washing) surfactant on the performance of the graft.

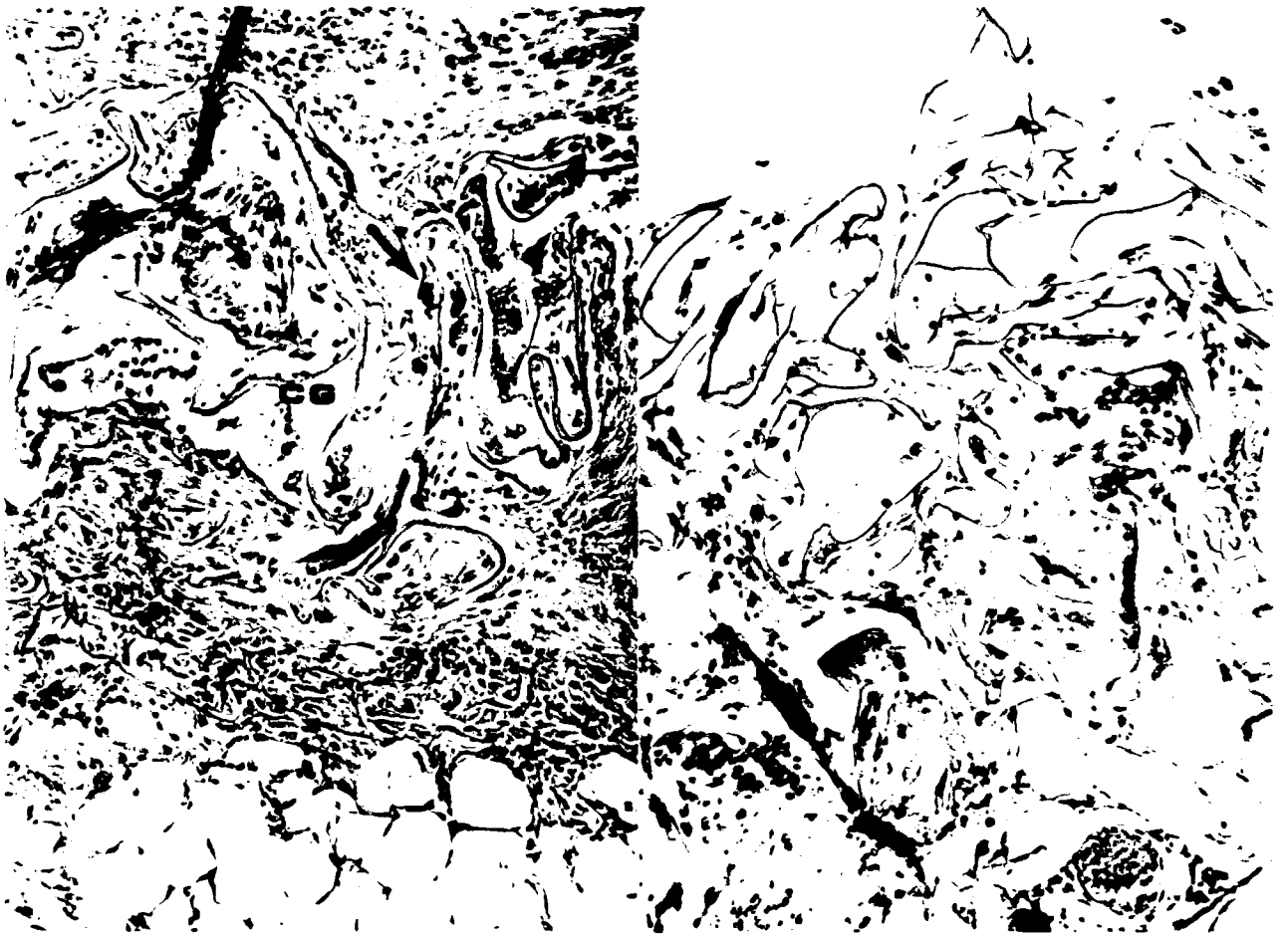
A preliminary effort was made to determine the effectiveness of skin grafts applied on the wound as dry (not hydrated) CG membranes. These grafts were much more rigid than the hydrated controls but were flexible enough to be grafted on 1.5 x 3.0 cm full-thickness skin wounds on the back of guinea pigs. Preliminary observations show that exudate from the wound bed may effectively hydrate the graft but additional animal studies are necessary to study this new graft configuration.

3. Development of methods for reducing the immunogenicity of heterologous epidermal cell preparations. This task consisted in preparing Stage 3 artificial skin, i.e. a donor-independent membrane which performs as well as a Stage 2 artificial skin without use of immunosuppressive agents.

The preliminary strategy consisted in first preparing a standard epidermal cell preparation from guinea pig skin; treating such a preparation in various biophysical modes which would be designed to reduce the immunogenicity of the preparation; developing an assay (the skin lymphocyte reaction, SLR) to determine the effects of such treatments on the immunogenicity of the basal cell population; and eventually grafting full thickness skin wounds on guinea pigs with basal cell populations that showed a sufficiently weak SLR reaction [15].

As described in the literature the skin lymphocytes reaction (SLR) uses skin lymphocytes as the in vitro equivalent of the recipient's immune system [16]. Blastogenesis of the lymphocytes occurs when the latter are mixed with heterologous epidermal cells and the reaction is characterized by increased DNA synthesis which can be measured by incorporation of a labeled DNA precursor, $^{3}$H-thymidine, as described by others [19].

The sharp comparison between the value of the SLR assay when used with human cells and when used with guinea pig cells can be made by use of the "response index", the ratio of stimulated DNA



A B

Figure 1. Comparison of freeze-dried CG membranes 14 days after grafting. The final freeze-drying step was out of 10% isopropanol (A) and 0.05 M acetic acid (B). Note that in (A) the CG fibers are much thicker and are surrounded by multinucleated giant cells (arrow), 300X. Histological sections prepared at the Research Animal Diagnostic Laboratory, MIT.

synthesis to nonstimulated DNA synthesis in an equal number of cells. The value of the response index was as high as 6.08 ± 8.80 in a mixed lymphocyte reaction using peripheral blood lymphocytes from unrelated human donors; and the response index was 2.26 ± 0.14 in SLR assays utilizing human peripheral blood lymphocytes and basal cell preparations from unrelated human donors. By comparison, the value of the response index was not significantly different from 1 (indicative of no response) when guinea pig cells were used. As described in detail previously [15], three different sources of guinea pig lymphocytes were tapped in an effort to identify a lymphocyte population which would be both active and easily accessible. Neither peripheral blood lymphocytes nor peritoneal exudate lymphocytes nor lymph node lymphocytes were found to give a response index significantly higher than 1 when lymphocytes from two strains of guinea pigs, English and Hartley, were mixed. Nor was there a significant reaction observed in the guinea pig SLR assay.

Our results show that whereas the SLR assay appears to be as useful as reported in the literature for the in vitro study of human skin graft rejection [19] adaptation of the published assay to guinea pig cells can not be done without major modification.

As an alternative to removing the Langerhans cells, guinea pig cornea were utilized as a source of Langerhans-cell free [11] epidermal preparations. The results of the treatment of cornea with the standard isolation used for skin are shown in Figures 2a and 2b. The basal epidermal layer is easily removed, and preliminary studies in our laboratory indicate that these cells are viable and can be grown as cell cultures (Figure 2c).

4. Development of ultrastructural procedures to assess the efficacy of removal of Langerhans cells from heterologous basal cell preparations and assessment of the efficacy of formation of neodermis and neoepidermis in Stage 3 grafts. In the effort to develop biophysical and other procedures for reducing the immunogenicity of basal cell populations which are used to "seed" Stage 1 membranes we have initiated development of specialized ultrastructural procedures to detect the degree to which certain types of cells, e.g., Langerhans cells, have been depleted from epidermal cell preparations following treatment of the cell suspension. Morphological observations by light and electron microscopy are also being developed to detect histological changes brought about by allograft rejection. It is expected that development of these microscopic techniques will allow detection of allograft rejection which may occur at a subclinical level and may not be detectable by gross observation. Thirdly, morphological procedures are being developed to detect the efficacy of formation of physiologic neoepidermis and physiologic neodermis following grafting with Stage 3 artificial skin.

Currently, our new Co-Investigator, Dr. George Murphy, Brigham and Women's Hospital, Boston is involved in a detailed electron microscopic observation of Stage 2 skin grafts, i.e., artificial skin which has been seeded with autologous cells prior to grafting. Since the methodology which is currently utilized by Dr. Murphy and his coworkers to study Stage 2 grafts will, to a large extent, be relied upon to study the performance of Stage 3 grafts we describe below some of the preliminary observations obtained.

Ultrastructural observation of Stage 2 skin grafts has been performed on a limited number of guinea pig biopsies. These have been compared with normal guinea pig skin and with scar tissue resulting from healed beds formed as a result of full thickness removal of skin and with full-thickness autografts. Specifically, we have attempted to ascertain, in graft that were older than 200 days, the cellular constituents of the epidermis and dermis. We are concerned with how precisely the neoepidermis and neodermis architecturally and cytologically recapitulate normal skin. Our preliminary results are as follows (see also Figure 3):

a) Keratinocytes. The neoepidermis is virtually identical to that of normal skin and the epidermis of autografted skin. All four layers of skin are present in normal proportions (stratum corneum, stratum granulosum, stratum spinosum and stratum basalis). In addition, the lowermost portion of the basal keratinocytes shows an undulating contour which is identical to that observed in normal skin. The maturation of keratinocytes as well as their morphologic capabilities to produce keratin protein, as is evidenced by the sequential production of tonofilaments, keratohyaline and eventually of mature stratum corneum, is normal in ultrastructural appearance. In addition, the formation of a ten-nm electron-dense


Figure 2. Guinea pig cornea (A) showing epithelium (E) and stroma (S). After being treated with trypsin (B), the epithelial cells are easily dislodged (300X). These cells can easily be grown in culture (C) as shown at 14 days. Photos (A) and (B) obtained at the Research Animal Diagnostic Laboratory, MIT. Photo (C) obtained at the Cell Culture Center, MIT.

## Figure 3 Legends

Figure 3A. Normal guinea pig skin. The epidermis is formed by keratinocytes (K) joined by junctional complexes (desmosomes – enclosed). Note characteristic unmyelinated nerve fiber (N) within superficial dermis (6,000X).

Figure 3B. Stage 2 artificial skin at 14 months after grafting. The neoepidermis is formed by keratinocytes (K) that are identical to normal control skin (panel A). Desmosomes (enclosed) and basal lamina (arrows) are well-formed and normal in appearance. Melanocytes (M, inset) containing characteristic melanosomes (arrows, inset) also repopulate the neoepidermis, where they normally function in pigment production and donation. A dermal nerve fiber (N), similar to those of normal skin, is also observed (10,000X; inset 6,000X).

Figures 3C and 3D. Normal dermal collagen (C) is composed of evenly-spaced fibers in longitudinal (L) and transverse (T) orientation. Similar relationships are observed in the neodermis (D), and at this magnification, neodermal collagen is architecturally indistinguishable from normal dermal collagen (4,000X).

Figure 3E. Neodermis (14 months), showing transversely-sectioned collagen (C) and newly-synthesized elastic fibers (E). The morphology and spacing (central dots) of the collagen fibers and extent of elastic tissue deposition is similar to normal dermis, and in contrast to scar tissue (45,000X).

Figure 3F. Anatomicall normal vessels lined by endothelial cells (En) and forming patent lumina (L) are characteristic of neovascularization within the newly-synthesized dermis of stage 2 grafts. The anatomical distribution of the neovascular plexes is normal (7,500).

Photos A through F obtained by Dr. George F. Murphy and co-workers at the Cutaneous Pathology Laboratory,Brigham and Women's Hospital, Boston.

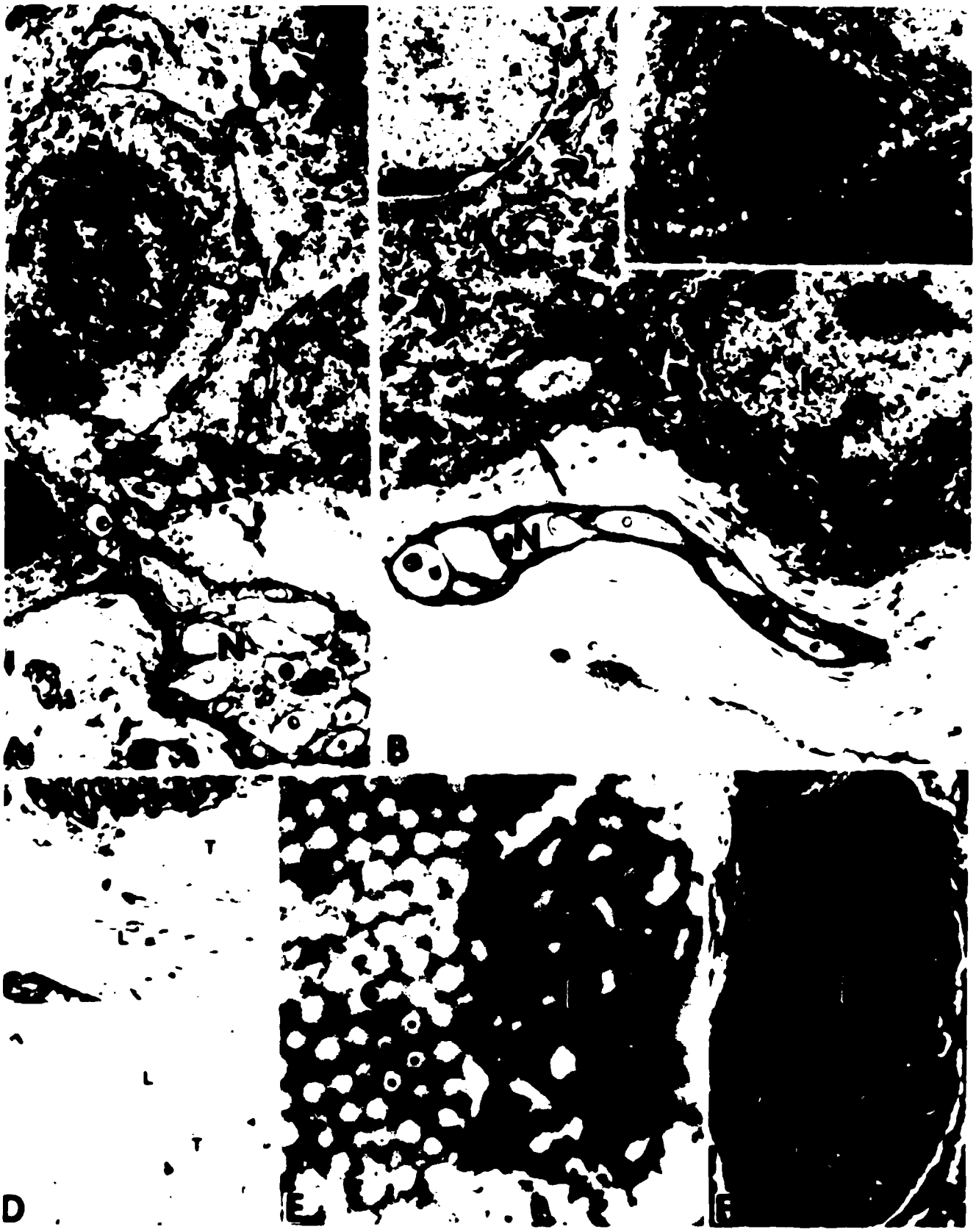



Figure 3

marginal band directly beneath the cell membrane of stratum corneum cells, an ultrastructural correlate of the cross-linked envelope which typifies terminally differentiated keratinocytes, is observed in the Stage 2 grafts. All of these data suggest that the neoepidermis of Stage 2 grafts is morphologically indistinguishable from that of normal skin and appears to be normal in function, as is evidenced by sequential production of keratin protein.

b) Melanocytes. Basally-located melanocytes are observed in Stage 2 skin grafts and are similar in number to those observed in normal controls. These cells contain variable numbers of premelanosomes, all of which are non-melanized. This, again, is indistinguishable from control tissue and is compatible with the tyrosinase deficiency characteristic of albino animals.

c) Langerhans Cells. Occasional mid-epidermal mononuclear cells with many morphological characteristics of Langerhans cells are observed in Stage 2 grafts. However, definitive identification of these cells as Langerhans cells is not possible on the basis of present data, owing to the absence in these cells of characteristic Langerhans cell granules. Further studies are under way to elucidate whether these cells are indeed Langerhans cells depleted of granules (structurally immature) or whether this cell represents a modified epidermal dendritic cell potentially subserving the function of Langerhans cells. Interestingly, our recent observation of Langerhans cell depletion by ultraviolet light has documented the replacement of Langerhans cells by mononuclear cells devoid of Langerhans cell granules within the human epidermis. We additionally observed that these cells which replace Langerhans cells are capable of subserving their immunologic function. We plan to pursue analogies of such phenomena within this rodent model of artificial skin grafting. The guinea pig model represents an excellent system in which to test and elucidate the cellular mechanisms responsible for repopulation of epidermal dendritic cells after their experimental depletion (in this case, surgical removal).

d) Dermal Vessels. The formation of a neovasculature within the dermis of grafted Stage 2 artificial skin has remarkable similarities to that of normal skin. Whereas in scar tissue dermal vessels are haphazardly arranged, the vasculature of the neodermis in Stage 2 grafts, as well as in normal skin, shows a discrete superficial plexus separating the superficial and mid-dermal collagen. From this plexus, largely composed of venules, small capillary loops extend into forming dermal papillae which interdigitate with rete ridges forming by the overlying epidermis. Detailed morphometric analysis of vessel caliber, basement membrane formation, and cellular organelles within lining endothelial cells are underway to further compare and contrast these newly-formed vascular structures of the neodermis with those of the normal skin.

e) Dermal Nerves. Stage 2 artificial skin shows marked ingrowth of non-myelinated cutaneous nerves 200 days post grafting. As in normal control skin, these nerves are situated predominantly in perivascular foci within the superficial dermis and extend as small twigs into dermal papillae. Focally, they even extend into the basal layer of the epidermis where they interdigitate between basal keratinocytes. Myelinated nerves within the deep dermis, as yet, have not been observed.

f) Dermal Collagen and Elastic Fibers. The morphology of the dermal collagen in the Stage 2 graft is strikingly similar to that of normal skin. The collagen fiber diameter and pattern of striation is quantitatively similar; the spacing between adjacent collagen fibers, however, is significantly less than that observed in normal skin and similar to that seen in scar tissue. Nonetheless, the cellularity, constituent vasculature, and neural structures, as described above, allow for easy separation of the neodermis from that of scar. A striking finding within the neodermis is the synthesis of newly-formed elastic fibers. This is a uniform and consistent finding throughout the neodermis and is similar to elastic fiber formation with embryonic skin. The degree of elastic tissue is more akin to that in forming normal skin and is dissimilar to that seen in scar tissue.

The above findings, although preliminary, provide some insight into the qualitative and quantitative constituents of the neoepidermis and neodermis of Stage 2 artificial skin. Further studies in larger number of biopsies are necessary to corroborate these impressions. Furthermore, immunocytochemical analyses of various cells within the artificial skin will supplement our understanding of the structural integrity of the newly formed epidermis and dermis.

The findings described above form a database which we will use to interpret the development of a neoepidermis and a neodermis in Stage 3 grafts. The procedures described above for detecting Langerhans cells will also be used to detect depletion of such cells following several treatments.

5. Development of an efficient procedure for cryopreservation of the epidermal cells with dimethylsulfoxide or glycerol. The long term storage of epidermal basal cells is a necessary step in the development of a Stage 3 artificial skin, as envisaged in this work.

We have demonstrated that basal epidermal cells can be efficiently preserved at -196°C for 6 months without loss of the ability of the cells to act as "seed" in Stage 2 artificial skin grafting (Figure 4). Results of animal studies with cells which had been cryopreserved in 7.5% DMSO over different periods are shown in Table 1.

TABLE 1

Viability and Confluence of Cryopreserved Epidermal Cells

| Storage time at -196°C | % Viability after thawing | Growth of a confluent epidermis in animals within 14 days |
|---|---|---|
| 0* | 79 | yes |
| 3 days | 75 | yes |
| 6 months | 71 | yes |

*Control grafts; cells were not cryopreserved.

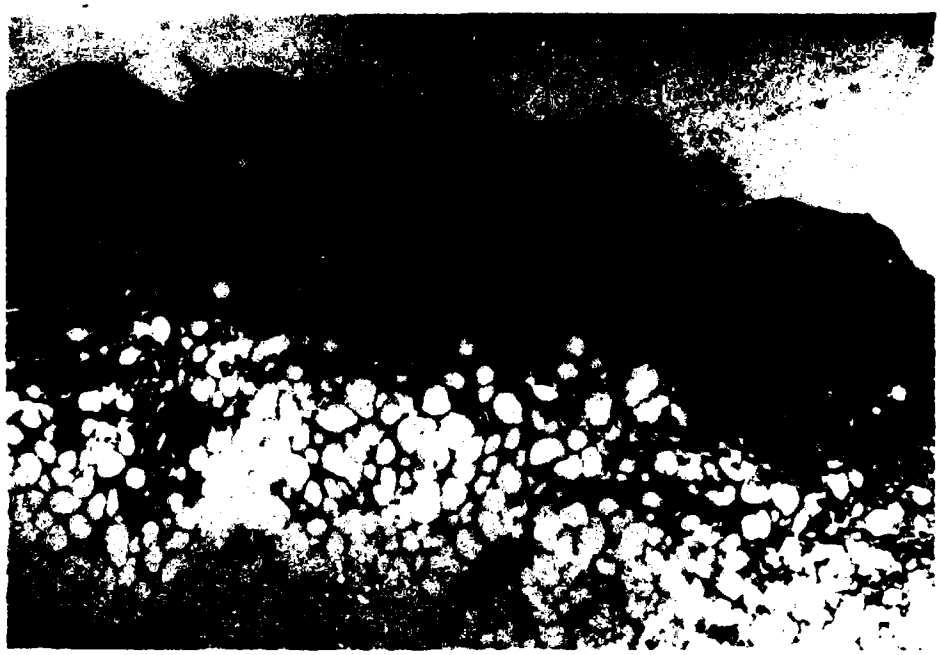
Efforts to preserve at -80°C, i.e. at mechanical refrigeration temperatures, yielded preliminary results which were reported earlier [15]. An apparently optimal cryopreservation medium was fetal calf serum containing 12% acid-citrate-dextrose (ACD) and 7.5% DMSO. Following storage at -80°C for 3 days in this medium, cell viability was 66%, somewhat lower than observed at a preservation temperature of -196°C (Table 1).

Animal studies with grafts prepared by seeding autologous epidermal cells cryopreserved at -80°C for 3 days showed that the contraction rate was significantly faster than with grafts seeded with cells that had not been cryopreserved. In addition, formation of a neoepidermis was imperfect by the time that contraction was almost complete. There was a reduced number of epidermal clusters; however, at the center of the graft there were a few clear colonies of neoepidermis.

A

B

Figures 4A and 4B. Epidermal cells were cryopreserved at -196°C for 6 months and then seeded into a CG membrane or placed into cell culture. (A) shows a Stage 2 wound 12 days after grafting where the neoepidermis appears to have covered about 75% of the wound (2X actual size). Similarly in (B) there are numerous epidermal colonies (E) seen in culture after 2 weeks. Photograph (A) obtained at the Research Animal Diagnostic Laboratory, MIT. Photograph (B) obtained at the Cell Culture Center, MIT.



C

D

Figures 4C and 4D. Low powere histology (C), 75X of center of the graft shows complete neoepidermal (arrow) confluence at day 12 and a richly vascularized neodermis (D). High power view (D) shows a rapidly dividing neoepidermis (N) and also epidermal clusters (arrow), see text. Histological sections obtained at the Research animal Diagnostic Laboratory, MIT.

Previously we have observed such undesirable wound healing kinetics when autologous fibroblasts had been deliberately added to the basal cell population prior to seeding [10]. A working hypothesis is that in the presence of seeded fibroblasts the collagen-GAG membrane is digested more rapidly than in the absence of seeded fibroblasts. Cell culture studies are consistent with this hypothesis. The cultures showed satisfactory fibroblast growth but poor epidermal cell growth.

Although these results were not optimal they showed that epidermal cells can survive freezing to -80°C and form epidermal colonies in vitro as well as clusters of neoepidermis in vivo. To overcome the problem of low epidermal colony formation, we conducted preliminary studies to purify the epidermal cell preparation extensively prior to freezing, in an effort to remove the greatest number of contaminating fibroblasts from the preparation. Our preliminary results indicate that Percoll (Silica Particles coated with polyvinylpyrrholidone) gradients can be utilized to separate the total cell population into sub-populations of cells which form either primarily fibroblasts or primarily epidermal colonies in culture (Figure 5). This technique utilized centrifugation of the epidermal cell preparation to separate the cells. Freezing rate and optimal cryopreservative concentration will be determined for these enriched epidermal cell populations.

6. Reduction of the time required to achieve hemostasis. Previous work done by I.V. Yannas and coworkers [6,20,21] has shown that the structure of collagen fibers can be manipulated to prepare collagens which cover a very wide range in their ability to aggregate platelets. Experiments in our laboratory show that, as currently produced, our collagen-GAG membranes do not aggregate human platelets in plasma. Our ongoing effort to design devices which can be used to control healing of deep skin wounds encouraged members of our research group to consider the possible merits of an artificial skin which could control capillary bleeding, thereby shortening significantly the time spent in the operating room (OR). In one version of such a device a thin layer of porous, thrombogenic collagen would be bonded onto a layer of porous, nonthrombogenic collagen and the latter would, in turn, be bonded onto a layer of silicone rubber. Alternately, a layer of porous, nonthrombogenic collagen would be impregnated with a population of thrombogenic collagen particles.

We present below a summary of our basic research findings which provide support for our future research in the direction of reducing the time required to achieve hemostasis in the OR.

We have found that native collagen fibrils lose their banding pattern rerversibly when exposed to certain aqueous solutions below pH 4.25 ± 0.30 [6]. We have studied collagen structure above and below this transition by transmission electron microscopy, small-angle x-ray scattering, and infrared spectroscopy. During the "melting" transition, which occurs by time dependent swelling of fibrils, the packing order in collagen fibrils is abolished but the triple helical structure of the constituent collagen molecules remains intact.

When fibrils which have lost their banding following incubation at pH levels below 4.25 ± 0.30 are subsequently exposed to neutral pH, banding is recovered almost fully (recrystallization). Several collagen dispersions were swollen in the pH range 1.6 - 7.0 and were treated with glutaraldehyde to prevent recrystallization during dialysis to neutral pH. The intensity with which these dialyzed collagens reacted with human platelets in plasma was studied by aggregometry and scanning electron microscopy [6].

We conclude that platelet aggregation is suppressed effectively when preparations of collagen particles are first treated below pH 4.25 ± 0.30 and are subsequently treated with glutaraldehyde below that critical pH level. Collagen preparations treated as described above show, in addition, no ability to activate either the extrinsic or the intrinsic coagulation cascade of human blood and therefore contrast vividly in their hematological behavior with collagen preparations that have not been treated below pH 4.25 ± 0.30 [6,20,21].

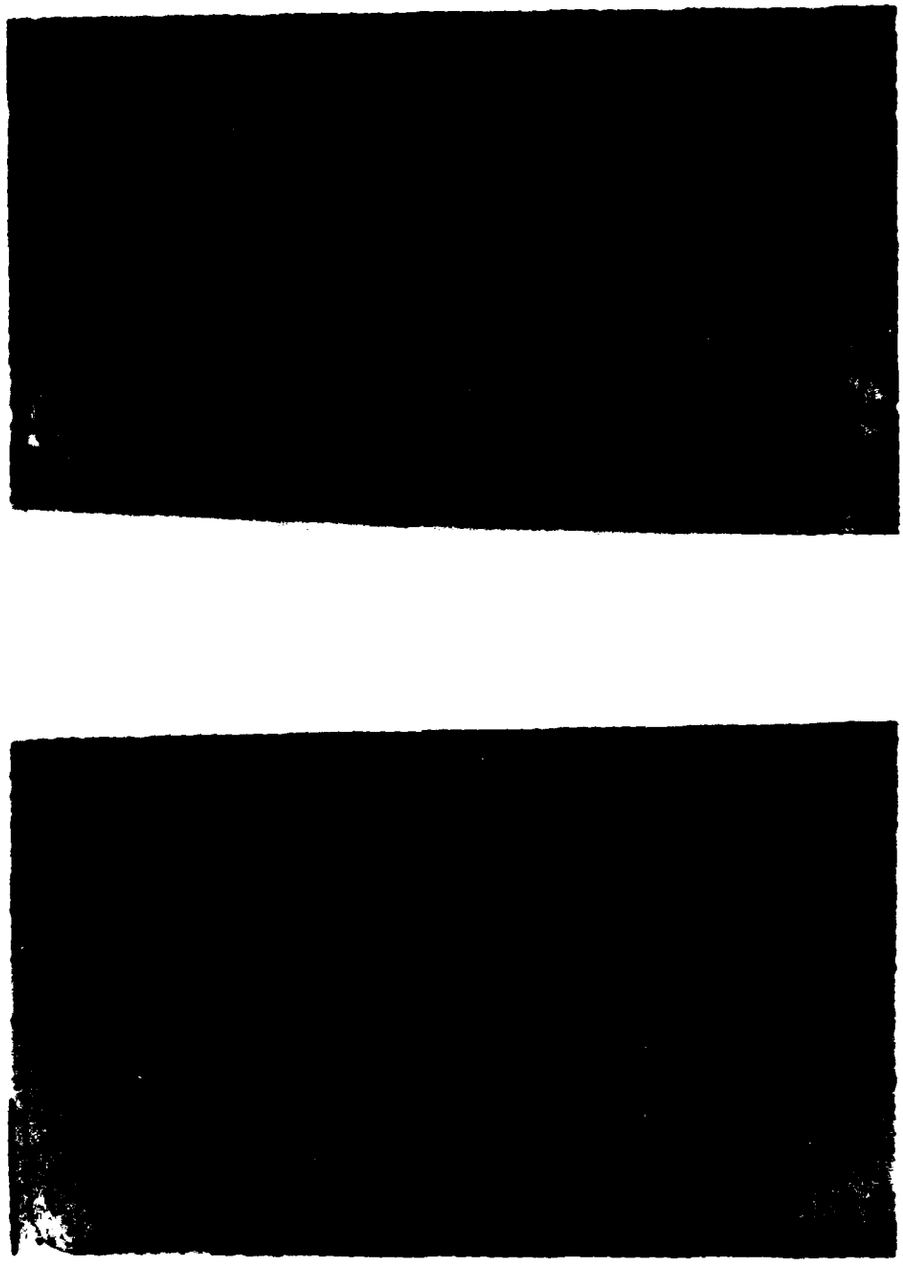



Figure 5. Comparisons of cell culture at 2 weeks obtained from a Percoll gradient and stained with rhodamine blue (2X actual size). Each culture plate was inoculated with $3.5 \times 10^6$ viable cells (trypan blue exclusion). The pellet obtained from this gradient yielded abundant epidermal colonies with few fibroblasts (A), whereas the interface yielded abundant growth of fibroblasts with few epidermal colonies (B).

## E. REFRENCES


1. Yannas, I.V. and Burke, J.F., J. Biomed. Mater. Res. 14, 65 (1980).
2. Yannas, I.V., Burke, J.F., Gordon, P.L., Huang, C. and Rubenstein, R.H., J. Biomed. Mater. Res. 14, 107 (1980).
3. Dagalakis, N., Flink, J., Stasikelis, P., Burke, J.F. and Yannas, I.V., J. Biomed. Mater. Res. 14, 511 (1980).
4. Chen, E.H., M.S. Thesis, MIT, 1982.
5. Yannas, I.V., Burke, J.F., Orgill, D.P., and Skrabut, E.M., Polymeric Materials and Artificial Organs, Gebelein, G. (ed.) ACS Books, Amer. Chem. Soc., Washington, D.C. 1984
6. Yannas, I.V., Salzmman, E.W., Sylvester, M.F. and Forbes, M.J., Polymer Preprints, Div. Polymer Chem., Amer. Chem. Soc., 14(1), 17 (1983).
7. Forbes, M.J., Engineers Thesis, MIT, 1980.
8. Sylvester, M.F., M.S. Thesis, MIT, 1982.
9. Yannas, I.V., Burke, J.F., Orgill, D.P. and Skrabut, E.M., Science 215, 174 (1982).
10. Orgill, D.P., Ph.D. Thesis, MIT, 1983.
11. Streilein, J.W. and Bergstresser, P.R., Transplant. 30, 319 (1980).
12. Streilein, J.W., McCulley, J. and Niederkorn, J.Y., Invest. Ophthalmol. 23, 489 (1982).
13. Sun, T-T. and Green, H., Nature 269, 489 (1977).
14. Catsimpoolas, N., Griffith, A.L., Skrabut, E.M., and Valeri, C.R., Analyt. Biochem. 87, 243 (1978).
15. Yannas, I.V. and Skrabut, E.M., Annual Summary Report, DOD Contract No. DAMD17-83-C-3203, Sept. 1984.
16. Yannas, I.V., Burke, J.F., Warpehoski, M., Stasikelis, P., Skrabut, E.M., Orgill, D. and Giard, D.J., Trans. Am. Soc. Artif. Intern. Org. 27, 19 (1981).
17. Yannas, I.V., in: The Surgical Wound, Dineen, P., ed. Lead and Febiger, Philadelphia, 1981 .
18. Burke, J.F., Yannas, I.V., Quinby, W.C., Bondoc, C.C. and Jung, W.K., Ann. Surg. 194, 413 (1981).
19. Morhenn, V.B., Starr, E.D., Terrill, C., Cox, A.J. and Engleman, E.G., J. Invest. Dermatol. 78, 319 (1982).
20. Silver, F.H., Yannas, I.V. and Salzman, E.W., Thromb. Res. 13, 267 (1978).
21. Silver, F.H., Yannas, I.V. and Salzman, E.W., J. Biomed. Mater. Res. 13, 701, (1979).

END

2-87

DTIC