

QUESTION 2

THE EXAMINATION PAPERS FOR QUESTION 2 CONSIST OF:

Paper 1: Text of Question (8 sheets)

Paper 2: Examples from client (3 sheets)

Paper 3: European Patent No. 0 243 132 (5 sheets)

Paper 4: Journal of Tissue Trauma, October 1988 (5 sheets)

QUESTION 2

PAPER 1

Page 1 of 8

TEXT OF QUESTION

You are consulted by a Professor Finn, a well-known, and respected skin specialist on yet another possible invention. He is a most prolific inventor.

Professor Finn tells you that his latest developments relate to living skin equivalents. These find use as replacement for human skin for skin grafts, and also as test skin for determining the effects of pharmaceutical substances, cosmetics, and other agents on skin.

Professor Finn explains to you that a major difficulty in pharmacological, chemical and cosmetic testing is that associated with determining the efficiency and safety of products on skin, and that one real advantage of his new proposal is that his skin equivalent can be used to demonstrate the effects of these agents as though they were tested on an individual's skin.

Professor Finn says that the whole area of skin grafts is a real problem notwithstanding advances over recent years. Grafting of skin onto denuded areas of wounds and Finn still presents major healing problems. The skin grafts often show limited tissue expansion, require repeated surgical operations and protracted hospitalisation to give rise to desired cosmetic results. He explains that one reason for this is that skin taken from other areas of the body often "takes" badly at the site of tissue damage.

QUESTION 2

PAPER 1

Page 2 of 8

Professor Finn also says to you that skin grafts from a patient take time to be produced, have a relatively low success ("take") rate of between 30-50%, often form spontaneous blisters, and are fragile and difficult to handle. He goes on to say that skin grafts are of little use in the treatment of very deep Finn where both the dermis (inner skin layer) and epidermis (outer skin layer) have been destroyed. You indicate to Professor Finn that you are not too sure on the structure of skin, and he says that the dermis is the cellular layer underneath the epidermis, which is the outermost portion of the skin.

You ask Professor Finn about alternative approaches to skin grafts. He says one alternative treatment is so-called "epidermal allografts" using cultured keratinocytes (skin cells from the outer skin layer). He says that American researchers have treated patients with second degree Finn by grafting these allografts onto wounds with some success. He says the benefits of this procedure includes a ready supply of grafts can be maintained through culture, patients can be treated in a single procedure and Finn covered with cultured allografts heal well. However, Professor Finn says that epidermal allografts still experience many of the limitations of convention skin grafts.

Professor Finn says there is a real need for the development of living skin equivalent grafts which have both an epidermal layer corresponding to the outermost layer of skin, and a dermal layer corresponding to the inner layer of cells in human skin which can be easily prepared and maintained in sufficient quantities to enable a single treatment of skin wounds such as Finn, and for testing.

QUESTION 2

PAPER 1

Page 3 of 8

Professor Finn tells you that his proposal for a skin equivalent has an epidermal layer of cultured keratinocyte cells which corresponds to the outermost layer of the skin. Professor Finn tells you that the epidermal layer of cultured keratinocyte cells can be readily prepared by known methods. These involve taking a skin sample, treating the skin sample enzymatically to separate the epidermis from the dermis, treating the epidermis enzymatically to release the keratinocyte cells, culturing the keratinocytes until they have multiplied extensively, whereafter the keratinocytes cells spread and eventually coalesce to give rise to an epidermal layer.

Professor Finn says that he also uses a layer of high purity, non-porous collagen. Professor Finn tells you that the collagen is highly purified. He says the collagen is preferably type 1 collagen, type 2 collagen, or mixtures of type 1 and type 2 collagen. Professor Finn tells you that human or bovine collagen is commercially available and either can be used in the skin equivalents.

Lastly, Professor Finn tells you that his skin equivalent includes a dermal layer which is in the form of a porous, cross-linked collagen sponge. The collagen sponge contains cultured fibroblasts. Any commercially available cross-linked collagen sponge can be used. Professor Finn tells you that cultured fibroblasts are prepared from a dermal sample of skin using the enzyme collagenase to release the fibroblast cells. The released fibroblast cells are recovered, and inoculated into culture flasks to allow cell division and multiplication. The collagen sponge is inoculated with the cultured fibroblast cells, at a preferred density of about 4×10^6 cells per ml. Professor Finn tells you that the inoculated sponge is incubated using standard methods to enable the growth of

QUESTION 2

PAPER 1

Page 4 of 8

the fibroblast cells throughout the collagen matrix. He says that this can be readily achieved by incubation, for example at 30°C for about 5 to 10 days in an appropriate cultured medium. Professor Finn gives you an example of cultured medium which is available under the commercial acronym BEMBEM. He explains that this medium is simply a physiologically stable isotonic medium which encourages cell growth.

Professor Finn tells you that after the sponge has been colonised by the fibroblast cells, it is inverted and the upper surface is laminated or treated with the non-porous collagen. The cultured keratinocytes may then be inoculated onto the collagen layer. Professor Finn indicates to you that the cells are preferably inoculated onto the collagen at a density of about 1×10^6 cells per drop. Professor Finn tells you that the resultant skin equivalent is incubated at around 33°C in a suitable growth medium for about 3 to 12 days, with the skin equivalent remaining immersed in the cultured medium throughout this incubation period.

Professor Finn tells you that after the incubation period, the resultant skin equivalent is essentially indistinguishable from human skin.

Because of the way the skin equivalent is produced a large surface area of skin equivalent can be prepared, to enable the coverage of a wide area of Finn.

On application to a wound or burn Professor Finn tells you that the collagen sponge provides a matrix which enables rapid and sustained adherence to the surface of the wound or burn that allows blood vessel and tissue ingrowth from

QUESTION 2

PAPER 1

Page 5 of 8

the wound's surface thus integrating the skin equivalent to the transplant site. Professor Finn indicates to you that it is most desirable that the collagen sponge is cross-linked. This is because the sponge does not contract and therefore the skin equivalent does not contract when drafted onto a wound leaving gapes or gaps which cause infection or are otherwise unsightly.

Professor Finn tells you that the non-porous collagen layer prevents invasion of the collagen sponge by the cultured keratinocyte cells and thus ensures the compartmentalisation of the skin equivalent into epidermal and dermal layers. Professor Finn again tells you that the laminate layer is gradually broken down to allow the normal interface between the dermis and epidermis to form.

You ask Professor Burn how thick the skin equivalent is and he says to you that it is in the order of 0.5 to 0.9mm thick and therefore much stronger than conventional skin grafts which facilitates ease of handling and grafting. He says that the take rate for these skin equivalents is in the order of 95% which he says is at least in part attributed to the presence of the collagen sponge dermal layer which promotes rapid integration and vascularisation of the graft.

Professor Finn tells you that the skin equivalent may be frozen, this allowing ready transport and overall use of the skin equivalent.

You ask Professor Finn if he has any examples and he says that he has. These form Paper 2.

QUESTION 2

PAPER 1

Page 6 of 8

Professor Finn ask you to carry out a patent search and to then go ahead and file a patent application. He asks you to prepare the patent application taking into account what you find in your search.

As an afterthought Professor Finn mentions to you several things.

Firstly Professor Finn says that he is aware of some proposals which involve the use of chondroitin-6-sulphate (GAG). He says that these proposals, as far as he is aware, have the potential to cause unforeseen long-term problems by virtue of the GAG being released into the wound and causing scar formation.

Professor Finn says he is also aware of some proposals which involve preparing collagen sponges by cross-linking with glutaraldehyde, whereas collagen sponges that are used in Professor Finn' invention are prepared by naturally cross-linking in air, such as by preparing a collagen solution at physiological pH, and blowing air or CO₂ through the solution of collagen until a sponge forms. Professor Finn tells you that the cross-linking agent glutaraldehyde is well-known to inhibit cell growth. He says glutaraldehyde is probably cytotoxic, that is, it kills cells in tissue culture and for this reason it is important to avoid this agent.

In relation to the fibroblast cells which colonise the collagen sponge, Professor Finn tells you that he gets exceptional results when these fibroblast cells are obtained from human foreskin, that is following the standard operation of circumcision in baby boys. He says that fibroblasts recovered by enzyme (collagenase) treatment of the foreskins obtained about 6 to 12 days after birth

QUESTION 2

PAPER 1

Page 7 of 8

have exceptional properties which give an outstanding character to the skin equivalent. Having said this, Professor Finn says that fibroblasts prepared from other areas of skin perform quite adequately.

Professor Finn also mentions to you that when he mixed type 1 and type 2 collagen together he also got excellent results compared with skin equivalents using type 1 or type 2 collagen alone.

Professor Finn says he does not really know why he gets these potentiated effects. He suspects that there was some totally unexpected interaction flowing from the use of the type 1 and type 2 collagen and/or the use of cultured fibroblasts from human foreskin.

In line with Professor Finn' instructions you carry out a patent search which reveals two articles, attached marked Paper 3 and Paper 4.

QUESTION 2

PAPER 1

Page 8 of 8

**PLEASE READ THE FOLLOWING STANDARD
INSTRUCTIONS CAREFULLY**

Candidates are required to draft a complete specification for an Australian Standard patent. Candidates should accept as correct the information given in the question relating to technical aspects of the prior art and the client's development, but candidates should exercise their own judgement in relation to non-technical statements made by the client.

Candidates should judge the novelty of the client's development against the prior art supplied and should not have regard to any personal knowledge they may have concerning similar subject matter.

The specification should comply with Sub-Sections (2) and (3) of Section 40 of the Patents Act 1990, and should address the prior art and advances made.

All claims must be novel over the prior art and should exhibit at least a scintilla of invention. Marks will not be awarded for claims directed to features which are not material to the invention under consideration and the inclusion of such claims may affect the Examiners' assessment of the candidate's answer paper.

Care should be taken with expression in all aspects of the paper and legible writing in ink will greatly assist the Examiners to assess candidate's answer papers.

(100 marks)

END OF QUESTION 1

QUESTION 2

PAPER 2

Page 1 of 3

EXAMPLES FROM CLIENT

Example 1

Composite skin equivalents were made from separate, parallel cultures of human keratinocytes (HK) and human fibroblasts (HF) and a cellular bovine collagen sponge. The collagen sponge was modified using type 1 bovine collagen to provide a planar surface for cultured HKs.

Human skin was obtained from surgical specimens. After the excision the skin was placed in a sterile container with Dulbecco's modified Eagle's medium (DMEM). Specimens were delivered within a short time to the tissue culture laboratory where the epidermis was separated from the dermis enzymically according to standard procedures.

Keratinocytes were cultured as a single cell suspension by the method of Smith, as follows: HKs were cultured in DMEM supplemented with 2% foetal bovine serum, epidermal growth factor, insulin and hydrocortisone at pH 7.2. Confluent cultures were ready for harvest after 12 to 14 days either for subculture or for inoculation onto the collagen surface.

Fibroblasts were released from dermal fragments by digesting these with the enzyme collagenase. The fibroblasts were then grown in DMEM using standard methods.

The collagen sponge membranes (6 cm diameter), stored frozen in Petri dishes and prepared by gentle crosslinking of collagen fibrils, for example at pH 7.2 in the presence of CO₂, were washed with sterile water. The sponges were then

QUESTION 2

PAPER 2

Page 2 of 3

incubated overnight in DMEM supplemented with conditioned medium. Cultures of fibroblasts were inoculated at a density of 5×10^5 cells/ml onto the surface of the sponge. The sponge was then placed in DMEM in an incubator at 37°C , 5% carbon dioxide and saturated humidity for 10 days.

Medium was changed every second day. At the end of that period the sponges were turned over and a non-porous collagen surface was prepared to provide a planar surface for cultured keratinocytes.

Lamination was performed by applying a thin film of non-porous, sterile type 1 bovine collagen. The films were prepared by commercially available purified type 1 collagen which was sterilised by Gamma radiation. The collagen solution was brought to neutral pH and applied as a thin film onto the surface of the collagen sponge and incubated for 60 minutes at 37°C .

Cultured keratinocytes were then inoculated in drops at a density of 1×10^5 per drop onto the nonporous surface of the sponge and incubated for 10 days in DMEM with supplements mentioned above. Vaseline gauze was placed over the cultured graft to facilitate the transporting and securing of the graft to the wound bed.

In 8 operations performed on 5 children using the living skin equivalents of the present invention, the success rate was 90%. This was raised to nearly 100% using human foreskin fibroblasts or a non-porous collagen layer which is a mixture of type 1 and type 3 collagen.

QUESTION 2

PAPER 2

Page 3 of 3

Example 2

The skin substitute of Example 1 was used to test four cosmetics for skin safety. The cosmetics were applied onto the epidermal layer. All agents were found to be well tolerated and caused no adverse reaction. This obviated animal testing.

QUESTION 2

PAPER 3

Page 1 of 5

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Artificial skin and its production

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QUESTION 2

PAPER 3

Page 2 of 5

Description

The present invention relates to an artificial skin, for transplanting onto a wounded skin surface such as a burned or scratched skin surface, and to its production.

Conventionally, artificial skins made of collagen and are used in practice as temporary wound-covering materials.

Cell-incorporating artificial skins having biocompatibility are proposed by E. Bell (Science 211, 1052 (1981)) and I.V. Yannas *et al* (Science 215, 174 (1982)). However, it takes a long cell culture period (about one month) to obtain an artificial skin by this method and the size of the artificial skin piece obtainable is limited since the agar-like collagen serving as the cell carrier is soft; accordingly these artificial skins are not used clinically.

Currently, a method for fast culturing cells is proposed by Gallico G.G. *et al* (NEJM, 311, 448-451 (1984)). According to this method, epidermal cells isolated from a skin piece are cultured using 3T3 (an established cell strain obtained from a Swiss mouse subcutaneous tissue) as supporting cells, and the cultured epidermal sheet is treated with dispase and removed from the supporting cells. The sheet is applied onto an ointment-coated gauze for transplantation.

In this case, although the cell culture period is shortened, the supporting cells remaining in the epidermal sheet adversely affect the recipient's body. This method also has the following defects:

QUESTION 2

PAPER 2

Page 3 of 5

1. The epidermal sheet must be removed by dispase treatment.
2. The are of the epidermal sheet is reduced to about $\frac{1}{2}$ the original area upon removal.
3. A carrier, such as a gauze, for the epidermal sheet is additionally required.
4. Scarring remains.

According to the present invention an artificial skin comprises an insoluble crosslinked atelocollagen sheet and an epidermal cell layer (comprising keratinocytes) associated therewith.

The insoluble atelocollagen sheet used in the present invention is obtainable by air-drying an atelocollagen solution and treating the resulting sheet by molecular crosslinking by chemical crosslinking agents such as glutaraldehyde treatment to give a sponge-like material. The term "atelocollagen" used herein means a collagen which is obtained by treating collagen with protease, such as pepsin, in an aqueous acid solution, such as aqueous acetic acid.

The artificial skin of the present invention can be prepared by the following method:

An atelocollagen solution is poured into an appropriate container, air-dried, and subjected to chemical crosslinking with glutaraldehyde to form an insoluble

QUESTION 2

PAPER 2

Page 4 of 5

atelocollagen sheet. Epidermal cells are inoculated only on one surface of the sheet. Subsequently, the resultant sheet is suspended in a liquid culture medium and the epidermal cells are cultured to form an epidermal cell layer, thereby producing an artificial skin of the present invention.

The atelocollagen sheet used preferably has a high permeability in order to shorten the culture period for the epidermal cells, that is allow nutrient flow to the epidermal cells (keratinocytes).

In the artificial skin according to the present invention, the epidermal cell layer corresponds to the epidermal layer of the living body, and the atelocollagen sheet layer corresponds to the dermis layer of the living body.

The artificial skin according to the present invention can have the following advantages:

- (a) The supporting cells do not adversely affect the recipient's body.
- (b) There is no need for enzymatic treatment such as dispase treatment, with consequent loss of artificial skin area.
- (c) No additional supporting body is required for the epidermal sheet.

The present invention will be more clearly understood by reference to the following example; however, the example is intended to illustrate the invention is not to be construed to limit the scope of the invention.

QUESTION 2

PAPER 3

Page 5 of 5

Example

An acidic solution containing 0.3 to 1.0% of a bovine dermis atelocollagen prepared in a germ-free manner was poured into a polystyrene mould frame (9 x 18 x 1 cm) such that the solution level was 1 to 5 mm, and was crosslinked using gluteraldehyde and air dried. This sheet was cut to give a circular sheet with a diameter of 25 mm. About 1×10^5 epidermal basal cells isolated from the skin of a newborn rat were inoculated only on one surface of the circular sheet. The circular sheet was suspended in a liquid culture medium ("medium 199") containing a 10% foetal bovine serum, streptomycin, and a phosphoric acid buffer, and was allowed to stand in an incubator at a CO₂ concentration of 5% and a temperature of 37°C to culture the epidermal basal cells. In one week the epidermal cells covered one entire surface of the sheet. An artificial skin was thus obtained.

The artificial skin obtained was transplanted onto a skin deficiency portion of a rat of the same type as the donor rat described above. After about three weeks the skin deficiency portion exhibited good healing with minor skin contraction.

QUESTION 2

PAPER 4

Page 1 of 5

JOURNAL OF TISSUE TRAUMA

July 1987

Roos Hiss, M.D.

Early coverage of the burn wound remains an important goal. Even temporary closure of the wound, be it partial or full-thickness in depth, can result in a reduction in fluid losses, decrease in infection and improved healing. In addition, wound coverage promotes the rate of epidermal and dermal repair processes. However, the identification and development of completely satisfactory skin substitutes remains elusive. To begin the discussion: what are the characteristics to look for regarding an artificial skin and what is the status of currently available skin substitutes?

In the future, hopefully, synthetic skin replacements will be developed which will allow for final wound coverage and avoid the need for subsequent autografting.

The ideal properties of skin substitutes have been discussed before. It has become clear that both physical and biologic properties of these materials must be considered if a completely satisfactory material is to be developed. The most important criteria merit discussion here.

The material should adhere to the burn wound soon after placement on the wound surface. The material should control evaporative fluid losses from the wound and also avoid the build-up of fluid between the wound and the dressing material. It should be flexible, and should be durable and resistant to tearing. The material should act as a barrier to microorganisms and prevent bacterial

QUESTION 2

PAPER 4

Page 2 of 5

invasion from the external environment into the burn wound, as well as limit the growth of microorganisms already present in the wound. It should not provoke an inflammatory or foreign body reaction in the burn wound, which may lead to granulation tissue.

The material should have safety. It should be sterilisable and should be non-toxic. If the material is only a temporary wound covering, it should be easily removable and not cause damage upon removal. The material should be cost effective, which is going to be, of course, much more important in coming years.

The list of available materials has not changed markedly in the past 5 years. As far as biologic dressing materials for temporary wound coverings, viable human homograft, either fresh or cryopreserved, remains the standard for comparison, as evidenced by the proliferation of skin banks in this country. Amniotic membrane is still used as a number of groups but lack of durability and availability and high vapour transmission remain major problems.

Porcine skin, either fresh or frozen, continues to be used for temporary wound coverage, although some have questioned its utility. Problems with pigskin include poor adherence and infection control, early rejection, and occasional incorporation into the wound. The efficacy of pigskin in controlling infection may be improved by the incorporated of antimicrobial agents such as silver nitrate into the material. Various tissue biologic derivatives such as collagen sheets, mats, fabric, or sponges have been studied as wound dressings. Although many collagen materials adhere well initially to the wound, collagen

QUESTION 2

PAPER 4

Page 3 of 5

is not an effective burn wound covering for several reasons. Collagen stimulates the development of granulations tissue and elicits a chronic inflammatory response before being biodegraded. Collagen sheets are not elastic and dislodged with shear stress. Also, collagen dressings fail to control the growth of microorganisms in the wound.

Finally, there are available the synthetic and composite materials which have been intensely studied in the last few years. Solid silicone membranes fulfil some of the criteria for wound coverings, and are particularly valuable because of their control of water vapour transmission. Silicone membranes alone fail to control infection in the burn wound. Various plastics such as the polyurethanes (e.g. Op-Site) and polyvinyl chlorides, in various physical forms – foams and sheets, solid or microporous – are also partially effective but generally fail to adhere to the wound or control bacterial growth.

The composite materials offer a useful alternative to wound coverings. These materials generally combine two different substrates in order to achieve the overall qualities desirable in an artificial skin covering; generally a porous material is used against the wound with a semi-permeable membrane on the exterior. One of the first of the composite dressings used was cotton gauze bonded to silicone membrane.

Over the past year the use of the composite material Biobrane (Hall-Woodroof) has been investigated for temporary wound coverage. Biobrane is a knitted nylon mesh covered with a very thin silicone membrane; both layers are bonded with collagen peptides to improve adherence. Biobrane has been shown

QUESTION 2

PAPER 4

Page 4 of 5

to been an excellent temporary covering for both partial-thickness and full-thickness wounds and has many of the desirable characteristics of a temporary skin covering, particularly properties of water vapour transmission and adherence. It appears to control bacterial proliferation in clean, debrided wounds and appears to be associated with minimal inflammation in the burn wounds. The combination of antimicrobial agents with Biobrane may prove to control bacterial growth and make this material more useful for contaminated wounds.

As mentioned previously, native collagen is not an effective wound covering material. Dr Burke and Dr Yannas have developed an "artificial skin" consisting of glutaraldehyde collagen and glycosaminoglycan (GAG) artificial dermis covered with a Silastic (a silicone rubber material) "epidermal" component. This material, first and very importantly, has biochemical characteristics of controlled rate biodegradation and it is neither inflammatory nor immunogenic, and second, possesses physical characteristics, namely, proper pore size and pore orientation of the crosslinked collagen GAG sponge that are equally important to allow for cell migration into the material and provide proper subsequent collagen fibre orientation. Vascularization of the collagen framework occurs in 3 to 5 days to form a "neodermis"; at a later time the Silastic epidermis is peeled off and the neodermis is covered with a thin skin graft to provide the epidermal component for the wound. Wound contracture and subsequent scar formation appear to be minimal, although long-term follow-up results are not yet available.

QUESTION 2

PAPER 4

Page 5 of 5

The limitations of this material, however, have also been discussed by Dr Burke. The first limitation is premature loss of the Silastic layer. This leads to subsequent damage of the neodermis below, followed by the development of granulation tissue, which is undesirable, and ultimately leads to a suboptimal functional and cosmetic result. Secondly, subsequent autografting of the wounds covered with the collagen-GAG neodermis is necessary to restore the epidermal layer and achieve permanent wound closure. GAG may also leak from the structure.

In other experiments, made possible by the very generous donation of samples of the collagen-GAG membrane by Drs Burke and Yannis to this laboratory, the growth of epidermal cells on that membrane have been studied. The area of the collagen-GAG membrane covered by cultured epidermal cells has been quantitated and is shown to be excellent.

Finally, we have observed that when the epidermal cells are inoculated onto the collagen-GAG membrane, they don't all remain on the surface. It appears that a substantial fraction of these epidermal cells migrate down into the collagen-GAG membrane, which is an undesirable result. It appears that we must make further efforts to insure that the cells grow only on the surface of the membrane.

In conclusion, it is believed that cultured keratinocyte and collagen-GAG composites are a "feasible alternative" for the development of an autograft substitute. However, further modifications of the culture conditions and of the collagen-GAG membrane will be required to fully optimise keratinocyte growth on the membrane.