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WOUND COVERAGE BY CULTURED SKIN CELLS

ANNUAL/FINAL SUMMARY REPORT

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Our methodology included using different tissue culture techniques. <u>In vitro</u> quantitative data were obtained by cell counts and by incorporation of radioactively labelled precursors. We also obtained qualititative data by light and electron microscopy. Studies <u>in vivo</u> were performed on experimental animals -- swine, horses, and dogs. Careful visual observations were documented histologically and by photography. They were supplemented by immunological approaches such as immuno-rosetting and immunoprecipitation, and molecular probing using <u>in situ</u> hybridization.

We evaluated the use of collagen matrices for growth of epidermal cells <u>in vitro</u> and <u>in vivo</u>. The plating efficiency of epidermal cells was about four times higher on collagen than on plastic. Epidermal cells grown on collagen sponges formed four to five layers of nucleated cells, compared to only one layer on plastic surfaces. The use of collagen did not impair the take of epidermal grafts.

Fibroblasts grown in collagen promoted healing of deep wounds. Clinical and histological observations suggested that fibroblasts grown <u>in vitro</u> can be transplanted allogeneically without noticeable rejection.

Autologously grown epidermal cells were transplanted to wounds of different depths. Wounds over 75/1,000 inch also needed dermal support to heal without scarring.

Studies evaluating the effects of dermal extracts suggested that for successful allogeneic transplantation of epidermal cells, the dermal extract has to be derived from the prospective recipient. In addition, the dermal extract, in 20% concentration, has to be added 48 hours after initiation of cultures.

Successful transplants of allogeneic epidermal cells were followed by split thickness skin transplants from the same donor, resulting in allogeneic graft take. The results suggested that allogeneically transplanted "treated" epidermal cells can induce specific tolerance. Allogeneic transplantation of epidermal cells "treated" by a third party donor resulted in a significant prolongation of allograft survival, but rejection occured 22 days post transplantation.

Pig and human epidermal cells grown <u>in vitro</u> were shown to contain and secrete factors (EDF) that stimulate proliferation of keratinocytes, but inhibit fibroblasts. <u>In</u> <u>vitro</u>, the effect of EDF on epidermal cells resulted in an increased number of rapidly proliferating colonies composed mainly of basal keratinocytes. Control cultures grown in the absence of EDF had a high proportion of terminally differentiated cells.

In fibroblast cultures, EDF inhibited the ability of fibroblasts to cause contraction of collagen sponges by 90%. Epidermal growth factor, basic fibroblast growth factor, platelet-derived growth factor, transforming growth factor Beta, nerve growth factor, and extracts of WI-38 cells (human embryonic lung fibroblasts) did not have this inhibitory activity.

Application of EDF to surgical wounds stimulated extensive migration and proliferation of keratinocytes from remnants of glands, hair follicles, and wound edges. The restoration of complete epidermal coverage of wounds treated with EDF occurred twice as rapidly as that of control wounds. In addition, regenerating dermis in the EDF-treated wounds contained 1/5 to 1/15th as many cells as wounds treated with epidermal growth factor, urogastone, transforming growth factor, or phosphate-buffered saline. The role of this factor in preventing scar formation is now investigated.

When we studied skin specific antigens to understand the mechanisms of allograft rejection, we found that three minor histo-compatibility antigens are expressed on keratinocytes, but not on lymphocytes, fibroblasts, or melanocytes. Two of these antigens were lost during cultivation <u>in vitro</u>. We also found no correlation between antigen expression and age, sex, cell differentiation or MHC.

We also examined the potential role of Ia antigen expression in graft rejection. Using a new <u>in vitro</u> "transplantation" model, we found that epidermal cells grown in tissue culture and exposed to allogeneic or xenogeneic dermis can be induced to Ia expression. Induction by allogeneic dermis occured in 48 hours, peaking in 4 days, while induction by xenogeneic dermis peaked in 8 days. While an autologuous combination induced 2 - 5% of the cells to express Ia, an allogeneic or xenogenic combination induced 20 -80% expression. Using <u>in situ</u> hybridization, we showed transcription of the gammainterferon gene. This novel finding further established the possible immune role of keratinocytes in graft rejection.

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#### SUMMARY

At the conclusion of a three year study on ways to improve wound healing by cultured epidermal grafts, we have found that:

- We were able to grow epidermal cells on collapsed collagen sponges. As a result, we can create a new transplant, of better quality and easier to handle in clinical settings, with a quarter of the donor's skin tissue previously required.

- We identified a factor in epidermal cells shown to stimulate wound healing and to prevent scar formation.

- We can successfully transplant epidermal cells autologously and allogeneically.

- We have identified three skin specific antigens, and have been able to temporarily eliminate two of them in pig epidermal cells during cultivation in vitro.

- Class II antigens, which contribute to skin graft rejection, are autoinduced by gamma interferon, rather than by lymphocytes, as previously thought.

Our methodology included using different tissue culture techniques. <u>In vitro</u> quantitative data were obtained by cell counts and by incorporation of radioactively labelled precursors. We also obtained qualititative data by light and electron microscopy. Studies <u>in vivo</u> were performed on experimental animals -- swine, horses, and dogs. Careful visual observations were documented histologically and by photography. They were supplemented by immunological approaches such as immuno-rosetting and immunoprecipitation, and molecular probing using <u>in</u> <u>situ</u> hybridization.

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Studies evaluating the effects of dermal extracts suggested that for successful allogeneic transplantation of epidermal cells, the dermal extract has to be derived from the prospective recipient. In addition, the dermal extract, in 20% concentration, has to be added 48 hours after initiation of cultures.

Successful transplants of allogeneic epidermal cells were followed by split thickness skin transplants from the same donor, resulting in allogeneic graft take. The results suggested that allogeneically transplanted "treated" epidermal cells can induce specific tolerance. Allogeneic transplantation of epidermal cells "treated" by a third party donor resulted in a significant prolongation of allograft survival, but rejection occured 22 days post transplantation.

Pig and human epidermal cells grown <u>in vitro</u> were shown to contain and secrete factors (EDF) that stimulate proliferation of keratinocytes, but inhibit fibroblasts. <u>In vitro</u>, the effect of EDF on epidermal cells resulted in an increased number of rapidly proliferating colonies composed mainly of basal keratinocytes. Control cultures grown in the absence of EDF had a high proportion of terminally differentiated cells.

In fibroblast cultures, EDF inhibited the ability of fibroblasts to cause contraction of collagen sponges by 90%. Epidermal growth factor, basic fibroblast growth factor, platelet-derived growth factor, transforming growth factor Beta, nerve growth factor, and extracts of WI-38 cells (human embryonic lung fibroblasts) did not have this inhibitory activity.

Application of EDF to surgical wounds stimulated extensive migration and proliferation of keratinocytes from remnants of glands, hair follicles, and wound edges. The restoration of complete epidermal coverage of wounds treated with EDF occurred twice as rapidly as that of control wounds. In addition, regenerating dermis in the EDF-treated wounds contained 1/5 to 1/15th as many cells as wounds treated with epidermal growth factor, urogastone, transforming growth factor, or phosphate-buffered saline. The role of this factor in preventing scar formation is now investigated. When we studied skin specific antigens to understand the mechanisms of allograft rejection, we found that three minor histocompatibility antigens are expressed on keratinocytes, but not on lymphocytes, fibroblasts, or melanocytes. Two of these antigens were lost during cultivation <u>in vitro</u>. We also found no correlation between antigen expression and age, sex, cell differentiation or MHC.

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#### FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

Studies with human subjects are not a part of this contract. Results in this report obtained in collaboration with other investigators are only for illustration and are quoted with the approval of the collaborators.

#### A. <u>GROWTH OF EPIDERMAL CELLS ON COLLAGEN SPONGES</u>

We prepared sheets of epidermal cells used for transplantation by growing single cell suspensions of human or pig epidermal cells in tissue culture vessels. Upon reaching confluency, the cultures began to stratify, becoming multilayered in 2-3 weeks.

Such cultures in general displayed 1-2 layers of viable epidermal cells, morphologically resembling basal and suprabasal cells of normal epidermis, and 12-14 layers of keratinized anucleated cells. In following four to five months, numbers of keratinized cells increased, reaching up to 60 layers.

Multilayered sheets of epidermal cells were either peeled from the plastics, or detached from the surface of tissue culture flasks, using enzymes such as Dispase. They were subsequently applied to surgically prepared full thickness wound beds.

Both procedures required much time and highly trained personnel

In an attempt to simplify these procedures, we at first evaluated growth of epidermal cells on different types of collagens. In collaborative approach with F. Silver of Rutgers University, we coated plastic Petri dishes with type I, III and IV collagens and evaluated epidermal cell attachment and growth. These preliminary experiments showed all above collagens to be suitable for growth of epidermal cells, and led to experiments using collagen sponges as a substrate.

When single cell suspensions of primary epidermal cells were seeded on prefabricated collagen sponges made from type I bovine collagen having interstices of a pore size from 3 to 100 um, epidermal cells were lost in interstices and a confluent layer was not obtained.

We therefore attempted to coat such collagen sponges with collagen Type III and IV, assuming that the collagen could provide a more suitable environment and fill the interstices. In a series of experiments in which we used different amounts of coating material, we have shown that even with coating of collagen sponges with Type III or IV collagen, epidermal cells did not attach and spread. We later coated collagen sponges with human or porcine plasma. Although this coating improved the plating of epidermal cells, it did not do so sufficiently.

In different series of experiments, collagen sponges both uncoated and coated with type III and IV collagen, were collapsed and subsequently used to grow epidermal cells. Labeling experiments, measuring <sup>3</sup>H-thymidine uptake by epidermal cells revealed that uncoated collapsed collagen sponges were equal to coated collapsed sponges in their support for growth of epidermal cells. Since coating of collagen sponges adds cost to preparation of such materials while not offering growth advantage for epidermal cells, we used collapsed type I collagen sponges. (Documentation for these findings was provided in our Annual Report dated January 9, 1987.)

We have shown by electron microscopy that cells grown on collapsed collagen sponges form multilayered structures containing 3-4 layers of live nucleated cells adjacent to the collagen matrix. These are combined with an additional 6-7 layers of keratinized cells. Such grafts are therefore superior to the ones grown on tissue culture plates in which only one layer of nucleated cells was generally found.

Furthermore, when cells grown on non-collapsed collagen sponges were transplanted and punch biopsies taken a week after transplantation, we observed epidermal cells growing within the collagen matrices and forming epidermal cysts. Epidermal cells grown on collapsed collagen sponges provided uniform epidermal cover of the surface of a third degree wound. Their morphological features resembled normal epidermis (documented in year's Annual Report dated January 9, 1987). Autologous epidermal cells grown on collapsed collagen sponges were also successfully used for treatment of patients with epidermolysis bullosa (3b).

We recommend the use of the cells grown on collapsed collagen sponges over the use of epidermal sheets grown on plastic for the following reasons:

1) Only 1 x  $10^6$  cells are needed to cover an area of 38.5 cm<sup>2</sup>, compared to 3 x  $10^6$  cells needed to cover 25cm<sup>2</sup> tissue culture plastic,

2) Cells grown on the collagen contain multiple layers of

viable, nucleated cells, compared to only one layer found in multilayered sheets of epidermal cells grown on plastic surfaces,

3) It is much simpler to prepare collagen-epidermal cells as grafts for transplantation, compared to preparation of epidermal sheets grown on plastic.

4) They can be better handled in a clinical setting.

#### B. GROWTH OF FIBROBLASTS IN COLLAGEN SPONGES

To assess provision of dermal elements for treatment of deep wounds (corresponding to third degree of burn), we have studied growth of fibroblasts in collagen sponges and their transplantability.

To shorten the incubation time required for growth of fibroblasts into a three dimensional matrix, and to avoid partial degradation of collagen by the collagenase produced by fibroblasts, we have chosen high seeding density of fibroblasts for the collagen sponges. A collagen sponge (2 mm thick) 80 mm in diameter was seeded with 3-4 x  $10^6$ fibroblasts. (Number of cells seeded in these sponges is 6-8 times higher than used for seeding a plastic dish of the same size.)

As documented in Annual Report dated January 9, 1987, human fibroblasts seeded onto collagen sponges underwent DNA synthesis 3-4 days after initial seeding. This DNA synthesis was partially inhibited by ascorbic acid, added to such cultures to increase collagen synthesis.

Growth of fibroblasts in collagen sponges resulted also in collagen shrinkage. The capability of fibroblasts to cause shrinkage of collagen slightly varied between individuals, but to a greater extent differences were observed between human and pig fibroblasts. Human fibroblasts seeded into a collagen sponge caused ~ 30% contraction while pig fibroblasts seeded at the same density caused ~50% shrinkage

When shrunken collagens were transplanted into full thickness wounds on the pig (93/1000 inch) and the control area was covered by collagen without cells, the wound with collagen and cells was filled with connective tissue in three days and looked well vascularized. The control area was still depressed and did not appear to be as well vascularized. In seven days the area which received fibroblasts was starting to have epidermal ingrowth from the side of the wound, while the control was covered by fibrin and was grayish and still slightly depressed. The difference in the healing was obvious even 16 days post grafting.

There was however, no significant difference in wound closure whether the cells were present or collagen itself was used. The wound originally covered with collagen and cells shrunk from  $28 \text{cm}^2$  to  $15 \text{cm}^2$ , and the one with collagen and cells from  $54 \text{cm}^2$  to  $27.5 \text{cm}^2$ . These experiments were repeated two times with similar results.

It was reported (1, 29, 15) that fibroblasts can be allogeneically transplanted. We therefore attempted to use allogeneic fibroblasts grown into collagen sponges in two pigs and found that they were not rejected up to 70 days post transplantation, based on clinical and histological observations. However, in these experiments we have not proven that the allogeneic cells were present in the wound after transplantation. These observations need to be confirmed and extended.

## C. <u>ATTEMPTS TO IMPROVE AUTOLOGOUS TRANSPLANTATION OF TISSUE CULTURE</u> <u>GROWN EPIDERMAL CELLS</u>

To date, over 30 pigs have been transplanted with autologous epidermal grafts. The depth of the wounds varied from 55/1000 to 110/1000 inch. All wounds were freshly excised, and the epidermal grafts were applied immediately.

Based on these experiments, we concluded that wounds ranging from 55-75/1000 inch healed very well when epidermal coverage was applied to freshly excised wounds. Wound contracture did not exceed 25% of the total surface area.

Deeper wounds (75/1000-110/1000 inch) contracted with noticeable scar formation both clinically and histologically. These findings led to attempts to use "dermal grafts" -- collagen sponges with ingrown fibroblasts -- to fill the deep wounds prior to application of the epidermal sheets. As described on page 7 of this report, "dermal graft" itself did not affect wound closure, but seemingly enhanced filling of the wound with connective tissue cells. However when wounds so treated were covered by autologous epidermal cells grown in tissue culture, the epidermal grafts "melted". The obvious conclusion was that large numbers of active fibroblasts prevented epidermal graft take. In this context it is important to realize that our previous experiments in covering freshly excised wounds vs. granulated wounds have shown superior success in epidermal graft take in freshly excised wounds.

Further evidence suggesting that combination of a "dermal" graft followed by epidermal graft does not increase dermal graft take came from studies of 10 patients with chronic ulcerous wounds (in collaboration with Dr. M. Carter, Rockefeller University). In this study one patient with traumatic injury, two patients with rheumatoid arthritis, two patients with scleroderma and 5 patients with venostasis ulcer were treated by application of autologous dermal grafts followed by autologous epidermal grafts on collagen sponges. All these patients had ulcerations of the lower extremities for several years. Their ages ranged from 39-69 years. Their ulcers failed to heal in the past with conventional treatment which included bed rest, leg elevation, supportive stockings, topical and systemic antibiotics, synthetic dressings, unna boots and split thickness skin grafts. The ulcers ranged from 3.5  $cm^2$  to 46.7  $cm^2$ .

Bacterial isolates recovered from the wounds were a mixture of gram positive and negative bacteria that included: S. aureus, E. coli, K. pneumoniae, P. mirabilis, P. aeruginosa and E. cloacae. Before applying the initial dermal graft, wounds were prepared with daily debridement and hydrotherapy until all nonviable tissues were removed and wound bed appeared vascularized. The day prior to application of the dermal graft, the wound bed was soaked with a broad spectrum antibiotic (Cefamandol 50 mg/ml) for two hours to reduce the bacterial flora. The graft was then placed on the wound under sterile techniques and firm, uniform pressure to the wound bed for 48 hours was provided by sterile pressure dressing.

Both dermal and epidermal grafts were repeated in seven day intervals if the take seemed incomplete or absent.

As can be seen from the enclosed Table (No. I) success in complete wound closure was achieved in 3 patients with a single application of a dermal graft of a combination of dermal and epidermal grafts. The three patients all had venostasis ulcers. Two of them remained healed, but the one which had the largest surface area of 46.7  $\rm cm^2$  reopened in 6 months. In the patients who have not healed completely, partial reduction in the size of the wound (~50%) was achieved. These later results are difficult to interpret as extensive care and rest might have been a contributing factor.

Because factors that inhibit fibroblast activity and stimulate epidermal engraftment are needed to increase the success rate in covering granulating wounds with epidermal grafts, further studies related to the combined application of dermal and epidermal grafts were postponed. These studies awaite the availability of epidermal derived factors we had discovered (9, 9a).

#### D. EPIDERMAL CELL DERIVED FACTOR(S)

We described in detail (9,9a) that epidermal cells grown in tissue culture secrete a factor(s) that both stimulated epidermal cell proliferation in <u>vivo</u> and in <u>vitro</u>. The factor also inhibited fibroblast proliferation/migration in <u>vivo</u> and collagen contraction by fibroblasts in <u>vitro</u>. This factor was found both in epidermal cell extracts and supernatant fluid from tissue culture grown epidermal cells.

Further studies of the effects of supernatant fluids obtained from cultured epidermal cells revealed that DNA synthesis (as shown by  ${}^{3}$ H-thymidine incorporation studies) or growth (shown by cell counts and growth curves) of fibroblasts cultured on plastic surfaces were not inhibited by this factor(s). However, addition of the factor to fibroblasts plated in collagen inhibited collagen contraction as well as migration of fibroblasts from the collagen onto plastic surfaces.

In the later experiments fibroblasts were mixed with a collagen gel (Type I-rat tail collagen) and plated in a non-tissue culture 35 mm Petri dish. In the presence of  $1 \times 10^5$  fibroblasts (suspended in 2.0 ml of tissue culture medium containing 10% FCS and 0.7ml collagen), contraction of collagen occured in 2-3 days. ~40% contraction of collagen occured in 3 days and ~70% in 7 days.

When epidermal cell supernatant was added to such cultures at the time of plating contraction was inhibited by  $\sim 67\%$  in 7 days. To assess

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whether fibroblasts maintained in collagen for 5-7 days retained their capability to migrate, contracted collagens were transferred to tissue culture coated Petri dishes and fed with fresh medium. Fibroblasts migrated from the collagen onto plastic surfaces.

However, cells exposed to the factor in collagen for 5-7 days lost their capability to migrate from collagen onto a plastic dish. To assure that fibroblasts in such gels were still viable, they were recovered from the gel by collagenase treatment and examined by trypan blue dye exclusion test. Over 97% of cells were viable. Moreover fibroblasts so recovered retained their capacity to attach to plastic surfaces and resume division.

These simple experiments provided additional evidence that the epidermal derived factor(s) affects the motility apparatus of fibroblasts, but that the effect of the factor is more profound in the presence of extracellular collagen. This finding is not suprising, as other extracellular matrix materials have been shown to either enhance or suppress growth factor activity. Naturally, it is now important to examine the effects of the epidermal derived factor(s) on the fibroblasts in collagen matrices. First, we want to measure the collagenase activity of fibroblasts in the presence of the factor. Second, we want to measure the de novo production of collagen, asking whether type I or Type III collagen is being produced.

Further in <u>vivo</u> studies with the supernatant fluids from epidermal cells also revealed that its use in combination with epidermal sheets grown in <u>vitro</u> enhances their attachment and results in formation of rete ridges already 5 days post transplantation. This finding is very important for the potential clinical use of tissue culture grown epidermal grafts . It was previously reported that the take of epidermal grafts varies largely, depending on the depth of the wounds. Most successful engraftment has been reported on wound beds corresponding to the second degree burns or donor sites.

Even in our limited clinical experience (mainly with epidermolysis bullosa patients), grafting had to be repeated 4-5 times to achieve wound coverage. We therefore believe that our findings about the effects of EDF on autologous epidermal graft take will contribute to the successfull use of tissue culture grown epidermal grafts in clinics. The experiments have thus far been performed on two pigs, with 3 additional pigs currently being grafted to confirm these preliminary observations.

#### E. TRANSPLANTATION OF ALLOGENEIC EPIDERMAL CELLS.

It has been shown previously, that Langerhans cells, which comprise a minor cell population within normal epidermis, are the main antigen presenting cells of the skin (35,36). We and others have evaluated the presence of Langerhans cells in epidermal cell cultures and shown that Langerhans cells were not detectable in epidermal cell cultures after 5 days of growth in <u>vitro</u>. This evaluation was based on morphological markers (electron microscopy), presence of cell surface markers (Ia and OKT6) and functional tests (capability of epidermal cells to act as stimulators in a keratinocyte-lymphocyte mixed reaction).

It was therefore believed, that epidermal cells grown in <u>vitro</u> may be allogeneically transplanted. Reports by Hefton et al (14, 21) and Thivolet et al (38) supported this view.

Our observations (7, 8), however, did not support these findings. Instead we found, that transplanted tissue culture grown allogeneic epidermal cells are gradually replaced by the recipient epidermal cells. This accords with the beliefs of many scientists today.

However, there is universal agreement that cultured allogeneic epidermal cells provide a good temporary cover. This cover may also deliver factors that stimulate migration and replication of the recipients epidermal cells. To this point, our recent findings about epidermal cell derived factor(s) might provide a feasible explanation (see page 10).

In view of the urgent need to provide a permanent skin coverage for patients with extensive skin damage, particularly those with over 50% of surface area burns and 3rd degree burns, we investigated the possibilities of modifying tissue cultured epidermis to achieve permanent engraftment. The studies were based on our findings that a dermal extract of the recipient can render allogeneic cells to be transplantable allogeneically. Three important questions related to the future applicability of treated epidermal grafts for patient treatment needed to be answered:

1) How important is the concentration of the dermal extract used in preparation of allogeneic epidermal grafts grown in tissue culture?

2) Can the allogeneic cells be conditioned by dermal extracts for a short period of time, just prior to transplantation?

3) Can the cells be conditioned by third party dermal extracts?

1) To evaluate whether 10% (v/v) dermal extract added to tissue culture medium 48 hours after the initiation of cultures and afterwards at each medium change will be sufficient, we transplanted four pigs with allogeneic cells.

Due to technical problems, two of the four transplanted pigs could not be evaluated The other two were followed in regular 3-5 day intervals for two months, with partial rejection observed one month after transplantation.

In previous experiments of 10 pigs and recently of two pigs, we have not observed rejection if the cells were grown in the presence of 20% dermal extracts. It therefore appears that concentration of the dermal extract is important in the induction of changes on epidermal cells required for successful allogeneic transplantation.

2) To evaluate whether epidermal cells can be conditioned by the dermal extracts for a short period of time prior to transplantation we have done the following experiments. Two pigs were transplanted with epidermal cells grown <u>in vitro</u> for six weeks and conditioned with the recipient's dermal extracts for the last two weeks of their growth <u>in vitro</u>. The grafts were rejected 8-10 days post transplantation. These results suggest that the conditioning might be most effective at the times when the cells are logarithmically growing, which is in the first three weeks after the initiation of the cultures.

An alternative explanation might be that cells which grew into multilayered sheets cannot be sufficiently exposed to the dermal extract, since keratinized layers of cells might constitute a barrier.

3) Finally, two pigs were transplanted with epidermal cells grown <u>in vitro</u> for six weeks in the presence of a dermal extract derived from an unrelated pig. The rejection was significantly delayed, occurring 22

days post transplantation. These experiments verified our original findings done two years before on two pigs.

#### F. INDUCTION OF TOLERANCE BY "TREATED" EPIDERMAL CELLS

We have observed, in the past, that pigs which accepted allogeneic epidermal cells "treated" by the dermal extract of the recipient could be transplanted with split thickness skin of the donor of epidermal cells. These experiments were done more than two years ago on three outbred pigs.

Recently, one outbred pig transplanted by conditioned epidermal cells of another outbred pig was further transplanted with split thickness skin from the same donor six weeks later . Clinical and histological observations were the following: The recipient pig RR was transplanted with epidermal cells of pig BP grown in the presence of 20% dermal extract for eight weeks. Three  $25 \text{cm}^2$  pieces of epidermis were transplanted, covered by petrolatun gauze and a pressure stent.

Autologous epidermal cells were also applied at the same time to an additional wounded area. The grafts were observed in regular 3 day intervals up to 28 days post transplantation. Both grafts took, and clinically no rejection was noticed throughout observation time.

Histological observations confirmed clinical findings; both areas covered by autologous and allogeneic cells were covered by epidermis. The area covered by autologous cells 13 days post transplantation had no signs of inflammatory response while allogeneic transplant areas had spaces occupied by inflammatory cells. There were a few randomly distributed lymphocytes but most of the inflammatory cells were macrophages.

Histological observations on biopsies taken 15 days post transplantation were similar. Forty two days post transplantation by allogeneic epidermal cells from pig BP, we applied a split thickness graft from BP covering a  $32.5 \text{ cm}^2$  area. The graft was accepted and the area looked healed four days post transplantation.

Histologically, four days post transplantation the epidermal cells looked in good condition, both well defined and tightly attached to the underlying dermis. There was a layer of inflammatory exudate on the surface which was separated from the underlying connective tissue by 2-3 layers of contiguous epidermis.

Within the dermis there were some focal areas of inflammatory cells and some scattered regions with massive infiltration of macrophages and polymorphonuclears. There was no apparent attack on epidermal cells, and no substantial number of lymphocytes present among the inflammatory cells.

Eight days after the transplantation, the split thickness graft consisted mostly of original dermis with very little new connective tissue growing from it. The surface was completly covered by a thick layer of epidermal cells sending long projections into the dermis. The surface of the epidermis was still covered by inflammatory exudate and a crust.

There were few scattered macrophages and almost complete absence of polymorphs. There were only one or two lymphocytes without apparent significance. Clinically, the split thickness graft did not reject up to three months of observation. The described results strongly suggest that acceptance of tissue culture treated cells results in induction of tolerance.

Two months after the first split thickness graft, we applied a second split thickness graft from donor BP (to which the recipient was tolerized), as well as a graft from an unrelated donor F. Clinical observations on day 4 and day 6 post transplantation showed excellent acceptance of the graft from donor BP. The graft from donor F was well attached on day 4 and was of pink color. On day 6 the color was changed to light purple.

Histological examination of samples taken 6 days post transplantation confirmed clinical observations. The graft from BP was covered by epidermal cells with a mild infiltrate of neutrophils, macrophages and giant cells, but no lymphocytes. The graft from donor F still had epidermis, but also had large infiltration of neutrophils and macrophages and obstructed blood vessels.

On day 8 post transplantation, the grafts from pig BP were healed, but graft from pig F was being rejected. The blood vessels were occluded and there was a heavy cellular infiltrate of neutrophils, macrophages and lymphocytes. Since no rejection of the graft from BP was observed for a month, we planned to perform a kidney graft from BP into RR. The operation was successful, but we lost the animal at the time of recovery from anaesthesia.

To assure that pig BP and RR were not compatible, pig BP was transplanted with a split thickness graft from pig RR. This graft was rejected by 6 days post transplantation.

The experiments performed and data obtained strongly suggest that "conditioning" of epidermal cells with the recipient's dermal extracts induces specific changes on the cell surface, rendering these cells to be acceptable as allografts. "Conditioning" also leads to induction of specific tolerance.

To investigate what changes are induced on such cells we initiated study of skin specific antigens and regulation of induction of Ia on keratinocytes.

#### G. <u>SKIN SPECIFIC ANTIGENS</u>

Based on extensive review of the literature and our own findings, it is clear that matching at the major histocompatibility complex, at HLA-DR and by MLC delays but does not prevent rejection of allogeneic skin grafts. These data suggest that MHC class I and class II antigens play an important role in skin transplantation. Merely matching these antigens, however, is obviously not sufficient for acceptance of skin grafts.

Results obtained with kidney transplantation demonstrate that matching for these antigens results in a long survival or acceptance of grafts (39, 31, 4, 13, 28). It is therefore possible that additional factors play an important role in the acceptance of skin grafts.

Billingham and Brent (2) were the first to describe rejection of skin grafts persistent hemopoietic chimeras. Ten years later Boyce and Old (3) suggested that in chimeras, where host hemopoietic cells coexist with donor hemopoietic cells, skin grafts are rejected because the skin cells express differentiation or "SK" alloantigens not expressed by lymphocytes. Tanaka et al (37) studied cytotoxic responses of regional lymph node cells in <u>vitro</u> after skin allografting in the MHC-compatible Fisher to Lewis rat strain combinations. They observed higher cytotoxic response against donor epidermal cells than donor spleen cells, and suggested that skin specific alloantigens could be accounted for that result.

Steinmuller et al (20,21), showed that injection of mouse epidermal cells that are matched for H-2 (major histocompatibility of mice) but differ at numerous minor H loci into C3H strain mice, evoke a marked cytotoxic T cell response against the donor epidermal cells, as opposed to that toward the donor lymphocytes. They assumed that cytotoxic T cells generated by immunization with epidermal cells consist of multiple clones, some with specificity for alloantigens shared by epidermal and lymphoid cells, others with specificity for alloantigens unique to epidermal cells i.e., tissue specific antigens.

Most attempts to prolong survival of allografts have involved treatment to impair the host's capacity to reject them. Attempts have been made in the past decade to alter the immunogenicity of the allografts.

Organ culture prior to transplantation can greatly prolong its survival in an allogeneic host. This phenomena was demonstrated for thyroid gland by Lafferty et al (18, 19, 5, 17) and for Langerhans islet cells of pancreas by Simeonovic et al (30). The effect was attributed to the loss of stimulator cells required for the presentation of antigens to the host's immune system in an effectively immunogenic form.

However, skin allografts are quite different. Similar treatment of swine epidermal cells in culture prior to transplantation resulted in prolongation of graft survival (7), but did not result in acceptance of graft. Extensive work on the role of passenger leukocytes in the immunogenicity of skin allograft by Steinmuller et al (32) suggest that passenger leukocytes and other bone marrow derivatives such as Langerhans cells probably do not contribute significantly to the immunogenicity of skin allografts.

Most previously reported transplantation experiments used mice or rats and occasionally monkeys. The need for a large animal system for such studies resulted in the development of a MHC matched swine colony (27).

There, it was shown that major histocompatibility complex (MHC) was of dominant importance for kidney transplantation (41, 16). However, non-MHC linked immune response gene has been shown to further regulate survival of completly matched grafts (23).

Identification of recombinant haplotype swine (24) has enabled Pescovitz and his coworkers (25) to test the effect of isolated class I and class II MHC antigen matching on graft survival in the miniature swine.

In this study, Pescovitz et al showed that overall survival of class II-only matched kidneys is clearly prolonged over completely mismatched or class I-only matched kidneys. They also showed tolerance that appeared to be systemic after renal transplantation.

They observed prolongation of donor specific skin graft survival but not of a third party skin survival. The rejection of the skin that finally did ensue was possibly related to skin specific antigens, since there was no associated evidence of renal dysfunction.

It was therefore the aim of our studies to establish whether antigens detectable on epidermal cells but not on lymphocytes or other skin cells can be defined in the pig model system.

(For derivation of antibodies and methodology see enc. Methods I). As outlined in the enclosure, antibodies were obtained by two consecutive skin transplants between animals matched at the MHC, and also given a kidney graft. As illustrated in Fig. 1, eleven sera were tested on a panel of single cell suspensions obtained from skins of 21 pigs. They were derived from 3 experimental animals. Series  $S_1$  (both donor and recipient of dd haplotype) shows that 2 weeks following second skin transplant and two weeks post kidney transplant, the sera from transplanted animals contained antibodies reacting with cell surface antigens of epidermal cells. The reactivity was observed on cells derived from 12 pigs out of 21 tested.

 $S_2$  series are antibodies obtained by reciprocal immunization of the same pigs, and  $S_3$  series by skin grafting between pigs of a cd haplotype. Antigens detected by  $S_2$  antibodies were found in 11 pigs and  $S_3$  in 12 pigs of the 21 tested. The highest titer of antibodies in all

3 series was found in sera obtained post second skin grafting, and a decline in the antibody titer was seen post kidney grafting, suggesting that antigens expressed on keratinocytes were not shared by kidney cells.

The sera collected prior to immunization and two weeks after a first skin transplant tested negatively on all keratinocytes. All antibodies were of IgG class of immunoglobulins.

Fig 2 summarizes results of the immunorosetting assay for detection of surface antigens on swine keratinocytes, showing the pattern of reactivity with sera generated by the three immunization series. Titers of reactivities shown are those for the sera of  $S_1$ ,  $S_2$  and  $S_3$  series from the third and fourth bleedings (as shown in Fig. 1). The titers of each serum are the average of at least three determinations on a skin sample.

Antigens were detected on the epidermal cells of eleven inbred swine out of seventeen and on four of the five outbred swine tested. Seven different patterns of reactivity were found:

1) Those positive only with antibodies of  $S_1$  series which was only in one case out of 22 tested;

2) Those positive with antibodies of  $S_2$  series.

3) Positive with  $S_1$  and  $S_2$  antibodies;

- 4) Positive with  $S_1$  and  $S_3$ ;
- 5) positive with  $S_2$  and  $S_3$  antibodies;
- 6) positive with all antibodies;
- 7) negative with all antibodies.

The largest groups are those expressing antigens detected by  $S_1$  and  $S_3$  (group 4), and those expressing all three antigens or none (groups 6,7). There was no obvious correlation of reactivity between any two of the immunizations and no correlation with SLA type or sex.

The immunorosetting assay was found to be useful to detect the SLA antigens on keratinocytes as shown in Fig. 2. In general, the method confirmed original SLA typing results based on routinely used complement-dependent lymphocytotoxicity (27). The sole exception was the presence of cross-reactivity between the anti-C antiserum and antigens expressed on the keratinocytes of pig 1413, SLA type ad. The determination of the specific SLA antigens on keratinocytes served as a control for tests of the reactivity of skin specific antisera.

In order to analyze patterns of reactivity of the antigens detected by  $S_1$ ,  $S_2$  and  $S_3$  for possible SLA linkage, cells from SLA identical siblings of pig 1413 and pig 1618 were tested.

Fig 3 shows the differing reactivities of the sera tested with cells of pigs 1412 and 1413, siblings which are SLA type ad, and with cells of pigs 1617, 1618, 1620 and 1621, siblings of SLA type dd. The two sets of siblings show that there is no linkage of the skin specific sera with the major histocompatibility complex.

To establish whether the expression of antigens detected by  $S_1$ ,  $S_2$  and  $S_3$  antibodies is modulated by their growth in <u>vitro</u> we tested cultures of pig epidermal cells. We maintained keratinocytes obtained from the skin of 6 pigs in culture up to 140 days. These keratinocytes were tested in culture at 15 day intervals for the first 30 days, and in monthly intervals afterwards.

Already, at 15 days in culture at the time when the cells were still rapidly growing, cell surface antigens  $S_2$  and  $S_3$  showed rapid decline, with a slight decrease in the reactivity of the cells with  $S_1$ antibodies. As shown in Fig. 4, by 30 days of maintenance in culture, the antigens reactive with  $S_2$  and  $S_3$  were not detectable at serum dilutions higher than 1:20. The expression of  $S_1$  did not change.

Further examinations revealed that  $S_1$  antigens were expressed continuously during 140 days of cultivation <u>in vitro</u>, and  $S_2$  antigen was not detectable throughout this period.

It therefore appears that these two antigens are not differentiation antigens. This suggestion comes from our observations that cultures up to 20 days are composed mainly of basal cells while older cultures contain up to 40 layers of differentiated cells and only one layer of basal cells.

The third antigen detected by  $S_3$  antibody showed cyclic reexpression at 40 and 90 days in culture. However it was not detectable at 30, 80, 120 and 140 days in culture.

In contrast to the skin specific antigens, there were no significant changes in the expression of SLA antigens on the pig

keratinocytes at early times, after establishment in  $\underline{vitro}$  and after prolonged maintenance up to 140 days.

To assure that the antigens detected by  $S_1$ ,  $S_2$  and  $S_3$  series are expressed only on keratinocytes, we also tested lymphocytes from the epidermal cell donor. As shown in fig. 5, lymphocytes derived from 17 pigs of those listed in Figs. 2 and 3 were all negative when tested with the above sera. However, the appropriate SLA haplotypes were detected on the lymphocytes by the immunorosetting assay.

In addition we tested melanocytes and dermal fibroblasts from pigs #1412, #1413, and #1414. They were maintained in culture for 28 days. Although they expressed the appropriate MHC antigens, antigens  $S_1$ ,  $S_2$  and  $S_3$  were not detectable.

Since antigen  $S_2$  is not detectable on keratinocytes after culture, and  $S_3$  is only occasionally detectable, the fact that  $S_1$  was not detected is of special importance, since this antigen is always expressed on keratinocytes.

To confirm and extend the findings of tissue specificity, sera  $S_1$ ,  $S_2$  and  $S_3$  were absorbed with non-cultured lymphocytes and keratinocytes or cultured fibroblasts from pigs whose cells were positive for the respective antigens. The absorbed sera were tested on keratinocytes, lymphocytes and fibroblasts by the protein A immunorosetting assay. Following absorption with lymphocytes or fibroblasts the skin specific sera,  $S_1$ ,  $S_2$  and  $S_3$  retained positive reactions with keratinocyte antigens (titers decreased by 1/2 to1/8), but following absorption with keratinocyte all the reactivity was removed.

As a control, appropriate antisera to the SLA antigens a and were absorbed similarly with lymphocytes or keratinocytes. Lymphocytes and keratinocytes were equally potent in removing the specific activity of each sera.

The results presented provide evidence for expression of skin specific antigens on keratinocytes. Further studies are necessary to establish their role in allograft rejection. (5a)

#### H. Induction of Ia Antigens on Human and Pig keratinocytes in vitro

To further our understandings of epidermal graft rejection, we also investigated induction of class II MHC antigens. The studies were performed using a novel in <u>vitro</u> transplantation system partially described in our second year report.

(For materials and methods, see encl. Methods II).

A series of pig and human keratinocytes cultures, initiated from single cell suspensions, were grown in vitro for a period of 3 - 10 weeks. At this time all such cultures were multilayered, ranging from 10 - 40 layers of cells.

In most instances there were only 1 - 2 layers of nucleated cells and varying numbers of layers of keratinized epidermal cells. Cultures were free of detectable Langerhans cells as was determined by OKT6 and Ia antibodies. They contained 1 - 2% melanocytes, as shown by DOPA staining, and occasionally a few fibroblasts visualized by leucine aminopeptidase staining procedure.

Multilayered sheets of human and pig epidermal cells, following enzymatic removal from the plastic surfaces, were placed on a pre-washed piece of pig dermis. Attachment of epidermal cells to the dermis occured within 12 - 24 hours and epidermal cells were seen to be tightly associated with the dermis in 2 - 3 days. Three - four layers of nucleated epidermal cells were seen on biopsies taken at day 3 - 5.

Histological examination of multiple repeated biopsies showed that fresh dermis following extensive washing prior to its use for the in <u>vitro</u> experiments contained sparse fibroblasts, and very rarely dispersed mononuclear cells with typical morphology of lymphocytes. No capillaries and red blood cells were noted.

After 2 days in culture, the population of fibroblasts were decreasing and occasionally fibroblast outgrowth was noticed on the bottom of the Petri dish, underneath the stainless steel grid, resulting from the few fibroblasts shed from the dermis.

Some lymphocytes were seen in the tissue culture fluid. However they numbered too few to be collected for detailed characterization. The lymphoid population was noted in only a few experiments and up to 5 - 6 days in culture. As summarized in table 2, when pig and/or human keratinocytes were grown in culture for 3 - 10 weeks and then exposed to fresh allogeneic or xenogeneic pig dermis, 95% of the epidermal cultures were induced to Ia antigen expression within 3 - 10 days. The percent of responding cells ranged from 5 - 80%.

In 10% of the experiments, only 5% of the cells were induced to express Ia. Epidermal cells derived from the same donor varied in their response when "transplanted" onto dermis obtained from different animals. Similarly, quantitative differences were observed in responses of epidermal cells obtained from different donors when "transplanted" on to the dermis from the same animal.

The age of the animal or the time in culture did not obviously affect the induction process. Expression of Ia antigens on epidermal cells was demonstrated in most experiments after 48 hours exposure of the cells to fresh dermis, with a few exceptions, in which peak induction was noted at 24 hours. Usually, the maximal expression ensued in 5 and 8 days for pig and human epider 1 cells respectively.

A characteristic time course of Ia antigen detection on pig and human epidermal cells is demonstrated in fig 6. When pig keratinocytes were exposed to an allogeneic pig dermis, induction of Ia on the pig keratinocytes ocurred within 48 hours, reaching maximum at 4 days. A gradual reduction in the population of cells expressing the Ia occurred within the following 3 days.

However, a complete disappearance of the Ia antigen was not demonstrated even in experiments carried up to 33 days. When human epidermal cells were exposed to pig dermis, time of the initial induction followed the same pattern as for pig cells.

Peak induction was usually delayed compared to time course observed with pig epidermal cells, however. Based on the results of different experiments, average peak induction time was 4.57 days for pig cells, and 8 days for human cells.

Fig 7a represents a series of experiments in which pig epidermal cells were examined for Ia induction following in vitro transplantation onto allogeneic pig dermis. In only one instance out of 11 experiments was induction not demonstrated. Therefore, in 84% of the experiments

induction was achieved, and on the average 41% of the cells were positive.

Experiments in which human keratinocytes exposed to pig dermis were induced to Ia expression are shown in Fig 7b. In two out of 8 experiments an induction of less than 5% was demonstrated. Therefore in 75 % of the experiments, human epidermal cells were induced to Ia expression, and the average number of positive cells was 33%.

# Exposure of cultured pig keratinocytes to autologous or allogeneic dermis.

Table 3 summarizes the results of 5 experiments in which pig keratinocytes cultured in <u>vitro</u> for 3 - 10 weeks were exposed to autologous or allogeneic pig dermis. In each of the experiments designated A, B, and C keratinocytes were derived from the same donor. They were grown in <u>vitro</u> simultaneously and under the same conditions, and placed on a dermis derived from the donor of keratinocytes or from an unrelated donor.

In one case (B), the autologous combination was substituted (IP-9 versus IP 10) by the use of the dermis from an SLA matched animal. In all three instances the combination of the same epidermal cells with allogeneic dermis gave 6-8 fold higher induction of Ia antigens than the autologous combination.

When epidermal cells obtained from different donors grown in vitro for the same length of time were exposed to the same dermis, epidermal cells in combination with allogeneic dermis showed increased number of Ia+ cells. In one case, (E) both autologous and allogeneic combinations gave identical results. The time course of induction was the same in both autologous and allogeneic combinations, with maximum Ia expression at 3 - 5 days following "transplantation" of epidermal sheets onto pig dermis.

Fig 8 represents a graphical evaluation of percentages % of the Ia cells in autologuous and allogeneic combinations, and compares it to the control cells prior to transplantation. The values for the controls were 0, epidermal cells in autologous combinations were induced to an

average of 1.5% with the exception of one case in which 30% induction was obtained. The allogeneic combination gave an average 43% induction.

Attempts to induce Ia expression on human or pig keratinocytes by agents other than the pig dermis.

A variety of reagents were tested to analyze the inducibility of human and pig keratinocytes on Ia expression. Human natural and recombinant gamma interferon were shown to induce Ia on human keratinocytes in a dose dependent fashion. Minimum concentration of interferon capable of induction was 40 u/ml. Optimal induction in 90% of cells was obtained with 200 u/ml within 5 days (data not shown). These findings confirm results previously published (14).

Human fibroblasts were inducible by 1,000 u/ml, showing 95% of the cells to be Ia+ within 48 hours. (data not shown). On the other hand pig keratinocytes or pig fibroblasts were not induced by human gamma interferon at doses shown to be optimal for human cells.

Natural INF-B<sub>2</sub> and recombinant INF-Alpha did not induce human or pig keratinocytes when tested at different concentrations (ranging from 100 pg to 2.5 ng/ml). Similarly, TNF at 50 u/ml, IL-1 at .2 ng/ml, JL-2 at 1 - 16 u/ml, and CON-A at 10 mg/ml, all gave negative results.

Tests of supernatant fluids from human keratinocytes, fibroblast, and human or pig dermis, all yielded negative results. Only supernatants from concanavalin-A stimulated peripheral blood leukocytes were capable of inducing Ia on human but not on pig keratinocytes.

#### <u>Autoinduction of Ia by gamma interferon:</u>

All our results as well as those of others provided evidence that induction of Ia antigen was mediated by gamma interferon. As we could not prove the presence of gamma interferon in pig dermis, we hypothesized that keratinocytes might be induced to gamma interferon production when exposed to allogeneic or xenogeneic dermis.

To prove this hypothesis, cells exposed to allogeneic or xenogeneic dermis were tested both for Ia induction and interferon production. Supernatants from cocultivated epidermis - dermis were collected, concentrated, and tested by radio-immune assay for gamma interferon. All such tests yielded negative results.

As it was plausible that amounts of interferon produced are very small and may be reutilized by epidermal cells in an autocrine fashion, we also tested epidermal cells by immuno rosetting using antibodies to gamma interferon (obtained from Dr. J. Vilcek, N.Y.U.). These tests revealed that cells expressing Ia also had Gamma interferon attached to their membrane.

To verify these findings, we performed <u>in situ</u> hybridization on histological sections of cocultivated human epidermal cells on pig dermis using a <sup>3</sup>H labelled single stranded RNA probe, and templates of gamma interferon. Hybridization, as measured by the appearance of grains over specific cells, revealed positive reactions on human epidermal cells grown on pig dermis. This finding is very interesting for two reasons:

1). Epidermal keratinocytes are the first non-T cells shown to produce Gamma (immune) interferon.

2) It further suggests that the mechanism of Class II antigen induction by epidermal cells may play an important part in skin allograft rejection. Manuscripts and patents resulting from this work.

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METHODS I.

#### MATERIALS AND METHODS

<u>Animals</u>: Swine, selectively inbred to identity at the major histocompatibility complex, were obtained from the laboratory of David H. Sachs at NIH (27). All were blood group 0.

Antisera: Antisera to non-major histocompatibility complex (MHC) antigens were generated by skin transplantation between swine leukocyte antigen (SLA) identical swine (16). Recipients 88 and 91, SLA type <u>dd</u>, were reciprocally immunized. Recipient 145, SLA type <u>cd</u>, was immunized by donor 119, type <u>cd</u>. Sera were obtained from each immunized pig at four different times: Preimmunization; two weeks after a first skin transplant; two weeks after a second skin transplant and two weeks after a kidney transplant from the same donor.(25) Specific antisera against the SLA haplotypes <u>a</u>, <u>c</u> and <u>d</u> were generated by immunizations between swine of the appropriate genotype (20).

<u>Cells</u>:

Fresh keratinocytes lymphocytes, and cultured keratinocytes, melanocytes, and dermal fibroblasts were tested for their expression of SLA and skin specific antigens. Skin cells were obtained by punch biopsies from normal pig skin. The epidermis was separated from the dermis after 18 h incubation at  $4^{\circ}$ C in 0.25% trypsin (Difco, Detroit, Michigan). Epidermal sheets were dissociated by gentle pipetting in a trypsin/EDTA solution with a pasteur pipette.  $1\times10^{6}$  epidermal cells were obtained from each 6 mm punch biopsy. Keratinocytes were tested within 48 hours or cultured as previously described by Eisinger et al. (6). Following growth in vitro for more than 14 days, confluent sheets of cells were harvested by trypsinisation, collected in fetal calf serum (FCS) and resuspended in Eagle's minimum essential medium (MEM) with Earle's salts containing: 0.01 mM nonessential amino acids; 2mM L-glutamine; penicillin (100 unit/ml); streptomycin (0.1 mg/ml); Fungizone (0.25 ug/ml); and ten percent

Fibroblasts were generated by explant cultures and fetal bovine serum. melanocytes were cultured after the method of Eisinger and Marko. (10). For preparation of lymphocytes, 10-20 ml of blood collected into heparin, (0.3 ml of sodium heparin, Elkins-Sinn, Inc. Cherry Hill, NJ) were diluted 1:2 into complete medium (RPMI 1640 containing: 25 mM Hepes; 2mM L-glutamine; 100 U/ml penicillin and 100 ug/ml streptomycin) plus heparin lml/100ml (Monoparin, Weddel Pharmaceuticals Limited, Wrexham, U.K.). Lymphocytes were obtained as the mononuclear cell fraction of heparinized blood by density separation. Diluted heparinized blood (20-40 ml) was carefully layered onto 15 ml Ficoll-Hypaque (Lymphoprep, Accurate Chemical Company, Hicksville, N.Y.) in a 50 ml Following centrifugation at 2000 rpm for 30 minutes, the centrifuge tube. cloudy interfacal layer was collected into another 50 ml tube, washed and centrifuged ( at 1.000 rpm for 30 minutes) three times in heparinized complete medium to remove ficoll-hypague and platelets. Yields of mononuclear cells were approximately  $5 \times 10^6$  cells/m] of blood with viabilities above 90%. The cells were suspended at  $5 \times 10^{5}$ /ml in complete medium without heparin for testing in the protein A immunorosetting assay.

#### Protein A Immunorosetting Assay:

Fresh and cultured cells were stored at  $4^{\circ}$ C in MEM + 10% FCS for 24 hours before plating on concanavalin A coated Terasaki plates according to the method of Mattes et al (22) at a concentration of  $5x10^5$  cells/ml. The Terasaki plates were spun for 5 min at 1000 rpm. washed with phosphate buffered saline (PBS) + 5% gamma-globulin (GG) free FCS (Gibco Laboratories) and blotted. The antisera were serially diluted 1:4 with a starting concentration of 1:20 for the skin specific antisera and a 1:50 concentration for the SLA typing sera. The target cells were incubated with the serial serum dilution for 1 hour at room temperature and subsequently washed twice with PBS (Ca + Mg free) containing 5% GG-free FCS. Negative controls included the incubation of target cells with pre-immunization sera and with the dilution medium alone. Lymphocytes and keratinocytes from the same animal were tested concurrently. As positive control, anti-SLA antisera were used in the same assays. Protein-A conjugated erythrocytes (indicator cells) were added at 0.2% dilution, 10 ul/well. The indicator cells had been previously prepared by conjugation of staphylococcal Protein-A (26) to the surface of human 0 erythrocytes with 0.01% Chromium-Chloride. After 45 min incubation at room temperature, nonadherent indicator cells were removed by two washings with PBS containing 10% FCS. Plates were scored under the light microscope for the percentage of positive, rosetted cells.

To define whether the specific antibody within the sera are of IgG or IgM subclass, we used immunorosetting assay. Indicator cells were coated with anti-pig IgG or anti-pig IgM. Each serum was screened therefore with 3 indicator cells: PA cells; Human O blood cells coated with Rabbit anti-pig IgG; and Human O blood cells coated with Rabbit anti-pig IgM.

#### Absorption Tests:

To examine the specificity of the sera, absorption tests with lymphocytes and keratinocytes were carried out. Fresh keratinocytes were screened for the titer of reactivity of each sera on the day of the absorption test. The dilution chosen for the absorption was ond before the last to be positive. Lymphocytes were pelleted in 15 ml tube and the pellet was marked and measured. The pellet was then resuspended with media and transferred equally into the required number of eppendorf tubes. The cells were pelleted again and all the supernatant collected very carefully. Into each tube, a volume of antiserum equal to the cell pellet volume was added, the cells resuspended, and incubated for 1 hour at room temperature. In the same manner fresh keratinocyte pellets were prepared, divided and incubated. After one hour at roon temperature, the eppendorf tubes were centrifuged, the antisera was collected and used for immunorosetting assay.

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#### Material and Methods

#### METHODS II.

#### Preparation of Fresh Dermis:

The dermis was obtained from swine inbred at the major histocompatibility locus (27) or domestic outbred swine. Animals were anesthetized using ketamine hydrochloride and anaesthesia was maintained by a mixture of halothane, nitrous oxide and oxygen. At first split thickness wounds 0.015 inch were created using a Brown Dermatome on the sides of the thorax. This was followed by a second shave 0.02-0.035 inch in the same area. The dermis from the second shave was used for the induction system. To alleviate pain and accelerate healing the first shave was placed over the wounded area after expanding it with a skin mesher (Genetic Lab., St. Paul, MN). After surgery the pig was given Tylenol for the next 2-3 days. The fresh dermis was then washed three successive times and kept at  $4^{\circ}$  C for 1 hour each in minimal essential medium with Earle's salts (MEM) containing: 1000 units/ml of penicillin, 1 mg/ml streptomycin and 2.5 ug/ml fungizone. This was followed by an extensive wash with MEM containing 1/10 the concentration of antibiotics, 2 mM L-glutamine and .01 mM non-essential amino acids (cMEM). The dermis was cut into  $5 \times 3$  cm pieces under sterile conditions, and placed on stainless-steel grids (custom made by the Rockefeller University shops) in 100 mm glass petri dishes (Corning Lab Products, NY).

#### The in vitro transplantation:

Human and/or pig epidermal cells were grown in tissue culture from single cell suspensions as previously described (Eisinger PNAS 1979). Multi-layered primary epidermal cell cultures (15-30 layers) grown in <u>vitro</u> for 3-5 weeks were removed from tissue culture flasks as previously described (11). Briefly, confluent cultures were washed twice with Dulbecco's phosphate buffered saline without  $Ca^{2+}, Mq^{2+}$ (PBS). The flasks were cut open with a hot scapel and the cells were incubated for 30-45 minutes in 0.5% Dispase (Bohringer Mannheim, West Germany) at 37°C. When the edges of the sheet of epidermis began to lift, the dispase was removed, the cultures were washed 5x with PBS and a precut piece of petrolatum gauze was placed on the epidermal sheet. The cells were peeled off the flask using watchmaker's forceps and were immediately positioned on a fresh, moist piece of dermis already placed on a grid in a petri dish. The petrolatum gauze was gently removed leaving the basal layer of epidermal cells adjacent to the dermis. The petri dish was then gently filled with cMEM containing 15% fetal calf serum (FCS) for pig epidermis or 10% FCS for human epidermis, the fluid reaching the dermis but not covering the epidermis. Using this system keratinocytes were kept at the liquid air interfase resembling conditions in <u>vivo</u>. The in <u>vitro</u> transplanted cultures were fed with fresh medium twice a week and examined up to 2 months.

#### Mixed hemadsorption assays

At a desired time, in order to follow the induction of Ia antigens, pieces of dermis containing transplanted epidermal cells were washed twice with PBS and incubated in a trypsin-EDTA mixture at  $37^{\circ}$ C. Epidermal single cell suspensions were prepared by gradual tripsinization and gentle pipetting. Viability of trypsinized cells ranged from 70-80%. Epidermal cultures established at the same time as those used for transplantation but not exposed to dermis were trypsinized and served as controls. Mixed hemadsorption assays were performed as previously described (22). Indicator cells were prepared as described (26). Briefly, Rabbit anti mouse Ig (Accurate Chemical and Scientific Corp, Westbury, NY) or protein A (PA) (Pharmacia fine chemicals, Uppsala, Sweden) was conjugated to type 0 human erythrocytes using 0.01% chromium chloride. Serological assays were carried out in 60 well terasaki plates (Falcon #3034). The plates were coated with concanavalin A (Con A) (Pharmacia fine chemicals, Uppsala, Sweden) and 10ul of the epidermal single cell suspensions (4 x  $10^5$  cells/ml) were plated to each well. Serial dilutions of the specific antibodies were added to the cells and incubated at room temperature (RT) for 1 hour. The plates were washed twice with PBS, indicator cells were added to each well and the plates were incubated at room temperature for 45 min. The percentage of positive cells (rosetted) was determined for each well. A positive control and negative controls of preimmune mouse sera and PBS were used in every experiment.

#### Peripheral Blood Mononuclear Cells:

Peripheral blood mononuclear cells (PBMC) from normal human donors or swine, were isolated by density separation using Ficoll-Hypaque. Isolated, the PBMC's were cultivated for 3 days in RPMI 1640 (Gibco, Grand Island, NY). For human or pig PBMC's the medium contained 10 % FCS or 10% fetal pig serum (FPS) (Gibco) respectively. In addition either 10 ug/ml Con A or 0.1% phytohemagglutinin (PHA) (Difco, Detroit Mich.) were added to such cultures. The supernatants from these activated PBMC's were used as an additional source of Gamma-interferon

#### Sources of reagents:

Polyclonal SLA typing sera were kindly provided by Dr. D. Sachs (NIH). The following mouse monoclonal antibodies were used for serological testing: MSA.3 detecting swine Ia, was generously given by Dr. Joan Lunney (NIH); L243 detecting monomorphic determinant of Ia (DR only) was purchased from Beckton-Dickinson. TV-39, detecting monomorphic determinant of Ia (DR, DQ, DP) was the generous gift of Dr. Robert Knowles (Sloan Kettering Institute, NY).

IL-1 was kindly provided by Citron Technology; IL-2 was a gift from Dr. Karl Welte (Sloan Kettering Institute NY); recombinant INF alpha was generously provided by Dr. Michell Ravel (Weizmann Institute, Israel); natural and recombinant a INF gamma was obtained from Dr. San (Sloan Kettering Institute, NY); Human recombinant TNF was obtained from Dr. Nabuko Satomi (Tokyo University, Japan).

## DETECTION OF ANTIBODIES TO KERATINOCYTE SURFACE ANTIGENS IN SERA FROM SLA IDENTICAL SWINE IMMUNIZED BY SKIN GRAFTING.

Immunization Series	Serum Collection	Reciprocal Titer 500 1000 1500 2000 2500
S1	.     . 2   . 3   . 4	
<b>S</b> 2	2.1 2.2 2.3 2.4	
<b>S</b> 3	3.1 3.3 3.4	

.1 = PRE-IMMUNIZATION

- .2 = POST 1ST SKIN GRAFT
- .3 = POST 2ND SKIN GRAFT

.4 = POST KIDNEY TRANSPLANT

TITERS AN AVERAGE OF 25 TESTS OF DIFFERENT SKIN SAMPLES,

DETECTION OF SURFACE ANTIGENS ON SWINE KERATINOCYTES BY PROTEIN A IMMUNOROSETTING ASSAY.

Fig.2

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Animal 1°		Non	-SLA Antib	odies	SLA	Antibod	ies z	
Number	Sex	Haplotype	S1	<b>\$</b> 2	<b>S</b> 3	Anti A	Anti C	Anti D
1567	F	CC	•	0	0	NT		0
1105	F	aa	0		0		0	0
8	F	outbred	0		0			
1413	F	ad			0		6	
1414	F	ac		0				0
1110	M	<u> </u>		0	•	Q		<u> </u>
1 5 8 2	M	ad		0	6	NT	0	
1453	F	outbred	6	0	6			
1 5 8 3	M	ad	0			NT	0	
1618	F	d d	0	6	Ø	NT	0	•
1454	F	out bred	6	6		Ø		
1455	F	outbred	6	6	0			
1973	M	aa –				NT	NT	NT
1974	M	a a	Ø	6		NT	NT	NT
1977	F	d d				NT	NT	NT
1978	F	d d		0		NT	NT	NT
1563	M		0	0	0	NT	NT	NT
1566	F	00	0	0	0	NT	•	
1611	M	d d	0	0	0	NT	0	•
1136	F	c d	0	0	0	NT	NT	NT
1145	F	22	O	0	0	•	0	0
1	M	outbred	0	0	0		$\bigcirc$	

EACH CIRCLE REPRESENTS A SKIN SAMPLE TESTED. ALL TITERS ARE THE AVERAGE OF THREE DETERMINATIONS.

- $\bigcirc$  = NEGATIVE OR TITER BELOW 1:20.
- $\bigcirc$  = TITER 1:20 1:80.
- $\bigcirc$  = TITER 1:80 1:320.
- = TITER GREATER THAN 1:320.
- NT = NOT TESTED

1°. SLA HAPLOTYPES DETERMINED AS PREVIOUSLY DESCRIBED ( )

2. SLA TYPING SERA PREVIOUSLY DESCRIBED ( )

PATTERN OF SWINE SK ANTIGEN EXPRESSION ON EPIDERMAL CELLS OF SLA-IDENTICAL SIBLINGS

Animal			No	Non-SLA Antibodies		
Number	Sex	Haplotype	<b>S</b> 1	<b>S</b> 2	<b>S</b> 3	
1413	F	ad			0	
1412	F	ad		0		
1618	F	d d	0	0		
1617	F	d d		0		
1620	F	d d	0	6	0	
1621	F	d d	0	0	0	

EACH CIRCLE REPRESENTS AN AVERAGE OF 3 TESTS OF SWINE KERATINOCYTES WITH THE APPROPRIATE ANTISERA BY IMMUNOROSETTING.

- = NEGATIVE OR TITER BELOW 1:20.
- = TITER 1:20 1:80.
- a = TITER 1:80 1:320.
  - = TITER GREATER THAN 1:320.

EXPRESSION OF SWINE SK ANTIGENS ON KERATINOCYTES BEFORE AND AFTER CULTIVATION IN VITRO.



■ AFTER 30 DAYS IN TISSUE CULTURE

Fig. 4

TISSUE SPECIFIC EXPRESSION OF SWINE SK ANTIGENS

Fig. 5

Lymphocytes	000000000000000000000000000000000000000
Fibroblast	000000
Keratinocytes	000000000000000000000000000000000000000
Melanocytes	000

EACH CIRCLE REPRESENTS A SAMPLE DERIVED FROM A DONOR-ANIMAL, TESTED IN TRIPLICATE, LYMPHOCYTES WERE OBTAINED BY FICOLL - HYPAQUE SEPARATION AND KERATINOCYTES BY ENZYMATIC SEPARATION AS DESCRIBED IN THE METHODS. FIBROBLASTS AND MELANOCYTES WERE TESTED FOLLOWING CULTIVATION IN VITRO.

○ = NEGATIVE OR TITER BELOW 1:20.

- = TITER 1:20 1:80.
- = TITER 1:80 1:320.
- = TITER GREATER THAN 1:320.

TIME COURSE OF IA EXPRESSION BY PIG AND HUMAN KERATINOCYTES EXPOSED TO ALLOGENEIC OR XENOGENEIC DERMIS.



= PIG KERATINOCYTES
 = HUMAN KERATINOCYTES

## MAXIMAL INDUCTION OF IN ANTIGEN ON CULTURED KERATINOCYTES.



A. PIG KERATINOCYTES EXPOSED TO ALLOGENEIC (PIG) DERMIS.B. HUMAN KERATINOCYTES EXPOSED TO PIG DERMIS.

MAXIMAL INDUCTION OF IA ANTIGENS ON CULTURED PIG KERATINOCYTES AFTER EXPOSURE TO AUTOLOGOUS OR ALLOGENEIC DERMIS.



- = CONTROL (CULTURED KERATINOCYTES NOT EXPOSED TO DERMIS),

AUTOLOGOUS COMBINATION (CULTURED EPIDERMIS AND DERMIS WERE DERIVED FROM THE SAME DONOR).

ALLOGENEIC COMBINATION (TISSUE CULTURE GROWN EPIDERMAL CELLS EXPOSED TO DERMIS FROM A DIFFERENT DONOR).

Table 1			
PATIENTS NAME	# OF DERMAL GRAFTS	# OF EPID. GRAFTS	HEALING
R.W.		2	+/-*
E.L.	1	6	-
A.W.	6	2	-
E.B.	2	1	+
P.P.	1	1	+
L.A.	1		+
T.A.	6	3	-
L.N.I	1	2	-
L.N.II	3	2	-
B.H.	3	3	-
Q.	4		-
Ζ.	2	1	-

+ = healed

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- = not healed
- +\* = healed and reopened

### Table 2

Maximal Induction of Ia antigens on Pig and Human cultured Keratinocytes after exposure to pig dermis.

	Epidermal	Time in	Dermis	% of cells
	cells origin	culture/weeks	origin	induced to Ia
				expression
Α.	0P-1	8	0P-2	70%
Β.	0P-1	10	0P-3	15%
C.	0P-1	10	0P-4	50%
D.	0P-5	9	0P-6	30%
Ε.	0P-5	6	0P-6	40%
F.	0P-7	9	0P-5	20%
G.	0P-7	4	0P-8	15%
Н.	0P-7	6	0P-6	00%
Ι.	IP-9	5	IP-12	60%
J.	IP- <b>9</b>	4	IP-11	80%
К.	IP-9	3	IP-11	30%
L.	Hu-20	4	OP-13	40%
Μ.	Hu-21	3	IP-11	30%
N.	Hu-22	3	OP-14	10%
0.	Hu-23	3	OP-14	05%
Ρ.	Hu-24	4	IP-09	25%
Q.	Hu-25	3	OP-15	05%
R.	Hu-26	3	0P-16	60%
S.	Hu-27	4	OP-16	30%

OP = outbred pig IP = inbred for MHC Hu = human

Tab	le.	3
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	EPID Cells	Time in	Dermis	Maximum % of	F Ia + cells
		culture in		Induction in	
		weeks		days	
Α	IP-9	3	IP-11	3	30%
	IP-9	3	IP-09	3	5%
В.	IP-9	5	IP-12	4	60%
	IP-9	5	IP-10	4	8%
C.	OP - 1	10	OP - 3	3	15%
	OP - 1	10	OP - 1	3	2%
D	IP-9	4	IP-11	4	80%
	IP-11	4	IP-11	4	15%
E.	OP - 5	9	OP - 6	5	30%
	OP - 6	9	OP - 6	5	30%

OP = Outbred Pig IP = Inbred Pig