Cultured allogeneic keratinocyte transplantation in the Large White pig

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This thesis represents the results of work that I personally undertook as a research fellow at the Blond McIndoe centre, East Grinstead between 1998 and 2000. The work is entirely my own unless otherwise stated. No part of this work has been or is currently being submitted for any other degree or qualification at another university.

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Abstract

Introduction: *Banked cultured Langerhans cell (LC) free allogeneic keratinocytes, could be a readily available resource for the treatment of burns. The key questions that have not been resolved regarding the natural history of allotransplanted cultured keratinocytes, are when, how and why they are eliminated. Previous work at the Blond McIndoe Centre in a Large White pig model has demonstrated the survival of allotransplanted keratinocytes at three weeks in a "chimeric" context (transplanting mixtures of autologous and allogeneic keratinocytes). The cultured cells were labelled by retroviral gene transfer to introduce a lacZnls histochemically detectable marker. This work however, involved grafting onto de-epidermalised dermis that was shown to be capable of regenerating an epidermis without the aid of transplanted keratinocytes. Additionally, the keratinocyte labelling efficiency was low. The purpose of this study was to investigate the fate of cultured allogeneic transplanted keratinocytes, when they are the only source of epidermal tissue in a wound incapable of epidermal regeneration. To accomplish this, the aim was to develop a more efficient means of porcine keratinocyte labelling with the lacZnls marker and to immunohistochemically characterise the cellular infiltrate with regard to allogeneic split skin graft rejection and compare this with the immune cell infiltrate associated with lacZnls labelled LC-free cultured allogeneic keratinocytes grafted onto Integra™ (a non-regenerative dermal template).*

Methods: *The Large White pig was used in view of the similarity of its skin architecture to humans. Retroviral gene transduction of cultured porcine keratinocytes was conducted with a MFGlacZnls construct in the PT67*

amphotropic packaging cell line. Leucocyte phenotypes infiltrating porcine skin were characterised immunohistochemically using a panel of monoclonal antibodies specific to porcine antigens: CD1, CD3, CD4, CD5, CD6, CD8, CD25, CD45, SWC3, SWC6, SLA DP, SLA DP, SLA DR and IgλLC with ABC staining. Porcine jejunum was employed as a positive control. LC depletion with Anti-MHC II and complement was monitored using flow cytometry. MFGlacZnls labelled LC-depleted cultured keratinocytes sheets and keratinocyte-dermal composites were auto and allografted onto Integra™ and onto deep fascia respectively. LacZnls β-galactosidase expression was detected using X-Gal staining of tissue biopsies at different time points up to 21 days following grafting and a reduced panel of monoclonal antibodies (omitting CD6, SLA DP and SLA DQ) was used to immunophenotype the infiltrate at these time points.

***Results**: The new PT67 producer line succeeded in achieving close to 100% of keratinocyte transduction with the MFG lacZ construct following three passages in a cell culture system containing irradiated retroviral producer cells and 3T3 fibroblasts as feeder cells. Stability was demonstrated with no apparent reduction in lacZ nls expression by porcine keratinocytes following a further seven passages on irradiated 3T3 fibroblasts. A reduction was demonstrated however, both in the number and size of colonies formed by keratinocytes following transduction. LCs were shown to be successfully eliminated from disaggregated epidermal cells using antibody and complement. We also demonstrated that overnight incubation results in up regulation of MHC class II antigen and increases the efficiency of LC elimination. As with retrovirally transduced keratinocytes a reduction was*

demonstrated however, both in the number and size of colonies formed by keratinocytes that had undergone treatment with antibody and complement. The differential distribution of leucocyte phenotypes as determined by the panel of antibodies within normal porcine skin was shown for the first time. The cell phenotypes examined were CD1 (mainly dendritic cell), pan leucocyte determinant CD45, pan T-cell determinants CD3, CD5 and CD6 as well as CD4 and CD8, MHC class II determinants SLA DP DQ and DR, SWC3 (found on cells of myeloid extraction including macrophages and granulocytes), SWC6 (null cell population), immunoglobulin light chain (B cell) and CD25 (IL-2 receptor). CD1-positive cells were found mainly in the epidermis. The remainder were concentrated in different proportions within the papillary dermis and around hair follicles. Counts of all determinants except for CD1 (and SWC6) were considerably raised in split skin graft undergoing rejection. With regard to cultured allogeneic keratinocytes, results suggest that they are rejected after 18 to 21 days when grafted onto biointegrated Integra™. Keratinocyte dermal composite grafts surviving at three weeks, however showed no sign of rejection. This difference in survival may be related to the 'proinflammatory nature of Integra™' alluded to by others. Any inference relating to the afore mentioned results must be however, tempered with caution as a high percentage of grafts were lost due to problems relating to the wound chamber. The staining pattern with regard to cultured keratinocytes grafted onto Integra™ suggests infiltration by dendritic cells by day 7. The infiltrate at the time of graft loss was comprised mostly of T-cells (SLA DR staining suggests many are activated) with CD4 and CD8 phenotypes present in equal number. With regard to the

keratinocyte dermal composites, although host dendritic cells were seen to have infiltrated the graft bed by day 14, the character of infiltrate did not suggest rejection at any of the time points.

***Conclusions**: This study has succeeded in characterising the differential immunoinfiltrate invading LC-free cultured allogeneic as opposed to autologous cultured porcine keratinocytes. The residence of the autologous and allogeneic cells was demonstrated unequivocally using retroviral gene marking. Due to problems relating to the wound chamber model a high percentage of grafts were lost. This has meant that results can only be suggestive and that it is very difficult to draw meaningful conclusions. The immune rejection of keratinocytes grafted onto Integra™ was however not of great vigour and was not evident with respect to the composite grafts at 21 days. This is consistent with a promising potential for LC-free cultured allogeneic skin products in the treatment of burn injuries.*

List of abbreviations

AB	antibody
ABC	avidin biotin complex
Ag	antigen
AKT	allogeneic keratinocyte transplantation
AMCA	aminomethylcoumarin
APC	antigen presenting cell
BSA	body surface area
CaCl	calcium chloride
CD	cluster of differentiation
CEA	cultured epidermal autograft
CEG	cultured epidermal graft
CJD	Creutzfeldt-Jakob disease
CTL	cytotoxic T lymphocyte
DAB	diaminobenzidine
DC	dendritic cell
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
DPX	distyrene, plasticiser (tricresyl phosphate), xylene
ECM	extracellular matrix
Fc	fragment crystallisable
FITC	fluorescein isothiocyanate
FACS	fluorescence activated cell sorting
HBSS	Hanks buffered saline solution
H&E	haematoxylin and eosin
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
γ-IFN	gamma interferon
IgλLC	immunoglobulin lambda light chain
IMS	Industrial methylated spirits
K	keratin
IL-2	interleukin 2
IL-2R	interleukin 2 receptor
LC	Langerhans cell
MELR	mixed epidermal cell lymphocyte response
MHC	major histocompatibility complex
MoAB	monoclonal antibody
NaCl	sodium chloride
NK	natural killer
NHS	national health service
OCT	optimum cutting temperature
PBL	peripheral blood leucocytes
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PI	propidium iodide
PTFE	polytetrafluoroethylene
RNA	ribonucleic acid
SLA	swine leucocyte antigen

SSG	split skin graft
TA	tranisent amplifying
TCR	T-cell receptor
Th	T helper cell

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1.1 Early burn wound resurfacing

Intact skin serves as a barrier preventing microbial invasion as well as fluid and nutrient loss. Destruction of skin due to thermal insult results in loss of this barrier capability. The degree to which this may impact on survival has been shown to be related to the amount of body surface area (BSA) burnt (Bull, 1971). Wounds that are greater than 20% BSA pose a significant threat due to fluid loss and potential infection and do best with early excision and resurfacing (Burke et al., 1974).

Earlier resurfacing of wounds also helps limit systemic inflammatory and catabolic responses (Press, 1997).

1.2 Split skin grafts

Autologous split thickness skin grafts remain the gold standard for resurfacing burns. In an injury more extensive than 30% BSA however, limitations in available skin graft donor area often necessitate multiple operations. Dermis does not regenerate. Repeat harvesting from the same donor area results in progressive dermal thinning and may result in poor healing and scarring (Place et al., 1997). There is also the additional burden of blood loss that occurs with skin harvest and the act of harvesting a graft creates a new wound, which further adds to the metabolic burden.

1.3 Cultured epithelium

In 1975 Rheinwald and Green succeeded in producing sheets of cultured human keratinocytes using a 3T3 murine fibroblast feeder layer and a growth factor-enriched medium (Rheinwald and Green, 1975). The introduction in 1979 of an enzyme (Dispase^{TM}) that selectively disrupted bonds between keratinocytes and glass or substrate plastic, but not intercellular bonds, allowed the sheets to be lifted out and used as grafts (Green et al., 1979). Since then there have been a number of reports detailing the use of cultured autologous epithelium as sheet grafts in burns with mixed success (Hefton et al., 1983; Madden et al., 1986; Thivolet et al., 1986; Pandya et al., 1998). In spite of advances in culture technology it still takes between 3 to 6 weeks to produce confluent keratinocyte sheets ready for grafting, thus limiting their usefulness.

1.4 Allogeneic keratinocyte transplantation

Allogeneic keratinocyte transplantation (AKT) (Hammond et al., 1987; Brain et al., 1989) refers to the grafting of cultured keratinocytes between genetically different members of the same species. Keratinocytes may be cultured and cryopreserved in great numbers. 'Banked' allogeneic keratinocytes could thus be used to provide sufficient confluent sheets for immediate use as an elective procedure.

AKT may in addition to the provision of epithelial cover in burns, be useful in a variety of clinical settings. AKT may be of benefit in the promotion of healing

within chronic leg ulceration (Leigh et al., 1987), skin graft donor sites, wounds created by removal of congenital giant naevi and in chronic fragile skin conditions such as epidermolysis bullosa (Hill et al., 1992). Genetically modified allogeneic keratinocytes may also be used to deliver gene therapy (Bevan et al., 1999).

A number of difficulties have been encountered with cultured sheet grafts that potentially compromise their take as well as long term survival of the graft. Fragility of cultured keratinocyte sheets (making them difficult to handle and easily damaged) is a technical problem common to both autologous and allogeneic grafts. Additionally, optimal health and vascularity of the bed is required to ensure graft take.

Viral transmission such as with HIV by infected allogeneic keratinocytes is a potential problem for the recipient of allogeneic (or xenogeneic) keratinocytes (Clarke, 1987);(Rappersberger et al., 1988; Chesebro et al., 1990; Pirnay et al., 1997; Kim et al., 1992; Ramarli et al., 1995). Reports regarding the transmission of new variant CJD through transplantation of allogeneic keratinocytes or any other transplanted organ are yet to emerge.

The majority of reports indicate that transplanted cultured allogeneic epithelium are eventually lost (Aubock et al., 1988; Burt et al., 1989; Brain et al., 1989), although there have been isolated reports of their long term survival (Otto et al., 1995). Aside from the aforementioned potential technical obstacles, which may be surmountable, rejection remains a paramount consideration.

1.5 Rejection of allogeneic skin grafts

Acute rejection of allogeneic skin graft refers to the destruction of transplanted skin that occurs following a period of transplant acceptance. It was characterised in detail by Medawar and Gibson (Gibson and Medawar, 1943). Following early experience with second set skin allograft reactions in human burns they concluded that an active acquired or specific immune process caused graft rejection after an initial period of take (Gibson and Medawar, 1943). Medawar observed the survival of autologous skin surrounded by allograft undergoing rejection (Medawar, 1944), reinforcing the view that rejection is cell-specific. He further concluded that antigen-antibody interactions were responsible for graft destruction rather than direct lymphocyte involvement (Medawar, 1944; Medawar, 1945).

In the late 1940's and early 1950's evidence began to accumulate (particularly with regard to tumour antigens) suggesting that lymphocytes were intimately involved with and mainly responsible (in acute rejection) for killing allogeneic cells (Snell, 1957).

Within the last two to three decades advances in three principle areas have contributed to the current understanding of the rejection process. The first concerns work in the field of dendritic cells. Work in this field contributed to understanding how the rejection process is initiated. The second involves the development of monoclonal antibodies. Early work using cytotoxicity assays (Kisielow et al., 1975), helped to delineate T from B cells and discover certain T-cell subsets. The advent of hybridoma technique in the development of

monoclonal antibodies (Kohler and Milstein, 1975) in the mid 1970s, facilitated subsequent identification of discrete T and B cell phenotypes. This also enabled the study of rejection occurring following T-cell depletion by prior administration of particular monoclonal antibodies (Tinois et al., 1989).

Development of mutant mouse strains expressing discrete structural mutations in products of the major histocompatibility complex (MHC) comprised the third principle area. This allowed for study of rejection across specific histocompatibility barriers (Rosenberg et al., 1991).

1.5.1 Sensitisation phase of acute rejection

Acute rejection of transplanted allogeneic split skin grafts is initiated following the migration of mature Langerhans cells (LC) to regional nodes where they fulfil their role as APCs. The LC present both class I (expressed normally by keratinocytes) and class II (expressed by LCs but not normally by keratinocytes) MHC antigens (Romani and Schuler, 1992; Rosenberg and Singer, 1992).

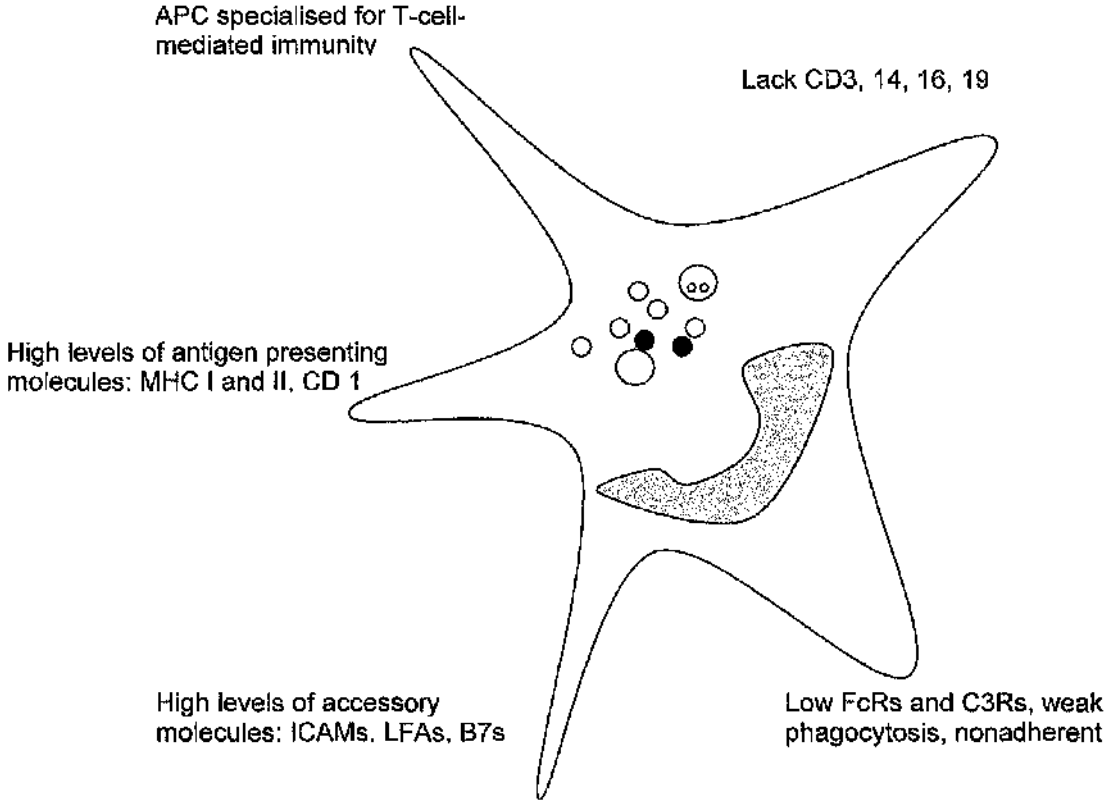
1.5.2 Langerhans cells

LC (see Figure 1.1) first described by Paul Langerhans in 1868 were originally thought to be a cell type of the nervous system (Romani and Schuler, 1992). They then were regarded as worn out melanocytes and only after the demonstration by electron microscopy of a specific cytoplasmic organelle, the Birbeck granule, did they assume an identity as a distincT-cell type. In the 70's MHC II molecules as well as Fc and complement receptors were

demonstrated on the cell surface confirming their nature as immunocompetenT-cells (Romani and Schuler, 1992). Although direct evidence is still lacking it now seems most probable that the LC lineage is myeloid and might be derived from precursors found in bone marrow (Steinman, 1991). *In vitro* studies established that LCs mature into potent immunostimulatory dendritic cells (Schuler and Steinman, 1985). In this state they lose the ability to further take up antigen. Cell expression of MHC II and MHC I complexes in which the antigen is presented increases dramatically with maturation (Witmer-Pack et al., 1988; Banchereau and Steinman, 1998).

Figure 1.1:

Schematic diagram illustrating features of dendritic cells (adapted from Steinman et al., 1995).



These LCs have been shown to be powerful stimulators of T-cell proliferation (Schuler and Steinman, 1985; Banchereau and Steinman, 1998). *In vivo* the life cycle of LCs is thought to originate in the bone marrow (Romani and Schuler, 1992). Within the epidermis antigen may be phagocytosed and processed, following which the LC will commence the process of maturation by migration to a regional node. The regional node is thought to be their 'final resting ground' where the LC mature into dendritic cells where they may present antigen to naïve T-cells (Knight and Stagg, 1993; Steinman et al., 1995).

Interaction between the T-cell CD (cluster of differentiation) 28 and the APC B7 costimulatory molecule (as well as that between the T-cell receptor (TCR) and major histocompatibility molecules) is thought to be necessary for effective T-cell alloactivation (Abraham et al., 1992); (Allison, 1994). Other cells besides dendritic cells (DCs) are capable of presenting antigen but in spite of this are unable to alloactivate naïve Th cells. These cells tend to lack the necessary CD28 ligands (Nickoloff et al., 1995).

Although certain other cells such as macrophages and B cells may be capable of stimulating proliferation of memory T-cells once they themselves are activated, by far the most potent stimulator of naïve T-cells are DCs (Knight and Stagg, 1993; Janeway and Bottomly, 1994). Keratinocytes although not capable of antigen processing, can be induced to express both MHC II molecules as well as the CD28 ligand B7-3 (Nickoloff et al., 1995). The significance of this is not yet clear.

Within skin there are now thought to be three pools of dendritic cells. One pool, represented by LCs, is contained within the epidermis. A further pool

exists within the dermis. These differ from LCs in their cell surface expression as well as a lack of Birbeck granules. In culture however, they behave like dendritic cells in terms of their shape and motility, cell surface phenotype and potent T-cell stimulating activity. The third pool exists within afferent lymph and are thought to be shorter lived and have a higher turn over than the epidermal LCs (Steinman et al., 1995). In comparison to epidermal LCs the immune surveillance function of afferent and dermal dendritic cell pools are not as well defined.

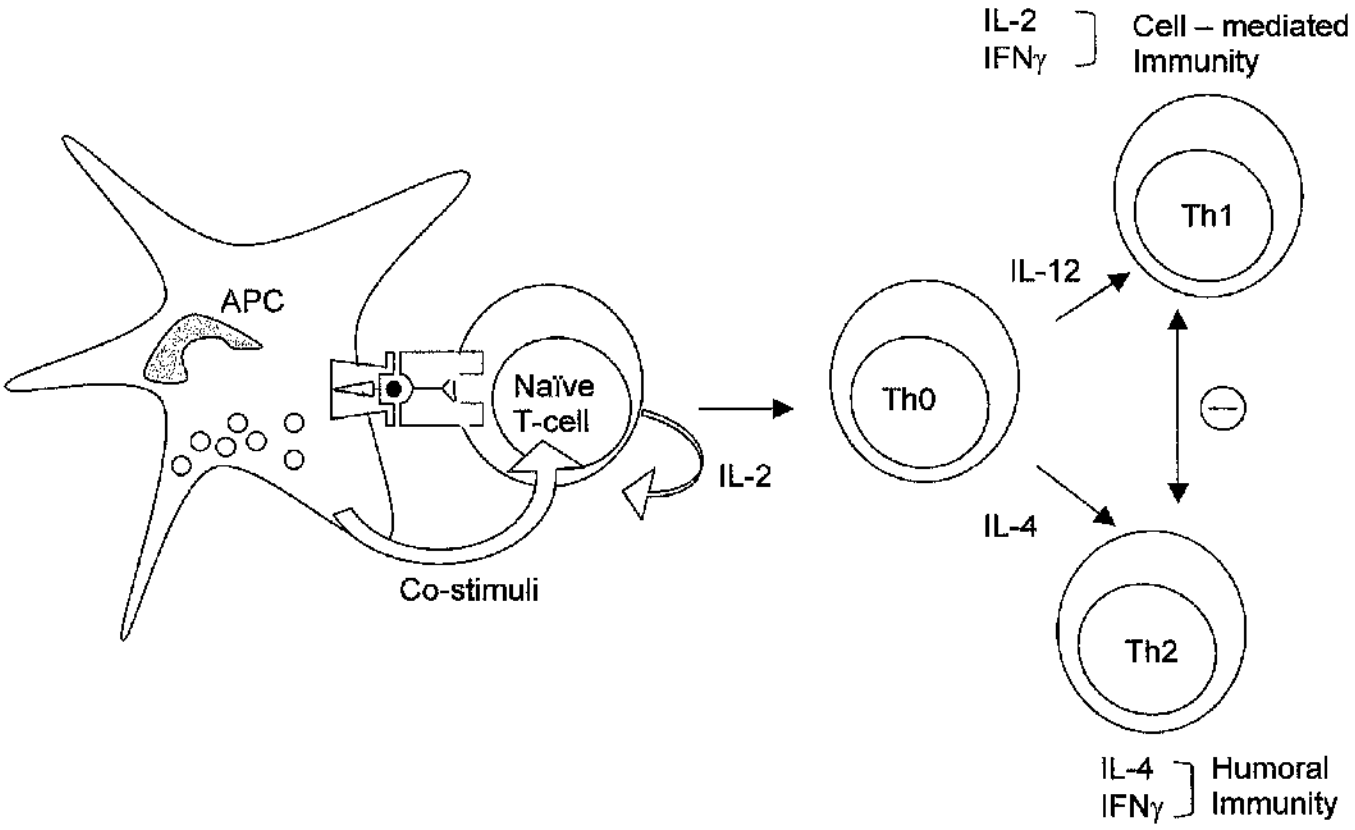
Following primary allografting of skin there are two principal ways in which naïve T-cells become activated. The 'direct' pathway is defined as the processing, migration to a regional node maturation and presentation of alloantigen by a graft derived APC. The classical or 'indirect' pathway is the phenomenon whereby host LCs migrate into the graft, process antigen and then migrate back out to regional lymph nodes to alloactivate naïve T-cells (Schuurman, 1994). Although the direct pathway is thought to be important to the initiation of the acute rejection process in sensitising the host to graft antigens the indirect pathway may have a role to play with regard to driving the destruction of transplanted tissue once underway (Benichou, 1999). Naïve T-cells distribute preferentially from blood to lymphoid tissues via the high endothelial venules (Knight and Stagg, 1993). This is consistent with much of the evidence to date which supports central (i.e. within a regional draining node) presentation as essential for the initiation of acute rejection (Barker and Billingham, 1967; Tilney and Gowans, 1970; Rosenberg and Singer, 1992). Memory T-cells are distributed to non-lymphoid tissues (Knight and Stagg, 1993) and consequently may be open to activation at these sites (in

secondary immune responses or late rejection) by indirect means (host derived APCs) (Benichou, 1999).

1.5.3 T-cells

Following activation by primed APCs phenotypically distinct T helper (Th) cells functionally differentiated by their cytokine expression profile may be demonstrated (see Figure 1.2). Th0 cells (cytokine profile: IL-2, IL-4 IFNγ, lymphotoxin) may be induced to differentiate into Th1 cells (cytokine profile: IL-2, IFNγ, lymphotoxin) specialising in cell-mediated immunity, by exogenous IL-12 or into Th2 cells (cytokine profile: IL-4, IL-5, IL-6, IL-10) specialising in humoral immunity, by exogenous IL-4 (Nickerson et al., 1994).

Figure 1.2: Schematic diagram illustrating the Th1/Th2 paradigm (adapted from Nickerson et al., 1994).



IL-2 is required for a further complex interaction to occur between resting cytotoxic T lymphocytes (CTL) and APCs transforming them into mature CTL cells (Rosenberg and Singer, 1992).

The sensitisation phase initiated by APCs encompasses therefore Th cell activation as well as the generation of CTL cells.

Th and resting CTL cells tend to differ with respect to their CD4/CD8 phenotypes and hence their receptor specificities. Th cells express CD4; an accessory molecule involved in the recognition of processed foreign antigen presented within the MHC II molecule cleft (Barclay et al., 1997). CD8 is a co-receptor with MHC I restricted T-cell receptors and is associated with resting CTL function (Barclay et al., 1997). Although the above is the general rule, there is a population of T-cells, of varying proportion depending on species, that express dual CD4/CD8 antigens and appear able to assume both T helper and cytotoxic functions (Rosenberg and Singer, 1992).

1.5.4 B cells

B cells are no longer thought to play the dominant role in acute skin graft rejection (Rosenberg and Singer, 1992). It is now thought that during acute rejection, antibody activity is principally targeted at endothelial MHC II antigens (Mason and Morris, 1986).

1.5.5 Effector phase

The effector phase is characterised by a (principally) cell-mediated response involving CD8-expressing CTL cells (Rosenberg and Singer, 1988) (Rosenberg and Singer, 1992).

CTL induced cell death is allogeneic cell-specific (Chandler and Passaro, 1993), sparing neighbouring non-allogeneic cells. Acute rejection also results in a degree of non-specific damage to non-allogeneic neighbouring cells. This is thought to be caused by infiltrating inflammatory cells such as activated macrophages which may be mediated by inflammatory cytokines secreted by activated Th or CTL cells (Chandler and Passaro, 1993; Rosenberg and Singer, 1992). The extent of bystander tissue damage relates directly to the degree of associated inflammation (Doody et al., 1994; Rosenberg and Singer, 1992). The way in which the rejection response is initiated may be important to the degree of inflammation. Were the mechanism for direct presentation to be bypassed and indirect presentation to proceed gradually and late and in addition to be targeted only at MHC I antigens, the accompanying inflammatory response may be much dampened. Damage to neighbouring autologous tissue may thus be reduced.

1.6 Rejection of cultured keratinocyte allografts

There have been conflicting reports concerning the behaviour of LCs *in vitro* when disaggregated epidermal cells are co-cultured. Certain investigators failed to demonstrate the presence of LCs (Hefton et al., 1984; Morhenn et

al., 1982; Thivolet et al., 1986); in addition the cultured grafts appeared to survive transplantation. Others demonstrated the presence of LCs in human epidermal cell culture at three weeks (Demidem et al., 1986). It has since become apparent that LCs in culture can alter phenotype considerably depending on culture conditions (Teunissen et al., 1990; Witmer-Pack et al., 1988; Schuler and Steinman, 1985; Romani and Schuler, 1992) which may partly explain the above.

Initial reports in the 80's failed to demonstrate an overt immune response mounted against cultured epidermal allografts (Thivolet et al., 1986; Hefton et al., 1983; Madden et al., 1986; Gielen et al., 1987). The models used however, were human partial thickness wounds of uncontrolled depth, such as tangentially excised partial thickness burns (Thivolet et al., 1986; Madden et al., 1986) or chronic ulcers (Hefton et al., 1983; Gielen et al., 1987)), which may well have contained residual regenerative epithelial elements.

Several investigators have been unable to demonstrate residual donor keratinocytes in the healed wound. This despite the absence of an overt rejection response and have therefore suggested that allogeneic donor cells undergo replacement by host epithelial elements (Gielen et al., 1987; Burt et al., 1989); (Brain et al., 1989); (van der Merwe et al., 1990); (Zhao et al., 1992) (Phillips et al., 1993). This is consistent with the notion of spontaneous cell death or cell-specific targeting by CTL cells with minimal accompanying inflammatory destruction of neighbouring elements.

Others (Aubock et al., 1988) have however demonstrated abrupt rejection of cultured epidermal allografts with a 14.5 day (mean) survival time.

All the above studies concentrated on chronic ulcers or burns in humans. In the late 1980's and early 90's increasing use was made of animal models to study rejection of allogeneic elements in a detailed and controlled manner (Fabre and Cullen, 1989; Doody et al., 1994; Carver et al., 1991; Suzuki et al., 1995; Rouabhia, 1996; Rouabhia et al., 1995).

Fabre working with inbred rats demonstrated that acute rejection of cultured keratinocyte allografts does not appear to be associated with antibody production (Fabre and Cullen, 1989). Rejection however, was delayed only by a few days when compared with split thickness allografts (supporting Aubock's earlier work (Aubock et al., 1988)). Carver et al., (1991) similarly reported only a brief delay in rejection of cultured allogeneic epithelial grafts in a pig model.

1.7 Chimeric grafts

The chimera in classical Greek mythology was a beast with the head of a lion, the body of a goat and tail of a serpent (Allen, 1990). The term has been adopted to represent tissues, organs or whole animals engineered to contain elements of differing parentage. The effect on autologous elements that a potential immune response mounted against foreign elements might have can thus be observed. Within the last few years attention has focused on the design of chimeras of cultured keratinocyte sheets consisting of syngeneic and allogeneic keratinocyte elements of varying proportions (Rouabhia et al., 1995; Suzuki et al., 1995). Using inbred mouse strains one group reported replacement of allogeneic keratinocytes by 30 days post grafting (Rouabhia et

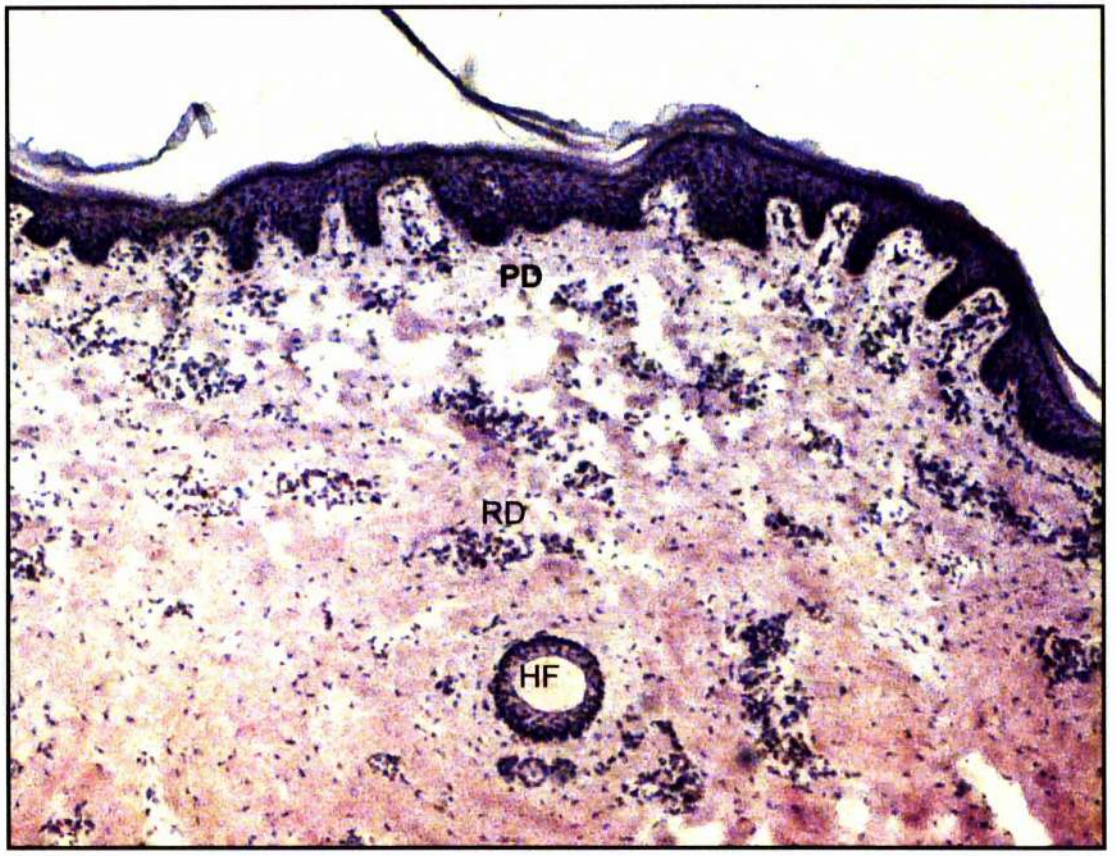
al., 1995). In comparison to control allogeneic grafts only a weak immune response was mounted against the chimeric graft. A further group using similar inbred mouse strains looked at chimeric grafts with a syngeneic to allogeneic ratio of: 1:15 and demonstrated similar success (Suzuki et al., 1995). The study ended at 14 days post grafting. Both studies showed complete replacement of autologous by syngeneic keratinocytes at the study end point. In a further study Rouahbia looked at chimeras consisting of cultured xenogeneic (human) and syngeneic keratinocytes. Results were similar to those in the previous experiment, with the chimeric grafts surviving at 30 days almost as well as the isograft with similar ultrastructural characteristics (Rouabhia, 1996). These results suggest (at least in the particular inbred mouse populations studied), a highly selective or cell-specific rejection response sparing syngeneic elements.

1.8 Porcine model

The domestic pig has been used to study wound healing for at least 4 decades (Winter, 1962). Porcine skin resembles human skin more closely than other common small laboratory animals. The epidermis and dermis are of similar thickness and there is a distinct dermal papillary layer (see Figure1.3). Both man and pig rely on subdermal fat rather than fur for insulation. Its epidermal appendages consist of hair follicles, sebaceous and apocrine glands. One way in which the pig differs from humans is in its lack of eccrine sweat glands (Winter, 1972) as it does not regulate body heat with surface sweating.

Figure 1.3: Porcine Skin

Photomicrograph (X40) H&E stained, formalin fixed cryostat sections.
PD = papillary dermis; RD = reticular dermis; HF = hair follicle



Porcine keratinocytes may be cultured with relative ease to produce sheet grafts (Bevan et al., 1997). Resultant sheets show similar ultrastructural characteristics to human cultured epithelial sheets (Bevan et al., 1997); (Kangesu et al., 1993). The creation of wounds that extend down to muscle fascia, reduces the possibility of re-epithelialisation by residual host keratinocyte elements. Take of autologous cultured sheets has been demonstrated on a wound bed prepared with the non-regenerative dermal template Integra™ (Grant and Martin, 1998). In this model a 4cm diameter disc of Integra™ was stitched to the wound bed and left to take for 10 days prior to receiving the cultured epidermal sheet. The graft was encompassed within an open cylindrical PTFE chamber as described by Kangesu and colleagues in 1993. The graft was covered by non occlusive saline-soaked gauze dressing facilitating easy access to the wound bed as well as reducing contamination by host keratinocytes from the peripheral wound edge. Although Integra™ has in the past been investigated with respect to autologous cultured epithelium in the porcine model (Grant and Martin, 1998), there are no reports of its use in conjunction with cultured allogeneic epithelium. Integra™, a dermal substitute consisting of bovine collagen and chondroitin-6-sulfate and an epidermal layer of synthetic polysiloxane polymer (Silastic) is known to encourage a mild inflammatory response (Stern et al., 1990). This presence of inflammatory cells at the time of allografting may prejudice graft take.

Swine have been used as a model for allotransplantation for more than three decades (Calne et al., 1969). The similarities in physiology and immunology have lead researchers to experiment with kidney, liver, heart, pancreas and

small bowel transplantation (Kenmochi et al., 1994). Their size, laboratory adaptability and breeding characteristics are further advantages. Serious consideration has in recent years been accorded the pig as a potential xenograft donor with regard to a variety of organs (Sachs, 1994). There has been intensive work in the last decade in the production of porcine-specific monoclonal antibodies (Saalmuller et al., 1998a), which has made it possible for in depth phenotyping of cells of the porcine immune system to be carried out.

1.9 Cell labelling

Various attempts have been made in the past to label cultured cell populations in order to track their fate after grafting. Methods used with regard to the human model are varied. Typing of ABO antigens as expressed by epidermal cells in the upper Malpighian layers has been attempted (Thivolet et al., 1986). Others have looked at MHC class I expression by human keratinocytes (Gielen et al., 1987), or Y chromosome detection (male to female recipient) (Burt et al., 1989; Brain et al., 1989), and DNA fingerprinting (van der Merwe et al., 1990). In the pig model neither blood group nor MHC class I characterisation of keratinocytes is established as yet. Female Large White pigs adapt more easily to the animal house and laboratory environment and are easier to handle. We have therefore used them in preference to males and so could not consider looking at Y chromosome detection. We were interested in a method that allowed monitoring of autologous (as well as

allogeneic) keratinocyte sheet grafts which therefore precluded the use of DNA fingerprinting.

Using retroviral transduction techniques the reporter gene MFG lacZnls coding for the marker enzyme β-galactosidase has been successfully introduced into porcine keratinocytes (Bevan et al., 1997; Ng et al., 1996a; Ng et al., 1996b; Ng et al., 1997a). The nuclear localised β-galactosidase enzyme causes the nucleus to turn blue when fixed and then incubated with X-Gal or magenta when incubated with M-Gal.

1.10 Thesis objectives

This thesis sets out to examine the effector immune response to allogeneic keratinocytes within the Large White pig model (see Figure 1.4).

My objectives were:

1. To establish a means of tracking keratinocytes *in vivo*. Work therefore concentrated initially on the development of a labelling technique employing retroviral vectors of nuclear localised genes. It was then important to ascertain whether the method was intrinsically damaging to keratinocytes.
2. To attempt to ensure that grafted cells were free of LCs. As discussed above, investigators had previously determined that the LC phenotype *in vitro* contrasts strongly with that *in vivo* maturation (Teunissen et al., 1990; Witmer-Pack et al., 1988; Schuler and Steinman, 1985; Romani and Schuler, 1992). Little is known about the behaviour of LCs in the type of medium we use to culture porcine keratinocytes which is designed to

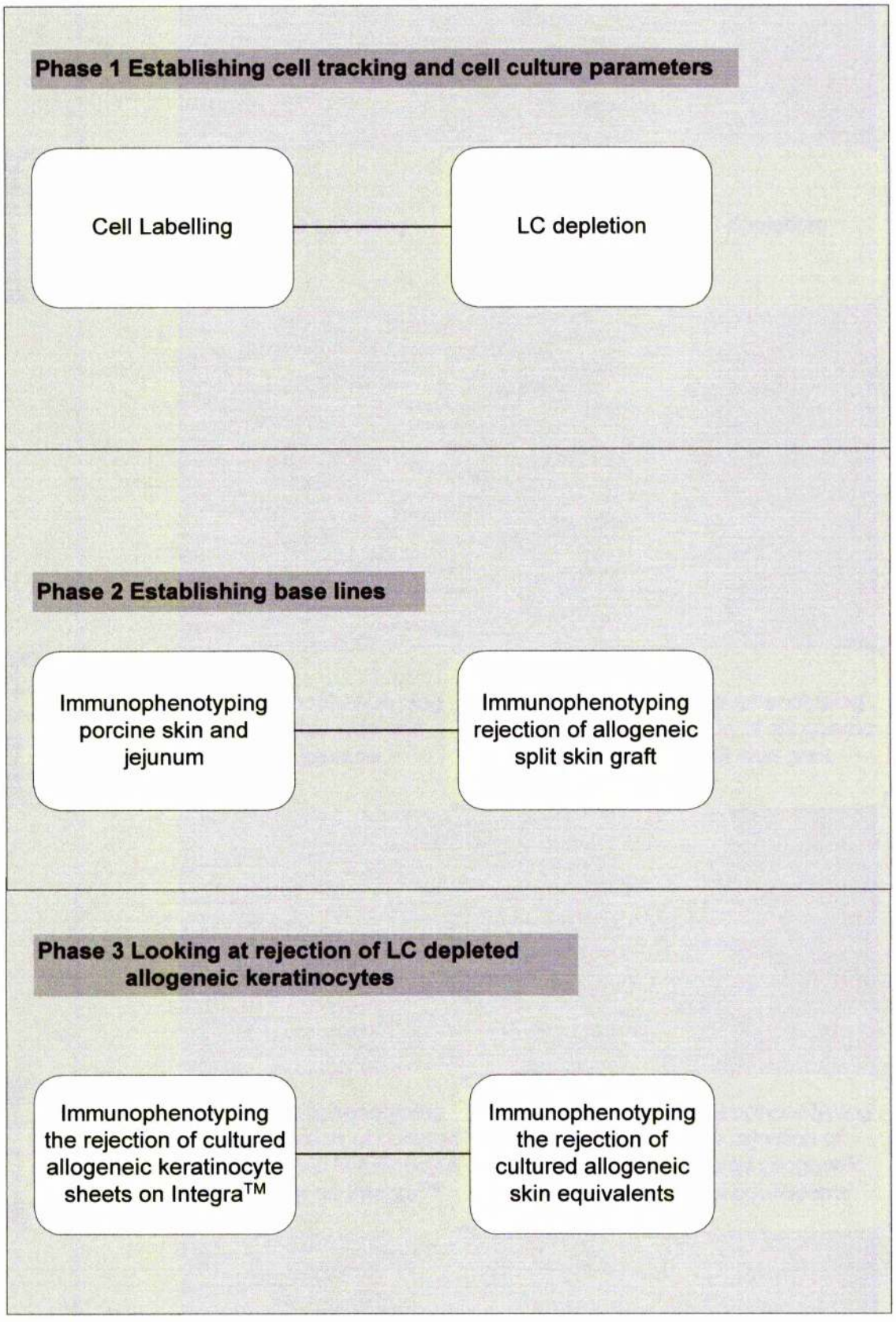
prevent early differentiation. Most researchers (excluding (Demidem et al., 1986)) have had difficulty detecting LCs (Hefton et al., 1984; Morhenn et al., 1982; Thivolet et al., 1986). It was decided therefore to proceed down the road of direct elimination. Here too it was felt important to examine whether the eliminatory process (antibody complement lysis) was detrimental to keratinocyte replication.

3. Examine the leucocyte population within normal Large White pig skin using a range of porcine-specific monoclonal antibodies. The few monoclonal antibodies that had been tested thus far were used to determine the immune cellular population within porcine jejunum. The majority of those now available had however, not previously been tested with respect to porcine skin. Having assembled a panel of recently developed monoclonal antibodies I sought first to examine the distribution of CD-specific cells within normal porcine skin and jejunum. Here we were seeking to establish a baseline for future investigations.
4. Evaluate leucocyte-infiltrating allogeneic split skin grafts undergoing rejection (the classical skin transplant model). Here too, the aim was to examine the population for comparison with later investigation.
5. Immunophenotype the response to cultured allogeneic keratinocytes grafted onto Integra™.
6. Establish a 'living skin equivalent' (keratinocyte dermal composite) and then examine the immune response following allografting using immunophenotyping techniques.

The long term fate of grafted cultured allogeneic keratinocytes remains uncertain. The host immune response to AKT has thus far not been well characterised.

As outlined above there are a variety of clinical settings in which AKT may be useful. Through elucidating the host response to AKT it is hoped that it will one day be possible to devise techniques that allow maximally effective use of AKT thus helping to minimise patient morbidity.

Figure1.4: Flow diagram illustrating general outline of thesis



Chapter 2

Materials & Methods

2.1 MATERIALS

2.1.1 Cell culture

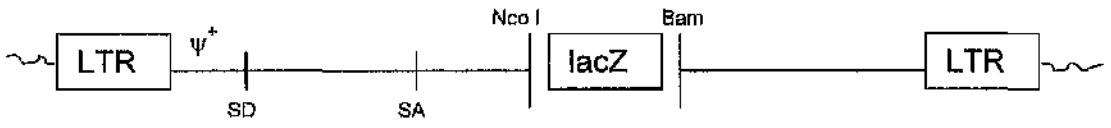
2.1.1.1 Cell Lines

3T3 Swiss Mouse fibroblasts were obtained from the Imperial Cancer Research Fund, London.

The ecotropic retroviral producer cell line TELCeB which produces the MFG lacZ nls retroviral vector (Ferry et al., 1991) (see Figure 2.1) was obtained from D. Winton & S. McCutcheon, Cambridge.

Figure 2.1: Schematic diagram of MFG vector construct.

MFG recombinant retrovirus encoding lacZ. Moloney murine leukaemia virus (MoMuLV) long terminal repeat sequences are used to generate both a full length viral RNA (for encapsidation into virus particles) and a subgenomic mRNA, which is responsible for expression of inserted sequences. No selectable marker exists in the vector (adapted from (Dranoff et al., 1993)).



The retroviral packaging cell line GP+EnvAm12 (Markowitz et al., 1988) was obtained from Genetix Pharmaceuticals, New Jersey USA.

The amphotropic retroviral packaging cell line PT67 was obtained from Clontech, California 94303-4230, USA.

Porcine keratinocytes were freshly obtained following disaggregation of partial thickness skin harvested from Large White pigs.

2.1.1.2 Cell culture media and supplements

MATERIAL	**SUPPLIER**
Dulbecco's Modified Eagle's Medium DMEM (Glucose 1g per l)	GIBCO BRL, Life Technologies, Paisley
Dulbecco's Modified Eagle's Medium DMEM with Glutamax™ (Glucose 1g per l)	
Dulbecco's Modified Eagle's Medium DMEM with Glutamax™ and Glucose 4.5g per l	GIBCO BRL, Life Technologies, Paisley
Calcium-free Opti-MEM®1	
Calcium-free Opti-MEM®1 with Glutamax™	
Hank's balanced salt solution (HBSS)	Sigma-Aldrich Co. Ltd., Poole, Dorset.
Fetal Calf Serum (FCS) Batch No. 72189	Imperial Laboratories Ltd, West Portway, Andover, Hant's, England

Penicillin/streptomycin 5000units per ml per 5000μg per ml	GIBCO BRL, Life Technologies, Paisley
Gentamicin 50 mg per ml	
Amphotericin B 250μg per ml	
L-Glutamine 200mM	
Calcium Chloride 0.1M	
Ciprofloxacin 2 mg per ml	BAYER AG, Levrkusen, Germany
NUTMIX F12 (HAM with Glutamax™)	GIBCO BRL, Life Technologies, Paisley
Recombinant human epidermal growth factor	Sigma-Aldrich Co. Ltd., Poole, Dorset.
Hydrocortisone	
Cholera toxin	
Actrapid® (recombinant human insulin)	Novo Nordisk Pharmaceutical Ltd, Crawley, West Sussex
Triiodothyronine (T3)	Sigma-Aldrich Co. Ltd., Poole, Dorset.
Adenine	

2.1.1.3 Culture materials (miscellaneous)

MATERIAL	SUPPLIER
25 cm^2 filter capped tissue culture flasks	Greiner Labortechnik Ltd, Dursley, Gloucestershire
75 cm^2 filter capped tissue culture flasks	
Petri dishes tissue culture grade 100mm X 20mm	
Petri dishes 60mm X 15mm	Corning Glass Works, Corning, NY 14831
Universal flasks (20ml)	Becton Dickinson Labware, Becton Dickinson Labware Europe,
Falcon® conical flasks (50ml)	
24 well tissue culture plates	
6 well tissue culture plates	
Syringes 1ml, 2ml, 5ml, 10ml, 20ml	
Rat tail collagen type I	Sigma-Aldrich Co. Ltd., Poole, Dorset.
Trypsin (0.25%)	Difco Laboratories, Detroit Michigan
Trypsin/EDTA (0.05%/0.02%)	GIBCO BRL, Life Technologies, Paisley
Dispase® II	Boehringer Mannheim, Lewes, Sussex
Tegapore®	3M Health Care, St.Paul, MN USA

Stainless steel grids used in the culture of composites were constructed in-house with stainless steel mesh of 106μm pore size obtained from

Glenammer Engineering, Glenammer, Dalrymple, Ayrshire, Scotland. A 'Mitutoyo' dial thickness gauge used to gauge the thickness of de-epidermalised dermis was obtained from Alan G Smith Ltd, Gravesend, Kent (see Figure 2.2).

All inverted phase contrast microscopy was carried out using an Olympus CK2 microscope.

Figure 2.2: Mitutoyo™ dial thickness gauge used to gauge the thickness of de-epidermalised dermis.

2.1.2 Retroviral transduction

Cell lines used in these studies have been outlined above. The polyanion, Polybrene™ (Hexadimethrine Bromide), which promotes retroviral transduction (Jiang et al., 1991) was obtained (as *Sequabrene*) from Sigma-Aldrich Co. Ltd., Poole, Dorset. Acrodisk® *PF* syringe filters (0.2μm, 0.4μm and 0.2/0.8μm) were obtained from Gelman Sciences, Ann Arbor, MI, USA.

Chemicals used in the staining of MFG *lacZ* nls transduced cells were:

Magnesium chloride, obtained from FSA laboratory supplies, Loughborough, Leicestershire; potassium ferricyanide and potassium ferrocyanide obtained from Sigma-Aldrich Co. Ltd., Poole, Dorset.

X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactoside), the substrate for the lacZ expressed enzyme β-galactosidase, was obtained from GIBCO BRL, Life Technologies, Paisley.

In preparation for flow cytometry, saponin (used in the permeablisation of β-galactosidase expressing cells prior to labelling) was obtained from Sigma-Aldrich Co. Ltd., Poole, Dorset, as was the sheep anti - mouse FITC secondary reagent. The primary monoclonal antibody to β-galactosidase was obtained from Boehringer Mannheim, Lewes, Sussex.

Alamar Blue™ an indicator dye used in the determination of cell proliferation was obtained from Serotec, Kidlington, Oxford, UK. The change in colour (from blue to red) on reduction was monitored using an Anthos 2001 platereader (serviced by Labtech International, Ringmer, East Sussex).

Flow cytometry was undertaken under the supervision of Mrs. J. Sargent, senior scientist, Dept. of Haematology Research, Pembury Hospital, Pembury, Kent.

2.1.2 Langerhans cell elimination

The monoclonal antibody (moAB) used against the SLA DR epitope was an IgG2a isotype (mouse anti-pig) derived from the cell line MSA3 obtained from VMRD Inc., Pullman WA 99163, USA. The secondary reagent sheep anti-mouse FITC was obtained from Sigma-Aldrich Co. Ltd., Poole, Dorset, England. Lyophilised sheep serum was obtained from ICN Biomedicals, Inc., Aurora, Ohio. Isotype control moAB was gifted by Dr WC Davis, WSU Monoclonal Antibody Centre, Veterinary Microbiology and Pathology, Washington State University, Pullman WA 99164.

Low-Tox®-M rabbit complement (*Cedarlane*) was obtained from VH BIO Ltd, Gosforth, Newcastle upon Tyne, who also donated samples of Low-Tox®-M, Low-Tox® guinea pig and baby rabbit complement. Goat serum was obtained from Sigma-Aldrich Co. Ltd., Poole, Dorset, England. Sodium azide was obtained from BDH laboratory supplies, Poole, Dorset, England

Peripheral blood lymphocytes for use as positive controls were obtained from whole blood harvested from experimental pigs at termination. Both Lympho-paque™ and Lymphoprep™ (similar products) were used in density gradient separation. Lympho-paque™ density gradient separation medium, (Density: 1.086 +/- 0.002g per ml, batch no. 807697), was supplied by Nyegaard & Co., Oslo, Norway. Lymphoprep™ (density 1.077 +/- 0.001g per ml, batch no. 808743) was supplied by NYCOMED AS, Diagnostica, Oslo, Norway. Although the expiry dates were 07 1993 and 08 1993 respectively neither appeared to show signs of deterioration, and functioned as expected.

The following isotype control antibodies were donated by Dr MJ Hamilton WSU Monoclonal Antibody Centre:

Cell Line	Isotype
ColiS69A	IgG1
ColiS205D1	IgG2a
ColiS169A	IgG2b

2.1.3 Histochemistry and Immunohistochemistry

2.1.4.1 Histochemistry

Paraformaldehyde and glutaraldehyde used as fixatives, as well as haematoxylin, eosin and DPX mountant were obtained from BDH laboratory supplies, Poole, Dorset, England. To facilitate cryostat sectioning OCT compound was used, obtained from Tissue-Tek, Miles Inc, Elkhart, IN, USA.

2.1.4.2 Immunohistochemistry

Primary Antibodies: (porcine-specific unless otherwise stated)

Monoclonal Antibody Specificity	**Cell Line / Clone**	**Isotype**	**Source**
CD1a (human)	NA1/34-HLK	IgG2a	Serotec, Kidlington, Oxford, UK

CD1	76-7-4	IgG2a	VMRD, Inc., Pullman, WA, USA
CD2	PG168A	IgG3	VMRD, Inc., Pullman, WA, USA (Gift)
CD2	MSA4	IgG2a	Dr. J.K. Lunney, Beltsville, USA
CD3	8E6	IgG1	VMRD, Inc., Pullman, WA, USA
CD3	FY1H2	IgG1	Dr. H. Yang, Pirbright, UK
CD4	PT90A	IgG2a	VMRD, Inc., Pullman, WA, USA
CD4a	b38c6	IgG1	Labor B. Glatthaar, Reutlingen, Germany
CD4	STH 293	IgG1	Dr. M. Shimizu, Ibaraki, Japan
CD5	PG114A	IgG1	VMRD, Inc., Pullman, WA, USA (Gift)
CD6	PG90A	IgG1	VMRD, Inc., Pullman, WA, USA (Gift)
CD8	PT81B	IgG2b	VMRD, Inc., Pullman, WA, USA
CD25	PGBL25A	IgG1	VMRD, Inc., Pullman, WA, USA (Gift)
CD25	231.3B2	IgG1	Serotec, Kidlington, Oxford, UK
CD45	K252.1E4	IgG1	Serotec, Kidlington, Oxford, UK
SLADR	MSA3	IgG2a	VMRD, Inc., Pullman, WA, USA
SLADR	TH14B	IgG2a	VMRD, Inc., Pullman, WA, USA
SLADP	H42A	IgG2a	VMRD, Inc., Pullman, WA, USA
SLADQ	TH81A5	IgG2a	VMRD, Inc., Pullman, WA, USA
IgλLC	K139.3E1	IgG2a	Serotec, Kidlington, Oxford, UK

SWC3	74-22-15	IgG1	VMRD, Inc., Pullman, WA, USA
SWC6	MAC320	IgG	Serotec, Kidlington, Oxford, UK
Cytokeratin 14 (human)	LL001	IgG	I. Leigh, Dept. Exp. Dermat. The Royal London Hospital (Gift)
Cytokeratin 10 (human)	LHP2	IgG	I. Leigh, Dept. Exp. Dermat. The Royal London Hospital (Gift)
Cytokeratin 6 (human)	LHK6	IgG	I. Leigh, Dept. Exp. Dermat. The Royal London Hospital (Gift)
Collagen VII (human)	LH7.2	IgG	I. Leigh, Dept. Exp. Dermat. The Royal London Hospital (Gift)
Collagen IV (human)	PHM12	IgG1	Serotec, Kidlington, Oxford, UK
Laminin (human)	Rabbitαhuman polyclonal		Serotec, Kidlington, Oxford, UK

Secondary reagents:

For single immunofluorescent labelling, fluorescein isothiocyanate (FITC) conjugated sheep anti-mouse or FITC-conjugated goat anti-rat was obtained from Dako Ltd, High Wycombe, Buckinghamshire England. Cy3 conjugated goat anti-mouse was obtained from Amersham Life Science Ltd., Little Chalfont, Buckinghamshire, England.

For dual and triple immunofluorescent labelling IgG2b-specific FITC-conjugated goat anti-mouse, IgG1 and IgG2a-specific phycoerythrin conjugated goat anti-mouse secondary reagents were obtained from Serotec, Kidlington, Oxford, UK. Biotin conjugated goat anti-mouse with IgG1, IgG2a or IgG2b specificities were also obtained from Serotec, Kidlington, Oxford, UK.

FITC-conjugated to avidin, texas red conjugated to avidin and FITC-conjugated to avidin as well as biotinylated anti-avidin D were obtained from Vector Laboratories, Peterborough, East Anglia. Lyophilised normal sheep serum was obtained from ICN Biomedicals, Inc., Aurora, Ohio. Normal goat and horse serum were obtained from Sigma-Aldrich Co. Ltd., Poole, Dorset. Propidium iodide (1mg per ml) fluorescent nuclear stain was supplied by Molecular Probes, Oregon, USA.

For immunoperoxidase staining using the nickel-enhanced avidin-biotin complex (ABC) method, biotinylated horse anti-mouse IgG and the avidin-biotin complex were obtained from Vector Laboratories, Peterborough, East Anglia. Hydrogen peroxide (30% w/v) was obtained from BDH laboratory supplies, Poole Dorset. Diaminobenzidine (DAB), glucose oxidase, α-D(+)-glucose and normal horse serum were obtained from Sigma-Aldrich Co. Ltd., Poole, Dorset.

Porcine jejunum used as a positive control for many of the antibodies was obtained from a freshly terminated experimental animal. 2-methyl-butane (isopentane) used to control the freezing rate of the jejunum was obtained from Sigma-Aldrich Co. Ltd., Poole, Dorset, England. Fine quartz sand (-50+70 mesh) used to construct a sand bath was also obtained from Sigma-Aldrich Co. Ltd., Poole, Dorset.

Both brightfield and fluorescent microscopy were performed using an Olympus BH2 microscope.

2.1.5 Animal work

2.1.5.1 Animal source and housing

Animal experiments were performed under a Home Office personal licence, No. PIL60/6453, under project licence PPL70/4100.

14 outbred female Large White pigs aged 6-7 weeks and weighing 20-25 kgs were obtained from Bury Farm, Edgware, Middlesex, and from the Royal Veterinary College, Pond Street. Pigs were fed on 'super grade pig pellets' (a weaning diet suitable for pigs up to 6 months of age) obtained from J&W Atlee Ltd, Parsonage Mills, Dorking, Surrey England. The delousing agent ivermectin (Ivomec®), was supplied by MSD-AGVET, Division of Merck Sharp and Dome Ltd. Hoddleston, Hertfordshire, England.

2.1.5.2 Anaesthesia and perioperative care

Drug	Source
Ketamine (Vetalar®)	Parke-Davies Veterinary Ltd, Pontypool, Gwent
Xylazine (Rompun®)	Bayer UK Ltd, St. Edmunds, Suffolk
Halothane (Fluothane®)	Zeneca Ltd, Macclesfield, Cheshire
Nitrous Oxide	BOC Ltd, Guilford, Surrey
Oxygen	BOC Ltd, Guilford, Surrey
Buprenorphine (Temgesic®)	Reckitt & Colman Products Ltd, Hull
Lignocaine gel (2%)	Biorex Laboratories Ltd, London
Amoxycillin/Clavulanic acid for	Smith Kline Beecham Animal Health, Surrey

injection (Synulox®)	
Phenobarbitone (Expiral®)	Civa Ltd, Watford, Hertfordshire

2.1.5.3 Wound chambers and protective jackets

Wound chambers were constructed from polytetrafluoroethylene (PTFE) by the Bioengineering Department at the Northwick Park Institute for Medical Research (see Figure 2.3). The chambers were protected by jackets made from thermoplastic Spectrum ® and secured with Velcro® straps (see Figure 2.4). These were obtained from Promedics Ltd, Blackburn. Medium density furniture foam used to line the jackets and foam adhesive was obtained from Southern Foam, Crawley, Sussex.

Figure 2.3: Polytetrafluoroethylene (PTFE) wound chamber constructed by the Bioengineering Department at the Nothwick Park Institute for Biomedical Research

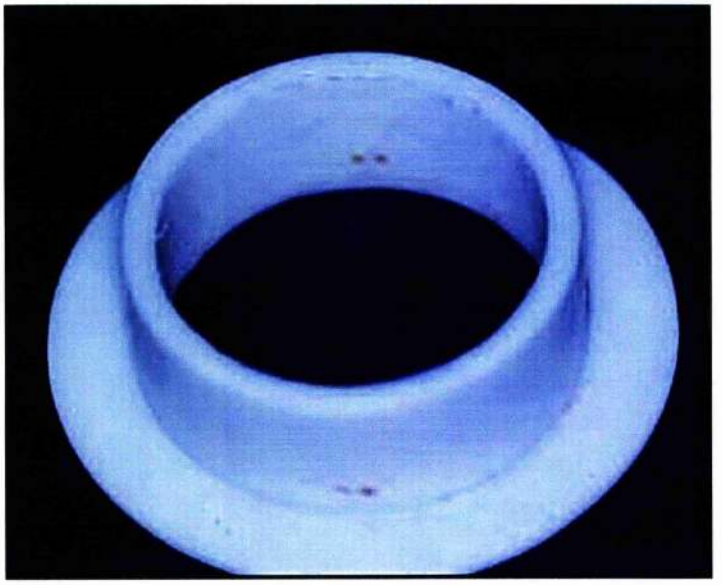
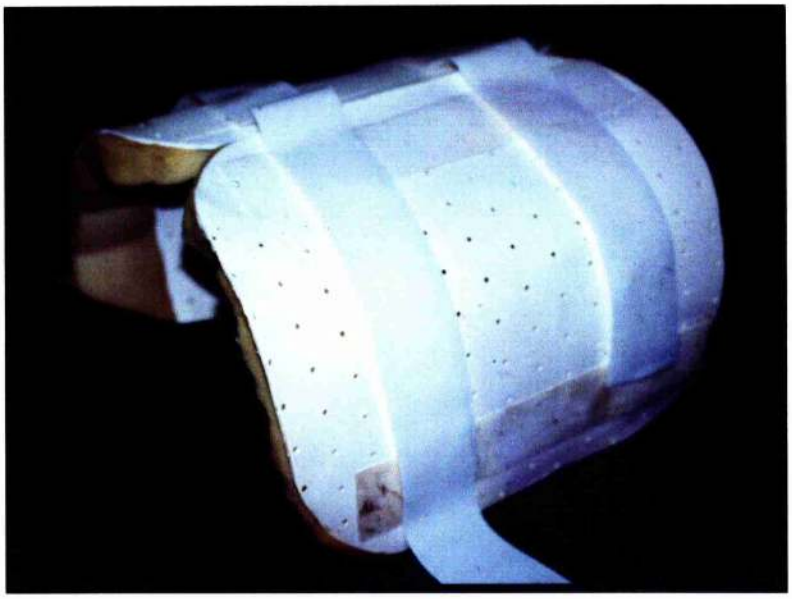


Figure 2.4: Thermoplastic Spectrum® jackets secured with Velcro® straps protect the chambers. Medium density furniture foam secured with foam adhesive was used to line the jackets.



2.1.5.4 Theatre Consumables

Consumables	**Source**
Silver nitrate in aqueous solution (0.25%)	Eastbourne District General Hospital, Eastbourne, England
Chlorhexidine (4%) surgical scrub (Hydrex®)	DePuy Healthcare Ltd, Leeds, England
Chlorhexidine 0.5% in spirit	
10% povidone iodine in aqueous solution (Betadine®)	Seton Healthcare, Oldham, Lancashire, England
Fucidin ointment (2% fusidic acid)	Leo Laboratories, Buckinghamshire, England
Sterile normal saline	Steripak Ltd, Runcorn, Cheshire,

(sodium chloride Ph. Eur. 0.9% w/v)	England
Liquid paraffin	The Pharmacy, Northwick Park Hospital, Harrow, England
Synthetic wool bandage (Velband®)	Johnson & Johnson, Ascot, Berkshire, England
Silicone coated dressings (N-A Ultra®)	
Paraffin gauze (Jelonet®)	Smith & Nephew, Chessington, Surrey
Adhesive bandage (Elastoplast®)	
2/0 silk sutures	Ethicon Ltd, Edinburgh
5/0 Proline sutures	

The Zimmer® pressurised air powered dermatome was purchased from Zimmer, Dover, Ohio, USA.

Integra™ artificial skin was obtained from Integra Life Sciences Corporation, Plainsboro, New Jersey, USA.

2.1 METHODS

2.2.1 Cell culture

All cells were cultured in a standard manner. A Jouan class II tissue culture hood was used at all times. Cells were grown in a LEEC incubator kept at a constant 37°C, 98% relative humidity and 10% CO_2 concentration. Cells were generally subcultured just prior to confluence.

2.2.1.1 3T3 Swiss mouse fibroblasts

The medium used for culturing 3T3 fibroblasts consisted of the following:

Ingredient	**Volume**
DMEM (1g per l glucose) with Glutamax™	500ml
Penicillin/Streptomycin	10ml *(100 units per ml/100 μg per ml)*
FCS	56ml *(10% of total volume)*

Prior to the availability of DMEM containing Glutamax™ (the dipeptide L-alanyl-L-glutamine which is substituted on a molar equivalent basis for L-glutamine), L-glutamine (200mM per ml, thawed from frozen stock) was added to give a final concentration (5ml in 500ml) of 2mM.

Cells were culture in 75cm^2 tissue culture flasks in which 10ml of culture medium was sufficient. Medium was changed every 2-3 days. To subculture a confluent flask, culture medium was aspirated using a sterile pasteur pipette. 5ml of HBSS was added, the flask gently agitated and the HBSS then aspirated. 3ml of trypsin/EDTA (0.05%/0.02%) was added and the flask gently agitated and then placed in the incubator. The flask was checked at 5 minute intervals. Following detachment of the cells 5ml of culture medium is inserted. All 10ml were then withdrawn and placed in a universal container. The universal was then spun for 5 minutes at 1200-1500rpm (approximately 250-300g) in a Jouan C312 centrifuge. Supernatant was then aspirated leaving a small volume covering the residual pellet. Medium was added and with repeated cycles of aspiration and then release, the cells were resuspended and could be subdivided into flasks containing fresh medium. Cells from one confluent flask were usefully subdivided in up to 6 further flasks and were

subcultured between 15 and 20 passages before infection or significant morphological change necessitated their discarding.

2.2.1.2 Large White pig keratinocytes

Under anaesthetic a 5cm wide and 10cm long strip of partial thickness skin was harvested from the paravertebral region. The Zimmer™ dermatome was set at 12 to ensure a partial skin thickness of approximately 200μm (as determined with the Mitutoyo dial gauge). The harvested skin was placed in a 45ml conical tube containing 35ml of transport medium, wrapped in parafilm and placed on ice for transportation to the laboratory. Transport medium contained the following:

Ingredient	**Volume**
DMEM (1g per l glucose) with Glutamax™	500ml
Penicillin/Streptomycin 600 units per ml/600 μg per ml	60ml
FCS	28ml *(5% of total volume)*
Gentamicin 250 μg per ml	2.5ml

Prior to the availability of DMEM containing Glutamax™, L-glutamine (200mM per ml, thawed from frozen stock) was added to give a final concentration (5ml in 500ml) of 2mM.

In the laboratory the harvested skin was washed 4 times in HBSS. It was then placed in 35ml of trypsin (0.25%, Difco Laboratories) containing penicillin/streptomycin (100 units per ml/100 μg per ml) and amphotericin B

(1.25 μg per ml) for 2 hours in a water bath set at 37°C. At the end of 2 hours the epidermis was carefully separated from the dermis and minced finely. The minced epidermis was then placed back into the trypsin solution and shaken to further disaggregate the cells. The solution was then passed through a stainless steel sieve (pore size ~ 100μm), then transferred to a conical tube and a further 10-15 ml of 3T3 growth medium added. Following centrifugation the pellet was resuspended in porcine keratinocyte growth medium the ingredients of which were:

Ingredient	**Volume**
Calcium-free Opti-MEM®1	500ml
Penicillin/Streptomycin	10ml *(100 units per ml/100 μg per ml)*
0.1M Calcium Chloride	0.75ml *(final concentration 0.5mM)*
FCS	5ml *(1% of total volume)*

Prior to the availability of DMEM containing Glutamax™, L-glutamine (200mM per ml, thawed from frozen stock) was added to give a final concentration (5ml in 500ml) of 2mM. The final $[Ca^{2+}]$ is adjusted to 0.5mM by the addition of 0.1M $CaCl_2$.

0.1ml of resuspended keratinocytes were mixed with 0.1ml 1% trypan blue and the viable cells then counted using a haemocytometer. $4X10^6$ viable keratinocyte per $75cm^2$ tissue culture flask ($5.3X10^4$ per cm^2) were then plated out into a prepared flask.

Flasks were prepared for keratinocyte culture by collagen coating and plating with $2X10^6$ (per $75cm^2$ tissue culture flask) irradiated 3T3 cells. Collagen

coating was performed by treating each flask with 7 ml of rat-tail collagen type 1 (50 μg per ml dissolved in 0.02M acetic acid) at room temperature for 2 hours. The flasks were then dried for one hour in the hood and then rinsed in HBSS before use. Collagen stock was made by dissolving 5 mg rat tail collagen type 1 in 20 ml of 0.1M acetic acid which was then diluted to 100mls with culture grade H_2O. The solution was sterilised with 10 mls chloroform and stored overnight in the fridge. The chloroform was aspirated on the following day with a sterile glass pipette. The collagen stock was subsequently stored at 4 °C.

Irradiated 3T3 cells were prepared up to a day before keratinocyte plating by irradiating with 6,000 rad (from a shielded ^{137}Cs source) and were plated at a density of $2X10^6$ per $75cm^2$ flask in the keratinocyte growth medium. As outlined in Chapter 3 the number of irradiated 3T3 fibroblasts were correspondingly reduced if irradiated PT67 retroviral producer cells are used as feeder cells (but no alteration in medium was necessary).

An evaluation was made of both keratinocyte sheets and composites grown in a modified medium described by Green (Green et al., 1979) and compared to the keratinocyte growth medium described above (see Chapter 6). The ingredients of this modified medium included:

Ingredient	**Volume (ml)**
DMEM (glucose 1 g per l) with Glutamax™	63
Hams F12	22
FCS	10 *(10%)*

Insulin Actrapid®	0.143 *(5 μg per ml)*
Epidermal growth factor (in DMEM with 10% FCS)	1 *(10 ng per ml)*
Hydrocortisone (in DMEM)	1 *(0.4 μg per ml)*
Cholera toxin (in DMEM with 10% FCS	1 *(10^{-10}M)*
Triiodothyronine (T3 10^{-8}M)	0.1 *($2X10^{-11}$M)*
Adenine ($1.8X10^{-1}$M)	0.1 *($1.8X10^{-4}$M)*
Penicillin/streptomycin	2ml *(100 units per ml/100 μg per ml)*

2.2.1.3 TELCeB, GP+EnvAm12 and PT67 cell lines

These lines were cultured in a similar fashion to Swiss 3T3 fibroblasts. The TELCeB and PT67 cell lines required DMEM with a glucose concentration of 4.5g per l. The PT67 clone that was selected out for use in transduction experiments required 15-20ml of medium (per $75cm^2$ tissue culture flask) for optimum growth.

2.2.1.3.1 Determination of TELCeB as ecotropic or amphotropic producer

2.2.1.3.1.1 TELCeB transduction of 3T3 cells

TELCeB cells were cultured in $75cm^2$ flasks until just confluent. The supernatant was removed and filtered. $1.6X10^5$ 3T3 cells in 0.5 ml were plated out in duplicate in 6 well plates to which were added the following dilutions of TELCeB supernatant, 3T3 culture medium. 8 μg per ml of polybrene was also added to each well.

	Amounts inserted into wells in duplicate (ml)		
TELCeB supernatant	1.5	0.15	0.015
3T3 culture medium	---	1.35	0.485

After 24 hours the medium was changed. After 4 days of culture plates were fixed and stained with X-Gal.

The experiment was repeated with 10^5 3T3 cells in 0.5 ml per plate and the following supernatant and culture medium concentrations:

	Amounts inserted into wells in duplicate (ml)		
TELCeB supernatant	1.5	0.15	0.015
3T3 culture medium	---	2.35	1.485

2.2.1.3.1.2 TELCeB transduction of porcine keratinocytes

TELCeB cells were cultured in 75cm^2 flasks until just confluent. The supernatant was removed and filtered through a 0.02μm Acrodisk™ filter. 2X10^5 irradiated 3T3 cells in 0.5 ml and 3.05X10^5 porcine keratinocytes were plated out in duplicate in 5X 6 well plates to which were added the following dilutions of TELCeB supernatant and porcine keratinocyte culture medium.

	Amounts inserted into wells in duplicate (ml)		
TELCeB supernatant	1.0	0.1	0.01
Porcine keratinocyte culture medium	1.0	1.9	1.99

To each of the five 6 well plates the following concentration of polybrene was added.

6 well plate number	A	B	C	D	E
Polybrene (μg per ml)	0	2	4	8	12

The medium was changed after 24 hours and cultured for a further 3 days before staining.

2.2.1.3.2 3T3 transduction with GP+Env Am12 producer cell line

From a just confluent (with GP+Env Am12 'producer' cells) 75cm^2 flask supernatant was extracted and filtered (0.02μm Acrodisk™ filter). 5 ml was diluted 1:2 in 3T3 growth medium and inserted into a flask containing 3T3 cells plated the previous day (2X10^5 cells into the 75cm^2 flask). The medium was changed after 24 hours and fixed and stained with X-Gal after 3 days of culture.

2.2.1.3.3 Porcine keratinocyte transduction with GP+Env Am12 producer cell line

Approximately 2X10^6 irradiated GP+Env Am12 cells were plated out per 75cm^2 flask which then received 2X10^6 P4 porcine keratinocytes. A control flask containing irradiated 3T3 cells and keratinocyte at equivalent numbers was set up in parallel. The medium was changed every 2-3 days and after a week of culture both flasks were fixed and stained with X-Gal.

2.2.1.4 Cryopreservation of cells

Following trypsinisation as described above, cells were spun down, counted with a haemocytometer. Up to $5x10^6$ cells were then resuspended in 1ml of DMEM (1g per l glucose) with Glutamax™ and 2% penicillin/streptomycin (100 units per ml / 100 μg per ml), 20% FCS and 10% dimethyl sulphoxide (DMSO). The cells were frozen at –70°C for 24 hours in insulated containers and then kept in liquid nitrogen for long term storage. Vials of cells were defrosted by rapid thawing in a 37°C water bath, resuspended in 10ml of 3T3 culture medium spun down in order to pellet the cells and the medium drawn off. The pellet of cells was resuspended in culture medium appropriate to the cells and centrifuged a second time prior to resuspension and plating out.

2.2.2 Keratinocyte sheet grafts

Following confluence, keratinocytes were cultured for a further five days to allow a second layer to form thus enhancing the mechanical integrity of the sheet. A fine balanced was maintained between culturing long enough to allow for a degree of mechanical integrity and over long resulting in keratinocyte differentiation and loss of viability.

2.2.3 Preparation of sheets for grafting

6-8 hours prior to grafting, the sheets were detached, stored in keratinocyte culture medium and transported from laboratory to operating theatre. In order to detach the sheets medium was aspirated from the flask and 3ml of Dispase® (2mg per ml in DMEM) inserted. The flask was gently agitated and incubated for 30 minutes. When the sheet began to detach the Dispase® was washed off with HBSS and the sheet gently encouraged to further detach and rolled a little way towards the flask centre with a sterile glass rod. Sterile backing material Tegapore® (fashioned to form a disc of 3.0cm diameter) was laid down in the centre of the flask. Using a pair of sterile fine forceps the redundant edges of the keratinocyte sheet were lifted centripedally onto the backing material. The sheet and backing material were then placed in a well in a 6 well plate into which 1ml of keratinocyte growth medium was placed. 6 well plates were wrapped in cling film in readiness for transportation from laboratory to operating theatre.

2.2.4 Assessment of feeder cell transduction potential

In experiments involving retroviral transduction of keratinocytes intended for use as sheet grafts, the feeder cell population contained retroviral producer cells until the last passage when the feeder cells were entirely composed of irradiated 3T3 fibroblasT-cells. To make doubly certain that no retroviral producer cells were included in the sheet graft, supernatant was harvested and tested on 3T3 fibroblasT-cells. 10^5 3T3 fibroblasT-cells per well were

plated out in triplicate in 6 well plates one day prior to the detachment of keratinocyte sheet grafts. On the following day supernatant was added to the medium in a series of dilutions ranging from 0.5 to 10-3 with 8μg per ml polybrene added. After 48 hours the medium was changed and after a further 24-48 hours the plates were stained with X-Gal. The results were consistently negative.

2.2.5 Composites

2.2.5.1 Preparation of dermis

Strips of partial thickness skin 5X10cm were harvested using the Zimmer dermatome at settings ranging from 8 to 18. The skin was placed in a 50 ml conical tube containing 35ml of 'composite' transport medium wrapped in parafilm and transported back to the laboratory.

The composite transport medium contained:

Ingredient	**Volume**
HBSS	500ml
Penicillin/streptomycin 600 units per ml/600 μg per ml	60ml
Gentamicin 250 μg per ml	2.5ml

The skin was subsequently washed 4 times with HBSS and then cut into sections of ~ 6X5cm (larger than is actually necessary to allow for contraction (Ralston et al., 1997)). These were then placed into separate 100mm X 20mm

petri dishes and incubated for 96 hours (medium was changed at 48 hours) at 37°C in 1M NaCl containing antibiotics. The antibiotic cocktail consisted of amphotericin B 2.5μ per ml (1/100 dilution from stock), penicillin/streptomycin 100iu per ml or 100μg per ml (1/50 dilution), ciprofloxacin 10μg per ml (1/200 dilution) and gentamicin 100μg per ml (1/50 dilution). The epidermis was then easily separated from the dermis and lifted off. Before the epidermis had been entirely lifted off a central stitch (6.0 proline) was placed with the knot overlying the papillary dermis. The dermis was then placed in fresh 100mm X 20mm petri dishes containing distilled water and antibiotics (as above) at 37°C for a further 10-12 days.

2.2.5.2 Preparation of keratinocyte – dermal composites

Following preparation of dermal sheets as above the distilled water was replaced with HBSS and the sheets incubated for one hour at 37°C. The sheets were then washed twice more with HBSS before being gently transferred to 60mm X 15mm petri dishes. The dermis was laid flat with the papillary surface uppermost, covering most of the surface area of the dish, surface tension between dermis and dish plastic helping to keep the dermis flat against the plastic. Keratinocytes were seeded in 2 ml of keratinocyte culture medium at densities of $1.38X10^5$ per cm^2, $4X10^5$ per cm^2 and $1.38X10^6$ per cm^2 for *in-vitro* studies (see Chapter 6), and ~$1.38X10^6$ per cm^2 when culturing composites to be used as grafts. One day later the composites were gently transferred onto stainless steel grids in 100mm X 20mm petri dishes and 25 ml of keratinocyte culture medium added to cover the composites. The

composites were cultured for a further seven days with medium changes every 2 days. As with keratinocyte sheet grafts, sterile backing material Tegapore® (fashioned to form a disc of 3.5cm approximate diameter) was laid down in the centre of the composite. Using a pair of sterile fine forceps the redundant edges of the composite were lifted centripedally onto the backing material. The composite and backing material were placed in a well in a 6 well plate into which 1ml of keratinocyte growth medium were placed. 6 well plates were then wrapped in cling film in readiness for transportation from laboratory to operating theatre.

2.2.6 Determination of cellular metabolism using Alamar Blue™ indicator dye method

Alamar Blue™ (obtained as a kit from Serotec, Kidlington, Oxford, UK) is an oxidation-reduction indicator dye that both fluoresces and changes colour (from blue to red) in response to chemical reduction of growth medium resulting from cellular metabolic activity. The reagent is water-soluble and non-toxic to cells. Alamar Blue™ was added to test cultures during the log phase growth stage, in an amount equal to 10% of the culture volume. Cells were returned to the incubator for 4 hours. 400μl from each test flask was added to each of 3 wells in a 96 well plate. Using the Anthos 2001 platereader absorbance was measured at a wavelength of 570nm. Background absorbance was measured at 600nm and subtracted.

2.2.7 Antibody complement pre-treatment (complement lysis)

Epidermal cells (suspended in porcine keratinocyte growth medium) were aliquoted into universals at a concentration of 10^7 cells per ml and incubated on ice with 400µl per 10^7 cells MSA3 or ColiS205D1 (porcine SLA DR-specificity, IgG2a isotype, IgG2a isotype control respectively) at a dilution of 1/100. After 45 minutes filter-sterilised complement was added at a final dilution of 1/10 and incubated for a further 45 minutes on ice. Cells were then washed twice and counted.

2.2.8 Flow cytometry

Flow cytometry involves the measurement of specific parameters of a single particle flowing past a point. In this study the particles concerned were single cells which were either epidermal cells, peripheral blood lymphocytes or retroviral producer cells (PT67 cell line).

2.2.8.1 Preparation of single cell suspensions

The first stage in preparation for flow cytometry is to generate a single cell suspension. Cultured keratinocytes (1^{st} to 3^{rd} passage) and PT67 retroviral producer cells were trypsinised (trypsin/EDTA (0.05%/0.02%) as for subculture. Following centrifugation the supernatant was discarded, the pellet resuspended in PBS with 0.01% sodium azide and 0.2% fetal calf serum and counted using a haemocytometer. Freshly disaggregated epidermal cells

(either immediately, following antibody-complement pretreatment or following overnight incubation) were similarly suspended in keratinocyte growth medium and counted and then centrifuged (this process repeated twice). Following final counts, cells were suspended in PBS with 0.01% sodium azide and 0.2% fetal calf serum at a concentration of $5X10^6$ cells per ml.

2.2.8.1.1 Lymphocytes

Peripheral blood lymphocytes were used as positive controls throughout experiments looking at SLA DR expression in epidermal cells, as the expected proportion of SLA DR-positive lymphocytes is between 10 and 20% (personal communication Dr K Haverson, Dept. of Clinical Veterinary Immunology, University of Bristol).

Blood taken from experimental pigs at termination was stored in CPD bags (350ml capacity bags containing a citrate phosphate dextrose solution, obtained from the blood bank at Queen Victoria Hospital, East Grinstead, UK) at room temperature for up to 3 days prior to use. 20ml aliquots of blood were diluted with a further 10ml of HBSS. The dilute blood was then underlayed with 10ml of lymphopaque or lymphoprep and centrifuged at room temperature at 700g (~2000rpm) for 45 minutes. The interface layer was carefully removed and resuspended in HBSS. The suspension was then washed twice in HBSS with centrifugation at 400g (~1600 rpm) for 5 minutes at room temperature. The pellets were then resuspended and used immediately or cryopreserved in liquid nitrogen for later use. Following

washes and counts, cells were suspended in PBS with 0.01% sodium azide and 0.2% fetal calf serum at a concentration of 10^8 cells per ml.

2.2.8.2 Indirect labelling for SLA DR

200 μl of keratinocyte cell suspension or 100μl of lymphocyte suspension were placed in 1.5ml Eppendorf® vials. Test and control vials were matched in duplicate or triplicate. Three types of control were generally used. These were peripheral blood lymphocytes as a positive control, substitution of the primary antibody with isotype control (ColiS205D1, IgG2a isotype) and labelling with the secondary reagent alone was a further negative control. 40μl (half this amount for the lymphocyte suspensions) of MSA3 (porcine SLA DR-specificity, IgG2a isotype) or ColiS205D1 at a dilution of 1/100 was added. The vials were gently agitated and incubated for 30 minutes at 4°C. Cells were then washed twice with PBS with 0.01% sodium azide and 0.2% FCS by microcentrifugation for 2 minutes at 1200g (4000rpm). 200μl (100μl for lymphocyte suspensions) of sheep anti-mouse FITC diluted 1/100 was added and the vials gently agitated and incubated for a further 30 minutes in the dark at 4°C. Cells were then washed twice with PBS with 0.01% sodium azide and 0.2% FCS by microcentrifugation for 2 minutes at 1200g (4000rpm). In later studies, cells were first incubated for 2 minutes with propidium iodide (1mg per ml) at a dilution of 1/100 after the first wash. After the second wash cells were resuspended in 0.5ml PBS with 0.01% sodium azide and 0.2% FCS and kept at 4°C. Cells underwent flow cytometry within 12 hours of labelling.

2.2.8.3 Staining for β-galactosidase expression

Following a protocol described recently (Mouawad et al., 1997) PT67 retroviral producer cells were first fixed as single cell suspensions in acetone/PBS (65:35) at 20°C for 10 minutes. Subsequent processing was similar to that for SLA DR detection with the following differences. The primary antibody (test and control) was diluted 1/20 in PBS containing 0.1% of saponin (a detergent whose function is to permeablise the cell and nuclear membrane). Cells were incubated for 45 minutes at each stage on ice and were processed as described earlier (2.2.13.2) with the omission of propidium iodide.

2.2.8.4 Data access

The software used to access and statistically represent data was WinMDI (windows multiple document interface) version 2.8, freely available from Dr J Trotter at http://facs.scripps.edu/.

2.2.8.5 Gating

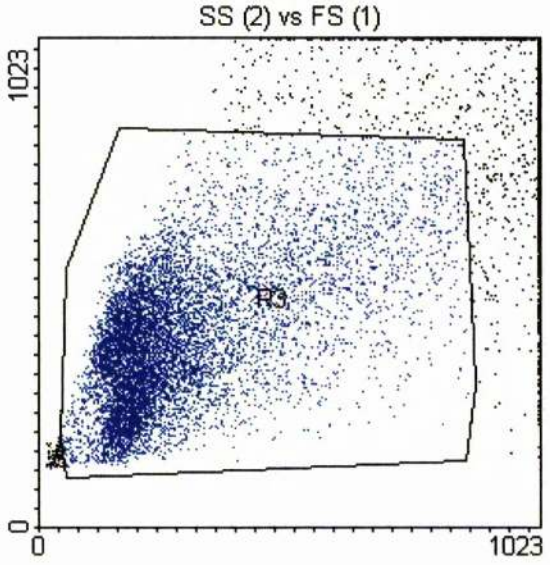
A light scatter dot plot of forward scatter (FS) versus side scatter (SS) was used to gate out debris and clumps (see Figure 2.5 (a)). Gating was cruder early on in the study but as processing improved it was possible to gate in an increasingly refined manner.

Statistics (calculated by the WinMDI® software) allow relative quantification of events within specified gates. A second cytogram is created in the form of a dot plot looking at gated events with LFL1, a filter allowing measurement of green fluorescence, as the X axis against LFL2 or LFL3 (filters for red fluorescence) along the Y axis (see Figure 2.5 (b). This protocol was followed in spite of the fact that a second indirect label was not used. This allowed for easier identification of subgroups both in terms of relative quantification as well as density of expression (geometric mean fluorescence). A histogram of log fluorescence may alternatively be plotted looking at gated events only and markers set between log10 and log1000 (see Figure 2.5 (c)). The geometric mean of gated events lying between these markers may then be calculated.
In later studies propidium iodide was incorporated which enabled gating out of dead cells using the LFL3 filter (see Figure 2.6).

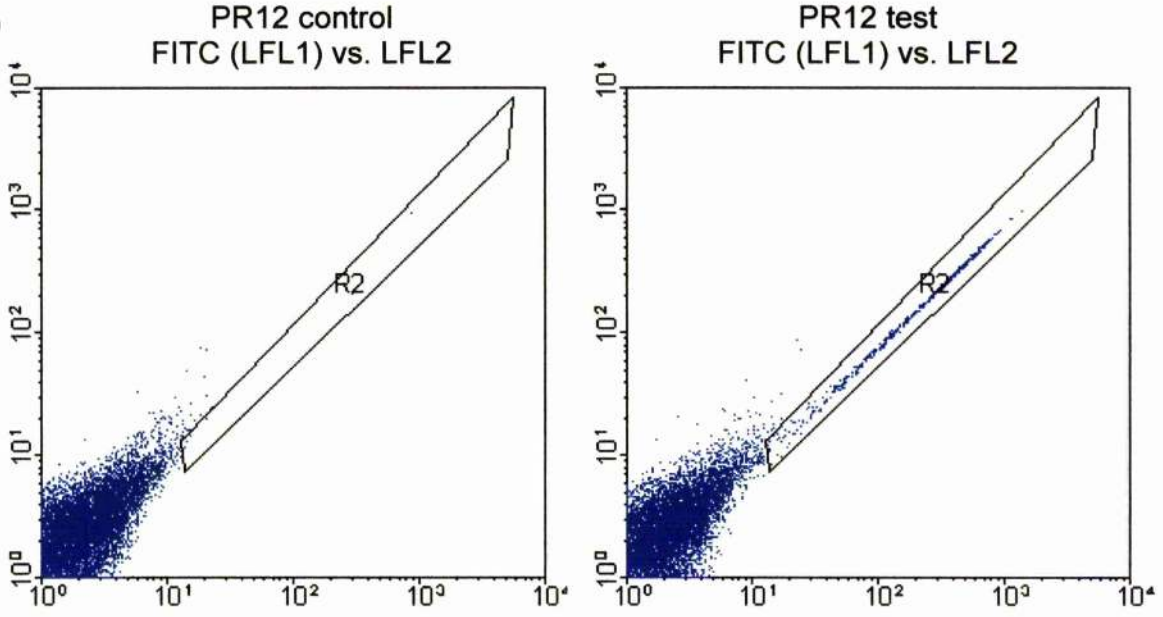
Figure 2.5: Flow cytometry gating

Dotplot 2.5 (a) is a light scatter plot of the test sample consisting of freshly trypsinised porcine epidermal cells. Clumps and debris were (crudely) gated out. Dotplots 2.5 (b) are plots of R3 gated events (from dotplot 2.5 (a)) sorted by filters LFL1 and LFL2. The LFL1 filter was poorly compensated. A gate R2 was then placed around a clear group of cells seen only in the test sample. The histogram 2.5 (c) shows the events delineated in R2 (b) plotted against log fluorescence of FITC (LFL1). The histogram shows the isotype control labelled sample in solid red with the SLA DR labelled cells in black line.

(a)



(b)


PR12 control
FITC (LFL1) vs. LFL2
R2
PR12 test
FITC (LFL1) vs. LFL2
R2

(c)

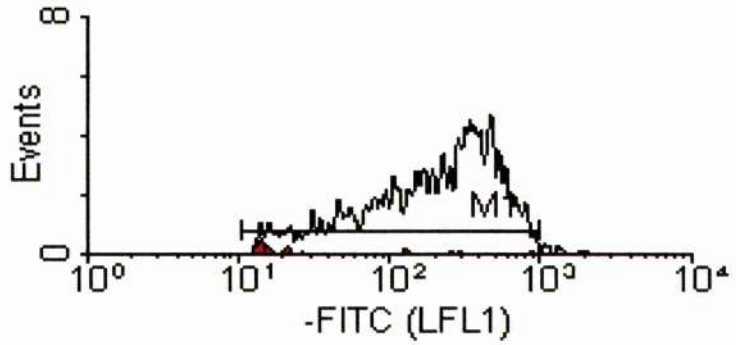
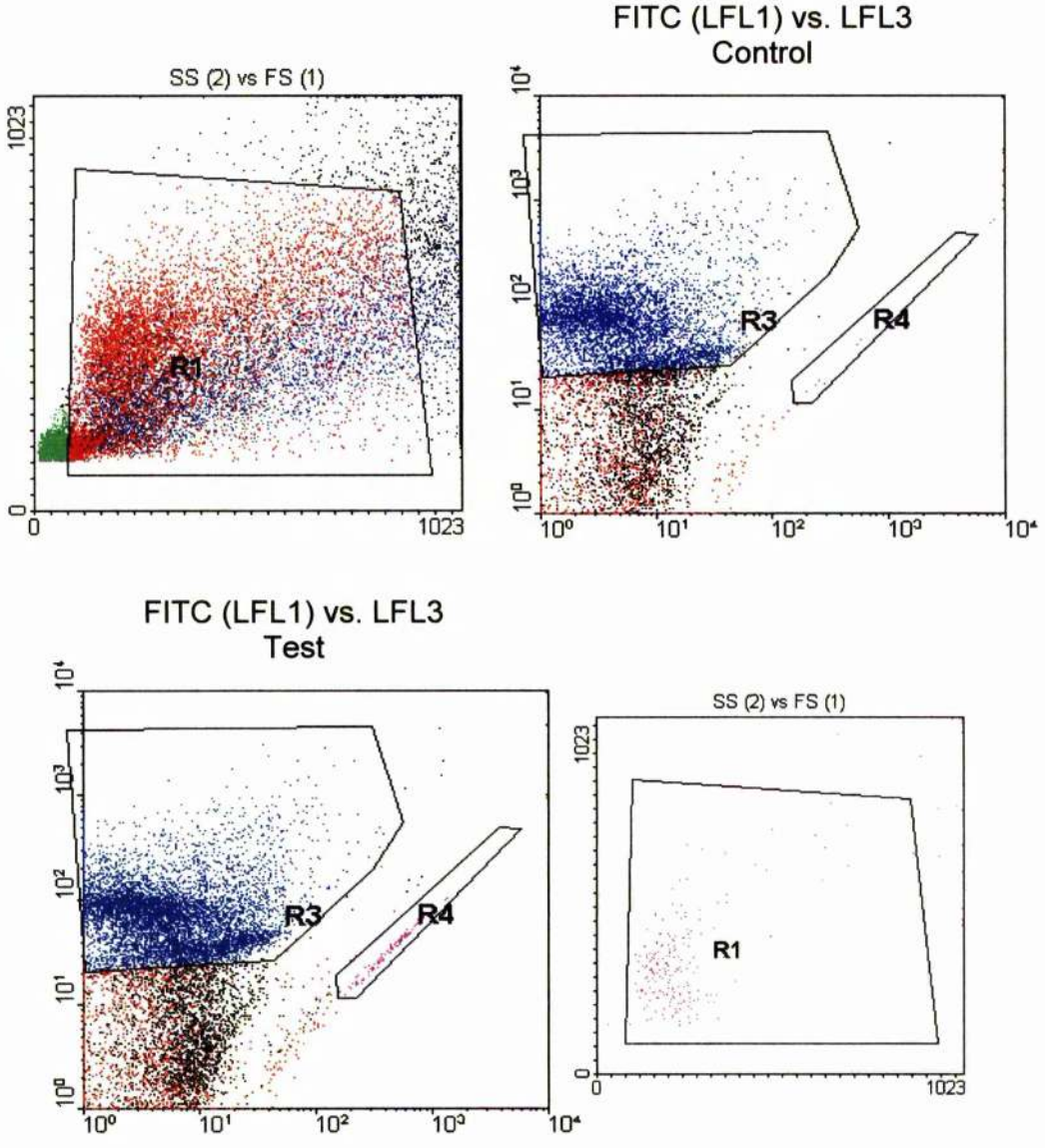

Events
8
0
M1
-FITC (LFL1)

Figure 2.6: Gating with the addition of propidium iodide (using LFL3)

The light scatter plot (SS vs FS) illustrates the plotting of region 1 crudely excluding debris (coloured green) and clumps. The following two dot plots illustrate test and control samples with FITC (LFL1) plotted against propidium iodide (LFL3). The dead cells (densely stained with propidium iodide) are gated (coloured blue). Region 4 (R4) displays a group of cells brightly fluorescing green (FITC labelled), albeit poorly compensated, that appears only with test sample and not with the control. The WinMDI® software was used to calculate the proportion of cells in R4 expressed as a percentage of cells in region 1 (R1) having excluded dead cells (R3). The last dot plot illustrates back gating and shows the light scatter characteristics of the group of cells of interest (R4).



2.2.9 Histochemistry

All sections were cut in the cryostat to a thickness of 15μm. Slides were left to dry at ambient room temperature for at least two hours prior to fixing and staining. Prior to staining with haematoxylin and eosin slides were fixed for 30 minutes in 4% paraformaldehyde on ice. Slides were then immersed twice in phosphate buffered saline (PBS) for periods of five minutes. Slides were then covered with haematoxylin for between 20-30 seconds (less time is required at higher cellular densities) before rinsing in tap water. The slides were then left in running tap water for 8-10 minutes. They were then covered in eosin for 6 minutes, rinsed and dehydrated by immersion in ascending concentrations of IMS (30, 70 and finally 100%) for 1 minute at a time, before a final immersion in xylene for at least 1 minute. Slides were then mounted in DPX, covered with a coverslip and left on a rack to dry.

2.2.10 Immunohistochemistry

2.2.10.1 Immunoperoxidase staining by the Avidin Biotin Complex (ABC) Method

15μm sections were cut with the cryostat at -18°C. 2-3 sections were placed on each vector-bonded slide depending on the size of section. Slides were kept in the cryostat for up to a week in a -40°C freezer until ready for drying, fixation and staining.

Slides were dried for an hour using a small fan, which delivered cool air at a fairly constant pressure. The fan was placed 12 inches away from the slide rack which was situated in almost upright position at an angle of approximately 30° to the vertical. A hydrophobic pen (DAKO Ltd, High Wycombe, Buckinghamshire) was used to isolate sections on the slide.

Recommendations were received for fixation of the membrane antigens from a number of sources. One source advised pure ethanol on ice (Dr WC Davis Washington State University) another advised pure acetone on ice for most with heat fixation for detection of the CD4 epitope (Dr K Haverson, Dept. of Clinical Veterinary Immunology, University of Bristol). A comparison was made between the different fixation methods and it appeared that ice cold ethanol for 20 minutes was better than ice cold acetone for fixation of most epitopes. Ice cold acetone for 20 minutes was preferred for fixation of CD2, CD3 and IgλLC antigens. CD4 was best fixed with heat. This was achieved with the construction of sand bath consisting of a shallow pyrex dish filled with fine quartz sand to a depth of 1cm. This was placed on a hot plate, which was set to the lowest setting, and the bath was allowed over several hours to equilibrate to a temperature of 60°C. Slides were placed with care directly on to the sand for 30 minutes.

Following fixation slides were immersed in PBS twice for 3-5 minutes at a time.

The slides are then immersed in 0.05% hydrogen peroxide for 30 minutes (to inactivate endogenous peroxidase). Following 2X5 minute washes in PBS, nonspecific binding sites were then blocked by incubation with 5% normal horse serum for 30 minutes. The sections to be labelled with the monoclonal

antibody MAC320 (SWC6 specificity) were incubated with 7.5% goat serum rather than horse serum (as the primary antibody is of rat origin and the second layer antibody therefore biotinylated goat anti-rat). The serum was then drained off and primary antibody directly applied (50μl per section). Primary antibodies were diluted in PBS. The dilutions are listed in Table 2.1.

Table 2.1: Primary monoclonal antibodies used in immunophenotyping.

Monoclonal Antibody Specificity	**Cell Line / Clone**	**Dilution (ABC)**
CD1	76-7-4	1/50
CD2	MSA4	1/2
CD3	FY1H2	1/5
CD4	STH 293	1/5
CD5	PG114A	1/30
CD6	PG90A	1/50
CD8	PT81B	1/250
CD25	231.3B2	1/3
CD45	K252.1E4	1/3-1/5
SLADR	MSA3	1/250
SLADR	TH14B	1/50
SLADP	H42A	1/50
SLADQ	TH81A5	1/50
IgλLC	K139.3E1	1/2
SWC3	74-22-15	1/100
SWC6	MAC320	1/3

Most of the monoclonal antibodies sourced have only recently become available (Saalmuller et al., 1998b) and used mainly in cell labelling for flow cytometry. Antibodies were therefore tested by staining jejunal sections at

dilutions ranging from 1/50 to 1/1500 for antibodies produced as mouse ascites fluid (those antibodies purchased from VMRD), and neat to 1/5 for those monoclonal antibodies grown in a cell culture system. The antibodies listed in Table 2.2 were found not to be optimally effective with ABC and alternatives found to stain consistently well were therefore used.

Table 2.2: Primary monoclonal antibodies that on testing (with ABC and immunofluorescence) did not prove efficient at immunohistochemical labelling.

Monoclonal Antibody Specificity	**Cell Line / Clone**
CD2	PG168A
CD3	8E6
CD4	PT90A
CD4a	b38c6
CD25	PGBL25A

The CD4 antigen was best displayed with immunofluorescent staining rather than with ABC.

Following incubation at 4°C overnight, the primary antibody was washed off by 2X5 minute immersions in PBS. The second layer antibody was biotinylated horse anti-mouse IgG diluted 1/100 in PBS (except for sections labelled with MAC320 (SWC6 specificity) which is of rat origin and the second layer antibody was therefore biotinylated goat anti-rat IgG diluted 1/100 in PBS). Following incubation with the biotinylated second layer antibody for one hour, the sections were washed by 2X5 minutes immersions in PBS and then

incubated at room temperature for a further hour with avidin-biotin complex. During this time the DAB (diaminobenzidine) developing solution was made up. This was prepared in the following way. 125mg of DAB was dissolved in 5ml of distilled water. This was filtered and added to 300ml of 0.1M sodium acetate into which 7.35g of ammonium nickel sulphate and 600mg of α D-glucose had been dissolved. This quantity of solution is sufficient for one rack of slides.

Slides were further washed in PBS (2X5 minutes immersions) and then equilibrated in 0.1M sodium acetate buffer at pH 6 by 2X5 minutes immersions. The DAB developing solution was activated immediately prior to the immersion of the slides by the addition of 6mg of glucose oxidase to the solution. Slides were immersed and their development monitored. After 3-4 minutes the degree of staining was checked under the light microscope. A fine balance was sought between over staining (poorly specific) and under staining (poorly sensitive). When staining was judged to be optimal, usually taking between 4.5 and 5 minutes, slides were removed and washed in 0.1M sodium acetate (2X5 minutes) and then PBS (2X5 minutes). They were then dehydrated by immersion in ascending concentrations of IMS (30, 70 and finally 100%) for 3 minute at a time, before a final immersion in xylene for at least 1 minute. Slides were then mounted in DPX mountant, covered with a coverslip and left on a rack to dry.

During staining room temperature was maintained at a temperature of between 18 and 21°C.

2.2.10.2 Indirect labelling with a single fluorochrome

Slides were air dried and sections isolated with a hydrophobic pen as before. Following fixation (acetone, ethanol or heat), nonspecific binding sites were blocked by incubation for 30 minutes with 10% serum (sheep or goat to match the origin of the secondary reagent). The serum was then drained off and 50 μl of primary antibody applied (see Table 2.3 for dilutions) and the sections incubated overnight at 4°C.

Table 2.3: Primary monoclonal antibodies used in indirect immunofluorescent single or dual labelling.

In dual labelling of sections the two primary monoclonal antibodies were of different isotype so as two allow for isotype-specific secondary labels attached to different fluorochromes.

Monoclonal Antibodies	Isotype	Concentration
CD45	IgG1	1/5
CD3 (8E6)	IgG1	1/10 – 1/15
CD1	IgG2a	1/15
SLA DR (MSA 3)	IgG2a	1/40-1/50
CD8	IgG2b	1/40

On the following day slides were washed by 2X 5minute immersions in PBS. The secondary antibody (sheep anti-mouse FITC or goat anti-mouse Cy3) was applied at a dilution of 1/100 and the sections incubated for 1 hour within a humid chamber in the dark. The slides were then washed by 2X 5min. immersions in PBS. They were then mounted in glycerine mountant and stored at 4°C.

2.2.10.3 Indirect dual fluorochrome labelling

The primary steps for fixation and section isolation were as described earlier for indirect single fluorochrome labelling. Following incubation for 30 minutes with 10% goat serum as described above, the serum was drained off sections and cocktails of two primary monoclonal antibodies of differing isotype (50 μl per section, see Table 2.3 for dilutions) were applied. The sections were incubated at 4°C overnight. On the following morning they were washed twice in PBS. Secondary dual fluorochrome labelling was subsequently achieved in one of several ways (see Tables 2.4a and 2.4b). One method involved combining two different fluorochrome-labelled isotype-specific secondary antibodies. FITC of IgG2b specificity in combination with Phycoerythrin (PE) of IgG1 or IgG2a specificity was used for this method. These are combined in a cocktail with staining steps as for single fluorochrome labelling. The intensity of the IgG1 isotype-specific PE label was seen to diminish within hours of staining (not shown). A more effective way to achieve dual labelling was to secondarily label using biotin with IgG1 or IgG2a isotype-specificity. This was followed up with a cocktail consisting of Texas red – avidin (or

aminomethylcoumarin (AMCA) - avidin) and IgG2b-specific FITC (see Table 2.4b for steps). The Texas red or AMCA staining may be enhanced by additional steps of isotype-specific biotin followed by a cocktail consisting of Texas red – avidin (or AMCA - avidin) and IgG2b-specific FITC, (see Table 2.4b for description of steps). This however generally gave an overly intense Texas red or AMCA stain with reduced sharpness.

Unless stated otherwise sections were incubated at each stage following primary labelling, for 1 hour at room temperature within a humid chamber in the dark. Between each step slides were washed by 2 X 5 minute immersions in PBS. Following completion of staining slides were mounted in glycerine mountant and stored at 4°C.

Table 2.4: Steps in dual fluorochrome labelling

Dual fluorochrome labelling was achieved following initial labelling with monoclonal antibodies of differing isotype following which, secondary labelling may either be achieved through the use of different fluorochromes attached to isotype-specific secondary antibodies (a), or by combining an isotype-specific fluorochrome-labelled secondary antibody with an isotype-specific biotin labelled secondary antibody (b).

(a)

Stage I	Sections are incubated overnight at 4°C with a cocktail of primary antibody isotype 1 (eg CD3 IgG1) and primary antibody isotype 2 (eg CD8 IgG2b).
Stage II	Slides are washed X3 in PBS then secondarily labelled with PE anti-isotype 1 (1/10) and FITC – anti-isotype 2 (1/20)and incubated for 1 hour at room temperature.

(b)

Stage I	Sections are incubated overnight at 4°C with a cocktail of primary antibody isotype 1 (eg CD3 IgG1) and primary antibody isotype 2 (eg CD8 IgG2b).
Stage II	The slides are washed X3 in PBS then labelled with either PE anti-isotype 1 (1/10) or FITC anti-isotype 1 (1/20).
Stage III	After 30 minutes incubation (at room temperature) biotin anti-isotype 2 (1/100) is added and incubated (at room temperature) for a further 30 minutes and then washed X2 in PBS.
Stage IV	Sections are then further labelled with either a cocktail of PE anti-isotype 1 (1/10) and FITC avidin(1/100) anti-isotype 2 or AMCA avidin(1/33) anti-isotype 2 or a cocktail of FITC anti-isotype 1 (1/20) and Texas Red (1/100) anti-isotype 2 or AMCA avidin (1/33) anti-isotype 2 and incubated for 45 minutes at room temperature

Optional futher steps

Stage V	Sections are washed X2 in PBS then biotinylated anti-avidin 1/100 is added and incubated at room temperature for 30 minutes
Stage VI	Sections are washed X2 in PBS and stage IV is then repeated

2.2.10.4 Triple fluorochrome labelling

The primary steps for fixation and section isolation were as described earlier for indirect single fluorochrome labelling. Slides were incubated for 30 minutes with 10% goat serum as described above. The serum was then drained off sections and 50 μl per section of cocktails of three monoclonal antibodies each with different isotype-specificities (see Table 2.3 for dilutions) were applied. This was followed by a cocktail of IgG2b-specific FITC with IgG2a or IgG1-specific PE with the addition of an IgG2a or IgG1-specific biotin (specificity of biotin being different to that of PE). A further step involved labelling with AMCA – avidin (see Table 2.5 for description of steps). The incorporation of an additional multiplication step as with 2.2.10.3 was found to be counterproductive with either over intense or more often loss of intensity of AMCA staining. In discussion with the manufacturers it was thought that stearic hindrance and distortion might account for the effects seen.

Sections were incubated at each stage (following primary labelling) for 1 hour at room temperature within a humid chamber in the dark. Between each step slides were washed by 2 X 5min. immersions in PBS. Following completion of staining slides were then mounted in glycerine mountant and stored at 4°C.

Table 2.5: Steps in triple fluorochrome labelling

Stage I	Sections are incubated overnight at 4°C with a cocktail of primary antibody isotype 1 (eg CD3 IgG1), primary antibody isotype 2 (eg CD8 IgG2b) and primary antibody isotype 3 (eg SLA DR IgG2a)
Stage II	The slides are washed X3 in PBS then labelled with PE anti-isotype 1 (1/10) and FITC – anti-isotype 2 (1/20)
Stage III	After 30 minutes incubation (at room temperature) biotin anti-isotype 3 (1/100) is added and incubated (at room temperature) for a further 30 minutes and then washed X2 in PBS
Stage IV	Sections are then further labelled with a cocktail of PE anti-isotype 1 (1/10), FITC anti-isotype 2 (1/20) and AMCA avidin (anti-biotin) (1/33) and incubated for 45 minutes at room temperature

Optional futher steps

Stage V	Sections are washed X2 in PBS, biotinylated anti-avidin 1/100 is then added and incubated at room temperature for 30 minutes
Stage VI	Sections are washed X2 in PBS and stage IV is then repeated

2.2.10.5 Wet preparations of immunofluorescent labelled lymphocytes

Lymphocytes were prepared in the manner described in 2.2.8.11. 200µl of lymphocyte suspension was placed in each Eppendorf® vial. 40µl of primary antibody (see Table 2.6 for antibodies and dilutions) was added. The vials were gently agitated and incubated for 30 minutes at 4°C. Cells were then washed with PBS with 0.01% sodium azide and 0.2% FCS by microcentrifugation for 2 minutes at 1200g (4000rpm). Cells were then incubated for 2 minutes with propidium iodide at a dilution of 1/100. They were then washed again with PBS with 0.01% sodium azide and 0.2% FCS by microcentrifugation for 2 minutes at 1200g (4000rpm). 200µl (100µl for lymphocyte suspensions) of sheep anti-mouse FITC diluted 1/100 was then added and vials gently agitated and incubated for a further 30 minutes in the dark at 4°C. Cells were then washed twice with PBS with 0.01% sodium azide and 0.2% FCS by microcentrifugation for 2 minutes at 1200g (4000rpm). Following resuspension in PBS with 0.01% sodium azide, two drops of 20 µl were placed at either end of a slide and a coverslip gently placed over the slide. Sides were stored at 4°C in the dark. Photomicrographs were taken within 24 hours of staining.

Table 2.6: Primary MoAB dilutions in wet preparations of labelled cell suspensions

Antibodies	Dilutions
CD1	1/50
CD2	1/2
CD3	1/3
CD4	1/2
CD5	1/50
CD6	3/50
CD8	1/10
IgλLC	1/2
CD25	1/2
CD45	1/50
SLA DR (MSA3)	1/50
SLA DP (H42A)	1/50
SLA DQ (TH81A5)	1/50
SLA DR (TH14B)	1/50
SWC6	Neat

2.2.11 X-Gal staining

2.2.11.1 Cultured cells

Medium was aspirated and the flask washed in 3 times with PBS. The cells were then fixed with 7ml (for a 75cm^2 tissue culture flask) of 2% formaldehyde and 0.02% gluteralehyde in PBS for five minutes. The flask was then rinsed 3 times with PBS and then incubated in X-Gal solution for two hours at 37°C. The X-Gal solution consists of 5mM potassium ferricyanide, 5mM potassium ferrocyanide, 2mM magnesium chloride and 0.05% X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactoside). Following two hours the X-Gal solution was aspirated and the flask rinsed 3 times with PBS. Finally 5ml of filtered PBS containing 0.01% sodium azide was placed in the flask which was then stored at 4°C.

2.2.11.2 Wound blocks

Following sectioning of the harvested wounds (see Section 2.2.22 and Figure 2.5), blocks were placed in 2% formaldehyde in PBS for 48 hours. Following this the formaldehyde solution was removed and the blocks washed and then incubated for a further two days in sucrose solution (containing 150g sucrose and 0.1% sodium azide in PBS) with a medium change every day. The blocks were then rinsed twice in PBS and covered with X-Gal solution and incubated as with cultured cells. Following incubation the blocks were rinsed and stored as with cultured cells.

2.2.11.3 Wound sections

Following drying and fixing (as for H&E) slides were immersed for five minutes twice in PBS and then covered in X-Gal solution and incubated for two hours at 37°C. Following incubation, slides were immersed for five minutes twice in PBS and then stained with haematoxylin and eosin.

2.2.12 Rhodamine B staining

To enable identification of colonies in the studies looking at colony forming efficiency, 6 well plates were stained with X-Gal as described above. Following this 1ml of 1% rhodamine B solution was placed in each well and left at room temperature for 3 minutes. The wells were then twice rinsed carefully with distilled water.

2.2.13 Image analysis

In the studies looking at colony forming efficiency, 6 well plates were stained with X-Gal and rhodamine B as described above. Images were then digitally recorded using a Seescan CCD (Charged Couple Device) computerised camera imaging system and analysed using Image Pro Plus® software with respect to number and size of colonies. Identical settings were used in all cases.

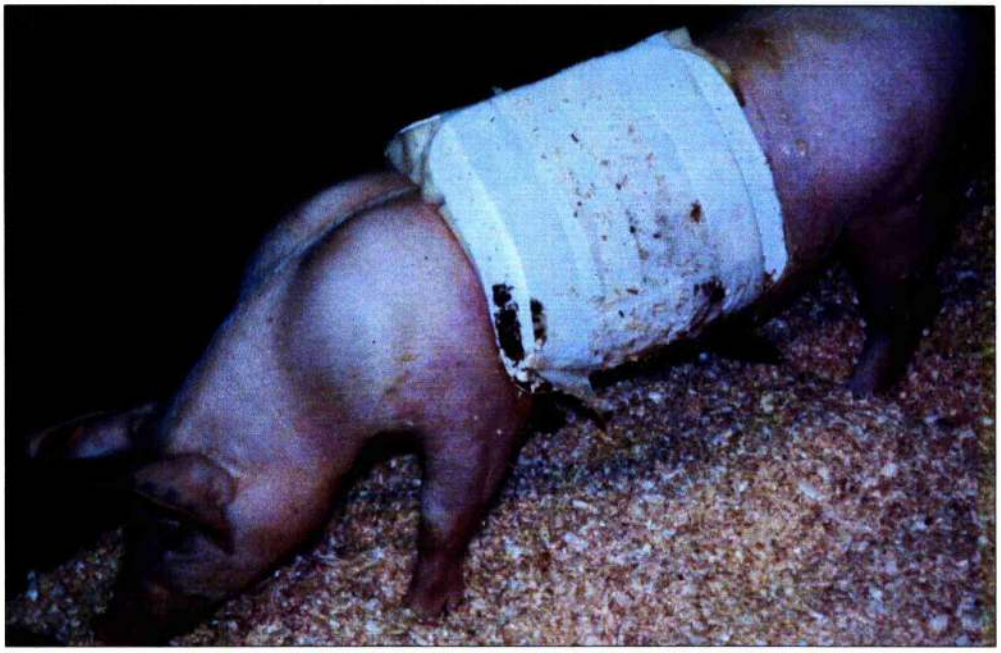
2.2.14 Statistics

All statistical analysis was carried out using the computer software package Sigmastat® (Jandel Scientific Software, Jandel Gmbh, Erkrath, Germany).

2.2.15 Animal work

On arrival animals were marked with indelible ink according to litter source, and additionally were numbered with an ear tag under the first anaesthetic. They were housed together in a single large pen for 5-7 days prior to surgery to facilitate their acclimatisation. Following surgery the pigs were housed in separate pens (see Figure 2.7).

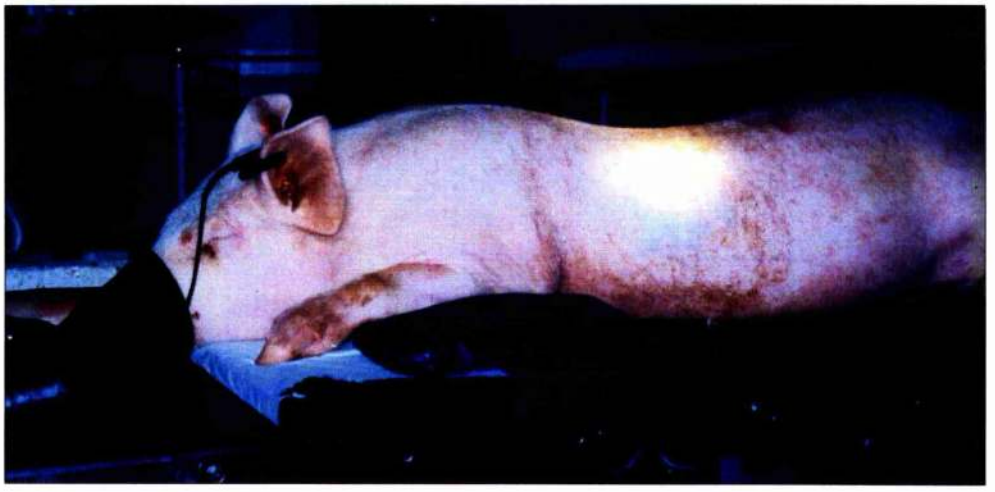
Figure 2.7: Large White pig returned to pen following a dressing change following which thermoplastic Spectrum® jackets were applied.



2.2.15.1 Anaesthesia, analgesia and euthanasia

As all procedures were performed under general anesthetic (see Figure 2.8), the pigs were not fed on the morning of surgery. Premedication consisted of an intramuscular injection of ketamine (Vetalar®) and xylazine (Rompun®) at 5 mg per kg and 1mg per kg respectively. The inhalational anesthetic (once the pigs were sufficiently drowsy) consisted of 2-5% halothane (Fluothane®) and a 50:50 mix of nitrous oxide and oxygen administered at a rate of 3-5l per min.

Figure 2.8: Large White pig under general anaesthetic in preparation for chamber implantation



Postoperative analgesia consisted of topical lignocaine gel BP 2% w/v to donor sites and a subcutaneous injection of 4μg per kg buprenorphine (Temgesic®).

Antibiotic prophylaxis employed at each procedure consisted of an intramuscular injection of 350mg amoxycillin/87.5mg clavulanic acid (Synulox®) given perioperatively.

The pigs were killed following the final procedure with an intravenous or intracardiac injection of pentobarbitone (Expirol®) 25mg per kg.

2.2.15 Tissue harvesting

2.2.15.1 Harvesting Skin

The pig was anaesthetised and laid in a prone position on the operating table. The pig's paravertebral region was washed with dilute chlorhexidine (4%) surgical scrub (Hydrex®). Hair in the paravertebral region was then clipped short and the skin shaved. The paravertebral region was then further cleaned with chlorhexidine 0.5% in spirit. Liquid paraffin was used to grease the skin and the blade within the Zimmer dermatome (on setting no. 12) which was then used to harvest the skin. The donor site was dressed in vaseline impregnated gauze (Paranet®) and treated with topical lignocaine gel BP 2% w/v. The site was then further dressed with gauze and circumferentially with Elastoplast®. For assessment of dermal thickness (see Chapter 6), the setting on the dermatome varied between 8 and 18. Following de-epidermalisation dermal thickness was assessed with the dial gauge. The harvested skin was placed directly into transport medium, wrapped in parafilm and placed on ice.

2.2.15.2 Harvesting Blood

Blood was harvested from experimental animals just prior to termination. Under general anaesthetic, with the pig lying supine on the table, the needle attached to a CPD (citrate phosphate dextrose) blood collection bag was inserted directly into the pigs heart. This was achieved with the needle inserted into the chest in the mid axillary line at just above a line parallel with heart apex and directed towards the heart. Blood then flowed directly into the bag under gravity. This was kept at room temperature until processing.

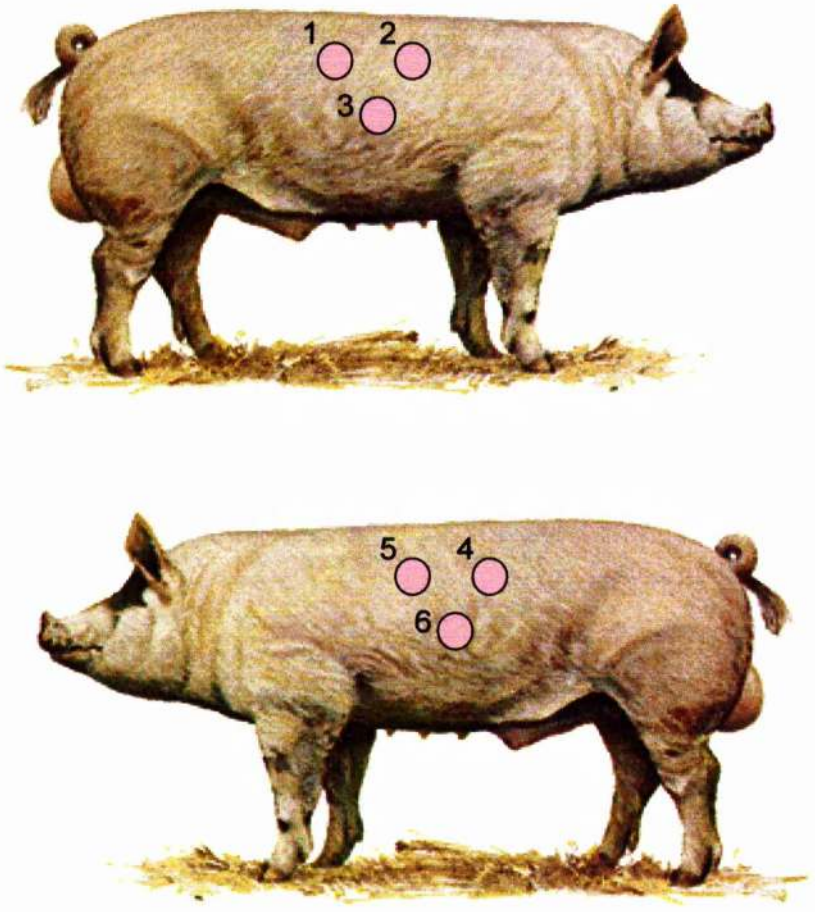
2.2.15.3 Harvesting Jejunum

Following termination of an experimental animal, a midline laparotomy incision was performed to enter the abdominal cavity. The duodenojejunal flexure was located and jejunum traced for 30cm. The distal 20cm of this length of jejunum was extracted and washed. The extracted jejunum was then divided into 3cm lengths. One end of each length was then tied around the neck of a disposable plastic Pasteur pipette and then everted around the bulb of the pipette (such that the previously luminal villi were now exteriorised). The bulb was then placed in a foil boat containing OCT compound and the pipette cut off at the neck. The foil boat was frozen in isopentane in the vapour phase of liquid nitrogen.

2.2.17 Wound preparation

The pig was anaesthetised and laid in a prone position on the operating table. Each flank and paravertebral region was then cleaned with chlorhexidine (4%) surgical scrub and then meticulously shaved with electric clippers and a fresh razor. The flanks were then scrubbed with chlorhexidine 0.5% in spirit and the pig draped with sterile drapes. Three 4cm diameter circles were marked out on each flank. Two were situated in a paravertebral position and the third was situated beneath and between the other two (see Figure 2.9). The circles were then excised down to deep fascia with meticulous haemostasis and with removal of all fat overlying the deep fascia. 2 cm of undermining at the level of deep fascia was then undertaken circumferentially to facilitate chamber implantation.

Figure 2.9: Diagram showing the placement of wounds on the pig's flank adopted as standard throughout this study. Two were placed in a paravertebral position and a further one situated beneath and between them. These three were mirrored on the on the opposite flank (wound numbering system as shown).



2.2.18 Grafting Integra™

Prior to the induction of anaesthesia, Integra™ artificial skin was removed in a sterile fashion from its foil container and placed in a sterile basin. It was then successively rinsed (in order to remove traces of isopropanol) in two changes of at least one litre of normal saline per change. 4.5 cm diameter circles of Integra™ cut using a sterile paper template (of PTFE chamber base) were placed in a basin containing sterile saline in preparation for implantation.

With wounds prepared as above, the Integra™ discs were stitched in place using a circumferential running stitch of 5.0 proline (Ethicon). Chambers were then implanted and the Integra™ dressed with a 4cm diameter disc of N-A Ultra® and 0.25% silver nitrate soaked gauze. The thermoplastic Spectrum® jackets were applied and secured with Velcro® straps (see Figure 10). Dressings were changed thereafter every 3-4 days for the ensuing 10 days, during which time the Integra™ was biointegrating (Grant et al., 1998).

2.2.19 Chamber implantation

In order to facilitate chamber implantation 1 – 2 cm incisions were made through skin, down to deep fascia at the upper and lower poles of each circular wound. This method rather than a longer incision made at either of the poles was adopted to reduce the extent of incised tissue and subsequent scarring. Sterile chambers were then implanted, the 2cm-phalange extending beneath the undermined area and secured (as well as incisions closed) with 2.0 silk through preprepared holes at the upper and lower poles of the

chamber. Only the upper and lower poles of the chamber were stitched in place (rather than four quadrants) in an effort to minimise tracking of pathogens along the braided silk stitch.

2.2.20 Grafting keratinocyte sheets

10 days after grafting of Integra™ the silicone top layer was easily separated off the underlying biointegrated bed. Keratinocyte sheets were detached and processed as described earlier (2.2.3 and 2.2.4). Using sterile technique each graft was in turn lifted out of the 6 well plate and placed on the biointegrated Integra™-neodermal bed. The wound was then dressed in a 4cm diameter disc of N-A Ultra® and normal saline-soaked gauze. The thermoplastic Spectrum® jackets were applied and secured with Velcro® straps. Dressing changes were generally undertaken every three or four days.

On one occasion the dressing change took place after a 5 day interval and all three pigs involved were found to have wound infections and surrounding cellulitis. These settled down after several days following intramuscular injection of 350mg amoxycillin/87.5mg clavulanic acid (Synulox®).

2.2.21 Grafting composites

Composites were processed as described earlier (see Section 2.2.5), and transported to the laboratory. Using sterile technique each graft was in turn lifted out of a 6 well plate and placed on the newly prepared fascial bed. Composites were secured circumferentially with acrylic glue and a 5ml vial was sufficient for 3 wounds. Dressing of wounds (including timing) were performed as for sheet grafts.

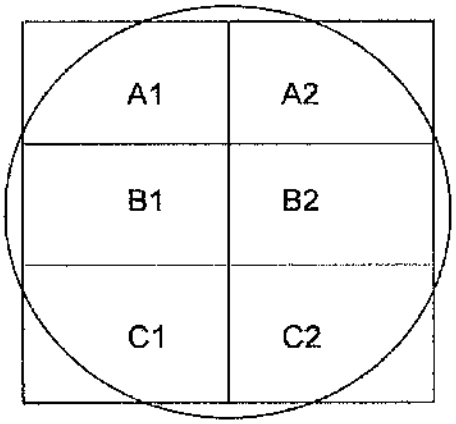
2.2.22 Processing of harvested grafts

Immediately following termination of animals on predetermined days grafts were harvested by excising the entire wound down to muscle. These were then washed in saline, photographed, wrapped in saline-soaked gauze and placed in a labelled petri dish. The dishes were then wrapped in cling film and then placed in a polystyrene box containing an ice slurry, in preparation for transportation to the laboratory (reaching the laboratory 2-3 hours later).

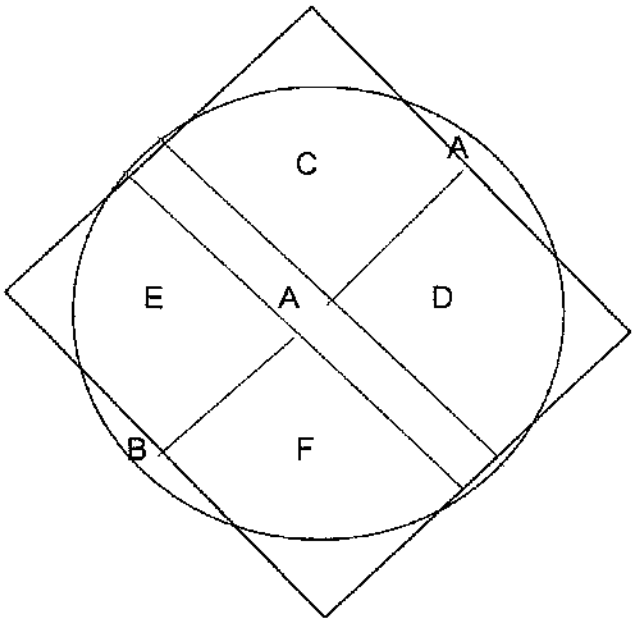
In the laboratory harvested wounds were divided as per Figure 2.10 (a) or Figure 2.10 (b). Blocks were immersed longitudinally (side on) in OCT compound in labelled foil boats and held in the vapour phase of liquid nitrogen until the OCT compound was completely opaque. Foil was then removed and the block inserted in a labelled compartment box and then placed in a -40°C freezer compartment.

Figure 2.10: Schematic representation of harvested wound processing. Circular harvested wounds were carefully pinned to a board, the pins marking out the grid patterns shown in (a) or (b). The edges were squared off (as shown in (a)) to facilitate easier processing. The wounds were then sectioned as shown and labelled accordingly. Only the wounds pertaining to experimentation with cultured composite grafts (chapter 8) were sectioned as shown in (b). The protocol was altered to facilitate analysis of the wound centre and edges.

(a)



(b)



Chapter 3

Development of a PT67 MFG lacZnls amphotropic retroviral producer cell line

3.1 Introduction

In order to evaluate the short, medium and long term biological efficacy of transplanted allogeneic components, the source of keratinocytes within a regenerated epithelium following allografting must be determined. This may be investigated by various means.

There are two principle approaches:

(a) Analysis of keratinocytes for an intrinsic differentiating feature. Examples of this include: detection of ABO antigens (Thivolet et al., 1986; Mauduit et al., 1987; Zhao et al., 1992), molecules of the major histocompatibility complex (Gielen et al., 1987) and chromosomal differences in cases of cross gender grafting (Brain et al., 1989; Burt et al., 1989). Detection of Y chromosomes by gross DNA hybridisation or polymerase chain reaction (PCR) techniques that involve disruption of tissue architecture makes it then difficult for further analysis of immune or other events occurring in relation to the keratinocyte under consideration. Other techniques such as in situ DNA hybridisation or the use of monoclonal antibodies with immunohistochemistry can be highly sensitive, and would preserve tissue architecture. They cannot however, be used to track the fate of autologous grafted cells.

(b) Labelling keratinocytes with a marker. Ideally, identification of marked keratinocytes should not necessitate tissue disaggregation thus allowing for further analysis of tissue in the vicinity of the cells under analysis.

Using an *in vitro* marking technique, autologous as well allogeneic grafted cells may be tracked subsequent to grafting.

Ideally the marker should be stable over the required number of cell divisions, easily detectable, non-toxic to host or neighbouring cells and incapable of contaminating non labelled cells.

Labelling can be achieved by the use of radioactive probes (such as ^{125}iodine and ^{51}chromium) or fluorescent probes (such as fluorescein, rhodamine isothiocyanate, bisbenzimide and PKH26) that colour either membrane or nuclear material. A variety of problems have been associated with the above. The radioactive probes have been used in lymphocyte tracking; however toxicity, internal radiation effects, poor uptake and rapid elution (Horan et al., 1990) render them unsuitable for anything but short term *in vivo* work. Disadvantages associated with the fluorescent probes include rapid escape with some, during the first 5 hours of tracking (Olszewski., 1987). With others, a dilution of intensity of labelling with each cell division and a tendency with some dyes to leach out and contaminate neighbouring (previously unlabelled) cells (Mosahebi et al., 1999), make them unsuitable for *in vivo* transplantation in which the probed cells are expected to undergo a series of cell divisions.

To overcome many of the deficiencies of radioactive and fluorescent probes, increasing use has been made of labelling by the insertion of a marker gene. The marker typically codes for an enzyme that on incubation with a suitable substrate results in identifiable colour staining. The gene having been isolated

in plasmid form is appropriately packaged in preparation for cell entry and then host DNA integration.

There are a variety of methods for insertion of a gene into a mammalian cell. These include physical and chemical gene transfer techniques, which give predominantly transient gene expression and the use of viruses. Retroviruses have highly efficient mechanisms for genome integration and consequently gene expression tends to be more stable.

The E. coli β-galactosidase lacZ gene has been used extensively with respect to keratinocyte tracking (Garlick et al., 1991; Jiang et al., 1991; Setoguchi et al., 1994; Stockschlader et al., 1994; Jensen et al., 1994; Ng et al., 1997).

The lacZnls marker gene has been modified, as the name implies, by the insertion of a nuclear localisation signal (nls) to ensure that the product coded for is nuclear localised (Ferry et al., 1991). This helps differentiate between endogenous expression and that due to gene marking, because endogenous β-galactosidase can sometimes be seen in the cytoplasm (but not the nucleus) of control tissues. The encoded β-galactosidase enzyme gives rise to dense nuclear localised blue staining on incubation with the substrate X-Gal and magenta when incubated with M-Gal.

Retroviral transduction is achieved using a replication incompetent retroviral particle containing only the marker gene of interest and the packaging signal Ψ^+. The particle itself does not contain the gag (core structural proteins), pol (reverse transcriptase) and env (coat glycoproteins) genes and is therefore replication incompetent. The envelope coating the genetic material needs to

be amphotropic in order to transduce cells of a different species. Markers that are currently available aren't always packaged within envelopes that can enter the particular species for which they are required. Hence they need to be tested and may need to be further packaged by a packaging cell line that provides a glycoprotein coat capable of entering the target species.

There are two principle ways of targeT-cell transduction. One using supernatant taken from retroviral producer cells, the other, rendering producer cells replication incompetent and using them as feeder cells. There are advantages and disadvantages with both methods.

Supernatant transduction involves using the medium harvested from a viable retroviral producer cell culture and utilised directly or indirectly for transduction. Direct usage of the supernatant involves culturing the recipienT-cells in the presence of supernatant. Indirect usage of the supernatant involves the use either of resuspended retroviral particles following ultracentrifugation of the supernatant or the use of dialysis techniques. For direct supernatant transduction to be most effective, the viral titre within the supernatant needs to be minimally diluted. Keratinocytes are susceptible however, to small changes in medium constituents. In particular they tend to differentiate (losing the ability for further cell division) with rising calcium concentration. For optimal keratinocyte culture the biochemical make up of supernatant from retroviral producer cells therefore needs to be similar to keratinocyte growth medium. This requirement may conflict with the nutritional needs of retroviral producer which may be very different to those of keratinocytes. Poor ability of retroviral producer cells to thrive in keratinocyte

culture medium makes their culture in this medium and subsequent successful direct supernatant transduction more difficult.

Using retroviral producer cells as feeder cells for keratinocytes will get round the problems delineated with respect to direct supernatant transduction. Producer cells (such as PT67), rendered replication incompetent by irradiation survive in keratinocyte culture medium for between 2 and 5 days producing retroviral particles.

Retroviral producer cells may package particles in an envelope that is ecotropic (capable of entering cells pertaining only to the producer species) or amphotropic (will enter cells in a variety of species). A producer line that packages retroviral particles in an ecotropic envelope may be used to transduce an amphotropic packaging cell line transforming it into an amphotropic producer cell line.

Production efficiency by retroviral producer cells may drop with repeated cell division as the retroviral producer cell line population becomes increasingly heterogeneous. There are two principle means of restoring efficiency. The first is to use selective media that favour cells with the marker gene linked to a sequence whose expression allows for selection within the selective medium. The second is to plate ouT-cells at low density and to pick a series of colonies, grow them up and test in order to detect the most efficient clones.

With respect to their efficacy as feeder cells for keratinocytes, producer cells may differ from 3T3 cells (Swiss mouse fibroblasts – the traditional feeder cell line as used by Rheinwald and Green (Rheinwald and Green, 1975)) in

several respects which may serve to limit their performance. Irradiated producer cells may be more limited both quantitatively and qualitatively in terms of their cytokine output as well as a markedly reduced survival in comparison to 3T3 cells. This may effect porcine keratinocyte phenotype, clonal expansion, colony forming efficiency and keratinocyte intercellular dynamics in a detrimental manner.

Mammalian cell expression of genes inserted by transduction, may be transient if translation alone of retroviral RNA has occurred without reverse transcription and incorporation into host DNA. Expression may not remain stable even following successful marker gene transduction through successive cell divisions and may disappear altogether (Fenjves, 1994) or be seen to vary with cell phenotype (Altmann and Trachsel, 1993). Prior to the use of labelled cells *in vivo*, it was useful therefore to examine the *in vitro* behaviour of transduced keratinocytes.

The aims of this study included the following:

- To establish a retroviral producer line capable of efficient porcine keratinocyte transduction
- To determine the effect of irradiated PT67 feeder cells on porcine keratinocyte colony forming efficiency
- To determine the stability of expression of lacZnls transduced porcine keratinocytes *in vitro*

- To examine the nature of porcine keratinocyte lacZnls expression in relation to confluence in culture

3.2 Results

3.2.1 Cloning TELCeB:

Retroviral producer lines in use by the laboratory were of very low titre and hence poorly transduction efficient. The first objective was therefore, to clone the retroviral producer line TELCeB (Cosset et al., 1995) in an effort to increase it's efficiency, and to verify if it could transduce porcine keratinocytes. The TELCeB cell line is derived from the human cell line TE671 (Takeuchi et al., 1994) infected with the MFGlacZnls retroviral vector (see Figure 3.1), (forming TEL) and transfected with CeB. CeB is a Moloney murine leukaemia (MoMLV) viral gag-pol expression plasmid derived from pCRIP (Takeuchi et al., 1994; Cosset et al., 1995) (see Figure 3.2).

Figure 3.1: Schematic diagram of MFG vector construct

MFG recombinant retrovirus encoding lacZ. Moloney murine leukaemia virus (MoMuLV) long terminal repeat sequences are used to generate both a full length viral RNA (for encapsidation into virus particles) and a subgenomic mRNA, which is responsible for expression of inserted sequences. No selectable marker exists in the vector (adapted from Dranoff et al., 1993).

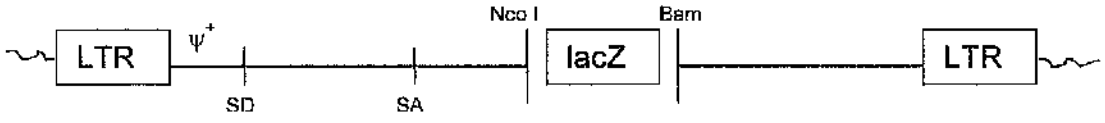
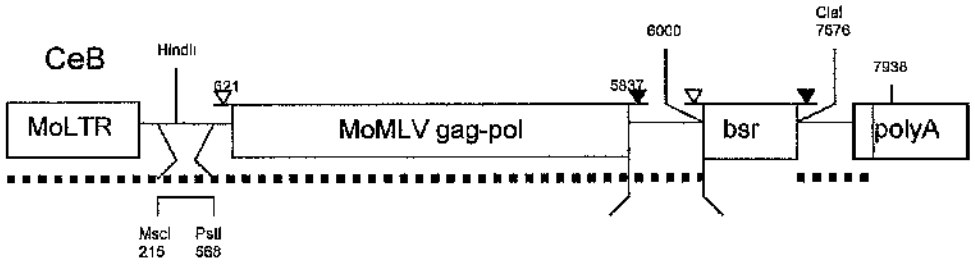


Figure 3.2: Schematic diagram of CeB construct

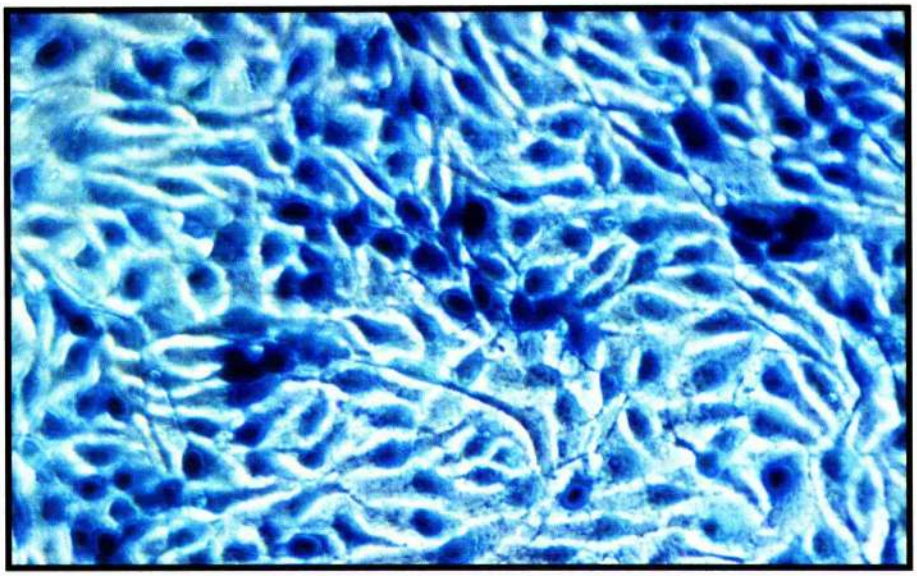
Initiation (▽) and termination (▼) codons are shown. The thick dotted line shows MoMuLV derived sequences (adapted from Cosset et al., 1995).



The cell line was grown up from frozen state to subconfluence, then plated out in 6cm tissue culture grade petri dishes in concentrations of 100, 300, 500 and 1000 cells per dish. On the 11th day healthy looking colonies had grown up in dishes plated with 300 and 500 cells. A dish plated with 500 cells was stained with X-Gal (see Figure 3.3). There was marked heterogeneity of β-galactosidase expression with 80% of cells staining blue in most colonies, a few colonies showing approximately 95% blue staining cells and some showing 30-40% staining.

Figure 3.3: X-gal staining of TELCeB cells

A 9cm diameter Petri dish plated with 500 TELCeB cells was stained after 11 days. The image (X20 objective) shows cells within a colony displaying the nuclear localised blue staining. The colony photographed shows a high proportion (approximately 95%) of cells with nuclear localised β-galactosidase expression.

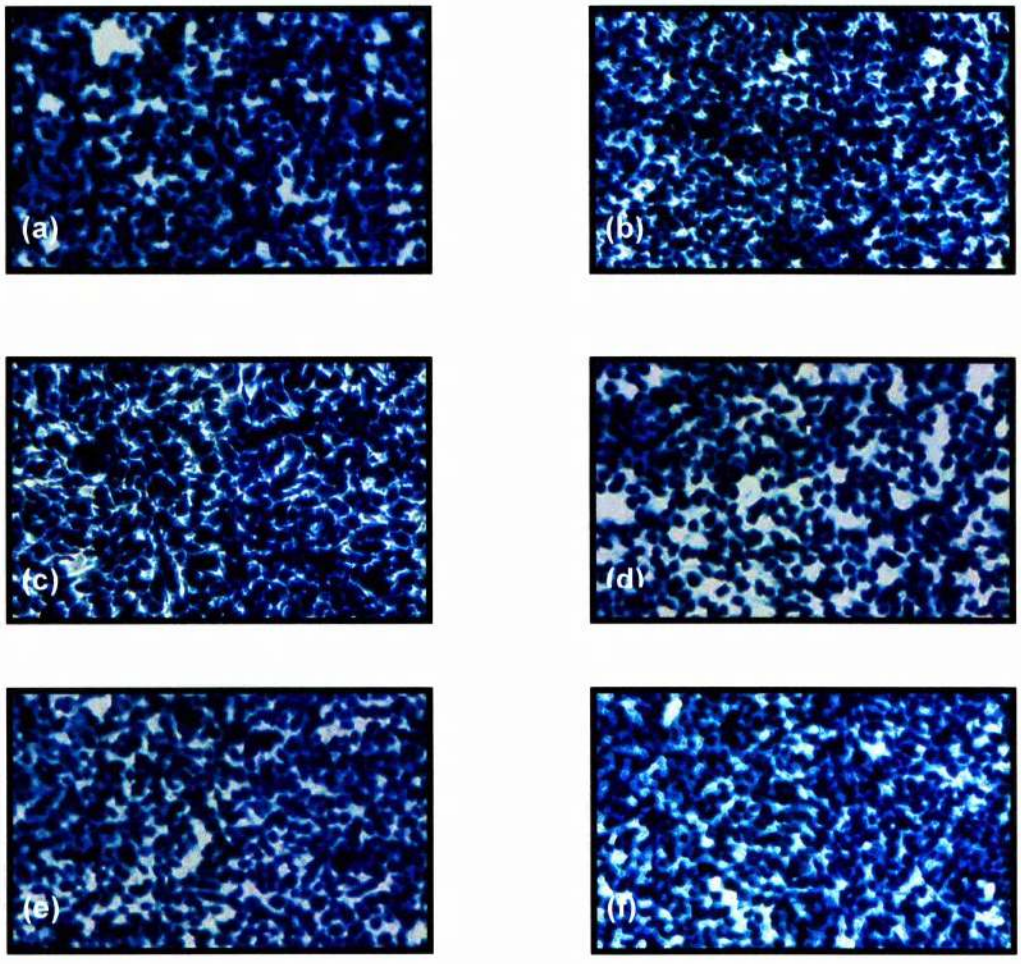


Disks of filter paper (0.75mm diameter) soaked in Trypsin/EDTA (0.05%/0.02%) and sterilised by irradiation with 66,000 rads/hour for 18hours, were used to transfer numbered colonies (from a petri dish plated initially with 300 cells) to a 24 well plate.

On the following day the paper disks were removed. Microscopic examination revealed homogenous looking colonies. When most colonies became just confluent, 80% of the cells were trypsinised and transferred to corresponding 6 well plates. The 24 well plate was stained with X-Gal 4 days later (see Figure 3.4) Judged according to percentage of cells showing blue nuclear staining and density of nuclear staining, the best colonies were nos. 5,11,15,18,22 and 24. These colonies were transferred from 6 well plates to 25cm^2 flasks. When just confluent the cells were transferred to 75cm^2 flasks.

Figure 3.4: X-gal staining of TELCeB clones grown up in 24 well plate

The 24 well plate containing TELCeB clones was fixed and X-Gal stained. 5 of the wells did not have any cells, 4 of the wells were difficult to interpret in that cells were seen only on the periphery of the well. Of the remaining 15 wells, 2 displayed close to 100% of cells staining blue, 3 had approximately 95% staining one of which did not appear to have as dense nuclear staining as the rest, 1 well had 90-95%, 4 had approximately 90% staining, two of which did not appear to have as dense nuclear staining as the rest, 3 had 80%, one had 60% and the remaining well displayed only 40% of cells staining blue. The images (X100) show the most prolifically and intensely staining clones from well no. 5 (a); well no. 11 (b); well no. 15 (c); well no. 18 (d); well no. 22 (e); well no. 24 (f);).



3.2.2 Determination of TELCeB as ecotropic or amphotropic producer

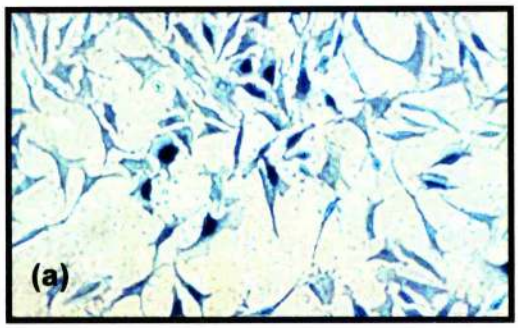
There was uncertainty as to the capacity of TELCeB to transduce both 3T3 cells and porcine keratinocytes. 2 consecutive attempts at 3T3 transduction (see Chapter 2 for method) were carried out with supernatant from clones from well numbers 5, 11, 15, 18, 22 and 24 in order to determine which of these was superior. These were determined (and confirmed by an independent assessor) to be from wells 11 and 24 (see Table 3.1 and Figure 3.5).

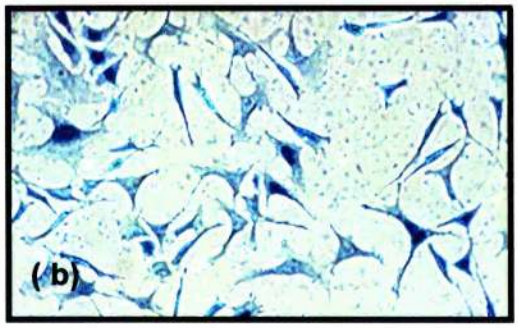
Table 3.1: X-Gal staining results following transduction of 3T3 Cells in 6-well plates by selected colonies

Flask number	***Neat supernatant*** *% transduction*		***1/10 supernatant*** *% transduction*		***1/100 supernatant*** *% transduction*	
	1st Study	***2nd Study***	***1st Study***	***2nd Study***	***1st Study***	***2nd Study***
*3T3 **(control)***	*0*	*0*	*0*	*0*	*0*	*0*
5	*50*	*40*	*~10*	*2*	*~1*	*<1*
*5 **(duplicate)***	*40*		*15*		*1*	
11	*unreliable*	*30-40*	*~5%*	*4-5*	*unreliable*	*unreliable*
15	*5*	*5*	*<1*	*<1*	*<1*	*unreliable*
18	*40*	*10-15*	*4*	*<1*	*<0.5*	*<1*
19	*10*	*10-15*	*3*	*<1*	*<1*	*<1*
22	*20-30*	*10*	*10*	*2*	*-*	*<1*
24	*30*	*40-50*	*5-10*	*<5*	*1*	*<1*

Figure 3.5: X-Gal stained 3T3 cells

Following supernatant transduction of 3T3s in 6-well plates by TELCeB clones flasks were stained with X-Gal to enable comparison between clones. The best clones (see Table 3.1) were from well numbers 11 and 24, photomicrographs (X200) (a) and (b) with approximately 30% and 40% 3T3 cell nuclei staining blue respectively.



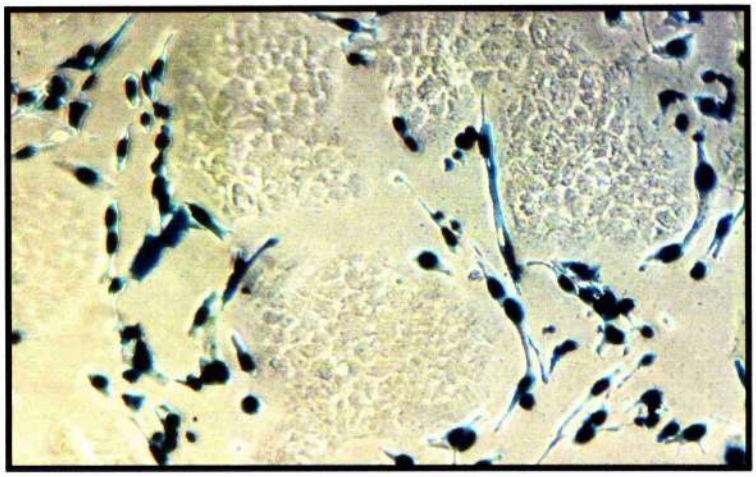


Attempts were then made to transduce porcine keratinocytes (see Chapter 2 for method) in 6 well plates with 0, 2, 4, 8, or 12 μg per ml of polybrene in each of the plates. Staining with X-Gal demonstrated a complete lack of keratinocyte transduction at any of the polybrene concentrations by the TELCeB lines (see Figure 3.6).

This experiment demonstrated that this TELCeB construct was not producing amphotropically packaged retrovirus.

Figure 3.6: X-gal stained keratinocytes

Photomicrograph (X200) of X-gal stained keratinocyte and TELCeB clone 11 feeder cells. The TELCeB cells stain blue as expected; however the keratinocytes show a complete lack of MFGlacZ expression, indicating a lack of transduction and ecotropicity of the TELCeB produced envelope protein.



3.2.3 Transduction of GP+EnvAm12 (amphotropic packaging cell line) with MFGlacZnls

TELCeB although a good ecotropic producer of MFGlacZnls was not able to package retroviral particles in envelopes capable of entering porcine keratinocytes. An attempt was made therefore, to use TELCeB in order to transduce the amphotropic packaging cell line GP+Env Am12 (Markowitz et al., 1988), hoping to covert it to an amphotropic producer cell line capable of transducing porcine keratinocytes.

GP+EnvAm12 is a clone isolated from an NIH/3T3 derived cell line containing the MoMLV gag and pol genes and transfected with the H070A amphotropic env gene (Markowitz et al., 1988).

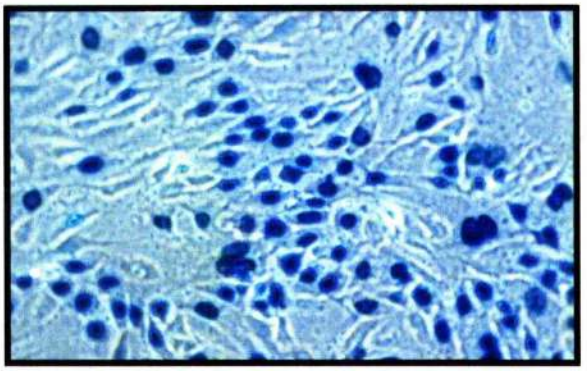
Flasks containing the packaging cell line GP+Env Am12 at 30-40% confluence were exposed on consecutive days to medium with a 50% concentration of supernatant from 'just confluent' TELCeB (clone number 11) cultures. 1 flask received growth medium alone, as a control. When the GP+Env Am12 flasks reached 80-90% confluence, an attempt was made to transduce 3T3 cells and P4 porcine keratinocytes (P4 denotes cells that have undergone 4 passages, with P0 referring to cells straight off the pig and P1 to cells that have undergone 1 passage). The method used for transducing 3T3 cells was by direct supernatant transduction and for transducing P4 keratinocytes utilised the GP+Env Am12 cells as feeder cells (see Chapter 2 for method).

Four days later both test and control keratinocytes were plated once more onto irradiated 3T3 cells. 3 days later the keratinocytes, 3T3s and a separate flask of transduced GP+Env Am12 cells were stained with X-Gal.

Approximately 80% of the GP+EnvAm12 cells transduced expressed the lacZ gene (see Figure 3.7). There was no detectable expression of β-galactosidase by the target keratinocytes or 3T3 cells (with X-Gal staining). A colleague working in tandem with the same line experienced similarly negative results. Others, including workers at this laboratory have in the past been moderately successful with GP+EnvAm12 (Markowitz et al., 1988; Ng et al., 1997a; Mathor et al., 1996). The line that had previously been found to work in our laboratory had become infected and the newly ordered cells we subsequently used turned out to be greatly inferior.

Figure 3.7: X-gal stained GP+EnvAm12 cells

Photomicrograph (X200) of GP+EnvAm12 cells stained with X-gal. The cells had undergone transduction by MFGlacZ following exposure to TELCeB clone 11 supernatant. Up to 80% of the cells stained blue, although they failed to produce retroviral particles when subsequently tested with 3T3 cells and keratinocytes.



Favourable reports were obtained of a different amphotropic packaging cell line offered for sale by Clontech Laboratories Inc. known as PT67 (Miller et al., 1994, Miller et al., 1996) I therefore proceeded to attempt transduction of this packaging cell line.

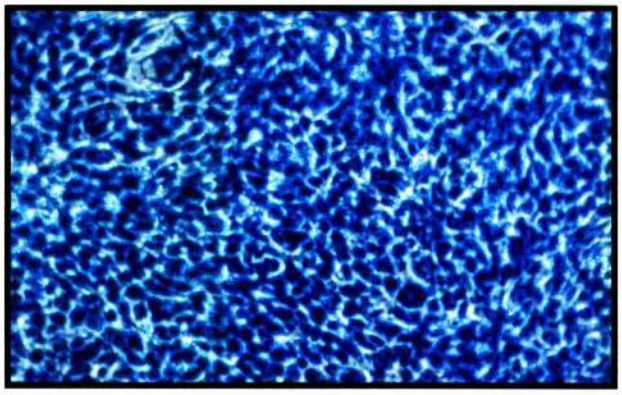
The PT67 cell line is an NIH/3T3 based line infected with the MoMLV gag and pol genes and expressing the 10A1 envelope protein. This envelope facilitates entrance of cells via both the amphotropic and the GALV (Gibbon Ape Leukaemia Virus) receptors thus broadening the host range.

3.2.4 Transduction of PT67 packaging cell population with MFG lacZnls

Newly obtained PT67 was grown up to confluence and then split 1:5. On the following day the PT67 cells received two exposures, 8 hours apart, to TELCeB clone 11 supernatant (at 50% concentration) with 4μg per ml polybrene. The medium was changed at 24 hours. On staining on day 4 the PT67 population looked heterogeneous with respect to both proportion of nuclei stained and intensity of nuclear staining (see Figure 3.8).

Figure 3.8: X-Gal stained PT67 cells

Photomicrograph (X100) of PT67 cells post transduction. Parallel flasks of the PT67 packaging cell line were exposed twice to TELCeB clone 11 and one flask was stained with X-Gal on day 4. Heterogeneity of staining density is evident, so the newly transduced producer cell line was cloned.



3.2.5 Testing the newly formed PT67 producer cell population

In order to confirm that the transduced PT67 cells were producing virus capable of amphotropic transduction it was necessary to test it on keratinocytes and 3T3 cells. The method used for transducing 3T3 cells was by direct supernatant transduction and for transducing porcine keratinocytes utilised the PT67 cells as feeder cells (as for testing of GP+EnvAm12 see Chapter 2 for method).

On staining with X-Gal, approximately 30% of the keratinocytes stained blue but there was no positive staining of 3T3 cells. The results indicated that an amphotropic producer cell line capable of porcine keratinocyte transduction had been established. The negative 3T3 result may have been due to premature fixing and staining. Subsequent attempts to transduce 3T3s with this line have been successful, but only when staining is done after 3 days.

To increase the efficiency of viral output it was decided to clone out the more efficient producers from the parent TELCeB transduced PT67 producer population.

3.2.6 Cloning the PT67 producer cell population

Transduced PT67 cells were plated out on to petri dishes in concentrations of 20X, 10X, 1X, 0.3X and $0.1X10^3$ cells per dish. Nine days later, disks of filter paper (0.75mm diameter) soaked in Trypsin/EDTA (0.05%/0.02%) and sterilised by irradiation with 66,000 rads per hour for 18hours, were used to transfer numbered colonies (from a petri dish plated initially with 300 cells) to a 24 well plate. On the following day the paper disks were removed. Three

days later 50% of the cells in each well were transferred to duplicate wells in a 24 well plate. The cells from one of the two 24 well plates were transferred 2 days later to 6 well plates. The other plate was stained with X-Gal (see Table 3.2). Colonies with the most favourable staining profile in terms of homogeneity, degree and density of staining were from well numbers 1,8,9,10,13,14,17 and 23. These were transferred from 6 well plates to 25cm^2 flasks and thence to 75cm^2 flasks.

Table 3.2: X-Gal staining of 24 well plate containing PT67 clones

The wells were graded 1 to 5 with respect to increasing homogeneity, proportion and density of staining.

(1) 5	(5) 4	(9) 4	(13) 5	(17) 5	(21) 4
(2) 4	(6) 5	(10) 5	(14) 5	(18) --	(22) --
(3) 2	(7) 2	(11) 1	(15) 3	(19) --	(23) 5
(4) 4	(8) 4	(12) 2	(16) 2	(20) 4	(24) --

To determine which of these clones was the most efficient retroviral producer they were compared with respect to transduction of 3T3 cells. On staining with X-Gal, the clone from well 8 showed the highest percentage transduction of 3T3 cells (see Figure 3.9 and Table 3.3 for results).

Flow cytometry (see Chapter 2 for method) confirmed that E. coli β-galactosidase is present in close to 100% of the PT67 clone from well number 8 (see Figure 3.10).

Table 3.3: X-Gal staining results of 3T3 transduction by selected PT67 producer cell clones.

$2X10^6$ cells from each clone were plated out in a $75cm^2$ flask. 10^5 3T3 cells per well were plated out in 6 well plates. Supernatant (from each clone as well as from a flask of subconfluent 3T3 cells) at concentrations of 50%, 0.5% and 0.05% and polybrene (8 μg per ml) were added on the following day. One 6 well plate acted as a negative control. 24 hours later the medium was changed and 2 days later the plates were stained with X-Gal and graded according to percentage of 3T3 cells with blue nuclear staining.

Clone Number	**50% Supernatant** % transduction	**0.5% Supernatant** % transduction	**0.05% Supernatant** % transduction
3T3 (control)	0	0	0
1	5-10	<1	<1
8	50-75	5-10	<1
9	10-20	1-2	<0.01
10	10-20	1-2	<0.01
13	10-20	1-2	<0.1
14	10-20	1-2	<0.1
17	5	1-2	<0.1
23	2-5	<1	<0.1

Figure 3.9: X-gal stained PT67 producer cells (Clone 8) and transduced 3T3 cells

Photomicrograph ((a) X100, (b) X200) of well number 8 (24–well plate) containing a clone of MFGlacZ transduced PT67 cells (a) and 3T3 cells transduced by this clone (b). The clone from well number 8 achieved a 50-75% transduction rate of 3T3 cells (higher than any of the remaining clones within the 24–well plate).

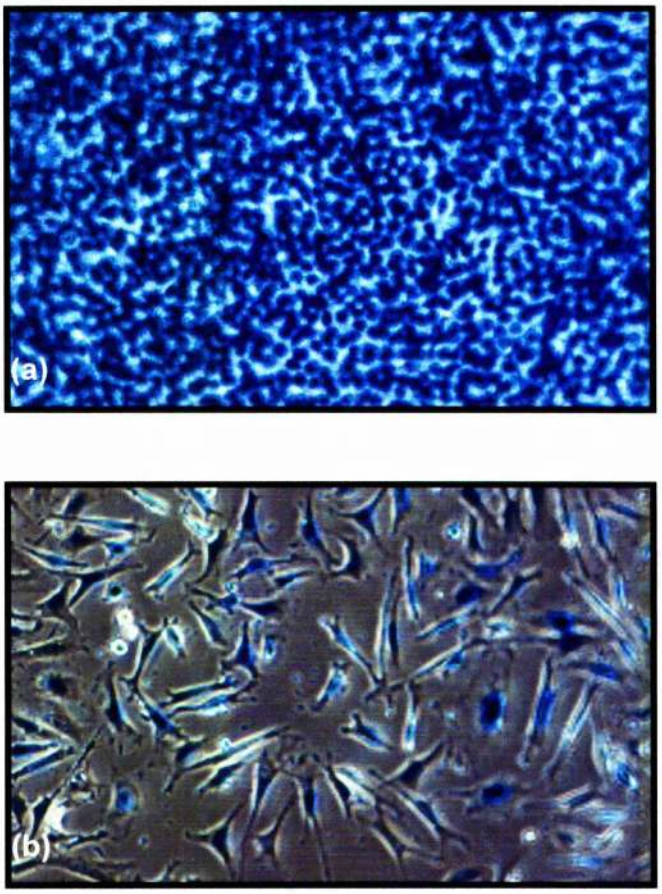
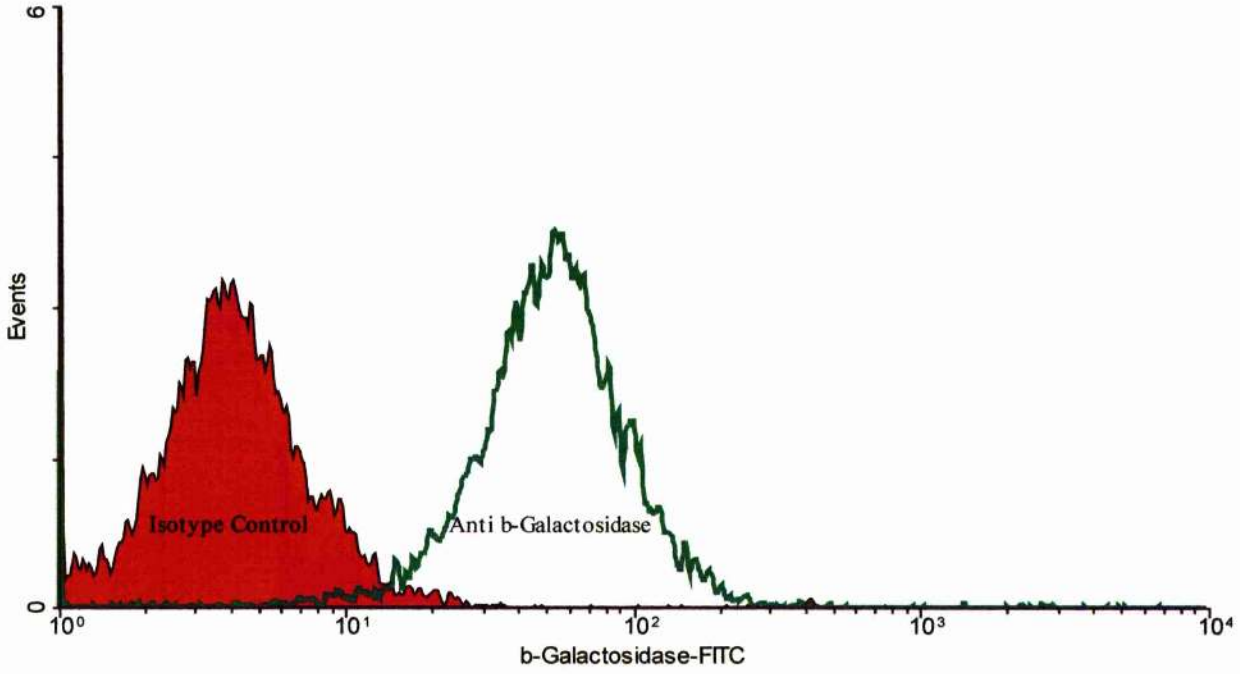


Figure 3.10: Histogram following flow cytometry showing anti-β-galactosidase-FITC labelling of PT67 clone 8 cells

One population was primarily labelled with an isotype control (IgG2b) and the other with anti-β-galactosidase (IgG2b). Secondary labelling with FITC (sheep anti-mouse IgG). Samples were processed in triplicate. The histogram illustrates close to 100% expression of β-galactosidase by PT67 clone 8.



3.2.7 Determination of PT67 clone (8) producer cell line titre

10^4 3T3 cells per well were plated out in triplicate into 6 well plates. 7 serial dilutions of PT67 clone 8 supernatant ($5X10^{-1}$, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}) and 1 control (3T3 medium) were added to the wells 24 hours later together with 8µg per ml per well of polybrene. The medium was changed after 24 hours and stained with X-Gal 2 days later.

The titre was determined for each of the 3 highest dilutions (see Figure 3.11). The mean of the titres at the top two dilutions (10^{-5}, 10^{-6}) is $1.7X10^6$ colony forming units per ml (cfu per ml). High titre > 10^6cfu per ml retroviral producer lines are reportedly capable of approaching 100% transduction (Carroll et al., 1993).

Consistently, close to 100% of keratinocytes were now found to have been transduced following repeated passage (3 times) on irradiated PT67 feeder cells (see Figure 3.12).

Figure 3.11: Graph showing the distribution of viral titre against supernatant dilution

The titre (virus particles per ml of supernatant) is determined as the number of blue staining colonies multiplied by the relevant dilution factor (divided by the well volume). In this experiment 20 ml of supernatant was diluted to a 10^{-6}, 10^{-5} or 10^{-4th} part of the total volume (4ml) within each well in 6 well plates. The wells were prepared in triplicate. Count results are shown.

Dilution	**10^{-4}**	**10^{-5}**	**10^{-6}**
Blue staining colony counts	131	23	8
	245	34	5
	225	20	20

The viral titre was determined to be 1.7X10^6 colony forming units per ml (cfu per ml) calculated from the mean of the titres determined at the top two dilutions (10^{-5}, 10^{-6}). Error bars represent the standard deviation (n=3).

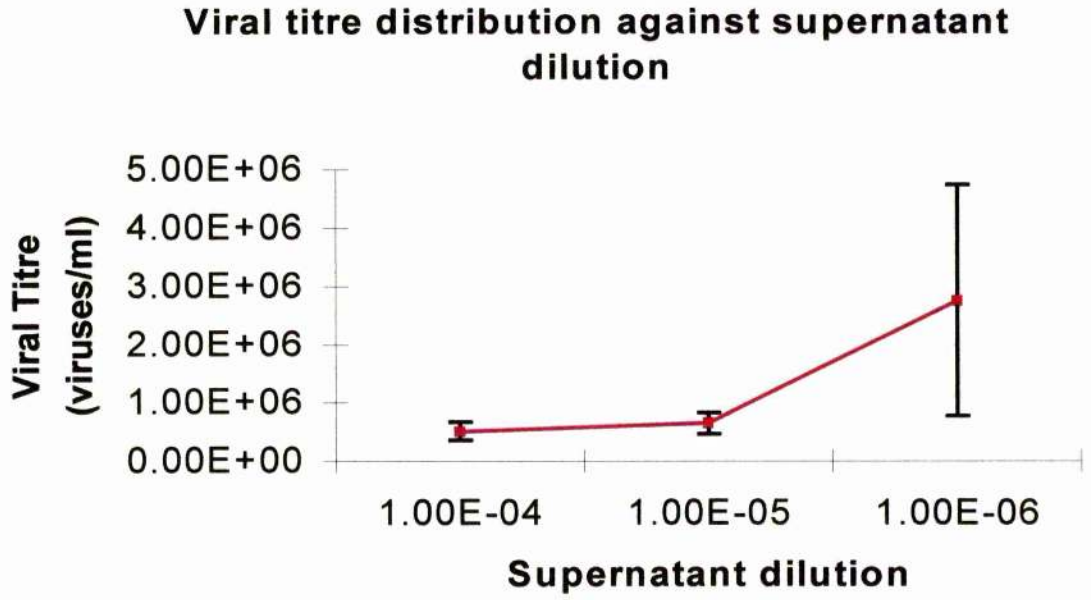
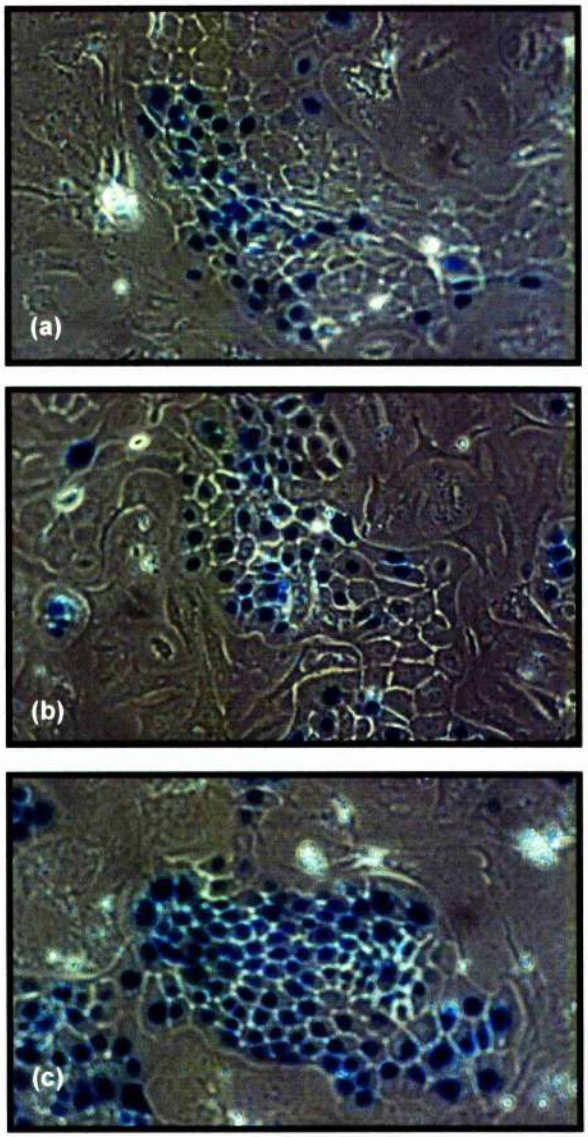


Figure 3.12: X-gal stained porcine keratinocytes cultured on PT67 clone 8 producer feeder cells

The keratinocytes initially passaged on PT67 producer feeder cells were subsequently passaged onto irradiated 3T3 cells before fixing and staining with X-gal. The percentage of keratinocytes staining blue (illustrated by the photomicrographs X200) were approximately 30% after 1 passage (a), 60% after 2 passages (b) and approaching 100% following 3 passages (c).



3.2.8 Effect of PT67 clone 8 supernatant on metabolic activity of 3T3 cells

Having observed that high concentrations of PT67 supernatant appeared to inhibit 3T3 cell replication during viral titre experiments, an attempt was made to see whether progressive dilutions of PT67 supernatant produced significant 3T3 cell replication inhibition and hence β-galactosidase expression. This could lead to a misleading estimate of viral titre.

Alamar blue™, a non-toxic redox indicator dye (see Chapter 2 for method) was added (prior to X-Gal staining) to the same set of 6 well plates used to determine PT67 (clone 8) titre. Absorbance was measured after 4 hours (see Figure 3.13).

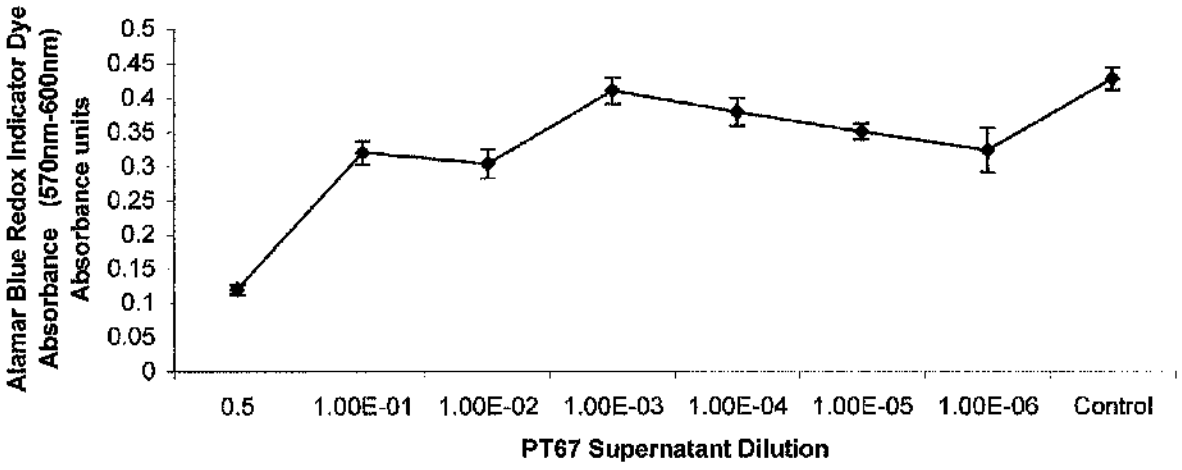
Using an all pairwise multiple comparison procedure (Tukey test) within a one way analysis of variance test there was a significant difference ($P<0.05$) in metabolic activity of between 60.7 and 72.0% between the cells that had 50% supernatant and the cells that received higher dilutions. There was also a significant ($P<0.05$) difference between the control and the cells receiving $1/10^{-1}$ and 10^{-2} dilutions of between 25.0 and 28.8% but not between the control and the cells receiving 10^{-3}, 10^{-4} or 10^{-5} dilutions.

PT67 cells are highly metabolically active and require frequent changes of medium. It might not be surprising that there could be a negative effect on cell metabolism seen with concentrated supernatant. This may be unrelated to the concentration of viral particles but instead be due to deficiency of nutrients within the supernatant. This however, does not adequately explain the negative effects on cell metabolism seen with the 10^{-1} or 10^{-2} dilution. An

explanation may lie in a phenomenon observed repeatedly with respect to PT67 cells. If within a flask of sparsely plated PT67 cells there is an isolated clump of (highly confluent) cells the rest of the cells will tend not to divide. The explanation may be that highly confluent PT67 cells secrete a substance that even in small concentrations inhibits cell metabolism.

Figure 3.13: Effect of PT67 supernatant on metabolic activity of 3T3 cells

10^4 3T3 cells per well were plated out in triplicate in 6 well plates. 7 serial dilutions of PT67 clone 8 supernatant ($5X10^{-1}$, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}) and 1 control (3T3 medium) were added to the wells 24 hours later together with 8µg per ml per well of polybrene. The medium was changed after 24 hours. 2 days later Alamar Blue was added to the wells at a concentration of $1/10^{th}$ of the volume of medium contained within the well (prior to staining with X-Gal). 4 hours later 200µl were transferred in triplicate from each well to a 96 well plate and the absorbance at 570nm minus the absorbance at 600nm determined (shown below with standard error bars, n=3).
Using an all pairwise multiple comparison procedure (n=3, Tukey test) within a one way analysis of variance test there was a statistically significant difference ($P<0.05$) in metabolic activity of between 60.7 and 72.0% between the cells that had 50% supernatant and the cells that received higher dilutions.



3.2.9 Effect of irradiated PT67 feeder cells on keratinocyte colony forming efficiency

The purpose of generating and cloning the PT67 producer line was to achieve highly efficient MFGlacZ keratinocyte labelling. Although NIH/3T3 cell-derived, PT67 cells have been altered genetically and repeatedly cloned. Their use instead of irradiated 3T3 cells as feeder cells may therefore deleteriously effect keratinocytes. Phenotypically, keratinocytes look different when grown on irradiated PT67 cells in that the cytoplasm appears less dense and intercellular attachments appear to be fewer. In terms of growth, although they initially appear to multiply faster, the colonies formed appear to be smaller and the rate of growth subsequently slower (after the initial spurt) than when grown on 3T3s. Irradiated PT67 cells die after 2-4 days while irradiated 3T3 cells often appear to survive for over a week on collagen-coated flasks. It therefore was imperative to investigate irradiated PT67 cells as feeder cells with respect to colony forming efficiency in terms of numbers and size of colonies formed (Barrandon and Green, 1987).

The study was carried out with keratinocytes from 3 separate Large White pigs. Using P0 keratinocytes that had undergone SLA DR antibody complement pre-treatment (see Chapter 2 for method), $1.1X10^2$ per cm^2 were plated out in triplicate with one of the following feeder cell regimens:

PT67 $1.4X10^4$ per cm^2	(1)
3T3 $1.4X10^4$ per cm^2	(1)
3T3 $1.4X10^4$ per cm^2+ PT67 $1.4X10^4$ per cm^2	(1) + (1)
3T3 $6.9X10^3$ per cm^2+ PT67 $2.1X10^4$ per cm^2	(0.5) + (1.5)
3T3 $6.9X10^3$ per cm^2+ PT67 $1.4X10^4$ per cm^2	(0.5) + (1)

3T3 $6.9X10^3$ per cm^2+ PT67 $6.9X10^3$ per cm^2 (0.5) + (0.5)

The surface area of a well (within a 6 well plate) is $9.4cm^2$. $2.67X10^4$ per cm^2 is the number of irradiated 3T3 cells generally used as feeder cells when culturing porcine keratinocytes. In this experiment, the number of keratinocytes plated was very low ($1.1X10^2$ per cm^2), a minimum number of $1.4X10^4$ per cm^2 was therefore judged to be more than sufficient to reduce bias due to differences in cell numbers and focus the study in the direction of analysis of qualitative differences between the cell types.

Cells from the 3 pigs were assessed in triplicate.

After 1 week the wells were stained with rhodamine B and X-Gal.

The number of colonies were counted both manually and confirmed with an automated image analyser count (see Figures 3.14 and 3.15). In addition the image analyser was used to assess the size (area) of colonies (see Chapter 2 for method).

Figure 3.14: Study of colony forming efficiency of feeder cell types and combinations (scanned images)

The images show examples of wells (6 well plate) that were scanned for image analysis following fixation and staining of keratinocyte colonies from pig number 17. The wells correspond to the following feeder cell types: PT67 (a); 3T3 (b);PT67+3T3 (c); 0.5X3T3 + 1.5XPT67 (d); 0.5X3T3 + PT67 (e); 0.5X3T3 + 0.5XPT67 (f).

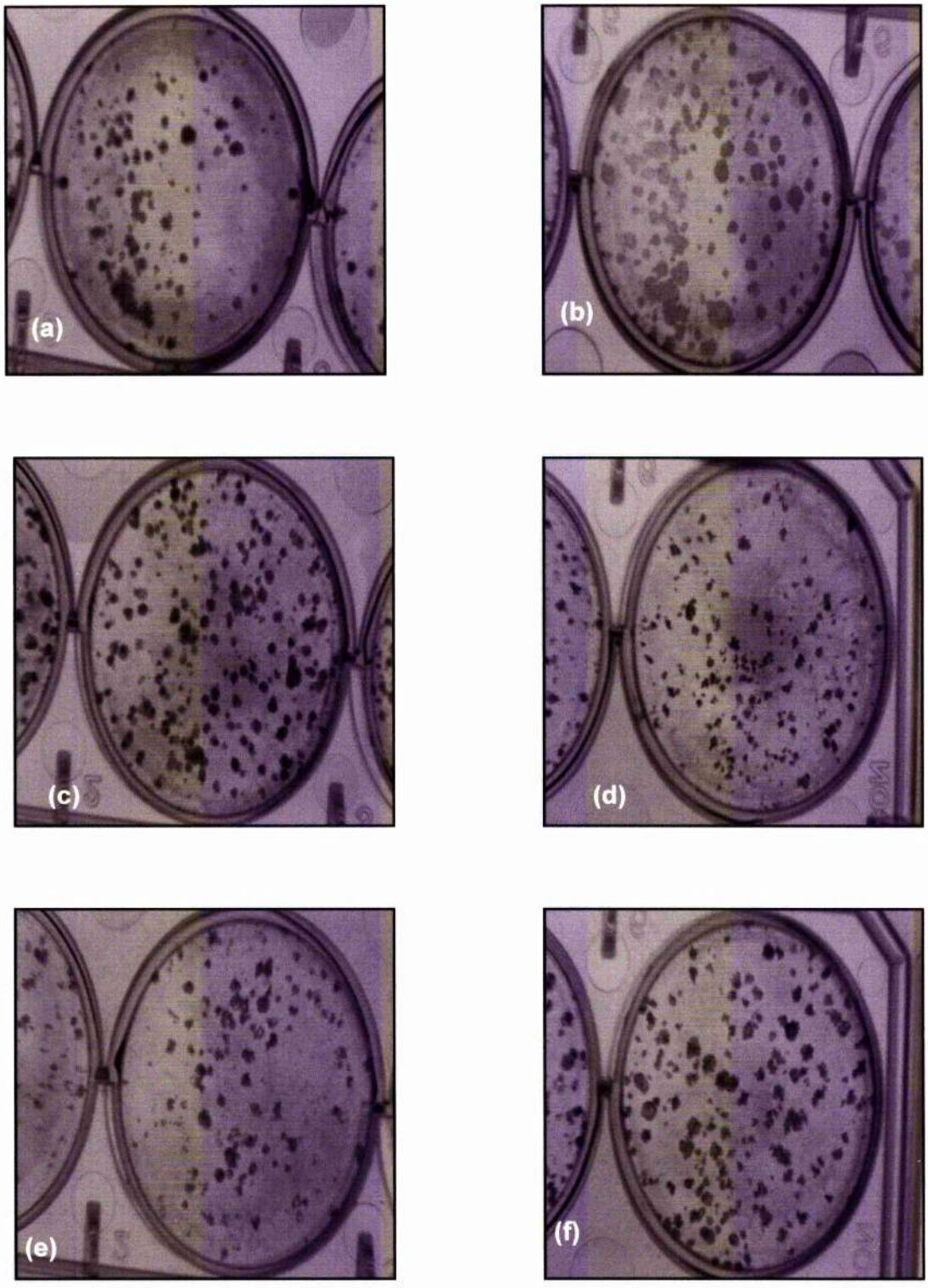
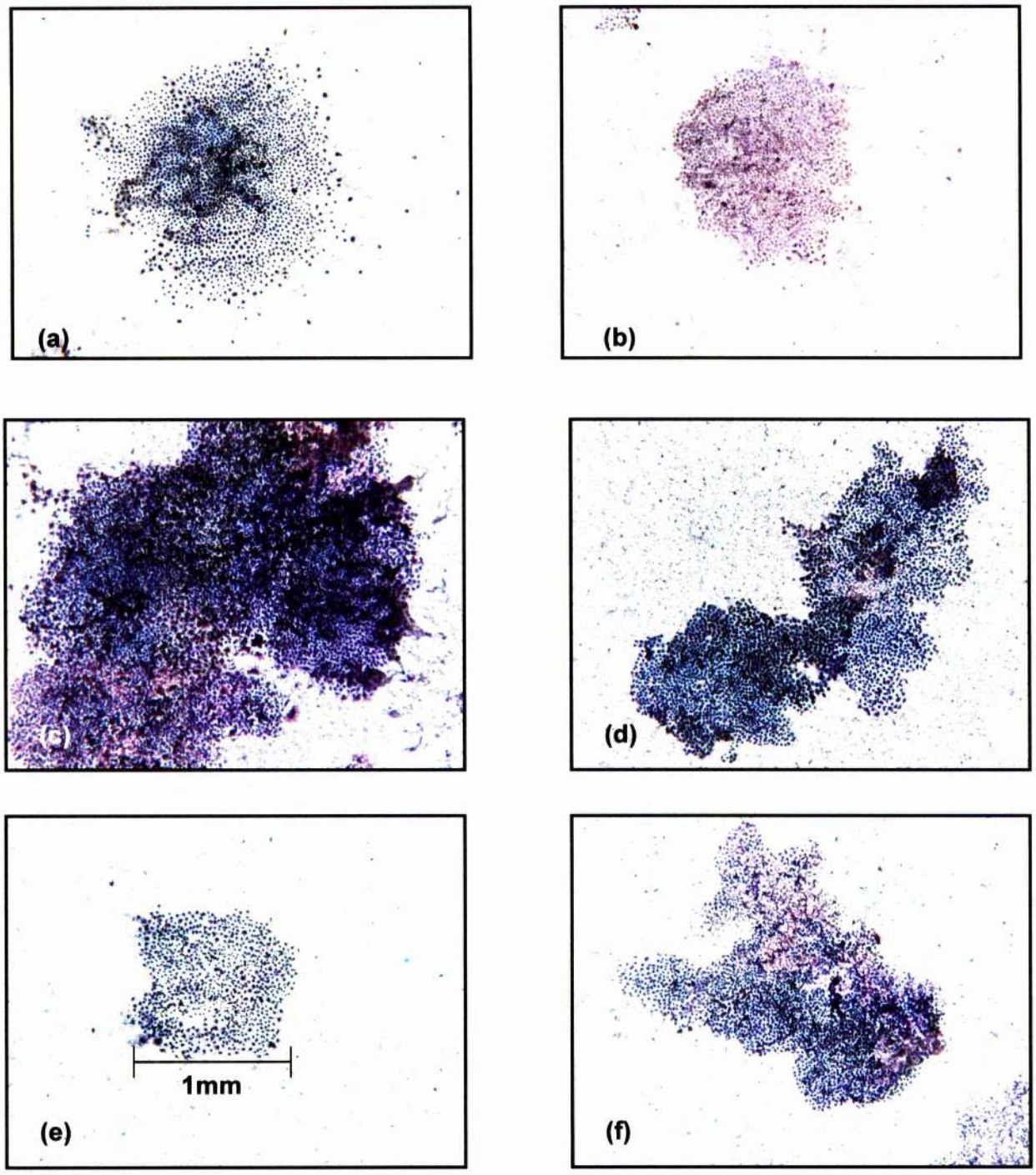


Figure 3.15: Study of colony forming efficiency of feeder cell types and combinations

Photomicrographs (X 40) showing typical keratinocyte colonies from pig number 17 grown up on the different feeder cell types. PT67 (a); 3T3 (b);PT67+3T3 (c); 0.5X3T3 + 1.5 X PT67 (d); 0.5X3T3 + PT67 (e); 0.5X3T3 + 0.5 X PT67 (f), following fixation and staining with X-gal and rhodamine B.



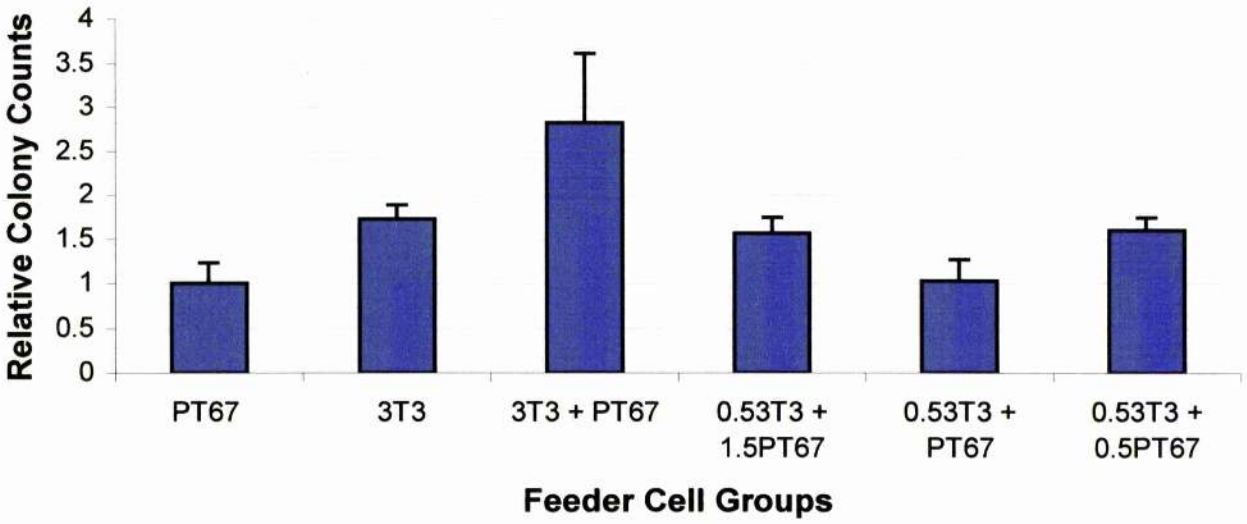
3.2.9.1 Quantitative analysis of colony forming efficiency

Manual Count: The results were analysed using a one way repeated measures analysis of variance. This demonstrated (Tukey test) a significant decrease of between 38 and 60% in colony numbers in all groups (with the exception of the 0.53T3 + 1.5PT67 group), as compared to the 3T3 + PT67 group ($P<0.05$) (see Figure 3.16). Note, that although the absolute colony numbers vary between each of the three pigs, the pattern due the different feeder cell regimens is consistent.

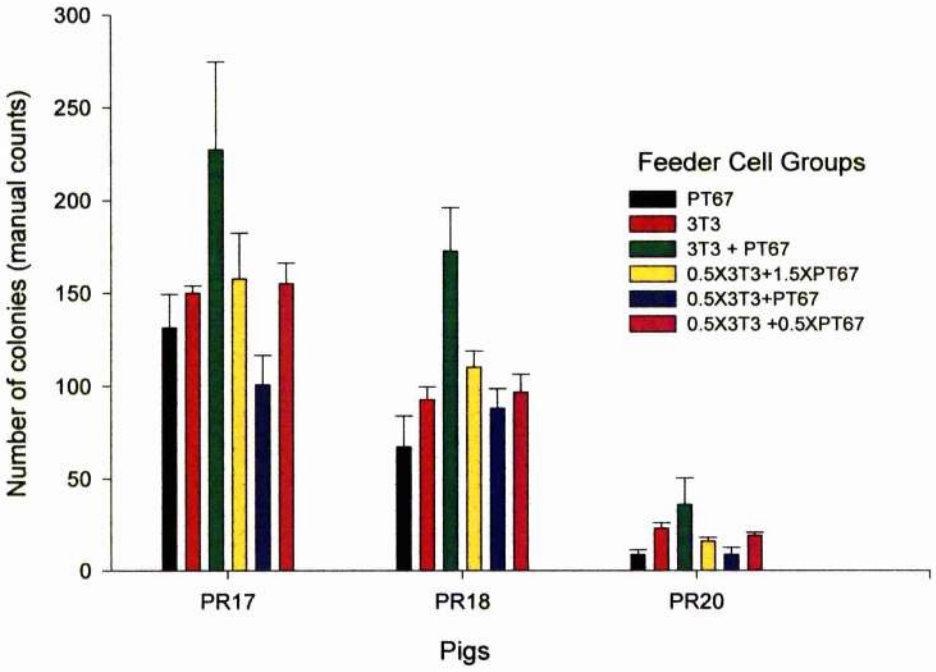
Figure 3.16: Feeder cell study, manual count of colony numbers

$1.1X10^2$ per cm^2 P3 keratinocytes (following complement lysis see Chapter 2 for method) from three different pigs plated in triplicate onto collagen-coated 6 well plates (well surface area = $9.4cm^2$) with any of 6 possible combinations of feeder cells: PT67 $1.4X10^4$ per cm^2 (PT67); 3T3 $1.4X10^4$ per cm^2 (3T3); 3T3 $1.4X10^4$ per cm^2 + PT67 $1.4X10^4$ per cm^2 (3T3 + PT67); 3T3 $6.9X10^3$ per cm^2 + PT67 $2.1X10^4$ per cm^2 (0.5X3T3 + 1.5XPT67); 3T3 $6.9X10^3$ per cm^2 + PT67 $1.4X10^4$ per cm^2 (0.5X3T3 + PT67); 3T3 $6.9X10^3$ per cm^2 + PT67 $6.9X10^3$ per cm^2 (0.5X3T3 + 0.5XPT67).
Cultures were assessed with rhodamine B and X-Gal staining after one week. Colonies were counted manually.
One way repeated measures analysis of variance of the absolute values showed that there were statistically significant differences ($P=0.005$) in the mean values among the feeder cell groups (b). To isolate the groups that differ an all pairwise multiple comparison procedure (Tukey test) was used. This showed that the following differences were ($P<0.05$) statistically significant. 59.7% fewer colonies in the PT67 group, 38.8% fewer colonies in the 3T3 group, 60.2% fewer colonies in the 0.5X3T3 + PT67 and 41.0% fewer colonies in the 0.5X3T3 + 0.5XPT67 group than in the 3T3 + PT67 feeder cell group.
One way analysis of variance and Tukey test of the relative values (calculated as relative to those obtained for the PT67 feeder cell group) was performed (a). This confirmed that the differences seen with one way repeated measures analysis of variance of the absolute values were significant ($P<0.05$). The error bars refer to standard deviations.

(a) Distribution of colony counts (performed manually) for each feeder cell group (n=3 pigs) shown relative to those obtained for the PT67 group



(b) Distribution of absolute colony numbers (manual count) for each feeder cell group (n=3 wells) shown for each pig.



Automated Count: The absolute values were analysed using a one way repeated measures analysis of variance. This demonstrated (Tukey test) a significant decrease of between 41 and 50% in colony numbers in the PT67 group, as compared to the 3T3 group, 3T3 + PT67 group and 0.53T3 + 1.5PT67 feeder cell group ($P<0.05$) (see Figure 3.17).

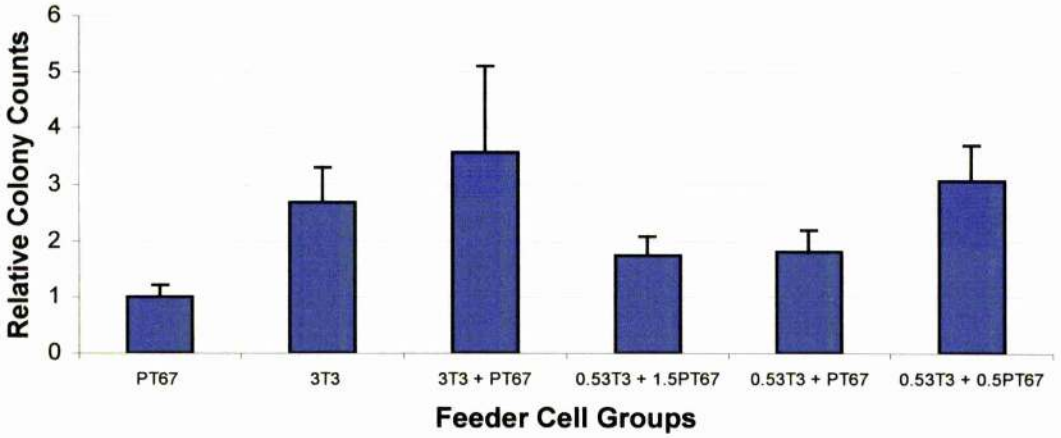
The colony counts (absolute values) observed manually tended to be higher than those observed by the image analyser (see Figures 3.16(b) and 3.17(b)). These differences may be explained in that with the naked eye it was possible to determine whether a single collection of cells was in fact several colonies that had become confluent. Attempting to manipulate the image analyser to do this was extremely difficult giving rise to inconsistent results and was therefore abandoned. Random wells were double checked to further ensure consistency. In spite of the differences seen in absolute values between the two methods of counting, the relative differences between groups appeared very similar (see Figure 3.18)

The results of counting the colonies tend to confirm the observed phenomenon that PT67 cells on their own are not as beneficial to keratinocytes in terms of colony forming efficiency as 3T3 cells on their own or when PT67 cells are combined with 3T3 cells.

Figure 3.17: Feeder cell study; Automated count of colony numbers

Following staining (see Figure 3.16 legend) wells were scanned and digitally analysed with Image Pro Plus® software with respect to numbers and size of colonies.
One way repeated measures analysis of variance of the absolute values showed that there were statistically significant differences (P=0.005) in the mean values among the feeder cell groups (b). To isolate the groups that differ an all pairwise multiple comparison procedure (Tukey test) was used. This showed that the following differences were statistically significant (P<0.05) with respect to colony numbers in the PT67 group in relation to the other groups. 50.4% fewer colonies than in the 3T3 + PT67 feeder cell group, 42.2% fewer colonies in the 3T3 group and 40.7% fewer colonies than in the 0.53T3 + 1.5PT67.
One way analysis of variance and Tukey test of the relative values (calculated as relative to those obtained for the PT67 feeder cell group) was performed (a). This confirmed that the observed reduction in numbers of colonies in the PT67 group (50.4% fewer) with respect to the number observed with the 3T3 + PT67 feeder cell was significant (P<0.05). The error *bars refer to standard deviations.*

(a) Distribution of colony counts (automated digital count) for each feeder cell group (n=3 pigs) shown relative to those obtained for the PT67 group



(b) Distribution of absolute colony numbers (automated count) for each feeder cell group (n=3 wells) shown for each pig.

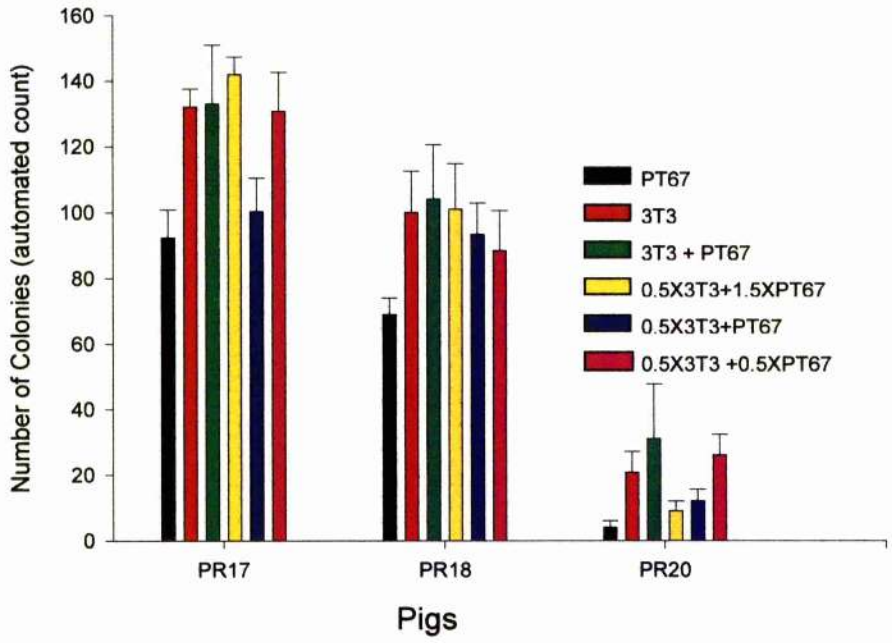


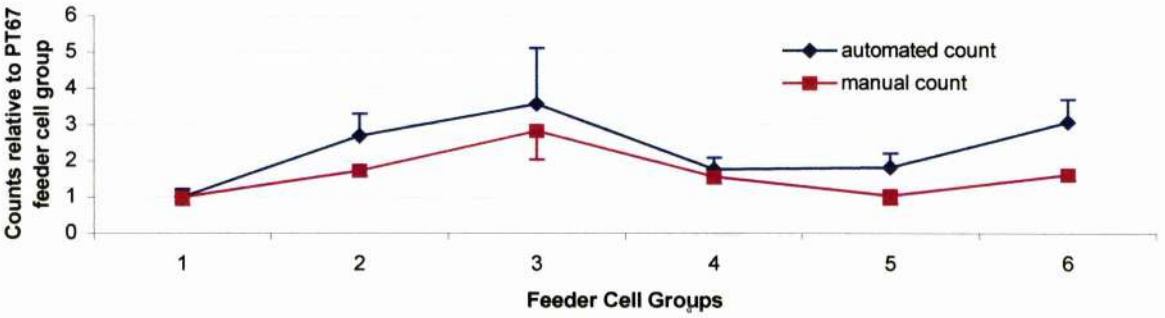
Figure 3.18: Comparison of automated and manual counts

The values for each feeder cell group have been averaged and made relative to those for the PT 67 group. The error bars refer to standard deviations.

The feeder cell groups denoted by the X-axis are:

1 = PT67; 2 = 3T3; 3 = 3T3 + PT67; 4 = 0.53T3 + 1.5PT67;

5 = 0.53T3 + PT67; 6 = 0.53T3 + 0.5PT67



3.2.9.2 Analysis of colony size

To further analyse the effects of different feeder cell regimens on keratinocyte colony forming efficiency, the different size of the colonies was measured by image analysis software (see Figures 3.19-3.22).

For both pigs nos. 17 and 18 there are significant differences in the mean values of colony areas (as measured by the image analyser) among the feeder cell groups. In both, the mean colony areas are significantly greater (Tukey Test $P<0.05$) with the 3T3, 3T3 + PT67, or with the 0.5 3T3 + 0.5 PT67 feeder cell combination than with the PT67 or the 0.5 3T3 + 1.5 PT67 feeder cell combinations. The pattern of differences suggests two things: first, that the addition of 3T3 cells may have a positive impact on the size of colonies formed; second, that the addition of PT67 cells may have a negative impact on the size of colonies formed perhaps favouring early clonal abortion. The Tukey test did not reveal significant differences amongst the groups for pig no. 20 (see Figures 3.23 and 3.24), but the less conservative Fisher LSD method revealed significantly greater mean colony areas ($P<0.05$) in the 3T3 + PT67 and the 0.5 3T3 + 0.5 PT67 feeder cell combination than the 0.5 3T3 + 0.5 PT67 feeder cell combination.

Figure 3.19: Frequency distribution of pig no. 17 keratinocyte colony surface areas per feeder cell type

The graph illustrates the frequency distribution of colony surface areas in each feeder cell group. 20 bins were created with the value of each bin rising incrementally from 100 to 2000 surface area units. The surface areas for each feeder cell group with values corresponding to particular bins were assigned to the corresponding bins. The number of surface area values within each bin were plotted against the bin designated value to give a frequency distribution histogram. The histogram illustrates the general trend of significantly greater(Tukey Test $P<0.05$) mean colony areas with the 3T3, 3T3 + PT67, or with the 0.5 3T3 + 0.5 PT67 feeder cell combination than with the PT67 or the 0.5 3T3 + 1.5 PT67 feeder cell combinations.

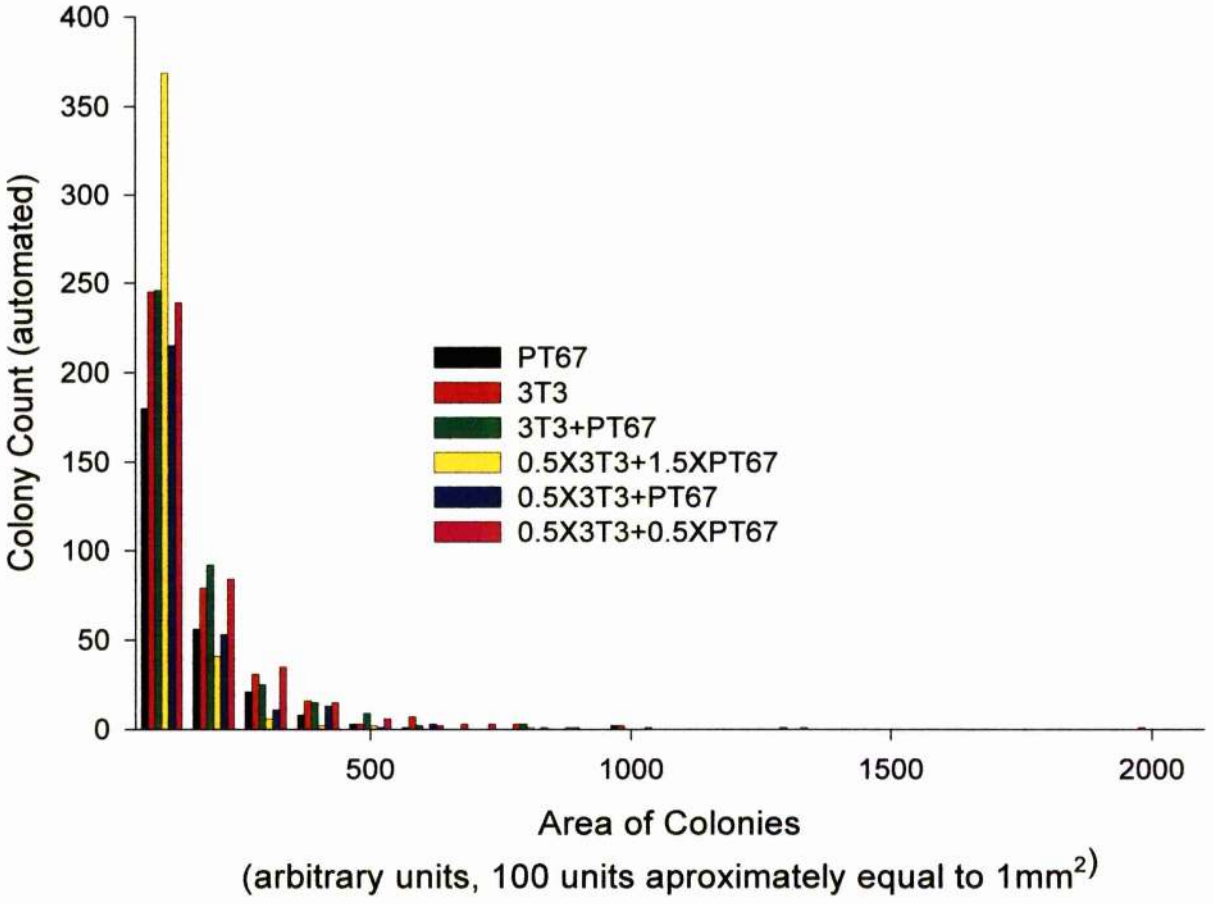


Figure 3.20: Distribution of pig no. 17 keratinocyte colony surface areas per feeder cell type

The graph illustrates the distribution of colony surface area for the largest 100 colonies from each feeder cell group. The smaller colonies have been eliminated from this graph in order to focus attention on the pattern of distribution of the larger colonies. The trend is suggestive of consistently more colonies with a larger surface area in the 3T3 and 3T3 + PT67 groups, (although the mean colony areas taken over the entire range are significantly greater(Tukey Test $P<0.05$) with the 3T3, 3T3 + PT67 and with the 0.5 3T3 + 0.5 PT67 feeder cell combination than with the PT67 or the 0.5 3T3 + 1.5 PT67 feeder cell combinations).

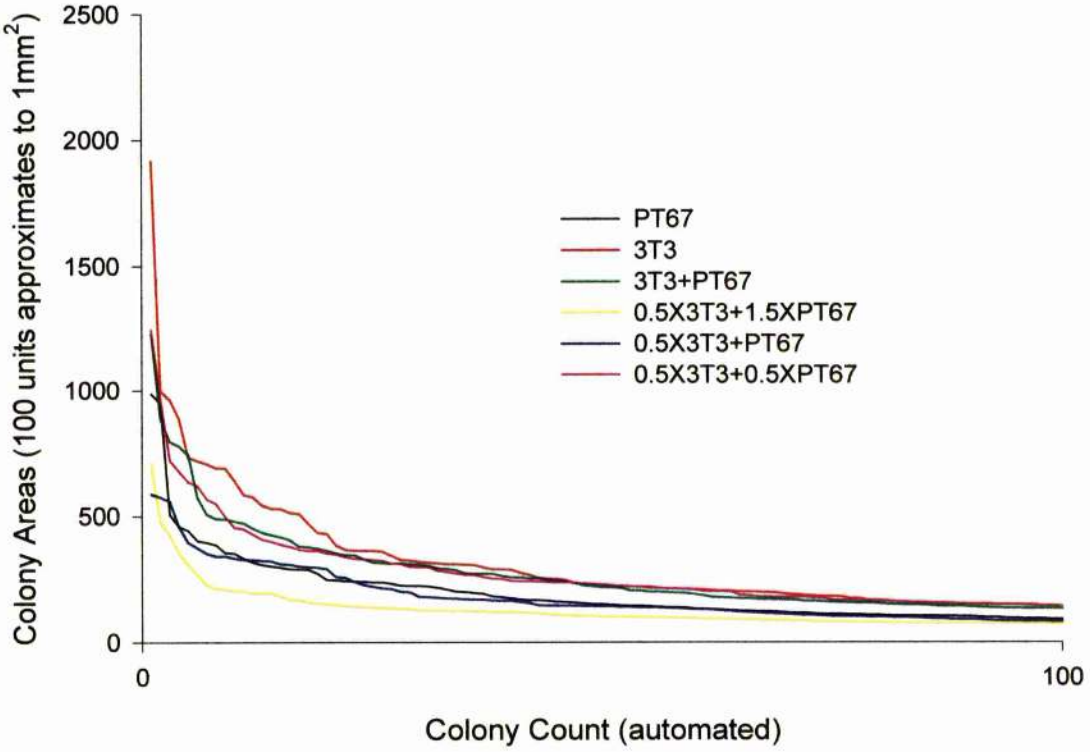


Figure 3.21: Pig no. 18 frequency distribution of colony surface area per feeder cell type

The graph illustrates the frequency distribution of colony surface areas in each feeder cell group. 20 bins were created with the value of each bin rising incrementally from 100 to 2000 surface area units. The surface areas for each feeder cell group with values corresponding to particular bins were assigned to the corresponding bins. The number of surface area values within each bin were plotted against the bin designated value to give a frequency distribution histogram. The histogram illustrates the general trend of significantly greater (Tukey Test $P<0.05$) mean colony areas with the 3T3, 3T3 + PT67, or with the 0.5 3T3 + 0.5 PT67 feeder cell combination than with the PT67 or the 0.5 3T3 + 1.5 PT67 feeder cell combinations.

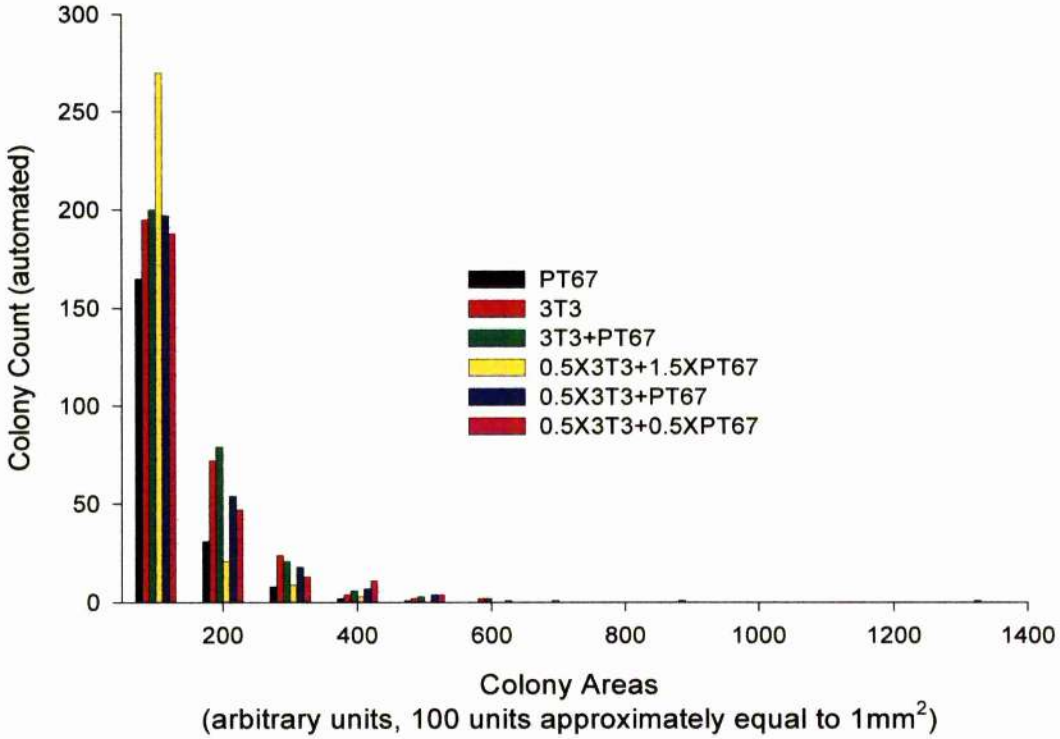


Figure 3.22: Distribution of pig No. 18 keratinocyte colony surface area per feeder cell type

The graph illustrates the distribution of colony surface area for the largest 100 colonies from each feeder cell group. The smaller colonies have been eliminated from this graph in order to focus attention on the pattern of distribution of the larger colonies. The trend is suggestive of consistently fewer colonies with a larger surface area in the PT67 or the 0.5 3T3 + 1.5 PT67 feeder cell combinations).

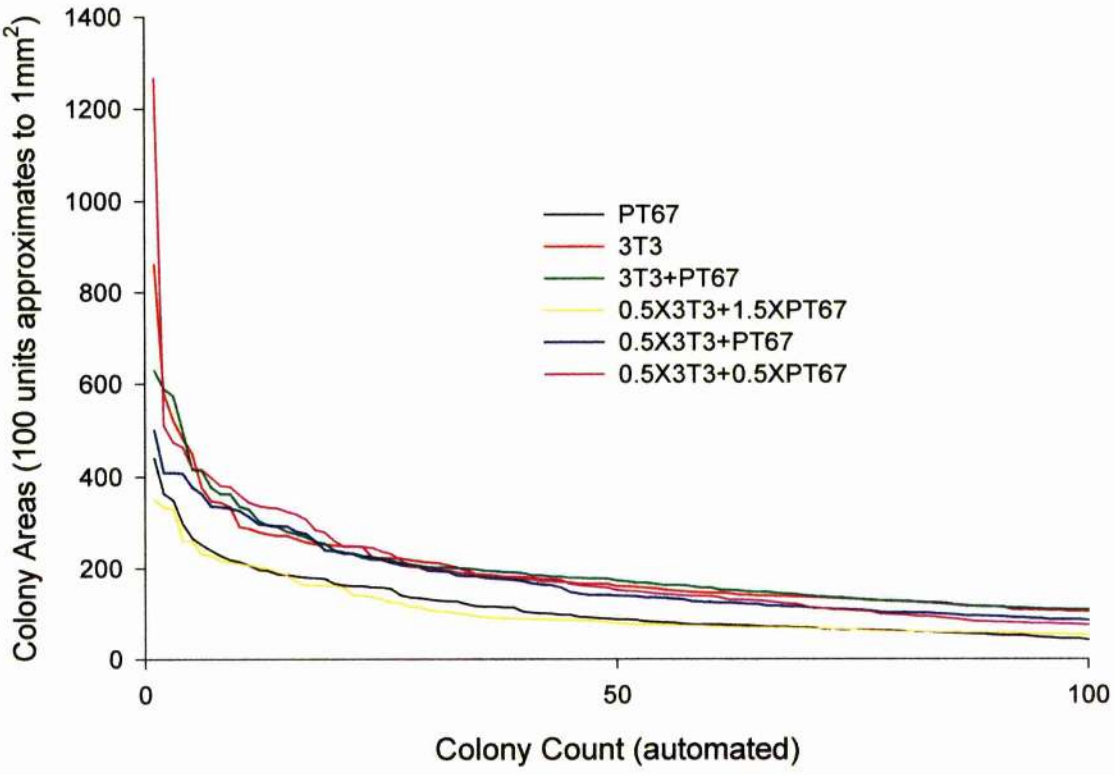


Figure 3.23: Frequency distribution of Pig No. 20 keratinocyte colony surface area per feeder cell type

The graph illustrates the frequency distribution of colony surface areas in each feeder cell group. 20 bins were created with the value of each bin rising incrementally from 100 to 2000 surface area units. The surface areas for each feeder cell group with values corresponding to particular bins were assigned to the corresponding bins. The number of surface area values within each bin were plotted against the bin designated value to give a frequency distribution histogram. The histogram illustrates the general trend of significantly greater (Tukey Test <0.05) mean colony areas with the 3T3, 3T3 + PT67, or with the 0.5 3T3 + 0.5 PT67 feeder cell combination than with the PT67 or the 0.5 3T3 + 1.5 PT67 feeder cell combinations.

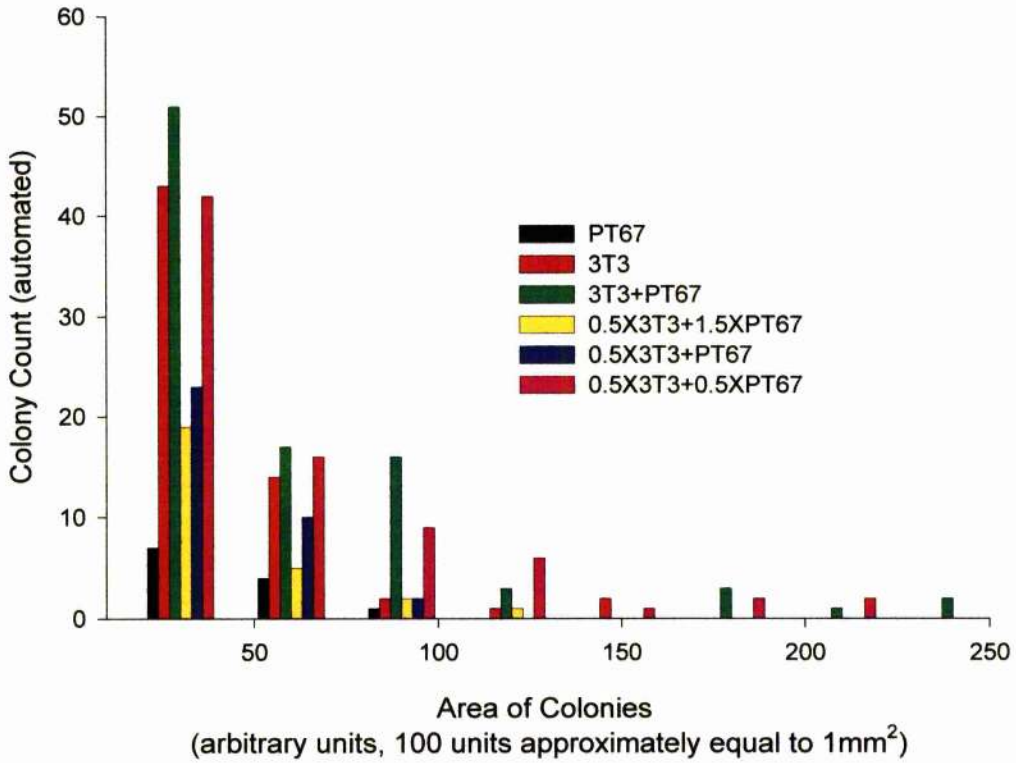
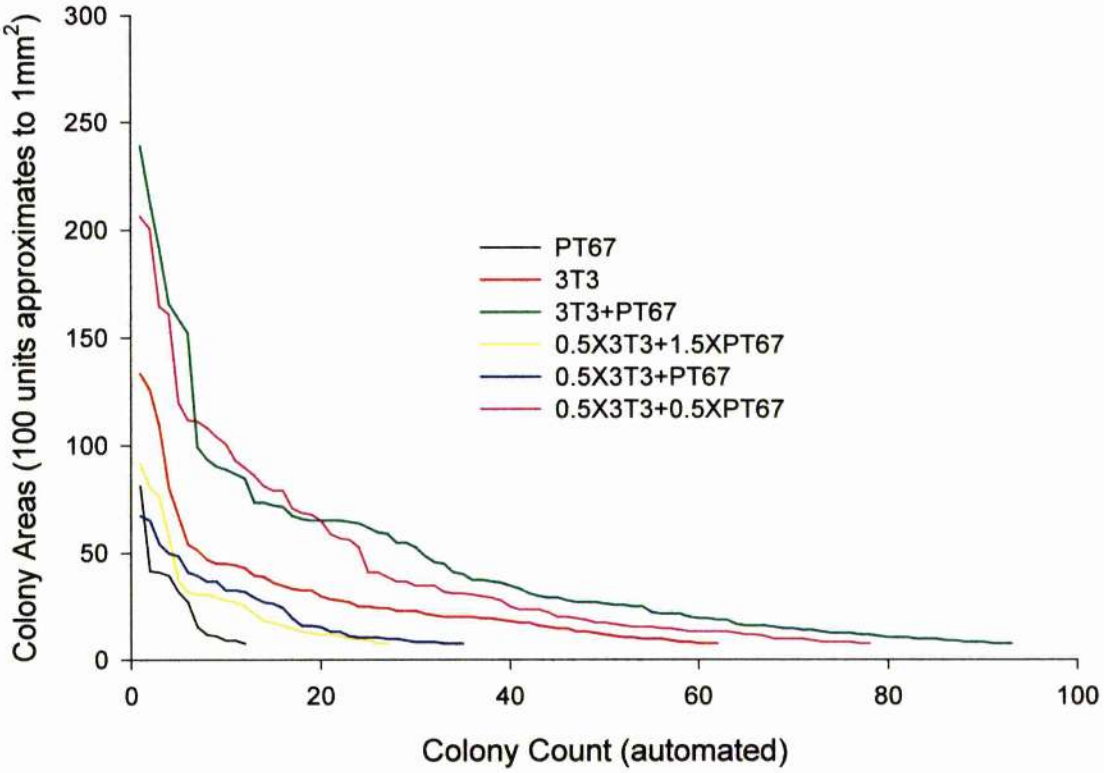


Figure 3.24: Distribution of pig no. 20 keratinocyte colony surface areas per feeder cell type

The graph illustrates the distribution of colony surface area for the largest 100 colonies from each feeder cell group. The smaller colonies have been eliminated from this graph in order to focus attention on the pattern of distribution of the larger colonies. The trend is suggestive of consistently fewer colonies with a larger surface area in the PT67, the 0.5 3T3 + 1.5 PT67 and 0.5 3T3 + PT67 feeder cell combinations.



3.2.10 *In vitro* stability of lacZnls transduced keratinocytes

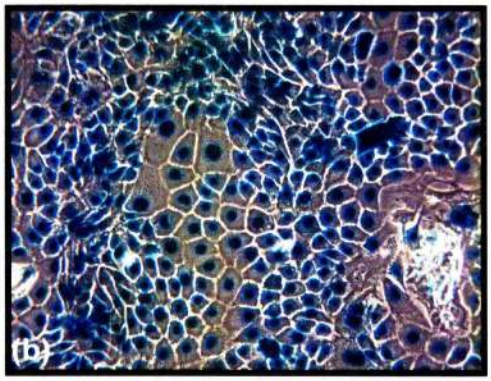
Various reports of retrovirus transduced genes in keratinocytes have indicated falling expression of β-galactosidase *in vivo*, with time (Fenjves et al., 1996; Vogt et al., 1994) It was pertinent therefore, having achieved close to 100% β-galactosidase expression (see Figure 3.12), to assess its stability *in vitro*.

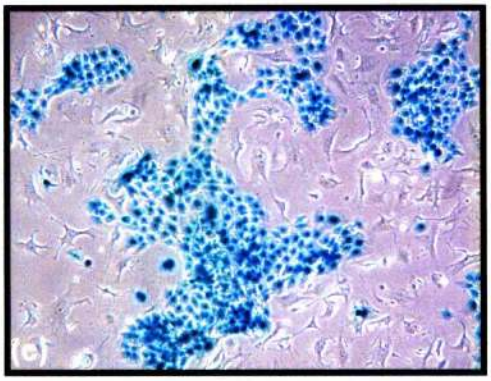
Po keratinocytes from 3 separate pigs were passaged three times on irradiated PT67 clone 8 cells and these underwent 7 further passages on irradiated 3T3 cells and were stained with X-Gal every 2nd passage.

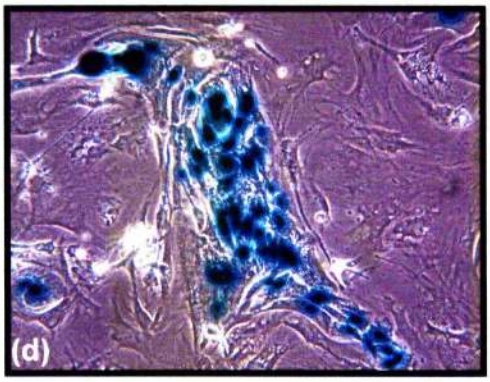
Staining after the first three passages on 3T3 cells showed 100% keratinocyte transduction. After the fourth passage on 3T3 cells the growth rate of the keratinocytes dropped dramatically and marked clonal abortion was seen. However the β-galactosidase expression continued to approximate 100% (see Figure 3.25).

Figure 3.25: X-gal stained porcine keratinocytes illustrating stability of MFGlacZ expression

This study assessed the in-vitro stability of MFGlacZ expression within transduced Large White pig keratinocytes. The keratinocytes were passaged 3 times (from P0) on PT67 producer cells then onto irradiated 3T3 cells. The photomicrographs (after 1 passage on 3T3 cells (a) X100 and (b) X200; after 3 passages on 3T3 cells (c) X100; after 7 passages on 3T3 cells (d) X200) illustrate close to 100% transduction even after 7 passages on 3T3 cells. A diminution in the size of colonies and capacity for replication is evident in successive passages.







3.2.11 Change of transduced keratinocyte lacZ expression with confluence

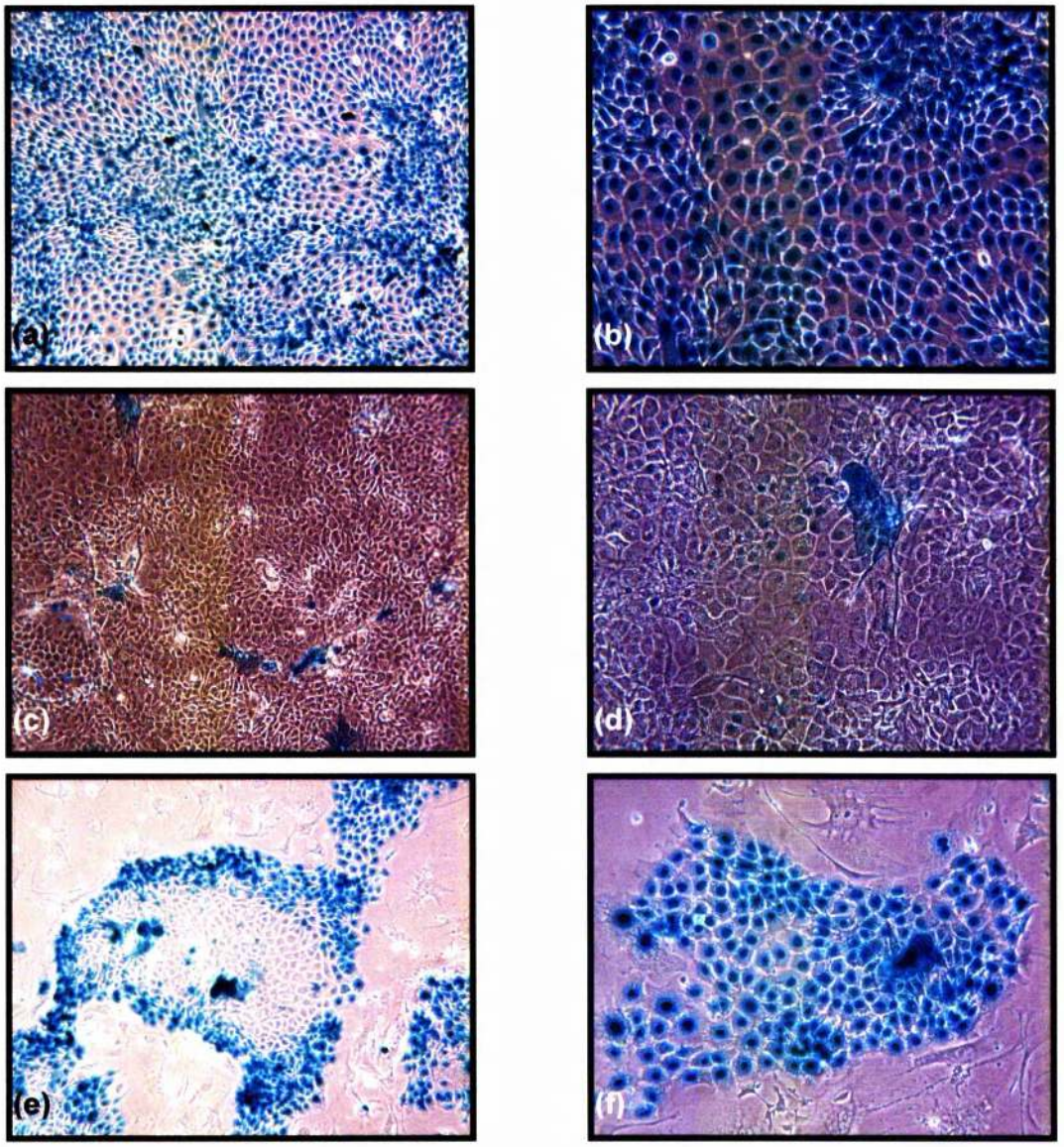
One method of grafting cultured keratinocytes is to transfer them having grown them up as sheets. An experiment was therefore devised to assess *in vitro*, in terms of continued MFGlacZ expression, the consequences of replication of labelled cells having first allowed them to form sheets.

Po keratinocytes from 3 separate pigs were passaged three times on irradiated PT67 clone 8 cells. Each flask was then divided 1:3 and underwent a further passage on irradiated 3T3 cells. After 2 days one flask was stained and the other 2 were left to reach confluence. At this time one of the remaining flasks was stained with X-Gal and the remaining was split 1:3 and plated on irradiated 3T3 cells. One of these flasks was then stained with X-Gal 2 days later.

Pre confluenT-cells showed 100% expression of the lacZ gene (see Figure 3.26(a) and (b)). As they became confluent the number of cells expressing β-galactosidase diminished (see Figure 3.26(c) and (d)). When confluent sheets were passaged onto irradiated 3T3 cells β-galactosidase expression returned, but expression tended to predominate in the cells at the periphery of the colony at the interface between keratinocytes and irradiated 3T3 (feeder) cells (see Figure 3.26(e) and (f)).

Figure 3.26: X-gal Stained Porcine Keratinocytes Illustrating the Nature of MFGlacZ Expression in Relation to Confluence

Keratinocytes from three pigs were passaged 3 times (from P0) on PT67 producer cells then onto irradiated 3T3 cells in triplicate. 1 set of flasks were stained when they were subconfluent, two parallel set of flasks were left to reach confluence and 1 further set was then stained. The remaining set was passaged onto 3T3 cells and stained with X-gal after 2 days. Photomicrographs ((a), (c) and (e) at X100 and (b), (d) and (f) at X200 magnification), illustrate level of expression in relation to confluence Sub confluent cells showed 100% expression of the MFGlacZ nls gene (a) and (b). As they became confluent the number of cells expressing β-galactosidase diminished (c) and (d). When confluent sheets were passaged onto irradiated 3T3 cells β-galactosidase expression returned (e), but tended to predominate in the cells at the periphery of the colony at the interface between keratinocytes and irradiated 3T3 (feeder) cells (f).



3.3 Discussion

One of the aims of this part of the study was to achieve high levels of stable transduction. This appears to have been successful *in vitro*. Following three passages on PT67 feeder cells, close to 100% of keratinocytes expressed β-galactosidase. This level of expression persisted over a series of 7 further passages. Other researchers have achieved high *in vitro* transduction rates, but these have not always been borne out following grafting (Fenjves et al., 1996). To achieve long term transduction it would be necessary to transduce the stem cells and not just the transient amplifying (TA) cells (Kolodka et al., 1998; De and Pellegrini, 1997). Stem cells are postulated to be slow cycling cells that give rise to the epidermal keratinocyte population (De Luca and Pellegrini, 1997). Using clonal analysis, three types of keratinocytes have been identified that have different capacities for multiplication. These are holoclones, meroclones and paraclones (Barrandon and Green, 1987). Holoclones have the greatest capacity for multiplication. A single holoclone can generate more than $1X10^{40}$ progeny. The holoclone in culture behaves characteristically like a stem cell. The paraclone is generated by a transient amplifying cell and is limited to no more than 15 divisions. Subcultivated paraclones do not generate colonies indicating their terminality (Barrandon and Green, 1987). The meroclone has an intermediate capacity for replication and conforms to the generally conceived notion of transient amplifying cell (Barrandon and Green, 1987). Viral integration is dependant on cell division (Miller, 1990). It is thus far easier to achieve stable transduction in rapidly dividing cells than it is in slowly cycling cells. Perceived wisdom is that the keratinocyte stem cell population is characterised by its relative quiescence

and the greatest proliferative capacity, compared with the TA cells characterised by reduced proliferative potential and more rapid terminal differentiation in culture (Barrandon and Green, 1987). This may not be true in our culture conditions where unlike with Green's medium, the culture conditions for porcine keratinocytes, deliberately maintain them in an immature, non differentiating state (Bevan et al., 1997). This may favour continued replication by the meroclonal TA cells rather than terminal differentiation. It is possible that the holoclonal stem cells may remain quiescent in these conditions. The stem cells represent a very small percentage of the total keratinocyte population. Stem cells may escape transduction and not be detected even in situations where apparently high percentage transduction rates prevail. Moreover, the cultured keratinocyte population may appear to be stably transduced over ensuing cell cycles and multiple passages *in vitro* because the meroclonal transiently dividing cells have been successfully transduced. Following grafting, marker gene expression may be far shorter lived owing to terminal differentiation ('turning paraclonal') of the transduced meroclones with reactivation of the non-transduced stem cells.

Loss of expression *in vivo* (following *in vitro* transduction) has been noted in a variety of other cell types. These include fibroblasts (Scharfmann et al., 1991), and myoblasts (Dai et al., 1992). Precise mechanisms are still being characterised but selective inactivation of viral promoters including by methylation of specific bases in the promoter has been proposed (Challita and Kohn, 1994).

As well as achieving stable long term keratinocyte transduction, it is important that the clonal potential of these cells are not compromised by highly efficient

multiple retroviral transductions. We found that the incorporation of 3T3 cells into the feeder cells is necessary to avoid the appearance of small irregular colonies which would suggest premature senescence. Others using a similar system have been able to demonstrate a healthy 150 cell divisions of β-gal transduced keratinocytes before losing proliferative ability (Mathor et al., 1996).

Having optimised keratinocyte lacZ transduction, exciting possibilities are opened up with respect to tracking the fate of grafted cultured allogeneic keratinocytes.

Chapter 4

Evaluation of antibody complement pre-treatment as a method of Langerhans cell elimination in Large White pig keratinocyte cultures

4.1 Introduction

Skin allotransplantation, since the days of Medawar (Gibson and Medawar, 1943; Medawar, 1944; Medawar, 1945) has provided a powerful paradigm for acute organ allograft rejection. Over the years however, it has become apparent that skin allografts may behave differently when compared to organ grafts. Rejection of skin grafts can occur across minor histocompatibility barriers that do not result in organ graft rejection (Mahabir et al., 1969; White et al., 1969; Fabre and Morris, 1975). Immunosupression preventing graft rejection is much more difficult to achieve with skin grafts (Fabre and Morris, 1975). Long surviving recipients of cardiac or renal transplants reject donor skin in a normal (Jenkins and Woodruff, 1971; Kawabe et al., 1972), or only slightly delayed fashion (Marquet et al., 1971; Stuart et al., 1970; Fabre and Morris, 1972; Mullen et al., 1973). Cultured keratinocytes that were grafted beneath the renal capsule in two rat strains, responded to cyclosporin in a manner typical of skin grafts rather than renal grafts (Fabre and Cullen, 1989). In spite of the eccentric behaviour of skin grafts there are major similarities to the model of acute organ rejection that remain valid. Initiation of the effector mechanism of graft rejection, involves complex interactions between naive T helper cells (Th cells) and APCs expressing the appropriate antigenic ligand and providing requisite costimulatory signals (Singer et al., 1987). Lymphoid organs provide anatomical structures in which critical intercellular interactions are properly guided and where appropriate cells are likely to make contact sufficiently frequently and in a critical milieu (Zinkernagel et al., 1997). Central sensitisation occurs when APCs migrate out of the 'peripheral' graft to draining nodes where an immune response is generated. The evidence for

this has accumulated since the skin flap experiments of the 1950s (Frey and Wenk, 1957; Barker and Billingham, 1967; Tilney and Gowans, 1970). In these, the necessity for intact afferent draining lymph vessels and nodes for skin graft rejection was demonstrated. Further evidence came from studies in which APCs had been depleted prior to grafting (Lafferty et al., 1976; Talmage et al., 1976; Lafferty and Woolnough, 1977; Faustman et al., 1984). In both thyroid and pancreatic grafts survival was demonstrated until the reintroduction of donor APCs (Talmage et al., 1976; Lafferty and Woolnough, 1977; Faustman et al., 1984).

Over the years, strong evidence has accumulated concerning the role played by epidermal dendritic cells (LC), in an antigen presenting capacity. Phenotypically, the mature LC are stellate with many fine dendritic processes that extend in all directions from its cell body which appear highly motile (Winzler et al, 1997). This shape and motility suggest a specialised central antigen presenting role. Resting epidermal LC are less dendritic in appearance and are functionally better suited to antigen capture and processing. They are weak T - cell stimulators and have fewer MHC and accessory molecules but many more antigen-capturing Fcγ and Fcε receptors (Banchereau and Steinman, 1998). These resting LC can be mobilised by the stimulus of transplantation (both *in vivo* and as an explant). The evidence suggests that maturation is associated with a marked increase of MHC II gene and B7 molecule expression (Larsen et al., 1990b; Larsen et al., 1994). Following migration to draining nodes, maturation is completed and the mature LC becomes specialised in clustering and activating T-cells (Romani and Schuler, 1992; Knight and Stagg, 1993). In classical skin allograft rejection the assertion that the donor (rather than recipient) LC are

responsible for antigen presentation is synonymous with the 'passenger leucocyte' phenomenon described by Snell (Snell, 1957). This assertion was lent credence by the skin flap and alymphatic pedicle experiments of the 1950s and 60s (Frey and Wenk, 1957; Barker and Billingham, 1967; Tilney and Gowans, 1970). More recently, Larsen and coworkers demonstrated that within the classical skin and cardiac allorejection scenario, no host dendritic cells were found to migrate into the graft (Larsen et al., 1990a).

The centrality of donor APCs to the picture of classical acute allograft rejection led many to experiment with dendritic cell depleted allografts. These showed a survival advantage following APC cell depletion and included thyroid epithelial cell and pancreatic isleT-cell grafts (Lafferty et al., 1976; Talmage et al., 1976; Lafferty and Woolnough, 1977; Faustman et al., 1984).

The picture with regard to APC-depleted cultured keratinocyte grafts is more mixed. In the 1980s, studies involving cultured epidermal cells demonstrated, that increasing time spent in culture, resulted in diminishing MHC II detection within the cultured population. Additionally, there was decreased ability of this population to activate T-cells within an MELR (mixed epidermal cell lymphocyte response) test as well the inability to detect typical LC with electron microscopy (Morhenn et al., 1982; Hefton et al., 1984; Demidem et al., 1986; Thivolet et al., 1986).

The sum of these studies was taken by many as proof that LC do not survive keratinocyte culture. The results however were mixed with one study showing persistence of 30% of original level of HLA DR expression remaining after three weeks in culture (Demidem et al., 1986). The LC phenotype following culture is known to alter quite profoundly (Larsen et al., 1990b; Teunissen et al., 1990; Witmer-Pack et al., 1988; Banchereau and Steinman, 1998). The culture

conditions used in our laboratory for porcine keratinocyte is designed to maintain keratinocytes in a relatively dedifferentiated state and encourage proliferation (Bevan et al., 1997). This raises the question as to whether in this medium, LC may also revert to an immature state, resulting in its reduced detection and loss of ability to stimulate the proliferation of allogeneic T-cells.

The clinical picture is mixed too. Early workers claimed success with prolonged survival of cultured allogeneic keratinocyte grafts with no overt evidence of rejection (Hefton et al., 1983; Thivolet et al., 1986; Madden et al., 1986; Gielen et al., 1987). Subsequently overt rejection of cultured allogeneic keratinocyte grafts at just over 2 weeks was demonstrated (Aubock et al., 1988). Studies using DNA fingerprinting to identify donor keratinocytes failed after 21 days to detect any donor cells (van der Merwe et al., 1990). Studies using sex mismatched donor-recipient groups failed to detect donor cells, using in situ DNA hybridisation techniques, after 1 week (Brain et al., 1989; Burt et al., 1989). In the Large White pig initial reports were of acute rejection, as with Aubock's study only slightly delayed, occurring at 14 days (Carver et al., 1991). Subsequent studies have demonstrated survival of porcine allogeneic keratinocytes at 14 days with no evidence of rejection, using marker genes to identify donor and recipienT-cells. At 21 days, both autologous and allogeneic labelled cells were hard to find (Ng et al., 1996b). One difference between the two studies may lie in method of keratinocyte culture and frequency of passage, which may affect the probability of survival and or the phenotype of surviving LC within the culture.

From the above it would appear that the key to delayed rejection of cultured allogeneic keratinocytes may lie in the complete elimination of the LC

population. It has been argued that culture and repeated passage may eliminate a substantial proportion of LC (see above). Culture of LC with porcine keratinocytes however, may merely alter LC phenotype with detection made more difficult, although there is as yet no direct evidence to support this view. Yet even if LC were greatly reduced in number, they may still theoretically pose a significant survival threat to the graft. This study has looked at whether active methods of elimination may be used to increase the probability that a cultured graft is rendered APC cell free.

Active elimination of LC may be achieved in a variety of ways. Ultraviolet B radiation and glucocorticoid steroids have been shown to reduce the numbers of LC detected within cadaver split skin (Alsbjorn, 1983; Alsbjorn and Sorensen, 1985). More recent evidence suggests that cryopreserved skin loses its LC population (Abe et al., 1995). Other techniques include the use of immunomagnetic bead separation (magnetic beads coated with antibody specific for certain LC determinants) (Nakano, 1998); flow sorting using electrostatic droplet deflection (Carter, 1996); and by antibody and complement (antiserum) pre-treatment (Nakano, 1998). In the antibody and complement pre-treatment method, antibody specific to LCs are added to a mixed epidermal cell suspension. Following a period of incubation during which time antibody will bind to specific antigen, complement is added. Complexing of antibody with antigen induces conformational change in the Fc portion of the antibody molecule that exposes a binding site for the C1q molecule within the macromolecular C1 component of the complement system. Activation of the complement cascade via the classical activation pathway is achieved following the binding of two such Fc sites by the C1q molecule. The membrane attack complex formed following complement

activation results in cells lysis (Kuby, 1997), through disruption of cell membrane integrity such that osmotic and chemical equilibrium cannot be maintained (Frank, 1991).

Successful transplantation following antiserum pre-treatment to deplete passenger lymphocyte populations, without the need for immunosupression, has been reported with a variety of tissues including pancreatic isleT-cell (Faustman et al., 1981; Faustman et al., 1982a; Faustman et al., 1982c; Faustman et al., 1982c; Faustman et al., 1984)., heart (Faustman et al., 1982b; Sone et al., 1987) and kidney (Brewer et al., 1989a; Brewer et al., 1989b).

The use of antibody and complement to deplete Large White pig epidermal cell populations of LC has not previously been evaluated. The process of antibody complement pre-treatment may adversely affect keratinocytes, which appear to be sensitive to culture conditions. Criteria to be evaluated therefore include the efficacy of this method and its effect on Large White pig keratinocytes. Media used for keratinocyte culture (Green et al., 1979; Bevan et al., 1997) have been optimised to minimise clonal abortion and promote maximal growth and proliferation. Hence there is a need to examine the effect of antiserum pre-treatment on size and numbers of colonies produced.

Various techniques are available for detecting the presence of LC. Flow cytometry (or fluorescence activated cell sorting, FACS) involves the detection of specific antigens using labelled antibodies. It conveniently enables semi quantitative (proportional) analysis to be performed.

AIMS:

- Define an antibody-complement pre-treatment method for eliminating the majority of LC from disaggregated Large White pig epidermal cells
- Determine the effect of antibody-complement pre-treatment on colony forming efficiency of Large White pig keratinocytes.

4.2 Results

4.2.1 MHC II expression in freshly trypsinised epidermal cells

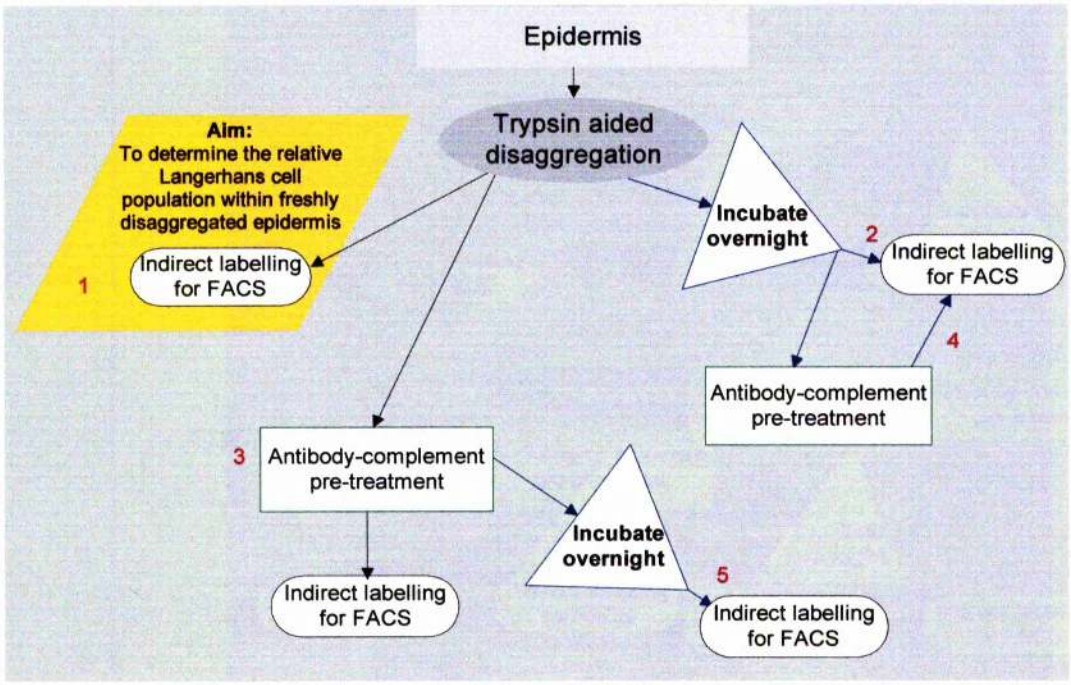
The aim of this study was to determine the percentage of LC found in freshly trypsinised epidermal cells relative to the remaining epidermal cells.

For this study, cell suspensions were prepared from freshly trypsinised epidermal cells from n=4 pigs (see Chapter 2 for method). These were labelled in duplicate with either test antibody (MSA3 specific for SLA DR) or isotype control (IgG2a) at a concentration of 1/100 (see Chapter 2 for method).

The results (see Figure 4.1) indicate there is a mean difference in cell labelling of 0.732 between test and control (t-test, P=0.042). Other studies contain estimates of LC within the epidermis of between 1 - 4% (Hefton et al., 1984; Demidem et al., 1986). The observed difference may relate to our method of preparing epidermal cell suspensions as well as FACS.

In this study and in all subsequent experiments, peripheral blood lymphocytes extracted from peripheral blood (see Chapter 2 for method) labelled in an identical fashion to epidermal cells functioned as a positive control (see Figures 4.2 and 4.3).

Figure 4.1: MHC II expression in freshly trypsinised epidermal cells



Flow cytometry results following labelling of freshly trypsinised epidermal cells with either test antibody (MSA3 specific for SL:A DR) or isotype control (IgG2a) at a concentration of 1/100. Cells have been gated to exclude debris and clumps as well to exclude dim or non fluorescing cells (see Chapter 2 for method for method). All test and control cells have been gated identically. Four pigs (n=4) were used in this study represented by the blue dots, the black dots the mean and the vertical lines the standard deviation. The blue lines connect the test and isotype control results for each pig. There is a mean difference of 0.732 between test and control (t-test, P=0.042).

Figure 4.1: MHC II expression in freshly trypsinised epidermal cells *(continued)*

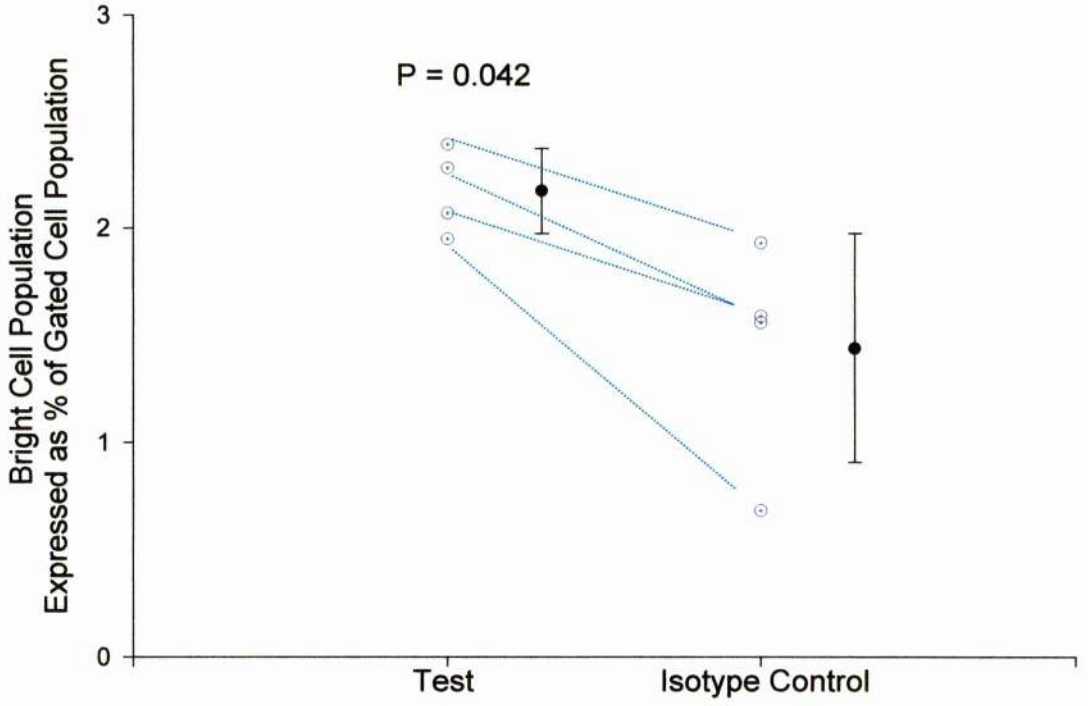


Figure 4.2: MHC II expression in porcine peripheral blood lymphocytes

Assessment of SLA DR expression in peripheral blood lymphocytes (PBLs) in n=3 pigs. MSA 3 was the antibody used and IgG2a the isotype control. Peripheral blood lymphocytes were prepared with density gradient separation and then used as positive controls in experiments to assess porcine epidermal cell or cultured keratinocyte SLA DR expression. The PBLs from the three pigs were used as controls in three separate studies and were not therefore processed in parallel. They were however, all processed, gated and analysed in exactly the same way.

The mean difference between the groups is 14.7 (P=0.03). The vertical lines indicate the standard deviation.

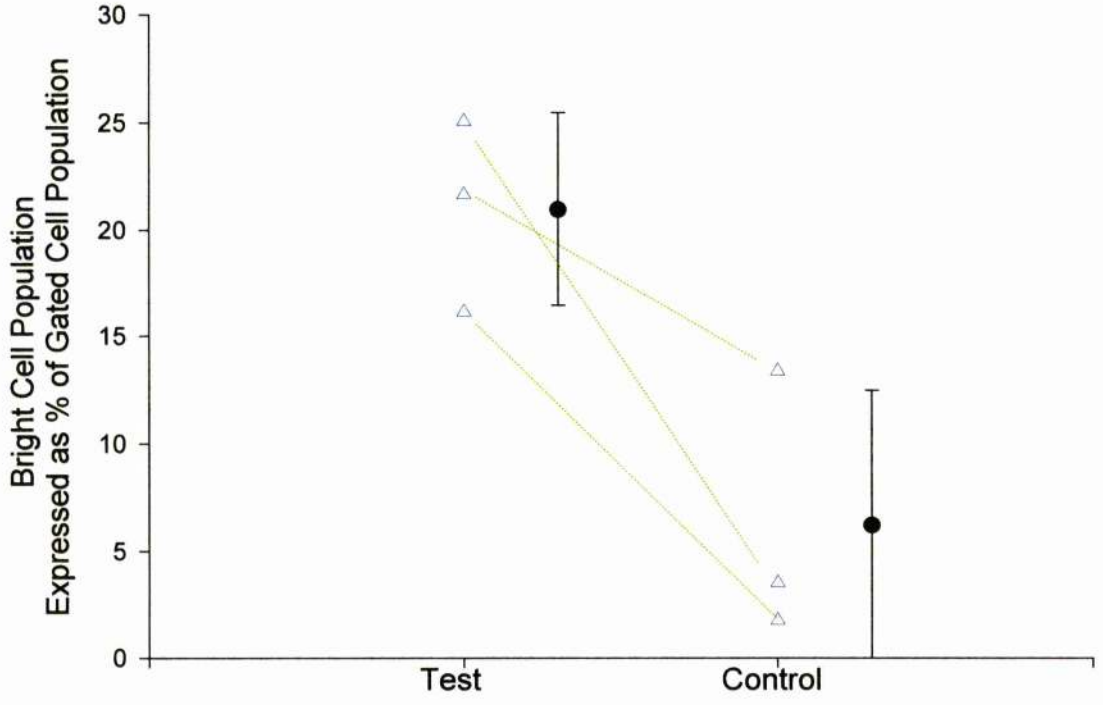
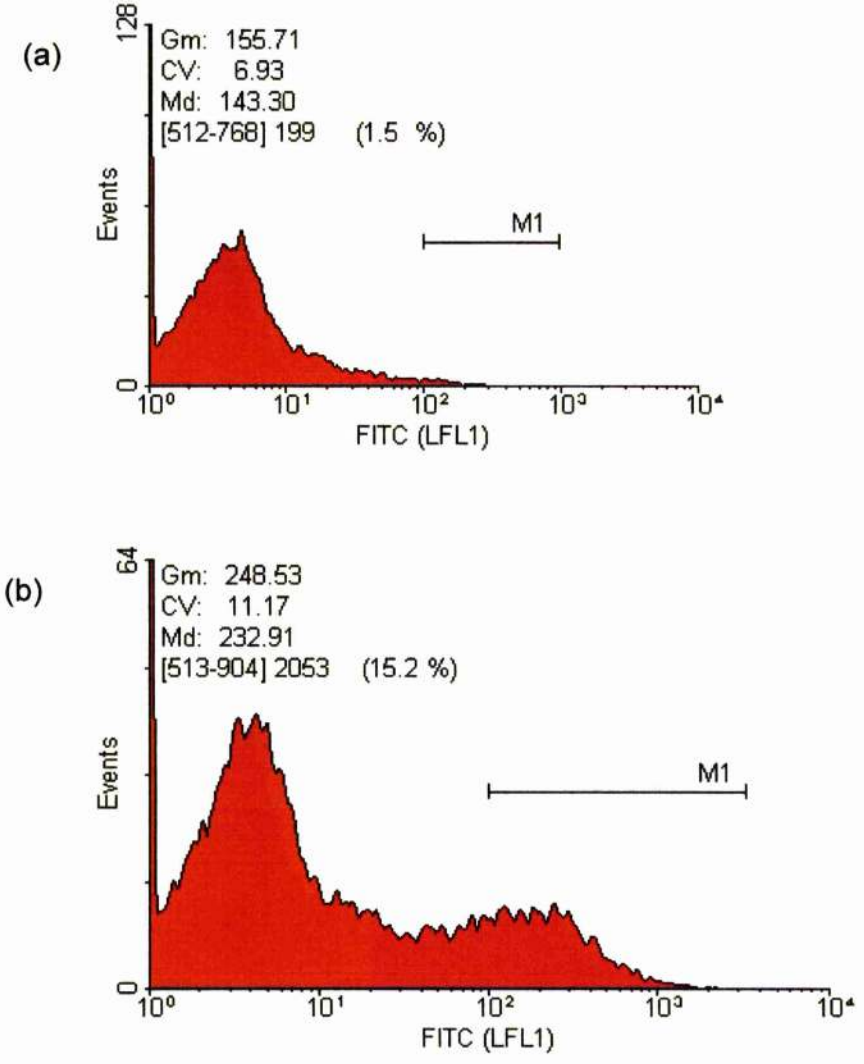


Figure 4.3: Histogram representation of SLA DR expression in peripheral blood lymphocytes in the Large White pig

Flow cytometric histograms of MSA3-FITC labelling (b) and IgG2a (a) FITC labelling (isotype control) of peripheral blood lymphocytes. One population was primarily labelled with the isotype control (IgG2a) and the other with anti-SLA DR (MSA3) (IgG2a). Secondary labelling was achieved with FITC (sheep anti-mouse IgG). The histogram illustrates a population of lymphocytes with 13.7% SLA DR expression. Note the M1 marker delineating the population of brighter (higher Geometric mean fluorescence value) SLA DR labelled cells.



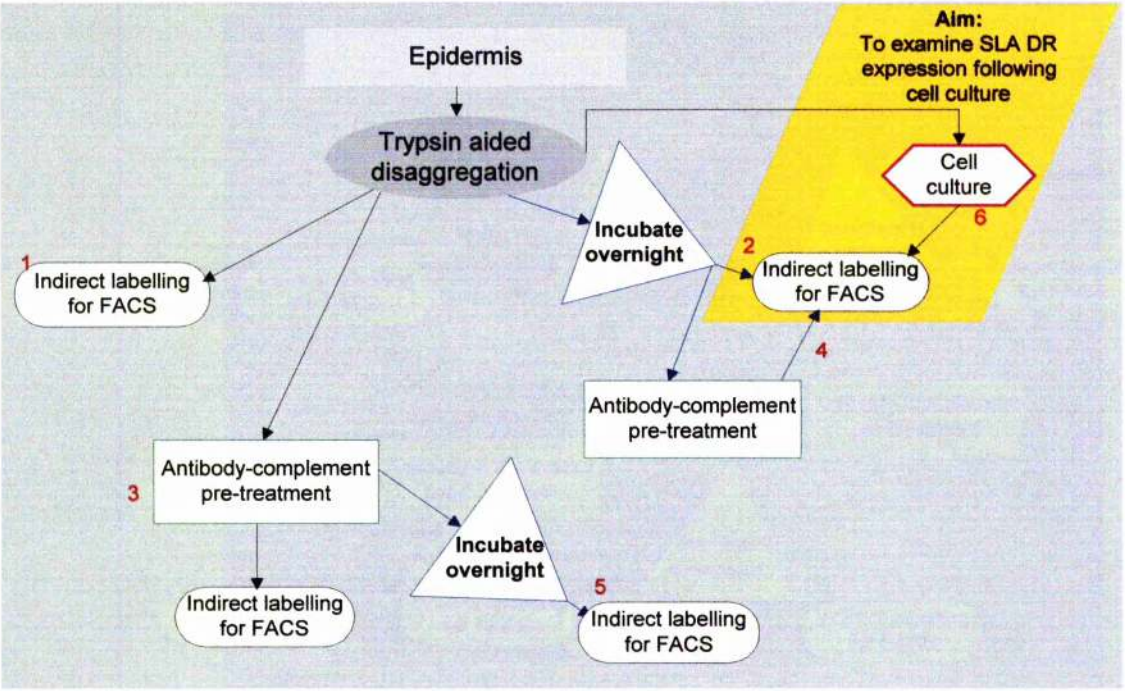
4.2.2 MHC II expression in P1 cultured epidermal cells

Previous studies have suggested that the LC population appears to diminish with each successive passage such that they are thought not to pose a threat with regard to allograft take (Demidem et al., 1986; Hefton et al., 1984; Morhenn et al., 1982; Thivolet et al., 1986).

Even very low numbers of LC may however pose a threat to allograft survival. The aim of this study was therefore to determine, using FACS, the presence of LC in mixed epidermal Large White pig cell culture.

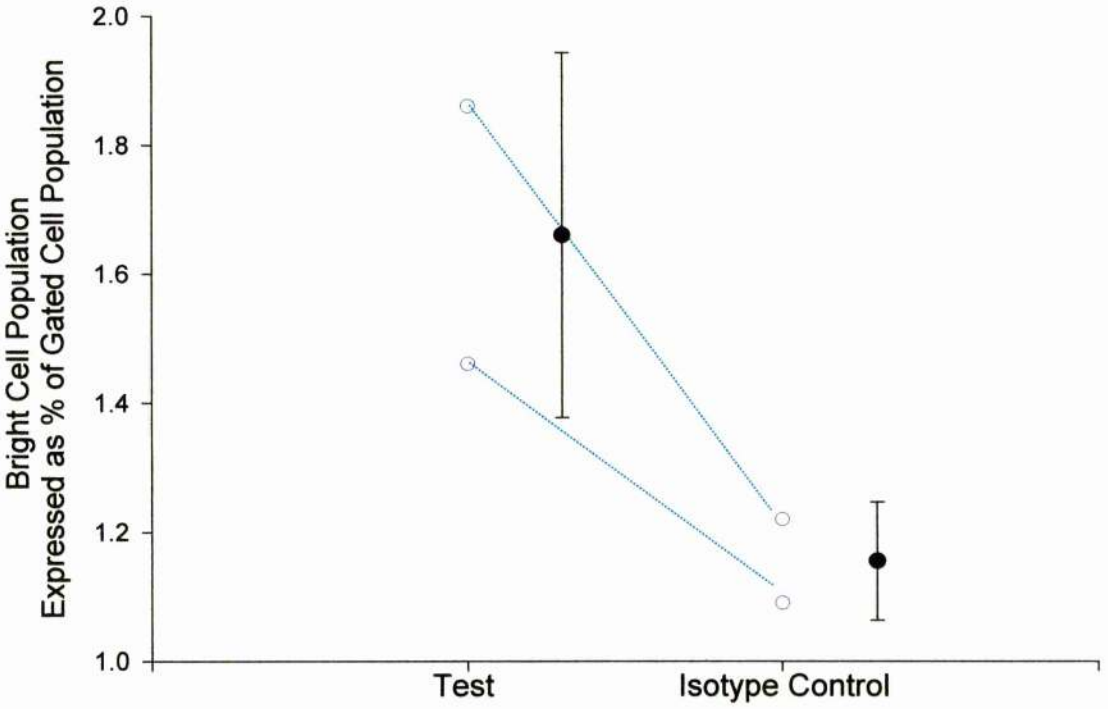
The results show a 0.5% difference between test and control results following the first passage (see Figure 4.4). The figures although not statistically significant are similar to those seen with non incubated P0 epidermal cells (see Figure 4.1) which raises the possibility that LC may indeed have survived for over a week in culture with keratinocytes. Although repeated passage is thought by most to passively eliminate LC (Demidem et al., 1986; Hefton et al., 1984; Morhenn et al., 1982; Thivolet et al., 1986) there remains a small risk of some surviving (Demidem et al., 1986) to act as passenger lymphocytes initiating rejection in subsequent allografting. Additionally poor detection rates of LCs might be accounted for through cell phenotype alteration in the keratinocyte culture medium with marked downgrading of SLA DR expression. Indeed following further passages I failed to detect LCs. This may reflect how difficult it is to determine very low percentages given that there is a degree of non-specific binding.

Figure 4.4: SLA DR expression in P1 cultured epidermal cells



The point plot illustrates flow cytometry results following labelling of epidermal cells with test antibody (MSA3 SLA DR-specificity) or isotype control (IgG2a). The antibody concentration was 1/100. The cells which had not previously undergone antibody complement pre-treatment, had been passaged once (P1). Cultures from n=2 pigs (represented by blue dots) were used in this study. Cells were processed simultaneously and identically for FACS and subsequently analysed in a similar fashion to the freshly trypsinised cell study (section 4.1). Mean values are represented by the black dots and the vertical lines indicate the standard deviation. The blue lines connect the test and isotype control results for each pig. The observed difference between test and control was not significant (t-test and Mann-Whitney Rank Sum).

Figure 4.4: SLA DR expression in P1 cultured epidermal cells *(continued)*



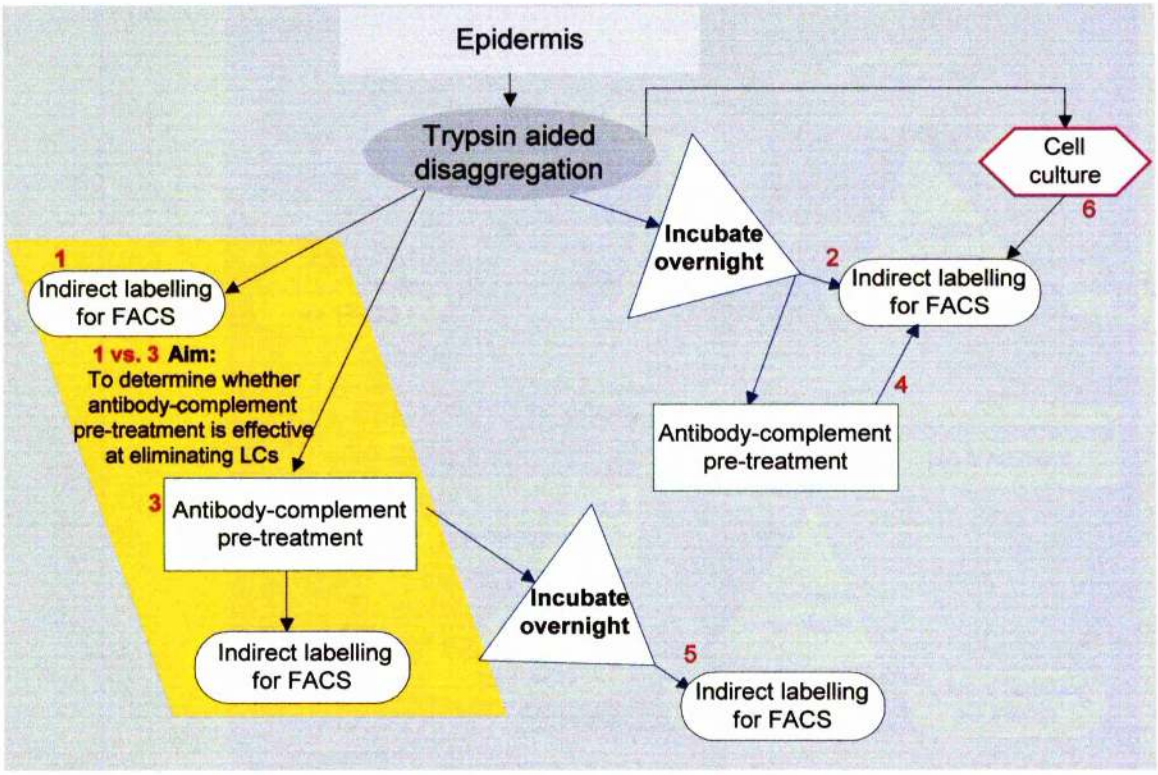
As discussed in the introduction, antibody and complement pre-treatment of epidermal cells with selective targeting of SLA DR-expressing cells (i.e. LC) induces complement mediated lysis of targeted cells. To ensure elimination of the LC population, antibody and complement pre-treatment of freshly trypsinised epidermal cells was therefore undertaken.

4.2.3 Determination of the effect of antibody complement pre-treatment on SLA DR expression in freshly trypsinised epidermal cells

Freshly trypsinised cells from three pigs were divided into two groups for comparison. One group underwent antibody complement pre-treatment (see Chapter 2 for method) and the other did not. All groups were then prepared for flow cytometry (see Chapter 2 for method).

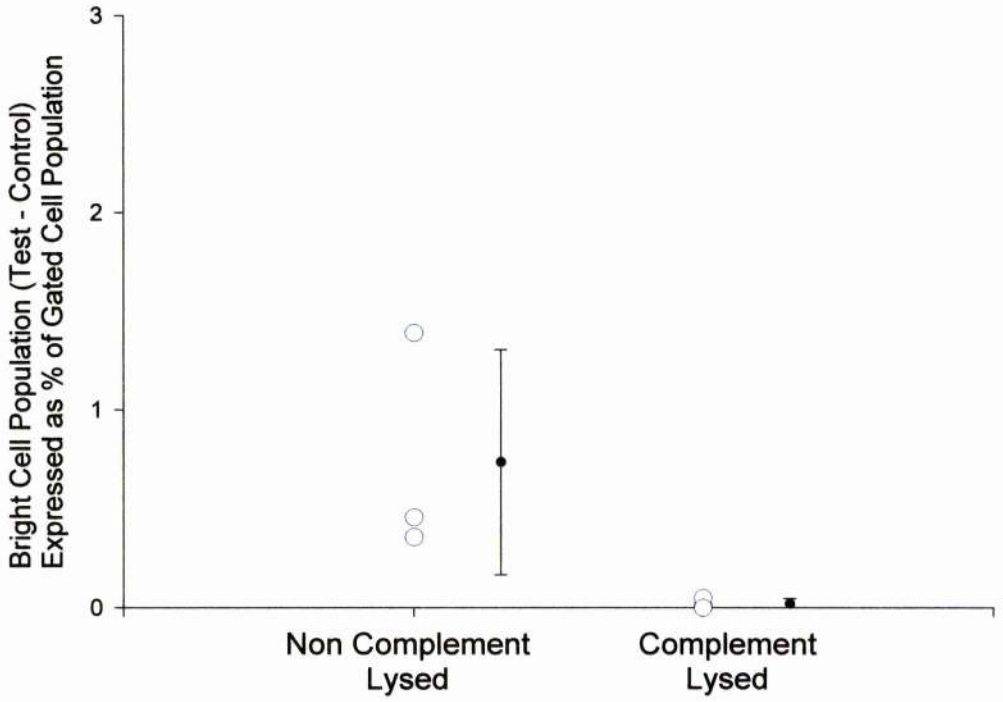
The results (see Figure 4.5) indicate a difference of 0.72 between the means of the two groups. The values for SLA DR expression within the antibody-complement pre-treated group approximate zero. These results suggest that antibody complement pre-treatment is effective at eliminating LC.

Figure 4.5: Determination of SLA DR expression in freshly trypsinised epidermal cells following antibody complement pre treatment



The point plot illustrates flow cytometry results following labelling of epidermal cells with test antibody (MSA3 SLA DR-specificity) or isotype control (IgG2a). Following trypsinisation of cells from three pigs they were divided into two groups. One group underwent antibody complement pre-treatment and the other did not. The group that underwent antibody complement pre-treatment contained a test limb which was incubated with MSA3 at a concentration of 1/100 and then incubated with complement and a control limb which had isotype control and complement. All groups both were subsequently prepared (with test or control antibodies) for flow cytometry without any further incubation. Preparation for FACS as well as subsequent gating and analysis used the same procedures and parameters as the previous studies.
The difference between the means of the two groups is 0.72.

Figure 4.5: Determination of SLA DR expression in freshly trypsinised epidermal cells following antibody complement pre-treatment *(continued)*



4.2.4 Determination of the effect of incubation on SLA DR expression in porcine epidermal cells

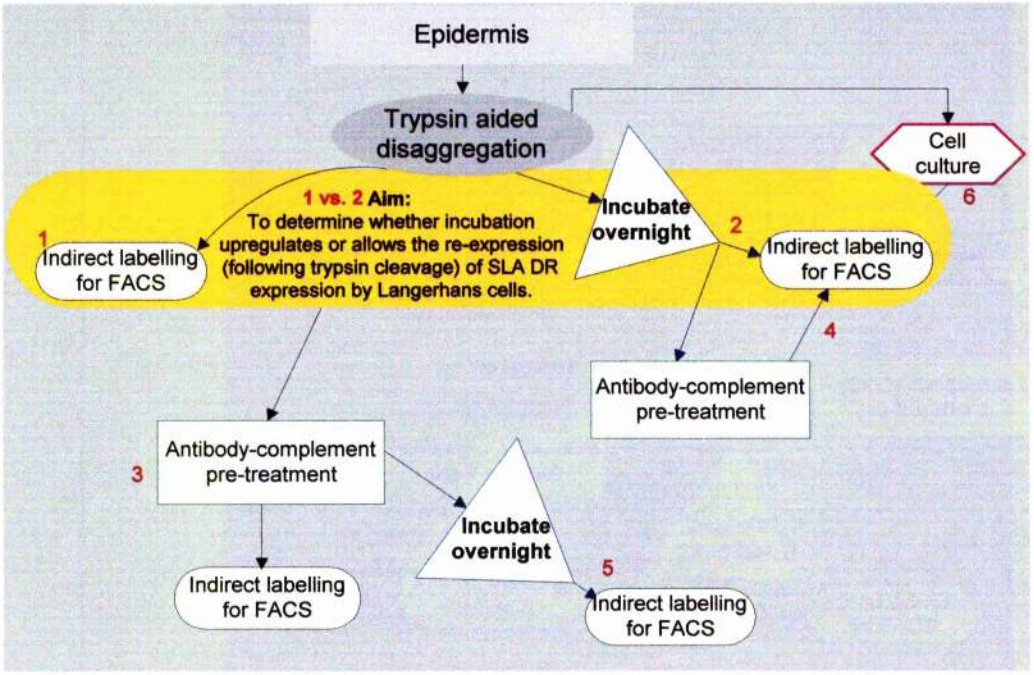
Trypsinisation of porcine cells strips off membrane antigens (personal communication M Bailey, Division of Molecular and Cellular Biology, Department of Clinical Veterinary Science, University of Bristol) and may result in cleavage of SLA DR determinants from LC. The cleaved membrane determinant may subsequently regenerate following a period of incubation. It has additionally been observed that immature LC evolve a more mature phenotype after a relatively short period in culture (Witmer-Pack et al., 1988; Larsen et al., 1990b; Teunissen et al., 1990) with markedly increased SLA DR expression. Bearing these observations in mind, antibody and complement pre-treatment might be more effective in eliminating LC following a period of incubation in culture than immediately following trypsinisation.

To test this hypothesis, freshly trypsinised cells from three pigs were incubated overnight in keratinocyte culture medium (see Chapter 2 for method) at 37°C in 10% CO_2, and were prepared for flow cytometry on the following morning. These were compared with results obtained previously from freshly trypsinised cells from four pigs prepared in the same way except that fixing and staining was performed without prior incubation. FACS results were similarly gated and analysed (see Chapter 2 for method).

The results (see Figure 4.6 and 4.7) suggest that following a period of incubation there is increased SLA DR expression (a mean of 4.1% of gated cells in the incubated group compared to 0.73% in the non incubated group, P=0.047). In addition, the density of expression is markedly increased (5 fold,

P =<0.001) as demonstrated by the geometric mean fluorescence values (see Figure 4.7).

Figure 4.6: Determination of the effect of incubation on SLA DR expression in porcine epidermal cells



The point plot illustrates flow cytometry results following labelling of epidermal cells with test antibody (MSA3 SLA DR-specificity) or isotype control (IgG2a). This study compares epidermal cells that were incubated overnight following trypsinisation (n=3) and subsequently fixed and stained to those that were fixed and stained immediately following trypsinisation (n=4), in terms of their SLA DR expression. Each group of n=3 pigs and n=4 pigs was processed separately, although the same procedures and parameters were used in preparation for FACS as well as subsequent gating and analysis.

The results suggest that an increased number of cells express SLA DR, a mean of 4.1% in the incubated group compared to 0.73% in the non incubated group, following a period of incubation (P = 0.047, t-test).

Figure 4.6: Determination of the effect of incubation on SLA DR expression in porcine epidermal cells *(continued)*

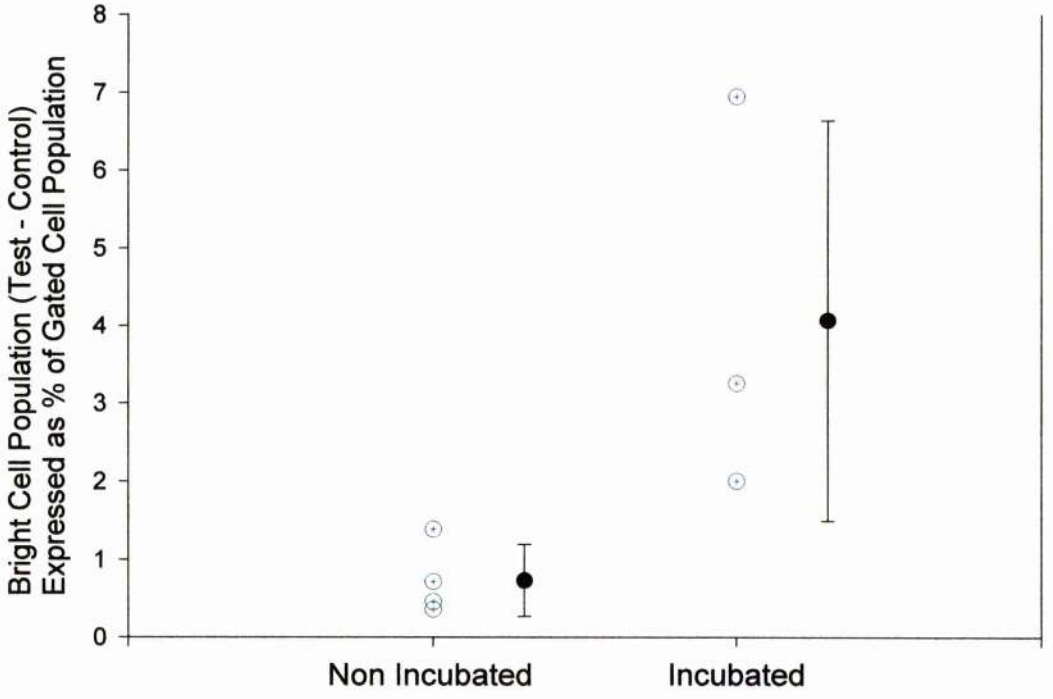
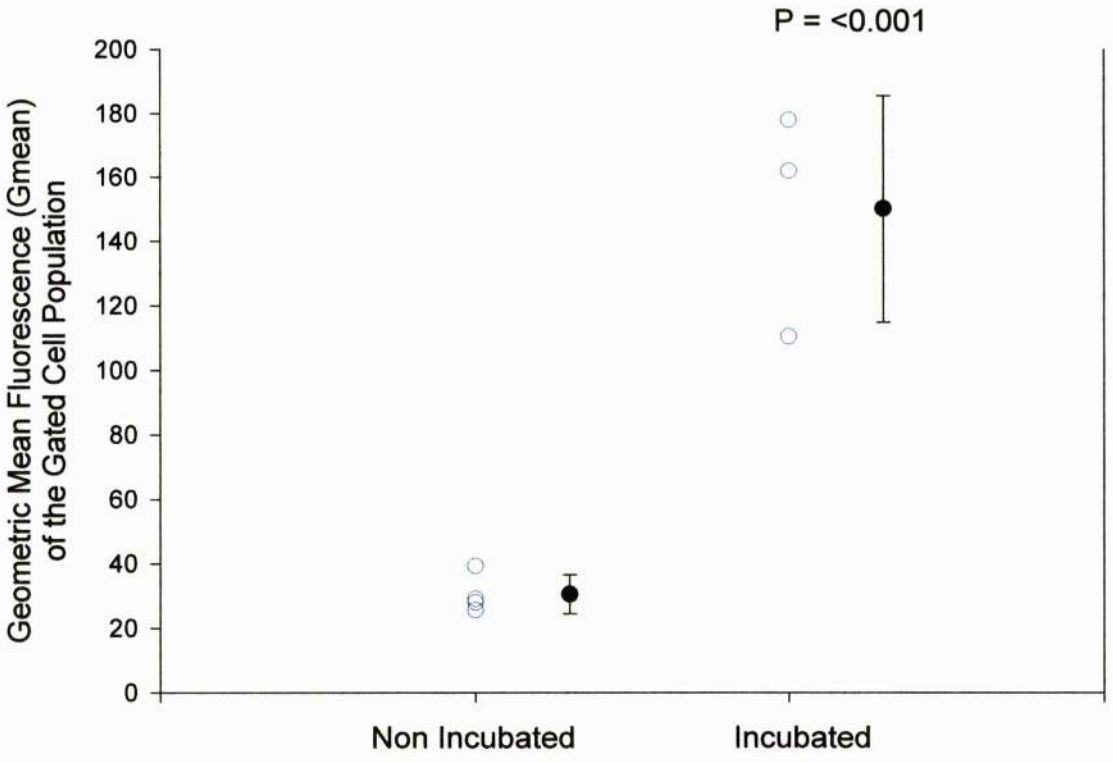


Figure 4.7: Determination of the effect of incubation on the brightness of SLA DR expression in porcine epidermal cells

The point plot illustrates the same flow cytometry results resulting from the study described in Figure 4.6 in terms of geometric mean fluorescence (G mean) rather than relative quantification of 'positive' cells (as in Figure 4.6). The geometric mean fluorescence is five times greater in the incubated group (t-test, P = <0.001. This suggests that incubation results in an upgrading of SLA DR expression. Hence, antibody - complement pre-treatment may be more efficient following a period of incubation.



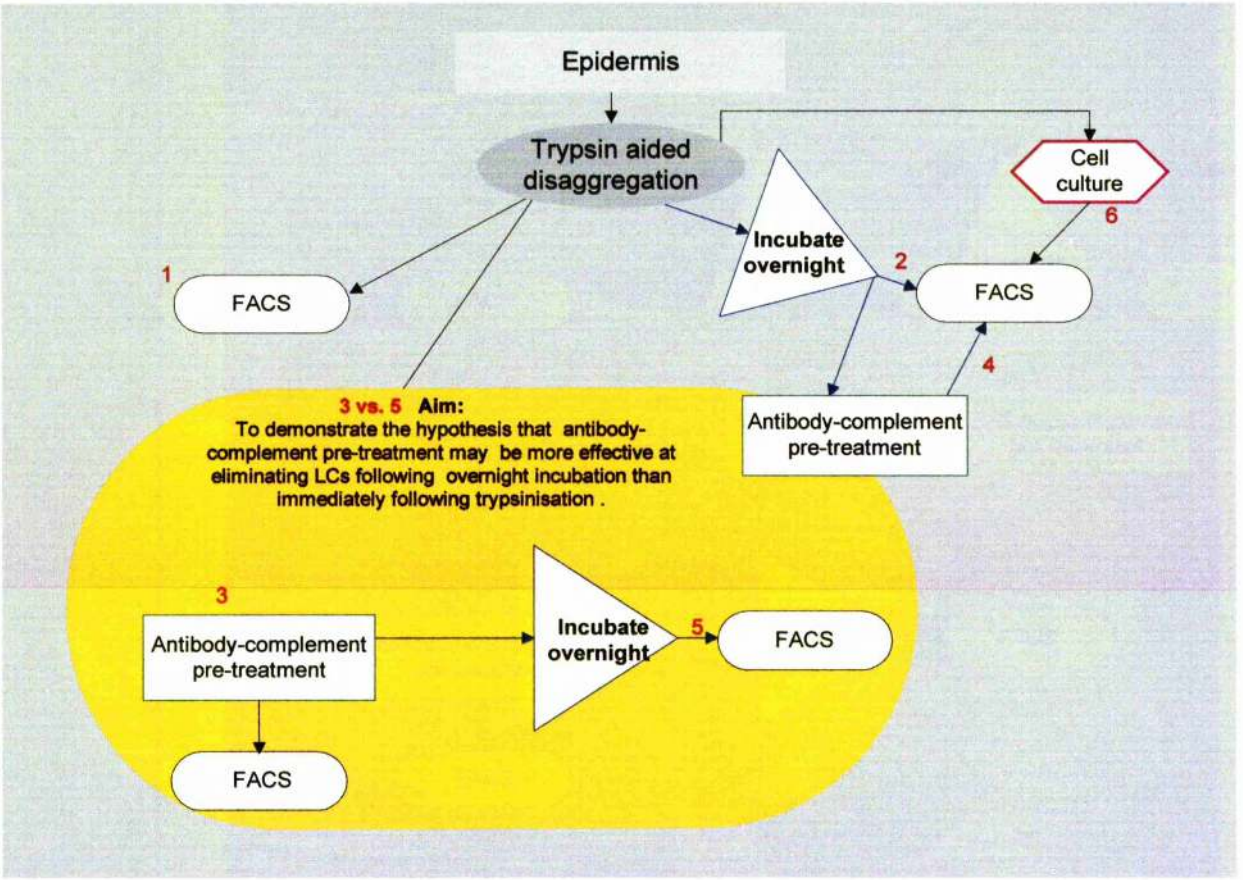
4.2.5 SLA DR expression following incubation of antibody – complement pre treated porcine epidermal cells

Incubation following trypsinisation results in the detection of a higher number of cells expressing SLA DR antigens (see 4.2.4). It follows therefore that antibody complement pre-treatment might be more effective at eliminating LC following a period of incubation. This is because antibody may fail to combine with (and complement fail to lyse) LC poorly expressing SLA DR (the antigens having been cleaved by trypsin).

To test the hypothesis that increased numbers of SLA DR-expressing cells may be detected following incubation of antibody-complement treatment of freshly trypsinised cells, cells from two pigs were incubated overnight following antibody-complement treatment and then prepared for FACS the following morning. The results were compared with results previously obtained from cells from three pigs similarly treated but which were not incubated prior to FACS (see Figure 4.5). Results from both studies were similarly gated and analysed (see Chapter 2 for method).

The results (see Figure 4.8 where a difference in mean SLA DR expression between the two groups of 2.2%% is apparent (P = 0.037, t-test)) suggest that lysis immediately following trypsinisation may not eliminate all the LC. The LCs that are not eliminated, if allowed to persist in culture, may act as passenger lymphocytes following allografting of keratinocyte sheet allografts, and initiate an alloimmune response.

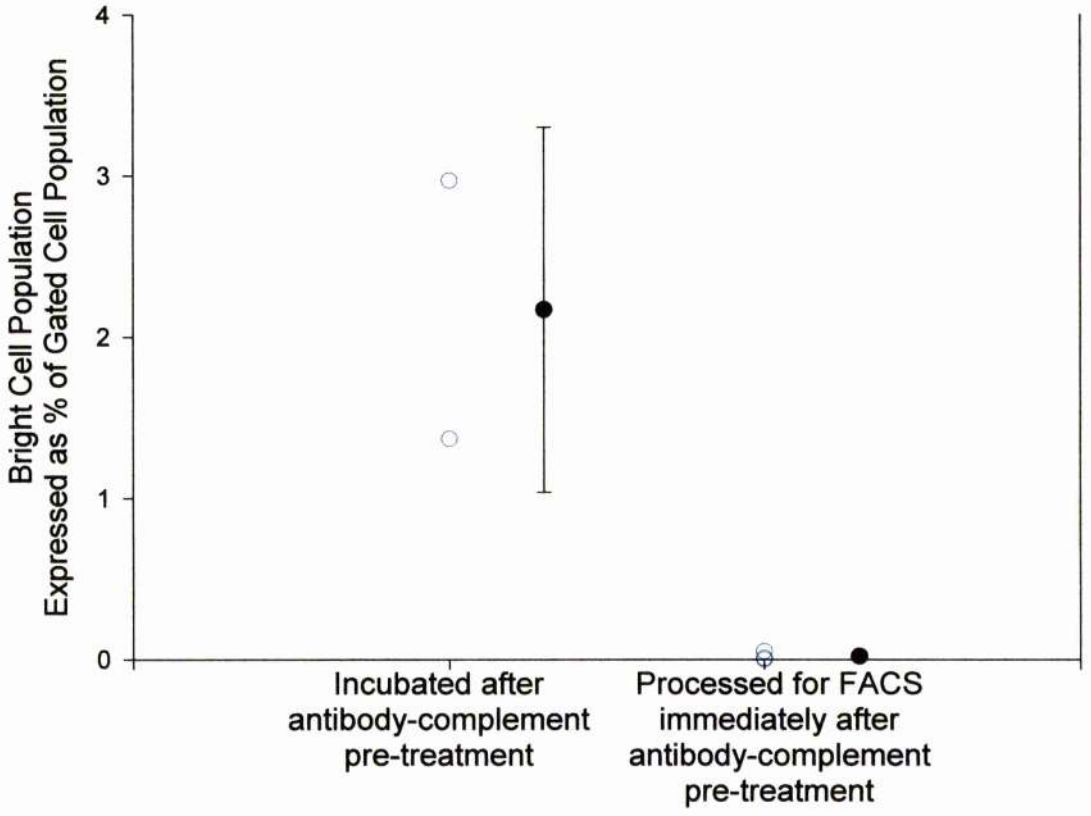
Figure 4.8: Determination of the effect of incubation following antibody complement pre-treatment on SLA DR expression in porcine epidermal cells



The point plot illustrates flow cytometry results indicating SLA DR expression, following labelling of epidermal cells with test antibody (MSA3, SLA DR-specificity) or isotype control (IgG2a). This study compares freshly trypsinised epidermal cells from (n=2 pigs) that underwent antibody complement pre-treatment and were incubated overnight prior to fixing and staining, to those (n=3), that were fixed and stained immediately following antibody complement pre-treatment (without prior incubation). Each group (of n=2 pigs and n=3 pigs) was processed separately, although the same procedures and parameters were used in preparation for FACS as well as subsequent gating and analysis.

The difference between the means of the two groups is 2.2% (P = 0.037, t-test) which suggests that incubation may allow Langerhans to reform SLA DR antigens following their cleavage by the trypsinisation process.

Figure 4.8: Determination of the effect of incubation following antibody complement pre-treatment on SLA DR expression in porcine epidermal cells *(continued)*



4.2.6 Comparison of different sources of complement

Low Tox H® rabbit complement (from Cedarlane Laboratories) was used in all the experiments described above. This product is designed to have a relatively low rate of non-specific lysis of human lymphocytes and it was hypothesised that it would be similarly less toxic than other complement types to porcine keratinocytes.

In previous experiments involving antibody complement pre-treatment, to identify live cells, propidium iodide was included in preparation for FACS (propidium iodide binds to DNA but can only enter dead cells whose cell membrane quickly becomes permeable). This had revealed a high proportion of dead epidermal cells, often as high as 40-60%.

Additionally, antibody-complement pre-treatment performed immediately following trypsinisation (see Section 4.2.5) may not be effective in generating lysis of LC whose membrane determinants are poorly expressed.

An experiment was designed to compare 4 different types of complement in terms of efficacy as well as to compare the proportion of non-specific cell death.

The four different types of complement-tested, included: the afore mentioned Low Tox H® rabbit complement (with low human lymphoid toxicity); Low Tox M® rabbit complement (designed to be of low toxicity to mouse lymphocytes); baby rabbit complement and guinea pig complement.

Following trypsinisation the epidermal cells from one pig were incubated overnight. The cells were then treated with isotype control or SLA DR-specific

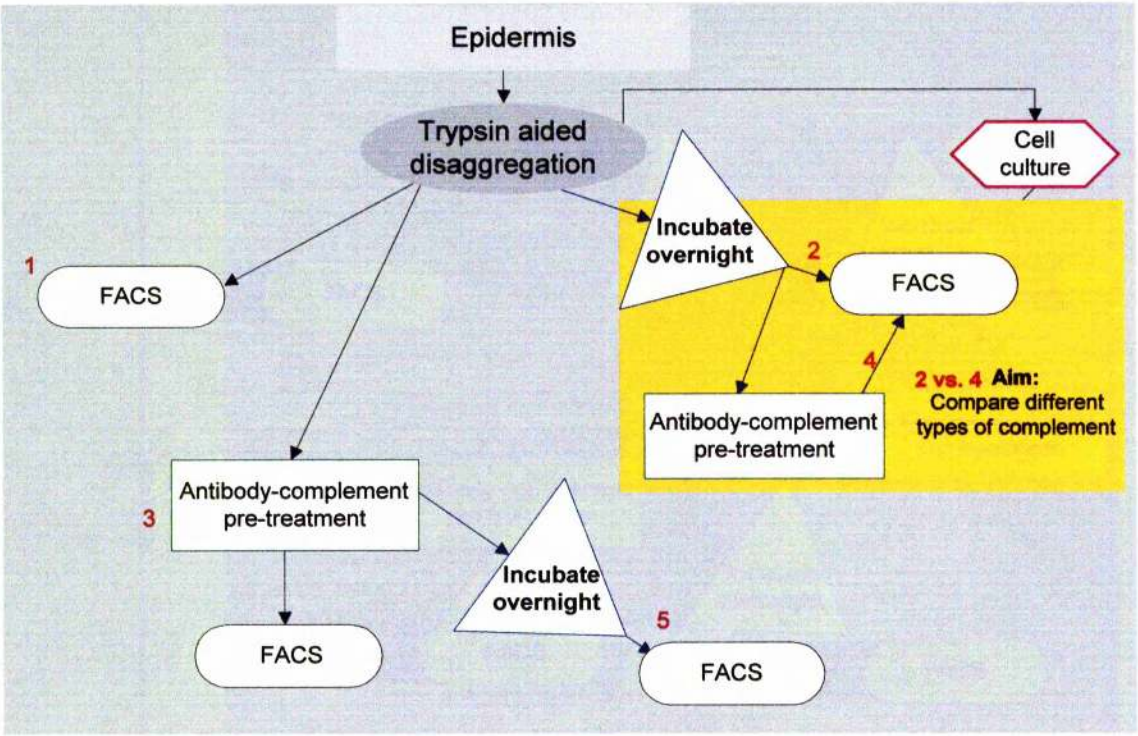
antibody and complement on the following morning and immediately processed for FACS (with test and control antibody-FITC labelling).

The results suggest (see Figures 4.9 and 4.10) that antibody – complement pre-treatment following overnight incubation on freshly trypsinised epidermal cells is effective at eliminating SLA DR-expressing cells. They do not however, suggest that any particular type of complement is more effective than another.

A consistent finding was that where (as in this experiment) harvested skin had been refrigerated for a couple of days prior to processing, the proportion of cells expressing SLA DR antigens diminished. This may account for values that are lower than previously seen (see Figure 4.6).

With regard to relative toxicity to porcine keratinocytes of the varieties of complement the results (see Figure 4.11) suggest that there is no difference between the complement types. There is also no significant difference in proportion of dead cells between the isotype-specific control antibody (IgG2a) or MSA 3 groups. One limb of this experiment involved a sample being placed on ice for 90 minutes (as a further control to samples undergoing incubation on ice with complement) but did not have antibody or complement added. The mean proportion of dead cells within this sample was 13.4% higher (~59%) than within those samples that had antibody and complement added (~46%). One explanation for this may be the increased number of times the 'complement added group' undergo centrifugation and resuspension, which may fragment and eliminate dead cells.

Figure 4.9: Comparison of complement types (a)



This graph illustrates a study involving freshly trypsinised epidermal cells from one pig in a comparison of complement types. Cells were divided into 6 groups. 4 of these groups were incubated overnight and then underwent antibody complement pre-treatment with one of four sources of complement. In each of the four groups cells either received isotype control and complement or anti-SLA DR and complement. Following antibody complement pre-treatment the cells were stained in duplicate with either isotype-FITC or SLA DR-FITC. The remaining two groups consist of cells that did not undergo antibody complement pre-treatment and were either incubated or not.

The groups, apart from the one that one not incubated and was processed 12-18 hours before the other groups, were all processed together. The same procedures and parameters were similarly used in preparation for FACS as well as subsequent gating and analysis.

The results suggest that antibody – complement pre-treatment following overnight incubation on freshly trypsinised epidermal cells is effective at eliminating SLA DR-expressing cells. They do not however, suggest that any type of complement is more effective.

Figure 4.9: Comparison of complement types (a) *(continued)*

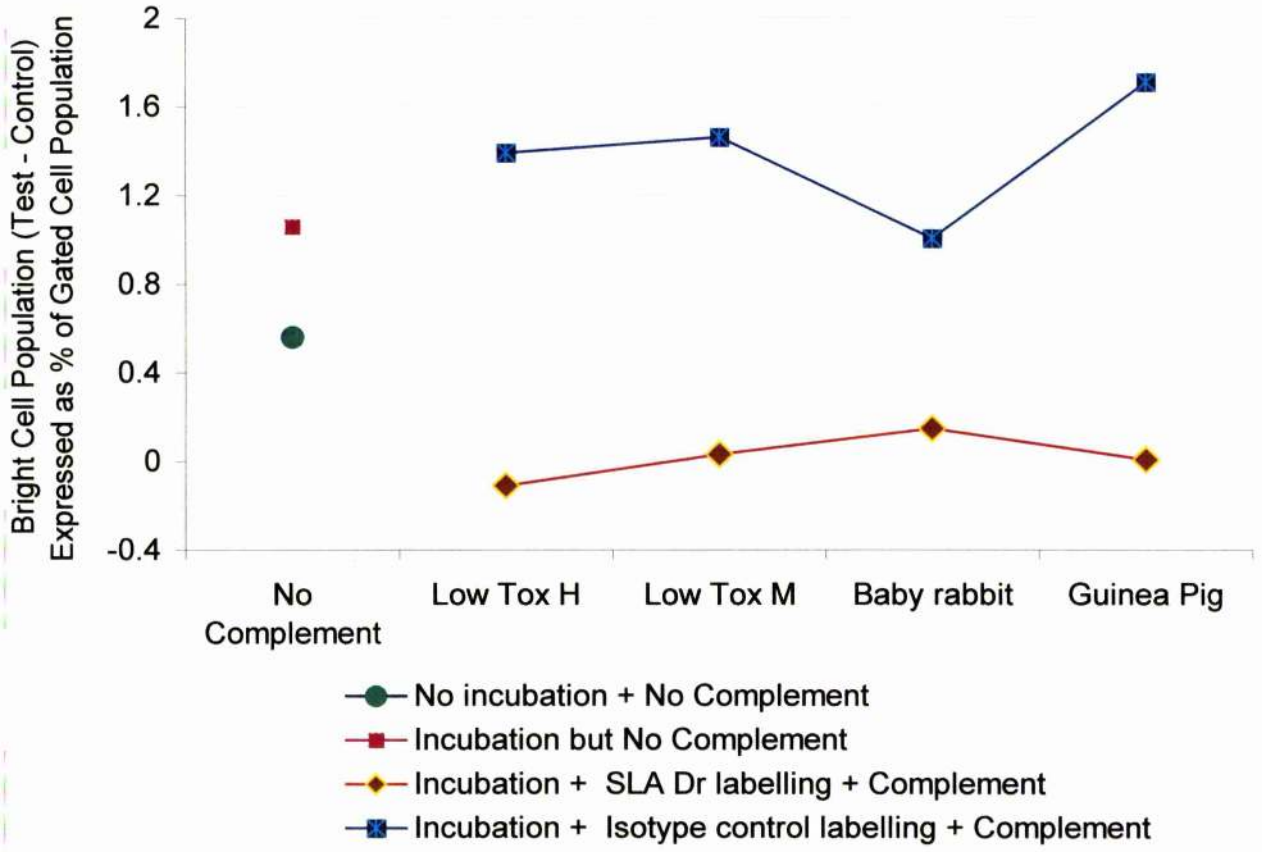


Figure 4.10: Comparison of complement types (b)

The point plot illustrates the same flow cytometry results resulting from the study described in Figure 4.9, in terms of geometric mean fluorescence (G mean) rather than relative quantification of 'positive' cells (as in Figure 4.9).

As with Figure 4.9 this graph illustrates several points. The first is that incubation appears to result in upgrading of SLA DR expression. The second is that antibody – complement pre-treatment following overnight incubation on freshly trypsinised epidermal cells is effective at eliminating SLA DR-expressing cells. The third is that all four sources of complement appear to be effective.

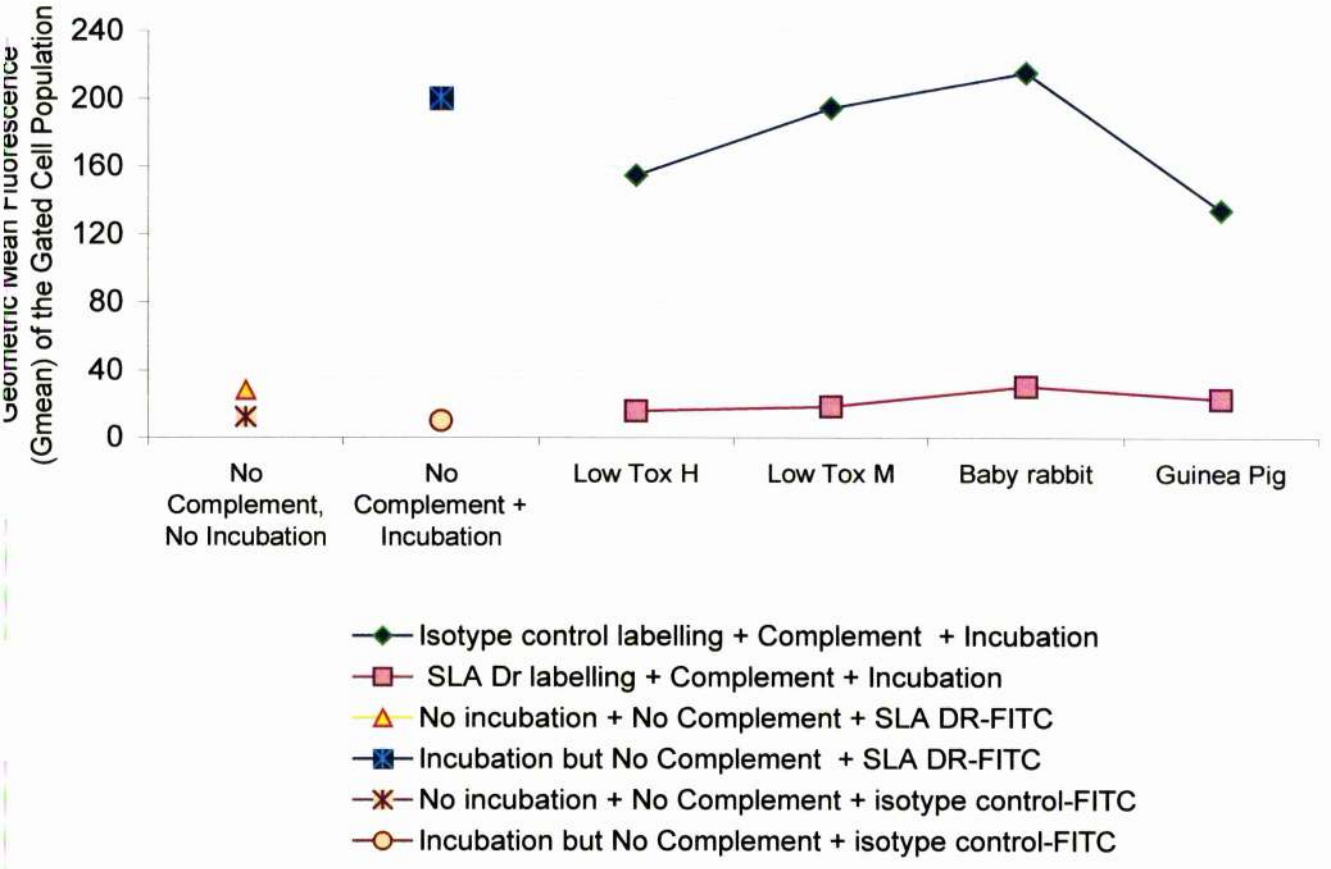
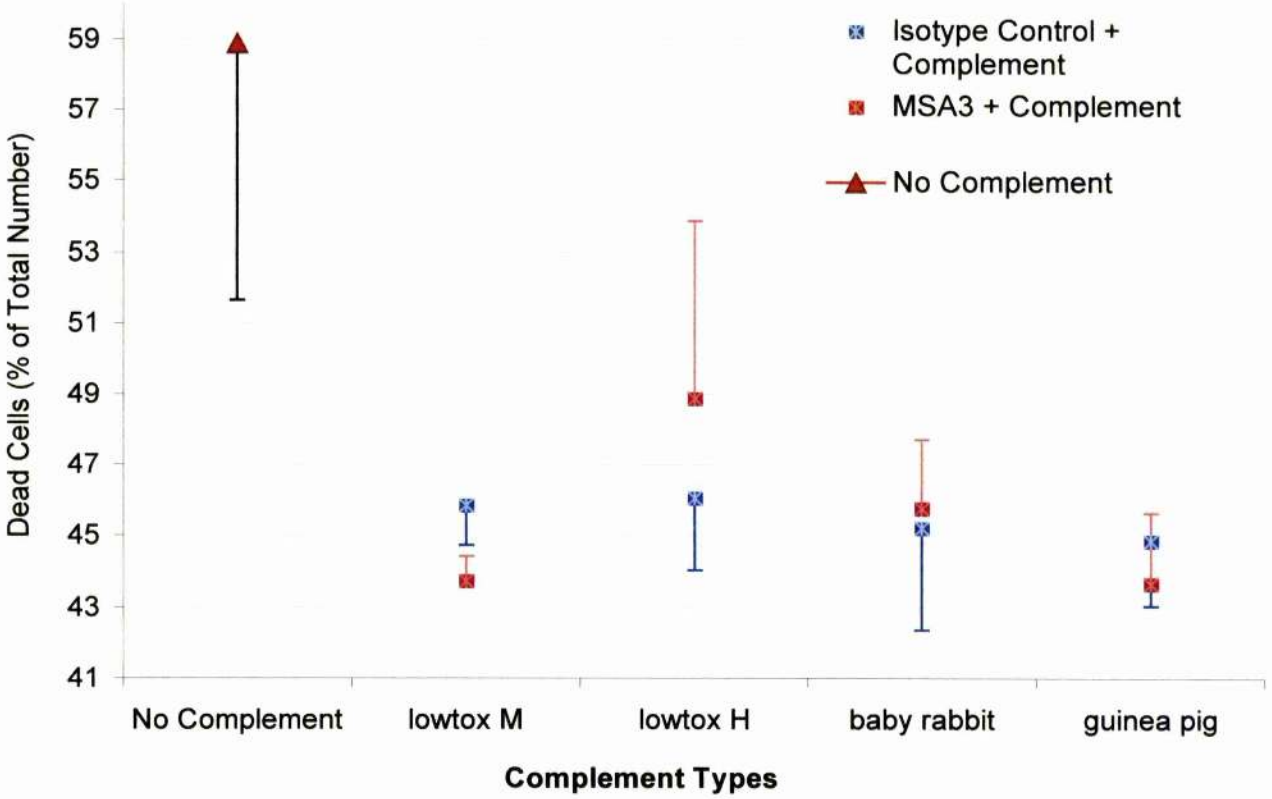


Figure 4.11: Comparison of complement type with regard to non specific cytotoxicity

This graph illustrates a comparison of complement types with regard to cell death. In the one pig study described earlier (see Figure 4.9), propidium iodide (1/50) was added after the first wash to the cells in the five groups that were incubated. Propidium iodide allows identification and quantification of dead cells with red fluorescence that can be detected at flow cytometry. All samples were prepared in duplicate and a mean taken (hence the vertical standard deviation lines). The value for the group that did not undergo antibody-complement pre-treatment (red triangle) is a mean of four values (isotype-FITC and anti-SLA DR-FITC limbs duplicated).

The results indicate that there is no significant difference in this sample between the complement types. There is also no significant difference in proportion of dead cells between the isotype-specific control antibody (IgG2a) or MSA 3 groups. The cells not subjected to complement appear to paradoxically have more dead cells (see text).



4.2.7 Determination of the effect of antibody complement pre treatment on colony forming efficiency of porcine keratinocytes

The process of antibody complement pre-treatment involves incubation for 90 minutes on ice with complement. The cells are washed several times prior to counting and plating. The resultant keratinocyte 'soup' appears cleaner, containing less debris which undoubtedly results from a higher number of cell washes than is normally employed. Keratinocytes are however, extremely sensitive to handling and culture environment. It is conceivable therefore that the manipulation and conditions involved in the antibody complement pre-treatment process may have an undesirable effect on keratinocyte colony forming efficiency in terms of the numbers and size of colonies formed.

Skin from three pigs was used in this study. Freshly harvested skin was trypsinised. The disaggregated epidermal cells were divided into two groups. One group underwent treatment with antibody and complement (using Low Tox H® rabbit complement) whilst the other had identical treatment except that isotype control antibody was substituted for the anti-SLA DR antibody.

Using P3 cells from each group, $1.1X10^2$ per cm^2 were plated in triplicate onto 6 well plates (seeded with $2.67X10^4$ irradiated 3T3 cells per cm^2). Medium was changed every 2-3 days. After 1 week of culture, the wells were stained with rhodamine blue.

The number of colonies were counted manually and confirmed with an automated image analyser count (Figures 4.12 – 4.16). In addition the image analyser was used to assess the size (area) of colonies (Figures 4.17 -4.23).

Figure 4.12: Scanned 6 well plates (colony forming efficiency / feeder cell study)

The digitally scanned images show examples of the wells (6 well plate) containing keratinocyte colonies that were manually counted as well as scanned for image analysis following fixation and staining (see Figure 4.14 for description of study).
The wells correspond to the following pigs: Pig no. 17 (a) and (b); Pig no. 18 (c) and (d); Pig no. 20 (e) and (f). Wells (a), (c) and (e) contain antibody-complement pre-treated keratinocytes; Wells (b), (d) and (f) contain keratinocytes that were not antibody-complement pre-treated.
These images demonstrate the trend for colonies from the non antibody-complement pre-treated group to be more numerous, larger and less eccentric in shape.

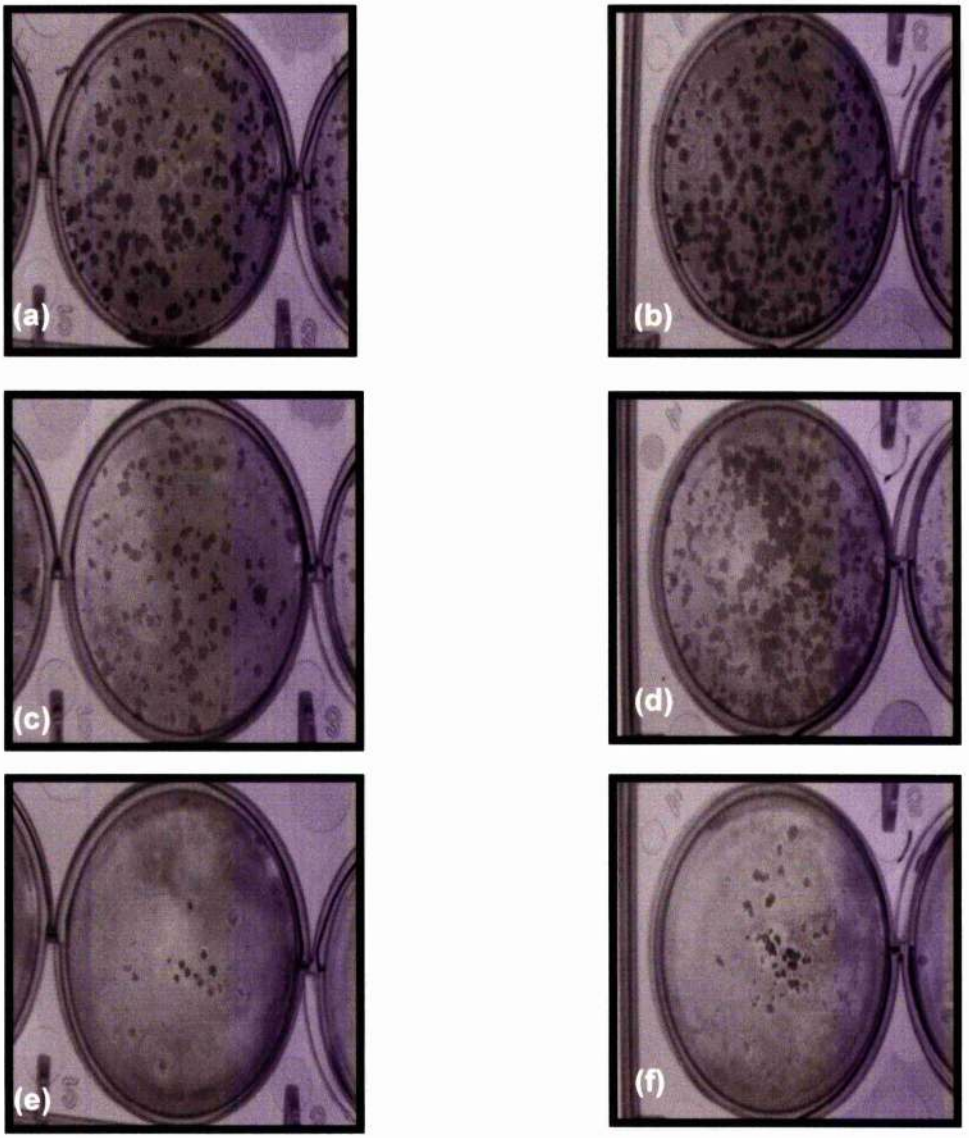


Figure 4.13: Cultured keratinocyte colonies (colony forming efficiency / feeder cell study)

The photomicrographs (X40) show examples of keratinocyte colonies (pigs 17-20) that were manually counted as well as scanned for image analysis following fixation and staining (see Figure 4.14 for description of study).
The wells (stained with rhodamine blue) correspond to the following pigs: Pig no. 17 (a) and (b); Pig no. 18 (c) and (d); Pig no. 20 (e) and (f). Wells (a), (c) and (e) contain antibody-complement pre-treated keratinocytes; Wells (b), (d) and (f) contain keratinocytes that were not antibody complement pre treated.
As with Figure 4.12, these images demonstrate the trend for colonies from the non antibody-complement pre-treated group to be more numerous, larger and less eccentric in shape.

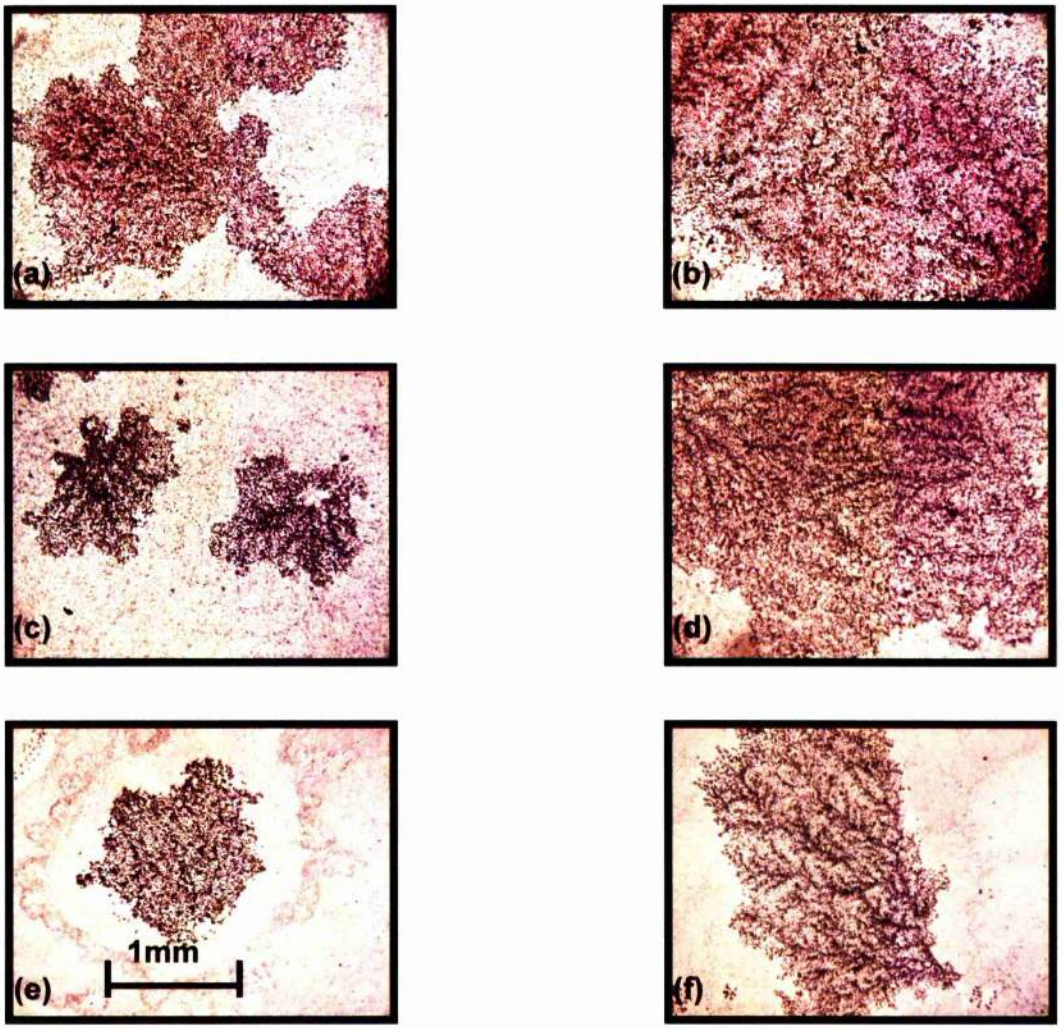


Figure 4.14: Antibody - complement pre-treatment: colony forming efficiency study - manual count of colony numbers

Freshly trypsinised disaggregated epidermal cells from three pigs were divided into two groups. One group underwent treatment with antibody and complement (using Low Tox H® rabbit complement) whilst the other did not. At the P3 stage, cells from each group, $1.1X10^2$ per cm^2 were plated in triplicate onto 6 well plates (seeded with $2.67X10^4$ irradiated 3T3 cells per cm^2). After 1 week the wells were stained with rhodamine blue. Colonies were manually counted (as well as digitally scanned and analysed see Figure 4.15).

Using the t-test the differences between the means of the absolute values (graph (c)) between the 'antibody complement pre-treatment group' and the 'no antibody complement pre-treatment group' in each pig were shown to be significant (p=0.002 for pigs 17 and 18 and 0.0026 for pig no. 20). The one way repeated measures analysis of variance (Tukey test) confirmed that the relative increase in numbers of colonies seen in the non complement lysed group (from 26% in pig no. 17 to 226% in pig no. 20) is significant (p=0.004).

One way analysis of variance and Tukey test of the relative values (graphs (a) and (c)) confirmed that the differences seen with one way repeated measures analysis of variance of the absolute values were significant (P=0.008).

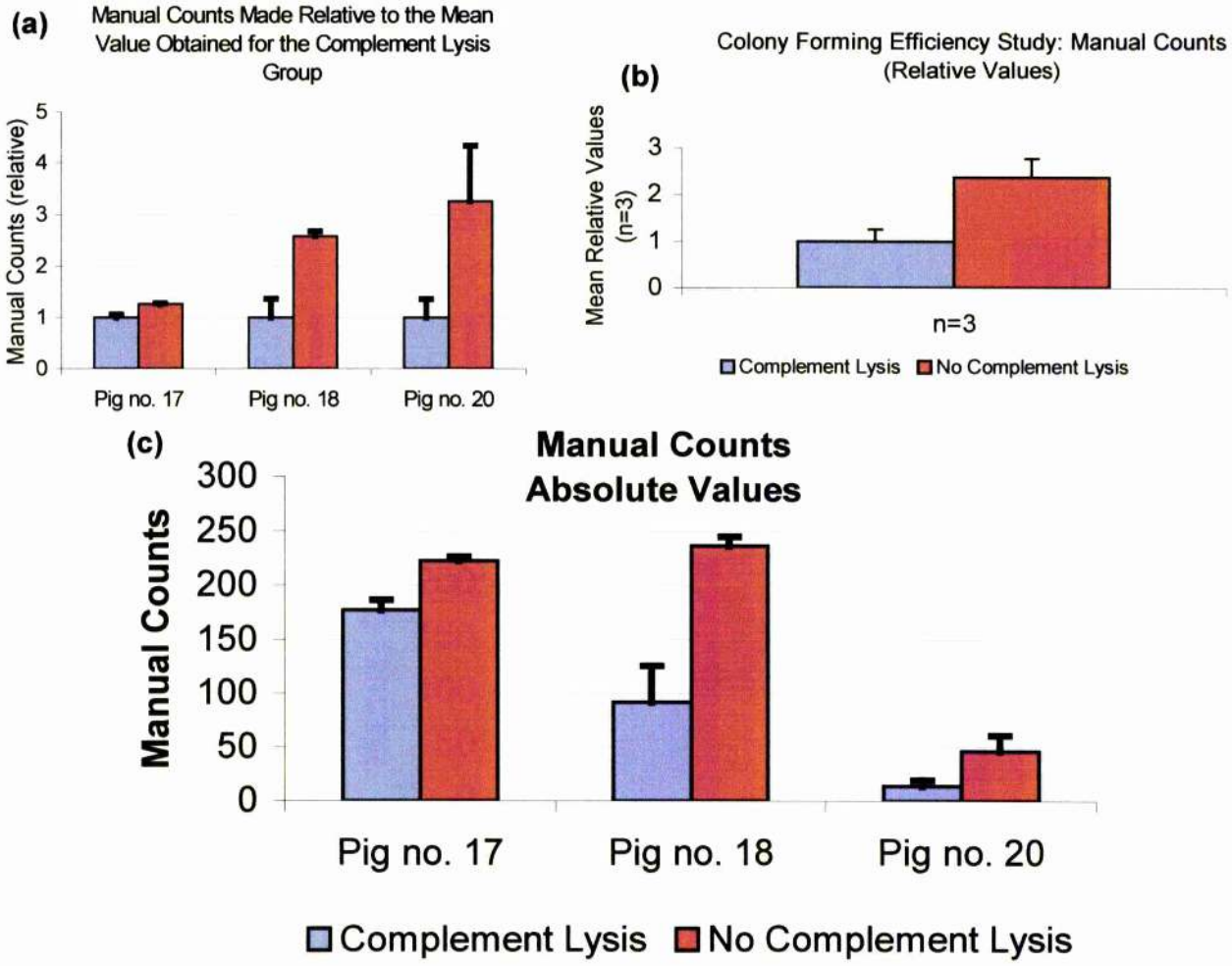


Figure 4.15: Antibody - complement pre-treatment:
colony forming efficiency study - automated count of colony numbers

Following the experiment delineated in the legend to Figure 4.14 wells were scanned and digitally analysed with Image Pro Plus® software with respect to numbers and size of colonies.
One way repeated measures analysis of variance of the absolute values (graph (c)), showed that there were significantly (P=<0.001) higher colony counts among the non complement lysed group (of between 18% higher in pig no.17 to 176% higher in pig no. 20) than the complement lysed group. This was confirmed with the t-test in pigs 17 and 18 (P=0.035 and P=0.02 respectively).
This was further confirmed by a one way analysis of variance (P=0.038) and Tukey test of the relative values (graphs (a) and (b)).

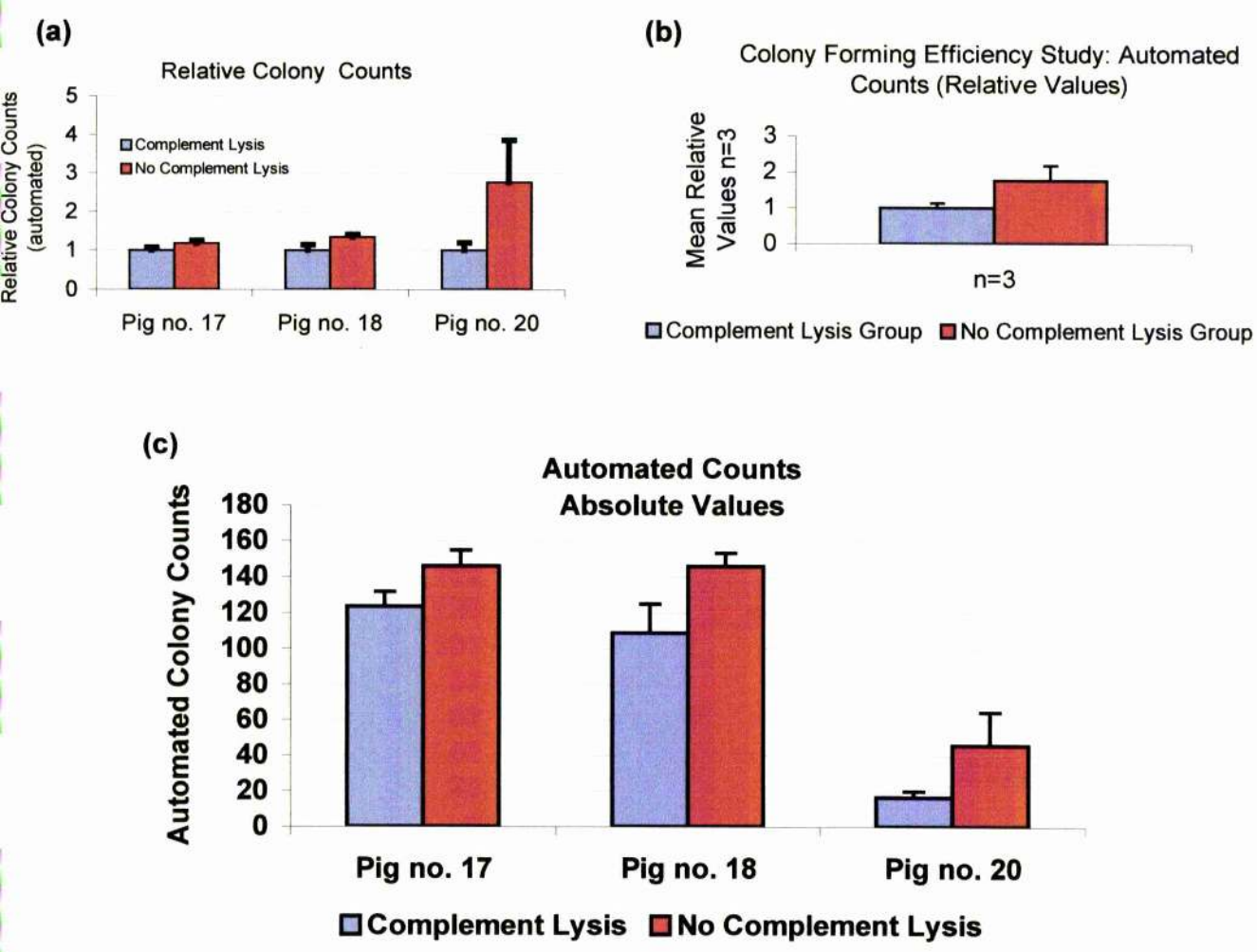


Figure 4.16: Antibody - complement pre-treatment:
colony forming efficiency study - comparison of automated and manual count of colony numbers

The relative values for both manual and automated counts (see Figures 4.15 and 4.16) have been made relative to those for the antibody complement pre-treatment group and the overall mean calculated pertaining to the method of colony counting. The error bars refer to standard deviations. The results indicate no significant differences between the mean relative values (obtained with both manual and automated methods) of the 'no antibody complement pre-treatment group'

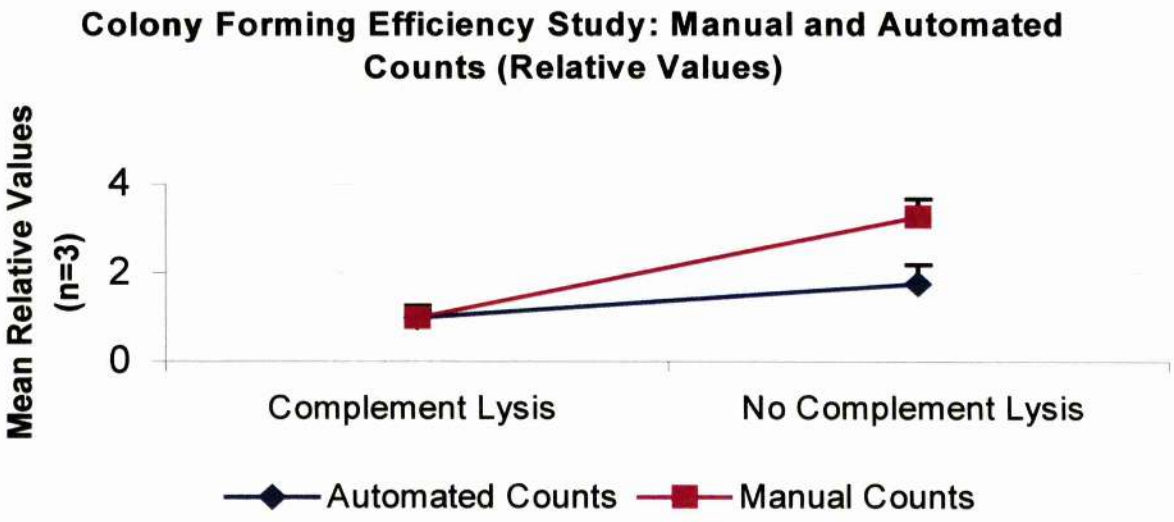


Figure 4.17: Antibody - complement pre-treatment:
colony forming efficiency study (pig no.17) – analysis of colony surface areas (frequency distribution)

Following the experiment described in the legend to Figure 4.14, wells were scanned and digitally analysed with Image Pro Plus® software with respect to numbers and size of colonies. The graph illustrates the frequency distribution of colony surface areas for keratinocyte colonies from pig number 17 in both the antibody-complement pre-treatment and the non pre-treatment groups.
9 bins were created with the value of each bin rising incrementally from 100 to 3000 surface area units. The surface areas for each feeder cell group with values corresponding to particular bins were assigned to the corresponding bins. The total number of surface area values within each bin were plotted against the bin designated value to give a frequency distribution histogram.
The histogram illustrates the general trend of significantly greater (t-test P=0.021) mean colony areas with the no antibody-complement pre-treatment groups than with antibody-complement pre-treatment group.

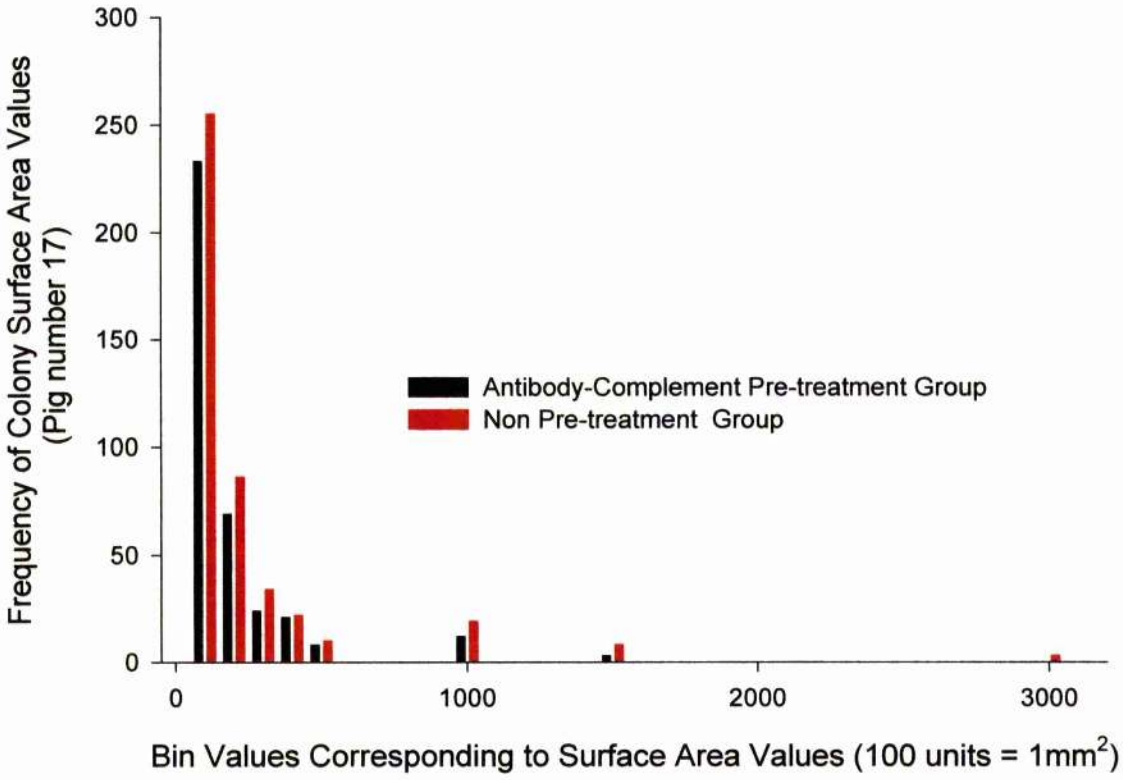


Figure 4.18 : Antibody - complement pre-treatment:
colony forming efficiency study (pig no.17) – analysis of colony surface areas

Following the experiment described in the legend to Figure 4.14, wells were scanned and digitally analysed with Image Pro Plus® software with respect to numbers and size of colonies. The graph illustrates the distribution of colony surface areas from pig number 17 for the largest 100 colonies from each of the antibody-complement pre-treatment and the non pre-treatment groups. The smaller colonies have been eliminated from this graph in order to focus attention on the pattern of distribution of the larger colonies. The trend is suggestive (t-test, P=<0.001) of consistently more colonies with a larger surface area in the non pre-treatment group, which follows the general trend (see Figures 4.20 and 4.22).

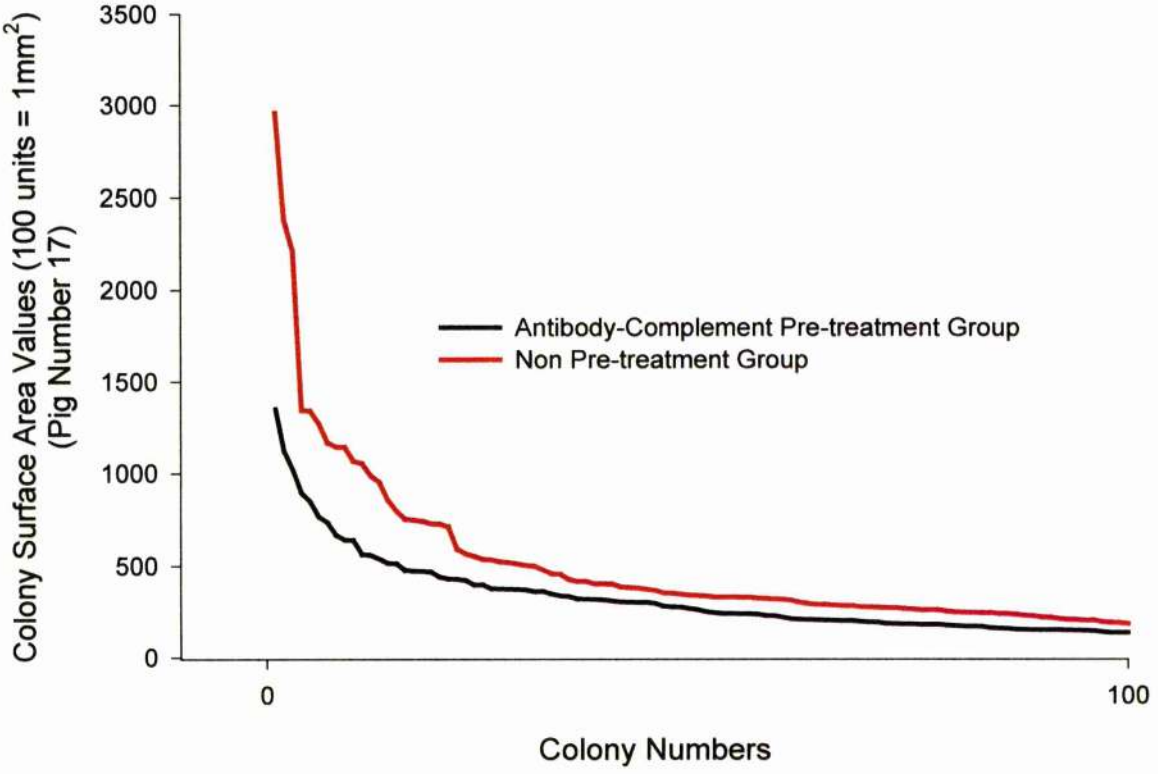


Figure 4.19: Antibody - complement pre-treatment:
colony forming efficiency study (pig no.18) – analysis of colony surface areas (frequency distribution)

Following the experiment described in the legend to Figure 4.14, wells were scanned and digitally analysed with Image Pro Plus® software with respect to numbers and size of colonies. The graph illustrates the frequency distribution of colony surface areas for keratinocyte colonies from pig number 18 in both the antibody-complement pre-treatment and the non pre-treatment groups.
11 bins were created with the value of each bin rising incrementally from 100 to 3300 surface area units. The surface areas for each feeder cell group with values corresponding to particular bins were assigned to the corresponding bins. The total number of surface area values within each bin were plotted against the bin designated value to give a frequency distribution histogram.
The histogram illustrates the general trend of higher (t-test P=<0.001) mean colony areas with the non pre-treatment groups than with antibody-complement pre-treatment group.

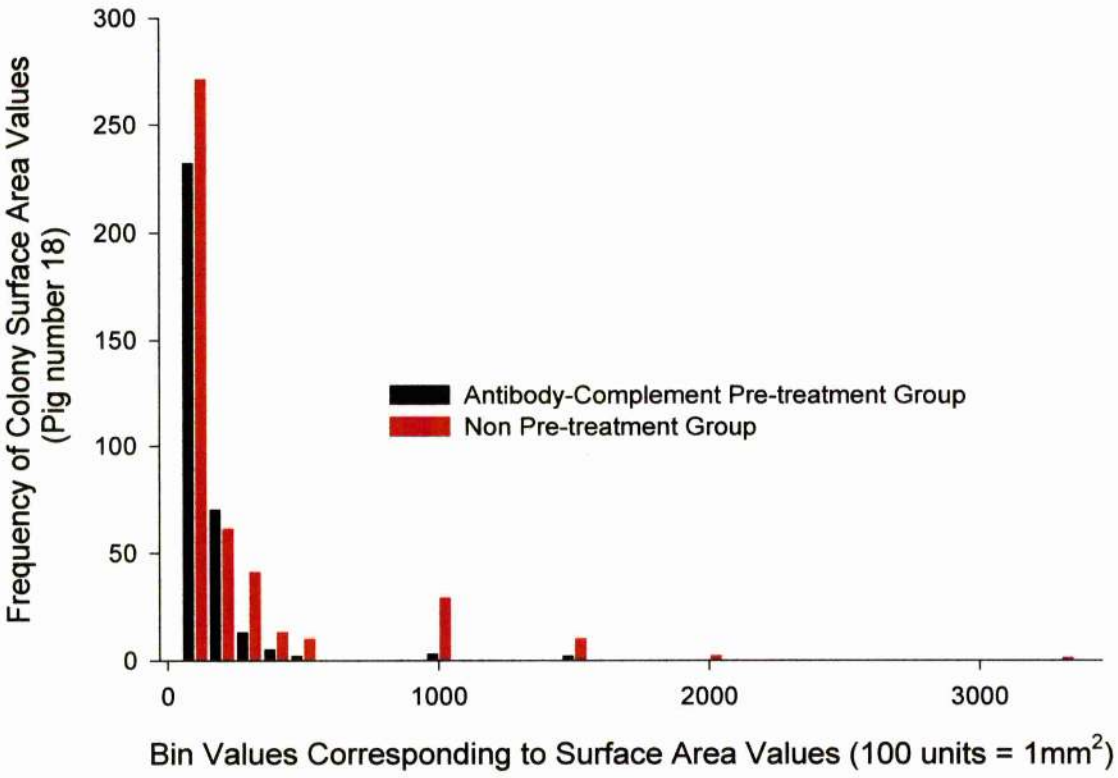


Figure 4.20: Antibody - complement pre-treatment:
colony forming efficiency study (pig no.18) – analysis of colony surface areas

Following the experiment described in the legend to Figure 4.14, wells were scanned and digitally analysed with Image Pro Plus® software with respect to numbers and size of colonies. The graph illustrates the distribution of colony surface areas from pig number 18 for the largest 100 colonies from each of the antibody-complement pre-treatment and the non pre-treatment groups. The smaller colonies have been eliminated from this graph in order to focus attention on the pattern of distribution of the larger colonies.

The trend is suggestive (t-test, P=<0.001) of consistently more colonies with a larger surface area in the no antibody-complement pre-treatment group, which follows the general trend (see Figures 4.18 and 4.22).

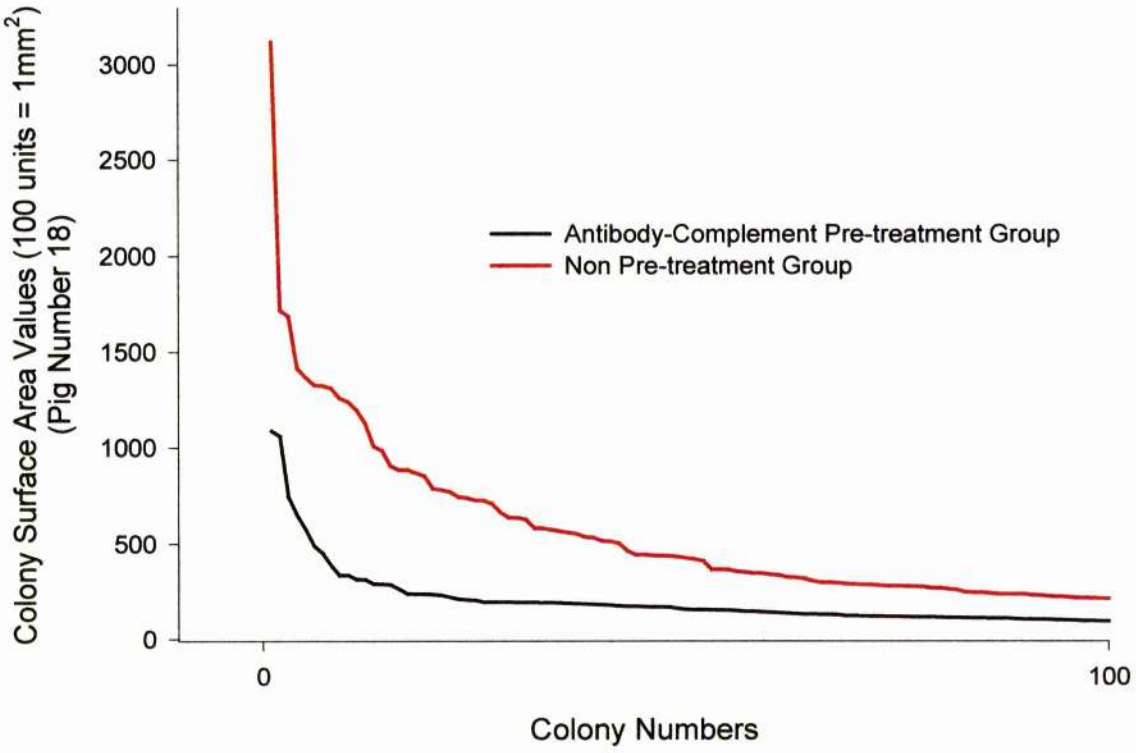


Figure 4.21: Antibody - complement pre-treatment:
colony forming efficiency study (pig no. 20) – analysis of colony surface areas (frequency distribution)

Following the experiment described in the legend to Figure 4.14, wells were scanned and digitally analysed with Image Pro Plus® software with respect to numbers and size of colonies. The graph illustrates the frequency distribution of colony surface areas for keratinocyte colonies from pig number 20 in both the antibody-complement pre-treatment and the non pre-treatment groups.
11 bins were created with the value of each bin rising incrementally from 50 to 550 surface area units. The surface areas for each feeder cell group with values corresponding to particular bins were assigned to the corresponding bins. The total number of surface area values within each bin were plotted against the bin designated value to give a frequency distribution histogram.
The histogram is suggestive of the general trend of significantly greater mean colony areas with the nom pre-treatment groups than with antibody - complement pre-treatment group (see Figures 4.17 and 4.19).

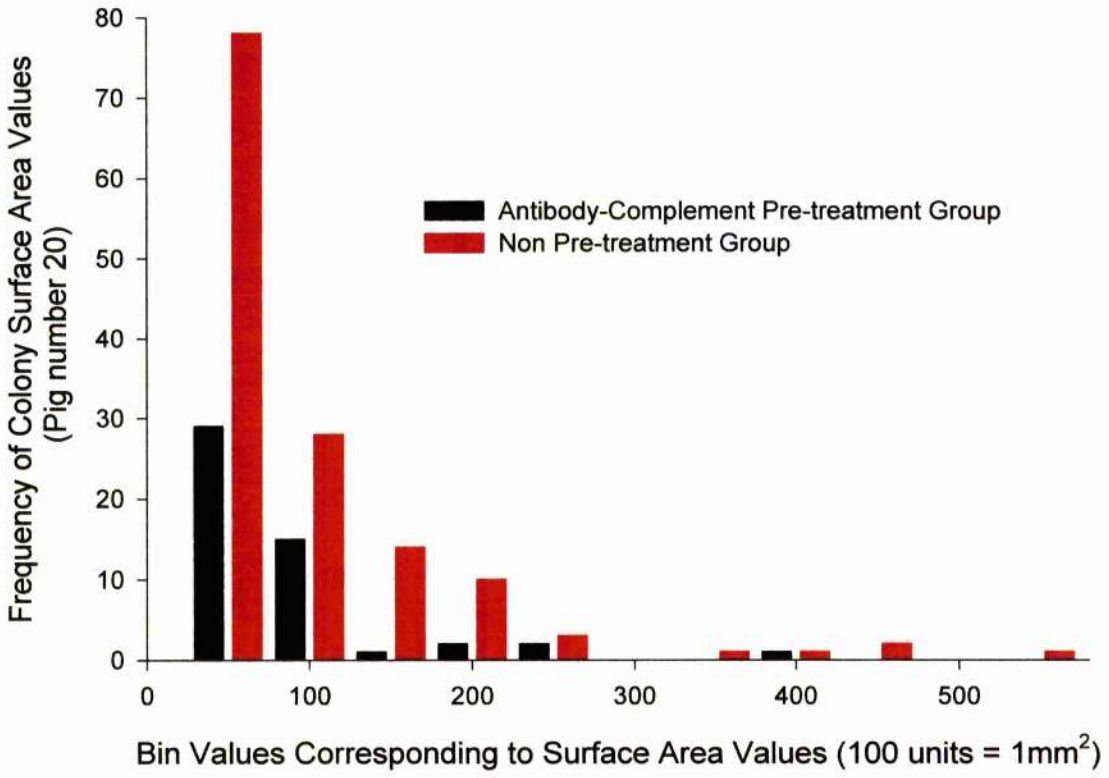


Figure 4.22: Antibody - complement pre-treatment:
colony forming efficiency study (pig no. 20) – analysis of colony surface areas

Following the experiment described in the legend to Figure 4.14, wells were scanned and digitally analysed with Image Pro Plus® software with respect to numbers and size of colonies. The graph illustrates the distribution of colony surface areas from pig number 20 for all the colonies from the antibody-complement pre-treatment group and the largest 60 colonies from the non pre-treatment group. The smaller colonies are eliminated from this graph in order to focus attention on the pattern of distribution of the larger colonies.

As seen with pig numbers 17 and 18 (Figures 4.18 and 4.20) the trend is suggestive of consistently more colonies with a larger surface area in the non pre-treatment group.

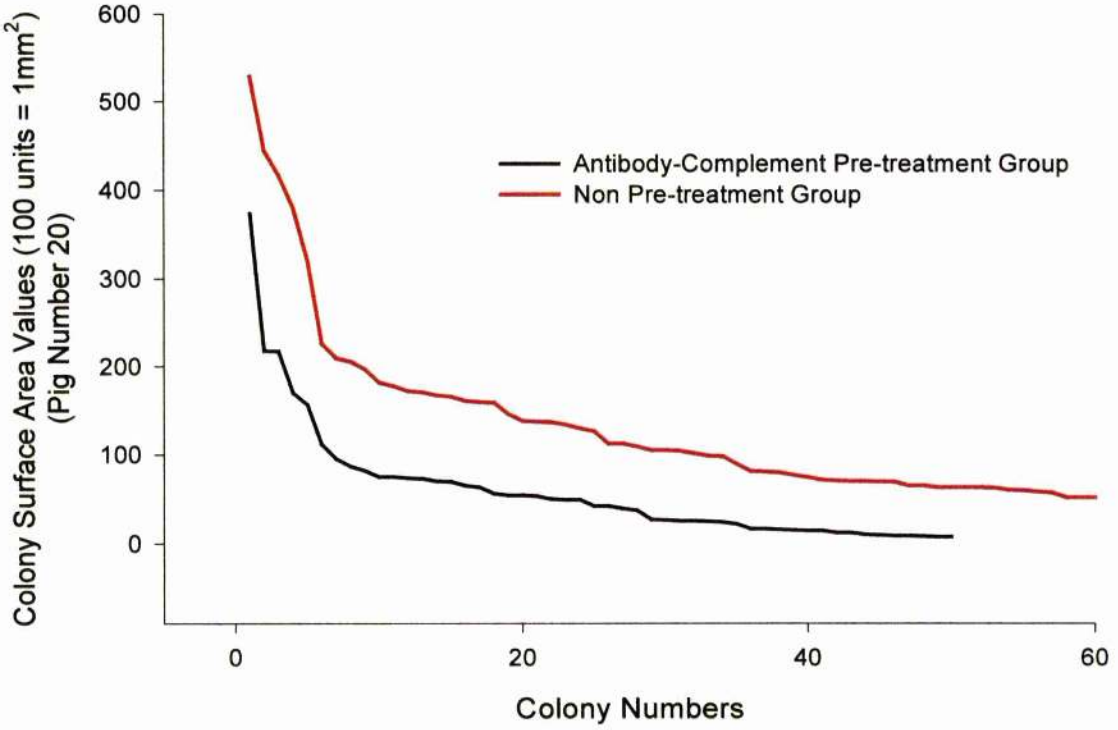
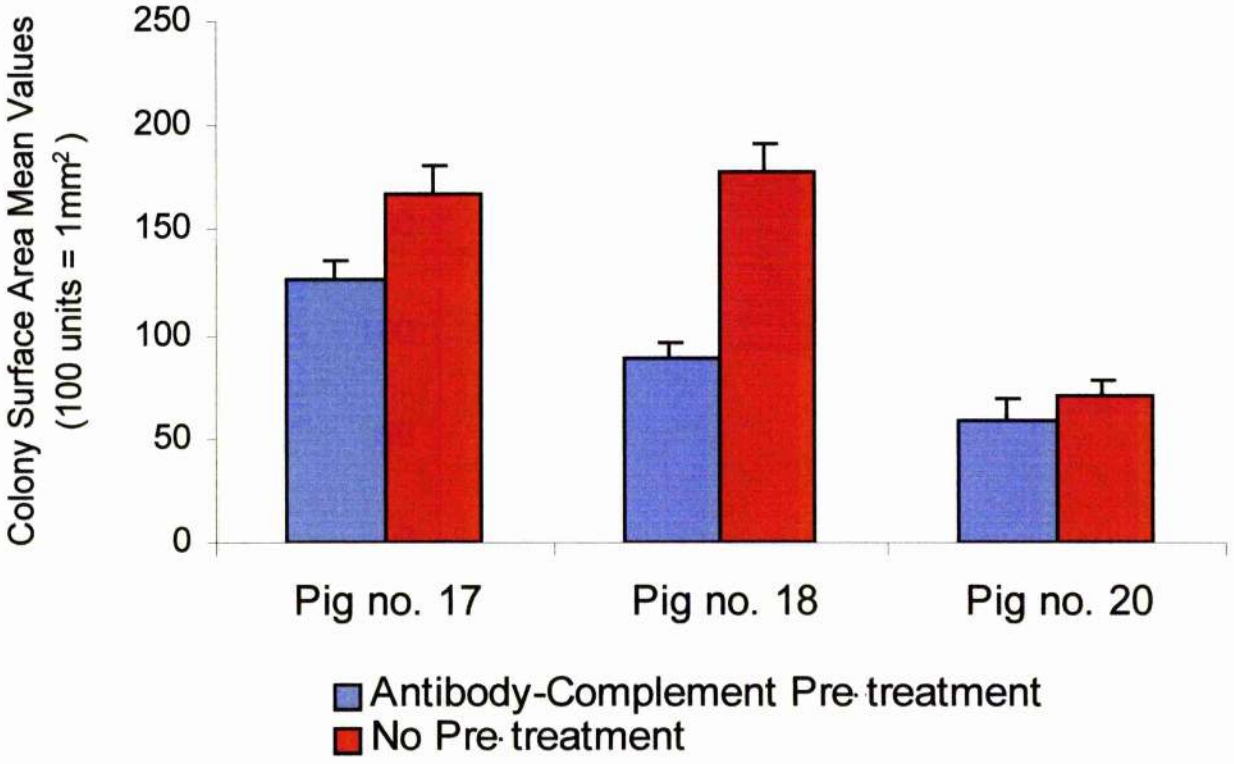


Figure 4.23: Antibody - complement pre-treatment:
colony forming efficiency study: comparison of mean surface area values

This histogram compares mean values of colony surface areas (pig numbers 17-20, experiment described in the legend to Figure 4.14). The wells were scanned and digitally analysed with Image Pro Plus® software with respect to size of colonies.

Using a one way analysis of variance where P is shown to = <0.001, the Tukey test confirms significant differences between the area means of the pre-treatment and non pre-treatment groups in pigs 17 and 18 (31% and 99% higher in the non pre-treatment groups in pigs 17 and 18 respectively). This is confirmed by individual t-tests (P=0.021 for pig no. 17 and P=<0.001 for pig no. 18).



4.2.7.1 Quantitative analysis of colony forming efficiency

Manual Count: The results were analysed (Figure 4.14) using a t-test looking aT-cells from each pig separately and using a one way repeated measures analysis of variance. The t-test demonstrated significant (P =0.002 for pigs 17 and 18 and 0.0026 for pig no. 20) differences between the means of the absolute values between the 'antibody complement pre-treatment group' and the 'no antibody complement pre-treatment group' in each. The one way repeated measures analysis of variance (Tukey test) confirm the t-test results that the increase in numbers of colonies seen in the non complement lysed group (from 26% in pig no. 17 to 226% in pig no. 20) is significant (P=0.004). This was also confirmed by a one way analysis of variance (P=0.008) and Tukey test of the relative values.

Automated Count: Following scanning of the wells (Figure 4.12), they were digitally analysed with Image Pro Plus® software with respect to numbers and size of colonies.

Results suggest (Figure 4.15) significant differences between the means of the two groups (absolute values) for two out of the three pigs (t-test, P=0.035 and P=0.02). One way repeated measures analysis of variance looking at all three pigs demonstrated a significantly (P=<0.001) increased numbers of colonies in the non complement lysed group (of between 18% in pig no.17 to 176% in pig no. 20). This was also confirmed by a one way analysis of variance (P=0.038) and Tukey test of the relative values.

As with a different study (comparing feeder cell types see 3.3.1.1) the colony counts observed manually were higher than those observed by the image analyser (Figures 4.14 - 4.15). These differences may be explained in that with the naked eye it is possible to differentiate between genuinely large colonies and those that *appear* to the image analyser to be single and large but are in fact several small confluent colonies. Attempting to manipulate the image analyser to obtain a higher level of discrimination was extremely difficult giving rise to inconsistent results and was therefore abandoned. Random wells were double checked to further ensure consistency. In spite of the apparent differences in absolute values between the two methods of counting, there are no significant differences in the relative values between the groups (Figure 4.16).

The results of counting the colonies suggest strongly that keratinocytes that have undergone treatment with antibody and complement have an inferior colony forming ability as result.

4.2.7.2 Analysis of colony size

Following scanning of the wells (Figure 4.12), they were digitally analysed with Image Pro Plus® software with respect to numbers and size of colonies. For both pigs nos. 17 and 18 there are significantly greater mean values of colony areas, as measured by the image analyser, of the no antibody-complement pre-treatment groups (31% and 99% respectively greater than the antibody-complement pre-treatment groups). This is confirmed using the t-test (P=0.021 for pig no. 17 and P=<0.001 for pig no. 18) and the one way

analysis of variance between the groups (P=<0.001, Tukey test) (Figures 4.17 - 4.20 and Figure 4.23).

The control and treatment groups were treated identically except that the anti-SLA DR monoclonal antibody was replaced by an isotype control. This would suggest that antibody-complement pre-treatment negatively impacts the colony forming efficiency of keratinocytes in culture both in terms of numbers of colonies and size of colonies formed.

4.3 Discussion

The use of antibody-complement pre-treatment to eliminate passenger lymphocytes is not new (Faustman et al., 1981; Faustman et al., 1984; Sone et al., 1987; Brewer et al., 1989a). Its use however with regard to cultured epithelial allografts has not previously been reported.

The above series of experiments demonstrate that antibody-complement pre-treatment is efficacious with regard to epidermal cell suspensions. They demonstrate the need for incubation in order to maximise LC upregulation of MHC II membrane determinant expression. Complement from a variety of animal sources were tested and found to be equally efficacious. Equally, there was no apparent difference between complement types in terms of non-specific cell death.

The effect of antibody-complement pre-treatment on the ability to form and expand colonies does however appear to be detrimental. Both the number and the type of colony formed in terms of size and shape were affected. This would suggest that keratinocyte clonogenic potential (Barrandon and Green, 1987) is affected by pre-treatment. The keratinocytes tested were P3 (passaged 3 times) following complement exposure. One explanation might be that the antibody-complement pre-treatment process prematurely ages the keratinocytes. This may be related to damage caused by released C5b67 complexes formed following complement activation which can bind to "innocent bystander" keratinocytes (Kuby, 1997). This may have implications for grafting in that the pre-treated grafts might survive for a significantly shorter period. Premature ageing of the keratinocytes would defeat the

purpose of antibody-complement pre-treatment which is to prolong survival following grafting by eliminating the APCs.

Some earlier studies have demonstrated the capacity for significant Langerhans survival in human epidermal cell culture (Demidem et al., 1986). Future work might focus on whether or not LC have the capacity to survive under the particular keratinocyte culture protocols relevant to the porcine model in the present study. If they do survive, it will be important to determine any alterations in LC phenotype (Teunissen et al., 1990; Witmer-Pack et al., 1988) and whether these are reversible. It remains unclear as to the mechanism by which the anti-SLA DR antibody and complement affects keratinocytes. It would therefore be of interest to isolate the particular element within the process of antibody-complement pre-treatment that may be detrimental to keratinocyte population expansion and clonal growth.

Chapter 5

Immunophenotyping skin in the Large White pig

5.1 Introduction

It is generally well accepted that acute rejection of allogeneic skin grafts occurs following 'direct' sensitisation, that is by donor dendritic cells, centrally in a regional draining lymph node, and involves an allogen directed response (Schuurman, 1994; Rosenberg and Singer, 1992). Besides allospecific cytotoxic T-cells, a highly destructive non-specific inflammatory infiltrate is also thought to be involved (Doody et al., 1994) consistent with the effect of inflammatory cytokines released by activated T-cells (Rosenberg and Singer, 1992).

Cultured allogeneic keratinocyte grafts are thought to be devoid of LC (Morhenn et al., 1982; Hefton et al., 1984; Demidem et al., 1986; Thivolet et al., 1986). Some researchers felt that rejection therefore probably did not occur (Hefton et al., 1986; Thivolet et al., 1986; Madden et al., 1986; Gielen et al., 1987). Subsequently overt rejection was demonstrated in humans (Aubock et al., 1988) and pigs (Carver et al., 1991c), and others demonstrated early loss of donor DNA from recipient tissue (Burt et al., 1989; Brain et al., 1989).

These apparently conflicting reports are consistent with variable graft bed and culture conditions and the difficulty of differentiating between an immunologically mediated event and keratinocyte death due to natural differentiation pathways with failure of donor stem cells to function effectively.

In the absence of donor dendritic cells, infiltration of the graft bed by host APCs may initiate rejection. These may then present antigen either to locally infiltrating memory T-cells (peripheral sensitisation) or more likely migrate to regional draining nodes to present there (central sensitisation) (Mason and

Morris, 1986). In either case the evidence from CD4 and CD8 T-cell depleted models suggests that cytotoxic CD8-positive cytotoxic T-cells are the principle effector cells generated (Rosenberg and Singer, 1992). Where foreign MHC class II determinants are present a highly destructive massive inflammatory infiltrate is generated (Doody et al., 1994; Rosenberg and Singer, 1992). Where they are not, the cytotoxic T-cell response may be delayed and attenuated (Rosenberg and Singer, 1992). The cytokines released by infiltrating activated T-cells giving rise to the inflammatory response may thus also be reduced. This may result in prolonged graft survival. Residual host epidermal remnants within a dermal graft bed may thus very gradually replace graft elements.

To help understand the events associated with rejection of cultured keratinocyte allografts it may be useful to immunophenotype or examine the phenotype of infiltrating cells at meaningful time points.

There are many different ways of identifying infiltrating cell types. These include light and electron microscopic morphological examination, cytokine profiling, functional studies including observations of cell-cell interactions and stimulus response studies and examination of cell membrane molecules. This last method has been used extensively in recent decades to classify leucocytes according to their surface molecule expression. An understanding of the function of these surface molecules helps elucidate the function of cells that express them.

Early work on immunophenotyping included cytotoxicity assays that helped delineate T from B cells and discover T-cell subsets (Kisielow et al., 1975; Barclay et al., 1997; Shiku et al., 1975). Further advances were made with

affinity purification of $F(ab')_2$ antibody fragments which allowed saturate binding and the development of flow cytometry (Bonner et al., 1972) and Western or immunoblotting (Barclay et al., 1997; Shiku et al., 1975). These enabled binding serology to replace cytotoxicity assays to facilitate a quantitative and biochemical approach to the cell membrane.

A major remaining problem was how to produce specific antibodies against a single cell surface molecule. Immunisation across a species barrier tended to produce antibodies active against the variety of xenogeneic cell surface molecules. This problem was resolved in 1975 with the development of the hybridoma method which enabled production of monoclonal antibodies with predefined specificity (Kohler and Milstein, 1975; Shiku et al., 1975). Use of the hybridoma method quickly expanded to include analysis of xenogeneic cell surfaces using monoclonal antibodies. Flow cytometric studies on human antigens formed the basis for grouping antigens in "Clusters of Differentiation" or CD antigens (Shiku et al., 1975). Monoclonal antibodies (moAB) resulting from different hybridoma cell lines were considered to label the same CD antigen if they produced the same pattern of staining on differenT-cell types (Barclay et al., 1997). Workshops were co-ordinated to standardise and formalise the testing and naming of moABs against CD determinants on human leucocytes.

Monoclonal antibodies reactive with porcine leucocytes were first reported in 1984 (Pescovitz et al., 1984; Jonjic and Koszinowski, 1984). The First International Swine CD Workshop took place between 1989 and 1992 with proceedings published in 1994 (Lunney et al., 1994b). Although pigs had served as allotransplantation models with regard to a variety of organs

(Kenmochi et al., 1994) including skin (Carver et al., 1991), the search for moABs was boosted by renewed interest in the pig as a potential xenograft donor (Sachs, 1994). The need for porcine-specific reagents is exemplified by the relative paucity of species cross reactivity between human and pig (Sopp et al., 1998). Two further International Swine CD Workshops have been organised and proceedings published (Saalmuller et al., 1998b; Haverson et al., 2001). We now have reagents to 27 porcine homologues of human CDs and to 9 swine-specific clusters, as well as to other cell surface molecules such as MHC determinants and surface immunoglobulin. As agreed at the Second International Swine CD Workshop: "*swine CD numbers are given to clusters of moAb to swine orthologues of human CD molecules when homology is proven by (1) suitable tissue distribution and lymphoid cell subset expression, (2) appropriate molecular mass of the antigen recognized by the moAbs, and (3) reactivity of moAbs with the cloned swine gene products, or cross- reactivity of the moAb on the human gene products. In some cases, this reactivity would not be fully proven, mainly due to the lack of cloned gene products; for these CD antigens, the respective clusters will be assigned by the prefix 'w' which will lead to 'wCD' antigens*" (Saalmuller et al., 1998b).

The two principle means of immunophenotyping a graft bed infiltrate are flow cytometry or immunohistochemistry. Advantages of flow cytometry include sensitivity and reliability of the technique (all available swine moABs have been tested with this technique) as well as the relative lack of background staining. One disadvantage is the lack of spatial visualisation of cellular relationships.

Immunohistochemistry on the other hand allows differential examination of cells within a 2-dimensional (or 3-dimensional with con-focal microscopy) context. A disadvantage is that not all the moABs that work well with fresh flow cytometric preparations, work with fixed cryostat preparations. The available panel of monoclonal antibodies available for immunohistochemistry is therefore smaller than that for FACS.

One of the eventual goals of this study was to examine the wound infiltrate beneath LC depleted cultured allogeneic grafts at various time points immunohistochemically, employing a panel of porcine-specific moABs in order to understand the immunological events involved in their rejection. Immunohistochemistry might thus allow quantification of infiltrating leucocyte subtypes as well as representation of spatial interrelationships between leucocyte subtypes in allografted skin wounds. Before this however, it was important for two principle reasons to determine the immunohistochemical activity of each antibody in the present population of animals.

The first is that all the available antibodies were shown to be effective with flow cytometry, but only a proportion have been demonstrated to be effective immunohistochemically. Indeed, antibodies from several sources were tested to determine an effective monoclonal antibody for certain of the antigen-specificities (CD2, CD3 and CD4).

The second reason concerns swine heterogeneity. Although these moABs were determined to be effective in swine it was felt important to ensure, particularly as they are so newly established, that they label effectively in the Large White pig population employed in the present study.

Porcine jejunum was used as a positive control since this has a well characterised lymphoid population (Haverson et al., 2000) (Vega-Lopez et al., 1993; Stokes et al., 1996). Additionally, peripheral porcine blood leucocytes were used as a positive control.

Following confirmation that staining with the panel of moABs conforms to the established pattern, it was important to characterise the baseline leucocyte population in normal porcine skin so as provide a basis for comparison with regard to later analyses of grafted wounds.

The aims of this study were therefore:

- To confirm the immunohistochemical activity of each antibody, using porcine jejunum and PBLs in the population of animals used in this research program.
- To type the baseline leucocyte population within porcine skin.

5.2 Results

Jejunum, a positive control for many of the determinants tested (Vega-Lopez et al., 1993); (Haverson et al., 1997; Haverson et al., 2000) was harvested from a female Large White pig aged 11 weeks following the termination of an experiment (see Chapter 2 for method).

Blood was harvested from female Large White pigs aged 12-13 weeks and weighing approximately 65Kg at termination. Peripheral blood mononuclear leucocytes were extracted from the blood kept in citrated phosphate dextrose at room temperature for up to 48 hours.

Whole skin was obtained from two female Large White pigs aged 12-13 weeks and weighing approximately 65Kg (see materials and methods).

Following primary labelling with each of the sixteen monoclonal antibodies (table 5.1) the jejunal and skin sections were then secondarily stained using the immunoperoxidase avidin biotin complex (ABC) method (with or without Xgal). Sections stained with moABs specific for CD4 and IgλLC were however secondarily stained using immunofluorescent techniques (see Chapter 2 for method). Skin sections were always stained in conjunction with jejunal sections (positive controls) as well as with skin section negative controls. All peripheral blood mononuclear leucocyte samples were stained secondarily using FITC and counterstained with propidium iodide (see Chapter 2 for method). Table 5.2 gives the expected cellular distribution as well as function of the CD antigens (which may be detected using the monoclonal antibodies in Table 5.1).

Table 5.1: This table indicates the cell lines and their source for the "panel" of monoclonal antibodies tested

moAB	CELL LINE	SOURCE
CD1	76-7-4	VMRD
CD2	MSA4	Dr. J.K. Lunney, Beltsville, USA
CD3	FY1H2	Dr. H. Yang, Pirbright, UK
CD4	STH293	Dr. M. Shimizu, Ibaraki, Japan
CD5	PG114A	VMRD
CD6	PG90A	VMRD
CD8	PT81B	VMRD
IgλLC	K139.3E1	Serotec
CD25	K231.3B2	Serotec
CD45	K252.1E4	Serotec
SWC3	74-22-15	VMRD
SWC6	MAC320	Serotec
SLA DP	H42A	VMRD
SLA DQ	TH81A5	VMRD
SLA DR	TH14B	VMRD
SLADR	MSA3	VMRD

Table 5.2: This table summarises the expected cellular distribution and function of those membrane determinants tested for with the "panel" of monoclonal antibodies indicated in Table 5.1.

Membrane Determinant	Cellular Distribution	Function
CD1	Dendritic cells, activated monocytes, some B cells, LC	Domain organisation similar to that of MHC Class I may serve to present lipids and peptides to T-cells
CD2	Pan α/βT-cell, NK cells, subset of γ/δ Tcells, (B cells)	Enhances T-cell Antigen recognition
CD3	α/β and γ/δ T-cells	Part of TCR complex (Ag recognition leads to signal transduction and ultimately T-cell activation)
CD5	CD5- population: all the NK activity $CD5^{dim}$ cells: γ/δ cells and the α/β CD4-8^{dim} population $CD5^{bright}$ population: contain the rest	Postulated role in T-cell signal transduction (stimulatory effects of moABs)
CD6	α/βT-cell subsets (all CD4+ cells and the $CD8^{hi}$ cells)	Postulated role in T-cell signal transduction
CD4	Subsets of α/βT-cells	Involved in the recognition by T-cells of foreign antigen associated with MHC class II antigens

Membrane Determinant	Cellular Distribution	Function
CD8	high expression: α/β T-cells with the T suppressor phenotype low expression: up to 60% of peripheral CD4+T-cells (helper T-cells with memory to viral antigens); subset of γ/δ T and NK cells	co-receptor with MHC class I restricted TCRs in antigen recognition
CD45	All cells of haemopoeitic origin except red blood cells	T-cell receptor signalling, may effect activation threshold
CD25	Activated cells including T-cells, B cells NK cells and monocytes	component of the IL-2 receptor expressed on a variety of activated cells
SWC3	High expression: monocytes, macrophages and granulocytes Low expression: dendritic cells	signal regulatory protein receptor associated with SHP-1
SWC6	Null cell: $CD2^-$ surface Ig^- γ/δ T-cell	Not yet known
SLA DR (MSA3)	dendritic cells, B cells, monocytes, macrophages, activated T-cells and endothelium	Present exogenously derived antigen to CD4+ T lymphocytes
SLA DR (TH14B)		
SLA DQ	similar to SLA DR	
SLA DP (equivalent)		

Each of the antibodies in Table 5.1 was tested using porcine jejunum and peripheral blood mononuclear leucocytes. Negative controls were always carried out (not shown).

5.2.1 Immunohistochemical confirmation of monoclonal antibodies using porcine jejunum and peripheral blood mononuclear leucocytes

The results of these tests are detailed below for each antibody.

Panleucocyte (Anti-CD45)

Almost all the PBLs stained positive with the monoclonal antibody (clone K252 1E4, Serotec Ltd, Oxford, UK; (Zuckermann et al., 1998)), (see Figure 5.1(a)).

There was ubiquitous positive staining of cells within all compartments of jejunal villi, (see Figures 5.2 and Figure 5.3(a)).

SWC 3

SWC3 is an epitope conserved on cells of myeloid extraction including macrophages and granulocytes, SWC3 has recently been identified as a signal regulatory protein receptor associated with SHP-1 (Alvarez et al., 2000). The staining pattern of the monoclonal antibody 74-22-15 in PBL appears to be consistent with a study carried out by the source laboratory for the monoclonal antibody in which it was found to stain 12-27% of PBL (Pescovitz et al., 1984).

Within porcine jejunal villi SWC3-positive cells were found principally in the subepithelial region of the lamina propria (see Figure 5.3(a)). Other researchers have reported SWC3+ cells in crypts and to a lesser extent in the villous core (Vega-Lopez et al., 1993). Flow cytometric studies have shown that eosinophils, macrophages and dendritic cells express this marker and are found in this site (Haverson et al., 1994).

T-cells

Staining of PBL for the pan T-cell antigens CD2, CD3, CD5 and CD6 was variable and may reflect their differential distribution (Pauly et al., 1996; Saalmüller and Bryant, 1994; Saalmuller et al., 1994; Yang and Parkhouse, 1996) (Figure 5.1(a) and Figure 5.5).

A differential staining pattern of porcine jejunal villi was also seen. Both CD5 and CD2 were seen to strongly stain cells both within the intraepithelial compartment and lamina propria (core). CD6 and CD3-positive cells were mainly in the intraepithelial compartment (Figures 5.3(b) and 5.3(c)).

Staining of the PBL with antibodies specific for CD 4 and CD8 revealed low proportions of CD4 and CD8-positive cells of varying staining intensity (Figure 5.1(b)) consistent with previously published reports (Yang and Parkhouse, 1996).

The pattern of staining within porcine jejunum proved similar to that reported by others (Vega-Lopez et al., 1993). CD8-positive cells were concentrated around the basement membrane associated with the intraepithelial compartment. CD4-positive cells were located mainly within the core of the lamina propria (Figure 5.3(c)). This was confirmed by double and triple

staining studies (see Figure 5.4) in which CD8-positive cells were mainly intraepithelial with CD45-positive but CD8-negative cells mainly within the lamina propria, with some more apically placed within the intraepithelial compartment.

CD1

Staining of PBL with anti-CD1 revealed low numbers of low intensity staining cells (Figure 5.1(b)). Within the specimens of porcine jejunum examined, cells expressing the CD1 antigen were found to be sparsely dispersed throughout the lamina propria core and additionally were seen around the crypts (Figure 5.3d).

Immunoglobulin (Ig) Light Chain

Although a variety of recent studies have revealed proportions of B cells within PBL of between 8.4% (Boeker et al., 1999) and 17.3-21.3% (Denham et al., 1994), staining of PBL with the antibody derived from K189.3E1, revealed few positively staining cells (Figure 5.1(b)). Recent reports, not available at the time of this study, have found that this antibody is specific for only a subset of Ig lambda chains (Sinkora et al., 2001). In porcine jejunum plasma cells are located mainly in the crypts (Lackovic et al., 1999) (Figure 5.3(d)) (Bianchi and van der Heijden PJ, 1994).

SWC 6

SWC6 is a marker specific for porcine $\gamma\delta$ T-cells, originally termed 'null cells' (Davis et al., 1998). Very few of the PBL stained positively for the SWC6

determinant (Figure 5.1(b)). Other workers however, have reported a population of 16.2 +/- 9.9 % (Boeker et al., 1999) and 30-65% (Binns et al., 1992; Binns et al., 1994) SWC6-positive cells within peripheral blood lymphocytes in young pigs (Binns, 1994).

In porcine jejunum there are few identifiable null cells (Figure 5.3(d)) which accords with the findings of others concerning non circulating SWC6+ cell numbers (Boeker et al., 1999; Binns et al., 1994).

CD25

Within PBL only a small proportion of cells stained positively (Figure 5.1(c)). Published data reveals 5-7% of circulating B cells to be positive (Denham et al., 1994) Within porcine jejunum, CD25-positive cells were evenly distributed within the lamina propria (Figure 5.3(f)) and this is consistent with reported studies (Vega-Lopez et al., 1993).

MHC class II

Staining of PBL with antibodies reactive to different SLA class II determinants revealed an apparently (not formally counted) higher proportion of PBL staining positive for the SLA DR than the other two determinants (Figure 5.1(c)). This is consistent with the findings of others (Lunney, 1994).

Within porcine jejunum MHC class II (SLA DP, DR, DQ see Figure 5.3(e)) expression was found almost exclusively within the lamina propria. It is apparently expressed mainly on endothelium and on cells situated between the capillary network and the core of the lamina propria, identified as

functional dendritic cells (Haverson et al., 2000). There is also low level expression on $CD2^+$ cells in this site (Wilson et al., 1996).

Figure 5.1: Immunolabelling Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells were obtained from fresh whole (Large White pig) blood using density gradient separation medium (see Chapter 2 for method). Cells were then immunolabelled (as described in Chapter 2) using the panel of monoclonal antibodies (see Table 5.1).

Figures (a) – (c) contain photomicrographs (all at X400 except for those of mononuclear cells stained for CD45, SWC3 and SLA DR (MSA3) which are shown at both X200 and X400) of immunolabelled mononuclear cells taken on the day of staining.

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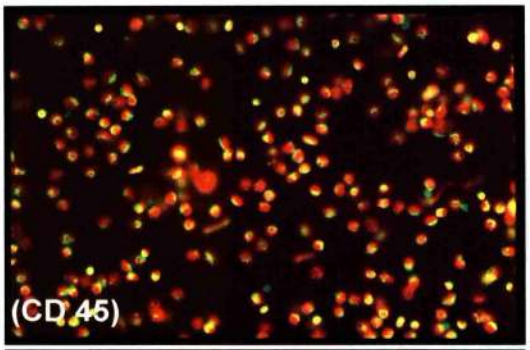

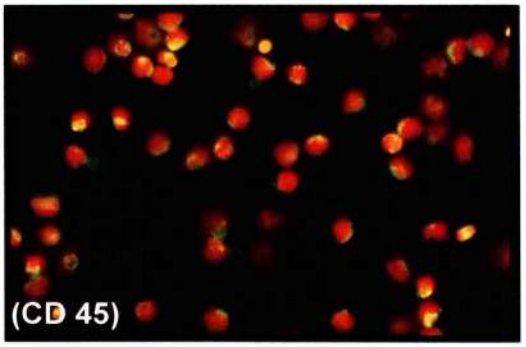

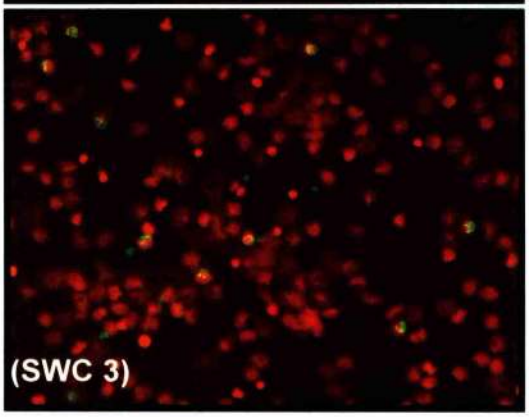

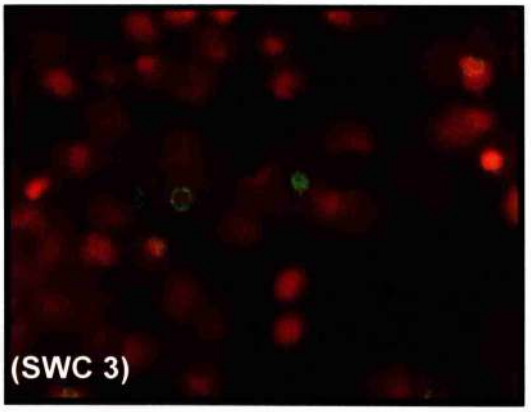

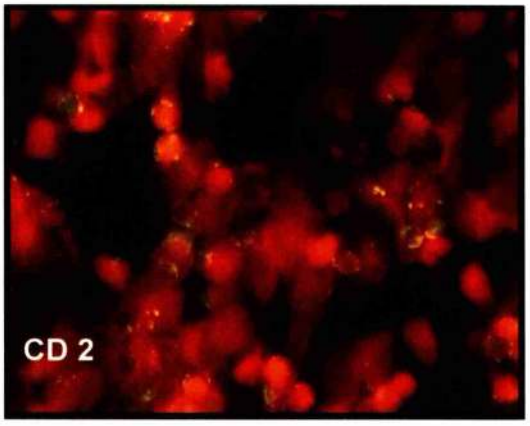

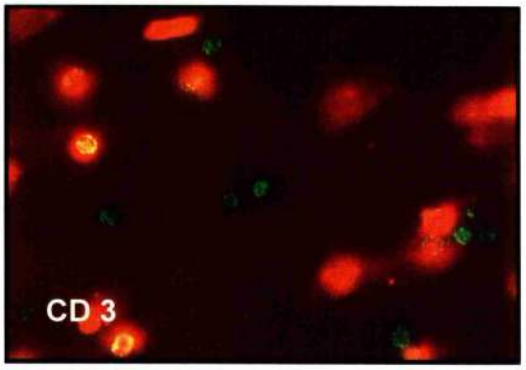

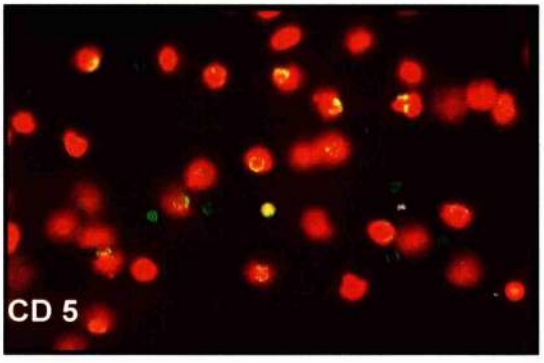

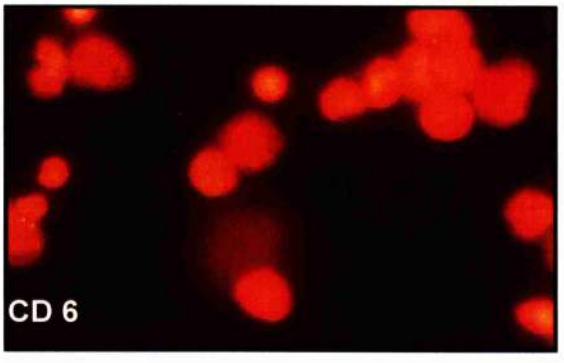

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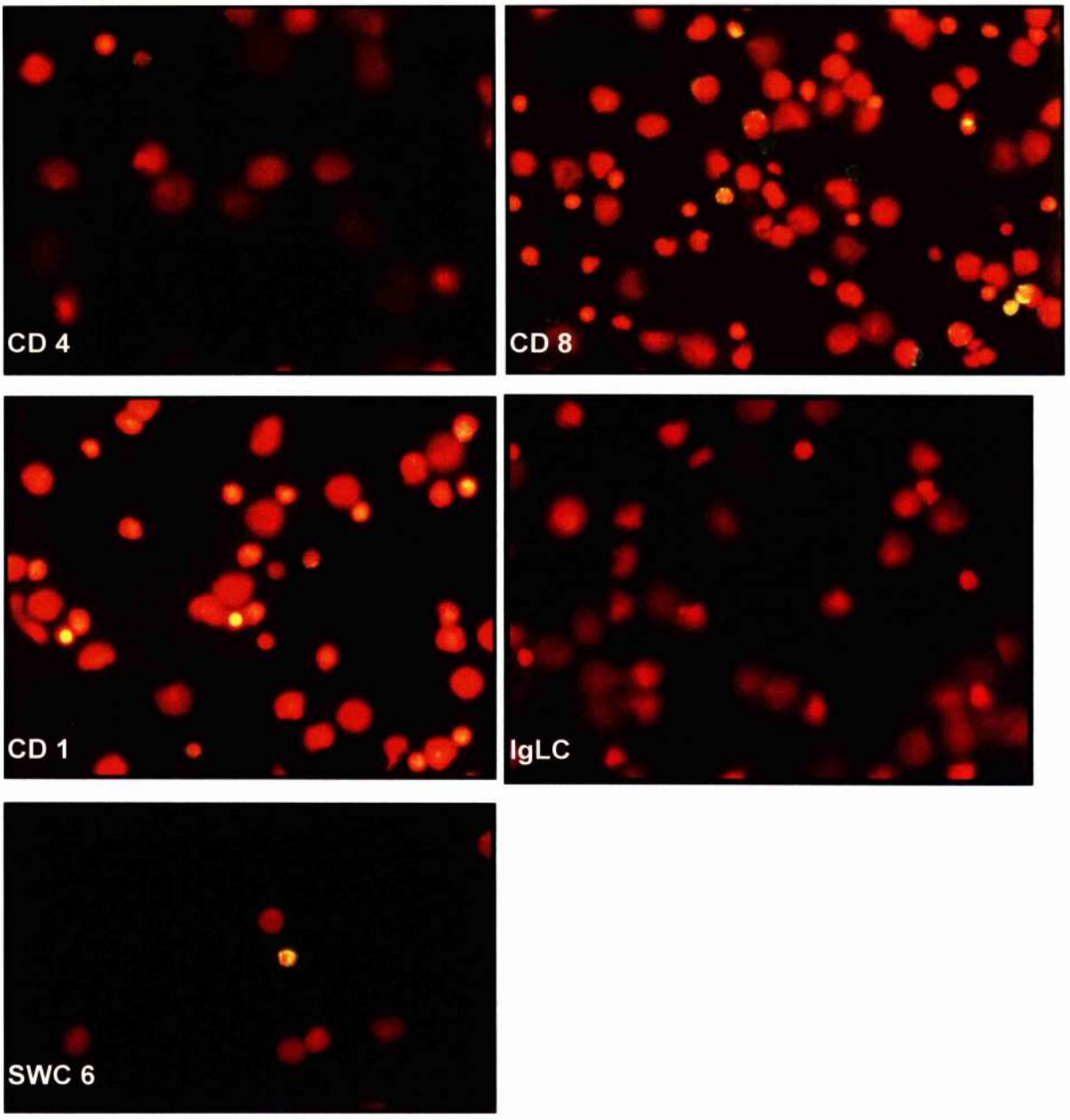

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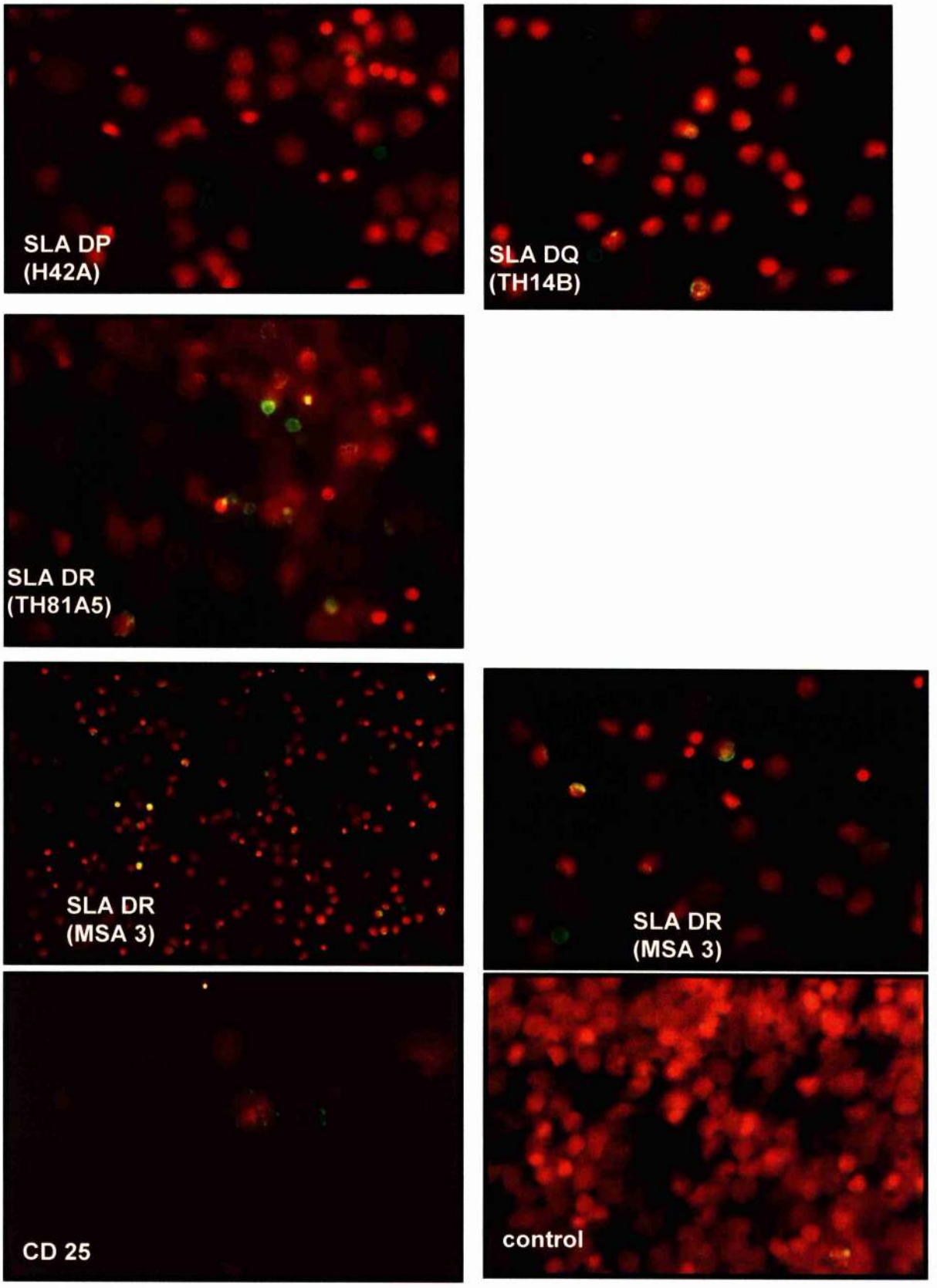

SLA DP
(H42A)
SLA DQ
(TH14B)
SLA DR
(TH81A5)
SLA DR
(MSA 3)
SLA DR
(MSA 3)
CD 25
control

Figure 5.2: Photomicrograph (X40) of an H&E cryostat section of porcine jejunal villus. The adjacent cartoon diagram illustrates the different compartments.

Capillaries lie immediately beneath the enterocytes (epithelium) with small lymphatics draining into lacteals situated closer to the core of the lamina propria. Studies have demonstrated MHC II negative CD2+CD8+ cells lying mainly in the intraepithelial compartment (below the enterocytes adjacent to the basement membrane) with MHC II positive CD2+CD4+ cells lying mainly within the core of the lamina propria (Vega-Lopez et al., 1993). MHC II positive capillary endothelium is present in the sub epithelial lamina propria (Wilson et al., 1996).

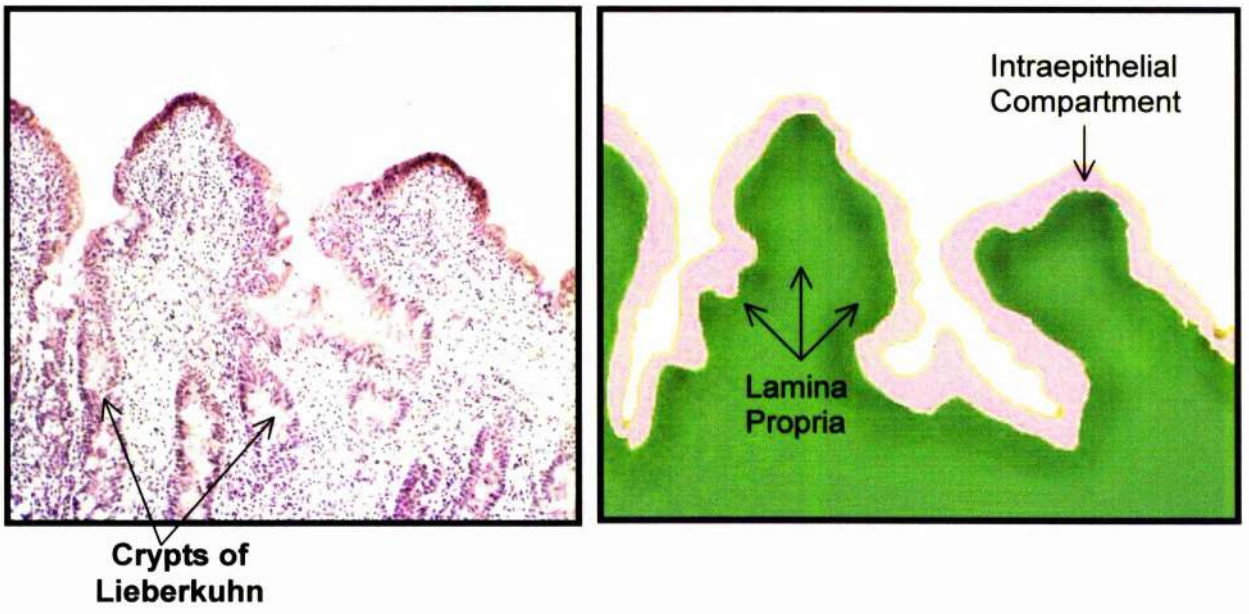
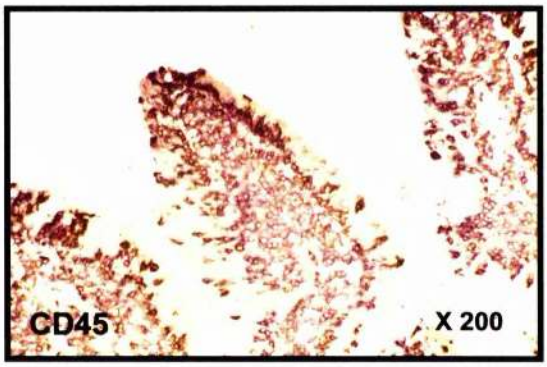


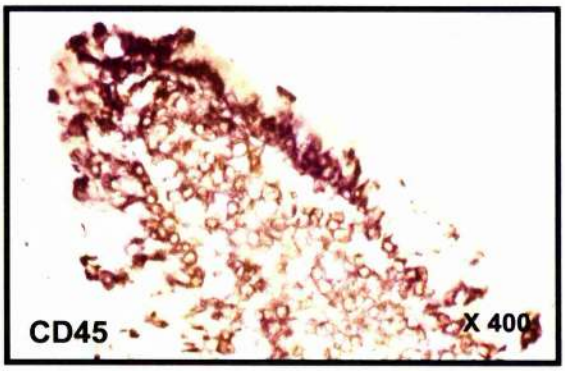
Figure 5.3: Immunolabelling Porcine Jejunum

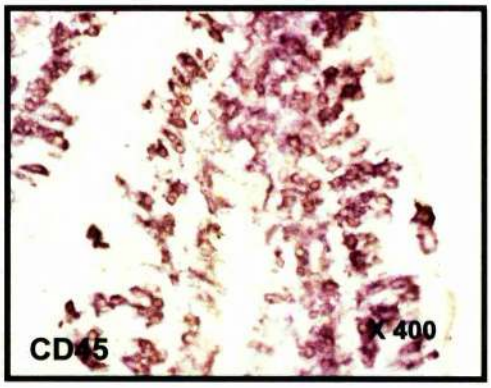
The jejunum was obtained from a single Large White pig approximately 11 weeks old and prepared as described in Chapter 2. Frozen sections were immunolabelled (as described in Chapter 2) using the panel of monoclonal antibodies (see Table 5.1).

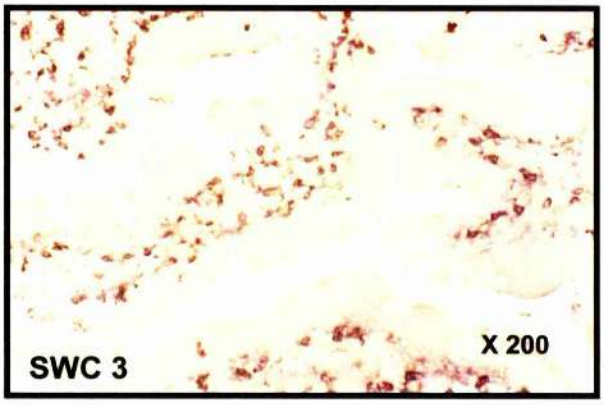
Figures (a) – (g) contain photomicrographs (magnification as labelled) of the immunolabelled porcine jejunal villi (and jejunal crypts with regard to immunoglobulin light chain staining).

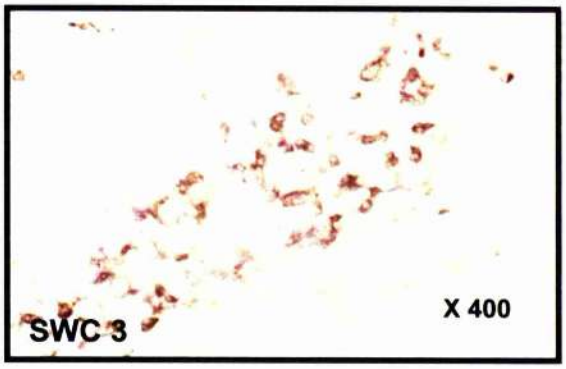
5. 3 (a)











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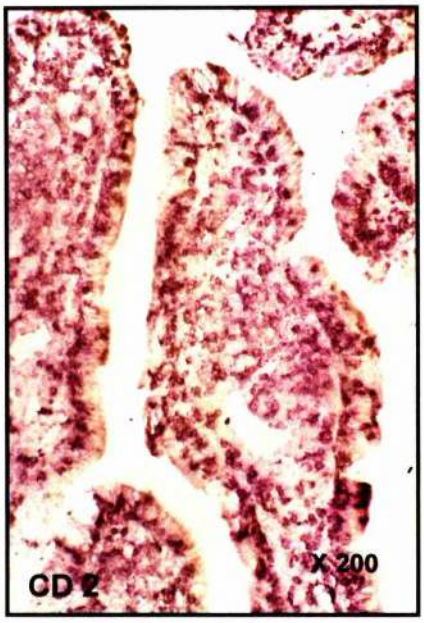

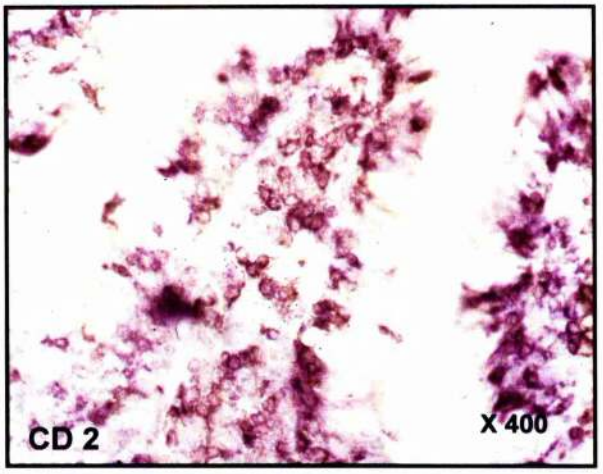

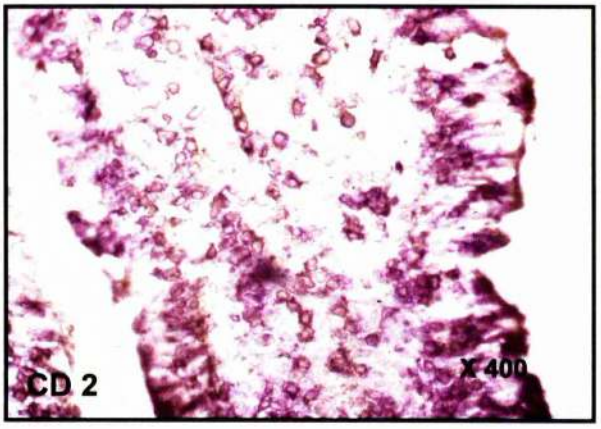

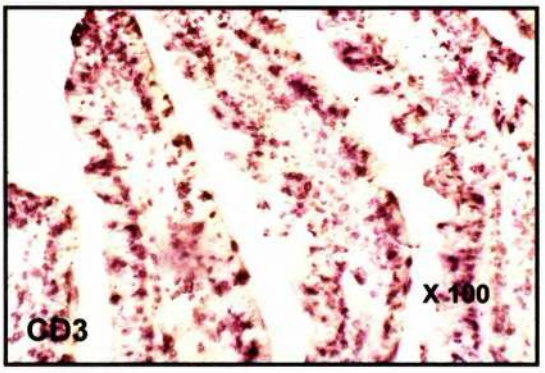

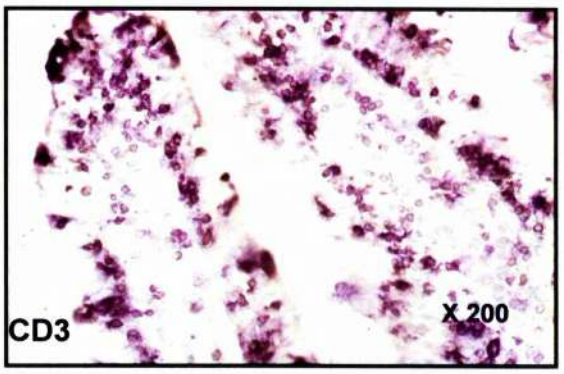

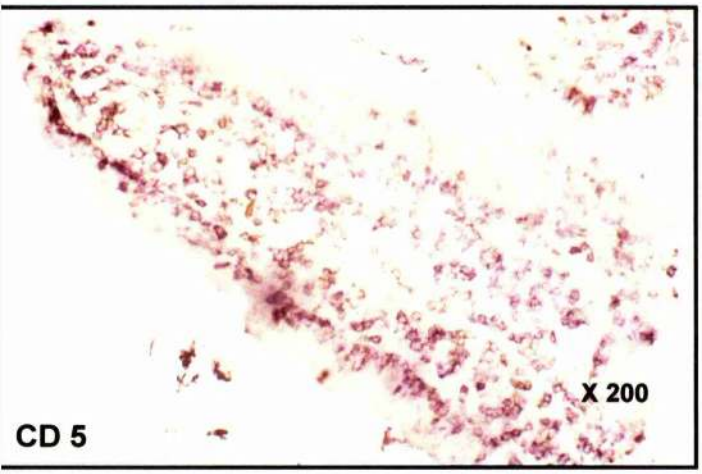

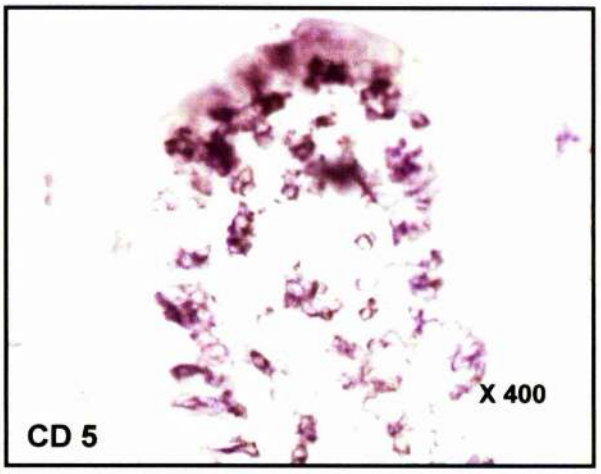

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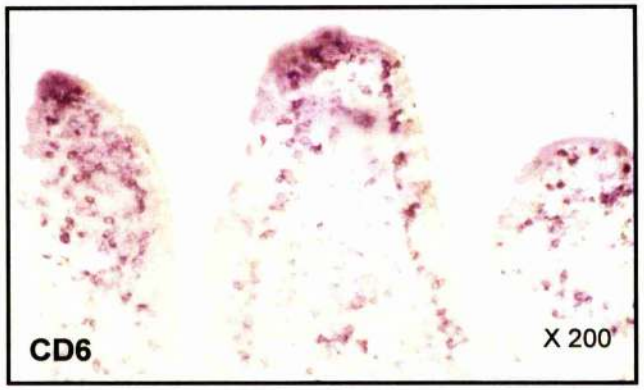

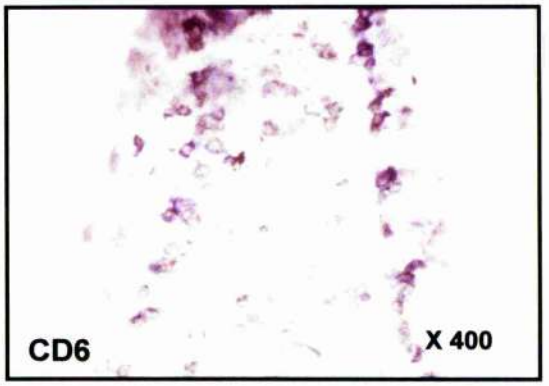

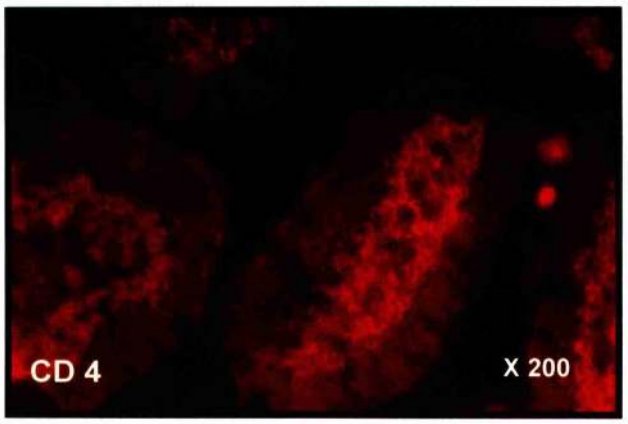

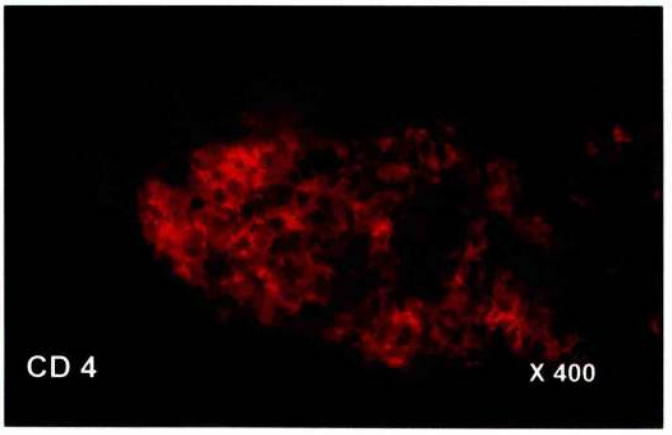

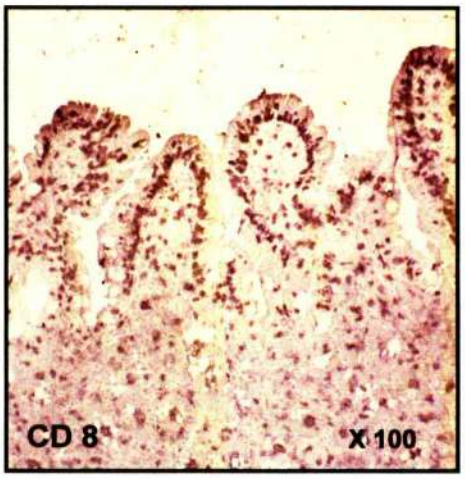

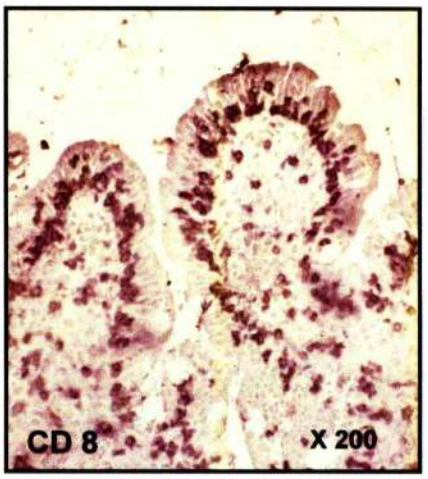

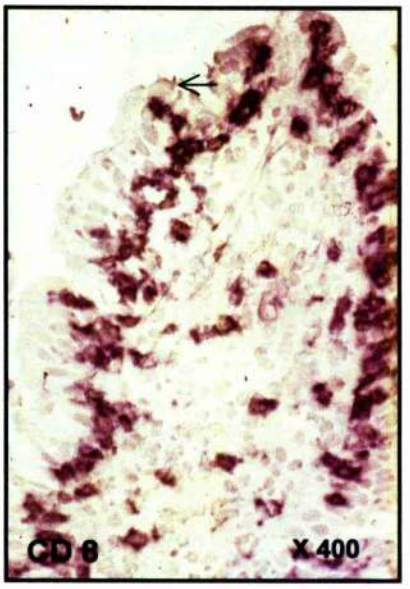

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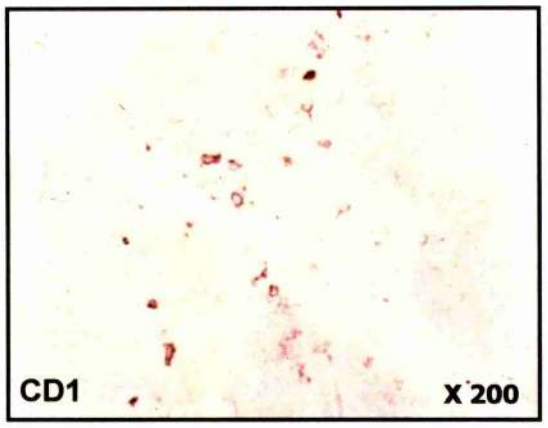

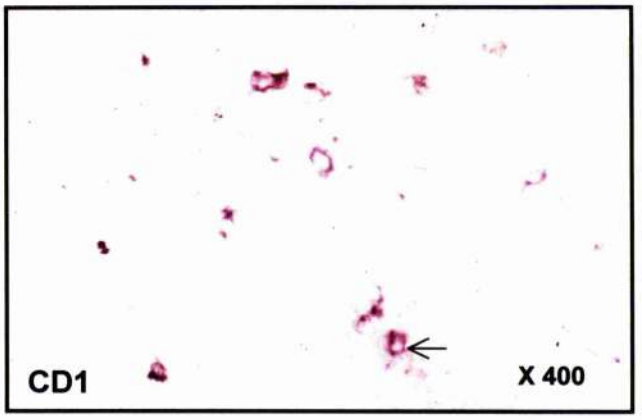

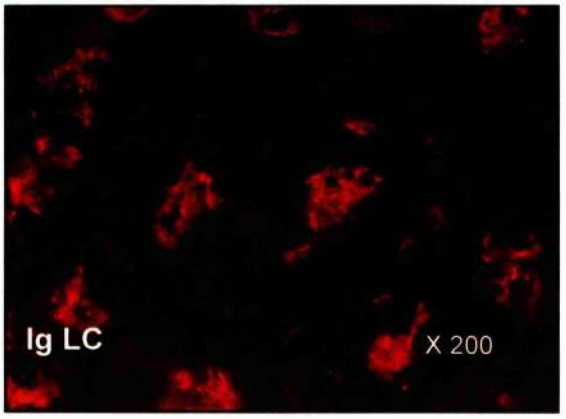

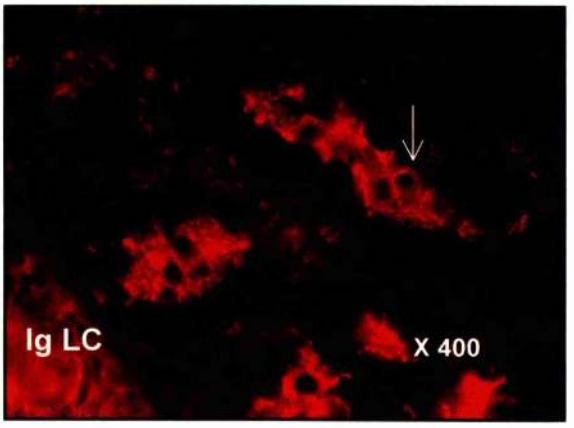

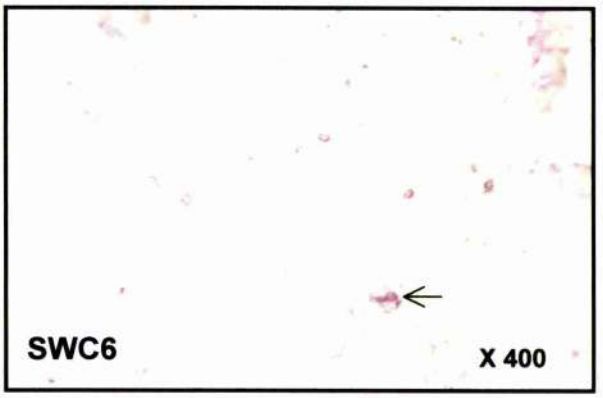

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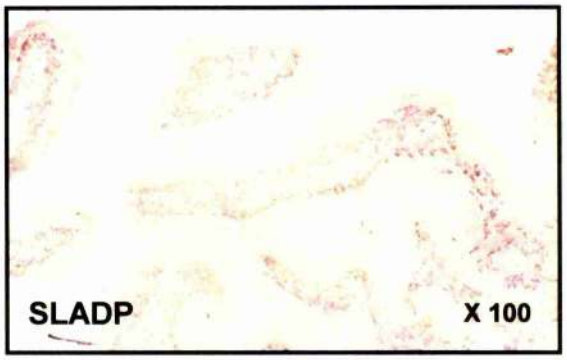
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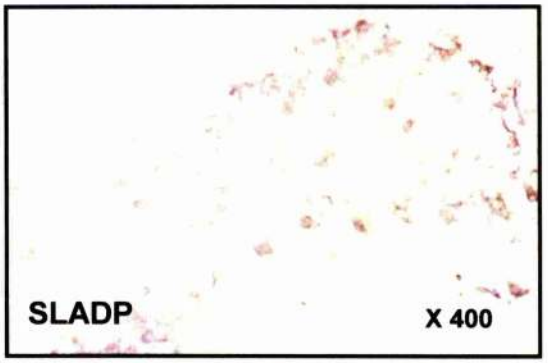
X 100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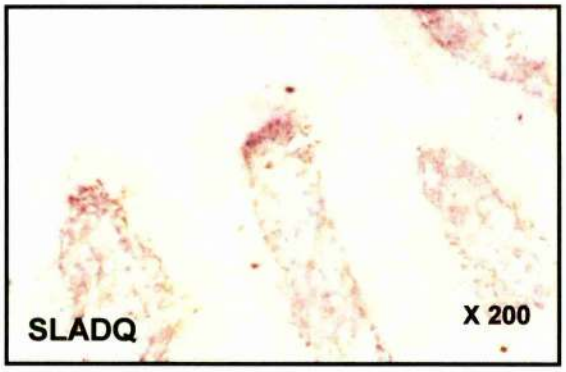
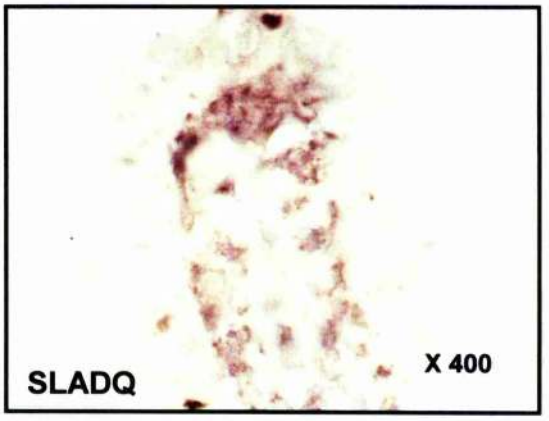
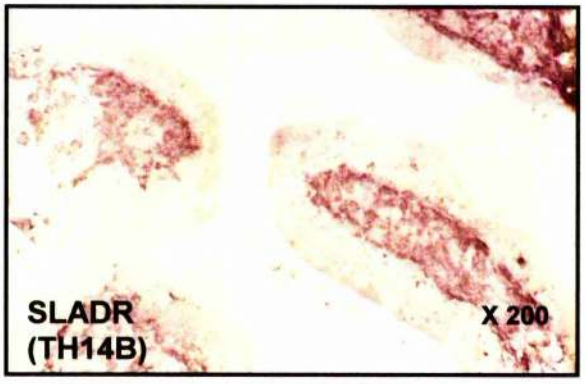
X 400


SLADQ
X 200


SLADQ
X 400


SLADR
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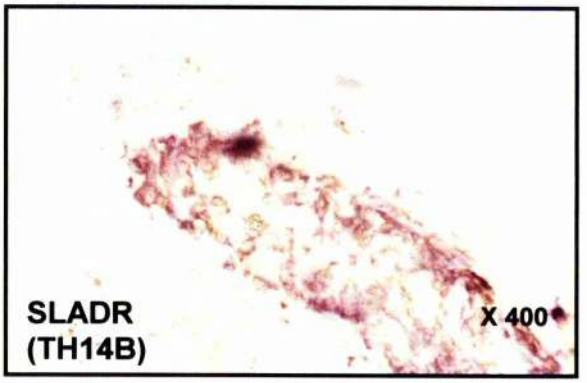
X 200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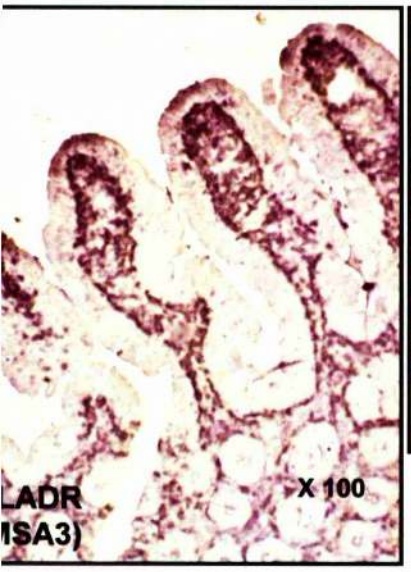
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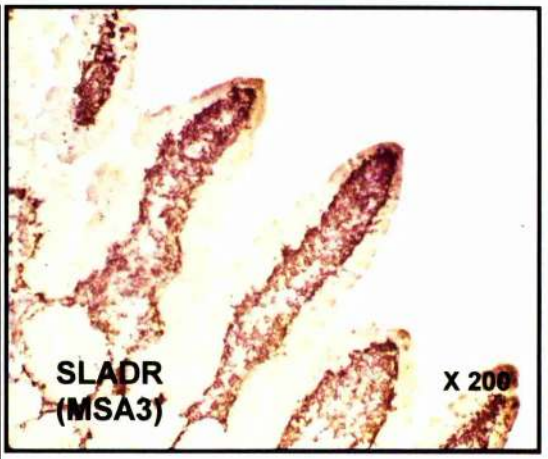
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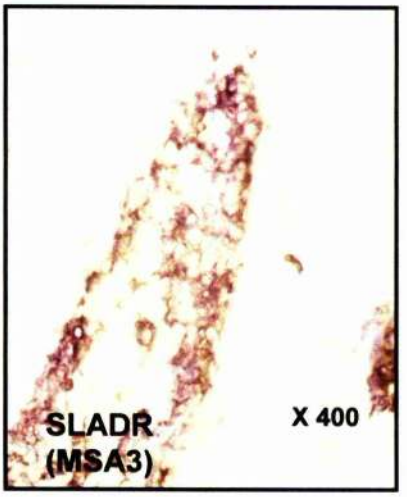
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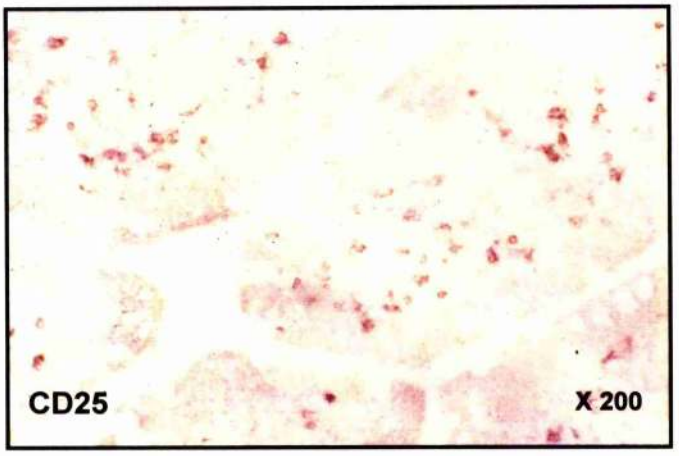
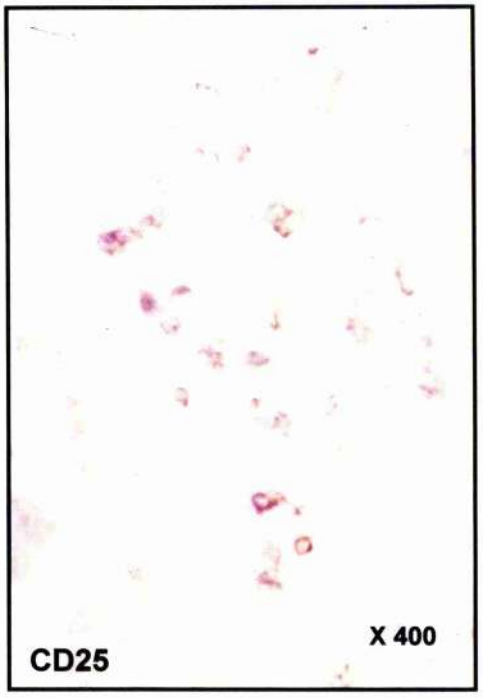
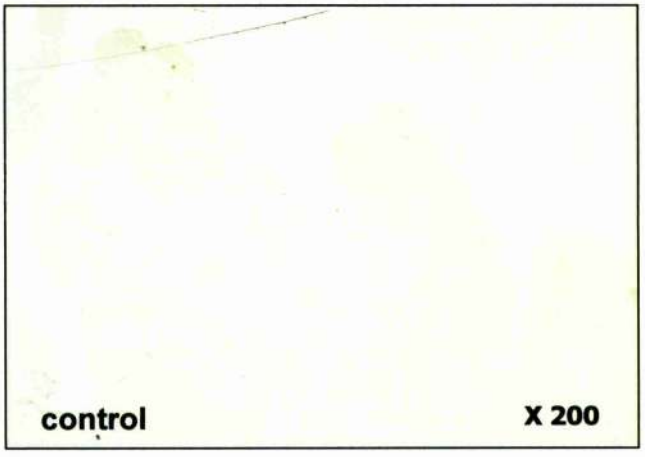
(MSA3)
X 400

5.3 (f)







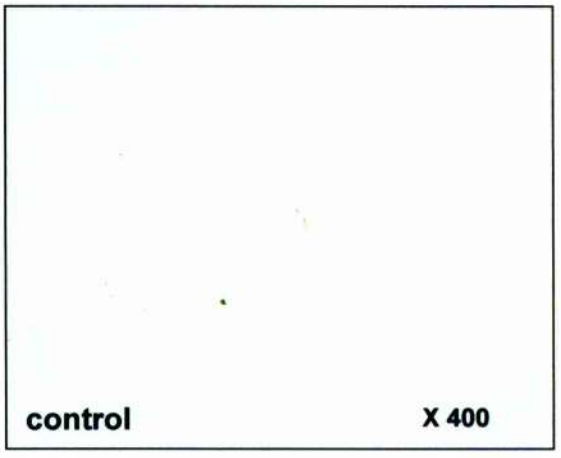


Figure 5.4: Photomicrographs (X400) of dual ((a)-(f)) and triple (g)-(i) labelled porcine jejunum.

Double staining (c) and (f) was achieved with CD45-FITC (b) and SLA DR-Phycoerythrin (a), and CD45-Texas Red (d) and CD 8-FITC (e). Double stained cells (arrows) are yellow-orange ((c) and (f)) and indicate dual CD45 SLA DR (c) or dual CD45 and CD8 expression (f).

Triple staining ((g)-(i)) is shown with CD8-FITC (g), SLA DR-Phycoerythrin (h) and CD45-AMCA (not shown). Cells show either dual CD45 and SLA DR expression (purple) or dual CD45 and CD8 expression (turquoise) or single SLA DR expression (red) or single CD45 expression (blue – not easily seen). This confirms earlier findings with SLADR-positive cells confined to the lamina propria, CD8-positive cells mainly intraepithelial and CD45 cells found in all compartments. Intraepithelial leucocytes (CD 45 expressing) that do not express CD8 (green) are seen in images (f) and (l) (arrow) and these tend to be more apically positioned than the intraepithelial CD8-positive cells.

5.4 (a)-(f)

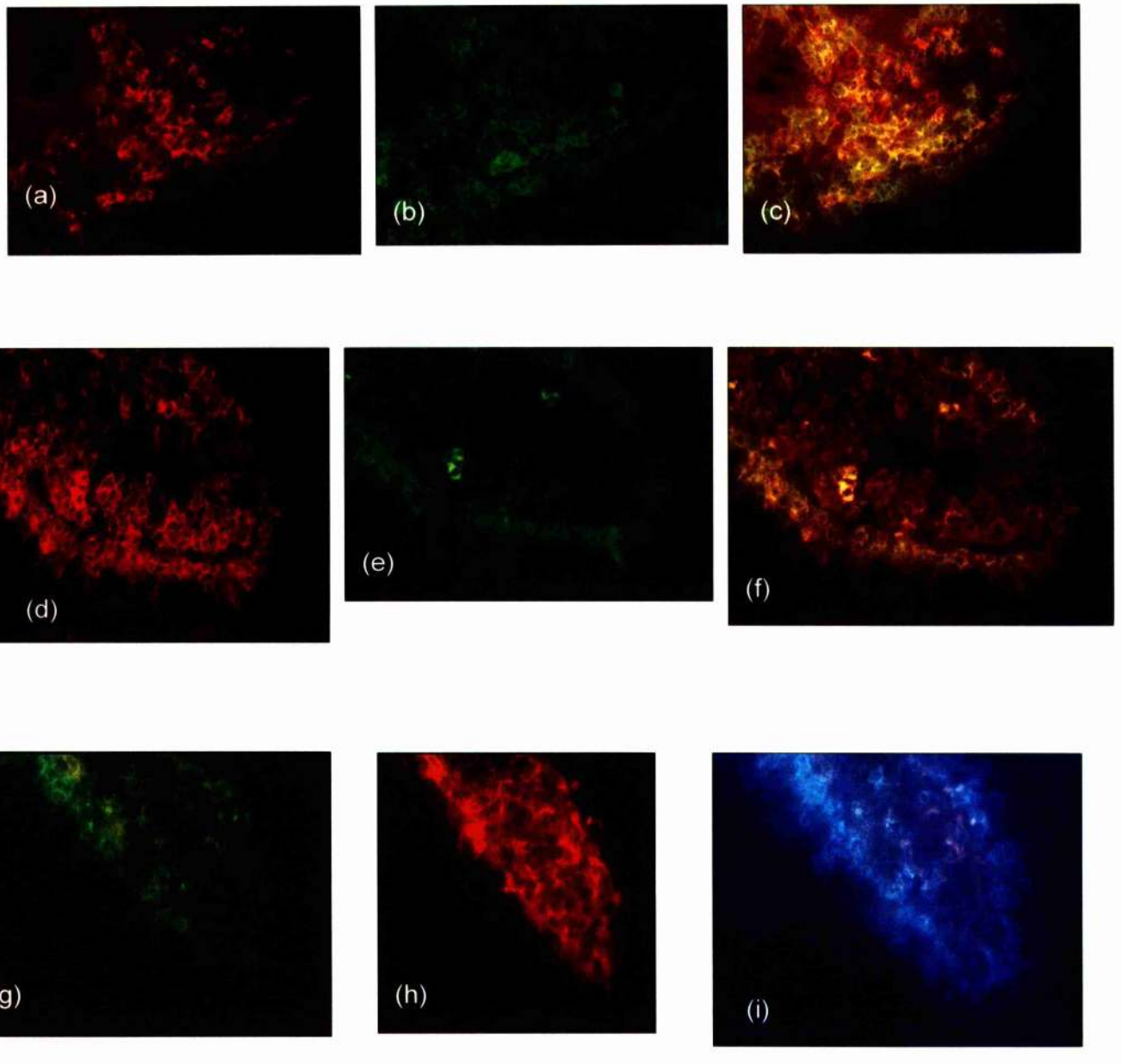
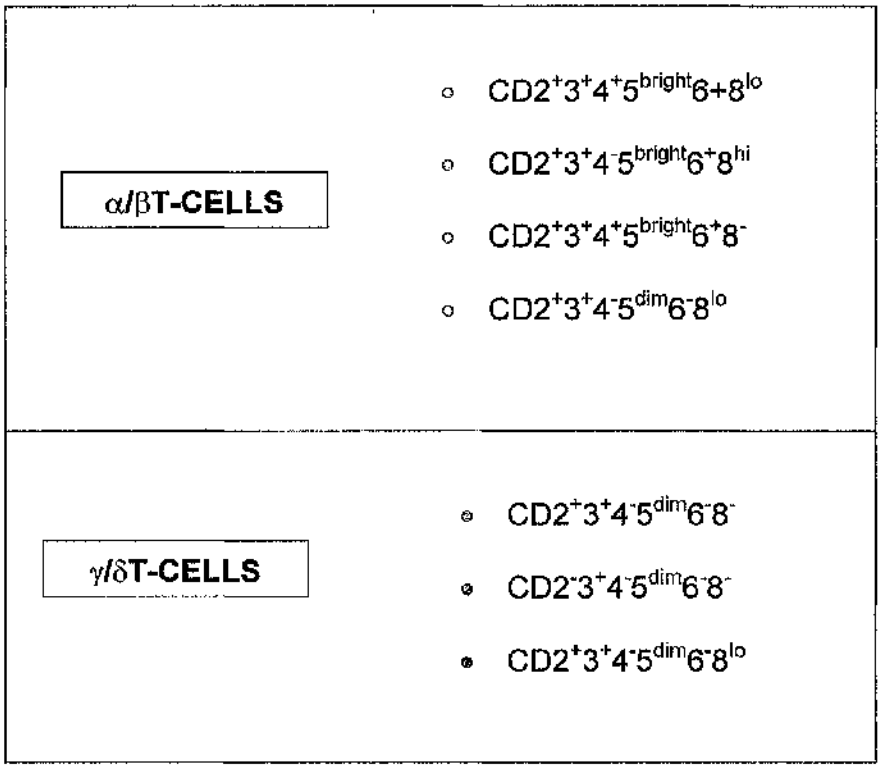


Figure 5.5: Diagrammatic representation of distribution of CD2, CD3, CD4, CD5, CD6 and CD8 antigen expression in porcine peripheral blood lymphocytes.

All the α/β T-cells express CD2 as well as a subset of the γ/δ T-cell population and in addition non-T-non-B lymphocytes with CD2+3-4-8lo surface immunoglobulin-negative phenotype containing natural killer (NK) activity (Pescovitz et al., 1988; Saalmüller and Bryant, 1994; Saalmuller et al., 1994; Saalmuller et al., 1994a; Saalmuller et al., 1994b; Pescovitz et al., 1988).



NK cells $CD2^{+}3^{-}4^{-}5^{-}6^{-}8^{lo}$

In summary all the monoclonal antibodies in "the panel" were shown to label a proportion of peripheral blood mononuclear leucocytes. Table 5.3 contains a summary of the results following labelling and staining of porcine jejunal villi. With minor exceptions these reagents gave results from immunostaining that were comparable to those reported by other investigators (see above).

Table 5.3: This table summarises the distribution of membrane determinants within porcine jejunal villi tested for with the "panel" of monoclonal antibodies indicated in Table 5.1.

Membrane Determinant	**Porcine Jejunal Distribution**
CD45	within lamina propria and epithelium
SWC3	Lamina propria only
CD2	Both within Lamina propria and Intraepithelial compartments;
CD3	predominantly in Intraepithelial compartments, low intensity in LP
CD5	Similar distribution to CD2
CD6	mostly intraepithelial
CD4	Lamina propria almost exclusively
CD8	Mostly adjacent to/Intra epithelial
CD1	Dispersed in Lamina propria
SWC6	Sparse Distribution
SLA DR (MSA3)	Lamina propria only SLADR>DQ>DP equivalents
SLA DQ	
SLA DR (TH14B)	
SLA DP equivalent	
CD25	Lamina propria

5.2.2 Immunophenotyping skin in the Large White pig

Each of the antibodies checked through PBL and jejunal staining were in turn used to examine normal skin from 2 pigs.

Table 5.4 contains a summary of the distribution of cells expressing the antigens described in Table 5.2, following labelling and staining of normal Large White pig skin. The histogram in Figure 5.7 illustrates the relative density of leucocytes following manual counts, with staining patterns illustrated in Figure 5.6.

Table 5.4: This table summarises the distribution of membrane determinants within porcine skin tested for with the "panel" of monoclonal antibodies indicated in Table 5.1, as well as the number of pigs, sections and random fields looked at for each antibody. All were counts were at X200 magnification except where indicated (IgλLC, CD4 and CD1 in blue).

Membrane Determinant	• **Porcine Cutaneous Distribution**	**Number of pigs**	**sections**	**fields**
CD45	• Intraepidermal: similar to CD1, • Dermal: throughout dermis however papillary dermis >> reticular dermis • Also present within sheath of cells around hair follicles	2	3	15
SWC3	• Papillary dermis • Intraepidermal LC cell population	2	2	10
CD2	• Mainly papillary dermis • Occasional Intraepidermal cells	2	2	10
CD3		2	2	10
CD5		2	2	10
CD6		1	1	5
CD4	• Mainly papillary dermis	2	2	11
CD8	• Mainly papillary dermis	2	4	20

Membrane Determinant	• Porcine Cutaneous Distribution	Number of pigs	sections	fields
CD1	• Basal and suprabasal epidermis • Mainly papillary dermis	2	2	11
CD1		2	2	12
IgλLC	• Subepidermal papillary dermis	2	3	15
SWC6	Sparse, seem more concentrated around hair follicles and in papillary ridges	2	2	10
CD25	Sparse, mainly papillary dermis	2	2	10
SLA DP (H42A)	• Intraepidermal LC (but only 'weakly' positive for SLADP • Mainly papillary dermis and around hair follicles • Appearance consistent with expression by endothelial cells in the sub epidermal plexuses (cf. jejunal villi) • Within the reticular dermis SLA DR>DQ>DP	1	2	10
SLA DQ (TH14B)		1	1	5
SLA DR (TH81A5)		1	1	5
SLA DR (MSA3)		1	1	5

Figure 5.6: Immunolabelling Porcine Skin

The skin was obtained from two Large White pigs approximately 11 – 13 weeks old and prepared as described in Chapter 2. Frozen sections were immunolabelled (as described in Chapter 2) using the panel of monoclonal antibodies (see Table 5.1) and subsequently photomicrographed (Figures (a) – (h), power of magnification as labelled). Negative control sections without primary antibody were always performed.

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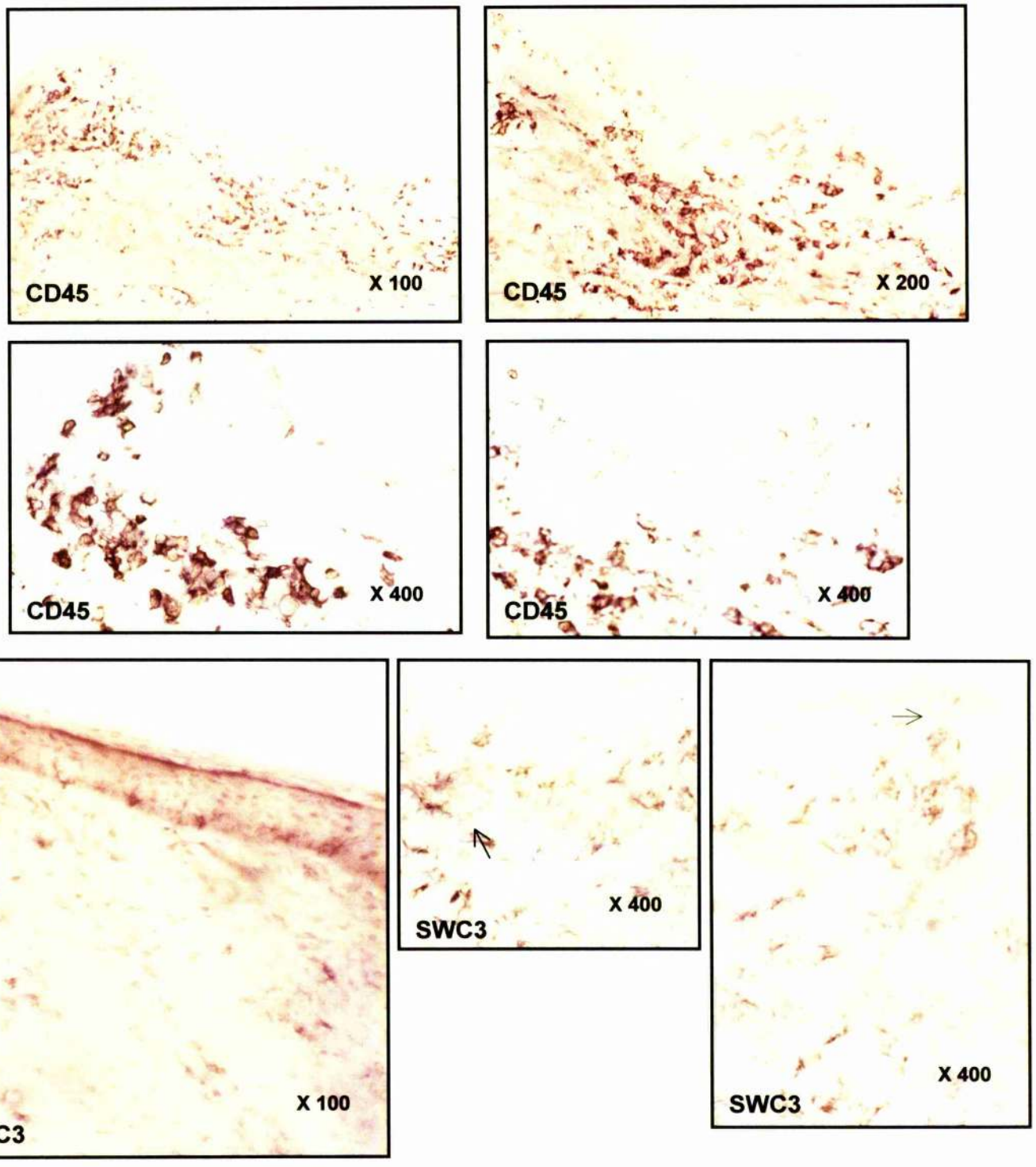
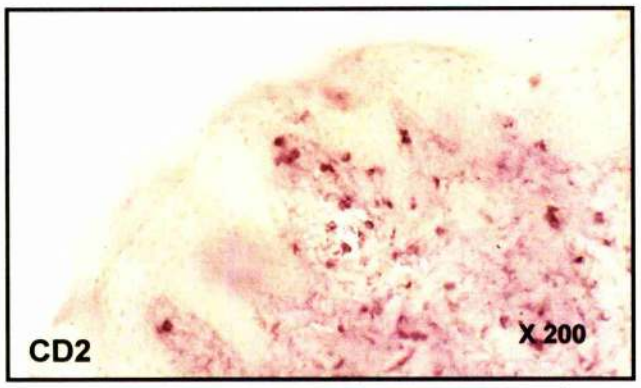
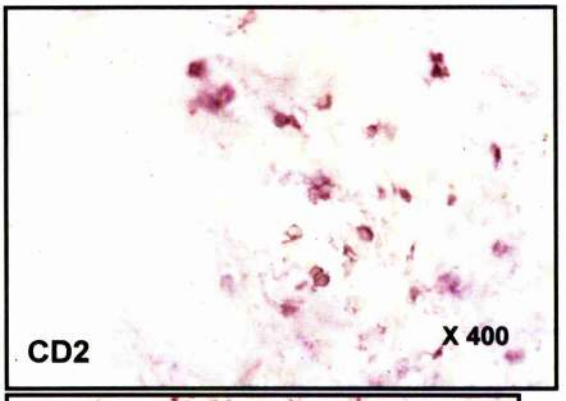
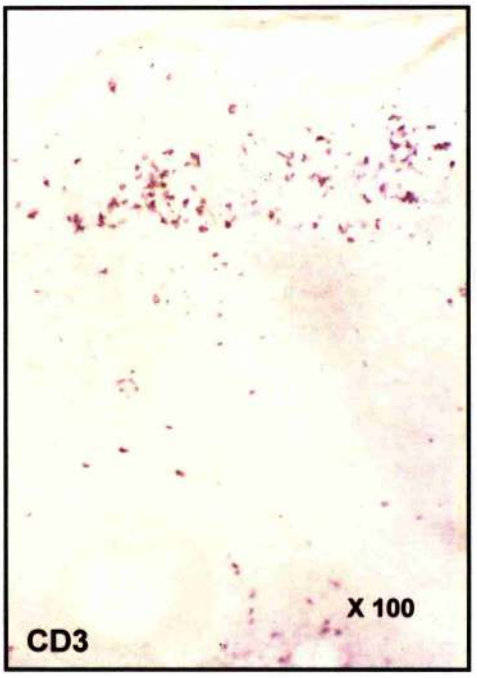
5.6 (a)

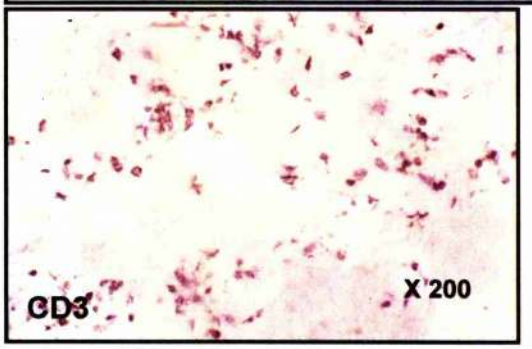

CD45
X 100
CD45
X 200
CD45
X 400
CD45
X 400
WC3
X 100
SWC3
X 400
SWC3
X 400

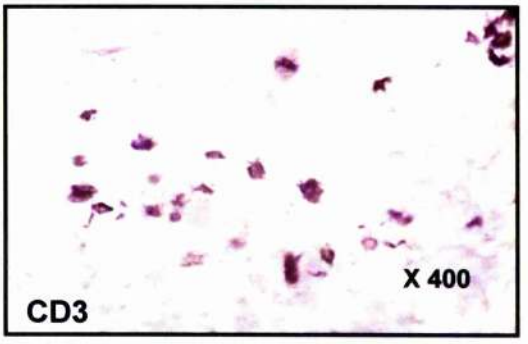
5.6 (b)

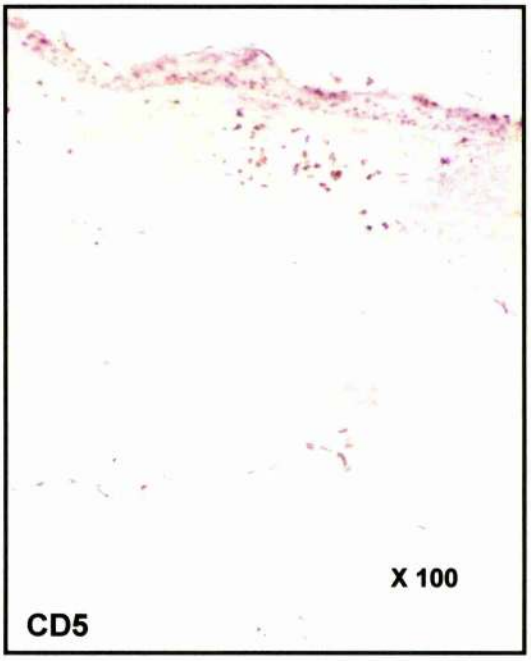


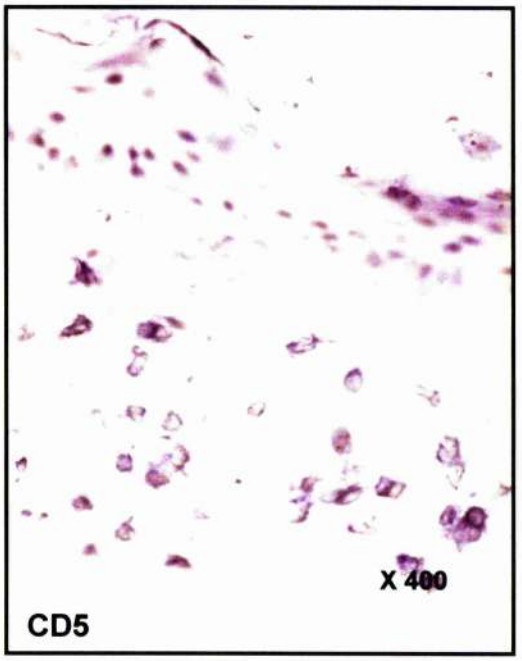












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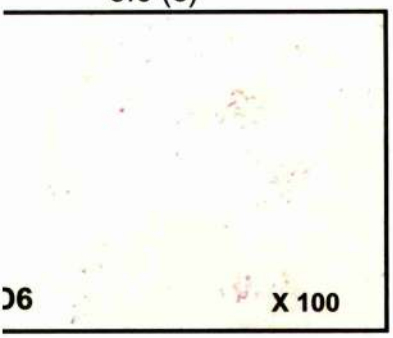

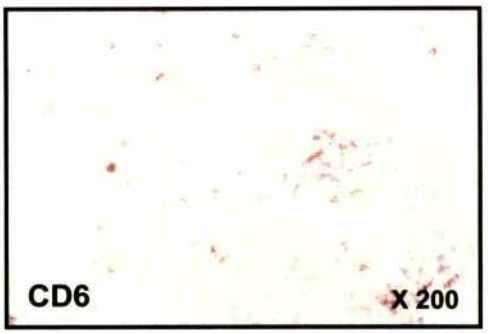

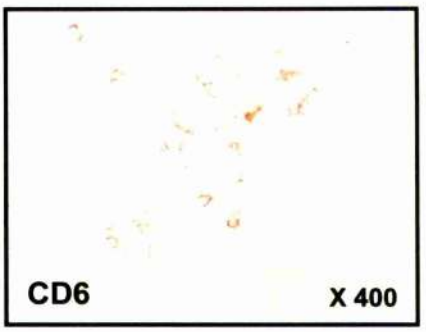

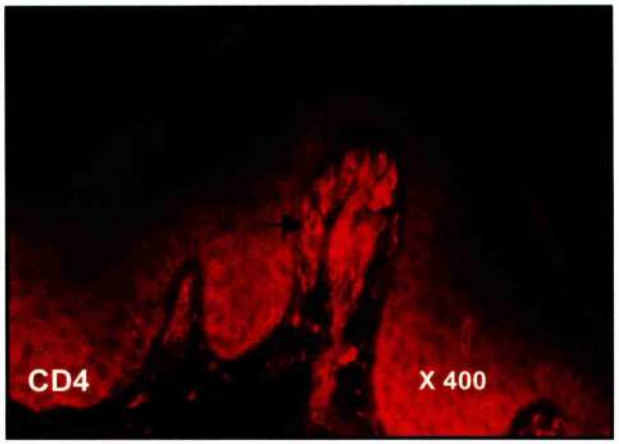

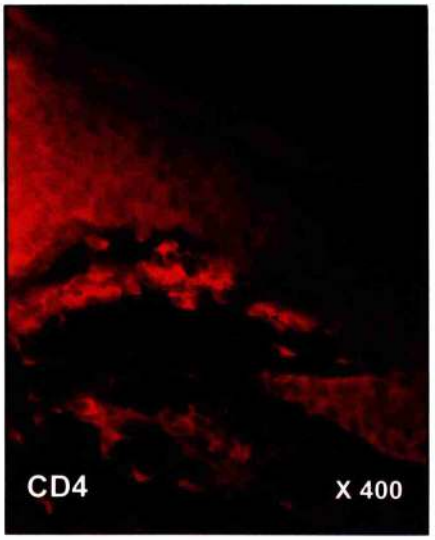

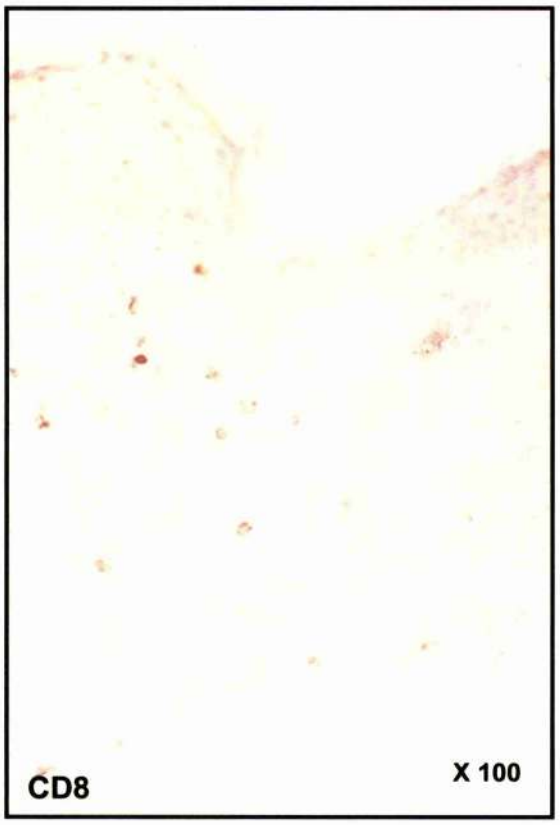

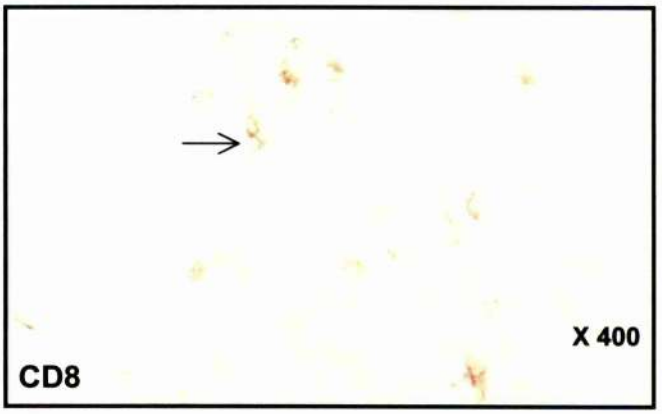

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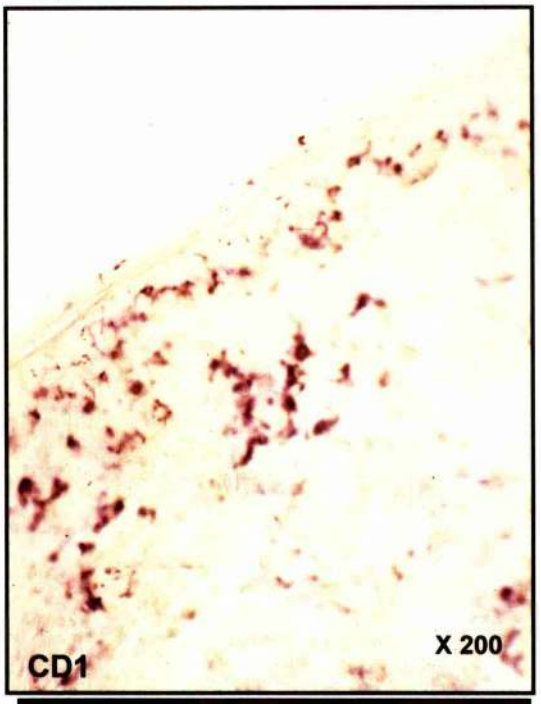

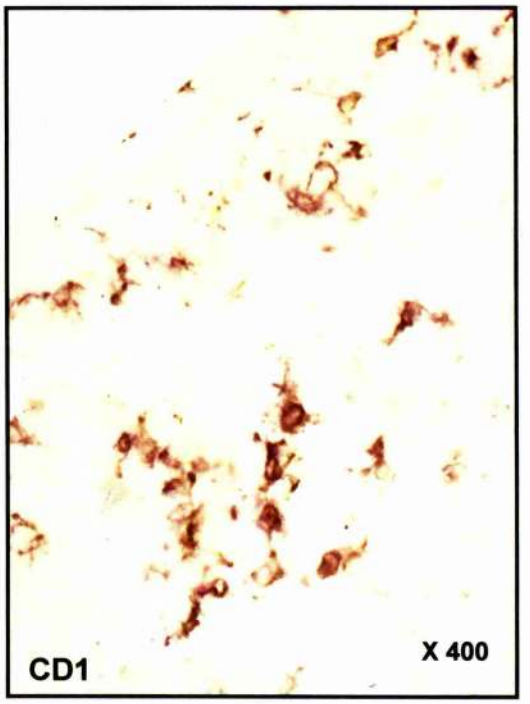

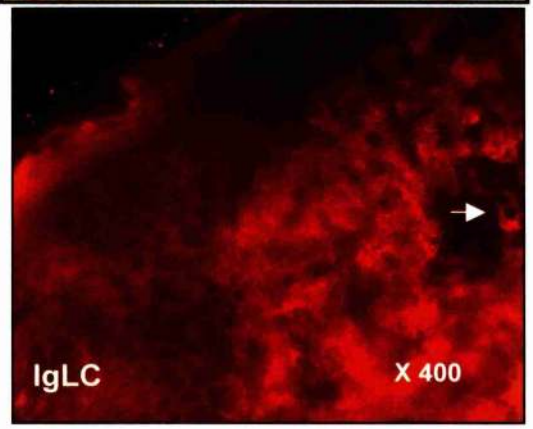

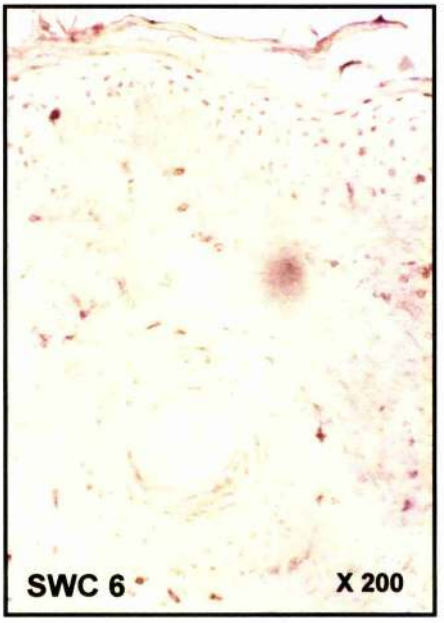

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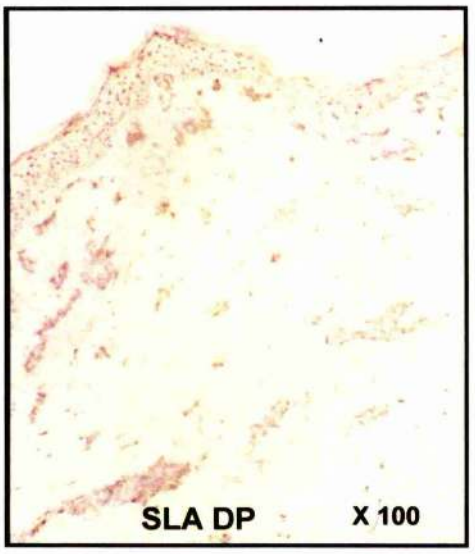

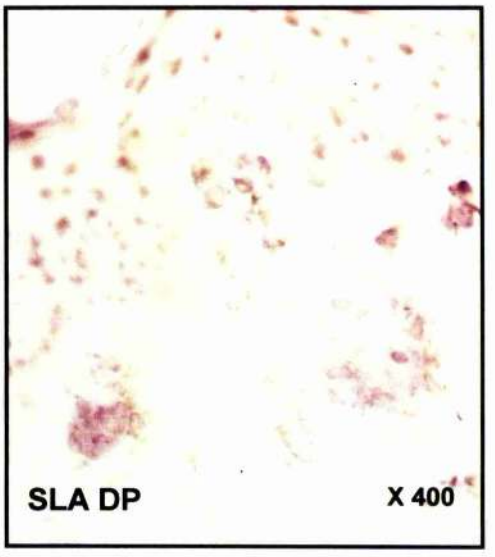

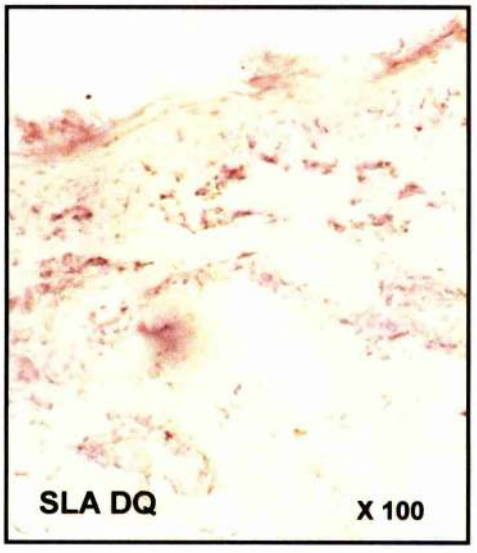

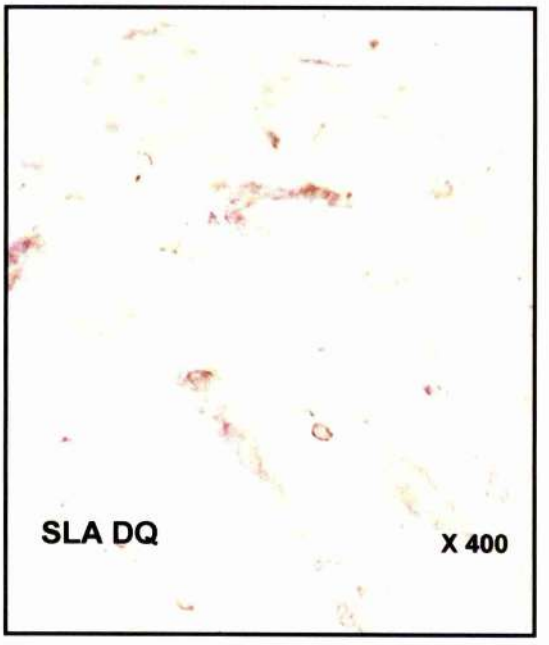

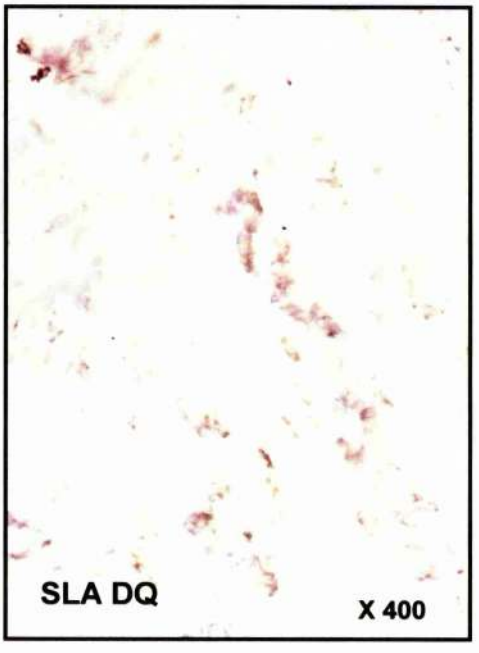

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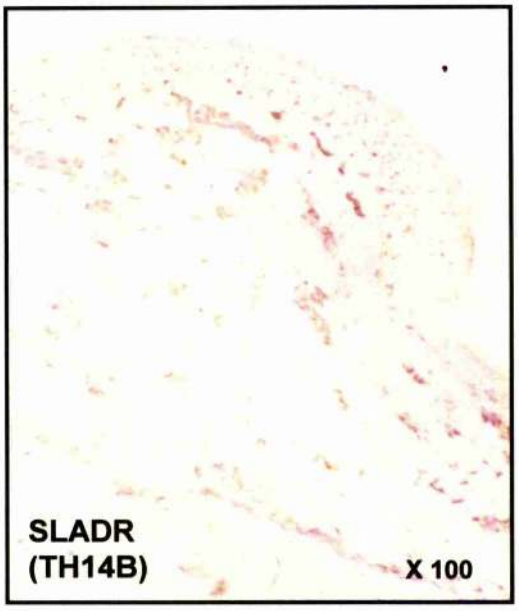

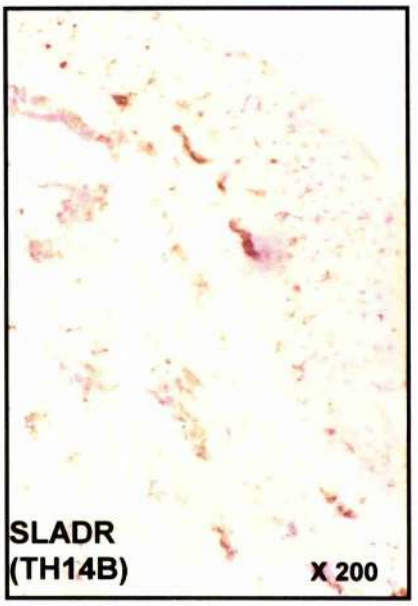

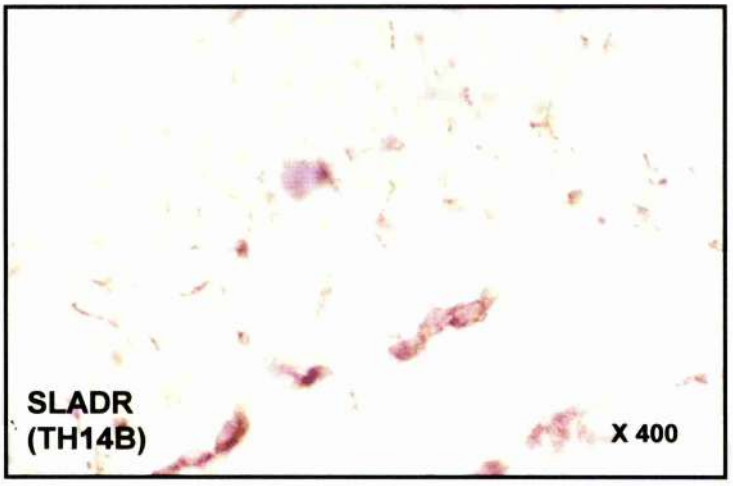

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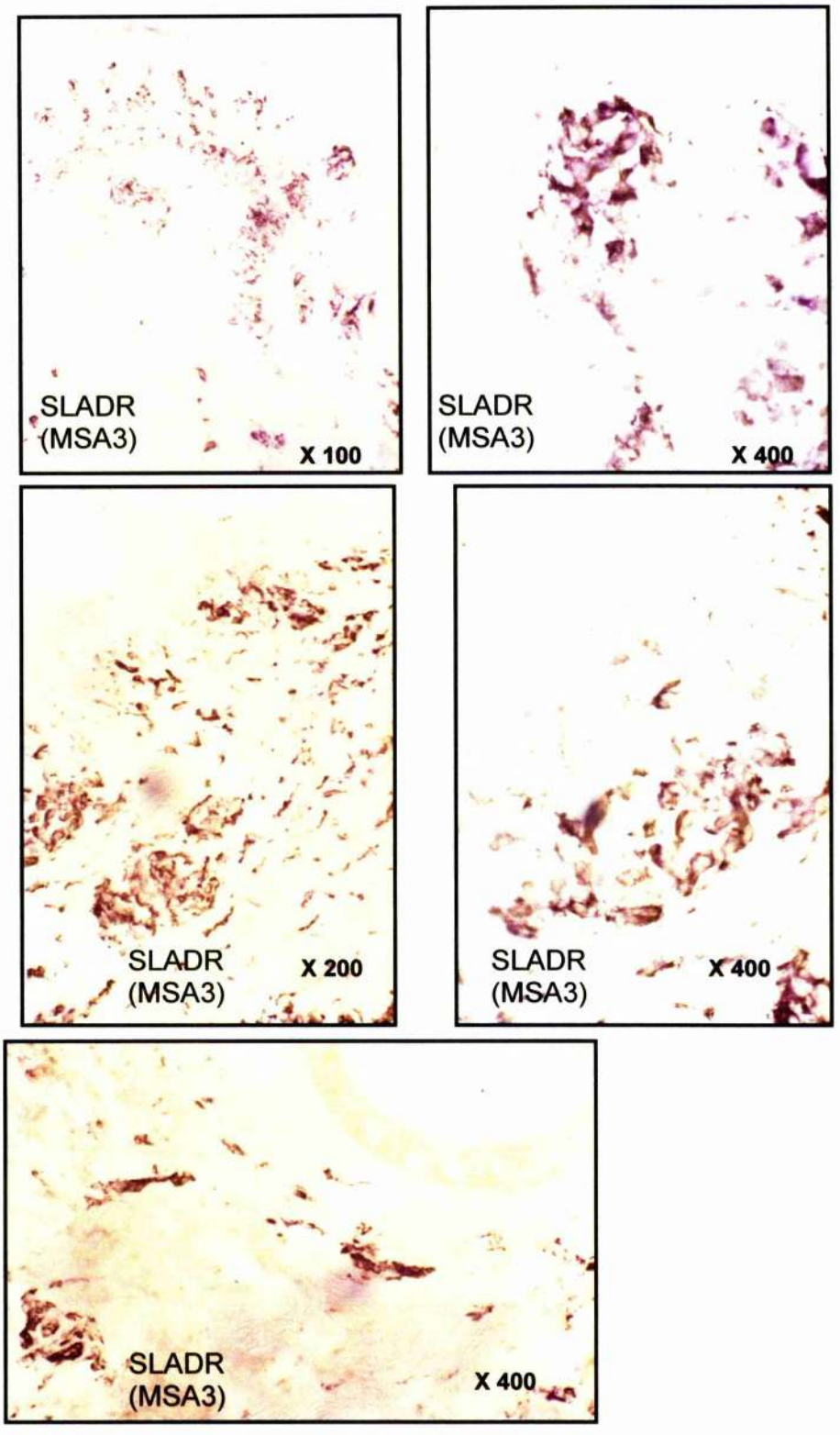
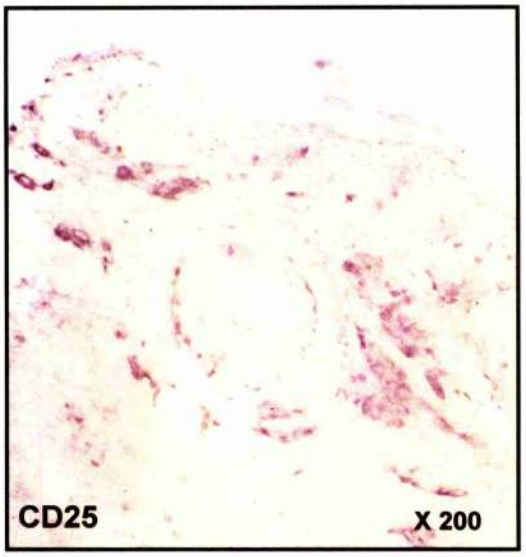
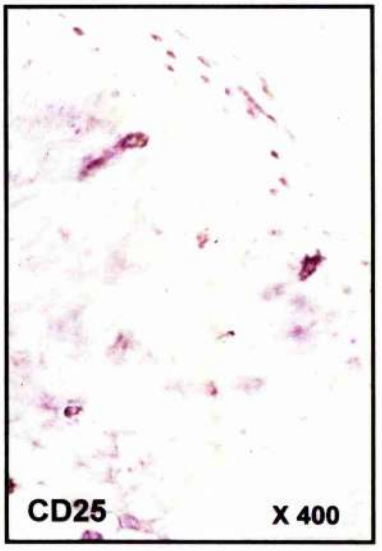
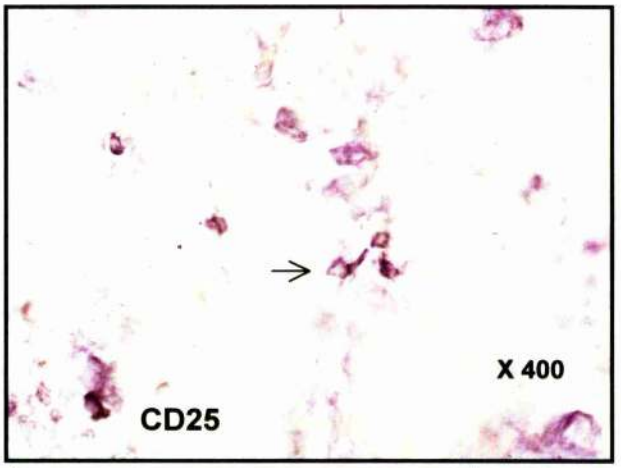

SLADR
(MSA3)
X 100
SLADR
(MSA3)
X 400
SLADR
(MSA3)
X 200
SLADR
(MSA3)
X 400
SLADR
(MSA3)
X 400

5.6 (h)


CD25
X 200


CD25
X 400


X 400
CD25

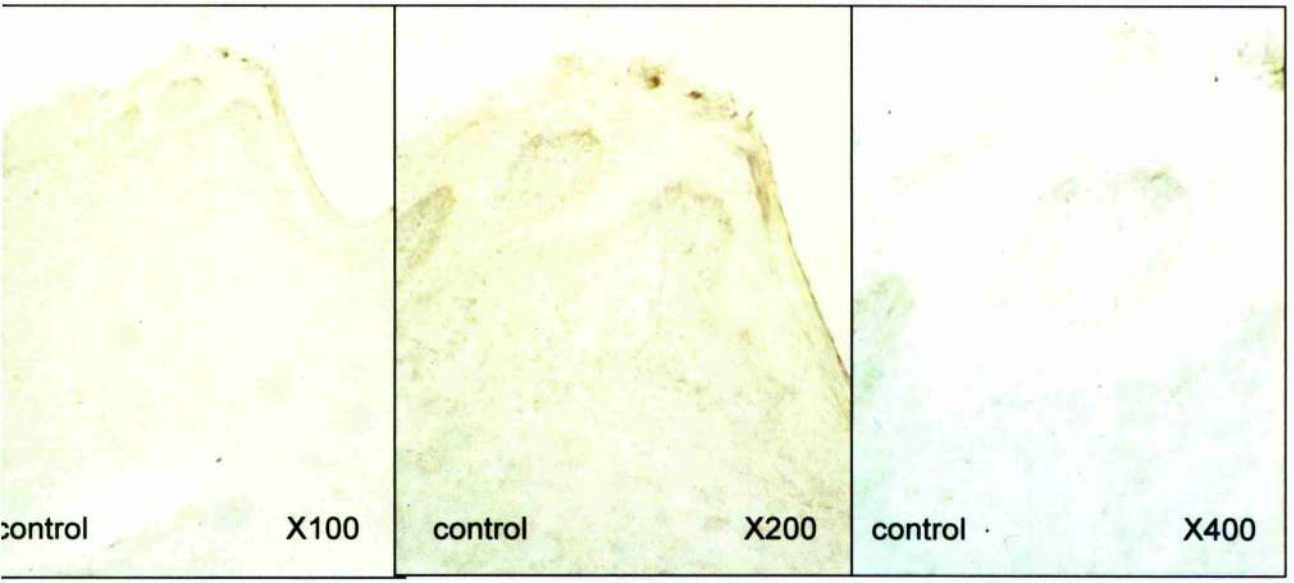
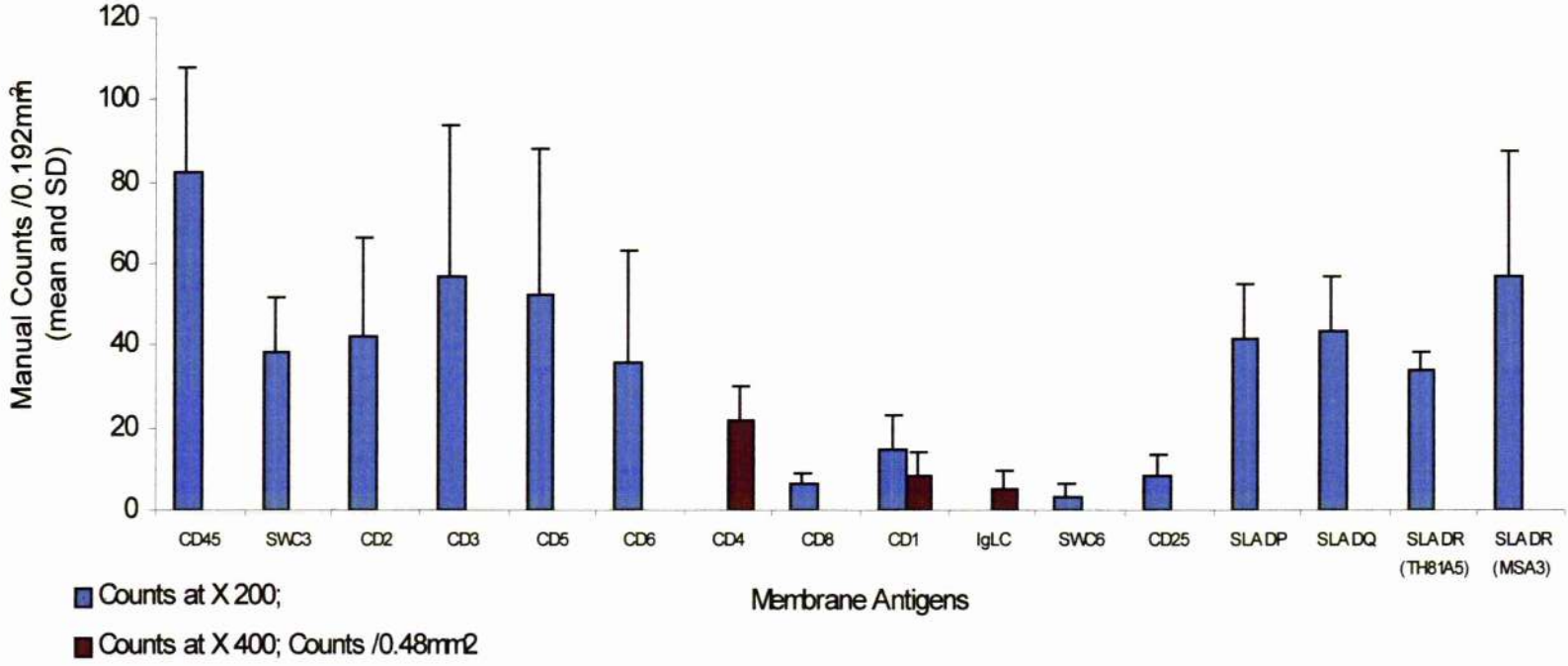

control
X100
control
X200
control
X400

Figure 5.7: Leucocyte densities within porcine dermis (n=2 pigs for all antigens except CD6, SLA DP, DQ and DR). Between 1 and 3 sections with 5-6 fields per section were examined. For most determinants (see graph) positive cells were counted at X200 magnification within 12 X 20 squares (each square is 40μm X 40μm) within fields chosen at random in the papillary and upper reticular dermis using a calibrated graticule. The remainder were similarly counted at X400 magnification within 12 X 20 squares (each square is 20μm X 20μm).



5.3 Discussion

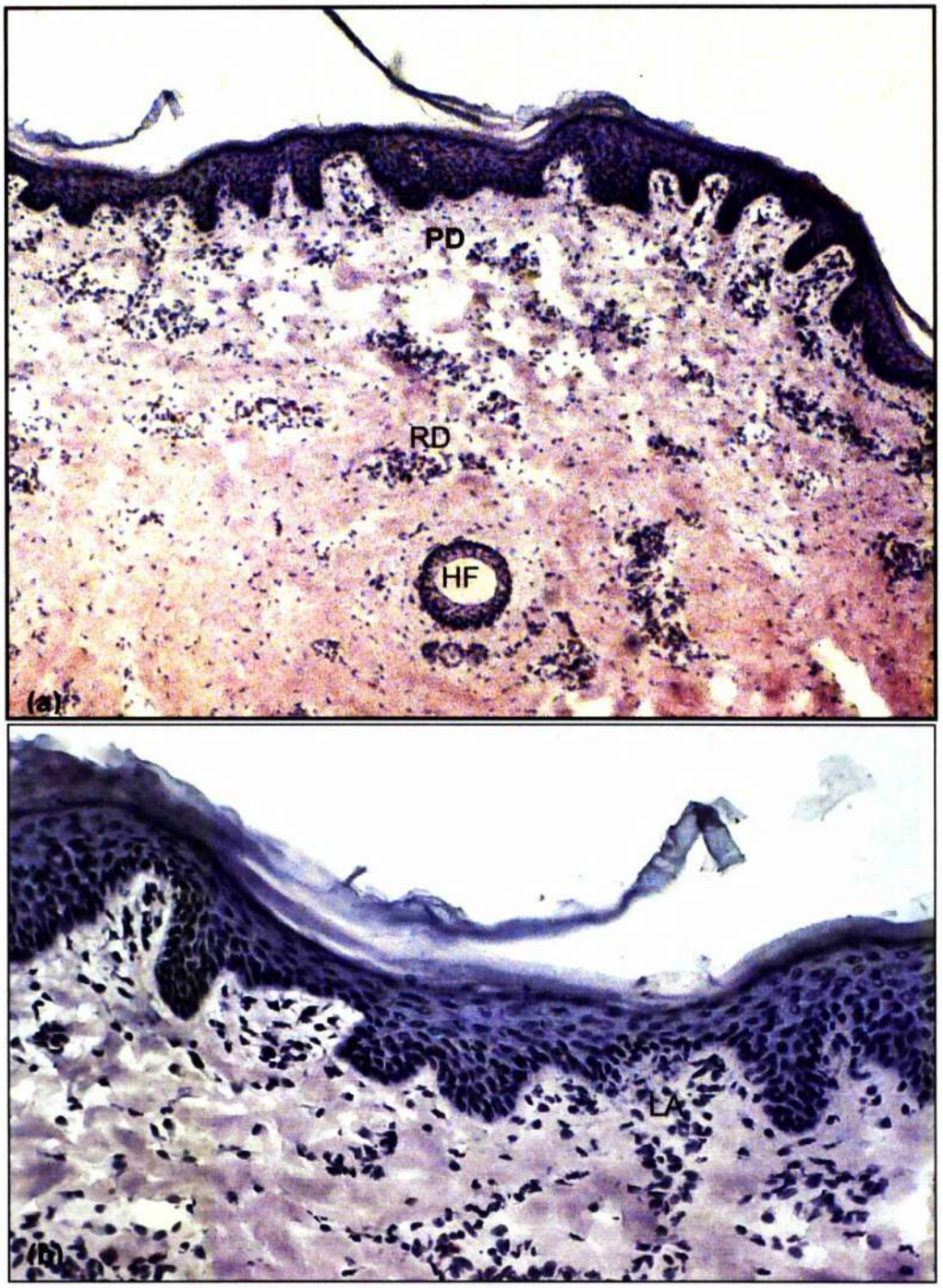
It is important to note that there are important differences between immunohistochemistry and flow cytometry, so that results based on these two techniques are not always correlated. In contrast to flow cytometry, immunohistochemistry will also label antigens expressed intracellularly, such as immunoglobulins in plasma cells and MHC class II in immature dendritic cells. However, immunohistochemistry can be less sensitive and can only provide information abouT-cells expressing a particular antigen in a high enough density for it to be seen following immunohistochemical staining. Cells seen to express an antigen 'dimly' by flow cytometry may have a low density of expression and not show up as positive upon immunohistochemical staining. Where cells are shown immunohistochemically to express certain antigens, the distribution of these cells within tissues may be demonstrated as well as their anatomical relationships with neighbouring cells.

Most of the antigen labelling work in this chapter utilised skin from 2 pigs (a few were tested on only 1 pig, see Section 5.2 and Table 5.4).

Within the epidermis, LCs accounted for approximately 4.7% of nucleated epidermal cells (n=2 pigs, staining with clone 76-7-4 (anti-CD1) combined with haematoxylin counter staining). The LCs stained positively with a number of other monoclonal antibodies including SWC3 (Figure 5.6(a)), which is expressed on cells of myeloid origin including monocytes, macrophages and granulocytes. LCs also stained positively for MHC II antigen expression although SLA DP staining was not as intense as SLA DQ or SLA DR (Figure 5.6(e)). Keratinocytes did not stain positively for MHC II antigens.

The majority of leucocytes within the dermis were found within the papillary dermis, principally in the region of subepidermal vascular plexuses (Figure 5.8).

Figure 5.8: Porcine Skin; Photomicrographs (X40 (a), X200 (b)) H&E stained formalin fixed cryostat sections. PD = papillary dermis; RD = reticular dermis; HF = hair follicle; LA = leucocyte aggregates in the region of the subepidermal plexuses



Approximately one fifth of dermal leucocytes appeared to be CD1-positive, although it is not possible to determine what percentage of these were dermal dendritic cells.

The human CD2 antigen is expressed on virtually all thymocytes, T lymphocytes and NK cells (Barclay et al., 1997). As in other species the porcine analogue is a pan-α/βT-cell antigen characterised by its ability to bind to xenogeneic red blood cells (E-rosette receptor) (Hammerberg and Schurig, 1986). CD2 in pigs is ubiquitous, it is also expressed on a subset of γ/δ Tcells (Saalmuller et al., 1990), comprising $CD2^{+}4^{-}8^{lo}$ and $CD2^{+}4^{-}8^{-}$ subsets (Davis et al., 1998; Yang and Parkhouse, 1996)) and at low intensity on B cells (Sinkora et al., 1998). In addition there are non-T-non-B lymphocytes with $CD2^{+}3^{-}4^{-}8^{lo}$ surface immunoglobulin-negative phenotype containing natural killer (NK) activity (Yang and Parkhouse, 1996) (see Figure 5.5).

The CD3 antigen is closely associated with the T-cell receptor. Functionally, it is involved in signal transduction leading to T-cell activation following antigen recognition (Barclay et al., 1997). Within pigs the CD3 antigen is expressed on both α/β and γ/δ T-cells. The density of CD3 on γ/δ T-cells tends to be at a higher and more homogeneous level than on α/β T-cells (Yang et al., 1996) the significance of which is unclear.

The CD5 antigen in humans and swine expressed on all mature T-cells and on a subset of mature B cells is involved in signal transduction (Saalmüller and Bryant, 1994; Appleyard and Wilkie, 1998; Barclay et al., 1997).

Within the peripheral blood cell population there are three differential levels of CD5 expression. The CD5- population contains all the NK activity, the $CD5^{dim}$ cells contain the γ/δ cells and the α/β $CD4^{-}8^{dim}$ population and the $CD5^{bright}$

contain the rest (see Figure 5.5) (Saalmuller et al., 1994b; Saalmuller et al., 1994).

The CD6 antigen is expressed by 76% of porcine peripheral T-cells (Saalmuller et al., 1994a). Only the α/β T-cell receptor subset expresses CD6. Within these, all the $CD4^+$-positive cells express CD6, and only the ($CD4^-$) $CD8^{hi}$ cells express CD6 (see Figure 5.5) (Pauly et al., 1996; Saalmuller et al., 1994a). Functionally, CD6 may have a role in intrathymic T-cell development as well as signal transduction (Barclay et al., 1997)

There does not appear to be any significant difference between expression of different pan T-cell antigenic determinants. This would be consistent with the majority of T-cells being of the α/βsubset. SWC6-positive cells ('null' cells) which have been shown to consist of $CD2^-3+$ γ/δ T-cells (Binns et al., 1992) were seen in only small numbers.

B cells labelled by IgλLC were also only sparsely seen, this may be due to the fact that this antibody is specific for only a subset of lambda chain, unfortunately not known at the time of these experiments. A proportion of these dermal B cells may stain positively for CD1. Additionally a proportion of resting B and T-cells stained positively for CD25 albeit at low intensity (upregulated upon activation) (Denham et al., 1994; Stokes et al., 1996; Bailey et al., 1998). The CD25 antigen forms a component of the IL-2 receptor (IL-2R), which is expressed on a variety of activated cells including T-cells, B cells NK cells and monocytes via which IL-2 induces their activation, maturation and proliferation (Barclay et al., 1997).

In the human the CD4 antigen is expressed on two thirds of peripheral blood T lymphocytes, expression of CD4 is predominantly mutually exlusive to CD8

expression (Barclay et al., 1997). In pigs, CD4+ cells are a more heterogeneous population with significant proportions of the population co-expressing low levels of CD8 (see Figure 5.5) (Yang and Parkhouse, 1996). The CD4 antigen functions as an accessory molecule involved in the recognition of foreign antigens in association with MHC class II by T-cells (Barclay et al., 1997).

The CD8 antigen acts as a co-receptor with MHC class I restricted antigen recognition. In the pig in peripheral blood the CD8 antigen is expressed on a variety of cell types (see Figure 5.5). These include α/β T-cells with the T suppressor phenotype and up to 60% of peripheral T-cells expressing dual helper/suppressor ($CD4^{+}CD8^{+}$) phenotypes (Pescovitz et al., 1994), as well as on a subset of γ/δ T-cells (Davis et al., 1998) and those with natural killer (NK) activity (Yang and Parkhouse, 1996). The density of surface antigen expression as indicated by degree of fluorescence in flow cytometry varies between the groups (Yang and Parkhouse, 1996). Low surface antigen expression (denoted as $^{'dim'}$ or $^{'lo'}$ in relation to flow cytometric measurement of fluorescence) detectable on flow cytometry is more difficult to interpret immunohistochemically.

Results suggest a higher proportion of CD4-positive cells than CD8-positive cells within porcine dermis. It is difficult do draw any meaningful conclusions however, as skin from only two pigs was examined. In addition different staining techniques were used, with ABC being the optimum staining method for all the antigens bar CD4 and IgλLC which were better revealed with immunofluorescence. Lastly it was necessary to perform the counts of CD4-positive cells at the higher magnification of X400.

MHC class II molecules are expressed on dendritic cells, B cells, monocytes, macrophages and activated T-cells in the human and present exogenously derived antigen to $CD4^+$ T-cells (Barclay et al., 1997). Monoclonal antibodies reactive with SLA DR (swine leucocyte antigen – HLA equivalent) and SLA DQ are well characterised (Lunney, 1994). There is evidence to suggest that a further moAB (H42A) reacts with SLA DP (HLA DP equivalent) (Ababou et al., 1995); and personal communication from Davis WC, Department of Veterinary Microbiology and Pathology, College of Veterinary Medicine, Washington State University). It reacts with a unique allomorph in mice that appears to be equivalent to DP, and it appears the determinant is conserved in humans on DP (Davis WC personal communication).

Within porcine dermis the results (Figure 5.6) suggest there is no significant difference in expression of the different MHC class II antigens. Without dual staining it is not possible to determine what percentage of T-cells are MHC II expressing, although it would be reasonable to assume that the majority of expression is seen on SWC3-positive cells with some occurring on dermal dendritic cells. The moAB 74-22-15 reactive against swine myeloid cells was found to recognise macrophages and granulocytes and assigned a swine workshop cluster (SWC) number 3 in the first international workshop (Pescovitz et al., 1984); (Blecha et al., 1994). The pattern of cellular binding reactivity differs from any known human CD antigen hence a swine workshop cluster number was assigned, SWC3 has recently been identified as a signal regulatory protein receptor associated with SHP-1 (Alvarez et al., 2000).

Within porcine jejunal villi, a network of dendritic cells as well as capillary endothelium have been shown to express SLA DR and SLA DQ antigens and

this is occurring in close association with T-cells (Haverson et al., 2000). The expression of MHC molecules appears ubiquitous on porcine endothelium and has also been found in many other tissues (Wilson et al., 1996). Suggestions have been made regarding the possible role of endothelial cells in lymphocyte trafficking and the maintenance of local tolerance (Wilson et al., 1996). The staining studies suggest that the subepidermal capillary plexus endothelium does express MHC class II antigens. Similarities have been proposed between gut-associated lymphoid tissue (GALT) and skin-associated lymphoid tissue (SALT) (Streilein, 1983). There are however marked differences in localisation of white cells in porcine gut and skin.

There are no published reports of immunohistochemical staining of porcine dermis with the vast majority of the antibodies tested within the above study. It has been a useful exercise therefore, to establish a baseline distribution of numbers and types of cells bearing different CD antigens within normal skin in the Large White pig.

Chapter 6

The immuno cellular response to split skin allografts in the Large White pig

6.1 Introduction

The Large White pig is well established as a model in the field of allotransplantation (Kenmochi et al., 1994). Indeed, epidermal regeneration in the Large White pig was first characterised nearly forty years ago (Winter, 1962). In the last decade, studies using allogeneic cultured porcine keratinocyte sheets were initiated (Carver et al., 1991), and a model using semi-isolation chambers in the Large White pig was developed (Kangesu et al., 1993).

Acute rejection of a split thickness skin graft follows sensitisation and is principally mediated by antigen-specific effector cells (Rosenberg and Singer, 1992). Sensitisation is thought to be dependant upon the presence of APCs which migrate to regional nodes (Lafferty et al., 1983), mature and give rise to a sequence of events that result in the generation of Class I restricted ($CD8^+$) cytotoxic T lymphocytes (Rosenberg and Singer, 1992). These infiltrating activated lymphocytes that are primarily responsible for effecting the rejection process are thought to produce cytokines promoting influx of non-specific inflammatory cells that result in a degree of non cell-specific damage or 'bystander damage' (Lafferty et al., 1983).

It has been demonstrated that the duration of survival of split skin allografts is related to degree of heterogeneity at certain leucocyte antigen loci (Koch et al., 1971). The Large White pigs in use in this series of experiments came from one of two farms. Due to previous inbreeding these may be related, although in these experiments care was taken to use non-littermates. With the degree of inbreeding that may have occurred there was concern to ensure the

validity of the model with regard to the degree of allogenicity. Indeed it required only 5 generations of in breeding to generate the Babraham herd as opposed to the usual 20 generations of brother X sister matings (Signer et al., 1999). If indeed the Large White pig populations in use in my (and other) experiments were too closely related, then one may expect allogeneic split skin to be rejected only after a prolonged period of take. Additionally, one may expect rejection to be a more subdued process (more akin to sub acute or chronic rejection).

A study was set up therefore to test the hypothesis that non-litter mate 'outbred' Large White pigs reject split skin allograft acutely within the classical time frame (Koch et al., 1971).

Although attempts to characterise the infiltrate with respect to the overlying allogeneic epithelium have been made in other species (Mayer et al., 1988), there has been no such study to date in the pig. With the steady development in the last decade of porcine-specific monoclonal antibodies that have been raised against membrane antigens (Lunney et al., 1994; Lunney, 1994; Saalmuller, 1996; Saalmuller et al., 1996; Saalmuller et al., 1998b) it has become possible to immunophenotype the cells contained within a porcine wound infiltrate.

The aims of this study were

- To show that the non-litter mate Large White pig population in this series of experiments reject split skin allograft acutely within the classical time frame.
- To develop a positive control as a basis for comparison of wound infiltrates beneath skin substitutes containing cultured cells.

6.2 Results

One Large White pig received four allogeneic split skin grafts from two non-littermate donors and two autologous split skin grafts. By day 10 each of the four allogeneic grafts had been rejected whilst the autologous grafts remained healthy (see Figures 6.1-6.2 and Table 6.1).

Table 6.1:

(a)

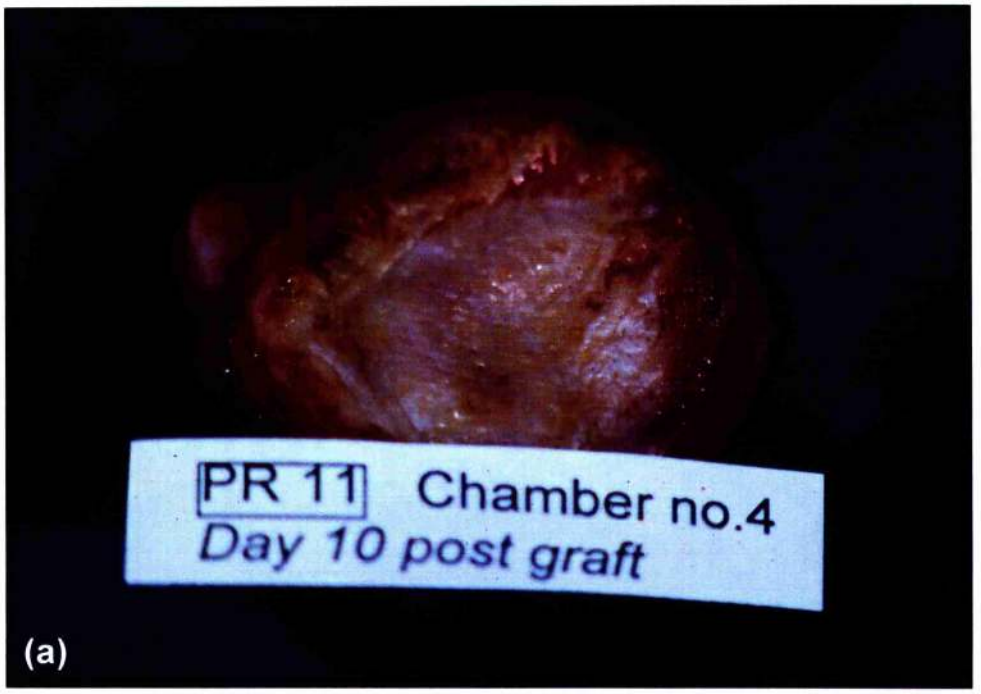
Wound Harvest Time Points (Days)		**10**
Types of Graft Applied to wounds (no. of wounds)	Autologous Split Skin	2
	Allogeneic Split Skin	4

(b)

Wound Harvest Time Points (Days)		**10**
Fraction of wounds that showed epithelium as identified histologically with H&E staining at time of harvest	Autologous Split Skin	2/2
	Allogeneic Split Skin	*4/4

* Grossly and histologically the epithelium (although still present) was observed to be undergoing acute rejection.

Figure 6.1: Photographs of harvested wounds 10 days after grafting with autologous split skin (a) and allogeneic split skin (b). Whilst the autologous grafted wounds remained healthy, the wounds grafted with allogeneic skin showed signs of early necrosis and graft rejection.



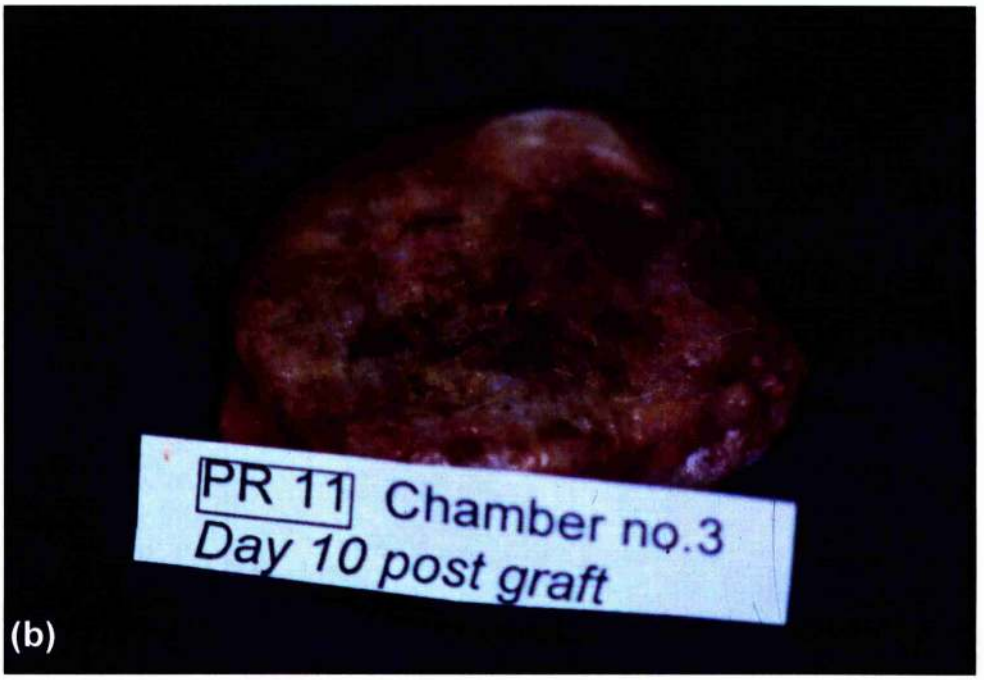
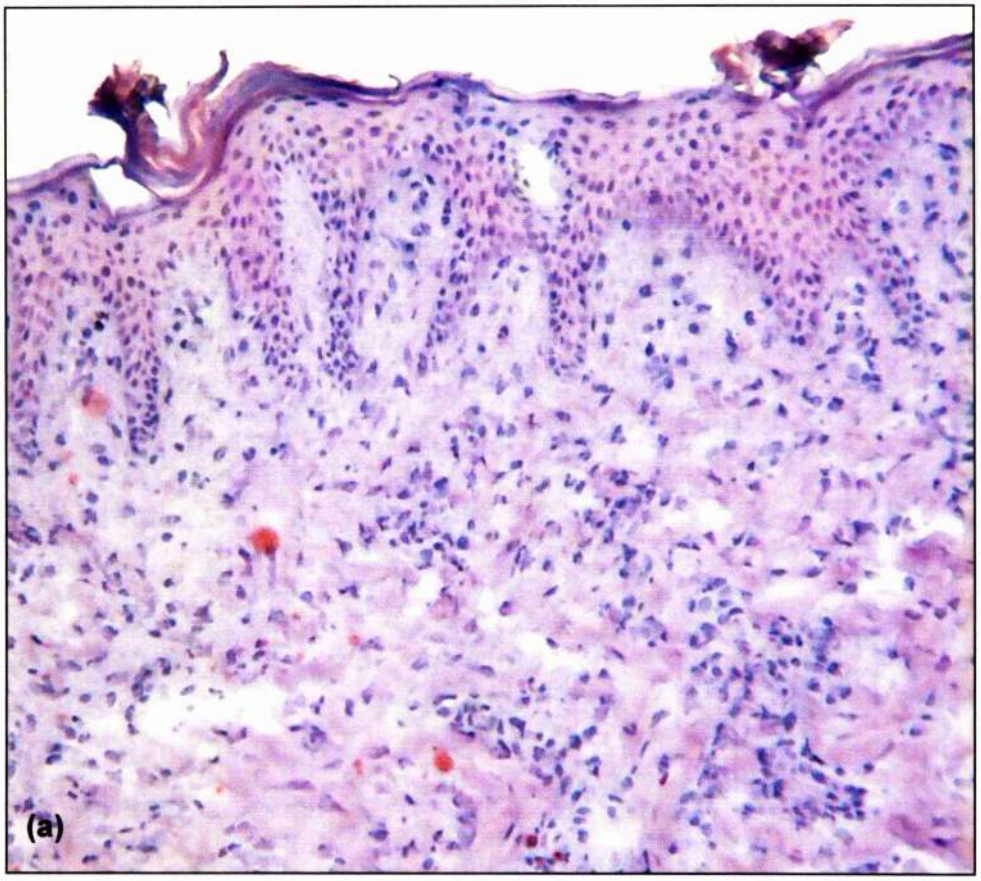
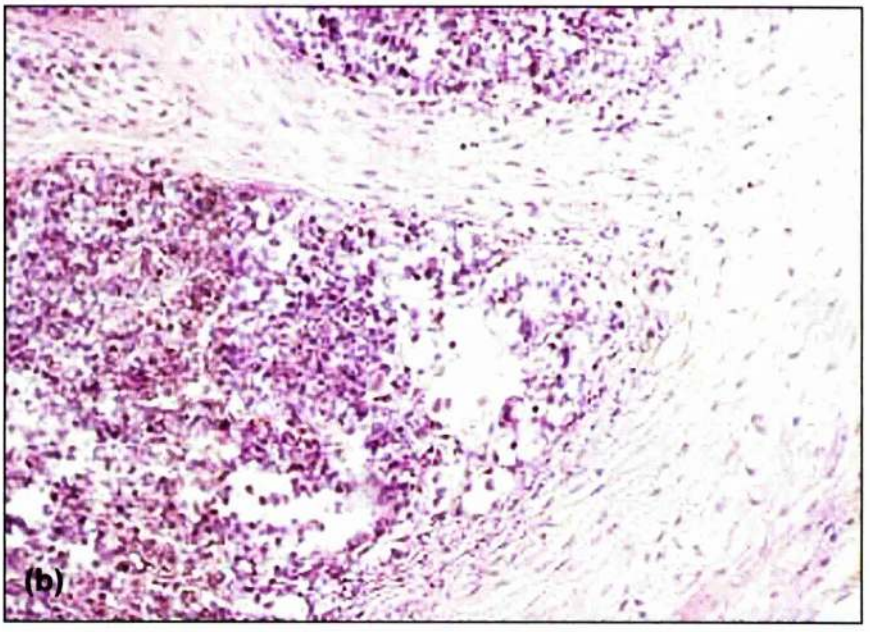


Figure 6.2: Photomicrographs (X100) of wounds harvested at 10 days following grafting with autologous and allogeneic split skin ((a) and (b) respectively) and stained with H&E. Note the intense infiltrate in (b).





6.2.1 Immunophenotyping of split skin grafted wounds

Blocks from allogeneic and autologous wounds were examined (as was normal porcine paravertebral skin) with each of the twelve monoclonal antibodies (see Table 6.2 and Figure 6.3). Two to five sections per wound were stained using the nickel-enhanced ABC method (apart from CD4 and IgλLC which were detected using Cy3 – an immunofluorescent dye). Using an optical grid, counts were taken from approximately five random fields per section at X200 magnification to a depth of 0.5mm below the epidermis, apart from CD4 and IgλLC which were counted at X400 magnification (see Table 6.3(a)-(c)).

All antibody counts within the allogeneic split skin grafted wounds were consistently higher than in wounds grafted with autologous split skin or within normal pig skin with the exception of SWC6 (null cells) which was not convincingly seen in any of the wounds (see Figures 6.4 and 6.5). The majority of white cells (CD45) were shown to be SWC3-positive (macrophage/monocyte and granulocytes) with the remainder mainly T-cells (CD2, 3 and 5) which consist both of T helper (CD4) and T cytotoxic/suppressor (CD8) phenotypes (many of which may well be of dual CD4 - CD8 phenotype).

Density counts of the SLADR determinant (the human equivalent may be expressed by monocytes, macrophages and activated T-cells as well as dendritic cells and some B cells) were increased, as were those for the CD25

(IL-2 determinant which denotes activation) in the wounds grafted with allogeneic split skin graft.

The Igλ LC determinant (B cell) appeared to be increased in the allogeneic split skin graft section examined. The null cell determinant SWC6 was difficult to detect in any section.

The number of cells staining with the CD1 determinant was not significantly increased in wounds grafted with either allogeneic or autologous split skin graft and equated with that found in normal skin.

Table 6.2: Summary of the monoclonal antibodies used in wound infiltrate immunophenotyping.

ANTIBODY SPECIFICITY	PHENOTYPE	CELL LINE	SOURCE
CD1	Dendritic Cell	76-7-4	VMRD
CD2	T-cell	MSA4	Dr. J.K. Lunney, Beltsville, USA
CD3	T-cell	FY1H2	Dr. H. Yang, Pirbright, UK
CD4	T helper	STH293	Dr. M. Shimizu, Ibaraki, Japan
CD5	T-cell	PG1114A	VMRD
CD8	T Cytotoxic/Supressor	PT81B	VMRD
IgλLC	Plasma Cell/Mature B cell	K189.3E1	Serotec
CD25	IL-2 Receptor	281.3B2	Serotec
CD45	Pan Leucocyte	K.252.1E4	Serotec
SWC3	Macrophage & Granulocyte	74-22-15	VMRD
SWC6	Null Cell	MAC320	Serotec
SLADR	Macrophages, Monocytes, Dendritic cells, B cells Activated T-cells (in humans and rats)	MSA3	VMRD

Table 6.3: Wound processing log (a)

Monoclonal Antibody Specificity	Microscope magnification	Field Surface Area		**Skin**	**Auto SSG**	**Allo SSG**
CD1	200X	40,000μm^2	sections	1	2	2
			Animals	1	1	1
			Wounds	1	2	2
			Blocks	1	2	2
			Non littermate donors	--	--	2
			Random fields	5	10	10
			Fields/section	5	5	5
CD2	200X	SSG: 40,000μm^2 Skin: 192,000μm^2 *	sections	2	1	2
			Animals	2	1	1
			Wounds	2	1	1
			Blocks	2	1	1
			Non littermate donors	--	--	1
			Random fields	10	5	10
			Fields/section	5	5	5
CD3	200X	SSG: 40,000μm^2 Skin: 192,000μm^2 *	sections	2	1	1
			Animals	2	1	1
			Wounds	2	1	1
			Blocks	2	1	1
			Non littermate donors	--	--	1
			Random fields	10	5	5
			Fields/section	5	5	5
CD5	200X	SSG: 40,000μm^2 Skin: 192,000μm^2 *	sections	2	1	1
			Animals	2	1	1
			Wounds	2	1	1
			Blocks	2	1	1
			Non littermate donors	--	--	1
			Random fields	10	5	5
			Fields/section	5	5	5
CD4	400X	SSG: 10,000μm^2 Skin: 48,000μm^2 *	sections	2	1	1
			Animals	2	1	1
			Wounds	2	1	1
			Blocks	2	1	1
			Non littermate donors	--	--	1
			Random fields	11	5	5
			Fields/section	5.5	5	5

*to equate skin with SSG, skin values were therefore multiplied by 0.208

Table 6.3: Wound processing log (b)

Monoclonal Antibody Specificity	Microscope magnification	Field Surface Area		**Skin**	**Auto SSG**	**Allo SSG**
CD8	200X	SSG: $40,000\mu m^2$ Skin: 1 section $40,000\mu m^2$ and 2 sections $192,000\mu m^{2*}$	sections	4	2	2
			Animals	2	1	1
			Wounds	2	2	2
			Blocks	3	2	2
			Non littermate donors	--	--	2
			Random fields	20	10	10
			Fields/section	5	5	5
SLADR	200X	SSG: $40,000\mu m^2$ Skin: 1 section $40,000\mu m^2$ and 2 sections $192,000\mu m^{2*}$	sections	3	2	2
			Animals	1	1	1
			Wounds	1	2	2
			Blocks	2	2	2
			Non littermate donors	--	--	2
			Random fields	15	10	10
			Fields/section	5	5	5
CD45	200X	SSG: $40,000\square m^2$ Skin: 1 section $40,000\square m^2$ and 2 sections $192,000\mu m^{2*}$	sections	3	2	2
			Animals	2	1	1
			Wounds	2	2	2
			Blocks	3	2	2
			Non littermate donors	--	--	2
			Random fields	15	10	10
			Fields/section	5	5	5
SWC3	200X	SSG: $40,000\mu m^2$ Skin: 1 section $40,000\mu m^2$ and 2 sections $192,000\mu m^{2*}$	sections	3	2	2
			Animals	2	1	1
			Wounds	2	2	2
			Blocks	3	2	2
			Non littermate donors	--	--	2
			Random fields	15	10	14
			Fields/section	5	5	5-9

*to equate skin with SSG, skin values were therefore multiplied by 0.208

Table 6.3: Wound processing log (c)

Monoclonal Antibody Specificity	Microscope magnification	Field Surface Area		**Skin**	**Auto SSG**	**Allo SSG**
IgλLC	400X	SSG: $10,000\mu m^2$ Skin: $48,000\mu m^2$ *	sections	3	1	2
			Animals	2	1	1
			Wounds	2	1	2
			Blocks	2	1	2
			Non littermate donors	--	--	1
			Random fields	15	5	11
			Fields/section	5	5	5-6
CD 25	200X	SSG: $40,000\mu m^2$ Skin: $192,000\mu m^2$ *	sections	2	1	1
			Animals	2	1	1
			Wounds	2	1	1
			Blocks	2	1	1
			Non littermate donors	--	--	1
			Random fields	10	5	5
			Fields/section	5	5	5
SWC6	200X	SSG: $40,000\mu m^2$ Skin: $192,000\mu m^2$ *	sections	2	1	1
			Animals	2	1	1
			Wounds	2	1	1
			Blocks	2	1	1
			Non littermate donors	--	--	1
			Random fields	10	6	5
			Fields/section	5	6	5

*to equate skin with SSG, skin values were therefore multiplied by 0.208

Figure 6.3: Photomicrographs of normal porcine skin labelled with anti CD45 ((a) X200 and (b) X400); and wounds harvested at 10 days following grafting with allogeneic split skin labelled with anti CD45 ((c) X400 and anti SWC3 (d) X400), control ((e) X400). All (bar the H&E stained section) were stained using the ABC method. The arrow indicates positive cells

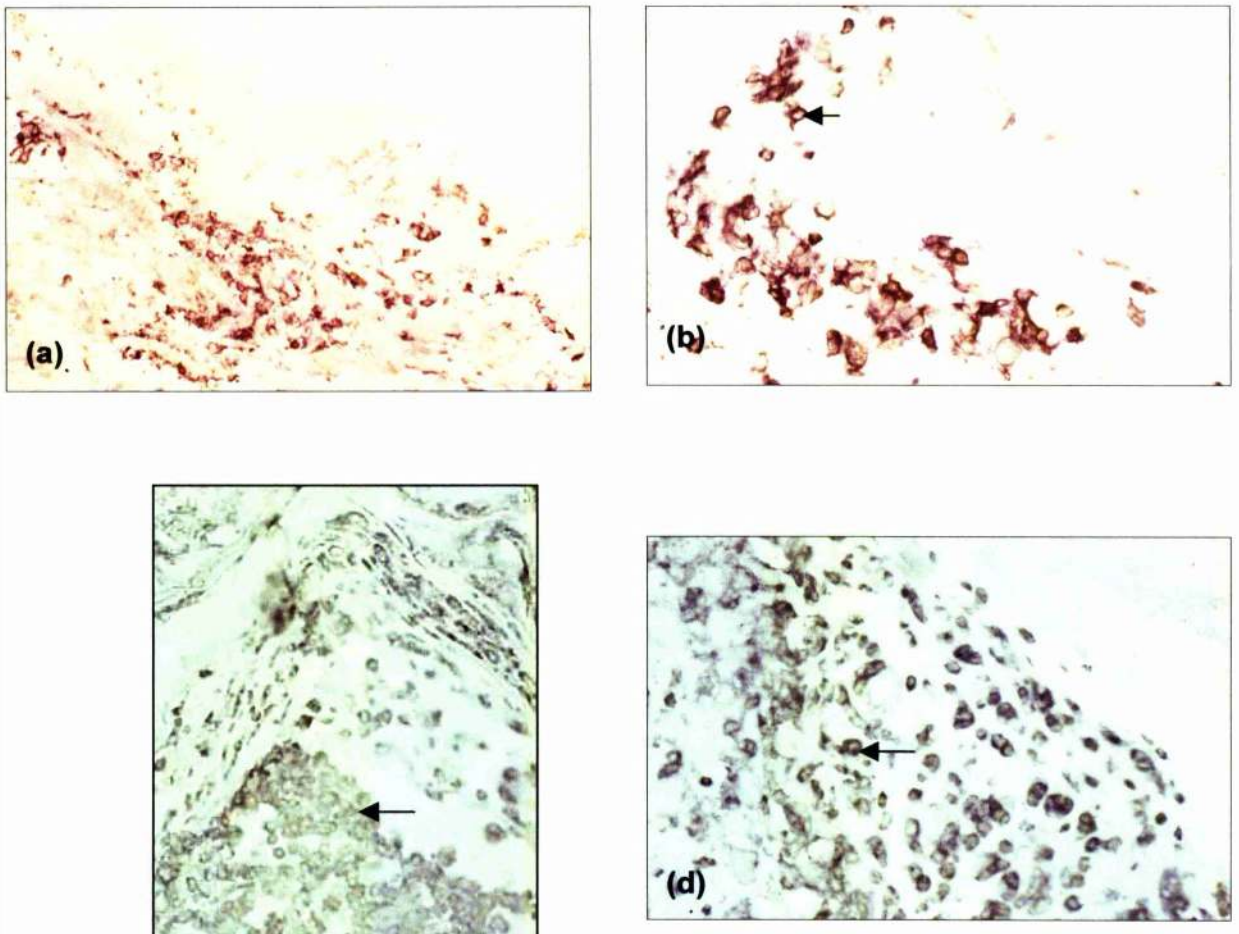


Figure 6.4: Bar charts illustrating manual counts of positively staining cells within the infiltrate of wounds grafted with allogeneic or autologous split skin graft (SSG), harvested at day 10 as well as in normal porcine skin. One Large White pig received four allogeneic split skin grafts from two non-littermate donors and two autologous split skin grafts. By day 10 all four allogeneic grafts had been rejected unlike the autologous grafts which remained healthy. Sections (see Table 6.2 (a)-(c)) from allogeneic and autologous wounds were stained with monoclonal antibody with antigen-specificities as delineated in table 6.1 and then stained using the avidin biotin complex (ABC) immunoperoxidase technique. All determinants were consistently seen in greater numbers in the wounds grafted with allogeneic split skin (the results for normal porcine skin are the same as those in Figure 5.5). The majority of white cells (CD45) were shown to be macrophages (SWC3) with the remainder mainly T-cells (CD2, 3 and 5) which consist both of T helper (CD4) and T cytotoxic/suppressor (CD8) phenotypes (and probably of dual CD4 and 8 phenotypes too).

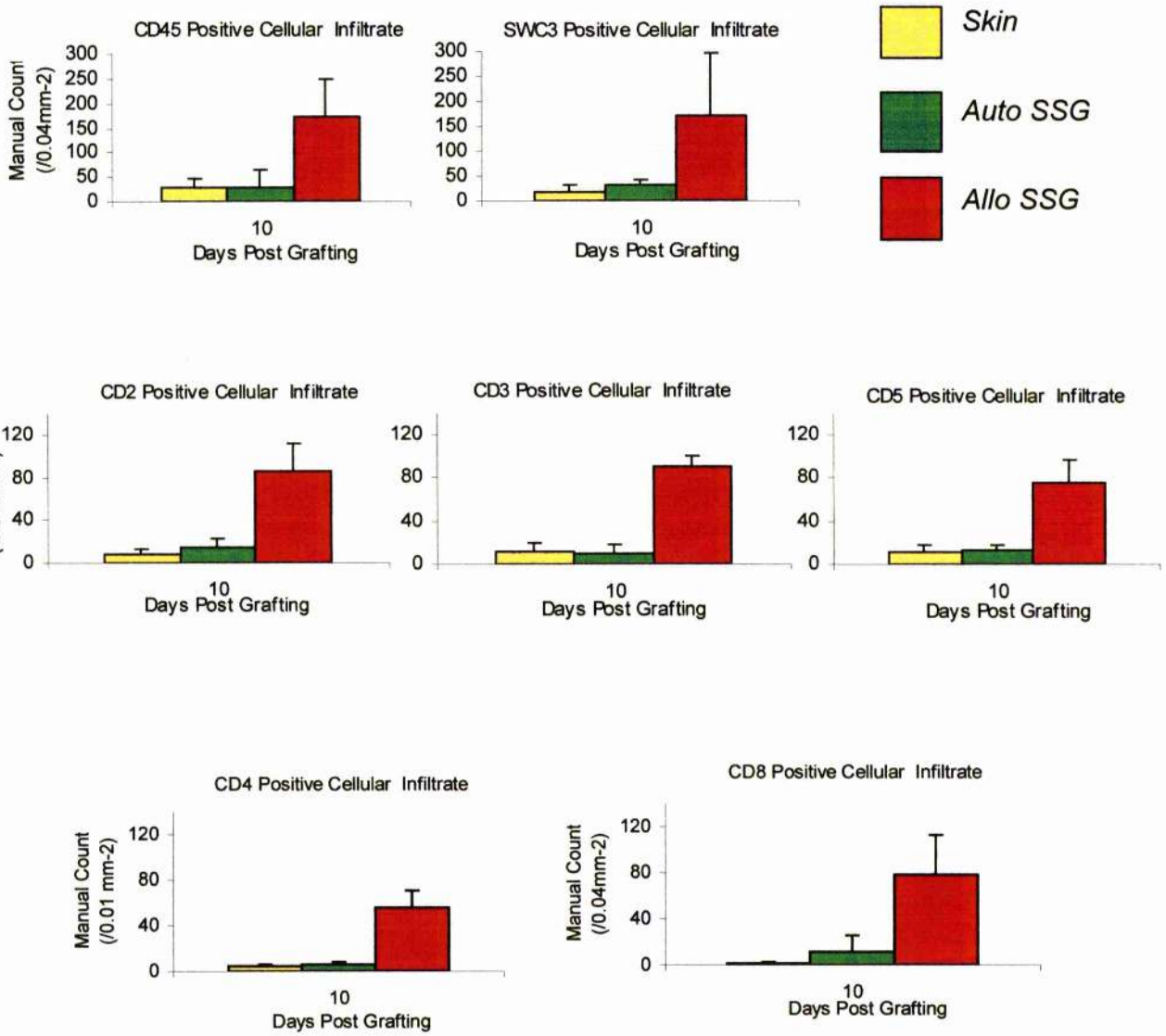
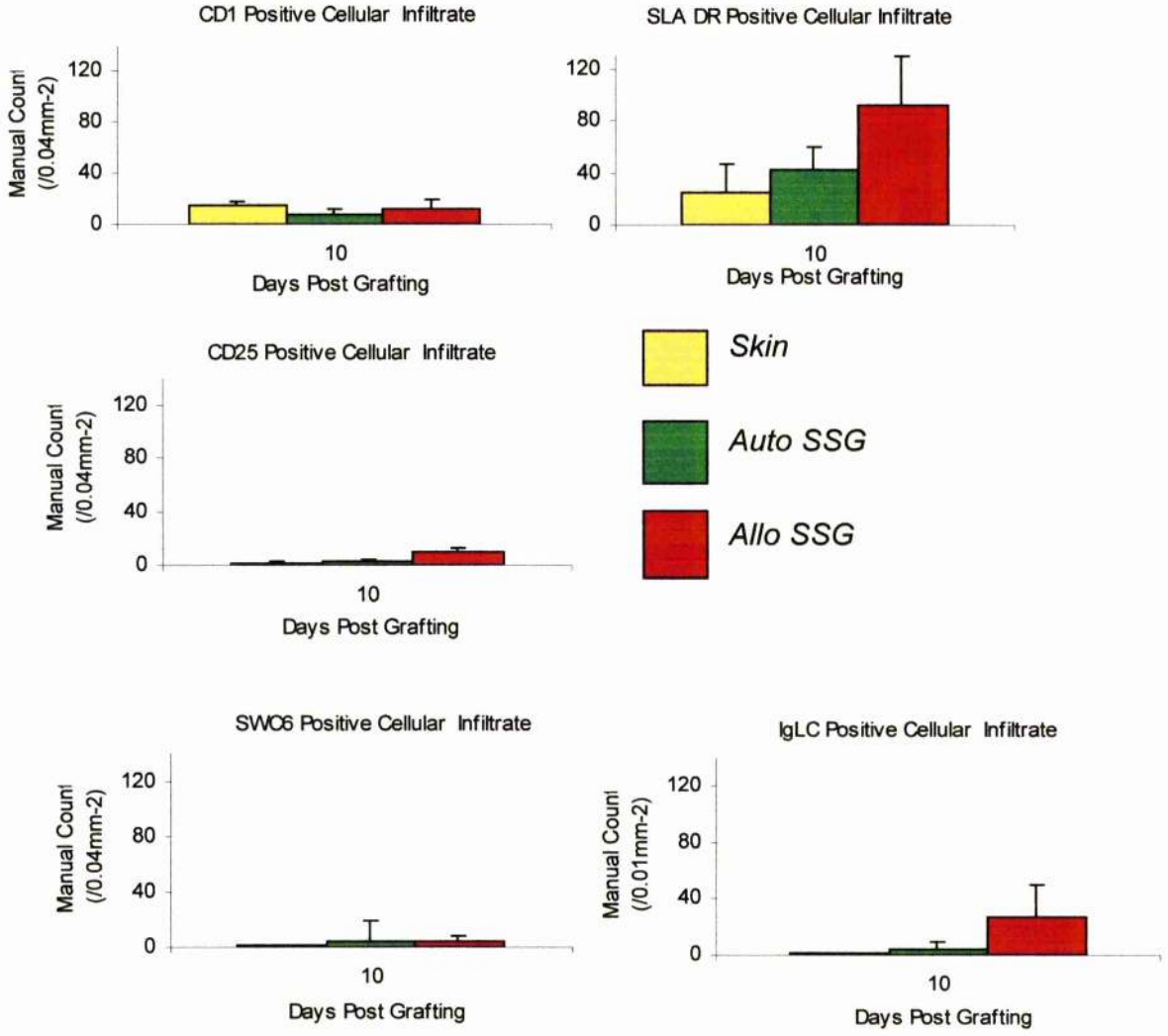


Figure 6.5: Bar charts illustrating manual counts of positively staining cells within the infiltrate of wounds grafted with allogeneic or autologous split skin graft (SSG), harvested at day 10 as well as in normal porcine skin. One Large White pig received four allogeneic split skin grafts from two non-littermate donors and two autologous split skin grafts. By day 10 all four allogeneic grafts had been rejected unlike the autologous grafts which remained healthy. Sections (see Table 6.2(a)-(c)) from allogeneic and autologous wounds were stained with monoclonal antibody with antigen-specificities as delineated in table 6.1 and then stained using the avidin biotin complex (ABC) immunoperoxidase technique.
The CD1 determinant (Langerhans/dendritic cells), is not significantly increased in any of the sections (the results for normal porcine skin are the same as those in Figure 5.5). The SLADR determinant (dendritic cells, B cells, monocytes, macrophages and activated T-cells) is increased as expected, as is the CD25 (IL-2 determinant) in the wounds grafted with allogeneic split skin graft (SSG). The IgλLC (B cell) determinant appears to be increased in the allogeneic split skin graft (SSG section) section examined. The null cell determinant SWC6 was difficult to detect in any section.



6.3 Discussion

It was important to examine the immune infiltrate associated with acute allorejection as well as to demonstrate that the Large White pig population used for this series of experiments was capable of mounting a classical allorejection reaction. As a positive control therefore, allogeneic split thickness skin was grafted directly onto fascia and was paired with wounds that had autologous split skin applied.

Classically allogeneic split skin grafts in unrelated humans are rejected at approximately 10 days after grafting (Koch et al., 1971). By day 10 gross inspection (followed by histological confirmation) revealed that all the autologous split skin grafts had taken whilst the allogeneic grafts were acutely rejecting which is consistent with the work of others (Carver et al., 1991).

Additionally the cellular infiltrate involved in acute rejection classically consists of T-cells (positive for CD4 and CD8 antigens) and a non-specific inflammatory infiltrate consisting mainly of macrophages (Rosenberg et al., 1988). The pattern seen beneath rejecting allogeneic split skin grafts in this experiment demonstrated a rise in numbers of all immunophenotypes looked for except for CD1 (dendritic cell) and null cell phenotypes. In humans the CD1 determinant may be expressed on some dendritic cells, activated monocytes and some B cells (Barclay et al., 1997). In the present study in pigs, within the infiltrate associated with rejecting allogeneic split skin grafts, SWC3 counts and SLA DR (an activation marker) counts were considerably increased with a moderate increase in Igλ (LC)-positive B cell infiltration. The CD1-positive cellular infiltrate however, was similar to normal porcine skin and autologous split skin grafts. This pattern of staining suggests that the CD1

antigen is expressed in highest density by porcine dendritic cells and that these are the cells staining positive for CD1.

Chapter 7

The fate of cultured allogeneic keratinocyte sheet grafts in the Large White pig

7.1 Introduction

In 1995 an estimated 24,986 patients were hospitalised due to burn injuries in Europe (Wedler et al., 1999). In the UK, in the 1980's, there were reportedly over 10,000 burns patients requiring hospital treatment with 600 deaths a year (Muir et al., 1987). The deaths are due to a variety of causes, not least smoke inhalation (Muir et al., 1987). The evolution of critical care techniques in combination with a more aggressive excisional approach has however led to enhanced survival in patients with massive burns (Herndon et al., 1987).

Hampering further progress is a lack of suitable skin substitutes. This is acutely felt in massive burns where there is insufficient autologous skin available for grafting. The aim of achieving a good long term outcome in terms of contracture-free, stable healing is still far off. New wound healing related products emerge commercially on a regular basis although few have achieved widespread acceptance. Cost effectiveness (for the NHS as well as for research and development departments of biotechnology companies) is a further issue that blights progress (Shakespeare, 1999).

The ideal skin substitute would display properties (see Table 7.1) similar to those of a medium thickness autologous split skin graft which forms the gold standard. Although some may claim to have achieved certain of the properties listed, there are no contemporary skin substitutes that conform to the aforementioned ideal.

Table 7.1: Properties of an 'ideal' skin substitute

Manufacture & Storage	Early Biological Function	Late Biological Function	Surgical Considerations
Cost effective	Non immunogenic	Non immunogenic	Pliable
Easy to store	Durable	Durable	Conforms well to surfaces
Long shelf life	Skin-like barrier functions (microorganisms, water loss)	Minimal contracture	Good take results in the average surgeons hands
Ready availability	Non-toxic	Minimal hypertrophic scarring	Single stage procedure
		Non-toxic	

Ready availability (within 7-10 days of injury) of the skin substitute is important both to the patient and the health service. With regard to the patient, ready availability facilitates earlier treatment with the attendant advantages of a reduction in sepsis and organ failure and the psychological benefits of earlier mobilisation. With regard to the health service it may imply cost effectiveness with reduced in-patient and time spent in critical care.

Integra™ is one such skin substitute that is engineered to form a neo-dermis. It is a non-regenerative dermal template consisting of a dermal substitute of bovine collagen and chondroitin-6-sulfate and an epidermal layer of synthetic polysiloxane polymer (Silastic) (Stern et al., 1990). The silicone epidermal membrane is removed following biointegration of the dermal component which takes approximately 10 days in the pig (Grant et al., 1998), and 3-6 weeks in humans.

Epidermal replacement options include cultured autologous keratinocytes (Boyce et al., 1993), cultured allogeneic keratinocytes (Falanga et al., 1998) or a mixture (chimera) of the two (Rouabhia et al., 1995; Rouabhia, 1996; Suzuki et al., 1995). These may be delivered in a confluent, subconfluent (Myers et al., 1997; Harris et al., 1998) or dispersed form (Stark and Kaiser, 1994; Harris et al., 1998). Only examination of the application of split skin and cultured autologous keratinocyte grafts to Integra™ has occurred to date (Lorenz et al., 1997; Pandya et al., 1998; Boyce et al., 1999).

To generate sufficienT-cells to cover the adult body surface area at a cell density of 5-8X10^4 per cm^2 in a dispersed form would take over three weeks starting from a 1cm^2 sample of skin (see Table 7.2). This time delay severely limits the use of autologous keratinocytes. The delay is further exacerbated if keratinocytes are to be used in a confluent sheet form, as extra culture time is necessary to develop additional sheets to compensate for sheet contracture that occurs with detachment of sheet from plastic flasks. Additionally further culture time is necessary to develop bilayered or multilayered sheets that have sufficient integral strength to allow for manipulation. The delay in cultured sheet grafting is less of a limitation when cultured grafts are used in the context of Integra™, in view of the 3-6 weeks necessary in humans for biointegration during which time the silicone membrane remains in place.

Keratinocytes from an allogeneic source may be cultured, frozen and banked in advance for later use. Using the techniques described earlier (see Chapter 4), the cultures may be rendered free of 'passenger lymphocytes', and thus potentially prove a useful resource.

Table 7.2: This table illustrates the maximal cover available from a 1 cm^2 piece of split skin from which $5X10^6$ viable epidermal cells may be obtained based on pig culture methods. It is assumed that the number of cells required to primarily seed a $75cm^2$ flask is $4X10^6$ and that thereafter subconfluent flasks may be subcultured 1:3 every 3 days (Bevan et al., 1997). In order to generate sheets for grafting, extra culture time of between 5 and 7 days is required to develop a sheet with integral strength and more than one layer deep. Additionally, more flasks than indicated will be required because the graft contracts as it is separated from the flask plastic. Its surface area will therefore be less than $75cm^2$.

Passage No.	Day	Cell Nos. (10^6)	# of $75cm^2$ flasks that could be seeded	Cover (cm^2) (area of confluent flasks)	% Body Surface Area Cover (BSA = $1.8m^2$)
P0	0	5	1	0	0
P1	8	6	3	75	0.4
P2	11	18	9	225	1
P3	14	54	27	675	4
P4	17	162	81	2025	11
P5	20	486	243	6075	34
P6	23	1458	729	18225	101
P7	26	4374	2187	54675	304

Host sensitisation to allogeneic skin is thought to be initiated directly (by donor LC) and take place centrally within draining regional lymph nodes, as opposed to peripherally within the graft itself (Rosenberg and Singer, 1992). Host sensitisation to cultured keratinocyte grafts if devoid of LC, is thought to occur as a result of infiltration by host APCs (Mason and Morris, 1986). In mice Larsen and coworkers demonstrated that host dendritic cells do not infiltrate allogeneic split grafts within the first eight days (Larsen et al., 1990a). The rate of APC infiltration (Aubock et al., 1988) may vary with the model used and site of grafting. The observed delay in rejection may therefore reflect delayed APC infiltration.

Within allogeneic split skin grafts, rejection is thought to principally be effected by class I restricted ($CD8^+$) cytotoxic T lymphocytes (Rosenberg and Singer, 1992). These infiltrating activated lymphocytes that are primarily responsible for effecting the rejection process, are thought to produce cytokines promoting influx of non-specific inflammatory cells that result in a degree of non cell-specific damage or 'bystander damage' (Rosenberg and Singer, 1992).

Reports indicate (Doody et al., 1994) that the degree of selective but non antigen-specific inflammatory infiltrate may be related to the extent of foreign class II expression within the graft. Where there is no foreign class II antigen apparent (as in a cultured graft that is presumed to be LC deficient), reports differ as to whether there is acute graft rejection as seen in the pig model (Carver et al., 1991; Aubock et al., 1988) the rat model (Fabre and Cullen, 1989) and in the murine model (Rouabhia et al., 1995; Suzuki et al., 1995), or a more gradual replacement by host elements as in human patients (Gielen et al., 1987; Otto et al., 1995), or even no rejection at all in one report in mice

(Hammond et al., 1987). The differences between these reports may reflect a number of factors including the species involved, the method of culture and elimination of 'passenger lymphocytes' and the bed to which the graft was applied. The reports suggesting more gradual graft replacement undetectable clinically, may reflect either a rejection process in which the non-specific bystander damage was minimal or a process by which the grafted keratinocytes are gradually lost by a means other than rejection.

Further evidence of gradual allogeneic keratinocyte loss with minimal bystander damage emerged with regard to chimeric grafts consisting of allogeneic and autologous cells co-cultured in predetermined proportions in murine species (Rouabhia et al., 1995; Suzuki et al., 1995).

Classically allorejection is thought to occur directly, via donor dendritic cells present within the graft (Rosenberg and Singer, 1992). Immunosupression is generally required to minimise the rejection response to allotransplantation of tissue containing cells capable of presenting antigen. Where these cells have been eliminated prior to transplantation, take is prolonged without the need for immunosupression (Faustman, 1995). This is consistent with work showing delayed entry of host APCs into these tissues (Larsen et al., 1990a).

The system used in this series of experiments is devoid of APCs upon grafting (see Chapter 4). With no antigen presentation, graft take may be expected to be prolonged (Faustman, 1995). Additionally, one would expect infiltrating host APCs to be responsible for sensitisation (indirect presentation) (Mason and Morris, 1986). If this was the case one might expect early detection of infiltrating host dendritic cells.

Ordinarily keratinocytes do not express MHC class II antigens. Within cultured keratinocyte allografts (processed as detailed in Chapter 4) therefore, only foreign MHC Class I antigens are expressed and presented by host APCs. Thus class I restricted CD8-positive cytotoxic T-cells would be expected to be generated as the principle effector cell of the rejection process (Rosenberg and Singer, 1992).

This chapter brings together the techniques evolved previously (including lacZnls keratinocyte labelling, LC elimination by complement lysis and immunophenotyping) to examine the fate of allogeneic keratinocytes within grafts.

The aims of this chapter are to examine the following hypotheses:

(a) that LC depleted allogeneic keratinocyte sheet grafts applied to Integra™ in the Large White pig are rejected after a more prolonged period of take than allogeneic skin grafts

(b) that host APCs are seen to infiltrate the graft bed prior to rejection and therefore may be involved in (indirect) antigen presentation

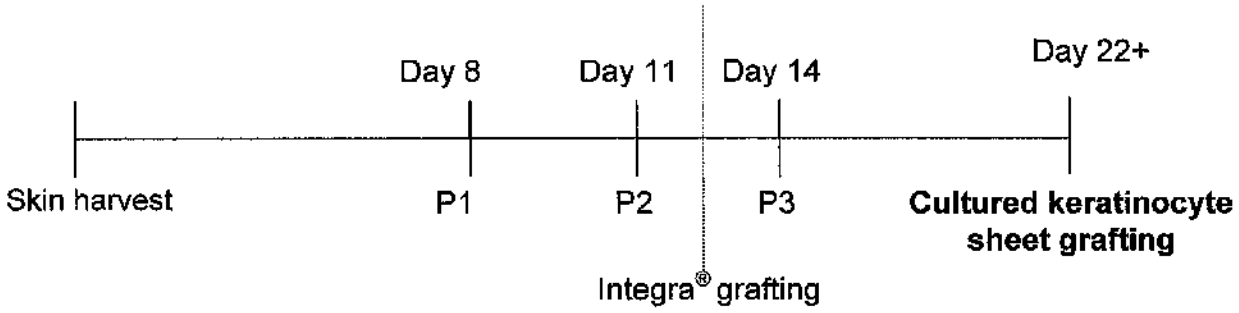
(c) that rejection is primarily effected through allo specific cytotoxic T lymphocytes.

7.2 Results

Seven pigs were used in this study. Six wounds per pig were fashioned and were randomly allocated to receive either autologous, allogeneic or no sheet graft (see Chapter 2 for method). All wounds were grafted with Integra™ ten days prior to the application of cultured sheet graft (see Figure 7.1).

Figure 7.1:

Time line showing progression from split skin harvest to cultured keratinocyte sheet grafting



Wounds were harvested at six time points: days 7, 10, 14, 18, 21 and 28 post grafting (see Table 7.3 (a)). The amount of surviving epithelium (see Table 7.3(b)) as well as MFGlacZ*nls* expression was assessed following sectioning and staining (see Figures 7.2 and 7.3) of each wound in a pre-specified fashion (see Chapter 2 for method). The immunoinfiltrate was assessed using a panel of porcine-specific monoclonal antibodies (see Chapter 6 table 6.1).

Table 7.3: Table (a) illustrates the types of wounds harvested at particular time points. Table (b) illustrates the fraction of wound types harvested that were shown to have epithelial cover.

a)

Wound Harvest Time Points (Days)		**7**	**10**	**14**	**18**	**21**	**28**
Types of Graft Applied to wounds (no. of wounds)	Autologous Sheet	2	2	4	2	2	2
	Allogeneic Sheet	3	3	6	3	3	3
	Control	1	1	2	1	1	1

(b)

Wound Harvest Time Points (Days)		**7**	**10**	**14**	**18**	**21**	**28**
Fraction of wounds that showed epithelium as identified histologically with H&E staining at time of harvest	Autologous Sheet	1/2	1/2	3/4	1/2	0/2	2/2
	Allogeneic Sheet	2/3	1/3	1/6	1/3	0/3	0/3
	Control	0/1	0/1	0/2	0/1	0/1	0/1

Figure 7.2:

(a) Photograph of a block from a wound grafted with cultured allogeneic keratinocyte sheet graft applied to Integra™. The block has been incubated in X-gal in order to detect β-gal expression (see Chapter 2 for method). The blue stain confirms the presence of β-galactosidase indicating that the epithelial cover is due to MFG lacZnls transduced cultured allogeneic keratinocytes. The photomicrograph (b) illustrates a section from the same wound stained with H&E (at X100 magnification) in addition to incubation in X-gal.

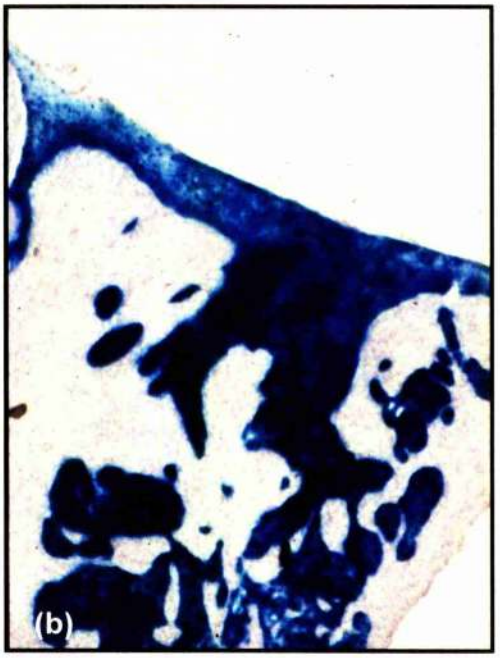
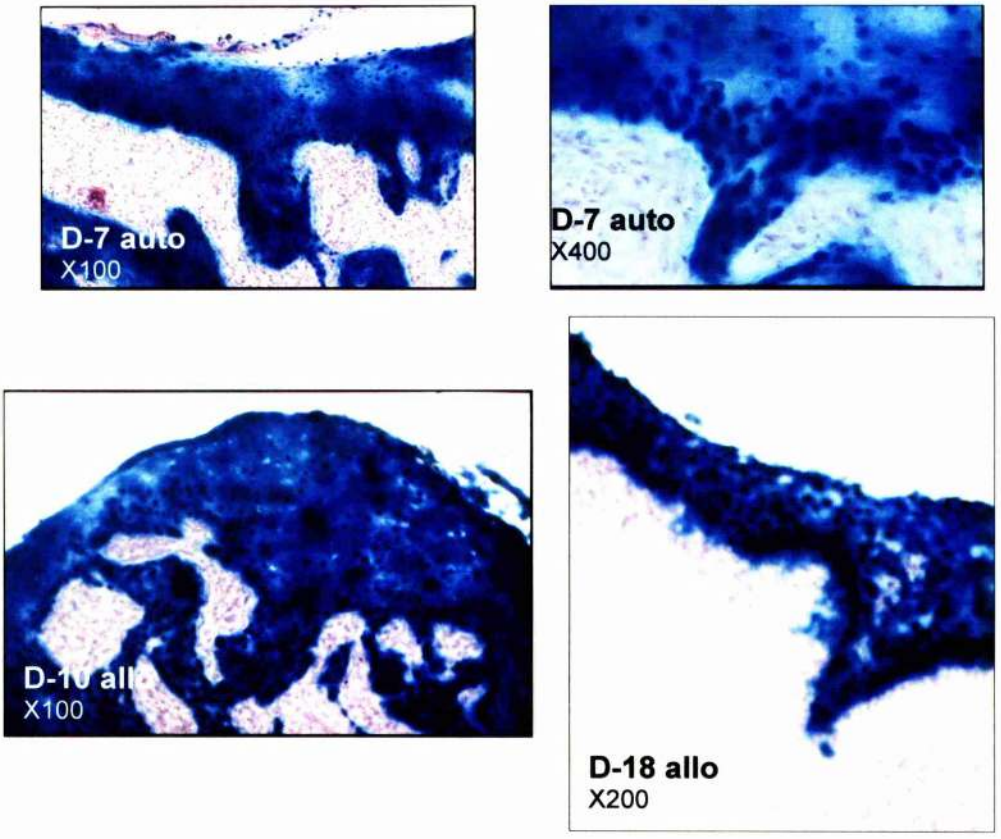


Figure 7.3:

Photomicrograph of an H&E and X-Gal stained sections from wounds grafted with an autologous or allogeneic cultured keratinocyte sheet grafts 7, 10 or 18 days previously. The blue stain confirms the origin of the keratinocytes. Note that within the allogeneic sheet graft β-gal expression is still evident at day 18.



7.2.1 Survival of cultured autologous and allogeneic keratinocyte grafts applied to Integra™ in the Large White pig

Early studies performed with Integra™ reported infiltration with macrophage derived giant cells and eosinophils but this did not appear to have clinical significance (Stern et al., 1990). Additionally a significant humoral response to bovine collagen was not detectable (Michaeli and McPherson, 1990). Good results have been reported following the clinical use of Integra™ and split thickness autograft (Lorenz et al., 1997) and the use of Integra™ with cultured autologous sheets in acute burns has also been reported (Pandya et al., 1998). Within the experimental setting moderate success was reported with the use of Integra™ with cultured autologous sheets in the Large White pig (Grant et al., 1998) using wound isolation chambers devised a few years previously (Kangesu et al., 1993).

The use of cultured allogeneic sheets as applied to Integra™ has so far not been examined.

In this series of experiments both the processes of repeated retroviral transduction and antibody complement pre-treatment were employed. These appeared to compromise the *in vitro* colony-forming efficiency of porcine keratinocytes (see Chapters 3 and 4) which in turn may impact on take and *in vivo* survival. The efficiency of initial take therefore ought to be considered, in order to appreciate the context in which the fate of allogeneic keratinocytes is explored. In this series of experiments early graft biopsies to confirm initial take were not undertaken. The reason for this omission was that it was

thought that trauma resulting from early punch biopsies may have been responsible for the disappointing graft survival that was experienced in prior pilot studies. These inflict a degree of wound trauma, result in exposure of a part of the wound that is then more vulnerable to infection and additionally may encourage a local inflammatory response. Additionally it was thought advisable to maximise the material available for later studies.

Survival of grafted epithelium at the time of wound harvest was however assessed, and was detected on only 37% of grafted wounds (see Table 7.3b).

7.2.2 Lac Z marking of cultured keratinocytes forming autologous and allogeneic keratinocyte grafts applied to Integra™ in the Large White pig

P0 keratinocytes underwent 3 passages on feeder cells comprising the retroviral producer cells PT67 clone 8 and 3T3 fibroblasts in a 2:1 ratio. This was followed by a further passage on feeder cells consisting of 3T3 fibroblasts alone.

Marking was intense at the day 10 time point and evident still at the day 18 time point (see Figure 7.3). At days 21 and 28 no allogeneic epithelium could be detected (in 6 wounds – 2 animals). Two wounds grafted with autologous keratinocyte sheets had epithelial cover when harvested at day 28 although MFG lacZ expression was not detected. It is likely therefore that on pre-grafted Integra™ cultured allogeneic keratinocytes are lost through immunorejection at anything from between 18 – 28 days, whereas allogeneic SSGs are in the process of rejection by 10 days.

7.2.3 Immunophenotyping of infiltrates beneath autologous and allogeneic keratinocyte grafts appiled to Integra™ in the Large White pig

Seven pigs were used in this study in which autologous and allogeneic cultured sheet grafts were applied to wounds which were harvested at six time points: 7, 10, 14, 18, 21 and 28 days post grafting (see Table 7.3 (a) for wound allocation). Graft survival was variable (see Table 7.3(b)) as assessed following sectioning and staining (see Figure 7.2) of each wound in a pre-specified fashion (see Chapter 2 for method). Autologous and allogeneic blocks with the best cover were selected for analysis (see Table 7.4(a)-(f) and Figures 7.4-7.6) at these (post grafting) time points.

Table 7.4: Log of animal and wound processing (cultured keratinocyte sheet applied to Integra™) (a)

Monoclonal Antibody Specificity	Microscope magnification	Field Surface Area		**D-7 allo**	**D-7 auto**	**D-10 allo**	**D-10 auto**	**D-14 allo**	**D-14 auto**	**D-18 allo**	**D-18 auto**
CD1	200X	40,000μ^2	**Animals**	1	1	1	1	2	2	1	1
			Wounds	3	1	1	1	2	2	1	1
			Blocks	3	1	1	1	2	2	1	1
			Sections	3	1	1	1	2	2	1	1
			Fields	15	5	5	5	10	10	5	5
			Non littermate donors	2		1		1		1	
			Fields /section	5	5	5	5	5	5	5	5
CD2	200X	40,000μ^2	**Animals**	1	1	1	1	1	2	1	1
			Wounds	1	1	1	1	1	2	1	1
			Blocks	1	1	2	1	1	2	1	1
			Sections	1	2	3	2	2	2	1	1
			Fields	5	11	15	10	10	10	5	5
			Non littermate donors	1		1		1		1	
			Fields /section	5	5-6	5	5	5	5	5	5

Table 7.4: Log of animal and wound processing (cultured keratinocyte sheet applied to Integra™) (b)

Monoclonal Antibody Specificity	Microscope magnification	Field Surface Area		**D-7 allo**	**D-7 auto**	**D-10 allo**	**D-10 auto**	**D-14 allo**	**D-14 auto**	**D-18 allo**	**D-18 auto**
CD3	200X	40,000μ^2	**Animals**	1	1	1	1	1	2	1	1
			Wounds	1	1	1	1	1	2	1	1
			Blocks	1	1	2	1	1	2	1	1
			Sections	1	1	2	1	1	3	1	1
			Fields	5	5	10	5	5	15	5	5
			Non littermate donors	1		1		1		1	
			Fields /section	5	5	5	5	5	5	5	5
CD5	200X	40,000μ^2	**Animals**	1	1	1	1	1	2	1	1
			Wounds	1	1	1	1	1	2	1	1
			Blocks	1	1	1	1	1	2	1	1
			Sections	1	2	1	1	1	2	1	1
			Fields	5	10	5	5	5	10	5	5
			Non littermate donors	1		1		1		1	
			Fields /section	5	5	5	5	5	5	5	5

Table 7.4: Log of animal and wound processing (cultured keratinocyte sheet applied to Integra™) (c)

Monoclonal Antibody Specificity	Microscope magnification	Field Surface Area		D-7 allo	D-7 auto	D-10 allo	D-10 auto	D-14 allo	D-14 auto	D-18 allo	D-18 auto
CD4	400X	10,000μ^2	**Animals**	1	1	1	1	1	2	1	1
			Wounds	1	1	1	1	1	2	1	1
			Blocks	1	1	2	1	1	2	1	1
			Sections	1	1	2	2	1	2	1	1
			Fields	5	5	10	10	5	10	5	5
			Non littermate donors	1		1		1		1	
			Fields /section	5	5	5	5	5	5	5	5
CD8	200X	40,000μ^2	**Animals**	1	1	1	1	2	2	1	1
			Wounds	2	1	1	1	2	2	1	1
			Blocks	2	1	1	1	2	2	1	1
			Sections	2	1	1	1	2	2	1	1
			Fields	10	5	5	5	10	10	5	5
			Non littermate donors	1		1		2		1	
			Fields /section	5	5	5	5	5	5	5	5

Table 7.4: Log of animal and wound processing (cultured keratinocyte sheet applied to Integra™) (d)

Monoclonal Antibody Specificity	Microscope magnification	Field Surface Area		D-7 allo	D-7 auto	D-10 allo	D-10 auto	D-14 allo	D-14 auto	D-18 allo	D-18 auto
SLADR	200X	40,000μ^2	**Animals**	1	1	1	1	2	1	1	1
			Wounds	3	1	1	1	2	1	1	1
			Blocks	3	1	1	1	2	1	1	1
			Sections	3	1	1	1	2	1	1	1
			Fields	15	5	5	5	10	5	5	5
			Non littermate donors	2		1		2		1	
			Fields /section	5	5	5	5	5	5	5	5
SWC3	200X	40,000μ^2	**Animals**	1	1	1	1	2	2	1	1
			Wounds	3	1	1	1	2	2	1	1
			Blocks	3	1	1	1	2	2	1	1
			Sections	3	1	1	1	2	2	1	1
			Fields	15	5	5	5	10	10	5	5
			Non littermate donors	2		1		2		1	
			Fields /section	5	5	5	5	5	5	5	5

Table 7.4: Log of animal and wound processing (cultured keratinocyte sheet applied to Integra™) (e)

Monoclonal Antibody Specificity	Microscope magnification	Field Surface Area		**D-7 allo**	**D-7 auto**	**D-10 allo**	**D-10 auto**	**D-14 allo**	***D-14 auto***	***D-18 allo***	***D-18 auto***
CD45	200X	40,000μ^2	**Animals**	1	1	1	1	2	2	1	1
			Wounds	2	1	1	1	2	2	1	1
			Blocks	2	1	1	1	2	2	1	1
			Sections	2	1	1	1	2	2	1	1
			Fields	10	5	5	5	10	10	5	5
			Non littermate donors	1		1		2		1	
			Fields /section	5	5	5	5	5	*5*	*5*	*5*
IgλLC	400X	10,000μ^2	**Animals**	1	1	1	1	1	2	1	1
			Wounds	1	1	2	1	1	2	1	1
			Blocks	1	1	2	1	1	2	1	1
			Sections	1	1	2	1	1	3	1	1
			Fields	5	5	10	5	7	15	5	5
			Non littermate donors	1		1		1		1	
			Fields /section	5	5	5	5	5	*5*	*5*	*5*

Table 7.4: Log of animal and wound processing (cultured keratinocyte sheet applied to Integra™) (f)

Monoclonal Antibody Specificity	Microscope magnification	Field Surface Area		**D-7 allo**	**D-7 auto**	**D-10 allo**	**D-10 auto**	**D-14 allo**	**D-14 auto**	**D-18 allo**	**D-18 auto**
CD25	200X	40,000μ^2	**Animals**	1	1	1	1	1	1	1	1
			Wounds	1	1	1	1	1	1	1	1
			Blocks	1	1	2	1	2	1	1	1
			Sections	2	2	2	1	2	1	1	1
			Fields	10	10	10	5	10	5	5	5
			Non littermate donors	1		1		1		1	
			Fields /section	*5*	*5*	*5*	*5*	*5*	*5*	*5*	*5*
SWC6	200X	40,000μ^2	**Animals**	1	1	1	1	1	1	1	1
			Wounds	1	1	1	1	1	1	1	1
			Blocks	1	1	1	1	1	1	1	1
			Sections	1	1	1	1	1	1	1	1
			Fields	5	5	5	5	5	5	5	5
			Non littermate donors	1		1		1		1	
			Fields /section	*5*	*5*	*5*	*5*	*5*	*5*	*5*	*5*

Figure 7.4:

Photomicrographs illustrating ABC and X-Gal staining showing a blue staining epithelium in association with CD45 phenotypes (a)-(c); CD8 (d)-(e); SWC3 phenotypes (f)-(g) and SLA DR phenotypes (h). The sections shown were taken from a wound (10 days after grafting) that had cultured allogeneic sheet applied to Integra™. Images (a), (d) and (f) were photographed at X200 and the rest at X400 magnification.

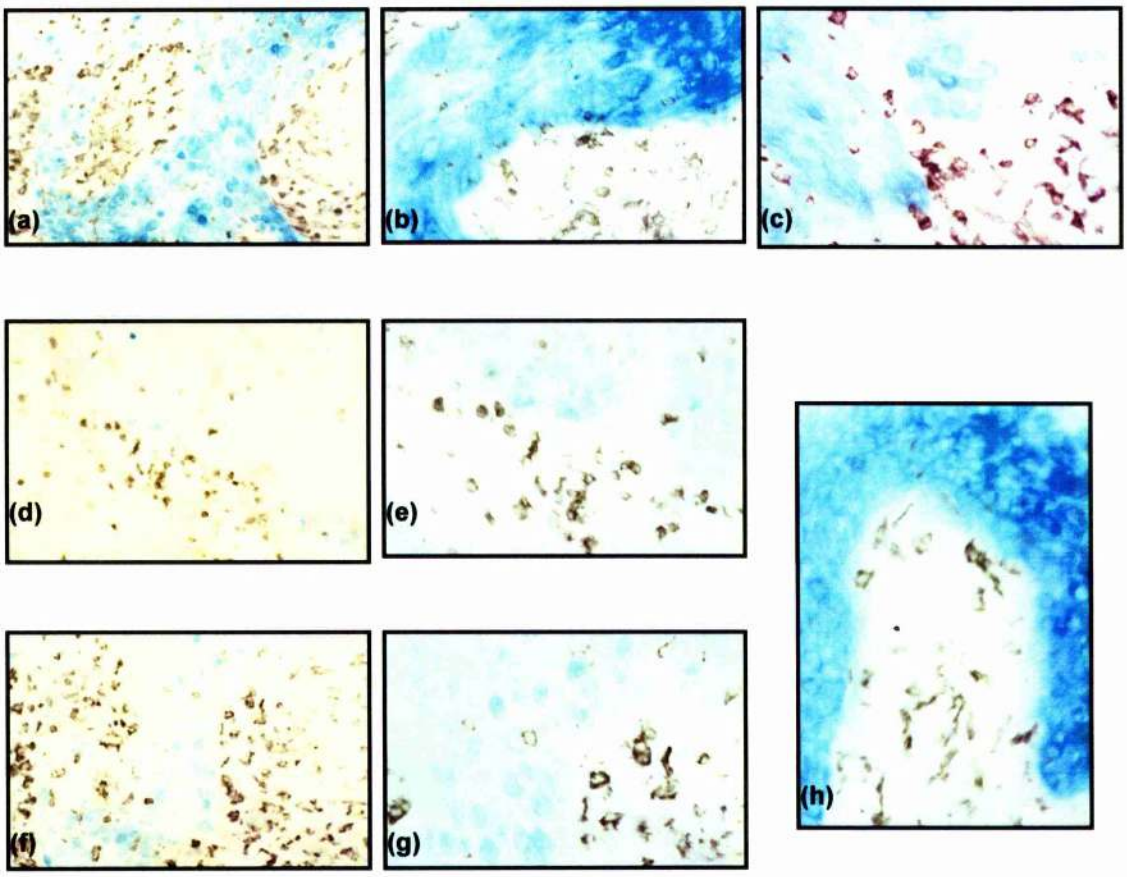


Figure 7.5: Bar charts illustrating manual counts of positively staining cells within the infiltrate of wounds grafted with allogeneic or autologous cultured porcine keratinocyte sheet graft onto Integra™ and harvested at days 7, 10, 14 and 18 (see 7.2.3). Blocks from all allogeneic and autologous grafted wounds were examined and sections (see Table 7.4(a)-(e)) from wounds with surviving epithelium were stained with monoclonal antibody with antigen-specificities delineated in chapter 6 table 6.1 and then secondarily stained using the avidin biotin complex (ABC) immunoperoxidase technique (except for CD4 and IgλLC labelling which was detected with immunofluorescence). Included on the right hand side of each graph are the results for normal porcine skin and autologous and allogeneic split skin graft 10 days post grafting (adapted from chapter 6 Figures 6.4 and 6.5).

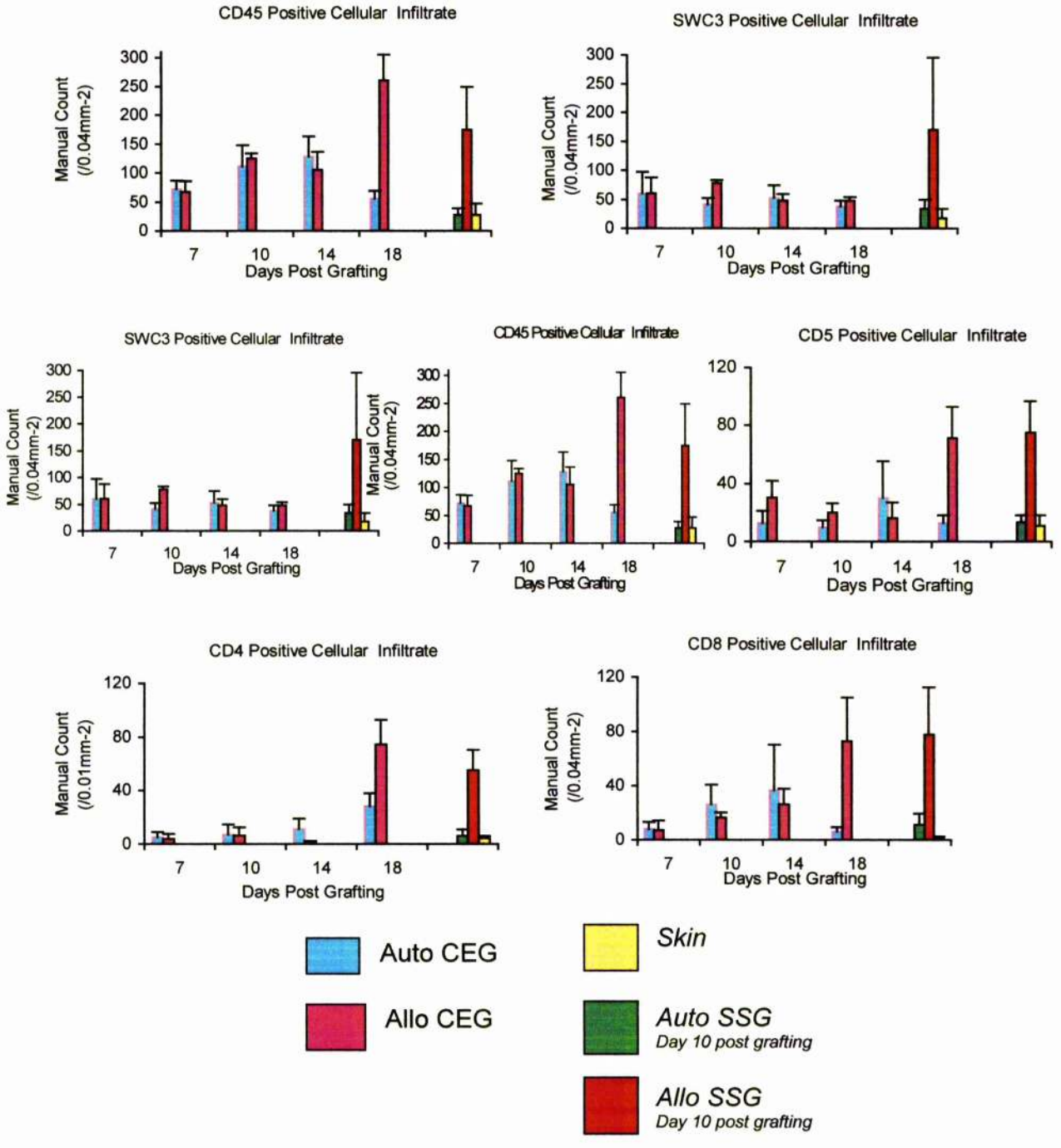
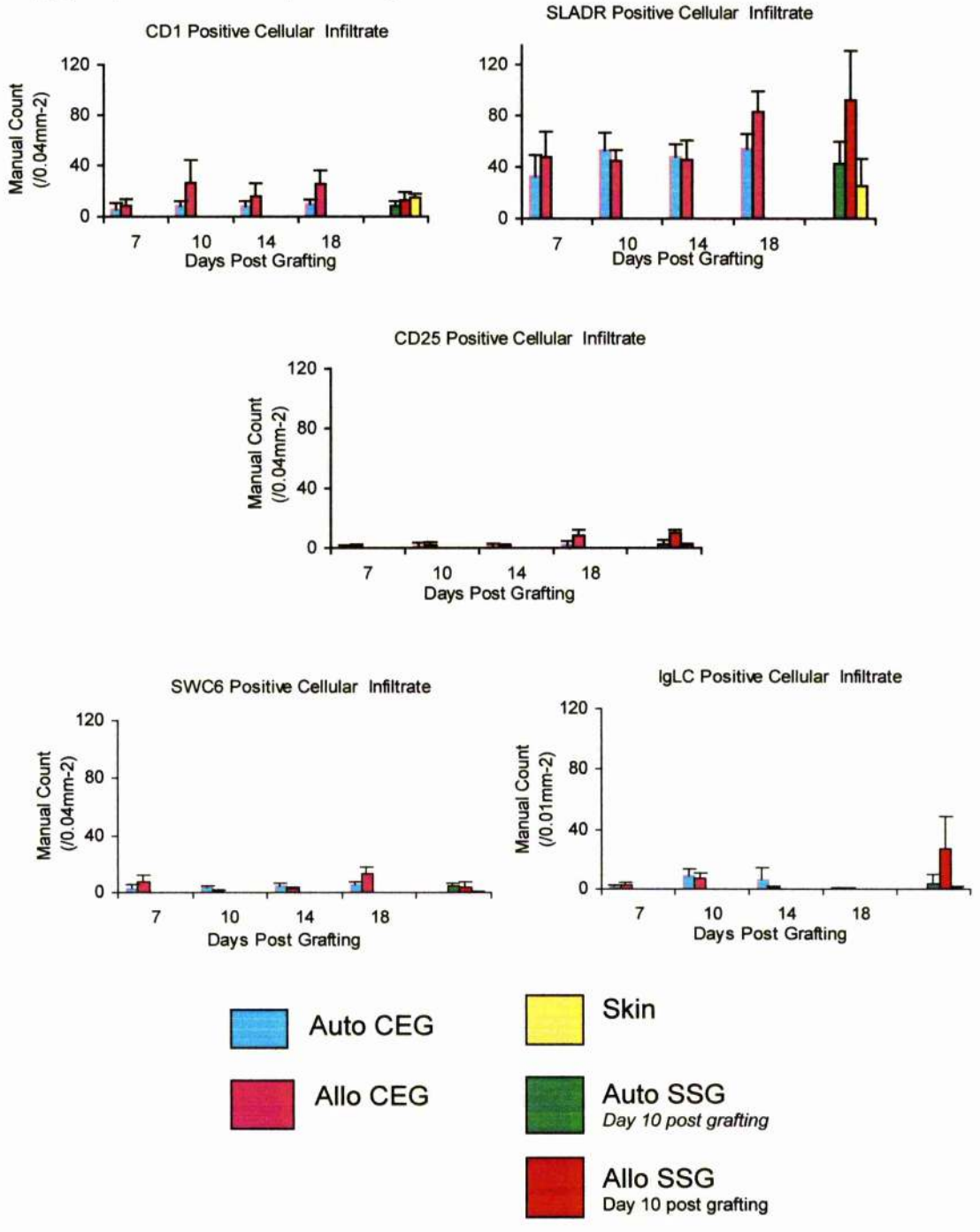


Figure 7.6: Bar charts illustrating manual counts of positively staining cells within the infiltrate of wounds grafted with allogeneic or autologous cultured porcine keratinocyte sheet graft onto Integra™ and harvested at days 7, 10, 14 and 18 (see 7.2.3). Blocks from all allogeneic and autologous grafted wounds were examined and sections (see Table 7.4(a)-(f)) from wounds with surviving epithelium were stained with monoclonal antibody with antigen-specificities delineated in chapter 6 table 6.1 and then secondarily stained using the avidin biotin complex (ABC) immunoperoxidase technique (except for CD4 and IgλLC labelling which was detected with immunofluorescence). Included on the right hand side of each graph are the results for normal porcine skin and autologous and allogeneic split skin graft 10 days post grafting (adapted from chapter 6 Figures 6.4 and 6.5).



The study was initially designed so as to generate sufficient material for analysis to enable significant conclusions to be drawn. Poor graft survival unfortunately, precludes the drawing of significant conclusions. Sufficient material was available with epithelial cover at the various time points however, to allow for useful analysis of their infiltrates.

A panel of 12 porcine-specific monoclonal antibodies was used (see Chapter 6 table 6.1). This included markers for pan leucocyte (CD45), pan T-cell (CD2, 3 and 5), plasma cell, mature or activated B cell (IgλLC), macrophage/granulocyte (SWC3), APC (CD1), null cell (SWC6) phenotypes. Additionally markers for CD4, usually associated with the T helper phenotype, CD8, usually associated with the T suppressor/cytotoxic phenotype, the IL–2 receptor (CD25) and SLA DR expression were used (see Chapter 5 for a more in depth discussion of the markers).

Two to five sections per wound were stained using the nickel-enhanced ABC method and additionally incubated for twenty minutes in X-Gal (apart from CD4 and IgλLC which were detected using Cy3 – an immunofluorescent dye). Using an optical grid counts were taken from five random fields ($0.04mm^2$) per section at X200 magnification to a depth of 0.5mm below the epidermis apart from CD4 and IgλLC which were counted at X400 magnification and there each field was $0.01mm^2$ (see Tables 7.4(a)-(f)).

Infiltration by CD1-positive cells was seen to occur early. It appeared to be moderately increased in wounds grafted with allogeneic keratinocyte sheet graft, although the density of infiltrating cells as early as day 7 was close to

that of normal porcine skin as well as autologous or allogeneically grafted wounds on day 10 (see Figure 7.6).

Pan leucocyte staining (CD45) showed remarkably little difference in infiltrate density until day 18 (see Figure 7.5). Staining for the SWC3 (macrophage/monocyte), pan T-cell, CD8, CD4 and SLADR antigens allowed an appreciation of the components that made up this observed increase in panleucocytic infiltrate in wounds grafted with allogeneic keratinocyte sheet graft.

Staining with SWC3 (see Figure 7.5) revealed that macrophages/granulocytes did not appear to form a significant proportion of the increased pan leucocyte infiltrate seen at day 18.

Pan T-cell (CD2, CD3 and CD5 see Chapter 5) staining (see Figure 7.5) revealed a variable pattern. There was little difference in density of CD2+ or CD3+ infiltrate at any of the time point. With CD5 however, a three to fourfold increase in infiltrate density was seen at the day 18 time point, in wounds grafted with allogeneic keratinocyte sheet graft. This increase in infiltrate density was further seen in CD4 and CD8 labelling. CD5 is expressed at high density in CD4+ and CD8+ α/βT-cells, and thus might be detected immunohistochemically more readily than CD2 or CD3 antigens (local experience of this study with staining for these antigens tends to support this). The density of SLA DR (porcine equivalent of HLA DR) staining approximated that for SWC3 until day 18 when there was a 50% increase in infiltrate density in wounds grafted with allogeneic keratinocyte sheet graft.

Staining for B cells (Igλ LC) and null cells (SWC6) detected only small numbers of positive cells beneath both types of graft at all time points.

CD 25 is an antigen associated with the IL-2 receptor and hence with cell activation (Barclay et al., 1997). Few cells stained positive for CD25 although there was an increase in detected expression at day 18 consistent with infiltration by activated T-cells.

7.3 Discussion

This study has succeeded in developing protocols for immunophenotyping the leucocytic infiltrate beneath MFG lacZnls marked autologous and allogeneic cultured keratinocyte grafts in the pig. Overall, the number of surviving grafts at different time points was low for both autologous and allogeneic cultures. Nevertheless differences were detectable between autologous and allogeneic grafts.

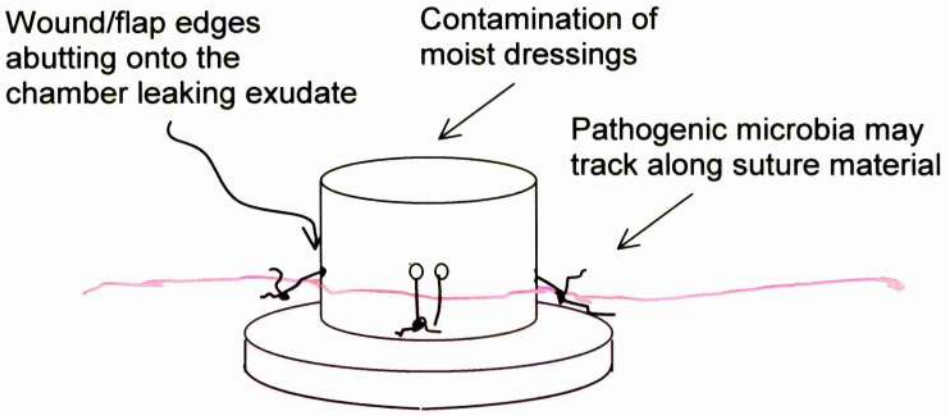
Reasons for disappointing graft survival Figures may be due to a combination of keratinocyte compromise through *in vitro* manipulation; graft trauma; wound infection; chamber extrusion and the pro-inflammatory nature of Integra.

There are several routes by which grafts may have become infected (see Figure 7.7). Wounds were dressed every 4 days. The dressings are required to protect the graft both mechanically and from infection, whilst at the same time not leeching any substances that may be toxic to keratinocytes. Due to the protrusive nature of the chambers, it proved difficult to fashion an occlusive dressing. Microorganisms may have contaminated the dressings or may have tracked along silk stitches that secured the chamber to skin,

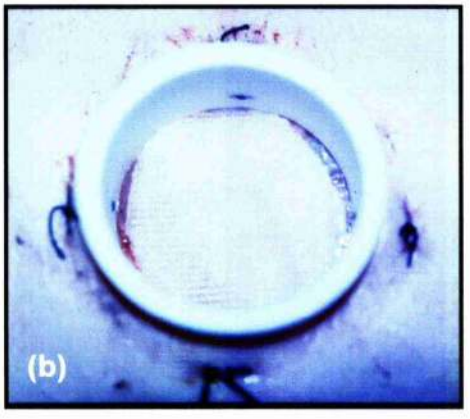
contaminated and then infected the graft. The model is additionally flawed in that the raw wound edge circumferentially abuts onto the chamber leaking copious exudate that readily finds its way to the graft. Although swabs were not routinely taken, infection characteristic of Pseudomonas was intermittently seen affecting the outer dressings and gross cellulitis affected 3 pigs (15 grafts) in which routine dressings changes were delayed due to a long Bank holiday weekend. Swabs taken for culture by a colleague using an earlier version of the model indicated rapid colonisation by coliforms (Martin R., personal communication).

Figure 7.7:

Diagram (a) illustrates various routes by which grafts applied to the wound bed (b) may become infected.



(a)



Between three and four weeks the flaps surrounding chambers contract (the term flap here referring to the skin and subcutaneous tissue surrounding the PTFE chamber that has been undermined in order to insert the chamber). With progressive flap contraction the chamber is gradually extruded. This on occasion was associated with partial flap necrosis constituting a further potential source of graft or wound bed infection.

Cultured sheet grafts are fragile and easily damaged. Additionally, when the graft is first applied to Integra™ there is no basement membrane and so graft adherence is initially tenuous. Removal of dressings therefore, may easily have resulted in some graft loss.

Integra™ is engineered to encourage cellular infiltration and may encourage a mild inflammatory reaction (Stern et al., 1990). Consequently a more severe reaction may occur to wound infection. Similarly with APCs forming a proportion of the infiltrative inflammatory response to Integra™, allorejection may occur earlier with consequent graft loss. Autologous controls were present on all pigs and the same cells were used on other animals as allogeneic grafts.

In preparation for grafting, keratinocyte sheets were lifted from culture flask plastic following incubation in Dispase. Sheets were in Dispase for between 25 minutes and an hour if slow to detach, following which they were incubated in 1-2 ml of culture medium. It took between 3 and 6 hours to detach all the necessary sheets, depending on the numbers of wounds needing grafts, after which the sheet grafts were transported in sealed 6 well plates at room temperature to the animal facility 70 miles away by car prior to grafting. Conditions for maintaining grafts optimally were thus compromised.

Lastly both the processes of retroviral transduction and active elimination of LC with antibody complement pre-treatment were seen to negatively impact on *in vitro* colony forming efficiency (chapters 3 and 4). *In vivo* efficiency of epidermal development may have similarly been compromised. Significantly, both auto and allo cell populations had undergone MFG lacZnls gene transduction and antibody complement pre-treatment.

Positive lacZ staining seen at day 18 within the epithelium covering a wound grafted with allogeneic sheet graft demonstrated the contribution of allogeneic keratinocytes to that epithelium. The presence of allogeneic keratinocytes at day 18 after grafting onto Integra™ suggests that the hypothesis that LC depleted allogeneic keratinocyte sheet grafts are rejected after a more prolonged period of take than allogeneic skin grafts may be valid.

Expression of the CD1 antigen as discussed earlier (Chapter 5), by dendritic cells (including LC) may be related to their role in antigen presentation (Barclay et al., 1997). In normal porcine skin there appear to be two main populations of CD1-positive cells. One population includes the epidermal LC population and the other is present in the papillary dermis, mostly to be found in relation to the superficial vascular plexuses (see Chapter 5).

Infiltration by CD1-positive cells was seen as early as day 7 in this experiment (see Figure 7.6). There is no direct evidence to suggest that the CD1-positive cells are dendritic cells. This is however suggested by the staining pattern with regard to autologous and allogeneic split skin grafts (see Chapter 6.3). This would imply that infiltration by APC cells such as dendritic cells occurs as early as 7 days. This suggests that the hypothesis 'that host APCs are seen to

infiltrate the graft bed prior to rejection and therefore can be involved in (indirect) antigen presentation' may be true.

The staining patterns (see Figure 7.5) with regard to the pan leucocyte (CD45), T-cell (CD2, CD3 and CD5), T helper (CD4), T cytotoxic (CD8) and macrophage/granulocyte (SWC3) markers suggests that most of the observed day 18 pan leucocytic infiltrate is comprised of T-cells; moreover these consist of approximately equal proportions of CD4 and CD8-positive cells.

The pattern of SLA DR staining suggests that it may at least partly be accounted for by up-regulation of SLA DR expression by activated T-cells. The day 18 pattern would be consistent with infiltration by activated (and hence SLA DR upregulated) cytotoxic T-cells. Thus there is no evidence from this series of experiments to refute the hypothesis that rejection is primarily effected through allo specific cytotoxic (CD8-positive) T lymphocytes.

The low numbers of B cell (Igλ LC) and null cell (SWC6) staining (see Figure 7.6) is consistent with expected infiltrate densities as neither cell is thought to play a significant role in acute rejection.

CD25 (an antigen associated with the IL-2 receptor) was detected at disappointingly low rates. A small increase in expression consistent with infiltration by activated T-cells was seen at day 18. There might however be a greater number (undetected) CD25-positive T-cells which might not be expressing CD25 at a high enough density to be seen immunohistochemically, and were therfore not seen with allo SSGs either, but may be detected with FACS.

Chapter 8

The fate of cultured chimeric and allogeneic keratinocyte-dermis composite grafts in the Large White pig

8.1 Introduction

Skin is a complex organ in which both epidermal and dermal elements are interdependent and are both necessary for optimal function. The dermal ECM consists of an ordered insoluble matrix and attached to it are soluble proteins. The ECM has a variety of functions which include maintaining epidermal strength in the face of shearing forces, providing a cushion to forces of stress, as well as in mediating cutaneous wound healing (MacNeil, 1994). The evidence suggests that ECM function is related inherently both to the three dimensional structure of the insoluble matrix and the soluble proteins found within it (MacNeil, 1994).

Composite grafts are composed principally of dermal and epidermal elements. A number of centres have developed composites using a variety of techniques (Bell et al., 1981; Ghosh et al., 1995; Ralston et al., 1997; Dawson et al., 1996; Ghosh et al., 1997; Medalie et al., 1998). With regard to the dermal component, different types of dermal equivalent have been produced and these may be grouped into one of two categories.

The first consists of a biopolymer which serves to act as a three dimensional substitute for the insoluble elements present within extra cellular matrix (MacNeil, 1994). One example is Integra™ a non-regenerative dermal template consisting of a dermal substitute of bovine collagen and chondroitin-6-sulfate and an epidermal layer of synthetic polysiloxane polymer (Silastic) (Stern et al., 1990). Many have incorporated fibroblasts into such collagen-GAG (Hansbrough et al., 1989; Boyce et al., 1993; Cooper et al., 1990; Cooper and Hansbrough, 1991; Cooper et al., 1993; Eaglstein et al., 1995; Meana et al., 1998) and fibrin

gel (Meana et al., 1998) biopolymers in an effort to reintroduce soluble proteins present within ECM that are conducive to maintaining a healthy integument (MacNeil, 1994).

The second approach is to use natural dermis that has been rendered acellular (Ghosh et al., 1997; Medalie et al., 1996a; Medalie et al., 1996b). The dual aims when using this approach are to rid the dermis of any cellular elements that may prove to be immunogenic, whilst at the same time attempting to maximise the preservation of basement membrane elements. With keratinocyte seeding, an epidermal-dermal structure resembling normal skin is apparently achieved in a far shorter space of time than in biopolymer alternatives (Cooper et al., 1993; Medalie et al., 1996a; Medalie et al., 1997).

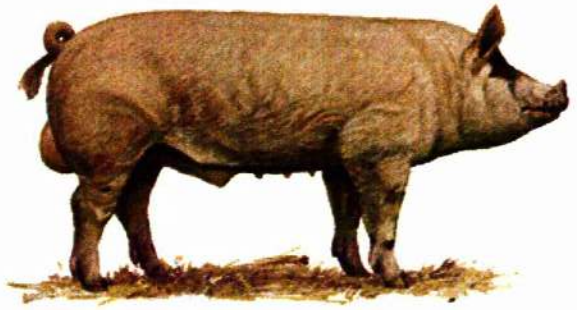
The model described earlier (see Chapters 2 and 7) utilised skills previously developed in the centre which involved the application of cultured keratinocyte sheets onto Integra™ (Grant et al., 1998). The methods used for culturing keratinocyte sheets were modified, to include a different feeder cell combination, PT67/3T3 to enable highly efficient MFG lacZnls transduction and SLA DR mediated complement lysis, to reduce LCs as described earlier (see Chapters 2, 3 and 4). The resulting keratinocytes had reduced colony forming efficiency (see Chapters 3 and 4), a more limited ability to withstand successive passage and sheets that seemed inferior in terms of mechanical strength. Additionally, Integra™ appears to be mildly pro-inflammatory (Stern et al., 1990) and it was therefore perhaps not surprising that poor graft survival and relatively early loss resulted (see Chapter 7).

In an effort therefore, to produce a more robust graft with better handling properties and which already had basement membrane elements in place,

methods were sought to develop a skin equivalent or composite graft with which to conduct further assessments of immune cell infiltrates.

There are a minimum of five steps involved in composite production: harvesting partial thickness skin, keratinocyte culture, de-epidermalisation of the harvested skin, rendering the dermis acellular, culturing keratinocytes with the dermis as substrate (see Chapter 2 for method and Figure 8.1).

Figure 8.1:

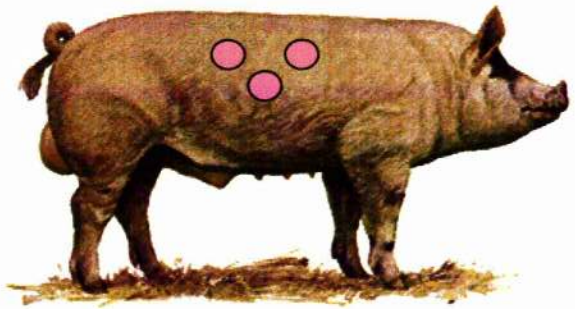


Split Skin Harvest

Keratinocyte Culture
- Langerhans Cell Depletion
- MFGlacZ*nls* transduction

Dermis
- De-epidermalise
- Render Acellular

Cultured Composite Graft



Wound Harvest

With regard to partial thickness skin harvest it is important to achieve a consistent and optimum dermal thickness. Dermis that is too thick may compromise the early nutrition of keratinocytes. In contrast dermis that is harvested too thin tends be lacy and difficult to handle. Accordingly the relationship between Zimmer® dermatome setting and resultant dermal thickness (in a single operators hands) was examined.

Several of the reported ways in which partial thickness skin may be de-epithelialised (Medalie et al., 1996a; Takami et al., 1996; Ghosh et al., 1997; Ralston et al., 1997) were evaluated. Ghosh and coworkers compared the use in human cadaver skin, of 1M NaCl (for 2-4 days) with PBS (7-14 days) and Dispase (for 1-2 hours). That study looked at the effectiveness of de-epidermalisation and the preservation of basement membrane elements and concluded that 1M NaCl and PBS are equally effective and both preserve basement membrane elements (Ghosh et al., 1997). An advantage with using 1M NaCl over PBS is that de-epidermalisation is achieved far more quickly. Dispase was shown to remove basement membrane components (Ralston et al., 1997). Medalie and colleagues subjected partial thickness human cadaver skin to three freeze-thaw cycles prior to a week of immersion in PBS in order to de-epithelialise the skin (Medalie et al., 1996a).

Accordingly in this study, a comparison of methods of de-epithelialising partial thickness porcine skin (immersion in 1M NaCl both on its own and preceded by three freeze-thaw cycles) was undertaken.

Previous researchers have immersed de-epithelialised human dermis in distilled water (Ralston et al., 1997), or PBS (Ghosh et al., 1997; Medalie et al., 1996a) for 4 weeks to render it acellular. Accordingly a study comparing

the effectiveness of PBS in rendering porcine dermis acellular against distilled water was undertaken.

The results obtained using the keratinocyte-Integra™ model described in Chapter 7 were inefficient owing to difficulties in maintaining the grafts for a sufficient period to obtain statistically valid results when significant numbers of grafts were lost. The aim therefore with this series of experiments was to develop a more robust model in the form of viable keratinocyte - dermal composites and to then test the hypotheses set out in the last chapter, within this new context.

To re-capitulate, the hypotheses were:

(a) that LC depleted allogeneic keratinocytes (within composite grafts) are rejected after a more prolonged period of take than allogeneic skin grafts

(b) that host APCs are seen to infiltrate the graft bed prior to rejection and therefore may be involved in (indirect) antigen presentation

(c) that rejection is primarily effected through allo specific cytotoxic T lymphocytes.

Additionally I sought to examine the fate of chimeric composite grafts. These are keratinocyte-dermal composites with the epidermal element consisting of allogeneic and autologous cells co-cultured in predetermined proportions. There is evidence emerging (in murine species), that these may have a considerable survival advantage (Rouabhia et al., 1995; Suzuki et al., 1995). This may be due to only gradual allogeneic keratinocyte loss with minimal bystander damage. The hypothesis to verify was therefore, that composites containing allogeneic and autologous keratinocyte elements in a 50:50 ratio

may last indefinitely with gradual replacement of the allo keratinocytes by the cultured autologous elements.

In developing the composite model the techniques of lacZnls keratinocyte labelling were utilised and LC elimination by complement lysis evolved previously. The immunohistochemical immunophenotyping techniques employed were as previously described (Chapter 2 for method and Chapters 5-7). In order to focus just on the host animal response to cultured allogeneic keratinocytes an important aspect of the study was to ensure that autologous dermis was used. It was essential though that this was rendered acellular, otherwise regeneration of autologous epidermal elements would complicate the analysis for immuno infiltrates, as might the survival of dermal dendritic cells.

8.2 Results

8.2.1 Evaluation of methods for deepidermalisation of split thickness skin and methods for rendering the resultant dermis acellular

Four regimes were compared, two of which commenced with 96 hours in 1M NaCl following which the epidermis was peeled off. This was then followed either by immersion for four days in distilled water and antibiotics and incubated at a constant 37°C, 98% relative humidity and 10% CO_2 concentration or by immersion for an identical period under the same conditions in phosphate buffered saline (PBS) and antibiotics. The other two regimes were identical except for initially undergoing 3 freeze thaw cycles in liquid nitrogen (see Chapter 2 for method).

At day eight, the processed dermis was assessed for the presence of cellular elements (propidium iodide and H&E), and for the presence of basement membrane zone collagen VII (see Figures 8.2 - 8.3).

Freeze thawed strips appeared to have reduced anti-collagen VII staining, possibly indicating a relative reduction in basement membrane integrity. Of the non freeze thawed preparations, the distilled water treated group appeared to be rendered more acellular, with less evidence of cellular remnants, with no impairment in collagen VII staining. It was therefore apparent that the method of choice is to use distilled water in order to render the dermal strips acellular, without prior freeze thawing so as to maximally preserve basement membrane elements.

Figure 8.2: Photomicrographs of processed dermis stained with H&E

All sections were taken from dermis harvested with the Zimmer® dermatome at setting numbers 8 (a-d), and 16 (e-h). Dermal strips were initially subjected to three freeze thaw cycles and immersed in 1M NaCl for 4 days followed by 4 days of PBS (a and e) or immersed in 1M NaCl for 4 days followed by 4 days of distilled water (b and f); (c and g) and (d and h) were treated in an identical manner to (a and e) and (b and f) respectively,. with the exception of the initial freeze thaw cycles. All media had 2.5μg per ml of amphotericin, 100iu per ml penicillin, 100μg per ml streptomycin and 10μg per ml ciprofloxacin added.

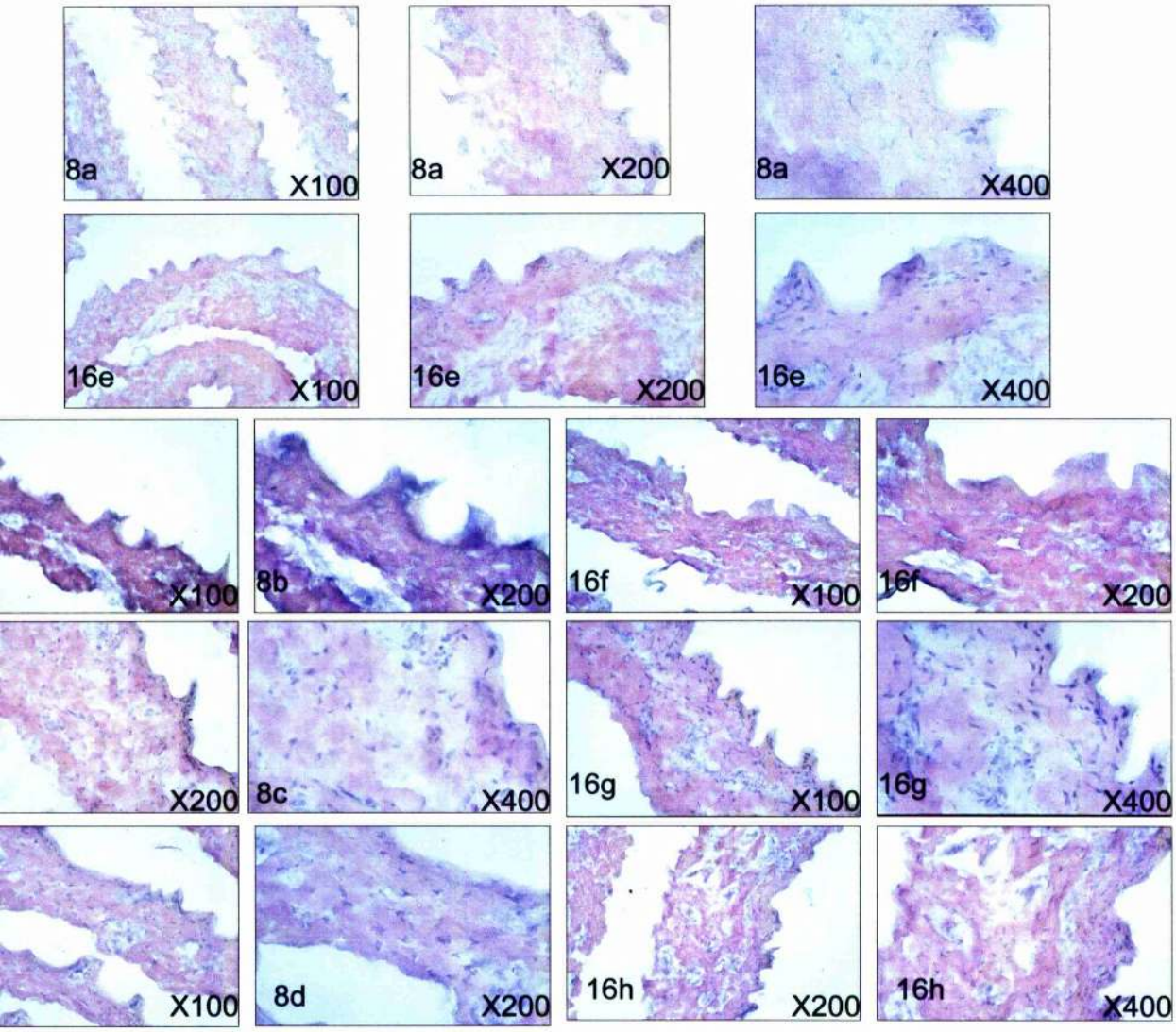


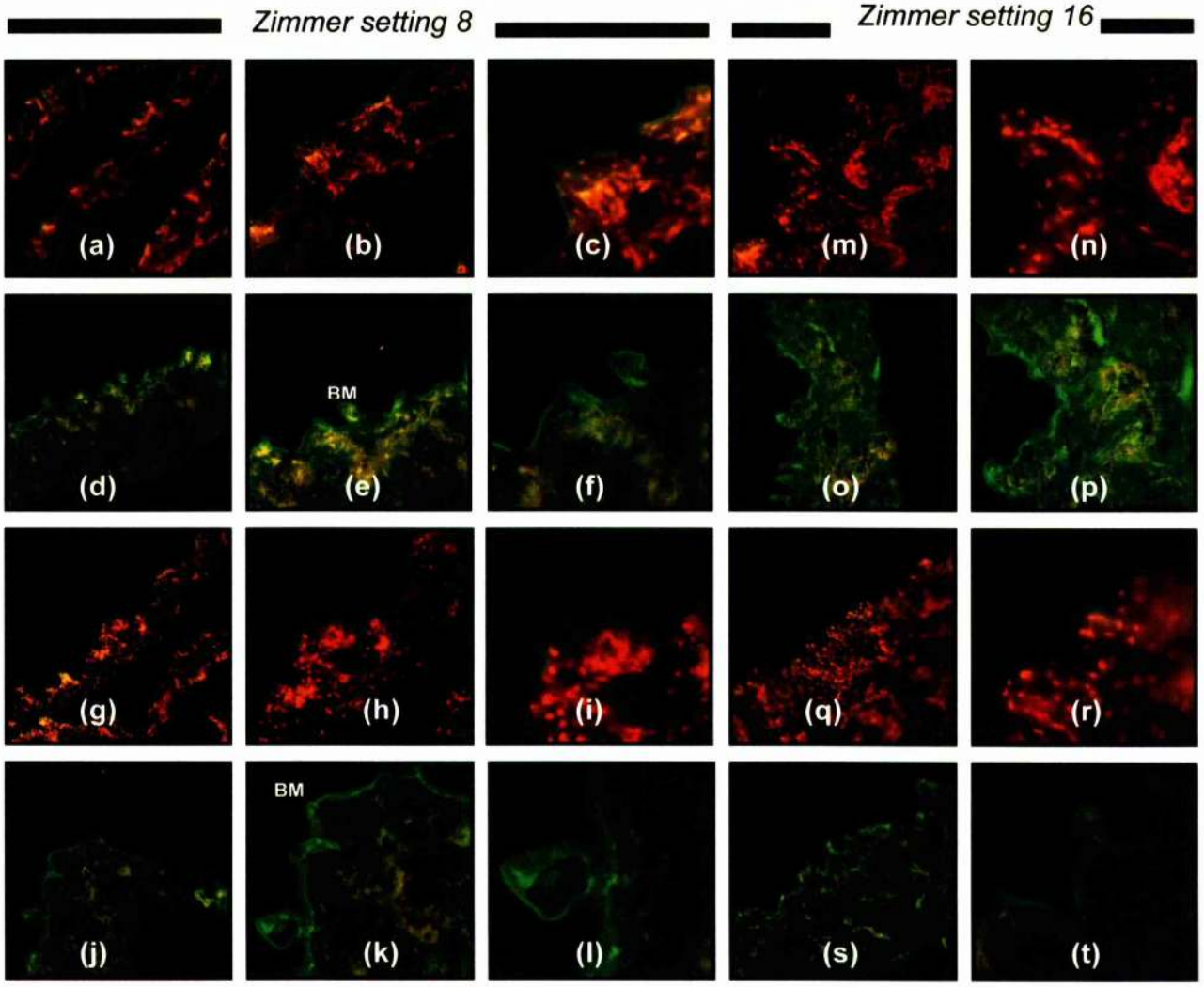
Figure 8.3: Processed dermis stained with FITC anti-collagen VII and propidium iodide

Photomicrographs were taken at X 100 ((a), (d), (g), (j), (m), (o), (q) and (s)), X 200 ((b), (e), (h), (K)) and X400 ((c), (f), (i), (l), (n), (p), (r) and (t)) magnification.

All sections were taken from dermis harvested with the Zimmer dermatome at setting numbers 8 ((a)-(l), and 16 ((m)-(t)).

Dermal strips were initially subjected to three freeze thaw cycles and immersed in 1M NaCl for 4 days followed by 4 days of PBS ((a)-(c) and (m)-(n)) or immersed in 1M NaCl for 4 days followed by 4 days of distilled water ((d)-(f) and (o)-(p)); Dermal strips ((g)-(i) and (q)-(r)) and ((j)-(l) and (s)-(t)) were treated in an identical manner to ((a)-(c) and (m)-(n)) and ((d)-(f) and (o)-(p)) respectively, with the exception of the initial freeze thaw cycles. All media had 2.5μg per ml of amphotericin, 100iu per ml penicillin, 100μg per ml streptomycin and 10μg per ml ciprofloxacin added.

The two principle conclusions from this study were that freeze thaw cycles appeared to diminish the quality of basement membrane zone collagen VII (BM) but not the degree of dermal cellularity. Additionally treatment with distilled water is superior to PBS in rendering dermis acellular (denoted by lack of propidium iodide staining) in the time period examined (without causing gross damage to the quality of basement membrane zone collagen VII).



8.2.2 Evaluation of the variation of dermal thickness with Zimmer® dermatome setting

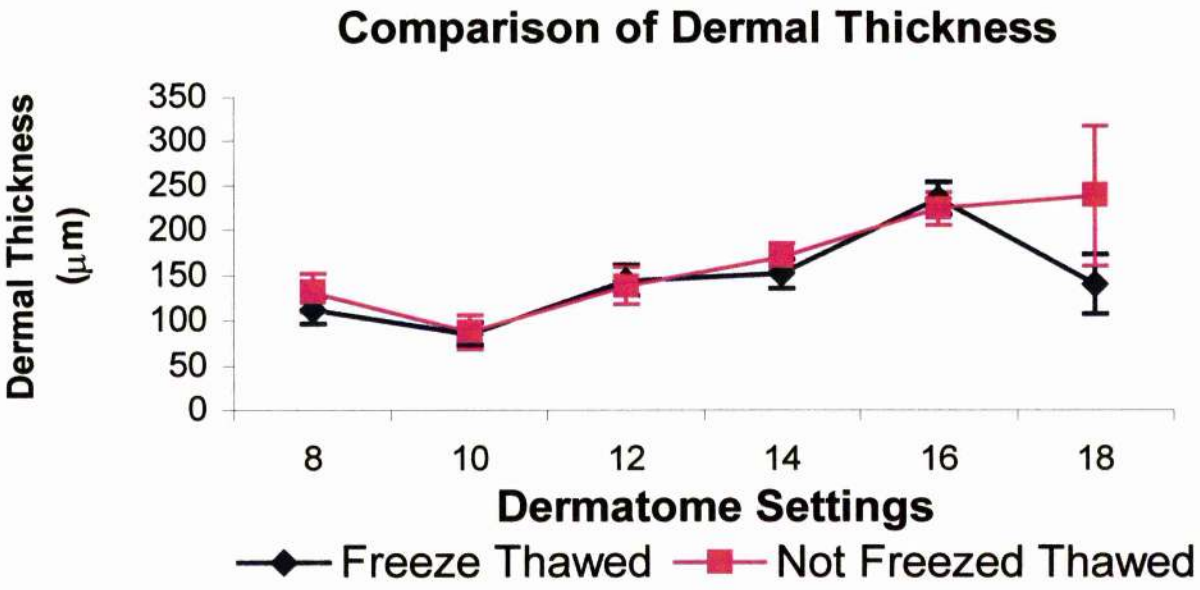
The thickness of the dermal element needs to be such that nutrition both *in vitro* and *in vivo* in the days prior to 'bio-integration', and consequently survival of the epidermal elements is not compromised. Skin harvested too thin however, tends to be 'lacy' (containing large holes), and to be difficult to handle. In the following evaluation therefore, partial thickness skin harvested from the paravertebral region using the dermatome at 3 different settings were compared. The lowest setting was 8, below which harvested skin was too thin and 'lacy'. The highest setting was 16 above which skin was too deeply harvested amounting almost to full thickness.

After the first four days (in 1M NaCl) the epidermis was peeled off and thickness of dermis gauged (see Figure 8.4). The was no significant difference in measurements between the freeze thawed and non freeze thawed groups. The dermatome setting of 12 which produced dermis of approximately 140μm following processing was adopted for future use. This dermatome setting consistently produced thin dermis with workable mechanical strength. Additionally, holes (occurring at regular intervals and relating to emerging hair follicles) were not too numerous or too large as to render the dermis difficult to use.

Figure 8.4: Assessment of processed dermis thickness

Following 96 hours in 1M NaCl epidermis was stripped off and the thickness of the dermis measured using a dial gauge micrometer. Dermal thickness was measured in 3 places after the gauge had settled (the pressure of the spring dial gauge tended to reduce the initial reading by at least 10 μm by compressing the dermis). The measurement was repeated and an average recorded. There were two groups compared, (n=2 in each group). One which underwent three freeze thaw cycles (placed in cryostat tube and immersed in liquid nitrogen for 3 minutes followed by immersion in a 37°C water bath) and one which did not. The measurements between the two groups are not significantly different. Inconsistencies seen at settings 10,14 and 18 may be related to the pressure applied when harvesting the split skin (lower pressure = thinner strips) and to minor inconsistencies in the area from which partial thickness skin was obtained from (harvesting the flanks away from the paravertebral region tends to result in less even and thinner strips).

The dermatome setting of 12 which produced dermis of approximately 140μm was adopted for future use as dermis excised at a lower setting consistently proved too full of holes and flimsy for use.



8.2.3 Evaluation of keratinocyte seeding numbers onto acellular dermis and culture media

Plating efficiency of pig keratinocytes (using Opti-MEM®1 with 1% fetal calf serum at 0.5mM calcium on collagen-I and 3T3 feeders) varies from approximately 1.2% for primary and secondary keratinocytes to between 11 and 17% for multiply passaged keratinocytes and varies considerably with medium and plating substrate (Bevan et al., 1997). The composites under investigation did not have prior seeding of fibroblasts and differ in surface structure from collagen-coated plastic flasks. The quality of epithelium achieved at different seeding densities was compared. Seeding densities used by other researchers in the manufacture of composites range from $5X10^4$ to $1.3 X 10^6$ per cm^2 (Bell et al., 1981; Ghosh et al., 1997; Medalie et al., 1996a; Ralston et al., 1997).

P4 porcine keratinocytes that had not undergone complement lysis or retroviral transduction were seeded onto acellular dermis (previously treated with 1M NaCl followed by distilled water with no freeze thawing – see Section 8.2.1) at densities of $1.38X10^5$ per cm^2, $4X10^5$ per cm^2 and $1.38X10^6$ per cm^2 (see Figure 8.5). Composites were cultured for eight days with either with the traditional Rheinwald and Green's medium (Rheinwald and Green, 1975) or with Opti-MEM®1 with 1% fetal calf serum at 0.5mM calcium (Bevan et al., 1997).

Composites were assessed by H&E staining (see Figure 8.6) and immunohistochemically with moABs specific for collagen VII (basement membrane) (see Figure 8.7), keratin 14 (expressed by most keratinocytes)

(see Figure 8.8), keratin 6 (expressed by proliferating keratinocytes) (see Figure 8.9), and keratin 10 (expressed by mature epidermal keratinocytes).

Quality of cover was assessed in terms of numbers of epithelial layers and adherence to the underlying papillary dermis. Prior to freezing, the composites were folded (rather than rolled) into a tube. A single section taken longitudinally from the tube therefore allowed assessment of the composite at multiple levels.

Results (Figure 8.6) suggest that the best quality of cover in terms of numbers of epithelial layers and adherence to the underlying papillary dermis is achieved at a seeding density of $1.38X10^6$ per cm^2 when cultured with Opti-MEM®1 (with 1% fetal calf serum at 0.5mM calcium).

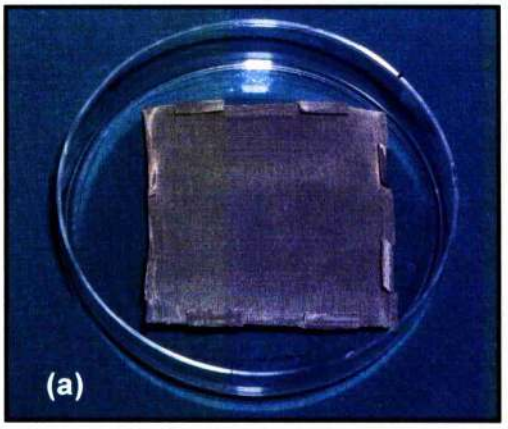
The marker for keratinocyte differentiation, cytokeratin 10, could not be detected in any of the systems (although it was seen in skin sections used as the positive control).

There was more even and apparently increased expression of cytokeratin 14 and 6 (Figures 8.8 and 8.9) with higher seeding densities.

Collagen VII is normally present at the dermo epidermal junction (as part of the basement membrane complex). Some reports have suggested that keratinocytes alone may synthesise collagen VII (Konig and Bruckner-Tuderman, 1991; König and Bruckner-Tuderman, 1994). Results here suggest (although tests were not quantitative) that collagen VII expression (Figure 8.7) did not appear to grossly increase or diminish with any particular seeding density.

Figure 8.5: Development of composite grafts

When epidermis is separated from dermis a marker stitch (s) is placed to indicate the papillary aspect. It is then placed in a 5cm diameter petri dish held to the bottom with suction and seeded with keratinocytes and culture medium made up to 3ml. On the second day following seeding, the sheets were gently transferred to and stretched over sterile stainless steel meshes (a) and held in place with integral clips (b). The meshes or grids are placed in 9cm diameter petri dishes and culture in 20-30ml of medium.



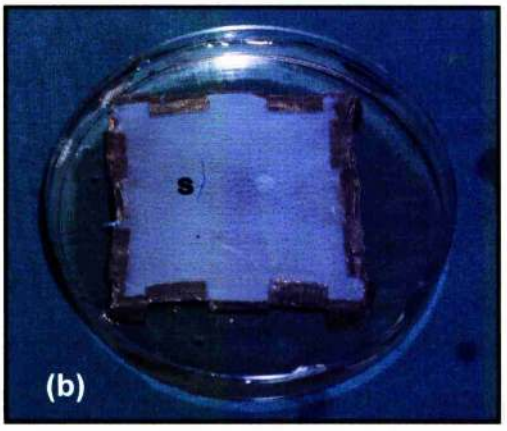


Figure 8.6: Comparison of keratinocyte seeding densities and culture media

Photomicrographs of H&E stained sections of acellular dermis seeded with keratinocytes at varying seeding densities and cultured with either Green's ((g)-(k)) or Opti-MEM®1 ((a)-(f)) (with 1% fetal calf serum and 0.5mM CaCl) for 10 days. Images (a), (e), (h) and (j) were photographed at X100, (c), (g), (i) and (k) at X200 and (b), (d) and (f) at X400 magnification. Composites were cultured in duplicate (in terms of culture medium and seeding density). Quality and consistency of cover was poorest following a seeding density of $1.38X10^5$ per cm^2 with either Green's (g) or Opti-MEM®1 (a) and (b), and best at a seeding density of $1.38X10^6$ per cm^2 with a more consistently multilayered epithelium when grown with Optimem (1% fetal calf serum and 0.5mM CaCl) (e) and (f) than with Green's (j) and (k).

The most stark difference in quality of epithelial cover depending on the culture medium used was seen with composites seeded at a density of $4X10^5$ per cm^2 , with superior cover in terms of numbers of layers (2-3 with >3 in places) and quality of adherence to underlying dermis seen when cultured with Opti-MEM®1 (c) and (d) as opposed to Green's (h) and (i).

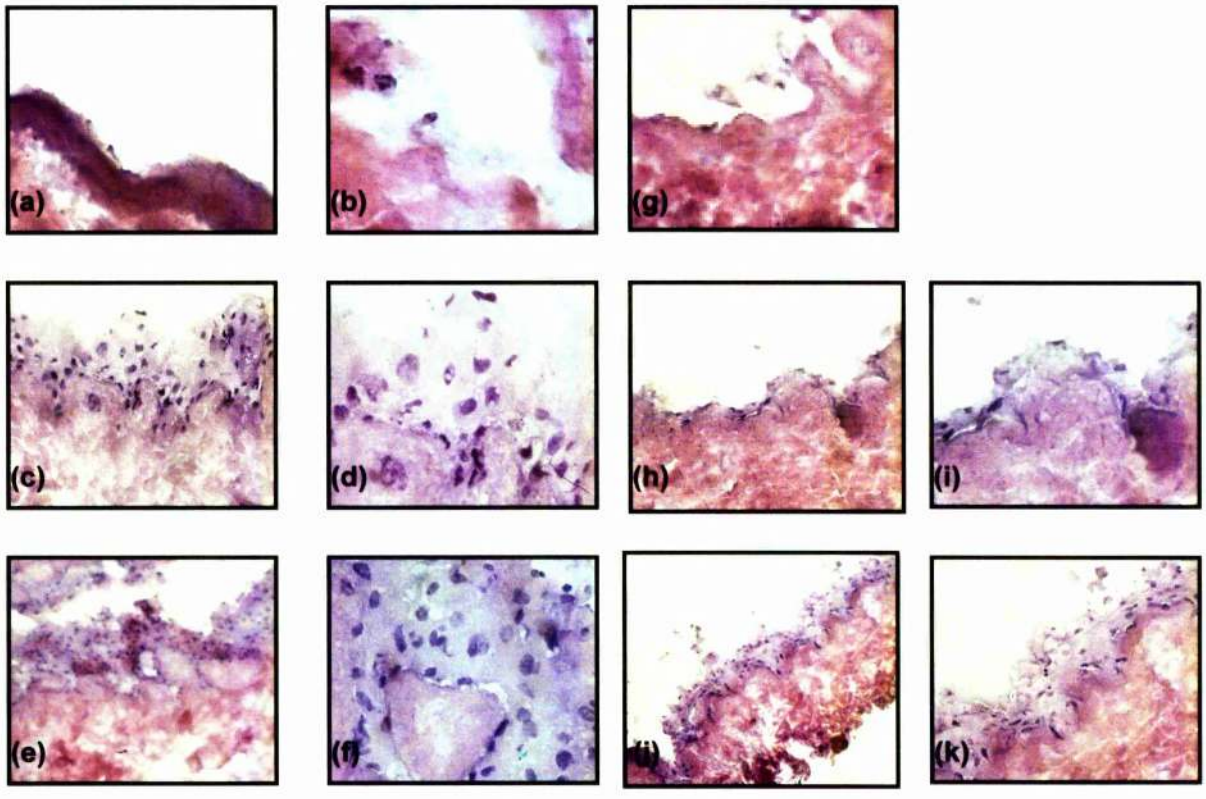


Figure 8.7: Comparison of keratinocyte seeding densities and culture media with regard to collagen VII

Photomicrographs of collagen VII - FITC stained sections of normal porcine skin ((a) and (b)) and acellular dermis seeded with keratinocytes at varying seeding densities ($1.38X10^5$ per cm^2 (c) and (h); $4X10^5$ per cm^2 (d), (e), (i) and (j) and $1.38X10^6$ per cm^2 (f), (g) (k) and (l)) and cultured with either Green's ((h), - (l)) or Opti-MEM®1 (with 1% fetal calf serum and 0.5mM CaCl) ((c) - (g)) for 10 days. All images were photographed at X200 magnification with the exception of (b) and (l) (both at X400). Composites were cultured in duplicate (in terms of culture medium and seeding density). There was no discernible difference in consistency of collagen VII expression at any seeding density or with any medium.

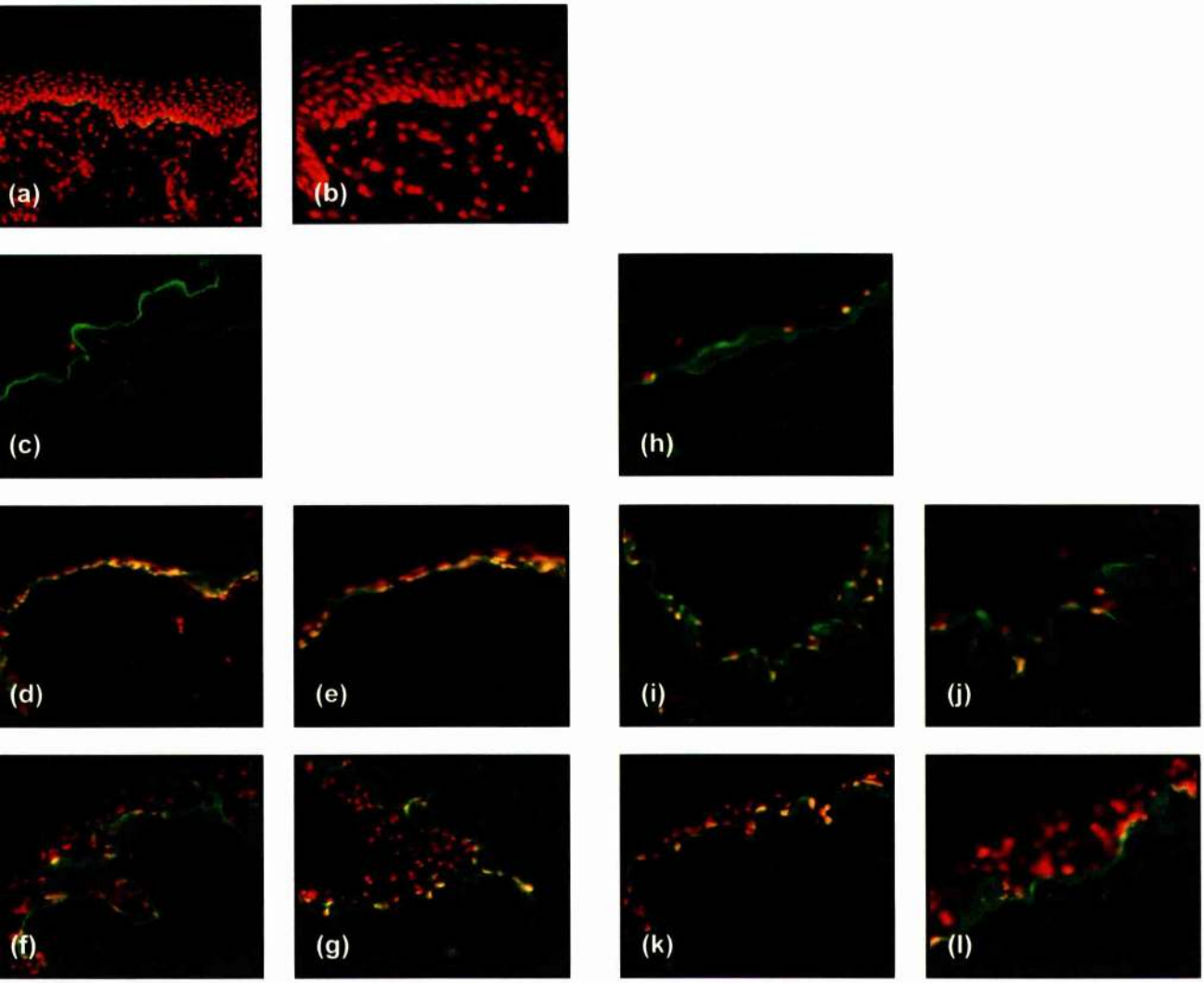


Figure 8.8: Comparison of keratinocyte seeding densities and culture media with regard to cytokeratin 14

Photomicrographs of Cytokeratin 14 - FITC stained sections of normal porcine skin ((a) and (b)-hair follicle) and acellular dermis seeded with keratinocytes at varying seeding densities ($1.38X10^5$ per cm^2 (c), (d), (i) and (j); $4X10^5$ per cm^2 (e), (f), (k) and (i) and $1.38X10^6$ per cm^2 (g), (h) (m) and (n)) and cultured with either Green's ((i), - (n)) or Opti-MEM®1 (with 1% fetal calf serum and 0.5mM CaCl) ((c) - (h)) for 10 days. Images (a), (b), (c) and (l) were photographed at X100, (j), (k) and (l) at X200 and (d), (e), (f) and (h) at X400 magnification. Composites were cultured in duplicate (in terms of culture medium and seeding density). As with collagen VII, there was no discernible difference in consistency of K-14 expression at any seeding density or with any medium.

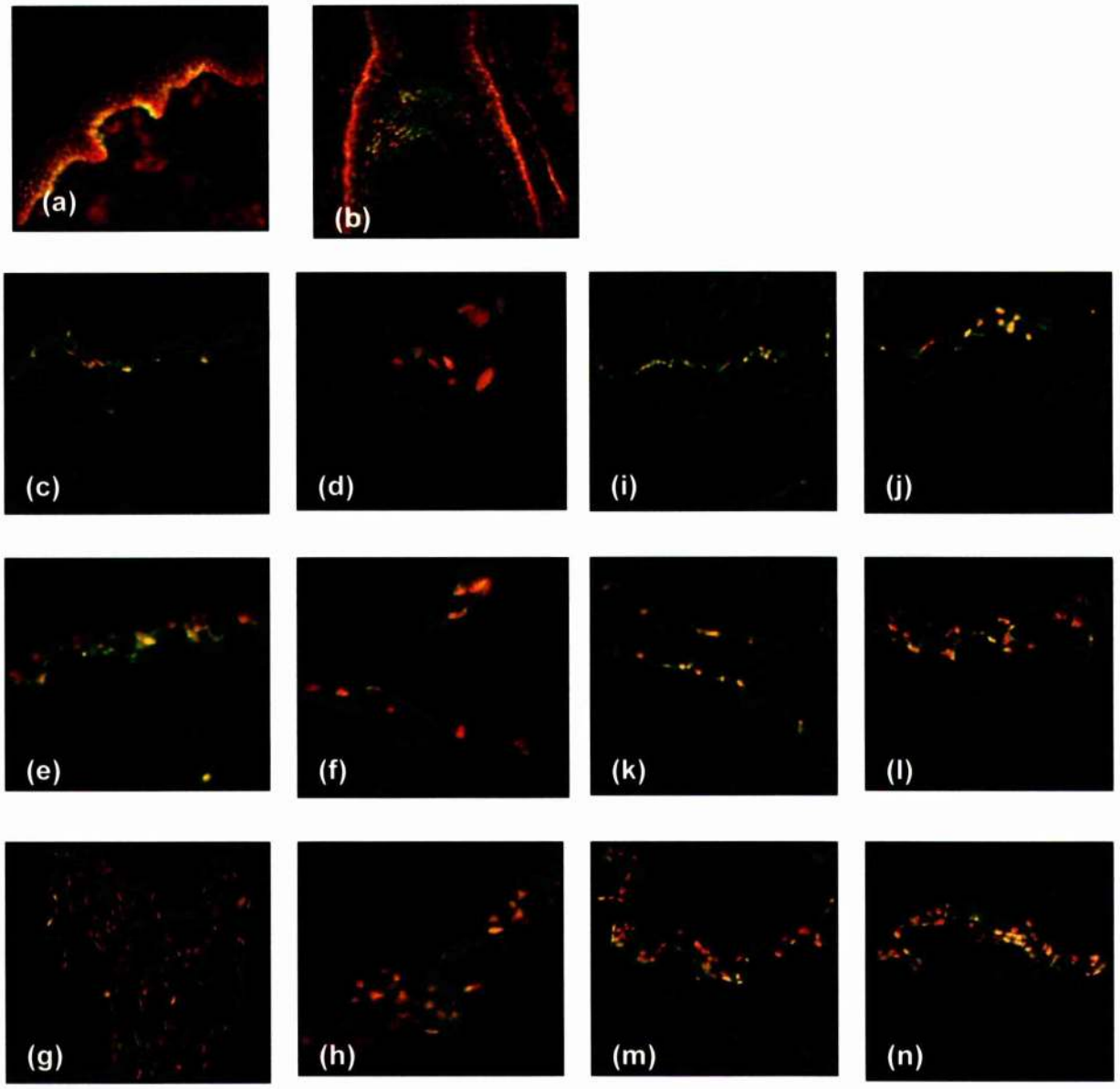


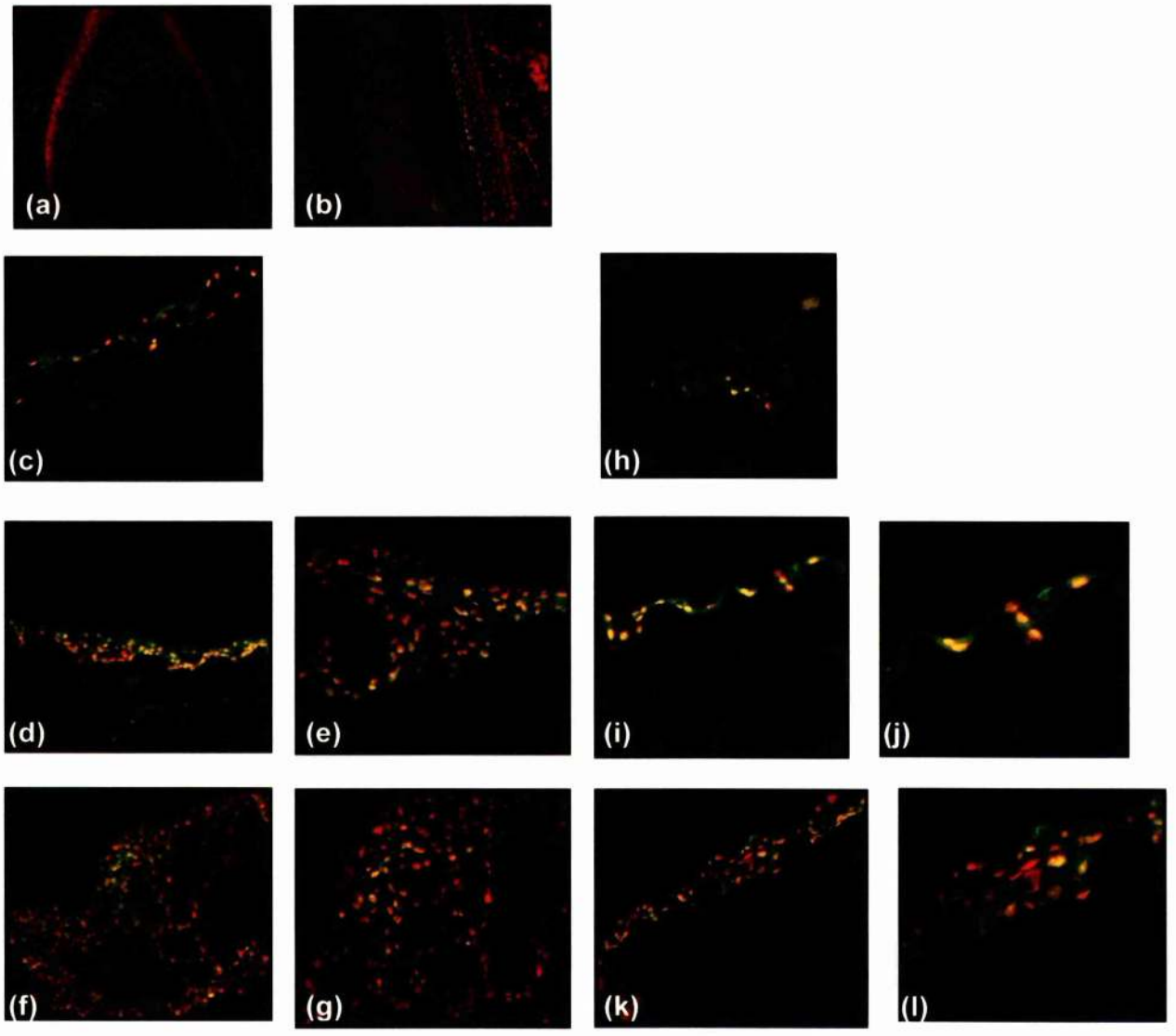
Figure 8.9: Comparison of keratinocyte seeding densities and culture media with regard to cytokeratin 6

Photomicrographs of cytokeratin 6 - FITC stained sections of normal porcine skin ((a) and (b)) and acellular dermis seeded with keratinocytes at varying seeding densities ($1.38X10^5$ per cm^2 (c) and (h); $4X10^5$ per cm^2 (d), (e), (i) and (j) and $1.38X10^6$ per cm^2 (f), (g) (k) and (l)) and cultured with either Green's ((h), - (l)) or Opti-MEM®1 (with 1% fetal calf serum and 0.5mM CaCl) ((c) - (g)) for 10 days. .

Images (a), (b), and (f) were photographed at X100, (c), (d), (e), (g), (i), (h) and (k) at X200 and (j) and (l) at X400 magnification.

Composites were cultured in duplicate (in terms of culture medium and seeding density).

As with K-14 and collagen VII there was no discernible difference in consistency of K-6 expression at any seeding density or with any medium.



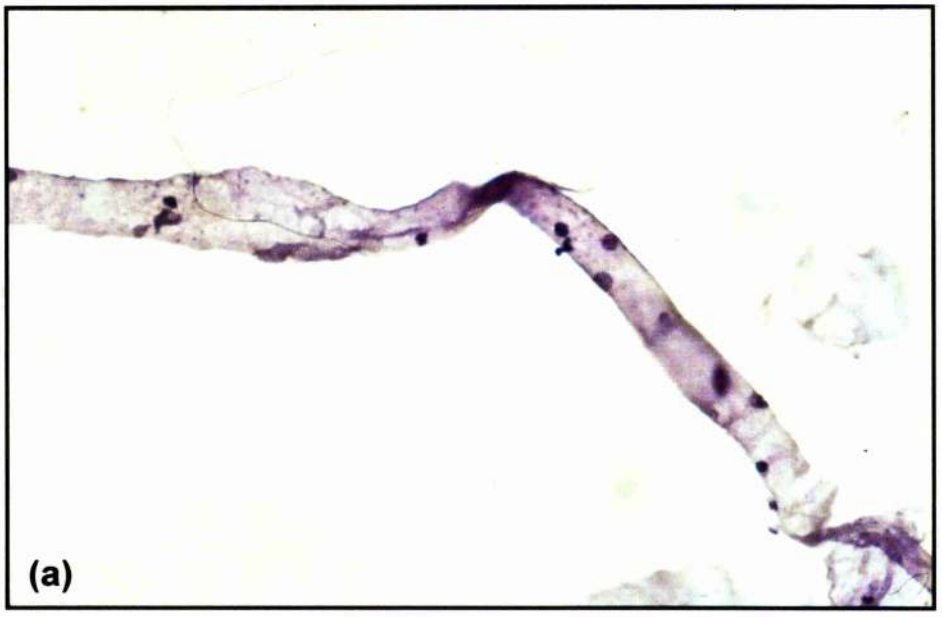
8.2.4 Survival of keratinocyte-dermal composite grafts in the Large White pig

The composite graft was developed (Figure 8.5) in order to evolve a model that was robust, easier to handle than Dispase released sheet grafts (see Figure 8.10), incorporated a basement membrane and did not encourage an inflammatory reaction. *In vitro* the best quality of epithelial cover was achieved with a seeding density of $1.38X10^6$ per cm^2 (see 8.2.3).

Six pigs were grafted with composites (see Table 8.1a and Figure 8.11). Only 13% of wounds at time of harvesting were found to have surviving epithelium (Table 8.1b). None of the autologous composites survived to 28 days. One allogeneic composite survived to 21 days with nuclear localised blue staining on incubation with X-Gal confirming that the epithelium originated with the graft (Figure 8.12). One chimeric composite graft was seen at 14 days (Figure 8.13) and two at 21 days

Figure 8.10: Cultured keratinocyte sheet graft

Photomicrograph showing cultured sheets following dispase treatment, stained with H&E (a); and anti cytokeratin 14 (b). Note that the epidermal sheets are only 1 – 2 cells deep. In addition in separate sections collagen VII was sought but was not detected (not shown as there was no fluorescence and therefore could not be seen or photographed). All images were photographed at X400 magnification.



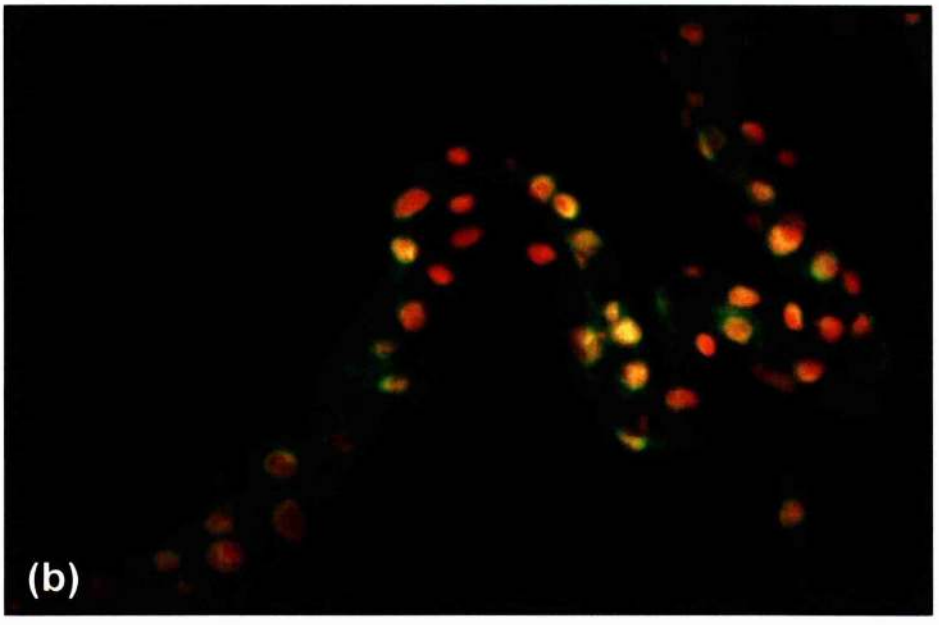


Figure 8.11: Time line showing progression from split skin harvest to composite grafting

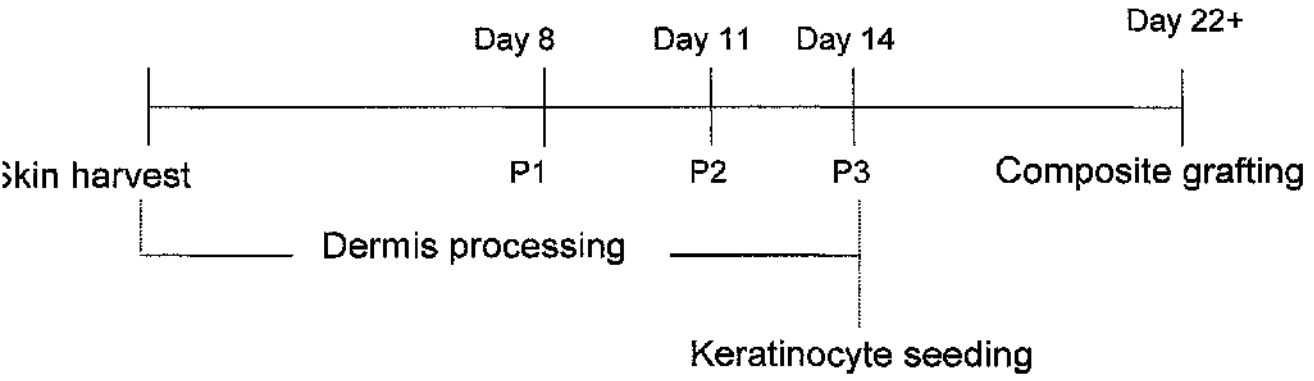
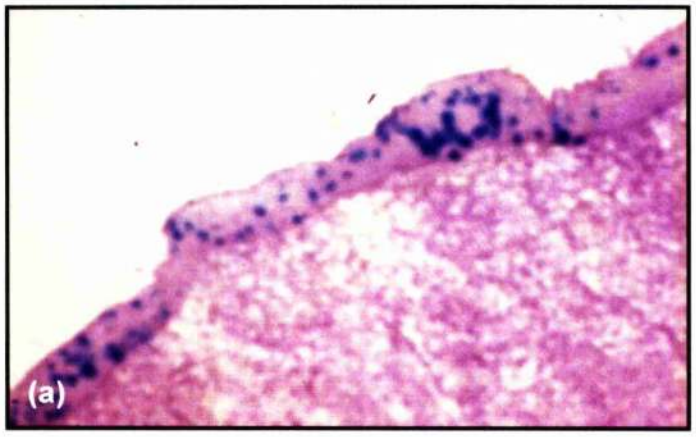


Figure 8.12: Allogeneic composite graft

Photomicrograph ((a) X 100, (b) X 400) of an H&E and X-Gal stained section from a wound grafted with an allogeneic composite graft 21 days previously. The dermis is autologous although rendered acellular. The blue stain confirms the origin of the keratinocytes. There is a characteristic 'fall off' in the proportion of β-gal expressing keratinocytes.



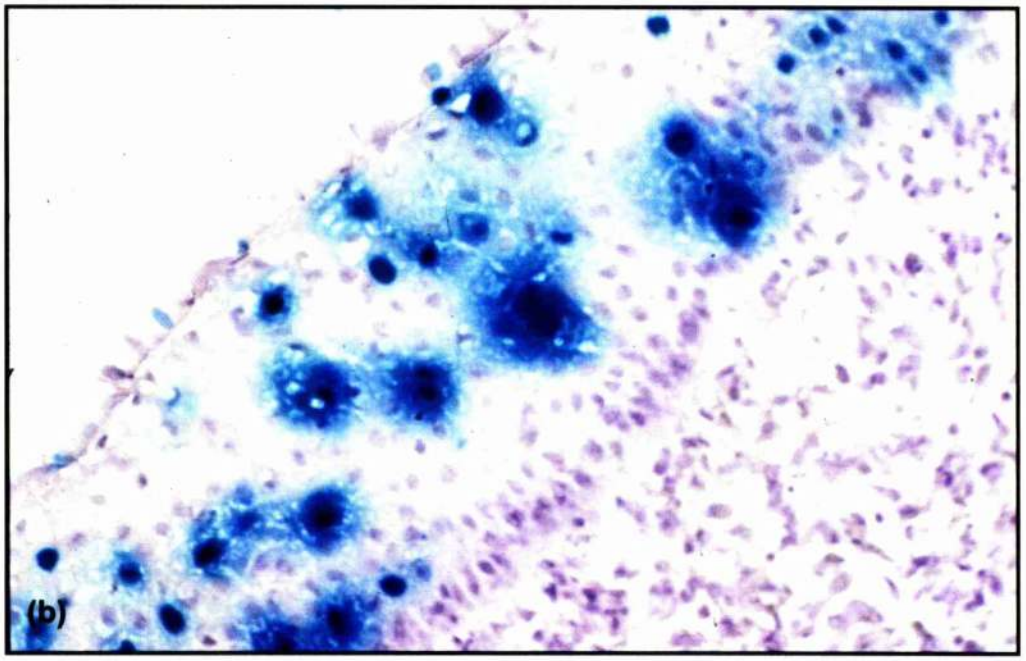


Table 8.1: Table (a) illustrates the types of wounds harvested at particular time points. Table (b) illustrates the fraction of wound types harvested that were shown to have epithelial cover.

(a)

Wound Harvest Time Points (Days)		14	21	28
Types of Graft Applied to wounds (#)	Autologous Composite	4	6	
	Allogeneic Composite	3	4	3
	Chimeric Composite	3	5	2
	Control	2	3	1

(b)

Wound Harvest Time Points (Days)		14	21	28
Fraction of wounds that showed epithelium as identified histologically with H&E staining at time of harvest	Autologous Composite	0/4	0/6	----
	Allogeneic Composite	0/3	1/4	0/3
	Chimeric Composite	1/3	2/5	0/2
	Control	0/2	0/3	0/1

8.2.5 Lac Z marking of cultured keratinocytes within composite grafts in the Large White pig

As with keratinocyte sheet grafts (grafted onto Integra™) the keratinocytes had undergone 3 passages on feeder cells comprising the retroviral producer cells PT67 clone 8 and 3T3 fibroblasts in a 2:1 ratio. This was followed by a further passage on feeder cells consisting of 3T3 fibroblasts alone.

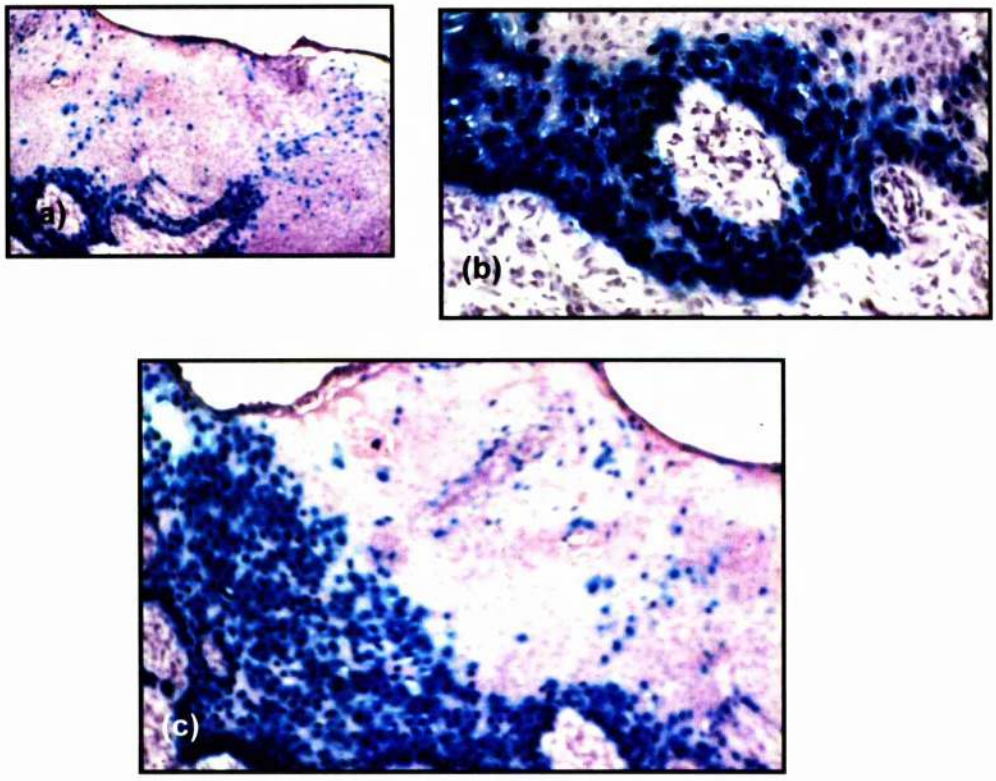
At 14 days the pattern of positive lacZ staining within epithelium on wounds grafted with chimeric composite delineated groups of proliferative keratinocytes that were β-gal expressing, and therefore clearly of allogeneic origin, and those that were not and may therefore have been of autologous origin (see Figure 8.13).

At day 21 lacZ-positive staining, evenly distributed within epithelium, covering a wound grafted with allogeneic composite (see Figure 8.12) clearly demonstrates the contribution of allogeneic keratinocyte to that epithelium.

The day 21 surviving allogeneic and chimeric composite grafts appeared healthy. By day 28 (1 pig with 2 wounds grafted with chimeric composite and 3 with allogeneic composite) however, epithelium was not detected.

Figure 8.13: Chimeric composite graft

Photomicrograph ((a) X 100; (b) X 200; (c) X 400) of an H&E and X-Gal stained section from a wound grafted with a chimeric composite graft 14 days previously. The dermis is autologous and rendered acellular. The chimeric graft is constructed with equal proportions of keratinocytes from allogeneic and autologous origins seeded onto the dermis. The allogeneic component was transduced and the blue stain confirms their origin. The division between blue and non blue staining epidermal cells may reflect the mixed origin of cells seeded (although the proportion of β-gal expressing keratinocytes *in vivo* appears to fall with time). Note the multilayered hyperproliferative epithelium.



8.2.6 Immunophenotyping results

As with the previous study (see Chapter 7) poor graft survival precludes the drawing of significant conclusions. Sufficient material was retrieved at days 14 and 21 however, to allow for useful analysis of graft bed infiltrates.

A panel of 12 porcine-specific monoclonal antibodies was used (see Chapter 6 Table 6.1, for brief description and Chapter 5 for a more in depth discussion of the markers) in the immunohistochemical analysis of graft bed infiltrates.

As with the previous study, two to five sections per wound were stained using the nickel-enhanced ABC method and additionally incubated for twenty minutes in X-Gal (apart from CD4 and IgλLC which were detected using Cy3 – an immunofluorescent dye). Using an optical grid, counts were taken from five random fields ($0.04mm^2$) per section at X200 magnification to a depth of 0.5mm below the epidermis (apart from CD4 and Igλ LC which were counted at X400 magnification and there each field was $0.01mm^2$) (see Table 8.2 and Figures 8.14-8.15).

Table 8.2: Log of animal and wound processing (composite grafts applied to deep fascia) (a)

Monoclonal Antibody Specificity	Microscope magnification	Field Surface Area μ^2		Day 14 Chimeric Composite	Day 21 Chimeric Composite	Day 21 Allogeneic Composite
CD1	**200X**	**40,000**	Animals	1	1	1
			Wounds	1	1	1
			Blocks	1	1	1
			Sections	1	1	1
			Fields	5	5	5
			Non littermate donors	**1**	**1**	**1**
			Fields/section	**5**	**5**	**5**
CD2	**200X**	**40,000**	Animals	1	1	1
			Wounds	1	1	1
			Blocks	1	1	1
			Sections	1	1	1
			Fields	5	5	5
			Non littermate donors	**1**	**1**	**1**
			Fields/section	**5**	**5**	**5**
CD3	**200X**	**40,000**	Animals	1	1	1
			Wounds	1	1	1
			Blocks	1	1	1
			Sections	1	1	1
			Fields	5	5	5
			Non littermate donors	**1**	**1**	**1**
			Fields/section	5	5	5
CD5	**200X**	**40,000**	Animals	1	1	1
			Wounds	1	1	1
			Blocks	1	1	1
			Sections	2	1	1
			Fields	10	5	5
			Non littermate donors	**1**	**1**	**1**
			Fields/section	5	5	5

Table 8.2: Log of animal and wound processing (composite grafts applied to deep fascia) (b)

Monoclonal Antibody Specificity	Microscope magnification	Field Surface Area μ^2		Day 14 Chimeric Composite	Day 21 Chimeric Composite	Day 21 Allogeneic Composite
CD4	**400X**	**10,000**	Animals	1	1	1
			Wounds	1	1	1
			Blocks	1	1	1
			Sections	2	1	1
			Fields	10	6	5
			Non littermate donors	**1**	**1**	**1**
			Fields/section	**5**	**5**	**5**
CD8	**200X**	**40,000**	Animals	1	1	1
			Wounds	1	1	1
			Blocks	1	1	1
			Sections	1	1	1
			Fields	5	5	5
			Non littermate donors	**1**	**1**	**1**
			Fields/section	**5**	**5**	**5**
SLADR	**200X**	**40,000**	Animals	1	1	1
			Wounds	1	1	1
			Blocks	1	1	1
			Sections	1	1	1
			Fields	5	5	5
			Non littermate donors	**1**	**1**	**1**
			Fields/section	5	5	5
SWC3	**200X**	**40,000**	Animals	1	1	1
			Wounds	1	1	1
			Blocks	1	1	1
			Sections	1	1	1
			Fields	5	5	5
			Non littermate donors	**1**	**1**	**1**
			Fields/section	5	5	5

Table 8.2: Log of animal and wound processing (composite grafts applied to deep fascia) (c)

Monoclonal Antibody Specificity	**Microscope magnification**	**Field Surface Area μ^2**		Day 14 Chimeric Composite	Day 21 Chimeric Composite	Day 21 Allogeneic Composite
CD45	**200X**	**40,000**	Animals	1	1	1
			Wounds	1	1	1
			Blocks	1	1	1
			Sections	1	1	1
			Fields	5	5	5
			Non littermate donors	**1**	**1**	**1**
			Fields/section	**5**	**5**	**5**
IgλLC	**400X**	**10,000**	Animals	1	1	1
			Wounds	1	1	1
			Blocks	1	1	1
			Sections	1	2	1
			Fields	5	10	5
			Non littermate donors	**1**	**1**	**1**
			Fields/section	**5**	**5**	**5**
CD25	**X200**	**40,000**	Animals	1	1	1
			Wounds	1	1	1
			Blocks	1	1	1
			Sections	1	1	1
			Fields	5	5	5
			Non littermate donors	**1**	**1**	**1**
			Fields/section	5	5	5
SWC6	**X200**	**40,000**	Animals	1	1	1
			Wounds	1	1	1
			Blocks	1	1	1
			Sections	1	1	1
			Fields	5	5	5
			Non littermate donors	**1**	**1**	**1**
			Fields/section	5	5	5

Figure 8.14: Bar charts illustrating manual counts within the infiltrate of wounds grafted with chimeric or allogeneic cultured porcine keratinocyte-dermal composite grafts onto deep fascia and harvested at days 14 and 21 (see 8.2.6). Sections were examined with each monoclonal antibody. Blocks from all chimeric or allogeneic cultured composite grafted wounds were examined and sections (see Table 8.2(a)-(c)) from wounds with surviving epithelium were stained with monoclonal antibody with antigen-specificities delineated in chapter 6 table 1 and then secondarily stained using the avidin biotin complex (ABC) immunoperoxidase technique (CD4 and IgλLC secondarily labelled with Cy3). Included on the right hand side of each graph are the results for normal porcine skin and autologous and allogeneic split skin graft 10 days post grafting (adapted from chapter 6 Figures 6.4 and 6.5).

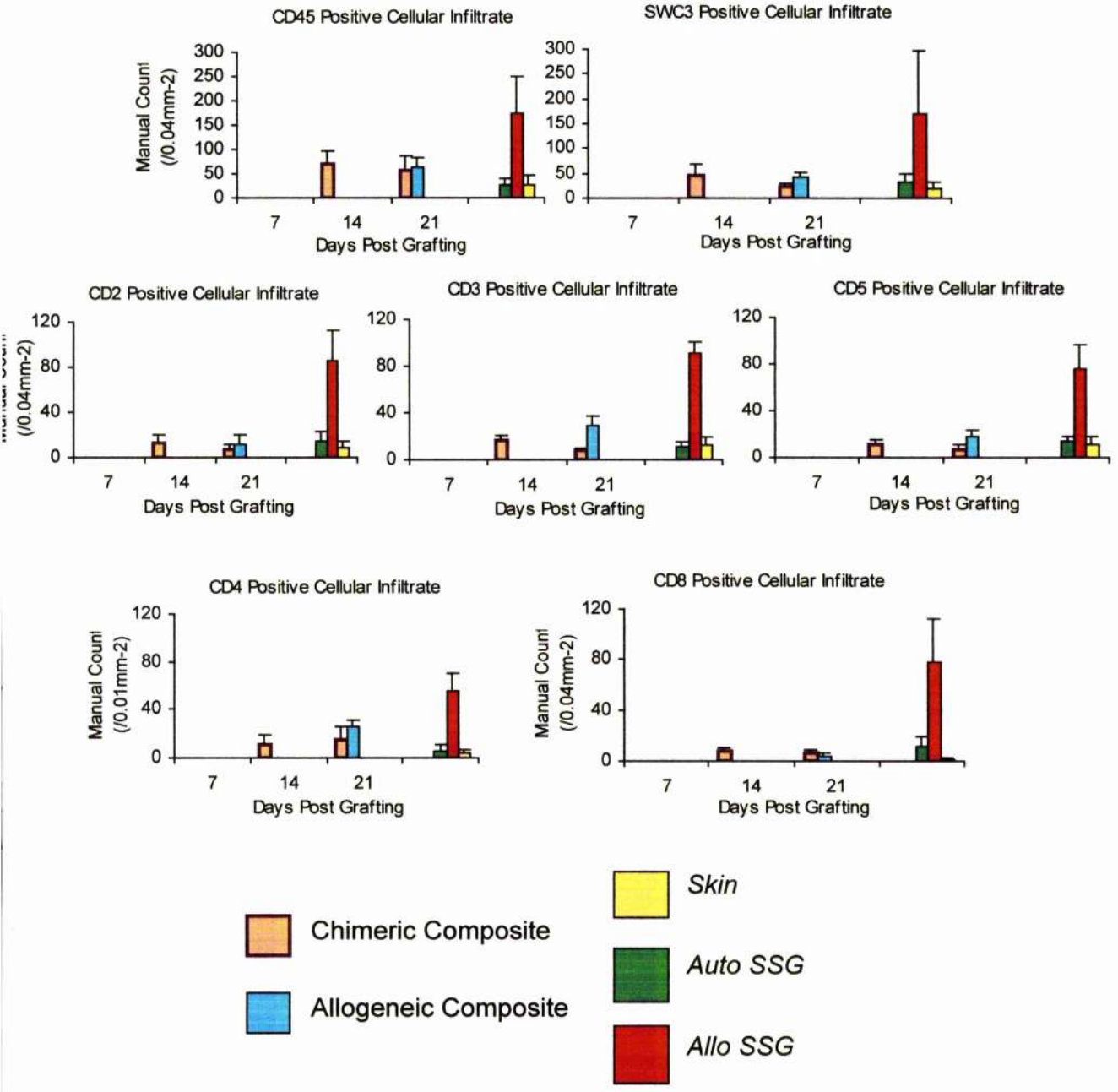
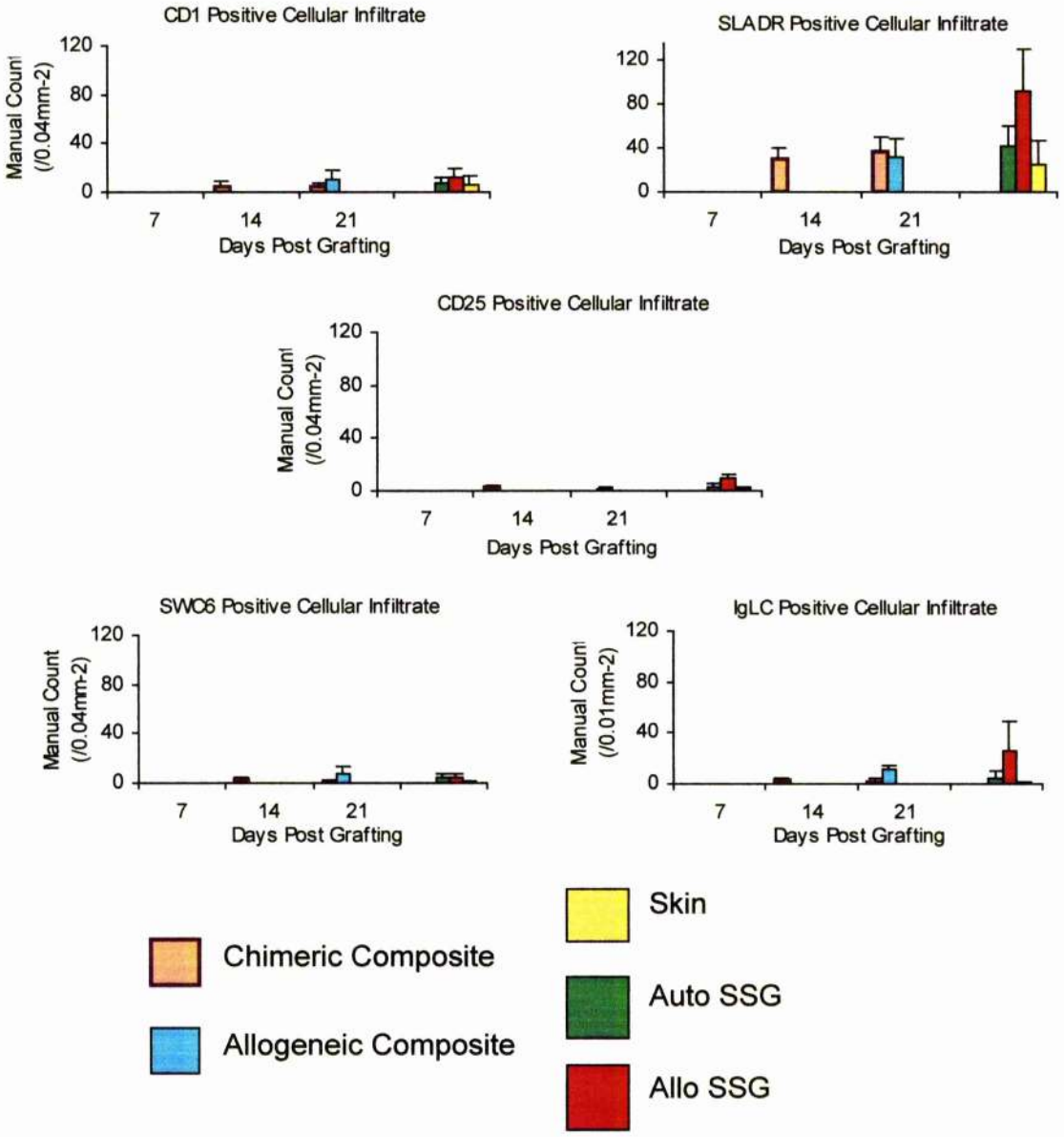


Figure 8.15: Bar charts illustrating manual counts within the infiltrate of wounds grafted with chimeric or allogeneic cultured porcine keratinocyte-dermal composite grafts onto deep fascia and harvested at days 14 and 21 (see 8.2.6). Sections were examined with each monoclonal antibody. Blocks from all chimeric or allogeneic cultured composite grafted wounds were examined and sections (see Table 8.2(a)-(c)) from wounds with surviving epithelium were stained with monoclonal antibody with antigen-specificities delineated in chapter 6 table 1 and then secondarily stained using the avidin biotin complex (ABC) immunoperoxidase technique (CD4 and IgλLC secondarily labelled with Cy3). Included on the right hand side of each graph are the results for normal porcine skin and autologous and allogeneic split skin graft 10 days post grafting (adapted from chapter 6 Figures 6.4 and 6.5).



8.2.6.1 Immunophenotyping of cultured chimeric and allogeneic keratinocyte-dermal composites grafted onto deep fascia in the Large White pig

The density of the panleucocyte (CD45) infiltrate is similar below allogeneic and chimeric grafts at both time points (Figure 8.14). It is similar or only slightly increased than that seen with normal porcine skin and autologous split skin graft (at day 10). This infiltrate (unlike that seen with allogeneic keratinocyte sheet graft - see Chapter 7 Section 7.2.3) consisted of SWC3-positive cells (macrophages/granulocytes) and T-cells (CD2, 3,and 5-positive cells), with SWC3 cells predominating. Additionally, the T-cell population contained both CD4 and CD8-positive cells (with CD4 numbers apparently predominant within day 21 allogeneic graft infiltrate) but it is not possible to come to any conclusion concerning the proportion of single vs. dual expression.

The density of infiltrating SLA DR-positive cells was similar to that seen with normal porcine skin and autologous split skin graft (at day 10). The density of infiltrating dendritic cells (CD1) is similarly comparable with infiltrate densities within normal porcine skin and following grafting of autologous split skin (at day 10) (see Figure 8.15).

As with earlier studies the densities of CD25 (IL-2)-positive cells, null cells (SWC6) and B cells (Igλ LC-positive) within graft bed infiltrates remained very low (see Figure 8.15).

8.3 Discussion

Many of the reasons for poor take of sheet grafts given in the previous chapter are equally applicable here. They include the predisposition to infection and chamber extrusion and laboratory manipulation of keratinocytes. An advantage with grafting onto Integra™ is that the wound bed is well vascularised, consisting effectively of controlled granulation with a degree of collagen support. In the present study, the composites were grafted onto freshly excised muscle fascia rather than biointegrated Integra™. The model may therefore have performed disappointingly due to poor early vascularisation of the dermal component. The model may therefore be criticised in an over reliance on the reasonable take results reported by others (Medalie et al., 1996a; Medalie et al., 1996b) and not performing smaller pilot studies to evaluate the system *in vivo*. Incorporation of cultured fibroblasts into the processed dermis as described in other skin equivalent systems (Ghosh et al., 1997) may have improved keratinocyte attachment, proliferation (Maruguchi et al., 1994; Boyce et al., 1993; Boyce et al., 1995) and differentiation (Krejci et al., 1991; Demarchez et al., 1992; MacNeil, 1994).

In conclusion, although the take of these composites was not any better than cultured sheet grafts, they were considerably easier to handle and the resulting epithelium looked more robust.

β-galactosidase expression by both chimeric and allogeneic composites at 21 days following grafting suggests that the hypothesis stating that LC depleted allogeneic keratinocytes within composites are rejected after a more

prolonged period of take than allogeneic skin grafts (see Chapter 6) may be valid.

Host APCs were seen to have infiltrated the graft at day 14. Hence, the hypothesis that '*host APCs are seen to infiltrate the graft bed prior to rejection and therefore could be involved in (indirect) antigen presentation*' may be valid.

Beneath a graft bed undergoing classical acute rejection an infiltrate dense with activated cytotoxic T-cells and macrophages are expected (Rosenberg and Singer, 1992). The character of the infiltrate beneath all composites examined, judging by the numbers of SLA DR, SWC3, CD8 and CD25-positive cells within the infiltrate (Figure 8.14 and 8.15), is not suggestive of an acute rejection effector response. A rejection response was not overtly evident. Many other factors may have contributed to composite graft loss. This experiment can not therefore address the hypothesis that '*rejection is primarily effected through allo specific cytotoxic T lymphocytes*'.

Chapter 9

Discussion

This series of experiments explored, within a Large White pig model, the rejection of transplanted cultured allogeneic keratinocytes from which LCs had been actively eliminated. The study evolved from being principally concerned with immunological events surrounding transplantation of allogeneic keratinocytes to one additionally involved with the bioengineering of skin equivalents in the search for a suitable model.

Initial experiments were concerned with gene transduction studies the aim being keratinocyte labelling that would allow tracking following transplantation. Experiments were concerned with attaining 100% keratinocyte transduction and for this to remain constant following multiple cell replication. The process of transduction was examined for its effect on keratinocyte clonogenic potential.

Following cloning and transduction studies with several different packaging and producer cell lines, a high titre producer cell line was formed which proved capable of almost 100% transduction of porcine keratinocytes. The process resulted unfortunately, in an *in-vitro* reduction in both size and numbers of colonies formed when compared with non-transduced keratinocytes that had undergone an equivalent numbers of passages. Keratinocyte replication potential also appeared markedly diminished after 7-10 passages.

The effect of the transduction process on keratinocytes *in vivo* following grafting remains unclear. In the first set of grafting experiments in which cultured keratinocyte sheets were grafted onto Integra™, both autologous and allogeneic keratinocytes were transduced with the MFG lacZnls construct. Neither was seen by 21 days following grafting. In the next set of experiments

involving the grafting of epidermal-dermal composites directly onto deep fascia, transduced cells were evident 21 days after grafting. The majority of keratinocytes had by that stage lost the nuclear localised β-galactosidase gene. Further study of the transduction method would be useful to understand which aspects of the transduction protocol are harmful to keratinocytes. These may be modified so as to evolve a method that is stable and does not damage keratinocyte clonogenic potential.

LC are considered key to the initiation of an acute rejection response to allotransplanted skin (Rosenberg and Singer, 1992). In an attempt to prolong survival of allotransplanted cultured epithelium, this study sought to actively eliminate LCs. It is widely asserted that LCs are not detected following plating and passage within a keratinocyte culture system (Morhenn et al., 1982; Hefton et al., 1984; Thivolet et al., 1986). The presence of LCs within a keratinocyte culture system has however been demonstrated, even following several weeks of culture (Demidem et al., 1986), although morphological and functional antigens appear to be progressively lost. As the keratinocyte culture system developed in our Centre is designed to prevent keratinocytes differentiating (Bevan et al., 1997), in this environment LCs might also de-differentiate making their detection difficult. Additionally, the phenotypic behaviour of LCs in our porcine keratinocyte culture system has not as yet been characterised and it is possible that a significant number of LCs survive the culture process all be it in a de-differentiated state. To therefore produce a LC-free culture, a step involving LC elimination was incorporated into the keratinocyte culture protocol. This was found to be most efficient if following

enzymatic disaggregation of epidermal cells the cells are incubated overnight in culture medium. The method used was similar to that used by researchers involved in the elimination of dendritic cells prior to the transplantation of other cell types (Faustman, 1995). It involved incubation with SLA DR-specific monoclonal antibody followed by incubation with complement. The moAB bound to the SLA DR-expressing LC to which complement was then bound inducing lysis. Following treatment there were no detectable LCs. There was little apparent difference between complement types from four different sources in the effectiveness of LC elimination or levels of non-specific cell death. Antibody - complement pre-treatment was however shown to affect, in a detrimental manner, colony-forming efficiency (both size and numbers of colonies formed).

Since the first international workshop to define swine cluster of differentiation (CD) antigens in the late 80's, porcine-specific moABs have become increasingly available. Immunohistochemical techniques have not been evaluated with regard to many of these and very few indeed have been used in relation to normal porcine skin. Indeed there are no published reports describing immunohistochemical staining of normal porcine skin. It was important therefore to establish a baseline in terms of the density of the white cell population within normal porcine skin.

The infiltrate within normal porcine skin was therefore examined using a panel of (mostly) recently available monoclonal antibodies. Two positive controls were incorporated. These were peripheral blood white cells and jejunal villi both from Large White pigs. LCs were found to account for approximately

4.7% of epidermal cells and stained positively with a variety of moAB including SWC3 (stains cells of myeloid origin), CD1, CD45 (pan-leucocyte stain) and moABs specific for SLADP, SLADQ and SLADR (MHC class II antigens). The papillary dermis was most densely populated with white cells. A variety of moABs detecting different 'pan T-cell' determinants (present on majority of T-cells) were used with apparently similar rates of T-cell detection. Approximately 20% of the dermal leucocytes were shown to be CD1-positive. Within the T-cell population a greater number of T-cells expressed the CD4 than the CD8 antigen. In immunological terms this implies more T-cells with helper phenotype than suppressor or cytotoxic (or dual helper/suppressor) phenotypes.

The density of MHC II expression is consistent with levels of CD1 (dendritic cell) and SWC3 (cells of myeloid origin) staining. Igλ LC (B cell), null cell determinant and IL-2R antigen were all detected in low numbers mostly found in the papillary dermis although null cells were also seen clustered around hair follicles deep in the reticular dermis.

Porcine skin was found to differ in terms of its immune cell population in certain respects from porcine jejunum. Within porcine jejunum CD8-positive T-cells were almost entirely located within or adjacent to the intraepithelial compartment reflecting the relatively precise localisation and compartmentalisation of white cells. Leucocyte distribution within the dermis however, appears less rigorously organised and may reflect very different environmental conditions and functions.

The study was next concerned with the immune cell infiltrate in acute allorejection of split skin graft in the Large White pig. This helped establish a baseline-positive control. It also confirmed that a true allorejection response was possible in this Large White pig population. By day 10 grafts were seen to have been rejected. Immunohistology at this time demonstrated the classical pattern of increased numbers of both T-cells and macrophages within the infiltrate. CD1-positive cell numbers were not raised above baseline expression in normal porcine skin although numbers of MHC II and SWC3-positive cells as well as T-cells and IgλLC expressing cells were increased.

The study next proceeded to examine allotransplantation (through grafting) of cultured keratinocyte sheets onto Integra™ (a non-regenerative dermal template). Keratinocytes had undergone antibody complement pre-treatment to eliminate LCs and gene transduction with the MFG lacZnls construct in order to facilitate identification of keratinocyte origin. Keratinocyte sheets were grafted onto biointegrated Integra™ that had been grafted onto deep fascia 10 days previously.

Wounds were harvested up to four weeks following grafting. In only 37% of all wounds was epithelium detected. Wounds harvested after 18 days failed to demonstrate any epithelium at all. 52% of wounds harvested up to 18 days demonstrated epithelium. As discussed at length in Chapter 7, the disappointing epithelium detection rate may be related to problems with both the pig chamber model and method of keratinocyte culture, which require further investigation.

β-galactosidase staining confirmed the presence of allogeneic epithelium 18 days following grafting. At days 21 and 28 no allogeneic epithelium could be detected (in 6 wounds – 2 animals).

Infiltration by CD1-positive cells was seen to occur as early as day 7 with numbers of infiltrating CD1-positive cells close to that of normal porcine skin. There was little change in infiltrate density beneath allografted wounds with regard to the remaining antigenic determinants until day 18 where a rise was apparent. Staining for the SWC3 determinant suggests that macrophages/granulocytes do not form a significant proportion of the increase in the day 18 infiltrate. A three to fourfold increase in CD5-positive infiltrate density (pan T-cell determinant) in allografted wounds was seen at the day 18 time point. This increase in infiltrate density was further reflected with CD4 and CD8 labelling. SLA DR staining approximated that for SWC3 until day 18 when there is a 50% increase in infiltrate density in wounds grafted with allogeneic cultured keratinocyte sheet graft.

B cells (Igλ LC) and null cells (SWC6) were sparsely detected at all time points. There was an increase in detected CD25 expression at day 18 consistent with infiltration by activated T-cells.

Results from this experiment suggest acute rejection occurring between 14 and 18 days effected principally by activated T-cells which is preceded by dendritic cell infiltration approximately 10 days previously.

Keratinocyte-dermal composite grafts were devised in an effort to improve the mechanical robustness of the model. These composite grafts consisted of autologous dermis upon which was cultured allogeneic, chimeric or

autologous epithelium. The keratinocytes making up the epithelium underwent active LC elimination and had then been passaged 4 times (allowing MFG lacZnls transduction) in an identical manner to the keratinocytes used with Integra™.

Epithelium was seen on fewer wounds (13% at day 28, 16% at day 21) than with cultured keratinocyte sheet graft onto Integra™. However the surviving epithelium looked healthier and mechanically more robust. β-galactosidase staining of allogeneic epithelium was demonstrated at 21 days following grafting.

Analysis of the immune infiltrate at 21 days revealed similarities to skin and the wound bed beneath an autologous split skin graft. This result and the robust healthy appearance of the grafts suggest that rejection had not occurred and was not happening at that stage. Lack of epithelium seen at 28 days may be related to flaws inherent in the model, with graft loss due to causes other than rejection.

The aim when resurfacing a massive burn is to achieve a permanent stable durable skin as rapidly as possible. A prerequisite for skin stability and durability is the presence of a basement membrane. Permanence is engendered by a lack of rejection, rapidity, by not having to spend weeks building up keratinocyte numbers.

There are two ways currently to achieve numbers of cultured keratinocytes sufficient to graft large body surface areas. One way is to harvest sufficient split skin to enable rapid culture of large numbers of keratinocyte. The other is

to have a banked store of allogeneic keratinocytes for use in transplantation either on there own or mixed with autologous keratinocytes as chimeric grafts. Use of cultured allogeneic keratinocytes initially met with enthusiasm, lost favour and has only recently been revived as a method for resurfacing chronic venous ulcers (Eaglstein et al., 1999). Research into skin equivalents can be seen as having gone through a series of developments. Keratinocyte culture techniques advanced significantly in the 1970's (Rheinwald and Green, 1975; Green et al., 1979). This was followed (mid 1980s) by studies looking at allogeneic cultured keratinocyte transplantation (Aubock et al., 1988; Hammond et al., 1987; Leigh et al., 1987; Gielen et al., 1987; Thivolet et al., 1986). Phase three (late eighties early nineties) was characterised by the development of dermal substitutes or 'neo-dermal' equivalents (Yannas et al., 1989). The latest phase although pioneered in the early eighties (Bell et al., 1981) marries the previous three phases in attempts to produce allogeneic skin equivalents (Falanga et al., 1998). Apligraf™ is the only skin equivalent commercially available to date and is licensed for and has been used clinically in the treatment of chronic venous ulcers (Falanga et al., 1998). Apligraf™ is a bilayered construct with the dermal element manufactured from bovine type I collagen impregnated with human neonatal foreskin fibroblasts and the epidermal layer manufactured from human neonatal foreskin keratinocytes. To date although trials involving their use suggest benefit in terms of faster wound healing over controls, there is as yet no evidence of the persistence of grafted keratinocytes. In humanised SCID mice Apligraf™ was seen to persist for a week longer than human foreskin (Briscoe et al., 1999).

The studies reported in this thesis are the first to provide unequivocal proof of the persistence of a skin equivalent system for more than twice as long as an allogeneic skin graft in a fully immune competent model.

Cloning out holoclonal or stem cells as described by others (Barrandon and Green, 1987; Mathor et al., 1996) may greatly improve functional survival of the skin equivalent. Incorporation of fibroblasts within the neodermis would allow for lower numbers of seeding keratinocytes. More fastidious preparation of and attention to the graft bed might also improve take. From a purely immunological standpoint we have succeeded in demonstrating early infiltration by dermal dendritic cells – and this was not previously reported. Additionally we have shown that allogeneic keratinocytes are not rejected for at least twice the time taken for rejection of allogeneic split skin grafts. We have not shown that this is due to LC elimination; our model however sought to actively eliminate LCs and the lack of early rejection is consistent with the work of others with regard to other cell types (Faustman, 1995). It remains to be clarified with regard to our keratinocyte culture system whether or not LCs need to be actively eliminated. On explant LCs are seen to further differentiate and to upgrade MHC II expression (Witmer-Pack et al., 1988). These findings may not however pertain to the conditions in which we culture porcine keratinocytes.

Future work would concentrate on several key areas. The techniques whereby wound infiltrates are analysed require further study. As outlined earlier (Chapter 5), immunohistochemistry is limited in several respects. In order to appreciate cell phenotypes (for example activated cytotoxic T-cells)

more completely, staining of dual or triple antigenic determinants is necessary. This may provide additional information about intercellular relationships. The use of flow cytometry would additionally provide relative quantitative information abouT-cell types, the qualitative content of which can be enhanced with the use of dual or triple staining techniques.

Keratinocyte gene transduction technology is improving and there have been recent reports of increasingly stable and persistent gene transduction (Mathor et al., 1996). Gene transduction for the purposes of labelling or otherwise needs to be rigorously tested to ensure that the process is not detrimental to the keratinocyte population (De Luca and Pellegrini, 1997; Mathor et al., 1996). In our work it became clear that fewer irradiated 3T3 cells producing fewer growth factors, or harmful growth factors produced by the irradiated producer cell line, resulted in loss of stem cells or possibly 'clonal conversion' from holoclones and meroclones to paraclones with fewer, smaller and less regular colonies.

The phenotypic behaviour of LCs in the particular keratinocyte culture environment used needs further study. If LCs are truly shown to die rather than merely de-differentiate then their active elimination may be superfluous. Were active elimination thought to be necessary further work is required to demonstrate a process by which LCs are eliminated which is not detrimental to co-cultured keratinocytes.

For cultured keratinocytes to take effectively a wound bed that provides stability and firm adherence is necessary. With regard to keratinocyte delivery there are those who favour a pre-confluent delivery system. In this system, pre-confluent keratinocytes are dispersed in a less differentiated state and

therefore a greater proportion may still have proliferative potential than those in sheet grafts. The numbers of keratinocyte therefore required to achieve epithelial cover may be reduced (Stark and Kaiser, 1994; Harris et al., 1998). Others favour the production of a neo-epidermis with the final few days of culture at an air liquid interface so as to produce a differentiated multi layered stratified epidermis with the uppermost layers cornified (Medalie et al., 1997; Ghosh et al., 1997). Regardless of the delivery system or substrate chosen, it is important to determine the clonogenic nature of the resultant keratinocytes.

Thus we are lead to several conclusions. First, it is important to ensure that the model chosen will support and not confound hypothesis through inherent flaws in design. The model used in this study was designed by my predecessors working in the field for shorter term use in the early 90's (Kangesu et al., 1993). Its flaws include the fact that it is not a closed system and as such is easily contaminated. The chamber used is vigorously expelled three to four weeks after implantation by a mixture of wound contraction and creep of skin flaps beneath chamber walls. PTFE chambers are used to isolate grafts and prevent inadvertent in growth by surrounding keratinocytes. Highly efficient labelling of keratinocyte with retroviral transduction may render the chambers unnecessary. Chamberless wounds would be flat and could be dressed with semiocclusive dressings thus reducing the likelihood of external contamination and wound infection. They also simulate the clinical situation more closely.

The future of research into skin equivalents needs to address the quality of dermis, epidermis and the basement membrane interface.

Epidermal elements need testing *in vitro* to ensure a stable population of holoclonal and meroclonal cells that does not substantially alter with processing.

Rigorous assessment of the immunogenicity of allogeneic keratinocytes needs to be addressed. If they are found not to be rejected as some claim (Falanga et al., 1998) laboratory engineering of allogeneic neo equivalents could take place on an industrial scale with rapid generation and immediate benefit to the massively burnt. Were allogeneic keratinocytes to be rejected in a delayed fashion, permanent cover might still be achieved with a composite graft in which the epidermal element results from co-culture of allogeneic with autologous keratinocytes in predetermined proportions. The resulting neo skin equivalent might then survive long enough to allow the autologous elements to replicate and so fill the void vacated by lysed allogenic cells rejected in a highly targeted fashion with minimal damage to surrounding non allogenic elements.

Ababou, A., Femenia, F., Crespeau, F., Fontaine, J.J., Davis, W.C., and Levy, D. (1995). A novel BoLA class II molecule with a tissue distribution different from BoLA-DR or BoLA-DQ molecules. Vet Res. *26*, 319-327.

Abe, S., Fujita, T., and Takami, Y. (1995). Disappearance of Langerhans cells and melanocytes after cryopreservation of skin. Br J Plast Surg. *48*, 405-409.

Abraham, R.T., Karnitz, L.M., Secrist, J.P., and Leibson, P.J. (1992). Signal transduction through the T-cell antigen receptor. Trends Biochem Sci. *17*, 434-438.

Allen, R.E. (1990). The concise Oxford dictionary (Oxford university press).

Allison, J.P. (1994). CD28-B7 interactions in T-cell activation. Curr Opin Immunol. *6*, 414-419.

Alsbjorn, B.F. (1983). A depression technique of the epidermal Langerhans cell in cadaver split skin. Scand J Plast Reconstr Surg. *18*, 62-64.

Alsbjorn, B.F. and Sorensen, B. (1985). Grafting with epidermal Langerhans cell depressed cadaver split skin. Burns Incl Therm Inj. *11*, 259-263.

Altmann, M. and Trachsel, H. (1993). Regulation of translation initiation and modulation of cellular physiology. Trends Biochem Sci. *18*, 429-432.

Alvarez, B., Sanchez, C., Bullido, R., Marina, A., Lunney, J., Alonso, F., Ezquerra,A., and Dominguez,J. (2000). A porcine cell surface receptor

identified by monoclonal antibodies to SWC3 is a member of the signal regulatory protein family and associates with protein-tyrosine phosphatase SHP-1. Tissue Antigens *55*, 342-351.

Appleyard, G.D. and Wilkie, B.N. (1998). Characterization of porcine CD5 and CD5+ B cells. Clin Exp Immunol. *111*, 225-230.

Aubock, J., Irschick, E., Romani, N., Kompatscher, P., Hopfl, R., Herold, M., Schuler, G., Bauer, M., Huber, C., and Fritsch, P. (1988). Rejection, after a slightly prolonged survival time, of Langerhans cell- free allogeneic cultured epidermis used for wound coverage in humans. Transplantation *45*, 730-737.

Bailey, M., Plunkett, F., Clarke, A., Sturgess, D., Haverson, K., and Stokes, C. (1998). Activation of T-cells from the intestinal lamina propria of the pig. Scand J Immunol. *48*, 177-182.

Banchereau, J. and Steinman, R.M. (1998). Dendritic cells and the control of immunity. Nature *392*, 245-252.

Barclay, A.N., Brown, M.H., Alex Law, S.K., McKnight, A.J., Tomlinson, M.G., and van der Merwe, P.A. eds. (1997). The leucocyte antigen facts book. (Oxford: Academic Press), pp. 132-593.

Barker, C.F. and Billingham, R.E. (1967). The role of regional lymphatics in the skin homograft response. Transplantation *5*, 962-974.

Barrandon, Y. and Green, H. (1987). Three clonal types of keratinocytes with different capacities for multiplication. Proc Natl Acad Sci. U.S.A. *84*, 2302-2306.

Bell, E., Ehrlich, H.P., Sher, S., Merrill, C., Sarber, R., Hull, B., Nakatsuji, T., Church, D., and Buttle, D.J. (1981). Developement and use of a living skin equivalent. Plast Reconstr Surg. *67*, 386-392.

Benichou, G. (1999). Direct and indirect antigen recognition: the pathways to allograft immune rejection. Front Biosci. *4*, D476-480.

Bevan, S., Woodward, B., Ng, R.L.H., Green, C., and Martin, R. (1997). Retroviral gene transfer into porcine keratinocytes following improved methods of cultivation. Burns *23*, 525-532.

Bevan, S., Martin, R., and McKay, I.A. (1999). The production and applications of genetically modified skin cells. Biotechnol Genet Eng Rev. *16*, 231-256.

Blanchi, A.T. and van der Heijden, P.J. (1994). Antigen presenting cells and B-cells in the pig. Vet Immunol Immunopathol. *43*, 63-68.

Binns, R.M., Duncan, I.A., Powis, S.J., Hutchings, A., and Butcher, G.W. (1992). Subsets of null and gamma delta T-cell receptor+ T lymphocytes in the blood of young pigs identified by specific monoclonal antibodies. Immunology *77*, 219-227.

Binns, R.M. (1994a). The Null/gamma delta TCR+ T-cell family in the pig. Vet Immunol Immunopathol. *43*, 69-77.

Binns, R.M., Bischof, R., Carr, M.M., Davis, W.C., Licence, S.T., Misfeldt, M., and Pospisil, R. (1994b). Report on the behaviour of monoclonal antibodies in the First International Pig CD Workshop identifying the Null cell families. Vet Immunol Immunopathol. *43*, 279-287.

Blecha, F., Kielian, T., McVey, D.S., Lunney, J.K., Walker, K., Stokes, C.R., Stevens, K., Kim, Y.B., Chu, R.M., and Chen, T.S. (1994). Workshop studies on monoclonal antibodies reactive against porcine myeloid cells. Vet Immunol Immunopathol. *43*, 269-272.

Boeker, M., Pabst, R., and Rothkotter, H.J. (1999). Quantification of B, T and null lymphocyte subpopulations in the blood and lymphoid organs of the pig. Immunobiology *201*, 74-87.

Bonner, W.A., Hulett, H.R., Sweet, R.G., and Herzenberg, L.A. (1972). Fluorescence activated cell sorting. Rev Sci Instrum. *43*, 404-409.

Boyce, S.T., Greenhalgh, D.G., Kagan, R.J., Housinger, T., Sorrell, J.M., Childress, C.P., Rieman, M., and Warden, G.D. (1993). Skin anatomy and antigen expression after burn wound closure with composite grafts of cultured skin cells and biopolymers. Plast Reconst Surg. *91*, 632-641.

Boyce, S.T., Goretsky, M.J., Greenhalgh, D.G., Kagan, R.J., Rieman, M.T., and Warden, G.D. (1995). Comparative assessment of cultured skin substitutes and native skin autograft for treatment of full-thickness burns. Ann Surg. *222*, 743-752.

Boyce, S.T., Kagan, R.J., Meyer, N.A., Yakuboff, K.P., and Warden, G.D. (1999). The 1999 clinical research award. Cultured skin substitutes combined with Integra Artificial Skin to replace native skin autograft and allograft for the closure of excised full-thickness burns. J Burn Care Rehabil. *20*, 453-461.

Brain, A., Purkis, P., Coates, P., Hackett, M., Navsaria, H., and Leigh, I. (1989). Survival of cultured allogeneic keratinocytes transplanted to deep dermal bed assessed with probe specific for Y chromosome. BMJ *298*, 917-919.

Brewer, Y., Bewick, M., Palmer, A., Severn, A., Welsh, K., and Taube, D. (1989a). Prevention of renal allograft rejection by perfusion with antileukocyte common monoclonal antibodies is dependent on good uptake of antibody by interstitial dendritic cells. Transplant Proc. *21*, 1772-1773.

Brewer, Y., Palmer, A., Taube, D., Welsh, K., Bewick, M., Bindon, C., Hale, G., Waldmann, H., Dische, F., and Parsons, V. (1989b). Effect of graft perfusion with two CD45 monoclonal antibodies on incidence of kidney allograft rejection. Lancet *2*, 935-937.

Briscoe, D.M., Dharnidharka, V.R., Isaacs, C., Downing, G., Prosky, S., Shaw, P., Parenteau, N.L., and Hardin-Young, J. (1999). The allogeneic response to cultured human skin equivalent in the hu-PBL- SCID mouse model of skin rejection. Transplantation *67*, 1590 -1599.

Bull, J.P. (1971). Revised analysis of mortality due to burns. Lancet 2, 1133

Burke, J.F., Bondoc, C.C., and Quinby, W.C. (1974). Primary burn excision and immediate grafting: a method shortening illness. J Trauma *14*, 389-395.

Burt, A.M., Pallett, C.D., Sloane, J.P., O Hare, M.J., Schafler, K.F., Yardeni, P., Eldad, A., Clarke, J.A., and Gusterson, B.A. (1989). Survival of cultured allografts in patients with burns assessed with probe specific for Y chromosome. BMJ *298*, 915-917.

Caine, R.Y., Sells, R.A., Pena, J.R., Davis, D.R., Millard, P.R., Herbertson, B.M., Binns, R.M., and Davies, D.A. (1969). Induction of immunological tolerance by porcine liver allografts. Nature *223*, 472-476.

Carroll, J.M., Fenjves, E.S., Garlick, J.A., and Taichman, L.B. (1993). Keratinocytes as a target for gene therapy. In Molecular Biology of the skin: The Keratinocyte. M. Darmon and M. Blumenberg, eds. (San Diego: Academic Press Inc.), pp. 269-284.

Carter, N.P. (1996). Flow sorting. In Flow Cytometry: A practical approach. M.G. Ormerod, ed. (New York: Oxford University Press), pp. 55-65.

Carver, N., Navsaria, H.A., Green, C.J., and Leigh, I.M. (1991). Acute rejection of cultured keratinocyte allografts in nonimmunosuppressed pigs. Transplantation *52*, 918-921.

Challita, P.M. and Kohn, D.B. (1994). Lack of expression from a retroviral vector after transduction of murine hematopoietic stem cells is associated with methylation *in vivo*. Proc Natl Acad Sci. U.S.A. *91*, 2567-2571.

Chandler, C. and Passaro, E.J. (1993). Transplant rejection. Mechanisms and treatment. Arch Surg. *128*, 279-283.

Chesebro, B., Buller, R., Portis, J., and Wehrly, K. (1990). Failure of human immunodeficiency virus entry and infection in CD4-positive human brain and skin cells. J Virol. *64*, 215-221.

Clarke, J.A. (1987). HIV transmission and skin grafts [letter]. Lancet *1*, 983

Cooper, M.L., Hansbrough, J.F., and Foreman, T.J. (1990). *In vitro* effects of matrix peptides on a cultured dermal-epidermal skin substitute. J Surg Res. *48*, 528-533.

Cooper, M.L. and Hansbrough, J.F. (1991). Use of a composite skin graft composed of cultured human keratinocytes and fibroblasts and a collagen-GAG matrix to cover full-thickness wounds on athymic mice. Surgery *109*, 198-207.

Cooper, M.L., Andree, C., Hansbrough, J.F., Zapata-Sirvent, R.L., and Spielvogel, R.L. (1993). Direct comparison of a cultured composite skin substitute containing human keratinocytes and fibroblasts to an epidermal sheet graft containing human keratinocytes on athymic mice. J Invest Dermatol. *101*, 811-819.

Cosset, F.L., Takeuchi, Y., Battini, J.L., Weiss, R.A., and Collins, M.K. (1995). High-titer packaging cells producing recombinant retroviruses resistant to human serum. J Virol. *69*, 7430-7436.

Dai, Y., Verma, I.M., and Naviaux, R.K. (1992). Gene therapy via primary myoblasts: long-term expression of factor IX protein following transplantation *in vivo*. Proc Natl Acad Sci. U.S.A. *89*, 10892-10895.

Davis, W.C., Zuckermann, F.A., Hamilton, M.J., Barbosa, J.I., Saalmüller, A., Binns, R.M., and Licence, S.T. (1998). Analysis of monoclonal antibodies that recognize gamma delta T/null cells. Vet Immunol Immunopathol. *60*, 305-316.

Dawson, R.A., Goberdhan, N.J., Freedlander, E., and MacNeil, S. (1996). Influence of extracellular matrix proteins on human keratinocyte attachment, proliferation and transfer to a dermal wound model. Burns *22*, 93-100.

De Luca, M. and Pellegrini, G. (1997). The importance of epidermal stem cells in keratinocyte-mediated gene therapy. Gene Therapy 381-383.

Demarchez, M., Hartmann, D.J., Regnier, M., and Asselineau, D. (1992). The role of fibroblasts in dermal vascularization and remodeling of reconstructed human skin after transplantation onto the nude mouse. Transplantation *54*, 317-326.

Demidem, A., Faure, M., Dezutter-Dambuyant, C., and Thivolet, J. (1986). Loss of allogeneic T-cell activating ability and Langerhans cell markers in human epidermal cell cultures. Clin Immunol Immunopathol. *38*, 319-326.

Denham, S., Shimizu, M., Bianchi, A.T., Zwart, R.J., Carr, M.M., and Parkhouse, R.M. (1994). Monoclonal antibodies recognising

differentiation antigens on porcine B cells. Vet Immunol Immunopathol. *43*, 259-267.

Doody, D.P., Stenger, K.S., and Winn, H.J. (1994). Immunologically nonspecific mechanisms of tissue destruction in the rejection of skin grafts. J Exp Med. *179*, 1645-1652.

Dranoff, G., Jaffee, E., Lazenby, A., Golumbeck, P., Levitsky, H., Brose, K., Jackson, V., Hamada, H., Pardoll, D., and Mulligan, R.C. (1993). Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific and long-lasting anti-tumor immunity. Proc Natl Acad Sci. U.S.A. *90*, 3539-3543.

Eaglstein, W.H., Iriondo, M., and Laszlo, K. (1995). A composite skin substitute (graftskin) for surgical wounds. A clinical experience. Dermatol Surg. *21*, 839-843.

Eaglstein, W.H., Alvarez, O.M., Auletta, M., Leffel, D., Rogers, G.S., Zitelli, J.A., Norris, J.E., Thomas, I., Irondo, M., Fewkes, J., Hardin-Young, J., Duff, R.G., and Sabolinski, M.L. (1999). Acute excisional wounds treated with a tissue-engineered skin (Apligraf). Dermatol Surg. *25*, 195-201.

Fabre, J.W. and Morris, P.J. (1972). The mechanism of specific immunosuppression of renal allograft rejection by donor strain blood. Transplantation *14*, 634

Fabre, J.W. and Morris, P.J. (1975). Passive enhancement of renal allografts in presensitised rats: theoretical and clinical implications. Transplantation *19*, 121

Fabre, J.W. and Cullen, P.R. (1989). Rejection of cultured keratinocyte allografts in the rat. Clinical implications and a possible clue to the enigma of skin graft rejection. Transplantation *48*, 306-315.

Falanga, V., Margolis, D., Alvarez, O., Auletta, M., Maggiacomo, F., Altman, M., Jensen, J., Sabolinski, M., and Hardin-Young, J. (1998). Rapid healing of venous ulcers and lack of clinical rejection with an allogeneic cultured human skin equivalent. Arch Dermatol. *134*, 293-300.

Faustman, D., Hauptfeld, V., Lacy, P., and Davie, J. (1981). Prolongation of murine islet allograft survival by pretreatment of islets with antibody directed to Ia determinants. Proc Natl Acad Sci. U.S.A. *78* , 5156-5159.

Faustman, D., Hauptfeld, V., Lacy, P., and Davie, J. (1982a). Demonstration of active tolerance in maintenance of established islet of Langerhans allografts. Proc Natl Acad Sci. U.S.A. *79*, 4153-4155.

Faustman, D., Kraus, P., Lacy, P.E., Finke, E.H., and Davie, J.M. (1982b). Survival of heart allografts in nonimmunosuppressed murine recipients by pretreatment of the donor tissue with anti-Ia antibodies. Transplantation *34*, 302-305.

Faustman, D., Lacy, P., Davie, J., and Hauptfeld, V. (1982c). Prevention of allograft rejection by immunization with donor blood depleted of Ia-bearing cells. Science *217*, 157-158.

Faustman, D., Lacy, P.E., and Davie, J.M. (1982d). Transplantation without immunosuppression. Diabetes *31 Suppl 4*:11-16.

Faustman, D.L., Steinman, R.M., Gebel, H.M., Hauptfeld, V., Davie, J.M., and Lacy, P.E. (1984). Prevention of rejection of murine islet allografts by pretreatment with anti-dendritic cell antibody. Proc Natl Acad Sci. U.S.A. *81*, 3864-3868.

Faustman, D. (1995). Strategies for circumventing transplant rejection: modification of cells, tissues and organs. Trends Biotechnol. *13*, 100-105.

Fenjves, E.S. (1994). Approaches to gene transfer in keratinocytes. J Invest Dermatol. *103 (Suppl)*, 70S-75S.

Fenjves, E.S., Yao, S.N., Kurachi, K., and Taichman, L.B. (1996). Loss of expression of a retrovirus-transduced gene in human keratinocytes. J. Invest.Dermatol. *106*, 576-578.

Ferry, N., Duplessis, O., Houssin, D., Danos, O., and Heard, J.M. (1991). Retroviral-mediated gene transfer into hepatocytes *in vivo*. Proc Natl Acad Sci. U.S.A. *88*, 8377-8381.

Frank, M.M. (1991). Complement and kinin. In Basic and clinical immunology. D.P. Stites and A.I. Terr, eds. (Lange medical publications), pp. 161-174.

Frey, J.R. and Wenk, P. (1957). Experimental studies on the pathogenesis of contact eczema in the guinea pig. Int Arch Allergy Appl Immunol. *11*, 81-94.

Garlick, J.A., Katz, A.B., Fenjves, E.S., and Taichman, L.B. (1991). Retrovirus-mediated transduction of cultured epidermal keratinocytes. J Invest Dermatol. *97*, 824-829.

Ghosh, M.M., Boyce, S.G., Freedlander, E., and MacNeil, S. (1995). A simple human dermal model for assessment of *in vitro* attachment efficiency of stored cultured epithelial autografts. J Burn Care Rehabil. *16*, 407-417.

Ghosh, M.M., Boyce, S., Layton, C., Freedlander, E., and MacNeil, S. (1997). A comparison of methodologies for the preparation of human epidermal- dermal composites. Ann Plast Surg. *39*, 390-404.

Gibson, T. and Medawar, P.B. (1943). The fate of skin homografts in man. J Anat. *77*, 299-316.

Gielen, V., Faure, M., Mauduit, G., and Thivolet, J. (1987). Progressive replacement of human cultured epithelial allografts by recipienT-cells as evidenced by HLA class I antigens expression. Dermatologica *175*, 166-170.

Grant, I. and Martin, R. Use of IntegraTM in conjunction with cultured keratinocyte sheet grafting in Large White pigs. 1998. Personal Communication

Grant, I., O'Byrne, A., Green, C., and Martin, R. (1998). The successful use of Integra™ artificial skin as a non-regenerative dermal substrate for cultured autologous sheets in the Large White Pig. Wound Repair Regen. *6*, A 501

Green, H., Kehinde, O., and Thomas, J. (1979). Growth of cultured human epidermal cells into multiple epithelia suitable for grafting. Proc Natl Acad Sci. U.S.A. *76*, 5665-5668.

Hammerberg, C. and Schurig, G.G. (1986). Characterization of monoclonal antibodies directed against swine leukocytes. Vet Immunol Immunopathol. *11*, 107-121.

Hammond, E.J., Ng, R.L., Stanley, M.A., and Munro, A.J. (1987). Prolonged survival of cultured keratinocyte allografts in the nonimmunosuppressed mouse. Transplantation *44*, 106-112.

Hansbrough, J.F., Boyce, S.T., Cooper, M.L., and Foreman, T.J. (1989). Burn wound closure with cultured autologous keratinocytes and fibroblasts attached to a collagen-glycosaminoglycan substrate. JAMA *262*, 2125-2130.

Harris, P.A., Leigh, I.M., and Navsaria, H.A. (1998). Pre-confluent keratinocyte grafting: the future for cultured skin replacements? Burns *24*, 591-593.

Haverson, K., Bailey, M., Higgins, V.R., Bland, P.W., and Stokes, C.R. (1994). Characterization of monoclonal antibodies specific for monocytes, macrophages and granulocytes from porcine peripheral blood and mucosal tissues. J Immunol Methods *170*, 233-245

Haverson, K., Stokes, C.R., and Bailey, M. (1997). Immunological structure and antigen-presenting cells in the intestinal lamina propria. Period Biol *99*, 335-341.

Haverson, K., Singha, S., Stokes, C.R., and Bailey, M. (2000). Professional and non-professional antigen-presenting cells in the porcine small intestine. Immunology *101*, 492-500.

Haverson, K., Saalmuller, A., Alvarez, B., Alonso, F., Bailey, M., Bianchi, A.T., Boersma,W.J., Chen, Z., Davis, W.C., Dominguez, J., Engelhardt, H., Ezquerra, A., Grosmaire, L.S., Hamilton, M.J., Hollemweguer, E., Huang, C.A., Khanna, K.V., Kuebart,G., Lackovic, G., Ledbetter, J.A., Lee,R., Lianes,D., Lunney,J.K., McCullough,K.C., Molitor, T., Nielsen, J., Niewold, T.A., Pescovitz, M.D., de la Lastra, J.M., Rehakova, Z., Salmon, H., Schnitzlein, W.M., Seebach, J., Simon, A., Sinkora, J., Sinkora, M., Stokes, C.R., Summerfield, A., Sver, L., Thacker, E., Valpotic, I., Yang, H., Zuckermann, F.A., and Zwart, R. (2001). Overview of the Third International Workshop on Swine Leukocyte Differentiation Antigens. Vet Immunol Immunopathol *20;80*, 5-23.

Hefton, J.M., Madden, M.R., Finkelstein, J.L., and Shires, G.T. (1983). Grafting of burn patients with allografts of cultured epidermal cells. Lancet *ii*, 428-430.

Hefton, J.M., Amberson JB, Biozes DG, and Weksler ME (1984). Loss of HLA-DR expression by human epidermal cells after growth in culture. J Invest Dermatol *83*, 48-50.

Hefton, J.M., Caldwell, D., Biozes, D.G., Balin, A.K., and Carter, D.M. (1986). Grafting of skin ulcers with cultured autologous epidermal cells. J Am Acad Dermatol *14*, 399-405.

Herndon, D.N., Gore, D., Cole, M., Desai, M.H., Linares, H., Abston, S., Rutan, T., Van Osten, T., and Barrow, R.E. (1987). Determinants of mortality in pediatric patients with greater than 70% full-thickness total body surface area thermal injury treated by early total excision and grafting. J Trauma. *27*, 208-212.

Hill, J.C., Grimwood, R.E., and Parsons, D.S. (1992). Treatment of chronic erosions of junctional epidermolysis bullosa with human epidermal allografts. J Dermatol Surg Oncol. *18*, 396-400.

Horan, P.K., Melnicoff, M.J., Jensen, B.D., and Slezak, S.E. (1990). Fluorescent T-cell labeling for *in vivo* and *in vitro* cell tracking. Methods Cell Biol. *33:469-90.*, 469-490.

Janeway, C.A.J. and Bottomly, K. (1994). Signals and signs for lymphocyte responses. Cell *76*, 275-285.

Jenkins, A.M. and Woodruff, M.F.A. (1971). The effect of prior administration of donor strain blood or blood constituents on the survival of cardiac allografts in rats. Transplantation *12*, 57

Jensen, U.B., Jensen, T.G., Jensen, P.K.A., Rygaard, J., Hansen, B.S., Fogh, J., Kolvraa, S., and Bolund, L. (1994). Gene transfer into cultured human epidermis and its transfer onto immunodeficient mice: an experimental model for somatic gene therapy. J Invest Dermatol. *103*, 391-394.

Jiang, C.K., Connolly, D., and Blumenberg, M. (1991). Comparison of methods for transfection of human epidermal keratinocytes. J Invest Dermatol. *97*, 969-973.

Jonjic, S. and Koszinowski, U.H. (1984). Monoclonal antibodies reactive with swine lymphocytes. I. Antibodies to membrane structures that define the cytolytic T lymphocyte subset in the swine. J Immunol. *133*, 647-652.

Kangesu, T., Navsaria, H.A., Manek, S., Shurey, C.B., Jones, C.R., Fryer, P.R., Leigh, I.M., and Green, C.J. (1993). A porcine model using skin graft chambers for studies on cultured keratinocytes. Br J Plast Surg. *46*, 393-400.

Kawabe, K., Guttmann, R.D., Levin, B., Merril, J.P., and Lindquist, R.R. (1972). Renal transplantation in the inbred rat: effect of cyclophosphamide on acute rejectionand long survival of recipients. Transplantation *13*, 21

Kenmochi, T., Mullen, Y., Miyamoto, M., and Stein, E. (1994). Swine as an allotransplantation model. Vet Immunol Immunopathol. *43*, 177-183.

Kim, C.M., Vogel, J., Jay, G., and Rhim, J.S. (1992). The HIV tat gene transforms human keratinocytes. Oncogene *7*, 1525-1529.

Kisielow, P., Hirst, J.A., Shiku, H., Beverley, P.C., Hoffman, M.K., Boyse, E.A., and Oettgen, H.F. (1975). Ly antigens as markers for functionally distinct subpopulations of thymus-derived lymphocytes of the mouse. Nature *253*, 219-220.

Knight, S.C. and Stagg, A.J. (1993). Antigen-presenting cell types. Curr Op Immunol. *5*, 374-382.

Koch, C.T., Eysvoogel, V.P., Frederiks, E., and Van Rood, J.J. (1971). Mixed-lymphocyte-culture and skin-graft data in unrelated HL-A identical individuals. Lancet *2*, 1334-1336.

Kohler, G. and Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. Nature *256*, 495-497.

Kolodka, T.M., Garlick, J.A., and Taichman, L.B. (1998). Evidence for keratinocyte stem cells *in vitro*: long term engraftment and persistence of transgene expression from retrovirus-transduced keratinocytes. Proc Natl Acad Sci. U.S.A. *95*, 4356-4361.

König, A. and Bruckner-Tuderman, L. (1991). Epithelial-mesenchymal interactions enhance expression of collagen VII *in vitro*. J Invest Dermatol. *96*, 803-808.

König, A. and Bruckner-Tuderman, L. (1994). Transforming growth factor-β promotes deposition of collagen VII in a modified organotypic skin model. Lab Invest. *70*, 203-209.

Krejci, N., Cuono, C.B., and Langdon, R.C. (1991). *In vitro* reconstitution of skin: Fibroblasts facilitate keratinocyte growth and differentiation on acellular reticular dermis. Soc Invest Dermatol. *98*, 843-843.

Kuby, J. (1997). The complement system. In Immunology. (New York: WH Freeman and Company), pp. 335-355.

Lackovic, G., Tomaskovic, M., Njari, B., Vrbanac, I., Krsnik, B., Rode, B., and Valpotic, I. (1999). Distribution of immune cells expressing CD3a, CD21 and S-100 protein markers in the porcine gut-associated lymphoid tissues. Eur J Histochem. *43*, 39-46.

Lafferty, K.J., Bootes, A., Dart, G., and Talmage, D.W. (1976). Effect of organ culture on the survival of thyroid allgrafts in mice. Transplantation *22*, 138-149.

Lafferty, K.J. and Woolnough, J. (1977). The origin and mechanism of allograft rejection. Immunol Rev. *35*, 231-262.

Lafferty, K.J., Prowse, S.J., and Simeonovic, C.J. (1983). Immunobiology of Tissue Transplantation: A Return to the Passenger Leukocyte Concept. Ann Rev Immunol. *1*, 143-173.

Larsen, C.P., Barker, H., Morris, P.J., and Austyn, J.M. (1990a). Failure of mature dendritic cells of host to migrate from the blood into cardiac or skin allografts. Transplantation *50*, 294-301.

Larsen, C.P., Steinman, R.M., Witmer-Pack, M., Hankins, D.F., Morris, P.J., and Austyn, J.M. (1990b). Migration and maturation of Langerhans cells in skin transplants and explants. J Exp Med. *172*, 1483-1493.

Larsen, C.P., Ritcie, S.C., Hendrix, R., Linsley, P.S., Hathcock, R.J., Lowry, R.P., and Pearson, T.C. (1994). Regulation of immunostimulatory function and costimulatory molecule (B7-1 and B7-2) expression on murine dendritic cells. J Immunol. *152*, 5208-5219.

Leigh, I.M., Purkis, P.E., Navsaria, H.A., and Phillips, T.J. (1987). Treatment of chronic venous ulcers with sheets of cultured allogenic keratinocytes. Br J Dermatol. *117*, 591-597.

Lorenz, C., Petracic, A., Hohl, H.P., Wessel, L., and Waag, K.L. (1997). Early wound closure and early reconstruction. Experience with a dermal substitute in a child with 60 per cent surface area burn. Burns *23*, 505-508.

Lunney, J.K. (1994). Current status of the swine leukocyte antigen complex. Vet Immunol Immunopathol. *43*, 19-28.

Lunney, J.K., Walker, K., Goldman, T., Aasted, B., Bianchi, A., Binns, R., Licence, S., Bischof, R., Brandon, M., and Blecha, F. (1994). Overview of the First International Workshop to Define Swine Leukocyte Cluster

of Differentiation (CD) Antigens. Vet Immunol Immunopathol. *43*, 193-206.

MacNeil, S. (1994). What role does the extracellular matrix serve in skin grafting and wound healing? Burns. *20 Suppl. 1*: S67-S70

Madden, M.R., Finkelstein, J.L., Staiano Coico, L., Goodwin, C.W., Shires, G.T., Nolan, E.E., and Hefton, J.M. (1986). Grafting of cultured allogeneic epidermis on second- and third- degree burn wounds on 26 patients. J.Trauma. *26*, 955-962.

Mahabir, R.N., Guttmann, R.D., and Lindquist, R.R. (1969). Renal transplantation in the inbred rat: a model of "weak histoincompatibility" by major locus matching. Transplantation *8*, 369

Markowitz, D., Goff, S., and Bank, A. (1988). Construction and use of a safe and efficient amphotropic packaging cell line. Virology *167*, 400-406.

Marquet, R.L., Heyster, G.A., and Tinbergen, W.J. (1971). Specific inhibition of organ allograft rejection by donor blood. Transplant Proc. *3*, 708

Maruguchi, T., Maruguchi, Y., Suzuki, S., Matsuda, K., Toda, K., and Isshiki, N. (1994). A new skin equivalent: Keratinocytes proliferated and differentiated on collagen sponge containing fibroblasts. Plast Reconstr Surg. *93*, 537-544.

Mason, D.W. and Morris, P.J. (1986). Effector mechanisms in allograft rejection. Ann Rev Immunol. *4*, 119-145.

Mathor, M.B., Ferrari, G., Dellambra, E., Cilli, M., Mavilio, F., Cancedda, R., and De Luca, M. (1996). Clonal analysis of stably transduced human epidermal stem cells in culture. Proc Natl Acad Sci U.S.A. *93*, 10371-10376.

Mauduit, G., Ohrt, C., Faure, M., Kanitakis, J., Demidem, A., and Thivolet, J. (1987). Expression of blood group antigens by cultured human epidermal cells used as allografts. Acta Derm Venereol. *67*, 93

Mayer, T.G., Bhan, A.K., and Winn, H.J. (1988). Immunohistochemical analyses of skin graft rejection in mice. Kinetics of lymphocyte infiltration in grafts of limited immunogenetic disparity. Transplantation *46*, 890-899.

Meana, A., Iglesias, J., Del, R.M., Larcher, F., Madrigal, B., Fresno, M.F., Martin, C., San, R.F., and Tevar, F. (1998). Large surface of cultured human epithelium obtained on a dermal matrix based on live fibroblast-containing fibrin gels. Burns. *24*, 621-630.

Medalie, D.A., Eming, S.A., Tompkins, R.G., Yarmush, M.L., Krueger, G.G., and Morgan, J.R. (1996a). Evaluation of human skin reconstituted from composite grafts of cultured keratinocytes and human acellular dermis transplanted to athymic mice. J Invest Dermatol *107*, 121-127.

Medalie, D.A., Tompkins, R.G., and Morgan, J.R. (1996b). Evaluation of acellular human dermis as a dermal analog in a composite skin graft. Am Soc Art Int Organs J. *42*, M455-M462

Medalie, D.A., Eming, S.A., Collins, M.E., Tompkins, R.G., Yarmush, M.L., and Morgan, J.R. (1997). Differences in dermal analogs influence subsequent pigmentation, epidermal differentiation, basement membrane, and rete ridge formation of transplanted composite skin grafts. Transplantation *64*, 454-465.

Medalie, D.A., Tompkins, R.G., and Morgan, J.R. (1998). Characterization of a composite tissue model that supports clonal growth of human melanocytes *in vitro* and *in vivo*. J Invest Dermatol *111*, 810-816.

Medawar, P.B. (1944). The behaviour and fate of skin autografts and skin homografts in rabbits. J Anat. *78*, 176-199.

Medawar, P.B. (1945). A second study of the behaviour and fate of skin homografts in rabbits. J Anat. *79*, 157-177.

Michaeli, D. and McPherson, M. (1990). Immunologic study of artificial skin used in the treatment of thermal injuries. J Burn Care Rehabil. *11*, 21-26.

Miller, D.G. (1990). Gene transfer by retrovirus occurs only in cells that are actively dividing at the time of infection. Mol Cell Biol. *10*, 4239-4242.

Morhenn, V.B., Benike, C.J., Cox, A.J., Charron, D.J., and Engleman, E.G. (1982). Cultured human epidermal cells do not synthesize HLA-DR. J Invest Dermatol. *78*, 32-37.

Mosahebi, A., Woodward, B., Green, C., Martin, R., and Terenghi, G. (2000). Long term effect of vital labelling on mixed Schwann cell cultures Histochem J. *32*(6):337-43

Mouawad, R., Khayat, D., Zerrouqi, A., Ghoumari, A.M., and Soubrane, C. (1997). A flow cytometric method for the rapid detection of β-galactosidase transfected cells: an *in vitro* and *in vivo* study. J Immunol Methods *204*, 51-56.

Muir I.F.K., Barclay T.L.K., and Settle J.A.D. (1987). Burns and their management. (London: Butterworths), pp. 1-14.

Mullen, Y., Takasugi, M., and Hildemann, W.H. (1973). The immunological status of rats with long-surviving (enhanced) kidney grafts. Transplantation *15*, 238

Myers, S.R., Grady, J., Soranzo, C., Sanders, R., Green, C., Leigh, I.M., and Navsaria, H.A. (1997). A hyaluronic acid membrane delivery system for cultured keratinocytes: clinical "take" rates in the porcine kerato-dermal model. J Burn Care Rehabil. *18*, 214-222.

Nakano, Y. (1998). Antigen-presenting cell function of epidermal cells activated by hapten application. Br J Dermatol. *138*, 786-794.

Ng, R., Bevan, S., Woodward, B., Green, C., and Martin, R. (1996a). Genetic marking as a means of identifying grafted autologous keratinocytes in a porcine model. (Abstract) British Association of Plastic Surgery Summer meeting, Leicester

Ng, R., Shah, K., Winton, D., Green, C., and Martin, R. (1996b). Genetically marked allogeneic keratinocytes within a chimaeric epidermis-evaluation of the host response. (Abstract) British Association of Plastic Surgery Winter meeting, London.

Ng, R.L.H., Woodward, B., Bevan, S., Green, C., and Martin, R. (1997). Retroviral marking identifies grafted autologous keratinocytes in porcine wounds receiving cultured epithelium. J Invest Dermatol. *108*, 457-462.

Nickerson, P., Steurer, W., Steiger, J., Zheng, X., Steele, A.W., and Strom, T.B. (1994). Cytokines and the Th1/Th2 paradigm in transplantation. Curr Opin Immunol. *6*, 757-764.

Nickoloff, B.J., Turka, L.A., Mitra, R.S., and Nestle, F.O. (1995). Direct and indirect control of T-cell activation by keratinocytes. J Invest Dermatol *105*, 25S-29S.

Olszewski W.L. (1987). *In vivo* migration of immune cells (Boca Raton, Florida: C.R.C. Press).

Otto, W.R., Nanchahal, J., Lu, Q.L., Boddy, N., and Dover, R. (1995). Survival of allogeneic cells in cultured organotypic skin grafts. Plast Reconstr Surg. *96*, 166-176.

Pandya, A.N., Woodward, B., and Parkhouse, N. (1998). The use of cultured autologous keratinocytes with integra in the resurfacing of acute burns. Plast Reconstr Surg. *102*, 825-828.

Pauly, T., Weiland, E., Hirt, W., Dreyer-Bux, C., Maurer, S., Summerfield, A., and Saalmüller, A. (1996). Differentiation between MHC-restricted and non-MHC-restricted porcine cytolytic T lymphocytes. Immunology *88*, 238-246.

Pescovitz, M.D., Lunney, J.K., and Sachs, D.H. (1984). Preparation and characterisation of monoclonal antibodies reactive with PBL. J Immunol. *133*, 368-375.

Pescovitz, M.D., Lowman, M.A., and Sachs, D.H. (1988). Expression of T-cell associated antigens by porcine natural killer cells. Immunology *65*, 267-271.

Pescovitz, M.D., Sakopoulos, A.G., Gaddy, J.A., Husmann, R.J., and Zuckermann, F.A. (1994). Porcine peripheral blood CD4+/CD8+ dual expressing T-cells. Vet Immunol Immunopathol. *43*, 53-62.

Phillips, T.J., Provan, A., Colbert, D., and Easley, K.W. (1993). A randomized single-blind controlled study of cultured epidermal allografts in the treatment of split-thickness skin graft donor sites. Arch Dermatol. *129*, 879-882.

Pirnay JP, Vandenvelde C, Duinslaeger L, Reper P, and Vanderkelen A (1997). HIV transmission by transplantation of allograft skin : a review of the literature. Burns *23*, 1-5.

Place, M.J., Herber, S.C., and Hardesty, R.A. (1997). Basic techniques and principles in plastic surgery. In Grabb and Smith's Plastic Surgery. J.S. Aston, R.W. Beasley, and C.H.M. Thorne, eds. (Lippincott - Raven)

Press, B. (1997). Thermal, electrical and chemical injuries. In Grabb and Smith's Plastic Surgery. J.S. Aston, R.W. Beasley, and C.H.M. Thorne, eds. (Lippincott - Raven)

Ralston, D.R., Layton, C., Dalley, A.J., Boyce, S.G., Freedlander, E., and MacNeil, S. (1997). Keratinocytes contract human dermal extracellular matrix and reduce soluble fibronectin production by fibroblasts in a skin composite model. Br J Plast Surg. *50*, 408-415.

Ramarli, D., Giri, A., Reina, S., Poffe, O., Cancedda, R., Varnier, O., Tridente, G., and De, L.M. (1995). HIV-1 spreads from lymphocytes to normal human keratinocytes suitable for autologous and allogenic transplantation. J Invest Dermatol. *105*, 644-647.

Rappersberger, K., Gartner, S., Schenk, P., Stingl, G., Groh, V., Tschachler, E., Mann, D.L., Wolff, K., Konrad, K., and Popovic, M. (1988). Langerhans' cells are an actual site of HIV-1 replication. Intervirology *29*, 185-194.

Rheinwald, J.G. and Green, H. (1975). Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinising colonies from single cells. Cell *6*, 331-344.

Romani, N. and Schuler, G. (1992). The immunological properties of epidermal Langerhans' cells as part of the dendritic cell system. Immunopathology *13*, 265-279.

Rosenberg, A.S., Mizuochi, T., and Singer, A. (1988a). Cellular interactions resulting in skin-allograft rejection. Ann N Y Acad Sci. *532:76-85*, 76-85.

Rosenberg, A.S. and Singer, A. (1988b). Evidence that the effector mechanism of skin allograft rejection is antigen-specific. Proc Natl Acad Sci. U.S.A. *85*, 7739-7742.

Rosenberg, A.S., Mintz, T.I., Maniero, T.G., and Singer, A. (1991). Cellular basis of skin allograft rejection across a class I major histocompatibility barrier in mice depleted of CD8+ T-cells *in vivo*. J Exp Med. *173*, 1463-1471.

Rosenberg, A.S. and Singer, A. (1992). Cellular basis of skin allograft rejection: an *in vivo* model of immune- mediated tissue destruction. Annu Rev Immunol. *10:333-58*, 333-358.

Rouabhia, M., Germain, L., Bergeron, J., and Augh, F. (1995). Allogeneic-syngeneic cultured epithelia. Transplantation *59*, 1229-1235.

Rouabhia, M. (1996). Permanent skin replacementusing chimeric epithelial cultured sheets comprising xenogeneic and syngeneic keratinocytes. Transplantation *61*, 1290-1300.

Saalmüller, A., Hirt, W., and Reddehase, M.J. (1990). Porcine gamma/delta T lymphocyte subsets differing in their propensity to home to lymphoid tissue. Eur J Immunol. *20*, 2343-2346.

Saalmüller, A. and Bryant, J. (1994). Characteristics of porcine T lymphocytes and T-cell lines. Vet Immunol Immunopathol. *43*, 45-52.

Saalmüller, A., Hirt, W., Maurer, S., and Weiland, E. (1994). Discrimination between two subsets of porcine CD8+ cytolytic T lymphocytes by the expression of CD5 antigen. Immunology *81*, 578-583.

Saalmüller, A., Aasted, B., Canals, A., Dominguez, J., Goldman, T., Lunney, J.K., Maurer, S., Pauly, T., Pescovitz, M.D., and Pospisil, R. (1994a). Analyses of monoclonal antibodies reactive with porcine CD6. Vet Immunol Immunopathol. *43*, 243-247.

Saalmüller, A., Aasted, B., Canals, A., Dominguez, J., Goldman, T., Lunney, J.K., Maurer, S., Pescovitz, M.D., Pospisil, R., and Salmon, H. (1994b). Analyses of monoclonal antibodies reactive with porcine CD5. Vet Immunol Immunopathol. *43*, 237-242.

Saalmüller, A., Denham, S., Haverson, K., Davis, B., Dominguez, J., Pescovitz, M.D., Stokes, C.C., Zuckermann, F., and Lunney, J.K. (1996). The Second International Swine CD Workshop. Vet Immunol Immunopathol. *54*, 155-158.

Saalmüller, A. (1996). Characterization of swine leukocyte differentiation antigens. Immunol Today *17*, 352-354.

Saalmüller, A., Pauly, T., Aasted, B., Jensen, K.T., Sachs, D.H., Arn, S., Davis, W.C., Park, Y.H., McCullough, K., Summerfield, A., Murtaugh, M., Pampusch, M.S., Burger, K.D., Laber, J., Nielsen, J., Pescovitz, M.D., Stokes, C., Haverson, K., Boyd, P., and Lunney, J.K. (1998a).

Summary of the first round analyses of the Second International Swine CD Workshop. Vet Immunol Immunopathol *60*, 237-249.

Saalmüller, A., Pauly, T., Lunney, J.K., Boyd, P., Aasted, B., Sachs, D.H., Arn, S., Bianchi, A., Binns, R.M., Licence, S., Whyte, A., Blecha, F., Chen, Z., Chu, R.M., Davis, W.C., Denham, S., Yang, H., Whittall, T., Parkhouse, R.M., Dominguez, J., Ezquerra, A., Alonso, F., Horstick, G., Howard, C., and Zuckermann, F. (1998b). Overview of the Second International Workshop to define swine cluster of differentiation (CD) antigens. Vet Immunol Immunopathol. *60*, 207-228.

Sachs, D.H. (1994). The pig as a potential xenograft donor. Vet Immunol Immunopathol. *43*, 185-191.

Scharfmann, R., Axelrod, J.H., and Verma, I.M. (1991). Long term *in vivo* expression of retrovirus mediated gene transfer in mouse fibroblast implants. Proc Natl Acad Sci U.S.A. *88*, 4626-4630.

Schuler, G. and Steinman, R.M. (1985). Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells *in vitro*. J Exp Med. *161*, 526-546.

Schuurman, H.-J. (1994). Molecular mechanisms of transplant rejection. Clin Investig. *72*, 715-718.

Setoguchi, Y., Jaffe, H.A., Danel, C., and Crystal, R.G. (1994). *Ex vivo* and *in vivo* gene transfer to the skin using replication-deficient recombinant adenovirus vectors. J Invest Dermatol. *102*, 415-421.

Shakespeare, P.G. (1999). Cost effectiveness of skin substitutes. Burns *25*, 179-181.

Shiku, H., Kisielow, P., Bean, M.A., Takahashi, T., Boyse, E.A., Oettgen, H.F., and Old, L.J. (1975). Expression of T-cell differentiation antigens on effector cells in cell- mediated cytotoxicity *in vitro*. Evidence for functional heterogeneity related to the surface phenotype of T-cells. J Exp Med. *141*, 227-241.

Signer, E.N., Jeffreys, A.J., Licence, S., Miller, R., Byrd, P., and Binns, R. (1999). DNA profiling reveals remarkably low genetic variability in a herd of SLA homozygous pigs. Res Vet Sci. *67*, 207-211.

Singer, A., Munitz, T.I., Golding, H., Rosenberg, A.S., and Mizuochi, T. (1987). Recognition requirements for the activation, differentiation and function of T-helper cells specific for class I MHC alloantigens. Immunol Rev. *98*, 143-170.

Sinkora, J., Rehakova, Z., Sinkora, M., Cukrowska, B., Tlaskalova-Hogenova, H., Bianchi, A.T., and De Geus, B. (1998). Expression of CD2 on porcine B lymphocytes. Immunology *95*, 443-449.

Sinkora, J., Rehakova, Z., Samankova, L., Haverson, K., Butler, J.E., Zwart, R., and Boersma, W. (2001). Characterization of monoclonal antibodies recognizing immunoglobulin kappa and lambda chains in pigs by flow cytometry. Vet Immunol Immunopathol. *80*, 79-91.

Snell, G.D. (1957). The homograft reaction. Ann Rev Microbiol. *11*, 439-458.

Sone, Y., Sakagami, K., and Orita, K. (1987). Effect of ex vivo perfusion with anti-Ia monoclonal antibodies on rat cardiac allograft survival. Transplant Proc. *19*, 599-604.

Sopp, P., Redknap, L., and Howard, C. (1998). Cross-reactivity of human leucocyte differentiation antigen monoclonal antibodies on porcine cells. Vet Immunol Immunopathol. *60*, 403-408.

Stark, G.B. and Kaiser, H.W. (1994). Cologne Burn Centre experience with glycerol-preserved allogeneic skin: Part II: Combination with autologous cultured keratinocytes. Burns *20 Suppl 1*, S34-S38

Steinman, R.M. (1991). The dendritic cell system and its role in immunogenicity. Annu Rev Immunol. *9:271-96.*, 271-296.

Steinman, R., Hoffman, L., and Pope, M. (1995). Maturation and migration of cutaneous dendritic cells. J Invest Dermatol *105*, 2S-7S.

Stern, R., McPherson, M., and Longaker, M.T. (1990). Histologic study of artificial skin used in the treatment of full- thickness thermal injury. J Burn Care Rehabil. *11*, 7-13.

Stockschlader, M.A.R., Schuening, F.G., Graham, T.C., and Storb, R. (1994). Transplantation of retrovirus-induced canine keratinocytes expressing the β-galactosidase gene. Gene Therapy *1*, 317-322.

Stokes, C.R., Haverson, K., and Bailey, M. (1996). Antigen presenting cells in the porcine gut. Vet Immunol Immunopathol. *54*, 171-177.

Streilein, J.W. (1983). Skin-associated lymphoid tissues (SALT): origins and functions. J Invest Dermatol. *80 Suppl:12s-16s*, 12s-16s.

Stuart, F.P., Fitch, F.W., and Rowley, D.A. (1970). Specific suppression of renal allograft rejection by treatment with antigen and antibody. Transplant Proc. *2*, 483

Suzuki, T., Ui, K., Shioya, N., and Ihara, S. (1995). Mixed cultures comprising syngeneic and allogeneic mouse keratinocytes as a graftable skin substitute. Transplantation *59*, 1236-1241.

Takami, Y., Matsuda, T., Yoshitake, M., Hanumadass, M., and Walter, R.J. (1996). Dispase/detergent treated dermal matrix as a dermal substitute. Burns *22*, 182-190.

Takeuchi, Y., Cosset, F.L., Lachmann, P.J., Okada, H., Weiss, R.A., and Collins, M.K. (1994). Type C retrovirus inactivation by human complement is determined by both the viral genome and the producer cell. J Virol. *68*, 8001-8007.

Talmage, D.W., Dart, G., Radovich, J., and Lafferty, K.J. (1976). Activation of transplant immunity: Effect of donor leucocytes on thyroid allograft rejection. Science *191*, 385-388.

Teunissen, M.B., Wormmeester, J., Krieg, S.R., Peters, P.J., Vogels, I.M., Kapsenberg, M.L., and Bos, J.D. (1990). Human epidermal Langerhans cells undergo profound morphologic and phenotypical changes during *in vitro* culture. J Invest Dermatol. *94*, 166-173.

Thivolet, J., Faure, M., Demidem, A., and Mauduit, G. (1986). Long-term survival and immunological tolerance of human epidermal allografts produced in culture. Transplantation *42*, 274-280.

Tilney, N.L. and Gowans, J.L. (1970). The sensitization of rats by allografts transplanted to alymphatic pedicles of skin. J Exp Med. *133*, 951

Tinois, E., Cobbold, S., Faure, M., Yeoman, H., and Stanley, M. (1989). Cultured keratinocyte grafts are recognized, but not rejected by CD8+ T-cells *in vivo*. Eur J Immunol. *19*, 1031-1035.

van der Merwe, A.E., Mattheyse, F.J., Bedford, M., van Helden, P.D., and Rossouw, D.J. (1990). Allografted keratinocytes used to accelerate the treatment of burn wounds are replaced by recipienT-cells. Burns *16*, 193-197.

Vega-Lopez, M.A., Telemo, E., Bailey, M., Stevens, K., and Stokes, C.R. (1993). Immune cell distribution in the small intestine of the pig: immunohistological evidence for an organized compartmentalization in the lamina propria. Vet Immunol Immunopathol. *37*, 49-60.

Vogt, P.M., Thompson, S., Andree, C., Liu, P., Breuing, K., Hatzis, D., Brown, H., Mulligan, R.C., and Eriksson, E. (1994). Genetically modified keratinocytes transplanted to wounds reconstitute the epidermis. Proc Natl Acad Sci. U.S.A. *91*, 9307-9311.

Wedler, V., Kunzi, W., Burgi, U., and Meyer, V.E. (1999). Care of burns victims in Europe. Burns *25*, 152-157.

White, E., Hildemann, W.H., and Mullen, Y. (1969). Chronic kidney allograft rejection in rats. Transplantation *8*, 602

Wilson, A.D., Haverson, K., Southgate, K., Bland, P.W., Stokes, C.R., and Bailey, M. (1996). Expression of major histocompatibility complex class II antigens on normal porcine intestinal endothelium. Immunology *88*, 98-103.

Winter, G.D. (1962). Formation of the scab and the rate of epithelisation of superficial wounds in the skin of the young domestic pig. Nature *193*, 293-294.

Winter, G.D. (1972). Epidermal regeneration studied in the domestic pig. In Epidermal wound healing. H.I. Maibach and D.T. Rovee, eds. (Chicago year book medical publishers), pp. 71-112.

Winzler, C. et al (1997). Maturation stages of mouse dendritic cells in growth factor-dependant long-term cultures. J Exp Med. *185*, 317-328.

Witmer-Pack, M.D., Valinsky, J., Olivier, W., and Steinmann, R.M. (1988). Quantification of surface antigens on cultured murine epidermal Langerhans cells: rapid and selective increase in the level of surface MHC products. J Invest Dermatol *90*, 387-394.

Yang, H., Oura, C.A., Kirkham, P.A., and Parkhouse, R.M. (1996a). Preparation of monoclonal anti-porcine CD3 antibodies and preliminary characterization of porcine T lymphocytes. Immunology *88*, 577-585.

Yang, H. and Parkhouse, R.M. (1996b). Phenotypic classification of porcine lymphocyte subpopulations in blood and lymphoid tissues. Immunology *89*, 76-83.

Yannas, I.V., Lee, E., Orgill, DP., Skrabut, E.M., and Murphy, G.F. (1989). Synthesis and characterisation of a model extracellular matrix that induces partial regeneration of adult mammalian skin. Proc Natl Acad Sci. U.S.A. *86*, 933-937.

Zhao, Y.B., Zhao, X.F., Ngao, L.A., Lu, S.Z., Wang, X., Huang, S.Z., and Zhuo, X.T. (1992). Clinical observation and methods for identifying the existence of cultured epidermal allografts. Burns *18*, 4-8.

Zinkernagel, R.M., Ehl, S., Aichele, P., Oehen, S., Kundig, T., and Hengartner, H. (1997). Antigen localisation regulates immune responses in a dose- and time-dependant fashion: a geographica view of immune reactivity. Immunol.Rev. *156*, 199-209.

Zuckermann, F.A., Peavey, C., Schnitzlein, W.M., Schabacker, D., Husmann, R.J., Yang, H., Saalmuller, A., and Lunney, J.K. (1998). Definition of the specificity of monoclonal antibodies against porcine CD45 and CD45R: report from the CD45/CD45R and CD44 subgroup of the Second International Swine CD Workshop. Vet Immunol Immunopathol. *60*, 367-387.