

ANTISEPTIC CYTOTOXICITY AND THE CUTANEOUS WOUND: AN IN VITRO STUDY

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ABSTRACT

Concern has been expressed about the possible harmful effects of antiseptics on cutaneous wounds. There are few clinical data to support this view but animal studies suggest that certain agents are toxic.

To simplify the investigation of antiseptic toxicity investigations have been conducted in vitro using cell culture. This study set out to establish an in vitro cytotoxicity assay which is simple, accurate and reliable, so that a number of cell types and agents could be easily studied. Basal keratinocytes and fibroblasts were studied as these cells are essential to the wound healing process. A transformed keratinocyte line (SVK 14 cells) was also compared.

The cells were exposed to hydrogen peroxide, chlorhexidine, sodium hypochlorite and cetrimide for 15 minutes and cell viability was assessed with a colorimetric assay which utilizes the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT).

Comparison of 50% survival levels showed that the three cell types showed similar susceptibilities to the agents tested. These findings suggest that the transformed cell line, which has the advantage of ready availability and immortality, can replace both fibroblasts and keratinocytes in an antiseptic cytotoxicity assay.

Comparison of 50% survival concentrations to the standard therapeutic concentrations showed the following ranking order of toxicity: sodium hypochlorite > cetrimide > hydrogen peroxide > chlorhexidine. These findings are in line with in vivo studies on inhibition of wound healing by antiseptic agents. The assay devised appeared to be of value in predicting the relative toxicities of antiseptic agents in vivo.

Reported experimental conditions for antiseptic cytotoxicity assays vary. The effect on toxicity of following conditions was studied: increasing exposure time; the effect of medium or serum; increasing cell number. Increasing cell number and diluting the agents in medium and serum reduced the toxicity of all agents but had the greatest effect on sodium hypochlorite. Increasing the exposure time increased the toxicity of all

agents but had the greatest effect on hydrogen peroxide. Changes in experimental variables clearly alter the toxicity of individual agents. The results of in vitro testing should be interpreted in the context of experimental conditions.

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ABBREVIATIONS USED IN TEXT

DMEM	Dulbecco's Modified Eagle's Medium
PBS-a	Phosphate Buffered Saline
PBS-abc	Phosphate Buffered Saline supplemented with calcium and magnesium salts
KCM	Complete Keratinocyte Culture Medium
KCM-Ca	Complete Keratinocyte Culture Medium depleted of calcium
FCS	Foetal Calf Serum
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
OD	Optical Density
EDTA	Ethylenediaminetetraacetic acid
h	hour

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CHAPTER 1

GENERAL INTRODUCTION

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1.1 DEFINITIONS

Antiseptic - a substance that kills or prevents the growth of microorganisms. This term is used for preparations applied to living tissues

Disinfectant - an agent that prevents infection by the destruction of pathogenic organisms. It is a term used in reference to substances applied to inanimate objects.

1.2 INTRODUCTION

Topical antibiotics and antiseptic agents have been widely used by dermatologists, surgeons and family practitioners to prevent bacterial contamination of a wound, in the belief that preventing bacterial growth can enhance wound healing. The desirable properties of antiseptic agents are outlined in **Table 1.1**. Antiseptics exert their antimicrobial effects through direct toxic effects on microbial cell walls, nuclei or cytoplasm and for many years concern has been expressed that these agents, in addition to killing or inhibiting bacterial proliferation, may exert a toxic effect on host cells in a wound. This study is concerned with the use of topical antiseptics in cutaneous wounds healing by secondary intention and their possible harmful effects. There is a wealth of information concerning the antibacterial efficacy of these agents but relatively few published data on their effects on wound healing. Herein follows a careful examination of the available literature on the toxic effects of antiseptics on wound healing, both in vivo and in vitro.

In general, in vivo studies are conducted in animal wound models. These studies require large numbers of animals to produce statistically significant results. Such studies are expensive in terms of animal life and cost. Because the use of animals for experimentation has become politically unacceptable, many pharmaceutical companies are looking for alternatives to animals in which to study new agents. In recent years, the advent of better cell culture techniques has led to the evaluation of antiseptic cytotoxicity in vitro. Cell culture systems may be used not only to assess the

TABLE 1.1 Desirable properties of antiseptic agents

1)	Agents are lethal to organisms
2)	Broad antimicrobial spectrum
3)	Bacteriocidal and fungicidal properties more important than virucidal or protozocidal action
4)	Low surface tension to allow spreading over skin and wound surfaces
5)	Potency retained in the presence of organic material
6)	Action must be rapid and sustained
7)	No adverse effects
	Non-irritant
	No inhibition of wound healing
	Non-sensitizing
	No systemic absorption

possible toxicological potential of individual agents, but also to establish modes of action. However such techniques are in their infancy. The purpose of this study was to establish a simple method for assessing antiseptic toxicity in cell culture and to estimate the value of cell culture systems in the assessment of antiseptic cytotoxicity.

In reviewing antiseptic toxicological data it is also necessary to review the practice of using antiseptics in healing wounds. It has been stated that when antiseptics are applied to a wound 'the chances are that they will largely be inactivated by serum, blood, necrotic tissue and dilution' (Altemeir, 1983). Although such views may be held, only a small number of studies are available in which attempts are made to critically evaluate the efficacy of antiseptic agents. In addition recent changes in wound management practice have emphasized the creation of an optimal wound healing environment and less emphasis has been placed on the eradication of bacteria. In examining the possible toxic role of antiseptics it is also necessary to question whether the application of these agents is either efficacious or necessary.

In the first section a brief history of antiseptics will be outlined, providing a background to current usage.

1.3 HISTORICAL ASPECTS OF ANTISEPTIC USAGE

Although the scientific application of disinfectants and antiseptics is limited to the past 150 years, empiric practices go back to ancient times. In the first century Dioscorides advocated the use of sulfur ointment as an antiseptic. The use of wine and vinegar in the dressing of wounds dates back to Hippocrates. Compounds of mercury were used to prevent sepsis in wounds by Arabian physicians in the Middle Ages.

In the prevention of human disease the first half of the nineteenth century was a period of awakening (Block, 1983). In 1825 Labarraque used chlorinated soda solution in treating infected wounds and recommended it for general disinfection. In 1829, Robert Collins employed solutions of chlorine compounds in an attempt to check an epidemic of puerperal fever. In 1830, the American physician Oliver Wendell Holmes

indicated that puerperal fever was contagious and transmitted by nurses and in 1835 he reported success on preventing transmission of infection by washing the hands in chlorine of lime solution following each visit to an infected patient. Semmelweis used chlorine successfully against childbed fever in 1847. He published a careful analysis of his work in the book 'Aetiologie'. This account showed a dramatic fall in the death rate from puerperal sepsis. However neither Holmes nor Semmelweis could gain acceptance for their principles of basic hygiene or for the use of antiseptics.

Phenol was first used in wounds in 1860 by Lemaire. However it was Lister who gave world fame to antiseptics through the use of phenol even though he succeeded Semmelweis and Holmes in the use of antiseptics by decades. He used phenol to successfully treat wounds and published his findings in 1867 (Lister, 1867). He then introduced the application of antiseptics to surgery and is credited with introducing the age of disinfection.

Other antiseptics whose use was introduced in the nineteenth century were: iodine, first used by Davies in 1839 and widely used in the American Civil War (1862); alcohol, shown to have germicidal activity by Reinicke in 1894 and Epstein in 1897; mercuric chloride which gained wide use after Koch demonstrated in vitro activity against anthrax spores. In the late nineteenth century aniline dyes, and newer chlorine, iodine and organic mercurials were introduced. The flavines appeared in the 1920's, cationic detergents in the 1930's, halogenated quinolones in the 1940's, hexachlorophene, chlorhexidine and iodophors in the 1950's and triclosan and benzoyl peroxide in the 1960's.

The evaluation of antiseptic antibacterial activity began in 1881 when Koch (1881) devised and employed the methods of modern bacteriology and used them to test the action of disinfectants. Kronig and Paul (1897) furthered this work by attempting to standardise conditions of testing for disinfectants in vitro. The phenol coefficient method of testing was introduced by Rideal and Walker in 1903 (Rideal and Walker, 1903). They standardised all conditions of testing and employed specific bacteria in a prescribed broth medium. Phenol formed the basis of comparison for all other agents

tested. Chick and Martin (1908) improved the methods of testing by introducing organic material to the test conditions to ensure that the test system mimicked the 'in use' situation. Although modifications to these methods have been introduced, they formed the basis of disinfectant testing procedures.

In the early part of the twentieth century, Fleming began to question the use of antiseptics in wound therapy stating that 'antiseptics will only exercise a beneficial effect in a septic wound if they possess the property of stimulating or conserving the natural defensive mechanism of the body against infection' (Fleming, 1919). Fleming recognized that antiseptic agents may have harmful effects on neutrophils which played a major role in eradicating bacterial infection. Early attempts to study the toxicity of antiseptics used rabbit spleen tissue (Lambert and Meyer, 1926) and cultures of spindle cells and periosteum from embryonic chicks (Bucksbaum and Bloom, 1931). Later attempts were made to establish a toxicity index (the ratio of the concentration necessary to produce cell/tissue damage to the concentration necessary to achieve an antibacterial effect) in an attempt to establish safe topical antiseptics. In 1937 Salle, McOmie and Schechmeister (1937) described a toxicity index in which the highest dilution of the germicide killing a strain of bacteria within 10 minutes was compared to the highest dilution preventing growth of an embryonic chick heart during a 48 hour incubation. This measure of toxicity was criticized principally because it was felt that chicken heart was unrepresentative of a cutaneous wound and that exposure for 48 hours was too long as agents were unlikely to persist in tissue for such long periods. A further study examined the effect of the antimicrobials on bacteria and leukocytes in vitro, and the effect of intradermal injection of antiseptics (Nye, 1937). This study showed iodine to be the most powerful antibacterial agent when compared to chlorine-releasing agents and mercury solutions, but equally it was the most toxic to neutrophils and skin. Others continued to use leukocytes to generate a toxicity index (Welch and Hunter, 1940). Later studies used mouse liver cells to evaluate tissue toxicity (Bronfengrenner et al, 1938). In reviewing the toxicity index generated by a number of studies Reddish (1954) concluded that such indices were of little value since they

generated such conflicting results with agents being given a high index by some investigators and a low index by others. This emphasizes the pitfalls of in vitro testing of antiseptic toxicity.

Interest in evaluating the possible harmful effects of antiseptics on tissues in vitro seems to have waned in the 1950's whilst the uncritical use of antiseptics continued. However in the 1980's interest was reawakened in both the usefulness of these agents in wound care and their possible harmful effects.

1.4 METHODS OF TESTING ANTISEPTIC ANTIBACTERIAL ACTIVITY

For new products, in vitro data are required to show that the ingredient is truly antimicrobial and the probable range of activity. The standard techniques and the methods of calculating the various coefficients of disinfectants are fully described in British Standard Specifications (Reynolds, 1989 i). The principle tests used are: The Rideal-Walker Test which requires distilled water dilutions of the disinfectant to be tested against broth cultures of the specified micro-organisms; the Chick Martin Test requires the disinfectant to be tested in the presence of a high concentration of organic matter. A chemical whilst showing positive results in vitro may not show significant activity when formulated and used topically. Undue emphasis has been placed on in vitro testing for topical antimicrobials. Current thinking demands that basic in vitro data is supported by in vivo tests showing effectiveness for a given indication.

It is widely recognised that clinical trials are an inadequate way of assessing new antimicrobial products (Leyden et al, 1979). A properly controlled study for assessing prophylaxis of infection of abrasions, for several formulations of a new agent on a population practising good hygiene would require observations of thousands of individuals, because only a small number of injuries would become infected. Additionally, adequate control of subjects and standardisation of wounds is impossible in clinical trials. For this reason the following in vivo human models (Leyden, Stewart and Kligman, 1979) have been established to evaluate the efficacy of

topical antimicrobial agents in intact skin and in wounds: 1) The occlusion test measures the ability of an agent to prevent the expansion of resident microflora which occurs when an impermeable dressing is applied to the forearm and measurements are made at 24 and 48 hours. 2) The expanded flora test measures the ability of an agent to suppress a dense population of micro-organisms produced by expansion of the resident flora of the forearm by application of an impermeable occlusive dressing and measurements are made at 10 minutes, 6 hours, 24 hours and 48 hours. 3) The persistence test measures the ability of an agent to establish a reservoir in the skin and exert an antimicrobial effect up to 3 days after the last application of the test material. 4) The ecological shift test determines any major alteration in cutaneous microbial ecology following several applications of the material under occlusive dressing. 5) The serum inactivation test determines whether the presence of serum proteins interferes with antimicrobial activity of an agent.

Human models have not replaced clinical testing as yet, however such studies may prove useful as screening tests prior to full-blown clinical testing. Doubts have been cast on the ethics of using artificially infected wounds in human volunteers to study the efficacy of these agents. Over emphasis on the in vitro testing for antimicrobial activity of antiseptic agents may have led to the situation where a large number of agents are available for a variety of indications, but doubt is cast on their efficacy. Below the efficacy of these agents is evaluated in clinical studies and animal and human models.

1.5 CLINICAL APPLICATIONS AND EFFICACY OF ANTISEPTICS

The principal uses of topical antiseptics are 1) for pre-operative skin preparation 2) as a surgical hand scrub 3) as a prophylactic wound cleanser, to prevent possible bacterial infection of a surgical wound 4) for cleansing infected wounds.

1.5.1 PROPHYLACTIC ANTISEPSIS OF THE SKIN

The surface bacterial counts of normal skin are shown in **Table 1.2**. Since an inoculum of 5×10^6 *S. aureus* cells is needed to cause infection in a healthy adult (Harvey, 1985)

routine antiseptic prophylaxis is unnecessary. However in the compromised host this inoculum may be much smaller and prophylactic antiseptics may play an important role in preventing infection. A greater emphasis is now placed upon suppression of environmental sources of pathogens than upon preoperative preparation. Preoperative preparation of the skin with antiseptics, although reducing surface pathogens markedly, does not always decrease the incidence of infections. Bathing with hexachlorophene did not reduce the postoperative infection rate among 5760 patients, despite a substantial reduction in the cutaneous bacterial population (Ayliffe et al, 1983). Antiseptic showers that reduce the number of staphylococci on the scrotum were not found to alter the incidence of infections after vasectomy (Randall et al, 1983), however preoperative showering with chlorhexidine decreased the incidence of postoperative infections in patients undergoing vascular surgery (Brandberg et al, 1979).

1.5.2 PROPHYLAXIS AGAINST SURGICAL WOUND INFECTION

Clinical studies

Surgical wounds at high risk of infection are irrigated with antiseptic prior to closure in the hope that this will prevent wound infection. Some studies have shown benefit from this procedure. The use of povidone iodine (Disidine spray, 0.5% povidone iodine) pre and post wound closure in 456 appendicectomy wounds significantly reduced wound infection rates (Gilmore and Martin, 1974). Similar findings were obtained in a further study in which 500 patients undergoing abdominal surgery had their wounds irrigated with either 10% povidone iodine or saline, used as a control, prior to closure (Sindlar and Mason, 1979). The antiseptic treatment significantly reduced infection in potentially infected abdominal wounds from 14.3% to 2% and in contaminated wounds

Table 1.2 Surface bacterial counts on normal human skin (Harvey, 1985)

Glabrous skin	100-5000/cm ²
Face and scalp	200,000/cm ²
Axilla	300,000/cm ²

from 20% to 6.8%. Two studies have shown that wound irrigation with chlorhexidine (0.05%w/v) reduced postoperative infection rates (Shepherd and Kinmonth, 1962).

Other studies have shown no benefit from the prophylactic use of antiseptics in surgical wound management. Galle and Homesley (1980) compared povidone iodine and saline irrigation of wounds in operations for gynaecological malignancies and showed that povidone iodine had no effect on reducing wound infection rates when compared to saline controls. Pollock, et al (1978) obtained similar results in a study which compared the effect of wound irrigation with povidone iodine, saline or cephaloridine prior to closure in abdominal operations considered at high risk of wound infection. Rogers, et al (1983) could not show statistical differences between rates of wound infection when comparing wound irrigation with povidone iodine or saline. Crosfil (1969) found that irrigation of appendectomy wounds with chlorhexidine 0.5% w/v did not reduce wound infection rates when compared to irrigation with normal saline inspite of using a concentration of chlorhexidine which is ten times that recommended for use in wounds (Reynolds, 1989 ii). Use of such a high concentration may induce tissue damage making secondary infection more likely. In one study an attempt was made to establish a safe concentration of povidone iodine which would not impair healing and would reduce wound infection rates (Viljanto 1980). Children undergoing appendectomy were treated with 5% povidone iodine spray prior to closure and this produced higher infection rates than in the untreated control group. In a subsequent study a Cellstic drain, comprising a silicone rubber tube containing a piece of viscose cellulose sponge, was placed between the wound edges for 24 hours. Cells moving with the exudate attached themselves to the sponge and subsequently the sponge was examined histologically. Prior to insertion the tubes were influxed with one of three povidone iodine solutions. When 5% povidone iodine was used leukocyte migration into the sponge was inhibited and many cells appeared dead; use of 5% aqueous iodine showed increased numbers of leukocytes and small aggregates of fibroblasts. When 1% aqueous povidone iodine was infused into the Cellstic tube there was little difference in cellularity between the control saline tube and the test agent tube. A further study

examined the effect of 1% aqueous povidone iodine instilled into appendicectomy wounds prior to closure; this showed that at this concentration wound infection rates were lower than in untreated controls, although the numbers in the test group were small (test=38, control=82). Viljanto (1980) concluded that a safe and effective concentration of an agent could be established.

Animal studies

Because of the practical difficulties entailed in clinical studies some workers have turned to animal models to evaluate the use of prophylactic antiseptic treatment.

Rodeheaver et al (1982) created surgical wounds in guinea pigs and inoculated the wounds with *Staphylococcus aureus*. Prior to closure the wounds were irrigated with 1% aqueous iodine, aqueous povidone iodine 10%, povidone iodine surgical scrub or normal saline. Aqueous iodine and povidone iodine surgical scrub caused infection rates in wounds which were higher than control. The authors concluded that this may be related to the toxic effects of these agents on tissues, which may make wounds more susceptible to infection, views which are in line with Viljanto (1980). Aqueous povidone iodine did not reduce wound infection rates compared to the control treatment. Platt and Bucknall (1984) used wounds in guinea pigs infected with *Staphylococcus aureus* to assess the efficacy of a range of antiseptic agents (chlorhexidine gluconate 0.01%, 0.02%, 0.05%; benzalkonium chloride 0.1%; noxythiolin 2.5%; povidone iodine 10%). The antiseptics were applied 15 minutes after inoculation of the bacteria or 45 minutes before. This design simulated surgical practice of treating the skin with antiseptics prior to starting surgery or cleansing the wound prior to surgical closure. Infection was judged according to the amount of pus and induration present in the wound on the sixth post-operative day. Irrigation 15 minutes after the bacteria were inoculated with either saline or noxythiolin did not reduce the incidence or degree of infection. Benzalkonium chloride and to a lesser degree povidone iodine significantly reduced the infection rate, but were inferior to chlorhexidine which eliminated all overt signs of infection. When irrigation was

carried out 45 minutes before bacterial inoculation, chlorhexidine was the only treatment which reduced infection rates.

In summary, there is no convincing clinical evidence that wound irrigation with topical antiseptics is effective in reducing post-operative wound infections and this practice has largely been abandoned. Limited animal data appears to support this view.

1.5.3 TREATMENT OF CHRONIC CUTANEOUS WOUNDS

Clinical studies

Cutaneous ulcers Although antiseptic agents are regularly used to treat chronic cutaneous ulcers, particularly venous ulcers, very few studies have systematically evaluated their efficacy as antibacterial agents or their effect on wound healing. The effect of benzoyl peroxide 10% was evaluated in thirteen patients with leg ulcers (Lookingbill et al, 1978). Bacterial counts were made pre- and two weeks post-treatment; the antiseptic agent had no effect on bacterial counts. Two patients out of six treated with benzoyl peroxide developed cultures dominated by a growth of *Pseudomonas aeruginosa* which was not present at the start of the trial and which was resistant to benzoyl peroxide. Daltry and Cunliffe (1981) performed a double blind trial of the effects of benzoyl peroxide 20% and Eusol and liquid paraffin on the microbial flora of ulcers. The Eusol solution had no significant antibacterial effect on the number of pathogenic bacteria but the number of pathogenic bacteria present increased in some wounds. Species of pseudomonas and streptococci and enterobacteria which were not initially present were subsequently isolated. Only a small number of wounds became free from bacteria following Eusol treatment. The effect of antimicrobial agents on the microbial flora of 75 varicose ulcers was studied by Henderson et al (1980). Bacterial counts were taken pre-treatment and 48 hours after the application of the antiseptic/antibiotic agents which were formulated into cream preparations. The agents tested were: silver sulphadiazine, gentamicin, neomycin/bacitracin,

chlorhexidine, sodium fusidate, and Viscopaste bandage was used as a control. Only chlorhexidine and gentamicin significantly reduced bacterial counts. This study was performed to assess the most effective antimicrobial agent in reducing bacterial counts prior to grafting. A study by Burke, (1971) showed that within 24 hours of covering burn wounds by xenografts the bacterial counts fell; this emphasizes the importance of the wound environment in reducing contamination rather than the ritual use of antiseptics and antibiotics.

The effect of antiseptics on bacteria in pressure sores has also been studied; povidone iodine 10% was no more effective than saline washes in reducing bacterial counts in human pressure sores (Kucan et al, 1981).

Burns The incidence of invasive burn wound infection has decreased significantly with the use of effective topical antimicrobial therapy. Many studies have evaluated the effect of these agents. Povidone iodine ointment applied four times daily to 50 patients with up to 90% burns showed 75% of cultures taken to be negative and 95% to be below the critical level for invasive sepsis (a surface swab giving giving a bacterial colony count $>10^5$) (Georgiade and Harris, 1973). This was an uncontrolled study and no information is given on how this treatment affected morbidity or mortality. Use of povidone iodine is limited by systemic absorption. A chlorhexidine plus silver sulphadiazine preparation was found to be effective in the treatment of burns; only 7 blood cultures were positive out of 900 patients treated (Clarke, 1975).

The topical agents currently in most frequent use are: silver sulphadiazine, silver nitrate solution, mafenide and chlorhexidine. Topical antimicrobial agents can prevent or minimize burn infections and use is advised in all patients who are at significant risk from sepsis. All of the currently available agents have short-comings. The recent shift in emphasis toward early surgical closure of extensive deep burns in great part has resulted from the appreciation of the inadequacies of currently available topical agents (Monafo and Freedman, 1987).

In summary, topical antiseptic agents appear to be effective in the treatment of burns and their use is often only limited by their systemic toxicity. Antiseptics used on chronic cutaneous ulcers appear to be less effective; this may be related to the persistence of an adverse wound environment created by such factors as venous hypertension, pressure and ischaemia. Bacteria may persist and recolonize in the presence of such wound conditions in spite of antibacterial measures.

Animal and human wound models

Cutaneous ulcers have been created to study the effect of antiseptic agents in vivo. Mertz et al (1984) examined the effect of antiseptics in bacterially contaminated partial thickness wounds in pigs. The wounds were inoculated with *Staphylococcus aureus*. The agents tested (povidone iodine and 70% alcohol) were then applied to the wound for 1 or 3 minutes or 24 hours and bacterial counts were assessed at these time points. At 1 minute neither treatments reduced the number of pathogens; at three minutes both agents produced a slight reduction and after 24 hours only povidone iodine reduced the number of pathogenic bacteria that could be cultured but the count remained above 1×10^5 , the number considered as evidence of infection (Mailbach and Hildick Smith, 1965). The authors conclude that the model is an accurate simulation of the clinical situation and that the results suggest that both 70% alcohol and povidone iodine are of limited efficiency in the treatment of superficial wounds.

Leyden et al (1979) devised a human model for evaluating antimicrobial agents on human skin. The serum inactivation test determined how the presence of serum proteins may interfere with antimicrobial activity. Three agents were tested: povidone iodine surgical scrub, hyamine 10X (a quaternary ammonium compound), and neomycin sulphate. The model comprised a de-roofed ammonium hydroxide induced blister which was contaminated with *Staphylococcus epidermidis*. The effect of these agents, which were applied under occlusion for 24 hours, was compared to their antibacterial effect when tested on intact skin. The iodine compound showed no antibacterial activity. The quaternary ammonium compound was still capable of

suppressing a dense population of bacteria but was not as effective as on intact skin. Neomycin showed no reduction in activity when used on an eroded surface as compared to intact skin. Leyden and Bartelt (1987) used the same human model to study both the efficacy of antiseptics and their effects on wound healing. The blister bases were contaminated with *Staphylococcus aureus* and bacterial counts were made 16 hours after two applications of the antimicrobial agents were made. The agents tested were: neomycin ointment, Polysporin ointment, benzalkonium chloride, thiomersal, hydrogen peroxide 3%, merbromin, camphor and phenol. Only the antibiotic ointments were able to significantly reduce bacterial contamination compared to the untreated control.

Chlorine-releasing agents, chlorhexidine and povidone iodine are amongst the most commonly used antiseptic agents in clinical practice. The majority of data suggests that chlorhexidine is an effective antiseptic both in the animal model and in clinical trials. Data on povidone iodine are less consistent; animal model data suggest that this agent is only partially effective in suppressing bacterial counts and frank wound infection and these findings are in keeping with variable effects on wound infection in clinical trials. Although chlorine-releasing agents have good antibacterial activity when tested against purely bacteria, available data from clinical studies suggest that these agents have poor antimicrobial activity in wounds.

A study which examines a wide range of agents regularly used for minor cuts and abrasions suggests that antiseptic agents are of very little benefit in reducing bacterial contamination (Leyden and Bartelt, 1987). Unfortunately the agents used in this study are all 'over the counter' preparations, and clearly further studies are necessary to evaluate all agents used regularly in hospital practice. However available data suggest that the efficacy of many agents in reducing bacterial contamination of a wound should be questioned.

1.6 **ROLE OF BACTERIA IN WOUND HEALING**

Does the presence of bacteria in a wound inhibit healing, have no effect on healing or enhance healing?

Invasive bacterial infection in a wound has long been recognised to delay healing (Carrel and Hartmann, 1916) but it has also been argued that optimal wound healing rates cannot be achieved unless bacteria are eliminated from open wounds (Burke, 1971). A healthy wound environment is known to contribute to wound healing, and the importance of local measures such as wound bebridement, removal of foreign bodies, and improving local circulation is considered of greater value than the topical application of drugs (Altemeier, 1983). A study by Burke (1971) showed that cutaneous wounds contaminated by *Staphylococcus aureus* and covered with conventional dressings did not heal; wounds covered with xenografts shed the bacterial contamination and heal faster. It was concluded that bacteria must be eradicated before a wound can heal. An alternative interpretation might be that this study emphasizes the role of the wound environment in eradicating bacteria and enhancing healing, rather than the role of bacteria in inhibiting it.

1.6.1 **EFFECT OF INFECTION ON SURGICAL WOUNDS**

Certain studies on surgical wounds confirm the view that invasive bacterial infection inhibits wound healing; Smith and Enquist (1976) infected sutured incisions in rats with *Staphylococcus aureus* and showed from the sixth post-operative day the infected wounds were weaker when compared to controls. However, other studies have shown that certain bacterial infections may enhance wound healing. Tenorio et al (1976) inoculated abdominal wounds in rats with pure or mixed cultures of gram negative bacteria and a gram positive coccus. The tensile strength of the wounds was measured and the infected wounds were found to be significantly stronger than controls. This effect was related to the inflammatory response elicited by the infection. The inflammation induced by pseudomonal infection was strong and this wound was not significantly stronger than the control, whereas the enterococci infected wounds had a

minimal inflammatory response and equally did not differ significantly from controls. However intermediate inflammatory responses, induced by certain bacteria, appeared to enhance wound healing. Raju et al (1977) infected surgical wounds with *Escherichia coli* and showed that the infected wounds were significantly stronger than uninfected controls. These latter studies used gram negative bacteria as the inoculum because these organisms have supplanted gram positive cocci as the cause of most surgical infections.

From these studies it might be concluded that certain bacteria evoke the proper inflammatory response and result in stronger wounds while others provoke too much inflammation leading to weakening.

1.6.2 CHRONIC CUTANEOUS WOUNDS AND OCCLUSIVE DRESSINGS

An important step forward in the management of wounds was made when Winter (1962) reported enhanced epithelialization in occluded wounds. The principal property of occlusion is to keep tissues moist. Although these findings were reported in the early sixties, occlusive dressings have only been introduced into clinical practice in recent years, principally because it was feared that occlusion might promote bacterial infection. Studies of wound healing using occlusive dressings in both clinical trials and animal models have led to a better understanding of the role of bacteria in wound healing. Although bacteria may proliferate under occlusive dressings, recent evidence suggests that the presence of large numbers of certain bacteria does not inhibit healing. Leaper et al. (1984) produced full thickness wounds in rats and 1×10^8 organisms, either *Staphylococcus aureus*, *Escherichia coli* or *Pseudomonas aeruginosa*, were placed into each wound and the wounds were occluded. At intervals up to 10 days, rats were killed from each group and wound size measured. The granulation tissue from the wounds was excised and cultured. There was a rise in bacterial counts for the *Escherichia coli* and *Staphylococcus aureus* infected wounds up to six days and then counts fell. Counts in the pseudomonas infected wounds continually rose until ten days. The *Escherichia coli* and *Staphylococcus aureus* infected wounds healed normally but the pseudomonas

infected wounds did not. This study suggests that with the exception of pseudomonas infections, large numbers of pathogenic bacteria do not inhibit wound healing. Alper et al (1983) reported a clinical study in which cutaneous ulcers in 18 patients were treated with an occlusive dressing. In 16 of the 18 patients the ulcers healed in spite of gross bacterial contamination, however here there was no quantitation of bacterial data.

Although large numbers of bacteria may be present under an occlusive dressing, it has been shown that, with time, the numbers fall (Leaper et al, 1984). Wound fluid has antibacterial activity (Buchan et al, 1981). This activity may be produced by large numbers of actively bacteriocidal neutrophils and the high concentrations of globulins and lysozyme which may prevent bacterial growth. Wound fluid may also contain growth factors which promote healing and proteases which will enhance debridement. Persistent use of antiseptic solutions to cleanse a wound may dilute growth promoting and antibacterial activity of wound fluid.

The data presented here suggest that eradication of bacteria from a wound is not essential to wound healing, although in the presence of certain bacteria the use of an antimicrobial agent may be advised.

1.7 COMPLICATIONS OF ANTISEPTIC THERAPY

Much attention has been focused on the antimicrobial, resorptive and potential allergenic properties of antiseptic agents. Adverse systemic and local effects from these agents are well documented.

Local adverse effects include irritant and allergic contact dermatitis. Irritancy is well recognised with sodium hypochlorite and severe irritant reactions to povidone iodine have been reported (Okano, 1989). Unlike topical antibiotics, allergic contact dermatitis to antiseptics is rare; amongst those agents used in current clinical practice contact dermatitis has been reported to chlorhexidine (Berqvist-Karlsson, 1988), cetrimide (Sharvill, 1965), and povidone-iodine (Marks, 1982). Contact urticaria to

chlorhexidine has been documented (Berqvist-Karlsson, 1988; Fisher, 1989). Photocontact dermatitis has been reported to halogenated salicylanides.

Toxicological studies on topical agents concentrate on the possible adverse systemic effect which may occur if the compound is absorbed. Iodine compounds are associated with a number of systemic adverse effects. Hypersensitivity reactions occur which lead to fever and a widespread rash. Absorption of iodine may lead to hypothyroidism and has been described following bathing of neonates, and the topical treatment of burns. Absorption of iodine from povidone iodine applied to patients with burns with impaired renal function has led to the production of a metabolic acidosis (Pietsch and Meakins, 1976) and the use of this agent has not been recommended in patients with more than 20% burns. Of the phenolic compounds, hexachlorophene is noted for its neurotoxicity; burns patients treated with 3% hexachlorophene developed spongy degeneration in the brain and spinal cord. Neonates washed with this antiseptic agent developed demyelination of the brain and spinal cord (Powell et al, 1973). The triphenylmethane dyes have been widely used for many years on wounds, however recent reports of mutagenicity in animals (Anon, 1987) has led to the recommendation that these dyes should not be used on mucous membranes or broken skin. The treatment of pressure sores with hypochlorites has been complicated by acute oliguric renal failure which was believed to have been caused by the release of endotoxins from bacteria present in the wounds (Barton and Barton, 1981). Judging by the literature, this complication of hypochlorite treatment would seem to be extremely rare.

Whilst antiseptics have been used for many years to combat infection recent reports have shown that these agents may become contaminated with bacteria, particularly pseudomonas species. Such contamination has been described for almost all agents in current use (Rutala, 1984) and has been associated with the introduction of major systemic infection.

- Although great interest in systemic and local adverse effects has been shown, until recently little interest has been shown on the effect of antiseptic agents on wounds. Whilst toxicity has been suspected on theoretical grounds there is little clinical

evidence to support this view, with the exception of the triphenylmethane dyes where tissue toxicity, particularly to mucosal surfaces, has been clearly documented (John, 1968). Hence the possible toxic effects of these agents have been studied experimentally both *in vitro* and *in vivo*.

1.7.1 ANTISEPTICS AND INHIBITION OF WOUND HEALING - EFFECT OF TOPICAL ANTIMICROBIALS *IN VIVO*

A limited number of studies have examined the effect of antimicrobials on wounds *in vivo*. Both the wound models and the agents used vary from study to study and there is much inconsistency in the findings. The results of these studies are detailed below and summarized in **Tables 1.3, and 1.4.**

Effect on epithelialization of cutaneous wounds (Table 1.3)

The rate of re-epithelialisation in surgically created wounds has been studied. Gruber et al (1975) used partial and full thickness wounds in rats and donor sites in humans to study the effect of acetic acid, povidone iodine and hydrogen peroxide. The agents were applied 4 times daily until the wounds were re-epithelialised. None of the agents in either the rat models or the human model caused a delay in healing. Geronemus et al (1979) used partial thickness wounds in pigs to study the effects of povidone iodine, Neosporin ointment, nitrofurazone and 1% silver sulphadiazine cream. As in the previous study no toxicity for povidone iodine (10%) could be demonstrated, but nitrofurazone significantly delayed healing; both Neosporin ointment and silver sulphadiazine cream significantly enhanced healing rates. Lineaweaver et al (1985) used full thickness wounds in rats to study the inhibition of wound healing by the thrice daily application of povidone iodine 1%, acetic acid 0.25%, sodium hypochlorite 0.5%, hydrogen peroxide 3%. Re-epithelialisation was significantly delayed at 8 days by acetic acid, sodium hypochlorite and povidone iodine but only by sodium hypochlorite at 16 days. Leyden and Bartelt (1987) reported the only human-model study in which blisters were produced by ammonium hydroxide and the blister base

AUTHOR	MODEL	AGENTS /CONCENTRATIONS	EFFECT ON HEALING		
			DELAYED	NO EFFECT	ENHANCED
Gruber et al, 1975	1) Rat-FT	Acetic acid (0.25%)		Acetic acid	Hydrogen peroxide
	2) Human donor site	Povidone iodine (10%) Hydrogen peroxide (3%)		Povidone iodine	
Geronemus et al, 1979	Pig-PT	Neosporin	Nitrofurazone	Povidone iodine	Silver sulphadiazine Neosporin
		Povidone iodine 10% Nitrofurazone Silver sulphadiazine			
Lineaweaver et al, 1985	Rat-FT	Povidone iodine (1%)	Povidone iodine Acetic acid } At 8/7 NaOCl NaOCl at 16/7	Hydrogen peroxide	
		Acetic acid (0.25%) Sodium hypochlorite -NaOCl (0.5%) H ₂ O ₂ (3%)			
Brennan et al, 1986	Rat-FT	Chloramine T 1%	ChloramineT	Chlorhexidine	
		Chlorhexidine 0.05%			
Leyden et al, 1987	Human infected blister base	Neosporin Hydrogen peroxide (3%) Benzalkonium chloride Thimerosal Tincture of iodine Camphor & phenol Merbromin	Tinct of I2 Camphor-phenol	Benzalkonium chloride Hydrogen peroxide Thimerosal Merbromin	Neosporin

TABLE 1.3 Reported studies of the effect of antiseptic agents on re-epithelialisation in wounds healing by secondary intention (FT=full thickness; PT=partial thickness; H₂O₂=hydrogen peroxide; NaOCl=sodium hypochlorite)

infected with *Staphylococcus aureus*. Two hours after inoculation the wounds were treated twice daily with one of the following test products: neomycin-polymyxin B-bacitracin ointment, polymyxin B-bacitracin ointment, benzalkonium chloride spray, Merbromin, thimerosal, hydrogen peroxide, tincture of iodine and camphor-phenol. Wounds treated with the neomycin-polymyxin B-bacitracin ointment healed significantly faster than untreated wounds; tincture of iodine and the camphor-phenol preparation significantly delayed wound healing when compared to no treatment. Two applications of neomycin-bacitracin-polymyxin B ointment eliminated bacterial contamination in the first 16 - 24 hours. No other test agent produced comparable results.

More complex analysis of wound healing, assessing both the rate of reepithelialisation and the amount of granulation tissue formed was made by Brennan, Foster and Leaper (1986). Two agents, chlorhexidine (0.05%) and chloramine T 1%, were applied to full thickness wounds in rats. Granulation tissue was excised three, five and seven days post-operatively and assayed for hydroxyproline content, a measure of collagen synthesis, and for DNA as a measure of tissue cellularity. Re-epithelialisation was assessed histologically. Wounds treated with chloramine T showed significantly less collagen but significantly higher levels of DNA when compared to those treated with chlorhexidine or the saline control. Histological examination showed marked hypercellularity of the chloramine treated group (mainly polymorphs). Epithelialization was significantly delayed in the chloramine T treated group.

Studies on blood vessels and granulation tissue (Table 1.4)

Effects on the cutaneous wound bed can be studied by examining an immediate, direct effect on blood vessels microscopically; longer-term effects can be evaluated by examining granulation tissue histologically, looking for induction of inflammation or simply measuring destruction of granulation tissue by thickness.

Using vital microscopy Branemark et al (1966) studied the microcirculatory effects of a range of commercial compounds on hamster cheek pouch, a peripheral nerve in

AUTHOR	MODEL	EXPOSURE	AGENT	ADVERSE EFFECT	NO EFFECT
Niedner, 1986	Guinea pig Formation of granulation tissue in FT wound- histological measurement	Continuous for 7/7	Pycatanin 0.5% Brilliant green 0.5% Eosin 0.5% Chlorhexidine 0.5% Chloramine T 10%	Pycatanin= brilliant green> chlorhexidine	Chloramine T Povidone iodine Silver nitrate Eosin
Brennan, 1986	Rat-FT Collagen content of GT	Continuous for 7/7	Chloramine T 1% Chlorhexidine 0.05%	Chloramine T	Chlorhexidine
Brennan, 1985	Rabbit ear chamber	Flooded with agent	Eusol Povidone iodine (5%) Povidone iodine (1%) Chloramine T (1%) H ₂ O ₂ (3%) Chlorhexidine (0.05%)	Chloramine T =Eusol> Povidone iodine5% Povidone iodine 1% Chlorhexidine	Hydrogen peroxide Povidone iodine 1% Chlorhexidine
Branemark, 1966	1)Cheek pouch hamster 2)Rabbit ear chamber-two agents tested. Microcirculatory effect observed- vital microscopy	Irrigation for 5 min, 60 min	Quaternary ammonium compounds Povidone iodine Hexachlorophene	Cheek pouch- all agents Ear chamber Quat ammon Co	Ear chamber Hexachlorophene

TABLE 1.4 Comparative effect of antiseptics on granulation tissue in wound healing by secondary intention
(Key: FT=full thickness; GT=granulation tissue; H₂O₂=hydrogen peroxide)

mouse, the rabbit's ear chamber and skin capillaries in man. In the hamster cheek pouch, all agents produced cessation of blood flow after a 60 minute exposure which was irreversible; 5 minute exposure caused cessation of flow which was reversible. Studies in the rabbit ear chamber (5 minute exposure) showed extensive delay in revascularisation of the chamber (14 days versus 6-7 days in control) with the quaternary ammonium compounds but only slight delay with hexachlorophene.

Twenty years later Brennan and Leaper (1985) returned to the rabbit ear chamber model to study the toxic effects of antiseptic agents. The chambers were flooded with the following agents: Eusol, povidone iodine 5% and 1%, hydrogen peroxide 10 volumes, chloramine T 1% and chlorhexidine 0.05%. Effects on granulation tissue were studied by examining capillary circulation with a dissecting microscope and by measuring capillary blood flow using a laser Doppler flowmeter. The chlorine releasing solutions caused cessation of blood flow within vessels and these vessels never reopened. On the fifth day after application new vessels started to grow in from the periphery. By 14 days the chamber had recovered to its pretreatment state. Povidone iodine (5%) caused cessation of blood flow and it took 72 hours for the chamber to recover. Chlorhexidine, hydrogen peroxide and povidone iodine caused no sustained adverse effects.

Niedner and Schopf (1986) examined the formation of granulation tissue in a full thickness wound in guinea pigs. Antiseptics and dyes (pyoctanin, eosin, brilliant green, chloramine T 10%, povidone iodine 5%, chlorhexidine 0.5%, silver nitrate 1%) were held in contact with full thickness wounds for seven days, and re-epithelialisation was prevented by insertion of a teflon ring. The wounds were excised and the thickness of the granulation tissue measured; pyoctanin and brilliant green caused marked inhibition of the formation of granulation tissue, chlorhexidine caused a 60% reduction and the other agents produced a small reduction that was not statistically significant. A study by Brennan et al (1986) uses a similar wound model to investigate the toxicity of antiseptic agents on granulation tissue (**Table 1.4**), although the methods for measuring toxic effects on granulation tissue were different. Here chloramine T 1% was toxic to granulation tissue and chlorhexidine 0.05% was not, reversing the findings

of Neidner and Schopf (1986). Comparison of these two studies emphasizes the difficulty of interpreting antiseptic toxicity data.

The toxicity of the triphenylmethane dye crystal violet was investigated by Mobacken and Zederfeldt (1973). Viscose cellular sponges were implanted into surgical wounds in rats. The sponges were either soaked in sterile water or crystal violet (three concentrations 1 in 100, 1 in 1000, 1 in 10,000). The sponges were examined histologically at 10 days; in those treated with water there was an ingrowth of capillaries, fibroblasts and collagen but in all dye-soaked sponges no connective tissue was seen. These findings support those of Neidner and Schopf (1986) which suggest that these agents are toxic to granulation tissue.

Effect on tensile strength of wounds

The effect of antiseptic agents on surgical wound models can be measured by estimating the tensile strength of wounds. The tensile strength of a wound in the first few days is ascribed to epithelialisation and production of immature collagen but by two weeks tensile strength is correlated with collagen production. Soaking dorsal wounds in rats with povidone iodine 1% for 15 minutes had no effect on the tensile strength of the wounds at 1, 2 and 6 weeks (Mulliken et al, 1980). In contrast, in a further study, wounds irrigated with povidone iodine (1%) three times daily were significantly weaker (21% of control) than wounds irrigated with saline, acetic acid 0.25%, sodium hypochlorite 0.5% or 3% hydrogen peroxide (Lineaweaver et al, 1985). Exposure of incisional wounds to one of two concentrations of chlorhexidine (0.02% or 0.1% w/v) for five minutes prior to closure led to a reduction in breaking strength at seven days compared to control wounds (Mobacken and Wengstrom, 1974).

Effects on other tissues

Faddis et al (1977) used the rabbit knee to study tissue injury induced by povidone iodine (Betadine Prep and Betadine scrub) and hexachlorophene (PhisoHex and 3% solution). These agents were injected into joints at 48 h intervals on three occasions.

The solutions containing detergents, Betadine scrub and pHisoHex caused severe gross and histologic damage to articular cartilage, synovia and muscle. The hexachlorophene solution caused moderate histological damage and loss of articular ground substance. Betadine Prep caused only minimal gross and histologic damage, without biochemical evidence of articular cartilage damage. It was suggested that if an antiseptic solution is to be used for packing joints, Betadine Prep is recommended. Much of the damage was attributed to the detergent base of the antiseptic agent emphasizing that the vehicle may also contribute to the overall toxicity of an agent.

These studies provide conflicting evidence for the tissue toxicity of antiseptics, which is perhaps in keeping with the variety of wound healing models used and the wide choice of antiseptic agents tested. The choice of agents partly reflects prescribing practices both over a period of 20 years and in different countries. Branemark et al (1966) tested quaternary ammonium soaps because 'invert soaps are the most commonly used disinfectants nowadays'. Superior agents are now available and Block (1983) states that there is little reason to use cationic surfactants as disinfectants now. In spite of this, their use persists and hence Leyden and Bartelt (1987) in a study of 'over the counter' antiseptic preparations chose to test benzalkonium spray. Although the choice of wound healing model varies, there are broadly two types of experiment: those which examine reepithelialization in full and partial thickness wounds, and those which examine the toxic effects on granulation tissue. Only in one human study (Leyden and Bartelt, 1987) is an infected wound created, and although the infected blister base probably provides the best way of assessing the toxic effects of antiseptic agents doubts have been expressed about the ethics of using an infected human model. Although the animal models are broadly similar, the methods of applying the tested agents vary. In several studies the wounds were irrigated at intervals, reflecting common clinical practice for cleansing cutaneous wounds. In other studies the antiseptics were formulated so that exposure was continuous for a period of days, which may in the case of some agents greatly increase toxicity.

In reviewing studies on the toxicity of antiseptics to wounds it is possible to find some consistent findings. All in vitro studies show that the triphenylmethane dyes are toxic to components of the healing wounds. In contrast, in all studies traced 3% hydrogen peroxide is non-toxic to granulation tissue and does not inhibit reepithelialisation (Lineaweaver et al, 1985; Brennan and Leaper, 1985; Gruber et al, 1975; Leyden and Bartelt, 1987). For other agents testing gives less consistent results, although some trends are apparent. Povidone iodine (10%) showed no adverse effect on wound reepithelialisation or upon rabbit knee cartilage; only one study has shown an adverse effect of povidone iodine 5% on the formation of granulation tissue. The majority of data, however, suggest that this agent does not inhibit healing. However tincture of iodine does inhibit re-epithelialisation; this agent is known to have much greater antibacterial effect than povidone iodine and it well known that it is also toxic to tissues (Harvey, 1985) and the application of tincture of iodine is not recommended to wounds. A series of studies by one group (Brennan and Leaper, 1985; Brennan et al, 1986) have consistently shown that chlorhexidine (0.05%) is non-toxic to epithelium and granulation tissue. Most available in vivo data suggest that chlorine-releasing agents are toxic to cutaneous wounds.

1.7.2 ANTISEPTICS AND INHIBITION OF WOUND HEALING: EFFECT OF TOPICAL ANTIMICROBIALS IN VITRO

This study is concerned with ways of looking at antiseptic toxicity in vitro. Only a limited number of published studies have examined the toxicity of antiseptics in vitro and most of these reports have been confined to the study of these agents in cell culture (single cells), although a few reports have been published which examine the effects of these agents in tissue culture (whole tissue) (Lawrence, 1970; Mobacken, Ahonen and Zederfeldt, 1974). As with the in vitro methods for testing the antibacterial activity of antiseptics, problems are encountered in designing a test system which reflects the 'in use' situation. In vitro studies are designed to assess the potential harmful effect of antiseptics on healing wounds and it is well known that certain agents are inhibited by

the presence of organic matter within a wound; particular problems are encountered in judging the amounts of organic material that are representative of the 'in use' situation and this has never been answered satisfactorily for either antibacterial studies or toxicological work. As with the in vivo studies, comparison of results between in vitro studies is difficult because of differences in choice of agent and differences in study design.

Studies in tissue culture

Lawrence (1970) studied the effect of silver nitrate, Sulfamylon, mercuric chloride and copper sulphate on whole skin using skin explants. Toxicity was evaluated by measuring skin respiration and histology, where the degree of migration of the epithellum in the epibole was measured. Results showed that silver nitrate, Sulfamylon and other sulphonamides were relatively non-toxic to skin but mercuric chloride and copper sulphate were highly toxic. The skin explant used in this method is cultured in 50% serum which poses a disadvantage when testing agents which are inhibited by serum. In vitro studies on granulation tissue have been performed by Mobacken, Ahonen and Zederfeldt (1974); this study used granulation tissue grown in cylinders in vivo and examines the effect of a single agent, crystal violet, on tissue respiration and collagen production (measured by incorporation of ^{14}C -proline and production of ^{14}C -hydroxyproline, which is taken as measure of capacity to produce collagen). A five minute exposure to two concentrations of crystal violet (0.001% and 0.0001% w/v) led to a striking dose-dependent reduction in oxygen consumption and ^{14}C -proline incorporation.

Studies in cell culture

Most in vitro studies of antiseptic toxicity have been performed in cell culture using single cell types. In comparison to tissue culture techniques, cell culture has the advantage of simplicity and reproducibility.

In a small number of published studies the toxicity of multiple antiseptic agents to cells in culture has been compared (**Table 1.5**) but most studies have examined the effect of a single agent (**Table 1.6**). Because fibroblasts and cell lines are easy to culture, these

TABLE 1.5 Published data comparing the toxicity of antiseptic agents in cell culture

AUTHOR	CELL TYPE	TOXICITY ASSAY	EXPOSURE	DILUENT	AGENTS	NO EFFECT DILUTION
Lincauweaver, 1985	Human fibroblasts (2.5x10 ⁸ cells)	Staining with vital dye	15 min	Normal saline x 10 dilution	1.Sodium hypochlorite 0.5% 2.Acetic acid 0.25% 3.H ₂ O ₂ (3%) 4.Povidone I ₂ (1%)	x 100 x100 x1000 x1000
Leaper, 1986	Fibroblasts (2X10 ⁶)	Vital staining + cell counts X10 dilution-medium	24 h	Medium	1.Chloramine T 2.Phenol 1% 3.Chlorhexidine 0.05% 4.Hexachlorophene = H ₂ O ₂ = 5.Hydrogen peroxide Povidone 6.Povidone iodine iodine	Chlor T< Chlorhexidine= Phenol< Hexachlorophene = H ₂ O ₂ = Povidone iodine
Deas, 1986	Fibroblasts (5x10 ³)	Viable cells stained with methylene blue	24 h	Medium + 10% FCS	Savlodil 1.5% H ₂ O ₂ (3%) Chlorhexidine 0.05% Povidone iodine 10% Phenol Chloramine T 2%	x 1000 x1000 x10,000 x10,000 x100,000 x1,000,000
Cooper, 1990	Keratinocytes	Growth effect -cell counting	4 days	Medium (MCDB 153)	Neosporin GU Acetic acid Gentamicin Dakin's 25% Sulfamylon	Non-toxic x 10 x10 x100 x100
Shakespeare, 1988	Oral keratinocytes	Growth inhibition H ³ thymidine Cell membrane damage (LDH levels)	2h, 22h	Medium	Chlorhexidine (C) 0.02% Hexetidine (H) 0.1% Benzylamine (B) 0.15%	22h-growth inhibition.x 1000 all agents 2h-growth inhibition B>x100 C+H>x250 LDH activity Bx50, Cx100, Hx250

TABLE 1.5

AUTHOR	CELL TYPE	TOXICITY ASSAY	EXPOSURE	DILUENT	AGENT	NO EFFECT DILUTION	COMMENT
Helgeland, 1971	Human skin epithelial cells (NCTC strain 2544) (4-8X10 ⁵ cells)	1.Cell growth counting 2.Cell viability vital stain 3.Enzyme activity*	5 min	Saline but serum compared	Chlorhexidine Concentrations- 0.02mM 0.05mM 0.10mM	0.02 mM Serum no effect conc ⁿ 10-20 X higher	*NADPH ₂ and, effect- NADH ₂ diaphorases, Akaline phosphatase, 5'nucleotidase.
Jamieson, 1972	Hela cells (1X10 ⁵)	1.Vital staining + cell counts 2.Growth inhibition- cell counting	4 h at 37°C	Parkers medium 199	Noxythiolin 1%	X 1000	
Kozol, 1988	Neutrophils Fibroblasts (F) Endothelial cells (E)	1) Leucocyte migration 2) E and F- electron microscopic changes	30 min 1 h	Medium (MEM) x 10 dilutions	Sodium hypochlorite Concentrations 0.025%-0.000025%	2.5x10 ⁻⁵ %- 90% inhibition of leucocyte migration 2.5%x10 ⁻⁴ and above toxic to E and F.	

Table 1.6 Studies of the toxic effects of a single antiseptic agent tested on cultured cells

cells have been the target cell (**Table 1.5 and 1.6**). Keratinocytes are obviously a key cell in the evaluation of antiseptic toxicity to wounds in vitro. The ability to culture keratinocytes has only become available in recent years (Rheinwald and Green, 1975) and studies using this cell type are in their infancy. Culture techniques, unlike those for cell lines and fibroblasts are difficult. Some recent studies have attempted to examine the susceptibility of normal human keratinocytes to these agents, using either cutaneous (Cooper et al, 1990) or oral cells (Shakespeare et al, 1988) (**Table 1.5**). These studies have examined the effect of antiseptic agents on cell growth. The use of oral cells may be judged particularly important because of the wide use of antiseptics on oral epithelium. As culture techniques have become more sophisticated it has become possible to culture most cell types. This has allowed investigators to study the effect of antiseptic agents on a number of cell types, essential to wound healing. Thus, in one study the effect of sodium hypochlorite was evaluated on endothelial cells and fibroblasts, whilst parallel data were produced for the effect of this agent on neutrophil function (Kozol et al, 1988) (**Table 1.6**).

The toxicity of antiseptics to mammalian cells has been considered advantageous in certain instances; wounds have been irrigated with agents such as cetrimide to eradicate malignant cells from a wound (Gibson, 1966; Stephens and Gibson, 1966). This property of antiseptic agents was evaluated in vitro by Blenkarn (1987) who studied the effect of two antiseptics, chlorhexidine and noxythiolin, on a range of neoplastic and non-neoplastic cell lines. Noxythiolin was judged to have greater cytotoxic action against the neoplastic lines than to the normal lines, whilst no differences could be determined for the toxic effect of chlorhexidine on either cell type. (**Table 1.7**). The authors conclude that the activity of noxythiolin is such that it may be suitable for use in the prevention of anastomotic recurrence in colorectal carcinoma caused by implantation of exfoliated cells.

Toxicological methods have usually examined cell killing using vital staining and cell counting, or inhibition of growth, using either a cell counting method or the incorporation of tritiated thymidine. For cultured keratinocytes only growth assays

AUTHOR	CELLTYPE	TOXICITY ASSAY	EXPOSURE TIME	AGENTS	NO EFFECT DILUTION	COMMENT
Blenkarn, 1987	1.Laryngeal carcinoma (Hep2) 2.Embryonic Fb (MRC 5) 3.Embryonic Fb (Flow 2002) 4.Skin Fb (CRL 1510) 5.Skin Fb (Malme-3)	1.Cell viability vital stain 2.Microscopy	1 min, 5 min, 10 min, 20 min, 30 min,	1.Noxythioline:1% 2.chlorhexidine 0.05%	Cell % kill Hep2 70 50 MRC5 70 50 Flow 40 40 2002 CRL 40 40 1510 <20 40 Malme <20 30 -3	Conclusion:1987 Malignant cells are more susceptible to noxythioline than chlorhexidine but normal and neo-plastic cells equally affected by Cx.

TABLE 1.7 Study showing increased susceptibility of neoplastic cells to noxythioline

have been used. The reported methods for establishing cell viability are time-consuming and inaccurate. In all studies the data generated is crude, with no attempt to generate LD 50 values for more accurate comparison of agents. The number of ten-fold dilutions to eradicate toxicity is usually examined. Some attempts have been made to examine the possible mode of action of the toxic agent on the cells (Shakespeare et al, 1988) (**Table 1.5**).

Available data shows that at concentrations recommended for use in a healing wound all agents tested are toxic in the various cell culture systems in which they have been tested. How should this be interpreted when, at least for some agents, in vivo data is available which suggests the agent is not toxic? In two studies (Lineaweaver et al, 1985; Leaper and Brennan, 1986) in vivo and in vitro data toxicity were reported. Although in both studies the in vivo data showed the chlorine-releasing containing agents to be the most toxic to the healing wound, the parallel in vitro data showed these agents to be the least toxic. What is noticeable from these and other in vitro studies is that there is no attempt to interpret the data. This is perhaps because such forms of testing are relatively novel and difficult to perform; most authors have been happy to conclude that if the agents tested are toxic to cells in vitro they should not be used in vivo.

1.8 AIMS OF THIS STUDY

For practical reasons, cell culture may offer the way forward in testing the potential toxicity of antiseptic agents on cutaneous wounds. These procedures are, however in their infancy. The aims of this study were as follows:

- 1) To devise an assay which was sufficiently simple and reliable to allow for an accurate comparison of the toxicity of a range of antiseptic agents on a number of cell types. Since keratinocytes and fibroblasts, are fundamental to the cutaneous wound healing process it was decided to examine their susceptibility to antiseptic agents.

Keratinocytes demand complex culture conditions and in order to simplify the investigation of toxicity on cutaneous epithelium it was proposed to compare the effect of the agents on keratinocytes to those on a transformed keratinocyte line (SVK 14 cells) available to the Department of Experimental Dermatology at the London Hospital and to establish whether these cells might be substituted for normal keratinocytes. These transformed cells have the advantage of ready availability and an infinite lifespan, and grow in the absence of a fibroblast feeder layer. Use of this cell line would simplify the investigation of the adverse effects of antiseptic agents on cutaneous epithelium.

The experimental conditions initially used are briefly outlined in **Figure 1.1**. It was proposed to use an attached cell assay and to expose the cells for a short period of time to the test agent. The cells would then be recultured to allow death or regeneration to occur prior to the assay of cell survival/death.

The cytotoxic effect (cell death) would initially be measured using a vital stain. Subsequently it was hoped to establish a simpler more accurate technique to measure toxicity.

2) The susceptibility of the three cell types would be evaluated and compared. Comparison of the toxicity of each agent would be made by comparing the number of dilutions from the standard concentration to reach an effective dose 50% (ED50) concentration, measuring either survival or cell death. The results would be compared to published in vitro and in vivo data. It was important to establish whether such in vitro assays have any predictive value for in vivo toxicity.

3) Published in vitro studies give conflicting results for the relative toxicities of individual agents. It was proposed to examine the factors which affect the toxicity of antiseptic agents in vitro.

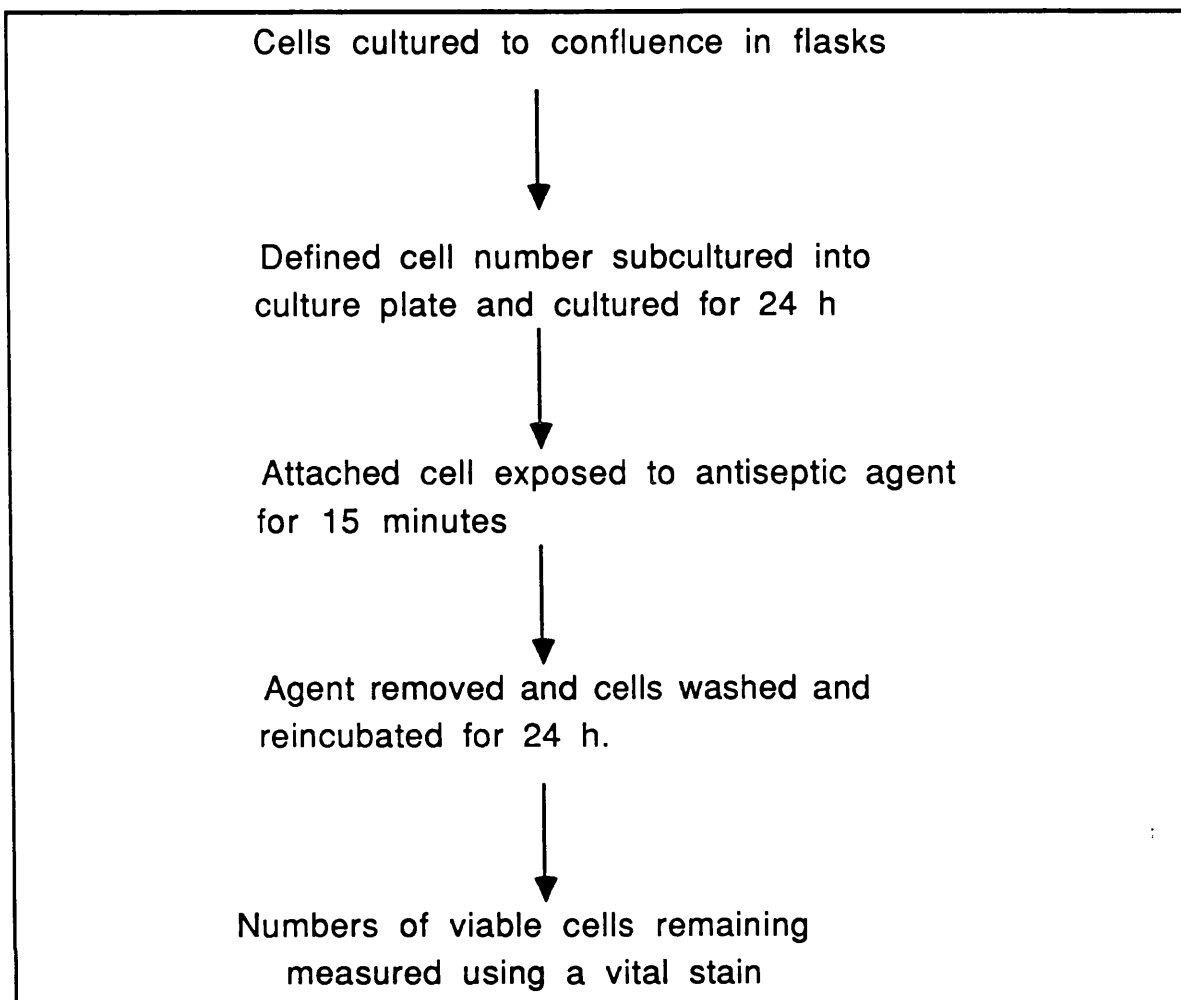


FIGURE 1.1 Outline of initial experimental procedure to be used in antiseptic cytotoxicity assay

CHAPTER 2

METHODS - ESTABLISHING CELL CULTURE TECHNIQUES

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2.1 INTRODUCTION

In this study it was proposed to use three human cell types: basal keratinocytes, normal fibroblasts and a transformed keratinocyte line, SVK 14 cells. Basal keratinocytes and fibroblasts were chosen because they are essential to the cutaneous wound healing process (**Figure 2.1**) (Woodley et al, 1985). The SVK 14 cell line has many advantages over the normal or non-transformed phenotype. Whilst the ability to culture fibroblasts is well established, improved techniques for culturing keratinocytes are still being generated. In this chapter the culture characteristics of the three cell lines are discussed ultimately defining the methods to be used in this study.

2.2 HISTORICAL ASPECTS OF CELL CULTURE

Tissue culture was devised at the beginning of the century (Harrison, 1907) as a method for studying the behavior of animal cells free from systemic variations that might arise in the animal. Culture of cells from primary explants (establishing a cell culture from whole tissue) dominated tissue culture for 50 years. Cell culture began in the 1940's with culture of mouse fibroblasts (Earle, 1943). In the 1950's interest turned to human material, and continuous cell lines were grown from human tumour (HeLa: Gey, 1952). The routine culture of human fibroblast through many generations as unchanged diploid cells was demonstrated by Hayflick and Moorhead (1961). In the early 1970's many mammalian cell types still resisted cultivation and included amongst these were epidermal keratinocytes. Studies on epidermal growth were therefore conducted in organ culture (Fell, 1964). However in the 1970's and '80's methods for culturing a variety of cell types became available. Keratinocyte culture began in 1967 when Briggaman et al (1967) noted that keratinocytes could be isolated from split thickness sections of human skin and that these cells undergo limited multiplication in vitro. Others studies followed (Karasek and Charlton, 1971; Fusenig, 1971; Fusenig and Worst, 1974; Yuspa et al.,

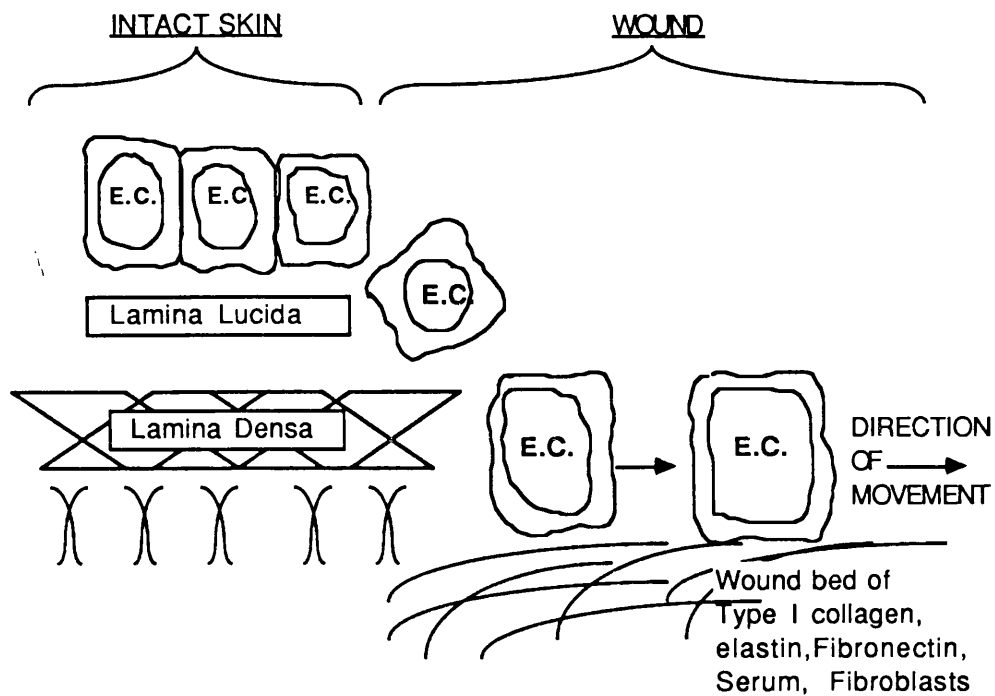


Figure 2.1 Schematic representation of wound-healing state showing epidermal cell (EC) response to damage to the basement membrane zone. In contrast to the usual vertical migration during differentiation, basal keratinocytes begin to move horizontally over the wound-bed matrix composed of collagen, fibronectin, serum, elastin and fibroblasts.

1970) but until the mid 1970's techniques for growing disaggregated epidermal cells could only demonstrate that the cells grew in monolayers to a limited extent and could not be satisfactorily subcultured. A great advance came in 1975 when Rheinwald and Green (1975) showed that successful growth of epidermal cells in serial culture is dependent upon the presence of fibroblasts. Provision of a fibroblast population could be made by using lethally irradiated 3T3 mouse fibroblasts which both promoted epithelial growth and inhibited growth of contaminating dermal fibroblasts. Colonies form from single cells which ultimately stratify. The keratinocytes had a finite culture lifespan of 20-50 cell generations, and 4-6 passages. Since this description a number of modifications to the culture method have been made. Rheinwald and Green (1975) suggested that cultured keratinocytes may have a variety of uses, namely for the study of: 1) growth and differentiation of the keratinocyte 2) the effect of viruses, including oncogenic viruses 3) the behaviour of epidermal cells involved in human disease 4) the testing of drugs affecting the human epidermal cell 5) the use of cultured keratinocytes for skin grafts. Many of these predicted uses have been fulfilled.

2.3 KERATINOCYTE CULTURE

2.3.1 REVIEW OF CULTURE METHODS

Rheinwald and Green (1975) showed that human epidermal keratinocytes could be cultured on feeder layers of irradiated 3T3 cells and could mature to form differentiated squames (Green, 1977). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) which was supplemented by 20% fetal calf serum and hydrocortisone, 0.4 µg/ml. Modifications to this original method will be outlined in the following section.

Isolation of keratinocytes

Keratinocytes are usually obtained from neonatal foreskins or from skin removed at surgery (cosmetic corrections). If sufficient quantities of skin are available full thickness skin may be trypsinised for 16 hours at 4°C, or for 2 hours at 37°C; keratotomed or partial thickness skin may be treated with trypsin for 1 hour at 37°C.

Substrate

The proliferative capacity and growth requirements of keratinocytes can be greatly affected by the type of substrate on which they are plated and maintained. Polystyrene flasks are generally used for cell culture. Polystyrene, as manufactured, is hydrophobic, and does not provide a suitable surface for cell growth; tissue culture plastics are therefore treated by irradiation, chemically, or with an electric arc to produce a charged surface which is then wettable. Fibroblasts and melanocytes will attach to such a charged plastic or glass surface; keratinocytes will attach to a plastic substrate in the presence of feeder cells but without these cells keratinocyte attachment is poor and cells must be plated at high density to obtain attachment and growth. Attachment can be facilitated by collagen coating dishes, type IV collagen being more effective than type I (Kleinman et al, 1978).

Feeder layer

Attachment and growth of primary keratinocytes is greatly enhanced by the use of irradiated or mitomycin C treated 3T3 cells. The factors synthesized by these cells are unknown. Media conditioned by the 3T3 cells are unable to substitute for cells. Passaged cells, if seeded at high density, will attach and grow readily in the absence of a feeder layer. The presence of the 3T3 cells within the keratinocyte cultures may interfere with certain experimental procedures. However, the feeder cells can be removed by vigorous washing with ethylenediaminetetraacetate (EDTA), leaving

the more firmly attached keratinocytes. In my hands this procedure did not work and it was necessary to wash the culture with trypsin 0.05 % and to observe the detachment of feeder cells microscopically; inevitably this results in some detachment of keratinocytes also. When the keratinocyte culture is about 10 days old the 3T3 cells begin to shed, so that cultures which are just subconfluent will contain few feeder cells. Alterations in the constituents of the medium enabled Peehl and Ham (1980) and Tsao et al (1982) to culture keratinocytes selectively without a feeder layer.

Physical properties

pH - Reduction of the pH of growth media to pH 5.8 has been reported to facilitate growth of keratinocytes (Eisinger et al, 1979), and promotes optimal growth in the absence of a feeder layer. However, in most studies on keratinocyte growth, media with a pH 7.2-7.4 have been found to produce adequate growth.

Temperature - 37°C supports adequate growth, but 31-34°C is optimal for rodent keratinocytes.

Growth media and supplements

Media and supplements - Rheinwald and Green (1975) originally cultured keratinocytes in media supplemented with hydrocortisone and 20% fetal calf serum. Growth was found to be enhanced by the addition of epidermal growth factor 20 ng/ml, cholera toxin 10^{-10} M, transferrin 5 µg/ml, insulin 5 µg/ml, and triiodothyronine 2×10^{-11} M (Rheinwald and Green, 1977; Green, 1978; Rheinwald, 1980; Watt and Green, 1981). The following modifications greatly improved growth and extended lifespan (Wu et al, 1982): 1) a 3:1 mixture of DMEM and Ham's F12 was used as the defined medium component 2) fetal calf serum was reduced to 5% 3) adenine 1.8×10^{-4} M was added.

Cholera toxin exerts its growth enhancing effects by increasing intracellular cyclic AMP concentrations in cultured keratinocytes (Okada et al, 1982). This effect can only be demonstrated in the early phase of cell culture.

Modulation of calcium ion concentration - Calcium ion concentrations influence proliferation, differentiation and cell attachment (Patel et al, 1981). Amongst the factors known to influence keratinocyte differentiation, extra cellular calcium has been extensively studied. Increased levels of calcium have been shown to result in the expression of several markers of differentiation such as increased transglutaminase activity and subsequent formation of cornified envelopes (Hennings et al, 1980; Hennings et al, 1981; Boyce and Ham, 1983; Hennings and Holbrook, 1983). The effect of calcium levels upon proliferation is less clear-cut. At concentrations below 0.1 mM Ca^{++} , cells do not stratify and it has been suggested that low calcium concentrations promote growth rather than differentiation. Hennings et al (1980) and Fairley et al (1985) reported that mouse keratinocytes grew faster in low calcium medium (0.05-0.1 mM and 0.02 mM respectively) than in normal medium containing 1-1.5 mM calcium. Boyce and Ham (1983) using chemically defined serum-free media showed that the growth rate of keratinocytes in culture increases as extra cellular calcium levels increase between 0.03 mM and 0.3 mM; at an extracellular calcium concentration of 1.0 mM keratinocyte growth rate is reduced. A further study showed that maximally stimulatory concentrations vary from 0.05 mM to 1.8 mM (Praeger et al, 1987) with responses varying with samples from different donors.

Serum - Keratinocytes require complex media for growth in culture and this must be supplemented with fetal calf serum (FCS) rather than the cheaper calf or horse serum. Although keratinocytes were originally grown in 20% FCS (Rheinwald and Green, 1975), changes in both the defined media used and the supplementation, have reduced the requirement to 5% (Wu et al, 1982).

Serum free media - There are a number of disadvantages to using serum and attempts have been made to replace essential components in serum with defined constituents to create a selective medium for a particular cell type. Such media are important to obtain consistent and defined conditions for the investigation of the factors governing growth and differentiation of cells. MCDB 153 (Boyce and Ham, 1983) is a medium which will support the growth of keratinocytes in the absence of serum and a 3T3 feeder layer, and additionally inhibits fibroblast growth. The medium is supplemented with epidermal growth factor, insulin, hydrocortisone, ethanolamine, and phosphoethanolamine. A small amount of bovine pituitary extract (BPE) is added for initiation of the primary cultures, for frozen storage and for serial culture. Optimal growth could be achieved with an extracellular calcium level of 0.3 mM. Growth is much slower in serum-free media and the life-span of the cells may be reduced. Although this medium has the advantage of being serum-free it still contains a biological constituent, BPE, whose activity may vary from batch to batch and which is capable of transmitting viral infections. However desirable serum free conditions may be, culture is much simpler in the presence of serum. MCDB 153 is very expensive and is of limited supply. Hence for bulk culture of keratinocytes, as is necessary in a keratinocyte grafting programme, practical considerations necessitate using a culture method which employs serum and a 3T3 feeder layer.

Fibroblast contamination

Contamination of a keratinocyte culture by dermal fibroblasts can usually be inhibited by the presence of the 3T3 feeder layer provided that the keratinocytes exhibit good growth. Fibroblast growth may be discouraged by the use of selective media e.g. MCDB 153. Once fibroblast growth is established these cells may be removed by selective trypsinisation (Milo et al, 1980) or by selective attachment (Freeman et al, 1976).

Characteristics of keratinocytes grown on a plastic substrate

Suspensions of keratinocytes when inoculated with 3T3 feeder cells onto a plastic substrate will attach, spread and proliferate and colonies will be visible in 24-48 hours. The cells become confluent in about 10 days but stratification takes place before confluence is reached. Differentiation is less complete than in vivo; stratification is poor with only four to six cell layers formed. The cells keratinize with minimum participation of keratohyalin; cells in the outer layer have a cornified cell envelope and retain their nucleus. Greater differentiation can be obtained by creating culture conditions which reflect the in vivo situation e.g. culturing on a collagen substrate; in such conditions a stratum corneum may develop (Baden and Kubilus, 1982; Mackenzie and Fusinig, 1983). Epidermal differentiation in cell culture has been likened to that of fetal epidermis as it first begins to keratinize (Holbrook, 1979).

2.3.2 ESTABLISHING A CULTURE OF BASAL CELLS FOR USE IN AN ANTISEPTIC CYTOTOXICITY ASSAY

Basic keratinocyte culture is well established in the Department of Experimental Dermatology, at The Royal London Hospital. The method used is that described by Rheinwald and Green (1975), adapted to include changes in media, serum and supplements which were described in a series of subsequent publications (Rheinwald and Green, 1977; Green, 1978; Rheinwald, 1980; Watt and Green, 1981; Wu et al, 1982). The culture conditions ultimately described by Wu et al (1982) are followed with the exception of the supplementation of the media by insulin; for the routine culture of keratinocytes insulin has not been noted to improve growth, although no controlled studies on keratinocyte growth rate, with and without insulin have been performed.

- There are two possible ways of establishing a culture of basal cells. The first is to create a primary culture in MCDB 153 (discussed in Section 2.3.1, serum free medium, p 56), a serum free medium with a low calcium level (0.3mM). In this

medium keratinocytes will grow but will not differentiate and stratify. The principal objections to this are the cost of MCDB 153 which is very expensive and its availability. When this project was started this medium was not commercially available in the UK. An alternative is to culture the cells in the standard way to produce a stratified culture, and then to switch the culture to low calcium medium to induce destratification of suprabasal cells (F. Watt, personal communication). It was decided to opt for this method.

A further problem that was addressed was the removal of the 3T3 fibroblasts from the cultures so that a pure population of basal cells remained. Techniques have been described for removing fibroblasts and these were explored. Other possibilities are the use of keratinocyte cultures at confluence, when all 3T3 cells have been shed or to passage primary cultures, since passaged keratinocyte cultures will grow in the absence of 3T3 cells.

Primary keratinocyte culture - Materials and methods

Chemicals, media and solutions - All chemicals were obtained from the Sigma Chemical Company, (Poole, UK) unless otherwise stated. The constituents of the solutions are shown in the **Reagent Appendix**.

Phosphate buffered saline - a (PBS-a)

Phosphate buffered saline-abc (PBS-abc) .

EDTA (0.02% w/v) - for removal of 3T3 cells

Trypsin (0.25% w/v) - for separation of epidermal cells

Trypsin (0.05% w/v) in EDTA (0.02% w/v)- for removal of 3T3 cells

Keratinocyte culture medium (KCM) used was that described by Wu et al (1982) with the omission of insulin. KCM contains a 3:1 mixture of DMEM and Ham's F12 medium. Fetal calf serum 5%, (FCS) (Flow, High Wycombe, UK) is added to the media which is supplemented with: epidermal growth factor 20 ng/ml (Flow Laboratories, High Wycombe, UK), cholera toxin 10^{-10} M (ICN Flow, High Wycombe, UK),

transferrin 5 µg/ml (Gibco Ltd, Paisley, UK), and triiodothyronine 2×10^{-11} M (Koch light Laboratories), adenine 1.8×10^{-4} M (Flow Laboratories, High Wycombe, UK).

Low calcium keratinocyte culture medium (KCM-Ca) was formulated using a method suggested by F. Watt (F. Watt, Imperial Cancer Research Fund, London, UK; personal communication). The medium was prepared by omitting calcium salts from the DMEM and Ham's F 12 formulations, adding 5% FCS which was depleted of calcium (see below), hormonal and biochemical additives as for KCM, and finally, 1% KCM was added to increase the final calcium level as required.

The FCS was depleted of divalent ions using the method described by Brennan et al (1975). Chelex 100 resin 20 g (Biorad, Hemel Hempstead, UK) was mixed with 500 ml of distilled, deionised water and the pH adjusted to 7.5 by the addition of 6M HCl. The slurry was filtered and the washed resin transferred to second flask containing 50 ml of serum. After continuous mixing for 1 hour, the serum was removed from the resin and sterilized by passage through 0.2 µm pore diameter filter (Nalgene). Two hundred millilitres of serum was treated and pooled. The treated serum was then frozen at -20°C in 25 ml aliquots

The calcium content of the medium was measured by atomic absorption photometry (Dr Houghton, Department of Chemical Pathology, The Royal London Hospital, E1.). The calcium content of the standard growth medium (KCM) was 1.62 mM. The calcium concentration of the basal medium minus calcium containing 5% calcium depleted FCS plus supplements and 1% KCM was 0.05 mM. The addition of 2% KCM rather than 1% KCM to the previous medium raised the calcium content to 0.07 mM. At this concentration keratinocytes are capable of growth but do not stratify.

Cell culture - Keratinocytes were isolated from foreskins (children under 7 years) and adult breast skin removed removed during cosmetic surgery. Preparation of the foreskins involved removal of the subcutaneous tissue with a fine surgical sissors. The skin was cut into strips and put into trypsin 0.25% for 2

hours at 37°C to separate the epidermis. Partial thickness skin was removed from the breast tissue using a keratome. Pieces of the separated skin were then placed in trypsin 0.25% for 2 hours at 37°C. The pieces of skin were removed from the trypsin and medium (DMEM plus 10% FCS) added to inactivate the trypsin. Separated epidermal cells were then scraped from the surface of the dermis with a scalpel. The suspension of epidermal cells and dermis was then filtered through a gauze to remove dermal fragments. The remaining suspension of epidermal cells was then centrifuged at 1500 rpm for 5 minutes. The pellet of cells was washed in KCM and recentrifuged as before. The cells were then resuspended in a known volume of medium and counted in haemocytometer. 2×10^7 epithelial cells in 20 ml KCM were inoculated into a 75 cm² flask (Falcon) which has been seeded on the previous day with 3T3 cells. The keratinocyte cultures were fed twice weekly with KCM.

Mitomycin C treated feeder cells were prepared by suspending 5×10^6 Swiss 3T3 cells (ATCC CCL-92) in 20 ml DMEM containing 10 µg/ml mitomycin C for 2 hours. The mitomycin C solution was prepared in 200 ml aliquots and stored at 4°C; it will remain stable provided that it is protected from light. Following exposure the cells were spun down at 1500 rpm, for 5 minutes, washed in DMEM plus 10% FCS and recentrifuged. The cells were resuspended in a known volume of KCM and counted in a haemocytometer. 2×10^6 feeder cells were inoculated into a 75 cm² flask and then incubated at 37°C, 8% CO₂ overnight. Division of the feeder layer can also be inhibited by irradiating a suspension of 3T3 cells with 6000 rad of γ radiation from a ¹³⁷Ce source.

When the cultures were 75% confluent the 3T3 cells were removed. The first method employed was exposure to EDTA 0.02%; prior to exposure the cells were washed twice with EDTA and then exposed at room temperature for 5 minutes. This failed to remove the 3T3 cells. The second method used was exposure to trypsin 0.05% w/v in EDTA; the cells were washed twice in this solution and then exposed for 5 minutes at 37°C.

Attempts were made to destratify subconfluent (75% confluent) and confluent cultures. The medium from flasks containing subconfluent and confluent cultures was removed and the cells were washed twice with PBS-a. The cells were then cultured in low calcium KCM (KCM-Ca) to induce destratification and examined daily by phase microscopy to establish when all suprabasal cells had detached.

Results

A primary stratifying culture of keratinocytes (**Figure 2.2 a and b**) could be established from foreskin and breast skin, although it was found that breast tissue from patients over 40 years of age failed to grow. Fibroblast contamination occasionally occurred with cultures from foreskins. The methods discussed in Section 2.3.1, Fibroblast Contamination were used to attempt remove them but were unsuccessful; it is well known that fibroblast contamination is extremely difficult to eradicate from a cell culture. Keratinocyte cultures became confluent in 2 to 3 weeks. Remaining 3T3 cells could be readily removed by incubating the cultures which were 75% confluent with trypsin 0.05% in EDTA, although some detachment of keratinocytes at the periphery of the colonies was observed.

Destratification occurred after 3 days in the subconfluent cultures; at 7 days the confluent cultures had still not completely destratified.

Comment

Primary cultures were readily established using standard culture techniques. It was proposed to set up an attached cell assay using basal keratinocytes to study the toxicity of antiseptics on keratinocytes. Primary keratinocyte cultures, for use in the antiseptic cytotoxicity assay, were judged unsuitable for the following reasons:

- 1) To establish a cytotoxicity assay using basal keratinocytes it is essential to establish good replication between wells in multiwell plates or in culture dishes; as destratification can only be induced when cultures are subconfluent it is essential to remove 3T3 cells using the trypsin method (see Section 2.3.1 - Feeder layer)

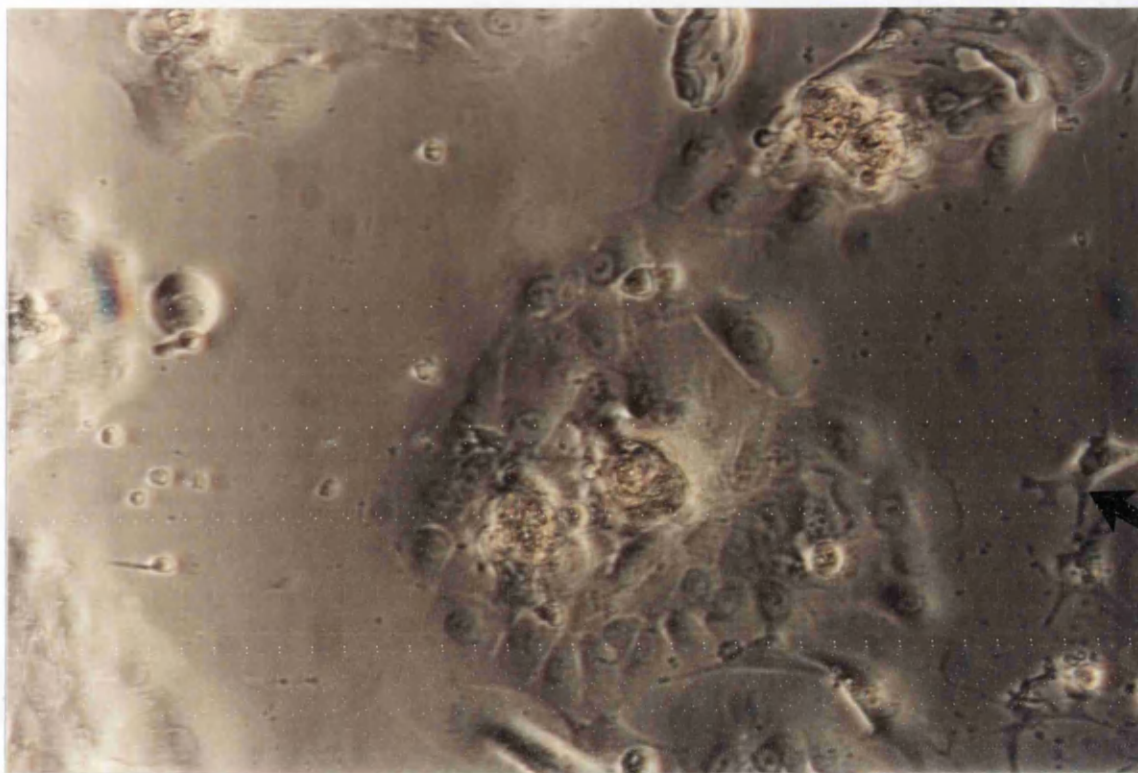
**a****b**

Figure 2.2a and b

- a) Early human keratinocyte culture (x1000) showing keratinocyte colonies and 3T3 cells (arrow).
- b) Confluent stratifying human keratinocyte culture. The cells are tightly opposed to each other and 3T3 cells have been shed (x250).

which results in some detachment of keratinocytes. Comparison of equal cell numbers could therefore not be guaranteed.

2) Growth of primary cultures is not always successful with 20% of cultures failing to grow, this would result in wastage of culture plates/ dishes which are expensive.

Subculture to establish a population of basal cells - Methods

Cell culture - Primary keratinocyte cultures (derived from foreskin and breast skin) were subcultured at about 75% confluence, at about 10 days. By this time many of the 3T3 cells had detached. Any remaining cells were removed with trypsin 0.05% w/v in EDTA as detailed above. If confluent cultures were used subsequent growth was impaired as sub-cultured cells derived from these cultures tend to differentiate rather than grow. Attempts to subculture the primary keratinocytes directly into low calcium medium to produce a basal cell population were unsuccessful, as the cells failed to attach and spread.

The medium was removed from the flask and the cells were washed twice with trypsin 0.25% and 3ml of trypsin 0.25% was added to the cells which were then incubated at 37°C for 10 minutes. KCM was then added to cell suspension to inactivate the trypsin and the cells were spun down and resuspended in 40 ml of KCM. 1 ml of the cell suspension was counted using a Coulter counter. The cell suspension was inoculated into 3, 24-well plates (which represented an approximate 1 to 3 split), 0.5 ml per well, without feeder cells. Plating in a low volume of medium allowed the cells to spread and attach evenly across the well. The multiwell plates were initially placed in a humidified incubator in 8% CO₂ at 37°C. The following day 1 ml of KCM was added to each well and medium was changed after 3 days. Using these culture conditions microscopic observation showed that the wells at the periphery of the dish grew less well than those at the centre. This was attributed to evaporation of water from the medium in the wells at the edge of the plate, leading to an increase in osmolarity. To correct this the multiwell plates were placed in plastic sandwich boxes and the medium was changed on alternate

days. The passaged cells were cultured in KCM for 5 days, washed twice with PBS-a, and then switched to low calcium medium (KCM-Ca) for three days to induce destratification. Prior to use in experiments, the cells were washed with PBS-abc to remove the detached or loosely attached suprabasal cells. KCM then replaced the low calcium medium and the cells were left in this medium for 30 minutes prior to use. The basal cell attachment to the plastic substrate is dependent on the extracellular calcium concentration; if the basal cells are exposed to calcium free media or calcium free balanced salt solutions, even for the briefest period, the basal cells detach. Once washed, the basal cell cultures, which were 80-90% confluent, were ready for experimental use. The wells from one 24-well plate were counted in a Coulter counter to estimate the number of basal cells per well. The wells were washed twice with PBS-abc and 1 ml of trypsin 0.05% in EDTA 0.02% was added. The cells were left at room temperature for 5 minutes to allow the cell to detach. 5 μ l FCS was added to each well to inactivate the trypsin; the suspension from each well was passed through a 16 G needle to produce a single cell suspension which was added to 9 ml of Isoton (Coulter Electronics, Luton, UK) and counted in a Coulter counter.

Results

Phase-contrast microscopy showed the basal cultures to be a monolayer with individual cells widely separated (**Figure 2.3**). This appearance is produced because desmosomal connections are lost at extra cellular calcium concentrations below 0.1 mM.

Cell counts showed that the approximate seeding density of keratinocytes per well was 1×10^5 ; this includes a mixture of basal and suprabasal cells which have limited growth potential. Transferring cultures from screw topped flasks to culture plates obviously leads to problems with evaporation and poor cell growth at the edge of the dish, where this is greatest. This can be corrected by putting the plates in plastic boxes and by frequent medium changes. Basal cell counts on replicate wells



Figure 2.3

Culture of basal cells produced by switching stratifying culture to low calcium keratinocyte culture medium. A monolayer with loss of cell contact is seen as desmosomal connections are lost in low calcium medium (x250).

using the culture technique devised gave the following findings: mean cell count per well = $98,900 \pm 60$. The distribution of cell counts per well over the multiwell plate showed no particular pattern of variation at the edge of the plate.

Comment

The culture method described represents an easy method of obtaining a population of basal cells. Use of a 24-well plate allows the operator to work with a small number of cells, which is important since sources of keratinocytes are limited and are required for many grafting and experimental projects. Using the culture modifications described it can be shown that replication for cell numbers across the wells of 24 well plate is good and therefore satisfactory for a cytotoxicity assay. The problems encountered with keratinocyte culture in a multiwell plate were not encountered with the other cell types since a long period of culture, prior to experimental use, was not required .

2.4 ESTABLISHING A CULTURE OF HUMAN SKIN FIBROBLASTS FOR USE IN AN ANTISEPTIC CYTOTOXICITY ASSAY

Culture of dermal fibroblasts is a well established technique. Culture can readily be established using a tissue explant from a small skin biopsy. Human skin fibroblasts have a limited replicative life span (Hayflick, 1965; Martin et al, 1970), senescence arising between 30 and 60 population doublings with cultures lasting 10 to 20 weeks. Human fibroblasts remain predominantly euploid throughout their lifespan and never give rise to continuous cell lines (Hayflick and Moorhead, 1961).

Materials and methods

Media - DMEM plus 10% FCS was used for the culture of fibroblasts.

Initiating explant culture - Dermal remnants, derived from foreskins from which the epithelium had been removed for keratinocyte culture, were cut into 1

mm² pieces. The bottom of a 25 cm² culture flask was scored with a glass Pasteur pipette to aid attachment of the dermal fragments. The tissue was placed in the flask and left dry for 15 minutes to help fix the tissue to the flask. To prevent the tissue floating off the bottom of the flask a small volume of DMEM plus 10% FCS (2 ml) was then added. The culture was left for 48 hours to allow attachment; at this stage the medium was changed and increased in volume to 5 ml. Following this the medium was changed twice weekly.

Subculture - At three weeks the cells were about 50% confluent. These were washed twice with trypsin 0.05% w/v in EDTA and then detached by adding 2 ml of the trypsin/EDTA. at 37°C for 10 minutes. 3 ml of DMEM plus 10% FCS was then added to the flask and the cell suspension placed in a universal container and centrifuged at 1500 rpm for 5 minutes. The cell pellet was then resuspended in 5 ml of DMEM plus 10% FCS and placed in a 25 cm² flask. The medium was changed twice weekly and the cells became confluent in one week and were then subcultured into a 75 cm² flask. Thereafter the fibroblasts were subcultured when they reached confluence and this occurred at about 10 day intervals.

For the purpose of the cytotoxicity assay cells of passages 3 to 7 were used. Cultures from several tissue samples were used. The cells were subcultured into 24-well plates in DMEM plus 10% FCS, 1×10^5 per well.

Results

Examination by phase contrast microscopy showed the cells to have bipolar or multipolar shapes (**Figure 2.4**), typical of fibroblast appearance which contrasts with epithelial cells which form confluent sheets of polygonal cells with a clearly defined edge. By seeding the cells at high density a confluent culture could be obtained in 24 hours.

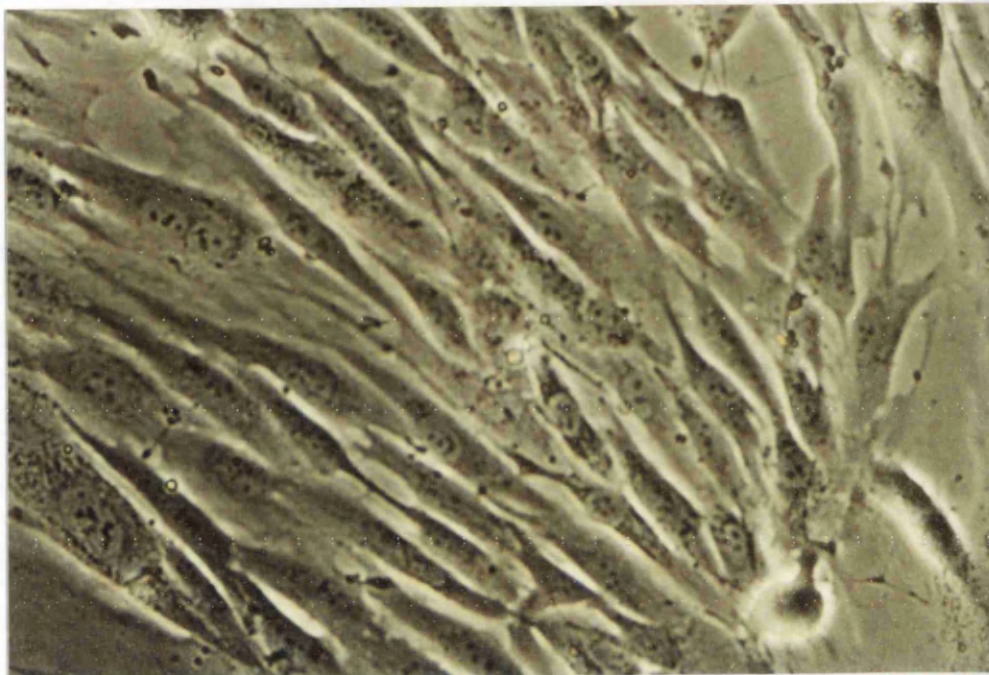


Figure 2.4

Normal human fibroblasts in culture (x1000). These cells have bipolar or multipolar shapes, which contrast with the epithelial cell types which form confluent sheets of polygonal cells with a clearly defined edge.

Comment

Cultures of dermal fibroblasts were readily established from foreskin dermal tissue and could be passaged easily to produce large numbers of cells for experimentation. Seeding at high density gave rise to confluent culture in 24 hours, unlike the basal keratinocytes where a near confluent culture took 8 days to obtain. Problems with evaporation of medium were, therefore not encountered.

2.5 TRANSFORMED HUMAN KERATINOCYTES (SVK 14 CELLS)

Keratinocyte culture is complex and expensive. One of the aims of this study is to establish whether a transformed keratinocyte line (SVK 14 cells, kindly given by Dr J. Taylor-Papadimitriou, Imperial Cancer Research Fund, London, UK.) could substitute for normal human keratinocytes in the antiseptic cytotoxicity assay. Here the known characteristics of the SVK 14 cells are compared to normal human cultured keratinocytes. Finally the culture technique is described.

2.5.1 CELL TRANSFORMATION

The term transformation was originally coined by microbiologists for the induced uptake of new genetic material. In mammalian cells the term transfection is used for this process. Tissue culture transformation implies a phenotypic modification not necessarily involving the uptake of new genetic material. The primary alteration is usually considered to be genetic and irreversible. The most important methods for transforming cell in vitro are exposure of the cells to: 1) physical stimuli (ultraviolet and γ irradiation) 2) chemical carcinogens 3) viruses - human epithelial cell transformation can be performed using Simian Virus 40.

The characteristics that are considered to be indicative of cell transformation are outlined in **Table 2.1**. These markers were originally developed to help define the transformed rodent fibroblast phenotype, but it has become evident that many of these characteristics are not suitable markers of the transformed human

TABLE 2.1 Comparison of general characteristics of transformed cells to those of SVK 14 cells.

Characteristics of the transformed phenotype	Characteristics of SVK 14 cells
Extended or indefinite lifespan	+
Altered cell morphology	+
A reduced serum requirement	+
Enhanced growth rate	+
Growth factor independent	+
Reduced density limitation of growth	+
Alteration of karyotype from diploid to aneuploid	+
Anchorage independent growth	+
Alteration of cell surface antigens	Not known
Enhanced protease secretion	Not known
Impairment or loss of terminal differentiation	+
Tumorigenicity in susceptible hosts	No

phenotype. Immortality is the commonly found phenotype in rodent cells transformed in vitro, however transformed human cell lines usually exhibit an extended lifespan. Alterations in cell surface moieties e.g. glycoproteins, may contribute to a decrease in cell-cell adhesion and cell-substrate adhesion. This leads to a loss of density limitation of growth and allows the cells to grow detached from a substrate. The cells are described as anchorage independent, and growth can be demonstrated in semisolid media such as agar. Cultures of transformed cells exhibit reduced density limitation of growth by growing to a higher saturation density than their normal counterparts. Aberrations in individual chromosomes and changes in ploidy may be found in transformed cells and flow cytometry reveals a concomitant change in DNA content. Proliferation of transformed cells is less dependent on both serum and growth factors than their normal counterparts. This may be because these cells secrete their own growth factors. Some transformed cells may release a factor which induces blood vessel proliferation. Other cell products which may alter include proteolytic enzymes which have been implicated in spread of tumour cells. The ability of cells to induce tumours in nude mice is often used as a criterion for malignant transformation of rodent and other mammalian cells. However, it is not clear whether this criterion is applicable to human cells since many human tumours or cell lines are not tumorigenic in nude mice.

Since transformed cells may exhibit properties of malignant cells in vivo, their principal use has been in examining the nuclear mechanisms of malignant transformation and defining parameters related to growth and differentiation which may be altered by transformation, and which may be relevant to malignant changes in vivo. Studies on transformed human epithelial cells, from which the majority of solid tumours in man derive, have been limited because of the problems of growing normal cells in culture. However, since the problems of culturing various cell types are being overcome, an increasing range of transformed epithelial lines has become available. Other important uses for transformed

epithelial cell lines are the study of epithelial physiology. The availability of cell lines which retain functions characteristic of and specific to the epithelial cell type from which they are derived should simplify investigation of the mechanisms underlying these functions.

2.5.2 VIRALLY TRANSFORMED HUMAN KERATINOCYTES

In 1979, Steinberg and Defendi reported that the growth pattern and differentiation of keratinocytes could be altered by infecting them with SV40 virus. The transformation of mammalian cells by SV40 virus is known to require the expression of only the early viral genome, which encodes for two proteins, large T-antigen and small t antigen (Rigby and Lane, 1983). Infection of trypsinized keratinocytes with SV40 by Steinberg and Defendi (1979) produced a culture which contained T antigen positive cells. As these increased in number the culture lost its dependence on high serum concentrations and became independent of a fibroblast feeder layer. The cells grew as a monolayer but retained their capacity to differentiate. After 10-15 passages the cells entered a 'crisis', after which some cells survived to become established cell lines. These cells no longer exhibited capacity to differentiate under exposure to 12-o-tetradecanoylphorbol acetate.

An alternative to SV40 infection as a method for establishing strains of SV40-transformed human cells is calcium phosphate mediated DNA transfection. Banks-Schlegel and Howley (1983) transformed foreskin keratinocytes by transfecting them with subgenomic segments containing the transforming early region of SV40. The transfected cultures became immortalised without a crisis stage. A line they derived, known as HE-SV, stratifies and differentiates to some degree, and also retains feeder and anchorage dependence.

2.5.3 THE SVK 14 CELL LINE

Original characterisation

The SVK 14 cell line was established from foreskin keratinocytes by Taylor-Papadimitriou et al (1982). The keratinocytes were cultured on a 3T3 feeder layer in RPMI 1640 medium supplemented with 10% FCS, 50 ng/ml cholera toxin, and 5 ug/ml hydrocortisone. The cells were infected with wild type SV40 virus and then cultured in the above medium on a feeder layer. Single cells were picked and cloned in the presence of feeders. When the clones became established feeders were no longer required. One of the clones was labelled the SVK14 cell line. At no stage did the cells enter a crisis, which is characterized by a much decreased growth rate from which cells may emerge as a permanent cell line. After six passages the cells grew independently of feeders, and with passage they became less dependent on exogenous growth factors; at passage 45 growth rates were comparable in RPMI 1640 which contained either 1% serum, 10% serum or 10% serum with cholera toxin and hydrocortisone.

The characteristics of the SVK14 cell line are shown in **Table 2.1**. The epithelial nature of the SVK 14 cell line was demonstrated by positive staining with an antiserum to human keratins which had already been characterised (Sun et al, 1979). The presence of the large T antigen in the nuclei of the transformed cells was demonstrated using the monoclonal antibody (MAb) DL3C5 (Clark et al, 1981). The cytokeratin expression in the transformed cells differed from the normal keratinocytes; the SVK14 cells showed positive reactivity with the MAb LE 61. The tissue reactivity and biochemical characteristics of this antibody were reported by Lane (1982). When this antibody was reported its exact antigenic determinant was not known, however this antibody is now known to react with keratin 18. Original characterization of LE 61 showed that it reacted with simple, non-keratinizing epithelia and is not detectable in normal keratinocytes. Since this antibody also shows positive reactivity in cutaneous malignancy (Markey et al, in press), it has recently been suggested that expression of this antigen reflects a major alteration

in keratin synthesis after transformation of keratinocytes to a malignant phenotype.

Differentiation of the SVK 14 cells was impaired; at late passages the cells failed to stratify and the level of spontaneous envelope formation was very low. Treatment with a calcium ionophore induced envelope formation in a small percentage of cells (17%) at passage 6 and this fell with passage. The cells showed anchorage independence by forming colonies in agar; this ability increased with passage.

The SVK 14 cell line does not form invasive tumours in nude mice, but will form transient cysts (Chang, 1986)

2.5.4 COMPARISON OF SVK 14 CELLS TO NORMAL CULTURED KERATINOCYTES

Culture characteristics

SVK 14 cell, unlike normal keratinocytes, can be cultured in the absence of feeder cells and added mitogens. They have an indefinite lifespan and an enhanced growth rate (**Table 2.1**).

Morphology

The cells show an epithelial cell morphology, with regular, polygonal cells with a clearly defined edge. Occasional large cells are seen.

Chromosome and DNA content

Chromosome studies have shown that the SVK 14 cells are tetraploid. This was confirmed by measuring the DNA content of these cells using flow cytometry (Dr. J. Newton, M.D. Thesis, 1987).

Cell metabolism

SVK 14 cells share with normal epidermal cells the metabolic peculiarity that glutamine constitutes one of the major respiratory energy sources (Reichert et al, 1983), whereas glucose is mainly oxidised by the pentose shunt (Freinkel, 1960). Reichert (1986) used the SVK 14 cell line to study the toxicity of dithranol on cell respiration by measuring $^{14}\text{CO}_2$ release from ^{14}C -glutamine and ^{14}C -glucose.

Ponec et al (1984) studied lipid metabolism in SVK 14 cells and normal keratinocytes. Normal keratinocytes do not obtain cholesterol by low density lipoprotein metabolism, but instead synthesize cholesterol de novo. SVK14 cells also have a high rate of de novo synthesis, which was found not to be controlled by serum lipoproteins. These cells can therefore serve as models in studies on the mechanism underlying the regulation of cholesterol synthesis in human keratinocytes.

Immune function

As with normal human keratinocytes in culture, SVK 14 cells will express class II antigen when treated with γ interferon and can be used to study this cell function in vitro (Kerr et al., 1990).

Differentiation

SVK 14 cells showed impaired ability to differentiate; unlike normal keratinocytes in culture they do not stratify. Ponec et al (1984) were able to show that SVK 14 cells showed an impaired ability to differentiate spontaneously into cornified cells (1-3%) compared to 25% of cells in confluent cultures of normal keratinocytes. Involucrin is the protein precursor of the cross linked envelope and is a marker of commitment to terminal differentiation; SVK 14 cells are unable to accumulate involucrin, but this protein is expressed suprabasally in cultured keratinocytes.

Basement membrane proteins

Laminin and bullous pemphigoid (BP) antigen are both synthesized by keratinocytes in culture (Prunieras et al, 1983; Wu et al, 1982). Fibronectin is synthesized in small amounts by cultured keratinocytes (Clark, 1983). SVK 14 cells showed positive perinuclear reactivity with antilaminin antisera, and strong cytoplasmic staining with an anti-fibronectin antibody, but staining for BP antigen was negative (Bernard et al, 1985).

Intermediate filament proteins

Keratins are the intermediate filament proteins of epithelia (**Table 2.2**). In human epithelia 19 different keratins have been defined (Moll et al, 1982) and usually 2 to 10 of these keratins are found in any particular epithelium, the subset varying according to the epithelial cell type and its stage of differentiation and development. Keratins are classified into two types, type I keratins are of lower molecular weight with an acidic isoelectric point and type II keratins are of higher molecular weight and neutral to basic (**Figure 2.5**) (Sun et al, 1984). Keratin filaments are constructed from a tonofilament which consists of a type I and a type II keratin heterodimer. The pattern of keratins expressed by a particular epithelium is specific to that type (Moll et al, 1982; Fuchs et al, 1981), but individual keratins are rarely organ specific. Keratins 1/2 and 10 are expressed mainly in cornified stratified epithelia; keratins 3 and 12 in cornea; keratins 4 and 13 in internal non-cornified stratified epithelia e.g. oesophagus; keratins 5 and 14 in all keratinocytes; keratins 6 and 16 in hyperproliferative keratinocytes, such as those found on the sole, in psoriasis and in wound healing. The smaller keratins, 7, 8, 18, 19 are found mainly in simple epithelia. The expression of keratins is related to three factors: 1) the epithelial type, simple or stratified 2) in stratified epithelia, the degree of differentiation 3) the proliferative state, normal or hyperproliferative.

The SVK 14 cells retain their epithelial character as shown by the presence of keratin filaments and numerous desmosomes (Bernard et al, 1985). SVK 14 cells express simple epithelial keratins i.e. keratins 8, 18, 19, which are normally found in fetal epidermis and simple epithelia and that none of the larger keratin pair, keratins 5 and 14, which are normally expressed by keratinocytes, were present in SVK 14 cells. Unpublished observations from this Department have shown that SVK 14 cells, like basal keratinocytes, do express keratin 14 (**Figure 2.6**), but this can only be demonstrated by immunocytochemical techniques, suggesting that this cytokeratin is present in only small amounts. This finding suggests that SVK 14

Table 2.2. Classes of intermediate filament and the cells they characterize

Type I	keratins	epithelia
Type II	keratins	epithelia
Type III	vimentin desmin glial fibrillary acidic protein	mesenchymal cells muscle astroglial cells
Type IV	neurofilaments	neurons

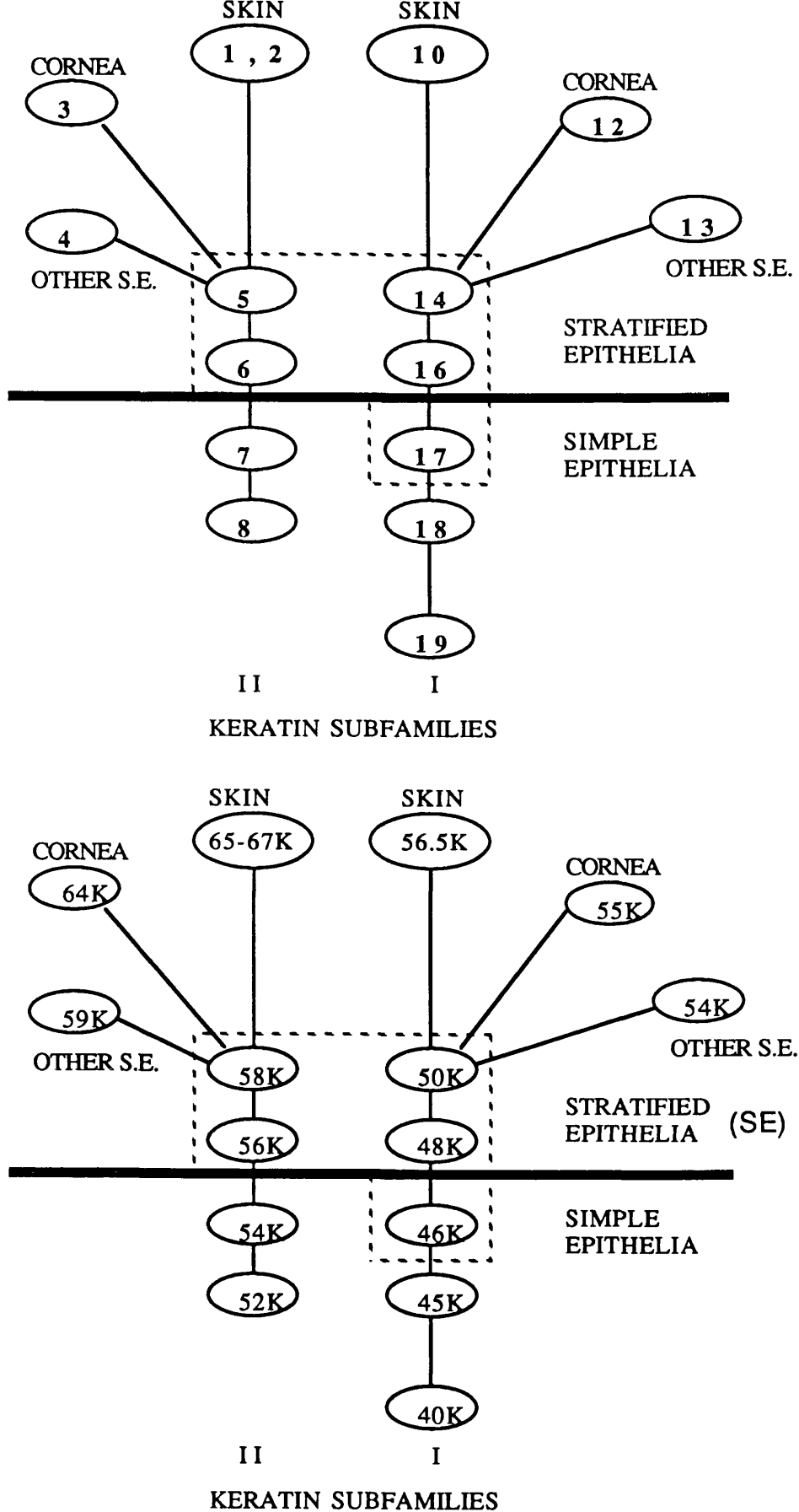


Figure 2.5

Classification of human keratins (Moll et al, 1982)

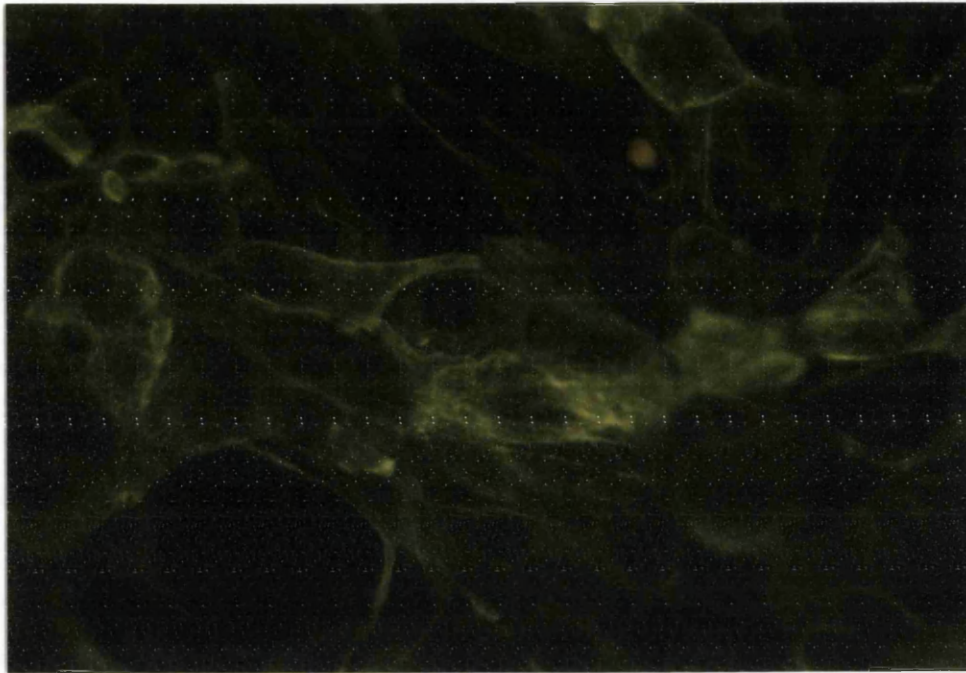


Figure 2.6

SVK 14 cells reacted with Mab LLO01 (x1000). This antibody reacts with keratins 5 and 14 which are expressed by normal human basal keratinocytes. Positive filamentous cytoplasmic staining by the transformed keratinocytes suggests that these cells retain keratin expression which is characteristic of the cells from which they derive.

cells retain some keratin expression characteristic of the keratinocyte from which they derive.

2.5.5 SVK 14 CELL CULTURE METHOD

As outlined earlier, once the original clones of the SVK 14 cell line were established they became independent of a 3T3 feeder layer and with passage became independent of exogenous growth factors. At high passage (passage 45 and above) the cells will grow in RPMI 1640 supplemented with 1% FCS. For routine culture it is recommended that the cells are grown in RPMI 1640 plus 10% FCS (J. Taylor-Papadimitriou, personal communication), although colleagues have grown these cells satisfactorily in DMEM plus 10% FCS.

Materials and methods

Media and solutions - RPMI 1640 (Flow, High Wycombe, UK) plus 20% FCS was used to initiate culture of the cells from the frozen state.

RPMI 1640 plus 10% FCS was used for the routine culture of SVK 14 cells

Trypsin 0.05% (w/v) in EDTA 0.02% (w/v) was used to detach the SVK 14 cells

Cell culture - The SVK 14 cells were cultured in a 75 cm² culture flask (Becton Dickinson UK Ltd, Oxford, UK). The medium was changed twice weekly and the cells were passaged when they were just subconfluent (at approximately weekly intervals).

For experimental purposes passage 7 to 12 cells were used. A 75 cm² flask of cells was trypsinized using trypsin 0.05% w/v as described above. The trypsin was inactivated using 5ml of RPMI 1640 plus 10% FCS and the cells centrifuged. The cell pellet was resuspended in 10 ml of RPMI 1640 plus 10% FCS. Like normal human keratinocytes the SVK 14 cells are prone to clumping and the cells were therefore passed through a 16 G needle prior to counting, so that a single cell suspension was produced. 1 ml of the needled cells suspension was then added to 9 ml of Isoton (Coulter Electronics, Luton, UK) and counted in a Coulter counter. The

concentration of the cell suspension was adjusted so that 1 ml contained 1×10^5 cells. Wells in a 24 -well plate were inoculated with 1×10^5 cells. The cells were cultured for 24 hours at 37°C in 8% CO_2 and then examined by phase contrast microscopy to judge whether this seeding density was too high.

Results

Examination of the SVK 14 cell culture by phase-contrast microscopy showed a monolayer with an epithelial cell morphology, with regular polygonal cells which had clearly defined edge (**Figure 2.7**); occasional large cells were seen.

Seeding the multiwell plate with 1×10^5 cells per well gave cultures which were just sub-confluent ie. 80 to 90% confluent.

Comment

The SVK 14 cell line is easy to culture which makes it particularly useful for assays which require multiple experiments, as were anticipated in this study. In the experiments proposed it was essential that equal cell numbers of each cell type were used. Using 1×10^5 SVK 14 cells produces a subconfluent culture in a 24-well plate, which could be satisfactorily compared to 1×10^5 fibroblasts and a sub-confluent culture of basal cells (approximately 1×10^5 cells).

SVK 14 cells retain many features of normal basal keratinocytes. They express keratin 14 which is characteristic of a basal keratinocyte phenotype. As they do not stratify, these cells may represent a valuable alternative to basal cells for in vitro experimentation.

Equally there are clear differences in keratin expression between normal keratinocytes and SVK 14 cells. Such differences may affect the susceptibility of each cell type to the antiseptic agents. The value of the SVK 14 cells in replacing normal basal keratinocytes for cytotoxicity testing was evaluated in this study.

Other transformed keratinocyte lines are available which are also non-stratifying. The HaCAT line was derived from a spontaneously transformed

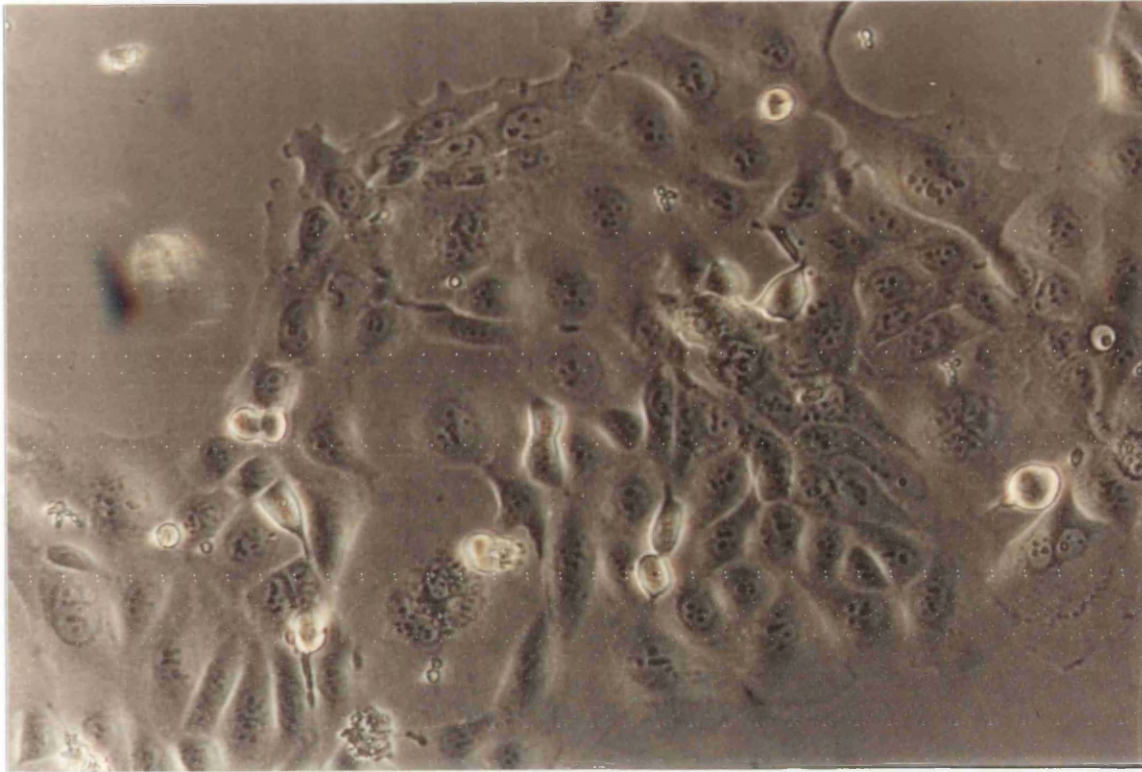


Figure 2.7

Monolayer of SVK 14 cells. These cells were originally isolated from SV40 infected foreskin keratinocytes. They show impaired ability to differentiate and stratify, but maintain functions characteristic and specific to their epithelial cell type (x1000).

keratinocyte culture (an exceedingly rare event) (Boukamp et al, 1986). Such lines may also prove valuable substitutes for basal cells but were not studied here.

2.6 SUMMARY

Basal cells and fibroblasts are considered essential to the cutaneous wound healing process. It was proposed to study these cells in an antiseptic cytotoxicity assay. Culture of fibroblasts was established using available techniques. Basal keratinocytes were cultured by destratifying passage 2 keratinocyte cultures using low calcium medium. The characteristics of a transformed keratinocyte line were described (SVK 14 cells). Unlike normal keratinocytes, these cells do not demand complex culture conditions and it was proposed to study this cell line as a possible replacement for normal keratinocytes in the cytotoxicity assay.

CHAPTER 3

METHODS - CHOICE OF AGENTS AND EXPERIMENTAL DESIGN

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3.1 INTRODUCTION

Although relatively little work has been done on studying the toxic effects of antiseptic agents on mammalian cells in vitro, a wealth of information about their antimicrobial activities in vitro is available. The shortcomings of these in vitro tests of antimicrobial activity has always been recognised, since experimental conditions may not reflect those encountered in the 'in use' situation. A wide range of methods have been developed to test the activity of antiseptics and disinfectants in vitro (see Section 1.4.1). Each author advocates specific microbial strains, experimental test conditions or methods according to the activity criteria for the test disinfectant and according to the specific use envisaged. Most countries have no official rules for evaluating disinfectants and, at present, no international standardized method is available.

In vitro testing of antiseptic toxicity in tissue culture places constraints upon methodology and the choice of agents used. Published studies of antiseptic toxicity on mammalian cells and tissue rarely address experimental design and hence the methods and results vary (**Tables 1.5, 1.6, 1.7**).

There are no available data on the fate of these agents when applied topically to wounds and application to target cells therefore tends to reflect the known properties of the agents and their use in clinical practice.

3.2 CHOICE OF AGENTS

Only those agents which are used on wounds will be used in this study; this excludes alcohols which because of their irritancy are only used on intact skin, hexachlorophene (a phenolic compound) whose use on eroded skin or mucous membranes is contraindicated because of systemic toxicity (see Section 1.6 - Complications of antiseptic therapy), certain triphenylmethane dyes eg. crystal violet, are now only recommended for application to intact skin because of possible mutagenicity (Reynolds, 1989 iii).

The agents to be considered and their characteristics are listed in **Table 3.1**.

Table 3.1 Properties of antiseptics considered for study in the antiseptic cytotoxicity assay.
GNB=gram negative bacteria; GPB=gram positive bacteria.

AGENT	MODE OF ACTION ON BACTERIA	ANTIBACTERIAL ACTIVITY	RAPIDITY OF ACTION	SUSTAINED ACTION	AFFECTED BY ORGANIC MATTER	
		GPB	GNB	Rapid, intermediate slow		
Chlorhexidine	Cell wall	V. good	Good	Intermediate	Excellent	Minimal
Povidone iodine	Oxidation substitution by free I ₂	V. good	Good	Intermediate	Fair	Yes
Sodium hypochlorite	? formation of toxic N-chloro compounds	V. good	V. good	Rapid	None	Yes
Hydrogen peroxide	Free radical attack	Good	Good	Rapid	None	Yes
Cetrimide	Cell wall	V. good.	Good - Pseudomonas resistance	Rapid	Fair	Yes

3.2.1 CHLORHEXIDINE

Chlorhexidine is a cationic bisbiguanide which derives its antimicrobial action by causing disruption of microbial cell membranes and precipitation of cell contents. This agent combines a high level of antibacterial activity, strong binding to the skin, and low systemic toxicity; these factors have led to its principal use as a disinfectant for skin, mucous membranes and wounds. Although it has a broad spectrum of activity, it is more active against gram positive than gram negative bacteria and is bactericidal at concentrations ranging down to about 100 µg/ml, below that it is bacteriostatic (Gardner and Gray, 1983). Chlorhexidine acts rapidly but also exhibits persistent action. It is a strong base virtually insoluble in water which reacts with acids to form salts; the hydrochloride has low solubility, but the acetate is soluble to 1.9% w/v. Chlorhexidine gluconate cannot be isolated as a solid and is produced as 20% w/v aqueous stock solution. At a concentration of 0.05% chlorhexidine is incompatible with borates, bicarbonates, carbonates, chlorides, citrates, phosphates, and sulphates forming salts of low solubility. At dilutions of 0.01% or more, these salts are soluble. In this study chlorhexidine gluconate will be used.

3.2.2 POVIDONE IODINE

Povidone iodine is an iodophor, a complex of iodine and a carrier molecule, polyvinylpyrrolidone. Iodophors have similar antimicrobial effects to iodine whilst being less irritant. The spectrum of antibacterial activity is very good against gram positive bacteria and good against gram negative bacteria. Their microbicidal effects are the result of cell wall penetration, oxidation and substitution of microbial contents with iodine. In pure aqueous iodine solutions hypiodic acid (HOI), and molecular I₂ have strong germicidal properties and the triiodide (I₃⁻) and hypiodite (OI⁻) ions have weak antibacterial properties. Povidone iodine is an iodophor, which is a loose complex of elemental iodine with a carrier (polyvinyl pyrrolidone) which serves to increase the solubility of the

iodine and also to provide a sustained release reservoir. In aqueous solution iodophors form the same antimicrobial chemicals as do the pure iodine solutions.

Iodine reacts with organic material although iodine binds less rapidly than chlorine with dissolved proteins (blood, pus); this comparatively low reactivity with proteins is however, sufficient to kill microorganisms, and gives iodine its excellent germicidal properties.

Caution must be exercised when using this agent in experiments because the concentration of free iodine available in a 10% solution of povidone iodine will rise ten-fold when this solution is diluted 1:100 (Gottardi, 1983). On further dilution the free iodine behaves as might be predicted in that it decreases, and below 0.01% w/v the povidone iodine solution can be regarded as simple aqueous solution of iodine. Recently it has been recognised that low dose iodophors (0.05%), rather than the standard concentrations of between 5 and 10% povidone iodine, have good antimicrobial activity (Berkelman et al, 1982) which is in keeping with recent observations on available free iodine from the lower concentrations. Surprisingly, when toxic effects from povidone iodine can be demonstrated, they disappear with dilution; hence 5% povidone iodine has been shown to be toxic to granulation tissue *in vivo* and a 1% solution was not (Brennan and Leaper, 1985).

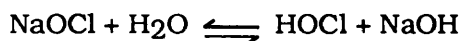
Povidone iodine has been used in *in vitro* testing (**Table 1.5**), where only the effect of ten-fold dilutions from the therapeutic concentrations have been quoted. For the purposes of this study it was not judged possible to use povidone iodine because serial dilution does not give rise to predictable concentrations of either povidone iodine or available iodine.

3.2.3 CHLORINE RELEASING COMPOUNDS

Hypochlorites are the oldest and most widely used of active chlorine compounds. Sodium hypochlorite 0.5% w/v is considered a useful antiseptic, particularly for dissolving and deodorising necrotic tissue and is a powerful antibacterial agent.

The concentration of available chlorine required to kill *Staphylococcus aureus* in 30 seconds is 0.00008% w/v (Dychdala, 1983).

The mode of action of both hypochlorites and chlorine is thought to be through the formation of hypochlorous acid. In solution hypochlorites are in the following equilibrium:



Hypochlorous acid (HOCl) in its undissociated form is highly microbicidal. Hypochlorite solutions are unstable and stability may be affected by temperature, concentration, pH, presence of catalysts, and light. Therefore the most stable chlorine-releasing solutions are those having the following characteristics: low chlorine concentration, absence of cobalt, nickel copper or other catalysts, high alkalinity, low temperature, absence of organic material and are stored in the dark. Similarly the factors which affect the biocidal activity are:

- 1) pH - an increase in pH substantially decreases the biocidal activity of chlorine, and a decrease in pH increases this activity
- 2) Concentration - Increase in concentration of available chlorine increases antibacterial activity.
- 3) Temperature - an increase in temperature produces an increase in bactericidal activity
- 4) Organic material in chlorine solutions consumes available chlorine and reduces its capacity for bactericidal activity. Sugars and starches do not affect the germicidal activity of chlorine but tyrosine, tryptophan, cystine, egg albumin, peptone, body fluids, tissues, and microbes will consume chlorine.

3.2.4 HYDROGEN PEROXIDE

Hydrogen peroxide was initially found to be a poor disinfectant because manufacture produced a dilute, highly unstable solution that decomposed rapidly in the presence of slight traces of impurities or on exposure to light or heat. Modern manufacturing procedure have led to the production of hydrogen peroxide of high

purity. Discovery of the factors that cause its decomposition led to the development of effective stabilisers that deactivate contaminating materials. The stability of hydrogen peroxide can be retained when diluted to 3% if this step is carried out with clean equipment and good grade deionized water.

Hydrogen peroxide is thought to kill by the production of hydroxyl free radicals (.OH) and this reaction is catalysed by ions of aluminium, iron, copper, manganese, and chromium. The hydroxyl free radical can attack lipid membranes, DNA and other essential cell components. Defences against free radicals are present in all respiring cells. These consist of catalase and peroxidases which inactivate hydrogen peroxide, and superoxide dismutases which scavenge superoxide radicals preventing interaction and the resultant formation of the destructive hydroxyl free radical. It has been stated that tissue catalase causes rapid decomposition of hydrogen peroxide which protects cells and prematurely inactivates antibacterial activity (Harvey, 1983) and hence hydrogen peroxide is an inadequate disinfectant. Others believe that this defence mechanism for metabolically produced hydrogen peroxide is overwhelmed by the concentrations used in antiseptics (Turner, 1983). Neither view is supported by experimental data.

3.2.5 QUATERNARY AMMONIUM COMPOUNDS

The most common quaternary ammonium compound applied to wounds in this country is cetrimide, which consists chiefly of decyltrimethylammonium bromide together with small parts of dodecyl- and hexadecyltrimethylammonium bromide. The agents act by disruption of the bacterial membrane. Cetrimide is active against gram positive and gram negative bacteria, but gram positive organisms are more sensitive and some gram negative organisms are resistant, particularly *Pseudomonas* species. Growth inhibitory concentrations of cetrimide for *Staphylococcus aureus* are 0.0007 % w/v and for *Escherichia coli* 0.0225% w/v. The activity of quaternary ammonium compounds increases with temperature and decreases in the presence of organic material. These agents are absorbed to a

significant degree by cotton, rubber, and other porous materials in addition to polyethylene and polypropylene.

The properties of the above agents allow introduction into a cell culture system. Povidone iodine will be excluded for the reasons outlined in Section 3.2.2.

3.3 **AGENTS AND CONCENTRATIONS**

The agents tested were as follows: 1.) chlorhexidine 2.) sodium hypochlorite 3.) hydrogen peroxide 4.) cetrimide. In most instances the highest concentration tested was that used clinically on cutaneous wounds. Initially, a wide range of concentrations using ten-fold dilutions were used, with a narrower range at subsequent attempts, based on the indications of the first. Clearly for the purposes of scientific correctness it is essential to quote molar concentrations, yet for clinical usage percentage weight for volume values are widely quoted. In **Table 3.2** standard use concentrations are shown with their molar equivalents; thereafter results will be shown in molar concentrations. All solutions were freshly prepared prior to use.

3.3.1 **CHLORHEXIDINE**

Chlorhexidine gluconate (molecular weight = 897.8) as a 20% aqueous solution (Sigma Chemical Company, Poole, UK) was used and diluted appropriately. Chlorhexidine is incompatible with a number of inorganic bases at concentrations of 0.05% w/v but at dilutions of 0.01% or more these salts are soluble. Since any diluent used is likely to contain one or more of the above bases the highest concentration tested must initially be 0.01% w/v.

The available concentration of chlorhexidine can be assayed using ultraviolet spectroscopy (Moffat, 1986).

Table 3.2 Test agents and concentrations recommended for use in cutaneous wounds shown in percentage w/v and molar equivalent.

AGENT	CONCENTRATION	CONCENTRATION
	(Percent w/v)	(Molar)
Chlorhexidine	0.05	5.60×10^{-4}
Sodium hypochlorite	0.5	1.4×10^{-1}
Hydrogen peroxide	3.0	8.8×10^{-1}
Cetrimide	0.1	3.0×10^{-3}

3.3.2 SODIUM HYPOCHLORITE

The concentration of sodium hypochlorite is calculated according to the available chlorine concentration (molecular weight of chlorine = 35.5). Sodium hypochlorite was obtained as a 15% w/v solution (Aldrich Chemical Co. Ltd., UK.) and was stored in a dark container at 4°C. The starting test concentration was 0.05% w/v available chlorine. Because sodium hypochlorite is unstable the concentration of the stock solution was checked at regular intervals by analysing the available chlorine using the method described in the British Pharmacopoea (British Pharmacopoea, 1987 i) for assay of strong sodium hypochlorite solution. The decay in available chlorine concentration over the study period is shown in **Table 3.3**.

3.3.3 HYDROGEN PEROXIDE

Hydrogen peroxide (molecular weight = 34.01) was obtained as a 30% w/v solution (Sigma Chemical Company, Poole, UK). This agent was used at starting concentration of 3% w/v. The concentration of the stock solution was checked at regular interval (assay for 30% hydrogen peroxide, British Pharmacopoea, 1987 ii), but remained highly stable (29.0-31.0 % w/v) over a two-year period.

3.3.4 CETRIMIDE

Cetrimide (molecular weight of alkyltrimethylammonium bromides = 336.4; these make up more than 96% w/v of cetrimide) was obtained in powder form from the Sigma Chemical Company, Poole, UK. A 1% w/v solution was prepared for cytotoxicity testing and sterilized by filtration. The solution was filtered by drawing it into a plastic syringe, the plunger of which was rubber, and then passing it through a 22 µm filter (Nalgene). Rubber is known to absorb cetrimide, and in order to assess whether any absorbance of cetrimide occurred, the concentration of the cetrimide 1% w/v solution was checked pre- and post-filtering. The concentration of cetrimide was measured using the method described in the British Pharmacopoea (1987 iii). Results showed that the filtration procedure had no effect

Table 3.3 Decay in available chlorine concentration (% w/v) over time in stock solution of sodium hypochlorite.

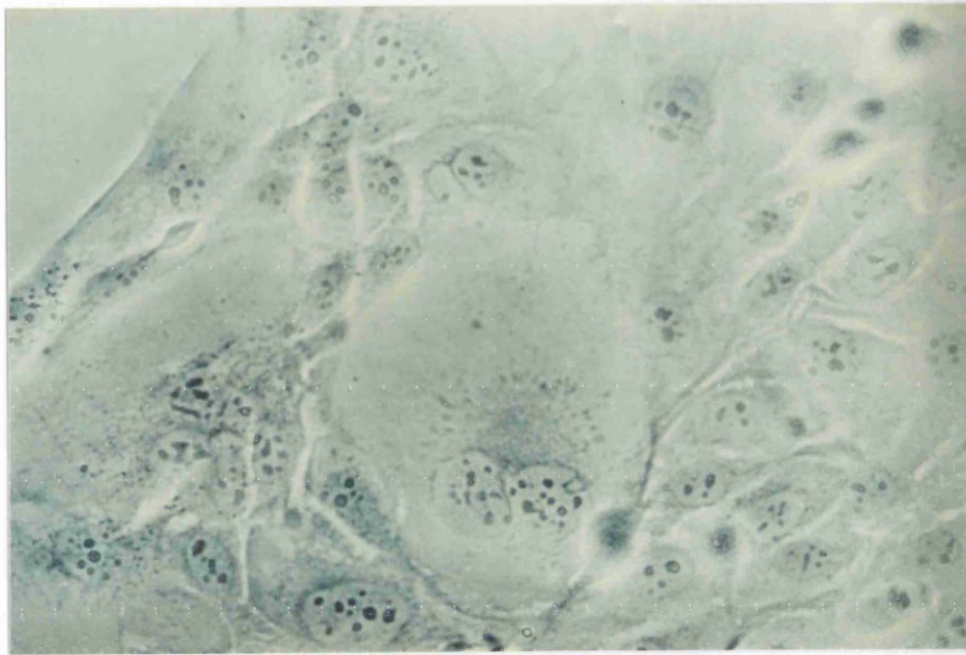
DATE	AVAILABLE CHLORINE CONCENTRATION (% W/V)
4/5/86	12.4
29/7/86	7.62
14/11/86	7.20
19/6/87	6.00
22/2/88	5.90
6/5/88	5.40
1/11/88	5.20

on the concentration of cetrimide; results of the assay showed that a predicted concentration of 1% cetrimide which was unfiltered had a concentration of 1.05% w/v and the filtered solution also had a concentration of 1.05% w/v.

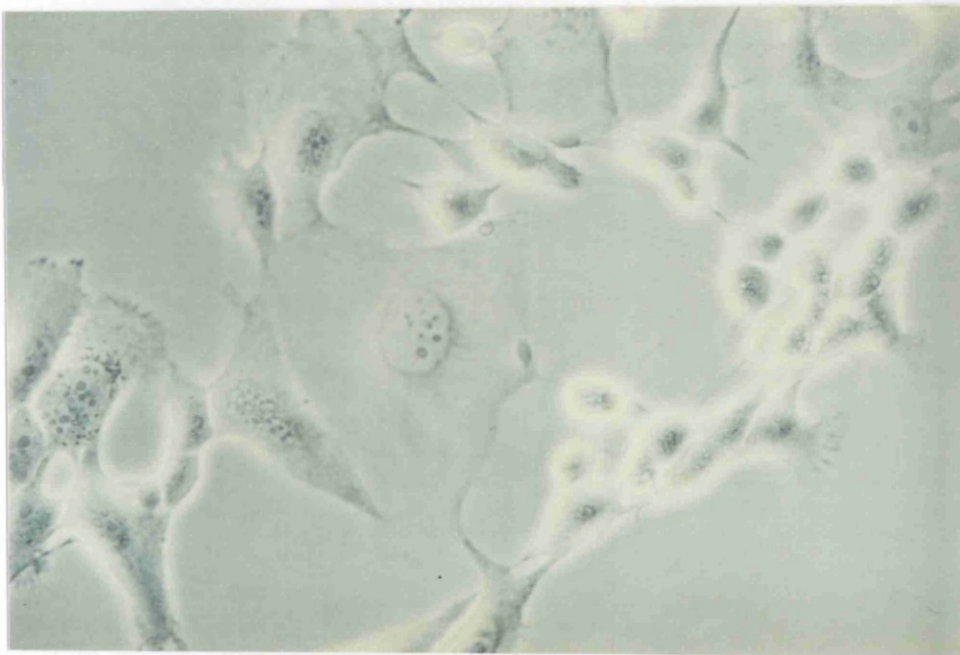
3.4 DILUENT

All agents are soluble in aqueous solution. The ideal diluent must obviously be compatible with the cell cultures, but the nature of the diluent may depend upon the length of exposure of the cells to the test agent, and whether or not such a diluent is compatible with the test agent. For short exposures to a drug, adherent cells or cells in suspension can be maintained in phosphate buffered saline or normal saline. However, when the adherent transformed keratinocyte line and the basal keratinocytes were used, these cells loosened their attachment within 10 minutes when maintained in either PBSa or saline and subsequent washings caused detachment of cells. This phenomenon was observed microscopically; within 10 minutes the polygonal epithelial cells began to round up (**Figure 3.1**). This problem could be corrected by using a phosphate buffered saline solution which contained calcium and magnesium salts (PBS-abc, see Reagent appendix). Long exposures, such have been used when studying the effect of antiseptics on keratinocyte growth (Shakespeare et al, 1988; Cooper et al, 1990), necessitate dilution of the test agent in medium with serum (**Table 1.5**). It was not proposed to use a long exposure in this study because it is not considered appropriate (see Section 3.5); however the effects of media and serum on the toxicity of antiseptics were studied and discussed in Chapter 6.

At a concentration of 0.05% (w/v), chlorhexidine is not compatible with phosphate buffered saline, since it interacts with chlorides and phosphates forming insoluble salts; at concentrations 0.01% (w/v) these salts are soluble (Reynolds, 1989 ii). Preliminary studies had shown that the lowest concentration producing 100% killing of both the normal and transformed keratinocytes was



a



b

Figure 3.1

- a) Monolayer of SVK 14 cells maintained in phosphate buffered saline supplemented with calcium (x1000).
- b) SVK 14 cells maintained in phosphate buffered saline without calcium supplements for 10 minutes. The cells lose their desmosomal attachment and attachment to the culture plate (x1000).

0.01% (w/v) but for fibroblasts this concentration was between 0.01 and 0.02% (w/v). To establish whether chlorhexidine gluconate was fully soluble in PBS-abc at concentrations of 0.02% (w/v) and below, the concentration of chlorhexidine was measured using ultraviolet spectroscopy measuring absorbance at 254 nm (Moffat, 1986). The 20% (w/v) chlorhexidine solution was diluted to 2.0% (w/v) in distilled water; subsequent dilution to 0.02% (w/v) was made in PBS-abc for the test solution and in distilled water for the control. Concentrations from 0.02% (w/v) to 0.004% (w/v) were compared. For the chlorhexidine solution diluted in PBS-abc results showed that there was a linear relationship between predicted concentration and absorbance. Comparison of the absorbance values for the agent diluted in distilled water and PBS-abc showed identical results (**Figure 3.2**). Results show that there is no difference in chlorhexidine concentration when this agent is diluted in PBSabc as opposed to distilled water and it can be concluded that concentrations up to 0.02% (w/v) may be tested.

Section 3.5 **LENGTH OF EXPOSURE**

An essential feature of antiseptic agents is their rapid antibacterial action. It is also desirable that their action is sustained, particularly for the skin preparation prior to surgery. Certain agents exhibit sustained activity; chlorhexidine exhibits good substantivity on intact skin, with 26% remaining on the skin after 29 hours (Harvey, 1983). Povidone iodine 10% also has a sustained effect on intact skin which is less marked than chlorhexidine. Data on whether antiseptics exhibit a sustained effect in wounds is not available, but it seems likely that, because of the vascularity of a wound bed, the active agent may be rapidly absorbed. In previous studies exposure times have varied from 5 minutes to 4 days (**Table 1.5, 6,7**), although a short exposure time has usually been chosen ie. less than 1 hour.

Since inactivation by organic material, absorption and dilution are likely to terminate the activity of an antiseptic agent within a wound, a short exposure time of 15 minutes was initially chosen. In a subsequent study the effect of an exposure

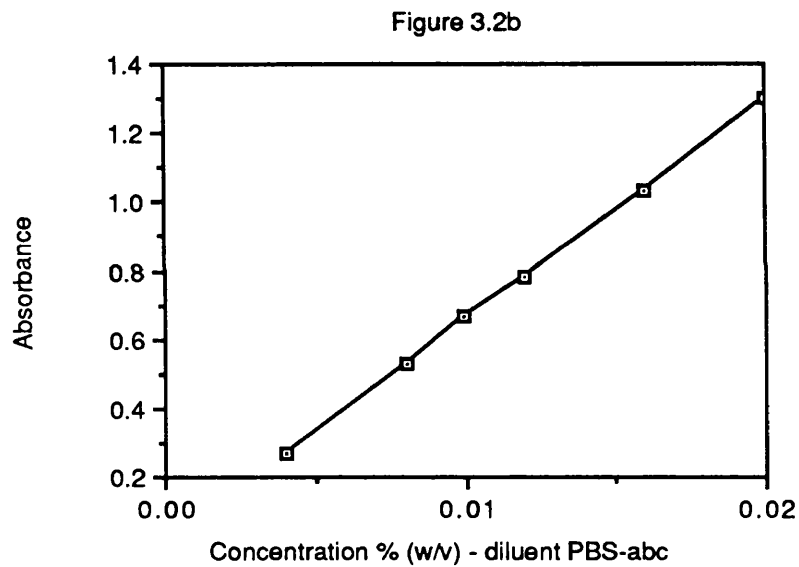
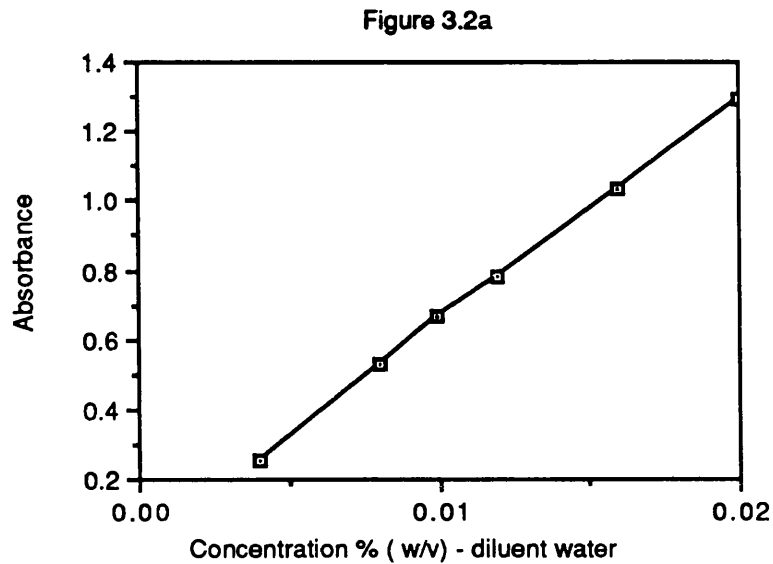


Figure 3.2 a and b

Comparison of concentration of chlorhexidine gluconate when diluted in distilled water and PBS-abc. The chlorhexidine concentration was assayed by measuring UV absorbance at 254 nm. The range of concentrations tested was 0.02%w/v to 0.004%w/v and the results show that diluting in PBS -abc gives identical results to the control experiment. These findings show that at concentrations between 0.01 and 0.02% w/v chlorhexidine is fully soluble in PBS-abc.

time of one hour was studied (Chapter 6). Long exposures of 24 hours seemed inappropriate, since the antiseptic agents were unlikely to persist in contact with a wound for such a period of time. Long exposures have generally been used to study the effect of antiseptic agents on cell growth. It may be more appropriate to expose the cells to the agent for a short period of an hour or less, and then return the cells to normal culture conditions to evaluate growth inhibition.

3.6 CELL NUMBER

The number of cells to be studied has been discussed and defined in Chapter 2. Cell number is constrained by certain factors: smaller numbers of cells in multiwell plates facilitate handling of large numbers of samples; cell culture equipment is expensive and use of large numbers of culture dishes or flasks would be costly. The cell number established for comparison of all cell types was 1×10^5 . Comparison of cell numbers used in published antiseptic cytotoxicity assays is shown in **Tables 1.5, 1.6, and 1.7**. Studies comparing the effect on antiseptic cytotoxicity of varying cell number are described in Chapter 6.

Most antiseptic cytotoxicity assays have used attached cell assays. For ease of handling it was decided to do likewise. When a cell suspension is used exposure time is difficult to control, and washing steps, which involve centrifugation, increase the amount of manipulation and allow for introduction of error. Additionally, it was noted that if SVK 14 cells were incubated in plastic centrifuge tubes for more than 1 hour, the cells attached to the surface; this would interfere with any counting/staining procedures.

3.7 DURATION OF EXPERIMENT

Although antiseptic agents are rapidly acting, their toxic effect may continue when the agent is removed. Lineaweaver, Howard and Soucy et al (1985) examined the

effect of a range of antiseptics on fibroblasts in culture. The cells were exposed for 15 minutes and cell viability was evaluated at the end of exposure and 24 hours later. This study showed that at the end of the recovery period the numbers of surviving cells was reduced when compared to the values obtained from immediate analysis. Initially it was decided to use the 24 hour recovery period in order to demonstrate the maximum toxicity of these agents.

3.8 DEFINING EXPERIMENTAL CONDITIONS

Experimental design for experiments conducted in cell culture are constrained by practical factors. However, acknowledging the known properties of the agents to be tested, experimental conditions for an initial set of experiments have been devised and the important features are summarized below:

- 1) A small number of cells (1×10^5) so that 24 well plates can be used which allow ease of handling for a number of experiments at any time and within each experiment to facilitate replicate sampling.
- 2) A short exposure to the test agents to reflect the rapid action of these drugs, with a long recovery period to demonstrate maximum toxicity.
- 3) The agents to be initially diluted in PBS-abc to demonstrate maximum toxicity of the agents.

An understanding of how alterations in variables discussed may alter results is important in interpreting data generated and the results of other authors' studies. These issues are addressed in Chapter 6.

3.9 SUMMARY

The properties of certain antiseptic agents limit their introduction into a cell culture assay. The properties of those agents which can be used were described. Theoretical and practical considerations allowed the exposure time, cells number and the duration of the experiments to be defined.

CHAPTER 4

METHODS - ESTABLISHING A CYTOTOXICITY ASSAY

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4.1 INTRODUCTION

Human cells in culture are being used with increasing frequency as in vitro models for studying drug induced toxicity. The choice of assay for measuring the toxic effect of a drug will depend on the agent under study, the nature of the response, and the particular target cell. Assays can be divided into two major classes: 1) those which measure a short term response 2) those which measure long-term survival, usually by ability to proliferate, or survival in an altered state e.g., the cells express a genetic mutation. The long term tests are necessary for agents which may show an effect hours or days after exposure. Many assays have been established to study the mechanisms of drug induced toxicity. Changes in cellular properties such as the adhesiveness of anchorage-dependent cells to their culture support, cell membrane permeability, proliferation, respiration and overall metabolic activity, have been monitored under the influence of a given drug by measuring cell detachment, release of intracellular material (ATP, proteins, DNA), exclusion of trypan blue or uptake of vital dyes, inhibition of thymidine incorporation and protein biosynthesis, as well as gas exchange and heat production (Freshney, 1987).

4.2 METHODS OF MEASURING CYTOTOXICITY AND CELL VIABILITY

Below the well established methods for measuring cytotoxicity are outlined.

4.2.1 SHORT-TERM ASSAYS

Short term assays measure cell viability. Most viability tests rely on a breakdown in membrane integrity in non-viable cells which leads to the uptake of a dye to which the cell is normally impermeable (e.g., trypan blue, nigrosin) or in viable cells release of a dye or isotope normally taken up or retained e.g., diacetyl fluorescein or 51 chromium.

The most commonly used methods are:

1.) Dye exclusion - Viable cells are impermeable to nigrosin and trypan blue. Trypan blue is the most commonly used dye and it has been shown that non-viable cells which take up this dye do not respire, glycolyse or extend cellular processes when reintroduced into a tissue culture system. A sample of the treated cell population is examined and counted in a haemocytometer, and the number of non-viable stained cells is noted.

Total cell counts can be made with either a haemocytometer or electronic particle counting. Determining the concentration of a cell suspension in a haemocytometer involves placing the cells in an optically flat chamber under a microscope; the cell number within a defined area is counted and the concentration is derived from the count. Haemocytometer counting is cheap and gives the opportunity of viewing what is counted. If the cells are mixed with a viability stain e.g. trypan blue, an assessment of viability can be made at the same time. The procedure is slow and prone to error, most particularly because of sampling errors; counting in a haemocytometer requires a minimum of 1×10^5 cells per ml and lower concentrations require volume reduction increasing the number of manipulations and further increasing the risk of introducing error.

Electronic particle counting provides a more accurate, automated method. Although a number of different automatic methods have been developed for counting cells in suspension, the system devised by Coulter Electronics is the most widely used. The Coulter counter works on the principle that when cells are drawn through an orifice, they change the current flow through the orifice producing a series of pulses which is sorted and counted. Electronic particle counting is rapid and has a low inherent error because of the high numbers of cells counted. Difficulties may arise with this procedure if the cells are not in a single cell suspension, and if debris is contained in the suspension since each particle will be recognised as a single cell. Obviously this technique cannot distinguish between dead and live cells and must be combined with vital staining of a representative sample of the total cell count to give an estimate of viability.

2.) Dye uptake - Viable cells take up diacetyl fluorescein and hydrolyse it to fluorescein to which the cell membrane of viable cells is impermeable. Live cells fluoresce green and dead cells do not. Non-viable cells will fluoresce red when stained with ethidium bromide or propidium iodide. The numbers of viable/non viable cells can be counted microscopically. Although this appears to be an excellent method for evaluating cell viability its use in conjunction with microscopy is likely to be laborious and inaccurate; Cells stained with fluorescent dyes can also be counted by flow cytometry but this method may require large numbers of cells which make it impractical for use in assaying a range of concentrations on a cell line which has a limited life-span such as normal keratinocytes.

3.) Chromium release - Reduced $^{51}\text{Cr}^{3+}$ is taken up by viable cells and oxidised to $^{51}\text{Cr}^{2+}$ to which the membrane of viable cells is impermeable. Dead cells release the $^{51}\text{Cr}^{2+}$ into the medium. A reduction in viability is detected by counting aliquots of medium from cultures labelled previously with $\text{Na}_2^{51}\text{CrO}_4$ for released ^{51}Cr . This method is widely used but facilities for handling radioactive chromium material must be available.

4.2.2 LONG-TERM ASSAYS

Long-term tests measures survival by measuring the number of cells or amount of cellular activity remaining after prolonged exposure to an agent or short exposure and prolonged recovery in the absence of an agent. The objective is to measure cell survival rather than short-term toxicity which may be reversible.

1) Plating efficiency - This is the best method of measuring survival and proliferative capacity. The cells are treated with the experimental agent at a range of concentrations for 24 hours. They are then trypsinized seeded at low density and and reincubated for 1-3 weeks. At the end of the experiment the colonies are stained and counted. This method is time consuming and the duration of each experiment may be up to four weeks.

2) Other methods - After a period of incubation of the cells, with and then without, the drug cell survival can be measured in the following ways:

- i) [^{35}S] methionine labelling and analysis by autofluorography and scintillation counting. This method measures active protein synthesis which implies that the cells are still alive.
- ii) Direct staining and cell counting in situ or by densitometry.
- iii) Measurement of dehydrogenase activity.
- iv) Labelling with [^3H]-thymidine which measures DNA synthesis or [^3H]-uridine which measures RNA synthesis with analysis by scintillation counting or autofluorography.

4.3 REVIEW OF CYTOTOXICITY ASSAYS USED FOR THE IN VITRO STUDY OF ANTISEPTIC TOXICITY

Antiseptic agents are chosen for their rapid antimicrobial action, although certain agents also possess sustained action e.g. chlorhexidine. Most studies have used short term assays which measure viability (**Tables 1.5, 1.6, 1.7**). The simplest way of assessing viability is by microscopy looking for characteristic changes such as toxic granulation and vacuolation but this technique is subjective and is now rarely used (Fleury et al, 1984). The majority of studies have measured cell viability using dye exclusion tests, in most instances trypan blue although use of safranin has been reported (Helgeland et al, 1971). Capin et al (1985) used three different dye techniques to study cellular injury induced by hydrogen peroxide: 1) fluorescein diacetate uptake 2) nigrosin exclusion 3) acridine orange uptake /ethidium bromide exclusion. In this study the labelled cells were counted visually but analysis can be performed in a flow cytometer.

A methylene blue assay was originally described by Olsson, et al (1982) in a study of the cytotoxic effect of macrophages on cancer cells. It has since been applied to the study of antiseptic toxicity in cell culture. Fleury et al, (1984)

compared microscopic evaluation of the cytotoxic effects of glutaraldehyde and dimethyl sulphoxide on vero cells (hamster fibroblasts) with the methylene blue method and showed good correlation. Deas et al (1986) used the same assay to evaluate the toxicity of a range of agents, also using a fibroblast line.

Effects of antiseptics on cell growth have been measured by cell counting methods, either using a haemocytometer or electronic particle counting (Coulter counter) (Cooper et al, 1990) or by uptake of tritiated thymidine (Shakespeare et al, 1988).

Attempts at establishing the mechanisms of action of antiseptics on human cells have been made. Shakespeare et al, (1988) studied the effect of chlorhexidine, benzyldiamine hydrochloride, and hexetidine on lactate dehydrogenase (LDH) levels in oral keratinocytes; hexetidine had a profound effect on the intracellular levels of LDH, whereas chlorhexidine showed no reduction in enzyme activity at an equivalent dilution but 90% inhibition of ^3H thymidine incorporation. No reduction of enzyme activity was seen with benzyldiamine hydrochloride. The authors concluded that hexetidine had a damaging effect on cell membranes permitting soluble cytoplasmic enzyme to leak into the culture medium. Helgeland et al, (1971) studied levels of intracellular enzymes (NADPH₂ and NADH₂ diaphorases, 5' nucleotidase and alkaline phosphatase) in cells treated with chlorhexidine. Decreasing intracellular enzyme activity was found after treatment with increasing concentrations of chlorhexidine. These findings were attributed to release or denaturation of enzyme proteins and it is possible that both mechanisms were operative since at high concentrations chlorhexidine fixes cells but at low concentrations this agent causes leakage of material from bacteria (Hugo and Longworth, 1964). These findings do not concur with the study by Shakespeare et al, (1988) where at high dilutions of chlorhexidine a cytotoxic effect was apparent, as measured by reduction of ^3H thymidine incorporation, but membrane function was normal.

4.4 ESTABLISHING AN IN VITRO CYTOTOXICITY ASSAY - INITIAL EXPERIMENT USING DYE EXCLUSION AND CELL COUNTING

It was essential to establish a cytotoxicity assay which was easily performed, giving reliable and reproducible results. Dye exclusion was initially chosen because it is a cheap and simple method of evaluating cell viability. The following experimental conditions were initially chosen:

- 1) For initial experiments the SVK 14 cells were used.
- 2) In line with bacteriological testing of antiseptic agents a short exposure time of 15 minutes was utilized.
- 3) An attached cell assay rather than a suspension assay was used to reduce the number of manipulations, particularly centrifugation. 3×10^5 cells were plated in culture dishes so that a sufficiently large sample was available for viability counting in a haemocytometer. It was initially assumed that following exposure to the drug the non-viable cells would detach, and that viable cells would remain attached and could be counted.
- 4) The cytotoxicity assay chosen to measure viability was dye exclusion (trypan blue) and the total cell counts were made using a Coulter counter. Following treatment both the viability of the remaining attached cell and those cells which had detached was studied. It was assumed that non-viable cells would detach and that viable cells would remain attached and this needed to be confirmed.
- 5) To establish the cytotoxicity assay only three agents were chosen for testing: cetrimide, sodium hypochlorite and hydrogen peroxide (see Section 3.3).

Materials and methods

Cell culture - SVK 14 cells, 3×10^5 cells per well were seeded into 30 mm culture dishes and cultured in RPMI 1640 plus 10% FCS at 37°C, 8% CO₂ for 24 hours. For each test concentration of an agent three dishes were set up for analysis by Coulter

counting and three for staining with trypan blue to assess the percentage of non-viable cells in the total cell count.

Exposure to antiseptic agents - The effect of three agents on these cells was examined. The concentration used in cutaneous wounds is shown in parentheses: 1) hydrogen peroxide (0.88 M) 2) cetrimide (0.003 M) 3) sodium hypochlorite (0.14 M). Each agent was freshly prepared prior to use; the agents were serially diluted in PBS-abc (see Section 3.4) from the concentration recommended for use in cutaneous wounds (see **Table 3.1**). Ten-fold dilutions were used to establish the range of concentrations which exerted a toxic effect.

Prior to exposure of the cells to the agents the culture medium was removed and the cells were washed twice in PBS-abc. The cells were then exposed to the antiseptic agent for 15 minutes, after which the drug was removed and the cells were washed twice with PBS-abc. Control wells were exposed to PBS-abc alone. The medium was then replaced and the cells reincubated at 37°C, in 8% CO₂ for 24 hours.

The removed drug and washings for each concentration were immediately centrifuged at 1000 rpm for 5 minutes, the supernatant removed and any cells were resuspended in 100µl of trypan blue 0.4% (Gibco Laboratories, Paisley, UK) diluted to 0.2% in PBS-a. The suspension was examined in a haemocytometer to determine the number of cells detaching at this stage and whether or not they were viable.

Analysis - viability stain and cell counting - After the 24 hour period of reincubation the medium containing detached cells was removed from three dishes; the attached cells were washed twice with PBS-a and the washings were added to the removed medium and spun down at 1000 rpm. The pellet of cells was resuspended in 100 µl of trypan blue 0.4% diluted to 0.2% in PBS-a for 2 minutes prior to examination in a haemocytometer, where numbers of non-viable cells were counted.

For the attached cells a total cell count using a Coulter was made on three dishes, and an estimate ^{of} viability was made by staining cells from three further dishes with trypan blue and counting the non-viable cells in a haemocytometer.

X

The attached cells were trypsinized with 1 ml trypsin 0.05% in 0.2% EDTA at 37°C for 10 minutes. Once the cells were detached 5 µl of FCS was added to the trypsin to inhibit its action and prevent cell aggregation. For three dishes the percentage viability of the cells was assessed using trypan blue as follows. The trypsinized cells were spun down and the pellet resuspended in 100 µl trypan blue 0.2% for two minutes. The cells were gently pipetted up and down in a 100 µl Gilson pipette to disaggregate clumps and counted in a haemocytometer. From the three counts a mean percentage non-viability was calculated.

The trypsinized cells in the other three dishes were syringed through a 16 gauge needle and the suspension from each dish added to 9 ml of Isoton II (azide-free isotonic diluent based on 9g/l sodium chloride in water; Coulter Electronics Ltd., Luton, UK) in a cuvette. Prior to this addition, the dish was washed twice with Isoton II from the cuvette to which the washings were then returned. The cells were then counted in the Coulter counter, which sampled 500 µl at each count. Each sample was counted twice. From the mean counts of three samples, the percentage of non-viable cells was subtracted (as established from the vital staining). The final viability count for each concentration was expressed as a percentage of the control, to give a percent viability. Each experiment was repeated three times.

Results

Viability of detached cells - From the washings and drug removed immediately after exposure, no cells could be counted so that cell numbers detaching at this point must have been equal to or less than 1000 cells i.e. 0.33% of the originally seeded cell number. The analysis of detached cells at 24 hours is shown in **Tables 4.1 a,b,c**. No statistical analysis was applied to the comparison of cells numbers between test and control values because replication between experiments was poor. This was likely to have arisen because number of cells counted was small. However, examination of the data (**Tables 4.1 a,b,c**) suggest that viable cells were detaching in numbers broadly similar to the control values. The highest concentrations of

Hydrogen peroxide Concentration (M)	Percent viability	Percent non-viable
8.8×10^{-2}	0	100
8.8×10^{-3}	100 (4×10^3 , 6×10^3 , 11×10^3)	0
8.8×10^{-4}	100 (4×10^3 , 6×10^3 , 8×10^3)	0
8.8×10^{-5}	100 (4×10^3 , 5×10^3 , 10×10^3)	0
8.8×10^{-6}	100 (3×10^3 , 6×10^3 , 10×10^3)	0
Control	100 (3×10^3 , 5×10^3 , 8×10^3)	0

Table 4.1a

Sodium hypochlorite Concentration (M)	Percent viability	Percent non-viable
1.4×10^{-1}	0	100
1.4×10^{-2}	0	100
1.4×10^{-3}	0	100
1.4×10^{-4}	100 (3×10^3 , 5×10^3 , 11×10^3)	0
1.4×10^{-5}	100 (6×10^3 , 10×10^3 , 11×10^3)	0
Control	100 (3×10^3 , 5×10^3 , 8×10^3)	0

Table 4.1b

Cetrimide Concentration (M)	Percent viability	Percent non-viable
3×10^{-3}	0	100
3×10^{-4}	0	100
3×10^{-5}	0	100
3×10^{-6}	100	0
Control	100 (3×10^3 , 5×10^3 , 8×10^3)	0

Table 4.1c

TABLES 4.1 a,b,c Analysis of viability of detached cells 24 h after treatment of SVK 14 cell cultures with hydrogen peroxide, sodium hypochlorite, and cetrimide in comparison to untreated controls. The numbers of cells detaching from an original seeding density of 3×10^5 are shown in parentheses, the values representing the results of three separate experiments.

hydrogen peroxide tested, 8.8×10^{-1} M and 8.8×10^{-2} M, produced a detached cell population which was 100% non-viable. At lower concentrations of hydrogen peroxide the detached cells were all viable and the numbers did not differ from control values. For sodium hypochlorite concentrations of 1.4×10^{-1} M and 1.4×10^{-2} M detached cells were fragmented and non-viable. At a concentration of 1.4×10^{-3} M the detached cells were intact but non-viable. For the lowest concentrations of sodium hypochlorite tested (1.4×10^{-4} M and 1.4×10^{-5} M) the detached cells were 100% viable and numbers did not differ from control values. The highest concentration of cetrimide tested was 3×10^{-3} M and this gave rise to detached cells which were fragmented and non-viable. At a concentration of 3×10^{-4} M the cells were intact but non-viable. At the lowest concentrations tested (3×10^{-5} M and 3×10^{-6} M) the detached cells were all viable and numbers did not differ from control values.

Cell counting and viability of attached cells - The results are summarized in **Table 4.2 a,b,c**. For hydrogen peroxide at a concentration of 8.8×10^{-1} M, there were no attached cells, at 8.8×10^{-2} M the attached cells were all non-viable; at concentrations of hydrogen peroxide of 8.8×10^{-3} M and below the attached cells were all viable. Percentage survival for each concentration compared to control values, based on cell counts and viability stains are shown in **Table 4.2a**. Exposure to sodium hypochlorite 1.4×10^{-1} M and 1.4×10^{-2} M left no attached cells (**Table 4.2b**); at a 1.4×10^{-3} M concentration of sodium hypochlorite the attached cells were 100% non-viable. A further ten-fold dilution produced a mixed population of viable and non-viable cells; viability stains showed that 20% of the cells were non-viable. The lowest concentration tested (1.4×10^{-5} M) gave rise to an attached cell population which were 100% viable. Absolute percentage survival rates compared to control values are shown in **Table 4.2b**. For cetrimide a concentration of 3×10^{-3} M left no attached cells; at a concentration of 3×10^{-4} M attached cells were present and were 100% non-viable. Two further ten-fold dilutions produced populations of

Hydrogen peroxide Concentration (M)	Percent viable (of treated cells)	Percent non-viable (of treated cells)	Percent viability treated cells cf control
8.8×10^{-2}	0	100	0
8.8×10^{-3}	100	0	52 ± 6
8.8×10^{-4}	100	0	70 ± 8
8.8×10^{-5}	100	0	90 ± 6
8.8×10^{-6}	100	0	100 ± 5

Table 4.2a

Sodium hypochlorite Concentration (M)	Percent viable (treated cells)	Percent non-viable (treated cells)	Percent viable treated cells cf control
1.4×10^{-1}	0	100	0
1.4×10^{-2}	0	100	0
1.4×10^{-3}	0	100	0
1.4×10^{-4}	70 ± 15	30 ± 15	52 ± 8
1.4×10^{-5}	100	0	104 ± 7

Table 4.2b

Cetrimide Concentration (M)	Percent viable (treated cells)	Percent non-viable (treated cells)	Percent viable treated cells cf control
3.0×10^{-3}	0	100	0
3.0×10^{-4}	0	100	40 ± 7
3.0×10^{-5}	0	100	104 ± 6
3.0×10^{-6}	100	0	98 ± 8

Table 4.2c

TABLES 4.2 a,b,c Analysis of viability of attached cells 24 h after treatment of SVK 14 cell cultures with hydrogen peroxide, sodium hypochlorite, and cetrimide in comparison to untreated controls. With the exception of sodium hypochlorite, at the higher concentrations tested the attached cells were all non-viable, and at lower concentrations were all viable. For sodium hypochlorite, mid-range concentrations gave a population of cells of mixed viability. The third column shows the final estimate of viability based on total cell counts (mean of 3 replicates \pm 1 SD) minus the mean numbers of non-viable cells. Values show the mean of 3 replicates \pm 1 SD.

attached cells which were all viable. Total percentage survival rates for each concentration are shown in **Table 4.2c**.

Comment

The initial assumption that attached SVK 14 cells would detach if killed by the antiseptic agent under test was incorrect. At the lowest concentration tested which allowed no survival of cells, many of the non-viable cells remained attached. With the exception of sodium hypochlorite, further ten-fold dilutions produced populations of attached cells which were all viable; dilution from the 'no-cell-survival' concentration of sodium hypochlorite produced an attached population of cells which was of mixed viability.

For all three agents tested viable cells were only shown to detach at the lower concentration tested but did not differ from numbers detaching from control dishes. These detaching viable cells are likely to be cells in mitoses, which round up and weaken their attachment to the plastic substrate prior to division. These findings negate the assumption that for treated cells, viable cells which have lost their ability to attach will be shed and therefore excluded from the assay. It appears that viable cells from treated plates do not detach more than those in the control plates, and it is therefore acceptable to continue with an assay which examines viability of attached cells alone.

When an attached cell assay is used non-viable cells remain attached, therefore a counting procedure such as electronic particle counting cannot be used in isolation and must be supported by a method which estimates viability of the counted cells. Electronic particle counting gives great speed and accuracy to cell counting, and replication of samples is good, showing a coefficient of variation of less than 10%. Haemocytometer counting with viability staining produces poor replication and is very time consuming. As the attached cell assay devised cannot be performed with electronic particle counting alone, it was decided to abandon

counting procedures, and to seek an alternative assay which was both accurate and quick.

The attachment of non-viable cells seen with the higher concentrations tested but not with lower strengths, may be related to the different modes of action of these agents at differing concentrations. For bacteria it has been established that at high concentrations of an antiseptic agent, which are rapidly bacteriocidal, bacterial membranes and cytoplasmic constituents are precipitated; at lower concentrations specific modes of action are operative e.g. disruption or damage to cell membranes. Equally these agents may act differently at different concentrations in mammalian cells, giving rise to the differing responses seen.

4.5 ESTABLISHING AN IN VITRO CYTOTOXICITY - COLORIMETRIC ASSAY USING METHYLENE BLUE

Methylene blue is a basic dye which stains both the nucleus and cytoplasm of cells. A photometric assay utilising methylene blue was originally described by Olsson et al (1982) to evaluate the cytotoxic effect of activated macrophages on tumour cells. In this study the methylene blue assay correlated well with cell counting and was more sensitive than a ^3H -thymidine assay. Its advantages were deemed to be that it was quick, sensitive and reproducible. To facilitate rapid handling experiments could be performed in microtitration plates and analysed in an automatic photometer. The use of this assay in the evaluation of antiseptic cytotoxicity has been reported twice (Fleury et al, 1984; Deas et al, 1986). Both studies used an attached cell assay which utilized fibroblasts. A preliminary experiment was performed to assess the suitability of this method for evaluating cytotoxicity using SVK 14 cells.

Materials and methods

Cell culture - SVK 14 cells were grown to confluence in flasks and sub-cultured in 30 mm dishes, 3×10^5 cells per dish. The cells were cultured in RPMI 1640 with 10%

FCS. Nine dishes were set up for each concentration, three to be analysed by cell counting, three for estimation of cell viability with trypan blue and three to be analysed by the methylene blue colorimetric assay. The dishes were incubated for 24 hours at 37°C in 8% CO₂.

Exposure to antiseptic agent - The cells were exposed to ten-fold dilutions of sodium hypochlorite, appropriate ranges having been previously established. Control wells were exposed to PBS-abc alone. The cells were reincubated for 24 hours at 37°C in 8% CO₂.

Cytotoxicity assays - For the methylene blue assay, the medium was aspirated and the cells washed twice with PBS-a. The residual adherent cells were then fixed with phosphate buffered formalin 10% for 10 minutes. The plates were washed in 0.01 M borate buffer, pH 8.4, then stained for 30 minutes in a solution of 1% methylene blue (BDH, UK) in borate buffer. Prior to eluting the dye the cells were examined microscopically. The plates were then washed four times with borate buffer, then left to dry at room temperature. Then each well was filled with 2 ml of 0.1 M hydrochloric acid which eluted the dye from the cells. 0.2 ml of this solution was added to 1.8 ml of deionised water and the absorbance was read at 644 nm in a spectrophotometer. One dish received no cells in order to control dye fixation of the plastic substrate. The OD of this dish was subtracted from the readings of the test samples. The OD of the test samples were expressed as a percentage of the control dishes to give an estimate of percentage viability.

The attached cells in three dishes were counted in the Coulter counter, and an estimate of the percentage viability of these cells was made by trypsinizing the cells in the remaining three plates, staining them with trypan blue and counting the numbers of non-viable cells in a haemocytometer. The methodology for cell counting and viability staining is detailed in Section 4.4 - Materials and Methods. The number of viable cells remaining for each concentration was expressed as a percentage of the control so that a percentage viability for each concentration was established.

Results

Gross observation - During the washing steps it was noted that the methylene blue dye eluted from cells into the borate buffer solution.

Microscopy - At the concentration at which trypan blue staining showed no cell survival, it was noted that there was some uptake of methylene blue by remaining attached cells. At a concentration of 1.4×10^{-4} M sodium hypochlorite trypan blue analysis showed that there were mixed populations of viable and non-viable cells; parallel plates stained with methylene blue showed that the majority of cells stained darkly with methylene blue, whilst paler staining was noted in a smaller number of cells. The paler cells were presumed to be the non-viable cells. At the lowest concentration tested, all the cells stained darkly.

Methylene blue and cell counting/viability assay - The results of the methylene blue assay in comparison to the cell counting/viability assay are shown in **Table 4.3**. Both methods showed a dose dependent reduction in cells survival. Correlation between the two methods was good (correlation coefficient= 0.99, p value = 0.03). The mean estimates of percentage viability compared to control values obtained using the methylene blue method were consistently higher than the numbers of surviving cells measured using the vital stain. Replication for the methylene blue assay was good, with a coefficient of variation of less than 10%.

Comment

Two problems were encountered with this assay: after fixing and staining the cells with methylene blue it was noted that the dye eluted during the washing steps. The effect on overall results is impossible to predict. Although the elution from the control plates may be proportional to that from the test plates, making no difference to the result when the OD for a given concentration is expressed as a percentage of the control, this cannot necessarily be assumed and was not tested.

The second problem relates to that already encountered in the first experiment (see Section 4.4); non viable cells may not detach and these cells stain, albeit

Sodium hypochlorite	Counting/viability stain	Methylene blue colorimetric assay
Concentration (M)	Percent viability cf control	Percent viability cf control
1.4×10^{-1}	0	0
1.4×10^{-2}	0	0
1.4×10^{-3}	0	10 ± 6
1.4×10^{-4}	55 ± 8	60 ± 9
1.4×10^{-5}	108 ± 6	101 ± 9

Table 4.3

Comparison of the counting/viability stain with the methylene blue colorimetric assay. Results of the first column show the mean of three replicate counts ± 1 SD minus the mean (n=3) percentage of non-viable cells with the final count expressed as a percentage of the control values. Results of the second column are the mean of three replicate counts ± 1 SD expressed as a percentage of the control value.

to a lesser degree than those judged to be viable. This phenomenon may have contributed to the greater 'viability' observed at the highest concentration tested using the methylene blue assay when compared to the results for the viability/cell counting method. In one published study problems were encountered with dead cells not detaching and taking up the stain such that for one agent, noxythiolin, a dose dependent cytotoxic effect could not be demonstrated (Deas et al, 1986). This phenomenon was only observed with noxythiolin and was attributed to release of formalin from this agent which acted as a fixative. With the other agents tested (chloramine T, chlorhexidine, Savlodil, phenol, povidone-iodine, hydrogen peroxide) this problem did not arise. This is in contradistinction to the observations in this study where, at high concentrations non-viable cells do not detach. The observed differences may relate to the length of exposure to the agent, since in the study by Deas et al (1986) the cells were exposed for 24 hours. In a second study which uses the methylene blue method exposure was for three days (Fleury et al, 1984). This may allow the cells to both die and detach. Long exposures were not considered appropriate for the testing of antiseptic agents [see Section 3.7].

The methylene blue assay was not pursued because of the problems encountered with elution of the dye during washing steps and uptake of the dye by non-viable attached cells.

4.6 ESTABLISHING AN IN VITRO CYTOTOXICITY ASSAY - MTT COLORIMETRIC ASSAY

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was originally described by Herrmann et al (1960) where it was used to study virus induced cytopathy. In 1983, Mosmann described the use of the MTT colorimetric assay to study proliferative responses in lymphocytes and complement mediated cytotoxicity. The assay measures viable cells and is dependent upon the reduction of the MTT, which is a yellow tetrazolium salt, to a blue insoluble formazan product

by mitochondrial succinate dehydrogenase (**Figure 4.1 a,b,c**) (Slater, 1963). The assay has been widely used in the study of lymphocyte proliferation and survival in vitro (Denizot and Lang, 1986; Green et al, 1984; Gallagher et al, 1987). More recently the method has been applied to the study of anticancer drugs in vitro (Freshney, 1987). Its use in keratinocyte culture has not been well documented. The original protocols describe the use of the MTT assay for non-adherent cells; the protocol used in this study was described by Ware (1985) and is applicable to adherent cells.

4.6.1 RELATIONSHIP BETWEEN CELL NUMBER AND MTT DYE REDUCTION

Initial experiments were performed to establish that there was a direct relationship between cell number and dye reduction for the three cell used in this study, namely basal keratinocytes, normal fibroblasts and SVK 14 cells.

Materials and methods

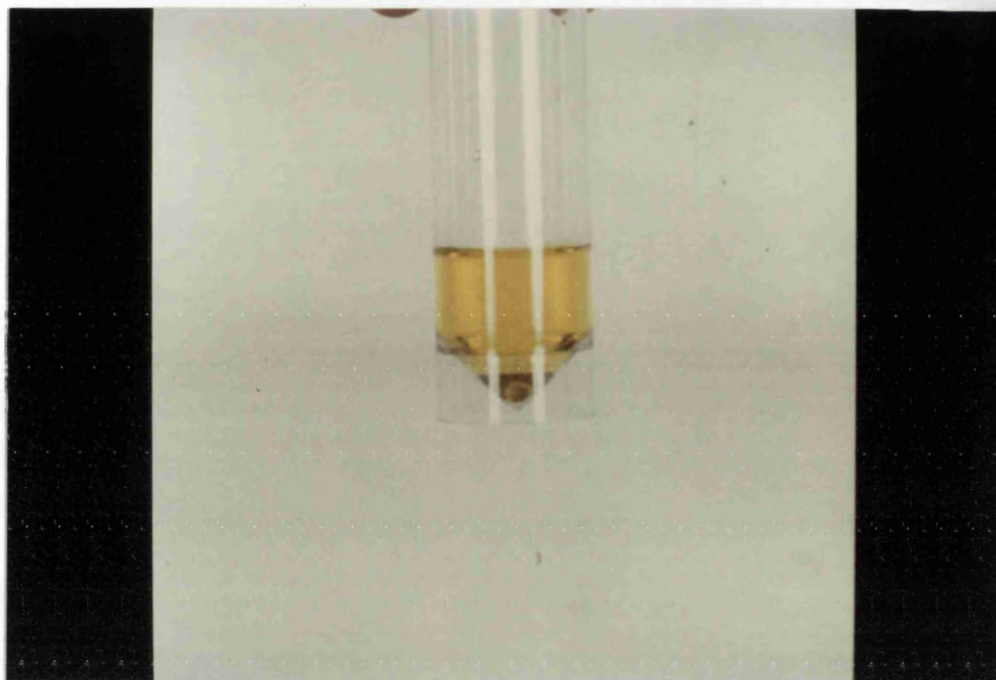
Reagents MTT dye (Sigma Chemical Co., Poole, UK) - stock solution is prepared by dissolving MTT dye in PBS-a at 5 mg/ml. The MTT was made up in a universal container, wrapped in foil to protect the dye from light.

Dye solubilization reagents - isopropyl alcohol acidified by addition of concentrated HCl to 0.04 M.

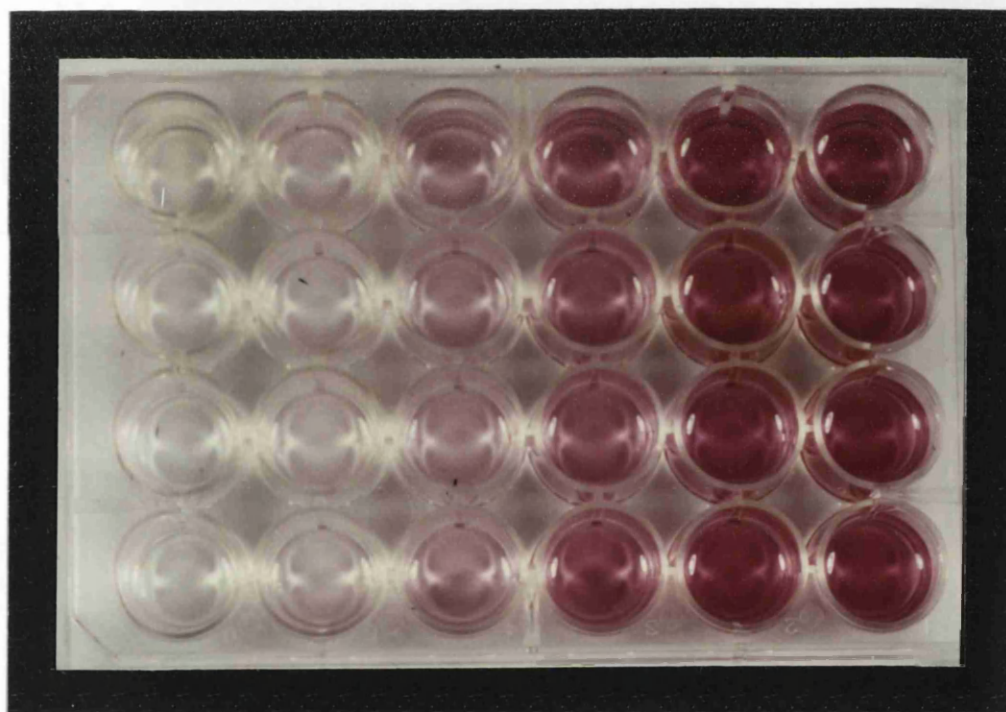
Preparation of target cells SVK 14 cells were seeded at various cell numbers (1×10^4 to 1.6×10^5) in 30 mm dishes, two replicates per cell number. The cells were cultured in RPMI 1640 plus 10% FCS and incubated for 24 hours at 37°C, 8% CO₂.

The fibroblasts were seeded at various cell numbers (1×10^4 to 2×10^5) in 30 mm dishes, two replicates per cell number. The cells were cultured in DMEM plus 10% FCS for 24 hours at 37°C, in 8% CO₂.

Passage 2 basal cells were used to study the relationship between cell number and MTT reduction. Primary keratinocyte cultures were established from breast



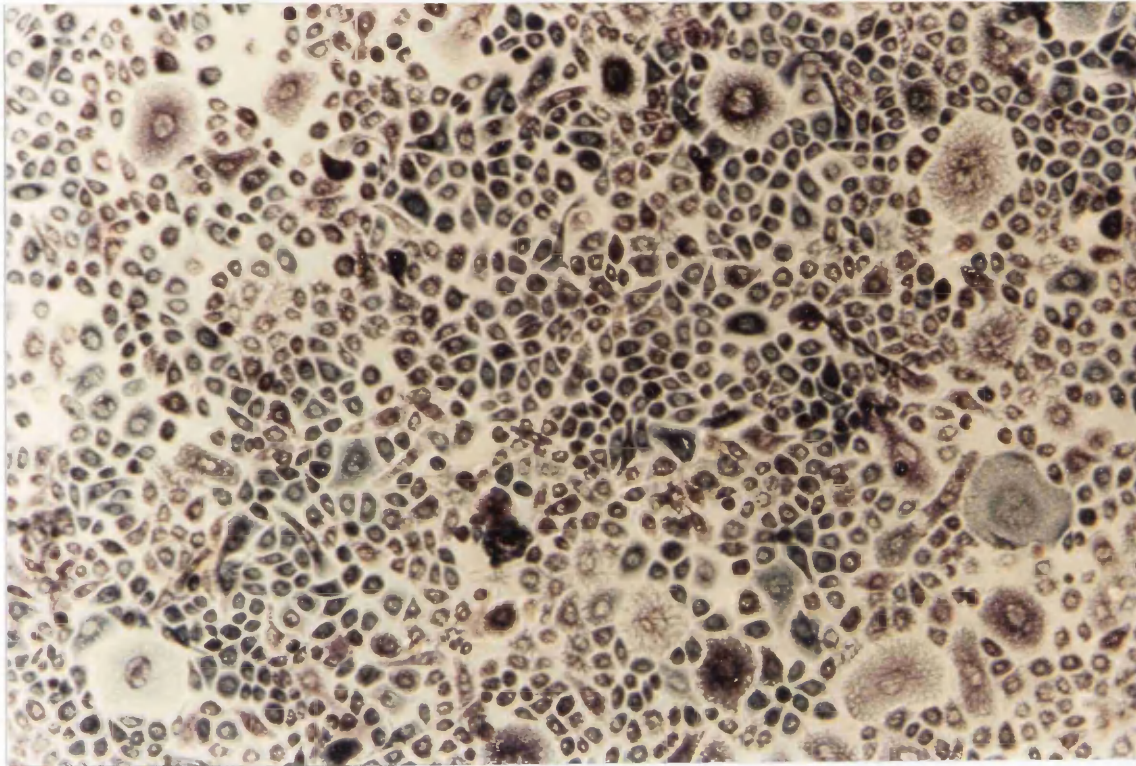
a



b

Figure 4.1 a,b Cell viability was assessed using the MTT colorimetric assay

- a) MTT is a yellow tetrazolium salt
- b) The compound is reduced to an insoluble blue formazan product by viable cells. The figure shows increase in formazan product with increase in SVK 14 cell number (row 1 [left] no cells; row 2-5 increasing cell number up to 1×10^5 [row 5 and 6 have equal cell numbers]).



C

Figure 4.1 c Reduction of yellow MTT dye by basal keratinocytes to an intracellular, insoluble blue formazan product. (x250)

skin using the Rheinwald and Green (1975) method as described in Section 2.3.2. In order that the number of seeded cells would approximate to the number counted, a population of basal cells was used, rather than a stratifying culture. The basal cells were obtained in the following way: primary keratinocyte cultures were established in 50 cm² flasks. When the cells were just subconfluent the cells were switched to low calcium medium, KCM-Ca (see Section 2.3.2). The keratinocytes were cultured in the low calcium medium for three days and then trypsinized in 0.05% trypsin, spun down, resuspended in normal keratinocyte culture medium and counted in a Coulter counter. Three 24-well plates were set up. Half the first plate contained 1.5×10^5 cells per well, the other half, 1×10^5 cells per well. In the second plate twelve wells contained 0.75×10^5 cells per well, and twelve 5×10^5 per well. The third plate contained 0.25×10^5 cells in twelve wells. The cells remained in KCM for 24 hours to allow attachment and were then switched to the low calcium medium for three days prior to counting.

Two wells/dishes containing culture medium alone served as blanks for each cell type to act as controls for non-specific binding of the dye.

Dye reduction and assay - The medium was removed and the cells were washed twice with PBS-abc. RPMI 1640 1ml per well was added to the keratinocytes, and DMEM 1 ml per well was added to the fibroblasts. To each well 200 μ l of MTT dye (5 mg/ml) were added. The plates were wrapped in foil to prevent exposure to light. These plates were then reincubated at 37°C for 1-4 h, depending on cell type (1 hour for basal keratinocytes, 2 hours for fibroblasts, 4hours for SVK 14 cells), to allow viable cells to reduce the dye to the purple formazan. The formazan product is insoluble in aqueous solvents and hence the dye stays associated with the cell. The unreacted dye was then removed from the wells and 1 ml of acidified isopropyl alcohol was added. Solubilisation of the dye was achieved by shaking the plates on a cell shaker. The optical density was then read in a spectrophotometer, and the optical density (OD) of each well read at 570 nm. The OD of the blank wells was subtracted to give a final OD for each cell count.

Cell counting - For each cell type half the wells/dishes were analysed by the MTT assay and half by Coulter counting. Prior to cell counting the medium was aspirated and the cells washed twice with PBS-abc. The cells were then trypsinized with 1 ml of trypsin, and 5ml FCS was added before the cell suspension was syringed through a 16 gauge needle. The cells were then added to 9 ml of Isoton and counted in a Coulter counter.

Results

The utility of MTT as a quantitative marker for viable cells was tested by determining the relationship between target cell number and the amount of MTT formazan product. Using simple regression analysis, for all three cell types a linear relationship was observed between absorbance of dye and cell number over the range of cell numbers used (basal keratinocytes, 0.13×10^5 - 1.1×10^5 ; fibroblasts, 0.105×10^5 - 2.06×10^5 ; SVK 14 cells, 0.122×10^5 - 2.07×10^5) (**Figures 4.2 a,b,c**). For basal keratinocytes using simple regression analysis, comparison of cell counting and the MTT assay produced a correlation coefficient of 0.99, ($p=0.00002$); for fibroblasts the correlation coefficient was 0.99 ($p=0.00008$) and for SVK 14 cells the correlation coefficient was 0.99 ($p=0.000035$). 1×10^5 cells of each cell type yielded 0.8 OD units when exposed to the MTT dye for an appropriate period of time (1 hour for basal keratinocytes, 2 hours for fibroblasts, and 4 hours for SVK 14 cells).

Comment

Cell counts of attached basal keratinocytes, fibroblasts and SVK 14 cells show good correlation with the MTT assay over the range of cell numbers tested. The results suggest that the MTT assay may be used instead of cell counting. These findings have generated a valuable method for measuring proliferation of keratinocytes, now in use in the Department of Experimental Dermatology, at The Royal London Hospital.

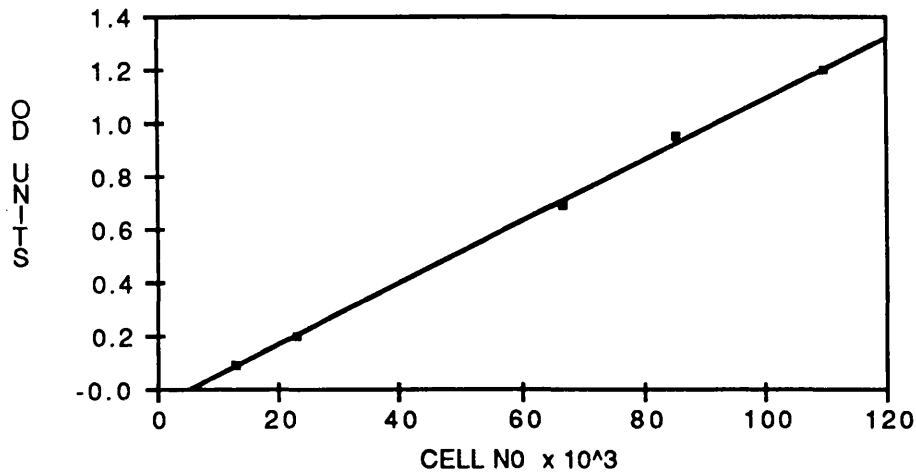


Figure 4.2 a

Relationship between mean basal keratinocyte cell number and mean optical density (OD) generated by the MTT assay. Each point represents the mean of 6 replicates. The straight line plotted is the best fit line calculated using all points from 0.13×10^5 to 1.1×10^5 cells per well.

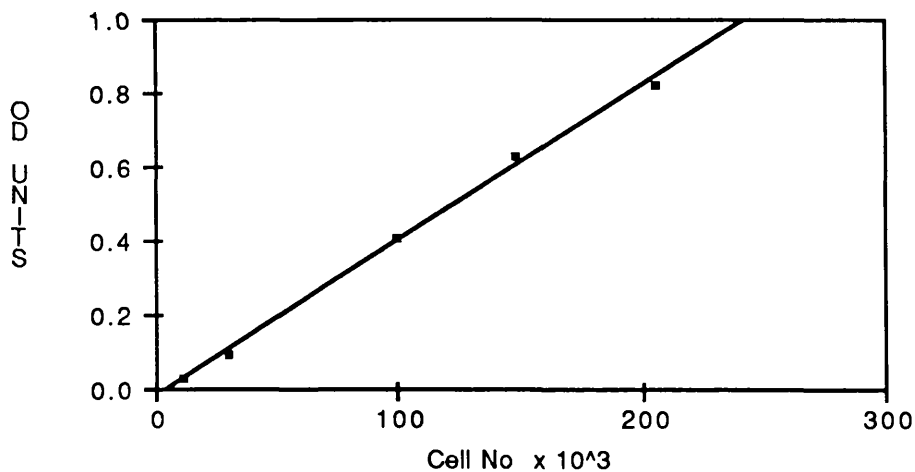


Figure 4.2b

Relationship between mean fibroblast cell number and mean optical density (OD) generated by the MTT assay. Each point shows the mean of 3 replicates. The straight line plotted is the best fit line calculated using all points from 0.105×10^5 to 2.05×10^5 cells per well.

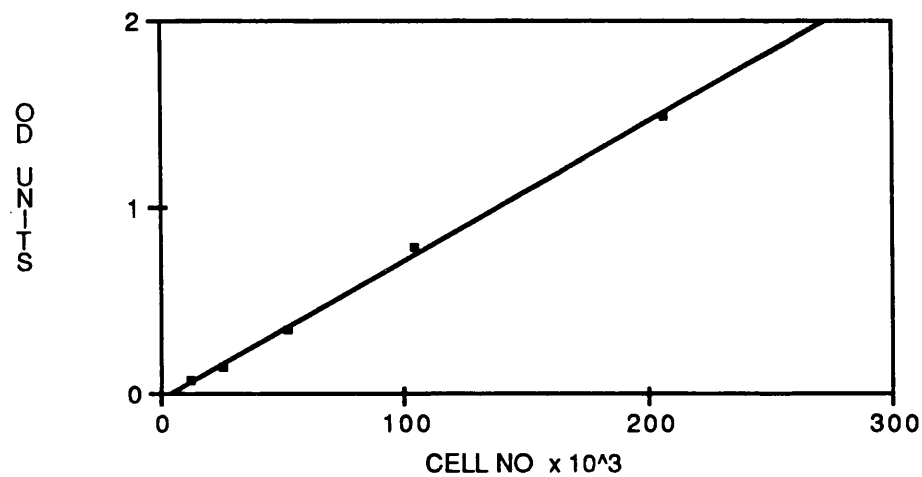


Figure 4.2.c

Relationship between SVK_14 mean cell number and mean optical density (OD) generated by the MTT assay. Each point shows the mean of 3 replicates. The straight line plotted is the best fit line calculated using all points from 0.12×10^5 to 2.07×10^5 cells per well.

4.6.2 ANTISEPTIC CYTOTOXICITY ASSAY: CORRELATION OF MTT ASSAY WITH THE CELL COUNTING/VIABILITY METHOD

Although there is a linear relationship between cell number and MTT reduction, this may not be maintained when cells are damaged, but not killed by toxic agents. In order to establish that the numbers of viable cells remaining following treatment with an antiseptic agent show correlation with the MTT assay, comparison of the two methods were made following treatment with three agents, cetrimide, sodium hypochlorite and hydrogen peroxide. For this study only the SVK 14 cells were used.

Materials and methods

Cell culture - SVK 14 cells were seeded in 30 mm dishes, 1.2×10^5 cells per dish, in RPMI 1640 medium with 10% FCS and incubated overnight at 37°C, in 8% CO₂. Nine dishes were set up for each test concentration, three for MTT dye reduction, and six for Coulter counting and trypan blue staining.

Antiseptic cytotoxicity assay - Three agents were tested: cetrimide, sodium hypochlorite and hydrogen peroxide. These agents were serially diluted in PBS-abc and the range of concentrations tested are shown in **Tables 4.4 a,b,c**. The cells were washed twice prior to exposure to the antiseptic agent for 15 minutes. The agent was then removed, the cells washed twice, and the medium replaced prior to reincubation at 37°C, in 8% CO₂.

MTT dye reduction - The medium was removed from the dishes and 1ml of RPMI 1640 and 200ul of MTT dye was added to each dish. The cells were incubated with the dye for 4 hours. The unreacted dye was then removed and 1 ml of acidified isopropyl alcohol added. The OD of the isopropyl alcohol eluate was read in a spectrophotometer at 570 nm.

Cell counting/viability stain - Of the remaining six replicates, the cells in three were trypsinized and counted in a Coulter counter as outlined in Section 4.4, Materials and methods; the cells in the last three dishes were trypsinized and

Hydrogen peroxide Concentration (M)	Percent viability cf control			
	Experiment 1 Cell counting	MTT	Experiment 2 Cell counting	MTT
8.8×10^{-2}	0	0	0	0
8.8×10^{-3}	50 ± 6	47 ± 5	55 ± 7	50 ± 4
8.8×10^{-4}	80 ± 7	77 ± 4	84 ± 8	81 ± 5
8.8×10^{-5}	91 ± 3	88 ± 4	93 ± 5	89 ± 4
8.8×10^{-6}	104 ± 4	96 ± 4	99 ± 8	96 ± 5

Table 4.4a

Sodium hypochlorite Concentration (M)	Percent viability cf control			
	Experiment 1 Cell counting	MTT	Experiment 2 Cell counting	MTT
1.4×10^{-3}	0	0	0	0
1.4×10^{-4}	55 ± 7	51 ± 4	± 56	52 ± 6
1.4×10^{-5}	104 ± 8	99 ± 3	98 ± 9	102 ± 7

Table 4.4b

Cetrimide Concentration (M)	Percent viability cf control			
	Experiment 1 Cell counting	MTT	Experiment 2 Cell counting	MTT
1.5×10^{-4}	0	0	0	0
3.75×10^{-5}	51 ± 8	45 ± 4	51 ± 9	51 ± 5
9.4×10^{-6}	84 ± 7	78 ± 4	83 ± 7	81 ± 3
2.3×10^{-6}	93 ± 6	95 ± 3	98 ± 5	94 ± 4
5.8×10^{-7}	97 ± 5	99 ± 4	106 ± 5	101 ± 4

Table 4.4c

TABLES 4.4 a,b,c Comparison of the results of the cell counting/viability assay to the MTT assay for the effects of cetrimide, sodium hypochlorite and hydrogen peroxide on SVK 14 cells. Values show the mean of three replicates \pm 1 SD. Comparison of results for hydrogen peroxide showed a correlation coefficient (r) of 0.99, $p < 0.01$ for experiment 1; for experiment 2 $r = 0.99$, and $p < 0.001$. Comparison of results for sodium hypochlorite showed: experiment 1, $r = 0.01$; experiment 2, $r = 0.99$, $p < 0.05$. Comparison of results for cetrimide showed: experiment 1 $r = 0.99$, $p < 0.001$; experiment 2 $r = 0.99$, $p < 0.001$.

stained with trypan blue and counted in a haemocytometer (see Section 4.4. Materials and methods), so that a percentage viability of the total count could be established. Cell viability counts for each concentration were expressed as a percentage of the control to give a percent viability.

The experiments were repeated twice.

Results

The results of the experiments are shown in **Tables 4.4 a,b,c**. Comparison between the results of the MTT and counting assays using regression analysis gave the following results: for cetrimide correlation was good (experiment 1, correlation coefficient (r) = 0.99, $p < 0.005$; experiment 2 $r = 0.99$, $p < 0.001$) and similarly for hydrogen peroxide (experiment 1, correlation coefficient $r = 0.99$, $p < 0.001$; experiment 2 $r = 0.99$, $p < 0.001$). For sodium hypochlorite experiments 1 and 2 also showed good correlation (Experiment 1 $r = 0.99$, $p = 0.01$; experiment 2 $r = 0.99$, $p = 0.045$). The coefficient of variation for the replicates used in the MTT assay was consistently below 5%.

Comment

There was a linear correlation between SVK 14 cell number and the reduction of the MTT dye by the cells. This correlation was maintained in the antiseptic cytotoxicity assay. The MTT assay had several advantages over counting techniques; the manipulations were simpler and therefore the method was less laborious with a reduced chance of introduction of error. The methodology for the MTT assay outlined here could be further improved by the introduction of a microtitre plate and a microplate spectrophotometric measurement, such as an ELISA reader. This was not judged suitable for this study, because of the timed exposure to a range of concentrations of the test agent, which is easier to conduct in larger wells, and experiments were, therefore, subsequently performed in 24-well plates. It was concluded that the MTT colorimetric assay is as reliable as counting techniques but

has the advantage of greater simplicity and rapidity. Hence for all further experiments only this technique was used.

4.7 SUMMARY

An attached cell assay (SVK 14 cells) was used to establish the cytotoxicity assay. In initial experiments, viability was assessed using trypan blue, which is simple and cheap. It was assumed that, following treatment, dead cells would detach and only viable cells remain attached. Results showed that only non-viable cells detached, and therefore, these cells could be ignored in subsequent assays. However for the attached cells, high concentrations of certain test agents produced populations of cells which were of mixed viability. The vital staining technique was laborious, but any further assay must clearly include a measure of viability. A methylene blue assay was subsequently tried; although a dose-dependent response could be demonstrated with this technique, it was noted that non-viable cells took up the dye, albeit to a lesser degree than the viable cells. Additionally, it was noted that dye eluted from the cells during washing steps. A third cytotoxicity assay, the MTT assay, was investigated. This proved to be simple and reliable and correlated well with the cell counting/viability assay and it was proposed to use this for subsequent studies.

CHAPTER 5

A COMPARATIVE STUDY OF THE CYTOTOXIC EFFECTS OF
ANTISEPTIC AGENTS ON BASAL KERATINOCYTES,
TRANSFORMED KERATINOCYTES AND FIBROBLASTS

CONTENTS

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5.1 INTRODUCTION

The purpose of this project was to establish an in vitro cytotoxicity assay which would allow comparison of the effect of a range of antiseptic agents on a number of cell types. Basal keratinocytes and fibroblasts are fundamental to the wound healing process and whilst a number of studies have examined the effect of antiseptic agents on fibroblasts (**Table 1.5, 1.6, 1.7**), few data were available on the susceptibility of cutaneous keratinocytes. This study is designed to compare the cytotoxic effects of antiseptics on fibroblasts and basal keratinocytes, and to assess whether the epithelial cells are more or less sensitive to the adverse effects of these agents. Many human cells require exacting conditions for culture and this is particularly so of keratinocytes; therefore the agents studied were tested on normal keratinocytes and compared to their effect the SVK 14 cells. The demonstration that both normal keratinocytes and the SVK 14 cells show similar susceptibilities to the cytotoxic effects of the antiseptic agents would allow the transformed cells to be substituted for normal keratinocytes, thereby simplifying the investigation of antiseptic cytotoxicity on cutaneous epithelium in vitro.

A further purpose of this study is to compare the toxicity of individual agents in the cytotoxicity assay devised, and compare the findings to published in vivo and in vitro data.

5.2 METHODS

To study the susceptibility of basal keratinocytes, fibroblasts and SVK 14 cells to antiseptic agents a restricted range was initially used (hydrogen peroxide, chlorhexidine and sodium hypochlorite). Cetrimide was subsequently tested on the SVK 14 cells alone. The agents were studied using the methods defined in Chapters 2, 3 and 4.

Cell culture - Primary epidermal keratinocyte cultures were obtained by culturing keratinocytes from adult breast skin and foreskin. The primary cultures

were subcultured without feeder cells into 24-well plates at a density of 1×10^5 cells per well and grown for 5 days in KCM at 37°C in 8% CO_2 . The cells were then washed twice in PBSa and switched to low calcium medium (KCM-Ca) for 3 days to induce destratification. At the time of exposure the cells were 80% confluent. Prior to exposure to the antiseptic agents the cells were washed twice in PBS-abc to remove all traces of suprabasal cells.

The SVK 14 cell line was used between the 10th and 20th passages. The cells were subcultured into a 24-well plate with 1×10^5 cells per well and cultured in RPMI 1640 plus 10% FCS for 24 hours at 37°C in 8% CO_2 prior to exposure to the antiseptic agents.

Fibroblasts between passages 3 to 7 were used for experiments. For the cytotoxicity assay the cells were subcultured into a 24-well plate, 1×10^5 fibroblasts per well and cultured in DMEM plus 10% FCS for 24 hours at 37°C in 8% CO_2 prior to exposure to the test agents.

For each cell type two wells contained no cells and were treated identically to the cells. These served as blanks to measure non-specific absorption of the MTT dye.

Exposure to antiseptic agents - Hydrogen peroxide (obtained as a 30% solution, Sigma Chemical Company, Poole, UK.), sodium hypochlorite (Aldrich Chemical Co. Ltd., U.K.) and chlorhexidine digluconate (obtained as 20% solution, Sigma Chemical Company, Poole, UK.) were tested on all three cell types. Cetrimide (Sigma Chemical Company, Poole, UK) was tested on the SVK 14 cell line alone and was prepared as a 1% w/v solution in PBS-abc which was sterilized by filtration. The agents were all freshly prepared prior to use and serially diluted in PBS-abc. The range of concentrations used was based on the ranges established in the preliminary experiments previously described, with narrower ranges at subsequent attempts (Chapter 4, Section 4.4). The cells were washed twice in PBS-a, and exposed to serial dilutions of the antiseptic agents for 15 minutes. Each concentration was

tested on four replicate wells. Control wells were treated with PBS-abc alone. The agents were removed, the cells washed twice with PBSa and the appropriate medium replaced. The cells were then reincubated at 37⁰C in 8% CO₂ for 24 hours. Each experiment was repeated four times.

Cytotoxicity Assay - The number of viable cells remaining in each well was evaluated using a colorimetric assay based on the tetrazolium salt, MTT (for method see Section 4.6). The optical density (OD) of the isopropyl alcohol in each well was read in a spectrophotometer at 570 nm; the final OD value for each well was generated by subtracting the mean OD for the blank wells. The OD values for each concentration were expressed as a percentage of the control values. The inferred percentage viability, based on the mean OD reading for each concentration compared to the mean OD for the control, was used to establish dose-response curves.

The MTT assay measures cell survival. The effect dose 50 percent (ED 50) was defined as the concentration allowing 50% survival of cells and was determined by linear regression analysis.

Statistical analysis - The significance of the difference of response of each cell type to the different agents was based on the Mann Whitney U test. This test was chosen because the samples were small and did not necessarily represent a normal distribution.

5.3 RESULTS

Log-dose response curves were established for each agent and cell type. **Figure 5.1** shows representative log-dose response for the effect of sodium hypochlorite and chlorhexidine on basal keratinocytes, fibroblasts and SVK 14 cells. **Figure 5.2** shows representative dose response curves for the effect of hydrogen peroxide on the

Figure 5.1

Log dose-response curves for chlorhexidine (Cx) and sodium hypochlorite (S) on basal keratinocytes, SVK 14 cells and fibroblasts. The figure shows one representative experiment for each agent and cell type. Points represent the mean of 4 replicate wells, with a coefficient of variation of less than 10%.

KEY

Sodium hypochlorite/SVK 14 cells = +

Sodium hypochlorite/keratinocytes = O

Sodium hypochlorite/fibroblasts = —

Chlorhexidine/SVK 14 cells = ×

Chlorhexidine/keratinocytes = ◇

Chlorhexidine/fibroblasts = ----

Therapeutic concentration of each agent -----

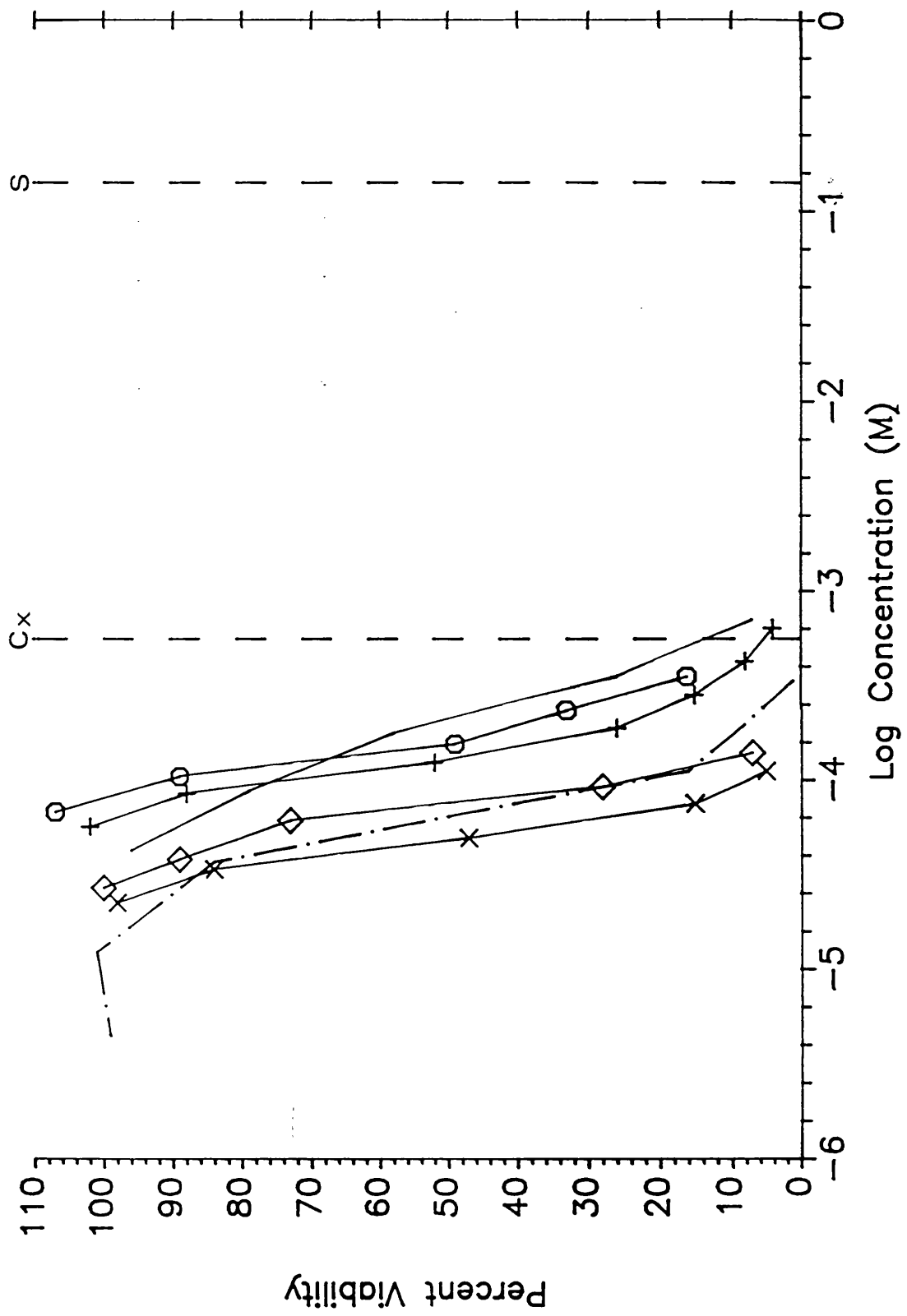


FIGURE 5.1

Figure 5.2

Log dose-response curves for hydrogen peroxide (H) and cetrimide (C) on basal keratinocytes, SVK 14 cells and fibroblasts. The figure shows one representative experiment for each agent and cell type. Points represent the mean of 4 replicate wells, with a coefficient of variation of less than 10%.

KEY

Hydrogen peroxide/SVK 14 cells = □

Hydrogen peroxide/keratinocytes = □

Hydrogen peroxide/fibroblasts = —

Cetrimide/SVK 14 cells = ◇

Therapeutic concentration of each agent -----

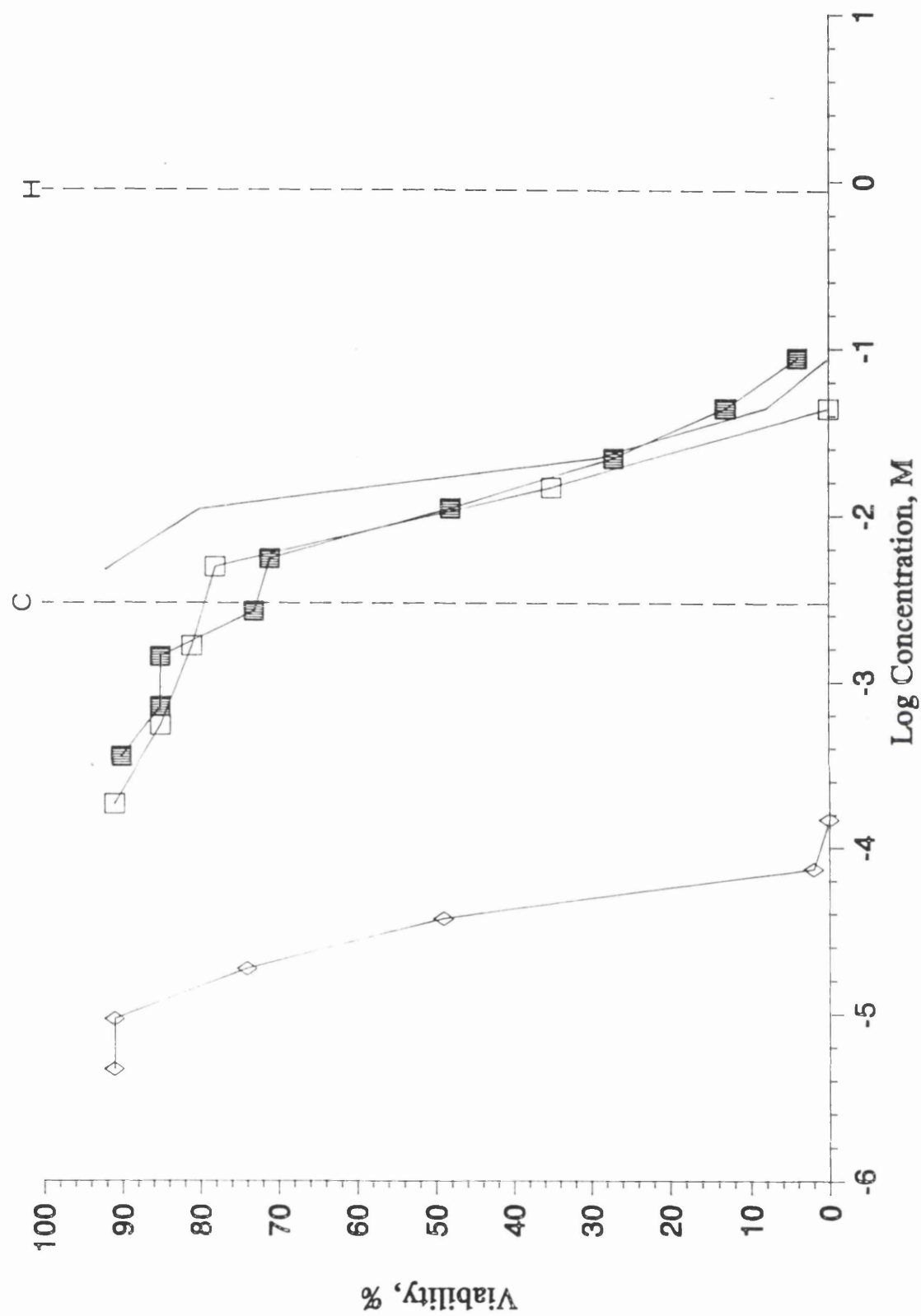


FIGURE 5.2

three cell types and for cetrimide tested on SVK 14 cells alone. With the exception of hydrogen peroxide on SVK 14 cells and basal keratinocytes, all agents and cell types showed a sigmoid dose response curve. The effect of hydrogen peroxide on SVK 14 cells and basal keratinocytes gave a biphasic log-dose response curve (**Figure 5.2**).

At therapeutic concentrations all of the antiseptics were 100% cytotoxic to all cell types. The median ED50 values and ranges for each agent and cell type are shown in **Table 5.1**. Statistical comparison of mean ED50 values (Mann-

Whitney U test) showed that keratinocytes and fibroblasts were equally susceptible to all agents. SVK 14 cells and keratinocytes were equally sensitive to hydrogen peroxide but the SVK 14 cells were more susceptible to this agent than fibroblasts. The transformed keratinocytes showed a small, but statistically significant, increase in susceptibility to chlorhexidine and sodium hypochlorite when compared to both fibroblasts and keratinocytes. All comparisons were tested at the 5% level of significance.

A direct comparison of the median ED50 concentrations for the three agents tested on all cell types produces the following toxicity ranking order: Chlorhexidine > sodium hypochlorite > hydrogen peroxide. Analysis of the SVK 14 data alone produced the same ranking but included the cetrimide data as follows: Cetrimide > chlorhexidine > hydrogen peroxide > sodium hypochlorite.

Previous studies have compared the toxicity of agents by analysing the number of ten-fold dilutions from the therapeutic concentration necessary to eradicate toxicity. When the data generated here were further compared by examining the dilution from the standard concentration (**Table 5.2**) necessary to allow 50% cell survival the ranking order of toxicity was as follows: sodium hypochlorite > cetrimide > hydrogen peroxide > chlorhexidine.

As cell type did not affect this toxicity ranking, comparison of only the SVK 14 cell data are shown in **Table 5.2**.

AGENT	SVK 14	Keratinocyte	P value	SVK 14	Fibroblast	P value	Fibroblast	Keratinocyte	P value
Hydrogen peroxide	9.5x10 ⁻³ (9.0x10 ⁻³ , 11.0x10 ⁻³)	10.7x10 ⁻³ (9.9x10 ⁻³ , 13.0x10 ⁻³)	0.19	9.5x10 ⁻³ (9.0x10 ⁻³ , 11.0x10 ⁻³)	14.5x10 ⁻³ (12.7x10 ⁻³ , 17.9x10 ⁻³)	0.016*	14.5x10 ⁻³ (12.7x10 ⁻³ , 17.9x10 ⁻³)	10.7x10 ⁻³ (9.9x10 ⁻³ , 13.0x10 ⁻³)	0.058
Chlorhexidine	4.8x10 ⁻⁵ (4.3x10 ⁻⁵ , 5.4x10 ⁻⁵)	7.7x10 ⁻⁵ (6.7x10 ⁻⁵ , 7.8x10 ⁻⁵)	0.016*	4.8x10 ⁻⁵ (4.3x10 ⁻⁵ , 5.4x10 ⁻⁵)	8.9x10 ⁻⁵ (5.4x10 ⁻⁵ , 9.6x10 ⁻⁵)	0.026*	8.9x10 ⁻⁵ (5.4x10 ⁻⁵ , 9.6x10 ⁻⁵)	7.7x10 ⁻⁵ (6.7x10 ⁻⁵ , 7.8x10 ⁻⁵)	0.11
Sodium hypochlorite	1.25x10 ⁻⁴ (1.1x10 ⁻⁴ , 1.3x10 ⁻⁴)	1.7x10 ⁻⁴ (1.4x10 ⁻⁴ , 1.8x10 ⁻⁴)	0.028*	1.25x10 ⁻⁴ (1.1x10 ⁻⁴ , 1.3x10 ⁻⁴)	1.7x10 ⁻⁴ (1.7x10 ⁻⁴ , 2.0x10 ⁻⁴)	0.028*	1.7x10 ⁻⁴ (1.7x10 ⁻⁴ , 2.0x10 ⁻⁴)	1.7x10 ⁻⁴ (1.4x10 ⁻⁴ , 1.8x10 ⁻⁴)	0.255
Cetrimide	3.0x10 ⁻⁵ (2.7x10 ⁻⁵ , 3.1x10 ⁻⁵)	-	-	3.0x10 ⁻⁵ (2.7x10 ⁻⁵ , 3.1x10 ⁻⁵)	-	-	-	-	-

TABLE 5.1 Comparison of median ED 50 values (M) for three agents tested on basal keratinocytes, fibroblasts and SVK 14 cells. Ranges are shown in parenthesis; n=4. * indicates significantly different results at the 5% level (p<0.05, Mann Whitney U test)

TABLE 5.2 Comparison of median ED 50 values for SVK 14 cells to the therapeutic concentration (TC) used in cutaneous wounds.

AGENT	TC (M)	DILUTION FROM TC TO ACHIEVE 50% CELL SURVIVAL
Chlorhexidine	5.6×10^{-4}	x 12
Hydrogen peroxide	8.8×10^{-1}	x 90
Cetrimide	3×10^{-3}	x 100
Sodium hypochlorite	1.4×10^{-1}	x 1,145

5.4 COMMENT

The results of this study showed that human cultured keratinocytes and fibroblasts were highly susceptible to the effects of commonly used antiseptics at concentrations used on cutaneous wounds, and to free-chlorine agents at high dilution. Whilst keratinocytes and fibroblasts showed equal susceptibility to the antiseptic agents tested, the sensitivity of the SVK 14 was slightly increased. Such a comparison has not been previously documented, but in a brief report Capin et al. (1985) described the differential susceptibility to injury by hydrogen peroxide of fibroblasts, keratinocytes, endothelial cells and melanocytes and showed the following results: fibroblasts < keratinocytes < endothelial cells < melanocytes. Although, in this study no statistical differences in susceptibility to hydrogen peroxide were shown between fibroblasts and keratinocytes ($p=0.058$, Mann Whitney U test), the results showed an obvious trend in which the fibroblasts appear less sensitive to hydrogen peroxide than the keratinocytes.

Although the basal keratinocytes and SVK 14 cells showed differences in their sensitivity to chlorhexidine and sodium hypochlorite, it is of interest that these cells appeared equally susceptible to hydrogen peroxide and showed similar dose-response curves (**Figure 5.2**). This suggests that the transformed cell type responds similarly to basal keratinocytes, in response to treatment with natural killing substances and confirms the hypothesis that the SVK 14 cells may be of value in studying keratinocyte physiology. The effect of hydrogen peroxide on basal cells and transformed keratinocytes gave a biphasic dose-response curve; the second phase of the response, seen at lower concentrations, could be attributed to an effect on a specific populations of cells, possibly in the proliferative compartment. Alternatively, at low concentration, hydrogen peroxide may inhibit keratinocyte cell growth, since proliferation is possible during the 24 hour recovery period following exposure to the test agent.

The mode of action of most antiseptic agents is poorly understood; in bacteria at low concentration, chlorhexidine acts by damaging the semi-permeable cell

membrane (Gardner and Gray, 1983). It is possible to attribute the increased chlorhexidine sensitivity of SVK 14 cells to differences in membrane structure between the transformed and normal cells. However this specific mode of action of chlorhexidine can only be determined at low concentration since, at high concentrations, where agents are rapidly bacteriocidal, membrane leakage does not occur because membrane and cytoplasmic contents are precipitated. Hydrogen peroxide is thought to kill by production of the hydroxyl free radical which can attack cell membranes, DNA and other cell components. Defences against free radicals are present in all respiring cells and differences in enzyme activity may account for differences in susceptibility between the cell types, however it is more likely that these defence mechanisms are over whelmed by the volume of hydrogen peroxide used in this assay. It is likely that at high concentrations all of the agents tested kill rapidly by non specific means and the differences observed probably cannot be attributed to physiological differences between cell types. At low concentrations specific modes of actions may be operative, as is observed in bacteria.

A possible explanation for the observed differences in sensitivity between the various cell types may be based on the amounts of organic material that the cell culture system represents. It is well recognised that iodine and free-chlorine agents are highly influenced by the presence of organic material and hydrogen peroxide is unstable in the presence of organic material. Organic material has a small effect on the activity of chlorhexidine (Ulrich, 1981). Although the different cell culture systems contain equal cell numbers they may not represent equal amounts of organic material. The most striking differences were noted between fibroblasts and SVK 14 cells and since fibroblasts are larger cells (comparative cell diameters [range]: basal keratinocyte 10-13 μm ; fibroblasts 12-14 μm ; keratinocytes 10-40 μm ; personal communication Dr C. Ling, Imperial Cancer Research Fund, London) than the transformed keratinocytes they may represent a greater organic load, giving rise to decreased susceptibility to the agents tested.

Differences between ED50 values for the normal cells and the transformed keratinocytes, whilst statistically significant at the 5% level, are of small orders of magnitude and do not affect the ranking order of the agents i.e. as with other cell types the SVK 14 cells are most susceptible to the effects of sodium hypochlorite and least sensitive to chlorhexidine. For the purposes of studying cytotoxicity of individual agents in vitro, the three mammalian cell types have equal predictive value. The choice of cell type therefore concentrates on practical considerations. Transformed keratinocytes have the advantage of ready availability and an indefinite lifespan. However although keratinocytes demand complex culture conditions and will only survive to the fourth, fifth or sixth passage, fibroblasts can be easily cultured to the fiftieth passage, although their characteristics may change with higher passage. However, in this study comparison of ED50 concentrations showed that the transformed cells gave the least inter-experiment variation, whilst fibroblasts gave the most (**Table 5.1**). For this reason the cells SVK 14 cells were used in all future assays. ✓

Comparison of ED50 values to the concentration in clinical usage shows that sodium hypochlorite is the most toxic agent and chlorhexidine the least. Cetrimide and hydrogen peroxide are intermediate in their toxicity, relative to other agents. Comparison of data from other studies is difficult as no attempt at accurate comparison i.e LD50 or ED50 values for the agents tested has been made; however several authors have examined the number of ten-fold dilutions necessary to eradicate the toxicity of an agent (Lineaweaver et al, 1985; Leaper and Brennan, 1986; Deas et al, 1986). Whilst a number of in vitro studies of antiseptic toxicity have been published (**Tables 1.5, 1.6, 1.7**), few have examined a range of commonly used antiseptics, and only three studies have been traced in which this was done (**Table 5.3**). By comparing the number of ten-fold dilutions necessary to eradicate toxicity, two studies show that the free-chlorine agents are the least toxic agents and hydrogen peroxide the most; parallel in vivo data do not support these findings. A study by Deas et al (1986) shows findings which are in agreement with those

Table 5.3 Relative toxicities of a range of agents tested in three separate studies

REFERENCE	AGENTS TESTED AND TOXICITY RANKING
Lineaweaver et al, 1985	Sodium hypochlorite=Acetic acid< Povidone iodine=Hydrogen peroxide
Leaper et al, 1986	Chloramine T< Phenol 1=Chlorhexidine< Hexachlorophene=Hydrogen peroxide=Povidone iodine
Deas et al, 1986	Savlodil=Hydrogen peroxide< Chlorhexidine=Povidone iodine< Phenol< Chloramine T

presented here i.e. hydrogen peroxide and chlorhexidine are less toxic than the chlorine releasing agent, chloramine T.

Reviewing the relevant literature in which the *in vivo* toxicity of antiseptic agents has been studied, reveals that the majority of studies report findings which suggest that chlorine-releasing agents are more toxic to cutaneous wounds than chlorhexidine, observations which are in line with those presented here. Lineaweaver et al (1985) examined the effect of sodium hypochlorite 0.5%, povidone iodine 1%, acetic acid 0.25% and hydrogen peroxide 3% on rat full thickness wounds; reepithelialisation was delayed at 8 days by acetic acid, povidone iodine and sodium hypochlorite but at 16 days only by the sodium hypochlorite. In a further study, Brennan, Foster and Leaper (1986) evaluated the effect of chlorhexidine 0.05% and chloramine T 1% on epithelialisation and formation of granulation tissue; both of these parameters were delayed by chloramine T but chlorhexidine had no adverse effect. Brennan and Leaper (1985) used the rabbit ear chamber to study the effect of Eusol, povidone iodine 5% and 1%, hydrogen peroxide 3%, chloramine T 1% and chlorhexidine 0.05% on blood vessels and formation of granulation tissue. The chlorine-releasing compounds caused destruction of granulation tissue. Chlorhexidine, hydrogen peroxide and povidone iodine 1% had no sustained adverse effects. Whilst one group of investigators have consistently shown that chlorhexidine is non-toxic and chlorine-releasing compounds adversely affect wounds, (Brennan and Leaper, 1985; Brennan, Foster and Leaper, 1986), Niedner and Schopf (1986) showed that chlorhexidine 0.5% substantially inhibited the formation of granulation tissue but chloramine T 10% had no significant effect. These findings are the reverse of the previous studies detailed here. The toxicity of chlorhexidine may be explained by the use of a concentration of 0.5% w/v, which is ten times the recommended concentration for use in a wound (Reynolds, 1989 ii).

When evaluating relative *in vitro* toxicities it is important to note that all agents are toxic at *in 'use'* concentrations, which is not necessarily the case *in vivo*.

At the concentration used in a wound chlorhexidine is toxic in cell culture but several studies have shown that this agent does not inhibit wound healing. When antiseptics are applied to a wound model and their immediate effects on vasculature are studied, all agents show an adverse effect (Branemark, 1966; Brennan and Leaper, 1985; see Chapter 1, Section 1.6.1). For certain agents this effect is reversed over a period of hours or days such that there is no effect on the parameter of wound healing that is being measured. Clearly there is no capacity for recovery in the short term assay described and all agents are toxic at the 'in use' concentration.

The demonstrated toxicity of antiseptics on basal keratinocytes is of particular importance to the clinical application of cultured keratinocytes (culture grafts) to enhance wound healing. Sheets of cultured keratinocytes have been used successfully to treat chronic cutaneous ulcers and burns. Although the best cover for a skin defect is normal skin using conventional grafting techniques, a substantial donor site may be required which may be slow to heal or there may be insufficient donor skin in a badly burned patient. Recent advances in culture techniques allow sheets of keratinocytes to be grown from very small skin samples. Both cultured keratinocyte allografts and autografts have been used to enhance wound healing (Leigh and Purkis, 1986; Leigh et al, 1987) Available data suggest that allografted keratinocytes do not 'take' (Brain et al, 1989), but promote wound healing. Some workers have claimed that cultured keratinocytes have no benefit in wound healing but poor outcome has been attributed to the concurrent use of antiseptic agents (IM Leigh, personal communication). The findings presented here suggest that the application of antiseptics prior to or following the application of a culture graft is contraindicated.

5.5 SUMMARY

- 1) Basal keratinocytes and SVK 14 cells showed similar responses to hydrogen peroxide, a natural killing substance, adding support to the view that the transformed keratinocyte line may represent an alternative to basal keratinocytes in the study of keratinocyte biology.
- 2) Fibroblasts and basal keratinocytes showed equal susceptibility to the cytotoxic effects of hydrogen peroxide, chlorhexidine and sodium hypochlorite. SVK 14 cells were more susceptible to these agents but have the same predictive value when the toxicity of the individual agents are ranked by comparing the ED50 concentrations to the concentration in clinical usage. The SVK 14 cells have practical advantages over the normal cell types and can, therefore, usefully replace them in an antiseptic cytotoxicity assay.
- 3) Comparison of the relative toxicities of the agents tested showed that sodium hypochlorite was the most toxic agent and chlorhexidine the least. These findings are in line with the results of a number of studies on the adverse effects of antiseptics on cutaneous wounds.
- 4) All agents were toxic to basal keratinocytes at concentrations applied to a wound. These findings suggest that antiseptic agents should not be used in conjunction with keratinocyte cultures used for grafting.

CHAPTER 6

ASSAY OF ANTISEPTIC AGENTS IN CELL CULTURE - CONDITIONS AFFECTING CYTOTOXICITY

CONTENTS

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6.1 INTRODUCTION

In Chapter 5 data for the relative toxicities of antiseptic agents in vitro were generated and compared to available in vitro data in the literature, with the discovery that the results were conflicting. Results from this study suggest that chlorine releasing antiseptics are the most toxic agents, in line with the majority of in vivo studies of the effects of antiseptics on wound healing. However, data from other cell culture studies suggest that chlorine-releasing agents are less toxic than other commonly used antiseptics. These reported differences in the toxicity of individual agents may be related to variation in experimental conditions. Whilst the methods of assessing antibacterial activity of antiseptic agents in vitro has been standardized to some extent (Bruch, 1983), the choice of experimental conditions for evaluating the cytotoxicity of these agents has varied greatly. Virtually all studies have used fibroblasts and exposed cell numbers have varied from 5×10^3 (Deas et al, 1986) to 2×10^8 (Lineaweaver et al, 1985). Length of exposure varies from 5 minutes (Blenkarn, 1987) to 24 hours (Leaper and Brennan, 1986) and four days (Cooper et al, 1990). Agents have been diluted in saline (Lineaweaver et al, 1985) and medium (Leaper and Brennan, 1986; Deas et al, 1986); in some studies effect on cell growth has been examined and the agents have been diluted in medium plus serum (Shakespeare et al, 1988; Cooper et al, 1990).

In the next study a series of experiments were performed which examined the effect of a number of variables on the toxicity of individual agents. In the previous study 1×10^5 cells were exposed for 15 minutes to an agent diluted in PBS-abc. Here, the following factors which may influence the toxicity of individual agents within a cell culture assay were examined: 1) Duration of exposure 2) The nature of the antiseptic diluent 3) increasing cell number.

The antiseptic cytotoxicity assay is confined to the SVK 14 cell type, because these cells can predict the relative toxicities of antiseptic agents in vitro on human basal keratinocytes and fibroblasts, cells which are crucial to the wound healing process.

6.2 METHODS

Cell culture - SVK 14 cells were cultured and subcultured as previously described.

Cytotoxicity assay - The following procedure was used for all experiments with individual modifications as noted below: 1×10^5 SVK 14 cells were seeded into 24-well plates and cultured in RPMI 1640 plus 10% FCS for 24 hours. Concentrations of three antiseptics, hydrogen peroxide, sodium hypochlorite and chlorhexidine, were prepared by serially diluting the agent (half or third dilutions) in PBS-abc from the concentration in clinical use. Each concentration was tested on four replicate wells. The cells were washed twice prior to the addition of 1ml of the agent for 15 minutes. Control wells were exposed to PBS-abc alone. The drug was then removed, the cells washed and RPMI 1640 plus 10% FCS replaced for a further 24 hours.

The standard conditions outlined above were compared to the following test conditions: 1) diluting the test agent in PBS-abc containing 10% FCS, the exposure time and cell number maintained as for standard conditions 2) diluting the test agent in RPMI 1640, the exposure time and cell number maintained as for standard conditions 3) increasing the number of exposed cells to 5×10^5 , maintaining the exposure time at 15 minutes and using PBS-abc as a diluent 4) Increasing exposure time to 1 hour, diluting the agent in PBS-abc and maintaining the cell number at 1×10^5 as per the standard conditions. For the experiments which used a variation in cell number 6-well plates were used for the standard and test experiments to accommodate the larger cell number. For hydrogen peroxide alone the effect of exposing the cells for 24 hours was studied; for this experiment ten-fold dilutions from the standard concentration were used and the agent was diluted in RPMI medium for standard and test experiments. Diluting in medium is essential because the SVK 14 cell line cannot be maintained in PBS-abc for more than 2 hours. Each experiment was repeated 3 to 4 times, with the exception of the experiment using a 24 hour exposure to hydrogen peroxide which was only performed twice.

The number of viable cells remaining in each well was evaluated using the MTT assay ([see Section 4.6](#)). The percentage viability, based on the MTT reading for each

concentration in comparison to the control wells was used to establish log-dose response curves. The ED 50 value was defined as the concentration allowing 50% survival of cells when compared to control wells and was determined by regression analysis.

For the 24 hour exposure of SVK 14 cells to hydrogen peroxide the lowest ten-fold dilution producing 100% killing, and the concentration allowing 100% survival of cells were compared to the same values generated for the experiments using a 15 minute exposure.

Statistical analysis - The significance of the difference between test and control experiments was determined using the Student paired *t* test.

6.3 RESULTS

The toxicity of each agent was evaluated under standard (cell number of 1×10^5 , exposure time of 15 minutes, the agent diluted in PBS-abc) and test conditions (1) diluting the test agent in PBS containing 10% FCS, the exposure time and cell number maintained as for standard conditions 2) diluting the test agent in RPMI 1640, the exposure time and cell number maintained as for standard conditions 3) increasing the number of exposed cells to 5×10^5 , maintaining the exposure time at 15 minutes and using PBS-abc as a diluent 4) Increasing exposure time to 1 hour, diluting the agent in PBS-abc and maintaining the cell number at 1×10^5 as per the standard conditions). The ED 50 was defined as the concentration allowing survival of 50% of cells and the values for test and standard experiment were compared. Mean ED 50 values for test and standard experiments are summarized in **Table 6.1**, and the ratio of the test and standard values is shown in **Table 6.2**. An increase in ED 50 concentration for a given test condition when compared to the control value reflects decreased sensitivity of the cells and therefore, decreased toxicity of the test agent. Representative dose-response curves are shown in **Figures 6.1, 6.2, and 6.3**, comparing test and standard conditions for each agent.

TABLE 6.1

MEAN ED 50 concentrations (M) \pm 1 SD (n=3-4) for three agents comparing values for standard experiments to those of test conditions. * indicates significantly different results at the 5% level ($p < 0.05$, paired Student T test). Standard conditions were as follows: 1×10^5 SVK 14 cells were exposed for 15 minutes to a test agent which was diluted in PBS-abc. Standard conditions were compared to the following test conditions: (1) diluting agent in PBS-abc containing 10% FCS (2) diluting agent in medium (RPMI 1640) (3) increasing cell number to 5×10^5 (4) increasing exposure time to 1 h

VARIABLE	AGENT					
	HYDROGEN PEROXIDE		SODIUM HYPOCHLORITE		CHLORHEXIDINE	
	test	standard	p value	test	standard	p value
10 % FOETAL CALF SERUM	$1.44 \pm 0.25 \times 10^{-2}$	$9.3 \pm 0.8 \times 10^{-3}$	0.0030*	$1.6 \pm 0.1 \times 10^{-3}$	$9.0 \pm 0.47 \times 10^{-5}$	0.00007*
MEDIUM	$1.10 \pm 0.04 \times 10^{-2}$	$9.5 \pm 0.9 \times 10^{-3}$	0.0340*	$2.8 \pm 0.13 \times 10^{-3}$	$8.2 \pm 0.6 \times 10^{-5}$	0.0007*
CELL NUMBER	$1.30 \pm 0.08 \times 10^{-2}$	$7.7 \pm 0.8 \times 10^{-3}$	0.0005*	$4.6 \pm 0.8 \times 10^{-4}$	$1.1 \pm 0.1 \times 10^{-4}$	0.018*
TIME	$0.18 \pm 0.014 \times 10^{-3}$	$8.4 \pm 0.5 \times 10^{-3}$	0.0015*	$7.9 \pm 0.1 \times 10^{-5}$	$9.1 \pm 0.5 \times 10^{-5}$	0.041*
				$7.5 \pm 1.4 \times 10^{-5}$	$4.1 \pm 0.12 \times 10^{-5}$	0.015*
				$3.7 \pm 0.1 \times 10^{-5}$	$3.6 \pm 0.4 \times 10^{-5}$	0.5
				$3.7 \pm 0.1 \times 10^{-5}$	$2.8 \pm 0.06 \times 10^{-5}$	0.25
				$2.1 \pm 0.2 \times 10^{-5}$	$3.9 \pm 0.07 \times 10^{-5}$	0.002*

TABLE 6.2

Ratio of mean ED 50 values for test and standard experiments. Standard conditions were as follows: 1×10^5 SVK 14 cells were exposed for 15 minutes to a test agent which was diluted in PBS-abc. Standard conditions were compared to the following test conditions: (1) diluting agent in PBS-abc containing 10% FCS (2) diluting agent in medium (RPMI 1640) (3) increasing cell number to 5×10^5 (4) increasing exposure time to 1 h

VARIABLE	AGENT		
	HYDROGEN PEROXIDE Test/standard	SODIUM HYPOCHLORITE Test/standard	CHLORHEXIDINE Test/standard
10% FOETAL CALF SERUM	1.55	17.8	1.8
MEDIUM	1.15	33.5	1.0
CELL NUMBER	1.72	4.2	1.0
TIME	0.21	0.89	0.54

Effect of foetal calf serum

Comparison of mean ED 50 values showed that diluting hydrogen peroxide in PBS-abc containing 10% FCS significantly reduced the toxicity of hydrogen peroxide and chlorhexidine in this assay. However the effect of 10% FCS on sodium hypochlorite was more dramatic, the ED 50 concentrations in standard experiments being 17.8 times lower than test values (**Table 6.2**), indicating that serum substantially reduces the toxicity of chlorine releasing agents.

Effect of medium (RPMI 1640)

Using medium as a diluent had no significant effect on the toxicity of chlorhexidine and only a small but significant decrease in the toxicity of hydrogen peroxide (**Table 6.1**). Again, the greatest reduction in toxicity was seen with sodium hypochlorite, where the concentration allowing 50% cell survival for the standard conditions was 33.8 times lower than the test value (**Table 6.2**).

Effect of cell number

In the standard and test experiments which examined the effect of cell number on toxicity the assays were conducted in 30 mm dishes, rather than 24-well plates, to accommodate the larger cell number. It was notable that the ED50 values generated for the standard conditions for this series of experiments differed from those of the other experiments. Altering the size of the culture dish increased the toxic effects of hydrogen peroxide and chlorhexidine and decreased the toxicity of sodium hypochlorite.

Whilst chlorhexidine was as active on 5×10^5 cells as 1×10^5 cells, both hydrogen peroxide and sodium hypochlorite showed reduced toxicity in the presence of an increased cell number. This effect was greatest for sodium hypochlorite where there was a four-fold increase in ED 50 concentration compared to a 1.7-fold increase for hydrogen peroxide.

Figure 1

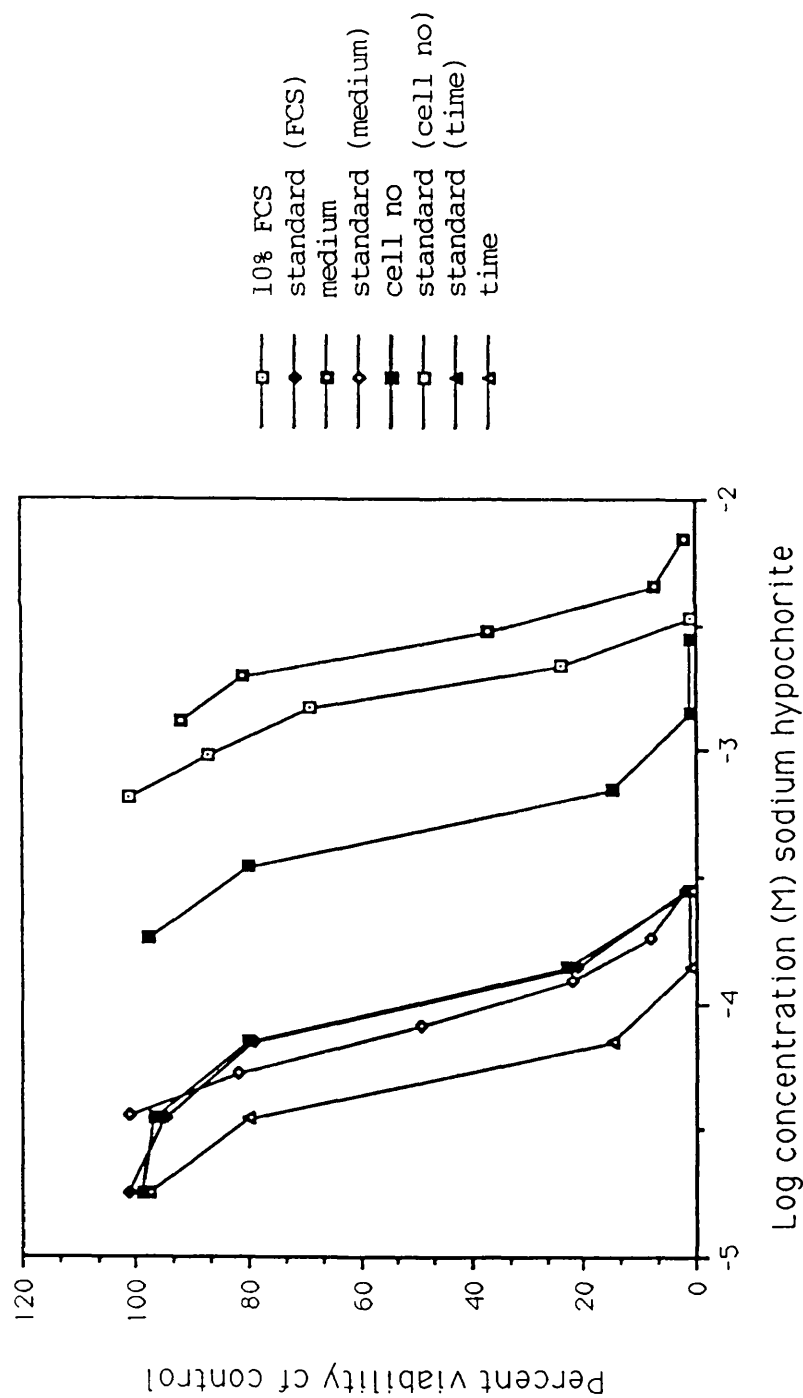


Figure 6.1

Dose response curves for the effect of sodium hypochlorite on SVK 14 cells showing one representative experiment. Test conditions are as follows: 1) diluting agent in PBS-abc containing 10% FCS 2) diluting agent in medium 3) increasing cell number from 1×10^5 to 5×10^5 4) increasing exposure time from 15 minutes to 1 h. For each experiment the test conditions were compared to the following standard conditions: 1×10^5 cells were exposed for 15 minutes to the test agent which was diluted in PBS-abc. Standard time and standard cell number are superimposed.

Figure 2

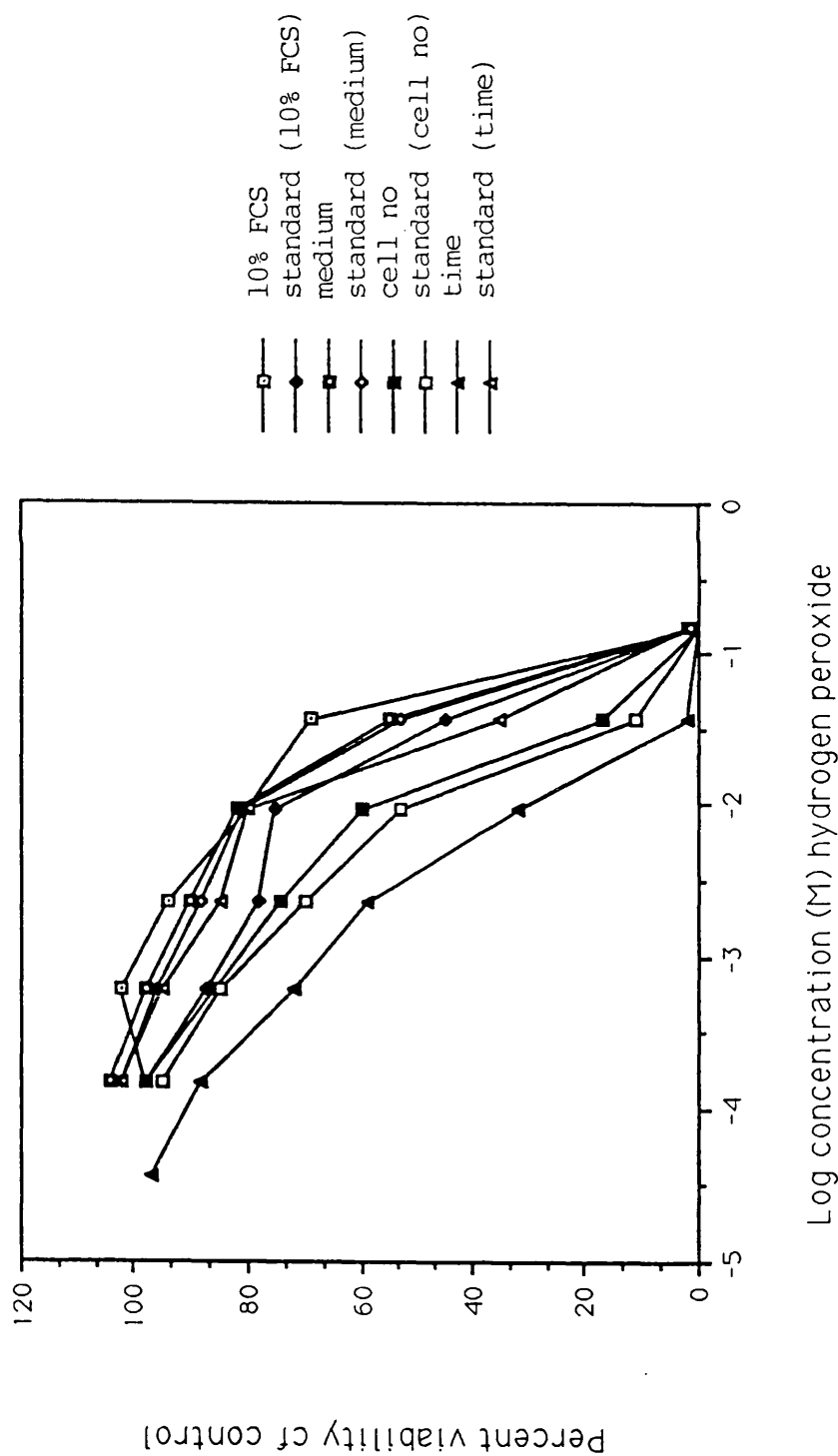


Figure 6.2

Dose response curves for the effect of hydrogen peroxide on SVK 14 cells showing one representative experiment. Test conditions are as follows: 1) diluting agent in PBS-abc containing 10% FCS 2) diluting agent in medium 3) increasing cell number from 1×10^5 to 5×10^5 4) increasing exposure time from 15 minutes to 1 h. For each experiment the test conditions were compared to the following standard conditions: 1×10^5 cells were exposed for 15 minutes to the test agent which was diluted in PBS-abc

Figure 3

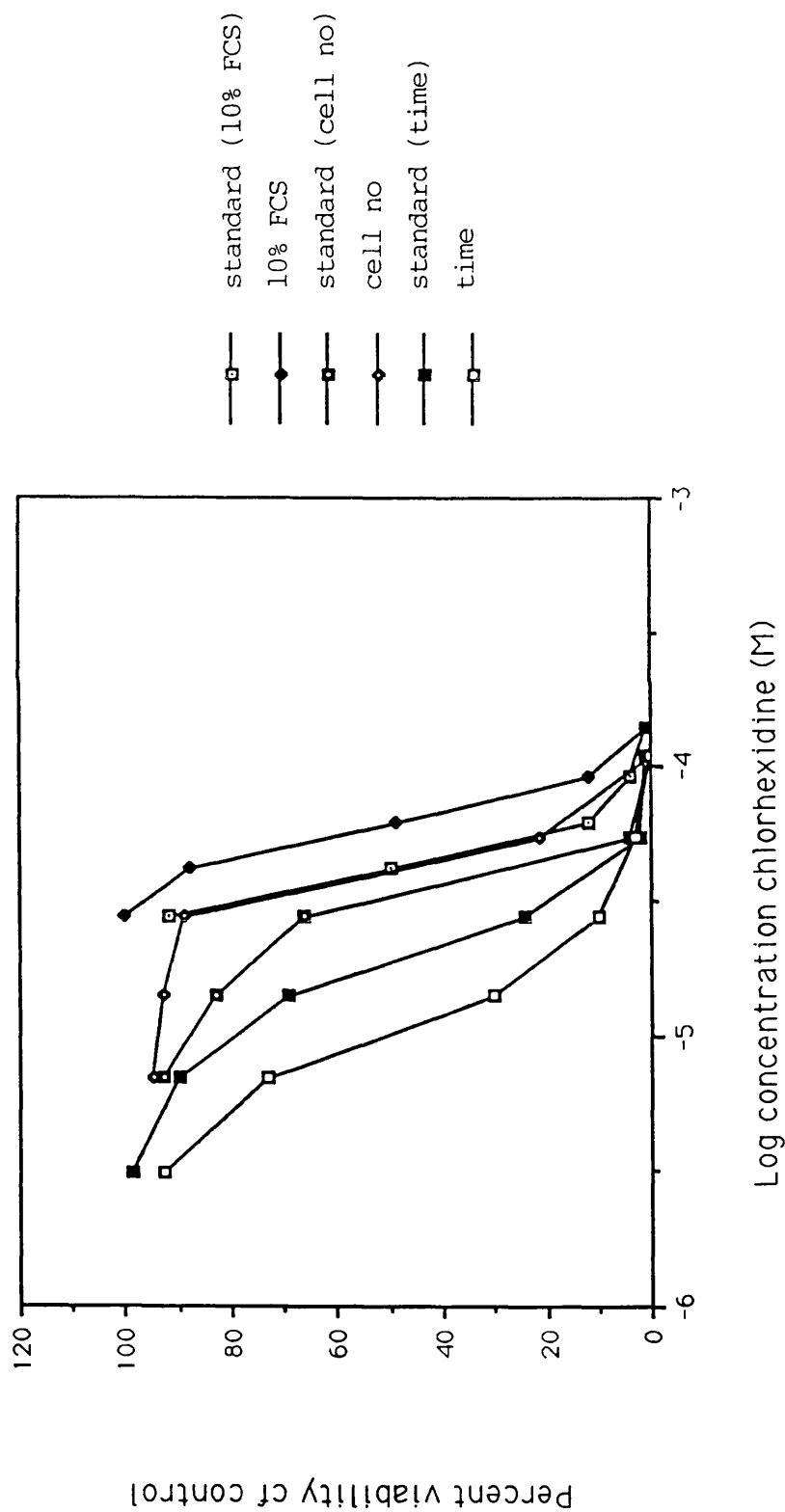


Figure 6.3

Dose response curves for the effect of chlorhexidine on SVK 14 cells showing one representative experiment. Test conditions shown are as follows: 1) diluting agent in PBS-abc containing 10% FCS 2) increasing cell number from 1×10^5 to 5×10^5 3) increasing exposure time from 15 minutes to 1 h. For each experiment the test conditions were compared to the following standard conditions: 1×10^5 cells were exposed for 15 minutes to the test agent which was diluted in PBS-abc. Data for diluting agent in medium are not shown, as responses for test and standard conditions were similar.

Effect of increasing exposure

Increasing exposure time increased the toxicity of all agents. The least effect was on sodium hypochlorite where a longer exposure produced an ED 50 value which was close to the standard result; for chlorhexidine the ED 50 value was half of that generated for the standard conditions. The greatest effect was on hydrogen peroxide, where increasing exposure four-fold decreased the ED 50 concentration to 20% of the control experiment.

Comparison of highest ten-fold dilution that gave 100% killing for the 15 minute and 24 hour exposure of SVK 14 cells to hydrogen peroxide showed no cell survival at 8.8×10^{-2} M for a 15 minutes exposure, compared to 8.8×10^{-7} M for the 24 hour exposure. The lowest dilution allowing 100% survival of cells was 8.8×10^{-6} M for the 15 minute exposure and 8.8×10^{-7} M for the 24 hour exposure. Comparison of 15 minutes and 24 hour exposures showed the 100% killing concentration for the shorter exposure to be a thousand times greater than the 24 h exposure. The dose response curves are shown in **Figure 6.4**.

The mean ED50 concentrations for test and control experiments were compared to the 'in use' concentration (**Table 6.3**) and a ranking order of toxicity produced for each experimental variable as previously described (see Section 5.3) (**Table 6.4**). When compared to the standard conditions, the ranking order of toxicity was only altered by diluting the test agents in medium, where the ranking order was as follows: hydrogen peroxide > sodium hypochlorite > chlorhexidine.

Figure 6.4

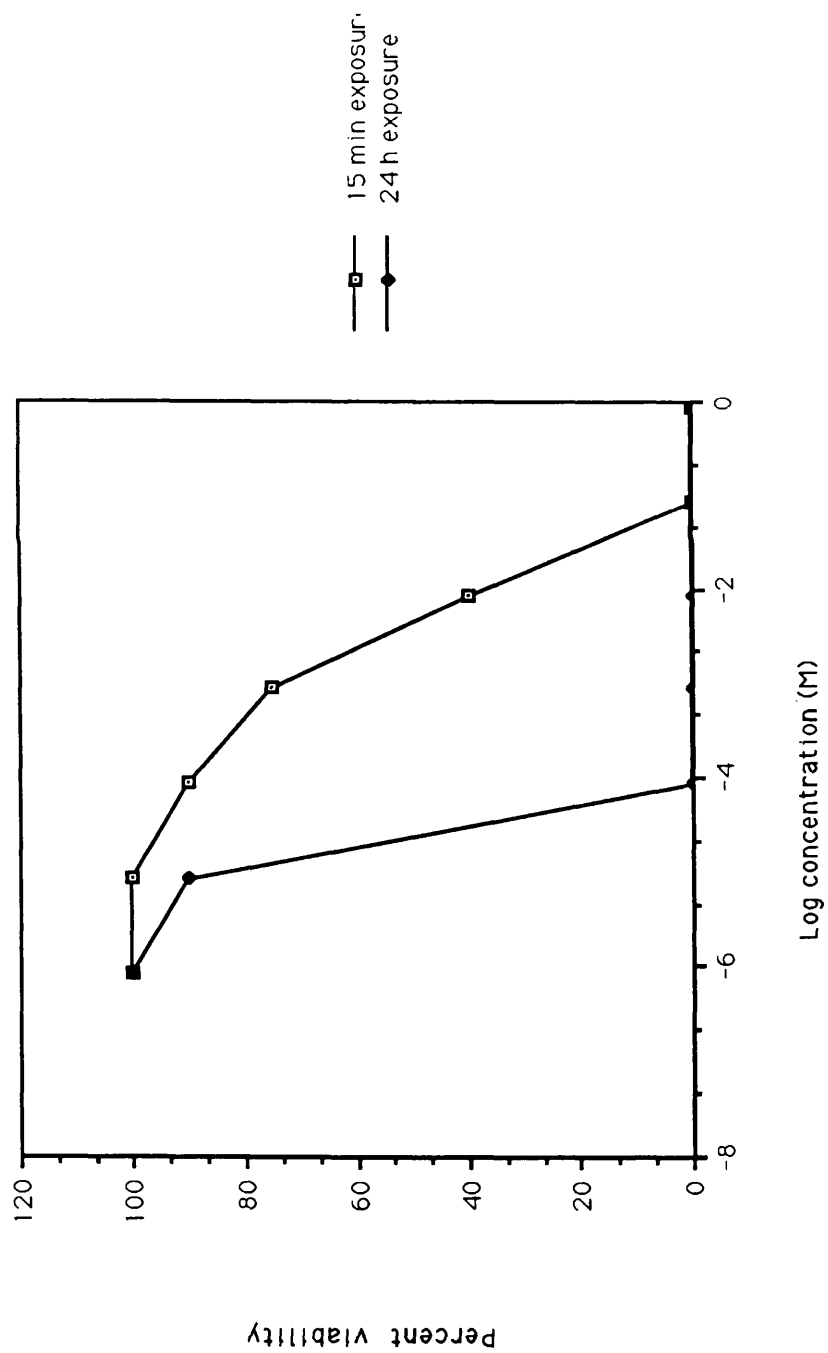


FIGURE 6.4 Log-dose response curve from one representative experiment showing the effect of exposing SVK 14 cells to hydrogen peroxide for 15 minutes and 24 hours. Points show the mean of three replicates.

TABLE 6.3 Comparison of mean ED50 concentrations for test and standard experiments to therapeutic concentration (shown in parentheses), showing the dilution necessary to allow 50% survival of cells.

AGENT	DILUTION FROM THERAPEUTIC CONCENTRATION TO ALLOW 50% CELL SURVIVAL									
	CELL NUMBER		10% FCS		MEDIUM		TIME			
	Test	Standard	Test	Standard	Test	Standard	Test	Standard	Test	Standard
Hydrogen peroxide (0.88M)	x 67	x 114	x 63	x 94	x 80	x 92	x 555	x 104		
Sodium hypochlorite (0.14M)	x 304	x 1272	x 87	x 1555	x 50	x 1707	x 1722	x 1406		
Chlorhexidine (0.00056M)	x 15	x 20	x 7	x 13	x 15	x 15.5	x 26	x 14		

TABLE 6.4 By comparing the mean ED50 values to the therapeutic concentration a ranking order of toxicity can be obtained. Only diluting the agents in medium alters the ranking originally produced using the standard conditions.

TOXICITY RANKING ORDER	
Standard conditions	Sodium hypochlorite > hydrogen peroxide > chlorhexidine
Diluent =PBSabc with 10% FCS	Sodium hypochlorite > hydrogen peroxide > chlorhexidine
Diluent=medium	Hydrogen peroxide > sodium hypochlorite > chlorhexidine
Increasing cell number	Sodium hypochlorite > hydrogen peroxide > chlorhexidine
Increasing exposure time	Sodium hypochlorite > hydrogen peroxide > chlorhexidine

6.4 COMMENT

A number of studies have examined antiseptic toxicity in cell culture, and whilst it has been shown that all agents tested are toxic to the various cell types used, scant regard has been paid to the interpretation of these results in comparison to available data for in vivo toxicity. Experimental conditions vary from study to study (**Tables 1.5, 1.6, 1.7**). Conditions for studies which have examined a comprehensive range of agents, including the data generated in this study, are summarized in **Table 6.5**.

The observations presented may explain why the toxicity of an individual agent varies between studies. Increasing cell number, and using medium or serum as diluent decreases the toxicity of chlorine-releasing agents to a greater degree than for chlorhexidine or hydrogen peroxide, and it seems likely that the combination of medium and serum used routinely to culture cells would decrease the toxicity of chlorine releasing agents further. Lengthening the period of exposure increases the toxicity of hydrogen peroxide, but only has a small effect on the toxicity of sodium hypochlorite. Cell culture systems which test agents using a high cell number, dilute the agents in serum or medium, and use long exposure times are likely to increase the toxicity of hydrogen peroxide relative to chlorine-releasing agents.

The high toxicity of hydrogen peroxide and relatively low toxicity of sodium hypochlorite, which have been reported in vitro, reverse in vivo findings, where hydrogen peroxide, unlike chlorine-releasing agents, has no adverse effects on the healing process (Brennan and Leaper, 1985; Lineaweaver et al, 1985; Gruber et al, 1975; Leyden and Bartelt, 1987). Lineaweaver et al (1985) examined the effect of a range of antiseptic agents on fibroblasts; the toxicity of sodium hypochlorite could be eradicated at one hundred times the 'in-use' concentration, whereas the toxicity of hydrogen peroxide was abolished at one thousand times the in 'in-use' concentration. Other data for hydrogen peroxide are in line with the data generated for fibroblasts in this study (Lineaweaver et al, 1985) and it seems likely that the differences in toxicity of sodium hypochlorite are related to the use of a higher cell

number (2×10^8 cells), since other aspects of the experimental conditions are comparable. Leaper and Brennan (1986) found that the toxicity of chloramine T and hydrogen peroxide could be eradicated at a one hundred-fold dilution and a ten-thousand fold dilution respectively from their 'in-use' concentrations. Here the high toxicity of hydrogen peroxide may be related to the length of exposure (24 hours). This view is supported by the observation that the 100% killing concentrations can be reduced a one hundred thousand-fold by increasing exposure time from 15 minutes to 24 hours. The relatively low toxicity of the chlorine-releasing agent in the Leaper and Brennan study (1986) may be attributed to the use of both a high cell number and medium as a diluent.

Only using medium as a diluent changed the ranking order of toxicity (**Table 6.3 and 6.4**) and for all other variables, the chlorine-releasing agent remained the most toxic antiseptic. Clearly if further experiments were performed which further increase cell number and amounts of serum the toxicity of sodium hypochlorite might be further decreased and an alteration in the ranking order for other variables could be seen.

The findings presented by Deas, Billings, Brennan et al (1986) are more comparable to those presented here, in spite of the fact that the experimental conditions are very different. By comparing ten-fold dilutions from the standard concentration, this group showed that chloramine T was the most toxic agent (toxicity eradicated at a dilution of 1×10^6 from the standard concentration), chlorhexidine was of intermediate toxicity (toxicity eradicated at a dilution of 1×10^4 from the standard concentration) and hydrogen peroxide of least toxicity (toxicity eradicated at a dilution of 1×10^3 from the standard concentration). This study comes from the same department as that of Leaper and Brennan (1986) and emphasizes how changes in experimental conditions can alter results. The key difference between these studies is the cell number (**Table 6.5**), Deas et al (1986) opting for a very small cell number in relation to volume. The volume of antiseptic in relation to cell number may be of great importance, and is often not quoted in

TABLE 6.5 Experimental conditions used in studies which have compared the toxicities of a range of antiseptic agents

CELL TYPE	LENGTH OF EXPOSURE	DILUENT	CELL NUMBER	ANTISEPTIC VOLUME	REFERENCE
Fibroblast (baby hamster)	24 h	Eagle's medium	2 x 10 ⁶	?	Leaper and Brennan, 1986
Fibroblast	15 minutes	Saline	2 x 10 ⁸	?	Lineaweaver et al, 1985
Fibroblast (baby hamster)	24 h	Eagle's medium plus 10% FCS plus 10% tryptose phosphate broth	5 x 10 ³	220 µl	Deas et al, 1986
Transformed keratinocytes (SVK 14 CELLS)	15 minutes	PBS-abc	1 x 10 ⁵	1 ml	Tatnall et al, 1990

studies (**Table 6.5**). This is, perhaps, a further important experimental parameter to evaluate. In Deas et al's study (1986), although chlorhexidine is of low toxicity in relation to other agents (**Table 5.3**), toxicity ^{was} eradicated at 10,000 times the standard use concentration, and 50% survival occurs at 1000 times this concentration (roughly gauged from the dose response curves presented); in this study 50% survival for fibroblasts occurs at a six-fold dilution of the standard concentration. The toxicity of chlorhexidine increases with length of exposure. This has been shown here and by other workers (Blenkarn, 1987). The long exposure may give rise to the very low concentration at which toxicity is eradicated.

The reduction of toxicity induced by serum, medium and increased cell number is likely to be related to the increase in organic material present within the test system. It is well known that chlorine releasing compounds are inhibited by organic material. Medium has the greatest effect on the activity of these agents. The likely inhibitory components are: amino acids, putrescine which is a polyamine, and the pH indicator phenol red. Hydrogen peroxide is unstable in the presence of organic matter decomposing to water and oxygen, and this may account for the small reduction in toxicity that was observed with increased cell number, medium and serum. However, the decrease in toxicity of hydrogen peroxide associated with an increase in cell number may be attributed to an increase in catalase and peroxidase activity, which will inactivate hydrogen peroxide, and superoxide dismutases which scavenge super oxide free radicals. Organic matter has only a small effect on the activity of chlorhexidine (Ullrich, 1981) and this was confirmed in this study.

The subject of an antiseptic toxicity index was addressed in the 1930's and 40's (see Chapter 1, Section 1.3) and was reviewed by Reddish (1954) who concluded that the toxicity index was valueless since the results were so conflicting. However, Lineaweaver et al (1985) resurrected interest in this subject and reported the use of cell culture to establish a safe concentration at which antiseptics can be administered to wounds i.e. a concentration which does not harm cutaneous cells but kills bacteria. Such a

concentration could be established for povidone iodine and sodium hypochlorite, agents whose toxicity is highly influenced by the amounts of organic material present. Although equal numbers of bacteria and cells (fibroblasts) were used in that study, these may not represent equal organic loads, bacteria being smaller than fibroblasts. Hence, at a given concentration, hypochlorites may be rendered less toxic to fibroblasts than to bacteria. Such differences might otherwise be attributed to different modes of action on bacteria and mammalian cells. However at high concentrations it is more likely that most antiseptic agents kill by non-specific means, without species differences (Gardner and Gray, 1983). Similar factors may be important when comparing the susceptibility of different types of mammalian cells (see Chapter 5, Section 5.4 - Comment).

The findings of Lineaweaver et al (1985) have been discussed by others (Kozol et al, 1988) who studied the effect of sodium hypochlorite on endothelial cells, fibroblasts and neutrophils using electron microscopy. These authors comment on the observation that concentrations of sodium hypochlorite below 0.05% were safe (Lineaweaver et al. 1985), since in their own study sodium hypochlorite toxicity could be demonstrated down to concentrations of 0.00025%, findings which are comparable to those in observed in this study. Kozol et al (1988) take issue with the safe concentration as defined by Lineaweaver et al (1985) and attribute the differences in findings between the two studies to their use of a sensitive technique to estimate cell damage. Alternatively this difference may be attributed to the differences in cell numbers used: 2×10^8 (Lineaweaver et al, 1985) versus 1×10^6 (Kozol et al, 1988).

Comparison of the 100% killing values for the SVK 14 cells to published bacteriocidal concentrations for antiseptic agents is of interest. For chlorhexidine the lowest concentration that is 100% cytotoxic to SVK 14 cells was 0.01%; concentrations of chlorhexidine which are bacteriocidal range down to 0.01% w/v (Gardner and Gray, 1983). The findings are remarkably similar. If such a comparison is made for sodium hypochlorite, the lowest concentration which will produce 100% killing of SVK 14 cells is 0.001% w/v, whereas the concentration of

available chlorine required to kill *Staphylococcus aureus* is 0.00008% w/v (Dychdala, 1983) i.e. approximately ten times less. These comparisons emphasize that when an agent is little inhibited by organic matter no safe and effective concentration can be generated; for those agents that are inhibited by organic material a toxicity index can readily be generated.

The differences in toxicity of individual agents in cell culture may be extrapolated to the study of antiseptics in wound models. Brennan and Leaper (1985) showed that chlorine-releasing agents have a dramatic effect on granulation tissue, resulting in its complete destruction. Another study has shown that chlorine-releasing agents are of low toxicity to granulation tissue showing 20 % inhibition and not significantly different from the control (Neidner and Schopf, 1986). In these studies the agents were tested in in vivo wound models; the amounts of organic material present within the wound and the volume maintained in contact, may be important factors which give rise to differences in results. In the rabbit ear chamber (Brennan and Leaper, 1985), the granulation tissue was in contact with phosphate buffered saline prior to exposure to the agents and was thus 'well washed'; the volume of antiseptic that was used to flood the chamber was clearly much greater than can be maintained in contact with a wound such as a cutaneous ulcer. This model may be likened to the in vitro model used in this study and gives very comparable results for the relative toxicities of the agents tested. Neidner and Schopf (1986) maintained contact between the antiseptic agent and the wound bed by formulating it in a polyacrylamide gel. Both the amounts of organic material in a wound model and the volume of antiseptic maintained in contact with the wound remain equally relevant to the study of antiseptic toxicity in vivo and again this has not been critically evaluated.

6.5 SUMMARY

- 1) Increasing cell number, using medium and serum as a diluent for the antiseptic agent decreased the toxicity of the chlorine releasing agents relative to hydrogen peroxide and chlorhexidine. Lengthening exposure increased the toxicity of hydrogen peroxide relative to sodium hypochlorite and chlorhexidine. These findings emphasize the ways in which assay in cell culture may alter the relative toxicities of individual agents and may explain why hydrogen peroxide has been shown to be of high toxicity and sodium hypochlorite of low toxicity in cell culture, findings which are the reverse of in vivo studies.
- 2) In vitro data which generates safe concentrations at which antiseptic agents are antibacterial and non-toxic are to be questioned.
- 3) Comparison of results of various antiseptic cytotoxicity assays emphasize the lack of standardization of experimental conditions. These may be better defined for each agent if the pharmacology of agents applied to wounds is studied.

CHAPTER 7

CONCLUSIONS

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7.1 INTRODUCTION

Review of the literature suggests that antiseptic agents have been used uncritically for many years. Efficacy and possible harmful effects have not been questioned. In the area of dermatological practice (venous ulcers, pressures sores) data on whether antiseptic usage aids or inhibits healing is particularly lacking, because very few controlled clinical trials have been performed.

To study the toxicity of antiseptics, investigators have turned to wound models in animals and in vitro cytotoxicity assays. Methods of studying antiseptic agents in vivo vary greatly and the results are conflicting, however the majority of studies suggest that chlorine-releasing agents are more toxic than other commonly used agents.

Most in vitro studies use cell culture to evaluate the cytotoxicity of antiseptic agents and the methods used are unstandardized. The data generated are equally conflicting when the relative toxicities of the individual agents are compared. The published studies have largely used fibroblasts, and toxicity has been measured using crude and laborious techniques.

The purpose of this study was to set up an in vitro cytotoxicity assay which was simple, accurate and reliable, allowing comparison of fibroblasts and basal cells, which are considered fundamental to the cutaneous wound healing process. Culture of normal human keratinocytes is complex, and these cells were therefore compared to a transformed keratocyte line (SVK 14 cells) which shares many features in common with the normal human basal keratinocyte, but is relatively simple to culture. The study set out to compare the relative toxicities of the agents tested using a set of experimental conditions which on theoretical ground were judged to best reflect the 'in use' situation. Additionally the absolute toxicity of the agents on the basal keratinocytes was of interest because the role of cultured keratinocytes is being evaluated in wound healing.

- Below the findings of this study are summarized.

7.2 METHODS

One of the main purposes of this study was to establish an in vitro cytotoxicity assay which was simple, accurate and reliable.

The experimental procedure chosen was in line with some other published studies. The attached cells (1×10^5) were exposed to the test agent diluted in PBS-abc for 15 minutes. The cells were then reincubated overnight to demonstrate maximum toxicity of the test agent, as cell death may take place some hours after exposure. Numbers of viable attached cells remaining were then measured.

Cell culture - Cultures of fibroblasts and SVK 14 cells could readily be established for use in the assay and merely required seeding at the appropriate cell density into a multiwell plate 24 hours prior to use. To establish a suitable culture of basal keratinocytes for use in the assay was difficult. The important problems to be overcome were:

- 1) the removal of the 3T3 fibroblast feeder cells used in the primary culture; this was done by using passaged cells which grew in the absence of a feeder layer

- 2) obtaining a culture of basal cells; this was achieved by passaging a primary stratifying of keratinocytes into multiwell plates, allowing these cells to grow and stratify until they reached 70-80% confluence and then inducing destratification with low calcium keratinocyte growth medium. This medium was made by omitting calcium salts from the basal medium, and depleting FCS of calcium. This medium, which was found to have a calcium concentration of 0.05 mM, will destratify the culture in three days, but washing procedures tended to detach the loosely attached cells. This problem could be corrected by raising the calcium level by adding 1% KCM (elevating the calcium level to approximately 0.07 mM) a concentration which still allowed destratification in three days with good hemidesmosomal attachment of the basal cells.

- 3) Initial basal cell cultures showed poor well-to-well replication, particularly at the edge of the plates; this was attributed to evaporation of water from the medium and

could be corrected by frequent medium changes and by enclosing the plates in plastic containers.

Agents and diluents - The agents initially chosen as clinically relevant to study were: povidone iodine, a chlorine-releasing agent (sodium hypochlorite), cetrimide, chlorhexidine, and hydrogen peroxide. Povidone iodine was excluded because serial dilution generates concentrations of both free-iodine and povidone iodine which are unpredictable. The remaining agents could all be introduced into a cell culture system diluted in PBS-abc.

The choice of diluent for the antiseptic agents had to be modified to include calcium salts (PBS-abc) because the basal keratinocytes and SVK 14 cells quickly detached in calcium-free solutions.

Cytotoxicity assay - The assay used attached cells, rather than cells in suspension. An attached cell assay has less experimental manipulation with less opportunity for introduction of error, and is more representative of the 'normal' cell state. It was initially assumed that, following exposure to the test agent, the non-viable cells would detach and viable cells would remain attached. Viability was initially determined by counting the remaining attached cells and estimating percentage viability using a vital stain. If the remaining cells were all viable only the counting procedure would be necessary to estimate the numbers of viable cells. This assumption that all attached cells remaining following exposure were viable was incorrect. Although detachment of viable cells did not exceed that occurring in control plates, non-viable cells remained attached, particularly at the higher concentrations tested. It was therefore essential that the assay included a measure of cell viability. Preliminary experiments were performed using cell counting and the vital stain. This was inaccurate unless large cell numbers were used and laborious. The MTT assay was evaluated as an alternative, this measures viable cells which can reduce MTT to an insoluble blue formazan product. The MTT assay correlated well with cell number for fibroblasts, basal keratinocytes and

SVK 14 cells, which implied that this method can be used to measure proliferation for these three cell types. In addition, the MTT assay correlated well with the cell counting/viability assay in measuring cell viability following treatment with antiseptic agents. The MTT assay provided a simple, reliable and accurate method for measuring cell viability and from this assay accurate comparison of agents could be made using ED50 concentrations, which has not been previously done.

7.3 SUSCEPTIBILITY OF BASAL CELLS, FIBROBLASTS AND SVK14 CELLS TO ANTISEPTIC AGENTS

The initial experiments compared the susceptibility of three cell types to a range of antiseptic agents. Basal cells and fibroblasts, which play a key role in the wound healing process, were equally susceptible to the effects of chlorhexidine, hydrogen peroxide and sodium hypochlorite. The SVK 14 cells were more susceptible to chlorhexidine and sodium hypochlorite than the other cell types. Although these cells were more sensitive to hydrogen peroxide than fibroblasts, basal keratinocytes showed equal susceptibility. Because antiseptic agents kill at high concentration by non-specific means and are all inhibited to some degree by organic material, it is likely that the differences in sensitivity observed between cell types may simply be related to the organic load that equal numbers of cells of different types represent.

Whilst the transformed keratinocytes and the basal keratinocytes differed in their response to chlorhexidine and sodium hypochlorite, the response to hydrogen peroxide was similar. Both cell types showed a biphasic dose-response curve following treatment with this agent. Hydrogen peroxide is a natural killing agent and these findings support the assumption that the SVK 14 transformed keratinocyte line may represent an alternative to normal human keratinocytes in the study of keratinocyte physiology.

Although the ED50 concentrations for the agents tested on SVK 14 cells were significantly different from the other cell types, the differences were of small orders of magnitude. In particular, use of the SVK 14 cell line did not affect the ranking order of

*The maximum difference observed between mean SVK14 ED50 values and those of other cell types was for chlorhexadine, where SVK14 ED50 values were 50% of fibroblast values. For all other cell types and agents the differences between SVK14 ED50 values was less than 50% (see Table 5.1, p.140).

toxicity for the agents, which was generated by comparing the ED50 concentration to the standard 'in use' concentration. SVK 14 cells have the advantages of ease of culture, ready availability and an indefinite lifespan. Additionally when these transformed cells are used in a cytotoxicity assay the results of experiments are highly reproducible when compared to the normal cell lines. The SVK 14 cell line, in addition to sharing biological similarities to normal human cultured keratinocytes, offer many practical advantages over normal cell lines, and can usefully replace these cells in an antiseptic cytotoxicity assay. The SVK 14 cells were therefore used for all further studies.

7.4 COMPARISON OF THE TOXICITY OF ANTISEPTIC AGENTS IN A CELL CULTURE ASSAY

In line with other studies that have evaluated the toxic effect of antiseptic agents in cell culture, at standard 'in use' concentrations all agents tested were 100% cytotoxic to all cell types. Reports which have evaluated inhibition of cutaneous wound healing by antiseptic agents have consistently shown that some agents are non-toxic, and hydrogen peroxide is the best example. However, if the toxicity of antiseptic agents is assessed by examining the effects on blood vessels, all agents will show an immediate adverse effect, from which recovery may take place. This clearly does not occur in a cell culture system. So the toxicity of all antiseptic agents seen *in vitro* can be demonstrated in the short term *in vivo*.

The absolute concentrations of an agent which produce a particular end-point in an *in vitro* cytotoxicity assay may be of less interest than the comparative data generated for a number of agents. These data were generated by comparing the number of dilutions from the standard concentration necessary to achieve the ED50 concentration and the findings were as follows; sodium hypochlorite > hydrogen peroxide > cetrimide > chlorhexidine. The use of chlorine-releasing agents has been surrounded by controversy in recent years and this has prompted studies both *in vivo* and *in vitro* to evaluate this. It was noted that the results obtained from this *in vitro* assay were

broadly in line with in vivo studies in wound models which show that chlorine releasing agents are toxic to wounds and chlorhexidine is not.

7.5 CONDITIONS IN A CELL CULTURE CYTOTOXICITY ASSAY WHICH INFLUENCE TOXICITY

In comparing the results obtained for the relative toxicities of the agents tested to those of other studies, it was clear that there were discrepancies. Comparison of an end-point (usually the lowest dilution which allows 100% survival of cells) to the standard concentration shows chlorine-releasing agents to be less toxic than other agents in some studies and more toxic in others. It was clear from the initial experiments that minor modifications in the assay, such as variation in cell number produced by poor cell attachment, could alter ED 50 values. Therefore, an analysis of the factors which might influence the toxicity of agents was made. The variables that were studied were: 1) increasing cell number 2) diluting the agent in medium (RPMI 1640) or PBS-abc containing 10% FCS 3) increasing the length of exposure. It was shown that increasing cell number, diluting the test agent in serum or medium reduced the toxicity of sodium hypochlorite relative to other agents, whereas increasing the length of exposure increased the toxicity of hydrogen peroxide relative to other agents. In the light of these findings it was possible to interpret other studies where using long exposures, high cell numbers and diluting agents in medium were likely to have decreased the toxicity of chlorine-releasing agents and increased the toxicity of hydrogen peroxide, producing results which are the reverse of in vivo findings. The effect of the presence of serum, medium or a high cell number within the test system is likely to be related to an increase in the amount of organic material present. This study establishes the importance of standardized conditions in the evaluation of cytotoxicity in vitro. Results can only be interpreted by understanding how these conditions alter findings.

7.6 USES AND LIMITATIONS OF A CELL CULTURE ANTISEPTIC CYTOTOXICITY ASSAY

Uses - The principal advantage of studying the cytotoxic effect of any drug in vitro is the ultimate reduction in the numbers of animal experiments performed.

Whilst cell culture techniques have been used enthusiastically to evaluate the possible harmful effects of antiseptics on wounds, little emphasis has been placed on interpretation of the results and the usual conclusion drawn is that all antiseptic agents tested are toxic to cells and should, therefore, not be applied to wounds. In this study it was also shown that at standard concentration all agents were toxic. However, it is judged that the most valuable data that can be derived from such studies is the comparative data i.e. which agent is the most toxic and which is the least.

The cell culture system is useful for studying factors which influence toxicity; variables, such as length of exposure and the effect of serum, can easily be studied in isolation. Such studies are particularly valuable in the interpretation of in vivo studies in wound models, many of which create an artificial wound environment.

Although not studied here, the cell culture system can be used to study the specific mechanisms of toxicity in mammalian cells, and this has already been attempted (Shakespeare et al, 1988; Helgeland, 1971). The specific mode of action of many antiseptic agents on bacteria is poorly understood, with different mechanisms likely to be operative at high and low concentration. The study of mammalian cells may contribute to the general understanding of how antiseptics work.

The cell culture assay is useful for evaluating the toxicity of topical antimicrobials. The results are directly applicable to the clinical use of cultured cells for grafting.

Limitations - Establishing a test system for evaluating the antibacterial effects of antiseptics in vitro which is representative of the 'in use' situation has always been difficult. Similarly problems are encountered in in vitro studies, and the test conditions devised are largely based on theoretical considerations. Should serum be present and how much? What is a representative cell number? How long should contact

with the test agent be maintained? It is quite possible that different agents require different test conditions. At the present point in time these questions cannot be answered because little is known of what happens to antiseptic agents when they are placed on a wound. The biggest drawback to *in vitro* testing is lack of realism in the conditions, as has been stressed throughout this study. Measurement of toxicity *in vitro* is a purely cellular event, and should be interpreted with the understanding that clear differences exist between *in vivo* and *in vitro* measurement. Within the limitations of an *in vitro* cytotoxicity assay, it is believed that the standard experimental conditions outlined in this study may reflect conditions in cutaneous wounds and, importantly, the assay appears to predict the *in vivo* toxicities of the agents tested.

In addition cell culture may introduce its own constraints on experimental conditions for reasons of both practicality and cost. An assay which is used to study a large number of agents repeatedly, using a rapid method of analysis such as a colorimetric assay read by an ELISA plate reader, may necessitate using a small number of cells in relation to the volume of the agent.

7.7 FUTURE STUDIES

In vitro studies - Cultured cells are in an abnormal environment relative to their tissue of origin. It would be valuable to create an *in vitro* culture system which was more representative of a cutaneous wound. Efforts are being made to establish keratinocyte cultures which are more representative of normal skin. In organotypical culture an attempt is made to create an epidermis and dermis; keratinocytes are cultured on type I collagen which is impregnated with dermal fibroblasts (Mackenzie and Fusenig, 1983). This may provide a useful model in which to further study antiseptic toxicity.

The *in vitro* model which best represents a cutaneous wound is organ culture. Organ culture of the skin was used by Lawrence (1970) to study the effects of various antimicrobial agents and is established by placing a small piece of whole skin in

culture medium containing 50% serum. Over a period of days the epithelium will grow over the undersurface of the dermis. In the assay used by Lawrence (1970) the test agents were added to medium and serum and maintained in continuous contact with the skin explant. Continuous contact is artificial particularly in a volume of medium which contains such a high serum content. An alternative approach would be to immerse the skin explant in the test antiseptic for a period of minutes and then return the explant to the culture medium; this would reproduce the routine cleansing of a wound and could be done daily over the period of study. A growth inhibitory effect is measured by examining the skin histologically; toxicity can be measured by the inhibition of epithelial growth over lower dermis. Similar studies can be performed on skin explants cultured on collagen (Coulomb et al, 1986); here new epithelium grows out on the collagen raft.

In vivo studies - Whilst valuable comparative data can be obtained ⁱⁿ _^ vitro, it is essential to continue to improve and define test conditions for antiseptic agents in vivo. Little is known of the pharmacokinetics of an antiseptic applied to a cutaneous wound. A possible way of studying this is by using a suction blister in a human volunteer. A suction blister is produced by applying 500 mm Hg pressure to the skin. In normal skin it takes about 2 hours to induce a blister and with the standard equipment available the blister is about 7.5 mm in diameter. The blister base represents a cutaneous wound and, following removal of blister fluid, small amounts of a test agent could be introduced and the concentration of the agent measured at intervals. Such a study would give some indication of the inactivation and absorption of the agent over time.

Clinical studies - What is clearly lacking in the literature of antiseptic toxicity to cutaneous wounds is clinical data. In the area of chronic cutaneous wounds are these agents effective in reducing bacteria? In so doing do they enhance wound healing? Studies which evaluate the effect of antiseptics on wound healing use donor sites which heal rapidly in a normal healthy individual; no studies exist which show that wound

healing is inhibited in chronic cutaneous wounds by the application of antiseptics. Whilst *in vivo* and *in vitro* data point to the potential toxicity of chlorine-releasing agents, this data should now be supported by appropriate clinical studies to avoid the continuing confusion which surrounds the use of antiseptic agents on cutaneous wounds.

7.8 CLINICAL RELEVANCE OF CELL CULTURE STUDIES

Chronic cutaneous wounds - In this study chlorine-releasing agents have been shown to be of high toxicity. This is supported by *in vivo* work which use models of cutaneous wounds. Not all wound models may be representative of the 'real' cutaneous wound in terms of slough/exudate and volume of the test agent maintained in contact with the wound surface. In a chronic cutaneous wound it is likely that chlorine-releasing agents will come in contact with fibrin and exudate on the wound surface and will kill surface bacteria, but are unlikely to be in contact with fibroblasts in granulation tissue and migrating epithelium. However in other situations the toxicity of chlorine-releasing agents may be relevant; if in a cutaneous wound such as a venous ulcer attention has been paid to the underlying pathology of the wound (e.g. correction of venous hypertension), it becomes healthy and exudate decreases from the surface. Such a wound may be treated by a dermatologist using a grafting technique called 'pinch grafting', whereby small islands of skin are dotted over the wound bed. In this instance the wound is clean with delicate islands of epithelium grow out from the transplanted skin. The toxicity of the applied antiseptic may well inhibit wound healing.

The debate on the toxicity of chlorine-releasing agents to cutaneous wounds rages on, probably because no-one has any controlled clinical data to support their viewpoint. The chronic cutaneous wound seen in the community is usually covered with exudate and it is very doubtful that any antiseptic agent has any adverse effect on wound healing. Equally, beneficial effects are doubtful. Concern about the toxicity of antiseptic agents would be better replaced by addressing the pathogenic factors which

prevent wound healing such as venous hypertension in venous ulcers. It has always been emphasized that a healthy wound environment is more important than eradicating bacteria.

Culture Grafts - Cultured keratinocytes are being evaluated as a replacement for conventional grafts, particularly in the treatment of burns. This study shows that standard concentrations of antiseptic agents are toxic to cultured keratinocytes and the findings suggest that these agents should not be used in conjunction with this form of treatment. However these cells are very vulnerable to infection and it may be appropriate to use topical antibiotics, many of which are non-toxic to cultured keratinocytes (Cooper et al, 1990; Tatnall, Leigh and Gibson, 1987).

REAGENT APPENDIX

Phosphate buffered saline

<u>Solution a</u>	<u>Concentration</u>
NaCl	0.17 M
KCl	0.003 M
Na ₂ HPO ₄	0.01 M
KH ₂ PO ₄	0.0018 M

Solution b and c

CaCl ₂ (2H ₂ O)	0.009 M
MgCl ₂ (6H ₂ O)	0.0049 M

Trypsin 0.25% in tris saline

NaCl	0.137 M
KCl	0.005 M
Na ₂ HPO ₄	0.0007 M
Dextrose	0.0055 M
Tris (hydroxymethyl) amino methane	0.0247 M
Trypsin	2.5 g/l
Penicillin	60 mg/l
Streptomycin	100 mg/l
Phenol red	0.0015%

Trypsin 0.05% in EDTA

Made by diluting trypsin 0.25% in EDTA 0.2%

EDTA 0.2%

NaCl	0.137 M
KCl	0.0027 M
Na ₂ HPO ₄	0.08 M
KH ₂ PO ₄	0.0015 M
EDTA	0.0005 M
Phenol red	0.002% M

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