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Influence of microbial antigen formulation and delivery route on the immune response

Submitted by

Joanne Claire Bowen

for the degree of

Doctor of Philosophy

The University of Aston in Birmingham

September 1990

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SUMMARY

Recent technological advances have resulted in the production of safe subunit and synthetic small peptide vaccines. Unfortunately, these vaccines are weakly or non-immunogenic in the absence of an immunological adjuvant (agents that can induce strong immunity to antigens). In addition, in order to prevent and/or control infection at the mucosal surface, stimulation of the mucosal immune system is essential. This may be achieved *via* the common mucosal immune system by exposure to antigen at a mucosal surface remote from the area of infection.

Initial studies investigated the potential of multiple emulsions in effecting oral absorption and the subsequent immune responses to a lipopolysaccharide vaccine (LPS) after immunisation. Nasal delivery of LPS was carried out in parallel work using either aqueous solution or gel formulations. Tetanus toxoid vaccine in simple solution was delivered to guinea pigs as free antigen or entrapped in DSPC liposomes. In addition, adsorbed tetanus toxoid vaccine was delivered nasally free or in an aerosil gel formulation.

This work was extended to investigate guinea pigs immunised by various mucosal routes with a herpes simplex virus subunit vaccine prepared from virus infected cells and delivered in gels, multiple emulsions and liposomes. Comparable serum antibody responses resulted but failed to produce enhanced protection against vaginal challenge when compared to subcutaneous immunisation with alhydrogel adjuvanted vaccine. Thus, immunisation of the mucosal surface by these methods may have been inadequate.

These studies were extended in an attempt to protect against HSV genital challenge by construction of an attenuated *Salmonella typhimurium* HWSH *aro*A mutant expressing a cloned glycoprotein D-1 gene fused to the *Escherichia coli lac z* promoter. Preliminary work on the colonisation of guinea pigs with *S. typhimurium* HWSH *aro*A mutants were carried out, with the aim of using the guinea pig HSV vaginal model to investigate protection.

Key words: mucosal, formulations, herpes simplex virus, *Salmonella typhimurium*, liposomes

To Mom, Dad and Su, with love

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Abbreviations

aa	Amino acids
APC	Antigen presenting cell
BGSD	Brilliant green sodium sulphadiazine agar
BSA	Bovine serum albumin
°C	Degrees centigrade
COOH	Carboxy terminal
DNA	Deoxyribonucleic acid
DSPC	Distearoyl-phosphatidylcholine
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
g	Gram
xg	Multiples of gravity
GALT	Gut associated lymphoid tissue
gD	Glycoprotein D
h r	Hour
HRP	Horseradish peroxidase
HSV	Herpes simplex virus
IATS	Inernational Antigenic Typing Scheme
Ig	Immunoglobulin
i.m	Intra-muscular
i.n.	Intra-nasal
IPTG	Isopropyl-ß-D-thiogalactopyranoside
kb	Kilobases
L	Litre
LPS	Lipopolysaccharide
M	Molar solution
mg	Milligram
m l	Millilitre
mM	Millimolar
MOPS	Morpholinopropane sulphonic acid
N	Normal solution
NH_2	Amino terminal
OD	Optical density
p	Plasmid
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PMSF	Phenyl-methylsulphonyl fluroride
RAPP	Rappaport broth
SBG	Selenite brilliant green broth
s.c	Subcutaneous
S.D.	Standard deviation
SDS	Sodium dodecyl sulphate
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Tc	Liquid-crystalline phase transition temperature
$TCID_{50}$	50% tissue culture infective dose
TEMED	N,N,N',N'-tetramethylethylene diamine
TMB	3,3',5,5',-tetramethylbenzidine
v	Volts
v/v	Volume per volume
w/v	Weight per volume
µg	Microgram
µ l	Microlitre

CHAPTER 1

INTRODUCTION

1 ORIGIN AND SCOPE OF THE WORK

The delivery route and formation of a vaccine can effect the level and type of immune response elicited (Mowat, 1987; Gregory *et al*, 1986). The success of new purified vaccines, either natural or synthetic, will largely depend on the parallel development of safe and effective immunoadjuvants.

Work in this laboratory is aimed at purifying iron regulated membrane proteins (IRMPs) of *Pseudomonas aeruginosa* and delivering them as an experimental vaccine, in order to evaluate their potential as protective antigens. It has been demonstrated that bacteria adapt to the iron-restricted environment imposed on them during an infection by producing IRMPs and iron-chelating agents (Shand *et al*, 1985; Anwar *et al*, 1984). IRMPs have been shown to be antigenically important surface accessible immunogens recognised early on during the course of an infection (Anwar *et al*, 1985). However, difficulties in the purification of these IRMPs were encountered and therefore model antigens were chosen to investigate potential adjuvants and delivery routes of vaccines.

Preliminary investigations were started with a *Pseudomonas* vaccine PEV-O1 supplied by Porton Down. However, the goat anti-human secretory component used in the ELISA method to detect specific secretory IgA (sIgA) was found to be cross reactive with the lipopolysaccharide used to coat the ELISA plate. Therefore, the guinea pig model of herpes simplex virus (HSV) type 2 intravaginal infection was chosen for several reasons. Genital herpes infection has been successfully established and extensively studied in female guinea pigs following intravaginal inoculation with HSV-2 (Scriba and Tatzber, 1981; Scriba, 1982; Scriba, 1975; Stanberry *et al*, 1982; Lucia *et al*, 1983). Much understanding of the pathophysiology of genital infection with HSV in humans has been derived from studies with the guinea pig model of genital herpes (Scriba and Tatzber, 1981; Stanberry *et al*, 1985; Scriba, 1976; Hsiung *et al*, 1984). In addition, recurrent genital HSV infection in female guinea pigs

shares many features with recurrent genital herpes in human females (Overall, 1984). Compared to the primary infection, recurrent genital herpes in both humans (Corey *et al*, 1983) and guinea pigs is milder, shorter in duration and without evidence of systemic complications. Both humans (Corey *et al*, 1983; Guinan *et al*, 1981) and guinea pigs demonstrate a declining rate of recurrence over time. Finally, latent HSV is demonstrable in the sacral ganglia from both guinea pigs (Stanberry *et al*, 1982) and humans (Baringer, 1974).

Thornton *et al* (1984) suggested that the gunea pig model allows close observation of many facets of the disease process and comparisons may be made between treated and control animals to assess the efficacy of vaccines in reducing the effects of infections with HSV-2 (Scriba, 1978; Stanberry *et al*, 1987; Stanberry *et al*, 1988; Stanberry *et al*, 1989). However, in common with all animal models of human disease, care must be exercised when using the results obtained in the model to predict expected events in human patients. In addition, HSV infects at a mucocutaneous site and therefore, one desirable property of any vaccine would be the ability to induce immunity at the site of viral entry ie the mucosal surface.

Tetanus toxoid was also chosen as a model antigen to investigate adjuvants such as DSPC liposomes, gels and their effect upon the immune system using different routes of delivery. Tetanus toxoid being a water soluble molecule, comparisons could be made with the work carried out using the Skinner HSV subunit vaccine entrapped in liposomes.

1.1 VACCINE DELIVERY SYSTEMS

Living microorganisms are often superior to inactivated or subunit vaccine preparations in their ability to stimulate protective immune responses, though in general the reasons for this remain unclear. One of the main problems with using live organisms as vaccines is related to the way in which they are derived ie by

attenuation of virulent strains and thus reversion may occur. The introduction of recombinant-DNA technology, together with the increasing availability of information on the genes that enable pathogens to survive and grow within the host has rekindled interest in the development of live vaccines. In addition, potentially protective antigens from heterologous pathogens can now be cloned and expressed in existing live vaccine strains making possible the development of multivalent vaccines.

Live vaccines do not require the addition of an immunological adjuvant, since the microorganism multiplies in the host cells and elicits an immune response in a manner analogous to the wild type infection. Vaccines composed of whole, inactivated microorganisms, although unable to replicate, still have the advantage of their particulate nature, which enhances immunogenicity due to better antigen presentation (Wold *et al*, 1989).

Development of vaccines consisting of proteins and peptides (or other macromolecules) purified from natural sources, produced by recombinant DNA methodology or by chemical peptide synthesis, has posed a major challenge, to enhance the overall immunogenicity of these purified antigens. An ideal adjuvant should have certain characteristics including non-toxic, biodegradable, non-immunogenic nature, the ability to elicit both cell-mediated immunity and humoral immunity to antigens administered by a variety of routes, a potential for selective interaction with populations of immunocompetent cells, and synergistic action with other adjuvants if needed. In addition, practical criteria include stability in storage, inexpensive raw materials, and simplicity to manufacture. This section will review the development of immunological adjuvants for such vaccine antigens.

1.1.1 Liposomes

Liposomes are biodegradable vesicular membranes of varying sizes consisting of one or more concentric phospholipid bilayers separated from each other

by an aqueous compartment (Bangham *et al*, 1965). They differ with respect to their dimensions, composition (different phospholipids), charge (neutral, positive or negative), and structure (multilamellar and unilamellar). Many phospholipids, alone or in combination with other lipids (including lipid extracts from membranes), will form liposomes. Depending on their gel-liquid crystalline transition temperature (Tc-the temperature at which hydrocarbon regions change from a quasicrystalline to a more fluid state), phospholipids determine bilayer fluidity and stability with respect to permeability to solutes *in vitro* and *in vivo.*

A variety of liposome preparation methods have been established. Firstly, multilamellar vesicles (MLV) can be prepared by rotoevaporation producing liposomes with a size range of 400-several nm (Bangham *et al*, 1965). Secondly, single unilamellar vesicles (SUV) can be prepared by sonication (25nm), detergent dialysis (100-200nm), or ether vaporisation (100-200nm) (Husang, 1969; Kagawa and Racker, 1971; Deamer and Bangham, 1976). Thirdly, large unilamellar vesicles (LUV) can be prepared by cochleate cylinder/chelation producing liposomes with a size range of 200-1,000nm (Papahadjopoulos *et al*, 1975). Finally, reverse phase evaporation vesicles (REV) can be made using the method of reverse phase evaporation (Szoka and Papahadjopoulos, 1978) producing liposomes with a size range of 100-1,000nm. In addition, a more recent method has been suggested by Kirby and Gregoriadis (1984) to avoid harsh conditions during entrapment of antigens and other sensitive agents. This method results in the production of dehydration-rehydration vesicles (DRV). Antigen-containing DRV can be freeze-dried in the presence of a cryoprotectant. Most of the antigen contents are retained within intact vesicles on reconstitution with saline (Gregoriadis *et al*, 1987).

When liposomes are prepared in an aqueous solution of antigen, the antigen may be entrapped either in the aqueous compartments of the liposomes, or it may be associated with the phospholipid bilayers themselves. If the antigen is bound to

liposomal phospholipid, part of it will probably be exposed on the outer surface of the liposome. This may be recognised by lymphocytes with surface receptors for that antigen, and an immune response can be initiated. When the antigen is entrapped within the aqueous compartments and not associated with the phospholipid bilayers, it is masked and so rendering recognition by antigen specific lymphocytes impossible. In this case macrophages are required to process the liposome-entrapped antigen. On the basis of information on liposomal fate *in vivo* (outlined later), it can be surmised that humoral immunity to liposomal antigens is in part the result of the system's ability to function as an antigen depot, wherein antigen is supplied to macrophages at rates conducive to optimal processing. The participation of macrophages in adjuvant enhancement of responsiveness has been shown by the lack of enhancement in macrophage depleted animals (Su and Van Rooijen, 1989).

Liposomes can act as immunological adjuvants for medically relevant antigens, for example, diphtheria toxoid (Allison and Gregoriadis, 1974) and cholera toxin (Pierce and Sacci, 1984). In addition, studies by Manesis *et al* (1979) with hepatitis B surface antigens have shown that the immune response can also be cell-mediated, an event that plays a major role in protection against most viral infections. Proteins coupled to lipids are known to induce delayed type hypersensitivity in proportion to the latter's hydrophobicity. Gregoriadis (1990) suggested that the increased internalisation of hydrophobic antigen lipids by macrophages ultimately improves antigen presentation to T cells.

Experiments by Davis *et al* (1986/87) using tetanus toxoid suggest that the adjuvanticity of liposomes is reflected in most antibody subclasses and that there is no shift in subclasses compared to the response obtained with free antigen, thereby establishing liposomes as a type 1 adjuvant (Asherson and Allwood, 1969). They also found that liposome adjuvanticity is due to events following primary immunisation and no differences were observed (IgG_1 and IgG_{2b}) between entrapped

and surface-linked toxoid.

Immunity to antigens can be drastically improved, in some cases selectively, through the administration of liposomes together with other adjuvants. For example, muramyl dipeptide (MDP) and lipophilic derivatives (Kersten *et al*, 1988b; Alving *et al*, 1986), *B. pertussis* (Manesis *et al*, 1979) and lipid A (Alving *et al*, 1986). This synergism of adjuvants can be effected by the co-entrapment of adjuvant together with antigen in the same vesicle, entrapment of adjuvant and antigen in separate vesicles or, by the simple addition of adjuvant.

Liposomes have been delivered by various routes (reviewed by Patel and Ryman, 1981). Conventional drug-containing liposomes become unstable and leaky upon contact with blood after intravenous delivery (Gregoriadis, 1981; Yatvin and Lelkes, 1982). This may be due to plasma high density lipoproteins (HDL) which remove phospholipid from the bilayers (Kirby *et al*, 1980), the destabilised liposomes are then removed by the reticuloendothelial system (RES). Phospholipid molecules can be made resistant to HDL attack by incorporating excess cholesterol into the liposome structure or using 'high melting' phospholipids or phospholipids which form inter-molecular bonds with other lipids present (Gregoriadis, 1984). Large liposomes bearing a negative charge are cleared rapidly from the circulation. Within minutes of intravenous injection they are found largely in the fixed macrophages of the liver and spleen (Ryman and Tyrrel, 1980; Gregoriadis, 1981; Yatvin and Lelkes, 1982). Circulation time can be prolonged to some extent by preparing smaller liposomes (Gregoriadis, 1984; Deamer and Uster, 1983). Once in the circulation properties such as permeability, size and surface charge are altered rapidly, and liposomes can be broken down by lipoproteins, circulating phospholipases or complement (Patel and Ryman, 1981).

Subcutaneously delivered liposomes of large size disintegrate locally, probably upon attack by infiltrating macrophages. In contrast, smaller vesicles enter

the lymphatic system and localise avidly into the lymph nodes (Gregoriadis, 1981 and Ryman and Tyrrell, 1980). This has stimulated research using liposomes either as reservoirs for sustained release of drugs, or vaccines (Gregoriadis, 1981).

The use of liposomes as an oral dosage form has been considered, since they may either protect the entrapped drugs from digestive degradation (Bangham *et al*, 1965) or increase absorption of poorly absorbed drugs from the gastrointestinal tract (Sessa and Weissman, 1970). The oral route of administration using liposomes has been employed in a limited number of studies performed in animal models. Dapergolas and Gregoriadis (1976) have shown that when insulin was entrapped within liposomes and administered orally, blood glucose levels were reduced in both normal and diabetic animals. However, some conflicting results were reported concerning the efficiency of the absorption of liposomal insulin from the gastrointestinal tract. Patel and Ryman (1977) and Hashimoto and Kawada (1979) were unable to show hypoglycaemic effects in normal rats. They did however note a significant reduction of blood glucose level, after oral administration of liposomally-associated insulin to diabetic animals.

Patel and Ryman (1981) and Patel *et al* (1982) have shown that liposomes protected entrapped antigens from proteolytic digestion and facilitated their absorption from the gastrointestinal tract. Experiments by Patel *et al* (1985) suggest that liposomes which escape degradation by residual phospholipases and bile in the lumen, can be taken up by mucosal cells of the intestine, but that they are not transported into the venous circulation as intact vesicles. The rate of uptake and intracellular digestion of liposomes is dependent on the degree of saturation and the fatty acid side chain length of the phospholipids from which they are prepared. In addition, work by Weingarten *et al* (1985) investigated the degradative action of three digestive enzymes against insulin-associated with or entrapped in positively-charged liposomes. Both insulin preparations were protected against enzymatic degradation.

The nature of the interaction between the external phospholipid bilayer and insulin was found to result from an ionic association.

Work by Wachsmann *et al* (1985) has illustrated that rats immunised by the intragrastic route with liposome-associated soluble antigen extracted from *Streptococcus mutans* cell wall showed a significantly higher IgA (and IgG) response than did rats infected with the soluble antigen alone. In addition, Wachsmann *et al* (1986) have shown that the immunogenicity of *S. mutans* polysaccharide could be improved by chemical coupling with a carrier cell surface protein. When orally administered with liposomes, a local IgA response was induced which was directed against both a polysaccharide antigen and a cell surface protein.

The stability of liposomes in the gastrointestinal tract is essential to their success and again there are conflicting results. Rowland and Woodley (1980) conducted *in vitro* studies to determine the extent to which low and high pH, bile salts and pancreatic lipase cause release of an entrapped macromolecule from liposomes. Liposomes composed of DSPC and cholesterol were the only ones 'stable' in the presence of extremes of pH, bile salts and pancreatic lipase. It has been suggested that intact liposomes which contain drugs can be endocytosed by the absorptive cells of the gastrointestinal tract (Rowland and Woodley, 1981), thereby facilitating transport of molecules which could not traverse membranes in their free state. However, the combined *in vitro* and *in vivo* results of Chiang and Weiner (1987a; 1987b) suggest that liposomes do not influence the oral absorption of entrapped drug. Furthermore, even the most 'stable' liposomes (DSPC-as suggested by Rowland and Woodley, 1980) do not retain entrapped material in the gastrointestinal tract and hence will not protect it from the environment.

Gregoriadis (1990) suggests that the adjuvanticity of liposomes is due to the system's vesicular structure and, perhaps, of its lipoid nature, rather than of the identity of its lipid components or other secondary characteristics. However, the

latter have been shown to control effectively the behaviour of liposomes *in vivo* (Gregoriadis, 1988) and may therefore aid in the way immunoadjuvant activity is expressed. Optimisation of liposomal vaccines has been reviewed by Gregoriadis (1990).

1.1.2 Iscoms

Quil A micelles are used for the presentation of membrane proteins. Quil A is an extract obtained from the bark of *Quillaja saponaria molina*. These micelles have hydrophobic regions which can form complexes with hydrophobic portions of membrane proteins, resulting in immune-stimulating complexes called ISCOMs (Morein *et al*, 1984). They are formed by solubilising the viral membrane with a detergent followed by exchanging the detergent with saponin by ultracentrifugation on a saponin-containing sucrose gradient (Morein *et al*, 1984). However, with the use of more highly purified membrane proteins these centrifugation procedures resulted in the formation of aggregates and micelles instead of the ISCOM particles. It was found that the highly purified membrane proteins lacked the small amounts of membrane lipids that had allowed ISCOM formation with the less highly purified membrane antigens. Subsequently, Lovgren and Morein (1988) found that approximately equimolar ratios of purified protein, cholesterol, and phosphatidylcholine are optimal for ISCOM formation.

ISCOMs have been prepared with a variety of membrane proteins. For example, surface glycoproteins from bovine herpes virus type 1 were shown to be approximately five to 10 fold more potent in eliciting antibody in rabbits when in ISCOMs, compared to non-adjuvanted micellar formulations (Trudel *et al*, 1987). In addition, ISCOM formulations of feline leukemia virus (FeLV) membrane glycoprotein (gp) 70/85 antigens were shown to induce antibody responses in and to protect six vaccinated kittens from viraemia 10 weeks after challenge with FeLV (Osterhaus *et al*,

1985). The immunisation experiment was extended to include 140 cats (Osterhaus *et al*, 1987;1989) and approximately 85% of the ISCOM-immunised cats developed virus neutralising antibodies. Preliminary immunisation experiments in mice with the FeLV ISCOMs elicited a clear cut immune response against gp70 after a booster (Akerbolom *et al*, 1989).

Trudel *et al* (1989) have evaluated the immunogenicity of respiratory syncytial (RS) virus subunit vaccines in guinea pigs. ISCOMs made from the surface proteins of both human and bovine strains absorbed to the adjuvant *Quil A*, were assayed for their capacity to induce neutralising antibodies, compared with experimental live virus vaccines. Sera from animals vaccinated with either the human or bovine RS subunit vaccines were equally efficient in neutralising human or bovine RS virus. Merza *et al* (1989) showed that the glycoprotein gp51 of bovine leukaemia virus (BLV) in ISCOM form induced considerably higher response in mice than purified gp51.

ISCOMs have also been shown to increase the humoral immune response in mice and guinea pigs to the influenza viral envelope glycoprotein haemagglutinin and neuraminidase approximately 10 fold as compared to the micellar protein antigens alone. They also elicited a higher and more long-acting antibody response in horses as compared to the conventional whole-virus vaccines (Sundquist *et al*, 1988). ISCOMs have been shown to elicit cell-mediated immunity against cytomegalovirus in monkeys (Wahren *et al*, 1987).

The optimal adjuvant for a protein antigen, must be determined by experiment. Kersten *et al* (1988b) have provided morphological and chemical data for ISCOM structures containing an amphiphilic protein. They have shown that ISCOMs are much more immunogenic than liposomes and protein detergent complexes, but are more toxic. In addition, the influences of *Neisseria gonorrhaeae* protein 1B content of liposomes and ISCOMs, and the bilayer composition of liposomes have been investigated

by comparing the humoral response in mice (Kersten *et al*, 1988a). Decreasing the *Quil A* to protein ratio from 12 to 6 reduced the secondary antibody response approximately two-fold. Changes in the protein content of liposomes did not influence the IgG response, and bilayer composition only influenced the primary IgG response; immunological memory was not affected. However, all the ISCOM formulations were better than the liposomal formulations both for primary and secondary antibody responses.

The disadvantages of ISCOMs include a relatively complex method of formulation, and reaction at injection sites (Morein, 1988). In addition, the presence of the heterogenous, ill-defined and haemolytic saponin mixture *Quil A* may prevent the use of ISCOMs in man. However, these disadvantages are less serious if the oral route can be used, *Quil A* is accepted as a food additive. Kersten *et al* (1990) immunised rats with pore protein I of *Neisseria gonorrhaeae* incorporated in ISCOMs (Kersten *et al*, 1988b). Induction of mucosal and systemic immune responses was noted using rats with a chronically isolated intestinal (Thiry-Vella) loop. The duration of the IgA response was short, but the booster effect suggested the induction of memory.

Letvin *et al* (1987) compared the adjuvant effects of ISCOMs and a threonyl-muramyldipeptide/Pluronic/Squalene adjuvant (SAF). Rhesus monkeys vaccinated with ISCOMs prepared from detergent solubilised Simian immunodeficiencey virus (SIV) elicited good anti-envelope antibody responses but variable responses to the core antigens. Monkeys vaccinated with inactivated SIV adjuvanted with a SAF formulation developed approximately four-fold higher anti-envelope titres, as well as a good anti-core response, compared with the ISCOM-vaccinated monkeys.

1.1.3 Emulsions

Multiple emulsions are complex, inherently unstable systems, which

are unlikely to be commercially acceptable as drug delivery systems until the problems of their instability *in vitro* and *in vivo* are solved. A number of factors have been identified as affecting the stability of w/o/w emulsions (reviewed by Matsumoto, 1985). These include method of preparation, nature of entrapped materials, effects of electrolytes, phase volumes, concentration and types of emulsifiers (Florence and Whitehill, 1981).

Florence and Whitehill (1981;1985) attempted an analysis of the possible mechanisms of instability. For example, under the influence of an osmotic gradient, the oily lamellae of the multiple drops act as 'semi-permeable' membranes resulting in the passage of water across the oil phase. This leads to either swelling or shrinkage of the internal droplets, depending on the direction of the osmotic gradient. Another possible breakdown mechanism may be coalescence of the internal aqueous droplets within the oil phase. In addition, according to Florence and Whitehill (1981) a combination of these mechanisms may take place; the likelihood of events taking place may be predicted by analysis of the van der Waal's attractive forces and free-energy changes in these systems. Attempts have been made to improve the stability of multiple emulsions for sustained drug release by interfacial complexation between non-ionic surfactant and macromolecules (Law *et al*, 1984; Florence *et al*, 1985; Omotosho *et al*, 1985). The formation of a complex interfacial membrane at the primary w/o interface was found to enhance the stability of the emulsions and to slow down the release of solute from the emulsion droplets. A number of techniques have been developed to assess the stability of multiple emulsions. For example, Omotosho *et al* (1986) looked at the release of NaCl and 5-fluorouracil (5-FU) separately entrapped in the aqueous phase of a w/o/w emulsion. They suggest the main factor in determining the differences in rates of release from hydrocarbon emulsions appears to be the droplet size of the internal aqueous phase.

The different types of emulsion systems and their application have been

reviewed in detail by Davis *et al* (1985). Oil-based adjuvants have been used to increase humoral responses of farm animals to many inactivated bacterial and viral vaccines (McKercher, 1986). Freund (1956) developed his incomplete and complete adjuvants (FIA and FCA). The incomplete adjuvant is mineral oil emulsion stabilised with the detergent Arlacel A. In general, protein antigens in FIA elicit antibody formation but not delayed type hypersensitivity (DTH); protein antigens in FCA also elicit DTH.

Freunds adjuvants have been widely used in laboratory animals to elicit high levels of antibodies, cell-mediated immunity and protection against challenge with viable microorganisms. However, FCA has not been approved by regulatory authorities for human or animal use because it elicits tuberculin hypersensitivity and granulomatous reactions at infection sites.

There has been considerable development of emulsion formulations with vaccines. A vaccine consisting of antifertility antigens (ß-HCG) coupled to diphtheria toxoid in a squalane-Arlacel A emulsion with nor-MDP (N-acetyldesmethyl muramyl-L-alanyl- D-isoglutamine) was tested in women in Australia (Jones *et al*, 1988). One batch of the squalane emulsion was rather unstable, suggesting that it may be necessary to develop a different vehicle preparation (Eppstein *et al*, 1990). All the women given the vaccine with the stable emulsion batches made antibodies to ß-HCG, the titres of which increased with increased antigen and MDP dose.

Sanchez-Pescador *et al* (1988) vaccinated guinea pigs with gD of herpes simplex virus with several adjuvants. MTP-PE (Muramyltripeptidiphos-phatidyl-ethanolamine) in a squalene/Tween-80 emulsion proved to be an effective adjuvant especially if injected in the footpad. However, MDP conjugated to gD provided little protection against challenge with live virus. Eppstein *et al* (1990) suggested on the basis of experimental data in humans and animals that MDP analogs as adjuvants will only be effective with the development of a stable formulation with properties

comparable to those of FIA, but which are acceptable to regulatory authorities.

Byars *et al* (1989) have developed a stable oil-in-water emulsion of squalane with Pluronic polymer L-121 which allows threonyl-MDP (N-acetyl-muramyl-L- threonyl-D-isoglutamine) to exert its adjuvant activity; the complete adjuvant is known as SAF. SAF has proven to be an effective antigen for a wide variety of antigens including hepatitus B virus surface antigen (Byars *et al*, 1989), ovalbumin (Byars and Allison, 1987) and inactivated SIV (Desrosiers *et al*, 1989).

1.1.4 Microspheres

Biodegradable albumin microspheres appear to be a useful delivery system for sustained and controlled release of drugs (Morimoto and Fujimoto, 1985). The factors which can modify the localisation and distribution of particles in the body are (Davis and Illum, 1986), route of administration, particle size, and particle surface characteristics. Therefore, depending on the surface characteristics, colloidal carriers can be recognised and subsequently taken up by various cells of the reticuloendothelial system, especially those residing in the liver. Albumin microspheres have been proposed as drug delivery systems for targeting to various organs and tissues, including tumors and cells of the reticuloendothelial system (Kramer, 1974; Illum and Davis, 1982; Widder *et al*, 1979).

A practical application of the oral immunisation route to the defence against pathogenic infection has in the past been hampered by vaccine degradation through gastric acidity and the proteolytic enzymes of the gut, and by the extremely large doses required to achieve adequate immunity. Le Fevre *et al* (1989) has shown that latex microspheres are absorbed from the intestinal lumen into the Peyer's patches. Jani *et al* (1989) have shown absorption of intact polystyrene latex particles in the 50nm to 1µm size range. Particles from animals fed orally for several days were taken up by the mesenteric lymph vessels and transported towards the lymph

nodes. Alpar *et al* (1989b) described the transport of microspheres from the gastro-intestinal tract to inflammatory air pouches in the rat. In addition, Eldridge *et al* (1989) have described the use of biodegradable microspheres as an oral delivery system which protects vaccine antigens from degradation in the gut, targets their delivery to the Peyer's patches, and provides sustained vaccine release in a manner which induces a strong secretory IgA (sIgA) response at diverse mucosal tissues (providing evidence also for the common mucosal immune system; see section 1.2.1.2).

1.1.5 Immune stimulants

This class of adjuvants is composed of factors that for their adjuvant effect stimulate T and/or B cells either directly or indirectly (review see Eppstein *et al*, 1990). For example, interleukins have been investigated; Weinberg *et al* (1986a) have shown that human recombinant interleukin 2 (rh IL-2) has a protective effect against acute herpes simplex virus (HSV) type 2 genital infection in guinea pigs, manifested by a decreased rate of infection, less severe acute disease, and lower mortality. Protection appears to be mediated principally by natural killer (NK) activity (Weinberg *et al*, 1986b). Weinberg *et al* (1987) showed that rh IL-2-induced immune stimulation was as protective against recurrent HSV-2 disease in guinea pigs as the viral suppression achieved with acylovoir. Weinberg and Merigan (1988) were able to increase resistance of immunised guinea pigs to challenge with HSV-2 by additional treatment with rh IL-2. The incidence of lesions in the rh IL-2-treated group was lower and they tended to be less severe. While there was little enhancement of antibodies to HSV, cytotoxic activity of monocytes from IL-2 treated animals was enhanced.

While these and other observations (see for example Eppstein *et al*, 1990) are interesting, the practical application of cytokines as adjuvants presents

several difficulties. For example, if a cytokine from another species is used it may be immunogenic; cytokines are not without toxic side effects; multiple doses may be required and it is likely that a cocktail of cytokines will be necessary, which has not yet been defined.

An alternative strategy is to use bacterial products as adjuvants which induce the production of cytokines, or preferably use analogs with adjuvant activity and fewer side effects (review, see Eppstein *et al*, 1990.)

1.1.6 Live attenuated *Salmonella* strains as vectors for antigen delivery

Since most pathogens colonise on or invade through a mucosal surface it is appropriate to develop an immunisation strategy that elicits a mucosal immune response to serve as a first line of defence. It would also be desirable if such an immunisation strategy caused production of serum IgG and a cellular immune response. The review so far has discussed adjuvants that can be potentially used for any route of delivery. However, attenuated *Salmonella* have been investigated for use by intra-peritoneal or oral immunisation. This section will concentrate on the oral use of *Salmonella* due to the aim of the PhD which is to investigate mucosal delivery and the production of mucosal responses.

Several lines of investigation have led to the use of avirulent *Salmonella* to express antigens and elicit secretory, humoral and cellular immune responses against the pathogen supplying those genes. Firstly, there is considerable evidence for a common mucosal immune system upon delivery of antigens to GALT (Mestecky *et al*, 1980; Lamm, 1976: Cebra *et al*, 1976 and Bienenstock *et al*, 1983a). More recently, it has been found that antigens can lead to humoral and cellular immune responses (Brown *et al*, 1987; Curtiss *et al*, 1987). Secondly, orally administered *S. typhimurium* initially attach to, invade and persist in the GALT in mice before

colonising the liver and spleen (Carter and Collins, 1974). Thirdly, *Salmonella* can be attenuated by introduction of mutations (Curtiss *et al*, 1987; Curtiss and Kelly, 1987; Hoiseth and Stocker, 1981) which do not alter the initial colonisation of the GALT (Curtiss, 1986; Curtiss and Kelly, 1987; Curtiss *et al*, 1988a; Curtiss *et al*, 1988b and Curtiss *et al*, 1989). Lastly, gene cloning and other genetic and biochemical procedures have been used to identify colonisation and virulence attributes of a large number of human and animal bacterial pathogens.

Attenuated *Salmonella* mutants have been used as vectors for expression of cloned genes for colonisation and virulence antigens from various pathogens to deliver these antigens to the GALT (Curtiss, 1986; Curtiss *et al*, 1986).

There are a number of features which the ideal attenuated vaccine strain should possess. It must be completely avirulent and highly immunogenic. However, because of genetic diversity in the population to be immunised, this balance will be difficult to achieve. It is also desirable to achieve protection after a single immunising dose. Avirulent *Salmonella*, must still be able to colonise the intestine and GALT without causing disease. There must be two or more attenuating deletion mutations to ensure reversion or gene transfer does not result in loss of the traits. The system must provide stable high-level expression of the cloned genes in the immunised animal host and it must be easy to grow, store and administer (Curtiss *et al*, 1989).

The following sections will review information on the isolation and characterisation of avirulent *Salmonella* strains and the construction and properties of recombinant avirulent vaccine strains to achieve stable high-level expression of cloned gene products.

1.1.6.1 Avirulent Salmonella mutants

Any *Salmonella* strain considered for use as a carrier should be attenuated in a genetically defined manner and be well characterised *in vivo*.

Bacterial pathogens require various gene products to establish an infection and, ultimately, cause disease in a host. These genes might encode classical virulence factors such as adherence determinants required for attachment to and colonisation of host tissues, or toxins which damage host defences and impair immune responses. Alternatively, genes involved in metabolic pathways or in the regulation of gene expression could play vital roles in adapting a pathogen to the environment *in vivo*.

Ideally, vaccine strains should harbour (review, see Charles and Dougan, 1990):-

1) mutations that cannot be suppressed by secondary mutations in other genes.

2) mutations in more than one gene, these should be localised in different regions of the chromosome to minimise the risk of reversion due to genetic exchange mechanisms

3) mutations carrying a defined genetic lesion, again minimising the possibility of reversion.

Different mutations can attenuate pathogens to varying extents. The degree of attenuation can be measured in several ways.

1.1.6.1.1 Plasmid-less mutants

It has been demonstrated that plasmid-less strains of *S. typhimurium* and *S. dublin* are capable of attaching to, invading and persisting in the GALT while being defective in either reaching or surviving in the liver or spleen (Gulig and Curtiss, 1987). Although there is disagreement about the virulence attributes encoded by the plasmids in *Salmonella* strains, progress is being made in defining the biochemical basis for plasmid-specified virulence by the use of gene cloning and transposon mutagenesis (Gulig and Curtiss, 1988).

Eliminating the virulence plasmid is not sufficient to develop a safe, efficacious vaccine strain. Gulig and Curtiss (1987) demonstrated that plasmid-less

strains can have an LD_{50} approaching that of wild-type strains when administered by the intraperitoneal route.

However, absence of the virulence plasmid would contribute to the safety of strains attenuated by additional means to be used for oral immunisation.

1.1.6.1.2 Auxotrophic mutants

One example of auxotrophic mutations concerns the *aro* gene cluster. These genes encode enzymes involved in the pre-chorismate biosynthetic pathway responsible for synthesis of aromatic compounds including aromatic amino acids, para-aminobenzoic acid (pABA) and dihydroxybenzoic acid. *Aro* mutants are dependent on these compounds for growth *in vitro*. Mammals do not possess this pathway and it is likely that the availability of some of these compounds *in vivo* is limiting. Following observations made by Bacon *et al* (1950;1951) that mutants unable to synthesise pABA, aspartate and purines were avirulent, Hoiseth and Stocker (1981) described the construction of *S. typhimurium* strains harbouring transposon insertions in the *aro*A gene. *Salmonella aro*A strains are attenuated and effective vaccines in mice (Hoiseth and Stocker, 1981), calves (Smith *et al*, 1984) and sheep (Lascelle *et al*, 1985).

Extensive studies with single and double auxotrophic mutants constructed by introducing different stable *aro* and *pur* deletion mutants into a *S. typhimurium* virulent in mice showed that strains harbouring single and different combinations of auxotrophic mutations differed substantially in their immunogenicity (O'Callaghan *et al*, 1988a; Dougan *et al*, 1988; Miller *et al*, 1989). It was concluded that strains harbouring single and double combinations of mutations in the pre-chorismate biosynthetic pathway such as *aro*A, *aro*D or *aro*C, were similar with respect to attenuation and *in vivo* persistence, and were all excellent single dose oral and parenteral vaccines. In contrast *pur*A mutations were too attenuated to provide

protection against Salmonellosis after a single oral dose, but they were able to stimulate local secretory immune responses.

1.1.6.1.3 Mutants defective in global gene expression

S. typhimurium mutants with deletion (Δ) mutations in the adenylate cyclase gene (*cya*) and the cyclic AMP receptor protein (*crp*) were avirulent and highly immunogenic (Curtiss and Kelly, 1987). Strains harbouring these deletion mutations can colonise and persist in the GALT at levels equivalent to that observed with wild-type strains. However, they had a diminished ability to reach or survive in mesenteric lymph nodes and spleen (Curtiss and Kelly, 1987). Loss of the 100kb *S. typhimurium* virulence plasmid further diminishes the virulence of *S. typhimurium* without imparing its initial tissue tropism or persistence in the GALT (Gulig and Curtiss, 1987).

In addition, it has recently been shown that *S. typhimurium* strains harbouring stable mutations in *omp*R, a positive regulator of porin expression, are attenuated following oral or parenteral administration to mice (Dorman *et al*, 1989). These strains were also effective oral vaccines.

Salmonella strains harbouring mutations in another regulatory gene *pho*P, have recently been recently shown to be attenuated (Fields *et al*, 1989). Also mutations in *pho*P impaired the ability of *Salmonella* strains to survive intracellularly inside macrophages.

1.1.6.1.4 Mutants defective in virulence attributes

Progress in basic understanding of *Salmonella* colonisation, invasion, intracellular multiplication and intraorgan spread has been slow. Nevertheless, the approach of rendering *Salmonella* strains avirulent by introducing mutations in genes of known function has potential. Moreover, it can be anticipated that some completely

avirulent, but highly immunogenic strain constructions may ultimately arise from this line of investigation.

1.1.6.2 Heterologous antigens expressed in live vaccine strains

Most attenuated variants can be considered as carriers, although certain strains may offer advantages or disadvantages for particular antigens. Heterologous antigens expressed in *Salmonella* vaccine strains have been reviewed recently by Charles and Dougan (1990).

Table 1.1 lists some of the heterologous antigens that have been expressed in *Salmonella* vaccine strains. Such strains have been used in immunisation studies; sIgA and humoral IgA and IgG antibodies have been detected. Brown *et al* (1987) recorded cellular immune responses against the expressed antigens although induction of protective immunity against the majority of these antigens has not been demonstrated. Exceptions include work by Fairweather *et al* (1990) who demonstrated oral immunisation against tetanus in mice using a *Salmonella aro*A mutant as a carrier of fragment C. Poirier *et al* (1988) demonstrated that a *Streptococcus pyogenes* M protein induced protecive immunity to mice against subsequent challenge with *S. pyogenes.*

Most of the antigens were introduced into *Salmonella* on recombinant plasmids using expression systems designed primarily for use in *E. coli* K12. Salmonella and *E. coli* are sufficiently related that most promoters active in *E. coli* will also function in *Salmonella.* However, in many cases the gene expression control systems are not present in *Salmonella* (eg *Salmonella* is *lac* negative). For this reason most heterologous antigens studied so far have been constitutively expressed.

Table 1.1 Heterologous antigens expressed in *Salmonella* spp.

Antigen	Plasmid/host	Reference
Schistosoma mansoni antigen	pUC-based plasmid, *S. typhimurium aro*A or *S. typhimurium gal*E host	Taylor *et al*, 1986
ß-Galactosidase	pBR322-based plasmid, *S. typhimurium aro*A host	Brown *et al*, 1987
M5 protein from *Streptococcus pyogenes*	pSC101-based plasmid, *S. typhimurium aro*A host	Poirier *et al*, 1988
Protective C fragment of tetanus toxin of *Clostridium tetani*	pUC-based plasmid, *S. typhimurium aro*A host, *aro*C-chromosomal vector	Strugnell *et al*, 1990

adapted from Charles and Dougan (1990)

Possible reasons for the lack of protective immunity after vaccination with recombinant avirulent *Salmonella* strains include:- 1) the recombinant strains were unable to stably maintain the cloned gene. 2) some hyperattenuated strains do not persist in a viable form long in the vaccinated animal to stimulate an adequate immune response. 3) the foreign antigen may not be capable of producing a protective immune response. 4) strains failed to produce the foreign colonisation or virulence antigen to the extent observed when the cloned gene was in typical *E. coli* K-12 cloning hosts (Charles and Dougan, 1990).

Although these constructs are useful experimental vaccines, they would be unacceptable as live vaccines. This is mainly due to the plasmids encoding antibiotic-resistance determinants, which because of the widespread use of antibiotics in agriculture and medicine, might lead to selective survival or enhanced multiplication of live avirulent vaccine strains. Therefore, a practical vaccine would need to meet rigorous manufacturing standards covering reproducibility of viability

and immunogenicity, lack of antibiotic resistance and other safety aspects.

1.2 NON-PARENTERAL IMMUNISATION

1.2.1 The immune response to mucosal immunisation

1.2.1.1 The secretory immune system

Active immunisation against infectious agents which enter the body through mucous membranes may depend on the successful induction of an immune response in the external secretion. Immunoglobulin A (IgA) is produced by many species, including humans, in quantities exceeding those of all other immunoglobulin classes combined. Most IgA is secreted onto mucosal surfaces, becoming the principal protection at these sites. In addition to external secretory tissues, large amounts of IgA are produced in human bone marrow, but its rapid catabolism relative to that of IgG results in lower serum levels of IgA than of IgG (Heremans, 1974; Mestecky and McGhee, 1987). Although both external secretions and serum contain molecules of the IgA isotype, the secretory and serum IgA systems appear to be independent with respect to molecular properties of IgA and cellular origins (Heremans,1974; Mestecky and McGhee, 1987). For example, while serum levels of IgA mature slowly over 15 years, the concentrations of IgA in external secretions may reach adult levels in as little as 4 to 6 weeks (Gleeson *et al,* 1982).

Under normal conditions, serum IgA occurs primarily in monomeric form with a pronounced predominance of the IgA1 subclass (Heremans, 1974). In contrast, IgA in glandular secretions and in gastrointestinal, respiratory and genitourinary fluids is almost exclusively dimeric to which the carbohydrate rich secretory component (SC) is attached (Tomasi *et al,* 1965; Hanson, 1961). The distribution of IgA1 and IgA2 in external secretions reflects the proportion of plasma cells in the corresponding tissues (Mestecky and Russell, 1986; Kemp *et al,* 1980). However, observations by Kett *et al* (1986) and Mestecky and Russell (1986)

indicate that there are notable differences between different anatomical sites. Whilst IgA2 cells may account for up to 60% of total sIgA in the colon, the proportions in nasopharyngeal tissues are more like those in bone marrow.

Extensive immunohistochemical studies (for review see Brandtzaeg, 1985) suggest that polymeric IgA is assembled from monomeric subunits within subepithelial IgA-secreting plasma cells before acquisition of SC and transport into external secretions. In addition, these studies resulted in the currently accepted model for the origin and transport of IgA. Polymeric IgA produced locally by plasma cells is taken up by SC present as a receptor on the surface of epithelial cells and transported into external secretions. The details of SC biosynthesis and molecular and subcellular events operational during transepithelial transport have been reviewed by several investigators (Mestecky and McGhee, 1987; Brandtzaeg, 1985; Solari and Kraehenbuhl, 1985; Ahen *et al*, 1985).

If polymeric configuration is required for selective IgA transport, could circulating endogenous or passively injected polymers also be efficiently transported into external secretions to enhance protection on mucosal surfaces? Experiments yielding controversial results have been reviewed by Mestecky (1987). The current consensus regarding the origin of sIgA in human external secretions favours local synthesis over transport from the circulation. Therefore, passive immunisation with polymeric IgA is an unlikey route for providing immunity on mucosal surfaces in humans.

1.2.1.2 Common Mucosal Immune System

The induction of immune responses at a mucosal surface was originally considered to be predominantly a local phenomenon: specific IgA antibodies were induced mainly at the site of antigen encounter (Ogra *et al*, 1968; Centifanto *et al*, 1970). However, animal studies of the origin and homing patterns of IgA precursor

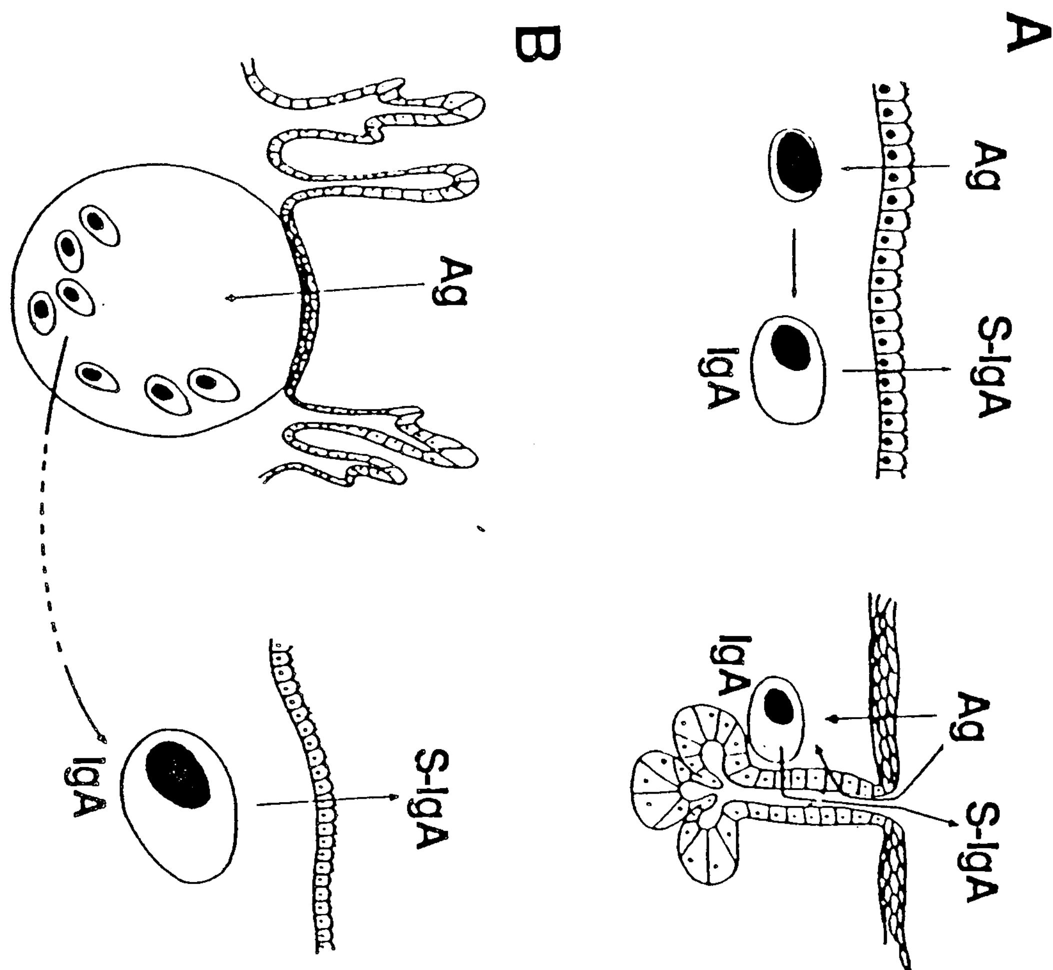
cells, including those from orally immunised animals, have suggested the existence of a central pathway for the induction of a generalised secretory immune response (Craig and Cebra, 1971; Michalek *et al*, 1976; Bienenstock, 1982; Lamm *et al*, 1982). These two pathways for stimulation of the sIgA-associated immune response are illustrated in fig 1.1.

There are three lines of indirect evidence in humans suggesting, with certain limitations, that induction of a generalised immune response may be used for prevention of human infectious diseases in which the agent enters through mucosal surfaces (Mestecky and McGhee, 1987). These are described below.

Firstly, secretions of exocrine glands not directly stimulated by antigens contain natural antibodies to microbial and environmental antigens (Mestecky *et al*, 1985). Secretory IgA (sIgA) from human colostrum and tears contains antibodies to the oral bacterium *Streptococcus mutans* (Arnold *et al*, 1976; Allansmith, 1982) as well as other bacterial and food antigens (Ogra *et al*, 1983).

Secondly, sIgA antibodies in tears, parotid or whole saliva, milk and nasopharyngeal secretions have been induced as a consequence of oral ingestion of bacterial and viral antigens (Mestecky, 1987). For example, oral immunisation with a polyvalent bacterial vaccine containing *Haemophilus influenzae* and *Staphylococcus aureus* led to an increase in specific antibodies of IgA, IgM and IgG isotypes to *H. influenzae* in the saliva but not in the serum of 55% of immunised volunteers (Clancy *et al*, 1983).

Fig 1.1 Two pathways for stimulation of the sIgA-associated immune response. A-local sIgA response restricted to the site of antigen application; B-sIgA response expressed in secretory tissues remote from the site of antigen application.



Waldman *et al* (1986) and Bergmann *et al* (1986) used orally-administered, influenza virus in enterically coated capsules in human volunteers. The results indicated that the specific IgA response was induced in tears, saliva, and nasal wash, but not in serum. Although an effective response, common to several external secretions, may be useful in preventing a wide spectrum of infectious and allergic diseases, the large doses of antigens and prolonged immunisation necessary may be a limiting factor in the efficacious application of oral ingestion of antigens as a primary route of immunisation.

Finally, because lymphoid tissues, including lymph nodes and spleen cannot be obtained, cellular studies are limited to examination of lymphocytes from peripheral blood. However, in the IgA cell cycle, antigen- and IgA-committed precursor cells from the gut associated lymphoid tissue (GALT) should be present in the peripheral blood before their migration into secretory glands and tissues. It has been shown that mitogen-stimulated peripheral blood lymphocytes secrete predominantly polymeric IgA and the intracellular distribution of IgA subclasses resembles that of secretory tissues and not that of bone marrow or spleen (Kutteh *et al*, 1980; Mestecky and Russell, 1986). Based on these results it was proposed that a large proportion of such cells in the peripheral circulation are precursors of IgA-producing plasma cells with the apparent potential to populate mucosal tissues (Kutteh *et al*, 1980).

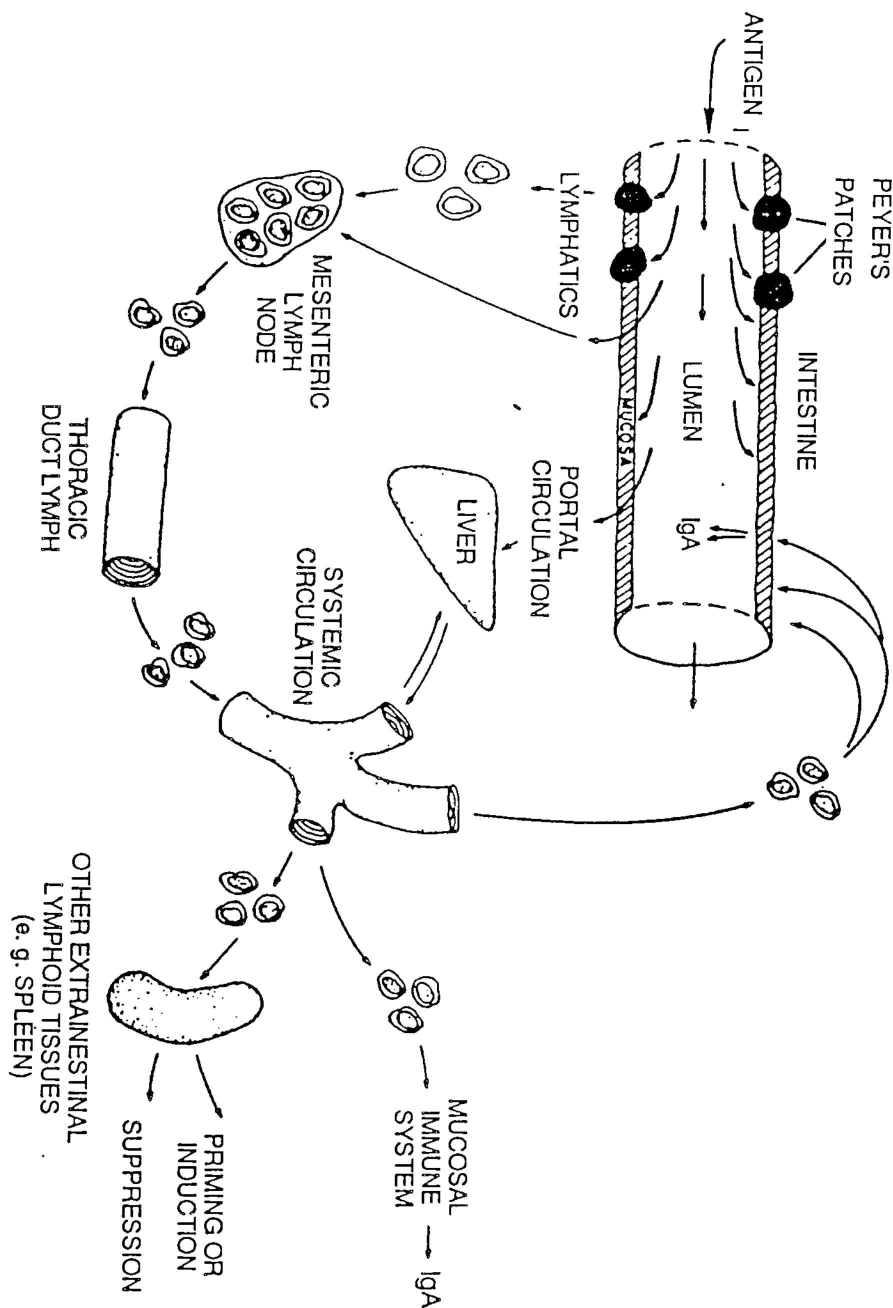
Subsequent experiments carried out with peripheral blood lymphocytes from volunteers orally immunised with a single dose of combined cholera B-subunit/whole-cell vaccine secreted IgA antibodies to the ingested antigen into tissue culture supernatants (Lycke *et al*, 1985). Peripheral blood lymphocytes that secreted specific IgA antibodies were detected in four of six volunteers who had been orally immunised with enterically coated capsules containing *S. mutans.* These cells were detectable in peripheral blood before the appearance of specific sIgA antibodies in

external secretions including saliva and tears (Czerkinsky *et al*, 1987). These results taken together indicate that the peripheral blood contains lymphocytes that have the potential to produce IgA antibodies specific for the ingested antigen.

The concept of a common mucosal immune system can be described as follows and as shown in fig 1.2. Lymphoid cells from the bone marrow enter the Peyer's patches (GALT) through high endothelial venules. Under the local influence of T cells and accessory cells they express surface IgA. Environmental antigens enter Peyer's patches through pinocytotic and phagocytotic M cells, and interact with resident accessory, T, and B cells. IgA-committed and antigen-sensitised B cells and lymphoblasts leave Peyer's patches and enter the regional lymph nodes, then the lymph and circulation. Finally, these cells populate various exocrine glands and mucosa-associated tissues, where terminal differentiation into IgA-secreting plasma cells occurs. Bronchus-associated lymphoid tissue (BALT) apparently plays a role analogous to that of GALT (Mestecky *et al*, 1980; Lamm, 1976; Cebra *et al*, 1976 and Bienenstock *et al*, 1983b).

Many factors are claimed to be responsible for remote-site cellular localisation of IgA precursor cells, including the presence of antigens. The role of antigen has been controversial. Husband and Gowans (1978) demonstrated extravasation *per se* is antigen-independent, but presence of antigen determines retention and proliferation of IgA cells in the mucosal lamina propria. The homing phenomenon has been shown to occur in the absence of antigen stimulation (Rudzik *et al*, 1975), but requires the local presence of T helper cells for IgA production (Bienenstock *et al*, 1983b). More recent results support a role in homing and recirculation for membrane glycoproteins and/or glycolipids on the migrating lymphocytes and on endothelial cells (Hooghe and Pink, 1985).

Fig 1.2 Diagram to illustrate cell traffic in the common mucosal immune system.



1.2.1.3 Mucosal cell-mediated immunity

The existence of local cellular immunity on secretory surfaces was recognised initially from studies in which animals were sensitised to an antigen by the parenteral route and found to react to local challenge with the recall antigen in the form of an aerosol (Miyamoto *et al*, 1971). Henney and Waldman (1970), assaying macrophage migration inhibition factor (MIF), demonstrated the presence of respiratory tract cell mediated immunity (CMI) and its independence from systemic CMI. In addition, Galindo and Myrvík (1970) have demonstrated CMI in lymphocytes from the lungs of rabbits immunised with Bacillus Calmette-Guerin (BCG). They showed that pulmonary CMI was independent of systemic CMI and that it varied with the route of immunisation. The duration of local CMI is dependent upon the nature of the antigen employed. For example, local cellular immunity arising from application of live attenuated mycobacteria is of longer duration compared to that generated from killed vaccine (Spencer *et al*, 1974).

The dichotomy between local and systemic CMI can be overcome by administration of a large amount of antigen. This has been shown in guinea pigs immunised either systemically or locally into the respiratory tract (Waldman *et al*, 1972a). Influenza virus vaccine adjusted by weight equivalent to that used in human immunisation, resulted in circulating antibody and CMI as determined by MIF assay in splenic lymphocytes after parenteral immunisation. Local immunisation with nasal drops resulted in local cellular and humoral immunity with only a slight systemic immune response. After a large dose of antigen, equivalent to 10 times the usual dose given to immunise humans, CMI developed in both bronchial washings and splenic lymphocytes in the parenterally-immunised animals. Humoral immunity, however, remained the same in the animals which received the lower dose of vaccine. The inhibition of macrophage migration activity had disappeared or was greatly reduced in both splenic and bronchial lymphocytes by 28 days; however, the

influenza-neutralising antibody persisted through this time. These data indicate that local or systemic CMI may be relatively short-lived compared to antibody response.

Morag *et al* (1974) studied local and systemic CMI following rubella immunisation in guinea pigs. The results indicate the independence of local CMI and its short duration. Stimulation of the mucosal surface by a non-cross-reactive protein is an additional method for stimulating a local reponse following injection of the usual dose of antigen (Nash and Holle, 1973).

1.2.2 Oral immunisation

1.2.2.1 Antigen uptake and processing

The intestine is the most important port of entry for foreign antigens and also contains the majority of the lymphoid effector cells of the body, collectively referred to as the gut-associated lymphoid tissue (GALT) (Brandtzaeg *et al*, 1984).

The mucosa-associated lymphoid tissue may deviate from its systemic counterpart in being able to discriminate between microbial and nonmicrobial antigens. Wold *et al* (1989) suggested that a prerequisite for a strong mucosal antibody response is that the GALT encounter the antigen within microorganisms capable of effective presentation. In addition, DeAizpura and Russel-Jones (1988) have identified classes of antigens that, when orally administered to an animal at low doses, were effectively taken up by the cells of the gastrointestinal mucosa and were then able to elicit serum and/or secretory antibodies without induction of systemic tolerance (section 1.2.2.3).

The absorption of proteins and macromolecules occurs *via* several mechanisms (review see O'Hagan, 1987). Nonspecific absorption of proteins and macromolecules occurs by cellular uptake *via* an endocytic process, by direct absorption *via* intercellular tight junctions or an intracellular pathway, and by M cells of the Peyer's patches.

The primary function of the M cells is to provide the immune system with samples of gut antigen in order to stimulate the body's defense mechanisms against future absorption of the antigen. The oral administration of antigens to M cells may prove to be a highly efficient method of priming all secretory sites for increased IgA response. This would be of great benefit in immunisation protocols which call for IgA antibody production.

1.2.2.2 Immune response to oral immunisation

In comparison to systemic immunisations, induction of sIgA antibodies by oral ingestion generally requires doses of antigens that may be higher by several orders of magnitude. This is due to the limited absorption from the intestinal lumen of antigenic material that escapes digestive enzymes or complexing with preexisting antibodies and other substances such as mucin (for review see Hemmings, 1978). Living microorganisms multiplying within the intestinal lumen or gut-associated tissue (GALT) provide a more effective stimulus for an immune response in secretions than killed bacteria and viruses (Ogra *et al*, 1976; Rubin *et al*, 1981). To maximise the secretory and systemic immune responses induced by oral administration of antigens, several possible approaches have been investigated

Firstly, the use of genetically engineered bacteria may preferentially colonise the GALT. For example, the use of live attenuated *Salmonella* strains as vectors for antigen delivery (see section 1.1.6). Secondly, to protect antigenic determinants on killed microorganisms or other immunogens from acid pH and proteolysis by gastric enzymes, orally ingested antigens have been given withy a large amount of sodium bicarbonate to neutralise hydrochloric acid in the stomach (Black *et al*, 1983). A more sophisticated approach involves coating antigens in gelatin capsules with substances that are soluble only at the alkaline pH of the small intestine (Czerkinsky *et al*, 1987; Waldman et al, 1986; Black *et al* 1983). This method of

antigen protection has been used successfully in human oral immunisation trials with both bacteria (Czerkinsky *et al*, 1987; Black *et al*, 1983) and viruses (Waldman *et al*, 1986). SIgA responses were induced in secretions of glands anatomically distinct from the site of immunisation.

Alternative possibilities for oral delivery of antigens, such as incorporation into liposomes (see section 1.1.1) or ISCOMs (see section 1.1.2) have also been explored. In addition, the use of orally administered adjuvants such as muramyl dipeptide (MDP) (Morisaki *et al*, 1983) and cholera toxin ß subunit (see section 1.5.2.4) has been used for boosting sIgA responses. MDP is nonimmunogenic by itself, exhibits a low toxicity and has none of the side effects inherent to other adjuvants. These characteristics are also manifested when MDP is given by the oral route (Morisaki *et al*, 1983). Rats receiving MDP and *S. mutans*-derived glucosyl transferase (GTF) orally showed elevated sIgA responses (Taubman *et al*, 1983). However, rabbits given muramyl dipeptide (MDP) and antigen chronically by inhalation produced an increase in serum, but not in intestinal IgA response (Butler *et al*, 1983).

The major unresolved problem associated with the oral administration of 'broad-spectrum' adjuvants concerns the undesirable potentiation of an immune response to a large number of bystander antigens in the gastrointestinal tract.

1.2.2.3 Oral tolerance

Oral tolerance is the state of specific immunological unresponsiveness induced by prior oral administration of antigen. Systemic unresponsiveness occurs after feeding a wide range of thymus-dependent antigens, including inactivated bacteria (Stokes *et al*, 1979). Thymus-independent antigens, however, usually prime the animal for an active immune response, even when presented in association with a T-dependent antigen (Stokes *et al*, 1979). It is also generally more difficult to induce

oral tolerance to a particulate antigen than to a soluble antigen (Challacombe and Tomasi, 1980), while oral administration of live allogeneic cells or virus (Rubin *et al*, 1981) generates local and systemic immunity. These findings may provide an experimental basis for the apparent ability of the intestinal immune system to prevent hypersensitivity to soluble, non-replicating antigens while mounting active immunity to particulate, thymus-independent antigens of the kind presented by potentially invasive organisms (Wold *et al*, 1989).

Specific systemic unresponsiveness in mice after protein feeding has been demonstrated for IgG and IgE antibody responses, antigen-specific T cell proliferation, and delayed skin reactions (Richman *et al*, 1978; Ngan and Kind, 1978; Miller and Hanson, 1979). In addition, a human volunteer study by Clancy *et al* (1983) using an oral polyvalent bacterial vaccine resulted in the selective appearance of antibody in saliva in the absence of serum antibody. This is consistent with previous observations in humans (Mestecky *et al*, 1978) and with the observation that systemic tolerance may follow the presentation of antigen to mucosal sites (Richman *et al*, 1981; Challacombe and Tomasi, 1980). Alternatively, ingested antigen can stimulate high levels of circulating antibody (Clancy and Pucci, 1978), and this may be enhanced in patients with abnormal mucosa or impaired local defence mechanisms (Shorter, 1974). A number of possible approaches to overcome oral tolerance have been investigated (section 1.2.2.3).

A number of different mechanisms have been found to operate in oral tolerance. These vary depending on the type of antigen used for feeding (review Mowat, 1987). After protein feeding, suppression of antibody reponses can be transferred with cells obtained from the Peyer's patch or spleen of fed animals (Richman *et al*, 1978; Ngan and Kind, 1978) but cannot be transferred by serum. The cell responsible is a radiation-sensitive T cell with a Ly-1−2+3+ surface phenotype. These suppressor T cells are present in Peyer's patches as early as 3 days after feeding

ovalbumin and are first demonstrable in spleen 7 days after feeding, suggesting that they are first stimulated in gut-associated lymploid tissue (GALT) and subsequently migrate to other lymphoid tissues (Richman *et al*, 1981). It has also been shown that splenectomised mice can be tolerised by ovalbumin feeding (Hanson *et al*, 1979). Although unresponsiveness after protein feeding occurs regularly in most mouse strains tested, NsB/W and C3H/HeJ strains do not develop oral tolerance; in both instances, this appears to be due to defects in the generation of suppressor cells (Carr *et al*, 1983; Kiyono *et al*, 1982).

Investigators have demonstrated antigen-specific T cell help for IgA responses and T cell suppression for IgG responses shortly after a single feeding of ovalbumin (Richman *et al*, 1981), diphtheria toxoid (Seman and Morisset, 1982), or sheep red blood cells (SRBC) (Mattingly, 1983). This effect is transient and is not the predominant one, at least not after multiple feedings (Elson and Ealding, 1984) and is supported by the work of Challacombe (1983).

It has been postulated that lipopolysaccharide (LPS) stimulates suppressor T cell precursors in GALT, which are stimulated by antigen to become the antigen-specific suppressor T cells mediating oral tolerance (Michalek *et al*, 1983; Babb and McGhee, 1980; Kiyono *et al*, 1982). In addition, Elson and Ealding (1984) have inferred that the lack of oral tolerance to cholera toxin and its ß subunit may be due to binding to or cross-linking of Gm1 ganglioside molecules on the cell surface membrane of lymphoid cell in GALT (Holmgren *et al*, 1973).

Therefore, several immunoregulatory mechanisms are implicated in the induction of oral tolerance. Mowat (1987) proposed that, after feeding proteins, systemic delayed type hypersensitivity responses are inhibited by antigen-specific T cell suppressors (Ts) which are activated by specialised I-J+ antigen presenting cells in the mucosa, Peyer's patches or in systemic lymphoid tissues. In addition, it has been suggested that intestinal epithelial cells may themselves present tolerogenic antigens

to Ts (Mowat, 1987).

1.2.2.4 Factors influencing the immune response

A major factor determining the response is the form in which the antigen is presented. The differences in response to living and dead antigen have been discussed. Interactions between protein antigen and the enterocyte membrane appear to promote antigenicity. As mentioned earlier, cholera toxin (CT) through its ß subunit interacts with ganglioside Gm1 on the surface of enterocytes. CT can serve as an adjuvant to enhance gut immune responses to co-administered antigen, including soluble proteins (Elson and Ealding, 1984) and hapten conjugates (Cebra *et al*, 1986).

Pierce (1978) showed that the ß subunit of CT, which binds to the enterocyte was more immunogenic than the formalin-treated toxin. The entire toxin was even more immunogenic. Nedrud *et al* (1987) used oral immunisation to induce immunity against Sendai virus, a common pathogen of mice. Inactivated virus was a poor gut immunogen whereas co-administered CT dramatically enhanced the antivirus immune response. However, the inherent toxicity of CT is a hinderance to its use in humans. Liang *et al* (1989) have dissociated adjuvanticity from the toxicity of CT by treating it with gluteraldehyde. Covalent cross-linking of toxoid adjuvant and virus resulted in a further enhancement of the adjuvant effect.

1.2.3 Nasal immunisation

Systemically active drugs such and proteins and peptides are poorly absorbed orally and are extensively metabolised in the gastrointestinal tract. The nose is well suited for absorption of drugs since it has a large epithelial surface area available due to numerous microvilli. In addition, the subepithelial layer is highly vascularised and the venous blood from the nose passes directly into the systemic

circulation, by-passing the liver. Various approaches have been attempted to increase the absorption and bioavailability of drugs administered intranasally. For example, Illum *et al* (1987) have shown that by selecting a delivery system in the form of microspheres that have good bioadhesive characteristics and which swell easily on contact with the nasal mucosa, it is possible to control the rate of clearance of the delivery system from the nose.

Insulin administered intra-nasally in animals by means of a nebuliser or in drop form has a negligible effect on plasma insulin and blood glucose levels; absorption occurs when the insulin is dissolved in an acid medium (pH 3.1), or is combined with surfactants like sodium glycholate, saponin or 9-lauryl ether, usually at pH of 7.4 (Aungst *et al*, 1988; Hirai *et al*, 1977). Surfactants are also required in humans and the use of a spray seems to be more effective than the drop form (Moses *et al*, 1983; Paquot *et al*, 1988). Insulin has a molecular weight of 6000 and forms high molecular weight complexes in solution. The adjuvants used above to increase absorption are surface-active agents which probably reduce the complex formation. McMartin *et al* (1987) suggest the adjuvants modify junctions between cells and therefore, if these junctions are formed between proteins embedded in the membranes, it is likely that at least part of the binding energy is provided by contact between apposed hydrophobic surfaces. The surface active adjuvant could sit on these surfaces rendering them hydrophilic and thus either enlarge the size of gaps or increase the number of hydrophilic channels. The nasal route has been shown to be suitable for efficient, rapid delivery of many molecules of molecular weight <1000. With the use of adjuvants, this limit can be extended to atleast 6000 (Pontiroli and Pozza, 1989; Huang *et al*, 1985). In addition, McMartin *et al* (1987) found that the relative absorption of cyclic and cross-linked peptides and proteins was significantly greater than that of linear peptides.

Perkins *et al* (1969) studied the antigenicity and protective action of

the inactivated rhinovirus type 13 vacine in adult male volunteers. After intra-nasal administration, 14 of thge 17 volunteers developed a 4 fold or greater rise in nasal and/or serum antibody. The men were challenged with type 13 virus and significant protection was achieved with only 4 of 12 vaccinees developing illness, whereas 11 of 13 seronegative controls became ill.

Parainfluenza viruses are a major cause of lower respiratory infections in children, which may be life threatening. An inactivated parainfluenza type 2 vaccine was used to vaccinate 56 volunteers by subcutaneous injection or intra-nasal aerosol spray and the antibody levels in the serum and in the respiratory secretions compared (Wigley *et al*, 1970). Both groups yielded a significant rise in the serum level of antibody. A greater rise was observed ink the s.c. group, which was not statistically different from the i.n. group.

1.2.4 Vaginal immunisation

The epithelial lining of certain secretory tissues (salivary, nasal, lacrimal, gut, bronchus) secretes locally-produced immunoglobulin A (IgA) which may serve a protective function (see sections 1.2.1.1 and 1.2.1.2). The work of Widders *et al* (1985) support the concept of a well developed local immune system in the equine genital tract. This emphasis on local production distinguishes the horse from a number of other species. Although there is evidence of local immunoglobulin production in the reproductive tracts of human (Ogra and Ogra, 1973; Waldman *et al*, 1972b), cow (Corbeil *et al*, 1976), sow (Hussein *et al*, 1983) and rabbit (McAnulty and Morton, 1978), the systemic immune system in these species is a major source of the immunoglobulins in genital tract secretions.

The relative contributions of serum or local production are an important consideration in any mucosal vaccination programme. Studies of the bovine reproductive tract (Schurig *et al*, 1975) have demonstrated the local protective

capacity of systemic vaccination, and in the rabbit (Collins *et al*, 1979), parenteral immunisation has a significant local effect. Work by McBride *et al* (1988) indicates that subcutaneous injection of the Skinner vaccine (Skinner *et al*, 1982a) induces a secondary response both in the serum and at the vaginal mucosa of subsequently challenged guinea pigs. This response was associated with a significant reduction in the degree of oedema and number of genital lesions produced after challenge. In contrast, Widders *et al* (1985) suggest that systemic immunisation in the mare would have little protective capacity for the equine genital tract.

1.3 PARENTERAL IMMUNISATION

1.3.1 The immune response to parenteral immunisation

Humoral response of the immune system to antigen is the proliferation of antigen-specific B lymphocytes and their maturation to antibody (immunoglobulin, Ig)-secreting cells (Burnet, 1957). Antigen selects from the repertoire of resting B cells, each expressing one of the diverse sets of variable regions of heavy and light chains Ig on their surface (Moller, 1961; Dutton and Eady, 1964). When B cells mature they not only increase their rate of Ig synthesis and begin to secrete Ig, they also switch the class of heavy chains that carry the variable regions involved in antigen recognition. A single dividing clone of B cells may switch at any division (Anderson *et al*, 1978). Switching occurs in individual cells of the clone. Within an expanding clone of B cells some cells may return to rest and remain in the immune system as long-lived memory cells (Sprent, 1977).

Antigens that elicit B cell responses can be split into 2 categories:

1) Thymus-independent.

T cells are not required for an effective immune response, examples include carbohydrate antigens and lipopolysaccharides.

2) Thymus-dependent.

T cells are required for an effective immune response, examples include red cells, protein antigens and hapten protein conjugates.

T cells respond to antigen only on the surface of another cell, the antigen-presenting cell. Moreover, in most cases it appears that the antigen must be taken up and 'processed' by that cell in endosomes, a step that is still incompletely understood (Unanue, 1984; Berzofsky, 1985). Fragments of the antigen are then presented to the T cell, not alone, but in association with a molecule encoded by the major histocompatibility complex (MHC) of the presenting cell (Berzofsky *et al*, 1987; Berzofsky, 1988; Berzofsky *et al*, 1986).

1.3.2 Mucosal immune response associated with parenteral immunisation

Parenteral immunisation with various antigens does not usually stimulate a sIgA immune response in external secretions (Heremans, 1974; Ogra *et al*, 1976) due to the apparent compartmentalisation of systemic and secretory immune systems (Heremans, 1974; Mestecky *et al*, 1986). The systemic humoral response is manifested primarily by IgM and IgG antibodies and only infrequently induces serum or sIgA (as described in section 1.3.1). However, this rule may not be generally applicable: systemic immunisation of individuals who had previously been exposed to the immunising antigen by the mucosal route could elicit a sIgA response in external secretions. This possibility has been demonstrated with systemically injected bacterial vaccines. For example, parenteral immunisation of lactating Pakistani women who had been presumably naturally exposed to *Vibrio cholerae* induced specific sIgA antibodies in external secretion. Subcutaneous immunisation with polyvalent pneumococcal vaccine led to the appearance of antibody forming cells in the peripheral blood, with IgA as the predominant isotype (Kehrl and Fauci, 1983; Steinitz *et al*, 1986). For reasons explained in section 1.2.1.2, such IgA cells may represent precursors of IgA

plasma cells that home to various secretory tissues. Generally, however, in the absence of previous antigen exposure, systemic immunisation leads to systemic rather than mucosal antibody; for instance, oral immunisation with cholera toxin is far more effective in stimulating intestinal immunity than is intravenous injection (Lange and Holmgren, 1978). Therefore, the initial mucosal exposure profoundly influences the outcome of subsequent systemic immunisation and appears to favour, at least with some antigens, an IgA response in external secretions. These findings may be of great importance in the design of efficient immunisation protocols in naturally exposed or previously unexposed individuals.

Keren *et al* (1988) have demonstrated that giving rabbits a parenteral priming dose of heat-killed shigellae 1 day before the oral dose of live shigellae resulted in an enhanced early IgA anti-shigella response measured in intestinal secretions. Pierce and Gowans (1975) found that a single intraperitoneal injection (100μg) of cholera toxin in complete Freund's adjuvant followed after 2 weeks with a 15mg intestinal boost of toxin produced a maximal IgA antitoxin-containing cell response in the lamina propria which peaked 4 days after the boost.

Unfortunately, these attractive results obtained using this combination of systemic priming and oral boosting are not universal, and prior subcutaneous priming of rabbits followed by an oral boost has been shown to suppress IgA antibody responses (Hamilton *et al*, 1979). In an attempt to explain these differences, Pierce and Koster (1980) showed that priming was obtained if antigen was given to rats intraperitoneally but not intravenously or subcutaneously and that it was enhanced by Freund's complete adjuvant. It is clear, then, that the use of regimes consisting of a combination of systemic and oral routes involves complex immunological interactions and must be characterised for each antigen and species studied.

1.4 HERPES SIMPLEX VIRUS

Herpes simplex virus (HSV) types 1 and 2 are responsible for a wide range of clinically important mucosal infections. These viruses can cause acute infections of the genital tract in human subjects and may result in the establishment of latent infection and, in some cases, recurrent disease.

1.4.1 Pathogenesis of infection

Understanding the pathogenesis of HSV infection is critical to the development of strategies for its control. From work in the early 1930's it became clear that there were three stages in HSV infection; 1) acute infection 2) recrudescent lesion, without systemic lesion 3) latent phase during which the virus persists unnoticed despite evident immunity to it.

Latency and pathogenesis can be reproduced in animal models, but few truly represent the sequence of phenomena noted in man. Scarification of virus inoculum onto the flank of a mouse is thought to mimic peripheral infection in man (Blacklaws *et al*, 1987). Mice reproduce all the features of HSV infection in man (Simmons and Nash, 1985). Guinea pigs differ in some respects; virus may become latent in sensory ganglia, but it also seems to persist in peripheral tissues. However, the animal provides an excellent model for studying recrudescence and the role of immunity (Scriba, 1975; Donnenberg *et al*, 1980; Stanberry *et al*, 1986; Stanberry *et al*, 1982; Stanberry *et al*, 1985).

The factors regulating the latency and recurrence of HSV infection are complex and have been recently reviewed (Klein, 1985; Wildy, 1986; Klein and Czelusniak, 1987). Latent virus may be reactivated by various stimuli, including ultra-violet irradiation, immunosuppression, trauma of the skin or infected nerves (Corey and Spear, 1986).

Reactivation of virus in the ganglion may allow virus replication and

transport along the sensory nerve to the skin. Replication at this peripheral site is termed recurrence and a recurrent infection may lead to recrudescence. The development of clinically apparent lesions is probably influenced by the amount of virus, the nature of the virus, virus cell interactions and the rapidity of the host's immune response (Notkins, 1974; Lopez, 1985).

1.4.2 Immune response to Herpes simplex virus

1.4.2.1 Natural resistance

The immune response to HSV infections involves the host's natural resistance mechanisms and the proliferative (specific) immune system. Natural resistance to HSV infection involves interactions between macrophages, natural killer (NK) cells and interferon (IFN). These mechanisms form the first line of defence against invading pathogens. The macrophages interact with NK cells and induce them to lyse infected cells (Yasukawa and Zarling, 1983) and stimulate IFN production. The IFN prevents macrophage maturation, further enhances NK lytic activity and stimulates mature, infected macrophages to increase the presentation of foreign antigens to the proliferative immune system, specifically to the T-lymphocytes (Kirchner *et al*, 1983; Hall and katrak, 1986). The presentation of viral antigens to T-lymphocytes is the most important function of macrophages. Antigens are taken up and 'processed' by the antigen presenting cell in endosomes (Unanue, 1984), and fragments of the antigen are then presented to the T-cell in conjunction with self antigens, known as the Major Histocompatibility Complex (MHC) antigens (Berzofsky *et al*, 1986); classes 1 and 2 are important in immune responses to HSV infections. It seems likely that the T-lymphocyte-mediated responses, restricted in activity by a particular set of MHC antigens, are the most important defence against HSV infections (Sethi *et al*, 1983).

1.4.2.2 T-lymphocyte responses

The cytotoxic T-lymphocytes (CTL) and the delayed hypersensitivity T-lymphocytes (T-DH) are the more important T-cell subsets in HSV infections (Nash *et al*, 1981b; Lawman *et al*, 1980; Larsen *et al*, 1983; Schrier *et al*, 1983a; Larsen *et al*, 1984). Several HSV glycoproteins elicit good CTL responses in experimental animals (Lawman *et al*, 1980; Marsden *et al*, 1987; Johnson *et al*, 1988; Meignier *et al*, 1988). However, Martin *et al* (1988) showed that a significant proportion of cytotoxic T-cell populations obtained from draining lymph nodes of mice acutely infected with HSV-1 also recognised immediate-early gene-expressing target cells, indicating the importance of nonstructural HSV proteins to antiviral immunity *in vivo*.

Recent analyses of the characteristics of virus-specific CTL have shown that two distinct CTL populations exist. One is $CD8^+CD4^-$ and restricted by HLA class I antigen (Sethi *et al*, 1980). The other is $CD4^+CD8^-$ and is restricted by HLA class II antigen (Yasukawa and Zarling, 1984a). The $CD4^+$ virus-specific CTL have been found to be multifunctional in that they exhibit both cytotoxic and virus-induced proliferation responses as well as helper activity, as evidenced by their production of interleukin-2 (Yasukawa and Zarling, 1984b) and helper factors to B cells for Ig production (Yasukawa *et al*, 1988). Yasukawa *et al* (1989) demonstrated that HSV-specific $CD4^+$ and $CD8^+$ CTL precursors are both present in peripheral blood of HSV-seropositive individuals. In addition, $CD4^+$ CTL are preferentially activated by cell-free HSV and $CD8^+$ CTL are predominantly activated by HSV-infected fibroblasts. Therefore, they suggest that *in vivo* $CD4^+$ CTL play major roles in resistance and recovery from conditions in which many cell-free HSV exist, such as viraemia and disseminated HSV infection, and that the spread of local HSV infections such as labial and genital HSV infection is prevented by $CD8^+$ CTL in addition to $CD4^+$ CTL.

Delayed type hypersensitivity (T-DH) responses are thought to be

responsible for rapid, early clearance of the virus (Nash *et al*, 1980; Nash *et al*, 1981a/1981b), yet once other immune mechanisms are established (CTL and antibody), the T-DH cells are thought to be superfluous to control of infection. This conclusion is based on the finding that T suppressor (TS) cell-mediated 'split tolerance' (affecting T-DH but not antibody or cytotoxic T lymphocyte responses) can be induced by intravenous virus inoculation (Nash and Ashford, 1982). Tolerance is mediated by Ts and occurs in two different forms. The first, acting early in infection to block establishment of the T-DH response, is thought to be mediated by Ts cells induced directly by virus particles or soluble viral proteins. The second set of Ts cells appears a few days later and gradually increases in numbers to become the more prolific of the two subsets. These cells are thought to be stimulated by processed viral antigens in conjunction with MHC antigens on the surface of infected cells, and appear to prevent the functioning of existing T-DH (Nash and Gell, 1983; Schrier *et al*, 1983b; Nash and Gell, 1981).

Intravenous inoculation of HSV is an artificial system. Altmann and Blyth (1984/1985) developed a complementary model for activation of specific suppressor cells by attempting to manipulate the immune response in the mouse ear model of herpes simplex. It is inferred from their findings in both models of Ts cell activation that during some stages of virus spread through the central nervous system, D-TH mediated pathology may be more harmful than the direct, cytopathic effect of virus. In this case, Ts cell activation has a beneficial effect.

1.4.2.3 Antibody response

Serum antibody responses to HSV infections are not thought to be as important as the cellular immune responses and good clearance is effected in their absence (Simmons and Hash, 1985). In the host, the virus spreads from cell to contigous cell by way of an intercellular mechanism and as a result of this the virus is

not generally available outside the cells for neutralisation by serum antibody. Antibody induced by HSV infections (mostly IgG and IgM; Kurtz, 1973) is able to neutralise viral infectivity *in vitro*, and in the host various antibody-dependent cytotoxic effects are thought to restrict viral spread. These include antibody-dependent cellular cytotoxicity and complement-mediated cytotoxicity, and their role in hindering virus entry into the nervous system may modify the appearance and severity of virus-induced neurological illness (McKendall *et al*, 1979).

1.4.3 Vaccine prospects

There is considerable interest at present in the production of a safe, DNA- free vaccine both for the prevention or modification of recurrent disease episodes (Allen and Rapp, 1982). An important consideration of such a mucosal infection is to induce immunity at the site of viral entry, i.e. the mucosal surface.

1.4.3.1 Non live vaccines

Subunit preparations must be of high purity and contain no viral DNA or toxic substances. They have been tested in animal models but open studies in humans yielded conflicting results regarding their ability to prevent disease recurrence (Skinner *et al*, 1982a; Woodman *et al*, 1983) and protect consorts of infected patients (Skinner *et al*, 1982b). A number of subunit vaccines against HSV are already undergoing clinical trials in man (Mertz *et al*, 1984; Kutinova *et al*, 1988). The procedures involved in obtaining sufficient quantities of pure subunit preparations are costly. In addition, subunit or peptide vaccines are weakly immunogenic. The influence of different adjuvants on the immune response has been investigated using a synthetic peptide of HSV type gD (amino acids 9-21) (Geerligs *et al*, 1989) and gD-1 from CHO cells (Sanchez-Pescador *et al*, 1988).

Attention has been focussed on expression of glycoprotein genes using

molecular cloning, whereby gene products could be expressed in virtually unlimited quantities. Glycoprotein D of HSV has been cloned and expressed in *E. coli* (Watson *et al*, 1982, Weis *et al*, 1983) and eukaryotic cells (Berman *et al*, 1985; Lasky *et al*, 1984) and shown to be antigenic as well as immunogenic in several animal models. Guinea pigs and mice were protected against challenge by alum-precipitated mixtures of glycoproteins prepared by affinity chromatography from infected vero cells (Meignier *et al*, 1987a). Cloned gD, lacking 93 amino acids from the carboxyl terminus was transfected into CHO cells, expressed, glycosylated, processed and secreted. Mice given 3-6µg of purified gD were protected against HSV challenge (Lasky *et al*, 1984). In addition, clinical signs were reduced by 90% in guinea pigs inoculated with gD-1 produced in yeast (aa 20-354) but recurrence was not significantly affected (Stanberry *et al*, 1987).

Too little is known of the determinants of immunity in humans and of the differences in pathogenesis of HSV infection in man and animal to be sure that protection in animal will be predictive of efficacy in man.

1.4.3.2 Live vaccines

Live vaccines are expected to mount quantitatively stronger immune responses in vaccinees and also, qualitatively to provide better stimulation of cellular mediated immunity.

One possibility involves expression of glycoprotein genes in a vector system such as vaccinia virus (Zarling *et al*, 1986; Paoletti *et al*, 1984; McLaughlin-Taylor *et al*, 1988). Glycoprotein D expressed in vaccinia protected mice (Cremer *et al*, 1985) and guinea pigs (Wachsman *et al*, 1987) against challenge and latent infection. Immunity induced by vaccina recombinants has been shown to be long-lasting and stimulated by booster injections (intra-dermal), but was inhibited by previous exposure to vaccinia (Rooney *et al*, 1988).

The most specific answer to the problem would be to engineer HSV that would lack unwanted properties such as neurovirulence, ability to establish latency, transforming potential and yet still be capable of limited replication in humans to trigger the broad array of humoral and cellular responses which take place in natural infections (Meignier *et al*, 1987b; Roizman *et al*, 1984).

1.5 AIMS OF THE STUDY

The delivery route and formulation of a vaccine can effect the level and type of immune response elicited (Mowat, 1987; Gregory *et al*, 1986). The success of new vaccines, either natural or synthetic, will largely depend in the development of safe and effective immunoadjuvants. The aim of the work was to investigate novel antigen delivery systems for the stimulation of immune responses. There is convincing evidence for a common mucosal immune system (Mestecky, 1987) whereby non-parenteral immunisation leads to the induction of local and generalised immune responses at sites distant from the immunistation site.

To maximise the secretory and systemic immune responses induced by non-parenteral immunisation, several possible approaches were undertaken to establish experimental vaccination strategies and the specificity of immune response initiated.

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CHAPTER 2

NON-PARENTERAL DELIVERY OF PEV-O1

PSEUDOMONAS VACCINE

2.1 INTRODUCTION

The stimulation of antibody production is influenced by: the route and schedule of administration, the vaccine preparation, and the adjuvant used (Keren *et al*, 1985;1988). Immune responses can be induced by non-parenteral routes, for example oral, and in some cases the orally induced immune responses can be almost identical to those obtained by parenteral immunization (Thomas and Parrot, 1974; Rothberg *et al*, 1967). Non-parenteral vaccine delivery offers many advantages such as ease of administration, better cost effectiveness and producing improved immune responses at the mucosal surfaces. Further work is required to fully understand the factors controlling the production of the different immune responses and to assess the potential of these processes for non-parenteral routes especially oral and nasal immunisation.

The lipopolysaccharide (LPS) obtained from *P. aeruginosa* is of interest for the serologic classification and for the development of vaccines against infections due to *P. aeruginosa* (Fisher *et al*, 1969; Wilkinson and Galbraith, 1973; Hanessian *et al*, 1971). It has also been well established that antibody directed at the serotype determinant located on the LPS can be a major host defense against infections due to *P. aeruginosa* (Young *et al*, 1970; Crowder *et al*, 1972; Pollack and Young, 1979). It has been clearly established that antibody directed against LPS protects mice against infection by homologous strains of *P. aeruginosa* (Cryz *et al*, 1983; Cryz *et al*, 1984). High anti-LPS titres have been correlated with increased rates of survival among patients with pseudomonal bacteremia (Pollack and Young, 1979). In addition, during a 5 year clinical trial, the rate of *Pseudomonas* related mortality among severely burned patients was found to be greatly reduced after immunisation with a heptavalent LPS-based vaccine called Pseudogen (Alexander and Fisher, 1974; Hanessian *et al*, 1971). Severe adverse reactions to this vaccine however, were common (Alexander and Fisher, 1974 ; Pennington, 1974). Wood *et al* (1983) administered the

Pseudogen vaccine *via* intra-nasal spray to cystic fibrosis patients in an attempt to induce development of local antibody without the side effects associated with parenteral administration of the vaccine. Although slight increases in serum antibody titres were noted, there was no appreciable change in specific antibody levels in parotid saliva or sputum following intra-nasal administration. The PEV-01 is a polyvalent vaccine which is composed of pooled surface antigen extracted under mild conditions from viable cells of 16 different serotypes of *P. aeruginosa* (Miler *et al*, 1977). This vaccine has been tested in volunteers (Jones *et al*, 1976) and has undergone preliminary clinical trials (Jones, 1979; Roe and Jones, 1983). The results indicate that immunization of burn patients with either the polyvalent vaccine or human anti-PEV immunoglobulins provides protection with few adverse side effects against fatal infection by *P. aeruginosa* (Roe and Jones, 1983). In addition, work by Klinger *et al* (1983) has shown successful protection against chronic pulmonary challenge with *P. aeruginosa* in rats immunized with PEV-01.

The aim of this section was to exploit the opportunities offered by the mucosal immune system by delivering various formulations of PEV-O1 vaccine *via* different mucosal surfaces (oral and nasal) and optimise the delivery of this model antigen.

2.2 MATERIALS AND METHODS

2.2.1 Materials

2.2.2 Bacteria

Representative strains of the sixteen serotypes of *Pseudomonas aeruginosa* described by the International Antigenic Typing Scheme (IATS), were obtained from the Aston culture collection. *P. aeruginosa* strain PAO1 (ATCC 15692) was used for LPS extraction. Strains were maintained on nutrient agar slopes at 4°C and subcultured monthly.

2.2.3 Media

Chemically defined medium (CDM) for growth of *P. aeruginosa*.

Table 2.1 CDM for growth of *P. aeruginosa*

Nutrient	Final concentration (mM)
NaCl	5.0
KCl	0.62
$MgSO_4.7H_2O$	0.4
$K_2HPO_4.3H_2O$	3.2
$(NH_4)_2SO_4$	40.0
$FeSO_4.7H_2O$	0.062
MOPS	50.0
Glucose	40.0

2.3 Methods

2.3.1 Lipopolysaccharide extraction

Lipopolysaccharide (LPS) was extracted by the hot-phenol method of Westphal and Jann (1965) and adapted as follows: log phase *P. aeruginosa* cells were harvested by centrifugation at 5000 xg for 10 min at 4°C. The pellet was resuspended in approximately 30-40 ml of Tris buffer (40mM Tris, pH 8.0) and then sonicated in an ice bath (10x20s pulses, with 20s intervals for cooling). Deoxyribonuclease (Bovine pancreas type III), ribonuclease (Bovine pancreas type 1-AS) and lysozyme (to digest the DNA, RNA and peptidoglycan respectively) were added to a final concentration of 0.1 mg ml^{-1} and the preparation incubated in the orbital shaker for 2 hr at 37°C. Ten ml of 0.5M tetrasodium ethylenediamine-tetraacetic acid (EDTA) to remove cations binding the LPS together, and protease (*Strep. griseus* type X1V; final concentration 1mg ml^{-1}) to digest protein, were added and incubated at 37°C overnight with constant shaking. Protease was destroyed by heating at 70°C for 20 min. Phenol (90% w/v) was preheated to the same temperature and an equal volume mixed with the digested cell suspension and stirred for 10 min. Centrifugation at 5,000 xg for 30 min allowed phase separation to occur. The upper aqueous layer containing LPS was carefully removed without disturbing any remaining proteinaceous material at the interface.

Two further extractions were carried out by reheating the phenol layer to 70°C and adding a further 50 ml of water at the same temperature. The pooled aqueous layers were dialysed against tap water for 48 hr to remove the phenol. The contents of the dialysis tubing were emptied into a flask and 50mM $MgCl_3$ was added. The LPS was pelleted by ultracentrifugation at 100,000 xg for 4 hrs at 4°C and the supernatant discarded. The pellet was resuspended in double distilled water, recentrifuged, and finally resuspended in 20 ml of distilled water and then lyophilised.

2.3.2 Formulations

2.3.2.1 Preparation of Emulsions

Emulsions were prepared as described by Alpar *et al* (1989a). Arachis oil and Arlacel 83 (8:1) were heated to 37°C and vigorously mixed. Water, containing 3750µg of LPS also at 37°C, was then added and mixed to form a water-in-oil emulsion. The preparation was homogenised and Tween 80, as the secondary emulsifier was slowly added followed by distilled water (1:6 v/v) while mixing which resulted in formation of a water-oil-water emulsion.

2.3.2.2 Stability tests on emulsions

The stability of the LPS emulsion in acid and buffer solutions was investigated by adding 1 ml of emulsion to 5 ml of 0.1M HCl of pH 1 and to 5 ml of 0.02M phosphate buffer pH 7.4. The vials were left for 10 days, the emulsions examined under a light microscope and the extent of creaming noted.

2.3.2.3 Preparation of aerosil gel

Freeze dried vaccine was reconstituted and diluted with iosotonic phosphate buffered saline (PBS) so that the required amount of LPS per dose (25µg) was contained in 0.15ml. Aerosil 200 gel (Degussa, Frankfurt) was added to give a final concentration of 0.4% (pH 7.5) and a total volume for each dose of 0.2 ml.

2.3.3 Vaccine preparation

The polyvalent cell extract vaccine PEV-O1 (equal portions of EDTA-glycine extracts from each of 16 *P. aeruginosa* serotypes) was used as the model antigen and was a kind gift of Professor J. Melling Porton Down, Salisbury. The main component of the vaccine was LPS (Miler *et al,* 1977).

2.3.4 Immunisation schedule

Pigmented guinea pigs (n=5 per group) were dosed on weeks one and two for three consecutive days *via* three routes. The first group were dosed orally (0.5 ml, 250μg LPS) with saline or a water-oil-water emulsion, using a gavage needle directly into the stomach. The second group were dosed nasally (i.n.) (0.2 ml, 25μg LPS) with saline or an aerosil 200 gel using a needle with tubing attached. The third group were dosed (0.2 ml, 20μg LPS) *via* the subcutaneous route.

2.3.4.1 Serum and bronchial wash samples

Serum samples were obtained by cardiac puncture periodically during the experiment. The animals were anaesthetised using 50% CO_2 and 50% O_2 mixture. The blood was alloed to clot, centrifuged at 1,000 xg for 10 min at 22°C and the serum collected and stored at -20°C.

Bronchial washes were also collected from the killed animals at the end of the experiment by exposing the trachea, making a small cut and inserting a needle with tubing attached. Wash fluid was introduced slowly using a 5 ml syringe and withdrawn, while massaging the chest of the guinea pig. This process was repeated using a further 5 ml of wash fluid. The washes were centrifuged at 1,000 xg for 10 min at 22°C, the supernatant collected and stored at 4°C.

2.3.5 Enzyme linked Immunosorbent Assay (ELISA)

The method used was that of Baumgartner *et al* (1987). Polyvinyl tissue culture plates (Dynatech) were coated with LPS at a concentration of 1μg ml^{-1} as determined in a preliminary checkerboard titration assay. Poly-l-lysine (Sigma) was diluted to 10μg ml^{-1} in phosphate buffered saline (PBS), and 50μl was added to each well and incubated for 30 min at room temperature. This procedure improved the adherence of LPS to the plastic wells and decreased the background optical density

(OD).

After washing the wells 3 times with PBS, 100µl of LPS (16 serotypes) was added at a concentration of 1µg ml^{-1} diluted in 0.1M sodium carbonate buffer pH 9.6 and the plates incubated overnight at 4°C. The wells were washed as before and filled with 150µl of 10% horse serum in PBS (horse serum buffer). The wells were washed after incubation for 1 hr at room temperature, and 100µl aliquots of guinea pig serum diluted in horse serum buffer were added to the wells. The wells were washed after incubating for 1 hr at 37°C, and 100µl of rabbit anti guinea pig horseradish peroxidase-conjugate (ICN, Pharmaceuticals), diluted 1:1000 in horse serum buffer, was added to each well and incubated for 1 hr at 37°C.

Peroxidase substrate (0.15mM 3,3',5,5'-tetramethyl-benzidine (TMB) in 0.1M sodium acetate pH 6.8 containing 0.003% v/v H_2O_2) was added to each well (100µl). The reaction was stopped after 10 min by adding 50µl of 2M H_2SO_4 to each well and the OD read at 450nm using a microplate reader and the titre defined as the highest dilution yielding an OD of 0.1.

2.3.6 ELISA to detect secretory IgA (sIgA)

LPS specific sIgA antibody levels were estimated in bronchial washes by an indirect ELISA (McBride *et al*, 1988). Dynatech Immunlon plates (96 well) were coated with LPS antigens (16 serotypes) 1µg ml^{-1} in 0.1M sodium carbonate buffer pH 9.6.

Bronchial fluids were diluted 1:100 in 5% (v/v) normal sheep serum, 0.1% (v/v) bovine serum albumin (BSA), 0.05% (v/v) Tween 20 and 0.9% (w/v) sodium chloride and incubated in the antigen coated wells for 2 hr at 37°C. The LPS specific sIgA was detected with a rabbit antiserum reactive with human secretory component (ICN, Pharmaceuticals) diluted 1:100 in the same buffer.

After 1 hr at 37°C bound rabbit immunoglobulin was detected with a

goat anti rabbit IgG horseradish peroxidase (HRP) conjugate (Sigma) diluted 1:2,000 in the above buffer. After 30 min incubation at 37°C the enzyme substrate TMB (Miles) was added (see section 2.3.5) and the absorbance at 450nm measured.

2.3.7 Statistical methods

Comparisons between the s.c. vaccination group and other groups, using ELISA data, were analysed for significance using Student's unpaired t test. Differences with $p<0.05$ were considered to be significant.

2.4 RESULTS AND DISCUSSION

It was possible to formulate the LPS antigen (PEV-O1 vaccine) as a multiple emulsion. The emulsion was of low viscosity and could easily be injected through a fine bore needle. Creaming of the emulsion did occur, but the cream could be easily dispersed by shaking (Table 2.2), and the emulsion as a whole was of good stability, but the stability decreased when added to media of low pH, although higher pH solutions did not have a very marked effect.

The above mentioned stable w/o/w emulsion was used to investigate the potential of multiple emulsions in effecting oral absorption and resulting immune responses (adjuvanticity) to PEV-O1 *Pseudomonas* vaccine. Nasal delivery of LPS was also carried out in parallel work using either drop or gel formulations. Serum IgG responses following oral administration of aqueous solution and w/o/w emulsions as well as nasal gel and drop administration of LPS are shown in table 2.3 together with parenteral (s.c.) delivery of the same antigen. The immune response to LPS when presented in emulsion form was markedly higher than that of the aqueous form. The data also indicated that nasal delivery of the antigen in simple liquid form gave higher antibody titres compared to subcutaneous delivery whereas nasal gel responses were poorer than the subcutaneous route (Table 2.3). However, the ELISA data was found to have high variation among the animals of each group, and therefore high standard deviations were observed for the mean values indicated in table 2.3. One reason may be that there was no standard serum available to use on each plate. This meant the samples from each group were analysed in one day to try and overcome the day to day variability.

The higher immune response to LPS when presented in emulsion form compared to the aqueous form may possibly be attributed to:- a) the inhibitory effect of the oil on gastric emptying processes producing a prolonged absorption phase, b) accumulation of oil globules at the mesenteric lymph nodes, c) less degradation of the

antigenic macromolecule entrapped in multiple emulsion and passage through gastro-intestinal mucosa at the water-oil interfaces of emulsion globules.

One of the main aims of the work was to investigate mucosal responses to non-parenteral delivery. Therefore, the lack of secretory IgA data, due to non-specific binding to the lipopolysaccharide of the goat anti-human secretory component used in the enzyme linked immunosorbent assays and the high variability found with the ELISA technique to assay serum response, resulted in a change of direction. Collaboration was arranged between Pharmaceutical Institute, Aston University and Public Health Laboratory Services, Porton Down to investigate non-parenteral immunization of a herpes simples virus subunit vacccine.

Table 2.2 Stability of multiple water-oil-water emulsions in acid and buffer solutions.

System	Creaming	Appearance under microscope
Emulsion	[a]+; easily dispersed	mostly very small some large globules
Emulsion at pH 1	+++; quickly reformed after dispersion	many large globules and floccules, very dense cream
Emulsion at pH 7.4	+; easily redispersed	mostly small globules, few floccules

a-extent of creaming, +++ high, + low

Table 2.3 Titres of IgG antibody to lipopolysaccharide as determined by ELISA in the sera of guinea pigs before and after vaccination with PEV-O1 vaccine.

	Weeks after immunisation					
Route of delivery	0	2	4	6	8	11
S.C.	1:130	1:170	1:500	1:530	1:480	1:2000
i.n. drop	1:490	1:1200	1:1200	1:1900	1:650	1:1200
i.n. gel	1:250	1:580	1:250	1:300	1:330	1:1200
oral liquid	1:300	1:800	1:1200	1:1600	1:2200	1:1100
oral emulsion	1:130	1:1800	1:1860	1:2000	1:2600	1:1600

CHAPTER 3

NON-PARENTERAL DELIVERY OF A TETANUS TOXOID VACCINE

3.1 INTRODUCTION

Vaccination programs against tetanus, which use formaldehyde-inactivated tetanus toxin pruified from *Clostridium tetani*, are highly effective in the prevention of tetanus in Western countries (Bizzini, 1984). However, some countries are unable to implement fully these programs, resulting in significant annual mortality from neonatal tetanus (Stanfield and Galazka, 1984). It is possible that the use of an effective oral tetanus vaccine may help to alleviate some of the problems associated with widespread vaccination in developing countries.

Liposomes are microscopic phospholipid vesicles composed of one or more concentric phospholipid bilayers, and were first described for their ability to function as adjuvants for protein antigens by Allison and Gregoriadis (1974). The physiochemical properties of liposomes can influence their utility as vaccine adjuvants. Variables considered, include the liquid-crystalline phase-transition temperature (Tc) of the lipids, the charge of the lipids, inclusion of cholesterol, ratio of protein to lipid, and of the liposomes in the preparation (Eppstein *et al*, 1990).

The aim of this section was to investigate non-parenteral delivery of tetanus toxoid using liposomes and gels in order to induce systemic and mucosal immunity.

3.2 MATERIALS AND METHODS

3.2.1 Vaccine preparation

Tetanus toxoid vaccine in simple solution (purchase from The Wellcome Foundation Ltd, London) was prepared by formalin detoxification of *Clostridium tetani* exotoxin either in simple solution or adsorbed to aluminium hydroxide.

3.2.2 Formulations

3.2.2.1 Preparation of aerosil gel

Adsorbed tetanus toxoid (600µg in 2 ml of PBS) was mixed with 0.08g of aerosil 200 (Degussa, Frankfurt) gel to give a final concentration of 4% (pH of 7.5) and a total volume for each dose of 0.2 ml.

3.2.2.2 Liposome preparation

Multilamellar dehydration-rehydration (DRV) vesicles composed of equimolar phospholipid (66µmoles) and cholesterol were prepared as described previously (Kirby and Gregoriadis, 1984). Distearoylphosphatidylcholine (DSPC) (Lipid Products, S. Nutfield, Surrey) and cholesterol (Sigma) were dissolved in 10 ml of chloroform. The resulting solution was placed in a 250 ml pear shaped flask and evaporated under reduced pressure at 58°C to dryness. The lipid film was dried under nitrogen for 10 min and then dissolved in 2 ml distilled water by gentle agitation in a water bath at 58°C. The mixture was sonicated at 58°C resulting in the formation of single unilamellar vesicles (SUV). The liposomes were centrifuged at 2,500 xg for 15 min to remove larger lipid aggregates and titanium particles released from the sonicator probe. Two ml of SUV was mixed with 2 ml distilled water containing 1000µg of tetanus toxoid vaccine in a 50 ml round-bottomed flask. The mixture was flash-frozen as a thin shell. After freezing, the preparations were lyophilised overnight. The preparation was rehydrated with distilled water, using a volume

equivalent to one-tenth of the total volume of SUV used (i.e. 0.2 ml in the present case). The rehydration procedure was aided by vortexing lightly and then left to stand for 30 min at 58°C. Isotonic PBS pH 7.4 equivalent to one-tenth of the total volume of SUV used was added to the preparation, vortexed lightly and left to stand for 30 min at 58°C. A further volume of PBS equivalent to eight-tenths of the total volume of SUV used was added, vortexed and allowed to stand for 30 min at 58°C.

The liposomes were separated from non-entrapped material by diluting with PBS to 20 ml followed by centrifugation at 10,000 xg for 30 min. The supernatant was retained and a further 20 ml of PBS added followed by centrifugation. The pellet was resuspended in 1 ml PBS and the supernatants were used to indirectly estimate the percentage entrapment of Skinner vaccine in the liposomes using the Lowry protein assay (section 3.2.2.3).

3.2.2.3 Lowry (Folin-Ciocalteau) protein assay

The assay used was that developed by Lowry *et al* (1951). Bovine serum albumin standards (containing 0 to 300 $\mu g\ ml^{-1}$, Sigma) and samples were made up to 0.5 ml with distilled water, 0.5 ml of 0.1N NaOH was added and the solutions boiled for 10 min at 100°C. On cooling, 2.5 ml of a freshly prepared solution made by mixing 1 ml of 0.5% w/v copper sulphate solution in 1% w/v sodium potassium tartrate with 50 ml of 5% w/v sodium carbonate in 0.1N NaOH was added to each sample. After 10 min at room temperature, 0.5 ml of Folin-Ciocalteau reagent (BDH) diluted 1:1 with water was added to each tube and the resulting blue colour developed for 30 min before the absorbance was read at 750nm. A calibration curve of albumin concentration against OD_{750} (optical density) was used to determine the protein content of the samples.

3.2.2.4 *In vitro* stability assay of liposomes

BSA (Sigma) was radiolabelled with ^{125}I (Na salt) (Amersham International) by the chloramine-T method based on that of Hunter and Greenwood (1962). Briefly, 10µl of 2mg protein ml^{-1} PBS was mixed with 15µl PBS and 10µl (1mCi) ^{125}I. To this was added 25µl chloramine-T solution (4mg ml^{-1} PBS), 100µl sodium metabisulphite (2.4mg ml^{-1} PBS) and 20µl sodium iodide (10mg ml^{-1} PBS). Radiolabelled protein was separated from free ^{125}I salt on a Sephadix G-25 column (Pharmacia) and the activity assessed using a 1282 Compugamma counter (LKB Instruments Ltd., Croyden, U.K.).

Liposomes containing BSA with trace ^{125}I-BSA were prepared and diluted with PBS to 3mg BSA ml^{-1}. Aliquots (2.5 ml) of liposome suspension were mixed with 0.1M PBS pH 7.4, 0.1M Walpoles acetate pH 4.0, or glycine/HCl pH 2.2. The mixtures were incubated at 37°C or 4°C. At the start (t_0) and at intervals throughout the 2 hr incubation, 400µl aliquots were taken from each mixture. A portion (100µl) was retained and the remainder was centrifuged to separate free and liposome-entrapped BSA. The volumes of the supernatants and pellets were measured and the BSA content of each of the fractions was assessed by gamma emission.

3.2.3 Non-parenteral delivery of tetanus toxoid vaccine using DSPC liposomes

Pigmented guinea pigs (5 months old) were immunized *via* the intramuscular, oral and nasal routes. A vaccine dose of 60µg tetanus toxoid was given nasally (0.2 ml) and orally (0.5 ml) on weeks 1, 2, 4 and 20 in phosphate buffered saline (PBS) or entrapped in DSPC liposomes. In addition, 6µg of tetanus toxoid vaccine in PBS was given *via* the normal parenteral route of delivery (i.m. 0.2 ml) on weeks 1, 4 and 20.

Pigmented guinea pigs (6-8 weeks old, 200-400g) were immunised

via the i.m., oral and nasal routes. A vaccine dose of 60μg tetanus toxoid entrapped in DSPC liposomes was given nasally and orally on weeks 1, 2, 4 and 20. In addition, 6μg of tetanus toxoid as free or entrapped in DSPC liposomes was given *via* the i.m. route on weeks 1, 4 and 20.

3.2.4 Nasal delivery of adsorbed tetanus toxoid vaccine using an aerosil gel

Pigmented guinea pigs (10 weeks old) were immunized *via* the i.m. and nasal route using adsorbed tetanus toxoid vaccine. A dose of 6μg in PBS was given *via* the normal parenteral route of delivery (i.m. 0.2 ml) on weeks 1, 4 and 20. A vaccine dose of 60μg was given nasally (0.2 ml) on weeks 1, 2, 4 and 20 in PBS or an aerosil 200 gel formulation.

3.2.5 Non-parenteral delivery of tetanus toxoid (adsorbed and simple solution) vaccine; dose response

Pigmented guinea pigs (8 weeks old) were immunised *via* the intramuscular (i.m.), nasal and oral routes using adsorbed tetanus toxoid. A dose of 6μg in PBS was given *via* the normal parenteral route of delivery (i.m. 0.2 ml) on weeks 1 and 4. A vaccine dose of 60μg was given orally (0.5 ml) and 15 or 30 μg was dosed nasally (0.2 ml) on weeks 1 and 4.

In addition, pigmented guinea pigs were dosed following the same protocol just described using simple solution tetanus toxoid, except for the oral dosage, whereby cimetidine (Tagamet, Smith Kline and French Laboratories) was administered 2 hrs before giving tetanus toxoid orally at a dose of 50mg/kg body weight.

3.2.6 Enzyme Linked Immunosorbent Assay (ELISA)

Antibody responses to the tetanus toxoid in immunized guinea pigs were

monitored by a microplate ELISA (Davis and Gregoriadis, 1989). Tetanus toxoid (5 μg ml^{-1}) in 0.05 M Na carbonate-bicarbonate buffer (pH 9.6) was added to Dynatech microelisa plates and incubated overnight at 4°C. The wells were washed three times with 9.5 mM sodium phosphate buffer containing 0.8 % NaCl and 0.05 % Tween 20 (PBST), pH 7.4.

Dilutions of guinea pig serum in PBST + 0.1 % bovine serum albumin (Sigma) were incubated in the plates for 2 hours at room temperature. The total amount of guinea pig IgG bound was estimated by the addition of rabbit anti guinea pig IgG-horseradish peroxidase conjugate (ICN, Pharmaceuticals).

Anti-tetanus immunoglobulin (supplied by The Wellcome Foundation Ltd, London) purified from the sera of healthy human donors known to have high levels of tetanus antitoxin following active immunisation with tetanus vaccine, was used as a standard serially diluted from 1:1,00 on each plate. Protein A peroxidase (concentration 0.5μg ml^{-1}) estimated the total amount of Ig bound to the tetanus toxoid.

After 1 hour incubation at room temperature the enzyme substrate 3,3',5,5'-tetramethylbenzidine (TMB, Miles) was added (0.15mM TMB in 0.1 M sodium acetate pH 6.8 containing 0.003 % v/v H_2O_2) and the reaction allowed to proceed for 10 min. The enzyme reaction was stopped by the addition of 2 M H_2SO_4 and the absorbance at 450 nm measured (Titertek Multiscan, Flow Labs). The endpoint of each titration was defined as the dilution at which the absorbance (450nm) was 0.2 (instrument zeroed on the reagent blank). This dilution was expressed as a titre after correction by reference to a standard on every plate.

3.2.7 Liposome uptake by the gut

Tetanus toxoid liposomes were prepared as described in section 3.2.2.2 and dosed orally to rats. Tissue sections were taken from the ileum and prepared at

Aston University for examination using the electron microscope in the School of Biochemistry, The University of Birmingham by Deryck Mills.

3.2.8 Statistical methods

Comparisons between the i.m. vaccination group and other groups, using ELISA data, were analysed for significance using Student's unpaired t test. Differences with $p<0.05$ were considered to be significant.

3.3 RESULTS AND DISCUSSION

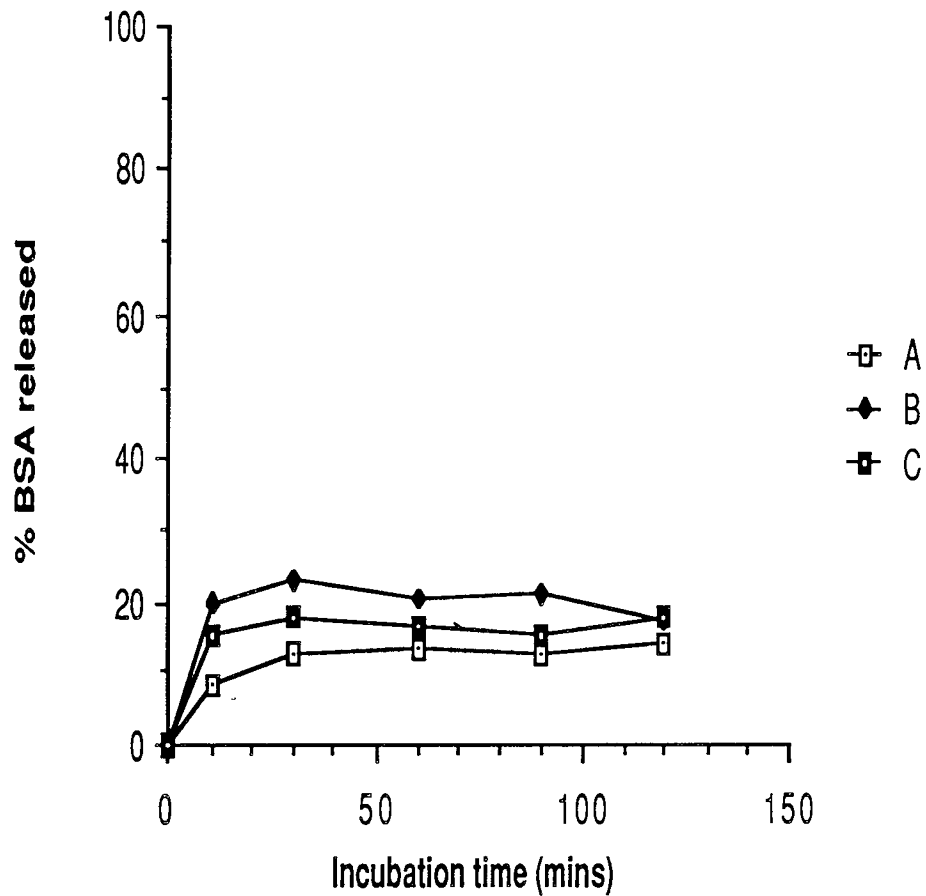
3.3.1 Non-parenteral delivery of tetanus toxoid vaccine using DSPC liposmes

The study was aimed at investigating the potential adjuvant effect of liposomes on tetanus toxoid, when delivered *via* the nasal, oral and i.m. routes compared to delivery in simple solution. In addition, 5 months old and 6-8 weeks old pigmented guinea pigs were used to investigate if age would effect the immune response after immunisation.

Entrapment values (% +/- S.D. of toxoid used) for the DSPC liposomes used in this study prepared using dehydration-rehydration procedure (Kirby and Gregoriadis, 1984) were 68% +/- 8.37 (6 preparations). These values are a little lower compared to the results of Davis *et al* (1986) which were 82.3 +/- 3.4 (8 preparations). The Lowry assay used to indirectly measure the % entrapment of tetanus toxoid in the liposomes was found to be reproducible. A known concentration of tetanus toxoid was used as a standard each time and gave a value of 34μg ml^{-1} (+/- 5). A more direct method would have been to use ^{125}I (Kirby and Gregoriadis, 1984). The phospholipid to antigen mass ratio for these liposomes was calculated to be 80:1. The liposome *in vitro* stability assay involved measurement of the gamma emission from the fractions of the aliquots taken at timed intervals during the incubations this allowed the percentage of BSA released from the DSPC liposomes to be calculated (Figs 3.1 and 3.2). The DSPC liposomes steadily released BSA, until at the end of the 2 hr period they had released about 10% at 4°C and 15% at 37°C of the entrapped BSA. Incubation in the presence of glycine/HCl pH 2.2 and Walpoles acetate pH 4.0 resulted in the steady release of BSA entrapped in DSPC liposomes until at the end of the 2 hr incubation they had released about 20% at both 4°C and 37°C. Therefore, the results suggest that the DSPC liposomes would be stable in the acid environment of the gastrointestinal tract (Rowland and Woodley, 1980;1981). In addition , after oral

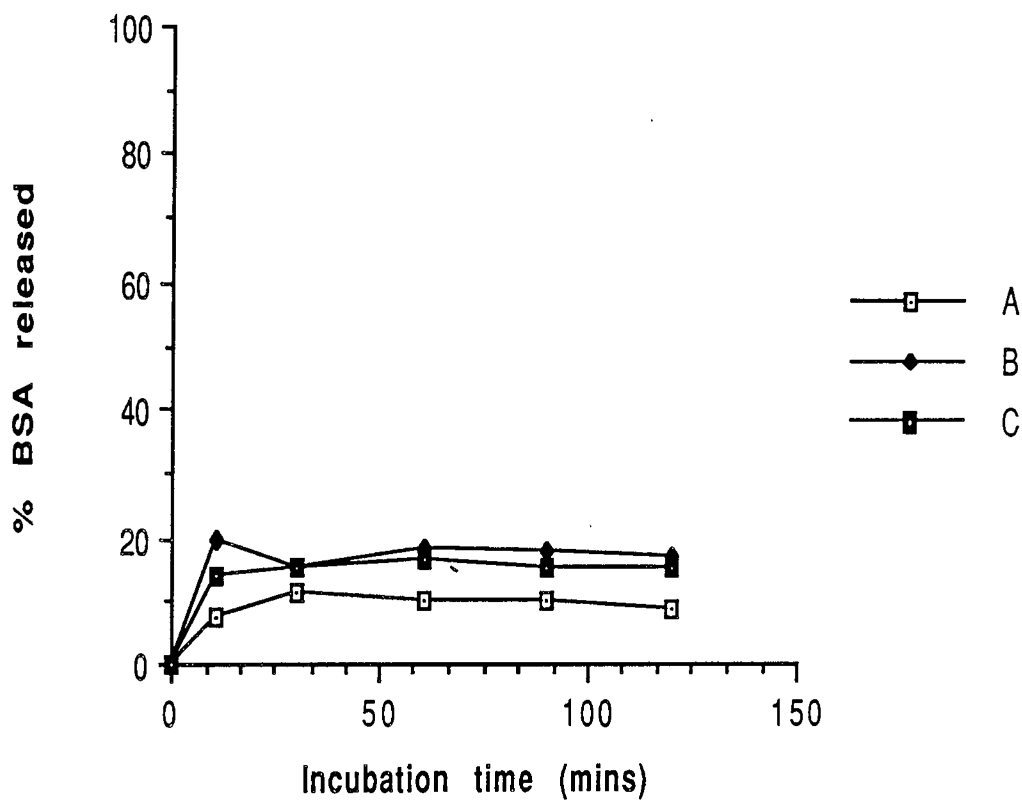
dosing of DSPC liposomes containing tetanus toxoid to rats, subsequent electron microscope examination of gut tissue sections showed liposome structures which appeared to be intact (Fig 3.3).

Fig 3.1 BSA release from DSPC liposomes at physiological temperature in the presence of varying pH solutions.



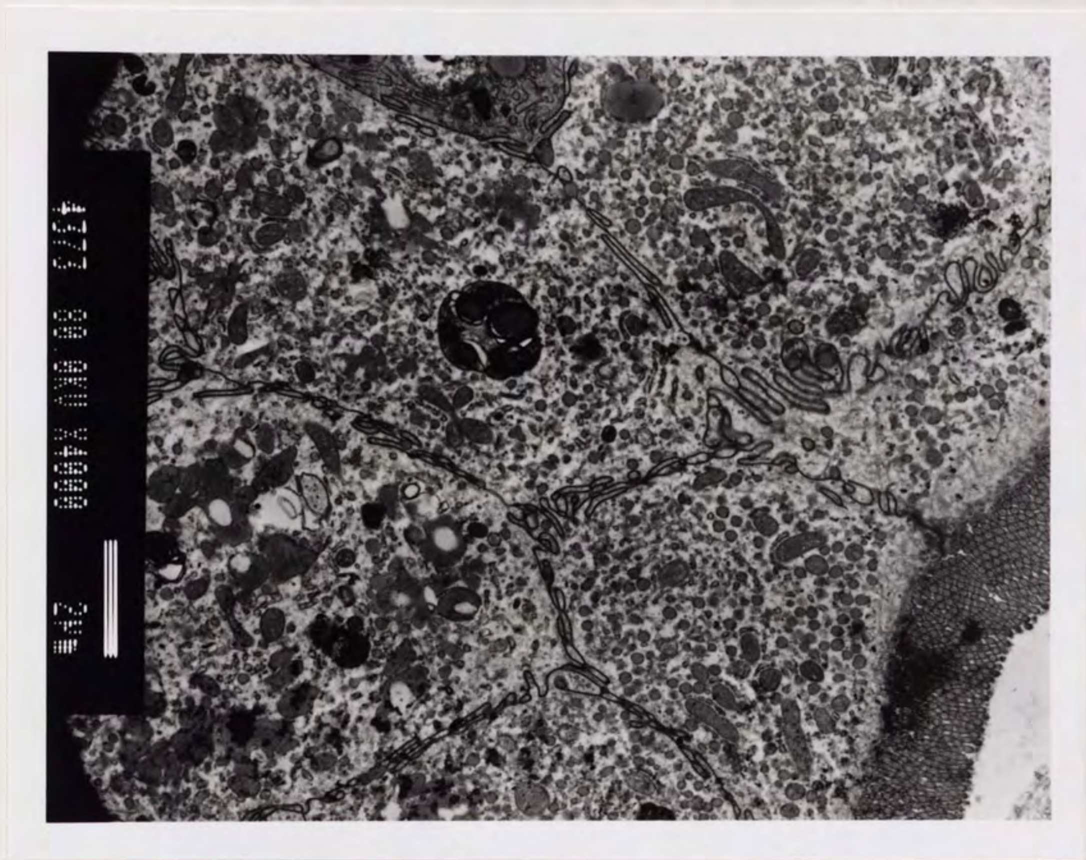
A-0.1M phosphate buffered saline pH 7.4
B-0.1M Walpoles Acetate pH 4.0
C-Glycine/HCl pH 2.2

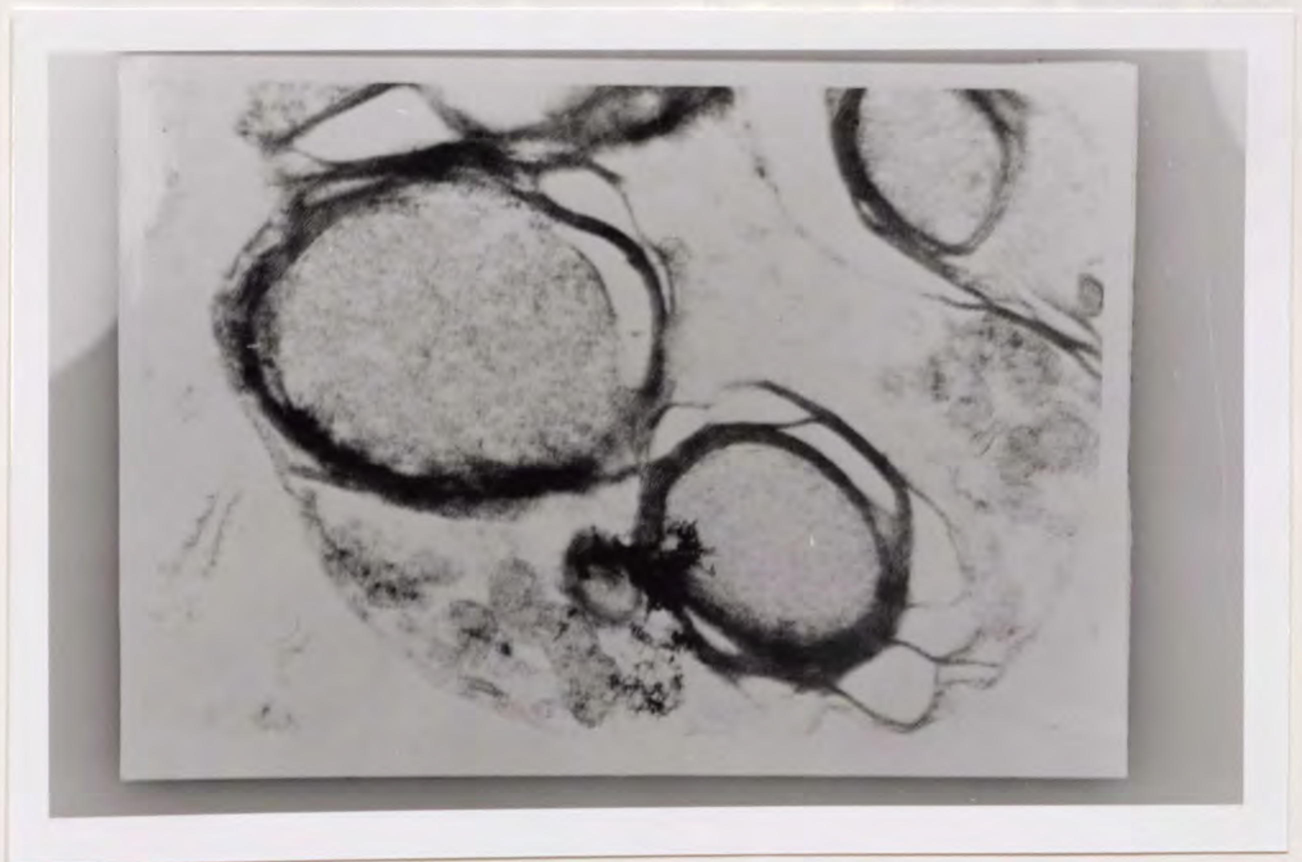
Fig 3.2 BSA release from DSPC liposomes at 4°C in the presence of varying pH solutions.



A-0.1M phosphate buffered saline pH 7.4
B-0.1M Walpoles Acetate pH 4.0
C-Glycine/HCl pH 2.2

Fig 3.3 Electron microscope photographs of rat gut tissue sections taken after oral delivery of tetanus toxoid entrapped in DSPC liposomes





Specific serum IgG antibodies measured after vaccination of guinea pigs with tetanus toxoid as free or entrapped in DSPC liposomes *via* the nasal and oral routes are shown in table 3.1. At week 6 post vaccination specific IgG antibody levels were significantly higher for all vaccinated groups except oral using DSPC liposomes compared to control animals. However, by week 22 all vaccinated groups were significantly higher than the control titres. In addition, there was no significant difference when tetanus toxoid was used for vaccination *via* the oral and nasal route as free or entrapped in DSPC liposomes.

At week 6 IgG antibody levels were significantly higher for immunisation of tetanus toxoid *via* the i.m. route compared to oral delivery using DSPC liposomes. However, there was no significant difference compared to the other groups. At week 22 i.m. antibody levels were significantly higher compared to the oral and nasal delivery of tetanus toxoid as free antigen. Whereas, there was no significant difference compared to the other groups.

Therefore, to summarise the results, delivery of tetanus toxoid entrapped in DSPC liposomes did not improve the immune response compared to free antigen when delivered *via* the nasal or oral routes. Similar, antibody responses were achieved by the nasal and oral routes (60µg/dose) compared to the i.m delivery (6µg/dose).

Table 3.1 Antibody titres to tetanus toxoid determined by ELISA in the sera of guinea pigs vaccinated on weeks 1, 2, 4 and 20 (oral and nasal routes of delivery) or weeks 1, 4 and 20 (i.m. delivery).

		Geometric mean (log_2) ELISA titre of serum IgG antibody					
route and dose[a]	formulation	pre-bleed	week 3	week 6	week 11	week 20	week 22
i.m.[b] 6	PBS[c]	<6.6 (0.18)[d]	<7.0 (0.49)	11.7 (0.99)	10.7 (1.51)	11.4 (1.48)	14.26 (0.86)
oral 60	DSPC[e] liposomes	<6.4 (0.13)	<6.4 (0.13)	8.5 (2.06)	<8.1 (2.96)	9.7 (2.2)	11.86 (2.42)
nasal 60	DSPC liposomes	<6.8 (0.27)	<6.8 (0.27)	10.5 (0.72)	<8.5 (1.7)	9.6 (0.56)	13.82 (2.0)
oral 60	PBS	<7.3 (0.55)	<7.3 (0.55)	10.0 (1.81)	<7.6 (1.63)	9.0 (0.83)	12.22 (0.96)
nasal 60	PBS	<6.1 (0.61)	<6.2 (0.42)	11.4 (1.5)	<9.2 (2.39)	9.5 (1.21)	12.83 (0.57)
control		<6.3 (0.85)	<6.3 (0.87)	<6.6 (1.07)	<6.4 (0.36)	<6.4 (0.93)	<8.7 (0.38)

a-µg of protein, b-intramuscular, c-phosphate buffered saline, d-standard deviation, e-distearoylphosphatidylcholine.

Serum tetanus toxoid specific IgG antibody levels were measured after vaccination of guinea pigs (6 months old) with tetanus toxoid entrapped in DSPC liposomes *via* the nasal, i.m. and oral routes (table 3.2). At weeks 11 and 22 post vaccination specific serum IgG antibodies were significantly higher for the nasal and i.m. routes of delivery compared to the non-vaccinated group. However, the oral delivery was not significantly different.

Tetanus toxoid entrapped in DSPC liposomes did not improve the immune response compared to i.m. delivery of free antigen. In addition, i.m. delivery of tetanus toxoid in simple solution gave significantly higher post vaccination IgG antibody levels compared to the nasal and oral delivery of tetanus toxoid entrapped in DSPC liposomes. Whereas, i.m. immunisation of tetanus toxoid entrapped in DSPC liposomes gave a comparable response to the oral and nasal post-vaccination IgG titres.

To summarise the results from these 2 experiments, firstly the age of the animal did not result in a significant difference of post-vaccination IgG antibody levels. Secondly, a 10x larger dose than the i.m. route given nasally and orally in simple solution or entrapped in DSPC liposomes reached levels comparable to the i.m. delivery suggesting that non-parenteral routes are promising for further investigation. Thirdly, the liposome formulation did not improve the immune response compared to free antigne when delivered *via* the nasal, oral or i.m. routes. However, the physiochemical properties of liposomes can influence their utility as vaccine adjuvants (Eppstein *et al*, 1990).

Table 3.2 Antibody titres to tetanus toxoid determined by ELISA in the sera of guinea pigs vaccinated on weeks 1, 2, 4 and 20 (oral and nasal routes of delivery) or weeks 1, 4 and 20 (i.m. delivery).

		Geometric mean (log_2) ELISA titre of serum IgG antibody					
route and dose[a]	formulation	pre-bleed	week 3	week 6	week 11	week 20	week 22
oral 60	DSPC[b] liposomes	<6.4 (0.58)[c]	<6.5 (0.45)	8.1 (2.32)	10.5 (2.55)	9.6 (1.87)	10.2 (3.6)
nasal 60	DSPC liposomes	<6.0 (0.35)	<6.0 (0.35)	8.4 (0.6)	10.16 (0.58)	9.96 (0.84)	13.7 (0.89)
i.m.[d] 6	PBS[e]	<7.3 (0.25)	<7.9 (0.41)	11.4 (0.78)	12.53 (1.05)	10.48 (0.62)	15.15 (0.1)
i.m. 6	DSPC liposomes	<7.28 (0.13)	<7.5 (0.53)	12.47 (0.81)	11.7 (0.66)	10.7 (0.84)	14.9 (0.69)
control		<8.2 (0.28)	<8.08 (0.46)	<8.08 (0.46)	<8.2 (0.62)	<5.8 (0.11)	<6.0 (0.2)

a-µg of protein, b-distearoylphosphatidylcholine c-standard deviation, d-intramuscular, e-phosphate buffered saline.

Davis *et al* (1987) showed that tetanus toxoid entrapped in liposomes composed of low Tc immunised *via* the i.m. route gave a high secondary immune response in mice but fell off dramatically when the more rigid distearoyl-phosphatidylcholine (DSPC) liposomes were used. Soluble antigens must be processed by antigen presenting cells (APCs) prior to exposure on their surface (Allison and Byars, 1986). This could be interfered with by high melting phospholipids such as DSPC (Davis *et al*, 1987) at any of the stages between liposome internalisation by cells and peptide migration to their membrane.

The ratio of protein to lipid can also significantly influence the immunogenicity of the liposomal-protein preparation (Davis and Gregoriadis, 1987;1989). Davis and Gregoriadis (1987) also showed an equally improved response to toxoid for DSPC liposomes with similarly high ratios (for example, $2x10^3$). It was tentatively proposed (Davis and Gregoriadis, 1987) that, because of the relatively large amount of lipid involved, disintegration of DSPC liposomes at the site of injection (and antigen release) would be slower, leading to a more efficient presentation of antigen to APC. This could override any inhibitory effect of DSPC on the immunogenicity of antigen taken up by the cells in the liposome form. However, when phospholipid to antigen mass ratios in liposomes were even higher, for example, $>10^4$ (Davis and Gregoriadis, 1987; Davis and Gregoriadis, 1989), adjuvanticity was abolished, possibly because the concentration of antigen in individual vesicles is too low for it to be immunogenic.

3.3.2 Nasal delivery of adsorbed tetanus toxoid vaccine using an aerosil gel

The study was aimed at investigating the delivery of adsorbed tetanus toxoid via the i.m. and nasal route. The potential adjuvant effect of aerosil 200 gel when given nasally with tetanus toxoid was alsojinvestigated. The IgG antibody levels obtained prior to and after immunisation of guinea pigs *via* the i.m. and nasal route using adsorbed tetanus toxoid vaccine and a aerosil (200) gel formulation for the nasal route are shown in table 3.3. At weeks 6 and 22 post-vaccination the IgG antibody levels were significantly higher for the vaccinated groups compared to the control animals. In addition, there was no significant difference in the IgG antibody levels of the nasal delivery of adsorbed tetanus toxoid as free or with aerosil gel. Therefore, the aerosil gel did not significantly increase the immune response compared to free antigen.

Table 3.3 Antibody titres to tetanus toxoid determined by ELISA in the sera of guinea pigs vaccinated on weeks 1, 2, 4 and 20 (nasal) or weeks 1, 4 and 20 (i.m. delivery).

		Geometric mean (log_2) ELISA titre of serum IgG antibody					
route and dose[a]	formulation	pre-bleed	week 3	week 6	week 11	week 20	week 22
i.m.[b] 6	PBS[c]	<7.6 (0.98)[d]	12.0 (2.09)	15.1 (1.59)	14.75 (0.92)	14.1 (10.8)	>15.8 (0.15)
nasal 60	PBS	<8.6 (0.15)	<9.1 (0.56)	12.37 (1.71)	9.37 (1.97)	9.47 (1.79)	11.8 (4.6)
nasal 60	aerosil gel	<8.4 (0.07)	<8.6 (0.36)	10.8 (1.94)	9.33 (2.29)	8.53 (1.36)	15.03 (0.95)
control		<8.26 (0.27)	<8.2 (0.26)	<8.2 (0.26)	<7.36 (0.52)	<7.76 (0.62)	<7.12 (0.23)

a-µg of protein, b-intramuscular, c-phosphate buffered saline, d-standard deviation.

3.3.3 Non-parenteral delivery of tetanus toxoid (adsorbed and simple solution) vaccine; dose response

The previous work showed that comparable immune responses to that achieved *via* the conventional i.m. parenteral route could be obtained using the nasal and oral routes of delivery of tetanus toxoid as free antigen or entrapped in DSPC liposomes. However, the nasal and oral dosage was 10 times greater than that used for i.m. delivery. Therefore, the aim of this study was to investigate lower dosage for the nasal route and also delivering cimetidine orally prior to tetanus toxoid oral delivery to reduce the acidity of the gut and hopefully improve the immune response.

The post-vaccination IgG antibody titres prior to and after vaccination of guinea pigs *via* the i.m., nasal and oral routes using adsorbed tetanus toxoid or in simple solution are shown in table 3.4. Post-vaccination IgG antibody levels were significantly higher for delivery of tetanus toxoid (adsorbed) compared to nasal and oral delivery of the adsorbed vaccine. The i.m. delivery of tetanus toxoid in simple solution, however, showed no significant difference in post-vaccination IgG antibody levels compared to nasal and oral delivery of tetanus toxoid in simple solution. These results suggest that a lower dose of tetanus toxoid in simple solution can be used for nasal delivery but not the adsorbed vaccine.

In addition, the aim was that with the use of cimitidine which neutralises the acidity in the gut, the oral delivery of tetanus toxoid would result in a higher IgG post-vaccination level. However, the cimetidine did not appear to have any affect. Several approaches have been used to protect antigens from acid pH and proteolysis by gastric enzymes, for example orally ingested antigens have been given with a large amount of sodium bicarbonate to neutralise hydrochloric acid in the stomach (Black *et al*, 1983).

Table 3.4 Antibody titres to tetanus toxoid determined by ELISA in the sera of guinea pigs vaccinated on weeks 1, 2 and 4 (oral and nasal routes of delivery) or weeks 1 and 4 (i.m. delivery).

		Geometric mean (log_2) ELISA titre of serum IgG antibody			
route and dose[a]	formulation	prebleed	week 3	week 6	week 9
i.m.[b] 6	PBS[c] adsorbed	<6.88 (0.43)[d]	11.42 (0.99)	>15.62 (0.49)	>14.8 (0.56)
nasal 15	PBS adsorbed	<6.82 (0.47)	<6.82 (0.47)	<8.52 (2.25)	<7.74 (1.36)
nasal 30	PBS adsorbed	<6.94 (0.51)	<8.16 (2.08)	10.2 (3.57)	10.1 (3.57)
oral 60	PBS adsorbed	<6.82 (0.47)	<6.82 (0.47)	<7.62 (2.01)	<7.28 (1.24)
i.m.[e] 6	PBS ss[f]	<6.8 (0.25)	<9.58 (2.48)	<11.82 (4.55)	11.42 (4.27)
nasal 15	PBS ss	<6.8 (0.25)	<8.16 (0.91)	13.46 (0.68)	12.2 (1.07)
nasal 30	PBS ss	<6.72 (0.25)	8.25 (1.54)	12.98 (1.62)	11.74 (1.61)
oral 60	ss-cim[g]	<6.6 (0.12)	<7.0 (0.90)	8.2 (2.43)	<7.36 (1.34)

a-μg of protein, b-intramuscular, c-phosphate buffered saline, d-standard deviation, e-intramuscular, f-simple solution, g-cimetidine.

CHAPTER 4

NON-PARENTERAL IMMUNISATION WITH A HERPES SIMPLEX VIRUS SUBUNIT VACCINE AGAINST INTRAVAGINAL CHALLENGE IN THE GUINEA PIG

4.1 INTRODUCTION

Herpes simplex virus (HSV) types 1 and 2 are responsible for a wide range of clinically important mucosal infections including oral and genital lesions and aseptic meningitis. There is considerable interest at present in the production of a safe, DNA-free vaccine both for the prevention or modification of recurrent disease episodes (Allen and Rapp, 1982; Weijer *et al*, 1988). Immunity to HSV entails aspects of both humoral and cellular immunity, and optimal responses of both types seem to require exposure to live virus (Watari *et al*, 1987; Lawman *et al*, 1980; Nash *et al*, 1981b). However, living vaccines will probably be unacceptable because of the lingering uncertainty about oncogenicity of HSV DNAs and their possible disease producing activity especially in immunologically compromised individuals. Synthetic peptides representing protective antigens provide an alternative and theoretically ideal approach, providing adjuvants can be found to attain acceptable immunogenicity. An important consideration with such a mucosal infection is to induce immunity at the site of viral entry, ie the mucosal surface. There is extensive evidence for a common mucosal immune system (Mestecky *et al*, 1980) and therefore immunisation at a distant, particularly a mucosal site, is likely to induce a specific response at the genital surfaces.

Testing of this hypothesis was the aim of this section. Therefore, experiments were designed to investigate the production of HSV specific vaginal and serum antibodies in guinea pigs vaccinated *via* the subcutaneous, oral, nasal and vaginal routes with the Skinner vaccine (Skinner *et al*, 1982a), followed by challenge with HSV-2 at the genital surface (Thornton *et al*, 1984). Initially, various delivery systems such as gels, multiple emulsions and liposomes were used to show the effects of different formulations on this model system.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of Skinner Vaccine

The HSV subunit vaccine was prepared as described by Skinner *et al* (1982a;1982b) at Porton Down, Salisbury. MRC 5 human embryo lung cells were infected with strain troisbel of herpes simplex virus. Cell nuclei were removed by low speed centrifugation after treatment of the cells with Nonidet NP40, which solubilises the virus membrane; formaldehyde was added to inactivate any residual virus. It has been proposed by Skinner *et al* (1982a) that formaldehyde treatment makes the vaccine more immunogenic. Virus core particles, although inactivated might contain biologically active DNA, which was removed by ultracentrifugation over 20% sucrose at 85,000 xg for 5 hrs. The protein constituents of the vaccine preparation were precipitated by cold acetone and the precipitate washed in acetone, which was then removed by evaporation. The dried pellet was stored at -70°C in a dehydrated container or for short term storage at 4°C prior to freeze drying.

4.2.2 Formulations

4.2.2.1 Preparation of emulsions

Emulsions were prepared as described by Alpar *et al* (1989a). See chapter 2 section 2.3.2.1.

4.2.2.1.1 Stability tests on emulsions

Stability tests on emulsions were carried out as described in chapter 2 section 2.3.2.2.

4.2.2.2 Preparation of aerosil gel

Freeze dried vaccine was reconstituted and diluted with isotonic phosphate buffered saline (PBS) so that the required amount of protein per dose

(60µg) was contained in 0.15 ml. Aerosil 200 gel (Degussa, Frankfurt) was added to give a final concentration of 0.4% (pH 7.5) and a total volume for each dose of 0.2 ml.

4.2.2.3 Preparation of carbopol gel

Freeze dried vaccine was reconstituted and diluted with PBS so that the required amount of protein per dose (60µg) was contained in 0.15 ml. Carbopol 940 (BF Goodrich, Cleveland, Ohio) was presoaked in PBS for 24 hrs and added to give a final gel concentration of 0.1% (pH 7.5) and a total volume for each dose of 0.2 ml.

4.2.2.4 Preparation of alhydrogel

Freeze dried vaccine was reconstituted and diluted with PBS so that the required amount of protein per dose (6µg) was contained in 0.15 ml. Aluminium hydroxide suspension (alhydrogel, Superfos) was added to give a final concentration of 0.5% and a total volume for each dose of 0.2 ml.

4.2.2.5 Liposome preparation

Multilamellar dehydration-rehydration vesicles composed of equimolar phospholipid (66µmoles) and cholesterol were prepared as described previously in chapter 3 section 3.2.2.2 by the method of Kirby and Gregoriadis (1984). For each preparation6mg of Kinner HSV subunit vaccine in 2 ml of distilled water was used. The % entrapment of Skinner vaccine in the liposomes was measured indirectly using the Lowry protein assay (section 4.2.2.6).

4.2.2.6 Lowry (Folin-Ciocalteau) protein assay

The assay used was that developed by Lowry *et al* (1951) described in chapter 3 section 3.2.2.3.

4.2.2.7 *In vitro* stability assay of liposomes

The assay has already been described in chapter 3 section 3.2.2.4.

4.2.3 Immunisation studies using gels and emulsions

Dunkin-Hartley guinea pigs, 300-350g (Porcellus Animal Breeding Ltd, Sussex, U.K.) were immunised with the Skinner vaccine (Skinner *et al*, 1982a and Skinner *et al*, 1982b) *via* the subcutaneous (0.2 ml), oral (0.5 ml), nasal (0.2 ml) and vaginal (0.1 ml) routes, five and three weeks prior to challenge with HSV-2 (Thornton *et al*, 1984).

The mucosally-vaccinated guinea pigs were given a vaccine dose of 300µg on 3 consecutive days, 5 and 3 weeks prior to challenge with HSV-2. Gel formulations were used for the nasal (aerosil 200) and vaginal (carbopol 940) routes and an emulsion (water-oil-water) for the oral route, as well as a PBS delivery in all cases. The subcutaneously vaccinated guinea pigs were given a single 30µg dose 5 and 3 weeks prior to challenge with HSV-2. The vaccinated groups contained 16 guinea pigs, the infected only and uninfected control groups contained 8 and 4 guinea pigs, respectively.

4.2.4 Immunisation studies using liposomes

Dunkin-Hartley guinea pigs (300-350g) were immunised with the Skinner vaccine (Skinner *et al*, 1982a and Skinner *et al*, 1982b) *via* the subcutaneous, oral and nasal routes, five and three weeks prior to challenge with HSV-2 (Thornton *et al*, 1984).

The guinea pigs vaccinated *via* the oral and nasal routes were given a vaccine dose of 300µg on 3 consecutive days, 5 and 3 weeks prior to challenge with HSV-2. DSPC liposome preparations containing the vaccine were pooled and diluted with PBS to give a total volume of 0.5 ml and 0.2 ml for each oral and nasal dose,

respectively. The subcutaneously vaccinated guinea pigs were given a single 30 μg dose 5 and 3 weeks prior to challenge with HSV-2. DSPC liposome preparations containing the vaccine were pooled and diluted with PBS to give a total volume of 0.2 ml for each subcutaneous dose.

4.2.5 Guinea pig Infection

Guinea pigs were infected by placing small pieces of absorbable gelatin sponge BP (Sterispon, Allen and Hanburys) soaked in 50μl of virus suspension containing approximately 4 x 10^5 $TCID_{50}$ (50% tissue culture infective dose) of HSV-2, into the vagina with blunt forceps (Thornton *et al*, 1984).

During the following 10 days, the clinical effects of virus challenge were recorded. Vaginal oedema was estimated by considering the vagina as an ellipse. Orthogonal measurements were used to calculate the cross sectional area with the formula A.B.π/4. Vaginal oedema was expressed relative to the pre-challenge state (index of oedema, IO). The area of the external genitalia covered by herpetic lesions (combined lesion score, CLS) and erythema on a scale of 1 to 5 were estimated. Urinary retention was also recorded when present (Thornton *et al*, 1984). Animals which became severely ill were sacrificed by injection of 0.2 ml pentabarbatone (200mg ml^{-1} solution) into the heart and their scores assumed to remain unchanged until the end of the experiment. All animals were sacrificed at the end of the observation period.

4.2.6 Serum and vaginal wash samples

Serum samples were obtained by cardiac puncture before vaccination, immediately prior to virus challenge (day 0) and on day 10 post challenge.

The animals were anaesthetised using a 50% CO_2 and 50% O_2 mixture. The blood was allowed to clot in serum separation tubes (Becton-Dickinson Ltd, UK),

centrifuged at 1,000 xg for 10 min at 22°C and the serum collected and stored at -20°C.

Vaginal washes were collected from the killed animals at 10 days post challenge by introducing 0.5 ml of wash fluid (0.9% w/v NaCl, 0.05% v/v Tween 20, 1mM phenyl-methylsulphonyl fluoride (PMSF) and 0.1% w/v sodium azide) into the vagina using a blunt plastic pipette and collected after several refluxes. Vaginal washes were stored at 4°C.

4.2.7 Enzyme linked immunosorbent assay (ELISA)

4.2.7.1 ELISA antigen preparation

ELISA antigen was kindly prepared as described by Jeansson *et al* (1983) by M. Welch at Porton Down. ERSF cells were grown in 20 x 850 cm roller bottles until lightly confluent, in minimum essential medium (MEM, Gibco Europe Ltd) with HEPES, 0.25% bicarbonate buffer, L-glutamine, newborn calf serum (NBCS, Imperial Laboratories) and kanamycin. The bottles were then drained of their medium. The virus (HSV-1, nominal titre $10^{8.4}$ $TCID_{50}$ ml^{-1}) was diluted with MEM containing 5% NBCS and 1% glucose (0.5 ml virus + 2.45 ml medium). One ml of this virus dilution was added to each bottle, together with 24 ml of the medium/diluent. The bottles were then incubated on the roller at 35°C. After 2 hrs 75 ml medium was added to each bottle and incubation continued.

The next day a 100% cytopathic effect (cpe) should be observed. The cells were detached from the surface of the bottles and suspended in the medium using glass balls. The contents of the bottles were centrifuged at 1,500 xg for 10 min. The pellets were resuspended in 250 ml of PBS and spun again. The supernatant was discarded and the pellet frozen at -70°C.

This procedure was repeated except the step of infection of the culture with HSV-1 was not included for the production of control antigen.

The antigen was prepared as follows: the pellets were thawed quickly, and their volume made up to 40 ml with 0.025M Tris-hydrochloride (pH 8.0), homogenised and centrifuged at 1,500 xg for 15 min at 4^oC. The supernatant was then centrifuged at 160,000 xg for 1 hr at 4^oC. The pellet was washed by suspension in 0.1M glycine-sodium hydroxide buffer (pH 8.0) and centrifuged at 160,000 xg for 1 hr to obtain washed membrane. The pellet was resuspended up to a volume of 15 ml in 0.1M glycine-sodium hydroxide buffer (pH 8.8) and homogenised again to properly resuspend the membrane proteins.

Sodium desoxycholate (DOC) was added to a final concentration of 0.33%. The mixture was centrifuged again at 160,000 xg for 1 hr. The supernatant was used in the ELISA technique and was stored at -70^oC for long term storage.

4.2.7.2 IgG titre of antibody to HSV

Detection of IgG titre of antibody to HSV has been described by Phillpotts *et al* (1988). An indirect ELISA was used to estimate the IgG titre of antibody to HSV-1 in guinea pig sera. Plates (Dynatech Microelisa, 129A) were coated with deoxycholate solubilised HSV-1 antigen prepared in ERSF cells (Jeansson *et al*, 1983) or with control antigen prepared from uninfected ERSF cells (see section 4.2.7.1). Checkerboard titrations were carried out to find the optimum dilution of ELISA antigen (1:1,000).

Dilutions of guinea pig serum in PBS containing 0.1% v/v Tween 20 (PBST) were incubated in the coated, washed plates for 2 hrs and the total amount of guinea pig IgG bound was estimated by the addition of rabbit anti guinea pig IgG-horseradish peroxidase conjugate (ICN Pharmaceuticals).

After 2 hrs incubation at 37^oC the enzyme substrate 3,3',5,5'-tetramethylbenzidine (TMB, Miles) was added (0.15mM TMB in 0.1M sodium acetate pH 6.8 containing 0.003% v/v H_2O_2) and the reaction allowed to

proceed for 10 min. The enzyme reaction was stopped by the addition of 2M H_2SO_4 and the absorbance at 450nm measured (Titertek Multiskan, Flow Labs.). The ELISA data were plotted as OD_{450} vs log_2 serum dilution. The endpoint of each titration was defined as the dilution at which the OD_{450} was 0.2 (instrument zeroed on the reagent blank). This dilution was expressed as a titre after correction by reference to a standard serum titrated on every plate.

4.2.7.3 Secretory IgA titre of antibody to HSV

Detection of secretory IgA (sIgA) antibody to HSV-1 has been described previously by McBride *et al* (1988). The plates were coated as above. Guinea pig vaginal washes were diluted in 5% normal sheep serum, 0.1% w/v bovine serum albumin (BSA), 0.05% Tween 20 and 0.9% w/v sodium chloride and incubated in the antigen coated wells for 2 hrs at 37°C. HSV specific sIgA was detected with a rabbit antiserum, reactive with human secretory component (ICN, Pharmaceuticals) and diluted 1:100 in the above buffer. After 1 hr at 37°C, bound rabbit immunoglobulin was detected with a goat-antirabbit IgG horseradish-peroxidase (HRP) conjugate (ICN, Pharmaceuticals) diluted 1:2,000 in the above buffer. After 30 min incubation, the substrate was added as described in section 4.2.7.2.

4.2.7.4 Non-specific sIgA titre of antibody

Plates (Dynatech Micoelisa, 129A) were coated with goat antiserum reactive with human secretory component (ICN, Pharmaceuticals), at a concentration of 10µg ml^{-1} in carbonate coating buffer. Guinea pig vaginal washes were incubated in the coated wells for 2 hrs at 37°C. Bound sIgA was detected with a rabbit anti guinea pig IgA (ICN, Pharmaceuticals) diluted 1:1,000 in PBST containing 0.1% BSA. After 1 hr incubation, the wells were washed 3x with PBST and then goat anti rabbit

horseradish peroxidase was added diluted 1:2,000 in the above buffer. After 1 hr the substrate was added as described in section 4.2.7.2.

Purified guinea pig sIgA kindly given by Dr B. McBride and prepared as described by McBride *et al* (1988) was serially diluted on each plate as a standard.

4.2.8 SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis)

The gel was typically made with a gradient of acrylamide concentration from T=7.5% at the top (large pore size), to T=20% (small pore size) at the bottom (Table 4.1). The gradient was pumped into the bottom of the gel cassette, light end first, and progressively displaced upwards by the increasingly dense liquid. This established a gradient of acrylamide concentration, as required, and a density gradient of sucrose to stabilise the acrylamide gradient against convection and diffusion. To protect further against convection; the upwardly increasing gradient of catalysts ensured that the gel set from the top downwards. Since the polymerisation process is exothermic, this eliminated convective disturbance. The surface of the gel was carefully overlayed with 0.1% SDS to exclude oxygen, inhibit polymerisation, and provide a flat meniscus. The gel set in 30-60 min at room temperature.

The stacking gel was prepared and poured between the plates on top of the running gel. Samples were mixed in a 1:1 ratio with either denaturing mix (50% glycerol, 5% v/v mercaptoethanol, 5% w/v SDS in 0.1M Tris buffer pH 8.4 containing 0.1mg of bromophenol blue in alcohol), and boiled for 5 min. The sample solutions were loaded and electrophoresis was carried out at 200 volts.

Table 4.1 Ingredients for SDS-PAGE gel

Components	7.5%	20%	Stacking gel
Distilled water	10.3	4.25	11.6
Acrylamide and bisacrylamide[1]	3.75	10.0	-
Acrylamide and DATD[2]	-	-	4.0
1.5M Tris/HCl pH 8.8[3]	5.0	-	-
1.5M Tris/HCl pH 8.8 (containing 50% sucrose)	-	5.0	-
1M Tris/ HCl pH 6.8	-	-	2.0
10% w/v SDS[4]	0.2	0.2	0.2
1% v/v TEMED[5]	0.7	0.5	2.0
10% w/v APS[6]	0.07	0.05	0.2

1 Acrylamide and bisacrylamide: 100 ml of stock solution contains 39.6g acrylamide and 0.4g N,N-methylenebisacrylamide ('bis'). T= 40% (total %= % acrylamide+ % bis)

2 Acrylamide and DATD (N,N' Diallyltartardiamide): 100 ml stock solution contains 21.25g acrylamide and 3.75g DATD. T= 25% (= 21.25+ 3.75)

6 Tris (hydroxymethyl) amino ethane

4 Sodium dodecyl sulphate

5 N,N,N',N'-tetramethylethylene

6 Ammonium persulphate

4.2.9 Immunoblotting

The transfer of antigens, separated by SDS-PAGE to a solid phase for immunoreaction was performed by the Western Blotting method of Towbin *et al* (1979). Nitrocellulose (NC) paper (pore size 0.45µm, Bio-Rad Laboratories Ltd) was soaked in blotting buffer (7.3mM $Na_2HPO_4.2H_2O$ and 17.5mM NaH_2PO_4) and laid on 2 sheets of chromatography paper. Following electrophoresis, the gel was washed for 20 min in blotting buffer with 3 changes, placed on top of the NC paper and overlayed with 2 more sheets of chromatography paper.

The NC paper, gel and chromatography paper were in turn sandwiched between scotchbrite pads in a transblot cassette. The cassette was then placed in a transblot cell filled with blotting buffer. Electrophoretic transfer of proteins was carried out at 15 volts for 3 hrs.

Qualitative transfer of proteins was confirmed by staining with 0.1% w/v amido black in 20% methanol-5% acetic acid, alternatively a total protein stain using colloidal gold (Bio-Rad) was used.

After transfer, the NC paper was soaked for 2 hrs in blocking solution (1% dried milk in PBS containing 0.1% v/v Tween 20 plus 0.02% sodium azide) to saturate nonspecific binding sites in the nitrocellulose. The NC paper was incubated for 2 hrs at room temperature in serum diluted 1:100 in blocking solution, and washed 3 times in PBS containing 0.1% v/v Tween 20 (PBST) for 20 min. The rabbit anti-guinea pig IgG horseradish peroxidase conjugate was added for 2 hrs at room temperature diluted 1:500 in PBST.

After several five min washes in PBST and a final wash in 100 ml of 2M sodium acetate buffer pH 5.0, the substrate (100 ml of 2M sodium acetate pH 5.0 containing 16mg 3-amino-9 ethylcarbazole (Sigma) dissolved in 2 ml of dimethylsulphoxide and 100µl of hydrogen peroxide) was added for 15 min. The colour reaction was terminated by removing the substrate and adding distilled water. The

blots were washed for 5-10 min, dried between paper and subsequently stored in the dark.

4.2.9.1 Determination of the molecular weights of HSV-1 proteins separated by SDS-PAGE

Denatured polypeptides bind SDS in a constant weight ratio and have essentially identical charge densities and therefore migrate in polyacrylamide gels according to size. A linear relationship exists between $\log_{10}$ of the polypeptide molecular weight and the relative mobility (R_f) values, where:

$$R_f = \frac{\text{Distance migrated by polypeptide}}{\text{Distance migrated by dye front}}$$

The apparent molecular weights of HSV-1 proteins separated by SDS-PAGE (section 3.2.8) transferred to nitrocellulose and recognised by serum (collected from guinea pigs vaccinated with HSV-1 and challenged intravaginally with HSV-2) in Western blots (section 3.2.9) were determined by comparing their R_f values with those of known protein markers. Molecular weight markers were purchased from Pharmacia. They consisted of:

	Subunit molecular weight
Phosphorylase b	94,000
Bovine serum albumin	67,000
Ovalbumin	43,000
Carbonic Anhydrase	30,000
Soybean Trypsin Inhibitor	20,100
∂-Lactalbumin	14,400

4.2.10 Statistical methods

The guinea-pig clinical data were processed and analysed using a dedicated software package 'PIGPROG' written by A.C. Walkland and designed to run on the BBC microcomputer. Significance tests performed by this program were based on the Mann-Whitney procedure.

Comparisons between the subcutaneous vaccination group and other groups, using ELISA data, were analysed for significance using Student's unpaired t test. Differences with $p<0.05$ were considered to be significant.

4.3 RESULTS AND DISCUSSION

4.3.1 Immunisation studies using gels and emulsion

4.3.1.1 HSV-specific antibody concentrations in serum

Titres of IgG antibody to HSV-1 as determined by ELISA in the serum of guinea pigs before and after vaccination with HSV-1 vaccine, *via* the s.c, oral, vaginal and nasal routes using gels and emulsions, and after challenge with HSV-2 are shown in table 4.2. The post vaccination HSV-specific serum IgG antibody levels were significantly higher for the vaccinated nasal (PBS, 10.67+/-1.65; aerosil gel, 10.8+/-2.23) and s.c. (11.86+/-0.86) groups compared to the non-vaccinated groups. However, antibody levels were not significantly different for the oral (PBS, 8.14+/-0.88; emulsion, <8.84+/-1.18) and vaginal (PBS, 8.4+/-1.27; gel, 8.47+/-1.03) compared to the non-vaccinated control group (7.98+/-0.55; 7.74+/-0.66).

The adjuvants used for the mucosal routes of delivery made no significant difference on the post vaccination antibody levels compared to the Skinner vaccine delivered in free form to the oral, nasal and vaginal surfaces.

The subcutaneous and nasal delivery of the Skinner vaccine resulted in significantly higher post-vaccination antibody IgG levels compared to the oral and vaginal delivery routes.

At 10 days post challenge, detectable levels of HSV specific IgG were present in the sera from the vaccinated and infected only (non-vaccinated) group of animals; however, all values were higher in the vaccinated groups than in the infected only group of animals (Table 4.2).

Analysis of the HSV-1 antigen prepared as described in section 4.2.7.1 by Western blotting (4.2.9) with post vaccination and post challenge serum collected from guinea pigs immunised with a subunit HSV-1 vaccine using gels and emulsions, and challenged intravaginally with HSV-2 are shown in fig 4.1. There are bands at

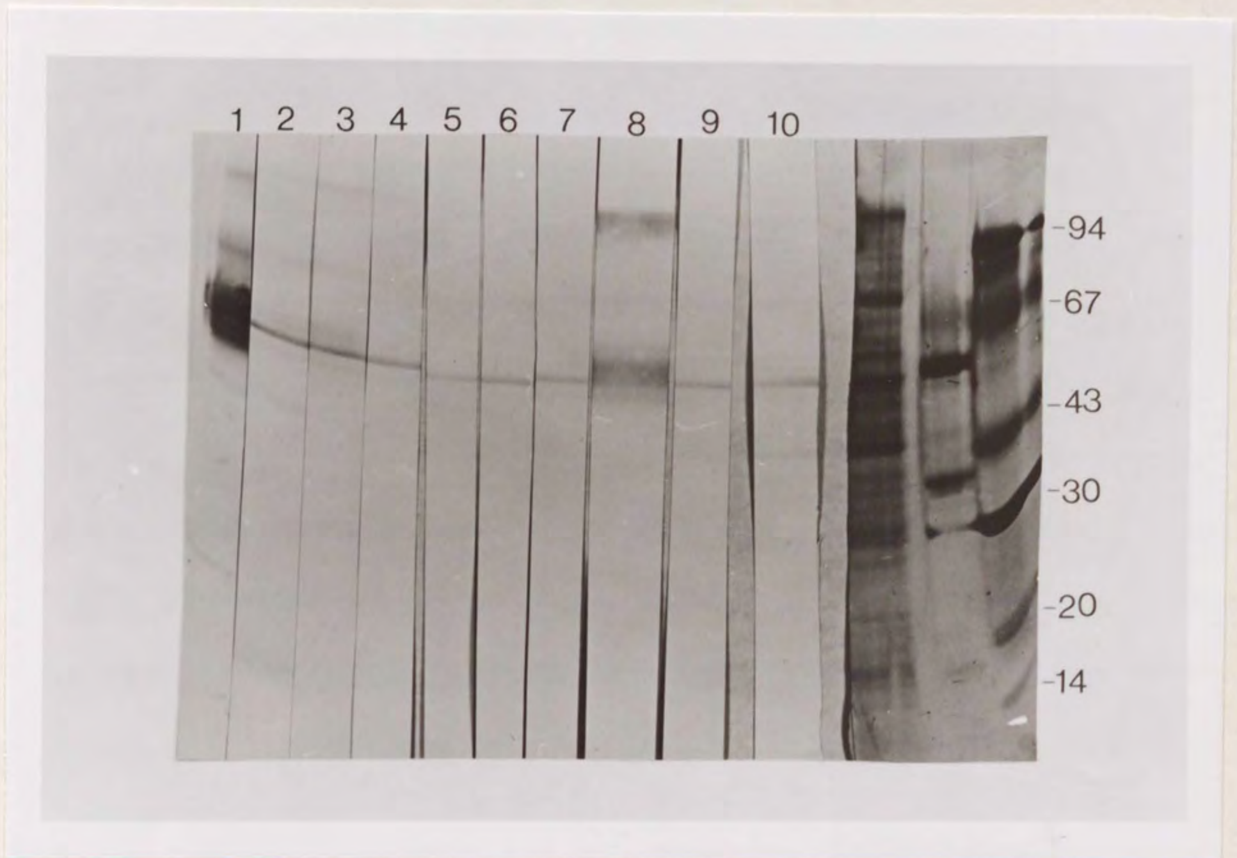
100-120kDa, faint bands at around 90kDa and strong bands between 50 and 58kDa. The post vaccination and post challenge serum recognised proteins of molecular weight 95, 41 and 50kDa determined as descibed in section 4.2.9.1. However, calculating R_f values has an inaccuracy of +/- 5 KDa. More conclusive proof would be achieved by carrying out Western blots using purified glycoproteins fractionated by SDS-PAGE. However, such purified glycoproteins were not available. The Western blots suggest that the serum had been raised against gB, gC, gD and possibly gE. The fainter bands that were often seen are probably precursors or degradation products of these major glycoproteins. The minor glycoproteins such as gH are very hard to detect.

Table 4.2 Titres of IgG antibody to HSV-1 as determined by ELISA in the serum of guinea pigs before and after vaccination with HSV-1 vaccine and after challenge with HSV-2.

		Geometric mean ($\log_2$) ELISA titre serum IgG antibody		
Route and dose[a]	formulation	pre vaccination	post vaccination	post challenge
S.C[b] 30	alhydrogel	<7.8 (8)[c] (0.52)[d]	11.86 (7) (0.86)	>15.41 (7) (2.42)
nasal 300	drop(PBS)[e]	<9.1 (16) (0.94)	10.67 (11) (1.65)	>16.07 (10) (2.8)
oral 300	PBS	<8.99 (16) (1.15)	8.14 (12) (0.88)	<10.64 (12) (3.17)
nasal 300	gel	<8.73 (15) (1.0)	10.8 (9) (2.23)	>13.9 (6) (3.37)
oral 300	emulsion	<9.23 (16) (0.94)	<8.84 (12) (1.18)	11.8 (10) (2.85)
vaginal 300	gel	<7.06 (16) (0.43)	<8.47 (12) (1.03)	>13.3 (11) (3.53)
vaginal 300	PBS	<7.65 (16) (0.55)	<8.4 (11) (1.27)	>14.01 (11) (2.84)
uninfected contols		<8.6 (4) (0.0)	<7.98 (3) (0.55)	<7.97 (3) (0.63)
infected controls		<7.76 (12) (0.65)	<7.74 (7) (0.66)	<10.47 (7) (2.05)

a-µg of protein, b-subcutaneous, c-no. of animals, d-standard deviation, e-phosphate buffered saline.

Fig 4.1 Western blotting of HSV-1 antigen prepared as descirbed in text (section 4.2.7.1) was fractionated by SDS-PAGE, transferred to nitocellulose and reacted in Western immunoblots with post vaccination and post challenge serum collected from guinea igs immunised with a subunit HSV-1 vaccine using gels and emulsions and challenge intravaginally with HSV-2. Protein size markers are indicated in kDa on the left margin.



4.3.1.2 Mucosal antibody responses

The results for the vaginal wash IgG antibody levels indicated that only those animals previously immunised with the Skinner vaccine had detectable HSV-specific IgG antibodies (Table 4.3).

Herpes simplex viruses (HSV) have an affinity for cells of ectodermal origin. Therefore a desirable property of a vaccine should be the ability to induce immunity at mucosal surfaces. This would be important for the prevention of primary disease and, whilst not preventing recurrences, may modify the duration of symptoms and period of viral shedding.

The results of this study indicate that an IgG mucosal immune response can be produced at a site distant from the site of vaccination. The non-vaccinated infected group of animals had no detectable HSV-specific antibodies present in the vaginal washes, even though there were high levels of specific antibodies present in the sera. This observation argues against the possibility that transudation of serum antibodies from sites of inflammation into the mucosa (Little *et al*, 1969) had occurred.

McBride *et al* (1988) suggested that absence of vaginal antibodies in non-vaccinated infected animals compared to vaccinated animals may be because local production of antibodies may require memory B lymphocytes (secondary response) in order to produce sufficiently high antibody levels to be detectable by day 10 post challenge. Alternatively, HSV-specific IgG plasmacytes may require more than 10 days from primary infection to become localised in the mucosal-associated lymphoid tissue.

The antigenic cross-reactivity between human and guinea pig secretory component, demonstrated by Western blotting (McBride *et al*, 1988) enabled the secretory IgA response at the vaginal mucosa to be measured using a rabbit anti-human secretory component anti-serum.

McBride *et al* (1988) demonstrated specific sIgA antibodies to HSV

after subcutaneous vaccination with the Skinner subunit vaccine and intravaginal challenge with HSV-2. However, in the immunisation studies described here no specific sIgA to HSV was detected. Various methods were tried to increase the sensitivity of the ELISA method used (see section 4.2.7.3), including the use of a biotin-streptavidin system. McBride *et al* (1988) used HSV-1 antigen to coat the ELISA plates prepared form HSV-infected and non-infected Chang cell monolayers as described by McBride and Ward (1987). Therefore, possibly the preparation of HSV antigen ie using ERSF or Chang cell monolayers affected the HSV antigens and their recognition by sIgA. The use of ELISA antigen prepared using Chang cell monolayers still did not enable detection of HSV specific sIgA.

Non-specific sIgA was detected in the vaginal wash samples (table 4.4) using an indirect ELISA (see section 4.2.7.4). There was no significant difference between the vaccinated groups and the control uninfected groups. However, the vaginally vaccinated groups had significantly higher non-specific sIgA levels compared to the infected only and than those animals vaccinated *via* the s.c. route.

Thornton *et al* (1984) suggested that an effective vaccine against HSV infection would probably have to exert its effect at the initial stage of the infection of vaginal epithelial cells before nervous tissue became involved by viral spread through the subepithelial tissues. This would require either neutralisation of infecting virus, or rapid elimination of infected epithelial cells before replicated virus could spread to surrounding tissue. They, further suggested that such a vaccine would, therefore, need to induce an appropriate immune response, humoral, cell-mediated or perhaps both, in the mucosa of the vagina.

The lack of HSV-specific sIgA results was disappointing especially when considering the aim of this PhD in relation to protection against HSV-infection. An alternative method known as the Enzyme linked immunospot assay (ELISPOT) involves the detection of specific antibody secreting cells. Briefly, nitrocellulsoe-bottomed

96-well plates are coated with antigen followed by isolated lamina propria cell suspensions which are incubated at 37°C for 4 hrs. Specific antibody production is then detected with the appropriate conjugate, the substrate added and the number of spots counted under low magnification. This method may have given more information and therefore will be used in any future work.

Table 4.3 Titres of IgG antibody to HSV-1 as determined by ELISA in the vaginal washes of guinea pigs at 10 days post challenge.

Route + dose[a]	formulation	IgG
S.C[b]	alhydrogel	0.68 (0.55)[c]
nasal 300	drop(PBS)[d]	1.10 (0.62)
oral 300	PBS	0.06 (0.07)
nasal 300	gel	0.66 (0.72)
oral 300	emulsion	0.14 (0.29)
vaginal 300	carbopol	0.40 (0.40)
vaginal 300	PBS	0.29 (0.32)
uninfected		0.00 (0.00)
infected only		0.061 (0.04)

a-μg of protein, b-subcutaneous, c-standard deviation, d-phosphate buffered saline.

Table 4.4 Vaginal wash non-specific sIgA antibody values (A_{450}) as determined by ELISA after vaccination with HSV-1 subunit vaccine and intravaginal challenge with HSV-2.

Route + dose[a]	formulation	non-specific antibody
S.C[b]	alhydrogel	0.18 (0.1)[c]
nasal 300	drop(PBS)[d]	0.37 (0.24)
oral 300	PBS	0.29 (0.12)
nasal 300	gel	0.38 (0.28)
oral 300	emulsion	0.24 (0.07)
vaginal 300	carbopol	0.48 (0.29)
vaginal 300	PBS	0.44 (0.24)
uninfected		0.19 (0.13)
infected only		0.25 (0.15)

a-µg of protein, b-subcutaneous, b-standard deviation, d-phosphate buffered saline.

4.3.1.3 Clinical scores

Figs 4.2-4.5 illustrate guinea pig infection and clinical signs of the disease after intravaginal challenge with HSV-2 (fig 4.2; uninfected guinea pig for comparison). The first signs of infection, which were present by day 3-4, were erythema of the vulva followed by oedema (fig 4.3). Appearance of vesicular lesions followed within the next two days (fig 4.4) and sometimes the transition from no clinical signs to vesicles occurred within 24 hrs. From days 6-7 onwards, the vesicles developed into pustules and then rapidly into ulcers covering 60-100% of the external genitalia (fig 4.5). Urinary retention was commonly observed and was relieved by pressing on the abdomen to cause emptying of the bladder. On the 10th day following challenge, animals were debilitated, lacked condition and were anorexic, and at this time were killed (Thornton *et al*, 1984).

The IgG titres for 8 guinea pigs out of a total of 78 studied; two from the oral PBS group, one from each of the other mucosal groups and one from the infected only group showed a drop in serum IgG titre (individual animals, therefore non tabulated data) on day 10 post challenge, compared to the post vaccination titre, and showed no sign of infection.

Therefore, the results have been evaluated in two different ways: firstly, not discounting the eight guinea pigs, on the assumption that they were protected by mucosal immunity, and secondly, discounting the 8 guinea pigs, on the assumption that they were not infected. The designs and pathology results of the experiment are summarised in tables 4.5 and 4.6 which gives the median cumulative score for index of oedema (IO), combined lesion score (CLS), and combined IO+CLS. Both IO and CLS were expressed cumulatively over the ten day observation period as the peak severity of illness does not necessarily coincide in all animals, and this would contribute to the variance of observations taken on a single day (Phillpotts *et al*, 1988).

Severe illness, requiring that an animal be killed, did not occur with sufficient frequency to allow distinctions to be made between treatments on this basis alone (Phillpotts *et al*, 1988). However, retention of urine and/or faeces was a frequent finding which was reduced in the subcutaneously vaccinated group compared to the infected only controls and the other vaccinated groups.

Fig 4.2 Normal external genitalia of uninfected guinea pig.

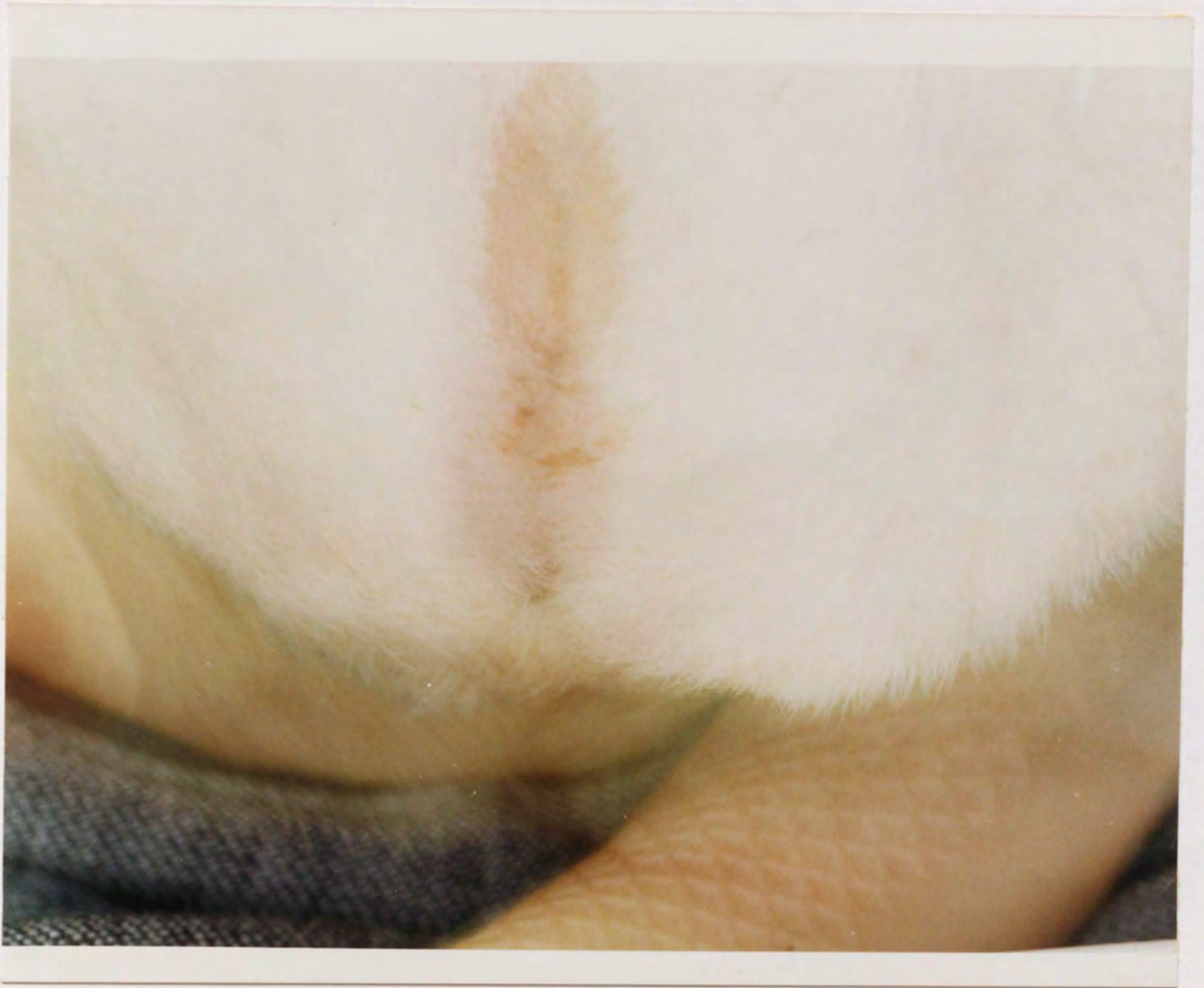


Fig 4.3 External genitalia of guinea pig 3 days after intrvaginal infection with HSV-2 showing erythema of the vulva and oedema.

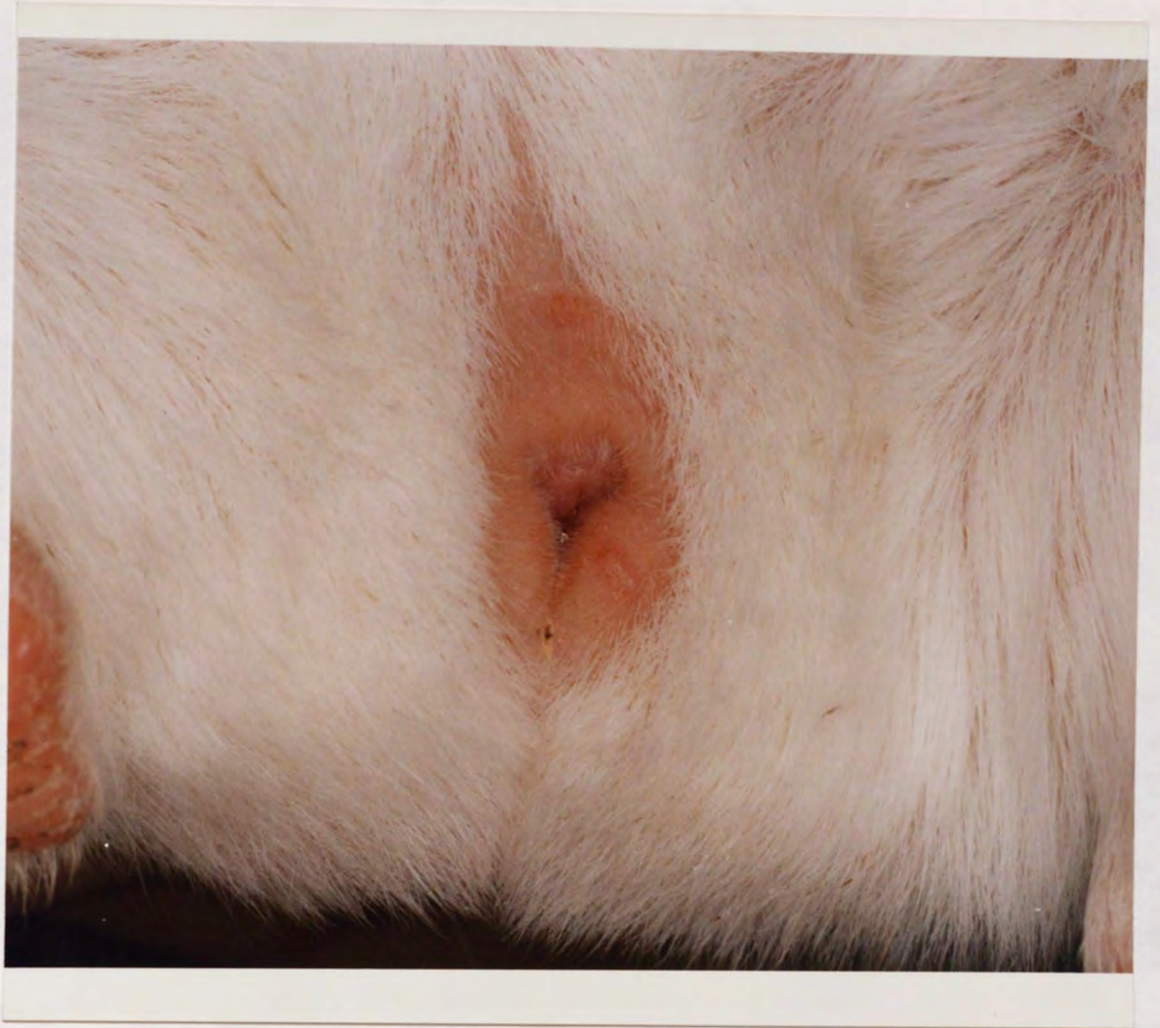


Fig 4.4 External genitalia of guinea pig 5-6 days after intravaginal infection with HSV-2 showing the appearance of vesicles.

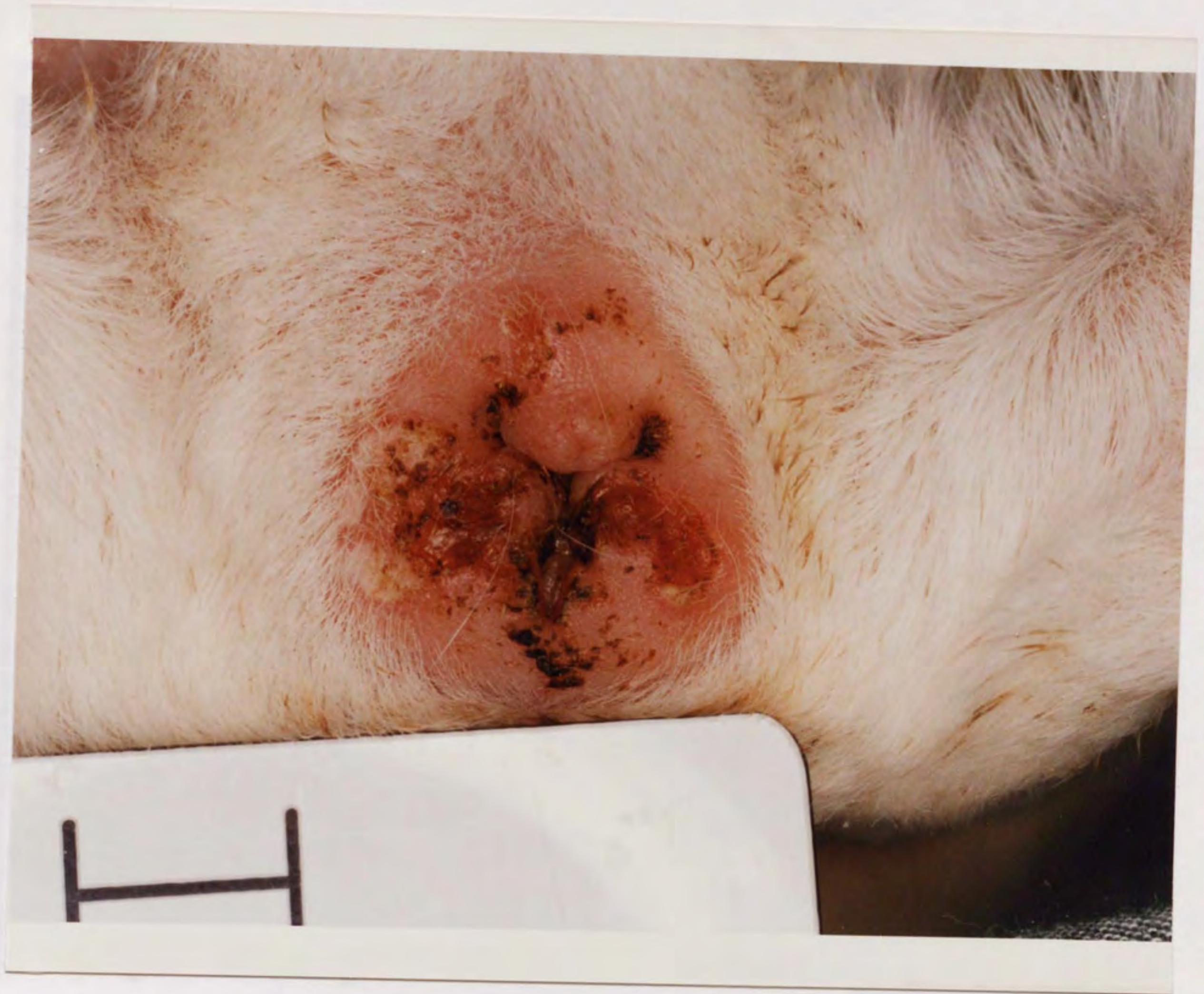


Fig 4.5 External genitalia of guinea pig 7-10 days after intravaginal infection with HSV-2 showing the appearance of pustules and ulcers covering 100% of the area.

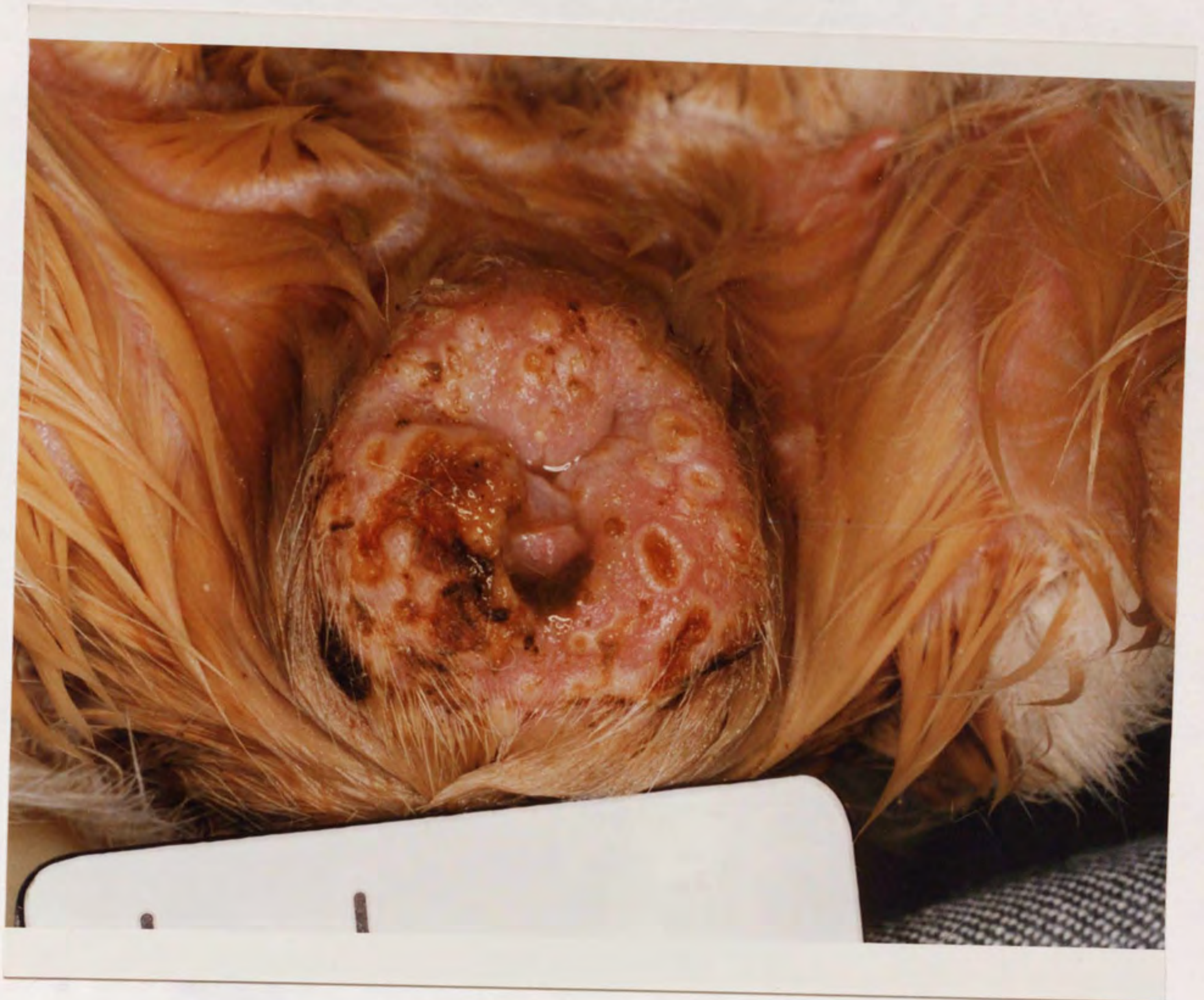


Table 4.5 Clinical scores obtained in which guinea pigs immunised with HSV-1 vaccine by four routes were challenged intravaginally with HSV-2.

			median cumulative score				
No. of animals	treatment	µg dose	IO[a]	CLS[b]	IO+CLS	A[c]	B[d]
7	S.C.[e] alhydrogel	30	12.5	2.0	13.1	0	0
10	nasal drop PBS[f]	300	22.4	11.5	35.6	6	0
13	oral PBS	300	25.5	24.0	49.2	9	1
8	nasal gel	300	24.8	17.0	40.8	5	2
12	oral emulsion	300	28.3	22.0	51.2	10	0
12	vaginal gel	300	25.9	23.5	50.6	7	0
12	vaginal PBS	300	29.8	27.5	57.6	10	1
3	uninfected controls		10.9	0.0	10.9	0	0
8	infected controls		25.9	27.5	54.9	6	1

a-index of oedema, b-combined lesion score, c-no. of animals with retention, d-no. of animals died earlier then 10 days after challenge, e-subcutaneous, f-phosphate buffered saline.

Table 4.6 Clinical scores obtained in which guinea pigs immunised with HSV-1 vaccine by four routes were challenged intravaginally with HSV-2. (Guinea pigs assumed to be non-infected on the basis of non-tabulated ELISA results are not included).

			median cumulative score				
no. of animals	treatment	µg dose	IO[a]	CLS[b]	IO+CLS	A[c]	B[d]
7	S.C.[e] alhydrogel	30	12.5	2.0	13.1	0	0
9	nasal drop PBS[f]	300	25.4	14.0	42.8	6	0
11	oral PBS	300	29.0	26.0	54.2	9	1
7	nasal gel	300	26.2	17.0	43.2	5	2
11	oral emulsion	300	29.9	25.0	52.4	10	0
11	vaginal gel	300	27.1	25.0	56.1	7	0
11	vaginal PBS	300	26.2	28.0	57.9	10	1
3	uninfected controls		10.9	0.0	10.9	0	0
7	infected controls		26.2	28.0	56.2	6	1

a-index of oedema, b-combined lesion score, c-no. of animals with retention, d-no. of animals which died earlier than 10 days after challenge, e-subcutaneous, f-phosphate buffered saline.

Phillpotts *et al* (1988) in considering the quantitative aspects of the guinea-pig model found IO (measurements of vaginal oedema) and CLS (visual estimates of the area of external genitalia covered by herpetic lesions) to be reliable, highly positively correlated scores for the severity of illness. In addition, they found that IO appeared to be a more useful score than CLS, and suggested that this was not surprising as IO was an objective measure made with a ruler, whereas CLS was a subjective visual estimate prone to error, even when a single observer was used. It was considered that combined IO+CLS was the optimal choice for estimating disease severity in human infection.

For both assumptions all the infected groups were significantly different for combined IO+CLS (Tables 4.7 and 4.8) when compared to the uninfected group. In addition, the mucosal routes gave no significant amelioration of the disease when compared to the infected only group, whereas the subcutaneous route did.

The results are presented graphically in figs 4.6 and 4.7, in which IO+CLS has been plotted against the day of observation. For both cases reductions in the severity of illness were produced by the subcutaneous and nasal vaccination.

Although the nasal delivery resulted in titres not significantly different compared to the s.c. route, no significant reduction of disease was seen compared to the infected only group whereas the s.c route did. Welch *et al* (1988) immunised guinea pigs with HSV-1 subunit vaccine and subsequently challenged intravaginally with HSV-2. No significant relationships were found between clinical score and virus specific serum antibody titre in functional assays (ELISA, neutralisation, complement-mediated cytotoxicity and antibody-dependent cellular cytotoxicity). They concluded it would seem unlikely that serum antibody plays a major role in vaccine-induced immunity to HSV-2 challenge in the model used which is the same as described in this work. However, Sanchez-Pescador *et al* (1988) used a recombinant, truncated HSV type 1 glycoprotein D secreted by Chinese hamster ovary cells (rgD1)

to compare the ability of several adjuvants to stimulate protective immunity in guinea pigs. They found that the anti-rgD1 antibody titres measured were very predictive of protective immunity. There was a linear relationship between the ELISA titre and total lesion score and there appeared to be a threshold ELISA titre that predicted protective immunity.

Vaginal immunisation using HSV-1 Skinner subunit vaccine did not show a very high antibody response and no protection was observed after intravaginal challenge with HSV-2. Small *et al* (1985) have demonstrated that in some instances, administration of a vaccine at the anticipated portal of viral exposure afford sadditional protection against subsequent challenge, presumably through stimulation of local defences such as mucosal antibodies. The non-specific sIgA antibody response after vaginal immunisation with the Skinner vaccine was significantly higher compared to the infected only, and those animals vaccinated *via* the s.c route, without significant protection against HSV-2 challenge. However, it has been argued that numerous aspects of acquired immunity must be induced including the cytotoxic T-lymphocytes (Lawman *et al*, 1980). Rooney *et al* (1989) showed that vaccination with vaccinia/gD by the nasal route resulted in neutralising antibody and protection against intranasal challenge.

Table 4.7 Satistical analysis of all possible pairwise comparisons between groups within the immunisation studies using gels and emulsions.

	p value for comparison*				
Score	1	2	3	4	5
CLS	<0.05	N/S	<0.05	N/S	N/S
IO	<0.05	N/S	N/S	<0.05	N/S
CLS+IO	<0.05	N/S	N/S	<0.05	<0.05

* Pairwise comparisons between the following groups

1-groups-AE/AG/AL/AP/BF/EF/FG/FL/FP

2-groups-BC/BD/BE/BG/BL/CD/CE/GG/CL/CP/DE/DG/DL/DP
EG/EL/EP/GL/GP/LP

3-groups-AB/AC/BP/CF/DF

4-groups-AD

5-groups-AF

Group A; subcutaneous route 30µg/dose, B; nasal phosphate buffered saline (PBS) 300µg/dose, C; oral PBS 300µg/dose, D; nasal aerosil gel 300µg/dose, E; oral emulsion 300µg/dose, G; infected only, F; control animals, L; vaginal carbopol 940 300µg/dose, P; vaginal PBS 300µg/dose. IO; index of vaginal oedema, CLS; combined lesion score. NS p>0.05 (Mann-Whitney U-test).

Table 4.8 Satistical analysis of all possible pairwise comparisons between groups within the immunisation studies using gels and emulsions. (Guinea pigs assumed to be non-infected on the basis of non-tabulated ELISA results are not included).

	p value for comparison*				
Score	1	2	3	4	5
CLS	<0.05	N/S	<0.05	N/S	N/S
IO	<0.05	N/S	N/S	<0.05	N/S
CLS+IO	<0.05	N/S	N/S	<0.05	<0.05

* Pairwise comparisons between the following groups

1-groups-AC/AD/AE/AG/AL/AP/BF/GF/DF/EF/FG/FL/FP

2-groups-BC/BD/BL/CD/CE/CG/CL/CP/DE/DG/DL/DP/EG/EL/EP/GL/GP/LP

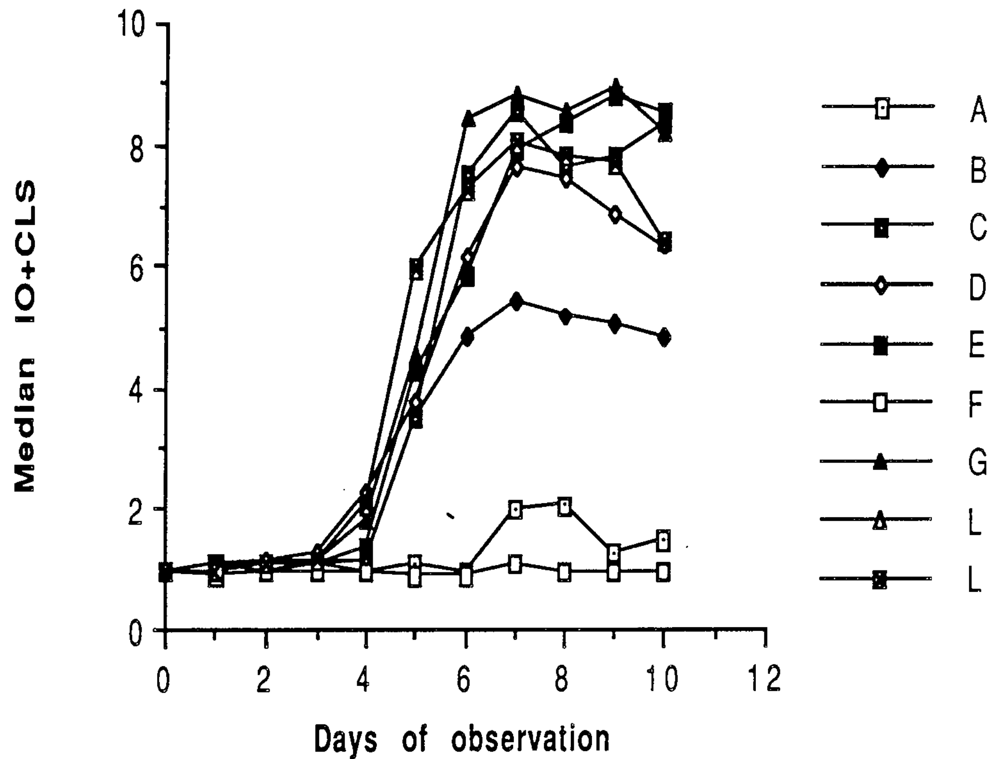
3-groups-BE/BG/BP

4-groups-AB

5-groups-AF

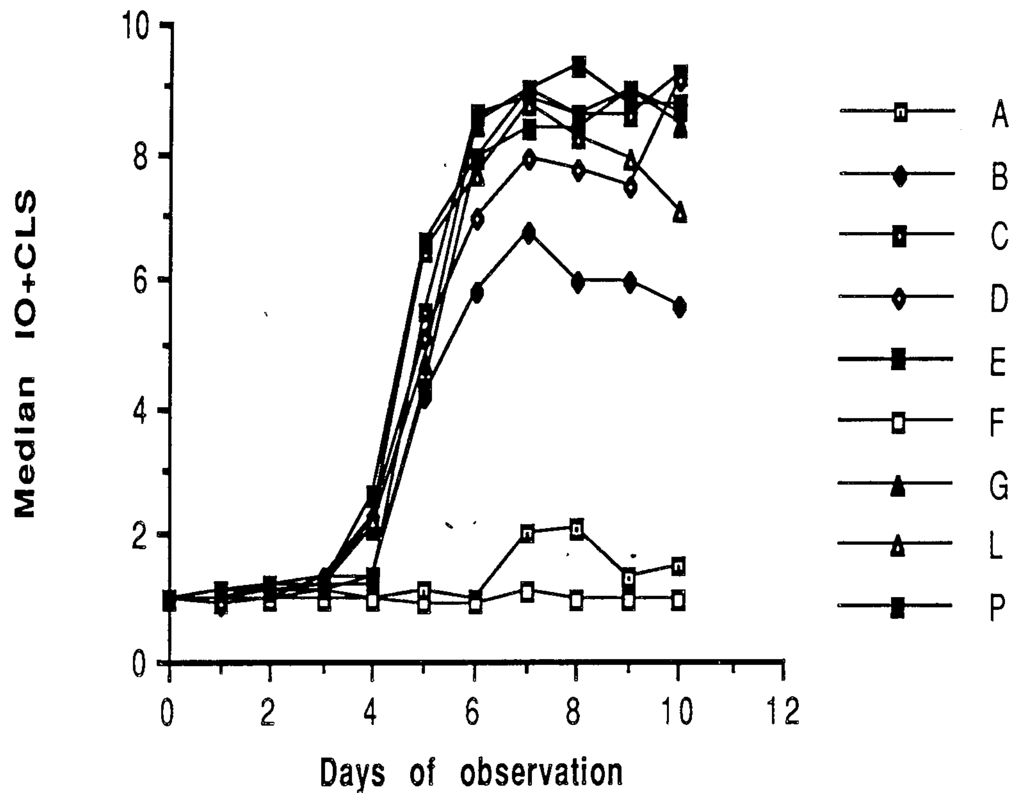
Group A; subcutaneous route 30µg/dose, B; nasal phosphate buffered saline (PBS) 300µg/dose, C; oral PBS 300µg/dose, D; nasal aerosil gel 300µg/dose, E; oral emulsion 300µg/dose, G; infected only, F; control animals, L; vaginal carbopol 940 300µg/dose, P; vaginal PBS 300µg/dose. IO; index of vaginal oedema, CLS; combined lesion score. NS $p>0.05$ (Mann-Whitney U-test).

Fig 4.6 Daily group mean index of vaginal and combined lesion score (IO+CLS) in guinea pigs vaccinated once (subcutaneous) or for 3 consecutive days (mucosal) five and three weeks prior to intravaginal challenge with HSV-2.



Subcutaneous, 30µg/dose (A); nasal phosphate buffered saline (PBS), 300µg/dose (B) or nasal aerosil gel, 300µg/dose (D); oral PBS, 300µg/dose (C) or oral emulsion, 300µg/dose (E); uninfected controls (F) or infected controls (G); vaginal carbopol 940 gel, 300µg/dose (L) or vaginal PBS, 300µg/dose (P).

Fig 4.7 Daily group mean index of vaginal and combined lesion score (IO+CLS) in guinea pigs vaccinated once (subcutaneous) or for 3 consecutive days (mucosal) five and three weeks prior to intravaginal challenge with HSV-2. (The assumed non-infected guinea pigs have been removed before analysis).



Subcutaneous, 30µg/dose (A); nasal phosphate buffered saline (PBS), 300µg/dose (B) or nasal aerosil gel, 300µg/dose (D); oral PBS, 300µg/dose (C) or oral emulsion, 300µg/dose (E); uninfected controls (F) or infected controls (G); vaginal carbopol 940 gel, 300µg/dose (L) or vaginal PBS, 300µg/dose (P).

4.3.2 Immunisation studies using liposomes

4.3.2.1 HSV-specific antibody concentrations in serum

Entrapment values (% +/- S.D. of Skinner HSV subunit vaccine used) for the DSPC liposomes used in this study prepared using dehydration-rehydration procedure (Kirby and Gregoriadis, 1984) were 44% +/- 12 (12 preparations). The Lowry assay used to indirectly measure the % entrapment of Skinner vaccine in the liposomes was found to be reproducible. A known concentration of HSV was used each time and gave a mean of 50μg (+/- 4.5). A more reliable method would have been to use ^{125}I (Kirby and Gregoriadis, 1984). The phospholipid to antigen mass ratio of these liposomes was 20:1. The liposome *in vitro* stability assay involved measurement of the gamma emission from the fractions of the aliquots taken at timed intervals during the incubations this allowed the percentage of BSA (model antigen) released from the DSPC liposomes to be calculated (Chapter 3 section 3.3.1; Figs 3.1 and 3.2). The DSPC liposomes steadily released BSA, until at the end of the 2 hr period they had released about 10% at 4°C and 15% at 37°C of the entrapped BSA. Incubation in the presence of glycine/HCl pH 2.2 and Walpoles acetate pH 4.0 resulted in the steady release of BSA entrapped in DSPC liposomes until at the end of the 2 hr incubation they had released about 20% at both 4°C and 37°C. Therefore, the results suggest that the DSPC liposomes would be stable in the acid environment of the gastrointestinal tract (Rowland and Woodley, 1980;1981).

Titres of IgG antibody to HSV-1 as determined by ELISA in the serum of guinea pigs before and after immunisation with HSV-1 vaccine *via* the s.c, oral and nasal routes using DSPC liposomes, and after challenge with HSV-2 are shown in table 4.9. The post vaccination HSV specific serum IgG antibodies were significantly higher for the vaccinated nasal (10.33+/-0.87) and s.c. (Alhydrogel, <13.01+/-1.51; DSPC liposomes, 11.91+/-1.65) groups compared to the non-vaccinated groups (<9.97+/-0.25; <9.15+/-0.69). However, the antibody levels for the oral group

<7.77+/-0.22) were significantly lower compared to the non-vaccinated and the other vaccinated groups (table 4.9).

The results presented show there was no significant difference in the post vaccination HSV specific serum IgG antibody levels between the s.c. delivery of the Skinner HSV subunit vaccine entrapped in DSPC liposomes or with alhydrogel adjuvant. Early studies on the effect of bilayer fluidity on the immunogenicity of membrane-soluble antigens (Bakouche *et al*, 1987; Kinsky *et al*, 1982) have shown that liposomes made of phospholipids with Tc higher than 37°C, for example DSPC liposomes, provoke strong antibody responses to the antigens.

At 10 days post challenge, detectable levels of HSV specific IgG were present in the sera from all the vaccinated groups except the oral dosed animals and the infected only group of animals.

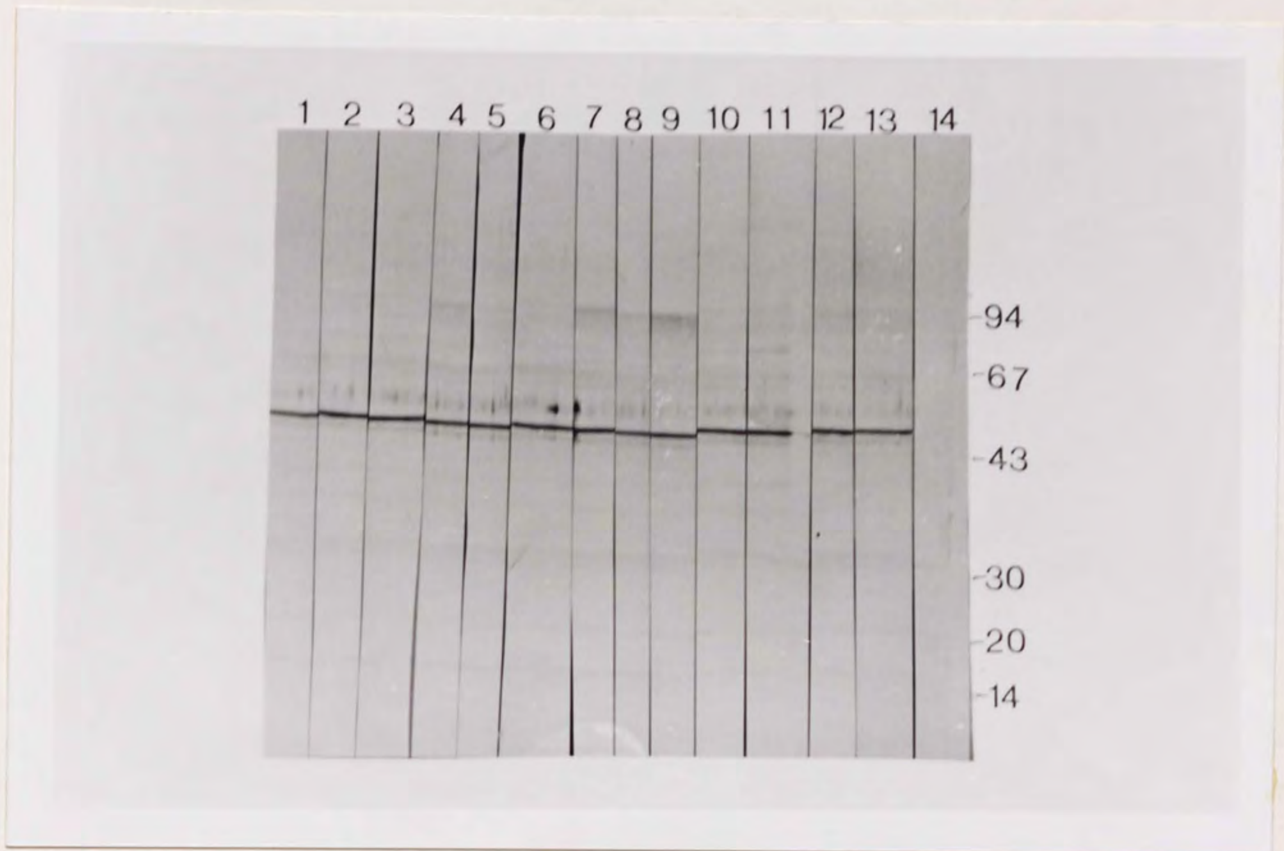
Analysis of the HSV antigen by Western blotting (4.2.9) with post vaccination and post challenge serum collected from guinea pigs immunised with a subunit HSV-1 vaccine using DSPC liposomes, and challenged intravaginally with HSV-2 are shown in fig 4.8. There are bands at 100-120kDa, faint bands at around 90kDa and strong bands between 50 and 58kDa. The post vaccination and post challenge serum recognised proteins of molecular weight 30, 38, 48, 50 and 90kDa determined as descibed in section 4.2.9.1. As already mentioned in section 4.3.1.1 calculating R_f values has an inaccuracy of +/- 5 KDa. More conclusive proof would be achieved by carrying out Western blots using purified glycoproteins fractionated by SDS-PAGE. However, such purified glycoproteins were not available. The Western blots suggest that the serum had been raised against gB, gC, gD and possibly gE (section 4.3.1.1). The fainter bands that were often seen are probably precursors or degradation products of these major glycoproteins. The minor glycoproteins such as gH are very hard to detect.

Table 4.9 Titres of IgG antibody to HSV-1 as determined by ELISA in the sera of guinea pigs before and after vaccination with HSV-1 vaccine and after challenge with HSV-2.

		Geometric mean (log_2) ELISA titre serum IgG antibody		
Route and dose[a]	formulation	pre vaccination	post vaccination	post challenge
oral	DSPC[b]	<8.66(10)[c]	<7.77(9)	8.5(7)
300	liposomes	(0.39)[d]	(0.22)	(0.4)
nasal	DSPC	<9.6(10)	10.33(8)	11.03(8)
300	liposomes	(0.36)	(0.87)	(2.06)
S.C.[e]	DSPC	<8.97(9)	11.91(9)	11.85(7)
30	liposomes	(0.41)	(1.65)	(0.85)
S.C.	alhydrogel	<8.91(10)	<13.01(8)	13.1(7)
30		(0.41)	(1.51)	(2.04)
infected		<9.09(10)	<9.97(9)	<8.37(9)
contols		(0.67)	(0.25)	(1.27)
uninfected		<8.94(10)	<9.15(7)	<8.2(7)
controls		(0.22)	(0.69)	(0.41)

a-µg of protein, b-distearoyl phosphatidylcholine, c-no. of animals, d-standard deviation, e-subcutaneous.

Fig 4.8 Western blotting of HSV-1 antigen prepared as described in text (section 4.2.7.1) was fractionated by SDS-PAGE, transferred to nitocellulose and reacted in Western immunoblots with post vaccination and post challenge serum collected from guinea pigs immunised with a subunit HSV-1 vaccine using gels and emulsions and challenge intravaginally with HSV-2. Protein size markers are indicated in kDa on the left margin.



4.3.2.2 Mucosal antibody responses

No specific sIgA to HSV was detected in the vaccinated or non-vaccinated control animals after intravaginal challenge with HSV-2. The possible reasons have already been discussed in section 4.3.1.2. However, non-specific sIgA antibody values (A_{450}) as determined by ELISA are shown in table 4.10. There was no significant difference between any of the groups except the HSV-2 infected only group have non-specific sIgA levels lower than the uninfected group. Therefore the vaccination schedules did not result in significant mucosal responses compared to the control groups. As already disscused in section 4.3.1.2 an ELISPOT method will be used in future experiments as it may yield more information.

Table 4.10 Vaginal wash non-specific sIgA antibody values (A_{450}) as determined by ELISA after vaccination with HSV-1 subunit vaccine and intravaginal challenge with HSV-2.

Route + dose[a]	formulation	non-specific
oral 300	DSPC[b] liposomes	0.17 (0.06)[c]
nasal 300	DSPC liposomes	0.27 (0.22)
S.C.[d] 30	DSPC liposomes	0.13 (0.06)
S.C. 30	alhydrogel	0.16 (0.08)
uninfected controls		0.22 (0.05)
infected only		0.12 (0.05)

a-µg of protein, b-distearoylphosphatidylcholine, c-standard deviation, d-subcutaneous

4.3.2.3 Clinical scores

Guinea pig infection following HSV-2 intravaginal infection and the relevance of the model has already been described (section 4.3.1.3). The IgG titres for 8 guinea pigs out of a total of 47 studied; 4 from the oral DSPC liposome group and 4 from the infected only group showed no increase in serum IgG titre (individual animals, therefore non-tabulated data) on day 10 post challenge, compared with the prebleed titres and showed no sign of infection.

Therefore, the results have been evaluated in two different ways: firstly, not discounting the 8 guinea pigs, on the assumption that they were protected by mucosal immunity, and secondly, on the assumption that they were not infected. The designs and results of the experiment are summarised in tables 4.11 and 4.12.

Table 4.11 Clinical scores obtained in which guinea pigs immunised with HSV-1 vaccine by three routes were challenged intravaginally with HSV-2.

			median cumulative score				
No. of animals	treatment	µg dose	IO[a]	CLS[b]	IO+CLS	A[c]	B[d]
7	oral DSPC[e] liposomes	300	12.9	0.0	12.9	0	0
8	nasal DSPC liposomes	300	12.2	0.0	12.2	0	0
8	S.C.[f] DSPC liposomes	30	11.9	0.0	11.9	0	0
7	S.C. alhydrogel	30	11.9	0.0	11.9	2	0
7	uninfected controls		11.8	0.0	11.8	0	0
9	infected controls		18.0	9.0	28.0	4	0

a-index of oedema, b-combined lesion score, c-no. of animals with retention, d-no. of animals died earlier then 10 days after challenge, e-distearoylphosphatidylcholine f-subcutaneous.

Table 4.12 Clinical scores obtained in which guinea pigs immunised with HSV-1 vaccine by three routes were challenged intravaginally with HSV-2. (Guinea pigs assumed to be non-infected on the basis of non-tabulated ELISA results are not included).

No. of animals	treatment	μg dose	median cumulative score IO[a]	CLS[b]	IO+CLS	A[c]	B[d]
7	oral DSPC[e] liposomes	300	20.2	14.0	34.2	0	0
8	nasal DSPC liposomes	300	12.2	0.0	12.2	0	0
8	S.C.[f] DSPC liposomes	30	11.9	0.0	11.9	0	0
7	S.C. alhydrogel	30	11.9	0.0	11.9	2	0
7	uninfected controls		11.8	0.0	11.8	0	0
9	infected controls		24.3	21.0	44.2	4	0

a-index of oedema, b-combined lesion score, c-no. of animals with retention, d-no. of animals died earlier then 10 days after challenge, e-distearoylphosphatidylcholine, f-subcutaneous.

Table 4.13 Satistical analysis of all possible pairwise comparisons between groups within the immunisation studies using liposomes.

	p value for comparison*	
Score	1	2
CLS	N/S	N/S
IO	N/S	<0.05
CLS+IO	N/S	<0.05

* pairwise comparisons between the following groups

1-groups-AB/AD/AE/BC/BD/BF/CD/CF/DF

2-groups-AC/AF/BE/CE/DE/EF

Group A; oral DSPC liposomes 300μg/dose, B; nasal DSPC liposomes 300μg/dose, C; subcutaneous DSPC liposomes 30μg/dose, D; subcutaneous alhydrogel 30μg/dose, E; infected only, F; control animals. IO; index of vaginal oedema, CLS; combined lesion score. N/S p>0.05 (Mann-Whitney U-test).

Table 4.14 Statistical analysis of all possible pairwise comparisons between groups within the immunisation studies using liposomes. (Guinea pigs assumed to be non-infected on the basis of non-tabulated ELISA results are not included).

	p value for comparison*		
Score	1	2	3
CLS	N/S	<0.05	<0.05
IO	N/S	<0.05	N/S
CLS+IO	N/S	<0.05	N/S

* pairwise comparisons between the following groups

1-groups-AE/BC/BD/BF/CD/CF/DF

2-groups-AB/AC/AF/BE/CE/DE/EF

3-groups-AD

Group A; oral DSPC liposomes 300μg/dose, B; nasal DSPC liposomes 300μg/dose, C; subcutaneous DSPC liposomes 30μg/dose, D; subcutaneous alhydrogel 30μg/dose, E; infected only, F; control animals. IO; index of vaginal oedema, CLS; combined lesion score. N/S p>0.05 (Mann-Whitney U-test).

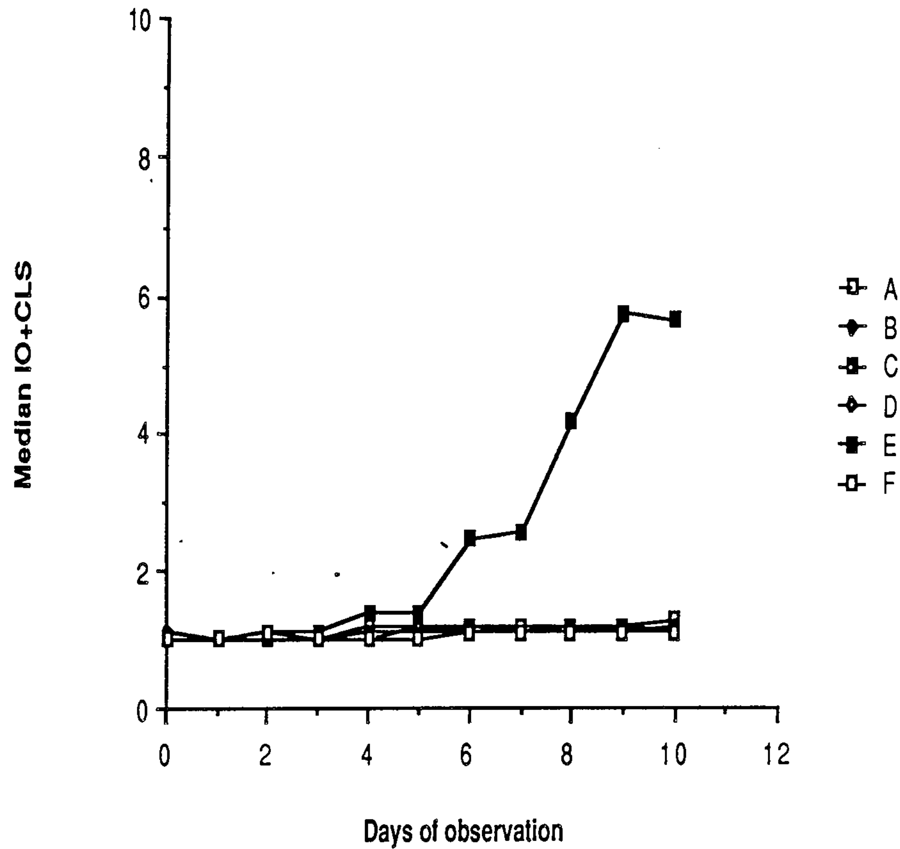
For both assumptions the guinea pigs vaccinated *via* the nasal and subcutaneous routes followed by challenge with HSV-2 at the genital surface were not significantly different with respect to combined IO+CLS (tables 4.13 and 4.14) when compared to the uninfected group. In addition, they showed a significant amelioration of disease when compared to the infected-only group.

However, the orally-vaccinated and infected only groups were significantly different for combined IO+CLS compared to the uninfected group. The orally vaccinated group gave no significant amelioration of the disease when compared to the infected only group.

The results are presented graphically in figs 4.9 and 4.10, in which IO + CLS is plotted against the day of observation. In both cases, reductions in the severity of the illness were produced by the subcutaneous and nasal treatments.

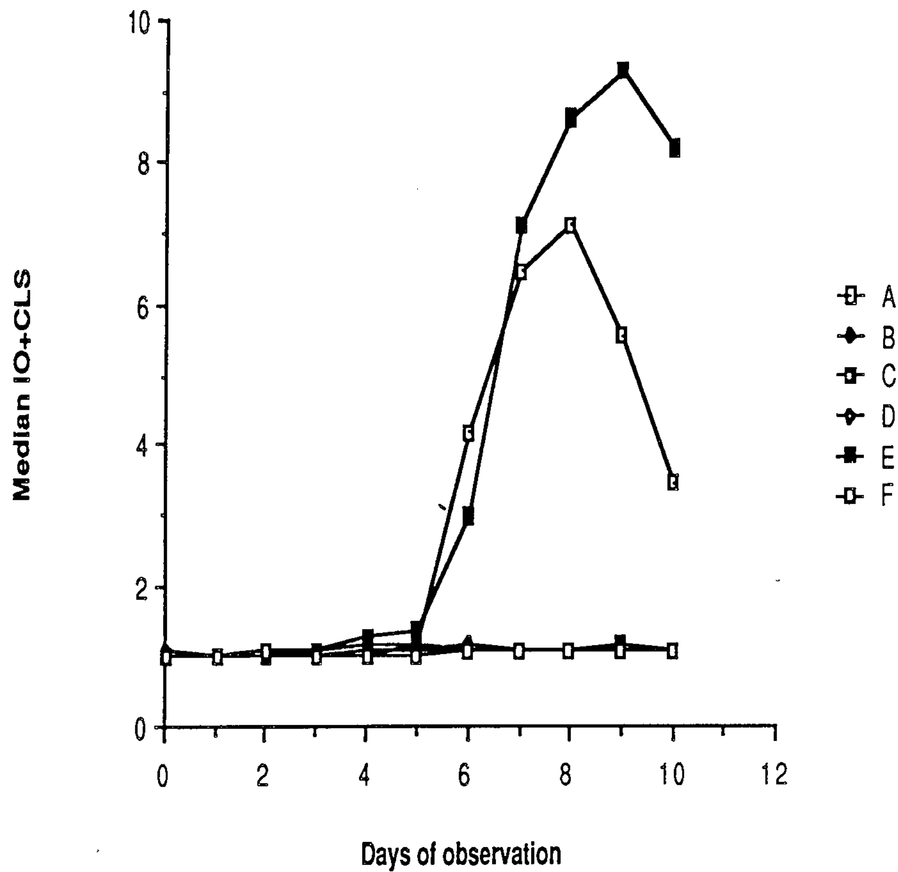
Therefore, nasal delivery of Skinner HSV subunit vaccine entrapped in DSPC liposomes resulted in significant reduction of disease after intravaginal challenge with HSV-2 compared to the infected only group. The nasal route has been used for several vaccines. For example, Robert *et al* (1979) have shown non-specific protection against infectious aerosols of influensa A virus in Swiss mice after vaccination by aerosols of bacterial ribosomes, together with membranal glycoproteins extracted from *Klebsiella pneumoniae* as the adjuvant. Fontages *et al* (1980) have shown significant protection in mice using ribosomal vaccines of bacteria of respiratory origin after vaccination *via* the subcutaneous or nasal (aerosol) route. In the previous study using gels and emulsions to deliver HSV subunit vaccine, the use of aerosil gel for nasal delivery did not result in a significant reduction of disease compared to the infected only group. However, for a direct comparison, these studies should be repeated together. The results will be discussed in more detail in the concluding discussion.

Fig 4.9 Daily group mean index of vagina and combined lesion score (IO+CLS) in guinea pigs vaccinated once (subcutaneous) or for 3 consecutive days (mucosal) five and three weeks prior to intravaginal challenge with HSV-2.



Oral distearoyl phosphatidylcholine (DSPC), 300ug/dose (A); nasal DSPC liposomes, 300ug/dose (B); subcutaneous DSPC liposomes, 30ug/dose (C); subcutaneous alhydrogel, 30ug/dose (D); infected only controls (E); uninfected controls (F).

Fig 4.10 Daily group mean index of vagina and combined lesion score (IO+CLS) in guinea pigs vaccinated once (subcutaneous) or for 3 consecutive days (mucosal) five and three weeks prior to intravaginal challenge with HSV-2. (The assumed non infected guinea pigs have been removed before analysis).



Oral distearoylphosphattidylcholine (DSPC), 300µg/dose (A); nasal DSPC liposomes 300µg/dose (C); subcutaneous alhydrogel, 30ug/dose (D); infected only controls (E); uninfected controls (F).

CHAPTER 5

CONSTRUCTION OF A gD-Lac z FUSION PROTEIN EXPRESSED IN *SALMONELLA TYPHIMURIUM* HWSH *AROA* MUTANTS

5.1 INTRODUCTION

Salmonella strains carrying non-reverting deletions in the *aro*A gene, making mutants dependent on certain aromatic compounds for growth, have been shown to be attenuated and effective as vaccines (for example in mice, Hoiseth and Stocker, 1981). In addition, there is evidence that attenuated *aro*A Salmonellae have potential as carriers for delivering heterologous antigens to the immune system (Maskell *et al*, 1987), resulting in stimulation of the systemic and mucosal immune system. Glycoprotein D (gD) is a virion envelope component of HSV types 1 and 2 which stimulates the production of high titres of virus-neutralising antibody and has been shown to protect animals from HSV challenge (Lasky *et al*, 1984).

This section describes experiments aimed at inserting the gene coding for gD of HSV-1 into *Salmonella typhimurium* HWSH *aro*A mutants and investigating expression.

5.2 MATERIALS AND METHODS

5.2.1 Materials

5.2.2 Media

All media were prepared as described by Sambrook *et al* (1989). L-broth: 50g Bactotryptone (Difco), 25g Bactoyeast extract, 25g NaCl made up to 1 litre with distilled water. L-agar: 10g, Bactotryptone, 5g Bactoyeast extract, 5g NaCl, 15g Difco agar made up to 1 litre with distilled water. B-agar: 4g Bactopeptone (Becton Dickinson), 3.2g NaCl, 6g Difco agar made up to 400 ml with distilled water. B-top agar: 0.4g Bactopeptone, 0.32g NaCl, 0.24g Difco agar made up to 40 ml with distilled water.

Soft-top agar: 0.8g Bactotryptone, 0.64g NaCl, 0.64g BBL agar made up to 80 ml with distilled water. 10x M9 salts: 60g Na_2HPO_4, 30g KH_2PO_4, 10g NH_4Cl, 5g NaCl made up to 1 litre with distilled water. Minimal media: 40 ml 10x M9 salts, Difco agar (6g in 360 ml of distilled water autoclaved separately), 2 ml 40 % glucose, 400µl 1M $MgSO_4$/Cl, 200µl Vitamin B1, 400µl 100mM $CaCl_2$.

5.2.3 Bacterial strains and plasmids

Escherichia coli strains JM101 *sup*E *thi* Δ(*lac-pro*AB)F' (*tra*D36 *pro*AB+ *lacI*q *lacZ* ΔM15) (Messing, 1979) and LE392 *sup*E44*sup* F58*hsd* R514*gal* K2*gal* T22 *met*B1 trpR55 *lacY*1 (Murray *et al*, 1977) were used as hosts for recombinant clones in the vectors pUC19 and M13mp18.

Plasmid pMW1 was constructed (in the Microbiology Department, Leicester University) by cloning the 1.7kb *Eco*RI fragment encoding the *lacI*q gene from pMC9 (Calos *et al*, 1983) into pACYC184 (Chang and Cohen, 1978).

M13mp18 and M13mp19 were used as vectors for preparing DNA templates for sequence analysis (Yanisch-Perron *et al*, 1985).

S. typhimurium strain SL3261, an *aro*A *his* derivative of strain

SL1344, was kindly provided by Dr Bruce A.D. Stocker (Stanford University, Stanford, CA) and has been described (Hoiseth and Stocker, 1981; Smith *et al*, 1984). In addition, *S. typhimurium* HSWH *aro*A was kindly provided by Dr G. Dougan (Wellcome Biotech, Beckenham, Kent) and has been described by O'Callaghan *et al* (1988a;1990). Minimal media with or without 10μg ml^{-1} each of paraminobenzoic acid (pABA) and 2,3-dihydroxybenzoic acid (Sigma) were used to confirm the strains were auxotrophic. *S. typhimurium* strain LB5010 is a *gal*E^- derivative of Bullas' strain LB5000, which is r^-m^+ for all three restriction-modification systems of *S. typhimurium* (Bullas and Ryo, 1983).

E. coli JM101 was maintained on minimal media. *E. coli* LE392 and the *Salmonella* strains were maintained on L agar.

5.3 Methods

5.3.1 DNA Manipulation

5.3.1.1 Restriction enzyme digests

These were carried out at 37°C, usually for 1-2 hrs or until complete digestion had occurred according to manufacturers' instructions (Bethesda Research Laboratories or Pharmacia LKB Biotechnology) and as described by Sambrook *et al* (1990). A typical digestion was as follows:-

DNA xμl, Nanopure water yμl (0.2-1μg of DNA with sufficient water to give a final volume of 18μl), Reaction buffer (x10) 2μl, and Restriction enzyme 1μl (1-2 units). For digestion of larger amounts of DNA, the reaction should be scaled up. Digestion was checked by running a small proportion on an agarose gel (see section 5.3.2).

5.3.1.2 Ligation Reaction

Bacteriophage T4 DNA ligase was used according to the manufacturers'

instructions (Pharmacia LKB Biotechnology) and as described by Sambrook *et al* (1990). For example, 10µl DNA (the concentration of vector and fragment to be subcloned will depend on the type of ends created by the restriction digests carried out), 1µl ligation buffer (x10) and 1µl ligase, and then left overnight in a 14°C water bath in the cold room.

The ligation buffer was freshly prepared as follows:-

10µl 1M Tris (pH 7.5), 2µl 1M $MgCl_2$, 2µl 0.1M ATP (Adenosine 5' triphosphate) and 6µl of nanopure water.

5.3.1.3 Rapid purification of DNA

Rapid purification of DNA preparations was performed by the method of Birnboim and Doly (1979). A single colony containing the required recombinant plasmid was transferred into 10 ml of L broth containing the appropriate antibiotic. The culture was incubated overnight at 37°C with vigorous shaking (300 cycles min^{-1} on a rotary shaker). From this culture, 1.5 ml was centrifuged at 12,000 xg for 1 min at 4°C in a large sterile eppendorf and the supernatant removed. The pellet was resuspended in 100µl of ice cold 40mM glucose, 25mM Tris.Cl (pH 8.0), 10mM EDTA, vortexed gently and left on ice for 30 min. Then, 200µl of fresh 0.2N NaOH, 1% sodium dodecyl sulphate (SDS) was added, mixed, left on ice for 5 min and 150µl of ice cold 3M Potassium acetate, pH 4.8 was added, mixed and left on ice for 5 min. The mixture was centrifuged at 12,000 xg for 5 min at 4°C and the supernatant transferred to a fresh sterile eppendorf. An equal volume of phenol/chloroform was added, the mixture was vortexed and centrifuged at 12,000 xg for 2 min at 4°C. Again, the supernatant was transferred to a fresh sterile large eppendorf and 2 volumes of ethanol at room temperature was added, vortexed and left to stand at room temperature for at least 2 min. The DNA was then centrifuged at 12,000 xg for 5 min at 4°C and the supernatant carefully removed. The DNA was washed with 70% ethanol, vortexed

briefly and centrifuged at 12,000 xg for 5 min at 4°C. The supernatant was removed and the pellet dried in a vacuum desiccator for 2 min. Finally 50µl of 10mM Tris: 1mM EDTA, pH 8.0 (TE) containing DNase free pancreatic RNase (20µg ml^{-1}) was added to the DNA pellet, vortexed briefly and stored at -20°C.

5.3.1.4 Caesium chloride preparation of plasmid DNA

Isolation of plasmid DNA by the caesium chloride method was carried out as described by Clewell and Helinski (1969). A 10 ml culture of the bacterial strain carrying the plasmid of interest was grown to late log phase in L broth containing the appropriate antibiotics. This culture was used to inoculate 400 ml of L broth containing the appropriate antibiotic in a 2 litre flask. The culture was incubated overnight with vigorous shaking (300 cycles min^{-1} on a rotary shaker), centrifuged at 5,000 xg for 10 min at 4°C and the pellet resuspended in 5 ml of ice cold 40 mM glucose, 25mM Tris.Cl (pH 8.0), 10mM EDTA. After 30 min on ice 10 ml 0.2N NaOH, 1% SDS was added, mixed gently and left on ice for 10 min. Next 7.5 ml of ice cold was added, mixed gently, left on ice for 10 min, and centrifuged at 20,000 xg for 30 min at 4°C. The supernatant was transferred to 30 ml corex tubes and 12 ml isopropanol added, mixed well and left at room temperature for 15 min. After centrifugation at 6,000 xg for 30 min at 20°C, the isopropanol was removed and the pellet resuspended in 8 ml of distilled water, transferred to a plastic universal and distilled water added to make a final volume of 17 ml. Next 17g of caesium chloride was added, dissolved and transferred to an ultracentrifuge tube containing 1 ml of ethidium bromide (10mg ml^{-1}). The ultracentrifuge tubes were then filled to the top with paraffin oil, balanced carefully and then centrifuged at 40,000 xg for 20 hrs at 20°C.

The tubes were removed and the DNA visualised under ultra-violet light in a dark room. The DNA band was collected through the side of the tube with a hypodermic needle. The ethidium bromide was removed by repeatedly mixing with an

equal volume of caesium chloride-saturated isopropanol until the solution was colourless. The DNA solution was dialysed against distilled water for 3-4 hrs, and stored at -20°C.

5.3.1.5 Polymerase chain reaction (PCR)

Polymerase chain reaction (Saiki *et al*, 1988) was carried out using GeneAmp ^{TM} DNA amplification reagent kit with Ampli*Taq*^{TM} Recombinant *Taq* DNA polymerase (Perkin Elmer Cetus) according to the manufacturers' instructions.

Preparation of stock solutions

Daily working solutions of a mix of dNTP's were prepared to a final concentration of 1.25 mM per nucleotide triphosphate. A 10-fold dilution of the control template in 10mM Tris-HCl pH 8.0 (room temperature), 1mM EDTA, 10mM NaCl was also prepared.

Reaction Mix component	addition order	volume	final concentration
Double distilled water, sterile	a	53.5µl	
(10x) Reaction buffer	b	10.0µl	(1x)
dNTP's Mix, 1.25mM each dNTP	c	10.0µl	200µM each dNTP
Contol Primer 1, 20µM	d	5.0µl	1.0µM
Contol Primer 2, 20µM	e	5.0µl	1.0µM
Control template, 1:10 dilution	f	10.0µl	1 ng/assay
Taq DNA Polymerase	g	0.5µl	2.5 units/assay

Reaction mix for amplifying other DNA segments

The conditions described above were also a useful starting point for amplification of different DNA targets using primers designed by the user. However, improvements were made by varying magnesium, primer and amplification conditions. The effects of these variations were monitored by examining the intensity and distribution of product samples run on agarose ethidium bromide gels.

The DNA segment to be amplified from the template can be up to 10kb long, although 50 to 5000 bases are more typical and easier to amplify. The single-strand DNA primers should be 15 to 30 bases in length and the %G+C of primers should be near 50%, to maximise specificity. High levels of primer can lead to amplification of non-target segments. Primer sequences should not complement within themselves or to each other, particularly at the 3' ends.

The most appropriate magnesium concentration was determined empirically, using a range of concentrations for each primer set. The concentrations of dNTP's in the reaction mix must be balanced; if the concentration of any one is significantly different from the rest, the *Taq* DNA Polymerase tends to misincorporate, slow down and terminate prematurely.

Optimal performance of the PCR technique was influenced by choice of temperature, time at temperature and length of time between temperatures for each step in the cycle. The length of the target sequence will affect the required extension time. Typically *Taq* DNA polymerase has an extension rate of 2,000 to 4,000 bases per min at 70-80°C. Polymerisation rates are significant even below 55°C and, with some templates, up to 85°C. As the amount of DNA increases in later cycles, the number of *Taq* DNA Polymerase molecules may become limiting for the extension time alloted. Lengthening the extension times in later cycles may be needed to complete polymerisation of the substrate cycle. Higher annealing temperatures (45-60°C or more) generally result in much more specific product; the optimum can be determined empirically by testing at 5°C or smaller increments until the maximum in specificity is reached. High G+C content DNA may need very high annealing (>60°C) and melting temperatures or the use of 7-deaza-2'-deoxy-GTP mixed with dGTP, to overcome secondary structure. The half life of *Taq* DNA Polymerase (35 min at 95°C) suggests 95°C as the maximum practical melting temperature. Low concentrations of target DNA may require up to 35 or more cycles to produce sufficient product for analysis.

5.3.2 Agarose gels

Agarose gels were prepared according to Sambrook *et al* (1990). Samples were mixed with 10x loading buffer (0.25% bromophenol blue, 25% Ficoll (type 400) in water, RNase final concentraion 10µg ml^{-1}) prior to loading.

5.3.3 Transformation procedures

5.3.3.1 Transformation using pUC

Competent cells were prepared as described below. Transformation were performed in large sterile eppendorf tubes according to Sambrook *et al* (1989), and left on ice for 1 hr:-

1) 100µl competent cells (cell control)

2) 100µl competent cells and DNA (ligation mixture)

3) 100µl competent cells and plasmid (competence control)

After this time the cells were put into a water bath at 42°C for 3 min (i.e. a temperature shock) and 500µl of L broth was added and left for at least 1 hr at 37°C.

The transformation was then plated out on L agar plates containing the appropriate antibiotics, using 100µl of transformation mixture for each plate and incubated overnight at 37°C.

5.3.3.2 Preparation of competent cells using calcium chloride

The following procedure is a variation of that of Cohen *et al* (1972). A single colony was picked from a plate freshly grown for 16-20 hrs at 37°C and transferred into 10 ml of L broth. The culture was incubated for approximately 3 hrs at 37°C with vigorous shaking until mid log phase. The culture was centrifuged at 5,000 xg for 10 min at 4°C. The supernatant poured off and the pellet resuspended in 4 ml of ice cold 10mM NaCl and centrifuged at 4,000 xg for 5 min at 4°C. The supernatant was removed, the pellet gently resuspended in 4ml of ice cold 100mM

$CaCl_2$, left on ice for 30 min and then centrifuged at 3,000 xg for 5 min at 4°C. The supernatant was poured off and gently resuspended in 1 ml of ice cold 100mM $CaCl_2$ and left on ice until required (up to 24 hrs).

5.3.3.3 Transformation using the Rubidium Chloride Method

A single colony, of the appropriate strain from a freshly streaked plate, was transferred into 10 ml of L broth and grown to mid log phase by vigorous shaking (300 cycles min^{-1} on a rotary shaker). To a large sterile eppendorf, 1.4 ml of the culture was added and centrifuged at 12,000 xg for 30 sec at room temperature. The supernatant was removed and the pellet resuspended in 0.5 ml 10mM MOPS pH 7.0, 10mM rubidium chloride. The cells were pelleted by centrifugation at 12,000 xg for 15 sec at room temperature, the supernatant removed and the pellet gently resuspended in 0.5 ml 100mM MOPS pH 6.5, 10mM rubidium chloride, 50mM calcium chloride. The cells were held on ice for 90 min and pelleted by centrifugation at 12,000 xg for 10 sec at room temperature. The cells were gently resuspended in 0.15 ml 100mM MOPS pH 6.5, 10 mM rubidium chloride, 50mM calcium chloride and 3µl of dimethyl-sulphoxide was added followed by the DNA; mixed, left on ice for 1 hr and heat shocked at 55°C for 30 sec. To each tube 1 ml of L broth was added, mixed and incubated at 37°C for 1 hr. The cells were plated out as described in section 5.3.3.1.

5.3.3.4 Transformation with M13

Transformation with M13 was carried out according to Sambrook *et al* (1989). Competent cells were prepared as described earlier (5.3.3.2). Plating bacteria was prepared as follows: a master culture of a bacterial strain carrying an F' episome (eg JM101) was used to streak a minimal (M9) agar plate and incubated for 24-36 hrs at 37°C. A single colony was used to inoculate 10 ml of L broth and

incubated for approximately 5 hrs at 37°C with vigorous shaking. Transformation was carried out as follows in large sterile eppendorf tubes and left on ice for 1 hr:-

1) 100µl competent cells (cell control)

2) 100µl competent cells and DNA-ligation mixture

3) 100µl competent cells and plasmid (competence control)

After 1 hr the tubes were put into a 42°C water bath for 3 min and then plated out as described below.

Sterile tubes containing 3 ml of melted top agar were labelled and kept at 42°C in a water bath. The following were added to each tube in turn:-

1) 200µl of plating bacteria

2) 20µl of 100mM IPTG

3)50µl of 2% X-gal (5-Bromo-4-chloro-3-indoly-ß-D-parano-galactoside)

4) All the transformed cells from one eppendorf

The contents were vortexed and poured onto a B agar plate, left at room temperature for 15 min to allow the top agar to set, and incubated at 37°C overnight.

5.3.4 Identification of bacterial colonies that contain recombinant plasmids

5.3.4.1 Restriction analysis of small-scale preparations of plasmid DNA

Independently transformed bacterial colonies were picked and grown in small-scale cultures with the appropriate antibiotics present. Plasmid DNAs, isolated from each culture by rapid DNA purification as described earlier (5.3.1.3), were analysed by digestion with restriction ensymes and gel electrophoresis. This procedure was the method of choice when there was a high chance of finding the desired recombinant within a small number of randomly chosen transformed colonies.

5.3.4.2 Colony blot hybridisation

Colonies were transferred to nitrocellulose filters (NC) and hybridisation was performed by established methods (Southern, 1975). A radiolabelled probe was generated by labelling the fragment directly in a molten agarose gel slice by extending random hexadeoxynucleotide primers using the Klenow fragment of DNA polymerase in the presence of (^{32}P) dCTP.

The NC was removed after overnight incubation and placed on top of approximately 8 Whatman filter papers soaked in 0.5M NaOH, 1.5M NaCl for 5 min in a square petri dish. The filters were transferred to a second square petri dish containing Whatman filter papers soaked in 1M Tris HCl pH 8.0, 1.5M NaCl for 5 min and then placed on dry Whatman filter paper for 15 min. The NC was then UV irradiated for 5 min and washed with polyallamer wool soaked in 5x saline sodium citrate (SSC) to remove all the bacterial remains leaving the DNA fixed to the NC.

Prehybridisation was carried out by washing the filters for 2 hrs at 65°C in the following:-

3x SSC, 5x Denhardts, 200μg ml^{-1} salmon sperm DNA (added just before use), 0.1% sodium dodecyl sulphate (SDS) and 6% polyethylene glycol (PEG) 8000 or 6000.

Hybridisation was carried out at 65°C in 20 ml of hybridisation solution (3x SSC, 2x Denhardts solution, 0.1% SDS and 6% PEG 8000 or 6000) mixed with the probe (boiled 5 min) and 200μg ml^{-1} salmon DNA immediately before use. The filters were transferred to the hybridisation chamber with the minimum of residual liquid. The chamber was sealed and incubated at 65°C overnight in a gently shaking water bath.

After this time the filters were removed separately to a plastic sandwich box containing wash 1 (2x SSC, 0.1% SDS) prewarmed to 65°C and then immediately transferred to a second box containing the same wash solution and incubated at 65°C in a shaking water bath for 20 min. The filters were then removed

to a fresh wash 1 solution and shaken at 65°C for 20 min and this last step was repeated once more. They were then transferred to 250 ml of wash 2 (0.5x SSC, 0.1% SDS) and shaken at 65°C for 20 min and this step was repeated once more. The filters were dried, wrapped in saran wrap and autoradiographed overnight at -70°C in a cassette containing intensifying screens. The film was developed and aligned with the filters and the positive colonies identified.

20x SSC 175.3g NaCl, 88.2g sodium citrate made up to 1 l with water

50x Denhardts 5g Ficoll, 5g polyvinyl pyrrolidine, 5g BSA (Pentax fraction V) made up to 500 ml with water.

5.3.5 Template preparation M13

DNA templates were prepared according to the method described by Sambrook *et al* (1989). An overnight bacterial culture of the appropriate strain was grown in L broth with vigorous shaking (300 cycles min-1 on a rotary shaker) and used to inoculate 5 ml of L broth. Sterile toothpicks were used to pick the plaques from the transformation, dropped into the L broth and incubated shaking (300 cycles min-1 on a rotary shaker) at 37°C for 6 hrs. The culture was then poured into sterile large eppendorfs, centrifuged at 12,000 xg for 3 min at 4°C, 800μl of supernatant was transferred into sterile eppendorf tubes and stored at 4°C until DNA rapid purification had been carried out on the pellet. Restriction digests were used to analyse the recombinant plasmids.

To the 800μl of supernatant, 200μl of 2.5 M NaCl 20% polyethlene glycol (PEG) 6000 was added, left at room temperature for 30 min and centrifuged at 12,000 xg for 5 min at 4°C. The supernatant was removed and the pellet recentrifuged for 2 min at 12,000 xg and the remaining supernatant removed. The pellet was resuspended in 300μl of 1.1M sodium acetate pH 7.0, followed by the addition of 300μl of Tris saturated phenol:chloroform:isoamyl alcohol (50:50:1). The mixture

was vortexed for 10 sec, left for 5 min at room temperature and centrifuged at 12,000g for 1 min at $4^{o}C$. Aliquots of 60µl of the top aqueous layer were removed to eppendorf tubes, 60µl of chloroform:isoamyl alcohol 50:1 added, mixed and left at $-20^{o}C$ overnight. The template was pelleted by centrifugation at 12,000 xg for 10 min and the supernatant removed. Finally, the pellet was dried, resuspended in 15µl of 10mM Tris: 1mM EDTA, pH 8.0 (TE) and stored at $-20^{o}C$.

5.3.6 Nucleotide sequencing analysis

Single stranded M13 DNA templates were sequenced by the dideoxy chain termination method (Sanger *et al*, 1977).

5.3.6.1 Materials for sequencing

Sequenase Buffer (5x concentrate)
200mM Tris.HCl pH 7.5, 100mM $MgCl_2$,

Control DNA M13mp18-0.2µg/µl

Primer (Universal)-0.5pmol/µl
5'-GTAAAACGACGGCCAGT-3'

Dithiothreitol (DTT)-0.1M

Labelling Mix (dGTP) 5x Concentrate
7.5µM dGTP
7.5µM dCTP
7.5µM dTTP

ddG Termination Mix
80µM dGTP, 80µM dATP, 80µM dCTP, 80µM dTTP
8µM ddGTP, 50mM NaCl

ddA Termination Mix
80µM dGTP, 80µM dATP, 80µM dCTP, 80µM dTTP
8µM ddATP, 50µM NaCl

ddT Termination Mix
80µM dGTP, 80µM dATP, 80µM dCTP, 80µM dTTP
8µM ddTTP, 50µM NaCl

ddC Termination Mix

80μM dGTP, 80μM dATP, 80μM dCTP, 80μM dTTP
8μM ddCTP, 50μM NaCl

Stop solution

95% Formamide
20mM EDTA
0.05% Bromophenol blue
0.05% Xylene Cyanol FF

5.3.6.2 Sequencing reactions

Annealing template and primer

For each set of reactions the following was made up:-Primer 1μl, sequencing buffer 2μl, DNA template (approximately 1-2μg) 7μl. The total volume should be 10μl; if a smaller volume of DNA solution was used, the balance was made up with distilled water. The tubes were heated to 65°C for 2 min and allowed to cool slowly to room temperature in the heating block. Once the temperature was below 35°C, annealing was complete.

Labelling reaction

For standard reactions (reading sequences up to 500 or so bases from the primer), the Labelling Mix (dGTP) was diluted 5-fold with distilled water. For sequencing within 30 bases of the primer, dilution should be about 15-fold and the amount of template DNA must be greater than 0.5pmol. Insufficient DNA (or primer) will reduce the labelling of the first few nucleotides from the primer. The Sequenase enzyme was diluted 1:8 in ice-cold 10mM Tris: 1mM EDTA, pH 8.0 (TE).

To the annealed template-primer the following was added:-1μl DTT 0.1M, 2μl diluted labeling mix, 0.5μl ^{35}S dATP and 2μl of diluted Sequenase, mixed and incubated for 5-10 min at room temperature.

Termination reactions.

For each reaction 4 tubes were labelled G, A, T and C; 2.5µl of each ddNTP termination mix was transferred to the appropriately labelled tubes and pre-warmed at 37°C for at least 1 min. When the labelling incubation was complete, 3.5µl was transferred to each labelled ddNTP tube, mixed, centrifuged and incubated for 5 min at 37°C. Next, 4µl of stop solution was added to each of the termination reactions, mixed and stored on ice until ready to load on the sequencing gel. The samples labelled with ^{35}S can be stored at -20°C for 1 week with little degradation. Before loading the samples onto the gel, the tubes were heated at 75°C for 2 min.

5.3.6.3 Denaturing gel electrophoresis

Two glass plates were carefully cleaned and the siliconised side of 1 plate was placed adjacent to the non-siliconised side of the second plate. Spacers (0.4mm) were inserted in between the long sides of the plates and then tape was used to hold the plates in position.

Solutions

x10 Tris:Borate:EDTA (TBE)

121.1g Tris base
55.0g Boric acid
7.4g EDTA

made up to 1 litre with distilled water, should be pH 8.3

40% acrylamide solution

38g acrylamide
2g bisacrylamide

made up to 100 ml with distilled water, 2.5g of amberlite was added to deionise, stirred for 10 min and filtered. Stored in the dark at 4°C.

x0.5 TBE

430g urea
50 ml x10 TBE
150 ml 40% acrylamide solution

made up to 1 litre with distilled water and stored at 4°C in the dark.

x5 TBE acrylamide urea mixture

430g urea
250 ml x10 TBE
150 ml 40% acrylamide solution
50g sucrose
50 mg bromophenol blue

made up to 1 litre with distilled water and stored at $4^{o}C$ in the dark.

10% ammonium persulphate solution(APS)

0.2g in 2 ml distilled water, made up fresh each time.

The following were aliquotted into 2 beakers:-

beaker 1-7 ml 2.5 or 5x TBE acrylamide, 45µl 10% APS, 2.5µl TEMED

beaker 2-40 ml 0.5x TBE acrylamide, 180µl 10% APS, 7.5µl TEMED

The gradient gel was poured by taking up 10 ml of the 0.5x mix and then 7 ml of the 2.5 or 5x mix. A few air bubbles were introduced into the pipette to generate a rough gradient. The gradient gel mixture was carefully poured down one side of the plates and then the remainder of the 0.5x mix was used to fill the plates. A sharkstooth comb was placed upside down at the top of the gel and clamped in position. The gel was left to polymerise for at least 1 hr.

The gel was pre-run (1x TBE poured into the bottom reservoir and 0.5x TBE into the top reservoir) for 30 min at 40 watts (power limiting) and then the samples were loaded into the appropriately labelled wells. The gel was run at 40 watts for approximately 3 hrs for a normal run or approximately 6 hrs for an extended run, depending on the sequence to be read.

The gel was dried at $80^{o}C$ in a gel drier for at least 1 hr, checked for radioactivity and autoradiographed overnight at room temperature.

5.3.7 Expression of cloned DNA

5.3.7.1 Colony Immunoblot

Colony immunoblotting was carried out as previously described (Sambrook *et al,* 1989). Bacterial colonies that had been positively identified to

contain the correct recombinant plasmid, by colony blot hybridisation and restriction digests, were streaked onto hybond C templates on L agar containing the appropriate antibiotics and isopropyl-ß-D-thiogalactopyranoside (IPTG) if necessary. After overnight incubation at 37°C the colonies were lysed in chloroform vapour for 1-2 hrs and then allowed to air dry. The filters were washed with TN (50mM Tris, 0.9% NaCl, pH 7.5) containing 0.02% sodium azide for 5 min; followed by a further 3x 5 min washes with TN only. The filters were incubated for 1 hr at room temperature with TN containing 3% bovine serum albumin (BSA), washed for 5 min with TN and incubated for 2 hrs at room temperature or overnight at 4°C with either a monoclonal or polyclonal antibody raised against the foreign DNA ie gD (P1M12; raised by P. Ridgeway in the Division of Biologics, Porton Down) diluted 1:500 in TN containing 3% BSA.

The filters were washed 4x 5min with TN and the relevant conjugate added to detect any bound antibody to the gD for 2 hrs at room temperature or overnight at 4°C diluted in TN. After 4x 5 min washes with TN a peroxidase substrate was added until colour developed for the positive controls. The filters were air dried and the results recorded.

5.3.7.2 Enzyme Linked Immunosorbent Assay using whole cells

Whole cell enzyme linked immunosorbent assay (ELISA) was modified from a method by Borowski *et al* (1984). Bacteria containing pJB1 were grown in L broth containing ampicillin and were induced for the synthesis of gD by the addition of IPTG, where necessary and grown to log phase. The cells were centrifuged, resuspended in coating buffer and the following dilutions made 1:250, 1:500, 1:1000, and 1:2000, 100µl was added to each well and incubated overnight at 4°C. The wells were washed 3 times with phosphate buffered saline containing 0.05% Tween 20 (PBST) and then blocked with PBST containing 3% BSA; 100µl was added to each well and

incubated at 37°C for 30 min.

After washing the plate 3 times with PBST 100µl of antibody was added to each well diluted in PBS containing 10% foetal calf serum and incubated for 2 hrs at 37°C. The plates were again washed 3 times with PBST and the correct conjugate added to detect any bound antibody to the gD, diluted in PBS containing 10% foetal calf serum. After 2 hrs incubation at 37°C the wells were washed 3 times with PBST and peroxidase substrate added for 10 min. The reaction was stopped by the addition of 50µl of 4M sulphuric acid. The optical density was read at 450nm.

5.2.7.3 ELISA to quantify production of gD-1

The same ELISA procedure as section 5.3.7.2 was used. However, after growth of the bacterial strains to log phase, a protein estimation (Biorad) of each bacterial strain was carried out so that the same concentration of each strain was used to coat the ELISA plates. Therefore allowing the level of gD-1 produced by each strain to be compared.

5.4 RESULTS AND DISCUSSION

5.4.1 Construction of a gD(aa 28-369)-Lac z fusion protein

Double stranded gD-1 in M13mp18 was provided by Dr D. Kilburne, University of British Columbia (figs 5.1 and 5.2). From this, the restriction sites *Pvu*II shown in fig 5.3 were used and the gD-1 structural gene was blunt end ligated in-frame into the pUC18 multiple cloning site (fig 5.4) to create a Lac z-gD-1 fusion protein. In order to create a compatible site a cut was made with *Sma*I in the polylinker cloning site of the vector pUC18, resulting in a blunt end. Ligation of the 1.8kb *Pvu*II DNA fragment into the *Sma*I site of pUC18 resulted in the deletion of sequences encoding the NH_2-terminal 27 amino acids of gD and brought the remaining 341 COOH-terminal gD-1 amino acids in-frame under the control of the *lac* promoter. This construction was used to transform *Escherichia coli* strain JM101 and screened for positive recombinants by colony blot hybridisation with a probe made by cutting out a 1.8kb fragment from gD-1 M13mp18 encoding the gD structural gene, using restriction sites *Hind*III and *Sst*I (fig 5.1 and 5.2). A radiolabelled probe was generated by labelling the fragment directly in a molten agarose gel slice by extending random hexadeoxynucleotide primers using the Klenow fragment of DNA polymerase in the presence of (^{32}P) dCTP (Feinberg and Vogelstein, 1983). Restriction endonuclease cleavage of the recombinant plasmids using *Eco*RI which cuts once in the polylinker of pUC18 and once in the 1.8kb *Pvu*II fragment encoding aa 28-369 (fig 5.5) revealed only one (lane 9, 1.6 and 2.9kb fragments) had the gD-1 structural gene cloned into pUC18 in the correct orientation. Restriction digests using the following polylinker enzymes, *Bam*HI, *Pst*I, *Sph*I and *Hind*III were made, which should have resulted in a 4.5kb fragment (ie gD (aa28-369)pUC18 cut once). However, after running out the restriction digests on agarose gels with uncut gD (aa 28-369)pUC18 it was noted that these polylinker enzymes were not cutting the recombinant plasmid. Therefore a deletion event had occurred in the polylinker.

Expression of the Lac z-gD fusion protein was investigated by colony immunoblots and enzyme linked immunosorbent assays (ELISA) methods after induction of the *lac* promoter with isopropyl-ß-D-thiogalactopyranoside (IPTG). No expression was detected using the following antibodies raised at Porton Down:-

1) rabbit anti-HSV-1 triosbel serum

2) C101 purified rabbit IgG, 5mg ml^{-1} from an antiserum raised against gD-1 purified by denaturing SDS-PAGE.

3) monoclonal antibody L35 raised against gD-1 group II continuous epitope (amino acids 272-279).

4) monoclonal antibody L101 raised against gD-1 group II continuous epitope (amino acids 272-279).

Nucleotide sequence analysis of a 1.6kb *Eco*RI fragment encoding gD(aa 28-369) cut from gD(aa 28-369)pUC18 and subcloned into M13mp18 using a -40 primer was carried out. The sequence analysis revealed that a 100 base *Pvu*II fragment from the M13mp18 containing two stop codons, had been ligated before the *Pvu*II gD-1 (aa 28-369) fragment in-frame (fig 5.6).

This was an initial simple approach to constructing a gD-Lac z fusion protein expressed in *S. typhimurium* HWSH *aro*A mutants. Previous work by Watson *et al* (1982) involved construction of a plasmid, pEH25, that encodes, under control of the lac promoter-operator, a hybrid protein composed of 342 amino acids of the HSV-1 (Patton)gD carboxy-terminus fused to the amino-terminal 24 amino acids of the bacteriophage lamda Cro protein. The yield was only 0.1% of the total cell protein which was insufficient for vaccine production. Weis *et al* (1983) found that ligation of a Cro-gD amino-terminal coding region contained in pEH25 to a DNA sequence encoding *E. coli* ß-galactosidase (ß-gal), resulted in expression of a chimaeric Cro-gD-ß-gal (gD-ß-gal) protein in *E. coli* at a higher level. In addition, removal of the entire membrane anchor sequence and up to half the transmembrane sequence resulted in

higher expression. This chimaeric protein elicited antibodies in rabbits that not only immunoprecipitated gD from cells infected with HSV-1 and HSV-2 but also neutralised HSV-1 and HSV-2 infectivity *in vitro*. It has been shown that antiserum prepared to HSV-1 gD polypeptide is type-common. Thus, polyvalent gD anti-serum (Cohen *et al*, 1978) and certain gD monoclonal antibodies (Pereira *et al*, 1980) recognise type common antigenic determinants (epitopes) and neutralise infectivity of both HSV-1 and HSV-2. The type common specificity of gD antiserum has been demonstrated *in vivo* by passively immunising mice with monoclonal antibody directed against HSV-1 gD (Dix *et al*, 1981). Such mice were protected against acute neurological disease induced by either HSV-1 or HSV-2.

Therefore, in this first approach the *Pvu*II site from gD-1(sequence McGeoch *et al*, 1988) was used with the aim of constructing a gD-expression plasmid making use of the previous work by Watson *et al* (1982) and Weis *et al* (1983).

The first construction was a compromise between the use of simple methods and the knowledge that gD(aa 28-369) contains regions with potential protective epitopes. For example, Wycoff *et al* (1988) have demonstrated regions 268-287 and 340-356 to be T cell targets inducing T-helper (Th) and T-suppressor (Ts) cells. In addition, Kocken *et al* (1988) have shown there may be a partial epitope, region 40-54. However, aa 1-27 are important, for example, a synthetic acylated peptide (1-23) liposome adjuvant mixture induced protective immunity in mice (Watari *et al*, 1987). This approach was not pursued further due to technical difficulties with the ligation. One possible explanation, is that the shot gun approach used did not allow optimum conditions for the ligation to work. However, the one positive gD-1 (aa 28-369)-lac z fusion did not show expression using enzyme linked immunosorbent assay and colony immunoblot assays; the reason was explained by nucleotide sequence analysis.

Fig 5.1 Construction of gD-1 M13mp18; gD-1 pUC18 was cut with *SstI* and *HindIII*, a 1.8kb fragment isolated containing the gD-1 structural gene and subcloned in *SstI* and *HindIII* sites of M13mp18.

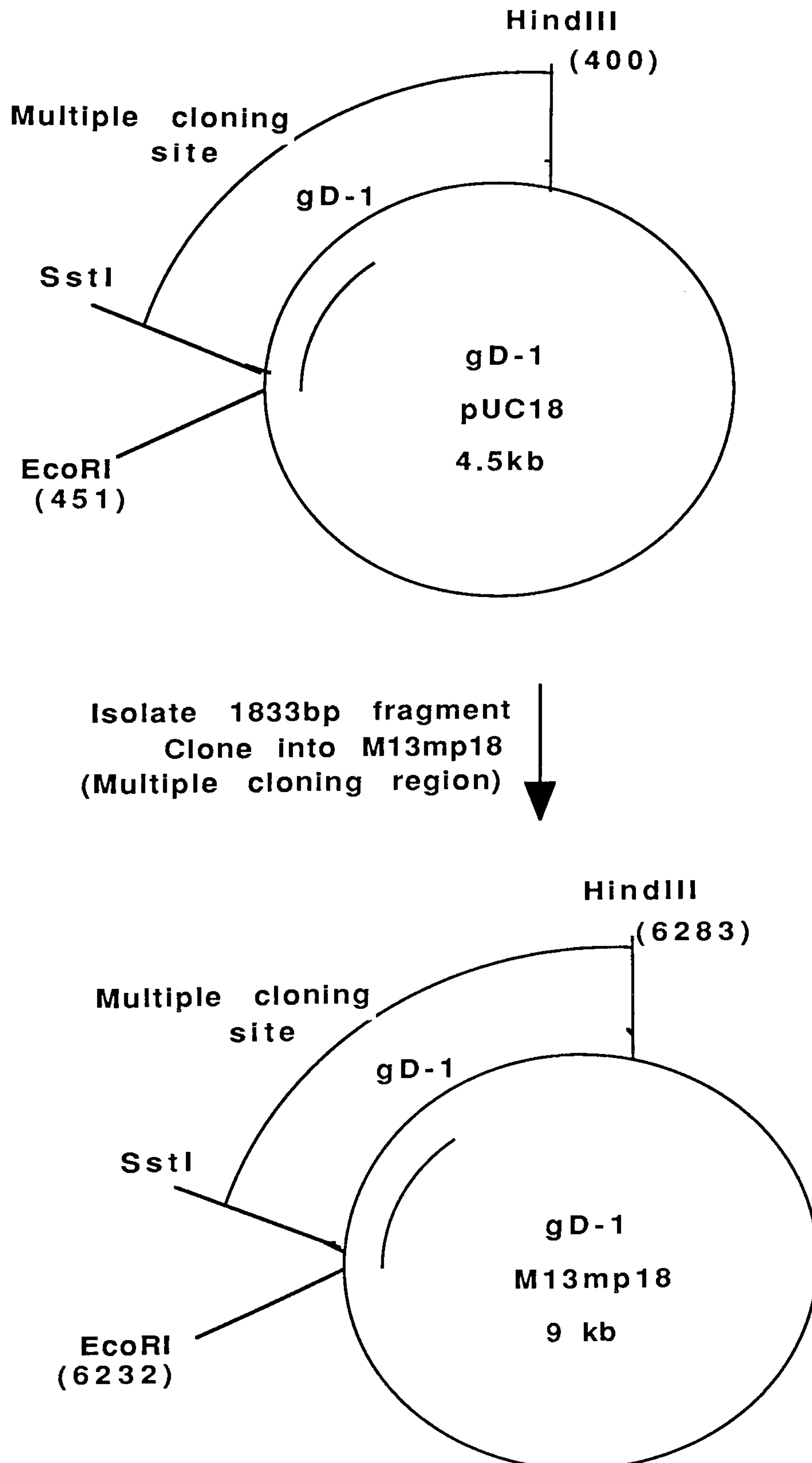


Fig 5.2 Construction of gD-1 M13mp18; gD-1 pUC18 was cut with *SstI* and *HindIII*, a 1.8kb fragment isolated containing the gD-1 structural gene and subcloned in *SstI* and *HindIII* sites of M13mp18.

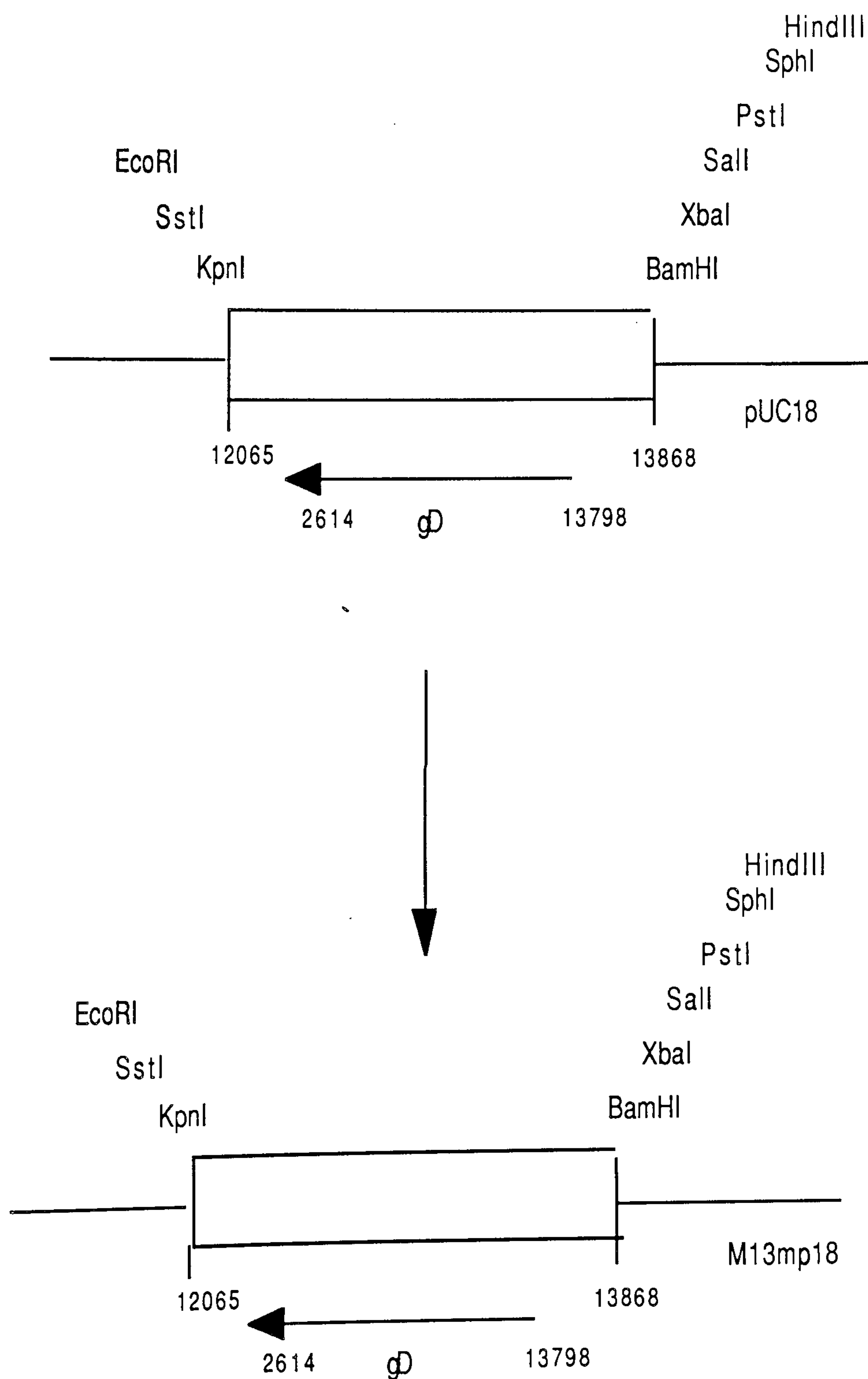


Fig 5.3 Diagram of gD-1 M13mp18 illustrating *Pvu*II restriction sites used to blunt end ligate in-frame the gD-1 structural gene (aa 28-369) into the pUC18 multiple cloning site (fig 5.4).

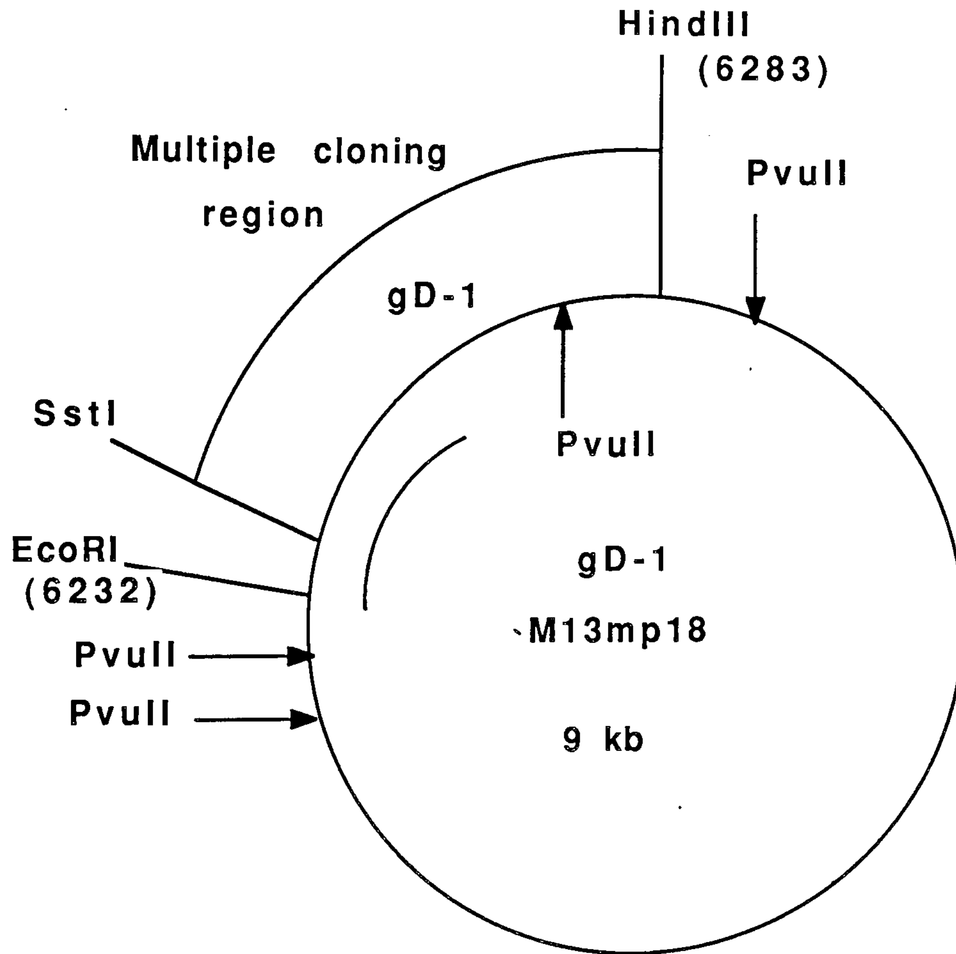


Fig 5.4 Multiple cloning regions of pUC18/M13mp18 and pUC19/M13mp19.

M13mp18/pUC18

5'	1	2	3	4	5	6	(1	2	3	4	5
	THR	MET	ILE	THR	ASN	SER	ser	ser	val	pro	gly
ATG	ACC	ATG	ATT	ACG	AAT	TCG	AGC	TCG	GTA	CCC	GGG

6	7	8	9	10	11	12	13	14	15	16	17
asp	pro	leu	glu	ser	thr	cys	arg	his	ala	ser	leu
GAT	CCT	CTA	GAG	TCG	ACC	TGC	AGG	CAT	GCA	GCA	TTG

18)	7	8
ala	LEU	ALA
GCA	CTG	GCC 3'

M13mp19/pUC19

5'	1	2	3	4	(1	2	3	4	5	6	7
	THR	MET	ILE	THE	pro	ser	leu	his	ala	cys	arg
ATG	ACC	ATG	ATT	ACG	CCA	AGC	TTG	CAT	GCC	TGC	AGG

8	9	10	11	12	13	14	15	16	17	18)	5	6
ser	thr	leu	glu	asp	pro	arg	val	pro	ser	ser	ASN	SER
TCG	ACT	CTA	GAG	GAT	CCC	CGG	GTA	CCG	AGC	TCG	AAT	TCA

7	8
LEU	ALA
CTG	GCC 3'

In pUC18, the *Eco*RI site lies immediately down stream from P_{lac}

In pUC19, the *Hind*III site lies immediately down stream from P*lac*

Fig 5.5 Restriction endonuclease cleavage using *Eco*RI of the positive recombinant plasmids determined by colony blot hybridisation after ligation of a 1.8kb *Pvu*II fragment encoding the gD-1 structural gene (aa 28-364) into the pUC18 multiple cloning site.

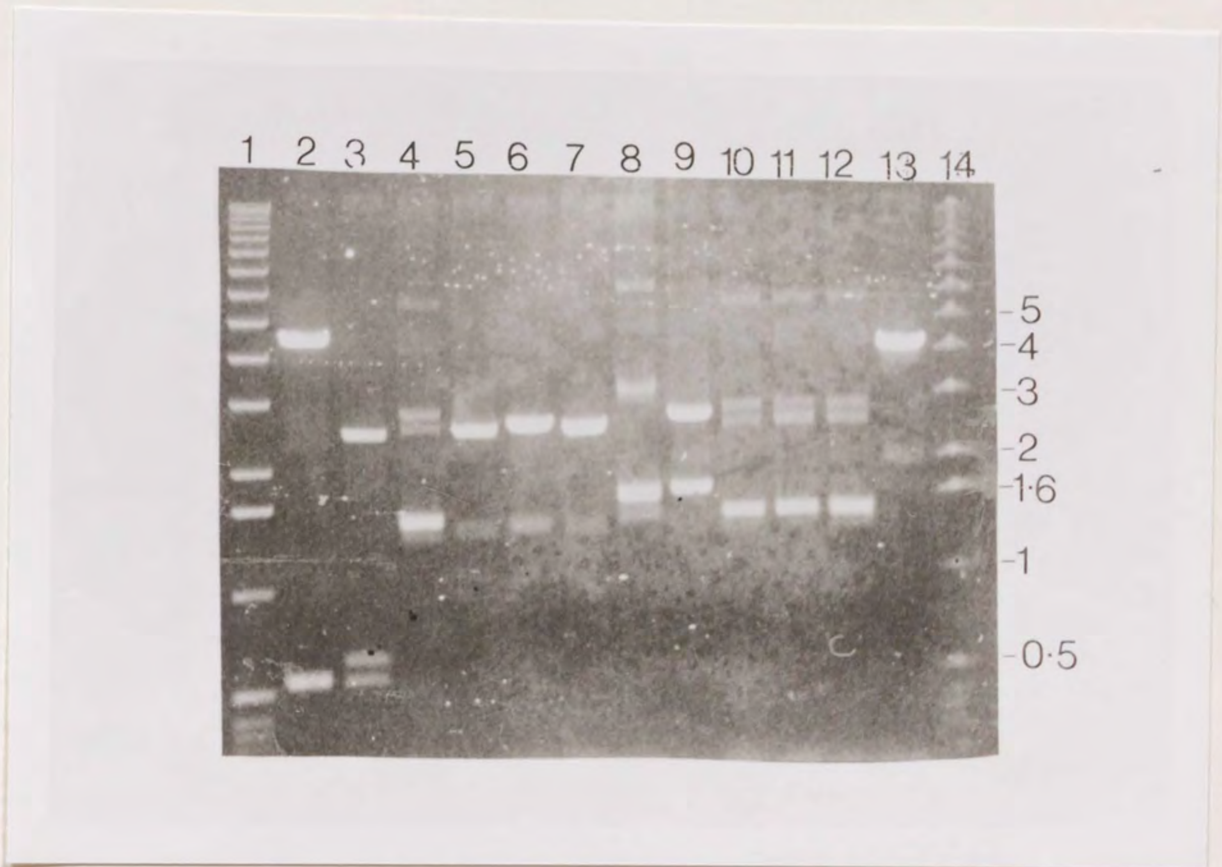


Fig 5.6 Nucleotide sequence analysis of single stranded M13 DNA templates. Prepared from a 1.6kb *Eco*RI fragment encoding gD-1 (aa 28-369) cut from gD-1 pUC18 and subcloned into M13mp18 *Eco*RI site.

5'

TCG AGC TCG GTA CCC CTG CAT TAA TGA ATC GGC

*Sst*I *Kpn*I

CAA CGC GCG GGG AGA GGC GGT TTG CGT ATT GGG

CGC CAG GGT GGT TTT TCT TTT CAC CAG TGA GAC

GGG CAA CAG CTG ACC GAC CCT

3'

A 100 base *Pvu*II fragment from gD-1 M13mp18 ligated in-frame into the polylinker of pUC18 at the cut *Sma*I site.

1.8kb *Pvu*II fragment from gD-1 (aa 28-369) M13mp18 blunt end ligated to the 100 base *Pvu*II fragment.

5.4.2 Construction of a gD-1 (aa 1-314)-Lac z fusion protein expressed in *Salmonella typhimurium* HWSH *aro*A mutants

Synthetic deoxyoligonucleotide primers were engineered with *Bam*HI restriction sites (fig 5.7) to amplify, using polymerase chain reaction (PCR) (Saiki *et al*, 1988), a 1 kb fragment of gD-1, amino acids 1-314 . The optimum magnesium concentrations for amplification were between 10-20mM, gD-1 M13mp18 (fig 5.1) was used as the template and the following temperatures:- 94°C, 1min (melt); 37°C, 1 min (anneal); 72°C, 2min (polymerise). The amplified gD-1 was cloned using the *Bam*HI restriction sites of pUC19 (fig 5.4) in-frame under the control of the *lac* promoter. This construction was used to transform *Escherichia coli* strain JM101. Restriction endonuclease cleavage with *Bam*HI showed that the 1kb fragment had been cloned into the pUC19 cut *Bam*HI site (fig 5.8). However, further digests using *Acc*I which cuts once in the polylinker of pUC19 and once in the amplified gD-1 (aa 1-314) fragment, resulting in 0.3kb and 3.4kb fragment when the gD has been cloned in the in-correct orientation (fig 5.8). However, if the amplified gD had been cloned in the correct orientation a 0.7kb and 3.0kb fragment would have resulted after digestion with *Acc*I. Restiction digests of the recombinant plasmids using *Acc*I revealed that in all cases the gD-1 encoding fragment had been cloned in the in-correct orientation for expression (fig 5.8).

The experiment was repeated and the ligation mixture used to transform *E. coli* strain LE392 harbouring the plasmid pMW1 which was constructed by cloning the 1.7kb EcoRI fragment encoding the *lacI^{q}* gene from pMC9 into pACYC184. Four recombinant colonies were detected expressing a gD-Lac z fusion protein by colony immunoblotting with a monoclonal antibody P1M12 at a concentration of 1μg ml^{-1} raised against gD-1 Eisenberg's group VII continuous epitope (amino acids 11-19) at Porton Down by Paul Ridgeway. Monoclonal antibodies L101 and L35 raised against gD-1 Eisenberg's group II continuous epitope (amino acids 272-279) did not bind to

the gD expressed by *E. coli*. Possible suggestions are that this region of the expressed gD was not in the correct conformation to be recognised, or the epitope was obscured due to folding of the protein. Restriction enzyme analysis of the recombinant plasmid DNA using *Bam*HI showed that the 1kb fragment had been cloned into pUC19 cut *Bam*HI site (fig 5.9). Further digests to reveal the orientation of the gD were complicated by the presence of plasmid pMW1. Therefore, the restriction enzymes *Hind*III and *Stu*I were chosen. *Hind*III cuts pJB1 once in the polylinker of pUC19 and cuts plasmid pMW1 once; *Stu*I cuts the amplified gD of pJB1 once and does not cut plasmid pMW1. Restiction digests of the recombinant plasmid revealed the gD-1 had been cloned in the correct orientation for expression (lanes 6-10 fig 5.9). One such plasmid termed pJB1 was used for further study. Nucleotide sequence analysis of single stranded M13 DNA templates prepared from a 1kb *Eco*RI and *Hind*III fragment encoding gD-1 (aa 1-314) from pJB1 subcloned into M13mp18 polylinker sites *Hind*III and *Eco*RI revealed that the gD-1 fragment had been cloned in-frame into pUC19 (fig 5.10). It is possible that the elevated levels of the Lac I repressor protein in strain LE392 (pMW1) are responsible for the increased stability of the recombinants expressing the gD-lac z fusion demonstrated by colony immunoblotting and enzyme linked immunosorbent assay (ELISA). Watson *et al* (1982) and Weis *et al* (1983) used *E.coli* strain NF1829 which is an overproducer of the *lac* repressor to transform plasmid pEH25 encoding gD. Therefore, the *lac* promoter of pJB1 was transcriptionally silent unless induced by isopropyl-ß-D-thiogalactopyranoside (IPTG).

In order to construct a *Salmonella* vaccine expressing gD-1 an intermediate strain, *S. typhimurium* LB5010, was transformed with plasmid pJB1. Colonies expressing gD-1 were detected by antibiotic selection followed by colony immunoblotting with P1M12 (described above). In addition, plasmid pJB1 appeared stable in the absence of plasmid pMW1 in strain LB5010. Bacteriophage P22 was used to transduce pJB1 from LB5010 into the smooth *S. typhimurium* vaccine strains

SL3261 and HWSH *aro*A. Only 2 colonies of pJB1 in strain HWSH *aro*A and 4 colonies of pJB1 in SL3261 would grow in L broth containing ampicillin (100μg ml^{-1}). Growth was very slow to begin with but after subculturing for 1 week pJB1 appeared stable in strains SL3261 and HWSH *aro*A. Therefore, selection with ampicillin appears to make the plasmid unstable. There are no reports in the literature, but several investigators working in this area have also encountered this problem, but without adequate explanation. Strains containing plasmid pJB1 were examined for their expression of gD-1 using colony immunoblotting (fig 5.11).

In order to use these strains to vaccinate guinea pigs, the plasmid carrying the gene has to be inherited in the absence of antibiotic selection. Plates of *S. typhimurium* SL3261 and HWSH *aro*A carrying pJB1 were grown overnight in L broth with and without ampicillin. Restriction digests of the plasmid preparations confirmed the presence of pJB1. Indicating that plasmid pJB1 is inherited in a stable manner under these conditions.

In summary, the gene coding for gD (aa 1-314) HSV-1 has been cloned into pUC19 and stably expressed in *S. typhimurium* HWSH and SL3261 *aro*A mutants. ELISAs were carried out to compare gD expression between the *E. coli* and *S. typhimurium* strains used. *S. typhimurium* HWSH *aro*A and SL3261 have the highest expression of gD (1.969+/-0.08 and 1.778+/-0.08 respectively). The *E. coli* strain LE392 harbouring plasmid pMW1 and *S. typhimurium* LB5010 produced significantly lower gD levels (1.286+/-0.09 and 0.925+/-0.11 respectively) compared to the *S. typhimurium* *aro*A strains. The results quoted were calculated as the mean OD_{450}+/-S.D. when a dilution of 0.02μg ml^{-1} protein was used for each strain to coat the ELISA wells. The four stains not harbouring plasmid pJB1 had OD_{450} values of 0.339+/-0.03.

It is attractive to consider *Salmonella* as a potential carrier for delivering heterologous antigens to the immune system. Potentially protective antigens

from many pathogens can now be expressed directly in *E. coli* or Salmonellae (review see Charles and Dougan, 1990).

A potential problem with such vaccines is the stability of the expression plasmid. During construction of pJB1 expressed in *S. typhimurium aro*A HWSH and SL3261 instability of the plasmid was found when selecting with ampicillin. Maskell *et al* (1987) used *S. typhimurium aro*A mutants as carriers of the *E. coli* heat-labile enterotoxin B subunit (LT-B) to the muriune secretory and systemic immune systems. Mice developed substantial levels of IgA and IgG anti-LT-B antibodies 14 days post infection in both serum and gut samples. However, they have also noted instability in a number of different plasmids directing the expression of recombinant antigens in salmonellae (their unpublished observations). They suggest that this may be a consequence of using strong unregulated promoters and high copy number plasmids. In addition, Dr Wallis (Microbiology Department, Leicester University) and Dr Hormaeche (Division of Microbioogy, University of Cambridge) have noted instability in a number of plasmids directing the expression of recombinant antigens in salmonellae (unpublished observations) when the ampicillin resistant gene present on the plasmid was used for selection.

There are no reports in the literature with respect to the instability due to ampicillin selection and discussion with several investigators working in this area did not lead to an adequate explanation.

Most available plasmid expression vectors are not suitable for *in vivo* use since they require specialised growth conditions in order to induce expression of the foreign protein. Maskell *et al* (1987) suggest one solution to the instability problem may be to introduce genes directly into the *Salmonella* chromosome. Strugnell *et al* (1990) have developed a versatile system using a new cloning vector which can serve as a vehicle for integrating DNA fragments, which direct the expression of heterologous antigens, into the *aro*C gene on the *Salmonella* chromosome. The vectors

were used to integrate nucleotide sequences into the *S. typhimurium* chromosome which directed the expression of tetanus toxin C and the *Treponema pallidum* lipoprotein. The expression of both antigens was detected by Western blotting.

A second potential problem with live carrier vaccines maybe that, unlike LT-B (Maskell *et al*, 1987), many of the heterologous antigens accumulate almost entirely in the cytoplasm of the cell and it is not clear whether antigens have to be surface located or outside of the bacterial cell in order to be recognised by the immune system.

With respect to plasmid pJB1, the gene coding for gD (aa 1-314) has been cloned in-frame into pUC19 under the control of the *lac* promoter. An in-frame stop codon at the C terminus of gD engineered into the oligonucleotide used for amplification of the gD using polymerase chain reaction, means the gD will not create a Lac z-gD-1 fusion protein at the carboxy terminus. In addition, the gD(aa 1-314) is minus the transmembrane and anchor sequence, suggesting the gD may be secreted from the *S. typhimurium aro*A mutants or collect in the cytoplasm. These are obviously only suggestions and further studies need to be carried out. However, as mentioned above, experiments infer that it may not be necessary to express the heterologous antigen on the bacterial cell surface to generate a strong immune response. However, Brown *et al* (1987) have shown that attenuated Salmonellae may be expected to elicit both humoral and cellular responses to intra-cellular cloned antigens. Thus, protein antigens may not need to be located at the cell surface or outside of the bacterial cell in order to stimulate an immune response. In addition, Tite *et al* (1988) have found that *Salmonella* expressing the influenzae virus nucleoprotein as an intra-cellular antigen can stimulate humoral and cytotoxic T-cell responses to the nucleoprotein in mice.

In contrast, some investigators believe that it is advantageous and several secretion systems have been developed for expressing foreign antigenic

determinants at the bacterial cell surface (Chatfield *et al*, 1989). In addition, Newton *et al* (1989) have recently described the use of flagella of *Salmonella* for presenting foreign antigens. They inserted a synthetic oligonucleotide encoding an epitope of cholera toxin subunit B into flagella of an *aro*A S. *dublin*. When given to mice this hybrid strain evoked a humoral antibody response to cholera toxin.

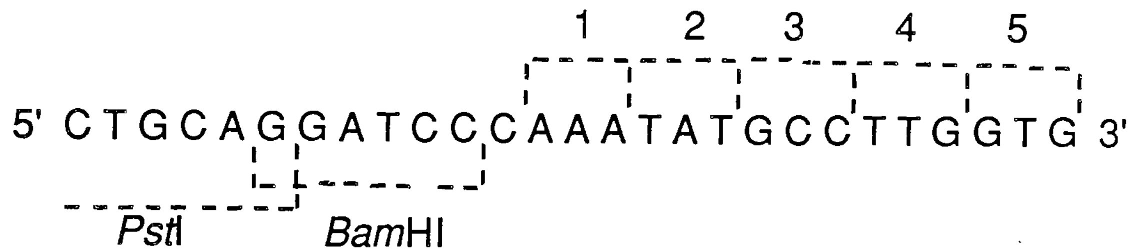
Induction of protective immunity against the majority of antigens expressed in *Salmonella* vaccine strains has not been demonstrated. There are several exceptions, for example, Poirier *et al* (1988) have shown that a *Streptococcus pyogenes* M protein induced protective immunity to mice against subsequent challenge with *S. pyogenes*.

One of the possible reasons for the lack of success is that the strains failed to produce the foreign colonisation or virulence antigen to the extent observed when the cloned gene was in typical *E. coli* K-12 cloning hosts (Charles and Dougan, 1990). This potential problem seems to be eleviated in *S. typhimurium* HWSH *aro*A expressing gD(aa 1-314). The ELISA data demonstrated that the gD was expressed at levels higher than in standard *E. coli* cloning hosts.

To conclude, this section has demonstrated expression of gD in 2 strains of *S. typhimurium aro*A mutants and deserves further investigation with the aim to a HSV vaccine.

Fig 5.7 Synthetic deoxyoligonucleotide primers engineered with *PstI* and *Bam*HI sites. Polymerase chain reaction primer 1 contains amino acid 1-5 of gD-1; polymerase chain reaction primer 2 contains amino acid 310-314.

Polymerase chain reaction primer 1



Polymerase chain reaction primer 2

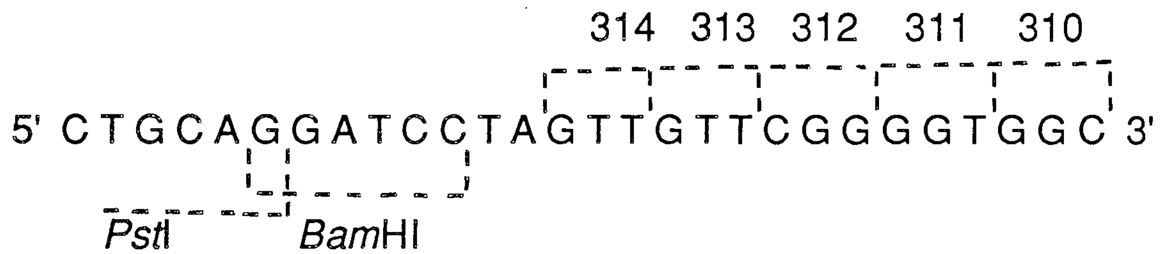
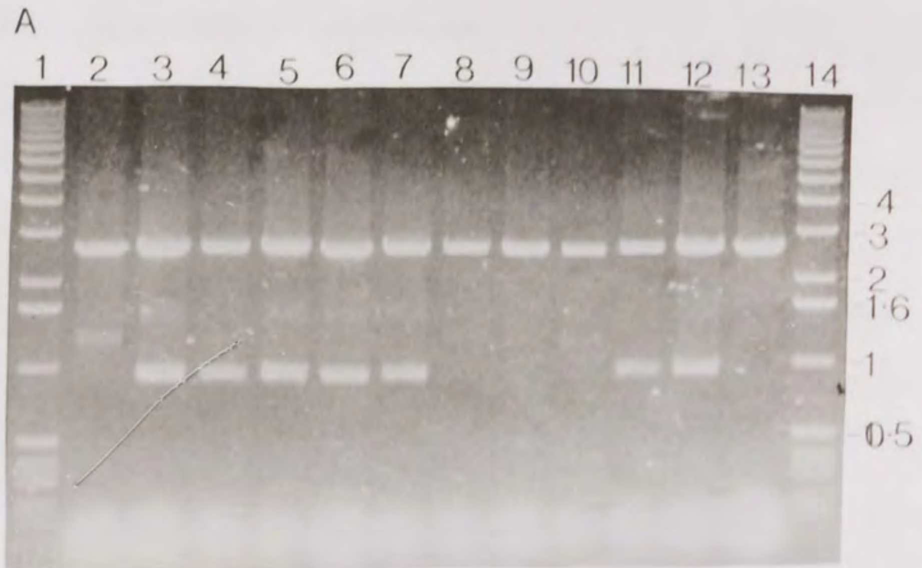


Fig 5.8 Restriction digests of the resulting recombinant plasmids after ligation of the 1kb *Bam*HI fragment encoding the gD-1 structural gene (aa 1-314) into *Bam*HI site of pUC19, show that the gD-1 had been cloned in the in-correct orientation for expression in *Escherichia coli* JM101. A-*Bam*HI restriction degests. B-*Acc*I restriction digests.



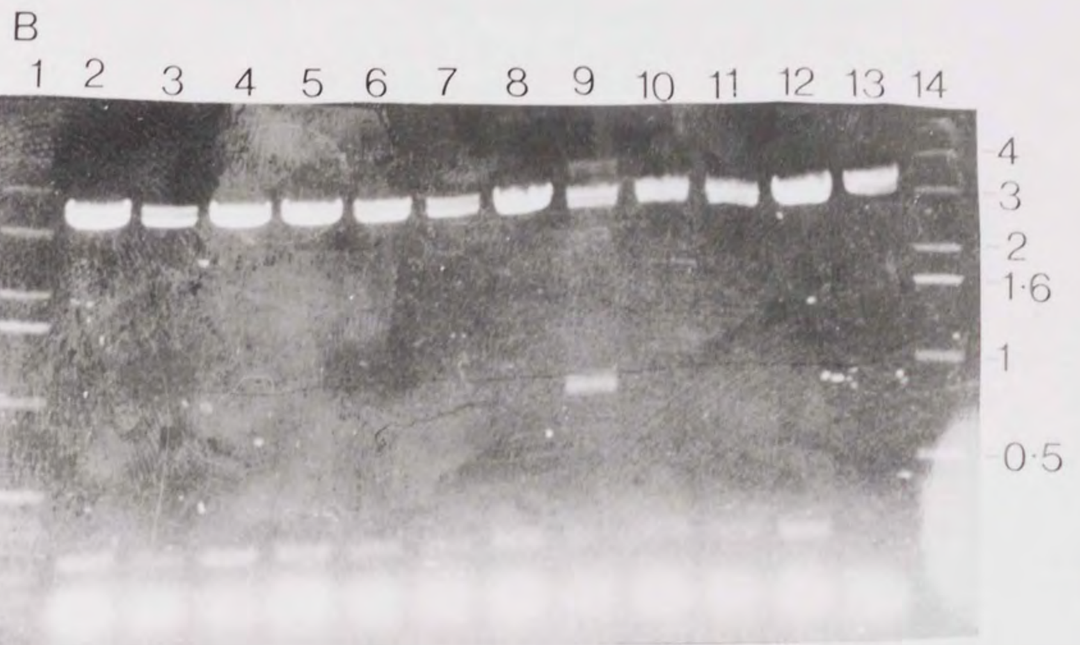
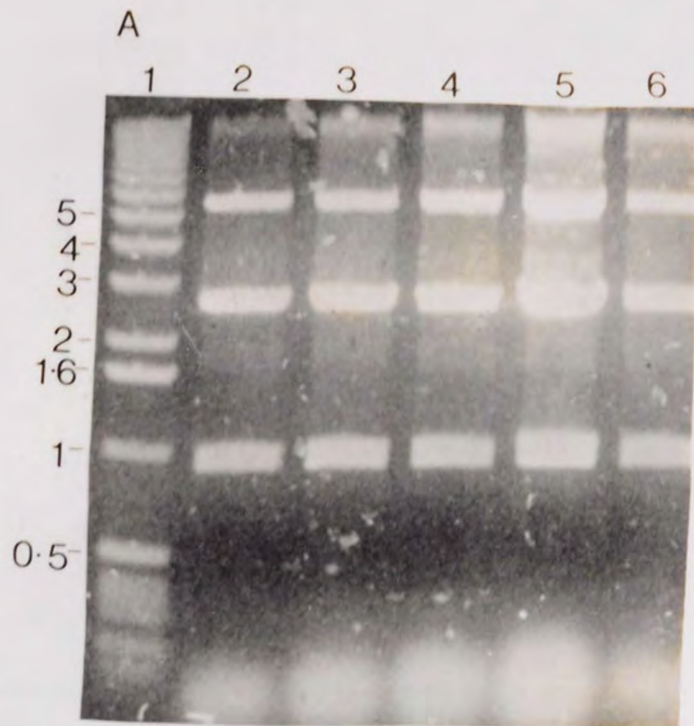


Fig 5.9 Restriction digests of the resulting recombinant plasmids after ligation of the 1kb *Bam*HI fragment encoding the gD-1 structural gene (aa 1-314) into *Bam*HI site of pUC19, show that the gD-1 had been cloned in the correct orientation for expression in *Escherichia coli* LE392(pMW1). A-*Bam*HI restriction digests. B-*Hind*III and *Stu*I restriction digest.



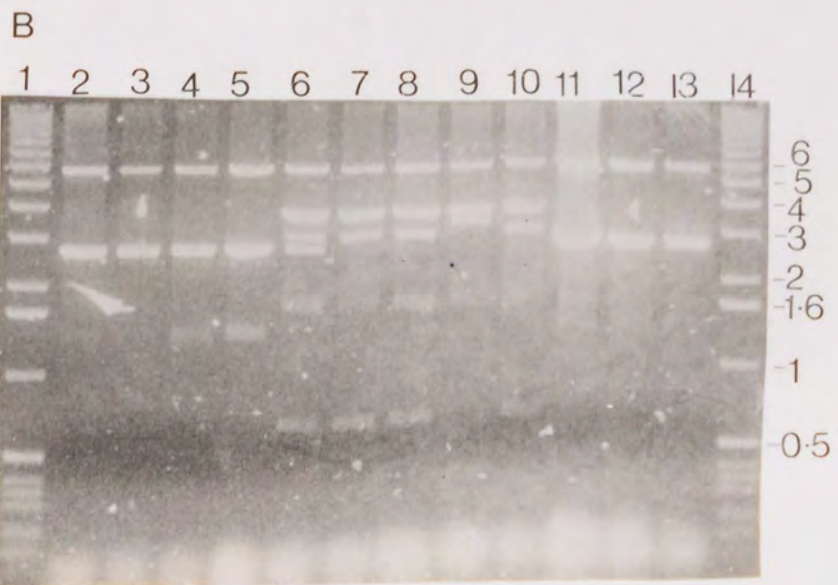
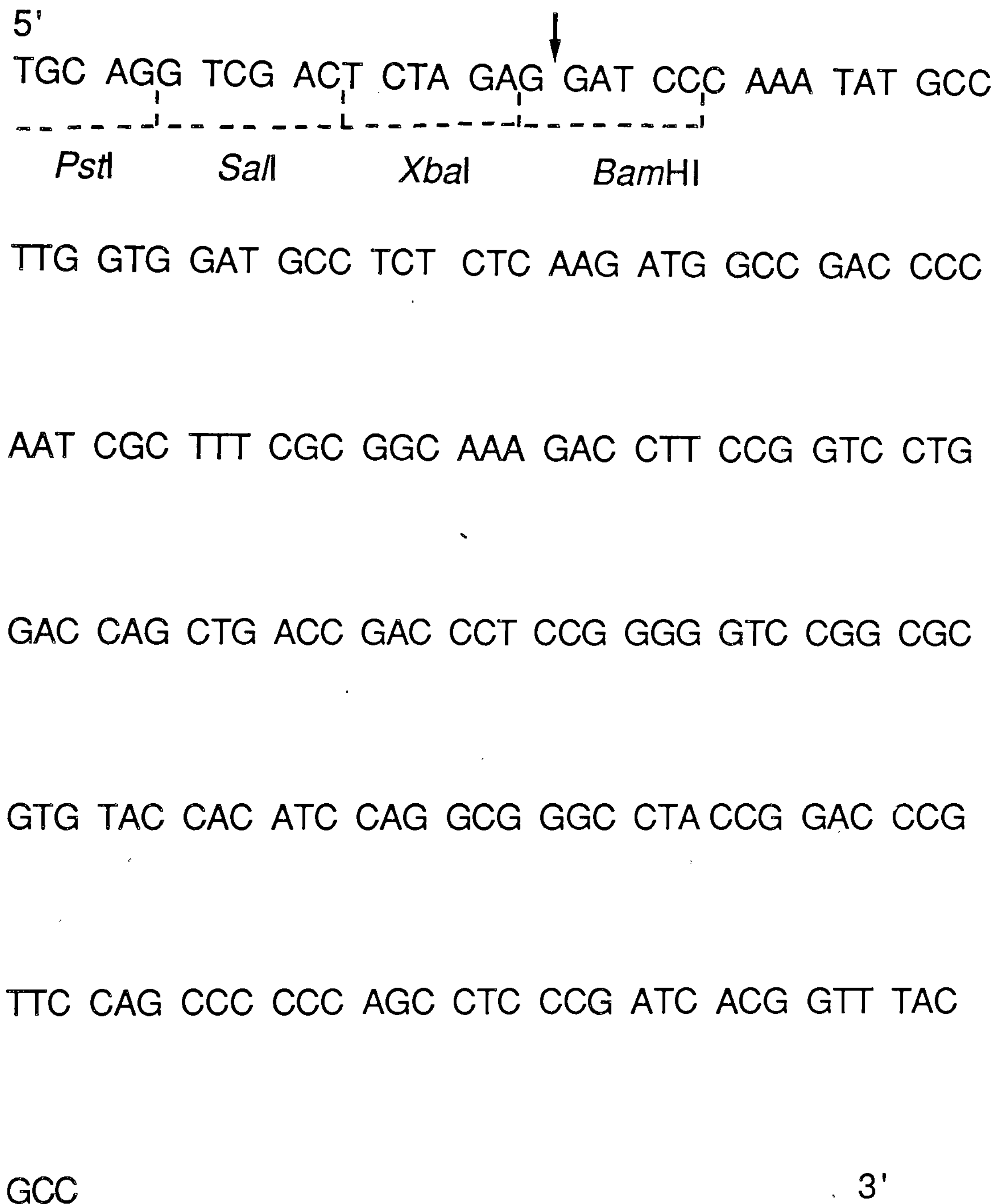
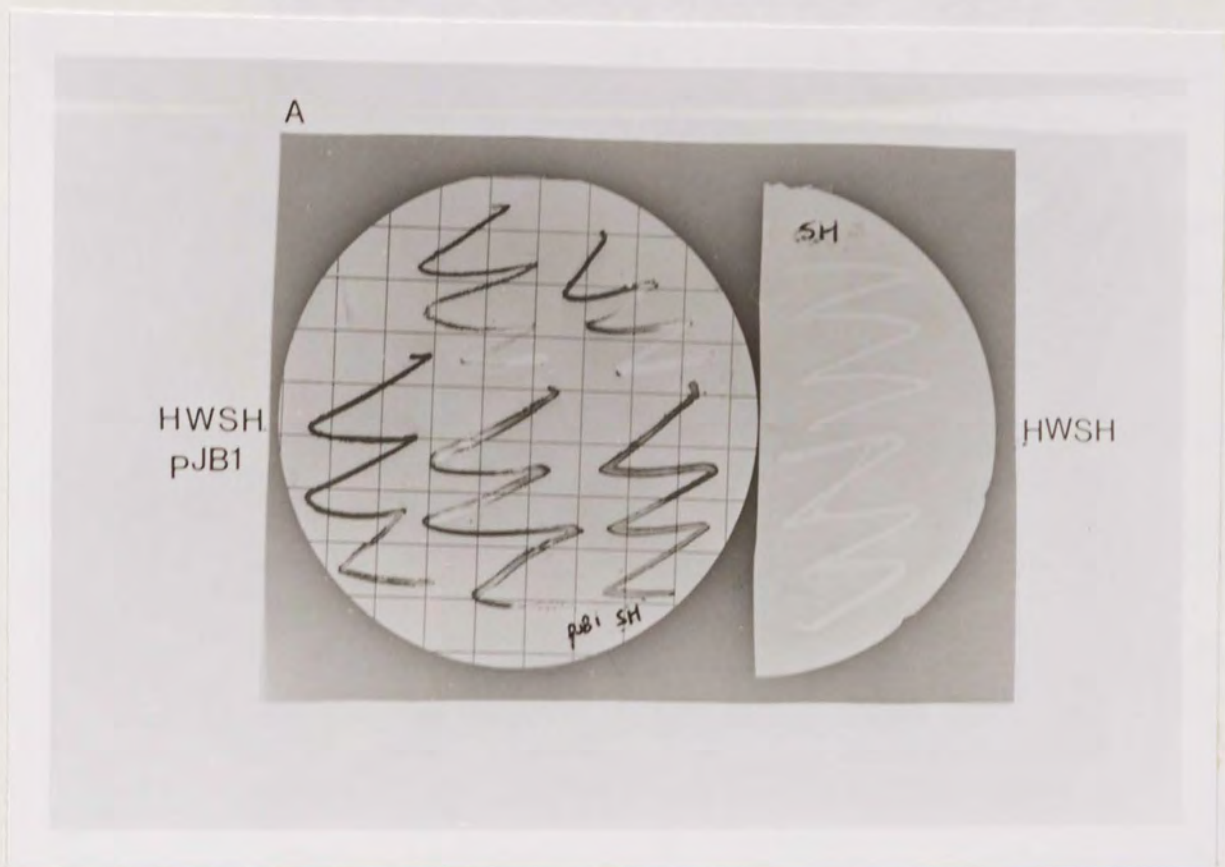


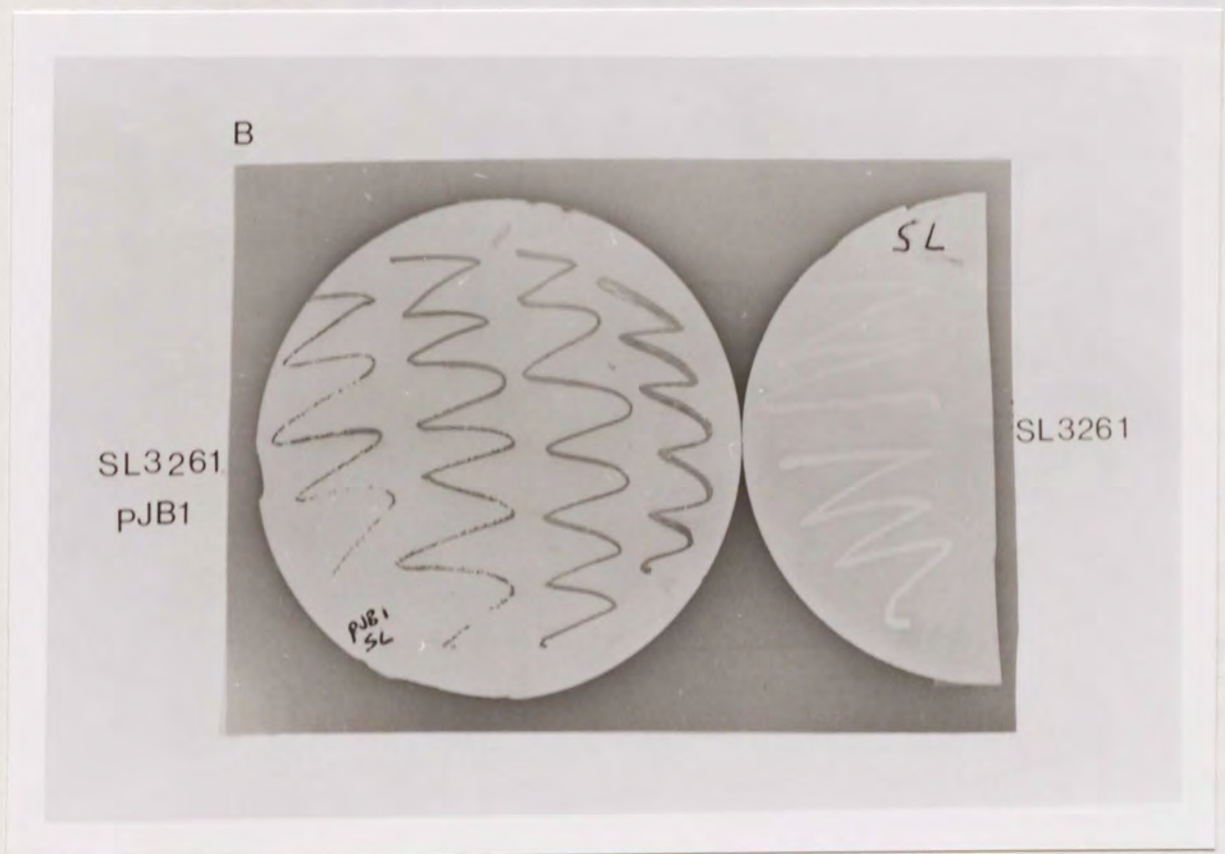
Fig 5.10 Nucleotide sequence analysis of single stranded M13 DNA templates, prepared from a 1kb *Eco*RI and *Hind*III fragment encoding gD-1 (aa 1-314) from pJB1 subcloned into M13mp18 polylinker sites *Hind*III and *Eco*RI.



↓ 1kb *Bam*HI fragment encoding gD-1 (aa 1-314) ligated in-frame into the polylinker of pUC19 at the cut *Bam*HI site.

Fig 5.11 *S. typhimurium* strains (A-HWSH; B-SL3261) harbouring plasmid pJB1 show expression of gD-1 detected with a monoclonal antibody raised against aa 11-19 of gD-1 (P1M12).





5.4.3 Construction of gD-1 (aa1-26)-Lac z fusion protein expressed in *Salmonella typhimurium* LB5010

A third approach was used to construct a gD expression plasmid whereby synthetic oligonucleotide primers (fig 5.12) were engineered, one with a *Bam*HI restriction site and the second with an *Eco*RI restriction site. These primers were used to amplify, using polymerase chain reaction (PCR) a 90 base fragment from gD-1 (ie aa 1-26). The same conditions described in section 5.4.2 were used in the PCR, but the template was a 1.8kb *Hind*III and SstI fragment cut from gD-1 M13mp18. The amplified gD-1 was cloned using the *Bam*HI and *Eco*RI restriction sites of pUC19 (fig 5.4) in-frame under the control of the *lac* promoter. This construction was used to transform *E. coli* strain JM101 and screened for positives by colony blot hybridisation (fig 5.13) with the amplified gD-1 (aa 1-26) fragment as a probe. Restriction endonuclease cleavage of the recombinant plasmids using *Bam*HI and *Eco*RI revealed the gD fragment had been cloned into pUC19 (fig 5.14). One such plasmid termed pJB2 was used for further study. Nucleotide sequence analysis of single stranded M13 DNA templates prepared from a 90 base *Hind*III and *Eco*RI fragment encoding gD-1 (aa 1-26) from pJB2 subcloned into M13mp18 polylinker sites *Hind*III and *Eco*RI confirmed this. It also showed that the PCR primer 1 (fig 5.12) had been cloned after the gD-1 (aa 1-26) fragment and therefore was not in-frame with the ß-galactosidase (fig 5.15). The reasoning behind engineering the PCR primers so that the amplified gD (aa 1-26) will be in-frame with the ß-Galactosidase is that small peptides do not usually elicit an immune response unless attached to a carrier molecule or delivered with an adjuvant. For example, immunisation studies with small peptides synthesised from HSV have used adjuvants such as liposomes or Freund's. In particular, a synthetic acylated peptide (aa 1-23 of gD) liposome adjuvant mixture induced protective immunity in mice (Watari *et al*, 1987). Therefore, if gD-1 was in-frame with the ß-galactosidase protein at the carboxy terminus ß-galactosidase

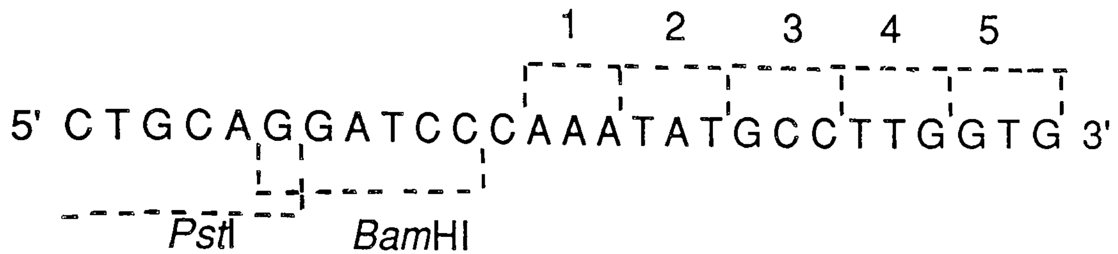
However, problems might arise if the gD is obscured by the Lac z protein.

Expression of the Lac z-gD-1 fusion protein was confirmed by colony immunoblots using a monoclonal P1M12 raised against gD-1 Eisenberg's group VII continuous epitope (amino acids 11-19). In order to construct a *Salmonella* vaccine expressing gD-1 an intermediate strain, *S. typhimurium* LB5010, was transformed with plasmid pJB2. Colonies expressing gD-1 were detected by antibiotic selection followed by colony immunoblotting using P1M12 monoclonal antibody.

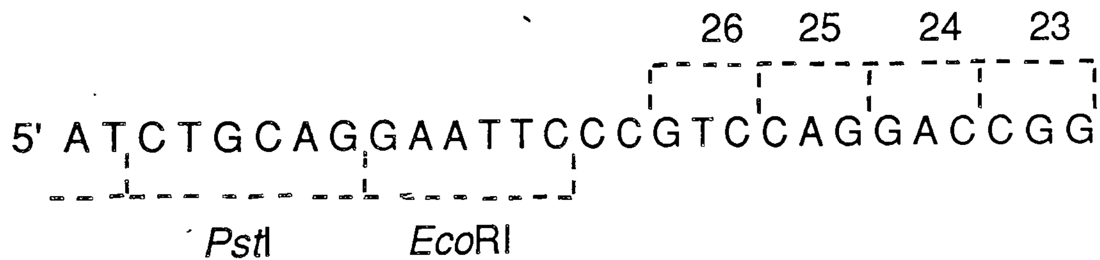
This approach was started due to the toxicity problems with pJB1 in *E. coli* JM101 and having to use *E. coli* strain LE392 harbouring pMW1. At this stage it was not known that pJB1 would be stable in *S. typhimurium* in the absence of pMW1. Section 5.4.2 described construction of gD-1 (aa 1-314)-Lac z fusion protein expressed in *S. typhimurium* HWSH and SL3261 *aro*A mutants. However, with this construction (pJB2) time did not allow for continuation with experiments to achieve expression in *S. typhimurium* vaccine strains. In addition, the same points already discussed in section 5.4.2 apply here.

Fig 5.12 Synthetic deoxynucleotide primers engineered with *Pst*I and *Bam*HI sites encoding amino acids 1-5 of glycoprotein D-1 (Polymerase chain reaction primer 1) and *Pst*I and *Eco*RI sites encoding amino acids 22-26 of glycoprotein D-1 (Polymerase chain reaction primer 2).

Polymerase chain reaction primer 1



Polymerase chain reaction primer 2



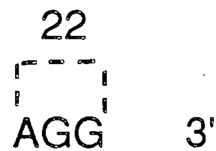


Fig 5.13 Positive colonies detected by colony blot hybridisation using the amplified gD-1 (aa 1-26) as a probe.

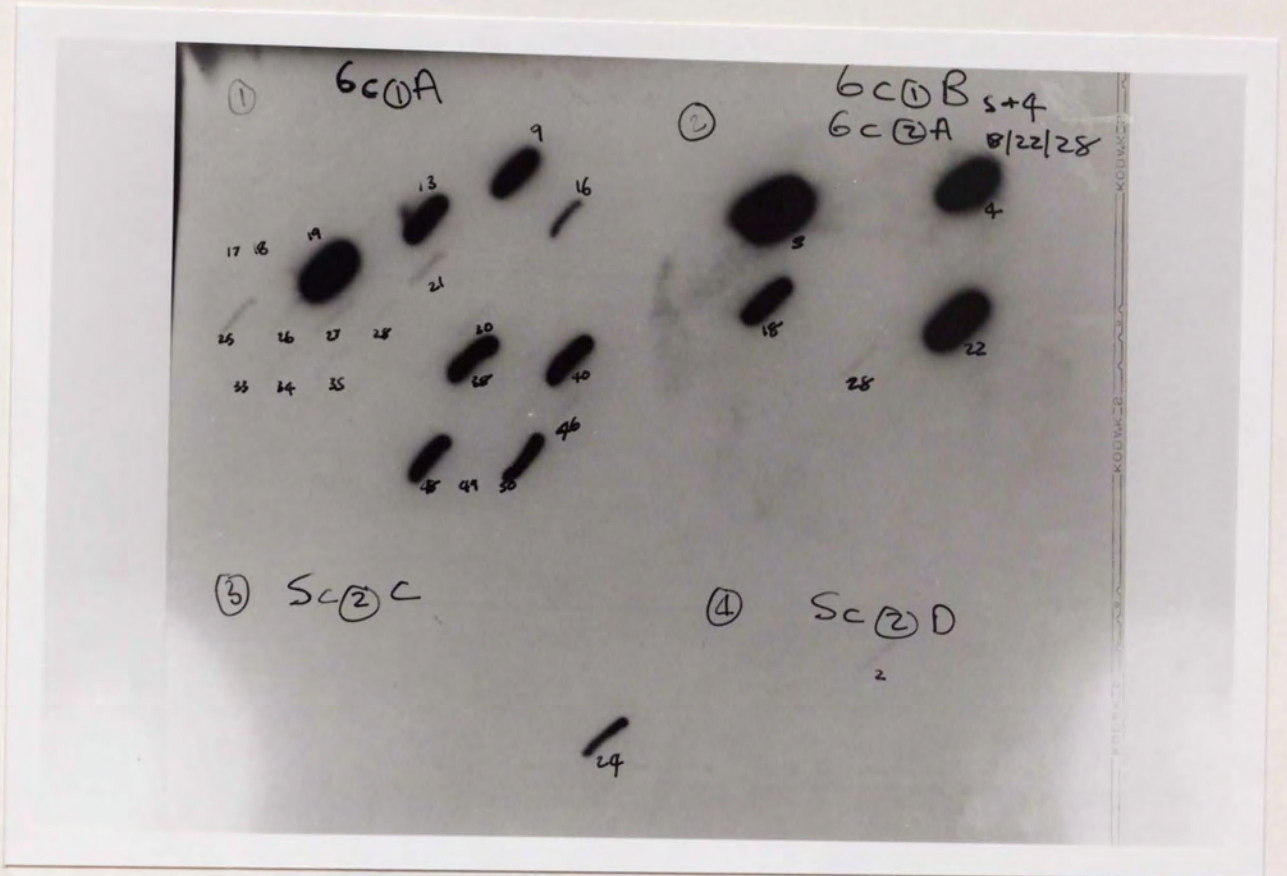


Fig 5.14 Restriction digests of the resulting recombinant plasmids after ligation of the 90 base *Bam*HI and *Eco*RI fragment encoding aa 1-26 of the glycoprotein D-1 structural gene into *Bam*HI and *Eco*RI sites of pUC19 show 2 positive recombinant plasmids.

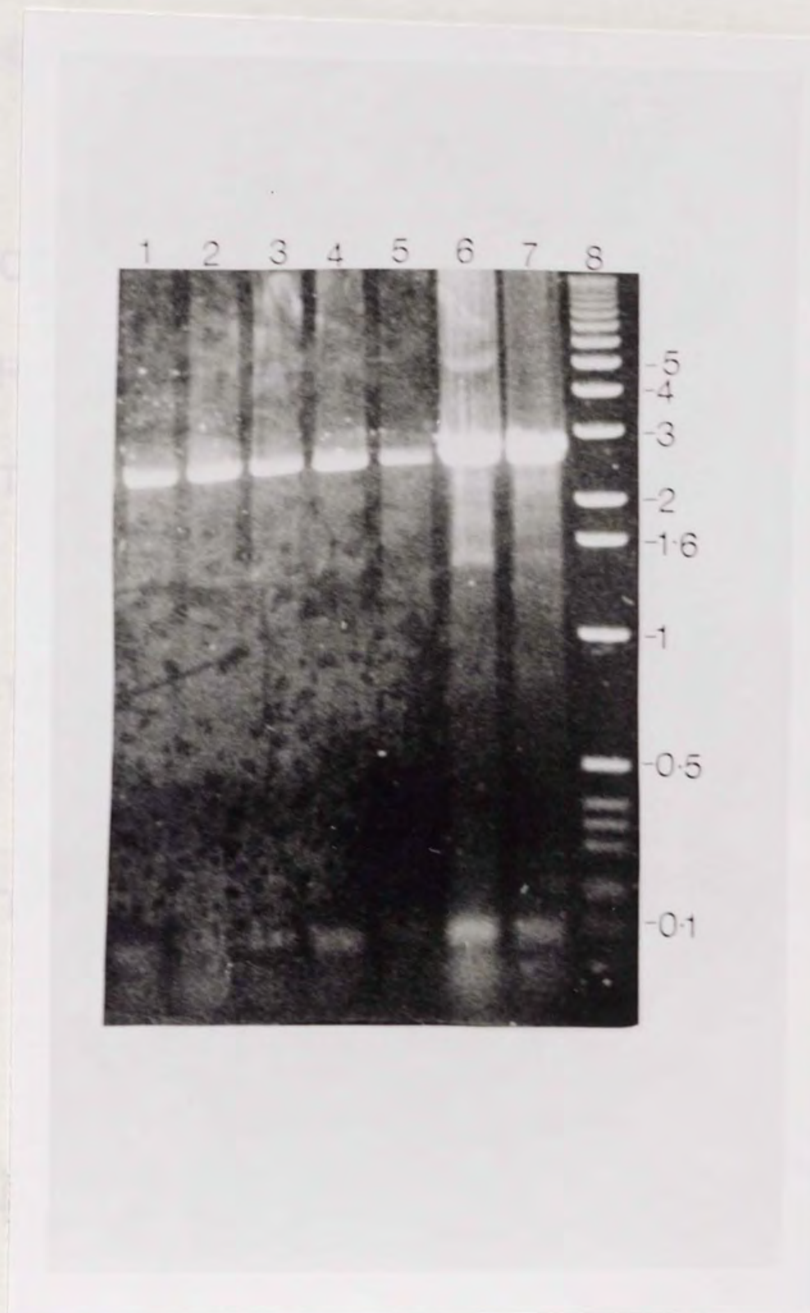
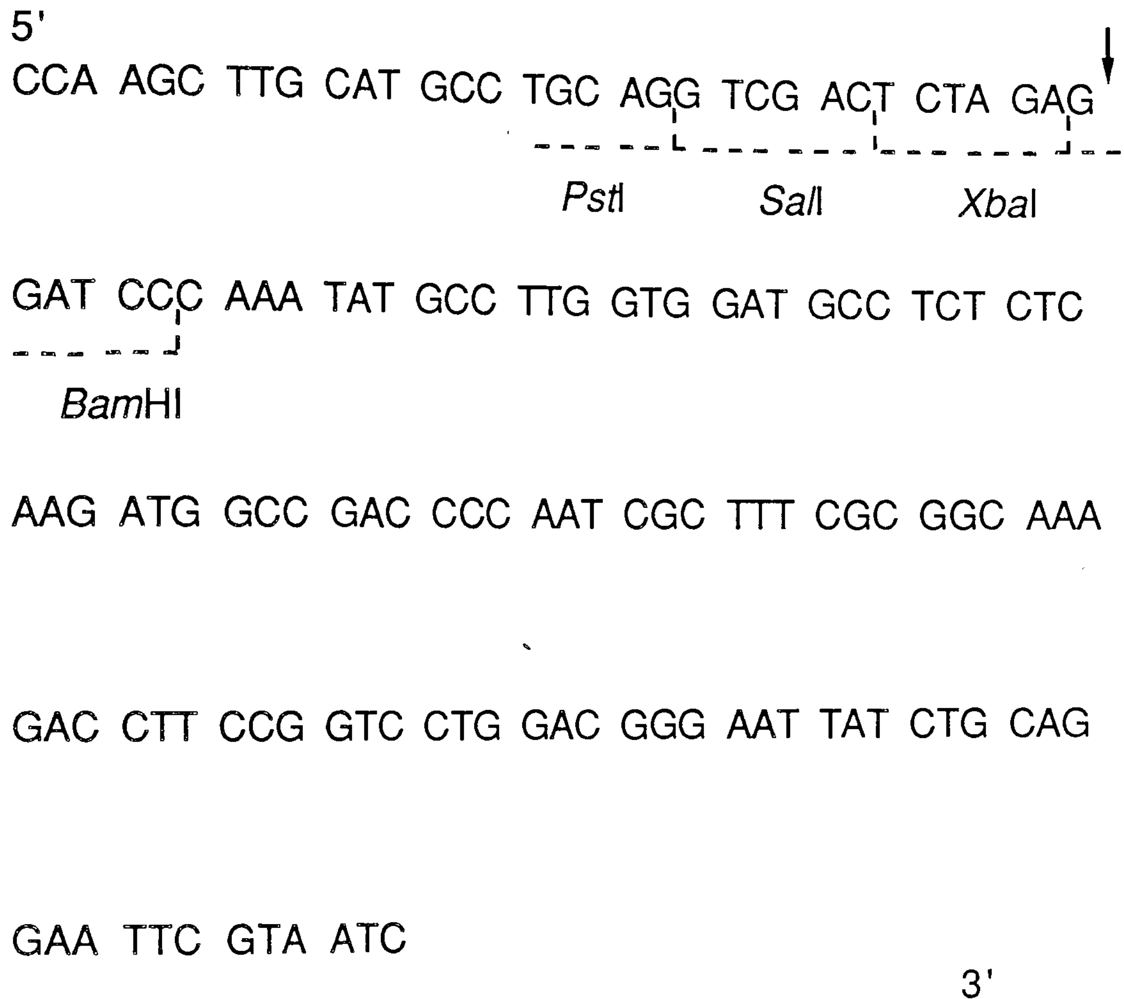
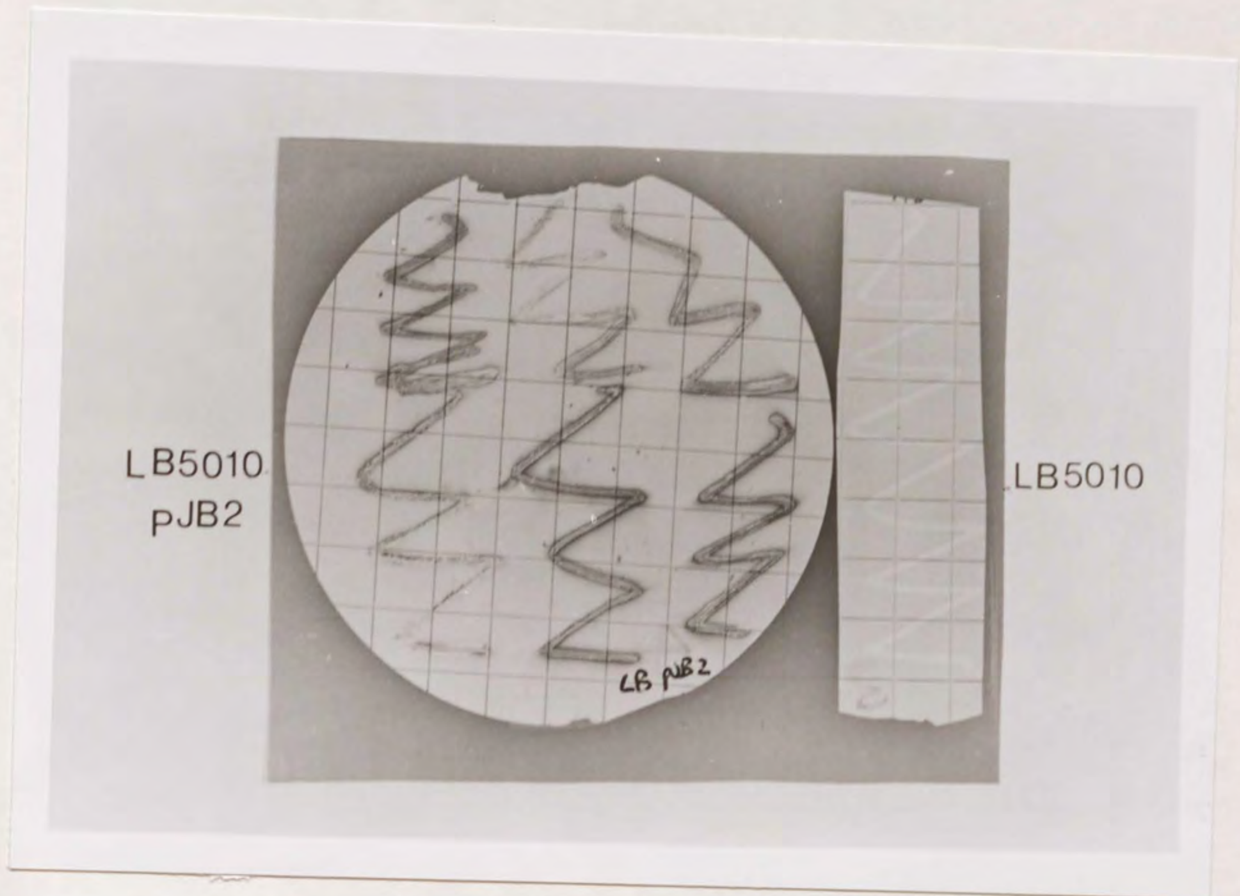


Fig 5.15 Nucleotide sequence analysis of single stranded M13 DNA templates, prepared from a 90 base *Hind*III and *Eco*RI fragment encoding gD-1 (aa 1-26) from pJB2 subcloned into M13mp18 polylinker sites *Hind*III and *Eco*RI.



90 base *Bam*HI and *Eco*RI fragment encoding gD-1 (aa 1-26) ligated in-frame into the polylinker of pUC19 at the *Bam*HI

Fig 5.16 *S. typhimurium* LB5010 harbouring plasmid pJB2 shows expression of gD-1 detected with a monoclonal antibody raised against aa 11-19 of gD-1 (P1M12).



CHAPTER 6

COLONISATION OF GUINEA PIGS WITH *SALMONELLA TYPHIMURIUM* HWSH *AROA* MUTANTS AFTER ORAL DELIVERY

6.1 INTRODUCTION

Orally fed *S. typhimurium* initially attach to, invade and persist in the gut-associated lymphoid tissue (GALT) in mice before colonising the liver and spleen (Carter and Collins, 1974). *Salmonella* can be attenuated without altering the initial colonisation of the GALT (Curtiss and Kelly, 1987; Curtiss *et al*, 1989). *S. typhimurium* HWSH *aro*A mutants were used to express glycoprotein D (as described in chapter 4) with the aim to immunise guinea pigs orally using this construction and to investigate the immune response and protection against intravaginal challenge with herpes simplex virus (HSV) 2. Therefore, before this can be achieved, the colonisation of guinea pigs with *S. typhimurium* HWSH *aro*A mutants after oral feeding was investigated as described in this next section.

6.2 MATERIALS AND METHODS

6.2.1 Materials

6.2.2 Media

Brilliant green agar (Oxoid, CM329) containing 120mg l^{-1} sodium sulphur diazine (BGSD), nutrient agar (NA) and broth (NB), minimal media consisting of 2% Noble agar (Difco), M9 salts, glucose and Ca/Mg salts as described by Sambrook *et al* (1990). Rappaport (RAPP) (Rappaport *et al*, 1956) and Selinite green enrichment broths (SGB).

6.2.3 Bacterial strains

S. typhimurium HWSH *aro*A was kindly provided by Dr G. Dougan (Wellcome Biotech, Beckenham, Kent) and has been described previously (O'Callaghan *et al*, 1988a;1990). Minimal media with or without 10μg ml^{-1} each of paraminobenzoic acid pABA) and 2,3-dihydroxybenzoic acid (Sigma) were used to confirm that the strain was auxotrophic.

6.3 Methods

6.3.1 Growth rate

The growth rate was determined by inoculating a 10 ml NB with 0.1 ml of a 10^{-5} dilution of an overnight 10 ml NB in 0.85% (w/v) saline and incubating for 24 hrs at 37°C. The broth was sampled when 0.1 ml aliquots were removed and diluted in 0.85% (w/v) saline after 2,4,8,12 and 24 hrs. Volumes of 0.1 ml of appropriate dilutions were spread over the surface of NA plates (3 replicates/dilution), incubated at 37°C and counted after 24 hrs. The mean doubling time was calculated according to the formula:-

$$\text{Growth rate } K = \frac{\log_{10}Nt - \log_{10}No}{0.301t}$$

$$\text{mean doubling time} = \frac{1}{K}$$

where t = time

No = population at a certain time

Nt = population at a subsequent time

6.3.2 Resistance to bovine serum albumin

The bacteria were grown in NB at 37°C for 18 hrs, and then 1 ml was removed and added to 10 ml of pre-warmed (37°C) NB in a 100 ml screw-capped bottle. This was incubated in a shaking incubator at 37°C for 1.5 hrs after which time the bacteria were removed by centrifugation at 2500 xg for 15 min, washed twice in 0.85% (w/v) saline and resuspended in 6 ml of 0.85% (w/v) saline. One ml of a 10^{-3} dilution of this suspension was added to 3 ml of pre-warmed serum (37°C). Volumes of 0.1 ml were removed and counted on NA (as above) and the serum bacterium mixture was incubated at 37°C for 3 hrs while being mixed continuously on a coulter roller. Further 0.1 ml volumes were removed and counted after 1, 2 and 3 hrs incubation.

6.3.3 Colonisation studies

6.3.3.1 Preparation of inocula

Bacterial suspensions for infecting guinea pigs were produced by inoculation of NB from an agar slope. The NB was incubated at 37°C for 18 hrs, after which time the *S. typhimurium* had multiplied to a concentration of approximately

10^8 ml^{-1}. The concentration of *S. typhimurium* in the inocula was determined by spreading 0.1 ml volumes of appropriate dilutions in 0.85% (w/v) saline over the surface of BGSD and NA. Plates were incubated at 37°C and colonies which developed were counted after 24 hrs.

6.3.3.2 Dosing schedule

A group of guinea pigs (Dunkin-Hartley, 300-350g), boxed singly, each received 10^9 *Salmonella typhimurium HWSH aro*A mutants mixed with 1 ml of antacid into the stomach and a second group received the same dose into the mouth. Two animals were sacrificed after 1/6/12/18 hrs and 3/6 days and post mortems carried out. Spleen, small intestine (contents and wall), caecum (contents and wall), reproductive tissues and lungs were removed aseptically and homogenised by grinding in Griffiths tubes. Tissues were weighed, appropriate dilutions in 0.85% (w/v) saline prepared (10^{-1} and 10^{-2}) and the concentration of *S. typhimurium* determined on BGSD as described previously.

Faecal samples were also collected at each time interval and approximately 1g was added to RAPP and SGB for 24 hrs at 37°C. BGSD plates were streaked and incubated for 24 hrs at 37°C to determine the presence or absence of *S. typhimurium*.

The remaining guinea pigs were observed up to 14 days at which time serum and faecal samples were collected and the antibody response determined by ELISA.

6.3.4 Enzyme linked immunosorbent assay (ELISA)

Lipopolysaccharide (LPS) antigen of *S. typhimurium* (Sigma) was dissolved in water at a concentration of 1µg ml^{-1}, mixed with an equal volume of 0.5%

sodium desoxycholate and incubated for 15 min at 37°C. It was then stored in aliquots of 0.2 ml at -20°C. Microtest plates (Falcon, 96 well flat bottom) were coated columns 1-10 with 50µl of 0.2 ml of the antigen in 20 ml of coating buffer (5x stock, 3.75g glycine, 0.209g NaF, 0.584g NaCl, 0.037g EDTA; for use dilute 1:5 and add 10% sodium deoxycholate to a final concentration of 0.1%. Adjust pH to 8.0) and incubated overnight at 4°C. The wells were washed with PBS containing 0.1% Tween 20 (PBST) and 200µl of PBST containing 0.1% BSA (PBST+BSA) was added to each well and incubated for 1 hr at 37°C.

The plates were then washed with PBST three times and 50µl of PBS+BSA was added to columns 2-10. The test sample was diluted in PBST + BSA (1:25) and added to columns 1,2,11, and 12 (50µl). Doubling dilutions were made from columnss 2-9, column 10 was left as an antigen control and columns 11 and 12 as sample controls.

After 1 hr at room temperature the plates were washed with PBS three times and 50µl of rabbit-anti-guinea pig horseradish peroxidase diluted 1:5000 in PBS+BSA from a stock 1:500 dilution kept at -20°C. After a further 1 hr at room temperature the plates were washed three times in PBS and 100µl of 3,3',5,5' tetramethyl-benzidine (TMB) substrate was added and left for 5-10 min. The reaction was stopped by the addition of 2M H_2SO_4 per well and the optical density at 450nm was read. The average of wells 11 and 12 was calculated and any well above that value was positive. Row 10 gave the background for the plate.

6.4 RESULTS AND DISCUSSION

6.4.1 Growth rate

The mean doubling time (MDT) was calculated for 0-4 hrs (29 min), 4-8 hrs (34 min), and 0-8 hrs (42 min), to account for both logarithmic growth rate and the lag phase of growth. Figure 6.1 shows that *S. typhimurium* HWSH *aro*A mutants did not achieve maximum growth until 24 hrs. The lag phase of growth was short, presumably because the inoculum was obtained from the same medium as the test medium. The most rapid period of growth was between 4 and 8 hrs after inoculation.

6.4.2 Resistance to bovine and guinea pig serum

The survival of bacteria added to bovine or guinea pig serum was measured after 1, 2 and 3 hrs; the results are shown in figure 6.2. The reproducibility of these results was determined by testing *S. typhimurium* HWSH *aro*A mutants on 3 separate occasions. The results show that *S. typhimurium* HWSH *aro*A is serum resistant and therefore a smooth strain.

Fig 6.1 Growth curve of *S. typhimurium* HWSH *aro*A mutants (A) grown in nutrient broth

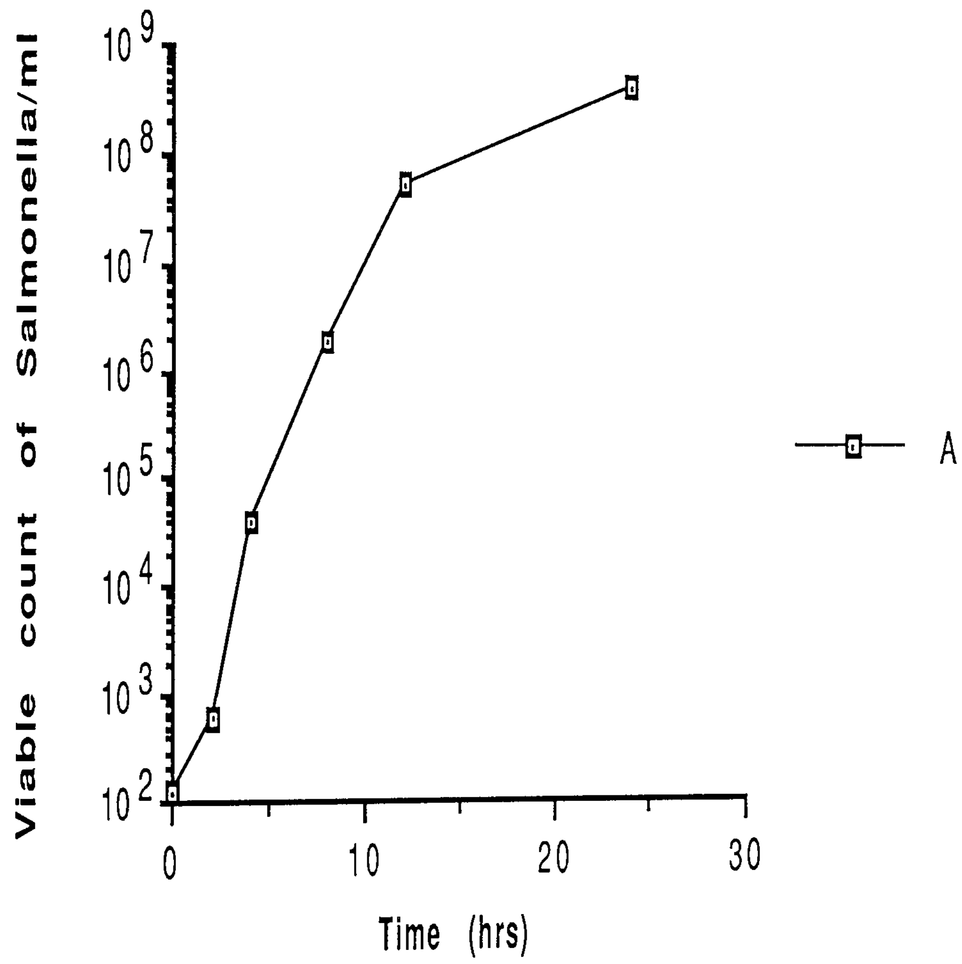
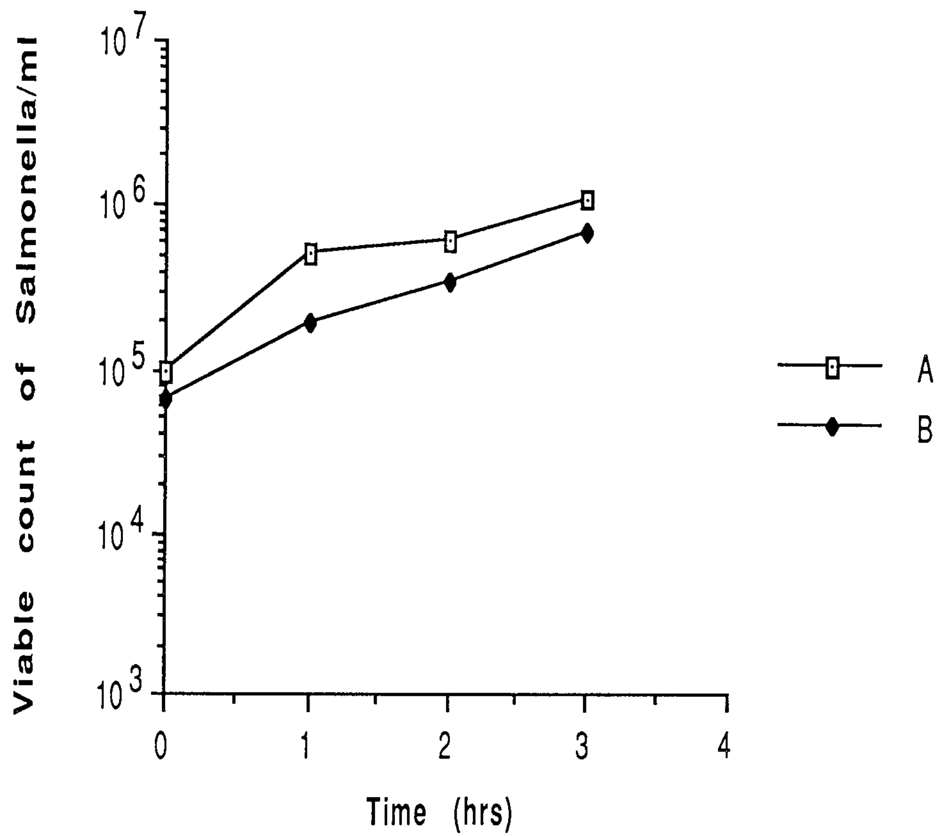


Fig 6.2 Serum sensitivity assay of log phase *S. typhimurium* HWSH *aro*A in bovine and guinea pig serum.



A-log phase *S. typhimurium* HWSH *aro*A in guinea pig serum
B-log phase *S. typhimurium* HWSH *aro*A in bovine serum

6.4.3 Colonisation studies

Colonisation of guinea pigs following oral inoculation of 10^9 *S. typhimurium* HWSH *aro*A mutants into the mouth are summarised in table 6.1. Organisms were recovered for several days after oral dosing from spleen, lungs, small intestine and caecum. Table 6.2 illustrates colonisation of guinea pigs following inoculation into the stomach using a gavage needle. Organisms were recovered for several days from small intestine and caecum. *S. typhimurium* HWSH *aro*A mutants were detected in the faeces of all animals at each sampling interval.

Methods used to infect any animal model by the oral route vary from introduction of bacteria to their drinking water to deposition of the inoculum in the stomach by means of a tube, and some techniques also involve the use of anaesthetics. Jones (1986 PhD) found that C57 mice were more susceptible to *S. dublin* infection when the inoculum was deposited at a depth of 1 cm from the incisors than when it was deposited at 2 cm, 3 cm or into the mouth. The effect was equally true with *S. typhimurium* or *S. dublin* LacG mice, and was not dependent upon the physical intrusion of the needle. In addition, variations such as inoculating the animals after starvation or the use of an antacid can affect infection using *Salmonella typhimurium*. The two methods used in these studies ie into the stomach using a gavage needle or into the mouth (Table 6.1 and 6.2) of the guinea pigs show a difference in infection.

Serum samples were obtained prior to and after 2 weeks from guinea pigs inoculated with *S. typhimurium* HWSH *aro*A mutants into the mouth. The post inoculation samples had detectable titres of IgG antibody raised against the *S. typhimurium* (log_{10} units +/- S.D.; 1.69+/-0.4). The prebleed samples had no detectable specific *S. typhimurium* IgG antibodies. O'Callaghan *et al* (1990) have shown that mice immunised with *S. typhimurium* HWSH *aro*A mutants intravenously induced both a rapid IgG and IgM anti-*S. typhimurium* response which was still

increasing 35 days after inoculation.

S. typhimrium fed to mice colonise the intestinal tract and persist and proliferate in the GALT as well as in the liver and spleen prior to establishing a fatal bacteremia (Carter and Collins, 1974). Maskell *et al* (1987) described colonisation of mice using *S. typhimurium aro*A SL3261 infected orally with 1.6×10^{10}; by day 7 post-infection *Salmonella* were detectable in livers and spleens at very low levels (10-100 bacteria per organ). Between days 7 and 14, bacterial counts increase in livers and spleens to just under 10^3 per organ on day 14. Thereafter counts fell until they were negative by day 42.

The results presented using *S. typhimurium* HWSH *aro*A dosed into the mouth with 1×10^9 organisms show a similar initial colonisation, being recovered for several days from spleen, lungs, small intestine and caecum. However, post mortems at later time points were not carried out. Work by O'Callaghan *et al* (1988a) using *S. typhimurium* HWSH *aro*A demonstrated attenuation in BALB/c mice, and persistence for several weeks in the livers and spleens of the mice after intravenous injection. They were also effective live vaccines given intravenously or orally (O'Callaghan et al, 1990).

Kent *et al* (1966) demonstrated the difficulty in infecting guinea pigs with virulent strains of *S. typhimurium.* Moore *et al* (1957) have shown that the natural route of infection for *Salmonella* in the guinea pig is the ocular route. In addition, there is a strain of *Salmonella* which naturally infects guinea pigs, *Salmonella grumpenis* (kindly provided by Dr D. O'Callaghan, Institut Pasteur, Paris). It was found that the strain could not be made *aro*A::Tn*10* using P22, suggesting it is not sensitive to this phage. An alternative method would be to attempt to attenuate it using *aro*C integration vector described by Strugnell *et al* (1990).

Several studies indicate that the longer the *Salmonella* can be recovered

the greater the immune response (Maskell *et al*, 1987; Brown *et al*, 1987; Fairweather *et al*, 1990). Therefore, a larger oral dose of *S. typhimurium* HWSH *aro*A should be investigated and the experiment extended to look at more time points. In addition, the ocular route for infection of guinea pigs would be worth investigating, as would the *Salmonella grumpenis* strain.

To conclude, these preliminary colonisation experiments suggest that the guinea pig model would be suitable to investigate immunisation of *S. typhimurium* HWSH *aro*A mutants expressing gD, subsequent immune respnses and protection against HSV-2 intravaginal challenge.

Table 6.1 Viable count of attenuated *Salmonella typhimurium* HWSH *aro*A mutants in guinea pigs following inoculation with $1x10^{9}$ organisms into the mouth.

Time after infection	GP[a] no.	viable count of *S. typhimurium*/g of: spleen	lungs	rep.[b] tissue	small intestine	small intestine contents	caecum	caecum contents
1 hr[c]	1	$1.4x10^{4}$	–	$3.0x10^{2}$	$5.8x10^{4}$	$2.1x10^{3}$	$4.3x10^{4}$	$3.8x10^{6}$
	2	$5.9x10^{2}$	$2.3x10^{7}$	$3.0x10^{3}$	$7.8x10^{4}$	–	$4.8x10^{6}$	$5.7x10^{6}$
6 hr	3	–	$9.0x10^{2}$	$4.1x10^{4}$	$3.0x10^{2}$	–	$6.5x10^{5}$	$2.7x10^{7}$
	4	$3.0x10^{2}$	$1.6x10^{6}$	$3.0x10^{2}$	–	–	$6.0x10^{3}$	$5.7x10^{4}$
12 hr	5	–	–	$6.0x10^{2}$	–	–	$3.0x10^{2}$	$3.9x10^{3}$
	6	–	–	–	$4.1x10^{4}$		$9.9x10^{5}$	$8.3x10^{5}$
18 hr	7	–	–	–	$2.3x10^{4}$	$1.2x10^{4}$	$3.8x10^{4}$	$5.2x10^{5}$
	8	–	$2.2x10^{5}$	–	–	–	$3.0x10^{3}$	$6.0x10^{3}$
3 d[d]	9	–	–	–	$6.9x10^{4}$	$5.6x10^{7}$	$8.3x10^{2}$	$9.0x10^{4}$
	10	$9.1x10^{2}$	–	–	–	$2.9x10^{2}$	–	$1.2x10^{3}$
6 d	11	$9.0x10^{2}$	$2.9x10^{4}$	–	$2.5x10^{4}$	$1.8x10^{3}$	–	$4.5x10^{2}$
	12	–	–	–	$4.7x10^{4}$	$9.8x10^{3}$	$1.0x10^{5}$	$1.8x10^{3}$

a-guinea pig; b-reproductive; c-hours; d-days.

Table 6.2 Viable count of attenuated *Salmonella typhimurium* HWSH *aroA* mutants in guinea pigs following inoculation with $1x10^9$ organisms into the stomach.

Time after infection	GP[a] no.	viable count of *S. typhimurium*/g of: spleen	lungs	rep.[b] tissue	small intestine	small intestine contents	caecum	caecum contents
1 hr[c]	1	–	$1.3x10^3$	–	$8.9x10^2$	$9.0x10^2$	$4.6x10^3$	$5.3x10^5$
	2	$1.4x10^4$	–	$1.4x10^5$	$1.3x10^3$	$2.2x10^5$	$9.8x10^5$	$8.7x10^6$
8 hr	3	–	–	–	$4.5x10^2$	–	$3.2x10^3$	$5.4x10^5$
	4	–	–	–	–	–	–	$7.2x10^3$
24 hr	5	–	–	–	$7.6x10^4$	$2.7x10^3$	$4.5x10^2$	$1.4x10^3$
	6	–	–	–	–	–	–	–
3 d[d]	7	$9.0x10^2$	–	–	$1.2x10^4$	$2.4x10^5$	$4.5x10^2$	$9.0x10^2$
	8	–	–	–	$3.1x10^4$	–	$1.1x10^4$	$1.4x10^3$
8 d	9	–	–	–	$1.3x10^2$	$1.5x10^2$	$3.2x10^2$	$2.9x10^2$
	10	–	–	–	–	–	–	–

a-guinea pig; b-reproductive; c-hours; d-days.

CHAPTER 7

CONCLUDING DISCUSSION

7.1 Parenteral delivery

There is a need for the development of new and effective delivery systems. The aim of this work has been to investigate liposomes, multiple emulsions, gels and attenuated *S. typhimurium* using tetanus toxoid, HSV and *Pseudomonas* vaccines as model systems.

Results presented in chapter 4 using Skinner HSV-1 subunit vaccine and alhydrogel show significant protection against intravaginal challenge with HSV-2 confirming the results of Welch *et al* (1988) and McBride *et al* (1988). Geeligs *et al* (1989) found that although immunisation of mice *via* the intraperitoneal route (i.p.) with a synthetic peptide of gD of HSV-1 (aa 9-21) coupled to ovalbumin combined with aluminium hydroxide stimulated induction of delayed type hypersensitivity, no significant protective immunity against i.p. challenge was generated. However, Thomson *et al* (1983) and Berman *et al* (1985) found that immunisation with HSV-1 antigens in combination with an aluminium adjuvant were protected against HSV infection. In addition, the tetanus toxoid vaccine adsorbed to aluminium hydroxide resulted in higher post vaccination IgG titres compared to immunisation of tetanus toxoid in simple solution, for both nasal and i.m. routes of delivery (chapter 3).

The results from chapter 4 using Skinner subunit HSV vaccine entrapped in DSPC liposomes to immunise guinea pigs *via* the s.c. route show that the liposomes reach post vaccination IgG antibody levels comparable to those produced by Skinner vaccine delivered with alhydrogel. In addition, both immunisation procedures resulted in a significant reduction of disease after intravaginal infection with HSV-2 compared to infected only control animals. Bakouche *et al* (1987) and Kinsky *et al* (1982) have shown that DSPC liposomes elicit strong antibody responses to membrane soluble antigens.

It has been argued that immunity to high dose virus challenge requires numerous aspects of acquired immunity to be induced including the cytotoxic

T-lymphocytes (Lawman *et al*, 1980). Unfortunately, CMI responses were not investigated however, the results using Skinner vaccine entrapped in DSPC liposomes (ie significant reduction of disease compared to the infected only group of animals) would suggest CMI responses were induced. However, this is only an assumption and needs to be investigated further. HSV-1 antigens and lipid A incorporated into PC liposomes induced high secondary antibody responses but no cell mediated immunity (Naylor *et al*, 1982).

Brynestad *et al* (1990) immunised mice i.p. using a chemically synthesised peptide corresponding to aa 1-23 of gD of HSV-1 and coupled to a fatty acid carrier. Incorporation of immunomodulators such as MTP-PE induced CMI responses and provided a significant level of protective immunity. Therefore, the use of liposomes to deliver HSV vaccines *via* the parenteral route is very promising and as more information becomes available on the use of liposomes they can hopefully be tailored to induce the desired response. With the increasing knowledge of pathogenesis of the infection and the technology for tailoring of the liposomes, induction of the desired immune response to protect against infection, is not an impossible aim.

The results from chapter 3 using tetanus toxoid either free or entrapped in DSPC liposomes to immunise guinea pigs *via* the i.m. route show that liposomes do not significantly increase the immune response to the tetanus toxoid vaccine. Davis and Gregoriadis (1987) demonstrated strong responses using liposomes entrapping tetanus toxoid with a low Tc (ie fluid liposomes), whereas responses were neglibible when the phospholipid was substituted with DSPC. Therefore, these results are the complete opposite to the response obtained with membrane antigens entrapped in DSPC liposomes (see above). Davis and Gregoriadis (1987) explained such different effects of DSPC on responses to the two types of antigen on the basis of evidence (Walden *et al*, 1985) that membrane antigens in solid (eg DSPC) liposomes are transferred into the plasma membranes of antigen presenting cells and associate

with major histocompatibility (MHC) molecules without first being processed. Soluble antigens on the other hand, must be processed by APCs prior to exposure on their surface (Allison and Byars, 1986). This could be inhibited or interfered with by high melting-point phospholipids such as DSPC (Davis and Gregoriadis, 1987) at any of the stages between liposome internalisation by cells and peptide migration to their membranes.

Gregoriadis and Panagiotidi (1989) and Xiao *et al* (1989) have shown that a phospholipid composition that produces high immune responses to a given water-soluble antigen, will not necessarily do so for another soluble antigen. Xiao *et al* (1989) suggest the need to tailor liposomal composition for individual antigens.

7.2 Oral delivery

Oral delivery of the Skinner HSV subunit vaccine to guinea pigs as free, entrapped in liposomes or in a stable w/o/w emulsion did not result in significant IgG post vaccination antibody levels or significant amelioration of the disease after intravaginal challenge with HSV-2 compared to the control infected only group (chapter 4).

Oral immunisation may result in the stimulation of local and systemic antibody-mediated and cell-mediated responses it may, under certain circumstances, result in the suppression of these same responses (chapter 1 section 1.2.2.3). This process known as oral tolerance induction has been most often demonstrated to protein antigens (Thomas and Parrott, 1974) and it has been proposed that this process protects against dietary hypersensitivities by suppressing harmful hypersensitivity responses to food antigens (Wold *et al*, 1989).

The lack of immune responses to HSV either free or entrapped in DSPC liposomes and delivered *via* the oral route may have been because the liposomes were not stable in the harsh environment of the gastrointestinal tract. The combined *in vitro*

and *in vivo* results of Chiang and Weiner (1987a; 1987b) suggest that liposomes do not influence the oral absorption of entrapped drug. Furthermore, even the most 'stable' liposomes (DSPC-as suggested by Rowland and Woodley, 1980) do not retain entrapped material in the gastrointestinal tract and hence will not protect it from the environment. However, the stability studies presented in chapter 3 section 3.3.1 suggest the liposomes would be stable in the acidic environment of the gastrointestinal tract. Therefore, an alternative explanation is that the HSV antigens, due to their nature were treated as dietary antigens and therefore no antigenicity was conferred on them when presented to the GALT (Wold *et al*, 1989). Presentation of antigen by MHC class II positive cells without a concomitant stimulation of T cells by IL's, may be tolerogenic (Markmann *et al*, 1988).

Oral delivery of tetanus toxoid vaccine as free or entrapped in DSPC liposomes resulted in comparable post vaccination IgG antibody levels to the i.m. delivery (chapter 3). It has been shown to be generally more difficult to induce oral tolerance to a particulate antigen than to a soluble antigen (Callacombe and Tomasi, 1980). The high antibody response to oral immunisation of tetanus toxoid could be due to specific receptors on the *Clostridium tetani* exotoxin, that have not been disrupted after formalin treatment, binding to the intestinal epithelium. The ability of cholera toxin and its ß subunit to prime the immune system upon oral feeding may be due to binding to or cross-linking of Gm1 ganglioside molecules on the surface membrane of lymphoid cells in the GALT (Holmgren *et al*, 1973). In addition, DeAizpura and Russell-Jones (1988) demonstrated that certain antigens, such as K99 and 987P pili (2 bacterial adhesins of enterotoxigneis *E. coli*) and lectins of varying binding specificities induced systemic and intestinal immune responses. Many molecules that have the capacity to bind to the intestinal epithelium can also be transported across the epithelial barrier, to enter the circulation and elicit an immune response. Alternatively, tetanus toxoid vaccine elicited a high immune response due to the GALT's

ability to discriminate between bacterial antigens and other soluble proteins (Wold *et al*, 1989).

The lack of improved immune responses to tetanus toxoid entrapped in DSPC liposomes and delivered *via* the oral route are firstly, that liposomes were not stable in the harsh environment of the gastrointestinal tract. However, after oral dosing of DSPC liposomes containing tetanus toxoid to rats, subsequent electron microscope examination of gut tissue sections showed liposome structures which appeared to be intact (chapter 3 section 3.3.1). In addition, the stability studies presented in chapter 3 section 3.3.1 suggest the liposomes would be stable in the acidic environment of the gastrointestinal tract. However, these are only preliminary results and further work is required to support them. Secondly, as already discussed the liposomes possibly prevented binding of receptors on the *Clostridium tetani* exotoxin to the intestinal epithelium and therefore the immune reponse was not improved.

The oral delivery of PEV-O1 *Pseudomonas* vaccine (chapter 2) resulted in comparable antibody levels to the normal parenteral delivery and the multiple w/o/w emulsion increased the antibody response compared to the the vaccine delivered in free form. Thymus-independent antigens, usualy prime the animal for an active immune response, even when presented in association with a T-dependent antigen (Stokes *et al*, 1979). Shichiri *et al* (1974) demonstrated using w/o/w emulsions a possible means of protecting insulin molecules from proteolytic destruction and of facilitating intestinal absorption of insulin after oral delivery.

To summarise, the results presented in this thesis and evidence from several studies described suggest that if non-bacterial vaccines are going to be delivered *via* the oral route they will need to be tagged onto bacterial antigens, such as cholera toxin ß subunit or lectins able to bind to the gastrointestinal tract. This will ensure presentation of the antigens in the Peyer's patches and therefore induction of an

immune response. Therefore the use of liposomes and microspheres to protect non-bacterial antigens (in particular water soluble antigens) from the harsh environment and improve absorption from the gut will need to used in association with immune stimulants such as muramyl dipeptides or interleukins if an immune response is to induced. However, this area is still wide open to question and needs further work before conclusive theories can be drawn and the way forward toward the development of oral vaccines is clear.

A different approach to pharmaceutical formulations is the use of live attenuated or genetically manipulated bacteria expressing heterologous antigens. The *Salmonella* live carrier system was investigated with the aim of immunising against HSV-2 intravaginal infection. Glycoprotein D (aa 1-314) was cloned into pUC19 to create a plasmid pJB1 which expressed gD in *S. typhimurium aroA* mutants detected by colony immunoblotting (see chapter 5) and has potential for investigating mucosal and humoral immune responses upon oral delivery. Essential preliminary studies to be carried out include colonisation of *S. typhimurium* vaccine strains harbouring pJB1, reisolation of the strains and demonstration that the plasmid pJB1 can be stably maintained *in vivo*. If the constructions prove to be stable, further experiments can be carried out to investigate if humoral an mucosal antibody responses can be elicited. In addition, the eventual aim would be to protect, after immunisation with the construction, against HSV challenge. This approach is ideal considering the evidence already discussed with regards to antigens taken up and their need to be presented in the Peyer's patches. Wold *et al* (1989) showed that OA and *E. coli* were both antigenic after immunisation directly into Peyer's patches. Therefore, attenuated strains of *Salmonella* which colonise the gut and reach the Peyer's patches means the heterologous antigens will be presented and as several investigators have shown, potentially induce protective immune responses (Poirier *et al*, 1988; Fairweather *et al*, 1989).

Several factors need to be considered in order to optimise live-carrier mediated targeting of heterologous antigens to the immune system. For example, the choice of promoter which coordinates and regulates expression of the heterologous antigens in *Salmonella* will probably ultimately depend on the nature of the protein to be expressed. However, promoters are often unregulated in *Salmonella*, and as a consequence may contribute to the instability of plasmids containing them.

One solution for the controlled expression of heterologous antigens would be to use native *Salmonella* promoters, particularly those regulated by the changes in environmental conditions that occur on entry of *Salmonella* into the target host. Recently, a class of proteins has been identified by Finlay *et al* (1989) that are induced in *Salmonella* in response to adhesion to eukaryotic cells. Charles and Dougan (1990) suggest it may be possible to design vectors based on promoters controlling expression of these genes so that heterologous antigens are produced when the *Salmonella* comes into contact with eukaryotic cells.

Other problems which may have to be considered (Charles and Dougan, 1990) include the following:-

1) heterologous expression may result in the incorrect folding and/or processing of proteins; this may be improtant for the correct localisation and immunogenicity of proteins.

2) the codon usage of the gene encoding the heterologous antigen may be suboptimal for the host, resulting in low or poorly regulated translation, or the gene may contain DNA motifs that are recognised by the host as translation pause or stop sites.

7.3 Nasal delivery

Intra-nasal immunisations may not represent a purely local response because part of the antigenic dosage may be swallowed or inhaled and lead to the stimulation of a more general immune response (Mestecky, 1987).

The Skinner HSV subunit vaccine entrapped in DSPC liposomes and delivered *via* the nasal route resulted in significant amelioration of the disease after intravaginal challenge with HSV-2 compared to the infected only group (chapter 4). The nasal route has been shown to be suitable for efficient, rapid delivery of many molecules of molecular weight <1000. With the use of adjuvants, this limit has been extended to at least 6000 (Pontiroli and Pozza, 1989; Huang *et al*, 1985). In addition, McMartin *et al* (1987) found that the relative absorption of cyclic and cross-linked peptides and proteins was significantly greater than that of linear peptides. The results presented demonstrate that large molecular weight glycoproteins of HSV entrapped in DSPC liposomes can be taken up *via* the nasal route. Examples of nasal immunisation using vaccines include, administration of Pseudogen vaccine *via* intra-nasal spray to cystic fibrosis patients (Wood *et al*, 1983). This resulted in slight increases in serum antibody titres but no appreciable rise in specific antibody levels in saliva or sputum. Reynolds *et al* (1974) showed that intra-nasal immunisation of rabbits with *Pseudomonas* LPS induced both cellular and humoral immunity in rabbits.

HSV delivered nasally in PBS resulted in high post vaccination antibody titres but no significant reduction of disease after intravaginal challenge with HSV-2 compared to the infected only group. However, as already mentioned in chapter 3 for direct comparison with nasal delivery using liposomes the two immunisation procedures should be carried out in the same experiment. With this in mind, possible reasons for the increased protection when the vaccine was delivered entrapped in DSPC liposomes are firstly due to protection of the vaccine from the enzymes present in the nasal cavities. In the previous section conflicting results were quoted on the stability of liposomes in the gastrointestinal tract. The environment of the nasal cavity has been shown to be less harsh and used to absorb peptides that are usually digested and broken down in the gut. The present results suggest the liposomes were stable in the nasal cavity and taken up more readily by the nasal epithelium compared to free antigen.

Liposomes taken up intact will pass directly to the circulation. 'High melting' phospholipids such as DSPC are more resistant to plasma high density lipoproteins (HDL) which remove phospholipid from the bilayers (Kirby *et al*, 1980). Gregoriadis (1984) found that by making liposomes smaller their circulation time could be prolonged to some extent. However, these are only assumptions and more work is needed before logical conclusions can be drawn.

Nasal delivery of HSV using aerosil 200 gel did not improve the immune response compared to free antigen. However, other studies using gels have shown increased uptake of the antigen being investigated. For example, Morimoto *et al* (1987) have studied nasal absorption of nifedipine from polyethylene glycol (PEG) 400, aqueous carbopol gel and carbopol-PEG gel. Nasal administration of nifedipine in PEG resulted in rapid absorption and a high maximum concentration. However, the elimination of nifedipine from plasma was very rapid. Carbopol-PEG gel (containing 50% w/v PEG) showed a relatively high nifedipine concentration and a prolonged action. Nasal administration of nifedipine in an aqueous carbopol gel resulted in very low plasma concentration.

Nasal delivery of tetanus toxoid as simple solution or adsorbed to aluminium hydroxide resulted in comparable post vaccination IgG antibody levels to the normal i.m. parenteral route (chapter 3). The aerosil gel used with the adsorbed tetanus toxoid did not improve the IgG response compared to free antigen. However, the nasal route was technically difficult to deliver the vaccine and ensure the correct dose was administered. The nasal delivery of adsorbed tetanus toxoid with aerosil resulted in a high immune response and a high standard deviation, suggesting that some of the animals were immunised and others were not. Therefore, from individual results (not tabulated) the aerosil gel did improve the immune response, although not significantly. A lower dosage of adsorbed tetanus toxoid (15 or 30μg) delivered *via* the nasal route resulted in IgG post-vaccination antibody levels that were lower than those obtained

via i.m. delivery of 6μg. However, a dosage of 15 or 30μg of tetanus toxoid in simple solution resulted in comparable antibody levels to the i.m. delivery. Therefore, a lower dose of tetanus toxoid in simple solution (15μg) will give a response comparable to the i.m. delivery (6μg). Tetanus toxoid has been used to immunise animals by several investigators. For example, Wigley *et al* (1969) immunised human volunteers with soluble tetanus toxoid given as an aerosol (particle size 1μm) which resulted in an increase in serum antibody titre comparable to that resulting from conventional s.c. administration. No antibody titres were found in nasal secretions probably because the particles were not large enough to lodge in the nasal passages. Lower respiratory tract antibody production did occur; probably because of the deep penetration of the small particles into the respiratory tract. Therefore, secretory antibody can be produced in response to an antigen that normally does not enter the respiratory tract.

Gerbrandy and van Dura (1972) immunised mice with a high dose of tetanus toxoid (64Lf) and $5x10^8$ cell equivalents of a desoxycholate extract of *Bordetella pertussis* cells which resulted in high antibody responses in sera, bronchial and nasal washes. Tetanus toxoid can obviously induce a high immune response *via* the nasal route of delivery which is comparable to the normal i.m. route.

The use of tetanus toxoid entrapped in DSPC liposomes did not improve the response compared to delivery of free antigen. As already mentioned the stability of the lipsomes is a very conflicting area. One assumption, is that the liposomes are not stable and therefore make no improvement compared to the delivery of free antigen. However, the results presented in this thesis suggest tetanus toxoid entrapped in DSPC liposomes are taken up intact in the gut. The conditions in the nasal cavity are less harsh, suggesting they would be stable. In addition, if the liposomes were taken up intact, the subepithelial layer of the nose is highly vascularised and therefore liposomes will pass directly into the systemic circulation. However, liposome properties such as permeability, size and surface charge are altered rapidly and they

can be broken down by lipoproteins, circulating phospholipases or complement (Patel and Ryman, 1981).

To conclude, the work presented in this thesis has attempted to investigate non-parenteral delivery systems in order to induce systemic and mucosal immunity. Promising results such as nasal immunisation with HSV subunit vaccine entrapped in DSPC liposomes and subsequent reduced infection after challenge with HSV-2 support the need for further investigation into the nasal route of delivery. In addition, the work presented has confirmed the difficulties in delivering vaccines *via* the oral route, but also suggesting that pharmaceutical approaches can be tailored to induce the desired immune response, especially with the increasing knowledge of antigen uptake and presentation to the gut associated lymphoid tissue. Alternatively, the use of attenuated or genetically manipulated bacteria expressing heterologous antigens is a promising are of study for oral delivery. This thesis has demonstrated expression of gD of HSV-1 in *S. typhimurium aro*A mutants and we can look forward to hopefully, some interesting results from future work with these constructs.

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