STUDIES OF MEMBRANE FUSION BY INFLUENZA HAEMAGGLUTININS, AND THEIR APPLICATION TO LIPOSOME DELIVERY SYSTEMS IN GENE THERAPY

Thesis submitted in accordance with the
requirements of the
UNIVERSITY OF LONDON
for the degree of
DOCTOR OF PHILOSOPHY
by

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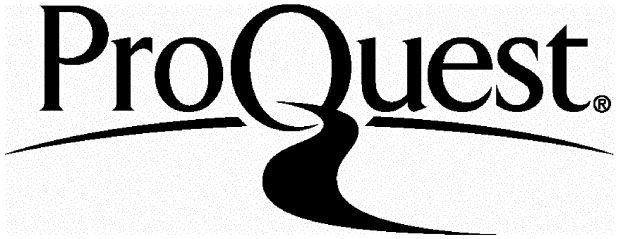

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BHA	Bromelain released haemagglutinin
BSA	Bovine serum albumin
Buffer I	100 mM NaCl/2 mM EDTA/100 mM borate/50 mM citrate/0.01% (w/v) NaN_3
$C_{12}E_8$	Octaethylene glycol monododecyl ether
CFTR	Cystic fibrosis transmembrane conductance regulator
CHA	Complete haemagglutinin
Chol	Cholesterol
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra - acetic acid
ELISA	Enzyme linked immuno sorbent assay
GFP	Green fluorescent protein
gp	glycoprotein
HA	Haemagglutinin
HEPES	N-[2-Hydroxyethyl]piperazine - N' -[2-ethanesulfonic acid]
HIV	Human immunodeficiency virus
N-NBD-PE	N-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine
N-Rho-PE	N-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine
NA	Neuraminidase
OG	Octyl-glucoside
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PDP-PE	N-3-(Pyridyl-2-dithio) Propionyl Phosphatidylethanolamine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PS	Phosphatidylserine
R.E.T	Resonance energy transfer
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SM	Sphingomyelin
SPDP	N-Succinimidyl 3-(2-Pyridyldithio) Propionate
Tris	tris[hydroxy methyl] aminomethane

1 ABSTRACT

The aim of the work presented in this thesis was to investigate the membrane fusion activity of influenza haemagglutinin (HA), particularly with regard to the contribution of receptor binding to the efficiency of the membrane fusion process. Since the membrane fusion potential of HA could provide an efficient mechanism to deliver molecules to the cell cytoplasm the ability of HA - containing liposomes to fuse with membranes was also established.

The role of receptor binding by HA with regard to the efficiency of membrane fusion has been extensively investigated. Liposome coupled anti-HA monoclonal Fab' fragments with various specificities towards the HA molecule were used as surrogate receptors for HA. Electron microscopy (EM) studies of HA-receptor complexes using liposome coupled Fab' fragments as surrogate receptors are reported.

On the basis of results from EM, membrane fusion experiments were done between HA containing lipid vesicles (virosomes) and anti HA Fab' coupled liposomes. The virosomes used contained two antigenically distinct strains of HA, which underwent their acid induced conformational change at a significantly different pH from each other. Anti HA Fab' fragments which recognised one of the two HA strains were used as surrogate receptors. These experiments concluded that HA bound to receptor can be more efficient at causing membrane fusion than a HA molecule held close to but not directly bound by the target membrane. These results have implications for the design of a HA based delivery vector which are discussed.

Various procedures were investigated for the reconstitution of HA containing lipid vesicles (virosomes), using purified HA and purified lipids. Using the detergent octaethylene glycol monododecyl ether virosomes with a lipid composition of

phosphatidylcholine and cholesterol (2:1 molar ratio) were made. These virosomes could participate in HA mediated membrane fusion. Virosomes made using the detergent octylglucoside were not fusion - active. The fusion efficiency of reconstituted vesicles containing HA was shown to be lower than that of influenza virus. Initial experiments using virosomes as vehicles for DNA delivery are also presented.

2 INTRODUCTION

2.1 INTRODUCTION

Influenza haemagglutinin (HA) is a membrane glycoprotein which binds to sialylated cell surface receptors, and following receptor mediated endocytosis undergoes an irreversible conformational change triggered by low endosomal pH which causes fusion of the viral and endosomal membranes. As a consequence the viral nucleocapsid enters the cell and virus replication begins (Wiley & Skehel, 1987). It is proposed to use the membrane fusion potential of HA to develop a vector able to deliver substances efficiently to specific cell types. With the development of a HA based delivery vector in mind, methods for reconstituting HA into lipid vesicles and the role of receptor binding by HA with regard to the efficiency of membrane fusion have been investigated.

This introduction will discuss the problems faced when delivering therapeutic molecules to cells and the potential advantages of the proposed HA delivery vector will be discussed. The introduction will also discuss influenza HA in detail.

2.2 THE PROBLEMS ASSOCIATED WITH THE SPECIFIC AND EFFICIENT DELIVERY OF THERAPEUTIC MOLECULES TO CELLS

By reconstituting HA into lipid vesicles and entrapping therapeutic molecules within the lipid vesicle it may be possible to deliver efficiently the entrapped molecules to the cell cytoplasm. Alternatively, hydrophobic therapeutic agents could be incorporated into the lipid bilayer of the vesicles. Cell delivery of therapeutic molecules entrapped within the lipid vesicles would result from HA mediated cell

attachment and following receptor mediated endocytosis, low pH induced HA mediated membrane fusion between the lipid vesicle and endosomal membrane, resulting in release of the therapeutic molecules into the cell cytoplasm. This mechanism of delivery is obviously analogous to the cytoplasmic delivery by influenza virus of the viral nucleocapsid. It may be possible to deliver efficiently a wide variety of drugs, proteins or nucleic acid using the proposed HA delivery vector, in the later case using the subsequent expression of the nucleic acid for some therapeutic purpose. If used to deliver nucleic acid the proposed HA delivery vector would fall within the definition of a gene therapy vector (Smith, 1995). To illustrate the problems associated with the delivery of therapeutic molecules to cells, examples of other gene therapy vectors are cited, which will lead to a discussion concerning the potential advantages of a HA based delivery vector.

Effective gene therapy not only requires the identification of an appropriate therapeutic gene for treatment of a disease, but also depends on the development of vectors which can deliver therapeutic genes to the intended target cell efficiently and specifically. The ideal vector would deliver to a high proportion of target cells, resulting in the expression of the gene it carries for as long as required in an appropriately regulated fashion without toxicity or significant anti-vector inflammatory or immune responses even after multiple administrations. The vector would be stable, easy and reproducible to produce and purify in large quantities and at high concentration. The criterion for a successful vector varies depending on the proposed application and it is likely that a collection of vectors will be used, each being suitable for a specific application.

2.2.1 Viral gene therapy vectors

Viral gene therapy vectors are based on replacing non essential parts of the viral genome with the therapeutic gene of interest, producing replication deficient infectious virions.

2.2.1.1 Retroviral gene therapy vectors

Retroviral vectors have been widely used for *ex vivo* gene therapy trials and have been recently reviewed (Jolly, 1994; Smith, 1995; Gunzburg & Salmons, 1996; Vile *et al.*, 1996).

Retroviruses are enveloped viruses, which contain oligomeric membrane spike glycoprotiens (Env proteins) which mediate cell attachment and entry. Retroviral vector delivery results in the integration of the therapeutic gene into the host genome (Coffin, 1996), it is possible that integration of vector DNA into the host genome within or near critical cellular genes could cause neoplastic transformation of the patients cells, although theoretical models suggest the overall risk to be low (Moolten & Cupples, 1992). Moloney Murine Leukemia Virus (Mo-MLV) is the prototype retroviral vector and is presently the most efficient vector identified for gene therapy (Miller, 1992).

There is a small risk that replication deficient recombinant retrovirus released from packaging cells could become contaminated with replication-competent virus formed by recombination (Scadden *et al.*, 1990). Second- and third- "generation" cell lines have been developed which make this possibility very unlikely although still possible (Miller & Buttimore, 1986; Danos & Mulligan, 1988; Dougherty *et al.*, 1989).

In many cases for *in vivo* gene therapy "effective" retrovirus titres are too low which is a major obstacle to the effective use of retroviral vectors. "Effective" *in vivo*

retrovirus titres are relatively low compared to *in vitro* titres due to sensitivity of Mo-MLV vectors to complement; Mo-MLV vectors also require that cells must be mitotically active for productive infection to occur. Approaches to these problems have included production of vectors that are resistant to human complement inactivation (Takeuchi *et al.*, 1994; Cosset *et al.*, 1995; Rigg *et al.*, 1996), vectors based on human immunodeficiency virus (HIV) which can infect non-dividing cells (Lewis *et al.*, 1992; Akkina *et al.*, 1996; Naldini *et al.*, 1996 a & b) and the production of more stable pseudotyped retroviruses which can be produced at titres of $>10^9$ infectious particles /ml (Emi *et al.*, 1991; Burns *et al*, 1993; Yee *et al.*, 1994; Ory *et al.*, 1996). For *ex vivo* protocols, which have been the major application of retroviral vectors the problems of low titre are less important.

Wild type MLVs do not show restricted tropism at the level of cell surface binding and use of retroviral vectors for *in vivo* gene therapy has been limited because of the risk of infecting bystander cell types. Designing retroviral vectors with restricted tropism is currently complex (Miller, 1996), as the structure of the receptor binding subunits of retroviral Env proteins is not known, and for some retroviruses two different cell surface receptors are needed for cell entry (Deng *et al.*, 1996; Dragic *et al.*, 1996).

A strategy to restrict retrovirus tropism using information concerning receptor binding and virus entry (Battini *et al.*, 1992; Ott & Rein, 1992; Morgan *et al.*, 1993) has been to incorporate hybrid retroviral Env protein into the retroviral envelope. For example a Mo-MLV containing a chimeric Env protein composed of the polypeptide hormone erythropoietin (EPO) and part of the Mo-MLV Env protein showed a modified tropism (Kasahara *et al.*, 1994). Other reports have described envelopes

containing single chain antibody-Env chimeras (Somia *et al.*, 1995; Hua *et al.*, 1997) and CD4-Env chimeras (Matano *et al.*, 1995).

2.2.1.2 Adenoviral gene therapy vectors

Adenoviral vectors unlike retroviruses remain stable when purified, high titre adenovirus containing 10^{12} particles/ml can be easily obtained. Adenoviral vectors can infect quiescent and dividing cells and have been widely used for *in vivo* gene therapy trials and have been recently reviewed (Ali *et al.*, 1994; Jolly, 1994; Smith, 1995; Descamps *et al.*, 1996).

Adenoviruses are a family of non-enveloped icosohedral double stranded DNA viruses with the ability to infect many cell types (Stratford-Perricaudet *et al.*, 1992). Viral replication is in the nucleus of the cell, normally without integration into the host genome. The genome is organised into four early regions (E1-E4) and one major late region (Shenk, 1996).

The capsid, which contains the viral DNA is composed of three protein subunits, the hexon, the penton base and the penton fibre. The penton fibre, alone mediates viral attachment to the cell surface (Philipson *et al.*, 1968; Henry *et al.*, 1994) following which the penton base binds $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors which mediate virus internalization into cells via receptor-mediated endocytosis (Wickham *et al.*, 1993; Shenk, 1996).

Recombination during the production of first "generation" E1 replacement adenoviral vectors produced replication competent adenoviruses (Smith, 1995). Development of adenoviral vectors containing no viral genes and only essential cis elements has been reported (Haecker *et al.*, 1996; Kochanek *et al.*, 1996; Kumar-Singh & Chamberlain, 1996; Fisher *et al.*, 1996; Chen *et al.*, 1997), which are very

Has he heard of the dodecahedron?

unlikely to undergo homologous recombination. Adenoviral vectors are presently based on human adenoviruses which could be present in patients, leading to possible recombination in target cells.

Transient gene expression of 2-3 weeks has been reported for genes delivered by first generation adenoviral vectors (Rosenfeld *et al.*, 1992; Grubb *et al.*, 1994; Zabner *et al.*, 1993; Knowles *et al.*, 1995; Crystal *et al.*, 1994; Mastrangeli *et al.*, 1993). The relatively short time of gene expression observed with first "generation" vectors was not due to the episomal nature of the vector but has been shown to be due to low level adenoviral vector gene expression causing the target cell to be identified by viral specific cytotoxic T-lymphocytes (CTL) causing destruction of the transformed cell (Yang *et al.*, 1994 & 1996). CTL responses to "non-self" therapeutic gene products have in some cases also contributed to destruction of transformed cells (Tripathy *et al.*, 1996). Strategies to avoid viral specific CTL responses have included the production of adenovirus vectors which do not give rise to viral gene expression, for example vectors deleted in both E1 and E2a (Gorziglia *et al.*, 1996) or lacking all viral genes (Chen *et al.*, 1997).

Administration of adenoviral vectors leads to non specific inflammation followed by humoral responses that produce neutralizing antibodies against the capsid proteins of the vector (Crystal *et al.*, 1994; Yang *et al.*, 1995a), this prevents or substantially reduces the efficacy of repetitive administration of adenoviral vector (Yei *et al.*, 1994). Possible strategies to avoid humoral responses include transient immunosuppression at the time of initial exposure to adenoviral vectors (Yang *et al.*, 1995b; Smith *et al.*, 1996) or use of a different serotype of adenovirus vector at each administration (Kass-Eisler *et al.*, 1996).

But probably not different from HA ciprosomes, see pg 11 re HA subtypes.

To produce a targetable vector it is proposed to limit adenoviral tropism by modification to the penton base and or penton fibre. Strategies to achieve restricted tropism have included addition of short peptide ligands at the carboxyl terminus of the fibre protein (Michael *et al.*, 1995) and use of bispecific antibodies that bind the penton base at one end and at the other end contain a specificity which targets adenovirus to a specific cellular receptor (Wickham *et al.*, 1996).

2.2.2 Non-viral gene therapy vectors

The safety concerns and the difficulty of obtaining a large quantity of certain recombinant viral vectors have prompted efforts to develop non-viral gene therapy vectors.

2.2.2.1 Cationic liposomes

Cationic liposomes form complexes with DNA through charge interactions. DNA-liposome complexes bind to the negatively charged cell surface due to presence of excess positive charge in the complex, resulting in efficient transfection of many cell types (Felgner *et al.*, 1987; Gao & Huang, 1995; Egilmez *et al.*, 1996). It has been proposed that the liposome-DNA complexes enter the cell via an endocytotic pathway followed by destabilization of endosomal membrane (Zhou & Huang, 1994; Zabner *et al.*, 1995; Xu & Szoka, 1996). It has also been suggested cationic liposomes deliver DNA directly across the plasma membrane (Felgner & Ringold, 1989). In contrast to viral nucleic acid the DNA delivered to the cytoplasm by cationic liposomes has no inherent mechanism to target it to the nucleus (Felgner, 1993), as a consequence most of the DNA is destroyed by cytoplasmic degradation (Capecchi, 1980) resulting in decreased efficacy.

which viral nucleic acid? adenoviral DNA? What is the mechanism

Liposome-DNA complexes have minimal toxicity when applied systemically (Stewart *et al.*, 1992) and to the airways of mice (Alton *et al.*, 1993) and humans (Caplen *et al.*, 1995) which is a major advantage over adenoviral vectors.

Short term transient transgene expression has been achieved using cationic liposomes (Stewart *et al.*, 1992; Alton *et al.*, 1993; Nabel *et al.*, 1993; Caplen *et al.*, 1995). The transfection efficiency *in vivo* of cationic liposomes is relatively low in comparison with what can be achieved with adenoviral vectors since substances in the body fluids such as serum proteins and mucus significantly reduce the transfection efficiency (Gao & Huang, 1995). A major problem with cationic liposome vectors is the lack of target specificity.

2.2.2.2 Incorporation of proteins into liposome vectors

It has been shown that DNA can be delivered to cells *in vitro* by using negatively charged phosphatidylserine containing liposomes which contained entrapped DNA (Fraley *et al.*, 1980; Schaefer-Ridder *et al.*, 1982). However, problems associated with the delivery of DNA entrapped within liposomes include low DNA encapsulation efficiency as discussed by Felgner (1993) and lysosomal degradation of phagocytically internalized liposomes. To avoid the latter phenomenon pH sensitive liposomes have been developed (Horwitz *et al.*, 1980; Wang & Huang 1989; Budker *et al.*, 1996). Alternatively, incorporation of surface glycoproteins from enveloped viruses into liposomes could result in an efficient transfection method by enabling efficient cell entry. Various studies have used sendai virus glycoprotein incorporation into liposomes. Sendai virus contains two glycoproteins in its envelope, haemagglutinin-neuraminidase which binds to sialoglycoproteins and sialoglycolipids and the fusion (F) glycoprotein which induces

membrane fusion with the plasma membrane at neutral pH (Lamb & Kolakofsky, 1996). It has been demonstrated that liposomes containing the sendai F protein can deliver their liposomal contents to the cell cytoplasm via fusion with the cell plasma membrane (Bagai & Sarkar, 1993; Dzau *et al.*, 1996).

Viral glycoproteins, such as haemagglutinin-neuraminidase of sendai virus do not have receptors that are restricted to a limited cell type, therefore inclusion of a wild type viral glycoprotein within a liposome will not serve as a specific binding molecule to target liposome vectors (Blumenthal & Loyter, 1991).

2.2.3 Sterically stabilized liposomes

Uptake by tissue macrophages mainly in the liver and spleen is responsible for the rapid removal from circulation of conventional liposomes, such as those composed of phosphatidylcholine and cholesterol (Gregoriadis & Ryman, 1972). This severely limits their application as systemic gene therapy vectors. Macrophages ingest and digest non-self and altered-self particulate material as a mechanism of homeostasis. Liposomes clearance is thought to occur by a two step mechanism, opsonization of liposomes by blood proteins followed by macrophage uptake of the marked liposomes (reviewed by Woodle & Lasic, 1992).

Sterically stabilized liposomes (S-liposomes) containing ganglioside GM1 or lipid derivatives of poly(ethylene glycol) have significantly reduced affinity for macrophages (Allen & Chonn, 1987; Allen *et al.*, 1991). It has been suggested that increased hydration of the liposome surface of S-liposomes reduces electrostatic and hydrophobic interactions with a variety of blood components, thus explaining the reduced affinity of S-liposomes for macrophages (Lasic *et al.*, 1991; Woodle & Lasic, 1992). The size of S-liposomes also effects their half life *in vivo* (Litzinger *et al.*,

1994). S-liposomes have been investigated for their potential to entrap and deliver cytotoxic drugs (Allen, 1994; Gregoriadis, 1995), coupling of ligands to the liposome surface can induce target specificity (Flasher *et al.*, 1994; Allen *et al.*, 1995).

2.2.4 Development of an influenza haemagglutinin (HA) - liposome based delivery vector

As illustrated above a problem common to all the present delivery vectors is the lack of target specificity when delivering to cells *in vivo*. Delivery of nucleic acid is most efficient when using viral vectors, but problems associated with viral vectors include contamination of viral vector with replication competent virus, immune responses to the vector or transfected cell and possible insertional mutagenesis when using retroviral vectors. Non viral vectors generally avoid the safety concerns associated with viral vectors but have significantly lower transfection efficiencies *in vivo* when compared to viral vectors.

By entrapping therapeutic molecules, such as DNA within S-liposomes together with introduction of influenza haemagglutinin (HA) into the liposomal membrane a gene therapy vector with potentially significant advantages could be developed. Due to the lipid composition of S-liposomes the proposed vector would be expected to have low affinity for tissue macrophages and due to the influenza HA, have a cell entry mechanism with comparable efficiency to that of a viral vector. The proposed vector avoids some of the major safety concerns associated with viral vectors.

It will be necessary to develop a HA delivery vector that can bind to specific cell types. HA mediates cell attachment by binding sialic acid residues which are present on the surface of many cell types. Due to the extensive characterization of HA

(Section 2.3) it may be possible to produce a modified HA, containing a specific ligand binding domain in place of the sialic acid binding domain. Following receptor mediated endocytosis it is proposed that the modified HA could mediate low pH induced membrane fusion resulting in release of the liposomally entrapped molecules to the cytoplasm.

An alternative vector design would be to produce mutant HAs in which the sialic acid binding site was mutated in such a way as to inhibit sialic acid binding, preventing wide spread binding of HA to sialic acid residues. To produce specificity of binding antibodies could be coupled to the HA - liposomes to enable specific cell attachment. The antibody should mediate efficient cell attachment and following receptor mediated endocytosis HA could mediate low pH induced membrane fusion of the liposomal and endosomal membranes.

The proposed HA-liposome vector would be expected to elicit the production of neutralizing antibodies directed against HA when applied *in vivo.* Such a response has already been observed against the capsid proteins of adenoviral vectors which prevents or substantially reduces the efficacy of repetitive adenoviral administration *in vivo* (Yei *et al.*, 1994). There are currently fifteen antigenically distinct influenza A HA subtypes (Rohm *et al.*, 1996), all of which could be used in subsequent applications of a HA-liposome vector, therefore avoiding the problems associated with repeated vector application. A potential problem remains that any specific binding domains engineered within HA could result in neutralizing antibodies which bind to epitopes contained within this domain, repeated administration of the vector could be inhibited by this antibody response.

The lipid composition of a HA-liposome vector will be very important as conventional liposomes have very short half lives in circulation (Gregoriadis & Ryman,1972). A lipid composition including moieties such as the ganglioside GM1 or lipid derivatives of poly(ethylene glycol) will be necessary to reduce the affinity of the liposomes for tissue macrophages.

A modified HA molecule containing a specific ligand binding domain could alternatively be used to produce pseudotyped retroviral vector. HA in the retroviral envelope could provide the retrovirus with binding specificity, a cell entry mechanism and structural stability. Lack of stability in purified retrovirus has been a major limitation in the use of retroviral vectors.

In the following sections influenza HA is described in detail.

2.3 INFLUENZA HAEMAGGLUTININ (HA)

2.3.1 Influenza virus

Influenza viruses are members of the orthomyxoviridae family of viruses, which have been comprehensively reviewed (Krug, 1989; Lamb & Krug, 1996). Influenza viruses are divided into types A, B and C based on differences between their nucleoprotein and matrix protein antigens. Influenza A viruses are assigned to specific subtypes based on the antigenicity of the major surface glycoproteins haemagglutinin (HA) and neuraminidase (NA). As previously explained there are currently fifteen influenza A HA subtypes (Rohm *et al.*, 1996), three of which H1, H2 and H3 are known to have infected humans. and H5?

The lipid envelope of the influenza A virion contains three integral membrane proteins - HA, NA and M2 a proton channel protein. The virion matrix protein M_1 is thought to underlie the lipid bilayer and also interact with the ribonucleoproteins.

HA has two functions in the initial stages of virus infection. It binds to sialylated cell surface receptors, and following receptor mediated endocytosis undergoes an irreversible conformational change triggered by low endosomal pH which causes fusion of the viral and endosomal membranes. As a consequence the viral nucleocapsid enters the cell and virus replication begins (Wiley & Skehel, 1987). HA belongs to a large class of fusion glycoproteins, including the fusion glycoproteins of alphaviruses, rhabdoviruses, retroviruses and paramyxoviruses (Gaudin *et al.*, 1995).

2.3.2 Native haemagglutinin

Native X-31 HA from influenza virus A/Aichi/2/68 (H3 subtype) has a relative molecular mass of 220,000 and is a homotrimer. Each subunit contains two glycopolypeptide chains, HA_1 (328 residues) and HA_2 (221 residues) which are linked

by a single disulphide bond. HA_1 and HA_2 are formed by proteolytic cleavage of the precursor HA_0. HA_2 is anchored in the viral membrane by its carboxy terminus, with eleven carboxy-terminal amino acids internal to the virus membrane (Wiley & Skehel, 1987).

Treatment of native HA with the protease bromelain cleaves HA_2 at its C-terminus after residue 175 (Brand & Skehel, 1972; Skehel & Waterfield, 1975; Ward & Dopheide, 1980) which releases the soluble trimer BHA. Native BHA has been crystallized and the structure solved by X-ray diffraction (Wilson *et al.*, 1981; Weis *et al.*, 1990; Watowich *et al.*, 1994) (Figure 2.3.1). The HA_1 chains form three membrane distal globular domains containing the receptor binding site (Weis *et al.*, 1988; Sauter *et al.*, 1992) and the sites to which neutralizing antibodies bind (Wiley *et al.*, 1981). A highly conserved hydrophobic sequence at the N terminus of HA_2 is referred to as the "fusion peptide" which is buried in the native structure. BHA_2 forms the majority of the α-helical stem domain which forms the centre of the molecule.

2.3.3 The acid induced conformation of haemagglutinin

Low pH treatment induces a major conformational change in the HA_2 subunit, leading to exposure of the "fusion peptide" (Skehel *et al.*, 1982; Ruigrok *et al.*, 1988). Low pH induces the HA_1 subunits to come apart (Godley *et al.*, 1992), but the monomeric structure of HA_1 is not significantly modified as shown by the crystal structure of "HA top" released by Endoproteinase LysC digestion of the low pH conformation of X-31 HA complexed with a Fab of a neutralizing antibody (Bizebard *et al.*, 1995). Low pH HA can still bind to viral receptors (Sauter *et al.*, 1989) and is

recognised by most monoclonal antibodies against native HA (Daniels *et al.*, 1983; Webster *et al.*, 1983; Yewdell *et al.*, 1983).

BHA in the low pH conformation aggregates via exposure of the "fusion peptide" (Skehel *et al.*, 1982; Ruigrok *et al.*, 1988). Aggregates can be solubilized by successive digestion with trypsin and thermolysin removing residues 28-328 of HA_1 and 1-37 of HA_2 (Ruigrok *et al.*, 1988; Bullough *et al.*, 1994b). The solubilized trimeric fragment $TBHA_2$ produced has been crystallised and the structure solved (Bullough *et al.*, 1994 a & b) (Figure 2.3.1).

The $TBHA_2$ structure implies that the conformational change of HA facilitates membrane fusion by insertion of the "fusion peptide" into the target membrane as hypothesised previously (Skehel *et al.*, 1982; Wiley & Skehel, 1987; Carr & Kim, 1993), although as yet there is no conclusive evidence for this hypothesis. Some evidence that the "fusion peptide" inserts into the target membrane comes from photolabelling studies, in which it has been shown that upon lowering the pH the "fusion peptide" of HA molecules becomes labelled by probes in the target membrane (Stegmann *et al.*, 1991; Tsurudome *et al.*, 1992; Durrer *et al.*, 1996). "Fusion peptide" insertion into the endosomal membrane would result in a bridge between the endosomal and viral membranes. It has been suggested that flexibility within the HA_2 chain would allow the gap between the two membranes to be narrowed, perhaps enabling membrane fusion to proceed (Skehel *et al.*, 1996).

2.3.4 Irreversible acid inactivation of haemagglutinin

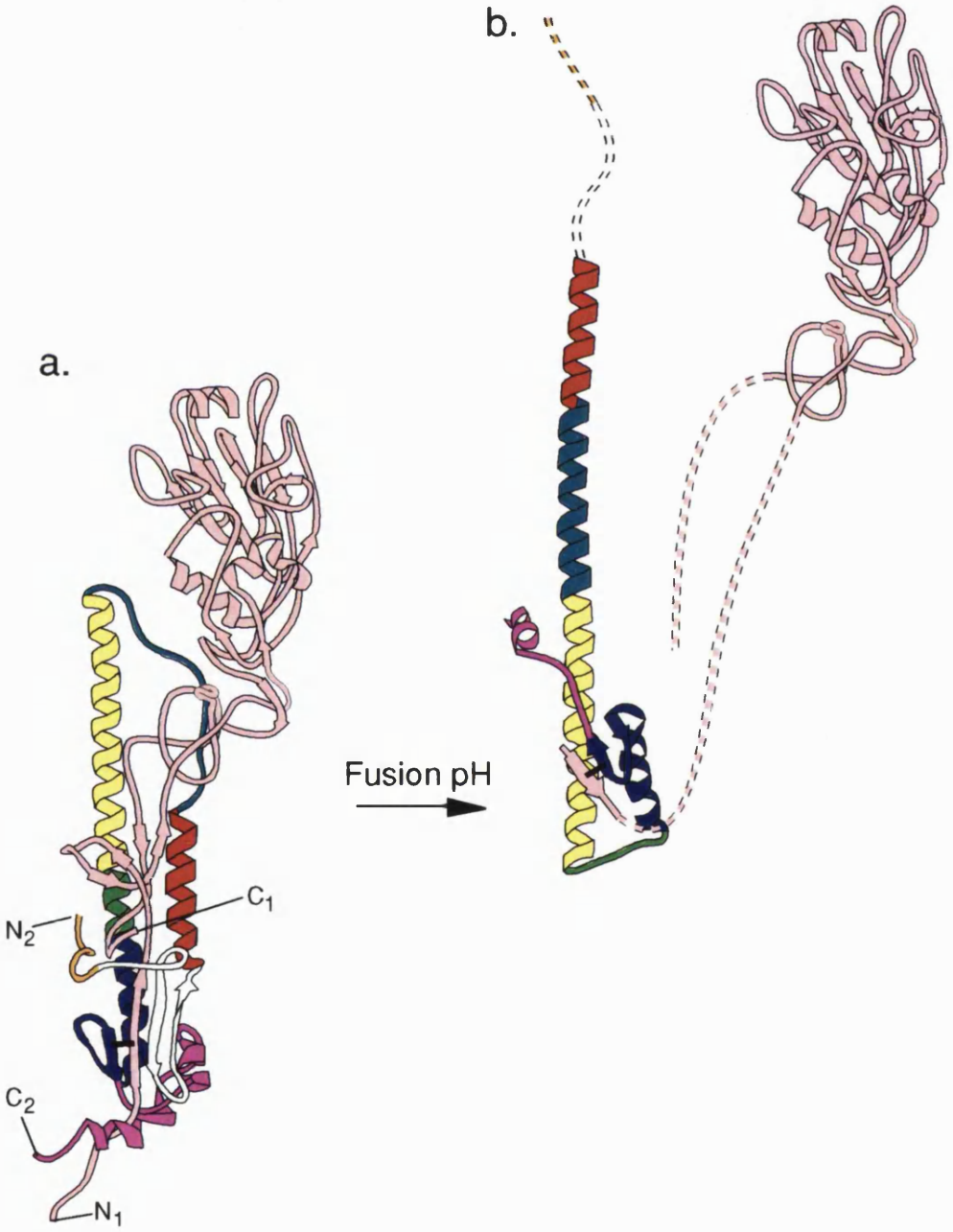
Low pH treatment of influenza virus in the absence of target membrane leads to irreversible inactivation of fusion activity (White *et al.*, 1982). Electron microscopy of $TBHA_2$ and the low-pH structure of HA_2 in virosomes orientated in

Figure 2.3.1

Shown in (a) is the structure of a monomer of native X-31 BHA (Wilson *et al.*, 1981), HA_1 is shown in pink. BHA_2 is shown in multicolour, with the fusion peptide in orange, 56_2-75_2 in turquoise, 76_2-105_2 in yellow and 106_2-112_2 in green. At fusion pH the HA_2 chain undergoes a major refolding as shown in (b).

(b) The structure of $TBHA_2$ is shown in multicolour (Bullough *et al.*, 1994a), the yellow region is unaffected by the conformational change, the turquoise region adopts a helical conformation, conversely the green region refolds into a loop. The low pH structure of HA_1 (Bizebard *et al.*, 1995) is shown in pink. Dotted lines indicate components of the structure that are unknown.

Figure 2.3.1 The structure of native X-31 BHA monomer and the structural changes induced at fusion pH



relation to the crystal structure of $TBHA_2$ strongly suggests that the amino termini of HA_2 can insert into the viral membrane (Wharton *et al.*, 1995). Further evidence suggesting that inactivation of HA leads to insertion of the "fusion peptide" into the viral membrane comes from electron microscopy of complete HA rosettes (Ruigrok *et al.*, 1986b) and photolabelling experiments (Weber *et al.*, 1994). The inverted structure of HA where both the carboxy-terminal membrane anchor and the amino terminal "fusion peptide" of HA_2 are inserted into the same membrane is also the situation expected after membrane fusion is complete.

2.3.5 Fusion pH mutant haemagglutinins

HA_2 in the fusion-pH conformation is more stable than its conformation in native HA (Ruigrok *et al.*, 1986a; Chen *et al.*, 1995); lowering the pH is thought to reduce the energy barrier between the two states and thereby enable the conformational change. HAs that undergo the acid induced conformational change at a higher pH than wild type HA have been isolated by selecting viruses able to grow in cells treated with amantadine hydrochloride, which raises the endosomal pH (Daniels *et al.*, 1985).

Fusion pH mutants of HA contain amino acid substitutions that further destabilize the native structure, resulting in the conformational change occurring at a higher pH. Sequencing of the HA mutants showed that the mutations fall into two groups, firstly mutations that destabilize the location of the "fusion peptide" and secondly mutations that result in alteration of intersubunit contacts (Daniels *et al.*, 1985 & 1987). All of the mutated residues selected identify interactions that are lost in the low pH structure; residue interactions that are maintained in the low pH conformation such as those in the native coiled coil region are not selected.

2.3.6 Studies of haemagglutinin mediated membrane fusion

By monitoring the dilution of fluorescent or spin labelled phospholipids fusion of influenza virus with target membrane has been followed *in vitro*. Liposomes have been used as model endosomal membranes (Wharton *et al.*, 1986; Stegmann *et al.*, 1989; Kawasaki & Ohnishi, 1992; Nussbaum *et al.*, 1992; Bron *et al.*, 1993a; Alford *et al.*, 1994; Stegmann *et al.*, 1995).

Acid induced HA mediated fusion of influenza virus with liposomes composed of phosphatidylcholine has been demonstrated and efficiency of fusion was increased by the presence of unsaturated acyl chains (Kawasaki & Ohnishi, 1992) and by cholesterol (Nussbaum *et al.*, 1992). Fusion of influenza virus with liposomes composed of negatively charged phopholipids such as phosphatidylserine is thought to involve a non-physiological mechanism not involving the acid induced conformational change of HA (Stegmann *et al.*, 1989).

At temperatures $<30^0$C a lag phase between the exposure of the "fusion peptide" of HA, that leads to immediate hydrophobic attachment to target liposomes and membrane fusion between virus and target membranes has been reported (Stegmann *et al.*, 1990; Clague *et al.*, 1991; Ludwig *et al.*, 1995; Stegmann *et al.*, 1995).

Studies using synthetic peptides corresponding to the sequence of the HA "fusion peptide" have shown that synthetic peptides can fuse cholesterol free liposomes at neutral as well as acidic pH, but synthetic peptides can only fuse cholesterol containing liposomes below pH 6 (Wharton *et al.*, 1988). Studies concerning the membrane fusion activities of "fusion peptide" mutants of HA and the corresponding synthetic peptides have shown that the "fusion peptide" is essential for

the membrane fusion activity of HA (Gething *et al.*, 1986; Steinhauer *et al.*, 1995). Synthetic peptides corresponding to the sequence of the HA "fusion peptide" have been incorporated into molecular conjugate gene therapy vectors, the HA "fusion peptide" was shown to be active in endosomal disruption (Wagner *et al.*, 1992).

Fusion between influenza virus and liposomes has shown that blocking of influenza M2 with amantadine reduces the rate of membrane fusion of drug sensitive viruses. Further, the proton ionophore monensin when inserted in the viral membrane increases the rate of fusion with liposomes. It has been proposed that M2 is a proton channel, acidifying the virion interior and facilitating virus uncoating (Hay, 1989; Martin & Helenius, 1991; Sugrue & Hay, 1991; Greber *et al.*, 1994; Wharton *et al.*, 1994)

2.3.7 The role of receptor binding in haemagglutinin mediated membrane fusion

HA binds to sialylated cell surface receptors, and following receptor mediated endocytosis undergoes a low pH induced conformational change which causes fusion of the viral and endosomal membranes. The role of receptor binding in attaching influenza virus to the cell surface is clear but reports concerning the role of receptor binding in the membrane fusion process are conflicting.

The extent of influenza virus - erythrocyte ghost fusion was significantly reduced by neuraminidase pre-treatment of erythrocyte ghosts which removed sialic acid residues (Stegmann *et al.*, 1986). Sialic acid containing ganglioside receptors have been shown to enhance the rate (Stegmann *et al.*, 1989 & 1995) and extent (Kawasaki & Ohnishi, 1992; Stegmann *et al.*, 1995) of influenza virus fusion with liposomes. In a situation contrary to that observed for influenza virus - erythrocyte

ghost fusion, liposomes containing no sialic acid receptors can undergo significant HA mediated fusion with influenza virus (Stegmann *et al.*, 1989).

2.3.7.1 Evidence that receptor binding by HA facilitates insertion of "fusion peptide" into the target membrane

Stegmann *et al.* (1995) reported that the rate and extent of influenza virus - liposome fusion was increased by the presence of liposomal ganglioside receptor. It was concluded that the increased rate and extent of influenza virus - liposome fusion was due not only to increased prefusion binding of the virus to the target membrane, but also that binding of HA to sialic acid containing receptor facilitated correct insertion of the HA "fusion peptide" into the target membrane.

Pedroso de Lima *et al.* (1995) reported studies of fusion between influenza virus and human T lymphocytic leukemia (CEM) cells. It was concluded that sialic acid containing receptors were required not only for efficient binding of the virus to the cell surface but also for efficient fusion of influenza virus with CEM cells.

In a study of fusion between influenza virus and planar lipid bilayers with and without receptor it was proposed that binding of HA to sialic acid containing receptors fundamentally alters the rate of fusion and that bound HA facilitates fusion (Niles & Cohen, 1993).

2.3.7.2 Evidence that unbound HA can facilitate membrane fusion

Schoen *et al.* (1996) have reported reconstituted influenza envelopes (virosomes) containing biotin-phosphatidylethanolamine were able to bind to target liposomes lacking HA receptor via streptavidin/biotin interactions. HA mediated virosome - liposome fusion where prefusion binding was streptavidin/biotin mediated showed that HA not directly bound by receptor could induce membrane fusion.

Fusion kinetics were very similar to virosome - liposome fusion where liposomes contained ganglioside receptor, suggesting that binding of gangliosides by HA does not influence the low pH induced conformational change of HA or the characteristics of the membrane merger itself (Schoen *et al.*, 1996). This is in agreement with previous conclusions (Stegmann *et al.*, 1989; Stegmann *et al.*, 1995). At present the relative efficiency of membrane fusion mediated by HA bound or unbound to receptor is not reported.

2.3.7.3 Evidence that HA bound to receptor does not participate in fusion

Ellens *et al.* (1990) have reported experiments based around two cell lines expressing HA at different surface densities, HAb-2 cells had a 1.9-fold higher plasma membrane surface density of HA than GP4F cells. Both cell lines had equal binding constants for glycophorin containing liposomes, indicating that for both cell lines binding of a liposome to the cell surface involved the same number of HA-glycophorin interactions. A known number of liposomes were attached to the cell surface of both cell lines via HA-glycophorin binding. Following low pH treatment the proportion of liposomes that had undergone membrane fusion with the plasma membrane was determined by assaying the amount of liposome encapsulated toxin delivered to the cytoplasm. It was found that an increase of 1.9-fold in the HA surface density of HAb-2 cells resulted in 4.4 times more fusion per bound liposome.

Ellens *et al.* (1990) assumed that if HAs bound to glycophorin were involved in the fusion process then the HAb-2 cells should have fused with the same fraction of bound liposomes as the GP4F cells. As 4.4 times more fusion was observed with HAb-2 cells it was proposed that the binding and fusion functions of HA are not

check this!

performed by the same trimer. It was also suggested that one HA is not sufficient to induce fusion.

The effect of increasing the liposomal concentration of ganglioside GD1a on influenza virus-liposome fusion has been investigated. High ganglioside GD1a concentrations resulting in a higher fraction of HAs bound to sialic acid residues led to a decrease in extent of virus-liposome fusion. The conclusion from these experiments has been that HAs bound to sialic acid bearing receptors do not participate in fusion (Alford *et al.*, 1994).

2.3.8 Proposed mechanisms for haemagglutinin mediated membrane fusion

Little is known about the mechanism by which the viral and endosomal membranes merge after the proposed insertion of HA "fusion peptide" into the target membrane.

Tatulian *et al.* (1995) have provided evidence using attenuated total reflection Fourier transform infrared spectroscopy that HA trimers in the low pH conformation are in a tilted conformation with respect to the viral membrane and that this tilting is reversible (Tatulian & Tamm, 1996). A model of a pre-pore fusion intermediate is proposed where several HA molecules with their "fusion peptides" inserted into the target membrane assemble at a fusion site, the tilted conformation resulting in a closer apposition of the viral and target membranes.

Studies following the membrane fusion between HA expressing fibroblasts and erythrocytes suggest that the HA mediated fusion is co-operative (Danieli *et al.*, 1996) and involves the formation of a fusion pore structure (Spruce *et al.*, 1989; Zimmerberg *et al.*, 1994; Blumenthal *et al.*, 1996). The fusion pore structure has also

been investigated by following membrane fusion between HA expressing cells and planar membranes (Melikyan *et al.*, 1993; Melikyan *et al.*, 1995 a & b).

Results obtained using cells expressing glycophosphatidylinositol-anchored HA (GPI-HA) showed that GPI-HA induced hemifusion, in which the outer but not the inner leaflets of the two fusing membranes have merged. Hemifusion is induced by GPI-HA without the formation of fusion pores. It is suggested that the ectodomain of wild type HA induces hemifusion and the carboxy-terminal transmembrane domain of HA_2 induces full fusion (Kemble *et al.*, 1994; Melikyan *et al.*, 1995c).

2.3.9 Reconstituted influenza envelopes (virosomes)

2.3.9.1 Production of influenza virosomes

To investigate the mechanism of HA mediated membrane fusion the reconstitution of functional influenza virus envelopes (virosomes) has been attempted.

Production of fusogenic virosomes containing a viral lipid composition with properties very similar to intact virions has been reported. Influenza virus was solubilized with the detergents octaethylene glycol monododecyl ether ($C_{12}E_8$) or Triton X-100, the viral nucleocapsid was sedimented by centrifugation leaving the viral lipids and surface glycoproteins in the supernatant, removal of the detergent from the supernatant was with biobeads resulting in HA containing vesicles with a viral lipid composition (Holloway, 1973; Nussbaum *et al.*, 1987; Stegmann *et al.*, 1987; Bron *et al.*, 1993b). Influenza virosomes prepared using this method have been reported not to contain the M2 proton channel protein and monensin does not stimulate virosome-liposome fusion (Bron *et al.*, 1993a).

Kawasaki *et al.* (1983) reconstituted HA into vesicles composed of phosphatidylcholine and cholesterol using the detergent Triton X-100. The vesicles

may have contained significant amounts of residual detergent, and although they underwent low pH induced membrane fusion it was not established whether the observed fusion was HA mediated.

Stegmann *et al.* (1987) using the readily dialysable detergent octylglucoside to solubilize influenza viral envelopes reported that after dialysis to remove the detergent the HA appeared predominantly as rosettes with very little incorporation into reconstituted vesicles.

Reconstituted influenza envelopes have been produced using the "protein-cochleate method". Influenza envelopes were solubilized in octylglucoside followed by addition of phosphatidylserine and cholesterol. Dialysis against Ca^{2+} containing buffer resulted in removal of octylglucoside and a calcium-phospholipid-protein precipitate. Addition of EDTA containing buffer results in large unilamellar proteoliposomes (Mannino & Gould-Fogerite, 1988). These reconstituted vesicles have been used to deliver encapsulated material (Section 2.3.9.2) but there have been no reports concerning their membrane fusion properties.

At present it has not been established whether purified HA and specific purified lipids can be reconstituted to form influenza virosomes, which are then able to cause HA mediated membrane fusion.

2.3.9.2 Use of influenza virosomes for delivery to cells

Influenza virosomes containing a viral lipid composition with subunit A of diphtheria toxin (DTA) encapsulated in the virosomal lumen have been produced using $C_{12}E_8$ by the method of Stegmann *et al.* (1987). These virosomes fused efficiently with the membranes of the endosomal cell compartment of BHK-21 cells *in vitro* resulting in cytoplasmic delivery of the virosome contents (Bron *et al.*, 1994).

Plasmid DNA encapsulated at high efficiency into large unilamellar reconstituted influenza envelopes using the "protein-cochleate method" has been reported (Mannino & Gould-Fogerite, 1988; Gould-Fogerite *et al.*, 1989). Vesicles produced by this method are too large to be endocytosed by most cell types. Delivery to cells *in vitro* has been described which involved low pH treatment of HA vesicles which were bound to the cell plasma membrane (Mannino & Gould-Fogerite, 1988; Gould-Fogerite *et al.*, 1989). There have been no reports concerning the membrane fusion properties of these vesicles so it is not known whether the observed delivery was HA mediated.

HA has also been used to deliver the cystic fibrosis transmembrane conductance regulator (CFTR) protein to cells *in vitro*. One successful approach involved co-reconstitution of HA and CFTR into vesicles with a viral lipid composition using octylglucoside detergent. After HA mediated binding of the vesicle to the cell surface membrane, fusion between the plasma membrane and the vesicle was initiated by transient low pH activation of HA, leading to the detection of functional CFTR in the recipient cells (Scheule *et al.*, 1995).

2.4 WORK PRESENTED

Work is presented that has investigated different procedures for the production of influenza virosomes, the virosomes produced were characterised in terms of their ability to cause HA mediated fusion and the efficiency of the process was directly compared to influenza virus fusion efficiency. It was demonstrated that HA reconstituted into vesicles composed of purified phosphatidylcholine and cholesterol could undergo HA mediated fusion, showing that it may be possible to modify the

lipid composition further to produce functional virosomes with a relatively long half life *in vivo*. Initial experiments using virosomes as vehicles for DNA delivery are also presented.

The role of receptor binding with respect to the efficiency of HA mediated fusion was extensively investigated. Liposome coupled anti-HA monoclonal Fab' fragments with various binding specificities towards the HA molecule were used as surrogate HA receptors. Electron microscopy studies which characterised various HA-receptor complexes are reported.

On the basis of results from electron microscopy membrane fusion experiments were done between virosomes and Fab' liposomes. These experiments concluded that HA bound by receptor can be more efficient at causing membrane fusion than a HA molecule held close to but not directly bound by the target membrane. These results imply that a HA-liposome vector will be most efficient at inducing membrane fusion when the binding specificity is introduced directly into the HA molecule rather than by other molecules coupled to the liposomal membrane. The results also have implications for design of a HA molecule containing a specific ligand binding domain.

3 MATERIALS AND METHODS

All chemicals except where otherwise stated were obtained from BDH or Sigma and were of analytical reagent grade.

3.1 GENERAL METHODS

3.1.1 Influenza growth and purification

Influenza A viruses was grown in the allantoic cavity of 10 day old embryonated chickens eggs and purified by sucrose gradient centrifugation as described by Skehel & Schild (1971).

3.1.2 Bromelain released HA (BHA)

BHA was prepared from gradient - purified virus, as described by Brand & Skehel (1972).

3.1.3 Complete HA (CHA)

X-31 CHA (also containing NA) was prepared in 2% (w/v) octyl-glucoside (OG, ICN) based on the method described by Ruigrok *et al.* (1986b). 3 ml of virus (viral protein ~10 mg/ml) was added to 30 ml of 0.5% (w/v) Brij 36T / 15 mM tris[hydroxy methyl] aminomethane (Tris) - HCl pH 8 and incubated for 30 mins at 4^0C. The viral nucleocapsid was removed by centrifugation for 30 mins at 150000g at 5^0C, and the supernatant concentrated to ~7 ml using an Amicon filtration device (PM10 membrane). Brij 36T was exchanged for OG by centrifugation through a 5-25% (w/v in 0.15 M NaCl / 10 mM sodium phosphate pH 7.4 (PBS) containing 0.01% (w/v) NaN_3) sucrose gradient containing 2% (w/v) OG for 60 h at 50000g at 5^0C.

The gradients were harvested in 1 ml fractions and the concentration of HA estimated by absorbance at 280 nm using a 10 mm cuvette. When absorbance = 1 HA concentration = 0.625 mg/ml (Ruigrok *et al.*, 1986a). Fractions were analysed by SDS PAGE (Section 3.1.4) and CHA containing fractions were pooled. CHA was used to produce virosomes by Method B (Section 3.2.2) or dialysed at 4^0C for two periods of 24 h against two times 5 l of PBS / 0.01% (w/v) NaN_3 containing ~30 g of Amberlite XAD-2 beads (biobeads) (BDH Cat No. 15088 4G) to produce HA rosettes.

3.1.4 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed as described by Laemmli (1970), with a 4% stacking and 12% resolving gel unless otherwise stated.

Unless otherwise stated gels were stained for 30 minutes in 10% (v/v) acetic acid / 40% (v/v) methanol / 50% (v/v) H_2O / 0.5% (w/v) page blue and destained in 10% (v/v) acetic acid / 40% (v/v) methanol / 50% (v/v) H_2O. Alternatively when the amount of protein present was too low to be visualised using page blue, gels were silver stained (Silver Stain Plus Kit, Bio-Rad Cat No. 161-0449)

Large molecular weight rainbow protein molecular markers (220000-14000 Da, Amersham) were used unless otherwise stated.

3.1.5 Western blotting

Proteins resolved by SDS-PAGE were transferred onto Immobilion PVDF membrane (pore size 0.45μm, Millipore) using a modification of the system described by Towbin *et al.* (1979). Electroblotting was done in transfer buffer containing 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid pH 11 and 10% (v/v) methanol. Four sheets of filter paper (Whatman 3MM) wetted in transfer buffer, one sheet of PVDF membrane wetted with 100% methanol then soaked in transfer buffer,

polyacrylamide gel, and 4 sheets of filter paper wetted in transfer buffer, were piled, in this order, on the anode of the semi-dry electroblotting apparatus (Ancos). Transfer was carried out for 75 mins at 12V.

The PVDF membrane was soaked in methanol for 30 secs and air dried for at least 30 mins at room temp. The membrane was then soaked for 1 h at room temp. in 20 ml of 3% (w/v in PBS) milk powder (Marvel) containing a specified primary antibody. The membrane was washed in 100 ml of 3% (w/v in PBS) milk powder for 5 mins. The membrane was then soaked for 45 mins at room temp. in 20 ml of 3% (w/v in PBS) milk powder containing 1 in 1000 parts "Protein A Horseradish Peroxidase Conjugate" (Bio-Rad). The membrane was then washed in PBS for 5 mins. The reactive protein bands were then detected using the ECL detection system (Amersham).

3.1.6 Purification of cellular lipid

Lipid of cellular origin was purified from washed packed human erythrocytes using the method of Folch *et al.* (1956). Chloroform was removed by rotary evaporation and the amount of lipid present estimated by weight. Stocks of 10 mg/ml lipid in chloroform were stored under N_2 at -20^0C.

3.1.7 BCA protein assay

The "BCA Protein Reagent" (Pierce) was used to determine protein concentration using the "standard protocol" supplied. Bovine serum albumin (BSA) solutions of known concentration were used to produce a calibration curve.

Question

3.1.8 Phospholipid assay

The concentration of phospholipid was determined using the method of King & Wootton (1956). The lipid extracts (Folch *et al.* 1956) containing at least ~0.5 mg

Does he know the chemical reaction path

of lipid were put into boiling tubes and all the solvent removed under a stream of N_2. way 1 ml perchloric acid 60% (v/v) was added to each tube which was stoppered with a glass bubble. The tubes were digested at ~140^{0}C on a heating block for 3 h. After cooling, 10 ml distilled water was added followed by 1 ml of 5% (w/v) ammonium molybdate. The resulting yellow complex was converted to a deep blue by the addition of 0.5 ml of reducing agent (0.2 g of 1,2,4-amino-naphthyl-sulphonic -acid, 12 g sodium metabisulphite and 2.4 g sodium sulphite were ground together. The reducing agent consisted of 1.46 g of this mixture per 10 ml of distilled water). After 10 mins the optical density at 660 nm was measured against a reagent blank. A standard curve was prepared using KH_2PO_4. The weight of the phospholipid was taken to be 25 times that of the phosphorus determined.

What is measured?

3.1.9 Electron microscopy (EM)

Samples were absorbed onto carbon films and negatively stained with 1% sodium silicotungstate (pH 7.0). Micrographs were taken under minimum dose and accurate defocus conditions to preserve detail to ~1.5 nm (Wrigley *et al.*, 1983a). The JEOL 1200 EX microscope was operated at 100 kV and the magnification was regularly calibrated with catalase crystals.

Question
- image formation
- phase contrast
- why is defocus important
- CTF

3.2 VIROSOME PRODUCTION

3.2.1 Method A virosomes

The method used was that of Metsikko *et al.* (1986) as described by Stegmann *et al.* (1987). 250 µl of a specified influenza virus (viral protein ~10 mg/ml) was added to 250 µl of 200 mM octaethylene glycol monododecyl ether ($C_{12}E_8$)(Fluka) / 145 mM NaCl / 0.1 mM ethylenediaminotetra acetic acid (EDTA) / 5 mM N-[2-

What is the CMC

very low, was pH checked

why HEPES, and not Tris or PBS?

Hydroxyethyl]piperazine - N' -[2-ethanesulfonic acid] (HEPES) pH 7.4 and incubated on ice for 30 mins. The insoluble viral nucleocapsid was pelleted by centrifugation for 30 mins at 200000g at 5^0C. The supernatant (0.4 ml) was transferred to a 1.5 ml Eppendorf tube containing 113 mg of wet biobeads and vigorously shaken in a Eppendorf 5432 Mixer for 1 h at room temp. A further two 65 mg aliquots of biobeads were added and shaking resumed for two periods of 8 mins. The solution became turbid at this point, indicating formation of vesicular structures. The virosome suspension was then overlaid on a discontinuous sucrose gradient (3.5 ml of 5% overlaid on 0.75 ml of 40% (w/v in 145 mM NaCl / 0.1 mM EDTA / 5 mM HEPES pH 7.4) sucrose) and centrifuged for 90 mins at 200000g at 5^0C. The virosomes appeared as a thin opalescent band at the interface, and were collected in 1 ml. The virosomes were then dialysed at 4^0C overnight against 5 l of PBS / 0.01% (w/v) NaN_3 containing ~30 g of biobeads. Virosome preparations were routinely inspected by EM.

3.2.1.1 Incorporation of fluorescent lipids into Method A virosomes

The fluorescent lipids N-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (N-NBD-PE) and N-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine (N-Rho-PE) (Avanti Polar Lipids Inc.) were supplied in chloroform. A specified amount was evaporated to dryness under N_2 and lyophilized, the lipid was then dissolved in 200 mM $C_{12}E_8$ / 145 mM NaCl / 0.1 mM EDTA / 5 mM HEPES pH 7.4 which was used to solubilize the influenza virus.

3.1.2.2 Determination of the level of residual detergent in Method A virosomes

Triton-X100[phenyl ^{3}H(N)] (Dupont) has very similar chemical properties to $C_{12}E_8$ and was used to determine the level of detergent remaining in Method A

virosomes. 25 μCi of Triton-X100[phenyl ^{3}H(N)] was included in the 250 μl of 200 mM $C_{12}E_8$ / 145 mM NaCl / 0.1 mM EDTA / 5 mM HEPES pH 7.4 used solublize the influenza virus. The amount of detergent in a 10μl sample was determined by addition of 5 ml of liquid scintillation cocktail (Beckman) and quantifying the ^{3}H radioactivity using the Beckman LS 5000CE liquid scintillation system. The phospholipid concentration of the virosome preparation was determined as previously described (Section 3.1.8), enabling a molar ratio between phospholipid and residual detergent to be calculated.

Results showed that biobead treatment reduced the detergent level from 100 mM to 1.7 mM and the virosome band removed from the sucrose gradient contained 40 μM detergent. After dialysis the detergent level was 3 μM. The final detergent : phospolipid molar ratio was approximately 1 : 35 respectively.

Yes but assuming that $C_{12}E_8$ interacts identically with the biobeads as the TX100

3.2.2 Method B virosomes

Method B[1] was based on that described by Ruigrok *et al.* (1986b). Briefly 0.33 mg, 1 mg or 1.66 mg of a lipid mixture of specified composition containing trace amounts of [1α,2α(n)-^{3}H] cholesterol (Amersham) was prepared in chloroform and evaporated to dryness under N_2 and lyophilized. 0.33 mg of purified X-31 CHA in 2% (w/v) OG (Section 3.1.3) was added to the lipid and the volume adjusted to 1 ml with 2% (w/v in PBS) OG. The mixture was sonicated in a bath sonicator for 100 secs and incubated for 15 mins at 37^{0}C. The mixture was then dialysed at 4^{0}C, twice against 1 ml of PBS for 1 hour, then 3 ml of PBS for 1 hour, and finally overnight against 5 l of PBS / 0.01% (w/v) sodium azide containing ~30 g of biobeads.

For Method B[2] 1 mg of a lipid mixture of specified composition was added to 0.33 mg of X-31 CHA in 2% (w/v) OG as described for Method B[1]. OG removal,

using 400 µl of the above solution was done as described for Method A (Section 3.2.1), using biobeads, sucrose gradient centrifugation and dialysis. Virosome preparations were routinely inspected by EM.

3.2.3 Method C virosomes

Purified X-31 CHA (also containing NA) was prepared in 100 mM $C_{12}E_8$ / 145 mM NaCl / 5 mM HEPES pH 7.4. 3 ml of X-31 virus (viral protein ~10 mg/ml) was dissolved in 3 ml of 200 mM $C_{12}E_8$ / 145 mM NaCl / 0.1 mM EDTA / 5 mM HEPES pH 7.4 and incubated (in) ice for 30 mins. The insoluble viral nucleocapsid was removed by centifugation for 30 mins at 200000g at 5°C. The supernatant was overlaid on top of a 5-25 % (w/v in 100 mM $C_{12}E_8$ / 145 mM NaCl / 0.1 mM EDTA / 5 mM HEPES pH 7.4) sucrose gradient and centrifuged for 60 h at 50000g at 5^0C. Gradient fractions were analysed by SDS PAGE (Section 3.1.4) and CHA containing fractions were pooled. The protein concentration was estimated by absorbance at 280 nm (Section 3.1.3) using 100 mM $C_{12}E_8$ / 145 mM NaCl / 0.1 mM EDTA / 5 mM HEPES pH 7.4 as a blank.

0.37 mg, 0.75 mg or 1.1 mg of a lipid mixture of specified composition containing trace amounts of [1α,2α(n)-^{3}H] cholesterol was prepared in chloroform and evaporated to dryness under N_2 and lyophilized. 500 µl of 0.38 mg/ml CHA in 100 mM $C_{12}E_8$ / 145 mM NaCl / 0.1 EDTA / 5 mM HEPES pH 7.4 also containing sucrose from the gradient was added to the lipid, the mixture was sonicated in a bath sonicator for 100 secs and incubated for 15 mins at 37^0C. 400 µl of this solution was removed and virosome formation was as described for Method A virosomes (Section 3.2.1). Due to the sucrose present, the virosome suspension did not overlay a 5% sucrose gradient. To ensure residual detergent removal dialysed virosomes were

subjected to a further round of centrifugation on a discontinuous sucrose gradient followed by dialysis. Virosome preparations were routinely inspected by EM.

3.2.4 Acid and trypsin treatment of influenza virosomes

The pH of the solution was lowered to a specified pH by adding aliquots of 0.15 M citrate buffer (pH 3.5) and incubated for 5 mins at 37^0C. The pH was then readjusted to pH 7.4 by adding 0.5 M HEPES (sodium salt pH 10).

1 mg/ml trypsin in PBS was then added to HA in a ratio of 1:40 (w/w) and incubated for 45 mins at room temp. The digestion was terminated by addition of an equal weight of trypsin inhibitor.

3.2.5 Determination of the HA:lipid ratio of virosome preparations

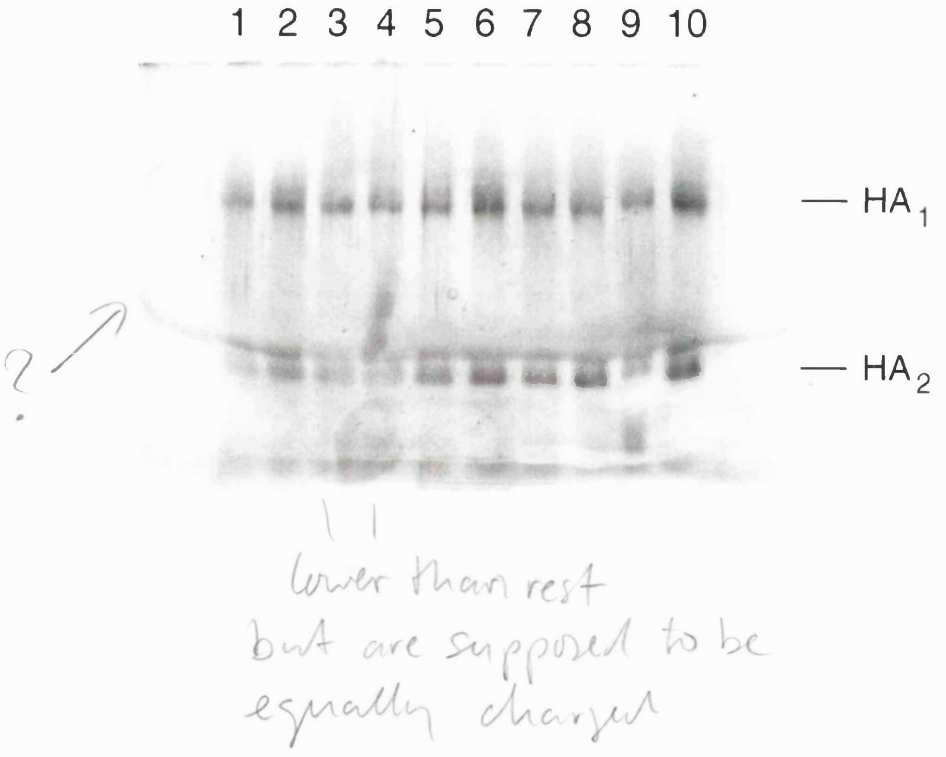
The virosomal concentrations of HA and lipid were determined and used to calculate a HA:lipid (w/w) ratio. The concentration of HA rosettes prepared using OG purified CHA was estimated by absorbance at 280 nm (Section 3.1.3). Using SDS PAGE followed by silver staining (Section 3.1.4) the HA band intensity was equalized between HA rosettes and various virosomes preparations. The HA concentration of the virosome preparations was then estimated from the known concentration of HA rosettes (Figure 3.2.1). The HA concentration of virus was also determined relative to a known concentration of HA rosettes. Western blotting (Section 3.1.5) using R186 anti-HA polyclonal primary antibody was used to detect HA.

When virosomes were labelled with [1α,2α(n)-^{3}H] cholesterol the ^{3}H radioactivity was quantified by scintillation counting. The level of radioactivity in virosomes was compared to that present in lipid stocks of known concentration used to make the virosomes and hence a lipid concentration was deduced. When virosomes

Figure 3.2.1

The procedure for the determination of virosomal HA concentration was done as described in Section 3.2.5, using SDS PAGE and silver staining (Section 3.1.4).
In Lanes 2 and 7 a constant, known amount of HA rosettes were loaded. Shown in all other lanes are various HA virosome preparations, in each case the amount of virosomes loaded was adjusted to give an equal band intensity to that of the HA rosettes. From the amount virosomes loaded and the known amount of HA rosettes loaded, a virosomal HA concentration was estimated. Samples were run under reducing conditions.

Figure 3.2.1: Determination of the HA concentration of virosomes using SDS PAGE and silver staining



were not labelled with [1α,2α(n)-^{3}H] cholesterol, the phospholipid concentration was determined directly (Section 3.1.8). The phospholipid concentration was then used to estimate a total lipid concentration. When phospholipid assays and ^{3}H cholesterol labelling were used together to estimate total lipid concentration results were in good agreement.

3.3 CHARACTERISATION OF THE FUSION PROPERTIES OF VIROSOMES

3.3.1 Haemolysis to assay membrane fusion

HA mediated haemolysis of human erythrocytes was done as described by Daniels *et al.* (1985). Haemolysis was measured after 30 mins by determining the optical density at 520 nm of the cell supernatant.

3.3.2 Resonance energy transfer (R.E.T) to assay membrane fusion

3.3.2.1 Preparation of sealed erythrocyte ghosts

Fresh heparinised human blood was used to produce erythrocyte ghosts as described by Steck & Kant (1974). Membrane protein concentration was determined using the BCA protein assay (section 3.1.7), and adjusted to 1 mg/ml with PBS.

3.3.2.2 Neuraminidase treatment of erythrocyte ghosts

Two 350 µl aliquots of erythrocyte ghosts were centrifuged for 5 mins at 14000g at 5^{0}C. The erythrocyte ghost pellets were resuspended in 350 µl of 150 mM NaCl / 2 mM CaCl / 10 mM HEPES pH 7.4. One aliquot was treated with 35 µl of *Clostridium perfrigens* neuraminidase (50 mU/ml, type X, Sigma). Samples were incubated for 3 h at 37^{0}C after which erythrocyte ghosts were pelleted as before and resuspended in 350 µl of PBS. The untreated and treated samples were then analysed

by the enzyme linked immuno sorbent assay (ELISA) using the method of Bos *et al.* (1981). The biotinylated lectins elderberry bark lectin, maackia amurensis lectin II and peanut agglutinin (Vector Laboratories) which bind to 2,6-sialic acid, 2,3-sialic acid and mannose respectively were used. Mannose is exposed after sialic acid removal. Results showed that neuraminidase treatment of erythrocyte ghosts removed sialic acid. EM showed there was no difference in the appearance of untreated and treated erythrocyte ghosts (data not shown).

3.3.2.3 Preparation of ganglioside containing unilamellar liposomes

5 mg of lipid (phosphatidylcholine (PC) / sphingomyelin (SM) / phosphatidylethanolamine (PE) / phosphatidylserine (PS) / phosphatidylinositol (PI) / Cholesterol (Chol) in molar ratios 10 : 3 : 3 : 1 : 0.5 : 12.25 respectively) containing 0.6 mol% of each of the fluorescent dyes N-NBD-PE and N-Rho-PE and 3 mol% gangliosides (Type V from bovine brain, Sigma) was prepared in chloroform and evaporated to dryness under N_2 and lyophilized. 5 ml of PBS was added to the lipid and unilamellar liposomes were prepared by the sonication method as described by Wharton *et al.* (1986). The mixture was sonicated for six 3-minute periods at 4^0C using a probe sonicator (MSE) set at 12 μM peak to peak. Multilamellar liposomes and debris were removed by centrifugation for 30 mins at 140000g at 5^0C.

3.3.2.4 Assaying membrane fusion by resonance energy transfer (R.E.T).

The method used to assay membrane fusion was that of Struck *et al.* (1981) as described by Stegmann *et al.* (1987). The method involves the nonexchangable probes N-NBD-PE as fluorescence donor and N-Rho-PE as fluorescence acceptor. Fusion of labelled membrane with unlabelled membrane resulted in dilution of the fluorescent probes and consequent decrease in R.E.T. Fluorescence was measured at

37^0C with a Perkin Elmer LS-50 Luminescence Spectrometer with excitation and emission slit widths set at 10 nm. Excitation of the donor probe N-NBD-PE was at 465 nm, membrane fusion was measured by following an increase in N-NBD-PE fluorescence at 530 nm due to decreased R.E.T. Unless otherwise stated filters BG37 and GG495 (Schott) were used in the excitation and emission paths, respectively, so as to minimise the effect of light scattering.

The initial fluorescence of the virosomes and target lipid at 530 nm (F_0) was set to zero and the fluorescence at infinite probe dilution was set to 100% (F_{max}). The latter value was determined by addition of 10% (w/v in PBS) Brij 36T (0.2% w/v final conc.), the percentage change in fluorescence was calculated as

$$\%\frac{\Delta F}{\Delta F_{max}} = 100\left(\frac{F - F_0}{F_{max} - F_0}\right)$$

Data were analysed using Microsoft Excel Version 5 and were corrected for dilution caused by addition of 0.15 M sodium citrate pH 3.5 and 10% (w/v in PBS) Brij 36T.

Fluorescence due to the N-Rho-PE at 590 nm unlike N-NBD-PE fluorescence at 530 nm was found to be unpredictable and did not reliably indicate the process of membrane fusion. N-Rho-PE but not N-NBD-PE fluorescence was shown to be affected by HA in the low pH conformation (data not shown), in agreement with Wharton *et al.* (1986).

Virosome - erythrocyte ghost fusion was followed by incorporating NBD-PE and N-Rho-PE into virosomal membranes. Virosome - ganglioside liposome fusion was followed by incorporating NBD-PE and N-Rho-PE into the liposome membranes, which allowed direct comparison of the fusion efficiency of different virosome preparations. Using a quartz fluorescence cuvette a specified virosome preparation

and erythrocyte ghosts or liposomes were added to PBS prewarmed to 37^0C to produce a final volume of 1 ml. The pH of the solution was adjusted to a specified pH using 0.15 M sodium citrate pH 3.5.

3.4 LIPOSOME COUPLING OF Fab' FRAGMENTS

The method used for liposome coupling of Fab' fragments was essentially that of Martin *et al.* (1981) with some minor modifications from the method of Shahinian & Silvius (1995). A summary of the method used is shown in Figure 3.4.1.

3.4.1 Synthesis of N-[3-(2-pyridyldithio)propionyl]phosphatidylethanolamine (PDP-PE)

The synthesis of PDP-PE was as described by Martin *et al.* (1981). 37.5 mg of PE was dissolved in 3 ml of anhydrous methanol containing 5 µl of trimethylamine and 25 mg of N-succinimyl 3-(2-pyridyldithio) propionate (SPDP) (Pharmacia) (Figure 3.4.1). The mixture was incubated for 5 h under a N_2 atmosphere at room temp. Methanol was removed by rotary evaporation and the products redissovled in an equal volume of chloroform. PDP-PE was purified by applying the reaction mixture to a 10 ml silica gel-60 column (Merck) equilibrated in chloroform, the column was then washed with 20 ml of chloroform followed by 20 ml of each of the following chloroform - methanol mixtures 40:1, 30:1, 25:1, 20:1, and 15:1, and finally with 60 ml of 10:1 chloroform : methanol.

Thin layer chromatography (TLC) (silica gel H; solvent chloroform - methanol - acetic acid, (v/v) 60:20:3) of the reaction mixture and the column fractions was done using ninhydrin to visualize PE and ultra violet light to visualize SPDP and PDP-PE. TLC showed the fractions eluting in 15:1 and 10:1 chloroform - methanol contained

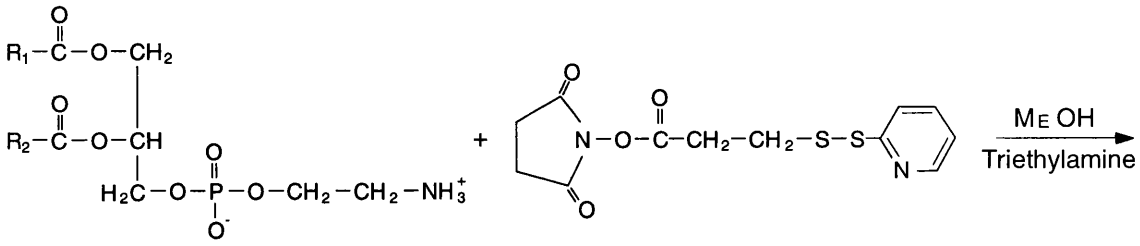
Figure 3.4.1

Upper diagram: Synthesis of N-3-(Pyridyl-2-dithio) Propionyl Phosphatidylethanolamine (PDP-PE)

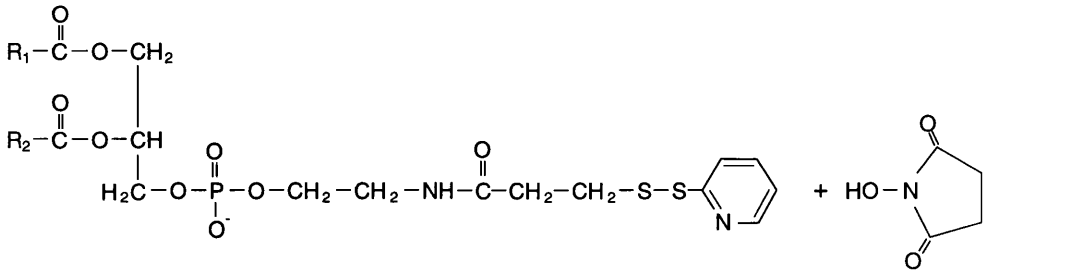
Lower diagram: Covalent coupling of Fab' fragments to PDP-PE liposomes

Adapted from Martin *et al.* (1981)

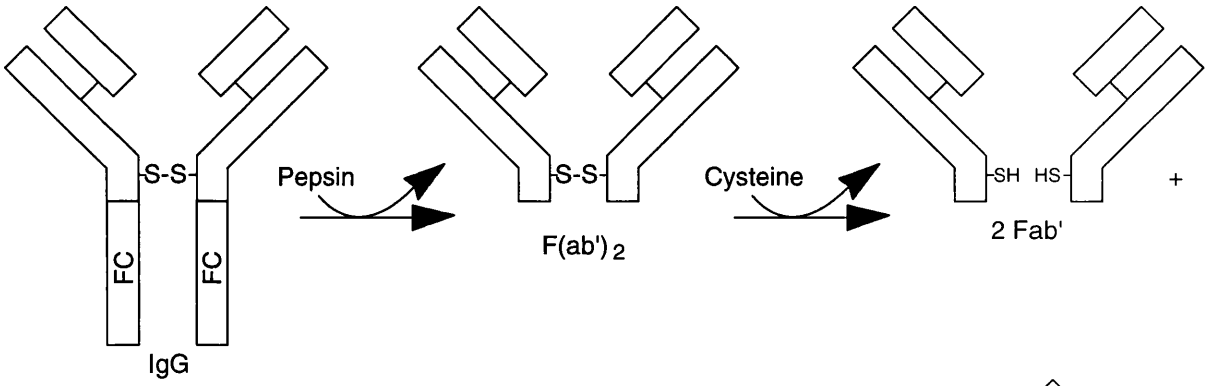
Figure 3.4.1 : Scheme to show the synthesis of PDP-PE and the covalent coupling of Fab' fragments to liposomes

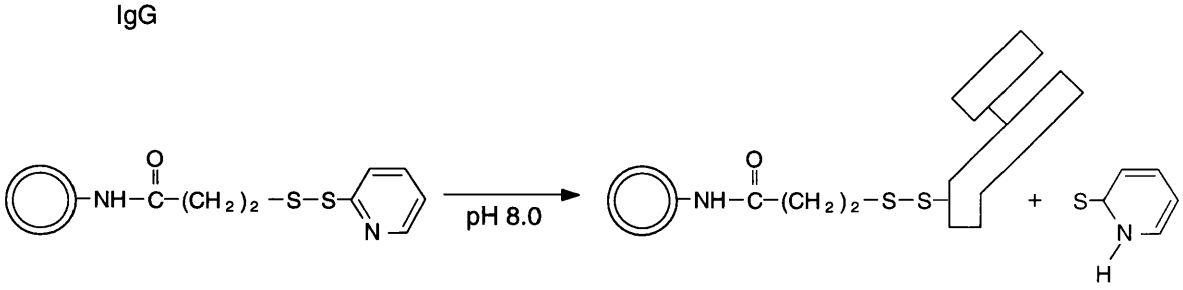


Phosphatidylethanolamine (PE) N-Succinimidyl 3-(2-Pyridyldithio) Propionate (SPDP)



N-3-(Pyridyl-2-dithio) Propionyl Phosphatidylethanolamine (PDP-PE) N-Hydroxysuccinimide





PDP-PE vesicle Fab' - Vesicle 2 - Thiopyridone

only PDP-PE. These fractions were pooled and the solvent removed by rotary evaporation and the amount of PDP-PE present estimated by weight. PDP-PE was stored in chloroform (10 mg/ml) under N_2 at -20^{0}C.

3.4.2 Preparation of PDP-PE containing unilamellar liposomes

PDP-PE (2.0 mg), PC (13.5 mg), cholesterol (6.0 mg) and 20 μCi of [1α,2α(n)-^{3}H] cholesterol was prepared in chloroform and evaporated to dryness under N_2 and lyophilized. 5 ml of Buffer I (pH 6) (100 mM NaCl / 2 mM EDTA / 100 mM borate / 50 mM citrate / 0.01% (w/v) NaN_3) was added to the lipid and unilamellar liposomes were prepared by the sonication method (Section 3.3.2.3)

3.4.3 Antibody preparation

Anti-HA monoclonal antibodies (Table 3.4.1) were purified from ascitic fluid or cell supernatant using Protein A Sepharose 4B (Sigma) affinity chromatography. Purified antibody was eluted using 0.15 M sodium citrate pH 4, and concentrated to 2 mg/ml using an Amicon filtration device (PM10 membrane). Antibody concentration was estimated by absorbance at 280 nm using a 10 mm cuvette, using the relationship that when absorbance =1 antibody concentration was 0.8 mg/ml.

3.4.4 F(ab')$_2$ preparation

Intact antibody (2 mg/ml) was digested with pepsin (60 μg/ml) in 0.15 M sodium citrate buffer (pH 4.0) for 4 h at 37^{0}C to produce F(ab')$_2$. The reaction was terminated by raising the pH by overnight dialysis at 4^{0}C against 145 mM NaCl / 0.01% (w/v) NaN_3 / 10 mM Tris-HCl pH 7.4. Undigested antibody was removed by passage through a 5-ml Protein A-Sepharose column at pH 7.4. F(ab')$_2$ was detected by absorbance at 280nm, positive fractions were pooled and concentrated to 5 mg/ml using a centriprep 30 concentrator (Amicon) and at the same time the buffer was

Table 3.4.1

Unless otherwise stated antibodies were purified from ascitic fluid. Antibody purity was assessed by SDS PAGE (Section 3.1.4). Antibody concentration was determined by absorbance at 280 nm using a 10 mm cuvette. It was estimated that absorbance1≡ 0.8 mg/ml.

Table 3.4.1 Summary of the monoclonal antibodies used to make Fab' liposomes

Monoclonal antibody	**HA_1 residue(s) mutated in escape mutant (if known)**	**Antibody subtype**	**Antibody binds to low pH conformation of HA ?**
Hc73 (anti X-31 HA)	134 G>W / 145 S>N	IgG2a	YES
Hc3 (anti X-31 HA)	144 G>D	IgG2a	YES
Hc19 (anti X-31 HA)	157 S>L	IgG1	YES
Hc68 (anti X-31 HA)	193 S>R	IgG2a	NO
SFA 9B-2.1 (anti X-31 HA)	53 N>D	IgG2a	YES
H100 (anti JHB HA)	Unknown, see results-chapter 2 which shows binding site by EM	IgG2a	YES
H8 (anti JHB HA)*	Unknown, see results-chapter 2 which shows binding site by EM	IgG2a	YES

* Purified from cell supernatant

changed to 100 mM Tris-HCl pH 7.6. $F(ab')_2$ concentration was estimated using the BCA protein assay (Section 3.1.7). $F(ab')_2$ analysis by SDS-PAGE showed Fab' was also present (Figure 3.4.2), at levels which depended on the particular monoclonal antibody. The binding properties of intact antibody and $F(ab')_2$ were assessed by ELISA which was done as described by Bos *et al.* (1981), using goat anti mouse antibody - peroxidase conjugate (Bio-Rad) and 3,3',5,5' tetramethylbenzidine as a substrate.

3.4.5 Fab' preparation

All solutions used for Fab' production and liposome coupling were degassed by N_2 purging before use. Fab' was produced by cysteine reduction of $F(ab')_2$. 1 ml of 5 mg/ml $F(ab')_2$ in 100 mM Tris-HCl pH 7.6 / 0.01% (w/v) NaN_3 was reduced with 30 mM cysteine for 15 mins at 37^0C under a N_2 atmosphere. Cysteine removal and buffer exchange was by passage through a NAP-10 desalting column (Pharmacia Biotech) equilibrated with degassed buffer I (pH 5.5). Fab' appearing in the void volume (~1.2 ml) was maintained under N_2 and used immediately in coupling experiments described below.

3.4.6 Liposome coupling of Fab' fragments and separation of liposomes from uncoupled Fab'

1 ml of the liposomes were mixed with ~1.2 ml of the Fab' fragments prepared as above. The pH was adjusted to pH 8 with 10 M NaOH and the mixture gently stirred under a N_2 atmosphere for 2 h at room temp.

An equal volume of 60% (w/v in PBS) sucrose was added to the liposome - Fab' mixture which was then overlaid with 20% (w/v in PBS) sucrose and centrifuged for 24 h at 110000g at 5^0C. Analysis of the gradient by SDS PAGE and scintillation

Figure 3.4.2

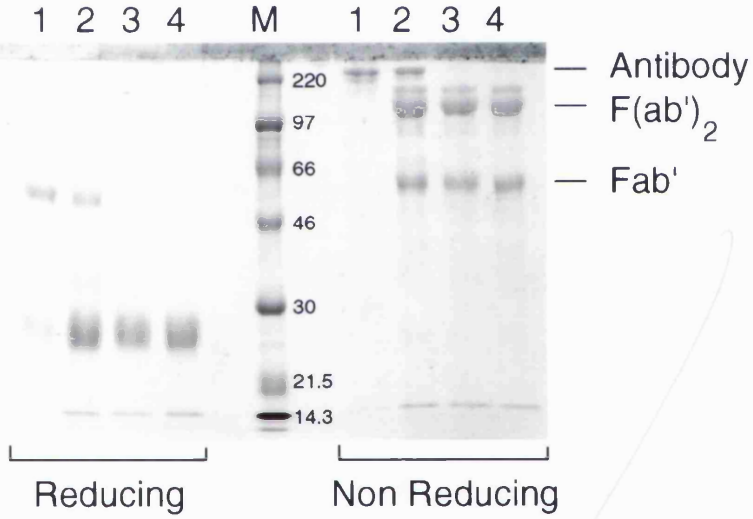
Shown is a typical F(ab')$_2$ preparation (Section 3.4.4), using the Hc73 monoclonal antibody. SDS PAGE was done as described in Section 3.1.4.

Lane 1 shows Hc73 antibody purified using Protein A Sepharose affinity chromatography (Section 3.4.3). Lane 2 shows antibody following pepsin digestion (Section 3.4.4), after which undigested antibody was removed by passage through a Protein A Sepharose column (Lane 3). Lane 4 shows the F(ab')$_2$ preparation after concentration to 5 mg/ml. It should be noted that although the major product was F(ab')$_2$, significant amounts of Fab' were also observed.

M = Large molecular weight rainbow marker, with the apparent molecular weights marked in kilodaltons

Large difference in reducing and non reducing running of Fab'

Figure 3.4.2: SDS PAGE showing the preparation of $F(ab')_2$ from purified antibody



counting to detect the ^{3}H labelled liposomes showed that the relatively less dense liposomes moved to the top of the gradient and the uncoupled Fab' remained at the bottom of the gradient (Figure 3.4.3). Fractions containing purified Fab' liposomes were pooled.

3.5 STUDIES OF BHA BOUND TO LIPOSOME COUPLED Fab' FRAGMENTS

3.5.1 Electron microscopy (EM) of BHA bound to liposome coupled Fab' fragments

Unbound BHA was separated from BHA bound to liposome coupled Fab' fragments by self forming density gradient centrifugation using "optiprep" (the trade name of Iodixanol, Nycomed Pharma). An excess of BHA was added to liposome coupled Fab' fragments and incubated for 30 mins at room temp. 0.5 ml of the BHA - Fab' liposome mixture was mixed with 2.5 ml of 22% optiprep (w/v in 1 mM EDTA / 1.3% (w/v) sucrose / 20 mM tricine pH 7.4) and centrifuged for 3 h at 300000g at $5^{0}C$. Analysis of the gradient by SDS PAGE and scintillation counting to detect the ^{3}H labelled liposomes showed unbound BHA at the bottom of the gradient and liposomes at the top of the gradient.

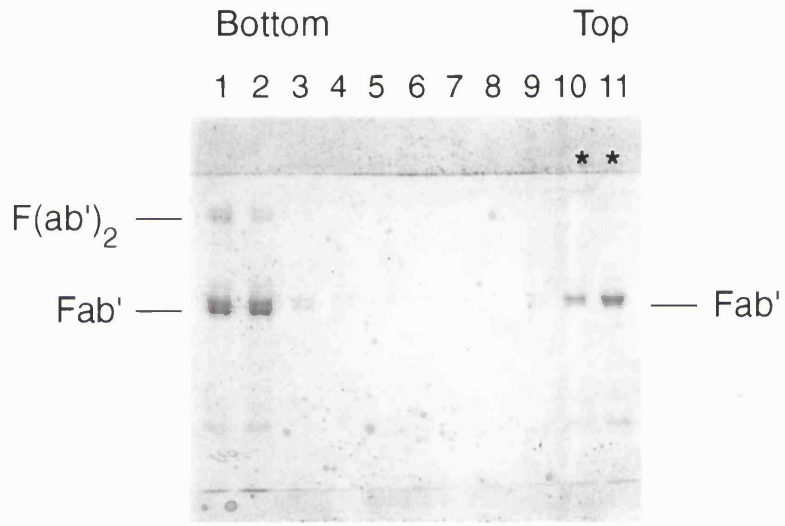
BHA bound to liposome coupled Fab' fragments was low pH treated and trypsin digested as previously described (Section 3.2.4). Electron microscopy was as described in Section 3.1.9.

IIF4 antibody (Vareckova *et al.*, 1993) was a kind gift of Dr. F. Kostolansky (Institute of Virology, Bratislava). IIF4 binding to liposome inserted BHA_2-HA_1 1-27 was investigated by gold labelling of IIF4. Low pH and trypsin pre-treated Fab'

Figure 3.4.3

Shown is a typical liposome coupling of Hc73 Fab'. Fab' was coupled to liposomes and purified from uncoupled Fab' by sucrose density gradient centrifugation as described in Section 3.4.6. As shown the gradient was fractionated and analysed by SDS PAGE (Sections 3.1.4). Detection of ^{3}H labelled liposomes by scintillation counting showed the liposomes were at the top of the gradient. Uncoupled Fab' and a small amount of non reduced $F(ab')_2$ remained at the bottom of the gradient and liposome coupled Fab' was at the top of the gradient.

Figure 3.4.3: SDS PAGE showing Fab' coupled to liposomes, purified from uncoupled Fab' by sucrose density gradient centrifugation



* liposome containing fractions

coupled liposomes were used as a control preparation. IIF4 antibody bound to liposome inserted BHA_2 was absorbed onto carbon grids, and blocked in 1% (w/v in PBS) BSA for 10 mins. Grids were then transferred to 10% (v/v in 1% (w/v in PBS) BSA) immunogold conjugated goat $F(ab')_2$ anti-mouse IgG (British BioCells International) and incubated for 30 mins at room temp. Grids were then washed with PBS and stained with 1% sodium silicotungstate and inspected by EM.

3.5.2 Attempts to quantify the proportion of BHA_2-HA_1 1-27 insertion into the liposome membrane

BHA bound by liposome coupled Fab' fragments was acid treated at pH 5 and trypsin treated as described in Section 3.2.4. Attempts to separate non-liposome associated BHA_2 aggregates from liposome associated BHA_2 by optiprep gradient centrifugation or sucrose gradient centrifugation were done as described in Sections 3.5.1 & 3.4.6. Analysis of the gradient fractions by scintillation counting to detect ^3H labelled liposomes and western blotting (Section 3.1.5) using the primary anti HA polyclonal antibodies R-186 and R-17 to detect BHA_2 indicated that >90% of BHA_2 was liposome associated.

At a range of BHA concentrations from 40 μg/ml to 500 μg/ml at a constant HA:lipid ratio of 1:4 (w/w) BHA was mixed with "bare" liposomes (containing no coupled Fab' receptors). Acid and trypsin treatment of the mixtures followed by gradient analysis also indicated that >90% of BHA_2 was liposome associated. It was thought that the actual proportion of liposome associated BHA_2 in this situation could have not been greater than ~30% (unpublished observations, S. A. Wharton, N.I.M.R). It was therefore concluded that the density gradient centrifugation systems used were

not reliably separating non-liposome associated BHA_2 aggregates from liposome associated BHA_2.

A second approach to separate non-liposome associated BHA_2 aggregates from liposome associated BHA_2 was to use gel filtration. Initial experiments used sephacryl S-300-HR, sephacryl S-400-HR or sepharose 6B (Sigma) to pack a 30cm x 7 mm column equilibrated in PBS. Analysis of the column fractions by scintillation counting to detect ^3H labelled liposomes and western blotting (Section 3.1.5) to detect BHA_2 showed that BHA_2-$HA_1$1-27 aggregates mixed with bare liposomes both eluted in or very close too the void volume and a large proportion (>50%) of liposomes were retained on the column matrix. For these reasons separation of non-liposome associated BHA_2 aggregates from liposome associated BHA_2 using gel filtration was not pursued further.

3.6 VIROSOME - Fab' LIPOSOME FUSION EXPERIMENTS

3.6.1 Production of virosomes

X-31 virus, Ab4 virus (containing X-31 HA in which histidine at $HA_1$17 was replaced by an arginine (X-31 $H17_1R$)) and Res vir 8 virus (containing A/JHB/33/94 HA - also H3 subtype) was grown and purified as described in Section 3.1.1 and the concentration of viral protein determined using the BCA protein assay (Section 3.1.7). Virosomes labelled with 0.6 mol% of N-NBD-PE and N-Rho-PE were made from the above viruses by Method A (Section 3.2.1). To make mixed HA virosomes Ab4 virus and Res vir 8 virus were mixed in equal amounts (w/w of viral protein) and the concentration adjusted to 10 mg/ml of viral protein. 250 µl of this mixture was then used to produce Method A virosomes (Section 3.2.1).

Was there detergent in this assay ??

Does this mean that the BCA assay does not give same results for two viruses?

SDS PAGE of mixed HA virosomes resolved A/JHB/33/94 HA from X-31 $H17_1R$ HA which enabled estimation of the relative proportion of A/JHB/33/94 HA compared to X-31 $H17_1R$ HA in virosome preparations. This enabled subsequent adjustments to be made to produce mixed HA virosomes containing A/JHB/33/94 HA and X-31 $H17_1R$ HA in equimolar amounts as judged by SDS PAGE (Figure 3.6.1).

3.6.2 Virosome - Fab' liposome fusion assay

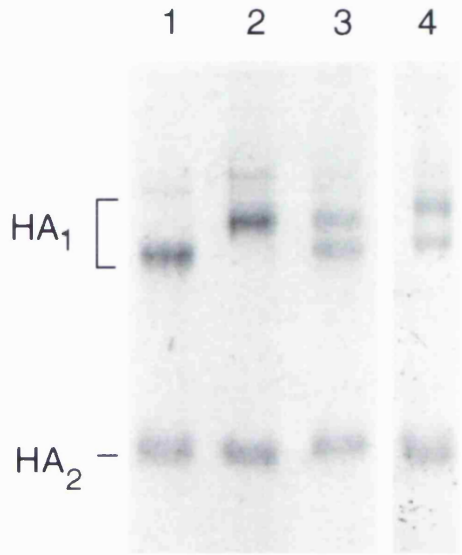
Anti X-31 Hc73 Fab' and anti Res vir 8 H100 or H8 Fab' (Table 3.4.1) were coupled to the same liposome preparation and purified from uncoupled Fab' as previously described (Section 3.4.6). The relative concentration of each Fab' liposome preparation was determined by quantifying the ^3H radioactivity. 10 µl of liposomes was added to 5 ml of scintillation fluid and the amount of ^3H was quantified using the Beckman LS 5000CE liquid scintillation system. Measurements were repeated at least 10 times for each Fab' liposome preparation and the liposome concentration equalized using 20% (w/v in PBS) sucrose.

In each assay 40 µl of N-NBD-PE and N-Rho-PE labelled virosomes and a fixed amount of Fab' liposomes (an amount that gave rise to 30000 cpm using the Beckman LS 5000CE liquid scintillation system - in the range of 30-50µl of liposomes depending on the liposome preparation) was added to PBS pre-warmed to 37^0C to produce a final volume of 1 ml. The pH of the solution was adjusted to a specified pH using 0.15 M sodium citrate pH 3.5. The extent of fusion was estimated by the increase in fluorescence at 530 nm as described in Section 3.3.2.4. Analysis of the relative levels of liposome coupled Fab' and virosomal HA present in the above fusion assays was assessed by SDS PAGE. Fab' liposomes saturated with bound BHA purified from uncoupled Fab' and unbound BHA was used as a standard.

Figure 3.6.1

Virosomes were produced by Method A (Section 3.2.1) as described in Section 3.6.1 and analysed by SDS PAGE (Section 3.1.4). Mixed HA virosomes contained both X-31 $H17_1R$ HA and JHB HA.

Figure 3.6.1: SDS PAGE showing mixed HA virosomes containing JHB HA and X-31 H17$_1$R HA



1) X-31 H17$_1$R HA virosomes
2) JHB HA virosomes
3) Mixed HA virosomes
4) "Uneven" mixed HA virosomes containing more JHB HA than X-31 H17 R HA (Section 4.3.4.2)

Results indicated that in all fusion assays there was an excess of Fab' relative to the amount of HA present when compared to the described standard (data not shown).

The rate of increase in fluorescence at 530 nm was analysed using the "Kfit Programme, Version 1.1" (By Neil C. Millar) which was kindly provided by Dr. S. Martin (N.I.M.R). The rate of fusion induced by some strains of HA could be fitted to a single exponential, for some HA strains the fusion kinetics were more complex and not fitted by a single exponential, however, in this case the apparent $t_{1/2}$ was estimated.

4 RESULTS

4.1 CHAPTER ONE - RESULTS: CHARACTERISATION OF VIROSOMES PRODUCED BY METHODS A, B AND C

4.1.1 Introduction

As discussed in Section 2.3.9 there have been various attempts to produce functional, reconstituted influenza envelopes (virosomes). Stegmann *et al.* (1987) showed that HA containing lipid vesicles reconstituted from viral lipid and HA could cause HA mediated fusion. There have been no reports conclusively showing that purified HA and purified lipids can be reconstituted to form virosomes able to cause HA mediated fusion.

Conventional liposomes are quickly removed from circulation by tissue macrophages, mainly in the liver and spleen (Gregoriadis & Ryman, 1972), by a similar mechanism virosomes containing a viral lipid composition may be expected to be cleared quickly from circulation by tissue macrophages. This would severely limit the ability of a virosome based delivery vector to deliver efficiently to any other cell type. The aim of the following experiments was to investigate whether HA containing virosomes made with purified HA and specified mixtures of purified lipids were able to mediate fusion with target membrane, the target membranes used were liposomes or erythrocyte ghosts. Development of virosomes containing specified lipid compositions that can function in HA mediated fusion might allow preparation of fusion - active virosomes containing moieties such as the ganglioside GM1 or lipid derivatives of poly(ethylene glycol) which would reduce the affinity of virosomes for

tissue macrophages and therefore increase the half life *in vivo* of any virosome based delivery vector and thus increase the delivery efficiency of the vector.

Virosomes produced by Method A had a viral lipid composition, the method used was that of Stegmann *et al.* (1987). This involved solubilization of influenza virus with octaethylene glycol monododecyl ether ($C_{12}E_8$). Removal of the nucleocapsid was by centrifugation, $C_{12}E_8$ was removed by hydrophobic biobeads resulting in formation of virosomes, residual detergent was removed by centrifugation down a discontinuous sucrose gradient followed by dialysis (Section 3.2.1).

Method B was designed to produce virosomes containing a specific lipid composition. Purified HA in octylglucoside (OG) was added to lipid in a specified ratio, OG removal was by dialysis for Method B^1 and when using Method B^2 OG was removed by the method described for Method A (Section 3.2.2).

Method C was an alternative approach to Method B to produce virosomes containing a specific lipid composition. Purified HA in $C_{12}E_8$ was added to lipid in a specified ratio, detergent removal was the same as that described for Method A (Section 3.2.3).

4.1.2 Electron microscopy of virosome preparations

4.1.2.1 Virosomes produced by Method A

Electron microscopy (EM) showed vesicles produced by Method A which contained a viral lipid composition were densely covered with HA (Figure 4.1.1a). EM showed variable levels of HA rosettes in different preparations, but the level of HA rosettes was generally less than 5% compared to the amount of HA incorporated into vesicles. EM of HA in the low pH conformation results in spikes that are less distinct and more "fuzzy" compared to native HA. EM of acid treated Method A

virosomes showed the vast majority of HA in the low pH conformation (Figure 4.1.1b).

4.1.2.2 Virosomes produced by Method B

Method B^1 involved removing OG by dialysis. Virosomes made with lipid of cellular origin (Section 3.1.6) resulted in vesicles densely covered with HA. EM showed these preparations contained less than 5% HA rosettes compared to the amount of HA reconstituted into lipid vesicles (Figure 4.1.1d). Virosomes produced by Method B^1 using mixtures of purified lipids composed of PC/SM/PE/PS/PI/Chol (molar ratios 10:3:3:1:0.5:12.25 respectively) or PC/Chol (molar ratio 2:1 respectively) produced lipid vesicles which were sparsely covered with HA, with at least 70% of the HA present in the form of HA rosettes, as judged by EM (Figure 4.1.1c).

Method B^2 involved removal of OG by biobeads. Virosomes produced by Method B^2 using lipid of cellular origin produced vesicles densely covered with HA but EM showed about 40% of the HA present was in the form of rosettes (Figure 4.1.1f). Virosomes produced using Method B^2 using a mixture of purified lipids composing of PC/SM/PE/PS/PI/Chol resulted in vesicles sparsely covered with HA, with at least 50% of the HA in the form of rosettes, as judged by EM (Figure 4.1.1g).

In all cases EM of acid treated virosomes showed the vast majority of HA in the low pH conformation (Figure 4.1.1e). Within the range of HA:lipid ratios used to make virosomes by Method B (Section 3.2.2) there was no significant difference in the ratio of HA incorporated into vesicles compared to the amount of HA rosettes as judged by EM (data not shown).

Not so clear on this figure! Comes back at pg 84! Should have done other analyses - trypsin etc. to see if identical to HHA

4.1.2.3 Virosomes produced by Method C

Virosomes produced using Method C made with lipid of cellular origin were highly variable. EM often showed about half the vesicles being sparsely covered with HA, together with vesicles which were densely covered with HA, the levels of HA rosettes generally less then 5% compared to the amount of HA reconstituted into vesicles (Figure 4.1.1h). In contrast to virosomes made with lipid of cellular origin, virosomes produced by Method C using mixtures of purified lipids composed of PC/SM/PE/PS/PI/Chol or PC/Chol reproducibly resulted in vesicles densely covered with HA with less than 5% HA rosettes relative to the amount of HA reconstituted into vesicles, as judged by EM (Figure 4.1.1i&j).

In all cases EM of acid treated virosomes showed the vast majority of HA in the low pH conformation (Figure 4.1.1k). Within the range of HA:lipid ratios used to make virosomes by Method C (Section 3.2.3) there was no significant difference in the ratio of HA incorporated into vesicles compared to the amount of HA rosettes as judged by EM.

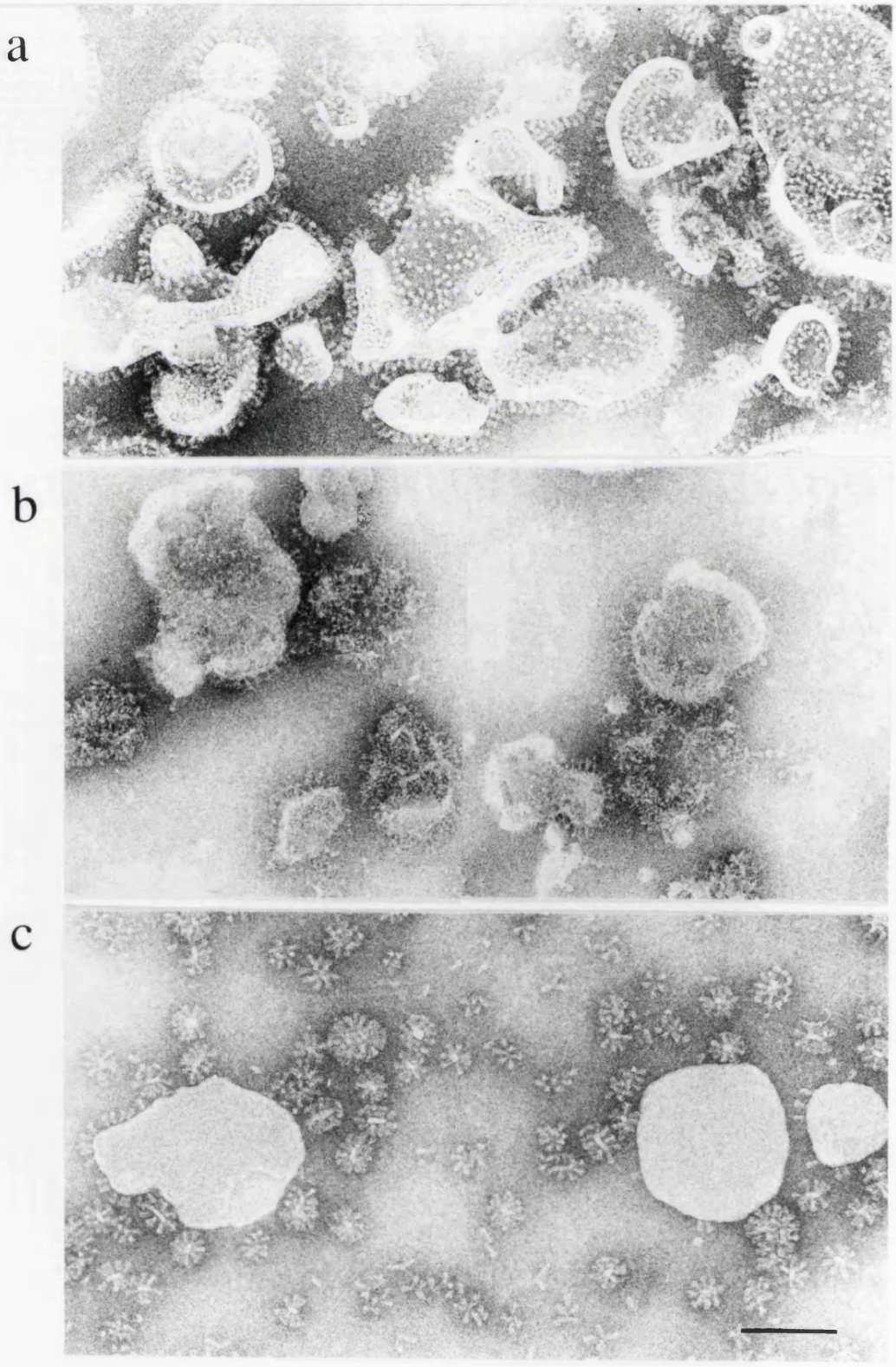
4.1.3 Membrane fusion activity of virosome preparations

The fusion activity of the virosome preparations described above was assayed by two methods, haemolysis of human erythrocytes (Section 3.3.1) and resonance energy transfer (R.E.T) (Section 3.3.2). For R.E.T, fusion between a membrane labelled with N-NBD-PE and N-Rho-PE and an unlabelled membrane resulted in dilution of the fluorescent dyes and an increase in N-NBD-PE fluorescence at 530 nm. When using R.E.T to monitor membrane fusion two types of target membrane were used, either erythrocyte ghosts or liposomes. When using erythrocyte ghosts as target membrane, virosomes were labelled with the fluorescent probes and the erythrocyte

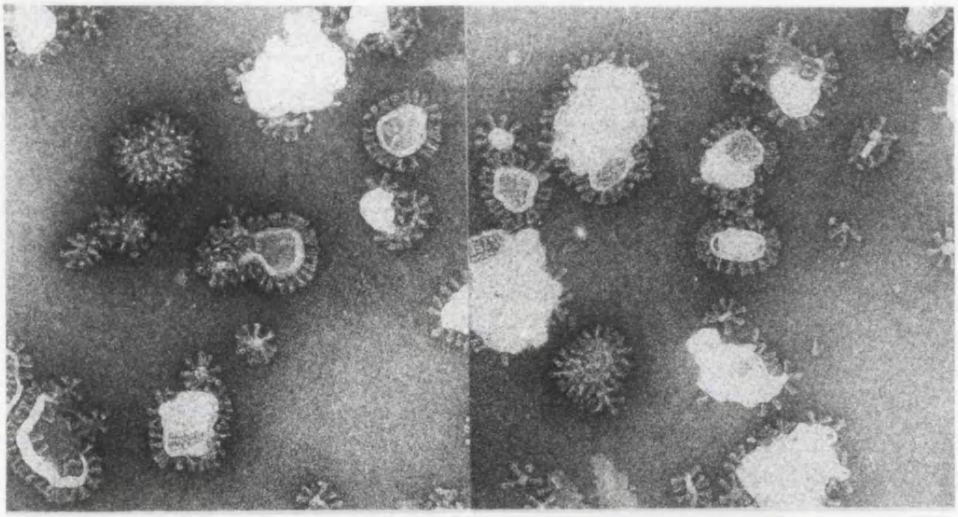
Figure 4.1.1

Virosomes were produced by Methods A, B and C as described in Section 3.2. Electron microscopy of virosome preparations was done as described in Section 3.1.9. **a)** shows virosomes produced by Method A, vesicles were densely covered with HA, with more than 95% of the HA present incorporated into vesicles. Pre-treatment of virosomes shown in a) at pH 5 caused the vast majority of HA to undergo a characteristic low pH induced conformational change, as shown in **b)**. **c)** shows virosomes produced by Method B^1 using 1mg of a PC/SM/PE/PS/PI/Chol lipid mixture (molar ratios 10:3:3:1:0.5:12.25 respectively) (HA:lipid ratio 1:2.9). Vesicles were sparsely covered with HA, with at least 70% of the HA present in the form of HA rosettes. **d)** shows virosomes produced by Method B^1 using 1mg of lipid of cellular composition (HA:lipid ratio 1:2.6), vesicles were densely covered with HA, with more than 95% of the HA present incorporated into vesicles. Pre-treatment of virosomes shown in d) at pH 5 caused the vast majority of HA to undergo a characteristic low pH induced conformational change, as shown in **e)**. **f)** shows virosomes produced by Method B^2 using lipid of cellular composition, vesicles were densely covered with HA , but it was estimated at least 40% of the HA present was in the form of rosettes. **g)** shows virosomes produced by Method B^2 using a PC/SM/PE/PS/PI/Chol lipid mixture (molar ratios 10:3:3:1:0.5:12.25 respectively), vesicles were only sparsely covered with HA with approximately half the HA present in the form of rosettes. **h)** shows virosomes produced by Method C using 0.75mg of lipid of cellular composition (HA:lipid ratio 1:3.1), vesicles which were both sparsely and densely packed with HA were observed, the level of HA rosettes was estimated to be less than 5% of the total HA present. **i)** shows virosomes produced by Method C using 0.75mg of a PC/Chol lipid mixture (molar ratio 2:1 respectively) (HA:lipid ratio 1:3.2), vesicles were densely covered with HA with less than 5% of the HA present in the form of HA rosettes. **j)** shows virosomes produced by Method C using 0.75mg of a PC/SM/PE/PS/PI/Chol lipid mixture (molar ratios 10:3:3:1:0.5:12.25 respectively) (HA:lipid ratio 1:5.3), vesicles were densely covered with HA, with more than 95% of the HA present incorporated into vesicles. Pre-treatment of virosomes shown in j) at pH 5 caused the vast majority of HA to undergo a characteristic low pH induced conformational change, as shown in **k)**. **bar ≡ 100nm**

Figure 4.1.1: Electron microscopy of X-31 HA - containing virosomes produced by Methods A, B and C



d



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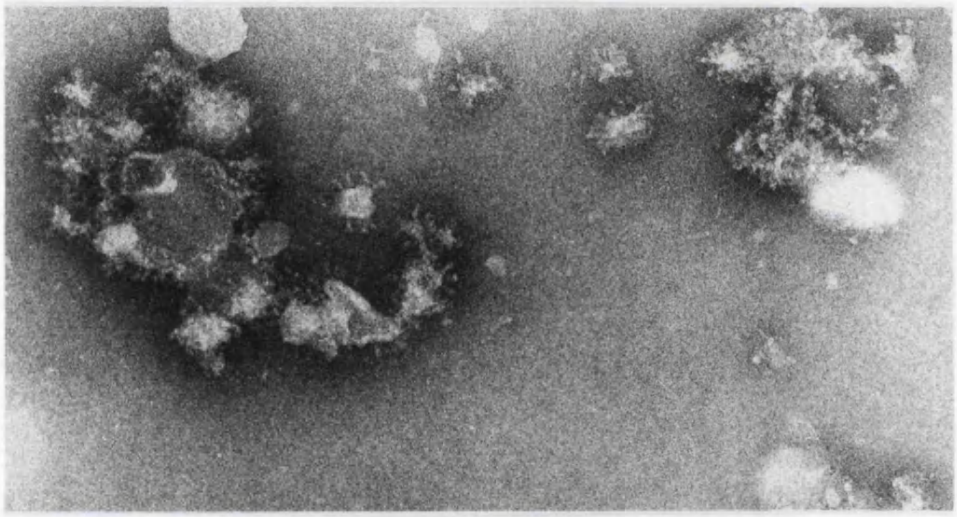

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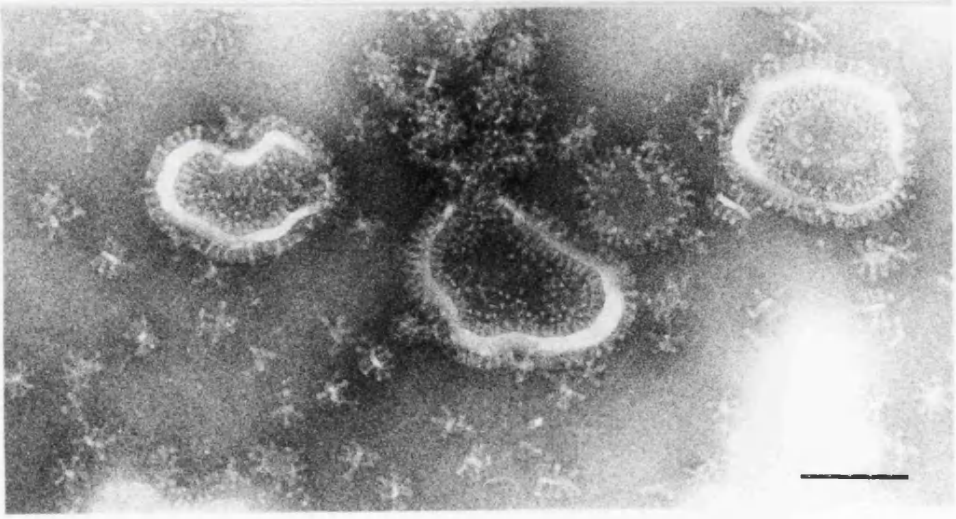

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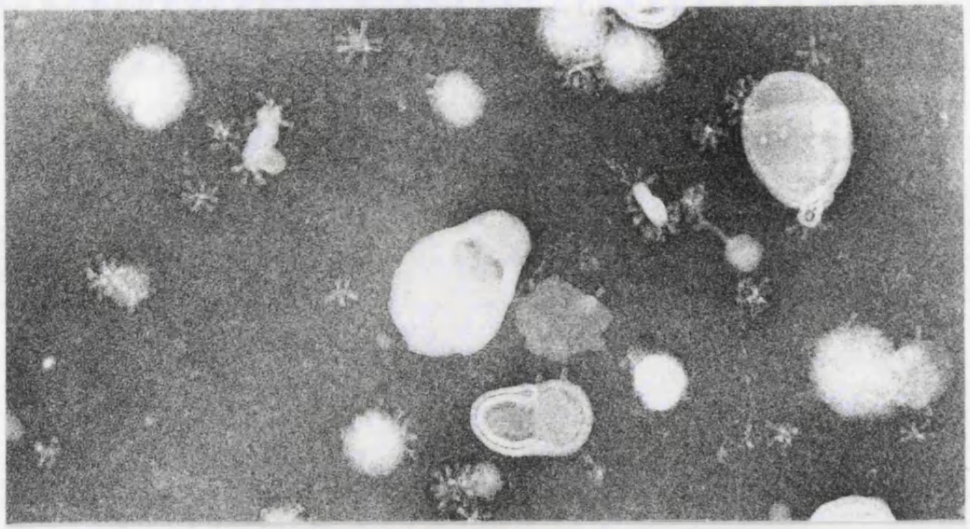

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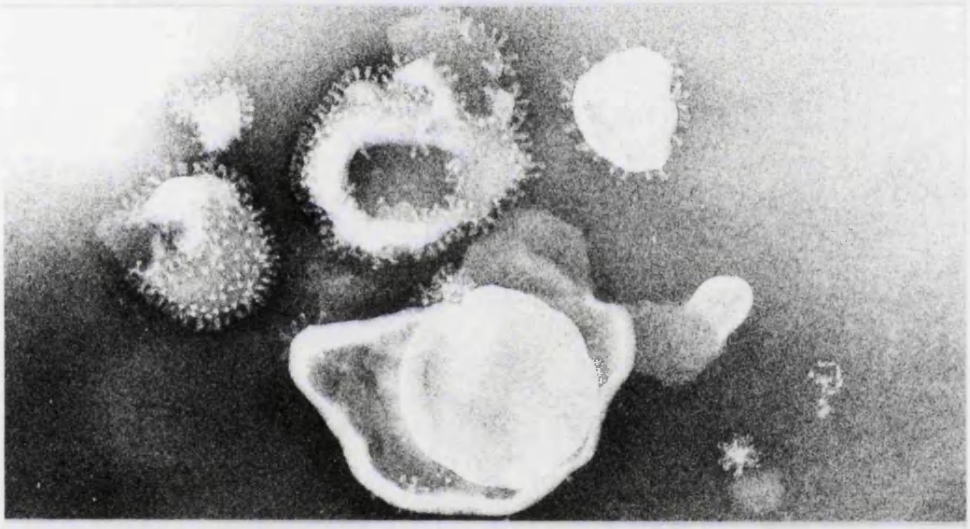

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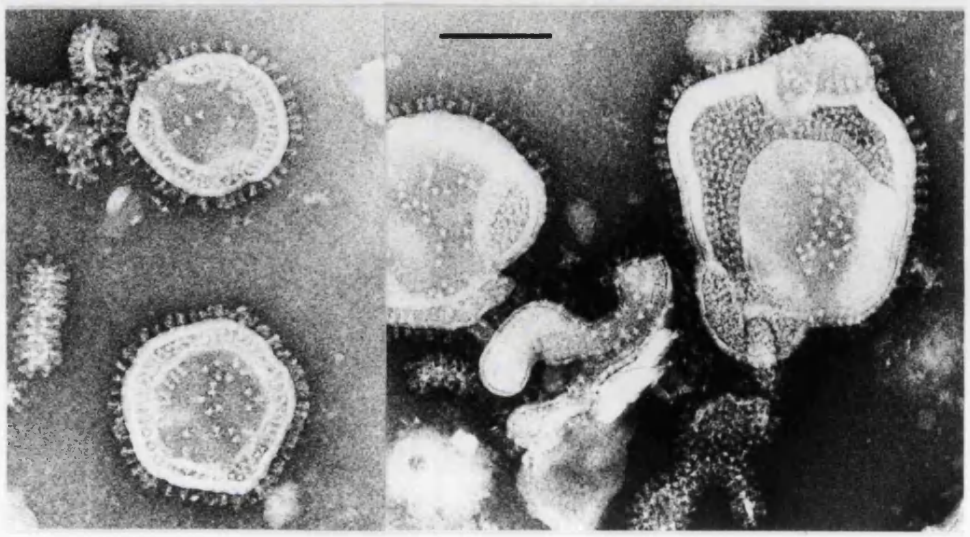

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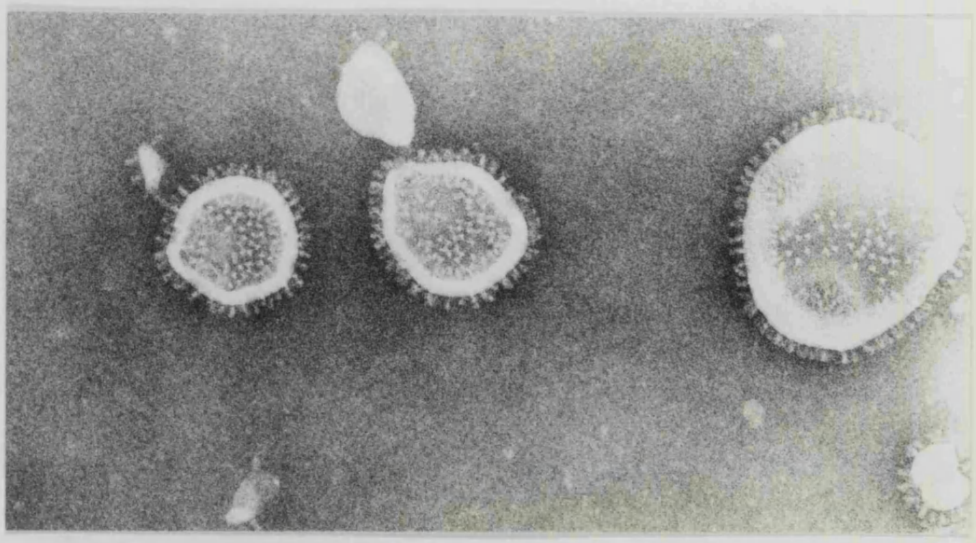

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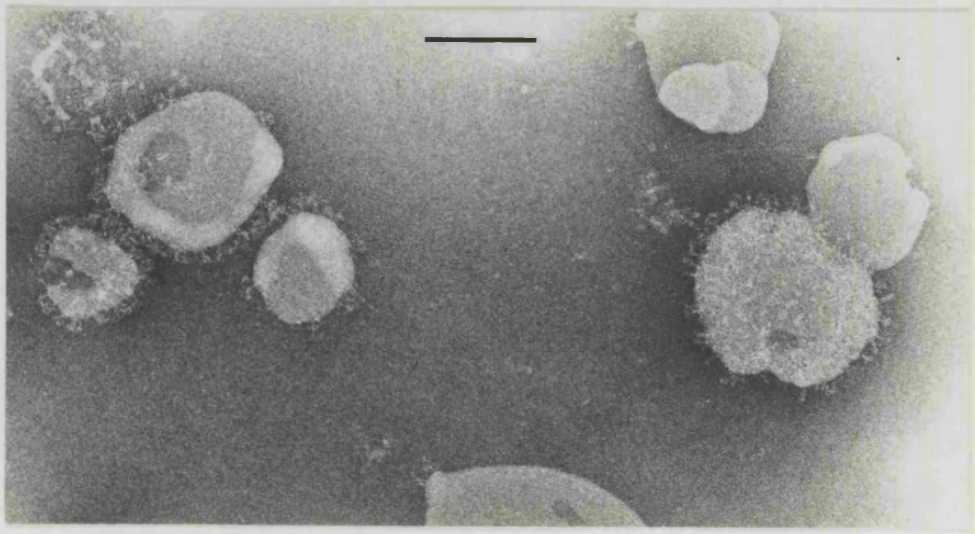


ghosts were unlabelled. The precise level of fluorescent dyes varied in different virosome preparations, which resulted in slightly different levels of R.E.T in different virosome preparations. This made direct comparison of the fusion efficiency between different virosome preparations inaccurate. When the fusion efficiency of different virosome preparations was directly compared, a constant amount of fluorescently labelled liposomes was used as target membrane, and virosomes were unlabelled.

Under the assay conditions specified the percentage increase in fluorescence at 530 nm of virosome - erythrocyte ghost fusion was higher than that for virosome - liposome fusion (Section 3.3.2.4). Virosome - erythrocyte ghost fusion was therefore a more sensitive assay to detect membrane fusion than virosome - liposome fusion. As described above virosome-erythrocyte ghost fusion could not be used to directly compare the membrane fusion efficiency of different virosome preparations and for this reason virosome - liposome fusion was also used.

4.1.3.1 Virosomes produced by Method A

Virosomes labelled with N-NBD-PE and N-Rho-PE produced by Method A induced membrane fusion with erythrocyte ghosts at pH 5 but not at pH 7, virosomes pre-treated at pH 5 inducing the irreversible conformational change of HA did not fuse with erythrocyte ghosts at pH 5. The extent of membrane fusion was significantly reduced by removal of sialic acid residues from the erythrocyte ghost membrane, showing HA receptor on the target membrane significantly enhanced fusion efficiency (Figure 4.1.2).

High level haemolysis of human erythrocytes (95%) was induced by Method A virosomes at pH 5 but not at pH 7, virosomes pre-treated at pH 5 did not induce haemolysis at pH 5 (Table 4.1.1).

Table 4.1.1

Virosomes preparations were made as described in Section 3.2 using X-31 HA in all cases. The HA:lipid ratio of virus and virosomes were determined as described in Section 3.2.5.

Haemolysis was done as described Section 3.3.1. For all samples 195 ng (in 50 μl) of HA was added to 500 μl of 2 % human erythrocytes and the percentage of haemolysis calculated relative to 100 % haemolysis induced by the addition of Brij 36T (final concentration 0.5% w/v).

Table 4.1.1: Haemolysis of human erythrocytes by various X-31 HA containing virosome preparations and intact X-31 influenza virus

Virosome preparation	HA:lipid ratio (w/w)	% of haemolysis at pH5		% of haemolysis at pH 7
		Virosomes untreated	Virosomes pre-treated at pH5	Virosomes untreated
X-31 influenza virus	1 : 0.831 (*)	67	6	6
Method A virosomes, viral lipid	1 : 3.1 (*)	95	5	7
Method B[1] virosomes, lipid of cellular composition	1 : 0.9 (H)	9	6	6
	1 : 2.6 (H)	7	7	6
	1 : 4.9 (H & *)	8	7	6
Method B[1] virosomes, PC/SM/PE/PS/PI/Chol lipid[#]	1 : 0.9 (H)	40	6	7
	1 : 2.9 (H)	33	6	7
	1 : 4.6 (H & *)	40	5	6
Method B[1] virosomes, PC/Chol lipid[~]	1 : 1.0 (H)	36	6	6
	1 : 3.5 (H)	34	7	7
	1 : 4.8 (H)	21	6	7
Method B[1], CHA rosettes	no lipid (*)	79	6	7
Method C virosomes, lipid of cellular composition	1 : 3 (H)	27	7	6
	1 : 3.1 (H)	60	6	6
	1 : 4.5 (H & *)	20	6	6
Method C virosomes, PC/SM/PE/PS/PI/Chol lipid[#]	1 : 3.4 (H)	44	6	6
	1 : 5.3 (H)	70	6	6
	1 : 6.5 (H)	31	6	5
Method C virosomes, PC/Chol lipid[~]	1 : 3.2 (H)	67	6	5
	1 : 4.6 (H)	55	6	7
Method C, X-31 CHA rosettes	no lipid (*)	67	6	6

* = Total lipid concentration calculated from phospholipid concentration determined by method of King and Wootton (1956) (Section 3.1.8).

H = Total lipid concentration calculated from final ^{3}H cholesterol concentration.

=PC/SM/PE/PS/PI/Chol in molar ratios 10:3:3:1:0.5:12.25

~ =PC/Chol in molar ratio 2:1

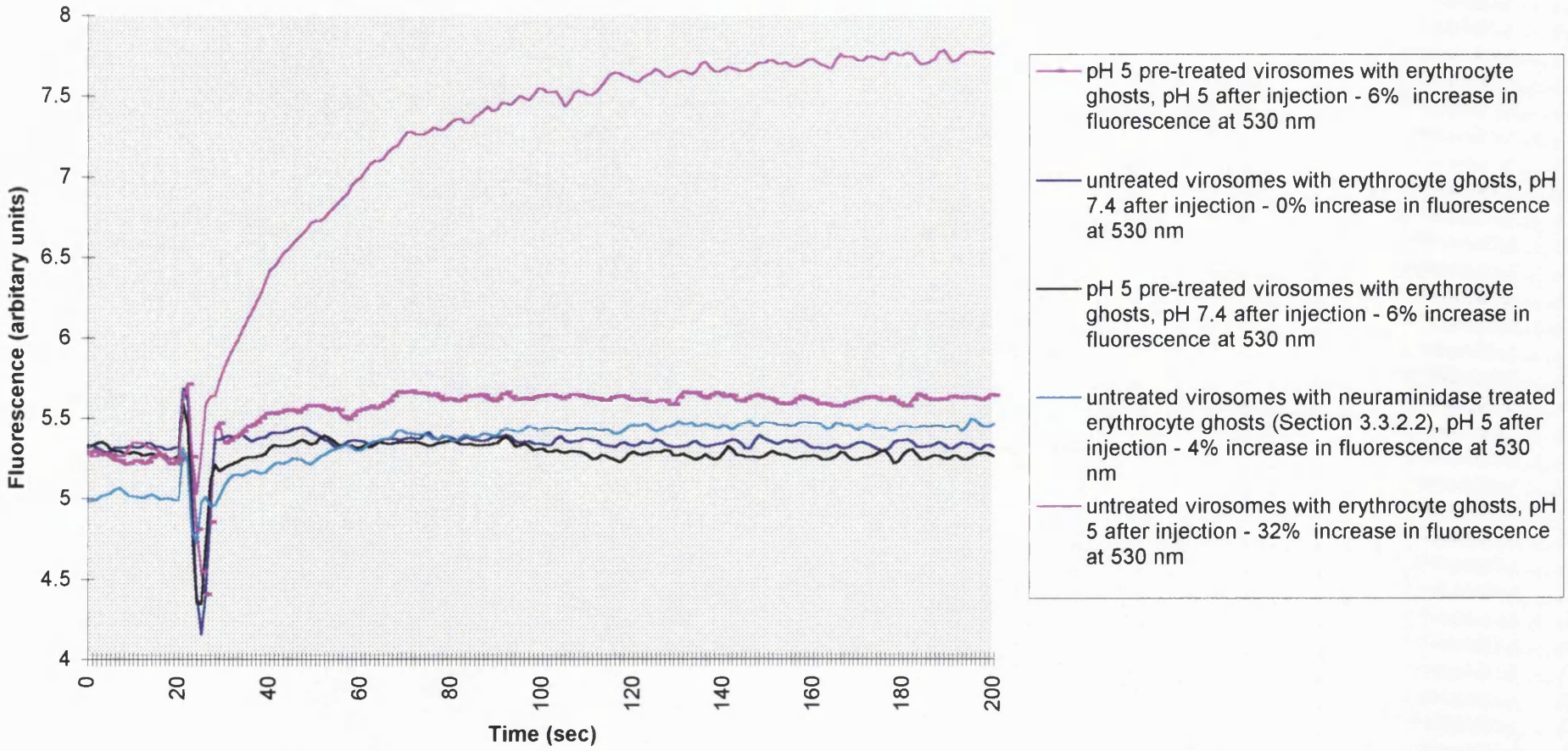
Figure 4.1.2

X-31 HA containing virosomes produced by Method A were labelled with 0.6 mol% N-NBD-PE and N-Rho-PE (Section 3.2.1). 50µl of labelled virosomes and 40 µl of unlabelled erythrocyte ghosts (Section 3.3.2.1) were added to PBS prewarmed to 37^0C to produce a final volume of 1 ml. The R.E.T membrane fusion assay was done as described in Section 3.3.2. At 20 seconds after incubation of the virosomes with the erythrocyte ghosts the pH was lowered to pH 5 by injecting 0.15 M sodium citrate pH 3.5 into the cuvette. Alternatively an equal volume of PBS was injected.

No wavelength filters were used.

Ex. λ = 465 nm, Em. λ= 530 nm Temp. = 37^0C

Figure 4.1.2: Virosome - erythrocyte ghost fusion assayed by R.E.T (virosomes produced by Method A - viral lipid composition)



These results indicate that Method A virosomes participated in HA mediated fusion, the level of HA rosettes in the preparation was less than 5% compared to the amount of HA reconstituted into lipid vesicles as judged by EM and it is therefore unlikely that HA rosettes contributed significantly to the observed fusion activity .

4.1.3.2 Virosomes produced by Method B

Methods B^1 and B^2 involved removal of OG by dialysis or biobeads respectively. Virosomes produced by Method B^1 made with lipid of cellular origin, or mixtures of purified lipids composed of PC/SM/PE/PS/PI/Chol or PC/Chol were labelled with N-NBD-PE and N-Rho-PE. These virosomes did not induce detectable membrane fusion with erythrocyte ghosts at pH 5 or pH 7 as assayed by the R.E.T assay.

Virosomes made with lipid of cellular origin produced using Method B^1 which contained less than 5% HA rosettes did not induce haemolysis of human erythrocytes (Table 4.1.1), in agreement with negative results obtained using the R.E.T. assay. Virosomes with a PC/SM/PE/PS/PI/Chol or PC/Chol lipid composition produced using Method B^1 induced significant haemolysis of human erythrocytes (~30-40%) at pH 5 but not at pH 7 (Table 4.1.1), contrary to the results obtained using the R.E.T. assay. The apparent discrepancy can be explained by the observation that HA rosettes produced by Method B^1 induced high levels of haemolysis (79%) (Table 4.1.1) which was in agreement with Sato *et al.* (1983). At least 70% of the HA present in Method B^1 virosomes made with PC/SM/PE/PS/PI/Chol or PC/Chol lipid was in the form of HA rosettes, it is therefore highly likely that the high level of rosettes was responsible for the observed haemolysis.

Virosomes produced using Method B^2 made with lipid of cellular origin or PC/SM/PE/PS/PI/Chol were labelled with N-NBD-PE and N-Rho-PE. These virosomes did not fuse with erythrocyte ghosts at pH 5 or pH 7 as assayed by the R.E.T assay. Using Method B^2 virosomes made with lipid of cellular origin or PC/SM/PE/PS/PI/Chol lipid significant haemolysis (~30%) of human erythrocytes was observed at pH 5 but not at pH 7 (data not shown). Virosomes pre-treated at pH 5 did not induce haemolysis indicating that the haemolysis was HA mediated. Due to the absence of membrane mixing as assayed by R.E.T it is likely that the observed haemolysis was due to the contaminating HA rosettes present in Method B^2 virosome preparations, in which ~40-50% of the HA present was in the form of HA rosettes, as shown by EM (Figure 4.1.1f&g).

Method B irrespective of the method of OG removal, produced virosomes which were unable to cause HA mediated fusion as shown by the R.E.T. assay, any haemolysis observed was likely to be due to contaminating HA rosettes.

4.1.3.3 Virosomes produced by Method C

Virosomes produced by Method C containing PC/SM/PE/PS/PI/Chol lipid were labelled with N-NBD-PE and N-Rho-PE and the amount of fusion with erythrocyte ghosts assayed by R.E.T. As shown in Figure 4.1.3 virosomes containing PC/SM/PE/PS/PI/Chol lipid participated in HA mediated fusion with erythrocyte ghosts. The results in Table 4.1.2 show that Method C virosomes made with lipid of cellular composition, PC/SM/PE/PS/PI/Chol or PC/Chol lipid can cause HA mediated fusion with liposomes as assayed by R.E.T.

Virosomes produced using Method C made with lipid of cellular origin, PC/SM/PE/PS/PI/Chol or PC/Chol lipid induced HA mediated haemolysis of human

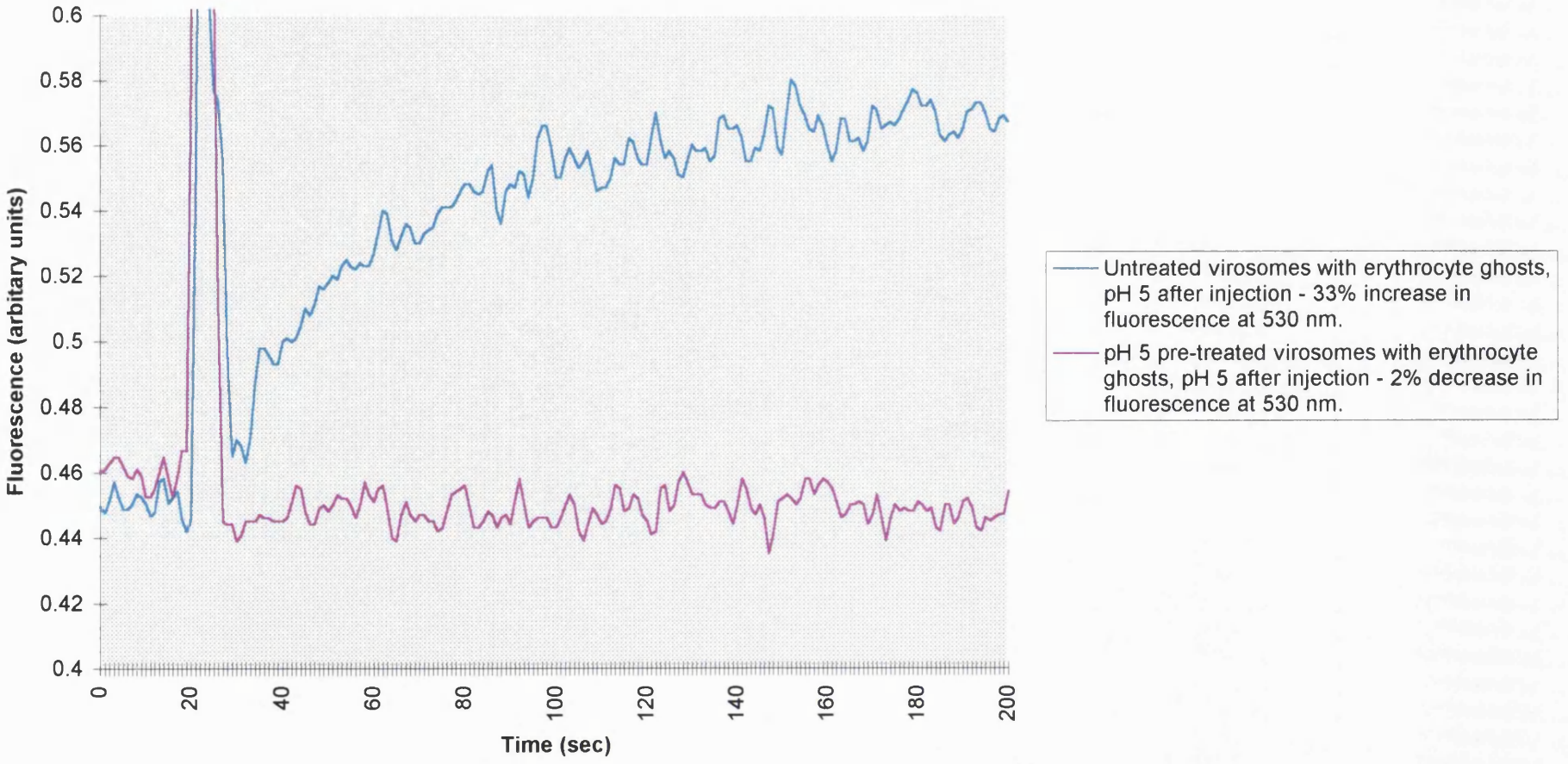
Figure 4.1.3

X-31 HA containing virosomes produced by Method C were made with 0.75 mg of a lipid mixture containing PC/SM/PE/PS/PI/Chol in molar ratios 10:3:3:1:0.5:12.25 containing 0.6 mol% N-NBD-PE and N-Rho-PE (Section 3.2.3). The HA:lipid ratio (w/w) of the virosome preparation was approximately 1:5 (Section 3.2.5).

50 µl of labelled virosomes produced by Method C and 40 µl of unlabelled erythrocyte ghosts (Section 3.3.2.1) were added to PBS prewarmed to 37^0C to produce a final volume of 1 ml. The R.E.T membrane fusion assay was done as described in Section 3.3.2. At 20 seconds after incubation of the virosomes with the erythrocyte ghosts the pH was lowered to pH 5 by injecting 0.15 M sodium citrate pH 3.5 into the cuvette.

Ex. λ = 465 nm, Em. λ= 530 nm Temp. = 37^0C

Figure 4.1.3: Virosome - erythrocyte ghost fusion assayed by R.E.T (virosomes produced by Method C - PC/SM/PE/PS/PI/Chol lipid composition. HA:lipid ratio (w/w) ~ 1:5)



erythrocytes at pH 5 (Table 4.1.1), in agreement with the results using the R.E.T assay. EM showed virosomes produced by Method C contained less than 5% HA rosettes compared to the amount of HA reconstituted into lipid vesicles. Due to the relatively low level of rosettes and the fact that Method C virosomes mediated membrane mixing as judged by the R.E.T assay it is therefore assumed the vast majority of the observed haemolysis was due to virosome-erythrocyte fusion.

It can be concluded that using Method C to produce virosomes was a successful approach to reconstituting purified HA and purified lipid into vesicles which can then cause HA mediated fusion. Fusion - active virosomes produced by Method C made with mixtures of purified lipids composed of PC/SM/PE/PS/PI/Chol or PC/Chol reproducibly resulted in uniform lipid vesicles, approximately 150 nm in diameter which were densely covered with HA. The level of HA rosettes in these preparations estimated by EM was less than 5% of the total HA present (Figure 4.1.1). The morphology and fusion activity of Method C virosomes made with lipid of cellular origin was less reproducible compared to virosomes made with PC/SM/PE/PS/PI/Chol or PC/Chol lipid.

4.1.4 Fusion efficiency - A comparison between virus and virosomes

The HA concentration of virus and various virosome preparations was equalized as described in Section 3.2.5. A liposome preparation composed of PC/SM/PE/PS/PI/Chol, containing 3 mol% gangliosides and labelled with N-NBD-PE and N-Rho-PE was used as target membrane (Section 3.3.2.3). In each assay an equal amount of HA was added to a constant amount of target liposomes and the decrease in R.E.T caused by acid induced membrane fusion was followed. The extent of the decrease in R.E.T depends on the dilution of the fluorescent probes within the viral or

virosomal membranes following membrane fusion. The amount of dilution and therefore decrease in R.E.T is dependent on the efficiency of the virus or virosome at inducing membrane fusion but also on the amount of viral or virosomal lipid available for probe dilution. For example, assuming that the availability of target membrane does not limit the amount of fusion, if at a fixed HA concentration a virosome preparation with a specified HA:lipid ratio gave rise to the same R.E.T decrease as a virosome preparation with a lower HA:lipid ratio the fusion efficiency in the first instance must have been higher compared to the virosome preparation with a lower HA:lipid ratio. Different lipid compositions also effected the level of R.E.T between NBD-PE and N-Rho-PE probes, for example R.E.T was more efficient in membranes composed of PC/PE (2:1 molar ratio, respectively) compared to membranes composed of PC/PE/Chol (2:1:2.1 molar ratio, respectively) which each contained 0.6 mol% of NBD-PE and N-Rho-PE (data not shown).

Virus was significantly more efficient at inducing membrane fusion than any virosome preparation inducing 20 % compared to at most 11% increase in fluorescence at 530 nm with Method C virosomes containing PC/Chol lipid (Figure 4.1.4 & Table 4.1.2). The difference was even more significant after accounting for the fact that virus had the highest HA:lipid ratio, and therefore the lowest amount of lipid available for probe dilution at the fixed HA concentration.

In agreement with Section 4.1.3.2 virosomes produced by Method B showed no significant membrane fusion. Virosomes produced by Methods A or C caused HA mediated fusion in agreement with previous experiments (Sections 4.1.3.1 and 4.1.3.3). Due to the relatively inaccurate method used to determine HA concentration by SDS PAGE (Section 3.2.5) and the other factors outlined above which effect the

Figure 4.1.4

Liposomes were prepared as described in Section 3.3.2.3 which contained 3 mol% gangliosides and 0.6 mol% N-Rho-PE and N-NBD-PE. Unlabelled X-31 influenza virus and X-31 HA containing virosomes were produced and the HA:lipid ratio estimated as described in Sections 3.1.1 & 3.2.

6 μl of labelled liposomes and virosomes or virus containing 4.8 μg of HA were added to PBS prewarmed to 37^0C to produce a final volume of 1 ml. The R.E.T membrane fusion assay was done as described in Section 3.3.2. At 20 seconds after incubation the pH was lowered to pH 5 by injecting 0.15 M sodium citrate pH 3.5 into the cuvette.

During analysis of the data the initial fluorescence has been adjusted to the same value to show clearly differences in fusion activity between preparations. The results shown are also summarised in Table 4.1.2.

Ex. λ = 465 nm, Em. λ= 530 nm Temp. = 37^0C

Figure 4.1.4: Comparison between the membrane fusion efficiency of various virosome preparations and intact virus

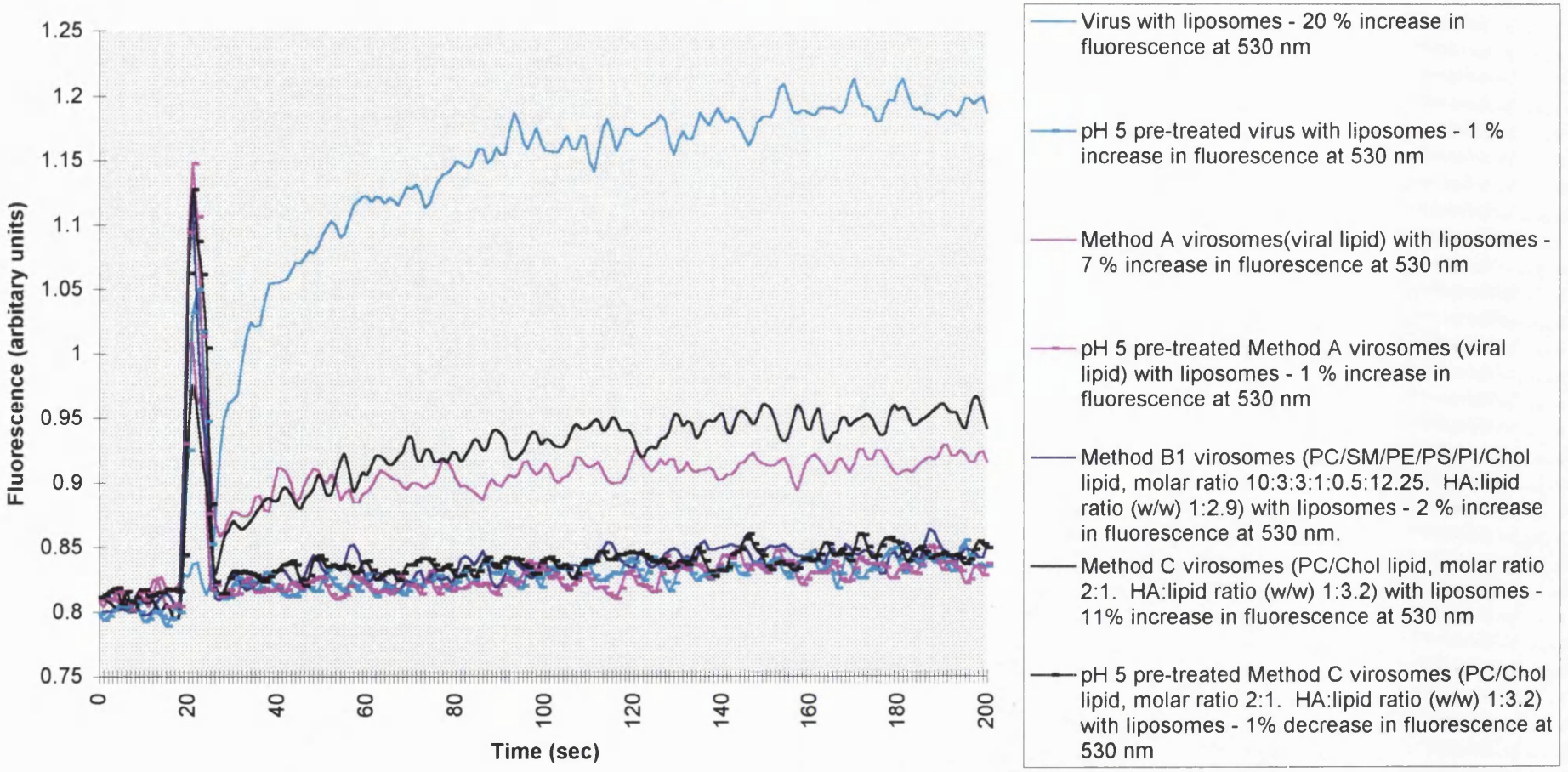


Table 4.1.2

Unlabelled virosomes preparations were made as described in Section 3.2 using X-31 HA in all cases. The HA:lipid ratio of virus and virosomes were determined as described in Section 3.2.5. Liposomes were prepared as described in Section 3.3.2.3 which contained 3 mol% gangliosides and 0.6 mol% of N-Rho-PE and N-NBD-PE.

6 μl of labelled liposomes and unlabelled virosomes or virus containing 4.8 μg of HA were added to PBS prewarmed to 37^0C to produce a final volume of 1 ml. The R.E.T membrane fusion assay was done as described in Section 3.3.2. At 20 seconds after incubation the pH was lowered to pH 5 by injecting 0.15 M sodium citrate pH 3.5 into the cuvette.

Ex. λ = 465 nm, Em. λ= 530 nm Temp. = 37^0C

Table 4.1.2: Comparison between the membrane fusion efficiency of various X-31 HA containing virosome preparations and intact X-31 influenza virus using the R.E.T assay.

Virosome preparation	**HA:lipid ratio (w/w)**	**Percentage increase in fluorescence at 530nm**	
		Virosomes untreated	**Virosomes pre- treated at pH 5**
X31 influenza virus [x]	1 : 0.831 [*]	20.1	1.2
Method A virosomes, viral lipid [x]	1 : 3.1 [*]	6.8	1.4
Method B[1] virosomes, lipid of cellular compostion	1 : 0.9 [H]	1.9	ND
	1 : 2.6 [H]	0.7	ND
	1 : 4.9 [H & *]	2.	ND
Method B[1] virosomes, PC/SM/PE/PS/PI/Chol lipid[#] [x]	1 : 0.9 [H]	0.7	ND
	1 : 2.9 [H]	2.3	ND
	1 : 4.6 [H & *]	2.1	ND
Method B[1] virosomes, PC/Chol lipid[~].	1 : 1.0 [H]	0.9	ND
	1 : 3.5 [H]	-1.1	ND
	1 : 4.8 [H]	1.6	ND
Method C virosomes, lipid of cellular composition.	1 : 3 [H]	3.8	0.5
	1 : 3.1 [H]	7.4	-0.1
	1 : 4.5 [H & *]	2.3	0.1
Method C virosomes, PC/SM/PE/PS/PI/Chol lipid[#]	1 : 3.4 [H]	6.7	-1.5
	1 : 5.3 [H]	10.7	0.0
	1 : 6.5 [H]	7.8	0.2
Method C virosomes, PC/Chol lipid[~] [x]	1 : 3.2 [H]	10.9	0.5
	1 : 4.6 [H]	10.6	-1.2

[*] = Total lipid concentration calculated from phospholipid concentration determined by method of King and Wootton (1956) (Section 3.1.8).
[H] = Total lipid concentration calculated from final ^{3}H cholesterol concentration.
[x] = As shown in Figure 4.1.4
ND = Not determined
[#] =PC/SM/PE/PS/PI/Chol in molar ratios 10:3:3:1:0.5:12.25
[~] =PC/Chol in molar ratio 2:1

Causing HA/lipid, virus 10X more active than virosomes.

R.E.T assay it can be concluded that no significant differences in the fusion efficiency of virosomes produced by Methods A or C were detected.

4.1.5 Discussion

4.1.5.1 Evaluation of Methods A, B and C for virosome production

Virosomes made by Method A produced vesicles with a viral lipid composition which mediated HA dependent fusion as previously reported by Stegmann *et al.* (1987).

Virosomes produced by Method B using OG did not mediate HA dependant fusion with liposomes or erythrocyte ghosts assayed using R.E.T. In the case of virosomes produced by Method B using PC/SM/PE/PS/PI/Chol or PC/Chol lipid HA rosettes accounted for ~50-70% of the HA present. The rosettes present were shown to induce haemolysis of human erythrocytes. The report of Wharton *et al.* (1986) demonstrated HA rosette mediated liposome-liposome fusion, it might therefore have been expected that the HA rosettes present in these Method B virosomes could have caused rosette mediated fusion. In fact, no HA rosette mediated fusion between the sparsely packed virosomes and liposomes or erythrocyte ghosts was detected by the R.E.T assay. The ratio of HA, unlabelled lipid and labelled lipid used was comparable to the report of Wharton *et al.* (1986), the reason for the apparent discrepancy can perhaps be explained by the differences in the HA and lipid concentrations used. The report of Wharton *et al.* (1986) worked at HA rosette concentrations of 100 μg/ml and lipid concentrations of 250 μg/ml, compared to the presented data which was determined at ~5μg/ml total HA, which included the HA incorporated into the sparsely packed vesicles and ~15 μg/ml lipid. The relatively low concentration of HA rosettes may have reduced the efficiency of rosette mediated

fusion and therefore explained why HA rosette mediated fusion was not detected by the R.E.T assay.

EM of Method B virosomes pre-treated at pH 5 (Figure 4.1.1e) showed HA that had appeared to have undergone a characteristic acid induced conformational change. It is therefore unknown why Method B virosomes made with lipid of cellular composition which were densely covered in HA were unable to cause HA mediated membrane fusion at any detectable level. It is possible that the process of OG removal, whether by dialysis or biobeads resulted in a non-functional insertion of the trans-membrane domain of HA into the lipid bilayer, which blocked successful HA mediated fusion. Scheule *et al.* (1995) reported that virosomes with a viral lipid composition made using OG were able to undergo HA mediated fusion, which is contrary to the presented results.

Fusion active virosomes were reproducibly made by Method C using PC/SM/PE/PS/PI/Chol or PC/Chol lipid mixtures, which resulted in densely packed vesicles with low levels of HA rosettes. The virosomes produced were shown to mediate HA dependent fusion with both liposomes and erythrocyte ghosts.

The results obtained using virosomes produced by Method C showed that it is possible to take purified lipid and purified HA and reconstitute lipid vesicles that can cause HA mediated fusion. This significantly improves the prospects of further experiments which will attempt to reconstitute fusion - active HA into lipid vesicles containing a lipid composition that increases the circulation half life *in vivo*, that have the potential to improve the delivery efficiency of a HA-liposome based delivery vector.

There was no M1 present in virosomes produced by Methods A B and C as judged by SDS PAGE.

The amount of NA in virosomes (methods A B and C) was relatively low compared to HA as judged by SDS PAGE and EM (<5% of membrane glycoprotein was NA, the rest being HA)

M2 could not be detected in virosomes produced by Methods A B or C. The presence of M2 was investigated by western blotting, detecting M2 using R54 as a primary antibody. Loading of an equivalent amount of virosomes and virus (as judged by the amount of HA) onto the gel - M2 could be detected in virus but not virosomes.

4.1.5.2 Comparison between virus and virosome fusion

There are several differences between influenza virus particles and reconstituted influenza envelopes which could be responsible for the lower fusion efficiency of virosome fusion.

The most obvious difference which could effect fusion efficiency is the surface density of HA. The HA:lipid ratio of virus particles is approximately 4 times higher than that of virosomes and therefore viral HA is more densely packed in the membrane, increased surface density of HA has been reported to increase fusion efficiency (Ellens *et al.*, 1990) and thus could explain why viral fusion is more efficient. Attempts to make virosomes with a HA:lipid ratio similar to that of virus resulted in virosome preparations with high levels of HA rosettes (data not shown).

The structure of the influenza virion, where the inner leaflet of the viral membrane is covered with matrix (M1) protein could contribute to the high efficiency of virus fusion. Virosomes do not contain M1 protein and this could contribute to the relatively low efficiency of virosome fusion.

The rate of virus-liposome fusion is increased by the incorporation of the proton ionophore monensin into the viral membrane and decreased by blocking the proton channel M2 with amantadine (Bron *et al.*, 1993a; Wharton *et al.*, 1994). Virosomes produced by Methods A, B or C do not contain M2 (data not shown) and it is possible that the presence of M2 in the viral membrane somehow contributes to high fusion efficiency. If M2 acts to increase the extent of membrane fusion it seems not to be simply by acidification of the virion interior because incorporation of monensin into the membrane of Method A virosomes had no effect on the rate or

extent of virosome fusion (data not shown) which was in agreement with the report of Bron *et al.* (1993a).

4.1.5.3 Receptor binding by HA and the efficiency of membrane fusion

The fusion efficiency of Method A virosomes with erythrocyte ghosts was strongly dependent on sialic acid residues attached to the target membrane in agreement with Nussbaum *et al.* (1987). ~~The presence of gangliosides within liposomal membranes increased the extent of Method A virosome - liposome fusion compared to liposomes without gangliosides (data not shown).~~ These data clearly show that receptor binding by HA increases fusion efficiency.

why not ??

Apart from prefusion binding of virosomes to target membrane which results in apposition of the virosomal and target membrane the precise role of receptor binding by HA with respect to the efficiency of HA - mediated fusion is unknown. Some reports have suggested that binding of HA to sialic acid containing receptors facilitated correct insertion of the fusion peptide into the target membrane (Niles and Cohen, 1993; Pedroso de Lima *et al.*, 1995; Stegmann *et al.*, 1995), contrary to these suggestions there have also been reports that proposed that the binding and fusion functions of HA are not performed by the same trimer (Ellens *et al.*, 1990; Alford *et al.*, 1994).

A HA based delivery vector will be required to bind to specific cell types. HA binds to sialic acid which is present on most cell types, it is therefore proposed that specific binding domains could be inserted within HA in place of the sialic acid binding. Alternatively specificity of binding could be incorporated into the vector by coupling other molecules, such as antibodies to the vector. Obviously, more information concerning the role of receptor binding by HA with regard to the

efficiency of membrane fusion will aid the design of a HA based delivery vector as well as providing more information regarding the mechanism of HA mediated fusion.

The role of receptor binding with regard to the efficiency of membrane fusion has been investigated using surrogate HA receptors. The binding properties of various liposome coupled Fab' fragments, derived from various monoclonal anti HA antibodies have been investigated by EM. Using results based on EM, liposome coupled surrogate receptors have then been used in virosome - liposome fusion assays to determine the fusion efficiency of HA when bound or unbound to receptor.

4.2 CHAPTER TWO - RESULTS: ELECTRON MICROSCOPY OF HA - RECEPTOR COMPLEXES

4.2.1 Introduction

The importance of receptor binding with regard to the efficiency of membrane fusion has been investigated using monoclonal Fab' fragments as surrogate receptors. Binding of sialic acid residues to HA has been shown to be relatively weak, with a dissociation constant (K_d) in the mM range (Sauter *et al.*, 1992), making single molecule HA - receptor studies very difficult. Monoclonal antibodies raised against HA on the other hand have a high affinity for HA, with a K_d in the nM range. Fab' fragments produced from monoclonal anti-HA antibodies which had different specificities for the HA molecule (Figure 4.2.1) were coupled to liposomes (Section 3.4) and used as surrogate receptors for HA. This procedure has enabled electron microscopy (EM) studies of HA - receptor complexes to be done and the importance of receptor binding for membrane fusion to be assessed.

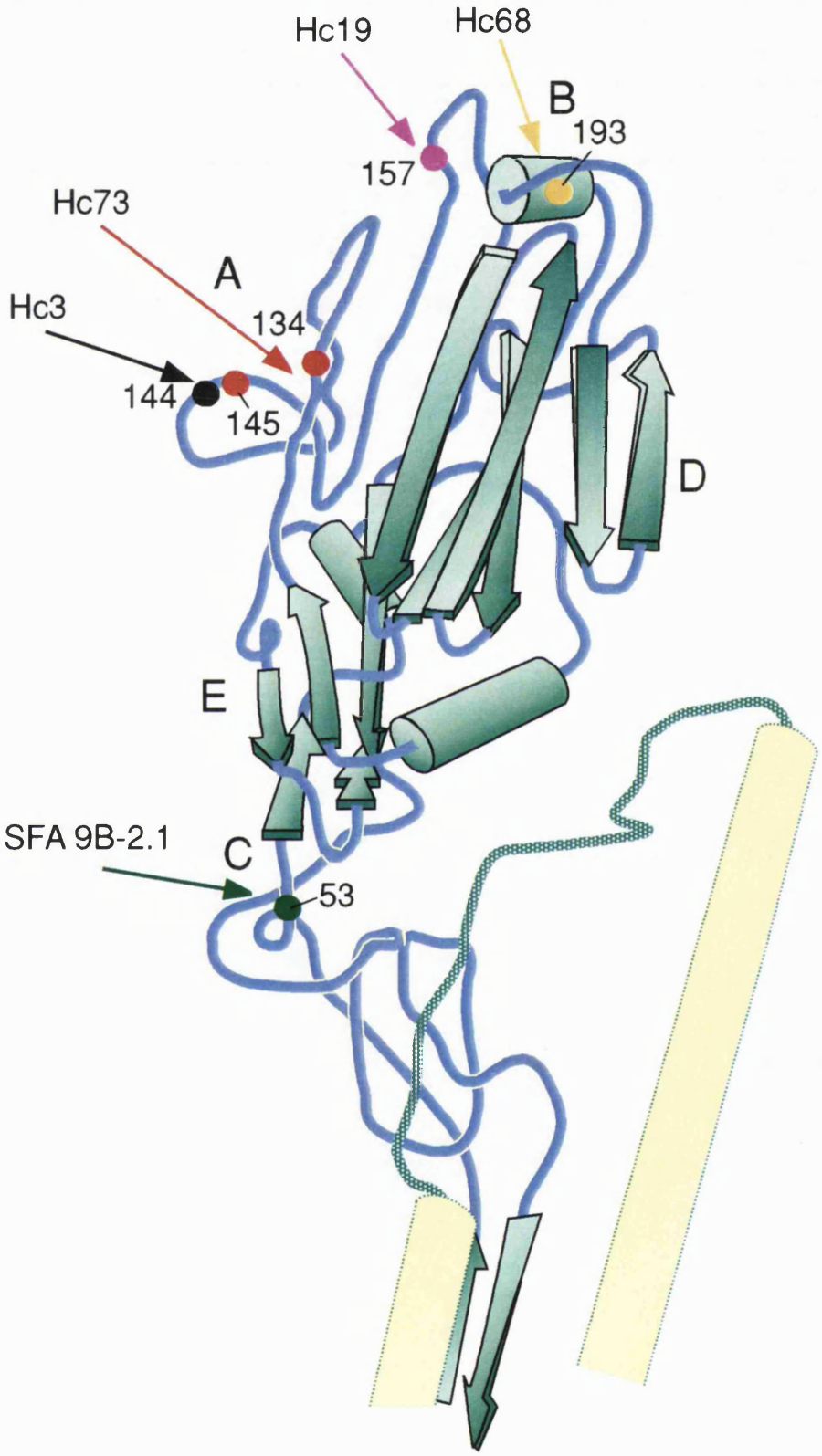
The HA - surrogate receptor complexes investigated by EM consisted of BHA bound to liposome coupled monoclonal anti-HA Fab' fragments. The effect on the low pH induced conformational change of HA when bound to various surrogate receptors was investigated. The monoclonal Fab' receptors used, except one (Hc68), recognised HA in both the native and low pH conformations.

The aim was to identify surrogate receptors which could be subsequently used in Fab' liposome - virosome fusion experiments, to directly investigate the effect of receptor binding on the efficiency of membrane fusion.

Figure 4.2.1

Arrows indicate binding positions of the five anti X-31 HA antibodies used to make Fab' coupled liposomes. The dots, the colour of which corresponds to the colour of the arrows, show the locations of the amino acid substitutions in HA_1 which occur in escape mutants of X-31 virus when grown in the presence of the antibody concerned. Labels A to E refer to the antigenic sites of X-31 HA (Wiley *et al.*, 1981).

Figure 4.2.1 The binding sites of various anti X-31 HA monoclonal antibodies



4.2.2 Liposome coupled anti X-31 Hc73 Fab' as a surrogate receptor for X-31 HA

Hc73 antibody binds to antigenic site A of X-31 HA (Table 3.4.1). EM of Hc73 antibody bound to BHA showed each Fab region formed a constant angle of about 130^0 with the BHA (Figure 4.2.2a). EM of Hc73 Fab' liposomes showed Fab' coupled to the liposome surface (Figure 4.2.2b). Due to the angle of Hc73 Fab' bound to BHA and flexibility within the Fab' fragment, and / or the flexibility around the attachment between the Fab' fragment and the PDP-PE it was possible for at least two liposome coupled Fab' fragments to bind per BHA trimer, as shown by EM (Figure 4.2.2c).

Low pH treatment of Hc73 Fab' liposomes did not result in liposome aggregation as judged by EM (data not shown). However, low pH treatment of BHA bound to Hc73 Fab' liposomes resulted in aggregation of the liposomes, mediated by BHA which had appeared to have undergone a characteristic low pH induced conformational change (Figure 4.2.2d). Low pH treatment of BHA bound to Hc73 Fab' liposomes in the presence of approximately three times the concentration of unbound BHA resulted in significantly less liposome aggregation (Figure 4.2.2e). Trypsin treatment of HA results in cleavage at HA_1 27 and 224, with the release of soluble HA_1 28-328 fragments. Trypsin treatment of low pH induced liposome aggregates resulted in the disaggregation of liposomes (Figure 4.2.2f). Trypsin treatment of Fab' liposomes alone did not result in cleavage of Fab' as judged by SDS-PAGE (data not shown).

It is therefore proposed that the low pH induced liposome aggregates resulted from BHA in the low pH conformation attached to one liposome by HA_1 binding to

liposomally coupled Hc73 Fab' and attached to other liposomes via hydrophobic interaction of the exposed "fusion peptide". Liposome aggregation could have resulted from the exposed "fusion peptide" interacting with other exposed "fusion peptides" or by inserting into a different liposome than that to which the BHA was bound to by Fab' fragments.

The presence of unbound BHA significantly inhibited liposome aggregation, presumably by interaction of "fusion peptide" exposed by unbound BHA with "fusion peptide" exposed by Fab' fragment bound BHA. This would block subsequent interaction of "fusion peptide" exposed by bound BHA with "fusion peptide" exposed by other bound BHAs and also block "fusion peptide" insertion into other liposomal membranes.

The observation that trypsin treatment reversed the aggregation is consistent with the above interpretation. Cleavage at HA_1 27 results in dissociation of HA_1 28-328 which contains the binding site for Hc73 Fab' from BHA_2 - $HA_1$1-27 which contains the "fusion peptide" at the amino terminus of BHA_2.

Trypsin cleavage of low pH BHA inserted via its "fusion peptide" into a liposomal membrane results in release of soluble HA_1 28-328, which leaves BHA_2 - $HA_1$1-27 associated with the liposomal membrane via the "fusion peptide" at the amino terminus of BHA_2. Liposome associated BHA_2 spikes have been identified by EM (Wharton *et al.* 1995). Trypsin treatment of low pH induced liposome aggregates resulted in disaggregated liposomes which probably contained BHA_2 spikes (Figure 4.2.2f) which resulted from bound BHA inserting its "fusion peptide" into the liposomal membrane. Identification of BHA_2 spikes in these experiments was complicated by the presence of liposome coupled Fab'.

remark p93 - no length measurements done of Fab'-liposomes and the resulting possible BHA_2 liposomes?

Figure 4.2.2

Electron microscopy was done as described in Section 3.1.9. Interpretations show BHA as open shapes and antibody or Fab' in black, liposomal membrane is shown as hatched.

BHA was prepared as described in Section 3.1.2. Hc73 antibody was purified as described in Section 3.4.3. Fab' liposomes were prepared as described in Section 3.4. X-31 BHA was bound to Hc73 Fab' liposomes and purified from unbound BHA as described in Section 3.5.1. Acid and trypsin treatment of BHA bound to Fab' liposomes was done as described in Section 3.2.4.

For a) bar ≡ 50nm, otherwise bar ≡ 100nm

a) shows Hc73 antibody - BHA complexes, The Fab region of Hc73 antibody formed a constant angle of ~ 130^0 with X-31 BHA. ($n = 25$ S.D. $= \pm 4.1$)

b) shows liposomes which contained Hc73 Fab' coupled to the liposome surface.

c) shows the Hc73 Fab' liposomes shown in b) after addition of X-31 BHA followed by purification from unbound BHA (Section 3.5.1).

d) shows liposomes previously shown in c) following treatment at pH 5. At least in some cases the low pH induced conformational change of bound BHA led to the exposure of the hydrophobic "fusion peptides" of BHA which led to "fusion peptide-fusion peptide" interactions between BHA's bound to different liposomes, which in turn resulted in the observed liposome aggregation.

e) shows liposomes previously shown in c) which have been treated at pH 5 in the presence of approximately three times the concentration of unbound BHA relative to the amount of liposomally bound BHA. Liposome aggregation was significantly reduced compared to d) presumably due to "fusion peptide-fusion peptide" interactions between bound and unbound BHA which reduced "fusion peptide" interactions between BHA's bound to different liposomes. Consistent with this low pH conformation BHA aggregates were visible around the liposomes.

f) shows liposomes previously shown in d) following trypsin treatment, this resulted in disaggregated liposomes. Liposomally inserted BHA_2 spikes were provisionally identified, although identification was complicated by the presence of liposomally coupled Fab' and therefore BHA_2 spikes are not directly labelled.

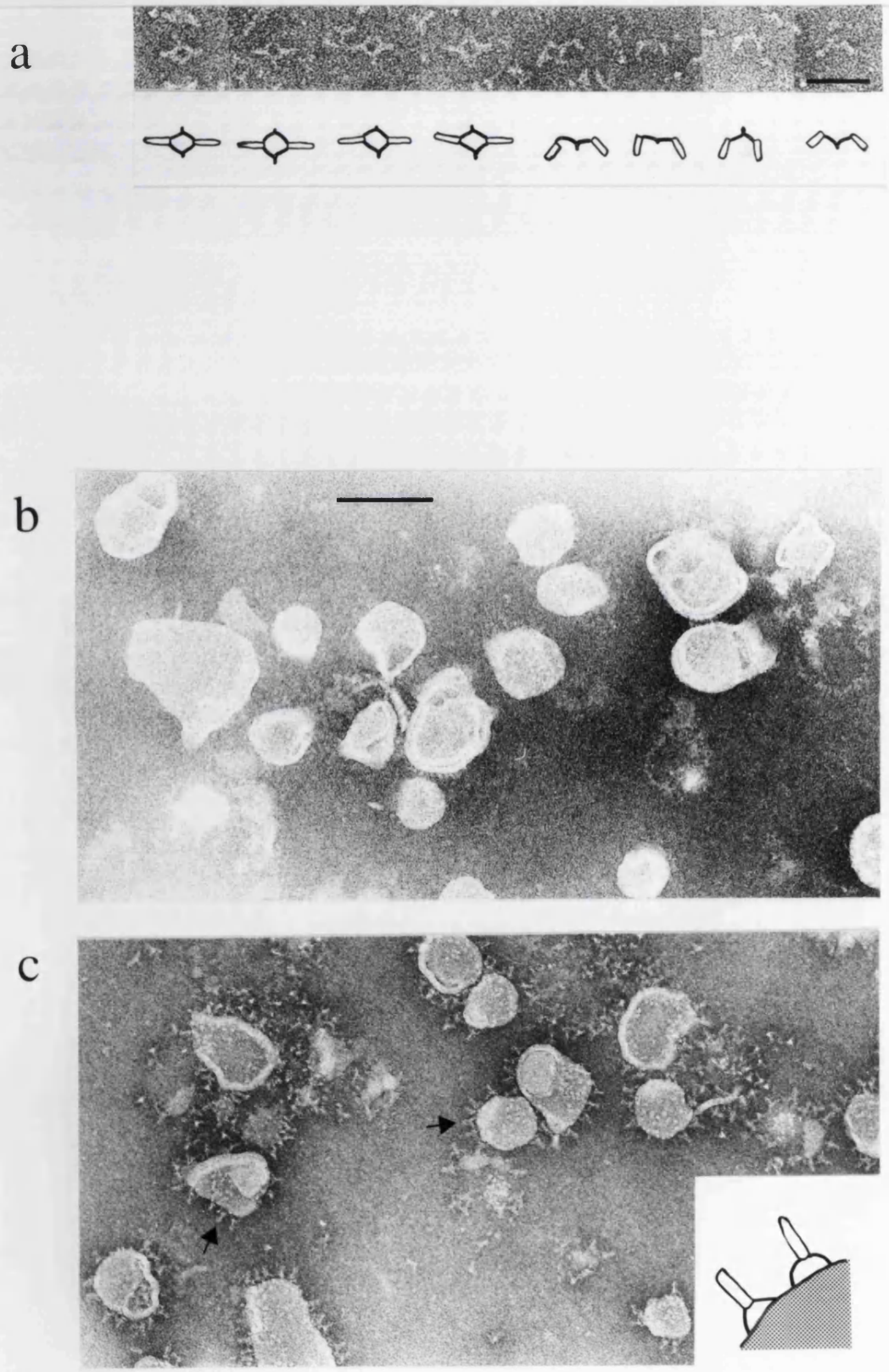
Not a very good picture (a print)

any measurements done?

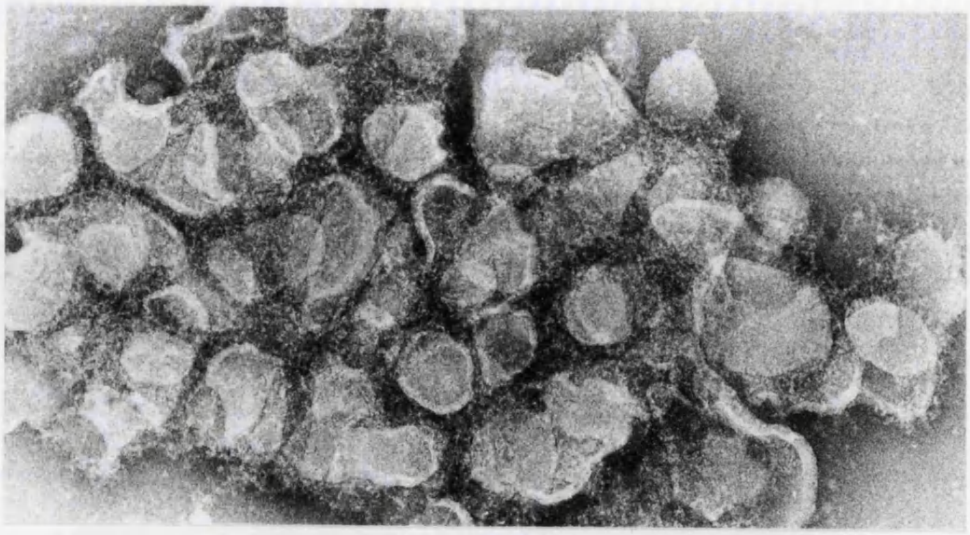
remarks p92

(2) is again not such a good print but the lipis seem to be different from those in the bad (b) print

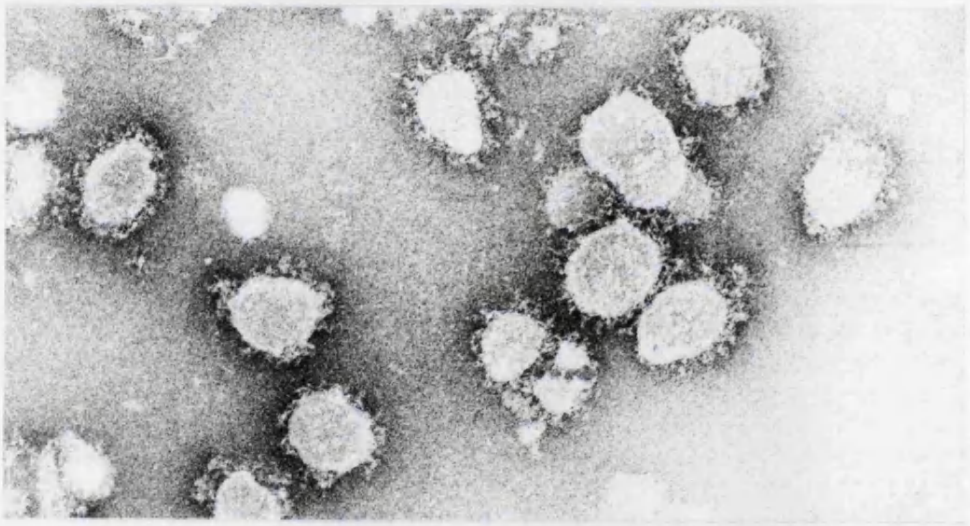
Figure 4.2.2: Electron microscopy of X-31 BHA bound to Hc73 antibody and liposome coupled Hc73 Fab'



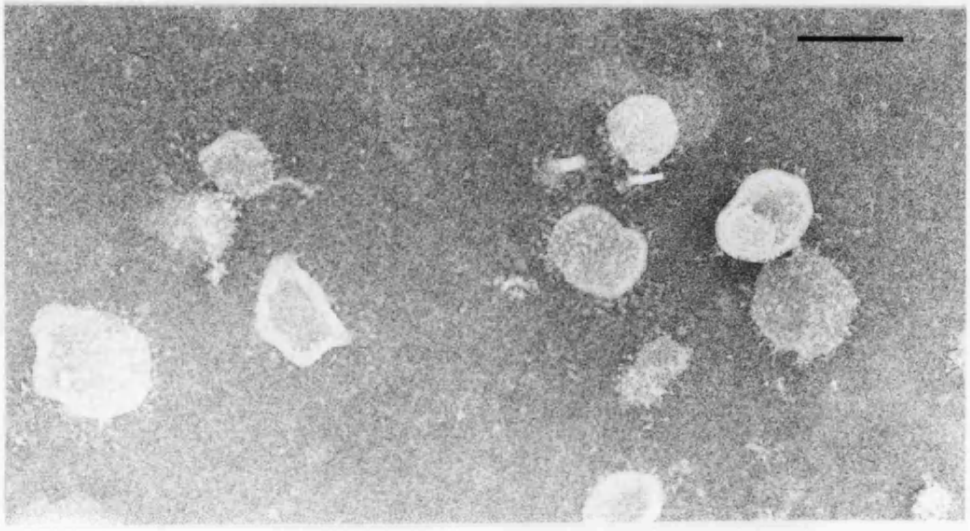
d



e



f



BHA bound to liposome coupled Hc73 Fab' fragments was held in an approximately perpendicular orientation with respect to the liposomal membrane, with the globular HA_1 domains of BHA held nearest to the liposomal membrane, as would be expected if HA was bound to liposomal sialic acid containing receptors. Low pH treatment of BHA bound to Hc73 Fab' surrogate receptor resulted in BHA undergoing a characteristic conformational change as judged by EM. These two observations made Hc73 Fab' a suitable surrogate receptor for subsequent Fab' liposome - virosome fusion assays in which the liposomal membrane acted as a model target membrane (Section 4.3)

4.2.3 Liposome coupled anti X-31 Hc19 Fab' as a surrogate receptor for X-31 HA

Hc19 antibody binds to antigenic site B of X-31 HA (Table 3.4.1). EM of Hc19 antibody bound to BHA showed each Fab region formed a constant angle of about 140^0 with the BHA (Wrigley *et al.*, 1983b). Due to the angle of Hc19 Fab' bound to BHA and flexibility within the Fab' fragment, and / or the flexibility around the attachment between the Fab' fragment and the PDP-PE it was possible for at least two liposome coupled Fab' fragments to bind per BHA trimer, as shown by EM (Figure 4.2.3b).

EM of low pH and trypsin treated BHA bound to liposome coupled Hc19 Fab' receptors (Figure 4.2.3c&d) resulted in the same observations and interpretation of results as discussed for liposome coupled Hc73 Fab' receptor (Section 4.2.2). Therefore it was concluded Hc19 Fab' receptor was equally as suitable a candidate as Hc73 Fab' for subsequent Fab' liposome - virosome fusion assays. Hc73 Fab' and not Hc19 Fab' was used for subsequent Fab' liposome - virosome fusion assays.

Figure 4.2.3

Electron microscopy was done as described in Section 3.1.9. Interpretations show BHA as open shapes and Fab' in black, liposomal membrane is shown as hatched.

BHA was prepared as described in Section 3.1.2. Fab' liposomes were prepared as described in Section 3.4. X-31 BHA was bound to Hc19 Fab' liposomes and purified from unbound BHA as described in Section 3.5.1. Acid and trypsin treatment of BHA bound to Fab' liposomes was done as described in Section 3.2.4.

bar ≡ 100nm

a) shows liposomes which contained Hc19 Fab' coupled to the liposome surface.

b) shows the Hc19 Fab' liposomes shown in a) after addition of X-31 BHA followed by purification from unbound BHA (Section 3.5.1).

c) shows liposomes previously shown in b) following treatment at pH 5. At least in some cases the low pH induced conformational change of bound BHA led to the exposure of the hydrophobic "fusion peptides" of BHA which led to "fusion peptide-fusion peptide" interactions between BHA's bound to different liposomes, which in turn resulted in the observed liposome aggregation.

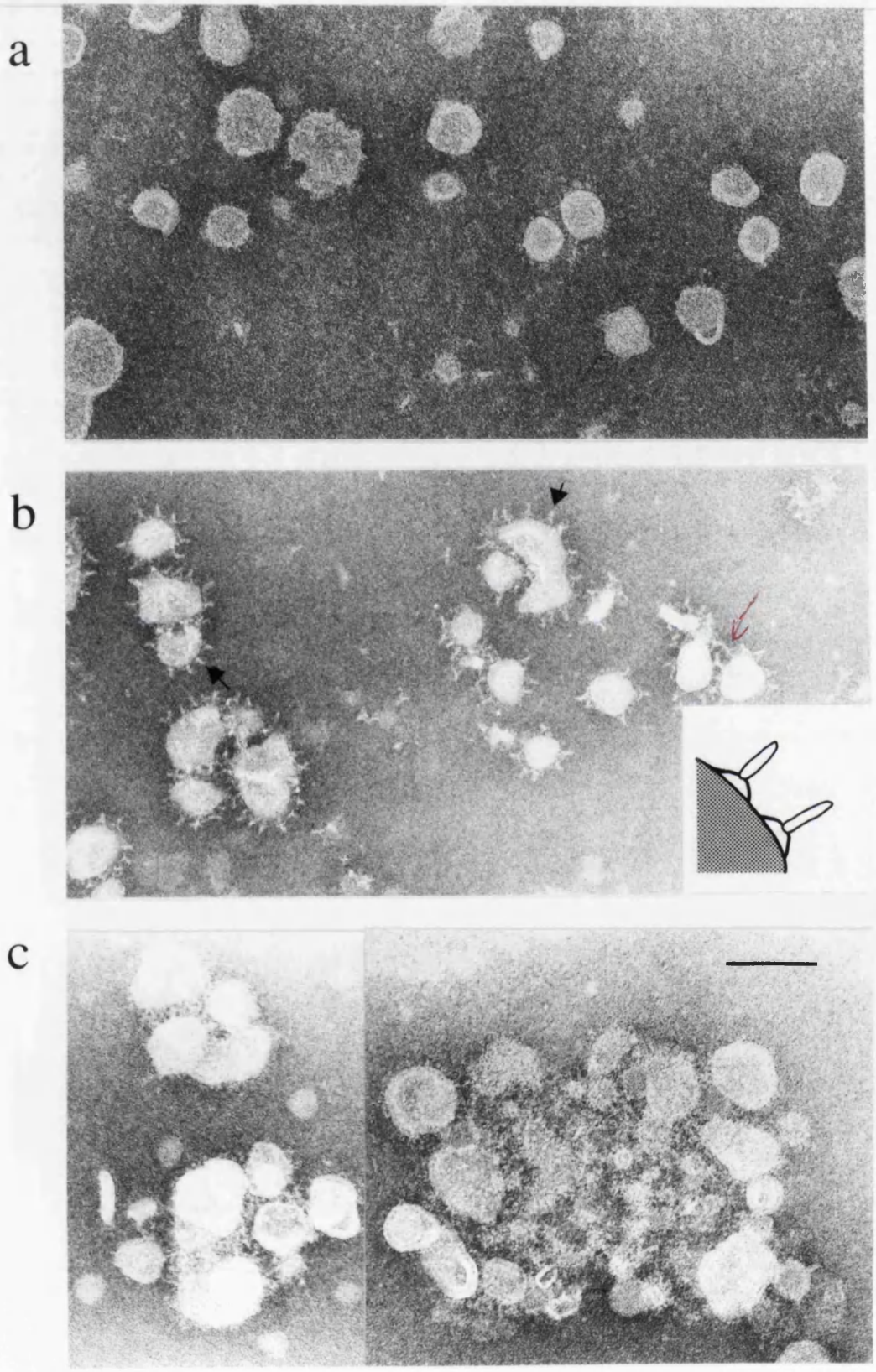
d) shows liposomes previously shown in c) following trypsin treatment, this resulted in disaggregated liposomes. Liposomally inserted BHA_2 spikes were provisionally identified, although identification was complicated by the presence of liposomally coupled Fab' and therefore BHA_2 spikes are not directly labelled.

but there is already aggregation in b:

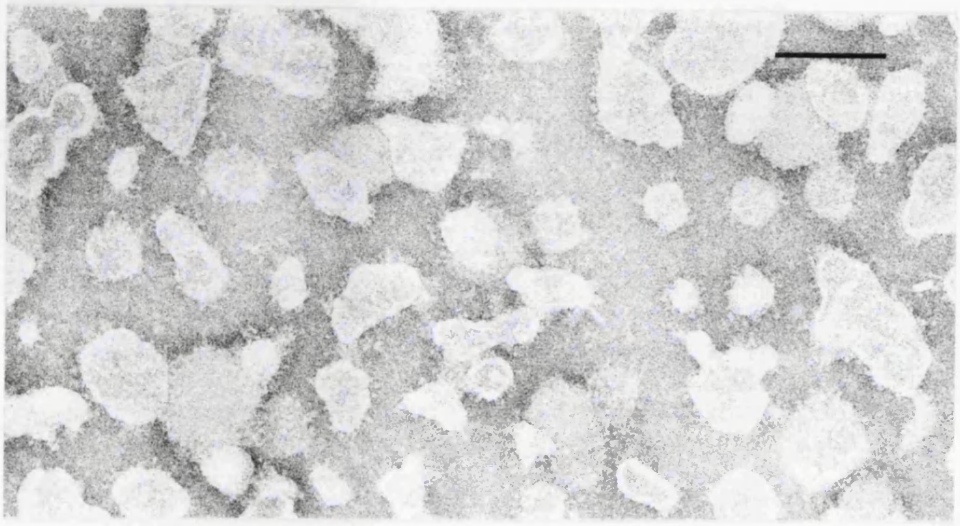
also bad picture. There seems to be a lot of free Fab' in background!

and (d) is also bad again. Much too light
doesn't also have tips and trypsin in background?

Figure 4.2.3: Electron microscopy of X-31 BHA bound to liposome coupled Hc19 Fab'



d



4.2.4 Liposome coupled anti X-31 Hc3 Fab' as a surrogate receptor for X-31 HA

Hc3 antibody binds to antigenic site A of X-31 HA (Table 3.4.1). EM of Hc3 antibody bound to BHA showed each Fab region formed a constant angle of about 110^0 with the BHA (Wrigley *et al.*, 1983b). Due to the angle of Hc3 Fab' bound to BHA and flexibility within the Fab' fragment, and / or the flexibility around the attachment between the Fab' fragment and the PDP-PE it was possible for at least two liposome coupled Fab' fragments to bind per BHA trimer, as shown by EM (Figure 4.2.4b).

Low pH treatment of BHA bound to liposome coupled Hc3 Fab' receptors resulted in aggregation of liposomes (Figure 4.2.4c), mediated by a population of BHA molecules that appeared to have undergone a characteristic low pH induced conformational change resulting in exposure of the "fusion peptide". Trypsin treatment reversed liposome aggregation (Figure 4.2.4d). This type of liposome aggregation was previously described for Hc73 and Hc19 Fab' receptors (Sections 4.2.2 & 4.2.3).

Low pH treatment of BHA bound to liposome coupled Hc3 Fab' receptors also resulted in a population of BHA spikes that were significantly thinner than native BHA (Figure 4.2.4c). These thinner spikes were not characteristic of BHA that had undergone a characteristic conformational change. Native BHA is resistant to trypsin digestion, however, trypsin treatment of low pH treated liposomes showed that the "thin" spikes produced by low pH treatment were susceptible to trypsin digestion (Figure 4.2.4c&d). This implied that a conformational change of native BHA bound to liposome coupled Hc3 Fab' had taken place at low pH, producing "thin" spikes.

"Thin" BHA spikes were not involved in aggregation of any sort, this suggested that they were not involved in "fusion peptide - fusion peptide" interactions.

The position of Hc3 Fab' binding to HA_1 is not suggestive of a mechanism by which it may partially block the conformation change of HA, but it can be postulated that Hc3 Fab' binding to BHA somehow blocked a stage in the conformational rearrangement of BHA resulting in the observed "thin spikes". This block to the conformational change was not absolute as demonstrated by the fact that a significant proportion of BHA bound by liposome coupled Hc3 Fab' appeared to undergo a complete characteristic conformational change, which resulted in liposome aggregation.

It is possible that low pH treatment of BHA molecules that were bound by three Hc3 Fab' receptors were blocked from undergoing a full conformational change, which resulted in "thin" spikes. However, BHA bound by only two Hc3 Fab' receptors may not have been blocked from undergoing a characteristic conformational change.

EM of BHA bound to Hc3 Fab' liposomes which had been acid and trypsin treated did not convincingly show liposome associated BHA_2 - HA_1 1-27 (Figure 4.2.4d). The low pH conformation specific antibody IIF4, which binds at one end of the BHA_2 trimer, at the opposite end of the molecule to the "fusion peptides" (Vareckova *et al.*, 1993; Wharton *et al.*, 1995) was added to trypsin treated liposomes and its binding to liposome associated BHA_2 detected by gold labelling (Section 3.5.1). These experiments showed that liposome inserted BHA_2 spikes were present (data not shown), indicating that at least in some cases low pH treatment of BHA

Figure 4.2.4

Electron microscopy was done as described in Section 3.1.9. Interpretations show BHA as open shapes in b) and as a black line in c). Fab' is shown in black and the liposomal membrane is shown as hatched.

BHA was prepared as described in Section 3.1.2. Fab' liposomes were prepared as described in Section 3.4. X-31 BHA was bound to Hc3 Fab' liposomes and purified from unbound BHA as described in Section 3.5.1. Acid and trypsin treatment of BHA bound to Fab' liposomes was done as described in Section 3.2.4.

bar ≡ 100nm

a) shows liposomes which contained Hc3 Fab' coupled to the liposome surface.

b) shows the Hc3 Fab' liposomes shown in a) after addition of X-31 BHA followed by purification from unbound BHA (Section 3.5.1).

c) shows liposomes previously shown in b) following treatment at pH 5. At least in some cases the low pH induced conformational change of bound BHA led to the exposure of the hydrophobic "fusion peptides" of BHA which led to "fusion peptide-fusion peptide" interactions between BHA's bound to different liposomes, which in turn resulted in the observed liposome aggregation. In addition "thin" BHA spikes were observed as indicated, which were not involved in liposome aggregation (Section 4.2.4).

d) shows liposomes previously shown in c) following trypsin treatment, this resulted in disaggregated liposomes. Liposomally inserted BHA_2 spikes were not readily visible, although the presence of BHA_2 spikes was confirmed by gold labelling (Section 4.2.4).

— How much thinner? Not much thinner than left of (b). Could it be due to staining a focus? Were they layer as well?

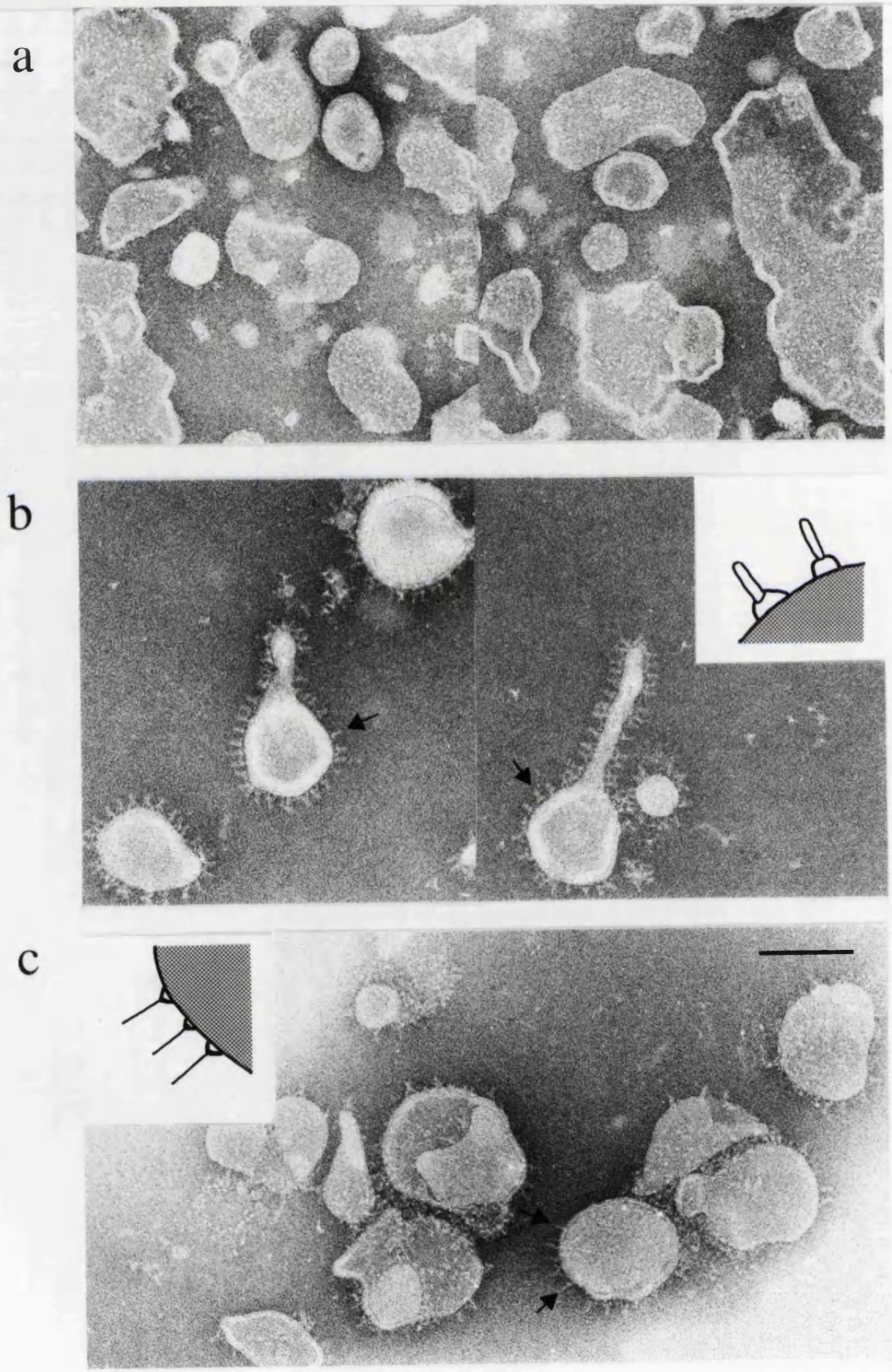
— I do not agree with this remark

What about tube formatic in (b)?

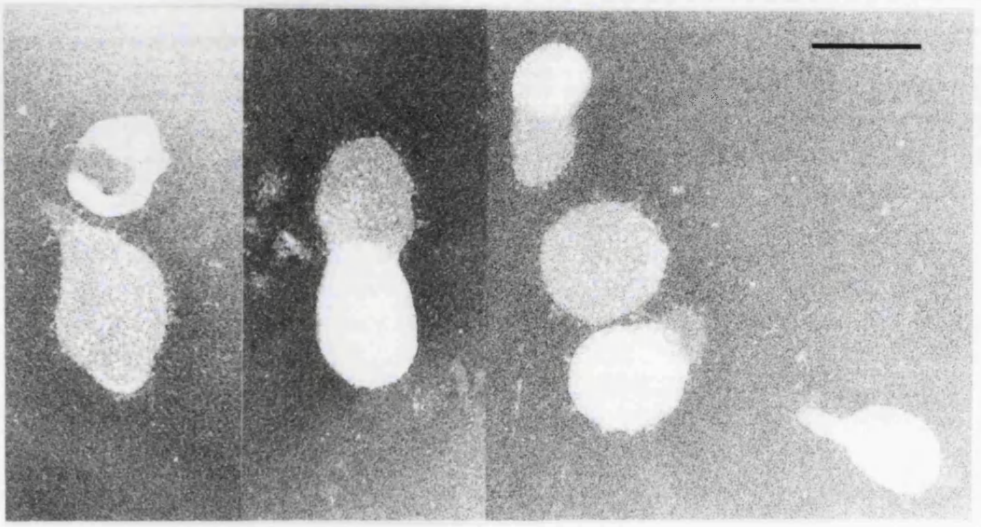
— Why would Hc3 be so different from Hc73 although they bind to the same site?

— Did you do any SDS PAGE on digested lipos?

Figure 4.2.4: Electron microscopy of X-31 BHA bound to liposome coupled Hc3 Fab'



d



bound to liposome coupled Hc3 Fab' receptor resulted in liposomal insertion of "fusion peptide".

BHA bound to liposome coupled Hc3 Fab' fragments was held in an approximately perpendicular orientation with respect to the liposomal membrane However, in some cases it appeared low pH treatment of BHA bound to Hc3 Fab' surrogate receptors resulted in BHA being partially blocked from undergoing a characteristic conformational change. It is for this reason Hc3 Fab' was not a suitable surrogate receptor for subsequent Fab' liposome - virosome fusion assays.

4.2.5 Liposome coupled anti X-31 Hc68 Fab' as a surrogate receptor for X-31 HA

Hc68 antibody recognises HA_1 193 in antigenic site B of X-31 HA (Table 3.4.1), which is at the membrane distal end of the HA molecule. EM showed at least two liposome coupled Fab' fragments were able to bind per BHA trimer (Figure 4.2.5b).

EM of low pH treated BHA bound to liposome coupled Hc68 Fab' receptors showed that a very high proportion of bound BHA molecules were blocked from undergoing the acid induced conformational change (Figure 4.2.5c). Consistent with this no liposome aggregation was observed. Trypsin treatment showed that acid treated BHA spikes were not trypsin susceptible (Figure 4.2.5d), confirming that the BHA was in its native conformation. Western blot analysis of the tryptic digestion products of low pH treated BHA bound to Hc68 liposomes also showed that BHA was blocked from undergoing the low pH induced conformational change (data not shown).

Figure 4.2.5

Electron microscopy was done as described in Section 3.1.9. Interpretations show BHA as open shapes and Fab' black, liposomal membrane is shown as hatched.

BHA was prepared as described in Section 3.1.2. Fab' liposomes were prepared as described in Section 3.4. X-31 BHA was bound to Hc68 Fab' liposomes and purified from unbound BHA as described in Section 3.5.1. Acid and trypsin treatment of BHA bound to Fab' liposomes was done as described in Section 3.2.4.

bar ≡ 100nm

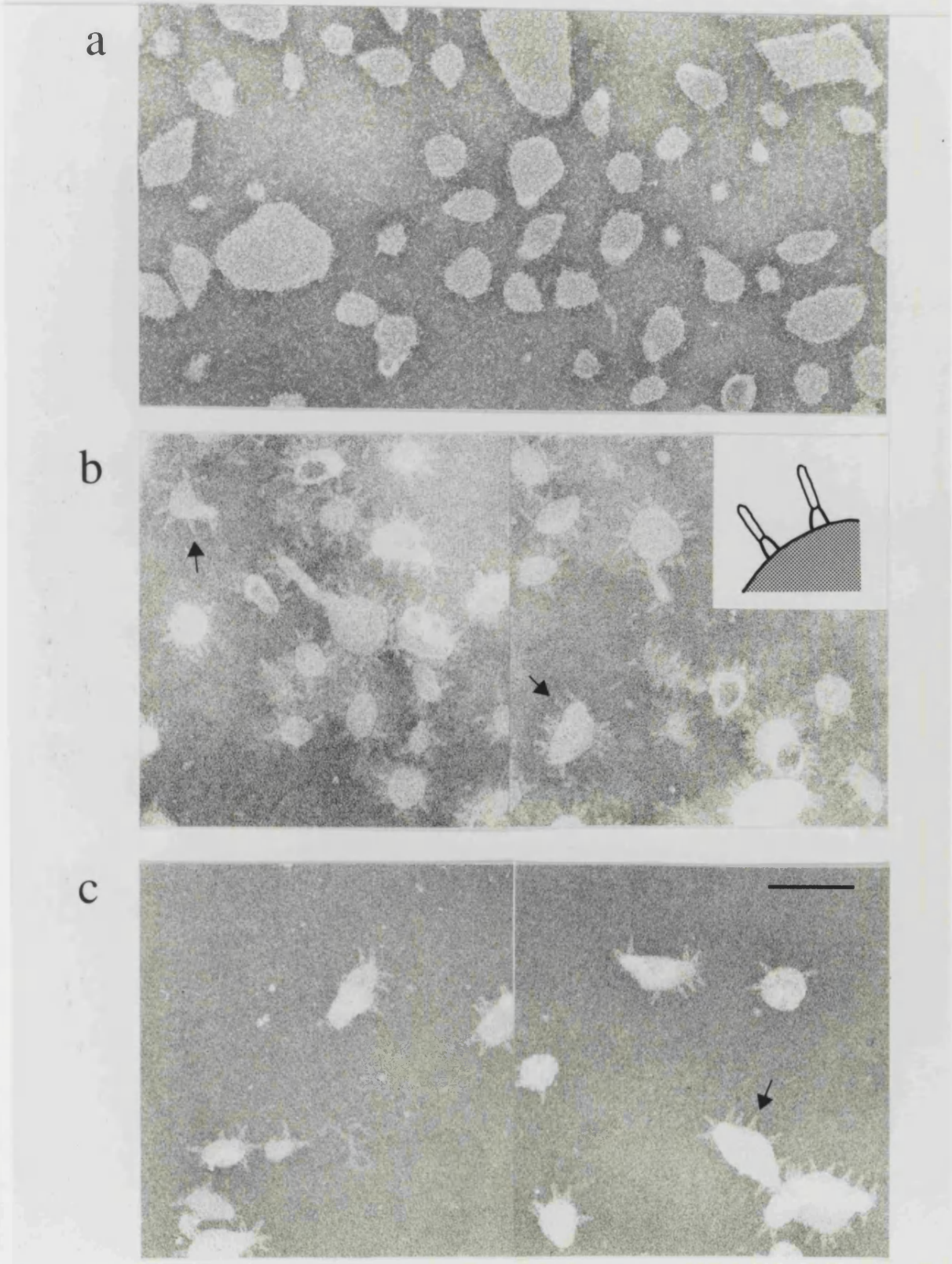
a) shows liposomes which contained Hc68 Fab' coupled to the liposome surface.

b) shows the Hc68 Fab' liposomes shown in a) after addition of X-31 BHA followed by purification from unbound BHA (Section 3.5.1).

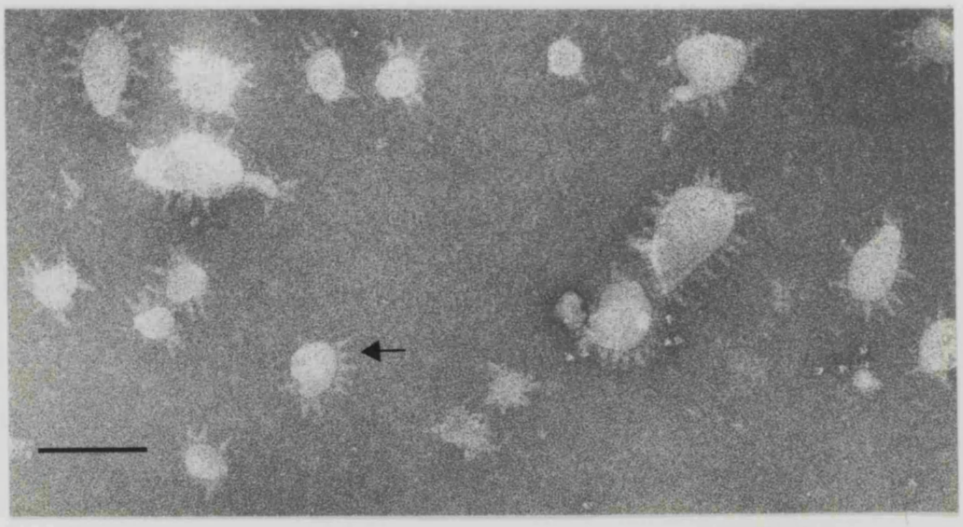
c) shows liposomes previously shown in b) following treatment at pH 5. No liposome aggregation was observed. BHA bound to liposome coupled Hc68 Fab' receptor appeared to be blocked from undergoing an acid induced conformational change. The arrow indicates a native BHA molecule bound to liposome coupled Hc68 Fab'.

d) shows liposomes previously shown in c) following trypsin treatment, this showed that the BHA spikes were not trypsin susceptible strongly suggesting that bound BHA was in its native conformation. The arrow indicates a native BHA molecule bound to liposome coupled Hc68 Fab'.

Figure 4.2.5: Electron microscopy of X-31 BHA bound to liposome coupled Hc68 Fab'



d



Does HcBP have the same blocking effect as soluble BHA? or a HA on vitro?

Hc68 Fab' receptor blocked the conformational change of BHA, probably by preventing the HA_1 subunits detrimerizing at low pH. It was for this reason Hc68 Fab' was not a suitable surrogate receptor for subsequent Fab' liposome - virosome fusion assays.

4.2.6 Liposome coupled anti X-31 SFA 9B-2.1 Fab' as a surrogate receptor for X-31 HA

SFA 9B-2.1 antibody recognises HA_1 53 in antigenic site C of X-31 HA (Table 3.4.1), which is on the side of the HA molecule. Due to the position of site C on the side of HA two Fab' fragments coupled to two different liposomes were able to bind to a single BHA trimer. Addition of BHA to SFA 9B-2.1 Fab' liposomes therefore resulted in liposome aggregation, as shown by EM (Figure 4.2.6).

BHA bound to liposome coupled SFA 9B-2.1 Fab' receptors was held in an approximately parallel orientation with respect to the liposomal membrane, a situation not expected when HA is bound to liposomal sialic acid containing receptors. It was for this reason that SFA 9B-2.1 Fab' was not a suitable surrogate receptor for subsequent Fab' liposome - virosome fusion assays, although EM showed BHA bound by SFA 9B-2.1 Fab' was not blocked from undergoing a characteristic change (data not shown).

4.2.7 Liposome coupled anti Res vir 8 H100 Fab' as a surrogate receptor for A/JHB/33/94 HA

Fab' liposome - virosome fusion assays investigating the role of receptor binding with regard to the efficiency of membrane fusion (Section 4.3) required the selection of surrogate Fab' receptors with similar properties to anti X-31 Hc73 Fab', but which recognised a strain of HA which was antigenically distinct from X-31 HA.

Figure 4.2.6

Electron microscopy was done as described in Section 3.1.9. Interpretations show BHA as open shapes and Fab' black, liposomal membrane is shown as hatched.

BHA was prepared as described in Section 3.1.2. Fab' liposomes were prepared as described in Section 3.4. X-31 BHA was bound to SFA 9B 2.1 Fab' liposomes and purified from unbound BHA as described in Section 3.5.1.

bar ≡ 100nm

a) shows liposomes prior to coupling with SFA 9B 2.1 Fab'.

b) shows liposomes which contained SFA 9B 2.1 Fab' coupled to the liposome surface.

c) shows the SFA 9B 2.1 Fab' liposomes shown in b) after addition of X-31 BHA followed by purification from unbound BHA (Section 3.5.1). As indicated in the interpretation, due to the binding site of SFA 9B 2.1 Fab' on the side of the HA molecule it was possible for two Fab' fragments coupled to different liposomes to bind to a single HA molecule. This therefore explains the observed liposome aggregation.

interpretation in c is wrong!

you mostly see

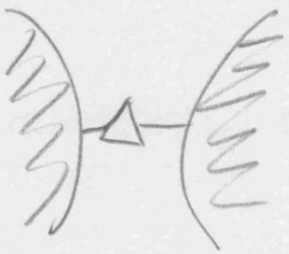
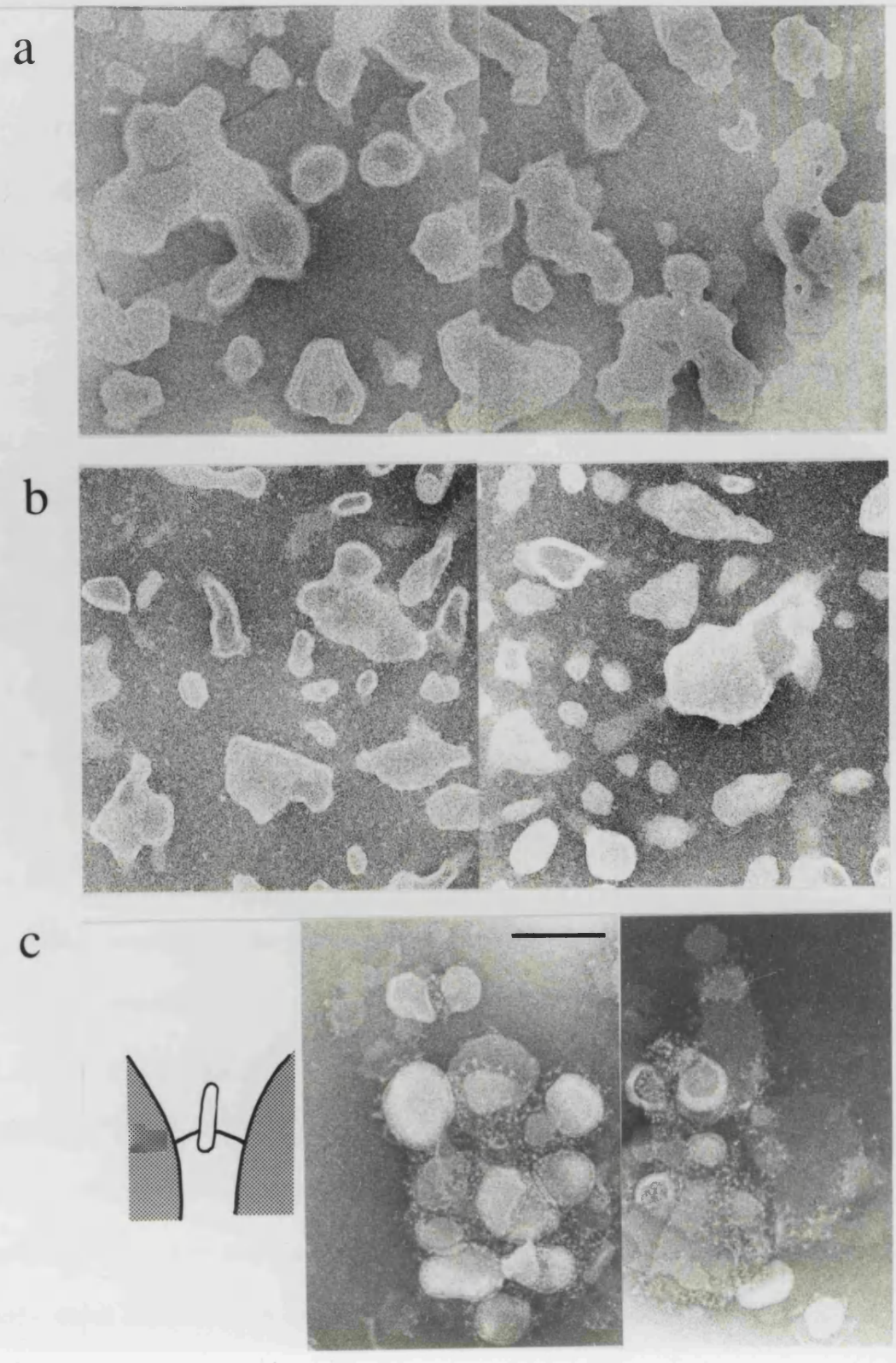


Figure 4.2.6: Electron microscopy of X-31 BHA bound to liposome coupled SFA 9B 2.1 Fab'



For this reason the binding specificities of a variety of anti Johannesburg (JHB) HA monoclonal antibodies were investigated by EM to select antibodies with similar binding properties to the anti X-31 HA Hc73 antibody. To keep variables to a minimum, only antibodies of the same subtype as Hc73 (IgG2a) were considered.

EM of H100 antibody bound to JHB BHA showed it bound to a site on JHB BHA at a similar position to that recognised on X-31 HA by the Hc73 antibody, each Fab region formed a constant angle of about 155^0 with the BHA (Figure 4.2.7a). Due the angle of H100 Fab' bound to JHB BHA and flexibility within the Fab' fragment, and / or flexibility around the attachment between the Fab' fragment and the PDP-PE it was possible for at least two liposome coupled Fab' fragments to bind per JHB BHA trimer, as shown by EM (Figure 4.2.7c).

Figure 4.2.7d&e shows low pH and trypsin treatment of JHB BHA bound to liposome coupled H100 Fab' resulted in the same observations and interpretation of results as discussed for liposome coupled Hc73 Fab' or Hc19 Fab' fragments when acting as surrogate receptors for X-31 BHA (Sections 4.2.2 & 4.2.3).

As was the case for Hc73 and Hc19 Fab' receptors it was concluded that H100 was a suitable surrogate receptor for subsequent Fab' liposome - virosome fusion assays, which investigated the role of receptor binding by HA with regard to the efficiency of membrane fusion.

4.2.8 Liposome coupled anti Res vir 8 H8 Fab' as a surrogate receptor for A/JHB/33/94 HA

To identify antibodies with similar binding properties to anti JHB H100 antibody Res vir 8 virus (containing JHB HA) was grown in the presence of H100 antibody which resulted in an escape mutant virus V100 Res vir 8 which was not

Figure 4.2.7

Electron microscopy was done as described in Section 3.1.9. Interpretations show BHA as open shapes and antibody or Fab' in black, liposomal membrane is shown as hatched.

BHA was prepared as described in Section 3.1.2. H100 antibody was purified as described in Section 3.4.3. Fab' liposomes were prepared as described in Section 3.4. JHB BHA was bound to H100 Fab' liposomes and purified from unbound BHA as described in Section 3.5.1. Acid and trypsin treatment of BHA bound to Fab' liposomes was done as described in Section 3.2.4.

For a) bar ≡ 50nm, otherwise bar ≡ 100nm

a) shows H100 antibody - BHA complexes, The Fab region of H100 antibody formed a constant angle of ~ 155^0 with JHB BHA. (n = 27 S.D. = ± 4.2)

b) shows liposomes which contained H100 Fab' coupled to the liposome surface.

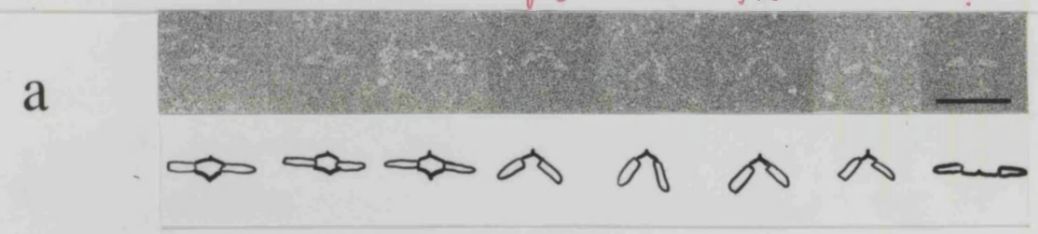
c) shows the H100 Fab' liposomes shown in b) after addition of JHB BHA followed by purification from unbound BHA (Section 3.5.1).

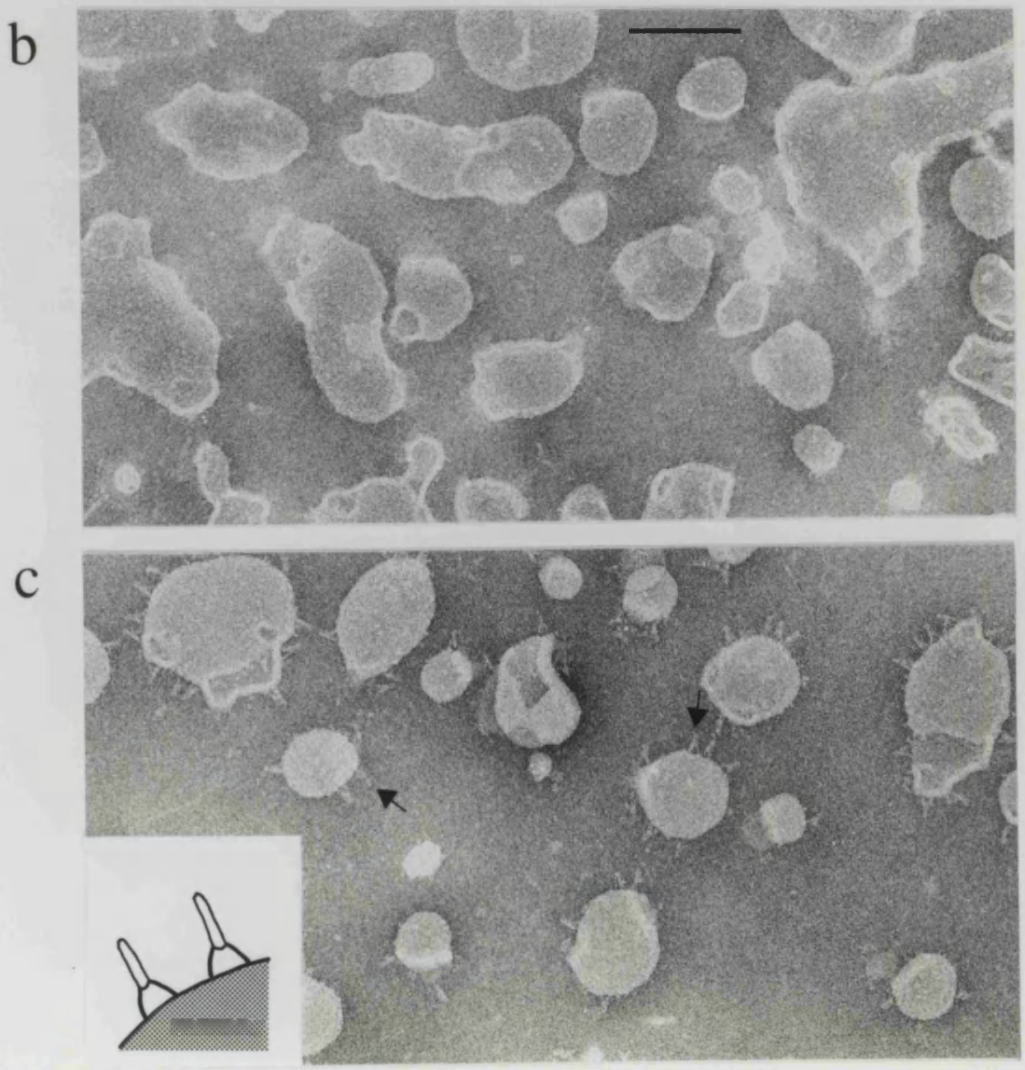
d) shows liposomes previously shown in c) following treatment at pH 5. At least in some cases the low pH induced conformational change of bound BHA led to the exposure of the hydrophobic "fusion peptides" of BHA which led to "fusion peptide-fusion peptide" interactions between BHA's bound to different liposomes, which in turn resulted in the observed liposome aggregation.

e) shows liposomes previously shown in d) following trypsin treatment, this resulted in disaggregated liposomes. Liposomally inserted BHA_2 spikes were provisionally identified, although identification was complicated by the presence of liposomally coupled Fab' and therefore BHA_2 spikes are not directly labelled.

You cannot say this from the pictures shown!

Figure 4.2.7: Electron microscopy of JHB BHA bound to H100 antibody and liposome coupled H100 Fab'





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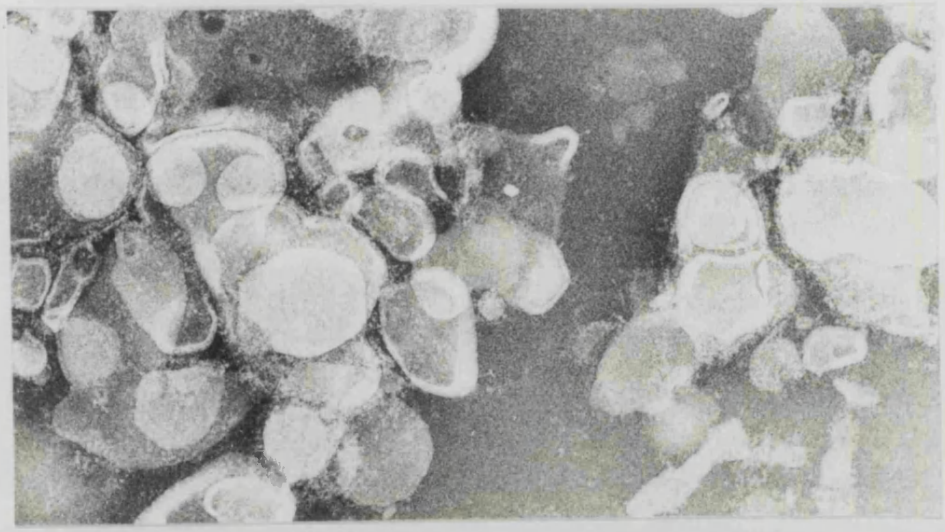

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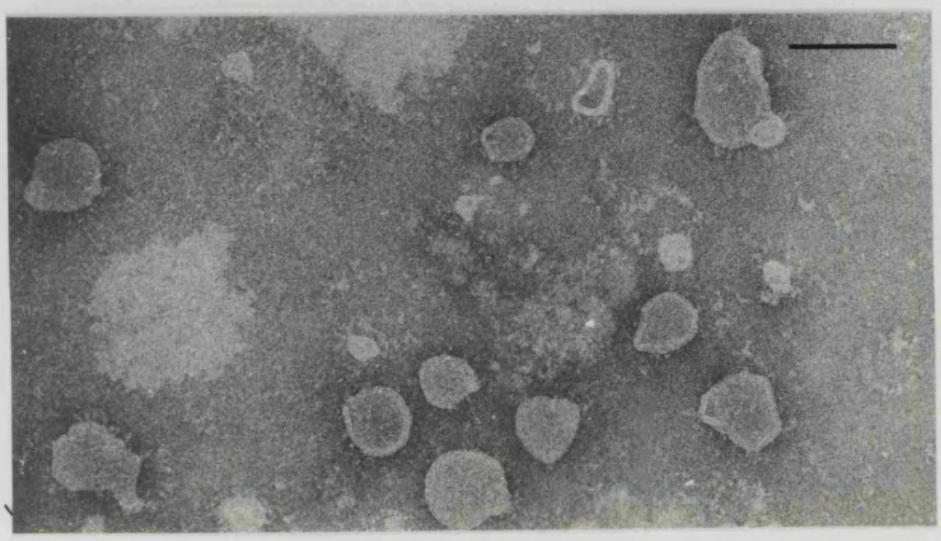


recognised by the H100 antibody. Anti Res vir 8 H8 antibody did not recognise V100 Res vir 8 virus, this indicated that both H100 and H8 antibody bound to a similar site on JHB HA. EM of H8 antibody bound to JHB BHA showed the Fab region formed a constant angle of about 115^0 with BHA and confirmed H8 antibody bound to a similar site on JHB BHA compared to the H100 antibody (Figure 4.2.8a). Due the angle of H8 Fab' bound to JHB BHA and flexibility within the Fab' fragment, and or the flexibility around the attachment between the Fab' fragment and the PDP-PE it was possible for at least two liposome coupled Fab' fragments to bind per BHA trimer, as shown by EM (Figure 4.2.8c).

The JHB BHA molecules held approximately perpendicular to the liposomal membrane were bound by at least two H8 Fab' fragments which were coupled to the same liposome. In addition, JHB BHA added to liposome coupled H8 Fab' fragments resulted in liposome aggregation (Figure 4.2.8c). The observed liposome aggregation was probably due to JHB BHA bound by two H8 Fab' fragments which were coupled to different liposomes. This type of liposome aggregation was described for liposome coupled SFA 9B-2.1 Fab' when acting as a surrogate receptor for X-31 HA (Section 4.2.6)

No liposome aggregation was observed when X-31 BHA was added to Hc3 Fab' liposomes (Section 4.2.4). Hc3 Fab' binds to X-31 HA at a similar angle (110^0) compared to H8 Fab' which binds to JHB HA at 115^0. Compared to the Hc3 Fab' liposomes it was less favourable for two H8 Fab' fragments coupled to the same liposomal membrane to bind to a single BHA molecule. Unlike liposome coupled Hc3 Fab' two H8 Fab' fragments which were coupled to different liposomes were observed to bind to a single BHA molecule . These observations indicate that factors

Figure 4.2.8

Electron microscopy was done as described in Section 3.1.9. Interpretations show BHA as open shapes and antibody or Fab' in black, liposomal membrane is shown as hatched.

BHA was prepared as described in Section 3.1.2. H8 antibody was purified as described in Section 3.4.3. Fab' liposomes were prepared as described in Section 3.4. JHB BHA was bound to H8 Fab' liposomes and purified from unbound BHA as described in Section 3.5.1. Acid and trypsin treatment of BHA bound to Fab' liposomes was done as described in Section 3.2.4.

For a) bar ≡ 50nm, otherwise bar ≡ 100nm

a) shows H8 antibody - BHA complexes, The Fab region of H8 antibody formed a constant angle of ~ 115^0 with JHB BHA. ($n = 34$ S.D. = $\pm$ 4.3)

b) shows liposomes which contained H8 Fab' coupled to the liposome surface.

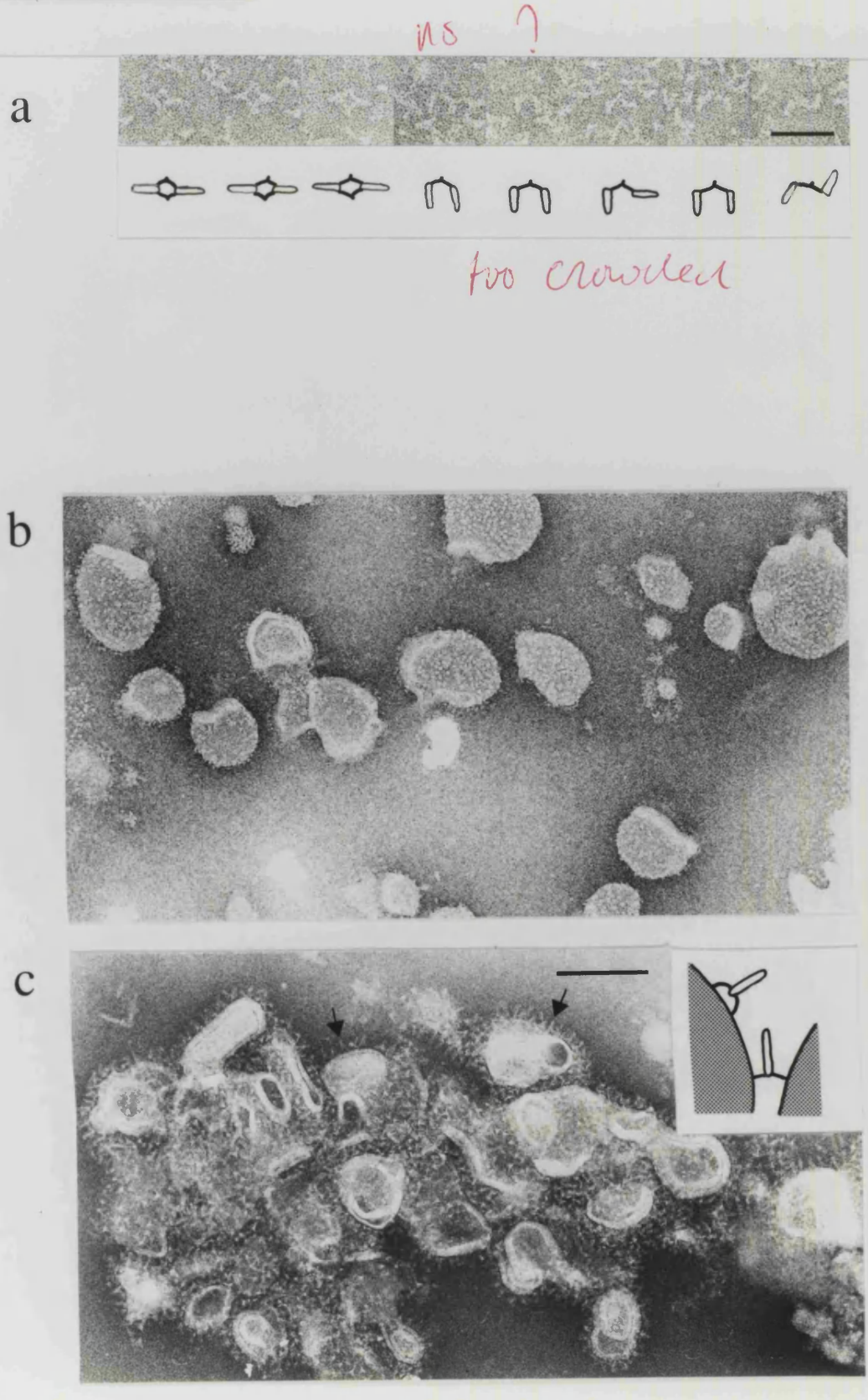
c) shows the H8 Fab' liposomes shown in b) after addition of JHB BHA followed by purification from unbound BHA (Section 3.5.1). As shown by the arrows it was possible for at least two liposome coupled H8 Fab' fragments coupled to the same liposomal membrane to bind to a single HA molecule. In addition liposome aggregation was observed which was probably due to JHB BHA bound to two H8 Fab' fragments which were coupled to different liposomes.

d) shows liposomes previously shown in c) following treatment at pH 5. The BHA has appeared to have undergone a characteristic low pH induced conformational change. It was unclear whether further liposome aggregation took place as liposomes were already aggregated in c).

e) shows liposomes previously shown in d) following trypsin treatment, this resulted in disaggregated liposomes. Liposomally inserted BHA_2 spikes were provisionally identified, although identification was complicated by the presence of liposomally coupled Fab' and therefore BHA_2 spikes are not directly labelled.

- I think that here again you overinterpret – this leads to statements that H8 should bind to JHB BHA ~~but~~ as HC3 to A31 BHA but it doesn't.
- Another difference may be that the density of the Fab' on the lipo may be much higher than elsewhere – from the pic it seems so
Is indeed case – pg 153-154

Figure 4.2.8: Electron microscopy of JHB BHA bound to H8 antibody and liposome coupled H8 Fab'



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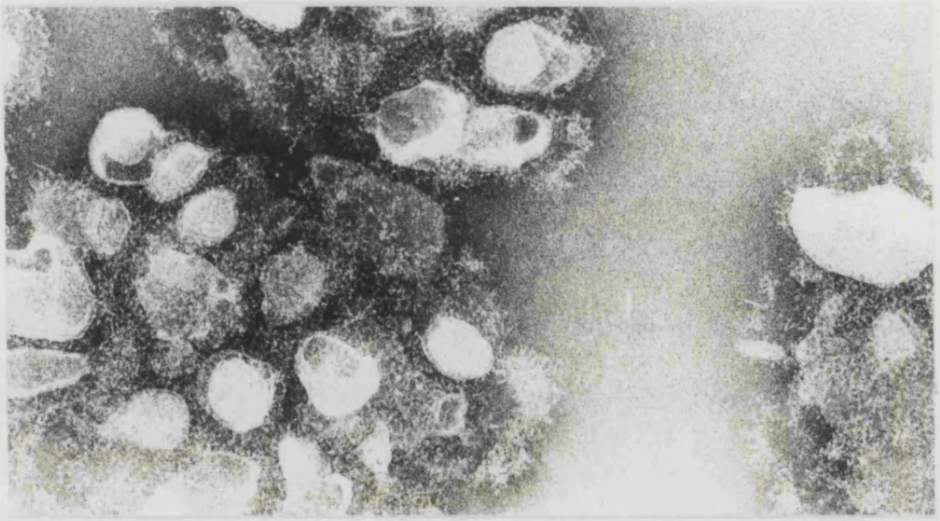

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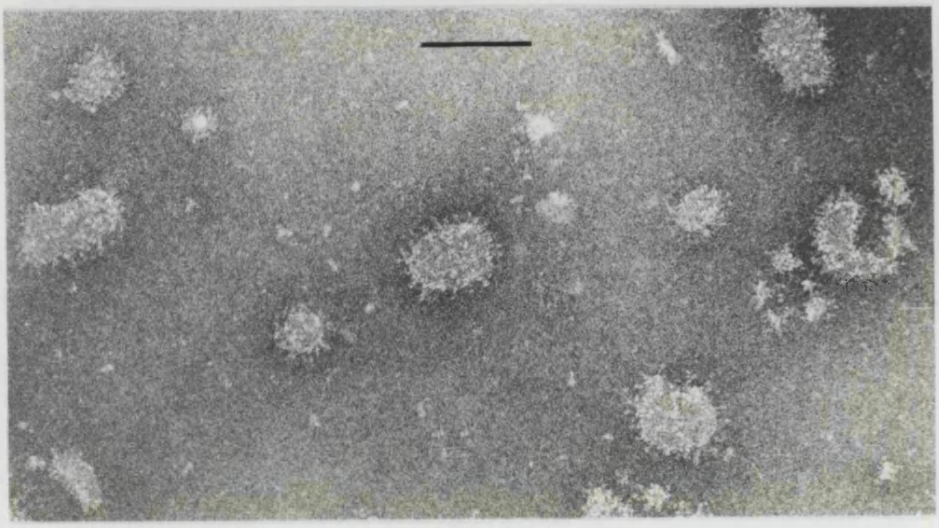


other than the angle of binding between the Fab' and the HA affect the orientation of the bound HA with respect to the liposomal membrane. Possible factors could include differences in the precise orientation of the Fab' when bound to the HA and or possible differences in flexibility within different Fab' fragments.

Low pH treatment of JHB BHA bound to liposome coupled H8 Fab' receptors resulted in BHA undergoing a characteristic acid induced conformational change as shown by EM (Figure 4.2.8d). Due to the liposome aggregation induced by native BHA, it was unclear whether further liposome aggregation took place upon low pH treatment. EM of disaggregated trypsin treated liposomes (Figure 4.2.8c) showed liposome inserted BHA_2, this indicated that upon acid treatment, at least in some cases, BHA bound to H8 Fab' receptor inserted its "fusion peptide" into the liposomal membrane.

JHB BHA bound to liposome coupled H8 Fab' receptor was not blocked from undergoing a characteristic low pH induced conformational change. The significantly different binding angles of H100 Fab' and H8 Fab' when bound to BHA resulted in BHA being held in a significantly different orientation with respect to the liposomal membrane when bound by either liposome coupled H8 or H100 Fab' receptor (Figures 4.2.7, 4.2.8 & 4.3.16). The effect on the membrane fusion efficiency of JHB HA bound to either to H8 or H100 Fab' receptor was investigated (Section 4.3). Differences in the orientation of JHB BHA bound to H8 or H100 Fab' receptor are discussed in Section 4.3.5 and illustrated in Figure 4.3.16.

4.2.9 Quantification of "fusion peptide" insertion into liposomal membranes

Acid induced inactivation of influenza virus is thought to result from HA undergoing a conformational change which results in insertion of "fusion peptide"

into its own viral membrane (Ruigrok *et al.*, 1986b; Weber *et al.*, 1994; Wharton *et al.*, 1995). In the case of BHA bound by liposome coupled Hc73, Hc19 or H100 Fab' receptors, low pH treatment resulted in exposure of "fusion peptide" which resulted in liposome aggregation. The exposure of "fusion peptide" leading to liposome aggregation may have resulted from a process comparable to virus inactivation in which the "fusion peptide" "inverts" into its own viral membrane. As BHA is cleaved from the viral membrane, "inverted" "fusion peptide" could have become involved in hydrophobic interactions leading to liposome aggregation.

BHA bound to Hc73, Hc19 or H100 Fab' receptors was held in an approximately perpendicular orientation with respect to the liposomal membrane, with the globular HA_1 domains of BHA held nearest to the liposomal membrane. Considering the proposed hypothesis concerning the low pH induced conformational change of HA (Section 2.3) it was expected that low pH treatment of HA may have resulted in insertion of "fusion peptide", at least a some cases, directly into the liposomal membrane to which the BHA was bound. Attempts were made to determine the number of "fusion peptides" of Fab' - bound BHA that inserted into liposomes compared with the number that formed "fusion peptide -fusion peptide" aggregates.

Trypsin treatment of low pH treated liposome aggregates would be expected to produce a mixture of liposome associated BHA_2 resulting from BHA that had inserted its "fusion peptide" into the liposomal membrane and BHA_2 aggregates resulting from BHA that had become associated in "fusion peptide - fusion peptide" interactions. Separation of liposome associated BHA_2 from BHA_2 aggregates would allow determination of the relative levels of "fusion peptide" insertion into the liposomal

membrane compared to the proportion of "fusion peptide - fusion peptide" interactions.

The detection of BHA_2 by western blotting following separation procedures was successful, but despite repeated efforts using density gradient centrifugation or gel filtration, a reliable method for separating liposomes from BHA_2 aggregates could not be developed (Section 3.5.2). It was therefore not possible to make an estimate of the proportion of bound BHA which upon acid treatment inserted its "fusion peptide" into the liposomal membrane. Determination of the proportion of liposome associated BHA_2 would of course not have resolved the question of what proportion of "fusion peptide" had inserted into the liposomal membrane to which the BHA had been bound by Fab' fragments, compared to the amount of association to other liposomes.

The precise orientation of BHA with respect to the target membrane was a direct consequence of the binding specificity of the surrogate Fab' receptor, the angle at which the Fab' fragment bound to the HA, and the flexibility of the Fab' fragments. It was likely that the precise orientation of BHA with respect to the target membrane effected the efficiency of insertion of "fusion peptide" into the liposomal membrane. Due to the difficulties stated above it was not possible to quantify any differences in the efficiency of liposome insertion of "fusion peptide" when using different surrogate receptors.

4.2.10 Summary

EM showed that the liposome coupled surrogate receptors anti X-31 HA Hc73 Fab' and anti JHB HA H100 Fab' had similar properties when acting as surrogate receptors for BHA. As these two receptors recognised two antigenically different strains of HA, this enabled Fab' liposome - virosome fusion assays to be done which

compared the membrane fusion efficiency of HA when bound or unbound to receptor (Section 4.3). Anti JHB HA H8 Fab' receptor, which bound JHB BHA in a different orientation to H100 Fab' receptor was also used in Fab' liposome - virosome fusion assays to investigate the membrane fusion efficiency of JHB HA when bound at a different orientation with respect to the target membrane (Section 4.3).

4.3 CHAPTER THREE - RESULTS: STUDIES OF THE EFFECT OF HA RECEPTOR BINDING ON THE EFFICIENCY OF MEMBRANE FUSION USING LIPOSOME COUPLED ANTI-HA Fab' FRAGMENTS AS SURROGATE RECEPTORS FOR HA

4.3.1 Introduction

In Section 2.3.7 the role of HA receptor binding with regard to the efficiency of membrane fusion was discussed. Certain experiments have concluded that binding of HA to sialic acid residues facilitates correct insertion of the HA "fusion peptide" into the target membrane (Niles and Cohen, 1993; Pedroso de Lima *et al.*, 1995; Stegmann *et al.*, 1995). Contrary to these suggestions there have been reports which propose that the binding and fusion functions of HA are not performed by the same trimer (Ellens *et al.*, 1990; Alford *et al.*, 1994).

The following experiments investigated the role of receptor binding in virosome - liposome fusion with regard to the efficiency of membrane fusion. Due to the problems associated with the study of low affinity receptor complexes of HA bound to sialic acid, liposome coupled anti-HA Fab' fragments were used as surrogate receptors for HA. On the basis of electron microscopy studies described in "results-chapter 2" it was decided to compare the amount of virosome fusion induced with Fab' liposomes when coupled with anti-X-31 HA Hc73 Fab' when acting as a surrogate receptor for X-31 $H17_1R$ HA (H3 subtype) or anti-JHB HA H100 or H8 Fab' when acting as a surrogate receptor for A/JHB/33/94 (JHB) HA (H3 subtype). As discussed in Section 4.2.8 H100 Fab' and H8 Fab' recognise a similar site on JHB HA but bind at a significantly different angle, this resulted in a different orientation of bound JHB BHA with respect to the target membrane. The first experiment described

compared H100 Fab' with Hc73 Fab' and the second experiment described compared H8 Fab' with Hc73 Fab' when acting as surrogate receptors for HA.

The results presented strongly suggest that direct binding by HA to receptor can increase membrane fusion efficiency. It is also suggested that the orientation of bound HA relative to the target membrane effects the ability of bound HA to increase the efficiency of membrane fusion, these findings have significant implications for design of a HA based delivery vector.

4.3.2 Characterisation of virosomes made using X-31, X-31 $H17_1R$ and JHB HA

Virosomes were made using Method A which was based on the method of Stegmann *et al.* (1987). Virosomes were produced with a viral lipid composition. Influenza virus was solubilized with octaethylene glycol monododecyl ether ($C_{12}E_8$), removal of the nucleocapsid was by centrifugation, $C_{12}E_8$ was removed by biobeads resulting in formation of virosomes, residual detergent was removed by centrifugation down a discontinuous sucrose gradient followed by dialysis (Sections 3.2.1 & 3.6.1).

4.3.2.1 Characterisation of fusion activity

X-31, X-31 $H17_1R$, JHB and a 1:1 mixture of X-31 $H17_1R$ and JHB HA (mixed HA) was used to prepare virosomes labelled with N-NBD-PE and N-Rho-PE (Section 3.6.1). The virosomes exhibited HA mediated fusion with erythrocyte ghosts as assayed by resonance energy transfer (R.E.T) (Figure 4.3.1). X-31 HA virosomes had 50 % maximal membrane fusion activity at ~ pH 5.7, JHB and X-31 $H17_1R$ HA virosomes had 50% maximal activity at +0.3 and +0.7 pH units higher respectively. The pH profile of membrane fusion induced by virosomes containing a 1:1 mixture of X-31 $H17_1R$ and JHB HA (mixed HA) showed that both JHB and X-31 $H17_1R$ HA

contributed to the fusion activity of the mixed HA virosome - erythrocyte ghost fusion (Figure 4.3.1)

Pre-treatment at pH 6 of single strain HA virosomes containing JHB or X-31 $H17_1R$ HA resulted in inactivation of X-31 $H17_1R$ HA virosomes but not JHB HA virosomes (data not shown). All virosome preparations pre-treated at pH 5 showed inactivation of membrane fusion activity (data not shown). Consistent with these observations mixed HA virosomes when pre-treated at pH 6 at 37^0C for 5 mins showed reduced but significant fusion activity with erythrocyte ghosts with a pH profile characteristic of JHB HA (Figure 4.3.1). This indicated native JHB HA was present after pH 6 treatment of mixed HA virosomes and inactivation of the majority of X-31 $H17_1R$ HA had taken place.

The rate of virosome - erythrocyte ghost fusion was analysed by following the increase in N-NBD-PE fluorescence at 530 nm. This showed that X-31 $H17_1R$ HA virosomes induced membrane fusion with erythrocyte ghosts significantly faster than JHB HA virosomes. For example, at pH 5.7 the extent of membrane fusion for X-31 $H17_1R$ and JHB HA virosomes was measured at 61% and 58 % increase in fluorescence at 530 nm, the time taken for the fluorescence at 530 nm to increase to half its final value was ~ 8 sec. with X-31 $H17_1R$ HA and ~26 sec with JHB HA.

Haemolysis of human erythrocytes by the same virosome preparations gave essentially the same pH profiles as those obtained with the virosome - erythrocyte ghost fusion followed by R.E.T (data not shown).

4.3.2.2 Electron microscopy of mixed HA virosomes

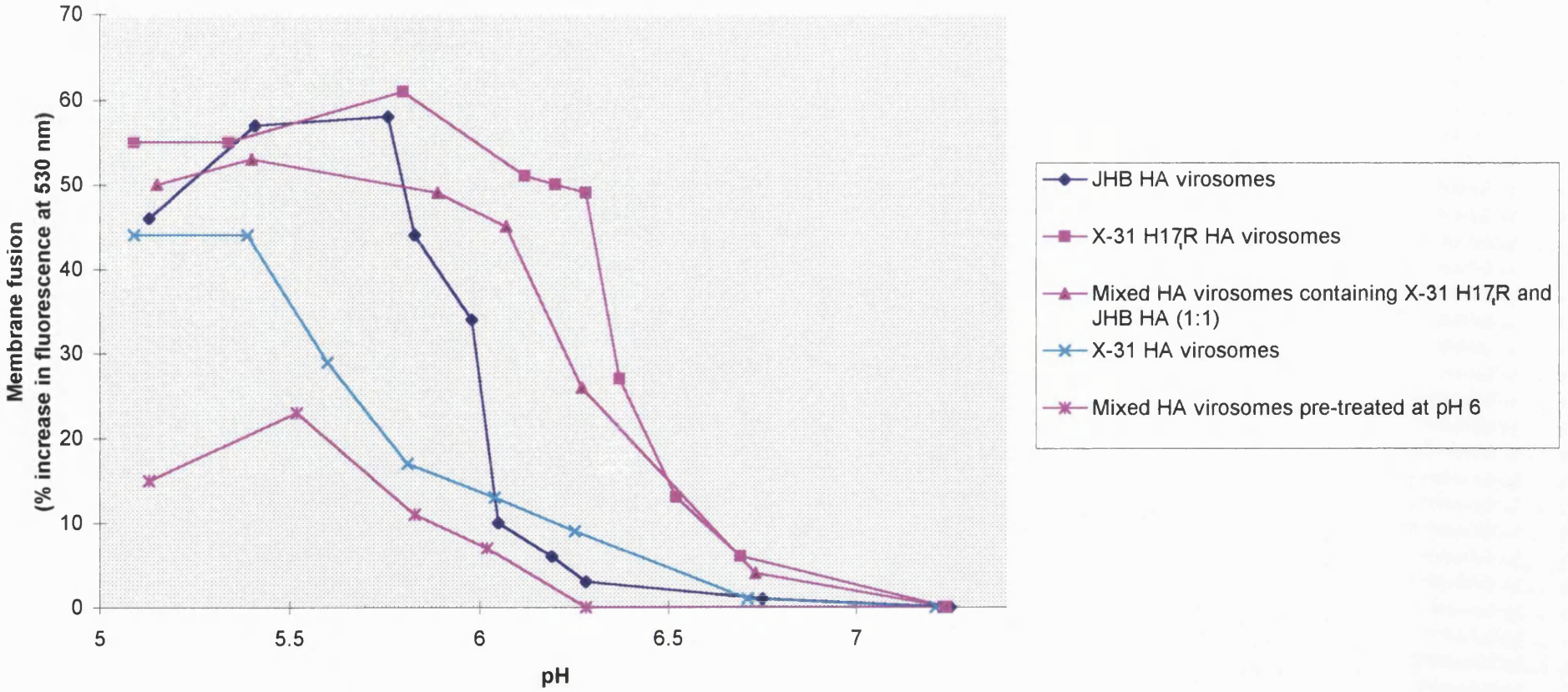
It was assumed that mixed HA virosomes would contain both X-31 $H17_1R$ and JHB HA reconstituted into the same membrane vesicle. To investigate whether this

Figure 4.3.1

Virosomes were produced by Method A containing various strains of HA (Section 3.6.1). Virosomes were labelled with 0.6 mol% N-NBD-PE and N-Rho-PE (Section 3.2.1). 40µl of labelled virosomes and 40 µl of unlabelled erythrocyte ghosts (Section 3.3.2.1) were added to PBS prewarmed to 37^0C to produce a final volume of 1 ml. The R.E.T membrane fusion assay was done as described in Section 3.3.2. After incubation of the virosomes with the erythrocyte ghosts the pH was lowered to a specified pH by injecting 0.15 M sodium citrate pH 3.5 into the cuvette and the percentage increase in fluorescence at 530 nm was calculated as described in Section 3.3.2 .

Ex. λ = 465 nm, Em. λ= 530 nm Temp. = 37^0C

Figure 4.3.1: pH profile of virosome - erythrocyte ghost fusion, comparison between virosomes containing X-31, JHB and X-31 H17$_1$R HA



assumption was correct pH 6 pre-treated X-31 $H17_1R$, JHB, and mixed HA virosomes were compared by EM. EM of pH 6 pre-treated mixed HA virosomes showed vesicles containing both native HA and HA in the low pH conformation in the same vesicle (Figure 4.3.2b). EM of pH 6 pre-treated X-31 $H17_1R$ HA virosomes showed the majority of HA in the low pH conformation, whereas pH 6 pre-treated JHB HA virosomes contained essentially only native HA (Figure 4.3.2e&h). From these observations it was clear that mixed HA virosomes contained both X-31 $H17_1R$ and JHB HA reconstituted into the same membrane vesicle.

4.3.3 Fusion of mixed HA virosomes with liposomes coupled to anti X-31 HA Hc73 Fab' or anti JHB HA H100 Fab' fragments

EM of BHA bound to liposome coupled Hc73 Fab' and H100 Fab' was discussed in Sections 4.2.2 & 4.2.7. In the following experiments liposomes coupled with anti-JHB HA H100 Fab' or anti X-31 HA Hc73 Fab' were used as target membranes in virosome - liposome fusion assays, in which the virosomes were labelled with N-NBD-PE and N-Rho-PE. Mixed HA virosomes were used to enable experiments which investigated whether HA bound by Fab' receptor was more or less efficient at inducing membrane fusion compared to unbound HA held close to the membrane by other HA - receptor interactions.

4.3.3.1 Determination of the relative amount of Fab' coupled to the liposome membrane

EM of BHA bound to liposome preparations coupled either with H100 Fab' or Hc73 Fab' showed slightly more BHA bound per Hc73 Fab' liposome compared to H100 Fab' liposomes, indicating that Hc73 liposomes contained slightly more coupled

Figure 4.3.2

Virosomes were produced by Method A (Section 3.2.1) as described in Section 3.6.1. Electron microscopy of virosome preparations was done as described in Section 3.1.9. Interpretation shows native HA as open shapes and low pH conformtion HA as black lines. Virosomal membrane is hatched. **Bar ≡ 100nm**

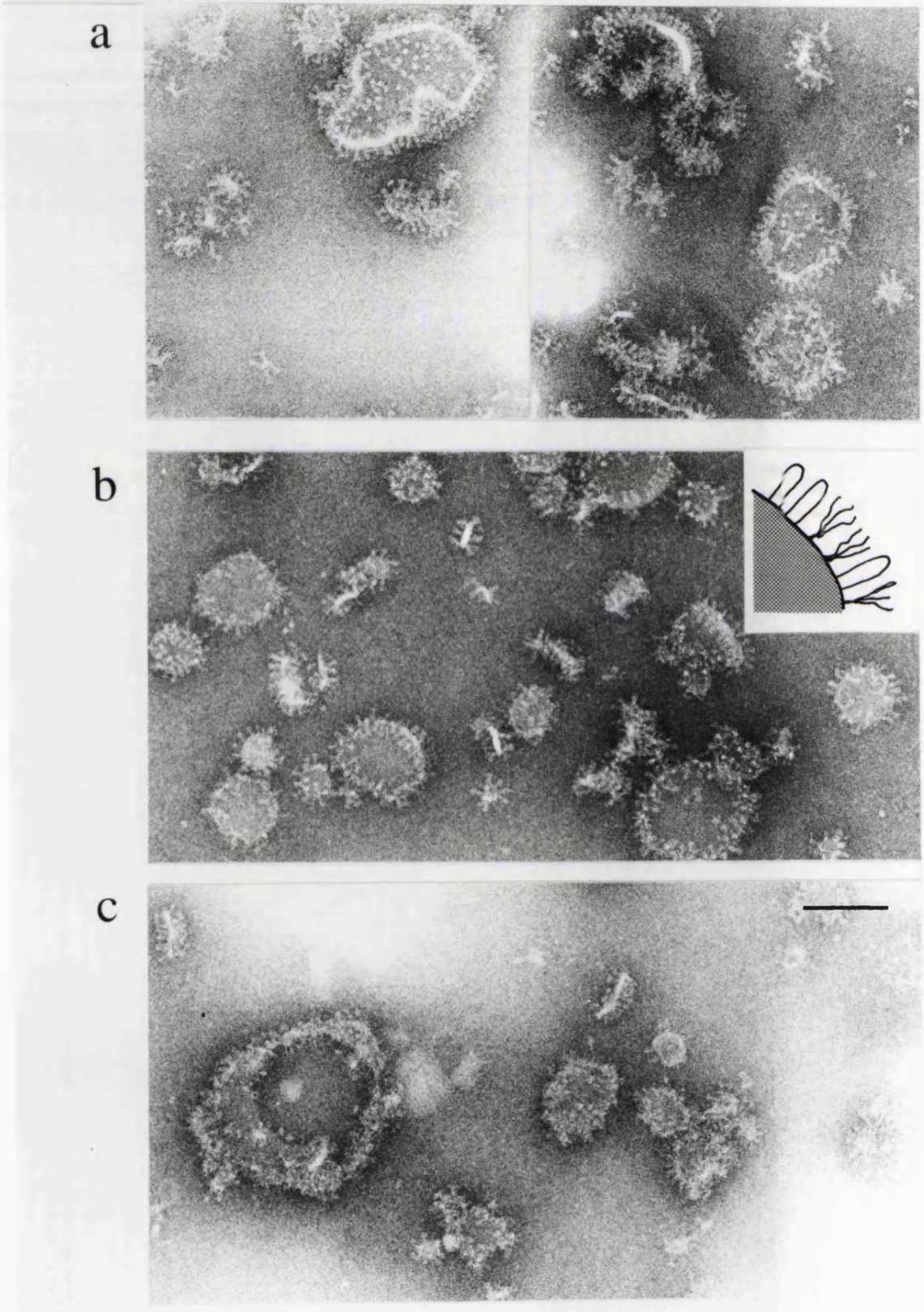
a) b) and **c)** show mixed HA virosomes containing JHB and X-31 $H17_1R$ HA in equal amount as judged by SDS PAGE (Figure 3.6.1). **a)** shows untreated mixed HA virosomes. The HA present, as expected was in its native conformation. **b)** shows mixed HA virosomes pre-treated at pH 6 (Section 3.2.4). Approximately half of the HA appeared to have undergone an acid induced conformational change and half remained in its native conformation, both native and low pH conformation HA was observed in the same vesicle. **c)** shows mixed HA virosomes pre-treated at pH 5. The vast majority of HA had appeared to have undergone an acid induced conformational change.

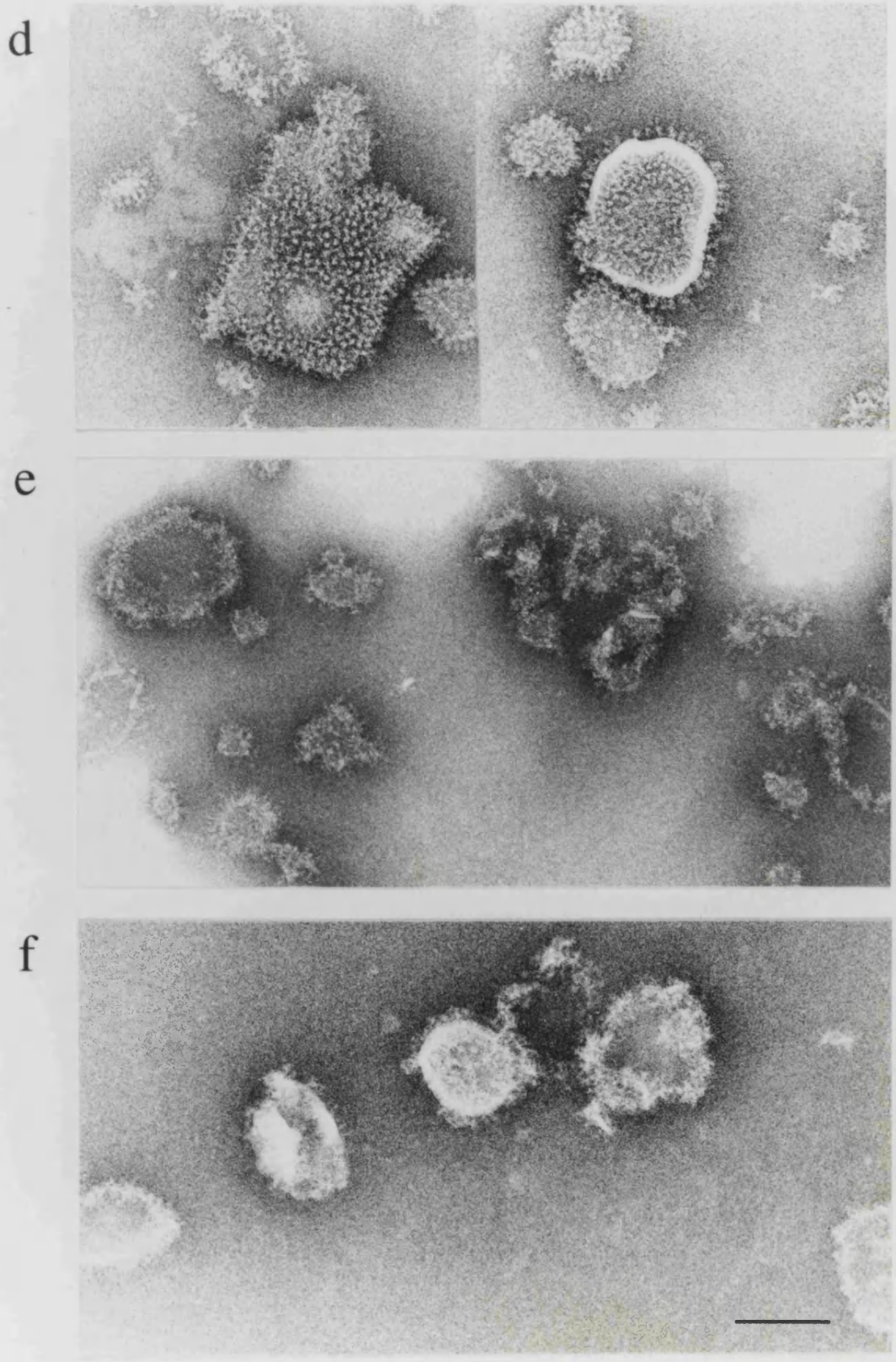
d) e) and **f)** show X-31 $H17_1R$ HA virosomes. **d)** shows untreated X-31 $H17_1R$ HA virosomes. The HA present, as expected was in its native conformation. **e)** shows X-31 $H17_1R$ HA virosomes pre-treated at pH 6. The vast majority of HA had appeared to have undergone an acid induced conformational change. **f)** shows X-31 $H17_1R$ HA virosomes pre-treated at pH 5.

g) h) and **i)** show JHB HA virosomes. **g)** shows untreated JHB HA virosomes. The HA present, as expected was in its native conformation. **h)** shows JHB HA virosomes pre-treated at pH 6. The majority of HA remained in its native conformation. **i)** shows JHB HA virosomes pre-treated at pH 5. The vast majority of HA had appeared to have undergone an acid induced conformational change.

pH 6 pre-treatment of X-31 $H17_1R$ HA virosomes but not JHB HA virosomes induced an acid induced conformational change of the HA. Mixed HA virosomes pre-treated at pH 6 contained approximately half native and half low pH conformation HA reconstituted within the same vesicle. It was therefore concluded that mixed HA virosomes contained both X-31 $H17_1R$ and JHB HA reconstituted into the same membrane vesicle

Figure 4.3.2: Electron microscopy of JHB, X-31 H17$_1$R and mixed HA virosomes

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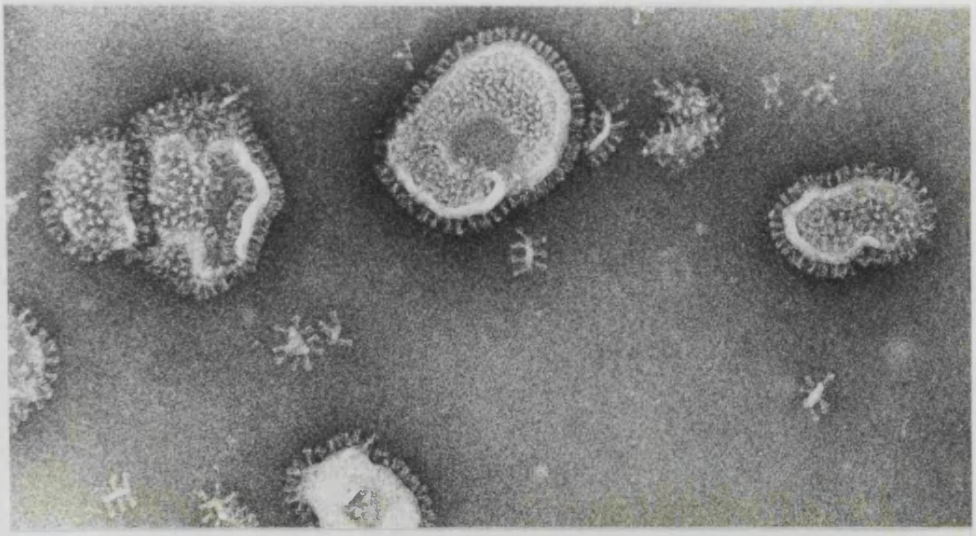

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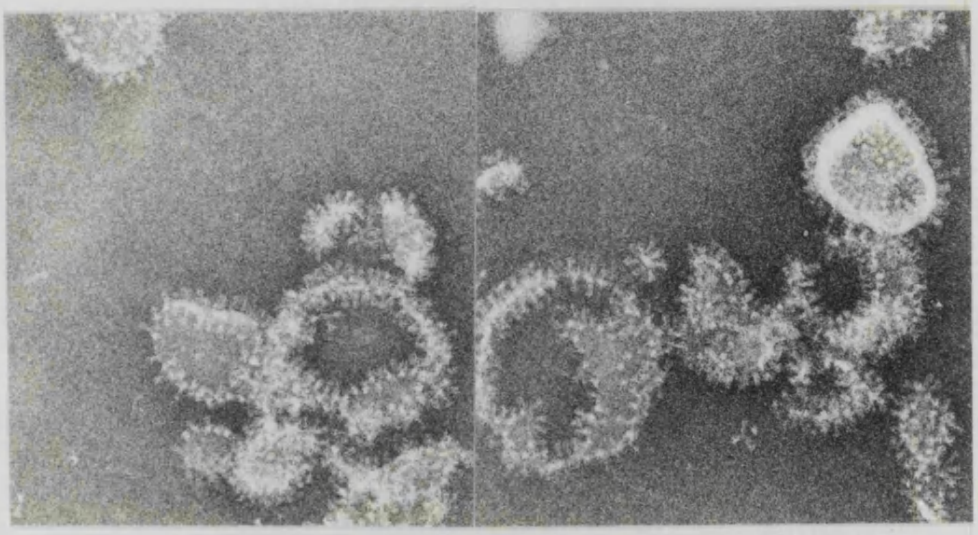

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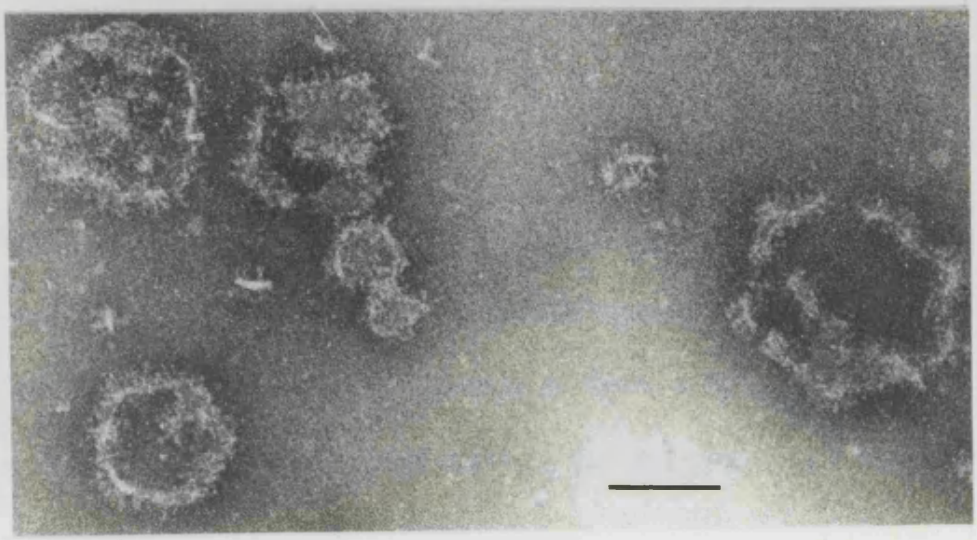


Fab' receptor and SDS-PAGE of Fab' coupled liposomes loading on the gels equal amounts of liposomes showed that this was the case (Figure 4.3.3).

The following experiments used the H100 Fab' and Hc73 Fab' liposome preparations shown in Figure 4.3.3. Interpretation of the results takes account of the observed difference in the level of liposome coupled H100 and Hc73 Fab' fragments.

4.3.3.2 Liposome coupled anti-HA Fab' receptors increased the efficiency of virosome - liposome fusion

H100 antibody but not Hc73 antibody recognises JHB HA as determined by ELISA (H100 - JHB HA = 25600. H100 - X-31 HA = <100. Hc73 - X-31 HA = 25600. Hc73 - JHB HA = <100. Values given are the reciprocals of the highest dilution of ascitic fluid scored as a positive).

JHB HA virosomes with anti-X-31 Hc73 Fab' liposomes exhibited only low levels of fusion. This was in contrast to JHB HA virosomes with anti-JHB HA H100 Fab' liposomes (Figure 4.3.4). These results showed that binding of HA to Fab' receptor significantly increased the extent of membrane fusion. The same conclusions can be made when comparing the extent of fusion between X-31 $H17_1R$ HA virosomes and H100 or Hc73 Fab' liposomes (Figure 4.3.4).

4.3.3.3 The membrane fusion activity of mixed HA virosomes when bound to liposome coupled Hc73 Fab' or H100 Fab' fragments

The pH profile of membrane fusion of X-31 $H17_1R$ HA virosomes with Hc73 Fab' liposomes and JHB HA virosomes with H100 Fab' liposomes was comparable to the pH profile of X-31 $H17_1R$ and JHB HA virosome fusion with erythrocyte ghosts (Figures 4.3.1 & 4.3.5) providing further evidence that virosome - Fab' liposome membrane fusion was HA mediated.

Figure 4.3.3

Fab' liposomes were prepared as described in Section 3.4.

Electron microscopy

Electron microscopy was done as described in Section 3.1.9. BHA was prepared as described in Section 3.1.2. BHA was bound to Fab' liposomes and purified from unbound BHA as described in Section 3.5.1.

Show is X-31 or JHB BHA bound to Hc73 Fab' or H100 Fab' liposomes respectively

bar ≡ 100nm

It was observed that more BHA molecules were bound per liposome in the case of Hc73 Fab' liposomes compared to H100 Fab' liposomes.

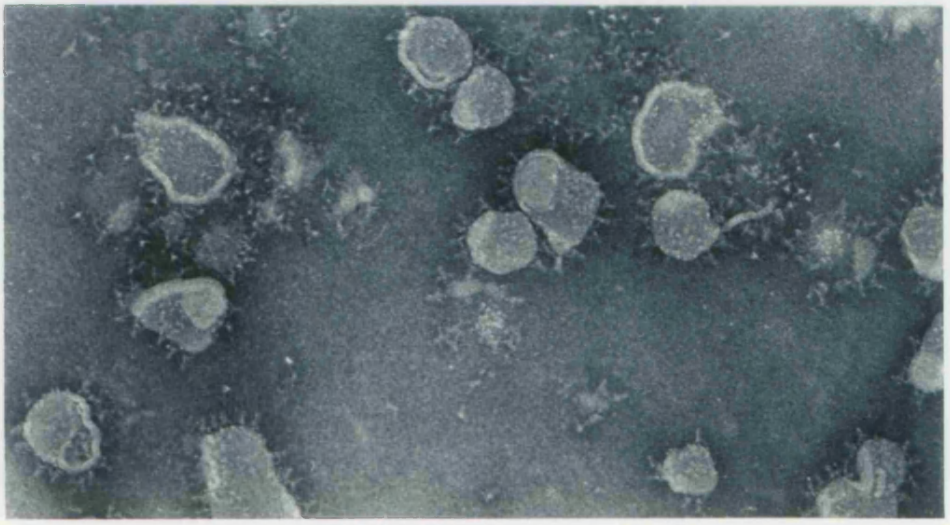
SDS PAGE of Fab' liposomes

The liposome concentration of the Hc73 Fab' and H100 Fab' liposomes preparations was equalized by quantifying the ^{3}H radioactivity (Section 3.6.2). SDS PAGE was done as described in Section 3.1.4, loading on the gel an equal amount of liposomes in each case. SDS PAGE showed that there was more Hc73 Fab' coupled per liposome compared to the H100 Fab' liposome preparation.

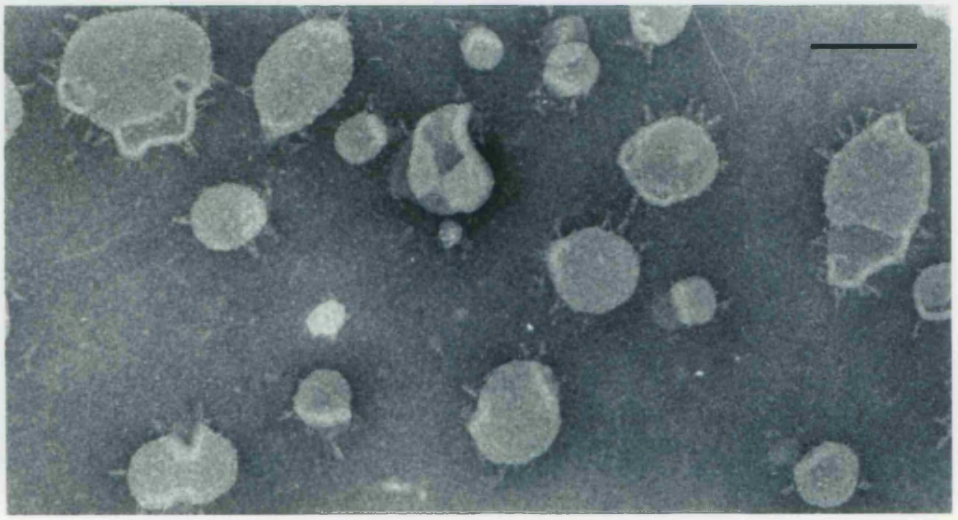
The results obtained by SDS PAGE and electron microscopy were consistent with each other.

Figure 4.3.3: Electron microscopy and SDS PAGE of the Hc73 Fab' and H100 Fab' liposome preparations: Determination of the relative amount of coupled Fab'

Hc73 Fab' liposomes + X-31 BHA



H100 Fab' liposomes + JHB BHA



SDS PAGE of Fab' liposomes

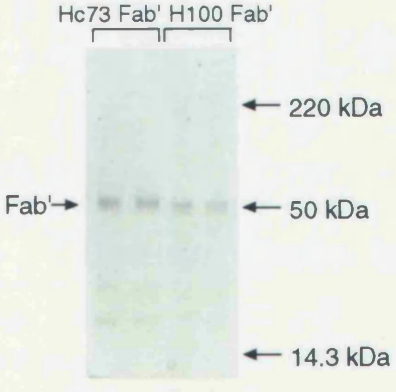


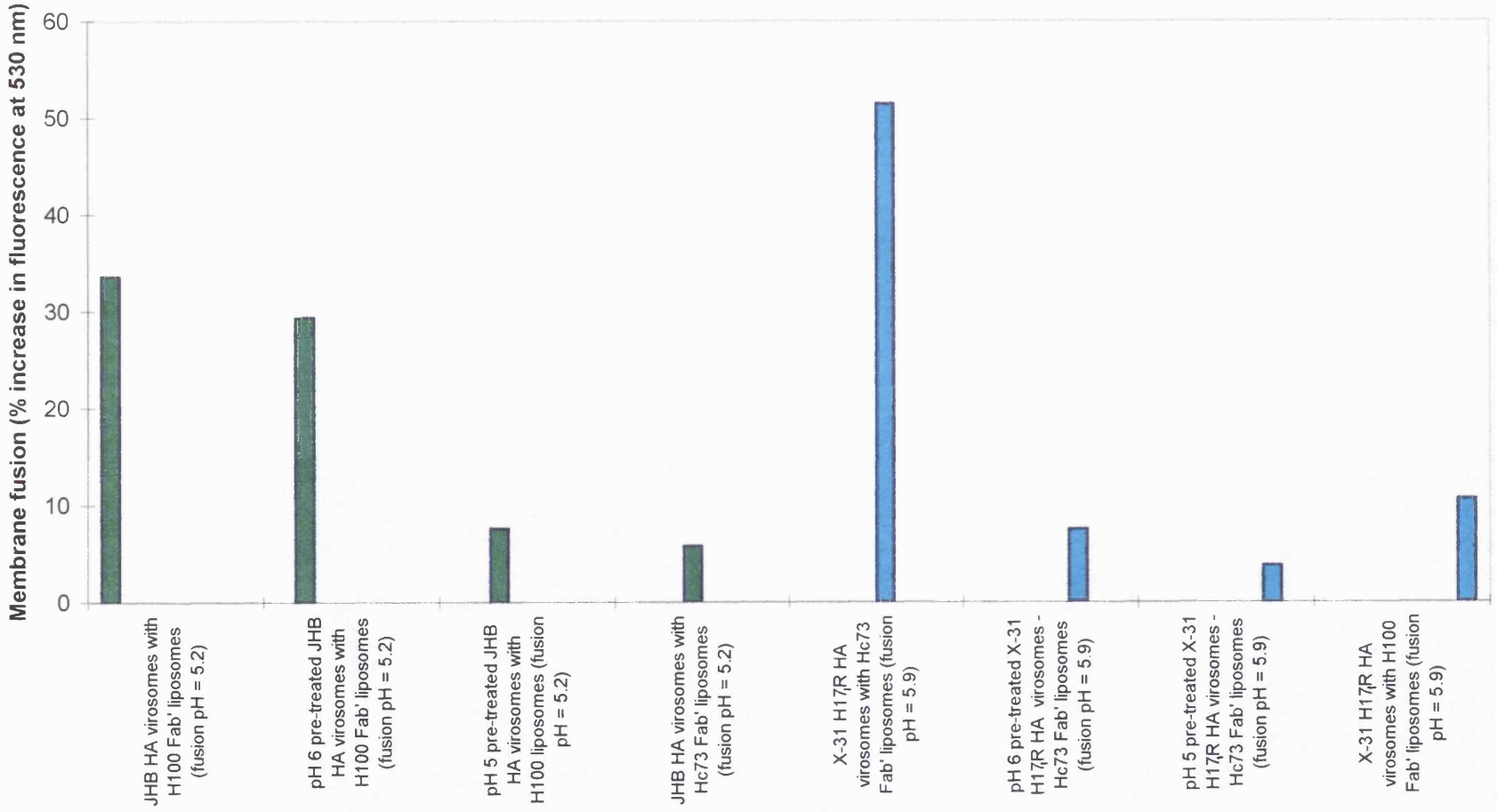
Figure 4.3.4

Virosomes were produced by Method A containing various strains of HA (Section 3.6.1). Virosomes were acid treated as described in Sections 3.2.4. Virosomes were labelled with 0.6 mol% N-NBD-PE and N-Rho-PE (Section 3.2.1). Fab' coupled liposomes were produced and purified as described in Section 3.4.

40µl of labelled virosomes and a constant amount of Fab' liposomes were added to PBS prewarmed to 37^0C to produce a final volume of 1 ml. The R.E.T membrane fusion assay was done as described in Section 3.6.2. After incubation of the virosomes with the liposomes the pH was lowered to a specified pH by injecting 0.15 M sodium citrate pH 3.5 into the cuvette and the percentage increase in fluorescence at 530 nm was calculated as described in Section 3.3.2 .

Ex. λ = 465 nm, Em. λ= 530 nm Temp. = 37^0C

Figure 4.3.4: Virosome - liposome fusion, effect of using JHB or X-31 H17$_1$R HA and anti-X-31 Hc73 or anti-JHB H100 Fab' receptors



To investigate the mechanism by which binding by HA to Fab' receptor increased the extent of membrane fusion, mixed HA virosomes containing X-31 $H17_1R$ HA and JHB HA in equal amounts were prepared (Section 3.6.1). Estimates of the pH of fusion of mixed HA virosomes with Hc73 or H100 Fab' liposomes resulted in different pH profiles. The pH profile when using Hc73 Fab' liposomes was similar to the pH profile of X-31 $H17_1R$ HA virosome - Hc73 Fab' liposome fusion (Figure 4.3.5). The pH profile of mixed HA virosome - H100 Fab' liposome fusion was less similar to the pH profile of X-31 $H17_1R$ HA virosome - Hc73 Fab' liposome fusion and was significantly different from the pH profile of JHB HA virosome - H100 Fab' liposome fusion (Figure 4.3.5). These observations indicated that for mixed HA virosome - Fab' liposome fusion when unbound X-31 $H17_1R$ HA was held close to the liposomal membrane via JHB HA - H100 Fab' interactions significant X-31 $H17_1R$ HA mediated fusion was observed. However, binding of X-31 $H17_1R$ HA to Hc73 Fab' increased its fusion efficiency compared to the situation where X-31 $H17_1R$ HA was held close to the liposomal membrane via JHB HA -H100 Fab' interactions.

The rate of fusion of mixed HA virosomes was analysed to investigate which strain of HA was responsible for the observed fusion activity. As described in Section 4.3.2.1 X-31 $H17_1R$ HA induced membrane fusion at a significantly higher rate than JHB HA when virosome - erythrocyte ghost was assayed by R.E.T. As shown in Figure 4.3.6 a similar difference in rate was also observed with virosome - Fab' liposome fusion. The rate of membrane fusion of mixed HA virosomes with either H100 or Hc73 Fab' liposomes at pH 5.9 was similar to that observed for X-31 $H17_1R$ HA virosomes (Figure 4.3.6), indicating that mixed HA virosome - liposome fusion at

Figure 4.3.5

Virosomes were produced by Method A containing various strains of HA (Section 3.6.1). Virosomes were labelled with 0.6 mol% N-NBD-PE and N-Rho-PE (Section 3.2.1). Fab' coupled liposomes were produced and purified as described in Section 3.4.

40μl of labelled virosomes and a constant amount of Fab' liposomes were added to PBS prewarmed to 37^0C to produce a final volume of 1 ml. The R.E.T membrane fusion assay was done as described in Section 3.6.2. After incubation of the virosomes with the liposomes the pH was lowered to a specified pH by injecting 0.15 M sodium citrate pH 3.5 into the cuvette and the percentage increase in fluorescence at 530 nm was calculated as described in Section 3.3.2 .

Ex. λ = 465 nm, Em. λ= 530 nm Temp. = 37^0C

Figure 4.3.5: pH profile of virosome - liposome fusion, comparison between Hc73 and H100 Fab' liposomes

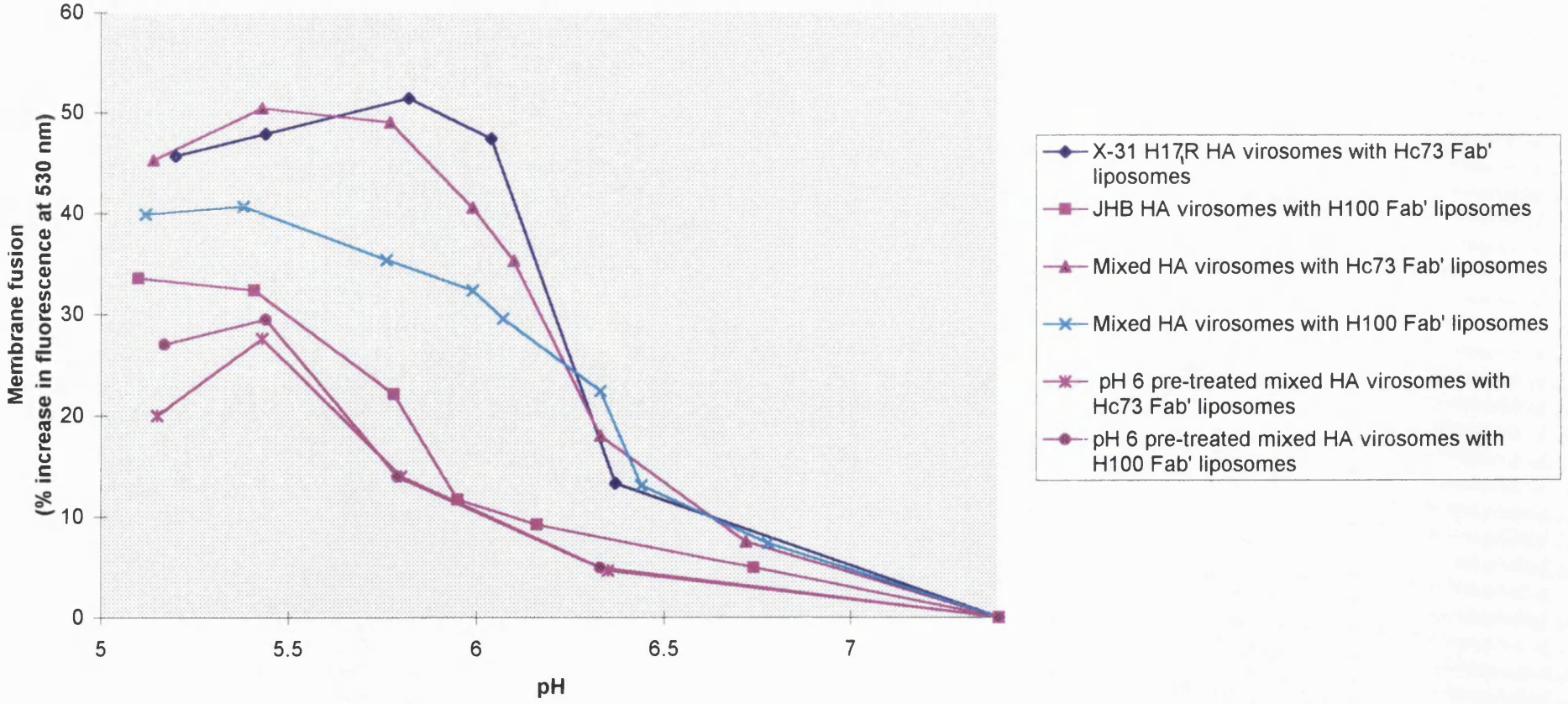


Figure 4.3.6

Virosomes were produced by Method A containing various strains of HA (Section 3.6.1). Virosomes were labelled with 0.6 mol% N-NBD-PE and N-Rho-PE (Section 3.2.1). Fab' coupled liposomes were produced and purified as described in Section 3.4.

40μl of labelled virosomes and a constant amount of Fab' liposomes were added to PBS prewarmed to 37^0C to produce a final volume of 1 ml. The R.E.T membrane fusion assay was done as described in Section 3.6.2.

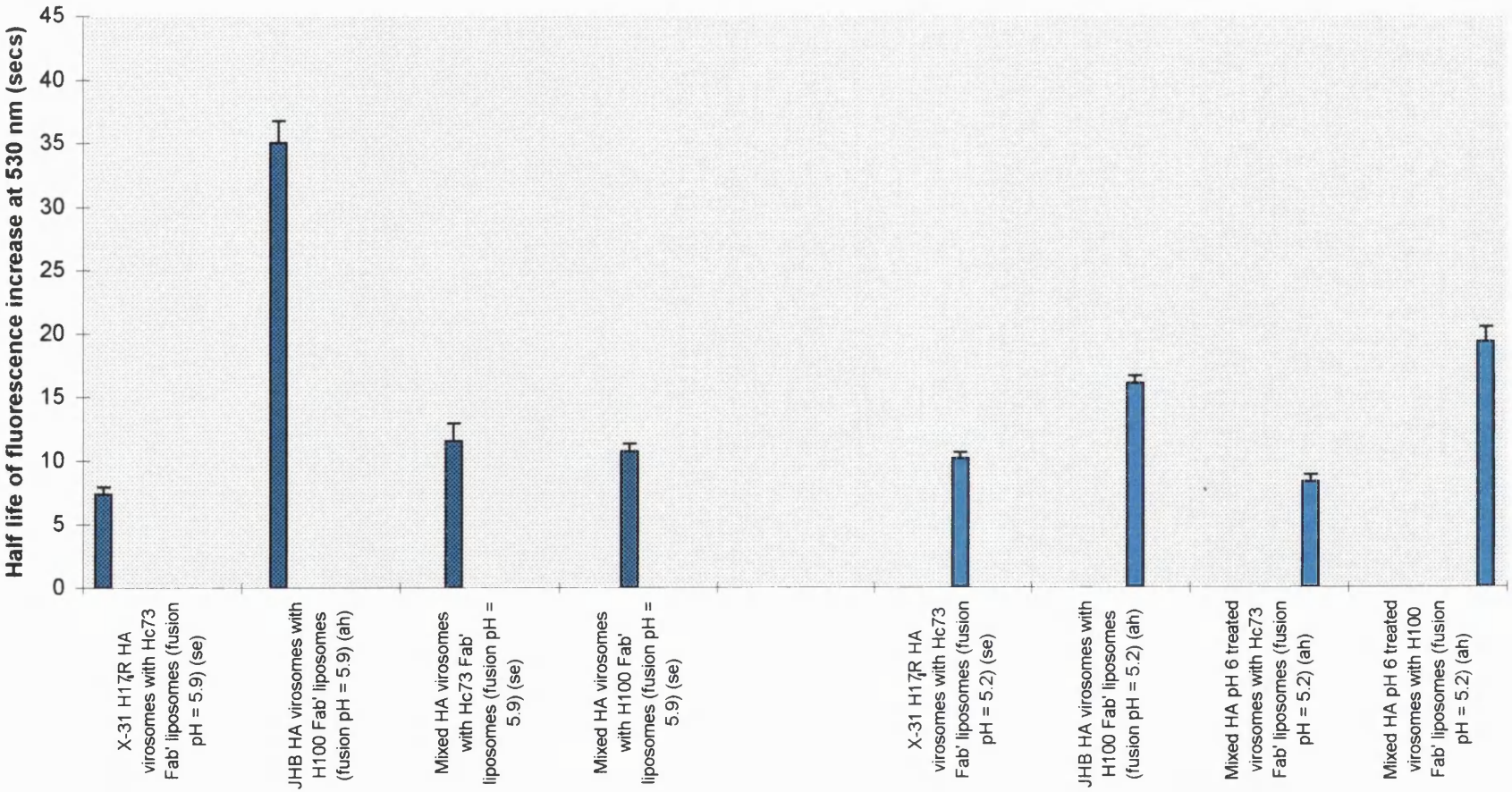
The rate of increase in fluorescence at 530 nm was analysed as described in Section 3.6.2. The time taken for the fluorescence at 530 nm to increase to half its final value is referred to as the "half life"($t_{1/2}$). Each assay was repeated at least 4 times. The mean half life is shown, the error bars show the standard deviation.

"se" indicates that the fusion kinetics were single exponential.

"ah" indicates that the fusion kinetics were more complex and did not fit to a single exponential, in which case an apparent $t_{1/2}$ was calculated.

Ex. λ = 465 nm, Em. λ= 530 nm Temp. = 37^0C

Figure 4.3.6: The rate of virosome - Fab' liposome membrane fusion, differences between X-31 $H17_1R$ and JHB HA (H100 / Hc73 Fab' liposome experiment)



pH 5.9 was induced predominantly by X-31 $H17_1R$ HA. JHB HA virosomes showed relatively low membrane fusion activity at pH 5.9 compared to X-31 $H17_1R$ HA virosomes (Figure 4.3.5). The relatively fast rate of membrane fusion induced by X-31 $H17_1R$ HA at pH 5.9 compared to JHB HA further indicates the decreased contribution of JHB HA to fusion of mixed HA virosomes at pH 5.9.

As described above the membrane fusion activity of mixed HA virosomes at pH 5.9 can be attributed to the activity of X-31 $H17_1R$ HA present in the virosomal membrane. At pH 5.9 the efficiency of membrane fusion induced by mixed HA virosomes was significantly higher with Hc73 Fab' liposomes compared to H100 Fab' liposomes (Figure 4.3.7a). This showed that X-31 $H17_1R$ HA directly bound to Hc73 Fab' was more efficient at inducing membrane fusion compared to the situation where X-31 $H17_1R$ HA was held close to the liposomal membrane via JHB HA - H100 Fab' interactions.

It was considered possible that the higher fusion efficiency of mixed HA virosomes when bound to Hc73 Fab' rather than H100 Fab' receptor may have been due to differing levels of coupled Fab' receptor. The slightly higher level of Hc73 Fab' coupled per liposome compared to H100 Fab' (Figure 4.3.3) may have resulted in a higher number of virosome - liposome binding interactions and the observed increase in fusion efficiency. Experiments described below (Section 4.3.3.4) and subsequent experiments comparing H8 Fab' and Hc73 Fab' HA receptors (Section 4.3.4) have shown that differing levels of liposome coupled Fab' were not solely responsible for the observed differences in the fusion efficiency of mixed HA virosomes.

4.3.3.4 The membrane fusion activity of pH 6 pre-treated mixed HA virosomes when bound to liposome coupled Hc73 Fab' or H100 Fab' fragments

Pre-treatment at pH 6 of mixed HA virosomes resulted in the irreversible low pH induced conformational change of the majority of X-31 $H17_1R$ HA (Figures 4.3.2 & 4.3.4). In the low pH induced conformation the HA_1 subunits come apart but are still recognised by Hc73 Fab' fragments. The amount of X-31 $H17_1R$ HA in pH 6 pre-treated mixed HA virosomes is equal to the level of native X-31 $H17_1R$ HA in untreated mixed HA virosomes as judged by SDS-PAGE (data not shown). The majority of JHB HA in pH 6 pre-treated mixed HA virosomes remained in its native conformation (Figures 4.3.2 & 4.3.4).

The pH profile of fusion of pH 6 pre-treated mixed HA virosomes with H100 and Hc73 Fab' liposomes was characteristic of JHB HA (Fig 4.3.5). This indicated that the vast majority of fusion was JHB HA mediated.

Trypsin treatment of HA in the low pH conformation results in removal of residues 28-328 of HA_1, thus removing the binding sites for both H100 and Hc73 Fab' fragments. Native HA is resistant to trypsin digestion. Trypsin treatment of pH 6 pre-treated mixed HA virosomes resulted in significantly less fusion with Hc73 Fab' liposomes compared to non-trypsin treated pH 6 pre-treated mixed HA virosomes (Figure 4.3.8), suggesting that trypsin treatment resulted in the removal of low pH X-31 $H17_1R$ $HA_1$28-328, resulting in decreased binding between Hc73 Fab' liposomes and virosomes and a subsequent drop in the efficiency of membrane fusion.

pH 6 pre-treated mixed HA virosomes, containing the vast majority of the X-31 $H17_1R$ HA in the low pH conformation induced significant JHB HA mediated fusion with Hc73 Fab' liposomes. This fusion was significantly reduced by trypsin

treatment of pH 6 pre-treated mixed HA virosomes, suggesting that the membrane fusion observed in the first instance was due to unbound native JHB HA being held close to the liposomal membrane via low pH X-31 $H17_1R$ HA - Hc73 Fab' binding.

The amount of fusion between pH 6 and trypsin pre-treated mixed HA virosomes and H100 Fab' liposomes was only slightly decreased compared to the amount of fusion with non trypsin treated pH 6 pre-treated mixed HA virosomes (Figure 4.3.8). This indicated that only a small proportion of JHB HA in pH 6 pre-treated mixed HA virosomes was in the low pH conformation. The results obtained by trypsin treatment of pH 6 pre-treated mixed HA virosomes were in good agreement with the results obtained by EM and acid treatment of virosomes (Figures 4.3.2 & 4.3.4) which also suggested that the majority of native HA remaining in pH 6 pre-treated mixed HA virosomes was that of the JHB strain.

X-31 $H17_1R$ and JHB HA induced fusion at different rates, therefore the rate of fusion of pH 6 pre-treated mixed HA virosomes was investigated in an attempt to gain further information concerning the HA strain responsible for the fusion activity. As described above the pH profile of fusion of pH 6 pre-treated mixed HA virosomes strongly indicated that the fusion activity was mainly due to JHB HA. The rate of fusion at pH 5.2 of X-31 $H17_1R$ HA virosomes with Hc73 Fab' virosomes was significantly faster than the rate of fusion at pH 5.2 of JHB HA virosomes with H100 Fab' liposomes (Figure 4.3.6). The rate of fusion at pH 5.2 of pH 6 pre-treated mixed HA virosomes with H100 Fab' liposomes was similar to the rate of fusion of JHB HA virosomes with H100 Fab' liposomes, indicating that the fusion was JHB HA mediated.

The rate of fusion at pH 5.2 of pH 6 pre-treated mixed HA virosomes with Hc73 Fab' liposomes was similar to the rate of X-31 $H17_1R$ HA virosomes with Hc73 Fab' liposomes. This suggested that the observed fusion had a larger contribution from residual amounts of native X-31 $H17_1R$ HA compared to the fusion of pH 6 pre-treated mixed HA virosomes with H100 Fab' liposomes (Figure 4.3.6). However, the pH profile of fusion of pH 6 treated mixed HA virosomes with Hc73 Fab' liposomes was characteristic of JHB HA (Fig 4.3.5). Therefore if the relatively fast rate of fusion of pH 6 pre-treated mixed HA virosomes with Hc73 Fab' liposomes was due to low levels of native X-31 $H17_1R$ HA, these levels were not sufficient to induce significant amounts of membrane fusion implying that the fusion process must have mainly been JHB HA mediated. A second explanation is that JHB HA when unbound induced fusion at a faster rate compared to when bound by liposome coupled H100 Fab' fragments. In any case it can be concluded the vast majority of the fusion activity of pH 6 treated mixed HA virosomes was due to JHB HA.

It can be concluded that the fusion activity at pH 5.2 of pH 6 pre-treated mixed HA virosomes is JHB HA mediated. The efficiency of membrane fusion induced by pH 6 pre-treated mixed HA virosomes was significantly higher with H100 Fab' liposomes than with Hc73 Fab' liposomes (Figure 4.3.7b). This indicates that JHB HA directly bound by H100 Fab' was more efficient at inducing membrane fusion than when JHB HA was held close to the liposomal membrane via X-31 $H17_1R$ HA - Hc73 Fab' interactions. This result was observed despite the fact that there was slightly less H100 Fab' coupled per liposome compared to Hc73 Fab' liposomes, which probably resulted in more virosome - Hc73 Fab' liposome binding interactions compared to H100 Fab' liposomes. It was probable that residual levels of native X-31

Figure 4.3.7

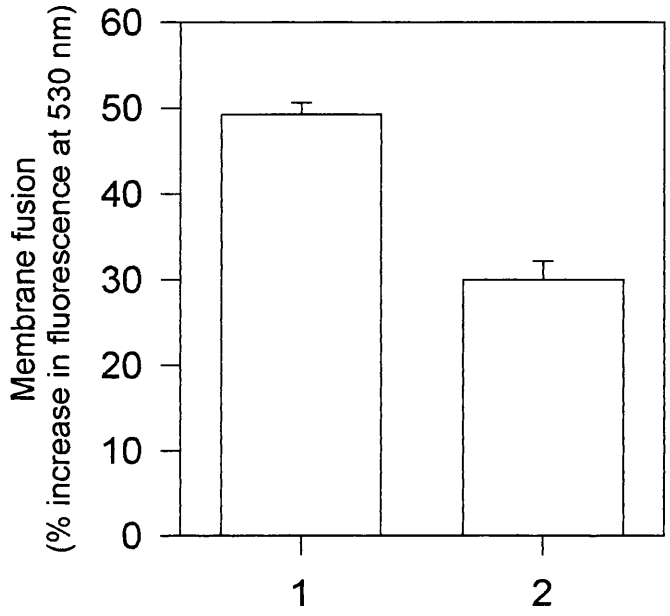
Virosomes were produced by Method A which contained equal amounts of X-31 $H17_1R$ HA and JHB HA (Section 3.6.1). Virosomes were labelled with 0.6 mol% N-NBD-PE and N-Rho-PE (Section 3.2.1). Fab' coupled liposomes were produced and purified as described in Section 3.4.

40μl of labelled virosomes and a constant amount of Fab' liposomes were added to PBS prewarmed to 37^0C to produce a final volume of 1 ml. The R.E.T membrane fusion assay was done as described in Section 3.6.2. Each assay was repeated at least 5 times. The mean increase in fluorescence is shown, the error bars show the standard deviation.

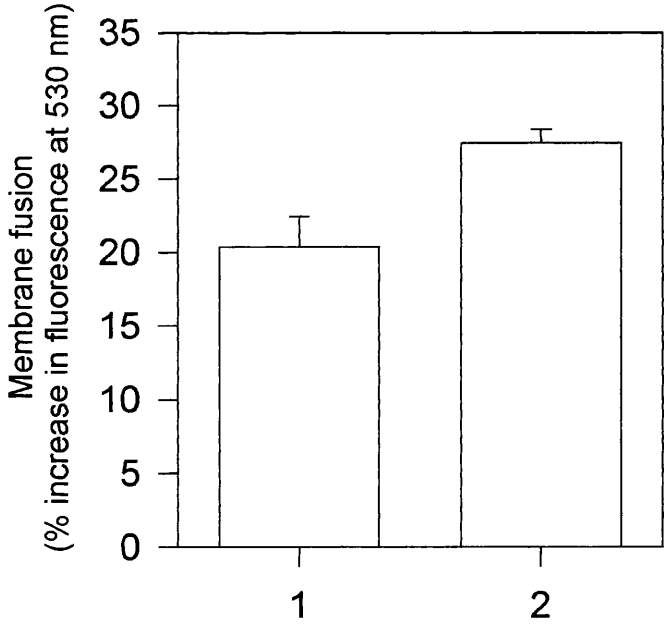
Ex. λ = 465 nm, Em. λ= 530 nm Temp. = 37^0C

Figure 4.3.7: Mixed HA virosome - liposome fusion, comparison between the membrane fusion efficency with Hc73 and H100 Fab' liposomes

a) Mixed HA virosome - liposome fusion at pH 5.9



b) Mixed HA virosome (pre-treated at pH 6) - liposome fusion at pH 5.2



1 = Hc73 Fab' liposomes
2 = H100 Fab' liposomes

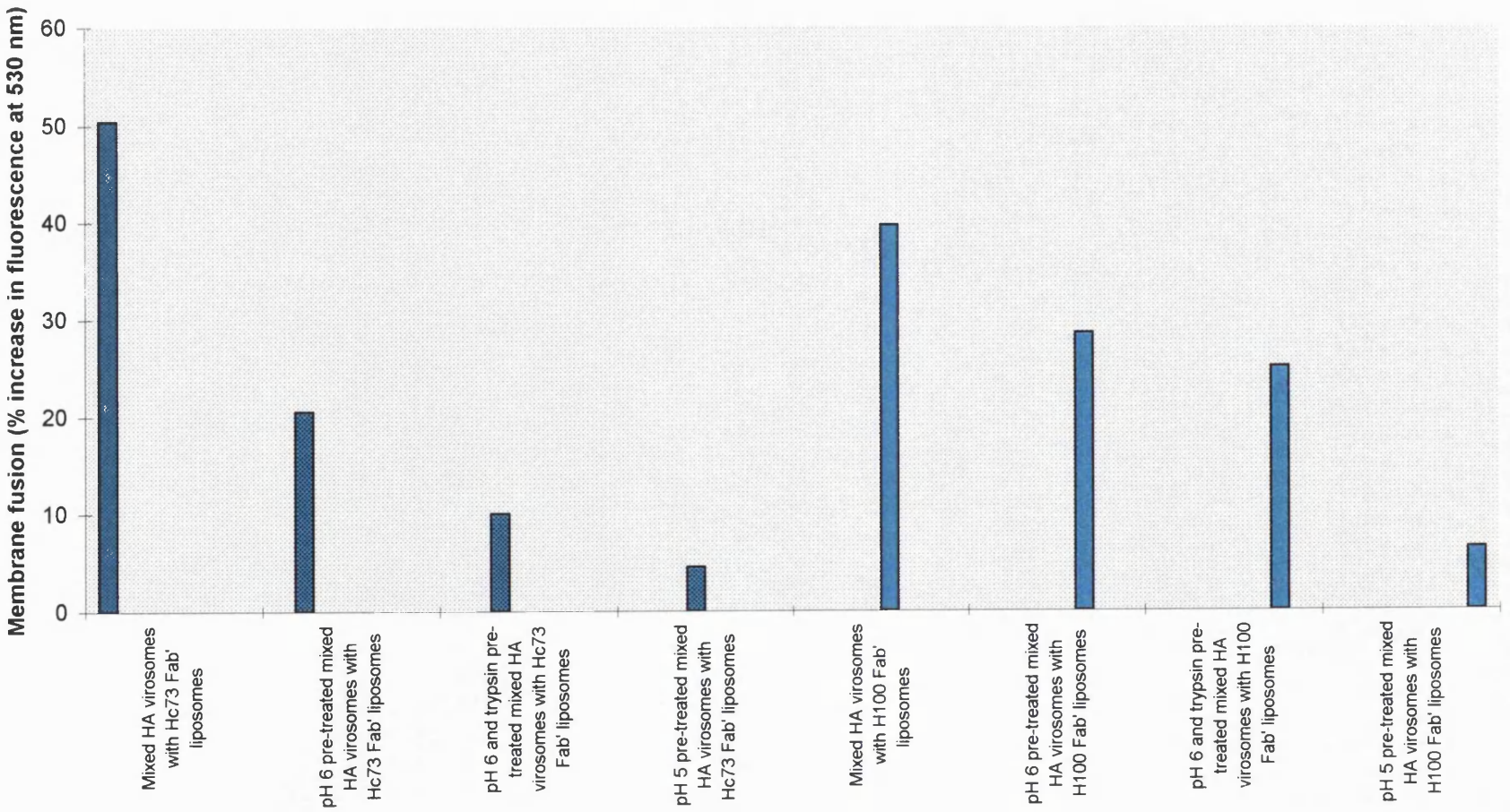
Figure 4.3.8

Virosomes were produced by Method A containing various strains of HA (Section 3.6.1). Virosomes were acid and trypsin treated as described in Sections 3.2.4. Virosomes were labelled with 0.6 mol% N-NBD-PE and N-Rho-PE (Section 3.2.1). Fab' coupled liposomes were produced and purified as described in Section 3.4.

40μl of labelled virosomes and a constant amount of Fab' liposomes were added to PBS prewarmed to 37^0C to produce a final volume of 1 ml. The R.E.T membrane fusion assay was done as described in Section 3.6.2. After incubation of the virosomes with the liposomes the pH was lowered to a pH 5.2 in all cases by injecting 0.15 M sodium citrate pH 3.5 into the cuvette and the percentage increase in fluorescence at 530 nm was calculated as described in Section 3.3.2 .

Ex. λ = 465 nm, Em. λ= 530 nm Temp. = 37^0C

Figure 4.3.8: Mixed HA virosome - Fab' liposome fusion, effect of acid and trypsin pre-treatment of virosomes (H100 / Hc73 Fab' liposome experiment)



$H17_1R$ HA directly bound by Hc73 Fab' contributed more strongly to the extent of pH 6 pre-treated mixed HA virosome - Hc73 Fab' liposome fusion than to the extent of pH 6 treated mixed HA virosome - H100 Fab' liposome fusion.

Comparing H100 Fab' liposomes and Hc73 Fab' liposomes, Hc73 Fab' liposomes were more efficient at fusing with mixed HA virosomes (Figure 4.3.7a) and less efficient at fusing with pH 6 pre-treated mixed HA virosomes (Figure 4.3.7b). It can therefore be concluded that the higher levels of liposome coupled Hc73 Fab' compared to H100 Fab' (Figure 4.3.3) were not solely responsible for the observed differences in fusion efficiency.

4.3.4 Fusion of mixed HA virosomes with liposomes coupled to anti X-31 HA Hc73 Fab' or anti JHB HA H8 Fab' fragments

Anti JHB HA H8 Fab' and H100 Fab' bind in a very similar position to JHB HA, but bind at a significantly different angle, 115^0 compared to 155^0 respectively (Sections 4.2.7 & 4.2.8 and Figure 4.3.16). The angle of H100 Fab' bound to BHA was such that at least two H100 Fab' fragments coupled to the same liposome bound to a single HA trimer and no liposome aggregation was observed at pH 7 (Section 4.2.7). Due to the angle of H8 Fab' binding to JHB HA and the possible lack of flexibility within the H8 Fab' fragment (Section 4.2.8), addition of JHB BHA to H8 Fab' liposomes resulted in liposome aggregation. This was explained by the fact it was possible for two Fab' fragments coupled to different liposomal membranes to bind to a single BHA trimer, resulting in liposomes being bound together. EM showed that at least two H8 Fab' fragments coupled to the same liposomal membrane could also bind to a single HA trimer, in a situation similar to that observed for the H100 Fab' receptor (Sections 4.2.7 & 4.2.8).

In the case of virosome - Fab' liposome fusion, due to the different binding angles of H8 Fab' and H100 Fab' to JHB HA it was likely that the liposome coupled H8 Fab' surrogate receptor resulted in a higher proportion of HA trimers being bound by a single H8 Fab' compared to H100 Fab' in which more of the HA trimers would be expected to be bound by at least two H100 Fab' receptors. As discussed further in Section 4.3.5 and illustrated in Figure 4.3.16 this obviously would have resulted in a different orientation of HA with regard to the target membrane. Steric considerations of virosome - liposome binding make it highly unlikely that two H8 Fab' fragments coupled to different liposomes could bind to a single HA trimer in a virosomal membrane, unlike the situation with BHA described above.

4.3.4.1 Determination of the relative amount of Fab' coupled to the liposome membrane

Electron microscopy of BHA bound to liposome preparations coupled either with H8 Fab' or Hc73 Fab' showed more BHA bound per H8 Fab' liposome compared to Hc73 Fab' liposomes, indicating that H8 Fab' liposomes contained more coupled Fab' receptor and SDS-PAGE of Fab' coupled liposomes loading on the gels equal amounts of liposomes showed that this was the case (Figure 4.3.9).

The situation therefore was that there were relatively more anti-JHB HA receptors than anti-X-31 HA receptors, the reverse of the situation in the H100 / Hc73 Fab' experiment (Section 4.3.3) where there was more Hc73 Fab' than H100 Fab' coupled per liposome.

as I remarked from the EM

Figure 4.3.9

Fab' liposomes were prepared as described in Section 3.4.

Electron microscopy

Electron microscopy was done as described in Section 3.1.9. BHA was prepared as described in Section 3.1.2. BHA was bound to Fab' liposomes and purified from unbound BHA as described in Section 3.5.1.

Show is X-31 or JHB BHA bound to Hc73 Fab' or H8 Fab' liposomes respectively

bar ≡ 100nm

It was observed that more BHA molecules were bound per liposome in the case of H8 Fab' liposomes compared to Hc73 Fab' liposomes.

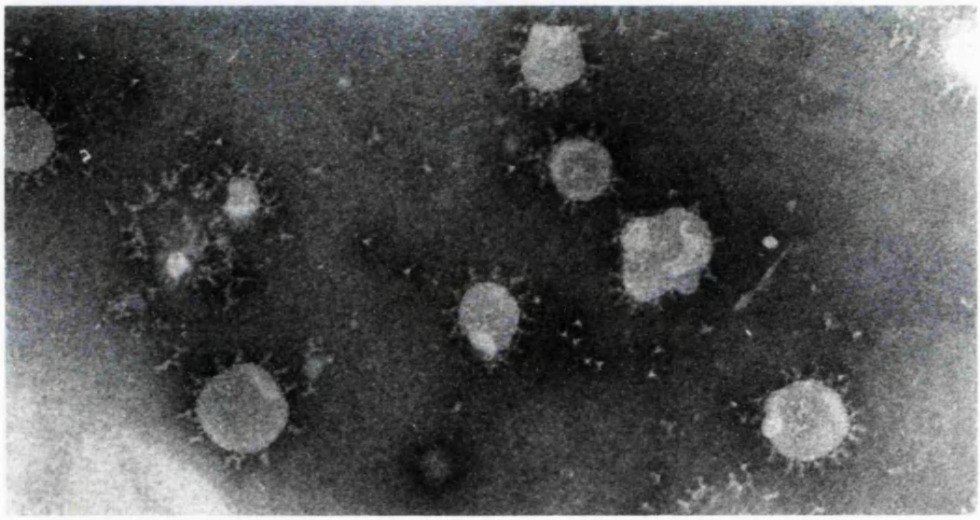
SDS PAGE of Fab' liposomes

The liposome concentration of the Hc73 Fab' and H8 Fab' liposomes preparations was equalized by quantifying the ^{3}H radioactivity (Section 3.6.2). SDS PAGE was done as described in Section 3.1.4, loading on the gel an equal amount of liposomes in each case. SDS PAGE showed that there was more H8 Fab' coupled per liposome compared to the Hc73 Fab' liposome preparation.

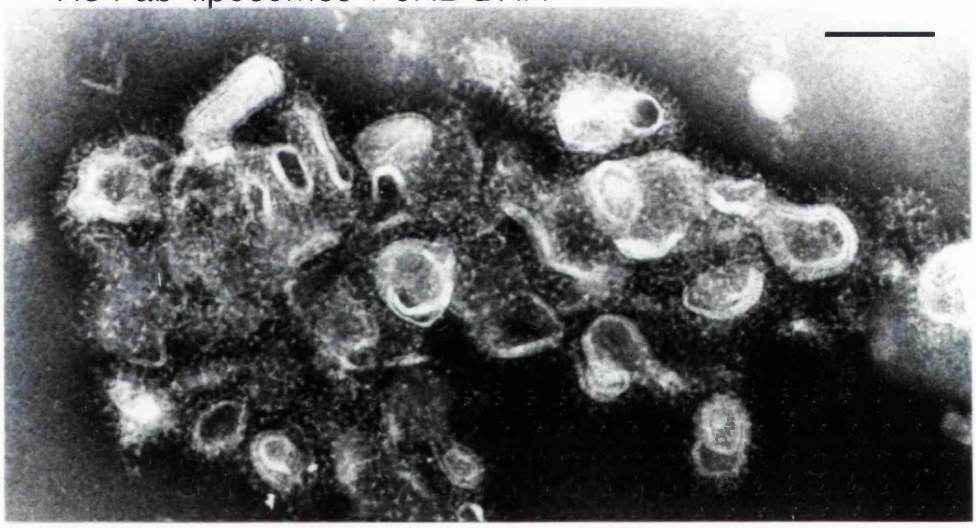
The results obtained by SDS PAGE and electron microscopy were consistent with each other.

Figure 4.3.9: Electron microscopy and SDS PAGE of the Hc73 Fab' and H8 Fab' liposome preparations: Determination of the relative amount of coupled Fab'

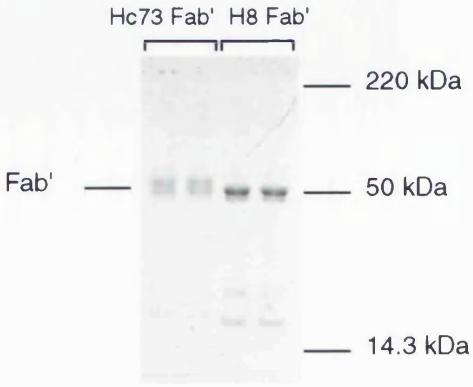
Hc73 Fab' liposomes + X-31 BHA



H8 Fab' liposomes + JHB BHA



SDS PAGE of Fab' liposomes



non reducing

4.3.4.2 The membrane fusion activity of mixed HA virosomes when bound to liposome coupled Hc73 Fab' or H8 Fab' fragments

The results shown in Figure 4.3.10 repeat those discussed in Section 4.3.3.2 showing that liposome coupled anti-HA Fab' receptors increased the efficiency of virosome - liposome fusion.

The pH profile of fusion of mixed HA virosomes with Hc73 or H8 Fab' liposomes again resulted in different pH profiles. The pH profile with Hc73 Fab' liposomes was similar to the pH profile of X-31 $H17_1R$ HA virosome - Hc73 Fab' liposome fusion (Figure 4.3.11). The pH profile of mixed HA virosome - H8 Fab' liposome fusion was less similar to the pH profile of X-31 $H17_1R$ HA virosome - Hc73 Fab' liposome fusion (Figure 4.3.11). NO - for opposite 160. the extent is less - profile the same!

Fusion of mixed HA virosomes at pH 5.9 was determined to be X-31 $H17_1R$ HA mediated as discussed in Section 4.3.3.3. This was due to the relatively low fusion activity of JHB HA at pH 5.9 compared to X-31 $H17_1R$ HA (Figure 4.3.11) and the rate of fusion of mixed HA virosome at pH 5.9 (Figure 4.3.12).

At pH 5.9 the efficiency of membrane fusion induced by mixed HA virosomes was significantly higher with Hc73 Fab' liposomes than with H8 Fab' liposomes (Figure 4.3.13a). An "uneven" mixed HA virosome preparation containing more JHB HA than X-31 $H17_1R$ HA (Figure 3.6.1) also fused more efficiently with Hc73 Fab' liposomes compared to H8 Fab' liposomes (33% compared to 23% increase in fluorescence at 530 nm respectively - fusion pH = 5.9). This indicated that X-31 $H17_1R$ HA directly bound to Hc73 Fab' was more efficient at inducing membrane fusion compared to the situation where unbound X-31 $H17_1R$ HA was held close to the liposomal membrane via JHB HA - H8 Fab' interactions. These results were

Figure 4.3.10

Virosomes were produced by Method A containing various strains of HA (Section 3.6.1). Virosomes were acid treated as described in Sections 3.2.4. Virosomes were labelled with 0.6 mol% N-NBD-PE and N-Rho-PE (Section 3.2.1). Fab' coupled liposomes were produced and purified as described in Section 3.4.

40μl of labelled virosomes and a constant amount of Fab' liposomes were added to PBS prewarmed to 37^0C to produce a final volume of 1 ml. The R.E.T membrane fusion assay was done as described in Section 3.6.2. After incubation of the virosomes with the liposomes the pH was lowered to a specified pH by injecting 0.15 M sodium citrate pH 3.5 into the cuvette and the percentage increase in fluorescence at 530 nm was calculated as described in Section 3.3.2 .

Ex. λ = 465 nm, Em. λ= 530 nm Temp. = 37^0C

Figure 4.3.10: Virosome - liposome fusion, effect of using JHB or X-31 H17$_1$R HA and anti-X-31 Hc73 or anti-JHB H8 Fab' receptors

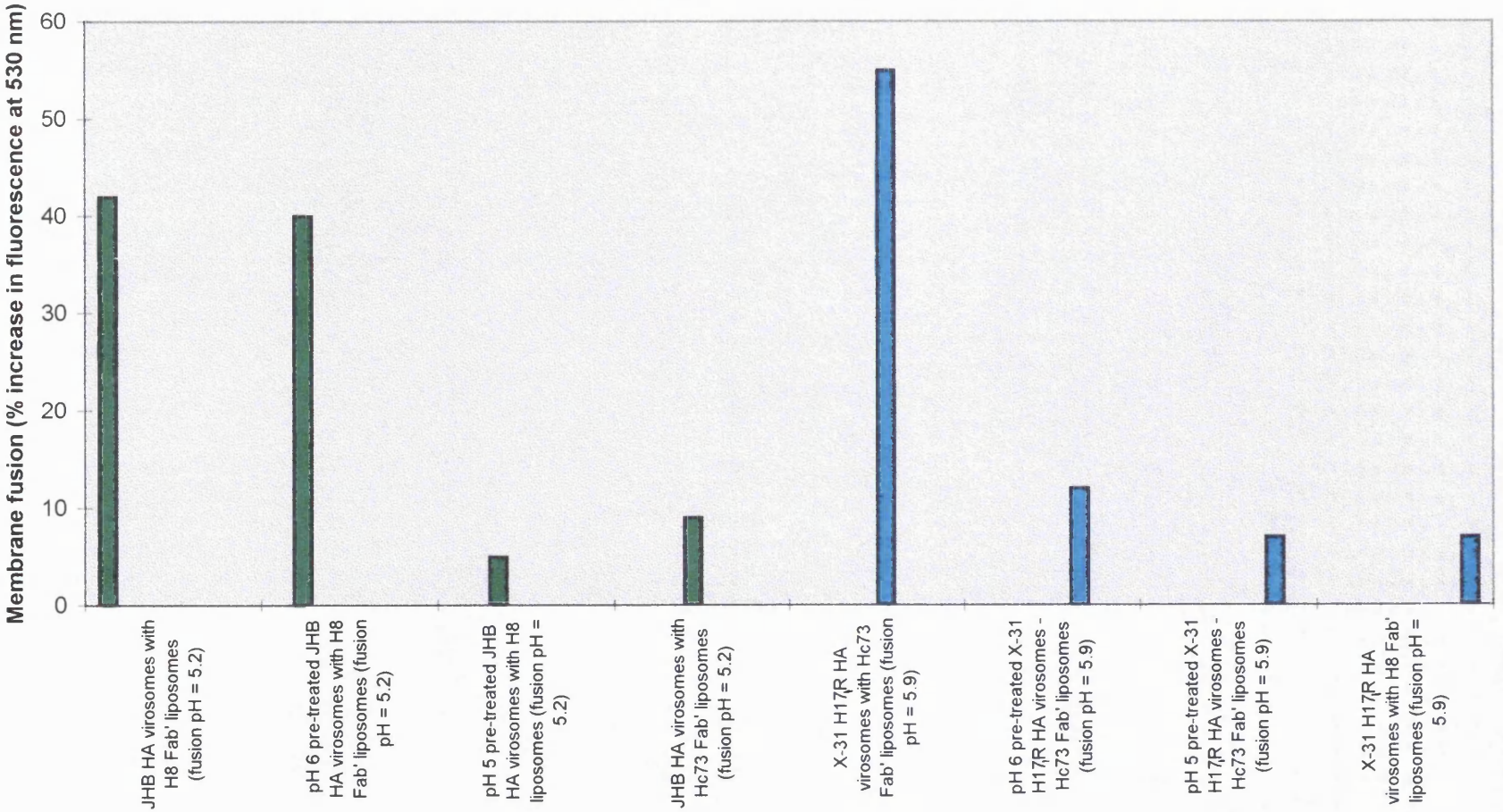


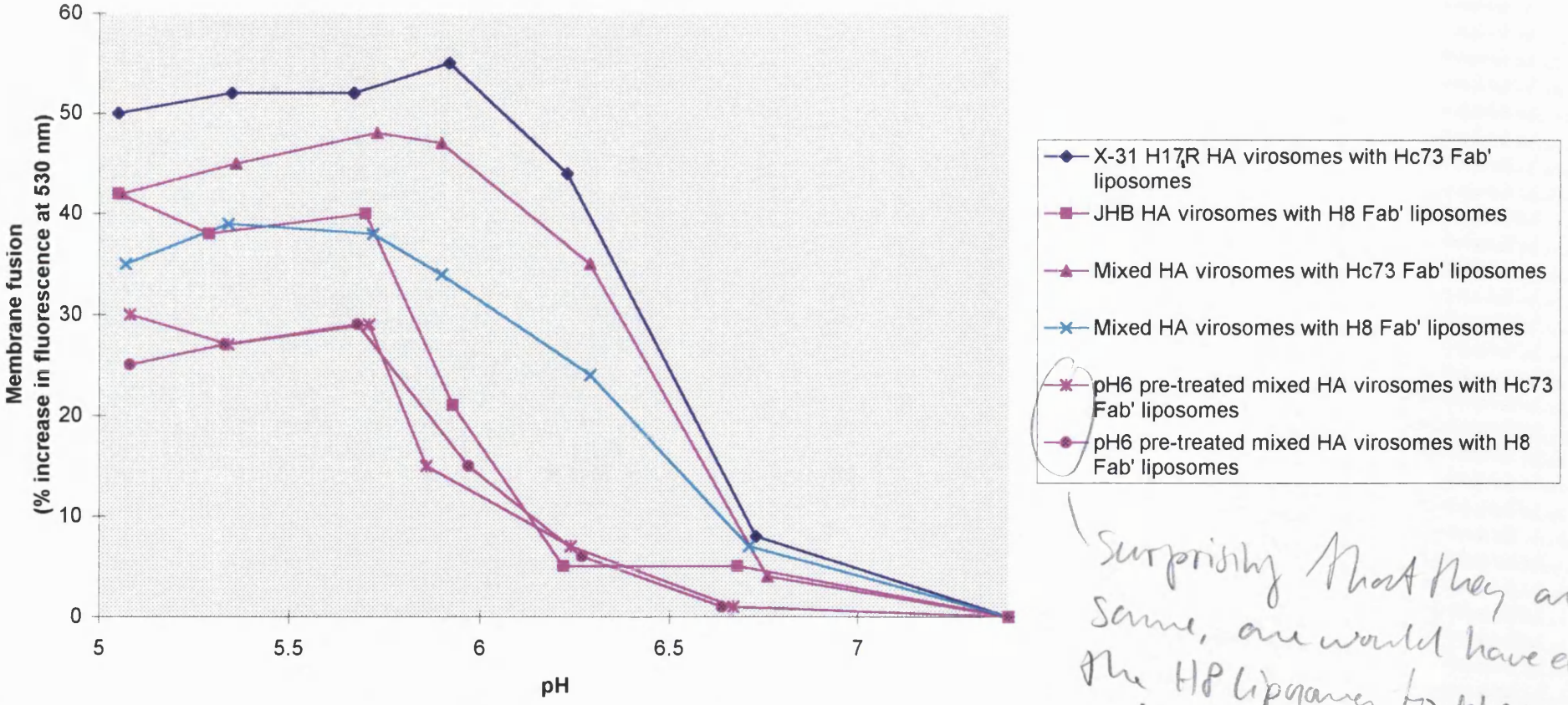
Figure 4.3.11

Virosomes were produced by Method A containing various strains of HA (Section 3.6.1). Virosomes were labelled with 0.6 mol% N-NBD-PE and N-Rho-PE (Section 3.2.1). Fab' coupled liposomes were produced and purified as described in Section 3.4.

40μl of labelled virosomes and a constant amount of Fab' liposomes were added to PBS prewarmed to 37^0C to produce a final volume of 1 ml. The R.E.T membrane fusion assay was done as described in Section 3.6.2. After incubation of the virosomes with the liposomes the pH was lowered to a specified pH by injecting 0.15 M sodium citrate pH 3.5 into the cuvette and the percentage increase in fluorescence at 530 nm was calculated as described in Section 3.3.2 .

Ex. λ = 465 nm, Em. λ= 530 nm Temp. = 37^0C

Figure 4.3.11: pH profile of virosome - liposome fusion, comparison between Hc73 and H8 Fab' liposomes



Surprisingly that they are the same, one would have expected the H8 liposomes to give more activity

Figure 4.3.12

Virosomes were produced by Method A containing various strains of HA (Section 3.6.1). Virosomes were labelled with 0.6 mol% N-NBD-PE and N-Rho-PE (Section 3.2.1). Fab' coupled liposomes were produced and purified as described in Section 3.4.

40μl of labelled virosomes and a constant amount of Fab' liposomes were added to PBS prewarmed to 37^0C to produce a final volume of 1 ml. The R.E.T membrane fusion assay was done as described in Section 3.6.2.

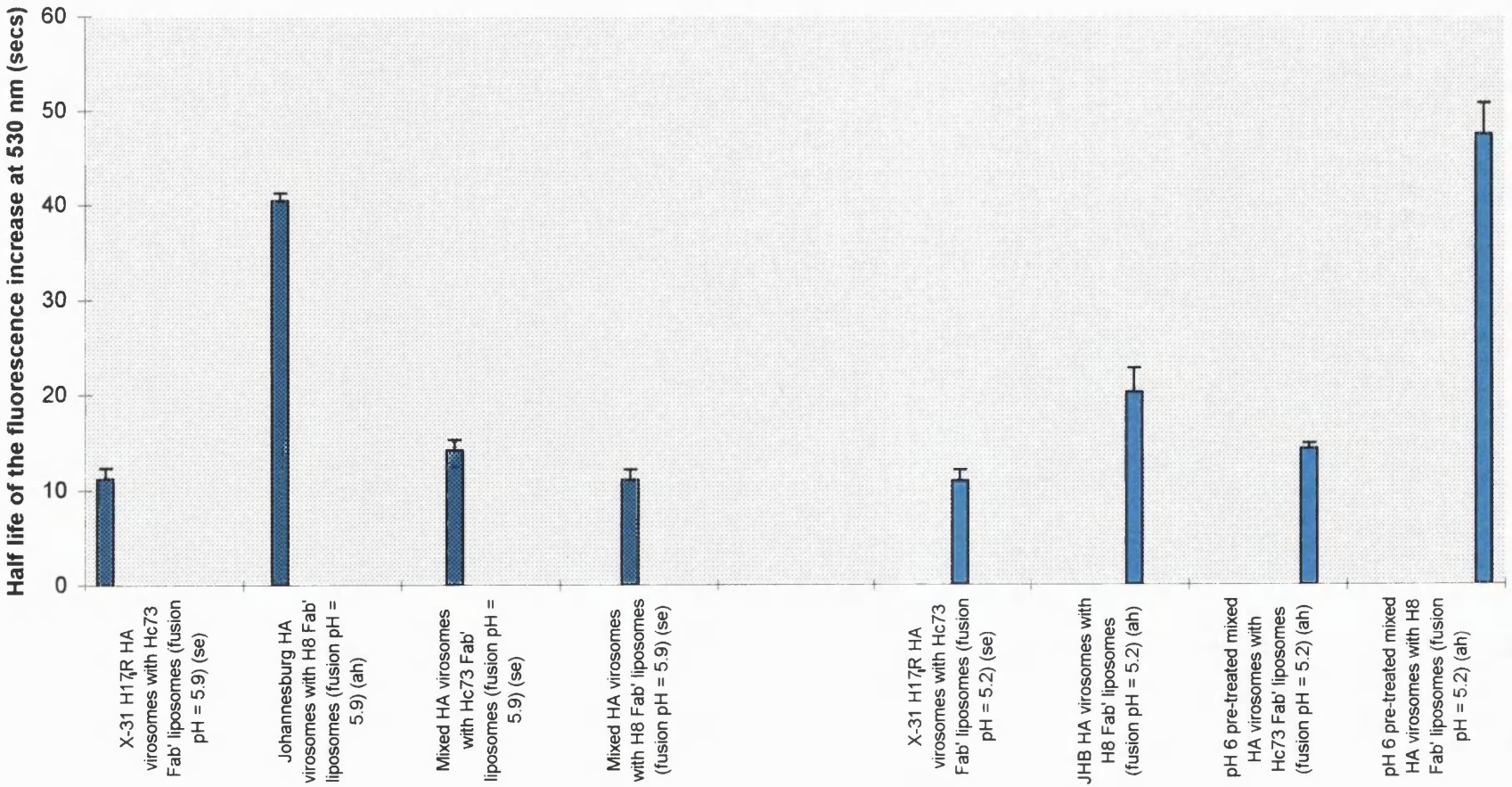
The rate of increase in fluorescence at 530 nm was analysed as described in Section 3.6.2. The time taken for the fluorescence at 530 nm to increase to half its final value is referred to as the "half life"($t_{1/2}$). Each assay was repeated at least 4 times. The mean half life is shown, the error bars show the standard deviation.

"se" indicates that the fusion kinetics were single exponential.

"ah" indicates that the fusion kinetics were more complex and did not fit to a single exponential, in which case an apparent $t_{1/2}$ was calculated.

Ex. λ = 465 nm, Em. λ= 530 nm Temp. = 37^0C

Figure 4.3.12: The rate of virosome - Fab' liposome membrane fusion, differences between X-31 $H17_1R$ and JHB HA (H8 / Hc73 Fab' liposome experiment)



observed despite there being more H8 Fab' coupled per liposome compared to Hc73 Fab' (Figure 4.3.9) which probably gave rise to more virosome - H8 Fab' liposome binding interactions compared to virosome - Hc73 Fab' liposomes, especially in the experiments using the "uneven" mixed HA virosome preparation.

4.3.4.3 The membrane fusion activity of pH 6 pre-treated mixed HA virosomes when bound to liposome coupled Hc73 or H8 Fab' fragments

Pre-treatment of mixed HA virosomes at pH 6 again resulted in the irreversible low pH conformational change of the majority of X-31 $H17_1R$ HA (Figures 4.3.2 & 4.3.10). In Section 4.3.3.4 it was concluded that the vast majority of the fusion activity of pH 6 pre-treated mixed HA virosomes was due to JHB HA. The pH profile of fusion of pH 6 pre-treated mixed HA virosomes with H8 Fab' and Hc73 Fab' liposomes (Figure 4.3.11) was consistent with this interpretation.

The rate of fusion of pH 6 pre-treated mixed HA virosomes was again investigated in an attempt to gain further information concerning the HA strain responsible for the fusion activity. The rates of fusion of pH 6 pre-treated mixed HA virosomes with H8 and Hc73 Fab' liposomes (Figure 4.3.12) were similar to those observed for the H100 / Hc73 Fab' fusion experiment (Section 4.3.3.4). The only significant difference was that the rate of fusion of pH 6 pre-treated mixed HA virosomes with H8 Fab' liposomes was significantly lower than with H100 Fab' liposomes (Figures 4.3.6 & 4.3.12). This could possibly be due to an effect of the difference in orientation of JHB HA when bound to either H100 Fab' or H8 Fab'.

Trypsin treatment of pH 6 pre-treated mixed HA virosomes resulted in a significant drop in the amount of fusion observed with Hc73 Fab' liposomes compared to non-trypsin treated pH 6 pre-treated mixed HA virosomes (Figure

4.3.14). Trypsin treatment had resulted in the removal of low pH X-31 $H17_1R$ HA_1 28-328, resulting in decreased binding between Hc73 Fab' liposomes and virosomes and a subsequent decrease in fusion efficiency. The amount of fusion between H8 Fab' liposomes and trypsin treated, pH 6 pre-treated mixed HA virosomes was only slightly decreased compared to the amount of fusion with pH 6 pre-treated mixed HA virosomes (Figure 4.3.14), indicating that only a small proportion of JHB HA in pH 6 pre-treated mixed HA virosomes was in the low pH conformation. These results were essentially the same as previously obtained in the Hc73 / H100 Fab' experiment (Figure 4.3.8).

The fusion activity of pH 6 pre-treated mixed HA virosomes can be attributed to the activity of JHB HA. At pH 5.2 the efficiency of membrane fusion induced by pH 6 pre-treated mixed HA virosomes was not significantly different with H8 Fab' liposomes or Hc73 Fab' liposomes (Figure 4.3.13b). Due to the relatively higher amount of H8 Fab' per liposome compared to Hc73 Fab' liposomes and the fact that the majority of fusion of pH 6 treated mixed HA virosome is JHB HA mediated, any increase in fusion efficiency due to direct binding of JHB HA to H8 Fab' would have been detected. The observed result indicates that JHB HA - H8 Fab' and low pH pre-treated X-31 $H17_1R$ HA - Hc73 Fab' receptor complexes are equally effective at increasing the fusion efficiency of pH 6 pre-treated mixed HA virosomes. This is in contrast to the result with Hc73 and H100 Fab' receptors which concluded that direct receptor binding of JHB HA to H100 Fab' receptor was more effective at increasing the fusion efficiency of the HA.

Figure 4.3.15 summarises the virosome - Fab' liposome fusion experiments presented.

Figure 4.3.13

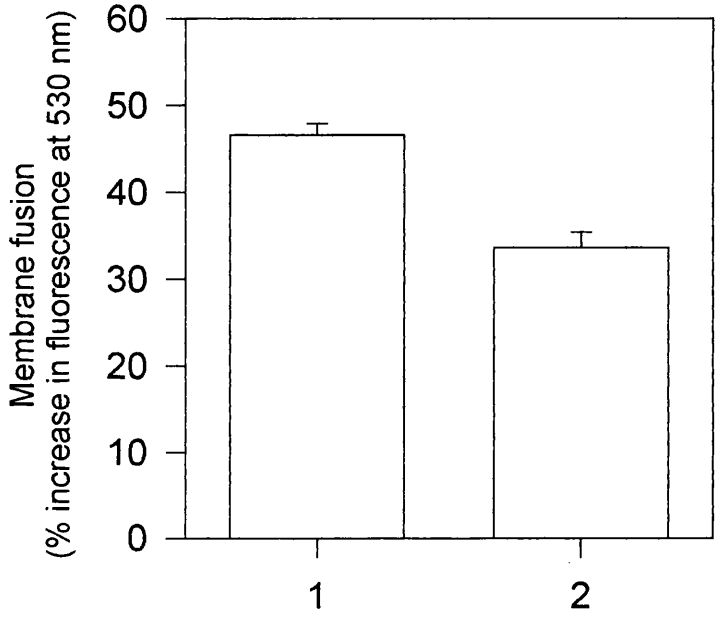
Virosomes were produced by Method A which contained equal amounts of X-31 $H17_1R$ HA and JHB HA (Section 3.6.1). Virosomes were labelled with 0.6 mol% N-NBD-PE and N-Rho-PE (Section 3.2.1). Fab' coupled liposomes were produced and purified as described in Section 3.4.

40μl of labelled virosomes and a constant amount of Fab' liposomes were added to PBS prewarmed to 37^0C to produce a final volume of 1 ml. The R.E.T membrane fusion assay was done as described in Section 3.6.2. Each assay was repeated at least 5 times. The mean increase in fluorescence is shown, the error bars show the standard deviation.

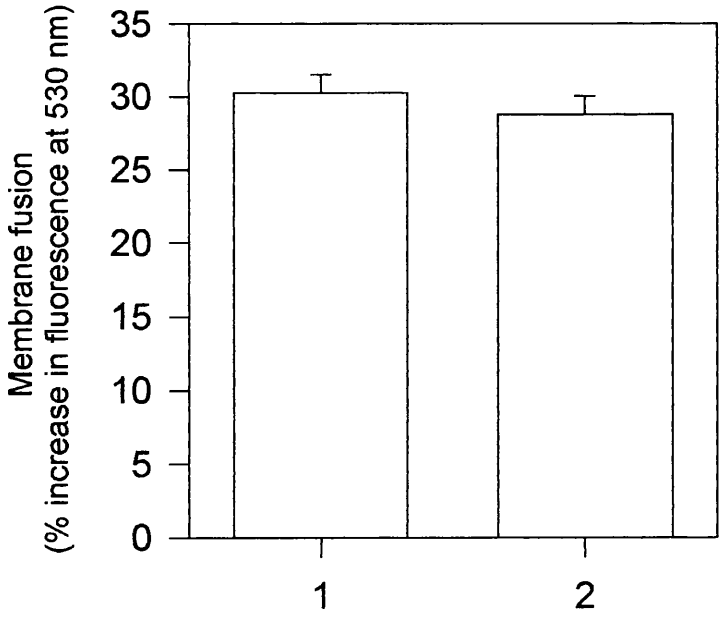
Ex. λ = 465 nm, Em. λ= 530 nm Temp. = 37^0C

Figure 4.3.13: Mixed HA virosome - liposome fusion, comparison between the membrane fusion efficency with Hc73 and H8 Fab' liposomes

a) Mixed HA virosome - liposome fusion at pH 5.9



b) Mixed HA virosome (pre-treated at pH 6) - liposome fusion at pH 5.2



1 = Hc73 Fab' liposomes
2 = H8 Fab' liposomes

Figure 4.3.14

Virosomes were produced by Method A containing various strains of HA (Section 3.6.1). Virosomes were acid and trypsin treated as described in Sections 3.2.4. Virosomes were labelled with 0.6 mol% N-NBD-PE and N-Rho-PE (Section 3.2.1). Fab' coupled liposomes were produced and purified as described in Section 3.4.

40µl of labelled virosomes and a constant amount of Fab' liposomes were added to PBS prewarmed to 37^0C to produce a final volume of 1 ml. The R.E.T membrane fusion assay was done as described in Section 3.6.2. After incubation of the virosomes with the liposomes the pH was lowered to a pH 5.2 in all cases by injecting 0.15 M sodium citrate pH 3.5 into the cuvette and the percentage increase in fluorescence at 530 nm was calculated as described in Section 3.3.2 .

Ex. λ = 465 nm, Em. λ= 530 nm Temp. = 37^0C

Figure 4.3.14: Mixed HA virosome - Fab' liposome fusion, effect of acid and trypsin pre-treatment of virosomes (H8 / Hc73 Fab' liposome experiment)

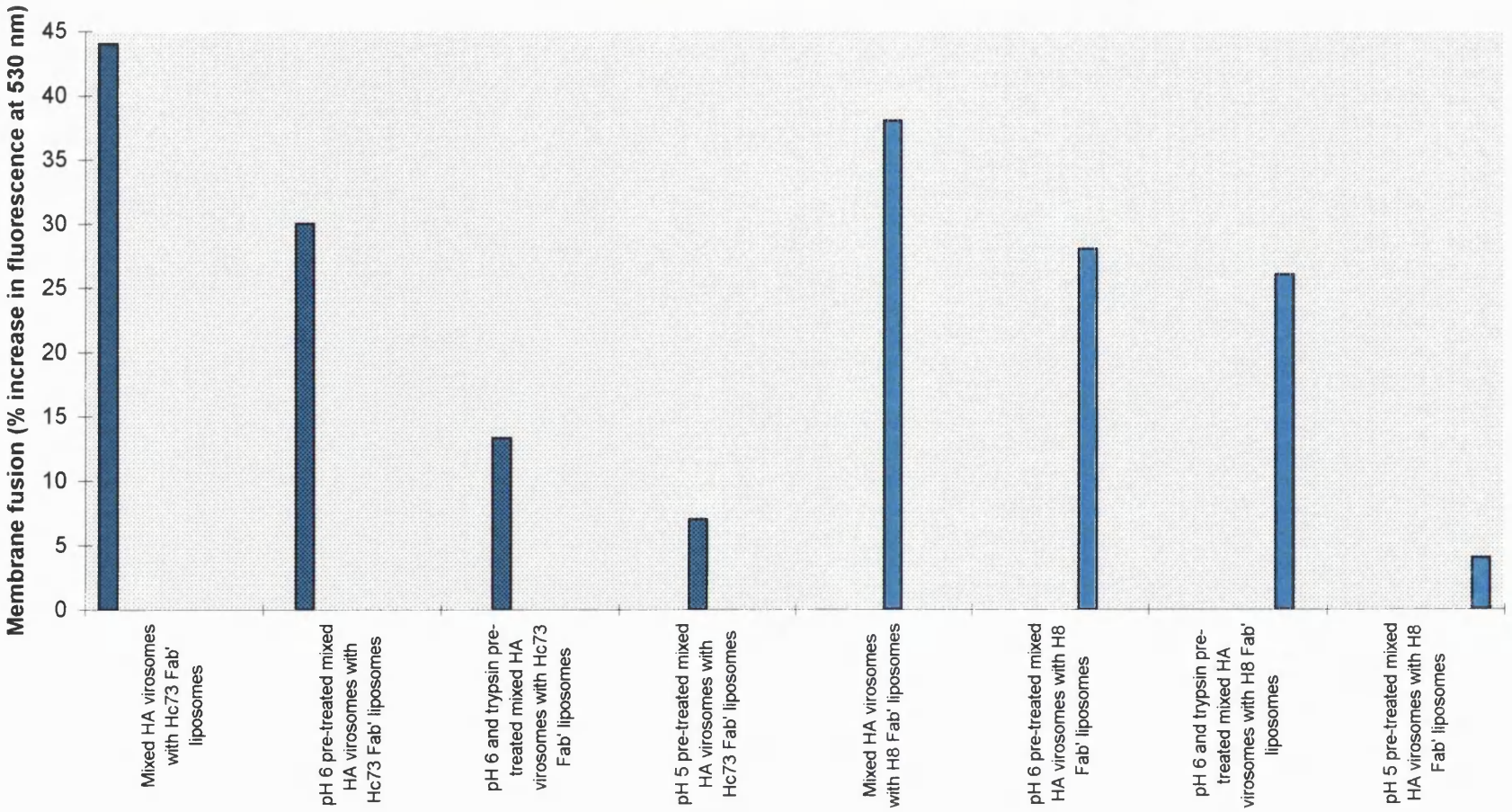
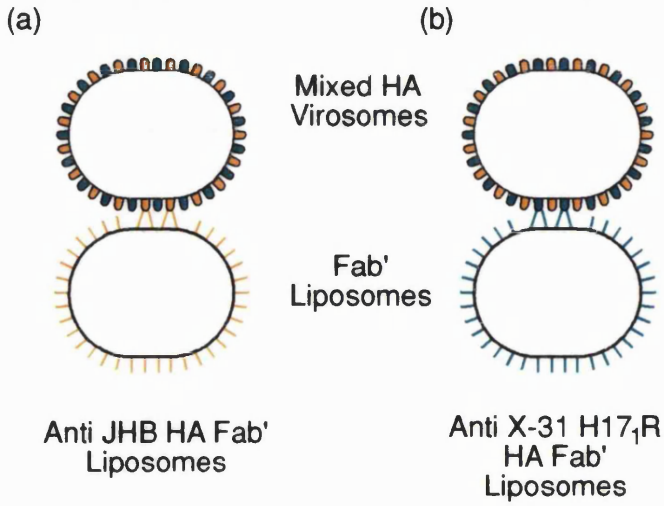
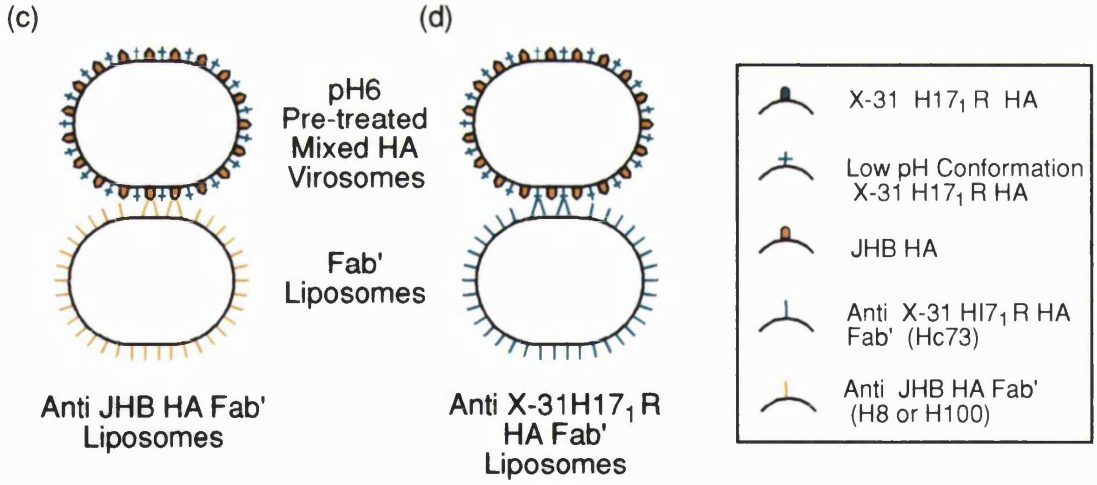


Figure 4.3.15 Summary of the Virosome - Fab' Liposome Fusion Experiments.



Fusion of mixed HA virosomes at pH5.9 was X-31 H17$_1$R HA mediated. Significantly more fusion was observed at pH5.9 in (b) when X-31 H17$_1$R HA was directly bound to Hc73 Fab' rather than (a) when X-31 H17$_1$R HA was held close to the liposomal membrane via H8 or H100 Fab' - JHB HA binding interactions.



Fusion of pH6 pre-treated mixed HA virosomes at pH5.2 was JHB HA mediated. Significantly more fusion was observed at pH5.2 in (c) when JHB HA was directly bound to H100 Fab' rather than (d) when JHB HA was held close to the liposomal membrane via Hc73 Fab' - X-31 H17$_1$R HA binding interactions. However, there was no significant difference in the fusion efficiency when JHB HA was bound directly to H8 Fab' ((c)) compared to when JHB HA was held close to the liposomal membrane by Hc73 Fab'-X-31 H17$_1$R HA binding interactions ((d)).

4.3.5 Discussion

The results presented show that liposome coupled anti-HA Fab' receptors significantly increase the extent of virosome - liposome fusion. HA unbound by receptor but held close to the liposomal membrane via low pH HA - Fab' binding was shown to induce significant levels of fusion. These findings were in agreement with Schoen *et al.* (1996) in which HA not directly bound by receptor, but held close to the target membrane via steptavidin/biotin interactions induced membrane fusion. Using the mixed HA virosomes it was possible to compare the relative fusion efficiency of HA directly bound by receptor and unbound HA held close to the target membrane by other HA - Fab' interactions.

The experiments described have shown that HA directly bound to Hc73 Fab' or H100 Fab' surrogate receptors fuse more efficiently compared to the situation where unbound HA is held close to the target membrane by other Fab' - HA interactions. It is proposed that following exposure to low pH, HA bound to Hc73 Fab' or H100 Fab' receptors inserted their "fusion peptides" into the target membrane more efficiently than HA held close to the target membrane by other Fab' - HA interactions. This interpretation is in general agreement with other reports (Pedroso de Lima *et al.*, 1995; Stegmann *et al.*, 1995) which suggested that sialic acid binding facilitates correct insertion of the HA "fusion peptide" into the target membrane.

The results presented in this thesis suggest that both HAs bound and unbound to receptor are able to induce significant membrane fusion. These results are contrary to the report of Ellens *et al.* (1990) which was described in Section 2.4.7. Two HA expressing cell lines were shown to have equal binding constants for glycophorin containing liposomes, an increase of 1.9 fold in the HA surface density of one cell line

(HAb-2) compared to another cell line (GP4F) resulted in 4.4 times more fusion per bound liposome. Ellens *et al.* (1990) assumed that HAb-2 cells should have fused with the same fraction of bound liposomes as GP4F cells if receptor bound HAs were involved in the fusion process. This led to the conclusion that the binding and fusion functions of HA were not performed by the same trimer. The assumption of Ellens *et al.* (1990) that HAb-2 cells should have fused with the same fraction of bound liposomes as GP4F cells if receptor bound HAs were involved in the fusion process did not account for the possibility that both HA bound and unbound to receptor may be able to mediate membrane fusion. In this case, as was observed by Ellens *et al.* (1990) HAb-2 cells and GP4F cells would not be expected to fuse with the same fraction of bound liposomes. It is therefore suggested that the analysis of the observed data by Ellens *et al.* (1990) and not the experimental results themselves are contrary to the results presented in this thesis. In addition, the approach of Ellens *et al.* (1990) of measuring the efficiency of HA membrane fusion by delivery of liposomally encapsulated material to the cell cytoplasm may have been prone to large errors due to the indirect nature of the experimental design.

Alford *et al.* (1994) showed that high ganglioside GD1a concentrations resulting in a higher fraction of HAs bound to sialic acid residues led to a decrease in the extent of virus - liposome fusion and suggested that bound HAs do not participate in fusion. One problem with these experiments was that changing the surface density of the ganglioside receptor unavoidably leads to a change in lipid composition of the liposome which may have resulted in a composition with properties that made membrane fusion with virus less favourable. It must also be considered that the reports of Ellens *et al.* (1990) and Alford *et al.* (1994) used sialic acid containing HA

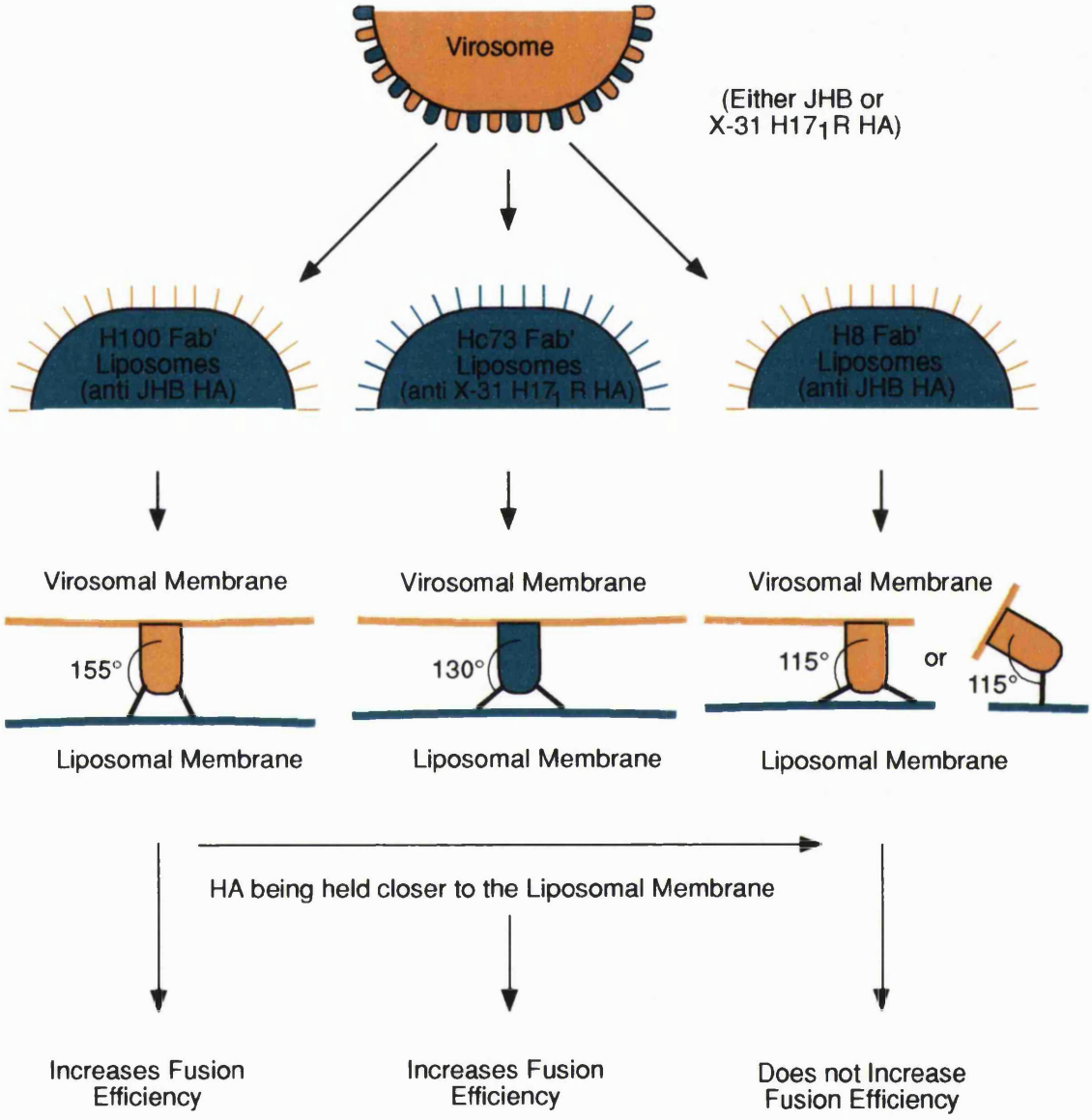
receptors, whereas the results presented here used liposome coupled anti HA Fab' fragments as surrogate receptors for HA to investigated the role of receptor binding in HA mediated fusion.

As already discussed JHB HA bound to H100 Fab' receptor was more efficient at inducing membrane fusion than JHB HA held close to the target membrane by other HA - Fab' interactions. However, JHB HA bound to H8 Fab' receptor was no more efficient at inducing membrane fusion than HA held close to the target membrane by other HA - Fab' interactions. As discussed in Sections 4.2.7 & 4.2.8 JHB BHA bound to H100 Fab' or H8 Fab' receptor resulted in HA being held in a different orientation with respect to the target membrane. Figure 4.3.16 shows an interpretation of how the orientation of virosomal JHB HA differed with respect to the liposomal membrane when bound to H100 Fab' or H8 Fab' receptor. It is proposed that the orientation of JHB HA with respect to the target membrane when bound by H100 Fab' receptors resulted in more efficient insertion of "fusion peptides" into the target membrane compared to the orientation of JHB HA when bound to H8 Fab' receptors.

The finding that HA bound in certain orientations is more efficient at inducing membrane fusion than unbound HA held close to the membrane has implications for the design of a HA based delivery vector. A HA molecule containing a specific binding domain within HA_1 has the potential to be more efficient at inducing membrane fusion and hence delivery compared to a delivery vector in which HA functions only to induce membrane fusion and the binding specificity is provided by other molecules.

Results using H8 Fab' as a surrogate receptor suggest that the orientation of bound HA with respect to the target membrane effects the efficiency of insertion of

Figure 4.3.16. Virosome-Fab' Liposome Fusion: An Interpretation of the Orientation of HA with respect to the Target Membrane when Bound to Liposome Coupled Hc73 Fab', H100 Fab' or H8 Fab' Surrogate Receptors.



the HA "fusion peptide" into the target membrane. This finding has implications for the design of a HA molecule containing a specific binding domain within HA_1. By altering the position of insertion of a ligand binding domain within HA_1 it may be possible to produce a HA molecule that when bound to the target membrane via its specific cell receptor will be held in an orientation similar to that observed for HA bound to H100 or Hc73 Fab' receptor, thus enabling efficient insertion of the "fusion peptide" into the target membrane during the acid induced conformational change of HA.

5 DISCUSSION SUMMARY

My thesis has been divided into two parts. Studies concerning the role of receptor binding in HA mediated membrane fusion and secondly, work aimed at developing a specific and efficient HA based delivery vector.

5.1 THE ROLE OF RECEPTOR BINDING BY HAEMAGGLUTININ (HA) IN HA MEDIATED MEMBRANE FUSION

The basic understanding of the role of HA in low pH induced HA mediated membrane fusion has come from structural analysis of native HA (Wilson *et al.*, 1981; Weis *et al.*, 1990; Watowich *et al.*, 1994), together with characterisation of the conformational changes in HA required for membrane fusion (Godley *et al.*, 1992; Bullough *et al.*, 1994a), which are thought to result in the insertion of "fusion peptide" into the target membrane (Stegmann *et al.*, 1991; Tsurudome *et al.*, 1992; Durrer *et al.*, 1996). It has also been shown that the "fusion peptide" of HA is essential for membrane fusion activity (Steinhauer *et al.*, 1995). Little is known concerning the mechanism by which "fusion peptide" insertion into the endosomal membrane leads to fusion between the viral and endosomal membranes.

The gp120/gp41 envelope glycoprotein of HIV-1 induces membrane fusion between the viral and plasma membranes at neutral pH (Stein *et al.*, 1987; McClure *et al.*, 1988). The precursor, gp160 is post-translationally cleaved into gp41 and gp120 which remain non-covalently associated (Allan *et al.*, 1985; Veronese *et al.*, 1985). gp41 is a transmembrane protein and in a situation analogous to HA_2 contains a hydrophobic "fusion peptide" at is N-terminus (Gallaher, 1987; Kowalski *et al.*, 1987; Bosch *et al.*, 1989). Binding by gp120 to its receptors result in a conformational

change in gp120, increased exposure of gp41 as judged by the availability of a gp41 epitope for antibody binding and ultimately dissociation of gp120 from gp41 (Kirsh *et al.*, 1990; Moore *et al.*, 1990; Hart *et al.*, 1991). It has been suggested that receptor binding by gp120 and the subsequent changes in its conformation enables gp41 to undergo a conformational change which results in exposure of its "fusion peptide" and subsequent membrane fusion (Chen *et al.*, 1995). The partial structure of gp41 (Chan *et al.*, 1997; Weissenhorn *et al.*, 1997) in the fusion conformation shows striking similarities to the low pH structure of HA_2, leading to the proposal that insertion of "fusion peptide" into the target membrane by influenza HA_2 and HIV-1 gp41 occurs by a common mechanism, whereas the triggers that induce the conformational changes in each case are different.

Sendai virus induces membrane fusion between the viral and plasma membranes at neutral pH and contains two envelope glycoproteins, haemagglutinin-neuraminidase (HN) and the fusion (F) protein (Scheid & Choppin, 1974). In a situation analogous to influenza HA the F protein is synthesized as an uncleaved, inactive form (F_0) and is cleaved into F_1 and F_2, which results in a hydrophobic "fusion peptide" at the N-terminus of F_1 (Scheid & Choppin, 1977). F protein contains two heptad repeats, one adjacent to the "fusion peptide" and another adjacent to the transmembrane anchoring domain (Chambers *et al.*, 1992). Heptad repeats are able to form triple stranded coiled coils (Pauling & Corey, 1953), mutations in both domains were shown to interfere with the fusogenic potential of the F protein (Buckland *et al.*, 1992; Sergel-Germano *et al.*, 1994). By analogy to the low pH induced conformational change of influenza HA it has been proposed that membrane fusion activity of sendai virus may result from the heptad repeats present in the F

protein being triggered to change conformation resulting in exposure of "fusion peptide" (Lamb, 1993; Rapaport *et al.*, 1995).

The other envelope glycoprotein of sendai virus, HN, binds to sialic acid residues and possesses neuraminidase activity (Morrison & Portner, 1991). Influenza A virus also has two envelope glycoproteins, one glycoprotein has neuraminidase activity (NA) while HA exhibits both receptor binding and fusion activities (Wiley & Skehel, 1987), with sendai virus the receptor binding / neuraminidase and fusion activities are present on different glycoproteins (Lamb, 1993). It has been reported that the fusion activity of some paramyxoviruses requires expression of both glycoproteins (Tanabayashi *et al.*, 1992; Heminway *et al.*, 1994), it has therefore been proposed that binding of HN to its receptor causes a conformational change that in turn triggers the conformational change in the F protein leading to exposure of the "fusion peptide" (Lamb, 1993). Consistent with this suggestion, identification of a domain of sendai virus HN which is thought to interact with the F protein has been reported (Tanabayashi & Compans, 1996). It has been reported that for the some paramyxoviruses, including simian virus 5 (SV5) that the F protein exhibits fusion activity in the absence of HN (Patterson *et al.*, 1985), in which case it has been suggested that a conformational change could be triggered in the F protein by contact of the F protein with a target membrane or after docking with an unidentified receptor located on the target membrane (Lamb, 1993).

As described above membrane fusion induced by HIV-1 is mediated by gp120/gp41. Membrane fusion is triggered by receptor binding by gp120 which leads to exposure of the "fusion peptide" of gp41, these observations indicate receptor binding and fusion in HIV-1 mediated fusion are directly linked, although it is

unknown whether gp120 is dissociated from gp41 during the fusion process. For paramyxoviruses, at least in some cases it is likely that membrane fusion is triggered by HN binding to cellular receptors which subsequently triggers the F protein to undergo a conformational change leading to exposure of "fusion peptide". This indicates that receptor binding and fusion of at least some paramyxoviruses are directly linked, although the receptor binding and fusion activities of paramyxoviruses are contained on separate glycoproteins.

The role of receptor binding in influenza HA mediated membrane fusion is unknown, it has previously been proposed that the binding and fusion functions of HA are not performed by the same trimer, suggesting HA unbound by receptor induces membrane fusion while HA bound by receptor does not participate in the fusion process (Ellens *et al.*, 1990; Alford *et al.*, 1994) (also see Sections 2.3.7.2 & 4.3.5).

The results reported in this thesis suggest the alternative, suggesting that both HA bound or unbound by liposome coupled Fab' surrogate receptors induced significant membrane fusion. When HA was bound by certain liposome coupled Fab' receptors (anti JHB HA H100 Fab' and anti X-31 HA Hc73 Fab') the fusion efficiency of HA bound to Fab' receptor was greater than that of unbound HA held close to the target membrane via other HA - Fab' binding interactions. In this situation it is possible that receptor binding acted to orientate the HA with respect to the target membrane and thus increase the efficiency of "fusion peptide" insertion into the target membrane, which subsequently led to the observed increase in fusion efficiency. This suggestion is in agreement with other reports (Pedroso de Lima *et al.*, 1995; Stegmann *et al.*, 1995) which suggested sialic acid binding by HA facilitated correct insertion of the HA "fusion peptide" into the target membrane. As discussed

previously (Sections 3.5.2 & 4.2.9) it has not been possible to investigate directly the efficiency of "fusion peptide" insertion into the liposomal membrane when comparing BHA bound or unbound by liposome coupled anti HA Fab' receptor.

5.2 THE POTENTIAL APPLICATIONS OF HA FOR USE IN DELIVERY VEHICLES

A delivery vector must, most importantly be efficient and be able to deliver to a specific cell type. Studies concerning the role of receptor binding by HA with regard to the efficiency of membrane fusion have concluded that a HA delivery vector containing a specific binding domain within the HA molecule itself has the potential to be more efficient at inducing membrane fusion compared to a delivery vector in which HA functions only to induce membrane fusion and the binding specificity is contained within other molecules (Section 4.3.5).

The results presented have shown that liposome coupled Fab' receptors such as Hc19 Fab' or Hc73 Fab' did not block the characteristic low pH induced conformational change of bound HA (Sections 4.2.2 & 4.2.3). It was also shown that HA bound by liposome coupled Hc73 Fab' was more efficient at inducing membrane fusion compared to unbound HA held close to the target membrane by other HA - Fab' interactions (Section 4.3.3). On the basis of these results it is proposed to design a HA molecule containing a specific binding domain around the area to which the surrogate receptors such as Hc19 Fab' or Hc73 Fab' bind. For example a specific binding domain could be inserted at $HA_1$157 of X-31 HA in an effort to produce a molecule that will be able to recognise specific cellular receptors and following receptor mediated endocytosis, undergo a characteristic acid induced conformational

- Not right - papers by Helenius group - endocytosis.

- also avedare. Many groups work a receptors and several have now been found - limited tropism of Adeno-ni interesting - differences Ad3 with 2+5

- "Fusion" mediated by pentan base

membrane penetration

change resulting in fusion with the endosomal membrane. The design of such HA - chimeric molecules will be aided by the precise structural information available for HA and the extensive characterisation of the low pH induced conformational change of HA.

The results obtained using anti JHB HA H8 Fab' as a surrogate receptor suggested HA bound in this orientation was no more efficient at inducing membrane fusion compared to unbound HA held close to the target membrane by other HA - Fab' interactions (Section 4.3.4). This indicates that the precise orientation of the bound HA relative to the target membrane effects the membrane fusion efficiency of bound HA upon exposure to low pH, this will therefore be an important consideration in the design of HA - chimeric molecules .

The fusion proteins that mediate cell entry in other gene therapy vectors are less well characterised. The entry of adenoviral vectors into cells is known to involve the penton fibre and the penton base (Philipson *et al.*, 1968; Wickham *et al.*, 1993; Henry *et al.*, 1994), but the mechanism of cell entry is unknown. There have been successful attempts to limit adenoviral tropism (Michael *et al.*, 1995; Wickham *et al.*, 1996), but these approaches have not involved the insertion of specific binding domains into either the penton base or penton fibre and an approach directed to do this is not feasible due to a lack of information concerning the entry mechanism of adenoviruses.

The entry of retroviral vectors into cells is mediated by the envelope (Env) glycoprotein. The structure of the receptor binding subunit of any retroviral Env protein is presently unknown. Despite this, hybrid retroviral Env proteins containing specific binding domains that alter viral tropism have been reported (Kasahara *et al.*,

1994; Matano *et al.*, 1995; Somia *et al.*, 1995; Hua *et al.*, 1997). Due to the extensive characterisation of the HA protein, the prospects for production of a functional HA molecule containing a specific binding domain are therefore relatively good compared to other gene therapy vectors in which the proteins that mediate cell entry are less well characterised.

The production of HA molecules containing specific binding domains is only one part of developing an effective delivery vector. A delivery vector in which the modified HA can be functionally reconstituted must also be developed. It has been previously shown that soluble material entrapped within Method A virosomes was efficiently delivered to the cell cytoplasm (Bron *et al.*, 1994). It has been shown here that HA reconstituted into vesicles composed of purified lipid can mediate membrane fusion (Results - chapter 1) and this substantially increases the prospects for development of influenza HA based delivery vectors. Production of virosomes containing moieties such as the ganglioside GM1 or lipid derivatives of poly(ethylene glycol) could reduce the affinity of virosomes for macrophages and therefore increase the half life *in vivo* of any virosome based delivery vector and thus increase the efficiency of delivery. It can presently be concluded that prospects for production of a modified HA which can be reconstituted into a functional delivery vector are promising.

5.3 FURTHER WORK

Further experiments will attempt to establish the efficiency of "fusion peptide" insertion into the liposomal membrane when comparing BHA bound or unbound by liposome coupled anti HA Fab' receptor. With regard to the low pH induced

conformational change of BHA bound to liposome coupled Fab', it is unknown what proportion of "fusion peptide" inserts into the liposomal membrane to which the BHA is bound compared to association with other liposomes. Experiments could be performed in which BHA bound to Fab' liposomes are mixed with bare liposomes, after low pH and trypsin treatment the amount of BHA_2 associated with Fab' liposomes and bare liposomes could then be compared.

It is proposed to investigate the effect of increased surface density of HA-receptor with respect to the efficiency of Fab' liposome - virosome fusion. A series of Fab' liposome preparations which have an increasing density of coupled Fab' will be produced. In each case the extent of Fab' liposome - virosome fusion will then be assayed. Unlike the experiments of Alford *et al.* (1994) which investigated the effect of increased surface density of HA-receptor with respect to the extent of liposome - influenza virus fusion, increasing the surface density of Fab' receptor will not modify the lipid composition of the target membrane.

The HA mediated fusion activity of virosomes produced by Method C which contain lipid derivatives of poly(ethylene glycol) will be investigated. Lipid derivatives of poly(ethylene glycol) have previously been shown to reduce the affinity of liposome for macrophages (Lasic *et al.* 1991; Woodle & Lasic, 1992). The next phase in the process of vector design will involve studies of entrapment into virosomes of either nucleic acid or proteins and their transfer into cells. The results of initial experiments on this topic are presented (Section 8).

It is proposed to obtain the HA gene sequence of V100 Res vir 8 and V8 Res vir 8 virus, thus ~~identif~~ identifying the position of the mutated residue(s).

6 ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. John Skehel for his guidance throughout my Ph.D. and for help when preparing this thesis. I would also thank him for encouraging me to broaden my scientific interests.

I would like to thank Dr Steve Wharton for his continuous patience, encouragement, advice and stimulating conversations. I am also indebted to Lesley Calder for teaching me various electron microscopy techniques.

I am also grateful to Dr Barry Ely for general advice and together with Margaret Goggin for large scale production of cell supernatant antibody. I would like to thank Rose Gonsalves, Kevin Booth and Dave Stevens for growing viruses used in this study. I would also like to thank Alan Douglas and Brian Thomas for providing ascitic fluid, Gordon Reid for help when synthesizing PDP-PE and my UCL supervisor Prof. Waterfield. Many thanks to the photographics section (N.I.M.R).

I am grateful to my Mother for her support throughout my education and finally, I would like to thank Roisean for making the time during my Ph.D. very happy.

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8 APPENDIX: INITIAL EXPERIMENTS INVESTIGATING DELIVERY TO CELLS USING INFLUENZA VIROSOMES

MOST OF THE EXPERIMENTS PRESENTED IN THIS SECTION WERE PERFORMED BEFORE THE EXPERIMENTS PRESENTED IN SECTION 4.1.

8.1 INTRODUCTION

Reports concerning the delivery potential of HA - containing lipid vesicles has been discussed in Section 2.3.9.2. Polypeptide entrapped within influenza virosomes produced by Method A (Section 3.2.1) was efficiently delivered to the cell cytoplasm *in vitro* (Bron *et al.*, 1994). Initial experiments presented here have investigated the potential of influenza virosomes to deliver substances to cells *in vitro*.

Virosomes were produced which contained fluorescent lipid in the virosomal membrane and the ability of virosomes to deliver the fluorescent lipid to cells *in vitro* was assessed. Experiments have also investigated procedures for entrapping DNA plasmid within virosomes, the virosomes produced were used to treat cells *in vitro* and expression of a reporter gene present on the DNA plasmid was assayed.

8.2 MATERIALS AND METHODS

All chemicals except where otherwise stated were obtained from BDH or Sigma and were of analytical reagent grade.

8.2.1 Incorporation of fluorescent lipids into the virosomal membrane

X-31 HA containing virosomes were made which contained 2 mol% of the fluorescent lipid N-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine (N-Rho-PE) (Advanti polar lipids Inc.). N-Rho-PE was incorporated into virosomes produced by Method A as described in Section 3.2.1.1. Virosomes produced by Method B[1] were made with 1 mg of lipid of cellular composition, N-Rho-PE was

included in the lipid mixture before it was evaporated to dryness under N_2 and lyophilized (Section 3.2.2).

8.2.2 Preparation of DNA

8.2.2.1 Growth and purification of DNA plasmids

pGL2 control vector (6 kilobase-pairs) (Promega) contained cDNA coding firefly luciferase under the control of a simian virus number 40 early promoter. pTR-UF2 was kindly provided by Dr Zolotukhin (Florida University) which contained a chemically synthesized "humanized" cDNA coding *Aequorea victoria* Green Fluorescent Protein (GFP) under the control of a cytomegalovirus early promoter (Zolotukin *et al.*, 1996). The humanized GFP sequence was engineered to contain codons which are more efficiently translated by mammalian cells, giving rise to higher levels of GFP.

The plasmids described above were used to transform competent DH5α *Escherichia coli* as described by Hanahan (1983). A single colony was used to inoculate 10 ml of LB-Medium (10 g bacto-tryptone, 5 g bacto yeast extract, 10g NaCl, made up to final volume of 1 l with distilled H_2O and adjusted to pH 7 with 5 M NaOH) containing 50 μg/ml ampicillin. Cultures were grown for 10 hours at 37^0C with vigorous shaking. The 10 ml pre-culture was then used to inoculate 5 l of LB-Medium containing 50 μg/ml ampicillin, which was then grown overnight at 37^0C with vigorous shaking.

Lysis of bacteria was by alkali and purification of plasmid was done using a mega kit for plasmid purification (Qiagen) using the protocol supplied. Plasmids were analysed by agarose gel electrophoresis as described by Sambrook *et al.* (1989), 0.8% agarose gels were made with electrophoresis buffer (0.04 M Tris acetate, 0.001

M EDTA) and run at 80 V. Gels were soaked in electrophoresis buffer containing 300 ng/ml ethidium bromide and the DNA bands visualized under ultra violet light.

8.2.2.2 Labelling of DNA plasmid with ^{32}P using nick translation

Deoxycytidine-$\alpha^{32}P$ triphosphate (dCTP$\alpha^{32}P$) (10mCi/ml, Amersham) was incorporated into the pGL2 plasmid using the "nick translation kit" from Promega. Briefly 70 μCi of dCTP $\alpha^{32}P$ was added to 1 μg of the pGL2 plasmid in the presence of DNA polymerase I / DNase I mix, nucleotide mix and nick translation buffer as specified by Promega. Non-incorporated nucleotides were removed using a Pharmacia NICK column using the protocol supplied.

8.2.3 Entrapment of DNA plasmid within X-31 HA containing virosomes

8.2.3.1 Methods used to entrap DNA plasmid within virosomes produced by Methods A and C

In the case of virosomes produced by Method C, 0.75 mg of PC/Chol (molar ratio 2:1) lipid was used. The pGL2 plasmid was grown and purified as described in Section 8.2.2.1. 5, 10, 40 or 80 μg of pGL2 plasmid on its own or in the presence of an equal weight of protamine (from salmon sperm, Sigma) or dextran sulfate (8000 average molecular weight, Sigma) was added to the HA lipid supernatant. The solution was incubated for 15 mins at 37^0C after which the biobeads were added and the preparation completed as described in Section 3.2.1, except NaN_3 was omitted from all solutions.

8.2.3.2 Methods used to entrap DNA plasmid within virosomes produced by Method B^1

1 mg of lipid of cellular composition was used to produce virosomes by Method B^1 as described in Section 3.2.2, except NaN_3 was omitted from all solutions.

5, 10, 40 or 80μg of pGL2 plasmid on its own or in the presence of an equal weight of protamine or dextran sulfate was added after sonication of the HA lipid mixture. The virosome preparation was then completed as described in Section 3.2.2,

8.2.3.3 Attempts to determine the entrapment efficiency of DNA plasmid within virosomes

Nick translated pGL2 labelled with ^{32}P (Section 8.2.2.2) was added to virosomes as described in Sections 8.2.3.1 and 8.2.3.2. The virosomes were pelleted by centrifugation for 1 h at 300000g at 5^0C and resuspended in a known volume of PBS. This procedure was repeated two times to remove plasmid which was not associated with the virosomes. The resuspended pellet was then treated with 1 μl of DNase I (Promega) for 30 mins at 37^0C to digest non entrapped DNA which was associated with the outside of the virosomes. The pelleting procedure was then repeated as described above and the amount of entrapped DNA was determined by quantifying the ^{32}P radioactivity using the Beckman LS 5000CE liquid scintillation system. This value was compared to the amount of ^{32}P originally added to the preparation enabling the efficiency of entrapment to be determined.

Virosomes made using Method A in the presence of nick translated ^{32}P labelled pGL2 (with no protamine or dextran sulfate added) showed that biobead treatment absorbed at most ~10% of the nick translated DNA (data not shown). However, agarose gel electrophoresis of pGL2 (or pGL2 in the presence of protamine- data not shown) following biobead treatment in the presence of $C_{12}E_8$ showed that ~100% absorption of the pGL2 had taken place (Figure 8.2.1a). These results indicated that for the purpose of determining the entrapment efficiency, nick translated

DNA was not equivalent to non - nick translated DNA plasmid and therefore the results obtained using ^{32}P labelled nick translated DNA are not presented.

Agarose gel electrophoresis of pGL2 and an equal weight of dextran sulfate following biobead treatment in the presence of $C_{12}E_8$ indicated that no significant absorption of DNA to the biobeads had taken place (Figure 8.2.1b). This suggests that dextran sulfate prevents the absorption of the DNA to the biobeads, the mechanism by which dextran sulfate acts is unknown.

8.2.4 Virosome delivery to cells

8.2.4.1 Cell maintenance

MDCK, CV-1, BHK, and COS-7 cell lines were maintained in Dulbecco's MOD eagle medium (DMEM) with sodium pyruvate (GibcoBRL) and CHO cells in MEM α medium (GibcoBRL). All medium contained 5% (v/v) foetal calf serum (GibcoBRL). Cells were seeded in 8-well glass chamber slides (Lab-Tek) or 35 mm tissue culture plates (Nunclon) and incubated at 37^0C in a CO_2 incubator.

8.2.4.2 Delivery of virosomes to cell monolayers.

When delivering pGL2 to cells using virosomes 35 mm tissue culture plates which contained ~ 40% confluent cell monolayers were used. Cells were washed twice with serum free medium and 10, 50, 200 or 500 μl of virosomes were overlaid per plate and the final volume adjusted to 500 μl with serum free medium. The cells were then incubated for 1 hour at 37^0C in a CO_2 incubator and the virosome suspension removed and 1 ml of serum medium overlaid. Cells were then incubated for 48-60 hours and assayed for luciferase expression as described in Section 8.2.4.3. As a positive control cells were transfected with pGL2 plasmid using lipofectin (Life Technologies) using the protocol supplied. Briefly 1 μg of pGL2 plasmid was

Figure 8.2.1

a) Lane 1 shows pGL2 prepared as described in Section 8.2.2.1. Lanes 2 to 5 show pGL2 after exposure to various treatments, in all cases 400µl of a specified solution containing 40µg of plasmid DNA was shaken in an Eppendorf 5432 Mixer for 75 mins at room temp. in an identical fashion to that specified during the production of virosomes by Methods A or C. Following this treatment 10µl of the solution was analysed by agarose gel electrophoresis (Section 8.2.2.1).

Lane 2) pGL2 in 145 mM NaCl / 0.1 mM EDTA / 5 mM HEPES pH 7.4

Lane 3) pGL2 in 100mM $C_{12}E_8$ / 145 mM NaCl / 0.1 mM EDTA / 5 mM HEPES pH 7.4

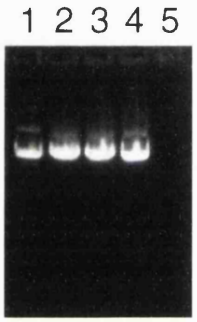
Lane 4) pGL2 in 145 mM NaCl / 0.1 mM EDTA / 5 mM HEPES pH 7.4 containing 113 mg of wet biobeads followed by two 65 mg aliquots of biobeads as described in Section 3.2.1

Lane 5) pGL2 in 100mM $C_{12}E_8$ / 145 mM NaCl / 0.1 mM EDTA / 5 mM HEPES pH 7.4 containing 113 mg of wet biobeads followed by two 65 mg aliquots as described in Section 3.2.1. Under these conditions pGL2 plasmid was removed from solution, presumably due to absorption to $C_{12}E_8$ / biobead complexes

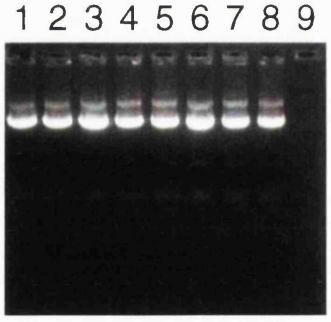
b) Lanes 1 and 9 shown in (b) are equivalent to Lanes 1 and 5 shown in (a). Lanes 2 to 8 shows pGL2 after shaking in 100mM $C_{12}E_8$ / 145 mM NaCl / 0.1 mM EDTA / 5 mM HEPES pH 7.4 containing 113 mg of wet biobeads followed by two 65 mg aliquots as described in Section 3.2.1. In addition the pGL2 solutions analysed in Lanes 2 to 8 contained 160, 120, 100, 80, 60, 40, or 20 µg of dextran sulfate (8000 average molecular weight) respectively, which in all cases blocked the removal of pGL2 from solution.

Figure 8.2.1: Agarose gel electrophoresis of pGL2: Removal of pGL2 from solution following biobead treatment and the effect of dextran sulfate

a)



b)



complexed with 17 µl of lipofectin reagent and the volume adjusted to 100 µl with OPTI-MEM reduced serum medium (GibcoBRL). 30 µl of this solution was added to each 35 mm plate and the final volume adjusted to 500 µl with OPTI-MEM. Cells were then incubated for 5 hours at 37^0C in a CO_2 incubator after which 1 ml of serum medium was overlaid. Cells were assayed for luciferase expression after 48-60 hours (Section 8.2.4.3).

When delivering virosomes labelled with 2 mol% N-Rho-PE 8 well glass chamber slides which contained a confluent MDCK cell monolayer were used. Cells were washed twice with serum free medium and 50 µl of virosomes were overlaid per well. The cells were then incubated for 1 hour at 37^0C in a CO_2 incubator and then the virosome suspension was removed. Cells were washed twice with PBS and 200µl per well of 1% paraformaldehyde (w/v in PBS) was overlaid, cells were then incubated for 1 hour at room temp. The cells were then washed twice with PBS and 200µl per well of 0.5 M ammonium chloride was added per well and incubated for 15 min at room temp to quench autofluorescence. The cells were then washed twice in PBS and viewed under a Nikon LABOPHOT-2 fluorescence microscope using a rhodamine filter set.

8.2.4.3 Assay to detect expression of luciferase

Luciferase expression was detected using a luciferase assay kit (Promega) using the protocol supplied. A Berthold CliniLumat LB9502 luminometer was used to measure the light emitted upon injection of the luciferase assay reagent to 20 µl of cell lysate.

But B' liposomes did not fuse!! —

So, B' lipos got into cells, into endosomes and were degraded, lipid into cells.

8.2.4.4 Expression of Green Fluorescent Protein (GFP)

pTR-UF2 was used to transfect cells using lipofectin as described for pGL2 in Section 8.2.4.2. Detection of GFP in transfected cells was by inspection under a Zeiss Axioskop fluorescence microscope using a fluorescein filter set.

8.3 RESULTS

8.3.1 Delivery to cells of N-Rho-PE by virosomes containing 2 mol% N-Rho-PE

Virosomes labelled with 2 mol% N-Rho-PE were used to treat MDCK cells as described previously (Sections 8.2.1 & 8.2.4.2). Figure 8.3.1 shows that MDCK cells became labelled with N-Rho-PE after treatment with virosomes produced by Method A, which had a viral lipid composition or virosomes produced by Method B^1 made with lipid of cellular composition. Virosomes produced by Methods A or B^1 were pre-treated at pH 5 (Section 3.2.4), which resulted in the irreversible conformational change of HA. Treatment of MDCK cells with pH 5 pre-treated virosomes resulted in the labelling of the outside of MDCK cells, in comparison, the labelling of cells which had been treated with virosomes which had not been pre-treated at pH 5 was not so concentrated around the outside of the cells (Figure 8.3.1).

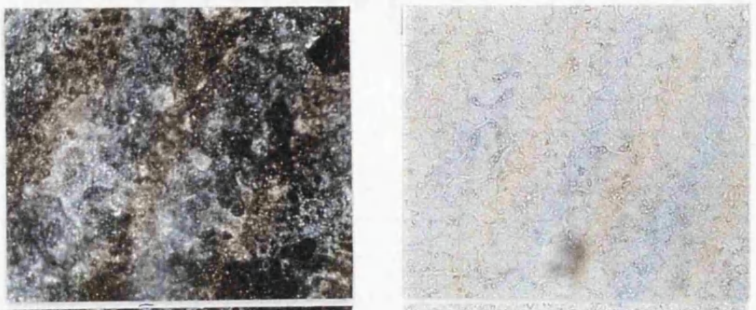
It is possible that the binding of untreated virosomes which contained native HA to the cell surface triggered receptor mediated endocytosis. HA in the low pH conformation present on acid treated virosomes was possibly less effective than native HA at triggering receptor mediated endocytosis which could account for the relatively high level of labelling of the outside of the MDCK cells. Previous results showed that virosomes produced by Method A but not Method B^1 can participate in HA mediated fusion (Section 4.1), it was therefore expected that Method A but not Method B^1

Figure 8.3.1

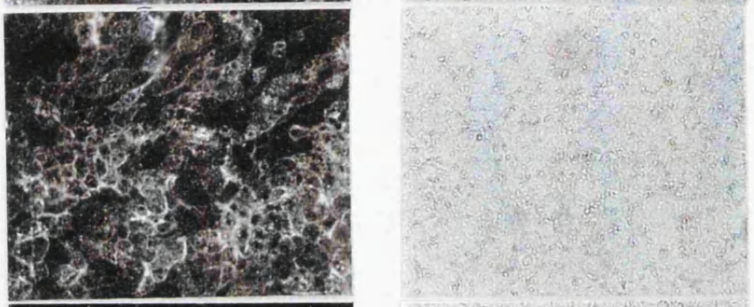
Virosomes containing 2 mol% N-Rho-PE were produced using Methods A & B[1] (Section 8.2.1) and used to treat a confluent MDCK cell monolayer as described in Section 8.2.4.2. Pictures of cells were taken with a Nikon LABOPHOT-2 fluorescence microscope, using a rhodamine filter set as shown in the left hand column. Pictures on the right show the same frame under bright field conditions. Magnification = x430

Treatment of MDCK cells with virosomes containing 2 mol% N-Rho-PE

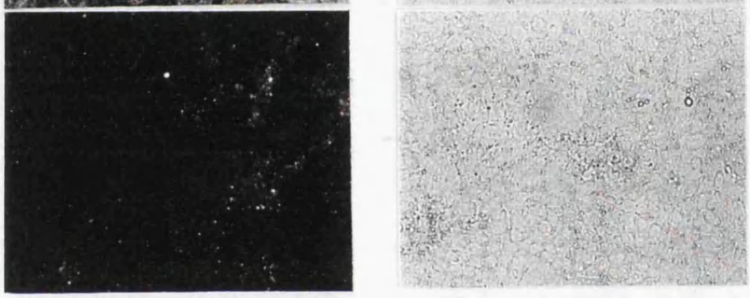
Untreated virosomes (Method A)



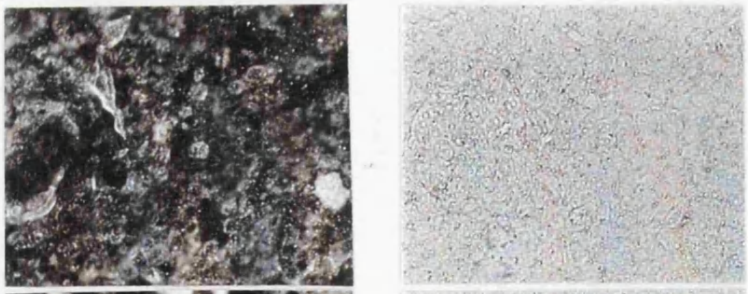
pH 5 pre-treated virosomes (Method A)



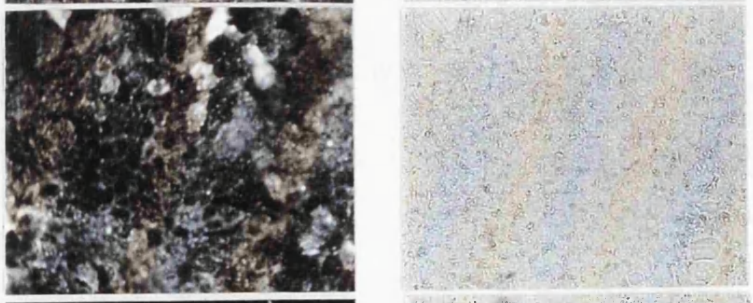
pH 5 and trypsin pre-treated virosomes (Method A)



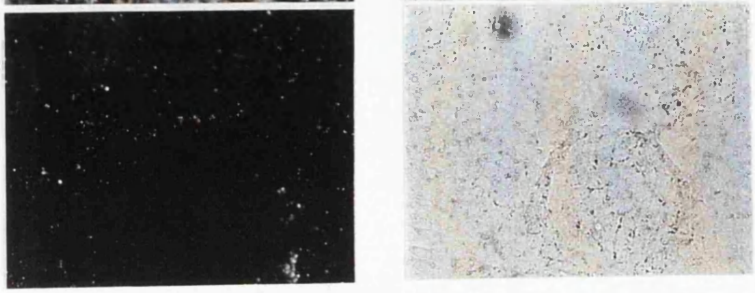
untreated virosomes (Method B[1])



pH 5 pre-treated virosomes (Method B[1])



pH 5 and trypsin pre-treated virosomes (Method B[1])



virosomes would have participated in membrane fusion with the endosomal membranes at low pH. It was not possible to conclude whether or not fusion between the endosomal and virosomal membranes had taken place when observing the labelling of MDCK cells with N-Rho-PE. There was no significant difference observed in the labelling of MDCK cells with N-Rho-PE when treated with virosomes produced by Methods A or B[1] when viewed with the Nikon LABOPHOT-2 fluorescence microscope. Further work could use confocal microscopy to investigate the N-Rho-PE labelling of MDCK cells.

Trypsin treatment of acid treated virosomes results in removal of HA_1 28-328 which contains the sialic acid binding site of HA. Treatment of MDCK cells with trypsin treated virosomes resulted in very low levels of N-Rho-PE being delivered to MDCK cells (Fig. 8.3.1). Due to the removal of HA_1 28-328 binding by trypsin treated virosomes to the cell surface via sialylated receptors was not possible, this therefore explains the observed lack of N-Rho-PE delivery to MDCK cells by trypsin treated virosomes.

8.3.2 Delivery to cells of DNA plasmid entrapped within virosomes

8.3.2.1 Characterisation of virosomes produced in the presence of DNA plasmid

Virosomes were produced in the presence of pGL2 plasmid (with or without protamine or dextran sulfate) as described in Sections 8.2.3.1 & 8.2.3.2. Electron microscopy of these preparations concluded that there was no difference in appearance of virosomes when prepared in the presence or absence of DNA plasmid (data not shown). Virosomes prepared in the presence or absence of DNA plasmid (with or without protamine or dextran sulphate) were shown to have equal fusion activity as judged by their ability to mediate low pH dependent haemolysis of human

erythrocytes (Section 3.3.1) (data not shown). Krumbiegel *et al.* (1992) have reported that low pH induced influenza virus fusion with erythrocytes is inhibited at concentrations of 0.5 mg/ml dextran sulfate (average molecular weight 8000), consistent with this low pH induced haemolysis of human erythrocytes by virosomes produced by Method A was inhibited by 0.5 mg/ml dextran sulfate (data not shown). The concentration of dextran sulphate when used in virosome preparations was at most 80 μg/ml, this concentration of dextran sulfate did not result in any observed inhibition of virosome fusion activity (data not shown).

8.3.2.2 Treatment of cells *in vitro* with virosomes produced in the presence of DNA plasmid

Table 8.3.1 summarises the initial experiments done. At the present time delivery of the pGL2 to cells by influenza virosomes has not been demonstrated. Transfection of all the specified cell types with pGL2 using lipofectin resulted in high amounts of luciferase expression. Luciferase expression was assayed as described in Section 8.2.4.3, luciferase expressing cell extracts gave rise to ~500000 counts per min. compared to ~200 counts per min. from a non luciferase expressing cell extract.

The plasmid pTR-UF2, expressing green fluorescent protein (GFP) will be used as an alternative plasmid to assay DNA delivery. Lipofectin mediated transfection of MDCK cells with pTR-UF2 caused approximately 15% of the cells to show strong fluorescence, due to the expression of GFP (data not shown). This system, unlike the assay to detect luciferase expression enables the proportion of transfected cells to be determined, simply by counting the number of fluorescent cells versus non-fluorescent cells. Determination of the proportion of transfected cells will

Table 8.3.1

Attempts to deliver pGL2 using influenza virosomes did not result in expression of the luciferase reporter gene in any case. For a particular cell line and each of the eight methods of virosome preparation specified the following experiments were done:-

As described in Sections 8.2.3.1 & 8.2.3.2 four X-31 HA containing virosome preparations were made in the presence of 5, 10, 40, or 80 μg of pGL2, together with an equal weight of protamine or dextran sulfate when specified. For each of the virosome preparations produced 10, 50, 200 or 500 μl was overlaid onto a ~40% confluent cell monolayer as described in Section 8.2.4.2. Cells were then incubated for 48-60 h and then assayed for luciferase expression as described in Section 8.2.4.3.

Table 8.3.1 Delivery of DNA to cells using virosomal vectors

	MDCK cells	**Cos-7 cells**	**BHK cells**	**CHO cells**	**CV-1 cells**
Method A virosomes (containing lipid of viral composition. HA:lipd ratio w/w ~1:3)					
Made in the prescence of DNA alone	NLEO	NLEO	NLEO	NLEO	NLEO
Made in the prescence of DNA + protamine	NLEO	NLEO	NLEO	NLEO	NLEO
Made in the prescence of DNA + dextran sulfate	NLEO	NLEO	ND	ND	ND
Method B[1] virosomes (containing lipid of cellular composition. HA:lipid ratio w/w ~1:3)					
Made in the prescence of DNA alone	NLEO	NLEO	NLEO	NLEO	NLEO
Made in the prescence of DNA + protamine	NLEO	NLEO	NLEO	NLEO	NLEO
Made in the prescence of DNA + dextran sulfate	NLEO	NLEO	ND	ND	ND
Method C virosomes (containing PC/Chol 2:1 molar ratio. HA:lipid ratio w/w~ 1:5)					
Made in the prescence of DNA alone	NLEO	NLEO	ND	ND	ND
Made in the prescence of DNA + dextran sulfate	NLEO	NLEO	ND	ND	ND

NLEO = No luciferase expression observed

ND = Not determined

be significant since the proportion of cells transfected in a cell population is likely to determine the success of any gene therapy method.

8.4 DISCUSSION

It was shown previously that virosomes produced by Methods A & C could cause membrane fusion with liposomes or erythrocyte ghosts and also mediate low pH induced haemolysis of human erythrocytes (Section 4.1). It is therefore reasonable to assume that virosomes produced by Methods A & C would cause low pH induced membrane fusion between the virosomal and endosomal membranes following receptor mediated endocytosis resulting in release of virosomal contents to the cytoplasm.

When MDCK cells were incubated with Method A virosomes which contained 2mol% N-Rho-PE the cells became fluorescently labelled (not done with Method C virosomes). This was consistent with the view that virosomes were taken up by the cell via receptor mediated endocytosis. Attempts to deliver DNA entrapped within virosomes produced by Methods A & C did not lead to expression of the DNA (Section 8.3.2.2).

A major problem which has been associated with the use of liposomes to deliver DNA to cells has been low DNA entrapment efficiency, as discussed by Felgner (1993). It is possible that the lack of DNA delivery by virosomes produced by Methods A & C was due to problems with DNA entrapment. Agarose gel electrophoresis following biobead / $C_{12}E_8$ treatment of DNA alone or DNA and protamine showed that no DNA remained in solution. It is therefore likely that virosomes produced by Methods A & C in the presence of DNA or DNA and

protamine did not contain entrapped DNA, which would therefore explain the lack of luciferase expression. As shown in Figure 8.2.1b agarose gel electrophoresis showed DNA in the presence of an equal weight of dextran sulfate remained in solution following biobead / $C_{12}E_8$ treatment. Due to the absence of an assay for the level of DNA entrapment within virosomes, the level of DNA entrapped within virosomes produced in the presence of DNA and dextran sulfate is unknown.

Virosomes produced by Method B^1 have been shown not to participate in HA mediated fusion (Section 4.1). It is suggested that virosomes produced by Method B^1 would not have caused low pH induced membrane fusion between the virosomal and endosomal membrane following receptor mediated endocytosis which would have blocked the release of virosomal contents to the cytoplasm and thus account for the absence of luciferase gene expression.

Problems which may have been associated with entrapment of DNA within virosomes has meant that the presented experiments have not demonstrated that substances entrapped within virosomes can be delivered to the cell cytoplasm. To demonstrate that virosomes are able to deliver entrapped substances to the cell cytoplasm it is proposed to entrap purified GFP within virosomes and investigate the ability of virosomes to deliver the entrapped GFP to the cytoplasm. Virosomes produced by Method B^1 unlike virosomes produced by Methods A & C are not fusion - active and therefore will not be pursued as delivery vectors.

Experiments will investigate further the procedures for efficient entrapment of DNA within virosomes produced by Methods A & C. A system which can accurately assay the DNA entrapment efficiency will be needed. DNA entrapment efficiency may be improved by first complexing DNA with nuclear DNA binding proteins. This

may act to reduce absorption of DNA onto biobeads, neutralize the negatively charged phosphate groups of DNA resulting in reduced repulsion between DNA molecules within the virosomal lumen and condense the DNA enabling more DNA to become entrapped within the relatively small virosomal lumen. The use of DNA binding proteins may also act to decrease cytoplasmic degradation of DNA by targeting DNA to the nucleus and hence increase the delivery efficiency. For example the nonhistone chromosomal protein, high mobility group-1 has been shown to increase significantly expression of DNA when included in DNA - containing vesicles delivered to cells *in vitro* (Kaneda *et al.*, 1989).