The Application of Inert Substrates as Carriers in Diagnostic Virology

Thesis submitted for

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of the

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by

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E R R A T A

(1) Insert p.47, following line 6:

Titration of conjugate
before use in IIF tests

Before being used in IIF tests for the detection of virus antibody, the conjugate component of the system was standardised as described by Grist et al, (1979). This was done by testing dilutions of the conjugate on a range of IIF substrates with standard antisera and by checking the specificity and intensity of immunofluorescence.

(2) Page 86, para 2, line 12:

Delete 'and reproducibility'.

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Acknowledgments

I would like to thank my supervisor Professor Morag C. Timbury for all her advice and encouragement during this study.

Declaration

I declare that this work was performed by me in pursuit of an M.Sc. The major part of the experimental work was performed by me, I received minor additional support from technical staff.

Summary

1) The use of liposomes in viral diagnostic serology was investigated. Virus-liposome mixtures were found to be as effective as conventional antigen preparations in CF, HAI, and IIF tests. They were equally stable and similar amounts of virus were required in liposome substrates as in conventional antigens.

2) The correlation between CF and IIF test results using virus-liposomes was found to be low. This was in agreement with the findings of other comparative studies. It was considered probable that the CF test was measuring different antibodies in certain sera to those detected by IIF.

3) In IIF conventional infected-cell substrates are often variable, and reproducibility of results is commonly a problem. Virus-liposome mixtures offered an advantage in that they were standardised test substrates that minimised the problems of non specific fluorescence and subjective interpretation of test results.

4) Purified influenza virus haemagglutinin subunits were prepared by cellogel electrophoresis of detergent-disrupted whole virus. Tests for purity showed them to be free from contamination by other viral proteins.

5) Liposomes combined with purified influenza virus haemagglutinin were as effective as conventional substrates in detecting influenza antibody by IIF. However, they did not detect strain-specific influenza virus antibody and reacted with antibody to haemagglutinin (H_1) of a different type from that (H_3) combined with the liposomes.

6) The adjuvant effect of liposome associated haemagglutinin subunits was tested in animals. Haemagglutinin - liposome mixtures although less immunogenic than whole virus, nevertheless induced higher levels of antibody than equivalent doses of haemagglutinin subunits alone. This showed that liposomes had adjuvant activity in the immune response to influenza virus produced in guinea pigs.

7) The liposome-haemagglutinin mixture showed a dose response relationship in the guinea pigs inoculated.

8) Haemagglutinin-liposome mixtures may therefore prove to be a non toxic pyrogen - and allergy-free vaccine for influenza. Clinical trials with human volunteers would be required to test this possibility.

Abbreviations

CPE	=	Cytopathic effect
IgA	=	Immunoglobulin A
IgD	=	Immunoglobulin D
IgE	=	Immunoglobulin E
IgG	=	Immumoglobulin G
IgM	=	Immunoglobulin M
SDS	=	Sodium Dodecyl Sulphate
PAGE	=	Polyacrylamide Gel Electrophoresis
PBS	=	Phosphate Buffered Saline
FITC	=	Fluorescein Isothiocyanate
IFA	=	Immunofluorescent Antibody Test
IIF	=	Indirect Immunfluorescent Test
HA	=	Haemagglutinin
HAI	=	Haemagglutination Inhibition
CF	=	Complement Fixation
EDTA	=	Ethyl-diamino-tetra-acetic acid
PTA	=	Potassium Phosphotungstate
EM	=	Electron Microscopy
IC	=	Virus infected cells
VL	=	Virus-liposomes

Chapter 1 INTRODUCTION

The object of this study was to determine if use could be made of an inert carrier of viral antigen, firstly in three routine diagnostic serology tests - Complement Fixation (CF), Haemagglutination Inhibition (HAI), and Indirect Immunofluorescence (IIF); and secondly as a possible adjuvant when injected with subunits of influenza virus into experimental animals. The inert carrier used throughout was the liposome.

There are several possible benefits that may be derived from the use of liposome associated test antigens in serology. For example they might provide a more economical use of virus in CF and HAI tests, and antigens prepared with liposomes might also be more stable and have a longer shelf life. In IIF use of liposomes attached to purified structural components or possibly single proteins - of the virus might provide an antigen substrate for detecting antibodies to a single virus component rather than whole virus. Such a technique would be of great importance in medical virology. Such IIF test substrates should also minimise the problem of non specific immunofluorescence - as unlike conventional infected cell substrates they may provide standardised test antigens containing only specified antigenic components of the virus - thus improving reproducibility in the IIF test for viral antibody.

This study also investigated the immunogenic potential of liposome associated influenza virus subunits. Subunit

preparations offer protection from influenza virus infection without causing pyrogenic side effects, by themselves however they are poorly immunogenic. Experiments were therefore set up to purify viral subunits and to determine if liposome associated subunits provoked a satisfactory antibody response in experimental animals.

Chapter 2 Historical and Literature Perspective

The immune responses elicited by animal viruses are of historical interest as well as current practical importance. Starting with Jenner's pioneering experiments in 1798 on the induction of immunity to smallpox by vaccination, and through the work of Ehrlich, Pasteur, Von Behring, Bordet, Metchnikoff and others; the nature of antibodies, their interactions with antigens, the dynamics of antibody metabolism, and the nature of the cells involved in the immune response, are now well understood. (Landsteiner, 1946; Burnet and Fenner, 1949; Porter, 1962; Burns and Allison, 1974; Hobart and McConnell, 1975).

Host response to infection

The response of human individuals to viral infection is known to be affected by a variety of different factors, for example, hormones, body temperature, nutrition, interferon, and - most important - the immune response. Antibodies are of importance both in the recovery from many viral infections, and in acquiring immunity to reinfection. The association of antibodies with the gamma globulin fraction of animal sera was first demonstrated by Tiselius and Kabat, (1939), in their electrophoresis experiments on serum proteins.

The basic four-chain structure of immunoglobulins with two light and two heavy chains cross-linked by disulphide bonds was proposed by Porter in 1962. Light chains having 213 or 214 amino acid residues of which 108 constitute a

'variable' region, the remainder being a 'constant' region, Intrachain disulphide bonds render these chains capable of loop-formation which is the basis of an intricate tertiary structure. In the variable portion this tertiary structure exposes certain portions to the surface for antigen-binding sites. One quarter of the length of the heavy chains constitutes the variable portion : the remainder is a constant portion which is characteristic in its amino acid sequence for each of the immunoglobulin classes : IgG, IgA, IgM, IgD, and IgE. Therefore variable lengths of the heavy chains and their carbohydrate content account for the different molecular weights of the five immunoglobulin classes. (Wittig, 1979).

In the process of virus infection many different antigens are produced, including structural components of the virion and virus-specific enzymes mostly required for viral mucleic acid synthesis. The important classes of antibody that result from infection include not only IgG and IgM, which are the major serum immunoglobulins; but also IgA, produced by lymphoid cells in the mucosa of the gastrointestinal and respiratory tracts. IgM are 19S serum macroglobulins and earliest antibodies formed. They persist for only a few weeks, IgG are 7S serum globulins which appear later than IgM antibodies, but persist for long periods of time. IgA are 7-11S non-serum globulins, important in affording immunity from respiratory virus infection. The serum antibodies neutralise viral

infectivity by inhibiting the normal process of viral attachment, penetration or uncoating; and may also attach to virus coded antigens on the surface of infected cells, thereby bringing about complement mediated cytolysis (Fenner and White, 1976).

Viral serology

Serological techniques have been developed to measure virus antibodies and are the principal way in which infection is diagnosed in the laboratory. This is usually done by the demonstration of the development of specific virus antibody at the same time as the patient's illness. The antibody titre is defined as the highest dilution of antiserum at which activity can be demonstrated: it is usually expressed as the reciprocal of the serum dilution. Serology thus provides the clinician with relevant information as to the likely cause of a recent infection, or of an individual's current immune status, and as such it is of great importance in the diagnosis of viral infection.

The most commonly used viral serology techniques include complement fixation and haemagglutination inhibition, but in recent years immunofluorescence and especially the indirect technique has become widely used (For example, Gardner and McQuillan, 1974; Griffiths, Buie and Heath, 1978). Neutralisation tests are less commonly performed now in routine viral serology, as they are more time consuming and extravagant in their use of materials than the other serological tests available.

Complement fixation

Complement fixation (CF) is the most generally useful serological method in diagnostic virology. (Grist, Bell, Follett and Urquhart, 1979). The technique orginates from observations in the late nineteenth century that immune haemolysis and immune bacteriolysis required, in addition to antibody, a heat labile cofactor that was present in normal unimmunised serum. Buchner (1889), therefore discovered that fresh rabbit antisera against sheep red cells could lyse the cells in high dilution, but on heating or ageing the antisera lost this haemolytic power; this activity could be restored by adding fresh normal serum from several species of animal, but not by old or heated serum. Buchner was probably the first to make this observation, but Bordet (1895), gave the first clear description of 'Alexin' or as it is now termed 'Complement'.

It was soon discovered that complement was not a single substance and Ferrata showed in 1907 that both the euglobulin and pseudoglobulin components of serum were required for the action of complement. By the middle of the twentieth century, at least four factors were recognised, but it was with the application of newly available biochemical separation methods, that details of the composition, action and mechanism of the system were elucidated. (Muller-Eberhard et al, 1966, 1968, 1971, 1972).

The CF Test

In diagnostic virology, the CF test for the detection of viral antibody is based on the fixation of complement by antigen-antibody aggregates. In commonly used methods, fixation of a known amount of complement is demonstrated by testing for residual complement with an indicator system comprising sheep red blood cells, sensitised by specific haemolysin (antiserum to sheep red blood cells). Absence of haemolysis indicates fixation of complement in the test system, showing that complement has been used up as an antigen-antibody reaction has taken place, from which it is concluded that viral antibody is present.

Most viruses can be used as complement fixing antigens, so that this useful serological test is applicable to all those viruses for which satisfactory CF antigens can be prepared. Many variations in technique have been described for example in the use of different volumes of the reagents, in test tubes or in plastic plates with moulded wells, or as small drops on flat plates. Fixation of complement may be allowed to take place at 37^{0}C for a short period (1-1½ hours) or at 4 ^{0}C for a long period (overnight).

The most widely used method and that used here for the detection of viral antibodies (see methods) employs cold overnight fixation. This results in increased sensitivity without a corresponding increase in anticomplementary reactions. The CF test in common with the Haemagglutination Inhibition

(HAI) test, does not distinguish between classes of immunoglobulin present in serum, unlike the indirect immunofluorescence technique.

Haemagglutination inhibition

The HAI test is another useful virus serological technique. The basis for the test lies in the fact that certain viruses possess the capacity to agglutinate red blood cells (Hirst, 1942) and that specific viral antibody prevents or inhibits this haemagglutination. The reaction can therefore be used for antibody assay or for virus indentification. The test is not however widely used for the demonstration of viral antibody, due to the presence of non specific inhibitors in sera, and the highly specific nature of the test. Non specific inhibitors may obscure genuine specific inhibition of haemagglutination, and have to be removed prior to testing. Inhibitors of influenza virus haemagglutination for example, are of two varieties:(i) serum glycoproteins - which may be removed by periodate, trypsin, or the receptor - destroying enzyme (RDE) of Vibrio cholerae;(ii) heat labile inhibitors destroyed by heating at 56^{0}C for 30 minutes.

The HAI test is therefore highly sensitive, and generally highly specific measuring only those antibodies that bind directly to viral haemagglutinin. It is however theoretically possible that antibodies capable of attaching to other antigens closely adjacent to the haemagglutinin molecule may inhibit haemagglutination by non-specific steric

hindrance. (Fenner and White 1976). Diagnostically this high degree of specificity can be an undesirable charac teristic of the test because if the antigen used for antibody determination in the test is different from contemporary strains involved in current outbreaks, (e.g. H_3N_2 and H_1N_1 strains of influenza virus A) then detection of antibody rises may be missed. (Robinson and Dowdle, 1969).

Immunofluorescence

The immunofluorescent antibody (IFA) technique, is in essence a method for the visualisation of an antigen-antibody reaction, made possible by conjugating one of the participating reagents with a dye which fluoresces when stimulated by light of an appropriate wavelength, Fluorescence being defined as 'the immediate emission of light from a molecule or atom following absorption of radiation' (Udenfriend 1962). With the technique, specific antigen or antibody can be detected, and located microscopically. The technique itself originates from work done in the 1930_s using chemically labelled antibody to identify a specific antigen. Marrack, (1934), discovered that serum proteins labelled with the salt of a diazo-benzidine-azo-R-dye, did not lose their ability to agglutinate specific antigen, and furthermore that the dye colour became associated with the final product.

Coons, Creech and Jones (1941), used antiserum conjugated with β-anthryl carbamide to distinguish between pneumococcus II and III. Improvements in the system followed in which

Fluorescein isocyanate (FIC), and fluorescein isothiocyanate (FITC), were used for conjugation. (Coons and Kaplan, 1950, Marshall, Eveland and Smith, 1958). Other dyes which have been used for conjugation include lissamine rhodamine B200 sulphonyl chloride (RB200SC), and tetramethylrhodamine isothiocyanate (TRITC). (Wick, Baudner and Herzog, 1978).

The IFA Test

Common modifications of the IFA technique include the direct and indirect tests. In the direct test, antibody to the fixed antigen is itself conjugated with the fluorochrome (e.g. FITC), and applied directly without any other intermediate reagents or stages. The antigen concerned is directly revealed by the binding to it of the specific, labelled antibody. In the indirect test, unlabelled antibody is applied directly to the fixed antigen, and visualised in a secondary reaction by treatment with a fluorochrome conjugated anti immunoglobulin serum. The principle of the test is shown in diagram form (figure one). The diagram also shows a third type of test-the 'sandwich test' - which is a double layer procedure designed to visualise specific antibody. This test however, is not used routinely in diagnostic virology.

The indirect test

The indirect immunofluorescence test (IIF) has several advantages over the direct test. Firstly, the fluorescence is brighter since several fluorescent anti-immunoglobulins

bind onto each of the antibody molecules present in the first layer (figure one). Secondly, it is necessary only to obtain a single FITC labelled reagent, the anti-immunoglobulin. Furthermore, the method has great flexibility, so that by using conjugates of antisera to individual immunoglobulin heavy chains, the distribution of antibodies among the various classes and subclasses can be assessed. (Roitt, 1977). However, as with most immunological techniques, as sensitivity is increased by use of the indirect test, specificity is reduced and there may be non specific staining for which careful controls are required.

The indirect test in diagnostic virology

In diagnostic virology, use is made of the indirect immunofluorescence test in identifying virus growing in tissue culture, or viral antigen in original specimens by using known high titred specific antisera; or in measuring viral antibody in patients' sera by using carefully-prepared virus infected cell substrates for the test. However, although the test is a sensitive one for the detection of viral antibody, the interpretation of test results is highly subjective, so that experience on the part of the operator and the use of careful controls are necessary to avoid false positive or negative test results. (Wick, Baudner and Herzog, 1978).

The role of an inert substrate in virology

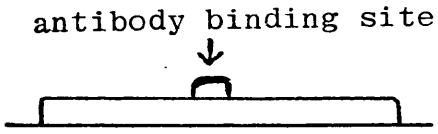
The object of this project was to investigate the use of

Figure One

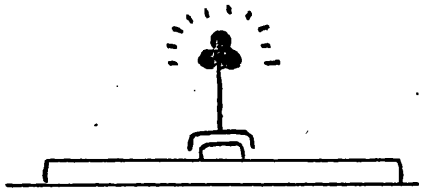
The IFA Test

unlabelled antibody

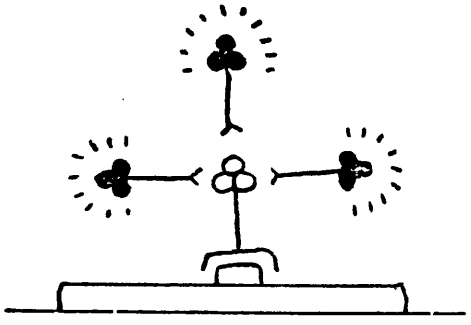
fluorescein - labelled antibody



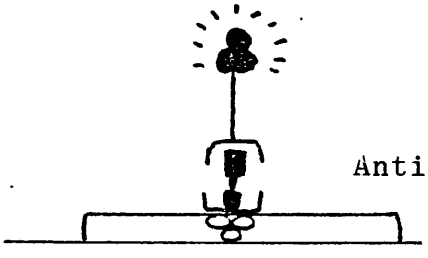
antigenic substrate



i) direct test



ii) indirect test



iii) sandwich test

(Roitt, 1977)

inert substrates as carriers in diagnostic virology. This involved a study of the use of an inert carrier of virus antigen in the serology test systems described above and also of the adjuvant activity of an inert carrier of virus antigen in experimental animals.

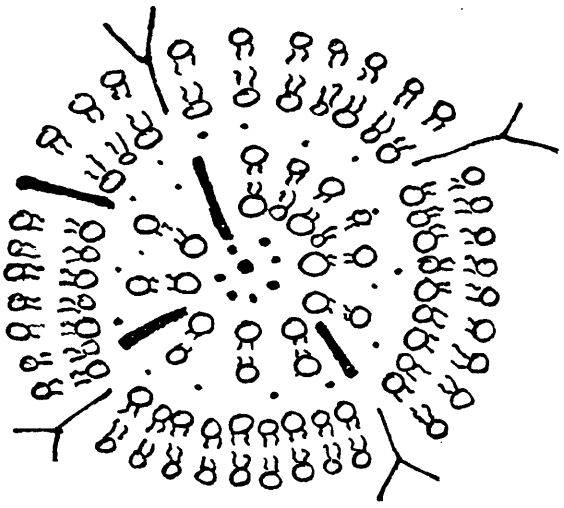
In the CF and HAI tests for example, it would be of interest to establish if a more economical use of test antigen were possible when antigen is associated with an inert carrier. Serology test antigens prepared with carriers might be more stable and have longer shelf lives. In addition, in indirect immunofluorescence if antigenically defined test substrates containing either purified virus or viral components could be prepared, these could provide a test for antibody directed against specific structural components of the virus.

Work carried out in recent years on the coupling of antigens to inert supports has been shown to provide more sensitive immunofluorescent tests. For example, Van Dalen, Knapp and Ploem (1973), prepared cyanogen bromide activated sephadex beads coated with bovine serum albumin as a defined antigenic substrate, and were able to detect 'marked photometrically quantifiable fluorescence on the surface of the particles', in immunofluorescent tests using FITC - anti bovine serum albumin conjugate prepared in rabbits. Similarly, Wick, Baudner and Herzog, (1978), found that coupling antigens to inert supports was a very promising development in respect of standardisation of antigenic preparations.

However in virology, where immunofluorescence is commonly used for diagnosis of infection, few investigations have been made into the possible applications of an inert carrier of viral antigen.

The liposome is a singularly suitable candidate for an inert carrier of viral antigen. The carrier potential in medicine of phospholipid suspensions had been predicted by various workers, (Sessa and Weissman, 1969; Bangham, 1971; Gregoriadis, Leathwood and Ryman, 1971; Fendler and Romero, 1977) since they were first described as 'liposomes' by Bangham, (1965). Liposomes are assemblages of one or several concentric lipid bilayers and form when water-insoluble polar lipids - namely phospholipids - meet with water (Bangham, Hill and Miller, 1974). The assemblages that result are stable in the presence of excess water and since they are associated with unfavourable entropy, become arranged finally in a system of concentric closed membranes each one of which represents an unbroken bimolecular sheet of lipid molecules (figure two). These multilamellar liposomes break up on sonication to form smaller monolamellar structures. Before closed structures form, there is unrestricted entry of water and solutes (containing substances for attachment) between the planes of polar head groups. Thus water-soluble substances can be entrapped in the aqueous compartments provided that such substances do not interfere with liposome formation and that their size is compatible with the dimensions of the aqueous space between the planes of the hydrophilic head groups (approx. 7.5nm in width) or of the space of monolamellar liposomes (approx. 8.5 nm in diameter), Gregoriadis (1976).

figure two



water soluble molecules

lipid soluble molecules

water soluble molecules with hydrophobic moiety penetrating lipid phase

Diagramatic representation of a liposome in which three bilayers of polar phospholipids alternate with aqueous compartments. The carrier potential of liposomes is related to their ability to accommodate water-soluble and lipid soluble substances in the aqueous and lipid phases respectively. Certain macromolecules can insert their hydrophobic regions into the lipid bilayers with the hydrophilic portions extending into water.

(Gregoriadis, 1976)

Technically, the entrapment of substances within or their attachment to liposomes is relatively easy. For entrapment, the appropriate lipids are dissolved in an organic solvent, which when later removed leaves a thin film. Liposomes with trapped solute form when the film with water containing the solute is dispersed - for example entrapment of the enzyme amyloglucosidase within liposomes as described by Gregoriadis, Leathwood and Ryman (1971). Substances may be attached to the liposome surface, rather than being entrapped within, simply by mixing the substance with preformed liposomes, and then sonicating the preparation. For example, the attachment of influenza virus A subunits - to the surface of liposomes as described by Almeida, Brand, Edwards and Heath (1975).

To date a variety of substances have been incorporated into inert liposome carriers, - proteins e.g. amyloglucosidase, (Gregoriadis, Leathwood and Ryman, 1971); influenza virus haemagglutinin and neuraminidase (Almeida et al, 1975); insulin (Dapergolas, Neerunjun and Gregoriadis, 1976); Cytochrome C, (Papahadjopoulos and Miller, 1967); and drugs e.g. actinomycin D and penicillin G, (Gregoriadis, 1973); viruses e.g. Sindbis (Mooney, Dalrymple, and Alving, 1975), and Sendai viruses (Haywood, 1974). Despite its theoretical potential however, as yet little use has been made of liposome carriers in diagnostic virology.

Viruses used:

The viruses under investigation for use with the inert liposome carrier included members of the paramyxovirus and orthomyxovirus groups. Namely, parainfluenza types 1 and 2, mumps, influenza A and B viruses.

The paramyxoviruses derive their name from a morphological resemblance to the orthomyxoviruses with which they were originally classified; however the two groups differ in several important characteristics, particularly in the nature of their viral nucleic acid and its replication (table one). Though few in number, all the human paramyxoviruses are important causes of infection in children. First isolated in the mid 1950_s, the parainfluenza viruses were originally known variously as 'influenza D', 'haemadsorption' viruses. and 'croup-associated' viruses. The parainfluenza viruses are recognised in cultured cells by haemadsorption and they constitute the main cause of croup (laryngo-bronchitis) in young children. In older children and adults they more commonly produce milder upper respiratory tract infections. Parainfluenza virus infections are confined to the epithelium of the respiratory tract; hence the incubation period is invariably short - 2 to 5 days. Mumps is a common contagious disease of children and young adults, it is a generalised infection caused by mumps virus which has a predilection for the central nervous system, and for glandular tissue. Unlike the parainfluenza viruses it has a relatively long incubation period of 18 to 21 days.

Table one

Members of the Paramyxovirus group ; parainfluenza types 1 - 4; mumps, measles.

Properties of Paramyxoviruses

Pleomorphic virion, diameter 150-300 nm.
Nucleocapsid with helical symmetry.
Envelope containing haemagglutinin and often neuraminidase. RNA, single-stranded, one molecule, molecular weight 7×10^6.
Transcriptase in virion.

Members of the Orthomyxovirus group: influenza viruses A, B and C

Properties of Orthomyxoviruses:

Filamentous or spherical virion, diameter 80 x 120 nm
Nucleocapsid with helical symmetry.
Envelope, containing haemagglutinin and neuraminidase
RNA, single-stranded, 7 molecules, total molecular weight 5×10^6.
Transcriptase in nucleocapsid.

(Andrewes, Pereira and Wildy, 1978)

The orthomyxoviruses comprise of three influenza virus types - influenza viruses A, B and C; influenza virus A is the principal cause of epidemic influenza, B is usually associated with a milder disease but can also cause winter epidemics, C is of low or doubtful pathogenicity in man. It has been known for many years that influenza is a viral disease affecting the respiratory tract, and that the appearance of the disease as pandemics at different times in human populations are caused by different strains of the virus. Although other infections can produce many of the symptoms of influenza, only influenza causes sudden epidemics that persist for a few weeks - and may equally suddenly disappear (Burnet and White, 1972). German records place the earliest recognisable epidemic of influenza in 1170, and from other historical records it has been possible to compile a fairly complete list of epidemics in Europe since the middle ages. (Burnet and Clarke, 1942; Burnet and White, 1972).

Importance of these viruses in diagnostic virology

Laboratory tests for serological evidence of recent viral respiratory infection form a major part of the work load of a routine diagnostic laboratory. Therefore attempts to improve such serological tests for example by increasing their sensitivity or specificity; simplifying the production or improving the effectiveness of test antigens; would be of some considerable value.

Potential adjuvant activity of liposome associated influenza virus antigens:

The possible adjuvant activity of the inert liposome carrier was suggested by Almeida et al in 1975, and also by Gregoriadis in 1976. Influenza virus has detachable surface antigens, haemagglutinin and neuraminidase which are by themselves poorly immunogenic (Webster, Glezen, Hannoun and Laver, (1977). Association of these antigens with an inert liposome carrier might be a means of inducing protective antibody to influenza (Almeida et al, 1975), by a vaccine which used less virus and perhaps produced less pyrogenic reaction.

Haemagglutinin subunits of influenza virus A

Influenza virus particles are known to be covered in 'spikes' as shown in the E.M. investigations of Horne, Waterson, Wildy and Farnham, (1960). These 'spikes' or surface projections are of two different types, haemagglutinin and neuraminidase (Laver and Valentine, 1969). The haemagglutinin (HA) enables influenza viruses to attach by combination with specific adsorption sites composed of neuraminic (sialic acid on the surface of red blood and other cells. Antibodies which neutralise virus infectivity are directed against the HA which is also a glycoprotein (Pereira, 1969), and recurrent influenza epidemics are associated with changes in its antigenic structure. The changes in structure that occur are of two kinds - antigenic shift (major changes) and antigenic drift (minor changes).

These alterations are most likely caused by point mutations in the RNA, and corresponding changes in amino acid sequences of the haemagglutinin polypeptides (Webster, 1972; Waterfield, Espelie, Elder and Skehel, 1979).

Neuraminidase subunits of influenza A

Neuraminidase subunits differ in function and appearance from those of the haemagglutinin. (Laver and Valentine, 1969). Neuraminidase was originally thought to be responsible for the penetration of virus into susceptible cells following attachment to neuraminic acid at the cell surface; the cleavage of neuraminic acid could be required for the entry of virion into the host cell (Webster, 1972). Several observations argue against such a role however, virus of which the neuraminidase had been heat inactivated still penetrated into cells (Fazekas de st. Groth, 1948). Such virus prevented the multiplication of active virus within the same cell by production of interferon, (Isaacs and Edney, 1950); and also participated in genetic recombination with other influenza viruses, (Burnet and Lind, 1954). However high titred antibody to neuraminidase modifies disease even if it does not prevent infection, and this 'second line' of defence may play some role in immunity when there have been antigenic changes in the haemagglutinin (Kilbourne, Schulman, Couch and Kasel. 1971).

Both the haemagglutinin and neuraminidase of influenza virus A are proteins which show antigenic variation, and antibodies to them can be used to distinguish different strains

of influenza viruses. Antibodies to the ribonucleoprotein (the 'S' or soluble antigen), and matrix protein are formed in the body after infection and are specific for types A, B, and C influenza viruses. They do not however distinguish between subtypes or strains within each type and are thought to be unimportant in affording protection against reinfection. (Slepushkin, Schild, Beare, Chinn and Tyrell, 1971; Hobson Curry, Beare and Ward-Gardner, 1972: Dowdle, Mostew, Coleman and Kaye, 1973).

Protection from influenza virus infection:

Since there is no generally applicable and effective antiviral chemotherapy, protection from infection by influenza virus depends on natural immunity or by vaccination against current strains. Vaccination protects against infection with the same serotype of virus on re-exposure to the strain of virus used in the vaccine; for example, by natural exposure in epidemic conditions.

Disadvantages of current vaccines:

Available vaccine preparations include whole virus vaccines, for example 'Admune", (Duncan Flockhart and Company); 'Influvac', (Duphar Labs Ltd.); and disrupted virus vaccines, 'MFV-ject'. (Servier Labs Ltd.); purified adsorbed surface antigen vaccine, 'Fluvirin', (Duncan Flockhart and Company). However vaccines containing intact virus particles sometimes cause adverse reactions both systematically and at the site of injection. The purity of these vaccines has improved

over the years (Reimer, Baker, Van Frank, Newton, Cline and Anderson, 1967); but even highly purified whole virus vaccine preparations cause reactions in sensitive individuals or young children. (Quilligan, Salgado and Alena, 1961; Peck, 1968). Because of this, influenza virus subunit vaccines have been developed and should offer protection from influenza virus infection with minimal adverse reaction. (Brady and Furminger, 1976; Potter, Jennings, Phair, Clarke and Stuart-Harris, 1977; Feery, Evered and Morrison, 1979; Eastwood, Jennings, Milner and Potter, 1979).

Hoever these vaccines may contain a small proportion of whole virus, and reduce the total required viral content to around one-tenth of that of whole virus vaccines. (Duncan Flockhart and Company, 1978). The potential risk of adverse reactions in sensitive individuals is therefore still present. In addition, influenza subunit vaccines were found to be poorly immunogenic in unprimed lower animals and man (Barry, Staton,and Mayner, 1975) and two doses of subunit vaccine were required to induce an acceptable level of antibodies. (Parkman, Galasso, Top and Noble, 1976).

This investigation into the possible applications of inert substrates as carriers, therefore included a comparison of the immunogenic potential of a purified influenza virus subunit prepartion, coupled to an inert liposome carrier compared to that of subunits without liposomes.

Methods of obtaining influenza virus A subunits:

Three methods of obtaining purified influenza virus A subunits will be described. Briefly these are:

i) Detergent disruption of whole virus, followed by sucrose density gradient centrifugation described by Almeida and her colleagues in 1975, and further detailed in a personal communication (see methods). The final product of this technique includes both haemagglutinin and neuraminidase subunits; haemagglutination activity remains unaffected by the purification procedures.

ii) Detergent disruption of a concentrated preparation of pure virus, followed by electrophoresis on cellulose acetate supports, as described by Skehel and his co-workers in 1975, and later demonstrated by Dr. Skehel to the author - see methods. The products obtained consists solely of the haemagglutinin class of subunit and haemagglutination activity is unaffected.

iii) Proteolytic digestion of whole virus with bromelain, as described by Skehel and Waterfield (1975). Although the product obtained consists solely of the haemagglutinin class of subunit, it is structurally modified, has no

haemagglutinin activity and was considered less likely to remain associated with the liposome carrier. This method of subunit preparation was therefore regarded as the least suitable of the three methods and was not used.

Methods i) and ii) were therefore chosen for experiments in this study.

Chapter 3 MATERIALS and METHODS.

Viruses used:

The viruses used were influenza virus A strains; X-31 (H_3N_2), Russia 77 (H_1N_1); influenza virus B strain Lee 40; parainfluenza virus type 1 strain Sendai, type 2 strain Greer, and mumps virus strain Enders. The X-31 influenza virus A strain reported by Kilbourne, (1969) to be a recombinant between A2/Hong Kong/68 virus and AO/PRS was obtained from the Medical Research Council Influenza Reference Laboratories, Mill Hill, London. All other virus strains were obtained locally.

Virus cultivation:

Virus preparations were obtained by inoculation of 0.2ml. virus per egg into the allantoic cavity of ten-day old embryonated eggs. The eggs were incubated at 35^0C for 48 hours and then chilled at 4^0C for one hour before harvesting the allantoic fluid. HA tests were then carried out and fluids with low titre (less than 128) discarded.

Purification of whole virus:

Virus preparations were purified by the method described by Skehel and Schild (1971) : Briefly allantoic fluid from infected eggs was clarified by centrifugation at 2000g for 60 minutes, to remove gross contaminants. The fluid was then centrifuged in a sucrose density gradient (20-60% W/V sucrose in PBS) at 100 000g for 16 hours. The virus was concentrated in a sharp band at a sucrose concentration of

45-48% W/V, and was removed as a pellet from the sucrose suspension by centrifugation (100 000g for 60 minutes) and then resuspended in PBS.

Isolation of virus subunits

Two methods were used to obtain preparations of influenza virus A subunits; method (i) as described by Almeida et al (1975), and later detailed in a personal communication; and method (ii) as described by Skehel and Waterfield (1975), and carried out by me at the Influenza Reference Laboratories in Mill Hill, London.

Method (i) yields both influenza A envelope proteins - namely the haemagglutinin and neuraminidase.

Method (ii) involves a more selective protein separation technique and results in the release of haemagglutinin sub-units: neuraminidase is lost with this method.

(i) Detergent disruption

The method described by Almeida et al (1975), yields a preparation of intact haemagglutinin and neuraminidase sub-units. Basically, a detergent is used to disrupt the virus and liberate its envelope proteins which are then purified by sucrose density gradient centrifugation. Purified virus is diluted to a protein concentration of 1mg/ml and the detergent NP40 added to make a final concentration of 0.2% W/V: the resultant preparation was then held at 37^{0}C for 15

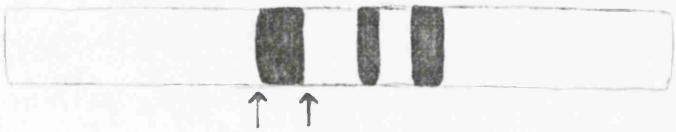
minutes before centrifugation at 10 000g for 3 hours. The supernatant was collected and concentrated (approximately 5:1) by vacuum dialysis and layered onto 20-60% sucrose gradients, before centrifugation at 100 000g for 18 hours. After this, approximately 35 fractions were collected and tested for HA titre: fractions with high HA titres were pooled and dialysed against PBS overnight to remove sucrose and detergent. The final preparation was assayed for protein concentration and again for HA activity, diluted to 1mg/ml and then finally filtered through 0.45 and 0.22 micron filters.

(ii) Cellogel electrophoresis

Purified virus was disrupted using the non-ionic detergent SDS and its protein components separated by electrophoresis on cellulose acetate slabs, as described by Laver, (1964); and Skehel and Waterfield (1975). Briefly, purified virus was suspended in PBS (50mg/ml) and disrupted by the addition of SDS to a final concentration of 2% W/V at room temperature for 10-15 seconds. Approximately 2mg of protein was applied to each slab of cellulose acetate (25 cm by 5 cm) and electrophoresis carried out at 8 V/cm for 4 hours. The electrophoresis buffer was 0.66M Tris; 0.12M boric acid; 0.015M EDTA; 0.5% SDS, pH 8.9. Following electrophoresis, the separated proteins were located by overlaying the cellulose acetate slab with a strip of cellulose acetate for 2-3 minutes, thus obtaining an imprint of the proteins present (figure 3). Proteins were stained in the strip with coomassie brilliant blue (0.75% W/V)

in methanol: hydrochloric acid (97.5%/2.5% V/V) followed by destaining in methanol. Virus envelope proteins were obtained by sectioning the cellulose acetate slab in line with the stained protein imprint, so that the proteins eluted into PBS. SDS was removed from the subunit preparation by overnight dialysis against PBS at 4^0C.

Figure three



A cellulose acetate strip stained with Coomassie Brilliant Blue showing the electrophoretic separation of the protein components of the recombinant influenza A virus, X-31, after disruption of the virus particles with SDS. Arrows show where companion portions of the cellogel slab were cut. The haemagglutinin subunits were eluted from the slab in PBS as described.

Assessment of subunit purity

Quality control analyses to check on the purity of the products obtained from methods (i) and (ii) were clearly of importance. The following were thought the most appropriate.

a) Infectivity in tissue culture; the subunit preparation should be non-infective, a negative result should exclude the presence of live virus.

b) Electrophoresis of subunit proteins in the presence of sodium dodecyl sulphate (SDS-page technique): comparison of the banding patterns produced with reference preparations would reveal contaminant protein.

c) HA activity: the preparation should be antigenically intact and capable of agglutinating red blood cells.

d) Protein concentration - estimated by a Lowry analysis for purposes of standardisation.

In addition, subunits satisfactorily purified associated with preformed liposomes would be investigated by negative stain transmission electron microscopy.

a) Infectivity in tissue culture

The subunit preparations obtained for use in this study were tested for the presence of live infectious virus. This technique would not of course detect contaminant inert whole virus. Subunit preparations were inoculated into tube cell cultures of African Green Monkey Kidney cells. These cell cultures were then examined daily, firstly for CPE formation and secondly for haemadsorption with human 'O' cells. This was repeated over a period of 21 days, with a blind pass at day 14. Haemadsorption positive tubes were further examined by indirect immunofluorescence for influenza virus A antigen. Controls consisted of viable virus, and virus which had been heat inactivated (56^{0}C for 30 minutes). If by day 21 no signs of viral growth had been detected in any tubes apart from the positive controls, it was concluded that the sub-unit preparation under test did not contain live influenza virus.

(b) Protein characterisation

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was the method of choice for detecting the presence of contaminant protein in subunit preparations. The conditions for dissociation and electrophoresis were essentially as described by Skehel and Schild. (1971). Samples containing approximately 100 µg of protein in 0.01M PBS, pH 7.2 were mixed with urea, SDS and β - mercaptoethanol to final concentrations of 5M, 1% and 2% respectively. The mixtures were then heated at 100°C for 1 minute and after cooling were applied directly to polyacrylamide slab gels containing 7.5% acrylamide and 0.26% bisacrylamide. They also contained sodium phosphate buffer (0.05M, pH 7.2), EDTA (0.01M), SDS (0.1%), and TEMED (0.1%): polymerization was catalysed with 0.1% ammonium persulphate. The electrophoresis buffer was 0.05M sodium phosphate, pH 7.2, containing 0.1% SDS and 0.01M EDTA. Electrophoresis was carried out at 2.5 Vs/cm for 14 hours, after which the banded proteins were fixed, stained with Coomassie brilliant blue and destained in methanol: water: acetic acid (50:50:7).

(c) Haemagglutination (HA) tests

HA tests were used to check the antigenicity of the subunit preparations produced. The technique used was that described by Grist et al, (1979). In brief, doubling dilutions (unit volume 0.025 ml.) were made in PBS in duplicate for each subunit preparation. One volume of PBS was then added to each dilution followed by two volumes of 0.5 per cent human '0' red blood cells, and left at room temperature for one hour. Appropriate red blood cell controls with PBS substituted for virus were included. The highest dilution of test preparation which gave complete (100 per cent) agglutination was taken as one haemagglutinin unit (1HU).

(d) Protein estimation

Protein content of subunits was assayed by the method of Lowry, Rosebrough, Farr and Randall (1951). Bovine plasma albumin was used as a standard.

Electron Microscopy

A loopful of the sample under investigation was placed on a carbon coated Formvar grid followed by a loopful of ethanol to facilitate spreading. A 4% solution of negative stain, potassium phosphotungstate (PTA) was then applied and the excess removed after 30 seconds. The sample was then inserted into the microscope for examination. This work was performed under the supervision of Dr. Parry at the M.R.C. Institute of Virology, on a Siemens EM 101 at 80 KV. The final magnification achieved was 100 000X.

Liposome preparation

Liposomes were prepared using methods described by Gregoriadis, Leathwood and Ryman (1971). Briefly, lecithin, cholesterol and dicetyl phosphate were mixed together in the molar ratios of 7:2:1, dissolved in chloroform (200:57:28.5 µ moles in 25 ml chloroform) and then evaporated to produce a thin film on the inside walls of a glass flask. 20 ml of 3.3mM PBS (pH 7.2) was added and the mixture gently shaken and sonicated in a Megason water bath sonicator at 50 KHz for one hour. Virus associated liposomes or subunit associated liposomes were obtained by adding purified virus or subunits to pre-formed liposomes in the ratio of 1:8 and further sonicated for 30 minutes. If 0.1 ml of a virus preparation containing 512 haemagglutinating units per 0.025 ml , was added to 0.7 ml pre-formed liposomes, the final virus

liposome preparation had an HA titre of 64 (i.e. 64 haemagglutinating units per 0.025 ml) at a lipid concentration of approximately 0.7g. lipid per dl.

Serological techniques

i) Complement fixation (CF) tests

CF tests were carried out by the method of Grist et al (1979), using the modification described by Zissis and Clinet (1974). Briefly this is a five volume modification of the normal CF technique, using dextrose gelatine veronal buffer (DGV), and microtitre 'U' plates. Red blood cells were used at a concentration of 0.25% in order to increase the sensitivity of the test: the dose of complement was 2 units instead of the conventional 4 units, and the titre of haemolysin was 4 units. This modification of the CF test is held by Zissis and Clinet (1974) to be advantageous in that it gives a stable result which can be read at different times with different incubation temperatures, is thought to be more reproducible, requires less antigen, and is more sensitive. Standardisation of conventional and liposome associated antigenic substrates, of complement, haemolysin and standard antisera, were all performed before the test proper.

ii) Immunofluorescence tests

The procedure used for indirect immunofluorescence testing was essentially as described by Liu (1969). Briefly heat inactivated (56^{0}C for 30 minutes) sera were diluted in

the microtitre system and applied directly to acetone fixed antigen substrates - either conventional infected cells or virus liposomes. These were incubated at 37^0C for 30 minutes, rinsed in PBS for 10 minutes, and specific FITC conjugated antiserum applied for a further 30 minute incubation period, before another rinse in buffered saline, and examination under the fluorescence microscope.

Preparation of infected cell material for use as antigenic substrate in the test

The method used for influenza virus A was as follows: Cultures of cell monolayers (African Green Monkey Kidney) in tubes were washed twice in sterile PBS and 0.2ml of a 10^{-1} dilution of virus (25.6HU_s per 0.025 ml) was added to each tube. Virus was allowed to adsorb onto cells for one hour at 4^0C to synchronise infection. 0.8 ml of serum free medium was added to each tube, and the cultures incubated at 35^0C for 2.5 hours. The monolayers were then rinsed with PBS. Virus infected cells were removed with 0.4% EDTA in PBS containing 0.1% W/V glucose and mixed with trypsin 0.25% W/V in the ratio of 5:1. The EDTA - Trypsin mixture was then removed by centrifugation of infected cells at approximately 600g for 10 minutes. Cells were resuspended in PBS and spotted onto glass slides (cleaned by overnight wash in decon before use) and the cell concentration checked microscopically. These antigen substrates were then air dried, fixed in acetone for 10 minutes at room temperature, and stored at -70^0C until required. The percentage of infected cells was then checked

to ensure that approximately 50% were infected: this was done by performing a standard indirect test with a positive control human test serum as described above. Antibody end points were assessed on conventional infected cell substrates as the highest dilution of serum at which 50% of the cells which fluoresced in the positive control were detectable.

Preparation of virus liposomes for use as antigenic substrate in the test.

Virus liposome mixtures were spotted and fixed onto glass slides as described above. The optimal ratio of viral haemagglutinating units to liposome content for immunfluorescent testing was estimated by varying the concentration of the purified virus component in virus liposome mixtures used as substrate in immunofluorescent tests. 64 HA units of virus per 0.7g. of lipid per dl of liposomes was found to be optimal.

On virus liposome substrates, the end point was taken as the highest dilution of serum at which bright particulate fluorescence could be detected.

Plates 1 - 6 are representative positive fields for the IIF substrates used. Magnification approximately 400 X.

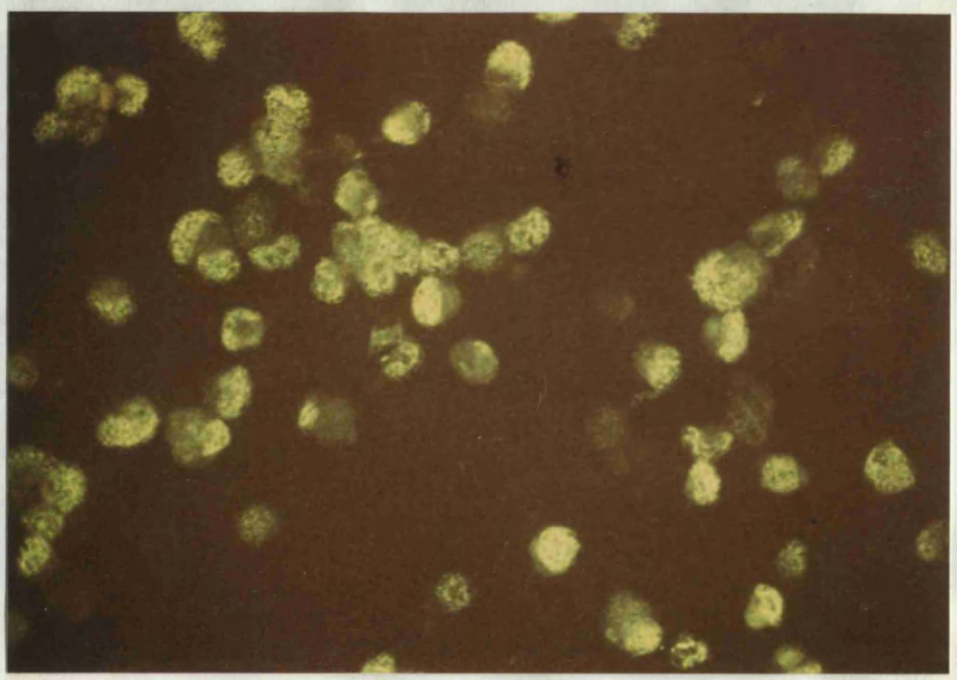


Plate 1

Influenza A
infected cell
IIF substrate

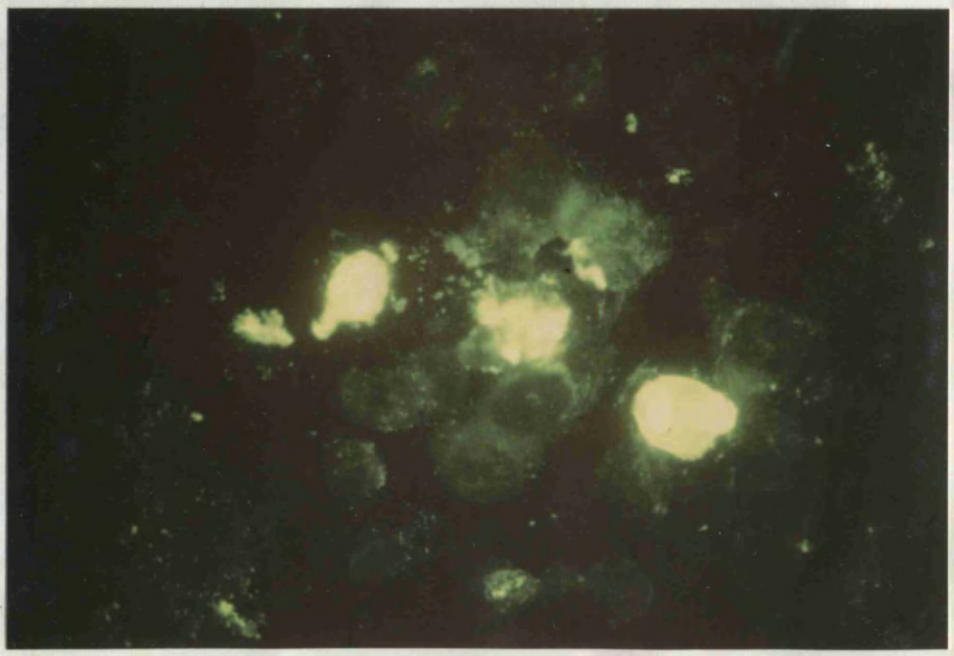


Plate 2

Influenza B
infected cell
IIF substrate

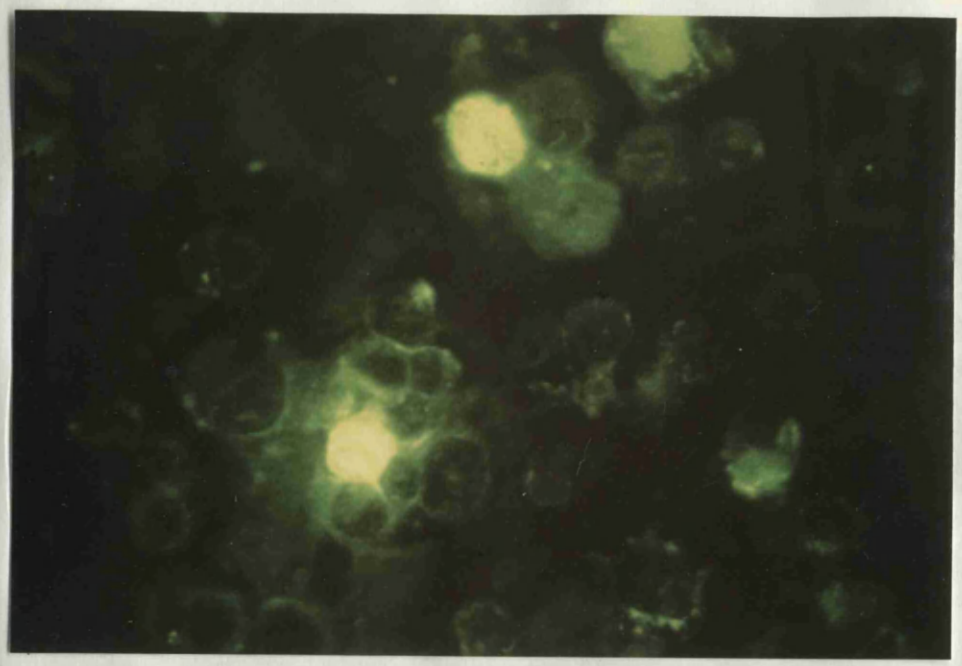


Plate 3

Parainfluenza type 1 infected cell IIF substrate

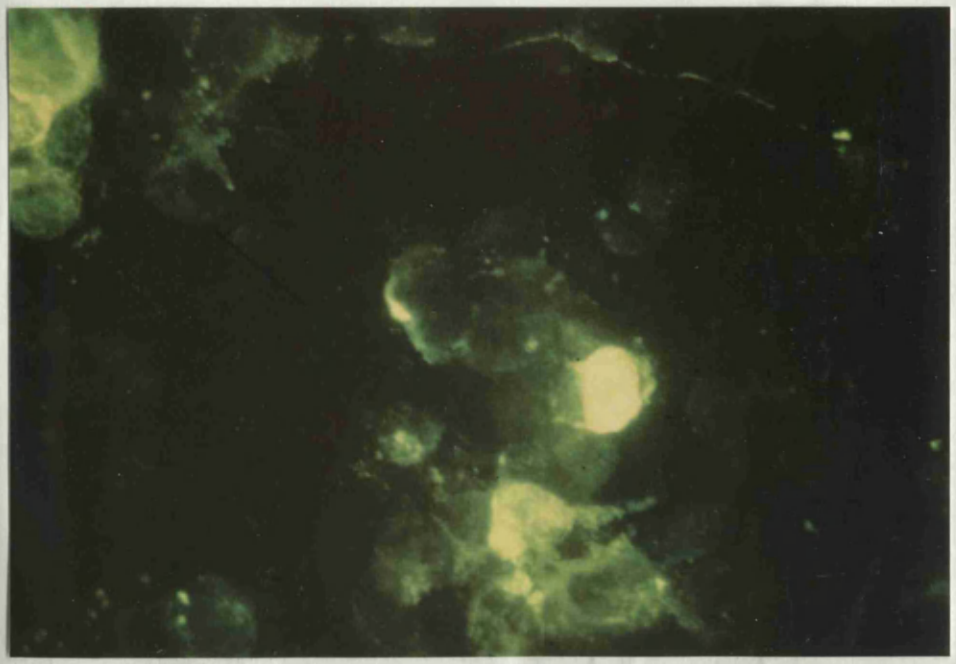


Plate 4

Parainfluenza type 2 infected cell IIF substrate

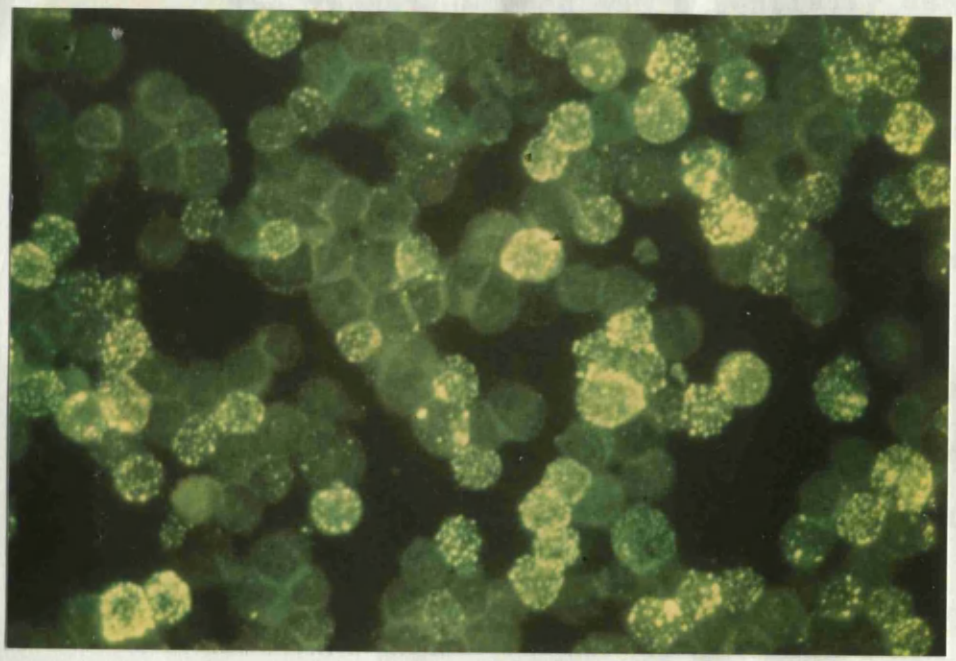


Plate 5

Mumps virus infected cell IIF substrate (note non specific fluorescence in uninfected cells).

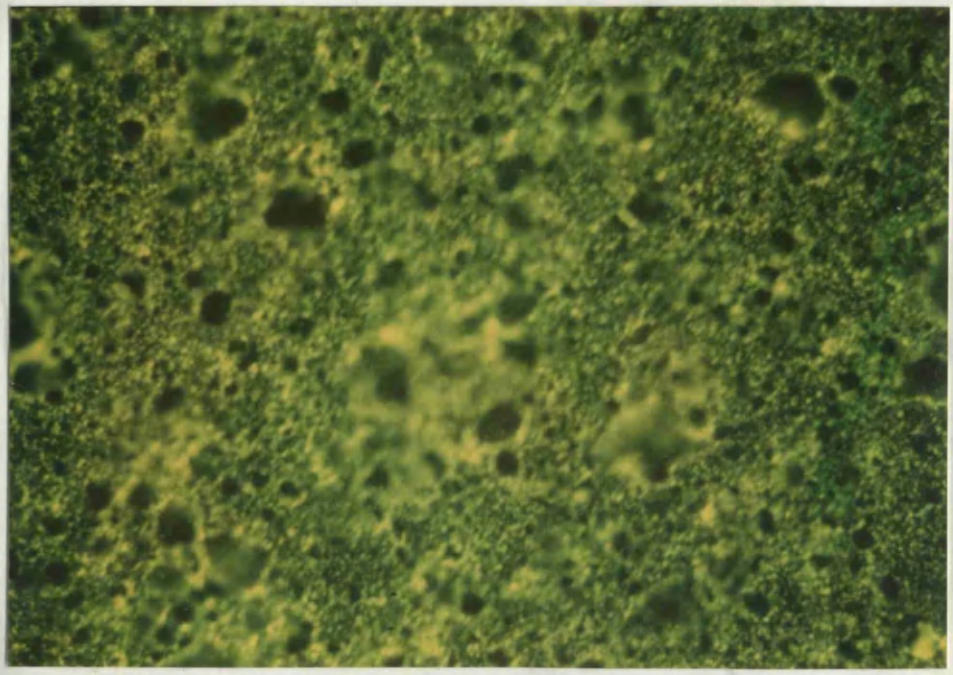


Plate 6

Standard virus-liposome IIF substrate

iii) Haemagglutination inhibition (HAI) test

The technique used was essentially as described previously for HA testing (Grist et al, 1979). Test sera were treated overnight with receptor destroying enzyme (RDE) to remove non specific inhibitors before titration in the microtitre system. Appropriate controls were included in all tests, i.e. positive and negative test sera, and red blood cell controls. Antigens were used at 4 HU_s, and human group 'O' red blood cells obtained from Blood Transfusion Service, were used at a standard concentration of 0.5%.

Collection of sera

The human sera tested were specimens received for the diagnosis of viral respiratory infection. In tests with a particular virus, all the sera examined were known to contain antibody to the virus.

Animal inoculation

Guinea pigs were inoculated by the intraperitoneal route, and blood collected at appropriate time intervals (see results) by cardiac puncture under Fluothane anaesthetic.

10-12 week old male guinea pigs, approximately 300g in weight were used throughout.

Statistical analyses

In order to establish the correlation that existed between sets of data, a desk calculator was programmed for linear regression analysis and appropriate data entered ($\log_2$ antibody titres). In this way linear lines which best represented the graph of all known data were obtained. This programme was also used to calculate the coefficient of determination (r^2) the square root of which (r) is the absolute value of the correlation coefficient.

Geometric mean titres were obtained using the following formula:

$$G = \text{antilog} \quad \frac{\log X_1 + \log X_2 + \log X_3 \ldots}{N}$$

where G is the geometric mean titre

X is the reciprocal antibody titre

and N is the number of samples.

Dextrose Gelatine Veronal Buffer (DGV)

Barbituric acid	0.58g
Gelatine	0.6 g
Sodium barbitol	0.38g
$CaCl_2$	0.027g
$MgSO_4\ 7H_2O$	0.12g
NaCl	8.5 g
Dextrose	10.0 g

Made up to 1 litre with deionised H_2O, pH to 7.2 then autoclaved at 115^0C for ten minutes.

Phosphate buffered saline (PBS)

Supplied by Oxoid in the form of tablets, which when reconstituted in distilled water (10 tablets per litre) converted to phosphate buffered saline (Dulbecco 'A'), this was then autoclaved at 115^0C for ten minutes.

Human red blood cells

Human group '0' red blood cells were supplied as a packed suspension, by the Blood Transfusion Service, and stored in this form for up to 14 days at 4^oC. Before use in serology tests, the cells were washed three times in PBS (centrifugation at 1000g for 15 minutes) and suspended to a concentration of 0.5% (V/V) in PBS.

Sheeps blood in Alsevers

50% whole sheeps' blood in 50% Alsevers solution, (obtained from Gibco biocult) was used.

Haemolysin

Rabbit haemolytic serum was obtained from Flow laboratories.

Guinea pig complement

One part of the reconstituted guinea pig serum was diluted with seven parts of distilled water, to give a dilution of 1/10 ; any further dilutions from this were made in DGV. Complement was obtained from Flow laboratories.

Microtitre equipment

Micro diluters and disposable test droppers were supplied by Titertek equipment and plastic U plates by Sterilin Ltd).

Tissue culture medium

Medium 199 supplied by Flow Laboratories, was used throughout unless otherwise stated.

Cell culture

African Green monkey kidney cells (primary culture) were supplied by Flow Laboratories.

Fluorescence Microscopy

A Leitz ortholux epi-illiminated microscope with an ultra-high pressure mercury lamp was used for the immunofluorescence studies. Photomicrographs were taken with a Leica camera attachment using Kodak Ektachrome high speed film.

Animals used

Adult male guinea pigs of the Dunkin-Hartley strain were supplied by A. Tuck & Sons, Exeter.

FITC conjugated antisera

i) anti-human IgG, produced in goats was supplied by Kallestad Laboratories.

ii) anti-guinea pig IgG, produced in sheep was supplied by Wellcome Reagents Ltd.

Chapter 4 RESULTS (a)

The use of an inert carrier of antigen in virus serology.

The use of an inert liposome carrier of viral antigen in diagnostic serology was tested in comparison to conventional antigen preparations in three test systems: namely, CF, HAI and IIF.

This was to find out if combination with liposomes would prove a more economical use of virus antigen. Virus liposome mixtures might also be more stable and have a longer shelf life than conventional virus antigen preparations. Furthermore in indirect immunofluorescence they could provide antigenically defined test substrates i.e. containing purified viral proteins, and so make possible tests for antibody directed against specific structural components of the virus.

In the follo wing experiments, purified virus or virus components were attached to liposomes and used as antigen in tests to detect virus antibody.

Complement Fixation

Preliminary experiments had shown that virus liposome mixtures could fix complement in the same way as conventional virus preparations. Before carrying out tests for viral CF antibody, two dimensional (chessboard) titrations of each antigen and its corresponding antiserum were set up to estimate optim al peak antigen dilutions. The results recorded in table two show that virus liposome antigens had similar CF activity to conventional virus antigens. Uninfected control antigen preparations confirmed the specificity of the antigen - antibody reaction.

When the optimal concentration of antigen had been determined, CF tests were carried out to compare the titres of CF antibody to five different viruses using conventional virus and virus liposome preparations as antigens. Tests were carried out on human sera known to contain antibody to the virus under test.

The results are summarised in table three.

table two

CF Chessboard Titrations

Preparation used as CF antigen :	Optim al Peak Dilution	Control antigen
influenza A virus	32	0
influenza A virus-liposomes	16	0
influenza B virus	16	0
influenza B virus-liposomes	16	tr
parainfluenza virus type 1	32	0
parainfluenza virus type1-liposomes	32	0
parainfluenza virus type 2	8	0
parainfluenza virus type 2-liposomes	8	0
Mumps virus	16	0
Mumps virus-liposomes	16	0

note: tr - trace of activity

table three Results of experiments to compare virus liposomes with conventional virus antigen preparations in complement fixation tests for viral antibody.

Virus	Number of sera tested:	Number sera (%) with same titre :*	Number of sera (%) with higher titres using virus liposome antigens	Number of sera (%) with lower titres using virus liposome antigens	Correlation coefficient r **
Influenza A	40	38 (95)	1 (2.5)	1 (2.5)	0.90
Influenza B	35	34 (98)	0	1 (2)	0.92
Parainfluenza type 1	25	24 (96)	0	1 (4)	0.86
Parainfluenza type 2	25	24 (96)	1 (4)	0	0.73
Mumps	25	25 (100)	0	0	0.95

* less than or only 1 $\log_2$ difference between tests

** $p < 0.001$ for all correlation coefficients

Tests using virus liposomes as antigen in CF tests.

The results summarised in table three show that virus liposomes are satisfactory antigens in CF tests to detect specific antibody to influenza viruses A & B, parainfluenza types 1 & 2, and mumps viruses. Overall the results were considered to be in close agreement and the correlation coefficients ranged from 0.73 to 0.95; p was < 0.001 in all cases showing these values to be highly significant.

Differences of 1 $\log_2$ in titre were commonly encountered, but this is within the limits of experimental error allowed in such tests. A few sera showed a 2 $\log_2$ difference in titre when tested with conventional and liposome-associated CF antigens but these were only a small proportion (3.3%) of the total number of sera tested.

Importantly when used as antigen, virus liposome mixtures were therefore found to give very similar results to conventional antigens in CF tests for virus antibody. The sensitivity of the test was neither improved nor impared. Identical amounts of virus were required to produce both standardised virus and virus liposome test antigens, so that virus liposomes are not more economical in their use of virus than conventional antigen preparations.

Haemagglutination inhibition

Preliminary work had also shown that virus liposome mixtures were able to agglutinate RBC_s in the same way as conventional virus preparations, and that equivalent dilutions of purified virus in buffer and in liposome mixtures had identical HA values. In other words virus attached to liposomes retained full HA activity.

Conventional and virus liposome antigens were therefore prepared at 4HA units per 0.025 ml., and tests were performed in parallel for HAI antibody to each of the five different viruses. The tests were carried out on human sera known to contain virus antibody, and included appropriate controls.

The results obtained are summarised in table four.

table four Results of experiments to compare virus liposomes with conventional virus antigen preparations in haemagglutination inhibition tests for viral antibody.

Virus	Number of sera tested:	Number of sera (%) with same titre: *	Number of sera (%) with higher titres using virus liposome antigens	Number of sera (%) with lower titre using virus liposome antigen	Correlation coefficient r **
influenza A (H_3N_2)	40	40 (100)	0	0	0.96
influenza A (H_1N_1)	40	40 (100)	0	0	0.95
influenza B	35	35 (100)	0	0	0.96
parainfluenza type 1	25	25 (100)	0	0	0.98
parainfluenza type 2	25	25 (100)	0	0	0.97
mumps	25	25 (100)	0	0	0.86

* less than or only 1 $\log_2$ difference between tests

* * $p < 0.001$ for all correlation coefficients.

Tests using virus liposomes as antigen in HAI tests.

Table four shows that virus liposomes are satisfactory antigens for HAI tests to detect specific antibody to each of the five viruses under test. The results on the two types of antigen were in close agreement and the correlation coefficients ranged from 0.86 to 0.98 ($p < 0.001$ in all cases).

There were occasional minor titre differences (≤ 1 $\log_2$) but these were less commonly encountered than in the preceding CF results; no serum showed a difference in titre greater than 1 $\log_2$. This may be because the HAI technique is less complex to perform than either CF or IIF, and the results therefore more reproducible.

Used as antigen, as in CF, virus liposome mixtures in HAI tests were found to give very similar results to those obtained using conventional antigen preparations. The sensitivity of the test was neither improved nor decreased. Identical amounts of virus were also required to produce standardised virus and virus liposome HA test antigens, so that as with CF, virus liposomes in HAI were not more economical in their use of virus.

Immunofluorescence tests

Preliminary experiments had shown that virus liposome mixtures, dried and acetone-fixed on glass slides were satisfactory antigenic substrates in IIF tests to estimate viral antibody. The optimal ratio of virus-measured in haemagglutinating units - to liposomes for immunofluorescence testing was estimated (as described in materials and methods) to be 64 HA units of virus per 0.7g. of lipid per dl. of liposomes. This concentration was used in all IIF tests.

Immunofluorescent tests to detect IgG viral antibody were carried out using standardised virus liposome substrates in parallel with conventional infected cell substrates. Appropriate controls included known positive and negative test sera and the results were evaluated blind - i.e. with no knowledge of preceding or corresponding results.

The results obtained are summarised in table five.

Table five Results of experiments to compare virus liposomes with conventional infected cell antigen preparations in immunofluorescent tests for viral antibody.

Virus	Number of sera tested:	Number of sera (%) with same titre: *	Number of sera (%) with higher titres on virus liposome substrates	Number of sera (%) with lower titres on virus liposome substrates	Correlation coefficient r **
influenza A	40	38 (95)	2 (5)	0	0.83
influenza B	35	33 (94)	1 (3)	1 (3)	0.91
parainfluenza type 1	25	24 (96)	1 (4)	0	0.86
parainfluenza type 2	25	25 (100)	0	0	0.88
mumps	25	25 (100)	0	0	0.93

* less than or only 1 $\log_2$ difference between tests.

* * $p < 0.001$ for all correlation coefficients

Tests using virus liposomes as antigen in IIF tests.

The results summarised in table five show that virus liposomes are satisfactory antigens in IIF tests to detect antibody. Five viruses were used in the tests. Overall the results were in close agreement and correlation coefficients ranged from 0.83 to 0.93 ($p < 0.001$ in all cases).

As in CF, in a proportion of sera there were differences of 1 $\log_2$ in titre encountered, but this is within the limits of experimental error. A small proportion of sera (4.7%) showed 2 $\log_2$ differences in titre using virus-liposome substrates in comparison to conventional infected cell preparations. However this was considered acceptable since it represents a similar degree of variability to that normally encountered in serological tests using conventional virus antigen preparations.

Virus liposome substrates therefore gave very similar results in IIF tests for viral antibody to those obtained with conventional infected cell preparations. The sensitivity of the test was the same i.e. neither improved nor diminished,

The data obtained on individual sera with the three types of serological tests are shown in detail in tables 6-10. The data are presented in full so that results using virus liposomes and conventional antigen preparations can be directly compared in three different tests.

table six

Reciprocal antibody titres to Influenza A

Serum Number	CFT V	CFT VL	HAI (H_3N_2) V	HAI (H_3N_2) VL	HAI (H_1N_1) V	HAI (H_1N_1) VL	IIF IC	IIF VL
11/07	<16	<16	512	512	<16	<16	16	16
11/107	<16	<16	128	128	16	16	32	64
11/412	32	32	256	128	128	128	32	16
2/78	128	64	128	256	64	64	128	64
12/145	256	356	256	256	16	<16	256	256
12/499	512	512	1024	1024	64	64	16	<16
12/845	128	256	512	512	64	64	64	64
2/887	16	32	64	64	64	64	16	16
1/2354	<16	<16	16	16	<16	<16	32	16
1/2841	256	256	512	256	128	256	<16	<16
2/198	256	128	1024	1024	256	256	64	128
2/642	256	256	128	256	16	16	64	128
2/713	64	128	512	512	256	128	16	32
2/775	128	128	512	512	64	128	32	32
2/890	256	256	1024	1024	128	256	32	32
2/1022	256	256	256	256	128	128	16	16
2/1041	64	128	256	256	1024	256	<16	<16
2/1042	16	32	128	128	64	64	<16	16
3/60	32	16	<16	<16	128	128	16	32
3/123	64	16	64	64	16	16	32	32
3/847	128	128	128	128	32	32	64	128
3/900	32	128	256	256	512	512	16	32
3/1553	32	32	1024	1024	256	128	32	64
3/1819	128	64	256	256	256	256	64	128
3/1996	64	128	32	64	16	<16	16	32
3/1997	64	64	512	512	128	64	16	32
3/2368	128	64	512	256	32	32	64	128
3/2399	256	128	512	512	128	128	128	64
3/2421	128	128	128	128	64	64	32	16
3/2501	<16	<16	<16	<16	32	32	16	16
3/2517	128	128	128	128	16	16	16	16

Note: V: conventional virus antigen. IC infected cell substrate antigen.
VL: liposome associated virus antigen.

table six cont'd.

Reciprocal antibody titres to Influenza A
cont'd

Serum Number	CFT		HAI (H_3N_2)		HAI (H_1N_1)		IIF	
	V	VL	V	VL	V	VL	IC	VL
3/3153	<16	<16	64	64	64	64	16	32
4/707	32	64	64	64	32	16	16	16
4/162	128	128	256	256	32	16	64	256
4/1183	128	128	1024	1024	128	128	64	256
4/1513	128	64	512	512	256	256	16	16
4/2622	128	256	256	256	512	512	128	64
4/2750	128	128	128	128	16	16	16	16
4/3535	128	128	16	16	64	64	32	32
5/312	128	128	128	128	16	16	16	16

table seven

Reciprocal antibody titres to influenza B

Serum Number	CFT		HAI		IIF	
	V	VL	V	VL	IC	VL
1/2812	64	64	64	64	16	16
2/772	<16	<16	128	64	<16	< 16
2/869	128	64	256	256	64	128
2/1602	16	16	256	256	256	256
2/2490	256	256	128	128	128	256
2/2638	64	128	16	32	16	16
3/586	128	128	32	32	32	32
3/656	512	512	512	512	128	64
3/2501	256	256	16	16	16	16
3/2699	128	256	64	128	64	64
3/2758	256	256	128	128	128	256
3/2763	<16	<16	16	32	< 16	<16
3/3117	64	64	128	128	64	32
3/3347	64	64	16	16	16	32
4/404	64	32	256	256	32	32
4/1450	64	64	128	128	16	64
4/1451	32	32	64	64	32	32
4/1795	512	512	2048	1024	512	256
4/2065	256	256	256	256	128	128
4/2497	128	128	256	256	128	128
4/2107	256	256	512	512	256	64
4/2110	64	128	32	32	64	64
4/3197	512	256	1024	1024	512	512
4/3233	256	256	256	256	256	512
5/235	512	512	32	32	256	512
5/978	512	512	1024	1024	128	256
5/1189	128	128	1024	1024	32	64
5/1544	256	128	512	512	64	64
5/1588	64	128	64	64	<16	< 16
5/2311	256	64	1024	1024	< 16	< 16
5/2493	256	128	128	128	64	64
6/62	128	128	512	1024	256	256
6/911	512	256	256	256	128	256
6/946	64	64	512	512	128	256
6/1160	128	128	64	64	256	128

table eight

Reciprocal antibody titres to parainfluenza type 1

Serum Number	CFT		HAI		IIF	
	V	VL	V	VL	IC	VL
1/2351	512	512	1024	1024	256	256
2/1748	256	256	32	32	64	32
3/2141	32	32	<16	<16	<16	<16
4/2330	32	32	64	64	16	16
4/2622	32	64	32	32	16	16
5/307	128	128	256	256	32	64
7/1103	32	64	16	32	<16	<16
7/1122	16	32	<16	<16	32	64
7/2976	128	32	<16	<16	64	64
8/456	32	64	256	128	8	8
8/454	32	32	32	32	<16	<16
8/457	32	32	128	128	16	8
8/883	16	32	32	32	<16	<16
8/884	16	16	<16	<16	16	8
8/1072	16	32	128	128	16	16
8/1235	16	16	16	16	16	16
8/1500	32	16	256	256	32	16
8/1524	16	16	64	64	<16	<16
8/1776	32	16	64	64	16	32
8/2950	16	<16	<16	<16	<16	<16
8/3029	16	16	16	16	<16	32
8/3220	32	32	<16	<16	<16	<16
8/2438	32	32	64	64	16	8
8/2912	<16	16	16	16	<16	<16
8/2863	<16	<16	<16	<16	<16	<16

table nine

Reciprocal antibody titres to parainfluenza type 2

Serum Number	CFT		HAI		IIF	
	V	VL	V	VL	IC	VL
1/2812	64	32	16	16	<16	<16
1/2805	<16	<16	<16	<16	<16	<16
2/881	64	128	<16	<16	16	16
2/1022	64	64	32	32	32	16
2/1880	32	32	16	32	32	32
2/2599	32	64	64	64	8	<16
2/2704	32	32	16	16	<16	<16
2/2242	32	32	128	128	16	32
3/204	64	128	256	256	128	256
3/344	32	32	16	16	16	16
3/1299	32	32	64	32	16	16
4/2065	32	32	32	32	8	16
4/2360	128	128	128	128	64	32
4/3083	64	64	256	256	32	64
5/862	32	32	16	32	16	8
5/936	32	32	16	16	8	16
5/3139	64	32	<16	<16	<16	<16
6/730	32	32	<16	<16	<16	<16
7/89	64	128	64	64	16	32
7/177	32	32	32	32	32	32
7/922	32	64	16	16	8	16
7/923	32	64	16	16	16	16
7/1509	32	32	<16	<16	<16	<16
7/1709	32	32	<16	<16	<16	<16
7/1716	32	128	256	256	128	128

table ten

Reciprocal antibody titres to Mumps

Serum Number	CFT		HAI		IIF	
	V	VL	V	VL	IC	VL
11/3310	32	32	256	256	256	128
12/1374	64	64	256	128	256	256
12/1375	32	32	128	256	256	256
12/1516	32	32	256	256	128	128
12/1579	16	16	64	64	128	64
12/2261	32	32	512	256	256	128
12/2394	64	64	512	512	256	256
1/35	64	64	128	128	256	512
1/180	64	64	128	128	256	128
1/682	<16	<16	128	128	128	64
1/1432	32	64	128	128	128	128
1/1607	32	64	128	64	256	256
1/2110	<16	16	256	256	128	128
2/519	<16	<16	16	<16	<16	<16
2/553	64	64	64	32	256	256
2/2886	32	32	256	128	128	128
2/2867	64	64	128	256	128	128
3/519	16	16	64	128	256	256
3/848	32	32	256	256	128	128
3/1472	16	16	64	64	128	256
4/95	<16	<16	128	128	128	128
4/277	16	16	256	256	64	32
4/1093	16	16	64	128	8	16
5/2211	32	32	512	512	256	256
6/1339	<16	<16	64	32	16	16

Serology results:

Of the three virus serology test systems used, HAI would appear to be overall the most sensitive since with all five viruses it gave generally higher titres and in some cases detected antibody which CF and IIF did not (e.g. sera numbers 1/2354, 1/2841, 3/2501 in table six). Occasionally CF detected antibody in sera which were negative by IIF and vice versa (e.g. sera numbers 2/1041, 3/3153 in table six, 5/1588 and 5/2311 in table seven). Some of the discrepancies can be explained by the fact that CF antibody tends to decline sooner than IIF or HAI, so that in many instances these two tests are detecting 'old' persisting antibody not demonstrable by CF. The observation that some sera with high CF and HAI titres showed little or no antibody detectable by IIF was unexpected, (e.g. sera numbers 1/2841, 2/1041 in table six, 7/1103, 8/454 in table eight). These sera may have been collected soon after the onset of illness, and so contained no IgG antibody detectable by the IIF technique at this stage and follow up serum samples would have shown the expected rise in antibody titre concomitant with a current infection.

Clearly, regardless of the technique used paired sera taken in the acute and convalescent stages of illness, in which rising titres can be demonstrated are of most value in the serological diagnosis of infection. However although there were some discrepancies in the results from the three kinds of test, these were found to be generally comparable,

and the use of liposome associated virus antigens instead of conventional virus antigens made little difference both overall and in the case of individual serum samples.

Figures a-e

The following scatter plots (figures a-e) represent a statistical analysis on the results obtained from each of the three serology test systems on sera with antibody to influenza A virus. These scatter plots were constructed with the object therefore of performing simple correlation analyses in order to determine the relationship that existed between several paired sets of variables i.e. antibody titre obtained using virus liposome and conventional antigens.

In figures (a) - (d) antibody titres estimated to influenza A using virus and virus-liposome antigens in CF, HAI and IIF tests were compared. In figure (e) the relationship that existed between two serology test systems, CF and IIF, using conventional antigens was established. The degree of association was measured by the use of coefficients of correlation - which can have values ranging from zero (indicating no correlation) to one (indicating perfect correlation).

Figure (a) illustrates the correlation in titre between CF test results using conventional and liposome associated antigens. A statistically significant high positive linear correlation was established ($0.91\ p < 0.001$) between titres estimated using conventional and liposome associated test antigens.

Figure (b) illustrates the correlation in titres between HAI test results using conventional and liposome associated

antigens for influenza A virus H_3N_2 strains. A statistically significant high positive linear correlation was established (0.96 $p < 0.001$) between titres estimated using conventional and liposome associated test antigens.

Figure (c) similarly illustrates the correlation in titres between HAI test results using conventional and liposome assoicated antigens - but for influenza A virus H_1N_1 strains. Again a statistically significant high positive linear correlation was established (0.96 $p < 0.001$), between titres estimated using conventional and liposome associated test antigens.

Figure (d) illustrates the correlation in titres between IIF test results using conventional infected cell and virus liposome substrates. A statistically significant high positive linear correlation was established (0.83 $p < 0.001$).

Figure (e) illustrates the correlation in titres between CF test results using conventional antigen, and IIF test results using conventional infected cell substrates. Low positive linear correlation not highly significant satistically was established (0.34 $p < 0.05$). Although correlation is clearly present between CF and IIF test results, the correlation coefficient is not especially high and shows a degree of variance between the two systems.

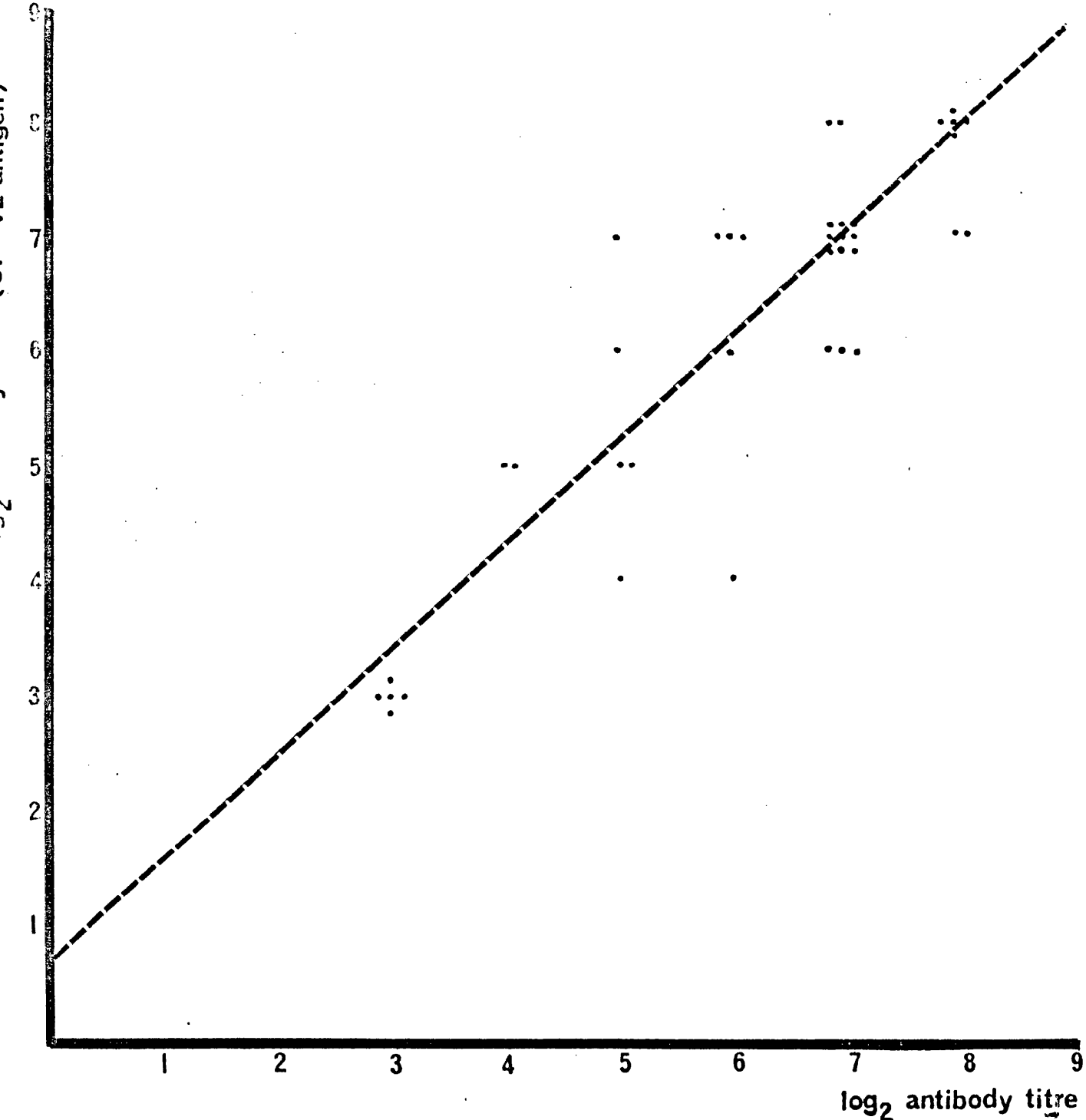


figure (a)

r = 0.91

slope = 0.89

n = 40

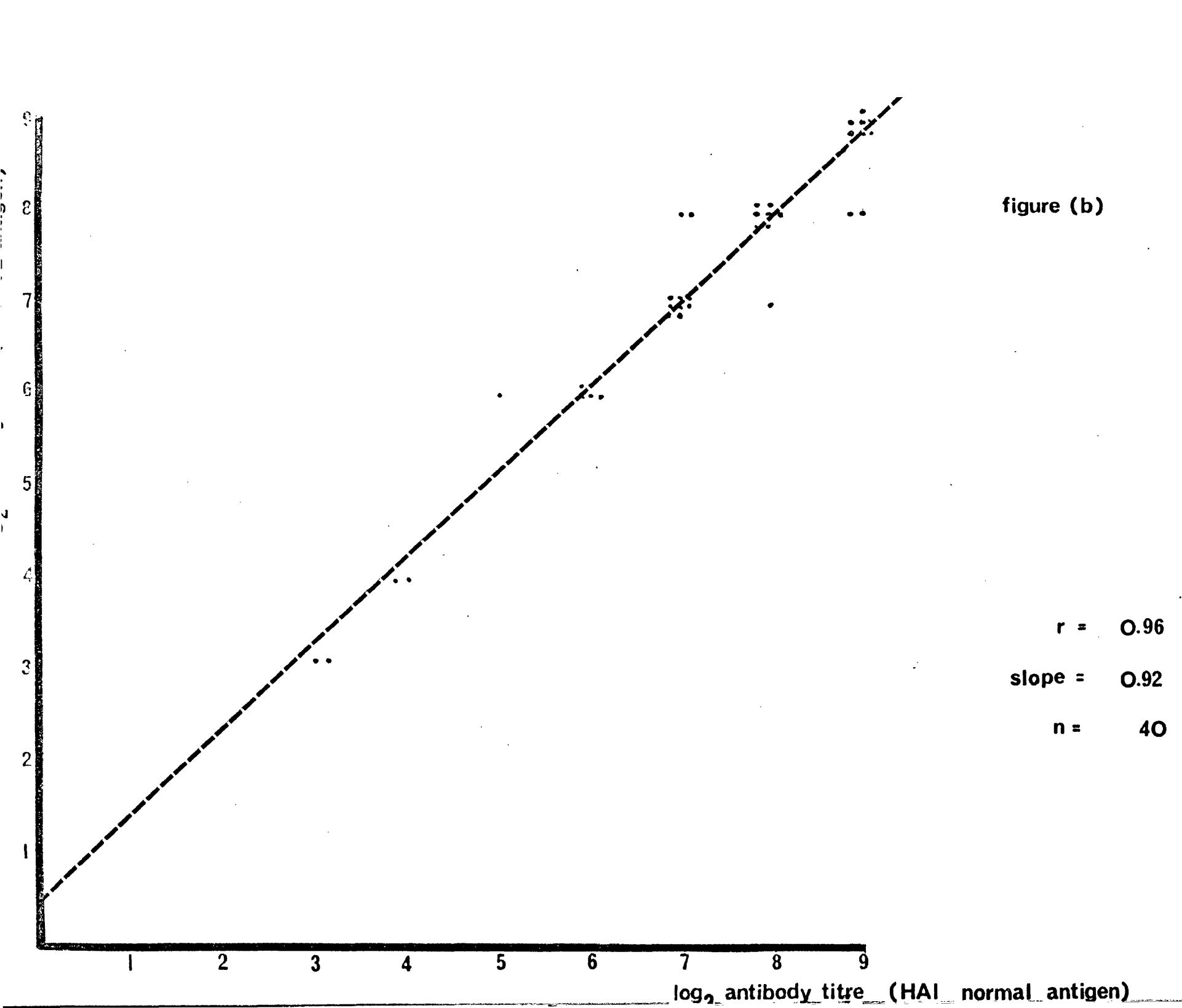


figure (b)
r = 0.96
slope = 0.92
n = 40
1
2
3
4
5
6
7
8
9
log2 antibody titre (HAI normal antigen)

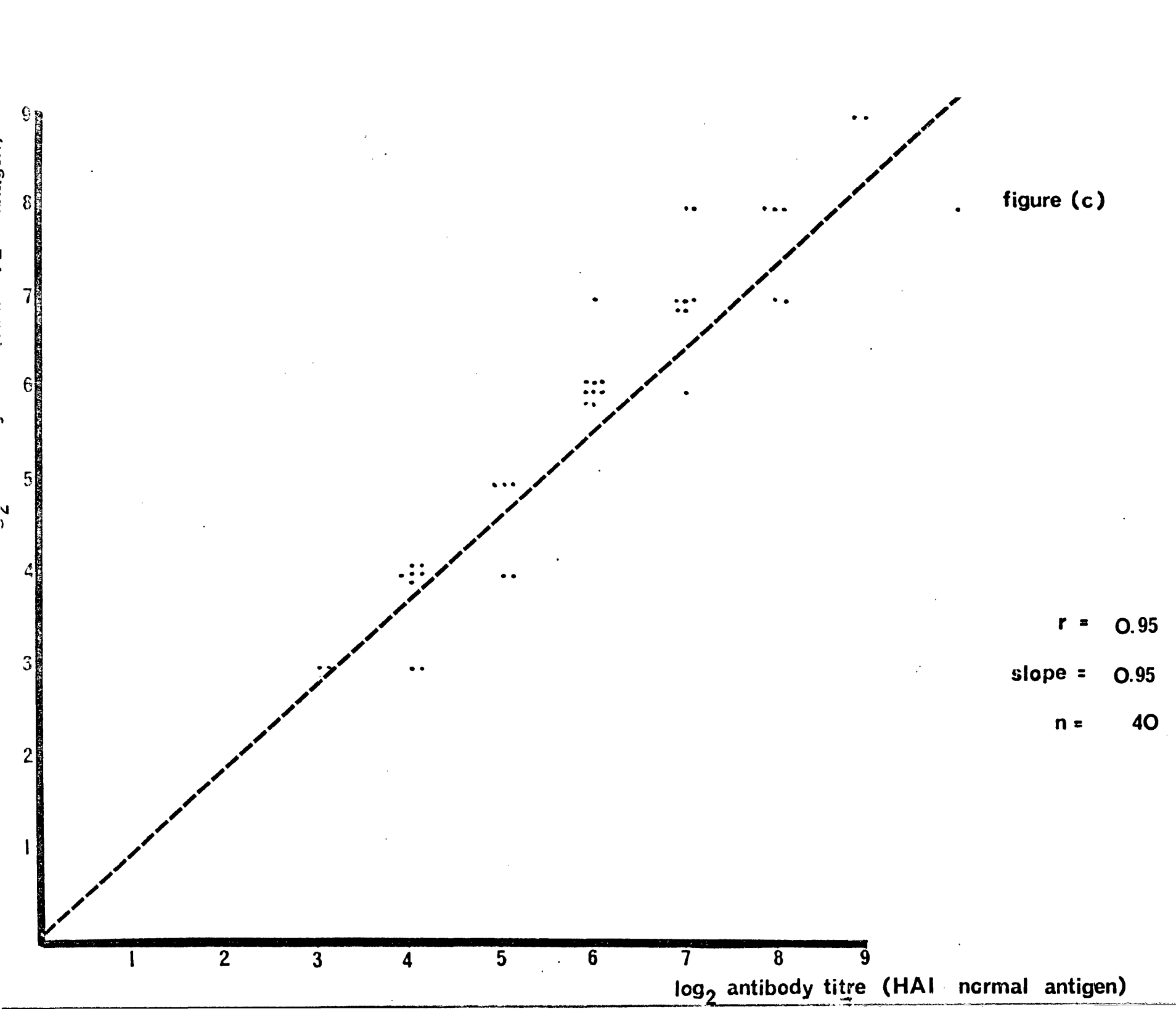


figure (c)
r = 0.95
slope = 0.95
n = 40
1
2
3
4
5
6
7
8
9
log2 antibody titre (HAI normal antigen)

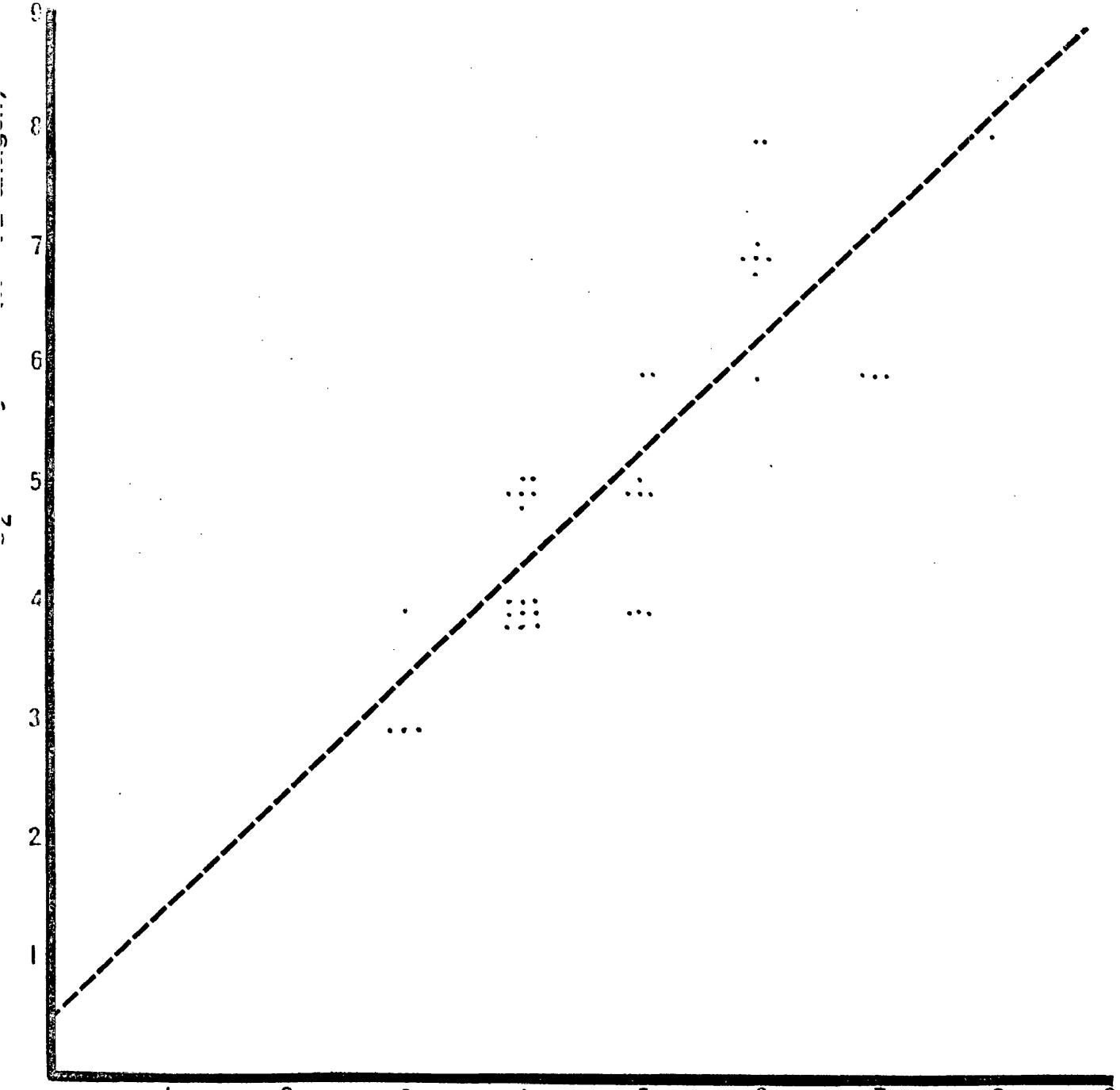


figure (d)

r = 0.83

slope = 0.95

n = 40

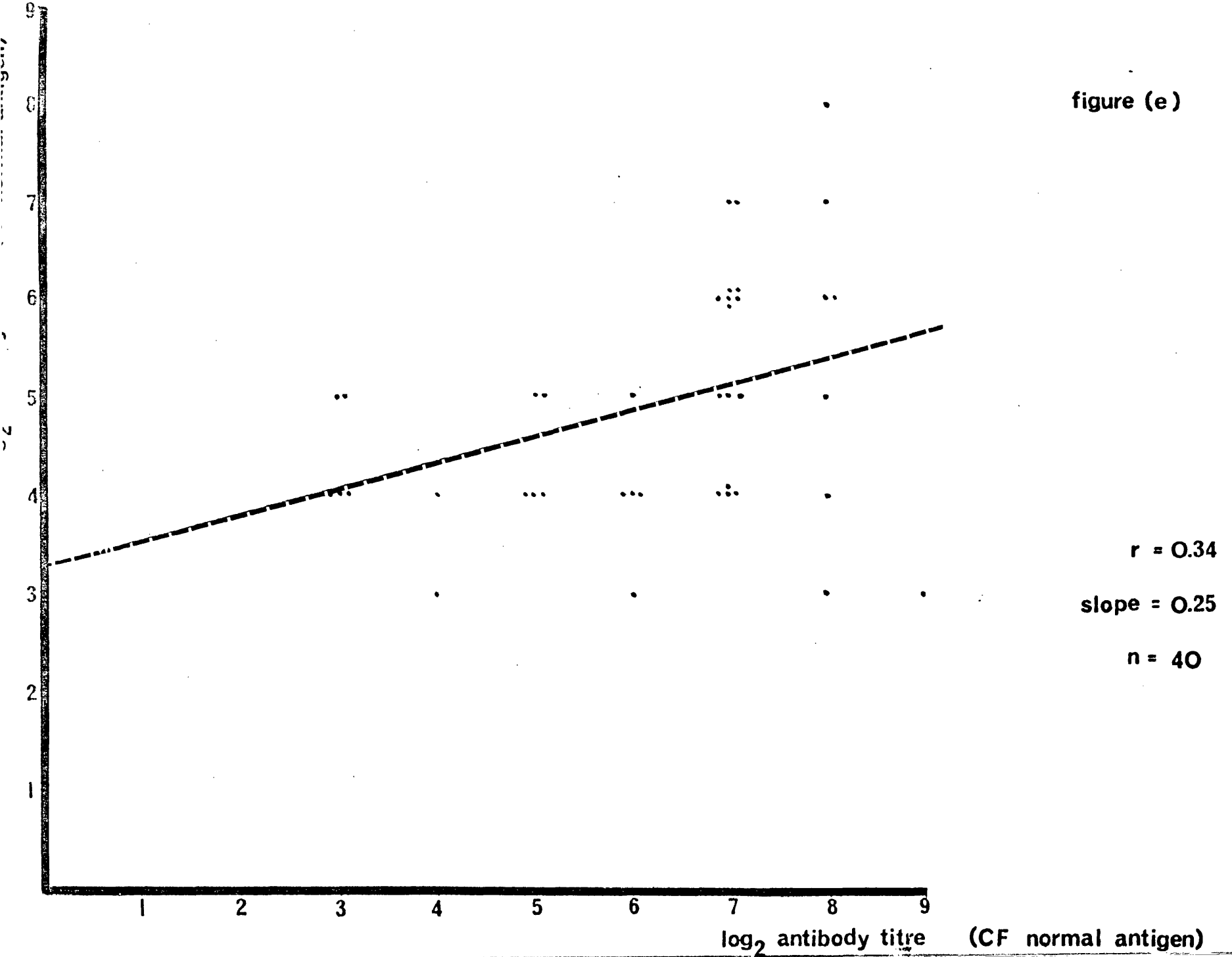


figure (e)
r = 0.34
slope = 0.25
n = 40
1
2
3
4
5
6
7
8
9
log2 antibody titre (CF normal antigen)

Preparation of influenza virus A subunits

Before experiments to investigate the use of liposomes as carriers for purified virus proteins as possible IIF substrates, and also as possible adjuvants - purified influenza virus A haemagglutinin had to be prepared. Two techniques were used to do this :-

(i) By detergent disruption of whole virus and purification of subunits by sucrose density gradient centrifugation, (Almeida et al, 1975; and personal communication).

(ii) by detergent disruption of whole virus and purification of subunits by cellogel electrophoresis, (Skehel and Waterfield, 1975). This work was carried out by me at the Influenza Reference Laboratories in Mill Hill, London.

The first method produces both envelope proteins of influenza virus, namely the haemagglutinin and neuraminidase; whereas the second produces only haemagglutinin protein.

Products from each method were checked for purity and activity by the following tests:

(a) Infectivity in tissue culture
(b) Electrophoresis in S.D.S. polyacrylamide gels
(c) Antigenicity - by haemagglutination
(d) Protein concentration by the Lowry technique

Purified subunits were also examined by negative stain electron microscopy - alone and associated with liposomes. Electron microscopy is not a quality control analysis since it could only detect gross contamination, but was nevertheless useful to confirm that purified subunits had associated with the inert liposome carrier.

table eleven

Results from quality control analyses on products recovered using method (i)

Fraction Number:	HA titre:	Protein content (µg/ml.)	No. of distinct bands in SDS page:	Growth in t/c:
1	64	1350	1	neg
2	64	1240	1	neg
3	128	1250	1	neg
4	128	1180	1	neg
5	64	1120	4	neg
6	64	1140	4	neg
7	64	1120	4	neg
8	128	1140	4	neg
9	64	1120	4	neg
10	256	1050	4	neg
11	64	740	4	neg
12	128	900	4	neg
13	64	900	4	neg
14	64	1120	4	neg
15	64	740	4	neg
16	128	900	4	neg
17	64	1050.	4	neg
18	64	600	4	neg
19	64	740	4	neg
20	64	820	4	neg
21	64	600	4 faint	neg
22	64	450	4 "	neg
23	64	670	4 "	neg
24	64	600	-	neg
25	16	520	-	neg
26	16	440	-	neg
27	16	380	-	neg
28	16	340	-	neg
29	16	280	-	neg
30	ND*	160	-	neg
31	ND	180	-	neg
32	ND	140	-	neg
33	ND	240	-	neg
34	ND	240	-	neg

*N.D. = not done; fractions 30-34 could not be assayed for HA activity as high sucrose concentrations lysed RBC_s.

Table eleven records the results from quality control analyses on the subunits prepared by method (i). It can be seen that HA activity was spread throughout fractions 1 - 24. Fraction 10 had a peak HA activity of 256, and had a protein banding pattern on SDS p.a.g.e. analysis similar to that of intact virus (figures 4 and 5). However, none of the fractions recovered from the gradient grew in tissue culture - suggesting that fraction 10 which had SDS p.a.g.e. bands similar to intact virus probably contained whole virus which had been inactivated by the detergent treatment.

Figure 4 method (i)

SDS page analysis of fractions 1 - 20

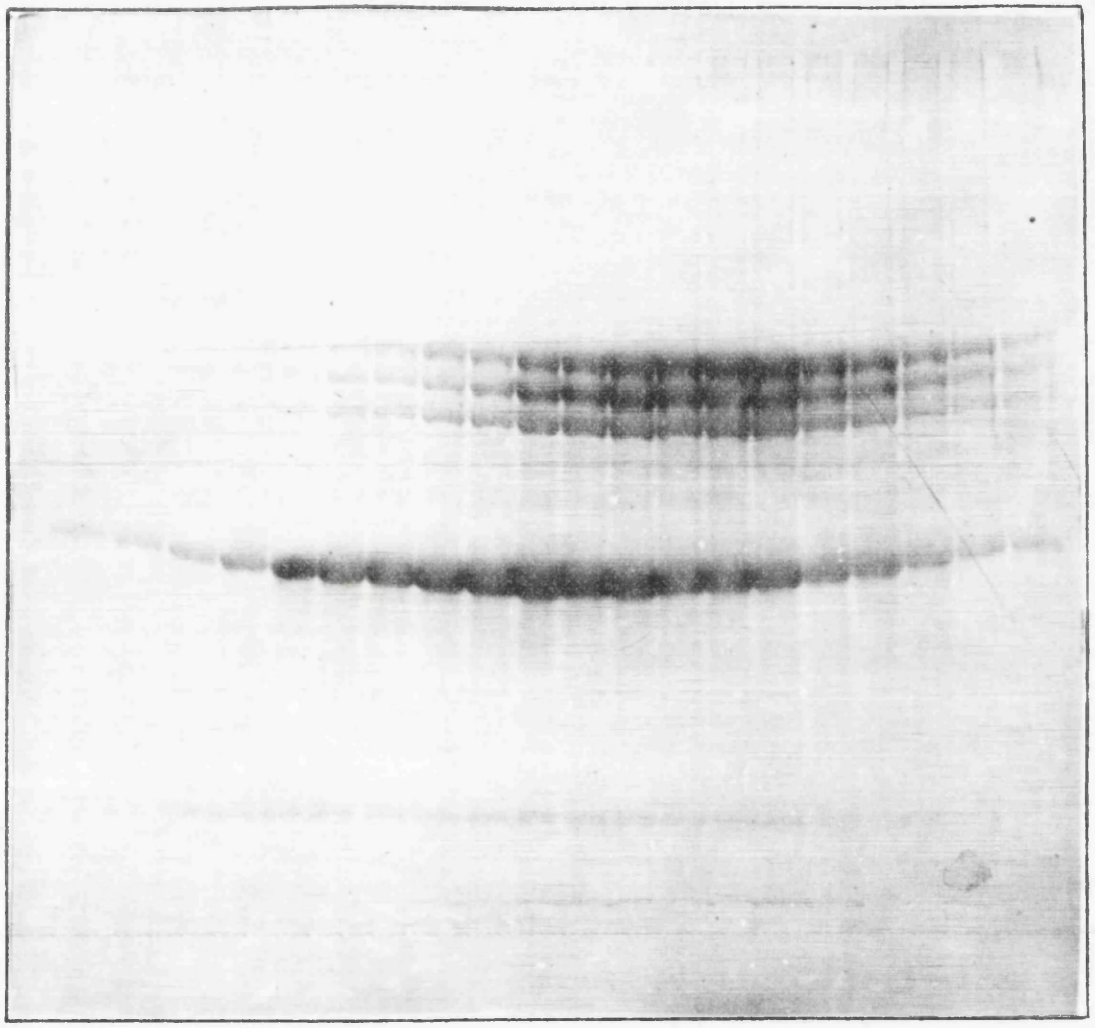
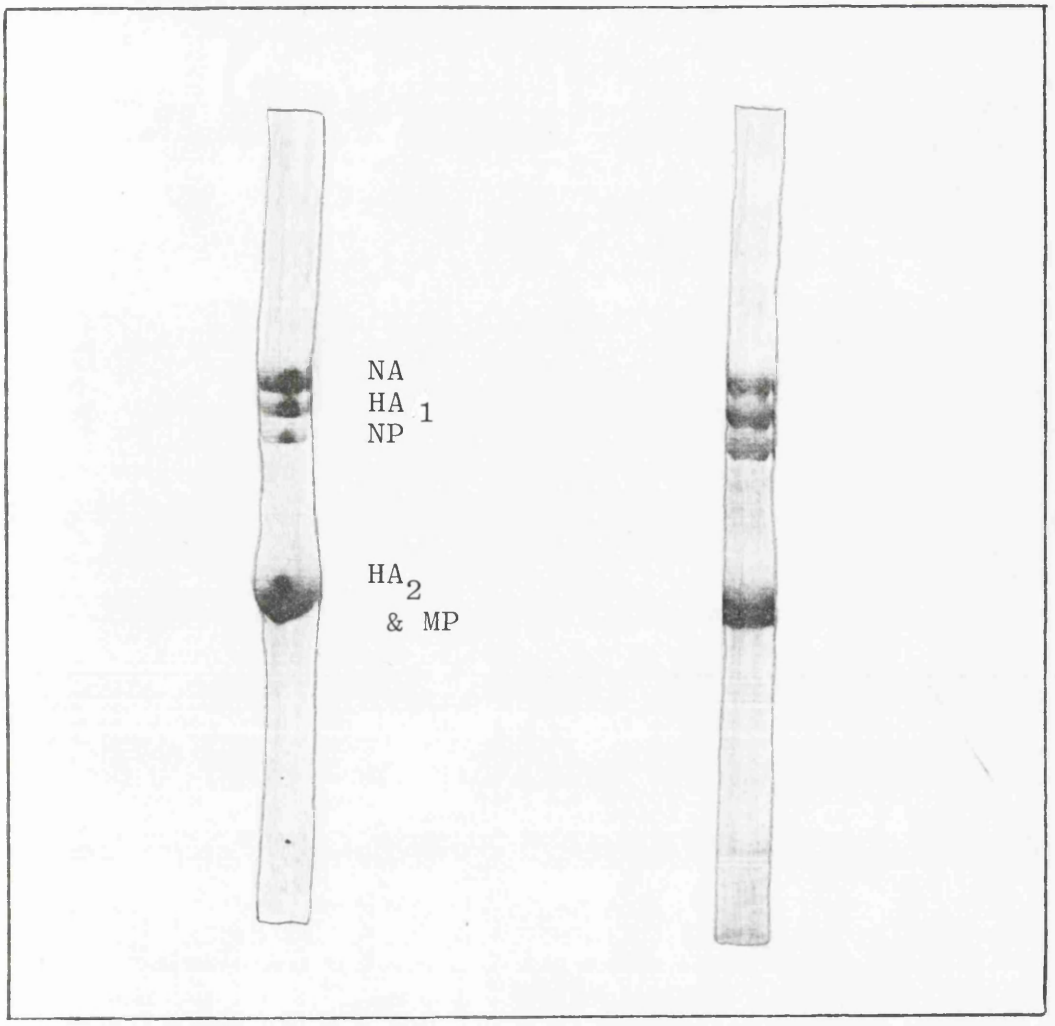


Figure 5 Results from SDS page analysis method (i)



(a) whole influenza virus

(b) peak HA (fraction 10) recovered from gradient in Almeida's method (i)

Note:-

NA	:	neuraminidase
HA_1	:	larger haemagglutinin subunit
NP	:	nucleoprotein
HA_2	:	smaller haemagglutinin subunit
MP	:	internal matrix polypeptide

Interpretation of quality control findings

The results of the quality control analyses recorded in table 11 and in figures 4 and 5, indicate that in my hands, Almeida's method (i) of detergent disruption of virus and isolation of subunit proteins by density gradient purification did not succeed in separating the haemagglutinin and neuraminidase subunit proteins from influenza A virus particles. This method of subunit isolation was clearly unsatisfactory in view of the contamination by proteins other than haemagglutinin and neuraminidase, possibly because of the low concentration of detergent used i.e. 0.2% as opposed to Skehel's method which uses a concentration of 2%. An alternative method of preparing subunits in which haemagglutinin subunits were prepared by detergent disruption and cellogel electrophoresis (method ii) was therefore used.

(ii) detergent disruption and cellogel electrophoreses

Unlike method (i), there was no sucrose density gradient fractionation of disrupted virus in method (ii). The results of quality control tests carried out on subunit preparations obtained by method (ii) are shown below:-

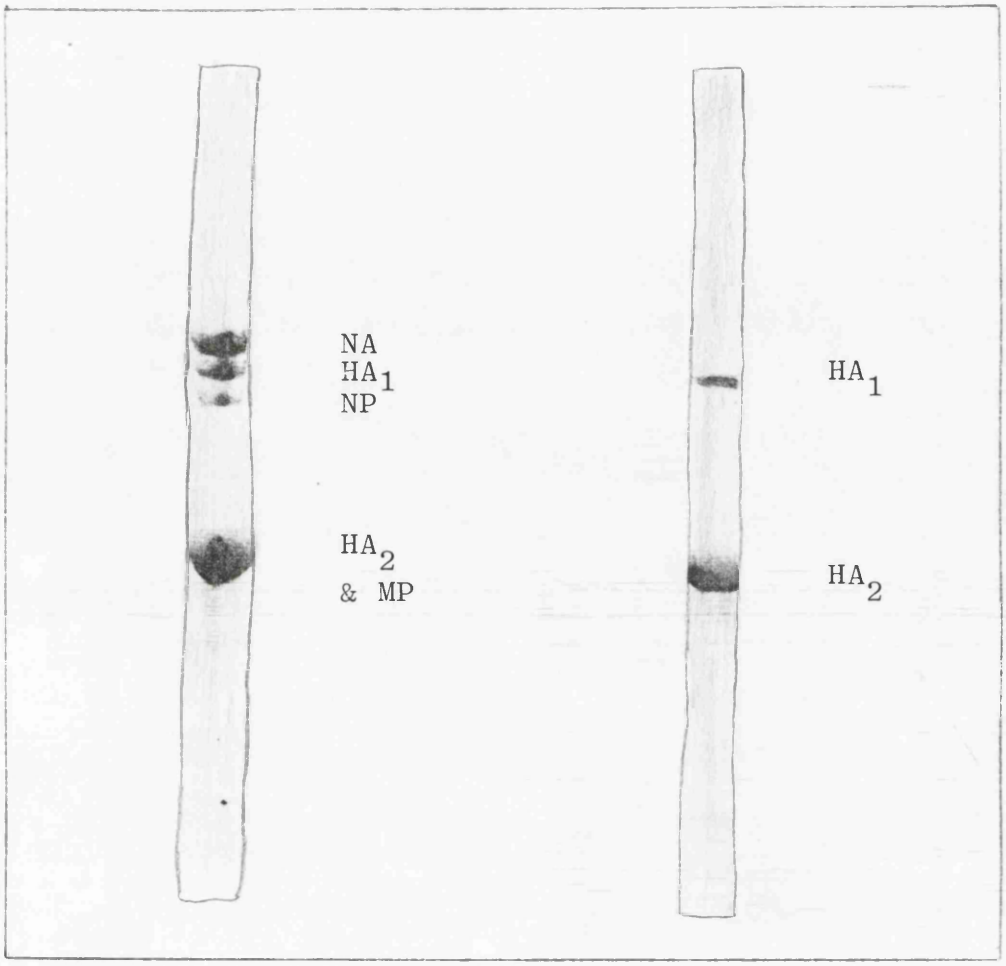
Infectivity in tissue culture: no cytopathic effect was seen after incubation for 21 days. It was concluded that the subunit preparation did not contain live influenza virus.

Protein characterisation: sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS p.a.g.e.) analysis showed that the haemagglutinin preparation was pure (figure 6), and that there was no contamination by other proteins.

Protein concentration: Lowry analysis showed a protein concentration of 1100 mg of protein per ml.

HA activity: HA tests on the purified haemagglutinin preparation showed a titre of 2048 per 0.025 ml. with human group O RBC_s.

figure 6 Results from SDS page analysis method (ii)



(c)

whole influenza virus

(d)

purified haemagglutinin polypeptides obtained using Skehel's method.

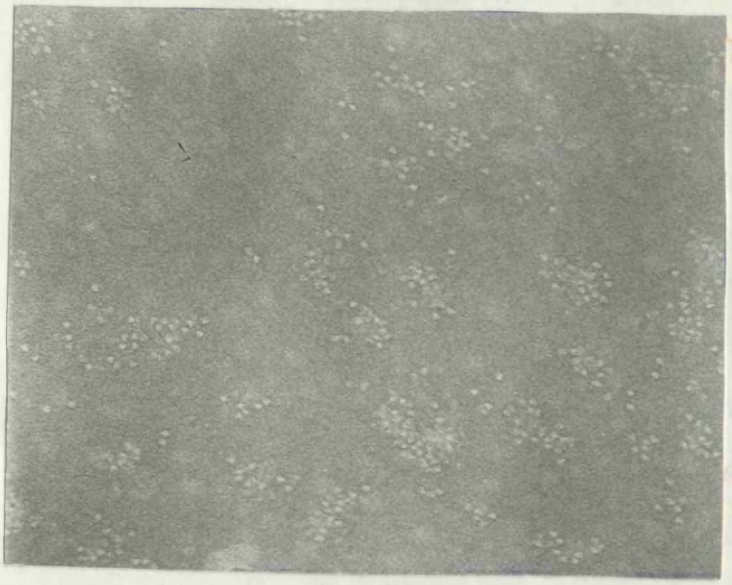


Plate 7. Electron Micrograph of purified haemagglutinin (PTA negative stain X100 000)

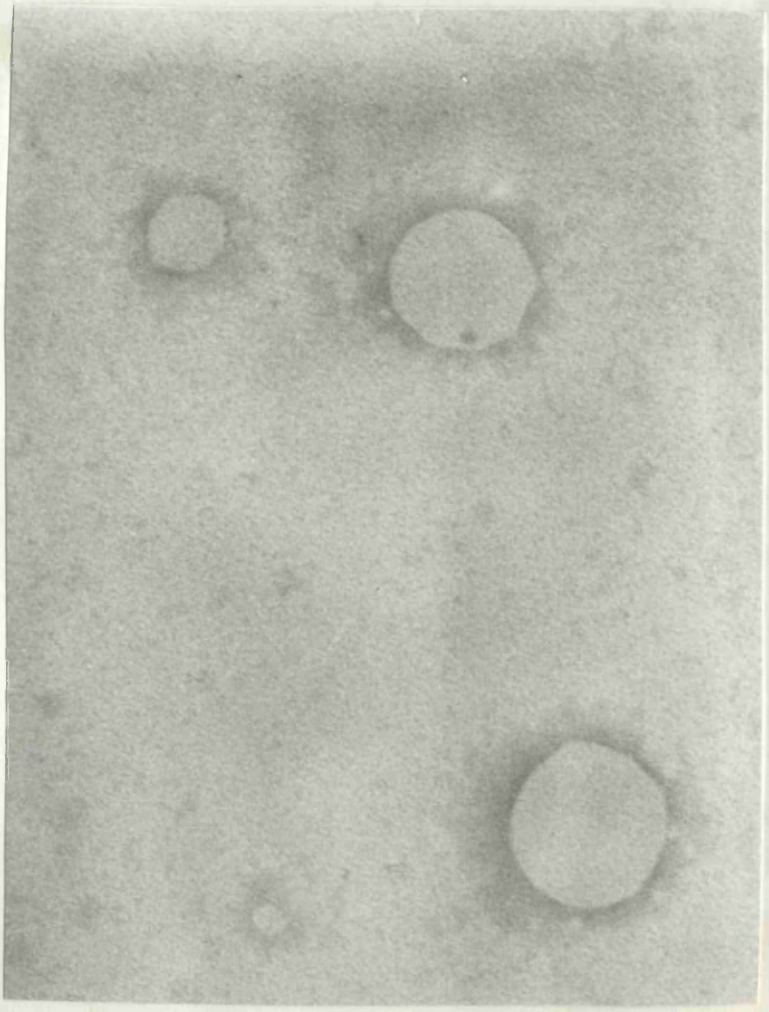


Plate 8. Electron Micrograph of liposome associated haemagglutinin (PTA negative stain X100 000)

The results of these quality control analyses confirmed that method (ii) had yielded a preparation of purified haemagglutinin subunits which was antigenically intact. The purified haemagglutinin was then examined, with and without liposomes, by electron microscopy (plates 7 and 8).

Plate 7 shows an electron micrograph of intact haemagglutinin after purification by method (ii). No contaminant debris was detected. Individual rods of haemagglutinin were difficult to discern at the maximum magnification (100 000X). However clusters of subunits were observed indicating that the haemagglutinin subunits had aggregated after the removal of SDS - as has been described by Laver and Valentine, (1969).

Plate 8 shows the association of purified haemagglutinin with preformed liposomes. Haemagglutinin subunits are attached to the liposome surface but are also present unattached in the background. Unlike the electron micrographs taken by Almeida et al, (1975), of influenza haemagglutinin and neuraminidase subunits attached to liposomes, complete orderly arrays of the purified haemagglutinin were not detected on the liposome surface. However, Almeida's method of preparing subunits results in production of both haemagglutinin and neuraminidase proteins - unlike method (ii) which yields only haemagglutinin. The presence of

both types of subunits may have resulted in this different arrangement on the liposome surface. In addition I found that Almeida's method resulted in a final product that contained all the viral components found in intact virus (figure 5).

Skehel's method of detergent disruption and cellogel electrophoresis was therefore used to produce purified haemagglutinin. The haemagglutinin obtained was used in serological tests as antigen, and also in experiments in guinea pigs to assess their immunogenic potential when liposome-associated.

Use of IIF to detect antibody to a specific virus component.

Experiments were also set up to determine if liposomes could be used as substrates for purified virus proteins - in tests to detect antibody against individual virus components by IIF. IIF normally uses infected cell substrates in which it is difficult and often impossible to determine the virus antigen to which the demonstrable antibody is directed. The results of the following experiment show that liposomes can be used to detect antibody to a specific virus component, in this instance, the purified haemagglutinin protein of influenza virus A.

In the tests, purified influenza A virus and one of its major antigenic determinants - the haemagglutinin protein, were coupled to inert liposome carriers and used as antigens in immunofluorescent tests on human sera. Two different antigenic types of influenza virus A were used - namely H_3N_2 and H_1N_1. The results are recorded in table twelve and compare titres obtained in IIF tests against conventional infected cell antigens to liposomes with purified virus as antigen. Purified haemagglutinin from influenza virus A, X-31 (H_3N_2), was included in the tests. The antibody detected was IgG.

table twelve

Use of IIF to detect antibody to a specific virus component

Serum Number:	Reciprocal Antibody Titres to Influenza A					$\log_2$ difference between titres
	(i)	(ii)	(iii)	(iv)	(v)	
11/07	16	16	16	16	16	-
11/107	32	64	32	32	64	1
11/412	32	16	32	16	64	1
12/78	128	64	128	128	64	1
12/145	256	256	125	256	256	1
12/499	16	<16	<16	<16	<16	1
12/845	64	64	32	32	32	1
12/887	16	16	16	32	32	1
1/2354	32	16	16	32	32	1
1/2841	<16	<16	16	16	16	1
2/198	64	128	128	128	64	1
2/642	64	128	64	64	64	1
2/713	16	32	32	32	32	1
2/775	32	32	32	32	16	1
2/890	32	32	64	64	64	1
3/1022	16	16	16	<16	16	1
2/1041	<16	<16	<16	<16	<16	-
2/1042	<16	16	<16	<16	<16	1
3/60	16	32	32	32	16	1
3/123	32	32	32	64	32	1
3/847	64	128	64	128	128	1
3/900	16	32	32	16	32	1
3/1553	32	64	64	32	32	1
3/1819	64	128	128	128	128	1
3/1996	16	32	32	64	32	1
3/1997	16	32	16	16	32	1
3/2368	64	128	64	128	128	1
3/2399	128	64	32	64	64	2
3/2421	32	16	32	16	32	1
3/2501	16	16	<16	<16	16	1
3/2517	16	16	16	16	16	-
3/3153	16	32	16	16	32	1
4/707	16	16	64	32	32	2

table twelve cont'd.

Serum Number:	Reciprocal Antibody Titres to Influenza A (i)	(ii)	(iii)	(iv)	(v)	$\log_2$ difference between titres
4/762	64	256	128	128	128	2
4/1183	64	256	256	128	128	2
4/1513	16	16	16	32	32	1
4/2622	128	64	64	64	16	3
4/2750	16	16	16	16	<16	1
4/3535	32	32	16	32	32	1
5/312	16	16	32	32	16	1

note: IIF substrates used in these experiments -

(i) influenza X-31 (H_3N_2) infected cells

(ii) influenza X-31 (H_3N_2) virus liposomes

(iii) influenza Russia (H_1N_1) infected cells

(iv) influenza Russia (H_1N_1) virus liposomes

(v) influenza X-31 haemagglutinin (H_3) - Liposomes

It was thought likely that this experiment might have shown variations in the levels of antibody detected to the two antigenically distinct strains of influenza virus used - X-31 (H_3N_2) and Russia (H_1N_1), as did HAI tests. That is in table six, sera numbers 11/07, 1/2354, 3/60, 3/2501 for example, showed marked differences in titre between HAI antibody to H_3N_2 and H_1N_1 influenza A strains. In addition there might have been greater differences in titre in tests in which the antibody measured was to one specific antigenic determinant - in this case the haemagglutinin of influenza A X31 (H_3N_2) virus. However with the possible exception of serum 4/2622 the titres were in reasonably close agreement and only minor differences in titre which were within experimental error were recorded.

In test substrates ((ii) and (iv)) which contained whole virus, four major antigens should be present, neuraminidase, membrane (or M protein), and nucleoprotein (or 'S' antigen). All these antigens should of course also be found in infected cells ((i) and (iii)). The membrane and nucleoprotein antigen are common to all influenza A viruses. Test substrate (V) contained purified haemagglutinin or V antigen, and should therefore be specific for antibody to influenza viruses containing that antigen - H_3.

The results recorded in table twelve show that similar titres were obtained using H_3N_2, H_1N_1 and H_3 - liposome substrates to detect influenza antibody.

Clearly several explanations could account for these findings. It is possible for example that the sera used actually contained antibody to both viruses, and were therefore capable of reacting with the two antigenic strains of influenza used. However, in view of the HAI results recorded in table six - where marked differences in titre to H_3N_2 and H_1N_1 influenza strains were noted - this would seem unlikely. More likely is the possibility of an antigen common to test substrates (i) - (V) in use in IIF tests (see discussion).

Shelf life and stability of Liposome associated influenza virus antigens in the HA Test.

Experiments were set up to compare the stability of the haemagglutinating activity of normal and liposome associated influenza virus A test antigens when stored at a range of different temperatures over a time interval of 50 days.

The results obtained are shown in table thirteen.

The results in table thirteen show that there is a marked variation in the shelf life of antigen stored at different temperatures when these are tested for haemagglutinating activity over a period of fifty days. Antigen stored frozen at $-20^{0}C$ or refrigerated at $4^{0}C$ remained antigenically viable for a considerably longer period of time than identical aliquots stored at room temperature or incubated at $37^{0}C$. However antigen associated with liposomes did not have a longer 'shelf life' in that it did not persist in an antigenically viable form for a longer period of time than equivalent unassociated preparations. On the other hand both types of preparation showed remarkable stability on storage.

table thirteen

Days stored:	Reciprocal HA titres of antigens stored at :-							
	-20^0C		4^0C		Room Temperature		37^0C	
	V	VL	V	VL	V	VL	V	VL
0	256	256	256	256	256	256	256	256
1	256	256	256	256	256	128	128	64
2	256	256	128	128	128	128	32	64
3	256	256	128	256	128	128	<8	16
4	256	256	256	256	32	64	<8	<8
5	256	256	256	256	16	16	<8	<8
10	256	128	128	256	<8	<8	<8	<8
15	256	256	256	256	<8	<8	<8	<8
20	256	128	256	128	<8	<8	<8	<8
25	256	256	256	256	<8	<8	<8	<8
30	128	128	128	256	<8	<8	<8	<8
35	256	256	128	128	<8	<8	<8	<8
40	256	256	128	128	<8	<8	<8	<8
45	256	256	64	128	<8	<8	<8	<8
50	256	128	64	128	<8	<8	<8	<8

CHAPTER 5 RESULTS (b)

Combination of purified virus protein with liposomes and tests of their antigenicity in guinea pigs.

Experiments were set up to determine if combining virus antigen with liposomes resulted in increased antigenicity - in other words if the liposomes acted as adjuvants. To do this purified haemagglutinin from influenza virus A was inoculated into guinea pigs with and without liposomes : geometric mean titres of antibody were calculated and compared to those in animals injected with whole virus.

First, purified subunits had to be prepared - this was done as described in the preceding results section using the method described by Skehel and Waterfield (1975).

Tests of Adjuvant Activity of liposome associated Haemagglutinin

In order to assess the adjuvant activity of liposomes, guinea pigs were inoculated by the intraperitoneal route with varying concentrations of purified haemagglutinin, haemagglutinin-liposomes, intact virus, and liposomes alone. The geometric mean antibody titres in HAI and IIF tests were calculated for each group of results, and are recorded in table 14. Details of the results obtained on sera from individual guinea pigs, are shown in tables, 15, 16 and 17.

The results in table 14 show that for each of the animal groups (1), (2), and (3) (i.e. antigenic doses of 4096, 2048 and 1024 (HU_S respectively), intact virus elicited the greatest antibody response. For example in group (1), geometric mean

table 14

Animal Group Number	Number of GPs:	Preparation used:	Total dose in HU_s First dose day 0 Second dose day 21	Geometric Mean Reciprocal Antibody Titres at day 35 IIF	HAI
(1)	6	influenza virus	4096	323	645
	6	HA-liposomes	4096	90	161
	6	HA	4096	23	40
	6	liposomes	-	<8	<8
(2)	6	influenza virus	2048	161	422
	6	HA-liposomes	2048	72	144
	6	HA	2048	18	40
	6	liposomes	-	<8	<8
(3)	6	influenza virus	1024	32	162
	6	HA-liposomes	1024	25	64
	6	HA	1024	11	14
	6	liposomes	-	<8	<8

note: first injection and test bleed at day 0
second injection and test bleed at day 21 (little or no antibody detected)
final test bleed at day 35 - geometric mean titres recorded above

HAI antibody titres obtained were 645, 161 and 40, for intact virus, HA-Liposomes, and HA alone respectively. The geometric mean titres for the IIF and HAI tests were also dose dependent. That is, the higher the antigenic dose, the higher the corresponding geometric mean titre of antibody produced.

The most interesting result from table 14 was that haemagglutinin-liposomes not only elicited a significant antibody response in each of the three groups of animals by both IIF and HAI tests, but this was greater than for haemagglutinin alone. The response of the animals in each group to injection with haemagglutinin-liposome preparations was less than that obtained using equivalent doses of whole virus (e.g. in group (1) a geometric mean HAI titre of 161 for those animals injected with HA-Liposomes compared with 645 for animals inoculated with whole virus). Nevertheless this geometric mean titre was significantly higher than that obtained using haemagglutinin subunits alone (e.g. in group (1) a geometric mean HAI titre of 161 for animals inoculated with HA-liposomes, compared with 40 for animals inoculated with haemagglutinin alone). These results are clear evidence that liposomes have adjuvant activity in that they increased the antibody reponse to the viral antigen with which they were combined.

Tables 15, 16 and 17 show results of tests on individual guinea pig sera. By day 35, after two doses of antigen, all three groups contained antibody to influenza A by IIF and HAI tests - except control animals inoculated with liposomes alone. Some sera contained no antibody to influenza virus A even after 35 days (e.g. animal numbers 1 and 8 in table 15) - but only 5 out of a total of 54 animals inoculated with antigen produced no immune response.

table 15

Data obtained for group (1)

(group (1) antigenic doses totalling approx. 4096 HU_S)

Animal Number:	Preparation	Total dose in HU_S First dose day 0 Second dose day 21	Reciprocal Antibody Titres Day 0 IIF	Day 0 HAI	Day 21 IIF	Day 21 HAI	Day 35 IIF	Day 35 HAI
1	HA	4096	<8	<8	<8	<8	<8	<8
2			<8	<8	<8	<8	64	256
3			<8	<8	<8	<8	64	128
4			<8	<8	<8	<8	32	128
5			<8	<8	<8	<8	16	32
6			<8	<8	<8	<8	64	32
7			<8	<8	<8	<8	512	1024
8	HA-liposomes	4096	<8	<8	<8	<8	<8	<8
9			<8	<8	<8	<8	64	1024
10			<8	<8	<8	<8	128	128
11			<8	<8	<8	<8	256	128
12			<8	<8	<8	<8	512	1024
13	liposomes	-	<8	<8	<8	<8	<8	<8
14			<8	<8	<8	<8	<8	<8
15			<8	<8	<8	<8	<8	<8
16			<8	<8	<8	<8	<8	<8
17			<8	<8	<8	<8	<8	<8
18			<8	<8	<8	<8	<8	<8
19	influenza virus	4096	<8	<8	<8	<8	128	512
20			<8	<8	<8	32	512	1024
21			<8	<8	<8	<8	256	512
22			<8	<8	<8	8	512	1024
23			<8	<8	<8	<8	256	256
24			<8	<8	<8	16	512	1024

note:- first injection and test bleed at day 0

second injection and test bleed at day 21

final test bleed at day 35 - individual titres recorded above

table 16

Data obtained for group (2)

(group (2) antigenic doses totalling approx. 2048 HU_s)

Animal Number:	Preparation	Total dose in HU_s First dose day 0 Second dose day 21	Reciprocal Antibody Titres Day 0 IIF	Day 0 HAI	Day 21 IIF	Day 21 HAI	Day 35 IIF	Day 35 HAI
1			< 8	< 8	< 8	< 8	64	128
2			< 8	< 8	< 8	< 8	8	16
3	HA	2048	< 8	< 8	< 8	< 8	16	64
4			< 8	< 8	< 8	< 8	16	8
5			< 8	< 8	< 8	< 8	32	64
6			< 8	< 8	< 8	< 8	8	64
7			< 8	< 8	< 8	< 8	64	256
8			< 8	< 8	< 8	< 8	16	128
9	HA-liposomes	2048	< 8	< 8	< 8	< 8	128	256
10			< 8	< 8	< 8	< 8	64	128
11			< 8	< 8	< 8	< 8	64	128
12			< 8	< 8	< 8	< 8	256	64
13			< 8	< 8	< 8	< 8	< 8	< 8
14			< 8	< 8	< 8	< 8	< 8	< 8
15	liposomes	-	< 8	< 8	< 8	< 8	< 8	< 8
16			< 8	< 8	< 8	< 8	< 8	< 8
17			< 8	< 8	< 8	< 8	< 8	< 8
18			< 8	< 8	< 8	< 8	< 8	< 8
19			< 8	< 8	< 8	< 8	256	512
20			< 8	< 8	< 8	< 8	64	256
21	influenza virus	2048	< 8	< 8	< 8	< 8	128	1024
22			< 8	< 8	< 8	< 8	256	256
23			< 8	< 8	< 8	< 8	256	512
24			< 8	< 8	< 8	< 8	128	256

note: first injection and test bleed at day 0
second injection and test bleed at day 21
final test bleed at day 35 - individual titres recorded above

table 17

Data obtained for group (3)

(group (3) antigenic doses totalling approx. 1024 HU_s)

Animal Number:	Preparation	Total dose in HU_s First dose day 0 Second dose day 21	Reciprocal Antibody Titres Day 0 IIF	Day 0 HAI	Day 21 IIF	Day 21 HAI	Day 35 IIF	Day 35 HAI
1			<8	<8	<8	<8	32	128
2			<8	<8	<8	<8	32	64
3	HA	1024	<8	<8	<8	<8	16	32
4			<8	<8	<8	<8	16	32
5			<8	<8	<8	<8	8	<8
6			<8	<8	<8	<8	<8	<8
7			<8	<8	<8	<8	128	256
8			<8	<8	<8	<8	32	128
9	HA-liposomes	1024	<8	<8	<8	<8	64	256
10			<8	<8	<8	<8	<8	<8
11			<8	<8	<8	<8	16	64
12			<8	<8	<8	<8	64	128
13			<8	<8	<8	<8	<8	<8
14			<8	<8	<8	<8	<8	<8
15	liposomes	-	<8	<8	<8	<8	<8	<8
16			<8	<8	<8	<8	<8	<8
17			<8	<8	<8	<8	<8	<8
18			<8	<8	<8	<8	<8	<8
19			<8	<8	<8	<8	32	128
20			<8	<8	<8	<8	64	256
21	influenza virus	1024	<8	<8	<8	<8	64	512
22			<8	<8	<8	<8	16	64
23			<8	<8	<8	<8	64	256
24			<8	<8	<8	<8	8	64

note - first injection and test bleed at day 0

second injection and test bleed at day 21

final test bleed at day 35 - individual titres recorded above

Chapter 6 DISCUSSION

This study was set up to investigate the use of inert substrates as carriers in medical virology. The inert substrates investigated were liposomes which were used as inert carriers of virus antigen in CF, HAI and IIF serodiagnostic tests. Experiments were set up to compare the tests with liposome-associated antigens to those with conventional antigen preparations run in parallel. Other experiments included a study of the possible adjuvant effect of liposomes in guinea pigs immunised with purified viral haemagglutinin of influenza virus A. The antibody titres induced by liposome-associated haemagglutinin were then assessed in comparison to viral haemagglutinin alone and to whole virus.

Liposomes in the Serological Diagnosis of Virus Infection

Much of the work of a routine diagnostic virus laboratory consists of serological tests on patients' sera; attempts to improve the sensitivity and economy of such tests are therefore of considerable potential value to the laboratory scientist. It had been hoped that liposome-associated antigens would be more economical in serological tests, both as regards the amount of virus required and their stability compared to conventional antigen preparations. However, experiments with three serological test systems - i.e. CF, HAI and IIF - showed that virus -liposomes gave results similar to those obtained with conventional antigens.

Furthermore, the same amount of virus was required for the liposome preparations as for standard antigens, so that liposomes did not achieve any economy in the use of virus. Antibody titrations were set up to compare conventional and liposome-associated antigens; however linear regression analyses showed no statistical difference in the titres obtained with the two different antigen preparations. Virus-liposome mixtures were neither more stable, nor did they have a longer shelf life than conventional preparations when tested for HA activity over a period of fifty days. The use of an inert carrier of viral antigen therefore offers no advantage with regard to virus economy, or improved stability in the serological systems tested. Nevertheless, liposome associated antigens have some advantages in these tests.

Correlation between IIF and CF results

Previous experience with the IIF technique had shown that the reproducibility was often low. In the experiments described here, IIF results on individual sera were found to differ significantly from those obtained by CF and HAI. Comparison with results from previous IIF tests also showed considerable variation on retesting. Although IIF tests on sera using virus-liposome and infected cell antigens were in close agreement, corresponding CF and HAI tests on the same samples often produced discordant results. The CF and IIF results for antibody to influenza A virus were analysed

statistically to determine the level of correlation between the two tests - but the correlation coefficient was of a low order. There are several possible explanations for this finding. Firstly the IIF technique used here measured only IgG antibody: CF, on the other hand, measures both IgM and IgG antibody. In addition CF antibody is known to decline earlier after infection than IIF and HAI antibody (Grist et al, 1979), so that in some instances IIF may be expected to detect antibody which will have declined to become undetectable by CF. This would not of course explain sera positive by CF but negative by IIF. IIF is further complicated by the non specific binding of IgG to the F_c receptors which have been shown to be produced in certain virus infected cell substrates - notably CMV (Cytomegalovirus) infected fibroblasts, (e.g. Keller, Peitchel, Goldman and Goldman, 1976). This can give rise to non specific fluorescence and therefore false positive results. Moreover many unstained tissue and cell preparations exhibit autofluorescence on irradiation with U.V. light. Considerable experience with the technique is therefore required before specific and non specific fluorescence can be reliably differentiated.

The difference in results on the same sera tested by different techniques emphasise the importance of testing paired sera in parallel and by the same serology test in order to diagnose current infection accurately.

Discrepancies between different serological techniques in virology have been previously reported by other workers. For example, Griffiths, Buie, and Heath in 1978 compared CF, IIF and ACIF (i.e. anti complement immunofluorescence) for the detection of antibody to CMV. They recorded discordant CF and IIF findings - similar to those described here - on some of the sera tested. They found that none of the tests used detected all the sera that contained specific antibody. Certain sera, for example, contained CF antibody but were negative by IIF tests and vice versa, Griffiths and his co-workers concluded that CF and IIF tests probably detect different antibodies.

The use of Liposome Associated antigens in IIF

The IIF technique for the estimation of viral antibody is often criticised on the grounds of lack of quantitation. This is because end points are difficult to read, and because there is inevitably a degree of subjectivity in reading results. Furthermore there are considerable technical difficulties in producing reliable test substrates and, in distinguishing specific from non specific immunofluorescence. In view of these difficulties, attempts have been made by various workers to standardise the principal variable component of the IIF test - namely the antigenic substrate. For example several reports have been published on the use of antigens immobilised on different kinds of inert supports

(Toussaint and Anderson, 1965; Camargo and Ferreira, 1970: Brandtzaeg, 1972; Van Dalen, Knapp and Ploem, 1973). Camargo and Ferreira (1970), for example, described a technique in which immunological reactions were obtained with soluble antigens covalently bound to cellulose particles of undefined dimensions. Van Dalen, Kanpp and Ploem (1973) found that antigens covalently bound to agarose beads were also suitable for microfluorometry. Their system could be used to test for conjugate specificity, i.e. to determine the exact specificity and titre of FITC conjugated antisera. It proved to be more sensitive for high dilutions of conjugate than immunofluorescence methods when tissue sections or cell preparations were used as a substrate. Their results therefore show that antigens covalently bound to agarose beads can act as a satisfactory, artificial substrate in immunofluorescence. Wick, Baudner and Herzog, (1978) have named this technique "the defined antigenic substrate spheres" or 'D.A.S.S.' method, and cite it as a promising development in the standardisation of IIF test substrates. However although a reduction in the subjective error in interpreting serological results with viruses could probably best be achieved by standardisation of test substrates, little effort has so far been made to do this.

The results reported here show that liposomes can be used as standardised IIF tests substrates with virus antigens. Virus liposome substrates can be standardised in terms of

virus haemagglutinin units (HU_s) and also lipid concentration. Conventional infected cell antigens on the other hand cannot be standardised quantitatively, so that the use of virus liposomes in this respect offers considerable advantage.

The comparative trial of virus liposome substrates in diagnostic serology showed a close correlation with results from parallel tests using conventional infected cell substrates. Virus liposome substrates were both as sensitive and as specific as infected cell preparations in detecting viral antibody. Furthermore by using a standardised test substrate in which non specific fluorescence was no longer a problem, the reliability and reproducibility of the IIF test was improved.

Virus liposome substrates prepared as described here for IIF tests could find a variety of uses. For example they might be particularly useful in diagnostic laboratories which have a small workload, and in which the preparation of satisfactory IIF infected cell substrates is both time consuming and difficult in terms of quality control. Virus liposomes might also be more suitable than conventional antigens for the rapid screening of large numbers of serum samples and because they can be accurately standardised, for the determination of the working titre and specificity of FITC conjugated antisera. Non specific fluorescence of cellular material often gives particular problems in reading IIF tests. Tests carried out with substrates which contain only those

antigens to which specific antibody is sought, would therefore be of great value in reading IIF tests. Virus liposome substrates should also be suitable for use in the automated microfluorometer system described by Van Dalen et al, (1973). If they proved satisfactory the speed and labour-saving efficiency of the IIF technique in viral diagnostic tests could be greatly improved.

Experiments on Liposomes combined with Purified Viral Protein: Preparation of influenza virus subunits

Experiments were also carried out to see if liposomes could be used with purified viral protein as antigens both in serological tests and in experimental animals in which liposomes might act as adjuvants. Before undertaking work on the attachment of a purified viral protein to liposomes it was necessary to produce a pure preparation of the protein subunit selected for study - i.e. free from contamination with either intact virus or other viral proteins.

Attempts to obtain purified haemagglutinin and neuraminidase subunits from influenza A virus using the method described by Almeida et al, (1975), and further detailed in a personal communication, were unsuccessful. The subunit preparation obtained showed a protein banding pattern similar to that of intact virus on S.D.S. polyacrylamide gel electrophoresis - indicating that the preparation of

haemagglutinin and neuraminidase subunits was not pure. It was possible that this failure was due to the low concentration of detergent used. The detergent concentration used to disrupt the virus by Almeida's method was only 0.2% compared to the 2% used by Skehel et al (1975) for the same purpose.

In view of these results, attempts were then made to use Skehel's technique to isolate influenza virus haemagglutinin. This method avoids the use of laborious sucrose density gradient fractionations and depends instead on cellogel electrophoresis to separate haemagglutinin subunits from detergent-disrupted whole virus. Skehel's technique gave satisfactory results in each of the quality control analyses described in the text, and including the SDS polyacrylamide gel pattern. A pure preparation of haemagglutinin subunits had therefore been obtained.

Use of IIF to detect antibody to a specific virus component

The IIF technique in diagnostic virus laboratories measures antibody directed against whole virus - rather than against the individual antigens of the virus particle. A technique that could measure antibodies to single virus components would be of considerable value, and possibly of great clinical importance in medical virology. Experiments were therefore set up to see if a purified virus antigen could be attached to liposomes and used as a test substrate for detecting antibody to the antigen by IIF. This was done by combining purified haemagglutinin from influenza A virus with liposome preparations. These haemagglutinin-liposome mixtures were used as substrates in IIF tests on human sera known to contain antibody to influenza A virus. Parallel tests using whole virus-liposome substrates and also virus infected cell substrates were included. The results showed that haemagglutinin-associated liposomes were able to detect antibody to influenza A virus by IIF. The use of this single viral component (i.e. the HA protein) attached to liposomes as an IIF substrate, gave similar antibody titres to those estimated using intact virus-liposome substrates and infected cell substrates. Although the technique was used here to detect antibody reacting with virus haemagglutinin (which can also be detected by HAI tests) clearly it could have applications with purified antigens or proteins of other viruses. For example, it might be used to detect antibody

to individual proteins of polioviruses, coxsackie B viruses (with which neutralisation tests are difficult to interpret due to cross reactions) herpes simplex, and possibly other human pathogenic viruses also.

Cross reaction between HA - Liposome IIF substrates with sera specific for a different viral haemagglutinin

Purified haemagglutinin successfully detected influenza virus antibody when used as antigen in IIF tests and the antibody measured was not markedly different in titre or specificity from that estimated on conventional substrates. However, unlike standard HAI tests, IIF tests with liposomes which contained intact influenza viruses with antigenically different HA_s (i.e. H_3N_2 and H_1N_1), could not distinguish antibody to the different haemagglutinins. Sera which had been shown by HAI tests to contain high levels of H_1N_1 strain specific antibody, reacted equally well in IIF with H_3N_2 and H_1N_1 intact virus - liposome substrates and with liposome substrates containing purified haemagglutinin protein from H_3N_2 virus. This raises the question as to whether the IIF test with whole virus antigen reacts with an antigen other than, or possibly in addition to the strain-specific haemagglutinin (of the antigen in HAI tests) - for example, the 'S' or soluble ribonucleoprotein antigen.

Possibility of a 'common' binding site on the influenza virus haemagglutinin

Sera - known from previous HAI tests to contain high levels of antibody only to H_1N_1 influenza virus - reacted by IIF with the purified haemagglutinin from H_3N_2 virus. These sera also reacted with H_3N_2 whole virus-liposome substrates. Most of the human sera used here contained different levels of antibody to H_3N_2 and H_1N_1 influenza virus strains - in general reacting in higher titre with H_3N_2 virus. The fact that the IIF tests using serum containing antibody to only one type of influenza virus reacted equally well with purified haemagglutinin from another type of virus, implies that the different influenza virus haemagglutinins may share a common antigen which is not strain specific.

Laver, Downie, and Webster (1974 and 1976) have put forward evidence that the V or haemagglutinin antigen of influenza virus A strains, contains at least two antigenic determinants with different specificities. One of these undergoes major antigenic change in different virus strains but the other remains relatively constant.

From their work on rabbits hyper-immunised with purified haemagglutinin subunits from different influenza viruses, Laver et al proposed that antibodies were formed to both ' common' and 'specific' antigenic determinants on the influenza virus

haemagglutinin. The 'common' binding sites were shared by haemagglutinins which were otherwise antigenically distint, but 'specific' sites were unique to antigenically different strains. In view of the results recorded here it seems possible that the two strains of influenza virus - namely X - 31 (H_3N_2) and Russia (H_1N_1) may share a common antigenic determinant on the haemagglutinin which reacts in IIF but not in HAI. The HAI technique detects strain specific antibodies to influenza virus A, and may do so by reacting only with the 'specific' sites on the haemagglutinin. The IIF technique on the other hand may react with the 'common' sites as well.

This hypothesis could be tested by preparing antibody in experimental animals to several antigenically distinct influenza virus A haemagglutinins (i.e. anti purified HA_s), and testing them on HA-Liposome IIF substrates for cross reactivity. If the sera were shown to cross react this would provide evidence for a 'common' binding site on the haemagglutinin which was not strain specific.

Adjuvant activity of liposome associated subunits

Previous work on the immunogenicity of influenza virus subunits had shown that the antibody response induced in both experimental animals and humans, was considerably less than that produced by preparations of intact virus. (Barry, Staton, and Maynor, 1974; Webster, Glezen, Hannoun and Laver, 1977). Some of the commercially available subunit vaccines in fact contain whole virus - although this has been reduced to approximately 10% of the original virus content in intact virus vaccines (Duncan Flockhart and Co., 1978). The risk of adverse reactions to subunit vaccines in sensitive individuals would therefore still be present. Such adverse reactions may be both systemic and at the site of injection and are due to either allergic or pyrogenic reaction. Allergic reactions are usually due to contaminating egg protein in the vaccine - this can be minimised by purifying the virus by sucrose density gradient centrifugation to remove contaminating egg protein. (Reimer et al., 1967). Pyrogenic reactions are caused by the presence of intact virus in the vaccine. Any factor which interferes with the structural integrity of viral lipids has been shown to reduce pyrogenicity (Webster and Laver, 1966), - even if the lipids themselves remain in the vaccine. However a vaccine which contains all the products of disruption will indiscriminately induce antibodies both to the extraneous viral components - as well as to the

protective surface antigen. The use as a vaccine of the major antigen (i.e. the haemagglutinin) responsible for inducing protective antibody to influenza - alone and in a purified state would be of considerable advantage.

Attempts have been made to enhance the immunogenicity of haemagglutinin and neuraminidase subunit preparations, by association with inert carriers (such as aluminium hydroxide) as adjuvants, (Jennings, Potter, McLaren and Brady, 1975: Brady and Furminger, 1975). This induced significant titres of antibody in both animals and volunteers, and there was a dose response relationship between the antibody produced and increasing doses of vaccine. (Potter et al, 1977). However no work has been done to date to determine if a purified haemagglutinin subunit preparation, containing no other viral proteins might, when associated with liposomes, induce significant levels of antibody either in experimental animals or volunteers. An adjuvant is by definition a substance which when incorporated into, or injected simultaneously with an antigen increases the immune response. (Roitt, 1977). Examples of work with other antigens have shown that liposomes have adjuvant activity (Gregoriadis and Allison, 1974; Allison and Gregoriadis, 1974; Heath, Edwards and Ryman, 1976). For example, the antibody response in mice challenged with diphtheria toxoid entrapped in negatively charged liposomes was found to be several times greater than when the antigen was given in the free form. Similar results were found with

entrapped albumin (Heathwood et al, 1976). The idea that antigens entrapped in lipid envelopes i.e. liposomes, may induce a higher antibody response has aroused wide interest (Gregoriadis, 1980). Lymph node localisation of injected liposomes is considered to be the basis of their immunopotentiating property, (Allison and Gregoriadis, 1974). This immunopotentiating effect has already shown promise in vaccine development - for example in conjunction with hepatitis B virus surface antigen for a vaccine against hepatitis (Manesis et al, 1979). If liposomes were to prove an effective and safe adjuvant in human beings, their use could reduce the amount of antigen required for effective immunisation.

The results reported here show that liposomes do indeed have adjuvant activity when combined with influenza virus haemagglutinin, and used to immunise guinea pigs. Two doses of liposome-associated haemagglutinin produced a significant antibody response in each of the groups of animals tested after 35 days. The response obtained was measured by IIF and HAI tests and was considerably less than that elicited by equivalent antigenic doses (measured in haemagglutinin units), of whole virus. However the most significant observation was that liposomes with haemagglutinin induced a significantly higher titre of antibodies than equivalent doses of haemagglutinin alone. These experiments therefore clearly showed that liposomes have adjuvant activity when combined with influenza

virus A haemagglutinin. A dose response relationship was established in that larger doses of haemagglutinin (of approximately 4096 HU_s) induced higher antibody titres than lower doses (of approximately 1024 HU_s). Although with each of the graded antigenic doses the antibody response to whole virus was several times greater than that in animals inoculated with haemagglutinin plus liposomes, it should be remembered that with inoculations of whole virus, antibodies are being induced to other viral components in addition to the protective surface antigen.

These results were similar to those of clinical trials in which human volunteers were inoculated with haemagglutinin and neuraminidase subunits adsorbed onto an inert alhydrogel carrier. In the human trials a dose response relationship was also established and carrier-associated subunits induced significant levels of serum antibody to influenza A virus. (Potter et al, 1977). However the liposome-associated subunit preparation used here contained the major antigen of influenza virus with no other viral proteins - and should thus be minimally reactogenic.

These findings suggest that liposome-associated haemagglutinin preparations might offer significant advantages over the present inactivated influenza virus vaccines. (Brandon, Barret, Hook and Lease, 1967; Phillips, Phillips, and Hodgkin, 1973). Volunteer studies would of course be

required to establish if the results found here in guinea pigs were also applicable to human beings. Unfortunately the experiments in guinea pigs indicated that two doses of haemagglutinin-liposomes were required to achieve significant antibody levels - a marked drawback if such a vaccine were to be used in man. This work had of course been carried out on unprimed experimental animals with no previous immunological experience of influenza A. It would therefore be of considerable interest to observe the response of human volunteers to graded antigenic doses of haemagglutinin liposome mixtures in similar experiments. As most people have had previous experience of influenza virus, their response to injection with haemagglutinin-liposome preparations might well be greater than in experimental animals. It would also be of interest to investigate the effect of varying the size and surface charge of the liposomes when used as an adjuvant for influenza virus. In this study multilamellar liposomes of negative charge and mixed size were used. According to Gregoriadis (1976), alteration of the carrier's physical characteristics can alter its circulatory properties in the host animal and improved retention would probably enhance the carrier's adjuvant effect.

The use of liposomes combined with purified virus protein offers the possibility of developing safer effective vaccines not only against influenza but against other viruses in which the antigen involved in protection can be identified e.g. members of the Paramyxo-virus group - possibly mumps and parainfluenza types 1-3.

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