A NOVEL ADJUVANT: POLYMERISED SERUM ALBUMIN BEADS

THESIS

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A B S T R A C T

Lee, T. et al (1981) proposed the encapsulation of hormones such as progesterone into serum albumin beads, such that their *in vivo* proteo= lysis would allow a gradual release of hormone at low levels, for ex= tended hormone action. It was proposed, in the Department of Micro= biology, Rhodes University, to replace the hormone component of the above bead formulation, with virus as antigen, in the development of a vaccine.

Beads optimally crosslinked at 1% final glutaraldehyde concentration, containing Nodamura virus, were shown to promote an adjuvant effect *in vivo*, analogous to the release of antigen from Freund's Complete Adjuvant (FCA), so that extended immunostimulation resulted.

It was shown that soluble antigen promoted a short-lived primary immune response, peaking around day 25 following inoculation. Antigen presented in beads, on the other hand, initially elicited a low humoral response, but this response gradually increased up to a peak around day 110 post inoculation, before decreasing.

No apparent adverse side-effects were noted following inoculation of antigen-containing serum albumin beads, compared to necrosis following antigen in FCA inoculation, supporting the proposal of using albumin homotypic for the test inoculee animal, so that the beads would themselves be non-immunogenic and would merely act as a vehicle in the vaccine for= mulation.

The indirect enzyme-linked immunosorbent assay (ELISA) was used to monitor the humoral response to antigen following inoculation. Results showed that

covalent crosslinking of albumin in the formation of the beads did not promote immunogenicity on the part of the chemically altered albumin.

The ELISA test was used to indicate the kinetics of the IgG response to Nodamura virus when presented in formulations such as: Freely soluble virus or its subunit; soluble intact virus inactivated by treatment with glutaraldehyde; intact virus entrapped in serum albumin beads cross= linked at different percentage final glutaraldehyde concentrations and also virus subunit prepared in albumin beads.

The presence of virus-neutralising antibodies was noted in serum obtained from rabbits inoculated with virus entrapped in albumin beads. Virus infectivity, titrated in mice, showed protection against virus challenge after incubation of virus with serum obtained above.

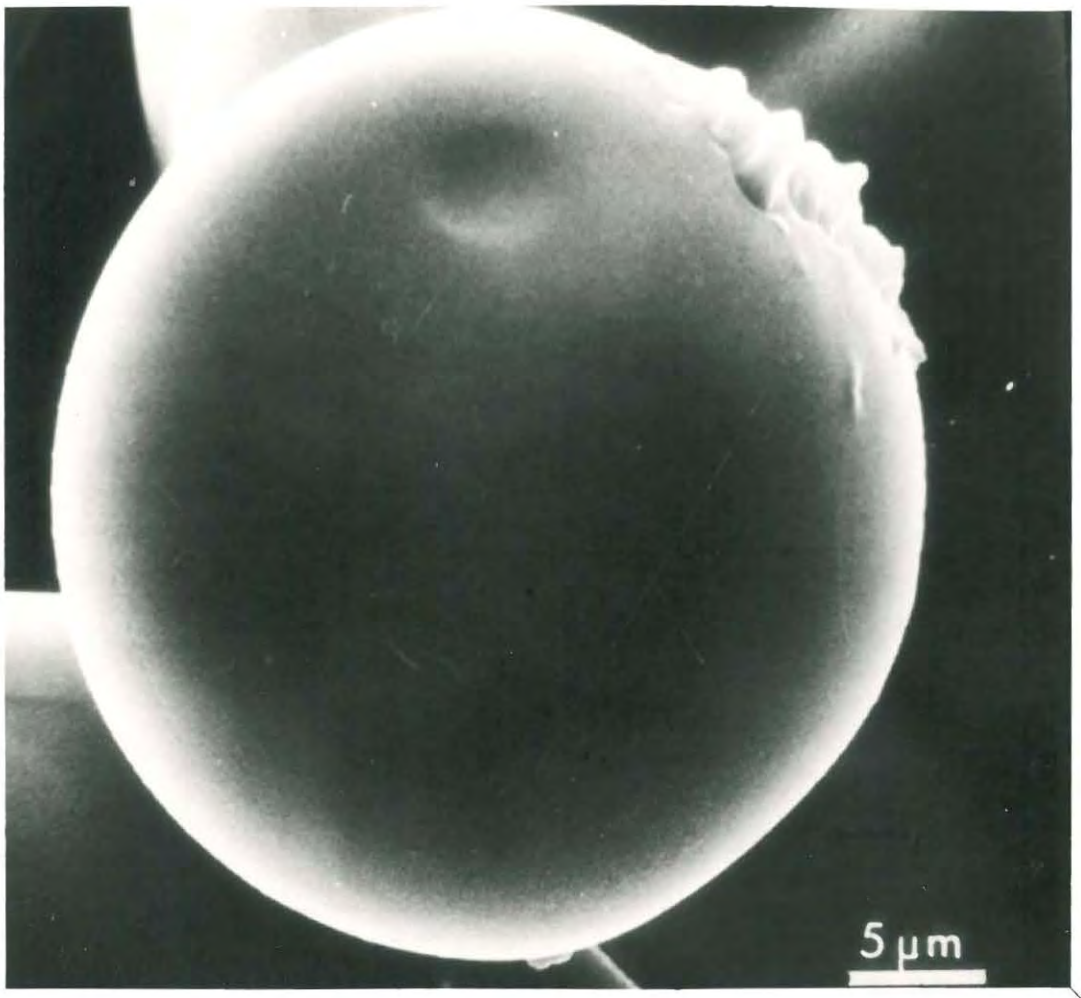


Figure 1: Scanning Electron Micrograph of a serum albumin bead, 50 µm in diameter.

INTRODUCTION

There is an ongoing need to develop new vaccine formulations. To com= plement accumulating knowledge on the structure of antigens and their interaction with cells of the immune system, new vaccine vehicles to optimise the immunogenic potential of the antigen must be developed. Associated with this adjuvanticity, should be minimal adverse side effects.

To these ends, this study was initiated to develop a vaccine vehicle, consisting of covalently cross-linked serum albumin beads (Lee, T. et al., 1981), themselves non-immunogenic, containing virus. Following ino= culation, in vivo proteolysis of the beads would allow a gradual release of antigen for sustained immunostimulation. An example of a scanning electron micrograph of a serum albumin bead is shown in Figure 1.

This system might have application in virus vaccine programmes to im= prove low immunogenic vaccines, such as Rift Valley Fever Virus (Kark, J. et al., 1982), to allow optimal delivery of the recently derived synthetic virus subunit peptides (Lerner, R., 1982; Shinnick, T. et al., 1981) as well as for inactivated virus vaccine preparations.

RECOGNISED VACCINES

Traditional virus vaccines are of two types: the killed or inactivated type, obtained by chemically altering and killing the vaccine virus; and the attenuated type, where genomic changes in the vaccine virus resulting from passage through cell culture, lead to avirulence.

A classic example of the effectiveness of these vaccines is shown in

the results obtained in the immunization programme against paralytic poliomyelitis. In 1954 the killed Salk vaccine was introduced and the incidence thereafter of poliomyelitis in the U.S.A., dropped from 10 to 12 cases per 100 000 population, to 0,5 cases per 100 000 popula= tion. Following the introduction of the attenuated Sabin vaccine strains (Sabin, A., et al., 1973) in 1961, the incidence of polio dropped further to 0,003 cases per 100 000 population (Robinson, D. 1982). This is dramatically illustrated in Figure 2 overleaf, indi= cating the efficacy of these vaccine formulations.

The methods used to develop these vaccines (Potash L.,1963) indicate some of the problems associated with these vaccines. Inactivated vaccines must be maintained in aqueous form at $4^{o}C$. Compared to lyo= phili sed vaccines, interruption in the refridgeration of these large volume vaccines could lead to reduced efficacy. Furthermore, this type of vaccine which requires boosters - a logistical problem is some un= derdeveloped countries - might be contaminated by residual live viru= lent virus.

Immunopathology has been shown with killed measles and respiratory syncytial virus vaccines (Chanock, R. et al., 1975), apparently due to insufficient stimulation of mucosal IgA production with concommi= tant reduction in local immunity. Severe reaction followed a subse= quent booster inoculation.

Problems such as these have resulted in a historical trend away from inactivated vaccines (only rabies and influenza virus vaccines are regularly prepared by inactivation) to attenuated virus vaccines, which emulate the process of natural immunization (Kit, M. & Kit, S., 1983).

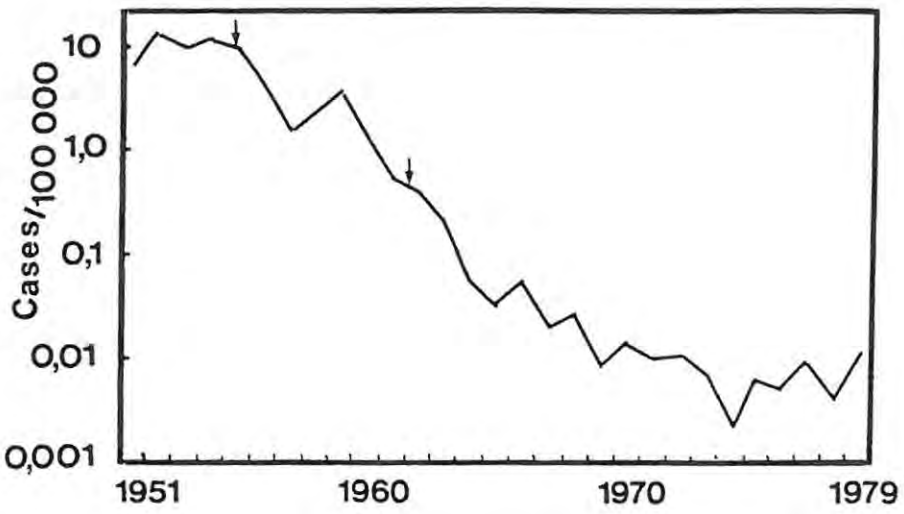


Figure 2: Reduction in paralytic poliomyelitits attack rate (1951-1979) since the introduction of the inactivated and attenuated vaccines (arrowed respectively).

There are problems, nevertheless, associated with these vaccines.

Live vaccines may be contaminated by adventitious virus co-infecting the propagating cell culture. Monkey kidney cells, possibly infected with Simian virus 40 (SV 40) have been therefore replaced with cell lines such as diploid WI-38 cells (Fenner, F. & White, D., 1976). Pri= mate cell lines are used to propagate inactivated vaccine strains against poliomyelitis (Salk, J. et al., 1982) and Rift Valley fever (Kark, J. et al., 1982). Post-vaccination loss of attenuation has been noted, resulting from only a small number of genome mutations, with poliomyelitis vaccine strains 2 and 3 (Kew, O. et al., 1981). Neuro= virulence has also resulted from smallpox vaccination (Heggie, A., 1983) as has measles encephalitis and rubella neuritis from their re= spective immunization (Fenichel, G., 1982).

Immunization with attenuated vaccines is contra-indicated in the case of immunocompromised individuals, whose immune system would be in= capable of preventing systemic spread of the vaccine virus.

Another problem associated with attenuated vaccines is the phenomenon of virus interference whereby superinfection of host cells is preven= ted by already replicating virus, thus blocking interaction between the superinfecting attenuated vaccine strain and the host immune system.

The effectiveness of immunization programmes in the U.S.A. has resulted in the eradication of, for example, smallpox. This has highlighted the necessity to re-evaluate immunization strategies.

Against the backdrop of problems associated with immunization, the increase in the risks/benefit ratio associated with immunization prompted the United States Public Health Service in 1971 to advise against routine smallpox immunization (Heggie, A., 1983). The resul= ting expanding pool of unimmunized individuals, vulnerable to viral attack, could be protected by the development and utilisation of novel vaccines, such as the subunit vaccines, to supplement existing vaccines.

SUBUNIT VACCINES

Ideally, a vaccine should contain only immunogenic material, sufficient to stimulate a protective immune response. This obviates many of the risks associated with attenuated vaccines. Part of the object of this study was to determine the immunizing potential of virus capsid protein released from serum albumin beads *in vivo*. If found to be effective, this formulation could be used to augment the immune response to poor immunogens such as haptenic peptides.

The parameters most crucial for the immunological properties of the antigen are its molecular size, spatial conformation and the accessi= bility of the antigenic determinants (Arnon, R., 1980). Morein, B. and Wun= ner, W. (1983) concluded from studies on influenza type 3 and rabies virus glycoprotein respectively, that multimeric polypeptides were highly immunogenic in comparison to the monomeric form. Furthermore, segments of polypeptides interacting with antibodies would be mainly hydrophilic in nature and externally situated in relation to the secon= dary and tertiary conformation of the polypeptide (Lerner, R., 1982). Accurate predictions of antigenic determinants from sequences rich in hydrophilic amino acids have been made (Hopp, T. & Woods, K., 1981).

Small synthesised peptides corresponding to immunodominant segments of virus capsid protein have been shown to elicit antibodies capable of interacting with intact virus (Lerner, R., 1982; Sutcliffe, J. et al., 1983). Bittle, J. et al. (1982) induced neutralising antibodies against foot and mouth disease virus (FMDV) following inoculation of a hydrophilic-rich dodecapeptide corresponding to amino acids 141 to 160 of FMDV virus protein 1 (VP1). Emini,E.et al (1983) used synthetic polio VP1 peptides to elicit priming, but not neutralising antibodies against intact virus challenge. The studies with these peptides involved linking them to carrier proteins such as keyhole limpit haemocyanin or bovine serum albumin so as to promote an immune response to the haptenic synthetic peptide. Chemically cross-linking peptides into serum albumin beads might emulate the effect of the large carrier proteins. Proteolysis of the beads in vivo might lead to the release of antigen attached to relatively large fragments of serum albumin. The linked molecules might then promote a potent immune response to the peptide antigen.

The above peptides were synthesized using the Merrifield method (Marglin, A. & Merrifield, R., 1970).Biosynthetically derived peptides have recently been produced. Kleid et al. (1981) cloned FMDV genome so that a hybrid VP1 protein, capable of eliciting neutralising antibodies, was synthesized. Hepatitis B surface antigen (HBsAg) and influenza virus haema=glutinin (HA) have also been synthesized in yeast and monkey kidney cells (Brown, F., 1984). Macket, M. et al. (1982) have replaced the non-essential regions of vaccinia virus genome with HBsAg and influenza HA genes. The resulting chimaeric virus elicits neutralising anti=bodies to homologous virus and may have potential for the development of polyvalent vaccines.

There is uncertainty, however, at this stage as to the relative im= portance of the humoral and/or cellular immune response to one type of virus over another. Thus the nature of subunit vaccines and their capacity to give rise to protective immunity have to be extensively studied before vaccines can be used for large scale vaccination pro= grammes (Pettersson, R., 1982).

IMMUNOPOTENTIATION

Immune stimulation results from an exceedingly complex interaction between the immunogen and the cells of the immune system. The repli= cation strategy of attenuated viral strains results in a natural immu= nopotentiation. The cycle of cell infection, promoting a cell mediated and hypersensitive response, followed by viremic release of progeny virions, stimulating a humoral response, virtually negates the inclu= sion of an adjuvant in the vaccine formulation.

Inactivated and subunit vaccines, usually inoculated subcutaneously or intramuscularly, require the addition of an adjuvant in their pre= paration however, to promote interaction between antigen and the cells of the immune system. Adjuvants may be regarded as substances which may increase the immunogenicity of a poor immunogen, or lead to an in= crease in the level of circulating antibodies, to an increase in cell= mediated immunity or to more effective protective immunity (White, R. G., 1976).

This study, involving the incorporation of soluble antigen such as inactivated virus, virus subunits or even bacterial toxoids, into serum albumin beads, hoped to show a comparable release of antigen - and their improved immunogenicity *in vivo* - as would be found with

the same antigen prepared in the recognised antigen depot adjuvants, such as Freund's complete and incomplete (FCA and FIA) and the gels of aluminium or calcium salts. While the latter gels are the most acceptable and widely-used adjuvants in human vaccines, they are inadequate as adjuvants for parasite antigens in animal vaccination systems (Bomford, R., 1980).

FCA and FIA are extremely effective in animal immunization studies. The adjuvants basically consist of mineral oil with emulsifier, containing or without dried Mycobacterium tuberculosis. Antigen in aqueous phase, is emulsified with the oil and the resulting water-in-oil emulsion, once inoculated, is thought to slow the absorption, destruction and elimination of the antigen (Talmage, D.W. & Dixon, F.J., 1953). These workers favourably compared the "antigen-depot" effect of water-in-oil emulsions over alum-precipitated antigens. They showed that 90% of bovine gamma globulin (BGG) as antigen, was released from the site of inoculation over a period of 30 days, when introduced as a water-in-oil emulsion, whereas only 1% of alum-precipitated BGG remained at the inoculation site after 7 days.

A disadvantage of the use of FCA, while stimulating a high and long-lasting antibody response, is that it's use is inevitably accompanied by extensive granuloma formation and, often, the production of an abscess at the inoculation site (Herbert, W.J., 1978).

These adverse reactions, preventing the use of FCA in human vaccines, are probably due to the extremely slow metabolism of the mineral oil and to inflammation caused by the mycobacteria. The wax D peptidoglycolipid component of human mycobacterial cell wall is thought to induce

this delayed hypersensitivity. Attempts to replace the mycobacteria with less damaging materials have shown that certain chemically de= fined compounds such as saponin and quaternary ammonium salts, characterised by long aliphatic chains and nitrogenous groups, as well as vitamin A derivatives, have adjuvant activity. These com= pounds have a membrane labilising effect and their adjuvanticity may be due to macrophage plasma and lysosome membrane destabilisation, resulting in the leakage of enzymes from the cell (White, R.G., 1972). Bacterial cell wall homologues, such as muramyl dipeptide (MDP) have also been used to replace mycobacterium (Chedid,L. et al 1976).

As alluded above, macrophages play a key crucial helper role in immune induction. They appear to be fundamentally involved in immune stimu= lation, antigen recognition and the control of lymphocyte prolifera= tion and differentiation (Unanue, E.R., 1978). Inoculated antigen would initially interact with circulating macrophages. Depending on the T-cell dependent or independent nature of the antigen (Burns, W., 1975), internalisation of most of the antigen by the macrophage and its subsequent expression on the macrophage cell membrane in a processed, highly immunogenic form, would promote B-cell and T-cell stimulation. Subsequent plasma cell secretion of antigen-specific antibody would allow antigen immobilisation and opsonisation. T-cell stimulation leading to secretion of lymphokines and other soluble factors, help focus the immune system on the antigen. A brief summary of the interaction between cells of the immune system and antigen, is provided in Figure 3.

From the viewpoint of vaccination strategy, much work remains toward elucidating the cell-antigen interaction so that immune stimulation

(and not tolerance) is induced with the minimum of harmful side-effects.

NOVEL VACCINES

This brief overview of vaccines and their formulations presently in use, indicate the need for ongoing research toward developing new (and improved) vaccines. This has led to many innovations toward optimising antigen presentation *in vivo*.

Higgins, P. (1983) used small volumes of primary precipitating antibody to promote antigenically stable agarose-immobilized immune complexes with tumour specific antigens. After processing and inoculation, these com=plexes act as suitable antigens leading to the formation of large quan=tities of highly specific antibodies. The development of virosomes (Van Rooyen, N. et al., 1983; Thibobeau,L. et al., 1984) consisting of lipo=somes prepared from readily metaboliseable lysolecithin, phosphatidyl=choline and cholesterol, onto whose surface was attached virus sub=units, have shown potential as vaccines. The emulation of intact virions by the subunit-studded liposomes, promotes effective interaction with cells of the immune system. Morein et al. (1984) developed an immuno=stimulating complex (ISCOM) resulting from hydrophobic interaction between virus protein, such as rabies, measles and parainfluenza type 3 subunits, and a glycoside, Quil A. The ISCOM has ten times the immunogenicity of equi=valent virus protein micelles.

Kreuter, J. & Liehl, E. (1981) demonstrated that killed influenza virus could be incorporated into photopolymerised poly(methylmethya=crylate) nanoparticles. This system stimulated an optimal antibody

response and was more stable to heat inactivation than aluminium hydroxide adjuvants.

Linking this to reports by Lee, T. et al. (1981), who showed that hormones were slowly released from polymerised serum albumin beads, crosslinked with glutaraldehyde, it was decided in this department to monitor the efficacy of this system as an adjuvant for the slow release of Nodamura virus an antigen.

To determine this, a number of parameters had to be monitored.

Of prime importance was the assurance that native serum albumin, homotypic for the host animal, once crosslinked into beads, would not undergo such chemical alteration as to render it immunogenic. This might have catastrophic effects following the development of auto-immunity to serum albumin in the host animal.

Brownlee, M. et al. (1983) indicated that associated with diabetic microvascular disease was the in situ formation of immune complexes attached to extracellular kidney matrix such as basement membrane or collagen.

Initially the matrix proteins are glycosylated by non-enzymatic interaction between glucose and protein lysine residues. Highly reactive carbonyl groups result capable of intermolecular crosslinking with plasma proteins such as albumin (Miller, K. and Michael, A., 1976).

The tightly bound albumin could then function as an antigen in the formation of immune complexes, leading ultimately to tissue damage.

Acquired immunogenicity to albumin beads prepared by crosslinking with GA, analogous to the activated molecules described above, must be deter= mined by extensive histopathological studies before the albumin beads may be considered acceptable for use in vaccine formulations.

Secondly, having determined the optimal percentage crosslinking of antigen-containing beads in vitro, would this system promote a suitable challenge on the host immune system by the optimal release of antigen from the proteolysed beads?

Thirdly, would this response lead to virus-neutralising antibodies or at least immune priming antibodies, suitable for immunoprophylaxis?

Finally, could this system, if successful for intact virus as antigen, be utilised with subunit antigens?

These parameters and the stages in the development of crosslinked beads containing antigen, as well as the monitoring of the release of antigen with resultant antibody formation, will be discussed in the following chapters.

CHAPTER 1

PREPARATION OF VACCINE FORMULATION COMPONENTS

1.1 INTRODUCTION

The vaccine formulation envisaged in this study involves the incorporation of virus particles into serum albumin beads, stabilised by a bifunctional cross-linking agent such as glutaraldehyde. This requires, therefore, a virus which can be readily propagated and isolated and which is non-pathogenic toward humans. Likewise, methods for optimal isolation of albumin from serum must be adopted.

The antigen of choice was Nodamura virus, a small icosohedral virus 28 nm in diameter containing a bipartite RNA genome (Newman, J. et al., 1978). This virus, readily isolated from the rear limbs of infected suckling mice, resembles picornaviruses in morphology, stability at pH 3 and its buoyant density in caesium chloride (Murphy, F. et al., 1969). Associated with this virus is its ability to replicate in insects such as mosquitoes (Bailey, L. et al., 1975), as well as in vertebrates such as suckling mice (Newman, J. & Brown, F. 1973). Antibodies to Nodamura virus occur in swine (Scherer, W. et al., 1968).

Serum albumin is found at high concentration in blood serum, averaging 46 mg/ml (Documenta Geigy, 1975), and is virtually

free of carbohydrates such as hexoses or acetylhexosamine and so will be degraded in vivo as pure protein. It is readily isolated from blood serum with techniques such as gel exclusion chromatography. The proteins in a serum sample would fractionate according to size during passage through a Sephadex G150 column, for example, so that typically IgM (MW = 970 000) would elute in the void volume, followed by IgG (MW = 150 000) and then albumin (MW = 69 000). Columns can be easily prepared and then regenerated after elution for re-use (Hudson, L. & Hay, F., 1983).

Larger volumes of serum can be fractionated using differentiated salt precipitation. Heide, K. et al. (1979) described the precipi= tation of the euglobulins (part of the IgG, IgM, IgA, C1, α_2HS and α_2M) at 28 to 33% ammonium sulphate ($(NH_4)_2\ SO_4$) saturation. At 33 to 50% saturation, the pseudoglobulins are precipitated, followed then by the albumins. Precipitation of albumin is facilitated by lowering the pH of the protein solution to pH = 4,9, the pI of albumin, where it has lowest solubility.

The methods of virus and albumin isolation used in this study will be described in the section to follow.

1.2 MATERIALS AND METHODS

1.2.1 Propagation and isolation of Nodamura virus

This involves the inoculation of suckling Swiss albino mice with stock virus, followed by the isolation of virus from infected tissue by means of a series of differential centrifugation steps.

1.2.1.1 Maintenance of mouse colony

The upkeep and non-inbreeding of the Swiss albino mice colony was maintained accor= ding to Lane-Petter, W. (1976). The mice were caged in an animal room maintained at 24°C. Ventilation allowed 10 changes of air per hour. The mice were checked and their water bottles and feed hoppers cleaned and filled on a daily basis. Mating was allowed between a male and groups of 3 females. The females were separated 14 days after mating to litter. Those females producing large litters (10 - 12 offspring) were remated to propagate the colony while any other offspring were inocu= lated with a Nodamura virus stock dilution.

1.2.1.2 Propagation of Nodumura virus

Methods for virus propagation were based on those described by Newman J.et al(1973). The stock virus suspension consisted of a 10% mouse muscle extract in phosphate buffer(PB) (0,01 M; pH 7,5) made 50% with respect to glycerol, stored at -30°C. Three to six day old suckling mice were inoculated intra= peritoneally with 0,05 ml of a $^{1}/_{5}$ stock virus dilution. When paralysis of the hind limbs became severe, usually after 5 - 6 days, the mice were killed and stored (-30°C).

To obtain radiolabelled virus, on days 3, 4 and 5 post-inoculation, each mouse was inoculated intraperitoneally with 15 µCi of α(^{35}S)methionine (Amersham - International).

1.2.1.3 Isolation of Nodamura virus

Frozen mice were thawed (T_{room}; 3 hours). Their rear limbs and pelvic girdles were dissected out and homogenised, using a pestle and mortar, with 15 ml PB and CCl_4 (ratio 4:1). The homogenate was then centrifuged (12 000 g; 10 minutes). The supernatant was removed and stored (4°C) and the sediment suspended and homogenised in 10 ml PB/CCl_4 (4:1) before being recentrifuged (12 000 g; 10 minutes).

Virus was sedimented from the combined supernatants by centrifugation (95 000 g; 60 minutes). The sediment was resuspended in 1 ml PB and stored (4°C; 17 hours). Insoluble debris was sedimented by low speed centrifugation (2 000 g; 5 minutes) and the clarified supernatant made 1% with respect to sodium dodecyl sulphate (SDS). The virus solution was then centrifuged (60 000 g; 3,5 hours) through a 28 ml 15% - 45% (w/v) sucrose gradient. 1,2 ml fractions

were collected using an ISCO Model 640 Density Gradient Fractionator. Those fractions which con= tained virus were pooled, diluted to 28 ml (PB) and centrifuged (95 000 g;; 60 min.). the virus sediment was resuspended in PB (1ml), made 0,02% with respect to NaN_3, and virus concentration (mg/ml) determined as described in section 1.3.1.
To determine radiolabel incorporation into virus, 10 µl of each fraction obtained above, was counted as described in section 2.2.1.2.

1.2.2 Isolation of rabbit serum albumin

Rabbits were to be used for this study, and so homotypic rabbit serum albumin, for reduced immunogenicity, had to be isolated.

1.2.2.1 Isolation of serum from whole rabbit blood

The protocol for serum isolation was adapted from Garvey, J. et al. (1977). Blood was collected from unimmunised rabbits and allowed to clot (T_{room}; 2 hours). The clot was care= fully separated from the side of the container with an applicator stick. The blood was then refridgerated (4^oC; 24 hours), to allow clot contraction.

The serum was decanted and centrifuged (1 000 g; 30 minutes; 4^oC) and then the clarified serum, stabilised against microbial growth by the addition of sodium azide (NaN_3) (0,02%), was stored (4^oC) in sterile flasks.

1.2.2.2 Isolation of serum albumin using gel exclusion chromatography

A column of Sephadex G150 was prepared and utilised according to Garvey, J. (1977) and to the pamphlet issued by Pharmacia Fine Chemicals (1981). The eluent buffer used was ammonium hydrogen carbonate (AHCB) (0,02 M; pH 7,8) stabilized with NaN_3 (0,02%).

10 g dry G150 gel was suitably swollen in buffer and then packed into a 100 cm x 1,8 cm I.D. glass column, the base of which was constricted into a capillary tube over= laid with sintered glass wool and sterile sand. At a hydrostatic head of 30 cmH_2O, the column was equilibrated with AHCB and then by eluting Blue Dextran 2000 (Pharma= cia Fine Chemicals Inc.), the column's void volume (Vo) was determined. 50 drop fractions were spectrophotometrically determined at 280 nm using a PYE UNICAM SP8-400 UV/VIS spectro= photometer. The column was then regenerated by passage of eluent buffer (300 ml).

To fractionate rabbit serum, an aliquot (2,5 ml) was loaded onto the column and eluted. 50 drop fractions were determined as above. The albumin fractions, identified as described in section 1.3.2.1, were pooled,

frozen and lyophilised using a Research Engineering Model 25 freeze-drying appa= ratus. Percentage recovery of albumin was then determined, before storage ($4^{o}C$).

1.2.2.3 Isolation of serum albumin by ammonium sulphate precipitation

The method used was adapted from Kabat, E. & Meyer, M. (1967). A serum sample (50 ml) was diluted to 100 ml with distilled water. The solution was slowly (15 minutes) made 50% with respect to saturated ammonium sulphate (SAS). Immunoglobulin precipita= tion was promoted by storing the solution on ice overnight.

The supernatant was carefully removed from the immunoglobulin pellet after sedimentation by centrifugation (2 000 g; 10 minutes), and acidified by the slow addition of acetic acid (0,5 N; 5 ml) and SAS (5 ml). The solution was then made 75% with respect to ammonium sulphate and left on ice (8 hours). Albumin was sedimented by centrifugation (2 000 g; 10 minutes). The albumin pellet was resuspended in 4 ml AHCB and then salt was removed by dialysis against 4 x 500 ml changes of AHCB ($4^{o}C$; 24 hours). The albu= min dialysate was frozen and lyophilised

as in section 1.2.2.2. above. After deter= mining the percentage recovery of albumin, it was stored ($4^{o}C$).

1.3 RESULTS

1.3.1 Isolation of Nodamura Virus

Figure 4 indicates the fractionation of a Nodamura Virus preparation through a 25% - 45% (w/v) sucrose density gradient. The single peak in the gradient suggests a pure viral isolation. Peak fractions were pooled and could be used to propagate the virus. This peak also coincides with a peak of radioactivity (data not shown) obtained after isolating virus from mice which had been inoculated with virus stock and $\alpha(^{35}S)$methionine (Section 1.2.1.2.). Gradient fractions were counted as in Section 1.2.1.3. This result indicates the incorporation of $\alpha(^{35}S)$methionine radio= label into Nodamura Virus.

The peak fractions were pooled and scanned in the UV range (220nm-320nm). Figure 5 illustrates this. The OD_{280}/OD_{260} = 0,60 ratio is typical for Nodamura Virus preparations (Adams E., Longworth J.1978). Multiplying OD_{280} by 0,35 derives the isolated virus concentration (mg/ml). Average yields of 30 µg of virus were obtained per mouse.

1.3.2 Isolation of rabbit serum albumin

1.3.2.1 Serum fractionation using gel exclusion chromatography

Figure 6 shows the elution profile of a 2,5 ml sample of rabbit serum loaded onto an 80 cm G150 column, consistent with published serum elution profiles (Hudson L. & Hay F., 1983).

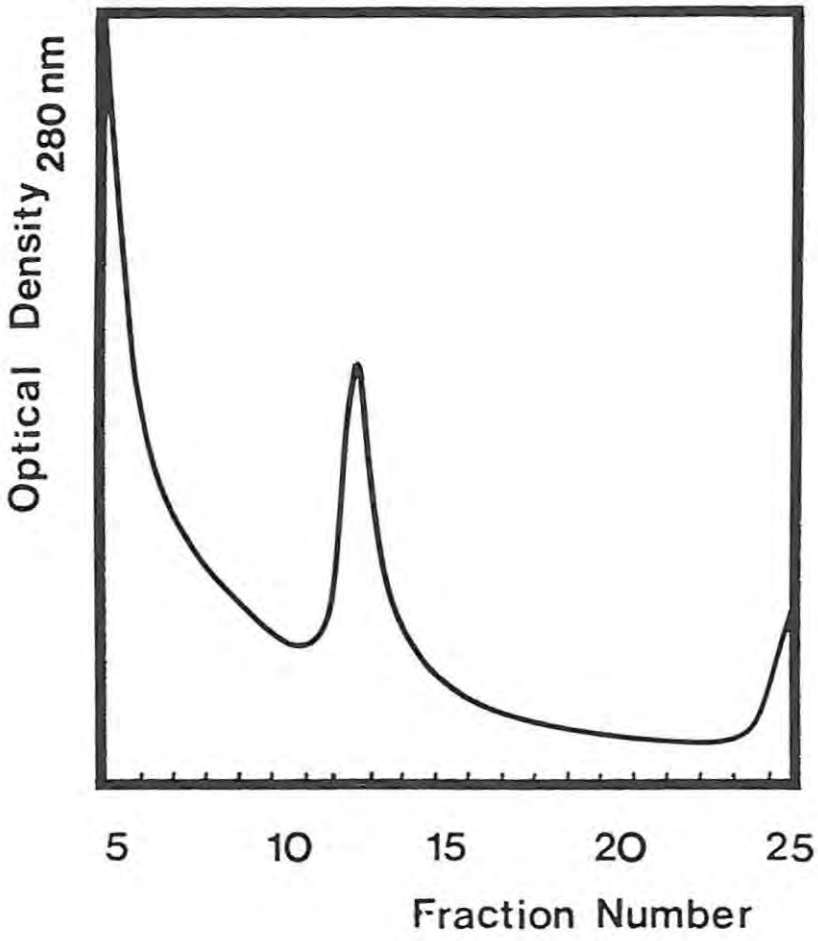


Figure 4: Fractionation of 25% - 45% (w/v) sucrose gradient, indicating the UV-absorbing virus peak around fraction 12.

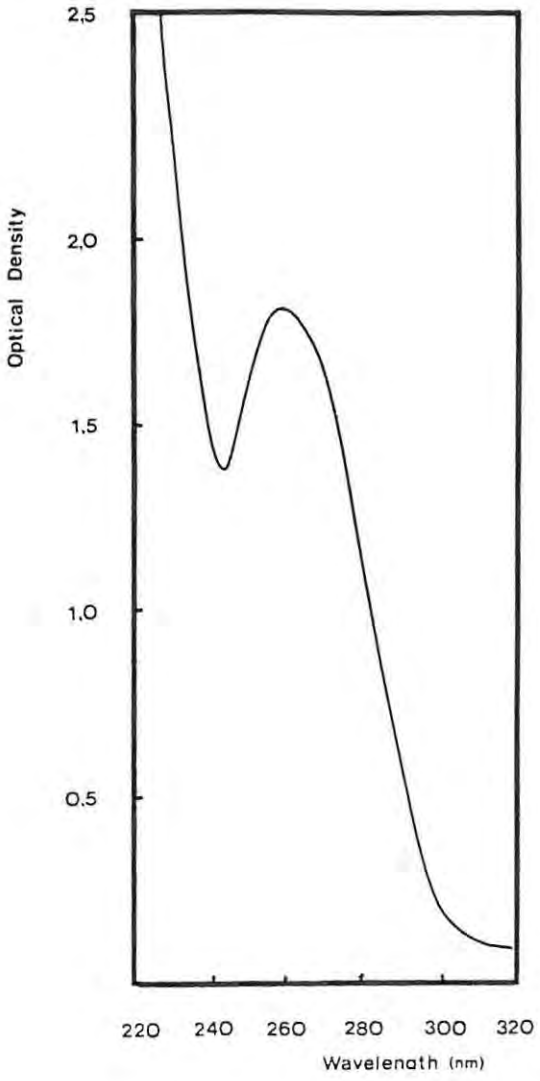


Figure 5: UV scan (220nm - 320nm) of a purified Nodamura virus sample.

Peak 1 corresponds to the V_o and consists of IgM and multimeric IgA molecules. This was suggested by the results of an experiment involving IgM precipitation. A serum sample (5 ml) was dialysed against distilled water. The resulting IgM precipitate was sedimented by centrifugation and the supernatant was loaded onto an 80 cm Sephadex G150 column. The elution profile of the IgM-free serum corresponded to Figure 6, with the notable absense of the peak equivalent to peak 1 in Figure 6.

Peak II consists of IgG, shown by elution of serum treated with SAS to 50% concentration. Comparison of the salt-treated serum elution profile with Figure 6 showed the absence of the peak corresponding to peak II. An UV scan of peak II showed an OD_{278}: OD_{250} ratio of between 2,3 and 2,7, characteristic of IgG (Steiner, L. & Lowey, S., 1966).

Peak III was identified as albumin. Comparison of the electrophoretogram obtained by electrophoresing a sample of peak III, untreated serum and molecular weight markers through a 10% polyacrylamide resolving gel, prepared according to Laemmli, U. (1970), showed peak III to resolve as a predominant band corresponding to a protein of 67 500 daltons.

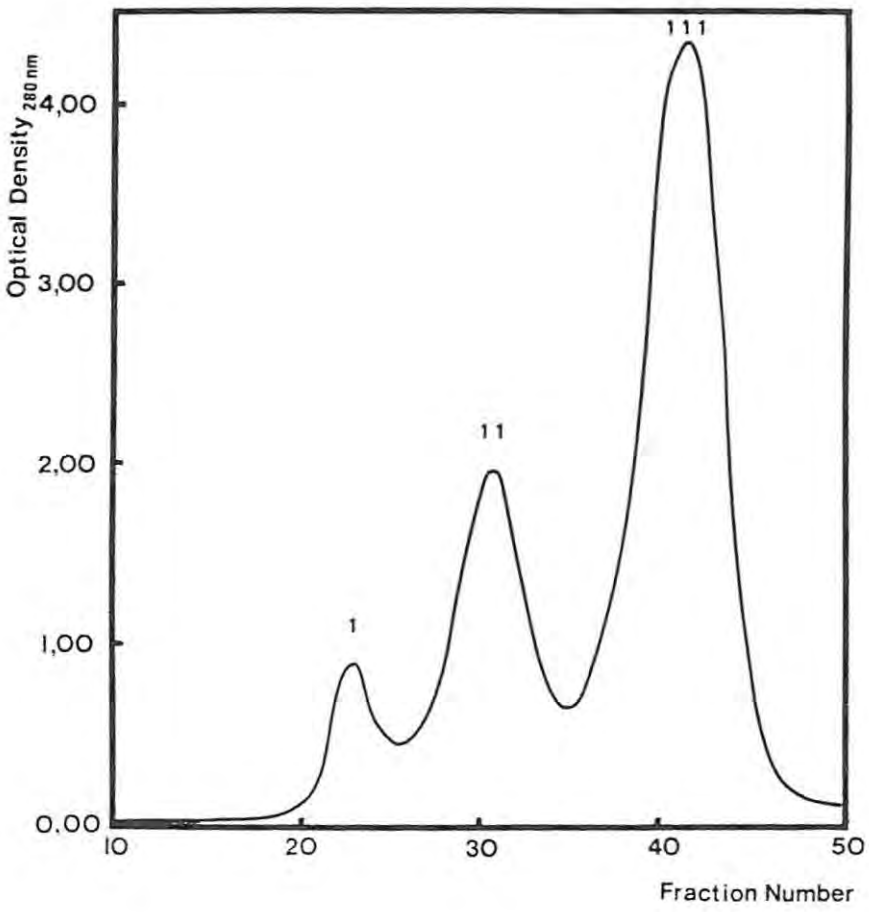


Figure 6: Elution profile of a 2,5 ml rabbit serum sample, loaded onto a Sephadex G150 column (50 drop fractions collected).

This also corresponded to a major protein band in the untreated serum track.

The mass of protein derived from the pooled, lyophilised peak III fraction equalled 100 mg. 2,5 ml serum could theoretically pro= vide 118 mg albumin, thus indicating an 85% yield of albumin using this technique.

1.3.2.2 Serum fractionation using differential salt precipitation

The mass of lyophilised salt-free protein precipitated after making serum 75% with respect to ammonium sulphate, corresponded typically to 60% of the expected theoretical yield. From 50 ml serum containing a theo= retical maximum of 2300 mg albumin, would be precipitated typically 1350 mg albumin.

Electrophoresis of a sample of the dialysate, as described above in section 1.3.2.1 also showed a single band corresponding to a pro= tein with a molecular weight of 67 100, con= sistent with results obtained above.

1.4 DISCUSSION

The methods described in this chapter lead to the ready isolation of the vaccine components. As serum albumin, homotypic for the inoculee, is fundamental to the formulation, it's easy isolation in good yields is important.

Serum can be readily obtained from a venous blood sample and depending on the albumin requirement, either of the two methods for its isolation as described in this chapter, gel exclusion chromatography or differential salt precipitation, can be utilised.

The gel exclusion technique allow rapid fractionation of serum, followed by immediate lyophilisation of the albumin component in high yield. The column can then be regenerated for re-use. The limitation to this tech= nique is the relatively small serum volume able to be loaded onto the column at any one time. As serum samples of only 1% to 5% of the column gel bed volume can be loaded for reasonable resolution of eluate components, longer columns or possibly larger diameter columns to prevent compaction of the gel, could be utilised for maximum albumin isolation.

For fractionation of larger volumes of serum, differen= tial salt precipitation was shown to be adequate. More steps in the process of producing salt-free albumin are involved with this technique, compared to the chromato=

graphic technique and so lower yields of albumin would be expected. Yields of only 60% albumin were noted, as opposed to 85% yields using gel exclusion chromatography.

The addition of salt to a protein solution results in competition between the inorganic ions and protein molecules, for the protein-hydrating water molecules. To promote protein-protein interactions over protein-water interactions, so that aggregation and precipitation can occur (Williams, B. & Wilson, K., 1975), addition of water must be minimised. Although the precipitation of albumin according to Kabat, E. & Meyer, M. (1967), involves the addition of SAS, it was found in this study that addition of solid ammonium sulphate had no adverse effects on albumin yields and kept volumes to a minimum, thus facilitating the procedure.

The techniques of differential centrifugation and precipitation, and gel exclusion chromatography, are basic biophysical methods for the isolation of (nucleo)proteins. The advent of genetically engineered subunits or chemically synthesised subunits as antigen sources, and chromatographic techniques such as affinity chromatography involving perhaps matrix-bound Cibacron Blue F3G-A, specific for albumin (Travis, J. et al., 1976), may possibly supplant the isolation methods described in this chapter.

CHAPTER 2

PREPARATION OF THE VACCINE FORMULATION

2.1 INTRODUCTION

When Lee, T. et al. (1981) indicated the sustained release of the hormone progesterone from serum albumin beads which were stabilised by the cross-linking agent glutaraldehyde (GA), this promoted the concept of replacing the small hormone molecules with entrapped antigen (Ag) in the beads, thus allowing Ag release following the gradual proteolytic dissolution, in vivo, of the beads. This system of antigen release is thought to mimic the action of "antigen-depot" adjuvants and so might be useful as an adjuvant.

This chapter will describe the action of GA, the preparation of the proposed vaccine formulation and some of the theoretical aspects of the possible adjuvanticity associated with this purported vaccine.

There has been widespread use of GA in medicine. It has been shown to be an effective biocidal agent, active against a broad spectrum of pathogens (Gorman, S.P. et al., 1980). The effec= tiveness of GA as a chemosterilizing agent is attributed to the stabilization of the surface of cells by the cross-linking mode of action of the bifunctional aldehyde groups of GA.

Commercially available GA is provided as a 25% solution at acid pH. Peters, K. and Richards, F. (1977) indicate that at this low pH (∿ pH 3) the GA molecule is in equilibrium with its cyclic hemiacetal and polymerised cyclic hemiacetal derivatives, as indicated in Figure 7(a) overleaf.

At neutral pH or slightly alkaline pH where most crosslinking experiments are performed, it is proposed that the GA molecule undergoes self aldol condensation followed by dehydration to form the αβ unsaturated aldehyde polymers , as indicated in Figure 7(b).

The aldehyde group of this derivative can now react with exposed protein amino groups, such as the ε-amino group of the lysyl side chain, in a Schiff base reaction, as shown in Figure 7(c). This product is resonance-stabilised by the conjugated double bonds. Lysine is the only amino acid which reacts with GA in this way.

Relyveld, E.H. and Ben-Efraim, S. (1983) indicated a number of bacterial, toxin, viral, allergen and cellular vaccines prepared by treatment with GA. Advantages of these chemically modified preparations are their intrinsic adjuvanticity, stability and short detoxification time when compared with formol-treated prepa= rations.

Bearing in mind acquired immunogenicity on the part of mole= cules treated with GA, care must be taken to ensure no altera=

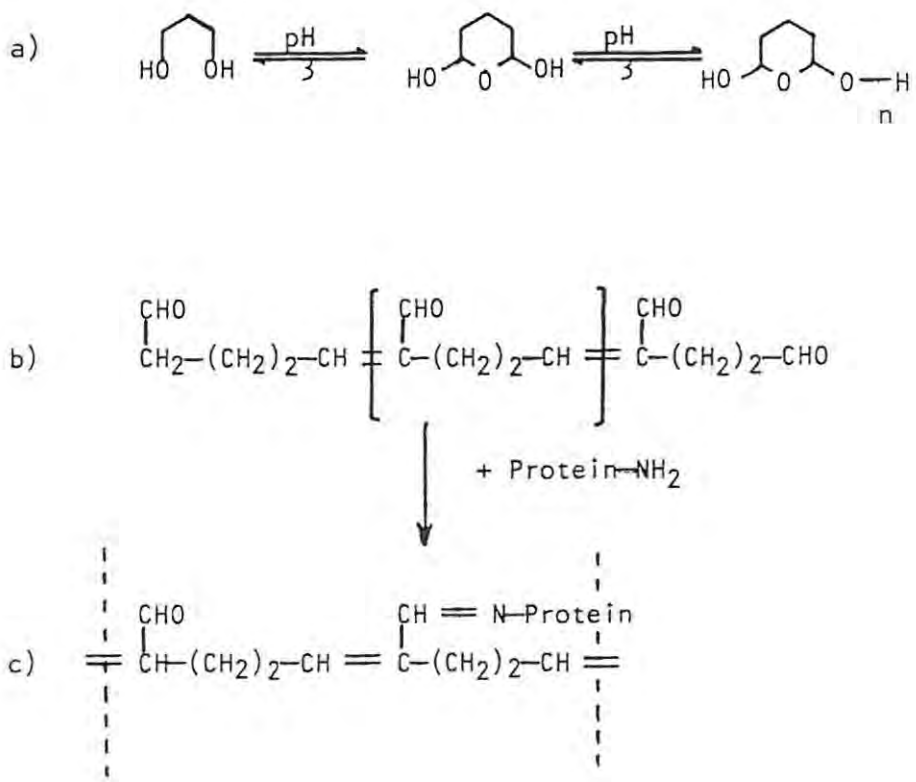


Figure 7: Activation of glutaraldehyde for reaction with proteins.

a) Equilibrium between cyclic hemiacetal and polymerised cyclic hemiacetal GA forms at pH 3.

b) Formation of αβ unsaturated aldehyde polymer at physiological pH.

c) Schiff base reaction with protein amino groups.

tion in the antigenic properties of the vaccine formulation albumin, used in this study. Acquired immunogenicity of albumin, originally obtained from the test animals, and then used in the vaccine formulation, could lead to autoimmunity. This requires testing. Lee, T. et al. (1981) indicated from physiological monitoring of test animals inoculated with GA-treated albumin, that no abnormal fluctuations in temperature and health of the animals occurred, however.

In the preparation of the beads, the albumin and antigen is dissolved in aqueous medium, mixed with GA before being expressed into a hydrophobic ethereal solution. Production of beads is promoted hydrodynamically by the formation of micelles in the resulting water-in-oil emulsion. The protein molecules are then linked by the polymerising action of GA, thus entrapping antigen molecules in the albumin matrix. The hydrophobic solution is stirred constantly throughout the polymerisation process, with the speed of stirring being inversely proportional to the size of the beads produced. The introduction of SDS, an ionic deter= gent, also promotes micelle formation.

Once introduced in vivo, the interaction between these beads and the host immune system is of paramount importance. Micellar bead formation in emulsified aqueous droplets would indicate that prior to bead stabilisation by GA, the albumin molecules would orient themselves so that their hydrophilic regions would be external to the hydrophobic portions of the molecules. This would impart an essentially hydrophilic nature to the outside of the beads, thus promoting attraction and interaction with

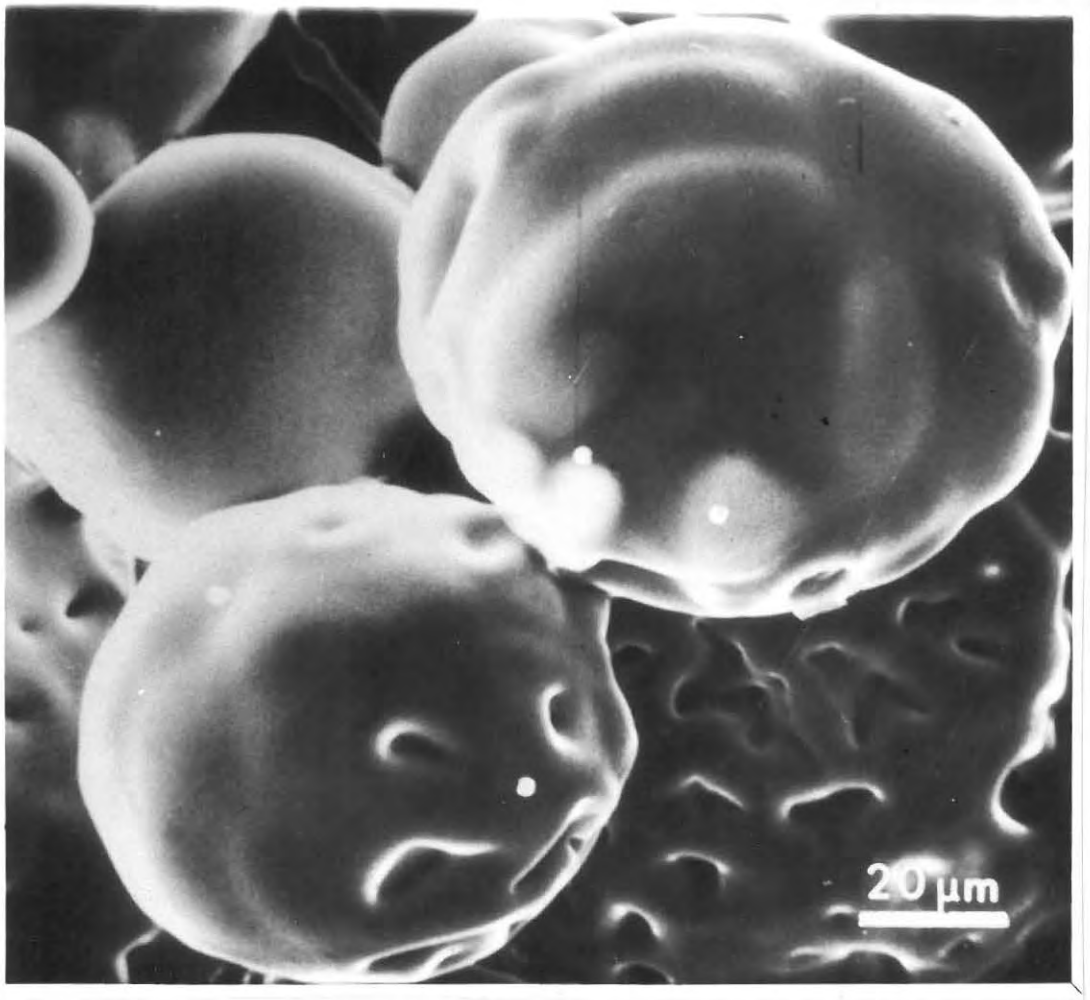


Figure 8: Scanning electron micrograph of albumin beads of various sizes.

cells of the host immune system.

Antigen would be randomly distributed throughout the beads, with a considerable proportion externally attached for imme= diate interaction with the host cells. A suitable balance can be maintained between immunostimulation, promoted by a relatively large proportion of surface bound antigen as would be found in small beads, and immunopotentiation, promoted by relatively less surface bound antigen and more interiorised antigen, found associated with large beads. In the preparation of a batch of beads, a variety of bead sizes result- see Figure 8. Large beads would require extended time for degradation and would therefore extend the release of entrapped antigen. The optimum proportion of particular bead sizes for maximum adjuvanticity, must be determined empirically, however.

These factors are the many others required for an effective vaccine formulation, will be considered in this chapter and in those following.

2.2 MATERIALS AND METHODS

2.2.1 Development of vaccine formulation

Nodamura virus as antigen and rabbit serum albumin was isolated as described in Chapter 1. An aqueous solution of antigen, albumin and glutaraldehyde (GA) was emulsified into an ethereal solution of maize oil. Protein beads were formed by the cross-linking stabili=

sing activity of GA. The percentage incorporation of antigen into the beads was determined by the uptake of radio-labelled virus.

2.2.1.1 Preparation of serum albumin beads

This method was adopted from Lee, T. et al. (1981). Figure 9 indicates the entire process.

To a solution (0,8 ml) of antigen in sodium phosphate buffer (PB) (1 mM; pH 7,5; NaN_3 0,02%) was added rabbit serum albumin (200 mg). Following albumin dissolution, polymerisation is initiated by the addition of GA (BDH Chemicals Ltd., Poole England) (0,2 ml) to a final concentration of 1%. The suspen= sion was dispersed (10 seconds) using a vortex mixer and then expressed through the 18 guage needle of a 2 ml syringe into a mixture (100 ml) of maize oil and petroleum ether (1:4 by volume). The emulsion was stirred (1650 rpm; 60 minutes). Beads were collected, by decanting the oil solution, and then washed (x 3 with petroleum ether) before being dried overnight using a freeze-drying apparatus. Beads were then stored in a vacuum desiccator ($4^{o}C$) prior to use.

The reaction vessel remains at T_{room} during the course of the polymerisation reaction.

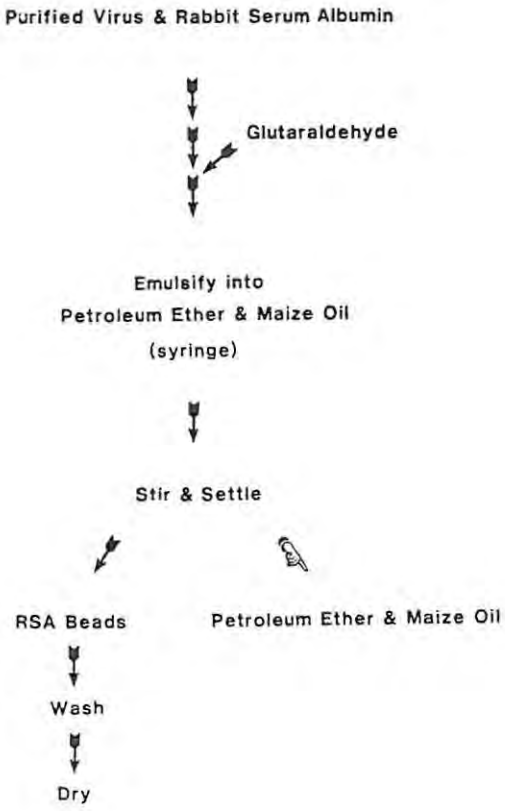


Figure 9: Flow diagram representing the process of serum albumin bead formation. (Reagent details pro= vided in text).

2.2.1.2 Determination of antigen incorporation into beads

^{35}S-methionine labelled Nodamura virus was isolated as indicated in 1.2.1.2 and 1.2.1.3. Triplicate aliquots (10 µl) of the antigen/albumin solution, described in 2.2.1.1 containing radiolabelled virus (50 µg) were dried (37°C) onto glass fibre disks. To each disk, placed in a scintillation vial , was added 10 ml of non-aqueous permafluor scintillant - consisting of 2,5 diphenyloxazole (5,0 g) and p-bis-(0-methylstyryl)-benzene (0,5 g) made up to 1 litre with toluene. Counts per minute were read using a Beckman (Model LS 3150T) liquid scintillation counter set at the ^{14}C channel, and an average count was obtained.

Beads were then prepared as described in 2.2.1.1. with the remaining antigen / albumin solution. Dried beads (50 mg) were then solubilised according to Madsen, N. (1969) by the addition of NaOH (1 N, 1 ml) followed by heating (100°C; 5 minutes). The cooled solution was neutralised with concentrated HCl (0,1 ml) before triplicate 10 µl aliquots were counted as above and an average count obtained.

Counting controls in both these experiments were PB (10 µl) and unradiolabelled albumin solution (section 2.2.1.1; 10 µl)

2.2.1.3 Effect of bead washing on antigen retention in beads

20 mg beads containing radiolabelled virus were prepared as in section 2.2.1.1. 10 mg of these beads were solubilised and triplicate aliquots (10 µl) counted as in section 2.2.1.2. An average count was then obtained.

The remaining 10 mg of beads were extensively washed with petroleum ether followed by PB, before being dried. The beads were then solubilised and triplicate aliquots (10 µl) counted and averaged as above. Counting controls are as those described in section 2.2.1.2. A comparison of the two averaged counts was then made.

2.3 RESULTS

2.3.1 Formation of serum albumin beads

Figure 1 shows a scanning electron micrograph of a typical serum albumin bead. The reference scale indicates its size to be between 50 µm and 100 µm. The procedure followed to form beads was found to be easy to perform and resulted in good bead yields. By weighing

the reaction vessel before and after bead formation, it was estimated that 5%-8% of the protein provided was not incorporated into bead form. This protein remained adhered to the side of the vessel after the beads had been decanted.

2.3.2 Incorporation of antigen into beads

The total radioactivity of the radiolabelled protein solution which could be formed into beads, showed an average count of 41104 cpm/µg. An average count of 35486cpm/µg was incorporated in the beads formed from this solution. The percentage incorporation is thus 86%. Considering the loss of protein by adhesion to the mixing vessel, averaging a minimum of 5%, greater than 90% of radiolabelled virus was incorporated into the beads.

2.3.3 Effect of bead washing on antigen retention in beads

The average count obtained for the unwashed beads (10 mg) was 25465 cpm/mg. This compared to an average count of 24923 cpm/mg for the petroleum ether and PB washed beads (10 mg), showing a loss of 2,2% of labelled virus following washing.

2.4 DISCUSSION

The formation of beads is dependent on a number of factors: The shape of the reaction vessel was important - parallel-walled beakers were found not to be suitable. A final choice was a 250 ml. Erlenmeyer flask (Pyrex, England), diameter 7,5 cm.

The dilution of commercially available GA with distilled water was necessary - dilution with sodium phosphate buffer (1 mM, pH 7,5) adversely affected bead formation possibly by the inter= action of buffer ions with the GA derivatives.

The size of beads is most critically influenced by several factors: The method of introduction of the protein plus glutaraldehyde mixture into the organic phase - rapid discharge through an 18 gauge needle - proved optimal as suitable droplet formation and hence water-in-oil emulsion resulted;

The speed of stirring of the organic phase is inversely propor= tional to the size of the resultant beads. Stirring by means of a 2,5 cm teflon-coated stirring rod at 1650 rpm was found to be optimal for the correct sized beads;

Protein solutions at temperatures below 20^{o}C were found to be too viscous, resulting in unacceptably large bead formation.

The results of the experiments to determine labelled virus uptake and stable incorporation into bead formation, indicate that this system efficiently entraps potential antigen.

Bearing in mind the losses of virus due to adhesion to the sides of the glass reaction and mixing vessels, as well as losses due to residual polymerised protein in the reaction vessel, the conditions for bead formation must be optimised. To this end, mixing of the polymerising protein solution should take place in a vessel from which it can be directly and efficiently

expressed into the hydrophobic solution, contained in a vessel treated to prevent protein attachment.

In summary, methods adapted from Lee, T. et al. (1981) have shown that large macromolecular structures such as intact virions may be stably incorporated into serum albumin beads, cross-linked at a final GA concentration of 1%. Having determined this, con= ditions for the optimal release of antigen, in vitro and in vivo, must be ascertained. This will depend to a large extend on the final GA concentration, controlling the stability of the polyme= rised structure.

CHAPTER 3

IN VITRO COMPARISON OF ALBUMIN BEAD STABILITY RESULTANT FROM VARIOUS PERCENTAGE FINAL GLUTARALDEHYDE CONCENTRATIONS

3.1 INTRODUCTION

Altering the amount of glutaraldehyde (GA) used to prepare serum albumin beads will effect their stability *in vivo* and hence the release of antigen from the beads. With proteolytic degradation being the main criterion for this release *in vivo*, it is necessary to optimise bead crosslinking such that the beads may be degraded over a period of time sufficient for the prolonged stimulation of the host immune system.

Lee T. et al. (1981) described that bovine serum albumin beads crosslinked at 2%, 3% and 4% final GA concentration were resistant to chymotryptic digestion. The compactness of these beads resul= tant from the close proximity of the interlinked polypeptide chains, presumably prevents the correct orientation of the substrate in the enzyme's active site.

This result indicates that GA at a lower concentration should be used to prepare beads such that the resultant beads should not be too rapidly degraded *in vivo*, but that their basic integrity be maintained, reduced by gradual proteolytic breakdown with concom= mitant gradual release of antigen.

Lee, T. et al. (1981) furthermore showed that 1% GA-prepared beads were biodegradable and released hormone at least ten times slower than the control which consisted of unentrapped hormone associated with albumin solution.

However, it might be anticipated that replacement of entrapped hormone by large protein structures such as virus would mean that for a particular percentage GA cross-linking, a comparative degree of bead destabilization would occur as interaction between macro= molecular antigen and GA would reduce the inter-albumin cross-linking.

To check this, in vitro experiments were conducted with various percentage GA cross-linked beads which were monitored as to their stability and release of entrapped antigen.

Initial bead degradation experiments were performed with readily available bovine serum albumin (BSA). Bead degradation was simply monitored by determining the protein content of the buffer in which the beads had been incubated. A subsequent experiment determined the release of radiolabelled virus from degrading beads into the incubation supernatant medium.

3.2 METHODS AND MATERIALS

3.2.1 In vitro degradation of bovine serum albumin beads

BSA beads were prepared at various final GA concentrations. Each group of beads was equally divided and incubated (37°C) with sterile phosphate buffered saline (PBS) (0,02 M;

pH 7,5; NaN_3 (0,02%)). At intervals the supernatant concentration of dissolved protein was determined using a modified Folin-Ciocalteau method. A comparison was then made of the variously cross-linked bead stabilities over a period of time.

3.2.1.1 Preparation and incubation of variously cross-linked beads

Batches of BSA beads (200 mg) at 0,5; 1; 2; 3 and 4% final GA concentration were prepared according to the protocol in Section 2.2.1.1. Sixteen 10 mg masses of each bead group were placed in detergent and acid cleaned test tubes. PBS (1 ml) was added to each tube. The stoppered tubes were then incubated (37°C) in an orbital shaker (Labotec). On days 0,75; 2; 4; 7; 11; 22; 36 and 60 following the start of the experiment, 200 µl aliquots of the duplicate incubation supernatants were removed and their protein concentration determined by means of the Folin-Ciocalteau method (Lowry, O. et al., 1951).

3.2.1.2 Folin-Ciocalteau (F-C) method

A standard protein curve was obtained by preparing a 2-fold serial dilution of BSA Fraction V (Miles Laboratories) (1 mg/ml) in sterile PBS. To duplicate 200 µl samples of each dilution was added Solution A (1,6 ml) and F-C reagent (200 µl) with mixing. Solution A is prepared by adding

Na_2CO_3 (2%) in NaOH (0,1 N) (50 ml) to $CuSO_4$. 5 H_2O (0,5%) in K Na Tartrate (1%) (1 ml). The solution were then incubated (T_{room}; 30 mins) before the protein was spectrophotometrically determined at 750 nm.

The 200 µl aliquots obtained in 3.2.1.1 were treated as above and their absorbances at 750 nm determined. The protein content of each was found by interpolation against the BSA standard curve. The results of the protein concentration determinations (µg/ml) were then tabulated.

3.2.2 In vitro release of radiolabelled virus from rabbit serum albumin beads

Rabbit serum albumin (RSA) beads containing radiolabelled virus, were prepared at various final GA concentrations. These were incubated in Ringers solution and at various intervals the radioactive content of the supernatant was determined, indirectly indicating bead stability by quantitation of the release of virus from degrading beads.

3.2.2.1 Preparation and incubation of radioactively-labelled rabbit serum albumin beads

^{35}S-methionine labelled Blue Tongue Virus (50 µl) was kindly provided by Miss Margot Humphrey.

An aliquot (10 µl) was diluted to 100 µl and 10 µl of this solution was then counted as described in

section 2.2.1.2. The remaining virus suspen= sion (40 µl) was diluted to 3200 µl and divided into 4 volumes of 800 µl each. RSA (200 mg) was dissolved in each volume and beads at final GA concentrations of 0,75 ; 1; 2 and 3% were pre= pared as described in section 2.2.1.1.

A 10 mg quantity of beads from each group was counted according to section 2.2.1.2. The balance from each group was divided into dupli= cate 10 mg quantities and these were separately placed in 1,5 ml Eppendorff microfuge tubes. Full strength Ringer's solution (1 ml) was added to each microfuge tube which was then incubated ($37^{o}C$) in an orbital shaker (Labotec). Separate tubes were marked to correspond with the date of proposed supernatant radioactivity determina= tion. Toluene (2 drops/tube) was added as preservative.

3.2.2.2 Determination of supernatant radioactivity

On days 0,5; 2,5; 11; 21 and 66 following the beginning of the experiment, appropriately marked tubes of each different bead preparation were centrifuged (5000 rpm; 1 minute) to sediment residual beads. Duplicate 100 µl supernatant aliquots were placed in scintillation vials. Scintillation mixture (15 ml), prepared by adding PPO (4 g/α) to toluene and triton x100 (2:1 by volume), was then added to the vials before being

counted.PB (100 µl) was included to obtain a background count. The duplicate counts (cpm/ml) were averaged and tabulated.

3.3 RESULTS

3.3.1 BSA bead degradation monitored by F-C method

During the experiment, the appearance of the variously cross=linked beads was noted. Those beads prepared with 0,5% GA solubilised rapidly and within 48 hours only a yellow-coloured, clear solution was evident in these tubes. On the other hand, beads prepared with GA at 2% or more, remained discrete throughout the experiment. Beads crosslinked at a final GA concentration of 1%, underwent partial degradation so that residual protein structures remained by the end of the experiment. The clarity of all the solutions by day 58, indicated that the beads were not degraded by the action of bacterial contaminants.

The following table indicates the concentration (µg/ml) of BSA solubilised in the supernatant PBS at a particular time following the start of the experiment. The percentage BSA solubilised compared to the original mass of beads is obtained by dividing these figures by 100.

	% GA	0,5	1	2	3	4
Supernatant BSA conc. (µg/ml)	Day 0,75	375	156	63	12	31
	2	850	260	60	42	50
	4	3550	350	70	50	60
	7	4000	500	100	60	70
	11	9375	690	150	100	210
	22	10000	910	150	225	150
	36	8075	1060	160	140	160
	58	8770	1100	150	150	160

A plot of Supernatant Protein Concentration (µg/ml) against Time (days) is provided in Figure 10.

3.3.2 RSA bead degradation monitored by supernatant radioactivity

The incorporation of radiolabel into beads (10 mg) of the various groups is as follows:

RSA BEADS (10 mg)

% GA	0,75	1	2	3
^{35}S-met count (cpm x 10-3)	210,5	201,0	213,8	195,7

The average count of radioactivity released into the super= natant, corrected for $t_{\frac{1}{2}}$ nuclide decay, at the indicated times following the start of the experiment, are tabulated below. Also indicated in brackets is the percentage radioactivity released into the supernatant relative to the original incorporated radioactivity. This data is illustrated in Figure 11 on Page 51.

RSA BEADS (10 mg)

% GA	0,75	1	2	3
Day 0,5	115,2 (54,7)	1,0 (0,5)	2,0 (0,9)	2,0 (1,0)
2,5	120,1 (57,1)	35,2 (17,5)	1,8 (0,8)	1,9 (0,9)
11	125,3 (59,5)	140,0 (69,7)	2,2 (1,0)	2,5 (1,3)
21	148,8 (70,9)	125,9 (62,6)	2,1 (1,0)	2,1 (1,1)
66	175,9 (83,3)	150,1 (74,7)	2,0 (0,9)	2,1 (1,1)

Supernatant radioactivity (cpm x 10^{-3}/ml) (% radioactivity released)

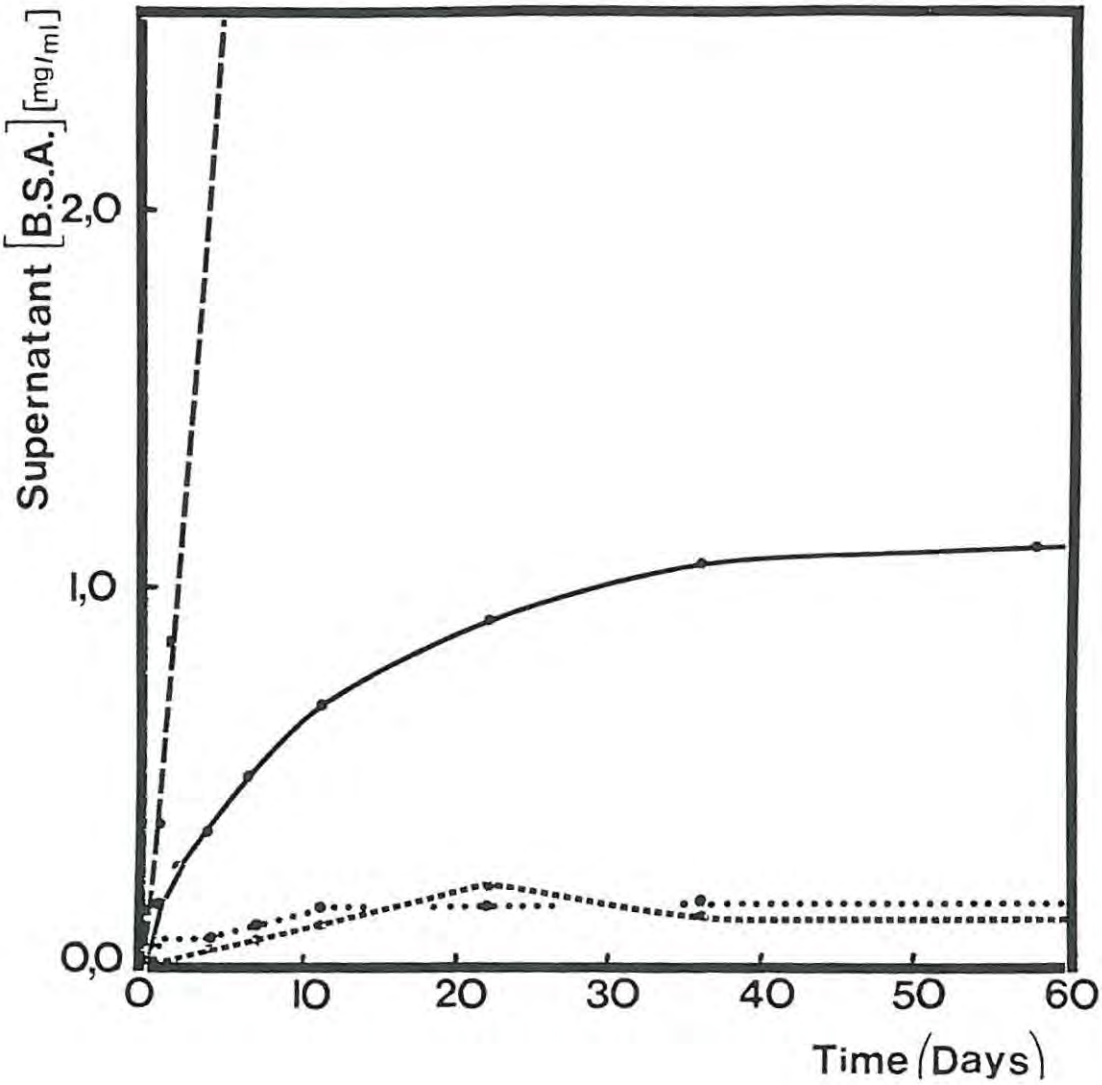


Figure 10: Albumin bead stability determined from supernatant albumin concentration.

– – – – –: solubilization of beads prepared at 0,5% final GA concentration

————: solubilization of beads prepared at 1% final GA concentration

.....: solubilization of beads prepared at 2% and 3% final GA concentration

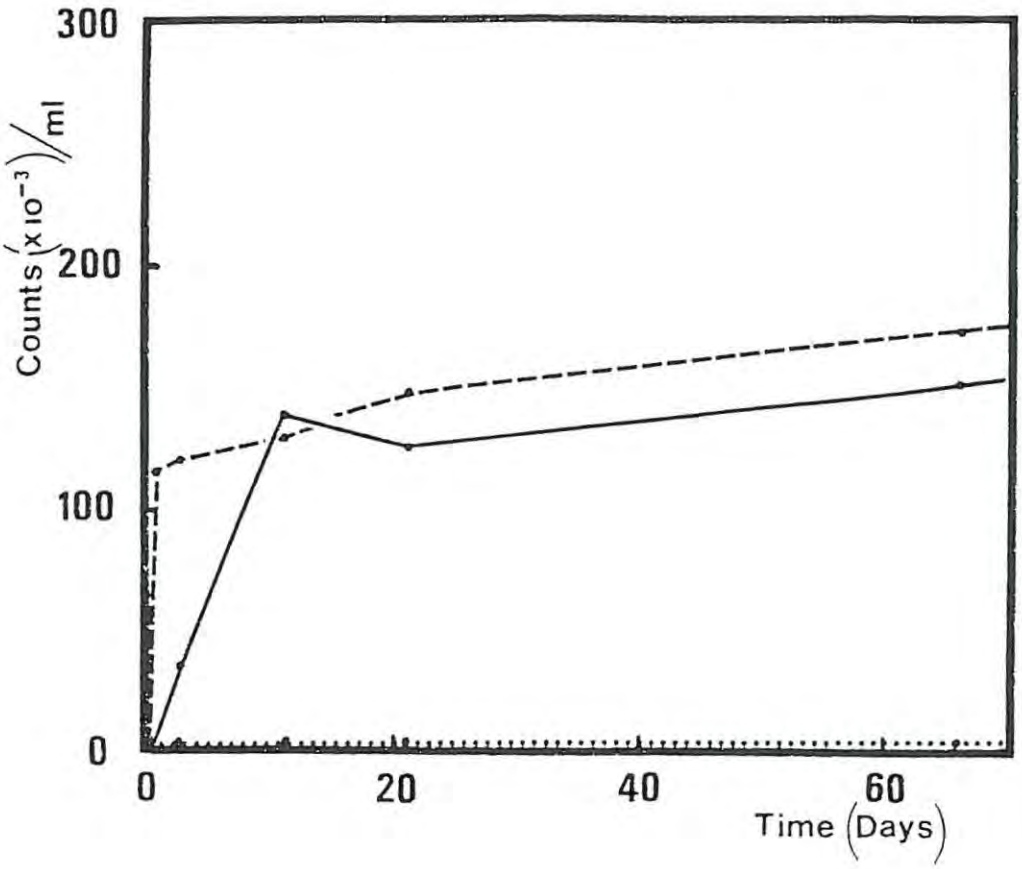


Figure 11: Albumin bead stability determined from supernatant radioactivity.

——— : release of radioactivity from beads prepared at 0,75% final GA concentration

———— : release of radioactivity from beads prepared at 1% final GA concentration

..... : release of radioactivity from beads prepared at 2% and 3% final GA concentration

The solubilisation of beads in this experiment was found to be similar to that noted in Section 3.3.1. By day 4, beads prepared at a final GA concentration of 0,75% had completely solubilised; the 2% and 3% prepared beads remained stable throughout the experiment, while partial degradation of the 1% GA prepared beads occurred by day 66. The clarity of the solutions during the experiment indicated the effectiveness of toluene as a preservative.

3.4 DISCUSSION

The results of BSA bead dissolution as plotted on Figure 10 indicate the inherent instability of beads prepared at 0.5% final GA concentration as well as the resistance to degradation of the beads prepared with GA at 2%, 3% and 4% final concentration. The 1% final GA concentration-prepared beads degrade between these extremes.

One must assume that *in vivo* degradation of beads will be accelerated by mechanical stress and enzyme activity. This underlines the inadequacy of the 0,5% prepared beads which show over 90% degradation *in vitro* by day 11. The 11% degradation by day 58 of the 1% GA-prepared beads allows for an increased degradation *in vivo* with extended adjuvanticity as antigen is slowly released.

Although this experiment allows only a crude estimate of bead degradation, its validity is indicated by the variable results obtained for the different percentage cross-linked beads as to this factor contributing toward bead stability.

Both Figure 10 and Figure 11 indicate similar optimal GA concentrations of 1%. Thus Figure 11 indicates that beads prepared at 2% or greater final GA concentration are too stable and insensitive to degradation. Little virus release, as indirectly monitored by radioactivity release from the beads, was noted. The preparation of 0,75% beads on the other hand still degrade too rapidly. Over 50% virus release had occurred 12 hours from the beginning of the experiment. This gradually increased to over 80% virus release over two months later.

Possibly the polymerisation reaction occurring from the centre of an emulsion droplet , promotes a stably polymerised core, relatively insensitive to degradation, with the outer bead layers more haphazardly cross-linked with remaining GA molecules. The outer layers are relatively readily degraded and therefore initially allow massive virus release from the incubated beads.

The 1% cross-linked beads, once again, are intermediate in stability. The high release of radioactivity from these beads (75% by day 66), compared to only 10% bead solubilisation as noted by the F-C method, indicates that the monitoring of the release of radioactivity into the supernatant, cannot distinguish between release of labelled virus as a result of bead degradation or by diffusion from the beads— in either case the desired effect is attained, i.e. a sustained release of virus.

An unexpectedly high virus release was noted on day 11, possibly due to the formation in any preparation of beads of an inhomogeneous population of bead sizes and stabilities as previously mentioned.

Beads with low stability will in these circumstances , release virus at high frequency, before the bulk of the consistently cross= linked, more stable beads have appreciably degraded.

If this reasoning was correct, this would have immunological signifi= cance in vivo, as an initial potent challenge of antigen would promote an intense stimulation of lymphocytes and phagocytes, similar to the action of dried Mycobacterium tuberculosis in Freund's Complete Adjuvant, and thereafter cellular stimulation could continue at a lower, but consistent level as the bulk of the more evenly cross= linked beads were more slowly degraded.

From an immunological point of view, various beads with varying antigenic presentation characteristics could be mixed together prior to inoculation to provoke optimal antigen challenge on the host immune system. Alternatively, lamellar beads, produced by sequential cross-linking reactions, could be produced such that a graded anti= genic release could be mediated from a single population of beads. This situation could be improved by the use of alternative bifunctional cross-linking agents to GA, allowing either a layering of antigen-containing albumin around a central bead core or by the attachment of antigen onto the surface of the bead to provoke maximal host cellular stimulation. A review of alternative cross-linking agents is provided by Freedman R. (1979).

CHAPTER 4

DETERMINATION OF SERUM ANTIBODY TITRE USING THE INDIRECT ENZYME-LINKED IMMUNOSORBENT ASSAY. (ELISA)

4.1 INTRODUCTION

The monitoring of antigen-dilution endpoints and serum antibody titres is fundamental to medical diagnosis. Hereby the etiology and the course of a particular disease can be determined. To these ends a number of techniques involving antigen-antibody interactions have been developed.

Berson, S. and Yalow, R. (1971) described the technique of radioimmuno= assay (RIA) involving radiolabelled ligands. While this technique is noted for extreme sensitivity (down to 10^{-17} mol), specificity and relative insensitivity to variations in the chemical composition of sample, drawbacks to this technique are the relatively short half-lives of the commonly used radionuclides, thus limiting reagent shelf life, the health hazards associated with the use of these radioactive materials and the problems associated with the disposal and release of radioactivity into the environment.

Partly in response to the challenge posed by these apparent draw= backs, a wide variety of nonisotopic immunoassay techniques have arisen. Included in this category are quantitative fluoroimmuno= assay, fluorescence immunoassay, free-radical immunoassay, viro= immunoassay, haemaglutination inhibition, and, of course, enzyme

immunoassay (Maggio, E., 1980).

Enzyme immunoassay for the determination of serum antibody titres is comparable in sensitivity to radioimmunoassays and negate the drawbacks associated with RIA. Furthermore, factors like lower cost of reagents, simplicity of protocol and concomitant reduction in technician time required per assay, availability of suitable instrumentation and potential of automation favour enzyme immunoassay over RIA.

For the purposes of this study, serum antibody titres were deter= mined using the indirect method of the enzyme-linked immunosorbent assay (ELISA) as describes by Engvall, E. and Perlmann, P. (1971; 1972) and Crook, N. and Payne, C. (1980).

This is a homogeneous single site non-competitive enzyme immuno= assay in which immobilized antigen is allowed to react with anti= body-containing serum. After washing, the immobilised antigen-antibody complex is incubated with enzyme-labelled antibody against immunoglobulin of the species in which the antibody has been elicited. Incubation with enzyme substrate follows washing and the concentration of enzyme product is directly proportion to the quantityof bound antigen-specific antibody. This sequence is indi= cated in Figure 12.

Carlsson, H. and Lindberg, A. (1978) applied this technique to diagnosing bacterial and fungal infections, while Voller, A. et al. (1978) diagnosed with this method viral and parasitic infections.

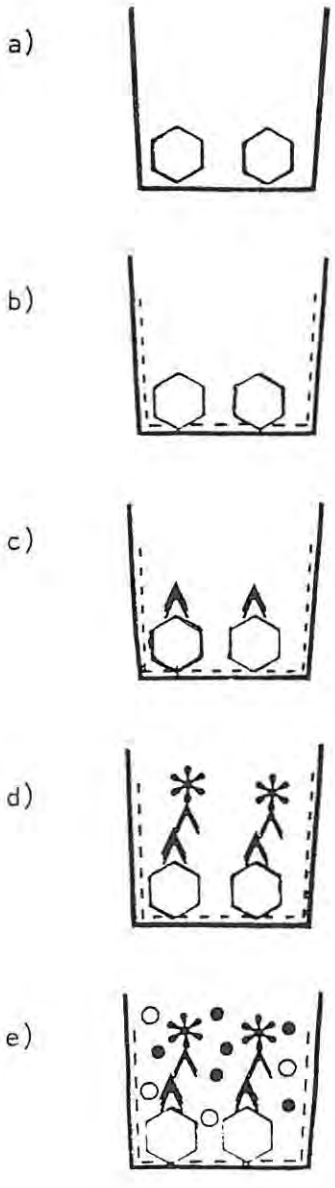


Figure 12: Diagrammatic representation of the indirect ELISA sequence.

As indicated in Figure 12 the first step in the procedure is the coating and immobilization of antigen to the polystyrene well walls in microtiter plates. This adsorption results probably from hydrophobic interactions between non-polar protein substruc= tures associated with the Ag and the non-polar plastic matrix of the well. The rate and extent of coating will depend on the diffu= sion coefficient of the absorbing molecule, its concentration in the solu= tion, the temperature and the duration of the adsorption reaction (Clark, B. and Engvall, E., 1980). Ehlers, U. and Paul, H. (1984) indicated that activation of polystyrene well walls with 3-(trie= thoxysilyl)-propylamine followed by treatment with glutaraldehyde allowed the covalent attachment of antigen to the well surface from solution with low antigen concentration and from solution containing inhibitory substances negatively influencing adsorption of antigen to untreated well surfaces.

Incubation time and temperature will depend on the Ag to be coated. Engvall, E. and Perlmann, P. (1972) described that optimal coating occurred at 37°C after 3 hours while Carlsson, H. and Lindberg, A. (1978) show optimal bacterial and fungal antigen coating following incubation at 25°C overnight. It was decided in this study to incu= bate Nodamura virus antigen at 37°C overnight, diluted in phosphate buffered saline (PBS) (0,02 M; pH 7,4).

The optimal concentration of antigen coating required for a specific antigen-antibody interaction must be empirically determined. Results of Engvall, E. and Perlmann, P. (1972) indicated the prozone phenomenon at low antigen concentration while reduced sensitivity was noted at higher antigen concentrations.

To prevent non-specific adsorption of antibody to the well surface which would lead to inaccuracies in antibody titre determination, exposed wall sites not occupied by antigen must be filled. Bittle, J. et al. (1982) incubated 3% BSA in PBS for 4 hours prior to antiserum introduction, while Ehlers, U. and Paul, H. (1984) intro= duced 2% polyvinyl pyrollidone plus 0,2% egg albumin in PBS made 0,05% with respect to Tween-20. The method for blocking unoccupied well sites in this study was provided by Emini, E. et al. (1983) who used 0,5% gelatin in PBS for this purpose.

The third stage in the procedure is the incubation of the immobilised antigen with serial dilution of antiserum in PBS containing Tween-20 (0,05%). Tween-20, a surfactant, is introduced in the dilution to reduce non-specific adsorption of antibodies to remaining open well sites.

To ensure adequate time for reaction between antigen and antibodies in the higher antiserum dilution, incubation at $37^{o}C$ was extended to 3 hours. A degree of automation in the introduction of a large number of dilutions into wells is required to standardise incubation time . Duplicate dilutions were prepared for improved accuracy.

The quantitation of antibody in the primary immune complex is pro= vided by the incubation of this complex with enzyme-linked anti- immunoglobulin specific for the complex antibody. Subsequent detec= tion of conjugate bound to the complexed Ab is detected colourimetrically by adding a suitable enzyme substrate.

The Ab to be conjugated is raised against IgG of the species in which the test Ab has been elicited. Enzymes such as alkaline phosphatase have been used, linked to goat anti-rabbit IgG with glutaraldehyde (Clark, M. and Adams, A., 1977). Barbara, D. and Clark, M. (1982) compared this conjugate with a conjugate prepared from horse radish peroxidase linked to IgG by a periodate oxidation method and found the latter to be more sensitive.

A variation to enzyme coupled to IgG was provided by Brown, D. et al., (1982) who linked protein A from Staphylococcus aureus to alkaline phosphatase in a two step glutaraldehyde reaction. The protein A has a high affinity for the Fc portion of IgG molecules of several mam= malian species.

In this study the conjugate of choice was alkaline phosphatase linked to goat anti-(rabbit IgG). This conjugate was found to be ex= tremely stable ($4^{o}C$)-at least one year. The introduction of conjugate in the test is readily automated allowing the standardising of incu= bation time (2 hours; $37^{o}C$) with the primary immune complex.

Bound conjugate is detected by adding enzyme substrate to the reac= tion wells. The substrate for alkaline phosphatase is p-nitrophenyl phosphate prepared at a concentration of 1 mg/ml in 10% diethanol= amine HCl (pH 9,8). This is hydrolysed to phosphate and yellow p-nitrophenolate which is measured in a spectrophotometer at 410 nm.

The hydrolysis reaction is stopped with the addition of 3 M NaOH, time of reaction being determined according to the temperature - usually room temperature - from a few minutes to an hour. A time of 30 minutes was chosen in this study.

Ab determination with ELISA is quantitative. However, because the serum to be tested contains polyspecific Abs of varying avidities between and within Ab classes, it is difficult to determine the absolute concentration of Ab (this may be realized using a reference Ab of known concentration). Therefore it is more convenient for routine Ab determinations to obtain a relative quantitation of Ab in terms of a titre. This titre or serum dilution end-point is indicated by an enzyme activity significantly different from the background. This can be obtained by monitoring the enzyme activity of a serum dilution series and preparing complete titration curves (Maggia, E., 1980). Controls indicating non-specific adsorption and therefore non-specific enzyme activity are subtracted from the above complete titration curves.

Successful application of the indirect ELISA technique for deter= mining Ab titres in serum samples is dependent on empiric evaluation of the parameters associated with each antigen-antibody system. The following sections will indicate some of these parameters and the experimental results promoting successful indirect ELISA investiga= tions.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of ELISA reagents

Nodamura virus, as antigen, was isolated according to the protocol in section 1.2.1.3. Goat anti-rabbit IgG was precipitated from serum and purified by passage through an anion exchange column. Alkaline phosphatase was then

linked to this IgG with glutaraldehyde (GA) and stored ($4^{o}C$). Enzyme substrate was prepared immediately prior to use.

4.2.1.1 Purification of goat anti-(rabbit IgG)

IgG was purified according to Clark, M. and Adams, A. (1977). An aliquot (0,5 ml) of reconstituted goat anti-(rabbit serum) (Miles-Yeda, Ltd., Israel) was diluted (to 5 ml) with distilled H_2O and made 60% with respect to S.A.S. The resulting precipitate, sedimented by centrifuging (6000 g; 10 minutes), was re-suspended in half strength PBS (HSPBS) (0,01 M; pH 7,4; 2 ml) before being dialysed against 3 x 500 ml PBS exchanges ($4^{o}C$; 24 hours).

A Whatman DE 22 (Whatman Ltd., Kent) column was prepared according to the Whatman information leaflet IL2. The dialysed IgG solution was eluted through the column with HSPBS and the optically dense fractions showing an $OD_{278}:OD_{250}$ of 2,3 to 2,7 were collected, pooled and adjusted to approximately 1 mg/ml with PBS, before being stored ($4^{o}C$).

4.2.1.2 Preparation of enzyme-linked IgG

This was prepared according to Clark, M. and Adams, A. (1977). Alkaline phosphatase (Type VII, Sigma Chemical Co., St. Louis, USA) (2 mg)

was added to IgG (1 mg), prepared according to section 4.2.1.1, and the solution dialysed (3 x 500 ml PBS; 4°C). To the ammonium sulphate free protein solution, was added GA (0,05%). This was incubated (22°C; 4 hours) before free GA removal with dialysis (4°C; 3 x 500 ml changes of PBS). The conjugate was then stored (4°C) following the addition of BSA (1%).

4.2.1.3 Preparation of enzyme substrate

A p-nitrophenylphosphate (Merck) solution (1 mg/ml) was prepared in diethanolamine (Merck) (10%), adjusted to pH 9,8 with concentrated HCl, immediately before use.

4.2.1.4 Dilution of test serum samples

Test serum isolated according to section 1.2.2.1 was diluted with PBS containing Tween-20 (Merck) (0,05%) (PBS-Tw). Duplicate 200 µl aliquots of each dilution were used in the indirect ELISA test.

4.2.2 Indirect enzyme-linked immunosorbentassay (ELISA)

Having prepared the ELISA reagents, according to protocols in section 4.2.1, and optimised ELISA reaction parameters, such as antigen concentration and conjugate dilution to be described in section 4.2.3, the determination of serum antibody titres can be accomplished following the protocols of Engvall, E. and Perlmann, P. (1971, 1972).

4.2.2.1 Indirect ELISA protocol

Aliquots (200 µl) of Nodamura virus at optimal concentration (5 µg/ml) were incubated (18 hours; 37°C) in each well of microtitre plates (Sterilin, Middlesex). The plate were then washed at least three times by flooding wells with PBS-TW and leaving for several minutes between washing. Then aliquots (300 µl) of gelatin (Merck) (0,5%) (Emini, E. et al., 1983) were incubated (8 hours; 37°C) in each well, following by washing as above. Aliquots (200 µl) of test serum diluted as described in Section 4.2.2.3, were added in duplicate to wells and incubated (3 hours; 37°C). After washing, each well was incubated (3 hours; 37°C) with 200 µl of a $^{1}/_{400}$ dilution in PBS-Tw of enzyme-linked goat anti-(rabbit IgG) conjugate, prepared in section 4.2.1.2. After washing as before, enzyme substrate (1 mg/ml; 200 µl), prepared in section 4.2.1.3, was added to each well followed, after incubation (T_{room}; 30 minutes) by 3 M NaOH (50 µl/well). Colour intensity was read at 410 nm using a Vitatron Densitometer (aperture set at 0,25 mm.), with interfaced Integrator. Subsequent results were obtained using a Minireader 11 (Series 2) photometer (Dynatech Lab., Inc., Virginia).

To detect any non-specific adsorption or inter= action of these test components, controls were

prepared concurrently as indicated in the following table:

	Control 1	Control 2	Control 3	Control 4	Control 5
Virus	+	+			
Gelatin		+	+		
Antiserum			+	+	
Conjugate	+	+	+	+	+
Substrate	+	+	+	+	+

Any spectrophotometric readings indicating positive enzyme activity in control 2 or control 3, were subtracted from the resultant complete titration curves

4.2.3 Optimising of ELISA reaction conditions

The optimal antigen concentration and conjugate dilution had to be ascertained prior to performing the initial indirect ELISA tests. For these determinations, Nodamura virus-specific antiserum was required.

4.2.3.1 Preparation and characterisation of anti-Nodamura virus serum

This was obtained by emulsifying a purified virus solution (0,5 ml; 200 µg/ml), isolated according to section 1.2.1.3, with an equal volume of Freund's Complete Adjuvant (Miles Laboratories, USA). On day 21 following the intramuscular inoculation of the emulsion into a rabbit, a blood sample (10 ml) was drawn and serum isolated

therefrom, according to section 1.2.2.1. This serum was stabilised with NaN_3 (0,02%) and stored (4^oC).

Four 0,5 ml serial doubling dilutions were prepared of a Nodamura virus isolate (200 µg/ml) as were eight 1,0 ml serial doubling dilutions of the above serum in PBS. Well patterns were punched in two 1% agar (Difco Detroit, USA) plates and the central wells were filled with antigen dilutions, surrounded by antiserum dilutions. The plates were then incubated (4^oC; 24 hours) before noting the precipitin band formation between the wells.

4.2.3.2 Determination of optimal antigen concentration

Seven 4 ml 2-fold serial dilutions were prepared in PBS of Nodamura virus (20 µg/ml), isolated according to the protocol in section 1.2.1.3, down to a virus concentration of 0,15 µg/ml.

200 µl aliquots of each dilution were incubated (18 hrs; 37^oC) in each well of duplicate rows of microtitre plates. After washing (PBS-Tw), to each well was added an aliquot (200 µl) of a 1/200 dilution of the serum isolated in section 4.2.3. Following washing (PBS-Tw), to duplicate rows of wells were then added aliquots (200 µl) of 1/400 dilution of the enzyme conjugate, pre=

pared in section 4.2.1.2. Enzyme substrate (200 µl) was then added to each well after washing, followed by NaOH (3 M; 50 µl). Each well was then determined spectrophotometrically at 410 nm. A plot of OD_{410} against antigen concentration (µg/ml) is shown in Figure 14.

4.2.3.3 Determination of optimal conjugate dilution

Aliquots of the enzyme-linked IgG conjugate, prepared in section 4.2.1.2, were diluted to 1/400 and 1/800 with PBS.

Parallel indirect ELISA tests were performed in two microtitre plates (as above) whose wells were coated with Nodamura virus (200 µl) at 5 µg/ml and 10 µg/ml respectively, the virus being purified according to section 1.2.1.3. The format of the test, described in section 4.2.2.1, was followed, using 200 µl aliquots of each dilution of a fourfold dilution series of the virus specific serum isolated in section 4.2.2. To duplicate rows of wells in the plates were added the respective conjugate dilutions (200 µl/well).

The averaged duplicate results of the spectrophotometric determination (410 nm) of the immune complexes formed were plotted as complete titration curves. This is shown in Figure 15.

4.3 RESULTS

4.3.1 Isolation of goat anti-(rabbit IgG)

Figure 13 indicates the successful elution of IgG from the anion exchange column. Pooled fractions showing $OD_{278}:OD_{250}$ = 2,3 to 2,7 were determined at 278 nm. Compared to $\Sigma^{1\ mg/ml}_{278}$ = 1,4 (Clark, M. and Adams, A., 1977) the pooled fractions contained a total of 4,2 mg IgG.

The theoretical yield of 6 mg IgG per 0,5 ml reconstituted serum, indicates a 70% IgG yield using this ion exchange chromatographic technique.

4.3.2 Optimal antigen concentration

An increase in bound Ag, showed an associated increase in the uptake of antibody by the bound antigen. This is indicated in Figure 14. For practical purposes, an upper concentration limit of 20 µg/ml virus was chosen.

As no definite antibody uptake maximum and hence optimal antigen concentration was indicated, antigen at 5 µg/ml was chosen as optimal. Increasing antigen concentration from 2,5 µg/ml to 5 µg/ml showed the greatest relative increase in OD_{410} and hence the greatest relative uptake of antibody by bound antigen.

4.3.3 Optimal conjugate dilution

Figure 15 indicates the results of the serum dilution end point titrations, indicating enzyme activity at conjugate

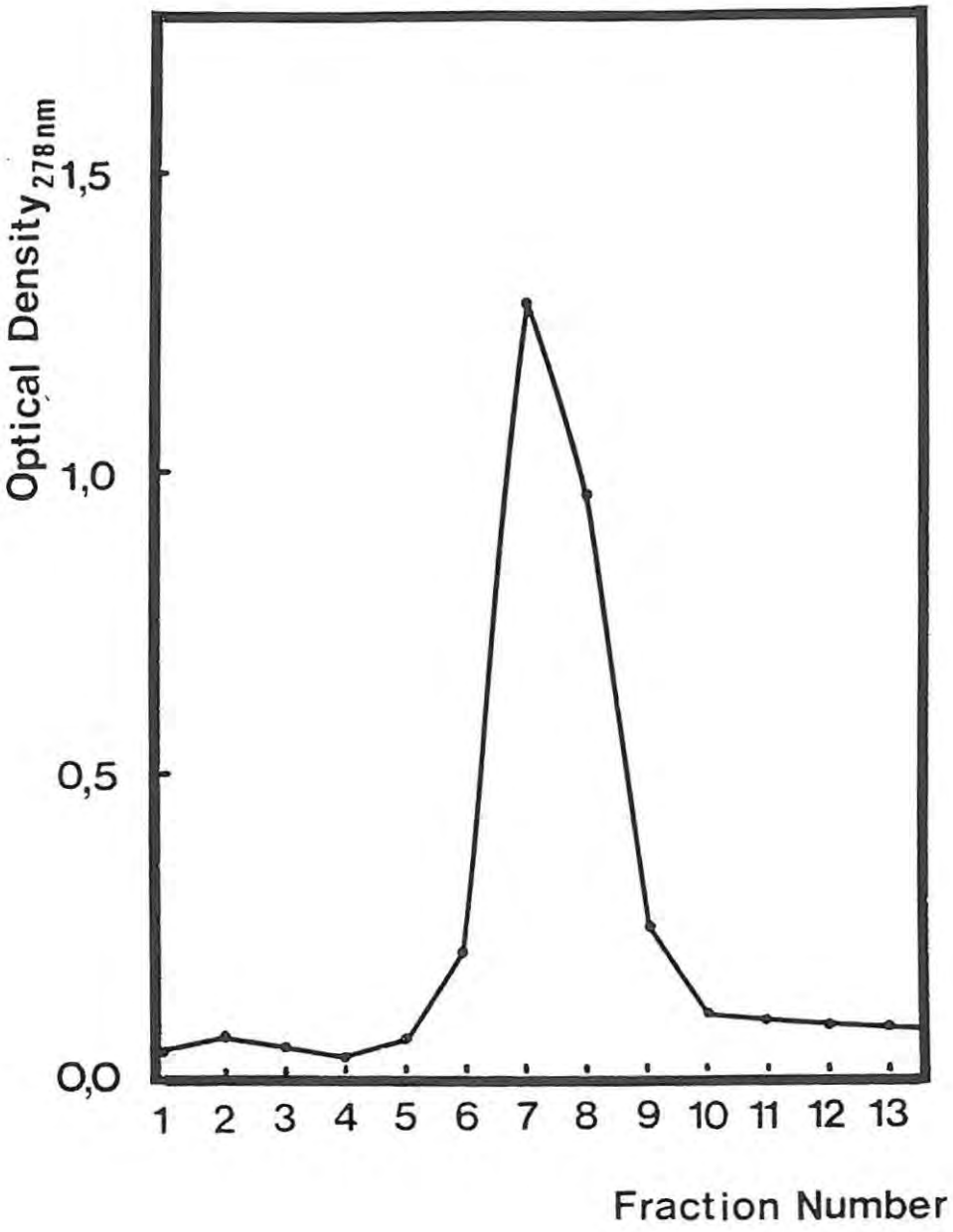


Figure 13: Elution profile of IgG from a DE 22 column.

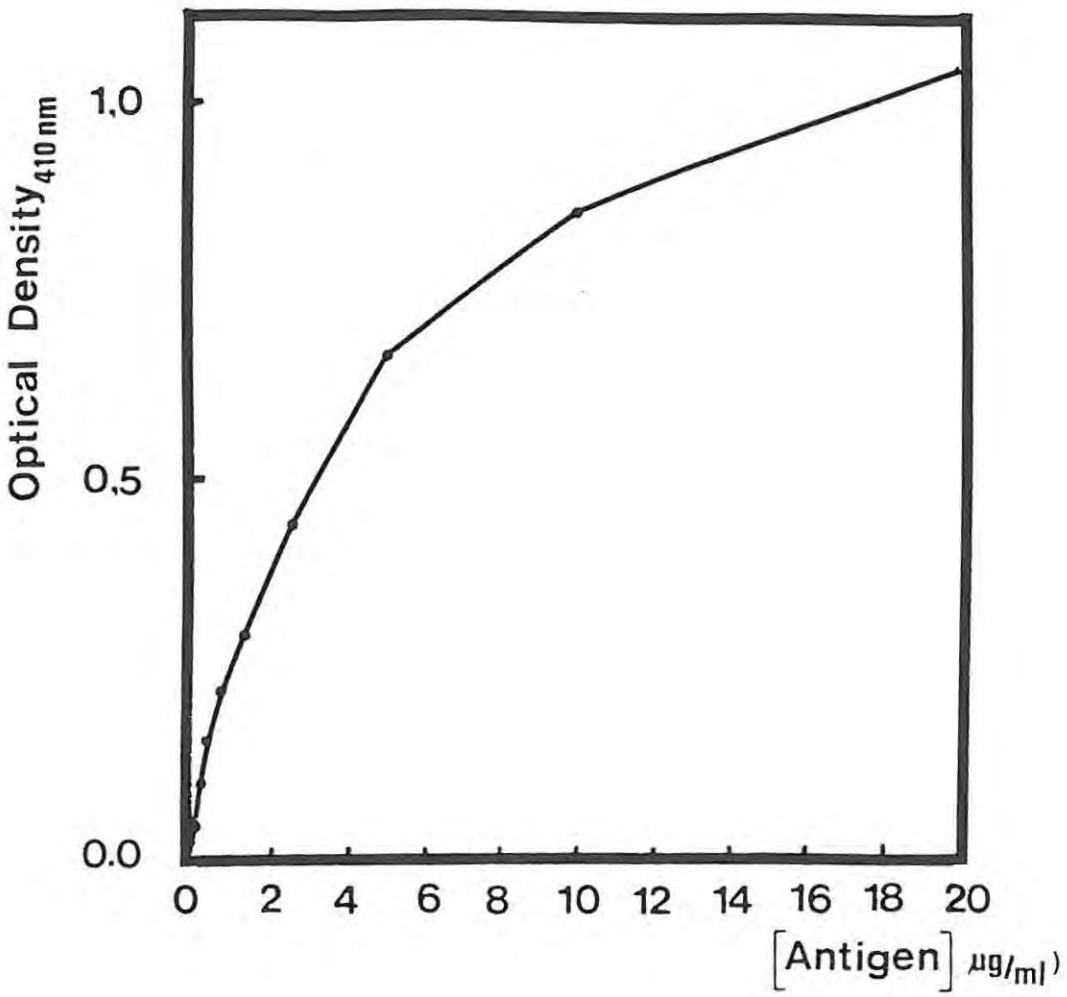


Figure 14: Determination of optimal antigen-coating concentration.

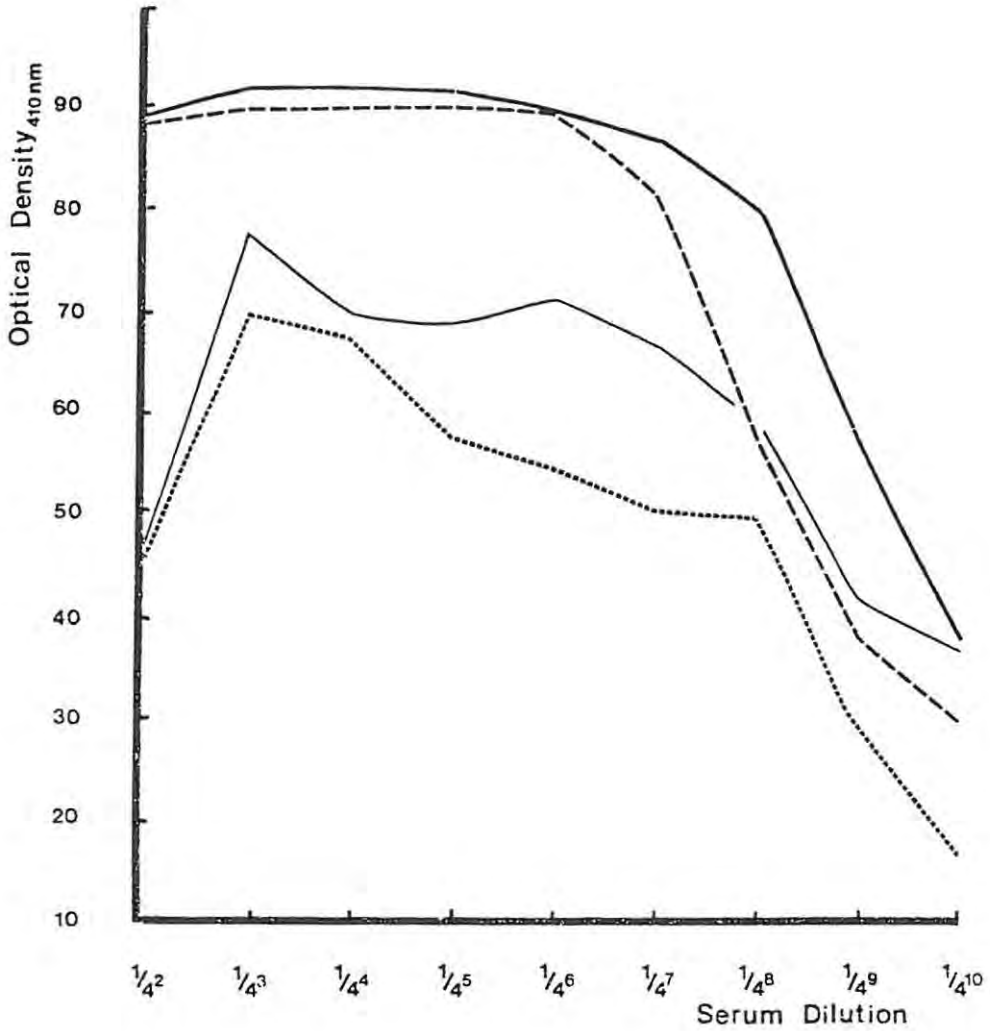


Figure 15: Complete titration curves for the determination of optimal conjugate concentration.

———: antigen (10 µg/ml) incubated with 1/400 conjugate

– – – –: antigen (5 µg/ml) incubated with 1/400 conjugate

————: antigen (10 µg/ml) incubated with 1/800 conjugate

.....: antigen (5 µg/ml) incubated with 1/800 conjugate

dilutions of 1/400 and 1/800. Little enzyme activity difference was noted at the lower dilution, whereas a marked reduction in enzyme activity at 1/800 dilution occurred, even with extended substrate incubation.

Efficient utilization of conjugate required maximal dilution and so a 1/400 dilution of conjugate was chosen.

Binding of antigen at 10 µg/ml showed relatively little difference in enzyme activity over antigen bound at 5 µg/ml, reinforcing the choice of antigen to be bound at a concen= tration of 5 µg/ml.

Storage of conjugate ($4^{o}C$) with BSA (1%) for over 12 months showed no adverse effects on the conjugate activity.

4.3.4 Indirect ELISA protocol

As will be indicated in subsequent chapters, this protocol allows a single method for reproducible determination of antibody titres. This system was found to be much more sensitive than the Ouchterlony agar immunodiffusion assay for antibody titre determination. Doubling dilutions of serum isolated in section 4.2.2 were prepared. These resulted in precipitin band formation with undilute virus antigen only, down to a serum dilution of only 1/16. Figure 15 indicates however (using the indirect ELISA system) an antibody titre in the same serum of more than 65 000.

Inconsistent formation of the ELISA immune complex was found when incubating bound antigen with undilute serum samples. The reduction in optical density and therefore reduction in immune complex formation, relative to the more dilute samples tested, is possibly due to competition between the antigen specific antibodies and relatively highly concentrated serum proteins for sites on the antigen.

4.4 DISCUSSION

An antigen concentration of 5 µg/ml was found to be suitable for optimal adsorption of antigen to the microtitre plate well walls. Carlsson, H. and Lindberg, A. (1978) indicate that optimal bacterial and fungal antigen concentrations for adsorption were in the range 0,1 - 10 µg/ml. So the Nodamura virus concentration is consistent with general economy of reagent utilisation.

Comparing Figure 14 with results from Engvall, E. and Perlmann, P. (1972), no prozone phenomenon was indicated at the higher antigen concentrations. Utilization of Nodamura virus at high concentrations, such as 100 µg/ml, might indicate the reduction in uptake of antibody by the bound antigen.

The enzyme-linked goat anti-(rabbit IgG) conjugate was shown to have efficient activity at a dilution of 1/400 with the indicated substrate and incubation conditions.

The enzyme-IgG linking takes place in a one-step reaction. Self-

linking of the components to be coupled is therefore possible. To reduce this, linking could take place in a two-step reaction with GA being used to activate one of the components before addition of the other component. Alternatively heterobifunctional cross-linking agents with specificity for each component at each reaction step can be used. Kitagawa, T. and Aikawa, T. (1976) used in this fashion, N-hydroxy-succinimidyl ma mallimidobenzoate (HSMB) to link the antigen insulin to β-galactosidase, thus eliminating self-linking, increasing enzyme activity and therefore improving assay sensitivity.

In the present study using the indirect ELISA test for the quantitative determination of serum antibodies, controls to detect any non-specific adsorption of or interaction between the reaction components were prepared. The only control which consistently gave a low positive enzyme activity was that consisting of serum dilution plus conjugate plus substrate, i.e. control 4. Only the most concentrated (least dilute) antisera showed false positive results, indicating an adsorption of the antibody molecules onto the well surface. Incubation of wells with 0,5% gelatin, as well as the use of surfac= tant, reduced this type of adsorption effectively.

This test has shown itself to be extremely simple to perform, reproducible and highly sensitive. Most proteins adhere to plastic, so the assay has wide application. Monodeterminant antigen can be used for determination of specific serum antibody quantitation. The indirect ELISA is economised by use of conjugate specific for immuno= globulin isotypes, precluding the necessity of linking enzyme to particular antigen-specific antibodies as is the case in the double antibody sandwich assay (Van Regenmortel, M. and Burchard, J., 1980).

Determination of enzyme activity by spectrophotometric means is facilitated by the use of photometers monitoring optical density at 410 nm directly in the wells, flat-based wells being the main requirement here.

The following chapters will indicate the use of the indirect ELISA technique for the quantitation of antibody titre elicited against antigen inoculated in various formulations.

With this method, one may monitor the efficacy of and improvement in serum albumin bead-presentation of antigen as a vaccine.

CHAPTER 5

IN VIVO IMMUNOGENICITY COMPARISON OF NODAMURA VIRUS PREPARED IN SERUM ALBUMIN BEADS, WITH NODAMURA VIRUS EMULSIFIED IN FREUND'S COMPLETE ADJUVANT

5.1 INTRODUCTION

The results of the *in vitro* experiments to determine the optimal stability of beads prepared with GA at various percentage final concentrations, reported in Chapter 2, indicate that the 1% GA cross-linked beads might be most suitable for the development of a vaccine formulation.

It was therefore decided to test *in vivo* the antigenic presenta= tion characteristics of virus-containing beads prepared at this percentage GA cross-linking. Vaccine efficacy could be compared to the response obtained against an equivalent mass of antigen prepared as a water-in-oil emulsion with Freund's complete adju= vant (FCA).

Not only would the relative serum antibody titres be monitored for the vaccine formulations, using the indirect ELISA method (Engvall, E. and Perlmann, P., 1972), as described in Chapter 4, but also physiological changes in the test animals during the course of the experiment, such as weight loss, reduction in general health and necrosis at the inoculation site, would be noted. Lee, T. et al. (1981) reported no adverse changes in the rabbits inoculated

with progesterone entrapped in homotypic serum albumin beads.

It would be anticipated that a single inoculation of antigen would result in a primary humoral response associated with a relatively low antibody titre compared to a secondary humoral response. For a viable vaccine effect, serum obtained from rabbits inoculated with serum albumin-entrapped antigen, should be tested as to their content of virus neutralising antibodies. These antibodies or at least immune system-priming antibodies would indicate some potential for this system as a vaccine formulation.

5.2 MATERIALS AND METHODS

5.2.1 Preparation, inoculation and monitoring of vaccine formulations

Nodamura virus was isolated and equal concentrations were prepared in rabbit-serum albumin beads and emulsified in Freund's complete adjuvant (FCA). These preparations plus an equal concentration of virus in phosphate buffered saline (PBS) (0,02 M, pH 7,5), were inoculated into three groups of 5 rabbits respectively. A pooled serum sample from rabbits inoculated with antigen in RSA beads was checked for virus neutralising antibodies. To ensure sterility, sodium azide (0,02%) was included in the PBS , while commercially available sterile syringes and needles (Terumo) were used for any inoculations.

5.2.1.1 Maintenance of test rabbits

Unimmunised New Zealand White rabbit does were obtained locally and were caged in wire as pairs or trios. The animals were checked daily as to their health and the filling of their water bottles and feed hoppers. The excrement trays

and cages were cleaned daily and weekly respectively. Each of the 5 rabbits in a group was marked with a distinctive dye for identification.

5.2.1.2 Preparation of vaccine formulations

Nodamura virus (1500 µg) was isolated according to the protocol in section 1.2.1.3. The re= suspended virus was then diluted to 2,4 ml with PBS and divided into 3 aliquots (0,8 ml). To each of 2 aliquots was added rabbit serum albumin (RSA) (200 mg), prepared according to section 1.2.2.2.

One of the albumin containing aliquots was diluted to 5 ml with PBS and then stored (4°C), while albu= min beads were prepared from the other aliquot according to the protocol in section 2.2.1.1. Resultant dried beads were then stored (4°C).

The third albumin-free virus aliquot was diluted to 2,5 ml with PBS and emulsified with an equal volume of Freund's Complete Adjuvant (Miles Labora= tories, USA) according to Herbert, W.J. (1978). The water-in-oil nature of the emulsion was checked by the non-dispersability of a drop of the emulsion in cold water.

Sterile PBS was used in this and subsequent vaccine experiments.

5.2.1.3 Inoculation and bleeding of rabbits

Prior to inoculation, PBS (5 ml) was added to the serum albumin beads which were then allowed to rehydrate (4°C; 2 hours).

A blood sample (5 ml - 10 ml) was obtained from each rabbit according to the protocol described by Herbert, W.J. (1973). The rabbit to be bled was placed in a rabbit box and hair was shaved from over the marginal ear vein and ear tip. Xylene was dabbed onto the ear tip, vaseline smeared over the vein, and after the vein had dilated, a blood sample (5 ml - 10 ml) was collected from a scalpel-incised nick of the vein. The ear was then cleaned of blood, xylene and vaseline. Serum was isolated from the blood sample according to section 1.2.2.1 and stored (-20°C).

Each rabbit was then inoculated intramuscularly in a 70% ethanol surface-cleaned hindquarter with 1 ml - containing approximately 100 µg virus - of the respective virus preparations.

On days 3, 7, 10, 14, 17, 26, 35, 50 and 64 following the initial inoculation, blood samples were obtained from the rabbits and serum isolated from each, as described above. Aliquots (2 ml) of

serum from a particular group of rabbits were pooled and stored (-20°C).

After the final blood sample was collected and serum isolated, the relative serum antibody titres were obtained by means of the indirect ELISA test described in section4.2.2., and read with the Vitatron Densitometer as described in section 4.2.2.1. Full titration curves of the group average results were plotted, the 50% dilution end point for each curve determined, and these were then plotted against time (days). See Figures 16 and 17 for the graphical repre= sentation of these results.

5.2.1.4 Serum neutralisation of virus

This test was adopted from Murphy, F. et al. (1970). An aliquot (0,5 ml) of pooled serum isolated on day 36 following inoculation with serum albumin bead-entrapped virus was mixed with stock virus solu= tion (0,5 ml) and incubated (37°C; 60 mins). An aliquot (0,5 ml) of PBS was mixed with day 36 serum (0,5 ml) or stock virus (0,5 ml) and incu= bated (37°C; 60 mins). Eight newborn mice were each then inoculated intraperitoneally with an aliquot (0,05 ml) serum/virus mixture, while two groups of 6 newborn mice each were inoculated with aliquots (0,05 ml) of the other two preparations respectively. The mice were checked daily for signs of paralysis.

5.3 RESULTS

5.3.1 General physiological condition of inoculated rabbits

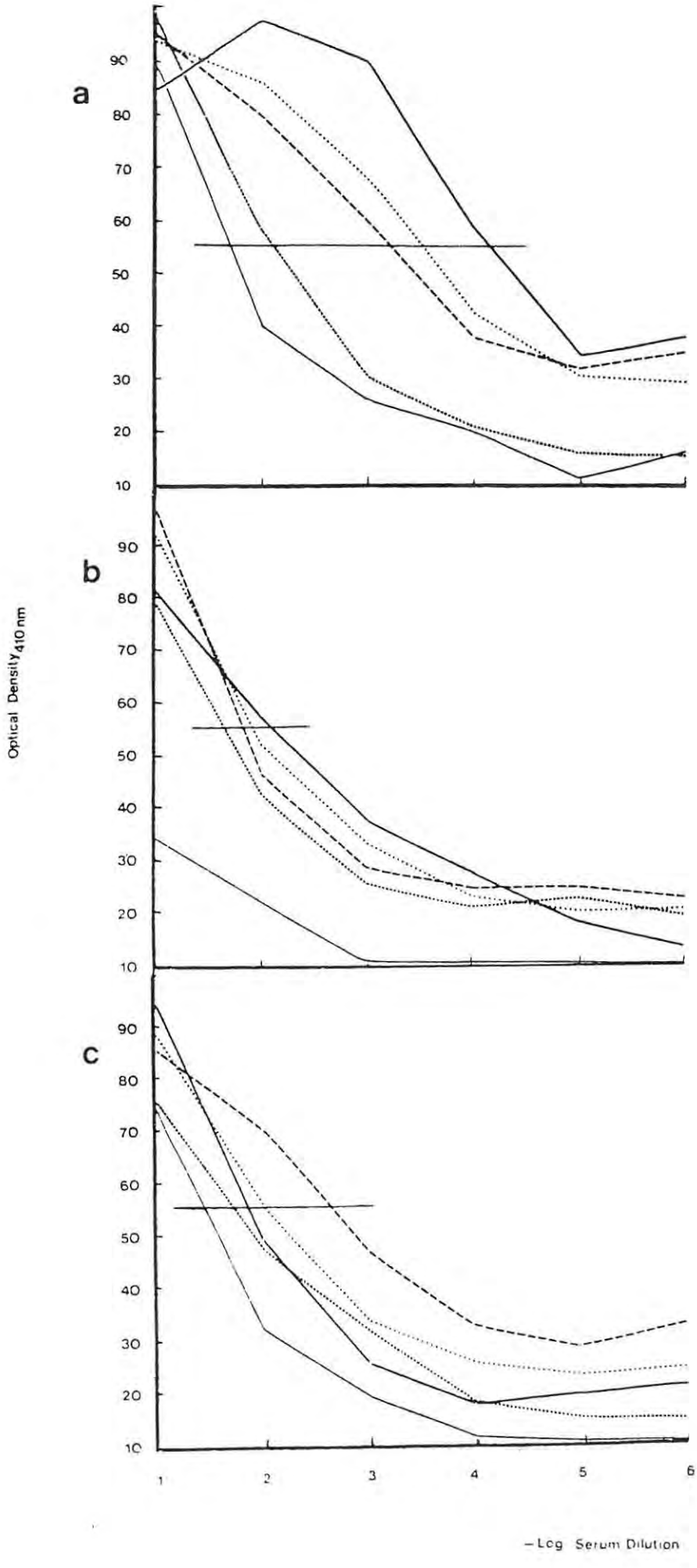
During the course of the experiment, the rabbits were checked daily. No weight or apparent appetite losses were noted and the rabbits looked active and healthy. While the groups of serum albumin-treated rabbits showed no adverse effects at the site of inoculation, swelling and tenderness occurred at the site of FCA inoculation, followed by ulceration in one of the 5 rabbits inoculated with FCA.

5.3.2 Interpretation of indirect ELISA results

Figure 16 represents the duplicate average complete titration curves for the pooled sera isolated on day 3, 14, 26, 35 and 64 post inoculation. These curves were plotted from a serum dilution of 1/16 (4^{-2}). Undiluted serum aliquots, rich in protein, indicated an interference in the consistent formation of the immune complex in the ELISA test, resulting in the reduction in the optical density of the wells concerned.

The 50% serum dilution end-point was determined from the intersection of the titration curves with the horizontal lines indicated. Any value obtained from the intersection of a group's day 0 serum titration curve with the horizontal line was subtracted from the values obtained for the other serum samples tested.

Examination of Figure 17 shows soluble antigen to be potently immunogenic - giving rise to a primary immune response which peaked around day 25 following inoculation and then dropped off gradually toward the end of the experiment.


a
b
c
Optical Density 410 nm
90
80
70
60
50
40
30
20
10
1
2
3
4
5
6
– Log Serum Dilution

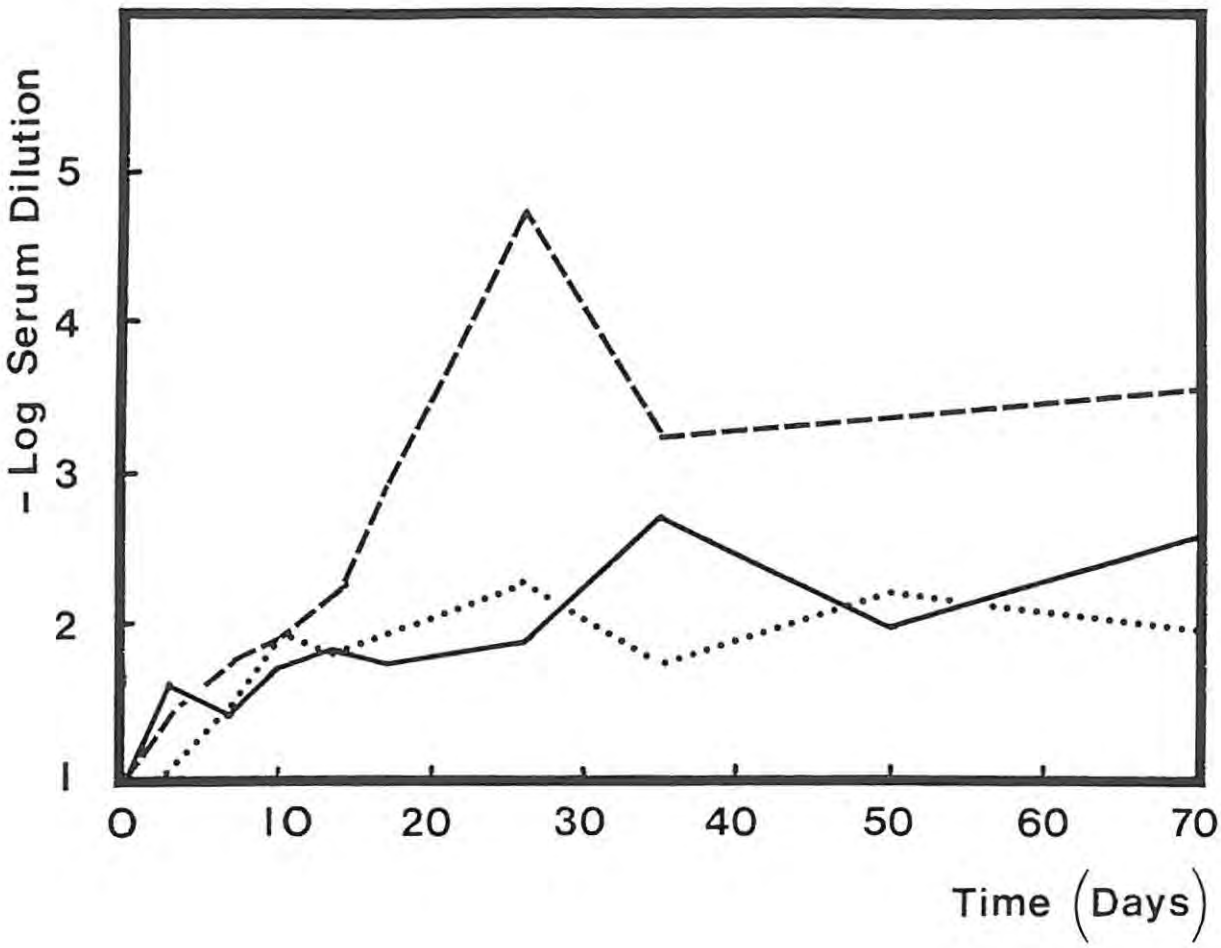


Figure 17: Kinetics of IgG response following immu= nization of antigen in FCA, in albumin beads and in solution.

– – – – –: antigen inoculation, in solution

.....: antigen inoculation, in FCA

————: antigen inoculation, in serum albumin beads

The humoral response to albumin bead- and FCA-entrapped antigen show essential similarities. An initial relatively low antibody response gradually increases with time, possibly reaching a plateau toward the end of the experiment (day64).

The fluctuations in the ELISA results as indicated in Figure 17, underline the inadequacy of testing pooled serum samples. Future tests should be performed on individual sera, so that extreme responses by individuals in a group, adversely effecting the group average trend, might be determined.

5.3.3 Serum neutralisation of Nodamura virus

Serum from rabbits inoculated with virus entrapped in albumin beads, was shown to contain virus- neutralising antibodies.

The 6 mice inoculated with virus/PBS developed disease symptoms 4 days post inoculation. The groups of 6 mice, inoculated with serum/PBS, and the other of 8 mice which was inoculated with virus/serum, showed no signs of infection by 10 days post inoculation.

It must be noted that undiluted virus stock was used in this experiment, compared to the 1/5 dilution of stock virus required to routinely propagate virus.

5.4 DISCUSSION

The inoculation of rabbits with virus antigen prepared in various formulations; freely soluble, entrapped in RSA beads and emulsified

in FCA, was performed to empirically determine the efficacy and potential of entrapping antigen in RSA beads as a vaccine formulation. This initial *in vivo* experiment, lasting 64 days, allowed a comparison of the primary humoral immune response to the variously presented antigen.

Solubilised antigen was shown to be extremely immunogenic, promoting a potent primary immune response. This peaked at about day 25 post inoculation, thereafter decreasing to a titre still superceding those generated against the "depot"-presented antigen. This high titre result effectively negates the concept of an "antigen-depot" type vaccine. As discussed, this result may be due to pooling serum samples prior to ELISA analysis. It was decided therefore to reduce the amount of challenge antigen from 100 µg virus per rabbit, to 10 µg virus per rabbit in future experiments and note differences in the kinetics of the resulting response to that illustrated in Figure 17.

Although the humoral response to the "antigen-depot" presented antigen was lower than that generated to soluble antigen, similarities can be noted between the humoral response to the antigen presented from either the FCA emulsion or the albumin beads.

Minimal humoral response to antigen inoculated as an emulsion in FCA was noted at day 3 and only became apparant at day 7. Thereafter a fluctuating, gradually increasing response was noted, beginning to stabilise from day 25. Herbert, W. (1978) indicates a comparable response in mice to ovalbumin in FCA which rises slowly to a peak over 2 - 3 months which then persists as a plateau, approximating the above results.

The humoral response to albumin bead-entrapped antigen showed an initial peak at day 3, possibly an artifact due to using pooled serum samples or possibly due to direct lymphocyte stimulation following the release of loosely bound surface-linked antigen after inoculation. The humoral response thereafter is very simi= lar to that obtained against the FCA formulation, with generally higher titres noted.

This strongly suggested an antigenic presentation strategy analo= gous to that found with FCA emulsions, i.e. the release of antigen at low levels is sustained over a period of time for extended immunostimulation. This is supported by the extended nature of this primary response. For logistical reasons, a booster inoculation was not administered to these rabbits to monitor the nature of a resulting secondary humoral response.

The results of the virus-neutralising experiment indicate the presence of virus-neutralising antibodies elicited by day 35 against antigen released from serum albumin beads. Mice were protected against virus challenge, by antibodies produced against virus released from serum albumin beads. This response is of prime import in determining the efficacy of a vaccine formulation.

The degree to which an infectious virus will be neutralized by anti= body is governed by the affinity of the antibody and the antibody con= centration (Taussig, M.J., 1976). The antibody titre for the serum (day 35) tested was only 2,6 log units. This means that efficient presentation of antigen to lymphocytes occurred so that mainly lym=

phocytes with high affinity receptors for the antigenic determinants were triggered, to produce high affinity antibodies. While the monitoring of high avidity IgM produced would give a more balanced representation of the humoral response to antigenic presentation from albumin beads, only the long-lasting IgG response was monitored.

So, in summary, the breakdown of albumin beads containing antigen, results in a release of antigen so that the resulting immunostimulation appears to mimic the action of an established vaccine formulation such as antigen emulsified in FCA. No adverse effects were noted relative to the necrosis associated with FCA. Virus-neutralising antibodies were elicited against virus released from albumin beads which were capable of protecting mice against potent virus challenge.

CHAPTER 6

IN VIVO IMMUNOGENECITY COMPARISON OF NODAMURA VIRUS PREPARED IN SERUM ALBUMIN BEADS CROSSLINKED AT VARIOUS FINAL GLUTARALDEHYDE CONCENTRATIONS

6.1 INTRODUCTION

Having established an association between the antigenic presen= tation characteristics of serum albumin beads and the "antigen-depot" adjuvants such as FCA (Chapter 5), it was necessary to determine optimal crosslinking of antigen in serum albumin beads.

An optimal ratio between bead stability and bead degradation, for optimal antigenic release, is central to the use of serum albumin beads as vaccine vehicles. The recalcitrance of the 1% GA crosslinked beads *in vitro*, as shown in Chapter 3 (Figures 9 and 10), combined with the low antibody titres obtained in the *in vivo* studies in Chapter 5 (Figure 17), indicates that *in vivo* studies on the presentation of antigen from albumin beads cross-linked at lower than 1% final GA concentration, should be performed.

Crosslinking the beads with reduced percentage GA not only would facilitate bead breakdown and antigen release, but would also affect the immunogenecity of the virus antigen itself.

At high GA concentrations, lysine residues in a relatively larger proportion of antigenic determinants might react with GA resulting

in increased epitope masking. This will result in the relative reduction in stimulation of lymphocytes capable of secreting virus-specific antibodies. This relative reduction in antigen "dose" might be counterbalanced by the stimulation predominantly of high-affinity antibody-secreting lymphocytes, thus promoting virus-neutralising immunoprophylaxis.

Also associated with relatively high GA concentrations would be the increased possibility of virus aggregation with virions linked by GA molecules. This massive and concentrated expression of antigen, once released from degraded albumin beads, could elicit a potent immune response.

The reduction in the crosslinking percentage of GA could lead to a reduction in the masking of virion antigenic determinants thus exposing those epitopes for stimulation of an expanded population of lymphocytes. Counterbalancing this would be the reduced likeli= hood of virion aggregation with concommittant reduction in viral immunogen city.

A basic determination of acquired immunogenicity by covalently cross-linked rabbit serum albumin, should be performed. This could be achieved by inoculating rabbits with albumin beads which contain virus as antigen. The presense of albumin-specific antibodies in the serum isolated thereafter, could be determined by an Indirect ELISA test involving rabbit serum albumin bound to the well walls.

To test these hypotheses, beads with incorporated virus were prepared at 0,8 ; 0,9 and 1% final GA concentrations and the humoral response

to these formulations were monitored using the indirect ELISA technique of antibody titre determination. Serum containing Nodamura virus-neutralising antibodies was also tested for its antibody recognition capability with respect to native rabbit serum albumin.

6.2 MATERIALS AND METHODS

6.2.1 Preparation, inoculation and monitoring of virus containing serum albumin beads

Albumin beads, containing bound Nodamura virus, were prepared at various final percentage GA concentrations. These were inoculated into groups of rabbits, each of which was bled at intervals thereafter. The serum antibody titres were determined using the indirect ELISA technique.

6.2.1.1 Preparation of virus-containing serum albumin beads

Nodamura virus (200 µg) was isolated according to the protocol in section 1.2.1.3. The resuspended virus solution was diluted to 3,2 ml with sterile PBS (0,02 M; pH 7,4), before being divided into four aliquots (0,8 ml each). Into each aliquot was dissolved rabbit serum albumin (200 mg), isolated according to section 1.2.2.2. Three aliquots were used to prepare beads at 0,8%; 0,9% and 1% final GA concentration. To each bead preparation was added sterile PBS (5 ml). The fourth

aliquot of virus/albumin solution was diluted to 5 ml with sterile PBS.

6.2.1.2 Bleeding and inoculation of rabbits

Unimmunized New Zealand white rabbits (20) were maintained according to section 5.2.1.1, divided into four groups of 5 rabbits, and then individually marked. Prior to inoculation, a blood sample (5 ml - 10 ml) was obtained from each rabbit and the sera therefrom were then isolated according to section 5.2.1.3 before being individually stored (-20°C).

Each rabbit in a particular group was inoculated with an aliquot (1 ml) of beads prepared at a particular % final GA concentration, while the fourth group of rabbits received virus/albumin solution (1 ml per rabbit).

Each rabbit received approximately 10 µg Nodamura virus contained in the albumin preparations.

The rabbits were then bled at intervals (days 7, 28, 48, 110 and 150) post inoculation and the individual sera isolated and stored (-20°C) as above.

After day 150, the Nodamura virus-specific antibody titre was determined in duplicate for each

serum according to section 4.2.1, using tenfold serial serum dilutions and the course of the humoral response to the virus/albumin prepara= tions (Figure 18), determined from the 50% serum dilution end-point of the complete titration curves.

6.2.1.3 Serum neutralisation of Nodamura virus

An aliquot (0,5 ml) of pooled sera isolated on day 110 following inoculation of virus entrapped in 1% GA crosslinked RSA beads, was incubated ($37^{o}C$; 60 minutes) with Nodamura virus (0,5 ml) according to the protocol in section 5.2.1.4. Controls of PBS-diluted serum and virus were prepared as described in that section.

Each suckling mouse in a litter of 8 was inocu= lated intraperitoneally with an aliquot (0,05 ml) of virus incubated with serum. Two litters of 7 mice each received inoculations (0,05 ml) of serum and virus respectively.

The mice were checked daily for paralysis.

6.2.1.4 Indirect ELISA test with serum albumin as antigen

Four serial 8 ml two-fold dilutions of rabbit serum albumin, isolated according to the protocol in section 1.2.2.3, were prepared in PBS, from a concentration of 100 µg/ml to 12,5 µg/ml.

To duplicate rows of wells, were added aliquots

(200 µl) of each dilution respectively, followed by incubation (18 hours; 37°C). Six serial 2 ml ten-fold dilutions of pooled sera isolated on day 110 following inoculation with virus entrapped in 1% GA crosslinked serum albumin beads were then prepared in PBS-Tw. Duplicate aliquots (200 µl) of each serum dilu= tion were added to respective wells of each duplicate albumin "antigen" and incubated (3 hours; 37°C). Primary immune complex formation was visualised as per the protocol in section 4.2.2.1. Figure 19 indicates the graph of OD_{410} vs log serum dilution for the four an= tigen preparations.

6.3 RESULTS

6.3.1 General physiological condition of rabbits

The rabbits inoculated with virus/albumin either in bead form or in solution, showed no adverse physiological effects during the course of the experiment. No necrosis or tender= ness was noted at the site of the inoculation, consistent with results obtained in Chapter 5 for rabbits inoculated with virus in serum albumin beads.

6.3.2 Interpretation of ELISA results

Figure 18 indicates the humoral response in the groups of rabbits to challenge by antigen, prepared in various formu= lations. Standard deviations of up to 0,73 $\log_{10}$ units between

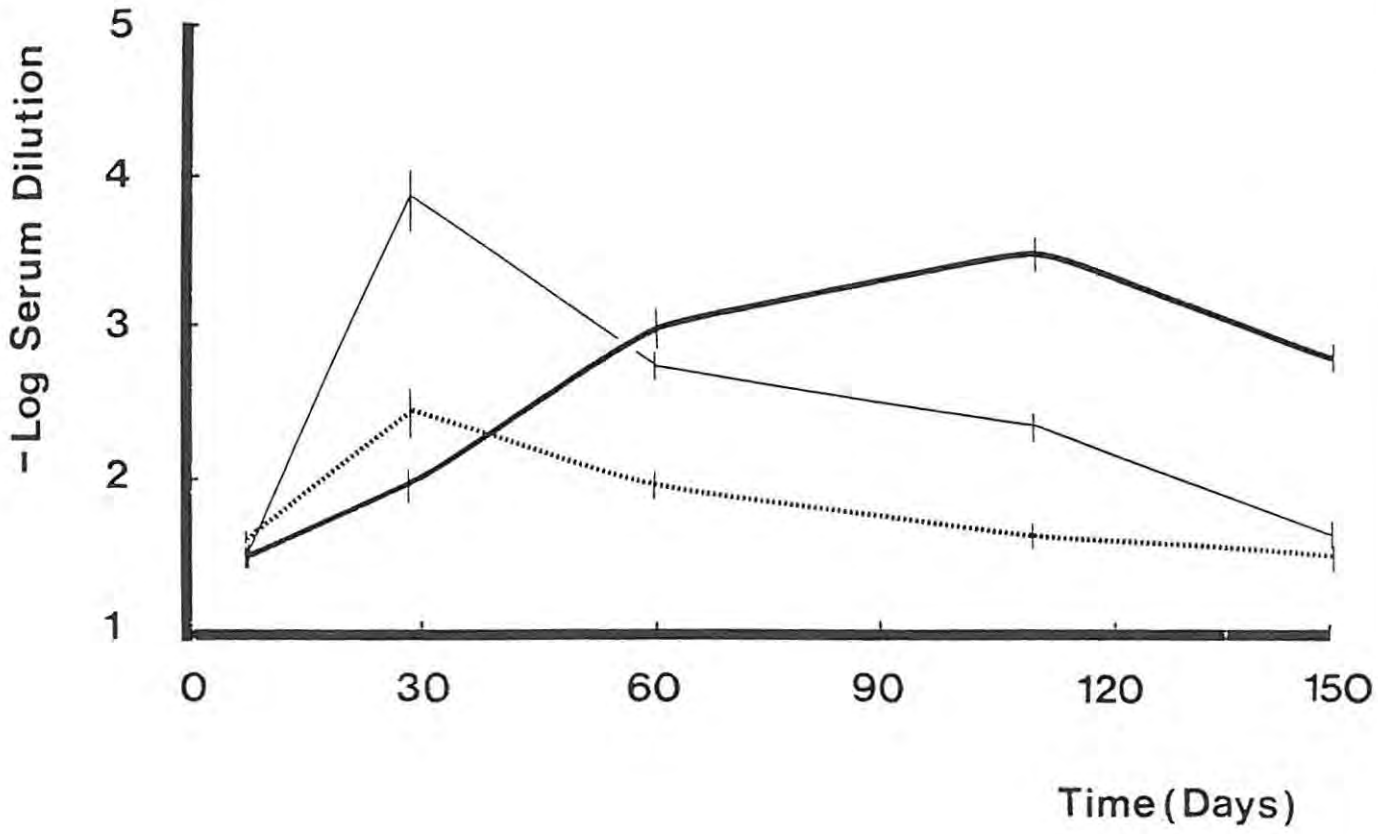


Figure 18: Kinetics of IgG response to Nodamura virus inoculated in albumin beads crosslinked at various final GA concentration (mean ± std. error; n = 5).

————: Antigen inoculated in solution

.....: Antigen inoculated in albumin beads cross= linked at 0,8% final GA concentration

————: Antigen inoculated in albumin beads cross= linked at 1% final GA concentration

individual titres within a group were noted.

Virus inoculated in solution once again showed itself to be potently immunogenic, with the primary response peaking around day 28. Thereafter the antibody titre dropped.

The antibody response to 0.8% and 0.9% (0.8% shown in Figure 18) final GA prepared beads shows essentially a reduced primary response, peaking around day 28 before levelling off and then decreasing.

The beads prepared at 1% final GA concentration resulted in a gradually increasing humoral response, peaking around day 110 before decreasing.

By day 7 post inoculation, the mice inoculated with virus/PBS were moribund. Those groups inoculated with serum/PBS and serum/virus respectively, showed no signs of disease by then. This indicated that virus neutralising antibodies were elicited against virus released from albumin beads prepared at a final GA concentration of 1%.

6.3.4 Interaction between antibodies and serum albumin as antigen

Figure 19 indicates only non-specific adsorption of serum antibodies to the well walls and no reaction with serum albumin as antigen. Decreasing the albumin concentration, allowed an increase in this non-specific adsorption. The "titres" obtained were lower than the only positive control,

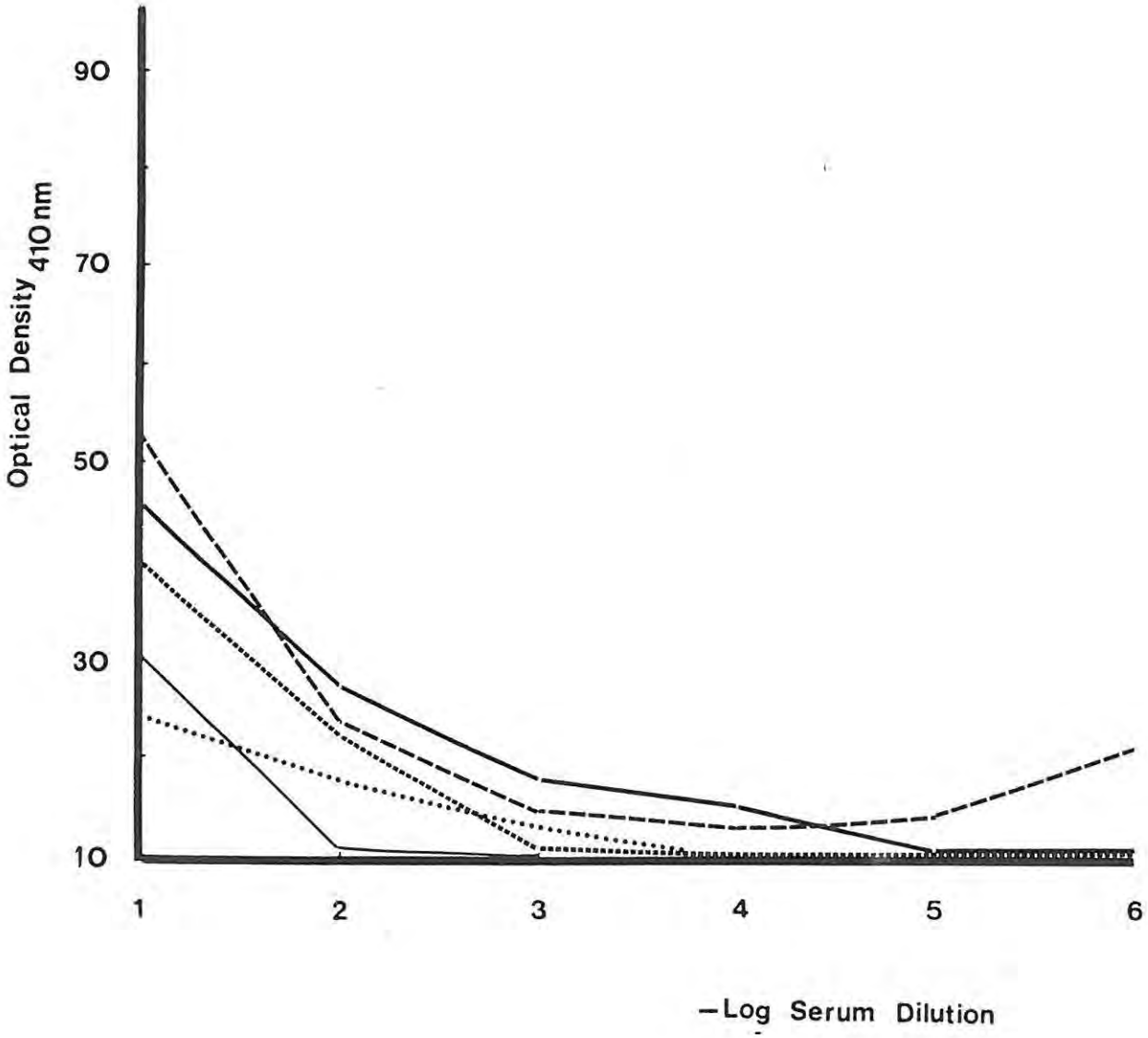


Figure 19: Use of native serum albumin as antigen.

———: Sero-negative serum control

———: Albumin at a concentration of 100 µg/ml

••••••••••: Albumin at a concentration of 50 µg/ml

.....: Albumin at a concentration of 25 µg/ml

– – – –: Albumin at a concentration of 12,5 µg/ml

that of serum dilution plus conjugate plus substrate.

6.4 DISCUSSION

The results of the vaccine experiment, illustrated in Figure 1 , support the results obtained in Chapter 5 - the incorporation of virus in albumin beads at 1% final GA concentration could be used as a vaccine formulation to elicit virus neutralising antibodies.

An extended primary response to antigen prescribed in this fashion resulted in an antibody titre of greater than 3 $\log_{10}$ units. Virus neutralising antibodies were demonstrated in serum obtained 110 days post inoculation - indicating that extended immunoprophylaxis results from antigen challenge presented from 1% final GA concentration crosslinked beads.

The antigen challenge presented from virus-containing beads crosslinked at a lower percentage GA concentration, elicits a humoral response similar to a reduced primary response against soluble antigen.

This indicates that antigenic masking by GA molecules might have occurred, leading to the reduced antibody titre (2,4 $\log_{10}$ units as opposed to 3,8 $\log_{10}$ units against free antigen), peaking around day 28 post inoculation.(See also Chapter 7, Figure 20).

Rapid breakdown of the 0,8% and 0.9% final GA concentration crosslinked beads appears to allow massive initial release of masked

antigen which thereafter rapidly decreases. This prevents an ex= tended antibody response being elicited.

Virus neutralisation studies were not conducted on the serum ob= tained against inoculation of antigen containing beads prepared at percentage GA final concentrations lower than 1%. However, the slight antibody titre peak noted around day 28 against these anti= gen formulations might make these formulations useful as priming agents, promoting an accentuated response to concommitantly ino= culated antigen entrapped in beads prepared in 1% final GA concen= tration.

A preliminary test for the presence of antibodies capable of recog= nising soluble serum albumin, showed that inoculation of crosslinked albumin beads had no effect on the humoral response. Notwithstanding the significance of this result, the cell-mediated immune response to the crosslinked albumin should be monitored in future studies for an overall indication of the safety of this formulation as a vaccine adjuvant.

In summary, the results of this experiment indicate that the degrada= tion of serum albumin beads crosslinked at 1% final GA concentration, has an adjuvant effect, stimulating an extended humoral response to antigen released from the beads with no immediately detectable adverse side effects. Covalent masking of epitopes at this % GA concentration does not adversely influence the total immunogenecity of the antigen once released from the bead.

CHAPTER 7

IN VIVO IMMUNOGENICITY COMPARISON OF NODAMURA VIRUS PREPARED IN SERUM ALBUMIN BEADS, WITH SOLUBLE NODAMURA VIRUS INACTIVATED WITH GLUTARALDEHYDE

7.1 INTRODUCTION

As indicated in the previous chapters, the effect on virus immunogenecity of covalently linking GA to the virions, should be determined. An excess of GA mixed with virus should inacti= vate the virus, by reaction with the free amino groups of lysine residues on the surface of the capsid.

GA has been used to inactivate poliomyelitis and foot-and-mouth disease virus in the preparation of vaccines (Relyveld, E. and Ben-Efraim, S. 1983). These workers incubated GA (0,0263%) with purified poliovirus type 3 (37^{o}C; 90 minutes) and FMDV strain O-Flandre (37^{o}C; 5 days) for complete virus inactivation. The inactivated FMDV preparation, diluted 1:3, induced neutralising antibodies in guinea pigs at a titre similar to that obtained after immunization with a commercial viral vaccine.

Virus neutralising antibodies will mainly be elicited against anti= genic determinants found on the surface of the virion capsid. In an aqueous environment, the tertiary conformation of the protein(s) comprising the capsid will be such that the exterior of the capsid will be rich in hydrophilic amino acid residues, such as lysine, arginine, glutamic and aspartic acid (Lerner, R., 1983).

It is likely therefore that lysine, associated with some of the virus epitopes, will be available for interaction with GA.

Newman, J. et al. (1978) described from peptide analysis of the Nodamura virus VP40 capsid protein, that the capsid consists of 180 protein chains, each chain of 370 amino acids containing 15 lysine residues. Nodamura virus consists of 80% protein and 20% RNA (Adams, E. and Longworth, J., 1978). Using Avogadro's number, 50 µg virus should contain 3.4×10^{12} VP40 polypeptides

$$\left(\frac{40 \times 10^{-6}\ g}{180 \times 40 \times 10\ 3\ _g} \times 6{,}023 \times 10^{23}\right)$$ with a total of 5.1×10^{13} lysine residues.

Interaction of virus (1 ml; 50 µg/ml) with GA (1%), representing 6×10^{17} GA molecules, should allow Schiff base reaction with capsid lysine residues for complete inactivation of the virus.

Nodamura virus was inactivated with GA, inoculated into a group of rabbits and the antibody titre elicited against this antigen was compared with the antibody titre elicited against purified virus entrapped in serum albumin beads.

The results of this study should indicate the effect of saturation GA linking to capsid lysine residues, relative to this influence on the immunogenicity of the capsid as a whole.

7.2 MATERIALS AND METHODS

7.2.1 Preparation, inoculation and titration of vaccine formulations

Two groups consisting of 5 rabbits each were prepared. Each rabbit in a group was inoculated with an equal quantity (10 µg) of GA-inactivated virus, while each rabbit in the other group was inoculated with virus (10 µg) prepared in rabbit serum albumin beads. Blood samples were obtained from the rabbits at intervals, before the serum antibody titres were determined, using the indirect ELISA method. A graph of antibody titre against time was then plotted.

7.2.1.1 Preparation and inoculation of vaccine formulations

Nodamura virus (100 µg) was isolated according to section 1.2.1.3 and the resuspended virus was diluted to 1,6 ml with sterile PBS (0,02 M; pH 7,4). This was divided into 2 aliquots.

One aliquot was made 1% with respect to 25% GA (BDH Chemicals, Poole England) and incubated (T_{room} ; 60 mins.). After incubation, the solution was diluted to 5 ml with sterile PBS and then 1 ml of this dilution was inoculated into each of a group of 5 rabbits.

To the remaining aliquot (0,8 ml) was added rabbit serum albumin (200 mg) as prepared in section 1.2.2.3.

Beads were then prepared according to section 2.2.1.1 following the addition of GA (0,2 ml) to a final concentration of 1%. To the resul= tant dried beads was added sterile PBS (5 ml) and then 1 ml of bead-containing solution was inoculated into each rabbit of the second group.

A sample of blood (5 ml - 10 ml) was obtained from each rabbit according to section 5.2.1.3 prior to inoculation and then on days 7, 14, 21, 28, 40 and 60 post inoculation. Serum was isolated from each blood sample according to section 1.2.2.1 and stored ($-20^{o}C$).

7.2.1.2 Determination of serum antibody titres

Following the isolation of serum from day 60 blood, the antibody titres of the isolated serum samples were individually determined in dupli= cate, according to the protocol for the indirect ELISA method in section 4.2.2.1.

The mean titres, with standard error, for each group of rabbits on the days indicated, were then plotted against time (days). (See Figure 21 for these results.)

7.3 RESULTS

7.3.1 General physiological condition of inoculated rabbits

During the course of the experiment the rabbits inoculated were checked daily and were found to be healthy and active.

7.3.2 Interpretation of ELISA graphs

Figure 20 indicates the kinetics of IgG formation elicited against the two virus preparations.

The curve representing the titre obtained against inactivated Nodamura virus, peaks around day 21 - at a value of 2,9 $\log_{10}$ units - before gradually falling off.

The response to virus released from serum albumin beads shows a gradual increase with time, still rising by day 60. After day 50, this curve indicates a greater titre to that obtained against inactivated virus.

7.4 DISCUSSION

The results of this experiment with GA-inactivated Nodamura virus shows a slower drop in antibody titre with time, compared to un= treated soluble virus (Figure 19). This is consistent with the use of GA-inactivated Polio and FMD virus as possible vaccines (Relyveld, E. & Ben-Efraim, S., 1983).

An overall lower antibody titre was obtained using GA-inactivated virus, as shown in Figure 20, compared to the response against the same mass of untreated virus, as shown in Figure 18.

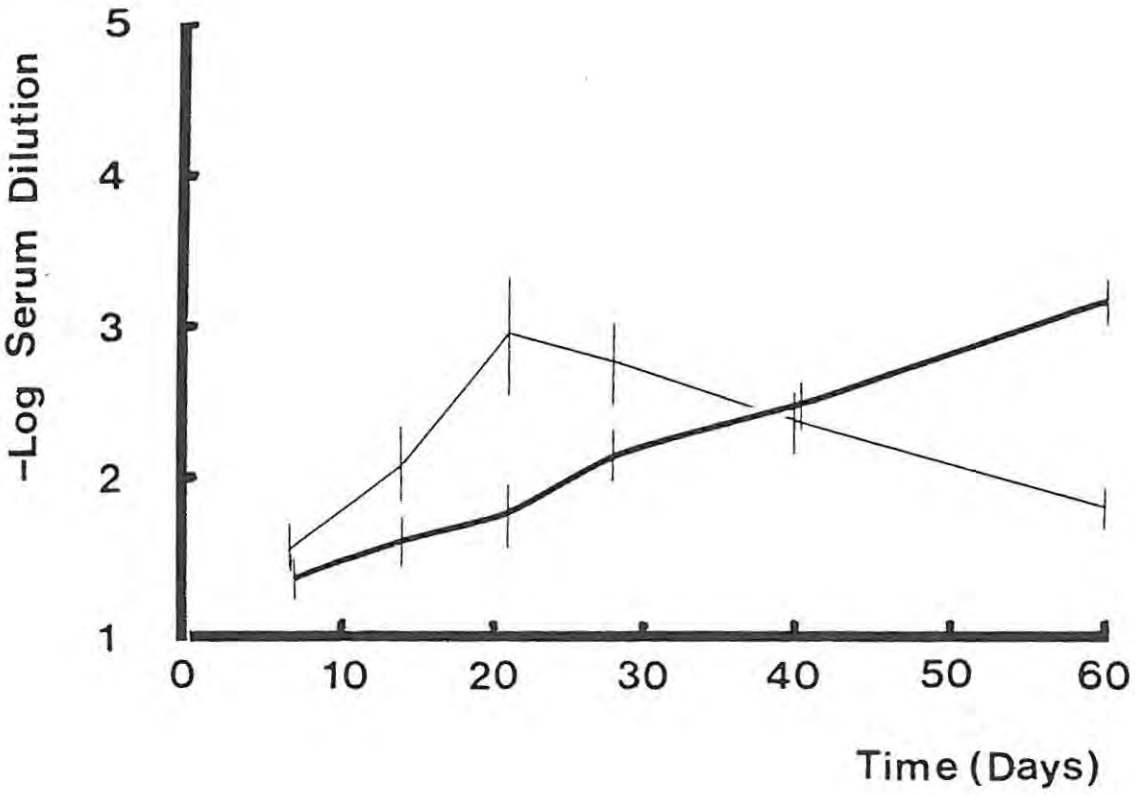


Figure 20: Kinetics of IgG response to Nodamura virus in albumin beads and to inactivated virus in solution (mean ± standard error; n = 5).

————: inactivated virus in solution

————: virus inoculated in albumin beads cross= linked at 1% final GA concentration.

This is possibly due to antigenic masking of epitopes by GA, resulting in a reduced stimulation of lymphocytes secreting Nodamura virus-specific antibodies.

However, during the reaction between GA and virion particles, virion aggregation might occur such that the close proximity of adjacent, linked virions might prevent saturated covalent bonding of free viral lysine residues by the GA molecules. This might result in incomplete virus epitope masking involving lysine residues so that the decrease in titre noted above would not be exceptionally reduced in comparison to the titre obtained against untreated, unaggregated virus.

A widely varied humoral response was noted in individual rabbits within the GA inactivated virus rabbit group. Standard deviations of up to 0,9 $\log_{10}$ units around a group median result was noted for individual rabbits in this group.

While fluctuations in humoral response was noted in individual rabbits in all groups tested in this study, variable epitope masking by GA would probably tend to increase the spread of titre results obtained for any particular group.

The antibody response to Nodamura virus released from serum albumin beads cross-linked at 1% final GA concentration in this experiment, was essentially similar to the response elicited against similarly prepared virus as shown in Figure 18. An initial low response is noted which gradually increases with time, presumably due to sequential

release of virus from degrading albumin beads, until an antibody titre is reached, which is greater than that obtained against soluble virus preparations.

The results of this experiment supported those obtained in the previous chapter and indicated that antigen entrapped in 1% final GA concentration cross-linked beads could undergo a maximum antigenic masking by GA in the process of bead formation and still leave free sufficient virus epitopes capable of eliciting a virus-neutralising humoral response.

This supported the proposal of using serum albumin beads at this percentage GA cross-linking as a vaccine adjuvant which for the suitable release of intact virions in vivo.

Having established this, it was necessary to test this vehicle as to its adjuvant effect when prepared with isolated Nodamura virus capsid protein (VP 40) as antigen, and subsequently for the optimal release of peptide antigens.

CHAPTER 8

IN VIVO IMMUNOGENICITY COMPARISON OF NODAMURA VIRUS CAPSID PROTEIN (VP 40) PREPARED IN SERUM ALBUMIN BEADS, WITH VP 40 PREPARED IN SOLUTION

8.1 INTRODUCTION

The many possible problems associated with inactivated and attenuated vaccines, have prompted research toward developing virus vaccines containing only the immunogenic capsid polypeptide of interest or portion thereof, free from infectious nucleic acid.

One such vaccine is that available against hepatitis B virus (Szmuness, W. et al., 1980; Prince, A., 1982). This vaccine is prepared by isolating Hepatitis B surface antigen (HBsAg) from the plasma of HB carriers. This is possible because of the excessive synthesis of surface capsid protein in infected liver cells.

This expensive vaccine has been shown to induce antibodies to HBsAg in 95% of healthy recipients (Editorial Lancet, 1982) and consists of HBsAg contained in the purified 22 nm particles isolated from infected carrier plasma, adsorbed to aluminium phosphate as adjuvant. A micellar vaccine consisting of the antigenic determinant of HB virus with a molecular weight of between 22 000 and 24 000 has more recently been developed (Skelly, J. et al., 1981) and found to be more immunogenic than the 22 nm particles.

The high cost of this vaccine and the potential danger associated with incomplete formalin inactivation of the virus material de= rived from infected carriers, has prompted the development of synthetic subunit vaccines (Dreesman, G. et al., 1982) who prepared peptides 20 amino acid residues in length. These workers found the synthetic peptide to be immunogenic without the necessity of attaching the peptide to a carrier protein. Lerner, R. et al. (1981) using smaller (14 - 15 residues) peptides, found the peptides to be immuno= genic only after their covalent attachment to a carrier protein such as keyhole limpit haemocyanin.

The use of carrier proteins to augment the immunogenicity of poorly immunogenic antigens allowed the natural progression in this study of linking Nodamura virus capsid protein (VP 40), isolated by phenol extraction of purified virus (Rueckert, R. et al., 1965) or by virus destabilization with chloride treatment (Newman, J. personal communications), into rabbit serum albumin beads.

The proteolysis of the beads could lead to albumin fragments attached to the VP 40. The attachment of antigen to carrier protein, could augment the host immune response to the antigen.
This immunogenic improvement would bolster the sustained immuno= stimulation resultant from the gradual release of immunogen from the degrading beads.

If this system of subunit antigen release elicited in suitable humoral response, the potential of peptide antigen release from albumin beads as a vaccine, inducing virus neutralising antibodies, could be tested.

8.2 MATERIALS AND METHODS

8.2.1 Preparation, inoculation and antigenic monitoring of Nodamura virus VP 40

Nodamura virus was isolated as described in section 1.2.1.3 VP 40 was initially prepared by the method of phenol extraction and then by chloride treatment. Half of the VP 40 prepared by these methods were prepared in serum albumin beads and the antibody titres resultant from their inoculation into rabbits compared to soluble VP 40 inocu= lated into another group of rabbits. The presence of virus neutralising antibodies was tested by virus challenge in mice co-inoculated with virus-specific serum.

8.2.1.1 Phenol extraction of VP 40

The protocol followed was described by Reuchert, R. et al. (1965). Nodamura virus (400 µg) was isolated according to section 1.2.1.3. To the virus, resuspended in sterile PB (0,02 M; pH 7,4; 1 ml), was added an equal volume of water-saturated phenol and the mixture was shaken (15 min; 20°C). The phases were separated by centrifugation (10 000 g; 10 min). The aqueous phase was then extracted with an half volume of phenol before the phenol phases were pooled and made 0,1 M with respect to ammonium acetate and 1% with respect to 2-mercaptoethanol. Protein was precipitated by mixing 6 volumes of ice-cold, absolute ethanol with the phenol and storing

(16 hours; -20°C). The protein was sedimented by centrifugation (10 000 g; 10 mins) and then washed once with 95% ethanol.

The protein precipitate was sedimented by cen= trifugation (10 000 g; 10 mins), dried in a freeze-drying apparatus before being weighed.

8.2.1.2 Chloride treatment isolation of VP 40

The protocol followed was described by Newman, J.(pers.comm.).

Nodamura virus (400 µg) was isolated according to section 1.2.1.3. The virus sediment was resuspended in acetate buffer (0,1 N; pH 6; 1,7 ml), made 0,1 M with respect to NaCl and incubated (30 mins; 20°C). After incubation, an aliquot (0,1 ml) was removed, diluted to 0,5 ml with sterile PBS and inoculated into 3 day old suckling mice (0,05 ml each) to test for virulence. The balance was divided into equal aliquots (0,8 ml each) and rabbit serum albumin (200 mg) iso= lated according to section 1.2.2.3, added to each aliquot. The solutions were then stored (-20°C) until it was proved that the mice received avi= rulent inocula.

8.2.1.3 Preparation and inoculation of phenol-prepared VP 40-containing serum albumin beads

VP 40 (200 µg) was resuspended in PBS (1,6 ml) and to equal aliquots thereof (0,8 ml each) was added rabbit serum albumin (200 mg). Albumin beads at 1% final GA concentration were prepared from an aliquot and then to the dried beads was added sterile PBS (5 ml).

The remaining aliquot was diluted to 5 ml with sterile PBS.

Two groups of 5 rabbits each were then inoculated with respective VP 40/albumin preparations, each rabbit receiving an 1 ml inoculation containing approximately 20 µg VP 40.

5 ml - 10 ml blood samples were obtained from each rabbit prior to inoculation and on days 7, 14, 21, 28, 40 and 60 thereafter. The serum was isolated from each sample according to section 1.2.2.1 and individually stored (-20°C).

8.2.1.4 Preparation and inoculation of chloride prepared VP 40-containing albumin beads

After 8 days, when it was shown that the VP 40 inoculated mice remained healthy, the stored VP 40/albumin aliquot were thawed.

One aliquot was used to prepare albumin beads at 1% final GA concentration. To the dried beads was added PBS (5 ml) and this preparation was inoculated into each of a group of 5 rabbits, each rabbit receiving 1 ml of preparation con= taining approximately 20 µg VP 40.

The remaining VP 40/albumin aliquot was diluted to 5 ml with sterile PBS and 1 ml of this solution was then inoculated into each of a group of 5 rabbits so that each received approximately 20 µg VP 40.

The rabbits were bled prior to inoculation and on days 7, 17, 36 and 50 thereafter. Serum was isolated from each blood sample according to section 1.2.2.1 and individually stored (-20°C) after stabilization with sodium azide (0,02%).

8.2.1.5 Monitoring of serum antibody titre

At the conclusion of each experiment, serial tenfold dilutions were prepared of the serum in PBS-Tw and their antibody titres determined using the indirect ELISA method according to section 4.2.2.1. Figures 21 and 22 were then prepared representing the kinetics of the IgG response to VP 40 prepared by the two methods described.

8.2.1.6 Determination of serum neutralising antibodies

A total of 1 ml of pooled day 60 serum obtained against phenol prepared VP 40 entrapped in al= bumin beads, was used in a serum virus-neutralising test according to section 5.2.1.4.

Eight mice in a litter were each inoculated (0,05 ml) with virus incubated with serum while litters of 6 and 7 mice each were inoculated with serum/ PBS and virus/PBS respectively.

The mice were checked daily for paralysis.

8.3 RESULTS

8.3.1 Phenol prepared VP 40 as vaccine antigen

Figure 21 indicates the humoral response to VP 40 prepared in albumin beads and to freely soluble VP 40. The reduced scale of the Y-axis (to a maximum of 2,0 $\log_{10}$ units) should be noted.

The kinetics of the response to the virus subunit is similar to that obtained against intact virus (cf. Figure 18). Untreated VP 40 elicits a primary immune response, peaking around day 25 post inoculation, while bead-bound VP 40 elicits a slowly increasing response with time.

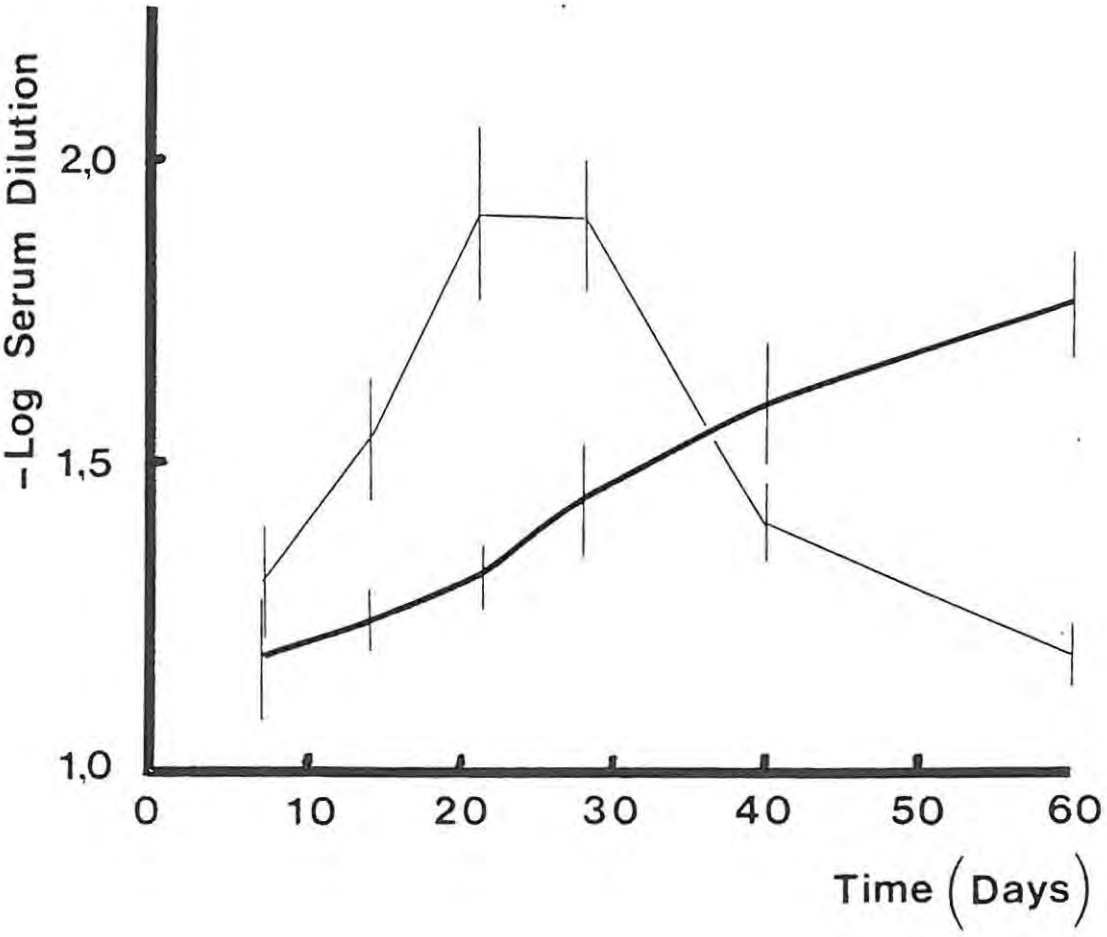


Figure 21: Kinetics of IgG response to Nodamura virus VP 40 prepared by phenol extraction (mean ± standard error; n = 5).

————: VP 40 inoculated in solution

————: VP 40 inoculated in albumin beads cross= linked at 1% final GA concentration.

A titre maximum of 1,9 log units was noted against soluble VP 40 between days 21 and 28 post inoculation.

The response to VP 40 presented from albumin beads gradually increased to over 1,7 $\log_{10}$ units by day 60, surpassing the decreasing primary response to soluble VP 40 by day 36 post inoculation.

8.3.2 Determination of serum neutralising antibodies

All the mice inoculated with virus preparations, according to section 8.2.1.6., developed paralytic symptoms of disease and then became moribund. The control mice which had been inoculated with serum/PBS remained healthy, even by day 10 post inoculation.

This indicated that the phenol-prepared VP 40 released from serum albumin beads either did not elicit virus neutralising antibodies, or only elicited them at a very low level.

8.3.3 Chloride treatment-prepared VP 40 as vaccine antigen

Reduced VP40 immunogenicity, as shown in Figure 22 indicates the need for the empirical determination of the optimal cross-linking of chloride-prepared VP40 into serum albumin beads.

Possibly proteolytic degradation of VP 40 occurred during the chloride treatment of virus or during the VP 40/albumin storage while waiting for the determination of chloride-treated virus inactivation.

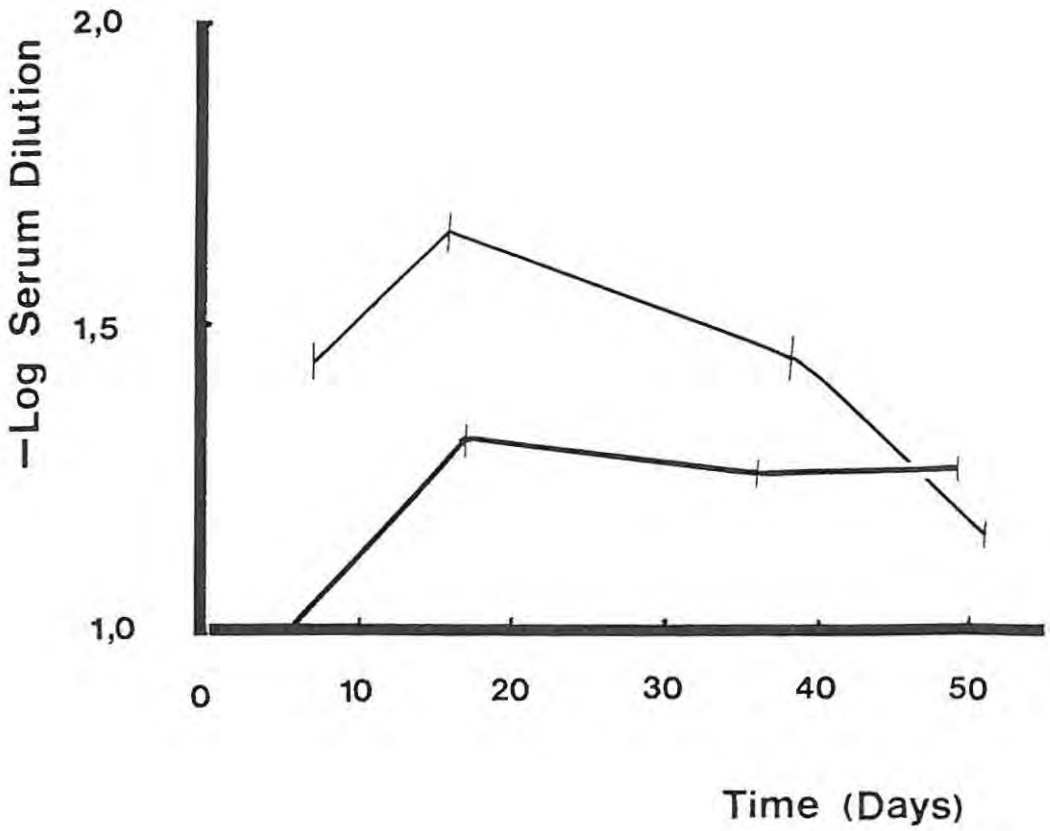


Figure 22: Kinetics of IgG response to Nodamura virus VP 40 prepared by chloride treatment (mean ± standard error; n = 5).

———: VP 40 inoculated in solution

———: VP 40 inoculated in albumin beads cross= linked at 1% final GA concentration.

8.4 DISCUSSION

The release of phenol prepared VP 40 from rabbit serum albumin beads was shown to elicit a humoral response in rabbits similar in kinetics to that elicited against intact virus released from albumin beads.

The VP 40 titre response was much reduced, however, compared to that elicited against intact virus, and was shown not to encompass virus neutralising antibodies. The amount of VP 40 ($\pm$ 20 µg) inoculated into each rabbit might be insufficient for effective immunogenicity.

Loss of antigenic dominant sites resultant from capsid destablization in the preparation of VP 40 could be the contributing factor in the reduction of immunogenicity. Analysis of antigenic determinants, by use of, for instance, monoclonal antibodies (Appleton, J. and Letch= worth, G., 1983; Emini, E. et al., 1983) would elucidate Nodamura virus immunogenicity, relative to it's native quarternary conformation.

An improvement in poorly immunogenic protein could be promoted by the attachment of the protein into heterotypic serum albumin beads. An experiment should be conducted whereby phenol prepared VP 40 is entrapped in bovine serum albumin beads prior to inoculation into rabbits. The bovine albumin could act as an immune system priming agent for a more vigorous response to the attached virus protein. This could lead to an autoimmune response, however.

Antigenic masking of available VP 40 epitopes might also occur when crosslinking the protein into albumin beads with GA at 1% final concentration. An experiment should also be conducted com= paring the humoral response to VP 40 entrapped in albumin beads crosslinked at lower GA final concentration.

Antigenic masking may also result from steric interaction between crosslinked albumin molecules, or peptide lengths thereof, so that intervening occluded virus protein is shielded from interaction with complementary surface receptors on lymphocytes capable of secreting virus specific antibodies.

While virus-neutralising antibodies were not elicited against albumin-entrapped VP 40, the response might be sufficient to prime the host immune system for an immediate response to a subsequent antigenic challenge. For logistical reasons, this possibility was not followed up in this study.

In summary, phenol prepared VP 40 was shown to be poorly immuno= genic when presented from serum albumin beads crosslinked at 1% final GA concentration. The proteolysis of the beads was shown to have an adjuvant effect, leading to an increased humoral response to presented VP 40. Virus neutralising antibodies were not elicited against VP 40.

CONCLUSION

These preliminary studies indicated that polymerised serum albumin beads have an adjuvant effect when inoculated as a vaccine formulation.

It has been shown that immunogenic structures may be incorporated into serum albumin beads, stabilised by glutaraldehyde crosslinking molecules. The *in vivo* proteolysis of these beads has an adjuvant effect by allowing gradual release of entrapped immunogen for extended immunostimulation.

Experiments show an essential similarity as to resultant immunopotentiation, when Nodamura virus, as immunogen, is presented entrapped in albumin bead form and when virus is presented as an emulsion with Freund's Complete Adjuvant. No adverse side effects were noted following bead inoculation.

It is proposed that the albumin beads, themselves homotypic for the host vaccine and therefore non-immunogenic, act as an "antigen-depot" so that immunogen is maintained *in vivo* for an extended period, prior to being degraded and processed by cells of the immune system.

Beads crosslinked at 1% final GA concentration were shown to allow optimal presentation of viral immunogen. Similarly crosslinked beads were shown to present virus subunit, VP 40, in such a fashion as to promote a gradually increasing humoral response.

The indirect ELISA method used to monitor the humoral responses in this study was shown to be simple to perform and to give reproducible results.

It was found necessary to determine the antibody titre of individual serum samples (in duplicate) for greatest accuracy as the individual humoral responses in a group of rabbits at a particular time to a particular immunogen, varied significantly.

Automation in the addition of ELISA reagent or test serum dilution to the wells would allow the standardising of times of incubation, leading to an improvement in results.

With longterm experiments being the norm in such studies, the consistent formulation of ELISA reagents is essential. The linking of isolated goat anti(rabbit IgG) to alkaline phosphatase is a case in point. This is the most variable procedure in the production of the ELISA reagents and should be performed so that consistently prepared conjugate is prepared when necessary.

The studies described here involved the use of Nodamura virus as antigen, with the consequences that there has been an emphasis on virus as antigen, to the exclusion of bacteria or their products.

However, this vaccine system could be extended by the entrapping of bacterial proteins such as the M proteins of group A haemolytic strepto= cocci (Fox, E. and Wittner, M., 1966) and bacterial fimbriae (Korhonen, T. and Rhen, M., 1982). Pneumococcal polysaccharide vaccines (Leinonen, M., 1982; Cano, F. et al., 1983) might also be improved following their incorporation in serum albumin beads.

Some dermatological problems associated with bacterial vaccination, such as the formation of keloid scar tissue at the tuberculosis inoculation

site (Sanders, R. and Dickson, M., 1982; De Souza, G. et al., 1983) as well as streptococcal abscesses following DTP immunization (Elsea, W., 1982) might be obviated by the use of this system.

Second generation beads may also be developed for improved vaccines. Albumin beads studded with antigen or molecules such as saponin or muramyl dipeptide could be mixed with beads incorporating antigen to test for improved immunostimulation. Furthermore, multilamellar beads for variable antigen release could be developed.

As an adjunct to this, the study of cells involved in the immune response could perhaps be extended by preparing antigen/bead-containing columns. The percolation of immune serum, containing antigen-specific lymphocytes, through this in vitro "immune system", might allow cellular fractionation or least cellular observation. Perhaps the use of an antigravity column, packed with beads, could be useful here. A comparison of cells entering the column with those leaving the column in the percolated serum could provide information as to their specificity and interaction with antigen.

This study involved the monitoring of the humoral response to virus antigen as presented from the bead formulation, and compared this to the B-cell response to virus antigen in other formulations. Work should be performed to monitor cell-mediated immunity or the T-cell response to the same antigen released from albumin beads. The measurement of lymphokines (Morley, J., et al.1974)as an indication of T-cell stimulation could be useful.

Once both the B-cell and T-cell responses can be routinely monitored so as to give an overall indication of immunostimulation by antigen released from

albumin beads, an animal model should be developed to determine this system's prophylactic capability against commercially important, local viruses, such as Blue-Tongue Virus.

This study has concentrated on the incorporation of virus as antigen into serum albumin beads, as a potential vaccine formulation. This system, however, could be extended by the incorporation of antibodies or other biologically active compounds into beads, so as to facilitate targeting of these molecules onto specific sites or cells in the body.

All these possibilities should be tested so as to realise the potential of incorporating biologically important molecules into serum albumin beads, as an adjunct in the fight against disease.

REFERENCES

Adams, E. Nomograph distributed by California Corporation for Biochemical Research, Los Angeles, California, USA.

Appleton, J.A. and Letchworth, G.J. (1983) Monoclonal antibody analysis of serotype-restricted and unrestricted blue tongue viral antigenic deter= minants. Virology: 124; 286-299.

Arnon, R. (1980) Chemically defined antiviral vaccines. Ann. Rev. Micro= biol.: 34; 593-618.

Avrameas, S. (1969) Coupling of enzyme to proteins with glutaraldehyde. Immunochemistry: 6; 43.

Bailey, L., Newman, J.F.E. and Porterfield, J. (1975) The multiplication of Nodamura virus in insects and mammalian cell cultures. J. Gen. Virol: 26; 15-20.

Barbara, D.J. and Clark, M.F. (1982) A simple indirect ELISA using $F(ab^1)_2$ fragments of immunoglobulin. J. Gen. Virol.: 58; 315-22.

Berson, S. and Yalow, R. (1971) Radioimmunoassay: A status report in immunobiology. (R.A. Good an D.W. Fisher, eds.) Sinauer, Stanford.

Bittle, J.B., Houghten, R.A., Alexander, H., Shinnick, T.M., Sutcliffe, J.G., Lerner, R.A., Rowlands, D.J. and Brown, F. (1982) Protection against FMDV by immunization with a chemically synthesised peptide predicted from the viral nucleotide sequence. Nature: 298; 30-33.

Blackshear, P. (1979) Implantable drug-delivery systems. Sci. Am.: 241 (6); 52-59.

Bomford, R. (1980) The comparative selectivity of adjuvants for humoral and cell-mediated immunity. I Effect on the antibody response to bovine serum albumin and sheep red blood cells of Freund's incomplete and complete adjuvants, alhydrogel, Corynebacterium parvum, Bordetella pertussis, muramyl dipeptide and saponin. Clin. Exp. Imm.: 39(2); 326-334.

Brown, D.A., Allen, C.J. and Bignell, G.N. (1982) The use of a protein A conjugate in an indirect enzyme-linked immunosorbent assay (ELISA) of four closely related Baculoviruses from Spodoptera species. J. Gen. Virol.: 62; 375-378.

Brown, F. (1984) Synthetic viral vaccines. Ann. Rev. Microbiol.: 3; 221-235.

Brownlee, M., Pongor, S. and Cerami, A. (1983) Covalent attachment of soluble proteins by non-enzymatically glycosylated collagen. J. Exp. Med.: 158; 1739-44.

Burns, W., Billups, L. and Notkins, A. (1975) Thymus dependence of viral antigens. Nature (London): 256; 654.

Cano, F.R., Querry, M.V., Tarrant, C.J., Ritchley, M.B. and Schiffmann, G. (1983) Antibody response after immunization with polyvalent pneumococcal vaccine containing 10, 25 or 50 µg of 14 polysaccharide types per dose. Proc. Soc. Exp. Biol. Med.: 173; 579-82.

Carlsson, H. and Lindberg, A. (1978) Application of enzyme immunoassay for diagnosis of bacterial and mycotic infections. Scand. J. Immunol.: 8 (Suppl. 7); 97-110.

Chanock, R.M., Richman, D.D., Murphy, B.R., Spring, S.B., Schnitzer, T.J. and Richardson, L.S. (1975) Current approaches to viral immunoprophylaxis. In: Viral Immunology and Immunopathology. A.L. Notkins (ed). Academic Press, New York.

Chedid, L., Audibert, F., Levrancier, P., Choay, J. and Lederer, E. (1976) Modulation of the immune response by a synthetic adjuvant and analogs. Proc. Natl. Acc. Sci.(USA).: 73; 2472

Clark, B.R. and Engvall, D. (1980) Enzyme-linked immunosorbent assay (ELISA): Theoretical and practical aspects. In: Enzyme Immunoassay. Edward T. Maggio (Ed). CRC Press.

Clark, M.F. and Adams, A.N. (1977) Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol.: 34; 475-483.

Crook, N.E. and Payne, C.C. (1980) Comparison of three methods of ELISA for Baculoviruses. J. Gen. Virol.: 46; 29-37.

De Souza, G.R.M., Sant'anna, C.C., Silva, J.R., Mano, D.B. and Bethlem, N.M. (1983) Intradermal BCG vaccination complications - analysis of 51 cases. Tubercle: 64; 23-7.

Documenta Geigy. (1975) Scientific Tables, Seventh Edition. Diem, K. and Lentner, C. (Eds) Ciba-Geigy Limited, Basle, Switzerland.

Dreesman, G.R., Sanchez, Y. and Ionescu-Matiu, I. (1982) Production of antibody to Hepatitis B surface antigen by single inoculation of uncoupled synthetic HBsAg peptides. Nature: 295; 158-60.

Editorial (1982) Costs and Benefits of Hepatitis B Vaccination. Lancet: 2 (8309); 1195-6.

Ehlers, U. and Paul, H. (1984) Binding of viruses from crude plant extracts to glutaraldehyde-treated plates for indirect ELISA. J. Virol. Methods: 8; 217-224.

Elsea, W.R. (1982) Group A streptococcal abscesses after DTP immunization - George. M.M.W.R.: 31(38); 519-520, 525-6.

Emini, E.A., Jameson, B.A. and Wimmer, E. (1983) Priming for and in= duction of anti-poliovirus neutralizing antibodies by synthetic peptides. Nature: 304; 699-703.

Emini, E.A., Kao, S-Y., Lewis, A.J., Crainic, R and Wimmer, E. (1983) Functional basis of poliovirus neutralization determined with monospecific neutralizing antibodies. J. Virol.: 46; 466-474.

Engvall, E. and Perlmann, P. (1971) Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G. Immunochemistry: 8; 874.

Engvall, E. and Perlmann, P. (1972) Enzyme-linked immunosorbent assay, ELISA. III Quantitation of specific antibodies by enzyme-labelled anti-immunoglobulin in antigen-coated tubes. J. Immunol.: 109 (1); 129-135.

Fahey, J.L. and Terry, E.W. (1979) Ion exchange chromatography and gel-filtration. In: Handbook of Experimental Immunology. Volume 1. Immuno=chemistry. Third Edition. D.M. Weir (Ed), Blackwell Scientific Publica=tions.

Fenichel, G. (1982) Neurological complications of immunization. Ann. Neurol.: 12 (2); 119-28.

Fenner, F. and White, D. (1976) Immunization against viral diseases. In: Medical Virology. Second Edition. Fenner, F. and White, D. (Eds.), Academic Press, Inc., New York.

Fox, E.N. and Wittner, M.K. (1966) Antigenicity of the M proteins of group A hemolytic streptococci. II Antibody response in rabbits to vaccines with oil emulsions and aluminium hydroxide. J. Immunol.: 97 (1); 86-94.

Freedman, R.B. (1979) Cross-linking reagents and membrane organization. Trends Biochem. Sci.: September; 193-197.

Garvey, J., Cremer, N. and Sossdorf, D. (1977) Separation and Preser=vation of Serum. In: Methods in Immunology: A laboratory text for instruc=tion and research. Third Edition. W.A.Benjamin Inc. Publishers.

Gorman, S.P., Scott, E.M. and Russell, A.D. (1980) Antimicrobial activity, uses and mechanism of action of glutaraldehyde. J. Appl. Bacteriol.: 48; 161-90.

Heggie, A.D. (1983) Immunization against infectious diseases. Practice and Problems. Med. Clin. North Am.: 67 (1); 17-38.

Heide, K. and Schwick, H. (1979) Salt fractionation of immunoglobulins. In: Handbook of Experimental Immunology. Volume 1, Immunochemistry. D.M. Weir (Ed). Blackwell Scientific Publications, Oxford.

Herbert, W.J. (1966) Antigenicity of soluble protein in the presence of high levels of antibody: A possible mode of action of the antigen adjuvants. Nature: 210; 747.

Herbert, W.J. (1973) Laboratory animal techniques for immunology. Appendix 3. In: Handbook of Experimental Immunology. Second Edition. D.M. Weir (Ed), Blackwell Scientific Publications, Oxford.

Herbert, W.J. (1978) Mineral-oil adjuvants and the immunization of laboratory animals. In: Handbook of Experimental Immunology. Volume 3. Application of Immunological Techniques. D.M. Weir (Ed), Blackwell Scientific Publications, Oxford.

Higgins, P.J. (1983) Preparation of polyclonal antisera to tumour-associated antigens using agarose-entrapped immune complexes as immuno=gens. Methods Enzymol.: 93; 78-83.

Higuchi, T. (1963) Mechanism of sustained-action medication. Theoretical analysis of rate of release of solid drives dispersed in solid matrices. J. Pharm. Sci.: 52; 1145-1149.

Hopp, T.P. and Woods, K.R. (1981) Prediction of protein antigenic determinants from amino acid sequences. Proc. Natl. Acc. Sci.:78(6);3824-8.

Hordern, J., Leonard, J. and Scraba, D. (1979) Structure of the Mengo virion. VI Spatial relationships of the capsid polypeptides as determined by chemical cross-linking analyses. Virology: 97; 131-140.

Hudson, L. and Hay, F. (1983) p 164. In: Practical Immunology. Second Edition. Blackwell Scientific Publications, Oxford.

Kabat, E. and Mayer, M. (1967) Crystalline serum albumins. In: Experimental Immunochemistry. Second Edition, C.C. Thomas (Publisher).

Kark, J.D. (1982) A Rift Valley Fever trial. I Side effects and serologic response over a six-month follow-up. Am. J. Epidem.: 116 (5); 808-20.

Kew, O.M., Nottay, B.K., Hatch, M.H., Nakano, J.H. and Obijeski, J.F. (1981) Multiple genetic changes can occur in the oral poliovaccines upon replication in humans. J. Gen. Virol.: 56; 337.

Kit, M. and Kit, S. (1983) Viral vaccines. Med. Clin. North Am.: 67 (5); 1129-45.

Kitagawa, T. and Aikawa, T. (1976) Enzyme-coupled immunoassay of insulin using a novel coupling reagent. J. Biochem.: 79; 233.

Kleid, D.G., Yansura, D., Small, B., Dowbenko, D., Moore, P.M., Grubman, M.J., McKercher, P.D., Morgan, D.O., Robertson, B.H. and Bachrach, H.L. Cloned viral protein vaccine for foot and mouth disease: responses in cattle and swine. Science: 214; 1125-8.

Korhonen, T.K. and Rhen, M. (1982) Bacterial fimbriae as vaccines. Ann. Clin. Res.: 14; 272-7.

Kreuter, J. and Liehl, E. (1981) Long term studies of microencapsulated and adsorbed influenza vaccine nanoparticles. J. Pharm. Sciences: 70(4); 368-71.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature: 227; 680-685.

Lane-Petter, W. (1976) The UFAW Handbook on the Care and Management of Laboratory Animals. Fifth Edition, UFAW (Eds), Churchill Livingstone, New York.

Lee, T.K., Sokoloski, T.D. and Royer, G.P. (1981) Serum albumin beads: an injectable, biodegradable system for the sustained release of drugs. Science: 213; 233-5.

Leinikki, P.O. (1982) Vaccination policies for the 80's. Ann. Clin. Res.: 14; 195-8.

Leinonen, M. (1982) Antibody responses against pneumococcal polysaccaride antigens in vaccinated persons. Ann. Clin. Res.: 14; 267-71.

Lerner, R.A., Green, N., Alexander, H., Liu, F-T., Sutcliffe, J.G. and Shinnick, T. Chemically synthesised peptides predicted from the nucleo= tide sequence of the Hepatitis B virus genome elicit antibodies reactive with the native envelope protein of Dane particles. Proc. Natl. Acc. Sci.: 78; 3403-3407.

Lerner, R.A. (1982) Tapping the immunological repertoire to produce antibodies of predetermined specificity. Nature: 299; 592-596.

Lerner, R.A. (1983) Synthetic vaccines. Sci. Am.: 248; 48-56.

Longworth, J.F. (1978) Small isometric virus of invertebrates. Adv. Virus Res.: 23; 103-157.

Lowry, O., Rosebrough, N., Farr, A. and Randall, R. (1951) Protein measurements with the folin phenol reagent. J. Biol. Chem.: 193; 265.

Macket, M., Smith, G.L. and Moss, B. (1982) Vaccinia virus: a select= able eucaryotic cloning and expression vector. P.N.A.S.: 79; 7415.

Madsen, N.P. (1969) Use of toluene/triton X-100 scintillation mixture for counting ^{14}C protein radioactivity. Anal. Biochem.: 29; 542.

Maggio, E. (1980) Homogeneous enzyme immunoassays. In: Enzyme-Immunoassay. Maggio, E. (Ed), CRC Press, Inc.

Mandel, B. (1979) Interaction of viruses with neutralizing antibodies. In: Comprehensive Virology. Fraenkell-Conrat, H. and Wagner, R.R. (Eds), Plenum Press, New York.

Marglin, A. and Merrifield, R. (1970) Chemical synthesis of peptides and proteins. Ann. Rev. Biochem.: 39; 841.

Miller, K. and Michael, A. (1976) Immunopathology of renal extracellular membranes in diabetics: specificity of tubular basement-membrane immuno= fluorescence. Diabetes: 25; 701.

Modern Approaches to Vaccines. Molecular and chemical basis of virus virulence and immunogenicity. R.M. Chanock and Lerner, R.A. (Eds.), Cold Spring Harbour Laboratory, 1984.

Morein, B., Sharp, M., Sundquist, B. and Simons, K. (1983) Protein subunit vaccines of parainfluenza type 3 virus: immunogenic effect in lambs and mice. J. Gen. Virol.: 64; 1557-1569.

Morein, B., Sundquist, B., Hoglund, S., Dalsgaard, K. and Osterhaus, A. (1984) Iscom, a novel structure for antigenic presentation of membrane proteins for enveloped viruses. Nature: 308; 457-60.

Morley, J., Walltencroft, R. and Dumonde, D.C. (1974) The measurement of lymphokines In: Handbook of Experimental Immunology. Second Edition. D.M.Weir (Ed) , Blackwell Scientific Publications, Oxford.

Murphy, F.A., Scherer, W.F., Harrison, A.K., Dunne, H.W. and Gary, J.W. Jr. (1970) Characterization of Nodamura virus, an arthropod transmissable picornavirus. Virology: 40; 1008-1021.

Newman, J.F.E. and Brown, F. (1973) Evidence for a divided genome in Nodamura virus, an arthropod-borne picornavirus. J. Gen. Virol.: 21; 371-384.

Newman, J.F.E., Matthews, T., Omilanowski, D.R., Salerno, T., Kaesberg, P. and Rueckert, R. (1978) In vitro translation of the two RNAs of Nodamura virus, a novel mammalian virus with a divided genome. J. Virol.: 25; 78-85.

Parish, H.J. (1968) Victory with vaccines. The start of immunization. Publishers: E. & S. Livingstone, Ltd.

Peters, K. and Richards, F.M. (1977) Chemical cross-linking: reagents and problems in studies of membrane structure. Ann. Rev. Biochem.: 46; 523-551.

Pettersson, R.F. (1982) Genetically engineered viral vaccines - prospects for the future. Ann. Clin. Res.: 14; 245-52.

Pharmacia Fine Chemicals (1981) Gel Filtration, Theory and Practice.

Potash, L. (1968) Methods in human vaccine preparation. In: Methods in Virology. (K. Maramarosch and H. Kaprowski, eds). Academic Press, New York.

Prince, A.M. (1982) Hepatitis B virus vaccine: A current appraisal of human plasma-derived vaccines. Ann. Clin. Res.: 14; 225-35.

Relyveld, E.H. and Ben-Efraim, S. (1983) Preparation of vaccines by the action of glutaraldehyde on toxins, bacteria, viruses, allergens and cells. Methods Enzymol.: 93; 24-60.

Robinson, D.A. (1982) Polio vaccination - A review of strategies. Trans. R. Soc. Trop. Med. Hyg.: 76(5); 575-81.

Rueckert, R. (1965) Studies on the structure of viruses of the Columbia SK group. Virology: 26; 345-58.

Sabin, A. and Boulger, L. (1973) History of sabin attenuated poliovirus oral live vaccine strains. J. Biol. Standards: 1; 115-8.

Salk, J., Stoeckel, P., Van Wezel, A.L., Lapinleimu, K. and Van Steenis, G. (1982) Antigen content of inactivated poliovirus vaccine for use in a one- or two-dose regimen. Ann. Clin. Res.: 14; 204-212.

Sanders, R. and Dickson, M. (1982) BCG vaccination scars: An avoidable problem? Br. Med. J. (Clin. Res.): 285 (6356); 1679-80.

Scherer, W.F., Verna, J.E. and Richter, G.W. (1968) Nodamura virus, an ether and chloroform-resistant arbovirus from Japan. Am. J. Trop. Med. Hyg.: 17; 120-8.

Shinnick, T., Sutcliffe, J., Green, N. and Lerner, R. (1983) Synthetic peptide immunogens as vaccines. Ann. Rev. Microbiol.: 37; 425-446.

Skelly, J., Howard, C. and Zuckerman, A. (1981) Hepatitis B polypeptide vaccine preparation in micelle form. Nature: 290; 51-4.

Steiner, L. and Lowey, S. (1966) Optical rotatory dispersion studies of rabbit γG-immunoglobulin and its papain fragments. J. Biol. Chem.: 241; 231.

Sutcliffe, J.G., Shinnick, T.M., Green, N. and Lerner, R.A. (1983) Antibodies that react with predetermined sites on proteins. Science: 219; 660-666.

Szmuness, W., Stevens, C.E., Harley, E.J., Zong, E.A., Oleszko, W., William, W.C., Sodvasky, R., Morrison, J.M. and Kellner, A. (1980) Hepatitis B vaccine. Demonstration of efficasy in a controlled clinical trial in a high risk population in the United States. N. Eng. J. Med.: 303; 833-41.

Talmage, D.W. and Dixon, F.J. (1953) The influence of adjuvants on the elimination of soluble protein antigens and the associated antibody response. J. Infec. Dis.: 93; 176.

Taussig, M.J. (1976) The antibody combining site. In: The Immune System. A course on the molecular and cellular basis of immunity. Hobart, M.J. and McConnell, I. (Eds), Blackwell Scientific Publications.

Thibodeau, L., Perrin, P. and Sureau, P. (1984) Rabies immunosomes: candidate for an RNA-free vaccine. In: Modern Approaches to Vaccines. Chanock, R.M. and Lerner, R.A. (Eds), Cold Spring Harbour Laboratory.

Travis, J., Bowen, J. and Tewksbury, D. (1976) Isolation of albumin from whole human plasma and fractionation of albumin-depleted plasma. Biochem. J.: 157; 301-6.

Unanue, E.R. (1978) The regulation of lymphocyte functions by the macro= phage. Imm. Reviews: 40; 227.

Van Regenmortel, M.H.V. and Burckard, J. (1980) Detection of a wide spectrum of tobacco mosaic virus strains by indirect enzyme-linked immunosorbent assays (ELISA). Virology: 106; 327-334.

Van Rooijen, N. and Van Nieuwmegen, R. (1983) Use of liposomes as biodegradable and harmless adjuvants. Methods Enzymol.: 93; 83-95.

Voller, A., Bartlett, A., Bidwell, D.E., Clark, M.F. and Adams, A.N. (1976) The detection of viruses by enzyme-linked immunosorbent assay (ELISA). J. Gen. Virol.: 33; 165-167.

Voller, A., Bidwell, D. and Bartlett, A.(1976) Enzyme immunoassays in diagnostic medicine: Theory and practice. Bull. W.H.O.: 53; 55-65.

White, R.G. (1971) Concepts of the mechanism of action of adjuvants. In: Immunogenicity. Fronteers of Biology. Borek, F. (Ed), Amsterdam.

White, R.G. (1976) The adjuvant effect of microbial products on the immune response. Ann. Rev. Microbiol.: 30; 579-600.

Williams, B. and Wilson, K. (Eds) (1975) A Biologist's Guide to Principles and Techniques of Practical Biochemistry.

Wunner, W.H., Dietzschold, B., Cortis, P.J. and Wiktor, T.J. (1983) Rabies subunit vaccines. J. Gen. Virol.: 64; 1649-1656.