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POLYMERIZED SERUM ALBUMIN BEADS FOR USE AS SLOW-RELEASE ADJUVANTS

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BY

MICHELLE ELIZABETH DENNY MARTIN

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ABSTRACT

Experimental vaccines have been made by covalently bonding virus particles into polymerized rabbit serum albumin beads. Using Nodamura virus as a model antigen, these model vaccines induced specific humoral antibody production, comparable with that achieved using Freund's adjuvants. Virus specific antibodies were also induced when Nodamura virus was covalently attached to the bead surface using different crosslinkers. However, when poliovirus type 2 (Sabin strain) was polymerized into beads, the levels of neutralizing antibodies were insignificant compared with control aqueous vaccines.

The synthetic immunostimulator, muramyl dipeptide, was included with bead vaccines in an attempt to potentiate the immune response. Immunostimulation is achieved by a slow release of antigen coinciding with the gradual breakdown of bead structure.

LIST OF ABBREVIATIONS

AAA	anti-albumin antibodies
BPB	bromophenol blue
BSA	bovine serum albumin
BTV	Blue tongue virus
CDI	1 ethyl-3(3-dimethyl-amino propyl)carbodiimide hydrochloride
CSA	colony stimulating activity
DMSO	dimethylsulphoxide
ELISA	Enzyme-linked immunosorbent assay
EP	endogenous pyrogen
FIA	Freund's incomplete adjuvant
FCA	Freund's complete adjuvant
GA	glutaraldehyde
GPDF	guinea pig distress factor
HA	haemaglutinin
HBV	hepatitis B virus
HBsAg	hepatitis B surface antigen
HSV	herpes simplex virus
i.m.	intramuscular
LAF	lymphocyte activating factor
MBS	m-maleimidobenzoyl-N-hydroxysuccinimide ester
m.p.	melting point
MDP	muramyl dipeptide
MEM	minimal essential media
MHC	major histocompatibility complex
NA	neuraminidase
NSA	non-specific adsorption
PAGE	polyacrylamide gel electrophoresis
PB	0.02M phosphate buffer, pH 7.4
PBS	phosphate buffered saline
PBT	0.02M phosphate buffer, pH 7.4 with 0.1% Tween 20
pI	isoelectric point
PI	protrusion index
PPB	1% peptone in 0.02M phosphate buffer, pH 7.4
PS	penicillin (0.1 mg/ml), streptomycin (0.2 mg/ml)
PSF	penicillin (0.1 mg/ml), streptomycin (0.2 mg/ml), fungisome (0.002 μg/ml)
RES	respiratory endothelial system
RIA	Radioimmunoassay

RSA	rabbit serum albumin
SDS	sodium dodecyl sulphate
SPDP	3(2-pyridyl-dithio)-propionic acid
TCA	trichloroacetic acid
TEMED	N N N N triethylene methyl diamine
V+B	virus admixed with bead vaccine

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CHAPTER 1

INTRODUCTION

The history of vaccination

The first report of protection against a viral disease described the somewhat dangerous practice, in ancient India and China, of "variolation", in which protection against smallpox was obtained by inoculating live organisms from disease pustules. In 1798, Edward Jenner, having observed that milkmaids were immune to smallpox, introduced the concept of vaccination where protection against smallpox infection was conferred by the nonvirulent cowpox. A century later, Louis Pasteur noted that serial passage of the rabies agent in rabbits resulted in a weakening of its virulence in dogs and that the virus retained its immunogenicity even after it was chemically inactivated. He was also responsible for making a vaccine against anthrax using attenuated organisms.

Early understandings of the mechanisms involved in disease prevention were minimal, with the first clues coming from studies by Emile Roux and Alexandre Yersin on diptheria. They discovered that the causative agent was a toxin. Emil von Behring and Shibasabuio Kitasato extended this investigation further and demontrated that normal animals could be passively immunized, with serum from animals resistant to diptheria. They concluded that sera from resistant animals contained a substance, antitoxin.

The current status of vaccines and vaccination

Today, vaccination constitutes one of the most successful means of preventing viral, bacterial and parasitic diseases. Existing vaccines which consist of killed or dead, live attenuated viral agents or

detoxified bacterial toxins, have diminished incidence, mortality and morbidity of a large number of infectious diseases and even completely eradicated some, i.e. smallpox.

Killed or dead virus vaccines are made by inactivating the virus with formaldehyde or an imine. Live virus vaccines are attenuated by passages in cells of an unnatural host which induces mutations that adversely affect viral reproduction in the natural host. There has been a great deal of controversy and discussion on the relative merits of killed and live attenuated vaccines (Lancet, 1977, 1982) or combinations of these for routine immunizations. If the antigen mass is sufficiently large, the amount of humoral antibody formed by the host is similar, whether live or inactivated virus is injected. In normal (immunocompetent) subjects, live viral vaccines strongly stimulate the production of virus-neutralizing antibodies, various types of cytotoxic white blood cells (cytotoxic T cells, antibody dependent cytotoxic T cells, natural killer cells, interferon, and other immune modulators (suppressor T cells, helper T cells). If the vaccinated individual is later infected with a pathogenic viral strain, the vaccine-induced antibodies and cytotoxic cells may significantly decrease the replications of the pathogenic virus at the port of entry and suppress its hematologic dissemination (Kit and Kit, 1983; Ada et al, 1981). Inactivated vaccines stimulate humoral antibodies and effector T cells, although the latter may sometimes even contribute significantly to the immunopathology (Ada et al, 1981).

Both killed and attenuated virus vaccines are effective, inducing humoral antibody production, however the immunity provoked by the

former is usually of shorter duration. In addition, attenuated vaccines induce intestinal immunity and promote herd immunity, which confers immunity to nonvaccinated contacts.

Certain advantages favour attenuated virus vaccines over killed ones; lower doses are required, usually administered orally and therefore no need for trained staff. However, the greatest disadvantage associated with its use is the possibility of reversion to virulence and the reported cases indicate that vaccinee contacts are at greatest risk (Authors cited in Hovi, 1986).

The phenomenon of intramolecular recombination between the RNA of different serotypes has been observed in the picornavirus group with both foot-and-mouth disease virus (FMDV) (King et al, 1982; McCahon et al, 1984) and poliovirus (Tolskaya et al, 1983; Agol et al, 1984; Emini et al, 1984b; Kew and Nottay, 1984; Minor et al, 1986b). This might be cause for concern with attenuated vaccines as ideal conditions for recombination occur. Since there has not been an increase in paralytic cases of poliomyelitis, due to the replacement of monovalent vaccines with the trivalent vaccines, intramolecular recombination is probably not a grave risk (Kew and Nottay, 1984).

Killed vaccines, provided that they have been adequately inactivated, are safer. However, killed vaccines are more expensive, since larger doses are required for immunity and the mode of administration (injection) necessitates trained medical staff. There have also been reports of side effects associated with killed vaccine use, i.e. anaphylaxis or neurological illness.

Live attenuated and killed vaccines can be used in child immunization programmes. Effective protective immunity usually requires booster injections, particularly with multivalent attenuated vaccines, since the phenomenon of interference, where some strains interfere with the replication of others, leads to deficient immunization.

Poliomyelitis has been completely eradicated from Sweden and (Leinikki, 1982) Finland, except for the recent outbreak in 1984 (Hovi, 1986; Hovi _et al_, 1986). Complete eradication of viruses, for which effective vaccines exist such as measles, mumps, etc., is presently only a theoretical possibility (Leinikki, 1982).

Despite the success of some vaccination programmes, there are many problems still associated with parasitic pathogens and with viral vaccine preparations (Arnon and Sela, 1985).

There are difficulties associated with virus vaccine production, some of which are mentioned below. Firstly, large quantities of starting material from natural sources are not available for some viruses, e.g. Hepatitis B virus (HBV) and Herpes Simplex virus (HSV). Secondly, some viruses do not grow successfully, if at all, in tissue culture, e.g. HBV and rotaviruses. Possible biological contamination of the inoculum may be introduced during large scale growth of the virus and labile viruses (e.g. Blue Tongue virus, BTV) present purification difficulties. Problems of oncogenicity and latent infections have to be overcome to make live attenuated vaccines (e.g. HSV) (Kit and Kit, 1983), whilst evolutionary variability reduces the efficacy of some vaccines, e.g. influenza.

There are also problems connected with the manufacture and application

of viral vaccines. Special containment facilities are required for growing and handling the large quantities of virus required for vaccine production. Elaborate safety testing, careful screening and rigid sterility controls form a crucial and costly part of the production process of vaccines. Viral lability limits its shelf life necessitating expensive storage and refrigeration facilities which, in turn, present difficulties under field conditions. The maintenance of the cold-chain limits the availability of vaccination to isolated and undeveloped regions and breaks in the cold-chain are responsible for ineffective vaccination leading to immunity deficits and subsequent epidemics. Other problems include the need for boosters and the limited number of antigenic determinants that can be administered at once. For example, protection against BTV which has 24 serotypes, of which 17 are prevalent in South Africa, would require several primary vaccinations. Immunization under these circumstances is time consuming, protracted over an extended period of time and extremely expensive. The duration of immunity is not necessarily uniform. It may be lifelong (measles, mumps, chicken pox) or fade with time (syphilis). Generally, killed vaccines fail to promote lasting immunity in the absence of subsequent exposure to infection since they do not induce cell mediated immunity.

New viral diseases (i.e. Aquired immune deficiency syndrome AIDS), variations of existing ones (influenza)·are replacing those whose incidence has diminished through vaccination and the many problems associated with the eradication of existing diseases have shown that the continuous development of vaccines is imperative.

New vaccines

Various strategies are being followed to supplement or replace the conventional methods of preparing vaccines. These include passive immunization with monoclonal antibodies, experimental attenuation (mutation and deletion of genes, reassortment of genes and selection with monoclonal antibodies), subunit vaccines and idiotype vaccines (Verwoerd, 1984).

Subunit vaccines

Because of the concern for the safety of inadequately inactivated or attenuated vaccines, attention has turned away from using the whole virion, toward designing and producing subunit vaccines, consisting of nonviable and noninfectious portions of the pathogenic agent that are capable of eliciting a protective immune response.

Genetic manipulation has generated one of the major breakthroughs in vaccine development and has far-reaching applications. Techniques of genetic recombination affect, on the one hand, the manufacture of essential vaccine epitopes and on the other hand, the construction of biological vectors for vaccination. The whole protein or the polypeptide moiety containing the major neutralizing epitope can be synthesized by cloning the appropriate DNA sequence into prokaryotes, yeast cells and mammalian cells. The immunogenic capsid protein VP1 of FMDV was successfully produced as a stable chimer using a plasmid/*E. Coli* system. This fusion protein elicited high levels of neutralizing antibody and protection against challenges with FMDV (Kleid *et al*, 1981). Mammalian cells constitute the most viable system since proteins are glycosylated, secreted from the cells in the correct conformation and are therefore easily purified, i.e. Hepatitis

B surface antigen (HBsAg). The only problem associated with cloning in mammalian cells is that all the successful recombinants have contained at least fragments of the transforming virus.

Vaccinia virus has received much attention as a viral vector (for review see Mackett and Smith, 1986). Being noninfectious and possessing nonessential DNA, which can be replaced with the gene of interest, makes it an attractive candidate. The use of vaccinia as a vector, for genes encoding proteins of pathogens, has already proved to be effective in the resistance to infections with rabies virus, influenza virus and HSV (Quinnan, 1985 in UytdeHaag et al, 1986). High antibody levels against HBsAg (Smith et al, 1983a) and influenza virus haemagglutinin (HA) protein (Smith et al, 1983b) have been induced using vaccinia vectors. Promotors and enhancers can be incorporated into the viral DNA to improve the efficacy of the protein antigen production.

Vaccinia virus, recombinant vaccines are characterized by low cost, ease of production, ease of administration and a high degree of stability. Long lasting immunity can be achieved by a single injection. However, Anderson and May (1985) described the concern associated with the use of vaccinia vaccines due to known adverse reactions to the virus itself; abnormal skin eruptions, disorders affecting the central nervous system and a variety of other lesser complications.

One of the disadvantages associated with genetically engineered vaccines, derived from cells or microorganisms, is that they are contaminated with antigenic determinants derived from elsewhere in the

virion or host cell (Kit and Kit, 1983). These contaminants may be responsible for autoimmune or hypersensitivity reactions and must be removed by difficult and expensive purification procedures (Kit and Kit, 1983). On the other hand, synthetic peptides are chemically well defined and homogenous.

Synthetic peptide vaccines

With the finding that chemically synthesized short peptides are capable of eliciting antibodies that react with the native protein (Arnon et al, 1971; Lerner et al, 1981; Baron and Baltimore, 1982; Lerner, 1983), attention was focussed on the production of synthetic peptide vaccines which would, hopefully, elicit antibodies capable of neutralizing viral infectivity.

The antigenicity of a protein resides in regions known as antigenic determinants or epitopes. Paratopes are those regions on the antibody which recognize and bind to epitopes (Figure 1.1).

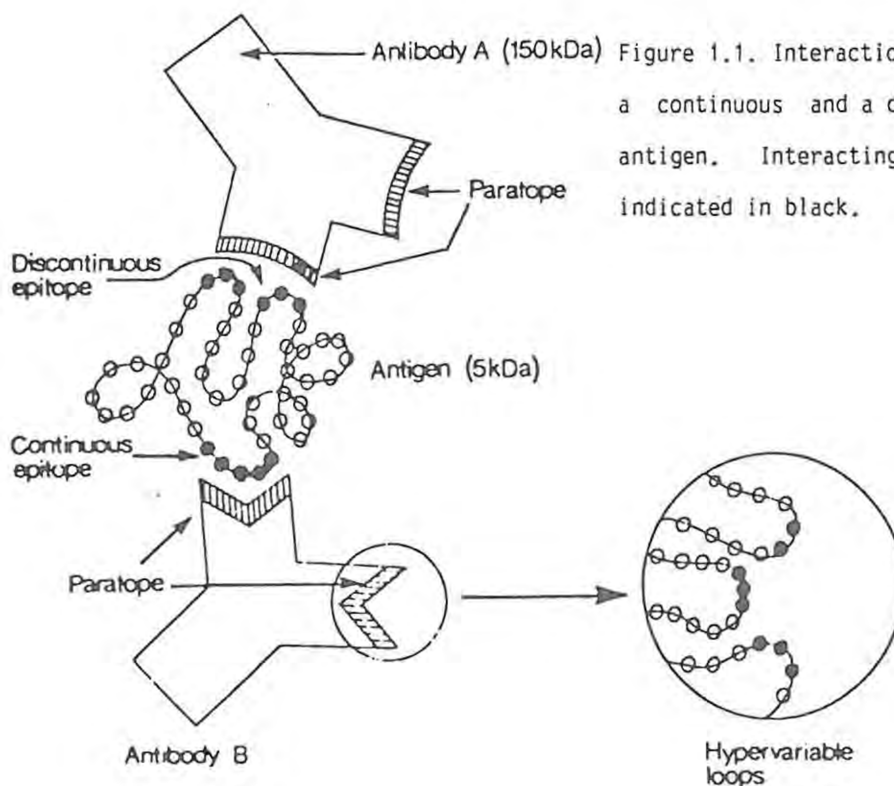


Figure 1.1. Interaction of the two antibodies with a continuous and a discontinuous epitope of an antigen. Interacting amino acid residues are indicated in black.

Epitopes can be defined either in terms of the nature of the amino acid sequence i.e. continuous or discontinous, or in terms of the epitope structure i.e. sequential or conformational. Sequential epitopes are defined as peptides that are specifically recognized in their unfolded random coil form, whereas conformational epitopes require specific conformations for binding to their paratopes. Continuous epitopes are defined as linear sequences of residues and discontinuous (or assembled) epitopes are made up of residues that are not contiguous in sequence but are brought together at the protein surface by the folding of the polypeptide chain (for review; Van Regenmortel, 1986).

The most popular view concerning the number of epitopes in a protein is that the entire, accessible surface of a protein is a continuum of overlapping epitopes that are potentially able to combine with appropriate paratopes on antibody molecules (Benjamin _et_ _al_, 1984 cited in Van Regenmortel, 1986). Most epitopes are likely to be discontinuous.

Recombinant DNA technology has made the synthetic peptide approach more feasible and practical since a prerequisite is the detailed knowledge of structure or at least the amino acid sequence of relevant antigenic proteins (Arnon and Sela, 1985). A protein has a large number of possible antigenic determinants of which a limited number are important for immunogenicity and even fewer for neutralizing immunity (Arnon and Sela, 1985).

Major antigenic regions can be identified by

1) fragmenting the pure protein and screening each fragment for

immunologic capacity,

2) use of monoclonal antibodies,
3) predicting potential determinants from crystallographic studies of the protein's tridimensional structure,
4) predicting the most feasible antigenic site,
5) evolutionary variability of the virus.

Several parameters have been described for predicting the major antigenic site from the primary sequence of a protein. These include: hydrophilicity (Hopp and Woods, 1981; Kyte and Doolittle, 1982), hydropathy (Kyte and Doolittle, 1982), solvent accessibility, mobility, peptide flexibility (Westhof *et al*, 1984; Kit and Kit, 1983; Tainer *et al*, 1984), protrusion index (Thornton *et al*, 1986) and chain termini (70% surface located) (Thornton and Sibanda, 1983).

Since antibodies bind to sites on the surface of the protein, characteristics associated with surface features will obviously have some predictive value.

Hydrophilic amino acid residues tend to be surface orientated rather than buried in the molecule. The point of highest local average hydrophilicity is invariably located in or immediately adjacent to an antigenic determinant. By assigning hydrophilic values to amino acid residues, averaging them over hexapeptides, optimal predictive results for antigenic sites have been obtained (Hopp and Woods, 1981). This rule is not absolute since not all antigenic determinants are associated with high points of hydrophilicity and not all maxima are associated with antigenic determinants. The correlation between hydrophilicity and antigenicity reflects the general correlation

between surface accessibility and antigenicity (cited in Van Regenmortel, 1986).

Terminal residues of proteins are predominantly surface orientated and N- and C- termini frequently possess antigenic activity (Thornton and Sibanda, 1983). Chain termini are also less restrained and more flexible than other regions of a polypeptide. Segmental mobility along the polypeptide chain has been correlated with antigenicity (Westhof et al, 1984; Tainer et al, 1984). Tainer et al (1984) examined the antigenic recognition of antibodies prepared against peptides from highly mobile or well-ordered regions of solvent accessible molecular surfaces of the protein, myohaemerythrin. The mobility of regions was determined from X-ray crystallographic temperature factors. The correlation between the mobility of sites in a protein and their reactivity with antipeptide antibodies suggests that molecular mobility, but not complete local disorder as suggested by Niman et al (1983) (cited in Van Regenmortel, 1986), is an essential part of the antigenic recognition process (Tainer et al, 1984). Westhof et al (1984), in recent studies showed that existing antibodies bind to regions of high mobility in tobacco mosaic virus coat protein, lysozyme and myoglobin. The correlation between mobility and antigenicity is not based solely on high surface accessibility (Tainer et al, 1984). The location of an epitope in a mobile region is advantageous because it makes it easier for the epitope to adjust to a preexisting paratope (on the antibody) and thus find a suitable antibody partner (Tainer et al, 1984). Antibodies act as a "sink", trapping particular, local conformations in mobile areas of proteins.

Mobility cannot be used exclusively since antibodies also recognize rigid, protein structures (Van Regenmortel, 1986). Mobility has little predictive value for antigenicity because mobility patterns are only known for a few proteins. However, it has been suggested that the flexibility of a protein could be predicted from its primary structure (Karplus and Schulz, 1985, cited by Van Regenmortel, 1986). The relative importance of flexibility versus antibody accessiblity has not been resolved and needs more well-defined, immunological and crystallographic data.

Recent evidence suggests that the protrusion index of the α-carbon coordinates of proteins has a better predictive value than hydrophilicity plots and is comparable with mobility (Thornton et al, 1986). Thornton et al (1986) showed that there is a marked correlation between high protrusion index (PI) values (PI $>$ 5) and the location of antigenic peptides. This method appears superior because most active peptides protrude furthest and cold nonactive peptides are least protrusive, although they may be accessible to the solvent as helical regions. The advantage of the α-carbon plots is that they highlight segments of the chain which protrude, regardless of the side-chain conformation, which will almost certainly be flexible in solution. Although PI, mobility, and accessibility have had broadly comparable success rates, none is definitive. At least one epitope in these and other methods (hydrophilicity) is not recognized (Thornton et al, 1986). In general, though not always, peptide determinants which protrude are hydrophilic with higher than average accessibility and are mobile (Thornton et al, 1986).

Although the synthetic peptide vaccine approach has been attempted with numerous pathogens, little success has been achieved; MS-2 coliphage (Langbeheim _et al_, 1976), FMDV (Bittle _et al_ ,1982, 1984), poliovirus (Emini _et al_, 1983b, 1984a,b; Chow _et al_, 1985; Ferguson _et al_, 1985) and cholera (Jacob _et al_, 1984).

Synthetic peptides can be most easily applied to sequential/continuous epitopes. However, since synthetic peptides are short and flexible, their use for inducing antibodies against the conformational epitopes on the relatively rigid protein surface presents more of a problem. Thus, there is a need for sophisticated mimicking of conformational determinants if practical immunogenicity is to be attained (Kit and Kit, 1983). The importance of length and conformation of the synthetic peptides for their immunological reactivity must be ascertained. It has been proposed that the reason why polypeptides are only weakly immunogenic is because they poorly mimic the tertiary structure of the native protein. In oligopeptides, the amino acids are free to rotate about the peptide bond with few restraints, whereas in the native protein there are severe steric restrictions. The synthetic peptide, in solution, exists in equilibrium with all possible conformations, with only some corresponding to those present in the native molecule (Kit and Kit, 1983).

Although some reports indicate that it is possible to immunize animals with 6 to 35 residue-long peptides without conjugating them to macromolecular carriers (Lerner _et al_, 1981; Atassi and Young, 1985), the usual method of raising anti-peptide antibodies is to immunize animals with peptide-carrier conjugates, using proteins such as bovine

serum albumin (BSA) or keyhole limpet haemocyanin. The most commonly used coupling agents are glutaraldehyde (Walter *et al*, 1980), carbodiimides (Goodfriend *et al*, 1964) and m-maleimidobenzoyl-N-hydroxy-succinimide ester (Sutcliffe *et al*, 1980).

These crosslinkers require specific reactive groups on the peptide and there is always a chance of modifying amino acids at the antigenic site. The production of coupling-agent-modified-residue (CAMR) antibodies, which react with neither the carrier nor the peptide (Briand *et al*, 1985), is a disadvantage associated with synthetic peptides.

To overcome the problems associated with conjugation, a universal strategy using photoaffinity, cross-linking reagents that are incorporated directly onto the peptide during synthesis, has been developed (Parker *et al*, 1984). No modification of any amino acids on the antigenic site occurs with this strategy. The benzophenone moiety (photo affinity crosslinker) has the additional advantage of being carrier-independent. This strategy has been successfully utilized to produce synthetic peptide HSV type I and II glycoprotein vaccines (Parker *et al*, 1984).

However, although the peptides' ability to elicit neutralizing antibodies may be weak or absent, they may be potent components of new vaccination programmes against infectious diseases.

Priming with synthetic peptides could be used to activate an immune response directed against neutralizing antigens that are less immunogenic than others. This may be of great advantage in viral or bacterial systems where the immunopotent neutralizing antigens undergo

rapid genetic changes leading to antigenic drifts or shifts (Emini *et al*, 1984b).

The future applicability of synthetic peptides, for vaccination, will depend on the development of new methodologies aimed at the improvement of their immunogenicity. The emphasis lies in the development of potent and safe immunogenic adjuvants.

Intertypic recombinant vaccines

Although picornaviruses are capable of recombination *in vivo* and, coinfection of cells with viruses carrying selectable markers has been used to produce recombinants of FMDV (King *et al*, 1982; McCahon *et al*, 1984) and poliovirus (Tolskaya *et al*, 1983; Agol *et al*, 1984; Emini *et al*, 1984c), these recombinants have been of little practical use. Variation in recombination frequency means that the location of markers is inaccurate and, because the crossover point cannot be accurately controlled, it is difficult to produce specific recombinants (Cooper, 1977). Recombination seems to occur near the middle of the genome and the absence of a range of crossover points suggests that this method is of limited usefulness (Agol *et al*, 1984; Emini *et al*, 1984c). It has been found that cDNA copy of poliovirus RNA, in recombinant DNA in cells, is infectious (Racaniello and Baltimore, 1981), and this has had a profound influence leading to production of specific recombinants.

To this end, one might produce intertypic recombinants between the three poliovirus serotypes, as they may be of use for further studies on antigenicity and may also have potential as novel poliovirus vaccines.

The trivalent oral poliovirus vaccine consists of the attenuated strains of the three serotypes. Many mutations at loci distributed throughout the genome, including the 5' noncoding region in Sabin 1 strain genome, are responsible for the attenuated phenotype. These conditions are compatible with the observation that Sabin 1 virus is a relatively stable vaccine, as multiple mutations would be required to regain the neurovirulent phenotype. In contrast, Types 2 and 3 are genetically unstable, and there is strong evidence that they can revert to neurovirulent phenotypes and produce paralytic disease in vaccine recipients or their contacts (Workers cited in Stanway _et al_, 1986).

There are only 10 nucleotide differences between Leon (Wild type 3) and Sabin 3, accounting for the 3 amino acid differences (Omata _et al_, 1986). Surface parameters, such as antigenicity and immunogenicity, are not main determinants of the attenuated phenotype of Sabin 1 strain. Stable vaccine strains of types 2 and 3 polioviruses might therefore be constructed _in vitro_, by the replacement of the sequence encoding the antigenic determinants of the Sabin 1 genome with the corresponding sequences of the types 2 and 3 genomes respectively (Omata _et al_, 1986). The recombinant would contain regions of type 1 genome conferring the stably attenuated phenotype (Stanway _et al_, 1986; Omata _et al_, 1986). Since not all intertypic poliovirus recombinants are viable, the nonviability may be dependent on detailed interaction of viral protein or RNA or both. This may limit their applicability.

Immunopotentiation

Immune stimulation results from an exceedingly complex interaction between the immunogen and the cells of the immune system. Figure 1.2.

The replication strategy of attenuated viral strains results in a natural immunopotentiation. The cycle of cell infection, promoting a cell mediated and hypersensitivity response, followed by viremic release of progeny virions, stimulating a humoral response, virtually negates the inclusion of an adjuvant in the vaccine formulation.

Adjuvants

Inactivated and subunit vaccines usually require an adjuvant to promote interaction between antigens 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 increase in both the level of circulating antibodies and cell mediated immunity, or to more effective protective immunity (White, 1976).

Adjuvants, presently available, fall into three categories:
(i) "antigen" depot adjuvants which include Freund's incomplete (FIA) and complete adjuvants (FCA), and alhydrogels which consist of aluminium or calcium salts; (ii) bacterial adjuvants; Corynebacterium parvum, Bordetella pertussis and muramyl dipeptide (MDP); and (iii) amphipathic and surface active adjuvants, i.e. saponin (Bomford, 1980).

The mechanism of adjuvant action is extremely complex and has been greatly under estimated. Edelman (1980) described a variety of cells influenced by adjuvants, the effect of the route of administration and many other factors contributing to this complexity.

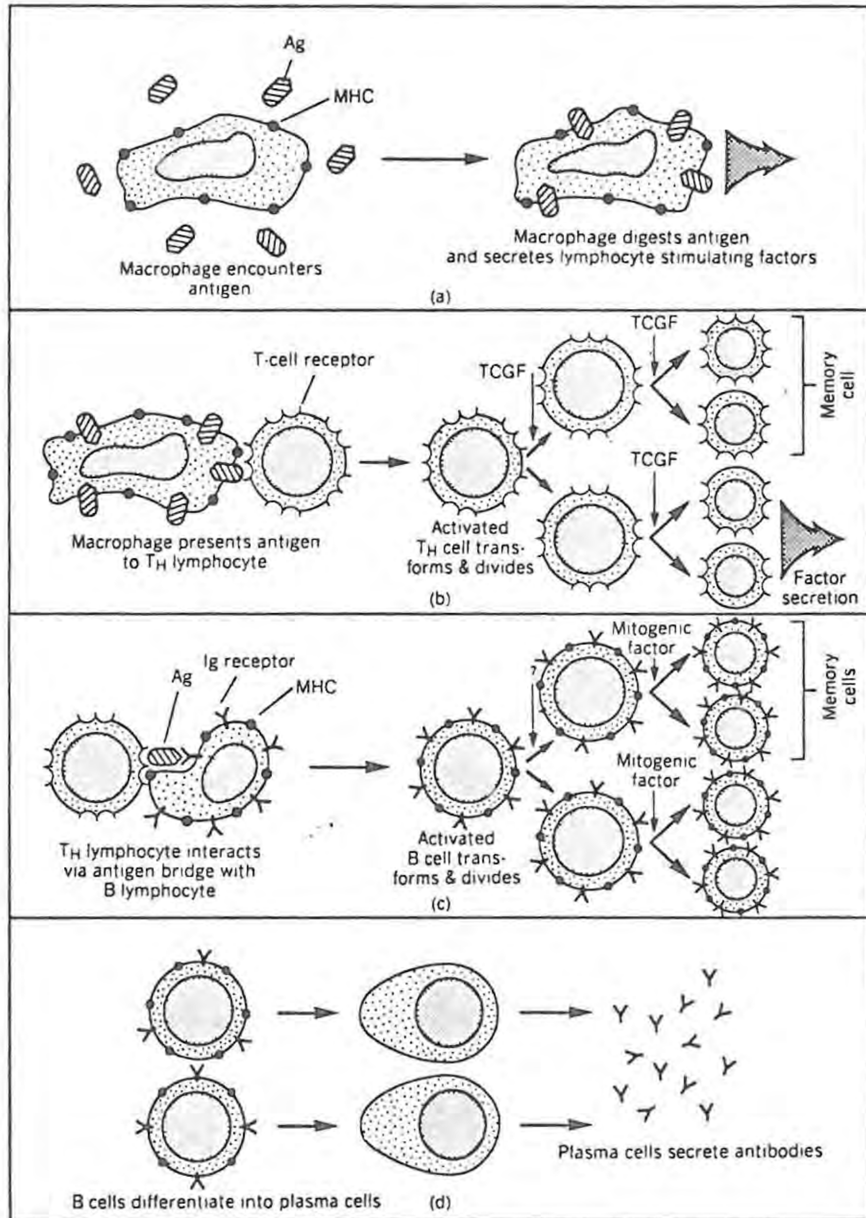


Figure 1.2. Postulated main steps leading to B-cell activation and differentiation into antibody-producing plasma cells. Ag, antigen; MHC, Major Histocompatibility Complex molecule class II; T_H, Helper T-cells; TCGF, T-cell growth factor.

The activity of solid depot adjuvants is partly due to antigen retention in the oil emulsion or hydrogel, resulting in a prolonged release of antigen, and partly due to their local implant nature which attracts cellular constituents of the immune system to the antigen.

MDP and the mycobacteria in FCA stimulate cells of the monocyte-macrophage lineage. Their exact effect on these cells is unclear but the monokines secreted generally enhance the immune response.

Saponin and quaternary ammonium salts, characterized by long aliphatic chains and nitrogenous groups, are thought to exert an adjuvant activity because of their membrane labilizing effect. Their adjuvanticity may be due to the destabilization of macrophage, plasma and lysosome membranes resulting in the leakage of enzymes from the cell.

An adjuvant's efficacy is often dependent on the antigen with which it is used (Bomford, 1980). For example, FCA, FIA and MDP were all effective with soluble antigens whereas saponin was most effective with particulate antigens.

FIA and FCA are particularly effective adjuvants. They are, however, not sanctioned for human use because the mineral oil base is not degradable and there are side effects associated with their use.

Another possible danger of the use of adjuvants is that in a minority of recipients, they may induce allergic reactions to the vaccines which they contain, especially if antigens are administered twice to boost immunity, or if the recipients are already sensitized to an antigen at the time of its administration. It would be an advantage

if the mode of presentation of antigens, in the adjuvant, were such as to avoid allergic reactions. If adjuvants consist of immunologic materials such as glycolipids or proteins, even in trace amounts, they might themselves induce allergic or even autoallergic responses, through sharing of antigens to those of the recipient or some other mechanism (Allison and Gregoriadis, 1974). In fact, FCA can activate autoimmune B cell clones to induce antibodies to autologous proteins (Paterson, 1966; Sakata and Atassi, 1981; Atassi *et al*, 1982).

There are many side effects associated with the bacterial adjuvants.

Although alhydrogels are widely used in humans as safe adjuvants, they occasionally produce sterile absesses and persistent nodules, particularly if they are injected subcutaneously rather than intramuscularly. They are, however, unlikely to cause harmful immune complexes, since they are non-immunogenic. Aluminium compounds have not always enhanced the antigencity of vaccines. They failed to enhance the protection of vaccines against whooping cough and typhoid fever (Authors cited in Edelman, 1980).

New adjuvants

Therefore, there is a real need to obtain a safe and effective adjuvant for use in human immunization programmes. Such an adjuvant could reduce the amounts of antigen required for immunization, with corresponding economies, especially relevant to the developing countries.

The designing of artificial vaccines has been in the forefront of vaccine research. The use of MDP as an immunostimulator will be

discussed in Chapter 4.

The synthetic, double-stranded, polyribonucleotide complexes, with adjuvant activity, include polyadenylic-polyuridylic acid (polyA:U), polyriboinosinic-polyribocytidylic acid (polyI:C), and polyI:C stabilized with poly-L-lysine and carboxymethylcellulose (polyICLC). The synthetic, RNA complexes enhance the antigenicity of inactivated viral vaccines and other biological activities associated with their use include, the induction of interferon and enhanced resistance to infectious agents and tumors in animals (Studies cited by Edelman, 1980).

A novel model of vaccines was described by Petrov et al (1985). Basically, it entails the enhancement of the immunostimulatory and protective activities of viral antigens in the form of virogates. Virogates are defined as conjugates consisting of viral antigens coupled to polyelectrolytes. The nontoxic, polyelectrolyte used in their study, as an immunostimulatory carrier polymer, was synthesized by radical copolymerization of acrylic acid with N-vinyl pyrrolidone. Petrov et al (1985) coupled influenza viral surface antigens, HA and HA+ NA, to the polyelectrolyte. The resultant virogates, with built-in adjuvanticity, stimulated a highly specific, intensive and thymus-independent immune response. The response was protective, as longlived antigen-specific memory cells necessary for the pronounced secondary response to challenge, were induced.

Other adjuvant active compounds, which induce cell mediated immunity and antibody formation, include the water-soluble derivatives of chitin. It was found that the immunological activity of the chemically

modified chitin is closely correlated to the degree of deacetylation (Nishimura et al, 1985).

The need for ongoing research towards developing new and improved vaccines has led to many innovative strategies which include liposomes (Allison and Gregoriadis, 1974; van Rooijen and van Nieuwmegen, 1979, 1980, 1983), idiotypic vaccines (Nisonoff and Lamoyi, 1981) and the use of major histocompatibility complex (MHC) (Sanderson, 1984) for improving the immune response.

Liposomes

Liposomes are artificially prepared spheres, of concentric phospholipid bilayers, separated by aqueous compartments. They form when water-insoluble, phospholipids are confronted with water. The hydrophilic groups orientate themselves in contact with water and with centre of the liposome, and the internalized hydrophobic groups are located directly opposite each other in the inner part of the bilayer (Figure 1.3).

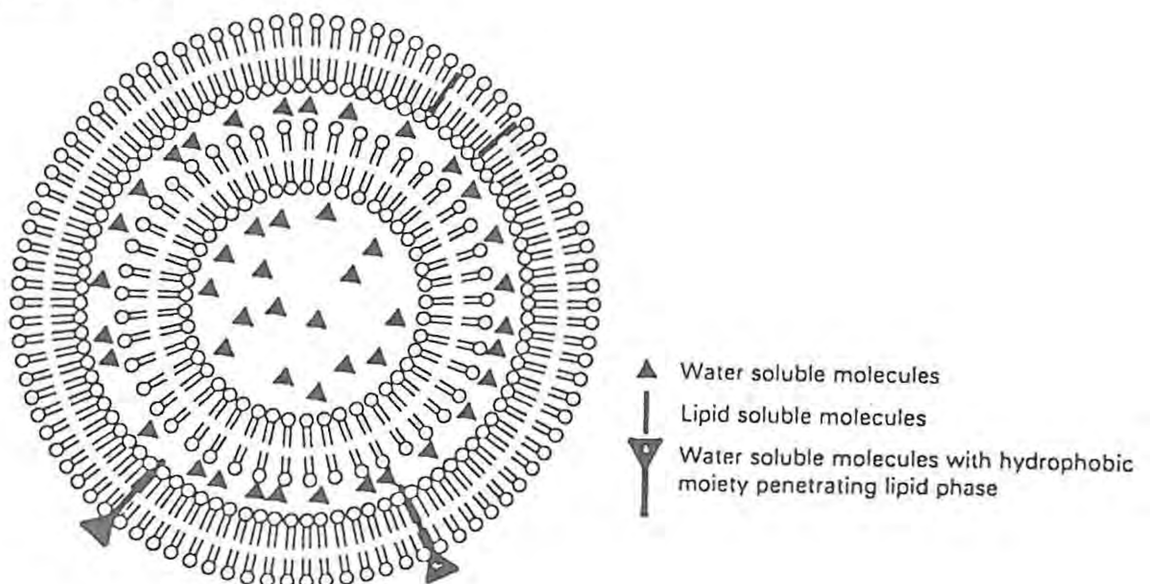


Figure 1.3. A model liposome with incorporated water and lipid-soluble molecules. These molecules can represent either antigens or synthetic adjuvants. The concentric circles represent phospholipid bilayers of the liposome.

Liposomes have been used as adjuvants in immune reactions (van Rooijen and van Nieuwmegen, 1980, 1983; Allison and Gregoriadis, 1974), inducing both a humoral immune response (van Rooijen and van Nieuwmegen, 1980; Allison and Gregoriadis, 1974) and cell mediated immunity (Lawman et al, 1981). Liposome vaccine formulations, containing virus subunits, were as effective as antigen in FCA and far superior to aqueous virus subunit vaccines (Edelman et al, 1980).

The adjuvant effect of liposomes is primarily dependent on antigen presentation. Antigen may be either exposed on the surface of liposomes or trapped within the liposome. Depending on the antigen to be used, several methods can be followed to produce liposomes with surface-exposed antigen. Antigen can be coated nonspecifically, attached covalently or bound via a receptor inserted in the phospholipid bilayer. Multilamellar, phosphatidylcholine liposomes are non-immunogenic, biodegradable, harmless, easily obtainable immunoadjuvants and may be used for intravenous administration. Their composition can be varied to give the most efficient antigen-liposome preparation. They also present a valuable, protective means for presenting potentially harmful antigens and/or immunostimulators (Fidler et al, 1985), if administered intravenously or in high concentrations to the immune system (Allison and Gregoriadis, 1974).

Idiotypic vaccines

Idiotypic vaccines were initially proposed by Nisonoff and Lamoyi (1981), and were based on Jerne's idiotypic cascade and internal image theory (Jerne et al, 1982). When the antibody 1 (Ab1), induced by external or internal stimuli (the antigen), is

inoculated into a host, there are 2 types of antiidiotypic antibodies (Ab2) that can be distinguished. These constitute the most promising candidates for idiotype vaccines; Ab2α defining interspecies, recurrent idiotypes Ab1 idiotype-bearing antibody or Ab2β constituting internal images of the antigen and mimicking antigenic structures (Nisonoff and Lamoyi, 1981; UytdeHaag et al, 1986).

The paratope of Ab2α antiidiotypic antibody reacts with an idiotope associated with the framework or paratope of Ab1. Ab2β antiidiotypic antibody binds, like the antigen with its epitope, through its idiotype to the paratope of Ab1. Consequently it can replace antigen in inducing antigen specific responses.

UytdeHaag et al (1986) evaluated the potential of monoclonal antiidiotype antibodies (MoAb2α) against either private, or recurrent idiotypes as vaccines. Briefly, the immune response elicited by injection of single MoAb2α, against either private (feline leukaemia virus as a model) or interstrain recurrent (rabies as a model) idiotopes, was significantly inferior to responses generated by viral antigens. Improvement in immune responses may be achieved by the sequential immunization with different MoAb2α in these systems.

In contrast, MoAb2β, defining a paratope-related idiotope of poliovirus type II neutralizing MoAb1 , seemed to behave according to the internal image (Ab2β) concept. MoAb2β reacted with hyperimmune sera from different animals and elicited neutralizing antibody in mice. It did not, however, confer protective immunity to a lethal viral challenge. This may be because internal images per se are poor immunogens and linking them to an immunogenic carrier might result in

the induction of protective immunity as found with other systems (McNamara et al, 1984). As structural data on Ab2β is not available, it is not possible to say whether antigen-specific responses, mediated by Ab2β, reflect real structural homology between Ab2β and epitopes (UydteHaag et al, 1986).

Major Histocompatibility Complex (MHC)

One of the most dramatic, primary immune responses elicited in a host is that associated with tissue graft rejection. There is a massive infiltration of the grafted tissue by T lymphocytes which are initially stimulated by MHC molecules. Therefore, allo-MHC molecules are superimmunodominant. Sanderson (1984) proposed that augmented primary immunogenicity will be conferred on any foreign epitope that is deliberately linked (i.e. prior to administration to an animal) to a mixture of MHC molecules of the species in question. Some of these MHC molecules will be recognized as self-MHC and others as allo-MHC. Together, these will elicit powerful immune signals; some mimicking the animal's normal immune physiology and others reflecting the signals that only occur naturally in pregnancy. Current understanding of immunophysiology suggests that MHC 2 molecules will be more efficacious in promoting immune stimuli, although MHC class 1 molecules (B2-myoglobulin) have been used successfully (Sanderson, 1984). The augmentation of the immune response was found to be superior to that provided by other molecules of similar size and seems promising as a general adjuvant strategy (Sanderson, 1984).

Research objectives

This research project will focus on the development of novel strategies for antigenic presentation and will involve the use of

polymerized serum albumin beads, incorporating the viral antigen, as an antigen depot possessing slow release properties.

This research forms part of an ongoing project developed in the Microbiology Department, Rhodes University. Dewar et al (1984) initiated studies using these slow release bead vaccines. Retention of the antigen and its subsequent release from the degrading beads, presented the antigen to the cells of the immune system for a prolonged period of time. The RSA beads caused no adverse, side reactions such as granulomas or abscesses at the injection site.

It is hoped that certain adjuvant requirements will be fulfilled by the serum albumin beads such as, prolonging the efficacy of immunization, avoiding the need for repeated injections and increasing the antigen's immunogenicity.

By using serum albumin, homotypic to the host, unwanted toxicity and immunological responses associated with the use of other adjuvants (Bomford, 1980; Edelman, 1980) will be avoided.

The efficacy of serum albumin beads, as a slow release depot, will be compared with conventional adjuvants, and the optimal conditions for adjuvant activity determined.

The stability of the bead vaccines will be determined in the hope of developing a vaccine which would remain effective under ambient temperature conditions.

The incorporation of immunostimulators into the beads, to augment their adjuvanticity, will be examined.

This model will be extended to include other viral systems, notably poliovirus and the potential of the bead vaccine model, to enhance the immunogenicity of synthetic peptides, will be discussed.

Finally, although autologous serum albumin, is not antigenic in its host, its treatment with glutaraldehyde might introduce new antigenic determinants which could be recognized by the host's immune system as non-self. It is therefore imperative to establish that, the beads themselves are nonantigenic, before the slow release bead vaccines can be introduced as a general vaccination strategy.

CHAPTER 2

MATERIALS AND METHODS

1.PREPARATION OF VACCINE COMPONENTS

1.1Antigens

1.1.1. Preparation and propagation of Nodamura virus

Nodamura virus, from the group Nodaviridae was used as a model vaccine. This is an unusual, mosiquitoe-borne, nonenveloped ribovirus containing 2 RNA molecules both of which are enclosed in the same icosohedron (diameter of 28 nm) and both are required for infectivity (Newman *et al*, 1978).

This viral system acts as an ideal model since large quantities of virus can be harvested from infected suckling mice, it does not infect humans (Scherer *et al*, 1968) and is stable under normal purification procedures. The virus is however, chloride and iodide labile and the lability is pH dependent. The addition of a protein e.g. 1% bovine serum albumin (BSA) confers stability except at that protein's isoelectric point.

1.1.1.1. Maintenance of mouse colonies

The upkeep and non-inbreeding of the Swiss Albino mice colony was maintained according to Lane-Petter (1976). The mice were caged in a ventillated animal room maintained at $27^{\circ}C$. The mice were checked and their water bottles and feed hoppers cleaned and filled daily. Mating was allowed between two females and a male. Once pregnant, the females were separated and caged alone to litter. Some litters were kept to propagate the colony whilst other offspring were inoculated with a Nodamura virus stock dilution.

1.1.1.2. Propagation of Nodamura virus

Methods for virus propagation were essentially those described by Newman and Brown (1973). The stock virus suspension was prepared from 5 mice hind legs ground using a mortar and pestle with sterile sand in 5ml 0.01M phosphate buffer, pH 7.5. Sand and muscular debris was pelleted by centrifuging for 10 minutes at 7000g. The supernatant was made 50% with glycerol and stored at -22°C. 2-3 day old suckling mice were inoculated intraperitoneally with 0.02 ml virus stock diluted 1:4. Mice were checked daily for hindleg paralysis. Once paralysed and monobound, the mice were sacrificed and stored at -22°C.

1.1.1.3. Isolation of Nodamura virus

Nodamura virus was extracted from the hind limbs of infected 5 day old mice and purified as previously described (Newman and Brown, 1973). Once the mice had thawed, hind limbs and pelvic girdles were dissected out.The tissue was homogenised in 0.01M phosphate buffer, pH 7.6 : carbon tetrachloride (4:1) for one minute in a Sorval Omni-mixer at 0°C. The insoluble debris was removed by centrifugation at 7000g for 10 minutes and reprocessed by grinding in a mortar and pestle with sterile sand. Insoluble debris was removed as described above. Cellular debris was removed by centrifugation at 12000g for 15 minutes. Supernatants were pooled and the virus was pelleted by centrifugation at 150000g (35000 rpm, SW 41 rotor in a Beckman L-2658 ultracentrifuge) for 2 hours. The pellet was resuspended overnight at 4°C in 0.8 ml 0.01M phosphate buffer, pH 7.6. The insoluble debris was removed by centrifugation at 2000g and was washed in 0.2 ml 0.01M phosphate buffer, pH 7.6 and pelleted. The pooled supernatants were made 1% with sodium dodecyl sulphate (SDS) and a 0.5 ml sample was centrifuged for 4 hours at 65000g at 10°C on a 10.4 ml 15-45% (w/v)

linear sucrose gradient in 0.01M phosphate buffer, pH 7.6. 0.6 ml fractions were collected from the top of the gradient using an ISCO (model 640) density fractionator with a flow rate of 3 ml/min. The concentration of the pooled peak fractions of purified virus was determined spectrophotometrically using a Pye Unicam SP 8-400 UV/VIS spectrophotometer and the concentration was calculated. The virus was stored at $4^{o}C$ for use in vaccines or titer determinations using enzyme-linked immunosorbent assay.

Figure 2.1 indicates the concentration of Nodamura virus in fractions 10, 11 and 12 after centrifugation through a 15-45% sucrose gradient. The UV scan (220-320nm) in Figure 2.2 of the virus containing fractions illustrates the purity of the viral preparation.(OD_{280}/OD_{260} = 0.6). The concentration of virus was calculated;

$$\text{conc (mg/ml)} = OD_{280nm} \text{ reading} \times 0.35$$

Approximately 0.1mg purified virus was routinely obtained from each mouse.

1.1.1.4. Radio-Iodination of Nodamura virus

^{125}I-iodine, having a half life of 60 days and a higher specific activity than many other radioactive compounds, allows for easy and rapid detection of picogram amounts of protein.

Chloramine-T, the sodium salt of the N-monochloro derivative of p-toluene sulphonamide, oxidises the $Na^{125}I$ in the presence of the protein to be labelled, with subsequent incorporation of ^{125}I-iodine into the tyrosine residues. Excess Chloramine-T is reduced by the addition of sodium metabisulphite and free iodine reduced to iodide

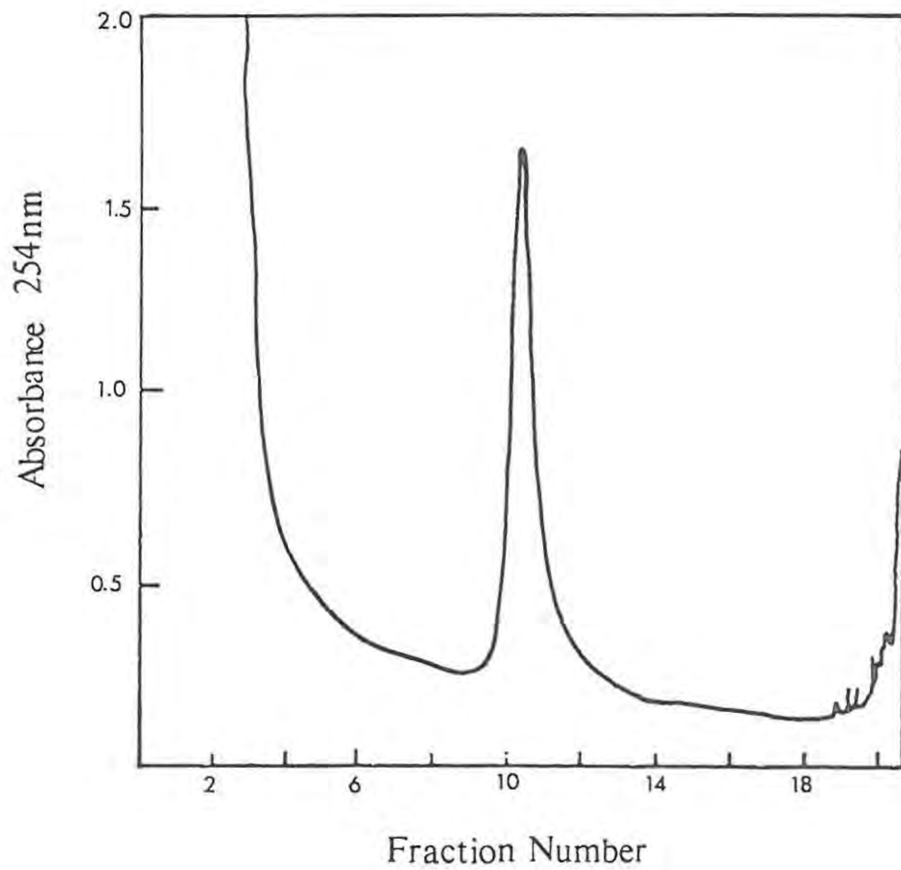


Figure 2.1. Absorbance at 254nm of Nodamura virus purified on 15-45% (w/v) sucrose gradient. Fractionated from the top of the gradient.

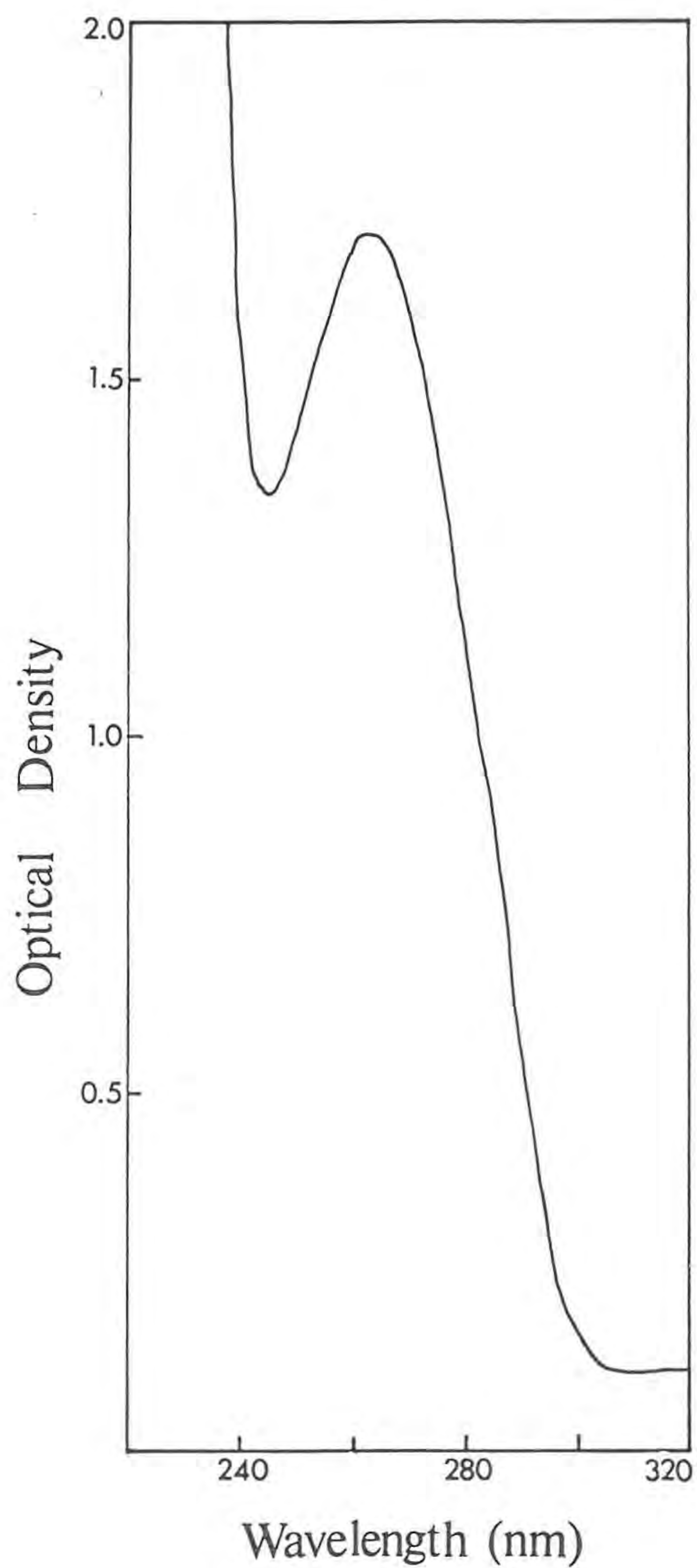


Figure 2.2. U.V. Scan (220-320nm) of a purified Nodamura virus sample.

(by adding potassium iodide).

Some iodine may react with histidine, tryptophan or sulphydryl groups, however tyrosine remains the principal amino acid involved, (Authors cited in Bolton, 1977).

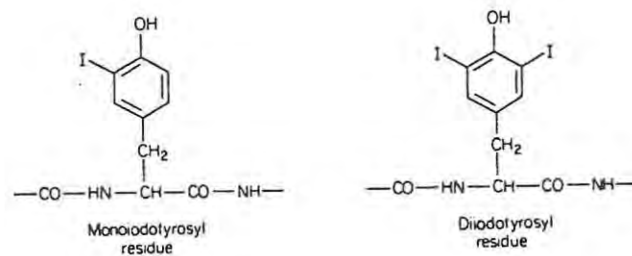


Figure 2.3. Structure of iodinated tyrosyl residues in a polypeptide. Under conditions normally used for iodination the mono-iodinated derivative predominates.

The major coat protein of Nodamura virus, vp40 (molecular weight 40000 daltons) has 180 chains (Newman and Brown, 1973) with 11 tyrosine residues per chain (Newman et al, 1978), therefore a high incorporation of iodine into the major coat protein should be obtained.

1.1.1.4.1. Radioiodination using the Chloramine-T method

The peak fractions containing virus from the sucrose density gradient were diluted up to 12 ml with 0.01M phosphate buffer, pH 7.5 and the virus was pelleted out of the sucrose by centrifugation at 150000g for 2.5 hours. Nodamura virus was labelled with carrier-free $Na^{125}I$ (10 µCi/mmol) (Amersham International plc, Amersham, U.K.) at 1 mCi/1 mg protein by the Chloramine-T method (Greenwood et al, 1963).

The resuspended pellet (40 µl in 0.05M phosphate buffer, pH 7.5) was added to 20 µl ^{125}I-iodine and 20 µl freshly prepared Chloramine-T (1

mg/ml). The solution was mixed well and incubated for 1 hour at room temperature. The reaction was stopped by the addition of 40 μl sodium metabisulphite (100 mg/ml). The effect of reducing the iodine to iodide with cold potassium iodide (1 mg/ml) was checked. Unreacted iodine was removed by dialysis against several changes of 0.01M phosphate buffer, pH 7.4. 3% BSA was added to the dialysing mixture to minimise losses due to adsorption onto the tubing. The sample was centrifuged through a sucrose gradient (1.1.1.3). The position of the virus was observed using oblique illumination and marked. 0.5 ml fractions were collected from the top and the radioactivity determined. 10 μl samples were mixed with 5 ml scintillation cocktail for aqueous solutions (Beckman) and the radioactivity was counted using the ^{14}C window channel of the Beckman LS3150T liquid scintillation counter. The virus concentration was determined spectrophotometrically. The % binding efficiency was determined by trichloroacetic acid (TCA) precipitation.

1.1.1.4.2. Determination of the radioiodination efficiency

Not all the total activity in a radiolabelled sample is covalently bound to the protein because of imperfections in labelling and fractionation procedures. The amount of radioactivity incorporated into the protein was determined by precipitating high molecular weight material (protein) with TCA.

Simply, an aliquot (10 μl) of ^{125}I-protein fraction was counted. 5 ml TCA (w/v) was added to the protein and incubated on ice for 30 minutes. The precipitate was pelleted (5000 rpm for 30 minutes), the supernatant discarded and the process repeated again. The final pellet was counted.

The binding efficiency was calculated;

$$\text{Binding efficiency} = \frac{\text{mean activity after acid precipitation}}{\text{mean activity before acid precipation}} \times 100\%$$

Figure 2.4A shows the position of ^{125}I-labelled Nodamura virus in fractions 11 to 15. The addition of potassium iodide resulted in complete degradation of the virus (Figure 2.4B). The presence of 0.1% BSA in the potassium iodide solution did not protect the virus. This step was not included in further iodinations.

The specific activity of ^{125}I-Nodamura virus was 3.57×10^5 cpm/mg virus. The binding efficiency was 97%.

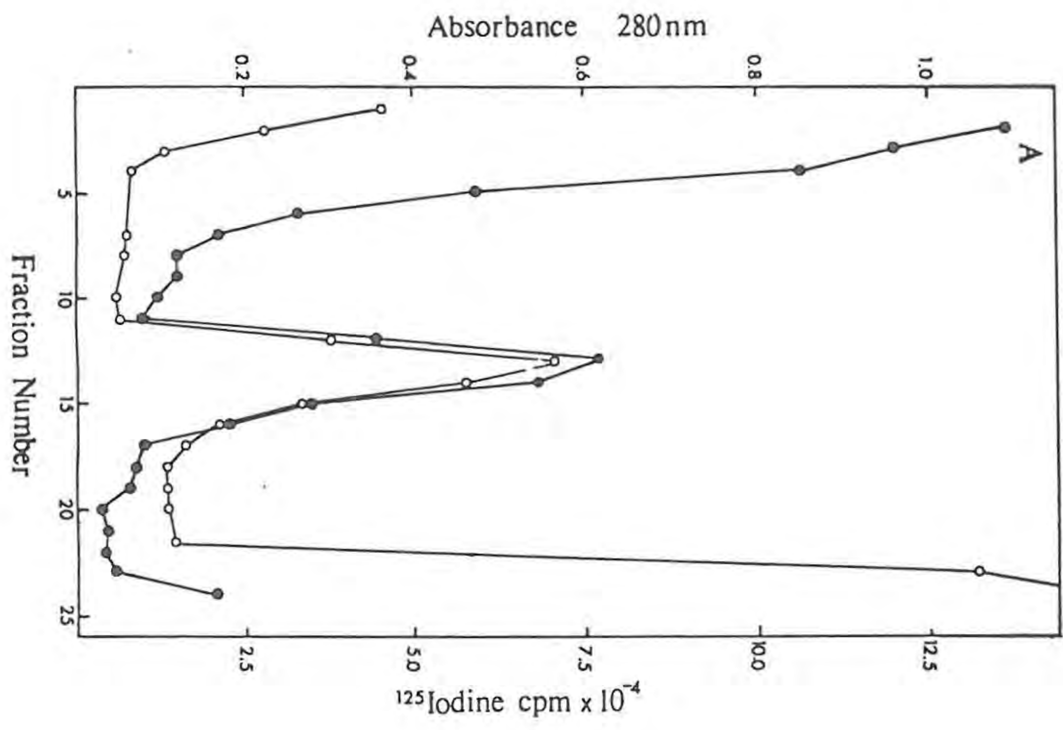
1.1.1.5. Conjugation of Nodamura virus to Muramyl dipeptide (MDP)

The immunostimulator MDP was conjugated to Nodamura virus using 1 ethyl-3(3-dimethyl-amino propyl) carbodiimide hydrochloride (CDI) which does not abolish the adjuvant activity, (Mozes et al, 1980).

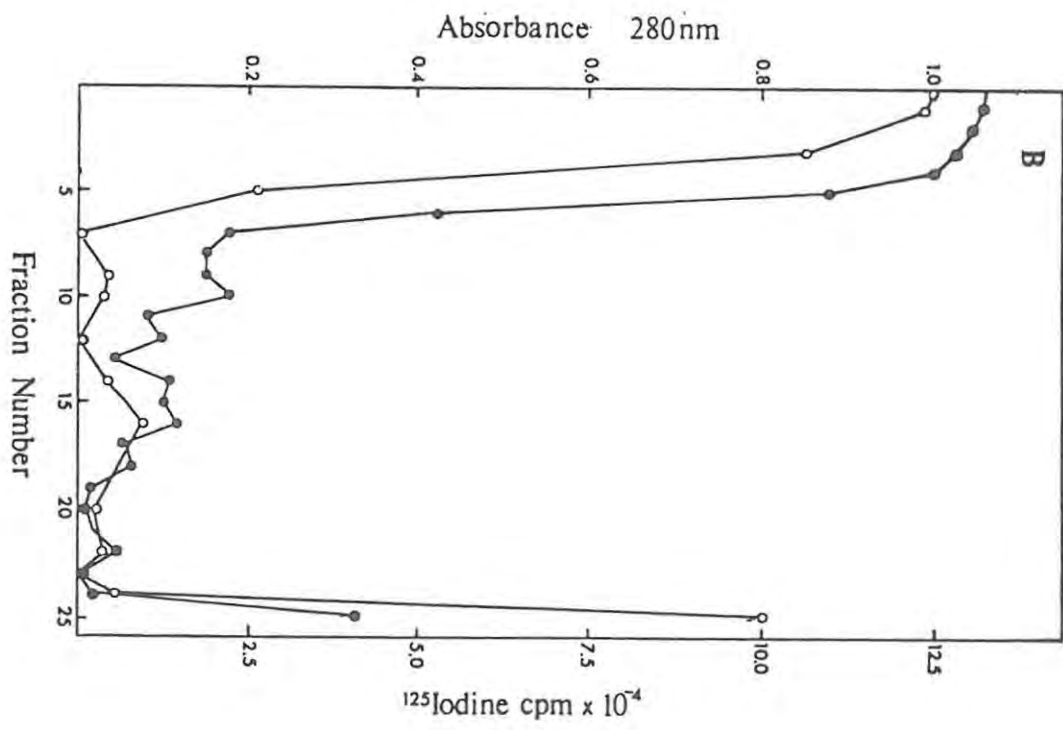
MDP, CDI and N- hydroxybenzotriazole were obtained from SIGMA Chemical Company, St. Louis, MO, U.S.A. Dimethylformamide was obtained from BDH Chemicals Ltd., Poole, England.

112.8 mg (0.229 mmol) MDP in 5 ml dimethylformamide, 38.9 mg (0.229 mmol) N hydroxybenzotriazole catalyst and 97.1 mg (0.229 mmol) CDI were mixed together and incubated for 1 hour at room temperature. Nodamura virus was dialysed extensively against 0.01M phosphate buffer, pH 7.4 to remove sucrose . To 200 µg virus, 1M sodium

Figure 2.4. Absorbance at 280nm of ^{125}I-labelled Nodamura virus on 15-45% (w/v) sucrose density gradient A) Potassium iodide excluded from iodination protocol. B) Potassium iodide (1mg/ml) included in iodination protocol. (Absorbance (○); cpm (●)).



A
Absorbance 280nm
0.2
0.4
0.6
0.8
1.0
Fraction Number
5
10
15
20
25
2.5
5.0
7.5
10.0
12.5
125Iodine cpm x 10-4



B
Absorbance 280nm
0.2
0.4
0.6
0.8
1.0
Fraction Number
5
10
15
20
25
2.5
5.0
7.5
10.0
12.5
125Iodine cpm x 10-4

bicarbonate buffer ($NaHCO_3$) was added until the pH was 8.5. The organic and aqueous solutions were mixed and stirred at room temperature for 24 hours. The sample was pressure dialysed against 1mM phosphate buffer, pH 7.5 containing 0.1% SDS, to remove free MDP and the catalyst and to reduce the volume, which was then further reduced under vacuum.

The MDP-Nodamura virus conjugate was used in polymerized serum albumin bead vaccines.

1.1.2. Propagation and isolation of poliovirus

1.1.2.1. Cells and virus strain

All cells were obtained from the National Institute of Virology. Hela S_3 cells and Vero cells were grown for preparation of large quantities of virus and for plaque assays respectively.

All cells were grown in Eagle's minimal essential media (MEM) (Eagle, 1959) supplemented with 5% fetal calf serum and penicillin (0.1 mg/ml) and streptomycin (0.2mg/ml) (PS). Infected Hela cells were grown in serum-free MEM with Penicillin (0.1 mg/ml), streptomycin (0.2 mg/ml) and fungisome (0.002 μg/ml) (PSF) supplemented with 0.22% bicarbonate, the latter being a requirement for Sabin vaccine virus growth. Vero cells were grown in 75cm^2 Falcon flasks to increase cell numbers prior to seeding the petri dishes for plaque assays. Further manipulations with the Vero cells are described in section 4.3.1.

The poliovirus strain used was Sabin type 2, p712, vaccine strain. This strain was chosen for these experiments because it is produced in high yields.

1.1.2.2. Production of poliovirus stocks

Once a confluent monolayer of Hela cells had formed in 75cm^2 Falcon flasks, the growth medium was discarded and 5 ml serum-free MEM was added. Each flask was inoculated with 0.2 ml virus, leaving one uninfected as a control. The cells were incubated at 37°C for 16 to 24 hours on a shaking platform. Once complete cytopathology had been observed (Zeiss inverted light microscope), the virus was harvested and stored at 4°C.

Stock virus grown in Hela cells was adapted for growth in Vero cells and stocks were frozen to ensure that there was no loss in infectivity (Bey and Golombick, 1984). For plaque assay, suitable dilutions of frozen virus were used either without further passage, or alternatively after two to three passages in monolayers of Vero cells in order to obtain a preparation of high infective titer.

1.1.2.3. Extraction and purification

The MEM, containing poliovirus, was centrifuged at 7000g for 10 minutes to pellet cellular debris. A reduction in virus recovery, due to adsorption onto the cellular debris, was observed if this step was delayed.(Result not shown). The virus was precipitated overnight at 4°C with an equal volume of saturated ammonium sulphate and centrifuged at 8000g for 30 minutes. The pellet was resuspended in 0.01M Tris-HCl buffer, pH 7.5 and centrifuged at 150000g for 2 hours, and the resultant pellet resuspended overnight in 0.01M Tris-HCl, pH 7.5 at 4°C. Insoluble debris was removed by centrifugation at 2000g for 10 minutes. A 10.4 ml linear gradient, 15-45% (w/v) sucrose in 0.01M Tris-HCl, pH 7.5 containing 0.1M NaCl, was prepared in a polycarbonate tube. The virus solution made 1% with SDS, to dissociate the virus from membranes, and was layered carefully onto the gradient

and centrifuged at 65000g for 4 hours.

The gradient was fractionated from the top. Purified virus was isolated in fractions 10, 11 and 12 (Figure 2.5). The concentration was determined using $E^{1\%}_{260nm}=77$. (Reuckert, 1976). Virus containing fractions were pooled and centrifuged at 15000g for 2.5 hours. The pellet was resuspended in 1mM phosphate buffer, pH 7.5, 0.1% SDS for vaccine preparation.

1.2.Rabbit serum albumin

Albumin is the most abundant serum protein, occurring at a concentration of 40-50 mg/ml serum and can even be prepared from out-dated blood. Small quantities of albumin can be isolated from serum by adsorption to Blue-Sepharose, followed by ion exchange chromatography on DEAE-cellulose. The serum-albumin monomer is then isolated after final gel filtration on sephadex G150 (Artursson et al, 1984).

However, when larger quantities are required, salt fractionation methods employing ammonium sulphate can be used, (Adair and Robinson, 1930 cited in Kabat and Mayer, 1961).

1.2.1.Isolation of rabbit serum albumin

The procedure of Adair and Robinson, (1930: cited in Kabat and Mayer, 1961) was used for preparing large quantities of rabbit serum albumin (RSA).

All manipulations were preformed at room temperature unless otherwise stated. Fresh rabbit serum (400 ml) was diluted in an equal volume of distilled water. 50% ammonium sulphate was added slowly with mechanical stirring. The globulin precipitate was removed by

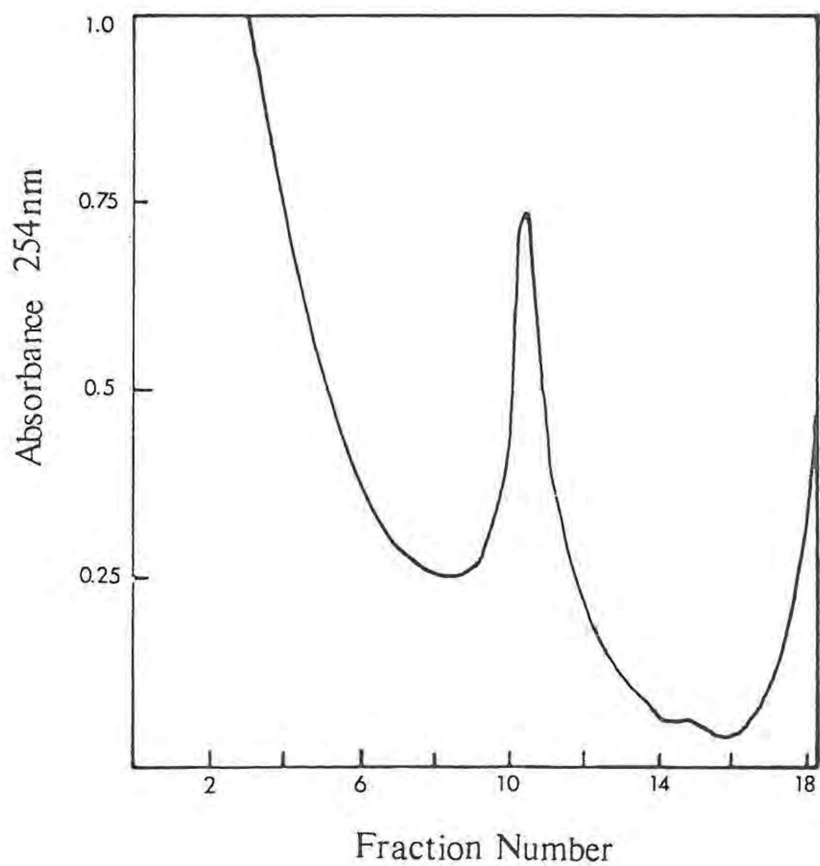


Figure 2.5. Absorbance at 254nm of Poliovirus type 2 (Sabin strain), p712, purified on a 15-45% sucrose density gradient. Fractionated from the top of the gradient.

centrifugation at 8000g for 30 minutes. The supernatant was acidified very slowly by the dropwise addition with mechanical stirring of 1 vol 0.5M acetic acid and 1 vol sat. ammonium sulphate (10% of volume). Crystallisation of RSA usually began during acidification at the final pH 4.9 and was complete after standing overnight. The solution was made 75% with ammonium sulphate crystals. The amount added was determined using an ammonium sulphate nomogram (Dixon, 1953 in Johnstone and Thorpe, 1982). After centrifugation at 8000g for 30 minutes, the pelleted albumin was resuspended in 0.01M ammonium carbonate buffer and dialysed at $4^{O}C$ against this buffer to remove ammonium sulphate. Any insoluble precipitate was removed by centrifugation and the supernatant was lyophilized (Labconco model 75035). The yield was determined. The purity of the isolated protein was determined by polyacrylamide gel electrophoresis.

Rabbit serum albumin yeilds of 20-30 mg/ml were obtained. The purity of freeze dried protein was checked by SDS-PAGE and compared to that of commercially supplied RSA (SIGMA) (Figure 2.6). The isolated RSA was relatively free of contaminating proteins and its purity was as high as the commercial protein.

1.2.2. SDS- Polyacrylamide gel electrophoresis (SDS-PAGE)

1.2.2.1. Sample preparation

The samples (0.05-0.1 ml; 0.5 mg/ml) containing the final concentrations of dissociation buffer; 0.0625M Tris-HCl, pH 6.8; 2% SDS; 10% glycerol; 5% 2-mercaptoethanol; 0.001% bromophenol blue as a dye were prepared. The samples, with 5 to 10 crystals sucrose (to increase the sample density), were boiled at $100^{O}C$ for 2 minutes, to ensure complete disruption of the secondary structure prior to loading onto the gel.

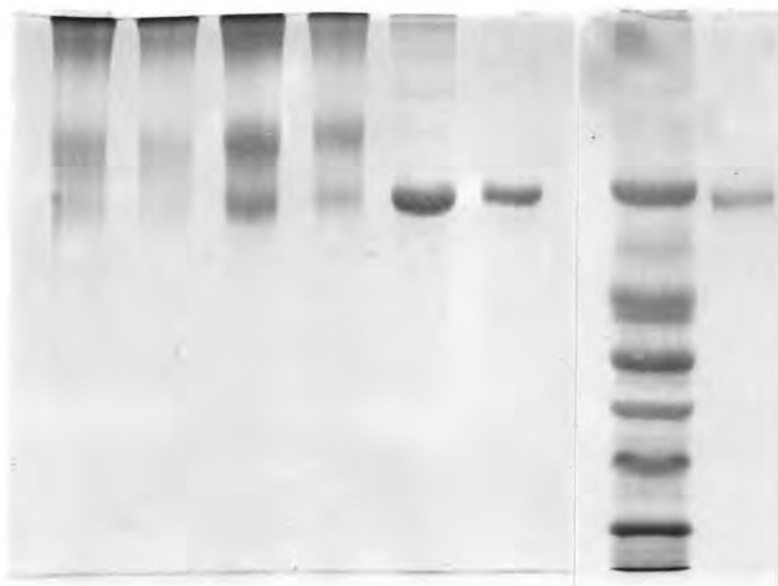


Figure 2.6. Determination of the purity of freeze dried rabbit serum albumin using SDS-PAGE and the electrophoretic mobilities of soluble RSA polymers. Track 1 and 2, RSAS 8/1; track 3 and 4, RSAS 20/1; track 5, RSA (SIGMA); track 6, RSA (isolated); track 7, marker proteins.

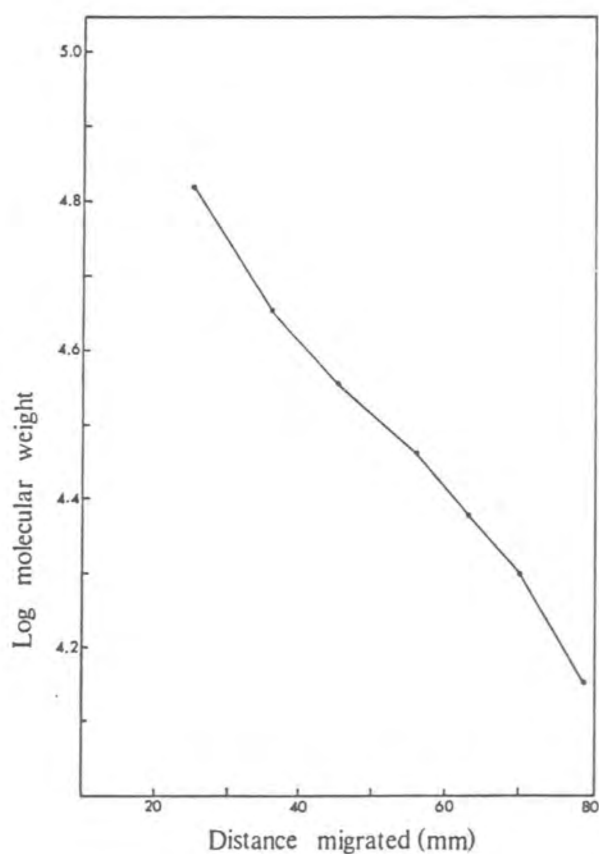


Figure 2.7. Standard curve of SIGMA Molecular weight markers.

SIGMA SDS molecular weight marker proteins were electrophoresed in wells adjacent to the sample proteins. Their mobilities were used to construct a calibration curve from which the molecular weights of the polypeptides were determined (Figure 2.7). MW markers were used on all PAGE analyses as they provide a measure of reproducibility between different gel runs. Table 2.1 shows how protein markers are prepared.

Table 2.1. Preparation of SIGMA molecular weight marker proteins.

Protein	Mol. Wt 10^{-3}	Optimal conc mg/ml
Albumin, bovine	66	0.71
Ovalbumin	45	1.0
Glyceraldehyde 3-Phosphate Dehydrogenase	36	0.67
Carbonic Anhydrase	29	0.5
Trypsinogen	24	1.0
Trypsin Inhibitor	20.1	1.0
Lactalbumin	14.2	0.5

1.2.2.2. Electrophoresis

Proteins were separated on 1.5 mm thick discontinuous SDS-polyacrylamide slab gels run on the Bio-Rad Model 220 dual vertical electrophoresis cell as described previously (Laemmli, 1970). Gels containing 4% acrylamide (stacking gel), 10% acrylamide (resolving gel) were prepared from a stock solution of acrylamide:N,N'-bis-methylene acrylamide (30:0.8 w/w). The final concentrations in the resolving gel were as follows; 0.375M Tris-HCl, pH 8.8 and 0.1% SDS. Gels were polymerized chemically by the addition of 0.075% freshly prepared ammonium persulphate and TEMED (20μl). The stacking gel contained 0.125M Tris-HCl, pH 6.8 and 0.1% SDS and was polymerized in

the same way as the resolving gel. Protein samples were loaded into the wells and "empty" wells recieved 10 µl dissociation buffer to prevent "smiling".

Electrophoresis was carried out in Laemmli bath buffer (0.025M Tris, 0.192M glycine, 0.1% SDS, pH 8.3) for 5 to 6 hours or until the tracking dye (BPB) had reached the bottom of the resolving gel.

Proteins were fixed and stained with Coomasie brilliant blue (45% methanol: 10% glacial acetic acid: 45% distilled water: 0.2% Coomassie brilliant blue) for 2 hours at $37^{o}C$ with shaking. The gel was destained with several changes of Destaining solution (45% methanol: 7% glacial acetic acid: 48% distilled water) until the background was faint and protein bands clearly visible. The gels were dried under vacuum onto Whatman No 3M paper using the slab gel dryer (Hoefer Scientific instruments, model SE540).

2. PREPARATION OF VACCINES

2.1. Preparation of polymerized serum albumin beads

RSA (200 mg) was added to a solution containing the antigen (100 µg) in 1mM sodium phosphate buffer, pH 7.5, 0.1% SDS. Polymerization was initiated by the addition of 0.2 ml glutaraldehyde (GA) (BDH) to a final concentration of 1%. After mixing for 10 seconds, the mixture was injected from a 2 ml syringe through a "bent" 18 gauge needle into a swirling mixture (100 ml) of petroleum ether (BDH) : sunflower oil (4:1 v/v). The rate of stirring was carefully checked with a stroboscope as this influences the size of the beads produced (1650 rpm). After stirring for 1 hour at room temperature, the beads were allowed to settle and the oil solution decanted. The beads were washed twice with petroleum ether and twice with ether before being dried for

2 hours under vacuum. The beads were stored in a vacuum desiccator at $4^{o}C$ prior to inoculation.

Structural features were analysed by scanning electron microscopy.

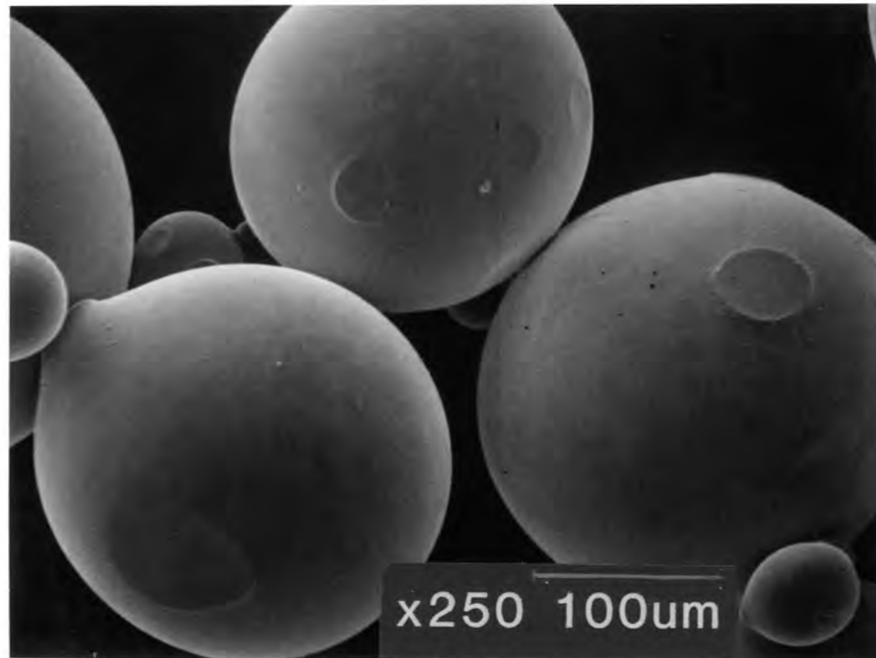
Figure 2.8A illustrates a scanning electron micrograph of RSA beads. The average diameter of the beads was 50-100 µm. The beads were well formed and spherical.

2.2. Soluble serum albumin polymers

Soluble albumin polymers were prepared using a modification of the method described by Onică et al, (1978b). Samples (0.9 ml) of RSA (20 mg in 1mM phosphate buffer, pH 7.5, 0.1% SDS) were mixed for one minute on a Vortex mixer with 0.1 ml amounts of 2.5 and 1 % GA. After incubating for 2 hours at room temperature, the solution was dialysed against 0.02M phosphate buffer, pH 7.4 , 0.9% NaCl (PBS) to remove unreacted GA. The resulting polymers RSAS 8/1 and RSAS 20/1 (albumin/GA w/w ratio), with final GA concentration of 0.25 and 0.1% respectively, were made. UV spectra and electrophoretic analyses were preformed on the polymers.

An electron micrograph of serum albumin polymers is indicated in Figure 2.8B. The electrophoretic mobilities of GA treated serum albumin polymers, in Figure 2.6, indicate two predominant fractions with molecular weights of approximately 64400 and 177800. There appears to be a component of the polymeric sample which does not migrate into the resolving gel. RSAS 8/1 has a greater proportion of this component. Figure 2.9 shows the UV absorption spectra of these polymers and untreated RSA. The shift of the absorption maximum 280 to 268 nm, and the accompanying increase in the absorption values is

A



B

Figure 2.8. Scanning Electron Micrograph of A) polymerized RSA beads (1% final conc).B) Soluble RSA polymers (0.1% final GA conc).

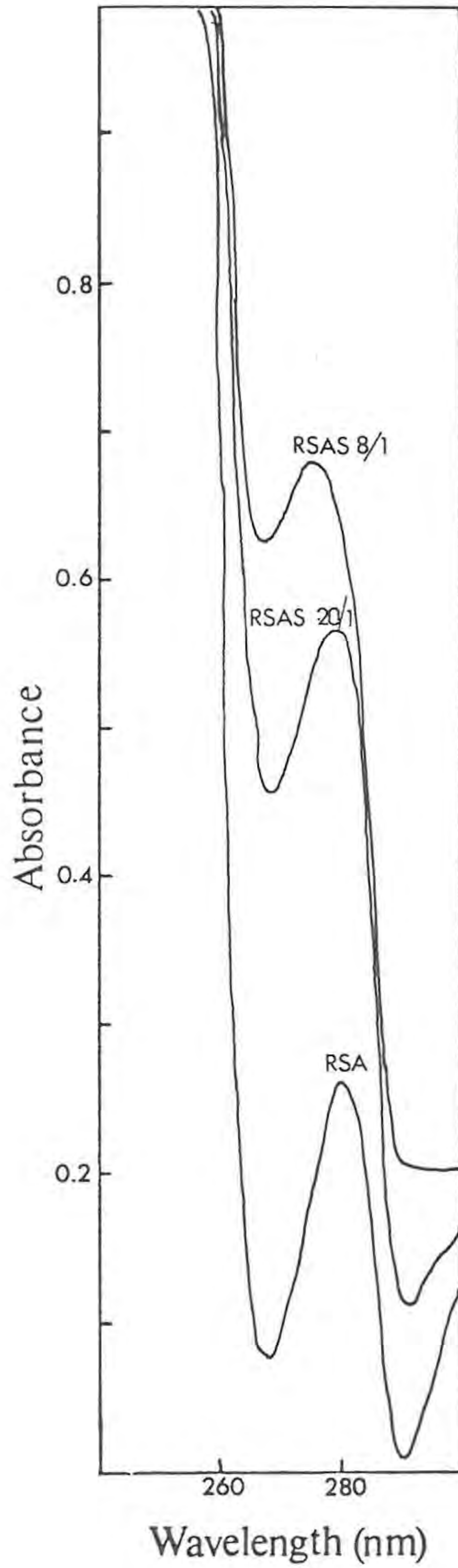


Figure 2.9. UV Absorption spectra of GA-treated and untreated RSA (protein concentration is 0.5mg/ml in PBS).

consistent with previous results (Onică *et al*, 1978b).

2.3. Freund's adjuvants and controls

The antigen solution was emulsified in an equal volume of Freund's incomplete or complete adjuvant (Miles Laboratories, Kankakee, IL, U.S.A.) immediately prior to immunization.

Control vaccines consisted of the virus solution made up to 2 ml with 5% glycerol in 0.02M phosphate buffer, pH 7.4. Each rabbit received 0.5 ml with an antigenic dose of 25 μg.

2.4. Bead vaccines with surface attached antigen

Beads were made as described in section 2.1. with no antigen incorporated.

2.4.1. Glutaraldehyde Coupling

All manipulations were preformed at $37^{o}C$. Beads (150 mg) were resuspended in 6 ml distilled water at $37^{o}C$. The beads were pelleted by centrifuging for 10 minutes at 12000g and the supernatant discarded. 10 ml 0.25% GA was added. After incubation for 1 hour with mechanical stirring, the beads were pelleted and washed twice with distilled water. Nodamura virus (0.075 mg) was added and the volume made up to 5 ml and the reaction was allowed to proceed for 1 hour with stirring. After the supernatant had been removed, unreacted sites were blocked with 10 ml 0.33% ethanolamine for 1.5 hours. The beads were washed 3 times with 20 ml aliquots of distilled water and dried under vacuum.

2.4.2. m-Maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) coupling

The following procedure was a modification of that of Sutcliffe *et al*

(1980). 63 μl MBS (15 mg/ml) (SIGMA) in N N'-dimethylformamide was added dropwise with mechanical stirring to beads resuspended in 10 mM potassium phosphate buffer, pH 7.0. The beads were stirred for 1 hour at room temperature. 0.25 ml Nodamura virus (0.3 mg/ml in 0.1M potassium phosphate buffer, pH 7.3, 5mM EDTA, 1% 2-mercaptoethanol) was heated at 100°C for 5 minutes to disrupt the virion and expose internal cysteine residues. The denatured protein solution and the activated beads were mixed, the pH adjusted to pH 6.5 and stirred for 6 hours at room temperature. The beads were then pelleted by centrifugation at 12000g for 10 minutes and the supernatant was discarded. After washing the beads 3 times with distilled water, pelleting between each step, the beads were dried prior to immunization.

2.4.3. Attachment using 3(2-pyridyl-dithio)-propionic acid (SPDP)

RSA beads (60 mg) were resuspended in 0.1M phosphate buffer, pH 7.4 at 37°C. 5 mg SPDP (SIGMA) was added to the beads with stirring. The reaction was allowed to proceed for 6 hours at room temperature with mechanical stirring. Nodamura virus (0.075 mg) was added and reacted overnight at room temperature. The beads were pelleted by centrifugation and the supernatant discarded. Unreacted reactive groups were blocked with 10 ml 0.33% ethanolamine for 1 hour at room temperature. The beads were washed as described for the other methods.

2.4.4. Attachment using 1-ethyl-3(3-dimethyl-amino propyl) carbodiimide hydrochloride (CDI)

The amine groups on the bead surface were firstly succinilated to convert them to carboxyl groups.

2.4.4.1. Synthesis of succinic anhydride

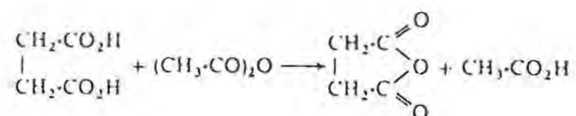


Figure 2.10. Reaction of succinic acid with acetic anhydride to form succinic anhydride.

0.5 mol succinic acid and 1 mol (94.5 ml) of redistilled acetic anhydride were added to a 500 ml round bottomed flask, attached to a reflux condenser, protected by a calcium chloride drying tube. The mixture was gently refluxed in a water bath with occasional shaking until a clear solution was obtained and then for a further hour to ensure the completeness of the reaction. The assembly was removed from the water bath, allowed to cool to room temperature and finally on ice. The succinic anhydride was collected by filtration through a Buchner funnel, washed twice with 40 ml portions of anhydrous ether and dried in a vacuum desiccator. The presence of succinic anhydride was confirmed by melting point determination (m.p. succinic anhydride 119-120°C). A yield of 90% was obtained.

2.4.4.2. Succinilation

Beads (60 mg) were resuspended in 2 ml dry dimethylsulphoxide (DMSO ; BDH). 420 mg succinic anhydride was added in small increments to the suspension with mechanical stirring. The reaction proceeded for 38 minutes. The supernatant was discarded and the beads were washed 3 times with distilled water, pelleting by centrifugation after each wash.

2.4.4.3. Conjugation

The beads were resuspended in 8 ml dry DMSO. Nodamura virus (0.075 mg)

and 150 mg CDI (SIGMA) were added to the beads. The mixture was stirred for 4.5 hours using a magnetic stirrer, after which the beads were pelleted and washed several times with distilled water. They were stored dry at $4^{O}C$ until inoculation.

MDP was also attached to the bead surface using this procedure.

3. IMMUNIZATION

3.1. Maintenance of test rabbits

Unimmunized New Zealand white rabbits were obtained locally and caged in wire cages with an unrestricted food and water supply. Excrement trays and cages were cleaned daily and weekly respectively. The animals' health was checked daily. The rabbits were identified by plastic tags punched through their left ear with a tagging gun (Allflex International Ltd, New Zealand.).

3.2. Inoculation and bleeding of rabbits

A preimmune blood sample was obtained from each rabbit. The rabbit to be bled, was placed in a box which restrained its head. The hair was shaved from the marginal vein in the right ear. Xylene was swabbed onto the vein to dilate it. The blood sample (2-5 ml) was collected using a gauge 2 needle attached to a glass syringe. The ear was then cleaned of xylene and blood.

Prior to inoculation, the RSA beads were rehydrated in sterile 5% glycerol in 0.02M phosphate buffer, pH 7.4 for 2 hours at $4^{O}C$. The volume was made up to 2 ml with the glycerol solution. Glycerol was used in bead inoculation as it prevented the beads from settling out during injection and thus improved the efficiency of inoculation. Each of the four rabbits, per experimental group, was inoculated

intramuscularly (i.m.) into the right hind limb with a 0.5 ml aliquot of the vaccine preparation.

The rabbits received antigenic doses of 20 mg of the soluble polymer vaccine.

The rabbits were subsequently bled at regular intervals over a 3 month period.

3.3. Isolation of serum

Blood samples were allowed to clot at room temperature. The clot was carefully separated from the glass wall and refrigerated overnight to allow clot contraction. Serum was collected and cleared of residual red blood cells (10 minutes at 10000 g). The serum was stored frozen in sterile bottles at -22°C, until the antibody titers were assayed.

4. ANTIBODY TITER ANALYSIS

4.1. Enzyme-linked immunosorbent assay (ELISA)

ELISA has been used extensively for the detection of viruses and for the determination of serum antibody IgG or IgM titers. Detection limits are within the nanogram and picogram range (Essink *et al*, 1985). This technique with its increased sensitivity and ease of performance has replaced the classical methods for antibody analyses.

The procedure outlined in section 4.1.5. was adopted with all sera analyses after the optimal conditions had been determined.

4.1.1. Experimental design

Several reports (Roggero and Pennazio, 1984; McLaughlin *et al*, 1981) have indicated that variations associated with row and column

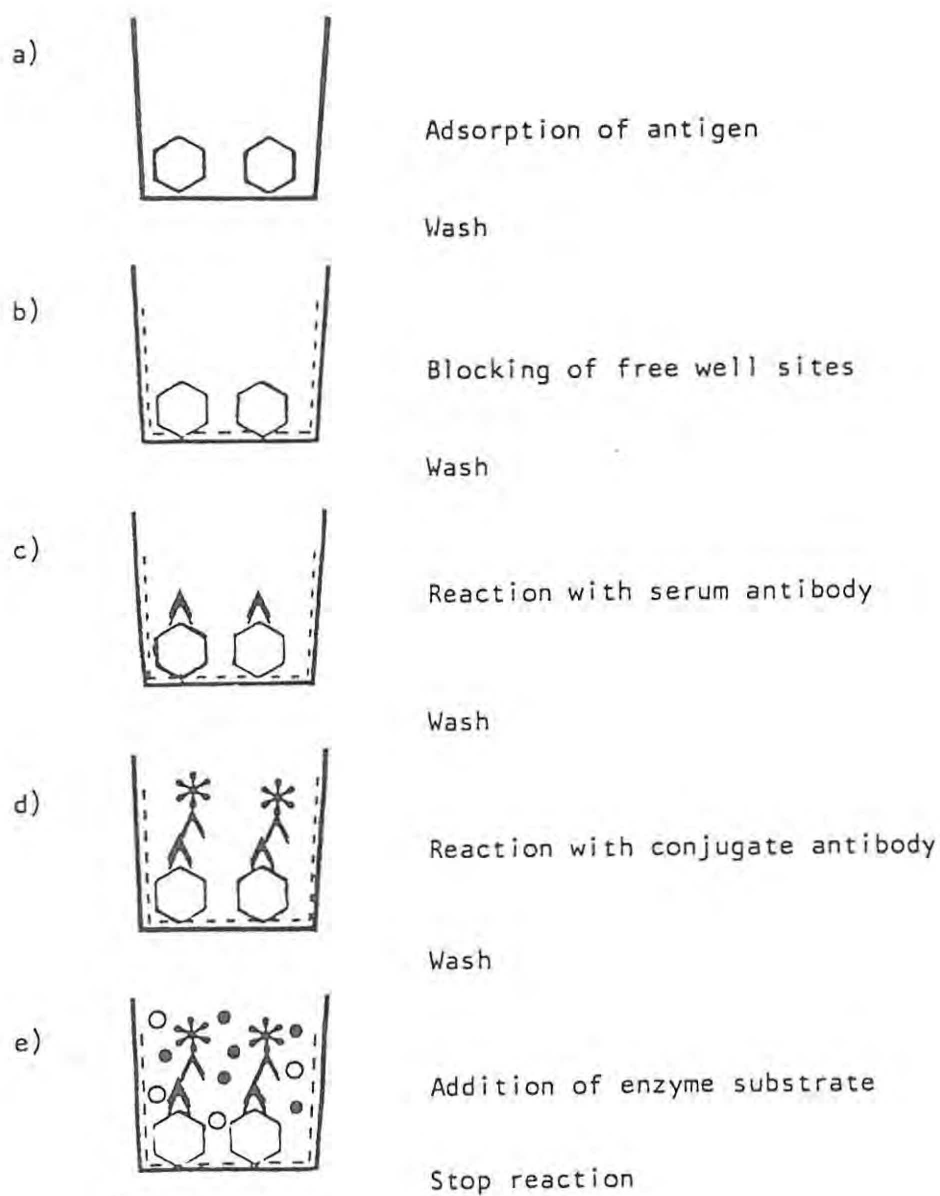


Figure 2.11. Diagrammatic representation of the indirect ELISA procedure.

positions within the plate (McLaughlin et al, 1981) and with edge and inner wells (Roggero and Pennazio, 1984) occur.

To check the variability in adsorption of wells, antigen (20 µg/ml) was incubated in each well and the standard ELISA protocol preformed. The absorbances, read at 410 nm, were analysed for edge and inner well effects, and row and column variations.

The plate effect was evident: the mean absorbance for the 36 edge wells was 1.755 (s= 0.348) whereas that of the 60 internal wells was 1.1865 (s= 0.1194). The difference between the two means was statistically significant (Student's t-test, P = 0.01). The absorbances of the internal wells appeared normally distributed. In all subsequent experiments the edge wells were not used.

Each sera dilution was tested in triplicate since this combination results in the lowest variability (Roggero and Pennazio, 1984).

4.1.2. Antigen coating

All solid phase immunoassays depend on the stable binding of the antigen/antibody to the solid phase support employed. Indirect ELISA (immobilized antigen) was used in sera analyses where Nodamura virus was the antigen. Figure 2.11 represents a schemmatic diagram of the procedure. Plastics or polystyrene plates have a small capacity for binding although this may be an advantage in limiting nonspecific adsorption (Kemeny and Challacombe, 1986). Since antigen adsorption is a passive process, particles which have come into contact with the surface of the plate, may drift away several times before a firm bond is formed by relatively weak electrostatic forces (Ehlers and Paul, 1984). Although adsorption is more rapid at higher temperatures

(Salonen and Vaheri, 1979), this equilibrium is less likely to be disturbed if stored in the cold (Engvall et al, 1971). Another problem associated with adsorption is that some particles may be eluted during washes (Ehlers and Paul, 1984), often as much as one third with viral antigens (Salonen and Vaheri, 1979).

The actual amounts of antigen needed for maximal reactivity in the final enzyme reaction are very small and these amounts represent only a minor fraction of the amount of antigen added. However, if supraoptimal amounts of antigen are used, the associated decrease in reactivity may be due to the high antigen concentration imposing steric hinderance in antigen-antibody reactions (Salonen and Vaheri, 1979).

4.1.2.1. Determination of optimal antigen concentration

The optimal antigen concentration was determined. Triplicate wells, coated with Nodamura virus (diluted two-fold; 50-0.01 μg/ml) were incubated with positive sera (1/200 dilution) for 2 hours at 37°C. The plate was then treated as described in 4.1.5. A plot of OD_{410nm} versus antigen concentration (μg/ml) is shown in Figure 2.12. The optimal concentration is 20 μg/ml, the point at which saturation is reached.

Since saturation of sites in the wells required a high concentration of viral antigen (20 μg/ml), two experiments were included in these initial studies in an attempt to conserve viral material; drying the antigen onto the well or covalently linking it using GA.

4.1.2.2. Drying the antigen

Doubling dilutions of Nodamura virus (50-0.1 μg/ml) were dried onto plates overnight at 37°C and processed further as described under

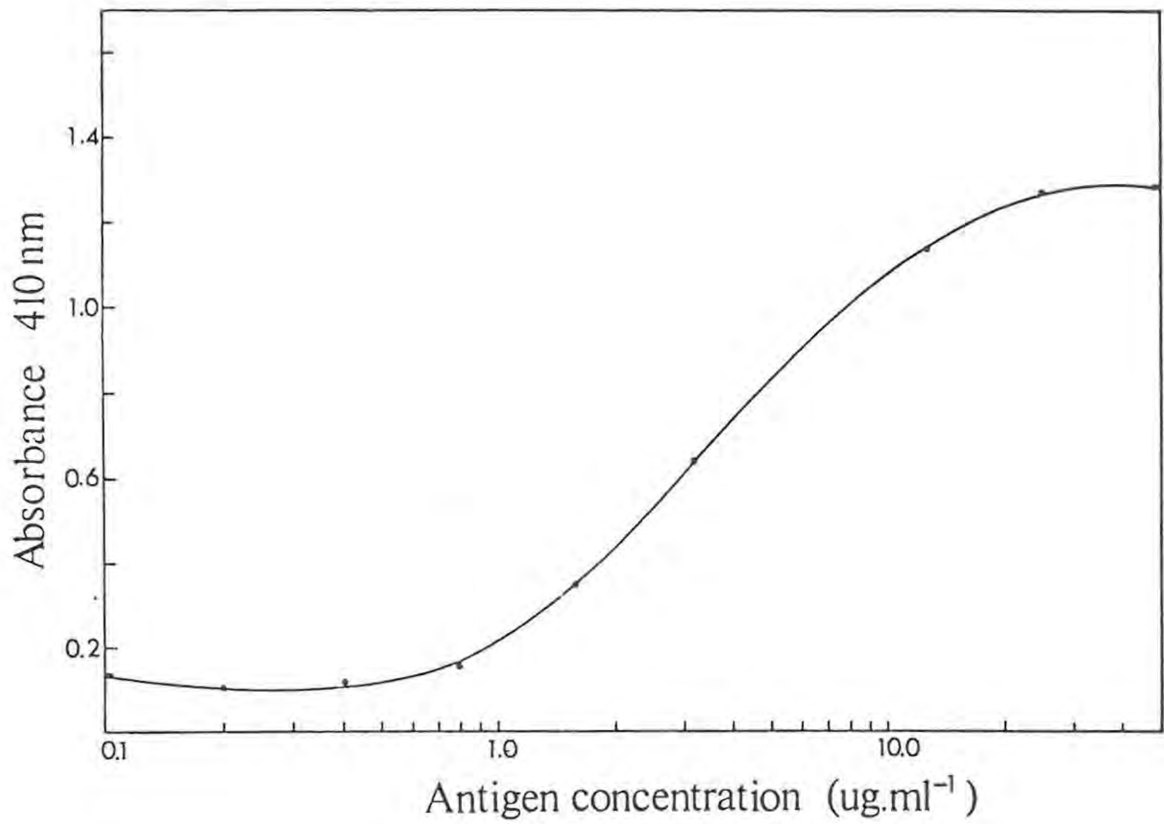


Figure 2.12. Determination of the optimal antigen concentration for ELISA. The antigen tested was Nodamura virus.

ELISA.

Drying the antigen onto the wells resulted in extremely variable results, probably due to inconsistent amounts of virus being leached off during washing stages (Figure 2.13).

4.1.2.3. Covalent bonding of the antigen

The method employed for activating ELISA plates with GA, for subsequent binding of proteins, was described by Ehlers and Paul (1984). They achieved more efficient and irreversible binding of viral particles.

To each plate, a 10% (v/v) solution of 3 (triethoxysilyl)-propylamine (SIGMA) in ethanol was added (0.2 ml/well) and emptied immediately afterwards as thoroughly as possible. The plates were then placed upside down on filter paper and dried at 85-90°C for 6 hours. A solution of 0.015% (v/v) GA in 0.3M phosphate buffer, pH 7.0 was added to the plates (0.2 ml/well), and the plates incubated for 1 hour at 56°C. The microtiter plates were thoroughly washed with distilled water and the virus sample (diluted two-fold; 50-0.01 μg/ml) was added to the wells. The plates were then treated as described in 4.1.5.

Covalently linking the virus to GA activated plates resulted in high nonspecific adsorption, with serum IgG molecules binding indiscriminantly to the plates.

Since neither drying nor cross-linking the antigen to the plates improved the sensitivity over the adsorption method, the preferred method employed was adsorption which was both simpler and easier.

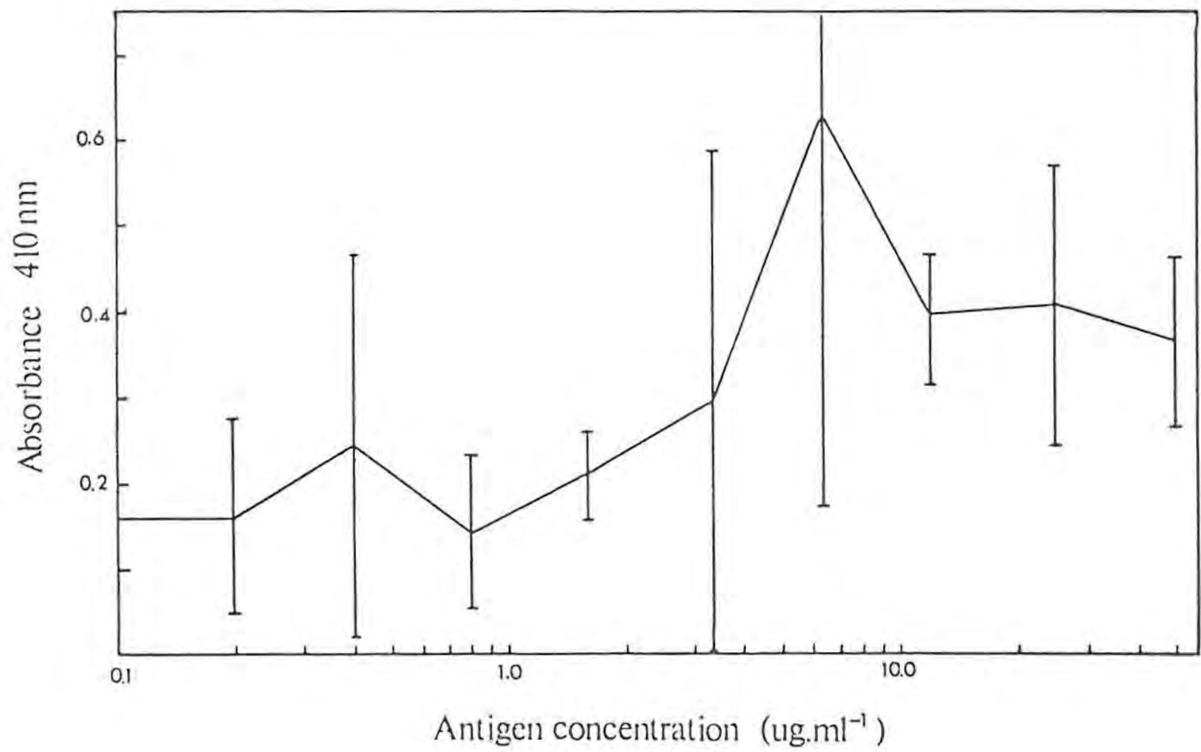


Figure 2.13. The effect of drying the antigen (Nodamura virus) onto the ELISA plate. I indicates standard deviation (n=3).

4.1.2.4. Antigens and antisera

In standard ELISA protocols, sucrose purified Nodamura virus was diluted to a concentration of 20 μg/ml with 0.02M phosphate buffer, pH 8.0. Test antisera were diluted in 0.02M phosphate buffer, pH 7.4 (PB) containing 0.05% Tween 20 from 10^0 to 10^{-6}.

4.1.3. Non-specific adsorption

Since the adsorption process is nonspecific, the inclusion of a neutral detergent (Triton X 100 or Tween 20) can be used to reduce non-specific adsorption (NSA) without interfering with antigen-antibody reactions (Crowther and Abu-el Zein, 1979). Generally a post antigen coating step is included to block unbound sites and in this way further reduce nonspecific effects.

The problem of NSA is widespread and researchers have employed several different alternatives to combat it. These include BSA (Salonen and Vaheri, 1979; Stechemesser et al, 1985), casein (Kenna et al, 1985), ovalbumin (Crowther and Abu-el Zein, 1979; Essink et al, 1985) and gelatin (Ionescu-Matiu et al, 1983).

Essink et al (1985) suggested that the presence of lysozyme in immunoassays plays a significant part in NSA, as it reduces the sensitivity by complexing with immunoglobulins and prevents their binding to the antigen. Cu^{+2} ions or proteins (pI<5; eg. BSA) can be used to overcome this interference.

Although some methods are effective in some systems, the success depends on the antigen being studied and all possibilities should be examined. Although, Tween 20 is generally used as the detergent in

washing steps, Kenna *et al* (1985) found that, in their assay, it actually increased NSA.

To determine the most effective blocking agent in this antigen-antibody system, the following were compared as post-coating treatments; 3% BSA, 1/5 human serum, 0.5% gelatin and 0.5% ovalbumin. The post-coating agents were incubated for 2 hours at 37°C in Nodamura virus coated wells and in control wells which had received 0.02M phosphate buffer, pH 8.0. After washing, 9 wells/coating agent received a 10^{-2} dilution of positive sera with the control wells (n=3) each receiving PB-Tween 0.05% and were incubated for 2 hours at 37°C. After washing, bound antibody was allowed to react with enzyme-IgG conjugate for 2 hours at 37°C. The reaction was quantified by the conversion of p-nitrophenyl phosphate to p-nitrophenol. The results depicted in Table 2.2 show that 3% BSA was the most effective post-coating agent and it resulted in the highest P/N ratio (positive/negative), which indicates a greater sensitivity over the other methods employed. There was also less variation between absorbance readings of BSA treated wells (s= 0.04) compared with untreated wells (s= 0.227). 0.5% ovalbumin and 0.5% gelatin were ineffective and actually enhanced non-specific reactions. 3% BSA has been used as a blocking agent in the assays preformed in this research programme. Although very low levels of anti-albumin antibodies (AAA) are present in normal serum (Lenkei, 1980; Mihăescu *et al*, 1981; Onică *et al*, 1983; Nardiello *et al*, 1985) which possess species cross reactivity (Sakata and Atassi, 1981) the inclusion of adequate controls ensured that erroneous results were avoided.

Table 2.2. Determination of the most effective agent for preventing non-specific reactions.

Treatment	Incubations*			
	V+S+C	PB+S+C	V+PB+C	P/N
3% BSA	1.49+0.04	0.21+0.02	0.03	6.99
1/5 Human serum	1.37+0.04	0.44+0.04	0.07	3.18
0.5% gelatin	2.39+0.19	0.91+0.05	0.08	2.64
0.5% ovalbumin	1.56+0.15	1.45+0.01	0.15+0.03	1.078
No treatment	1.31+0.23	0.33+0.09	0.07+0.01	4.00

* V, Nodamura virus; S, serum; C, conjugate; PB, 0.02M phosphate buffer, pH 7.4; P/N, positive/negative result.

4.1.4. Enzyme-immunoglobulin conjugate

The sensitivity of the antigen-antibody reaction is enhanced by employing an anti-immunoglobulin conjugated to an enzyme. The enzyme remaining in the wells after washing provides a measure of the amount of specific antibodies in the serum. Alkaline phosphatase (calf intestine) is very stable and hydrolyses its substrate, p-nitrophenyl phosphate, to yellow coloured p-nitrophenol which is detected spectrophotometrically at 410 nm.

Alkaline phosphatase has approximately 19 free amino groups that potentially may react with GA. Free IgG reduces the sensitivity and free enzyme enhances the specific activity of the conjugate and immunoassay blanks (Porstmann et al, 1985). Although previous workers (Engvall et al, 1971; Ionescu-Matiu et al, 1983) reported that, since GA reacts only with free amino groups, it would have no significant role in altering the enzymatic activity or the specific antibody binding, others (Porstmann et al, 1985) showed that alkaline phosphatase conjugate activity was 11% less than the free enzyme.

Therefore it is extremely important to access the activity, both enzymatic and immunologic, of the enzyme-IgG conjugate. Immunoelectrophoresis was used for this purpose.

4.1.4.1. Purification of goat anti-rabbit IgG

Goat anti-rabbit IgG was purified from sera (Cappel Laboratories, West Chester, PA, U.S.A.) with affinity chromatography on rabbit IgG-agarose (Affi-Gel 10; Bio Rad Laboratories, Richmond, California, U.S.A.) according to the manufacturer's instructions.

Affigel-10 is an N-hydroxysuccinimide ester of a derivatized cross-linked agarose gel bead support and contains a neutral 10-atom spacer arm. (Figure 2.14). The gel couples via a stable amide bond to the primary amino groups of the ligand, rabbit IgG under physiological conditions (Figure 2.14). The coupling is stable, rapid and highly efficient.

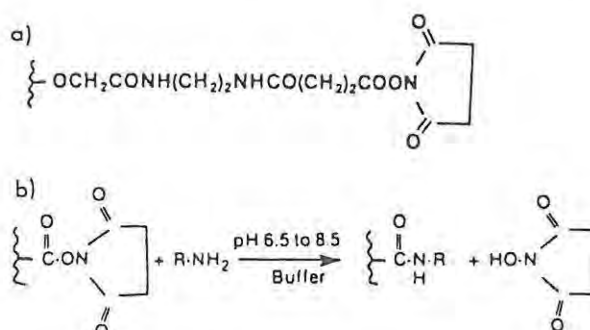


Figure 2.14. a) Chemical structure of Affi-Gel 10. b) Coupling reaction of Affi-Gel 10 with ligand containing free amino groups.

The rabbit IgG was purified by ion exchange chromatography.

4.1.4.1.1.Preparation of Rabbit IgG by Ion exchange chromatography

Whatman DE22 (Whatman Ltd., Kent), an ion exchange resin was activated by incubations for 2-3 hours with 0.5N HCl followed by 0.5N NaOH at $4^{0}C$. After each incubation, the gel was washed with distilled water until pH 7.0.

The activated gel was equilibrated in 0.01M phosphate buffer, pH 7.4. Rabbit serum (0.5ml), diluted two fold in distilled water, was applied to the DE22 column. Rabbit IgG was eluted from the column in the void volume with 0.01M phosphate buffer, pH 7.4 (Figure 2.15). 0.5 ml the fractions were collected and those with an $OD_{278}:OD_{250}$ of 2.3 to 2.7 were pooled. The concentration, estimated spectrophotometrically ($E_{280nm}^{1\ mg/ml}$ = 1.4), was 1.2 mg/ml.

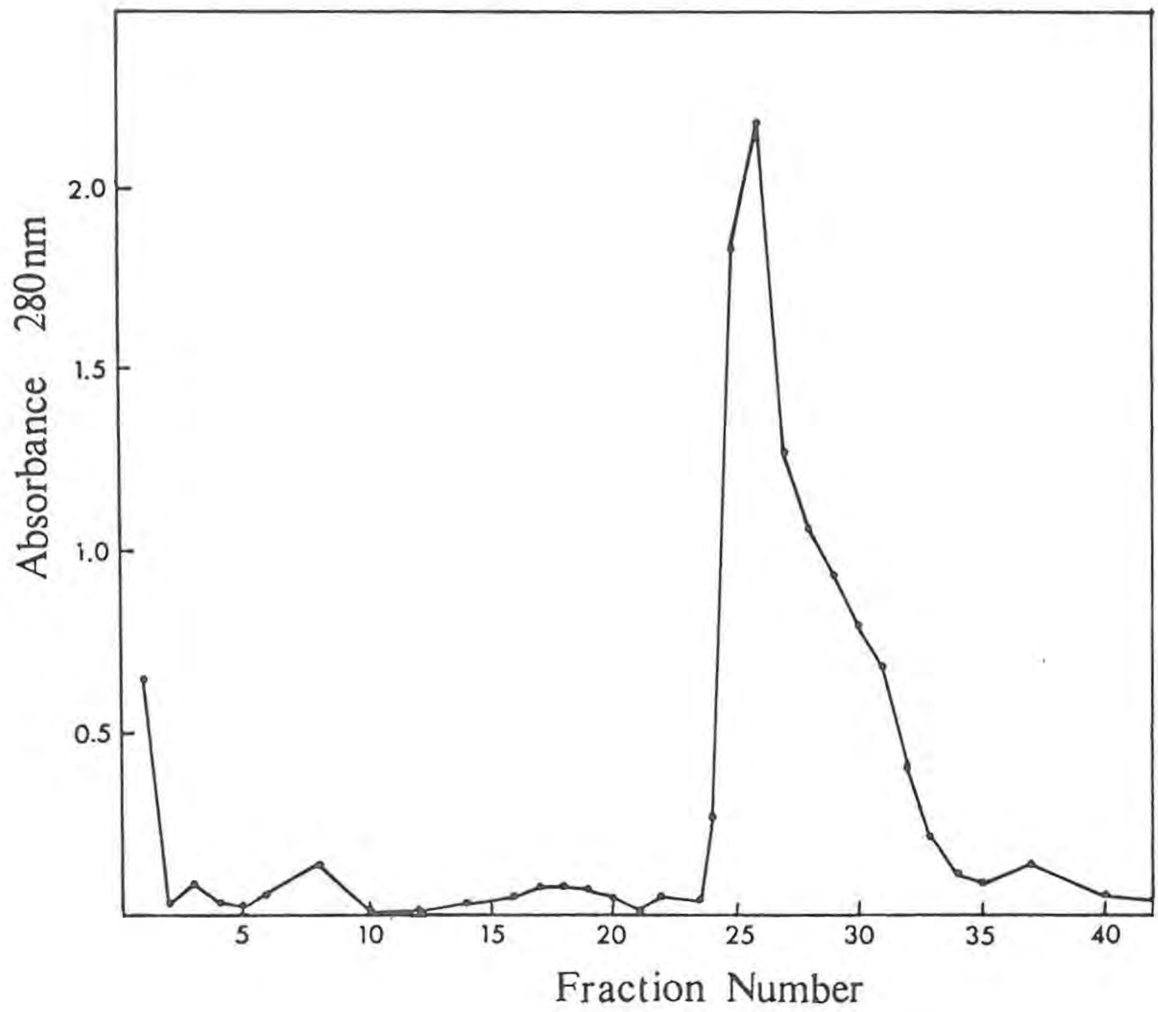


Figure 2.15. Separation of Rabbit IgG from serum proteins using ion-exchange chromatography (DEAE-22).

4.1.4.1.2. Purification of goat anti-rabbit IgG using affinity chromatography

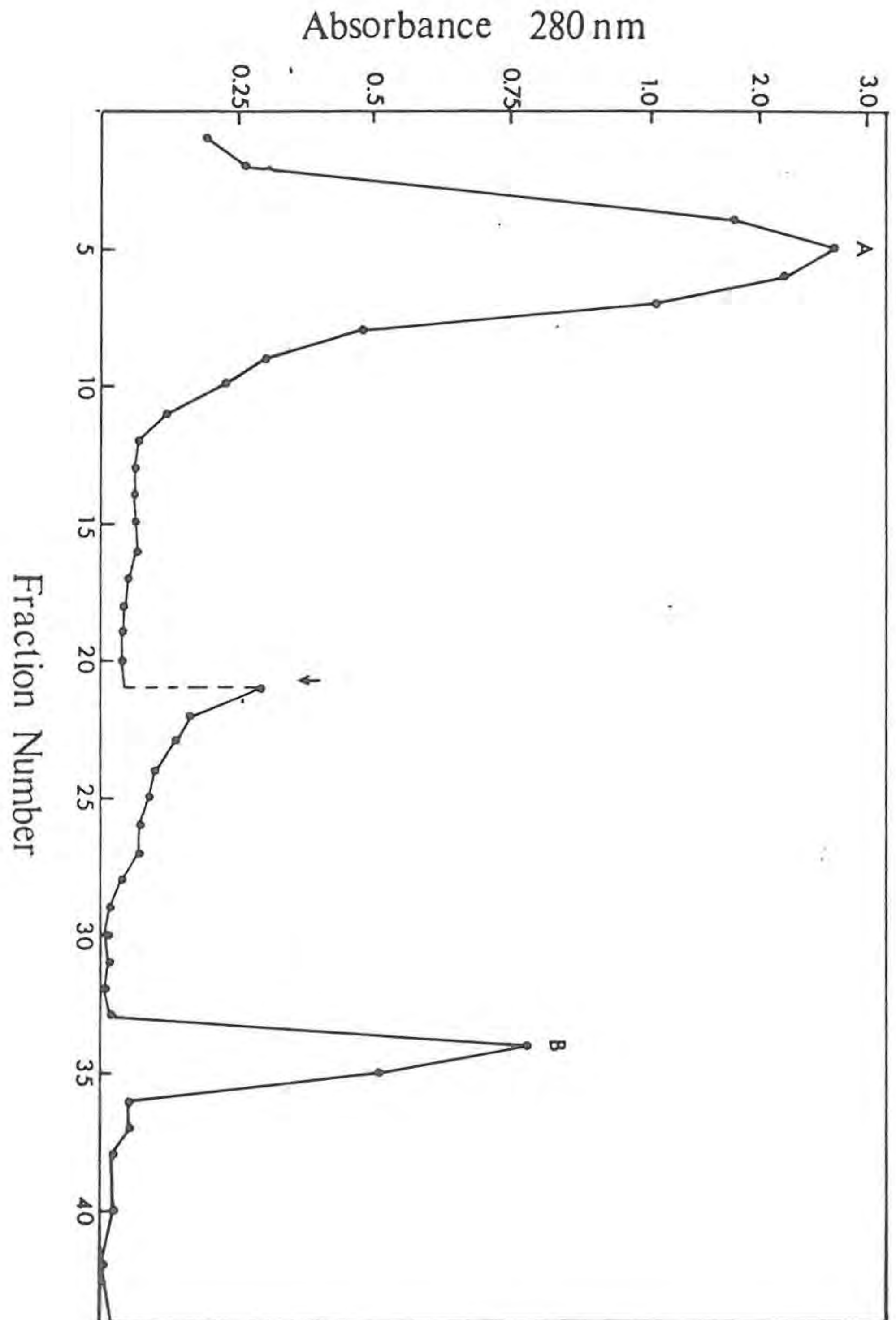
All solutions and apparatus were cooled to $4^{o}C$. The vial containing the gel was shaken up, the contents transferred to a Buchner funnel and the supernatant solvent was drained off. The gel (12.5 ml) was washed in 3 bed volumes of isopropyl alcohol followed by 3 bed volume washes with deionized water.

The dried cake was transferred to a flask. For coupling rabbit IgG (DEAE purified) to Affi-Gel, the ligand (7.5 mg) was added to the gel (0.5 ml ligand/ml gel) and an equal volume of coupling buffer 0.1M carbonate buffer, pH 8.0, as volume ligand was added. The gel was agitated gently at room temperature for 1 hour or at $4^{o}C$ for 4 hours. The remaining active esters were blocked by incubation for 1 hour at room temperature with 1M ethanolamine-HCl, pH 8.0 (0.1 ml/ml gel).

A column (1 cm by 15 cm) was poured at room temperature in 0.01M phosphate buffer, pH 8.0 (starting buffer) and washed with 0.1M carbonate buffer, pH 8.0 and 0.1M glycine-HCl, pH 2.8, and then equilibrated with starting buffer.

An aliquot (0.5 ml) of reconstituted goat anti-rabbit serum was applied to the column. The column was washed with starting buffer, fractions (0.5 ml) were collected using a LKB Ultrorac fraction collector 7000 until the absorbance (280 nm) had returned to baseline. Goat anti-rabbit IgG was eluted with 0.1M glycine-HCl, pH 2.8 (Figure 2.16). Fractions (0.5 ml) were immediately neutralized to pH 7.0 with 0.1M NaOH and assayed for protein concentration. The presence of goat anti-rabbit IgG in fractions 34 and 35, (peak B), was confirmed by double immunodiffusion, (4.1.4.1.3). One single precipitin arc was

Figure 2.16. The elution profile of goat anti-rabbit IgG from an Affinity Chromatography column (Affi-Gel 10). Peak A: non specific goat IgG. Peak B: specific goat anti-rabbit IgG, as confirmed by Double Immunodiffusion. ↓ indicates when 0.1M glycine-HCl, pH 2.8 was added.


Absorbance 280 nm
3.0
2.0
1.0
0.75
0.5
0.25
A
B
5
10
15
20
25
30
35
40
Fraction Number

formed with whole rabbit serum, (Figure 2.17). The pooled peak fractions were dialysed against PBS, pH 7.2. The concentration was adjusted to 1 mg/ml and stored at $4^{o}C$ with 0.1% sodium azide.

The gel was washed with PBS and stored at $4^{o}C$ for later use.

4.1.4.1.3. Double Immunodiffusion

This simple, yet informative, technique allows antibody to encounter antigen by diffusion alone.

The diffusion medium contained 1% purified Oxoid agar in 0.05M phosphate buffer, pH 7.4 containing 0.86% sodium chloride and 0.1% sodium azide. Ouchterlony plates were prepared by pouring 15 ml of sterile agar into each 8.5 cm diameter Petri dish. These were stored inverted at $4^{o}C$. Wells were cut in the agar with a gel punch. Serial two fold dilutions of rabbit antibody were loaded into the outer wells and goat anti-rabbit IgG dilutions into the inner wells. After incubation for 24 to 48 hours at room temperature, antigen-antibody recognition was detected by the formation of an immuno-precipitin line between the wells. The gel was removed from the petri dish and washed with several changes of 1% NaCl in PB for 48 hours. After removal of all uncomplexed proteins, the gel was dried onto gel bond. The precipitin bands were stained with Coomassie brilliant blue as described for protein detection with SDS-PAGE (1.2.2.2). An advantage with this technique is that concentration gradients of antigen and antibody are automatically formed by the diffusion process.

4.1.4.2. Preparation of enzyme-linked IgG

Goat anti-rabbit IgG was conjugated to alkaline phosphatase using the one step conjugation procedure with GA (Avrameas, 1969). The enzyme,

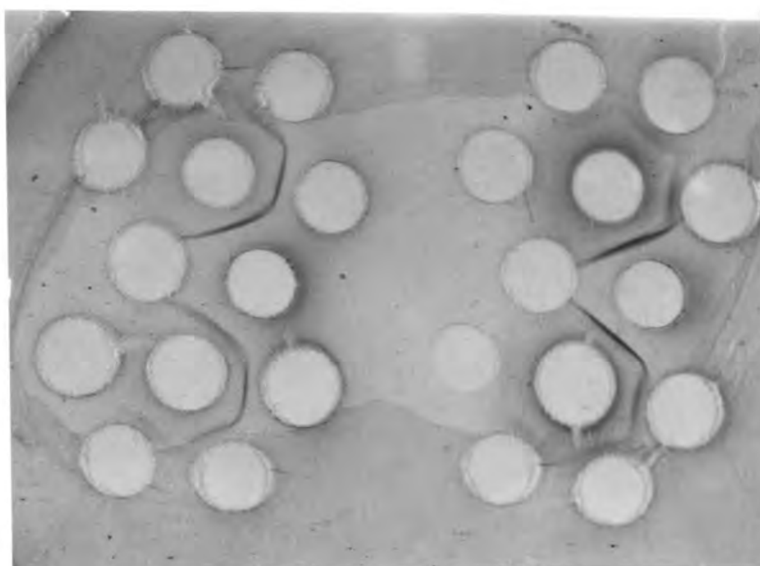


Figure 2.17. Confirmation of the isolation of specific goat anti-rabbit IgG (from peak B in Figure 2.16) using double immunodiffusion. Inner wells: dilutions of goat anti-rabbit IgG in PBS. Outer wells: Serial two fold dilutions of normal rabbit serum.

alkaline phosphatase (Boehringer Mannheim, West Germany) had a specific activity of 25000 units/mg. 2 mg alkaline phosphatase EC.3.1.3.1. (0.2 ml) was added to 1 mg affinity purified IgG. GA was added at a final concentration of 0.05%. The reaction was allowed to proceed for 4 hours at 22°C or overnight at 15°C. Free GA was removed by extensive dialyses against PBS. The conjugate was stored with 1% BSA (SIGMA) at 4°C in the dark until use. The conjugate was analysed using SDS-PAGE (1.2.2.) and immunoelectrophoresis (4.1.4.3.).

4.1.4.3. Immunoelectrophoresis

Microscope slides were coated with 1% agarose in 0.05M barbitone buffer, pH 8.6. Using the immunoelectrophoresis gel cutter, 3 wells and 2 troughs were stamped into the gel and the agar plugs and excess liquid removed from the wells. The slides were placed in the Shandon electrophoresis tank with the wells positioned nearest the cathode. 5 µl samples were added to each well and electrophoresed in 0.1M barbitone buffer, pH 8.6, for 1.5 hours at 6V/cm, as determined using a voltmeter.

The agar troughs were removed and rabbit IgG added to each trough. The slides were incubated at 37°C overnight in a humid chamber. Immunoprecipitin arcs were visible. Unprecipitated protein was removed by immersion in 1% NaCl for 48 hours and then for 1 hour in distilled water. The gel was dried onto the slide or gel bond at 37°C. For photographic purposes, the bands were stained for 5 minutes with 1% amido black 10B in rinsing solution (methanol 45%: acetic acid 10%: water 45%). Excess stain was removed by 4 baths in rinsing solution. The slides were dried and photographed.

To detect enzyme activity, the gel was dried onto the slide

immediately after electrophoresis without prior washing. The slide was then placed in a solution of 1 mg/ml p-nitrophenylphosphate in 10% diethanolamine-HCl, pH 9.8. Regions showing a yellow colour were recorded.

Figure 2.18 indicates that several high molecular weight conjugates were formed. This heterogenicity is characteristic of this uncontrolled one-step conjugation procedure (Ionescu-Matui et al, 1983). No free IgG was present after conjugation, however there was still unreacted alkaline phosphatase present (Figure 2.18). The conjugate possessed enzyme activity and reacted with rabbit sera (Figure 2.19).

4.1.4.4. Determination of optimal conjugate dilution

The importance of determining the optimal conjugate concentration and the use of optimal conditions was illustrated by Gadkari et al (1985), who showed that as the number of enzyme molecules increased per IgG molecule, the conjugate's preformance decreased. This might be due to a decrease in IgG affinity or specificity, indicated by lower P/N ratios. If the conjugate is too concentrated this leads to an increase in NSA.

The method followed was described by Voller (1980). The following recipricol conjugate dilutions were tested; 400, 800, 1000, 2000, 4000, 6000, 8000 and 9000.

Aliquots (0.2 ml) rabbit IgG (100 ng/ml) were incubated overnight at 37°C. The plate was washed with PB-Tween 0.05% 3 times. Duplicate dilutions of enzyme-labelled antiglobulin were incubated at 37°C for 2 hours. Substrate (1 mg/ml p-nitrophenyl phosphate in 10%

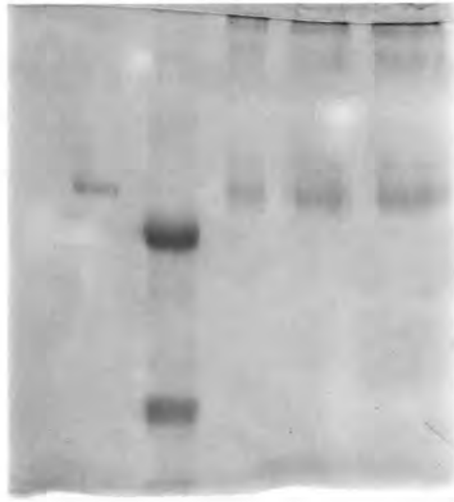


Figure 2.18. Electrophoretic mobility of Alkaline phosphatase-goat anti-rabbit IgG conjugate using SDS-PAGE. Track 1, alkaline phosphatase; Track 2, IgG; Track 3, 4 and 5, conjugate. The largest molecular weight polymers of the conjugate did not migrate into the stacking gel.

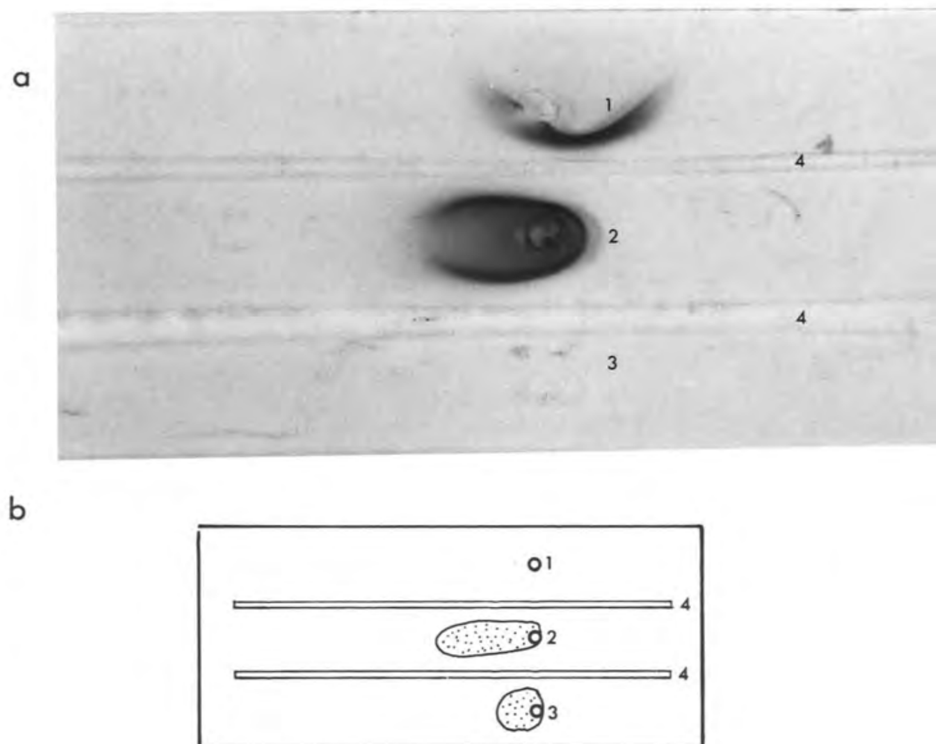


Figure 2.19. Immunoelectrophoresis of Alkaline phosphatase-IgG conjugate. a) Immunogenic activity b) Enzyme activity. 1, anti-rabbit IgG; 2, conjugate; 3, alkaline phosphatase; 4, rabbit serum.

diethanolamine-HCl, pH 9.8) was added and the absorbance at 410 nm read after 1.5 hours at room temperature. The optimal conjugate dilution was taken as that which gave OD_{410nm}= 1. The protocol described in section 4.1.5. was repeated using the postive test sera to ensure that it covered the range adequately. The optimal concentration of the conjugate was 1/4000.

4.1.5. Indirect Enzyme-linked Immunosorbent Assay

Once optimal concentrations of antigen and conjugate were determined, the following protocol was employed for the analyses of antisera, (Engvall and Perlmann, 1972).

0.2 ml aliquots of antigen (20 μg/ml) were coated for 24 hours at 37°C onto each well of flat bottomed Linbro microtiter plates (Flow Laboratories, Hamden, Conn., U.S.A.). Control wells were filled with 0.02M phosphate buffer, pH 8.0. The plate was washed 3 times by flooding with PB-Tween 0.05%. 0.3ml 3% BSA was incubated at 37°C for 2 hours to block any uncoated sites. The plate was washed 3 times with PB-Tween 0.1% (PB-T). Antisera tested in triplicate (0.2 ml/well) were incubated for 2 hours at 37°C. After washing with PB-T, 0.2 ml conjugate, diluted to 1/4000 with PB-Tween 0.05%, was added to each well followed by incubation for 2 hours at 37°C. The plates were washed with PB-T and 0.2 ml aliquots enzyme substrate (1 mg/ml p-nitrophenyl phosphate (Boehringer Mannheim) in 10% diethanolamine-HCl, pH 9.8) was added to each well. The reaction proceeded for 1.5 hours at room temperature and was stopped with 50 μl 3M NaOH. The colour intensity was read at 410nm with a Dynatech Minireader II (series 2) (Dynatech Laboratories, Inc., Alexandria, VA, U.S.A.). To detect any non-specific adsorption or interaction of these test components, the following controls were included with each sera tested; PB + serum

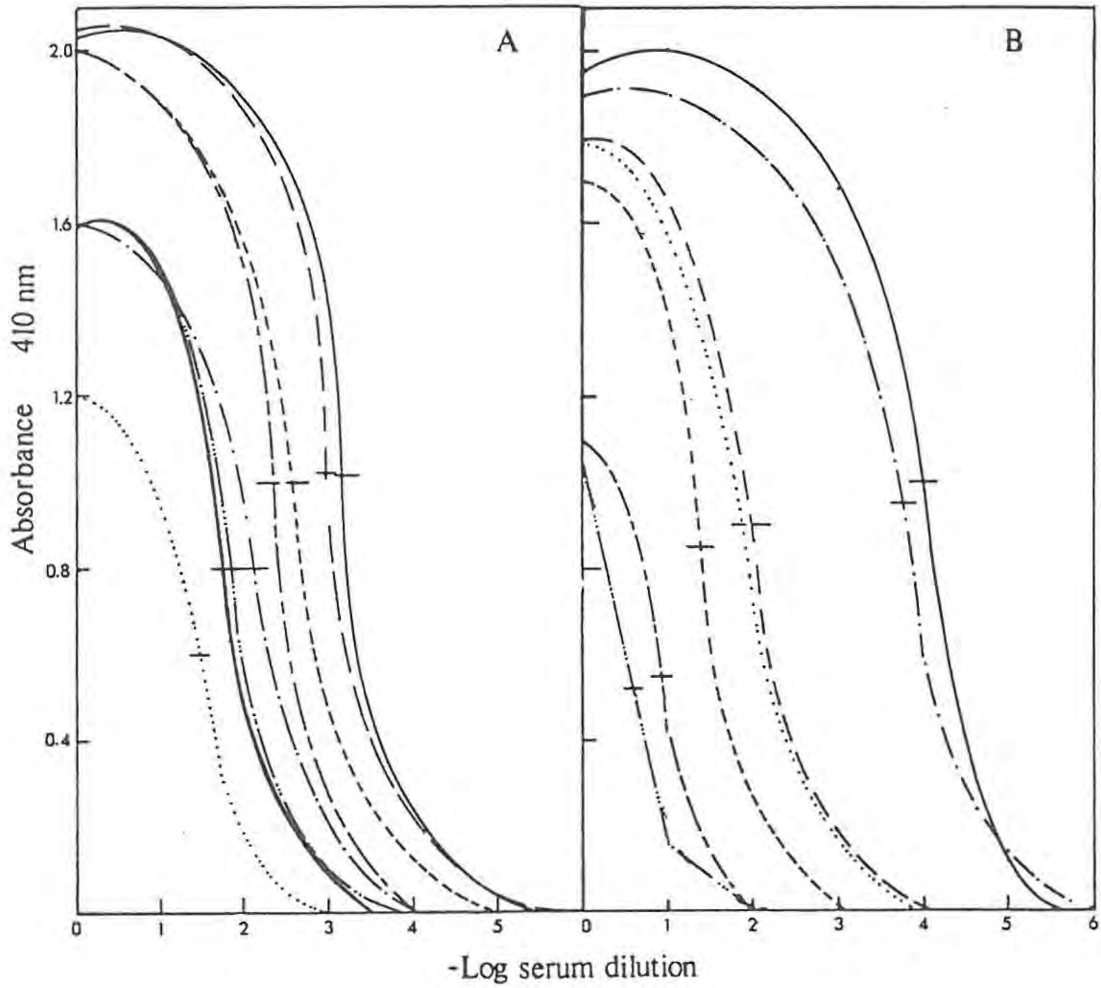


Figure 2.20. ELISA titration curves, indicating the serum antibody titers raised in rabbits immunized with Nodamura virus inoculated in A) PB; B) polymerized into RSA beads (1% final GA concentration). Rabbits recieved a boost on day 21 with the same vaccine as was used in the primary dose. Serum samples taken on day; 7 (·······); 14 (—--); 21 (—-—); 30 (---); 35 (——); 42(——); 63 (—·—); 86 (——).

+conjugate, virus + PB-Tween 0.05% + conjugate and PB + PB-Tween 0.05% + conjugate. All these controls were repeated using a post coating step of 3% BSA. Figure 2.20 depicts the titration curves of sera tested using ELISA. The log serum dilution was defined as the 50% end point.

Finally, one should be aware of all limitations associated with the techniques used. Apart from the fact that the antigen binding is weak and consequently, substantial amounts may be leached off. The nature of the interaction may be of significance since molecular distortion may occur in the immobilized antigen and antigenic epitopes in or near such sites can be obscured with the result that some antibodies are not detected.

4.2. Radioimmunoassay (RIA)

4.2.1. Coupling the antigen to CNBr-activated Sepharose 4B

Protein monomers and polymers were coupled to CNBr-Sepharose 4B (Pharmacia Fine Chemicals) to insolubilize them so that a direct comparison of the immunogenicity of the soluble antigens could be made with the insoluble beads.

CNBr-activated sepharose 4B was resuspended and washed for 15 minutes with 1mM HCl on a sintered glass filter to remove additives, (200 ml/g freeze dried powder).

Each ligand in coupling buffer, 0.1M $NaHCO_3$, pH 8.3, was mixed with the gel in a stoppered tube (5 ml ligand/g powder) and rotated for 2 hours at room temperature in an end-over-end mixer. Excess ligand was washed away with coupling buffer. The quantity of ligand coupled was calculated and found to be greater than 98%. The remaining active

ester groups were blocked with 0.1M ethanolamine-HCl buffer, pH 9.0 for 2 hours at room temperature. The gel was then washed with 3 cycles of alternating pH. Each cycle consisted of a wash with 0.1M acetate buffer, pH 4.0 containing 0.5M NaCl followed by a wash with 0.1M Tris-HCl, pH8.0 containing 0.5M NaCl. The product (beads) was stored at 4-8°C until immunoassays were preformed.

4.2.2. Iodination of goat anti-rabbit IgG (Greenwood et al, 1963)

0.1 mg goat anti-rabbit IgG (affinity purified)(1 mg/ml in 0.05M phosphate buffer, pH 7.5) and 125 µl chloramine-T (0.2 mg/ml; freshly prepared) were added simultaneously to carrier free $Na^{125}I$ (25 µCI ^{125}I 10 µCI/mmol) and incubated at room temperature for 10 minutes with occasional mixing. The reaction was stopped with 0.025 ml sodium metabisulphite (0.2 mg/ml). The whole sample was carefully layered onto a 1 X 15cm sephadex G100 column (Pharmacia Fine Chemicals AB, Uppsala, Sweden.) and the ^{125}I-labelled IgG was eluted with 0.05M phosphate buffer, pH 7.5. Fractions were collected and the aliquots (10 µl) were counted on the Beckman Gamma 110 gamma counter.

Good separation of ^{125}I-IgG from free iodine was achieved (Figure 2.21). The amount of radioactivity incorporated into the protein was determined as 2 X 10^{7} cpm/mg. In final preparations, 92% of the radioactivity was associated with TCA-precipitable material.

4.2.3. Radioimmunoassay protocol

The protocol followed was a modification of that described by Johnstone and Thorpe (1982).

The "antigen-gel" beads were devided into 2 mg aliquots in microfuge tubes and incubated for 2 hours at room temperature with 1 ml 1%

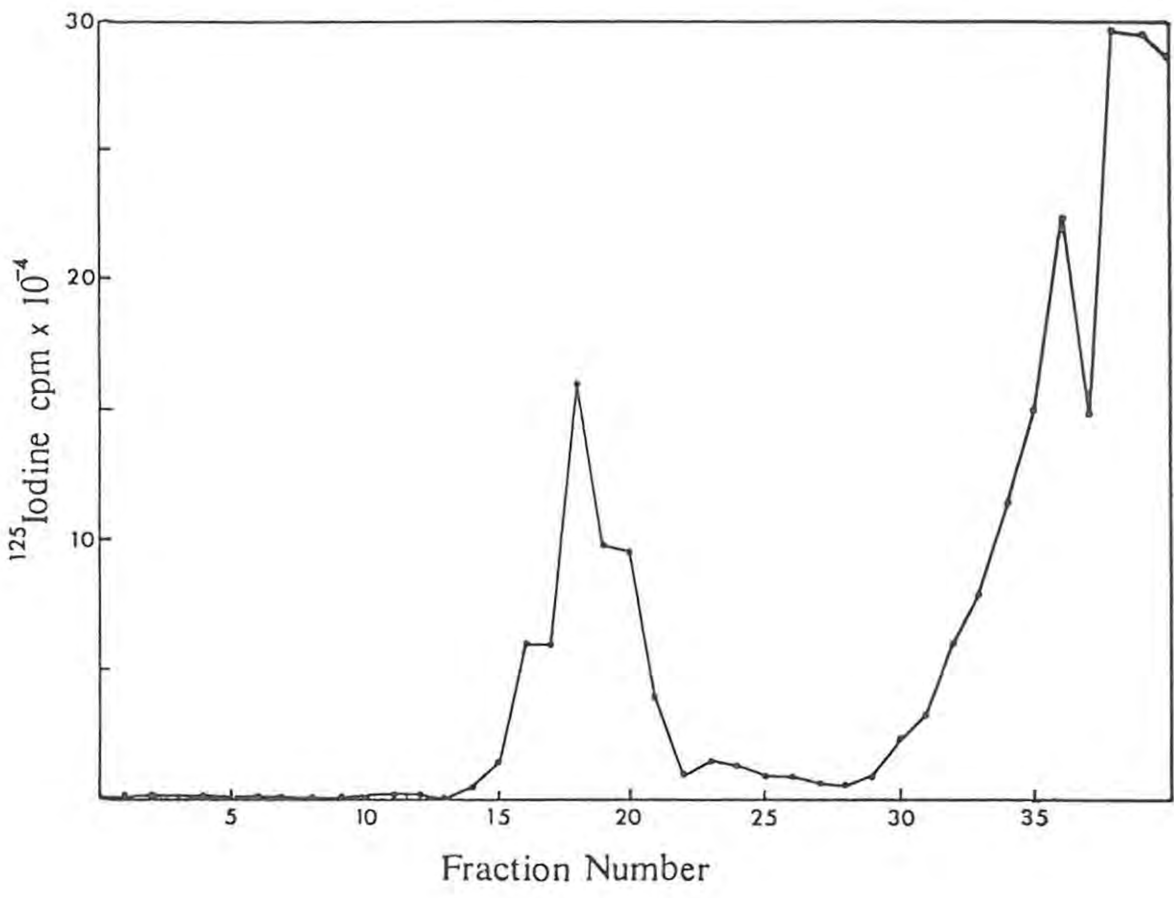


Figure 2.21. Separation of ^{125}I-labelled goat anti-rabbit IgG from free iodine using Sephadex G-100 gel filtration. Mass of protein iodinated was 100μg.

peptone in 0.02M phosphate buffer, pH 7.4 (PPB) to block binding sites on the plastic. The beads were pelleted by centrifugation using a Beckman microfuge. Duplicate 0.1 ml serum samples, diluted 10 fold in PPB, were added to the beads and allowed to react for 2 hours at room temperature with occasional mixing. The beads were pelleted, serum removed and washed 5 times with PPB. Washing entailed resuspending the beads in PPB, vortex mixing and centrifuging the beads prior to removing the washing fluid. Care was taken to ensure that no beads were lost during this step. 0.1 ml ^{125}I-IgG (~50000 cpm/0.1 ml) was added to each tube and incubated for 17 hours at room temperature with occasional mixing. After centrifugation, the supernatants were discarded and the beads washed extensively with PPB in a multi-well millipore filter. The bound radioactivity was determined using a gamma radiation counter.

Preimmune, immune and booster sera were analysed for each antigen. Insulin coupled to the CNBr-activated gel and lactalbumin beads prepared as described in 2.1. served as negative control antigens. BSA beads, with a final concentration of 1%, were prepared and immunized into rabbits and served as positive controls.

4.3. Neutralization Tests

4.3.1. Plaque reduction assays

Virus infectivity was measured by the number of plaques produced on Vero cell monolayers.

Vero cells (10^7) were planted on 60 mm diameter plastic petri dishes (Sterilin Ltd., Middlesex, England.) in MEM, 5% fetal calf serum and PS and left to form a confluent monolayer for 72 hours at 37°C. The

media was poured off. 100 µl virus aliquots, diluted 10^{-3} to 10^{-8} in serum-free MEM supplemented with 0.22% bicarbonate, was added to triplicate cell sheets. The virus was allowed to adsorb for 45 minutes at room temperature with shaking at intervals to prevent the cell sheet from drying out. Equal volumes of 2% agar (Biolab) in milli-Q water and 2 times concentrated MEM with PSF were mixed at 45°C. 5 ml agar medium was added to each monolayer and allowed to solidify at room temperature. The petri dishes were incubated inverted, to prevent condensation forming between the agar and cells at 37°C. CO_2 was flowed through the incubator to assist in pH control. The cells were examined daily and after 48 hours were fixed with 5% formaldehyde. The agar overlay was removed and plaque formation was observed by staining with 0.1% crystal violet in 50% ethanol (Racaniello and Baltimore, 1981). Excess stain was removed by rinsing gently with water. Plaque numbers were recorded and the virus titer calculated.

4.3.2. Neutralization tests

The titers of neutralizing antibodies induced by poliovirus vaccines were measured by a plaque reduction assay (Chow and Baltimore, 1982).

All sera were inactivated at 56°C for 30 minutes before use, to eliminate non-specific effects due to complement and other blood components.

Duplicate serial 2-fold dilutions of serum in sterile PBS were incubated with 100 plaque forming units (pfu) of poliovirus Sabin type 2 vaccine strain, p712, in a final volume of 0.2 ml for 2 hours at 25°C and then overnight at 4°C. The entire sample was placed undiluted on Vero cell monolayers in a standard plaque assay protocol.

Controls included; 0.1 ml virus + 0.1 ml PBS, 0.1 ml media + 0.1 ml sera, 0.1 ml virus + 0.1 ml preimmune serum and PBS + media. Neutralization titer was defined as the serum dilution required to reduce the number of plaques by 50%.

CHAPTER 3

THE IMMUNOGENICITY AND ADJUVANT ACTIVITY OF RABBIT SERUM ALBUMIN BEADS

Introduction

The formulation of a device for the controlled release of biologically active substances has been the goal of many researchers. Immobilized systems in the form of small beads or particles, (i.e. liposomes) have recently been introduced as carriers of enzymes and drugs *in vivo* (Allison and Gregoriadis, 1974; van Rooijen and van Nieuwmegen, 1979, 1980, 1983; Kreuter and Liehl, 1981; Edman *et al*, 1983; Artursson *et al*, 1983, 1984).

Several reports have described the immunostimulatory properties of small microparticles (Allison and Gregoriadis, 1974; Kreuter and Liehl, 1981; Artursson *et al*, 1985).

Ideally, the polymer matrix should be nonantigenic, biodegradable and applicable to a wide range of bioactive substances. It should also be readily available and relatively inexpensive. Serum albumin would be an ideal matrix; it is an abundant natural body protein (concentration in mammals: 40-50 mg/ml). The albumin polymers are biodegradable (Burdine *et al*, 1971) and would remain nonantigenic if the formulation procedures were nondenaturing (Royer *et al*, 1983).

Lee *et al* (1981) prepared serum albumin beads with entrapped progesterone by chemically cross-linking the protein with GA. These beads presented an effective device for the controlled release of progesterone in rabbits.

This work was subsequently applied to the administering of bacterial toxins (Royer *et al*, 1983) and viral antigens (Dewar *et al*, 1984) as

vaccines. Although no antibodies were raised when tetanus toxin was entrapped in the albumin bead (Royer et al, 1983), Dewar et al (1984) demonstrated that Nodamura virus particles, incorporated into rabbit serum albumin beads, were released slowly from the protein matrix and were able to stimulate the production of long lasting, virus-specific antibodies in rabbits.

This strategy of viral presentation will be repeated in order to formulate a sound basis on which to develop this technique further. The antigenic presentation characteristics of virus-containing beads, prepared at 1% GA final concentration, will be tested in vivo, and its efficacy will be compared with the response elicited by antigen emulsified in FIA. Once the adjuvant activity of the RSA beads has been demonstrated, the conditions for optimizing this adjuvant effect must be determined.

Albumin beads cross-linked at final GA concentrations of 2, 3 and 4% were resistant to chymotrypsin digestion (Lee et al, 1981; Royer et al, 1983). The compactness of these beads due to the close proximity of the interlinked polypeptide chains, presumably prevents the correct orientation of the substrate in the enzyme's active site (Lee et al, 1981).

This indicates that a lower GA concentration should be used, so that the resultant beads are degraded in vivo. However, if the concentration of GA is too low, the beads will be mechanically unstable and therefore degraded too rapidly in vivo to allow for sustained release.

The effect of the bead adjuvant on the secondary antibody response will be investigated. It is hoped that the beads will behave as other adjuvants, in stimulating both higher primary and secondary responses. Before the beads can be used as general vaccines, it must be shown that they do not suppress the generation of the immunological memory.

If vaccines which were stable even under hot ambient conditions were available, effective immunization programmes could be executed, particularly in undeveloped rural areas where the necessary facilities for storage and refrigeration of vaccines are minimal or nonexistent. Experiments will therefore be performed to investigate whether antigens incorporated into RSA beads retain their immunogenicity when stored under ambient conditions.

Results

Immunogenicity of RSA bead vaccines

The immunogenicity of RSA bead vaccines was ascertained and compared with other means of eliciting an immune response. Three experimental vaccines were injected i.m. into groups of 4 rabbits.

Each rabbit received 75/4 µg of purified Nodamura virus in one of the following formulations:

(1) 0.5 ml 0.02M phosphate buffer, pH 7.4, (PB).

(2) emulsified in FIA.

(3) polymerized into RSA beads with 1% GA.

The ELISA results in Figure 3.1. show that the control vaccine, Nodamura virus in PB, induced an initially high specific humoral response, which peaked at day 24 post inoculation and then declined rapidly.

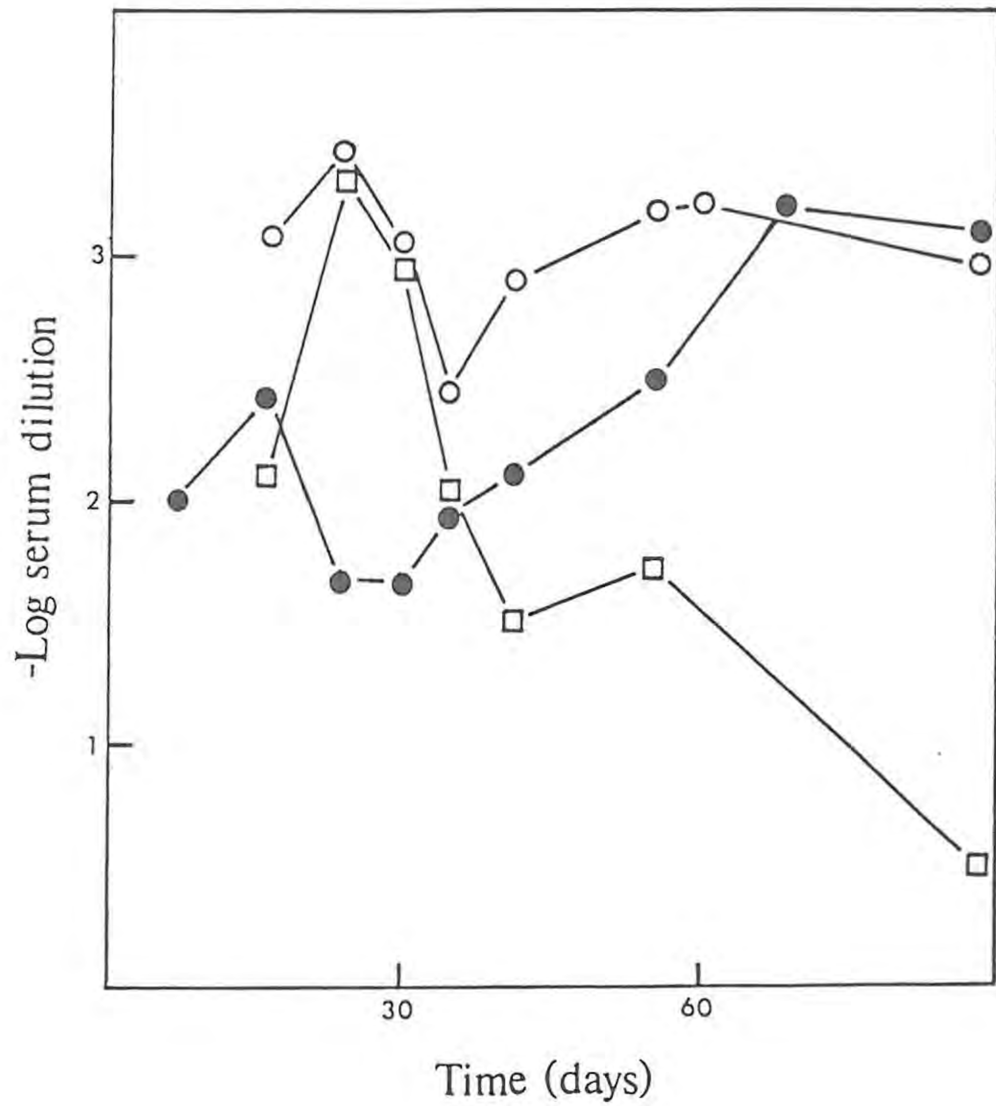


Figure 3.1. Comparison of specific IgG in serum from rabbits immunized with Nodamura virus in PB (□); emulsified in FIA(○); polymerized into RSA beads (●).

The virus preparations, in FIA or in the RSA beads, induced comparable antibody titers which remained high for the 90 day test period. The bead vaccine appeared to elicit its primary response at day 20, slightly earlier than the other two vaccine preparations, followed by a lag before serum titers rose and were maintained at a high level. This bimodal effect will be discussed later.

Optimal level of cross-linking for RSA bead vaccines

Antigen release from RSA beads relies upon breakdown or erosion in the animal tissue. The rate of degradation is dependent upon the level of cross-linking induced in their formation. The findings of Lee *et al* (1981) suggested that beads prepared using less than 0.7% GA had a short life span, possibly only a few days, whereas those prepared with a concentration greater than 1.5% did not break down for several weeks.

To determine the optimal level of cross-linking, aliquots containing Nodamura virus and RSA were mixed with the following final GA concentrations; 0.7, 0.9 and 1.1%. The bead vaccines were inoculated into groups of rabbits which were subsequently bled at appropriate intervals. A control vaccine consisted of Nodamura virus in 5% glycerol in PB.

The ELISA titers in Figure 3.2. show that each vaccine preparation induced the formation of virus specific antibodies. However, there were significant variations in the kinetics of the humoral response of the different vaccines. The bead vaccine prepared using 0.7% GA induced serum titers similar to those obtained with the control

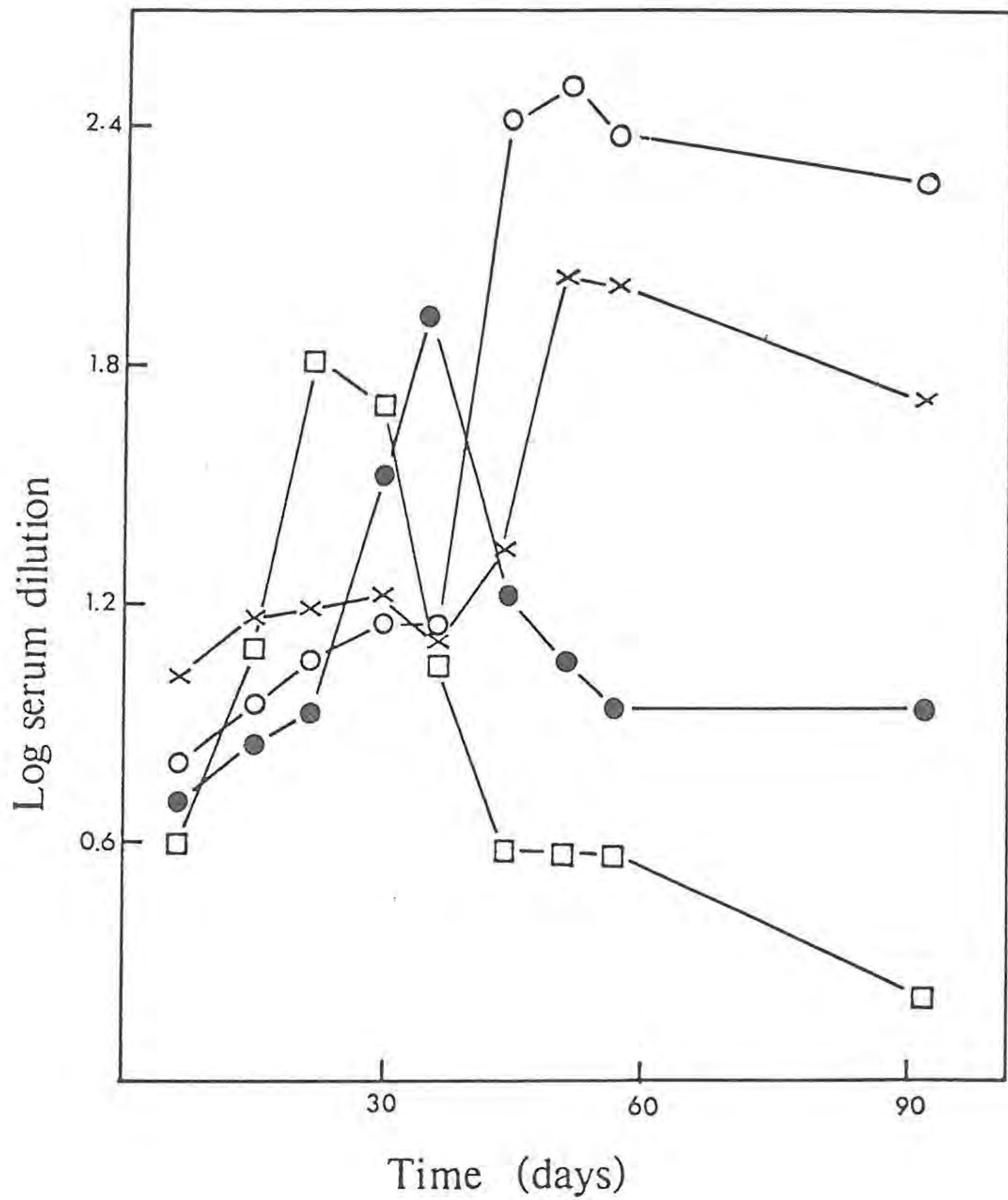


Figure 3.2. Comparison of specific IgG in serum from rabbits immunized with Nodamura virus in PB(□); and in RSA beads crosslinked with GA final concentrations: 0.7%(●);0.9%(×) 1.1%(○).

vaccine, but reached a maximum approximately 10 days later. In common with the control vaccine, this was followed by a rapid fall in titer until day 60 when it was maintained until the experiment was terminated. Vaccines prepared using 0.9% and 1.1% GA induced the formation of significantly higher IgG titers although maximum levels were not reached until about 60 days after inoculation.

After 60 days, the IgG titers in the 0.9% GA experiment fell slightly while those obtained using 1.1% remained high.

In vitro degradation of RSA bead vaccines

The differing rates of antibody production in Figure 3.2. suggest that antigen release coinciding with the gradual breakdown of beads is related to the extent of crosslinking. To investigate this interpretation further, ^{125}I-radiolabelled virus and RSA were polymerized into beads with 0.7, 1.0 and 1.3% final GA concentrations. The beads were incubated at 37°C in 25 ml PB in an orbital shaker (Gallenkamp, 40 rev/min). A preparation of beads (1% GA) was incubated with lysozyme (1 mg/ml, specific activity about 22 000 U/mg) (Boehringer, Mannheim.). Aliquots were withdrawn at various intervals and counted directly using a gamma radiation counter. Their breakdown was monitored by the release of radioactivity into the supernatant.

Although this experiment does not duplicate the *in vivo* situation, it illustrates that the rate of virus release is governed by the level of crosslinking, (Figure 3.3).

Several workers (Edman *et al*, 1983; Artursson *et al*, 1983, 1984) have shown that microparticles are rapidly taken up by cells of the respiratory endothelial system (RES) and introduced into the lysosomal

vacuome. Since albumin microspheres are biodegradable (Burdine et al, 1971) the effect of proteolytic enzymes, like lysozyme, on the bead vaccines was investigated. The presence of lysozyme accelerated the bead degradation (Figure 3.3.), indicating that the presence of this and other enzymes, possessed by macrophages (Klein, 1982) in the animal, would enhance the rate of antigen release.

The effect of the RSA bead adjuvant on secondary antibody response

To study the effect of the RSA bead adjuvant on immunological memory, rabbits received an antigenic dose of 75/4 µg Nodamura virus either incorporated into beads or as an aqueous solution in 5% glycerol in PB. On day 21, a booster injection of the same vaccine as used for the primary inoculation was administered. The rabbits were bled at regular intervals until day 90 when the experiment was terminated. A control group of rabbits were inoculated with a single dose of the bead vaccine.

The sera was assayed using ELISA and the effect of vaccination is shown in Figure 3.4. Both vaccines showed a strong secondary response. The bead vaccine response after the secondary dose rose more gradually, peaking around day 42, than the aqueous vaccine although the final specific serum antibody titers were superior. A comparison of the two bead vaccine experiments indicates the dramatic increase in antibody titers is due to the booster inoculation.

Immunogenicity of stored bead vaccines

RSA-Nodamura virus bead vaccines were stored under various conditions to assess their stability and subsequent immunogenicity. The RSA-

virus bead vaccines were stored at either -22°C or at room temperature in a vacuum desiccator. The beads stored at -22°C were injected into rabbits after 6.5 and 13.5 months and those at room temperature (20-25°C) after 1, 2, 3 and 5 months. Control vaccines, consisting of freshly prepared RSA-Nodamura bead vaccines, were inoculated into rabbits in parallel with the other vaccines.

The sera collected over the 3 month test period were assayed for virus specific antibodies using ELISA. Figure 3.5 indicates that beads retain their immunogenicity when stored at -22°C in the dry form after 6.5 and 13.5 months. One aspect which seems to be associated with storing beads at -22°C is that as the storage period increases the lag phase prior to the induction of specific viral antibodies lengthens. If this lag phase is of significance, it may be due to the partial degradation of the surface exposed antigen. However, comparable titers were obtained once the serum antibody levels plateaued at about day 42.

The results in Figure 3.6 illustrate the immunogenicity of the bead vaccines stored at room temperature. The beads are less stable at room temperature and their subsequent immunogenicity is influenced by the storage time. Storage for 1 month had no adverse effect on the immunogenicity of the bead vaccines while 2 month old beads were still able to potentiate the immune response, although the serum titer profile was more eratic and titers declined gradually after day 60. Beads stored for both 3 and 5 months were weakly immunogenic. The bead structure did not appear altered (Figure 3.7) after 5 months. Therefore, this loss of antigenicity is most probably due to the

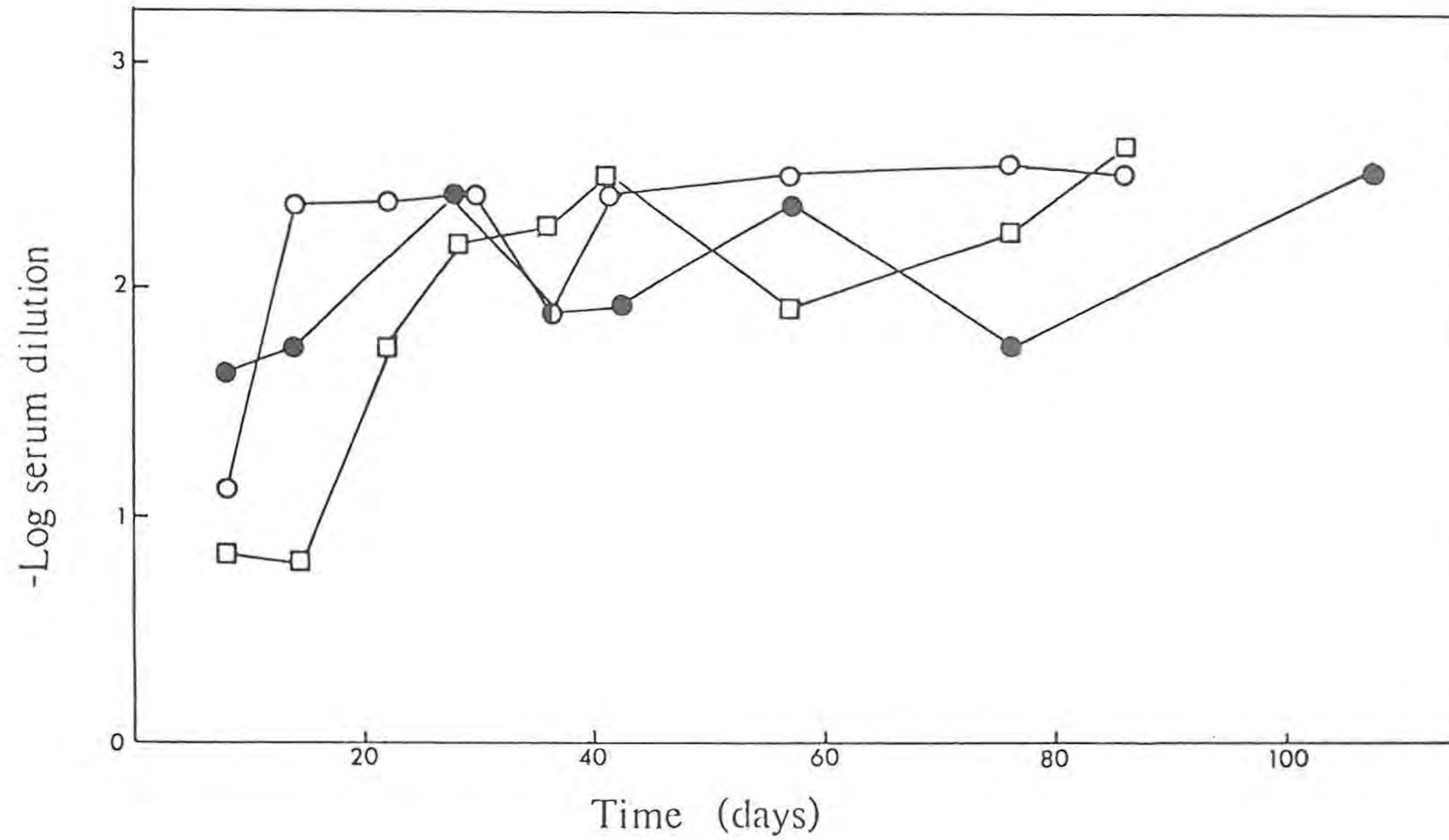


Figure 3.5. Comparison of virus specific antibodies induced by bead vaccines when stored at -22°C for; 0 months (○); 6.5 months (●); 13.5 months (□).

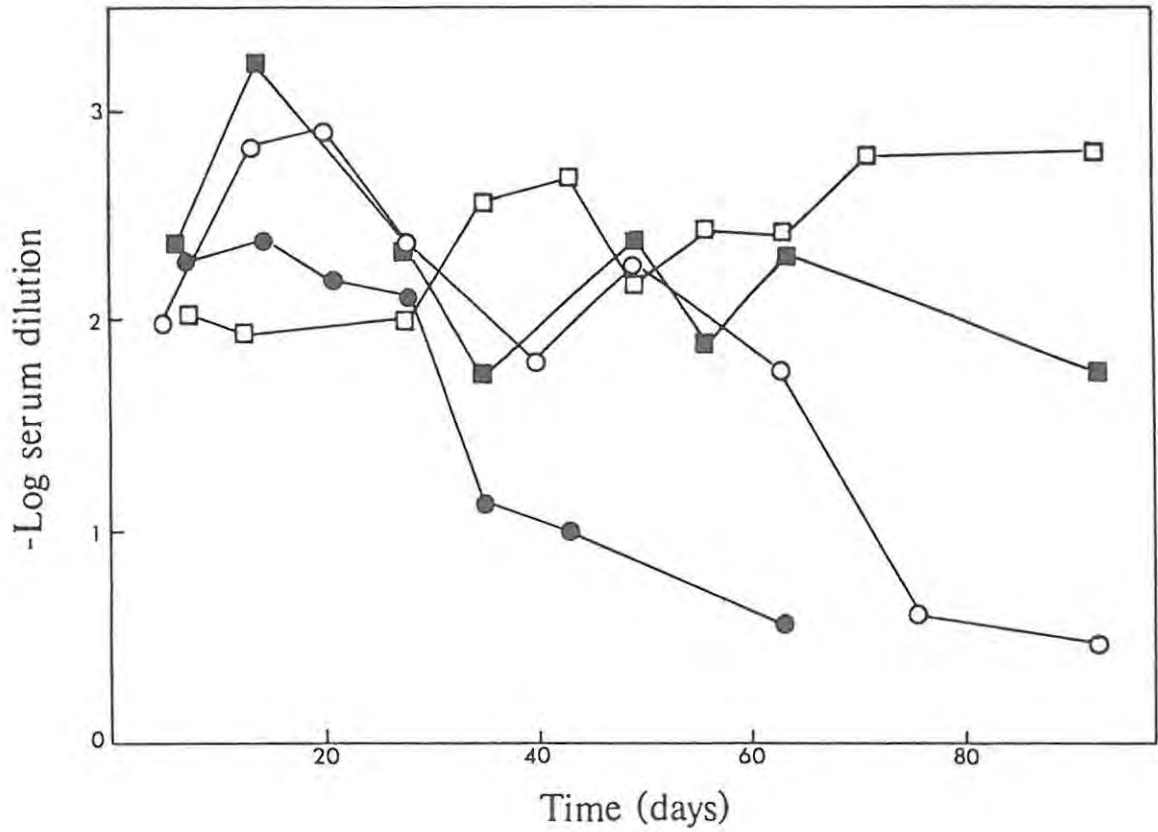


Figure 3.6. Comparison of virus specific IgG antibodies induced by Nodamura virus in RSA beads stored at room temperature for;1 month (□); 2 months (■); 3 months (○); 5 months (●)

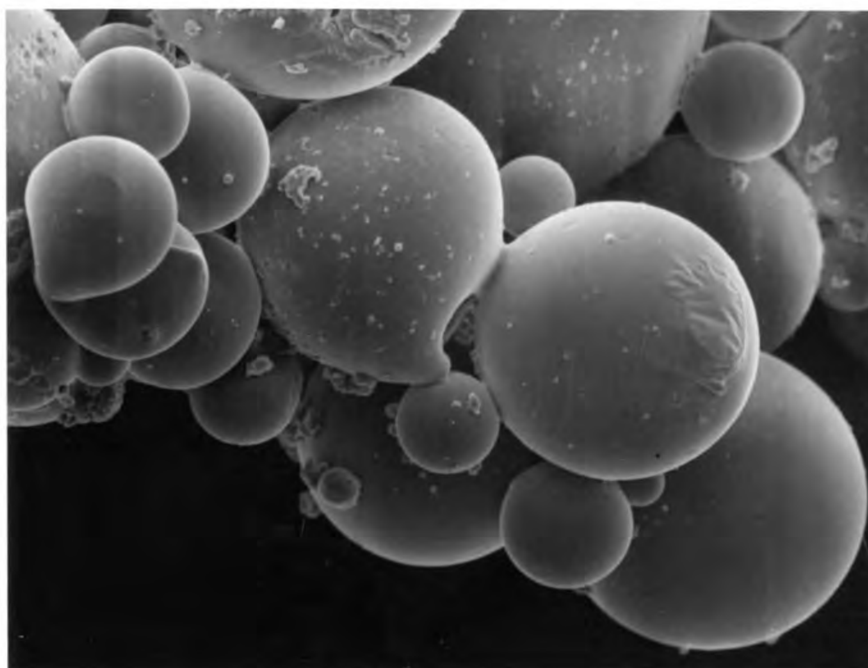


Figure 3.7. Scanning Electron Micrograph of polymerized RSA beads after storage at room temperature for 5 months.

instability of the viral antigen itself under these conditions.

Is immunostimulation by RSA beads due to the presence of free GA on the bead surface?

The adjuvant activity of the beads might be a result of free GA on the bead surface, linking host cells to the beads after inoculation, and in this way inducing a strong immunological response.

In order to examine this possibility, Nodamura virus-RSA beads were prepared and any free GA subsequently blocked. The GA was blocked by incubating the beads in 1M ethanolamine for 2 hours at room temperature with mechanical stirring. The beads were washed with several changes of distilled water and dried, prior to immunization into a group of rabbits. The control group each received 25μg Nodamura virus polymerized into RSA beads which were untreated.

The results in Figure 3.8 indicate that there was no significant difference in the kinetics of the virus specific humoral immune response whether the beads were treated or not. This indicates that immunostimulation is due to the slow release of the antigen from the bead as it is degraded.

Discussion

These experiments demonstrated that antigen incorporated into controlled release RSA beads is an effective system for stimulating long-lasting serum antibody production which is comparable with FIA.

The preparation of the RSA beads is simple, inexpensive and rapid. No specialized equipment is required. Furthermore, the polymerization

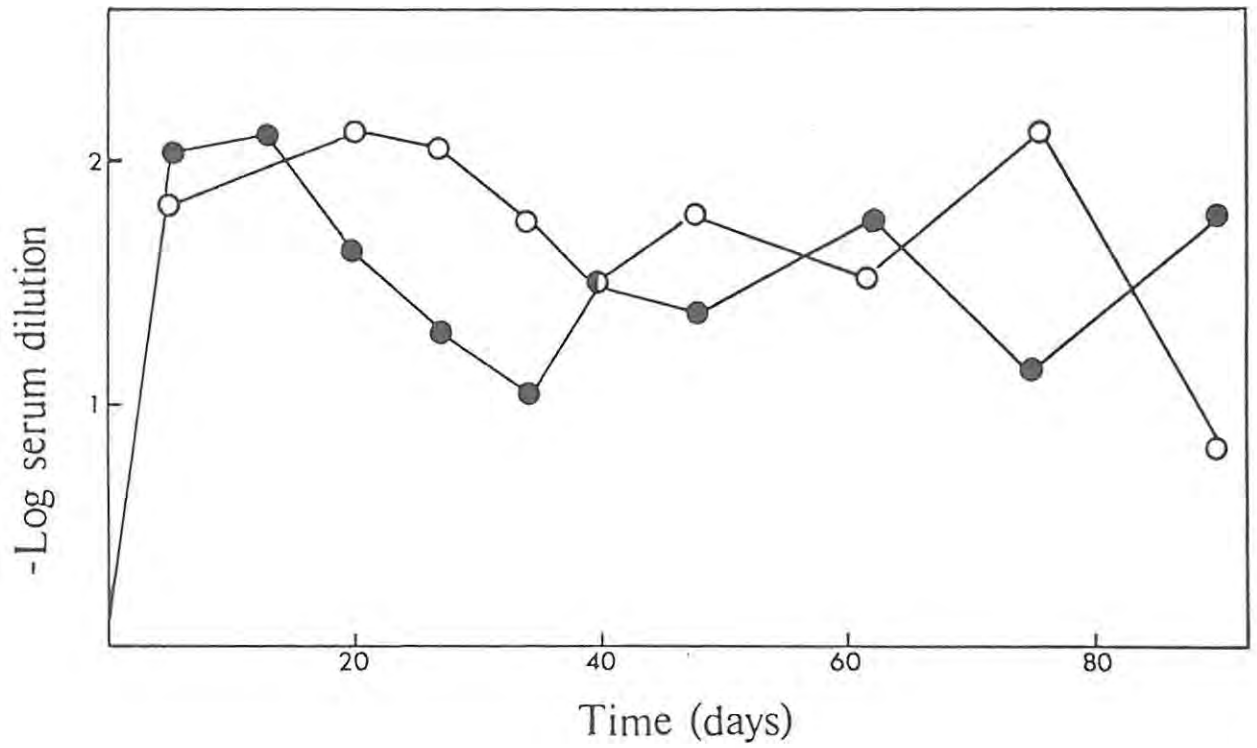


Figure 3.8. The determination of the effect on the virus specific IgG response of blocking free GA on the surface of RSA beads. Rabbits each received (i.m.) 25µg Nodamura virus in RSA beads; untreated (○); treated with 1M ethanolamine (●).

process is mild and nondenaturing. With crosslinks residing mainly in the interior, the surface of the bead seems to pass as native albumin (Royer et al, 1983) inducing no adverse immunological responses (Lee et al, 1981; Royer et al, 1983; Dewar et al, 1984). It should be noted, however, that these researchers only examined the animal for gross immunological changes (i.e., inflammation, granulomas, abscesses, body temperature and weight fluctuations). The vaccinated serum must therefore be examined, using sensitive techniques, for the presence of anti-albumin/matrix antibodies.

GA reacts with the ε-amine groups of lysine residues (Peters and Richards, 1977). Thus, the polymerization process will result in albumin-albumin, albumin-virus and virus-virus interactions. However, since the ratio of albumin to virus is 2.4×10^5 :1 (using Avogadros' number), the majority of interactions will be albumin-albumin, and therefore the proportion of lysine residues, which by virtue of their hydrophilic nature (Hopp and Woods, 1981) might lie in the neutralizing epitope(s) of the virus, involved in the polymerization process would be minimal. This was confirmed, since neutralizing antibodies were elicited when rabbits were immunized with Nodamura virus-RSA bead vaccines (Dewar, 1985).

The results have shown that the beads are biodegradable (Figure 3.3), and that the rate of antigen release is controlled by the extent of cross-linking. Since the viral antigens are of high molecular weight, the release of those entrapped, but not linked to the bead matrix, will depend on degradation rather than diffusion. 1.1% GA crosslinked beads appeared to be the most promising candidates for vaccination

protocols.

The results in Figures 3.5 and 3.6 indicate the potential use of the bead vaccines, as stable vehicles for vaccine delivery. When Clostridium botulinum D toxoid was incorporated into RSA beads and immunized into rabbits after being stored at room temperature for 4 months, it induced protective antibodies. (Colin Langheim, pers. comm.). Therefore antibodies might be raised to bead vaccines if a stable antigen (subunit or synthetic peptides) was incorporated into them. Their superior stability over aqueous vaccines means that they could be stored and transported in a dry form without refrigeration. This could represent significant savings on transport costs and reduce the necessity for a cold chain, particularly in poor countries. It would ensure the availiability of vaccines to isolated areas and thus eliminate the possibility of immunity deficits forming and the consequent epidemics occurring. The potential of these beads would be enhanced with the incorporation of more stable antigens such as synthetic peptides, into bead vaccines.

The bead vaccines elicited a normal secondary immune response (Figure 3.4), indicating that the beads do not exert a suppressive effect on the antigenic expression of the viral antigen. It still remains to be established whether immunization of an antigen eg. "A", in the beads results in the suppression of the specific immune response to antigen "B" in beads delivered subsequently to the individual.

It is speculated that the bimodal profile observed in Figure 3.1. is related to the antigenic processing of the bead vaccine. Some of the antigen incorporated into the beads will be partially exposed on the

surface of the bead, while the remainder will be entrapped within the bead matrix and effectively masked from the immune system.

Since the RSA beads are too large (50-100μm) to be removed via the lymphatics, which have openings as large as 10μm, they will remain at the injection site until they are degraded.

On inoculation, there will be an infiltration of nonspecific macrophages to the target site. The repeated exposure of the antigens on the surface will elicit a primary response which because of the repetition will actually enhance the immunogenicity, (Figure 3.1, the first peak at day 20).

Since the beads are too large for normal phagocytosis by macrophages, the macrophages probably fuse to form giant cells which can contain 20 or more nuclei. Normal encapsulation is followed by proteolysis by lysosomal enzymes, exposing previously masked antigenic determinants (Klein, 1982). A small part of the antigen ingested by macrophages is prevented from complete digestion by lysosomal enzymes and is associated with the membrane of the macrophage. This membrane-associated antigen is particularly immunogenic (van Rooijen and van Nieuwmegen, 1983). Antibody production is stimulated, and specific activated macrophages will converge at the injection site for further antigenic processing. The lag period observed (Figure 3.1.) might possibly correspond to the stages of initial proteolysis occurring before internal antigens are unmasked.

With the booster immunization, the surface exposure of the antigen will be of great importance. Antigen-specific antibodies in circulation can then bind to the bead associated antigen. Then the

opsonized beads will be available for interaction with Fc-receptors on the macrophages.

The processing of the bead vaccines is probably quite complex since their size would hinder normal phagocytosis by cells of the immune system. However, no inflammation or other side effects resulted from bead vaccine immunization, (Lee et al ,1981; Royer et al, 1983; Dewar et al, 1984).

The GA-polymer amine complex is self-limiting in size and can undergo internal rearrangement to become chemically inert (Cheung and Nimni, 1982). This theory explains why the antibody response, induced by bead vaccines which were treated to block free GA on the bead surface, was comparable with that of untreated beads. This experiment supports the hypothesis that the enhanced antibody response is achieved by the slow release of antigen, from the beads rather than free GA on the bead surface binding cells to the beads, and in this way causing an immunopotent response.

Since RSA bead vaccines represent safe and promising candidates for sustained release of antigen, the possibility of potentiating their adjuvanticity by the incorporation of synthetic immunostimulators will be examined.

CHAPTER 4

POTENTIATION OF THE IMMUNE RESPONSE WITH A SYNTHETIC IMMUNOSTIMULATOR

Introduction

The current trend, of increasing the purity of antigens decreases their side effects but usually also decreases their immunogenicity. This is characteristic of subunit and synthetic peptide vaccines. Therefore the need for effective adjuvants continues.

Until very recently, most immunopotentiating agents were complex and ill defined. This is changing.

Freund's complete adjuvant (FCA) is a water-in-oil emulsion containing killed mycobacteria. The immunostimulatory property of FCA is generally considered to be due to two distinct mechanisms. The mycobacterial cell wall component acts directly to stimulate cells of the immune system, while slow release from the oil emulsion is thought to ensure that the antigen is present in the animal tissue for an extended period of time. FCA can increase, very powerfully, both humoral and cell mediated immune responses (Freund, 1956). In fact, FCA is so efficient that it can induce autoimmune responses to autologous proteins (Paterson, 1966; Sakata and Atassi, 1981; Atassi et al, 1982) presumably by overcoming T cell requirements. It is, however, responsible for several undesirable effects such as granuloma formation, lymphoid hyperplasia, sensitization to mycobacterial antigens (tuberculin) and adjuvant polyarthritis. The therapeutic use of FCA is limited because of these side effects, inherent to mycobacteria, and also due to the presence of nonmetabolizable mineral

oil. Several studies were undertaken to characterize the minimal adjuvant unit of mycobacteria and to dissociate the various biological activities.

N-acetylmuramyl-L-alanyl-D-isoglutamine, denoted muramyl dipeptide or MDP, represents the smallest part of the repetitive unit of the bacterial cell wall peptidoglycan with adjuvant activity, that can substitute for mycobacteria (Ellouz et al, 1974; Kotani et al, 1976).

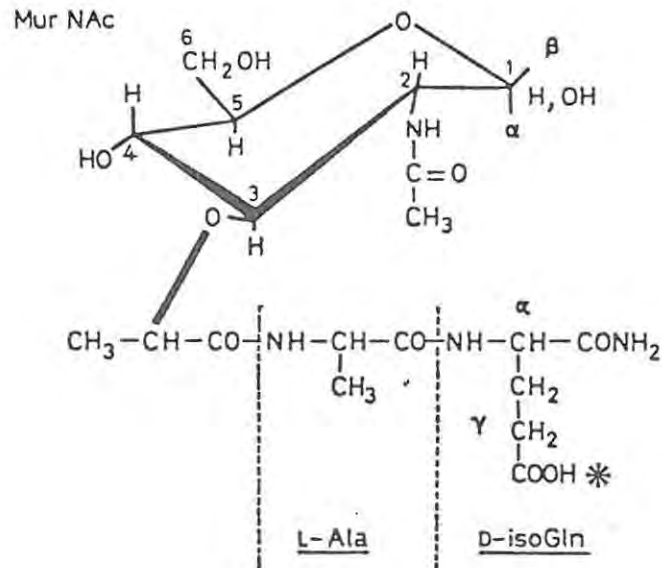


Figure 4.1. The chemical structure of muramyl dipeptide. * indicates the carboxyl group involved in the crosslinking reaction, with CDI, to Nodamura virus.

The adjuvant activity of MDP does not possess the adverse side effects associated with mycobacteria (Audibert et al, 1976; Chedid et al, 1978). No changes in blood pressure, in leukocyte and erythrocyte counts occurred and no inflammatory skin reaction was elicited (Masek,

K. quoted in Edelman _et al_, 1980). However, when MDP was administered with FIA, it induced delayed hypersensitivity and even autoimmune diseases (Chedid _et al_, 1978).

When MDP is conjugated to an antigen or carrier and administered in saline (Mozes _et al_, 1980), it elicits comparable or even higher titers than FCA (Audibert _et al_, 1976; Arnon _et al_, 1980). Conjugation of MDP to the antigen and/or a carrier, modified the rapid clearance of free MDP (Chedid _et al_, 1979) and resulted in higher antibody levels than when admixed with the antigen (Chedid _et al_, 1979; Arnon _et al_, 1980; Mozes _et al_, 1980). This has the advantage of reducing antigenic doses.

MDP is capable of inducing humoral immunity, when administered with an antigen in saline, with no adverse reactions (Audibert _et al_, 1976; Chedid _et al_, 1976). It induces an antibody response when given orally, or if the antigen is given by an independent route (Chedid _et al_, 1976). Timing studies have shown that adjuvant and antigen must be injected together to obtain an enhanced specific immune response to the antigen.

MDP has previously been shown to be nonimmunogenic when existing as an integral part of the cell wall (Audibert _et al_, 1976; Reichert _et al_, 1980). Its conjugation to carrier proteins (Reichert _et al_, 1980) or to synthetic peptides (Chedid _et al_, 1979; Arnon _et al_, 1980; Mozes _et al_, 1980) enhances its immunogenicity. Antibodies reacting with MDP are directed mainly against a determinant that includes both the dipeptide and N-acetyl muramic acid, with the latter being its

immunodominant part (Mozes et al, 1980; Reichert et al, 1980).

Since MDP can be obtained synthetically, many derivatives have been prepared by chemical modifications and the relationships between the chemical structure and the biological activity have been studied (Kotani et al, 1976; Lefrancier et al, 1977, 1978, 1979; for review see Chedid et al, 1978; Lefrancier and Lederer, 1981). Even though tentative studies to relate these parameters did not always result in strict correlations, many properties have already been dissociated (Lefrancier and Lederer, 1981).

In addition to enhancing specific immune responses, MDP and several of its derivatives stimulate nonspecific immunity (Chedid et al, 1977, 1978, 1979; Arnon et al, 1980; Ausobsky et al, 1984). Although Mitsuzawa et al (1984) reported that pretreatment with MDP in saline alone did not protect mice against experimental allergic encephalomyelitis, coupling of MDP to a macromolecule dramatically increases its capacity to enhance natural resistance in mice towards bacteria. Thus, MDP covalently attached to A--L, effectively protected mice from infection with Klebsiella pneumoniae (Chedid et al, 1979). Parant and Chedid (1985) reported a study, where MDP, administered 24 hours or more before the infectious challenge, was found to be optimal to ensure a protective effect. However, when MDP was injected prior to the antigen and at relatively high doses, it could even suppress the specific immune response (Leclerc et al, 1983).

Dissociation between adjuvant and anti-infectious activities has been shown, and increasing effectiveness was maintained with compounds

lacking the N-acetyl muramyl moiety that were usually devoid of adjuvant activity (Chedid _et al_, 1979).

The biological activity of MDP is less selective than had originally been assumed and this small molecular weight (496 daltons) glycopeptide still retains certain unwanted effects of the bacterial peptidoglycan.

One of the most commonly reported side effects of MDP and some of its derivatives is pyrogenicity in rabbits, by various routes even orally (Kotani _et al_, 1976; Riveau _et al_, 1980). This is even more pronounced once conjugated (Chedid _et al_, 1979). Adjuvanticity and pyrogenicity were initially postulated to be linked (Kotani _et al_, 1976). However, there are several derivatives of MDP that have adjuvant activity but are non-pyrogenic. These include murabutide or MDP-butyl ester (Chedid _et al_, 1982; Damais _et al_, 1982) and 3-n'-propyl-MDP (Byars, 1984). Murabutide has received favourable attention because repeated injections into animals produced no adverse reactions. It is well tolerated in humans and has been selected for clinical trials because of the absence of any side effects in several studies, and in toxicological assays conducted in various animal species (Studies cited in Parant and Chedid, 1985). Dissociation between toxicity and adjuvant activities have been demonstrated with 3-n'-propyl-MDP. This derivative is nontoxic, nonpyrogenic and adjuvant-active, and is also an attractive compound for use in human vaccines (Byars, 1984).

Monocyte-macrophage cells are the primary target and effector cells of MDP and its adjuvant active analogs (Wahl _et al_, 1979; Galelli & Chedid, 1983; Ausobsky _et al_, 1984; Byars, 1984). T and B lymphocytes

are also involved, (For review see Chedid et al, 1978; Ausobsky et al, 1984). Macrophages can display considerable influence on their environment through the secretion of regulatory molecules. The main mediator, that adjuvants induce macrophages to release, is lymphocyte-activating-factor or Interleukin-1 (LAF/IL1). Macrophage activation is also evidenced by stimulation of prostaglandins, cyclic AMP, collagenase and superoxide. (Studies cited in Byars, 1984).

MDP and its pyrogenic derivatives induce the production of endogenous pyrogen (EP). Dissociation studies have demonstrated EP and LAF are not the same molecules (Byars, 1984). Similarly the guinea pig distress factor (GPDF) and LAF stimulated by MDP and its toxic analogues (in guinea pigs) are not the same molecules, although GPDF may have LAF activity (Byars, 1984).

Therefore, it should be possible to avoid most of the pharmacological reactions elicited by MDP by the proper choice of a derivative that still displays similar immunostimulant properties.

To elucidate further the actions of MDP, Galelli and Chedid (1983) assessed the effects of MDP, an adjuvant-active derivative MDP-butyl ester and an adjuvant-inactive stereoisomer MDP (D-D), on the induction of serum colony-stimulating activity (CSA) and on multipotential (CFU-S) and committed (GM-CFC) hematopoietic stem cells. Results showed that both MDP and MDP-butyl ester induced a monocyte-macrophage CSA in serum, which stimulated the proliferation of CFU-S and increased the number of splenic GM-CFC. In contrast, MDP(D-D) induced only low levels of serum CSA.

A consequence of the effects induced by MDP would be the availability of greater numbers of macrophages, able to process the antigen thereby regulating humoral immune responses, or to enhance nonspecific resistance to infection. Increased phagocytic function of fixed macrophages of the RES was observed (Ausobsky *et al*, 1984).

MDP and several of its derivatives have been successfully employed to enhance the immunogenicity of viral and bacterial antigens as well as synthetic peptides (Audibert and Chedid, 1980; Arnon *et al*, 1980; Mozes *et al*, 1980; Audibert and Jolivet, 1982). The fact that different adjuvants enhance the immune response, by different effects, suggests that the adjuvant effect of serum albumin beads, by slow release of antigen, may be potentiated by combination with other immunostimulators like MDP.

MDP will be conjugated to Nodamura virus and incorporated into RSA beads. Its efficacy will be compared to a vaccine prepared in FCA. Unconjugated MDP, admixed with virus, will be incorporated into beads to determine whether conjugation of MDP to the virus elicits higher antibody responses, as previously reported (Chedid *et al*, 1979; Mozes *et al*, 1980; Arnon *et al*, 1980). The carbodiimide crosslinker, most commonly used to conjugate MDP to the antigen or carrier (Chedid *et al*, 1979; Arnon *et al*, 1980; Mozes *et al*, 1980; Reichert *et al*, 1980) will be employed. Conjugation does not abolish the adjuvant activity and the mild reaction conditions ensure that denaturation of the antigen does not occur.

Results

The possibility of incorporating the immunostimulator, MDP, into

polymerized serum albumin beads to enhance their adjuvanticity was investigated. Experimental vaccines were prepared with each rabbit receiving 25μg Nodamura virus in one of the following formulations; (i) resuspended in PB; (ii) polymerized into RSA beads at a final concentration of 1% GA; (iii) conjugated to MDP (100μg) with the crosslinker, CD1, before polymerizing into RSA beads (MDP-V); (iv) mixed with MDP (100μg) and polymerized into RSA beads without prior conjugation (MDP+V); and (v) emulsified in FCA.

MDP was conjugated to the antigen to avoid the stimulation of many different clones of immunocompetent cells and thus prevent possible non-specific or even adverse immunologic responses. Conjugation would therefore target the immunogen to a specific clone of responding immunocompetent cells.

The adjuvant effect of MDP was compared with both FCA and bead vaccines. The ELISA results depicted in Figure 4.2, indicate that, under the conditions used in this experiment, MDP did not potentiate the immune response. The initial serum titers, elicited by the MDP-V vaccine, were superior to those induced by the FCA adjuvant vaccine. This indicates the superior adjuvant properties of MDP over FCA as reported by other workers (Audibert _et al_, 1976; Arnon _et al_, 1980). However, after day 45, the titers fell rapidly whereas the serum IgG levels in the FCA experiment peaked at day 70 and were maintained for the duration of the experiment. This result could possibly be attributed to that portion of the MDP-virus conjugate exposed on the bead surface whereas the entrapped conjugate was inactive.

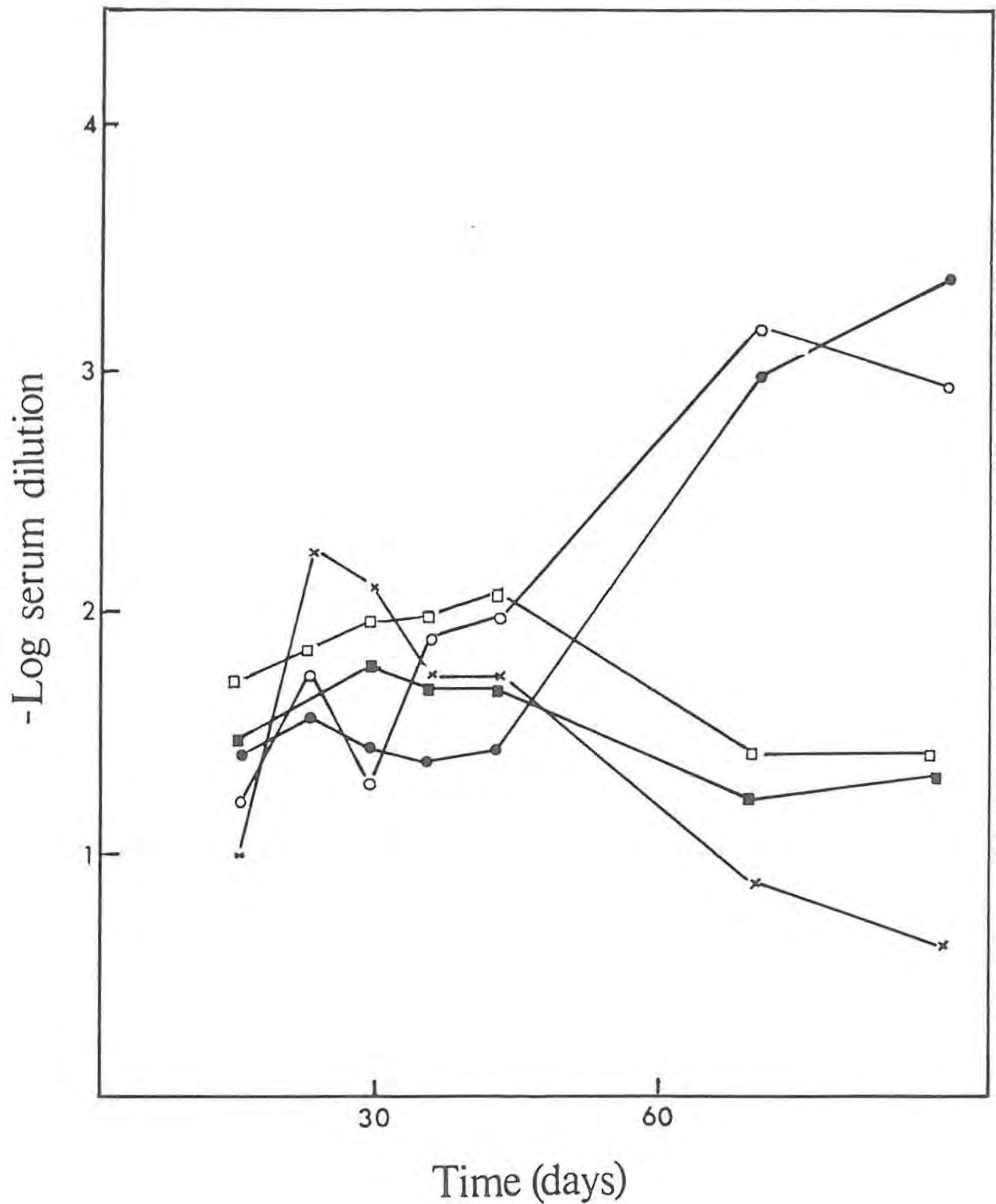


Figure 4.2. Comparison of specific IgG in serum from rabbits immunized with Nodamura virus in PB(×); emulsified in FCA (●); polymerized in RSA beads (○); crosslinked to MDP with CDI before polymerization into beads (□); admixed with MDP before polymerization into beads (■).

The bead vaccine was more effective than the MDP-V, inducing higher and longer lasting antibody titers (Figure 4.2). These results suggest that the immunostimulatory properties of MDP may have been blocked by the polymerization process involving GA, or that the virus antigenicity may have been blocked or suppressed. Conjugation of MDP to the antigen resulted in a higher specific antibody response than when admixed, an observation consistent with other work (Chedid et al, 1979; Arnon et al, 1980; Mozes et al, 1980). It is, therefore, unlikely that the conjugation of MDP to virus, blocked viral antigenic sites since the immune response of MDP+V was even lower than that elicited by MDP-V (Figure 4.2). The adjuvanticity of MDP was blocked whether presented in the form of MDP-V or MDP+V.

MDP was covalently linked to the bead surface, without involving it in the bead formation step, in an attempt to potentiate the immunogenicity of the beads. This might then establish whether the immunostimulatory properties of MDP were blocked during the bead polymerization process or whether MDP actively suppressed the immune response.

Two groups of rabbits were injected with Nodamura virus bead vaccines, one with MDP covalently attached to the surface of the beads with CDI and one without. Each rabbit recieved an antigenic dose of 25μg virus and the one group also received an MDP dose equivalent to 250μg. The virus specific antibody response was evaluated by the ELISA technique (Figure 4.3). These results clearly indicate that MDP had a suppressive effect on the immune response to the viral antigen.

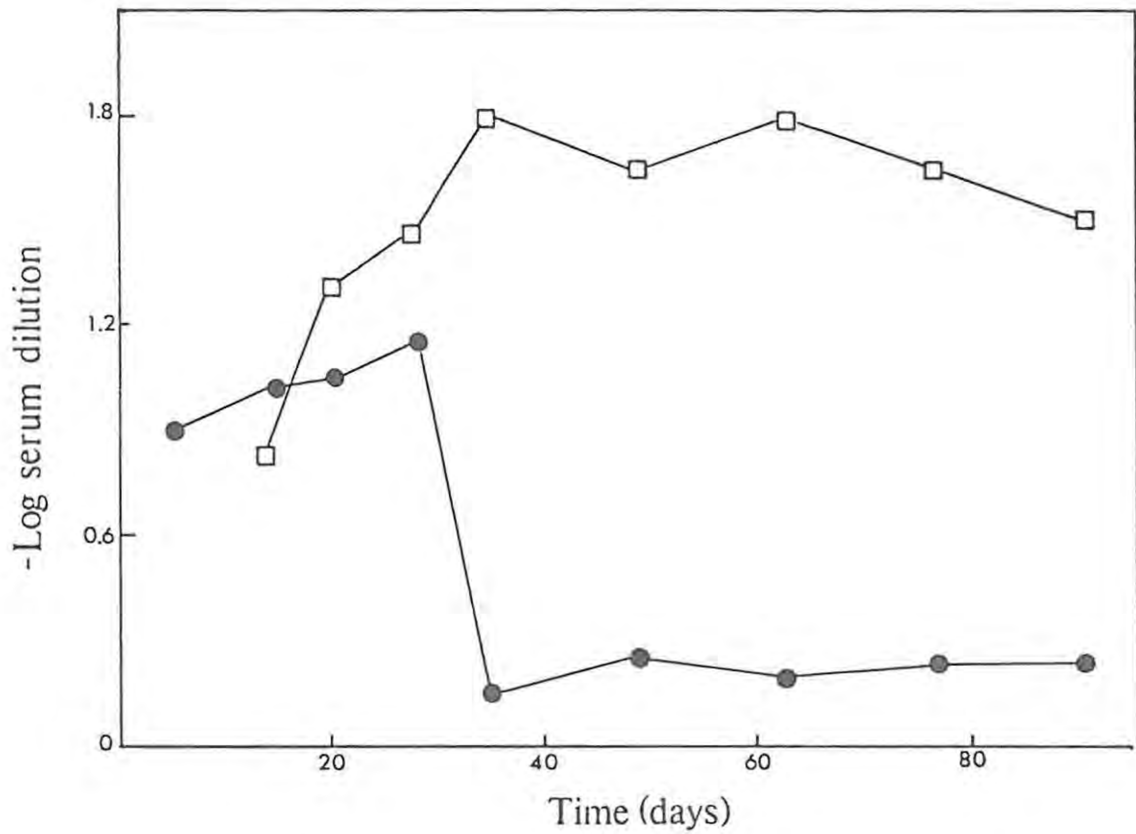


Figure 4.3. The effect of attaching MDP to the surface of Nodamura virus-RSA beads on the virus specific IgG response induced in rabbits immunized with; Beads (□); Bead with MDP attached using CDI (●).

Discussion

The involvement of MDP in the suppression of specific immune responses has been observed (Kishimoto *et al*, 1979; Bomford, 1980; Leclerc *et al*, 1983; Anderson, 1985).

Although FIA has been used with MDP without causing any suppression of the specific immune response (Audibert *et al*, 1976; Reichert *et al*, 1980), both Bomford (1980) and Anderson (1985) independently found that when antigen was injected with MDP, in combination with water-in-oil adjuvants, FIA or FCA, the immune response to the antigen was suppressed. The reason was unclear. However, one may speculate that since both water-in-oil emulsions and the bead vaccines exert their adjuvant action through the slow release of antigen, this may have in some way, influenced the activity of MDP (Figure 4.2).

Leclerc *et al* (1983) showed that when MDP was injected at relatively high doses, prior to the antigen, it suppressed the specific immune response. The results in Figure 4.3 may be explained in the following way. Since MDP is surface attached and the majority of the antigen is masked within the bead, the cells of the immune system would encounter MDP prior to the antigen, which would only be exposed as the bead degrades. Thus, MDP acting as a pretreatment, would stimulate suppressor cells with the result that the specific immune response to the entrapped antigen would be suppressed rather than enhanced.

A further possibility is that the activity of MDP may have been influenced as its movement was restricted by its association with the beads.

The results indicate that the mechanisms involved in adjuvant action are complex, and manipulations involving different adjuvants will not always necessarily produce the desired effect.

CHAPTER 5

MODE OF ANTIGENIC PRESENTATION

Introduction

Antigenic presentation to the cells of the immune system in the RSA beads described in previous chapters may be attributed initially to partial exposure of virus on the surface and, as the bead degrades, to the entrapped antigen. Although this mode of antigenic presentation has been shown to be highly efficient, the 'bead vehicle' could be extended in at least two ways which might potentially enhance its efficacy as a vaccine 'vector'.

Firstly, the antigen could be administered as a mixture of beads with antigen incorporated and free antigen. This mode of presentation may serve to regulate the immune response and eliminate the lag observed after the initial peak (see Chapter 3, Figure 3.1).

The second, more complicated method involves the covalent attachment of the antigen to the bead surface. The bead could also have virus incorporated but not necessarily. An advantage with this method is that it provides a means for administering antigens whose neutralizing epitopes are blocked by GA during the polymerization process.

There are generally two classes of crosslinkers, homofunctional reagents where the two reactive groups are identical (i.e. GA) and heterofunctional reagents where the two reactive groups are sufficiently different to permit well controlled sequential reactions of each group in turn.

The crosslinkers that will be utilized in this study, to attach Nodamura virus to the surface of RSA beads, are described below.

Glutaraldehyde (GA)

GA is one of the most useful and extensively studied protein reagents (Figure 5.1).

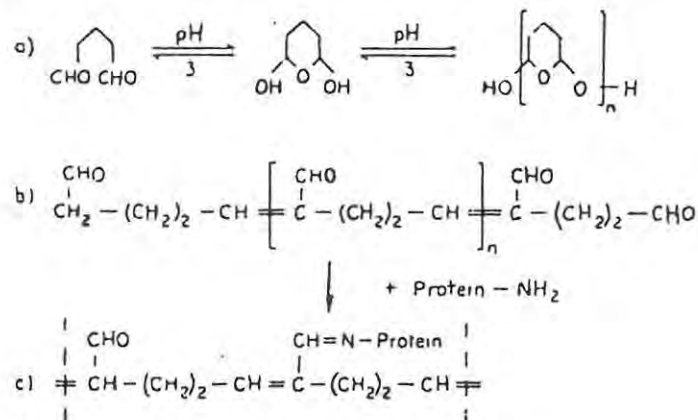


Figure 5.1. The structure and the simplified reaction of glutaraldehyde. The predominant forms of glutaraldehyde at (a) acid pH and (b) at neutral pH. (c). Example of a conjugated Schiff base formed between (b) and a protein amino group.

Although readily available commercially as a 25% aqueous solution, NMR studies showed that it contains virtually no free GA; 79%H_2O; 3% GA and 18% derivatives of higher molecular weight that could be broken down to GA.

GA only reacts with the ε-amino groups of lysine residues, however the mechanisms involved are more complicated than initially proposed (Figure 5.2). At acidic pH, GA is in equilibrium with its cyclic hemiacetal and polymers of the cyclic hemiacetal. When the pH is raised to the neutral or slightly basic range at which cross-linking is carried out, the dialdehyde undergoes an aldol condensation with itself, followed by dehydration, to generate $\alpha\beta$ unsaturated aldehyde polymers, whose length increases as the pH is raised until the polymer precipitates from solution (Peters and Richards, 1977)(Figure 5.1).

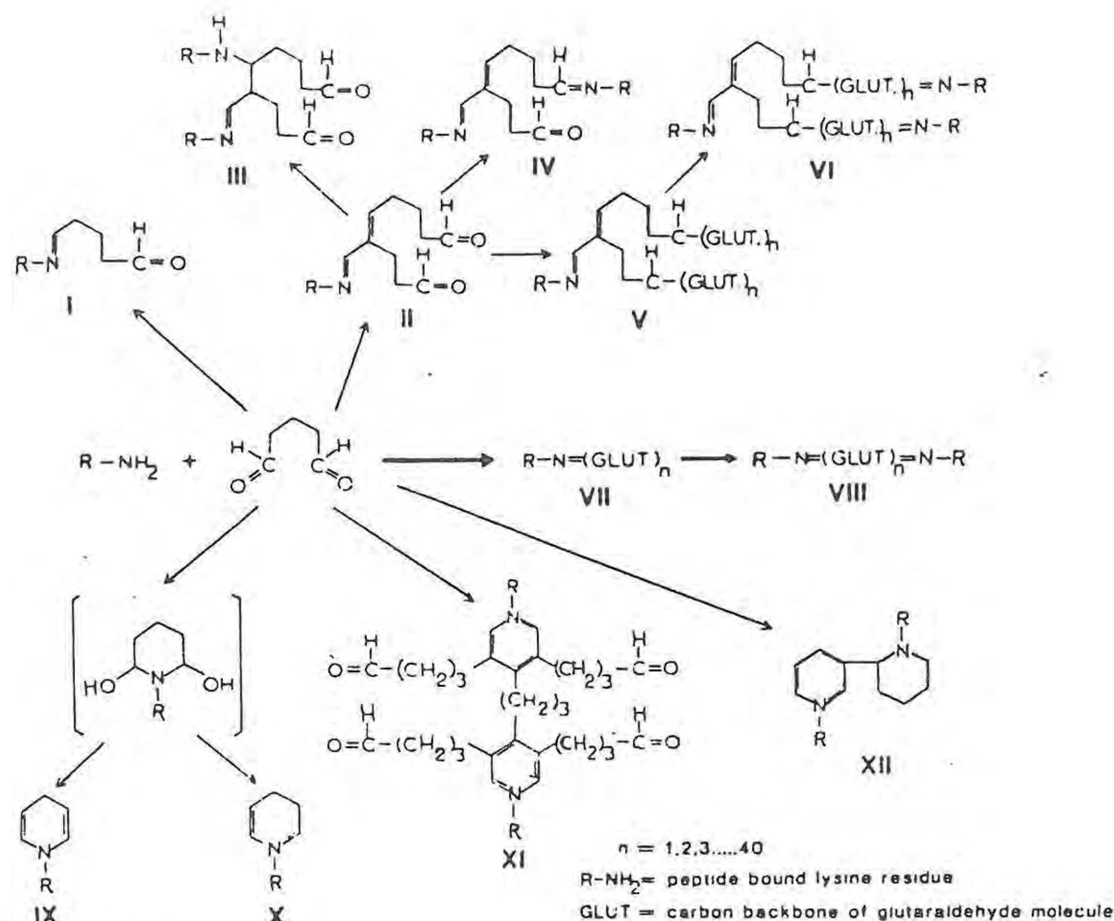


Figure 5.2. Schematic representation of possible reactions of the ε-NH_2 groups of peptide bound lysyl residues with glutaraldehyde. Compound I is a Schiff's base formed between glutaraldehyde and the amine. Compound II is the α,β-unsaturated (conjugated) Schiff's base, shown in Figure 5.1. Compound III is a subsequent Michael addition product of compound II. Compound IV is derived from the reaction between compound II and another amine. Compounds V and VI represent the glutaraldehyde polymer adducts or crosslinks which are also derived from compound II *via* Schiff's base catalyzed polymerization. Compounds VII and VIII may be structures similar to V and VI except through some other unknown reaction mechanisms. The exact chemical structures of these compounds are not yet understood. Only structures such as III, IV, VI and VIII represent the actual crosslinks formed between peptide chains. Compounds IX and X are possible dead-end dihydropyridine or dihydropyridinium products which follow the ring closure of an intermediate. Compound XI is an earlier model of the crosslink proposed by Hardy *et al*, 1953. Compound XII is their most recent suggested crosslink-anabilysine. These latter structures, however, do not reflect the larger molecular weight products observed by Cheung and Nimni(1982), (taken from Cheung and Nimni, 1982).

The predominant reactive species is the $\alpha\beta$ unsaturated aldehyde under the conditions used in this study.

The reaction is rapid and efficient, generating products of high stability. The success of GA as a cross-linking reagent is based on the large number of different types of molecules present simultaneously in the reagent solution.

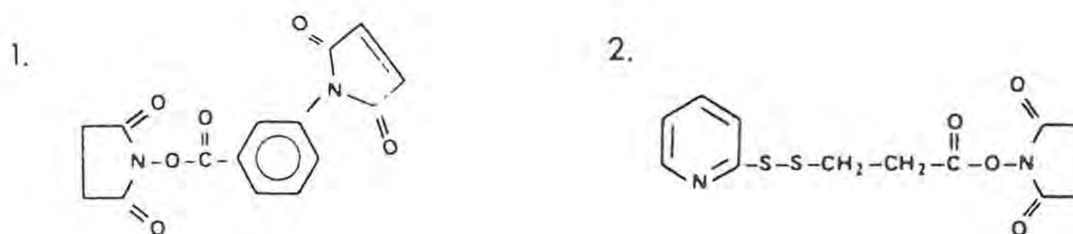
Cheung and Nimni (1982) studied the complex reactions occurring between GA and amine groups and showed that initially an unstable product absorbing at 265nm is formed which reflects the polymerization process taking place. Later, the 265nm absorbing material is converted to a 325nm absorbing product which appears to be formed as a result of internal rearrangement of the initial products.

m-Maleimidobenzoyl-N-hydroxy-succinimide ester (MBS).

MBS (Figure 5.3.1), a bifunctional cross-linking reagent is probably among the most specific protein reagents, reacting under mild conditions and with a minimum of side reactions. This cross-linker reacts with amino and sulphydryl groups. Acrylation of the protein's amino groups occurs via the active N-hydroxysuccinimide ester (Figure 5.3.3. a). A thioether is formed by the addition of a thiol to the double bond of the maleimide (Figure 5.3.3. c).

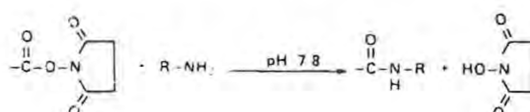
N-hydroxysuccinimide ester of 3(2-pyridyl-dithio) propionic acid (SPDP).

SPDP ia an insoluble bifunctional crosslinking reagent (Figure 5.3.2). Amine groups of the protein react with the N-hydroxysuccinimide ester at one end of the reagent with the elimination of the N-



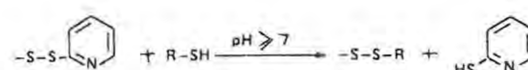
3. Amino Reacting Functions

a) n-Hydroxysuccinimide (NHS) active esters



Thiol Reacting Functions

b) Pyridyl disulfides



c) Maleimides

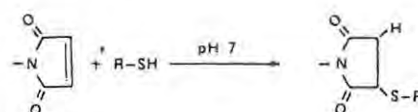


Figure 5.3. 1) Structure of m-Maleimidobenzoyl-N-hydroxy-succinimide ester.

2) Structure of 3(2-pyridyl-dithio) propionic acid.

3) The amino and thiol reacting functions of reagents, 1 and 2.

hydroxysuccinimide (Figure 5.3.3. a). The other end reacts with sulphydryl groups forming a disulphide bridge and 2-mercaptopyridine is liberated (Figure 5.3.3. b).

1 ethyl-3(3-dimethyl-amino propyl) carbodiimide hydrochloride (CDI).

Water soluble carbodiimide reagents provide an easy and rapid method, with mild conditions, for conjugating virus to RSA beads without interposing additional groups between the antigen and carrier. Carbodiimides (Figure 5.4) can couple compounds containing many types of functional groups including -COOH, $-NH_2$, $-PO_4$ and -OH. The amine groups on the bead surface were succinylated to provide more carboxyl groups to which the coupling agent would link the viral antigen (Figure 5.5.1). The one end of the carbodiimide reacts with carboxyl groups on the bead surface whilst the other end reacts with amine groups on the antigen (Figure 5.5.2). This procedure is fairly controlled with no self coupling occurring.

Results

Experiments were performed to examine the influence of antigenic presentation on the induction of an immune response. Two different approaches were taken.

The effect of adsorbed virus on the immune response

In order to enhance the immune response initially and to maintain it at a high titer, three experimental vaccines were compared; (i) Nodamura virus incorporated into RSA beads (25µg virus/ rabbit); (ii) Nodamura virus (25µg) in PB and (iii) Nodamura virus incorporated into RSA beads (25µg) and in free solution (25µg).

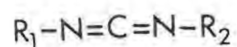


Figure 5.4. General formula of carbodiimides. R_1, aryl and R_2, alkyl groups.

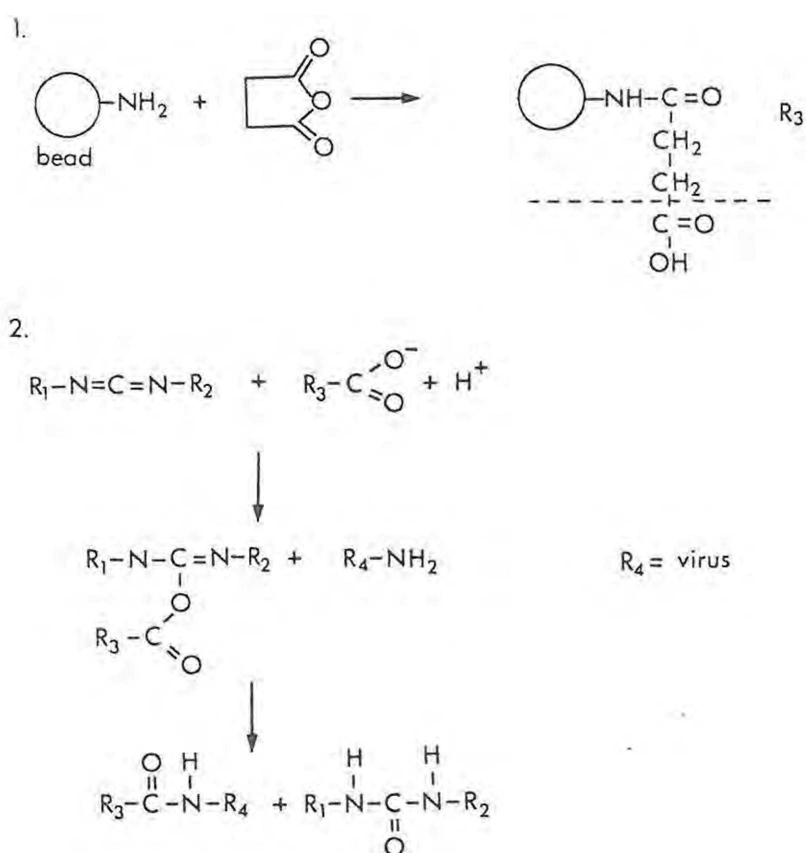


Figure 5.5. Coupling reactions involved in linking the virus to RSA bead. 1. Succinylation of RSA bead. 2. Conjugation of the modified bead to virus using 1 ethyl-3(3-dimethyl-amino propyl) carbodiimide hydrochloride.

The kinetics of the humoral responses shown in Figure 5.6 clearly indicate the superiority of the virus and bead mixture vaccine (V+B) over the other vaccine preparations. The V+B vaccine and the fluid vaccine preparation elicited comparable titers initially although V+B peaked about 6 days later. This might be explained by the retention of free virus due to adsorption onto the beads, hence localizing it at the injection site, whereas the fluid vaccine would have been able to circulate more freely or macrophages would have phagocytosed the free viral particles more easily and rapidly. The V+B titers then dropped until day 28, were maintained at this intermediate level and started to increase after day 50. The titer then remained high until the test period was terminated.

The control virus in PB dropped by day 20 and titers continued to gradually decline.

The bead vaccine displayed its characteristic profile with high, long lasting antibody titers, although no initial peak, due to partial exposure of the antigen on the bead surface, was observed (see previous chapters). It is thus possible that all virions were completely entrapped and thus masked from the immune system until their exposure once the beads were being degraded.

The kinetics of V+B humoral response displayed an additive effect combining a rapid, high initial response with sustained high antibody titers.

<u>The effect on the immune response of virus covalently attached to the bead surface</u>

To determine the efficacy of the beads as carriers with adjuvant

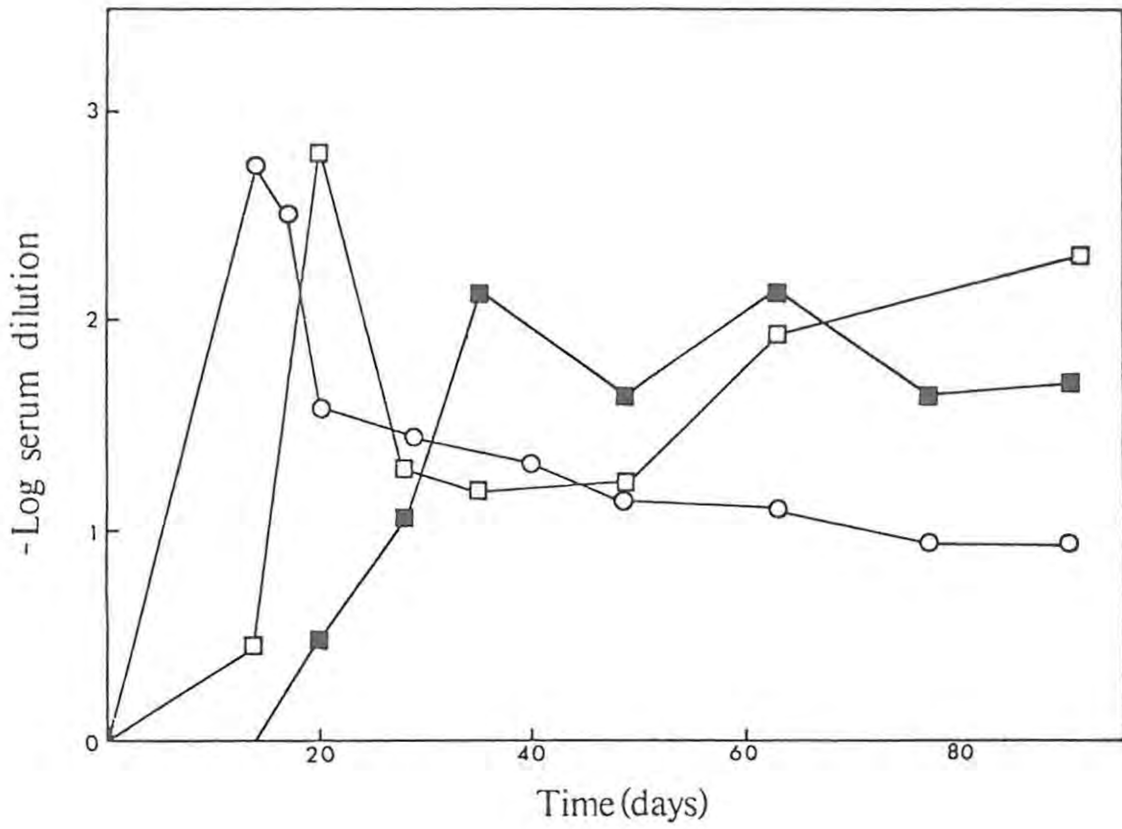


Figure 5.6. Comparison of virus specific IgG antibodies in serum of rabbits immunized with Nodamura virus in PB (○); polymerized into RSA beads (■); polymerized into RSA beads and in PB (□).

action, a second study was undertaken. A comparison was made of the virus specific antibodies induced by "empty" beads with Nodamura virus covalently attached to the surface using different crosslinkers; GA, CDI, MBS and SPDP.

The determination of the coupling efficiency utilized ^{125}I-radiolabelled Nodamura virus. The total radioactivity in the purified bead conjugate was determined (Table 5.1). CDI coupled the virus to the bead most efficiently. MBS and SPDP both displayed poor coupling efficiencies under the conditions used in this study.

Table 5.1. Determination of the efficiency for coupling Nodamura virus to RSA beads.

Crosslinker	% Radioactivity in bead
GA	10.37
MBS	2.95
SPDP	1.56
CDI	57.60

The virus specific antibody response was determined using ELISA and results are shown graphically in Figure 5.7. All vaccine formulations induced virus specific antibodies in rabbits, even that of SPDP which had only a fraction of the virus attached to its surface.

Beads, which had more virus coated on their surface, induced less eratic and more prolonged humoral antibody responses. When small quantities of virus were attached, the antibody response peaked but declined to baseline after about 30 days.

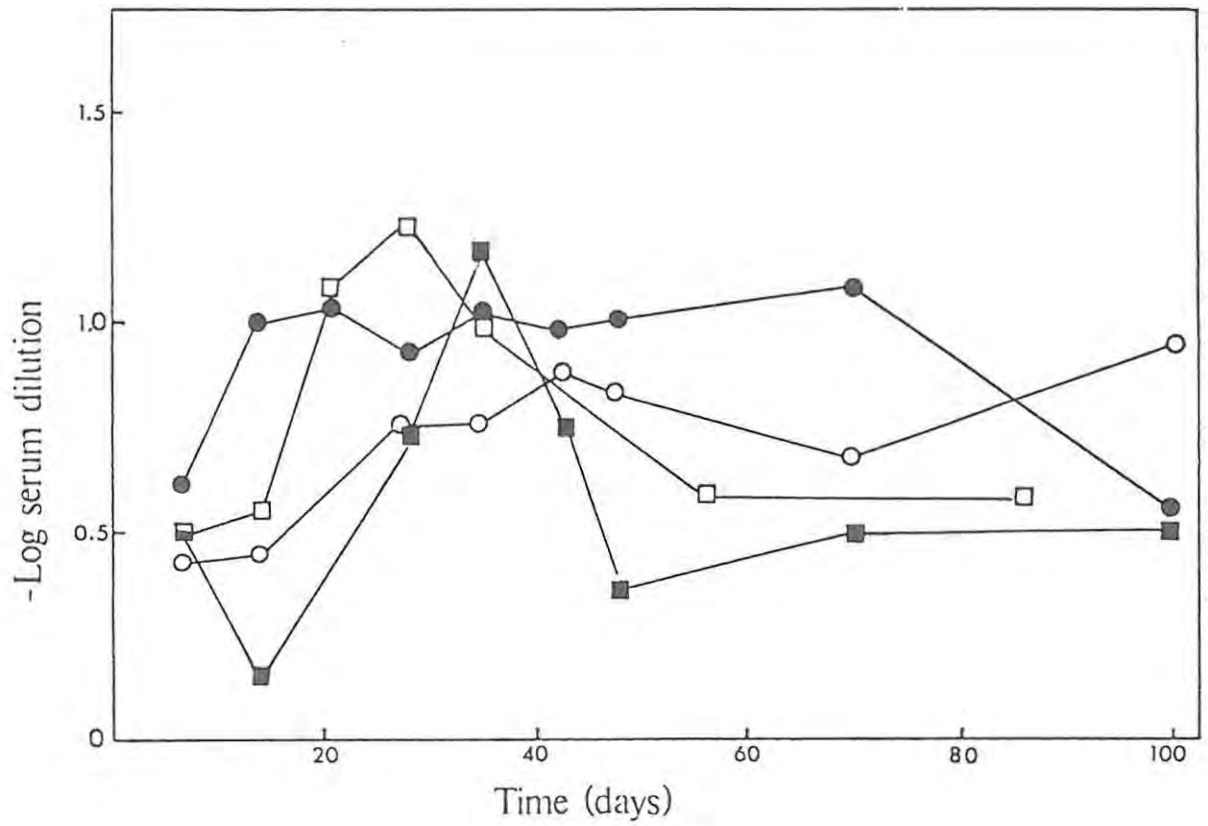


Figure 5.7. Comparison of the virus specific IgG antibody responses elicited in rabbits immunized with Nodamura virus covalently coupled to the surface of RSA beads with : CDI (●) ;GA(○); SPDP(■); MBS(□).

MBS and SPDP react with sulphydyl groups. Their use as crosslinking reagents would ensure that the antigenic sites would not be blocked because cysteine residues usually lie internally and are therefore unlikely to form part of the antigenic sites.

Nodamura virus was attached to the MBS beads in the form of subunit particles. The beads rendered the subunits more immunogenic although the antibody response was not prolonged.

Discussion

The two modes of antigenic presentation investigated in these studies allowed a higher initial exposure of antigen than would normally be achieved if the antigen was incorporated into the bead structure. Therefore, they provide alternative ways for overcoming the initial lag observed in IgG serum titers when the bead vaccine is used alone.

Although a higher antigenic dose was administered with the V+B formulation, the advantage of including virus which is not incorporated into the bead was apparent. Obviously, the 'external' quantity could be reduced to save material.

At least some of the virus in V+B vaccine would have adsorbed onto the bead surface. This high exposure of virus did not result in the suppression of the response towards the antigen within the bead as has been suggested to occur with liposomes (van Rooijen and van Nieuwmegen, 1983). Adsorbed antigen is more effective than when it is free (Kreuter and Liehl, 1981; van Rooijen and van Nieuwmegen, 1980) because it is delayed in the tissue longer.

One of the advantages, for covalent attachment of antigens to the

beads over adsorption, is that covalent bonds are stronger than adsorption bonds. Therefore the likelihood of determinants being split off is reduced.

There are a wide range of crosslinking reagents which could be employed which would not influence the protein's antigenicity. The choice is limited by the stability range of the proteins. When linking different proteins (P-Q), the pH and ionic strength of the reaction mixture should be adjusted to give maximum opposite charge on P^- and Q^+ to favour $P^- Q^+$ interactions and at the same time reduce the probability of self coupling $P^- P^-$.

One of the advantages of using these insoluble RSA beads as a matrix for attaching antigen, is that excess reagent can be removed easily by several washing steps, each of which would be followed by centrifugation to pellet the beads. This eliminates the need for comparatively lengthy and expensive processes such as gel filtration or chromatography, routinely used to separate soluble conjugates.

The optimal amount of antigen attached to the surface would be less than the maximum binding capacity which is limited by the surface area.

An advantage with the covalent attachment of antigens to beads is that one batch of beads could be used with different antigens. Since the beads themselves are stable under dry conditions, even at room temperature, many batches could be stored and antigen attached as needed. Different antigens could also be selectively linked to the surface to produce effective multivalent vaccines. Optimal

immunological reactions would be obtained if antigen was covalently attached to beads in which antigen had already been incorporated.

The 'empty' bead carriers could still be utilized if it is established that new antigenic determinants are introduced in the albumin by GA treatment. It might be possible to substantially mask these groups by covalent reaction, whilst leaving or creating bonding sites for the desired new antigenic determinants and then covalently bond them onto these sites. This suggestion is supported by the finding that, the immunological properties of BSA were altered/masked by the covalent attachment of polyethylene glycol (Abuchowski et al, 1977).

There are potential ways of improving coupling efficiencies. For example, the reaction with SPDP might be improved by introducing new sulfhydryl groups onto the protein by reacting the protein with thiolactones or with mercaptosuccinic anhydride as suggested by Wold (1972).

A good understanding of protein modification techniques would be highly advantageous so that the wide variety of crosslinkers available could be utilized to achieve optimal coupling efficiencies. This would enable the immunostimulatory properties of the bead vaccine to potentiate the immune response to those antigens for which only small quantities are available.

CHAPTER 6

POLIOVIRUS VACCINES

Introduction

Poliovirus, a picornavirus, occurs in three stable serotypes. Although both the Salk (dead) and the Sabin (live attenuated) vaccines have been effective in controlling poliomyelitis, only the Sabin vaccine is used in South Africa. Vaccines containing one strain of each serotype have proved extremely effective in controlling poliomyelitis, implying that immunization with one virus confers immunity to all strains of the same serotype. This suggests that polioviruses are not able to evade host immunity by antigenic drift (as observed with influenza virus). The reason for this stability is not known and it contrasts strongly with the potentially high degree of antigenic variability of poliovirus under immune pressure *in vitro* (Minor *et al*, 1983, 1985). Reversion to virulence is low, suggesting that the attenuated phenotype is stable *in vivo*.

In this light, what potential advantages would bead vaccines with poliovirus incorporated into them have over the Sabin virus vaccines?

Firstly, bead vaccines are genetically and physically more stable than the Sabin vaccines. In addition, the slow release mechanism would enable the vaccinee to elicit antibodies to all three serotypes with a single injection and hence eliminate the necessity for booster inoculations.

Genetic instability

Polioviruses are capable of rapid evolution upon replication in humans. This process is characteristic of both wild strains during

epidemic transmission and the live virus vaccines during growth in vaccinees (Kew et al, 1981; Kew and Nottay, 1984; Minor, 1980, 1982; Yoneyama et al, 1982; Minor et al, 1986b).

The genetic instability of Sabin types 2 and 3 has been well documented (Cann et al, 1984; Minor, 1982). In contrast, Sabin type 1 vaccine strain seems to be more genetically stable (Stanway et al, 1986) although the studies of Kew and Nottay (1984) have indicated otherwise.

Studies with infant vaccinees illustrate the genetic mutability of live polio vaccines during normal immunizations (Kew & Nottay, 1984; Evans et al, 1985; Minor et al, 1986b). These studies indicated that the observed increased neurovirulence could be attributed to either point or multisite mutations and to intertypic recombination.

It was suggested that a host response near days 8-10 exerts selective pressure upon the virus, which either clears the infection, as for the type 1 component studied by Minor et al (1986b) or selects novel strains, as for type 3 component of samples (Minor et al, 1986b) and type 1 components (Kew and Nottay, 1984). As recombination requires coinfection of a cell by two viruses, this implies a high multiplicity of infection in the gut.

Concern arises because the intramolecular recombinant strains may not only be amplified in humans, but also transmitted at low levels to others (Kew and Nottay, 1984).

Both synthetic peptide vaccines (Emini et al, 1983b, 1984b; Chow et al, 1985) and intertypic recombinant vaccines (Stanway et al, 1986)

have been studied as means of overcoming the genetic instability of Sabin vaccines strains. However as yet, little success has been achieved.

Synthetic peptides (No. 3 and No. 9) of neutralization epitopes on VP1 and VP2 proteins of type 1 poliovirus (Emini et al, 1983b, 1984a) elicited neutralizing antibodies. Although the other peptides (No. 1 and 5) did not induce neutralizing antibodies, they did prime the system for a secondary antiviral response. However, the success of synthetic peptides depends on the adjuvant and often, only FCA will induce neutralizing antibodies (Ferguson et al, 1985).

The recombinant vaccines were noninfective (Stanway et al, 1986).

Physical Lability

It is imperative that Sabin vaccines are refrigerated to maintain their infectivity. Immunization with titers lower than the accepted $TCID_{50}$ increases the chance of reversion to virulence.

The superior stability of the bead vaccines at ambient temperatures enables those individuals in remote, undeveloped regions where refrigeration facilities are limited or non-existent to be effectively immunized.

Booster immunizations

The interference between the three serotypes replicating in the gut necessitates booster inoculations, since very rarely does a vaccinee raise protective antibodies against all three serotypes after one immunization.

By incorporating all three serotypes into bead vaccines, an

individual's immune system would be able to mount a response against all three as they are released slowly into the tissue. In countries where primary vaccination not to mention recalling vaccinees for booster inoculations is difficult or impossible, these bead vaccines would provide a means of eliminating the booster requirement. In this way, the immunity deficits which tend to develope predominantly in isolated rural areas (Schoub et al, 1986) may be avoided.

The immunizing potential of an inactivated poliovirus vaccine in the form of albumin beads will be evaluated. Poliovirus Sabin type 2 vaccine strain, p712, is the representative antigen in these studies.

Results

To determine whether the model bead vaccine described in other chapters could be extended to other viral systems, the following experiment was performed using Sabin type 2 strain, p712. Groups of rabbits were each immunized with an antigenic dose of 25 μg virus either incorporated into RSA beads crosslinked with GA (1% final concentration) or in phosphate buffered saline (PBS).

The surface of the virion can be visualised as a continuum of epitopes of which only some are of immunological importance. Of these, even less are important as neutralizing sites.

Therefore, it was important to assess the adjuvant effect of the bead vaccines with respect to the induction of neutralizing antibodies. Each serum sample collected over a 3 month period was assayed separately for the presence of neutralizing antibodies. The results presented in Figure 6.1 indicate that the aqueous control vaccine

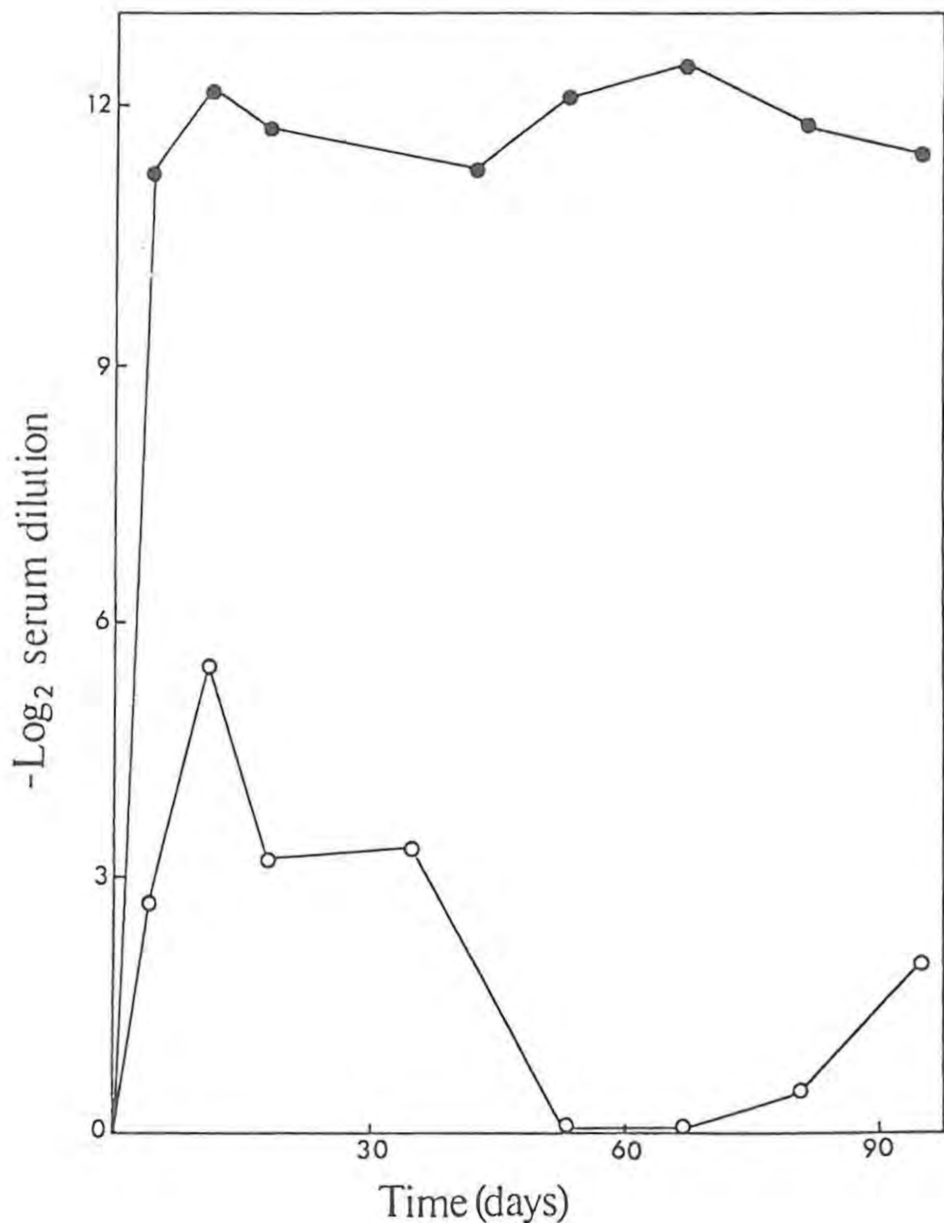


Figure 6.1. Kinetics of the neutralizing antibody response in rabbits immunized with Poliovirus type 2 Sabin strain, p712 in PBS (●) or polymerized into RSA beads (○).

induced significantly higher neutralizing antibody titers than the poliovirus bead vaccine (9 log units). The neutralizing antibodies induced by the control peaked at day 11 and were maintained at a high level over the 3 month test period. Neutralizing antibodies elicited by the bead vaccines peaked at day 11. However after day 32, the levels dropped rapidly and only at day 94 was any additional neutralizing activity observed.

Discussion

The results showed that the control vaccine administered in PBS elicited higher and longer lasting neutralizing antibodies than the bead vaccine. Since poliovirus only infects primates and humans, the high neutralizing antibody response of the control vaccine is only due to the antigen presence and not a result of the virus replicating in the animal.

The poor neutralizing response of the bead vaccine can be explained in retrospect by examining the antigenic structure of the poliovirus type 2. Minor et al (1986a) identified the antigenic sites in poliovirus of serotypes 1, 2 and 3 by selecting for mutants with monoclonal antibodies (Table 6.1).

The major viral protein, VP1, has been implicated as possessing the immunodominant antigenic site for polio type 2 (Minor et al, 1986a), and has also been shown to be the protein most exposed on the virion surface (Hogle et al, 1985). Therefore, it is most likely to be involved in virus-virus and virus-albumin crosslinking by GA.

Table 6.1. Location and occurrence of antigenic sites in poliovirus of serotypes 1, 2 and 3.

Site	Location	Serotype
1	VP1; 89-100	2,3,1*
2a	VP1; 220-222	1
2b	VP2; 164-172	1,3
3a	VP1; 286-290	3
3b	VP3; 58-60,70,71,77,79	1,3

*Site 1 on the VP1 protein of type 1 was identified as lying between amino acids 93-103 (Emini et al, 1983; Wychowski et al, 1983; Chow et al, 1985). Four synthetic peptides to regions on VP1 (61-80, 95-109, 182-201 and 222-264) induced serotype-specific, neutralizing antibodies. Although the regions are separated along the one dimensional structure of the polypeptide sequence, they could come together to form a single site when the polypeptide is folded up in the virion (Chow et al, 1985).

Since GA only reacts with the ε-amino groups of lysine, the amino acid sequence of poliovirus type 2, Sabin strain (Toyoda et al, 1984), corresponding to the antigenic site (aa 89-100 of VP1) (Minor et al, 1986a) was examined (Figure 6.2).

Figure 6.2. The region corresponding to the antigenic sequence on VP1 of poliovirus, Sabin type 2, p712, vaccine strain.

```
           aa 89                                          aa 100
N-terminus-Ile Ile Glu Val Asp Asn Asp Ala Pro Thr Lys Arg-C-terminus
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A lysine residue is found in position 99. One may infer from the result depicted in Figure 6.1, that the majority of neutralizing antigenic sites were blocked during the polymerization process. The neutralizing antibodies elicited initially may have arisen in response

to any virions on the bead surface with unaffected neutralization sites.

The future application of the RSA bead vaccines would be limited if only antigens which do not have lysine residues in their antigenic sites could be used. The antigenic sites must therefore be protected during the polymerization procedure (Figure 6.3).

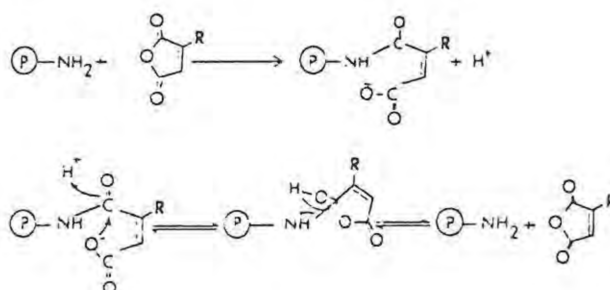

Figure 6.3. Amino group modification with citroconic anhydride ($R=CH_3$) or maleic anhydride (R=H).

Briand et al (1985) showed that a synthetic peptide retained its antigenicity if the amino groups were blocked by citraconylation prior to conjugation. The groups were then deprotected by dialysis against 5% acetic acid.

Protection of neutralization sites would enable the application of the bead vaccine model to all antigens, even synthetic peptides. Another crosslinker could be used to attach the antigens to RSA prior to polymerization. This would ensure that the antigen remains within the bead structure until its release during bead erosion. This might be particularly important when employing synthetic peptides as antigens, since their lower molecular weight might allow their diffusion from the bead. The antigenic release would then be not only controlled by the level of crosslinking with GA. Antigenic sites could also be

protected by using a different crosslinker for the bead polymerization, which reacts with functional groups outside the neutralization site.

The bead vaccines have the potential of providing an effective way of combatting infectious diseases provided that the immunodominant sites remain antigenic after polymerization.

The genetic and physical stability of the bead vaccines would ensure that the susceptible contacts of children undergoing immunization with their first dose of the attenuated Sabin vaccine would be effectively immunized. The bead vaccines would also enable populations living in isolated regions to be adequately immunized. Such an approach would not tamper with the highly successful immunization programme using Sabin vaccines.

CHAPTER 7

THE ANTIGENICITY OF THE RABBIT SERUM ALBUMIN BEADS THEMSELVES

Introduction

There are conflicting reports in the literature concerning the antigenicity of GA-treated albumin (Remy and Poznansky, 1978; Onică et al, 1978a, b, 1983; Lenkei, 1980; Mărgineanu et al, 1983; Nardiello et al, 1985).

Although albumin is a natural body protein, anti-albumin antibodies (AAA) of both IgG and IgM classes are present in normal healthy individuals and hepatic patients (Lenkei, 1980; Mihăescu et al, 1981; Onică et al, 1983; Nardiello et al, 1985). These AAA have shown specificity for the new antigenic determinants induced in albumin by GA treatment (Onică et al, 1983; Nardiello et al, 1985) which is dependent on the degree of polymerization (Onică et al, 1978b; Mărgineanu et al, 1983). Onică et al (1978a) and Lenkei (1980) found that AAA reacted with GA-treated monomeric albumin whereas Mărgineanu et al (1983) found evidence to the contrary. No reaction of AAA with homologous RSA occurred (Onică et al, 1978a, b; Lenkei, 1980; Mărgineanu et al, 1983).

Elevated levels of circulating AAA have been demonstrated on immunization of GA-treated albumin (Onică et al, 1978b) and in patients with chronic liver diseases (Lenkei, 1980) or hepatitis B viral infections (Nardiello et al, 1985).

In contrast, Remy and Poznansky (1978) found that polymerized albumin was nonantigenic. The concentration of GA, used in their studies, was greater than that used by Onică et al (1978b).

Immunochemical identity has been demonstrated between GA-treated albumin and albumin isolated from pathological sera , *in vitro* and *in vivo* aged albumin (Lenkei, 1980; Onică *et al*, 1983; Mărgineanu *et al*, 1983).

Little is known about the formation and function of AAA. However, it was assumed that by molecular ageing due to metabolic wear, albumin molecules acquired new antigenic determinants similar to those induced in albumin by GA-treatment, and that AAA in normal subjects represents physiological autoantibodies, involved in the selective recognition and removal, from circulation, of the *in vivo* aged albumin molecules (Gheţie *et al*, 1981).

GA treatment induces two types of antigenic determinants in albumin (Onică *et al*, 1978b, 1983). These new determinants are designated haptenic and structural determinants. Haptenic determinants are common in both monomeric and polymeric GA-albumin. A haptenic determinant is represented by the chromophoric pyridinium groups resulting from the interaction of GA with the lysine residues of albumin (Onică *et al*, 1978b). *In vivo* aged albumin acquires new determinants such as pyridinium structures. These pyridinium haptenic determinants are also present on different proteins which have been treated with GA (Onică *et al*, 1978a).

The formation of structural determinants found only on polymeric molecules is dependent on the structure of albumin and the polymerization process. The structural determinants are characterized by a great density of intermolecular links determining changes in the conformation of the molecules (Onică *et al*, 1978b).

Onică *et al* (1983) examined the immunochemical properties of AAA and separated them into two groups, AAA_1 and AAA_2 based on their differing affinities for the antigens; GA-treated albumin and horseradish peroxidase (haptenic determinants). AAA_1 had a higher affinity than AAA_2. It was also suggested that they might differ with respect to their specificity; AAA_1 might recognize haptenic determinants whilst AAA_2 might be specific for structural antigenic determinants.

The roles of the differing AAA may be explained. According to the hypothesis (Gheţie *et al*, 1981), in the presence of circulating autoantibodies, the modified albumin molecules cannot reach the B cells which have receptors for the new antigenic determinants of the aged albumin, since they become complexed by the corresponding autoantibodies as soon as they appear in circulation. In pathological cases (or immunization with GA-treated albumin), the concentration of modified albumin increases, stimulating active synthesis of AAA by immunocompetent cells. In the presence of excess circulating modified albumin, high affinity AAA_1 may be removed in a complexed form, leaving predominantly low affinity AAA_2 in circulation (Onică *et al*, 1983).

In view of the presence of circulating AAA, and the fact that they are boosted by immunization with polymerized albumin, it is important that the antigenicity of the polymerized RSA beads, used in this research project, is evaluated.

The albumin beads can only be successfully employed as an integral part of the vaccination strategy for humans if they are nonantigenic, since an essential requirement of adjuvants is that they do not induce

autoimmune responses.

Results

The bead vaccine formulation, described in this report, utilized albumin homotypic for the host. It was therefore important to determine whether the albumin possessed new antigenic determinants due to its reaction with GA.

The antigenicity of albumin treated with GA has been shown to be dependent on the degree of polymerization (Onică et al, 1978b; Mărgineanu et al, 1983).

A study was undertaken to determine whether polymerized homotypic serum albumin in the form of insoluble beads possessed significantly altered antigenic structures and whether their use would activate autoimmune clones.

RSA beads were prepared with 0.5, 0.667, 1, 2 and 4% GA (final concentration) which had the corresponding albumin/GA (w/w) ratios; 40/1, 30/1, 20/1, 10/1 and 5/1. Soluble GA treated polymeric albumin with final concentrations of 0.1% and 0.25% (RSAS 20/1 and RSAS 8/1) were prepared in PBS. The polymers were characterized using SDS-PAGE and spectrophotometry. BSA beads, with a final GA concentration of 1% and a corresponding albumin/GA ratio of 20/1, were prepared as a control experiment. Rabbits were immunized with these preparations and boosted on day 72 with the same inoculum as was used for the primary vaccination.

Complement fixation, immunoprecipitation and indirect immunofluorescence are not sensitive enough for detecting

autoantibodies (Stechemesser et al, 1985). Therefore, preimmune, immune and booster sera were assayed for the presence of AAA using RIA.

Soluble polymers and protein monomers (insulin as a negative control) were insolubilized by covalently coupling to CNBr-activated Sepharose 4B. This would enable a direct comparison of the antigenicity between RSA beads, RSA soluble polymers and RSA itself.

The technique, used here, was more involved than when antigen is adsorbed onto the solid phase because the beads/gel had to be pelleted by centrifugation after each incubation and wash. Careful washing was important to ensure that material was not lost.

Onică et al (1983) found that AAA were very sensitive and that their reactivity was greatly reduced after their purification. For this reason, in a simplified assay procedure, anti-albumin sera was allowed to react with antigen, without initial purification. Unbound antibodies were removed by extensive washing. The antigen/antibody reaction was quantified by utilizing an ^{125}I-labelled second antibody. The results obtained are shown in Table 7.1.

It was found that only RSA 5/1 beads elicited a very low level of antibodies after immune and booster immunizations. A low level of antibodies was detected for RSA 10/1 in the immune sera. No antibodies directed against RSA 20/1, 30/1 nor 40/1 were detected. BSA 20/1 beads induced significant antibody titers after the immune inoculation.

The antigenic properties of the soluble polymers, RSAS 8/1 and RSAS

20/1, were examined using both the immunodiffusion and RIA techniques. Both polymers failed to react with their respective antisera in the immunodiffusion test. The results obtained using RIA are shown in Table 7.2.

The results in Table 7.2 indicate that the soluble polymers are only weakly antigenic or nonantigenic with only 14.8% more antibody bound from the booster sera compared with the preimmune sera. The preimmune counts were consistently high even though successive experiments were undertaken. Although the participation of the naturally occurring AAA cannot be excluded, there still appears to be some non-specific reaction. The reason for this is unknown.

The high preimmune values prevent one from making an accurate comparison between the antigenicity of the beads and soluble polymers. However the differences, between the booster and preimmune sera, suggest that the soluble polymers may be marginally more antigenic than the beads.

Autoimmune clones were not activated because sera from rabbits immunized with the beads of different GA concentration all failed to react with RSA using the ELISA technique. (Results not shown).

Discussion

Although previous workers have detected AAA against GA-treated albumin (Onică et al, 1978a, b; Lenkei, 1980; Mǎrgineanu et al, 1983), these results and those of Remy and Poznansky (1978) have demonstrated to the contrary.

The soluble polymers demonstrated a shift into the UV absorption

Table 7.1. Binding of rabbit anti-albumin with the immunizing antigens.

Antigen[a]	Antibody bound (cpm)[c]		
	Preimmune	Immune	Booster
RSA 5/1	8625	10780	11712
RSA 10/1	8542	11132	9779
RSA 20/1	9186	8395	9798
RSA 30/1	8988	7636	9016
RSA 40/1	8832	8850	8905
BSA 20/1	8436	42480	ND[b]
Insulin	9600	9822	ND

a Albumin/ GA w/w ratio

b ND not determined

c Amounts of antibodies bound to the beads were determined by reacting with ^{125}I-labelled IgG (46 000 cpm). For experimental details see chapter 2.

Table 7.2. Binding of anti-albumin with the immunizing soluble polymers.

Antigen[a]	Antibody bound (cpm)		
	Preimmune	Immune	Booster
RSAS 8/1	15700	15700	22500
RSAS20/1	16100	13200	21500
Insulin	10000		10000[b]
			9800[c]

a Albumin/GA w/w ratio.

b reaction with booster anti RSAS 8/1 antiserum.

c reaction with booster anti RSAS 20/1 antiserum.

maxima from 280 to 268 nm with the concomitant increase in absorbance. SDS-PAGE analysis depicted two predominant bands corresponding to the monomeric and polymeric fractions. Therefore it appears that the soluble polymers displayed identical physiochemical characteristics to those described by Onică et al (1978b). However, although similar antigenic doses were administered, previous workers administered their samples emulsified in FCA and animals received three boosts whereas in these experiments saline protein solutions were injected. Each animal only received one boost.

It has been suggested that FCA can activate autoimmune clones (Yokota et al, 1980; Atassi et al, 1982), most likely through the by-passing of T cell suppression (Atassi, 1980). AAA have been found in normal sera (Mihăescu et al, 1981; Mărgineanu et al, 1983; Onică et al, 1983) and these antibodies have specificities for both haptenic and structural antigenic determinants (Onică et al, 1983). The numbers of circulating AAA are obviously under strict regulatory control. It is therefore possible that the "strongly provocative i.m. administration" used in previous studies, resulted in disruptions of the immune regulation by overcoming T cell suppression to induce the proliferation of B cell clones responsible for AAA production. The RSA beads and polymers used in this study did not appear to disturb the normal functioning of the immune system.

It appears that the beads do not possess any new structural determinants. This is not unfounded since the polymerization process is mild and albumin molecules are not denatured (Royer et al, 1983). The ELISA results suggest that the 'empty' bead vaccines do not induce

anti- RSA antibodies, implying that autoimmune clones were not activated.

The results could also be explained if one speculates that, as the beads are slowly degraded, the albumin molecules will be treated as aged albumin and removed from circulation before they can activate B cell clones. The soluble polymers, on the other hand, have a more random structure with a larger surface area (Figure 2.8B) than the insoluble beads. It is therefore likely that their degradation would be more rapid and hence the immune system would be "flooded" with modified albumin which could then activate B cell clones. Since aged albumin possesses both haptenic and structural antigenic determinants (Onică et al, 1983) which are recognized by AAA, these circulating natural AAA would be specific for both determinants in GA-treated albumin.

There is evidence to suggest that a nonantigenic carrier can become antigenic after conjugation to a heterologous protein (Drewes et al, 1978). However, studies with starch microparticles (Artursson et al, 1985) and RSA beads (Dewar, 1985) showed that no anti-matrix antibodies were induced when associated with antigen. The polyacrylamide conjugates used in their studies (Drewes et al, 1978) were both soluble and nondegradable, therefore either factor may have been contributory.

Since the immune system is highly complex and not fully understood, it is important that further immunological and pathological studies are undertaken. These studies do infer that the serum albumin bead vaccines have great potential as safe and promising adjuvants.

GENERAL DISCUSSION

The results of the studies presented in this manuscript indicate that when virus particles were covalently bound into polymerized serum albumin beads, they were potent antigens. The induction of virus specific IgG antibodies by the bead vaccines was superior to those induced by the aqueous vaccine and comparable to those achieved using Freund's incomplete and complete adjuvants. This adjuvant effect was achieved by a slow release of the antigen.

Although the serum albumin beads failed to induce significant levels of neutralizing antibodies when poliovirus was polymerized into them, the antigenicity would have been retained by protection of the free amino groups during polymerization.

If the albumin bead vaccines were to be employed as part of the vaccination policy, many problems associated with present-day vaccines would be overcome. The enhanced immunogenicity of the incorporated antigens implies a reduction in the amount of purified antigen and the number of doses required for effective immunization. Therefore, vaccine production would be more economical and feasible. The lag phase after administration could be overcome by introducing an aliquot of the aqueous vaccine with the beads. The slow release properties would ensure that antibodies were induced against each epitope in multivalent vaccines.

The enhanced stability of the antigen, conferred by the serum albumin beads, is not only significant for economical reasons, but also for greater distribution purposes, particularly in poor countries where vaccine storage and delivery are difficult.

To avoid an undersirable immunological response in animals inoculated with a protein adjuvant, homotypic serum albumin was used to produce the slow release beads. The use of serum albumin beads produced no adverse systemic effects or local inflammatory or necrotic reactions at the injection site. No significant fluctuations in body temperature or weight were observed (Lee et al, 1981; Royer et al, 1983: Dewar et al, 1984).

Preliminary studies have indicated that the serum albumin beads are nonantigenic and do not acquire new structural determinants through the polymerization process with GA. However, further immunological and pathological analyses should be made, so that every aspect involved in the bead degradation is understood.

Although serum albumin beads present a non-irritative adjuvant which could replace Freund's adjuvants, currently employed in laboratories, another alternative is ethylene-vinyl acetate copolymer (EVAc). EVAc discs have been used extensively in vivo as a drug delivery device (Langer et al, 1980), and they represent an effective system for stimulating antibody production over a long period of time, in response to antigen which has been incorporated into the EVAc matrix (Langer et al, 1980; Niemi et al, 1985). EVAc has the following advantages: stability when stored at -70°C, non-inflammatory, antibody levels comparable with FCA and only one dose is required for effective immunization (Niemi et al, 1985).

In contrast, the serum albumin beads have all the advantages of EVAc as a sustained release matrix but have greater practical potential. The EVAc discs are relatively large (diameter 5 mm, depth 3 mm)

compared with the sphereical RSA beads (diameter 50-100 µm). This physical difference could contribute to practical difficulties in vaccine administration. The discs have to be implanted, requiring surgery which in turn necessitates anaesthetics. Therefore with large scale laboratory vaccinations, the disc administration would be more expensive and time consuming than the simple intramuscular injection of the beads.

Although this research project concentrated on viral antigens, the bead model would provide effective immunization for antigens over a wide molecular weight range, to include bacterial polysaccharides, toxins, subunit proteins and even synthetic peptides. The incorporation of toxic compounds into beads reduces their toxicity without affecting their antigenicity (Colin Langheim, pers. comm.), as has been observed with liposomes (Fidler et al, 1985).

Nonreplicating, purified subunit or synthetic viral vaccines of the future are likely to be weak immunogens that will require immunopotentiation if they are to be effective. Repeated use of a carrier could induce hypersensitivity to the carrier. In addition, the administration of a vaccine epitope on a carrier to which the host has been previously exposed, such as tetanus toxiod, may lead to suppression of the new epitope (Herzenberg and Tokuhisa, 1982). This phenomenon called epitope specific suppression occurs when an animal inoculated with a carrier, subsequently receives an injection of the same carrier conjugated to an epitope. The antibody response to the epitope is specifically suppressed and the antibody response against the carrier proceeds normally. If the bead vaccines are, on further examination, nonantigenic as these initial studies have indicated, it

is unlikely that epitope specific suppression will occur with their repeated use. Therefore serum albumin beads would provide the ideal vehicle for synthetic peptide administration.

Certain avenues in this project should be further investigated. Ada *et al* (1981) showed that infectious influenza provided greater and more effective protection than the inactivated and subunit vaccines. Their results also showed that the form of antigen presentation influenced the induction and regulation of the different types of T cells; helper, suppressor and cytotoxic. Therefore, the serum albumin bead vaccines should be examined fully to determine whether cell-mediated immunity plays a role in the immune response induced by their use.

To investigate the *in vivo* degradation and elimination of the serum albumin beads, time course and tracer experiments, using radioactive beads in which the albumin and antigen are labelled with different isotopes, should be executed. This could shed light on antigen processing and any problems associated with the use of the bead vaccines.

Finally, since synthetic peptides are becoming increasingly important as the vaccines of the future, studies pertaining to their incorporation into bead vaccines will further increase the bead vaccine's usefulness.

REFERENCES

Abuchowski, A., van Es, T., Palczuk, N.C. & Davis, F.F. (1977). Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol. J. Biol. Chem. 252: 3578-3581.

Ada, G.L., Leung, K-N. & Ertl, H. (1981). An analysis of effector T cell generation and function in mice exposed to influenza A or Sendai viruses. Immunol. Rev. 58: 5-24.

Agol, V.I., Grachev, V.P., Drozdov, S.G., Kolesnikova, M.S., Kozlov, V.G., Ralph, N.M., Romanova, L.I., Tolskaya, E.A., Tyufanov, A.V. & Viktorova, E.G. (1984). Construction and properties of intertypic poliovirus recombinants: first approximation mapping of the major determinants of neurovirulence. Virology 136: 41-55.

Allison, A.C. & Gregoriadis, G. (1974). Liposomes as immunological adjuvants. Nature 252: 252.

Anderson, A.O. (1985). Multiple effects of immunological adjuvants on lymphatic microenvironments. 1. Role of immunologically-relevant angiogenesis in the mechanisms of action of CFA, MDP and avridine. Int. J. Immunotherapy 1: 185-195.

Anderson, R.M. & May, R.M. (1985). Vaccination and herd immunity to infectious diseases. Nature 318: 323-329.

Arnon, R., Maron, E., Sela, M. & Anfinsen, C.B. (1971). Antibodies reactive with native lysozyme elicited by a completely synthetic antigen. Proc. Natl. Acad. Sci. U.S.A. 68: 1450-1455.

Arnon, R., Sela, M., Parant, M. & Chedid, L. (1980). Antiviral response elicited by a completely synthetic antigen with built-in adjuvanticity. Proc. Natl. Acad. Sci. U.S.A. 77: 6769-6772.

Arnon, R. & Sela, M. (1985). Synthetic vaccines : present and future. Ann. Inst. Pasteur/Immunol. 136D: 271-282.

Artursson, P., Laakso, T. & Edman, P. (1983). Acrylic microspheres in vivo IX : Blood elimination kinetics and organ distribution of microparticles with different surface characteristics. J. Pharm. Sci. 72: 1415-1420.

Artursson, P., Edman, P. & Sjöholm, I. (1984). Biodegradable microspheres. 1. Duration of action of Dextranase entrapped in polyacrylstarch microparticles in vivo. J. Pharmcol. Exp. Ther. 231 : 705-712.

Artursson, P., Edman, P. & Sjöholm, I. (1985). Biodegradable microspheres II: Immune response to a heterologous and an autologous protein entrapped in polyacryl starch microparticles. J. Pharmacol. Exp. Ther. 234: 255-260.

Atassi, M.Z. (1980). Precise determination of protein antigenic sites has unravelled the molecular immune recognition of proteins and provided a prototype for synthetic mimicking of other protein binding sites. Mol. Cell Biochem. 32: 21-44.

Atassi, M.Z., Sakata, S. & Sakata, S. (1982). Immune recognition of serum albumin XVI: Role of adjuvant in the autoimmune response to mouse serum albumin. Mol. Immunol. 19: 1509-1512.

Atassi, M.Z. & Young, C.R. (1985). Discovery and implications of the immunogenicity of free small synthetic peptides : powerful tools for manipulating the immune system and for production of antibodies and T cells of preselected submolecular specificities. CRC Crit. Rev. Immunol. 5: 387-409.

Audibert, F., Chedid, L., Lefrancier, P. & Choay, J. (1976). Distinctive adjuvanticity of synthetic analogs of mycobacterial water soluble components. Cell Immunol. 21: 243-249.

Audibert, F. & Chedid, L. (1980). Recent advances concerning the use of muramyl dipeptide derivatives as vaccine potentiators. In Progress in Clinical and Biological Research, vol 47. New Developments with Human and Veterinary Vaccines. pp325-338, (Mizrahi, A., Klingberg, M.A., Hertzman, I. & Kohn, A. eds.), Alan, R. Liss. Inc, New York.

Audibert, F. & Jolivet, M. (1982). Immunization by a diphtheria toxin oligopeptide and MDP, a model for synthetic vaccines. Int. Congr. Ser. - Excerpta Med. 563, Immunomodulation by Microbial Products and Related Synthetic Compounds. pp.241-244 .

Ausobsky, J.R., Cheadle, W.G., Brosky, B.G., Polk Jr., H.C. (1984). Muramyl dipeptide increases tolerance to shock and bacterial challenge in mice. Br. J. Surg. 71: 151-153.

Avrameas, S. (1969). Coupling of enzymes to proteins with glutaraldehyde. Use of the conjugates for the detection of antigens and antibodies. Immunochemistry 6: 43-52.

Baron, M.H. & Baltimore, D. (1982). Antibodies against the chemically synthesized genome-linked protein of poliovirus react with native virus-specific proteins. Cell 28 : 395-404.

Bey, E. & Golombick, T. (1984). A comparison of three methods for titration of poliovirus vaccines. J. Virol. Methods 9: 123-130.

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

Bittle, J.L., Worrell, P., Houghten, R.A., Lerner, R.A., Rowlands, D.J., & Brown, F. (1984a). Immunization against foot-and-mouth disease with a chemically synthesized peptide. IN Modern Approaches to Vaccines: Molecular and Chemical Basis of Virus Virulence and Immunogenicity, pp. 103-107. (Chanock, R.M. & Lerner, R.A., eds.), Cold Spring Harbor Laboratory, New York.

Bolton, A.E. (1977). Radioiodination techniques. Review 18, pp. 12-13. Amersham International Ltd., Amersham, UK.

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. Immunol. 39: 426-434.

Briand, J.P., Muller, S., Van Regenmortel, M.H.V. (1985). Synthetic peptides as antigens: pitfalls of conjugation methods. J. Immunol. Methods 78: 59-69.

Burdine, J.A., Ryder, L.A., Sonnemaker, R.E., DePuey, G. & Calderon, M. (1971). ^{99m}Tc-human albumin microspheres (HAM) for lung imaging. J. Nuclear Med. 12: 127-130.

Byars, N.E. (1984). Two adjuvant-active muramyl dipeptide analogs induce differential production of lymphocyte-activating factor and a factor causing distress in guinea pigs. Infect. Immun. 44: 344-350.

Cann, A.J., Stanway, G., Hughes, P.J., Minor, P.D., Evans, D.M.A., Schild, G.C. & Almond, J.W. (1984). Reversion to virulence of the live attenuated Sabin type 3 oral poliovirus vaccine. Nucleic Acids Res. 12: 7787-7792.

Chedid, L., Audibert, F., Lefrancier, P., Choay, J. & Lederer, E. (1976). Modulation of the immune response by a synthetic adjuvant and analogs. Proc. Natl. Acad. Sci. U.S.A. 73: 2472-2475.

Chedid, L., Parant, M., Parant, F., Lefrancier, P., Choay, J. & Lederer, E. (1977). Enhancement of nonspecific immunity to Klebsiella pneumoniae infection by a synthetic immunoadjuvant (N-acetyl-muramyl-L-alanyl-D-isoglutamine) and several analogs. Proc. Natl. Acad. Sci. U.S.A. 74: 2089-2093.

Chedid, L., Audibert, F. & Johnson, A.G. (1978). Biological activities of muramyl dipeptide, a synthetic glycopeptide analogous to bacterial immunoregulating agents. Prog. Allergy 25: 63-105.

Chedid, L., Parant, M., Parant, F., Audibert, F., Lefrancier, F., Choay, J. & Sela, M. (1979). Enhancement of certain biological activities of muramyl dipeptide derivatives after conjugation to a multi-poly (DL-alanine) -- poly (L-lysine) carrier. Proc. Natl. Acad. Sci. U.S.A. 76: 6557-6561.

Cheung, D.T. & Nimni, M.E. (1982). Mechanism of crosslinking of proteins by glutaraldehyde 1: reaction with model compounds. Connective Tissue Research 10: 187-199.

Chow, M. & Baltimore, D. (1982). Isolated poliovirus capsid protein VPI induces a neutralizing response in rats. Proc. Natl. Acad. Sci. U.S.A. 79: 7518-7521.

Chow, M., Yabrov, R., Bittle, J., Hogle, J. & Baltimore, D. (1985). Synthetic peptides from four separate regions of the poliovirus type 1 capsid protein VP1 induce neutralizing antibodies. Proc. Natl. Acad. Sci. U.S.A. 82: 910-914.

Cooper, P.D. (1977). Genetics of picornaviruses. IN Comprehensive Virology 9, pp. 133-207, (Fraenkel-Conrat, H., & Wagner, R., eds.), Plenum, New York.

Crowther, J.R. & Abu-el Zein, E.M.E. (1979). Detection and quantification of foot and mouth disease virus by enzyme labelled immunosorbent assay techniques. J. Gen. Virol. 42 : 597-602.

Damais, C., Riveau, G., Parant, M., Gerota, J. & Chedid, L. (1982). Production of lymphocyte activating factor in the absence of endogenous pyrogen by rabbit or human leucocytes stimulated by a muramyl dipeptide derivative. Int. J. Immunopharmacol. 4: 451-462.

Dewar, J.B. (1985). A novel adjuvant: Polymerized Serum Albumin Beads. Master of Science Thesis.

Dewar, J.B., Hendry, D.A. & Newman, J.F.E. (1984). Biodegradable serum albumin polymers for the sustained release of virus antigens in vaccines. S.A. Med. J. 65: 564-565.

Drewes, P.A., Kamp, A.O. & Winkelman, J.W. (1978). Radioimmunoassay of polyacrylamide. Experientia (Basel) 34: 316-318.

Eagle, H. (1959). Amino acid metabolism in mammalian cell cultures. Science 130: 432-435.

Edelman, R. (1980). Vaccine adjuvants. Rev. Infect. Dis. 2: 370-383.

Edelman, R., Hardegree, M.C. & Chedid, L. (1980). Summary of an International Symposium on Potentiation of the Immune Response to Vaccines. J. Infect. Dis. 141: 103-112.

Edman, P., Sjöholm, I. & Brunk, U. (1983). Acrylic microspheres in vivo Vll : Morphological studies on mice and cultured macrophages. J. Pharm. Sci. 72: 658-665.

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

Ellouz, F., Adam, A., Ciorbaru, R. & Lederer, E. (1974). Minimal structural requirements for adjuvant activity of bacterial peptidoglycan derivatives. Biochem. Biophys. Res. Comm. 59: 1317-1325.

Emini, E.A., Dorner, A.J., Dorner, L.F., Jameson, B.A. & Wimmer, E. (1983a). Identification of a Poliovirus neutralization epitope through use of neutralizing antiserum raised against a purified viral structural protein. Virology 124: 144-151.

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

Emini, E.A., Jameson, B.A. & Wimmer, E. (1984a). Identification of a new neutralization antigenic site on poliovirus coat protein VP2. J. Virol. 52: 719-721.

Emini, E.A., Jameson, B.A. & Wimmer, E. (1984b). Identification of multiple neutralization antigenic sites on poliovirus type 1 and the priming of the immune response with synthetic peptides. IN Modern Approaches to Vaccines: Molecular and Chemical Basis of Virus Virulence and Immunogenicity, pp. 65-75. (Chanock, R.M. & Lerner, R.A., eds.), Cold Spring Harbor Laboratory, New York.

Emini, E.A., Leibowitz, J., Diamond, D.C., Bonin, J. & Wimmer, E. (1984c). Recombinants of Mahoney and Sabin strain poliovirus type 1: analysis of in vitro phenotypic markers and evidence that resistance to guanidine maps in the non-structural proteins. Virology 137: 74-85.

Engvall, E., Jonsson, K. & Perlmann, P. (1971). Enzyme-linked immunosorbent assay II. Quantitative assay of protein antigen, immunoglobulin G, by means of enzyme-labelled antigen and antibody-coated tubes. Biochim. Biophys. Acta. 251: 427-434.

Engvall, E. & 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: 129-135.

Essink, A.W.G., Arkesteijn, G.J.M.W., Notermans, S. (1985). Interference of lysozyme in the sandwich enzyme-linked immunosorbent assay (ELISA). J. Immunol. Methods 80: 91-96.

Evans, D.M.A., Dunn, G., Minor, P.D., Schild, G.C., Cann, A.J., Stanway, G., Almond, J.W., Currey, K. & Maizel, Jr., J.V. (1985). A single nucleotide change in the 5'non-coding region of the genome of the Sabin type 3 poliovaccine is associated with increased neurovirulence. Nature 314: 548-550.

Ferguson, M., Evans, D.M.A., Magrath, D.I., Minor, P.D., Almond, J.W. & Schild, G.C. (1985). Induction by synthetic peptides of broadly reactive, type-specific neutralizing antibody to poliovirus Type 3. Virology 143: 505-515.

Fidler, I.J., Brown, N.O. & Hart, I.R. (1985). Species variability for toxicity of free and liposome-encapsulated muramyl peptides administered intravaneously. J. Biol. Resp. Modif. 4: 298-309.

Freund, J. (1956). The mode of action of immunologic adjuvants. Adv. Tuberc. Res. 7: 130-148.

Gadkari, D.A., Fields, H.A. & Maynard, J.E. (1985). Enzyme-antibody conjugation by a heterobifunctional reagent and its application in enzyme-linked immunosorbent assay (ELISA) for the detection of Hepatitis B surface antigen. J. Virol. Methods 10: 215-224.

Galelli, A. & Chedid, L. (1983). Modulation of myelopoiesis in vivo by synthetic adjuvant-active muramyl peptides : Induction of colony-stimulating activity and stimulation of stem cell proliferation. Infect. Immun. 42: 1081-1085.

Gheţie, V., Onică, D., Lenkie, R. & Mărgineanu, I. (1981). An immunological hypothesis for plasma protein catabolism. Mech. Ageing Dev. 17: 27-40.

Goodfriend, T.L., Levine, L. & Fasman, G.D. (1964). Antibodies to bradykinin and angiotensin: a use of carbodiimides in immunology. Science 144: 1344-1346.

Greenwood, F.C., Hunter, W.M. & Glover, J.S. (1963). The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. Biochem. J. 89: 114-123.

Herzenberg, L.A. & Tokuhisa, T. (1982). Epitope-specific regulation. I. Carrier-specific induction of suppression for IgG anti-hapten antibody responses. J. Exp. Med. 155: 1730-1740.

Hogle, J.M., Chow, M. & Filman, D.J. (1985). The three dimensional structure of poliovirus at 2.9A resolution. Science 229: 1358-1365.

Hopp, T.P. & Woods, K.R. (1981). Prediction of protein antigenic determinants from amino acid sequences. Proc. Natl. Acad. Sci. U.S.A. 78: 3824-3828.

Hovi, T. (1986). Immunization against poliomyelitis: Still problems to solve in both developed and developing countries. Annals of Clin. Res. 18: 119-120.

Hovi, T., Huouilainen, A., Kuronen, T., Popry, T., Salama, A., Cantell, K., Kinnunen, E., Lapinlei, K., Roivainen, M., Stennik, M., Silander, A., Thoden, C-J,. Salminen, J. & Weckstrom, P. (1986). Outbreak of paralytic poliomyelitis in Finland: Widespread circulation of antigenically altered poliovirus type 3 in a vaccinated population. Lancet, June 21, 1427-1432.

Ionescu-Matiu, I., Sanchez, Y., Fields, H.A. & Dreesman, G.R. (1983). Development of sensitive immunoassays for detection of antibodies against Hepatitis B surface antigen. J. Virol. Methods 6: 41-52.

Jacob, C.O., Sela, M., Pines, M., Hurwitz, S. & Arnon, R. (1984). Both cholera toxin-induced adenylate cyclase activation and cholera toxin biological activity are inhibited by antibodies against related synthetic peptides. Proc. Natl. Acad. Sci. U.S.A. 81: 7893-7896.

Jerne, N.K., Roland, J. & Cazenave, P.A. (1982). Recurrent idiotopes and internal images. EMBO J. 1: 243-247.

Johnstone, A. & Thorpe, R. (1982). IN Immunochemistry in Practice, Blackwell Scientific Publications, Oxford.

Kabat, E.A. & Mayer, M.M. (1961). Crystalline Serum Albumins. IN Experimental Immunochemistry: Second edition. pp. 755-757. C.C. Thomas, Springfield, Illinois, U.S.A.

Kemeny, D.M. & Challacombe, S.J. (1986). Advances in ELISA and other solid-phase immunoassays. Immunol. Today 7: 67-68.

Kenna, J.G., Major, G.N. & Williams, R.S. (1985). Methods for reducing non-specific antibody binding in enzyme-linked immunosorbent assays. J. Immunol. Methods 85: 409-419.

Kew, O.M., Nottay, B.K., Hatch, M.H., Nakano, J.H. & Obijeski, J.F. (1981). Multiple genetic changes can occur in the oral poliovaccines upon replication in humans. J. Gen. Virol. 56: 337-347.

Kew, O.M. & Nottay, B.K. (1984). Evolution of the oral polio vaccine strains in humans occurs by both mutation and intramolecular recombination. IN Modern Approaches to Vaccines: Molecular and Chemical Basis of Virus Virulence and Immunogenicity, pp. 357-362. (Chanock, R.M. & Lerner, R.A., eds.), Cold Spring Harbor Laboratory, New York.

Kieny, M.P., Lathe, R., Drillien, R., Spehner, D., Skory, S., Schmitt, D., Wiktor, T., Koprowski, H. & Lecocq, J.P. (1984). Expression of rabies virus glycoprotein from a recombinant vaccinia virus. Nature 312: 163-166.

King, A.M.Q., McCahon, D., Slade, W.R. & Newman, J.W.I. (1982). Recombination in RNA. Cell 29: 921-928.

Kishimoto, T., Hirai, Y., Nakanishi, K., Azuma, I., Nagamatsu, A. & Yamamura, Y. (1979). Regulation of antibody response in different immunoglobulin G classes. VI. Selective suppression of IgE response by administration of antigen-conjugated muramylpeptides. J. Immunol. 123: 2709-2715.

Kit, M. & Kit, S. (1983). Viral vaccines. Med. Clin. North Am. 67: 1129-1145.

Kleid, D.G., Yansura, D., Small, B., Dowbenko, D., Moore, D.M. Grubman, M.J., McKercher, P.D., Morgan, D.O. Robertson, B.H. & Bachrach, H.L.(1981). Cloned viral protein vaccines for foot and mouth disease: Responses in cattle and swine. Science 214: 1125-1129.

Klein, J. (1982). Immune responses dominated by cells of the monocytic series. IN Immunology: The Science of Self-Nonself Discrimination, pp. 417-444, John Wiley & Sons, New York.

Kotani, S., Watanabe, Y., Shimono, T., Harada, K., Shiba, T., Kusumoto, S., Yokogawa, K. & Taniguchi, M. (1976). Correlation between the immunoadjuvant activities and pyrogenicities of synthetic N-acetylmuramyl-peptides or -amino acids. Biken J. 19: 9-13.

Kreuter, J. & Liehl, E. (1981). Long-term studies of microencapsulated and adsorbed influenza vaccine nanoparticles. J. Pharm. Sci. 70: 367-371.

Kreuter, J., Berg, U., Liehl, E., Soliva, M. & Speiser, P.P. (1986). Influence of the particle size on the adjuvant effect of particulate polymeric adjuvants. Vaccine 4: 125-129.

Kyte, J. & Doolittle, R.F. (1982). A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157: 105-132.

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

—— (1977) Poliomyelitis vaccines. (Leading article). Lancet 2: 21-22.

—— (1982) Poliomyelitis vaccines. Killed or live? Lancet 1: 84-85.

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

Langbehiem, H., Arnon, R. & Sela, M. (1976). Antiviral effect on MS-2 coliphage obtained with a synthetic antigen. Proc. Natl. Acad. Sci. U.S.A. 73: 4636-4640.

Langer, R.S., Rhine, W.D., Hsieh, D.S.T., & Bawa, R.S. (1980). Polymers for the sustained release of macromolecules: applications and control of release kinetics. IN Controlled Release of Bioactive Materials, pp. 83-98 , (Baker, R., ed.), Acad. Press, New York.

Lawman, M.J.P., Naylor, P.T., Huang, L., Courtney, R.J. & Rouse, B.T. (1981). Cell-mediated immunity to herpes simplex virus: Induction of cytotoxic T lymphocyte responses by viral antigens incorporated into liposomes. J. Immunol. 126: 304-308.

Leclerc, C., Morin, A. & Chedid, L. (1983). Potential use of synthetic muramyl peptides as immunoregulating molecules. IN Recent advances in clinical immunology, pp. 187-204. (Thompson, R.A. & Rose, N.R., eds.), Churchill Livingstone, Edinburgh.

Lee, T.K., Sokoloski, T.D. & Royer, G.P. (1981). Serum albumin beads: an injectable, biodegradable system for the sustained release of drugs. Science 213: 233-235.

Lefrancier, P., Choay, J., Derrien, M. & Lederman, I. (1977). Synthesis of N-acetyl muramyl-L-alanyl-D-isoglutamine, an adjuvant of the immune response, and some N-acetyl muramyl peptide analogs. Int. J. Pept. Protein Res. 9: 249-257.

Lefrancier, P., Derrien, M., Lederman, I., Nief, F., Choay, J. & Lederer, E. (1978). Synthesis of some new analogs of the immunoadjuvant glycopeptide MDP (N-acetyl muramyl-L-alanyl-D-isoglutamine). Int. J. Pept. Protein Res. 11: 289-296.

Lefrancier, P., Petitou, M., Level, M., Derrien, M., Choay, J. & Lederer, E. (1979). Synthesis of N-acetyl-muramyl-L-alanyl-D-isoglutamic-α-amide (MDP) or α-methyl ester derivatives, bearing a lipophilic group at the C-terminal peptide end. Int. J. Pept. Protein Res. 14: 437-444.

Lefrancier, P. & Lederer, E. (1981). Chemistry of synthetic immunomodulant muramyl peptides. Prog. Chem. Org. Natur. Prod. 40: 1-47.

Leinikki, P.O. (1982). Vaccination policies for the 80's. Ann. Clin. Res. 14: 195-198.

Lenkei, R. (1980). A new antigen/antibody system - the polymerized albumin and antialbumin antibodies. Rev. Roum. Med. - Med. Int. 18:129-147.

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

Lerner, R.A., Green, N., Alexander, H., Liu, F.-T., Sutcliffe, J.G. & Shinnick, T.M. (1981). Chemically synthesized peptides predicted from the nucleotide sequence of the hepatitis B virus genome elicit antibodies reactive with the native envelope protein of Dane particles. Proc. Natl. Acad. Sci. U.S.A. 78: 3404-3407.

Mackett, M. & Smith, G.L. (1986). Review article: Vaccinia Virus Expression vectors. J. Gen. Virol. 67: 2067-2082.

Mărgineanu, I., Medesan, C. & Onică, D. (1983). Anti-albumin antibodies in normal rabbit sera. Immunol. Letters 6: 45-50.

McCahon, D., King, A.M.Q., Saunders, K., Slade, W.R. & Newman, J.W.I. (1984). Use of genetic recombination for constructing novel strains of a picornavirus. IN Modern Approaches to Vaccines: Molecular and Chemical Basis of Virus Virulence and Immunogenicity, pp. 45-51. (Chanock, R.M. & Lerner, R.A., eds.), Cold Spring Harbor Laboratory, New York.

McLaughlin, M.R., Barnett, O.W., Burrows, P.M. & Baum, R.H. (1981). Improved ELISA conditions for detection of plant viruses. J. Virol. Methods 3: 13-25.

McNamara, M.K., Ward, R.E. & Kohler, H. (1984). Monoclonal idiotope vaccine against Streptococcus pneumoniae infection. Science 226: 1325-1326.

Mihăescu, S., Lenkei, R. & Gheţie, V. (1981). Radioimmunoassay of anti-albumin autoantibodies in human sera. J. Immunol. Methods 42: 187-193.

Minor, P.D. (1980). Comparative biochemical studies of Type 3 Poliovirus. J. Virol. 34: 73-84.

Minor, P.D. (1982). Characterization of strains of type 3 poliovirus by oligonucleotide mapping. J. Gen. Virol. 59: 307-317.

Minor, P.D., Schild, G.C., Bootman, J., Evans, D.M.A., Ferguson, M., Reeve, P., Spitz, M., Stanway, G., Cann, A.J., Hauptmann, R., Clarke, L.D. Mountford, R.C. & Almond, J.W. (1983). Location and primary structure of a major antigenic site for poliovirus neutralization. Nature 301: 674-679.

Minor P.D., Evans, D.M.A., Ferguson, M., Schild, G.C., Westrop, G. & Almond, J.W. (1985). Principal and subsidiary antigenic sites of VP1 involved in the neutralization of poliovirus type 3. J. Gen. Virol. 65: 1159-1165.

Minor, P.D., Ferguson, M., Evans, D.M.A., Almond, J.W. & Icenogle, J.P. (1986a). Antigenic structure of polioviruses of serotypes 1,2 and 3. J. Gen. Virol. 67: 1283-1291.

Minor, P.D., John, A., Ferguson, M. & Icenogle, J.P. (1986b). Antigenic and molecular evolution of the vaccine strain of Type 3 poliovirus during the period of excretion by a primary vaccinee. J. Gen. Virol. 67: 693-706.

Mitsuzawa, E., Yasuda, T., Tamura, N. & Ohtani, S. (1984). Pretreatment with antigen or adjuvant, and the preventability of allergic encephalomyelitis induced by basic protein with synthetic muramyl dipeptide in comparison with Freund's complete adjuvant. Japan. J. Exp. Med. 54: 97-107.

Mozes, E., Sela, M. & Chedid, L. (1980). Efficient genetically controlled formation of antibody to a synthetic antigen [poly(L Tyr, L Glu)-poly(DL Ala)--poly(L Lys)] covalently bound to a synthetic adjuvant (N-acetylmuramyl-L-alanyl-D-isoglutamine). Proc. Natl. Acad. Sci. U.S.A. 77: 4933-4937.

Nardiello, S., Pizzella, T., Russo, M., Galanti, B. (1985). Sensitive ELISA for IgG and IgM anti-albumin autoantibodies not influenced by HBsAg-associated polyalbumin receptors. J. Immunol. Methods 76: 121-127.

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

Newman, J.F.E., Matthews, T., Omilianowski, D.R., Salerno, T., Kaesberg, P. & 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.

Niemi, S.M., Fox, J.G., Brown, L.R. and Langer, R. (1985). Evaluation of ethylene-vinyl acetate copolymer as a non-inflammatory alternative to Freund's complete adjuvant in rabbits. Laboratory Animal Science 35: 609-612.

Nishimura, K., Nishimura, S., Nishi,N., Numata, F., Tone, Y., Tokura, S. & Azuma, I. (1985). Adjuvant activity of chitin derivatives in mice and guinea-pigs. Vaccine 3: 379-384.

Nisonoff, A. & Lamoyi, E. (1981). Implications of the presence of an internal image of the antigen in anti-idiotypic antibodies: possible application to vaccine production. Clin. Immunol. Immunopathol. 21: 397-406.

Omata, T., Kohara, M., Kuge, S., Komatsu, T., Abe, S., Semler, B.L., Kameda, A., Itoh, H., Arita, M., Wimmer, E. & Nomoto, A. (1986). Genetic analysis of the attenuation phenotype of Poliovirus Type 1. J. Virol. 58: 348-358.

Onică, D., Dobre, M-A. & Lenkei, R. (1978a). Immunogenicity of glutaraldehyde treated homologous monomeric albumin in rabbits. Immunochemistry 15: 941-944.

Onică, D., Lenkei, R. & Gheţie, V. (1978b). Immunogenicity of glutaraldehyde-treated homologous albumin in rabbits. Immunochemistry 15: 687-693.

Onică, D., Călugăru, A., Mărgineanu, I., Zamfir, G. & Belaşcu, I. (1983). Immunochemical characterization of anti-albumin antibodies in liver diseases. Clin. Immunol. Immunopathol. 26: 223-231.

Remy, M.H. & Poznansky, M.J. (1978). Immunogenicity and antigenicity of soluble cross-linked enzyme/albumin polymers: Advantages for enzyme therapy. Lancet, July 8: 68-70.

Riveau, G., Masek, K., Parant, M. & Chedid, L. (1980). Central pyrogenic activity of muramyl dipeptide. J. Exp. Med. 152: 869-877.

Roggero, P. & Pennazio, S. (1984). Quantitative determination by ELISA of tobacco necrosis virus from necrotic local lesions in tobacco. J. Virol. Methods 8: 283-291.

Royer, G.P., Lee, T.K. & Sokoloski, T.D. (1983). Entrapment of bioactive compounds within native albumin beads. J. Parent. Sci. Technol. 37: 34-37.

Rueckert, R.R. (1976). On the structure and morphogenesis of picornaviruses. IN Comprehensive Virology, vol. 6, pp. 131-213. (Fraenkel-Conrat, H. & Wagner, R.R., eds.), Plenum, New York.

Sakata, S. & Atassi, M.Z. (1981). Immune recognition of serum albumin-XIII. Autoreactivity with rabbit serum albumin of rabbit antibodies against bovine or human serum albumins and autoimmune recognition of rabbit serum albumin. Mol. Immunol. 18: 961-967.

Salonen, E.-M. & Vaheri, A. (1979). Immobilization of viral and mycoplasma antigens and of immunoglobulins on polystyrene surface for immunoassays. J. Immunol. Methods. 30: 209-218.

Sanderson, A.R. (1984). Use of MHC molecules to augment immunogenicity: widely applicable MHC1 purification technology and more evidence for superior carrier function. IN Modern Approaches to Vaccines: Molecular and Chemical Basis of Virus Virulence and Immunogenicity, pp. 379-383. (Chanock, R.M. & Lerner, R.A., eds.), Cold Spring Harbor Laboratory, New York.

Scherer, W.F., Verna, J.E. & Richter, G.W. (1968). Nodamura virus, an ether and chloroform-resistant arbovirus from Japan. Am. J. Trop. Med. Hyg. 17: 120-128.

Schoub, B.D., Johnson, S., McAnerney, J.M., Küstner, H.G.V. & van der Merwe, C.A. (1986). A comprehensive investigation of immunity to poliomyelitis in a developing country. Am. J. Epidemiol. 123: 316-324.

Smith, G.L. Mackett, M. & Moss, B. (1983a). Infectious vaccinia virus recombinants that express hepatitis B virus surface antigen. Nature 302: 490-495.

Smith, G.L., Murphy, B.R. & Moss, B. (1983b). Construction and characterization of an infectious vaccinia virus recombinant that expresses the influenza hemagglutinin gene and induces resistance to influenza virus infection in hamsters. Proc. Natl. Acad. Sci. U.S.A. 80: 7155-7159.

Stanway, G., Hughes, P.J., Westrop, G.D., Evans, D.M.A., Dunn, G., Minor, P.D., Schild, G.C. & Almond, J.W. (1986). Construction of poliovirus intertypic recombinants by use of cDNA. J. Virol. 57: 1187-1190.

Stechemesser, E., Scherbaum, W.A., Grossmann, T. & Berg, P.A. (1985). An ELISA method for the detection of autoantibodies to adrenal cortex. J. Immunol. Methods 80: 67-76.

Sutcliffe, J.G., Shinnick, T.M., Green, N., Liu, F.-T., Niman, H.L. & Lerner, R.A. (1980). Chemical synthesis of a polypeptide predicted from nucleotide sequence allows detection of a new retroviral gene product. Nature 287: 801-805.

Tainer, J.A., Getzoff, E.D., Alexander, H., Houghten, R.A., Olson, A.J. & Lerner, R.A. (1984). The reactivity of anti-peptide antibodies is a function of the atomic mobility of sites in a protein. Nature 312: 127-134.

Thornton, J.M. & Sibanda, B.L. (1983). Amino- and carboxy-terminal regions in globular proteins. J. Mol. Biol. 167: 443-460.

Voller, A. (1980). Herterogenous enzyme-immunoassays and their applications. IN Enzyme-Immunoassay, pp 193-194, Appendix 1, (Maggio, E.T. ed.), CRC Press, Boca Raton, FL, USA.

Wahl, S.M., Wahl, L.M., McCarthy, J.B., Chedid, L. & Mergenhagen, S.E. (1979). Macrophage activation by mycobacterial water soluble components and synthetic muramyl dipeptide. J. Immunol. 122: 2226-2231.

Walter, G., Scheidtmann, K-H., Carbone, A., Laudano, A.P. & Doolittle, R.F. (1980). Antibodies specific for the carboxy- and amino-terminal regions of simian virus 40 large tumor antigen. Proc. Natl. Acad. Sci. U.S.A. 77: 5197-5200.

Westhof, E., Altschuh, D., Moras, D., Bloomer, A.C., Mondragon, A., Klug, A. & Van Regenmortel, M.H.V. (1984). Correlation between segmental mobility and the location of antigen determinants in proteins. Nature 311: 123-126.

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

Wold, F. (1972). Bifunctional reagents. Methods Enzymol. 25: 623-651.

Yokota, S., David, C.S. & Atassi, M.Z. (1980). Genetic control of the immune response to myoglobin. 3. Autoimmune T-lymphocyte proliferative response to mouse myoglobin. Mol. Immunol. 17: 1079-1082.

Yoneyama, T., Hagiwara, A., Hara, M. & Shimojo, H. (1982). Alteration in Oligonucleotide Fingerprint patterns of the viral genome in Poliovirus type 2 isolated from paralytic patients. Infect. Immun. 37: 46-53.