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Comparison of the Immune Response against Polio Peptides Covalently-Surface-Linked to and Internally-Entrapped in Liposomes

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SUMMARY **The adjuvanticity of liposomes on two different modes of presentation of polio virus subunit peptides was demonstrated by incorporating the poorly immunogenic synthetic polio peptides, W1 and W2, into the internal space of and covalently-linked to the surface of dehydration-rehydration vesicles (DRV). It was found that for both peptides, liposome association in either mode boosted the primary and secondary IgG_1 responses against 5 μg peptide as compared to controls in which free peptide was administered. Surface-linkage of peptides (both W1 and W2) exhibited an initially more rapid rise in antibody levels, as compared to internal entrapment of the peptides, but elicited no observable secondary response. However, although encapsulated W1 showed a milder primary response when compared to the surface-linked formulation, it later elicited a strong secondary response. These results suggested that it may be advantageous to administer liposomal virus subunit vaccines in both surface-linked and internally entrapped formulations to achieve adequate initial antibody levels followed by an anamnestic response.**

(Abbreviations: MLV, multilamellar vesicles; SUV, small unilamellar vesicles; DRV, dehydration-rehydration vesicles; PC, phosphatidylcholine; DSPC, distearoyl phosphatidylcholine; Chol, cholesterol; ELISA, enzyme-linked immunosorbent assay.)

Synthetic antigens have been very useful in the study of various immunological phenomena, including the chemical basis of antigenicity and the genetic control of the immune response.[1] It has been demonstrated that synthetic peptides can elicit antibodies with the capacity to inactivate the respective viruses and to protect against a viral challenge.[2]

However, synthetic oligopeptides are non- or only weakly immunogenic and are presently used experimentally as haptens coupled to carrier proteins, often in admixture with Freund's adjuvant. Liposomes have been shown to have the potential of being versatile and non-toxic immunological adjuvants for a wide range of natural bacterial and viral products.[3,4] The presentation of synthetic virus subunit peptides associated with liposomes may represent a novel and clinically acceptable way of enhancing their immunogenicity.

In this paper, the adjuvanticity of liposomes on the polio virus peptides type 3-VP2 (designated W1) and type 2-VP2 (W2) is described. A comparison is made between two different modes of presentation of the peptides:- internally entrapped and surface-linked. Entrapped peptides may avoid conformational changes induced by coupling procedures and gives rise to immunological memory (for W1) whilst linking peptides to the surface of liposomes results in more efficient antigen presentation in the primary response without the disadvantage of introducing an immunogenic carrier protein.

MATERIALS AND METHODS

Preparation of small unilamellar vesicle (SUV) liposomes

Multilamellar vesicles (MLV) were prepared from equimolar egg phosphatidylcholine (PC) (Lipoid) and cholesterol as described.[5] Briefly, 32 μmoles egg PC were mixed with 32 μmoles cholesterol (British Drug

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Houses, U.K.) in a 50 ml round-bottomed flask (Quickfit). The lipids were dried to a thin film by evaporation of the solvent at a low speed in a rotary evaporator (Buchi) connected to a running tap water pump. Two ml of phosphate-buffered saline (PBS, pH 7.4) were added to the dried lipid film. The flask was lowered into a bath sonicator (Kerry) and subjected to burst of sonication while being manually rotated to form MLV. To convert MLV into small unilamellar vesicles (SUV), sonication of the MLV samples using a probe sonicator was performed at room temperature. A 1-minute burst of ultrasound was followed by 30-60 seconds of cooling. A total sonication time of 10 minutes gave a clear preparation as the predominantly large vesicles in the original preparation become small. After sonication, the preparation was left at room temperature for another hour.

W1 C F N K D N A V T S P K R E F C
(polio type 3)

W2 C F T P D D N Q T S P A R R F C
(polio type 1)

Fig. 1 Amino acid sequences of polio peptides W1 and W2.

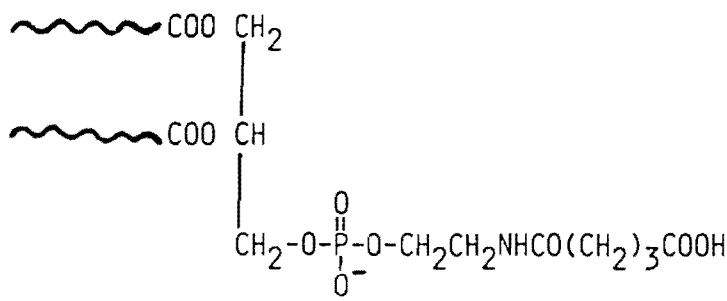

Fig. 2 Anchor: N–glutaryl phosphatidylethanolamine.

Entrapment of polio peptides in dehydration-rehydration vesicle (DRV) liposomes

The dehydration-rehydration method [6] was used to encapsulate the antigens. Briefly, small unilamellar liposomes composed of equimolar egg PC or DSPC (16 μmoles) and cholesterol were mixed with 1 ml of polio peptides W1 or W2 (gift from M. Ferguson, National Institute for Biological Standards and Control, Hertfordshire, U.K.; amino acid sequences shown in Fig. 1) at a concentration of 200 μg peptide/ml water and dehydrated overnight. The dry powder was then rehydrated with 0.1 ml distilled water followed by 0.9 ml PBS (pH 7.4). The suspension was diluted with 7 ml PBS and centrifuged at 10,000 × g for 30 minutes. The liposomal pellet was washed twice in 8 ml PBS by centrifugation. Polio peptide entrapment was estimated by measuring peptide concentrations in the supernatant using a protein assay from Bio-Rad (Watford, U.K.).

Covalent coupling of polio peptides to DRV liposomes

To covalently link the polio peptides to liposomes, the hydrophobic 'anchor', N-glutaryl phosphatidylethanolamine (structure shown in Fig. 2) prepared as described [7] was used. The acyl chains of the anchor intercalate between phospholipid molecules of the liposomal bilayer whilst the carboxyl group at the opposite extremity, after activation, binds to the amino groups on lysine residues present in peptides or proteins. Dehydration-rehydration vesicles were generated from SUV prepared form 16 μmoles egg PC, 16 μmoles cholesterol and 1.2-4.54 μmoles anchor mixed with tracer N-glutaryl(^{14}C) phosphatidylethanolamine (^{14}C-labeled anchor), both synthesised according to Weissig *et al.* [4] Incorporation of the anchor into DRV was measured by assaying ^{14}C radioactivity.

DRV (1.0 ml) incorporating various amounts of anchor were adjusted to pH 3.5 with 40 μl 0.01M HCl, and mixed with 8-15 mg 1-ethyl-3(3-dimethylamine propyl) carbodiimide. The mixture was incubated at 22°C for 5 minutes, supplemented with 1 ml 0.1 M $Na_2B_4O_7$, pH 8.5 (borate buffer) containing *p*-aminophenyl-D-mannopyranoside and incubatd at 22°C overnight. DRV were separated from non-bound polio peptides by centrifugation at 100,000 × g for 60 minutes and the liposomal pellet washed once with borate buffer. Control DRV were incubated with the peptides in the absence of carbodiimide.

Animal immunization experiments

BALB/c mice were primed in-

tramuscularly in groups of five with 0.1 ml containing 5 μl polio peptide free, entrapped in or covalently linked to DRV. Four weeks later, they were boosted with the same amount of free or liposome-associated antigen. Blood samples were obtained from the tail veins 1 day before and 14 days after priming and 10 days after the booster injection. Serum samples were assayed for anti-peptide (W1 or W2) IgG_1 by ELISA.

Enzyme-linked immunosorbent assay (ELISA)

Antibody responses to liposome-associated antigens in immunized mice were monitored by a microplate ELISA as follows: A solution of the polio peptide (W1 or W2, 30 μg/ml 0.05 M carbonate-bicarbonate buffer, pH 9.6) was added into each of the wells of a plastic microelisa plate (Dynatech) and incubated at 4°C overnight. The wells were washed three times in 9.5 mM sodium phosphate buffer containing 0.8% NaCl and 0.05% Tween 20, pH 7.4 (PBS-Tween buffer), allowed to dry and stored at room temperature for a maximum of four days. Serum samples from immunized mice were diluted twenty-fold in PBS-Tween buffer with 0.5% BSA. Fifty μl portions of diluted sera were dispensed into the microtiter plate wells, covered and incubated at room temperature for 3 hours. Each well was washed three times with PBS-Tween buffer with a minimum of 3 minutes between washes. Fifty μl of a horseradish peroxidase-labeled rabbit antimouse Ig serum diluted in PBS-Tween with 0.5% BSA and 5% fetal calf serum was dispensed into each well, covered and incubated at room temperature for 3 hours. The washing procedure was then repeated. Subsequently, 200 μl of the substrate (40 mg o-phenylenediamine in 100 ml 2.8 mM sodium phosphate/1.4 mM citric acid acid buffer, pH 5.0, into which 40 μl of 30% H_2O_2 had been added immediately before dispensing) was added to each well. After 30 minutes, the reaction was stopped by the addition of 50 μl 2.5 M H_2SO_4 and the colour change read spectrophotometrically at 492 mm. ELISA values were determined by subtracting the mean of two duplicate readings obtained with serum taken before injection of mice, from the mean of duplicate readings obtained with serum taken from the respective mice at various time intervals after the second injection.

Statistical analysis

Differences in absorbance readings, as determined by ELISA, between two groups of mice being compared were tested for significance by the Mann-Whitney non-parametric test for two samples. In this test, the statistic T was taken to be the lower sum of ranks of all observation in the two groups being compared.

Where serum samples were titrated, comparison of antibody titers between two groups of mice was made using Student's t test.

RESULTS AND DISCUSSION

Table 1 shows the percentage binding values of each of the polio peptides, derived from polio type 3VP-2 (designated Wl) and from type 1VP-2 (designated W2) to PC: Chol liposomes containing two different molar proportions of the anchoring ligand N-glutaryl phosphatidylethanolamine.

Fig. 3 compares the primary and secondary responses in BALB/c mice to 5 μg of both W1 and W2 in various preparations: free, covalently surface-linked to, or internally entrapped in PC liposomes.

Fig. 4 shows the serum IgG_1 titers obtained in the secondary responses to liposome-entrapped and surface-linked W1 and W2.

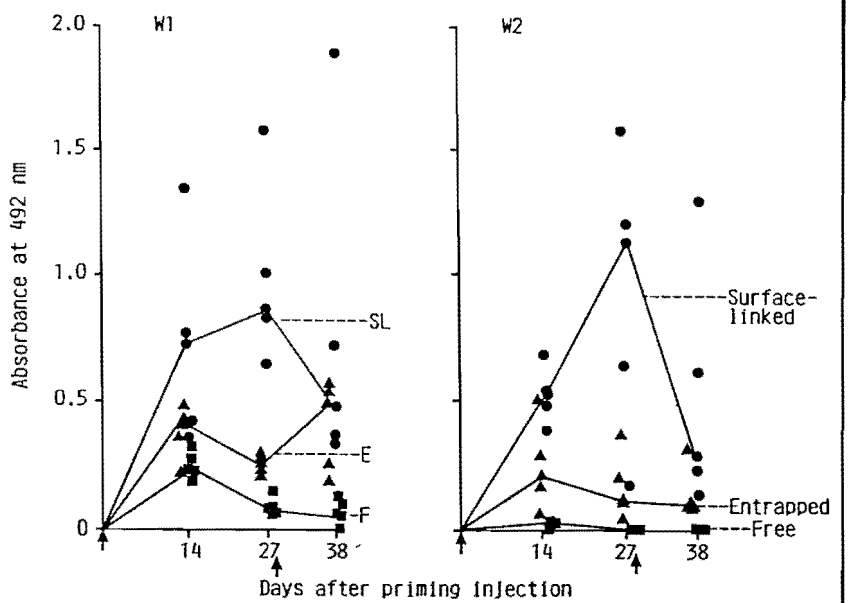

Fig. 3 Antibody responses (IgG_1) to free and liposome–associated polio peptides
BALB/c mice in groups of five were injected intramuscularly twice (with a 28–day interval between injections) with 5 μg free (■), covalently surface–linked to (●), or internally entrapped in (▲) PC DRV. Animals were bled one day before and 14,27 and 38 days after the priming injection.

Coupling values of polio peptides to DRV liposomes

The coupling reaction via the anchoring ligand N-glutaryl phosphatidylethanolamine of both polio peptides to the external surfaces of DRV liposomes was efficient. Binding values (Table 1) ranged from 21-70% (for W1) and 23-52% (for W2) of the total amount of polio peptide added. It is seen that increasing the molar ratio of anchor in relation to phospholipid and cholesterol did not result in increased binding values. Covalent coupling was specific as witnessed by the comparatively low binding values displayed by control liposomes in which the reactive carboxyl groups on anchor molecules were not activated by carbodiimide. These low values could possibly be due to non-specific adsorption onto the external surface of the liposomes.

Table 1. Polio peptide coupling to liposomes
DRV liposomes incorporating 'anchor' were interacted in the presence or absence (controls) of carbodiimide with 200 μg peptide. Peptide coupling was estimated from the amount measured in the supernatant. Numbers in parentheses denote control values.

Molar ratios of lipids (PC:chol:anchor)	Peptide bound (% of used)	
	Polio type 3VP–2	Polio type 1VP–2
1:1:0.11	21–70 (5–13)	23–52 (8–9)
1:1:0.28	51–65 (7–8)	41–63 (6–7)

Immune response to free and liposome-associated W1

In a study designed to compare the effects of immunizing mice with free, surface-linked and liposome-entrapped W1, BALB/c mice in groups of five were injected twice with 5 μg of the polio peptide in its various formulations with a 28-day interval between injections. Animals were bled on the days indicated in Fig. 3 and serum IgG_1 levels were determined by ELISA.

Results show that liposome-associated W1 elicited higher IgG_1 responses as compared to free W1 for both the primary and secondary immune responses (Fig. 3). Antibody levels against free W1 peaked at 14 days after the priming injection and fell to lower levels thereafter. No characteristic rapid rise in antibody levels as would be seen in an anamnestic response was observed even 10 days after the booster injection of 5 μg free W1. Comparing levels obtained for covalently surface-linked and internally-entrapped peptide, much higher levels were observed for the primary response when the antigen was surface-associated. IgG_1 levels against the latter rose steeply to attain a much higher level than that against entrapped W1 at day 14 and continued to rise and peak at day 27 before falling sharply thereafter even after the booster injection was given. Thus, it would appear that the immune response to surface-linked W1 was an exaggerated form of the response to the free peptide, probably induced by the carrier and adjuvant effect of the liposomes.

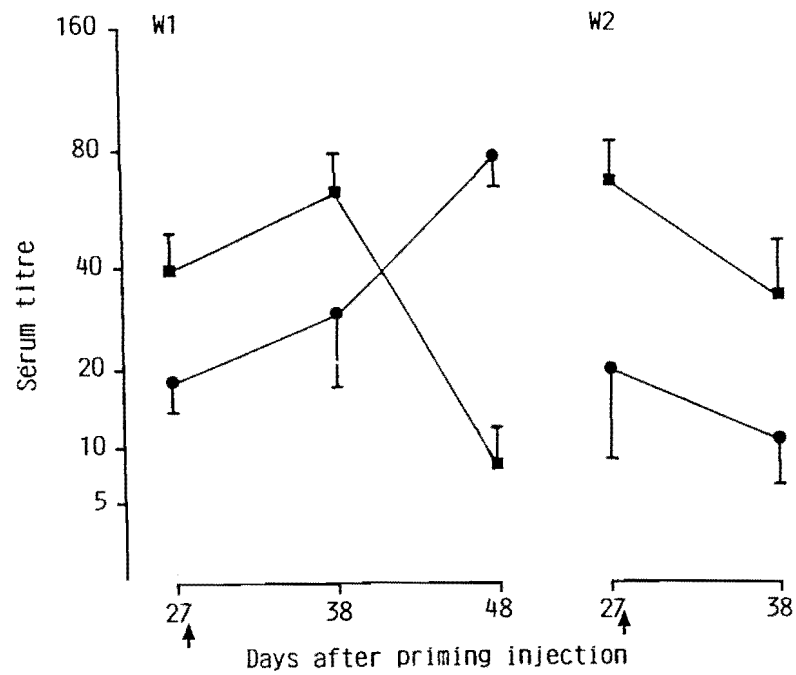

Fig. 4 Titration of antibody responses (IgG_1) to liposome–entrapped (●) and surface–linked (■) polio peptides.
Sera obtained from mice immunized as described in the legend to fig. 1 were titrated by doubling dilutions. Results are expressed as the highest titer of serum that yielded an optical density of 0.100 as determined by ELISA.

For the entrapped W1, even though IgG_1 levels in the primary response did not reach as high as those attained with surface-linked peptide, peaking at day 14 and declining in tandem with the response to free W1 to lower levels at day 27, a good anamnestic response was observed. Ten days after the booster injection, median IgG_1 levels rose to reach values higher than the peak at day 14.

Immune responses to free and liposome-associated W2

To determine the immune responses against various modes of presentation of the polio peptide W2, an identical experiment was performed using free, surface-linked and internally-entrapped W2 to immunize BALB/c mice.

Results for the primary response showed a similar pattern to that obtained for W1, with the notable exception that free W2 was intrinsically very poorly immunogenic, hardly eliciting any IgG_1 response at all under the assay conditions employed. As with W1, association of the peptide with liposomes resulted in a marked enhancement ($p < 0.01$, $T = 15$ where T is the lower sum of ranks of either surface-linked or entrapped W2 being compared to free W2 in the Mann-Whitney test) of immunogenicity with surface-linked W2 giving by far the highest response, with peak levels being reached at 27 days and falling precipitously thereafter. A more modest rise in IgG_1 levels is seen with entrapped W2, peaking at day 14 and decreasing gently after that.

An important difference between the secondary response to W2, compared to W1 is that no anamnestic response was observed, even 10 days after the booster injection of 5 μg of the peptide. IgG_1 levels of all three modes of presentation of the antigen fell to below peak values observed during the primary response. This suggests that the dose used may be a tolerogenic one and that unlike the case of the peptide W1, association of W2 with liposomes does not alter the situation, apart from exaggerating the primary response.

Titration of IgG_1 responses to surface-linked and liposome-entrapped polio peptides

To determine the relative amounts by which IgG_1 levels differed when immunized with either surface-linked or entrapped polio sub-unit peptides, serum samples were titrated by doubling dilutions and results expressed as the highest titer of serum that yielded an optical density of 0.100 as measured by ELISA.

Since even undiluted serum from mice immunized with free W1 or W2 did not give absorbance values of over 0.100, serum titers could not be reliably compared with those of liposome-associated peptides. Suffice it to say that presentation of either of the peptides W1 or W2 in a form associated with liposomes, be it covalently surface-attached of internally entrapped results in an increase in IgG_1 levels of greater than an order of magnitude.

For W1, pre- and post-booster IgG_1 levels indicated that antibody levels elicited by surface-linked peptide were about twice as great as those elicited by entrapped peptide (Fig. 4). The mean serum titer observed 10 days (day 38) post-booster injection with surface-linked W1 appeared to be greater than the mean titer of entrapped W1 at day 38 whereas median values of untitrated sera (Fig. 3) demonstrated the opposite relationship to be the case. This discrepancy is due to the fact that a single mouse from the group of five immunized with surface-attached W1 showed an exceedingly high response at day 38, leading to a highly-skewed distribution. Serum titrations done 20 days post-booster revealed that IgG_1 levels in mice immunized with internally-entrapped W1 continued their ascent such that these became more than ten times greater than surface-linked W1 IgG_1 levels. Thus, the relationship of median values of untitrated sera at day 38 give a more accurate reflection of the declining trend in surface-linked W1-elicited IgG_1 levels. In the case of W2, it is seen that pre- and post-booster levels of surface-linked peptide-elicited IgG_1 were about three times as great as those of entrapped peptide-elicited IgG_1. In contradistinction to the opposite movements of antibody levels obtained by the two different modes of presentation of W1, IgG_1 levels in both modes of W2 presentation exhibited a downward trend, indicating that no secondary response was being elicited.

There are several antigenic components in viruses, each of which consists of multiple antigenic regions. Some of these are relevant in inducing a protective immune response in the host. Identification of such regions and synthesis form the basis in the design of the new generation of vaccines.[8] Amongst the advantages of synthetic antiviral vaccines are the avoidance of problems associated with conventional vaccines (e.g. large-scale cultivation of viruses with attendent risks and cost of extraction of viral agents from infected donors), stability in freeze-drying and storage conditions and improved purity.

Generally, synthetic oligopeptides are thought to be too small to induce antibodies and they are thus coupled, via a variety of reagents, as haptens to larger carrier molecules such as keyhole limpet hemocyanin, diphtheria toxoid and albumin. However, this approach is undesirable and will need to be replaced: peptides will assume a number of conformations upon coupling and preparations are difficult to standardize. Immunological adjuvants will therefore be needed if enhancement of oligopeptide immunogenicity is to be achieved. Powerful adjuvants such as Freund's complete adjuvant cannot be used in man and clinically

acceptable adjuvants such as alum (which has proven effective with some synthetic peptides) can only be a temporary measure, in view of its deficiencies.[9] At present, muramyl dipeptide (MDP), especially some of its non-pyrogenic derivatives, appears to be promising.[10] MDP with added acyl chains or covalently linked to antigens have been incorporated into liposomes for further improvement of adjuvanticity. Liposomes thus seem a reasonable alternative, especially since no conformational changes in the peptides are expected to occur upon passive entrapment. Novel synthetic adjuvants such as the nonionic block copolymer surfactants[11] also have the potential of being formulated into liposome-like structures.

Amongst the controversies surrounding the mode of presentation of antigens in liposomes is the question of whether covalent surface linkage or internal entrapment is the most efficient way of capitalising on the adjuvant effect of liposomes.[12] A recent study[13] employing bovine serum albumin either free, encapsulated in liposomes or covalently linked to the liposomal surface suggested that although both types of liposome association are equally efficient in potentiating the humoral response, encapsulation mainly favours IgG isotype production with little or no effect on the IgM subset, while covalent linkage stimulates the production of both IgG and IgM. Our present study gives further insight into the time course of IgG production with the discovery that surface-linked peptides are more effective in eliciting an IgG_1 response after the priming injection while the encapsulated form is more effective (for W1) after the booster injection. Using liposomes formulated from phospholipids of varying acyl chain lengths, it was shown that DRV with bilayers of higher liquid-to-gel transition temperatures constructed of longer chain phospholipids (e.g. distearoyl phosphatidylcholine) were more effective in potentiating the secondary IgG responses to entrapped W1.[14] This contrasts with results obtained for encapsulated tetanus toxoid[15] in which 'fluid' liposomes with shorter acyl chain lenths were found to be better adjuvants for IgG production. Clearly, the often conflicting data amassed to date reveal that the factor of mode of antigen localisation can only be considered in isolation if all other parameters such as lipid composition, nature and dose of antigen, lipid-to-antigen ratio, time of antibody titer determination and vesicle size and structure are rendered equal in controls. This gives rise to the difficulty in comparing results from different studies which employ widely ranging conditions so that the conclusions drawn as to whether surface linkage or internal entrapment is more effective are rendered invalid. For the case of the polio peptide W1 at least, one may conclude that it would be more efficacious to administer the antigen in both covalently surface-linked and entrapped forms in liposomes constructed of long-chain phospholipids to elicit rapid primary and secondary antibody responses.

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