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Liposomes and their Potential in Oral Cholera Vaccines

Liposomes are concentric spheres of phospholipid bilayers with aqueous compartments which can entrap proteins and other antigens. [1] They are considered as new water-in oil emulsions of the Freund's adjuvant type, but suitable for human use since: being microscopic droplets, they will not cause granuloma formation at the injection sites; and liposomes using phospholipids naturally present in host cells are completely biodegradable. [2]

Uses of Liposomes

The potential of liposomes as carriers in medicine has been predicted as early as 1935 but such potential became apparent in experiments in which liposomes injected into animals were found to control both rate of clearance of liposome-entrapped agents and their destination in the body. [3]

1. Liposome as protective carriers of active substances: Experiments revealed that liposomes were suitable for delivery of biologically-active substances such as invertase enzyme to macrophages and fibroblasts in cell cultures, [4] or of cytotoxic drugs *in vivo*. [5] Liposomes not only protected their contents from the environment, but also prevented leakage of damaging substances to host tissues. It was also found in the experimental manipulation of liposomal contents that their surface properties can be altered to increase affinity to target cells. This has facilitated the selective uptake of drugs by diseased cells as has been demonstrated by the delivery of liposome-entrapped enzymes to defective cells in storage disease. [4] When proteins are loaded in liposomes and given orally, it could enable efficient transfer of proteins to the plasma in high concentrations. This was observed with the entrapment of blood clotting factors inside liposomes for oral treatment of haemophilia A. Gastrointestinal absorption of factor VIII in liposomes delivered the substance to the plasma. [6] With intravenous injection, liposome-entrapped substances reach the liver, spleen and other tissues, and are taken up by cells of the reticulo-endothelial system which are responsible for the rapid clearance of liposomes from the blood (see below). [3]

2. Liposomes as drug carriers: Intially-experimented as carriers of therapeutic agents, liposomes have been used as drug carriers in cancer chemotherapy with encouraging results. [5] There was increased survival of tumor-bearing mice treated with liposome-entrapped actinomycin D. In addition, entrapment of cancer drugs protected the tissues of the host from the cytotoxic effects of such drugs.

3. Liposomes as carriers and adjuvants in vaccines: The use of liposomes as antigen carriers, magnified by addition of substances with marked affinity for epithelial membranes, is a possibility for oral vaccination and provides a new and potentially-significant development for the future. [7]

Allison and Gregoriadis [1] reported that liposomes can be used as adjuvants to entrap antigens which are then released gradually to elicit antibody formation and perform the role of a booster. These authors explored this area by experimental immunization of mice with diphtheria toxoid (DT). DT administered intravenously in negatively-charged liposomes elicited stronger antibody (Ab) responses than in the free form. Subcutaneous (footpad) and intramuscular inoculation of the same significantly enhanced primary and secondary immune responses over those obtained when the antigen was administered freely. Without entrapment, DT caused serum sickness

and mortalities which were not observed with liposome-entrapped antigens. Another significant experiment was conducted by New *et al.*, [8] in the search for effective delivery of snake venom in immunization against this lethal antigen. Usually, immunity to snake venoms takes a long period to develop. However, entrapment of venoms in liposomes produced powerful, sustained, and protective antibody responses after subcutaneous, intravenous or oral administration of the preparation to mice, sheep, and rabbits. This was also the first report of oral liposomes which inspires their prospect in cholera vaccines.

Different Classes of Liposomes

Liposomes are formed when water-insoluble polar lipids, namely phospholipids, are confronted with water. [3] Held together by reinforcing, non-covalent interactions, phospholipid bilayers in aqueous medium have an inherent tendency to close on themselves, leaving no ends with exposed hydrocarbon chains, which results in the formation of a compartment. Lipid bilayers are self-sealing because a hole in the bilayer is energetically-unfavorable. [9] The following is a loose classification of liposomes that have been used in experimental studies:

1. Multilamellar vesicles (MLVs)

These were the original liposomes prepared by Bangham in 1965 which have been suitable in defining many properties of model membranes. [3,10] These vesicles form when phospholipids are suspended in water. The lipid bilayers arrange into concentric vesicles of several bilayers alternating with aqueous compartments. [3]

2. Unilamellar vesicles (ULVs)

As the name implies, these are vesicles having a single phospholipid bilayer enclosing an aqueous compartment. Like the MLVs, the ULVs have small aqueous space and limited ability to entrap large macromolecules. [10]

3. Dehydration- rehydration vesicles (DRVs)

Sonicated vesicles are freeze-dried, stored, and as the need arises, they are suspended in aqueous antigen solution. Davis and Gregoriadis [11] reported that antigen entrapment in DRVs using tetanus toxoid as antigen ranged from 39-82%.

4. Reverse-phase evaporation vesicles (REVs)

These are unilamellar vesicles about 200-1,000 nm, with large aqueous compartment and high percentage entrapment, prepared from "inverted" micelles suspended in organic solvents. [10]

Materials Used in the Preparation of Liposomes

Phospholipids and cholesterol are the most common materials used in the preparation of artificial membranes. Phospholpids are the major components of membrane lipids. Those derived from glycerol are called phosphoglycerides. The simples structure is that of phosphotidate which forms the backbone of the larger molecules like phosphatidylserine,phosphatidylethanolamine (cephalin), phosphatidylcholine (lecithin), diphosphatidylglycerol and phosphatidylinositol. The other groups of phospholipids are derived from sphingosine where the polar head is esterified to phosphorylcholine to form sphingomyelin. [9]

Cholesterol is a neutral lipid which is present in plasma membrane of eukaryotic cells but less seen in organelle membranes. It is included in liposomal preparation to increase the captured volume and lend fluidity to the liposomal membrane. [10]

Membrane lipids are amphipathic structures, *i.e.*, they possess both hydrophilic (polar) heads and hydrophobic (hydrocarbon) tails. In an aqueous medium, phospholipids form micelles or bilayers where the polar heads are at the outer layers due to their affinity to water. In organic solvents, these structures are consequently reversed to form "inverted micelles". [10]

Procedures in the Preparation of Liposomes

Multilamellar vesicles: Preparation of liposomes always starts with dried lipids. The simplest procedure consists of dispersing a dried film of lipids in an aqueous antigen solution. MLVs are formed this way, entrapping antigen molecules in the aqueous compartments as the lipid bilayers closed in. The MLVs can then be washed and used immediately as in the experiment of Bakouche and Gerlier. [12]

Unilamellar vesicles: These vesicles are derived by subjecting MLVs to one of the following methods :

1) Ethanol injection method. Vesicles are prepared by rapidly mixing a solution of lipids in ethanol with water and injecting the lipids through a fine needle. This procedure forms vesicles about 500 Å in diameter. [9]

2) Sonication method. Lipid suspensions in an aqueous phase are agitated ultrasonically using a sonicator machine. This procedure further breaks up MLVs into smaller, unilamellar structures. [3] The temperature and time of sonication depend on the purpose of the user and also upon the phase transition temperature of the lipids. Sonication is best done above the transition temperature, but not so high as to denature the molecules in the mixture.

3) Detergent dialysis method. Lipids are dissolved in buffered-saline solution containing sodium deoxycholate or other bile salts. [13] Bile salts seem to accomplish the purpose of sonication.

Combined detergent dialysis and solvent evaporation is a procedure which leaves detergent along with the preparation, which is unsuitable for biological use. [10]

Reverse-phase evaporation vesicles (REVs)

Slow evaporation of the organic solvent suspending the phospholipids produces large vesicles about 500 Å in diameter.[9] Szoka and Papahadjopoulos[10] developed a technique that can produce REVs with large internal space and high entrapment. The dried lipids are redissolved in organic solvent in which REVs are formed. The aqueous phase is added at this point and the mixture is sonicated. The organic solvent is dried by rotary evaporation to recover the vesicles. Finally, buffer is added to suspend the liposomes.

Liposomes prepared by any of these methods can be purified through dialysis, column chromatography, centrifuge washing, or by sterilization techniques.

Incorporation of Antigens in Liposomes

There are three ways by which liposomes can be used as carriers of antigens.[12] The antigen can be 1) entrapped in the aqueous compartment, 2) incorporated in the lipid bilayers, and 3) covalently attached to the liposome surface.

Antigen solutions are trapped inside liposomes by adding them to the phospholipid suspension before sonication.[9] The technical aspect of entrapment is simple: lipids are dissolved in organic solvent which is subsequently removed to leave a thin film of lipids. Upon reconstitution with aqueous antigen solution, liposomes are formed and the antigen solute is trapped in the aqueous compartment. Lipid-soluble substances, on the other hand, are incorporated into the lipid phase of liposomes by dissolving them together with the lipids in the organic phase.[3] Antigen molecules can be covalently attached to the liposomal surface by using bridging molecules such as SATA, SPDP or other substances. This chemical bonding renders soluble antigens more immunogenic than free antigens. Waldman *et al.*[14] used SPDP [N-succinimidyl 3-(2-pyridyldithio) propionate] to link antigens to the liposomal surface. On the same principle, Davis and Gregoriadis[11] linked tetanus toxoid to the surface of liposomes pretreated with $NaNO_2$. Toxoid was then added to the diazotized liposomes. In contrast, Shek and Sabiston[15] simply adsorbed bovine serum albumin to the surface of liposomes without using any bridging substances.

Transport and Uptake of Liposomes in the Body

Liposomes injected intravenously into rats are quickly removed from the blood and are found localized mainly in the liver and spleen, and to lesser extent in other tissues. Active substances and drugs in the aqueous compartment are carried unaltered in the circulation.[3] Removal of liposomes from blood is biphasic, consisting of a fast initial phase and a slower second phase. Actually the rate of removal was found to be related to size and surface charge. Negatively-charged liposomes are removed more rapidly than those of neutral or positive charge. However, many liposomes may acquire their negative charges in association with plasma proteins. Cells of the reticuloendothelial (RE) system, especially fixed macrophages, are responsible for the rapid initial clearance, while the slower second phase is linked to uptake of liposomes by other tissue cells, *e.g.*, parenchymal cells of the liver.[3]

Liposomes themselves lose their structure soon after endocytosis by cells of the RE system as the lipid substances are degraded in the phagolysosomes of the cell. The trapped substances are then released to the cytoplasm and nucleus.[5]

McDougall *et al.*[16] reported that the vesicles administered via the intraperitoneal route, were distributed in the same manner as those adminsitered by the intravenous route, after passage of the liposomes through the vascular barrier into the portal vein. The evidence suggested that passage through the endothelial membrane did not alter the structure of the vesicles. Subcutaneously-applied liposomes, especially the large liposomes, were cleared slowly from sites, suggesting their possible use as slow-release depots.

The possible fate of liposomes given orally has been studied both *in vivo* and *in vitro*. Glucose oxidase administered intragastrically associated with phosphatidylinositol liposomes reduced blood glucose; similar results have been reported with entrapped insulin in normal and diabetic animals. The integrity of the liposomes as carriers of active substances afforded both protection of the entrapped protein from the digestive enzymes of the gut and efficient penetration into the circulation.[3]

Using labeled lipid molecules as markers, Mutsch *et al.*[17] studied the interaction of liposomes with enterocytes *in vitro*. Lipid exchange rate depended on collision frequency between the vesicles and enterocytes, while fusion was a minor process. Less-soluble lipid molecules used exchange-proteins to increase the rate of exchange. These authors found that the contents of vesicles partially leak out during the process of lipid exchange. Whether this process is physiologically-aligned or whether there are protein catalysts *in vivo* were not covered by the experiment. In living animals, specialized antigen absorptive M cells overlying the Peyer's patches are actively involved in antigen uptake in the gut, and this may apply equally to uptake of oral liposomes. Owen *et al.*[18] have studied the mechanism of uptake of free antigens in the gut, but there have been no similar studies as yet for oral liposomes.

Mammalian cells such as mouse

3T3 and L929 cells and human erythrocyte ghost cells were used in a study for investigating *in vitro* uptake of liposomes.[19] Although the mechanism of fusion had been inferred from an earlier study,[20] the later study showed that several non-exclusive mechanisms played a role in uptake, such as: exchange diffusion of lipid molecules betwen vesicles and natural membrane, endocytosis and surface adsorption. These processes were temperature-dependent. "Solid" vesicles made from long-chain fatty acids were unable to fuse to cells but were absorbed through endocytosis, while "fluid" vesicles, prepared from less-complicated materials were incorporated by fusion. In endocytosis, vesicles were incorporated intact into the cells. Pagano *et al.*[21] found that ULVs were more efficient than MLVs in phospholipid transfer (and consequently, antigen transfer). During uptake processes, normal cell growth was undisturbed and the lipids were not altered.

Mechanisms of Liposome Adjuvanicity

Though the mechanisms of liposome adjuvanicity are poorly understood,[12,22] several studies shed light on this matter. Studies have shown that liposomes stimulate B cell proliferation *in vitro* and *in vivo*.[22,23] The B cell mitogenic effect is polyclonal, similar to that of LPS. Van Houte *et al.*[24] established that the response is T-cell independent, but could enhance T cell cytotoxicity. However, Walden *et al.*[25] found that inserting a combination of class II MHC and foreign antigens into liposomes activated the regulatory helper and suppressor T lymphocytes. Macrophages were found to be the main target of liposomes and were the cells that initiated the immune response.[12]

Factor affecting the adjuvanicity of liposomes :

Composition Liposomes of certain lipid compositions prepared by the detergent method induced the proliferation of spleen cells from different mouse strains.[13] Liposomes prepared from dimyristoyl lecithin (DML) and phospholipid-cholesterol combinations induced B cell proliferation, while egg lecithin was not mitogenic. Bakouche and Gerlier[12] observed that the length and saturation of the lipid material and inclusion of cholesterol affect the stability of liposomes in the system. The composition of lipids is related to the phase-transition temperature.

Phase-transition temperature In 1977, Yasuda *et al.*[26] reported that phospholipids with high transition temperatures, *e.g.*, beef sphingomyelin and distearoyl phosphatidyl choline, were more immunogenic than those with low transition temperatures like egg lecithin and dioleoyl phosphatidylcholine. However, Bakouche and Gerlier[12] reported that antibody responses were similar for phospholipids with transition temperatures ranging from -32 to 41.5° C.

Net surface charges The phospholipid composition influenced uptake by macrophages based on the net surface charge.[1,3,12] In the system, negatively-charged liposomes were favored, but whether the same holds true for oral liposomes is not yet known.

Mode of antigen incorporation The mechanisms by which liposomes enhance immunogenicity are likely to vary with different antigenic models, *i.e.*, entrapped, inserted in lipid bilayers, adsorbed to the surface, or as carrier for haptens.[12] Entrapped antigen was more immunogenic than adsorbed,[15] but combining entrapment and adsorption produced the highest adjuvant effect. However, Davis and Gregoriadis[11] reported that there is no difference in response to surface-adsorbed or entrapped antigens.

Phospholipid : antigen ratio There is a liposomal-toxoid mass ratio optimal for adjuvanicity.[11]

Liposomes as Carriers for Haptens or Low Antigenic Substances

The specificity of the immune response to an immunogenic protein can be ascertained by its specific determinants, while the rest of the molecule is regarded inert. Haptens are used to explore the influence of these molecules. If a hapten is attached to a heterologous protein carrier, it generates a hapten-specific response that is not observed with a carrier derived from the recipient species.[27] Uemura *et al.*[28] showed that liposomal membrane actively sensitized with 2, 4-DNP-aminocaproyl-phosphatidylethanolamine (DNP-Cap-PE) elicited the formation of anti-DNP antibodies in guinea-pigs without inducing significant antibody formation to other components of the liposome. Similarly, animals immunized with liposomes containing the fluorescein-isothiocyanyl conjugate of phosphatidylethanolamine (PE) produced anti-fluorescein antibodies.

Thus, liposomal model membranes may be novel carriers into which B cell determinants (hapten) can be incorporated within the lipid bilayers instead of the conventional covalent bonding of the hapten to a high molecular weight, water soluble carrier.[29]

Potential of Liposomes in Oral Cholera Vaccines

Cholera is essentially a local mucosal disease of the gut. Logically,the mucosal immune response is expected to be solely responsible for protection through secretory antibodies mainly SIgA which could be available at the lumen of the intestines.[7] Parenteral vaccination enhances the systemic immune response but suppresses the mucosal system,[30] yet still confers some degree of protection. The observed immunity in these parenteral vaccines was found to have resulted from incidental transudation of serum IgG antibodies to the intestinal lumen during

periods of high serum titres.[31] On the other hand, oral vaccination using killed whole cells, though showing promising safety could only stimulate the gut immune system if given in very large doses and over considerable periods. This is in order to overcome the local immunological tolerance mechanisms.[7] In antigen sampling in the gut, such mechanisms are necessary in order not to disturb normal digestive functions. Antigens must be powerful enough to evoke a response. In using large doses of weak antigens, the vaccine is predisposed to uncalculated risk.[31]

The above argument against the use of inactivated vaccines together with the fact that the vaccines only stimulated partial immunity focused attention to cholera toxin (CT) or its derivatives as immunogen. It has been shown that the CT alone can produce a clinical syndrome identical to cholera.[32] With the reports of its adjuvant properties[33,34] and strong antigenic characteristics when used orally,[35] the toxin was used as a monovalent vaccine. Different preparations of CT evoked different responses in rats.[35] The holotoxin stimulated a stronger response than the B subunit alone, while toxoids did not evoke a primary response. Procholeragenoid, obtained by heating the toxin at 65° C for at least 15 min[36] was as immunogenic as the parent toxin while retaining only 5% of its original toxicity. However, these antigens that stimulate purely antitoxic immunity have not sufficiently protected humans against cholera.[32] Subsequent experiments have been then involved in exploiting the synergistic effect of combining antibacterial and antitoxic immunity. It was found that combined antigen vaccines increased the immune response 100-fold.[37] The relative efficacies of combined antigen vaccines have been evaluated. Due to the synergism, there is a considerable reduction in antigen dose.[38,39] In field trials using a combination of procholeragenoid and whole cells of *Vibrio cholerae,* the vaccine produced 27-67% efficacy[40] when given in 3 doses. In 1984, Svenerholm *et al.*[41] compared oral and parenteral routes in immunization of volunteers using B subunit plus killed whole cells. The duration of mucosal response was longer and stronger than systemic response but the oral route required two doses while the intramuscular route needed only a single dose to produce a strong serum response. However, field trials of the vaccine proved only 67% efficacy after three doses.[42] Clemens *et al.*[43] conducted a field trial of oral killed whole cell-B subunit vaccine (WC-B) in comparison to killed whole cells (WC). The WC-B protected 85% while the WC had a 58% efficacy in a 6-month study. The vaccines were all given in three doses. A subsequent study[44] showed that for the first dose of the WC-B, about half of the vaccinees were protected, but only the third dose produced a sufficient 5-fold increase in titre, particularly among children. So far, none of the experimental vaccines tested in humans can be delivered in a single dose which is enough to be protective and of public health significance.[42]

In laboratories, much work have been done on the animal models. Antigens which had immunogenic properties have been identified and isolated using various techniques and tools. Lipoplysaccharides (LPS) of *Vibrio cholerae* have been isolated using the phenol-water extraction method[45] while the cell-bound haemagglutinin (CHA) has been extracted.[46-48] Combination of LPS and CHA together with procholeragenoid (P) has been tested in rats[39] and found to have marked synergism over vaccines using only one or two of these antigens. Although the results are encouraging, the oral application of such free antigens to humans is hindered both by ethical and safety consideration, hence there is a need to devise a technique with which to make this new combined antigen vaccine acceptable.

In light of these events, the development of ways to prolong and augment the protective immune response is a perceived necessity. The need for adjuvants and safe carriers for cholera vaccines has been underscored by WHO.[49] It is expected to lessen the number and size of doses, enhance the efficacy of oral cholera vaccines, and effect a significant improvement of response in populations, especially in low responders like children, who are most susceptible to the infection.

Liposomes offer the greatest promise for an oral adjuvant applicable to cholera vaccines for several reasons. First, they can be made of completely biodegradable materials which can be varied to suit specific purposes.[1] Second, there are various means by which the *V. cholerae* antigens can be carried in liposomes, with methods such as insertion in lipid bilayers,[29] entrapment in the aqueous phase, or combination of entrapment and surface-adsorption.[15] Since liposomes can leak out their contents only upon contact with target cells,[4] the ethical problem of orally administering free antigens to humans is by-passed. Third, antigens with affinity for mucosal epithelium such as *V. cholerae* cell-bound haemagglutinin or cholera toxin-B subunit can be attached to the liposome surface to enhance their effectiveness as oral adjuvants.[7] Fourth, use of liposomes and adjuvants as a whole, renders purified antigens more immunogenic. Laboratory and field trials showed that it is much better to use purified antigens in cholera than whole cells. Fifth, the mechanisms of action of liposomes such as B cell mitogenicity and enhancement of T cell cytotoxicity accord with the protective mechanisms of mucosal immunity against cholera. Lastly, with many studies exploring the adjuvanicity and immunogenicity of liposomes, a wealth of background information

is available for which to base further studies on the applicability of liposomes in cholera vaccines.

Conclusion

The existing commercially-available vaccines for cholera are killed whole cell vaccines applied through the parenteral route in at least two doses. Though some degrees of protection has been achieved with these vaccines, recent knowledge of immunological mechanisms involved in cholera showed that orally-administered vaccines are more preferable.

Antigens eliciting both anti-cell and anti-toxin antibodies have been combined, producing synergism which lowered the antigen doses in vaccines but not the number of boosters. Adjuvant acting through depot mechanisms can eliminate the need for a booster but produce severe side reactions. Hence, a new adjuvant which is safe and biodegradable, capable of carrying and enhancing the immunogenicity of combined purified antigens and applicable for oral stimulation is required for cholera.

Liposomes satisfy all these conditions. Extensive research generated on this synthetic adjuvant suggest that it offers a great deal of promise as adjuvant for oral cholera vaccines.

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