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Immunopotentiation of the humoral response by liposomes: encapsulation versus covalent linkage

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SUMMARY

Two different modes of antigen association with liposomes were compared for their stimulation of IgM- and IgG-producing cells in primary- and secondary-response experiments. The study was carried out on BALB/c mice using the antigen bovine serum albumin either free, encapsulated in liposomes or covalently linked to the liposomal surface. Our results indicate 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 results reconcile some apparently conflicting published data and suggest that the mode of antigen association with liposomes considerably influences the pathways by which stimulation occurs.

Liposomes are membranous vesicles essentially composed of naturally occurring phospholipids which, because of their numerous advantages, have rapidly been envisaged as potential *in vivo* carrier systems (Gregoriadis, 1983; Ostro, 1983; Koff & Fiddler, 1985). Although in many applications liposomes have not been as powerful tools as expected (Gregoriadis, 1978), they have been shown repeatedly, since the first report by Allison & Gregoriadis in 1974, to potentiate efficiently the humoral as well as the cell-mediated immune responses to different protein antigens (Gregoriadis, 1978; Shek, 1984; Warren, Vogel & Chedid, 1986; Rouse, Turtinen & Correa-Freire, 1986). A physical association between antigen and liposomes seems to be a prerequisite for the potentiation to be observed, but the nature of this association remains matter of debate (Six, Kramp & Kasel, 1980; Lifshitz, Gitler & Mozes, 1981; Shek & Sabiston, 1981; Tom *et al.*, 1982; Van Rooijen & Van Nieuwmegen, 1982; Gerlier, Bakouche & Dore, 1983; Snyder & Vannier, 1984; Shek & Heath, 1983; Gregoriadis, Davis & Davies, 1987). The differences reported in the literature on the most efficient type of antigen association to liposomes do not appear to reflect simply the different behaviour of the different antigens tested with the liposomal system, since conflicting results have also been reported in studies using the same antigen, serum albumin (Shek & Sabiston, 1982; Van Rooijen & Van Nieuwmegen, 1982). Part of the controversy, however, may originate from the different aspects of the immune response that have been tested and from the differences in the liposomal preparations used by each author. In order to re-evaluate this question, we examined the influence of the mode of association of bovine serum albumin (BSA) with liposomes on the number of cells secreting antigen-specific IgG and IgM in primary and secondary responses.

Abbreviations: APC, antigen-presenting cell; BSA, bovine serum albumin; DMPC, dimyristoyl phosphatidylcholine; DPPE, dipalmitoyl phosphatidylethanolamine; DTT, dithiothreitol; OGP, *n*-octyl-β-D-glucopyranoside; PBS, phosphate-buffered saline; PFC, plaque-forming cell; SPDP, 3-(2-pyridyldithio) propionic acid *N*-hydroxy succinimide ester; SRBC: sheep red blood cell.

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Liposomes, made of dimyristoylphosphatidylcholine (DMPC), cholesterol and dipalmitoylphosphatidylethanolamine (DPPE) (Sigma) in a molar ratio of 63:31:6, were prepared at 35° by a detergent dialysis technique according to the technique described by Mimms *et al.* (1981). Liposomes were kept frozen at −20° until use.

Encapsulation of BSA in the internal liposomal aqueous phase was done by suspending the dry lipid film obtained after roto-evaporation in a 150 mM OGP-PBS solution (Sigma) containing 10 mg BSA/ml, while covalent coupling to the liposomal surface was achieved following modification of both, DPPE and BSA, with the heterobifunctional reagent 3-(2-pyridyldithio) propionic acid (SPDP) (Sigma) according to the method described by Leserman, Machy & Barbet (1984).

Liposome-associated BSA, either encapsulated or surface-linked, was separated from free BSA by gel filtration on a Sepharose CL-4B column (Sigma) and the associated amount was estimated by gamma counting using BSA radioiodinated by the Chloramine T method (Garvey, Cremer & Sussdorf, 1977).

Unless otherwise specified, BALB/c mice of at least 8 weeks of age were injected i.p. with 25 μg of the different albumin preparations. In secondary-response experiments, mice were injected twice with the same preparation at an interval of 3 weeks. Spleen cells of immunized animals were assayed for

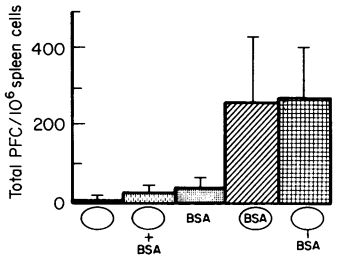

Figure 1. Total number of PFCs obtained the fourth day following the second immunization for spleen cells of BALB/c mice challenged twice with 25 μg of different BSA formulations at three weeks interval. The bars represent the standard deviations of the mean and values have been obtained from at least six animals. Statistical analysis of the data reveals that the differences observed in the total number of Ig-secreting cells between liposome-associated BSA and free BSA are highly significant ($P < 0.05$) while those between the two types of liposome-association are not. BSA, free BSA; (BSA), encapsulated BSA; ⬭ BSA, surface-associated BSA.

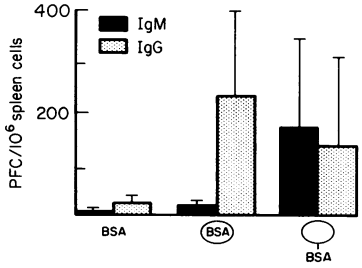

Figure 2. Comparison of BSA-specific IgM and IgG PFCs. IgM plaques were measured directly while the number of IgG plaques was calculated as the difference between the number of total plaques obtained in presence of rabbit anti-mouse IgG, Fc specific, antiserum and the number of direct IgM plaques. Each bar represents the mean response $\pm$SEM obtained the fourth day after the second immunization for at least six animals. The differences observed between BSA-associated liposomes and free BSA for each isotype are all highly significant ($P < 0.05$) except for the IgM response of encapsulated BSA. The differences observed for each isotype between the two modes of liposome association are all highly significant.

plaque-forming cells (PFC) by a modified version of the Jerne haemolytic plaque technique (Lefkovits & Cosenza, 1979), the fourth day following the last immunization (Shek & Sabiston, 1981). Indicator sheep red blood cells (SRBC) IAF production, Laval, Quebec were coated with BSA by the $CrCl_3$ method (Sweet & Welborn, 1971) and used at a final concentration of 4%. IgM-secreting cells were measured directly while IgG-secreting cells were revealed by using a rabbit anti-mouse IgG, Fc specific (Cedarlane Lab., Hornby, Ontario).

The liposomes that we obtained by the detergent dialysis technique are unilamellar vesicles of relatively homogeneous size with a mean diameter of 577–108 nm, as measured by quasi-elastic light scattering.

The mean efficiencies of encapsulation and covalent linkage were 105 μg BSA/μmole of phospholipids and 27 μg BSA/μmole of phospholipids, respectively. The covalent nature of the bond between BSA and liposomes was confirmed by treating the conjugated vesicles with 50 mM dithiothreitol (DTT) (Sigma) so as to break the disulphide linkage of the association. The results indicate that at least 80% of the BSA is disulphide linked and not merely adsorbed to the liposomal surface. The immunization of BALB/c mice with the different antigenic preparations was carried out using a fixed quantity of the antigen (25 μg). However, in control experiments, including immunization with free BSA, liposomes alone or free BSA + liposomes, a wide range of concentrations was tested. In primary response experiments, the number of IgM-secreting cells was very low for all types of antigen formulations studied, less than 10 per 10^6 cells, and no significant adjuvanticity could be demonstrated with liposomes. However, in secondary response the adjuvant character of liposomes becomes clearly evident. The results obtained for total Ig-secreting cells are shown in Fig. 1. As can be seen, liposomes alone do not stimulate any non-specific response while free BSA stimulates a low but significant number of anti-BSA-producing cells. Addition of liposomes to free BSA does not change its response pattern but when BSA is associated with liposomes, a dramatic increase in the number of Ig-secreting cells is observed, an increase which is about one order of magnitude greater than the response to free BSA for both encapsulated and surface-associated BSA. Although the number of Ig-secreting cells is equivalent for the two types of antigen association, an important difference in isotype distribution was also observed. As shown in Fig. 2, encapsulated BSA and free BSA behave as typical thymus-dependent antigen, stimulating almost exclusively IgG-secreting cells while covalently linked BSA stimulates the IgM subset to a degree equivalent to that of the IgG response. If one considers the number of PFCs obtained for each isotype with liposome-associated BSA, relative to the values obtained in response to free BSA, one obtains, for encapsulated BSA, an increase of 2 for IgM and of 16·2 for IgG, yielding a ratio IgG/IgM of 16·8, while for covalently linked BSA these increases are, respectively, 24·1 and 5·6, yielding a final IgG/IgM ratio of 0·8.

These results, although they confirm the efficient adjuvant character of liposomes on the humoral response and the absolute necessity of a physical association between antigen and liposomes for the potentiation to be observed, also indicate, in contrast to what has been reported in the literature (Six *et al.*, 1980; Lifshitz *et al.*, 1981; Van Rooijen & Van Nieuwmegen, 1982; Shek & Sabiston, 1982; Tom *et al.*, 1982; Gerlier *et al.*, 1983; Snyder & Vannier, 1984; Claassen, Kors & Van Rooijen, 1987), that both modes of antigen association with liposomes are equally effective as potentiators of the humoral response. However, by measuring simultaneously IgG and IgM, we also reconcile some of the conflicting data published and, in agreement with Shek & Sabiston (1982), we conclude that encapsulation is the most effective way of promoting an IgG response and at the same time, in agreement with Van Rooijen & Van Nieuwmegen (1983), we conclude that surface association promotes an important IgM response.

The difference in the dominant isotype produced by either encapsulated or surface-exposed antigen suggests that different mechanisms operate in both situations. The responses obtained with encapsulated BSA, as well as that obtained with free BSA, are typical of thymus-dependent antigens, with an increased secondary response suggestive of memory induction (Katz & Benacerraf, 1973) and a high ratio of IgG to IgM. A similar mechanism may be involved for both modes of antigen presentation, implicating the usual route of antigen processing

by antigen-presenting cells (APC), presentation to T cells in association with MHCII molecules and the consequent help for the differentiation and proliferation of B cells. The potentiation of the response by encapsulation of the antigen in liposomes therefore may be explained only by a more efficient targeting of the antigen to APC, resulting in a more efficient activation of immunocompetent cells.

The situation is different with surface-exposed antigen, which yields a secondary response atypical for either thymus-dependent or thymus-independent antigen. Even if a low ratio of IgG to IgM has been observed, a memory has also been induced as indicated by the significant increase in production of both isotypes. Although a more efficient targeting of the antigen to APC might still contribute significantly to the observed stimulation, a direct interaction between surface-exposed antigen and specific receptors on immunocompetent cells leading to direct activation may also be involved. The possible implication of this phenomenon is substantiated by the observation made by Walden, Nagy & Klein (1986) that antigen presented at the surface of liposomes may substitute for APC in the activation of T cells, provided the epitope density of the antigen is sufficiently elevated. At low epitope density, the activation requires the simultaneous presence on the liposome of the antigen and the appropriate MHCII molecule.

The similar levels of total stimulation obtained with encapsulated and covalently linked antigen raise the intriguing possibility that exposure of antigen at the liposomal surface may affect the switch of IgM to IgG normally occurring in the course of an humoral immune response without altering the helper signals for differentiation and proliferation. Although this hypothesis remains to be tested, our results nevertheless suggest that the mode of antigen association with liposomes profoundly influences the pathways by which lymphocyte activation occurs.

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