Immunology 1983 **49** 431

Induction of syngeneic tumour-specific immunity by liposomes reconstituted with L_2C tumour-cell antigens

A.J. SCHROIT & M.E. KEY, *Cancer Metastasis and Treatment Laboratory, Basic Research Program-LBI, Frederick Cancer Research Facility, Frederick, Maryland, U.S.A.*

Accepted for publication 19 January 1983

Summary. Liposomes composed of phosphatidylcholine and phosphatidylserine were reconstituted by detergent dialysis with 3 M KCl extracts of L_2C tumour cells. Liposomes containing L_2C antigens were as antigenic as intact tumour cells in the elicitation of delayed-type hypersensitivity responses in strain-2 guinea-pigs previously immunized against L_2C tumours. Soluble L_2C antigens were devoid of immunoprotective activity, whereas the reconstituted liposomes were capable of protecting animals against up to approximately 100 times the minimal lethal dose of tumour cells. Moreover, the reconstituted liposomes were as antigenic and immunoprotective as viable (irradiated) cells.

INTRODUCTION

Experimental and clinical applications of soluble tumour-specific antigens have attracted considerable interest in recent years. Indeed, soluble extracts prepared from various neoplastic cells have been shown to contain tumour-specific antigens, in that they are capable of inducing the development of tumour-specific immunity in certain systems (Pellis & Kahan, 1975; Hu *et al.*, 1978; Braun *et al.*, 1978).

However, a requirement for the induction of immunity in many of these systems is the inclusion of Freund's complete adjuvant (FCA). The use of FCA is frequently associated with severe allergic reactions and granuloma formation, and the development of alternative approaches to increasing immunogenicity may result in more amenable treatment methods.

The use of lipid vesicles (liposomes) as vehicles for the presentation of antigens to the immune system has generated considerable interest. Liposomes reconstituted with various membrane proteins have recently been used to delineate the role of several cell-surface components (Engelhard *et al.*, 1978; Littman, Cullen & Schwartz, 1979; Hollander *et al.*, 1979). Moreover, immune reactivity to a variety of haptens has been shown to be enhanced following the incorporation of the haptens into liposomes (Kinsky & Nicolotti, 1977; Kinsky, 1980) and vesicles reconstituted with H-2 have been shown to stimulate specific cytotoxic T lymphocytes (Sherman, Burakoff & Mescher, 1980; Hale, Ruebush & Harris, 1980; Hollander *et al.*, 1979). These findings prompted us to investigate whether liposomes reconstituted with syngeneic tumour antigens would be immunogenic. Studies by Sakai, Gerlier & Doré (1980) and Gerlier, Sakai & Doré (1980) showed that when antigens isolated from lymphoma cells are encapsulated in multilamellar

Abbreviations: BCG, *bacille* Calmette Guérin; FCA, Freund's complete adjuvant; DTH, delayed-type hypersensitivity; NBD-PE, l-acyl-2-*N*-4-nitrobenzo-2-oxa-1,3-diazole) aminocaproyl phosphatidylcholine; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PS, phosphatidylserine.

Correspondence: Dr A. J. Schroit, Cancer Metastasis and Treatment Laboratory, Basic Research Program-LBI, Frederick Cancer Research Facility, P.O. Box B, Frederick, Maryland 21701, U.S.A.

0019-2805/83/0700-0431$02.00
© 1983 Blackwell Scientific Publications

vesicles and injected subcutaneously into syngeneic hosts, consistently high levels of cytotoxic antibodies are produced in the recipient animals. These data suggested that the physical entrapment of solubilized tumour-cell surface antigens could augment host immune responsiveness. In light of these observations, we attempted to construct liposomes reconstituted with solubilized L_2C antigens that were both antigenic and immunogenic in syngeneic guinea-pigs.

MATERIALS AND METHODS

Animals

Specific-pathogen-free strain-2 guinea-pigs were obtained from the Animal Production Area of the NCI-Frederick Cancer Research Facility, Frederick, MD.

Reagents

Phosphatidylcholine (PC; derived from eggs), phosphatidylserine (PS; derived from beef brain) and NBD-PE were obtained from Avanti Polar Lipids (Birmingham, AL). All lipids were confirmed to be pure by thin-layer chromatography. Phipps fresh frozen BCG was obtained from Trudeau Institute, Saranac Lake, N.Y., sodium cholate from Sigma (St. Louis, MO) and ammonium molybdate from Aldrich (Milwaukee, WI).

Leukaemia cells

The L_2C B-cell leukaemia arose spontaneously in a strain-2 female guinea-pig and has been described by Congdon & Lorenz (1954). In our studies, tumours were established by injection of 5×10^6 tumour cells (maintained in liquid nitrogen) subcutaneously into the inguinal area of weanling strain-2 guinea pigs. Leukaemic animals were bled by cardiac puncture 10–14 days after tumour inoculation, and the tumour cells were separated from heparinized blood by gradient centrifugation with lymphocyte separation medium (Litton Bionetics Inc., Kensington, MD) for 20 min at 400 ***g***.

Solubilization of tumour antigens

Washed tumour cells were suspended in cold 0·15 M phosphate-buffered saline (PBS) pH 7·2 ($6{\cdot}8 \times 10^8$ cells/ml). Soluble antigens were then extracted using 3 M KCl (Reisfeld, Pellegrino & Kahan, 1971). Briefly, cold 3·5 M KCl in PBS was added drop by drop to the stirred cell suspension to effect a final concentration of 3 M KCl. The suspension was stirred at 4° for 16 hr. Insoluble material was removed by sequential centrifugation at 1000 ***g*** (15 min) and 100,000 ***g*** (1 hr). The KCl was then removed from the soluble preparation by dialysis against PBS. Small amounts of precipitable material formed during dialysis were removed by centrifugation (100,000 ***g*** for 1 hr). The protein concentration in this extract was estimated by the method of Lowry (Lowry *et al.*, 1951), using a standard curve generated with known amounts of bovine serum albumin. Typical yields were approximately 19 μg protein/10^6 cells.

Preparation of liposomes

Large unilamellar vesicles were formed by drying PC (70 mg) and PS (30 mg) in a small tube under nitrogen. In some of the experiments the fluorescent lipid NBD-PE (0·1% w/w) was included in order to quantify lipid yield. An amount (1·5 ml) of Na-cholate (10% w/v with trace amounts of [^{3}H]-cholate) and 5 ml of the KCl extract (10 mg total protein) were added to the lipid residue, and the mixture was sonicated in a bath-type sonifier at 2° until completely clear ($\sim$ 30 min). The solution was then dialysed for 3 days against three 5-litre changes of PBS, followed by chromatography on Sephadex G-100 to ensure complete removal of cholate. More than 99% of the detergent was removed during dialysis, as determined by the residual [^{3}H]-cholate.

Purification of L_2C-reconstituted liposomes

Liposomes reconstituted with L_2C antigens were isolated from unincorporated protein by flotation on sucrose gradients. To accomplish this, solid sucrose was added to the vesicle suspension to a final concentration of 45% (w/w). The vesicle suspension was placed on a 65% sucrose cushion and overlaid with 35% sucrose and then by PBS. It was centrifuged for 16 hr at 190,000 ***g*** at 4°. Fractions were collected from the bottom of the tube and dialysed against PBS to remove sucrose; lipid content was determined by the relative fluorescence of the NBD-PE (λex, 470 nm; λem, 525 nm) and protein by the method of Lowry *et al.* (1951).

Antigenicity of L_2C-reconstituted liposomes

The antigenicity of the various preparations was assessed in L_2C-immune animals by measuring specific delayed cutaneous reactivity (erythema) at the injection site at 24 hr. Guinea-pigs were immunized with a standard vaccine composed of 5×10^7 BCG

admixed with 5×10^7 fresh, irradiated L_2C cells or line-10 hepatocarcinoma cells at least 30 days before testing. This vaccine has been observed to be an effective immunization protocol for this system (unpublished observations).

Immunization to L_2C leukaemia
Strain-2 guinea-pigs were immunized with a single intradermal injection of the various antigen preparations (see 'Results'). Control groups were injected with liposomes alone (containing no antigens) or with soluble antigen. The immunized and control animals were challenged intradermally with different numbers of viable L_2C cells in the flank opposite the vaccination site 14 days after immunization. The immunogenic activity of the various preparations was assessed by animal survival.

Electron microscopy
Liposome suspensions purified by flotation on sucrose gradients were applied to formvar-coated grids and stained with 0·5% ammonium molybdate for 30 sec. Excess stain was filtered off, and the preparation was air dried before examination.

RESULTS

Purification of liposomes reconstituted with L_2C antigens

Liposomes reconstituted with L_2C KCl extracts and control liposome populations banded at the PBS/35% sucrose interface, whereas unincorporated protein did not migrate out of the 45% sucrose band (Fig. 1). An electron micrograph of the unilamellar vesicles obtained from the PBS/35% sucrose interface is shown in Fig. 2. These vesicles are approximately 0·2 μm in diameter and have a unilamellar structure. The amount of protein which became associated with the vesicles is shown as a function of the initial protein/lipid ratio in Fig. 3. Approximately 20% of the total available protein could be incorporated into the liposomes when an initial protein/lipid ratio of 1/1 was used. A 1/10 ratio increased the amount of protein associated with the liposomes to ~50%, whereas the addition of more lipid was without substantial effect.

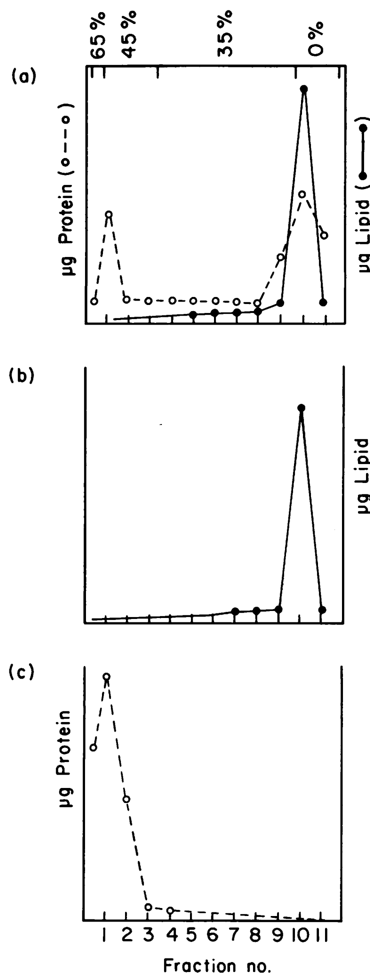

Figure 1. Insertion of protein antigens into liposome bilayers. (a) Liposomes prepared with L_2C KCl extract were centrifuged in a discontinuous sucrose gradient; 100% of the lipid and ~50% of the protein floated. (b) Flotation of liposomes alone; 100% of the lipid floated. (c) KCl extract alone centrifuged in the absence of lipid; 100% of protein remained at the bottom of the tube.

Antigenicity of liposomes reconstituted with L_2C antigens

The antigenicity of the reconstituted liposomes was assessed by testing their ability to elicit specific delayed-type hypersensitivity (DTH) reactions in strain-2 guinea-pigs that had been previously immunized with irradiated L_2C cells and BCG (Fig. 4). The DTH response elicited by the reconstituted vesicles was equal to the DTH response elicited by intact irradiated L_2C cells when they were compared on the basis of the yield of 3 M KCl-extractable protein/cell (19 μg/10^6 cells). In contrast, 10–15 times the amount

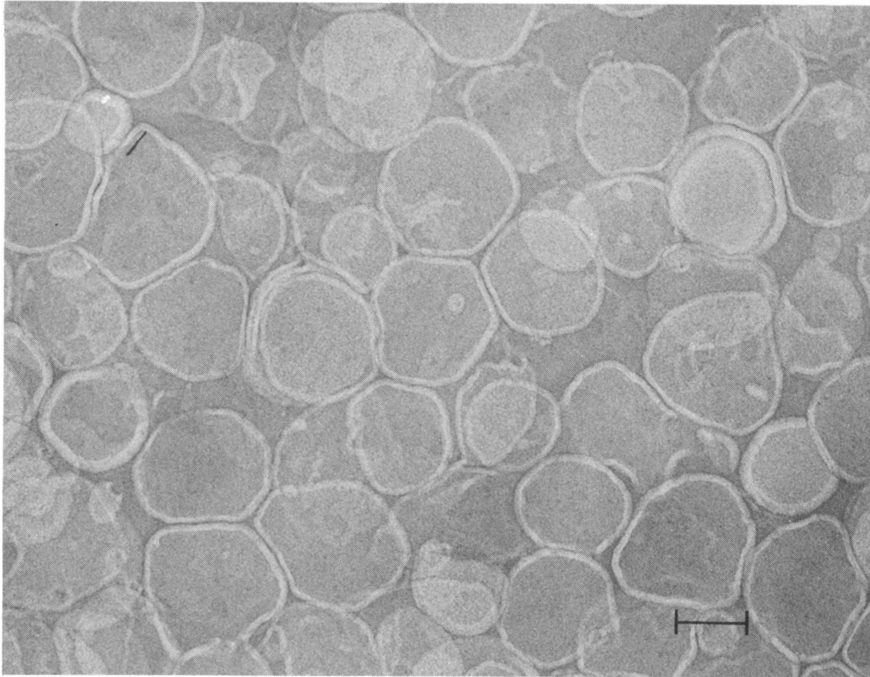

Figure 2. Electron micrograph of L2C antigen-containing liposomes purified by flotation on sucrose gradients. Bar = 0·1 μm.

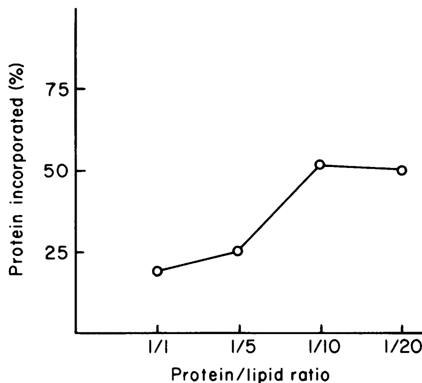

Figure 3. Effect of the initial protein/lipid ratio on the extent of antigen incorporation into liposomes.

of soluble antigen was required to induce the same response. As shown in Fig. 4, approximately 20 μg of protein reconstituted in liposomes (10^6 cell equivalents) resulted in 1 cm erythema, while ~300 μg soluble antigen was necessary to induce a similar response. These data indicate that liposomes containing L2C antigens are as antigenic as intact L2C tumour cells. Moreover, the DTH responses elicited by liposomes reconstituted with L2C antigens were tumour-specific. Line-10 hepatocarcinoma antigens encapsulated in liposomes (in an identical manner) failed to elicit detectable DTH in animals previously immunized against L2C tumours, and conversely, L2C liposomes failed to elicit DTH responses in line-10-immune animals.

Immunogenicity of liposomes reconstituted with L2C antigens

Based upon the observations that L2C-reconstituted liposomes are as antigenic as intact L2C cells, we have initiated a series of experiments to determine whether vesicles reconstituted with L2C antigens are also immunogenic (protective) in normal guinea-pigs. The ability of the various preparations to confer immunoprotection in syngeneic guinea-pigs against a lethal challenge of L2C cells was assessed and is summarized in Tables 1 and 2. The results show that normal guinea-pigs receiving a single intradermal injection of liposomes (0·5 mg L2C antigen corresponding to ~10 mg lipid) were capable of protecting the animals

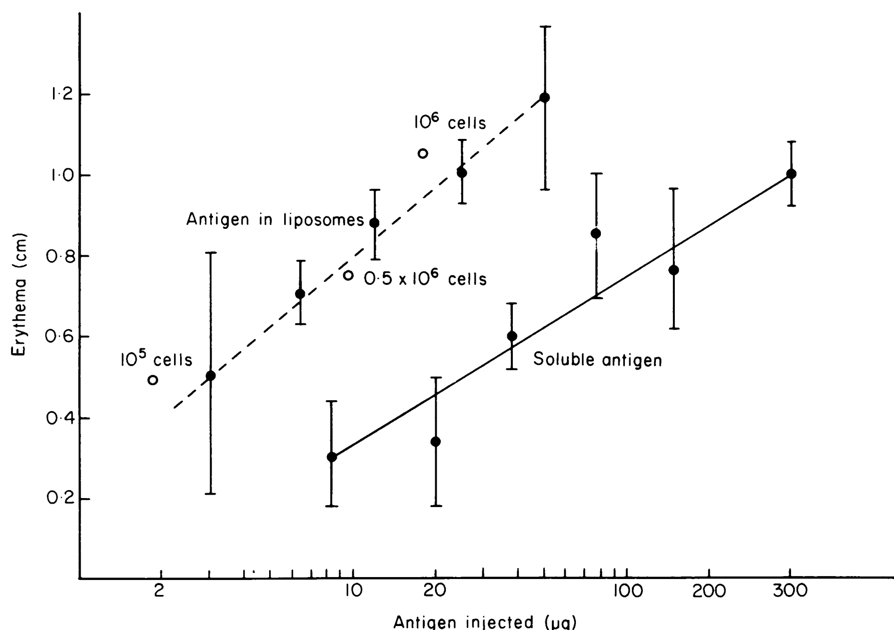

Figure 4. Evaluation of the local inflammatory reaction in L2C immune animals given L2C cells (O), liposomes reconstituted with soluble KCl extracts of L2C (- - - -) (protein/lipid ratio of 1/10–20, w/w), and soluble KCl extracts (——). Guinea-pigs were immunized with an intradermal inoculation (200 μl) of a vaccine composed of 5×10^7 *bacille* Calmette Guérin admixed with 5×10^7 fresh, irradiated L2C cells, at least 30 days before testing. DTH responses to the various antigen preparations were evaluated 24 hr after challenge by measuring the largest diameter of erythema and a second diameter perpendicular to the first. The mean diameter of erythema/injection site was taken as the average of the two measurements $\pm$ standard deviation; 5 animals/group.

Table 1. Immunogenicity of L2C antigens incorporated into liposomes

	Survival of animals challenged with†					
	10^3 L2C tumour cells		10^4 L2C tumour cells		10^5 L2C tumour cells	
Treatment of guinea-pigs*	Day 40	Day 90	Day 40	Day 90	Day 40	Day 90
None	0/5		0/5		0/5	
Liposomes alone	0/5		0/5		0/5	
KCl extract of L2C cells alone	Not done		0/5		0/5	
Liposome-entrapped L2C antigens	5/5	5/5	5/5	5/5	1/5	1/5
Viable L2C cells‡	5/5	5/5	5/5	5/5	1/5	1/5

* Guinea-pigs received no treatment or one intradermal injection of 10 mg lipid and/or 0·5 mg total antigen 14 days before tumour cell challenge.

† Animals were challenged intradermally with viable L2C tumour cells in the flank opposite the vaccination site. Surviving guinea-pigs were not resistant to a challenge of 10^4 viable cells with the non-cross-reacting but syngeneic line-10 tumour.

‡ L2C cells were irradiated with 20,000 rad prior to injection.

Table 2. Immunogenicity of L_2C antigens incorporated into liposomes admixed with BCG

	Survival of animals challenged with†					
	10^4 L_2C tumour cells		10^5 L_2C tumour cells		10^6 L_2C tumour cells	
Treatment of guinea-pigs*	Day 40	Day 90	Day 40	Day 90	Day 40	Day 90
None	0/5		0/5		0/5	
L_2C cells‡	5/5	5/5	2/5	1/5	0/5	0/5
L_2C cells + BCG‡	5/5	5/5	5/5	5/5	1/10	1/10
L_2C liposomes + BCG	5/5	5/5	5/5	4/5	0/5	

* Guinea-pigs received no treatment or one intradermal injection of 10 mg lipid and/or 0·5 mg total antigen 14 days before tumour cell challenge.

† Animals were challenged intradermally with viable L_2C tumour cells in the flank opposite the vaccination site. Surviving guinea-pigs were not resistant to a challenge of 10^4 viable cells with the non-cross-reacting but syngeneic line-10 tumour.

‡ L_2C cells were irradiated with 20,000 rad prior to injection.

against a lethal challenge with viable tumour cells injected 2 weeks after the initial immunization (Table 1). Whereas the injection of 10^3 viable tumour cells is uniformly fatal in this model system, guinea-pigs treated once with 0·5 mg vesicle-associated antigen were able to reject a challenge of 10^4 viable L_2C tumour cells. However, these L_2C-immune animals were sensitive to a challenge of 10^4 viable cells of the non-cross-reacting by syngeneic line-10 tumour, indicating that the immunization was specifically against the L_2C tumour. In addition, animals surviving at day 90 after L_2C tumour challenge were resistant to a subsequent challenge of up to 10^5 viable L_2C cells, whereas they remained sensitive to 10^4 viable line-10 tumour cells.

It should be noted that no additional adjuvants were used in the above experiment. However, because one can immunize strain-2 guinea-pigs with a vaccine composed of 5×10^7 BCG admixed with 5×10^7 fresh irradiated L_2C cells, we wanted to determine whether the admixing of BCG with the reconstituted liposomes would result in enhanced immunoprotection against a subsequent lethal challenge with viable L_2C cells. Immunization of animals with L_2C cells alone or L_2C-reconstituted liposomes did not protect the animals against a challenge of 10^5 viable L_2C cells (Tables 1, 2). However, when BCG were admixed with L_2C-reconstituted liposomes, the level of protection approached that obtained when BCG were admixed with intact L_2C cells, 80% and 100% protection, respectively (Table 2).

DISCUSSION

Synthetic membrane vesicles have been widely used in a variety of functional membrane systems (Burakoff & Mescher, 1982). The insertion of antigens into model membranes and the use of these membrane vesicles as functional antigens and immunogens could possibly provide a means for understanding the molecular requirements for the induction of specific tumour reactivity.

It has recently been shown that liposomes reconstituted with a variety of cell-surface antigens can replace intact viable tumour cells in the induction of specific allogeneic and xenogeneic cytotoxic T lymphocytes *in vitro* (Sherman *et al.*, 1980; Rafael & Tom, 1982). In addition, appropriately designed liposomes can replace intact viable tumour cells for the induction of high levels of antibody directed against a syngeneic tumour antigen (Sakai *et al.*, 1980; Gerlier *et al.*, 1980). Thus, the association of complex cell-derived antigens with liposomes appears to result in enhanced immune reactivity similar to that described originally for defined haptens by Kinsky and co-workers (Kinsky & Nicolotti, 1977; Kinsky, 1980).

In light of these observations, we have constructed liposomes by reconstitution with solubilized L_2C antigens in an attempt to determine whether such structures would serve as immunogens for cell-mediated immune reactivity in a syngeneic model system. Our results show that L_2C antigens incorporated into liposomes are both highly antigenic and

immunogenic in syngeneic hosts. Soluble L_2C antigens were completely devoid of immunoprotective activity, whereas the reconstituted vesicles were capable of protecting animals against up to approximately 100 times the minimal lethal dose of tumour cells. Moreover, the reconstituted liposomes were as antigenic (as detected by DTH) and immunoprotective as viable (irradiated) cells.

These results are consistent with the observations that 3 M KCl extracts of L_2C leukaemia cells are immunogenic only after emulsification with FCA (Hu *et al.*, 1978; Braun *et al.*, 1978). This finding suggests that the inability of the soluble extracts to induce immunity may be due to the lack of appropriate antigen density or appropriate orientation possibly required for inducing immunity.

In conclusion, incorporation of extracted tumour cell-associated antigens into synthetic bilayer membranes can greatly enhance their antigenicity and immunogenicity in syngeneic hosts, and replace the requirement for viable cells. We anticipate that the efficacy of the liposome-encapsulated antigens can be further enhanced by the inclusion of one or several lipophilic adjuvants such as lipid A (Alving *et al.*, 1980), trehalose dimycolate (McLaughlin *et al.*, 1980), and hydrophobic muramyl dipeptide analogues (Kotani *et al.*, 1977; Azuma *et al.*, 1978) and may provide an effective and reproducible means of immunization.

ACKNOWLEDGMENTS

Research sponsored by the National Cancer Institute, DHHS, under Contract No. NO1-CO-23909 with Litton Bionetics Inc. The contents of this publication do not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

REFERENCES

ALVING C.R., BANERJI B., CLEMENTS F.D. & RICHARDS R.L. (1980) Adjuvanticity of lipid A and lipid A fractions in liposomes. In: *Liposomes and Immunobiology* (eds B. H. Tom and H. R. Six), p. 67. Elsevier/North Holland, Amsterdam.

AZUMA I., SUGIMURA K., YAMANAK M., UEMIYA A., KUSUMOTO S., OKADA S., SHIBA T. & YAHAHURA Y. (1978) Adjuvant activity of synthetic 6-0-"mycoloyl"-n-acetyl-muramyl-L-alanyl-D-isoglutamine and related compounds. *Infect. Immun.* **20,** 600.

BRAUN D.P., HENGST J.C.D., MOKYR M.B. & DRAY S. (1978) Antitumour immunity in strain-2 guinea pigs immunized with potassium chloride extracts of L_2C tumour cells. *J. natn. Cancer Inst.* **60,** 899.

BURAKOFF S.J. & MESCHER H.F. (1982) Reconstituted membranes and liposomes in the study of lymphocyte interactions. In: *Membrane Reconstitution*, Cell Surface Reviews Vol 8, (eds G. Poste and G. L. Nicolson). Elsevier/North Holland, Amsterdam.

CONGDON C.C. & LORENZ E. (1954) Leukemia in guinea pigs. *Am. J. Pathol.* **30,** 337.

ENGELHARD V.H., GUILD B.C., HELENIUS A., TERHORST C. & STROMINGER J. (1978) Reconstitution of purified detergent-soluble HLA-A and HLA-B antigens into phospholipid vesicles. *Proc. natn. Acad. Sci. (U.S.A)*, **75,** 3230.

GERLIER D., SAKAI F. & DORÉ J.-F. (1980) Induction of antibody response to liposome-associated gross-virus cell-surface antigen (6/CSAa). *Br. J. Cancer* **41,** 236.

HALE A.H., RUEBUSH H.J. & HARRIS D.T. (1980) Study of the minimal molecular and cellular requirements for elicitation of anti-vesicular stomatitis virus cytotoxic and T lymphocytes using purified viral and cellular antigens incorporated into phospholipid vesicles. In: *Liposomes and Immunobiology* (eds B. H. Tom and H. R. Six), p. 211. Elsevier/North Holland, Amsterdam.

HOLLANDER N., MEHDI S.Q., WEISSMAN I.L., MCCONNELL H.M. & KRISS K.P. (1979) Allogeneic cytolysis of reconstituted membrane vesicles. *Proc. natn. Acad. Sci. (U.S.A.)*, **76,** 4042.

HU C.-P., KONEN T.G., HOES C. & GREEN I. (1978) Solubilization and partial characterization of a tumour-associated transplantation antigen of the guinea pig L_2C leukemia. *J. Immunol.* **120,** 1521.

KINSKY S.C. (1980) Factors influencing liposomal model membrane immunogenicity. In: *Liposomes and Immunobiology* (eds B. H. Tom and H. R. Six), p. 79. Elsevier/North Holland, Amsterdam.

KINSKY S.C. & NICOLOTTI R.A. (1977) Immunological properties of model membranes. *Ann. Rev. Biochem.* **46,** 49.

KOTANI S., KINOSHITA E., MURISAKI I., SHIMONO T., OKUNAGA T., TAKADA H., TSUJIMOTO M., WANATABE Y. & KATO K. (1977) Immunoadjuvant activities of synthetic 6-O-acyl-n-acetyl-muramyl-L-alanyl-D-isoglutamine with special reference to the effect of its administration with liposomes. *Biken J.* **20,** 95.

LITTMAN D.R., CULLEN S.E. & SCHWARTZ B.D. (1979) Insertion of I_a and H-2 alloantigens into model membranes. *Proc. natn. Acad. Sci. (U.S.A.)*, **76,** 902.

LOWRY O.H., ROSEBROUGH N.J., FARR A.L., & RANDALL R.J. (1951) Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193,** 265.

MCLAUGHLIN C.A., SCHWARTZMAN S.M., HORNER B.L., JONES G.H., MOFFATT J.G., NESTON J.J. & TEGG D. (1980) Regression of tumours in guinea pigs after treatment with synthetic muramyl dipeptides and trehalose dimycolate. *Science*, **208,** 415.

PELLIS N.R. & KAHAN B.D. (1975) Specific tumour immunity induced with soluble materials: restricted range of antigen dose and of challenge tumour load for immunoprotection. *J. Immunol.* **115,** 1717.

RAFAEL L. & TOM B.H. (1982) *In vitro* induction of primary and secondary xenoimmune responses by liposomes containing human colon tumour cell antigens. *Cell. Immunol.* **71,** 224.

REISFELD R.A., PELLEGRINO M.A. & KAHAN B.D. (1971) Salt extraction of soluble HL-A antigens. *Science*, **172,** 1134.

SAKAI F., GERLIER D. & DORÉ J.-F. (1980) Association of gross virus-associated cell surface antigen with liposomes. *Br. J. Cancer* **41,** 227.

SHERMAN L., BURAKOFF S.J. & MESCHER M.F. (1980) Induction of allogeneic cytolytic T lymphocytes by partially purified membrane gycoproteins. *Cell. Immunol.* **51,** 141.