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I am submitting herewith a thesis written by Sharon Carpenter-Green entitled "Covalent Coupling of Palmitate to Wheat Germ Agglutinin and Its Incorporation into Liposomes." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry.

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COVALENT COUPLING OF PALMITATE TO WHEAT GERM AGGLUTININ AND ITS INCORPORATION INTO LIPOSOMES

A Thesis

Presented for the

Master of Science

Degree

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ABSTRACT

In order to study cell to cell interaction a model system was designed utilizing liposomes containing a membrane-bound protein which binds to many cell surfaces. Specifically, wheat germ agglutinin (WGA), a plant lectin which binds specifically to N-acetylglucosamine and N-acetylneuraminic acid, was covalently modified with a fatty acid by a reaction with the N-hydroxysuccinimide ester of palmitic acid. The palmitoyl-WGA was subsequently incorporated into liposomes with a detergent removal procedure.

Liposome bound WGA could agglutinate chick erythrocytes at a concentration 8-fold lower than the native WGA. Liposome binding to mouse spleen cells was observed only when palmitoyl-WGA was incorporated; protein-free liposomes did not bind to spleen cells. These results indicate WGA remained active after derivatization and incorporation into liposomes. These observations are explained on the basis of a multivalent binding theory.

The second part of the project involves an in vitro system for the induction of cytotoxic T-lymphocytes (CTL) using liposomes containing trinitrophenol (TNP) haptens. CTL could be obtained by stimulating immune or non-immune mouse T-lymphocytes with hapten modified syngeneic cells. The effector cells could only lyse hapten-modified, syngeneic target cells, but not allogeneic target cells whether haptenated or not. Thus, a H-2 restricted, hapten-specific CTL response of mouse T-lymphocytes was established in vitro. Liposomes containing syngeneic plasma membrane proteins as a source of H-2 antigen and a lipid hapten,

trinitrophenyl-caproyl-phosphatidylethanolamine (TNP-CAP-PE), did not induce any CTL. Attempts to replace the plasma membrane proteins with palmitoyl-WGA as a non-specific binder in liposomes also failed to induce a CTL response. These results are inconsistent with the "dual recognition" model of H-2 restriction in CTL.

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ABBREVIATIONS

α BGT α-bungarotoxin

Con A Concanavalin A

cpm counts per minute

CTL cytotoxic T-lymphocytes

DNP-CAP-PE dinitrophenyl-caproyl-phosphatidylethanolamine

DOC deoxycholate

EYPC egg yolk phosphatidylcholine

FCS fetal calf serum

GlcNAc N-acetylglucosamine

H-2 anitgen mouse major histacompatability complex antigen

$H\text{-}2K^k$ H-2 antigen with haplotype "k"

HBSS Hank's Balanced Salt Solution

HLA human leukocyte antigen

IgG Immunoglobin G

^{125}I-pWGA ^{125}I-labeled palmitoyl-wheat germ agglutinin

^{125}I-WGA ^{125}I-labeled wheat germ agglutinin

L-PM/TNBS liposomes containing plasma membrane proteins treated with trinitrobenzene sulfonate after incorporation of membranes into liposomes

LPS lipopolysaccharide

LpWGA liposomes containing palmitoyl-wheat germ agglutinin

L-TNP liposomes containing 50 mole percent trinitrophenyl-caproyl phosphatidylethanolamine (TNP-CAP-PE).

L-TNP-PM liposomes containing 50 mole percent TNP-CAP-PE and plasma membrane proteins

L-TNP-pWGA liposomes containing 50 mole percent of TNP-CAP-PE and palmitoyl-wheat germ agglutinin

LUV large unilamellar vesicles

MLV multilamellar large vesicles

NHSP N-hydroxysuccinimide ester of palmitate

PBGT palmitoyl-bungarotoxin

PBS phosphate buffered saline

PM plasma membrane proteins

PMSF phenylmethylsulfonyl fluoride

PM/TNBS plasma membrane proteins treated with trinitrobenzene sulfonate

pWGA palmitoyl-wheat germ agglutinin

RBC red blood cells

SDS sodium dodecyl sulfate

SUV small unilamellar vesicles

TNBS trinitrobenzene sulfonate

TNP trinitrophenol

TNP-CAP-PE trinitrophenyl-caproyl-phosphatidylethanolamine

WGA wheat germ agglutinin

DEFINITIONS

Allogenic--between genetically different individuals of the same species.

B-lymphocytes--population of lymphocytes primarily involved in the humoral immune response (i.e., antibody production).

Effector--whole spleen cell population which has been sensitized to the antigen of which CTL are a subpopulation.

H-2 restriction--phenomenon involved with induction of CTL in a syngeneic system, CTL stimulated to one H-2 haplotype will kill targets containing that haplotype only.

Haplotype--genes making up a given H-2 complex.

Hapten--small molecule which by itself cannot stimulate antibody synthesis, but will combine with antibody once formed.

Percent specific lysis--assay of killing of ^{51}Cr-labeled target cells by CTL:

$$\% \text{ specific lysis} = \frac{\text{Experimental } ^{51}Cr \text{ release-Media } ^{51}Cr \text{ release}}{\text{Total } ^{51}Cr\text{- Media } ^{51}Cr \text{ release}} \times 100$$

where experimental release is ^{51}Cr released from targets in presence of CTL, media release is ^{51}Cr release from target cells without CTL, and total release of ^{51}Cr is amount of label in targets (obtained by solubilization of targets with 2 percent triton).

Primary CTL response--induction of CTL upon first introduction of antigen to lymphocytes.

Responder--spleen cells that are introduced to antigen that will become effectors.

Secondary CTL response--CTL induction upon second introduction of antigen to sensitized lymphocytes.

Stimulator--agent by which antigen is introduced to lymphocytes, usually in the form of whole cells containing the antigen or hapten.

Syngeneic--between genetically identical individuals.

Target--cell that is specifically identified by the CTL and killed.

T-lymphocytes--population of lymphocytes primarily involved in the cellular immune response (i.e., cell-mediated).

CHAPTER I

INTRODUCTION

A. Cell to Cell Interactions

Cell to cell interaction is a specific biochemical event occurring at cell surfaces. For example, the interaction between the egg cell membrane and the sperm cell membrane is a crucial event in fertilization (1). Studies in embryology have noted the preferential adhesion between cells at certain stages of development, indicating a specific cell surface binding event (2). In immune responses, lymphocyte activation is often initiated by cell to cell recognition consisting of ligand-receptor binding (3). Throughout biological systems cell to cell interactions are of great importance.

Although these examples do indeed require cell to cell interactions, the actual mechanisms involved are poorly understood. This is especially true of the physical chemical mechanisms operating at the membrane level.

One of the major problems involved with studying cell to cell interactions is the lack of well-defined systems. Recently, liposomes have been used to provide a means for establishing a more defined membrane system. Isolated and defined receptors have been incorporated into these model membranes and the subsequent interaction of these membranes with cell surface components has been studied *in vitro* (4). The present project is an attempt using liposome model membranes to study the molecular events involved in cell to cell interactions.

B. Liposomes

Liposomes are closed phospholipid bilayers which can be prepared by a variety of well established methods (5). Different methods of preparation will achieve a wide range of sizes and shapes of liposomes. There are three basic types of liposomes categorized by size. Small unilamellar vesicles (SUV) have one bilayer and are under 500 Å in diameter. Large unilamellar vesicles (LUV) have one bilayer and are about 1000 Å in diameter. Multilamellar large vesicles (MLV) have several concentric bilayers (lamellae) and are heterogeneous in size ranging from 0.1 to a few microns in diameter. Bangham et al. were the first to fully describe MLV preparation (6). Briefly, solvents are removed from lipids, resuspended in aqueous buffer, and are mechanically agitated resulting in an emulsive suspension. SUV are prepared by a variety of methods. Sonication of a MLV suspension results in a mixture of MLV and SUV (7). The two liposome types can be separated by gel filtration resulting in two homogeneous populations of liposomes. Other methods for preparation of SUV include ethanol injection (8), and cholate-dilution (9, 10). LUV can also be prepared by a variety of methods. The ether infusion method of Deamer and Bangham (1) is one of the most popular techniques for preparing LUV. Briefly, phospholipids dissolved in ether are injected into an aqueous solution at or above the boiling point of ether. Thus, the ether evaporates leaving the phospholipids in the aqueous solution, which will in turn form LUV. Other methods of preparing LUV include Ca^{+2}-fusion (12), and reverse phase evaporation (13).

It is possible to reconstitute hydrophobic membrane proteins into

lipid bilayers by mixing the proteins with lipids in the presence of detergent. By slow removal of the detergent either by dialysis or by gel filtration, lipids and proteins aggregate and subsequently form liposomes with proteins intercalated in the bilayers (7, 14).

C. Lectins as Specific Cell Surface Ligands

In search of a proper ligand to be used as a model for cell to cell interactions, plant lectins offer distinct advantages over other proteins. Lectins bind to specific sugar residues on cell surfaces. Lectins were originally isolated from plants. More recently some lectins have been isolated from various animal tissues (15, 16). Along with the specific sugar binding activities of lectins, some lectins have other properties as well. Concanavalin A is a lectin specific for D-mannose and is known to be a T-lymphocyte mitogen (17). Ricin is a lectin specific for lactose and is known to be very toxic to animal cells (17). One particular lectin, wheat germ agglutinin (WGA), is known to be neither mitogenic nor toxic. WGA binds specifically with high affinity to baby hamster kidney cells with a reported K_d of 1.6×10^{-6}M (18) and with a low affinity to N-acetylneuraminic acid with a reported K_d of 1.0×10^{-2}M (19). WGA has a molecular weight of some 36000 and in its native state consists of two identical subunits of molecular weight 17000. Each subunit consists of 2 binding sites with each binding site consisting of 3 to 4 extended subsites allowing WGA to bind tighter to chitotriose (a β-1,4 linked trisaccharide of GlcNAc) than to GlcNAc alone (20, 21). With these characteristics WGA might be a good choice for our purpose. However, WGA does not bind to liposomal

membranes in its native state. It would thus be necessary to derivatize WGA or chemically couple WGA to the liposomes. I have used the former approach. WGA was covalently modified with a fatty acid by a reaction with the N-hydroxysuccinimide ester of palmitic acid. The resulting palmitoyl-WGA (pWGA) could be incorporated into liposomes which resulted in a well defined membrane system consisting of a membrane-bound ligand specific for sugar residues on cell surfaces.

D. T-Lymphocyte as a Model System for Cell to Cell Recognition

The T-lymphocyte has membrane bound antigen receptors that recognize foreign antigens on the surface of other cells. Thus, this is a system where cell to cell recognition is a prerequisite for subsequent biochemical events. It has been observed in many cases that the stimulation of T-cells by the antigen-carrying cells can be studied in vitro. The result of in vitro stimulation is production of the cytotoxic T-lymphocytes (CTL). The experimental procedures and the assay protocols for CTL induction in vitro have been standardized (22). The induction of CTL is of particular importance in cellular immunology since it is responsible for the immunities against virus infection and against cancer. It is one of the central focuses for current studies. With these considerations in mind, we decided to choose CTL induction as a model system for cell to cell interactions.

One striking phenomenon observed in the induction of CTL is known as the H-2 restriction discovered in 1976 by Zinkernagel and Doherty (23, 24). Subsequently, many researchers have observed H-2 restriction in CTL induction. Components of the major histocompatibility antigen complex (MHC), i.e., the H-2 antigen in mice and the human leukocyte

antigen (HLA) in humans, are specifically recognized by CTL. For example, CTL stimulated by haptenated cells containing the H-$2K^k$ haplotype will kill only haptenated target cells bearing the same haplotype and cells of other haplotypes will not be recognized by the CTL. The mechanism of this recognition is not fully understood, however two models have been proposed to explain this H-2 restriction. The altered-self hypothesis (23, 25) states that the H-2 antigen and the foreign antigen (or hapten) associate to form a "neoantigen" on the target cell surface that is recognized by one receptor on the CTL. The dual recognition theory (23, 25) states that the foreign antigen and the H-2 antigen are recognized independently of each other by two receptors on the CTL. This project will attempt to further elucidate the phenomenon of H-2 restrictions.

E. Liposomes Containing WGA as a Model System to Study H-2 Restriction

The major question to be addressed involves the actual role of the H-2 antigen. Is the H-2 antigen recognized by the CTL in such a way that it is actually involved in the lysis of the target cell, or does the H-2 antigen act to enhance the binding between the CTL and the target cell so that through this interaction the antigen (or hapten) is recognized and subsequent target lysis occurs? If indeed the latter speculation is true, it should be possible to replace the specific H-2 antigen with a non-specific binder capable of binding the CTL and target.

Although the proposed models for H-2 restriction deal primarily at the CTL:target level, it can be proposed that the same mechanism exists at the stimulator:responder level whereupon the responder is

induced or stimulated to become a CTL. It has been observed that liposomes containing hapten and partially purified membranes as a source for the H-2 gene products can indeed stimulate responder cells to become CTL (22). It has also been reported that liposomes could not be used as a target for CTL-dependent lysis (26). It is therefore more feasible to examine the replacement of the H-2 antigen with a non-specific binder at the level of induction rather than at the level of target cell killing. Since WGA will bind to GlcNAc and sialic acid which are abundant on many cell surfaces WGA could be considered a non-specific binder. After derivatization of WGA with a fatty acid it is possible to incorporate this protein into liposomes. Thus, a membrane bound, non-specific binder protein can be constructed, which will hopefully be useful in testing the hypotheses described above.

F. Organization of Thesis

The first part of this thesis describes the formation of liposomes with WGA covalently attached to the surface. Some physical and biological activities of these liposomes in testing the various hypotheses for H-2 restriction are described. Finally, potential uses of the WGA-liposomes in other types of cellular recognition are suggested.

CHAPTER II

PREPARATION AND CHARACTERIZATION OF FATTY ACID DERIVATIZED WHEAT GERM AGGLUTININ

In order to develop a model membrane system to study cell to cell interactions, palmitoyl-wheat germ agglutinin (pWGA) was prepared and subsequently incorporated into liposomes, resulting in a defined membrane system containing a bound lectin.

A. Materials and Methods

1. Materials. Five-to-seven week old male and female C_3H/HeJ mice were purchased from Cumberland View Farms, Clinton, Tennessee. Egg yolk phosphatidycholine was isolated from chicken egg yolks and purified by chromatography on silicic acid as previously described (27). Hexadecy [^{3}H]-cholesteryl ether was synthesized from hexadecyl [^{3}H]-cholesterol and hexadecyl methane sulfonate (both from New England Nuclear) and purified by silicic acid chromatography (28). N-hydroysuccinimide ester of palmitic acid was synthesized (29) and then purified by recrystallization from ethanol (29).

Wheat germ agglutinin was purchased from Cal-biochem. Concanavalin A (Con A) was purchased from Pharmacia, and Lipopolysaccharide (LPS) was purchased from Sigma. Sodium deoxycholate and sodium cholate were purchased from Aldrich, and were recrystallized four times with ethanol.

2. Iodination of wheat germ agglutinin. Iodination of wheat germ agglutinin (WGA) was carried out by the chloramine-T method (30). Routinely, one milligram of WGA was labeled with $Na^{125}I$ or $Na^{131}I$. In 0.5M $NaPO_4$ buffer (pH 7.3) the protein, isotope, and freshly prepared chloramine-T (5ug/mg of protein) were mixed together in a plastic tube and incubated for 5 minutes at room temperature. Freshly prepared sodium metabisulfite (10ug/mg of protein) was added to the reaction mixture and the mixture was dialysed against PBS overnight at 4°C. Protein was determined by the method of Lowry (31). The radiolabeled protein was counted in a Beckman Biogamma II counter. Specific activity of the ^{125}I or ^{131}I-labeled protein ranged from 3,000-30,000 cpm/ug.

3. Covalent attachment of palmitic acid to wheat germ agglutinin. Derivatization of WGA with palmitate was carried out with a modified method established in this laboratory (32). Briefly, one milligram of ^{125}I-WGA in 0.1M N-acetylglucosamine (GlcNAc) was added to the N-hydroxysuccinimide ester of ^{3}H-palmitic acid (^{3}H-NHSP) in 2 percent sodium cholate at a NHSP to protein molar ratio of 5:1. The mixture was incubated at 37°C for 6 hours. After incubation, the mixture was applied to a Sephadex G-50 (superfine) column equilibrated with PBS containing 0.5 percent sodium cholate. Thirty drop fractions were collected and counted in a Beckman Biogamma II Counter. Fractions were also prepared for scintillation counting. The peak radioactive fractions were pooled and concentrated to approximately 1 ml by ultrafiltration in a Millipore Concentrator, using 13 mm filters with molecular weight cut-off of 2.5×10^4. Column recovery ranged from 65-85 percent. If the

derivatized palmitoyl-WGA (pWGA) was to be stored, the concentrated pWGA was dialysed against 0.5 percent sodium cholate buffer.

4. SDS-polyacrylamide gel electrophoresis. The discontinuous buffer gel system of Laemmli was used (33). Samples of unlabeled, native WGA, iodinated WGA, and derivatized WGA were prepared for electrophoresis by dissolving in 0.05M Tris-HCl, pH 7.0, 2 percent SDS, 5M urea. Samples were heated to 100°C for 2 minutes, and then applied to a 12 percent polyacrylamide slab gel (with approximately 1 cm of 3 percent stacking gel). Electrophoresis was carried out using a current of 12 mA per slab for approximately 4 hours. The gels were stained with Coomassie brilliant blue staining solution for approximately 30 minutes, after which the gels were destained overnight with a solution containing 5 percent methanol and 7.5 percent glacial acetic acid. Low molecular weight standards (BioRad, Richmond, CA) were prepared for electrophoresis as were the samples.

5. Liposome preparation. Egg yolk phosphatidylcholine (EYPC) and cholesterol were mixed in chloroform at a ratio of 7:2 (mole/mole). The lipids were dried under a stream of nitrogen gas and then vacuum desiccated for 1 hour. The dried lipids were resuspended in PBS to a concentration of 20mg/ml. The lipid suspension was sonicated to clarity at 25°C in a probe type sonifier (Heat Systems, W-375). Incorporation of pWGA was carried out by adding protein to the sonicated lipids at a lipid to protein ratio of 10:1 (w/w). Sodium cholate was also added so that the final sodium cholate to lipid ratio was 5:1 (mole/mole). These mixtures were dialysed against PBS supplemented with 0.02 percent

sodium azide and 1uM phenylmethylsulfonyl fluoride (PMSF) for 40 hours at 4°C. The dialysis buffer was then changed to PBS to remove azide and PMSF.

6. Sucrose density gradient centrifugation. Linear sucrose gradients (5 to 20 percent, w/v, with a 60 percent cushion) were centrifuged in a Beckman L3-50 ultracentrifuge at 200,000 x g for 5 hours at 4°C in a SW50.1 rotor. The gradients were fractionated from the bottom using a peristalic pump. Fractions were collected and counted using a Beckman Biogamma II Counter. When necessary an aliquot of each fraction was also prepared for scintillation counting.

7. Hemagglutination assays. Hemagglutination assays for WGA activity were carried out by adding 25ul of PBS to all but the first well of V-bottomed microtiter plates (Flow Laboratories). Protein or liposomes were added to the first well in a volume of 25 ul. The protein was serially diluted two-fold from well to well, and then 25ul of a 1 percent chicken red blood cell suspension was added to all wells. The last well contained no protein. The amount of liposomes to be added without incorporated protein was determined by calculating the amount of lipid associated with the same does of LpWGA. In controls, PBS containing 0.1M GlcNAc was added to the wells, and the protein was preincubated with 0.1M GlcNAc for 30 minutes at room temperature. The cells in the well were incubated at room temperature for 1 hour. Cloudy sediments of cells were marked as positive agglutination and distinct cell pellets were marked as negative agglutination. In some cases aliquots of these samples were viewed microscopically to check the reliability of this assignment. The results of hemagglutination are

expressed as the lowest protein concentration which causes positive agglutination of chicken red blood cells, i.e., the end-point titer.

8. Binding of liposomes containing WGA to spleen cells. Spleen cell suspensions from C_3H/HeJ mice were prepared aseptically in RPMI 1640 media as previously described (34). Erythrocytes were lysed by brief exposure to 0.83 percent NH_4Cl. The cells were washed twice with RPMI 1640 and the viable cells were enumerated by trypan blue exclusion. Quadruplicate samples of various doses of ^{3}H-liposomes containing ^{125}I-pWGA (LpWGA), ^{125}I-WGA, ^{3}H-liposomes, and ^{3}H-liposomes with exogenous ^{125}I-WGA were added to 2 X 10^6 cells in RPMI 1640 media to a final volume of 1 ml in disposable culture tubes. The mixture was incubated for 1 hour at 4 or 37°C. After incubation the mixture was diluted four-fold with ice cold media and centrifuged at 2,000 rpm for 2.5 minutes. The supernatant was carefully discarded and the pellet was washed 4 times in 1 ml of RPMI 1640 media. After washing, one tube of cells was counted for cell viability by trypan blue exclusion and the other three were counted in a Beckman Biogamma II Counter. The cell pellets were then solubilized in 2 percent sodium dodecyl sulfate (SDS) and were prepared for scintillation counting.

9. Mitogenicity assay of LpWGA. Spleen cells (2 X 10^5/well) were incubated in RPMI-1640 medium with varying doses (2-10 ug) or unlabeled WGA, ^{125}I-WGA, LpWGA, liposomes alone, and liposomes with exogenous ^{125}I-WGA in microtiter plates (Flow Laboratories). After 24 hours each well was supplemented with 5 percent dialysed fetal calf serum (dialysed against RPMI 1640, overnight, 2 changes). The

incubation was continued for a total of 62 hours at which time [^{3}H]-thymidine (0.2 uCi/well) was added and the culture was incubated for an additional 10 hours. The cells were harvested on a Titertek cell harvester (Flow Laboratories) using glass fiber filters. The filters were dried overnight and then soaked in ice cold trichloroacetic acid (5 percent) for 30 minutes, and then in 70 percent cold ethanol, and finally in cold acetone. After drying, the filters were prepared for scintillation counting. Each sample was repeated 5 times. Three wells were harvested; the other two were counted for cell number.

Con A and LPS were used as controls for positive mitogenesis.

B. Results

1. Purification of derivatized WGA. The derivatization mixture was applied to a Sephadex G-50 (superfine) column equilibrated with PBS containing 0.5 percent sodium cholate. The separation profile can be seen in Figure 1. The first peak represents iodinated and derivatized WGA as seen by the co-chromatographed ^{3}H and ^{125}I radioactivities. The second peak represents free ^{3}H-palmitic acid and the third peak represents residual ^{125}I incompletely removed after protein iodination.

It should be noted that earlier reports of this modification procedure (32) used sodium deoxycholate to solubilize the NHSP. However, it appeared that WGA was denatured at high concentrations of sodium deoxycholate, consequently sodium cholate was used instead. The concentration of sodium cholate required to achieve sufficient column recovery (i.e., at least 50 percent) was experimentally determined. Figure 2 shows a significant difference between 0.15 percent and 0.5

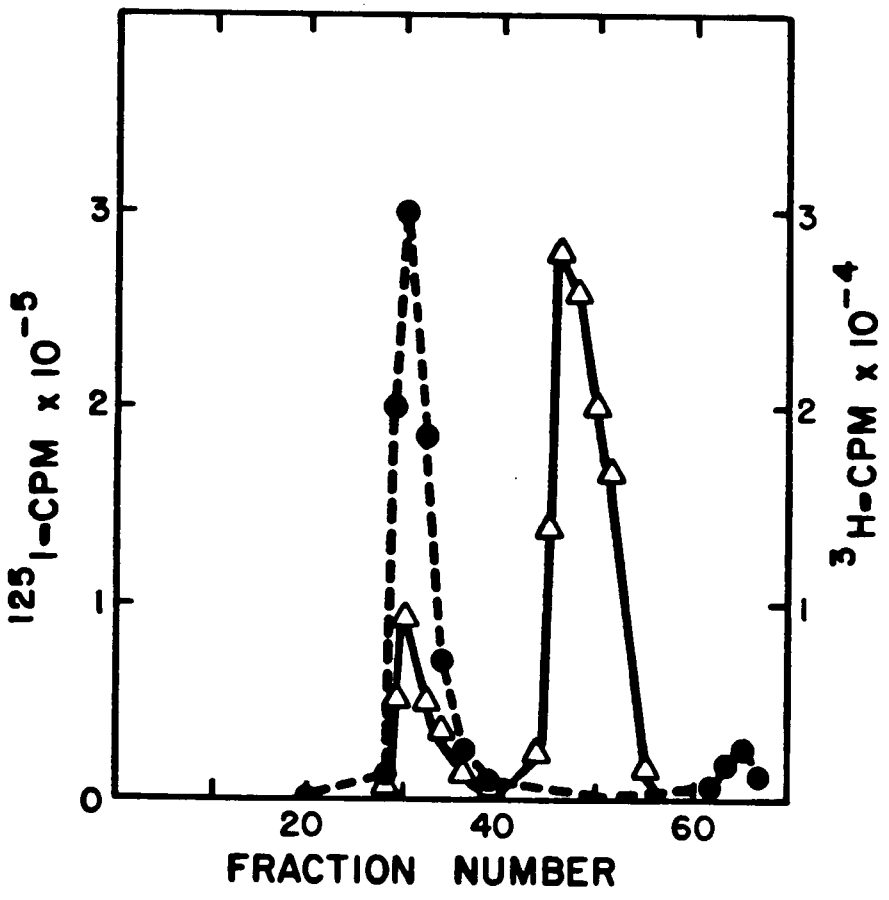

Figure 1. Sephadex G-50 profile after derivatization of WGA with palmitate.

Sephadex G-50 (superfine) column (1.5 cm X 52 cm) contained 50 ml of gel, equilibrated with PBS containing 0.5 percent cholate. Column flow rate was approximately 0.34 ml/minute. 800-900ug of pWGA was applied to column.

●---● ^{125}I-WGA △——△ ^{3}H-palmitate

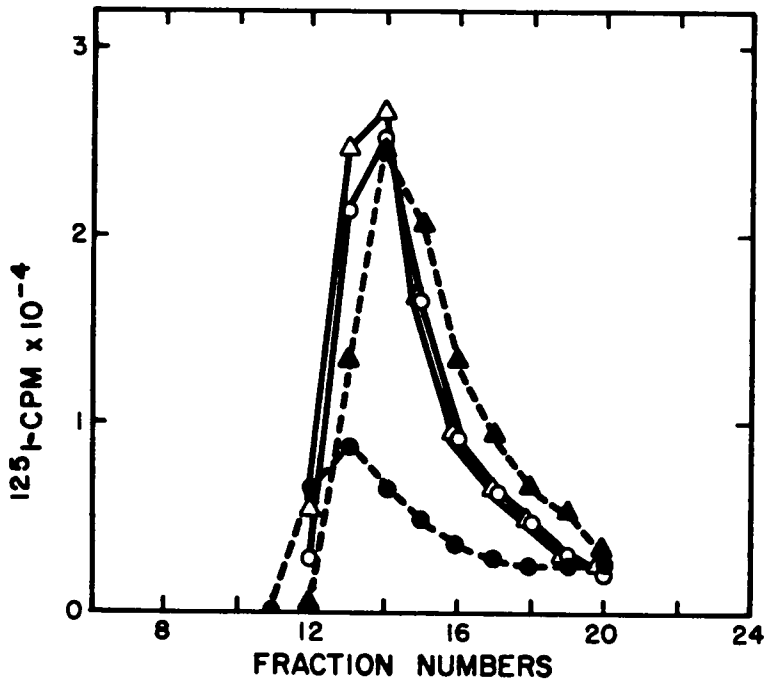

Figure 2. Sephadex G-50 profile of pWGA using different concentrations of sodium cholate.

Sephadex G-50 columns (0.5 cm X 18.5 cm) with bed volumes of 5 ml were equilibrated with PBS containing varying concentrations of sodium cholate. Approximately 0.5 mg of ^{125}I-labeled pWGA was applied to each column, and flow rate was approximately 0.5 ml/minute.

●---● 0.15 percent cholate
▲---▲ 0.5 percent cholate
△——△ 1.0 percent cholate
o——o 2.0 percent cholate

percent cholate. However, above 0.5 percent there seemed to be little difference in the recovery of the protein. Therefore, it was decided to use 0.5 percent cholate in PBS to elute the protein from the column. The palmitate to protein molar ratio in the eluted WGA peak was calculated to be 1.3 (palmitate/WGA) by comparing ^{3}H cpm and ^{125}I cpm in the eluted WGA peak.

Iodination and covalent modification did not cause gross structural alteration of the protein as seen by the SDS-gel electrophoresis profile (Figure 3). Lanes B,C,D represent native WGA, iodinated WGA, and derivatized WGA, respectively. They all showed identifical migration in the gel which corresponded to a subunit molecular weight of $\sim$ 17,000.

2. Liposome preparation. In order to demonstrate that pWGA was indeed incorporated into liposomes, ^{125}I-pWGA modified with ^{3}H-palmitate were reconstituted with lipids as described in Materials and Methods. Sucrose gradients were performed on the liposomes after the extended dialysis procedure. The fractions were collected and counted both for ^{125}I and ^{3}H. The turbidities of the fractions were also measured as absorbance at 450nm. Figure 4 shows the sedimentation profile of the various samples. Characteristically, liposomes floated at the top of the sucrose gradient. Palmitate appeared to be associated with the WGA as seen from co-sedimentation of the ^{125}I and ^{3}H counts. The turbidity measurements indicated the presence of liposomes in the fraction with the pWGA (panel A). Liposomes without WGA did not penetrate the gradient as did the LpWGA (panel B). ^{125}I-WGA entered the gradient slightly and showed no turbidity (panel C). Also, ^{125}I-WGA did not

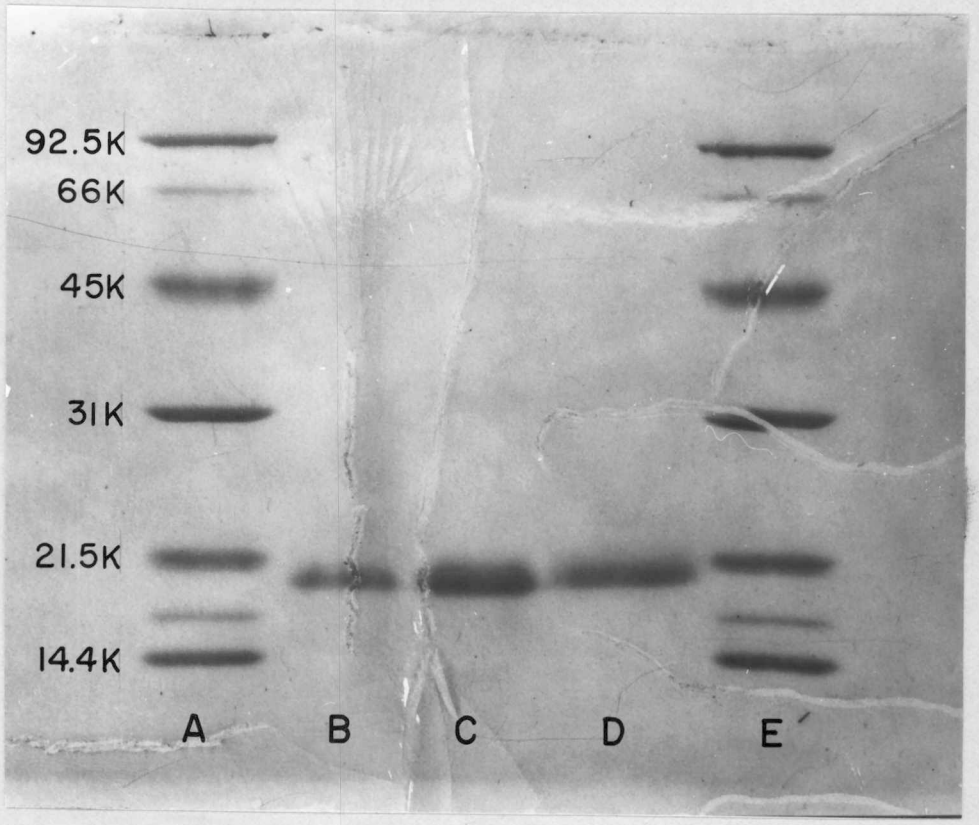

Figure 3. SDS-Gel electrophoresis of various preparations of WGA.

Unlabeled WGA(B), iodinated WGA (C), and derivatized WGA (D). Molecular weight standards (A) and (E) with indicated molecular weights. Approximately 15 ug of various WGA samples was applied to the gel.

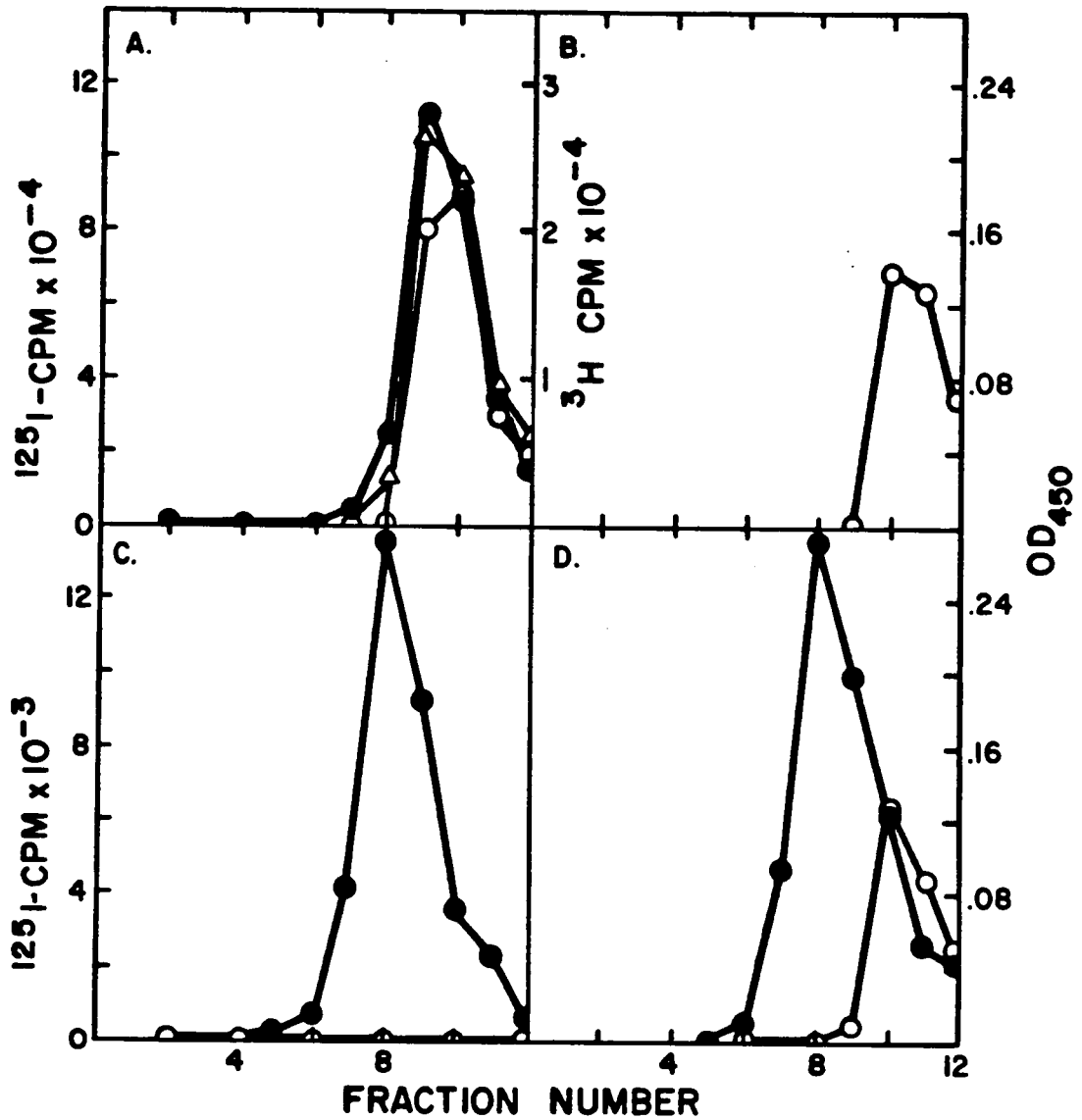

Figure 4. Sucrose density gradient centrifugation of liposomes containing pWGA

Liposomes containing ^{125}I-WGA modified with ^{3}H-palmitate (A), liposomes alone (B), ^{125}I-WGA alone (C), and ^{125}I-WGA plus liposomes (D). All fractions were collected from the bottom of the gradient, and each fraction was counted in a Beckman Biogamma II Counter and measured for absorbance at 450 nm. Sample A was also counted in a scintillation counter.

o—o OD_{450} ●—● ^{125}H Δ—Δ ^{3}H

associate with liposomes when mixed with preformed liposomes (panel D). No association of pWGA with preformed liposomes was observed unless under the conditions of reconstitution with detergent (data not shown). These results clearly indicated that pWGA was indeed incorporated into liposomes by the method used.

3. Hemagglutination assay. Although pWGA can be incorporated into liposomes its binding properties should remain active. This was tested by a hemagglutination assay. The results (Table 1) indicate that LpWGA not only retained their binding ability, but the binding actually appears to be enhanced. There was an 8-fold decrease in the end-point titer for LpWGA as compared to native WGA or ^{125}I-WGA. Agglutination by LpWGA could be inhibited by excess GlcNAc as could native WGA and ^{125}I-WGA. It would thus appear that the saccharide binding activity of pWGA incorporated into liposomes was not impaired. It should be noted that liposomes alone did not contribute to the agglutination of the red blood cells.

4. Binding of LpWGA to spleen cells. Although LpWGA appear to bind to RBC it was necessary to test their ability to bind to spleen cells. After a one hour incubation of spleen cells with varying doses of LpWGA and appropriate controls (as described in Materials and Methods), it can be seen from Figure 5 (panels A and C) that there was a marked enhancement in binding of liposome-bound WGA to spleen cells both at 37° and 4°C as compared to the undervatized WGA. Panels B and D show binding of liposomal lipids to cells. Liposomes did not associate with cells, either alone or when introduced to the cells in the presence of

TABLE 1

HEMAGGLUTINATION ASSAY

Sample	End-Point Titer (ug/ml)
WGA	1.25
WGA + GlcNAc[a]	5.0
^{125}I-WGA	1.25
^{125}I-WGA + GlcNAc	5.0
LpWGA	0.16
LpWGA + GlcNAc	1.25
Liposomes	>18.0
Liposomes + GlcNAc	>18.0

[a]0.1M N-Acetylglucosamine.

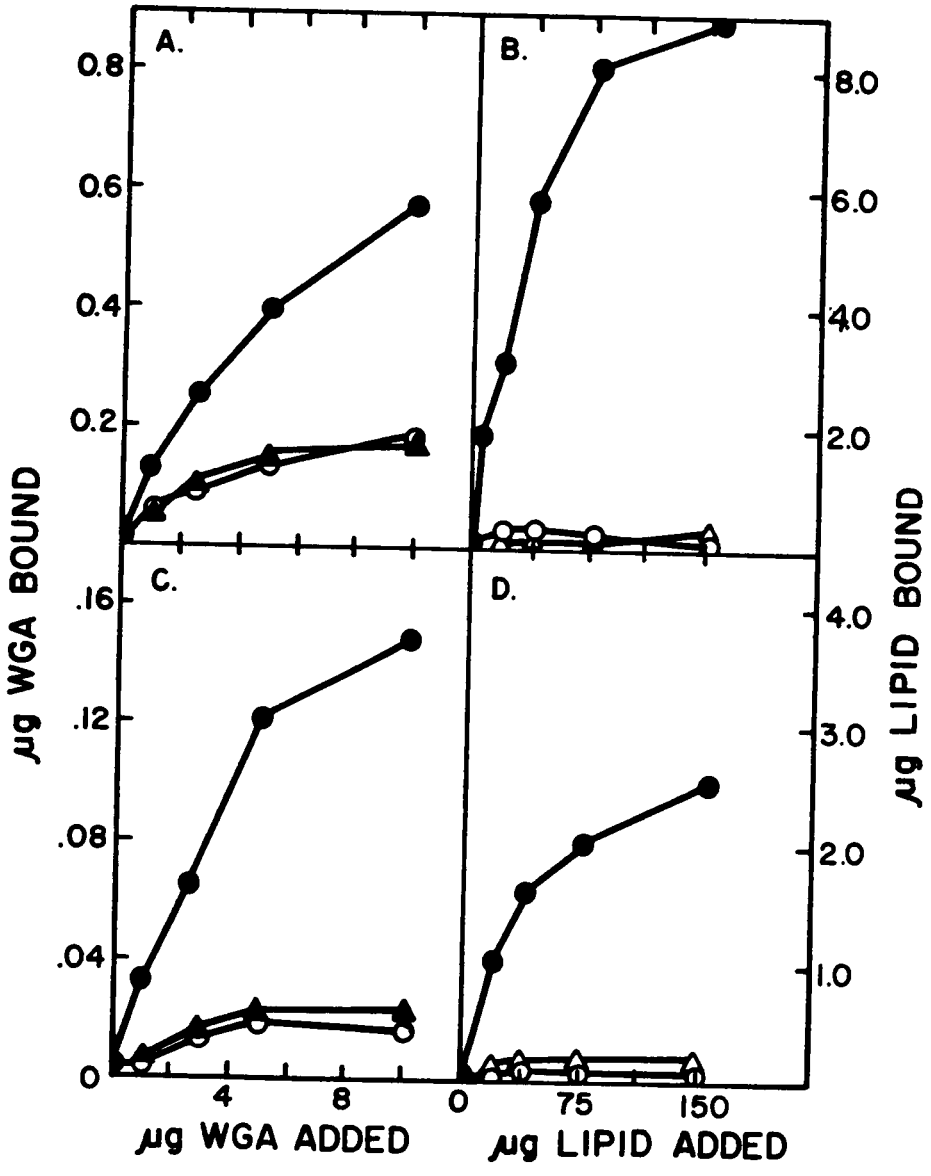

Figure 5. Binding of WGA and lipid to spleen cells.

0.1-10ug of WGA and equivalent amounts of liposomes were incubated with spleen cells for 1 hour at 4°C or 37°C. The amount of WGA or lipid bound to cells was determined from ^{125}I (WGA) and ^{3}H (lipid cpm associated with cells). Data from panels A and B were obtained at 37°C, and panels C and D at 4°C.

●—● LpWGA
▲—▲ WGA alone
o—o Liposomes plus WGA
△—△ Liposomes alone

underivatized WGA. Lipid binding occurred only when liposomes were introduced in the form of LpWGA.

Binding of both WGA and lipids were temperature sensitive; greater binding was observed at 37°C than at 4°C. This result suggests that binding at 37°C probably involves some metabolic processes, although this possibility was not investigated.

5. Mitogenicity of LpWGA. Although WGA is not mitogenic in its native state (35), it is important to know whether it remains non-mitogenic after it has been covalently modified by fatty acid and its subsequent incorporation into liposomes. This information is necessary for interpretation of the effect of LpWGA on spleen cell metabolism. An assay utilizing ^{3}H-thymidine incorporation was performed on the cells after a 3-day incubation with LpWGA (see Materials and Methods).

As can be seen in Figure 6, cells incubated with Con A and LPS, both established mitogens for lymphocytes, showed a significant uptake of ^{3}H-thymidine as well as an increase in the total number of cells. The discrepancy between the increase in cell number and ^{3}H-thymidine uptake for Con A (panel A) may be explained in that some cells are rapidly synthesizing DNA, without subsequently dividing. Thus, an increase in ^{3}H-thymidine would occur without an increase in cell number. Cells incubated with LpWGA as well as with other forms of the agglutinin, except ^{125}I-WGA, showed neither an appreciable incorporation of ^{3}H-thymidine nor an increase in cell number during the 3-day incubation. The slight increase seen in panel C was irreproducible. Thus, the derivatized and liposome bound WGA remained non-mitogenic.

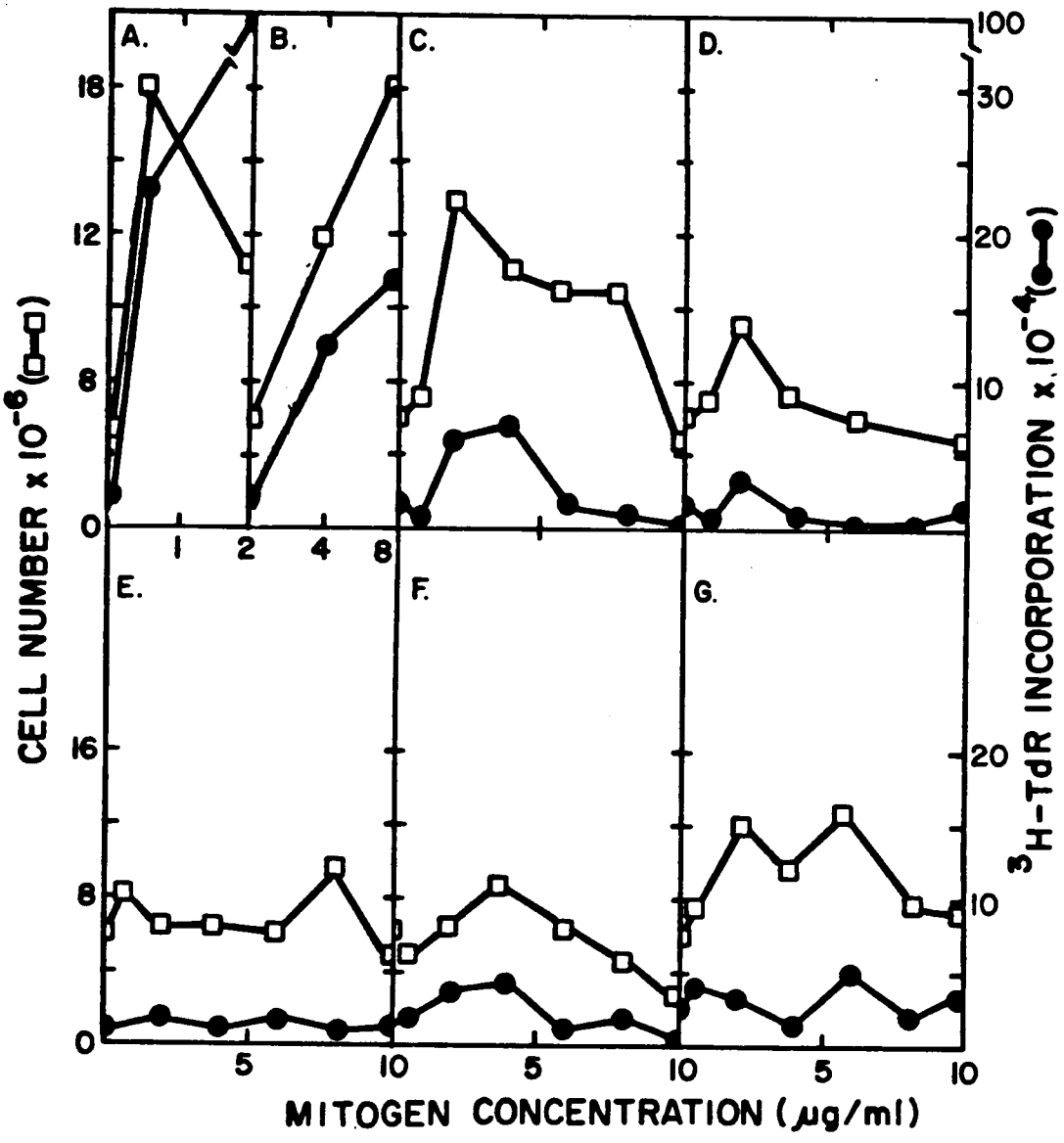

Figure 6. Mitogenicity assay of liposomes containing pWGA.

^{3}H-thymidine uptake. 2 X 10^{5} cells/well were incubated with 2-10ug of various samples of WGA, equivalent amounts of liposomes alone, 0.5ug/ml and 2.0ug/ml of Con A, and 4ug/ml and 8ug/ml of LPS.

A. Con A, B. LPS, C. ^{125}I-WGA, D. Liposomes alone.
E. LpWGA, F. Liposomes Plus ^{125}I-WGA G. Unlabeled WGA.

□—□ Cell number ●—● ^{3}H-cpm

C. Discussion

Covalent modification of WGA with a fatty acid has proven to be a method by which this protein can be incorporated into liposomes efficiently. This procedure has previously been reported (36) in which α-bungarotoxin and IgG have been covalently modified with palmitate. In both cases the binding ability of the two proteins was retained after being incorporated into liposomes. Similar to these two modified proteins, pWGA also retains its binding ability. In fact, the incorporation of pWGA into liposomes appears to enhance the binding of WGA to cells. In earlier reports sodium deoxycholate (DOC) was used to solubilize the NHSP during the derivatization reaction (32). In the present experiments, it was noted that WGA is fairly unstable in DOC. Nicholson et al. (37), have reported that at DOC concentrations greater than or equal to 1 percent, the binding of glycoproteins to WGA-sepharose columns is irreversibly impaired, and that "detergent inhibition of lectin activities" occurred when WGA was assayed for hemagglutination activity in the presence of DOC. As a result, sodium cholate was substituted for sodium deoxycholate. WGA appeared to be less sensitive to sodium cholate than to DOC. The concentration of sodium cholate required to achieve sufficient column recovery was experimentally determined (Figure 2, page 14).

It has been shown that native WGA binds specifically to erythrocytes and spleen cells (Table 1 and Figure 5); however, when WGA is incorporated into liposomes there appears to be an enhanced binding of WGA to cells. This can be attributed, presumably, to the multivalency of the liposomes containing pWGA.

The binding of LpWGA to spleen cells containing oligosaccharides

on their surface is mediated presumably by multiple bonds between individual WGA-saccharide pairs. The binding of a particular WGA molecule to a saccharide molecule upon random collision of liposomes with cells has brought the neighboring WGA and saccharides into close proximity, and therefore, the probability of establishing more bonds between the two membranes is higher. Dissociation of liposomes from cells would require the simultaneous breakage of all WGA-saccharide bonds between the two membranes. The possibility of dissociation drastically decreases when the number of WGA-saccharide bonds increases. This multivalent binding is well known in antibody-antigen binding. The apparent binding affinity of a bivalent antibody molecule (e.g., IgG) is about 1-2 orders of magnitude higher than the corresponding monovalent ones (e.g., Fab) (38). Multivalent binding is also responsible for the increased apparent affinity of liposomes containing palmitoyl-α-bungartoxin (PBGT) for microsac membrane vesicles containing the acetylcholine receptors (a specific receptor of α-BGT) (36). It was observed that by increasing the density of PBGT molecules on the liposomes, the apparent binding to microsacs increased by approximately 20-fold as compared to liposomes containing no PBGT. Therefore, it is possible to increase the apparent binding affinity by increasing the valency of the binder.

Although WGA is known to be a non-mitogenic lectin, it cannot be assumed that WGA remains non-mitogenic after covalent modification and after immobilization of WGA into a lipid matrix. However, data obtained from a ^{3}H-thymidine incorporation assay indicates there is no significant increase in DNA synthesis nor increase in cell number after

incubation with LpWGA. This would indicate that WGA remains non-mitogenic after chemical and physical modification.

This observation is important in view of the subsequent application of LpWGA in which liposomes containing a hapten, a H-2 antigen source, and pWGA are to be tested for their abilities to stimulate the production of cytotoxic T-lymphocyte. Endogeneous mitogenic activity of liposome-bound WGA would interfere with any possible lymphocyte activation by antigens. Thus, it appears that liposome-bound WGA is a suitable non-specific binder for experiments to be described in the next chapter.

CHAPTER III

STIMULATION OF CYTOTOXIC T-LYMPHOCYTES BY LIPOSOMES: A MODEL SYSTEM TO EXAMINE H-2 RESTRICTION

The stimulation of cytotoxic T-lymphocytes (Figure 7) by liposomes may serve as a potential model to study cell to cell interactions. Burakoff has summarized several reports of CTL stimulation by viral glycoproteins incorporated into liposomes (22). Viral glycoproteins would thus appear to be suitable antigens for our purpose. However, the relatively pure viral proteins are rather difficult to obtain, and as a result the search for an appropriate hapten system was necessary. The trinitrophenol (TNP) hapten system characterized by Shearer (39) was chosen since it was relatively easy to obtain and the significant CTL response with proper H-2 restriction has been reported (39). However, this hapten has never been used in liposome systems to stimulate T-lymphocytes. It was thus necessary to first test the activity of liposomes containing TNP haptens in the induction of CTL. The use of pWGA in liposomes is justified only after such activity has been established. In these experiments a crude plasma membrane preparation was used as a source for H-2 antigen. Liposomes containing hapten and H-2 gene products were used as a control to establish a positive CTL induction similar to the CTL response stimulated by haptenated whole cells.

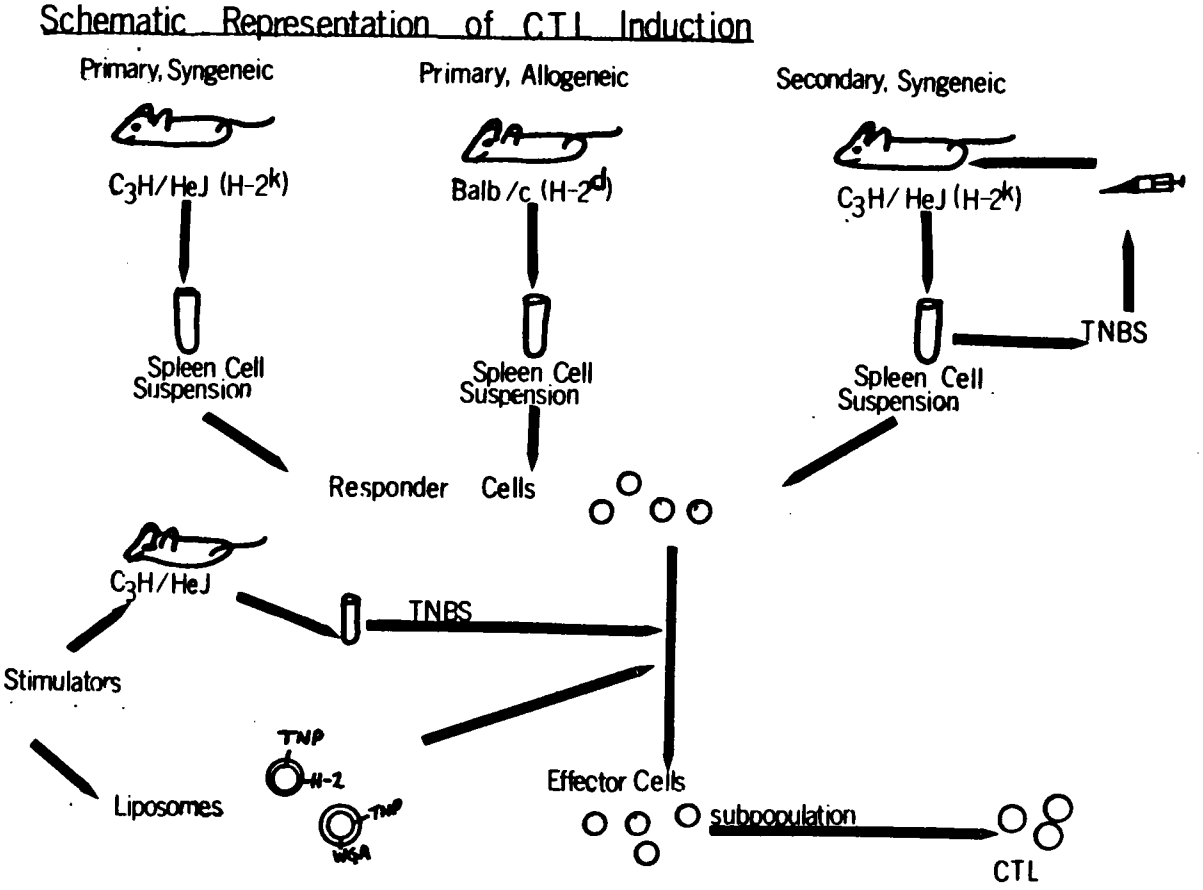

Figure 7. Schematic representation of CTL induction.

A. Materials and Methods

1. Materials. Five to seven week old female and male C_3H/HeJ and Balb/c mice were purchased from Cumberland View Farms, Clinton, Tennessee. Mouse fibroblasts were used as target cells. Mouse L-929 ($H\text{-}2^k$) target cells were obtained from Flow Laboratories and Balb/c 3T3 clone A31 cells ($H\text{-}2^d$) were obtained from Dr. Ray Tennant, Biology Division, Oak Ridge National Laboratory. Both types of cells were grown in McCoy's 5A medium supplemented with 5 percent calf serum.

Lipids were prepared or purchased as described in Chapter II, with the exception of trinitrophenyl-caproyl-phosphatidylethanolamine (TNP-CAP-PE) which was purchased from Avanti Polar Lipids, Birmingham, Alabama.

Trinitrobenzene sulfonate (TNBS) was a generous gift to Dr. Gene Shearer, National Institute of Health, Bethesda, Maryland.

2. Radiolabeling of plasma membrane. Whole spleen cells or isolated spleen cell membranes were labeled with ^{131}I by the lactoperoxidase labeling procedure of Hubbard and Cohn with some modifications (40). Briefly, 10^8 cells/2ml PBS, $Na^{131}I$, and 15-20 units (23.5 IU/mg) of lactoperoxidase were mixed together. Fifteen ul of 0.59 percent H_2O_2 was added to the mixture, agitated for 10 minutes, and another 15 ul of 0.59 percent H_2O_2 was added with subsequent agitation. The mixture was dialysed against PBS supplemented with 0.02 percent sodium azide and 1uM PMSF overnight at 4°C. Protein was determined by the method of Lowry (31).

3. Isolation of plasma membrane. Plasma membranes were isolated by modified procedures of Ferber et al., and Lawman et al. (41, 42). Spleens from C_3H/HeJ mice were removed aseptically and suspended in ice cold PBS. Single cell suspensions were prepared and RBC were lysed with 0.83 percent NH_4Cl. The cells were washed twice with Hepes buffer (0.02M Hepes, 0.13M NaCl, 0.5mM $MgCl_2$, pH 7.4), enumerated, and adjusted to 0.5-2.0 X 10^7 cells/ml. An equal volume of 17 percent sucrose was added to the cell suspension. The cells were ruptured by the nitrogen cavitation method at 735 psi for 15 minutes. The resulting cell homogenate was monitored microscopically to ensure cell disruption. The lysed cell suspension was centrifuged for 30 seconds at 1000 rpm to remove nuclei and whole cells. The supernatant was centrifuged at 100,000 x g for 90 minutes at 4°C, in Ti 60 rotor. The pellet was resuspended in approximately 2 ml of PBS and layered on a sucrose step gradient (20, 30, 40, 50, 60 percent, w/v). The gradient tubes were centrifuged at 100,000 x g for 14 hours at 4°C in an SW27.1 rotor. Routinely, 2 turbid bands appeared in the gradient. One band was associated with the 30 percent sucrose and one at the 45-50 percent interface. The upper (30 percent) band was collected and washed twice by resuspending the pellet in 5 ml PBS and centrifuging at 100,000 x g for 40 minutes. Protein was determined by the method of Lowry (31).

It was necessary in some cases to repeat nitrogen cavitation in order to obtain sufficient disruption. This was noted when large volumes of cells were used.

4. Liposome preparation. Liposomes containing 50 mole percent of TNP-CAP-PE were prepared as described in Chapter II, with the

TNP-CAP-PE being added with the initial EYPC and cholesterol in chloroform. Fifty mole percentage was calculated as 500 moles of TNP-CAP-PE/umole total lipsomal lipid. Incorporation of plasma membrane proteins into liposomes was carried out by adding the plasma membrane proteins to the sonicated lipids and sodium cholate as described in Chapter II at a lipid to protein ratio of 10:1.

5. Primary immunization (priming) of mice to TNP-hapten. Spleens from C_3H/HeJ mice were removed aseptically as previously described (34). The erythrocytes were lysed by treatment of the single cell suspension with 0.83 percent NH_4Cl for 3 minutes. The cells were washed twice with Hank's Balanced Salt Solution (HBSS) and the resulting pellet was incubated with 4 volumes of 10mM trinitrobenzene sulfonate (TNBS) in PBS, pH7.3, at 37°C for 10 minutes. The cells were washed twice with HBSS containing 10 percent fetal calf serum to remove unreacted TNBS, and then washed twice with HBSS and enumerated.

2.5×10^7 treated cells were injected intraperitoneally and 1.5×10^7 cells were injected subcutaneously to each C_3H/HeJ mouse. Spleens of the injected mice were routinely used 7 to 14 days post-immunization (43).

6. Determination of TNP groups per cell. The target L-cells were treated with TNBS under the same conditions as for the spleen cells described above. The treated cells were examined for the amount of trinitrophenylation. Cells were counted after treatment with TNBS and then solubilized with 10 percent SDS in a final voluume of approximately 1 ml. The viscous solution was applied to a pasteur pipette plugged

with glass wool. It was centrifuged at 2000 rpm for 5 minutes in a test tube. The resulting fluid (viscosity reduced) in the test tube was measured for absorbance at 348nm. The number of TNP groups per cell was calculated using the published molar extinction coefficient of 1.5×10^4 (44). The absorbance readings were corrected using a sample containing untreated cells and SDS.

7. TNP-haptenation of liposomes containing plasma membrane proteins. Liposomes containing plasma membrane proteins (L-PM) were mixed with 4 volumes of 10mM TNBS, pH 7.3, and incubated at 37°C for 10 minutes, after which a 10-fold excess of glycine (in PBS) was added to remove unreacted TNBS. The treated lipid suspension was dialyzed overnight against PBS (2 changes), and concentrated by ultrafiltration in a Millipore Concentrator (13mm) using filters with a molecular weight "cut-off" of 1×10^5.

8. Induction of CTL in vitro. Responder cell suspensions were prepared from spleens of primed C_3H/HeJ mice. Erythyocytes were removed by brief exposure to 0.83 percent NH_4Cl. The lymphocytes were washed twice with RPMI 1640 media, and enumerated. $4\text{-}6 \times 10^6$ cells in RPMI 1640 media supplemented with 10 percent fetal calf serum (FCS), 2mM glutamine, 100 units/ml penicillin, 100 g/ml streptomycin and 5×10^{-5} M 2-β-mercaptoethanol were added to 1.5 ml plastic culture wells (15mm, Flow Laboratories).

Stimulator cells were prepared from spleens of non-immune C_3H/HeJ mice. The cells were prepared as for primary immunization except after TNBS treatment, the spleen cell suspensions were treated with

Mitomycin C (25 ug/ml) for 25 minutes at 37°C to inactivate their DNA synthesis activities. These stimulator cells were washed 4 times with RPMI 1640 media to remove excess Mitomycin C and then enumerated. Routinely, 2 X 10^6 stimulator cells were used per well of responder cells. Liposomes or partially purified plasma membrane preparations to be used as stimulators were sterilized by a 30 minute exposure to UV irradiation prior to use. Stimulators were incubated with responders for 1 hour after which FCS was added to a final concentration of 10 percent. One ml cultures were incubated for 5 days in a humidified CO_2 incubator, after which cells were harvested, washed, and enumerated for cytotoxicity assays. These cells are termed effector cells.

9. Cytotoxicity assay. Cytotoxicity assays were performed as previously described (45). Briefly, varying amounts of effector cells suspended in RPMI media supplemented with 10 percent FCS, 2mM glutamine, 100 units/ml penicillin, 100 g/ml streptomycin and 5 X 10^{-5} M 2-β-mercaptoethanol were added to round-bottomed microtiter wells (Flow Laboratories) containing 10^4 targets. The ^{51}Cr labeling of targets was described (46). Briefly, targets were incubated with 100 uci of $Na^{51}Cr$ for 2 hours with repeated shaking. L-cells, L-cells treated with TNBS, A31 cells, and A31 cells treated with TNBS were used as targets. Microtiter plates were centrifuged at 1000 rpm for 1.5 minutes in a Damon/IEC PR-6000 centrifuge. Assays were incubated at 37°C in a humidified CO_2 incubator for 5 hours, after which 100 ul of the supernatant was counted on a Beckman Biogamma II Counter. Target cell killing was assessed by percent specific lysis,

$$\text{Percent specific lysis} = \frac{\text{Experimental } {}^{51}\text{Cr release-Media } {}^{51}\text{Cr release}}{\text{Total } {}^{51}\text{Cr release-Media } {}^{51}\text{Cr release}} \times 100$$

where experimental ^{51}Cr release is the average amount of label released from triplicate doses of the various samples tested for stimulation ability, media ^{51}Cr release is the amount of label released from cells without stimulators present, and total ^{51}Cr release is the amount of label present in the targets determined by solubilizing the targets with 2 percent triton.

10. Electron microscopy of liposome preparations. Liposomes were applied to carbon-coated, formvar grids and then negatively stained with 2 percent phosphotungstic acid. Liposomes were viewed at a variety of magnifications and electron micrographs were taken at 40,000 magnification.

B. Results

1. Trinitrophenol as a T-cell hapten. In order to establish TNP as a T-cell hapten, whole spleen cells modified with TNBS were used as stimulator cells to demonstrate primary and secondary syngenic response, as well as an allogeneic response. Table 2 summarizes these results. Whole cells modified with TNBS could elicit a primary CTL response which was H-2 restricted. As can be seen, effector cells derived from non-immune C_3H/HeJ ($H\text{-}2^k$) cells could effectively lyse TNP-modified targets bearing $H\text{-}2^k$ haplotype (TNP-L-cells), but to a much lower extent, the haptenated targets bearing $H\text{-}2^d$ haplotype (TNP-A31 cells). Unhaptenated targets could not be lysed efficiently. Primary allogeneic

TABLE 2

CTL INDUCTION BY TNP MODIFIED C_3H/HeJ SPLEEN CELLS

Responder	E:T[a]	Percent Specific Lysis Targets: L-cell-TNP ($H\text{-}2^k$)	L-cell ($H\text{-}2^k$)	A31-TNP ($H\text{-}2^d$)	A31 ($H\text{-}2^d$)
Non-immune $C_3HeJ(H\text{-}2^k)$	40	50 (12)[b]	18.5 (18)	20.5 (13)	18.6 (17)
	20	24 (7)	8.6 (9.0)	11.4 (8.0)	17.6 (15)
	10	14 (5)	3.0 (3.0	3.0 (3.0)	7.0 (7.0)
Non-immune Balb/c($H\text{-}2^d$)	40	37 (-1.0)	32.5 (-3.3)	0.46 (-5.5)	-0.84 (-1.1)
	20	25 (1.0)	19.7 (-2.2)	-0.62 (-1.0)	-0.37 (-0.63)
	10	16 (-1.0)	8.6 (-1.6)	2.2 (0.02)	-0.13 (-0.3)
Immune $C_3H/HeJ(H\text{-}2^k)$	40	66 (12)	13.6 (11)	13.4 (6.6)	ND[c]
	20	56 (12.5)	4.0 (10)	8.3 (3.0)	ND
	10	58 (8.4)	4.0 (12)	5.7 (3.0)	ND

Stimulators: 2×10^6 C_3H/HeJ spleen cells modified with 10mM TNBS as described in Materials and Methods.

[a]Effector cell to target cell ratio.

[b]Numbers in parentheses represent percent specific lysis by the responder cells which had not been stimulated.

[c]Not done.

response was seen with effectors derived from Balb/c ($H\text{-}2^d$) cells. These cells could only lyse targets of a different haplotype, no matter whether the targets were haptenated or not. Effector cells derived from immune C_3H/HeJ cells (secondary CTL response) gave similar results to those from non-immune cells except the specific killing was of a greater magnitude. These data are in agreement with literature reports (39). It would thus appear that TNP is an appropriate hapten which gives a significant CTL response with expected H-2 restrictions. Since the secondary syngeneic CTL response gave more clearly defined H-2 restriction, further experiments using liposomes were performed with pre-immune (or primed) mice.

2. Quantitation of TNP groups per cell. It was necessary to quantitate the actual number of molecules of cell-associated TNP for the stimulator and target cells used in the CTL experiments. It was done by an optical absorption method, using the published molar extinction coefficient. Table 3 shows the results of this determination. The two types of target cells contained 7-fold more TNP groups per cell than did the stimulator cells. This is probably because of the size difference between the cells. Spleen cells are "small" lymphocytes (about 6-9 um in diameter); whereas target cells are fibroblasts of larger size (about 10-15 um in diameter). One would expect more TNP groups appearing on the surfaces of target cells than on the spleen cells. It is also possible the cell surface components of the targets is much different than the spleen cells resulting in the higher number of TNP groups per cell.

TABLE 3

QUANTITATION OF CELL-ASSOCIATED TNP GROUPS

	OD_{348}	Number TNP Molecules/cell X 10^{-8}[a]
C_3H/HeJ spleen cells	0.017	3.0
L-cells	0.034	24.0
A31 cells	0.026	21.4

[a]Calculations were made using molar extinction coefficient 1.5×10^4.

3. Liposomes prepared with TNP-CAP-PE. It was necessary to incorporate as much TNP-CAP-PE into the liposomes as possible in order to mimic the stimulator cells that showed positive inducation of CTL. Liposomes were made with varying amounts of TNP-CAP-PE up to 50 mole percent. In order to show a proper incorporation of plasma membrane proteins into liposomes containing large amounts of TNP-CAP-PE, sucrose density gradient sedimentation of liposomes was carried out. As can be seen from Figure 8, panels B and D, liposomes containing either 30 or 50 mole percent TNP-CAP-PE could incorporate the plasma membrane proteins. The fast sedimenting liposome peak probably represented liposome aggregation. The presence of lipid (TNP-CAP-PE) was followed in this experiment by absorbance at 348 nm.

It was decided that 50 mole percent of TNP-CAP-PE was to be used in further experiments to best mimic the TNP-modified stimulator cells.

4. Incorporation of plasma membrane proteins, WGA, and TNP-CAP-PE into liposomes. Preparation of liposomes was attempted by incorporating plasma membrane proteins, WGA, and TNP-CAP-PE and various combinations of these constituents into liposomes. The incorporation of these constituents into liposomes was demonstrated by the sedimentation profiles of liposomes in sucrose density gradients. Figure 9 shows the profiles of these liposomes. Liposomes made with ^{125}I-pWGA (panel G) and liposomes made with ^{131}I-labeled plasma membrane proteins (panel B) showed characteristic co-sedimentation of most if not all of the proteins (^{125}I or ^{131}I) with lipids (^{3}H). It should be noted that plasma membrane alone will pellet in the gradient (data not shown). Liposomes containing plasma membrane proteins are probably quite hererogeneous since the profile suggests

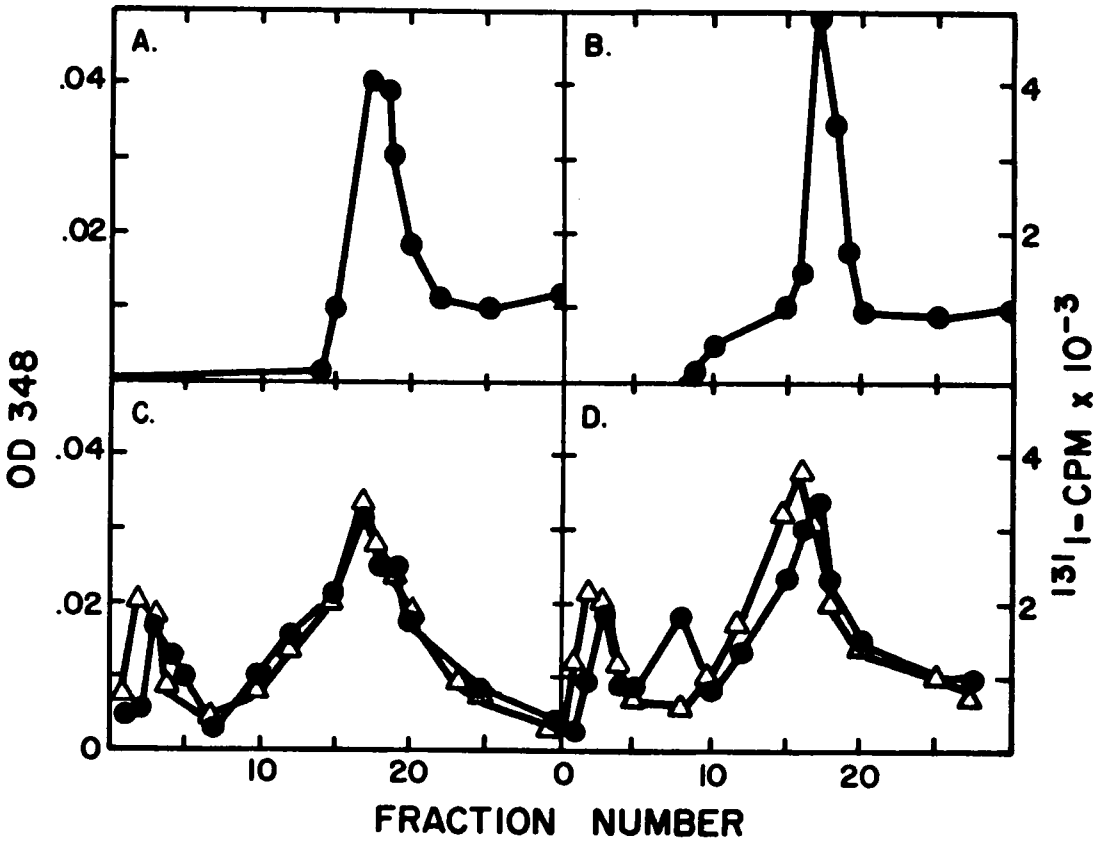

Figure 8. Sucrose density gradient centrifugation of liposomes containing TNP-CAP-PE and plasma membrane proteins.

All fractions were collected from the bottom of the gradient, and each fraction was counted for ^{131}I and measured for absorbance at 348 nm.

A. 30 mole percent TNP-CAP-PE B. 50 mole percent TNP-CAP-PE
C. 30 mole percent TNP-CAP-PE with PM D. 50 mole percent TNP-CAP-DE with PM

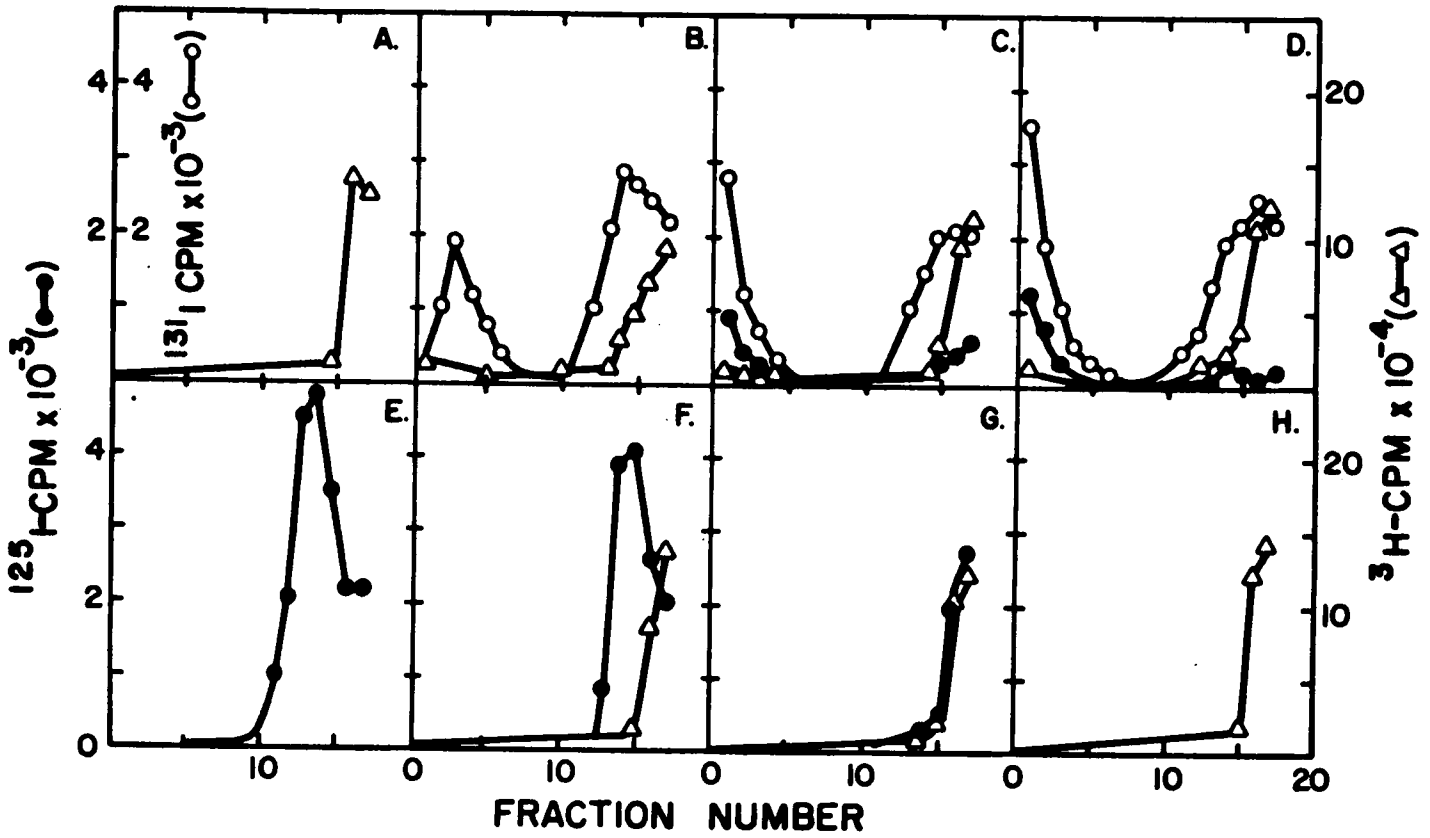

Figure 9. Sucrose density gradient centrifugation of liposomes containing PM, pWGA, and TNP-CAP-PE.

All fractions were collected from the bottom of the gradient and counted for ^{125}I, ^{131}I, and ^{3}H. All lipids were labeled with a trace amount of [^{3}H]-cholesteryl ether.

A. Liposomes alone B. L-TNP-PM C. LpWGA-PM D. L-TNP-PM-pWGA E. ^{125}I-WGA
F. ^{125}I-WGA plus liposomes G. L-TNP-pWGA H. TNP-liposomes alone

●—● ^{125}I o—o ^{131}I Δ—Δ ^{3}H

that some liposomes contain more plasma membrane proteins than others as seen by the different peaks of ^{131}I and ^{3}H cmp in Panel B. When pWGA and plasma membrane proteins were incorporated into the same liposomes (panels C and D) a significant amount of protein sedimented to the bottom of the gradient, indicating aggregation of the protein with little lipid association. This is probably because of the association of the glycosylated membrane components with the lectin. Thus liposomes can be made with WGA and with plasma membrane proteins when incorporated into separate liposomes, but liposomes containing both WGA and plasma membrane proteins are probably heterogeneous and ill-defined.

Figure 10 shows electron micrographs of three negatively stained preparations of liposomes. Photo C represents liposomes without protein. It can be seen that the liposomes were quite heterogeneous in size. Significant amounts of MLV were present. Photos B and A represent LpWGA and L-TNP-P , respectively. These liposomes showed a greater degree of homogeneity. Liposome sizes were calculated to be from 0.03 μm to 0.15 μm in diameter.

5. CTL stimulation by liposomes. It has been previously reported (47) that TNP-modified plasma membrane proteins indeed stimulate a secondary CTL response. It was my intention to stimulate such a response by using TNP-containing liposomes. Since a source of H-2 antigen is required to confer a proper H-2 restriction, I have used the crude plasma membrane proteins purified from spleen cells of C_3H/HeJ mice for this purpose. A similar preparation was used in liposomes containing viral glycoproteins by Dr. Paul Naylor in this laboratory. Successful stimulation of CTL by these liposomes was reported by him (42).

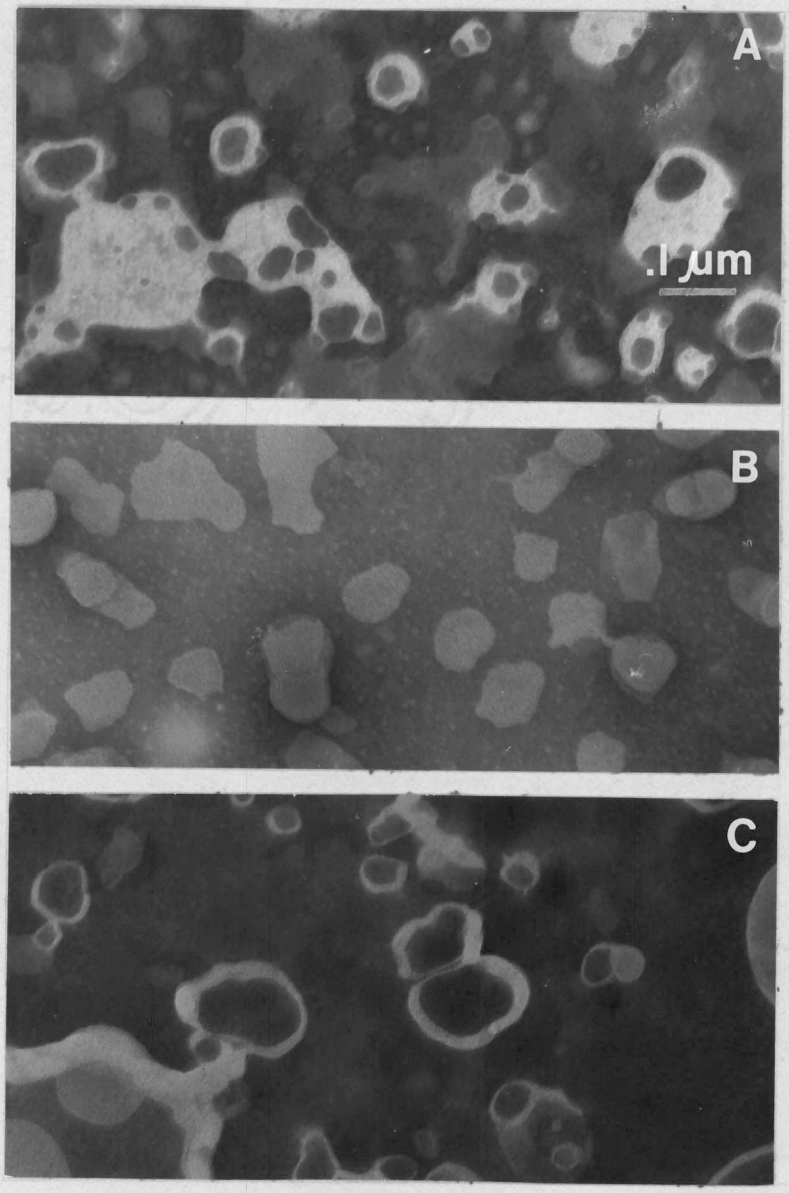

Figure 10. Electron micrographs of liposome preparations.

Bar is 0.1 microns.

A. L-TNP-PM B. LpWGA C. Liposome alone

Liposomes containing WGA and plasma membrane proteins were examined for their ability to stimulate a secondary CTL response. The secondary response was examined since the primary response was found to be less H-2 restricted (Table 2, page 34). Liposomes containing 10 ug WGA and liposomes containing 15 ug of plasma membrane proteins were tested for CTL induction activity. Table 4 summarizes the data. As can be seen there was no positive response to any liposome preparations while positive and H-2 restricted response was found for the TNP-modified spleen cells as a stimulator. It would this appear that the liposomes with the indicated doses of hapten, lectin or plasma membrane proteins failed to stimulate responder cells to become CTL. It was also observed that plasma membrane isolated from spleen cells and subsequently haptenated did not stimulate a positive response.

C. Discussion

This part of the project was an attempt to study a specific case of cell to cell interaction, i.e., the H-2 restriction of CTL induction, by the use of a defined model membrane system. In order to stimulate the production of CTL which is properly H-2 restricted, the antigen (or hapten) source must reside in the same membranes as the H-2 gene products (48, 49). We therefore constructed liposomes which contain both the TNP-hapten groups and plasma membrane proteins as a source for H-2 antigens. We have used TNP-CAP-PE as a hapten carrier because of its availability and well-defined chemical and physical properties. A similar haptenated lipid, DNP-CAP-PE has been used to stimulate antibody production in B-lymphocytes (50).

TABLE 4

CTL INDUCTION BY LIPOSOMES

Stimulators	Percent Specific Lysis — Targets: L-cell-TNP	L-cell	A31-TNP	A31
C_3H/JeJ TNP-modified spleen cells	15.5[a]	3.2	2.8	4.1
L^a-pWGA	-0.2	4.6	-0.78	2.2
L-PM[b]	-2.3	-6.0	-4.1	0.02
L-TNP	-5.3	-1.1	-0.34	3.3
Liposome	-2.1	-1.8	-4.1	0.44
L-TNP-PM	-2.0	0.12	-1.6	-5.7
L-TNP-pWGA	-2.9	-3.2	-2.4	3.1
L-PM/TNBS[c]	-3.6	-1.9	-2.8	2.2
PM/TNBS[d]	-1.1	-0.79	-3.3	3.2
None	0.65	7.0	2.4	4.0

[a]Percent specific lysis calculated at 40:1, E:T. Samples of 20:1 were also performed and corresponded qualitatively with 40:1 results.

[b]L = liposomes PM = plasma membrane isolated from C_3H/HeJ spleen calls.

[c]L-PM treated with TNBS after incorporation of PM into liposomes.

[d]PM treated with TNBS at a dose comparable to amount incorporated into liposomes. An increased amount of PM/TNBS was also examined and results were similar to those presented.

In the design of the experiments, TNP haptens and the H-2 antigens are presented in liposomes as separate molecular entities. We have reasons to believe that these liposomes would be good stimulators for CTL production, provided that the dual recognition hypothesis for H-2 restriction is correct. First of all, the dose of hapten offered to the responder cells as liposomes should be in great excess compared to that offered as TNP-modified spleen cells. In typical experiments, the responder cells were exposed to 6.82×10^{14} total TNP groups, since there were 3.41×10^{8} TNP groups per stimulator cell and 2×10^{6} of such cells were used. For liposome containing 50 mole percent TNP-CAP-PE there were 1.81×10^{17} TNP groups for each dose of liposomes added to the responder cells. Assuming that 15 percent of surface exposed lipids in multilamellar liposomes used in our experiment (51), the responder cells were exposed to 2.7×10^{16} total TNP groups presented as liposomes which was about 40-fold more than those presented as hapten-modified cells. Second, the surface density of TNP on the liposomes was approximately the same as that of the cells. Each phospholipid occupies a surface area of about 70 $Å^2$ in the lipid bilayer (5). For liposomes containing 50 mole percent TNP-CAP-PE, the surface density of TNP is calculated to be about 0.7×10^{6} TNP groups/um^2. The spleen cells contained 3.41×10^{8} TNP groups/cell (Table 3, page 36). Assuming an even distribution of these groups on the cell surfaces and the diameter of an average lymphocyte being 9.4 um, a surface density of 1.23×10^{6} TNP groups/um^2 is calculated. Thus, the haptens are presented at a comparable density in liposomes as in cells. Next, the amount of plasma membrane proteins in the liposomes was the same as those liposomes used

to stimulate a positive CTL response specific to the Herpes simplex viral antigens previously done in our laboratory (42). Therefore, it was expected that the liposomes used in the present experiments should be effective in the stimulation of CTL which is hapten specific and H-2 restricted.

The results described in Table 4 clearly indicate that liposomes containing TNP-CAP-PE and plasma membrane proteins were unable to stimulate any CTL response. Although a negative result is always insufficient to disprove any theory, our data are certainly inconsistent with the "dual recognition" hypothesis originally proposed by Zinkernagel (23, 24). Our data also offer little insight into the question of whether the TNP groups should be in some way associated with the H-2 antigens for a positive stimulation as depicted by the "altered-self" hypothesis, although the following attempt was made. Liposomes containing plasma membrane proteins (but not TNP-CAP-PE) were treated with TNBS under identical conditions as those for the haptenization of cells. These liposomes were then added to the responder cells for the stimulation of CTL. Again, no positive CTL was obtained (Table 4). Although the amount of TNP groups in these liposomes was not determined, we have noticed that the intensity of yellow color in these liposomes was much weaker than liposomes containing 50 mole percent of TNP-CAP-PE. Therefore, the dose and perhaps the density of the hapten in these liposomes were suboptimal.

Although data presented in the final experiments, an attempt to use liposomes containing various constituents to stimulate a secondary induction of CTL, show inconsistencies with the reported models for

interaction between the H-2 antigen, foreign antigen, and responder cell receptors, the author feels that by utilizing a system known to be stimulated by liposomes containing the foreign antigen and the H-2 gene products as in the case of many viral systems, the H-2 restriction mechanism would still provide a viable model to study cell to cell interactions. It would be possible to then investigate the ability for the non-specific binder to replace the H-2 antigen-receptor interaction.

Due to the failure in establishing a hapten containing liposome system that could stimulate a CTL response, the model system did not provide a solid foundation to study the H-2 restriction mechanism.

CHAPTER IV

SPECULATIONS

By engineering a molecular tool as in the case of LpWGA, there are other systems and uses that could be studied. For example, Burger has reported that WGA binds preferentially to polyoma virus-transformed 3T3 mouse cells over normal 3T3 cells (52). It may be possible to prepare LpWGA and trap anti-viral drugs in the liposome, resulting in a targeted drug carrier specific for virally infected cells. It is probable that other tumor cells have similar lectin preferences that could be examined using this same principle.

Another system that can be examined utilizes another lectin, Con A. Con A is a tetramer that binds specifically to D-mannose found on the surface of many cells. In its native state Con A is a known lymphocyte mitrogen which depends on the multiple binding sites of the lectin (53), thus the dimer has much less mitogenic activity than the tetramer, and the monomer is even less mitogenic if at all. By derivatizing the monomeric form of Con A with a fatty acid and subsequently incorporating the derivatized protein into liposomes the liposome-bound ligands are potent multivalent binders, thus, it is quite possible that the monomeric Con A incorporated into liposomes may be mitogenic. As a result it would be possible to study the possible physical chemical changes of the T-cell membrane upon binding of the membrane-bound ligand.

By using our knowledge of science and by utilizing one's imagination, this covalent modification of a molecule and subsequent incorporation into a lipid vesicle has unlimited uses as a molecular tool.

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