Liposome encapsulated tumor-associated antigens elicited humoral and cellular immune responses in mice bearing tumor

Anis Alam#, Longkumer Imliwati & Chowphi Raphap

Immunology Laboratory, Department of Biochemistry, North-Eastern Hill University, Shillong 793 022, India and

Vinod Singh

Hormone Biochemistry Laboratory, Institute of Self-Organizing Systems and Biophysics, North-Eastern Hill University, Shillong 793 022, India

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Chemically induced tumors in mice provide a system to investigate tumor-associated antigens (TAA). The cell surface glycoprotein antigens on such tumor cells have been identified as suitable targets for immune attack. The induction of immune responses against (TAA) in N-nitrosodiethylamine (DEN) exposed mice has been examined. In order to present antigens to the immune system, the liposome was used as vehicle to deliver the TAA. Liposomal-TAA formulation, elicited both humoral and the cellular immune responses, when administered intramuscularly in DEN-exposed mice. Presence of circulating antibodies against TAA and the induction of cellular responses in immunized mice were monitored using ELISA and in vitro cell proliferation assay of lymphocytes respectively. Specificity of antibody against TAA in immune sera was analysed using immunoblotting technique. Based on these results, it is proposed that the liposome encapsulated TAA may successfully be used to induce humoral and cellular immune responses against tumor.

Tumor-associated antigen (TAA) can serve an effective target for active immunotherapy against tumor. In this context, the preparations containing extracted TAA of tumor cells (TAA extract) and purified TAA have been explored for active immunization. Encouraging results have been reported in cancer patients for specific active immunotherapy with crude as well as highly purified TAA preparations1-3. In tumor immunotherapy, the cytotoxic T lymphocytes (CTL) are a critical component of the cellular immune response to tumor and sufficient to protect against tumors and also eliminate even established cancer in human and murine tumor models4. Induction of a strong antigen-specific CTL response is the major goal of tumor immunologist. Progress towards this goal has been aided by the identification of TAA on tumor cells. They are present at high levels on tumor cells and in trace amounts on normal cells. The self-like nature and low immunogenicity of these antigens have made it clear that other measures to enhance the effectiveness of the T cells reactivity to these antigens are essential for effective clinical immunotherapy. To overcome these problems, recently cell-based tumor vaccines have been developed on the hypothesis that genetically modified tumor cells will be effective antigen-presenting cells (APC) of TAA and that immunization with the modified cells will stimulate a potent anti-tumor immune responses in tumor bearing individual5-7. Some promising results have been shown in animal systems and several are being tested in clinical trials8-11. The present study uses tumor bearing animal model for TAA vaccination in an attempt to devise an effective vaccine formulation using liposomes that would elicit humoral and cellular immune responses against tumor.

Materials and Methods

Cell proliferation kit I (MTT) and 5-bromo-2'-deoxy-uridine (BrdU) labeling and detection kit III were obtained from Boehringer Mannheim, Germany. N-diethylaminoethylamine (DEN), protein A-oxidase conjugate, O-phenylenediamine dihydrochloride (OPD) substrate, fetal calf serum, dipalmityl phosphatidyl choline (DPPC), dimyristoyl phosphatidyl choline (DMPC), cholesterol (CHOL) and dicetyl phosphate (DCP) were purchased from Sigma Chemical Co., USA. All other reagents and chemicals used were of analytical grade.

*Correspondent author: Tel: +91-0364-250-107
Fax: +91-0364-250-076
Animals—Swiss albino mice, aged 6-8 weeks from inbred colony maintained in our animal house under controlled temperature (20°C ± 2°C), lighting (12 hr light/12 hr dark) conditions, standard mouse diet (NMC Oil Mills Ltd., Pune, India) and water ad libitum, were used in all experiments.

Tumour induction—Carcinogenesis was induced in mice by administering DEN (20 mg/kg body weight) through iv route at weekly intervals. The treatment was continued up to two months. Introduction of carcinogenesis in hepatocytes was followed by marker enzyme assays and histological studies of DEN-exposed livers.

Enzyme assays—Assays of γ-glutamyltransferase (GGT) and acetylcholine esterase (AcChE) are based on the methods described by Meister et al.,12 and Ott et al.,13 respectively. Assays were carried out in the supernatant fractions obtained from DEN-exposed and normal liver tissue homogenates.

Histological study—Microtomy technique was used for histological study of liver tissues in DEN-exposed and control mice. Briefly, the tissues were fixed in picric acid and then thoroughly washed under tap water. dehydration was carried out in increasing alcoholic grade from 30 to 100%. The tissues were further cleared using xylene and embedded in paraffin wax. Blocks prepared were trimmed and cut in ribbons. Ribbons were mounted on slides, dried and paraffin wax was removed by xylene. Rehydration was done with down grade alcohol from 100 to 30%. Haematoxyline and a counter stain, eosin stained the slides. Again, dehydration was done and after clearing by xylene, the slides were examined microscopically.

TAA extraction—Selective extraction by 1-butanol of membrane surface glycoprotein antigens from liver tissues of DEN exposed and age-matched normal mice was based on the method of Liao et al.14 Briefly, the normal saline washed tissues were minced over fine wire screen and washed in 0.02% EDTA followed by gentle dispersion in 0.25% trypsin EDTA solution. It was then washed twice in PBS (10 mM, pH 7.4) and incubated for 7 min. in 3% 1-butanol diluted in PBS at 22°C with occasional agitation. Finally, the mixture was centrifuged at 100,000 g for 1 hr at 4°C. The supernatant obtained was concentrated and used as TAA extract to resolve membrane glycoproteins using SDS-PAGE and for the induction of immune responses in DEN-exposed mice.

Gel electrophoresis—The membrane glycoprotein extracts were subjected to SDS-PAGE on 10% polyacrylamide with 0.05% SDS in tris-glycine buffer (pH 8.8). The gel was stained for resolution with Coomassie brilliant blue R-250.

TAA encapsulation into liposomes—Liposomes were prepared of two different lipid compositions using dry film method originally described by Bangham et al.15 Briefly, DPPC: CHOL: DCP and DMPC: CHOL: DCP were taken in a molar ratio of 1.0: 0.9: 0.25 and dissolved in a mixture of chloroform and methanol (ratio 2:1 v/v). The solvent was evaporated under nitrogen stream to make a lipid film. The film was dried under vacuum for 1 hr. The lipid film was then suspended in 1.0 ml of aqueous TAA extract (1 mg/ml glycoprotein in 10 mM tris-buffered saline pH 7.4) and kept for 2-3 min at 40°C in a water bath. The mixture was thoroughly vortexed for 10-15 min. till all the lipid film turned into lipid vesicles. It was then centrifuged for 30 min at 14000 rpm in a refrigerated centrifuge (Sorval RC 5C) at 4°C. The supernatant was carefully aspirated and the pellet was washed two times with buffer. After centrifugation the supernatants were pooled and the protein content was estimated by Lowry method16. The protein entrapped into liposomes was calculated by subtracting free from the total.

Immunization protocols—DEN-exposed mice described above were divided into three groups each of 8-10 animals. Group 1 and group 2 animals received three intramuscular injections of 10 μg equivalent of TAA extract or the TAA encapsulated into liposomes at fortnightly intervals. Whereas, the group 3 animals received a single dose of 10 μg equivalent of TAA emulsified with CFA (ratio 1:1, v/v) by the same route. One month after the last immunization the group 1 and 2 animals received a booster injection with TAA and the liposome encapsulated TAA respectively. However, group 3 animals received booster by TAA emulsification with incomplete adjuvant in equal dose after a month. Blood was collected on day 3 and day 15 after booster injection by retro orbital bleeding. The serum was stored at -20°C before antibody titration.

ELISA for anti-TAA antibodies—The presence of circulating antibodies against TAA in the test sera was determined by ELISA. Aliquots of 10 μg/ml of the antigen was used for coating the wells (1 μg/well) of the ELISA plates, flat bottom polystyrene (Corning, NY), and blocking of the wells was done with 2% BSA. To assess antibody binding, the test sera were diluted with PBS containing 2% BSA and
Western blot analysis—Aliquots of the test antigens containing 20 μg protein were electrophoresed on 7.5% SDS-PAGE and transferred onto nitrocellulose papers (Bio-Rad). The blots following washing with PBST were blocked with 2% BSA in PBS and incubated overnight with test serum diluted (1:10) in PBST. The washed strips were then incubated with Protein A-peroxidase conjugate followed by washing with PBST. The reactive bands were visualized using DAB as substrate.

Lymphocytes cell proliferation assay in vitro:
(i). Lymphocyte culture—Mice were killed by cervical dislocation on day 3 after giving the booster injection. Lymphocytes obtained from the harvested spleen of immunized and normal mice (age-matched) were suspended in DMEM (GIBCO) containing 10% heat inactivated FCS (fetal calf serum), 2 mM L-glutamine, 0.55 mM L-arginine, 0.24 mM L-asparagine-monohydrate supplemented with penicillin/streptomycin. Cells were counted on a Burker chamber under a Zena phase contrast microscope. Culture was performed in a humidified atmosphere (37°C and 6.5% CO₂).

(ii). BrdU incorporation assay—BrdU was added to the cells suspension or to the culture medium. It incorporates into DNA of the cells. Following fixation of cells, cellular DNA was partially digested by nuclease treatment. A peroxidase labeled antibody to BrdU was added that binds to BrdU. The peroxidase substrate was added and peroxidase enzyme activity was measured. The OD was determined at 440 nm on a Multiscan MS India ELISA reader.

(iii). MTT assay—MTT assay was performed to determine the metabolic activity of immune lymphocytes by the method described elsewhere.²⁷ Briefly, the suspended immune and normal lymphocytes were seeded (1×10⁷/100μl/wells) in 96 well flat bottom polystyrene microtiter plates. An aliquot of 10 μl of yellow MTT solution (final concentration 0.5 mg/ml) was added and the plate was again incubated for approximately 4 hr at 37°C. After the incubation, purple formazan salt crystals were formed. These salt crystals were insoluble in aqueous solution but were solubilized by adding 100 μl of solubilization solution (10% SDS in 0.01 M HCl) followed by overnight incubation in a humidified atmosphere (37°C, 6.5% CO₂). The solubilized formazan product was quantified spectrophotometrically at 550 nm using an ELISA reader.

Results
Carcinogenesis—Induction of carcinogenesis in DEN-exposed mice was followed by marker enzyme assays and histological examination of liver cells. The activity of GGT in liver of DEN-treated and normal mice was separately monitored in the supernatant fractions (Fig.1). The results indicate that GGT activity was markedly increased in mice exposed to DEN in comparison to age-matched normal untreated mice. AChE, an another membrane bound marker enzyme was assayed in the supernatants. More than two folds increase in the activity of AChE was observed in DEN-exposed mice in comparison to normal control mice (Fig. 2).

Histological examination of DEN-exposed liver cells exhibited marked distortion of cells and nucleus shapes and at various stages of cell necrosis (Fig. 3B), while the normal liver had well defined, symmetrical, mono and bi-nucleated cells (Fig. 3A).

The SDS-PAGE gel chromatogram showed
significantly high expression of a glycoprotein in hepatocytes membrane of DEN-exposed mice (Fig. 4B). This glycoprotein was also present in TAA-extract of normal mice but only in trace amount (Fig. 4A).

Liposomal-TAA formulation—A total of 8.0-8.5 mg of lipids of two different compositions was taken to entrap 1.0 mg of TAA. The entrapment efficiency of TAA into liposomes was observed to be 25-30% only (Table 1).

Induction of antibody responses against TAA in DEN-exposed mice—Antibody against TAA in immune serum was determined using ELISA. Mice immunized with TAA as such served as positive control. The results shown (Fig. 5), indicate that the liposomal-TAA formulation elicited significantly high antibody response in comparison to TAA alone. The response elicited by liposomal-TAA was also found equivalent to that of response elicited by CFA-TAA formulation (Fig. 5).

Lymphocyte proliferation in response to active immunization with liposomal-TAA formulation—Incorporation of 5-bromo-2'-deoxy-uridine (BrdU) in place of thymidine was monitored as a parameter for DNA synthesis and cellular proliferation. First, the log-diluted lymphocytes harvested from spleen of normal and immunized animals were assayed for BrdU incorporation. This experiment was led to determine the change in OD caused by increase in the numbers of cells. Approximately 0.2 to 0.4 OD difference was observed when the number of cells increased ten times both in control as well as DEN-treated (Fig. 6). Later, equal numbers of lymphocytes of both the normal and immunized animals were cultured for 0, 6, 12 and 24 hr respectively and then assayed for BrdU incorporation. Results indicate that immune lymphocyte numbers increased significantly during culture at 6 and 12 hr time points that is evident from the increase in absorbance (Fig. 7). However, a decrease in cell number observed when the lymphocytes were cultured up to 24 hr. This may be due to the death of cells when kept in culture for more than 12 hr. Lymphocytes obtained from normal mice did not show any increase in their numbers and thus no increase in absorbance (Fig. 7).

Metabolic activity of lymphocytes—The microtiter assay that uses the tetrazolium salt MTT was performed to measure the metabolic activities in immune and resting (control) lymphocytes as described in material and method section. In this assay the metabolically active viable cells cleave MTT and produce crystals of water-insoluble purple formazan salt, which were quantified spectrophotometrically after solubilization. Absorbance was measured at 550 nm (maximum absorbance).

Discussion
Changes of activity (inhibition or activation) of marker enzymes, viz. GGT and AChE are common findings in hepatic tissues during cellular transformation. In liver GGT has been recognized as a positive marker for hepatocytes which have undergone malignant transformation. This enzyme is

<table>
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<th>Liposomal phospholipids (μg)</th>
<th>Molar ratio</th>
<th>TAA (μg)</th>
<th>Unentrapped mean±SD (μg)</th>
<th>% Entrapment mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC: CHOL: DCP (5.0: 2.5: 1.0)</td>
<td>1.0: 0.9: 0.25</td>
<td>1000</td>
<td>692.86 ± 22.01</td>
<td>30.71 ± 2.20</td>
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<td>DMPC: CHOL: DCP (4.5: 2.5: 1.0)</td>
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<td>1000</td>
<td>745.71 ± 30.28</td>
<td>25.43 ± 3.03</td>
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diagnostically important because its activity is altered in chemically induced hepatomas. Thus, the hyperactivation of GGT in DEN-exposed mice signifies hepatocellular transformation (Fig. 1). On the other hand increase in the activity of AchE in DEN-exposed mice (Fig. 2) also support the development of a cancerous situation in hepatocytes as the cancer induction is accompanied by membrane changes and AchE is a membrane bound enzyme. Cancer induction in DEN-exposed mice was further confirmed by the histological studies of liver tissues. The DEN-exposed livers had distorted and ill-defined cells without clear nucleus and at various stages of cell necrosis (Fig. 3B), while the normal liver had well defined, symmetrical, mono- and bi-nucleated cells (Fig.3A).

Over expression of certain membrane protein/glycoprotein have been found very often in a
variety of cancers. We also observed here significant difference in expression of a cell surface glycoprotein in liver cells of DEN-exposed mice (Fig. 4), which had undergone malignant transformation. Such cell surface glycoproteins have been identified as suitable targets for immune attack by both active and passive immunotherapies. Different approaches have been adopted to induce immune responses against these antigens. These include vaccination with whole or lysed tumour cells TAA extract as such or purified tumour antigens.

Our study aimed to determine whether TAA of DEN-treated mice elicits immune responses in mice (exposed to DEN) or not. Liposomes are used as a carrier for the presentation of TAA to the immune systems and the formulation is self-contained without any further addition of other adjuvant materials or carrier molecules. The method selected to encapsulate TAA into liposomes showed only 25-30% efficiency, which is insignificant (Table 1). Poor entrapment efficiency by this method has also been reported earlier. Although, we have developed method where very high entrapment efficiency of protein was achieved, but this method could not be used as it was found unsuitable for entrapment of TAA. This is because it requires exposure of TAA to organic solvent, which may lead alteration of the antigens and eventually its immunogenicity. The method is simple, highly reproducible and more importantly that the immunogenicity of entrapped TAA remained unaltered (Fig. 5).

Liposome was chosen as carrier because of their ability to be rapidly internalized and degraded by the macrophages which are involved in antigen processing and presentation to T-lymphocytes. As shown in Fig. 5 the liposomal-TAA formulation elicited significantly high antibody response over TAA alone, which seems likely due to macrophage-mediated presentation of liposomal-TAA to the immune system. The importance of macrophage-mediated presentation of liposomal-TAA to the immune system has been investigated and a correlation found between the response (IL-2 production) of antigen-specific T cells and the uptake and processing of the liposomal antigen by macrophages in vitro.

Our findings are in agreement with these observations. Further, we also studied the efficacy of liposomal-TAA formulation over TAA emulsified with CFA and observed that the anti-TAA response elicited by liposomal-TAA formulation is in equivalence to the CFA-TAA formulation (Fig. 5). Specificity of antibodies to the high molecular weight glycoprotein of TAA-extract that expressed at high level in the DEN-exposed mice was identified by immunoblotting. Glycoproteins resolved on SDS-PAGE were transferred on to nitrocellulose strip and incubated with the immune sera. When it was allow to
react with protein A conjugate followed by substrate (DAB) reaction, cross reactivity with that specific glycoproteins was observed along with other major glycoproteins transferred on nitrocellulose (Figure not shown). These findings support that a good immune induction could be achieved with liposome entrapped antigens and therefore, this particular approach could be useful to elicit immune response to self-existing bio-molecules.

The immune response evoked by liposomal-TAA is likely to be the result of targeting to antigen presenting cells (APC). When the antigen presentation is in the appropriate MHC context it would result in T cell activation. It was therefore, interesting to study the induction of cellular responses in mice immunized with liposomal-TAA formulation. Cellular induction of immune responses is usually measured by delayed-type hypersensitivity reaction or lymphocyte proliferation. We monitored it by quantitating BrdU incorporation into the DNA of replicating cells. Since, the assay is based on a microtiter plate format and the color development is measured spectrophotometrically, it was desirable to know the change in ratio of absorbance on BrdU incorporation in the diluted lymphocytes. A difference of 0.2 to 0.4 in OD was found on log dilution (Fig. 6). Based on these observations, same numbers of the normal and immune lymphocytes were cultured for 0, 6, 12 and 24 hr and then assayed for BrdU incorporation to determine proliferation of cells on culture. The increase in absorbance observed for immune lymphocytes when they were cultured for 6 and 12 hr clearly indicates that the cells had undergone proliferation (Fig. 7). However, the normal lymphocytes (resting) did not show any significant change in absorbance when cultured for the same time periods (Fig. 7). Cell death was observed when cultured for more than 12 hr, both in normal and immune lymphocytes. Therefore, the decrease in absorbance at 24 hr time point may be due to cells death, since the BrdU incorporation occurs only in viable cells.

Change in absorbance due to BrdU incorporation in immune lymphocytes cultured for 12 hr (Fig. 7) signifies that the numbers of the immune lymphocytes increased by approximately ten folds. It is likely therefore that the immune lymphocytes may have undergone 3-4 cell division cycles in the culture (Figs 6 & 7). This is in agreement with the fact that the lymphocytes on antigenic stimulation undergo 3 to 4 cell division cycles every 24 hr up to 4 or 5 days in vivo. Since, the proliferating cells are metabolically more active than non-proliferating (resting) cells, the metabolic activity of both the cells were studied using cell proliferation kit I (MTT). MTT is cleaved to formazan by the "succinate-tetrazolium reductase" system that belongs to the mitochondrial respiratory chain and is active only in viable cells. The purple formazan salt crystals were dissolved and the solubilized formazan product was quantified spectrophotometrically. Immune lymphocytes were found metabolically more active than the resting ones (Fig. 8). This further supported that the lymphocytes of DEN-exposed mice were sensitized by the liposomal antigens in vivo and the cells became metabolically more active during cell proliferation.

Our observations, therefore, suggest that vaccine based on liposomal-antigen formulation in particular with non-fractionated cell extracts as a source of tumor antigen may be equally effective to induce immune response to that of other cell based modified tumor vaccines reported recently. Vaccination with tumor cell extracts circumvents the need for identifying specific tumor antigens and hence extends the use of active immunotherapy to the vast majority of cancers, in which specific tumor antigens have not been identified. Effects of immune responses against TAA on tumour regression are under investigation and the preliminary results are encouraging that need further confirmation.
Acknowledgement

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References