

## Anticancer Effect of Liposome Incorporated with Methotrexate and Antibody against Tumor Specific Surface Antigen of Rat Hepatoma

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*Antibody against tumor specific surface membrane protein was produced by immunizing a New Zealand White rabbit with antigen (66 kDa) prepared from the plasma membrane of rat hepatoma induced by feeding a diet containing 3'-methyl-4-dimethylaminoazobenzene, and was purified by protein A-Sepharose 6MB affinity chromatography. The purified antibody was incorporated into liposomes by a reverse phase evaporation vesicle method in order to prepare a tumor specific anticancer drug carrier. The effect of the antibody against tumor specific antigen was evaluated by comparing the inhibition of DNA synthesis in hepatoma cells with different preparations of methotrexate. Methotrexate encapsulated into liposome showed a stronger inhibitory effect on DNA synthesis (1.4-1.7 times) than free methotrexate. Liposomes having the antibody showed stronger inhibitory effect (3.1 times) on DNA synthesis than free methotrexate group in hepatic nodular area. From these results, it is concluded that tumor specific antibody inserted into liposomal membrane would be recognized by surface antigens which were expressed on the plasma surface membrane of rat hepatoma cells and thereby increase the carrying efficiency of drugs to the target cells. This could be useful in cancer chemotherapy.*

**Key Words:** Liposome drug delivery system, methotrexate, tumor specific surface antigen, hepatoma

Although chemotherapy, radiotherapy, immunotherapy or surgery are generally employed in the treatment of human cancer, no specific method for the killing of tumor cells is available yet. In treatment of the advanced tumor, chemotherapy and radiotherapy are commonly used, but both methods have cytotoxic problems which are serious to normal cells. Many attempts have been made to develop anticancer therapy which is specific for tumor cells, but has a less deteriorative effect on normal cells (Rubens 1974; Ghose *et al.* 1976; Gregoriadis 1977).

It has been reported that administration of a complex of amethopterin and serum globulin obtained from hamsters into a leukemic hamster produced a greater antitumor effect than that of amethopterin alone (Mathe *et al.* 1969). The beneficial effects of a

complex of anticancer drugs and serum protein against tumor cells have been recognized by many investigators (Magenat *et al.* 1969; Szekerke *et al.* 1972). Liposome (Weissman *et al.* 1975; Fendler and Romero 1977), albumin (Regers and Kornfeld 1971) and lectin (Nicolson 1974; Rapin and Burger 1974) have been used as carriers for anticancer drugs.

These carriers are not specific for cancer cells but they could mediate slow destruction, slow release and easy transport of drugs into cells (Ghose *et al.* 1962; Silverstein *et al.* 1977). It has been reported that new proteins are expressed on the surface of the tumor cells which are not expressed on the normal cells (Old and Boyse 1964; Klein 1968; Law 1969; Wu *et al.* 1969; Baldwin *et al.* 1971; Reutter and Bauer 1978; Vischer and Reutter 1978; Lim *et al.* 1982). Attempts to increase the efficacy of anticancer drugs have been made by conjugation of the tumor specific antigen and anticancer drugs such as chlorambucil (Israels and Linford 1962; Davies and O'Neill 1973; Flechner 1973; Rubens and Dulbecco 1974), bleomycin (Murakami *et al.* 1976), daunomycin and adriamycin (Hurwitz *et al.* 1975), methotrexate (Uadia *et al.* 1985), diphtheria toxin A chain (Moolten and Cooperbrand 1970), and A chain of ricin (Cilliland *et al.* 1978; Uatila *et al.* 1981;

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Jansen *et al.* 1982; Trowbridge and Domingo 1982; Vitetta *et al.* 1983a; Vitetta *et al.* 1983b).

Carbodiimide (Rebek and Feitler 1973, 1974) or dialdehyde (Avrameas 1969, 1976) is employed to conjugate the antibody and anticancer drugs. Also noncovalent modifications (O'Neill *et al.* 1975; Guglu *et al.* 1976) have been widely used. But these methods have the disadvantages of inactivation or denaturation of the drug after transport into the cells. In the present study, the antitumorigenic effect of encapsulated methotrexate in liposome bearing antibody against rat hepatoma specific plasma membrane surface antigen induced by 3'-methyl-4-dimethylaminoazobenzene (3'-Me DAB) was tested in order to evaluate the encapsulated methotrexate in liposome bearing the antibody.

## MATERIALS AND METHODS

### Animals and diet

One hundred and twenty Albino male rats (~80g B.W.) were divided into control group and tumor group. Both groups received a basal diet (Lim *et al.* 1982) for two weeks. The tumor group's diet was supplemented with 0.06% 3'-Me DAB (Eastman Organic Chemicals) for 12 weeks and then they were fed the basal diet for another 4 weeks. The control group received the basal diet for the entire experimental period (Table 1).

### Purification of tumor specific antigen from the plasma membrane of rat hepatoma

Extraction of surface proteins from the plasma

Table 1. The compositions of diets (per kg)

Constituent	Diet group	
	Control	3'-Me DAB
Casein, gm	180	180
Corn oil, gm	50	50
Glucose monohydrate, gm	770	770
Salt mixture, gm	40	40
Riboflavin, gm	0.001	0.001
Vitamin mixture*, ml	5	5
3'-Me DAB, gm	—	0.6

\* Contains vitamin A 20,000 unit, vitamin D 2,000 unit, choline chloride 1.5gm, and pteroyl glutamic acid 0.6mg, biotin 1.5mg, thiamine-HCl 20mg, pyridoxine-HCl 20 mg, menadione 50 mg, potassium parabenzoic acid 50mg, nicotinamide 50mg, calcium pantothenate 60mg, inositol 100 mg and cyanocobalamine 40µg.

membrane of normal rat liver and tumor bearing liver was carried out using 3M KCl according to the procedure of Stevens *et al.* (1981). Minced liver was washed three times by centrifugation at 5,000×g for 30 min with phosphate buffered saline (PBS), pH 7.4, and the extraction of surface proteins was performed with 3M KCl in PBS, pH 7.4 (2ml per g tissue) for 18 hours at 4°C with constant stirring. The mixture was centrifuged at 20,000×g for 30 min followed by centrifugation at 110,000×g for 90 min.

The supernatant was dialyzed against PBS, pH7.4 followed by 1mM EDTA, pH7.4. The dialysate was centrifuged at 110,000×g for 90 min and clear supernatant containing tumor specific antigen associated with rat hepatoma induced by 3'-Me DAB was separated by gel permeation column chromatography using Sephadex G-200 (Pharmacia Fine Chemicals) and ion exchange chromatography on a column of DEAE Sephadex A-50 (Pharmacia Fine Chemicals) as described in the previous report (Kim *et al.* 1988).

### Preparation of antibody against tumor specific cell surface protein

New Zealand White male rabbits (~2kg B.W.) were immunized with a plasma membrane surface protein (MW 66kDa) isolated from hepatoma induced by 3'-Me DAB (500µg protein/2 ml of complete Freund adjuvant) followed by a boost injection of 300µg protein in 1 ml of incomplete Freund adjuvant two weeks after the first injection. Antiserum was obtained two weeks after the boost injection.

To prepare absorbed antiserum, 50mg of surface protein of normal liver plasma membrane was dissolved in one ml of antiserum and the mixture was incubated for 2 hours at 37°C with constant stirring followed by centrifugation at 800×g for 10 min. Ten milliliter of supernatant (absorbed antiserum) was applied on a protein A-Sepharose 6MB affinity chromatography column (1×10cm) equilibrated with 0.1M sodium phosphate buffer (pH 8.0). After washing the column several times with the same buffer, antibody was eluted with 0.2M sodium citrate buffer, pH3.0. Fractions which absorbed more than 0.1 unit of UV light (280nm) were collected and saved for use (Fig. 1).

### Preparation of methotrexate encapsulated liposome bearing antibody against tumor specific cell surface protein

Preparation of methotrexate encapsulated liposome was carried out according to the reverse phase evaporation vesicle (REV) method of Szoka and Papahadjopoulos (1978).

The capacity of methotrexate encapsulation by liposome was tested by comparing the total lipid content (2.5, 5.0, 7.5, 10  $\mu$ mol) in the liposome preparation.

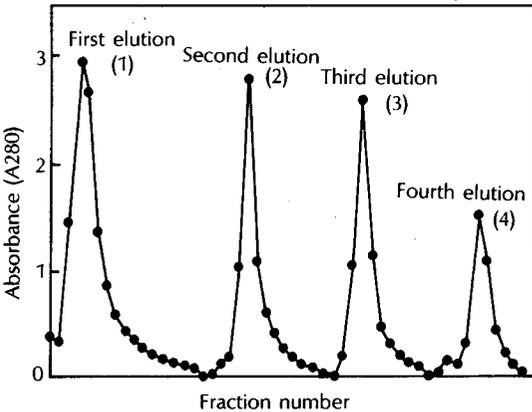
The efficiency of methotrexate encapsulation by liposome prepared by the reverse phase evaporation vesicle method with 3.8mg of egg lecithin and 1.9mg of cholesterol was much higher than that of the detergent dialysis method (Engelhard *et al.* 1978) or sonication method (Kasahara and Hinkle 1976). Therefore, the REV method was employed in this study (Table

2). Egg lecithin (3.8mg) and cholesterol (1.9mg) were dissolved in 2ml of chloroform/methanol (2:1, V/V) in a 15 ml glass tube equipped with a teflon cap. The contents were dried on the wall of the glass tube by a stream of  $N_2$  gas. The lipid layer was redissolved in 2ml of diethyl ether and 1.25mg of methotrexate, with or without the addition of 500 $\mu$ g of antibody against tumor antigen (dissolved in 0.25ml of PBS, pH 7.4). The mixtures were sonicated for 2 min 3 times by the Fischer sonic dismembrator at a setting of a relative output of 0.8 (Model 300) to obtain a lipid emulsion.

It was dried by  $N_2$  gas while vortexing until it became liquified. PBS buffer (pH 7.4) was added to this liposome preparation. The remaining ether was completely removed by vacuum pump. This liposome preparation was dialyzed for 24-36 hours against PBS, pH 7.4 at 4°C to remove the methotrexate that had not been encapsulated by liposome. Antibodies that had not incorporated into liposome in the dialyzed solution were removed by centrifugation using a swinging bucket rotor at 1,000xg for 30 min after mixing with an equal volume of 25% Ficoll solution (in PBS, pH 7.4) and overlaying with 2ml of 10% Ficoll solution and 1ml of PBS, pH 7.4. Liposome present at the interphase of the PBS and 10% Ficoll solutions was harvested by a serum separator and the content of encapsulated methotrexate was measured by the method of Chakrabarti and Bernstein (1969).

#### Measurement of DNA synthesis in rat liver and the effect of liposome incorporated with methotrexate and antibody against tumor specific plasma membrane surface protein

DNA synthesis in livers of control, regenerating and tumor bearing rats were estimated by measuring the incorporation of radioactivity into liver DNA 48 hours



**Fig. 1.** Affinity purification profile of antibody against hepatoma specific surface protein (MW 66,000) of plasma membrane of rat hepatoma induced by 3'-Me DAB. (1) First protein A-Sepharose 6MB affinity chromatography profile. (2) Second chromatography profile for unbound antibody in 15 ml of initial eluate. (3) Third chromatography profile for unbound antibody in 15ml of second eluate. (4) Fourth chromatography profile for unbound antibody in 15 ml of third eluate.

**Table 2.** The encapsulation efficiencies by methods of liposome preparations. 5 or 10  $\mu$ moles of total lipid and 1.25 mg methotrexate were contained in each preparation. The method of liposome preparations was described in the text.

Method of liposome preparation	Lipid concentration (mg) (egg lecithin/cholesterol)	% Encapsulation (metho trexate)
Detergent dialysis	1.9/1.0	0.013 $\pm$ 0.007*
	3.8/1.9	0.030 $\pm$ 0.010
Sonication	1.9/1.0	0.064 $\pm$ 0.012
	3.8/1.9	0.184 $\pm$ 0.090
REV**	1.9/1.0	3.2 $\pm$ 0.2
	3.8/1.9	8.0 $\pm$ 1.2

\* Mean  $\pm$  SD

\*\* REV: reverse phase evaporation vesicle

after injecting the  $^3\text{H}$ -thymidine (Giles and Meyers 1965).

For the regenerating liver group, rats were partially hepatectomized and used in the experiment after 4 days recovery. Partial hepatectomy was carried out by removing the mid and left lobes of livers of rats fed a basal diet for 12 weeks. Different preparations of methotrexate (MTX,  $50\mu\text{g}$ ) were injected into each group of rats after injecting  $10\mu\text{Ci}$  of [ $^3\text{H}$ ]-thymidine. After 24 hours, these MTX preparations were administered again into each group of rats.

For the DNA extraction, 0.5g of fresh liver from each group was homogenized in 5ml of saline with a glass homogenizer, and the homogenate was centrifuged at  $600\times g$  for 15 min. The nuclei-containing precipitate was suspended in 2ml of distilled water, then 3ml of 10% trichloroacetic acid was added, and the mixture was centrifuged at  $1,000\times g$  for 15 min.

To the precipitate, 3ml of 0.2% perchloric acid was added, and it was mixed and centrifuged at  $1,000\times g$  for 15 min. The precipitate was washed with 3ml of absolute ethanol followed by centrifugation as above. To each washed precipitate, 0.5ml of 1M perchloric acid was added and heated for 25 min in the boiling water bath, then centrifuged at  $1,000\times g$  for 15 min. The radioactivity in  $200\mu\text{l}$  of clear supernatant solution containing DNA hydrolysate was counted by a liquid scintillation counter. The DNA content in the final supernatant was measured by the method of Giles and Meyers (1965).

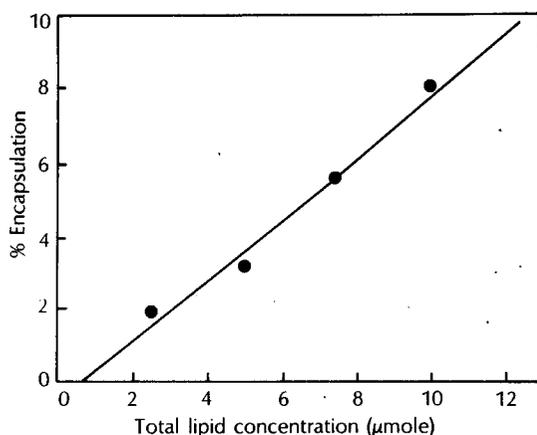
## RESULTS

### Purification of tumor specific antigen on the plasma membrane of hepatoma induced by 3'-Me DAB in rats and production of antibody against tumor specific plasma membrane surface protein

Plasma-membrane surface proteins were isolated from the hepatoma of rats fed a diet containing 3'-Me DAB for 12 weeks and then a normal diet for another 4 weeks.

Plasma membrane surface proteins of 3'-Me DAB induced rat hepatoma were eluted on a column of Sephadex G-200. The fractions corresponding to tumor specific antigen were pooled, concentrated, loaded onto a column of DEAE Sephadex A-50, and eluted with a step gradient formed with 0 to 0.4M NaCl in 0.05M Tris-HCl buffer, pH 8.3.

The tumor specific antigen of 66 kDa was separated and used for the preparation of antibody (Kim 1985; Kim *et al.* 1988). When the antibody against tumor specific surface protein (MW 66kDa) antiserum



**Fig. 2.** The effect of lipid concentration on the % encapsulation of methotrexate in egg lecithin/cholesterol (molar ratio 1:1). REV was prepared with the indicated concentration of lipid, 1.5ml of diethyl ether, 0.5ml of phosphate-buffered saline and 1.25 mg of methotrexate.  $Y=0.8X-0.5$ ,  $r^2=0.98$

was analyzed by Ouchterlony double immunodiffusion, a major precipitation band appeared between the antiserum and cell surface protein from 3'-Me DAB induced hepatoma. However, no detectable precipitation band appeared between the antiserum and surface protein from the control rat liver (Kim 1985; Kim *et al.* 1988). Antibody against tumor specific surface protein was purified by protein A-Sepharose 6MB affinity chromatography (Fig. 1) and the antibody was used in the preparation of liposome by the REV method (Szoka and Papahadjopoulos 1978).

### Effect of lipid concentration on the encapsulation of methotrexate into liposome prepared by REV method

When the total lipid concentrations were adjusted to 2.5, 5.0, 7.5, and 10  $\mu\text{moles}$  (egg lecithin/cholesterol, molar ratio 1:1, containing 1.25mg methotrexate) in each glass tube, the amounts of methotrexate encapsulated into liposomes were 1.9, 3.2, 5.6, and 8.0%, respectively (Fig. 2). There was a linear relationship between lipid concentration and the amount of methotrexate encapsulated into liposome ( $y=0.8x-0.5$ ,  $r^2=0.98$ ).

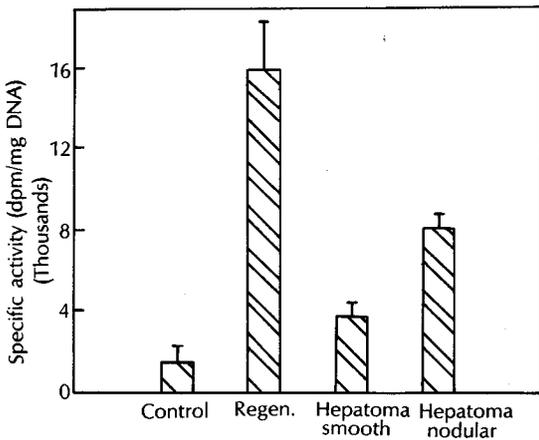
### Effect of methotrexate in different preparations on DNA synthesis in the control, regenerating, and 3'-Me DAB treated rat livers

Activities of DNA synthesis in rat livers of each

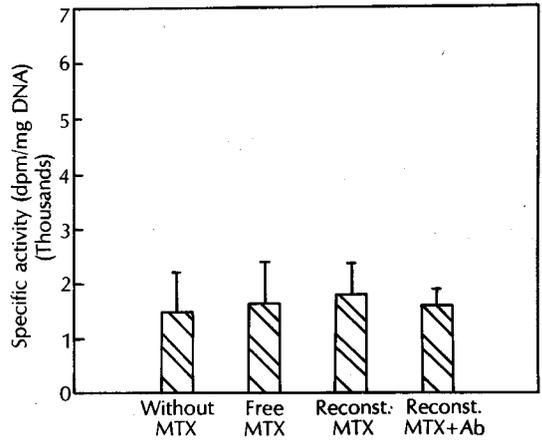
**Table 3. The effect of methotrexate treated with different methotrexate (MTX) preparations on the DNA synthesis**

Preparation of MTX Group liver	Specific activity (dpm/mg DNA)			
	Without methotrexate	Free methotrexate	Reconstituted methotrexate	Reconstituted MTX+antibody
Control	1,493±722*	1,627 ±769	1,799±522	1,575 ±235
Regenerating Hepatoma	15,865±2,340	11,879±1,232	8,884±944	9,579±3,011
smooth area	3,664±504	2,370±294	1,574±170	1,714±245
nodular area	8,029±504	6,464±610	5,419±678	3,130±475

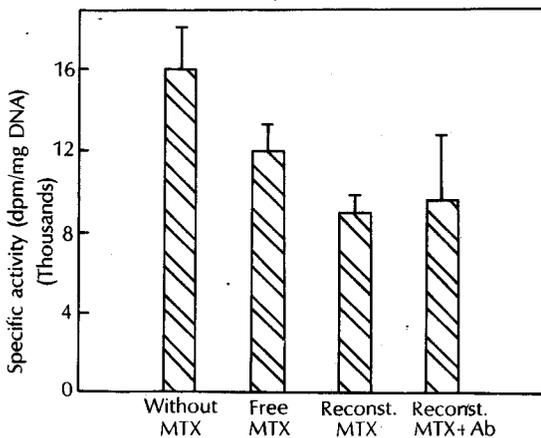
\*Mean±SD



**Fig. 3.** DNA synthesis in the liver of control, regenerating, hepatoma (smooth and nodular area) bearing rats.  $10\mu\text{Ci}$  of  $^3\text{H}$ -thymidine was injected through tail vein, respectively. After 2 days, rats were sacrificed and specific activities were measured as described in the text.



**Fig. 4.** The effect of methotrexate treated with different preparations on the DNA synthesis in the control group.  $10\mu\text{Ci}$  of  $^3\text{H}$ -thymidine and methotrexate were injected through tail vein. After 24 hours, each preparation containing  $50\mu\text{g}$  of methotrexate was injected again to corresponding group and specific activity measurements were described in the text



**Fig. 5.** The effect of methotrexate treated with different preparations on the DNA synthesis in the regenerating liver. Experimental methods are same as Fig. 4.

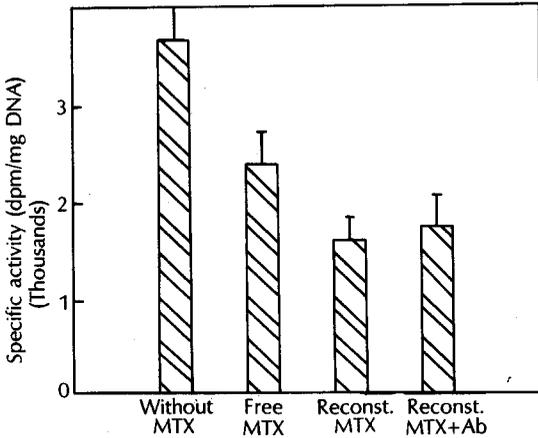


Fig. 6. The effect of methotrexate treated with different preparations on the DNA synthesis in the smooth area of hepatoma tissue. Experimental methods are same as Fig. 4.

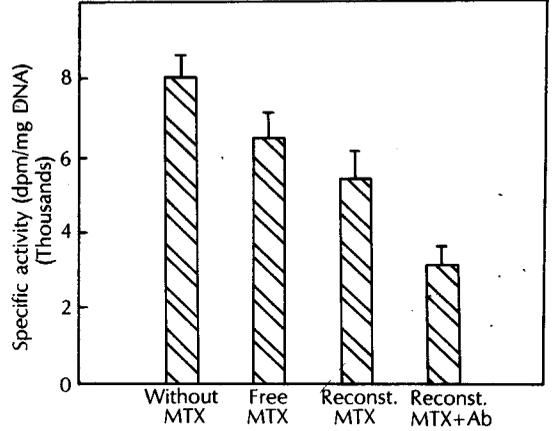


Fig. 7. The effect of methotrexate treated with different preparations on the DNA synthesis in the nodular area of hepatoma tissue. Experimental methods are same as Fig. 4.

Table 4. Differences of DNA synthesis between each group without methotrexate and corresponding group with methotrexate preparations

Preparation of MTX Group liver	Differences (dpm/mg DNA)		
	Free methotrexate	Reconstituted MTX	Reconstituted MTX + antibody
Regenerating liver	3,986	6,981 (1.7)*	6,106 (1.5)
Hepatoma smooth area	1,294	2,090 (1.6)	1,950 (1.5)
nodular area	1,565	2,160 (1.4)	4,988 (3.1)

\* Numbers in the parentheses indicate ratio of change as compared to corresponding free methotrexate group.

group decreased in the order of the regenerating, hepatoma (nodular), hepatoma (smooth) and control groups (Table 3, Fig. 3). Administration of methotrexate (50µg), an anticancer drug, in the forms of free, encapsulated into liposome or encapsulated into liposome with antibody against tumor specific cell surface protein, resulted in the inhibition of DNA synthesis in regenerating liver, rat hepatoma (Table 3). In the control group, methotrexate in all forms had no effect on DNA synthesis (Fig. 4). With the exception of the control group, methotrexate encapsulated into liposome had a stronger inhibitory effect on DNA synthesis than free methotrexate (Fig. 5,6,7). Methotrexate encapsulated into liposome incor-

porated with the antibody had a stronger inhibitory effect on DNA synthesis only in the nodular hepatoma area (Table 3), indicating that the antibody incorporated into liposome was tumor (nodule) specific. The net inhibitory effect of methotrexate in different preparations on DNA synthesis was measured by subtracting each value (dpm/mg DNA) in the methotrexate-treated group from the value in the corresponding non-treated group (Table 4).

In the nodular hepatoma area, methotrexate encapsulated liposome bearing antibody against tumor specific cell surface protein had the strongest inhibitory effect on DNA synthesis.

## DISCUSSION

After many studies on the morphological changes of rat liver during carcinogenesis by 4-dimethylaminoazobenzene (Orr 1949; Edwards and White 1942; Opie 1944), 3'-Me DAB was identified to cause early changes in the liver cells and the frequencies of tumor incidences as well as metastasis (Cunningham *et al.* 1950; Richardson and Nachtnebel 1951). Metabolism of 3'-Me DAB is also known to involve N-demethylation and N-hydroxylation followed by conjugation with a sulfate moiety to yield a strong electrophilic ester (Miller 1970).

Miller and Miller (1947) first reported that DAB, when metabolized, formed a covalent linkage with proteins of rat hepatocytes, and it was also reported (Sorof *et al.* 1970; Sorof and Young 1973) that the metabolic product of 3'-Me DAB induced the h2-5s azoprotein in rat liver whose molecular weight was 60,000-80,000 daltons. The specificity and function of the protein bound with metabolites of carcinogens were shown by presenting evidence of the receptor protein which carries the metabolites of 3'-Me DAB.

The presence of tumor specific antigen on the plasma membrane of DAB-induced hepatoma (D23) was first reported by Baldwin *et al.* (1971). They identified a tumor specific antigen on the surface of hepatoma by the indirect immunofluorescent antibody technique and noticed the change of the protein antigens during malignant transformation. The molecular weight of this tumor specific antigen was reported to be about 55,000 daltons (Baldwin *et al.* 1971; Baldwin *et al.* 1973). Pellis *et al.* (1976) separated two tumor specific antigens (MW 40,000 and 75,000 daltons) from a 3M KCl extract of methylcholanthrene induced fibrosarcoma in C3H/HeJ mice after 0.25% trypsin digestion. In our previous study (Kim 1985; Kim *et al.* 1988), tumor specific plasma membrane surface proteins induced by 3'-Me DAB were isolated using 3M KCl, purified and identified by the combined techniques of Disc-PAGE and SDS-PAGE. At least three tumor specific proteins induced by 3'-Me DAB were identified. This result supports the hypothesis that the altered immunoreactivity of tumor cells is caused by the tumor specific surface antigens appearing on the plasma membrane during the malignant transformation. Among the tumor specific surface proteins, a major protein (MW 66,000 daltons) has been purified and used as an antigen for the preparation of antibody against tumor specific surface protein in this study (Fig. 1).

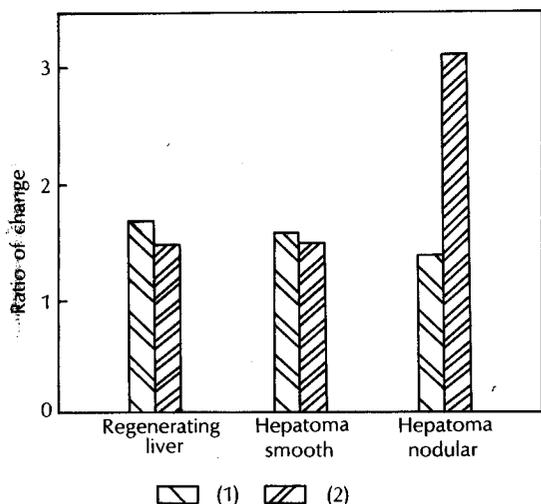
Due to the toxicities of anticancer drugs or radiation, there are limitations in the amount of application for the treatment of cancer. Ehrlich (1906) first insisted that molecules having an affinity for target tissue could be used as carriers for the transport of drugs to that tissue. Now, liposome (Weissman *et al.* 1975; Fendler and Romero 1977) and lectin (Nicolson 1974; Rapin and Burger 1974) are widely used as carriers for anticancer drugs, but these carriers are not specific for cancer cells.

Since then it has been reported that selective transport of anticancer drugs to the target tumor cells will minimize the cytotoxic effect on normal cells by reducing the drug dosage needed in the treatment of cancer patients (Rubens 1974; Ghose *et al.* 1976; Gregoriadis 1977). No specific method for the destruction of tumor cells is available now.

In the present study, a better antitumor effect of liposome reconstituted with methotrexate and antibody against tumor specific plasma membrane surface protein of rat hepatoma was obtained. In the liposome preparations, the detergent dialysis method, sonication method and reverse phase evaporation vesicle method were compared and the last one was employed in this study because it could encapsulate more methotrexate than the other two methods could.

DNA synthesis in normal hepatocyte was  $1493 \pm 722$  dpm/mg DNA which is not significantly different ( $p > 0.05$ ) from the values of methotrexate-treated groups in different preparations, indicating that any form of methotrexate could not inhibit DNA synthesis in hepatocytes, and the result agrees with the fact that 0.005-0.05% of the total cells are in the S-phase of the cell cycle in normal hepatocytes (Arias *et al.* 1982). When the liver has grown enough and part of the liver (even 70%) is removed, cell division is rapidly increased (Koch and Leffert 1980). In the regenerating liver, the treatment of free methotrexate could inhibit DNA synthesis (Table 3) by about 25%. The effect of methotrexate in the regenerating liver seems to be due to the rapid synthesis of DNA in the liver. A stronger inhibitory effect (44% inhibition, Table 4) of methotrexate encapsulated into liposome compared to free methotrexate indicates that methotrexate encapsulated into liposome moves fast into hepatocytes and non-specific endocytosis is active in the regenerating liver.

Antibody against tumor specific plasma membrane surface protein had no inhibitory effect on DNA synthesis in the regenerating liver because of the lack of tumor specific antigen on the surface of the regenerating hepatocytes. However, antibody against tumor



**Fig. 8.** Ratio of changes as compared to corresponding free methotrexate group.

(1) reconstituted methotrexate

(2) reconstituted methotrexate + antibody

specific surface protein inserted into liposome encapsulated with methotrexate showed a marked inhibitory effect on DNA synthesis in the hepatoma nodular area (3.1 times that of free methotrexate), while little change was observed in the hepatoma smooth area (Table 4, Fig. 8): The present result indicates that hepatoma cells in the nodular area are expressed with tumor specific antigen (66kDa) and antibody incorporated into liposomes against this antigen carries methotrexate selectively to hepatoma cells, where methotrexate inhibits DNA synthesis more effectively.

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