CHAPTER III

MATERIALS AND METHODS

Materials

1. Biological materials

1.1 Anti - hepatoma monoclonal antibodies (Laohathai, 1985, in press)

1.2 Hepatocellular carcinoma cell lines; S102, R12 (Laohathai, 1985), HepG2 (American Tissue Culture Collection; ATCC)

1.3 Pancreatic cancer; HS766T (ATCC)

1.4 Goat anti-mouse IgG conjugate FITC (8264, Sigma)

1.5 Protein A-conjugate gold 5 nm (RNP439, Amersham, UK)

2. Culture medium

2.1 RPMI 1640; Roswell Park Memorial Institute Medium 1640 (Seromed Co. Ltd.)

2.2 L - glutamine (G-1517, Sigma, USA)

2.3 D - glucose (Riedle - de - Haen)

2.4 Pennicillin G Sodium (M7780, M and H, Thailand)

2.5 Streptomycin (1A 70133, Dumex, Thailand)

2.6 Pyruvic acid (Sigma, USA)

2.7 Sodium bicarbonate; $NaHCO_3$ (S-5761, Sigma, USA)

3. Chemical

3.1 Electron microscopic chemical

3.1.1 Glutaraldehyde (G-6257, Sigma, USA)

3.1.2 Uranyl acetate (22400, EMS, USA)

3.1.3 Lead nitrate (17910, EMS, USA)

3.1.4 Sodium citrate (21140, EMS, USA)

3.1.5 Toluidine blue (22050, EMS,USA)

3.1.6 Osmium tetroxide,OsO_4 (R1017, EMS, USA)

3.1.7 Spurr

: 4- vinylcyclohexene dioxide, VCD (15000, EMS, USA)

: DER resin (13000, EMS, USA)

: Nonenyl succinic anhydride , NSA (19050, EMS, USA)

: Dimethylaminoethanal, DMAE (13300, EMS, USA)

3.1.8 Sodium hydroxide, NaOH (K 19742898, Merck, USA)

3.1.9 Ethanal; 35%, 60%, 85%, and 100% (414608, Analyticals, Italy)

3.1.10 Sodium carcodylate - trihydrate (12300, EMS, USA)

3.1.11 Agar (A-9915, Sigma, USA)

3.2 Chemicals using in preparing of the MAb

3.2.1 Protein A -sepharose (17-0963-03, Pharmacia Fine Chemicals, Piscataway, Sweden)

3.2.2 Citric acid; $C_6H_8O_7.1H_2O$ (L801, May and Baker, England)

3.2.3 Ammonium sulphate; $(NH_4)_2SO_4$ (420777, Analyticals, Italy)

3.2.4 Thimerosal; $C_9H_9HgO_2SNa$ (T-5125, Sigma, USA)

3.2.5 Tris- hydroxyaminomethane; $C_4H_{11}NO_3$ (T-1503, Sigma, USA)

3.2.6 Protease inhibitor

: Benzamidine (B-6506, Sigma, USA)

: Epsilon amino carproic acid (12548, Serva)

: Phenyl methyl sulfonyl Pluoride (32395, Serva)

3.3 Buffers

3.3.1 Di - sodium hydrogen phosphate, Na_2HPO_4 (D-3016, Riedel de Haen)

3.3.2 Sodium di-hydrogen phosphate, NaH_2PO_4 (K3481045, Merck, USA)

3.3.3 Sodium chloride , NaCl (479687, Analyticals, Italy)

3.3.4 Di-potassium hydrogen phosphate, (60355, Fluka AG, Swizerland)

3.3.5 Methyl ρ - hydroxy benzoate

3.3.6 Hydrogen peroxide, H_2O_2 (Fluka A.G., Swizerland)

3.3.7 OPD; O-phenylenediamine (Sigma, USA)

3.3.8 Potassium chloride, KCl

3.3.9 Phenol red (P-3532, Sigma, USA)

3.3.10 Potassium di- hydrogen phosphate (A682773, Merck, USA)

3.3.11 Hydrochloric acid, HCl

3.3.12 Pristane; 2,6,10,14 -tetramethylpentadecane (Sigma, USA)

3.4 Others

3.4.1 EDTA

3.4.2 Trypsin (Sigma, USA)

3.4.3 Trypan blue (BDH, England)

4. Instruments

4.1 FACS (Becton, Dickinson)

4.2 Light microscope (TMS; Nikon, Japan)

4.3 Transmission electron microscope (JEOL, Japan)

4.4 Centrifuge (MSE, England)

4.5 Laminar flow (HS 124; Dwyer, USA)

4.6 CO_2 Incubator (Yamato, Japan)

4.7 Recording spectrophotometer (UV 3100; Shimadzu, Japan

4.8 Refrigerate cantrifuge (Kubota, Japan)

4.9 Microcentrifuge (Tomy Seiko, Japan)

4.10 Vortex (Vortex-genie, USA)

4.11 Freezer (Sanyo, Japan)

Methods

1. Cell line cultures

Hepatocellular carcinoma (HCC), the S102 and R12 cell lines were established as cell lines from Thai patients at time of using the passage were 150 to 234 (Laohathai, 1985) and the Hep G_2; the passage was 60 to 81; and the pancreatic cancer, the HS 766T; the passage was 8 to 10; were purchased from the American Tissue Culture Collection (ATCC). All of them were maintained in RPMI 1640 (Seromed) supplemented with 5% heat - inactivated fetal calf serum, 10,000 units per ml of pennicillin and 0.01 gram per ml of streptomycin, and 0.1 mg/ml of L - glutamine. The cells were grown in a humidified atmosphere of 95% air / 5% CO_2 at 37° C. Cell were grown in monolayer cultures on tissue culture flask (Nunclon). Confluent cells were removed by 0.05% trypsin / EDTA. Thus, cancer cell lines have grown in double fold within 24 hr. The pH of culture media is also estimated at harvesting times, at appropriate pH 7.4.

2. The production of anti - hepatoma monoclonal antibodies

The selected hybridoma that secrete anti - hepatoma monoclonal antibodies, anti - hep. MAbs (Laohathai, 1985), was maintained in RPMI while was supplemented with 10% heat - inactivated fetal calf serum, 10,000 units per ml of pennicillin and 0.01 gram per ml of streptomycin, 0.1 mg/ml of L-glutamine, 2 mg/ml D-glucose and 0.11 mg/ml pyruvic acid. The cells were grown in a humidified atmosphere of 95% air/ 5% CO_2 at 37°C.

3. Methods in preparing the monoclonal antibodies

The selected hybridomas, individually, were injected into the peritoneal cavity of BALB/c mouse which were formuly stimulated by pristane. The hybridomas were expanded and secreted the anti - hepatoma monoclonal antibodies which accumulated in ascites form. The details of preparation was described as follows.

3.1 Preparing the mouse: The 6 to 8 weeks old BALB/C mice were injected intraperitoneally with 0.5 ml pristane at 14 days before injecting the hybridoma cells. The mechanism of action of pristane is not known, but it probably acts as an irritant to increase macrophage and ascites inducing in mouse rather than as an immunosuppressive agent (Brodeur, 1984). Male mouse produced significantly more antibody than female mouse. When hybridoma is injected into the abdominal cavity it will lead to the formation of ascites, a serum - like fluid in the peritoneal cavity. The amount of ascites which obtained from one mouse for this study was 3-10 ml of ascites and somtimes was up to 10 ml. Remark:

1) Mouse which have been pristane treated sometimes was reinjected after was left for 4 weeks, this time of reinjected with pristane be ready for injected the hybridoma 1 weeks later.

2) Each mouse was harvested the ascites before the abdomen distended very large because the mouse will become distressed as the volume of ascites increases, even with syngenic animals.

3.2 Preparing the expanded hybridoma: Hybridoma cells should be washed extensively in RPMI without serum priorly to remove the extraneous proteins as much as possible. The components of fetal calf serum are highly immunogenic, they can be antigenically dominant. Then, healthy hybridoma between 6 to 32×10^5 cells (Harlow, 1989, Zola, 1987) were injected intraperitoneally into the mouse. Mouse was checked frequently, at lease three times a week and daily in the later stages of tumor growth.

Remark:

1) Larger doses lead to shorter survival and smaller ascitic fluid volumes.

2) The mice will die earlier of systemic effects of the tumor if the hybridomas are injected excess in number.

3) Some hybridomas have a tendency to produce solid tumors that may have very high concentrations of antibody in the serum(Zola, 1988).

4) The degree of swelling which will give this yield of about 5 ml as a rough guide the mouse looks pregnant.

3.3 Harvesting the MAbs: Collect the ascites after injected the hybridoma 7 to 21 days. Protease inhibitor was immediately added in concentration of 10 μl per ml for prevent protein denature by protease enzyme. This collected ascites then was centrifuge at 3000 rpm for 10 min for removing the fibrins, debris and blood clot.

Remark:

1) Heat inactivation at this stage will help in the ascites may interfere the later tests, some MAbs lose significant amounts of activity on heat inactivation. In this study, heat inactivation of ascites is not necessary.

3.4 The stage recently used Abs, ascites was stored at -70 c. Some of them were kept in one to one ratio of MAbs to glycerine for the more stable (Harlow, 1989)

4. The purification of antibodies: MAbs in ascites form usually contaminated with others proteins. Ammonium precipitation and column chromatography were used to clean up in solving the problem.

4.1 Saturated ammonium sulfate precipitation. This is one of the most common, cheap and simple used methods for extracting the antibodies from serum and ascites.

One disadvantage of ammonium sulfate precipitation is that the antibodies will not pure. The high molecular weight proteins and non specific Ab could be seen. The factors that will effect the concentration at which a particular protein will precipitate include the number and position of the polar groups, the molecular weight of the protein, the pH of the solution, and the temperature at which the precipitation is performed. The preparations were all awared of this point. The procedure is described as follow:

4.1.1 Centrifuged fresh harvest ascites at 3000 g for 10 min to remove the fibrins, debris and blood cells.

4.1.2 Added 100% ammonium sulfate dropwise in ascites to bring the final concentration to 50% saturation, continuous mixing gently at 4°C for 1 hr. Most of the immunoglobulin was precipitated while other serum protein (albumin) remain in solution at 50 % saturation.

4.1.3 Centrifuged the precipitate at 3000 g for 30 min at 4°C.

4.1.4 Carefully removed and discarded the supernatant . Resuspend the pellet in 50% ammonium sulfate to bring the final concentration to 25% saturation , mix gently .

4.1.5 Centrifuged the precipitate at 3,000 g for 30 min at 4°C .

4.1.6 Carefully removed and discarded the supernatant, resuspended the pellet in 0.3 - 0.5 volumes of the starting volume in 0.01 M PBS pH 7.4 with 0.01% thimerosal. Avoid bubbles and frothing.

4.1.7 Transfered the antibody solution to dialysis tubing that have 30,000 of molecular weight cut out (Thomas) and dialyzed versus three changes of PBS with thimerosal overnight at 4°C to remove citrate buffer. Be sure to allow enough space for expansion of the antibody solution during dialysis. Normally twice the resuspended volume is sufficient.

4.1.8 Removed the antibody solution from the tubing. Remove any remaining debris by centrifugation.

4.1.9 Estimating the concentration of antibody by UV spectrophotometry.

4.1.10 Divided the antibody in small volume and kept at - 70°C.

4.2 Affinity chromatography. This well documented gel consist of protein A that isolated from structure of the bacterial cell wall of Staphylococcus aureus, coupled to sepharose CL - 4B (Pharmacia Fine Chemicals, Uppsala). Protein A actually bound to the Fc portion of the antibodies molecule. Protein A-sepharose column chromatography (Ey, 1978) provides rapid purification of immunoglobulins in a single step. Such as mouse immunoglobulins is possible to be isolated into IgG1, IgG2a, IgG2b, and IgG3 subclasses by just elute with citric buffer in different pH. The elution is simply result by stepping decrease the pH. The majority of mouse immunoglobulins bind to protein A at pH 8.1 and elute from the column at pH 6.0 for IgG1, pH 4.5 for IgG2a, pH 3.5 for IgG2b, and pH 3.0 for IgG3. In avoiding the denature of antibodies from harsh acidic elution, the receiving vessel was contained Tris - HCl pH at 8.5 which will immediately adjust the pH down tothe neutral pH. The protocol of this purification is described as follow:

4.2.1 Swoll protein A-sepharose was packed into a 10 ml chromatography column with 2 cm in diameter and this column was stored at 4°c until using. At time of start to use, the column was washed with citrate buffer, pH 3.0 to wash out the free bounded material completely.

4.2.2 Equilibrated the column with 0.1 M PB, pH 8.0 approximately 50 - 70 ml.

4.2.3 Determined total protein of sample (ascites) at OD280 and OD260 before purification.

4.2.4 Loading the 3 ml desired mouse ascites which was priorly mixed with 5 ml of 0.1 M PB, pH 8.0 and adjusted pH to 8.1 with 1.0 M Tris-HCl buffer, pH 9.0 This column then was washed through with 30 ml of 0.1 M PB, pH 8.0 (flow rate 0.4 - 0.5 ml/min) for washing out the unbound protein. The diluted ascites approximately composed with 30 to 70 mg protein per ml. The entire fractionation was performed at 4°c.

4.2.5 The IgG1 was first eluted with approximately 25 to 30 ml of 0.1 M citrate buffer, pH 6.0.

4.2.6 Washed the column with 25 to 30 ml of 0.1 M citrate buffer, pH 5.5.

4.2.7 As mention before, to minimize the denaturation of IgG_{2a} and IgG_{2b} antibodies which would be eluted by harch citrate buffer, 0.3 ml of 1.0 M Tris-HCl buffer ,pH 8.5 was added to the receiving tubes prior to collecting the eluted samples 1 ml per fraction.

4.2.8 The IgG_{2a} was eluted with 30 ml of 0.1 M citrate buffer, pH 4.5.

4.2.9 The IgG_{2b} was eluted with 25 to 30 ml of 0.1 M citrate buffer, pH 3.5.

4.2.10 The IgG3 was eluted with 0.1 M citrate buffer, pH 3.0.

4.2.11 The column was re - equilibrated to pH 8.0 with 0.1 M PB, pH 8.0 approximately 50 to 70 ml.

4.2.12 The eluted Abs was measured its concentration and transfered to the dialysis tubing and dialyze versus three changes of 0.01 M PBS, pH 7.4 with 0.01% thimerosal overnight at 4°c. The proteins containing Ab were tranversely calculated for exact concentration of Abs by the following formula:

$$\text{Ab concentration} = \frac{\text{absorbance at 280 nm}}{\text{extinction coefficient of IgG at 280 nm (13.6)}} \times 10 \text{ mg/ml}$$

Remark: The purified Abs were storage at -70°c. Antibody molecules has compact and stable protein domains beside the resistant to a broad mild denaturing conditions. It was found that Ab stored at -20 c for several years have shown no obvious deterioration but at 4 c may results in some reduction of activity for only a few days. The Abs should not thawed and refrozen repeatly because the freezing and thawing leads to measurable loss in activity of antibody.

5. Determined immunoreactivity of purified MAbs: Before and after affinity purification, the immunoactivity and specificity of MAbs has to be exammed, the most contribute method is the ELISA.

5.1 About 5×10^3 cells per well was seeded respectively in 96- well plates and maintaining in 5 % RPMI in a humidified atmosphere of 95% air/5%CO2 at 37 c for 2 days until the cell grew confluently. Then was fixed with 2% formalin at room temperature for 2 hr.

5.2 Before used, the cell was washed with 0.01 M PBS, pH 7.4 three times.

5.3 Reducing background by blocking with 0.5% BSA 300 µl per well at 37 c for 2 hr.

5.4 Washed culture plate which carrying the Ag, three times with 0.01 M PBS, pH 7.4.

5.5 Added the sample, (purifical MAb; 1 μg/ml), 100 μl per well(100 ng), incubated this primary Ab for overnight at 4°C.

5.6 After finished the incubation with first Ab, the cell was washed three times with 0.01 M PBS, pH 7.4.

5.7 The second antibodies used was Rat anti-mouse IgG_{2a} peroxidase conjugated Ab at dilution 1:1000 in 0.5% BSA. Each well was added 100 μl and incubated for 2 hr at 37°C.

5.8 After cleaned up the second Ab with buffer three times, added 150 μl per well of activated OPD substrate and incubated for about 5-10 min in the dark at room temperature.

5.9 Stoped reaction by adding 100μl of 2.5 M H_2SO_4 per well and the optical density of sample was read at 492 nm by ELISA reader.

6. Preparing the anti-hepatoma MAbs

6.1 Saturated MAb ; This was estimated by using ELISA and flow cytometry method.

6.1.1 ELISA method : The process was the same as former described in 5.

6.1.1.1 The concentration of all MAbs were set in serial dilution which starting concentration was 200 μg/ml such as 20,10,5,2.5,1.25 μg, 625,312,156,78,39,19.5 ng etc. per well of 96- well plate and incubated overnight or 12 hrs at 4°c.

6.1.1.2 Washed with PBS, pH 7.4 three times to removed excess first Ab.

6.1.1.3 The second Ab was rat anti-mouse IgG_{2a} peroxidate conjugated Ab at dilute in 0.5% BSA 1:1000 and added 100 μl per well then incubated 2 hr at 37°C. Then, following the conventional method of ELISA.

The saturated concentration of each MAbs were taken from the concentration which started to run the plateau.

6.1.2 Flow - cytometry method: This assay used indirect immunofluorescence method. The technique is automatic process and usable for quantitative determination. Instead of looking at cells randomly distributed around a half binding site of monolayer cells, the sensor read flowing cell in single file through a laser beam. The concept of mechanism is base on observing the way in which the laser beam (optical sensors) provides information when it interact with the physical change of cells properties. The fluorescence induced by the attachment of fluorescent dyes which the possible at present are phycoerythrin(PE), and FITC. To complete the immunoreactivity, this dyes has to be conjugated with antibody.

6.1.2.1 Preparing the antigens, various cell lines: sample cells were dispersed as single cell as possible by gentle trypsinization with 0.05% trypsin/EDTA.

6.1.2.2 Transfered the $1x10^5$ cells of each sample into the eppendorf vessle. Let the cells were incubated with anti-hepatoma MAbs (#27) at 0.01,0.05,0.1,0.5,1.0,10,20,50,and 100 µg for 30 min at 4°C the dark. The volume of Ab was 10 µl. After washed three rimes in 0.9% NSS, the cells were incubated with 1:10 dilution of FITC-Goat anti-mouse IgG for 30 min at 4°C in the dark. The treated cells were washed three times in 0.9% NSS and resuspended in Haemalin (Gibco) and were analysed by a cell sorter; FACScan.

7. Determination the tumoricidal effect of anti-hepatoma MAbs by estimating the cell viability and cell growth inhibition

7.1 Selected the best MAbs which have tumoricidal effect. As described in background that the anti-hepatoma MAbs were characterized and

devided into three groups; the MAbs recognized ODA,TAA and TSA. Each of the group were chosen for the best tumoricidal effect.

7.1.1 The screening anti-hepatoma MAbs were all together namely #16, #20, #27, #36, #43, #44, #54, #58, #75. The starting cells were seeded with 1×10^5 cells per sample. The concentration used were 50 and 100 μg. In this study, the HCC-S102 was used as model cell. The control MAb used non-specific NS-1 at the same concentration of Ig.

7.1.2 The cells were incubated at 37 c, 5%CO_2. The viability of treated cells were harvested at every 24 hrs for 3 days.

7.1.3 After washed with RPMI, cells were resuspened with 0.9 % NSS 900 μl and 0.4% trypan blue 100 μl (10:1 dilution), mix gently. The estimating for the tumoricidal effect % viability was performed within 3 to 5 min.

7.1.4 The unstained cells were taken as viable cells and the blue stained cells as dead cells. For greater accuracy, the cell was counted more than 200 cells of each sample. The percentage of viable cell or viability was calculated according to the formula as follow: (Paul, 1975)

$$\%\ \text{viable cells} = \frac{\text{number of viable cells}}{\text{number of viable cells + number of dead cells}} \times 100\%$$

7.2 Selected the appropriate concentration and incubated time of MAbs

7.2.1 HCC cell lines; S102 and HEpG2 and pancreatic cancer cell line; HS766T were trypsinized with 0.05% trypsin/EDTA and dispersed them into single cell as much as possible.

7.2.2 The started cells were seeded with 1×10^4 cells/well. The concentration of MAbs #27, #43 and NS-1 (non-specific) were 0.1, 1.5, 3.0, 5.0, 10.0, and 20.0 μg per well. The cells were incubated in 24 - well culture plate.

At time of medium change new MAbs were added and the activity of MAbs in those treated medium were also estimated.

7.2.3 Treated cells were harvested at every 24 hrs for 8 days. The media was changed after 4 days. The estimating for the tumoricidal effect %viability was calculated as the formular described in 7.1.4. The percent cell growth inhibition represented the relative percentage to the control-cell growth (non-MAb treated cell) was calculated by using the following formular: (Motoo, 1986)

$$\% \text{ Cell growth inhibition} = \frac{Cc-Ce}{Cc-Cs} \times 100\%$$

When Cc is the control cell count, Ce is the experiment cell count and Cs is the starting cell count.

7.3 Increasing the sensitivity of MAbs by increasing the MAbs binding site : As cancer cell, the cell lines, adhered the culture plate. This suggested that MAbs bind only to half of the sites that it could be concerned to this truth, the experiment was attempted by gave a condition of coating the plate with silicone before cell platting. The silicone will prevent the cell attachment. However, the silicone coating plate has also used for cell growth and producing the desirable product, before go on the experiment, the effect of silicone against cell growth and viability were tested. The negative result of this test provided the experiment going on as described as follow:

7.3.1 Method for coating plate; The 24-well plate was coated with 0.1 ml of silicone for 10 min before incubated with RPMI through overnight to detoxication of the silicone which may be have.

7.3.2 The starting HCC; S102 cells were seeded with 1×10^4 cells/well. The concentration of anti-hepatoma MAb#27 0.1, 1.5, 3, 5, 10, and 20 µg per well were selected for this study. The new media with MAb was changed after 4 days.

7.3.3 Treated cells were harvested at every 24 hrs for 8 days. The study had compared both the tumoricidal effect of MAb by culturing the treated cells in two different culture plate, non-silicone coated and silicone coated plate. The estimating tumoricidal effect %viability and cell growth inhibition were calculated by the formula as previous described.

8. The destructive change of MAbs treated cells and its cell-surface antigen observing under the electron microscope.

The preparation of this sample was followed the result gained from that was described in 7.1-7.3 that cells were obviously destroy by estimating %viability and %cell growth inhibition. The tumoricidal effect of 50 μg per 1×10^5 cells of MAb#27 and #43 were compared between the S102 and HepG2. For control the absence of the treatment with MAb were performed. After 3,6,12 hrs and 1, 2, 3 days incubations of MAb tratment, cells were then scrapped by police rubber and spinned into pellet before running on the EM process for observing the morphology and cell surface antigen change.

The protocol in preparing the sample for studying with transmission electron microscope are described as follow:

8.1 Preparing the sample

8.1.1 The MAbs-treated-cell pellet was fixed with 1% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.4 for 1 - 12 hr at 4 c .

8.1.2 The cells were rinsed in 0.1 M. sodium cacodylate buffer, pH 7.4 for 3 times, each for 5 mins.

8.1.3 The cell pellet was embedded in 1% agar. (Beware to avoid the high temperature of agar)

8.1.4 Embedded cells in agar were cut into 1-mm^3 pieces.

8.1.5 Then were post fixed in 2 % reduced osmium tetroxide for 1 hr at room temperature.

8.1.6 After washed in sodium cacodylatd buffer 1 time and subsequented in 0.11 M veronal acetate buffer 2 times, each for 5 mins.

8.1.7 Cells were prepared for increasing the contrast by incubating in the 1% Uranyl acetate for 1 hr.

8.1.8 Washed in 0.11 M. veronal acetate buffer 3 times.

8.1.9 The samples were dehydration in a graded series of alcohol which started from 35% through 65%, 80% and finished at absolute ethyl alcohol for 15 mins each except the absolute alcohol which had to complete with two change.

8.1.10 Infiltrated the samples with epoxy resin (spurr). The ratio of mixing between spurr resin and absolute ethanol were started with 1:3 and passed through the to 1:1 and 3:1 mixture. Then, each of mixture was incubated for 2 hr in vacuum except the 1:1 mixture can also incubated overnight.

8.1.11 The incubation in pure spurr resin was completed with two changes for 2 hr each.

8.1.12 Finally, the samples were embedded in mold which had some extra spurr resin.

8.1.13 Polymerized the spurr resin in oven at 70°C for 8 hr.

8.2 The preparation for section

8.2.1 Thick section:

8.2.1.1 A section was cut at about 1 μl thickness for selecting the appropriate area by trimming with LKB ultramicrotome.

8.2.1.2 Examined section was stained with toluidine blue and observing under light microscope.

8.2.2 Ultra-thin section:

8.2.2.1 After the triming was success, the appropriate area was selected, the sections were trimmed as called ultra-thin sections about 60 nm thickness, the sections were sticked on the 200 mesh copper grid.

8.2.2.2 Selection were stained with uranyl acetate and followed by lead citrate.

8.3 The effect of anti-hepatoma MAb on HCC surface antigen.

Paralled to the observation on effect of anti-hep MAbs intracellular, the effect on distribution of cell surface antigens were also studied, but the process of preparation the specimens was different at step of incubating the second antibodies. The specimens that studied for the intracellular changes were immediately fixed after the treatment, while the specimen for surface antigens were incubated with second antibody which conjugated with gold particle, 5 nm in size, for 6 hrs at 4 c before were fixed and went through the EM conventional method as others. The pilot studies had prooven that the 6 hrs incubation did not damage the cells and the antibody free control. The condition of setting the immunogold-labeling, the suitable fixative, the appropriate concentration of primary antibodies, the saturated antibodies were prior studied. Some of them were studied by Varunee (Varunee, 1994).

The procedure in preparing the specimens for immunoelectron microscopic study was as follow:

8.3.1 S102 cells were cultured in supplemented media with or without anti-hepatoma MAb#27 at concentration of 50 μg per $1x10^5$ cells and incubated in 37°C, 5% CO_2 incubator for 3, 6, 12 hr and 1 to 3 days (selected by the method as described in 7.2.

8.3.2 The cells were harvested at schedule times which were expected to show the time-sequence of damage change of anti-hepatoma MAbs-treated cells (see the details at method as described in 7.2). The cells at start of

each sample was at least $1x10^6$ number of cells. Control cells were incubated with absence of MAbs but in the same condition.

8.3.3 After finished the treatment the cells were harvested and washed, then were incubated with protein A linked to 5 nm colloidal gold particles for 6 hr at 4°C.

8.3.4 After finished the gold labelling the treated cells were washed two times in 0.1 M sodium cacodylate buffer, pH 7.4.

8.3.5 Then the pellets were fixed with 1% gluteralclehyde in 0.1 M sodium cacodylate, pH 7.4 in 4 c 1 hr, and passed through the process of preparing the conventional EM specimens as previously described above.