III. MATERIALS AND METHODS

1. Reagents

The following is a list of the regents used and their sources:
- RPMI 1640 (Gibco, Grand Island, NY)
- Iscove's Modified Dulbecco's Medium (IMDM; Gibco)
- Minimum Essential Medium (MEM; Gibco)
- Fetal Calf Serum (FCS; Gibco)
- Bovine serum albumin fraction V (Sigma, St. Louis, MO, USA)
- Sodium azide (Merck, Darmstadt, Germany)
- Gentamicin (Roussel, London, UK)
- Amphotericin B (Squibb, NJ, USA)
- Ampicillin (D[-]-α-Aminobenzylpenicillin) Sodium Salt (Sigma)
- Tetracycline Hydrochloride (Sigma)
- Polyethylene glycol M.W. 3,000-4,000 (PEG 4000; Gibco)
- Purified-protein derivative (PPD; Statens Seruminstitut, Denmark)
- Phytohemagglutinin (PHA; Murex, Dartford, UK)
- [^{3}H]thymidine (Amersham, Buckinghamshire, UK.)
- Hypoxanthine Aminopterin Thymidine (HAT; Gibco)
- Hypoxanthine Thymidine (HT; Gibco)
- Sodium chloride (Merck)
- 2- Mercaptoethanol (Merck)
- Lysozyme (Sigma)
- Heparin (Leo, Ballerup, Denmark)
- Histapaque-1077 (Sigma)
- Dextran in water (Macrodex; Thai-O-Suka, Thailand)
- Xho1 restriction endonucleases enzyme (Gibco)
- Ethylenediaminetetra- acetic acid (EDTA; Fluka, Switzerland)
- D (+)- Glucose (M&B, Dagenham, England)
- Glycerol (Merck)
- Paraformaldehyde (Fluka)
- Sulfuric acid (Baker, New Jersey, USA)
- Hydrochloric acid (Baker)
- Tween 20 (Fluka)
- Potassium chloride (Merck)
- Potassium dihydrogen phosphate (Merck)
- Sodium hydrogen phosphate (Merck)
- Acetic acid glacial (Merck)

- Sodium dodecyl sulfate (Sigma)
- Ethyl alcohol (Merck)
- Ethidium bromide (Sigma)
- Potassium acetate (Merck)
- Dimethyl popop (1, 4- bis [2[4- methyl-5-phenyl-oxazolyl]] benzene), (Sigma)
- 2, 5- Diphenyloxazole (PPO; Sigma)
- Toluene (BDH, Poole, England)
- Isopropanol (Merck)
- Cecium chloride (Sigma)
- Diethylether (Merck)
- Chloroquine diphosphate salt (Sigma)
- DEAE- Dextran M.W. 500,000 (Sigma)
- Dimethyl sulfoxide (DMSO; Sigma)
- Trizma (Tris [hydroxymethyl] amino-methane), (Sigma)
- Sucrose (BDH)
- Bromphenol blue (Merck)
- Ammonium choride (Fluka)
- Potassium hydrogen carbonate (Fluka)
- Sodium citrate (M&B)
- Agarose (FMC, Rockland, ME, USA)
- Blue /Orange loading buffer (Promega, Madison, WI, USA).

2. Antibodies

The following is a list of the antibodies used in this study:

- Anti-M6 mAb obtained from hybridomas derived from Balb/c mouse spleen cells after immunization with K-562 cell line
- Fluorescein isothiocyanate (FITC)-conjugated anti-CD3 (Leu 4), (Becton Dickinson, Sunnyvale, CA, USA). The CD3 molecules express on all T lymphocytes.
- Phycoerythrin (PE)-conjugated anti-CD19 (Leu 12), (Becton Dickinson). The CD19 molecules express on all B lymphocytes.
- PE-conjugated anti-CD56 (Leu 19), (Becton Dickinson). The CD56 molecules express on all NK cells.
- FITC-conjugated rabbit anti-mouse immunoglobulins (Dako, Carpinteria, Denmark)
- Anti-CD25 was obtained from Dr. O. Majdic (University of Vienna, Vienna, Austria). The CD25 molecule is interleukin 2 receptor which is expressed on activated lymphocytes.

- FITC- conjugated anti-CD45 and PE-conjugated anti-CD14 (Becton Dickinson). The CD45 molecules express on all leukocytes.
- Anti-CD14 (MEM 18) was a kind gift from Dr. Hannes Stockinger (University of Vienna, Vienna, Austria). The CD14 molecules express on all monocytes.

3. Cell lines

The characteristic of all cell lines used in this study are shown in Table 1 and 2. Human haematopoietic cell lines are K-562, U-937, Sup-T1, Daudi and Molt-4, and animal cell lines are COS and X63 Ag8.653 myeloma cells.

Table 1. Specification of all human haematopoietic cell lines used in this study

Cell line Designation	Cell Type	Origin	References
K-562	Erythroid/myeloid cell line	chronic myelogenous leukemia	Lozzio and Lozzio, 1975
U-937	Myeloid cell line	histiocytic lymphoma	Sundstrom and Nilsson, 1976
Sup-T1	T cell line	acute lymphoblastic leukemia	Smith et al., 1984
Molt-4	T cell line	acute lymphoblastic leukemia	Minowada et al., 1972
Daudi	B cell line	Burkitt' s lymphoma	Klein et al., 1968

Table 2. Specification of all animal cell lines used in this study

Cell line Designation	Cell type	Origin	References
X63 Ag8.653	Myeloma	Spleen, Balb/c mouse	Kearney et al., 1979
COS	SV40 transformed cell line	Kidney, African green monkey	Gluzman, 1981

All human haematopoietic cell lines were maintained in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal calf serum. COS was maintained in MEM, supplemented with 5% heat-inactivated fetal calf serum and the X63 Ag8.653 myeloma cell line was maintained in IMDM supplemented with 10% heat-inactivated fetal calf serum. All culture medium contained 40 mg/L Gentamicin and 2.5 mg/L Amphotericin B. These cultures were incubated in a fully humidified atmosphere of 5% CO_2 at 37°C.

4. <u>DNA</u>

- The DNA used in this study was cDNA encoding M6 molecule, which were constructed into an eukaryotic expression vector, πH3M. This DNA was named M6 DNA (Kasinrerk et al., 1992).
- The standard DNA marker used in this study was Lambda DNA/ EcoR I+Hind III marker (Promega, Madison, WI, USA).

5. Production of monoclonal antibodies to M6 molecule

5.1 Immunization of mouse

K-562 erythroid/myeloid cell lines, which express M6 molecule constitutively on their surface, were washed 3 times with sterile PBS and immunized into a 6 week old female Balb/c mouse using the immunization schedule as follows: Mouse was injected intraperitoneally (ip) with 10^7 of K-562 cells in 500 µl of sterile PBS using a 25-gauge needle on day 0. On day 14, the mouse was boosted ip with the same amount of K-562 cells as day 0. On day 24, blood from the immunized mouse was collected by tail bleeding and screened for anti-M6 antibodies by using the indirect immunofluorescence technique. On day 35, the mouse was finally boosted, intravenously at tail vein, with 10^6 of K-562 cells in 100 µl of sterile PBS. On day 38, the mouse was sacrificed and its spleen was removed for fusion.

5.2 Production of hybridomas

5.2.1 Preparation of myeloma cells for fusion

Three days prior to fusion, myeloma cells (X63 Ag8.653) were expanded to the log phase at the concentration of $5x10^4$ cells/ml, 10 ml were seeded into a 25-cm^2 tissue culture flasks (Nunc, Roskilde, Denmark). Approximately two 25-cm^2 tissue culture flasks of myeloma cells were required for the fusion of each mouse. On the day of fusion, all flasks were checked for contamination and healthy cell growth. Cell suspension was transferred to centrifuge tubes and centrifuged at 440g for 7 minutes. The supernatant was then discarded. Afterwards, cells were resuspend in 20 ml of IMDM and centrifuged at 440g for 7 minutes. The supernatant was then removed and the cells were resuspended in 10 ml of IMDM. The number and viability of cells were counted in a hemacytometer, using 0.2 % trypan blue. Myeloma cells were kept at room temperature until use.

5.2.2 Preparation of splenocytes for fusion

The immunized mouse was sacrificed and its spleen was aseptically removed and placed in a 6 cm sterile petri dish (Nunc) containing 4 ml of IMDM medium. Any contaminating tissue from the spleen was trimmed off and discarded. After rinsing, the spleen was transferred to a new petri dish containing 4 ml of IMDM, and the lymphoid cells were blown out with two 1.0 ml syringes fitted with 26-gauge needles. To achieve this, the spleen was anchored with one syringe, and while being punctured with the needle of the other, and blew out fluid into successive small areas of spleen. The spleen carcass was removed, the cell suspension was transferred to a 50-ml tube using a pasteur pipette and left behind any small lump of tissue which had settled on the base of the dish. The cell suspension was then left standing for approximately 5 minutes. After that, the cell suspension was carefully removed, while avoiding the sediment, and transferred to a new 50-ml tube. The cells were then collected by centrifugation at 440 g for 7 minutes and the supernate was removed. To lyse contaminating red cells, a cell pellet was resuspended in 5 ml of hypotonic ammonium chloride and incubated at room temperature for 5 minutes. The cells were subsequently washed twice with 30 ml IMDM, and later pelleted at 440g for 7 minutes and resuspended in 10 ml IMDM. The number and viability of cells were counted in a hemacytometer, using 0.2 % trypan blue.

5.2.3 Preparation of splenocyte feeder

Splenocyte feeders were prepared 1 day before the fusion or the single cell cloning, using a female Balb/c mouse which had the same genetic background as the hybridoma. Splenocytes were collected from the spleen as described in 5.2.2. The number of viable cells were counted in a hemacytometer, using 0.2 % trypan blue. The cell concentration was then adjusted to $5x10^5$ cells/ml with HAT or HT medium for fusion or single cell cloning, respectively. One hundred microliters of cell suspension were dispensed into each well of 96-well tissue culture plate (Nunc), by using a multichannel micropipette, and placed at 37° C in a CO_2 incubator.

5.2.4 Fusion

Spleen cells from 5.2.2 and myeloma cells from 5.2.1 were mixed together at a 5:1 ratio (5 spleen cells: 1 myeloma cell) in a 50 ml sterile centrifuge tube. These cells were centrifuged at 440 g for 7 minutes. The medium was then removed carefully and as completely as possible, after which, the tube with the cell pellet was placed in a 37°C water-bath for 5 minutes. Cells were fused with 50% PEG (MW 3000-4000), all PEG and medium were added in a dropwise fashion, continuously and very gently shaked, and time intervals were monitored by a stopwatch. The sequence of the addition of reagents were as follows: 2 ml of PEG was added dropwise into a tube for over 1/2 minute, then the cells were resuspended for over 1/2 minute and the tube was left standing for 1/2 minute. Five milliliters of serum free IMDM medium was added into the tube for over 2 minutes while gently shaken. Another 5 ml of serum free medium was added into the tube immediately afterward and shaken. Following that, the tube was left standing for 3 minutes and cell suspension was centrifuged at 440 g for 7 minutes. The supernatant was then aspirated as completely as possible and the cell pellet was resuspended in 70 ml HAT medium. One hundred microliters of cell suspension were then seeded into each well of 96-well plates which contained feeder cells. The plates were then placed in a CO_2 incubator for 7 days. After that, 150 µl of tissue culture medium was aseptically removed from each well using a multichannel micropipette. Later, 150 µl of HT medium was added to each well. Plates were then incubated in a CO_2 incubator. After 7-9 days of incubation, cells in the well were observed by an inverted microscope. When the hybrid or hybrids in a well were ready to be seen, 50 µl of tissue culture supernatant was aseptically removed from the top of the medium without disturbing the hybridomas at the bottom. After removing the supernatant, the well was refed with fresh medium. The collected supernatants were then used in the screening assay for anti-M6 antibodies.

5.3 Screening of hybridomas

Screening of hybridomas which produce anti-M6 antibodies, were firstly analysed by indirect immunofluorescence analysis, using haematopoietic cell lines, K-562, that are known to express M6 antigens on cell surfaces, as the tested cells.

5.3.1 Indirect Immunofluorescence analysis

Cells were washed twice with cold 1% BSA-PBS azide and the cell concentration was adjusted to $1x10^7$ cells/ml with the same reagent. To block non-specific Fc receptor binding, cells were incubated for 30 minutes at 4°C with 10% human AB serum before staining. Aliquot of 50 µl cell suspension was incubated with 50 µl tested antibodies (hybridoma culture supernatant) on ice for another 30 minutes. The cells were washed twice with cold 1% BSA-PBS azide and resuspended to 25 µl with the same reagent. Then, 25 µl of FITC conjugated rabbit anti-mouse immunoglobulins (Dako) were added and incubated on ice for a further 30 minutes. Finally, the stained cells were washed three times with 1% BSA-PBS azide and analyzed for fluorescent staining by a fluorescent microscope.

5.3.2 Screening of hybridoma by COS cell expression system

To confirm whether culture supernatants that showed a positive result with K-562 were anti-M6 antibodies, the COS cell transfection system was used. The experiments were carried out as follows:

5.3.2.1 Preparation of M6 DNA

5.3.2.1.1 Preparation of competent bacteria

E. coli strains MC 1061/p3 were streaked on PSI a plate and incubated at 37°C overnight. Then, 4 separated *E.coli* colonies were picked and each one was inoculated separately into a 50 ml tube containing 5 ml of PSI b broth, incubated at 37°C with shaking at 275 rpm for about 3-4 hours. After that, 2.5 ml of bacterial suspension was transferred into 50 ml of pre-warmed (37°C) PSI b broth and placed in a 37°C incubator. This was shaken at 275 rpm until the OD at 600 nm was 0.20. Flasks were then plugged into an ice-water bath immediately and left on ice for 15 minutes. All bacterial suspension was taken into pre-cooled 50 ml tubes and centrifuged at 850g for 10 minutes at 2°C. Supernatant was then poured off and the pellet was resuspended in 2 ml of ice-cold TfbI solution. Later, 18 ml of ice-cold TfbI solution was added into each tube and mixed thoroughly. The tubes were left on ice for 30 minutes, then centrifuged at 850g in 2°C for 5 minutes. The supernatant was drained

off and the cells were resuspended in 2 ml of ice-cold TfbII, mixed thoroughly, and then incubated on ice for another 15 minutes. Bacterial suspension was aliquoted into cryotubes and stored at -70°C.

5.3.2.1.2 Transformation of competent *E. coli* by M6 DNA

Competent bacteria were removed from -70°C and thawed on ice. Then, 100 µl of each competent bacteria was aliquoted into a pre-cooled 10 ml tube. M6 DNA (2.5 µl) was then added into the tube, mixed gently and incubated on ice for 30-40 minutes. The tube was put into a 42°C water bath for exactly 45 seconds, then, rapidly placed on ice for 2 minutes. LB broth (0.9 ml) was added into the tube and incubated at 37°C at 225 rpm while shaking for 1 hour. Ten microliters of bacteria suspension was then spread on LB plate containing tetracycline and gentamicin (LB antibiotic plate), and incubated at 37°C overnight. The resulting transformed bacterial colonies were screened for their M6 DNA containing.

5.3.2.1.3 Screening of transformed bacterial colonies

The standard method used to identify bacterial colonies that contain recombinant plasmid is the restriction analysis of plasmid miniprep. The sequence of work was performed as follows.

5.3.2.1.3.1 Isolation of plasmid DNA from transformed bacteria

Each bacterial colony was incubated separately in a 50 ml tube containing 5 ml LB-antibiotic broth and shaken at 275 rpm in 37°C, overnight. On the next day, 1.5 ml of bacterial suspension was added into a microtube and centrifuged with a microcentrifuge at full speed for 2 minutes. The medium was then discarded. One hundred microliters of pre-cooled 1x glucomix-lysozyme was then added into a bacterial pellet and resuspended by vortexing. The tube were left at room temperature for 5 minutes. Two hundred microliters of 1% SDS in 0.2 N NaOH was added into each microtube and samples were mixed by inverting the tube rapidly 2-3 times and left to stand on ice for 5 minutes. One hundred and fifty microliters of pre-cooled potassium acetate solution were then added into each tube, mixed by vortexing, and stored on ice for another 5 minutes. After that, samples were centrifuged at full speed for 10 minutes. Then 400 µl of the supernatant from each tube was transferred to a

new microtube containing 400 µl of phenol-chloroform solution, mixed thoroughly and centrifuged for 5 minutes at full speed. Three hundreds microliters of DNA aqueous phase from each tube was then transferred to a new microtube containing 300 µl of saturated diethylether, mixed thoroughly by vortexing before being centrifuged at full speed for 5 minutes. The diethylether (upper layer) was later discarded. To evaporate the remainder of the diethylether, the tubes were allowed to stand (cap opened) at 37°C for 30 minutes. Six hundred microliters of 96% ethanol was added into each tube and mixed by vortexing. The tubes were left to stand at room temperature for 2 minutes, then centrifuged for 10 minutes at full speed before the supernatant was discarded. One milliliter of 80% ethanol was added into the pellet, which was vortexed until it came up from the bottom of tube. It was then centrifuged for 5 minutes at full speed. The supernatant was aspirated and the pellet was dried at 37°C, then dissolved in 100 µl distilled water containing 100 µg RNAse A/ml, left to dissolved at room temperature for 15 minutes and finally stored at -20°C.

5.3.2.1.3.2 <u>Restriction analysis of plasmid DNA</u>

Two microliters of 10x react 2 buffer (Gibco), were added to 7 µl of distilled water and 1 µl of Xho1 restriction enzyme in a sterile microtube. The mixture was then mixed by tapping the tube and centrifuged at full speed for 2-3 seconds, then 10 µl of plasmid DNA was added and mixed by tapping the tube. The tube was centrifuged at full speed for 2-3 seconds and the mixture was incubated at 37°C for 1.5 hours. Afterwards, 2 µl of 10x sample buffer (loading buffer) were added and incubated at 65°C for 5 minutes. The tube was then plunged into an ice bath immediately, and left on ice for 5 minutes. Ten microliters of each sample were subsequently loaded into 1% agarose gel. The lid of the electrophoresis chamber was closed and the electrical leads were attached so that the DNA were migrated toward the anode by applying 150 volts. The sample ran until the bromphenol blue migrated to the appropriate distance through the gel. Later on, the electric current was turned off and the gel was removed from the gel tank. The gel was soaked in ethidium bromide solution (1 µg/ml) for 5 minutes, then, washed in water for further 10 minutes. It was later visualized for fragments of DNA with a UV light (302nm), photographed, and the band was compared with standard M6 DNA and standard DNA marker.

5.3.2.1.3.3 Large scale preparation of M6 DNA

To prepare a large amount of M6 DNA, a colony which had been proved to carry M6 DNA was inoculated with 5 ml of TB antibiotic broth medium in a 50 ml tube, incubated at 37°C and shaken at 275 rpm for about 3-5 hours. After that, 5 ml of bacteria suspension were transferred into 500 ml of TB antibiotic broth in a 1-liter flask, incubated at 37°C and shaken at 275 rpm for about 15-18 hours. The next day, the culture was poured into 50 ml tubes. Tubes, containing bacterial suspension, were then centrifuged at 750g for 15 minutes. The supernatant was discarded and the bacterial pellet was resuspended with 25 ml of 1x-glucomix-lysozyme before leaving to let stand at room temperature for 5 minutes. Then, 5 ml of 1% SDS in 0.2 N NaOH was added into each tube and mixed thoroughly several times by gently inverting the tubes. The tubes were later stored at room temperature for 5-10 minutes. Following that, 2.5 ml of ice-cold potassium acetate solution were added into each tube and mixed by shaking several times. The bacteria lysate was centrifuged at 750g for 30-40 minutes at 4°C and the supernatant was filtered through four layers of gauze into new 50-ml centrifuge tubes. Next, 0.6 volume of isopropanol was added, mixed thoroughly and the nucleic acids were recovered by centrifugation at 750g for 30 minutes. The supernatant was discarded and the pellet was washed twice with 70% ethanol in 100 mM Tris pH 7.5. Then, the remaining ethanol was discarded as much as possible and the pellet was dried in an incubator at 37°C for approximately 2 hours. The DNA was dissolved in 7.5 ml of 10 mM Tris pH 7.5, 1 mM EDTA solution ($T_{10}E_1$) at 4°C by stirring gently several times. To remove protein, the DNA solution was extracted once with 6 ml of phenol-chloroform solution, mixed by vortexing and centrifuged at 750g for 10 minutes, and the supernatant was then transferred to a new tube. To purify the DNA by Cesium Chloride-Ethidium bromide gradients ultracentrifugation, $T_{10}E_1$ was added to DNA solution until the total solution weight (DNA solution +$T_{10}E_1$) was 9 gm. Then, CsCl 10.2 gm was added and mixed well. Five hundred microliters of ethidium bromide solution (a mixture of 250 µl of 10 mg/ml Ethidium bromide and 250 µl $T_{10}E_1$) was then added and mixed immediately. The clear, red solution was subsequently transferred to a Backman ultracentrifuge tubes by using a disposable syringe and sealed. The sealed tubes were loaded into a Backman 70.1 TI rotor, and centrifuged at 55000 rpm for 24 hours at 25°C. On the next day, the band of circular plasmid DNA was monitored by transiluminator and collected

into a new 50 ml centrifuge tube. Five milliliters of distilled water was added into tube and mixed well. Then, 1.5 volume of absolute ethanol was also added and the two phase were mixed by vortexing. The mixture was transferred to sterile microtubes, centrifuged at full speed for 10 minutes, and the supernatant was removed by aspiration. Afterward, 80% ethanol was added into microtubes at 1 ml/tube, centrifuged at full speed for 5 minutes, and the supernatant was removed by aspiration. The plasmid DNA was then dried by incubation at 37°C for 1-2 hours. The DNA pellet was dissolved in 50 µl of $T_{10}E_1$ at 4°C by stirring gently several times. All plasmid DNA was then pooled and its concentration was calculated by measuring the OD at 260 nm (1 OD_{260} =50 µg of double stranded plasmid DNA/ml). An estimated purity of the nucleic acid was provided by measuring the ratio between the readings of 260 nm and 280 nm (OD_{260} /OD_{280}), a pure preparation of DNA should have the OD_{260}/ OD_{280} values of 1.8-2.0.

5.3.2.2 Transfection of M6 DNA into COS cells by DEAE-dextran method

Transfection mediated by DEAE-dextran is one of many methods used for introducing plasmid DNA to cultured mammalian cells such as COS cells. The procedures for transfection of M6 DNA into COS cells by DEAE-dextran were as follows. COS cells were collected from culture flasks using 0.5 mM EDTA-PBS. They were washed twice with MEM medium. The cell concentration was adjusted to $1x10^6$ cells in 4 ml of 5% FCS-MEM medium, plated into a 6 cm dish (Nunc) and incubated in a CO_2 incubator at 37°C. After overnight culture in a CO_2 incubator, the culture medium was discarded. Then, 4 ml of MEM medium were added into the COS cell dish and left in a CO_2 incubator. The transfection solution, which contained 2 ml MEM medium, 50 µl DEAE-dextran stock solution (10 mg/ml), 80 µl chloroquine diphosphate stock solution (10 mM) and 10 µl plasmid DNA (200 µg/ml), was prepared. Then the medium from the COS cell dish was discarded, and 2 ml of transfection solution was added and incubated in a CO_2 incubator for 3 hours. The transfection solution was aspirated and 2 ml of 10 % DMSO-PBS was added into the COS cell dish, left to stand for exactly 2 minutes at room temperature and then removed rapidly. The cells were washed once with 4 ml MEM medium, after that 4 ml of 5% FCS-MEM medium were added into the dish and the cells were cultured in a CO_2 incubator at

37°C. After overnight incubation, the medium was removed and 4 ml of fresh 5% FCS-MEM medium were replaced. The COS cells were then reincubated for another 2 days in a 5% CO_2 incubator.

5.3.2.3 Screening of hybridoma produced anti-M6 monoclonal antibodies

To confirm the specificity of mAb produced by hybridoma, indirect immunofluorescence method was used. M6 DNA was transfected into COS cells by means of DEAE-dextran transfection as described in 5.3.2.2. Then, M6 transfected COS cells were removed from the transfection dish by using 1.5 ml of 0.5 mM EDTA-PBS. All cells were collected and washed twice with 1% BSA-PBS azide. Finally, M6 expressing COS cells were used to test with hybridoma culture supernatants by indirect immunofluorescence analysis (as described in 5.3.1) and analyzed for fluorescent staining by a fluorescent microscope.

5.4 Single-cell cloning by limiting dilution

After the hybridoma which produced specific anti-M6 mAb had been identified, the anti-M6 antibody producing cells were cloned by limiting dilution. The procedures of single-cloning by limiting dilution were performed as follows. The hybridomas from the positive wells were collected and the cell concentration was adjusted to 5, 1, and 0.5 cells per 100 µl of HT medium. One hundred microliters of each dilution were added into 1/3 of 96 well plates, which already contained 100 µl of feeder cells. After 7-9 days of incubation, the cell growth was checked under an inverted microscope. Wells which contained a single colony were marked. About 50 µl of tissue culture supernatant was aseptically collected from the marked wells. The supernatant was analyzed for anti-M6 antibody activity by the indirect immunofluorescence technique using K-562 and M6 transfected COS cells as targets. Then the single hybridoma clone, which produced anti-M6 antibodies were grown to a large number of cells by transferring them to a fresh medium in 96 well plate, then to 24 wells plate with 2 ml medium per wells and finally to tissue culture flasks that contained more medium and allowed the cells to replicate freely. Some growing cells were aliquoted into 5-10 vials, and stored in liquid nitrogen.

5.5 Freezing and thawing of hybridoma and myeloma lines

Cells (in logarithmic phase) were centrifuged and resuspended to 5 $x10^6$-$1x10^7$cells/ml in IMDM containing 25% FCS and 15% DMSO. One milliliter of this cell suspension was then added to cryotubes. The cryotubes were covered with cotton and kept at -70°C for overnight. On the next day, the cryotubes were covered in liquid nitrogen tank. For thawing, freezing cells were thawed quickly at 37°C. As soon as they were liquefied, they were taken out with a pasteur pipette and transferred into a 50-ml tube. To dilute the freezing mixture, IMDM was added slowly over 1-2 minutes dropwise to the cell suspension. Then, the cells were centrifuged at 440g for 7 minutes and resuspened in IMDM containing 10% FCS. This cell suspension was seeded into a 25-cm^2 tissue culture flask and incubated in a CO_2 incubator.

5.6 Production of anti-M6 monoclonal antibodies

5.6.1 Collection of tissue culture supernatant

The hybridoma cells were grown in IMDM supplemented with 10% FCS at 37°C in a CO_2 incubator. They were allowed to grow until they died. The debris was removed by centrifugation at 500g for 10 minutes and the supernatant which contained anti-M6 mAb was collected and stored in small aliquots at -20°C.

5.6.2 Collection of ascitic fluid

A Balb/c mouse was injected with 0.5 ml of pristane (2, 6, 10, 14-tetramethyl pentadecanoic acid) into the peritoneal cavity. After 7-14 days, healthy hybridoma cells were collected from the culture flask, washed twice in sterile PBS and their concentration was adjusted to 5 $x10^6$-$10x10^6$ cells/500 µl with the same reagent. They were then injected into the peritoneal cavity of the mouse. Ascitic fluid began to build up within 1-2 weeks following the injection of the cells. When the abdomen of the mouse was noticeably large, and the mouse had difficulty in moving, it was killed by breaking the neck and the ascitic fluid was harvested by making an incision at the peritoneum to allow access. Ascitic fluid was sucked up into microtubes with a pasteur pipette and spun at 3000g for 10 minutes, and an oil layer was later discarded. The supernatant was collected, then aliquoted into microtubes and stored at -20°C.

6. Determination of the isotype of monoclonal antibodies

Tissue culture supernatant from anti-M6 monoclonal antibody secreting hybridoma cells were used to determine the isotype of the monoclonal antibodies by Capture ELISA. Each of the isotype specific antibodies (goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM, IgA) was diluted 1:1000 with carbonate-bicarbonate buffer pH 9.6. Fifty microliters of diluted antibody was applied to coat each well of a microtiter plate. The plate was incubated at 4°C overnight, then the coating solution was removed and the plate was washed 4 times with PBS containing 0.5% Tween (PBST). Seventy microliters of 5% BSA-PBS azide was filled into the wells and incubated at 37°C for 1 hour before the plate was washed once with PBST. Fifty microliters of the cultured supernatant to be tested was then added into each wells, and incubated at 37°C for 1 hour. The plate was washed 4 times with PBST. After that, 50 µl of peroxidase labeled goat anti-mouse immunoglobulins were added to each well and incubated at 37°C for 1 hour. Then the plate was washed again 4 times with PBST. After the final wash, 50 µl of 3′, 3′, 5′, 5′, tetramethylbenzidine (TMB) substrate solution were added and incubated for 10 minutes at room temperature. To stop a reaction, 50 µl of 2.5 N H_2SO_4 were added. The intensity of the reaction color was measured at wave length 450 nm by ELISA plate reader.

7. Detection of M6 antigen on the cell surface of white blood cells

7.1 Isolation of peripheral blood mononuclear cells

Human peripheral blood mononuclear cells (PBMC) were isolated from haparinized whole blood by Ficoll-Hypaque density gradient centrifugation. Briefly, 20 ml of heparinized whole blood were diluted with 20 ml of sterile PBS pH 7.2 in a 50 ml tube. Then, 10 ml of sterile Ficoll-Hypaque were underlayered and centrifuged at 400g for 30 minutes at room temperature. The PBMC rich interphase layer was collected. Prior to any experiment, the cells were washed three times with RPMI 1640 medium and resuspended in 10% FCS-RPMI 1640.

7.2 Isolation of peripheral blood granulocytes

Granulocytes were isolated by 6% dextran sedimentation. Briefly, the pellet from the Ficoll-Hypaque density gradient isolation was washed once with sterile PBS pH 7.2, resuspened with one volume of PBS in a 50-ml tube and one volume of 6% dextran was added. The tube was left to stand at room temperature for 45 minutes in a 45° angle position. The leukocyte rich fraction was then collected and washed one time with PBS. The washed cells were mixed with 5 ml of lysing buffer and left to stand at room temperature for 5 minutes. After that, the cells were washed twice with 1% BSA-PBS azide. The granulocyte rich population was evaluated for cell survival by using the trypan blue dye-exclusion test.

7.3 Detection of M6 antigen on leukocyte surface

PBMC and granulocytes were isolated as described above. The expression of M6 antigen on each cell type was determined by indirect immunofluorescent technique. The membrane fluorescence was analyzed by a flow cytometer.

8. Detection of M6 antigen on the cell surface of stimulated PBMC

Peripheral blood mononuclear cells were isolated from healthy donors by Ficoll-Hypaque density gradient centrifugation. The isolated PBMC were cultured in 10 %FCS-RPMI 1640 medium at a density of 1 $x10^6$ cells/ml in the presence or absence of 30 μg/ml PPD and 2 μg/ml of PHA at a final volume of 5.0 ml in 25-cm^2 tissue culture flasks. The cultures were incubated in a CO_2 incubator at 37°C for 5 and 7 days for PPD stimulation and 1, 3 and 5 days for PHA stimulation. The activated cells were collected and washed twice with 1% BSA-PBS azide. Cells were then analysed for M6 molecule expression by the indirect immunofluorescence technique. The membrane fluorescence was analyzed by a flow cytometer.

9. Detection of M6 antigen on the cell surface of haematopoietic cell lines

The haematopoietic cell lines used in this study were myeloid cell line (U-937), B cell line (Daudi), T cell lines (SUP-T1 and Molt-4), and

erythroid/myeloid cell line (K-562). Cells were collected and washed twice with 1% BSA-PBS azide. The M6 molecule expression was determined by the indirect immunofluorescence technique. The membrane fluorescence was analyzed by a flow cytometer.

10. Functional analysis of M6 molecule on the proliferation of haematopoietic cell lines

Molt-4 and K-562 were collected and washed once with RPMI 1640 medium. The cells were individually cultured at a cell density of $1x10^5$ cells/ml in a final volume of 150 µl/well in 96 well plates. This was performed in the presence or absence of 50 µl of various concentrations of anti-M6 mAb, including myeloma induced ascitic fluid control. In each culture, 50 µl of 4 µCi/ml of [^{3}H]thymidine were added, and the cultures were then incubated in a CO_2 incubator at 37°C for 5 hours. The cultures were subsequently harvested, and incorporated radioactivity was counted in a Beckman LS3801 liquid scintillation counter. The mean c.p.m of triplicate cultures of each condition were compared.

11. Optimization of the mitogen concentrations for PBMC stimulation

The culture was set up in 96 well plates in a final volume of 200µl/well. Triplicate aliquots of $1x10^5$ PBMC from healthy donors were cultured with various concentrations of PHA (0-2µg/ml) in 10% FCS-RPMI 1640 medium. The cultures were incubated in a CO_2 incubator at 37°C for 3 days. The cultures were pulsed with 0.4 µCi/well of [^{3}H]thymidine 18 hours before being harvested. Incorporated radioactivity was counted. Values were expressed in term of the means of the c.p.m. of triplicate cultures.

12. Functional analysis of M6 molecule on the proliferation of activated PBMC

PBMC were cultured in 96 well plates at a cell density of $1x10^6$ cells/ml in a final volume of 200 µl/well in the presence or absence of 0.03125 µg/ml of PHA and various concentrations of anti-M6 mAb. The cultures were incubated in a CO_2 incubator at 37°C for 3 days. [^{3}H]thy-

midine (0.4 μCi/well) was pulsed 18 hours before being harvested. The incorporated radioactivity was counted. The mean c.p.m of triplicate cultures were calculated and compared between each condition in the absence and presence of various concentrations of anti-M6 mAb.