V. DISCUSSION

M6 molecule is a leukocyte surface antigen which has unknown function (Kasinrerk et al., 1992). To study the function of M6 proteins, in this study, monoclonal antibody against this protein was firstly generated. K-562, which have M6 molecule expression on their surface (Kasinrerk et al., 1992), were used as an antigen for immunizing Balb/c mouse. When the mouse was well immunized, its spleen cells and myeloma cell line X63 Ag8.653, which is a murine myeloma line (Malik and Lillehoj, 1994), were fused. In this study, spleen cells and X63 Ag8.653 myeloma cells were mixed in a 5:1 ratio and PEG was added to facilitate fusion. Theoretically, even in the most efficient hybridoma fusion, only about 1% of the starting cells are fused, and only approximately 1 in 10^5 form viable hybrids (Harlow and Lane, 1988). This study obtained 511 out of 672 well (76%) containing hybridomas and demonstrate that a very good fusion procedure was performed.

Various factors were found to influence the fusion frequency or successful hybridization. These factors include the concentration of PEG, different molecular weight PEG, types of medium, a used of feeder cells and the stage of cell differentiation.

Virtually all current hybridoma protocol uses PEG as the fusing agent. It is a polymer which comes in a range of molecular weights, from 200 to 20000 (Butler and Dawson, 1992). The exact molecular weight of the PEG seems to be unimportant, but it was proven that when it is lower it was less effective and in some case, obviously toxic (Fazekas and Scheidegger, 1980). It is generally used within the range of 1000-6000 with good results (Harlow and Lane, 1988). In this study, PEG MW 3000-4000 was used. The typical concentration of PEG used in the fusion procedure normally ranges from 30 to 50%. Below 30%, the fusion frequency is low, and above 50%, toxicity becomes overwhelming (Goging, 1980). The PEG solution of 50% concentration was used in this experiment. In addition, some types of medium were also found to increase the fusion yield. Several investigators demonstrated that Iscove's modified Dulbecco's medium (IMDM) was suitable for hybridoma production and gave the best fusion yield (Iscove and Melchers, 1978; Sharon et al., 1980; Davis et al., 1982). Therefore, this study selected IMDM.

In this experiment, normal spleen cells were used as feeder cells. Hybridoma cells have a very low plating efficiency and the presence of a feeder cell increases the ability of culture cell to grow at very low densi-

ties. Good feeder cells should secrete the appropriate growth factors (Harlow and Lane, 1988), so to greatly enhance the survival and growth of hybridomas at the early stage after fusion. Therefore, they increase the viability of the hybrid. Normal spleen cells are commonly used as feeder cells (Levy et al., 1978). Furthermore, a cell culture medium addition of 2-ME increases the yield of hybrids and reduces the variability in outgrowth associated with variations in the capacity of serum supplements (Davis, 1995).

To screen hybrids, which produce anti-M6 antibody, supernatants from all hybridoma positive wells were tested by the indirect immunofluorescence technique. In the preliminary screening procedure, K-562 cell line was used as the antigen. It was found that the culture supernatant from 25 wells of hybridoma were positive with K-562. This meant that this entire culture supernate contained antibodies against K-562 cell-lines. This, however, could not conclude that these supernatants contained anti-M6 mAb, since there are many other molecules that also expressed on a K-562 cell surface. To verify which hybrids produce antibody specific to M6 molecule, the confirmatory test was needed. This confirmatory test used the same indirect immunofluorescent technique, but changed the target cell from K-562 to M6 transfected COS cell. Therefore, the DNA encoding M6 protein (M6 DNA) and vector DNA lacking in any expressible gene insert were needed.

The M6 DNA used in this study was cDNA encoding M6 molecule, which were constructed into an eukaryotic expression vector, πH3M. (Kasinrerk et al., 1992). The πH3M is a high efficiency expression vector, which was constructed for the expression of inserted cDNA in COS cells (Aruffo and Seed., 1987). This vector contains a simian virus 40 (SV40) origin of replication for replication of cDNA in SV40 transformed COS cells. Transcription of the inserted cDNA is driven by the human cytomegalovirus (CMV) promoter. Two features of this vector make it particularly suitable for this use: (1) the eukaryotic transcription unit allows a high-level expression in the COS cells of coding sequences placed under its control; (2) the small size and particular arrangement of sequences in the plasmid, permit high-level replication in COS cells. In this study, to prepare M6 DNA and vector DNA, plasmid DNA was firstly transformed into *E.coli* to increase the number. Then, plasmid DNA was isolated from the transformed bacterial colony. The isolated DNA were proved by restriction analysis to contain DNA insert. The result showed that M6 and vector plasmid DNA contained M6 and vector DNA

respectively, since these plasmid DNA showed the same fragment as standard M6 DNA and standard vector DNA.

The isolated M6 DNA was transfected into COS cells to test their capability in expressing M6 proteins. The M6 DNA transfected COS cells reacted strongly with standard anti-M6 mAb 1B2 (obtained from Dr. O. Majdic, University of Vienna), but were negative with the other control antibodies. The anti-M6 mAb 1B2, incontrast, were negative with mock transfected COS cells. The results indicated that the isolated M6 DNA could be transcribed and translated into M6 proteins in COS cells. This DNA can be used to prepare M6 transfected COS cells in confirmatory test to detect an anti-M6 mAb producing hybridoma clone.

For the introduction of M6-DNA into COS cells, the DEAE-Dextran transfection method, which has been demonstrated as the best, was selected (Kasinrerk et al., 1992). In this method, a DEAE-Dextran/ M6 DNA complex mixture was prepared and incubated with COS cells in culture. The complexes stuck to the cell surface, then, cells were exposed to the dimethyl sulfoxide to increase DNA uptake (Ausuble et al., 1990). After transfection, transfected DNA are normally degraded by enzymes contained in lysosomes (Alberts et al., 1983). Inhibition of the lysosomal enzymes is required for the maintenance of transfected cDNA and, therefore, increases transfection efficiency. Chloroquine, which has been described as an inhibitor of lysosomal enzymes (Alberts et al., 1983), is commonly used to increase transfection efficiency (Selden, 1987). However, it is generally agreed that chloroquine diphosphate is extremely cytotoxic if left on the cells for a long period of time. Generally, most cells cannot survive exposure to chloroquine for more than 4 hours (Ausuble et al., 1990). After introduction of the DNA, expression of the transfected gene can be analyzed between 1 and 4 days. Based on the highest transfection efficiency and number of COS cells after transfection, Kasinrerk (1992) found that DEAE-Dextran 250 μg/ml, chloroquine diphosphate 400 μM and 3 hours transfection time with $1x10^6$ COS cells in a 6 cm dish were the optimal conditions. These conditions were used in all transfections in this study.

In the screening of the hybridoma by using M6 transfected COS cells as an antigen, the supernatant from 3 wells, 2D1, 5H5 and 7H11, were positive. Hybridoma from clone 5H5, however, stopped producing anti-M6 mAb a few weeks after fusion, whereas hybrids 2D1 and 7H11 still produced anti-M6 mAb. The reasons for the instability of antibody production was due to the outgrowth of a non-secreting clone (Lemke et al., 1979; Goding, 1980). There was an abnormal number of chromo-

somes in the segregation dose that did not always deliver identical sets to daughter cells, which may cause chromosomes loss. If one of the chromosomes that carries a function rearranged immunoglobulin, a heavy or light chain gene, is lost and production of the antibody will stop (Harlow and Lane, 1988). Even with the utmost care, more than 50% of initial positive clones might be lost during the early cloning period. This represented one of the major problems in hybridoma technology (Yelton et al., 1980). The reasons for this instability were not completely clear. It was reported that one of the recommended antibiotics, gentamicin, could induce chromosome damage in mouse cells (Leonard and Botis, 1975) and this antibiotic was also added to the culture medium used in this experiment. To reduce the risk of instability and the overgrowth by non producing variants it was advised to initially clone as early as possible and always keep the culture in exponential growth phase. This would minimize stress and adverse selective pressure, which might favour the growth of a non producing variant (Goding, 1980).

Hybridoma cells from 2D1 and 7H11 that were still producing anti-M6 mAb were cloned in order to obtain a single-cell clone by limiting dilution, which is the easiest method of cloning techniques (Harlow and Lane, 1988). The healthy single clones of both 2D1 and 7H11 were selected and renamed as 2G11 and 1B9, respectively.

Normally, tissue culture supernatant is the most useful source of monoclonal antibodies. Supernatants are not contaminated with high levels of other antibodies (Harlow and Lane, 1988), but the low concentration, 10-100 μg/ml (Falkenberg et al, 1995), limits the use of monoclonal antibodies from culture supernatant. Therefore, ascitic fluid was produced because it contains a high concentration of monoclonal antibodies, typically, between 1 and 10 mg/ml (Harlow and Lane, 1988). In the ascitic fluid production, adult female mice were primed by injection of pristane into the peritoneum. This solution acted as irritants to the mice, which responded by secreting nutrients and recruiting monocyte and lymphoid cells into the area. This created a good environment for the growth of the hybridoma cells (Potter, 1972). The anti-M6 mAb producing hybridoma cells were then injected into the peritoneum of mice. This study showed that the induction of ascitic fluid, which contained a high concentration of anti-M6 mAb in all mice, was successful.

Monoclonal antibodies from clone 1B9 and 2G11 were the IgG3 and IgM isotype, respectively, after being tested by capture ELISA. In this capture ELISA, the hybridoma culture supernatant was used instead of ascitic fluid. Between 2% and 10% of antibodies from ascitic fluid

were from the current mouse antibody repertoire and not from inoculated hybridoma (Harlow and Lane, 1988). Therefore, the ascitic fluid contained with various isotype of antibodies, whereas, the culture supernatant from the hybridoma contained only one isotype which derived from the hybridoma cell.

A previously report demonstrated that M6 molecule express on various types of haematopoietic cell lines (Kasinrerk et al., 1992). It is interesting to study the expression of M6 molecule on haematopoietic cell lines by using the generated anti-M6 monoclonal antibodies. In this study, several haematopoietic cell lines, including the erythroid/myeloid cell line K-562, myeloid cell line U-937, T cell lines Sup T1 and Molt-4, B cell line Daudi were tested with the new generated anti-M6 mAb 1B9 and 2G11 by the indirect immunofluorescence technique. It was found that all haematopoietic cell lines tested, reacted with anti-M6 mAb, in different fluorescent intensities when analyzed by a flow cytometer and fluorescent microscope. These results suggested that cells in a continuous manner of proliferation and differentiation expressed M6 molecule on their surface. In a further study, the expression of M6 molecule on various types of white blood cell surfaces was investigated. Leukocytes were stained with anti-M6 mAb, 1B9 and 2G11, and several mAb for a positive and negative control by the indirect immunofluorescence technique. Monocytes showed a strongly positive with both 1B9 and 2G11, whereas, granulocytes and lympocytes, showed a weak positive. The results were similar to the previous report using anti-M6 mAb AAA6 (Kasinrerk et al., 1992).

In a further study, the expression of M6 molecule on activated peripheral blood lymphocytes was studied. PBMC isolated from 3 healthy donors were cultured in the presence of mitogen (PHA) for 1, 3 and 5 days, and, 2 PPD positive healthy donors were cultured in the presence of PPD antigen for 5 and 7 days. The activated PBMC were stained with anti-M6 mAb, 1B9 and 2G11, by using the immunofluorescence technique and analyzed for immunofluorescent reaction with a flow cytometer. It was found that in the freshly isolated lymphocyte population, cells, again, showed a weak positive with both anti-M6 mAb. However, cells became positive with anti-M6 mAb after PHA activation. The M6 expression on the first day of PHA activation was lower than after 3 days, but then stayed the same after 5 days activation. The results were similar those described by Kasinrerk et al. (1992). In PPD activation, the M6 expression increased on 7 days PPD activation compare to 5 days. These results show that the M6 molecule is lymphocyte activation associated.

The expression of M6 protein was time dependent manner. Several molecules on lymphocytes have been reported to be up-regulated by mitogen and antigen activation, for example, interleukin-2 receptor (Waldmann, 1989) or transferrin receptor (Schwarting and Stein, 1989). These activation associated molecules always involve in cell proliferation.

The expression of M6 molecule on activated T-lymphocytes and haematopoietic cell lines made it interesting to study whether M6 molecule might be involved in cellular proliferation. The functional study of M6 molecule was then performed by using generated anti-M6 mAb to find out the answer as to whether M6 molecule might be involved in the proliferation of activated PBMC and haematopoietic cell lines. In this study, the first experiment was to investigate the effect of anti-M6 mAb on the proliferation of activated PBMC. Firstly, the concentrations of mitogen used to stimulate PBMC were optimized. The mitogen used was PHA, and it was found that in all 3 healthy donors the suboptimal concentration of PHA was 0.03125 μg/ml. This concentration can, therefore, possibly investigate whether anti-M6 mAb suppresses or enhances the cell proliferation. The alteration in proliferation of PHA activated PBMC by anti-M6 mAb was then investigated. It was found that anti-M6 mAb inhibited PHA activated PBMC proliferation. The significant changes in [^{3}H]thymidine incorporation in a presence of anti-M6 mAb 1B9 and 2G11 compared to control ascitic fluid was observed ($p < 0.05$). These results indicated that the M6 molecule may be involved in cell proliferation.

To characterize the function of M6 molecule further, Molt-4 and K-562, both of which strongly express M6 molecule, were used. Firstly, the optimal cell concentration and incubation time used were studied. It was found that at a cell concentration of $1x10^5$ cells /ml and incubation of time 5 hours, the proliferation inhibition was clearly observed. These conditions were therefore used in all subsequent experiments. The effect of anti-M6 mAb on the cell proliferation of both cell lines was determined. The inhibition effect of anti-M6 mAb 1B9 and 2G11 in K-562 was 36.44% and 13.09%, respectively. As expected, this effect of anti-M6 mAb 1B9 and 2G11 was similar in Molt-4, with 33.94% and 27.43%, respectively. As demonstrated in this study that M6 molecule were involved in cell proliferation, one possible explanation was that it might be either one of the growth factor receptors or a signal transducing protein. Growth factors are small polypeptide proteins, which can act as mitogens for target cells *in vivo* or *in vitro,* either alone or synergistically with other factors, as a result of a primary interaction with specific cell recep-

tors (Franks and Teich, 1991), stimulation of cell proliferation and induction of cell differentiation. Growth factors interact with cells through specific receptors in the cell membrane. (Tannock and Hill, 1992). Cells generally express receptors for several different growth factors and the number and affinity of these receptors can be modulated by ligand binding and other mechanisms.(Franks and Teich, 1991). The largest group of growth factor receptors studied thus far has a cytoplasmic tyrosine kinase activity; these include the epidermal growth factor (EGF), insulin, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and macrophage colony stimulating factor (CSF-1) receptors (Franks and Teich, 1991). This group can be subdivided on the basis of sequence insertion within or at the C terminus of the kinase domain, allowing definition of subgroups related to either the EGF, insulin, or PDGF receptors. A number of other receptors contain seven transmembrane segments. These receptors are linked to signal transduction systems (Franks and Teich, 1991).

A sequence of a growth signal transduction pathway includes: growth signals that are transferred or transduced from outside the cell. The effect of the cell on the nucleus was by the transient activation of biochemical pathways. Peptide growth factors transfer the signal inside the cell by activation of a plasma membrane receptor. Inside the cell membrane, the signal is passed as a wave of phosphorylation through a chain of second messenger molecules to transcription factors in the nucleus. Activated transcription factors alter the transcription of the genes controlling passage through the cell cycle, which in turn, regulates the expression of genes needed for DNA replication and cell division (Yarnold, 1996).

In this study, the results indicated that anti-M6 mAb could recognized a protein on the surface of several haematopoietic cells and activated PBMC. In addition, anti-M6 mAb inhibit cell proliferation. M6 molecule might be an unclassified growth factor receptor. The biochemical characterization and mechanism involving cell proliferation are very interesting to clarify further.