V. DISCUSSION

Monoclonal antibodies have been used to establish a route for the discovery, purification and functional characterization of new leukocyte surface molecules. Full characterization of leukocyte surface molecules allow greater understanding of the immune system regulation. To define and characterize new leukocyte surface molecules, several monoclonal antibodies were generated, with 1B2 mAb being one of those. Preliminary analysis indicated that this 1B2 mAb reacted with several cell lines tested (Kasinrerk, unpublished data). Thus, it came to the following points of interest where, firstly, besides those cell lines tested, others, including both haematopoietic and non-haematopoietic, and normal blood cells in both resting and activating stages, also reacted with 1B2 mAb. The second point was to determine the molecular weight of 1B2 molecule. The last, and most important objective of this research, is to study the function of 1B2 mAb recognized molecules.

In the initial attempt to investigate the 1B2 molecule on several types of cells, an indirect immunofluorescent technique was employed. It was found that all seven haematopoietic cell lines tested reacted with 1B2 mAb, with different fluorescence intensities and patterns of staining. Some were homogeneous and others speckled. Three non-haematopoietic cell lines were also positive with 1B2 mAb. All results suggested that cells in a continuous manner of proliferation and differentiation expressed 1B2 molecules on their surface.

In a further study, the distribution of 1B2 molecules on normal blood cells was investigated. Sampling from 15 healthy blood donors was carried out. Leukocytes were stained with 1B2 mAb, and several mAb for positive and negative control. All kinds of peripheral blood leukocytes, including lymphocytes, monocytes and granulocytes, were negative with 1B2 mAb results obtained from both a fluorescent microscope and FACScan analysis. However, each blood population reacted with a mAb specific to their own surface molecules. MEM18, mAb specific to CD14 which was the molecules that expressed on monocytes (Goyert, et al., 1989), were all positive. Granulocytes were positive with 3E10, undefined mAb, but positive with granulocytes (Kasinrerk, unpublished data). The reaction of the lymphocyte population with Leu-4 (anti-CD3) were totally positive. The proper results using control mAb, suggested the working system of the test. Therefore, the results revealed that 1B2 molecules did not generally express on resting human peripheral blood leukocytes.

Since the data indicated that 1B2 mAb reacted exclusively with cell lines only, an investigation into the expression of this molecule on activated blood cells was also tried. Several cell surface molecules can be induced to express on PBMC by both mitogen and antigen (Knight, 1987). PPD-positive healthy donors were selected to evaluate the expression of 1B2 molecules from both mitogens and antigen-induced PBMC. PBMC was stimulated with an optimal concentration of stimulators i.e. PHA, ConA and PPD (Kasinrerk, et al., 1993) for 1 and 3 days. For PPD activation especially, another experiment with a 5-day incubation period was performed to obtained the greatest efficiency of activation. The activated cells were then stained with 1B2 mAb. The results revealed that activated PBMC did not express 1B2 molecules. In all experiments, the PBMC was positive with Leu-4 (anti-CD3) and MEM18 (anti-CD14), which suggested that the cells tested were of a lymphocyte and monocyte population respectively, and demonstrated how the experiment worked. In order to ensure that the activation was successful, the activated PBMC was also stained with CD25-8D8, which was specific for an epitope of the IL-2Rα molecule. This molecule was induced to express on activated cells (Kasinrerk, et al., 1993), and it was found that, activated PBMC was positive with CD25-8D8.

There were several studies indicated that cytokines could induce the expression of various molecules on the surface of leukocytes (Holter et al., 1985, Chantry et al., 1990). To further investigate the effect of cytokines on the expression of 1B2 molecules, PBMC was stimulated with rTNFα and rGM-CSF for 1 and 3 days (Kasinrerk et al., 1993). Stimulated cells were stained with CD25-8D8 in order to make certain that the stimulation system was successful. It was found that stimulated PBMC was positive for CD25-8D8, however, they were negative with 1B2 mAb.

Therefore, the 1B2 molecule might be one of which expressed only on cell lines, and the cells derived from human cancer cells with anomalies in both proliferation and differentiation.

As expected that this 1B2 molecule expressed only on continuous growing cell lines, the search to examine specimens from human hematological malignancies for evidence of abnormal 1B2 production commenced. Leukemic blood samples were collected from children aged between 6-10 years who were diagnosed with AML or ALL by both clinical symptoms and the results from blood smear features and histocytochemistry. These patients did not received any medical treatment. Firstly, the leukocytes were isolated from the whole blood by lysing out

histocytochemistry. These patients did not received any medical treatment. Firstly, the leukocytes were isolated from the whole blood by lysing out red blood cells with the hypotonic lysing solution, pH 7.2, but this technique could not lyse out red blood cells completely. In order to achieve this, the lysing procedure had to be repeated several times. In later experiments, red blood cells were lysed by NH_4Cl-Tris lysis buffer (Hunt, 1987), which proved more efficient, took less time, and was suitable for a small amount of blood samples. It was found that 10% of the total population of only one case (which was AML) from all six donors gave a positive result with 1B2 mAb. The results suggested that, the expression of 1B2 molecules might depend on the type of abnormality and/or stage of leukemic cells (Clendeninn et al., 1984).

These studies all suggested that 1B2 molecules expressed only on continueous growing cell lines and/or cells that had been uncontrollable in differentiation and proliferation. Whether they might became involved in cell proliferation was also of interest.

By the meaning of lymphocyte proliferation assay, the initial proposal was to investigate the effect of 1B2 mAb on activated PBMC. Firstly, the concentrations of antigen and mitogens used to stimulate PBMC were optimized. The mitogens used were PHA and ConA, and it was found that all 4 donors resulted in the optimal concentration of 0.0625 μg/ml and 5.0 μg/ml for PHA and ConA, respectively. Therefore, the suboptimal concentrations (0.03125 and 2.5 μg/ml for PHA and ConA, respectively) in stimulating the PBMC were used. It was therefore, possible to investigate whether 1B2 mAb suppressed or enhanced the cell proliferation. Similar to the mitogens, PPD was optimized and it indicated that the suboptimal concentration was 1.25 μg/ml. Activation of four PPD-positive donors with different concentrations of PPD showed different cell proliferation patterns. This might due to the difference in the number of PPD-recognized T-lymphocytes. In donor-3, however, the [^{3}H]thymidine incorporation fluctuated greatly. This might be due to a technical error in the experiment, such as the amount of cells or PPD added in the cultures.

The alteration in proliferation of activated PBMC, when cultured with 1B2 mAb in a total concentration of 5 μg/ml, was then investigated. Two healthy PPD-positive donors were individually examined, and it was found that there were no changes in [^{3}H]thymidine incorporation, compared with the condition of presence or absence of 1B2 mAb. It was concluded that 1B2 mAb has no effect on cell proliferation, because there was no expression of 1B2 molecules on the PBMC activated. No more

experiments were performed regarding the previous studies which altogether demonstrated that even with the high concentration of stimulators used to stimulate PBMC, there was still no expression of 1B2 molecules on the cells used.

To characterize the function of 1B2 molecules further, U-937 and K-562, both of which are strongly positive with 1B2 mAb, were used. The effect of 1B2 mAb on both cell lines was determined in three experiments with various cell concentrations, mAb concentrations, and incubation periods. A significant increase in the inhibition activity of 1B2 mAb in all experiments was observed ($p<0.05$). The inhibition effect of 1B2 mAb was up to 83% in U-937, and as expected, this effect on K-562 was similar with up to 94%.When comparing the percentage in inhibition effect of 1B2 mAb on both cell lines with any variation of cell concentrations, mAb concentrations and incubation periods to that of control mAb, the siginificant difference in percentage of inhibition was observed ($p<0.05$). 9C8 inhibited the proliferation of both cell lines slightly, however, the percentage of inhibition was quite small (not more than 26%), and was significantly less than that of 1B2 mAb ($p<0.05$). The inhibition effect of 9C8 was increased significantly when compared to the effect from mouse IgG2bκ at the same concentration ($p<0.05$). This lead to the interesting fact that 9C8 (undefined mAb) might be one of the surface molecules involved in cell proliferation, liked 1B2 mAb but its effect much lower.

Control mAb used in this study including, MEM18 (anti-CD14), 9C8 (undefined mAb) and mouse IgG2bκ isotype matched control. MEM18 is the mAb specific to CD14, which is an antigen expressed on monocytes. Although, U-937 and K-562 originated from monocytic and erythro/myeloid progenitors, respectively, they do not express CD14 on their surface. Therefore, MEM18 was used as an irrelevant non-specific mAb. In the presence of MEM18, there was small changes in radioactive incorporation, which were not significant ($p>0.05$).

9C8 (undefined mAb), which reacted with both cell lines (Kasinrerk, unpublished data), was also investigated as a control. The results from all experiments demonstrated that 9C8 mAb inhibited proliferation of both cell lines slightly, but in no comparison to 1B2 mAb. The percentage of inhibition was not more than 26%, and this might have occurred by chance, or been the direct effect of this molecule on cell proliferation.

Whether the inhibition effect of 1B2 mAb might be dependent on the nature of isotype of immunoglobulin was also interesting.The isotype of 1B2 mAb was mouse IgG2bκ, therefore, in order to address this question, mouse IgG2bκ as an isotype matched control was performed. Mouse IgG2bκ did not inhibit proliferation ($p>0.05$). Even when the concentration of mouse IgG2b isotype matched control was raised up to 40 μg/ml, there was still no significant inhibition effect on both cell lines.

Collectively, the data showed that 1B2 mAb suppressed the proliferation of both U-937 and K-562 that expressed 1B2 molecules. This effect was in a dose dependent manner and was not observed from either irrelevant non-specific mAb MEM18 or mouse IgG2b.

Another question was raised as to whether the decrease in the radioactive incorporation of cells in the presence of 1B2 mAb, might be caused by dead cells affected by toxicity from 1B2 mAb. This possibility was excluded. The experiment was performed by culturing U-937 and K-562 individually, in the presence or absence of 20 μg/ml of 1B2 mAb or mouse IgG2bκ. A viability test was determined after 5 hrs. of cultivation. Results from two independent experiments confirmed that the viability of cells in the presence or absence of mAb were similar, and all above 97%. This study demonstrated that suppressing the proliferation of both cell lines was an exclusively direct effect of the 1B2 mAb present in the cultures.

In order to determine the molecular weight of 1B2 molecules on U-937, immunoblotting and chemiluminescent techniques were performed. Firstly, the conditions of immunoblotting were optimized. Normal mouse serum was diluted five-fold into 1:500, 1:2500 and 1:12500, then separated by SDS-PAGE under reducing condition. Each separated protein was blotted and reacted with two different concentrations of biotinylated rabbit anti-mouse Igs, followed by three different concentrations of peroxidase-conjugated avidin. Immunoblotting revealed two bands of about 50 and 25 kDa for heavy chain and light chain of immunoglobulin, respectively. Each band was detected by a high sensitivity of enhanced chemiluminescent reagents. The comparison between different conditions, as shown in figure 21, indicated that in the concentration of 1:5000 biotinylated rabbit anti mouse Igs and 1:20000 peroxidase-conjugated avidin, each separate band was observed distinguishably, while having a clear background, which was the best possible condition.

The analysis of the molecular weight of U-937 was performed under the optimal condition investigated. It was indicated that 1B2 mAb recognized a protein from the U-937 lysate at the molecular weight of 23 kDa.

Since 1B2 molecules 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 that linked to a growth factor receptor.

It is known that, in the cell cycle, DNA synthesis is not continuous from one mitosis to the next, but takes place only during a specific period, known as the S phase (Howard and Pelc, 1951). There is a sequence of phase transitions that must be completed before a cell divides. Emerging evidence suggests that each of these transitions is regulated by cellular proteins. Subsequent stimulation with serum or growth factors leads to a resumption of cell proliferation, but there is a delay prior to each initiation of DNA synthesis, followed by a partially synchronized entry of cells into S phase (Pardee, 1989). These observations suggest that a signal to initiate proliferation occurs in the G_0/G_1 phase of the cell cycle. A "restriction point" in G_0/G_1 phase, beyond which a cell is committed to enter DNA synthesis, has been referred to as "R" in mammalian cells. A sequence of events is necessary before a resting G_0 cell is triggered to initiate a proliferation at R. These include (Pardee, 1989):

1. The binding of one or more growth factors to specific receptors
2. Transmission of growth factor signals to the nucleus
3. Activation of "early" genes to produce proteins
4. The binding of these proteins to DNA, which in turn regulates other genes whose products are necessary for the cell cycle to proceed.

Ordered cell growth and differentiation *in vivo* involves multiple levels of control, including physical parameters, extracellular matrix components, cell adhesion molecules, and membrane junctional complexes. A key role, however, is played by soluble factors including the diverse group of secreted molecules known as the polypeptide growth factors (Buick and Tannock, 1992). Growth factors are products of proto-oncogenes. The protein products of proto-oncogenes, and their transforming variants, can be grouped into distinct classes based on their subcellular localization and biochemical activity. These include (a) growth factors, (b) growth-factor receptors with tyrosine kinase activity, (c) cytoplasmic protein tyrosine kinases, (d) membrane-associated, guanine nucleotide-binding proteins, (e) soluble, cytoplasmic serine-

threonine-specific protein kinases, and (f) nuclear proteins (Minden and Pawson, 1992). Sporn and Tadaro (1980) proposed, and evidence has accumulated, that certain cells may not only produce and release a message, but may also respond to this same signal (an autocrine effect) through a receptor mechanism similar to that described for cells responding to endocrine and paracrine stimuli.

Growth factors are small polypeptide proteins that act at the cell surface by binding to specific receptors. Upon binding to a specific receptor, the growth factor may have a variety of effects, including maintenance of the viability of the cell, stimulation of cell proliferation, and/or induction of cell differentiation (Cross and Dexter, 1991). A common feature of the polypeptide growth factors is that they elicit their biological effects by binding to membrane receptors that are then activated to initiate intracellular signalling. The receptor proteins are thought to relay signals by certain mechanisms. In brief, the binding of the growth factor is followed by the receptor dimerization and activation of the tyrosine kinase domain. This is then followed by internalization and degradation of the receptor-ligand complex (Wells et al., 1990). The internalized, activated receptor sets in motion a number of changes in cytoplasmic biochemistry, including cytoplasmic alkalinization via the exchange of H^+ and Na^+ ions across the cell membrane, an increase in intracellular cAMP levels, and the degradation of phosphoinositides to produce inositol phosphates and diacylglecerol (Berridge et al., 1985; Berridge and Irvine, 1989). These latter products, in turn, effect the release of Ca^{2+} ions from intracellular stores and the activation of protein kinase C (PKC). Protein kinase C is thought to coordinate further protein modification, resulting in the regulation of the transcription of genes.

Results indicate that 1B2 mAb could recognized a protein on the surface of several cell lines: both haematopoietic and non-haematopoietic, by an indirect immunofluorescent technique. This suggested that 1B2 mAb-recognized molecules expressed on cell membrane. As mentioned previously, there is a protein product group of proto-oncogenes that are membrane proteins and act as growth factor receptors with tyrosine kinase activity. There are a number of transmembrane receptors that have protein kinase activity (Hunter, 1987). Examples include the epidermal growth-factor receptor (EGFR); platelet derived growth-factor receptor (PDGFR); insulin receptor; insulinlike growth factor-1 receptor; *kit*, the receptor for a recently identified stem-cell growth factor (Besmer et al., 1986; William's et al., 1990); *fms*, the colony-stimulating factor-1 (CSF-1) receptor; and

trk, the receptor for nerve growth factor (Martin-Zanca et al., 1986). In addition, there are several transmembrane tyrosine kinase whose ligands have not yet been identified, such as *nue* and *ret* (Bargmann et al., 1986; Takahashi and Cooper, 1987). Of course, a number of growth factor receptors still await investigation.The binding of a ligand to their respective receptor, induces the sequence of events necessary for the activation of an intracellular signalling pathway, as previously mentioned (Cantley et al., 1991). The reason that 1B2 molecules were membrane protein, and in addition, could inhibit cell proliferation, suggested that 1B2 molecules might be one growth-factor receptor which has the tyrosine kinase activity.

The molecular weight of 1B2 molecules assayed from U-937 lysate, indicated that its MW was 23 kDa. This is not similar to any growth factor receptor previously reported. It is possible that 1B2 molecule is a novel growth factor receptor. The main point of these interesting results is that this molecule expressed on both haematopoietic and non-haematopoietic cell lines. Thus, it can be used as a tool to study the expression and nature of anomalies in both cell types. Collectively, these results lead us to a better understanding of the nature of leukemic cells, and the strategy of how to control their growth.