II. LITERATURE REVIEWS

A. Leukocyte surface molecules

All nucleated cells have a great variety of proteins associated with their surface membranes. These can be attached in many ways. Some are loosely attached to the surface by hydrophobic bonding and may be easily removed. Others are integral membrane proteins. Integral membrane proteins are of five major types (Tizard, 1995). Type I transmembrane proteins are single-pass polypeptides that have their C-terminus in the cytoplasm and their N-terminus outside the cell. The domain that passes through the cell membrane has a hydrophobic sequence that enables it to bind to the membrane lipids. These are the most common forms of cell membrane protein on leukocytes. Type II transmembrane proteins have the opposite orientation to type I proteins. Their N-terminus is in the cytoplasm, and the C-terminus is extracellular. The third form of membrane protein is covalently attached to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor. The other two types of integral transmembrane proteins pass through the cell membrane several times. One type passes through four times and the other type passes through seven or more times. Both have hydrophilic and hydrophobic regions and orient themselves in the lipid bilayer so that the hydrophobic regions are associated with the interior of the membrane and the hydrophilic regions protrude into the aqueous tissue fluid. Some leukocyte surface proteins can be found on many types of cells, and others may be specific for the cells' function and state of development. Thus some are found only on neutrophils, and some only on monocytes or subpopulation of lymphocytes.

B. Cell surface receptors and signal transduction

The interaction between cells in the immune system, they must be able to sense what is occurring in their surroundings and react accordingly. These processes require that cells possess many different receptors on their surface membrane and that once these receptors bind to their ligands, they transmit signals to the cell nucleus. This conversion of an extracellular signal into a series of intracellular events is called signal transduction. The key components of these signal transduction pathways

include activation of a transducer protein by the receptor, secondary activation of other enzymes, generation of new transcription factors, and gene activation leading to altered cell behavior such as an increased metabolic rate, induction of and altered responsiveness to various cytokines, facilitation of cellular interactions, and finally, the development of an effector function (Sklar, 1986, Weiss and Imboden, 1987, Johnston, 1988). Most cell surface receptor proteins belong to one of four classes, based on their mode of action.

The first class of receptors is channel-linked receptors. It uses transmitter gated ion channels, which are involved in rapid signaling between nerve cells. Thus the receptor itself is a channel, and binding of the agonist opens the channel, allowing ions to pass through it. Channel-linked receptors are found in inflammatory and immune cells, but their roles are unclear (Tizard, 1995).

The second class of receptors consists of proteins that have a cytoplasmic domain that acts as a tyrosine specific protein kinase that become activated when the ligand binds. Thus, when the ligand binds to the extracellular domain, a conformational change occurs that activates the intracellular kinase domain. These kinases add a phosphate group to tyrosine residues on other cytoplasmic or membrane proteins or even the receptor itself (autophosphorylation). The phosphorylation modifies the protein structure and function and initiates a series of changes in cellular activities (Tizard, 1995).

The third major class of receptors is associated with one of a group of membrane-bound GTP-binding proteins, called G-proteins. G-proteins act as chemical switches. When inactive, they bind guanosine diphosphate (GDP). When active, they bind guanosine triphosphate (GTP). Thus, once these receptors bind their ligand, a conformational change in the receptor-G-protein complex results in the G-protein losing its GDP and binding GTP. The activated G-protein can then act on other substrates, causing their activation. More than 20 G-proteins have been identified. They are all $\alpha\beta\gamma$ heterotrimers, although there is considerable diversity between the subunits. All G-protein-linked receptors are closely related, single polypeptides that pass through the membrane several times. The ligand binds to multiple sites on the receptor. The targets of G-proteins can include ion channels, enzymes such as adenylate cyclase, phospholypase C, and some protein kinases. Modulation of adenylate cyclase activity alters the intracellular concentration of cyclic adenosine monophosphate (cAMP). cAMP is a second messenger that regulates a

wide variety of intracellular activities. Most important, increasing cAMP increases the activity of a cAMP-dependent protein kinase (protein kinase A), which then phosphorylates specific protein substrates (Tizard, 1995).

The fourth class of receptor mediates signal transduction through the sphingomyelin pathway. Ligand binding activates a neutral sphingomyelinase, which then hydrolyzes sphingomyelin in the cell membrane to ceramide. The ceramide then acts as a second messenger. It stimulates a serine threonine protein kinase that phosphorylates cellular proteins. These in turn generate transcription factors such as NF-κB. This mechanism of signal transduction is employed by the receptors of interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) (Mathias et al., 1993).

C. Cell adhesion molecules (CAMs)

Adherence between cells and between cells and connective tissue molecules mediates by cell membrane proteins. These adhesion molecules belong to four glycoprotein families, namely, the immunoglobulin superfamily, the cadherin family, the integrin family, and the selectin family.

The proteins of the immunoglobulin superfamily are the major regulators of lymphocyte function, and more than 35% of cell surface proteins belong to this family. All members of the immunoglobulin superfamily share, as a common binding block, a structure called an immunoglobulin domain. The domain consists of a peptide chain of about 100 amino acids folded over into two β-pleated sheets and stabilized by a single intrachain disulfide bond. Different members of the family have varying numbers of these immunoglobulin domains. The members of the superfamily fall into two functional groups. One group consists of proteins specialized for specific antigen recognition. Its members include the immunoglobulins, the MHC molecules, and the T-cell antigen receptor proteins. The other group includes cell surface receptor proteins that are not antigen specific. These proteins include CD2 (LFA-2), CD58 (LFA-3), CD54 (ICAM-1), CD102 (ICAM-2), CD106 (VCAM-1), CD80 (B7-7), CD3, CD4, and CD8. Many of these proteins form adhesive pairs. For example, CD4 on the Helper T lymphocytes binds to invariant parts of the MHC class II molecules on the antigen-presenting cells, and, CD8 on the Cytotoxic T lymphocyte binds to invariant parts of the MHC class I molecules on the target cells (Janeway, 1994). ICAM-1 on

antigen-presenting cells binds to CD11a/ CD18 on leukocyte (Sumida, 1995), B7-1 to CD28, and CD2 binds to CD58 specificity (van der Merwe et al., 1995).

The cadherin family consists of several calcium dependent transmembrane proteins involved in cell adhesion. They include L-CAM, E-cadherin, and P-cadherin. They are essential for normal tissue formation since they set up strong physical bounds between cells. They may also be involved in regulating cellular motility and migration. No cadherins have been found on leukocytes.

The integrin family are transmembrane proteins that mediate adherence between cells or between cells and extracellular matrix proteins such as collagen. In so doing they can also modulate cellular activities. They are all non covalantly linked heterodimers consisting of one α and one β chain. At least 14 α chains and eight β chains have been identified. They are divided into subfamilies, such as β_1 , β_2 and β_3 , each with a common β chain binding with one of several α chains (Hynes, 1992). More than 20 $\alpha\beta$ heterodimers have been identified, but this number continues to grow. Integrin binding activity depends on divalent cations. The β_1 subfamily is the largest of the integrin families. These integrins have been called VAL (very late antigen) integrins because some of them appear on activated T cells at two to four weeks after antigen stimulation. However, most cell types express one or more β_1 intrigrins constitutively (that is, they produce small amounts all the time). The β_1 chain is common to all members of the subfamily and is also called CD29. Each member has a different α chain. β_1 intrigrins are expressed on many different cell types and bind to extracellular matrix proteins such as fibronectin, laminin, and collagen. Resting T cells express these integrins at low levels. Activation of T cells increases this expression and enables them to bind to fibronectin or laminin. This binding acts as a costimulator of T-cell function and thus promotes their ability to proliferate. Memory T cells have three to four times as many intergrins on their surface as do naive T cells. The β_2 subfamily is the β_2 intrigrins that consist of three heterodimers: CD11a/CD18- the α chain is also called CD11a and the β chain is called CD18 ($\beta_2\alpha_L$ or LFA-1); CD11b/CD18 ($\beta_2\alpha_M$, Mac-1, or CR3); and CD11c/CD18 ($\beta_2\alpha_x$,p150, 95, or CR4). They are found only on leukocytes. When T cells interact with antigen-presenting cells, the initial adherence between the T cells and antigen-presenting cell is mediated by both CD11c/CD18 and CD2. These two molecules bind strongly to ICAM-1 and CD58, respectively, on the

antigen-presenting cell. The CD11a/CD18 adherence reaction is Mg^{2+} dependent and temperature dependent. The β_3 subfamily and the members of the β_3 subfamily of integrins are called cytoadhesins. One is a vitronectin receptor ($\beta_3\alpha_{IIb}$ or CD51/61), the other is a platelet receptor ($\beta_3\alpha_v$ or CD41/CD61). The vitronectin receptor may be involved in cell-cell interactions since antibodies to it may block the ability of macrophages to phagocytose dead neutrophils or lymphocytes. Vitronectin itself controls the activity of the terminal portion of the complement cascade (Tizard, 1995).

The selectin family comprises many molecules, which are found on lymphocytes or endothelium. These molecules regulate lymphocytes which circulate throughout the blood system when migrating through tissues. Especially, lymphoid organs by the interaction between these molecules on the surface of lymphocytes and receptors on vascular endothelial cells (Pardi et al., 1992). Three glycoproteins, P-selectin (CD62P), L-selectin (CD62L), and E-selectin (CD62E) are currently recognized as belonging to this family. All of them mediate leukocyte adhesion by binding to the carbohydrate side chains of cell surface glycoproteins (addressins). P-selectin is found in the secretory granules of endothelial cells in capillaries. The ligand for P-selectin is a glycoprotein side chain called CD15s (also called sialyl Lewis^x) (Etzioni, 1994). L-selectin is a glycoprotein found on lymphocytes and neutrophils surface. The ligands for L-selectin include three glycoproteins: GlyCAM-1, CD34 (or sialomucin), and a molecule found in intestinal lymphoid tissue called MAdCAM-1. E-selectin is a glycoprotein receptor for neutrophils found on endothelium. The ligand for E-selectin is also CD15s.

D. M6 molecule

The M6 molecule is a leukocyte surface antigen. Kasinrerk et al. (1992) demonstrated that this molecule is broadly expressed on human haematopoietic cell lines. On peripheral blood cells, M6 molecule are expressed on monocytes. Peripheral granulocytes from rheumatoid arthritis and reactive arthritis patients were found to express higher levels of M6 molecule, but granulocytes from healthy donors showed a weak positive (Felzmann, 1991). Peripheral lymphocytes do not significantly express M6 Ag, however, it appears on the surface of T blasts after 3 days of PHA stimulation (Kasinrerk et al., 1992).

The cDNA encoding M6 protein has been cloned and it was found that the M6 gene codes for a 269 amino acid sequence have a typical feature of a type I integral membrane protein with a predicted mass of 27.4 kDa (Kasinrerk et al., 1992). The M6 amino acid sequence indicated that M6 molecule is a member of the immunoglobulin superfamily. The extracellular region is organized into two Ig-like domains. Domain 1 of the M6 molecule relates substantially to domain 3 of the human myelin associated glycoprotein and domain 3 of the human IL-1R. Domain 2 of M6 Ag was found to be significantly related to domain 5 of the α-chain of the CD22 molecule. The putative transmembrane region of the M6 molecule contains glutamic acid, which is an acidic amino acid, interrupted between hydrophobic amino acid. This feature is usually found on membrane protein which always associate to other proteins and functions as a signal transducing protein such as the CD16 molecule (Letourneur et al., 1991) or the γ-, δ-, ε-chains of the CD3 complex (Williams and Barclay, 1988). Amino acid sequences comparison showed that human M6 protein is the species homologue of rat OX-47 Antigen (Fossum et al., 1991), mouse basigin (Altruda et al., 1989), and chicken antigen HT7 (Seulberger et al., 1990). Remarkably, the 21 amino acids of the putative transmembrane region of human M6 molecule are 100% identical to those of rat OX-47 Ag (Fossum et al., 1991), mouse basigin (Altruda et al., 1989), and chicken HT7 molecules (Seulberger et al., 1990). This strong conservation of human, rat, mouse, and chicken points to an important functional role for this region in these molecules. From all characterizations of M6 molecule, including the expression on human haematopoietic cell lines, it is the lymphocyte activation molecule and structure of the molecule that suggest the important function of this protein.

E. Monoclonal antibodies

1. The monoclonal antibody technology in theory

Serum contains many different types of antibodies that are specific for many different antigens. Even in hyperimmune animals, seldom are there more than one-tenth of the circulating antibodies specific for one antigen. The use of these mixed populations of antibodies creates a variety of different problems in immunochemical techniques. Therefore, the preparation of homogeneous antibodies with a defined specificity

was a long standing goal of immunochemical research. This goal was achieved with the development of the technology for hybridoma production (Köhler and Milstein, 1975).

In the animal, antibodies are synthesized primarily by plasma cells, a type of terminally differentiated B lymphocyte. Because plasma cells can not be grown in tissue culture, they cannot be used as an in vitro source of antibodies. In 1975 Köhler and Milstein developed a technique that allows the growth of clonal populations of cells secreting antibodies with a defined specificity. By this technique, an antibody secreting cell isolated from an immunized animal is fused with an immortal myeloma cell, a type of B-cell tumor. These hybrid cells or hybridomas can be maintained in vitro and will continue to secrete antibodies with a defined specificity (Köhler and Milstein, 1975). Antibodies that are produced by hybridomas are known as monoclonal antibodies. A broad overview is provided in Figure 1.

The success of this technique depended on the development of cultured myeloma lines that would grow in a normal culture medium but not in a selection medium because they lacked a functional gene required for DNA synthesis in this selection medium. Fusing normal spleen cells to these defective myeloma fusion partners would provide the necessary genes from the normal spleen cells, so that only the somatic cell hybrids would continue to grow in the selection medium. Moreover, genes from the myeloma cell make such hybrids immortal. Myeloma cell lines that can be used as fusion partners are created by inducing defects in the nucleotide synthesis pathway. Normal animal cells synthesize purine nucleotides and thymidylate via the de novo pathway from phosphoribosyl pyrophosphate and uridylate, respectively, in several steps, one of which involves the transfer of a methyl or formyl group from activated tetrahydrofolate. Anti-folate drug, such as aminopterin, block the reactivation of tetrahydrofolate, thereby inhibiting the synthesis of purine and thymidylate. Since these are necessary components of DNA, aminopterin blocks DNA synthesis via the de novo pathway. Aminopterin-treated normal cells can use a salvage pathway in which purine is synthesized from exogenously supplied hypoxanthine using the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT) and thymidylate from thymidine using the enzyme thymidine kinase (TK). Therefore, cells grow normally in the presence of aminopterin if the culture medium is also supplemented with hypoxanthine and thymidine, called HAT medium. Myeloma cell lines can be made defective in HGPRT if they are

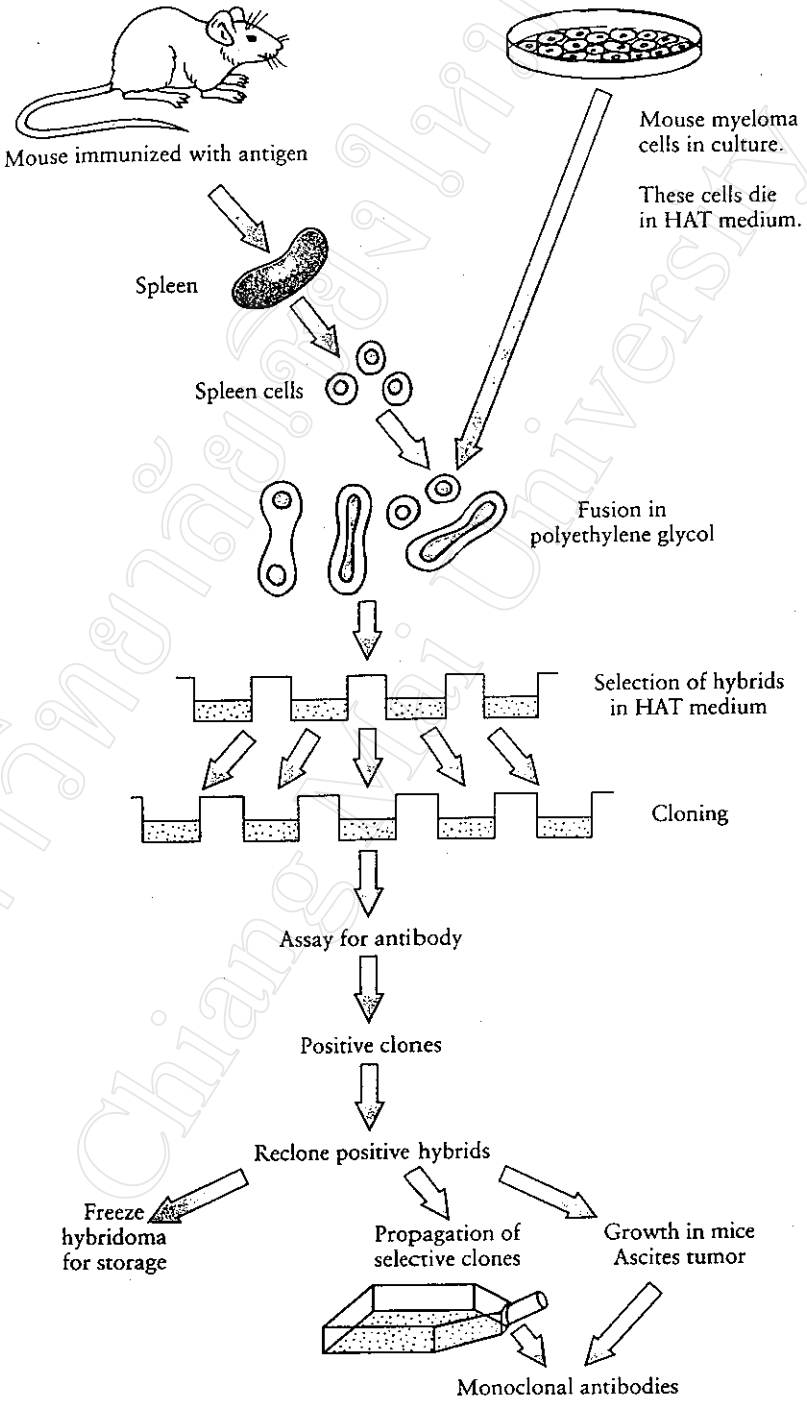

Figure 1. Basic protocol for derivation of monoclonal antibodies from hybridoma (Tizard, 1995)

mutagenized and selected in thioguanine or azaguinine, which are analogs of normal metabolites that function as substrates for HGPRT, but give rise to non functional purines. Similarly, myeloma cells can be made defective in TK by mutagenesis and selection in bromodeoxyuridine, which is metabolized by TK to form a light-sensitive, lethal product. Such HGPRT- or TK-negative myeloma cells can not use the salvage pathway and will die in the HAT medium. If normal spleen cells are fused to HGPRT-negative or TK-negative myeloma cells, the normal spleen cells provide the necessary enzymes, so that the hybrids synthesize DNA and grow in the HAT medium (Abbas et al., 1991).

In the technique for hybridoma production, an antibody secreting cell, isolated from an immunized animal, is fused with a HGPRT-negative myeloma cell, such as the myeloma cell X63 Ag8.653 which is not secreting antibody itself (Kearney et al., 1979), by using polyethylene glycol (PEG) to produce hybrid cell line called hybridoma. PEG fuses the plasma membranes of adjacent myeloma and/or antibody-secreting cells, forming a single cell with two nuclei. This heterokaryon retains these nuclei until the nuclear membranes dissolve prior to mitosis. During mitosis and further rounds of division, the individual chromosomes are segregated into daughter cells.

The HGPRT gene, contributed by the spleen cell, allows hybrid cells to survive in the HAT medium, and only hybrid cells can grow continuously in culture because of the malignant potential contributed by the myeoma cells. Therefore, unfused myeloma cells die in the HAT medium and unfused spleen cells have a limited lifespan and soon die. Individual hybridomas are then screened for antibody production, and cells that make antibodies of the desired specificity are cloned by growing them up from a single antibody-producing cell. The cloned hybridoma cells are grown in bulk culture to produce large amounts of antibody. As each hybridoma has descended from a single cell, all the cells of a hybridoma cell line make the same antibody molecule, which is called a monoclonal antibody.

2. Monoclonal antibodies are powerful immunochemical tools for the characterization of leukocyte surface antigens

The usefulness of monoclonal antibodies stems from three characteristics: their specificity of binding, their homogeneity, and their ability to be produced in unlimited quantities (Harlow and Lane, 1988).

These monoclonal antibodies can be used to identify, purify and functional analysis of different cell surface molecules. Some of the most common applications of hybridomas and monoclonal antibodies include the following:

1. Identification of phenotypic markers unique to particular cell types. The basis for the modern classification of lymphocytes and mononuclear phagocytes is the binding of population-specific monoclonal antibodies. The clustering of these antibodies into groups related by recognition of the same antigens has allowed the definition of the "cluster of differentiation", or CD nomenclature, for antibodies to human leukocyte cell surface balb/c (Barclay et al., 1993). For example, the T cell-associated markers are CD2, CD3 and CD7, together with the subset markers for T cells of predominantly helper function (CD4) and suppress or cytotoxic function (CD8). The **B** cell-associated markers are CD19 and CD20 (Janossy and Amlot, 1987), and the leukocyte common antigen (LCA) is a protein tyrosine phosphatase and is identified by the CD45 cluster of monoclonal antibodies (Lazarovits et al., 1992).

2. Immunodiagnosis. The diagnosis of many infectious and systemic diseases relies upon the detection of specific antigens and /or antibodies in the circulation or in tissues, using monoclonal antibodies in immunoassays.

3. Tumor diagnosis and therapy. Tumor-specific monoclonal antibodies are used for detection of tumors by imaging techniques and for immunotherapy of tumors *in vivo*. For example, the diagnosis of common acute lymphocytic leukemia (ALL), an early pre-B cell malignancy, is established by the strong reactivity with anti-class II MHC molecules and CD10 (cALL antigen) and the weaker, but still clear, reactivity with CD19. This patient's leukemic cells are reactive with CD34, a stem cell marker, and negative with CD20, a mature B cell marker (Stashenko et al., 1980).

4. Functional analysis of cell surface and secreted molecules. In immunologic research, monoclonal antibodies that bind to cell surface molecules, and either stimulate or inhibit particular cellular functions, are invaluable tools for defining the functions of surface molecules. Antibodies that neutralize cytokines are routinely used for detecting the presence and functional roles of these protein hormones *in vitro* and *in vivo*.

5. Use of monoclonal antibodies to induce immune suppression. In recent years, it has become apparent that autoreactive T cells can induce disease in humans. Autoimmune diseases are thought to be caused by tis-

sue destruction mediated by autoreactive T cell clones. In the field of transplantation, graft rejection is clearly mediated by host T cells recognizing foreign determinants on the allograft, while graft-versus-host disease (GVHD) is mediated by mature T cells within the donor bone marrow. Treatment for autoimmune diseases, graft rejection, and GVHD consists primarily of drugs that suppress immune function nonspecifically. Several investigators have attempted to use mAb against T cells to suppress the T cell response in the hope of inducing a more specific and effective immune suppression without the significant side effects associated with immunosuppressant drugs. For example, the use of antibodies to the T-cell CD3 antigen in immunosuppresssive treatment for organ transplantation (Barclay et al., 1993). OKT3, anti CD3 mAb, used to reduce T cell reactivity, is currently approved for clinical use. This mAb has been found to be effective in reversing acute graft rejection episodes in patients receiving renal (Schroeder et al., 1991) or hepatic allografts (Woodle et al., 1990). OKT3 is a mouse IgG2a antibody and its use is not without complications. Repeated use of this antibody usually provokes an antiglobulin response, which eventually negates the effectiveness of the antibody if repeated therapy proves necessary. As a possible solution to this problem, humanized CD3 antibodies have been constructed (Routledege et al., 1991). However, a greater problem is the observation of severe side effects associated with earlier doses in the use of CD3 antibodies, which are thought to be due to the triggering of cytokines release by T cells (Chatenoud et al., 1989). The *in vivo* cytokine release is thought to be dependent upon the binding of the antibody to accessory cells through Fc receptors. As a response to this problem, humanized forms of CD3 antibodies, in which the constant regions have also been engineered to alter Fc receptor binding, are now available (Alegre et al., 1992). It will be interesting to see if such engineered antibodies are as effective as OKT3, but without many of the problems currently associated with its use.

F. Expression of cloned genes in cultured mammalian cells

The introduction of DNA into cultured mammalian cell, transfection, has made it produce large amounts of proteins which are biologically interesting regarding cloned genes in mammalian cells. Expression of these proteins has been used for structural and biochemical studies (Sambrook et al., 1989) and can also be used to search for their natural

ligands. In addition, the 4th international conference on human leukocyte differentiation antigen in 1989 prescribed that the transfection was one of the standard methods for study the function and structure of leukocyte surface antigen, as well as study the specificity of monoclonal antibodies (Knapp et al., 1989).

There was considerable effort to develop a system to express mammalian proteins in mammalian cells. This system involved the use of viral expression vectors derived from simian virus 40 (SV40) (Elder et al., 1981). SV40 is a member of the papova group of small, nonenveloped DNA viruses, its genome is a covalently closed circular double-stranded DNA. This virus causes lytic infection of permissive monkey cells. A number of plasmid-based expression vectors carry individual regulatory regions derived from SV40, but they lack most of the coding region of the viral genome. After transfection into mammalian cells, foreign DNA cloned into these vectors are transiently expressed, but no virus particles are produced.

A good way to rapidly establish the feasibility of expressing a cloned gene in mammalian cells is to use a transient expression system such as that provided by the simian COS cell line (Gluzman, 1981). This cell line derived from the transformation of simian CV-1 cells with an origin defective SV40 genome (Gluzman, 1981). It constitutively expresses a wild-type SV40 T antigen, which is required to activated the SV40 origin of replication and all of the cellular factors required for the replication of DNA that contains the SV40 origin of DNA replication (Sambrook et al., 1989).

In this type of study, the clone sequence of interest is inserted into the appropriate expression vector, cloned in bacteria, amplified by replication, and then used to transfect mammalian cells (Sambrook et al., 1989).

Transfection mediated by DEAE-dextran is one of many methods to introduce DNA into cultured mammalian cells. This technique continues to be widely used for viral genome and plasmids carrying a viral sequence (Sambrook et al., 1989). The mechanism by which treatment with DEAE-dextran allows mammalian cells to take up DNA and transport it to their nuclei is poorly understood. Conventionally, it is assumed that large complexes containing DEAE-dextran bind to DNA and inhibit the action of nuclease, and/or bind to the cell surface and promote endocytosis of the DNA. Despite this paucity of mechanistic information, it is clear that DEAE-dextran mediated DNA transfection is a highly repro-

ducible and effective method (Ausubel et al., 1990, Kasinrerk, 1990). Over the course of a transfection experiment, COS cells accumulate over 10^5 copies per cell of recombinant expression plasmids containing the SV40 origin of replication (Mellon et al., 1981), and they express high levels of foreign DNA sequences. The transcription or replication of the transfected gene can be analyzed between 1 and 4 days after the introduction of the DNA (Ausubel et al., 1990). Expression in this system is transient because replication of the transfected plasmids continues unchecked until the cells die (at ~70-90 hours posttransfection), presumably because they cannot tolerate the high levels of extrachromosomally replicating DNA.