Chapter II

Literature Review

Tumor Marker

Tumor marker is a substance which can be measured quantitatively by biochemical or immunochemical means in tissue or body fluids to identify the presence of a cancer, possibly the organ where it resides and to establish the extent of tumor burden before treatment(9). It has several possible uses(1). Firstly, as a screening test for cancer. The test which shall be simple, rapid and versatile in order that the presence of cancer can be detected before the appearance of symptoms. Secondly, as a diagnostic tool for use in a patient suspected of having cancer. Thirdly, as a staging the extent of the malignant disease. Fourthly, as a therapeutic agent in monitoring the effects of treatment such as the development of recurrence after surgical excision or monitoring the response to chemotherapy. The changing level of tumor markers may not indicate a real change of the tumor burden. All markers do not behave similarly.

Many of tumor markers are not only present in the cell or on the cell surface, but are also secreted into the body fluids. These are usually identified by hetero antisera, monoclonal antibodies, or biochemical properties. Examples

of different types(10) of tumor markers are listed in Table 1. Since some tumors may produce more than one marker, the simultaneous measurement of more than one marker often provides information leading to a more precise diagnosis or prognosis than using one marker alone. The most useful tumor markers are monoclonal immunoglobulin, alpha-fetoprotein and carcinoembryonic antigen.

Table 1. Types of Tumor markers

Type	Example
Deletions of blood group markers	ABH
Exposure of backbone or blood group markers	Monosialoganglioside
Oncofetal proteins antigen	Carcinoembryonic
	Alpha fetoprotein
Enzyme alterations	Glycosyltransterases
Isozymes	Alkaline phosphatase
Ectopic hormone	Human chorionic gonadotropin
Tumor antigens	Melanoma proteins identified by monoclonal antibodies
Cytoskeletal elements	Epidermal tumors
Immunoglobulin gene-rearrangement	B cell tumors
Gene translocations (philadelpia-chromosome)	Lymphomas
Fragile sites	Leukemia

Alpha-fetoprotein (AFP)

1. Properties and structure

Human alpha fetoprotein (AFP) was first noted in the fetal sera of humans by Bergstrand and Czar(11) in 1956. In 1963, Abelev et al(12) indicated that the embryonal protein of mice was similar to one occurring in animals with spontaneous or graft-induced hepatomas. The finding of AFP in the serum of human hepatoma patients(13) stimulated widespread investigations of this protein. Since then, several reports have confirmed the usefulness of AFP for the diagnosis and prognosis of hepatomas. This protein is not restricted to hepatocellular cancer but is also found in the sera of patient with malignant teratoblastomas of the testis and ovary. The presence of AFP in the serum of patients with various malignancies, including those of primitive gut origin(14), has engendered a great deal of interest in developing sensitive immunological assays as an aid in detecting not only the appearance of the protein in significantly enhanced amounts but in quantitating the level as a possible index of the activity of the tumor.

AFP is a product of specific fetal tissues and of neoplastic cells of hepatocyte or germ cell origin in adults. It is a glycoprotein containing about 4 % carbohydrate with a molecular weight from 67,000 to 74,000, a sedimentation constant of 4.5 and an isoelectric point at 4.8. Electrophoresis of human AFP isolates in SDS gels usually show a single component ,even though multiple bands

are seen under nondenaturing conditions(15). AFP preparations almost invariably exhibit considerable degrees of charge heterogeneity. The presence of seven charged forms in AFP isolated from human amniotic fluid has been demonstrated by Ampholyte displacement chromatography using an anion exchange column and Ampholine(16) in the acidic range (pH 4-6). This separation is related to the isoelectric points of the proteins. On isoelectric focusing in agarose gels, blotting and immunological detection with polyclonal and monoclonal antibodies, it was possible to detect nine AFP electrophoretic variants (bands A-I) in the range of pH 4.5 to pH 5.2(17). It was found that the isoelectric points of human fetal AFP isolates were a function of their contents of fatty acid and they could be converted into essentially homogeneous material by removal of, or on reconstitution with fatty acid.

Amino acid sequence(18) analysis has established that the AFP of various species is a single-chain protein with about 590 amino acid residues. The complete amino acid sequence of human AFP obtained from the structure of its cDNA(19) is shown in Figure 1. From these results the similarities of the overall structures of AFP and serum albumin are apparent.

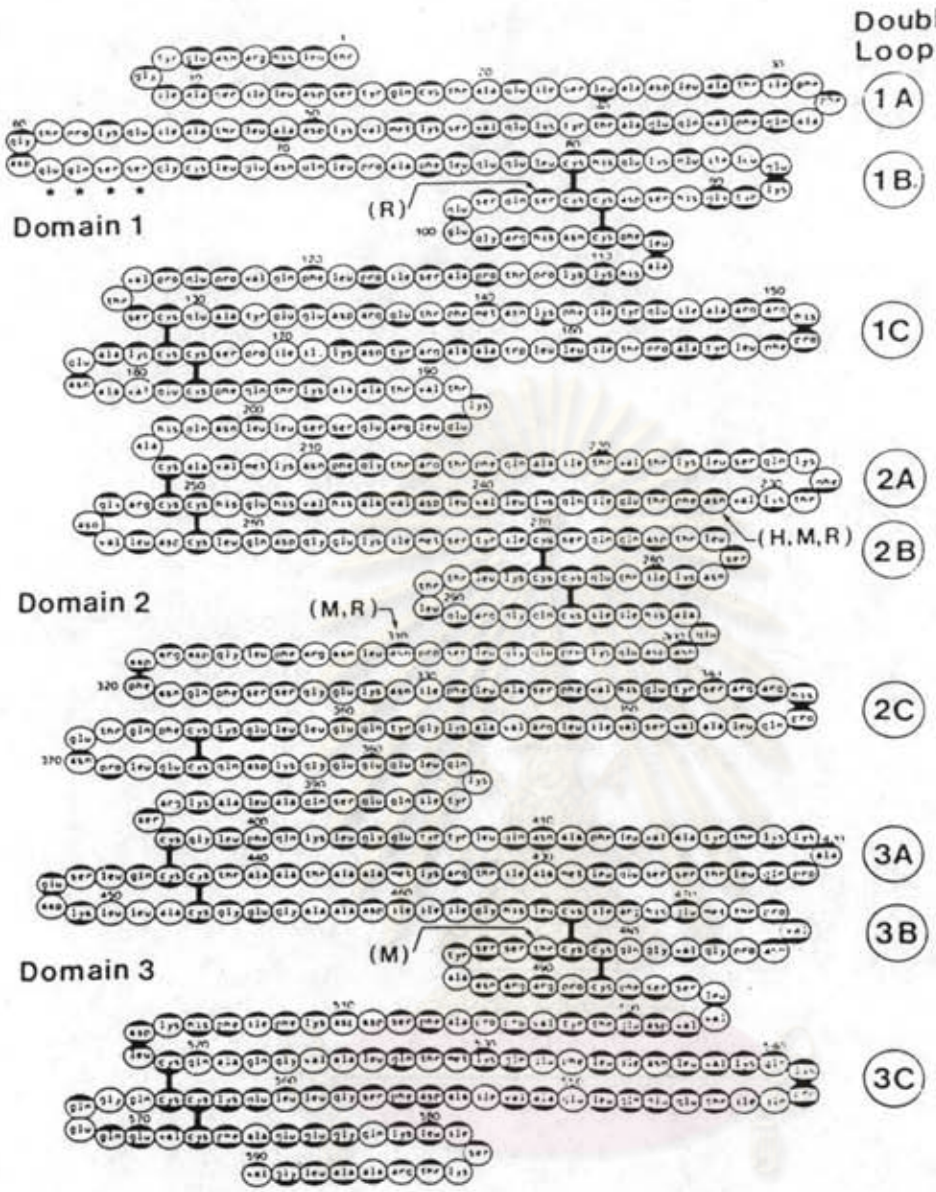

Figure 1 The amino acid sequence of human AFP arranged as proposed for serum albumin

The primary sequence of AFP departs most widely from serum albumin in the first 135 amino acid residues, with about 42% of the remaining 590 residues of the human proteins being identical. Some evidence exists that there are limited sequence differences in the AFP of a given animal species(20).

AFP differs from serum albumin in containing what appears to be variable amounts of carbohydrate(21). Variations in the carbohydrate contents of AFP of different species may reflect in part their variable glycosylations. Thus, the human protein appears to have a single point of attachment of glycoid material, whereas there are two in the case of bovine and rat AFP.

A representative structure of these carbohydrate chains of AFP isolated from human hepatoma and from a yolk sac tumor is presented in Figure 2A. The yolk sac-derived material also contains a bisecting N-acetylglucosamine(22) (GluNAc) and this form may also show variable fucosylation. Fetal calf AFP contains two glycoid chains. The biantennary chain form is similar to that of the human hepatoma AFP isolates, although it appears to contain only a small amount of fucose. The triantennary chain form of the calf AFP is shown in Figure 2B. The variations in extent and type of glycosylations are evidenced by differences in the binding to various lectins. These interactions are being extensively explored in attempts to differentiate the sources of the protein produced by various normal and neoplastic cells and may provide valuable diagnostic methods.

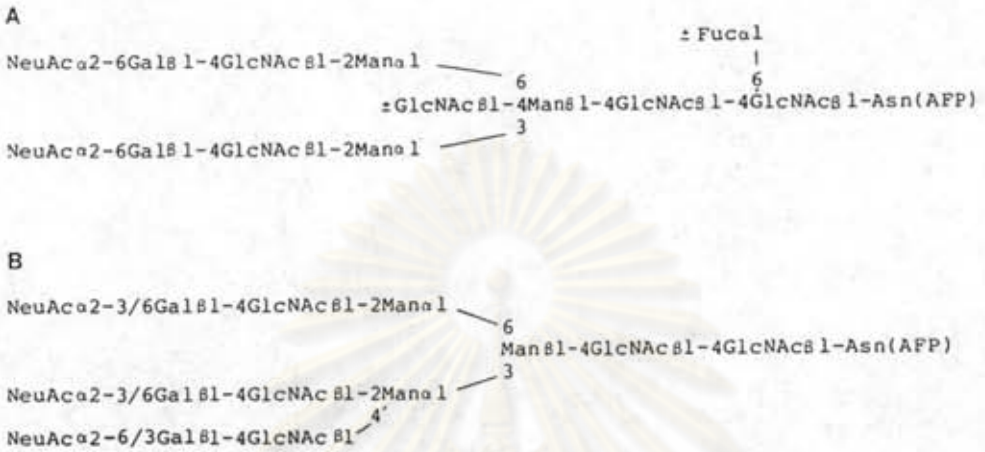

Figure 2 Representative structures of the glycoid moieties of AFP

2. Development of Biology and Physiology

Human serum AFP is normally a product of the conception which is synthesized principally by the developing liver as early as 29 days after conception(23,24). Aside from the fetal liver, AFP is also synthesized in the yoke sac and the fetal alimentary canal(23,25). However, AFP synthesis in the yolk sac is active only in early development since it becomes atretic toward the end of the 1st trimester and the amount of AFP synthesized by the GI tract is much less as compared to the equivalent amount of liver and early yolk sac(23). Trace amounts of AFP may also be produced by the kidneys in an occasional conceptus or even by the placenta, but synthesis

at either site does not seem to be common(23) and the quantities produced are very small indeed. The highest concentration in fetal serum appears during 12 to 14 weeks of gestation at the level of several mg per ml which accounts for almost a third of the total serum protein(26,27). All of the AFP present at this point is synthesized by the liver(24). After the 14th week the fetal AFP concentration decreases and the AFP synthesis is almost completely repressed at or near birth whether birth is premature or at term.

The AFP present in the circulation if the newborn then disappears with a half life of approximately 3.5 days during the first weeks of life and then decreases more slowly to the normal adults by 2 years of age(28). However, AFP synthesis does not cease entirely at or near term since trace amounts of AFP range from 2 to 25 ng/ml are demonstrated in normal human serum(29) which is almost one million times lower than the fetal level. This indicates that suppression of the AFP gene is not complete in human adults.

AFP enters the amniotic fluid through urination, passing through the placenta to enter the maternal circulation. At 12-14 weeks of gestation, amniotic fluid concentrations of AFP ordinarily peak at 15-20 mg/L(30), after which they decline rapidly. Like fetal serum AFP, amniotic AFP levels correlate inversely with gestational age(31). The significant correlation of decreasing AFP levels with advancing gestation makes AFP a useful marker

in the assessment of gestational age ie; low levels signify that a certain stage of fetal maturity has been reached, but high levels can not be used to indicate prematurity since the normal range is wide and fetal abnormalities may obscure the situation.

In normal pregnancy maternal serum AFP concentration gradually rises during gestation, reaching a maximum of up to 500 ng/mL by term(32), presumably by fetal maternal transfer via the placenta. The placenta becomes increasingly pervious to a number of plasma protein in the last half of gestation, possibly contributing to the gradual increase in maternal serum AFP during the last part of gestation(33). The maternal AFP level sometimes increases during delivery, probably reflecting fetomaternal transfusion. After delivery it decrease at a rate corresponding to a half-life of 4-5 days.

The serum AFP concentration continues to fall during the neonatal period, and by 6 to 12 months of age it has reached the normal adult level. Sensitive radioimmunoassay techniques(34) have demonstrated the presence of measurable levels of serum AFP during life and this circulating AFP is not significantly different from AFP of the fetus or that synthesized by hepatocellular carcinoma(35). The lower limit of the AFP concentration in normal sera is probably less than 1 ng/ml, the upper limit varies between 10 and 30 ng/ml, depending on the laboratory performing the assay. Healthy individuals appear to have stable levels of serum AFP so that elevation above the

individuals base line, even though it may still be within the arbitrary "normal range" has the potential for reflecting a disease process(36).

3. Clinical application

3.1. Alpha fetoprotein in hepatocellular carcinoma

Level of AFP greater than 2000 ng/ml are often considered of liver cancer in which such elevations are observed has depended upon the type of sensitivity of the technique used for the detection of AFP and also upon the ethnic group under investigation(37). The AFP concentration is apparently not related to the volume of the tumor nor to the duration of the illness(38). However, the potential usefulness of a screening programme was demonstrated in report by Koji and others(39) in 1975. The detection of elevations in serum AFP concentration led to the early diagnosis(40) and removal of the tumor in and otherwise clinically latent disease. The serum concentration of AFP in hepatoma varies widely, from normal to several mg/ml(41). Several factors may be involved in the quantitative variation of AFP secreting tumors. There are undoubtedly multiple etiologic and pathogenic factors involved in causing this carcinoma, including genetic and acquired chronic liver disease, mycotoxin and other environmental carcinogens and viral infection.

3.2. Alpha fetoprotein in other malignancies

Malignancies of yolk sac cell origin, including certain embryonic carcinomas of the ovaries and gonads, have a high frequency of AFP positivity. Serum AFP elevation has been reported in association with a number of other neoplastic disease; carcinomas of the pancreas, stomach and biliary system are where AFP is found with greatest frequency after hepatocellular carcinoma(42) and germ cell tumors (Figure 3). Patients with carcinoma of the kidney, lung, colon, and prostate have occasionally had elevated serum AFP(43,44). Compared with tumors of the liver and testis, the AFP levels in these other tumors are not as high on the average.

3.3. Alpha fetoprotein in nonmalignant disease

Various non malignant disorders also have been shown to involve increased production of AFP. Two such liver disorders are viral hepatitis type B and liver cirrhosis. The first case of elevated AFP concentration in hepatitis was reported by Alpert and others(45) in 1968. Unlike the persistently high levels of AFP detected in individuals with primary liver cancer, patients with viral hepatitis type B exhibit transient elevations and the maximum concentrations of AFP are in general much lower. Increase in AFP concentration during the course of viral hepatitis type B may be more common among younger patients(46). In 1975 Bloomer(47) et al found that 69% of patients below 30 years of age and 38% of those above 40 years exhibited transient elevations on AFP concentration.

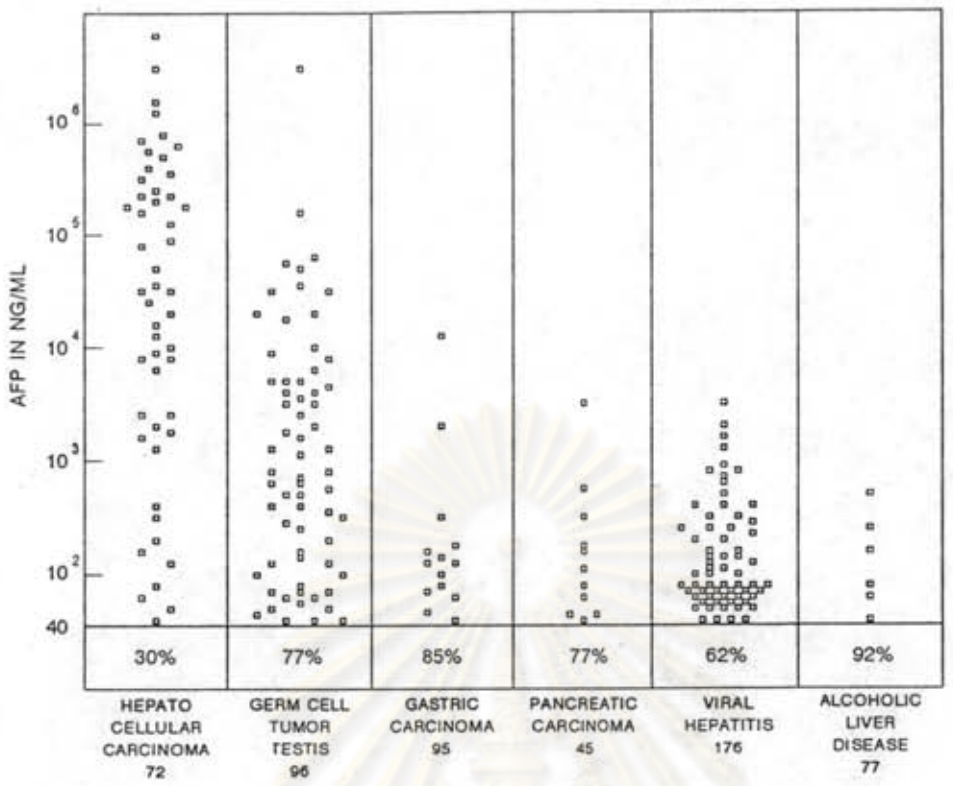

Figure 3 Comparison of AFP levels in four types of cancer and two non malignant liver diseases

A second benign liver disorder in which transient elevations in AFP concentration may be observed is cirrhosis(48,49). It is considered to be one of the precancerous conditions, and the majority of cases of liver cancer have a background of cirrhosis. Individual with cirrhosis should be regarded as a high risk population for the development of a liver tumor(50,51). Only in the case of primary liver cancer are the levels persistently high. Transient moderate elevations are observed in patients with acute viral hepatitis type B or with liver cirrhosis (Figure 3). An investigation of the concentration of serum AFP in such individuals may not only be important from a

clinical standpoint, but also to evaluate this possibility by performing a long term longitudinal study.

3.4. Alpha fetoprotein in fetal development anomalies

In the earliest stages of pregnancy, the highest concentration of AFP is in the amniotic fluid, whereas AFP in the fetal serum reaches a peak at the beginning of the second trimester; both then diminish in concentration through the remainder of the pregnancy (Figure 4). Abnormally high concentrations of AFP are found in the amniotic fluid and maternal serum of most women bearing a fetus with an open neural tube defect (NTD)(52,53). Most closed NTDs are not accompanied by abnormally high AFP concentrations and therefore are not detectable by AFP testing(54). The optimal practical time for detecting NTD by measuring maternal serum AFP (MS AFP) is at 16-18 weeks of gestation(55). MSAFP levels normally rise during pregnancy from a normal, non pregnant level of 0-20 ng/ml to a mean level of about 250 ng/ml at 32 weeks gestation(56). The AFP estimation in maternal serum can not serve as a specific diagnostic test but it seems to be a useful screening test so as to select certain symptom-free women for further diagnostic procedures(55). Several other conditions have been reported in which raised levels of AFP in amniotic fluid and/or maternal serum may be found such as multiple pregnancy, congenital nephrosis, chromosome aberrations, sacrococcygeal teratoma, Rhesus hemolytic disease and others(57).

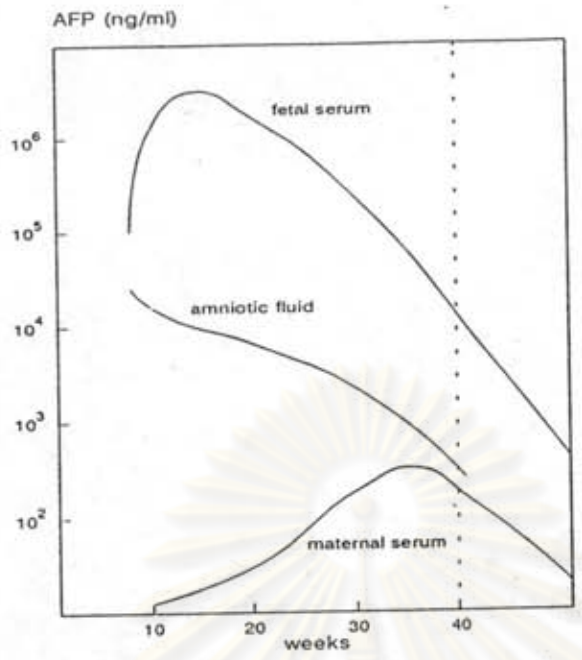

Figure 4 AFP concentration in fetal serum, amniotic fluid and maternal serum during normal pregnancies

More recently, AFP measurement has been used simultaneously to assess risk of Down's syndrome during pregnancy(58,59). While the former application screens for abnormally elevated values, the latter is based on values that may be low for any given gestational age. The reduced MSAFP and amniotic fluid AFP in Down syndrome are due to a decreased level of AFP in the fetus(60). Assuming the reduction in concentration of AFP does not vary with gestational age, then any reduction in fetal AFP will be more apparent by measurement of MSAFP in the second trimester when the contribution from fetal AFP to the total MSAFP will be proportionally higher (90-95%)(56).

4. Analytical Assay

In 1963 Abelev(61) et al developed the AFP assay technique using immunodiffusion. This technique is based on the principle that antigen and antiserum diffuse through a semisolid medium and form visible precipitin line. It is simple and practical but suffer from low sensitivity. At the level of sensitivity of this technique (1-3 ug/ml)(62), 50% to 80% of hepatoma patients can be detected(62,63).

In 1971 Ruoslahti(36) and Seppala described the determination of AFP using radioimmunoassay (RIA). It is one of a group of assays based on the principle of competition between a radioactively labelled AFP and unlabelled AFP for a binding protein. The binding protein in RIA is a specific antibody and antigen. The sensitivity of RIA is 5 to 10 ng/ml(34). It is capable of detecting trace amounts of AFP in normal adult serum and also assigns numerical values in AFP study(64,65).

In 1982 Belanger's group(66) developed enzyme immunoassay (EIA) to measure the quantitation of AFP. This technique is analogous to those in use for radioimmunoassay except that the molecule to be titrated is labelled with an enzyme instead of a radioisotope(67,68). The sensitivity of the assay depends largely on the amount of tracer required for proper signal detection. The present end point photometric conditions place the limit of sensitivity to around 5 ug/ml which suits all AFP levels of clinical interest(69,70).

4.1. Immunoradiometric Assay

In 1968, Mile and Hales(71) described an immunoradiometric assay (IRMA) which employs similar reagents and techniques but is fundamentally different in that a radiolabelled antibody (*Ab) is used to prepare a radiolabelled derivative (*AbAg) of the antigen (Ag) which is then separated from excess *Ab and measured directly in a gamma counter. This technique is summarized below (Figure 5).

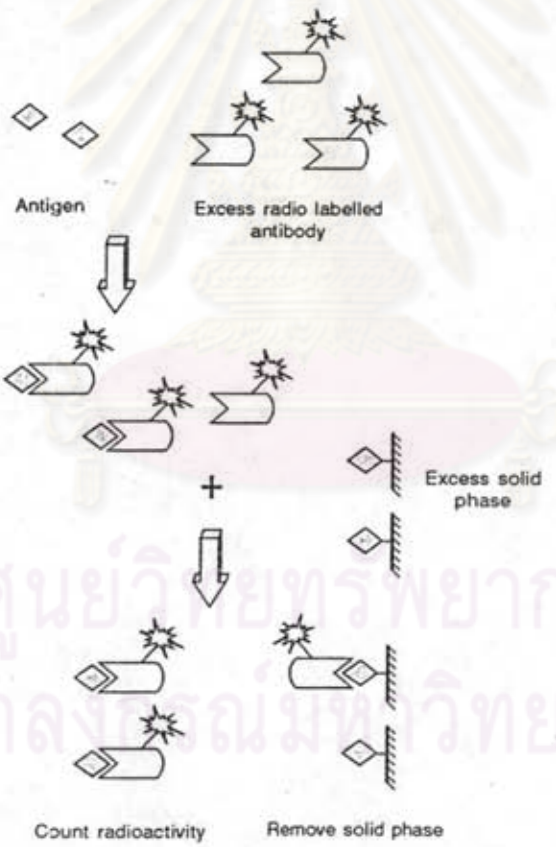

Figure 5 Immunoradiometric assay technique

4.2. Two-site immunoradiometric assay

IRMA may also be performed by the two-site immunoradiometric method. This procedure is illustrated in Figure 6. In the two-site immunoradiometric assay the complex of radiolabelled antibody and antigen is precipitated from the reaction mixture by the addition of a solid-phase antibody directed towards the second antigenic binding site on the antigen. Because of the requirement for two binding sites this refinement is termed the two-site IRMA.

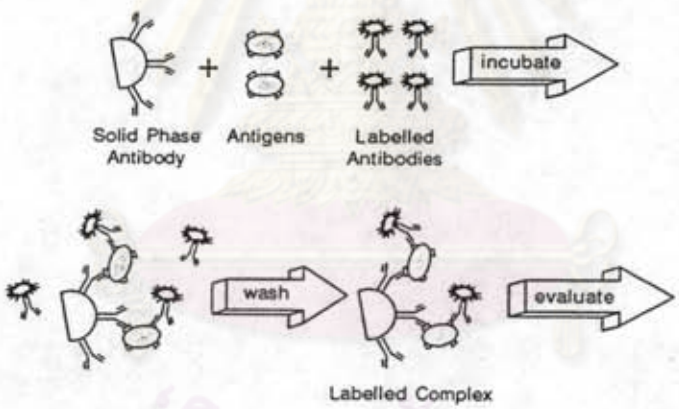

Figure 6 Two-site immunoradiometric assay

There are three designs that take advantage of the two-site concept. First, and most common is the " forward two step " method. Figure 7 illustrates this procedure. First, the antigen is allowed to react with the solid-phase adsorbent. After an appropriate incubation period, unbound antigen is removed by washing. Labelled antibody then reacts with the bound antigen during the second step. A subsequent buffer wash removes the unbound labelled antibody, and the label associated with the solid phase is detected proportional to the amount of antigen originally in the specimen. Monoclonal antibodies used in this " forward two-step " assay have a distinct advantage over polyclonal antisera : there is no need to affinity-purify the antibodies for use either as the solid phase or the label.

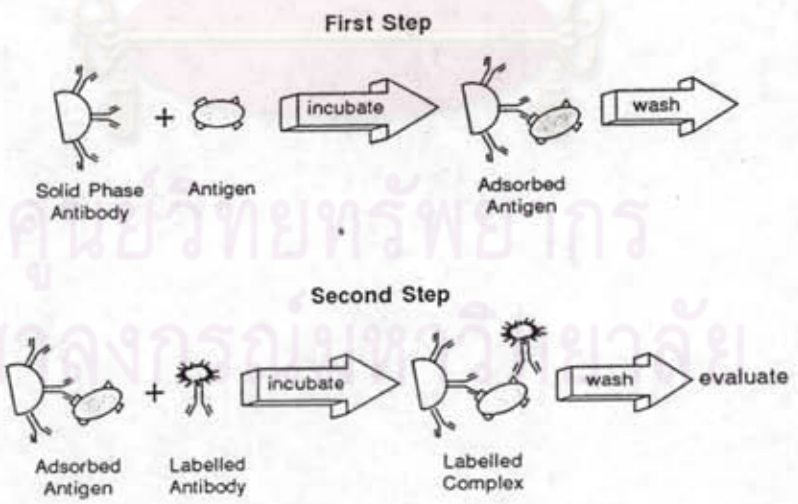

Figure 7 Forward two-step immunometric assay

The second immunometric design is the " reverse two-step " method. Here the antigen reacts with the labelled antibody in solution phase first (Figure 8). After an appropriate incubation, the solid phase antibody is added. A wash follows the incubation and again the amount of antigen originally present in the specimen is related to the quantity of label associated with the solid phase. In this system, monoclonal antibodies again enjoy a distinct advantage. During the first incubation, polyclonal labelled antibody will bind most sites on the antigen, rendering it less accessible to the solid phase, while the monoclonal antibody will bind a single site and leave the rest of the antigen free to bind the solid phase.

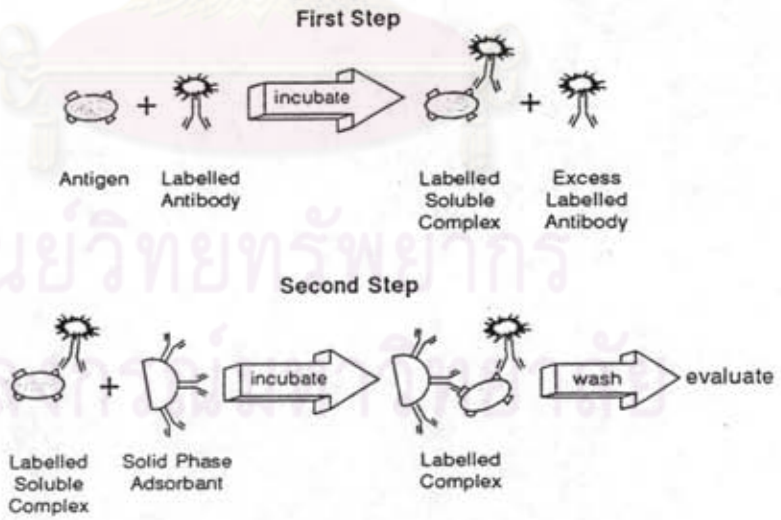

Figure 8 Reverse two-step immunometric assay

The third design, the " simultaneous " procedure, is by far the most simple (Figure 9) As the name implies, all the reagents and the specimen are introduced together in the reaction vessel. A single incubation and a single wash-step readies the solid phase for analysis. This design, like the " reverse two-step " system, will function better with monoclonal reagents, owing to the lack of competition between the binding sites. In addition, the higher-capacity monocolnal solid-phase antibody delays the saturation effect at extremely high antigen concentrations. This " high dose hooking effect " of the response may severely limit the use of low-capacity solid-phase adsorbents made with polyclonal antiserum and used in a simultaneous assay. While some clinically important molecules (e.g.large, polyvalent antigens) may be quantitated by using polyclonal antisera in the simultaneous assay, monoclonal-based reagents allow the general application of this procedure to all but monovalent antigens.

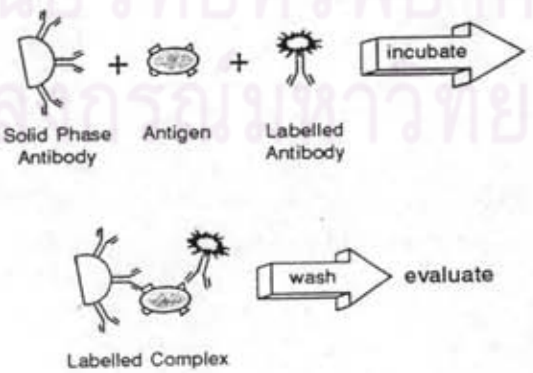

Figure 9 Simultaneous immunometric assay

4.3. Advantages of labelled antibody assays

In addition to continuous supply and homogeneous binding properties, labelled monoclonal antibodies used in immunometric assays circumvent major problems encountered with labelled antigens used in conventional competition immunoassay(72). First, antigen is measured directly, as opposed to estimates based on the antigen's ability to compete with a labelled antigen for a combining site. Second, precision relates essentially only to the addition of the specimen (other reactants including the labelled antibody are in excess) rather than a multiple function (addition of the specimen, the labelled antigen, and the antibody) as found in competition immunoassay. Thirdly, labelling the highly purified, sometimes precious, and possibly labile antigens for use as competitors is no longer required. This eliminates possible damage to the antigenic site, as well as shelf-life problems associated with the instability of the radiolabelled antigens.

Antibodies

The antibody as the specific reagent in immunoassay is crucial to performance. An antiserum with high specificity and a high affinity constant (K value) will confer the attributes of good specificity and sensitivity on any assay in which it is used. As the quality of the antibody will be more relevant to assay performance than the quality of any other reagent, it will be useful to critically evaluate the

techniques used to produce and prepare antibodies(73).

The production of antibodies is a specific event in the more general phenomenon known as the immune response. Following immunization or stimulation of the immune response with an immunogen, antibodies produced by the B lymphocytes will circulate in the blood system and may be present for periods of time from weeks to months. Serum taken from the blood of an immunized animal is called antiserum.

1. Properties of antibodies

1.1. antibody combination sites

The sites on antibody molecules which have specific binding activity for antigen determinants are antibody combining sites(73) (antigen binding sites). They are constructed from the folding of variable domains of light and heavy chains of antibody molecules to form three-dimensional binding species with an internal surface shape and charge distribution complementary to these features of antigen determinants.

1.2. antibody affinity

Operationally, antibody affinity relates to the exactitude of stereochemical fit of an antibody combining site to its complementary antigen determinant. This can be considered in thermodynamic terms as the strength of close range non-covalent force interactions between receptor (combining site) and ligand (determinant). Mathematically, affinity is expressed as an association

constant (K, L/mole) which may be calculated under conditions where equilibrium can be achieved between ligand bound and unbound in its reversible interaction with a homogeneous source of combining site. As most antibody preparations are heterogeneous in affinity towards a single antigen determinant, the derived association constant represents an average value. Antisera of multiple specificity, that is specific to many determinants on an antigen, cannot be assessed for affinity.

1.3. antibody specificity : cross reactivity

Each antibody molecule is specific to the antigen determinant to which it binds--this represents the most closely--defined level of specificity. The specificity of an antiserum, by contrast, reflects the many specificities of the constituent antibodies. Some antigen determinants are shared between molecules-especially if these are similar molecules of related species. In this case some antibodies induced in response to one antigen may bind to another antigen and are then said to be cross reacting.

2. Properties of polyclonal and monoclonal antibodies

In exploiting the specific binding properties of antibody one has a choice between two vary different forms of reagent-these are conventional polyclonal antisera and monoclonal antibodies (Mabs). These forms of reagent are prepared in a different way for requirement of specificity, the choice between them is often not arbitrary but depends

the choice between them is often not arbitrary but depends to a large degree upon matching the special properties of the antibodies to the assay requirement.

2.1. Polyclonal antisera

A polyclonal antiserum is the conventional serum product of an immunized animal. It contains many different antibody specificities to the various epitope of the structurally complex immunogen (Figure 10). The major advantage of polyclonal antisera lies in their capacity to form large insoluble immune complexes with antigen, so that the reactions can be seen and measured visually or determined photometrically(74). For all their value, animal antisera have certain limitations for exploitation in immunoassay, the main one being their heterogeneity in specificity, ever when reacting to small antigens and their variability between animals and batches. The significance of such heterogeneities within and between polyclonal antisera in practice means that each product is unique in specific antibody composition, in optimal binding conditions and in performance and requires to be separately assessed for its suitability in any particular immunoassay.

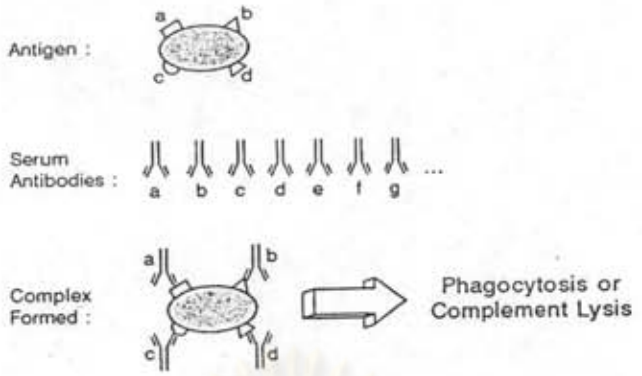

Figure 10 Polyclonal response to antigenic intruder.

2.2. Monoclonal antibodies

Hybridoma technique for the production of antibody of predefined specificity represents a recent major breakthrough in the field of immunological research. It has been successfully applied for the determination and analysis of a number of proteins and polypeptides with biological significance. In 1975, Kohler and Milstein(6) made the first monoclonal antibody of predefined specificity by fusing the right combination of cells : a spleen cell with the genetic information to produce specific antibody and a selectable myeloma cell that was adapted to continuous and vigorous growth in culture.

The general technique(75) (Figure 11) starts essentially the same as polyclonal antiserum generation : a mouse is immunized with the antigen of interest. But spleen cells, rather than serum, are removed from the animal at a carefully defined time subsequent to a final

booster immunization and fused with myeloma cells(76) in the presence of polyethyleneglycol. The resulting "hybridomas" are selected-- first for their ability to grow in a medium containing aminopterin, which poisons the unfused myeloma parent cells, and then for the hybridoma's ability to produce antibody of desired characteristics(77). Each selected hybridoma, if carefully managed, can make large quantities of homogeneous antibody (Figure 12) with a definable affinity for a single antigenic determinant.

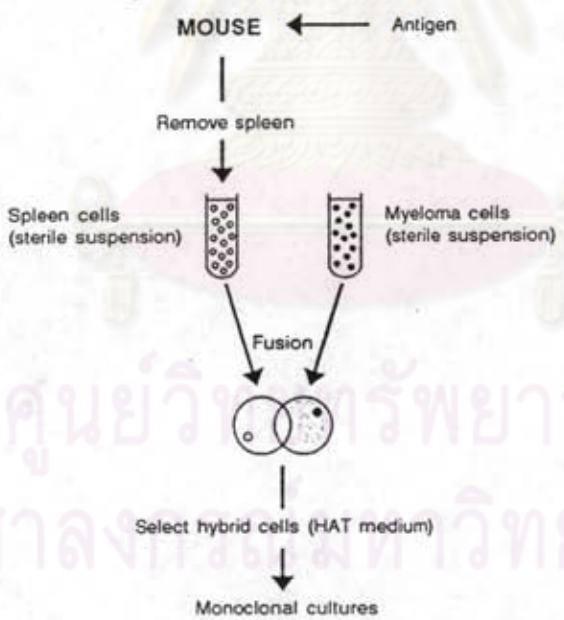

Figure 11 Production of monoclonal antibodies.

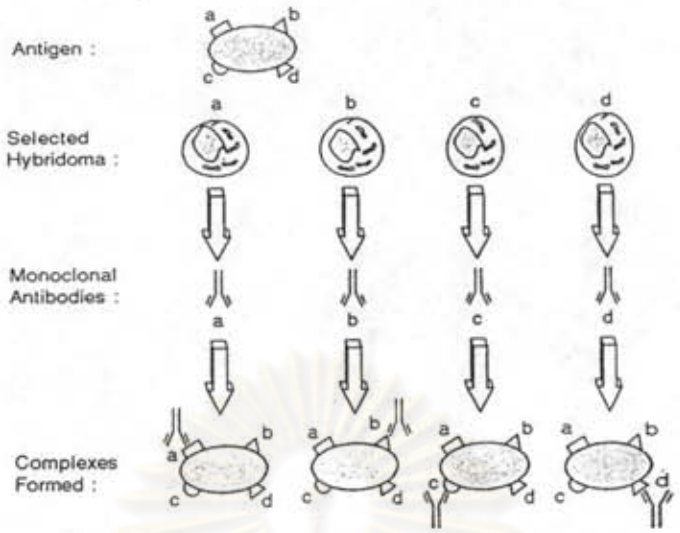

Figure 12 Monoclonal antibodies made by hybridoma cells.

3. Advantage of monoclonal antibody production

Monoclonal antibodies are produced by a colony of cells derived from a single hybridoma. Each cell contains identical genetic material derived from a single spleen cell and produces homogeneous immunoglobulin, which in turn recognizes a single antigenic determinant, and possesses a single affinity constant(77) for that determinant. The homogeneity of hybridoma--derived monoclonal antibodies should allow the development of highly selective immunodiagnostic(78) procedures that will be devoid of most problems associated with polyclonal sera. Hybridoma cell lines are easily generted, but selecting the proper clones and establishing them are quite difficult. A rapid and accurate screening procedure is the major requirement.

With hybridoma technology, a portion of any positive clone can also be frozen, in liquid nitrogen to avoid any possible loss of the cell line in culture(79). Due to stability of the frozen cell lines and stability of growth in appropriate culture medium, the supply of antibody is considered to be permanent. This means that antibodies can have the essential qualities that are common for many other analytical reagents.

In addition to the advantages already mentioned, the production of monoclonal antibody offers one considerable advantage. It is extremely useful for the production of specific antibodies to impure or mixed immunogens, unwanted reactivity may be eliminated by merely selecting against antibodies responsible for such cross reactivity during the screening phase.