1.0 Introduction

1.1 Natural Autoantibodies

1.1.1 Autoantibodies

The immune system is the major defence mechanism against substances that have gained entry. A substance capable of eliciting such a reaction from the immune system is called an antigen (Ag). One of the body’s reactions to the recognition of this antigen is to manufacture a protein called an antibody (Ab).

Antibodies (abs) are found in the globulin fraction of the proteins that circulate in the blood and are called immunoglobulins. Immunoglobulins (Ig) have a basic four-peptide structure of two identical heavy and two identical light chains joined by interchain disulphide links (Appendix 1). Based upon the structure of their heavy chain constant regions, immunoglobulins are classed into major groups termed classes which may be further subdivided into subclasses. In the human, for example, there are five classes: immunoglobulin G (IgG), IgA, IgM, IgD and IgE. They can be distinguished by differences in their constant (C) regions. Physical & biological properties of major immunoglobulin classes in the human are illustrated in Table 1.1 a, b.
Table 1.1 a: Biological Properties of Major Immunoglobulin Classes in Human

<table>
<thead>
<tr>
<th>Ig</th>
<th>IgA</th>
<th>IgD</th>
<th>IgE</th>
<th>IgG</th>
<th>IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biological properties</td>
<td>Secretory Ig at mucosal surface and in breast milk</td>
<td>Marker for mature B cells</td>
<td>Allergy, anti-parasite responses</td>
<td>Secondary antibody for most anti-pathogen responses</td>
<td>Primary antibody responses, marker for immature &amp; mature B cells</td>
</tr>
</tbody>
</table>
Table 1.1 b: Physical Properties of Major Human Immunoglobulin Classes

<table>
<thead>
<tr>
<th>Ig</th>
<th>IgA</th>
<th>IgD</th>
<th>IgE</th>
<th>IgG</th>
<th>IgM</th>
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</thead>
<tbody>
<tr>
<td>Molecular weight (KD)</td>
<td>160</td>
<td>185</td>
<td>200</td>
<td>150</td>
<td>950</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td></td>
<td></td>
<td></td>
<td>1150</td>
</tr>
<tr>
<td>No. of basic four-peptide units</td>
<td>1.2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Heavy chains</td>
<td>γ</td>
<td>α</td>
<td>μ</td>
<td>δ</td>
<td>ε</td>
</tr>
<tr>
<td>Light chains</td>
<td>κ + λ</td>
<td>κ + λ</td>
<td>κ + λ</td>
<td>κ + λ</td>
<td>κ + λ</td>
</tr>
<tr>
<td>Concentration range in normal serum</td>
<td>1.4-4 mg/ml</td>
<td>0-0.4 mg/ml</td>
<td>17-450 ng/ml</td>
<td>8-16 mg/ml</td>
<td>0.5-2 mg/ml</td>
</tr>
<tr>
<td>% total immunoglobulin</td>
<td>13</td>
<td>0 - 1</td>
<td>0.002</td>
<td>80</td>
<td>6</td>
</tr>
<tr>
<td>Subclasses</td>
<td>IgA₁, IgA₂</td>
<td>None</td>
<td>None</td>
<td>IgG₁, IgG₂, IgG₃, IgG₄</td>
<td>None</td>
</tr>
<tr>
<td>Forms</td>
<td>Monomer</td>
<td>Monomer</td>
<td>Monomer</td>
<td>Monomer</td>
<td>Pentamer</td>
</tr>
<tr>
<td></td>
<td>Dimer</td>
<td></td>
<td></td>
<td></td>
<td>Hexamer</td>
</tr>
</tbody>
</table>
Antibodies that can react with a large variety of cellular and humoral constituents are present in normal sera and it is a common concept that the development of antibodies against an autoantigen or drug is always undesirable. There are some antibodies that are present in the serum of healthy individuals in the absence of deliberate immunization with any antigen, they are referred to as natural antibodies (Coutinho et al., 1995). A vast majority of natural antibodies react with one or more self-antigens and are termed natural autoantibodies (NAA). Natural polyreactive autoantibodies (pNAA) are an important component of the normal B cell repertoire (Guilbert et al., 1882). They are present in sera of normal mammals and in lower phylogenetic species.

One intriguing characteristic of these Abs (NAA) is their binding to various dissimilar molecules (antigens, Ags) such as proteins, nucleic acids, polysaccharides (Dighiero et al., 1985; Prabhakar et al., 1984). It has been generally assumed that these Abs bind Ags with low affinity. The broad specificity of NAA may play a major role in primary defence against invading agents before specific Abs are produced by the immune system.

Autoantibodies are a hallmark of organ specific autoimmune diseases and paraneoplastic syndromes. Cell biologists have used autoantibodies as probes to define the structure and function of novel macromolecules. Some autoantibodies are disease specific markers and are an aid to establishing a diagnosis. Although it has been difficult to link autoantibodies to pathogenesis, they have been used to predict disease progression and outcome (Fritzler, 1997). NAA may be useful in their own right, that they may help the avoidance of autoimmune disease by binding the immune system to environmental epitopes cross-reactive with self (Cohen and Cooke, 1986).
1.1.2 Characteristics of Natural Autoantibodies

To study the autoreactivity of human NAA in a systematic way, it is necessary to study their interactions with a large panel of antigens including highly conserved antigens, such as actin, tubulin, myosin and cytochrome c. Analysis of antibodies isolated by adsorption to these antigens shows that they are of the IgM, IgG and IgA isotypes and that most can react with more than two antigens, although monospecific antibodies reacting with a single antigen are also present. A few were specific to only one antigen, whereas most antibodies could recognize two or more antigens (Avrameas, 1991). The bulk of NAA are IgM and have a short life in the body suggesting that they must be secreted continually. It has been shown without ambiguity that in humans, IgG NAA display low \(5 \times 10^{-13} \text{ M}\) to very high \(5 \times 10^{-11} \text{ M}\) affinity for various self antigens. Secretory immunoglobulin A (S-IgA) has been found in human secretions, acting as the first "immune barrier" to infection before induction or boosting of specific response. Significant levels of S-IgA Abs to human actin, myosin, tubulin, and spectrin have been found in normal human serum (Quan et al, 1997).

It has been reported that NAA exhibite low affinities and high avidities for self antigens (Nakamura et al; Ternynck 1986). NAA may exhibit a broad range of affinities with dissociation constant ranging from \(10^{-5}\) to \(10^{-8} \text{ M}\) (Bendtzen et al, 1990; Avrameas, 1991, Diaw et al, 1997). NAA with low affinity still can bind strongly when they bind to more than one epitope at the same time. This shows that NAA exhibit a high degree of avidity for self antigen.
The NAA characterized so far are, however, polyreactive in that the antibodies are capable of recognizing several self and 'foreign' antigens in man (Elson et al, 1979, Rossi et al, 1990). Depending on their specificity, natural IgG autoantibodies which have been affinity purified from healthy human serum exhibit similar or higher degrees of polyreactivity as compared with autoantibodies from patients with autoimmune diseases (Hurez et al, 1993). Polyreactivity of NAA towards self antigens does not correlate with their connectivity, i.e. their ability to interact with variable regions of other autoantibodies (Rossi et al, 1990; Hurez et al, 1993. Polyreactivity does not mean lack of specificity thus, each polyreactive NAA encompasses its own distinct set of epitopic CD5+ B cells which has been shown to be preferentially involved in the synthesis of these polyreactive Natural Autoantibodies (pNAA) but CD5+ B cells also participate in their production.

1.1.3 Functions of Natural Autoantibodies

Several functions have been proposed for NAA under physiological conditions (Table 1.2). NAA are involved in the natural 'non-specific' host defense against infection (Boyden, 1965) and thus serve as opsonins for pathogens (Michel et al, 1990). By binding to self constituents altered through the aging process, NAA contribute to the clearance of catabolic products from the organism. NAA bind to pathogens as a consequence of polyreactivity or cross-reactivity between self and 'foreign' epitopes.

NAA also serve in the clearance of metabolic waste and senescent cells. The role of natural IgG anti-band 3 autoantibodies for the clearance of senescent erythrocytes in healthy individuals has been extensively substantiated (Lutz et al,
1987). It has also been suggested that NAA endowed with anti-IgG Fc specificity facilitate the clearance of soluble immune complexes from the circulation. By binding to constant or variable regions of IgG and/or to available epitopes on antigens complexed with antibody, NAA modify the size, clearance and phylogistic potential of circulation immune complexes.

A role for NAA in immune surveillance against cancer has been hypothesized (Greenberg et al, 1983). Thus, the binding of NAA to cell surface antigens on malignant cells enhances or retards tumor development (Cahalon et al, 1992).

Another function of NAA is that they bind to antigens and thus modulate the processing of antigens and their subsequent presentation to T cells (Thornton et al, 1994). It has been shown by Boyden (1965) and Jerne (1984) that NAA function primarily to control autoreactivity and immune homeostasis in healthy individuals. As NAAbs exist that react with a variety of mediators and their receptors (cytokines, hormones, coagulation factors, etc), natural antibodies (NAAbs) could constitute a general homeostatic system that 'buffers' rapid changes in the concentrations of biologically active molecules and messages.

NAA play a role in preventing the occurrence of pathological autoimmunity by binding to microbial epitopes that are similar or identical to self (Cohen and Cooke, 1986).
Table 1.2: Functions of Natural Autoantibodies

- First line defense against infection
- Clearance of aged cells
- Antigen presentation to T cells
- Anti-tumoral surveillance
- Anti-inflammatory activity
- Selection of immune repertoires and homeostasis of autoreactivity
1.1.4 Therapeutic Role for Natural Autoantibodies

Intravenous immunoglobulin (IVIg) is a therapeutic preparation of pooled normal polyspecific human IgG obtained from large numbers of healthy donors. IVIg contains the wide spectrum of NAA and 'immune' antibodies expressed in normal human serum. Some of the examples of self antigen recognized by NAA in IVIg are CD4, CD5, idiotypes regions of antibodies, MHC class I molecule, autologous blood group antigen, etc. IVIg has been shown for the treatment of primary and secondary antibody deficiencies. Nowadays, IVIg is broadly used in the autoimmune diseases, namely autoimmune thrombocytopenic purpura and systemic inflammatory disorders (Kazatchkine et al, 1994; Dalakas 1997). The immunoregulatory properties of IVIg are primarily dependent on the variable regions (V regions) of infused antibodies. Variable region-dependent effects of IVIg include:

- the neutralization of pathogenic antibodies by anti-idiotypes present in IVIg
- the neutralization of superantigens
- the long-term control of the expansion and activation of lymphocyte subsets, and selection of immune repertoires.
1.2 Adrenal Hormones

Hormones are molecules synthesized and secreted by endocrine glands, acting on target cells in a manner specific to interaction of the molecule and the target and are released directly into the blood stream. These hormones exert profound effects on a wide range of processes related to basic bodily functions. The chemical activity of a hormone reflects its chemical structure.

The adrenal gland consists of two distinct organs, the adrenal medulla and the adrenal cortex. They are located next to the kidneys. They produce different hormones, but two hormone groups are involved in responses to stress - the medulla produces two peptides, adrenaline and noradrenaline, under nervous control (these are peptide hormones - fast response) to the muscle. They increase heart rate, raise blood sugar (ready availability of energy), and channel blood to the muscles.

The adrenal cortex produces steroids, being steroids, this is a long-term response to stress under central nervous system (hypothalamus) control. More than 40 steroids have been isolated from the adrenal cortex. It produces hormones that promote the release of sugar stored in the liver, and regulate the excretion of sodium and potassium. Hormones of the adrenal cortex are closely related structurally to cholesterol, which is a precursor of all the adrenal cortical hormones.

The reaction sequence of adrenal cortical hormone synthesis (Figure 1.1) from cholesterol is initiated when cholesterol is cleaved to yield a 21 carbon compound with a ketone group.
Figure 1.1: Pathways involved in the synthesis of major classes of adrenal hormones
Cholesterol is an amphipathic lipid. It is an extremely important biological molecule that has roles in structural component of membranes and of the outer layer of plasma lipoprotein. Apart from this, it is a precursor for the synthesis of steroid hormones, such as corticosteroids, sex hormones, bile acids and vitamin D. The parent compound from which all the steroid hormones are ultimately derived is cholesterol. This white, soft, waxy, crystalline compound has been known for many centuries and was originally isolated from gall stone; hence the name cholesterol, from chole, meaning bile and steros, solid. Cholesterol is a constituent of virtually every animal tissue (in the form of cholesteryl ester) and occurs partly as the free alcohol and partly esterified with the higher fatty acids. Elevated levels of cholesterol present as very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), or low density lipoprotein (LDL), and is associated with atherosclerosis, whereas high levels of HDL have a protective effect.

Cholesterol is like other fat-like substances in that it will not mix with water. Therefore, to carry cholesterol and fat ("lipid") in the blood, the body wraps them in protein packages. This combination is called a "lipoprotein". Lipoprotein transports free cholesterol in the circulation. It circulates in the blood and is essential to life for the formation of cells, production of hormones, and protection of joints and nerves.

Its chief role in pathologic process is as a factor in the genesis of atherosclerosis of vital arteries, causing cerebrovascular disease, coronary and peripheral vascular disease. Cholesterol comes from two sources. It is produced in our body, mostly in the liver (about 1,000 mg a day), and it is found in foods that come from animals, such as meats, poultry, fish, seafood and dairy products. Foods from plants (fruits, vegetables, grains, nuts and seeds) do not contain cholesterol.
Aldosterone is the most potent natural mineralocorticoid produced by the adrenal cortex and made exclusively in the zona glomerulosa of the suprarenal gland. It does not have a specific plasma transport protein. The half-life of aldosterone in normal subjects has been calculated as 14 minutes during the initial phase of rapid distribution in plasma and 35 minutes during the phase of slow diffusion.

Aldosterone is concerned primarily with electrolyte metabolism. Metabolism occurs mainly in the liver and a large number of aldosterone metabolites have been identified. It acts mainly on the sodium ion and by so doing maintains the level of sodium chloride in the body. Any fall in the level of circulating aldosterone will result in a decreased tubular reabsorption of sodium chloride and water and an increased reabsorption of potassium. Recently, natural antibodies have been suggested to also function as carriers (i.e. immunotransporters) for important bioactive molecules. Thus there could be an essential role in immunoregulation of physiologic functions.

Acetylcholine is a small molecule and an important neurotransmitter found throughout the central nervous system, autonomic nervous system, and at neuromuscular junctions (between motor neurons and muscle cells). Anything that interferes with the action of acetylcholine can produce paralysis. It functions as a neurotransmitter, sending electrical impulses between nerve cells and from nerve cells to muscle cells, causing the muscle cells to contract. It is associated with the regulation of numerous body processes including memory.

Angiotensin II is very important in the regulation of blood pressure. It has long been regarded as the major active component of the renin-angiotensin system. After its
demonstration in 1939 it was isolated, purified and sequenced by Skeggs, Peart and Bumpus between 1954 and 1957. Initially angiotensin was thought to have only powerful vasoconstrictor action, however, a number of other actions have since been attributed to it. Angiotensin II is degraded in peripheral capillaries with an extremely short disappearance time, about 30 seconds, which is more rapid than can be accounted for by circulating angiotensinases. It increases blood pressure by causing vasoconstriction of the arteriole and is a very potent vasoactive substance. It inhibits renin release from the juxtaglomerular cells and is a potent stimulator of aldosterone production.

Angiotensin II has been shown to stimulate the adrenal medulla, sympathetic ganglia and a number of sympathetic transmitter functions each tending to potentiate the effect of catecholamines. Angiotensin II directly inhibits the secretion of renin and may play a role in renal sodium reabsorption and synthesis of renal prostaglandins. This small peptide binds to cell surface receptors, initiating a wide diversity of physiologic responses. There are two major subtypes of angiotensin II receptors referred to as AT1 and AT2. Antibodies against AT1 receptor have first detected by Bernstein & Berg. 1993.

All components of the renin-angiotensin system are found in the brain and there is good evidence for Angiotensin II receptors in areas on the brain where the blood-brain barrier is absent. The effects of intraventricular angiotensin, which may stimulate drinking behaviour and vasopressin release, may represent only pharmacological actions.
Androstenedione is a natural androgenic pro-hormone which is naturally produced in the bodies of both men and women. It is a metabolite of dehydroepiandrosterone (DHEA) that serves as a direct precursor (one step removed) in the biosynthesis of testosterone. In all mammals, androstenedione is produced in the gonads & adrenal glands. This hormone increases the blood levels of testosterone and bring about effects such as increased energy and enhanced recovery result.

Androstenedione profoundly influences the development and expression of sexual and aggressive behaviour. The neural basis of these effects is, however, poorly understood. It has been shown that androstenedione and testosterone treatment were equally effective in preventing the reduction of vasopressin immunoreactivity associated with castration. Androstenedione may therefore be able to mimic the effects of testosterone on testo-responsive neural systems (Villalba et al, 1999).

Adrenaline is found in the central nervous system, especially in neurons whose cell bodies are in the brain stem. It is synthesized in the chromaffin cells of the adrenal medulla. The major product of the adrenal medulla is adrenaline. This compound constitutes about 80% of the catecholamines in the medulla, and it is not made in extramedullary tissue. Normal adrenaline value in plasma is <100 pg/ml. This plays a major role in sympathetic stimulation for the onset of acute arterial thrombosis (Goto et al, 1996).

Adrenaline is involved in a number of neuropsychiatric disorders and in hypertension. Many therapeutic agents have been devised on the basis of their ability to alter the synthesis, metabolism, or action of the catecholamines at their respective receptors.
Cortisol is the major member of the group of hormones known as glucocorticoids, produced by the zona fasciculata and zona reticularis of the cortex of the suprarenal gland. Cortisol is needed by the body to keep up normal energy levels, deal with physical stress and keep the balance of fluid and electrolytes. An excess of cortisol leads to a high blood glucose level and diabetes mellitus. The adrenal cortex of the normal human adult secretes about 20 mg of cortisol per day. Cortisol reduces the number of circulating eosinophils, consequently allergic response to aeroallergens. There is a greater need for cortisol during times of illness or physical stress.

**Estrogen**: A subpopulation of IgG in man interacts with the hormone-binding site of estrogen receptors (ER), competes with estradiol (E2) uptake, and decrease effective ER concentrations in cell cultures. Some natural antibodies in man can function like potent estrogens on ER and mammary cells (Borkowski et al, 1991).

**Noradrenaline** is a catecholamine, which is synthesized in the chromaffin cells of adrenal medulla from the amino acid tyrosine. It is found in the central nervous system and in the sympathetic nervous system. Most of the noradrenaline present in organs innervated by sympathetic nerves is made in other nerve endings and reaches the target sites via the circulation. It is important in increasing lipolysis in the tissue. Noradrenaline synthesis increases after acute stress. In normal plasma the value of noradrenaline is <500 pg/l.

**Progesterone** is a C 12 steroid secreted by the corpus luteum and the placenta. It is an important intermediate in steroid biosynthesis in all tissues that secrete steroid
hormones. Mainly the ovaries and placenta in female animals produce progesterone during the period when they are able to bear young. Progesterone is secreted into the bloodstream by luteal tissue during its short life span. Secreted progesterone is probably bound to protein, although little is known about the details of the binding. The circulating level of progesterone, like that of estrogens, is very low. This hormone can also be produced by the adrenal glands in both the females and males and by the testes in males. It plays an important role in the normal menstrual cycle and in pregnancy. When the ovum leaves its follicle in the ovary, the follicle is transformed into the corpus luteum. The corpus luteum manufactures progesterone, apparently from cholesterol. Progesterone then acts upon the uterine lining, which has already been primed by the female sex hormone, estrogen, in preparation for the egg. If the egg is not fertilized, progesterone production ceases, menstruation occurs, and the corpus luteum degenerates; if the egg is fertilized, the corpus luteum continues to secrete progesterone. This maintains pregnancy, prevents egg for maturing in ovary and inhibits menstruation. The compound 17-α- hydroxyprogesterone, plays a vital role in the ovarian synthesis of estradiol and estrone.

**Testosterone** is a hormone produced by men and women. It is not just a male sex hormone. It is primarily responsible for maintenance of the male reproductive system. In men, testosterone is primarily produced in the testes and to a lesser extent in the adrenal glands. In women, testosterone is produced in the ovaries, adrenal glands and to a lesser extent in the skin, brain, and liver. It belongs to a family of hormones called androgens. Testosterone is a steroid compound, which also promotes masculinization. In men, testosterone builds muscle, enhances sex drive, elevates
mood, prevents osteoporosis and increases energies. In women, testosterone enhances the sex drive, helps relieve menopausal symptoms, restores lost energy, strengthens bone, elevates mood. Reports indicate that adequate levels of testosterone can help prevent heart disease, stroke and vascular disorders such as diabetic blindness.
1.3 Apolipoprotein E

1.3.1 Plasma Lipoproteins and Lipoprotein Metabolism

Plasma lipids, except free fatty acids (FFA), in the extracellular fluids are transported by lipoproteins. Lipoproteins are spherical macromolecular complexes in which hydrophobic molecules of triglyceride and cholesteryl esters are enveloped within a monolayer of amphipathic molecules of phospholipids, free cholesterol, and apoproteins. Four major groups of lipoproteins have been identified that are important physiologically and in clinical diagnosis. These are chylomicrons, very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL). Apolipoproteins, protein moieties of the lipoproteins (constituting nearly 60% of some HDL and as little as 1% of chylomicrons), play a vital role in stabilizing lipid particles and conferring specificity on the particles. Ten major human plasma apolipoproteins are Apo AI, Apo AII, Apo A IV, Apo B-48, Apo B-100, Apo CI, Apo CII, Apo CIII, Apo E and Apo (a). Apolipoproteins carry out several roles:

- They are enzyme cofactors.
- They can act as lipid transfer proteins.
- They act as ligands for interaction with lipoprotein receptors in tissues.
- They act as structural proteins for the biosynthesis and secretion of plasma lipoproteins.

Apolipoproteins interact with enzymes or receptors on cell surfaces resulting in degradation of the lipoproteins and the uptake of the products into the cells. These processes may not result in complete degradation but rather in the transformation of one type of lipoprotein into another. Abnormalities of lipoprotein metabolism occur at
the sites of production or utilization of lipoproteins, causing various hypo-or hyperlipoproteinemias.

Lipoproteins transport lipids in 3 separate ways, the exogenous pathway, the endogenous pathway, and the reverse cholesterol pathway. The first 2 of these routes, by which cholesterol and the triglyceride-rich lipoproteins (chylomicrons and very low density lipoproteins) are metabolized, are focused here (Figure 1.2).

**Exogenous fat transport**

Dietary cholesterol is absorbed into the intestinal wall and packaged into particles called chylomicrons. Protein, which comprises 1% of the weight of the chylomicron, resides on the surface of the particle and consists of the intestinal form of apo B, B-48, apo A I, apo A IV. In the circulation, chylomicrons acquire apo C II, which is required cofactor for the lipoprotein lipase (LDL) enzyme. Apo C II catalyses triglyceride hydrolysis in the lipid core of the chylomicron and results in smaller particles known as chylomicron remnants. Apo E is required for recognition of these remnants by the hepatic receptors, called chylomicron remnant receptors.

**Endogenous fat transport**

Lipoprotein transport begins with the synthesis of VLDL by the liver. VLDL, which contains apo B100, CII and E, is also converted by LPL to a smaller particle or remnant, called intermediate density lipoprotein (IDL). As IDL is converted to LDL, apo C and E are transferred to HDL. These remnants can be removed directly by the hepatic LDL-receptors through apo E binding, whereas LDL is removed by LDL-receptors binding to apo B100. About two-thirds of the LDL is believed to be removed by this mechanism; peripheral tissues involving so-called low-affinity pathways take up the other one-third.
Figure 1.2: Schematic diagram of lipid transport pathways. TG, triglyceride; CE, cholesterol ester; A: exogenous fat transport; B: endogenous fat transport (From reslow, J.L. *Physiological Reviews*, 1988).
1.3.2 Characteristics of the Apolipoprotein E

Apolipoprotein E (apo E) was first identified in 1973 in human VLDL (Shore and Shore, 1973). Apolipoprotein E is a plasma protein involved in the transport of cholesterol and other hydrophobic molecules. Apo E is produced in most organs. Significant quantities are produced in liver, brain, spleen, lung, adrenal, ovary, kidney, cerebrospinal fluid and muscle (Blue et al, 1983; Roheim et al, 1979). It is synthesized with the sialic acid attached by α-glycosidic linkage and is subsequently desialated in plasma. Human plasma apo E concentrations are in the range of 0.03 - 0.05 mg/ml. The characteristics of apo E is shown below in Table 1.3.
Table 1.3: Characteristics of the Human Apolipoprotein E

<table>
<thead>
<tr>
<th>Mole. Mass (daltons)</th>
<th>Amino acid residues</th>
<th>Function</th>
<th>Major sites of synthesis</th>
<th>Major Lipoproteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>34,000</td>
<td>279</td>
<td>Cholesterol Clearance</td>
<td>Liver</td>
<td>VLDL, HDL, Chylomicrons. Chylomicron remnants</td>
</tr>
</tbody>
</table>
Apo E is one of the major protein constituents of VLDL (10 - 20%), HDL (1-2%), chylomicrons and chylomicron remnants. Table 1.4, showing lipoproteins in plasma of human.

**VLDL or pre-β - lipoprotein**: Plasma VLDL is heterogeneous in origin, size, and composition (Elisa et al, 1992). The predominant lipid present in VLDL is triacylglycerol. The synthesis of triacylglycerol provides the immediate stimulus for the formation and secretion of VLDL. Most of the plasma VLDLs is of hepatic origin. They are the vehicles of transport of triacylglycerol from the liver to the extrahepatic tissues. It is also degraded by lipoprotein lipase. VLDL transports endogenous triglyceride and cholesterol in lipoprotein synthesis (Gotto, 1990). Factors that enhance both the synthesis of triacylglycerol and the secretion of VLDL by the liver include:

- the fed state rather than the fasting state.
- the feeding of diets high in carbohydrate.
- high levels of circulating free fatty acids.
- ingestion of ethanol.
- the presence of high concentrations of insulin and low concentrations of glucagon.

**HDL or α - lipoprotein**: HDL is called the "good" cholesterol. HDLs are believed to take cholesterol away from cells in the artery wall and transport it back to the liver for reprocessing or removal from the body. A major function of HDL is to act as a repository for apolipoprotein C and E that are required in the metabolism of chylomicrons and VLDL. HDL contains approximately 50% cholesterol and
phospholipid. Nascent HDL particles can take up cholesterol from the peripheral tissues, after which the enzyme lecithin cholesterol acyltransferase, or LCAT, converts the cholesterol to cholesteryl ester. The buildup of cholesteryl ester within the HDL particle in turn transforms the nascent HDL into a mature spherical particle. There are at least 4 different sources of HDL (Figure 1.3):

- The chylomicrons and VLDL, which may be considered together.
- The intestine, which secretes the apolipoproteins associated with phospholipid that constitute a nascent form of HDL.
- The liver, which also secretes a nascent form of HDL.
- The macrophages within the arterial wall and phospholipid particle may serve as another nascent form of HDL.
Figure 1.3: Origin of high-density lipoprotein cholesterol. There are at least 4 different sources: 1) the chylomicrons and very low density lipoprotein, 2) the intestine, 3) the liver, and 4) arterial wall macrophages. CE = cholesterol ester; LCAT = Lecithin cholesterol acyltransferase. (From Gotto, A.M., 1990; *Am J Cardiol*).
Chylomicrons and chylomicron remnants: Chylomicrons originate at the small intestine and circulate in the blood. The chylomicrons transport exogenous triacylglycerol and cholesterol from the intestines to the tissues. When labeled chylomicrons enter in the triacylglycerol, fatty acids are administered intravenously, some 80% of the label is found in adipose tissue, heart, and muscle and approximately 20% in the liver. This lipoprotein is hydrolyzed at the capillary surface by the enzyme lipoprotein lipase. Lipoprotein lipase catalyzes the hydrolysis of triglyceride in the lipid core of these particles, producing smaller particles known as remnants (Gotto, 1990). Chylomicrons remnants are about half the diameter of the parent chylomicrons. In terms of the percentage composition, chylomicron remnants are relatively enriched in cholesterol and cholesteryl esters compared with chylomicrons. They are usually taken up by the liver, and the cholesteryl esters and triacylglycerol are hydrolyzed and metabolized.
### Table 1.4: Lipoproteins in Plasma of Human

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Source</th>
<th>Diameter (nm)</th>
<th>Density</th>
<th>Protein %</th>
<th>Total lipid %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylomicrons</td>
<td>Intestine</td>
<td>90 - 1000</td>
<td>&lt;0.95</td>
<td>1 – 2</td>
<td>98 - 99</td>
</tr>
<tr>
<td>VLDL</td>
<td>Liver</td>
<td>30 - 90</td>
<td>0.95 - 1.006</td>
<td>7 – 10</td>
<td>90 - 93</td>
</tr>
<tr>
<td>HDL</td>
<td>Liver &amp; Intestine</td>
<td>10 - 20</td>
<td>1.063-1.125</td>
<td>33</td>
<td>67</td>
</tr>
</tbody>
</table>
Apo E has been implicated in many physiological processes, including plasma cholesterol and triglyceride homeostasis, immune response, protection against oxidation and the control of neuronal growth. Apo E is thus believed to play a significant role in the pathophysiology of Alzheimer's disease and in the onset and development of coronary artery atherosclerosis (Davignon et al. 1988).

1.3.3 Functions of Apolipoprotein E

- Apo E mediates binding, internalization, and catabolism of lipoprotein particles
- Main apoprotein of the chylomicron.
- It can serve as a ligand for the LDL (Apo B/E) receptor and for the specific Apo E receptor (chylomicron remnants) of hepatic tissues.
- It also inhibits mitogen-stimulated lymphocyte proliferation and gonadotropin-stimulated ovarian theca / interstitial cell androgen production.
1.3.4 Apo E Receptor Binding Domain contains a Four-Helix Bundle

Apo E is a 299-residue monomeric protein that consists of two independently folded domains: An N-terminal domain that binds strongly to the LDL receptor but only weakly to lipid and a C-terminal domain that binds to the lipoprotein surface but lacks affinity for the LDL receptor. Proteolysis of apo E yields fragments corresponding to apo E's N-terminal domain (residues 1-191) and C-terminal domain (residues 216-199). The X-ray structure of the N-terminal fragment (Figure 1.4) reveals that this domain consists mainly of five \( \alpha \) helices, four of which form an elongated (65 Å) four-helix bundle. The helices of the four helix bundle are strongly amphipathic with their hydrophobic residues sequestered inside the protein, out of contact with solvent, whereas their hydrophilic residues are solvent-exposed. The structure appears to be further stabilized by numerous salt bridges on the protein's highly charged surface.
Figure 1.4: A ribbon diagram of the receptor-binding domain of human apolipoprotein E. [Based on an X-ray structure determined by David Agard, In: Biochemistry, 1995].
1.3.5 Apolipoprotein E in Cerebrospinal Fluid

The presence of lipoproteins in human cerebrospinal fluid (CSF) was first reported by Swahn et al (1961). Roheim et al (1979) subsequently identified specific apolipoproteins in human CSF and demonstrated that these were associated with the d< 1.210 g/ml fraction. CSF particles are composed of approximately one-third protein, one-third phospholipid, and one-third cholesterol. The function of CSF lipoproteins is to assay the cholesterol efflux and influx. Lipoproteins decreased intracellular levels of cholesterol in cholesterol-loaded fibroblasts, suggesting these particles can act to remove excess lipids from degenerating cells and deliver lipids to cells for new membrane synthesis or storage (Rebeck et al, 1998).

The most abundant CSF apolipoproteins are apo A-I and apo E. Apo E in the human CSF is derived from astrocytes in brain and not from plasma (Swahn et al, 1961; Linton et al, 1991). Apo E containing CSF apo's as well as the molecular distribution of CSF Apo II are significantly related to APO E genotype but not to Alzheimer's disease (AD) (Larbi et al, 1998). In vitro studies have been shown that CNS lipoproteins are delivered to neurons via apo E dependent mechanisms, that oxidized lipoprotein may be neurotoxic, and that CNS lipoproteins are oxidized in Alzheimer's disease patients (Montine et al, 1997; Kivatinitz et al, 1997).
1.3.6 Apolipoprotein E Polymorphism and Atherosclerosis

In addition to the influence of common apo E variants on lipoprotein metabolism in healthy individuals, apo E variants are also associated with some diseases. It has been reported that apo E phenotype is not an independent risk factor for coronary artery disease (CAD); the apo E polymorphism influences lipoprotein levels and possibly, in that way, indirectly also the risk for CAD (Stuyt et al, 1991).

The observed impact of allelic variation at the apo E locus on plasma lipid and lipoprotein levels in normal and dyslipidemic individuals has led to the hypothesis that the apo E polymorphism plays a major role in determining susceptibility to atherosclerosis in man. Characteristics of atherosclerosis are illustrated in Table 1.5.
**Table 1.5: Characteristics of Atherosclerosis**

- Chronic inflammatory response of the vascular wall to a variety of events
- Endothelial dysfunction
- Monocyte adhesion and infiltration
- Smooth muscle proliferation
- Extracellular matrix deposition
- Lipid accumulation
- Thrombosis
The biological significance of the apo E polymorphism in humans has emphasized the importance of gene-gene and gene-environment interaction in the pathogenesis of atherosclerosis. The apo E polymorphism involves the coding region of the apo E gene and results in alterations of the gene product which, in turn, either directly or secondarily affect the metabolic fate of the lipoprotein particles (Davignon et al., 1988). When lipoprotein transport is abnormal, levels can change in ways that predispose individuals to atherosclerosis (Ginsberg, 1998).

The ε2 allele is associated with lower levels of LDL cholesterol, but predisposes to hyperglyceridemia; it could, therefore, play a protective role, provided no other condition interacted to raise the plasma levels of atherogenic cholesterol-enriched lipoprotein remnants, and offset its beneficial effect on LDL. On the other hand, the ε4 allele, which is associated with higher levels of LDL cholesterol, could favor the development of atherosclerosis. This hypothesis has been tested in survivors of myocardial infarction, in-patients with angiographically documented coronary heart disease.

It has been reported that atherosclerosis is associated with a cellular immune response in the arterial lesions and a humoral immune response directed towards oxidized lipoproteins, certain microbes and other antigens. It has been shown that B cells participate in atherosclerosis. These cells may accumulate through vascular cell adhesion molecule-1 expression by surrounding cells and may produce antibodies and proinflammatory cytokines. These factors are likely to be important in the pathogenesis of atherosclerosis (Zhou & Hansson, 1999).

The genetic polymorphism of apo E is also associated with the age of onset and relative risk of Alzheimer's disease (AD). It has been shown that apo E isoform
affect the risk of developing AD (Clatworthy et al, 1999). In contrast to apo E3, the wild type allele, apo E4 confers an increased risk of late-onset AD (Winkler et al, 1999). In normal brain apo E efficiently binds and sequesters amyloid beta peptides (Abeta), preventing its aggregation. In AD, the impaired apo E-Abeta binding leads to the critical accumulation of Abeta, facility plaque formation (Russo et al, 1998).
1.4 Enzyme - linked Immunosorbent Assays (ELISA)

Engvall and Perlmann (1971) first introduced ELISA for the detection of IgG antibody. Since then, it has become a well-established tool in clinical and research laboratories (Schuurs and van weemen, 1977). It is a good tool for quantitation of single or multiple Antigen (Ag) or Antibodies (Abs) on a patient sample (usually serum). The test is performed in a 8 cm X 12 cm plastic plate which contains an 8 X 12 matrix of 96 wells, each of which are about 1cm high and 0.7cm in diameter. The assay fulfils the requirements of objectivity, simplicity and sensitivity previously accomplished only by RIA. In comparison with RIA, the ELISA employs stable and non-hazardous reagent, requires less expensive equipment and is more suitable for automation.

1.4.1 Principles of ELISA

The performance of ELISA and related solid phase assays depends on four major principles:

- A variety of enzymes, including horseradish peroxidase (HRP) and alkaline phosphatase (AP) – can be chemically coupled to either antibody or antigen under conditions which retain the biological properties (i.e. substrate interaction, antigen binding, antigenicity) of both components of the conjugate.

- Most antigens, for example proteins, peptides and polysaccharides, bind spontaneously to plastic surfaces such as the wells of the polysterene microtitre plates. Antibodies, as proteins, also attach whilst retaining their antigen-binding activity. Thus antigen- or antibody-coated plates can be prepared as the initial
step. Once antigens or antibodies applied to coat or ‘sensitize’ the solid phase are bound, they become resistant to vigorous washing in detergent buffer whilst excess unbound reagent is simply removed by this process.

- In subsequent steps one or more layers of a solid phase captured immune complex are formed, with unbound entities again efficiently washed away. This affords the basis for high specific to non-specific signal ratio when captured enzyme reacts with substrate.

- An enzyme conjugate of antibody or antigen when bound in the immune complex leaves the enzyme component available for substrate interaction. Addition of substrate, in the usual form of assay, results in a progressive substrate solution color change. The reaction can be stopped at an appropriate stage and the color signal determined by visual comparison with standards or by optical density measurement.

1.4.2 Indirect ELISA

In ELISA methods, an immunoreagent (antigen or antibody) is immobilized onto a solid phase (such as the wells of a microtiter plate) in such a manner that it retains its immunological reactivity. Two methods commonly used are the indirect method and the double sandwich method.

Indirect ELISA method is most often used to detect serum antibodies to infections and parasitic disease agents. The binding sequence for an indirect ELISA is illustrated in Figure 1.5.
After an antigen has adsorbed onto the solid phase, serum sample is added and incubated. If the serum contains antibody (Figure 1.6), it will bind to the adsorbed antigen. Following a washing step, an excess of enzyme-labeled antispecies antibody (conjugate) is added, where it binds to the antibody already attached to the antigen. One type of conjugate can be used for a variety of infectious agents that infect a given species because conjugates are directed toward bound antibody. Following a final wash step, substrate is provided for the bound enzyme, which in turn, is catalyzed to a colored reaction product. The color can be read spectrophotometrically. The sources of error encounter during indirect ELISA is illustrated in Table 1.6.

Figure 1.5: Binding Sequence of Indirect ELISA
Figure 1.6: Antibody Detection in an Enzyme-Linked Immunosorbent Assay
### Table 1.6: Sources of Error in Indirect ELISA

#### Sources of Error

**Low assay sensitivity**
1. Choice of solid phase.
2. Antigen concentration (e.g. over-coating).
3. Use of low-titer conjugates.
4. Use of substrates with poor solubility.
5. Presence of enzyme inhibitors in buffers, e.g. azide or phosphates, for peroxidase and phosphatase enzymes, respectively.
6. Inadequate mixing of highly diluted samples, e.g. serum and conjugates.

**Poor assay specificity**
1. Quality of antigen.
2. Choice of solid phase.
3. Extended incubation periods.
4. Use of unpurified conjugates containing free enzymes.

**Low assay precision**
1. Volumetric pipetting errors, especially critical in serum dilutions
2. Inadequate incubation periods (i.e. below equilibrium level).
3. Poor endpoint standardization.
4. Operator errors.
Coating of Antigen/Antibody to the solid phase:

The conditions used for the coating of the solid phase with Ag or Ab can seriously effect the outcome of the assay. Some of the variables to be considered include the

- temperature used for coating
- concentration of Ag
- type of buffer
- time of incubation of the antigen with the solid phase
- type of solid phase used for the ELISA
1.5 Epitope Mapping

1.5.1 Characteristics of epitopes

The first step in the production of anti-peptide antibodies is to design the peptide antigen that will be used to generate the antibodies. To do this, the concept of an epitope and what methods are available to help in choosing the epitope must be understood.

Antibodies are raised by the immune system against regions on the surface of a protein known as "epitopes". An epitope consists of short sequences of amino acids on the protein antigen which enable specific antigen-antibody binding. Immunogenic epitopes can be sequential (short linear sequence of amino acids) or may occur as assembled (Geysen, 1985). Usually there are more than one epitope per antigen molecule. Each of the epitopes may be recognized by different antibodies.

Protein antigenic determinants have been classified as continuous ("linear", "sequential") or discontinuous ("conformation", "assembled") (Benjamin et al, 1984). A continuous epitope is composed of a contiguous stretch of residues in a protein. A discontinuous epitope consists of a group of residues that are not contiguous in the sequence, but are brought together by the folding of the polypeptide chain, or by the juxtaposition of two separate peptide chains.

Epitopes must be exposed on the surface of the native antigen molecules (three dimension structures) to make contact with antibodies. In this case, epitopes should have high hydrophilicity so that they can interact with antibodies through polar interactions. For example, an anionic glutamic acid carboxyl group may complementarily bind to cationic lysine amino group on the antibody or vice versa (Berzofsky & Berkower, 1993). But it is also true that not all the hydrophilic regions
are necessarily epitopes, not all epitopes are hydrophilic or on the surface of antigen and that the major antigenic regions are not necessarily the most hydrophilic.

1.5.2 Choosing the epitope

In order to mimic a protein epitope with a small synthetic peptide, it is important to choose a sequence that is hydrophilic, surface-oriented, and flexible (Van Regenmortel, 1984) since:

- Most naturally occurring proteins found in physiological solutions have their hydrophilic residues on the surface and their hydrophobic residues buried.
- Antibodies bind to epitopes on the surfaces of naturally occurring proteins.
- Several known epitopes have a high degree of mobility (Westhof et al., 1984).

The N- and C- termini of proteins are generally surface-oriented since they contain charged groups, i.e. \( \text{NH}_3^+ \) and \( \text{COO}^- \). They often have a high degree of mobility as well, since they are located at the ends. These termini are often chosen as candidates for synthetic peptide because they possess all three properties.

The term epitope mapping has also been used to describe the attempt to determine all major sites on a protein surface that can elicit an antibody response, at the end of which we produce an epitope map of the protein antigen (Atassi, 1984).

Implicit in this view of epitopes is that they are fixed, concrete structures on protein sequences, which are few in numbers and are uniquely capable of stimulating the immune system. This kind of epitope map also confuses the important distinction between antigenicity (the ability to recognize a specific antibody) and immunogenicity (the ability to produce antibodies in a given animal species).
Epitope mapping is an efficient method, which allows the synthesis of overlapping amino acid sequence that may be tested. It also allows the study of proteins at their amino acid level and in turn allows the identification of epitope(s) or epitope clusters (Geysen et al., 1987).

1.5.3 The Epitopes of Synthetic Peptides of Apo E and Native Apo E

The synthetic peptides are the amino acid residues of Apo E present as linear sequences. As monoclonal antibodies recognize a small sample of all antigenic specificities, they are more likely to be directed against the numerous surface patches of a protein made up of residues distant in the sequence, rather than against continuous determinants. Attempts to locate protein epitopes by using monoclonal antibodies directed against the native molecule have led to the identification of mainly discontinuous epitopes (Smith-Gill et al., 1982; Van Regenmortel, 1984).

Antibodies reactive with the native protein would be produced only if the fragment used for immunization closely reproduced the tertiary conformation of the protein. It has been shown that many linear synthetic peptides corresponding to parts of proteins are able to elicit antibodies that react with the intact antigen (Lerner, 1982; Shinnick et al., 1983; Green et al, 1984). It is well known that synthetic peptides are able to mimic the corresponding regions of the native protein because one of the conformations adopted in solution approximates that in the native molecule (Lerner, 1982).

There is also an apparent contradiction between the finding that, on the one hand, monoclonal antibodies raised against a native protein are so conformation-specific that they usually do not bind to peptide fragments of the molecule, while, on
the other hand, the majority of monoclonal antibodies raised against a peptide fragment also recognize the intact molecule (Todd et al, 1982). It has been suggested that this contradiction might disappear if truly native, that is, correctly folded, intact, molecules were tested for their ability to react with anti-peptide monoclonal antibodies.

In general therefore, antibodies raised against a peptide would have a high probability of recognizing the corresponding epitope in the complete protein, whereas monoclonal antibodies raised against the intact protein would tend to be directed against the discontinuous epitopes, which are more numerous.
1.5.4 Peptide Synthesis

Once the peptide antigen is designed, the next step is to make it. There are two strategies for the chemical synthesis of peptides: solid phase and solution phase. The most common way peptides are synthesized for research use is solid phase synthesis.

Solid phase peptide synthesis, pioneered by Merrifield (1963), have proven to be extremely versatile tools for the study of protein-protein interactions. It involves the sequential addition of amino acids to create a linear peptide chain. Solid phase synthesis involves three steps:

- **Immobilization**: The C-terminal amino acid of the growing peptide chain is anchored covalently to a solid support or resin during the synthesis.

- **Chain assembly**: Each amino acids is then added to the peptide chain one at a time by a cyclic process involving three chemical reactions (Figure 1.7): activation, coupling, deprotection.

- **Cleavage**: After the synthesis is completed, the peptide must be removed from the resin and the protecting groups must be removed from the side chains.
Figure 1.7: The Peptide synthesis cycle
There are two types of strategies commonly used in the solid phase synthesis of peptides: F-moc/tBu and t-Boc. Fmoc chemistry was developed by Eric Atherton and Bob Sheppard at the Laboratory of Molecular Biology in Cambridge in the late 1970’s. Fmoc synthesis is mild, flexible and versatile and consequently offers more synthetic options than Boc chemistry. Boc solid phase chemistry was invented by Bruce Merrifield and was of such significance to the sphere of organic chemistry that he was awarded the Nobel Prize. It was, and in some laboratories still is, the method of choice for peptide synthesis, however, it can produce problems especially in inexperienced hands. Boc chemistry uses trifluoroacetic acid to remove the Boc group from the N-terminus of the elongating chain and uses hydrofluoric acid to cleave the peptide from the solid support. The recent basic chemistry is based on the F-moc amino acid derivatives. The Fmoc chemistry is commonly used now. Advantage of using F-moc chemistry is the avoidance of using liquid hydrogen fluoride (which is used in t-Boc chemistry to cleave off protecting groups and to remove the peptide from the solid support after synthesis is complete). Hydrogen Fluoride is potentially hazardous, so the great care must be taken when using it (Gullick et al, 1994). The F-moc/tBu approach is discussed here.

Deprotection: Deprotection is the removal of the N-terminal protecting group after coupling, in preparation for the addition of the next amino acid. Piperidine, a secondary base, is used to remove the Fmoc group (Appendix 2).

Activation: Before the incoming amino acid can be coupled to the growing peptide chain, the C-terminus of the incoming amino acid must be chemically activated.
Activation involves adding an activating agent that converts the amino acid into a highly reactive active ester (Appendix 2).

Since activated amino acids are highly reactive, they are susceptible to degradation and must therefore be generated immediately before the coupling step. There are numerous chemicals for converting amino acids into active esters. This method uses diisopropylcarbodiimide (DIC) as the activating agent.

**Coupling** : Coupling is the formation of an amide bond between the carboxyl of an incoming, activated amino acid and the amino terminal of the growing, resin-bound peptide chain (Appendix 2). The objective is to obtain the highest coupling efficiency possible, preferably greater than 99.5%.

**Protecting Groups** : Two different types of protecting groups are used in peptide synthesis to block reactive groups in the growing peptide chains. They are referred to as N-terminal (or amino terminal) and side-chain protecting groups. The C-terminal is protected by attachment to the resin.

**Amino terminal** : The amino terminal of the incoming amino acid must be protected so that only one amino acid is added to each growing peptide chain. There are two terminal protecting groups commonly used in peptide synthesis, Fmoc and Bmoc.

**Side Chain** : Since the side chains of amino acids may contain chemically reactive groups, they must be protected. To prevent any unwanted side reactions, these protecting groups must stay in place during the entire synthesis, but also must be
completely removed by the same method used to cleave the peptide from resin after synthesis is completed.

With Fmoc chemistry, the side chain protecting groups are stable to the piperidine base used for removal of the Fmoc group, but are removed by TFA, the acid used to cleave the peptide from the resin (Appendix 2). Thiols, such as ethanedithiol, are typically added to the TFA to scavenge the reactive groups generated when the side chain protecting groups are removed.

1.5.5 The Multipin Peptide Synthesis

The multipin method is an effective, low-cost, simultaneous multiple peptide synthesis technology which gives ready access to large numbers of peptides. Synthesis is performed with Fmoc-protected amino acids. Where required, side-chain functionality is protected with trifluoroacetic acid (TFA)-labile protecting groups, hence avoiding the need to use hydrogen fluoride. Synthesis is performed on the heads of polyethylene pins (crowns). The detachable crowns are radiation-grafted with an appropriate polymer and subsequently derivatized so that they are ready for synthesis. The pins are mounted on a plastic holder in an 8 X 12 matrix, which matches the common 96-well microtiter tray format.

In recent years, a number of methods for the simultaneous synthesis of many peptides have been developed. Some are capable of simultaneous synthesis of as many as 100 peptides resins but all these procedures are limited by the need for individual cleavage and purification which are invariably time consuming.

New methods for the rapid synthesis of large numbers of peptides have facilitated the mapping of antigenic determinants of protein. The pin technology
developed by Geysen and coworkers is particularly useful for probing the antigenic structure of an entire polypeptide sequence (Geysen et al., 1987). Short overlapping peptides, e.g., hexapeptides, covering the sequence of a protein antigen are synthesized onto polyethylene pins by the Merrifield solid phase method (Merrifield, 1963). The pin bound peptides are then tested for reactivity with antibodies against the protein antigen, using a standard ELISA procedure. Since peptides remain covalently linked to the solid support, bound antibodies can be removed and the peptides retested with other antisera.
1.6 Objectives

- Firstly, to detect the natural autoantibodies in normal human serum (NHS) against hormones, namely adreno-cortical (hydrocortisone, aldosterone, androstenedione), adreno-medullary (noradrenaline, adrenaline), and other related steroid hormones (progesterone, testosterone, β-estradiol). An ELISA to detect all three antibody isotypes (IgG, IgA and IgM) will be used to investigate natural autoantibodies (NAA) to these hormones.

- Secondly, to synthesize multipin non-cleavable peptides, to obtain overlapping amino acid peptides of the complete sequence of apolipoprotein E (317 aa).

- Lastly, to test for natural antibody epitopes using the apo E peptide multipin ELISA. All isotypes will be analysed for their epitope binding pattern. The epitope specificity of natural antibodies will also be compared with antibody binding to apo E sequences in atherosclerotic patients.