1

Introduction

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1.1 Introduction

The production of antibodies (also referred to as immunoglobulins, Ig) forms an integral part of an individual's immune response to an invading pathogen. The structure of these molecules is designed such that they are bifunctional: they carry specificity for their antigen and they activate a variety of effector functions that lead ultimately to the destruction of the specific target pathogen. These two properties mean that antibodies are critical to the successful defence of the host; they have also led to the use of antibodies as indispensible tools in experimental research, clinical investigation and diagnosis and, more recently, clinical therapy. However, there is a major difference between the role of antibodies in host defence and in the laboratory or clinic. Invading pathogens are complex organisms, carrying on their surface a multitude of different antigenic determinants (epitopes). It is therefore advantageous for the host to produce as wide a range as possible of antibody molecules to ensure that the pathogen is effectively targeted and destroyed. In contrast, laboratory and clinical reagents are usually required to recognize a single molecule and frequently a single epitope on that single molecule. It is with the production and use of such reagents that this book is concerned.

1.2 Generation of an immune response

Immune responses are generated as a consequence of the recognition of foreign material by antigen-specific lymphocytes. Activation of B lymphocytes leads to the production of antibody whereas activation of T lymphocytes leads to destruction of infected host cells by different mechanisms. A brief account of B cell activation will be given here as a knowledge of this will provide the framework within which the techniques of monoclonal antibody production can be placed.

Lymphocytes recognize antigen by means of specific cell surface receptors; for B cells these receptors consist of membrane-bound antibody molecules. Each cell carries approximately 50 000 receptor molecules on its surface, each

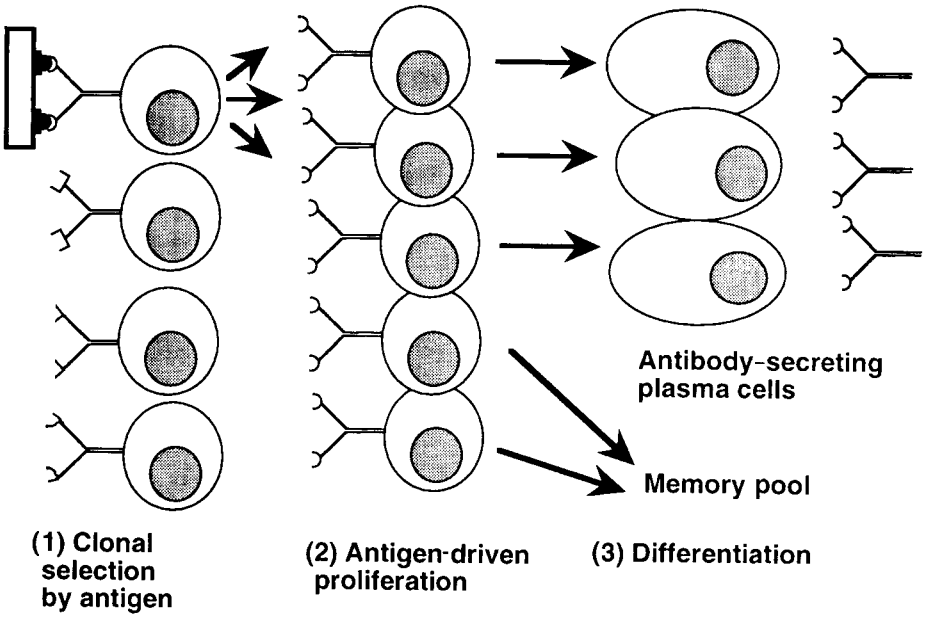

Figure 1.1. Clonal selection of B cells.

with an identical specificity. Hence, each B cell has a single specificity although at any one time an individual will possess many millions of different B cells as part of their immune repertoire. When an antigen enters the body it will bind to and activate any B cell whose receptor can recognize an epitope on that antigen. Activation leads to two main events (Figure 1.1). First, the B cell undergoes several mitotic divisions, thus giving rise to a 'clone' of identical cells. Second, these daughter cells differentiate to form either plasma cells or 'memory' B cells. The function of plasma cells is to secrete large amounts of soluble antibody of the same specificity as that which formed the cell surface receptor on the original, activated B cell. The function of memory cells is to mount an enhanced immune response on subsequent re-exposure to the same antigen. Moreover, the antibodies produced during this 'secondary' immune response are of higher affinity as development of memory B cells involves a process of hypermutation in the receptor gene loci followed by stringent antigen selection ('affinity maturation'). An individual, therefore, contains a repertoire of potential immune responses but it is the invading pathogen that directs and enhances the actual response against itself.

1.3 Antibody structure

Antibody molecules are glycoproteins whose basic unit comprises two identical 'heavy' and two identical 'light' polypeptide chains (Figure 1.2). Each chain is composed of domains: heavy chains contain four or five, according to their

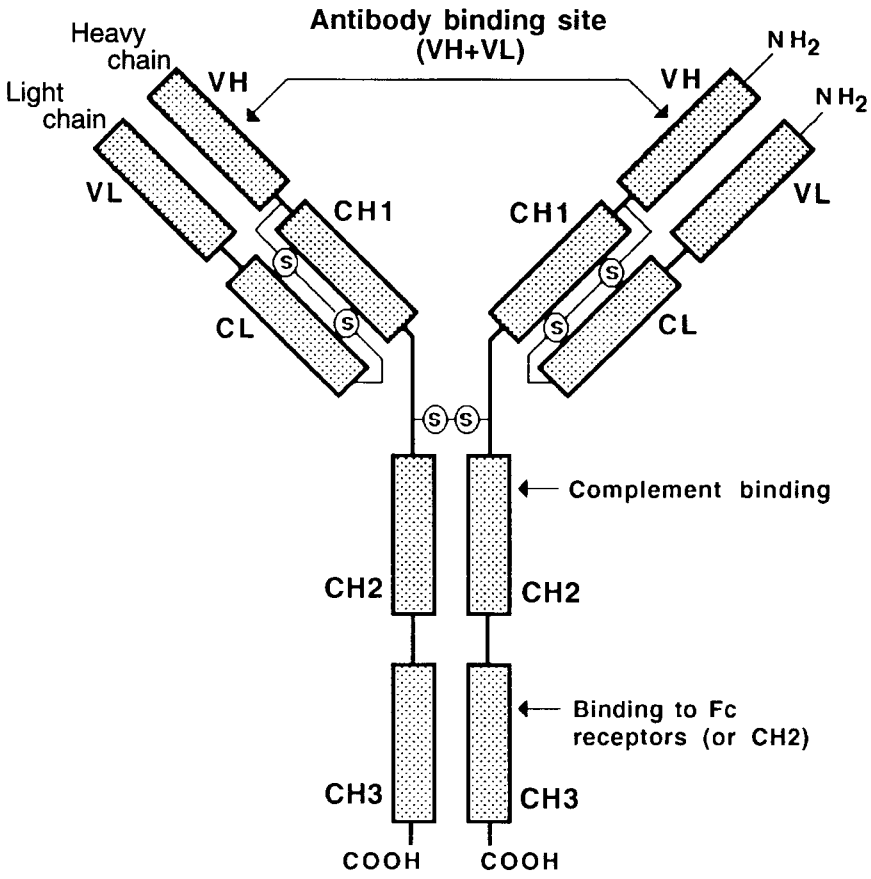

Figure 1.2. Basic structure of an antibody molecule.

class (4, IgG, IgA, IgD; 5, IgM, IgE) and light chains contain two. The ability to bind to antigen is controlled by the N-terminal domains, one binding site for antigen being created by the combination of one domain from the heavy and one from the light chain. The remaining domains control the effector functions of the antibody molecule (for example by activating the complement cascade or by activating phagocytosis via binding to Fc receptors on the surface of macrophages). This bifunctionality is controlled by differences in the primary structure of the various domains. Those at the N-terminus vary considerably between antibodies of differing specificities (and are therefore termed 'variable' or 'V' domains) whereas all other domains are relatively 'constant' ('C' domains). In a single antibody molecule, the two heavy chains are identical to each other as also are the two light chains, thus the antibody contains two identical binding sites for antigen.

How is the specificity of antibody determined? Variability in the V domains is not spread throughout the domain but is focused in three main areas that are termed the 'hypervariable' (or complementarity determining, CDR) regions.

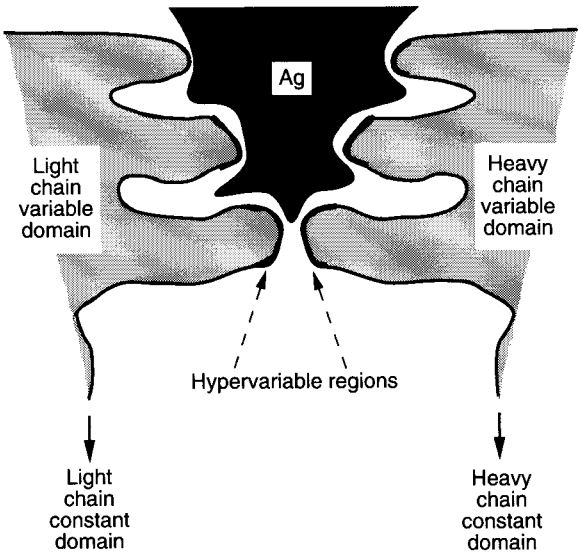

Figure 1.3. Interaction between antibody and antigen.

The β-pleated sheet folding pattern of the antibody molecule places these regions on the ends of the folds such that they are exposed to the outside of the molecule and are accessible for interaction with antigen (Figure 1.3). Variations in the amino acids in these three regions will alter the shape of the binding site for antigen and will also alter the potential chemical bonds that can form to hold antigen and antibody together. The alteration of the amino acid sequence in the hypervariable regions will control both antibody specificity and affinity (point mutations that occur in the genes encoding the V domains during B memory development can generate antibodies of enhanced affinity).

1.4 Generation of the B cell repertoire

How is the bifunctional antibody protein encoded in the genome? In the past, theories have ranged from the proposal that there is a single gene which encodes a single protein whose specificity is instructed by the antigen around which it moulds itself, to the idea that there is a separate gene for each antibody specificity. The first theory was discounted once the relationship between primary and secondary structure of proteins was understood; the impossibility of the second theory was appreciated once it had been calculated that, if true, antibody-encoding genes would occupy almost the entire mammalian genome. The truth, as often is the case, lies somewhere between these two extremes.

Each antibody polypeptide chain is encoded within a separate genetic locus (heavy chain locus, IgH; kappa light chain locus, κ; lambda light chain locus,

λ). Within each locus there are exons that encode the constant domains of the appropriate chain (these determine the isotype, i.e. κ and λ for the light chains, α, δ, ε, γ and μ for the heavy chains). In addition, each locus has clusters (families) of exons that the B cell uses to create its own unique V region encoding gene. Three rearranging gene families are present in the IgH locus (variable, V; diversity, D; joining, J), while the κ and λ have only two (variable, V; joining, J). During B cell development each B cell randomly 'selects' one V and one J exon within a light chain locus (from a choice of approximately 200 V and 4 J) and one V, one D and one J within the IgH locus (from a choice of approximately 100 V, 50 D and 4 J) to create its own unique variable region genes for its light and heavy chains (Figures 1.4 and 1.5). In this way each B cell creates its own unique specificity from a potential repertoire (total rearrangements and recombinations possible) of approximately 10^{10}. It is likely that around 10^8 specificities are present in an individual at any given time. The B cell receptor repertoire is created randomly; it is for antigen to select out those B cells of the appropriate specificity.

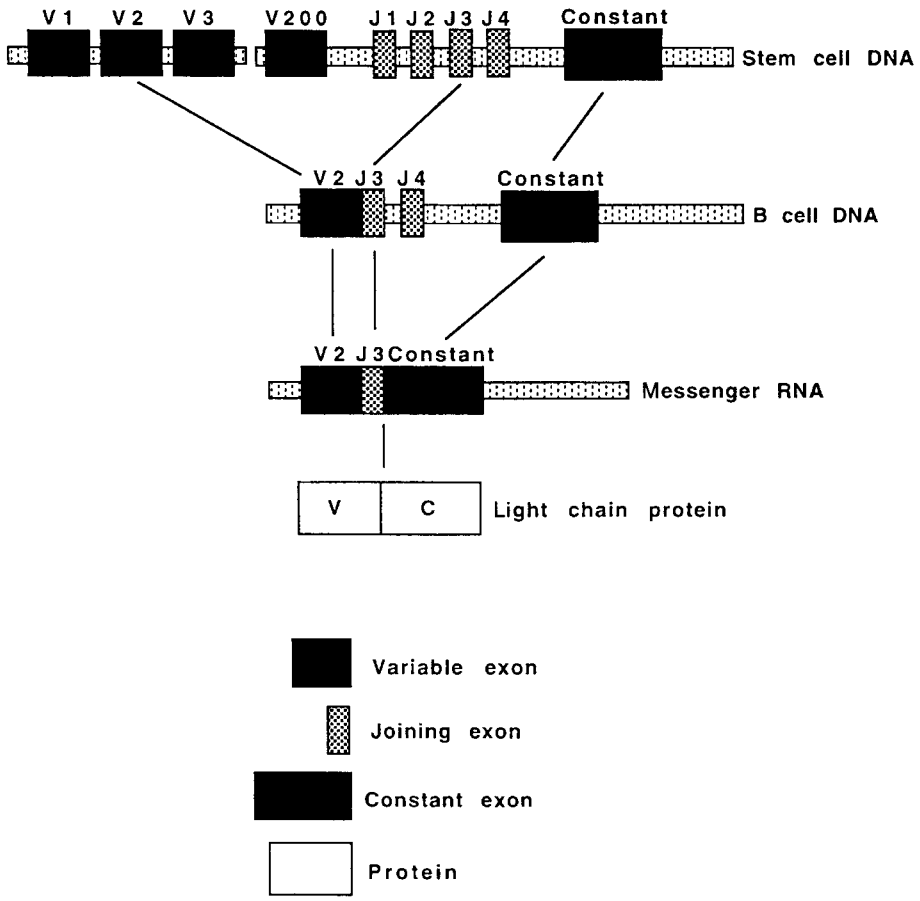

Figure 1.4. Gene rearrangements in the light chain locus of the B cell receptor.

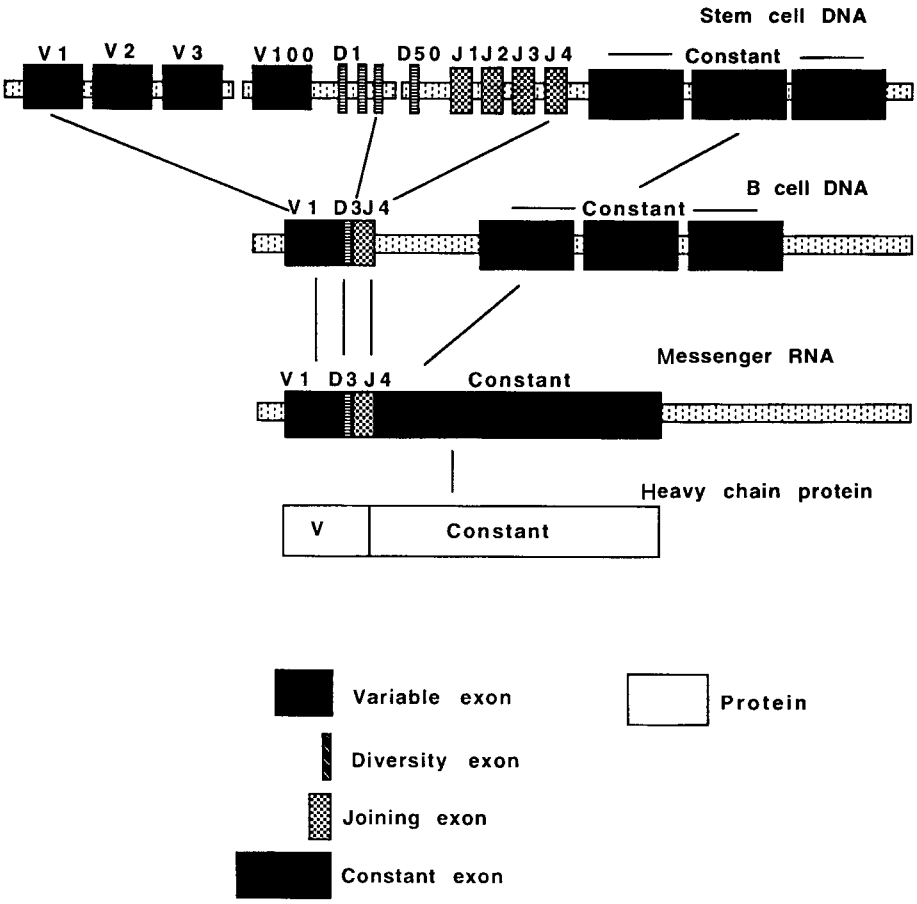

Figure 1.5. Gene rearrangements in the heavy chain locus of the B cell receptor.

1.5 What is a monoclonal antibody?

When antigen enters the body, there may be many B cells whose receptors can recognize some aspect of the foreign molecule/pathogen. These are likely to differ from each other both in the epitope to which they bind and in the affinity of their binding. This diversity will then be reflected in the secreted antibodies that are produced after clonal expansion and differentiation of the antigen-activated B cells. Many B cells will have undergone clonal expansion and so the antibodies will be the product of many clones: they are therefore 'polyclonal'. A polyclonal response, as discussed above, is highly advantageous in combating an invading pathogen, both in terms of the quantity of antigen–antibody binding and in the range of effector functions that can be activated. However, polyclonal antibodies do not in general provide clearly defined and reproducible reagents for use in the laboratory. (Although in fairness it should be

pointed out that polyclonality can be an advantage in certain experimental situations, such as anti-Ig antibodies for use as secondary reagents in immunostaining, where binding of several anti-Ig molecules to the first layer reagent will provide useful amplification of the signal.)

In the majority of situations, however, it is highly beneficial to have an homogeneous reagent that is of a single specificity and affinity and which is, therefore, predictable: the product of a single B cell clone, i.e. monoclonal antibody (mab). To obtain such an antibody it is necessary to isolate and propagate single antibody-secreting B cell clones, something that is not directly possible but which is made possible by the combination of methods of *in vitro* cell fusion, selection and cloning. This technique of monoclonal antibody production was developed by Georges Köhler and Cesar Milstein as part of their research into mechanisms underlying the diversity of antibodies (Köhler & Milstein, 1975). It is an excellent example of how basic academic research can lead to the more applied aspects of science, industry and clinical medicine. In recognition of the significance of their findings, Köhler and Milstein were awarded the Nobel prize for medicine in 1984 (Milstein, 1985).

In essence the technique involves the fusion of a B lymphocyte that has the property of specific antibody production, but which cannot live for more than a few days in culture, with a myeloma cell that has lost the ability to produce antibody but which has the property of immortality. The product of this union is a 'hybridoma' cell which can live forever (via mitotic cell division to give an ever expanding clone of identical cells) and which can secrete antibody of the desired specificity. The actual technique is obviously more complex than this as in reality the source of immune B cells (usually rodent spleen or lymph node) is a mixture of many different cell lineages. It is therefore necessary to put the hybridoma cells through a series of stringent selection procedures to eliminate hybrids that are either producing no antibody or, importantly, antibody that is of either the wrong specificity or of too low an affinity to be useful. The separation of individual clones to ensure monoclonality of the hybridoma cells and of the antibody that they secrete is also of crucial importance (Chapter 2).

More recently, molecular strategies have been devised so that, by manipulating the genes that encode the heavy and light chains, 'designer' mab can be produced with properties suited to their final use (Chapters 7 and 8). For example, antibodies can be 'humanized' by genetically grafting their hypervariable regions (CDRs) into a human antibody; these reagents can be then used *in vivo* without inducing an anti-mouse Ig response in the patient. 'Phage techniques can be used to create very small monovalent antibodies (single chain antibodies) with the property of excellent tissue penetration, while 'phage display libraries may make it possible to generate a vast array of unique antibody specificities without the need for active immunization. This is of considerable importance for the generation of novel reagents particularly in the human system where it has been difficult to apply conventional monoclonal techniques (Chapter 5).

1.6 The aim of this book

We have designed this book to provide our readers with a full perspective of mab in both the laboratory and clinical setting because there is an important interplay between the experimental development of new reagents and their subsequent application. The initial chapters deal with the use of cellular and molecular techniques for the production (Chapters 2, 5 and 7) and modification (Chapters 6 and 9) of monoclonal reagents, the mapping of the epitopes recognized by antibodies (Chapter 4) and the use of these reagents in novel and enhanced assay systems (Chapter 9). To complete this aspect of the book, Chapter 3 provides an important and 'user-friendly' consideration of the nature of antibody affinity and its practical implications for those who wish to use monoclonal antibodies in the laboratory and clinical setting. The second part of this book contains a series of chapters covering the many clinical areas in which mab are currently used, both in routine application as well as in experimental investigations.

We hope that after reading these chapters you will not only successfully raise your own monoclonal reagents but that you will have a good appreciation of the many and varied applications of these reagents to clinical medicine. Even if you do not go on to produce your own antibodies, we hope that a knowledge of how they are made will enhance your appreciation of these unique reagents.

1.7 References

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Milstein, C. (1985). From the structure of antibodies to the diversification of the immune response. *EMBO Journal*, **4**, 1083–1092.

2

Production of monoclonal antibodies

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2.1 Introduction

In this chapter we discuss the various techniques currently used during the production of monoclonal antibodies (mab), their application and their relative merits. The actual protocols are detailed in Chapter 16 (Technical appendix). As you will discover, although all are based on the original method of Kohler and Milstein (Kohler & Milstein, 1975; Galfre & Milstein, 1981) there are many variations in the methods used by different laboratories. The final design of such techniques is determined in part by logical consideration and in part by empiricism. When establishing the monoclonal system in your own laboratory it is important to try to dissociate these two aspects, so that you can 'filter' the often conflicting advice of others in the field (we all have our idiosyncracies). We hope that this chapter will help you to do this.

2.2 Antigens and immunization

2.2.1 Antigen

When planning the production of a mab you must first consider the antigen that you wish it to recognize. The method of antigen preparation, the immunization procedure and the choice of animal to immunize will differ according to the species of origin, chemical structure, size and source (e.g. whole cells, crude tissue extract, purified protein or peptide). You may wish to use mab techniques to discover novel molecules or you may be interested in producing reagents to a known molecule whose structure and function you wish to investigate further. Obviously the choice of antigen is a personal one, determined by your own particular interests, but certain general points are worth considering.

An antigen is a molecule that can be recognized by an antibody (or T cell receptor). The immunogenicity of a molecule is determined by its ability to activate the immune system. Not all antigens are immunogenic, although by definition they can be recognized by an antibody, because they may be too

small on their own to generate an immune response *de novo*. To initiate an immune response the molecule must obviously bind to and activate specific B lymphocytes such that clones of specific antibody-producing cells and memory B cells can be generated. On their own, B lymphocytes will only generate a primary immune response that is characterized by relatively limited clonal expansion, IgM antibody and no memory. To induce B cell memory and the consequent Ig class switching (switch from IgM to IgG, A or E), enhanced affinity and increased level of antibody production that characterize the secondary immune response to re-exposure to the same antigen, it is necessary to also activate T lymphocytes. This dependence on T cells is because they provide growth and maturation signals via cell–cell contact and secreted soluble molecules (cytokines). In contrast to B lymphocytes which can recognize whole macromolecules in their native configuration, T lymphocytes can only respond to enzymatically processed protein antigen that is presented to them on the surface of an 'antigen presenting cell' (the processing cell, e.g. dendritic cell, macrophage, B lymphocyte). This processed antigen is in the form of a small peptide (approximately 15 amino acids, for the T cells that help B cells) sitting in a specialized groove in class II major histocompatibility complex (MHC) molecules on the surface of the antigen-presenting cell. The two populations of lymphocytes, B and T, therefore have very different ways of looking at antigen and these will influence the outcome of immunization.

The major parameters that you should consider when selecting your immunogen are size, heterogeneity, chemical nature, quantity and conformation.

2.2.1.1 Size and quantity

Molecules whose molecular weight is less than 1000 are unlikely to be large enough to be able to stimulate both T and B cells and are therefore likely to generate only an IgM 'primary' type immune response. This is important to remember when trying to raise antibodies to peptide antigens (Chapter 4). The problem can be circumvented by coupling the peptide to a larger 'carrier' macromolecule such as bovine serum albumin (BSA) or keyhole limpet haemocyanin (KLH), both old favourites of immunologists owing to their very effective immunogenicity (Dadi *et al*., 1984). Conjugation to a carrier can also be useful for larger molecules which, although structurally immunogenic, may be available in only very small quantities.

2.2.1.2 Heterogeneity

Experiments with synthetic polymers have shown that molecular heterogeneity is an important component of immunogenicity. Homopolymers were shown to be non-immunogenic, copolymers were sometimes immunogenic while heteropolymers were regularly effective in generating an immune response. Heterogeneity will not be a problem with macromolecules or more complex cellular